JOTCS A
Journal of the Turkish Chemical Society
Section: A
Chemistry
TURKISH CHEMICAL SOCIETY

# Anti-Inflammatory Effect of Coxibs and their Compositions with Caffeine on the Level of Conjugated Dienes in the Formalin-Induced Edema Model

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**Abstract**: The development of novel pharmaceutical compositions, which are effective in the treatment of different inflammatory diseases and have a minimum number of side effects is very relevant. The aim of the present study was biochemical confirmation of anti-inflammatory activity of new pharmaceutical compositions comprising coxibs and caffeine. The level of conjugated dienes as primary products of lipid peroxidation has been evaluated in the plasma of rats at the acute inflammation caused by formalin. The white male rats of WAG line were used. The content of conjugated dienes was determined by spectrophotometric method. It was shown that combinations of caffeine and coxibs showed statistical significant decrease in the content of conjugated dienes in the rats' blood plasma. Caffeine enriched anti-inflammatory action of coxibs effectively.

**Keywords:** Celecoxib, rofecoxib, caffeine, conjugated dienes, non-steroidal anti-inflammatory drugs.

**Submitted:** December 31, 2021. **Accepted:** August 13, 2022.

**Cite this:** Syrova G, Petiunina V, Savelieva O, Tishakova T, Lukianova L. Anti-Inflammatory Effect of Coxibs and their Compositions with Caffeine on the Level of Conjugated Dienes in the Formalin-Induced Edema Model. JOTCSA. 2022;9(4):1029–34.

**DOI:** https://doi.org/10.18596/jotcsa.1051858.

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## INTRODUCTION

Continuous development of medical science causes the necessity of search for new medicinal products and carrying out the research for expanding the therapeutic potentialities of already known pharmaceutical preparations.

Nowadays new scientific research has been performing for one of the important groups of medicinal products – non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs have received great deal of attention compared to traditional analgesics because of their anti-inflammatory, pain-relieving and antipyretic effects. These medications are highly effective for treating musculoskeletal disorders (like osteoarthritis, rheumatoid arthritis) accompanied with pain syndrome. Application of these medicinal products improves quality of life (1-5). However, the usage of NSAIDs results in the development of serious gastrointestinal, liver and cardiovascular side effects (6-8).

That's why, a new perspective for scientists for improving efficiency, reduce toxicity and side effects of NSAIDs is to create combinations of medicinal products. Such investigations have been performed by the scientists of medical and bioorganic chemistry of Kharkiv National Medical University. Researchers of the department have studied antiexudative, analgesic and antipyretic actions of pharmaceutical compositions containing 2,4-dichlorobenzoic acid, caffeine (9), known NSAIDs and non-narcotic analgesics such as paracetamol, diclofenac, ibuprofen, meloxicam with adjuvant caffeine.

The results showed that caffeine potentiates antiexudative and analgesic effect of investigated

NSAIDs (10). Adjuvanticity of caffeine towards NSAIDs and non-narcotic analgesics is explained with its positive influence on bioavailability (11), as well as its structural similarity with adenosine that contributes to neurochemical blocking of “purine” receptors in the brain with caffeine (12).

Today, we have been paying attention on anti-inflammatory, analgesic and antipyretic effects of newer NSAIDs – coxibs (celecoxib and rofecoxib) (8, 9). It is known that these medicinal products belong to highly-selective inhibitor of cyclooxygenase-2 and their pharmacological action is based on the inhibition of the biosynthesis of prostaglandins – moderator of pain and inflammation. As cyclooxygenase-2 is a main trigger mechanism of inflammation and neoangiogenesis, these phenomena accompany a wide range of pathological conditions and diseases. That's why therapeutic properties of celecoxib have been studied in all fields of medicine and it found a wide application (13). Celecoxib is used to treat acute pathologies of musculoskeletal system, during surgery and postrheumatic syndrome (14) or as urgent analgesic (15, 16).

It has been proven that celecoxib is an efficacious and safe at the treatment of psychotic symptoms, particularly in first-episode schizophrenia (17, 18). Cyclooxygenase-2 inhibitors have demonstrated potential therapeutic effects in tumors (19). Celecoxib is one of the few drugs that can be taken for a long time, due to its high tolerability and low risk of gastrointestinal and cardio-vascular adverse effects.

Rofecoxib is widely used in clinical practice in Ukraine (20). It demonstrates pharmacological effect similar to celecoxib (21). This medication is used as analgesic and antipyretic for treating rheumatoid conditions, primary dysmenorrhea (22) and, moreover, rofecoxib exhibits chondroprotective properties.

We selected coxibs as objects of study because there are no data in the literature on the presence of combination medicinal products containing celecoxib and rofecoxib.

Inflammation is a protective reaction of the body against injury or infection. It is a known fact that in the first day’s inflammation leads to the changes in blood lipids and to the distress of liver protein synthesis (23, 24). Acute inflammation is marked by the changes in the level of markers of lipid peroxidation in the form of the primary products – conjugated dienes (25, 26).

Conjugated dienes are the primary products of lipid peroxidation and classified as toxic metabolites that impair lipoproteins, enzymes and nucleic acids. Further products of lipid peroxidation are aldehyde and ketones (malondialdehyde and others) that play an important role in the synthesis of prostaglandins, progesterone and other steroids. Lipid peroxidation can alter vital membrane protein structure and function; it could lead to cellular dysfunction and widespread tissue damage (27).

Therefore, the aim of our investigation was to evaluate the effect of coxibs (celecoxib and rofecoxib) and their composition with caffeine on the process of lipid peroxidation in the form of conjugated dienes by utilizing formalin-induced edema model in experimental animals.

## MATERIALS AND METHODS

### General

Biochemical investigations of rofecoxib (*4*-(*4*'-methylsulfonylphenyl)-3-phenyl-2-(5H)-furanone), celecoxib (*4-[5-(4-methylphenyl)-3-(*trifluoromethyl*)*-1H-pyrazol-1-yl]benzenesulfonamide) and their compositions with caffeine (1,3,7-trimethylxynthine) were performed to estimate their anti-inflammatory action in comparison to reference drug – sodium diclofenac (28, 29).

Anti-inflammatory action of abovementioned coxibs was studied using the animal experimentation and formalin-induced edema model. The white male rats of WAG line (weighing 180-200 g) were divided into eight equal groups (n = 6). The animals of group 1 received 3% starch mucus (2 mL/200 g body weight of the rat) (Control). Group 2 received subplantar injection of 2% freshly prepared formalin and was treated intragastrically with 3% starch mucus. Groups 3-8 received subplantar injection of 2% formalin and investigated medications, in particular: Group 3: rats were intragastrically injected with (*4*-(*4*'-methylsulfonylphenyl)-3-phenyl-2-(5H)-furanone) (rofecoxib) at a dose of 1.5 mg/kg in 3% starch mucus. Group 4: rats were intragastrically injected with (*4-[5-(4-methylphenyl)-3-(*trifluoromethyl*)*-1H-pyrazol-1-yl]benzenesulfonamide) (celecoxib) at a dose of 5 mg/kg in 3% starch mucus. Group 5: rats were intragastrically injected with caffeine at a dose of 5 mg/kg in 3% starch mucus. Group 6: rats were intragastrically injected with pharmaceutical composition of rofecoxib with caffeine at the same dose. Group 7: rats were intragastrically injected with pharmaceutical composition of celecoxib with caffeine at the same dose. Group 8: rats were intragastrically injected with reference drug – sodium diclofenac at a dose of 8 mg/kg in 3% starch mucus. The formalin-induced edema was produced by subplantar injection of 2% freshly prepared formalin in the right hind paw.

In 4 hours after the injection of formalin acute inflammation was produced in the right hind paw of each rat. Animals of groups 3-8 were treated by investigated medicinal products and their compositions with caffeine at 3 hours after the formalin injection taking into consideration their pharmacokinetics and pharmacodynamics. One

hour after the administration of medicinal products animals were decapitated under ether anesthesia (30). Samples of blood were collected. 1% solution of heparin was used as an anticoagulant. The blood plasma was separated by centrifugation at 1500 rpm/min for 15 minutes and used as biomaterial.

The animals were kept in the vivarium of Kharkiv National Medical University according to the rules of humane treatment of laboratory animals. The studies on animals were performed as per the principles of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes" (31) and the Decree of the First National Congress on Bioethics (32).

**Anti-inflammatory action assay**

Anti-inflammatory action of investigated substances was studied by the level of conjugated dienes as inflammatory markers. The content of conjugated dienes was determined by spectrophotometric method at 233 nm using the spectrophotometer SF-46. The conjugated diene moiety is a strong chromophore that can be detected spectrophotometrically. When present in fatty acids they show a characteristic absorption in the ultra violet region at around 233 nm (33).

*The principle of method.* Heptane-isopropanol mixture is used to extract plasma lipids. Because heptane is very non polar, it primarily extracts neutral lipids. Phospholipids have some charges and typically extracted by isopropanol. Because of these differences in charge, extraction of total lipids typically uses mixture of solvents and it becomes possible to estimate level of the lipid peroxidation product in different classes of lipids. Lipid extracts of every phase are analyzed by spectrophotometric method at about 233 nm.

*Procedure of determination.* 0.5 mL of blood plasma was mixed with 5 mL of heptane-isopropanol mixture at a ratio of 1:1 (v/v), agitated manually for 15 minutes to extract lipids and then centrifuged at 5000 rpm/min for 10 min at 20 °C. The aqueous phase was collected and subjected to two subsequent identical extraction steps. Such procedure is necessary to get optimal values of absorbance in both phases of extract. Then 2 mL of 1 M hydrochloric acid solution was added to lipid extracts to separate and to remove nonlipid impurities. The mixture was then centrifuged at 12000 rpm/min for 15 min at 4 °C. The upper layer (heptane phase) was collected into a dry test tube. Water-alcohol phase was mixed with 1 g of NaCl to separate isopropanol from its mixture with water by a salting out method. After the separation from water isopropyl alcohol was transferred into the dry test tube containing heptane phase. A blank solution was prepared according to the same procedure described above. 0.5 mL of 0.1% Ethylenediaminetetraacetic acid solution in 0.9% sodium chloride solution was used instead of plasma. Organic phase from a result of extraction was analyzed by spectrophotometer SF-46 at 233 nm. The content of lipid peroxidation products was calculated using the following formula:

$$\text{Conjugated dienes} = \frac{(A_0 - A_x) \times 15}{0.022} \frac{\mu mol}{L} \quad (1)$$

where $A_0$ is the absorbance of blank solution, and $A_x$ is the absorbance of investigated organic phase; and 0.022 is the extinction coefficient of conjugated dienes, and 15 is the dilution coefficient of plasma in organic extract.

Statistical processing of the obtained data was performed using application packages Microsoft® Excel 2000 (Microsoft®) and STATISTICA® for Windows 6.0 (StatSoft Inc.). Statistical analysis was carried out with one-way analysis of variance, ANOVA. Data at $P<0.05$ were considered statistically significant.

**RESULTS AND DISCUSSION**

It is known that exposure to pathogenic factors leads to a disturbance in the pro-oxidant to antioxidant balance, which is accompanied by the increase in intensity of free radical oxidation of membrane phospholipids (18).

The results of biochemical investigations of anti-inflammatory action of coxibs and their compositions with caffeine on the level of conjugated dienes in rats' blood plasma are presented in Figure 1.

Conducted biochemical research demonstrates that level of conjugated dienes in the blood plasma increased 4.5 times in the Group 2 (Formalin-induced edema) compared to the Control (Group 1). Administration of investigated coxibs showed statistically significant decrease in conjugated dienes content in the plasma of rats. It is worth noting, that rofecoxib was more efficacious than celecoxib.

Mono-administration of rofecoxib reduced the conjugated dienes level in rats' blood plasma by 2 times as compared with formalin-induced edema (Group 2). And in return, mono-administration of celecoxib decreased the conjugated dienes content by nearly 1.5 times.

Obtained data statistically significant differ from Control (Group 1) and from the reference drug – sodium diclofenac (Group 8). Administration of caffeine (Group 5) decreased level of conjugated dienes in rats' plasma by 1.8 times compared to formalin-induced edema that is statistically significant different from the second group of animals (formalin-induced edema). This favors efficiency of caffeine for reduction of lipid peroxidation primary products in the plasma of rats

under conditions of formalin-induced edema. Therefore, according the ability to decrease content of conjugated dienes in rats' blood plasma, coxibs and caffeine can be arranged in the following row: rofecoxib ˃ caffeine ˃ celecoxib.

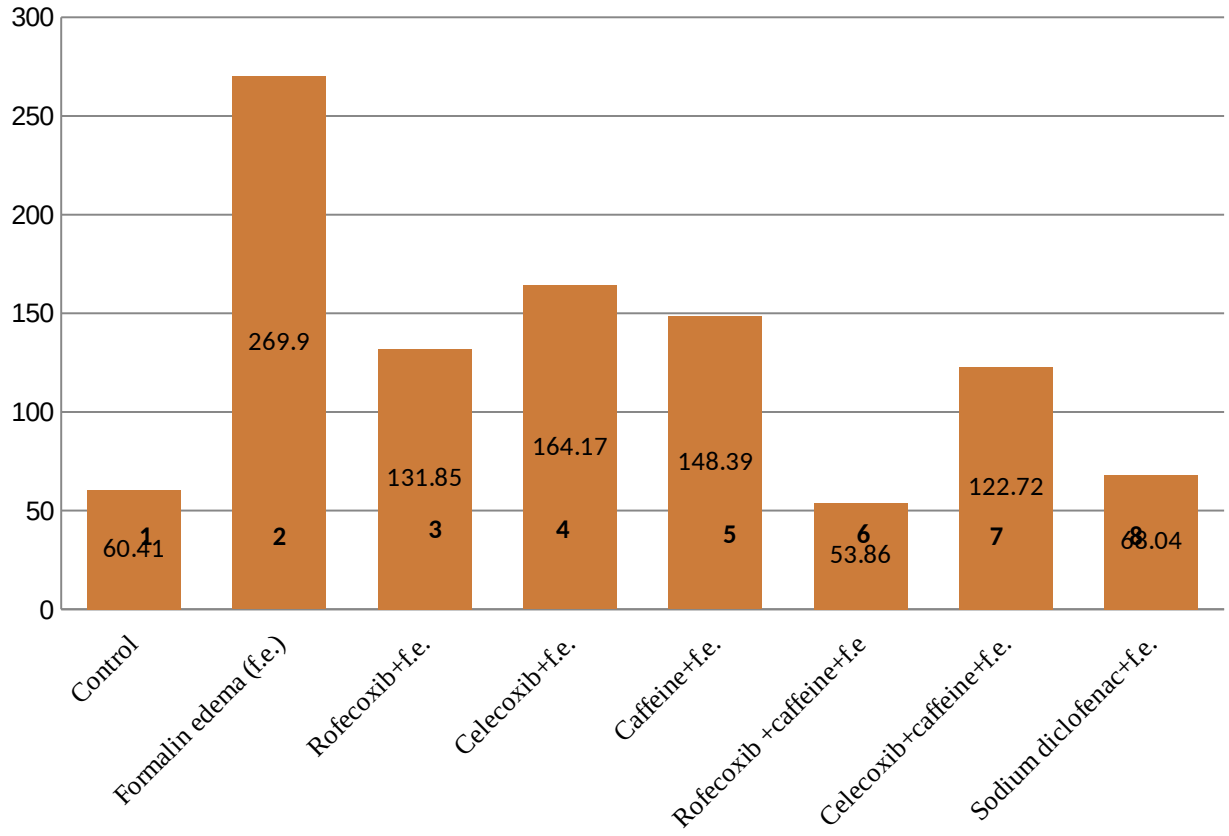


**Figure 1:** Effect of coxibs and their pharmaceutical composition with caffeine on the level of conjugated dienes in rats' blood plasma under conditions of formalin-induced edema. X axis shows the groups and Y axis shows the concentration of conjugated dienes in μmol/L.

Note 1. (mean ± SEM) * – the difference is significant compared to Control, $p < 0.05$;
Note 2. (mean ± SEM) ** – the difference is significant compared to formalin-induced edema, $p < 0.05$;
Note 3. (mean ± SEM) *** – the difference is significant compared to rofecoxib, $p < 0.05$;
Note 4. (mean ± SEM) **** – the difference is significant compared to celecoxib, $p < 0.05$;
Note 5. (mean ± SEM) ***** – the difference is significant compared to caffeine, $p < 0.05$.
Note 6. (mean ± SEM) ****** – the difference is significant compared to sodium diclofenac, $p < 0.05$.

Combination of adjuvant (caffeine) with coxibs (Groups 6 and 7) promotes the decrease in conjugated dienes level in the plasma of rats that was statistically significant compared to formalin-induced edema as well as compared to the mono-administration of coxibs. Pharmaceutical composition of rofecoxib with caffeine proved to be more efficient than reference drug (Group 8). Statistically significant differences between the results obtained in experimental group (Group 6) and intact control (Group 1) was not observed. It means that caffeine potentiates anti-inflammatory action of rofecoxib effectively.

Combination of caffeine with celecoxib (Group 7) resulted in significantly lower decrease of CD content. Pharmaceutical composition of celecoxib and caffeine (Group 7) exhibited statistically significant decrease in the level of conjugated dienes in the rats' blood plasma compared to formalin-induced edema (Group 2). However, obtained results were not statistically significant in comparison to the mono-administration of celecoxib (Group 4) but they showed statistically significant difference compared to reference drug (Group 8) and Control. Hence, according the anti-inflammatory activity proposed pharmaceutical compositions can be arranged in the following row: rofecoxib+ caffeine ˃ sodium diclofenac ˃ celecoxib + caffeine.

It is clear that proposed new pharmaceutical composition of caffeine and rofecoxib more effectively suppress the edema produced by

formalin and became the leader of biochemical studies.

## CONCLUSION

Based on the results obtained from the study it can be concluded that:
1. Rofecoxib, celecoxib, caffeine and their compositions exhibit anti-inflammatory activity against formalin-induced edema with different efficiency. Composition of rofecoxib and caffeine provided greater overall anti-inflammatory activity than sodium diclofenac but composition of celecoxib and caffeine has effect that is comparable to the reference drug. It was shown that monopreparations and caffeine act worse than the novel proposed compositions.

2. Caffeine potentiates anti-inflammatory action of investigated coxibs in the formalin-induced edema model. The level of conjugated dienes reduced as compared with the value in control group after administration of pharmaceutical composition containing rofecoxib and caffeine. At the same time, composition of celecoxib and caffeine did not significantly affect the formalin-induced edema.

3. The leader in biochemical studies is a two-component composition of rofecoxib and caffeine, which reduces the level of the LOP primary products – conjugated dienes in rats' blood plasma to the level of control group and proved to be better than the reference drug.

## CONFLICT OF INTEREST

The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

## ACKNOWLEDGMENTS

The study was supported by the budget topic of the department of Medical and Bioorganic chemistry of Kharkiv National Medical University 0119U002907 "Chemical-pharmaceutical substantiation of creation of biologically active compounds, conjugates and medicinal compositions with anti-inflammatory and analgesic activities". Applied scientific research" (2019-2023 academic years)

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