The Efficacy of Tramadol Combined with A Donor of NO, Glyceryl Trinitrate (GTN) Mixture on Cytokines, NF-KB Expression and Oxıdative Stress Marker in the Rat Model of Formalin-Induced Inflammation

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Authors' contributions

This work was carried out in collaboration between all authors. Author SS designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author FKD is assessed pathology preparations. Author MO helped during the experimental procedures. Author ONA helped during the experimental procedures. Author SS provided the surgical removal of the spleen and thymus. Author AK evaluated the biochemical data. All authors read and approved the study.

Research Article

ABSTRACT

In addition to well-known analgesic action of tramadol, its potential antinflammatory effects have not been thoroughly evaluated. On the other hand, effectiveness of antioxidants is also reported against inflammation. It is known that glyceryl trinitrate, as a

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nitric oxide donor, enhance the antioxidative and anti-inflammatory effects. In the present study, the efficacy of the tramadol mixtue with glyceryl trinitrate on cytokines, NF-kappa B expression and oxidative stress marker was examined on the formalin-induced inflammation in rats (Tramadol 5, 10 and 30 mg/kg + nitroglycerine 1 mg/kg). Cytokines (TNF-α, IL-6 and IL-10) and oxidative/anti-oxidative stress markers (MDA, GSH) were measured in blood samples. NF-kappa B expression was assessed immunohistochemically in spleen and thymus.

The results show that tramadol 30 mg/kg has both anti-inflammatory and anti-oxidative effects. Additionally, it was evidenced that glyceryl trinitrate improves the anti-inflammatory and anti-oxidative effects of Tramadol (30 mg/kg) on the formalin-induced inflammation in rats. In this framework, the present study provides a unique approach for the analysis of the efficacy of tramadol and additive effects of glyceryl trinitrate on the acute inflammations in rats.

1. INTRODUCTION

Tramadol is a centrally acting analgesic active substance with a double mechanism: it binds with low affinity to μ-opioid receptors and activates central monoaminergic pathways inhibiting the neuronal uptake of serotonin and noradrenaline. Although there are many studies about analgesic action of tramadol, its potential antinflammatory effects have not been thoroughly evaluated. Bianchi et al. [1] demonstrated the activity of tramadol may involve in the modulation of inflammatory mediators such as IL-6 in patients with knee osteoarthritis; norepinephrine is able to inhibit the secretion of proinflammatory mediators in synovial tissues of patients with osteoarthritis. Furthermore, Sacerdote et al. [2] found that tramadol can produce significant pain relief and suppress proinflammatory cytokine production in rats through serotonergic mechanism. It is also stated that the anti-inflammatory mechanism of Tramadol demonstrated in some models is not related to the direct inhibitory effect on the formation of prostanoids [3].

On the other hand, reactive oxygen species may cause inflammatory tissue injury; antioxidants have been reported to be effective against inflammation and acute inflammatory pain. Nuclear transcription factor kappa B (NF-kappa B) is a ubiquitous transcription factor involved in the immune and inflammatory response, however, reactive oxygen metabolites may also initiate and/or amplify inflammation via the up regulation of proinflammatory cytokines and NF-kappa B activation [4].

Glyceryl trinitrate, which is a nitric oxide donor, can also produce antioxidative affect [5,6]. In our previous study it was demonstrated that the addition of glyceryl trinitrate to lornoxicam for chronic treatments may prevent gastrointestinal and renal side effects and enhance antioxidative and anti-inflammatory effects, compared to the use of lornoxicam alone [7].

The present study concentrates on the combined effects of both tramadol and glyceryl trinitrate on cytokines, NF-kappa B expression and oxidative stress marker. The results indicate how the addition of glyceryl trinitrate changes the tramadol action in formalin-induced inflammation in rats. The approach in the present study is unique, to our knowledge,

as it specifically evaluates the anti-inflammatory effects of tramadol on inflammation when mixed with glyceryl trinitrate.

2. METHODS

2.1 Animals

All experimental protocols were approved by Animal Ethics Committee of Adnan Menderes University Medical Faculty. Wistar rats (48 male) weighting approximately 300-450 g were obtained from Adnan Menderes University Medical Faculty of Animal Research Laboratory Center (Aydin, Turkey). They were housed in polypropylene cages in groups of five per cage, and received standard laboratory chow and tap water ad libitum with 12/12 hours light/dark cycles.

2.2 Drugs

Tramadol (Contramal ®, Abdi Ibrahim, Istanbul, Turkey) and Glyceryl trinitrate (nitroglycerin, Perlinganit®, Adeka, Istanbul, Turkey) were used. Drug solutions were prepared so that the desired dose, expressed in terms of saline, was contained in a volume 10 ml kg−1 of body weight for each injection into intraperitoneal cavity.

Rats were randomly divided into 8 groups (n=6 for each group) and administered as follow: Group C (Control Group; isotonic NaCl 0.9%), Group T-5 (Tramadol 5 mg/kg), Group T-10 (Tramadol 10 mg/kg), Group T-30 (Tramadol 30 mg/kg), Group T5-N (Tramadol 5 mg/kg and Glyceryl trinitrate 1 mg/kg), Group T10-N (Tramadol 10 mg/kg and Glyceryl trinitrate 1 mg/kg), Group T30-N (Tramadol 30 mg/kg and Glyceryl trinitrate 1 mg/kg) and Group N (Glyceryl trinitrate 1 mg/kg). After the administrations, %5 formalin 50μl was injected into the dorsalsurface of the hind paw using a 27G needle.

Blood samples were collected from the rat tail under ether anesthesia before the drug injections and 30 minutes after the formalin injections in order to measure for cytokines and oxidative/anti-oxidative stress markers. Spleen and thymus were collected under anesthesia (Ketamine, 90 mg/kg+ Xylazine, 10 mg/kg; IM).

2.3 Biochemical Analysis

Blood samples from each rat were centrifuged at 1500 rpm for 15 minutes and the serum obtained was kept at -86ºC until the analysis.

2.3.1 Malondialdehyde (MDA) determination

Serum malondialdehyde concentration was measured, as an indirect marker of oxidative stress in terms of thiobarbituric acid reactive substances, spectrophotometrically [8]. Serum samples (0.125 ml) were mixed with 20% trichloroacetic acid (1.25 ml) and 0.67% thiobarbituric acid (0.5 ml). Mixture was then boiled at 95 ^{0}C for 30 minutes, immediately followed by cooling on ice. Reaction mixture was then vortexes, following the addition of n-Butanol (2ml). All vials were then centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was then measured at 535 nm. Concentration of lipid peroxidation products was calculated as malondialdehyde concentration using the extinction coefficient for malondialdehyde -thiobarbituric acid complex of 1.56 x 10^5 / mol/ cm.

2.3.2 Glutathione (GSH) determination

Reduced glutathione level was visually estimated regarding the reduction of DTNB (dithiobis-2-nitrobenzoic acid) that forms a yellow colored anion at 412 nm [9].

2.3.3 Interleukin (IL)-6, IL-10 and Tumor Necrosis Factor (TNF)-alpha analysis

Serum TNF-alpha and IL-6 concentrations were measured with Biosource enzyme-linked immunosorbent assay (ELISA) kit (for TNF-alpha Cat no: KRC3011; for IL-6, Cat no: KRC0061; BioSource Europe S.A.; Nivelles, Belgium). Results were expressed as pictograms in per milliliter (pg/ml) of serum.

## 2.4 Immunohistochemical Examination of NF-kappa B

For immunohistochemical evaluation, specimens were processed for light microscopy and sections incubated at 60ºC overnight and then de-waxed in xylene for 30 minutes. Following the rehydration in a decreasing series of ethanol, sections were washed with distilled water and PBS for 10 minutes.

Sections were then treated with 2% trypsin in 50 mMTris buffer (pH 7.5) at 37ºC for 15 minutes and washed with PBS. Then, sections were incubated with NF-κB/p65 (Rel A) Ab-1 (R-B-1638-R7, Neomarkers, Labvision, Fremont, California, USA). Ten micrometer sections were obtained using a cryostat. Sections prepared for each case of spleen and thymus was examined by light microscopy[10]. A minimum of 10 fields for each thymus and spleen slide were examined. Positive and negative controls were conducted in parallel with NF-κBstained sections. Staining of sections with commercially available antibodies served as the positive control. Negative controls included staining tissue sections with omission of the primary antibody (Fig. 1). Semiquantitative.analysis of the immunostaining results was performed. The percentage of positive cells was recorded as follows: 5% of cells, absent; 5–25% of cells, weak; 25–75% of cells, moderate and 75% of cells, intense. For statistical analysis, absent and weak group classified as negative, moderate and intense groups classified as positive [11,12].

2.5 Statistical Analysis

Data are reported as the mean ± SD when appropriate (95% confidence interval). Distribution of the groups was analyzed with the Kolmogorov-Smirnov one-sample test. Differences between means were evaluated by one-way analysis of variance test. Bonferroni's test was applied to perform the pair wise comparison of every combination of group pairs. Immunohistochemical evaluations of the groups were carried out by the chi-square test.

All analyses were conducted in SPSS 13.0 (SPSS Inc., Chicago, IL). Statistical significance was accepted as $P < 0.05$.
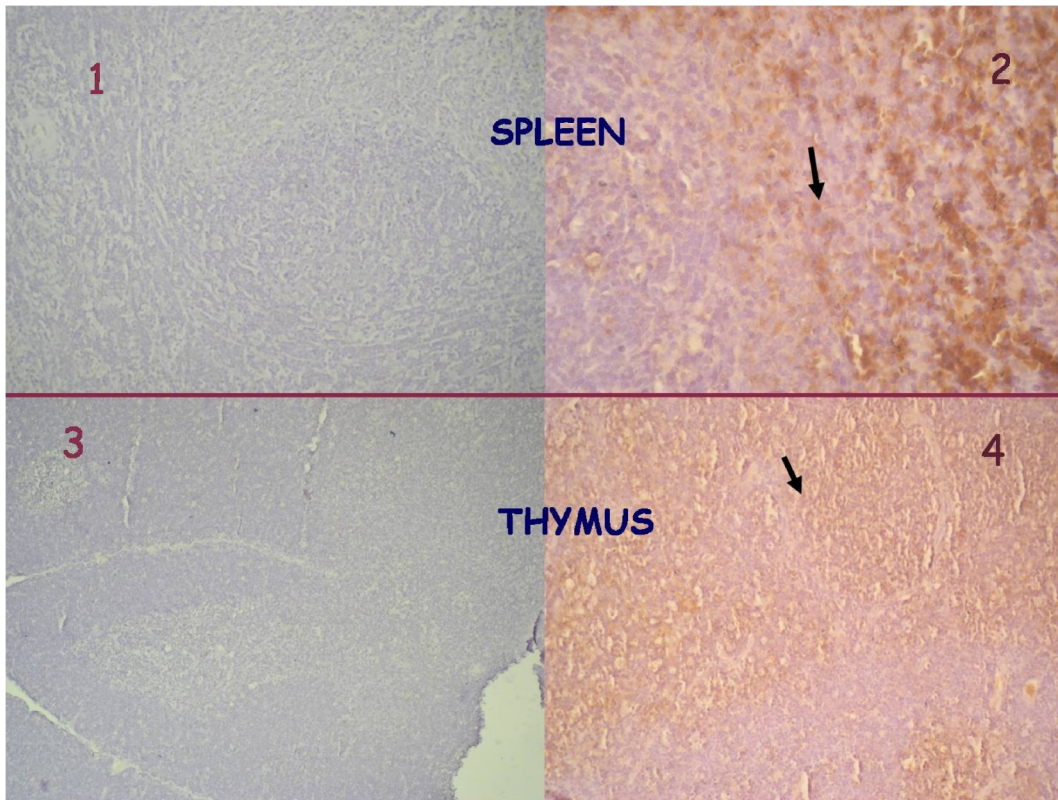
Fig. 1. NF-kB immunohistochemically activation in spleen (1 and 2) and thymus (3 and 4). Arrows show positive activation
(Magnification, ×20 or 40 in spleen and ×10 or 20 in thymus).
1: Spleen; NF-kBx20, negative , 2: Spleen; NF-kBx40, positive
3: Thymus; NF-kBx10, negative, 4: Thymus; NF-kBx20, positive

3. RESULTS

3.1 Biochemical Results

There was no significant difference between the groups regarding the baseline values (before drug injection) of TNF-α, IL-6 and IL-10 (Table 1). After the drug injections, TNF-α and IL-6 levels were increased and IL-10 levels were decreased in the Groups T5, T10, T5N, T10-N as well as in the Control Group (Table 1), compared to the baseline values (P<0.05).

In the Groups T30 and T30-N, TNF-α (P=0.032 and P=0.028 respectively) and IL-6 (P=0.041 and P=0.035respectively) levels were lower, while IL-10 (P=0.034 and P=0.026 respectively) levels were higher after formalin injections, compared to Group C. The results of TNF-α, IL-6 and IL-10 levels are summarized in Table 1.

Table 1. Changes in TNF-alpha, IL-6 and IL-10 levels before drugs administration and after formalin injection (n=6, for each groups)

	TNF-α (pg/mL)		IL-6 (pg/mL)		IL-10 (pg/mL)	
	Before	After	Before	After	Before	After
Group C	21.8±4.2	46.5±6.9	49.3±5.2	88.5±8.3	3.9±0.3	7.4±4.8
Group T5	23.5±5.1	44.3±3.8	47.4±3.4	87.7±7.2	4.0±0.4	9.6±7.0
Group T10	22.3±6.2	44.4±5.7	46.2±4.4	81.6±7.1	3.7±0.3	8.7±5.5
Group T30	21.6±4.2	35.3±3.4*	46.4±5.1	57.3±5.2*	3.8±0.4	19.4±7.7*
Group T5-N	24.2±7.3	43.8±3.9	47.3±5.9	86.3±7.7	3.6±0.6	8.2±5.7
Group T10-N	22.8±4.7	42.2±4.4	47.6±5.4	82.3±5.2	3.9±0.4	9.8±6.5
Group T30-N	24.2±4.3	30.6±4.4**,#	48.3±6.3	50.3±6.5**,#	3.7±0.5	26.4±7.3**,#
Group N	23.4±7.5	44.2±5.3	49.6±6.2	89.3±7.1	3.8±0.2	8.6±5..8

Values are means ± SD
*P<0.05 vs group control; **P<0.01 vs group control;
#P<0.05 compared to Group T30-N and Group T30

Regarding the malondialdehyde (MDA) and glutathione (GSH) levels after formalin injections, there was no significant difference observed between the Groups T5, T10, T5N, T10-N and the Control Group. In the Groups T30, T30-N and N, lower MDA levels (P=0.033, P=0.019 and P=0.048 respectively) and higher GSH levels (P= 0.041, P=0.001 and P=0.046 respectively) were observed than the levels in the Control Group. In comparison with the Group T30, lower MDA levels (P=0.045) and higher GSH levels (P=0.026) were measured in the Group T30-N. The results of MDA and GSH levels after formalin injections are summarized in Table 2.

Table 2. Changes in MDA and glutathione (GSH) levels before drugs administration and after formalin tests (n=6, for each groups) (Results are presented as mean ± S.D.)

	MDA (nmol/mL)		GSH (µmol/g Hb)	
	Before	After	Before	After
Group C	0.53±0.06	0.56±0.07	3.31±0.47	3.32±0.51
Group T5	0.52±0.1	0.55±0.1	3.25±0.56	3.31±0.57
Group T10	0.52±0.06	0.54±0.08	3.29±0.34	3.32±0.43
Group T30	0.54±0.07	0.50±0.07*	3.33±0.38	4.07±0.44*
Group T5-N	0.52±0.05	0.54±0.09	3.34±0.45	3.34±0.51
Group T10-N	0.54±0.1	0.53±0.08	3.32±0.47	3.51±0.47
Group T30-N	0.53±0.08	0.48±0.09**,#	3.25±0.56	6.88±0.93**,#
Group N	0.52±0.05	0.52±0.03+	3.29±0.34	3.66±0.44+

Values are means ± SD
*P<0.05 vs group control; **P<0.01 vs group control;
#P<0.05 compared to Group T30-N and GroupT30;
+P<0.05 vs group control

3.2 The Results of Immunohistochemical Evaluation of NF-kappa B in Spleen and Thymus

NF-kappa B immunohistochemical activations in spleen and thymus were shown in Fig. 1. A significant difference was observed in the Group T30-N where Tramadol was administered

with Glyceryl trinitratee, compared to the Control Group. The significance levels were P=0.006 and P=0.004 for spleen and thymus respectively (Table 3).

Table 3. The number of NF-KB expression positive rats in spleen and thymus

The number of NF-KB Expression positive rats (n=6, for each groups)	Spleen	Thymus
Group C	6	6
Group T5	6	6
Group T10	5	6
Group T30	5	6
Group T5-N	6	6
Group T10-N	5	5
Group T30-N	1[*]	2[*]
Group N	5	5

[*]$P < 0.001$ vs Group C

4. DISCUSSION

The results of our study indicate that addition of glyceryl trinitrate to tramadol enhances the both antioxidative and anti-inflammatory effects of Tramadol alone.

Tramadol creates a positive impact on immune functions such as the natural killer cell activation and proliferation of lymphocytes are increased while the production of cytokines are decreased [2,13]. However, the mechanism of Tramadol on modulating cytokine production is unknown. Tramadol is able to reduce peripheral inflammation in rat's subcutaneous carrageenin-induced inflammation [13]. Similarly, Liu et al. [14] show that IL-6 levels were lowest when 20 mg/kg of Tramadol administered, compared to the other doses (1mg/kg and 10 mg/kg) in the incision pain model in rats. In the present study, inflammatory cytokines such as TNF-α and IL-6 levels are lower, while anti-inflammatory cytokine such as IL-10 levels are higher in T-30 group. Therefore it is concluded that tramadol in the dose of 30 mg/kg may exert better anti-inflammatory effect than the lower doses.

Antioxidant activity (glutathione) is also higher and oxidative stress marker (MDA) is lower in the Group T-30, compared to the Controls in the present study. Similar to our results, Bilir et al. [15] show that tramadol has anti-oxidative effects and provide cardio-protective effect against myocardial ischemia-reperfusion in isolated rat heart. In contrast to our results, Atıcı et al. [16] demonstrate the chronic Tramadol treatment does not change MDA levels. The reasons of the difference between two similar researches can be explained as follow: the chronic administration, the use of different doses and the exclusion of inflammation conditions versus acute administrations and inflammation conditions in our study.

Antioxidative effect of glyceryl trinitrate are mentioned in many studies [7,17,18]. Sokolowska et al. [18] demonstrate that the use of 2.5 mg/kg Glyceryl trinitrate administered via intraperitoneal route to rats for 5, 10 and 17 days participates in antioxidant reactions without any hypotensive effects. Similarly in the present study, MDA levels are lower and glutathione levels are higher after the formalin injection in the Group N (Glyceryl trinitrate 1 mg/kg), compared to the controls.

Nitric oxide (NO), which is produced from glyceryl trinitrate, also acts as an oxygen radical scavenger [5]. Furthermore, 'NO-releasing' NSAIDs (CINODs) exhibit similar or even greater effects on the inhibition of inflammation than those of their parent NSAIDs, in various acute and chronic animal models [19]. In the present study, the use of glyceryl trinitrate with tramadol (in T-30N group) has similarly produced more antioxidative and anti-inflammatory effects compared to the use of tramadol alone (in T-30 group), which has been confirmed by the increase of glutathione and IL-10 levels.

The relationship between Tramadol and nitric oxide are often mentioned in the literature. The results of some studies support the involvement of the L-arginine/nitric oxide pathway in the antinociceptive and antidepressant-like effects of Tramadol in the rat model [20,21].

Nuclear factor-kappa B (NF-kappa B) plays an important role in inflammation, proliferation and regulation of apoptosis. NF-kappa B exists in a latent form in the cytoplasm of unstimulated cells comprising a transcriptionally active dimer bound to an inhibitor protein, IkB. Activation of NF-kappa B involves the phosphorylation and subsequent degradation of an inhibitory protein, IkB, and recently many of the proximal kinases and adaptor molecules involved in this process have been elucidated. Additionally, we now understand in detail the NF-kappa B activation pathway from cell membrane to nucleus for interleukin-1 (IL-1) and tumour necrosis factor (TNF) [22].

On the other hand, it is clear that the activity of NF-kappa B is under the control of an oxidant/antioxidant balance and anti-oxidants are demonstrated inhibiting the activation of NF-kappa B. Additionally, specific examinations indicate that some antioxidants such as vitamin E derivatives, N-acetyl-L-cysteine (NAC) and a-lipoic acid inhibit the NF-kappa B activation in some cell types. It is suggested that the effects of anti-oxidants on NF-kappa B activation are stimulus- and cell-specific [23,24]. Zhang and Guo [25] indicate tramadol may protect myocardium against acute myocardial ischaemic injury, which may be associated with the expression and activation of NF-kappaB. Yet, the result of tramadol administered alone in a dose of 30 mg/kg in our study has not significantly indicated the inhibition of NF-kappaB. But, the addition of glyceryl trinitrate to tramadol (30 mg/kg) has resulted the reduction in the NF-kappa B activation in spleen and thymus. It is remarkable that glyceryl trinitrate administered alone has not caused reduction in the NF-kappa B activation. We can interpret this result as follow: Glyceryl trinitrate may have increased anti-oxidative effect, therefore, it enhanced anti-inflammatory effect of tramadol through NF-kappa B mechanism.

The limitations of the present study are also worth to be considered for further research in the same field. Firstly, the present study concentrates on the inflammatory conditions only. As the research was specifically aimed at enhancing anti-inflammatory effects of tramadol, non-inflammatory conditions are not included in the analysis. Considering the unique insight in the present study, this shortcoming was accepted to be fulfilled in the future studies. Secondly, the inflammations are induced with plantar formalin injections for the analysis of the acute reactions only. According to the formalin test for the pharmacological analysis of the drug mechanisms, primary (neuronal effect, 0-5 minute) and secondary (inflammatory effect, 25-35 minutes) stage responses after the plantar formalin treatments are taken into account [26]. In this framework, the research was planned to evaluate the interference of a systemic substance on an acute inflammation. Therefore, the present study has analysed the first 30 minute period. In future research for a detailed analysis in this field, parental application of the inflammatory agent and recordings in 1 and 2 hours periods would provide information on the systemic effects. The final remark is that the doses administered in the

present study are in the range of 5-30 mg/kg which is widely used tramadol doses in research studies [13,14,27,28]. On the other hand, it was not possible to prepare a correlation curve for the dose/effect. Increasing the number of doses in similar range may achieve more distinct results as far as the efficacy is concerned.

5. CONCLUSION

We conclude that antioxidative effect of tramadol has also a role in its already known anti-inflammatory mechanisms. If these findings can be confirmed clinically, the additive effects of tramadol and glyceryltrinitrate together may offer several advantages over other pharmacological agents (such as NSAID) for acute or chronic inflammatory condition.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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