# Anti-inflammatory effects of riboflavin and morphine on zymosan-induced peritonitis in Swiss mice

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***Abstract***

*We have previously shown that in several strains of mice morphine abolishes inflammation-related pain symptoms already at 5 mg/kg, but inhibits inflammation only at 20 mg/kg. Other investigators have shown that also riboflavin (vitamin $B_2$) has antinociceptive and anti-inflammatory activities. In particular, highly purified riboflavin is a promising therapeutic agent for both bacterial infections and toxin-induced septic shock, mainly due to inhibition of pro-inflammatory cytokines/chemokines and nitric oxide production. The aim of present study was to examine anti-inflammatory effects of morphine, riboflavin and combined action of the both agents injected intraperitoneally (i.p.) 30 min before zymosan treatment in Swiss mice. Animals pre-injected with PBS formed the control group. At $4^{th}$ hour after PBS-Zymosan injection, the number of peritoneal PMNs reached $20 \times 10^6$. The number of PMNs was similar in animals pre-injected with low dose of morphine (5 mg/kg b.w.) or riboflavin (20 and 50 mg/kg b.w.) while intraperitoneal influx of PMNs was significantly inhibited in animals pre-injected with high dose of morphine (20 mg/kg b.w.) or riboflavin (100 mg/kg b.w.). Similar anti-inflammatory effect was achieved by pre-injection with the combined low dose of morphine (5 mg/kg b.w.) with riboflavin (50 mg/kg b.w.).*

***Key words:*** *peritonitis, inflammation, morphine, riboflavin, zymosan, cytokine, Swiss mice.*

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## Introduction

In several experimental models it has been shown that riboflavin (vitamin $B_2$) has antinociceptive [1, 2] and anti-inflammatory activities [1, 3-7]. In particular, application of highly purified vitamin $B_2$ is a promising therapeutic strategy for gram-positive and gram-negative bacterial infection, exotoxin or LPS-induced septic shock via inhibition of proinflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ), chemokines (MCP-1, MIP-2), and nitric oxide (NO) production [3-6]. Especially, vitamin $B_2$ protected animals against mortality due to *Eschericha coli*, *Staphylococcus aureus*, LPS or SEB injection [4]. The therapeutic effect of riboflavin was enhanced by its supplementation with amino acids, e.g. the dose of 2,5 mg/kg of riboflavin improved the survival rate when supplemented with 100 mg/kg of valine [5]. Moreover, riboflavin markedly enhances antinociceptive effects of morphine when administrated with its ineffective dose (2 mg/kg b.w.) [1].

The results of our previous investigations revealed that morphine co-injection with zymosan (yeast agent), not only abolished inflammation-related pain symptoms but, at the high dose (20 mg/kg b.w.), had also anti-inflammatory properties in several strains of mice via inhibition of influx of exudatory polymorphonuclear leukocytes (PMNs) [8]. Therefore the aim of further studies is to combine the appropriate doses of morphine and riboflavin to achieve both antinociceptive and anti-inflammatory effects on zymosan-induced peritonitis.

The aim of the present work was to study modulatory effects of morphine and/or riboflavin applied before ip

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zymosan injection on the pain symptoms and intaperitoneal accumulation of exudatory cells and inflammation-related cytokines/chemokies.

## Material and Methods

### Animals

Adult male Swiss mice (6-7 week-old, 25-30 g), were purchased from the Unit of Laboratory Animals (Collegium Medicum, Jagiellonian University, Kraków, Poland). All mice were housed 5 per cage in cages 20 × 13 × 18 cm under strictly controlled conditions (free access to food and water, 12-hr dark-light cycle, temperature 22°C). All procedures were approved by the Local Committee on Animal Care of the Jagiellonian University (license no. 23/OP/2005).

### Experimental model of zymosan induced peritoneal inflammation

After one-week adaptation to the laboratory conditions, the animals were divided into experimental groups (5-6 animals per group). Inflammation was induced by intraperitoneally (*i.p.*) injection of 0.5 ml/25 g b.w. zymosan A (Z40, 40 mg/kg b.w.) (Sigma, London, UK). Half an hour before zymosan injection (time – 30 min), mice received 0.9% PBS (Polfa, Kutno, Poland) or highly purified vitamin $B_2$ (riboflavin 5'-sodium phosphate; purity >97%) (R, 50 or 20 mg/kg b.w.) (Sigma, London, UK) or morphine hydrochloride (M, 5 mg/kg b.w.) (Sigma, London, UK). Some mice received combined doses of morphine (5 mg/kg b.w.) with riboflavin (50 or 20 mg/kg b.w.). Control animals were injected with PBS only. During the first 45 min after injection, body writhes (pain symptoms) were counted as previously described [8]. At the 4$^{th}$ hour of peritonitis animals were sacrificed by decapitation and peritoneal exudate (fluid and leukocytes) were retrieved by lavaged of peritoneal cavity with 1 ml of sterile PBS. Peritoneal leukocytes (PTL) and, among them, polymorphonuclear leukocytes (PMN) were stained with Türk solution (0.01% crystal violet in 3% acetic acid) and counted in a hemocytometer. The retrieved fluid was centrifugated at 1500 rpm for 10 min at 4°C and stored at -20°C until further analyses.

### Measurement of cytokines/chemokines by cytometric bead array

Cytometric Bead Array (Mouse Inflammation kit, CBA; BD Biosciences) was used to study cytokines and chemokine levels in peritoneal fluid frozen prior to analysis (-20°C). The mouse inflammation kit was used according to the manufactures instruction to measure simultaneously the levels of mouse IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 on a FACScan cytometer (FACSCaliburTM flow cytometer, BD Biosciences). Following acquisition of data by two-colour cytometric analysis, the results were analysed using CBA software (BD Biosciences). The range of detection for each cytokine measured by CBA kit was 20-5000 pg/ml. For that the samples were diluted (1:5) to ensure that their mean fluorescence values fall within the standard curves. The sensitivities of the CBA kit for IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 are 5.0, 17.5, 52.7, 2.5, 7.3, and 10,7 pg/ml, respectively.

### Statistical analysis

The data were analyzed using the Tukey's Test. The level of statistical significance was set at 0.05. All results are expressed as mean ± standard error (X ±SE).

## Results

Swiss mice *i.p.* injected with pure riboflavin exhibited several body writhes with a stretching of hind limbs (data not shown), considered to be the pain symptoms, common at the early stages of zymosan-induced peritonitis [8]. Similar injection of intact Swiss mice with PBS or morphine had no such effects. In the present paper we focus an antinociceptive activity of these factors on peritonitis induced 30 min later by *i.p.* zymosan injection (Figure 1, open bars). Body writhes were counted between 0 and 45 min after zymosan injection. The animals with zymosan-induced peritonitis preceded by PBS exhibited up to 10 pain symptoms mainly during the first 30 min. Pain symptoms were in practice absent in animals preinjected with morphine, either at 5 or 20 mg/kg. Low dose of riboflavin (20 mg/kg b.w.) did not reduce the number of body writhes while higher doses (50 and 100 mg/kg b.w.) caused significant reduction of pain symptoms. Addition of a low dose of morphine to riboflavin (M5R20 and M5R50) caused almost complete elimination of pain (Figure 1, open bars).

Intact mice had a low number of peritoneal leukocytes without PMNs among them (data not shown), while *i.p.* zymosan injection [8], or that preceded by PBS, induced a massive influx of immunocompetent cells, especially PMNs (up to 20 × $10^6$) into peritoneal cavity (Figure 1, solid bars). Preinjection with morphine at the low dose (M5) had no effect on the number of zymosan-induced PMNs, but morphine at the high dose (M20) inhibited PMN influx significantly. Preinjection with riboflavin at 20 or 50 mg/kg b.w. (R20, R50) had no anti-inflammatory effects, while riboflavin at 100 mg/kg inhibited PMN influx significantly, similarly to effects of a high dose of morphine. Addition of the low dose of morphine to riboflavin had anti-inflammatory effects only at the M5R50 dose combination.

The effects of morphine plus riboflavin pre-treatment on cytokine/chemokine levels at 4$^{th}$ hour of zymosan-induced peritonitis are shown on Figure 2. In comparison with intact animals, zymosan injection preceded by PBS induced prominent elevation of all analysed cytokines (TNF-α, IL-12p70, IFN-γ, IL-6, MCP-1, and IL-10) (data not shown). However, in animals from M5R20 or M5R50 groups, profound alterations of peritoneal cytokine profile

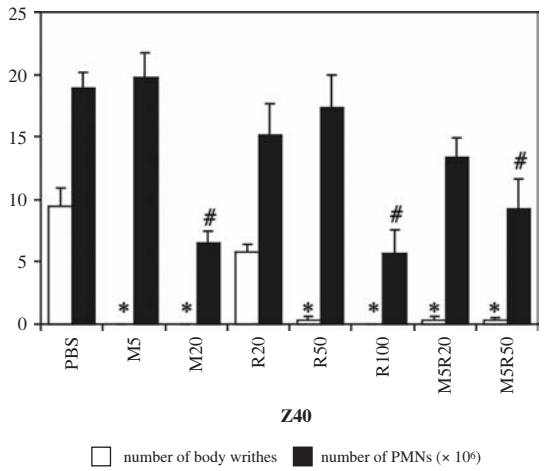


**Fig. 1.** Effects of morphine and/or riboflavin on pain symptoms (body writhes) and intraperitoneal PMN accumulation during zymosan-induced peritonitis in males of Swiss mice. 30 min before zymosan injection (40 mg/kg, Z40) animals were *i.p.* injected with PBS (controls), morphine at 5 or 20 mg/kg (M5, M20), riboflavin at 20, 50, or 100 mg/kg (R20, R50, R100) or morphine with riboflavin (M5R20, M5R50). Number of writhes were counted during the first 45 min of peritonitis (open bars); numbers of PMNs were counted at 4th hour of peritonitis (solid bars). Means ±SE, n=5-8

**, # means significantly different (according to Tukey's test) from the mean of the group pretreated with PBS (P<0.05)*

were observed. In particular, co-administration of morphine and riboflavin caused a significant decrease of the levels of early proinflammatory cytokines: TNF-α, IL-12p70 and IFN-γ. Only higher dose of riboflavin in M5R50 group caused decrease of IL-6 and MCP-1, while the level of anti-inflammatory IL-10 remained unaffected.

## Discussion

The results described by other investigators have shown that combination of riboflavin (25 mg/kg b.w.) with otherwise ineffective dose of morphine (2 mg/kg b.w.) induced an antinociceptive effect in the formalin test, comparable to that achieved by a high dose of morphine (8 mg/kg b.w.) [1].

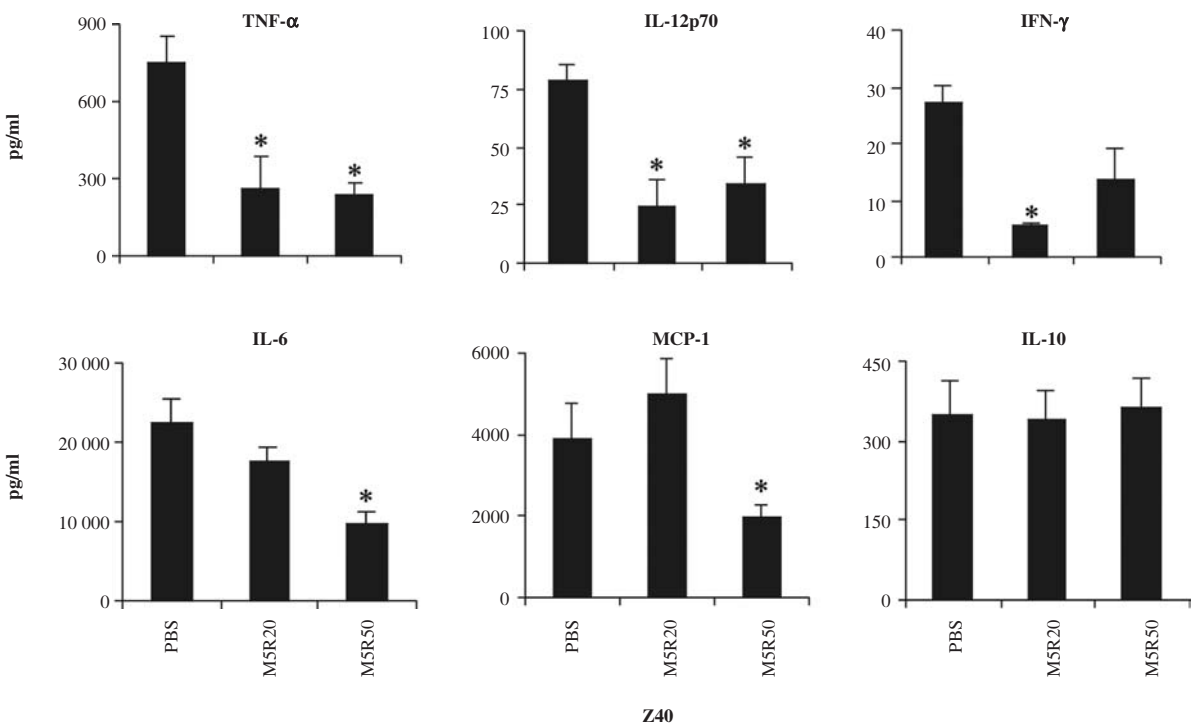


**Fig. 2.** Effects of morphine with riboflavin (MR) on cytokine/chemokine levels in peritoneal fluid at 4th hour of zymosan-induced inflammation (40 mg/kg b.w., Z40) in males of Swiss mice. 30 min before zymosan injection, animals were preinjected either with PBS (controls) or with morphine (M, 5 mg/kg) with riboflavin (R, 20 or 50 mg/kg). Means ±SE, n=4

**means significantly different (according to Tukey's test) from the mean of the group pretreated with PBS (P<0.05).*

As shown on Figure 1, in our experimental model, morphine at 5 mg/kg b.w. abolished pain symptoms but did not reduce the leukocyte influx, while at 20 mg/kg b.w. morphine had both antinociceptive and anti-inflammatory effects. The latter effects were achieved at the massive dose of riboflavin, 100 mg/kg. Lower doses of riboflavin had no anti-inflammatory effects but 50 mg/kg b.w. was antinociceptive. The low dose of morphine, 5 mg/kg b.w., inefficient per se, enhanced antinociceptive effects of riboflavin at 20 mg/kg b.w., while only combination M5R50 had both antinociceptive and anti-inflammatory effects.

Preliminary investigations on mechanisms of anti-inflammatory effects of combined action of both drugs strongly indicated that they act through inhibition of early proinflammatory cytokines, such as TNF-α, IL-12p70, IFN-γ, and facilitation of resolution of inflammation, as indicated by the relatively high level of anti-inflammatory IL-10 (Figure 2). Experiments on kinetics of proinflammatory and anti-inflammatory cytokines at various stages of inflammatory process are in progress. Anti-inflammatory effects of riboflavin applied at 6$^{th}$ hour of LPS-induced septic shock were described by Toyosawa et al. [4-6], who recorded decreased levels of TNF-α, IL-1β, MCP-1, IL-6 and nitric oxide. Also Kodama et al. [3] in mice with LPS induced sepsis recorded decreased levels of IL-6, MIP-2 after intravenous riboflavin injection.

Thus riboflavin, which is a safe drug already approved for clinical use, can exacerbate the antinociceptive and anti-inflammatory effects of morphine in numerous experimental models. Therefore it seems that this vitamin may represent an alternative to the high doses of analgesic opioids and, consequently, their side effects. Nevertheless, as the mechanisms of riboflavin and morphine action are poorly understood the successive studies will be necessary for explanation of the underlying molecular mechanisms.

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