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# **The Effects of $\beta$ -glucan isolated from *Pleurotus ostreatus* on Methotrexate Treatment in Rats with Adjuvant Arthritis.**

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**Short running title:** Effects of  $\beta$ -glucan on methotrexate treatment.

## Abstract

The purpose of this study was to evaluate the effect of  $\beta$ -(1,3/1,6)-D-glucan isolated from *Pleurotus ostreatus* ( $\beta$ -glucan-PO) on prophylactic treatment of adjuvant arthritis (AA) with methotrexate (MTX) in rats. Groups of rats with AA were treated with methotrexate (1 mg/kg/week),  $\beta$ -glucan-PO (1 mg/kg every second day) or their combination for the period of 28 days from adjuvant application. Body mass, hind paw swelling, arthrogram scores and a level of serum albumin were measured as markers of inflammation and arthritis. Treatment with low dose of MTX significantly inhibited the markers of both inflammation and arthritis. MTX and its combination with  $\beta$ -glucan-PO significantly increased body mass of arthritic rats.  $\beta$ -glucan-PO administered alone significantly decreased both the hind paw swelling and arthritic score. In combination with MTX,  $\beta$ -glucan-PO markedly potentiated the beneficial effects of MTX, which resulted in a more significant reduction of hind paw swelling and arthritic scores. The concentration of albumin in the serum of arthritic controls was significantly lower than in healthy controls. Both MTX alone and the combination treatment with MTX +  $\beta$ -glucan-PO significantly inhibited the decrease in serum albumin.  $\beta$ -glucan-PO increased the treatment efficacy of basal treatment of AA with MTX.

**Key words:**  $\beta$ -glucan, methotrexate, adjuvant arthritis.

## Introduction

Poly-branched  $\beta$ -1,3-(D)-glucans are naturally occurring polysaccharides, with or without  $\beta$ -1,6-(D)-glucose side chains, that are integral cell wall constituents in a number of bacteria, plants and yeasts.  $\beta$ -glucans from various sources are different in their structure, chemical, physical and biological properties (1). Moreover, they represent the conserved structure - pathogen-associated molecular pattern (PAMP) and are effective biological response modifiers, non-specifically enhancing the host immune system by multiple interactions within innate and adaptive mechanisms (2). The induction of cellular responses by  $\beta$ -glucans is likely to involve their specific interactions with several cell surface receptors, as complement receptor 3, lactosylceramide, selected scavenger receptors, and dectin-1 (3, 4, 5).  $\beta$ -Glucans increase host immune defense by activating the complement system, enhancing the function of macrophages, leucocytes and natural killer cells. The use of  $\beta$ -glucans alone or as vaccine adjuvants for viral and bacterial antigens has been shown in animal models to increase resistance to a variety of bacterial, fungal, protozoan and viral infections (6, 7, 8).  $\beta$ -Glucans also show anticarcinogenic activity. Its use as adjuvant to cancer chemotherapy and radiotherapy demonstrated the positive role in the restoration of hematopoiesis following bone marrow injury (9, 10, 11).

$\beta$ -(1,3/1,6)-D-glucan, is an insoluble polysaccharide isolated from the mushroom *Pleurotus ostreatus*. It is a safe and potent nutritional supplement with a profound systemic effect that can be described as nonspecific immune stimulation combined with antioxidant activity (11, 12, 13). Recently, Smiderle et al. (14) described the antiinflammatory and analgesic activity of  $\beta$ -(1,3/1,6)-D-glucan isolated from *Pleorutus ostreatus* on the acetic acid-induced writhing reaction in mice, a typical model for quantifying inflammatory pain. The authors suggested that the glucan had potent anti-inflammatory and analgesic activities, possibly due to the inhibition of pro-

inflammatory cytokines. In our previous studies with application of  $\beta$ -(1,3/1,6)-D-glucan isolated from *Pleurotus ostreatus* to rats with adjuvant arthritis we showed decreased activities of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 and IL-6 in the serum of arthritic rats, decreased oxidative stress and suppressed inflammatory and arthritic signs in rats (15, 16). Protective antioxidant activity and anti-inflammatory activities of carboxylated (1-3)-beta-D-glucan isolated from *Saccharomyces cerevisiae* were reported in adjuvant arthritis in Lewis rats (17).

Methotrexate is an antifolate that is widely used in the treatment of rheumatic disorders and malignant tumors. The efficacy of methotrexate is often limited by severe side effects, which includes also the development of oxidative stress. Sener et al. (18) showed that  $\beta$ -glucan can ameliorate the methotrexate-induced oxidative organ injury (liver or kidney) in rats via its antioxidant and immunostimulatory effects.

Our previous results showed the beneficial effects of  $\beta$ -(1,3/1,6)-D-glucan in rat adjuvant arthritis, the aim of the present study was to evaluate its effect on methotrexate treatment of rats with adjuvant arthritis.

## **Materials and methods**

*Materials.* In this study methotrexate injection solution 10 mg/ml in sterile saline from Medac Company, Hamburg, Germany was used. Beta-(1,3/1,6)-D-glucan is insoluble micronized pure compound isolated from *Pleurotus ostreatus* ( $\beta$ -glucan-PO). Beta-(1,3/1,6)-D-glucan (Imunoglukan®) was obtained from Pleuran s.r.o. company (Bratislava, Slovakia). *Mycobacterium butyricum* was purchased from Difco Laboratories Co. Ltd. (Detroit, USA) and incomplete Freund's adjuvant from Sigma-Aldrich Chemie GmbH (Germany).

*Animals.* Male Lewis rats (160 - 180 g) obtained from Charles River Wiga, Germany were maintained during the experiment in standard animal facilities that comply with European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The animals were fed pelleted food (TOP DOVO, Dobrá Voda, Slovak Republic) and had free access to both food and water. The State Veterinary Committee of the Slovak Republic and the Ethics Committee for Control of Animals Experimentation at the National Institute of Rheumatic Diseases approved the experimental protocol and all procedures.

*Induction of arthritis.* The rats were injected with 0.1 ml suspension of heat killed *Mycobacterium butyricum* (12 mg/ml) in incomplete Freund's adjuvant intradermally at the base of the tail.

*Treatment.* MTX and  $\beta$ -glucan-PO were administered in corresponding doses from day 0 (the day of immunization) to day 28 of the study. MTX was prepared by dilution with sterile saline to yield the desired concentration of 0.5 mg in 0.1 ml saline, and applied twice a week *per os* (1 mg/kg in total per week).  $\beta$ -glucan-PO was administered orally as suspension in saline every second day in dose 1 mg/kg body mass. The untreated groups received the vehicle (sterile saline) in the same manner daily for 28 days.

The animals were divided into the following five groups of 8 animals: group 1 - non-arthritic untreated healthy controls; group 2 - untreated rats with AA; group 3 - AA rats treated with  $\beta$ -glucan-PO; group 4 - AA rats treated with MTX; group 5 - AA rats treated with the combination of MTX +  $\beta$ -glucan-PO.

*Evaluated Parameters*

Body mass of rats was measured at the beginning of study and every week during the study.

Hind paw swelling. The volume of the hind paw swelling was measured with an electronic water plethysmographically (UGO BASILE, Comerio-Varese, Italy) on days 14, 21 and 28.

Arthrogram score. The severity of arthritis was quantified by scoring each paw from 0 to 5, based on increasing levels of swelling and periarticular erythema. The sum of the scores for the limbs was calculated as the arthritic index, with a maximum possible score of 20 per rat. Arthrogram scores were evaluated on days 14, 21 and 28.

Serum albumin levels were measured on days 14, 21 and 28 in the rat serum by spectrophotometric method, using SYS 1 kit (BM/Hitachi, Boehringer Mannheim, Germany) on a Hitachi 911 automatic biochemical analyzer.

Statistical analysis of the results. One-way analysis of variance (ANOVA) was used for statistical analysis of the results, and  $p < 0.05$  was considered as the significance limit for all comparisons.

## **Results**

*Body weight.* In the first 7 days of the treatment, the increment in body weight was similar in all groups of rats (Table 1). However, on day 14, the body mass of arthritic control rats and rats treated with  $\beta$ -glucan-PO alone was significantly lower than that of the healthy controls and arthritic rats treated with MTX and with combination of MTX +  $\beta$ -glucan-PO. The increase of body mass in rats treated with combination MTX +  $\beta$ -glucan-PO was similar to that treated with MTX alone.

*Hind paw swelling, arthrogram score.* The clinical signs of arthritis reflect both inflammatory and arthritic changes occurring in rats with AA. The volume of the swollen hind paws in arthritic rats was significantly higher compared to healthy controls on days 14, 21 and 28, as supported by the mean value for two hind paws (Table 2). Statistically significant decreases of both hind paw swelling and arthrogram scores were observed in the arthritic rats treated with MTX on post-immunization days 14, 21 and 28 (Table 3).  $\beta$ -glucan-PO administered alone significantly decreased both the hind paw swelling and arthritic score on day 21 and 28. The combination treatment MTX +  $\beta$ -glucan-PO reduced these parameters statistically more significantly than MTX treatment alone (MTX vs. MTX+  $\beta$ -glucan-PO,  $P < 0.05$ ).

*Serum albumin levels.* Serum albumin acts as a negative acute phase reactant in both rat and human arthritis. Lower levels of serum albumin correspond to higher levels of inflammatory activity. The concentration of albumin in the serum of arthritic controls was significantly lower than in healthy controls (HC vs. AA rats,  $p < 0.001$ ). Both MTX alone and the combination treatment with MTX +  $\beta$ -glucan-PO significantly inhibited the decrease in serum albumin (Table 4).

## **Discussion**

This experiment was focused on the effect of  $\beta$ -(1,3/1,6)-D-glucan isolated from *Pleurotus ostreatus* on the inflammatory and arthritic markers in rats with AA during basal treatment with MTX. The treatment was prophylactic, which means that the animals were treated immediately after administration of the adjuvant.

The results of our investigation confirmed the previously reported effect of MTX treatment in rats with AA (19, 20). Methotrexate at a dose of 1 mg/kg/week



suppressed, but did not prevent, arthritis development. In our study, MTX significantly suppressed the hind paw swelling and decreased arthrogram scores.  $\beta$ -glucan-PO alone decreased both the hind paw swelling and the arthrogram on days 21 and 28. The remarkable finding was that  $\beta$ -glucan-PO potentiated the beneficial effect of MTX; reduction of hind paw swelling and arthrogram scores on days 21 and 28 were more significant compared to the rats treated with MTX alone.

Serum albumin acts as a negative acute-phase reactant in rat arthritis. Decreased levels of serum albumin reflect the changes in synthesis of this protein in the liver secondary to the activation of hepatic cells by inflammatory cytokines, mainly interleukin-1 (20). Our results correlate with the observation that MTX markedly prevents the albumin decrease in AA rats. The combination of MTX with  $\beta$ -glucan-PO had no additional effect compared to MTX alone (Table 4).

Systemic administration of  $\beta$ -glucan to rats and mice has been demonstrated to protect against various infections by activation of macrophages and attenuation of pro-inflammatory cytokine release (13, 21, 22, 23). Hetland et al. (24) have showed that  $\beta$ -glucan reduced growth of *Mycobacterium tuberculosis* in macrophage cultures and had protective effect against *Mycobacterium bovis*, BCG infection in BALB/c mice (25). Certain microbes, such as fungi and viruses led to generation and activation of autoimmune T cells resulting in a development of a particular autoimmune disease in genetically susceptible individuals.  $\beta$ -(1,3/1,6)-D-glucan, an effective activator of immune system may be beneficial also in humans in preventing or eliminating bacterial infections which are known to induce reactive arthritis.

In our study we tested a food supplement, the commercially used  $\beta$ -glucan isolated from *Pleurotus ostreatus*. This  $\beta$ -glucan decreased the arthritis development in rats and had additional beneficial effect to methotrexate treatment.

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**Table 1. The effect of MTX,  $\beta$ -glucan-PO, and their combination on body mass of rats (g).**

Groups of rats	Day 1	Day 7	Day 14	Day 21	Day 28
Healthy controls	167 $\pm$ 6	219 $\pm$ 13	224 $\pm$ 13 **	239 $\pm$ 14 ***	250 $\pm$ 15 ***
Untreated AA controls	170 $\pm$ 8	217 $\pm$ 12	186 $\pm$ 11	179 $\pm$ 15	199 $\pm$ 13
AA rats treated with:					
$\beta$ -glucan-PO	171 $\pm$ 6	217 $\pm$ 7	193 $\pm$ 10	184 $\pm$ 9	212 $\pm$ 12
MTX-2	173 $\pm$ 6	227 $\pm$ 7	228 $\pm$ 17 ***	236 $\pm$ 14 ***	247 $\pm$ 15 ***
MTX-2+ $\beta$ -glucan-PO	171 $\pm$ 6	228 $\pm$ 8	231 $\pm$ 14 ***	230 $\pm$ 13 ***	244 $\pm$ 14 ***

Data represent mean value and standard deviation (mean value  $\pm$  SD) for groups of 8 rats.

Significantly different from arthritic control rats: \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

**Table 2. The effect of MTX,  $\beta$ -glucan-PO and their combination on hind paws swelling (mL) in AA rats**

Groups of rats	Day 14	Day 21	Day 28
Healthy controls	1.36 $\pm$ 0.04 ***	1.40 $\pm$ 0.02 ***	1.41 $\pm$ 0.05 ***
Untreated AA controls	2.25 $\pm$ 0.20	2.51 $\pm$ 0.18	2.34 $\pm$ 0.15
AA rats treated with:			
$\beta$ -glucan-PO	2.05 $\pm$ 0.14	2.31 $\pm$ 0.09 *	2.16 $\pm$ 0.24 *
MTX-2	1.85 $\pm$ 0.23 **	2.16 $\pm$ 0.22 **	2.04 $\pm$ 0.30 **
MTX-2 + $\beta$ -glucan-PO	1.73 $\pm$ 0.22 ***	1.94 $\pm$ 0.37 ***†	1.88 $\pm$ 0.28 ***†

Data represent mean value and standard deviation (mean value  $\pm$  SD) for groups of 8 rats.

Significantly different from arthritic control rats: \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001.

Significantly different from arthritic rats treated with MTX: † $p$ <0.05

**Table 3. The effect of MTX,  $\beta$ -glucan-PO and their combination on the arthrogram score in AA rats.**

Groups of rats	Day 14	Day 21	Day 28
Untreated AA controls	13.44 $\pm$ 1.81	17.22 $\pm$ 1.99	14.89 $\pm$ 2.26
AA rats treated with:			
$\beta$ -glucan-PO	11.30 $\pm$ 1.46	14.25 $\pm$ 0.53 *	12.14 $\pm$ 2.10 *
MTX-2	8.50 $\pm$ 1.52 **	13.33 $\pm$ 3.44 **	11.00 $\pm$ 2.53 *
MTX-2+ $\beta$ -glucan-PO	8.25 $\pm$ 2.25 ***	10.88 $\pm$ 2.85 ***†	9.88 $\pm$ 2.10 *** †

Data represent mean value and standard deviation (mean value  $\pm$  SD) for groups of 8 rats.

Significantly different from arthritic control rats: \* $p$ <0.05, \*\* $p$ <0.01, \*\*\*  $p$ <0.001.

Significantly different from arthritic rats treated with MTX: † $p$ <0.05



**Table 4. The effect of MTX,  $\beta$ -glucan-PO and their combination on serum albumin concentrations (g/L) in AA rats.**

Groups of rats	Day 14	Day 21	Day 28
Healthy controls	42.00 $\pm$ 2.70 ***	39.92 $\pm$ 2.45 ***	42.15 $\pm$ 2.14 ***
Untreated AA controls	27.68 $\pm$ 1.08	30.91 $\pm$ 2.10	34.28 $\pm$ 1.22
AA rats treated with:			
$\beta$ -glucan-PO	28.62 $\pm$ 1.26	30.66 $\pm$ 1.71	34.41 $\pm$ 2.04
MTX-2	32.01 $\pm$ 3.36 **	35.33 $\pm$ 1.79 ***	38.03 $\pm$ 2.19 **
MTX-2+ $\beta$ -glucan-PO	36.01 $\pm$ 3.44 ***	36.33 $\pm$ 3.39 ***	37.84 $\pm$ 2.02 **

Data represent mean value and standard deviation (mean value  $\pm$  SD) for groups of 8 rats.

Significantly different from arthritic control rats: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .