The effects of β-glucan isolated from *Pleurotus ostreatus* on the development of arthritis and methotrexate treatment in rats with adjuvant arthritis

**Summary**

**Aim of the study:** The purpose of this study was to evaluate the effect of β-glucan (1,3/1,6)-D-glucan isolated from *Pleurotus ostreatus* on the preventive treatment and therapy of adjuvant arthritis (AA) and methotrexate (MTX) treatment in rats.

**Material and methods:** Groups of rats with AA were prevenetively treated with methotrexate (1 mg/kg/week), β-glucan PO (1 mg/kg every second day) or their combination for a period of 28 days from adjuvant application. Methotrexate and β-glucan PO (15 mg/kg every day) were also applied therapeutically from day 13 to day 35 after immunization. Body mass, hind paw swelling, arthrogram scores, and the level of serum albumin were measured as markers of inflammation and arthritis.

**Results:** Preventive treatment with a low dose of MTX significantly inhibited the markers of both inflammation and arthritis. Methotrexate and its combination with β-glucan PO significantly increased the body mass of arthritic rats. β-glucan PO administered alone had no effect on body mass but significantly decreased both the hind paw swelling and arthritic score. In combination with MTX, β-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 the healthy controls. Both MTX alone and the combination treatment with MTX + β-glucan PO markedly potentiated the beneficial effects of MTX, which resulted in a more significant reduction of hind paw swelling and arthritic scores. PO markedly potentiated the beneficial effects of MTX, which resulted in a more significant reduction of hind paw swelling and arthritic scores.

**Key words:** β-glucan, methotrexate, adjuvant arthritis.

**Stwóra kluczowe:** β-glukan, metotreksat, adiuwantowe zapalenie stawów.

**Streszczenie**

**Cel pracy:** Celem badania była ocena wpływu β-glucan (1,3/1,6)-D-glucanu izolowanego z grzyba *Pleurotus ostreatus* na postępowanie profilaktyczne oraz leczenie adiuwantowego zapalenia stawów (adjuvant arthritis – AA), a także na leczenie metotreksatem (MTX) u szczurów.

**Materiał i metody:** W grupie szczurów z AA profilaktycznie podawano MTX (1 mg/kg m.c. na tydzień), β-glukan-PO (1 mg/kg m.c. co drugi dzień) lub ich kombinację przez 28 dni od aplikacji adiuwantu. Metotreksat i β-glukan-PO (15 mg/kg m.c. codziennie) stosowano także terapeutycznie od 13 do 35. dnia po immunizacji. Jako markery stanu zapalnego oraz zapalenia stawów oceniano masę ciała, obrzęk tylnej łapy, wynik oceny artrogramu oraz stężenie albuminy w surowicy.

**Wyniki:** Profilaktyczne podawanie małej dawki MTX znamiennie hamowało markery zarówno stanu zapalnego, jak i zapalenia stawów. Metotreksat i jego kombinacja z β-glukanem-PO znamiennie zwiększała mase ciała u szczurów z zapaleniem stawów. β-glukan-PO podawany w monoterapii nie wpływał na masę ciała, ale znamiennie zmniejszał zarówno obrzęk tylnej łapy, jak i wynik oceny zapalenia stawów. W skojarzeniu z MTX, β-glukan-PO znacznie wzmacniał korzystne działanie MTX, co powodowało wyraźniejsze zmniejszenie obrzęku tylnej łapy oraz obniżyło wskaźników oceny zapalenia stawów. Stężenie albuminy w surowicy u szczurów w grupie kontrolnej z zapaleniem stawów było znamiennie mniejsze niż w grupie kontrolnej zdro-
β-glucans are long-chain polysaccharides containing D-glucose monomers linked by β-glycosidic bonds, with or without β-(1,6)-(D)-glucose smaller side chains. They belong to the group of polysaccharides present in the cell wall of bacteria, fungi, yeast, but also in cereals such as barley and oat [1]. Glucans differ not only in the molecule length and branching, but also in their tertiary structure based on their source. They are regarded as immune response modulators, non-specific stimulators of the immune system with several interactions with inherent and adaptive immunity of the host [2]. The induction of cellular responses by β-glucans is likely to involve their specific interaction with several cell surface receptors, such as complement receptor 3, lactosylceramide, selected scavenger receptors, and dectin-1 [3–5]. β-glucans increase host immune defense by activating the complement system, enhancing the phagocytic and proliferation activity of professional phagocytes – granulocytes, monocytes, macrophages, and dendritic cells. The use of β-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, protozoal and viral infections [6–8]. β-glucans also show anticarcinogenic activity. Their use as adjuvants to cancer chemotherapy and radiotherapy demonstrated a positive role in the restoration of hematopoiesis following bone marrow injury [9–11].

β-(1,3/1,6)-D-glucan is an insoluble polysaccharide isolated from the mushroom Pleurotus ostreatus. It is a safe and beneficial nutritional supplement with a strong system effect that is manifested in particular through the stimulation of specific immunity combined with antioxidant activity [11–13]. Recently, Smiderle et al. [14] described the anti-inflammatory and analgesic activity of β-(1,3/1,6)-D-glucan isolated from Pleurotus 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 β-(1,3/1,6)-D-glucan isolated from Pleurotus ostreatus to rats with adjuvant arthritis (AA) we observed decreased activities of pro-inflammatory cytokine TNF-α (tumor necrosis factor α), IL-1 (interleukin 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)-β-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 also includes the development of oxidative stress. Sener et al. [18] showed that β-glucan can ameliorate the methotrexate-induced oxidative organ injury (liver or kidney) in rats via its antioxidant and immunomodulatory effects.

Our previous results showed the beneficial effects of β-(1,3/1,6)-D-glucan in an animal model of rheumatoid arthritis [15, 16]. The aim of this study was to evaluate its effect on the development of arthritis and methotrexate treatment in rats with AA in both the preventive and therapeutic regimen.

Material and methods

Material

Methotrexate 10 mg/ml solution for injection in sterile physiological saline was supplied by Medac Company, Hamburg, Germany. β-(1,3/1,6)-D-Glucan is an insoluble micronized pure compound isolated from Pleurotus ostreatus (β-glucan-PO), β-(1,3/1,6)-D-glucan (Imunoglobulan®) was obtained from Pleuran s.r.o. company (Bratislava, Slovakia). Mycobacterium butyricum was purchased from Difco Laboratories Co. Ltd. (Detroit, MI, USA) and Freund’s incomplete adjuvant from Sigma-Aldrich Chemie GmbH (Germany).
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**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 the 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 a 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**

Methotrexate and β-glucan-PO were administered preventively in corresponding doses from day 0 (the day of immunization) to day 28 of the study. Methotrexate 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). β-glucan-PO was administered orally as a suspension in saline every second day in a dose of 1 mg/kg body mass. Untreated animals received physiological saline in a similar manner. β-glucan-PO was administered therapeutically from day 13 of the study in a dose of 15 mg/kg every day (except Saturday and Sunday) up to day 35 of the experiment; the dose of MTX remained unchanged, but was only applied from day 13 of the experiment twice a week.

After a 14-day quarantine, the animals were divided into the following 8 groups of eight animals: group 1: healthy controls; group 2: untreated rats with AA (AA controls); group 3: AA rats preventively treated with β-glucan-PO; group 4: AA rats preventively treated with MTX; group 5: AA rats preventively treated with the combination of MTX + β-glucan-PO; group 6: AA rats therapeutically treated from day 13 with MTX; group 7: AA rats therapeutically treated with β-glucan-PO from day 13 of the experiment; group 8: AA rats therapeutically treated with the combination of MTX + β-glucan-PO from day 13.

**Evaluated parameters**

**Body mass** of rats was measured at the beginning of the study and on days 1, 7, 14, 21, 28 and 35 of the experiment.

**Hind paw swelling.** The volume of the hind paw swelling was measured with an electronic water plethysmograph (UGO BASILE, Comerio-Varese, Italy) on days 14, 21, 28 and 35.

**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, 28 and 35.

**Serum albumin levels** were measured on days 14, 21, 28 and 35 in the rat serum by the spectrophotometric method, using the SYS 1 kit (BM/Hitachi, Boehringer Mannheim, Germany) on a Hitachi 911 automatic biochemical analyzer. Blood was taken from the retro-orbital sinus and was then processed into serum. The serum was stored at –60°C until the albumin analysis.

**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 I). However, on day 14, the body mass of arthritic control rats and rats treated with β-glucan-PO alone was significantly lower than that of the healthy controls and arthritic rats preventively treated with MTX and with a combination of MTX + β-glucan-PO. The loss of body mass in rats treated with the combination MTX + β-glucan-PO was similar to that in rats treated with MTX alone. The therapeutic application of MTX, β-glucan-PO, and the combination of MTX + β-glucan-PO from day 13 of the experiment did not influence the body mass of arthritic animals.

**Hind paw swelling**

Hind paw swelling reflects both arthritic and inflammatory changes occurring in AA rats. The volume of the swollen hind paws in arthritic rats was 1.5–1.7 times higher than in healthy controls (Table II). Statistically significant decreases of hind paw swelling were observed in arthritic rats preventively treated with MTX, β-glucan-PO, and the combination of MTX + β-glucan-PO on day 14, 21, and 28 of the experiment. The mean volumes of both paws are shown in Table II. β-glucan-PO alone, administered preventively, significantly reduced the hind paw swelling on day 21 and 28 after immunization. The combination treatment MTX + β-glucan-PO reduced this parameter statistically more significantly than MTX treatment alone (MTX vs. MTX + β-glucan-PO, *p* < 0.05).

Tested preparations behaved differently following application from day 13 after immunization when clinical signs of arthritis are already manifested, such as hind paw swelling...
swelling and periarticular erythema. The application of β-glucan-PO from day 13 after immunization in a dosage of 15 mg/kg of body mass also significantly reduced the hind paw swelling on day 21 and 28 of the experiment. Methotrexate alone and combined therapy with MTX + β-glucan-PO in a therapeutic regimen had no significant effect on hind paw swelling.

Arthrogram score

Arthrogram score similarly as hind paw swelling was significantly reduced by β-glucan-PO and MTX applied preventively. The combination treatment with MTX + β-glucan-PO was more efficient than MTX alone (Table III). β-glucan-PO administered in a therapeutic dose of 15 mg/kg significantly reduced the arthrogram score, similarly to hind

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**Table I.** The effect of MTX, β-glucan-PO and their combination on the body mass of rats (g)

| Groups of rats          | Day 1        | Day 7        | Day 14       | Day 21       | Day 28       | Day 35       |
|-------------------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Healthy controls        | 167 ±6       | 219 ±13      | 224 ±13**    | 239 ±14***   | 250 ±15***   | 258 ±16***   |
| AA controls             | 170 ±8       | 217 ±12      | 186 ±11      | 179 ±18      | 199 ±13      | 216 ±13      |
| AA rats treated from day 0 with: |             |              |              |              |              |              |
| β-glucan-PO             | 171 ±6       | 217 ±7       | 193 ±10      | 184 ±9       | 212 ±12      |              |
| MTX                     | 173 ±6       | 227 ±7       | 228 ±17***   | 236 ±14***   | 247 ±15***   |              |
| MTX + β-glucan-PO       | 171 ±6       | 228 ±8       | 231 ±18***   | 230 ±20***   | 244 ±24**    |              |
| AA rats treated from day 13 with: |            |              |              |              |              |              |
| MTX                     | 172 ±8       | 217 ±8       | 195 ±13      | 191 ±10      | 204 ±8       | 221 ±10      |
| β-glucan-PO             | 173 ±6       | 220 ±8       | 193 ±15      | 190 ±13      | 201 ±16      | 219 ±15      |
| MTX + β-glucan-PO       | 171 ±8       | 222 ±6       | 191 ±13      | 192 ±11      | 202 ±15      | 220 ±16      |

*Data represent mean value and standard deviation (mean value ± SD) for groups of 8 rats.*

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

**AA** – adjuvant arthritis, **MTX** – methotrexate.

**Table II.** The effect of MTX, β-glucan-PO and their combination on hind paw swelling in AA rats (ml)

| Groups of rats          | Day 14      | Day 21      | Day 28      | Day 35      |
|-------------------------|-------------|-------------|-------------|-------------|
| Healthy controls        | 1.36 ±0.04*** | 1.40 ±0.02*** | 1.41 ±0.05*** | 1.43 ±0.04  |
| AA controls             | 2.25 ±0.20  | 2.51 ±0.18  | 2.34 ±0.15  | 2.08 ±0.22  |
| AA rats treated from day 0 with: |             |              |              |              |
| β-glucan-PO             | 2.05 ±0.14  | 2.31 ±0.09*  | 2.16 ±0.24*  |              |
| MTX                     | 1.85 ±0.23** | 2.16 ±0.22** | 2.04 ±0.30** |              |
| MTX + β-glucan-PO       | 1.73 ±0.22*** | 1.94 ±0.37***† | 1.88 ±0.28*** † |              |
| AA rats treated from day 13 with: |             |              |              |              |
| MTX                     | 2.22 ±0.22  | 2.42 ±0.20  | 2.39 ±0.28  | 1.98 ±0.22  |
| β-glucan-PO             | 2.14 ±0.18  | 2.09 ±0.23** | 1.97 ±0.22** | 1.95 ±0.22  |
| MTX + β-glucan-PO       | 2.24 ±0.21  | 2.31 ±0.44  | 2.28 ±0.47  | 2.18 ±0.47  |

*Data represent mean value and standard deviation (mean value ±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

**AA** – adjuvant arthritis, **MTX** – methotrexate.
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281 paw swelling. The therapeutic application of MTX and the combination of MTX + β-glucan-PO did not influence the arthrogram score.

**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 and the combination treatment with MTX + β-glucan-PO applied preventively significantly inhibited the decrease in serum albumin (Table IV). β-glucan-PO therapy reduced albumin decrease on day 21. Combined therapy with MTX + β-glucan-PO was efficient on days 21 and

**Table III.** The effect of MTX, β-glucan-PO and their combination on the arthrogram score in AA rats

| Groups of rats          | Day 14     | Day 21     | Day 28     | Day 35     |
|------------------------|------------|------------|------------|------------|
| AA controls            | 13.44 ±1.81| 16.22 ±1.99| 14.89 ±2.26| 12.84 ±2.14|
| AA rats treated from day 0 with: |            |            |            |            |
| β-glucan-PO            | 11.30 ±1.46| 14.25 ±0.53*| 12.14 ±2.10*|            |
| MTX                    | 8.50 ±1.52**| 13.33 ±3.44**| 11.00 ±2.53*|            |
| MTX + β-glucan-PO      | 8.25 ±2.25***| 10.88 ±2.85***†| 9.88 ±2.10***†|            |
| AA rats treated from day 13 with: |            |            |            |            |
| MTX                    | 14.10 ±1.54| 15.40 ±1.98| 13.92 ±2.18| 11.38 ±2.83|
| β-glucan-PO            | 13.80 ±2.10| 12.13 ±1.73**| 11.63 ±2.39*| 10.50 ±2.27*|
| MTX + β-glucan-PO      | 13.64 ±3.22| 14.38 ±4.72| 13.38 ±3.96| 12.13 ±3.64|

Data represent mean value and standard deviation (mean value ± 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

AA – adjuvant arthritis, MTX – methotrexate.

**Table IV.** The effect of MTX, β-glucan-PO and their combination on serum albumin concentrations (g/l) in AA rats

| Groups of rats          | Day 14     | Day 21     | Day 28     | Day 35     |
|------------------------|------------|------------|------------|------------|
| Healthy controls       | 42.00 ±2.70***| 39.92 ±2.45***| 42.15 ±2.14***| 41.82 ±2.6***|
| AA controls            | 27.68 ±1.08| 30.91 ±2.10| 34.28 ±1.22| 36.42 ±2.14|
| AA rats treated from day 0 with: |            |            |            |            |
| β-glucan-PO            | 28.62 ±1.26| 30.66 ±1.71| 34.41 ±2.04|            |
| MTX                    | 32.01 ±3.36**| 35.33 ±1.79***| 38.03 ±2.19**|            |
| MTX + β-glucan-PO      | 36.01 ±3.44***| 36.33 ±3.39***| 37.84 ±2.02**|            |
| AA rats treated from day 13 with: |            |            |            |            |
| MTX                    | 28.24 ±2.79| 31.42 ±2.14| 34.80 ±2.14| 37.22 ±2.45|
| β-glucan-PO            | 28.80 ±2.82| 36.21 ±4.67**| 35.30 ±2.79| 36.24 ±2.10|
| MTX + β-glucan-PO      | 27.62 ±2.70| 35.21 ±4.24**| 36.40 ±2.45*| 33.24 ±2.70*|

Data represent the mean value and standard deviation (mean value ± SD) for groups of 8 rats.

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

AA – adjuvant arthritis, MTX – methotrexate.
28 of the experiment, but on day 35 after immunization the serum albumin was even lower in this group than in untreated arthritic animals, which may indicate that the inflammatory process was augmented following previous suppression.

**Discussion**

In the study we investigated the effect of β-(1,3/1,6)-D-glucan isolated from *Pleurotus ostreatus* on inflammatory and arthritic parameters in rats with adjuvant arthritis and on the treatment with MTX. β-glucans, even in very high doses, are non-toxic and free of adverse reactions. The U.S. Food and Drug Administration classified β-glucan in the GRAS category (Generally Recognized As Safe), which means that there are no known dangerous interactions with other substances, adverse effects, or toxicity [19]. In rats, even doses of 2000 mg/kg of body mass did not lead to toxic effects. In our work, we are presenting the results of the preventive and therapeutic application of β-glucan and its effect on MTX treatment. Methotrexate, β-glucan, and their combination were preventively administered from the day of adjuvants application and from day 13 after immunization, when clinical signs of arthritis such as hind paw swelling and periarticular erythema are present. Doses of β-glucan in the preventive regimen were 1 mg/kg of body mass. Therapeutic doses of β-glucan were much higher, up to 15 mg/kg of body mass. These doses were applied alone and in combination with MTX.

The results of our investigation confirmed the previously reported effect of MTX treatment in rats with AA. Methotrexate at a dose of 1 mg/kg/week suppressed, but did not prevent, arthritis development [20, 21]. In our study, MTX applied preventively significantly suppressed hind paw swelling and reduced the arthrogram score. Therapeutically, its effect was not proved. β-glucan alone in the preventive regimen reduced both hind paw swelling and the arthrogram score on days 21 and 28 of the experiment. The remarkable finding was also that β-glucan-PO potentiated the beneficial effect of MTX; it reduced both the hind paw swelling and arthrogram score more significantly compared to the rats treated with MTX alone. β-glucan-PO, in contrast to MTX, was capable of reducing both the hind paw swelling and arthrogram score even in the therapeutic regimen. In this connection, it is remarkable that such high doses of β-glucan-PO (15 mg/kg of body mass) were immunostimulatory and capable of suppressing the already started arthritic process. 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 IL-1 [21]. Our results correlate with the observation that preventively applied MTX markedly prevents the albumin decrease in AA rats. The combination of MTX with β-glucan-PO had no additional effect compared to MTX alone. Therapeutically MTX had no effect on serum albumin. Interestingly, the combination of MTX with β-glucan-PO reduced albumin decrease also therapeutically, but at the end of the study on day 35 the serum albumin level decreased again, probably as a result of activation of suppressed inflammation.

Systemic administration of β-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, 22–24]. Hetland et al. [25, 26] showed that β-glucan reduced growth of *Mycobacterium tuberculosis* in macrophage cultures and had a protective effect against *Mycobacterium bovis* BCG infection in BALB/c mice.

Some microbes, such as fungi and viruses, lead to generation and activation of autoimmune T cells resulting in the development of a particular autoimmune disease in genetically susceptible individuals. β-(1,3/1,6)-D-glucan, an effective activator of the immune system, may also be beneficial in humans in preventing or eliminating bacterial infections which are known to induce reactive arthritis. The finding that β-glucan-PO, applied both preventively and therapeutically, has a beneficial effect on the clinical symptoms of arthritis, even at a high dose of 15 mg/kg, may be interesting also in prevention and treatment of various infections in humans with rheumatoid arthritis.

Authors declare no conflict of interest.

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