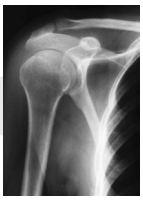

Effects of Purified Micronized Flavonoid Fraction (Detralex®) on Prophylactic Treatment of Adjuvant Arthritis with Methotrexate in Rats

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Key words: purified micronized flavonoid fraction, Detralex, methotrexate, adjuvant arthritis, microcirculation

Abstract

Background: In both adjuvant arthritis and rheumatoid arthritis, edema and inflammation appear in synovial joints. Edema or effusion reflects an imbalance in lymph dynamics. Purified micronized flavonoid fraction is mainly used in the treatment of chronic venous insufficiency. This compound improves lymphatic drainage with a signicant increase in lymphatic flow and lymphatic pulsality. It is suggested that the beneficial effect of purified micronized flavonoid fraction may be involved in the treatment of adjuvant arthritis in rats.

Objectives: To evaluate the effect of Detralex on methotrexate prophylactic treatment of adjuvant arthritis in rats.

Methods: Groups of rats with adjuvant arthritis were treated with methotrexate (0.6 mg/kg/week), Detralex (20 mg/kg/day) and their combination for 50 days from adjuvant application. Hind paw swelling, arthrogram scores, serum albumin level, serum nitrite/nitrate concentrations and whole body mineral density were evaluated as markers of inflammation and destructive changes associated with arthritis.

Results: Long-term prophylactic treatment with low dose methotrexate significantly inhibited the markers of both inflammation and arthritis. Detralex administered alone slightly decreased both the hind paw swelling and the arthritic score. Other inflammatory and arthritic markers were not significantly influenced. However, Detralex combined with methotrexate markedly potentiated the beneficial effects of methotrexate, which resulted in a more significant reduction in hind paw swelling, arthritic scores, and serum concentrations of nitrite/nitrate. Interestingly, the arthritis-induced decrease of BMD in AA rats was significantly lower only in the group treated with the combination of Detralex+methotrexate.

Conclusion: Detralex increased the therapeutic efficacy of methotrexate basal treatment in AA. We suggest that this may be related to the beneficial effect of Detralex on microcirculation, especially on venules and lymphatic vessels.

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Detralex® (Les Laboratoires Servier, France), a purified micronized flavonoid fraction, contains two flavonoids: diosmin (90%) and hesperidine (10%). Micronized flavonoid fraction is also known under other names: 5682 SE, daflon 500, arvenum 500, capiven or venotec. Daflon 500, like the majority of flavons, has antioxidant properties; it is an effective scavenger of reactive oxygen species *in vitro* and *in vivo* [1]. I*n vitro* daflon 500 inhibits both classic and alternative pathways of complement activation [2]. Activation of the complement system during the early phase of inflammation is partially responsible for increased capillary permeability.

Purified micronized flavonoid fraction is mainly used in the treatment of chronic venous insufficiency. The beneficial venotropic effect of daflon 500 on blood vessels in patients with chronic venous insufficiency was proven by clinical evaluations and intravital capillaroscopy [3-5]. The results of experimental studies in dogs and rats have shown that daflon 500 improves lymphatic drainage [4,6], with a significant increase in lymphatic flow and lymphatic pulsatility [6,7]. Micronized flavonoid fraction also significantly decreased the migration of leukocytes from post-capillary venules to the surrounding connective tissue during an experimentally induced venous occlusion and subsequent reperfusion [8,9]. A generalized abnormality in capillary function (an increase in capillary filtration rate) and some obstruction of lymphatic clearance are associated with rheumatoid arthritis [10,11]. Microscopic changes of post-capillary venules were also found in RA [12,13], and structural changes of lymphatic vessels in synovial membrane were observed in RA and juvenile idiopathic arthritis [14-16].

Adjuvant arthritis, an experimentally induced inflammatory disorder in rats, bears some features of rheumatoid arthritis. In both diseases edema and inflammation appear in synovial joints. Edema or effusion reflects an imbalance in lymph dynamics [17]. The animal model of AA is widely used for testing drugs with anti-inflammatory and anti-arthritic effects. The aim of the present study was to evaluate the effect of Detralex on clinical symptoms and certain laboratory markers of AA during basal treatment with methotrexate.

Materials and Methods

Methotrexate was purchased as pure substance from Pliva-Lachema Ltd., Brno (Czech Republic). Detralex was obtained from Les Laboratoires Servier (France). M*ycobacterium butyricum* in lyophilized form was purchased from Difco Laboratories Co.

BMD = bone mineral density
AA = adjuvant arthritis
RA = rheumatoid arthritis

Ltd. (Detroit, MI, USA) and incomplete Freund's adjuvant from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

Male Lewis rats (170 ± 10 g; Sulzfeld, Germany) were maintained during the experiment in standard animal facilities that comply with requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The animals were fed pelleted food (TOP DOVO, Slovak Republic) and had free access to food and water. The State Veterinary and Food Committee of the Slovak Republic and the Ethics Committee for Control of Animal Experimentation of the National Institute of Rheumatic Diseases approved the experimental protocol and all procedures.

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

Tested substances were administered in corresponding doses from day 0 (the day of immunization) to day 50 of the study. Detralex 20 mg/kg in the form of fine suspension in saline was administered daily per os. Methotrexate was prepared in sterile saline to yield the desired concentration of 0.3 mg/kg in 0.1 ml saline, and applied twice a week per os (0.6 mg/kg in total per week). Fresh solutions of the tested substances were prepared each day of administration. The untreated AA group received the vehicle (sterile saline) in the same manner daily for 50 days.

The animals were divided into five groups of eight animals: group 1: non-arthritic untreated healthy controls; group 2: untreated rats with AA; group 3: AA rats treated with methotrexate; group 4: AA rats treated with Detralex; group 5: AA rats treated with the combination of methotrexate+Detralex.

Evaluated parameters

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

Arthrogram score. The severity of arthritis was quantified by scoring each paw from 1 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. Serum albumin was measured on days 14, 21 and 28 in the rat serum by the spectrophotometric method using SYS 1 kit (BM/Hitachi, Boehringer Mannheim, Germany) on a Hitachi 911 automatic biochemical analyzer.

Serum nitrite/nitrate. Nitrite/nitrate concentration in deproteinized serum was determined by the method of Cortas and Waking [18] on days 14, 21 and 28. Cu-coated cadmium granules in glycine buffer at pH 9.7 reduced nitrate to nitrite. The total nitrite concentrations were determined by the Griess reaction.

Bone mineral density. BMD was measured by dual-energy X-ray absorptiometry using Hologic QDR®-4500 (Waldham, MA, USA) with the equipment for measuring small laboratory animals. The whole body BMD of rats was determined on day 50 after immunization.

Statistical analysis

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

Hind paw swelling and arthrogram score. These markers reflect both inflammatory and arthritic changes occurring in rats with AA. The volume of the swollen hind paws in arthritic rats on day 21 was about twice that found in healthy controls [Table 1]. A statistically significant decrease of both hind paw swelling and arthrogram scores were observed in rats treated with methotrexate compared to untreated arthritic controls. The reductions in both hind paw swelling and arthrogram scores were more pronounced in rats treated with the combination of methotrexate+Detralex than in those treated with methotrexate alone [Tables 1 and 2]. Detralex alone tended to reduce both these markers, but the decrease was not statistically significant in comparison with untreated AA rats.

Serum albumin concentration. 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 (non-arthritic untreated

Table 1. The effects of methotrexate (MTX), Detralex (DTX) and their combination on hind paw edema (ml) and on whole body BMD (g/cm^2) in AA rats

	Day 14	Day 21	Day 28	BMD
Healthy controls	1.34 ± 0.07 ***	1.41 ± 0.05 ***	1.41 ± 0.02 ***	0.1382 ± 0.0087 ***
AA controls	2.05 ± 0.29	2.46 ± 0.33	2.34 ± 0.27	0.1222 ± 0.0046
AA rats treated with				
MTX	1.59 ± 0.06 ***	1.85 ± 0.27 **	1.83 ± 0.26 *	0.1259 ± 0.0050
DTX	1.86 ± 0.31	2.28 ± 0.26	2.17 ± 0.22	0.1206 ± 0.0046
DTX+MTX	1.48 ± 0.25 ***	1.71 ± 0.35 ***	1.77 ± 0.30 **	0.1275 ± 0.0051 *

Data represent mean values ± SD for 8 rats.
Significantly different from arthritic control rats: * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

Table 2. The effects of methotrexate (MTX), Detralex (DTX) and their combination on the arthrogram score in AA rats

	Day 14	Day 21	Day 28
AA controls	15.33 ± 3.12	18.22 ± 1.66	17.00 ± 1.83
AA rats treated with			
MTX	8.80 ± 1.87 ***	12.70 ± 2.98 ***	13.60 ± 3.86 **
DTX	13.60 ± 3.86	18.00 ± 2.87	19.10 ± 2.23
DTX+MTX	6.90 ± 4.58 ***	10.80 ± 4.08 ***	12.10 ± 3.96 ***

Data represent mean values ± SD for 8 rats.
Significantly different from arthritic control rats: ** $P < 0.01$, *** $P < 0.001$.

Table 3. The effects of methotrexate (MTX), Detralex (DTX) and their combination on serum albumin concentrations (g/L) in AA rats

	Day 14	Day 21	Day 28
Healthy controls	42.53 ± 2.04 ***	42.34 ± 2.03 ***	43.80 ± 2.39 ***
AA controls	29.01 ± 1.75	31.23 ± 1.07	33.44 ± 1.76
AA rats treated with			
MTX	32.25 ± 3.46 *	33.38 ± 2.56*	35.21 ± 1.25*
DTX	28.83 ± 1.07	29.58 ± 1.28	31.51 ± 1.93
DTX+MTX	31.55 ± 2.25 *	33.79 ± 2.78*	35.50 ± 1.61*

Data represent mean values ± SD for 8 rats.
Significantly different from arthritic control rats: * $P < 0.05$, *** $P < 0.001$.

Table 4. The effects of methotrexate (MTX), Detralex (DTX) and their combination on serum nitrites/nitrates concentrations (nmol/ml) in AA rats

	Day 14	Day 21	Day 28
Healthy controls	25.07 ± 5.82 ***	29.44 ± 7.68 ***	32.58 ± 7.01 ***
AA controls	108.80 ± 26.15	78.25 ± 10.89	69.97 ± 11.63
AA rats treated with			
MTX	84.12 ± 19.87 *	62.63 ± 16.00 *	59.65 ± 9.92*
DTX	100.55 ± 29.79	90.78 ± 17.26	75.07 ± 11.56
DTX+MTX	68.57 ± 19.52 **	59.48 ± 9.18 **	57.52 ± 4.35**

Data represent mean values ± SD for 8 rats.
Significantly different from arthritic control rats: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

controls vs. AA rats, $P < 0.001$). Both methotrexate and the combination of Detralex+methotrexate significantly inhibited the decrease in serum albumin [Table 3]. Detralex without metotrexate did not influence this inflammatory marker.

Serum nitrite/nitrate concentrations (NO2-/NO3-). Serum concentrations of nitrite/nitrate reflect nitric oxide production in various tissues during inflammation. The clinical onset of AA was associated with a significant rise in nitrite/nitrate concentrations. Methotrexate alone and in combination with Detralex significantly decreased nitrite/nitrate concentrations compared to AA controls [Table 4]. Detralex had no significant effect on the serum nitrite/nitrate concentrations, however it did potentiate the beneficial effect of methotrexate during the whole experiment, which resulted in a more significant reduction of nitrite/nitrate concentrations in rats treated with the combination of methotrexate+Detralex compared to methotrexate administered alone ($P < 0.01$ vs. $P < 0.05$) [Table 4].

Densitometry. The development of arthritis in both humans and rats is associated with the development of osteopenia due to the activation of pro-inflammatory cytokines. At the end of the study (day 50) AA rats had markedly lower values of whole body BMD compared to healthy controls [Table 1]. Evaluation of different treatments showed that only the combination of methotrexate+Detralex significantly inhibited the whole body BMD decrease in AA rats.

Discussion

This experiment focused on the effect of Detralex on the inflammatory and arthritic markers in rats with AA during basal treatment with methotrexate. The treatment was prophylactic, which means that the animals were treated immediately after administration of the adjuvant. Since the effect on BMD was also investigated, the duration of the treatment was longer (50 days) than usual (10–14 days) to prevent exacerbation of arthritis after discontinuation of the therapy.

The results of our study confirmed observations on methotrexate treatment reported by Welles at al. [19] and Connoly at al. [20]. Methothrexate at a dose of 0.6 mg/kg/week suppressed but did not prevent the development of arthritis. In our study, it significantly suppressed the hind paw swelling and decreased the arthrogram scores. Detralex alone tended to decrease these parameters, but the decrease was not statistically significant compared to the group of untreated AA rats. The remarkable finding was that Detralex potentiated the beneficial effect of methotrexate; reductions in hind paw swelling and arthrogram scores on days 21 and 28 were more significant compared to rats treated with methotrexate alone [Tables 1 and 2]. We presume that Detralex might influence permeability of tiny blood vessels and improve lymphatic drainage of joints affected by synovitis. Several authors reported the beneficial effect of micronized flavonoid fraction on blood and lymphatic vessels in humans, dogs and rats [3-8]. They found a significant increase in the lymphatic flow and pulsatile activity of lymphatic vessels after Daflon 500 application in dogs and rats [6,7].

Serum albumin acts as a negative acute-phase reactant in rat arthritis, and the decrease in serum albumin levels reflects 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 [Table 3] correlate with the observation that methotrexate markedly prevents the albumin decrease in AA rats [20]. The administration of Detralex alone or in combination had no effect on this parameter [Table 3].

Nitric oxide, an unstable free radical produced by the action of the enzyme NO synthase on L-arginine, is a mediator of multiple physiological functions, and may also mediate local inflammation and tissue destruction [21]. Nitric oxide is involved in both initiation and development of AA in rats [22]. Moreover, inhibitors of NOS have been shown to suppress arthritis in several animal models [23,24] and increased NO levels have been found in patients with RA [25,26]. Omata and co-workers [27] reported that methotrexate suppresses *ex vivo* production of NO in macrophages of rats with AA. In our study, markedly increased serum nitrite/nitrate concentrations were found in AA rats. Flavonoid hesperidine (component of Detralex) markedly decreases the formation of inducible NOS in liposaccharide-activated macrophages *in vitro* at the level of NOS gene transcription [28]. We presume that hesperidine largely contributes to reducing serum nitrite/nitrate level in AA rats treated with the combination of Detralex+methotrexate also in our experiment [Table 4].

Marked osteopenia and extensive bone loss have been described in AA rats [29]. Suzuki and team [30] reported that

NO = nitric oxide
NOS = nitric oxide synthase

low dose weekly methotrexate therapy had a favorable effect on abnormal bone metabolism and osteopenia in rats with AA. In our study, methotrexate (0.6 mg/kg/week) alone had no effect on whole body BMD in rats. However, in our experiment the combination of methotrexate with Detralex significantly reduced the BMD loss in AA rats compared to the group treated with methotrexate only. We suggest that this may be due to the effect of the combination treatment on microcirculation in bone tissue. Administration of Detralex alone had no such effect.

Recently, flavonoid hesperidine was used to treat collagen-induced arthritis in mice [31]. The authors found a significant reduction in the clinical parameters and an improvement in the histological picture of the disease in arthritic mice treated with hesperidine. Shevchuk et al. [32] used Detralex to treat patients with RA and found an increased therapeutic effect of methotrexate and decrease in the adverse effects of methotrexate.

In conclusion, our results demonstrate that Detralex does not cause any worsening of the parameters evaluated; on the contrary, it mildly reduces some of the clinical symptoms of AA such as hind paw edema and arthritic score. Our findings showed that Detralex increased the efficacy of basal methotrexate therapy in AA rats. Furthermore, our study suggests that this may be related to the beneficial effect of Ddetralex on microcirculation, especially on venules and lymphatic vessels.

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