ORIGINAL ARTICLE

IMMUNOMODULATORY EFFECTS OF METHADONE FOLLOWING METHOTREXATE THERAPY IN A RAT MODEL OF ARTHRITIS

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disease causing inflammation, pain, swelling, stiffness and loss of function in the joints. The advantage of opioids use in RA treatment is the simultaneous pain control and immune response modulation. The effects of methadone (MTD) on the proliferation of splenic lymphocytes from Wistar rats treated *in vivo* with methotrexate (MTX) after induction of chronic inflammation arthritis were investigated. The animals were administered three doses of MTX solution for injection and sodium MTX ("hydrosoluble" MTX) or MTX *per se* ("hydrophobic" MTX) encapsulated in liposomes. Splenic lymphocytes were activated with concanavaline A and uridine incorporation method was used for the evaluation of lymphocytes proliferation. The results showed an immunosuppressive effect of MTD in rats treated with a low dose of MTX (solution for injection or "hydrosoluble" MTX-liposomes) and a persistent inhibition at high doses of MTX solution for injection. MTD can activate the splenic lymphocytes of animals treated with "hydrophobic" MTX liposomes. The study indicated that high doses of hydrosoluble and hydrophobic MTX could modulate cells reactivity to MTD.

Rezumat

Poliartrita reumatoidă (RA) este o boală autoimună cronică care provoacă inflamație, durere, edem, rigiditate și pierderea funcției articulațiilor. Avantajul utilizării opioidelor în tratamentul RA este controlul simultan al durerii și modularea răspunsului imun. Am investigat efectele metadonei *in vitro* (MTD) asupra proliferării celulelor mononucleare splenice izolate de la șobolanii tratați cu metotrexat (MTX) după inducerea inflamației cronice. Au fost investigate trei doze diferite de MTX sau lipozomi încărcați cu MTX hidrosolubil (MTX sare de sodiu) sau MTX hidrofob (MTX ca atare). Limfocitele splenice au fost activate cu concanavalină A și s-a utilizat pentru evaluarea proliferării limfocitelor metoda de încorporare a uridinei. Rezultatele au arătat un efect imunosupresiv al MTD la animalele tratate cu doze mici de MTX hidrosolubil (soluție injectabilă sau lipozomi). MTD exercită un efect inhibitor persistent la doze mari de MTX soluție injectabilă. MTD poate induce activarea limfocitelor splenice izolate de la animale tratate cu lipozomi încărcați cu MTX hidrofob. Rezultatele studiului sugerează că dozele mari de MTX hidrosolubil și hidrofob pot modula reactivitatea celulelor la MTD în ambele sensuri.

Keywords: methadone, rheumatoid arthritis, methotrexate, liposomes

Introduction

The immune and the nervous systems are the "super-systems" of the body. There is a complex functional dialogue between them, with a major role in maintaining the body homeostasis [2, 14, 15]. An important component of the neuro-immune network is the system composed of endogenous opioid peptides and related receptors, which controls a number of physiological processes [17, 18, 22, 30]. Immunomodulatory effects exerted by opioid appear to be relevant in a number of diseases with immune component [9, 26]. Given that μ and κ opioid receptors mediate immunosuppressive effects and δ receptors send immunosuppressant signals, pharmacologically targeting specific opioid receptors by agonists or selective antagonists may have therapeutic benefits in diseases characterized by pro-inflammatory immune response like rheumatoid arthritis [11, 23].

Rheumatoid arthritis (RA) has a complex pathogenesis and affects 1% of the general population. The therapy of RA is an ongoing challenge especially since traditional therapeutic protocols delay the disease progression without attaining the remission and the adverse effects are considerable.

Methotrexate is an antimetabolite used in the treatment of certain neoplastic diseases, severe psoriasis and rheumatoid arthritis [10]. Methotrexate remains the basic therapy for patients with rheumatoid arthritis, as it possesses immunosuppressive properties. Currently, methotrexate is accepted as the most effective and best-tolerated disease-modifying anti-rheumatic drug (DMARD) used in RA [25]. High dose methotrexate

therapy, accompanied by folinic acid (leucovorin) rescue therapy, is an important, well-established component of the intensive chemotherapy protocols used in the treatment of certain types of cancer. In order to have the therapeutic efficacy without unacceptable toxicity of methotrexate, plasma concentrations of the drug should be monitored [8].

Inflammatory pain is one of the main symptoms of rheumatoid arthritis. It has no solid correlation with the destruction of the joint, but it is one of the major causes of the malfunction and disability [19, 20]. It was also observed that the patients with rheumatoid arthritis show disturbances in the hypothalamic-pituitary-adrenal system, central nervous system and endogenous opioid systems [15, 24, 27]. All these disturbances are associated with the pain and the inflammation processes. It has been found that activated immune cells and the memory immune cells, which are characteristic for an inflammatory process, produce and locally release pro-inflammatory cytokines under the action of opioids [12, 17, 21, 28]. Locally, endogenous opioids are controlling the pain by binding to the sensitive nerves receptors and by inhibiting the pro-inflammatory immune response or by binding to receptors expressed on activated immune cells and by changing the cellular circulation [9, 31].

In 1997 WHO has agreed the use of opioids in treating severe non-malignant pain, especially in arthritis [32, 33]. Exogenous opioids control the pain and can also modulate the immune response as follows: immunosuppression, a relevant effect in rheumatoid arthritis and graft rejection or immunostimulation, a relevant effect in cancer. Exogenous opioids exert their effect indirectly (*via* the hypothalamic-pituitary-adrenal axis, central nervous system) or directly by interaction with opioid receptors expressed by immune cells. Exogenous opioids do not induce tolerance or notable adverse effects in the presence of an inflammatory process [29].

Consequently, the administration of κ and μ immunosuppressive agonist may be an alternative therapeutic strategy in rheumatoid arthritis, able to control the pain and the inflammatory process [31], in terms of controllable adverse effects. Methadone proved to have an important role in the management pain, in particular of cancer pain [16], although it is the main medication used in the substitution therapy for heroin addiction [3, 30].

In this context, the *ex vivo* effects of methadone (μ opioid agonist) on the proliferation of the splenic lymphocytes were investigated. The splenic cells were harvested from Wistar rats *in vivo* that received methotrexate, in an experimental model of chronic inflammation, arthritis type, induced with Freund adjuvant.

Materials and Methods

Chemicals. Biocol, RPMI1640 cell culture medium, foetal bovine serum, antibiotic-antimycotic solution were obtained from Promega Corporation. Methadone hydrochloride, methotrexate, sodium methotrexate and Concavaline A were purchased from Sigma-Aldrich, Germany. Complete Freund adjuvant (0.25 mg inactivated *Mycobacterium tuberculosis*) was purchased from DIFCO and Methotrexate solution for injection (0.2 mg MTX/mL) was purchased from a local pharmacy. Empty liposomes "hydrosoluble" MTX liposomes (0.2 mg MTX/mL), "hydrophobic" MTX liposomes (0.2 mg MTX/mL) were prepared "in house". Tritium-labelled uridine was kindly provided, by the "Horia Hulubei" Institute of Physics and Nuclear Engineering, Măgurele, Romania.

Animals. The *in vivo* experiments were performed on male Wistar rats (n = 10 animals/group), 12 weeks old, 175 - 280 g body weight (b.w.), with free access to granulated food and water. The animals were provided by an authorized breeding farm ("Cantacuzino" Institute, Băneasa Bio-basis, Bucharest, Romania). After induction of arthritis, 21 days after the injection of the Freund adjuvant in the left rear paw of the rats, the animals were distributed in ten groups based on the received treatment and on MTX concentration level as showed in Table I. The rats were treated with MTX solution for injection or MTX sodium salt ("hydrosoluble" MTX) and MTX itself ("hydrophobic" MTX) encapsulated in liposomes. Three different doses of methotrexate (0.2, 0.3 and respectively 0.4 mg/kg b.w.) have been administered i.v. weekly, for 21 days. The doses were selected based on methotrexate doses used in human rheumatoid arthritis (5 mg/kg b.w., 7.5 mg/kg b.w. or 10 mg/kg b.w. once a week) and taking into account the safety factor of 10, used to extrapolate doses from animal to human. In order to evaluate the low-dose efficacy of methotrexate encapsulated in liposomes, in this experiment, doses equivalent to approximately one-third of those used in human rheumatoid arthritis therapy were administered in rats. The preparation of the liposomes, as well as the investigation of the methotrexate effects in a murine model of arthritis have been previously described [5, 6, 7].

All researches were conducted in accordance with The European Directive 86/609/EEC/24.11.1986 and The Romanian Government Ordinance 37/30.01.2002 regarding the protection of animals used for experimental and other scientific purposes.

Table I

Rat groups codification based on the received treatment and on MTX concentration level

MTX dose (mg/kg b.w.)	Animal groups			
	Control	Hydrosoluble MTX – loaded liposomes	Hydrophobic MTX – loaded liposomes	MTX – injection solution
0	9			
0.2		1	4	7
0.3		2	5	8
0.4		3	6	10

In vitro experiments. The *in vitro* effect of methadone (20 ng/mL) on the lymphocyte proliferation was determined 7 days and 14 days after the last methotrexate dose.

For the *in vitro* experiments, the spleen cells were isolated after the aseptically harvested spleens were cut in RPMI 1640 supplemented with antibiotic-antimicotic (penicillin 10,000 UI/mL, streptomycin 10,000 UI/mL and amphotericin B 2.5 µg/mL) and 10 UI/mL heparin. The suspension was passed through sterile cotton wool filter, and then was centrifuged at 150 g, 10 minutes. The cell pellet was resuspended in complete RPMI (RPMI 1640 supplemented with antibiotic-antimicotic, glutamine 2 mM and 10% heat inactivates foetal calf serum) and the cell concentration was adjusted to 2 x 10^6 cells/mL.

The splenic lymphocyte proliferation was measured by the tritium-labelled uridine incorporation method as has been previously described [4, 13]. The splenic lymphocytes have been *ex vivo* activated with concanavaline A (Con A, Sigma; final concentration 2.5 µg/mL) as polyclonal mitogen for T lymphocytes. Briefly, triplicate test samples (200 µL final volume), containing mononuclear cell suspension (0.2 x 10^6 cells), were incubated in absence or presence of mitogens and/or methadone, in 96 well plates, for 72 h at 37ºC, in 5% CO_2 atmosphere. Six hours prior to harvesting, cell cultures were labelled with 1 µCi tritium-labelled uridine (21 Ci/mmol). Cells, harvested on glass filters, were evaluated for radio-activity in scintillation liquid, using a Canberra-Packard beta-counter (PerkinElmer Life and Analytical Science). The uridine incorporation was expressed in counts per minute (cpm).

Based on radioactivity readings, the *in vitro* effect of methadone was calculated according to the formula:

$$Methadone\ Effect = \frac{(sample) + methadone}{(sample) - methadone}$$

Results and Discussion

Methadone exerts *in vitro* an inhibitory effect on the proliferation of rat splenic lymphocytes activated *ex vivo* with Con A in groups 1, 7 and 10, 7 days after the last dose of methotrexate, as shown in Figure 1. The animals from those groups were *in vivo* treated with hydrosoluble methotrexate liposomes and respectively methotrexate injection. The expression of the inhibitory effect of methadone was observed for the treatment with a low-dose of hydrosoluble methotrexate, both for liposomes and injection solution (groups 1 and 7). These doses of methotrexate induced the increased of the basal activation/proliferation of spleen cells and did not affect or enhanced the response of splenocytes to ConA. It is possible that the up-regulation of the opioid receptor expression in the splenocytes to be induced by methotrexate therapy.

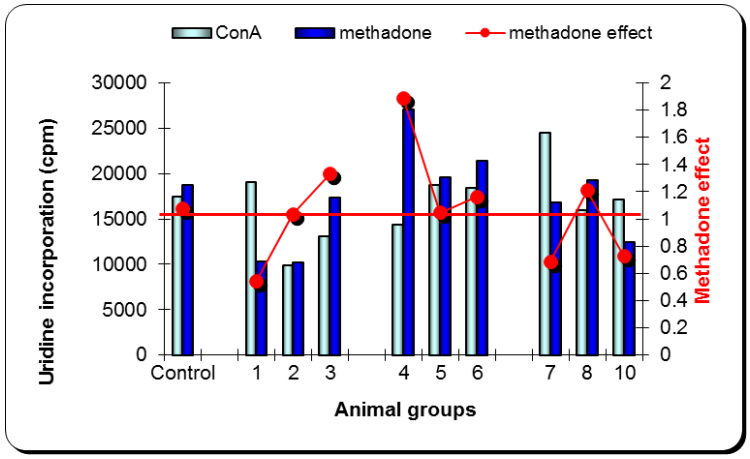

Figure 1.

The effect of methadone on the proliferation of splenic lymphocytes from rats administered with methotrexate (7 days after the last dose). The cells were activated *ex vivo* with ConA

Fourteen days after the last methotrexate dose, methadone, exerts an inhibitory effect only in the case of the highest dose of methotrexate solution treatment (group 10), as it is shown in Figure 2. For the same group, the inhibitory effect was also observed for methadone 7 days after the last methotrexate dose (Figure 1), so it seems that the enhance of opioid receptor expression in splenocytes is persistent at this high dose of hydrosoluble methotrexate.

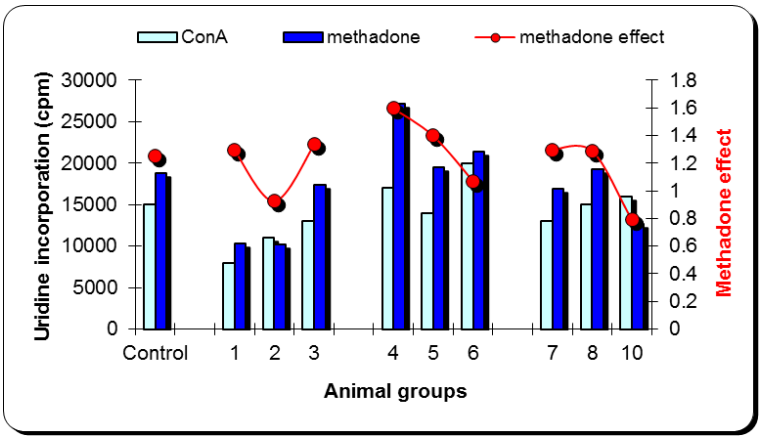

Figure 2.
The effect of methadone on the proliferation of splenic lymphocytes from rats administered with methotrexate (14 days after the last dose). The cells were activated *ex vivo* with ConA

At low doses of MTX solution for injection or liposomes with hydro-soluble MTX given *in vivo*, methadone exerts contrary effects on splenic lymphocyte proliferation, depending on the sampling time: inhibitory effect 7 days after the last MTX dose and slight stimulatory effect 14 days after the last MTX dose.

It is to underline that, for the treatment with hydrophobic methotrexate encapsulated in liposomes, methadone induced a certain stimulatory effect on the splenic mononuclear cell proliferation in the group receiving *in vivo* the lowest dose of methotrexate (group 4). This stimulatory effect manifests both at 7 days (Figure 1), and at 14 days after the last methotrexate dose administered (Figure 2). In this case, the mentioned methadone concentration seems to act by a non-opioid mechanism. There is a linear relation between methadone-stimulatory effect on splenic lymphocytes proliferation and the dose of hydrophobic MTX-loaded liposomes. Therefore, a linear decrease (R^2 = 0.9805) of the methadone stimulatory effect as the dose of hydrophobic methotrexate encapsulated in liposomes increased, was observed (Figure 3).

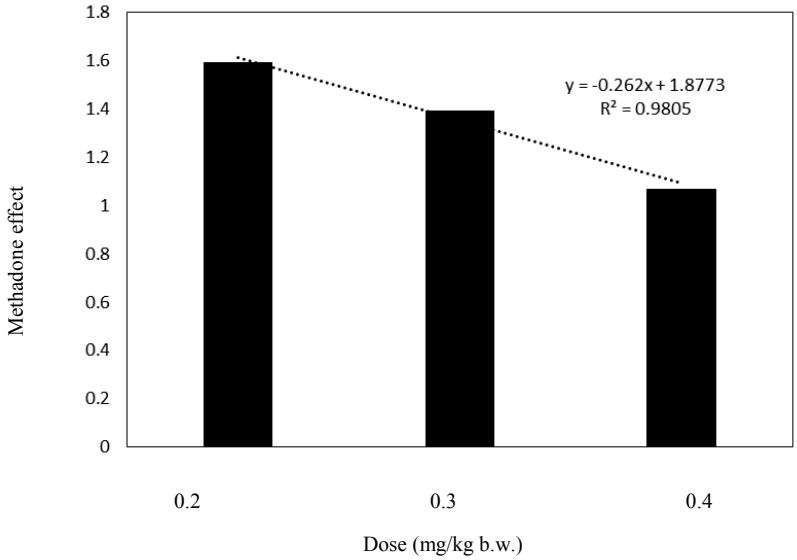

Figure 3.
The linear dependence between methadone effect and dose of hydrophobic methotrexate encapsulated in liposomes for groups 4, 5, 6

By comparing the results obtained in the two experiments (7 days and 14 days after the last methotrexate dose), it was observed that in the case of animals receiving *in vivo* low-doses of hydrosoluble methotrexate, both as liposomes and as injectable solution (group 1 and group 7, respectively), the *in vitro* effects of methadone are different: inhibitory effects occurred after 7 days (experiment 1) and a slight stimulation occurred after 14 days (experiment 2) as it is shown in Figure 4. This type of *in vitro* methadone behaviour in case of *in vivo* low-dose of methotrexate administration indicates that the splenocytes are no longer responsive to methadone at 14 days after the last dose of methotrexate, suggesting the possibility of opioid receptors loss (by internalization or desensitization).

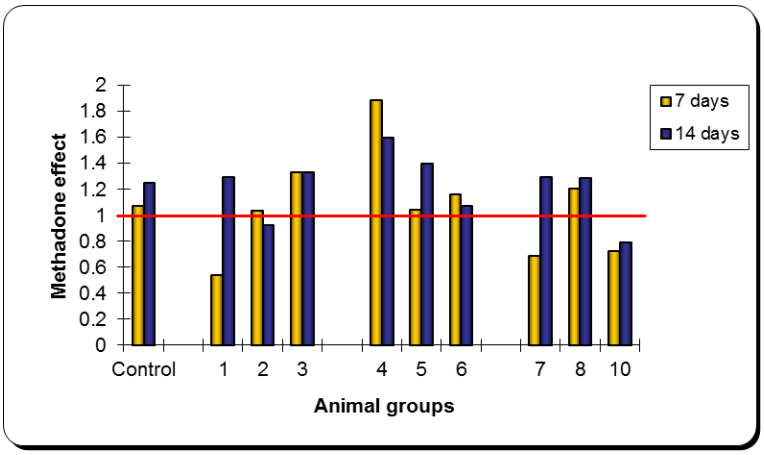

Figure 4.
The comparative effect of methadone on the proliferation of splenic lymphocytes from rats administered with methotrexate. The cells were activated *ex vivo* with ConA

The analysis of the results of the two series of experiments highlighted that methadone inhibits the proliferation of splenic mononuclear cells activated *ex vivo* with Con A and this effect was maintained only in the case of the *in vivo* administration of high doses of hydrosoluble methotrexate injectable solution (group 10) as it is shown in Figure 4. In addition, methadone exerted a similar inhibitory effect (Figure 4) in both Experiments 1 and 2 (7 days and 14 days after the last dose of methotrexate). This suggested that methotrexate injection therapy induces a persistent expression of opioid receptors at high doses of the drug.

Conclusions

The study showed a clear immunosuppressive effect of methadone in rats treated with low doses of hydrosoluble MTX (solution for injection or encapsulated in liposomes). The reversibility of the immunosuppressive effect is possible related to the therapy or to the immune state evolution between two doses of MTX. In rats treated with high doses of MTX solution for injection, methadone exerted a persistent inhibitory effect, while, in rats treated with hydrophobic MTX encapsulated in liposomes, methadone induced lymphocytes activation. This dual effect of an opioid is in line with recent findings of other authors [1, 23].

The therapy with high doses of MTX solution for injection highlights the persistent functional expression of the splenic opioid receptors. The results suggest the possibility of combining the injectable methotrexate therapy with agonists of opioid receptors, in order to simultaneously control the pain and the inflammatory process, in this case, limited by both methotrexate therapy and signalized by the opioid receptors.

Significant progresses have been made for understanding the effects of opioids on the immune responses, but further studies are needed in order to clarify the clinical relevance of these effects.

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