ORIGINAL ARTICLE

Veterinary Research Forum. 2018; 9 (4) 315 - 321
doi: 10.30466/vrf.2018.33110
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Intra-vitreal injection of methotrexate in experimental endotoxin-induced uveitis in rabbit

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Article Info

Article history:

Received: 08 November 2017
Accepted: 09 January 2018
Available online: 15 December 2018

Key words:

Inflammation
Intra-vitreal injection
Methotrexate
Uveitis

Abstract

Uveitis is a major cause of vision loss. Methotrexate (MTX) has been widely used in uveitis due to its relatively safe profile. The purpose of this study was to evaluate the effects of two different dosages of MTX via intra-vitreal administration for treatment of endotoxin induced uveitis (EIU) in an experimental model. Thirty-five healthy rabbits were randomly divided into four groups and all animals were tolerated intra-vitreal injections. The first group received normal saline (NS), the second group received normal saline plus *Salmonella typhimurium* lipopolysaccharide endotoxin (LPS), (NS+LPS), the third group received 400 µg MTX plus LPS (LPS+MTX 400) and the fourth group received 800 µg MTX plus LPS (LPS+MTX 800). Intra-ocular inflammation was evaluated by clinical examination scoring during 7 post-injection days and histopathological examination at the end of study. Kruskal-Wallis and Mann-Whitney U tests were used to compare the histopathological and clinical scores. According to the clinical examinations, all groups demonstrated higher uveitis score than group 1 on first post-injection day. Also, groups 2 and 3 showed greater uveitis score than group 4. On the third, fifth and seventh post-injection days, clinical uveitis score in groups 2, 3 and 4 was significantly higher than group 1. The mean histopathological inflammation intensity scores in groups 2, 3 and 4 were significantly higher than group 1. Single intra-vitreal injection of 400 µg and 800 µg of MTX did not show significant anti-inflammatory effects on EIU in rabbits.

تزریق داخل زجاجیه‌ای متوترکسات در یووییت تجربی ناشی از اندوتوکسین در خرگوش

چکیده

یووییت عامل اصلی از بین رفتن بینایی است. متوترکسات به واسطه ماهیت نسبتاً ایمن آن به طور گسترده در یووییت استفاده شده است. هدف این مطالعه بررسی آثار دو دوز مختلف متوترکسات از طریق تجویز داخل زجاجیه‌ای برای درمان یووییت ناشی از اندوتوکسین در یک مدل تجربی بود. ۳۵ خرگوش سالم به چهار گروه تقسیم شدند وتمام حیوانات تزریق داخل زجاجیه‌ای را متحمل شدند. گروه اول سالین نرمال، گروه دوم سالین نرمال به اضافه اندوتوکسین لیپوپلی‌ساکارید *سالمونلا تایفی‌موریوم* (لیپوپلی‌ساکارید)، گروه سوم ۴۰۰ میکروگرم متوترکسات به اضافه لیپوپلی‌ساکارید و گروه چهارم ۸۰۰ میکروگرم متوترکسات به اضافه لیپوپلی‌ساکارید دریافت کردند. التهاب داخل چشمی از طریق امتیاز بندی معاینه بالینی در طی هفت روز بعد از تزریق و بررسی هیستوپاتولوژیکی در انتهای مطالعه مورد ارزیابی قرار گرفت. آزمون‌های آماری کروسکال والیس و Mann-Whitney U برای مقایسه‌ی امتیازهای بالینی و هیستوپاتولوژیکی استفاده شد. بر اساس معاینات بالینی، تمام گروه‌ها امتیاز بالینی یووییت بالاتری را از گروه ۱ در روز اول پس از تزریق نشان دادند. همچنین، گروه ۲ و ۳ امتیاز یووییت بیشتری نسبت به گروه ۴ نشان دادند. در روزهای سوم، پنجم و هفتم پس از تزریق، امتیاز بالینی یووییت در گروه ۲، ۳ و ۴ به طور معنی داری بیشتر از گروه ۱ بود. میانگین امتیازهای شدت التهاب هیستوپاتولوژیکی در گروه های ۲، ۳ و ۴ به طور معنی‌داری بیشتر از گروه ۱ بود. تک تزریق داخل زجاجیه ای دوزهای ۴۰۰ و ۸۰۰ میکروگرم متوترکسات آثار ضدالتهابی قابل توجهی بر روی یووییت ناشی از اندوتوکسین در خرگوش‌ها نشان نداد.

واژه های کلیدی: التهاب، تزریق داخل زجاجیه‌ای، متوترکسات، یووییت

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Introduction

Uveitis is a major cause of vision loss in adults.[1] It is the third leading cause of blindness that is potentially treatable.[2] Uveitis is inflammation of the vascular uveal tract of the eye including iris, ciliary body and choroid; however, surrounding structures such as retina, optic nerve, vitreous and sclera can also be affected. Clinically, uveitis is classified into four categories of anterior, intermediate, posterior and panuveitis depending on which anatomical structures of the eye are involved.[3] Uveitis comprises a heterogeneous group of diseases with complex pathogenesis. In general, uveitis can be divided into two major forms of origin including infectious and non-infectious uveitis. Non-infectious (or autoimmune) uveitis can be a part of systemic diseases such as juvenile inflammatory arthritis, spondyloarthritis and Behcet's disease. However, in many cases, no underlying cause can be found and uveitis is considered idiopathic.[4]

In infectious uveitis, treating the underlying infection is a critical part of the ocular inflammation treatment. In non-infectious or immune-mediated cases of uveitis, the aim of treatment is to control the ocular inflammation.[5] Although the exact pathogenic mechanisms underlying uveitis are unknown, cytokines playing an important role as mediators of immunologic and inflammatory responses appear to be involved in this inflammatory disorder. Several cytokines including tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-6 and interferon-γ have been demonstrated in ocular tissues obtained from patients with uveitis.[6] Treatment of uveitis is important in order to avoid complications such as posterior synechiae, secondary glaucoma, cataract and macular edema.[4] The first treatment option for non-infectious uveitis is corticosteroids (CS).[7]

Uveitis and its sight-threatening consequences such as cystoids macular edema (CME) may be treated with local (topical, periocular or intra-vitreal) or systemic (oral or intravenous) forms of CS or other immunosuppressive drugs. Local treatment is preferred where possible, especially for unilateral disease. However, this method for CS is associated with an increase in intra-ocular pressure in some patients.[8] Also, it can cause cataract, glaucoma and delay wound healing.[9] In some cases, corticosteroid-sparing medications should be considered. Indications for the use of corticosteroid-sparing agents include the inability to control inflammation with high-dose CS, patients who require chronic oral corticosteroid therapy especially at doses greater than 10 mg per day and the occurrence of adverse effects that require tapering or discontinuation of CS.[7]

In recent years, methotrexate (MTX) has been increasingly used in the treatment of ophthalmic diseases, both locally and systemically.[8] Several reports have shown that MTX is an effective therapy for ocular inflammatory diseases.[10-18] To our knowledge, the effects of intra-vitreal MTX on ocular inflammation in an experimental model of uveitis have not been assessed previously. All the previous works associated with MTX and ophthalmic inflammation have been based on oral medication and the reports of intra-vitreal usage of MTX for subsiding ophthalmic diseases are scarce. To the best of the authors' knowledge, no previous study has investigated intra-vitreal administration of MTX in the management of experimental uveitis.

The EIU is a widely accepted animal model for some types of human uveitis. The inflammatory reaction peaks 24 hr after endotoxin injection and subsides after five to seven days. Some species including New Zealand rabbits, rats and mice are usually used in EIU experiments. *Salmonella typhimurium* endotoxin, the lipopolysaccharide component of the Gram-negative bacterial cell wall, is commonly used for the uveitis induction. The endotoxin is usually injected into the footpad or vitreous body of the animal.[19] This study was designed to evaluate clinical and histopathological effects of intra-vitreal administration of MTX. The purpose of the present study was to evaluate the effects of two different dosages of intra-vitreal MTX for the treatment of endotoxin-induced uveitis (EIU) in an experimental model.

Materials and Methods

Animals. Thirty-five male New Zealand white rabbits weighing between 2.70 and 3.30 kg were used in this study. All rabbits were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research (NRC2011 and AAALAC's)[20] and our study was approved by the Institutional Review Board at the Shiraz University of Medical Sciences (IACUC No: 4687/63).

Procedures. All procedures were carried out under general anesthesia with an intra-muscular injection of a mixture of 25 mg kg^{-1} ketamine hydrochloride 10.00% (Alfasan, Woerden, Netherlands) and 10 mg kg^{-1} xylazine hydrochloride 2.00% (Alfasan). Ophthalmic solution of tetracaine (0.50%; Sina Darou, Tehran, Iran) was administered for topical anesthesia.

Only one eye of each animal (right eye) was used in this experiment. Rabbits were randomly divided into four groups. The first group (n = 5) received 0.10 mL normal saline (NS) intra-vitreally, the second group (n = 10) received 2.00 µg 0.05 mL^{-1} *Salmonella typhimurium* lipopolysaccharide endotoxin (LPS), (L6511; Sigma Chemical, St. Louis, USA) plus 0.05 mL NS (NS + LPS), the third group (n = 10) received 400 µg 0.05 mL^{-1} MTX without preservative (Mylan V.N, Saint-Priest, France) plus 2.00 µg 0.05 mL^{-1} LPS (LPS + MTX400) and the fourth group (n = 10) received 800 µg 0.05 mL^{-1} MTX plus 2 µg 0.05 mL^{-1} LPS (LPS + MTX800). The aseptic preparation

was provided by 5.00% povidone–iodine solution to the conjunctival sac before any manipulation. Injections were performed 2.50 mm posterior to the limbus into vitreaous cavity using a 30-gauge needle. Topical ciprofloxacine 0.30% (Sina Darou) and timolol 0.50% (Sina Darou) were applied before and after the injections and the eyes were monitored by indirect ophthalmoscopy for post-injection complications. All injections were done by a single investigator in a blind fashion.

Clinical evaluations. All animals were examined for signs of clinical inflammation with slit lamp biomicroscopy (Sl 115; Carl Zeiss, Oberkochen, Germany). On days 1, 3, 5 and 7 after the intra-vitreal injection, the degree of the anterior uveitis intensity was clinically assessed in a masked manner by two ophthalmologists. All eyes were evaluated for inflammatory signs. The intensity of the signs of intra-ocular inflammation was graded by using the following clinical scoring system.[19] Iris hyperemia was scored for absence (0), mild (1), moderate (2) or severe (3), pupil was scored for miotic (1) or normal (0), anterior chamber exudate formation was scored for absence (0), mild (1) or severe (2) and hypopyon was scored for none (0) or positive (1). The maximum possible clinical uveitis score, the sum of four parameter scores, was 7.

Histopathological evaluations. On the seventh post intra-vitreal injection day, the rabbits were euthanized by an intra-cardiac injected overdose of sodium pento-barbital. The eyes were enucleated immediately after euthanasia. The enucleated globes were fixed in 10% formaldehyde for three days and processed in the standard manner for light microscopy using hematoxylin and eosin staining. Pupillo-optic sections were cut. All infiltrating inflammatory cells in six random, non-contiguous fields at 200× magnification in both anterior (iris, anterior chamber and ciliary body) and posterior (vitreous and retina) segment parts were counted by a single masked ocular pathologist. Also, pathological uveitis score, the sum of five parameters of anterior chamber, iris, vitreous and retinal inflammation, was evaluated. A semi-logarithmic grading scale using the following criteria,[21] was used to compare the median inflammatory cell infiltrate among the four groups; grade 0: no cells, grade 1: 1 to 10 cells, grade 2: 11 to 30 cells, grade 3: 31 to 100 cells and grade 4: 101 to 300 cells per field. Meanwhile, the ratio of polymorphonuclear cells (PMN) to mononuclear ones was estimated in each case and separately recorded as PMN dominant (PMNs > 80.00%) or mononuclear dominant (mononuclear cells > 50.00%).

Statistical analyses. A Kruskal-Wallis test was used to compare the grading of histopathological and clinical scores and p value of less than 0.05 was considered significant. Mann-Whitney with Bonferroni correction was used to detect which pairs have significant difference and values of $p < 0.01$ were considered significant. The statistical analysis was performed using SPSS software (version 21.0; IBM Corp., Chicago, USA). Comparison of percentage of PMN dominancy between experimental groups was performed by Fisher Exact test.

Results

Clinical findings. Twenty-four hr after intra-vitreal injection, some degrees of ocular inflammation with regard to iris hyperemia, miosis and anterior chamber reaction (ACR) were seen in all eyes injected with LPS (groups 2, 3 and 4). Iris hyperemia, ACR, pupil condition and hyperemia were the parameters that were assessed clinically. Iris hyperemia in all experimental groups (2, 3 and 4) was significantly more severe than group 1 in day 1 ($p < 0.01$). Also, iris hyperemia in groups 2 and 3 was significantly more severe than group 4 in day 1. Iris hyperemia in groups 2, 3 and 4 was significantly more severe than group 1 in day 3 ($p < 0.01$). Iris hyperemia in groups 3 and 4 was significantly more severe than group 1 in day 5 ($p < 0.01$).

The ACR in groups 2, 3 and 4 was significantly more severe than group 1 in days 1 and 3 ($p < 0.01$). Pupil miosis in group 4 was significantly more severe than group 1 in days three, five and seven. Also, pupil miosis in groups 2 and 3 was significantly more severe than group 1 in day 5. Hypopyon was not observed in any group. In subsequent examinations on the third, fifth and seventh post-injection days, rabbits in group 1 showed no clinical sign of ocular inflammation (clinical severity scores were 0), while in the other three groups, the degree of inflammation decreased gradually. Description of median clinical severity score according to the slit lamp examination grading in four groups and the details of comparing clinical uveitis score p value's between all groups are presented in Table 1.

According to the clinical examination, all experimental groups demonstrated higher uveitis score than group 1 on first post-injection day ($p < 0.01$). Also, groups 2 and 3 showed greater uveitis score than group 4 ($p < 0.01$). On the third, fifth and seventh post-injection days, comparison of clinical uveitis score between groups 1 and 2, 1 and 3 and 1 and 4 showed a statistically significant difference ($p < 0.01$). In addition, there was no statistically significant difference with regard to clinical uveitis score between other groups on the third, fifth and seventh post-injection days.

Histopathological findings. Anterior chamber inflammation was significantly higher in groups 2, 3 and 4 compared to group 1 ($p < 0.01$). Iris inflammation was also significantly higher in group 4 compared to group 1 ($p = 0.008$). Ciliary body inflammation and vitreous inflammation were significantly higher in groups 2, 3 and 4 compared to group 1 ($p < 0.01$). Retina inflammation was significantly higher in groups 2, 3 and 4 compared to group 1 ($p < 0.01$). The mean histopathological inflammation uveitis score (including anterior chamber, iris, ciliary body,

Table 1. Comparison of clinical uveitis scores according to slit lamp examination grading between four groups. All data are presented as median (inter quartile range).

Groups	Number	Day 1	Day 3	Day 5	Day 7
NS*	5	1 (1 to 1)	0 (0 to 1)	0 (0 to 0)	0 (0 to 0)
NS + LPS*	10	6 (5 to 6)	4 (3 to 5)	2 (1 to 3)	2 (1 to 3)
LPS + MTX* 400	10	5 (5 to 6)	4 (4 to 6)	3 (2 to 3)	1 (1 to 3)
LPS + MTX 800	10	4.50 (4 to 5)	4 (3 to 4)	3 (2 to 3)	2 (2 to 3)
***p* value†**		< 0.001	0.002	0.003	0.018
Pair-wise comparison ǂ					
NS vs NS + LPS		0.002	0.002	0.002	0.008
NS vs LPS + MTX 400		0.001	0.002	0.002	0.005
NS vs LPS + MTX 800		0.001	0.002	0.001	0.004
NS + LPS vs LPS + MTX 400		0.842	0.521	0.778	0.819
NS + LPS vs LPS + MTX 800		0.008	0.445	0.413	0.762
LPS + MTX 400 vs LPS + MTX 800		0.004	0.149	0.561	0.469

*NS, normal saline; LPS, lipopolysaccharide, MTX; methotrexate; † Calculated by the Kruskal-Wallis test ($p < 0.05$ considered as significant); ǂ Calculated by the Mann-Whitney U test ($p < 0.01$ considered as significant).

vitreous and retina) in groups 2, 3 and 4 was significantly higher than group 1 ($p < 0.01$), (Fig. 1).

Median histopathological uveitis score in groups 1, 2, 3 and 4 animals was 0.20 (range 0.20-0.40), 1.60 (range 1.40-1.80), 1.80 (range 1.80-2.20) and 1.80 (range 1.60-2.00), respectively. However, there were no significant differences between groups 2 and 3, groups 2 and 4 or groups 3 and 4. Histopathological examination (Figs. 2 and 3) revealed that MTX with either dose did not reduce inflammation after single intra-vitreal injection. Regarding type of inflammation, PMN dominated infiltration was seen in the majority of cases in groups 2, 3 and 4 except for three cases in group 2, one case in group 3 and three cases in group 4, in them, lymphocytes were predominant. However, the difference was not statistically significant ($p > 0.05$).

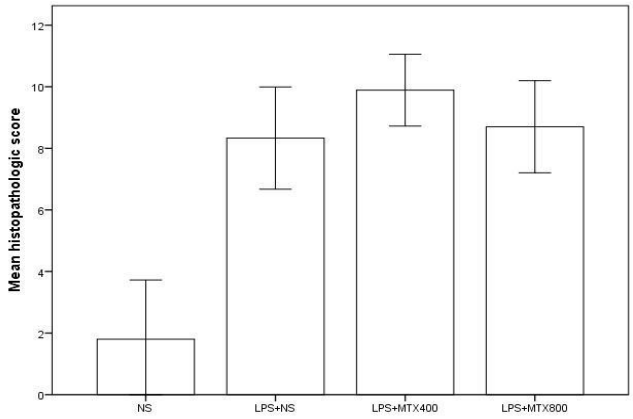

Fig. 1. Mean histopathological severity score of uveitis in groups 1 (NS), 2 (LPS + NS), 3 (LPS + MTX 400) and 4 (LPS + MTX 800) seven days after injection. The mean histopathological inflammation intensity score in groups 2, 3 and 4 was significantly higher than group 1. NS = normal saline; LPS = lipopolysaccharide, and MTX = methotraxte.

Median histopathological uveitis score in groups 1, 2, 3 and 4 animals was 0.20 (range 0.20-0.40), 1.60 (range 1.40-1.80), 1.80 (range 1.80-2.20) and 1.80 (range 1.60-2.00), respectively. However, there were no significant differences between groups 2 and 3, groups 2 and 4 or groups 3 and 4. Histopathological examination (Figs. 2 and 3) revealed that MTX with either dose did not reduce inflammation after single intra-vitreal injection.

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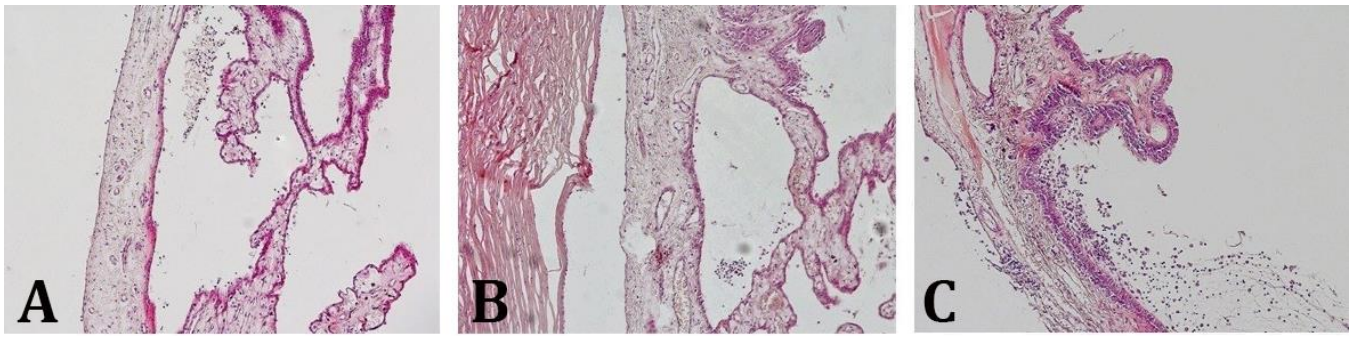

Fig. 2. Photomicrographs of ciliary body of each group seven days after injection. **A)** In group 2 (LPS + NS), ciliary body shows considerable inflammatory cells infiltration in the anterior chamber and ciliary processes. **B)** In group 3 (LPS + MTX 400), eyes show less inflammation in the anterior chamber; however, **C)** increasing dose of the drug in group 4 (LPS + MTX 800) only increased the inflammatory reaction, (Hematoxylin and eosin, 1000×).

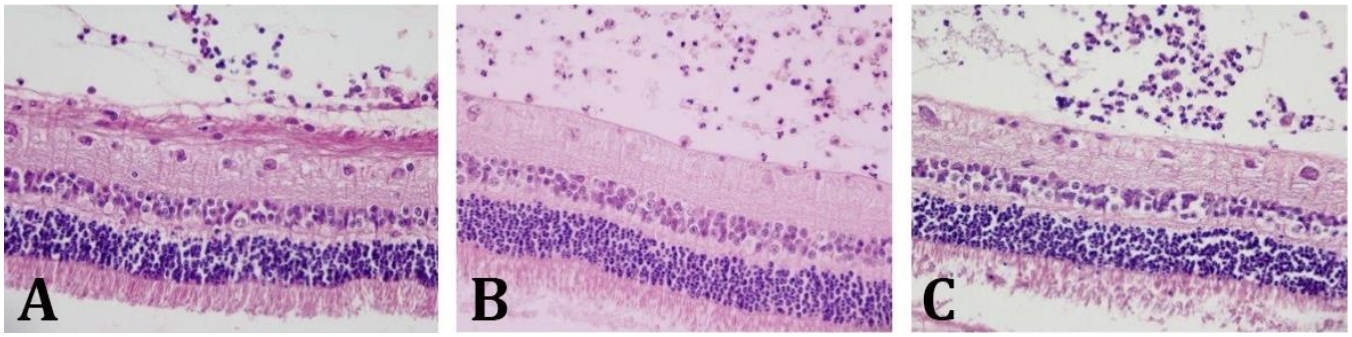

Fig. 3. Histopathological images of retina in different treatment groups. Note the presence of almost equal amounts of acute inflammatory cells and exudate in pre-retinal area and vitreous cavity in control (A) and group 3 (B: LPS + MTX 400) and group 4 (C: LPS + MTX 800), (Hematoxylin and eosin, 400×).

Discussion

The MTX is an antimetabolite agent with anti-inflammatory and immunosuppressive effects. The MTX was introduced in 1948 as an antineoplastic agent[22] and approved by the United States Food and Drug Administration as a treatment for rheumatoid arthritis in 1988.[12] The anti-inflammatory effects of MTX are primarily mediated by the release of adenosine into the extracellular space. Adenosine is a potent inflammatory inhibitor and induces vasodilatation.[23] Adenosine also suppresses the pro-inflammatory mediators such as TNF-α, IL-6, IL-8, macrophage inflammatory protein–1α, leukotriene B4 and nitric oxide while increasing the production of anti-inflammatory mediators IL-10 and IL-1 receptor antagonists. Finally, adenosine lessens the inflammation by decreasing macrophage activation and changing the T helper type 1 response to type 2 in body.[24]

According to the results of clinical examination in present study, all experimental groups demonstrated higher uveitis score than control group and a high dose MTX was able to decrease uveitis score comparing to a low dose MTX on first post-injection day. Clinical uveitis scores between all treated groups were significantly different with control group, on the third, fifth and seventh post-injection days. The largest cohort of patients with ocular inflammatory disease on MTX treatment was described by Gangaputra *et al*.[12] This retrospective study comprised 384 uveitis patients on MTX therapy (including 126 patients with anterior uveitis). In patients with anterior uveitis, a complete remission was achieved in 55.60% of patients within 6 months and 67.20% of patients within 12 months.

A retrospective study by Foeldvari and Wierk evaluated the effectiveness of MTX therapy in juvenile idiopathic arthritis associated with chronic uveitis.[11] The study group comprised 25 patients treated with MTX. Four of 25 patients did not show any significant improvement from MTX treatment and an additional immuno-suppressive medication was needed. Shah *et al*. reported a good clinical response of different inflammatory ocular diseases to low doses of MTX.[15] Kaplan-Kaplan-Messas *et al*. retrospectively studied 39 consecutive patients with uveitis, who were treated with MTX following inadequate control with CS lasting five years.[13] Full or partial control of inflammation was achieved in 23 (79.00%) patients. All patients were still on cortico-steroid therapy when MTX was started.

The first use of intra-vitreal MTX in uveitis was reported in 2006.[25] Hardwig *et al*. reported a retrospective study in patients with uveitis or advanced proliferative diabetic retinopathy, which 400 µg intra-vitreal MTX improved visual acuity (VA) in 75.00% of patients.[25]

Taylor *et al*. reported a prospective case series of 15 patients with uveitis and uveitic CME in 2009, in which intra-vitreal MTX was found to be effective in intra-ocular inflammation control and VA improvement.[8] The authors reported a retrospective study in 2013 which showed intra-vitreal MTX was effective in controlling intra-ocular inflammation and improving vision in 30 of 38 eyes (79.00%).[26] Khalil *et al*. have reported 80.00% improvement in VA after intra-vitreal MTX in patients with Behcet's disease-associated ocular inflammation with posterior segment involvement.[24] A retrospective study by Samson *et al*. suggested that inflammation was controlled by MTX in a large series of patients (76.00%) with chronic uveitis.[14] Inflammation did not respond in 17.00% of the cases. Seventy-two patients (45.00%) had previously taken prednisone or were receiving systemic prednisone at the time of being offered MTX therapy, either pulse oral therapy (58.00%) or chronic daily oral therapy (42.00%).

Bachta *et al*. evaluated prospectively the efficacy of MTX in the treatment of recurrent idiopathic acute anterior uveitis in 22 patients.[10] Remission was achieved in 84.00% of patients and reduced frequency of flares in 16.00% of patients. This study was the only one which focused on acute form of uveitis but shows systemic MTX therapy in doses considered optimal in rheumatic diseases controls inflammation in a one-month period. In contrast to this study, our results determined no clinical improvement after intra-vitreal injection of MTX in EIU, not even in 800 µg group. According to our results, there

was no difference between test and control groups regarding clinical and histopathological findings after treatment of EIU with intra-vitreal injection of MTX. The severity of uveitis in NS + LPS group decreased gradually in one week spontaneously without any medication. This evidence indicates that if the study had been continued by repeated injections of *Salmonella typhimurium* lipopolysaccharide into the vitreous to extend acute phase of uveitis more than one week, the anti-inflammatory effects of MTX might have been manifested by decreasing clinical signs or histopathological findings of uveitis. Short duration of the research was the limitation of our study. Uveitis could be prolonged experimentally for a longer period in order to extend the maximum three to five days of disease process.[27] In the previous studies, patients were treated with different doses of MTX. More importantly, in almost all previous studies, some patients continued other systemic drugs such as cyclosporine or prednisolone or were treated with a combination of two immunosuppressive drugs while being treated with MTX. Thus, the observed beneficial effect might not be attributed completely to MTX therapy. Also, in previous studies, the patients included were suffering from different types of ocular inflammation and the dosages used differed significantly between the studies. Intra-vitreal injection of MTX has been used in human studies for treatment of non-infectious uveitis. Bae and Lee have demonstrated that intra-vitreal injection of 400 µg MTX in patients with refractory retinal vasculitis was effective in inflammation control and was associated with a significant reduction of intra-ocular levels of IL-6 and IL-8.[28] In another study by Taylor *et al*., it was suggested that effect of administration of 400 µg MTX could be comparable with intra-vitreal prednisolone administration.[26] This dose and route and even doubled dose had no efficacy in our study in an EIU in rabbit. The acute nature of inflammatory reaction induced in this study explains why mononuclear dominancy wasn't observed in histopathological examination. Longer follow up may be needed to see the effects of injection in chronic uveitis and can be a base for future studies.

In conclusion, intra-vitreal injection of MTX did not show any beneficial effect in management of acute uveitis in an experimental model in rabbits. However, due to diversity in etiologies and types of uveitis, intra-vitreal MTX may be beneficial in certain cases. Clearly, there is a demand for future experimental and clinical studies in order to clarify the role of intra-vitreal MTX in uveitis management.

Acknowledgments

We are grateful to Mr. Omid Koohi for his assistance and cooperation in Laboratory Animal Center of Shiraz University of Medical Sciences, Shiraz, Iran during the present study.

Conflict of interest

The authors declare no conflict of interest.

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