Uterine leiomyomas (fibroids) are the most common benign tumors in women of reproductive age (1). Surgery, in the form of myomectomy or hysterectomy, is currently the primary treatment for uterine fibroids and so these tumors cause hysterectomy in the 77% of cases (2). The initiating factors that lead to the development of fibroids are not well understood.

However, some evidence supports that ovarian steroids, such as estrogen and progesterone, are the important factors for leiomyoma growth (3). Over the past decade, analogs of GnRH (Gonadotropin Releasing Hormone) have been commonly used in the conservative treatment of fibroids. Continuous application of GnRH analogs reliably and reversibly suppress the gonadal function causing the condition similar to hypogonadotropic hypogonadism and affect the reduction of the myoma size. Some literature data indicate that their effect is achieved by causing apoptosis in myomas (3), but the role of apoptosis in uterine myomas is still unknown. Our preliminary clinical study showed that local application of low dose of methotrexate (MTX) on the eighth day of menstrual cycle, during three consecutive cycles, reduced the volume of uterine myomas (20-30%), decreased menstrual bleeding, improved hematological status of patients and most importantly reduced incidence of hysterectomy and other invasive methods. This approach did not cause menopause and its side effects and was able to diminish the use of hormonal substitution therapy (4). MTX is used in the treatment of numerous lymphomas, osteosarcomas, breast, ovarian, lung and urinary bladder cancers (5-10). Furthermore, it serves as an immunosuppressive agent in the treatment of rheumatoid arthritis (11) and for hyperproliferative epidermal cell disorders (psoriasis) (12) that are often associated with the increased angiogenesis (13). MTX competitively inhibits dihydrofolate reductase (DHFR).

**METHOTREXATE AND MYOTREXATE INDUCE APOPTOSIS IN HUMAN MYOMA FIBROBLASTS (T hES CELL LINE) VIA MITOCHONDRIAL PATHWAY**

TATJANA KASTRATOVIĆ¹*, SLOBODAN ARSENIJEVIĆ¹, ZORAN MATOVIĆ², MARINA MITROVIĆ¹, IVANA NIKOLIĆ³, ZORAN MILOSAVLJEVIĆ⁴, ZORAN PROTRKA¹, MARIJA ŠORAK¹ and JANKO DURIĆ⁵

¹Department of Obstetrics and Gynaecology, Faculty of Medical Sciences, ²Faculty of Chemistry, ³Department of Biochemistry, Faculty of Medical Sciences, ⁴Department of Histology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

**Abstract:** Uterine leiomyomas (fibroids) are the most common benign tumors in women of reproductive age. Although the local application of low doses of methotrexate (MTX) is used as an effective treatment of the myomas, myotrexate could be a promising new drug. This study investigated the cytotoxic and apoptotic effects of both MTX and myotrexate in human fibroblasts derived from the uterine fibroids (T hES cell line). The myotrexate adduct is an aqueous solution of MTX and L-arginine. Cells were treated with a graded concentrations of both MTX and myotrexate (0.1-16 µM) for 24 h. The cytotoxicity was assayed by MTT test, apoptosis was evaluated by Annexin V-FITC assay and their possible role in apoptosis was determined by immunofluorescence. Both MTX and myotrexate induced apoptosis in T hES cells in a dose dependent manner (p < 0.001). Myotrexate significantly increased the percentage of AnnexinV positive cells, BAX/Bcl-2 ratio and subsequent caspase-3 activation compared to the MTX treated cells (p < 0.05). Both MTX or myotrexate treatment showed a diffuse staining of cytochrome c indicating its release from mitochondria to the cytosol, suggesting that their mechanisms of action most likely involves the mitochondrial apoptotic pathway.

**Keywords:** MTX, myotrexate, apoptosis, Bcl-2 family, cytochrome c
and it prevents folic acid’s participation in the de novo synthesis of nucleic acids and proteins (14), especially during the S-phase of the cell cycle. Thus, it achieves the greatest toxic effect on rapidly dividing cells. The exact mechanism of action of MTX is not fully understood. Recently, Spurlock et al. have shown that MTX did not induce apoptosis directly, but through indirect cells stimulation that led to the cells induced sensitivity to apoptosis, possibly through mitochondrial or death receptor pathway through Jun N-terminal kinase (JNK)-dependent mechanisms (15). Moreover, Savion et al. showed that MTX was directly involved with BAX regulation, a molecule of Bcl-2 family group (16), and key regulators of the apoptotic processes (17). One of MTX’s shortcomings is the ability of tumor cells to continually develop resistance to it, possibly due to the amplification of the DHFR gene, or gene mutation that impairs MTX’s binding. Using computer programs (Discovery Studio Visualizer and Hyperchem), we identified that by binding to DHFR, MTX is placed into a large hydrophobic niche (18, 19) where is predominantly surrounded by amino acid residues of L-arginine in a 1 : 5 ratio with which produces hydrogen and Van der Waals links which are most responsible for secondary and tertiary structure and hence activity of the substrate.

Based on this observation, in our laboratory we synthesized the adduct myotrexate as an aqueous solution of MTX and L-arginine. Combining the results of NMR spectroscopy, quantum mechanical modelling and molecular dynamics simulations we got 3D look of solvating myotrexate. The final results show that the drug compounds in solution are unaltered on a molecular level, but held each other with strong inter- and intra-molecular hydrogen bonding. Hydrogen bonds are particularly pronounced between the deprotonated carboxyl groups of MTX and guanidine group the three of the five molecules of L-arginine.

Here, we investigated the effect of myotrexate on the basic cell component of myomas fibroblasts. Additionally, knowing that Bcl-2 group molecules play a role in the MTX apoptotic signalling pathway, we investigated if that the same would apply to myotrexate.


The aim of this study was to investigate and compare both the cytotoxic and apoptotic effects of methotrexate and myotrexate and their potential role in apoptosis.

**MATERIALS AND METHODS**

**Cell line**

T hES cell line (T hES; ATCC®: CRL-4003tm) human fibroblasts derived from uterine fibroids, immortalized with human telomerase reversible transcriptase. Cells were grown in DMEM (Dulbecco’s modified Eagle’s medium, Sigma Aldrich, Germany) enriched with L-glutamine (2 mM/L, Invitrogen, USA), 1% nonessential amino acids (Sigma Aldrich, Germany), 1% ITS (insulin transferin supplement, BD Biosciences, USA), penicillin-streptomycin (1 mM/L, Sigma Aldrich, Germany) and 10% FBS (fetal bovine serum, Sigma Aldrich, Germany). Cells were incubated at 37°C, atmosphere of 5% CO₂.

Pharmaceutical composition (myotrexate) was prepared by dissolving 10 g of L- arginine in a 600 cm³ of distilled water and the vessel was softly shaken until the L-arginine was completely dissolved. After that, 5 g of methotrexate was added and the vessel was softly shaken until all methotrexate was dissolved, and following that another 385 cm³ of distilled water was added and the obtained solution was autoclaved. An aqueous solution of methotrexate and L-arginine prepared in this manner contains methotrexate and L-arginine in molar ratio of 1 : 5.

Cells were treated with pre-sterilized methotrexate (Sigma Aldrich, Germany) and/or myotrexate in concentrations of 16, 12, 8, 4, 2, 1, 0.1 and 0.01 µM and L-arginine (Sigma Aldrich, Germany) in concentrations 80, 60, 40, 20, 10, 5 and 0.5 µM.

Cytotoxic effect of these compounds was examined by MTT cytotoxicity assay. Briefly, in a 96-well microtiter plate, cells were resuspended in a medium (1.8 × 10⁴ cells/200 µL medium), treated with tested substances (MTX, myotrexate and L-arginine in appropriate concentrations for 24 h), and incubated with MTT solution (5 mg/mL MTT dissolved in PBS) for 4 h (37°C, 5% CO₂). After centrifugation (1000 rpm, 5 min) and removal of supernatant, cells were resuspended in 200 µL DMSO (Sigma Chemical, St. Louis, MO) per well and incubated for 30 min on a shaker, at room temperature, and away from light. The optical density (OD) was measured at wavelength 595 nm (multimode microplate detector, Zenuth 3100). The percentage of unviable cells was calculated by the formula: Cytotoxicity (%) = [1 - (experimental group (OD)) / (control group (OD))] × 100.

Investigation of apoptotic effect of these substances was performed by Annexin V-FITC assay.
Briefly, cells (5 × 10^5 cells/800 µL) were treated with tested substances (MTX, myotrexate and L-arginine in appropriate concentrations for 24 h), then washed two times with cold PBS, and resuspended in a binding buffer. Annexin V-FITC (5 µL) and propidium iodide (5 µL) was added in 100 µL of that solution. After 15 min, away from light, and at room temperature, 400 µL of binding buffer was added and cells were analyzed on FACS (Becton-Dickinson, FACS-Calibur, Mountainview, CA, USA) within one hour.

The mechanism of apoptotic effect was determined by fluorescence microscopy.

Briefly, cells (2 × 10^4 cells/400 µL) were seeded on sterile cover slips and incubated for 24 h (37°C, 5% CO₂) to achieve confluence of 80%. Cells were then treated with tested substances (MTX and myotrexate) for 24 h, washed in PBS, and fixed in 4% paraformaldehyde, 23 mM NaH₂PO₄ and 77 mM Na₂HPO₄ (pH 7.3). Cells were permeabilized for 10 min with 0.2% Triton-X/PBS, incubated for 30 min in a blocking buffer (10% FCS, 0.1% Triton X-100/PBS), washed and then incubated for one hour with different anti-rabbit primary antibodies: Bax (N20, sc-493, Santa Cruz Biotech. Inc.), Bcl-2 (DC21, sc-783, Santa Cruz Biotech. Inc.), caspase-3 (9661, Cell Signalling Technology, USA) and anti-mouse antibodies: cytochrome-c (G7421, Promega, USA), β-actin (A5316, Sigma Aldrich, Germany).

After the incubation, cells were washed three times in PBS, incubated and stained with fluorescent secondary antibodies Alexa 488 (11001, Invitrogen, USA) and Cy3 (C7604, Sigma Aldrich, Germany) for one hour away from light at room temperature. Fluorescence of the cells was observed at 100x and 400x magnifications on Olympus (model BX51). ImageJ was used for image analysis.

Student’s t-test or nonparametric Mann-Whitney’s sum rank tests were used for statistical analysis. Results were analyzed by commercial software package SPSS (version 13) and are presented as the mean ± SD (standard deviation) and statistical significance was determined by the level of p < 0.05.

Figure 1. Cytotoxic and apoptotic effects of MTX, myotrexate and L-arginine on fibroblasts. Cells were treated with various concentrations of MTX, myotrexate and L-arginine for 24 h at 37°C. A) represent cytotoxic effect of those agents and B) comparison of cytotoxic effect of MTX and myotrexate. Cell viability was determined by MTT assay. Results are presented as the mean values of five independent experiments for each concentration ± standard deviation (SD). Statistical analysis of the presented relationships was performed with Student’s t-test for independent samples and statistical significance was determined by the level of p < 0.05. C) represent apoptotic effect of those agents and D) comparison of apoptotic effect of MTX and myotrexate. FACS analysis was performed with Annexin V-FITC and propidium-iodide. Results are presented as the mean values of three independent experiments for each concentration ± SD. Statistical analysis of the presented relationships was performed with Student’s t test, the Mann-Whitney’s test for independent samples, and statistical significance was determined by the level of p < 0.05.
RESULTS

The cytotoxic effects of methotrexate (MTX), myotrexate and L-arginine on myoma fibroblasts

To investigate the cytotoxic activities of the MTX, myotrexate and L-arginine on cultured myoma fibroblasts, T hES cells were treated for 24 h with graded concentrations of those drugs (concentration range from 0.01 µM to 16 µM vs. from 0.5 µM to 80 µM) and the number of viable myoma cells was determined by MTT assay. Differences in the number of viable cells between the treated groups and the cells grown in complete medium were statistically significant (p < 0.05). MTX, myotrexate and L-arginine significantly decreased the cell numbers compared to control group (untreated cells) in dose dependent manner (p < 0.001). It was noted that L-arginine exerted the greatest cytotoxic effect on those cells. Cells treated with myotrexate at concentrations of 2, 8, 12, and 16 µM showed statistically significant decrease in the number of viable myoma cells compared to the cells treated with the same concentrations of MTX (p < 0.05); hence, myotrexate exerted the greater cytotoxic effect on T hES cells compared to methotrexate (Fig. 1).

MTX, myotrexate and L-arginine induce apoptosis in myomas fibroblasts

Since MTX, myotrexate and L-arginine demonstrated the cytotoxic effects on myoma fibroblasts, next we investigated the molecular type of cell death induced in these cells by those agents. Thus, T hES cells were treated with graded concentrations of MTX and myotrexate (from 1 to 16 µM) and L-arginine (from 5 to 80 µM) for 24 h and apoptosis was assessed with Annexin V/propidium iodide assay by flow cytometry. The apoptotic cells were defined as any cells colocalized with Annexin V in T hES cells. Induction of apoptosis by MTX,
myotrexate and L-arginine in THES cells was statistically higher than in the control cells in a dose dependent manner (p < 0.001). L-arginine had the greatest effect of apoptosis on this cell line. Furthermore, myotrexate treatment of THES cells, particularly at the concentrations of 8, 12 and 16 µM, significantly increased the percentage of AnnexinV positive cells compared to the MTX treated cells (Fig. 1).

Moreover, by examining the morphology of THES cells treated with both agents at the concentration of 16 µM, we observed typical apoptotic morphological changes, such as cell rounding, condensation and detachment of cells compared to control. Here, myotrexate displayed stronger apoptotic morphological changes in the cells compared to MTX treated cells (Fig. 2). These results suggest myotrexate to be more efficient apoptotic agent on the cultured myoma fibroblasts than MTX.

**MTX and myotrexate induce the decrease of Bcl-2 expression**

Cell survival is enabled by anti-apoptotic protein Bcl-2 through the inhibition of apoptosis. The effects of different cytotoxic stimuli that induce apoptosis in the cells also lead to the reduction of the expression level of Bcl-2. Thus, we examined the effects of MTX and myotrexate stimulation of THES cells on the total expression levels of endogenous Bcl-2 by immunofluorescence. THES cells treated either with 8 µM concentration of MTX or myotrexate demonstrated significantly reduced expression of Bcl-2 compared to untreated cells by 19.54 ± 11.57% in MTX and by 71.35 ± 8.7% in myotrexate (p < 0.05; Fig. 3).

**MTX and myotrexate treatment of myoma fibroblasts induces the activation of pro-apoptotic protein BAX**

Apoptosis is regulated by Bcl-2 family members such as proapoptotic BAX that upon induction of apoptosis is activated and recruited into the outer bilayer of mitochondria causing its permeabilization. To investigate the possible role of BAX in our study, we tested the effect of MTX and myotrexate on BAX activation and its recruitment into the mitochondrial outer membrane by immunofluorescence. Our results demonstrated that both MTX and myotrexate at 8 µM
concentration induced the activation and incorporation of pro-apoptotic BAX into mitochondria of hES cells compared to the control cells. Whereas the percent of cells expressing active BAX in cells treated with either MTX or myotrexate was 19.5 ± 3.4% and 32.42 ± 4.45%, respectively, the percent in untreated cells was only 3.19 ± 1.1% (Fig. 4). Therefore, both agents induced the activation of BAX; however, this effect was significantly greater in myotrexate treated cells compared to MTX treated cells.

MTX and myotrexate induce the release of cytochrome c from mitochondria

Following the stimulation of hES cells with either MTX or myotrexate, activation and recruitment of BAX occurred into the mitochondrial outer membrane. Therefore, we further examined the effect of these drugs on the cytochrome c release from mitochondria. This was examined by immunoflourescence in both untreated cells and cells treated with MTX and myotrexate at the concentration of 8 µM (Fig. 5). hES cells stimulated with either MTX or myotrexate showed diffuse staining of cytochrome c indicative of its release from mitochondria to the cytosol. However, the untreated hES cells demonstrated a punctuate staining typical for the cytochrome c localized in the intermembranous space of mitochondria.

MTX and myotrexate induce caspase-3 dependent apoptosis

To investigate the apoptotic mechanisms in the cells treated with MTX and myothrexate, next we analyzed the activation of caspase-3 by immunofluorescence. Our results demonstrated that both MTX and myotrexate treatment of the cells caused morphological changes in the cytoskeleton of fibroblasts as well as the activation of caspase-3, whereas the same changes were not detected in the untreated cells (Fig. 6). Percentage of cells expressing the active caspase-3 when stimulated with either MTX or myotrexate was 5.72 ± 2.05%, and 37.36 ± 5.3%, respectively (p = 0.008). The cell activation of caspase-3 in untreated samples was 3.41 ± 1.13%, a

Figure 4. Activation of pro-apoptotic protein BAX following MTX and myotrexate treatment. ThES cells were treated with 8 µM methotrexate and 8 µM myotrexate for 24 h. Cells were then immunostained and signal quantified. Top panel represents immunostaining of active form of pro-apoptotic BAX so that in A) are untreated cells, B) cells treated with MTX, and in C) cells treated with myotrexate. Cells were observed under magnification 100×. Bottom panel (D) represents quantified immunostaining results on the same number of cells in three different visual fields with ImageJ software. The activation of BAX was significantly greater in myotrexate-treated cells compared to MTX-treated cells. Statistical analysis was performed with Student’s t test for independent samples and statistical significance was determined by the level of p < 0.05.
Methotrexate and myotrexate induce apoptosis in human myoma fibroblasts. Caspase-3 activation in cells treated with myotrexate was statistically significantly higher ($p < 0.05$) when compared to the MTX treated cells.

**DISCUSSION**

Although methotrexate (MTX) is commonly used in the treatment of numerous neoplastic and hiperproliferative disorders, our preliminary clinical study showed that local application of low dose of this antimetabolite agent could be used for myomas treatment as well. However, growing resistance to MTX has been reported for various cancers (20, 21). To overcome the resistance we sought to investigate the possibility of improving MTX’s effects. Using computer programs, we identified that MTX binds L-arginine through hydrogen and Van der Waals forces in a 1:5 ratio inside a hydrophobic pocket of the DHFR enzyme. Thus, we synthesized myotrexate, an aqueous solution of MTX and L-arginine and tested its efficacy on T hES cell line.

Analyzing the percentage of apoptotic cells, it was observed that the adduct myotrexate exhibited greater apoptotic effect compared to MTX and the statistical significance of this difference rises with increasing concentrations of these substances. Spectrophotometric analysis of myotrexate showed that MTX created strong hydrogen bonding with three molecules of L-arginine leading to its conformational change. As the concentration increases, differences in their apoptotic effect also increase, so might be assumed that two molecules of L-arginine, which are free and the number of which increases with rising concentration, contribute to the better apoptotic effect of myotrexate. MTX, which is in clinical use, is actually the sodium salt of MTX. By replacing NaOH with L-arginine to make it soluble (because MTX is insoluble in water) we have contributed to the improvement of its efficiency.

L-arginine is a basic semi-essential amino acid serving as a precursor for many important molecules for cellular physiology (proline, glutamate, creatine, nitric oxide (NO) and polyamines), making L-arginine one of the most versatile amino acids (22). The plasma concentration of L-arginine is about 200 µmol/L in humans (23). In some cell types, L-arginine, NO, and polyamines stimulate cell proliferation and reduce apoptosis (24) and inhibit cell proliferation and promote apoptosis in others (25, 26). Chen et al. have shown that supraphysiological concentration of L-arginine led to apoptosis of human choriocarcinoma cells (cell line JAR) (27) but this study have shown that L-arginine added to the culture media at subphysiological concentrations induced apoptosis in T hES cells as well.

Methotrexate’s immunosuppressive, anti-inflammatory, anti-proliferative and cytotoxic effects are consequences of apoptosis (28, 29).

Some studies have shown that MTX could induce apoptosis through Fas / Fas- ligand system (30) and the others demonstrated that it activated apoptosis via the mitochondrial pathway (27). Morphological changes, including a notable decrease in cell size, increase in granularity - phenomena that may implicate apoptotic death, cell shrinkage and cytoplasmic condensation as well as Annexin V test showed that apoptotic process was notable in T hES cells after treatment by MTX and myotrexate. In cells treated with tested substances, the release of cytochrome c from intermembranous space of mitochondria into the cytosol was observed, suggesting that apoptosis in these cells was realized through, so called, internal, mitochondrial pathway. After release into the cytoplasm, cytochrome c forms a complex with an enzyme called apoptosis activating factor-1 (Apaf-1) with consumption of energy (ATP). The complex binds to caspase-9, forming the apoptosome - oligomeric complex of cytochrome c/Apaf-1/cas-
pase-9. This complex primarily activates caspase-9, then binds and activates the effector caspase-3 and -7 (31). Activation of effector caspase-3 further causes the splits of actin fibers, subsequently leading to the formation of apoptotic bodies confirming that cells are succumbed to irreversible apoptotic process. The statistically significant increase in Annexin V positive cells, levels of activated caspase-3 and pronounced morphological changes in myotrexate treated cells indicated a stronger apoptotic effect of this agent compared to MTX. One of the key regulators of the apoptotic process is the Bcl-2 family proteins consisting of two opposing groups: death antagonists (Bcl-2, Bcl-XL, Mcl-1) and death agonists (Bax, Bak, Bcl-XS) (32) so, cell apoptosis depends on the

Figure 6. Activation of effector caspase-3 apoptotic protein following MTX and myotrexate treatment. Cells were treated with 8 µM MTX and 8 µM myotrexate for 24 h and assayed for caspase-3 activation and their morphological changes by immunofluorescence. On top figure, panels (A-C) depict untreated cells, (D-F) cells treated with MTX, and (G-I) cells treated with myotrexate. Left column (A, D, G) was stained with β-actin, middle column (B, E, H) was stained for anti-active caspase-3, and left column (C, F, I) present an overlay of those two. Cells were observed under 400× magnification. Bottom figure (J) represents quantified immunostaining results on the same number of cells in three different visual fields with ImageJ software. Caspase-3 activation in cells treated with myotrexate was statistically significantly higher compared to the MTX treated cells. Statistical analysis was performed using the Mann-Whitney’s test for independent samples and statistical significance was determined by the level of p < 0.05
Methotrexate and myotrexate induce apoptosis in human myoma fibroblasts...

ratio of these protein groups (33). However, the exact mechanism of apoptosis regulation by Bcl-2 family is still unknown and being intensively investigated (34, 35). The fibroid cells have demonstrated increased Bcl-2 protein expression compared to the normal myometrial cells of the same uterus and the enlarged expression of Bcl-2 protein in leiomyoma cells is the molecular base for the increased proliferation of myocytes similar to the smooth-muscle cells of myometrium (36).

The influence of sex hormones (estrogen and progesterone) on the expression of pro- and anti-apoptotic proteins is well known (37-39). The treatment of myomas with GnRH was based on the indirect influence on these proteins and predominantly by reducing the level of sex hormones (40). However, in this in vitro investigation we presented a completely different approach to the treatment of fibroids by direct action on Bcl-2 protein family. Our results showed that MTX and myotrexate reduced the level of anti-apoptotic (Bcl-2), increased the level of pro-apoptotic proteins and the increase in BAX/Bcl-2 ratio led cells to apoptosis. Myotrexate was more efficient with the increase of this ratio; therefore, it could be a better potential apoptotic agent. So far, there are no known literature data on the effects of MTX and myotrexate on T hES cell line.

CONCLUSION

The findings presented in this study indicated that MTX and myotrexate exhibited cytotoxic effect on myoma fibroblasts (T hES cell line) and this effect was achieved by the induction of apoptosis via caspase dependent mitochondrial pathway and due to an imbalance in the expression of pro-apoptotic and anti-apoptotic proteins.

Myotrexate exhibited greater apoptotic effect compared to MTX partially due to conformational changes and partially due to the effect of free molecules of L-arginine.

This study opens up the possibility for further in vivo investigations that could indicate that myotrexate might be a potential new drug not only for the conservative treatment of myomas, but also as a possibility of applying this drug in the treatment of the other disorders where MTX is commonly used.

REFERENCES


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