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SENSITIVITY OF HUMAN LARYNGEAL SQUAMOUS CELL CARCINOMA HEP-2 TO METROTEXATE CHEMOTERAPY

A.L.S. Galbiatti[1,], H. C. Caldas[2], J. A. Padovani Junior[3], E.C. Pavarino[1], E.M. Goloni-Bertollo[1,*]*

[1]Genetics and Molecular Biology Research Unit, Upgem, FAMERP, São José do Rio Preto Medical School, Brazil

[2]Laboratory of Experimental Immunology and Transplantation, Litex, FAMERP, São José do Rio Preto Medical School, Brazil,

[3]Otorhinolaryngology and Head and Neck Surgery Department of University São José do Rio Preto Medical School, FAMERP, Brazil

Aim: **Methotrexate (MTX) is an antifolate agent that acts inhibiting purine and pyrimidine synthesis. The objective of the study was to evaluate the viability of Hep-2 human laryngeal cancer cells to the treatment with MTX chemotherapy *in vitro*. *Methods:* Cultured Hep-2 cells were treated with 0.25, 25.0 and 75 µM MTX for 24 h, and their viability was evaluated with Bcl-2-FITC antibody in flow cytometry. *Results:* The numbers of viable Hep-2 cells after 24 h treatment with 0.25, 25.0 and 75.0 uM MTX were 85.43%, 22.46% and 8.42%, respectively ($p < 0.05$). Therefore, MTX possesses a dose-dependent effect on viability of Hep-2 cells *in vitro*. *Conclusion:* The highest MTX concentration is associated with highest tumor cell sensitivity of human laryngeal cancer cells of Hep-2 line.**

Key Words: **cell line, laryngeal cancer, methotrexate, dose-response relationship, flow cytometry.**

Head and neck cancer (HNC) includes tumors of pharynx, oral cavity and larynx. The treatment of these tumors may be surgery, radiotherapy and chemotherapy [1–3]. Methotrexate (2,4-diamino,N10-methylpteroyl glutamic acid) (MTX) is an antiproliferative and immunosuppressive chemotherapeutic agent widely used against a broad spectrum of diseases, including HNC [4, 5]. It acts via inhibition of the synthesis and conversion of folate derivatives responsible for providing methyl groups for the nucleotides synthesis and DNA methylation reactions [6–13]. Although chemotherapy presents good results, tumors may develop resistance to antifolate agents. A number of factors are critical for a favorable clinical outcome for MTX therapy, in particular acute toxicity, side effects, and drug resistance development [5, 14–16]. The current study was undertaken to evaluate *in vitro* the human larynx squamous cell carcinoma Hep-2 cell line sensitivity to the MTX treatment. The cell line was cultured in Dulbecco Medium (D-MEN 00068 medium, Cultilab), supplemented with 10% fetal bovine serum (FBS, Cultilab), 2 µM glutamine (Cultilab), 100 U/ml of penicillin, 100 U/ml of streptomycin, 1 µM sodium pyruvate (Sigma–Aldrich) and 1 µM non-essential amino acid (Sigma–Aldrich) in a humidified 5% CO_2/95% air atmosphere at 37 °C. MTX concentrations of 0.25 µM, 25 µM, and 75 µM were calculated according to Pai et al. [13]. Hep-2 cells were incubated with the mentioned MTX concentrations for 24 h, while the control cells were cultured in MTX-free medium. Cell viability was measured by flow cytometry (FACS caliber- Becton Dickinson Immunocytometry Systems, San José, USA) with double staining with fluorescein isothiocyanate (FITC)/Bcl-2 according to manufacturer's manual (Santa Cruz Biotechnology, Inc). Each experiment was performed in triplicate. The normal distribution of the samples was verified with Normality tests (Shapiro — Wilk's test and Kolmogorov — Smirnov test). Statistical analysis was performed by nonparametric methods based upon the comparison between the groups. The effects for MTX concentrations in the cell viability were evaluated independently by Kruskal-Wallis test (Control group x 0,25 uM MTX concentration / Control group x 25 uM MTX concentration / Control group x 75 uM MTX concentration). For comparison of the variables between groups exposed with MTX and free-MTX group we used the Mann — Whitney test. The Spearman correlation degree between variables of interest was calculated by Spearman test.

The results of flow cytometry analysis with FITC/Bcl-2 double staining showed that 92.6% of control cells were vital, while the numbers of viable Hep-2 cells after 24 h treatment with 0.25; 25.0 and 75.0 uM MTX were 85.43; 22.46 and 8.42%, respectively (Figure, Table) ($p < 0.05$). The Shapiro — Wilk's test indicated that there was a normal distribution for the groups ($p = 0.180$). The Kolmogorov — Smirnov test confirmed that all samples presented significance level of 5% for groups (K-S = 0.383; $p = 0.008$) (Table). The Kruskal-Wallis test indicated a significant effect of MTX in the cell viability (H = 9.00, $p = 0.003$). The value of Mann — Whitney test showed significant results (p= 0.0304). The Spearman correlation between frequencies of cells exposed with MTX and unexposed showed an interaction between these cells (r = 0.50). Our study confirmed that cells were more sensitive and became less resistant to the MTX chemotherapy as dose was increasing. Moreover, there was a correlation between cells exposed frequencies with MTX and unexposed cells; literature

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*Correspondence: E-mail: analivia_sg@yahoo.com.br; eny.goloni@famerp.br
Abbreviations used: HNC – head and neck cancer; MTX – methotrexate.

data show MTX concentration correlates with drug therapeutic efficacy [17]. These results suggest that MTX has substantial antiproliferative activity and is used effectively as a chemotherapy agent in the treatment for solid-organ neoplasms and the treatment is more efficient when the dose is increased. However, the pilot study of Pai et al. [13] that evaluated the sensibility of oral cancer cells to MTX *in vitro* and its association with clinical response to MTX in oral cancer showed that there is differential sensitivity to MTX among the various tumor cells in the *in vitro* assay, and these data had significant correlation when compared with clinical outcome for 7 out of 10 patients. MTX is an antimetabolite, analogous to folate, that competitively inhibits diidrofolatoredutase (DHFR) enzyme activity, essential for nucleotides purines and thymidylic acid biosynthesis, interfering with DNA synthesis. [10, 18, 19]. Although we found that MTX treatment is highly effective in HNC cells, data confirm that high-dose MTX schemes may arrest normal epidermal cell proliferation and cause direct cell toxicity [20]. Toxicity is increased by folic acid deficiency or by medications such as barbiturates and nitrofurantoin, which impair folic acid absorption [10, 20]. However, it has been documented that folic acid (1 to 5 mg/day) supplementation helps to prevent MTX associated toxicities and concomitant use of either folic acid with methotrexate has no impact on the therapeutic efficacy of MTX in multiple clinical trials and meta-analyses [10, 21].

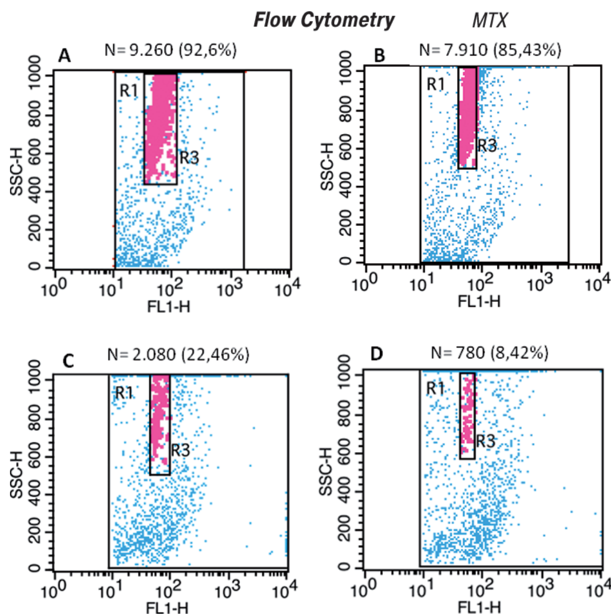

Figure. Flow cytometry analysis of Hep-2 cells treated with 0.25, 25 and 75 mM MTX (*b*, *c*, *d* respectively) and control cells (*a*). The cells in the "R1" block are cells non-viable and cells in the "R3" are viable. Cell viability was evaluated by double staining with fluorescein isothiocyanate (FITC) label Bcl-2 (100: sc-509); Pink cells are viable, blue cells — non-viable

Table. Viability of Hep 2 cells treated with MTX for 24 h

MTX dose Concentration	Viable cells, M ± SD
Control group	9,260 ± 50
0.25 mM	7,910 ± 26*
25 mM	2,080 ± 44*
75 mM	780 ± 30*

* The difference is significant compared to the control ($p < 0.05$).

Research about chemosensitivity is important to screen new therapeutic agents, identify patterns of sensibility for different tumor types, to select chemotherapy regimens to individual patients and improvement in life quality [22]. We conclude that the highest MTX concentration is associated with highest tumor cells sensibility; as a consequence, the knowledge of the drug sensibility can do significant impact in decision-making and treatment.

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