ANALYSIS

23(95), January - February, 2019

ISSN
2321–7359
EISSN
2321–7367

Medical Science

Comparison of the effects of all-*trans* retinoic acid, methotrexate, actinomycin D, and combined chemotherapy on different choriocarcinoma cell culture models

Görker SEL[1], Müge Harma[2], Mehmet İbrahim Harma[3], İshak Özel Tekin[4]

***Introduction*: Our objective was to investigate the efficacy of all-*trans* retinoic acid (ATRA) alone and in combination chemotherapy with methotrexate (MTX) and combined with actinomycin D (Act-D) in choriocarcinoma cell culture models (JAR, JEG-3). *Material and Methods*: JAR and JEG-3 cells were cultured. ATRA, MTX and Act-D trial groups were determined with different doses. DMSO was applied as control group. Drugs were administered to the cells simultaneously, and 72 hours after drug administration, the cells were detached using trypsin-ethylenediamine tetraacetic acid solution. The degree of apoptosis was determined by flow cytometry. Statistical analyses of the apoptotic ratios were performed using SPSS 19.0 and the Wilcoxon test. *Results*: The ratio of apoptosis was statistically significant when only ATRA was applied on JAR and JEG-3 cell culture lines versus control group, p<0.05. The ratio of apoptosis was increased on JAR and JEG-3 cell culture lines, when ATRA was added in the combination of MTX 2 µM, ACT-D 0.1 µM, p<0.05. *Discussion:* ATRA increased the apoptotic ratios in both JAR and JEG-3 cell cultures. The apoptotic ratios were increased with the higher ATRA doses. The application of ATRA, MTX and Act-D combination on the JAR and JEG-3, cell line models, is made in both cell lines for the first time in the literature. The apoptotic data reveal that: ATRA could be used in choriocarcinoma treatment. Also, the combination of ATRA, MTX, and Act-D had stronger apoptotic effects than did the combination of MTX and Act-D. Therefore, ATRA also could be used as a synergistic drug and an option to combat the multi-drug resistance often encountered in the treatment of choriocarcinoma.**

INTRODUCTION

Gestational trophoblastic disease (GTD) appears in the fetal chorion and involves a variety of interrelated diseases, ranging from benign hydatidiform moles (HM), which usually resolve spontaneously, to life-threatening (1). GTD usually develops from HM, but it has been observed in aborted, term, and ectopic pregnancies. Methotrexate (MTX) is conventionally known as a folic acid antagonist. It is a cytotoxic chemotherapeutic agent that inhibits the conversion of folic acid to folinic acid by inhibiting the enzyme dihydrofolate reductase in actively dividing cells (2, 3). Because its cytotoxic effect is not selective, it may damage hematopoietic cells and intestinal mucosal cells, which are highly proliferative (4). The MTX dosage must be reduced due to the possible side effects of enterocolitis or intestinal damage, affecting cancer treatment. Actinomycin-D (Act-D) is a transcription inhibitor (5). It attaches to DNA and inhibits RNA and protein synthesis, thereby suppressing cell proliferation (6; 7). Act-D has serious side effects, including bone marrow depression and gastrointestinal toxicity. All-*trans* retinoic acid (ATRA) is the carboxylic acid form of vitamin A. Retinoic acid affects angiogenesis in cell proliferation and induces apoptosis by regulating cell expression (8, 9). ATRA prevents the formation of free oxygen radicals by enhancing the effect of superoxide dismutase and reducing oxidative stress (8). ATRA also induces the differentiation of immature cells and the functioning of highly pluripotent tumor cells. Our objective was to investigate the efficacy of ATRA alone and in combination chemotherapy with MTX and combined with Act-D in choriocarcinoma cell culture models (JAR, JEG-3), adding new data to the insufficient literature on this topic.

[1]Görker Sel: Assistant Prof. Gynecologist, Adr: Bülent Ecevit University Hospital, Obstetrics and Gynecology Department, Zonguldak, 67100, Kozlu, Email: gorkersel@gmail.com; Tel: 00903722262573; [2]Müge Harma: Prof Dr. Gynecologiconcologist, Adr: Bülent Ecevit University Hospital, Obstetrics and Gynecology Department, Division of Gynecologic Oncology Zonguldak, 67100, Kozlu, Email: Obgyn.info@gmail.com; Tel: 00903722262569; [3]Mehmet İbrahim Harma: Prof Dr. Gynecologiconcologist, Adr: Bülent Ecevit University Hospital, Obstetrics and Gynecology Department, Division of Gynecologic Oncology Zonguldak, 67100, Kozlu; Email: Obgyn.info@gmail.com; Tel: 00903722262569; [4]İshak Özel Tekin: Assoc. Prof. Dr. Immunologist, Adr: Bülent Ecevit University Hospital, Immunology Department, Zonguldak, 67100, Kozlu, Email: ishaktek@yahoo.com; Tel: 00903722262569

✉*Corresponding author:* Görker Sel: Assistant Prof. Gynecologist, Adr: Bülent Ecevit University Hospital, Obstetrics and Gynecology Department, Zonguldak, 67100, Kozlu, Email: gorkersel@gmail.com; Tel: 00903722262573

MATERIAL AND METHODS

JAR and JEG-3 cell cultures

JAR and JEG-3 cell culture lines, shown in Figure 1 and 2, were obtained from the American Tissue Type Culture Collection. All cell cultures were maintained and cultured in RPMI-1640 medium (İnterlab) supplemented with 10% heat-inactivated fetal calf serum, penicillin-streptomycin, and L-glutamine in a 98% humidified, 5% CO_2 atmosphere at 37°C in a Nuve CO_2 incubator in 75-cm^2 flasks.

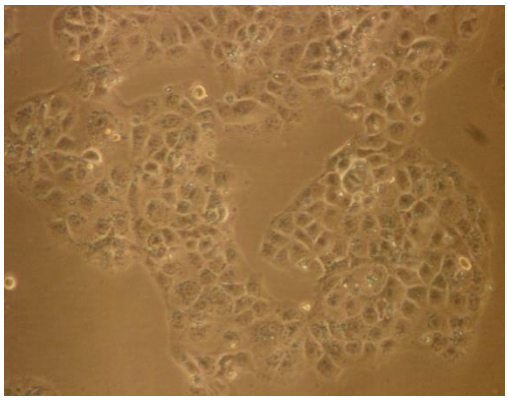

Figure 1 JAR cell culture

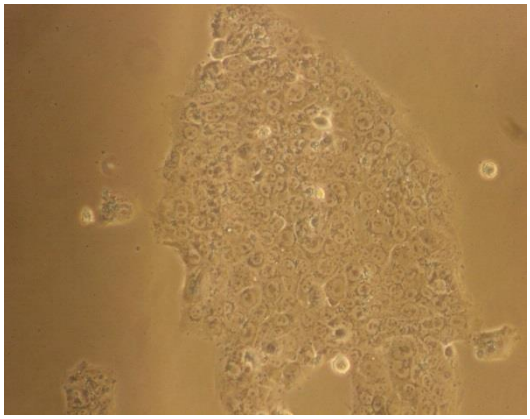

Figure 2 JEG-3 cell culture

Drugs

ATRA preparation

ATRA was purchased from Sigma, prepared in dosages of 0.1, 1, and 10 µM by diluting in dimethyl sulfoxide (DMSO), and diluted in RPMI-1640 to a maximum concentration such that DMSO formed less than 1% of the mixture.

MTX preparation

MTX was purchased from Sigma, prepared in dosages of 2, 4 and 8 µM by diluting in DMSO, and diluted by RPMI-1640 to a maximum concentration such that DMSO formed less than 1% of the mixture.

Act-D preparation

Act-D was purchased from Sigma, prepared at concentrations of 0.05, 0.1, and 0.2 µM by diluting in DMSO and then diluted in RPMI-1640 to a maximum concentration such that DMSO formed less than 1% of the mixture.

The dosages used in the ATRA, MTX and Act-D trial groups were as follows:

Single drug trial: MTX 2 µM, 4 µM, 8 µM; ATRA 0.1 µM, 1 µM, or 10 µM; and Act-D 0.05 µM, 0.1 µM, 0.2 µM.

Combined ATRA and MTX drug trial: ATRA 0.1 µM, 1 µM, or 10 µM; and MTX 2 µM.

Combined ATRA and Act-D drug trial: ATRA 0.1 µM, 1 µM, or 10 µM; and Act-D 0.1 µM.

Combined ATRA, MTX, and Act-D drug trial: ATRA 0.1 µM, 1 µM, or 10 µM; MTX 2 µM; and Act-D 0.1 µM.

Drugs were administered to the cells simultaneously, and 72 hours after drug administration, the cells were detached using trypsin-ethylenediamine tetraacetic acid (EDTA) solution. The degree of apoptosis was determined by flow cytometry (FCM). The supernatant was collected before trypsin application for only one set and stored in a deep freezer to investigate β-hCG levels. β-hCG levels were investigated using an immunoenzymatic method (DXI 600, Beckman Coulter, CA, USA). Statistical analyses of the apoptotic ratios were performed using SPSS 19.0 and the Wilcoxon test.

Detection of apoptosis using Annexin V

Annexin V is a protein that binds preferentially to phosphatidylserine, which is located at the outer surface of the cell membrane. This feature allows apoptotic cells to be observed after marking them with a fluorescent agent such as FITC (9). The binding ratio of FITC-annexin-V complex to phosphatidylserine at the cell membrane can be measured using flow cytometry.

RESULTS

After 72 hours, β-hCG levels were 1217 mlU/ml in the JAR cell cultures and 1811 mlU/ml in the JEG-3 cell cultures. The apoptotic ratio in the control group, in which only DMSO was used, was 11% in the JAR cell culture and 12% in the JEG-3 cell culture. After the application of 0.1 µM ATRA to JAR and JEG-3 cells, β-hCG levels were decreased to 450 mlU/ml and 810 mlU/ml, respectively (Table 1). The apoptotic ratio was 14% in the JAR cells and 15% in the JEG-3 cells. The increase in the apoptotic ratio was statistically significant ($p<0.05$) (Table 2). Additionally, increasing the dosage of ATRA (0.1, 1, 10 µM) in both JAR and JEG3 cells increased apoptotic ratios significantly ($p<0.05$) (Table 2). In the JEG-3 cells, the ratio of apoptosis was 40% after application of 2 µM MTX, and after application of 2 µM MTX combined with 0.1 µM, 1 µM, or 10 µM ATRA, the apoptotic ratios were 44%, 49%, and 51%, respectively. This incremental increase was statistically significant ($p<0.05$) (Table 3). However, in the JAR cell culture, only the 2 µM MTX and 10 µM ATRA combination resulted in statistically significant apoptotic ratios compared with 2 µM MTX ($p<0.05$) (Table 3). In the JAR cells, the apoptotic ratio was 13% after application of 0.1 µM Act-D, and after application of 0.1 µM Act-D combined with 0.1 µM ATRA, the apoptotic ratio increased significantly to 15% ($p<0.05$) (Table 4). In the JEG-3 cell culture, application of Act-D (0.1 µM) combined with increasing dosages of ATRA (0.1, 1, 10 µM) increased apoptotic ratios significantly ($p<0.05$) (Table 4). After application of 2 µM MTX + 0.1 µM Act-D in JAR and JEG-3 cell lines, the apoptotic ratios were 42% and 43%, respectively, and after application of 2 µM MTX + 0.1 µM ACT-D combined with 0.1 µM, 1 µM or 10 µM ATRA in JAR and JEG-3 cell lines, the apoptotic ratios were 43%, 45%, 48% and 52%, 57%, 68%, respectively. All of the apoptotic ratios increased significantly ($p<0.05$) (Table 5). Additionally, application of increasing dosages of ATRA (0.1, 1, 10 µM) combined with MTX 2 µM + Act-D 0.1 µM increased apoptosis ratios significantly

Table 1 Drug groups, β-Hcg levels (mIU/ml) in JAR and JEG-3 cell cultures

Drugs	β-Hcg(mIU/ml)	
	JAR	JEG-3
Control group	1217	1811
ATRA 0.1 μM	450	810
ATRA 1 μM	225	671
ATRA 10 μM	136	411
MTX 2 μM +ATRA 0.1 μM	827	474
MTX 2 μM +ATRA 1 μM	829	582
MTX 2 μM +ATRA 10 μM	874	899
MTX 2 μM +ACT-D 0.1 μM	752	671
MTX 2 μM +ACT-D 0.1 μM +ATRA 0.1 μM	728	72
MTX 2 μM +ACT-D 0.1 μM +ATRA 1 μM	210	54
MTX 2 μM +ACT-D 0.1 μM +ATRA 10 μM	125	51

Table 2 Apoptosis induced by ATRA in JEG-3 and JAR cell cultures (p values)
a Based on negative ranks

JEG-3	ATRA 0.1 μM Vs. Control	ATRA 1 μM Vs. ATRA 0.1 μM	ATRA 10 μM Vs. ATRA 1 μM
Z Asymp. Sig. (2-tailed)	-1,992 (a) ,046	-2,226 (a) ,028	-2,201 (a) ,028
JAR	ATRA 0.1 μM Vs. Control	ATRA 1 μM Vs. ATRA 0.1 μM	ATRA 10 μM Vs. ATRA 1 μM
Z Asymp. Sig. (2-tailed)	-2,201 (a) ,028	-2,201 (a) ,028	-2,201 (a) ,028

Table 3 Apoptosis induced by 2 μM MTX combined with 0.1 μM, 1 μM, or 10 μM ATRA in JEG-3 and JAR cell cultures (p values)
a Based on negative ranks. b Wilcoxon Signed Ranks Test

JEG-3	MTX 2 μM+ATRA 0.1 μM Vs. MTX 2 μM	MTX 2 μM +ATRA 1 μM Vs. MTX 2 μM	MTX 2 μM +ATRA 10 μM Vs. MTX 2 μM
Z Asymp. Sig. (2-tailed)	-2,207(a) ,027	-2,201(a) ,028	-2,201(a) ,028
JAR	MTX 2 μM+ATRA 0.1 μM Vs. MTX 2 μM	MTX2 μM +ATRA 1 μM Vs. MTX 2 μM	MTX2 μM +ATRA 10 μM Vs. MTX 2 μM
Z Asymp. Sig. (2-tailed)	-1,572 (a) ,116	-1,572 (a) ,116	-2,032 (b) ,028

Table 4 Apoptosis induced by 0.1 μM Act-D combined with 0.1 μM, 1 μM, or 10 μM ATRA in JEG-3 and JAR cell cultures (p values)
a Based on negative ranks. b Wilcoxon Signed Ranks Test

JEG-3	Act-D 0.1 μM +ATRA 0.1 μM Vs. Act-D 0.1 μM	Act-D 0.1 μM +ATRA 1 μM Vs. Act-D 0.1 μM +ATRA0.1 μM	Act-D 0.1 μM +ATRA 10 μM Vs. Act-D 0.1 μM +ATRA 1 μM
Z Asymp. Sig. (2-tailed)	-1,787 (a) ,074	-2,201 (b) ,028	-2,201 (b) ,028
JAR	Act-D 0.1 μM +ATRA 0.1 μM Vs. Act-D 0.1 μM	Act-D 0.1 μM +ATRA 1 μM Vs. Act-D 0.1 μM +ATRA0.1 μM	Act-D 0.1 μM +ATRA 10 μM Vs. Act-D 0.1 μM +ATRA 1 μM
Z	-2,207(a)	-1,367(a)	-2,201(a)
Asymp. Sig. (2-tailed)	,027	,172	,028

Table 5 Apoptosis induced by 2 μM MTX + 0.1 μM Act-D combined with 0.1 μM, 1 μM or 10 μM ATRA in JAR and JEG-3 cell cultures (p values)
a Based on negative ranks.

JAR	**MTX 2 μM + Act-D 0.1 μM +ATRA 0.1 μM** Vs. **MTX 2 μM + Act-D 0.1 μM**	**MTX 2 μM + Act-D 0.1 μM +ATRA 1 μM** Vs. **MTX 2 μM + Act-D 0.1 μM**	**MTX 2 μM + Act-D 0.1 μM +ATRA 10 μM** Vs. **MTX 2 μM + Act-D 0.1 μM**
Z	-2,023(a)	-2,201(a)	-2,201(a)
Asymp. Sig. (2-tailed)	,043	,028	,028
JEG-3	**MTX 2 μM + Act-D 0.1 μM +ATRA 0.1 μM** Vs. **MTX 2 μM + Act-D 0.1 μM**	**MTX 2 μM + Act-D 0.1 μM +ATRA 1 μM** Vs. **MTX 2 μM + Act-D 0.1 μM**	**MTX 2 μM + Act-D 0.1 μM +ATRA 10 μM** Vs. **MTX 2 μM + Act-D 0.1 μM**
Z	-2,201(a)	-2,201(a)	-2,201(a)
Asymp. Sig. (2-tailed)	,028	,028	,028

Table 6 Apoptosis induced by increasing doses of ATRA vs. combined doses in JAR cells (p value)
a Based on negative ranks.

JAR	**MTX 2 μM +Act-D 0.1 μM+ ATRA 1 μM** Vs. **MTX 2 μM +Act-D 0.1 μM+ATRA 0.1 μM**	**MTX 2 μM +Act-D 0.1 μM+ATRA 10 μM** Vs. **MTX 2 μM +Act-D 0.1 μM+ATRA 1 μM**
Z	-2,207(a)	-1,992(a)
Asymp. Sig. (2-tailed)	,027	,046
JEG-3	**MTX 2 μM +Act-D 0.1 μM+ ATRA 1 μM** Vs. **MTX 2 μM +Act-D 0.1 μM+ATRA 0.1 μM**	**MTX 2 μM +Act-D 0.1 μM+ATRA 10 μM** Vs. **MTX 2 μM +Act-D 0.1 μM+ATRA 1 μM**
Z	-2,214(a)	-2,201(a)
Asymp. Sig. (2-tailed)	,027	,028

in both JAR and JEG-3 cell cultures ($p<0.05$) (Table 6). In the JAR cells, the highest apoptotic ratio (55%) was observed with the combination of 2 μM MTX, 0.1 μM Act-D, and 10 μM ATRA, and the β-hCG level was decreased to 125 mlU/ml. In the JEG-3 cells, the highest apoptotic ratio (61%) was observed with the combination of 2 μM MTX, 0.1 μM Act-D, and 10 μM ATRA, and the β-hCG level was decreased to 51 mlU/ml.

DISCUSSION

Cell cultures are often used to evaluate new drugs, because they enable experimentation without requiring animal studies. Our present study showed the efficiency of ATRA on two different choriocarcinoma cell culture models: JAR and JEG-3. GTD appears in the fetal chorion and involves a variety of interrelated diseases, ranging from benign HMs, which usually resolve spontaneously, to life-threatening choriocarcinoma (1). ATRA and vitamin A analogues are widely used in dermatological, hematopoietic diseases and in cancer management (10; 11; 12; 13; 14). ATRA has chemotherapeutic effects by suppressing the activities of activation protein-1 and induction of transforming growth factor-β and by regulating gene expression specifically at the G1 phase (15). Retinoic acids are responsible for the growth and reproduction of epithelial tissues, preventing some types of cancer cells from proliferating. They are used in the treatment and prevention of lung cancer, leukemia, skin cancers, and prostate cancer, but it is still not entirely clear how they inhibit the growth of cancer cells (16).

ATRA is a potent antioxidant and decreases oxidative stress (8). Research has demonstrated that oxidative stress plays an important role in the ethiopathogenesis of GTD (17; 18; 19). These studies have demonstrated the importance of oxidative stress in GTD ethiopathogenesis, and our findings suggest that ATRA's anti-cancer effect on choriocarcinoma may be related to its ability to decrease oxidative stress. Drugs can cause two kinds of cytotoxic effects: necrosis and apoptosis. Cancer research generally focuses on apoptosis, because the main etiology in carcinogenesis is the deterioration of the apoptotic mechanism during the cell cycle. Therefore, we used FCM to calculate the apoptotic ratio and thereby investigated how ATRA affects choriocarcinoma cell cultures. ATRA affected the apoptotic ratios in JAR and JEG-3 cell cultures, and the effects were dose-dependent with statistical significance. Adding ATRA to the combination of MTX (2 μM) and Act-D (0.1 μM) increased the apoptotic ratios significantly. Additionally, the ratios increased incrementally with the ATRA dose, and these increases were significant ($p<0.05$). These results demonstrate an apoptotic effect of ATRA, alone or in combination with MTX and Act-D. Additionally, β-hCG levels were decreased, which may provide some information about the maintenance of choriocarcinoma cells. To the best of our knowledge, this is the first study to investigate the effect

of ATRA alone and in combination with MTX and Act-D chemotherapeutics in JAR and JEG-3 cell cultures. ATRA could be used alone for choriocarcinoma treatment, and a chemotherapeutic combination including ATRA could help combat the drug resistance that can occur during choriocarcinoma treatment.

CONCLUSION

For the first time, the application of the ATRA, MTX, and Act-D combination was explored in both JAR and JEG-3 human choriocarcinoma cell cultures. The synergistic apoptotic data reveal that: ATRA could be used as choriocarcinoma treatment. Also, the combination of ATRA, MTX, and Act-D had stronger apoptotic effects than did the combination of MTX and Act-D. Therefore, ATRA could be used as a synergistic drug and an option to combat the multi-drug resistance often encountered in the treatment of choriocarcinoma. However, the effects of the drugs and the combinations of drugs may be different in vivo systems, such that they should be tested initially using animal experiments and then in clinical trials.

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Article Keywords

All-trans retinoic acid; choriocarcinoma; JAR cell culture; JEG-3 cell culture; gestational trophoblastic disease

Funding

By the Project of Bulent Ecevit University. BAP 2012-20-00-02, the cell culture lines (Jarand Jeg-3) and chemotherapeutics for our research were obtained, no funding further more.

Ethical approval

2012-56-03/04-4

Consent for publication

Not applicable, my manuscript does not report on or involve the use of any animal or human data or tissue.

Competing interests

The authors declare no competing interests.

Authors' contributions

İshak Özel Tekin was participated in ATRA application on cell cultures, and Annexin V test made by him.
Görker SEL, Mehmet İbrahim Harma and Müge Harma analyzed and interpreted the apoptotic cell culture data and writing the manuscript.
All authors read and approved the final manuscript.

Article History

Received: 11 September 2018
Accepted: 04 November 2018
Published: January-February 2019

Citation

Görker SEL, Müge Harma, Mehmet İbrahim Harma, İshak Özel Tekin. Comparison of the effects of all-*trans* retinoic acid, methotrexate, actinomycin D, and combined chemotherapy on different choriocarcinoma cell culture models. *Medical Science*, 2019, 23(95), 19-23