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Investigation of Topotecan and Folic Acid Effect in Jar Choriocarcinoma Cell Culture

JAR Koryokarsinom Hücre Kültüründe Topotekan ve Folik Asit Etkisinin İncelenmesi

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ABSTRACT

Aim: The purpose of this study is to search the effects and treatment efficacies of topotecan and folic acid on the choriocarcinoma cultures generated in JAR cell culture.

Material and Methods: Human choriocarcinoma-like JAR cell is augmented in the 10% fetal bovis serum and RPMI-1640 media, 95% air at 37°C with the existence of penicillin and streptomycin. Groups to apply topotecan or folic acid are identified as below:Topotecan dissolved in dimethyl sulphoxide (DMSO) and prepared as separate doses of 1, 5, 10 and 50 µg/ml. Substances are given to cells simultaneously.

Results: In this study, the apoptotic effect of the use of topotecan only, in residual doses on JAR cell series is statistically significant. The use of folic acid only, in residual doses has found to decrease the apoptosis. In the combination of these two drugs in residual doses, the apoptotic effect of this drug is even more increased, and statistically significant.

Conclusion: This is the first study which use the topotecan and folic acid separately and combined on JAR, a human choriocarcinoma cell line model, and evaluation of β-hCG and h-hCG by the means of immunoenzymatic method on the both two cell lines. Synergistic and apoptotic data gathered indicates that topotecan and folic acid can be one of the treatment options in order to struggle multi-drug resistance which is an enormous obstacle on treatment. However, the efficacy of drugs and effects of combination therapies might be disparate in vivo; therefore data must be supported by animal experiments and after that by clinical evaluations.

Keywords: Topotecan, Folic acid, Choriocarcinoma

ÖZ

Amaç: Bu çalışmanın amacı, koryokarsinom tedavisine yeni bir bakış açısı sunarak daha efektif tedavi yöntemleri geliştirebilmek amacıyla topotekan ve folik asitin, JAR hücre kültüründe geliştirilen koryokarsinom modelleri oluşturulmuş hücre kültürleri üzerindeki etkilerini ve tedavi etkinliklerini araştırmaktır.

Gereç ve Yöntemler: İnsan koryokarsinom benzeri JAR hücrelerin RPMI-1640 ortamında % 10 fetal dana serumu, penisilin-steptomisin varlığında 37°C, % 95 hava, % 5 CO2 içeren atmosferde çoğaltıldı. Topotekan ve folik asitin uygulama grupları şu şekilde belirlendi: Topotekan, Dimetil sülfoksit (DMSO) içerisinde çözülerek 1, 5, 10, ve 50 µg/ml'lik dozlar halinde hazırlanmıştır. Folik asit, DMSO içerisinde çözülerek 1, 5 ve 10 µg/ml'lik dozlar halinde hazırlanmıştır. Maddeler hücrelere aynı anda verilmiştir.

Bulgular: Yaptığımız çalışmada topotekanın tek başına artan dozlarda kullanıldığında JAR hücre serileri üzerindeki apopitotik etkilerinin istatistiksel olarak arttığı görülmüştür. Folik asitin tek başına artan dozda apoptozisi azalttığı, bu iki ilacın kombine edilip artan dozlarda kullanıldıklarında JAR hücre serisi üzerinde apoptotik etkilerinin istatistiksel olarak daha da arttığı görülmüştür.

Sonuç: İnsan Koryokarsinom hücre hattı modellerinden biri olan JAR üzerinde topotekanın folik asit ile tek ajan ve kombine kullanımları ayrıca immünoenzimatik yöntemle β-hCG ve h-hCG ölçümü, literatürde ilk kez her iki hücre hattında birden yapılmıştır. Elde edilen sinerjistik apoptotik veriler topotekan ve folik asitin Koryokarsinom tedavisinde karşılaşılan çoklu ilaç direnciyle mücadelede kullanılabilecek seçeneklerden biri olabileceğini göstermektedir. Ancak ilaçların etkileri ve kombinasyonlarındaki etkilerin in vivo sistemlerde farklı olabileceğinden, veriler ilk olarak hayvan deneyleri ve sonra klinik araştırmalarla da desteklenmelidir.

Anahtar Sözcükler: Gestasyonel trofoblastik hastalık, folik asit, topotekan, koryokarsinom.

INTRODUCTION

Gestational trophoblastic diseases, which are of the trophoblastic origin of placenta, are a group of disease which characterized by the abnormal proliferation of the trophoblast. The etiology of GTD is multifactorial and the pathogenesis is not fully known (1). GTD are divided histopathologically into four groups. These are hydatiform (complete and partial), invasive mole, choriocarcinoma, and trophoblastic tumors originating from the placental site (2). Choriocarcinoma is the most malignant type of these diseases . Various hormones are secreted from the placenta, one of these hormones is human chorionic gonadotropin (hCG), which begins to be secreted after fertilization. The most widely used biochemical parameter in the diagnosis and follow-up of this disease is β-hCG. β-hCG values should be made serially during the diagnosis, treatment and post-treatment process of the disease. The hCG produced in choriocarcinoma is called hyperglycosylated hCG (H-hCG). It has a much larger oligosaccharide side chain than hCG synthesized during pregnancy (2).

Folate is a B-9 vitamin. Folate; It takes part in nucleotide biosynthesis, DNA replication, methyl group supply, cell growth and repair (3). Experimental studies so far have shown that folate deficiency affects the first stage of carcinogenesis. High dose FA level induces the growth of cancer cells (4-6).

FA has an important role in the preservation of body homeostasis and is involved in the remethylation step with vitamin B12 in the conversion of homocysteine to methionine (7).

Topotecan (Topo) is an inhibitor of topoisomerase I, a chemotherapeutic drug used to treat ovarian and small cell lung cancer. Topo hydrochloride, which has topoisomerase I inhibitory activity, is an antitumoral drug. Topoisomerase I induces the single-strand breakings in DNA to bind to the DNA complex by eliminating the tension around the axis of the DNA and inhibits single-strand breaks (8-11).

The purpose of this study is to search the effects and treatment efficacies of Topo and FA on choriocarcinoma cultures generated in JAR cell culture.

MATERIALS and METHODS

JAR Cell Culture Line

JAR cell culture line was 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.

Topotekan Preparation

Topotecan was dissolved in Dimethyl sulfoxide (DMSO) and prepared in 1, 5, 10 and 50 µM doses. At the highest concentration, the dilution was made with PPMI 1640 with a DMSO ratio of less than 1%.

FA Preparation

Folic acid was dissolved in DMSO and prepared in 1, 5 and 10 µM doses. At the highest concentration, the dilution was made with PPMI 1640 with a DMSO ratio of less than 1%.

Preparation of Drugs for Test, β-hCG and H-hCG Measurement

TOPO and FA were prepared as the following doses**;**

Single drug trial: Topo 1 µM, 5 µM, 10 µM and 50 µM; FA 1 µM, 5 µM, and 10 µM.

Combined Topo and FA drug trial: Topo 1 µM, 5 µM, 10 µM and 50 µM; and FA 10 µM.

Jar cell cultures were cultured 6 times separately, each for all doses of drug, including the control group. Cells were removed with trypsin-EDTA solution at the 48th hour follow-

ing drug administration and taken into apoptosis study. All experiment sets were collected before trypsin supernatant and stored in freezer for β -hCG and H-hCG measurement. β-hCG and H-hCG levels (Sunred Elisa Kit) were studied in DXI 600 device (Beckman Coulter, CA, USA) by immunoenzymatic method.

Apoptosis Detection

Annexin V is a protein that can bind to phosphatidylserine that migrates towards the outer surface of the cell, and due to this feature, we can make the apoptotic cell visible by lbeling it with a fluorescent substance (FITC) (12). The rate of binding of the FITC-Annexin-V complex to phosphatidylserine on the cell surface can be measured by flow cytometry.

Statistical Analysis

The statistical analysis of the study was done in SPSS 19.0 package program. Descriptive statistics of the continuous variables in the study are given with median, minimum and maximum values. Kruskal-Wallis test was used for comparisons of doses in groups of 3 or more, and Mann Whitney U tests with Bonferonni correction were used for subgroup comparisons of 2 between doses. Box-line graph was used in the graphical representation of the comparison results. In all statistical analyzes in the study, comparisons with a p value below 0.05 were considered statistically significant.

RESULTS

After 48 hours, β-hCG and h-hCG levels were respectively, 128 and 70 mIU/ml in the JAR cell cultures. The median rate of apoptosis in the control group using only DMSO was 17.7% in JAR cell line. In JAR cell lines; Median apoptosis rates after 1, 5, 10 and 50 µM TOPO application were found as 26.4%, 29.0, 38.7 and 60.5%, respectively. The rate of apoptosis was found to be statistically significant ($p < 0.05$) (Table 1, Figure 1). After the application of 1, 5, 10 and 50 µM TOPO to JAR cell lines, β-hCG levels were decreased to 124, 120, 118 and 98 mIU/ml, respectively; H-hCG levels were decreased to 64, 58, 57 and 54 mIU/ml, respectively (Table 2). Decrease in β-hCG and h-hCG levels were found to be statistically significant by increasing Topo dose. ($p<0.05$).

However, increasing the dosage of FA (1, 5, and 10 µM) in JAR cell cultures was not found to affect apoptotic ratios positively, even inversely affected in FA 1 µM application group in comparision with the control group significantly (17.7% versus 16.2%) (*p*:0.002). FA 5 and 10 µM doses were ineffective in comparision with the control group's apoptotic ratios (17.7 vs 17.5 and 17.9; p: 0.394 and p: 0.485; control group, FA 5 and 10 µM respectively) (Table 3, Figure 2).

Table 1: The Rates of Apoptosis Caused by Topo Given in Increasing Doses in JAR Cell Cultures

	Median (Min-Max)
JAR Control	17.7 (17.5-18.2)
JAR Topo_1 µg/ml	26.4 (26.0-27.1)
JAR Topo_5 µg/ml	29.0 (27.6-31.0)
JAR Topo_10 µg/ml	38.7 (38.0-39.4)
JAR Topo_50 µg/ml	60.5 (58.6-65.0)

Table 2: H-hCG and β–hCG Levels in JAR Cell Cultures When Topo is Used.

	H-hCG mIU/ml	β–hCG mIU/ml
JAR Control	70	128
JAR Topo_1 µg/ml	64	124
JAR Topo_5 µg/ml	58	120
JAR Topo_10 µg/ml	57	118
JAR Topo_50 µg/ml	54	98

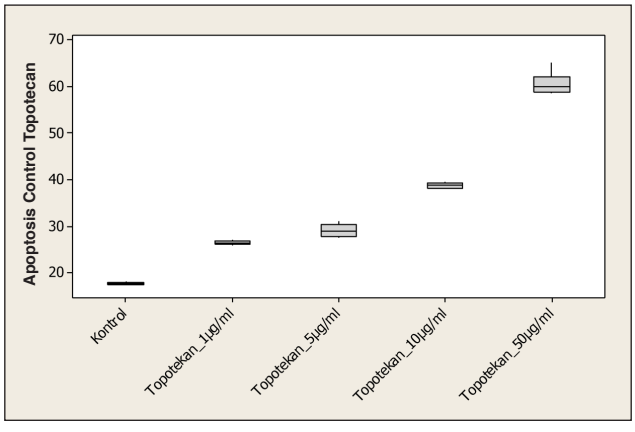

Figure 1: Evaluation of the Effect of Topo in JAR Cell Cultures.

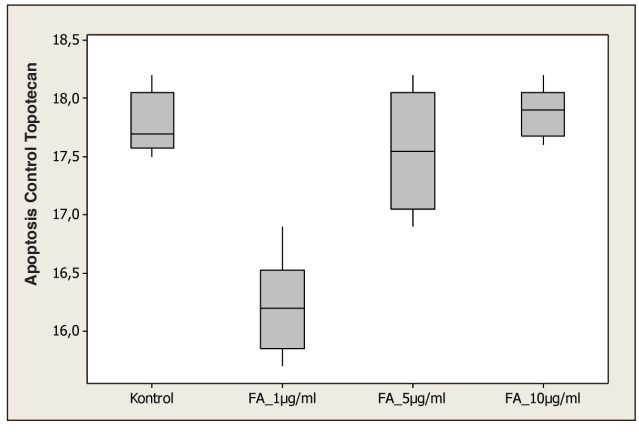

Figure 2: Evaluation of the Effect of Folic Acid in JAR Cell Cultures

According to the control group; It was observed that FA administration did not statistically affect β-hCG and hCG levels (Table 4) (p> 0.05).

The median ratios of apoptosis were 26.4, 29.0, 38.7, and 60.5% after application of 1 µM, 5 µM, 10 µM and 50 µM Topo, respectively; as mentioned above. This time 10 µM FA was added to those TOPO doses, and after application of 10 µM FA combined with 1 µM, 5 µM, 10 µM or 50 µM TOPO, the apoptotic ratios were 33.1%, 33.8%, 44.9% and 72.4%, respectively. This incremental increase was statistically significant in comparision when TOPO used alone (p<0.05) (Table 5, Figure 3). However combination of TOPO and FA did not affect on β-hCG and H-hCG levels were between 49-60 IU/mL for H-hCG and 112-123 IU/mL for β- hCG (p > 0.05) (Table 6).

Table 3: The Rates of Apoptosis Caused by Folic Acid Given in Increasing Doses in JAR Cell Cultures.

	Median (Min-Max)
JAR Control	17.7 (18.2-17.5)
JAR FA_1 µg/ml	16.2 (15.7-16.9)
JAR FA _5 µg/ml	17.5 (16.9-18.2)
JAR FA _10 µg/ml	17.9 (17.6-18.2)

Table 4: H-hCG and β–hCG Levels in JAR Cell Cultures When Folic Acid is Used

	H-hCG mIU/ml	β-hCG mIU/ml
JAR Control	70	128
JAR FA_1 µg/ml	65	123
JAR FA_5 µg/ml	67	125
JAR FA_10 µg/ml	68	124

Table 5: The Apoptosis Rates of Folic Acid and Topotecan Combination in JAR Cell Line Cultures

	Median (Min-Max)
JAR Control	17.7 (17.5-18.2)
JAR Topo_1 µg/ml+ FA_10 µg/ml	33.1 (30.3-35.0)
JAR Topo_5 µg/ml+ FA_10 µg/ml	33.8 (32.5-35.0)
JAR Topo_10 µg/ml+ FA_10 µg/ml	44.9 (42.0-46.1)
JAR Topot_50 µg/ml+ FA_10 µg/ml	72.4 (67.2-81.0)

DISCUSSION

The combination of Topo and FA on JAR, which is a human choriocarcinoma cell line model, and the measurement of β-hCG and H-hCG by immunoenzymatic method were performed for the first time in the literature.

Gestational tissue is responsible for the pathogenesis of all forms of GTH and β-hCG levels are increased in all forms. The h-HCG level is also increased in choriocarcinoma. Because of these reasons, the effects of FA and Topo on cell culture lines were measured by using β-hCG and H-hCG parameters and ratio of apoptosis.

Topotecan is one of the first topoisomerase I enzyme inhibitors to be approved in clinical use. Topoisomerase is present in all eukaryotic cells. They take part in DNA replication and repair. Topoisomerase reversibly binds to the DNA strand and resolves the double stranded DNA (13). Topo, topoisomerase I enzyme blocker, especially for the treatment of refractory ovarian cancer and refractory small cell lung cancer (14). In our study, TOPO was shown to decrease H-hCG and β-hCG levels while increasing apoptosis. There is no study in the literature on Topo-related choriocarcinoma, in this sense our study is a first.

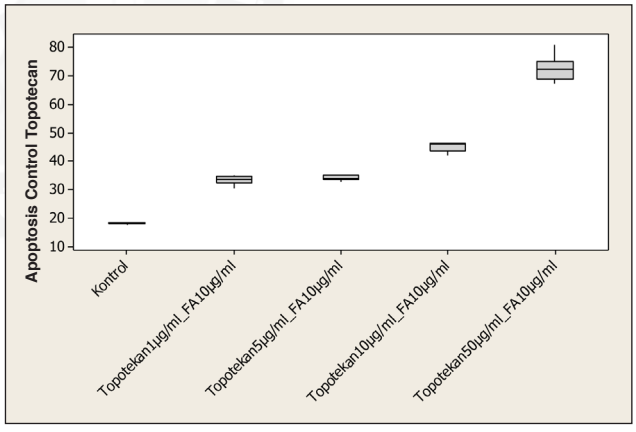

Figure 3: Evaluation of the effect of folic acid and topotecan combination in JAR cell cultures.

Table 6: H-hcg and β-hcg Levels in Jar Cell Cultures of Folic Acid and Topotecan Combination

	H-hCG mIU/ml	β-hCG mIU/ml
JAR Control	70	128
JAR Topo_1 µg/ml+ FA _10 µg/ml	60	123
JAR Topo_5 µg/ml+ FA _10 µg/ml	58	119
JAR Topo_10 µg/ml+ FA _10 µg/ml	54	111
JAR Topo_50 µg/ml+ FA _10 µg/ml	49	112

FA, which is used as a tumor-specific specific drug delivery agent in the treatment of cancer; connects to the folate receptor on the surface of tumor cells. This complex is taken into the cell by endocytosis and works as a cancer drug. FA, an exogenous antioxidant, plays a role in preventing damage caused by free radicals by keeping oxidants in metabolism.

Physiologically, the antioxidant system and radicals are in equilibrium. As a result of the shift of this balance to the free radicals, the organelles and the lipid and protein structure in the membrane are disrupted, the intracellular enzymes are inactivated, DNA damage occurs, the aerobic respiration in the mitochondria is disrupted, lytic enzymes are activated, platelet aggregation and the migration of phagocytes into the tissues increases. This phenomenon, called oxidative stress, forms the basis for many diseases from hypertension to osteoporosis and is mainly cancer and atherosclerosis. In recent studies, oxidative stress has been reported to increase in physiological conditions such as pregnancy. In our study, while FA did not increase apoptosis alone, it showed a synergistic effect when used with TOPO and increased the effect of TOPO.

Di Simone et al. (2004) found that homocysteine targeted human placenta and caused a series of events in trophoblasts. Homocysteine causes apoptosis in trophoblastic cells. FA has been found useful in preventing trophoblastic damage caused by homocysteine. The same researchers, in a different study, FA; They found that it reduced DNA fragmentation and hHcg secretion (15). Aytan et al. reported that maternal FA usage and β-hCG levels were correlated as well (16).

In our study only FA addition to the cell culture line did not found to be effective on cancer apoptosis nor β-hCG and H-hCG levels.

Although the effects of FA on oxidative stress have been extensively studied, it is not known whether it has an effect on GTD. Previously Sel et al. reported that, all Trans retinoic acid (ATRA) was an effective drug on choriocarcinoma cell line because of decreasing oxidative stress (17). Since FA is also has a positive effect on oxidative stress, it could be used in these kinds of oxidant situations such as GTD. However, probably we could not find the right dose to be effective on JAR cell lines, so FA alone in our study did not work.

In a similar study by Erol et al., They found that beta-carotene and PLD could be an option for choriocarcinoma treatment. And in the same study, they suggested that vitamin A supplementation could prevent the formation of choriocarcinoma (18).

Topo is used in lung tumors in the literature, but its effect on choriocarcinoma is unknown (14). In our study, apoptotic effects on JAR cell lines were found to be statistically increased when Topo was used alone. However FA was not found to be effective on choriocarcinoma cell culture lines when used alone, in comparision with the control group. Nonetheless, when FA was kept constant and the dose of Topo was increased, the apoptotic effect was found to be further increased statistically in the JAR cell line. According to this data, it could be postulated that, when FA and TOPO were combined together, thay had a synergistic affect on choriocarcinoma. Therefore, TOPO and FA 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 topotecan, folic acid, and combinations of these two drugs in vivo systems may differ, such that they must be tested initially using animal experiments and later in clinical trials.

Author Contrubitons

Surgical and Medical Practices: **İshak Özel Tekin,** Concept: **Aykut Barut, Müge Harma, Mehmet İbrahim Harma, İshak Özel Tekin,** Design: **Aykut Barut, Mehmet İbrahim Harma, Müge Harma,** Data Collection or Processing: **Rahşan Eyüp Doğan, Aykut Barut, Mehmet İbrahim Harma, Müge Harma, İshak Özel Tekin,** Analysis or Interpretation: **Rahşan Eyüp Doğan, Görker Sel, Aykut Barut, Mehmet İbrahim Harma, Müge Harma,** Literature Search: **Rahşan Eyüp Doğan, Görker Sel, Aykut Barut, Mehmet İbrahim Harma, Müge Harma,** Writing: **Rahşan Eyüp Doğan, Görker Sel, Aykut Barut, Mehmet İbrahim Harma, Müge Harma.**

Informed Consent

No informed consent was obtained due to cell culture study.

Conflicts of Interest

No conflict of interest is declared by the authors.

Financial Disclosure

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Ethical Approval

Ethics committee approval was received for this study from the Ethics Committee of Bülent Ecevit University School of Medicine (No: 2014/14 Date: 15.07.2014).

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