Original Investigation

The comparison of Pegylated Liposomal Doxorubicin and Beta-Carotene effects on JAR and JEG-3 choriocarcinoma human cell culture models
Erol et al. PLD and beta-carotene effects on JAR, JEG-3 cells

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Abstract

Objectives: The aim of this study was to investigate the effectiveness of pegylated liposomal doxorubicin (PLD), beta-carotene, and a combination of PLD and beta-carotene on JAR and JEG-3 human choriocarcinoma (CC) cell lines for the treatment of CC.

Material and Methods: JAR and JEG-3 cells were cultured. PLD, beta-carotene trial groups were determined with different doses (for single drug trial; PLD 1, 2, 5 µg/ml and beta-carotene 1, 5, 10 µg/ml, and for combined drug trial; all PLD doses combined with beta-carotene 5 µg/ml). Drugs were administered to the cells simultaneously, and 72 hours after drug administration, the cells were detached using trypsin-ethylenediamine tetraacetic acid solution. The percentage of apoptotic cells was determined by flow cytometry after Annexin V staining. One set of the supernatant was collected before trypsin application to investigate beta-human chorionic gonadotropin (β-hCG) and hyperglycosylated hCG (H-hCG) levels.

Statistical analyses of the apoptotic ratios were performed using SPSS 19.0, Shapiro Wilk, Mann Whitney U and Kruskal-Wallis tests.
Results: The apoptosis increased in JAR and JEG-3 human CC cell cultures after treatment with all doses of PLD ($p<0.05$). A single application of each beta-carotene doses increased the apoptosis in JAR cells ($p<0.05$) but had no apoptotic effects on JEG-3 cells. In the PLD and beta-carotene combination group, the apoptosis increased in both JAR and JEG-3 cells ($p<0.05$).
Conclusion: To our knowledge, this is the first investigation of the effectiveness of PLD, beta-carotene, and PLD + beta-carotene combination therapy in two different CC cell lines. PLD is a promising chemotherapeutic drug, and beta-carotene can be used as a novel non-chemotherapeutic agent for treatment of CC. Based on the results of this study, vitamin A supplementation as preventive measure can be considered in the future. However, these data need support from animal experiments and clinical trials.
Keywords: Pegylated liposomal doxorubicin, beta-carotene, choriocarcinoma, JAR cell culture, JEG-3 cell culture

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 lifethreatening. GTD usually develops from HM, but it has been observed in aborted, term, and ectopic pregnancies. Choriocarcinoma (CC) is the most aggressive histologic type of GTD (1). The most common clinical value in the diagnosis and follow-up of this disease is serum beta-human chorionic gonadotropin (β-hCG), which is the biochemical parameter. β-hCG follow-up should be carried out in series in terms of the course and behavior of the disease and after the treatment period. However, hCG produced in choriocarcinoma has a larger oligosaccharide side chain than hCG synthesized in normal pregnancy. Therefore, it is called hyperglycosylated hCG (H-hCG) (2).
There are many medical treatments for CC depending on the stage of the disease. Multi-agent chemotherapy protocol is the preferred treatment method if hCG levels increase during treatment, if metastasis develops, or a resistance develops to sequential single-agent chemotherapy protocol (3). The current evidence-based initial therapy in treatment of high-risk metastatic GTD is the EMA-CO (etoposide, methotrexate, actinomycin D, cyclophosphamide, vincristine) protocol. Drug resistance may develop during of after primary chemotherapy in approximately 20% of high risk gestational trophoblostic neoplasia patients. In patients who do not respond to first-line EMA-CO therapy or for those who relapse, the most appropriate second-line therapy is the EMA-EP protocol. However, this protocol is quite toxic (4). The International Society of the Study of Trophoblastic Diseases (ISSTD) has reported that different treatment regimens such as paclitaxel/etoposide may be effective in relapsing patients after paclitaxel/cisplatin based combination therapies, but more studies should be conducted in this regard (5).
Doxorubicin is an anthracycline antibiotic which intercalates between base pairs in the DNA helix, thereby preventing DNA replication and ultimately inhibiting protein synthesis (6). Pegylated liposomal doxorubicin (PLD) is a polyethylene glycol-coated form of doxorubicin that has fewer side effects than those of doxorubicin, and it is approved for the treatment of HIV-related Kaposi's sarcoma, metastatic breast cancer, advanced ovarian cancer and multiple myeloma (7).
Beta-carotene is a naturally-occurring retinol (vitamin A) precursor obtained from certain fruits and vegetables with potential antineoplastic and chemopreventive activities. It is particularly protective in chemical carcinogenesis by taking part in the detoxification of

peroxide radicals (8). It was reported that the incidence of complete hydatidiform mole decreased due to increase in carotene consumption. Parazinni et al. stated that low beta-carotene consumption is related to gestational trophoblastic disease (9).
The options that can be used against multiple drug resistance in the treatment of choriocarcinoma are limited in the literature. Therefore, the aim of this study is to investigate the effects of PLD and beta-carotene on choriocarcinoma models of cell cultures developed in JAR and JEG-3 cell cultures and treatment efficacy in order to provide more effective treatment methods by providing a new perspective in choriocarcinoma treatment.

Materials and Methods

JAR and JEG-3 cell culture lines

This study was planned as pre- and post-test study. JAR and JEG-3 cell culture lines 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.

Pegylated liposomal doxorubicin preparation

PLD was purchased from Sigma, prepared in dosages of 1, 2 and 5 µg/ml 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.

Beta-carotene preparation

Beta-carotene was purchased from Sigma, prepared in dosages of 1, 5 and 10 µg/ml by diluting in DMSO, and diluted by RPMI-1640 to a maximum concentration such that DMSO formed less than 1% of the mixture.

Preparation of chemotherapeutics for tests, β-hCG and H-hCG measurement

The dosages used in the PLD and beta-carotene trial groups were as follows:
Single drug trial: PLD 1, 2 and 5 µg/ml; beta-carotene 1, 5 and 10 µg/ml.
Combined PLD and beta-carotene drug trial: PLD 1, 2 and 5 µg/ml; and beta-carotene 5 µg/ml.
Drugs were administered to the cells simultaneously, and 72 hours after drug administration, the cells were detached using trypsinethylenediamine 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 H-hCG levels. H-hCG levels were investigated using an immunoenzymatic method (Sunred Elisa Kit and DXI 600, Beckman Coulter, CA, USA). All tests were repeated 6 times.

Statistical Analysis

Statistical analyses of apoptosis results were performed through SPSS 19.0 (Statistical Package for the Social Sciences) package program. Descriptive statistics related to continuous variables were indicated with their median, minimum and maximum values. The conformity of variances to normal distribution was evaluated through the Shapiro Wilk test. The Kruskal Wallis test was used in quaternary comparison of doses and the Mann Whitney U test and the Mann Whitney U test with Bonferonni correction were used in binary subgroup comparison between doses. In all statistical analysis in the study, comparisons below a *p* value of 0.05 were accepted as statistically significant.

Detection of apoptosis using Annexin V

The annexin V-binding assay is one of the most sensitive and widely used techniques to detect

and distinguish between early apoptosis and late apoptosis, as well as between apoptosis and necrosis. 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 (10). The binding ratio of FITC-annexin-V complex to phosphatidylserine at the cell membrane can be measured using flow cytometry.

Results

The median apoptotic ratio in the control group, in which only DMSO was used, was 0.3% in the JAR cell culture; was 3.7% in the JEG-3 cell culture. The median apoptotic ratios after the application of 1, 2 and 5 μg/ml PLD were; 1.3, 1.4 and 2% in the JAR cells, respectively. The increase in the apoptotic ratio was statistically significant ($p<0.05$) (Table 1, Graph 1). The median apoptotic ratios after the application of 1, 2 and 5 μg/ml PLD were; 16.1, 31.4 and 27.5% in the JEG-3 cells, respectively. The increase in the apoptotic ratio was statistically significant, as well ($p<0.05$) (Table 1, Graph 1).

After 72 hours, median β-hCG levels in JAR and JEG-3 cell cultures were respectively, 126 and 118 mlU/ml. Median H-hCG levels in JAR and JEG-3 cell cultures were respectively, 63 and 66 mlU/ml (Table 4). After the application of 1, 2 and 5 μg/ml PLD to JAR cell lines, median β-hCG levels were: 115, 106 and 117 mlU/ml, respectively; median H-hCG levels were: 64, 60 and 64 mlU/ml, respectively (Table 4); in JEG-3 cell lines, median β-hCG levels were: 127, 114 and 118 mlU/ml, respectively; median H-hCG levels were: 63, 61 and 59 mlU/ml, respectively (Table 4). Median β-hCG and H-hCG levels after application of PLD were found to be statistically insignificant by increasing PLD dose ($p>0.05$).

The median apoptotic ratios after the application of 1, 5 and 10 μg/ml beta-carotene were 0.35, 0.6, and 0.8% in the JAR cells, respectively. The increase in the apoptotic ratio was statistically significant ($p<0.05$) (Table 2, Graph 2). The median apoptotic ratios after the application of 1, 5 and 10 μg/ml beta-carotene were 3.7, 2.5, and 2.9% in the JEG-3 cells, respectively. The increase in the apoptotic ratio was found to be statistically insignificant by increasing beta-carotene dose. ($p>0.05$) (Table 2, Graph 2).

After the application of 1, 5 and 10 μg/ml beta-carotene to JAR cell lines, median β-hCG levels were: 107, 115 and 111 mlU/ml, respectively; median H-hCG levels were: 64, 68, and 56 mlU/ml, respectively (Table 4); in JEG-3 cell lines, median β-hCG levels were: 116, 121 and 115 mlU/ml, respectively; median H-hCG levels were: 63, 63, and 64 mlU/ml, respectively (Table 4). Median β-hCG and H-hCG levels after application of beta-carotene were found to be statistically insignificant by increasing beta-carotene dose. ($p>0.05$).

The median ratios of apoptosis were 1.3, 1.4 and 2% after application of 1, 2 and 5 μg/ml PLD in JAR cell cultures, respectively; as mentioned above. This time 5 μg/ml beta-carotene was added to those PLD doses, and after application of 5 μg/ml beta-carotene combined with 1, 2 and 5 μg/ml PLD, the median apoptotic ratios were 0.4%, 1% and 1%, respectively. This incremental increase of the combined doses were statistically significant in comparision with the control group ($p<0.05$), but the incremental increase of the PLD used alone doses were statistically significant in comparision to 5 μg/ml beta-carotene that combined with PLD indeed ($p<0.05$) (Table 3, Graph 3). However combination of PLD and beta-carotene did not affect on β-hCG and H-hCG levels were between 54-63 IU/mL for H-hCG and 107-111 IU/mL for β-hCG in JAR cell cultures ($p>0.05$) (Table 4).

The median ratios of apoptosis were 16.1, 31.4 and 27.5% after application of 1, 2, and 5 μg/ml PLD in JEG-3 cell cultures, respectively; as mentioned above. This time 5 μg/ml beta-carotene was added to those PLD doses, and after application of 5 μg/ml beta-carotene combined with 1, 2, and 5 μg/ml PLD, the median apoptotic ratios were 6.1%, 5.7% and 15.5%, respectively. This incremental increase of the combined doses were statistically significant in comparision with the control group ($p<0.05$), but the incremental increase of the

PLD used alone doses were statistically significant in comparision to 5 μg/ml beta-carotene that combined with PLD indeed ($p<0.05$) (Table 3, Graph 3). Also combination of PLD and beta-carotene β-hCG and H-hCG levels were between 52-63 IU/mL for H-hCG and 109-117 IU/mL for β-hCG in JEG-3 cell cultures ($p<0.05$) (Table 4).

Discussion

Cell culture studies are frequently employed methods especially in the evaluation of newer drugs. Therefore, it enables determining the effects caused by candidate drugs on molecular targets, which are common or are exptected to be effected by those drugs, and predicting the effects that the drug will have on target tissue. Recurrent tumor growth due to changes caused by developed cancer drug components on cell phenotype leads to unexpected results in clinical practices. Use of human monolayer cancer cell lines is fairly common and an important marker to understand the causes of these results obtained in clinical practices (11). JAR and JEG-3 cells are frequently used in choriocarcinoma cell culture studies, so these cells were preferred in our study.

Various drugs have cytotoxic effects on cells in two ways with necrosis and apoptosis. It is a preferred characteristic that drugs used or to be used in cancer treatment cause cytotoxic effects in apoptotic pathways, as one of the mechanism leading to cancer is disruption in apoptotic mechanism of a normal cell becoming cancerous, and domination of anti-apoptotic signal pathways of the cell on apoptotic pathways. Therefore, it is extremely important to analyze whether apoptotic effect due to the use PLD and beta-carotene alone or in combination. In this study, apoptosis level of JAR and JEG-3 cells was identified with FCM.

Gestational trophoblastic disease (GTD) is an interrelated group of tumor characterized with abnormal proliferation of plasental trophoblasts (12). Benign GTD consists of plasental site nodule, exaggerated plasental site and hydatidiform mole (complete or partial). There is potential for local invasion and distant metastasis. Malign GTDs are persistent GTD, choriocarcinoma evaluated under gestational trophoblastic neoplasia (GTN), plasental site trophoblastic tumor (PSTT) and epithelioid trophoblastic tumor (ETT), which is a variation of PSTT (13).

Complete molar pregnancy risk increases in those with vitamin A (carotene) and animal fat deficiency on a diet, while there is no increase in partial molar pregnancy incidance (9). Choriocarcinoma is seen once in every 50000 pregnancies (14). Advance maternal age, molar pregnancy experienced and blood type A carrier increase the risk (15). Patients with stage IV or stage II-III and risk score of >7 are accepted as high risk and they are aggressively treated with multipl agents chemotherapy and/or adjuvant radiotherapy/surgery. Remission and survival rates in high-risk patients improved with EMA-CO (etoposide, methotrexate, actinomycin D, cyclophosphamide, vincristine) protocol (16). EMA-CO protocol is first-line treatment based on recent evidence in metastatic GTD with high-risk. The optimum second-line treatment in patients not responding to first-line treatment (forming plateau in low hCG values) or relapsing (re-increase in hCG after complete response) is EMA-EP protocol. Cyclophosphamide and vincristine in EMA-CO protocol replace etoposide and cisplatin in EMA-EP protocol (4). Today, the clinical use of PLD on choriocarcinoma seems to be only in limited quantity and with chosen cases.

All forms of GTD are characterized by β-hCG increase due to the interrelated, heterogeneous structure of GTD, especially resulting from trophoblastic epithelium of the placenta, and gestational tissue is responsible for its pathogenesis. HCG is used in the diagnosis and follow-up of the disease and response to treatment. Hyperglycosylated hCG (H-hCG), is a glycosylation variant with a larger side chain of hCG produced by invasive cytotrophoblast cells in pregnancy implantation, GTD and choriocarcinoma. There is an increase in H-hCG levels in testicular and ovarian germ cell tumors (17). Because of these reasons, the effect of

beta-carotene and PLD on cell culture lines were measured by using β-hCG and H-hCG parameters and ratio of apoptosis. The combination of beta-carotene and PLD on JAR and JEG-3 which are human choriocarcinoma cell line models, and the measurement of β-hCG and H-hCG by immunoenzymatic method were performed for the first time in the literature.
Cole et al. showed in their study analyzing the biological function of H-hCG in BeWo, JAR, JEG-3 choriocarcinoma cell culture and isolated gravid cytotrophoblast cells that H-hCG values were 841 ng/ml, 126 ng/ml, 165 ng/ml and 2.3 ng/ml, respectively and H-hCG was a biological tumor marker indicating cell invasion in active choriocarcinoma (17).
Rubin et al. indicated in their study evaluating the usability of H-hCG in differentiation of benign parathyroid disease and parathyroid cancer that H-hCG levels in all of patient group with cancer were higher than 3.77 pmol/l that was maximum value for patient group with benign primary hyperparathyroid and thus, H-hCG could be a possible tumor marker (18).
PLD is a long-release formulation composing of embedded doxorubicin hydrochloride into pegylated liposomes. This formula is administered in breast cancer patients with increased cardiac risk, in advanced ovarian cancer cases with unsuccessful platinum-based chemotherapy and acquired immuno-deficiency syndrome with kaposi sarcoma. Embedding doxorubicin into liposomes causes changes in pharmacokinetics and biodistribution and decrease in toxic effects (19).
Soninen et al. indicated in their study on quantitative cellular intake and toxicities of doxorubicin and other liposomal forms with different concentrations (0.5 and 5 μM) in human plasental choriocarcinoma cell culture (BeWo) that fetal exposure decreased due to pegylated formulation with lower cellular intake and toxicity of PLD compared to doxorubicin (20).
Eetazadi et al. showed in their study evaluating the intratumoral penetration and efficacy of block copolymer micella doxorubicin (BMC-DOX) formulation with similar toxicity with PLD in terms of the effects on microscopic lesions that were especially invisible to the eye in two- and three-dimensional (2D and 3D) HEYA8, OV-90 and SKOV3 human ovarian cell cultures in second-line treatment of recurring ovarian cancer after cytoreduction that BCM-DOX formulation in increasing doses between 2 mg/ml and 7.6 mg/ml provided nine-fold monolayer cytotoxicity compared to PLD and suggested that the results needed to be supported with in vivo studies (21).
Hanf et al. evaluated oxidative stress, apoptosis, phosphoproteom and epigenom changes due to doxorubicin in cardiomyocyte cell culture model investigating cardiotoxicity related to doxorubicin and showed that caspase-3 and fractin, which are apoptosis markers, and 3-nitrotyrosine and malondiadehyde, which are oxidative stress markers, dose-dependently increased as a result of (H9c2) doxorubisin 24 and 48h 1 and 5 μM administration in cultured rat myoblasts and histone-3 acetylation decreased and indicated that in conclusion, apoptosis related to oxidative stress caused cell death (22).
Popadiuk et al. showed that there was a complete response especially against brain metastasis when PLD only adminisitered in 2-3 cycles in two patients with multiple organ metastatis recurring after stnadart chemotherapy and radiotherapy regimens and concluded that PLD was an active agent in high-risk choriocarcinoma (23).
Essel et al. stated that multiple salvage chemotherapy regimen was effective in patient group with gestational trophoblastic neoplasia (GTN) on which standart treatment regimen was unsuccessful and liposomal doxorubicin was in active treatment regimen (24).
Saul et al. showed the existence of folic acit receptors administering liposomal doxorubicin with a dose of 10 μM in KB, C6 glioma cells and E9 cortex cells (25). Similarly, Lee et al. identified the existence of folate pegylated liposome complexes using LD with a dose of 10 μM, 20 μM and 50 μM and 20 μM folic acit and stated that these complexes had higher affinity in targeting cancer cells (26).

Pariente et al. have evaluated the in-vitro effect of melatonin on the cytotoxic and pro-apoptotic actions of the chemotherapeutic agents such as cisplatin (CIS), 5-fluorouracil (5-FU) and doxorubicin (DOX) in cervical cancer HeLa cells in their study and shown that melatonin increases the cytotoxic effect of the chemotherapeutic agents, caspase-3 (a key downstream effector of apoptosis) activation in CIS- and 5-FU-challenged cells and also elevated the ratio of the cells which enter mitochondrial apoptosis due to the production of reactive oxygen species (ROS). It has been demonstrated in this study that cell viability in HeLa cells was approximately 73.1% after administration of 20 μM doxorubicin alone whereas a cell viability of 57.9% was found when administered in combination with 1 mM melatonin. Doxorubicin alone was found to cause an approximately 12-fold elevation in caspase-3 activity whereas a 17-fold elevation was shown when administered in combination with 1 mM melatonin. The administration of 20 μM CIS alone induced an approximately 53% apoptosis in HeLa cells whereas an apoptosis of 73% was demonstrated when 20 μM CIS and 1mM were concomitantly administered, it was concluded according to these statistically significant outcomes ($p<0.05$) that indoleamine can be applied as a potentially strong synergistic agent in the treatment of cervical cancer (27).

Similarly, it has been shown that in vitro melatonin potentiates cytotoxic and apoptotic effects of CIS and particularly 5-FU by stimulating MT3 receptor in the human colorectal adenocarcinoma cells (HT-29) and HeLa cells. In this study, cell viability rates in the HT-29 and HeLa cells were found respectively approximately 30.7% and 22.7% after administration of 1mM 5-FU alone, whereas these rates were shown to be approximately 11.1% and 10.7% after combined administration of 1mM 5-FU and 1 mM melatonin, respectively. Apoptosis rates in the HT-29 and HeLa were reported as respectively approximately 29% and 45% after administration of 20 μM CIS alone whereas these rates were found 45% and 50% after administered in combination with 1 mM melatonin, respectively. Apoptosis rates induced by 5-FU alone were reported to be respectively approximately 46% and 47% whereas these rates were found as 71% and 65% after concomitant treatment of 5-FU and 1 mM melatonin, respectively. These increased rates of apoptosis after administration in combination with melatonin was found statistically significant ($p<0.05$) (28).

It has been shown in another study that melatonin increases the efficacy of CIS and 5-FU in HT-29 cells. In this study, apoptosis rate after administration of 1mM 5-FU alone was reported as 24.1% whereas apoptosis rate reached 30% when administered in combination with 1 mM melatonin, it has been concluded that melatonin increases the sensitization of HT-29 cells to 5-FU treatment and thus indoleamine could be used as a potential chemosensitizing agent in the treatment of adenocarcinoma (29).

There are studies showing that carotenoids have protective effect on head, neck, mouth, skin, lung and other various malignancies and hematopoietic diseases. Furthermore, it was indicated that carotenoid (beta-carotene, alfa-carotene, lycopene, beta cryptoxanthin, lutein and zeaxanthin) intake with a large amount was related to low level of oesophagus cancer risk. Most of the in vitro studies recently focused on anti-carcinogenic mechanism of beta-carotene on lung, liver and blood cell. In some animal studies, alfa-carotene had higher suppressor activity on liver, lung, skin and colon carcinogenesis compared to beta-carotene (30).

In studies, molecular protective mechanisms of carotenoids in isolated human cell culture have been indicated as: (1) stopping on cell cycle in G1/G0 phase by decreasing cycline D1 levels; (2) apoptosis induction downregulating survivin levels; (3) increase in cellular gap junction communication and (4) angiogenic effect through modulation of various cytokines (decrease in IL-6, IL-1b, TNF-a and GM-CSF levels and increase in IL-2 and TIMP-1 levels) (30, 31). Chemotherapeutic effects with similar mechanisms for all-trans retinoic acid (ATRA) that is another vitamin A analog have been showed (32). Beta-carotene is a modified

antioxidant and reduces oxidative stress (33). Researches have indicated that oxidative stress plays a critical role in the ethiopathogenesis of GTD (34, 35, 36).
Dutta et al. showed that alfa and beta-carotene, with 5 and 10 µM doses respectively, synergisticly decreased cell proliferation and DNA synthesis in oesophagus epithelial cell culture (HEE) and squamous cancer cell culture (HESC) by using in combination and identified that early administration could be a new strategy for oesophagus cancer treatment in situations like Barret oesophagus and a lower dose of beta-carotene could be sinergisticly used with alfa-carotene in protection against oesophagus malignancy (30).
Wang et al. indicated that minimum beta-carotene concentration for significant cell proliferation of EC9706 cells was 40 µM, however, in our study minimum beta-carotene concentration for significant decrease in cell proliferation was 1 µg/ml. Hurst et al. reported that high doses of beta-carotene caused decreased mitochondrial function in unexplained mechanisms and human K562 erythroleukemic and 28SV4 retinal pigment epithelial cells and that, however, there were no sufficient clinical studies evaluating beta-carotene toxic effects on human (37, 38).
Gloria et al. identified in their cell culture study related to breast cancer that use of beta-carotene (β-carotene) doses of 0,5, 1, 2,5, 5, 10 µM had dose-dependent apoptosis and necrosis-enhancing effects in MCF-7, MDA-231 and MDA-235 cell lines (39). Moreover, Wang S. et al. showed doxorubicin use at doses starting from low ones as 0,5 µM induced apoptosis in PA-1 ovarian teratocarcinoma cells and MCF-7 cells (40). Osman Abdel-M. et al. indicated that doxorubicin 0.25 µg/ml caused early apoptosis at a rate of 78%, resveratrol 15 µg/ml caused early apoptosis at a rate of 76% and use in combination caused early apoptosis at a rate of 90% (41). Previously Sel et al reported that, all-trans retinoic acid (ATRA) was an effective drug on JAR and JEG-3 choriocarcinoma cell lines due to decreasing oxidative stress (42).
For the first time, the effect of beta-carotene has been studied in combination with PLD over JAR and JEG-3 cells. To the best of our knowledge, there has not been any study evaluating the role of beta-carotene over human choriocarcinoma cell cancer lines in-vitro. In our study, when beta-carotene was administered alone at increasing doses, its apoptotic effects on JAR cell series statistically increased, however, its apoptotic effects on JEG-3 cell series did not statistically increased. We identified that when PLD was administered alone at increasing doses its apoptotic effects on both JAR and JEG-3 cell series statistically increased. When both drugs were combined, beta-carotene dose was kept stable and PLD dose was increased, apoptotic effect on JAR and JEG-3 cell series statistically increased.

Conclusion

Beta-carotene and PLD combination on JAR and JEG-3 that are human choriocarcinoma cell line models and β-hCG and H-hCG tests with immunoenzymatic method on every two cell line together have been performed for the first time in literature. Synergistic apoptotic data acquired show that beta-carotene and PLD combination is one of the options to be used against multiple drug resistence during choriocarcinoma treatment. Based on the results of this study, vitamin A supplementation as preventive measure can be considered in the future. However, data should be primarily supported with animal trials and then with clinical studies, as the effects of the drugs and the effects in combination can be different in vivo systems.

Ethics

Ethics committee approval: Ethics committee approval was received for this study from the Ethics Committee of Bülent Ecevit University School of Medicine (No: 2014-68-25/03, Date:03/25/2014).

Informed Consent: No informed consent was obtained due to cell culture study.

Authorship Contributions

Surgical and Medical Practices: İ.Ö.T., Concept: M.İ.H., M.H., İ.Ö.T., Design: M.İ.H., M.H., Data Collection or Processing: S.A.E., M.İ.H., M.H., İ.Ö.T., Analysis or Interpretation: S.A.E., G.S., M.İ.H., M.H., Literature Search: S.A.E., G.S., M.İ.H., M.H., Writing: S.A.E., G.S., M.İ.H., M.H.

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Table 1. Ratio of apoptosis (%) on control group and after application of PLD to JAR and JEG-3 cell lines (p < 0.05)

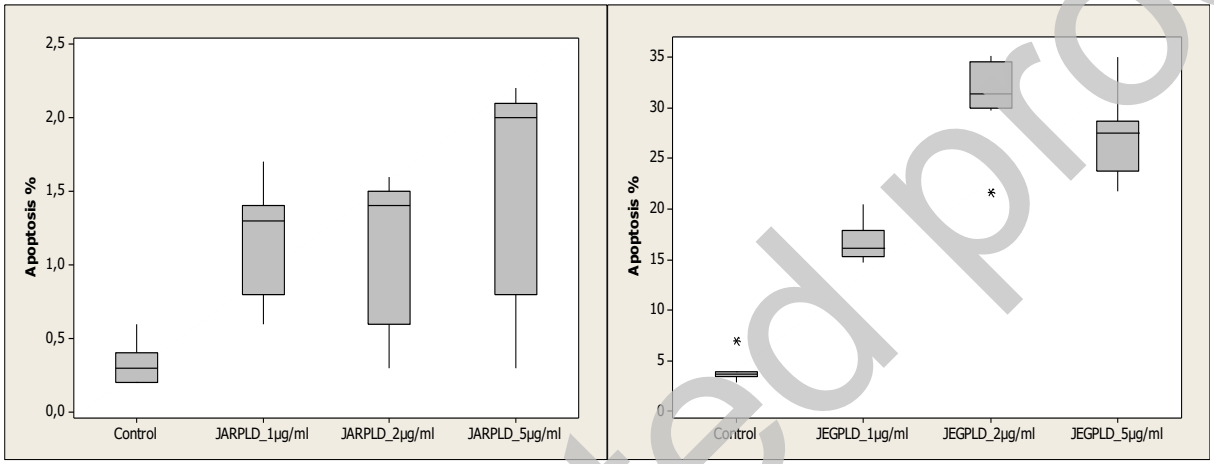
	Ratio of Apoptosis; Median (Min-Max) in JAR	Ratio of Apoptosis; Median (Min-Max) in JEG-3
Control	0.3 (0.6-0.2)	3.7 (2.9-7)
PLD 1 µg/ml	1.3 (0.6-1.7)	16.1 (14.7-20.5)
PLD 2 µg/ml	1.4 (0.3-1.6)	31.4 (21.6-35.1)
PLD 5 µg/ml	2 (0.3-2.2)	27.5 (21.8-35)
***** Shapiro Wilk, Mann Whitney U and Kruskal-Wallis tests.		

Table 2. Ratio of apoptosis (%) on control group and after application of beta-carotene to JAR and JEG-3 cell lines ($p < 0.05$, $p > 0.05$, respectively)		
	Ratio of Apoptosis; Median (Min-Max) in JAR	Ratio of Apoptosis; Median (Min-Max) in JEG-3
Control	0.3 (0.2-0.6)	3.7 (2.9-7)
Beta-carotene 1 µg/ml	0.35 (0.2-0.8)	3.7 (1-10.1)
Beta-carotene 5 µg/ml	0.6 (0.4-0.9)	2.5 (1-12)
Beta-carotene 10 µg/ml	0.8 (0.7-0.9)	2.9 (1-7.3)
***** Shapiro Wilk, Mann Whitney U and Kruskal-Wallis tests.		

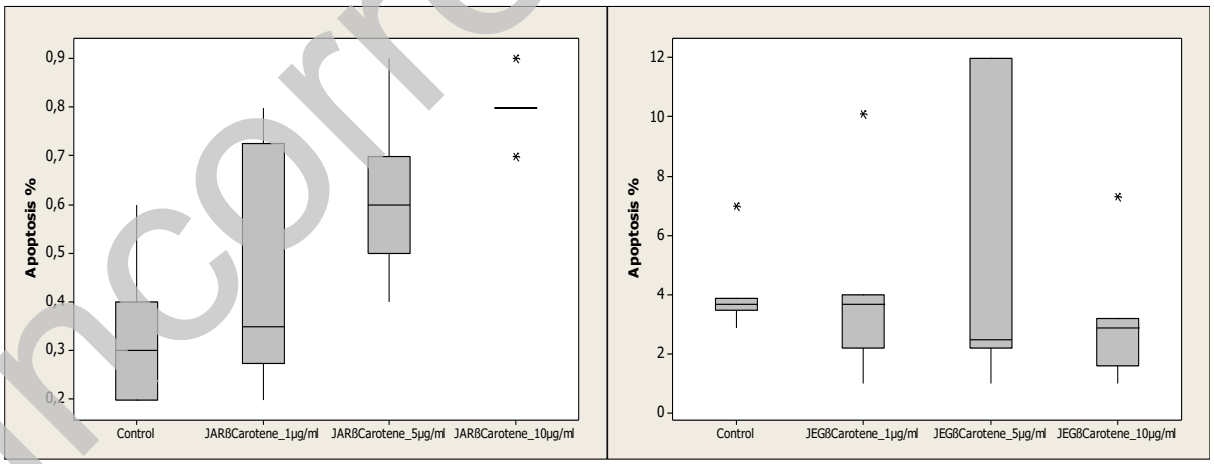
Table 3. Ratio of apoptosis (%) on control group and after application of combined doses of PLD and beta-carotene to JAR and JEG-3 cell lines ($p < 0.05$)		
	Ratio of Apoptosis; Median (Min-Max) in JAR	Ratio of Apoptosis; Median (Min-Max) in JEG-3
Control	0.3 (0.2-0.6)	3.7 (2.9-7)
PLD 1 µg/ml + beta-carotene 5 µg/ml	0.4 (0.3-0.8)	6.1 (5.2-7.1)
PLD 2 µg/ml + beta-carotene 5 µg/ml	1 (0.3-1.6)	5.7 (4.4-9)
PLD 10 µg/ml + beta-carotene 5 µg/ml	1 (0.4-1.1)	15.5 (5.5-17)
***** Shapiro Wilk, Mann Whitney U and Kruskal-Wallis tests.		

Table 4. H-hCG and β-hCG levels on control group and after application of PLD, beta-carotene and combined doses to JAR and JEG-3 cell lines ($p > 0.05$)		
	H-hCG mlU/ml	β–hCG mlU/ml
JAR Control	63	126
JAR PLD 1 µg/ml	64	115
JAR PLD 2 µg/ml	60	106
JAR PLD 5 µg/ml	64	117
JAR Beta-carotene 1 µg/ml	64	107
JAR Beta-carotene 5 µg/ml	68	115
JAR Beta-carotene 10 µg/ml	56	111
JAR PLD 1 µg/ml + beta-carotene 5 µg/ml	54	107
JAR PLD 2 µg/ml + beta-carotene 5 µg/ml	63	107
JAR PLD 5 µg/ml + beta-carotene 5 µg/ml	58	111
JEG-3 Control	66	118
JEG-3 PLD 1 µg/ml	63	127
JEG-3 PLD 2 µg/ml	61	114
JEG-3 PLD 5 µg/ml	59	118

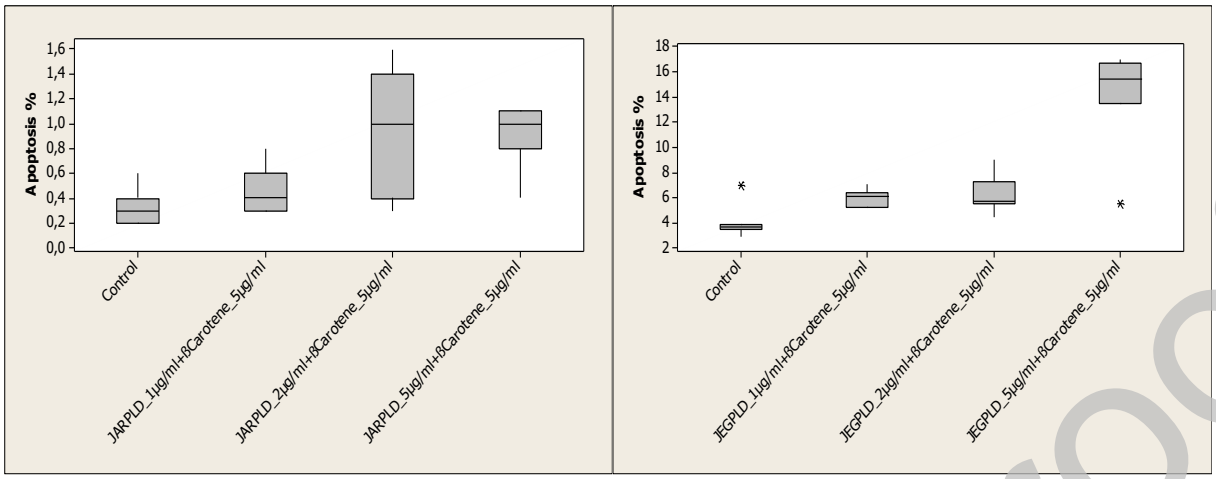
JEG-3 Beta-carotene 1 µg/ml	63	116
JEG-3 Beta-carotene 5 µg/ml	63	121
JEG-3 Beta-carotene 10 µg/ml	64	115
JEG-3 PLD 1 µg/ml + beta-carotene 5 µg/ml	63	109
JEG-3 PLD 2 µg/ml + beta-carotene 5 µg/ml	59	117
JEG-3 PLD 5 µg/ml + beta-carotene 5 µg/ml	52	111
***** Shapiro Wilk, Mann Whitney U and Kruskal-Wallis tests.		



Graph 1. Ratio of apoptosis (%) on control group and after application of PLD to JAR and JEG-3 cell lines ($p < 0.05$).
*****Shapiro Wilk, Mann Whitney U and Kruskal-Wallis tests.



Graph 2. Ratio of apoptosis (%) on control group and after application of beta-carotene to JAR and JEG-3 cell lines ($p < 0.05$, $p > 0.05$, respectively).
*****Shapiro Wilk, Mann Whitney U and Kruskal-Wallis tests.



Graph 3. Ratio of apoptosis (%) on control group and after application of combined doses of PLD and beta-carotene to JAR and JEG-3 cell lines ($p < 0.05$).
***** Shapiro Wilk, Mann Whitney U and Kruskal-Wallis tests.