Research Article / Araştırma Makalesi
doi: 10.18678/dtfd.1315416

Duzce Med J, 2023;25(3):225-230
Düzce Tıp Fak Derg, 2023;25(3):225-230

Muscarinic Receptors as Targets for Metronomic Therapy in Ovarian Cancer

Yumurtalık Kanserinde Metronomik Tedavi Hedefi Olarak Muskarinik Reseptörler

Fatma SÖYLEMEZ[1]
0000-0002-1939-8691
Çağatay Han TÜRKSEVEN[2]
0000-0002-0584-0661

[1]Department of Medical Biology and Genetics, Faculty of Medicine, Mersin University, Mersin, Türkiye

[2]Department of Biophysics, Faculty of Medicine, Mersin University, Mersin, Türkiye

Corresponding Author
Sorumlu Yazar
Çağatay Han TÜRKSEVEN
cagatayhanturkseven1923@gmail.com

Received / Geliş Tarihi : 15.06.2023
Accepted / Kabul Tarihi : 22.09.2023
Available Online / Çevrimiçi Yayın Tarihi : 23.10.2023

ABSTRACT

Aim: In this study, the effects of muscarinic acetylcholine receptor (mAChR) agonist carbachol on the proliferation of cisplatin-resistant (A2780cis) and cisplatin-free (SKOV-3) ovarian cancer cell line were for the first time investigated to further evaluate the potential therapeutic effect of metronomic chemotherapy.

Material and Methods: The inhibitory effect of carbachol on cell proliferation was detected using the xCELLigence Real-Time Cell Analyzer (RTCA) dual plate (DP) system. A preliminary study was conducted to determine the dose of carbachol 100 µM, cisplatin 1 µM, and two combination studies were carried out with 100 µM carbachol + cisplatin 1 µM and 100 µM carbachol + 10 µM atropine, over cancer cells without drugs was used as the control group. The cell proliferation curve was monitored for 96 hours. The cell index value of inhibition in cell proliferation was automatically measured every hour for each well using RTCA 1.2.1 software.

Results: Co-administration of carbachol with cisplatin caused a decrease in cell number in both A2780cis and SKOV-3 cell lines in a time-dependent manner ($p<0.001$). Substantial cell death was observed in both cisplatin-resistant (A2780cis) and cisplatin-free (SKOV-3) cell lines within 24 hours after carbachol with cisplatin application and this continued at the 96th hour.

Conclusion: The findings of this study confirm the notion that mAChRs can be considered as therapeutic targets for metronomic therapy in ovarian cancer, as well as the usefulness of a muscarinic agonist as a repositioning drug in the treatment of such tumors.

Keywords: Ovarian cancer; mAChR; carbachol; SKOV-3; A2780cis; cisplatin.

ÖZ

Amaç: Bu çalışmada, muskarinik asetilkolin reseptörü (mAChR) agonisti karbakolun, sisplatine dirençli (A2780cis) ve sisplatine dirençsiz (SKOV-3) yumurtalık kanseri hücre hatlarının proliferasyonu üzerindeki etkileri, metronomik kemoterapinin potansiyel terapötik etkisini daha ileri düzeyde değerlendirmek üzere ilk kez araştırıldı.

Gereç ve Yöntemler: Karbakolün hücre proliferasyonu üzerindeki inhibitör etkisi, xCELLigence Real-Time Cell Analyzer (RTCA) dual plate (DP) sistemi kullanılarak tespit edildi. 100 µM karbakol ve 1 µM sisplatin dozunu belirlemek için bir ön çalışma yapıldı ve 100 µM karbakol + 1 µM sisplatin ve 100 µM karbakol + 10 µM atropin olmak üzere iki kombinasyon çalışması yapıldı, ilaçsız yumurtalık kanser hücreleri ise kontrol grubu olarak kullanıldı. Hücre proliferasyon eğrisi 96 saat izlendi. Hücre proliferasyonundaki inhibisyonun hücre indeksi değeri, RTCA 1.2.1 yazılımı ile her kuyu için her saat otomatik olarak ölçüldü.

Bulgular: Karbakolun sisplatin ile birlikte uygulanması hem A2780cis hem de SKOV-3 hücre hatlarında zamana bağlı olarak hücre sayısında azalmaya neden olmuştur ($p<0.001$). Sisplatin ile karbakol uygulamasından sonraki 24 saat içinde sisplatine dirençli (A2780cis) ve sisplatine dirençsiz (SKOV-3) her iki hücre hattında da önemli düzeyde hücre ölümü gözlenmiş ve bu durum 96. saatte de devam etmiştir ($p<0.001$).

Sonuç: Bu çalışmanın bulguları, mAChR'lerin yumurtalık kanserinde metronomik tedavi için terapötik hedefler olarak kabul edilebileceği fikrini ve ayrıca bu tür tümörlerin tedavisinde bir muskarinik agonistin bir yeniden konumlandırma ilacı olarak kullanışlılığını doğrulamaktadır.

Anahtar kelimeler: Yumurtalık kanseri; mAChR; karbakol; SKOV-3; A2780cis; sisplatin.

INTRODUCTION

G protein-coupled receptors (GPCRs) are involved in tumorigenesis, including abnormal cell growth, increased cell viability, angiogenesis, and metastasis (1). These receptors control basic physiological functions such as neurotransmission, enzyme and hormone release, immune reactions, muscle contraction, and blood pressure regulation (2). Functional differences in these receptors cause some diseases including cancer and therefore, GPCRs are the target of therapeutic agents used for many diseases.

Three muscarinic receptor subtypes (M1R, M3R, and M5R) that stimulate cellular signaling when expressed in proliferating cells are conditional oncogenes (3).

Carbachol is a cholinomimetic drug that binds and activates acetylcholine receptors, and therefore it is classified as a cholinergic agonist. Some studies have shown that the cholinergic agonist carbachol induces cancer cell proliferation (4,5). Activation of muscarinic acetylcholine receptors (mAChRs) by the agonist carbachol has been reported to cause two opposite types of responses in breast tumor cells: short-term stimulation promotes tumor progression, while long-term treatment induces cancer cell death without affecting normal cells lacking mAChRs (4). The latter effect was thought to be an unfulfilled goal of conventional chemotherapy, consisting of systemic delivery of the highest effective and tolerable dose to kill as many of the tumor cells as possible. It also produces undesirable effects on the normal tissues of cancer patients and requires chemotherapy to be administered at relatively free and long intervals between doses to allow normal cells to heal. Therefore, the application of metronomic therapy has come to the fore to improve the treatment of cancer patients. Metronomic therapy relies on the administration of low doses of a chemotherapeutic drug, alone or in combination with other drugs, at short inter-dose intervals to provide both effective treatment and reduce side effects. Long-term treatment of breast cancer cells with the muscarinic agonist carbachol promotes cell death, it was investigated whether low doses of this agonist combined with paclitaxel (PX) used in the treatment of breast cancer inhibit disease progression in human MCF-7 tumor cells and PX plus carbachol has been shown to reduce cell viability and tumor growth in vitro (6). The results of this study confirm that mAChRs can be considered as therapeutic targets for metronomic therapy in breast cancer and that a muscarinic agonist would also be useful in the treatment of such tumors.

Gynecological cancers are among the important health problems in terms of mortality and morbidity in all women (7). Ovarian cancer is the seventh most common cancer in women and the most lethal gynecological cancer (8). It is the seventh most common cancer among women and the eighth leading cause of cancer-related death. The typical age of diagnosis with ovarian cancer is 60, and the average lifetime risk for women is about 1 in 70. Studies have shown that GPCRs are involved in the progression and metastasis of ovarian neoplasms. The presence of mAChRs has been detected in different types of tumor cells, including ovarian cancer, and they are associated with tumorigenesis (9,10).

In a single study that was identified in the literature, the effects of carbachol and histamine on changes in cytosolic-free calcium and cell proliferation have been characterized in human ovarian cancer (OVCAR-3) and non-tumorigenic Chinese hamster ovarian (CHO) cells. Both carbachol and histamine-induced cell proliferation in OVCAR-3 cells but did not affect CHO cells (11). It was observed that the effects of carbachol and histamine on cell proliferation and Ca^{2+} increase on OVCAR-3 cells were completely blocked by atropine and the selective H-1 histaminergic receptor antagonist pyrilamine, respectively. In this study, it was planned to investigate whether the muscarinic receptor agonist carbachol combined with PX used for cancer treatment inhibits the progression of the disease in tumor cells of cisplatin-resistant (A2780cis) and cisplatin-free (SKOV-3) ovarian cancer cell lines.

MATERIAL AND METHODS

Cell Culture

Cell culture A2780 was obtained from the European Collection of Authenticated Cell Cultures, (93112519) and SKOV-3 over cancer cells was obtained from the American Type Culture Collection (ATCC® HTB-77™, Manassas, VA, USA). These cells were cultured in RPMI medium (Catalog number: 11875093, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum, penicillin/streptomycin, and amphotericin-B 1%. Cell culture was performed at 37 °C in a 5% CO_2 incubator. In a study aiming to evaluate the potential association of M3 muscarinic receptors with proliferation and cell death in the human chronic myelogenous leukemia K562 cell line, it was determined that 100 µM carbachol application caused a decrease in cell number (5). Exposure of K562 cells to carbachol for 24 hours reduced the number of early apoptotic cells but had no change in the number of necrotic cells. 100 µM carbachol treatment for 48 hours increased the number of necrotic cells and decreased the number of apoptotic cells. Taking the results of this study as a reference, the carbachol 100 µM (CCh group), cisplatin 1 µM (Cis group), 100 µM carbachol + cisplatin 1 µM (CCh+Cis group), and 100 µM carbachol + 10 µM atropine (CCh+Atr group) combinations were carried out, over cancer cells without drug was the control group. Cancer cells were incubated with carbachol, atropine, and cisplatin for 96 hours.

xCELLigence Real-Time Cell Analysis

The xCELLigence Real-Time Cell Analyzer (RTCA) dual plate (DP) system (Roche Diagnostics GmbH, Penzberg) was used for real-time monitoring of cell viability without labeling on the cells. $3x10^3$ cells were seeded in each well of the E-plate and the cell proliferation curve was monitored for 24 hours after that carbachol, atropine, cisplatin, and their combinations were added to the E-plate systems and they were monitored in real-time for 96 hours. The cell index (CI) value was automatically measured every hour for each well with RTCA 1.2.1 software.

Statistical Analysis

All statistical analyses were performed using the SPSS software v.21. The assumptions of normality were tested by the Shapiro-Wilk test. The homogeneity of variances with normal distribution was tested by the Levene test. Homogeneous data were analyzed with the ANOVA test for comparisons between groups, and non-homogeneous data were analyzed with the Welch test. The Bonferroni

post hoc test was used for comparisons between subgroups of homogeneous data, while the Dunnett T3 test was used in non-homogeneous data. The data were expressed as mean±standard deviation, and the p value of <0.05 was defined as statistically significant.

RESULTS

The effect of the non-selective muscarinic agonist carbachol (100 μM) added to cisplatin-resistant (A2780cis) and cisplatin-free (SKOV-3) ovarian cancer cells on cell proliferation were examined in this study. The time period in which it showed the best effect was determined by comparing the groups at 0, 24, 48, 72, and 96 hours.

Results for Cisplatin-Resistant (A2780cis) Cell Line

Comparison of the cell proliferation between the groups for the cisplatin-resistant (A2780cis) cell line at 0, 24, 48, 72, and 96 hours were shown in Table 1 and Figure 1. Any statistically significant difference was not seen when the cell index values taken at the 0th hour were compared between the groups (p=0.165). At the 24th hour, it was observed that the control and CCh groups decreased significantly compared to the Cis group (p<0.001). At 48th and 72nd hours, the groups administered only carbachol were statistically significantly reduced compared to the group administered only cisplatin (p<0.001). At the 24th, 48th, and 72nd hours, a statistically significant decrease was detected in the CCh+Cis group compared to the control, CCh, and Cis groups (p<0.001). Additionally, when the CCh+Atr group was compared with the Cis and CCh+Cis groups, there was a significant increase in the Cis group, while a statistically significant decrease was detected in the CCh+Cis group (p<0.001). At the 96th hour, there was a statistically significant decrease in the CCh+Cis group compared to the control, CCh, and Cis groups (p=0.009).

Results for Cisplatin-Free (SKOV-3) Cell Line

Comparison of the cell proliferation between the groups for the cisplatin-free (SKOV-3) cell line at 0, 24, 48, 72, and 96 hours were shown in Table 2 and Figure 2. Any statistically significant difference was not seen when the cell index values taken at the 0th hour were compared between the groups (p=0.329). At the 24th hour, when the control group was compared with the group administered only with carbachol, a significant decrease in cell viability was detected in the CCh group (p<0.001). In addition, when the control and CCh groups were compared with the Cis group, a statistically significant decrease was found in the control and CCh groups (p<0.001). When the CCh+Cis group was compared with the CCh group, a statistically significant decrease was observed in the CCh group, while there was a significant increase in the Cis group (p<0.001). When the CCh+Atr group was compared with the CCh group, a statistically significant decrease was observed in the CCh and CCh+Cis groups, and a significant increase was found in the Cis group (p<0.001). At the 48th hour, when the control group was compared with the group administered only with carbachol, a significant decrease in cell viability was detected in the CCh group (p<0.001). In addition, when the control and CCh groups were compared with the Cis group, there was a statistically significant decrease in the control and CCh groups (p<0.001). When the CCh+Cis group was compared with the group administered only cisplatin, a significant decrease in cell viability was detected in the CCh+Cis group (p<0.001). When the CCh+Atr group was compared with the control and only cisplatin-administered groups, it was observed that there was a statistically significant decrease in the CCh+Atr group (p<0.001). At the 72nd hour, when the groups administered only carbachol and only cisplatin were compared, a significant decrease was detected in the CCh group (p<0.001). When the CCh+Cis group was compared with the control, only carbachol and only cisplatin applied groups, a statistically significant decrease was observed in the CCh+Cis group (p<0.001). When the CCh+Atr group was compared with the Cis and CCh+Cis

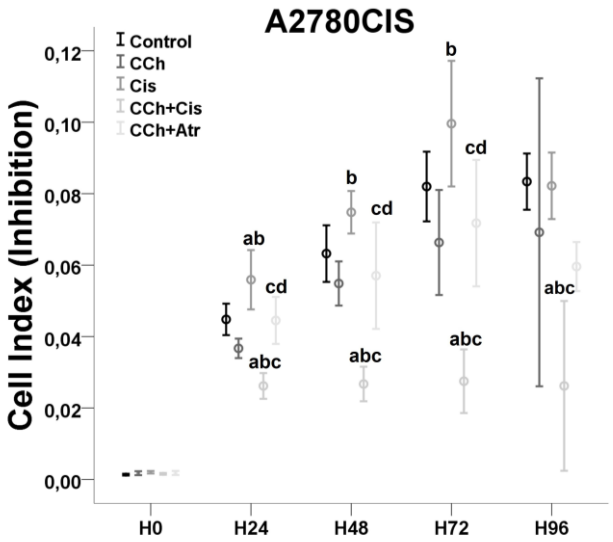

Figure 1. The effect of the cisplatin and carbachol on cisplatin-resistant (A2780cis) ovarian cell proliferation

CCh: carbachol applied group, Cis: cisplatin applied group, CCH+Cis: carbachol and cisplatin applied group, CCh+Atr: carbachol and atropine applied group, superscript letters denote significant differences of the group when compared with the [a]: control group, [b]: CCh group, [c]: Cis group, and [d]: CCh+Cis groups (p<0.001)

Table 1. Carbachol and cisplatin effects on cisplatin-resistant (A2780cis) ovarian cancer cell proliferation

	Control	CCh	Cis	CCh+Cis	CCh+Atr	p
H0	0.0013±0.0001	0.0017±0.0003	0.0020±0.0002	0.0015±0.0001	0.0018±0.0003	0.165
H24	0.0448±0.0022	0.0367±0.0014	0.0559±0.0041^{ab}	0.0261±0.0018^{abc}	0.0445±0.0032^{cd}	**<0.001**
H48	0.0632±0.0039	0.0548±0.0031	0.0747±0.0030^{b}	0.0267±0.0024^{abc}	0.0570±0.0074^{cd}	**<0.001**
H72	0.0820±0.0048	0.0663±0.0073	0.0996±0.0087^{b}	0.0275±0.0044^{abc}	0.0717±0.0088^{cd}	**<0.001**
H96	0.0833±0.0039	0.0691±0.0215	0.0821±0.0046	0.0261±0.0118^{abc}	0.0596±0.0034	**0.009**

CCh: carbachol applied group, Cis: cisplatin applied group, CCH+Cis: carbachol and cisplatin applied group, CCh+Atr: carbachol and atropine applied group, superscript letters denote significant differences of the group when compared with the [a]: control group (p<0.001), [b]: CCh group (p<0.001), [c]: Cis group (p<0.001), [d]: CCh+Cis group (p<0.001)

Table 2. Carbachol and cisplatin effects on cisplatin-free (SKOV-3) ovarian cancer cell proliferation

	Control	CCh	Cis	CCh+Cis	CCh+Atr	p
H0	0.0057±0.0002	0.0051±0.0003	0.0052±0.0004	0.0058±0.0005	0.0061±0.0004	0.329
H24	0.0390±0.0092	0.0116±0.0040[a]	0.0779±0.0039[ab]	0.0336±0.0026[bc]	0.0547±0.0073[bcd]	**<0.001**
H48	0.0551±0.0071	0.0394±0.0037[a]	0.0863±0.0045[ab]	0.0469±0.0038[c]	0.0324±0.0042[ac]	**<0.001**
H72	0.0740±0.0073	0.0606±0.0031	0.0849±0.0039[b]	0.0236±0.0034[abc]	0.0617±0.0030[cd]	**<0.001**
H96	0.0846±0.0077	0.0774±0.0071	0.0752±0.0025	0.0158±0.0033[abc]	0.0888±0.0038[d]	**<0.001**

CCh: carbachol applied group, Cis: cisplatin applied group, CCH+Cis: carbachol and cisplatin applied group, CCh+Atr: carbachol and atropine applied group, superscript letters denote significant differences of the group when compared with the [a]: control group (p<0.001), [b]: CCh group (p<0.001), [c]: Cis group (p<0.001), [d]: CCh+Cis group (p<0.001)

groups, there was a significant increase in the Cis group, while a statistically significant decrease was detected in the CCh+Cis group (p<0.001). At the 96th hour, when the CCh+Cis group was compared with the control, only carbachol applied and only cisplatin applied groups, a statistically significant decrease was determined in the CCh+Cis group (p<0.001). When the CCh+Atr group was compared with the CCh+Cis group, a significant decrease was observed in the CCh+Cis group (p<0.001).

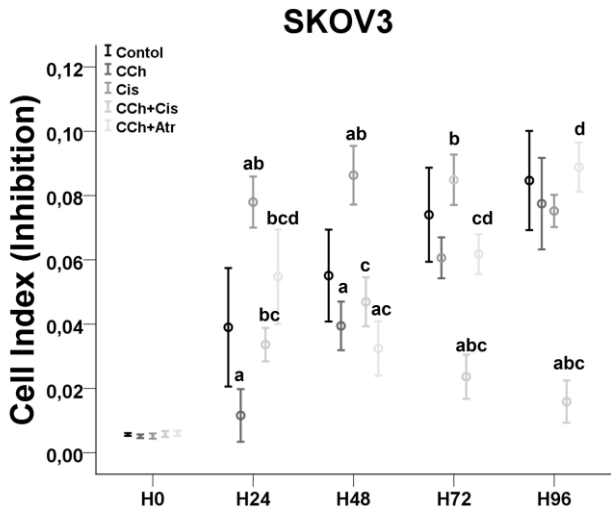

Figure 2. The effect of cisplatin and carbachol on cisplatin-free (SKOV-3) ovarian cell proliferation

CCh: carbachol applied group, Cis: cisplatin applied group, CCH+Cis: carbachol and cisplatin applied group, CCh+Atr: carbachol and atropine applied group, superscript letters denote significant differences of the group when compared with the [a]: control group, [b]: CCh group, [c]: Cis group, and [d]: CCh+Cis groups (p<0.001)

DISCUSSION

The term metronomic chemotherapy (MT) first used by Hanahan et al. (12), is the chronic administration of chemotherapy at low, minimal doses on a continuous schedule of administration (13). As ovarian cancer remains the most common cause of death from a gynecological malignancy, it is necessary to find better treatment strategies than traditional dosing of chemotherapy to cure this deadly disease. The traditional chemotherapy program requires episodic administration of a cytotoxic drug at the maximum tolerated doses (MTD) which targets tumor cells followed by periods of rest to allow non-cancer tissues to recover (14). However, these cytotoxic drugs also damage normal cells, especially in MTD (15). It has been reported that the success of chemotherapy may depend on the development of approaches for metronomic planning in order to minimize MTD toxicity and improve its anti-tumor effect (16). One of the main challenges in the treatment of ovarian cancer is the development of drug resistance. Metronomic chemotherapy also prevents the development of drug resistance as it reduces the extent of drug-free periods.

For the development of the MT strategy, some drugs were tested, especially those with an oral formulation. The common point of the studies conducted is that MT applications have promising results for ovarian cancer therapy. Nevertheless, the identification of the optimal dosage has yet to be established. Using an optimal metronomic dose of metronomic docetaxel has been shown to be effective in inhibiting tumor growth and prolonging survival using an ovarian cancer model (14). In addition, it has been reported that oral topotecan may be an ideal agent to be considered for clinical MT trial in ovarian cancer (17). In another study, pazopanib therapy in combination with metronomic topotecan therapy showed significant antitumor effects in preclinical ovarian cancer models (18). When the studies are reviewed, it is suggested that MT is a treatment option for ovarian cancer patients.

Muscarinic receptor agonists such as carbachol stimulate cell proliferation, survival, migration, and invasion, as shown by in vitro studies using human ovarian cancer cells. There is previous evidence suggesting that muscarinic receptors can regulate cell proliferation depending on the growth context of the cell. It has previously been reported that mAChRs are involved in ovarian cancer progression (9). mAChRs belong to the GPCR family which constitutes the largest family of cell surface receptors involved in signal transduction. The effects of carbachol and histamine on cytosolic-free calcium and changes in cell proliferation were characterized in OVCAR-3 cells and non-tumorigenic CHO cells (11).

No studies have yet been conducted on the effects of CCh in metronomic therapy applications for ovarian cancer. In a study evaluating cell proliferation has been reported that the cholinergic agonist carbachol inhibits the proliferation of human chronic myelogenous leukemia K562 cells, especially at the 48th hour after administration (5). A study investigating metronomic treatment approaches in breast cancer demonstrated that low doses of therapy combining PX with carbachol could be a useful strategy to treat triple negative (TN) breast tumors (19). To inhibit the action of

carbachol, cells also were treated with atropine at 10^{-9} M and this inhibitory effect was prevented in the presence of atropine.

In this study, it was investigated the action of a combination of low doses of the muscarinic agonist carbachol plus cisplatin, a chemotherapeutic agent frequently used in ovarian cancer treatment, in terms of effectiveness. Substantial cell death was observed in A2780cis and SKOV-3 cells within 24 h after carbachol application and this continued at 96th hour. It was observed that carbachol application caused a significant decrease in cell number at the 24th hour compared to cisplatin in cisplatin-free and cisplatin-resistant cell lines. As a result of atropine application, while carbachol decreased cell proliferation both alone and together with cisplatin, only cisplatin itself showed the opposite activity in the cisplatin-free cell line. The effect of carbachol on reducing cell proliferation was seen at the 72nd and 96th hours when it was administered together with cisplatin in the cisplatin-free cell line. In the cisplatin-resistant cell line, it was observed that the control and CCh groups significantly decreased compared to the Cis group at 24 hours. Carbachol with cisplatin significantly inhibited cell proliferation at the 24th, 48th, 72nd, and 96th hours. Co-administration of carbachol with cisplatin caused a more effective decrease in cell number than application of carbachol alone. Unlike other studies, the inhibitory effect of carbachol was not prevented by atropine application. It is demonstrated that low doses therapy combining cisplatin with a cholinergic agonist carbachol could be a useful strategy to treat ovarian tumors.

CONCLUSION

These results support the notion that the cholinergic agonist carbachol may have roles in cell death and affect cell proliferation by activating muscarinic receptors. The results of the study confirmed that mAChRs can be considered as therapeutic targets for metronomic therapy in ovarian cancer and the usefulness of a muscarinic agonist as a repositioning drug in the treatment of this type of tumor. However, in order to reach a definite conclusion, the results need to be supported by other methods. The next study is planned to evaluate the effects of carbachol on cell proliferation as well as its effects on cell cycle and apoptosis in breast, ovarian, and brain cancers.

Ethics Committee Approval: Since our study was not an experimental study including human or animal subject, ethics committee approval was not required.

Conflict of Interest: None declared by the authors.

Financial Disclosure: None declared by the authors.

Acknowledgments: The authors would like to thank to Mersin University Advanced Technology Education Research and Application Center.

Author Contributions: Idea/Concept: ÇHT; Design: FS, ÇHT; Data Collection/Processing: FS, ÇHT; Analysis/Interpretation: ÇHT; Literature Review: FS, ÇHT; Drafting/Writing: FS, ÇHT; Critical Review: FS, ÇHT.

REFERENCES

1. Dorsam RT, Gutkind JS. G-protein-coupled receptors and cancer. Nat Rev Cancer. 2007;7(2):79-94.
2. Zhang Q, Madden NE, Wong AST, Chow BKC, Lee LTO. The Role of endocrine G protein-coupled receptors in ovarian cancer progression. Front Endocrinol (Lausanne). 2017;8:66.
3. Gutkind JS, Novotny EA, Brann MR, Robbins KC. Muscarinic acetylcholine receptor subtypes as agonist-dependent oncogenes. Proc Natl Acad Sci USA. 1991;88(1):4703-7.
4. Español AJ, Jacob G, Dmytrenko G, Sales ME. Muscarinic activation enhances the anti-proliferative effect of paclitaxel in murine breast tumor cells. Anticancer Agents Med Chem. 2013;13(8):1273-9.
5. Aydın B, Tulunay A, Ekşioğlu-Demiralp E, Kan B, Cabadak H. Effects of carbachol on apoptosis in human chronic myelogenous leukemic K562 cell line. Marmara Med J. 2019;32(1):38-43.
6. Salem AR, Martínez Pulido P, Sanchez F, Sanchez Y, Rojo D, Español AJ. Effect of low dose metronomic therapy on MCF-7 tumor cells growth and angiogenesis. Role of muscarinic acetylcholine receptors. Int Immunopharmacol. 2020;84:106514. Corrigendum. Int Immunopharmacol. 2020;85:106701.
7. Uçar T, Güney E, Bal Z. Psychosocial aspects of gynecologic cancer. Sakarya Med J. 2018;8(4):678-85.
8. Cannistra SA. Cancer of the ovary. N Eng J Med. 2004;351(24):2519-29.
9. Oppitz M, Mobus V, Brock S, Drews U. Muscarinic receptors in cell lines from ovarian carcinoma: negative correlation with survival of patients. Gynecol Oncol. 2002;85(1):159-64.
10. Batra S, Popper LD, Iosif CS. Characterization of muscarinic cholinergic receptors in human ovaries, ovarian tumours and tumour cell lines. Eur J Cancer. 1993;29A(9):1302-6.
11. Popper L, Batra S. Muscarinic acetylcholine and histamine-receptor mediated calcium mobilization and cell-growth in human ovarian-cancer cells. Int J Oncol. 1994;4(2):453-9.
12. Hanahan D, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J Clin Invest. 2000;105(8):1045-7.
13. Munzone E, Colleoni M. Clinical overview of metronomic chemotherapy in breast cancer. Nat Rev Clin Oncol. 2015;12(11):631-44.
14. Kamat AA, Kim TJ, Landen CN Jr, Lu C, Han LY, Lin YG, et al. Metronomic chemotherapy enhances the efficacy of antivascular therapy in ovarian cancer. Cancer Res. 2007;67(1):281-8.
15. Su WH, Ho TY, Li YT, Lu CH, Lee WL, Wang PH. Metronomic therapy for gynecologic cancers. Taiwan J Obstet Gynecol. 2012;51(2):167-78.
16. Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. Nat Rev Cancer. 2004;4(6):423-36.
17. Hashimoto K, Man S, Xu P, Cruz-Munoz W, Tang T, Kumar R, et al. Potent preclinical impact of metronomic low-dose oral topotecan combined with the antiangiogenic drug pazopanib for the treatment of ovarian cancer. Mol Cancer Ther. 2010;9(4):996-1006.

18. Merritt WM, Nick AM, Carroll AR, Lu C, Matsuo K, Dumble M, et al. Bridging the gap between cytotoxic and biologic therapy with metronomic topotecan and pazopanib in ovarian cancer. Mol Cancer Ther. 2010;9(4):985-95.

19. Español AJ, Salem A, Di Bari M, Cristofaro I, Sanchez Y, Tata AM, et al. The metronomic combination of paclitaxel with cholinergic agonists inhibits triple negative breast tumor progression. Participation ofM2 receptor subtype. PLoS One. 2020;15(9):e0226450.