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Original Article

Carboplatin and epigallocatechin-3-gallate synergistically induce cytotoxic effects in esophageal cancer cells

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Abstract

Background and purpose: We aimed at evaluating the effects of combinatorial treatments with carboplatin and epigallocatechin-3-gallate (EGCG) on the KYSE-30 esophageal cancer (EC) cell line and elucidate the underlying mechanisms.
Experimental approach: EC cells were harvested and exposed to increasing concentrations of carboplatin and EGCG to construct a dose-response plot. Cell inhibitory effects were assessed by the MTT method and apoptosis-related gene expression levels (caspases 8 and 9) and Bcl-2 mRNA were detected using real-time polymerase chain reaction. The lactate levels in the various treated cases were analyzed using the colorimetric assay kit. In addition, total antioxidant capacity was measured.
Findings/Results: The results indicated that, following treatments with carboplatin in IC_{20}, IC_{25}, and IC_{10} concentrations when combined with EGCG in similar concentrations, synergistically decreased cell viability versus single treatments of both agents. Also, in combined treatments at IC_{20} and IC_{25} of both agents the gene expression ratio of caspases 8 and 9 upregulated significantly compared to monotherapies ($P < 0.05$). Bcl-2 gene expression ratios were decreased in double agents treated cells versus monotherapies. Following treatment of KYSE-30 cells with carboplatin and EGCG in double combinations, lactate levels were significantly decreased compared with the untreated cells and single treatments ($P < 0.05$). Also, in IC_{25}, IC_{20}, and IC_{10} concentrations of both agents the total antioxidant capacity levels were decreased versus monotherapies and untreated cells.
Conclusion and implications: The presented study determined that treatment with carboplatin and EGCG was capable of promoting cytotoxicity in EC cells and inhibits the cancer progress. Combined treatments with low concentrations of carboplatin and EGCG may promote apoptosis induction and inhibit cell growth. These results confirmed the anticancer effects of carboplatin and EGCG and providing a base for additional use of EGCG to the EC treatment.

Keywords: Carboplatin; Caspase; Epigallocatechin-3-gallate; Esophageal cancer.

INTRODUCTION

Esophageal cancer (EC) is one of the digestive tract cancer, particularly in men, with high rates of mortality worldwide (1).

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Recently, there are numerous cancer treatment options, that the most prevalent of these methods are surgery, chemotherapy, radiotherapy, or the combination of these therapies. Although their efficiency, because of systemic toxicity has been limited (2). Cisplatin-based adjuvant chemotherapy is considered as main treatment option utilized in EC treatment. Nevertheless, this chemotherapy type has numerous side effects that some patients are unable to tolerate (3,4). Indeed, even though the usage of multimodality treatment, the 5-year survival rate is not even 50% (5). Consequently, investigation of efficient and low-toxic chemotherapeutics for the EC treatment is necessary (3,4). Hence, despite conventional treatments that remain limited to chemo-radiotherapy and esophagectomy, evaluation of less-toxic treatment options with promising outcomes regarding treatment is essential (6). One of the main objectives of cancer treatment is to expect response toward chemotherapy drugs *via* response assays (7,8). It has been indicated that the cancerous cells may possibly be resistant to various treatment strategies (9). Moreover, targeted therapy in cancer is reflected as favorable treatment options (7,8,10-12). Consequently, new drug evaluation for EC is crucial (13). Recently herbal medication and alternative medicines in the treatment of malignancies have been utilized (14-16). Indeed, natural products are considered as active agents for cancer prevention, comprising epigallocatechin-3-gallate (EGCG). EGCG is the main catechin of green tea, which has antioxidant and anticancer effects (13). Nevertheless, studies that were evaluated the EGCG mechanisms in EC treatment are limited, and the EGCG role in EC therapy is poorly understood (13). In this study, the effects of EGCG in combination with low concentrations of carboplatin in EC cells and the related molecular mechanisms were studied.

MATERIALS AND METHODS

Materials

EGCG and carboplatin were purchased from Sigma-Aldrich (USA). Penicillin-streptomycin, Dulbecco's modified eagle's medium, (DMEM), and fetal bovine serum (FBS) were from Biowest (France). Dimethyl sulfoxide (DMSO) from Sigma-Aldrich (USA)*;* acetate buffer and phosphate-buffered saline from Sigma (USA).

Cell culture, drug treatment, and cell viability assay

The esophageal cancer cell line KYSE-30 was seeded in DMEM, that supplemented with 10% FBS, 0.1% of penicillin-streptomycin, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Intended for drug treatments, KYSE-30 cells were harvested in monolayer 24 h before treatments and then treated with or without carboplatin and EGCG in various single concentrations and combinations of these agents for 24 h. The concentrations of carboplatin were 0.5, 1, 2, 3, 4, 5, 10, 25 µmol/L, EGCG were 10, 15, 20, 30, 35, 40, 50, 100, 150 µmol/L. The cell viability was studied by MTT assay based on manufacture instruction (Kia zist, Iran). Based on dose-response plots, the IC_{50} of both carboplatin and EGCG was determined. The concentrations for the drug co-administration were IC_{25}, IC_{20}, and IC_{10} of each agent which were used in combination treatments for all experimental analysis. To study the cytotoxicity of different combinations, the combination index (CI) was determined based on Chou-Talalay method (Combosyn, Inc., Paramus, US). The CI < 1, CI = 1, and CI > 1 show synergism, additive effect, and antagonism, respectively (17). Fraction affected (fa) values show the cells fraction which has been affected through the double combination and dose reduction index (DRI) determines the dose-reduction level for each *agent once assumed in a synergistic combination ver*sus single-drug therapies.

Assessment of caspase 8, caspase 9, and Bcl-2 mRNA expression ratio

In order to evaluate the caspase 8, caspase 9, and Bcl-2 gene expression levels, total RNA from untreated and treated cells in different concentrations described above, were extracted with GeneAll RNA extraction kit based on the kit protocol (Seoul, South Korea).

Table 1. Forward and reverse sequences of primers used in real-time polymerase chain reaction (18-20).

Primers	*Sequences*
β-actin	Forward: GGACATCCGCAAAGACCTGTA
	Reverse: ACATCTGCTGGAAGGTGGACA
BCL-2	Forward: GTGGATGACTGAGTACCTGA
	Reverse: AGCCAGGAGAAATCAAACAGA
Caspase 8	Forward: CCAGAGACTCCAGGAAAAGAGA
	Reverse: GATAGAGCATGACCCTGTAGGC
Caspase 9	Forward: CGAACTAACAGGCAAGCAGC
	Reverse: ACCTCACCAAATCCTCCAGAAC

RNA integrity was confirmed by agarose gel electrophoresis and RNA purity was studied *via* calculating the ratio of optical density (OD) at 260/280 nm. In the next step, using Gene All Hyperscript™ first-strand synthesis kit (General, South Korea), RNA was reverse-transcribed into cDNA. Then, real-time polymerase chain reaction (RT-PCR) was carried out utilizing Real Q Plus 2x Master Mix Green (Ampliqon, Denmark) and specific primers (Table 1). The PCR conditions were as follow: 15 min at 94 °C, 35 cycles of 95 °C for 20 s; then 60 s at 59 °C (for caspases 8 and 9) and 60 °C (for Bcl-2) and 72°C for 5 min. The gene expression ratios of caspase 8, caspase 9, and Bcl-2 normalized to β-actin gene expression (the housekeeping gene) and measured by $2^{-\Delta\Delta Ct}$ method.

Lactic acid

About 10^6 KYSE-30 cells were grown 24 h before treatments and then exposed to various concentrations of EGCG and carboplatin as described above. After 24 h of treatment, the treated and untreated cell suspensions were collected. The lactic acid levels were measured by lactic acid (Lactate) assay kit (ZellBio GmbH, Germany) according to the kit instruction. Well's absorbance read throughout 30 min by a microplate reader at 546 nm. Lactate levels were detected in all samples using the equation below:

$$Lactate\ ({}^{mg}/_{dL}\ or\ {}^{mmol}/_{L}) = \frac{OD\ sample - OD\ blank}{OD\ standard - OD\ blank} \times standard\ concentration$$

Total antioxidant capacity

Total antioxidant capacity (TAC) was assessed in the cell culture supernatant according to the ferric reduction antioxidant power (FRAP) test (21). Indeed, at low pH condition utilizing acetate buffer (300 mM, pH 3.6), Fe^{3+}-TPTZ complex reduced to the ferrous form, that led to the formation of an intensive blue color which measured at 593 nm. The intensity of complex color subsequent to adding of the cell supernatants from un-treated and treated cells to a reducible solution of Fe^{3+}-TPTZ is correlated to the total reducing power of the electron-donating antioxidant. In this study, Fe^{2+} ($FeSO_4.7H_2O$) solution was utilized as standard solutions.

Statistical analysis

Data represent the mean ± SD. Statistical analysis was carried out with one-way analysis of variance (ANOVA) using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, California) followed by Tukey's posthoc test which was utilized to evaluate the differences between groups. P less than 0.05 was considered statistically significant.

RESULTS

The effects of carboplatin and EGCG on the growth of EC cells

KYSE-30 cell line was tested for sensitivity to single-agent carboplatin or EGCG using MTT cell viability method (Fig. 1). These cells responded to single agents of carboplatin and EGCG values in concentrations-dependent manners. The possible synergistic effect of combining EGCG with carboplatin was examined by adding carboplatin to cells with increasing concentrations of EGCG and comparing cell viability to treatment with carboplatin and EGCG alone. Nevertheless, significant synergistic effects were observed when the analysis was carried out for all the combinatorial cases (Table 2).

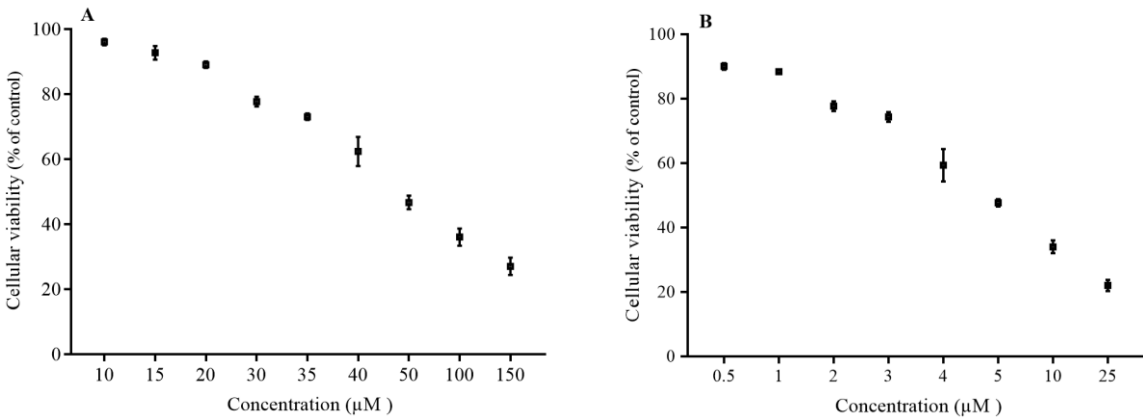

Fig. 1. Cytotoxic effects of (A) EGCG and (B) carboplatin in single treatments (various concentrations). In order to evaluate the cytotoxicity of EGCG and carboplatin in single treatments after 24 h, the cell viability was assessed in various concentrations by MTT assay. The cell viability decreased in dose-dependent manner (in both agents). Data represent mean ± SD. EGCG, epigallocatechin-3-gallate.

Table 2. Results of combination Index, fraction affected, and DRI for double treatment groups. DRI values indicate decreased levels of each drug dose in synergistic combination treatments.

Treatments	Combination index	DRI of EGCG	DRI of CP	Fraction affected	Pattern
Combination at IC_{25} of EGCG and CP	0.47	3.24	5.79	0.72	Synergism
Combination at IC_{20} of EGCG and CP	0.40	3.52	7.7	0.7	Synergism
Combination at IC_{10} of EGCG and CP	0.35	4.01	9.33	0.58	Synergism

CP, Carboplatin; DRI, dose reduction index; EGCG, epigallocatechin-3-gallate.

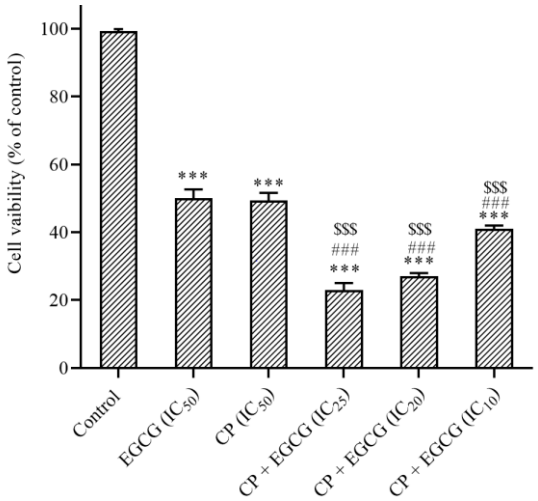

Fig. 2. Results of cell viability assay using MTT assay in single and combined treated cases with EGCG and CP after 24 h in esophageal cancer cells. Cellular viability assay after 24 h of various treatments by MTT test in KYSE-30 cell line was decreased versus control. Data are presented as mean ± SD. $^{***}P < 0.001$ indicates significant differences compared to the untreated control, $^{###}P < 0.001$versus EGCG (IC_{50}), and $^{\$\$\$}P < 0.001$ against CP (IC_{50}) group. CP, Carboplatin; EGCG, epigallocatechin-3-gallate.

Evaluation of the effect of co- or single-treatments of carboplatin and EGCG on cell viability

KYSE-30 cells were subjected to different treatment regimens: (1) treatment with carboplatin or EGCG at IC_{50}, (2-4) co-treatment with carboplatin and EGCG at IC_{25}, IC_{20}, or IC_{10}, respectively. The sequential regimen carboplatin and EGCG in IC_{25}, IC_{20}, and IC_{10} were significantly effective than the single treatments in IC_{50}. In addition, cell viability in all double combinations versus untreated control (Fig. 2) was decreased. According to the CI values indicated in Table 1, double combination cases of EGCG, and carboplatin represent synergistic effects in all examined concentrations of both drugs. The DRI values presented in Table 1 showed the reduction in drug concentration of double combinations as compared to single treatments.

Effect of co- or single-treatments of carboplatin and EGCG on caspase 8, caspase 9, and Bcl-2 genes expression

To evaluate the cellular mechanisms underlying the effects of combinations of EGCG and carboplatin in concentrations lower than IC_{50}, the gene expression ratios of caspase 8, caspase 9, and Bcl-2 were evaluated. Data showed that regimens containing carboplatin as a single agent and all double combinations upregulated the gene expression levels of caspase 8 versus untreated control; and also, there was a gene upregulation of caspase 8 in double combinations comprising EGCG and carboplatin in IC_{20} and IC_{25} versus single treatments (Fig. 3A).

The caspase 9 gene expression was increased in all single and co-treated cases in comparison with the control. Also, there were significant elevated caspase 9 mRNA levels in double combinations compared to the single treatments (Fig. 3B). Results of RT-PCR indicated downregulation of Bcl-2 mRNA in monotherapies and double combinations in all examined concentrations against the untreated control. Similarly, the Bcl-2 gene expression ratio was decreased in double agents treated cells versus monotherapies (Fig. 3C).

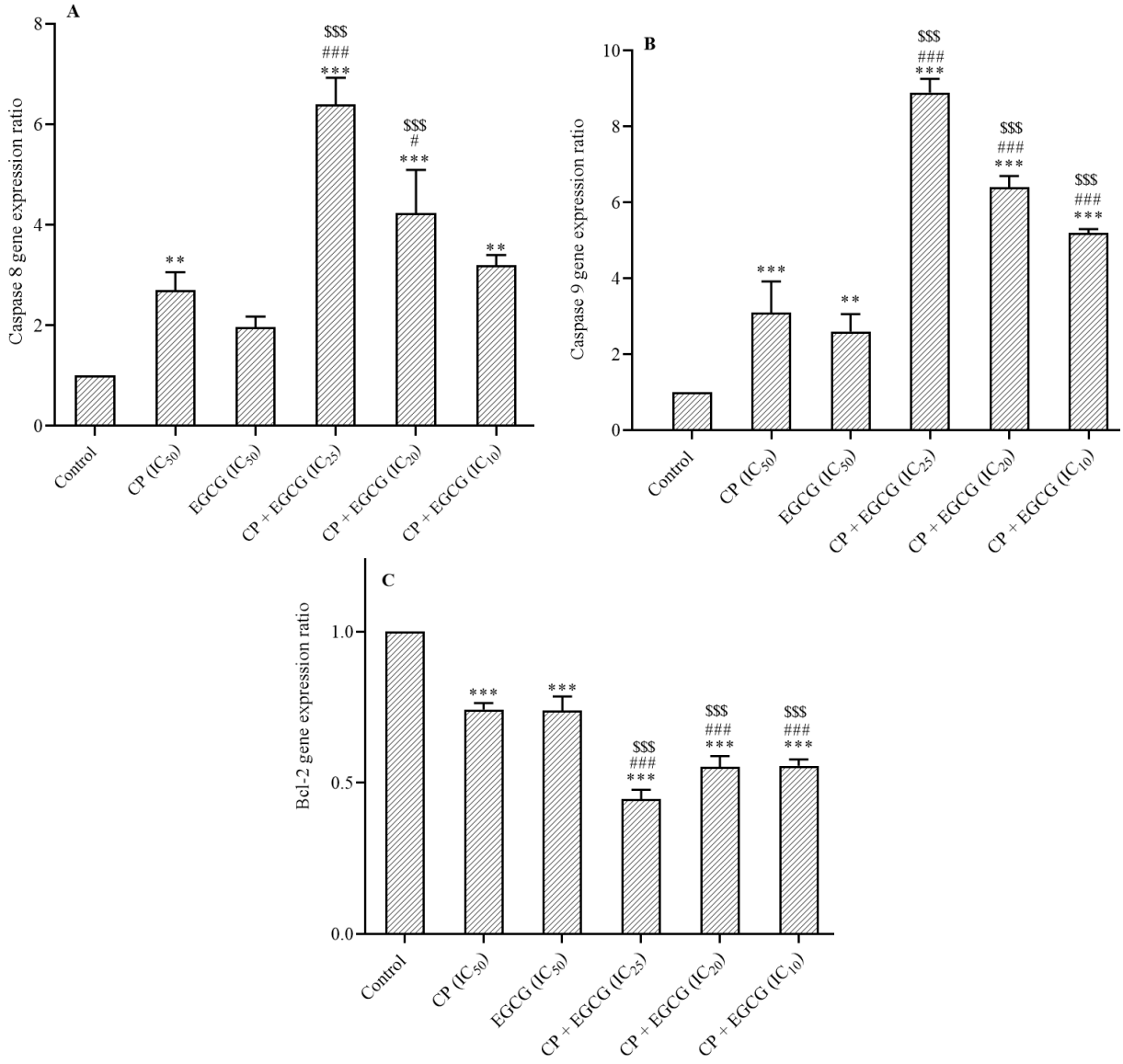

Fig. 3. Results of real-time polymerase chain reaction assay in single and combined treated cases with EGCG and CP after 24 h in esophageal cancer cells. Gene expression ratios of (A) caspase 8, (B) caspase 9, and (C) Bcl-2 is shown. Gene expression ratios in various treatments in KYSE-30 cells were evaluated and data were normalized to the housekeeping gene (β-actin) and presented as mean fold change ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences compared to the untreated control, $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ versus CP (IC_{50}), and $^{\$\$\$}P < 0.001$ against EGCG (IC_{50}) group. CP, Carboplatin; EGCG, epigallocatechin-3-gallate.

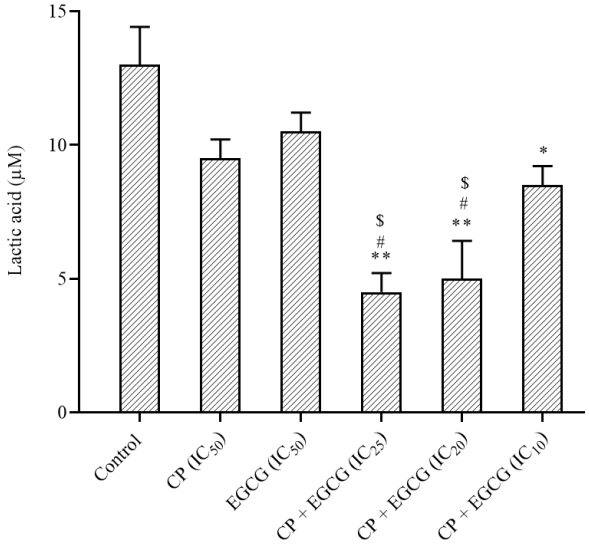

Fig. 4. Results of lactic acid (lactate) assessment in KYSE 30 cells following single and combined treated cases with EGCG and CP after 24 h in esophageal cancer cells. Data are presented as mean ± SD. $*P < 0.05$ and $**P < 0.01$ indicate significant differences compared to the untreated control, $^{\#}P < 0.05$versus EGCG (IC_{50}), and $^{\$}P < 0.05$ against CP (IC_{50}) group. CP, Carboplatin; EGCG, epigallocatechin-3-gallate.

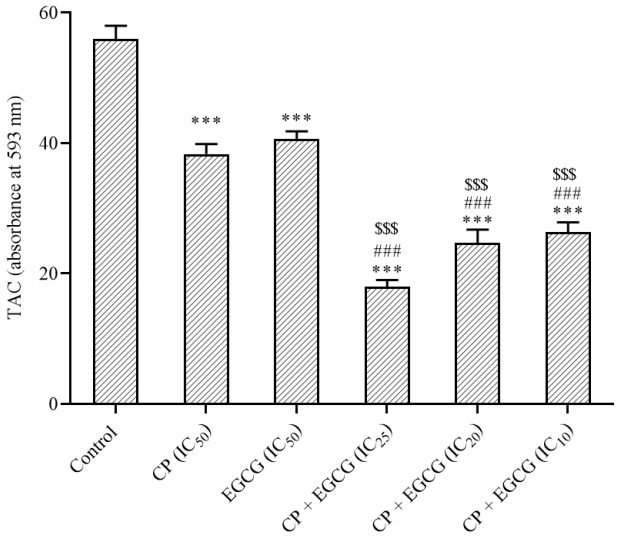

Fig. 5. Results of TAC assay in single and combined treated cases with EGCG and CP after 24 h in esophageal cancer cells. Data are presented as mean ± SD. $***P < 0.001$ indicates significant differences compared to the untreated control, $^{\#\#\#}P < 0.001$versus EGCG (IC_{50}), and $^{\$\$\$}P < 0.001$ against CP (IC_{50}) group. CP, Carboplatin; EGCG, epigallocatechin-3-gallate; TAC, total antioxidant capacity.

The effect of carboplatin and/or EGCG on lactate levels

In further analysis, we measured the lactate levels in treated and untreated cells with EGCG and carboplatin after 24 h of treatment. According to our results, the lactate levels were decreased in cells that were treated with EGCG + carboplatin at IC_{25} and IC_{20} versus monotherapies. Also, lactate levels in all double agents treated cells were decreased compared to untreated control cells. There were insignificantly decreased lactate levels in double combination at IC_{10} of both agents compared to the monotherapies (Fig. 4).

The effect of carboplatin and/or EGCG on TAC

TAC in KYSE-30 cells which were treated with both compounds either in combination or single treatments was decreased significantly compared to the untreated control cells. All three combined treatments exhibited the highest potency in the reduction of TAC versus single treatments (Fig. 5)

DISCUSSION

Platinum(II) complexes including carboplatin, oxaliplatin, and cisplatin are clinically appropriate for the treatments of different types of solid tumors (22). Indeed, chemotherapy is one of the frequently utilized cancer treatment techniques, nonetheless, the adverse effects of this treatment are unavoidable. Consequently, natural extracts can apply to decrease the adverse effects of traditional cancer therapies (1). Indeed, the tendencies towards the phytochemicals use in the treatment of diseases have increased (23,24). Previous report has indicated that people consuming tea showed decreased incidences of certain cancers. These effects are associated with the polyphenol content of the herbal tea(25).

EGCG in normal cells, acting as a natural antioxidant, has considerable potential scavenging activity for free radicals comprising hydrogen peroxide, superoxide anion and hydroxyl radical (1,26-30). In this study, combined effects of EGCG, and carboplatin significantly increased the anticancer efficiency and induced synergic effects as opposed to utilizing any one of both agents alone. These combinations inhibited the cellular growth and proliferation of EC cells, and decreased the lactate and also altered the gene expression of caspase 8 and caspase 9, as apoptosis main mediators, and Bcl-2 as anti-apoptotic genes in

comparison to single treated cases. In addition, these combinations reduced TAC to monotherapies.

Certainly, apoptosis is a regulated process of cell death that dysregulation of this process is related to pathologic states. Indeed, altered apoptosis regulation can lead to tumor growth and metastasis (31, 32). The extrinsic pathway of apoptosis is intermediated by caspase 8 through the intrinsic pathway triggered by caspase 9, and these two pathways initiate apoptosis *via* the downstream executioner proteins cleavage, caspases 7 and 3 (32,33). In this study, both caspases 9 and 8 were overexpressed in carboplatin and EGCG co-treatments at lower concentrations than IC_{50} compared to the single treatments at IC_{50} of each drug. These data indicated that at low concentrations, combined treatments of carboplatin and EGCG can increase the major mediators of apoptosis and inhibits EC growth. Also, Bcl-2 gene expression ratios were decreased in double agents treated cells versus monotherapies. It could be indicated that a decrease in cell proliferation is related to apoptosis induction by increasing the expression of apoptosis-related main markers and downregulation of Bcl-2 as the anti-apoptotic gene.

In a recent similar study, the combinations of EGCG with curcumin and lovastatin were capable of decreasing EC cell growth *in vitro*. These agents in combined cases also inhibited COX-2 expression. In this study combination of EGCG and curcumin with lovastatin upregulates caspase 3 expression, which is a major mediator of apoptosis (34). Based on previous studies EGCG can inhibit cancer cell growth and promote EC apoptosis (1,4,13,35). EGCG inhibited the anti-apoptotic protein expression including Bcl-2 in the NF-κB pathways, and upregulate the caspase 3 and Bax, which led to cell cycle arrest (1,13). In accordance with these reports, Bcl-2 downregulated in more efficient combinations in this study.

In another study, EGCG inversed multidrug resistance *via* decreasing the ABCG2 expression and elevate the drug concentration in cancer cells to increase the anticancer properties (13). Also, previous data proposed that EGCG may be a potential adjunct candidate for cisplatin (36). In addition, in another study, vitamin C could considerably increase the EGCG therapeutic effects by triggering the activity of caspase 3/9, exert apoptosis of cancer cells through MAPK pathways regulation (37).

Based on our data after combinatorial treatments of EGCG and carboplatin, the lactate levels were decreased. The lactate production ratio divided *via* glucose consumption reveals terminal glycolysis amounts versus total glucose metabolism. Indeed, glucose consumption/lactate production ratio is related to glycolysis rates. The glycolysis rates measurement that is assessed by lactate production/glucose consumption is associated with NADH α1 (38). It has been indicated that lactate is possibly the metabolic compound that is necessary for all foremost sequela for carcinogenesis, exactly: cell migration, angiogenesis, and metastasis. It has been hypothesized that lactogenesis for carcinogenesis is the description. Therefore, treatments to limit lactate exchange and signaling in cancerous cells should be important (39). In this regard decreased lactate levels after 24 h of treatments showed the therapeutic efficiency of EGCG and carboplatin combination in this study.

Correspondingly, the combined treatment with EGCG and carboplatin in examined concentrations decreased the TAC versus monotherapies in our study which might be related to the cytotoxic effects of both drugs. Indeed, several natural products have the capability to sensitize cancerous cells to oxidative stress exerted *via* chemotherapy through restraining the antioxidant capacity of cancer cells. Certainly, inhibition of antioxidant defense in cancers reduces their capability to balance oxidative insult and led to cell death (40). It seems that decreasing the TAC is one of the possible mechanisms of synergistic effects of EGCG and carboplatin and inducing cytotoxic effects.

The anticancer mechanisms are different based on the cell type, dose, and/or time of treatment. It has been indicated that EGCG pro-oxidant effects might be one of the potential mechanisms for anticancer action. Indeed,

EGCG-mediated mitochondrial ROS could increase cytochrome c release to the cytosol (41). Another comparable study showed that EGCG treatment increases cisplatin-mediated chemosensitivity through suppressing the ABCG2 and ABCC2 transporter genes, which are putative molecules of treatment resistance of cancer stem cells. In addition, the combination treatment of cisplatin and EGCG induced apoptosis in a xenograft model (42).

Based on author's knowledge there were no related studies to compare with our findings. Overall, EGCG in combination with carboplatin exerts synergistic effects by suppressing the TAC and lactate production and downregulate Bcl-2 anti-apoptotic gene and also upregulate intrinsic and extrinsic apoptosis main markers which promoted apoptosis.

CONCLUSION

It has been shown that the anticancer effects of single agents could be increased by combining them synergistically with chemically comparable or dissimilar compounds. Indeed, combinations might be efficient in the drug dosage reduction, resistance, and concurrently displaying higher therapeutic outcomes (43). In this regard, the present investigation determined that treatment with the combination of carboplatin and EGCG was capable of promoting cytotoxic effects in EC cells and inhibits the cancer progress. Additionally, the present results recognized some anticancer molecular mechanisms of carboplatin and EGCG and provided a base for additional use of EGCG to increase the carboplatin efficacy in EC treatment. Further study on the molecular mechanisms of EGCG is needed because these combinations could have a significant role in the successful treatment of EC.

Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

F. Taghvaei, S. Jafarzadeh Rastin and Z. Rostamzadeh Khameneh performed the experimental tests. RT-PCR assay. Cell culture was carried out by A. Toofani Milani. F. Hamini performed the MTT assay. M.A. Rasouli performed the statistical analysis. K. Asghari and A.M. Rekabi Shishavan performed the RT-PCR assay. M. Ebrahimifar wrote the manuscript draft. S. Rashidi was the supervisor of this study and wrote the manuscript draft.

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