Melatonin Increases the Anticancer Potential of Doxorubicin in Caco-2 Colorectal Cancer Cells

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Abstract

Colorectal cancer (CC) is an important human malignancy with high cancer related death worldwide. The chemotherapy using doxorubicin hydrochloride is one of the most common cancer therapeutic methods. However, drug resistance lowers the treatment efficacy in CC patients. The combination therapies seem to be more promising by taking the advantage of synergistic effects. The present study aimed to evaluate a new strategy to enhance the anticancer activity of doxorubicin in Caco-2 CC cell line by co-administration of melatonin. The effects of doxorubicin, melatonin, and their combinations (Dox-Mel) were investigated on the proliferation and viability, morphological alterations, and tumor spheroid formation. Flow cytometry was employed to compare the apoptotic situation of the cells in study groups. Changes in metastatic potential of the cells were assessed by wound healing assay and trans-well migration assays. Moreover, expression of *BAX*, *SMAC*, *BCL-2*, *SURVIVIN*, *MMP-2*, and *MMP-9* genes were evaluated by quantitative real time PCR (qRT-PCR) and western blotting. Our study showed that doxorubicin, melatonin, and Dox-Mel significantly decreased the proliferation and viability, tumor spheroid formation, invasion and migration. Furthermore, the changes were in a concentration and time dependent manner. There was an increase in apoptosis rate in the treatment groups. Expression of genes involved in apoptosis and cell motility were altered significantly. It was observed that anticancer activity of Dox-Mel combination was significantly more than doxorubicin and melatonin treatments alone. We showed an enhanced apoptotic and anticancer activity of doxorubicin and melatonin combination chemotherapy on CC cell line than doxorubicin or melatonin treatments alone. This combination could promote the treatment efficiency and alleviate the un-intended side effects by lowering the dose of doxorubicin prescription.

Introduction

Colorectal cancer (CC) is a highly prevalent malignancy and causative of cancer related death in the world. The incidence of CC is much lower in the developing countries of Africa and Asia compared with the United States and developed European countries (Hagggar and Boushey, 2009). Nowadays, surgery and chemotherapy are the most common methods for treatment of CC. However, due to development of drug-resistant CC cells the efficacy of chemotherapy is limited (Wang et al., 2017). Therefore, application of combinational methods seems to be more effective and result in prolonged survival of CC patients (Soheilyfar et al., 2018; Maroufi et al., 2020).

Doxorubicin is an anthracycline antibiotic, commonly used as a chemotherapeutic agent in treatment of patients with CC. This chemotherapy agent inhibits cancer cells through induction of apoptosis and inhibition of cell division. However, resistance to doxorubicin is frequently evident in several drug-resistant cancer cells (Nakanishi and Ross, 2012). The upregulation of efflux transporters decreases intracellular levels of doxorubicin through drug excretion. This leads to reduction of therapeutic efficacy and apoptosis rate in drug-resistant cancer cells. Accordingly, anticancer activity of doxorubicin decreases in cancer cells with inhibited apoptosis. Various molecular processes are involved in dysregulation of apoptotic pathways including: activation of anti-apoptotic factors (Bcl-2, Bfl1/A1, etc.), inactivation of pro-apoptotic factors (Smac, Bax, etc.), and reinforcement of survival signals (Survivin, NF- κ B, etc.).

Therefore, therapeutic efficacy of doxorubicin on cancer cells were decreased through overexpression of efflux transporters and inhibition of apoptosis (Hu et al., 2006). Combination of doxorubicin with different compounds and molecules to overcome the drug resistance has been found to be promising in various cancer cells (Menéndez et al., 2019; Fic et al., 2007).

Melatonin is a natural hormone secreted by the pineal gland as well as testes, retina, ovary, skin, and intestine (Chen et al., 2016). Previous studies reported that, melatonin plays an important role in regulation of several biological pathways such as secretion and modulation of hormones, reproduction, and circadian rhythms (Kim et al., 2012; Wang et al., 2012; Maroufi et al., 2020). In addition, melatonin has shown an appropriate anticancer activity without side effects on several cancer cells (Wang et al., 2012; Maroufi et al., 2020). Melatonin activities are mediated by receptor-dependent or -independent pathways (Maroufi et al., 2020; Witt-Enderby et al., 2006). The most important melatonin receptors are MT1 and MT2, which inhibit several signaling pathways involved in cancer progression including phosphoinositide 3-kinases (PI3K), mitogen activated protein kinase (MAPK) pathways and stimulate apoptotic pathways in several cancer cells (Kong et al., 2008; Mao et al., 2010). Moreover, the anticancer activity of melatonin has been largely described in hormone-dependent tumors, when administered alone or in combination with therapeutic agents (Menéndez et al., 2018; Najafi et al., 2019).

So far, several studies have been conducted to assess the anticancer effects of various natural compounds in combination with doxorubicin against cancer cells. However, this is the first study on the anticancer effects of doxorubicin-melatonin combination and underlying mechanisms in CC cell line which can help making decisions for clinical applications in the future.

Materials And Methods

Cancer cell culture

The CC cell line Caco-2 was obtained from Immunology Research Center, Tabriz University of Medical Sciences. The cell culture was performed using Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS) and appropriate concentrations of penicillin (5,000 units/mL)- and streptomycin (5,000 mg/mL). The cells were then incubated in standard conditions (5% CO₂, 95% humidity and 37 °C).

Cancer cell viability assay

The Caco-2 cancer cells were seeded in 96 well culture plates (1.5×10^4 cells/well) in 200 μ L culture medium, and then incubated for 24 hours. Next, cancer cells were treated with different concentrations of doxorubicin (1, 2, 4, and 8 μ M), melatonin (1, 2, 4, and 8 mM), and Dox-Mel (1, 2, 4, and 8 μ M-mM), and incubated for 24–72 hours. Then, 5 mg/mL of MTT reagent incomplete medium was substituted with previous medium and incubated for 4 hours. Then, culture medium removed, and 50 μ l dimethyl sulfoxide was added, and incubated for 30 minutes. The optical densities (OD) were measured at 570 nm

wavelength. Finally, viability of cancer cells was measured using this formula: $[(\text{treated sample OD} / \text{untreated sample OD}) \times 100]$.

Assessment of the morphological alterations

The Caco-2 cancer cells were seeded in a 6 well culture plates (1.5×10^5 cells/well) in 2000 μL culture medium. After 24 hours of incubation, the cells were then treated with doxorubicin (1 μM), melatonin (2 mM), and Dox-Mel (0.8 μM -mM), and then incubated for 24–72 hours. Finally, morphological alterations of cancer cells were monitored by an inverted phase contrast microscope.

Spheroid formation assay

The Caco-2 cells were seeded in a 6 well culture plates (1.5×10^5 cells/well) in 2000 μL culture medium containing 10 μM basic fibroblast growth factor (bFGF) and 10 μM epidermal growth factor (EGF), and then incubated for 24 hours. Subsequently, the cancer cells were treated with doxorubicin (1 μM), melatonin (2 mM), and Dox-Mel (0.8 μM -mM), and maintained in an incubator under standard conditions. The culture medium was substituted every 72 hours. Finally, formation of tumor sphere-like cells was monitored by an inverted phase contrast microscope, after 7–12 days

Wound healing assay

The Caco-2 cancer cells were seeded in a 6 well culture plates (1.5×10^5 cells/well) in 2000 μL culture medium, and then incubated. After reaching to more than 90% confluence, the cancer cell monolayers in each well were scratched with a sterile pipette tip. Then, cancer cells were treated with doxorubicin (1 μM), melatonin (2 mM), and Dox-Mel (0.8 μM -mM), and incubated for 0–20 hours. Finally, migration of cancer cells was monitored by an inverted phase contrast microscope.

Trans-well migration assay

The Caco-2 cancer cells were seeded in the upper trans-well chamber in serum free culture medium and treated by doxorubicin (1 μM), melatonin (2 mM), and Dox-Mel (0.8 μM -mM) for 10–20 hours. In parallel, supplemented culture medium was added to the bottom trans-well chamber, and incubated for 20 hours. Then, non-migrated cancer cells in the upper chamber were removed, and the migrated cancer cells in the bottom chamber were fixed by 4% paraformaldehyde and stained by 0.1% crystal violet. Finally, migration of cancer cells in 3 random fields was monitored by an inverted phase contrast microscope.

Apoptosis quantification by Annexin V-FITC/PI assay

The Caco-2 cells were seeded in a 6 well culture plates (1.5×10^5 cells/well) in 2000 μL culture medium. After 24 hours of incubation, the cells were treated with doxorubicin (1 μM), melatonin (2 mM), and Dox-Mel (0.8 μM -mM), and maintained for 48 hours. The cancer cells were detached, washed, and resuspended in binding buffer containing Annexin V-FITC (5 mL) and PI (10 mL), and incubated at room temperature in dark for 15 minutes. Finally, apoptosis rate in cancer cells was measured by a flow cytometry instrument (Becton Dickinson Bioscience).

Gene expression assay by real time PCR

The Caco-2 cancer cells were seeded in a 6 well culture plates (1.5×10^5 cells/well) in 2000 μ L culture medium, and then incubated overnight. Then, the cancer cells were treated with doxorubicin (1 μ M), melatonin (2 mM), and Dox-Mel (0.8 μ M-mM), and incubated for 48 hours. The cancer cells were detached, washed, and re-suspended in TRIzol reagent, and total RNA extraction was performed according to the manufacturer's instructions. Then, random hexamer primer was used for cDNA synthesis. The mRNA expression levels of *BAX*, *BCL-2*, *SMAC*, *SURVIVIN*, *MMP-2*, and *MMP-9* genes were evaluated using quantitative Real Time PCR (qRT-PCR). The used primers sequences were presented in Table 1. The Real Time PCR was performed in 20 μ l total volume comprising: 1 μ l cDNA, 0.5 μ l forward and reverse primers (5 μ M), and 10 μ l of PCR pre-Mix (Takara, RR820L). The cycling program included: 1 cycle of 1 minutes at 94 $^{\circ}$ C) followed by 45 cycles of denaturation at 94 $^{\circ}$ C for 10 seconds, annealing at 59 $^{\circ}$ C for 30 seconds, and extension at 72 $^{\circ}$ C for 20 seconds. The *ACTB* gene (β -actin) was considered as endogenous control, and calculations were conducted by $2^{-\Delta\Delta Ct}$ (Livak) formula.

Table 1
Sequences and characteristics of primers used for detection of genes mRNA expression.

Genes	Primer sequence	T _m	Products size
<i>BAX</i>	F-CCCGAGAGGTCTTTTCCGAG	60 $^{\circ}$ C	155 bp
	R-CCAGCCCATGATGGTTCTGAT	60 $^{\circ}$ C	
<i>BCL-2</i>	F-GATGGGATCGTTGCCTTATG	56 $^{\circ}$ C	223 bp
	R-GCGGAACACTTGATTCTGG	56 $^{\circ}$ C	
<i>SMAC</i>	F-CAGAGGAGGAAGATGAAGTGTG	55 $^{\circ}$ C	196 bp
	R-GCGGTTATAGAGGCCTGATCTG	56 $^{\circ}$ C	
<i>SURVIVIN</i>	F-CCCTTTCTCAAGGACCACCG	52 $^{\circ}$ C	172 bp
	R-GTTCCTCTATGGGGTCGTCA	51 $^{\circ}$ C	
<i>MMP-2</i>	F-CACATAGTGATGGTTCCCCTGTT	60 $^{\circ}$ C	177 bp
	R-CGGCCACTCAGTAGGTGTCTTT	62 $^{\circ}$ C	
<i>MMP-9</i>	F-ATTTCTGCCAGGACCGCTTCTAC	62 $^{\circ}$ C	187 bp
	R-ATCCGGCAAACCTGGCTCCTTC	63 $^{\circ}$ C	
<i>ACTIN</i>	F-AGAGCTACGAGCTGCCTGAC	61 $^{\circ}$ C	186 bp
	R-AGCACTGTGTTGGCGTACAG	59 $^{\circ}$ C	

Gene expression assay by western blotting

The Caco-2 cancer cells were seeded in a 6 well culture plates (1.5×10^5 cells/well) in 2000 μ L culture medium, and then incubated for 24 hours. Then, the cancer cells were treated with doxorubicin (1 μ M), melatonin (2 mM), and Dox-Mel (0.8 μ M-mM), and incubated for 48 hours. The cancer cells were detached, washed, and lysed in ice-cold RIPA lysis buffer containing protease inhibitor for 30 minutes. The cell lysates centrifuged and supernatant collected. The protein expression levels of Bax, Bcl-2, Smac, Survivin, MMP-2, and MMP-9 were evaluated using western blot analysis. Approximately 100 μ g of cells protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred on to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked using non-fat dry milk (5%) and then incubated with primary antibodies at 4 °C for 24 hours. The horseradish peroxidase (HRP) conjugated secondary antibody was used as secondary antibody at room temperature for 60 minutes. The protein detection was performed using enhanced chemiluminescent (ECL) detection system.

Statistical analysis

The all performed experiments were repeated for three times, and obtained data were presented as mean $\pm$ standard deviation (SD). The Student's ttest, Tukey (posthoc), and oneway analysis of variance (ANOVA) were used to statistical analysis using the Graph Pad Prism software. The p-value < 0.05 was considered as significant.

Results

Cancer cell viability

According to the results, both doxorubicin and melatonin demonstrated a time and concentration dependent anticancer activity. The viability of the cancer cells was significantly decreased in the high concentrations of doxorubicin and melatonin. In addition, the anticancer activity of Dox-Mel was significantly more than doxorubicin and melatonin. The half maximal inhibitory concentration (IC_{50}) of doxorubicin, melatonin, and Dox-Mel on CC cells after 48 hours were: 1 μ M, 2 mM, and 0.8 μ M-mM, respectively (Fig. 1).

Morphological alterations

The cancer cells treated with doxorubicin, melatonin, and Dox-Mel showed several morphological alterations including shrinkage, decreased cell size, fragmented nuclei, and membrane damages, which can result in programmed cell death. The cells treated with Dox-Mel revealed significantly more morphological alterations than the cells with doxorubicin and melatonin monotherapy (Fig. 2).

Tumor spheroid

The treatment of cancer cells with doxorubicin, melatonin, and Dox-Mel showed significant decrease in the number and size of the spheroids compared to the untreated cancer cells. The size and number of

tumor spheroids in the treated cancer cells with Dox-Mel were significantly less than in Caco-2 cells treated with doxorubicin and melatonin alone (Fig. 3).

Cancer cell migration and invasion

The trans-well assay showed that the migration and invasion of cancer cells treated with doxorubicin, melatonin, and Dox-Mel was significantly decreased in a time dependent manner (Fig. 4A). The wound healing showed a significant decrease in the migration and invasion of cancer cells treated with doxorubicin, melatonin, and Dox-Mel in a time dependent manner (Fig. 5A). However, inhibition of migration and invasion in the cancer cells treated with Dox-Mel was significantly more than doxorubicin and melatonin groups (Fig. 4B and Fig. 5B).

Cancer cell apoptosis

The Annexin V-FITC/PI assay showed that early and late apoptosis rate were significantly increased in cancer cells treated with doxorubicin, melatonin and Dox-Mel compared to untreated cells. Total apoptosis rate in the cancer cells treated with doxorubicin and melatonin were 34% and 29%, respectively; whereas the total apoptosis rate was 42% in cancer cells treated with Dox-Mel combination (Fig. 6).

Transcript quantification of migration and invasion related genes

The mRNA expression levels of *MMP-2* and *MMP-9* genes were significantly decreased in treated Caco-2 cells with doxorubicin, melatonin, and Dox-Mel. However, regulation of migration and invasion related genes expression in the treated cancer cells with Dox-Mel was significantly more profound than cells treated with doxorubicin and melatonin (Fig. 7A, B).

Expression analysis of migration and invasion related genes in protein level

The migration and invasion related MMP-2 and MMP-9 protein levels significantly decreased in Caco-2 cells treated with doxorubicin, melatonin, and Dox-Mel. However, regulation of migration and invasion related proteins levels in the treated cancer cells with Dox-Mel was significantly more profound than treated cancer cells with doxorubicin and melatonin (Fig. 8).

Transcript quantification of apoptosis related genes

mRNA expression of the apoptosis related genes including *BCL-2* and *SURVIVIN* significantly decreased in treated cancer cells with doxorubicin, melatonin, and Dox-Mel (Fig. 7C, D). Moreover, apoptosis related *BAX* and *SMAC* genes mRNA expression significantly increased treated cancer cells with doxorubicin, melatonin, and Dox-Mel (Fig. 7E, F). However, regulation of apoptosis related genes expression in the

treated cancer cells with Dox-Mel was significantly more profound than treated cancer cells with doxorubicin and melatonin.

Expression analysis of apoptosis related genes in protein level

The apoptosis related Bcl-2 and Survivin proteins levels significantly decreased in treated cancer cells with doxorubicin, melatonin, and Dox-Mel. Moreover, apoptosis related Bax and Smac proteins levels significantly increased in treated cancer cells with doxorubicin, melatonin, and Dox-Mel. However, regulation of apoptosis related proteins levels in the treated cancer cells with Dox-Mel was significantly more profound than treated cancer cells with doxorubicin and melatonin (Fig. 9).

Discussion

Resistance to chemotherapeutic drugs is one of the most important challenges in treatment of patients with cancer. Several molecular mechanisms are involved in progress of resistance to chemotherapeutic agents in patients with CC including drug efflux, drug inactivation, reduced prodrug activation, drug targets alteration, growth factors alterations, extracellular matrix, microRNAs and cytokines involvement, cell death and survival dysregulation, and hypoxia (Vahedian et al., 2020; Firouzi Amoodizaj et al., 2020). Recently, various natural compounds and their derivatives are being used to overcome the resistance to chemotherapeutic drugs through regulation of various mechanisms (Hajazimian et al., 2020; Mahdavi et al., 2019).

Doxorubicin is a widely used cancer drug for the treatment of a variety of human malignancies (Xu et al., 2018). However, overexpression of drug efflux transporters including multi drug resistance-associated protein 1 (MRP1), breast cancer resistance protein (BCRP), and P-glycoprotein (P-gp) has been reported to play important roles in mediating drug resistance in CC cells (Xu et al., 2018). Upregulation of these transporters results in decreased intracellular accumulation of anticancer drugs (Hu et al., 2016; Xu et al., 2018). Moreover, inhibition of apoptosis is a frequent event occurred in cancer cells resistant to doxorubicin. this happens through down-regulation of pro-apoptotic genes and/or up-regulation of anti-apoptotic genes (Xu et al., 2018). Several studies demonstrated that, anticancer effects of doxorubicin increases in combination with other natural compounds (Xu et al., 2018; Hosseinzadeh et al., 2011). Previous studies have also reported that melatonin has an appropriate anticancer activity through regulation of several pathways, such as p53, MAPK, AMPK, and NF- κ B as well as induction of apoptotic pathways (Kong et al., 2008; Mao et al., 2012). Therefore, it is important to evaluate whether melatonin enhances the anticancer effects of doxorubicin on CC cells.

In the present study, we assessed the anticancer effects of doxorubicin, melatonin, and Dox-Mel on CC cells and underlying mechanisms of action. We demonstrated that, melatonin increases the anticancer effects of doxorubicin against CC cells through regulation of several mechanisms involved in cell viability.

We evaluated the morphological alterations and tumor spheroid formation in CC cells treated with doxorubicin, melatonin, and Dox-Mel in comparison with untreated CC cells. According to the results, various morphological alterations such as cell shrinkage, chromatin condensation, and apoptotic body formation as indicators of apoptosis induction were increased in CC cells treated with doxorubicin, melatonin, and Dox-Mel. However, morphological alterations of CC cells treated with Dox-Mel significantly more increased, as compared to doxorubicin and melatonin monotherapy. Moreover, tumor spheroid formation was significantly decreased in CC cells treated with Dox-Mel, as compared with doxorubicin and melatonin monotherapy.

The high lethality in patients with CC is due to metastasis and tumor growth at distant tissues (Bakhshaiesh et al., 2015). In this study, wound healing and trans-well migration assays demonstrated that invasion and migration of CC cells treated with Dox-Mel decreased more significantly, as compared doxorubicin or melatonin monotherapy. Moreover, we evaluated them RNA and protein levels of MMP-2 and MMP-9, which is related to CC cells metastasis and invasion. Our results showed that doxorubicin, melatonin, and Dox-Mel significantly inhibited the mRNA and protein levels of MMP-2 and MMP-9 in CC cells. However, the mRNA and protein levels of MMP-2 and MMP-9 in CC cells treated with Dox-Mel combination decreased more significantly, as compared to doxorubicin and melatonin monotherapy. These results showed that doxorubicin and melatonin may inhibit the invasion and migration of CC cells through downregulation of MMPs.

The apoptosis induction is one of the most important mechanisms of cancer treatment with chemotherapeutic drugs. We demonstrated that the apoptosis rate of the CC cells treated with doxorubicin, melatonin, and Dox-Mel were increased, as compared with untreated CC cells. However, the apoptosis rates of CC cells treated with Dox-Mel indicated more significant increase in comparison with doxorubicin and melatonin monotherapies. Previous studies reported that doxorubicin and melatonin increased apoptosis rate in several cancer cells (Di Bella et al., 2013; Wakharde et al., 2018). Several factors have identified in the regulation of apoptotic pathways. In particular, the evidence showed that doxorubicin and melatonin are able to down-regulate and up-regulate the anti-apoptotic and pro-apoptotic factors, respectively (Di Bella et al., 2013; Wakharde et al., 2018). In this regards, we demonstrated that doxorubicin and melatonin were able to down-regulated the mRNA expression of *BCL-2* and *SURVIVIN* genes, as anti-apoptotic factors. Moreover, it was shown doxorubicin and melatonin up-regulated the mRNA expression of *BAX* and *SMAC* genes, as pro-apoptotic factors. As expected, combinational treatment with Dox-Mel resulted in the favor of apoptotic pattern of *BCL-2*, *SURVIVIN*, *BAX*, and *SMAC* genes mRNA expression. Furthermore, we demonstrated that levels of Bcl-2 and Survivin proteins were decreased via doxorubicin and melatonin treatment, which is related to increased viability and proliferation of CC cells, Moreover, the obtained results indicated that doxorubicin and melatonin significantly increased the levels of Bax and Smac proteins, which decreased viability and proliferation of CC cells. However, combinational treatment with Dox-Mel resulted in the favor of apoptotic pattern of Bcl-2, Survivin, Bax, and Smac proteins levels. These results showed that doxorubicin and melatonin could inhibit CC cells viability and proliferation through induction of apoptotic pathways.

The current study represents a novel combination chemotherapeutic approach to treatment of patients with doxorubicin-resistant CC. It was shown that, melatonin enhanced the anticancer activity of doxorubicin through induction of intrinsic apoptotic pathway and suppressed the invasiveness of CC cells. Therefore, the Dox-Mel can be used in the future to control and even treatment of patients with CC. However, further studies are required to identify the enhanced anticancer mechanisms of Dox-Mel to better management of patients with CC and prescription of lower doses of chemotherapeutic agents.

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Declarations

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Author contributions

Each author has contributed to the design of the study, interpretation of data, manuscript preparation, editing and has provided approval of the final version submitted for publication.

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Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The inhibitory effects of doxorubicin, melatonin, and Dox-Mel on viability of CC cells in 24 (A), 48 (B), and 72 (C) hours. The anticancer activity of Dox-Mel was significantly more than doxorubicin and melatonin alone.

Figure 2

The morphological alteration of CC cells treated with doxorubicin (1 μ M), melatonin (2 mM), and Dox-Mel (0.8 μ M-mM) in 24, 48, and 72 hours. Treatment with Dox-Mel caused more profound morphological alterations.

Figure 3

The tumor spheroid formation of CC cells treated with doxorubicin (1 μ M), melatonin (2 mM), and Dox-Mel (0.8 μ M-mM) after 7-12 days. Treatment of CC cells with Dox-Mel cause significant decrease in the size and number of tumor spheroids.

Figure 4

The migration and invasion of CC cells evaluated by trans-well migration assay following treatment with doxorubicin (1 μ M), melatonin (2 mM), and Dox-Mel (0.8 μ M-mM) for 10-20 hours (A). Treatment of CC cells with Dox-Mel caused more significant decrease of migration and invasion (B).

Figure 5

The migration and invasion of CC cells evaluated by scratch wound healing assay following treatment with doxorubicin (1 μ M), melatonin (2 mM), and Dox-Mel (0.8 μ M-mM) for 10-20 hours (A). Treatment of CC cells with Dox-Mel resulted in more significant decrease of migration and invasion (B).

Figure 6

The apoptosis rate of CC cells treated with doxorubicin (1 μ M), melatonin (2 mM), and Dox-Mel (0.8 μ M-mM). The induction of apoptosis in the CC cells treated with Dox-Mel was significantly more than doxorubicin and melatonin alone.

Figure 7

The mRNA expression analysis of migration and invasion related MMP-2 (A) and MMP-9 genes (B) and apoptosis related BCL-2 (C), SURVIVIN (D), BAX (E), and SMAC (F) genes by qRT-PCR following the treatment with doxorubicin (1 μ M), melatonin (2 mM), and Dox-Mel (0.8 μ M-mM). The regulation of migration and invasion and apoptosis related genes expression in the CC cells treated with Dox-Mel was significantly more than doxorubicin and melatonin alone.

Figure 8

The western blot analysis (A) of MMP-2 (B) and MMP-9 (C) proteins following the treatment with doxorubicin (1 μ M), melatonin (2 mM), and Dox-Mel (0.8 μ M-mM). The regulation of migration and invasion related genes expression in the CC cells treated with Dox-Mel was significantly more than doxorubicin and melatonin alone.

Figure 9

The western blot analysis (A) of Bcl-2 (B), Survivin (C), Bax (D), and Smac (E) proteins levels following the treatment with doxorubicin (1 μ M), melatonin (2 mM), and Dox-Mel (0.8 μ M-mM). The regulation of apoptosis related genes expression in the CC cells treated with Dox-Mel was significantly more than doxorubicin and melatonin alone.