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Research Article

Investigating the Effects of Morphine on Survival and Sensitivity to Cisplatin in Ovarian Cancer Cells

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Abstract

Morphine as an analgesic drug is used frequently in cancer patients. Contradictory results have been achieved from previous studies related to morphine effects in different concentrations. In current study, we examined the effect of clinical concentrations of morphine on A2780Cp cell line related to ovarian cancer. Moreover, its effect on the cytotoxicity of cisplatin was investigated. A2780CP cells were cultured in RPMI1640 medium and treated with clinical doses of morphine alone or in combination with cisplatin. The rate of cell proliferation was measured using MTT assay, morphological changes of nuclei were revealed by 4′,6-diamidino-2-phenylindole (DAPI) staining, and expression of B-cell lymphoma 2 (Bcl-2) was measured using flowcytometry. MTT assay results showed clinical concentration of morphine had no effect on viability of A2780CP cells and toxicity of cisplatin. DAPI staining revealed no chromatin condensation in presence of morphine, and flowcytometry analysis showed that the expression of Bcl-2 in treated cells did not differ from control cells. In accordance with findings in other kinds of cancer, our results demonstrated that morphine did not interact with the function of cispatin in ovarian cancer. This finding can be considered in clinical applications of morphine.

***Keywords*:** morphine, ovarian cancer, apoptosis, cytotoxicity, cisplatin

Introduction

Ovarian cancer is the fifth leading cause of cancer death in the world. This type of cancer develops in one of 70 women in their lifetime. DNA-damaging agents such as Cis-diammine dichloroplatinum II (cisplatin) are used for the treatment of those who suffer from ovarian cancer (Gonzalez et al., 2001). Actually pain occurs in more than 80% of cancer patients which can take place as a result of cancer or chemotherapeutic drugs. Morphine is the most analgesic drug which is used to relieve pain resulted from such conditions (Hatsukari et al., 2007). It is usually injected to patients about 50-100 mg/day for a 70kg human (Gupta et al., 2002). The most part of injected morphine is metabolized into active glucuronide metabolites in the liver. Plasma concentration of free morphine was reported about 1 µg/ml and moreover about 96 µg/ml in a dead cancerous patient (Sarah et al., 2004).

Previous studies demonstrated that the effects of morphine on growth of cancer cells depends on morphine concentration or type of the cells (Gach et al., 2011). Investigations which used extremely high concentrations have revealed that morphine reduces the growth of cancer cells *in vitro* (Maneckjee et al., 1990, Naoko et al., 1996) or tumor weight *in vivo* (Harimaya et al., 2002). However, there are some reports which show that low concentration of morphine produces early apoptotic markers in HL-60 and A549 tumor cell lines (Hatsukari et al., 2007), reduces the growth of HT-29 cancer cells (Tegeder et al., 2003), and arrests cell cycle progress in MCF-7 cells (Chen et al., 2017). In addition, other studies have shown that plasma concentration of morphine stimulates microvascular endothelial cell proliferation and angiogenesis *in vivo* or *in vitro* (Gupta et al., 2002) and promotes the tumor growth in breast cancer (Bimonte et al., 2015). Concerning the interaction of morphine with chemotherapy drugs, it may cause drug interference with chemotherapy agents routinely used to treat cancers such as cisplatin or naloxon and may affect drug cytotoxicity depending on its dosage (Cao et al., 2016, Chen et al., 2017). In 2016, Cao *et al*. showed that morphine affects the antitumor activity of cisplatin on tumor growth in nasopharyngeal carcinoma CNE-2 xenografts in nude mice (Cao et al., 2016).

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With respect to broad usage of morphine to relieve pain in patients with ovarian cancer, it is important to determine its exact mechanism of function, especially its effect on cancer cell viability and/or death and also its interference with chemotherapy drugs. Continuing the previous work, which examined the effect of high concentrations of morphine on ovarian cancer cells (Nabiuni et al. ,2015), in the current study we investigated the effect of clinical concentrations of morphine on ovarian cancer cell line A2780CP, and also studied its effect on the cytotoxicity of cisplatin. This is the first time that the effect of the plasma dose of morphine is being studied on an ovarian cancer cell line.

Materials and Methods

Cell culture and growth conditions

All experiments were performed on human ovarian cancer cell line A2780CP. Cells were purchased from NCBI (National Cell Bank of Iran, Pasteur Institute) and were cultured in RPMI-1640 (Gibco-Invitrogen, US) with 10% (v/v) Fetal Bovine Serum (FBS, Gibco-Invitrogen, US) and 1% antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) at 37˚C in a 5% (v/v) CO_2 and 95% humidified incubator. The medium was changed every 48 hours.

Chemicals

Morphine sulfate was purchased from Daroupakhsh Co. (Iran), cisplatin from Naprod (India), Dimethyl Sulfoxide (DMSO) from Gibco (US) and triton from Merck (US), 3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazolium bromide (MTT) and 4',6-diamidino-2-phenylindole (DAPI) from Sigma (US), and antibodies from Abcam (UK).

MTT assay

In this study, 1-50 µg/ml of morphine was regarded as clinical or plasma concentrations. To determine the percentage of viable cells and the proliferation rate, MTT assay was performed. Briefly, cells were cultured in 24-well plates, treated with morphine (0.1, 1, 10, 20 and 50 µg/ml) or cisplatin (1-40 µg/ml) or combination of them (2 µg/ml of cisplatin; 2 and 20 µg/ml of morphine). After completion of the treatment time, 100µl of MTT was added to each well and incubated for 4 hours in darkness at 37˚C; then, the medium was removed and formazan crystals were dissolved in DMSO. Finally, the optical density was read by spectrophotometer at 570nm wavelength.

DAPI staining

To observe the morphological changes of the nucleus, DAPI staining was performed. Briefly, A2780CP cells were cultured in 24 well plates, treated with 1 µg/ml of morphine, washed with PBS and fixed in formalin 4% for 20 minutes at 4˚C. The cells were washed with PBS and permeabilized with Triton X-100 for 20 minutes at room temperature. After washing with PBS, DAPI (1%) was added to each well for 30 seconds in darkness and room temperature. The additional staining was removed and wells were washed with PBS carefully. Finally, 0.5 ml of PBS was added to each well and cells were observed with a fluorescent microscope.

Measuring the expression of Bcl-2 by flowcytometry

In order to detect the apoptotic or anti-apoptotic effect of morphine, the expression of Bcl-2, as an anti-apoptotic protein, was measured. To do so, the cells were treated with 1 µg/ml of morphine up to 120 hours. Then, they were trypsinized and washed with PBS and centrifuged for 5 minutes in 2000 rpm and then were fixed with formalin 4% for 15 minutes at room temperature. After washing, the cells were permeabilized to antibodies with triton X-100 (0.4% v/v) for 20 minutes at room temperature, washed with PBS and centrifuged, then blocked with bovine serum albumin (BSA) 2.5% w/v in tween-PBS 0.1% v/v (TPBS) for 30 minutes at room temperature. In the next step, cells were treated with 50 µl primary antibody diluted 1:100 in diluting buffer (BSA 0.25% in TPBS 0.1% v/v) for 2 hours at room temperature, washed with PBS and centrifuged. Then, cells were incubated with 50 µl fluorescein-labeled goat antibody against rabbit IgG diluted 1:30 with diluting buffer for 30 minutes at 37˚C in darkness. The
additional antibody was washed and finally, the expression of Bcl-2 was measured by Partec PAS instrument and the data were analyzed by flow max software (Partec, Germany).

Statistical analysis

All experiments were repeated three times. Results were analyzed using one-way ANOVA with InStat-3 software. Values were expressed as Mean±S.E.M and *p*-value of <0.05 was considered significant.

Results

Plasma concentrations of morphine did not change the viability of A2780CP cells.

The A2780CP cells were grown for 3 and 5 days with plasma concentrations of morphine (1-50 µg/ml). Investigation of cell viability showed that the cells which treated with plasma concentrations of morphine, proliferated as same as cells in the control group. Although cells which treated with 1 and 20 µg/ml of morphine proliferated more than untreated cells, statistical analysis showed that differences were not significant at *P*-value of 0.05 (Figure 1).

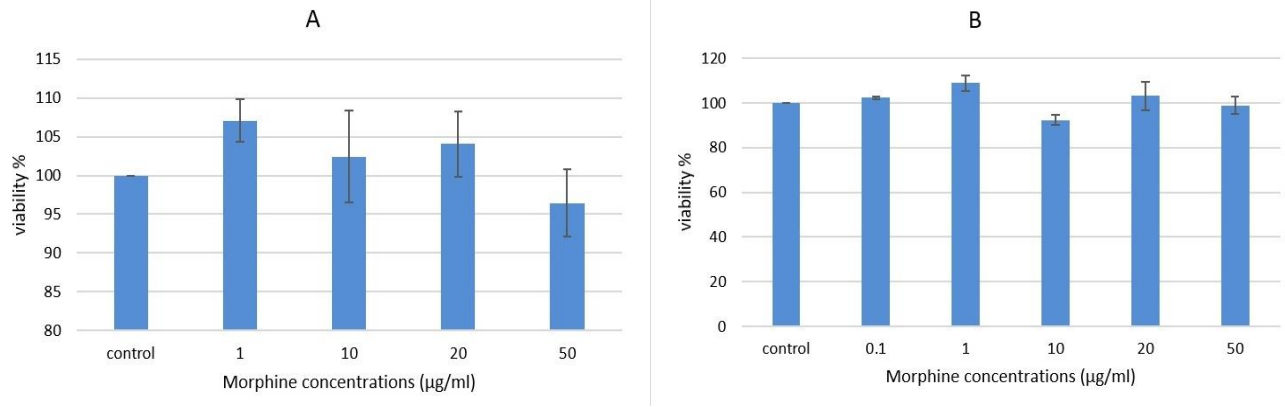

Figure 1. Percentage of viable cells under treatment with plasma concentrations of morphine compare to untreated cells after 3 days (A) and 5 days (B). The results are as Mean± SEM from three or more independent experiments.

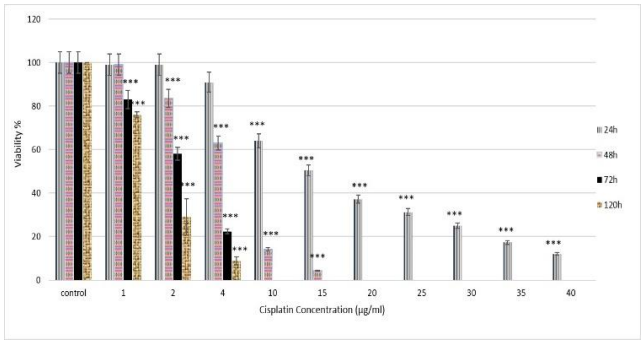

Figure 2. Percentage of viable cells under treatment with cisplatin. The results are as Mean± SEM from three independent experiments (* p<0.05, ** p<0.01, ***p<0.001).

The toxicity of cisplatin on A2780CP cells was not affected by morphine.

The effect of cisplatin (1-40 µg/ml) was examined by MTT assay after 1, 2, 3 or 5 days. As shown in Figure 2, cell viability was decreased after treatment with cisplatin in a dose and time-dependent manner. There was no viable cell after two days in doses more than 15 µg/ml. To evaluate the effect of morphine on the function of cisplatin, cells were treated with morphine and cisplatin. Co-treatment of the cells with 2 µg/ml of cisplatin in combination with 1 or 20 µg/ml of morphine showed that 20 µg/ml of morphine enhanced cytotoxicity of cisplatin slightly but not statically significant. The rate of cell viability in presence of 1 µg/ml of morphine and 2 µg/ml of cisplatin did not change compared to the cells which treated with 2 µg/ml of cisplatin (Figure 3).

Plasma concentration of morphine did not harm the nuclei of A2780CP cells.

DAPI staining was performed after 48 hours treatment of A2780CP cells with 1 µg/ml of morphine. Observation showed that the total number of nuclei in morphine treated cells was comparable with untreated cells and no fragmented nuclei were found in the presence of 1 µg/ml of morphine (Figure 4). These results were in accordance with MTT assay results.

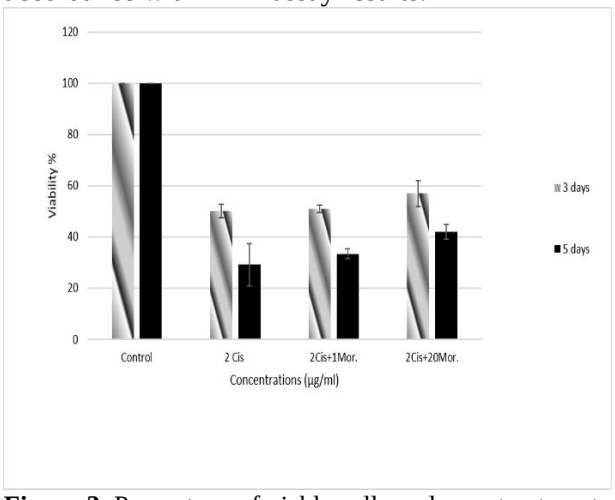

Figure 3. Percentage of viable cells under co-treatment with 2 µg/ml of cisplatin and 1 or 20 µg/ml of morphine. The results are as Mean± SEM from three independent experiments.

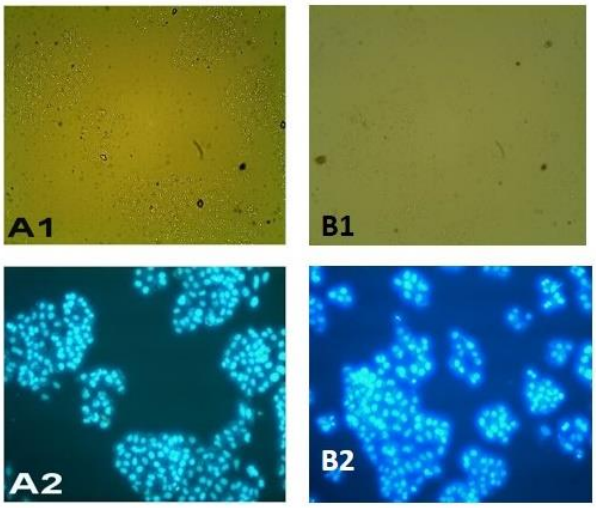

Figure 4. DAPI stained nuclei under light (part 1) and florescent (part 2) microscope. A: untreated (control), B: 1 µg/ml of morphine (× 100).

Anti-apoptotic Bcl-2 protein was expressed as control state

To investigate the effect of morphine on the function of apoptotic factors in A2780CP cells, the expression of Bcl-2 protein, as an anti-apoptotic protein, was evaluated. Briefly, cells were treated with predominant plasma concentration of morphine (1µg/ml) for 5 days. The results are shown in Figures 5. 1µg/ml of morphine enhanced the expression of Bcl-2 in the amount of 6.5%, so that the expression of Bcl-2 in control cells was 72.86% compared to 79.36% in morphine treated cells. The difference was not significant at P-value of 0.05.

Discussion

Several studies have shown that morphine administration can affect cancer cells growth (Bhat et al., 2004, Bimonte et al., 2013, Donahue et al., 2009); and there is some information about the mechanisms by which morphine acts through (Crawford and Bowen 2002, Diao et al., 2000, Donahue et al., 2009, Gach et al., 2011, Hsiao et al., 2009, Iglesias, 2003, Koodie et al., 2010). Morphine effects may be depend not only on the cell type and the system of investigation (*in vitro* or *in vivo*) but also on its doses. Investigation of high doses of morphine has been shown apoptotic cell death induction in a dose and time-dependent manner, however, these concentrations are not tolerable in animal bodies.

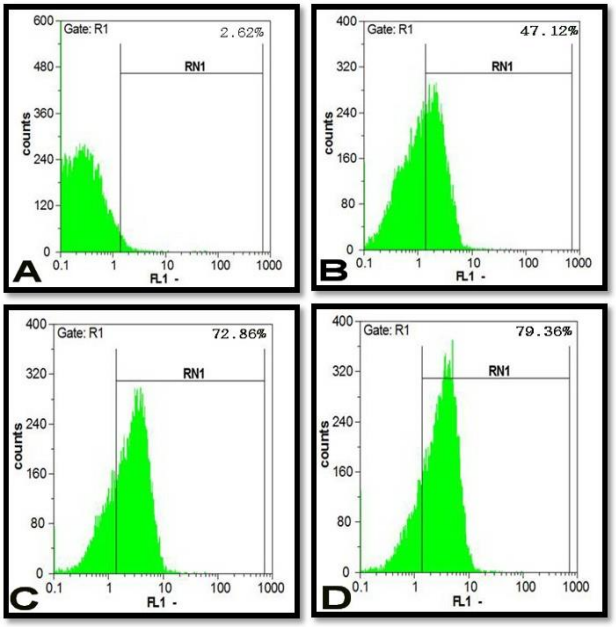

Figure 5. Measurement of Bcl-2 protein expression in the cells which treated with plasma concentration of morphine. A) Unstained, B) negative control, C) untreated (normal), D) cells treated with 1 µg/ml Morphine. The RN1 region shows the percentage of BCl-2 expression.

Herein, we studied the effect of clinical concentrations of morphine. Our findings showed that morphine did not increase the proliferation of A2780CP cells significantly in the range of 0.1-50 µg/ml, which was in agreement with Cao et al. findings which showed 0.1-100 µg/ml of morphine did not change the cell viability of human nasopharyngeal carcinoma CNE-2 cells significantly (Cao et al., 2016). Investigation of clinically relevant doses of morphine on human glioblastoma T98G cells (Lazarczyk et al., 2010) and human breast carcinoma cells (Bimonte et al., 2015) and bladder cancer cells (Harper et al., 2018) indicated that morphine increased proliferation of cancerous cells, that can be explained with differences in morphine concentration, duration of treatment and the type of cancer cells.

In the course of this study, we also examined the combined effect of morphine and cisplatin on A2780CP cell line. Cisplatin is a well-known DNA-damaging factor and is a common chemotherapeutic agent. It is used widely for head and neck, ovarian and non-small cell lung cancers. Previous studies have been demonstrated that cisplatin induces cell death through apoptosis and cell cycle regulating pathways (Agrez et al., 2011, Tanida et al., 2012). In previous study by Cao et al., it was indicated that morphine (1 µg/ml) abolished the 4 µg/ml cisplatin-induced loss of cell viability

only at 72 h, but not at 48 h; also, morphine (0.1 µg/ml, 10 µg/ml or 100 µg/ml) did not show any effect on the cisplatin-induced decrease in cell viability of CNE-2 cells at 24 h, 48 h and 72 h (Cao et al., 2016). In compare, our results showed that co-treatment of 1 and 20 µg/ml of morphine and 2 µg/ml of cisplatin did not lead to obvious increase in cell proliferation. From these findings, it can be concluded that the effects of morphine might be different based on cellular sensitivity or cisplatin dose.

Several investigations reported that high concentrations of morphine, induce apoptosis in cancer cells that are along with a decrease in Bcl-2 expression; which means morphine acts through an intrinsic pathway and promotes apoptosis (Cheng et al., 2006, Kapasi et al., 2004). On the other hand, studies in low doses of morphine have shown that morphine inhibits cell death with the inhibitory effect on the expression of p53 in MDA.MB231 cells (breast cancer cell line) (Bimonte et al., 2015) or through declining the expression of Bcl-2 in nasopharyngeal carcinoma (Cao et al., 2016). Although the combined treatment of morphine (1 µg/ml) and cisplatin (4 µg/ml) increased the expression of Bcl-2 in nasopharyngeal carcinoma cells (Cao et al., 2016), our finding indicated that in ovarian cancer cells, morphine did not have obvious effect on Bcl-2 expression in usual plasma concentration.

Conclusion

In conclusion, the present study demonstrated that plasma concentrations of morphine, in range of clinical doses, could not alter the proliferation of ovarian cancer cells and the cytotoxicity of cisplatin as well. In terms of its effect on the molecular pathway of apoptosis, morphine also did not affect the expression of anti-apoptotic factor, Bcl-2. These results can be considered for the potential effects of this drug in ovarian cancer patients.

Authors' contributions

All authors conceived design of study and basic concepts. MR and HJ performed laboratory tests and statistical analysis. All authors wrote the article and approved the final version of the manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

Ethical Statement

Not applicable.

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