Apomorphine induces mitochondrial-dysfunction-dependent apoptosis in choriocarcinoma

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Abstract

Apomorphine is a derivative of morphine that is used for the treatment of Parkinson's disease because of its effects on the hypothalamus. Therapeutic effects of apomorphine have also been reported for various neurological diseases and cancers. However, the molecular mechanisms of the antitumor effects of apomorphine are not clear, especially with respect to choriocarcinoma. This is the first study to elucidate the anticancer effects of apomorphine on choriocarcinoma. We found that apomorphine suppressed the viability, proliferation, ATP production, and spheroid formation of JEG3 and JAR choriocarcinoma cells. Moreover, apomorphine activated the intrinsic apoptosis pathway by activating caspases and inhibited the production of anti-apoptotic proteins in choriocarcinoma cells. Further, apomorphine caused depolarization of mitochondria, calcium overload, energy deprivation, and endoplasmic reticulum stress in JEG3 and JAR cells. We confirmed synergistic effects of apomorphine with paclitaxel, a traditional chemotherapeutic agent, and propose that apomorphine could be a potential therapeutic agent in choriocarcinoma and an important candidate for drug repositioning that could help overcome resistance to conventional chemotherapy.

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Introduction

Apomorphine, a quinoline alkaloid, is a degradation product of morphine. It is a dopamine receptor agonist and an alpha (α)-adrenergic receptor antagonist (Millan *et al.* 2002). Apomorphine has been used to treat Parkinson's disease, and because of its inhibition of amyloid β protein fiber formation, it has also been used to treat Alzheimer's disease (Himeno *et al.* 2011, Pessoa *et al.* 2018, Pieroni 2019). Besides neurological disorders, apomorphine can also be used to target tumors (Auffret *et al.* 2019). It inhibits invasion of human breast cancer (MCF-7) cells, suppressing inflammation regardless of the presence of dopamine receptors (Ding & Cui 2017). Additionally, it prevents metastasis of primary tumors, especially in the brain (Singh *et al.* 2018). Although its anticancer activity has been described for several tumors, there is no information about its therapeutic efficacy against choriocarcinoma cells.

Choriocarcinoma is a gestational trophoblastic cancer that usually originates in the placenta. It proliferates aggressively and metastasizes to other organs such as the lung, liver, or brain because of its hematogenous characteristics (Duong *et al.* 2018, Lee & Cho 2019). Treatment of this gestational trophoblastic

disease involves surgical resection, radiation, and chemotherapy; however, 25% of such cases have been reported to develop chemoresistance (Alazzam *et al.* 2016, Eysbouts *et al.* 2017). Although the combination of etoposide, methotrexate with folinic acid, and actinomycin D/cyclophosphamide and vincristine (EMA/CO) is used as first-line therapy, the development of chemoresistance results in treatment failure and even tumor recurrence (Alazzam *et al.* 2016). Thus, there is an urgent need to discover new therapeutic agents that have higher efficacy with lower side effects (Sato *et al.* 2020).

In this study, we aimed to study the anticancer effects of apomorphine on human JEG3 and JAR choriocarcinoma cells, especially with respect to synergy with traditional chemotherapeutic agents. Specifically, we sought to examine the effects of apomorphine on JEG3 and JAR cells with respect to (1) cell proliferation and viability, ATP production, and formation of spheroids; (2) caspase-dependent apoptosis; (3) mitochondrial function and energy metabolism; (4) endoplasmic reticulum (ER) stress; and (5) synergy with paclitaxel. Therefore, we suggest that apomorphine could be used as a pharmacological agent for the treatment of human choriocarcinoma after

the elucidation of its mechanism of anticancer activity. As apomorphine is already approved by the United States Food and Drug Administration to treat off-episode motor symptoms, our findings may contribute to its repositioning for choriocarcinoma treatment.

Materials and methods

Chemicals and antibodies

Apomorphine was purchased from Sigma-Aldrich. Hoechst 33342 (Cat. No: B2261) and propidium iodide (Cat. No: P4170) were purchased from Sigma-Aldrich, while carbobenzoxy-valyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone (Z-VAD-FMK, Cat. No: 627610) was purchased from Calbiochem. Antibodies purchased from Cell Signaling Technology for Western blotting include myeloid cell leukemia 1 (Mcl-1), B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra-large (Bcl-xL), poly (adenosine diphosphate (ADP)-ribose) polymerase (PARP), cleaved PARP, β -actin, glucose-regulated protein-78/binding immunoglobulin protein (GRP78/Bip), activating transcription factor 4 (ATF4), and CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP).

Cell maintenance and chemical treatment

Human choriocarcinoma JEG3 and JAR cells were purchased from American Type Culture Collection (ATCC). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin at 37°C in a 5% CO₂ incubator. Cells were seeded at a concentration of 2×10^4 cells/cm² and treated with various concentrations of apomorphine for 24 h. Dimethyl sulfoxide was used as vehicle control.

Measurement of cell viability and proliferation

To measure the viability and proliferation of JEG3 and JAR cells, the trypan blue exclusion assay was conducted to discriminate between viable and dead cells. ATP production was measured using a Cell Titer-Glo Luminescent assay (Promega) as an indicator of cell viability. This assay was performed according to the manufacturer's instructions.

Spheroid formation

For the spheroid formation assay, we seeded 100 μ L of JEG3 and JAR cells at a density of 1×10^5 cells/mL in each well of a round bottom plate. Cells were treated with vehicle or 30 μ M of apomorphine and incubated at 37°C in a 5% CO₂ incubator for 5 days. Three-dimensional (3D) graphics images were obtained by using Image J and Reconstruction and Visualization from a Single Projection (ReViSP) software.

Detection of apoptotic cells

Hoechst 33342 (Sigma-Aldrich) and propidium iodide (PI, Sigma-Aldrich) were used to detect apoptotic and necrotic cells. For measurement of caspase 3/7 enzyme activity, the

Caspase-Glo3/7 Assay (Promega) was performed according to the manufacturer's protocol. Cells were pretreated with 50 mM of Z-VAD-FMK, the caspase inhibitor, for 1 h. A fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit 1 (BD Biosciences) was used to analyze the cells using flow cytometry. Detailed protocols were described in previous studies (Lim *et al.* 2019).

Western blot analysis

Proteins were extracted from whole cells after treatment with apomorphine for 24 h, and their concentrations were determined by using a Bradford protein assay (Bio-Rad). After denaturation, proteins were separated by sodium dodecyl sulfate-PAGE (SDS-PAGE) and then transferred to nitrocellulose membranes. Blots were quantified by chemiluminescence detection (SuperSignal West Pico, Pierce) using a ChemiDoc EQ system and Quantity One software (Bio-Rad). Detailed procedures were described in previous studies (Lim *et al.* 2019).

Detection of mitochondrial membrane potential and relative mitochondrial calcium levels

A mitochondrial staining kit (Sigma-Aldrich) was used to detect loss of mitochondrial membrane potential and the acetoxymethyl ester of Rhod-2 (Rhod-2 AM reagent, Thermo Fisher Scientific) was used as an indicator of mitochondrial calcium levels. Fluorescence signals were measured and analyzed by flow cytometry. To perform these assays, 2×10^4 cells/mL of JEG3 and JAR cells were seeded in 6-well plates in 2 mL of complete media and incubated with apomorphine (0, 10, 30, and 50 μ M) for 24 h. Detailed procedures were described in previous studies (Lee *et al.* 2019b).

Seahorse assay

For mitochondrial stress analysis, we evaluated the oxygen consumption rate by studying oxidative phosphorylation. The sequential injection of oligomycin, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), rotenone, and antimycin A (mitochondrial complex I and III inhibitors) enabled the quantification of basal respiration, proton leak, maximal respiration, non-mitochondrial respiration, ATP production, and spare respiratory capacity using a Seahorse Analyzer and a mitochondrial stress analysis kit (Agilent Technologies). Detailed procedures were described in previous studies (Lee *et al.* 2018).

Statistical analysis

Data are expressed as mean $\pm$ s.d., and significance was estimated by one-way or two-way ANOVA, as described in the figure legends. *Post-hoc* analyses were performed using GraphPad Prism 7 software. Statistical significance was indicated by $P < 0.05$ and presented as follows in the legends: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; *post-hoc* analyses: Dunnett, Sidak). All histograms represent the mean $\pm$ s.d. of at least three independent experiments.

Results

Apomorphine suppressed viability and proliferation of human choriocarcinoma JEG3 and JAR cells

We evaluated the viability of JEG3 and JAR cells using trypan blue staining. Apomorphine gradually decreased the viability of JEG3 and JAR cells over 72 h in a dose-dependent manner (0 to 50 μ M) (Fig. 1A). Treatment with apomorphine (30 μ M) for 24 h decreased the viability of JEG3 and JAR cells to

57.5% and 69%, respectively (Fig. 1A). Proliferation of JEG3 and JAR cells also decreased in response to apomorphine treatment (0, 10, 30, 50 μ M) for 24 h (Fig. 1B). In addition, ATP production in both cell lines decreased significantly by >50% with apomorphine treatment (Fig. 1C). Apomorphine completely inhibited the formation of tumor spheroids by up to 33% and 59% in JEG3 and JAR cell lines, respectively (Fig. 1D, E and F). This study demonstrates that apomorphine has strong anticancer effects and inhibits cell viability

Figure 1 Antiproliferative effects of apomorphine on human choriocarcinoma JEG3 and JAR cells. (A) JEG3 and JAR cells were treated with apomorphine in a dose-dependent (0, 10, 30, and 50 μ M) and time-dependent manner (0, 24, 48, and 72 h). Viable cells were counted using trypan blue staining, and data from three independent experiments were subjected to statistical analysis. (B) Proliferation of JEG3 and JAR cells was measured by using the trypan blue exclusion assay after treatment with 30 μ M apomorphine for 24 h. (C) Intracellular ATP levels in JEG3 and JAR cells were measured by colorimetric methods, as a response to various concentrations of apomorphine for 24 h. (D, E and F) Vehicle or 30 μ M of apomorphine were incubated with JEG3 and JAR cells to study the effects on spheroid formation. 3D-structure images obtained using the ReViSP software are shown in the right panel. (F) Relative values of spheroid surface area are represented as bar graphs compared to control. Results are expressed as mean $\pm$ s.d. of three independent experiments. Asterisk indicates a statistically significant difference between control and treatment, as analyzed by Dunnett's test, one-way analysis of variance (ANOVA) (* P <0.05; ** P <0.01; *** P <0.001).

and proliferation of human choriocarcinoma JEG3 and JAR cells.

Apomorphine induced caspase-dependent apoptosis in human choriocarcinoma JEG3 and JAR cells

To verify the mechanism of cell death induced by apomorphine, we performed Hoechst 33342 and Annexin V/PI staining to detect apoptotic cells. Compared to vehicle-treated JEG3 or JAR cells, early and late apoptotic cells were detected based on their blue fragmented fluorescence and red fluorescence, respectively, after treatment with apomorphine (Fig. 2A and B). As indicated by the arrows, nuclear fragmentation was observed in both JEG3 and JAR cells in apomorphine treatments of 30 μ M and 50 μ M, but not in the control group. These suggest, a hallmark of apoptotic cell death, that nuclear DNA is degraded into nucleosomal

subunits. In JEG3 cells, flow cytometry using Annexin V and PI staining revealed significant increase in the number of early and late apoptotic cells (Fig. 2C and D). In JAR cells, the increase in late apoptotic and necrotic cells was induced by apomorphine treatment in a dose-dependent manner (0, 10, 30, and 50 μ M) (Fig. 2C and D). To confirm whether intrinsic apoptosis was induced in JEG3 and JAR cells by apomorphine treatment, we measured caspase 3 and 7 activity (Fig. 2E). In both apomorphine-treated choriocarcinoma cell lines, caspase 3/7 activity was dramatically increased ($>2\times$). This apomorphine-induced caspase 3/7 activity was significantly inhibited by pretreatment with the pan-caspase inhibitor, Z-VAD-FMK, which implies that apomorphine induced caspase-dependent apoptosis in both cell lines (Fig. 2E). Thus, we confirmed that apomorphine induced intrinsic apoptotic cell death in human choriocarcinoma cells.

Figure 2 Effects of apomorphine on cell death in JEG3 and JAR choriocarcinoma cells. (A and B) To determine changes in morphological characteristics caused by apomorphine, JEG3 and JAR cells were stained with Hoechst 33342 (upper panel, blue) and propidium iodide (PI, middle panel, red). (C) Cell death patterns in apomorphine-treated JEG3 and JAR cells were analyzed using Annexin V and PI staining and flow cytometry. (D) Relative cell populations of live (lower left panel), early apoptotic (lower right panel), late apoptotic (upper right panel), and necrotic (upper left panel) cells were analyzed using fluorescence-activated cell sorting (FACS) data. (E) Caspase 3/7 activity was measured in 30- μ M-apomorphine-treated JEG3 (upper panel) and JAR cells (lower panel) compared to vehicle-treated cells. Further, cells were pretreated with Z-VAD-FMK, a caspase inhibitor, for 1 h before incubation with apomorphine. Results are expressed as mean $\pm$ s.d. of three independent experiments. Asterisk indicates a statistically significant difference between control and treatment, as analyzed by Dunnett's test, one-way (D), or two-way (E) analysis of variance (ANOVA) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Apomorphine regulated levels of anti-apoptotic proteins, leading to intrinsic apoptosis in JEG3 and JAR cells

We performed Western blotting to detect the levels of proteins of the intrinsic apoptosis pathway in JEG3 and JAR cells. Levels of the anti-apoptotic Bcl-2 family proteins, Mcl-1 and Bcl-2, were significantly decreased by 10 μ M of apomorphine in JEG3 cells (Fig. 3A and B). Apomorphine decreased Bcl-xL levels in a dose-dependent manner (0, 10, 30, and 50 μ M) (Fig. 3A and B); even lower concentrations of apomorphine inhibited Bcl-2 protein levels in JEG3 cells to <30% of those in untreated cells (Fig. 3B). Apomorphine also decreased protein levels of Mcl-1, Bcl-2, and Bcl-xL in JAR cells (Fig. 3C and D). In contrast, levels of cleaved PARP, the pro-apoptotic marker, were increased in apomorphine-treated JEG3 and JAR cells (Fig. 3A, B, C and D). Thus, we demonstrate that apomorphine activated the intrinsic apoptosis pathway in JEG3 and JAR cells and regulated apoptotic protein levels.

Apomorphine caused mitochondrial dysfunction and metabolic changes in JEG3 and JAR cells

Mitochondrial function is crucial for cell survival and plays a major role in apoptotic cell death. Hence,

we investigated mitochondrial membrane potential, calcium uptake, and metabolic homeostasis in JEG3 and JAR cells. Relative mitochondrial membrane potential (MMP, $\Delta\Psi$) and calcium uptake of mitochondria were dramatically modulated by 50 μ M of apomorphine in JEG3 cells, leading to depolarization and increased calcium uptake, respectively (Fig. 4A and C). The extent of depolarization (decrease in MMP) and mitochondrial calcium levels were gradually increased by apomorphine in JAR cells (Fig. 4B and D). The effects of apomorphine on mitochondrial respiration and glycolysis in choriocarcinoma cells were examined by analyzing the bioenergetics profile using oligomycin, an ATP synthase inhibitor; FCCP, a depolarizer of plasma membrane potential; and rotenone and antimycin A, complex I and III inhibitors, respectively (Fig. 4E). In both JEG3 and JAR cells treated with 30 μ M of apomorphine for 24 h, basal/maximal respiration levels and ATP production decreased considerably compared to those of the vehicle-treated cells (Fig. 4F). The bioenergetics phenotype of JEG3 and JAR choriocarcinoma cells changed from energetic to quiescent with reduced glycolytic function after treatment of 30 μ M of apomorphine (Fig. 4G). This energy profile supports our data for the inhibition of proliferation and ATP reduction by apomorphine treatment. Therefore, as mitochondrial dysfunction is considered an important

Figure 3 Apoptosis-related changes in protein levels induced by apomorphine in human choriocarcinoma JEG3 and JAR cells. (A and C) Immunoblots of JEG3 and JAR cells treated with various concentrations of apomorphine (0, 10, 30, and 50 μ M) for 24 h. (B and D) Relative protein levels of Mcl-1, Bcl-2, Bcl-xL, PARP, and cleaved PARP in human choriocarcinoma cells (JEG3, JAR) were analyzed using Image Lab software and represented as bar graphs compared to control cells. Results are expressed as mean $\pm$ s.d. of three independent experiments. Asterisk indicates a statistically significant difference between control and treatment, as analyzed by Dunnett's test, one-way analysis of variance (ANOVA) (* P < 0.05; ** P < 0.01; *** P < 0.001).

Figure 4 Effects of apomorphine on mitochondrial function and energy metabolism in human choriocarcinoma cells. (A and B) Depolarization of $\Delta\Psi$ was detected by JC-1 dye staining in JEG 3 and JAR cells ($\Delta\Psi$; mitochondrial membrane potential). Cells detected in R² area of cytometry data were analyzed and represented as bar graphs (right panel) relative to vehicle-treated cells. (C and D) Increase in mitochondrial calcium levels in apomorphine-treated JEG3 and JAR cells were detected by Rhod-2 staining and analyzed using flow cytometry. (E) Metabolic profiles of apomorphine-treated JEG3 and JAR cells were analyzed by a Seahorse analyzer. (F) Basal respiration, ATP production, and maximal respiration values of JEG3 and JAR cells were measured based on the Seahorse assay. (G) Changes in energy phenotype of JEG3 and JAR cells treated with apomorphine (30 μ M) were analyzed based on the results of the Seahorse assay. Results are expressed as mean $\pm$ s.d. of three independent experiments. Asterisk indicates a statistically significant difference between control and treatment, as analyzed by Dunnett's test, one-way analysis of variance (ANOVA) (* P < 0.05; ** P < 0.01; *** P < 0.001).

representative feature of intrinsic apoptosis, these data may indicate apomorphine-induced activation of specific apoptosis pathways in choriocarcinoma cells. Taken together, apomorphine exhibited anticancer effects on human choriocarcinoma cells by inducing mitochondrial dysfunction, as evidenced by loss of MMP and disruption of calcium homeostasis and energy balance. Mitochondrial apoptosis is defined by mitochondrial outer membrane depolarization leading transcriptional regulation of BH3-only proteins, proteasomal degradation, apoptosome formation, and caspase activation, and our results suggested most of the characteristics (Lopez & Tait 2015). Considering mitochondria is a key organelle to regulate the energy process, we speculated that mitochondrial apoptosis may eventually cause metabolic shifting, in particular oxidative phosphorylation (OXPHOS)

(Porporato *et al.* 2018). In agreement with our hypothesis, apomorphine-induced systematic mitochondrial dysfunction triggers mitochondrial apoptosis, which contributes to metabolic alteration by loss of OXPHOS in placental choriocarcinoma cells.

Apomorphine induced endoplasmic reticulum stress in human choriocarcinoma JEG3 and JAR cells

As endoplasmic reticulum (ER) stress is known to be a major factor in the induction of apoptosis by interactions with mitochondria, we performed Western blotting to examine the protein levels of ER stress signaling molecules. Protein levels of an initiation marker of ER stress, GRP78/Bip, were increased 8-fold in JEG3 and 2-fold in JAR cells after treatment with 50 μ M apomorphine (Fig. 5A and C). Levels of ATF4 and CHOP,

Figure 5 Effects of apomorphine on ER stress signaling proteins in choriocarcinoma cells. (A and C) Immunoblots of JEG3 and JAR cells treated with various concentrations of apomorphine (0, 10, 30, and 50 μM) for 24 h. (B and D) Relative protein levels in immunoblots of GRP78/Bip, ATF4, and CHOP in choriocarcinoma cells (JEG3, JAR cells) were analyzed using the same methods used for the experiments depicted in Fig. 3. Results are expressed as mean $\pm$ s.d. of three independent experiments. Asterisk indicates a statistically significant difference between control and treatment, as analyzed by Dunnett's test, one-way analysis of variance (ANOVA) (* P < 0.05; ** P < 0.01; *** P < 0.001).

transcription factors and downstream signal molecules of ER stress increased to 2.1-fold and 3.7-fold in JEG3 cells, respectively (Fig. 5B). In JAR cells, apomorphine treatment increased ATF4 and CHOP levels (Fig. 5D). These results suggest that ER stress induced by apomorphine led to mitochondria-mediated intrinsic apoptosis of choriocarcinoma JEG3 and JAR cells.

Apomorphine exhibited synergistic anticancer effects with paclitaxel in human choriocarcinoma cells

To determine whether apomorphine could enhance chemosensitivity in choriocarcinoma cells, we added paclitaxel, a standard chemotherapeutic agent, to our experiments. In both JEG3 and JAR cells, the number of apoptotic and necrotic cells significantly increased in the combined treatment compared to single treatment of apomorphine or paclitaxel (Fig. 6A and B). Co-treatment with apomorphine (10, 30 μM) and paclitaxel (0.1, 0.5 μM) revealed synergistic effects on the viability of JEG3 and JAR cells (Fig. 6C and D). The combination index (CI) and fraction affected (FA) values of these combination treatments were calculated. In all co-treatment conditions, CI values were <1 in both JEG3 and JAR cells, which indicated that the relationship between paclitaxel and apomorphine was synergistic (Fig. 6E and F). These results suggest the efficacy of apomorphine as a therapeutic agent for human choriocarcinoma

cells, both for its anticancer effects and as an agent for those cancers that have become resistant to traditional chemotherapeutic agents.

Discussion

Apomorphine was initially developed for the treatment of Parkinson's disease for its effects on the hypothalamus and its activity as a dopaminergic receptor agonist. Besides these effects on the brain and neuronal disease, there is no comprehensive assessment of apomorphine that targets gynecological disease; however, there is interesting research that supports our hypothesis. Dopamine abrogates the secretion of human chorionic gonadotropin (hCG) in human placental culture (Macaron *et al.* 1979). Human chorionic gonadotropin plays a crucial role in angiogenesis via transforming growth factor-beta activation in the placenta at the maternal and fetal interface during early pregnancy and stimulates choriocarcinoma in the same biological frame (Berndt *et al.* 2013). Ectopic expression of beta-hCG (β-hCG) demonstrates a paradoxical function in its stimulation of cancer growth (Giovangrandi *et al.* 2001, Nagiraja *et al.* 2010). As the inhibition of hCG secretion in the human placenta and trophoblast by dopamine is well characterized, we hypothesized that a dopaminergic receptor agonist might effectively inhibit the growth of

Figure 6 Synergistic effects of combined treatment with apomorphine and paclitaxel on choriocarcinoma cells. (A and B) Hoechst 33342 and propidium iodide (PI) staining was carried out after combined treatment of JEG3 and JAR cells with apomorphine (30 μM) and paclitaxel (0.5 μM). (C and D) Different concentrations of apomorphine (10, 30 μM) and paclitaxel (0.1, 0.5 μM) were combined to confirm synergistic effects on the inhibition of cell viability of JEG3 and JAR cells. (E and F) Through CompuSyn software analysis, combination index (CI) and fraction affected (FA) values were calculated to indicate synergistic relationship between apomorphine and paclitaxel toward JEG3 and JAR cells. Results are expressed as mean $\pm$ S.D. of three independent experiments. Asterisk indicates a statistically significant difference between control and treatment, as analyzed by Dunnett's test, two-way analysis of variance (ANOVA) (* P < 0.05; ** P < 0.01; *** P < 0.001). Lowercase letters indicate statistically significant differences of treatments: a, compared with 10 μM apomorphine alone; b, compared with 30 μM apomorphine alone.

choriocarcinoma cells and contribute to increasing the susceptibility of these cells to chemotherapy.

Efforts to discover new antitumor drugs are ongoing and focus on combining standard chemotherapeutic agents, combining them with bioactive natural products or finding new indications of drugs already being used for other diseases (Lee *et al.* 2016, Chikara *et al.* 2018, Riedel *et al.* 2018). Apomorphine, a dopaminergic agonist and derivative of morphine, has been used as a remedial agent for Parkinson's disease and also for neurological disease, sexual disorders, and cancers (Auffret *et al.* 2019). A few decades ago, there were reports about the cytotoxicity of apomorphine toward brain glioma of rat (El-Bacha *et al.* 1999). Apomorphine also shows antitumor effects on human breast cancer and anti-metastatic effects on brain metastases (Jung & Lee 2017, Singh *et al.* 2018). Apomorphine, which shares the quinolone alkaloid structure of apomorphine, induces caspase-dependent apoptosis in head/neck squamous cell carcinoma (Rodrigues-Junior *et al.* 2020). Apomorphine also suppresses the binding of mouse double minute 2 homolog (MDM2) to p53 (Ishiba *et al.* 2017), which restores WT p53 function (Wiman 2006,

Vassilev 2007). The increase in p53 activity, as a tumor suppressor, leads to apoptosis with cell cycle arrest and inhibits cell migration by regulating microRNA in choriocarcinoma JEG3 cells, which supports our hypothesis of the anticancer activity of apomorphine toward choriocarcinoma cells (Drukeinis *et al.* 2005, Lu *et al.* 2020).

In this study, we demonstrate that apomorphine induced caspase-dependent apoptosis in human choriocarcinoma cells and caused global mitochondrial dysfunction. Serial caspase activation was initiated by the release of cytochrome *c*, leading to the formation of apoptosomes. This was accompanied by a decrease in MMP and in the production of anti-apoptotic proteins (Fu *et al.* 2017). Mcl-1 and Bcl-xL are pro-survival proteins belonging to the Bcl-2 family of proteins and are involved in drug resistance (Lee *et al.* 2019a). Mcl-1 is a key indicator of drug resistance in myeloma, prostate cancer, and fibroblast-like synoviocytes (Punnoose *et al.* 2016, Jiao *et al.* 2018, Pilling & Hwang 2019). Our results show decreased protein levels of Bcl-2 family members, Mcl-1, Bcl-2, and Bcl-xL, indicating the initiation of mitochondrial apoptosis in choriocarcinoma

Figure 7 Illustration about possible mechanisms of anticancer activity of apomorphine in human choriocarcinoma JEG3 and JAR cells. Mito, mitochondria; ER, endoplasmic reticulum; N, Nucleus; $\Delta\Psi_m$, mitochondrial membrane potential; $[Ca^{2+}]_m$, mitochondrial calcium level.

cells. Cleaved PARP levels, the last step of the caspase activation pathway, were also elevated, which indicated a reduction in DNA repair capacity (Li & Darzynkiewicz 2000). Loss of MMP and mitochondrial calcium overload are typical characteristics of intrinsic apoptosis, which were also observed in our study (Szabadkai & Rizzuto 2004, Fischer *et al.* 2008).

Further, we examined the metabolic effects of apomorphine in choriocarcinoma cells, which could be targeted to enhance drug sensitivity. For instance, reactive oxygen species-induced apoptosis in leukemia cells was increased by co-treatment with agents inhibiting mitochondrial respiration (Pelicano *et al.* 2003). Although there was no specific bioenergetics profile for choriocarcinoma, we observed the suppression of a shift to a glycolytic energy phenotype by apomorphine. Cancer cells prefer aerobic glycolysis to generate their energy rapidly; this phenomenon is known as the Warburg effect (Liberti & Locasale 2016). Targeting the Warburg effect has also been used as an anticancer therapeutic strategy (El Sayed *et al.* 2013, Chen *et al.* 2016), for example, the disruption of mitochondrial bioenergetics with the pharmacological inhibitor, mitochondrial division inhibitor-1 (MDIVI-1), efficiently induces breast cancer cell death (Lucantoni *et al.* 2018).

Mitochondria and ER are closely interacted with each other and share functions such as the regulation of calcium homeostasis or cellular stress (Szabadkai & Rizzuto 2004, Senft & Ronai 2015, Marchi *et al.* 2018). As Bcl-2 family proteins affect the release of calcium from ER (Kuwana & Newmeyer 2003), we examined whether apomorphine treatment could cause ER stress in choriocarcinoma cells. The GRP78/Bip protein regulates unfolded protein response in response to cellular stress. The protein kinase RNA-like endoplasmic reticulum kinase (PERK)/ATF4/CHOP pathway is activated as an ER stress signal, and ATF4/CHOP subsequently suppresses the production of Bcl-2 proteins, which is

also consistent with our observations (Rozpedek *et al.* 2016). Moreover, through mitochondrial-associated ER membranes (MAM), calcium is transferred from the mitochondria to the ER, leading to calcium overload, which then induces apoptosis (Pinton *et al.* 2008, Bravo-Sagua *et al.* 2013). Similarly, prolonged ER stress disrupts cellular metabolism and mitochondrial functions, which also eventually leads to apoptosis (Gupta *et al.* 2010, Bravo *et al.* 2012). Thus, in the present study, we show that apomorphine induced intrinsic apoptosis through mitochondrial dysfunction and ER stress in choriocarcinoma cells.

Paclitaxel is a member of the taxane family and had been used for the treatment of high-risk choriocarcinoma in combination with other chemotherapeutic agents (Marth *et al.* 1995, Joshua *et al.* 2004). However, paclitaxel possesses side effects, especially in pregnant patients, in whom it could cause birth defects (Berveiller & Mir 2012). Thus, combining paclitaxel with other chemotherapeutic agents can not only minimize its side effects because of its dose reduction, but also maximize the anticancer effects due to the combination of drugs. Therefore, our observations on the antiproliferative effects of apomorphine in combination with paclitaxel suggest a new therapeutic strategy to treat chemoresistant choriocarcinoma.

In the current study, we investigated apomorphine as an anticancer agent for choriocarcinoma cells, as illustrated in Fig. 7. Apomorphine showed antiproliferative effects on JEG3 and JAR cells and inhibited spheroid formation, a simulation of *in vivo* antiproliferative activity. Apomorphine caused mitochondria-mediated intrinsic apoptosis in choriocarcinoma via mitochondrial dysfunction and energy deprivation. We also demonstrated a reduction in the levels of anti-apoptotic proteins and induction of ER stress. Moreover, we confirmed synergistic effects of apomorphine in combination with paclitaxel, which could be used to treat chemoresistant choriocarcinoma. Taken together,

our findings suggest that apomorphine is a novel anticancer agent against human choriocarcinoma cells and set the stage for drug repositioning of apomorphine for choriocarcinoma treatment.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

G S, W L and J Y L conceived and designed the culture experiments, the cell culture methodology, and all other experiments; J Y L and J H collected experimental samples and conducted all experiments; J Y L, W L, and G S analysed, interpreted the data and contributed to the development of the manuscript. All authors contributed to its critical review and agreed on the final version.

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