REPRODUCTIVE ENDOCRINOLOGY AND INFERTILITY

Impaired steroidogenesis and apoptosis of granulosa-luteal cells in primary culture induced by cis-platinum

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OBJECTIVE: The purpose of this study was to test the hypothesis that the cytotoxic drug cis-platinum (CP) induces premature ovarian failure by reducing the viability of human granulosa cells.

STUDY DESIGN: We incubated cultured human granulosa-luteal cells (GLCs) with varying concentrations of CP for 48 hours. Steroidogenesis and apoptosis were assessed by progesterone and estradiol, annexin V/propidium iodide, phase contrast, and transmission electron microscopy.

RESULTS: CP caused impaired production of progesterone and estradiol in a dose- and a time-dependent fashion. The estradiol production was more pronounced than progesterone for each concentration of CP that was studied. The phase contrast microscopy of CP-treated GLCs showed loss of cell number with condensed nuclei. CP-induced apoptosis was maximum at 20 μg/mL compared with a 10-μg/mL concentration (79.9% $\pm$ 4.6% vs 58.3% $\pm$ 3.9%; $P < .01$). The hallmark of apoptosis (ie, nuclear condensation, cell size shrinkage) was seen in CP-treated cells by transmission electron microscopy.

CONCLUSION: CP induces apoptosis of human GLCs in culture with impaired steroidogenesis, which may be one mechanism by which a CP-containing regime induces premature ovarian failure.

Key words: apoptosis, cytotoxic drug, granulosa-luteal cell, premature ovarian failure, steroidogenesis

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Cis-platinum, cisplatin, or *cis*-diamminedichloroplatinum (CP) is a cytotoxic drug that is used widely as a part of multiagent high-dose chemotherapy regimen for the treatment of a variety of gynecologic malignancies (especially ovarian cancer) and refractory non-Hodgkin's lymphoma with good survival figures.[1,2] However, use of a CP-based regime is associated with long-term side-effects that include ovarian toxicity, with infertility, sexual dysfunction, and osteoporosis because of premature ovarian failure.[3,4]

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Although it is well-known that most chemotherapeutic agents such as CP damage malignant cells by apoptosis,[4] it is impossible to predict the lifespan of the chemotherapeutically damaged ovary because of dynamicity of the disease and the waxing and waning of ovarian reserve from multiagent chemotherapy (ovarian insufficiency, resistant ovarian syndrome, premature ovarian failure as we showed in another model of hematologic malignancy).[5]

Such information is important because granulosa cells are the somatic cells that nourish, provide metabolic support, and participate in intrafollicular communication with their accompanying germ cell (oocyte). The granulosa cells in response to endocrine, paracrine, and autocrine factors proliferate to differentiate and to luteinize. Further, cell cycle regulators have been shown to be crucial for normal fertility. This makes it a unique model to study effect of cytotoxic drugs in vitro.

The exact mechanism of damage of nonmalignant ovarian tissue by CP is still unclear. In mouse models, CP-induced follicular loss because of oocyte damage which was reported recently.[6,7] A number of studies in humans have examined ovarian histologic condition after chemotherapy. The most common observation has been ovarian atrophy with reduced follicle stores.[8,9] Similar changes are expected with CP-based regimen. But in those studies, the exact mechanisms of chemotherapy-induced damage to granulosa cells by CP have not been evaluated.

Although some investigators have used primary cells of animals, this approach demands costly, labor-intensive isolation procedures and yields cells with inconsistent functional capacity. Another caveat is the lack of similarities in phenotype to in vivo cells. Some of the literature on CP and granulosa cells include studies in chickens,[10] mice,[11] and rats.[12] Yoshida et al[13] have shown that CP can induce apoptosis by modulation of Bcl-2/Bax expression of human granulosa cells that were transformed by stable transfection with mutant p53 plus Ha-ras.[14] A recent study showed that, unlike other chemotherapeutic agents, CP causes apoptosis of oocytes by oxidative stress and the c-Abl pathway in mouse model.[15] However, this study did not address the effect of CP on steroidogenesis or gametogenesis in normal granulosa cells.

We previously developed and validated an in vitro model of human granulosa cell in culture, which was shown to be morphologically and functionally viable with intact steroidogenesis.[16] This in vitro system will permit dissection of gamatogenic from steroidogenic apparatus and is likely to improve understanding of the dynamics of cytosol and steroidogenesis (a predictor of menstrual function).

In this study, we aimed to investigate the effect of CP on human granulosa cells in culture to test the hypothesis that CP induces damage to the ovarian cells by apoptosis, which in turn can cause germ cell (oocyte) death. The results of the study are likely to have far reaching implications in understanding the exact mechanism of CP damage to the ovary and provide translational research to formulate the safest regimen for cancer treatment without compromising survival rates.

PATIENTS AND METHODS

Granulosa-luteal cell culture

Granulosa-luteal cells (GLCs) were isolated from the follicular fluid that was obtained from women who were undergoing ovarian stimulation for in vitro fertilization (IVF) for infertility. Details of the superovulation protocol that we used has been described previously.[16] This was obtained from patients who were undergoing a standard IVF protocol for unexplained or male factor infertility. Patients underwent pituitary down-regulation for 3 weeks with the use of a gonadotropin-releasing hormone analogue followed by 7-10 days of ovarian stimulation with high-purity follicle-stimulating hormone. Once there was a sufficient ovulatory response, patients were administered human chorionic gonadotropin for 36 hours after which any follicle >10 mm in diameter was aspirated under ultrasound guidance. Granulosa lutein cells were obtained from follicular fluid after oocytes were retrieved by ultrasound-guided needle aspiration. The assisted conception unit at University College London Hospitals has a blanket approval by the local research ethics committee for the collection of human follicular fluids and their use for research (Ref no: 03/0156).

The GLCs in the follicular fluid were disaggregated by being passed 30 times through a 25-gauge needle. The disaggregated GLCs were centrifuged through Ficoll-Paque (Amersham-Pharmacia Biotech, St. Albans, Herts, UK) and sedimented at the interface with the mononuclear cells. Contaminating blood cells were removed with immunomagnetic beads, which led to unacceptable losses of GLCs. As part of the purification procedure, the Ficoll-purified cells were cultured overnight on plastic slides in RPMI-1640 medium that contained 10% (volume/volume) fetal calf serum (Life Sciences, Paisley, Scotland, UK). These cells adhered to the plastic within 2 hours and became different morphologically in that they flattened and extended processes. To assess their purity, slides of each sample before and after purification were prepared by cytocentrifugation and stained with May-Grunwald-Giemsa stain. The GLCs were larger than the contaminating blood cells, were granular in appearance, and tended to form clumps. GLCs of >90% purity were used in the experiments that will be described later. Cells were counted with the use of the Trypan blue exclusion method on a hemocytometer. The mean viability of cells was approximately 95%.[16]

Experimental protocol

The purified GLCs were plated out into 96-well plates at a density of 5×10^4 cells per well in 2 mL of RPMI-1640 culture medium that was supplemented with 10% (volume/volume) fetal calf serum. The cells were maintained in culture for 48 hours with 5% CO_2 and 20% O_2 at 37°C in a humidified incubator. Experiments were performed by incubating cells in triplicate with (1) culture media alone (control) and (2) cells with various concentrations of CP (0.01 μg/mL, 0.1 μg/mL, 0.10 μg/mL, 20 μg/mL). Functional viability of the cells during the experiment period was evaluated by incubation of the cells with 10 mmol/L of pregnenolone and testosterone compared with controls (cells with culture media only). The concentrations of the CP that were used in this study encompassed the expected plasma concentrations that were likely to be achieved in clinical practice after the administration of the recommended dose of 50 mg to 120 mg/m^2 of body surface area of CP. Each experiment was repeated a minimum of 5 times. Aliquots of 100 μL were removed at 12 hour-intervals to measure progesterone and estradiol.

Measurement of estradiol and progesterone

The concentrations of estradiol and progesterone in the cell culture media were measured by radioimmunoassay with the use of tritiated tracers (New England Nuclear Life Science Products, Hounslow, UK) and highly specific sheep anti-estradiol and sheep anti-progesterone antisera (Bioclin Services International, Helsinki, Finland). All samples were assayed in the same batch; the intraassay variation was <5%.

Determination of apoptosis

Annexin V−propidium oxide detection in flow cytometry

Early apoptotic cells are recognizable through reversible binding of fluorescein-conjugated Annexin V to phosphatidylserine (a membrane phospholipid normally present in the inner membrane leaflet) that is transported to the cell surface early in the apoptotic process. It is fluorescent in the fluorecein conjugate form and emits green light at 523 nm. Propidium iodide (PI) binds to DNA by intercalating between the bases as a homodimer. It is actively excluded from live cells, but late apoptotic/necrotic cells are unable to actively exclude the dye, which enters the cell nucleus. The dimerization of PI results in a highly fluorescent entity, brightly staining these cells.

A commercial kit (human Annexin V-fluorescein isothiocyanate [FITC] Kit; Bender MedSystem, Vienna, Austria) was used. The purified GLCs were prepared for flow cytometry at time 0 and at the end of the experiment. Briefly, the cells were lifted off the plates, washed with phosphate-buffered saline solution and were stained with Annexin V-FITC and PI (1 μg/mL) labeling solution at