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Chemotherapy-Related Structural Changes in Cancer: Effect of GnRH Antagonist in the Ovarian Follicles

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Abstract

Objectives: The adverse effect of chemotherapy on the proliferation of granulosa cells has been indicated in recent studies. Gonadotropin-releasing hormone (GnRH) antagonists exert protective effects on granulosa cells against the side effects of chemotherapy. In the present study, we aimed to evaluate the impact of cetrorelix on the proliferation of ovarian granulosa cells following administration of thiotepa in the ovaries of female mice.

Materials and Methods: In this experimental study, 30 adult Balb/c female mice (5-8 weeks old, weighing 24-28 g) were divided into three groups (n=10/ each group) (Control group, T. group, and C. group). T. group received 2.5 mg/kg of thiotepa for four consecutive days. The C. group received cetrorelix (0.25 mg/kg) before and at the same time as thiotepa administration and a week after the end of thiotepa administration. Ovaries were used for quantitative and immunohistochemical studies at the end of the investigation.

Results: The mean numbers of follicles such as primordial, primary, secondary, and tertiary significantly decreased in the T. group than control group ($P=0.02$). Cetrorelix treatment exerted a protective effect against thiotepa-induced damage by increasing the mean numbers of follicles in the ovarian cortex ($P=0.04$).

Conclusions: As a GnRH antagonist, cetrorelix can be considered as one of the effective drugs to protect the granulosa cells against chemotherapy-induced damages in cancer disease.

Keywords: Chemotherapy, Gonadotropin-releasing hormone, Follicle-stimulating hormone, Luteinizing hormone, Thiotepa.

Introduction

Infertility is a phenomenon that has many individual and social complications. Multiple factors affect the process of spermatogenesis or ovogenesis in men and women, respectively. Among the factors, drugs used to treat cancer are of great importance. Chemo drugs have been shown to exert adverse effects on fertility in humans and laboratory animals. thiophosphoramide (thiotepa), a chemical drug, is widely used to treat cancerous tumors such as bladder, ovarian, and breast cancers (1,2).

Gonadotropin-releasing hormone (GnRH), a decapeptide, is responsible for producing and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the pituitary gland. GnRH antagonists can inhibit the effect of GnRH on gonadotropic cells by occupying GnRH receptors and ultimately inhibit the secretion of gonadotropins from these cells (3). GnRH antagonists have been used for many years to minimize the harmful effects of radiation therapy and chemotherapy on spermatogenesis as well as oogenesis. Cetrotide, a synthesized decapeptide, is known to inhibit the secretion of LH and FSH through binding to surface receptors of gonadotropin cells (4). Administration of GnRH antagonists in female mice receiving radiotherapy or anti-cancer drugs has been shown to reduce the destructive effects of anti-cancer drugs on the reproductive system and accelerate the return to normal condition. There are reports of adverse effects of anti-cancer drugs on cell proliferation in the reproductive system (5).

There are few studies about the role of GnRH antagonists in the cancer treatment. In the present study, we investigated the effects of cetrorelix (GnRH antagonist) on histological changes of ovarian follicles in thiotepa-treated female mice. In addition, we evaluated the proliferation of granulosa cells in animals after administration of thiotepa and cetrorelix.

Materials and Methods

In this experimental study, 30 female BALB/c mice (5-8 weeks old, weighing 24-28 g) were prepared from Applied Pharmaceutical Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. The experiment was performed from 15th March 2020 for two months until the end of May 2020 in Drug Applied Research Center, Tabriz University of Medical, Sciences, Tabriz, Iran. Animals were kept under 22-24oC for 12 hours light/dark cycle. Mice were randomly divided into three groups (n=10/each): control, T. group, and C. group. The control group only received the vehicle. The T. group received 2.5 mg/kg of thiotepa (Sigma, Germany) for four consecutive

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Key Messages

- Cetrorelix ameliorate thiotepa-induced histological changes in the ovarian follicles.

days, intraperitoneally (6). The C. group received 0.25 mg/kg cetrorelix (Serono, Germany), subcutaneously, one week before thiotepa (2.5 mg/kg) injection, at the same time, and one week after the end of it, for 3 weeks (5). Of note, thiotepa was administered only in the second week of the experiment. Two weeks after the last injection, the mice were anesthetized, their ovaries were removed and used for morphometric and immunohistochemical studies. The right ovary was used for immunohistological evaluation, and the left ovary was used for follicular number evaluation.

Morphometric Study

Left ovarian samples were taken to assess the number of follicles. The right ovary was also used to evaluate the proliferation of granulosa cells using kits related to the Ki-67 protein kit (BD Biosciences). Briefly, 5-µ tissues were prepared and stained with hematoxylin-eosin after tissue fixation. At least five sections of each tissue sample were randomly selected, and at least three fields of each section were examined under a light microscope (Olympus BH-2, Tokyo, Japan) (15 fields of each sample) (7). To prevent recounting, only follicles with specific nuclei were counted. In the present study, primordial follicles were considered to contain a layer of broad, squamous granulosa cells surrounding the oocyte. Primary follicles were considered follicles containing a layer of cubic granulosa cells an oocyte larger than the previous stage. Finally, the prenatal follicles were considered follicles containing three or more layers of granulosa cells. Normal follicles contain healthy, one-handed oocytes surrounded by normal granulosa cells, while abnormal follicles contain pycnotic oocytes, wrinkled cytoplasm, and irregular granulosa cells.

Immunohistochemical Studies

Ovarian tissue sections (about 3 µ thicknesses) fixed with formaldehyde. Ki67 antigen kit was used to detect proliferation (8). Ki67 is one of the nuclear proteins that appeared in stages M, G2, S, and G1. The background was then stained with hematoxylin-eosin. Using the kit, the nuclei of granulosa cells and ovarian stromal cells turned brown during the mitotic division; other nuclei became blue hematoxylin without the sign of mitotic division.

Statistical Analysis

The results were described as mean ± scanning electron microscope (SEM). Also, one-way analysis of variance (ANOVA) with Tukey's comparison test of the Statistical Package for the Social Sciences software (SPSS, version 23.0 for Windows; SPSS Inc., Chicago, IL) was used to determine the differences between the groups. A P value less than 0.05 was considered significant.

Results

Light and Electron Microscopy Evaluation

A. Histological Study

The light microscopic study revealed normal oogenesis and different types of follicles, including primordial, growing, and graphs in ovarian tissue in the control group. Corpus luteum was detected in some ovarian follicles sections (Figure 1a-c). Electron microscopic study showed that granulosa cells were arranged regularly without ruptures between them (Figure 1d). Light microscopic examination in the T. group showed ruptures in cortical ovarian cells and granulosa cells disruption (Figure 2a). Electron microscopic study showed that granulosa cells were separated from each other (Figure 2b). There were also several degenerated granulosa cells (Figure 2c). Likewise, an electron microscopic study showed a ruptured area between granulosa cells (S) in the ovarian cortex (Figure

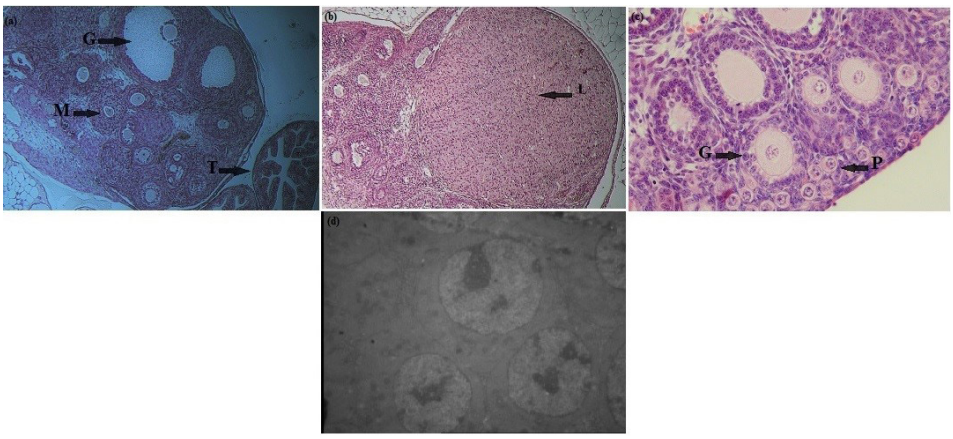

Figure 1. Ovarian Cortex and Follicles in the Control Group. (a) Photomicrograph of an ovarian section: Graafian follicle (G), Growing follicle (M), Uterine tube (T), ×400 (H & E), (b) Photomicrograph of an ovarian section: Corpus luteum (L), ×550, (H & E), (c) Photomicrograph of an ovarian section: Primordial follicle (P), Primary follicle (G), ×700, (H & E), (d) Electron micrograph of an ovarian section. Not the attachment of granulosa cells (G) ×2784.

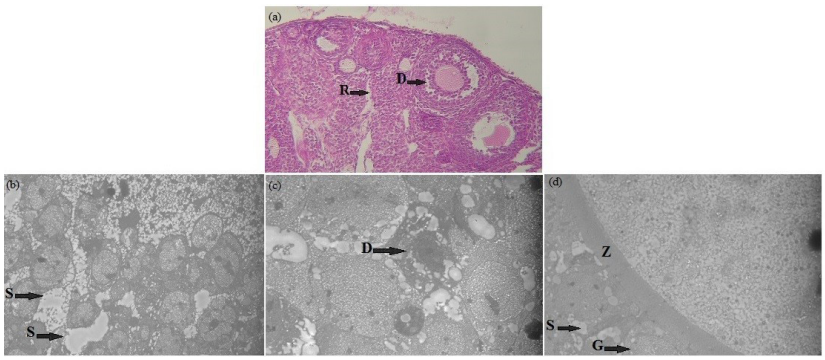

Figure 2. Ovarian Cortex and Follicles in Thiotepa-Treated Mice (T. group). (a) Photomicrograph of an ovarian section in the control group. Note Ruptures among cortical ovarian cells (R) and disruption on granulosa cells (D), ×750, (H & E), (b) Electron micrograph of an ovarian section in the control group: Note the separation of granulosa cells (S), ×1650, (c) Electron micrograph of an ovarian section: Note the degeneration of granulosa cell (D), ×2600, (d) Electron micrograph of an ovarian section in cetrorelix-treated mice (C. group). Not some ruptured area between granulosa cells (S), Granulosa cell (G), Zona pellucida (Z), ×2780.

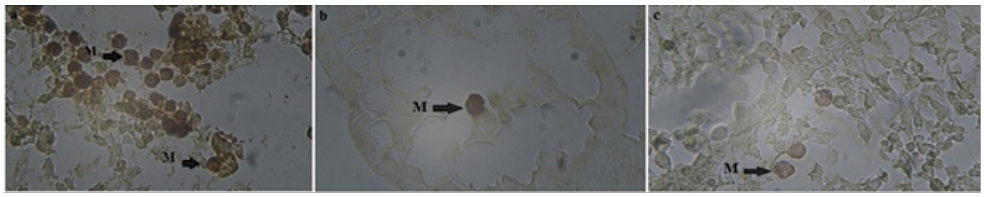

Figure 3. Ovarian Section in Control Mice (IHC staining), Photomicrograph of Ovarian Sections With IHC Staining. (a) Mitotic entering cells using ki 67- kit in control, ×750, (b) Mitotic entering cells using ki 67-kit in Thiotepa- treated mice (T. group), ×1650, (c) Mitotic entering cells using ki 67- kit in cetrorelix-treated mice (C. group), ×2600.

2d). In the C. group, a group receiving thiotepa + cetrorelix study with LM, ovarian tissue was similar to the control group. However, EM studies revealed still some rupture between the granulosa cells (Figure 3).

B: Morphometric Study

Morphometric evaluation of ovarian tissue revealed a significant reduction in the mean number of primordial, primary, secondary, and tertiary follicles in the T. group compared to the control ($P=0.02$). Cetrorelix treatment meaningfully increased the mean number of primordial, primary, secondary, and tertiary follicles in the C. group compared to the T. group ($P=0.04$) (Table 1).

C: Immunohistochemical Study

In histochemical evaluation using Ki 67 kit, the nuclei of granulosa cells entering mitosis turn brown, while cells not present in mitosis become colorless. The mean number of mitotic cells significantly decreased in the T. group compared with the control ($P=0.01$) (Figure 3b). Cetrorelix administration meaningfully increased the mean number of mitotic cells in the C. group in comparison to the T. group (Figure 3c) ($P=0.04$) (Table 1).

Discussion

This study describes the effect of cetrorelix on ovarian follicles in thiotepa-treated mice. In our research, thiotepa administration meaningfully reduced the number of primordial, primary, secondary, and tertiary follicles in the ovaries of female mice. Significantly, cetrorelix reversed these side effects of thiotepa administration in ovarian follicles. The activity of each ovary depends on the

Table 1. The Viability of Follicles in Different Stages of Experimental Groups

Variations	Control Group	T. Group	C. Group
Primordial follicles	1184 ± 21.590	692 ± 18.70*	1033 ± 12. 24**
Primary follicles	241.60 ± 26.26	202.50 ± 25.41*	231.10 ± 30.08**
Secondary follicles	58.60 ± 14.14	39.70 ± 7.32*	55.40 ± 13.32**
Tertiary follicles	42.10 ± 7.29	19.90 ± 2.92*	38.10 ± 7.29**
Cells entered in mitosis	9.1 ± 2.38	3.1 ± 1.52*	7.2 ± 1.99**

Data are presented as the means ± SEM. * $P < 0.05$ versus control group; ** $P < 0.05$ versus T. group.
T. group: Thiotepa-treated group; C. group: Cetrorelix-treated.

follicular reserve and the population of primordial follicles preserved. Granulosa cells, the dominant somatic cell of follicles, are the main cells involved in folliculogenesis and steroidogenesis. The side effects of cancer treatment on the reproductive system are well established. Among other factors, radiation therapy and chemotherapy are suggested to have several side effects on ovarian structure and function.

Chemo drugs, especially alkylating agents, affect rapidly dividing cells such as granulosa and hematopoietic stem cells (9). Accordingly, chemotherapy is recognized as an effective way to induce severe damage in ovarian tissue by reducing the number of primordial follicles, causing vascular and stromal damage and follicular atresia (10). Treatment with busulfan and cyclophosphamide, as chemotherapeutic agents, has been shown to reduce the content of follicles in the ovary of female rats (11). As a chemo drug, cyclophosphamide has been reported to reduce mitotic division in rapidly dividing cells, such as endometrial cells. The experimental study of cyclophosphamide administration has shown a significant reduction in the storage of ovarian follicles in a dose-dependent manner (12). Thiotepa, an alkylating agent, is widely used to treat various types of cancer. It has been suggested that ovarian damage in thiotepa-treated samples is time and dose-dependent. It has been reported that discernable destruction in the ovarian coat in *Agrotis ipsilon* larvae. They have also indicated that ovarian cell degeneration increases proportionally with increasing doses of thiotepa and the course of thiotepa treatment (13). The main mechanism of ovarian cell damage in chemotherapy is not fully understood. Chemo drugs induce apoptosis in granulosa cells. After chemo drug administration, laboratory studies on ovarian follicles have detected significant cell apoptosis in granulosa cells (14).

Thiotepa exerts apoptotic effects through DNA alkylation, causing cytotoxic DNA–DNA cross-links (15) and preventing suitable DNA replication. The majority of normal cells in the body are quiescent and live in the G1/G0 of cell phase; hence, chemo drugs selectively damage rapidly dividing tumor cells (16). It should be noted that long-term treatment with chemotherapy agents leads to potential apoptosis in rapidly dividing cells, resulting in damage in rapidly dividing normal cells (17). It has been demonstrated that mitotic granulosa cells are more sensitive to chemotherapy than non- mitotic granulosa cells. They proposed dose-dependent chemotherapy-induced apoptosis in human and rat granulosa cells (18). Consistent with the recent studies, we have observed a potential reduction in mitosis in granulosa cells and hyperchromicity of the nucleus in the follicular cells of mice treated with thiotepa. Also, the number of primordial, primary, secondary, and tertiary follicles in the ovary of thiotepa treated mice was significantly reduced compared to control mice.

Some similarly necessary treatments may minimize ovarian damage in women undergoing chemotherapy. The ideal treatment to prevent chemotherapy-induced infertility is medication. Decreased gonadotropin secretion and decreased ovarian function may prevent the side effects of chemotherapy. Previous studies have suggested that gonadotrophin-releasing hormone analogs (GnRH-a) may preserve fertility and are considered an effective concomitant treatment with chemotherapy (19). GnRH-a administration has been found to reduce the rate of follicular decline and the number of follicles lost during chemotherapy in parallel with cyclophosphamide (19). Pulsatile GnRH secretion induces the secretion of FSH and LH hormones. However, continuous administration of GnRH has been found to induce desensitization in pituitary gonadotropic cells. Also, the administration of a competitive GnRH antagonist is known to occupy pituitary receptors, causing desensitization in gonadotropic cells. In addition to its hypophysiotropic action, GnRH acts as a modulator in many cell types, including lymphoid and granulosa cells (20, 21).

Cetrorelix has been shown to have an anti-proliferative effect on granulosa cells. Administration of cetrorelix has been indicated to exert significant inhibition of ovarian tumor growth (22). In ovarian granulosa cells, GnRH acts as an autocrine growth factor in ovarian cancer (23). Taylor and colleagues demonstrated a significant reduction in the proliferation of granulosa and theca cells after Antarelix (GnRH antagonist) administration (24). Moreover, Meirow et al have reported that primordial follicle destruction due to cetrorelix treatment was decreased in cyclophosphamide treated young mice (25). Consistent with the previous works, we found less follicular damage in cetrorelix-treated mice than in the thiotepa- treated group. Administration of cetrorelix exerted a protective effect on the follicles of mice treated with thiotepa. The number of primordial, primary, secondary, and tertiary follicles was higher in cetrorelix-treated animals than in thiotepa- treated mice. Also, the mean number of cells entered in the mitotic division was higher in the C. group than the T. group. In our study, cetrorelix administration preserved ovarian follicles during the administration of chemo drugs. This finding suggests the protective effects of cetrorelix against chemotherapy-induced damage to the ovarian follicles. However, evaluation of cetrorelix therapy in other cancer treatments needs further evaluation.

Limitations of the Study

To determine the exact treatment mechanism with cetrorelix, we can evaluate the cellular level of GnRH receptors and the molecular levels of inflammatory and apoptotic markers in granulosa cells. However, it requires more costs. In future studies, the molecular mechanism of treatment of cetrorelix could be performed on granulosa and theca cells using various techniques such as western blotting and polymerase chain reaction (PCR).

Conclusions

In our study, cetrorelix administration preserved ovarian follicles during the administration of chemo drugs by preserving primordia, primary, and secondary follicles in the ovary. Also, cetrorelix treatment ameliorated the destruction of granulosa cells affected by thiotepa administration in ovarian tissues.

Authors' Contribution

DM and AH designed the study and conducted the research. FD monitored, evaluated, and analyzed the result of the study. Further, DM and AH reviewed the article. All authors approved the final manuscript and took responsibility for the integrity of the data.

Conflict of Interests

Authors declare that they have no conflict of interests.

Ethical Issues

This study was approved by the ethics committee of Tabriz University of Medical Sciences, Tabriz, Iran (Code: R.TBZMED.REC.1398. 854). .

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