Effects of Paclitaxel and Cisplatin on Ovarian Reserves in Rats

Paklitaksel ve Sisplatinin Ratlarda Over Rezervi Üzerine Olan Etkileri

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Abstract
Purpose: We targeted to investigate the effects of paclitaxel and cisplatin treatments, both solely and in combination with each other, on ovarian reserves in rats.
Materials and methods: Forty female Wistar albino rats were divided into 4 groups as to include 10 rats in each. While 7.5 mg/kg paclitaxel was given to group II, 5mg/kg cisplatin was administered to group III, 7.5 mg/kg paclitaxel and 5 mg/kg cisplatin combination therapy was applied to group IV via intraperitoneal route in a sterile way at single dose. Group I, which was assigned to be the control group, was subjected to intraperitoneal sterile saline in equal volumes. One week after the chemotherapy, ovaries of all the rats were oophorectomized. Hematoxylin-eosin stain was used to determine the follicles.
Results: Number of primordial, primary, and secondary follicles were found to show a statistically significant decrease ($p<0.001$). Most significant drop in number of follicles was determined to be in group 4.
Conclusion: Single and combined use of paclitaxel and cisplatin caused a decrease in number of primordial, primary, secondary, and tertiary follicles. Most significant drop regarding the number of follicles, was found to be in the combined chemotherapy group. Therefore, single-agent chemotherapy regimes may be considered as an alternative for patients asking for fertility.
Key Words: **Cisplatin; Ovarian follicles; Paclitaxel.**

Özet
Amaç: Paklitaksel ve sisplatinin tek başına ve kombine olarak uygulanmasının ratlarda over reservi üzerine olan etkilerini araştırmak amaçlandı.
Gereç ve Yöntemler: Wistar albino cinsi 40 adet dişi rat her grup 10 rat içerecek şekilde 4 gruba ayrıldı. Grup 2'ye 7.5 mg/kg paklitaksel, grup 3'e 5mg/kg sisplatin, grup 4 ise 7.5 mg/kg paklitaksel ve 5 mg/kg sisplatin kombine olarak steril şekilde intraperitoneal tek doz olarak uygulandı. Kontrol grubu olan grup 1'e ise eşit volümde steril serum fizyolojik intraperitoneal olarak uygulandı. Kemoterapiden 1 hafta sonra tüm ratlar ooferektomize edilerek overleri çıkarıldı. Hematoksilen ve eozin boyası folliküller tespit etmek için kullanıldı.
Bulgular: Kontrol grubuna göre tedavi alan gruplarda primordial, primer, sekonder follikül sayılarında istatistiksel olarak anlamlı derecede azalma tespit edildi. ($p<0.001$) Follikül sayılarında en fazla azalma dördüncü grupta tespit edildi.
Sonuç: Paklitaksel ve sisplatinin tek ve kombine uygulanması primordial, primer, sekonder ve tersiyer follikül sayılarında azalmaya neden olmuştur. Follikül sayılarında en fazla azalma kombine kemoterapi grubunda saptanmıştır. Bu nedenle fertilite isteği olan hastalarda tek ajanlı kemoterapi rejimleri alternatif olarak düşünülebilir.

Anahtar kelimeler: **Ovaryan folliküller; Paklitaksel; Sisplatin.**

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Introduction

As a result of the alterations in chemotherapy protocols and combined use of chemotherapeutic agents, average survival durations of cancer patients began to prolong. Use of chemotherapeutic agents at high doses and in combination therapies, may lead to complications such as amenorrhoea, premature menopause, and infertility by exhibiting a long term negative influence on ovarian reserve of cancer patients within reproductive period. While destroying the tumoral tissues, chemotherapeutic agents also cause damage over multiplying germinal epithelium of cells such as ovary. Whereas the damage inflicted by cytotoxic agents on rapidly multiplying cells such as included in bone marrow and thymus, is reversible; the damage induced over primordial follicles which do not have the ability to regenerate, is of progressive and irreversible character. Thus, a damage on ovarian follicles inevitably leads to premature ovarian insufficiency and infertility. Reproductive performance is not important in determining ovarian damage taking place following chemotherapy. Most important parameter is known to be the number of primordial follicles forming a significant portion of the follicular pool (1).

Alkylator agents such as cyclophosphamide and chlorambucil are the best known chemotherapeutics which have a cytotoxic effect on ovaries (2,3). There is little data in the literature on possible cytotoxic effects of paclitaxel (which is used in treatment of breast, lung, or lymphoma tumors) and cisplatin (which is applied in treatment of breast, lymphoma, bladder, and lung tumors) on ovary. Predicting cytotoxic influence of paclitaxel and cisplatin, which are also used as first-line treatment for adjuvant chemotherapy of ovarian cancer, particularly on patients subjected to fertility-preserving surgery along with sparing of at least one ovary, bears importance. In the present study, we aimed to investigate the effects of single or combined use of paclitaxel and cisplatin on ovarian reserve in rats.

Materials and Methods

The present study has been carried out after the approval of ethics committee. Forty mature wistar albino rats, each varying between 200-250g, were included in the study. The rats were kept in cages which were subjected to 12-hour light period and constant ventilation at 19±1C^{o} temperature alongside 50-60% mean moisture level. Rats were fed with atlibutum method. Rats were divided into 4 groups: 1 control group (Group I) and 3 study groups (Group II, III, IV). Group I was subjected to intraperitoneal saline. While 7.5 mg/kg single dose paclitaxel (Anzatax, Orna, Australia) was administered to Group II, 5mg/kg cisplatin (Cisplatin, Orna, Australia) was applied to the Group III, and 7.5mg/kg single dose paclitaxel and 5mg/kg cisplatin combination therapy was carried out in Group IV. Same volumes of sterile saline was given via intraperitoneal route to the control group (Group I). Anesthesia was established by combined intraperitoneal administration of 60mg/kg ketamine (Ketalar, Eczacibasi, Istanbul, Turkey) and 10mg/kg xylazine (Rompum 2%, Bayer, Germany). Following the removal of ovaries under anesthesia, rats were sacrificed by cervical dislocation.

In the present study, chemotherapeutic agents were applied to all the groups except the control group. Duration of the estrus cycle of the rats was recognized as 5 days and in order to evaluate the influence of chemotherapeutic agents over follicles, ovaries were planned to be removed 1 week after the chemotherapy. All the rats were oophorectomized 1 week after the chemotherapy. Removed ovaries were kept in Bouin solution for approximately 24 hours and paraffin blocks were prepared. Follicular toxicity was evaluated in a similar fashion to the previous studies (4) by carrying out morphometric analyses on 5 sections obtained in a randomized way from each ovary. All the sections were evaluated by a single investigator who didn't know the groups.

While the most important parameter for determining the ovarian reserve is known to be the number of primordial follicles, by considering that mature follicles have a better sensitivity against chemotherapy compared to the primordial follicles; primordial follicles, primary follicles, secondary follicles, and tertiary follicles in the obtained sections were counted seperately in this study. Follicles in the histological preparations were identified as follows:

Primordial follicle. Localized just below the cortex and wrapped within a simple squamous epithelium on basal lamina.

Primary follicle. By losing their simple squamous epithelium structure, follicle cells first form a simple cuboidal layer and as a result of eventual multiplication, generate 2 or 3 layered granulosa cell structure.

Secondary follicle (Antral follicle). Following the determination of antrum in histological examination, follicle is considered as a secondary follicle.

Tertiary follicle (Graaf follicle). The structure turns into a vesicle and join with the cell group referred to as cumulus oophorus. Follicle diameter reachs to 10-15mm. ANOVA test was used for statistical evaluation of the study results including the comparison of control and study groups. Tukey test was applied for evaluating the differences between groups. The power of the study was determined to be 0.99.

Results

Throughout the whole study, no complication associated with the applied chemotherapeutic agents, was detected in rats. The study was ended with 40 rats. Number of follicles and statistical results for the control and study groups are shown in Table-1.

The number primordial follicles after chemotherapy, was found to show a statistically significant reduction in study groups compared to the control group. The highest drop in number of primordial follicles was observed in group IV where paclitaxel and cisplatin were used together as a combination therapy (control: 11.6 ± 2.7; group IV: 3.1 ±1.4). No statistically significant difference was detected in terms of decrease in number of primordial follicles between paclitaxel group and cisplatin group ($p>0.05$). The paclitaxel + cisplatin combination therapy group exhibited a significant reduction in number of primordial follicles compared to those of single-agent paclitaxel and cisplatin groups ($p<0.05$).

A statistically significant drop was determined in the number of primary follicles in study groups compared to those of the control group. Cisplatin group was found to have the most affected number of primary follicles (Control 11.6 ± 2.7; Group III 2.4±0.5). There was no statistically significant difference in terms of the reduction in number of primary follicles between the paclitaxel group and the cisplatin group ($p>0.05$). The group of paclitaxel + cisplatin combination therapy, was not found to exhibit a statistically significant difference regarding reduction in the number of primary follicles compared to the groups including single use of cisplatin and paclitaxel.

The number of secondary follicles in study groups, showed statistically significant drops compared to that of control group. Most affected group in terms of the number of secondary follicles, was the paclitaxel + cisplatin combination group (Control: 11.6 ± 2.7; Group IV: 2.4 ±0.6; Table I). No statistically significant difference was found between paclitaxel group and the cisplatin group regarding decrease in the number of secondery follicles ($p>0.05$). Combination therapy of paclitaxel + cisplatin did not show any statistically significant drop compared to single-agent paclitaxel and cisplatin groups in terms of the number of decrease in secondry follicles ($p>0.05$, Table I).

A statistically significant amount of drop was determined in the number of tertiary follicles in paclitaxel+cisplatin combination therapy group ($p<0.001$). Number of tertiary follicles in paclitaxel and cisplatin groups were similar to that of control group (Control 4.5±2.2; Group II: 3.6±1.3; Group III: 3.7±0.9; Table I). No statistically significant difference was detected between paclitaxel group and cisplatin group in terms of the number of tertiary follicles ($p>0.05$). Combination use of paclitaxel + cisplatin, was found to reduce the number of tertiary follicles in higher amounts compared to the reductions in single-agent paclitaxel or cisplatin therapies ($p<0.05$, Table I).

Table I. Comparison of number of follicles between control group and the study groups

	Grup 1	Grup 2	Grup 3	Grup 4	F Value
PMF	11.6 ± 2.7	8.3 ± 1.4[a]	6.9±1.1 [a]	3.1 ± 1.4 [a,b,c]	37.702
PF	5.9 ± 2.4	4.0 ± 1.1 [a]	2.4±0.5 [a]	2.8 ± 1.3 [a]	10.470
SF	5,7 ± 2,6	4.1 ± 0.8 [a]	3.2±0.4 [a]	2.4 ± 0.6 [a]	9.616
TF	4.5 ± 2.2	3.6 ± 1.3	3.7±0.9	1.8 ± 1.3 [a,b,c]	5.954

PMF: Primordial follicle PF:Primary follicle SF: Secondary follicle TF: Tertiary follicle Group I: Control Group II: Paclitaxel Group III: Cisplatin Group IV: Paclitaxel and cisplatin groups
a.Different from Group I ($p<0.05$); b.Different from Group II ($p<0.05$); c.Different from Group III ($p<0.05$).

Discussion

Premature ovarian insufficiency occuring due to chemotherapy, depends on age, types of the used chematherapeutic agents, application dose and form. Application duration and route are known to be independent factors (5,6,7). Thus, high-dose and combined usage of alkylator chemotherapeutic agents among patients younger than 40 age, inflicts the highest damage on ovary.

Particularly in young patients, losses in follicular pool may be tolerated because of good ovarian reserve. In those patients, ovarian functions may not be affected within a short period of time following chemotherapy. However, on long-term, premature ovarian insufficiency is an inevitable outcome.

While the exact reason leading to a reduction in number of follicles alongside chemotherapy is unknown, many mechanisms have been proposed to explain the cytotoxicity occuring after chemotherapy. Most important of those is known to be apoptosis of oocytes. In vitro studies showed paclitaxel inducing apoptosis on granulosa cells, the most important component of follicles, and inflicting destruction over primordial follicles (8). Moreover, paclitaxel causes a delay in meiotic maturation of mature oocytes and spindle defects, leading to formation of aneuploid oocytes and a reduction in number of ovarian follicles (9). On the other hand, alkylator agents show their cytotoxic affects by causing an irreversible damage on granulosa cells which are known to be the most important component of developing follicles, particularly primordial follicles (10). Histologic studies showed that chemotherapy leads to; luminal stenosis by causing diffuse hyalinization in vessel walls, ovarian atrophy by inflicting diffuse cortical fibrosis, and significantly reduced number of primordial follicles (11). Regular menstruation periods, achievement of pregnancy, and normal hormone profile after chemotherapy, are parameters indirectly related to ovarian functions. Determination of those parameters within normal range, do not necessarily indicate inefficient therapy or non-affected ovarian reserves. The most important parameter for detecting ovarian damage is known to be the number of primordial follicles.

Paclitaxel is a chemotherapeutic agent commonly used in treatment of breast, ovarian, and lung cancer. Recently, it has been started to be successfully applied in treatment of cancers occuring at early ages such as lymphoma and germ cell tumors. Exhibits its antitumor function via microtubules. Binds to microtubules with a high affinity and prevents them from depolarization and binding to each other by stabilization. Thus, starts apoptosis by way of preventing cell division, proliferation, and migration (8). Paclitaxel starts apoptosis at low doses and prevents cell growth at high doses. It is combined with agents containing platinium and applied in treatment of ovarian cancer as a first-line chemotherapy.

Cisplatin is one of the first generation platinium compounds and is commonly used for treatment of ovarian, breast, testicular, and bladder cancers. Cisplatin binds covalently to guanine and adenine on DNA and thus prevents transcription and DNA replication (12). At the same time, exhibits anti-tumoral activity by increasing toxic influence and apoptosis as a result of binding with nuclear and cytoplasmic proteins within the cell (13).

Most determinative parameter for ovarian reserve is the number of primordial follicles rather than reproductive performance. However, in the present study, considering the fact that mature follicles show higher sensitivity against chemotherapy compared to the primordial follicles, cytotoxic effects of chemotherapeutic agents over both the primordial and the mature follicles, were evaluated by seperately counting the primordial, primary, secondary, and tertiaryfollicles in the histological sections.

Yucebilgin et al. applied paclitaxel and cisplatin on rats in one of their studies and found the number of primordial follicles significantly low in study group compared to that of controls (14). In the present study, we found the number of primordial follicles in single-agent cisplatin and paclitaxel groups significanly lower than that of control group. No statistically significant difference was determined between paclitaxel and cisplatin groups regarding the reduction capacity for number of primordial follicles. The influences of cisplatin + paclitaxel combination therapy over primordial follicles, has been investigated for the first time by this study in the literature. The number of primordial follicles in the combination therapy group was lower than those of single-agent cisplatin and paclitaxel groups.

In the present study, the effects of single or combined usage of paclitaxel and cisplatin over mature follicles, has been investigated for the first time. Number of primary and secondary follicles in single-agent or cisplatin + paclitaxel combination groups, were found to be lower than that of control group. While single-agent paclitaxel or cisplatin groups showed a close number of tertiary follicles compared to that of control group, combination therapy group exhibited a significantly lower value compared to that of control group. Generally, single or combined use of paclitaxel and cisplatin, shows toxic effect on mature follicles and decrease the number of follicles.

The results obtained from the present study indicate a higher drop in number of tertiary follicles for the combination therapy group than those of single-agent chemotherapeutic groups. While the number of tertiary follicles is not important for commenting on how it directly affects ovarian reserve (15), we believe that statistical difference might have occured due to induction effect as a result of increased follicular development caused by chemotherapeutic agents delivered in single-agent drug groups. However, because there is no clear data for neither cisplatin nor paclitaxel regarding the induction effect on ovary, we think this should be a subject of further investigation.

In conclusion, single or combined use of paclitaxel and cisplatin, reduces the number of primordial follicles which constitute a considerable portion of ovarian reserve. This reduction appears to be lower in single-agent therapies compared to that of treatment including combination therapy. Therefore, both single-agent and combination chemotherapies are expected to exhibit a negative influence on ovarian reserve in early stage ovarian cancer patients who asking for fertility and subjected to organ preserving surgery , and on cases of malignant diseases such as breast cancer, leukemia, or lymphoma arising during reproductive period.

Because our results indicated that ovarian reserve is affected at a higher level in combination therapy compared to the single-agent regimes, selection of single-agent chemotherapy as an alternative approach may be considered in patients asking for fertility. Moreover, in order to prevent the cytotoxic effect of chemotherapy on ovary and preserve the fertility performance of patients to receive cancer treatment, presence of several treatment modalities should be known and presented to the attention of the patients. Multiplication and storage (by freezing) of oocyte, embryo, and ovarian tissues before chemotherapy, and applying ovarian suppresion by gonadotropin releasing hormone (GnRH) analogs during chemotherapy, appear to be the most significant preservative options.

Kaynaklar

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