Assessment of the Effects of Chemotherapy on Ovarian Reserve in Rats

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OBJECTIVE: To determine the effect of cyclophosphamide and cisplatin on ovarian reserve in rats by the way of counting primordial follicle and assessing TNF-alpha, 8-OH deguanosine and oxidant/ antioxidant status.

STUDY DESIGN: Thirty Wistar rats were divided into three groups. Cisplatin was administered at a dose of 5 mg/kg (Cisplatin group, n=10), cyclophosphamide was administered at a dose of 6 mg/kg (Cyclophosphamide group, n=10) and control group was only given sterile saline (Control group, n=10) in Ege University Faculty of Medicine. All injections were administered intraperitoneally at a single dose. At the end of the 7th day blood samples were drawn by intracardiac aspiration and bilateral extirpation of ovaries were performed. The number of primordial follicles, levels of TNF-alpha, 8-OH deoxyguanosine, malondialdehyde (MDA) and catalase enzyme activities were evaluated.

RESULTS: The number of primordial follicles in the control group, cisplatin group and cyclophosphamide group were 27.5±4.7;17.3±2.1;17±1.4, respectively (p=0.156). While the levels of TNF-alpha were significantly higher in cisplatin group (p<0,001), 8-OH deoxyguanosine levels did not have significant difference among three groups (p=0.431). The levels of MDA were significantly lower compared to the other groups (p<0.001); however, the catalase enzyme activities did not show statistically significant difference.

CONCLUSIONS: Insignificant depletion of primordial follicles was observed by using specified doses of chemotherapeutics. An increase in TNF-alpha and MDA levels may play a role in the development of ovarian tissue damage.

Key Words: Chemotherapy, Ovarian reserve, DNA damage, Oxidative stress, Rats

Gynecol Obstet Reprod Med 2014;20:29-33

Introduction

Recently chemotherapies used in cancers of reproductive period achieve high chance of cure and long lifetime. As a result of cell damage, depletion of ovarian reserve may be logical when we consider the mechanism of action in chemotherapy especially for patients desiring fertility after chemotherapy.

Cisplatin and cyclophosphamide are the chemotherapeutic agents which can be used alone or in combined therapies. Cisplatin is used in the treatment of head and neck cancers, germ cell tumors, bladder, breast and lung carcinomas. On the other hand, cyclophosphamide is a multi-action drug which can be used both in the treatment of cancers and autoimmune diseases such as vasculitis, Behcet's disease, systemic lupus erythematosus, Sjögren, scleroderma and rheumatoid arthritis.[1]

In the previous animal studies based on the effects of cisplatin on germ cell damage, it was pointed out that both germ cells and Sertoli cells were damaged and germ cell damage was indirectly related to the Sertoli cell damage.[2] Also it was reported that cisplatin has nephrotoxicity and cyclophosphamide has scarification effects both on the bladder and rarely in the lungs.[3,4]

It was proved both in vivo and in vitro studies that cis-

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Submitted for Publication: *12. 11. 2013*
Accepted for Publication: *07. 01. 2014*

platin and cyclophosphamide has an influence of increment for active oxygen radicals.[5,6] It was shown that as a result of lipid peroxidation in such tissues, levels of malondialdehyde (MDA), protective enzymes and antioxidant substances had been changed. Formation of active oxygen radicals may be resulted in damage in cells and through some mechanisms of membrane lipid peroxidation it may cause necrosis, protein denaturation and DNA damage.[7]

8-OH DG (8-OH deguanosine) is used to determine the DNA damage which arise after being exposed to carcinogens. Previously it was used in many studies to determine the endogenous oxidative DNA damage.[8,9]

In the reproductive system; endometriosis, polycystic ovary syndrome, tubal obstruction, preeclampsia and recurrent abortions are related to the presence of inflammatory cytokines (TNF-alpha, IFN-gamma, IL-1) and high levels of free radicals damaging biological molecules, such as lipids, proteins or DNA.[10]

In the present study, we aimed to determine the effect of cisplatin and cyclophosphamide administration on ovarian reserve by counting primordial follicle and assessing the levels of TNF-alpha, 8-OH DG (known as a DNA damage marker) and oxidant/antioxidant markers.

Material and Method

This animal model study was approved by the Animal Ethics Committee of Ege University, Izmir, Turkey. Thirty Swiss Albino female rats weighing between 130-150 g were divided into three groups and to each group, cisplatin (Orna Ilac, David Bull Laboratories, Victoria, Australia) (n=10), cyclophosphamide (Endoxan, Eczacıbası Baxter, Turkey) (n=10) and sterile saline (n=10) was administered intraperitonealy at single doses of 5 mg/kg, 6 mg/kg and 5 ml, respectively. Seven days after the administration of drugs, rats were oopherectomized bilaterally and the blood samples were drawn via intracardiac route.

Pathological examination

The mean duration of rat oestrus cycle is 4 days. To assess the effects of the chemotherapeutic agents on primordial follicles, rats were oopherectomised 7 days after administration of the chemotherapeutic agents. Both ovaries were fixed in 10% formalin and embedded in paraffin. Serial sections were prepared and stained with haematoxylin and eosin to determine the number of primordial follicles. Primordial follicle toxicity is adequately defined by counting five random sections.[11] The primordial follicles are located near the outer surface of the ovary and distinguished by a single layer of flattened follicle cells.

Preparation of the Hemolysates

Blood samples were drawn into EDTA-containing tubes, and then plasma and erythrocyte lyzates (ELs) were prepared. After removal of plasma as aliquotes, the packed erythrocytes were washed two times with 0.9 g/L NaCl solution and hemolyzed in ice-cold distilled water (1/5, v/v). EL-MDA levels and EL-CAT activities were determined immediately in hemolysates.

Determination of MDA levels

MDA levels were measured spectrophotometrically by Yagi's method.[12] MDA couples to thiobarbituric acid to form pink chromogen compound, which has a maximum absorbance at 532 nm wavelength. Hemolysate MDA levels were expressed as micromoles per gram hemoglobin (µmol/g Hb).

Determination of Catalase (CAT) activities

Catalase activities were determined as defined by Aebi et al [13] in which the degradation of peroxide is recorded spectrophotometrically at 240 nm. One unit of catalase was defined as the amount of enzyme, which decomposes 1 µmol H_2O_2/min under specific conditions.

TNF- alpha

TNF-alpha was assessed with Invitrogen rat ELISA kit.

8-OH dg

8-OHdG-EIA (Bioxytech®) kit, a competitive enzyme-linked immunosorbent assay (ELISA) for quantitative measurement of 8 hydroxy-2'-deguanosine in plasma resulting from oxidative damage to DNA was used. The results were given as ng/ml.

Statistics

The data was expressed as mean ± Standard deviation. The differences in the mean number of primordial follicles, TNF-Alpha, 8-OH dG, MDA and CAT were compared by Kruskal Wallis test using Statistical Package for Social Sciences software (SSPS,version 18.0). $P<0.05$ was accepted as significant.

Results

After counting primordial follicles, mean number of primordial follicles in control, cisplatin and cyclophosphamide groups were found 27,5±4,7; 17,3±2,1; 17±1,4 respectively (p=0,156) (Figure 1). Although TNF–Alpha level was significantly higher in cisplatin group (p<0,001), it was at the minimum level in control and cyclophosphamide groups. There was no significant difference in 8-OH dG levels among three groups (p=0,431). In the cisplatin group, MDA levels were significantly lower (p=0,001) however catalase enzyme activity was insignificant compared to the other groups (p=0,09) (Table 1).

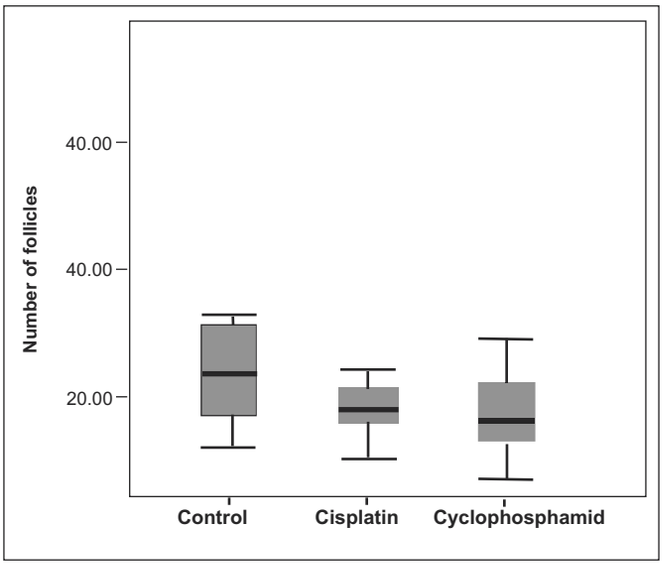

Figure 1: Box-plot graphical analysis of the mean number of primordial follicles at the end of the 7th day in three study groups

Discussion

Ovarian responses against chemotherapy are varied by multiple factors such as patient's age, type of chemotherapeutic agent and dose.[14] In older patients, primordial follicle reserve may be totally depleted after chemotherapy. Patient who are willing to preserve fertility after chemotherapy, pre-treatment GnRH agonists adjustment or embryo cryopreservation are the most proposed choices of treatment.[15,16] Cyclicity of menses after chemotherapy does not predict the ovarian reserve and exclude ovarian damage. Meirow et al.[17] observed the effects of cyclophosphamide on ovarian tissue through primordial follicle counting and they found that depletion of ovarian follicle reserve was related to dose dependent fashion. Also in this study investigators suggested that counting the number of primordial follicles were more accurate in determination of ovarian damage than reproductive performance. There have been many studies counting primordial follicles after administration of paclitaxel and cisplatin.[18,19] In these studies Gucer et al[18] suggested that increasing doses of paclitaxel causes depletion of ovarian reserve and 7,5 mg/kg, the highest dose of study, results in depletion of primordial follicles by 36%. Apart from this study Yucebilgin et al.[19] also found similar results and they suggested that both paclitaxel and cisplatin cause depletion of ovarian reserve. In the current study; although the number of primordial follicles in chemotherapy groups are less than the control group, results are not statistically significant.

Relationship between female reproductive system, antioxidants and reactive oxygen metabolites draw higher attention in recent years. These substances have such roles in many physiological processes as oocyte maturation, fertilization, embryo development and pregnancy.[20] Yang et al.[21] found that levels of H_2O_2 (hydrogen peroxide) were higher in fragmented embryos than in non fragmented embryos, and Paszkowski et al.[22] suggested that consumption of antioxidants were much more in poor quality embryos. Recent data was all compatible with the studies that observe induction of human oocyte apoptosis through oxidative stress.[23] Tamura et al.[24] found that concentrations of intrafollicular 8-OH dG were much more in women who has high rate of degenerated oocytes than who has lower rate of degenerated oocytes.

Both oxygen molecules and cytokines are in interaction with each other. Rupture of follicle also occurs through these molecules over the ovarian epithelium. More exposure, chronic inflammation as a result of repeated ovulation may have a role in ovarian cancer.[25] There may be an imbalance between oxidative stress and antioxidant system in some diseases after inflammation.[26,27] In our study; although we could not demonstrate any clinical evidence of cell damage, levels of TNF-alpha were found to be significantly higher in cisplatin group. Cell damage though reactive oxygen metabolites may arise after repetitive treatments and higher doses.

In the present study, even though number of primordial follicles were lower in chemotherapy groups, it was not statistically significant. In previous studies, investigators found that ovarian follicle reserve depletes through chemotherapy. The reason of discordance may be due to the absence of pretreatment primordial follicle count, single and poor choice of

Table 1: Number of primordial follicles, TNF-alfa, 8-OHdg, MDA levels and CAT enzyme activities in three study groups

	Control group (n=10)	Cisplatin Group (n=10)	Cyclophosphamide Group (n=10)	P Value
Number of primordial follicles	27.5±4.7	17.3±2.1	17±1.4	p=0.156
TNF- alfa (pg/ml)	0.001	3.11±1.7	0.001	p=0.001
8-OH dg (ng/ml)	1.43±0.74	1.82±1.08	1.83±0.82	p=0.431
MDA (nmol/g)	16.82±4.7	8.95±2.45	19.2±5.8	p=0.001
CAT (U/g)	6331.8±1028	5280.6±1436	6248.9±2647	p=0.098

CAT: Catalase, MDA: Maloniyldialdehyde, TNF: Tumour necrosis factor, 8- OH dg: 8 hydroxy-2'-deguanosine

chemotherapy doses as well as limited number of days (7 days) after injection that did not result in enough cell damage and increase in oxidative stress.

We concluded that short term of cisplatin and cyclophosphamide treatments in specified doses do not cause cell damage through oxidative stress and DNA damage and above all; it does not affect ovarian reserve significantly. We need more comprehensive studies to find out the effects of these chemotherapeutics on ovarian reserve consisting of repetitive or higher doses and also the relationship between cisplatin and inflammatory cell damage should be proved by long-term clinical investigations.

Ratlarda Kemoterapinin Over Rezervi Üzerine Etkilerinin Değerlendirilmesi

AMAÇ: Ratlarda siklofosfamid ve sisplatinin over rezervi üzerine etkisini; primordial follikül sayısı, oksidan/anti-oksidan durum, TNF-alfa ve 8-OH deguanozin ölçümleri ile değerlerlendirmektir.

GEREÇ VE YÖNTEM: Otuz Wistar rat üç gruba ayrılmıştır. Çalışma Ege Üniversitesi Tıp Fakültesi'nde yapılmıştır. Sisplatin 5 mg/kg dozda (sisplatin grup, n=10), siklofosfamid 6 mg/kg dozda (siklofosfamid grup, n=10) ve kontrol grubuna sadece steril salin (kontrol grup, n=10) uygulanmıştır. Tüm enjeksiyonlar tek doz ve intraperitoneal olarak uygulanmıştır. Yedinci günün sonunda intrakardiyak yoldan kan örnekleri alınmış ve her iki over çıkarılmıştır. Primordial follikül sayısı, TNF-alfa, 8-OH deoksiguanozin, malondialdehit (MDA) ve katalaz enzim aktivite düzeyleri değerlendirilmiştir.

BULGULAR: Primordial follikül sayısı kontrol grubunda, sisplatin grubunda ve siklofosfamid grubunda sırasıyla 27,5±4,7; 17,3±2,1; 17±1,4 idi (p=0,156). TNF-alfa düzeyi sisplatin grubunda anlamlı olarak yüksek olmasına rağmen (p<0,001), 8-OH deoksiguanozin düzeyinde üç grup arasında anlamlı farklılık yoktu (p=0,431). Diğer gruplar ile karşılaştırıldığında, sisplatin grubunda MDA düzeyi anlamlı olarak düşük (p<0,001) iken katalaz enzim aktivitesi istatiksel anlamlı farklılık göstermemekteydi.

SONUÇ: Belirtilen dozlarda kullanılan kemoterapötik ilaçlarda az düzeyde primordial folliküllerde azalma görülmüştür. Artmış TNF-alfa ve MDA düzeyleri, over dokusu hasarı gelişmesinde rol oynayabilir.

Anahtar Kelimeler: Kemoterapi, Over rezervi, DNA hasarı, Oksidatif stres, Ratlar

References

1. Guillevin L. Advances in the treatments of systemic vasculitides. Clin Rev Allergy Immunol 2008;35:72-8.
2. Boekelheide K. Mechanisms of toxic damage to spermatogenesis. J Natl Cancer Inst Monogr 2005;1:6-8
3. Mora, LO, Antunes, L.M, Francescato, HD, Bianchi, ML. The effects of oral glutamine on cisplatin-induced nephrotoxicity in rats. Pharmacol Res 2003;47:517-22.
4. Datta K, Chin A, Ahmed T, et al. Mixed effects of 2,6-dithiopurine against cyclophosphamide mediated bladder and lung toxicity in mice. Toxicology 1998;125:1-11
5. Naziroğlu M, Karaoğlu A, Aksoy AO. Selenium and high dose vitamin E administration protects cisplatin-induced oxidative damage to renal, liver and lens tissues in rats. Toxicology 2004;195:221-30.
6. Tsai-Turton M, Luong BT, Tan Y, Luderer U. Cyclophosphamide-induced apoptosis in COV434 human granulosa cells involves oxidative stress and glutathione depletion. Toxicol Sci 2007;98:216-30.
7. Kehrer, JP. Free radicals as mediators of tissue injury and disease. Crit Rev Toxicol 1993;23:21-48
8. Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 2009;27:120-39.
9. Alper G, Irer S, Duman E, Caglayan O, Yilmaz C. Effect of I-deprenyl and gliclazide on oxidant stress/antioxidant status and dna damage in a diabetic rat model Endocr Res. 2005;31:199-212.
10. Iborra A, Palacio JR, Martinez P. Oxidative stress and autoimmune response in the infertile woman. Chem Immunol Allergy 2005;88:150-62.
11. Smith BJ. Comparison of random and serial sections in assessment of ovarian toxicity. Reprod Toxicol 1991;5:379-83.
12. Yagi, K. A simple fluorometric assay for lipoperoxide in blood plasma. Biochem Res 1976;15:212-216
13. Aebi, H. E. Catalase in: Bergmeyer, H. U (Ed.): Methods of enzymatic analysis 1986;3:273-86
14. Sanders JE, Hawley J, Levy W, Gooley T, Buckner CD, Deeg HJ. Pregnancies following high-dose cyclophosphamide with or without high-dose busulfan or total-body irradiation and bone marrow transplantation. Blood 1996;87:3045-52.
15. Lemos CN, Reis FM, Pena GN, Silveira LC, Camargos AF. Assessment of fertility protection and ovarian reserve with GnRH antagonist in rats undergoing chemotherapy with cyclophosphamide. Reprod Biol Endocrinol 2010; 18:51.
16. Oktem O, Oktay K. Fertility preservation for breast cancer patients. Semin Reprod Med 2009;27:486-92.
17. Meirow D, Lewis H, Nugent D, Epstein M. Subclinical depletion of primordial follicular reserve in mice treated

with cyclophosphamide: clinical importance and proposed accurate investigative tool. Hum Reprod 1999;14:1903-7.

18. Gucer F, Balkanli-Kaplan P, Doganay L et al. Effect of paclitaxel on primordial follicular reserve in mice. Fertil Steril 2001;76:628-9
19. Yucebilgin MS, Terek MC, Ozsaran A, Akercan F, Zekioglu O, Isik E, Erhan Y. Effect of chemotherapy on primordial follicular reserve of rat: an animal model of premature ovarian failure and infertility Aust N Z J Obstet Gynaecol 2004;44:6-9.
20. Agarwal A, Gupta S, Sharma RK Role of oxidative stress in female reproduction Reprod Med Biol 2005;4:31-44.
21. Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW, Oh KS. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Hum Reprod 1998;13:998-1002.
22. Paszkowski T, Clarke RN. Antioxidative capacity of preimplantation embryo culture medium declines following the incubation of poor quality embryos. Hum Reprod 1996; 11:2493-2495
23. Zhang X, Li XH, Ma X, Wang ZH, Lu S, Guo YL. Redoxinduced apoptosis of human oocytes in resting follicles in vitro. J Soc Gynecol Investig 2006;13:451-458.
24. Tamura H, Takasaki A, Miwa I, et al. Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. J Pineal Res 2008;44:280-7.
25. Murdoch WJ, Martinchick JF: Oxidative damage to DNA of ovarian surface epithelial cells affected by ovulation: carcinogenic implication and chemoprevention. Exp Biol Med 2004;229:546-52.
26. D'Aiuto F, Nibali L, Parkar M, Patel K, Suvan J, Donos N. Oxidative stress, systemic inflammation, and severe periodontitis. J Dent Res 2010;89:1241-6
27. Maes M. The cytokine hypothesis of depression: inflammation, oxidative & nitrosative stress and leaky gut as new targets for adjunctive treatments in depression. Neuro Endocrinol Lett 2008;29:287-91.