CURRENT THERAPEUTIC RESEARCH

VOLUME 72, NUMBER 2, APRIL 2011

In Vivo Evaluation of the Genotoxic Effects of Gonadotropins on Rat Reticulocytes

Bulent Duran, MD[1]; Onder Koc, MD[1]; Safak Ozdemirci, MD[2]; Ata Topcuoglu, MD[1]; and Ozturk Ozdemir, MD[3]

[1]Department of Obstetrics and Gynecology, Faculty of Medicine, Abant Izzet Baysal University, Bolu, Turkey; [2]Department of Obstetrics and Gynecology, Simav Government Hospital, Kütahya, Turkey; and [3]Department of Medical Genetics, Faculty of Medicine, Canakkale Onsekiz Mart University, Canakkale, Turkey

ABSTRACT

BACKGROUND: Gonadotropins, as ovulation-inducing drugs, have been used widely to treat infertility. An epidemiologic correlation between infertility therapy and ovarian cancer development has been reported. However, the effect of gonadotropins in the formation of reproductive tract cancers is controversial.

OBJECTIVE: The aim of the study was to determine the in vivo genotoxic effects of gonadotropins on rat reticulocytes.

METHODS: In this prospective, randomized, controlled study, rats were randomly assigned to 1 of 5 groups. The calculated rat doses of 0.65 human menopausal gonadotropin (hMG), 0.95 hMG, 0.65 follitropin beta (FB), 0.95 FB, or normal saline (control group) were injected, respectively. These calculated rat doses (U/g) are based on average human gonadotropin doses of 150 and 225 IU/d for a 70-kg woman given in 2-mL saline (the control group received 2 mL of saline). Injections were administered once per day for 5 days, followed by 5 days of rest. Each treatment was repeated for 6 estrus cycles in the rats for a total of 12 estrus cycles. Six months after the last day of the 12^{th} cycle, the rats were euthanized. Bone marrow tissues were removed, and pluripotent reticulocyte cells with micronuclei, nuclear buds, and binuclear abnormalities were analyzed using an in situ micronuclei assay under light microscopy. The proportion of micronucleated cells, cells with anaphase bridge, nuclear buds, and other nuclear abnormalities were measured.

RESULTS: The number of cells with nuclear buds and binuclear abnormalities in the hMG 225 and FB 225 groups was significantly higher ($P < 0.05$) than that from the hMG 150, FB 150, and control groups in the cytogenetic analysis of bone marrow stem cells. An increased rate of genotoxicity in all gonadotropin groups versus that of placebo was found.

CONCLUSION: In rats, the micronucleus genotoxicity assay suggests a dose-dependent gonadotropin effect on genomic instability in bone marrow stem cells in

Accepted for publication March 10, 2011.

doi:10.1016/j.curtheres.2011.03.003

0011-393X/$ - see front matter

vivo. (*Curr Ther Res Clin Exp.* 2011;72:60-70)

KEY WORDS: genotoxicity, gonadotropins, infertility, micronuclei, rat.

INTRODUCTION

Ovulation-inducing drugs have been used widely to treat infertility since the 1960s. Ovulation induction regimens are the most widespread treatment for ovulation disorders, which account for approximately 33% of female infertility cases.[1] Clomiphene citrate (CC), bromocriptine, gonadotropins, and gonadotropin-releasing hormone analogs are the treatment options for infertile women.[2] An epidemiologic correlation between infertility therapy and ovarian cancer development has been reported[3]; in particular, human menopausal gonadotropin (hMG) was reported to increase the risk of developing epithelial ovarian tumors[4]; hence, the possible role of gonadotropins in ovarian carcinogenesis has received much attention. The effect of gonadotropins on epithelial ovarian carcinoma by stimulating cell proliferation and/or inhibiting apoptosis in ovarian surface epithelium is advocated for the histogenetic origin of carcinomas.[5,6] These data suggest that gonadotropins play an important role in the development, progression, and chemoresistance of ovarian carcinomas.[5,6] Although several recent studies have proposed a connection between fertility drugs and ovarian cancer,[7–9] no strong association has been firmly established as addressed in some reports.[10–12]

Micronuclei are pieces of extranuclear chromatin that originate when chromosome fragments or lagging whole chromosomes fail to be incorporated into daughter nuclei as cells divide. The occurrence of micronuclei increases following the exposure to clastogenic agents that cause double-strand DNA breaks and to aneugens that discontinue chromosomal segregation.[13,14] The in vivo rodent micronucleus (MN) test is used widely to identify or study chemicals with genotoxic potential.[15–17] Numerous studies have reported on the incessant ovulation hypothesis[18] and oxidative damage products in ovarian surface epithelium DNA as contributors to the risk of developing cancer.[19–21] The genotoxic potential of ovulation and genomic integrity of the ovarian surface epithelium have been investigated.[22] However, no research was carried out to explore the genototoxic effects of gonadotropins on rodent MN.

The purpose of this study is to evaluate the genotoxic effects of gonadotropins on rat reticulocytes in situ MN assay.

MATERIALS AND METHODS

This study was conducted at Abant Izzet Baysal University (AIBU), Faculty of Medicine, between March 2008 and January 2009. The Committee of Animal Care and Use at AIBU approved the procedures, which are in line with the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals. The rats were kept in the laboratory for at least 4 weeks before the experiments. A total of 40 sexually mature, nontransgenic female Wistar albino rats (body weight, 220–250 g) at 20 weeks of age were assigned to 1 of 5 groups: 0.65 IU intramuscular hMG

(menogon low [ML] group [n = 8]); 0.95 IU intramuscular hMG (menogon high [MH] group [n = 8]); 0.65 IU subcutaneous follitropin beta (recombinant low [RL] group [n = 8]); 0.95 IU subcutaneous follitropin beta (recombinant high [RH] group [n = 8]); and control rats (saline group [n = 8]). Follitropin beta (Puregon) was provided by Organon Laboratories Limited (Cambridge, United Kingdom) and menogon was provided by Ferring Pharmaceuticals (Copenhagen, Denmark).

These calculated rat doses (units/gram) were based on average human gonadotropin doses of 150 to 225 IU/d for a 70-kg woman and were given in 2-mL saline for infertility; control rats received only 2 mL of subcutaneous saline.

Before the study, the estrus cycle was confirmed by vaginal cytology, and only rats with an estrus cycle of 5 days were included.[23] The rats were injected for 5 days once daily, beginning on the first estrus cycle day, which was followed by 5 days of rest (an unmedicated cycle) during 1 course of therapy; 6 cycles of gonadotropins were given in a total of 12 estrus cycles. All the animals were bred, fed, and watered ad libitum under standard laboratory conditions in separate cages for 6 months after the last day of the 12th cycle until the euthanization by cervical dislocation.

Micronuclei Analysis

Different researchers blind to the treatment groups assessed the results and evaluated an equal number of slides from unmarked slide boxes that the groups were allocated during the study. Bone marrow was prepared according to the method described by D'Souza et al[24] and Schmid.[14] First the femurs were removed from each animal; bone marrow stem cells from the control and experimental groups were removed using a thin brush. The MN assay was completed as recommended by standard procedures[25–29] with some modifications appropriate for in situ evaluation.[29,30] Briefly, after a few deep strokes into the stem cell tissue with a brush, the cells were spread on clean slides, which were then coded. After the last superficial stroke had been diluted with Hank's balanced salt solution, spread slides were prepared. Four slides were prepared for each group. After the stem cells in the control and experimental groups were stained with 5% Giemsa dye in Sorensen's buffer (pH 6.8), the slides were rinsed twice with fresh Sorensen's buffer and air dried. Subsequently, MN frequencies were scored in the youngest fraction of reticulocytes. The immature polychromatic erythrocytes were validated from mature normochromatic erythrocytes cells and evaluated as an in vivo rat model assay. The proportion of micronucleated erythrocytes and cells with other features—mononucleated, binucleated, anaphase bridges, or nuclear buds—were counted in 1000 cells per slide using a lighted microscope (Zeiss Axioskop 2 plus; Carl Zeiss MicroImaging, Thornwood, New York) equipped with a ×100 immersion objective (final magnification: ×1000) and a charge-coupled device camera. The polychromatic erythrocyte cells were scored by a cytogeneticist to determine the percentages of micronucleated cells, and to compare the control group rats in the current observer-blinded study.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences 11.5 software (SPSS Inc., Chicago, Illinois). The distributional properties of metric discrete variables were determined with the Shapiro Wilk test. Nonparametric data were expressed as median (minimum–maximum range), and the differences among groups were evaluated by using the Kruskal-Wallis test. When the P value from Kruskal-Wallis test statistics was found to be statistically significant, group differences were identified by nonparametric multiple comparison tests. $P < 0.05$ was considered statistically significant.

RESULTS

The distribution of nuclear abnormalities in the ML, MH, RL, RH, and control groups are presented in the **Table**. No cells with attached MN, binucleated cells with MN, cells with anaphase bridges, or cells with nuclear buds were observed in the control group. Apart from the control group, varying degrees of nuclear abnormalities were detected in the ML, MH, RL, and RH groups.

The mean (minimum–maximum range) of mononuclear cells in the ML (145 [120–180]) and RL (60 [50–80]) groups was significantly lower than that of the control group (235 [180–345]); $P = 0.021$ and $P < 0.001$, respectively (**Table**). More abnormalities for cells with attached MN were found in ML (10 [5–14]), MH (34 [20–50]), RL(10 [6–14]), and RH (8 [2–20]) groups compared with the control group (0 [0–1]), $P < 0.05$. However, the number of cells with attached MN in RL and RH groups was significantly lower than that in the MH group ($P < 0.01$). The number of binucleated cells with MN in ML (1 [0–3]), MH (3 [1–6]), and RH (2 [0–6]) groups was significantly higher than that of the control group (0 [0–1]); $P = 0.029$ for ML versus control, and $P < 0.001$ for MH and RH versus control, respectively. The number of binucleated cells with MN in the MH group was significantly higher than that in both the ML and RL groups (1 [0–1]); $P = 0.006$ and $P < 0.01$, respectively. Moreover, the number of cells with nuclear bud abnormalities was higher in ML (2 [0–4]), MH (1 [0–4]), RL (1 [0–2]), and RH (4 [2–6]) groups than in the control group (0 [0–1]); $P = 0.012$, $P = 0.004$, $P = 0.031$, and $P < 0.001$, respectively. The nuclear bud abnormalities were the highest in the RH group and were significantly higher than the ML, MH, and RL groups; $P = 0.002$, $P = 0.007$, and $P < 0.001$, respectively.

Furthermore, no significant changes in the number of cells with anaphase bridges were noted in any of the groups. Some abnormal bone marrow cell profiles (cells with nuclear buds, cells with micronuclei, cells with anaphase bridge abnormality) of the experimental group of rats are depicted in the **Figure**.

DISCUSSION

Exposure to different substances in daily life requires an alternative technique for monitoring personal gene damage. The evaluation of the genotoxic safety of drugs and other chemical agents is an extremely important aspect of toxicologic work in biological systems. The combined use of chemical analysis techniques (biomonitoring

Table. Distribution of nuclear abnormalities in reticulocyte cells in experimental (ML, MH, RL, and RH) and control groups.

	Group				
	Control	ML	MH	RL	RH
Cell Feature	Mean (min–max)	Mean (min–max)	Mean (min–max)	Mean (min–max)	Mean (min–max)
Mononuclear cell	235 (180–345)	145 (120–180)*	323 (120–450)†	60 (50–80)*†‡	183 (90–375)§
Cell with BN	2 (0–3)	15 (8–22)*	24 (18–35)*†	7 (3–10)*†‡	15 (4–35)*§
Cell with attached MN	0 (0–1)	10 (5–14)*	34 (20–50)*†	10 (6–14)*‡	8 (2–20)*‡
Binucleated cell with MN	0 (0–1)	1 (0–3)*	3 (1–6)*†	1 (0–1)‡	2 (0–6)*
Cell with AB	0 (0–0)	1 (0–2)	1 (0–5)	0 (0–2)	1 (0–2)
Cell with NB	0 (0–1)	2 (0–4)*	1 (0–4)*	1 (0–2)*	4 (2–6)*†‡§

AB = anaphase bridge; BN = binucleated cell; MH = menogon high; ML = menogon low; MN = micronucleus; NB = nuclear bud; RH = recombinant high; RL = recombinant low.

*$P < 0.05$ vs control.

†$P < 0.05$ vs ML.

‡$P < 0.01$ vs MH.

§$P < 0.05$ vs RL.

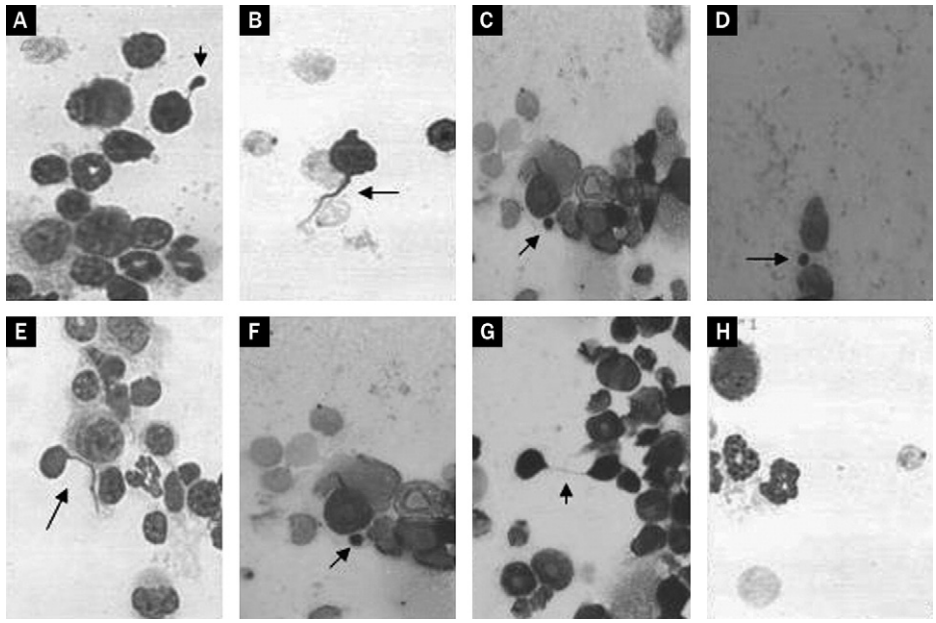

Figure. Some abnormal bone marrow cell profiles of experimental group rats. Arrows show the abnormal structures. A: Cell with nuclear bud; B and E: Cells with nuclear buds that derivated from anaphase bridge abnormalities; C, D and F: Cells with micronuclei; G: Cell with anaphase bridge abnormality; H: Normal cell.

assay) plays a crucial role in the identification of specific toxic and mutagenic damage caused by some environmental agents in the biological systems. In addition to biological samples such as blood, urine, and cerebrospinal fluid, bone marrow and solid tissues are used to evaluate the genotoxic and cytotoxic effects of agents. Because bone marrow is one of the incessantly proliferating tissues in adult animals, it has been preferred as a common target organ for cytogenetic studies. One of the most promising methods at present is the in situ MN assay that detects large chromosomal breakages in the early human biomonitoring system.[15–17] The rat bone marrow MN assay is a standard in vivo genotoxicity test that has been performed widely to assess the clastogenic potential of chemicals.[15–17] In the present study, a direct in situ MN assay technique was used to explore gonadotropin genotoxicity in a rat model. CC and gonadotropins are the most commonly used ovulation-inducing agents.[31–33]

The available data on the possible relationship between these agents and ovarian cancer are inconclusive, although theoretical considerations and clinical observations suggest such a relationship is likely. In addition, the association with fertility drugs, especially between gonadotropins and ovarian cancer, is supported by case reports and epidemiologic studies.[3,4,34–36] Research[37,38] supports the pituitary gonadotropin hypothesis[39] and mutagenic potential of gonadotropins. For example, Mertens-Walker et al[37] suggested that gonadotropins induce both ovarian cancer cell migra-

tion and proliferation by activation of extracellular signal-regulated kinase 1/2 signaling in a calcium-dependent and protein kinase C delta-dependent manner. A recent article reported use of cDNA microarray analysis on MCV152 cells with gonadotropin treatment, which revealed that gonadotropins promoted cell growth in ovarian surface epithelial cell lines.[38] Discordant with these reports, Ness et al[40] found no association between fertility drug use and the overall risk of ovarian cancer and concluded that neither duration nor unsuccessful use of fertility drugs was related to adjusted cancer risk. To the best of our knowledge, no experimental studies have investigated the genotoxic or cytotoxic effects of gonadotropins in rat in situ MN assay. In vivo erythrocyte MN assays in biological systems are extensively used to evaluate the potential genotoxic and cytotoxic effects of chemical and physical agents. Scoring of micronuclei has been proposed as an alternative to conventional chromosome aberrations analysis, because it is more sensitive and faster in eukaryotic cell transformation and tumorigenesis.[31,41,42] These assays are effective in detecting MN formation in target cell/tissue DNA material and indicate the genotoxicity and mutagenicity of such chemicals in biological systems.[43] Almost all agents that cause double-strand chromosome breaks (clastogens) induce micronuclei.[13,14] The induction of DNA damage and chromosomal breakages due to mutations is the primary mechanism by which cancers arises. In a previous study, no statistically significant difference was found between the CC and the control groups in terms of anaphase bridges and MN formation, which are strong indicators of genotoxicity.[30] However, statistically significant differences were observed between the CC and the control groups for nuclear buds and binuclear cells, which are weak indicators of genotoxicity. The results indicated that CC has a dose-dependent mutagenic effect on the nuclear genome of pluripotent stem cells in rats.[30]

In the current study, statistically significant differences were detected among all parameters of cell abnormalities and MN cells except for the anaphase bridge abnormality by using high doses of subcutaneous gonadotropins. Moreover, significant differences were seen in the high-dose hMG compared with low-dose hMG and control groups in term of cells with binucleated cells, cells with attached MN, and binucleated cells with MN. A statistically significant increase was monitored in the number of cells with nuclear buds and cells with MN in the high-dose follitropin beta group when compared with the low dose and control group rats.

The current results provide in vivo data from a rat model and suggest possible mutagenic effects of gonadotropins on rat reticulocytes. Results further suggest that the in situ evaluation of rat pluripotent stem cells in vivo works well as a method for accurately determining the genotoxic effects of gonadotropins. The validation efforts are based on the key role of in vivo rat MN testing in pharmaceutical safety assays, and mutagen sensitivity detected with the MN testing are also reported in various articles.[26–29,44] It is likely that hyperproliferative rat bone marrow stem cells are among the most sensitive targets for drugs such as gonadotropins and CC.[30] Based on the presented results here, it can be concluded that in vivo gonadotropin exposure serves as an important parameter for genomic instability in rat proliferative stem cells.

Furthermore, Chene et al[8] identified a relationship between ovarian epithelial dysplasia and ovulation-inducing drugs; Fathalla[18] described an incessant ovulation theory as well as the dose and the time effect of ovarian stimulation, which may be the possible explanation of this dysplasia.[18] In addition to dysplasia, peripheral blood lymphocytes exhibit DNA damage in most cases of cancer. The measurement of MN in peripheral blood lymphocytes is frequently used in molecular epidemiology as one of the preferred methods of assessing sporadic chromosomal damage resulting from environmental mutagen exposure; however, the current study puts forward the genetic evaluation of bone marrow reticulocytes.The MN assay has also been used to assess DNA damage before and after exposure to iodine-131 in thyroid cancer cases.[45] Results suggested that image analysis of MN can be used as a highly sensitive method of evaluating the effect of long-term gonadotropin use. In addition, it is observed that ovulation-inducing drugs are able to increase MN frequency in rat reticulocyte cells in vivo. The current method is acceptable for biomonitoring studies[46,47] focusing on the measurement of MN in assessing chromosomal damages as a result of environmental mutagens or medical applications.

The limitation of the current study could be stated as the possible influence of interindividual results due to the visual scoring system. An automated scoring system of slides for quicker and more reliable data acquisition that is not influenced by the interindividual and temporal variability of human scorers might enable consistent results to be obtained.

CONCLUSION

In conclusion, the data provide evidence that therapy with at least 6 cycles of gonadotropin could affect overall genomic instability in pluripotent reticulocytes (proliferative stem cells) and damage their DNA. These findings show increased basal DNA damage in high-dose gonadotropin-treated rat reticulocytes. Varying degrees of nuclear abnormalities were detected in ML, MH, RL, and RH groups compared with those in the control group. The study might play a precursor role for future human studies that will be performed using cytogenetic methods on MN, evaluating genotoxic effects of gonadotropin use for infertility treatment.

ACKNOWLEDGMENTS

The authors thank Salih Ergocen, MSc (Department of Biostatistics, Ankara University School of Medicine) for his contribution to the statistical analysis section of this study. The authors report no conflicts of interest. The authors also attest that this article has not been presented or published previously.

Dr. Duran was responsible for the protocol and project development, data collection and management, and data analysis. Dr. Koc was responsible for the protocol and project development, data collection and management, data analysis, and manuscript writing and editing. Dr. Ozdemirci was responsible for the data collection and management and data analysis. Dr. Topcuoglu was responsible for data collection and management. Dr. Ozdemir was reponsible for data collection and management, micronuclei testing, and figure creation.

REFERENCES

1. Franco C, Coppola S, Prosperi Porta R, Patella A. Ovulation induction and the risk of ovarian tumors. *Minerva Ginecol*. 2000;52:103–109.
2. Blacker CM. Ovulation stimulation and induction. *Endocrinol Metab Clin North Am.* 1992;21: 57–84.
3. Whittemore AS, Harris R, Itnyre J. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 us case control studies. II. Invasive epithelial ovarian cancers in white women. Collaborative Ovarian Cancer Group. *Am J Epidemiol.* 1992;136:1184–1203.
4. Shushan A, Paltiel O, Iscovich J, et al. Human menopausal gonadotropin and the risk of epithelial ovarian cancer. *Fertil Steril.* 1996;65:13–18.
5. Konishi I, Kuroda H, Mandai M. Gonadotropins and development of ovarian cancer. *Oncology*. 1999;57(Suppl 2):45–48.
6. Derman SG, Adashi EY. Adverse effects of fertility drugs. *Drug Saf.* 1994;11:408–421.
7. Celik C, Gezginc M, Aktan M, et al. Effects of ovulation induction on ovarian morphology: an animal study. *Int J Gynecol Cancer.* 2004;14:600–606.
8. Chene G, Penault-Llorca F, Bouedec LG, et al. Ovarian epithelial dysplasia after ovulation induction: time and dose effects. *Hum Reprod.* 2009;24:132–138.
9. Kosec V, Bukovic D, Grubisic G, Fures R. Ovarian cancer and ovulation induction drugs – is there a link? *Coll Antropol.* 1999;23:633–639.
10. Kashyap S, Davis OK. Ovarian cancer and fertility medications: a critical appraisal. *Semin Reprod Med.* 2003;21:65–71.
11. Hull ME, Kriner M, Schneider E, Maiman M. Ovarian cancer after successful ovulation induction: a case report. *J Reprod Med.* 1996;41:52–54.
12. Bristow RE, Karlan BY: Ovulation induction, infertility, and ovarian cancer risk. *Fertil Steril.* 1996;66:499–507.
13. Heddle J. A rapid in vivo test for chromosome damage. *Mutat Res.* 1973;18:187–190.
14. Schmid W. The micronucleus test. *Mutat Res.* 1975;31:9–15.
15. Hayashi M, MacGregor JT, Gatehouse DG, et al, for the In Vivo Micronucleus Assay Working Group, IWGT. In vivo erythrocyte micronucleus assay: III. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes with discussion of non-hematopoietic target cells and a single dose-level limit test. *Mutat Res.* 2007;627:10–30.
16. Organization for Economic Cooperation and Development (OECD). OECD guideline for the testing of chemicals. Mammalian erythrocyte micronucleus test. Guideline 474. July 1997. http://www.oecd-ilibrary.org/docserver/download/fulltext/9747401e.pdf?expires=1301368766&id=0000&accname=freeContent&checksum=5D5ACC0CFC7A9B1CA41D8D4F7319F2FC. Accessed March 28, 2011.
17. US Food and Drug Administration. Redbook 2000: IV.C.1.d Mammalian erythrocyte micronucleus test. July 2000. http://www.fda.gov/food/guidancecomplianceregulatoryinformation/guidancedocuments/foodingredientsandpackaging/redbook/ucm078338.htm. Accessed March 28, 2011.
18. Fathalla MF. Incessant ovulation—a factor in ovarian neoplasia? *Lancet*. 1971;2:163.
19. Marnett LJ. Oxyradicals and DNA damage. *Carcinogenesis*. 2000;21:361–370
20. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J.* 2003;17:1195–1214.
21. Valko M, Izakovic M, Mazur M, et al. Role of oxygen radicals in DNA damage and cancer incidence. *Molec Cell Biochem*. 2004;266:37–56.

22. Murdoch WJ. Carcinogenic potential of ovulatory genotoxicity. *Biol Reprod.* 2005;73:586–590.
23. Sharp PE, La Regina MC. *The Laboratory Rat.* Boca Raton, Fl: CRC Press; 1998.
24. D'Souza UJ, Zain A, Raju S. Genotoxic and cytotoxic effects in the bone marrow of rats exposed to a low dose of paraquat via the dermal route. *Mutat Res.* 2005;581:187–190.
25. Dobrzynska MM, Gajewski AK. Induction of micronuclei in mouse bone marrow after combined X-rays-cyclophosphamide and X-rays-mitomycin C treatments. *Teratog Carcinog Mutagen*. 1999;19:267–274.
26. Fenech M. The in vitro micronucleus technique. *Mutat Res.* 2000;455:81–95.
27. Fenech M, Chang WP, Kirsch-Volders M, et al, for the Human Micronnucleus project. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat Res.* 2003;534:65–75.
28. Abramsson-Zetterberg L, Grawe J, Zetterberg G. The micronucleus test in rat erythrocytes from bone marrow, spleen and peripheral blood: the response to low doses of ionizing radiation, cyclophosphamide and vincristine determined by flow cytometry. *Mutat Res.* 1999;423:113–124.
29. Coskun M, Coskun M, Cayir A, Ozdemir O. Frequencies of micronuclei (MNi), nucleoplasmic bridges (NPBs), and nuclear buds (NBUDs) in farmers exposed to pesticides in Canakkale, Turkey. *Environ Int.* 2011;37:93–96.
30. Duran B, Ozdemir I, Demirel Y, et al. In vivo evaluation of the genotoxic effects of clomiphene citrate on rat reticulocytes: a micronucleus genotoxicity. *Gynecol Obstet Invest.* 2006;61:228–231.
31. Terry V, Murphy CR, Shorey CD. Clomiphene citrate alters vaginal surface morphology in cycling rats. *Acta Anat (Basel).* 1992;145:212–215.
32. Kerin JF, Liu JH, Phillipou G, Yen SS. Evidence for a hypothalamic site of action of clomiphene citrate in women. *J Clin Endocrinol Metab.* 1985;61:265–268.
33. Mikkelson TJ, Kroboth PD, Cameron WJ, et al. Single-dose pharmacokinetics of clomiphene citrate in normal volunteers. *Fertil Steril.* 1986;46:392–396.
34. Rossing MA, Daling JR, Weiss NS, et al. Ovarian tumors in a cohort of infertile women. *New Engl J Med.* 1994;331:771–776.
35. Venn A, Watson L, Lumley J, et al. Breast and ovarian cancer incidence after infertility and in vitro fertilization. *Lancet*. 1995;346:995–1000.
36. Franceschi S, La Vecchia C, Negri E, et al. Fertility drugs and risk of epithelial ovarian cancer in Italy. *Hum Reprod.* 1994;9:1673–1675.
37. Mertens-Walker I, Bolitho C, Baxter RC, Marsh DJ. Gonadotropin-induced ovarian cancer cell migration and proliferation require extracellular signal-regulated kinase 1/2 activation regulated by calcium and protein kinase C{delta}. *Endocr Relat Cancer.* 2010;17:335–349.
38. Ji Q, Liu PI, Chen PK, Aoyama C. Follicle stimulating hormoneinduced growth promotion and gene expression profiles on ovarian surface epithelial cells. *Int J Cancer*. 2004;112:803–814.
39. Cramer DW, Welch WR. Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. *J Natl Cancer Inst.* 1983;71:717–721.
40. Ness RB, Cramer DW, Goodman MT, et al. Infertility, fertility drugs, and ovarian cancer: a pooled analysis of case-control studies. *Am J Epidemiol.* 2002;155:217–224.
41. Min W, Cortes U, Herceg Z, et al. Deletion of the nuclear isoform of poly(ADPribose) glycohydrolase (PARG) reveals its function in DNA repair, genomic stability and tumorigenesis. *Carcinogenesis.* 2010;31:2058–2065.

42. Fang CY, Lee CH, Wu CC, et al. Recurrent chemical reactivations of EBV promotes genome instability and enhances tumor progression of nasopharyngeal carcinoma cells. *Int J Cancer.* 2009;124:2016–2025.
43. Cândido Bacani PD, dos Reis MB, Serpeloni JM, et al. Mutagenicity and genotoxicity of isatin in mammalian cells in vivo. *Mutat Res.* 2011;719:47–51.
44. Speit G, Trenz K. Chromosomal mutagen sensitivity associated with mutations in BRCA genes. *Cytogenet Genome Res*. 2004;104:325–332.
45. Joseph LJ, Bhartiya US, Raut YS, et al. Micronuclei frequency in peripheral blood lymphocytes of thyroid cancer patients after radioiodine therapy and its relationship with metastasis. *Mutat Res.* 2009;675:35–40.
46. Von Tungeln LS, Churchwell MI, Doerge DR, et al. DNA adduct formation and induction of micronuclei and mutations in B6C3F1/Tk mice treated neonatally with acrylamide or glycidamide. *Int J Cancer.* 2009;124:2006–2015.
47. Verschaeve L, Juutilainen J, Lagroye I, et al. In vitro and in vivo genotoxicity of radiofrequency fields. *Mutat Res.* 2010;705:252–268.

Address correspondence to: Onder Koc, MD, Abant Izzet Baysal University, Izzet Baysal Medical Faculty, Department of Obstetrics and Gynecology, 14280 Gölköy, Bolu, Turkey. E-mail: onderkoc1@hotmail.com