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ORIGINAL ARTICLE

ASSESSMENT OF DNA NUCLEO BASE OXIDATION AND ANTIOXIDANT DEFENSE IN POSTMENOPAUSAL WOMEN UNDER HORMONE REPLACEMENT THERAPY

TÜLAY AKCAY, YILDIZ DINCER, E. ILKER SAYGILI[1], HAKAN SEYISOĞLU[2], ERDOĞAN ERTUNGALP[2]

ABSTRACT

BACKGROUND AND OBJECTIVE: *The aim of the present study was to evaluate oxidative stress byinvesting oxidatively damaged DNA AS Formamidopyrimidine DNA glycosylase (Fpg) -sensitive sites, glutathione peroxidase (GPx), superoxide dismutase (SOD) activities reduced glutathione (GSH) level and nitrite level as satble end product of in women receiving hormone replacement therapy (HRT).* ***MATERIALS AND METHODS:*** *127 healthy postmenopausal women receiving HRT and 25 healthy control postmenopausal women were included in this study. Women receiving HRT, comprised surgical menopausal women who underwent surgery for benign conditionsand received conjugated equine estrogen, 0.625 mg/day for 1year (group 1), 5 years (group 2) and more than 10 years (group 3), spontaneous postmenopausal women received conjugated equine estrogen, 0.625 (Premarin) mg/day and medroxyprogesterone acetate, 2.5 mg/day (Premelle) for 1 year (group 4), 5 years (group 5) and more than 5 years (group 6).We investigated in the present study the effects of HRT on nitrite level and GSH level, activities of SOD and GPx and oxidative damage to DNA by comet assays by measuring levels of Fpg-sensitive sites.* ***RESULTS:*** *Although no significant differences were found in the SOD activities, in total group receiving HRT, increased DNA oxidation ($P<0.001$) together with an increased GPx activity ($P<0.001$) and nitrite level ($P<0.001$) as well as a decreased GSH level ($P < 0.05$) as compared with controls were observed.* ***CONCLUSION:*** *Estrogen alone or oestrogen in combination with progesterone and duration of use did not significantly alter the results. We evaluated that caused oxidative stress by investigating oxidative DNA damage as Fp-sensitive sites and GSH.NO levels in women receiving HRT.*

Key words: *8-Hydroxy –2′-deoxyguanosine, estrogen, glutathione, glutathioneperoxidase, nitrite superoxide dismutase*

Department of Biochemistry, Cerrahpaşa Medical Faculty, University of Istanbul, Istanbul, [1]Universiy of Gaziantep Vocational School Of Higher Education For Health Services, [2]Department of Gynecology and Obstetrics, Cerrahpaşa Medical Faculty, University of Istanbul, Turkey

Address for correspondence:
Dr.Tülay Akçay,
Fahrettin Kerim Gökay Cd. No.156/4, Kadıköy, İstanbul, Turkey.
E-mail: tulakcay@yahoo.com

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DOI: 10.4103/0019-5359.92483

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INTRODUCTION

Hormone replacement therapy (HRT) is generally given after menopause to replace the hormones which are no longer produced by the ovaries. HRT is a therapy which involves the use of either oestrogen alone or oestrogen in combination with progesterone. Chlebowski *et al*,[1] reported that relatively short-term estrogen + progesterone use increases the risk for breast cancers, which are diagnosed at a more advanced stage compared with placebo use. As far as endometrial cancer is concerned, there is no doubt that oestrogen replacement therapy increases cancer risk by as much as 10-fold, especially with long-term(10-15 years) oestrogen use.[2] Holmberg *et al*.[3] reported that after extended follow-up, there was a clinically and statistically significant increased risk of a new breast cancer event in survivors who took HRT.

4-Hydroxyequilenin (4-OHEN) is a catechol metabolite of equilenin, which is a component of the oestrogen replacement formulation marketed under the name of premarin.[4] Unlike the endogenous catechol estrogens, 4-OHEN rapidly autoxidizes to the long-lived 4-OHEN-o-quinone.[5] As o-quinones are potent redox cycling agents, damages in cells may occur to DNA, lipids and proteins. Significant oxygen consumption was also detected, consistent with in vitro models which have shown that 4-OHEN –o-quinone significantly increases the amount of oxidative damage to DNA bases.[6,7] Pisha *et al*[8,9] reported that oxidative stress could be partially responsible for the carcinogenic effects caused by 4-OHEN and that 4-OHEN is a more potent transforming agent than the catechol metabolite of the endogenous oestrogen, 4-hydroxyestrone (4-OHE) *in vitro*.

Zhong *et al*.[9] suggested that oxygen free radicals, by interacting with genomic DNA, damage specific genes which control cell growth and differentiation. Major DNA oxidation product is 8-hydroxy –2'-deoxyguanosine (8-OHdG).[10] The 8-OHdG has a proto- mutagenic potential by mis-pairing with residues, leading increased frequency of spontaneous G:C→ T:A transversion unless repaired prior to DNA replication.[11] This mutation is generally observed in mutated proto-oncogene's and tumour suppressor genes.[12] To our knowledge, no investigation has been conducted to demonstrate the importance of 8-OHdG in postmenopausal women receiving HRT. Therefore, we proposed to investigate the frequency of 8-OHdG in DNA by using the comet assay. The comet assay is a rapid and sensitive fluorescence microscopic method.[13] The sensitivity of the comet assay is higher than the other DNA damage detection methods, such as chromatid exchange production, alkaline elution and micronucleus assay[14] It can be used not only for determination of strand breakage but also for detection of base oxidation on DNA by using damage specific-repair endonuclease.[15] Formamidopyrimidine DNA glycosylase (Fpg) isolated from Eschericia coli specifically removes 8-OHdG and ring-opened purines. 8-OHdG exerts the highest affinity and it is also believed to be the biologically most relevant substrate for this repair enzyme.[12] Therefore, the frequency of Fpg-sesitive sites provides a valuable tool to assess the extent of oxidatively damaged DNA[10,15,16]

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DNA strand breakage produced by NO has been shown in mammalian cells.[17] The metabolites of NO such as the potent mutagen peroxynitrite ($OONO^-$) can induce transversion mutations.[18]

Reduced glutathione (GSH) is a physiological constituent of the intracellular antioxidant defense system. The first line of defense is the antioxidants such as glutathione peroxidase (GPx) and superoxide dismutase (SOD). SOD converts $O_2^{\cdot-}$ to H_2O_2, which is further converted to H_2O with the help of GPx. SOD inhibits $^{\cdot}OH$ production. SOD also acts as an anti-carcinogen, and inhibitor at initiation and promotion/transformation stage in carcinogenesis.[18] The aim of the study was to evaluate oxidative stress by investigating oxidative DNA damage as Fpg-sensitive sites, GPx, SOD activities and GSH and NO levels in women receiving HRT.

MATERIALS AND METHODS

One hundred and twenty seven healthy postmenopausal women [BMI (kg/m^2): 27; age: 54±9 years] receiving HRT and 25 healthy control postmenopausal women [BMI (kg/m^2): 28; age: 50 ±3 years] were selected for this study from Gynaecology and Obstetrics Department of Cerrahpaşa Medical Faculty, İstanbul University. None of these women had smoking habit nor had taken vitamin supplements for the last 3 month). Women receiving HRT, comprised surgical menopausal women who underwent surgery for benign conditions and received conjugated equine oestrogen, 0.625 mg/day for 1year (group 1), 5 years (group 2) and more than 10 years (group 3), and spontaneous postmenopausal women who received conjugated equine estrogen, 0.625 (Premarin) mg/day and medroxyprogesterone acetate, 2.5 mg/day (Premelle) for 1 year (group 4), 5 years (group 5) and more than 5 years (group 6).

Institutional Ethical Committee approval was obtained in accordance with the principles of Declaration of Helsinki. Informed consent was obtained from each study subject.

Collection and preparation of blood samples

Ten milliliters of venous blood sample was collected in heparinized tubes for determination of DNA oxidation within nucleated blood cells, GSH level of whole blood, and GP_X and SOD activities. Following the centrifugation at 2000g for 15 minutes, plasma was removed and kept at -80°C until the time of NO analysis. As an indicator of NO, total nitrate and nitrite levels in the plasma were determined by kit from UK (catalogue no: DE1600) after the reduction of nitrate to nitrite by nitrate reductase. Activities of SOD and GPx were measured using spectrophotometric kits from Randox, UK (catalogue nos: SD125 and RS 504, respectively). For the separation of erythrocytes, blood samples were drawn into heparinized tubes, centrifuged at 700 g at 4°C for 15 min; plasma was carefully aspirated and packed erythrocytes were washed three times with 0.9 % saline solution for removal of the buffy coat. GSH measurements were performed immediately. Aliquots of erythrocytes were stored at – 80°C for determination of GPx and SOD activities. GSH levels were measured as described by Beutler *et al.*[19] and spectrophotometrically using metaphosphoric acid for protein precipitation and 5,5'- dithiobis 2-nitrobenzoic acid for colour development

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at 412 nm. GSH level was determined using the molar absorption coefficient of the GSH at 432nm($1.36x10^4$ l/mol/cm) and expressed as micromoles/gram hemoglobin (µmol/gHb).The haemoglobin concentration was measured by Drabkin's reagent as spectrophotometrically.[20]

The comet assay was performed using an adaptation of the method of Singh *et al*.[21] This method uses direct fluorescent microscopy of ethidium bromide-stained DNA in agarose gel. Briefly, a single-cell suspension of cells was embedded in agarose of low melting point an agarose sandwich on a microscope slide. Electrophoresis was then carried out at high pH, and loops of DNA extend toward the anode, giving the appearance of the tail of comet when stained and viewed by fluorescence microscopy. Undamaged DNA remained within the head. In order to evaluate the degree of damage, comet images were scored visually. Fifty cells per slide were examined and 100 cells were examined per case. Each comet was classified within five categories (0–4) according to the extension of DNA migration. The comets with bright heads and no apparent tails were assigned to category 0, and comets with very little heads and long diffused tails to category 4. Comets displaying features intermediate between categories 0 and 4 were divided and assigned to easily distinguishable categories 1, 2 and 3. The number of the comets in each category was counted and average DNA damage in the case of strand breaks was expressed as an arbitrary unit (a.u.), which is related to the percentage of DNA in the tail.[14]

In order to examine base oxidation, DNAs were incubated with Fbg, a bacterial endonuclease that generates additional breaks at sites containing 8-OHdG after the lysis step. Subtracting the percent DNA in tail without Fbg incubation gives the net amount of base damage represented by Fbg-sensitive sites.[14]

To evaluate the susceptibility to oxidation, slides were incubated with 100 µM H_2O_2 in phosphate buffered saline solution for 5 min on ice just before the lysis step.

Statistical analysis

The results were expressed as means ±S.D. Comparisons were performed by parametric one- way analysis of variance (ANOVA) and Kruska –Wallis test. Correlation analysis was performed using the Pearson-Pravais test. Significance was established at the $P<0.05$ level.

RESULTS

Fpg-sensitive sites in leukocytes, erythrocyte GSH level, GPx activitiy, SOD activity and plasma nitrite level in postmenopausal females before HRT as control groups and at 1,5 and 10 years after HRT are indicated in Table 1. Although no significant differences were found in the SOD activities, a significant increase in the Fp-sensitive sites occurred, and GPx activity and NO levels increased in all groups receiving HRT compared to controls. Also, decreased GSH levels have been detected in all groups receiving HRT. Estrogen alone or oestrogen in combination with progesterone and duration of use did not significantly alter the results. In toutal group receiving HRT, increased DNA oxidation together ($P<0.001$) with an increased GPx activity ($P<0.001$) and nitrite level($P<0.001$)

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Table 1: The parameters compared in post menopausal women under hormone replacement therapy and control groups†

	Fpg-sensitive sites (au)	*GSH (µmol/gHb)*	*SOD (U/gHb)*	*G-Px (U/gHb)*	*NO (nitrite) (µmol/l)*
Control (n =25)	127±19	5.7±0.6	1233±474	55.83±18.7	21±7
Toutal group receiving HRT n= 127	159±32**	5.1±0.6*	1323±441	80.8±20.5**	39±12**
Group 1 (n=20)	163±29*	5.0±0.5*	1583±450	88.3±22.9**	40±13**
Group 2 (n=22)	170±35**	5.2±0.3*	1145±180	82.1±18.4**	35±6*
Group 3 (n=21)	171±34**	5.1±0.4*	1492±462	81.6±21.5*	36±10**
Group 4 (n=21)	150±26*	5.2±0.6*	1120±263	75.5±11.1*	41±15**
Group 5 (n=23)	151±28*	5.1±0.4*	1629±550	77.0±16.8*	45±14**
Group 6 (n=20)	153±29*	5.0±0.6*	1132±215	81.5±24.4*	37±14*

*Comparision with the control group(P<0,05), ** Comparision with the control group (P<0,001), †Values are expressed as mean±SD. Group 1: Conjugated equine estrogen, 0.625mg/day for 1 year, Group 2: Conjugated equine estrogen, 0.625mg/day for 5 years, Group 3: Conjugated equine estrogen, 0.625mg/day for more than 10 years, Group4: Conjugated equine estrogen, 0.625mg/day and medroxyprogesterone acetate, 2.5mg/day for 1 year, Group5:Conjugated equine estrogen, 0.625mg/day and medroxyprogesterone acetate, 2.5mg/day for 5 years, Group6:Conjugated equine estrogen, 0.625mg/day and medroxyprogesterone acetate, 2.5mg/day for more than 5 years

as well as a decreased GSH level (P< 0.05) as compared with controls was observed. Groups 1- 6 had higer Fp-sensitive sites (P<0.05. P<.001, P<0.001,P<0.05, P<0.05and P<0.05 repectively) than the controls. In all groups lower GSH levels (P<0.05). Groups 1-6 had higer G-Px activities (P<0.001, P<0.001, P<0.05, P<0.05, P<0.05, and P<0.05respectively) than the controls. Groups 1- 6 had higher NO (nitrite) levels (P<0.001, P<0.05, P<0.001, P<0.001, P<0.001, and P<0.05 respectively) than the controls.

No relationship between Fpg-sensitive sites and GPx, SOD activities and GSH, NO levels in women receiving HRT was found in the present study.

DISCUSSION

The risk factors associated with cancer may exert their effects via generation of reactive oxygen species (ROS), which has already been recognized to induce oxidatively DNA damaged and neoplastic transformation.[22] There is some evidence supporting a role for estrogens in cancer etiology. Estrogens are converted to catecholestrogens and these produce oxygen radicals, which cause various types of DNA damage. 4-Hydroxyequileinin, a metabolite of equine estrogens has been revealed to induce cytotoxic and carcinogenic effects.[23] Significant increases in DNA oxidation in peripheral leukocytes which have been observed in the present study may be attributed to over production of ROS; in particular, estrogens induced by ROS generates products such as catecholesters. Enhanced oxidative DNA damage, unless repaired before the replication, leads to mutations in proto-oncogenes and tumor–suppressor genes and possible transformation of normal epithelium to a malignant phenotype.[23-25]

Diamanti-Kandarakis[26] presented a thorough review of the relationship between estrogen

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exposure and malignancy. Ozcaglı *et al*.[27] reported that significant DNA damage was observed with equine oestrogen 0.625mg/day and equine oestrogen 0.625mg/day +medroxyprogesterone. Their data suggest that women under oestrogen therapy exhibit more DNA damage in their leukocytes than women under oestrogen plus medroxyprogesteron therapy.[27] Ozcaglı *et al*[27] evaluated DNA damage as SSBs by the comet assay. Although SSBs are derived from ROS, it cannot be considered as a powerful index for oxidative DNA damage, because some environmental factors such as ionizing radiation, air pollutants, may also cause breaks on DNA strands.[24,28] Furthermore, SSBs may occur during the repair processes.[29]

In this study, we have investigated the effects of estrogen and progesterone + estrogen on oxidative damage to DNA by the comet assay, by measuring the frequency of Fpg-sensitive sites which indicates 8-OHdG adducts on DNA as a biomarker of oxidant-induced DNA damage in leukocytes of post menopausal women. Fpg-sensitive sites were found to be increased in all groups, receiving HRT for 1 year, 5 years and more than 10 years as compared to controls and no significant difference was found between estrogen and estrogen + medroxyprogesterone acetate groups.

Enhanced expression of GPx has been documented to inhibit ROS-induced apoptosis in human breast cancer cell lines. Apoptosis evasion a hall mark of malignant cells, is known to enhance the proliferative potential of tumor.[30] The increased activity of these enzymes is consistent with the reports of anti-oxidant enzyme over expression in tumours.[31] The increase in ROS-induced DNA oxidation in peripheral leukocytes observed in this study was associated with one of the antioxidant enzyme, GPx, reflecting the ability of the cells to counter oxidative stress. Like Bednarek-Tupikowska *et al*.[32] we did not observe any changes in SOD activity under the influence of hormone replacement. These authors also observed that GPx and GSH increased significantly after HRT.

GSH is a physiologically important non protein thiol with multiple functions ranging from antioxidant defense to modulation of cell proliferation. In particular, intracellular GSH maintains the reduced status of thioredoxin which activates ribonucleotide reductase, a key enzyme essential for DNA synthesis. GSH deficiency contributes to oxidative stress, and therefore may play a key role in aging and the pathogenesis of many diseases.[33] GSH in conjunction with GP_x documented in tumours is believed to be an early proliferative response that influences the cellular thiol redox status leading to activation of genes essential for G_1 to S transition.[34] In our study, in menopausal women receiving HRT, a significantly decrease in the level of GSH was found.

DNA strand breakage produced by NO has been shown in mammalian cells.[17] It has been suggested that NO-mediated oxidants play a fundamental role in the initiation promotion/progression of tumour by damaging the DNA strains.[35] In the present study, plasma level of nitrite was significantly elevated in all groups receiving HRT. Bednarek-Tupikowska *et al*.[36] reported that estrogen therapy and estrogen + progesterone therapy improve

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nitric oxide production synthesis.Our data are in accordance with the data of Bednarek-Tupikowska *et al*.[36]

Although it is now known that the use of HRT has been associated with increased risk of breast and endometrial cancer,[1,26,37,38] as far as we know, there is no report indicating oxidative damage to DNA by measuring Fpg-sensitive sites in post menopausal women under HRT. In this study, we determined that in all groups receiving HRT, DNA oxidation has increased together with an increased activity of GPx and nitrite level as well as a decrease in GSH level as compared to controls. Increased oxidative DNA damage in leukocytes shows increased mutagenicity. This phenomenon may also occur in breast or endometrial tissue. Blood samples can be easily obtained from the patients and are reliable for screening as well as for the follow-up of treatment. The future of HRT in postmenopausal women may lie in the individualization of the therapy based upon personal characteristics (antioxidant status, DNA repair enzymes).

ACKNOWLEDGEMENT

The study was supported by the research fund of the University of Istanbul, Project Number.12/27082002

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How to cite this article: Akcay T, Dincer Y, Saygili EI, Seyisoglu H, Ertungalp E. Assessment of DNA nucleo base oxidation and antioxidant defense in postmenopausal women under hormone replacement therapy. Indian J Med Sci 2010;64:17-25.
Source of Support: University of Istanbul, Project Number.12/27082002. **Conflict of Interest:** None declared.

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