20 7.

Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

European Journal of Experimental Biology, 2013, 3(6):207-211

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Assessment of DNA damage of women using oral contraceptives by high resolution melt analysis

Marianna Trebuňová[1], Galina Laputková[1] and Jozef Živčák[2]

[1]*Department of Medical and Clinical Biophysics, Faculty of Medicine, P.J. Šafarik University, Trieda SNP 1, Košice, 040 11, Slovak Republic*
[2]*Department of Biomedical Engineering and Measurement DBEaM, Faculty of Mechanical Engineeering, Institute of Technical Sciences, TUKE, Letná 9, 042 00 Košice, Slovak Republic*

ABSTRACT

The aim of the study was to evaluate the genotoxic effect of combined oral contraceptives with low concentration of estrogens: 0.03 mg ethinylestradiol and 3 mg desogestrel per day using HRM analysis of genomic DNA isolated from the whole unstimulated saliva. Our data suggest that there are not significant alterations in the melting temperature of DNA of the group exposed to the oral contraception in comparison with the control group. The sequenation (MDR1 gene, a forward primer 5′-TGGGGCTTTTAGTGTTGGAC-3′; a backward primer 5′-TGTGGAGAGCTGGATAAAGTCA-3′) confirmed identical nucleotide sequences for forward fragments of both groups. In spite of this, minor change of nucleotide content were observed when compared the sequences of DNA for the control group and the volunteers group taking oral contraceptives for backward fragments, when a deletion in the sequence on the 50 bp position for the group exposed to the oral contraception was observed.

Key words: Melting analysis, DNA, Saliva, Contraception, MDR1 gene

---

INTRODUCTION

Estrogen is a class of 18-carbon steroid indispensable for the growth and upkeep of reproductive and non-reproductive organs in the body. The level of estrogens in peripheral blood of non-ovulating women averages 199 pg/ml [1]. Although, there are several well-established risk factors identified for breast cancer such as the inherited genetic predisposition or reproductive history; it is uncertain whether prolonged use of oral contraceptives and hormone replacement therapy containing synthetic estrogens increases the risk of carcinogenesis in hormone-dependent organs, particularly breast and endometrium [2,3].

Estrogens are transformed to catechol estrogens and these, or the resulting quinones, interact with DNA producing oxidative damage to DNA and hence cause mutations [4]. A concept that reactive oxygen species can produce genotoxic effects as well as support the development of estrogen-dependent breast tumors is discussed in the review [5]. The endogenous estrogens and their metabolites by increasing genomic instability and by transducing signal through influencing redox sensitive transcription factors play important role in cell transformation, cell cycle, migration and invasion of the breast cancer.

Among various drug transporters, P-glycoprotein (Pgp), the MDR1 gene product, is one of the best studied and characterized. Pgp is an ATP-dependent efflux pump, which plays a key role in regulating absorption, excretion, and tissue distribution of various drugs. P-glycoprotein represents also an important prognostic factor in malignant diseases. Pgp is expressed in normal human tissues such as liver, kidney, intestine and the endothelial cells of the

blood-brain barrier. A growing number of preclinical and clinical studies have demonstrated that polymorphism of the MDR1 gene may be a factor in the overall outcome of pharmacotherapy for numerous diseases [6].

The great interest stimulated by high resolution melting (HRM) methods explains its rapid expansion to cope with broad spectrum of human genetic mutations [7] and polymorphisms [8]. HRM includes the precise monitoring of the variation in fluorescence initiated by the release of an intercalating DNA dye from a DNA duplex as it is denatured by increasing temperature.

Considering the fact that studies on the mutagenic effects of combined oral contraceptives with low concentration of estrogens are limited, the aim of our study was to evaluate the effects of oral contraceptives to detect potential associations between oral contraceptives and the genotoxic damage in healthy women using HRM analysis from the samples of the whole unstimulated saliva.

MATERIALS AND METHODS

A sample of the whole unstimulated saliva was obtained from 3 women (range: 31-35 years) taking an oral contraception containing 0.03 mg ethinylestradiol and 3 mg desogestrel per day for 5years at least. Furthermore, none of the three control individuals (range: 28-35 years) had history of oral contraceptive, cigarette smoking, and alcohol intake. Approximately 3-6 ml of the whole unstimulated saliva was collected by expectoration into sterile 50 ml polypropylene tube placed on ice. Volunteers were instructed to refrain from the morning oral hygiene, eating or drinking at least one hour before saliva collection. Saliva secretion during the collection of the sample was not stimulated mechanically or chemically. Non-soluble debris, food fragments and microbes were removed by centrifugation at 14000×g for 30 min at 4°C. The supernatant and pellet were separated, the pellet was discarded and the supernatant saved at -80°C for further analysis.

Genomic DNA samples were extracted from the unstimulated saliva using the Genomic DNA from Tissue kit (Macherey-Nagel, Biotech) according to the manufacturer´s instruction with some modifications. The concentration of DNA was approximately in the interval of 2.5 – 3.0 μg/μl.

Two primers, where DNA fragment is between exon 14 and intron 14 of MDR1 gene, were selected [9]. As a forward primer and a backward primer were used respectively 5´-TGGGGCTTTTAGTGTTGGAC-3´ and 5´-TGTGGAGAGCTGGATAAAGTCA-3´. HRM analysis was performed with the aid of Line-Gene K (KRD Molecular Technologies) using FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics) as recommended by the manufacturer. The cycling protocol started with one cycle of 50°C for 2 min followed by 1 cycle of 95°C for 10 min followed by 40 cycles of 95°C for 15 s (1°C/cycle), a touch down of 95°C to 61.2°C for 1min. The melting analysis step started at 61.2°C to target temperature of 95°C for 15 s (0.5°C/cycle). Fluorescence data were converted into melting peaks by the aid of fluorescence quantitative PCR detection software (Line-Gene K, Bioer) to determine binding efficiency of DNA from saliva.

PCR product was sequenced in Sequencing Centre of Department of Molecular Biology (University of Veterinary Medicine and Pharmacy in Košice, Slovak Republic). The concentration of primer was 1 pmol/μl. The template accomplished the volume of 20 μl. The volume of primer was 10 μl. The resulting percentage of nucleotide bases and amino acid of MDR1 gene were analyzed with the aid of BioEdit Sequence Alignment Editor.

RESULTS AND DISCUSION

The effect of the use of an oral contraceptive on the generation of DNA strand alterations derived from human saliva was evaluated using the HRM. Fig. 1 illustrates the melting curves and the derivative melting curves of control women, women using oral contraceptives, and women using oral contraceptives during the beginning of menstruation.

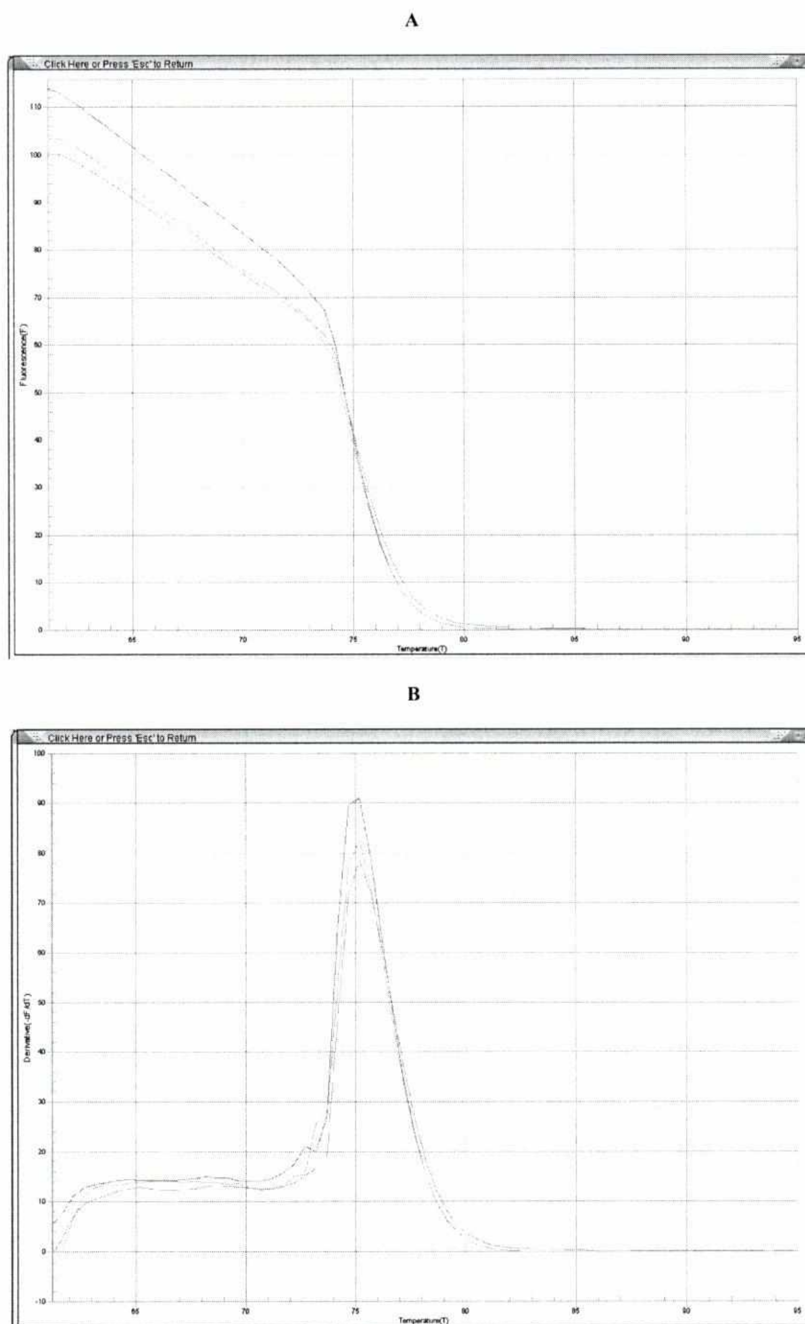

Fig. 1. Representative melting curve (A) and derivative melting curve (B) of DNA of control women (green), women using oral contraceptives (grey) and women using oral contraceptives during the beginning of menstruation (blue).

One clear melting transition for both amplicon duplexes melted at 65-90°C was observed. Based on 12 measurements, one melting peak was displayed at 75.2±0.5°C for control women, at 75.1±0.7°C for women using oral contraceptives and at 75.1±0.6°C for women using oral contraceptives during the beginning of menstruation, respectively. Nosignificant change at $p < 0.05$ were observed.

Using MDR1 primer pairs: forward 5′-CCCATCATTGCAATAGCAGG-3′, backward 5′-GTTCAAACTTCTGCTCCTGA-3′ it was possible to amplify fragments of genomic DNA and detect PCR products. In Fig. 2there is a verification of DNA sequence of MDR1 gene from saliva in BLAST database.

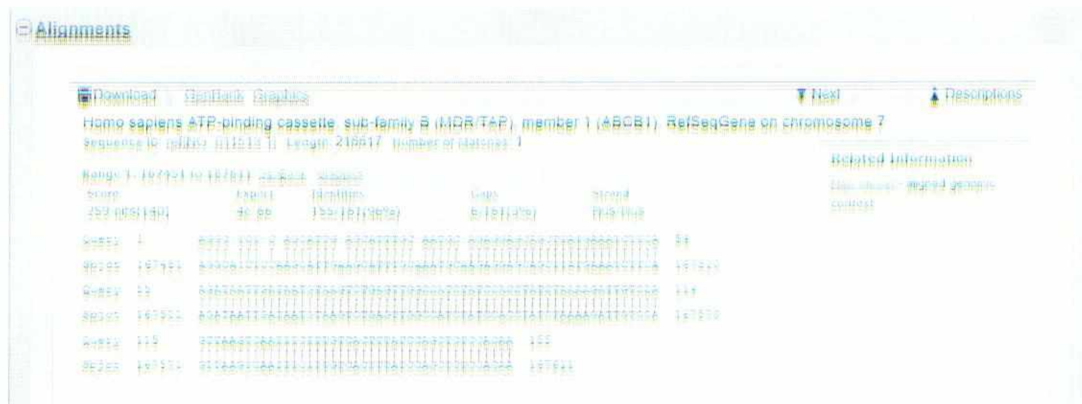

Fig. 2. Verification of DNA sequence of MDR1 gene from saliva in BLAST database

Comparing the relative content of G+C/A+T in the group of women taking oral contraceptives and the group of women using oral contraceptives during the beginning of menstruation, no change was observed. The sequencing results confirmed identical nucleotide sequences for both fragments of DNA. In spite of this, minor change of nucleotide content were observed when compared the sequences of DNA for the control group and the volunteers group taking oral contraceptives for backward fragments, when a deletion in the sequence on the 50 bp position for the group exposed to the oral contraception was occurred (Fig. 3).

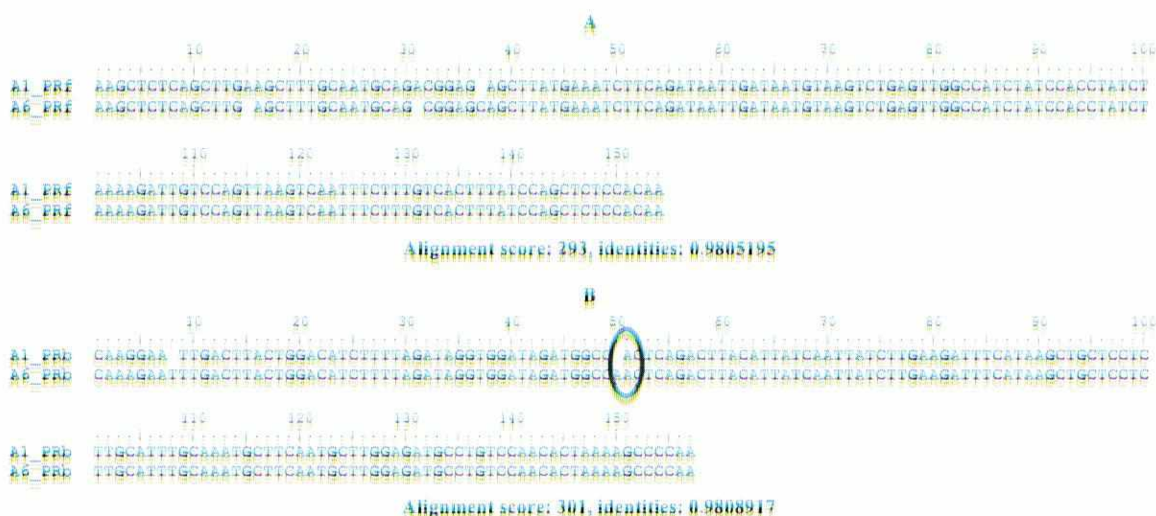

Fig. 3. The aligned nucleotide sequences of fragments of DNA of women taking oral contraceptives (A1) and the control group (A6) for both forward (A) and backward primers

Clues about a role of estrogen genotoxicity and an increased risk of breast cancer as a function of plasma estrogen levels in plasma were published earlier [14]. In a case-control study [15] was also demonstrated that elevated serum levels were positively associated with breast cancer risk. Evidence suggests that the genotoxic mechanism of estrogens and its metabolites in breast cancer involves their oxidation to 3,4-quinones and reaction with DNA to form depurinating N3Ade and N7Gua adducts. Authors of the study [10] examining estrogen genotoxicity of the female ACI rats revealed mutagenic estrogen-dependent effect on breast cancer. Mammary glands of both estrogen-responsive and resistant rats contained pre-existing mutations at frequencies of (39.8–58.8) × 10^{-5} , the majority (62.5–100%) of which were AT to GC transitions detected as GT heteroduplexes.

Estrogens inducing tumors in laboratory animals can be associated with breast cancer in humans as well. The authors of the study [12] examine formation of DNA adducts by reactive electrophilic estrogen metabolites, formation of reactive oxygenspecies by estrogens and the resulting indirect DNA damage by these oxidants, and, finally, genomic and gene mutations induced by estrogens. Several types of indirect DNA damage are caused by estrogen-induced oxidants, such as oxidized DNA bases, DNA strand breakage, and adduct formation by reactive aldehydes derived from lipid hydroperoxides were observed.

Our study did not show significant genotoxic effect of oral contraception containing 0.03 mg ethinylestradiol and 3 mg desogestrel. There was not observed the change of melting temperature of genomic DNA of the control group of women and the group taking oral contraception. Only minor change for backward fragments of DNA - a deletion in the sequence on the 50 bp position for the group exposed to the oral contraception - was occurred

It is obvious that there are many other individual factors that can cause the DNA damage, e.g. previous and current medication, excessive diagnostic radiation [14], exposure to toxicants [15,16,17], endogenous infection, diabetes [18], polymorphic metabolizing genes, and efficiency of DNA repair. Therefore it is not excluded that the minor change in the DNA sequence observed in our experiment at low oral contraceptive levels are not due to the taking of the drug, but the factors mentioned above can be responsible. Nevertheless, it would be advisable to take precautions while prescribing oral contraceptives as well and take into account all of these factors

CONCLUSION

In conclusion, we found no associations between the intake of the oral contraceptives with low estrogen content for a longer period and the genetic changes observed with the aid of HRM analysis. However, our results are expected tocontribute to the efforts to better understand the sporadic cancer risk of the oral contraceptives.

Acknowledgements
This study was supported by the grants VEGA 1/0515/13 (80 %) of the Ministry of education of the Slovak Republic and partially by the Agency of the Slovak Ministry of Education for the Structural Funds of the EU under project: ITMS: 26220 220143 (20 %).

REFERENCES

[1] N.S.Jiang, R.J.Ryan, A. Albert,*Clin.Chem.*,**1973**, 19, 740.
[2] D.Roy, Q.Cai, Q.Felty, S. Narayan,*J.Toxicol. Environ. Health B Crit. Rev.*,**2007**, 10, 235.
[3] P.A.Marchbanks, J.A.Mcdonald, H.G.Wilson, S.G.Folger, M.G.Mandel, J.R.Daling, L.Bernstein, K.E.Malone, G.Ursin, B.L.Strom, S.A.Norman, L.K. Weiss,*New Engl. J. Med.*,**2002**, 346,2025.
[4] R.P. Rubin; Modern Pharmacology, Little Brown & Co., Boston, **1990**.
[5] V.Okoh, A.Deoraj, D. Roy,*Biochim.Biophys.Acta*,**2011**, 1815, 115.
[6] I.Ieiri, H.Takane, K.Otsubo,*Clin.Pharmacokinet.*,**2004**, 43, 553.
[7] E.A.Takano, G.Mitchell, S.B.Fox, A.Dobrovic,*BMC Cancer*,**2008**, 8, 59, doi: 10.1186/1471-2407-8-59.
[8] G.H.Reed, C.T. Wittwer,*Clin.Chem.*,**2004**, 50, 1748.
[9] Y.Sung-Tsai, Ch.Tzer-Ming, T.Shih-Yun, Ch.Yen-Hui,*Biochem.Biophys. Res.Commun.*,**2007**, 358,79.
[10] T.Key, P.Appleby, I.Barnes, G. Reeves,*J. Natl. Cancer.Inst.*,**2002**,94,606.
[11] R.Kaaks, S.Rinaldi, T.J.Key, F.Berrino, P.H.Peeters, C.Biessy, L.Dossus, A.Lukanova, S.Bingham, K.T.Khaw, N.E.Allen, H.B.Bueno-de-Mesquita, C.H.van Gils, D.Grobbee, H.Boeing, P.H. Lahmann, G.Nagel, J.Chang-Claude, F.Clavel-Chapelon, A.Fournier, A.Thiébaut, C.A.González, J.R.Quirós, M.J.Tormo, E.Ardanaz, P.Amiano, V.Krogh, D.Palli, S.Panico, R.Tumino, P.Vineis, A.Trichopoulou, V.Kalapothaki, D.Trichopoulos, P.Ferrari, T.Norat, R.Saracci, E.Riboli,*Endocr.Relat.Cancer*,**2005**,12,1071.
[12] P. C.Mailander, J.L.Meza, S.Higginbotham, D.Chakravarti,*J. Steroid.Biochem.*,**2006**, 101, 204.
[13] E.Cavalieri, K.Frenkel, J.G.Liehr, E.Rogan, D.Roy,*J. Natl. Cancer. Inst.Monogr.*,**2000**,27,75.
[14] M.R. Usikalu, S.O. Rotimi, A.E. Oguegbu,*Euro. J. Exp. Bio.*, **2012**, 2, 2499.
[15] H.Farhadi, M.Siakuhian, H.Dolatkhah,S.Rahimifardin, S.N.P. Salemi,*Euro. J. Exp. Bio.*, **2013**, 3, 455
[16] K.M. Maria John, J. Rajesh,, A.K.A. Mandal, S. Natarajan, *Euro. J. Exp. Bio.*,**2011**, 1, 145.
[17] R. Elayaperumal, P. Dharmalingam, *Der ChemicaSinica*, **2013**, 4, 170.
[18] R. Bayatmakoo, H. Mobaiyen, A. Monfaredan, B.S. Katayoun,B. Ronak, *Euro. J. Exp. Bio.*, **2013**, 3, 512.