

EVALUATION OF DNA DAMAGE IN THE LYMPHOCYTES OF YOUNG, ELDERLY AND ALZHEIMER'S DISEASE PATIENTS TREATED WITH β -ESTRADIOL IN THE COMET ASSAY

EVALUACIJA OŠTEĆENJA DNK U LIMFOCITIMA MLADIH, STARIH I PACIJENATA OBOLELIH OD ALCHAJMEROVE BOLESTI TRETIRANIH β -ESTRADILOM U KOMET TESTU

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Summary

Background: The antioxidant activity of estrogen has a beneficial impact in Alzheimer's disease. A variety of clinical studies have demonstrated that estrogen replacement therapy in postmenopausal women results in a lower frequency of AD, delaying the onset of the neurodegenerative cascade. On the other hand, it has been demonstrated that estrogens may exhibit genotoxic effects, especially at elevated tissue concentrations. Therefore, the aim of this study was to determine whether β -estradiol induces DNA damage in the peripheral blood lymphocytes of healthy young females and males, healthy elderly females and males and females and males with Alzheimer's disease.

Methods: All experiments were performed using the alkaline version of the Comet assay (single cell gel electrophoresis), on six donors per each experimental group and controls.

Results: In the Comet assay, a significant increase of DNA migration was observed in the lymphocytes of all treated groups (young and elderly females, young and elderly males, AD females and AD males) at all β -estradiol concentrations (50 $\mu\text{mol/L}$, 100 $\mu\text{mol/L}$ and 250 $\mu\text{mol/L}$) used in this investigation. In all the experiments cell viability was over 80%.

Kratak sadržaj

Uvod: Antioksidativna svojstva estrogena imaju povoljan uticaj na Alchajmerovu bolest (AB). Brojne kliničke studije su pokazale da primena hormonske supstitucione terapije estrogenom kod žena u postmenopauzi smanjuje učestalost pojave AB, odlažući početak neurodegenerativnih procesa. S druge strane, pokazano je da estrogeni mogu ispoljiti genotoksičan efekat, naročito pri povišenim koncentracijama u tkivu. Shodno tome, cilj ove studije bio je ispitivanje sposobnosti β -estradiola da izazove oštećenja u limfocitima periferne krvi zdravih mladih žena i muškaraca, zdravih starih žena i muškaraca, kao i žena i muškaraca koji boluju od Alchajmerove bolesti.

Metode: Svi eksperimenti su izvedeni primenom alkalne verzije komet testa (gel-elektroforeza DNK pojedinačnih ćelija), na po šest subjekata u svakoj ispitivanoj grupi.

Rezultati: Upotrebom komet testa, zabeleženo je značajno povećanje migracije DNK u limfocitima ispitanika iz svih tretiranih grupa (mladih i starih žena, mladih i starih muškaraca kao i kod žena i muškaraca sa AB), pri svim koncentracijama β -estradiola (50 $\mu\text{mol/L}$, 100 $\mu\text{mol/L}$ i 250 $\mu\text{mol/L}$) koje su korišćene u ovoj studiji. U svim eksperimentima vijabilnost ćelija je bila iznad 80%.

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List of abbreviations: AD, Alzheimer's disease; CNS, central nervous system; ROS, reactive oxygen species; MCI, middle cognitive impairment; SAD, sporadic Alzheimer's disease; DMSO, dimethylsulfoxide; LMPA, low melting point agarose; TCS, total comet score.

Conclusions: Lymphocytes are sensitive to the test concentrations of β -estradiol in the Comet assay regardless of gender, age and health condition of the examined subjects. Therefore, the role of β -estradiol in cellular DNA damage has been confirmed.

Keywords: Alzheimer's disease, β -estradiol, Comet assay, DNA damage

Introduction

Alzheimer's disease (AD) is the most common form of dementia associated with ageing, manifested as a progressive loss of memory and other cognitive functions. It is a slow progressing disease, starting with mild memory changes and ending in severe brain damage (1). Genetic and environmental factors play an important role in the AD etiology (2). Some evidence suggests that oxidative stress is increasingly implicated in the pathogenesis of AD as a common feature (with abnormalities) in neurons, the glia cells, fibroblasts as well as vascular cells from sporadic AD patients but also from familial cases (3, 4). The etiological changes such as neuronal loss and selective neuronal degeneration affecting the hippocampus and other cortical regions reflect gender-based predispositions of female patients with AD, which is not the case in other neurological diseases such as Parkinson's disease and Huntington's chorea (5). The fact that the sporadic form of AD affects twice as many women as men, and that women develop AD mainly after the menopause, indicates that it is possible that hormonal factors may play an important role in the loss of the differentiated phenotypes in neurons (6, 7). These findings support the role of hormones, especially estrogen in AD pathophysiology (8). Estrogen has very complex effects on the CNS. A variety of clinical studies have demonstrated that estrogen replacement therapy in postmenopausal women results in a lower frequency of AD, delaying the onset of the neurodegenerative cascade (9, 10). However, other studies show that clinical trials of estrogen in females with mild cognitive impairment (MCI), the phase between normal ageing and early dementia, do not lead to cognitive improvement (11). Much evidence suggests that oxidative stress is related to age-

Zaključak: Limfociti periferne krvi osetljivi su na testirane koncentracije β -estradiola, bez obzira na pol, godište i zdravstveno stanje ispitanika. U skladu s navedenim nalazima, potvrđena je uloga β -estradiola u izazivanju oštećenja na ćelijskoj DNK.

Ključne reči: Alchajmerova bolest, β -estradiol, komet test, oštećenje DNK

ing and a variety of neurodegenerative disorders including AD. Oxidative stress implies the development of reactive oxygen species (ROS), which lead to excessive oxidation of cellular proteins, lipids and DNA resulting in oxidative changes and dysfunction, leading to neuronal cell death (3). It is proposed that estrogen might act as a neuroprotective and antioxidant molecule in a lower micromolecular concentration in ageing disorders such as Alzheimer's disease (12). On the other hand, the role of estrogens in the generation of ROS in cellular systems, leading to oxidative stress (13) and DNA damage, has received little attention.

Therefore, we have investigated the effects of various doses of β -estradiol on peripheral blood lymphocytes DNA stability in elderly and young donors of both sexes with Alzheimer's disease. For that purpose, the DNA Comet assay (single cell gel electrophoresis), a simple and sensitive technique, was used.

Materials and Methods

Participants

Blood samples were collected by venipuncture from sporadic Alzheimer's disease (SAD) patients, age-matched and young adult control participants. The Medical School Ethics Committee at the University of Belgrade approved the study and written informed consent was obtained from all participants or from their families (2492/1). Diagnosis was based on the National Institute of Neurology criteria. The following 6 groups ($n=6$ subjects/group) were included in the study: AD male, AD female, elderly control male, elderly control female and young adult male and young adult female. The participants' age

Table I General characteristics of Alzheimer's disease and control subjects. Values are means \pm SEM or min-max ($n=6$ /group).

	Controls				AD	
	Young adult		Elderly		Men	Women
	Men	Women	Men	Women		
Average age (yr)	22.5 \pm 0.7	21.8 \pm 0.5	74.5 \pm 1.4	73.2 \pm 2.5	74.3 \pm 2.3	72.3 \pm 2.7
Age range (yr)	20–24	20–24	70–80	66–81	67–83	65–81
Average duration of dementia (yr)	/	/	/	/	2.8 \pm 0.8	2.8 \pm 0.7

range, mean age and duration of dementia (for AD groups) are summarized in *Table I*. Elderly controls without a history of neurological disorders were selected. The donors in the control groups were also non-smokers and did not use antioxidant supplements or medications known to influence the level of oxidative stress and DNA damage. This was a double-blind, randomized study.

Comet assay and analyses

Preparation of slides for the Comet assay was carried out as previously described, using a modification of the methods of Singh et al. (14). Before each experiment, microscope slides were precoated with 1% normal agarose (Sigma, St Louis, MO) and allowed to air dry at room temperature for at least 48h. Heparinised blood samples (4 mL) were obtained by venipuncture from each donor. Lymphocytes were isolated from whole blood with Ficoll-Paque medium and centrifuged at 1900 g for 15 min. The lymphocytes forming a layer were directly above Ficoll-Paque. The isolated lymphocytes were washed twice in RPMI 1640 medium, and each wash was followed by centrifugation for 10 min at 1800 g. Finally, the supernatant was removed as carefully as possible without disturbing the pellet. An aliquot of 1 mL of RPMI 1640 was added and the pellet was resuspended. Then, 50 μ L of isolated lymphocytes was mounted in 50 μ L of 1% low melting point agarose (LMPA) and rapidly placed on precoated microscopic slides covered with a coverslip, and allowed to solidify for 5 min at 4 °C. The coverslip was removed and the cells were treated for 30 min at 37 °C to obtain the final experimental concentration of β -estradiol (Sigma Chemical Co., St Louis, MO, CAS No. 50-28-2), 50, 100 or 250 μ mol/L. Negative control was dimethylsulfoxide (DMSO), a solvent of β -estradiol. Hydrogen peroxide (H_2O_2) was used as the positive control at a final concentration of 25 μ mol/L. The cells were checked for their viability before and after exposure to β -estradiol using trypan blue dye. For each experiment, untreated control samples were made. After incubation, 90 μ L of 0.5% LMPA was added as the third layer, spread using a coverslip and allowed to solidify at 4 °C for 5 min and the slides were placed overnight in lysing solution (2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, 1% Triton X100 and 10% DMSO, pH 10 adjusted with NaOH). After the lysis, the slides were placed in electrophoresis buffer (10 mol/L NaOH, 200 mmol/L EDTA, pH 13) for 30 min at 4 °C in the dark to allow DNA unwinding. Electrophoresis was carried out for 30 min at 25 V and 300 mA. Finally, the slides were gently rinsed with neutralizing solution (0.4 mol/L Tris base, pH 7.5) three times, 5 min each time. Slides were stained with 50 μ L of ethidium bromide (20 μ g/mL) per each slide. The comets were observed and analyzed on an Olympus BX 50 microscope (Olympus Optical Co.,

GmbH, Hamburg, Germany), equipped with a mercury lamp HBO (50 W, 516–560 nm, Zeiss) at 100 \times magnification. For each sample, 100 cells (50 for each replicate slide) were analysed.

Evaluation of DNA damage was performed as described by Anderson et al. (15). Namely, cells were graded by eye into five categories corresponding to the following amounts of DNA in the tail: (A) no damage, <5%; (B) low level damage, 5–20%; (C) medium level damage, 20–40%; (D) high level damage, 40–95%; (E) total damage, >95%.

DNA damage is considered as DNA migration over 5% (B+C+D+E classes). DNA damage can be scored by using parameter total comet score (TCS) which is calculated from DNA damage classification and measured in arbitrary units. The total score was calculated by the following equation: (percentage of cells in class A \times 0)+(percentage of cells in class B \times 1)+(percentage of cells in class C \times 2)+(percentage of cells in class D \times 3)+(percentage of cells in class E \times 4) (16). Consequently, the total score was in the range from 0 to 400. Two comet slides were used for each donor, which makes a total of 100 comets.

Statistical analysis

χ^2 -test was used to assess the differences in the total level of DNA damage in peripheral blood lymphocytes between young, elderly subjects and AD patients. Wilcoxon non-parametric test was used to compare the effect of different concentrations of β -estradiol related to the level of DNA damage (TCS) in peripheral blood lymphocytes. A $p < 0.05$ was considered as significant. Statistical software GraphPad Prism (Version 5.0) was used.

Results

This study was conducted on human peripheral lymphocytes of three groups of individuals: AD patients, age-matched healthy controls (elderly control) and young adult controls (young control). In each group, subjects were subdivided according to gender. The AD patients were affected by the sporadic form of the disease. We evaluated the β -estradiol induced DNA damage *in vitro*. In each examined group, 600 cells were scored (100 cells per person). In all experiments the cell viability was over 80%. There was no difference between the DNA damage of the cells treated with the solvent (DMSO) and the untreated cells. The mean DNA damage of positive control in six experiments (533.16 ± 7.19) was statistically different from negative control (90.0 ± 29.62) ($p < 0.001$). The β -estradiol induced DNA damage in the examined group treated with different concentrations of β -estradiol is shown in *Table II*.

Table II DNA damage in the examined group, induced by different concentrations of β -estradiol. The Comet assay was performed 30 min after the treatment (100 comets counted per experiment).

	Negative control, N	Estradiol, 50 μ mol/L	Estradiol, 100 μ mol/L	Estradiol, 250 μ mol/L
DNA damage in young females	62	103 a**	110 a**	141 a**;b*;c*
DNA damage in old females	93	125 a*	141 a**	174 a**; b**; c*
DNA damage in AD females	127	162 a*	181 a**	210 a**, b*
DNA damage in young males	53	94 a**	102 a**	133 a**; b*;c*
DNA damage in old males	86	116 a*	128 a**	162 a**; b*;c*
DNA damage in AD males	119	156 a*	174 a**	201 a**, b*

*, $p < 0.05$; **, $p < 0.01$; a vs. negative control; b vs. 50 μ mol/L; c vs. 100 μ mol/L
Variables are compared using χ^2 -test.
N – the number of cells

Table III Total Comet Score of lymphocytes of the examined group treated with different concentrations of β -estradiol.

	Negative control	50 μ mol/L	100 μ mol/L	250 μ mol/L
Young females	26.83 \pm 10.62	48.00 \pm 12.65 a*	57.00 \pm 7.24 a*;b*	78.50 \pm 14.35 a*;b*;c*
Old females	41.17 \pm 15.19	58.17 \pm 16.91 a*	72.50 \pm 22.62 a*;b*	87.83 \pm 8.19 a*;b*;c*
AD females	57.17 \pm 10.92	77.67 \pm 13.22 a*	92.83 \pm 12.32 a*;b*	109.33 \pm 15.31 a*;b*;c*
Young males	23.50 \pm 16.57	45.00 \pm 15.51 a*	53.00 \pm 15.07 a*;b*	74.17 \pm 11.77 a*;b*;c*
Old males	42.33 \pm 10.35	57.00 \pm 16.36 a*	66.83 \pm 11.89 a*;b*	93.00 \pm 20.51 a*;b*;c*
AD males	54.83 \pm 20.67	71.33 \pm 18.65 a*	88.50 \pm 21.95 a*;b*	106.67 \pm 20.43 a*;b*;c*

*, $p < 0.05$; a vs. negative control; b vs. 50 μ mol/L; c vs. 100 μ mol/L
Variables are compared using Wilcoxon non-parametric test.

Evidently, analysis of the Comet assay revealed significant differences ($p < 0.01$) in DNA damage after treatment with β -estradiol concentrations of 50 μ mol/L, 100 μ mol/L and 250 μ mol/L compared to negative control in all the test groups (AD, old and young controls) (Table II). Also, there is a significant difference in DNA damage between concentrations of 250 μ mol/L and 50 μ mol/L ($p < 0.05$) in all the examined groups. It should be mentioned that a concentration of 250 μ mol/L significantly increased DNA damage compared to a concentration of 100 μ mol/L in young and old controls, but this increase was not present in AD males and females.

The TCS in peripheral blood lymphocytes is presented in Table III. In all the examined groups we found a statistically significant difference between the total Comet score of cells treated with DMSO (negative control) and the cells treated with β -estradiol concentrations of 50, 100 and 250 μ mol/L. A significant difference was detected in all the groups between the total Comet score of cells treated with β -estradiol concentrations of 50 μ mol/L and 100 μ mol/L. The DNA damage was statistically higher at a dose of 100 μ mol/L compared with a dose of 50 μ mol/L in all the studied groups. Also, a dose of 250 μ mol/L induced statistically greater DNA damage in comparison to 50 μ mol/L and 100 μ mol/L in all the examined groups.

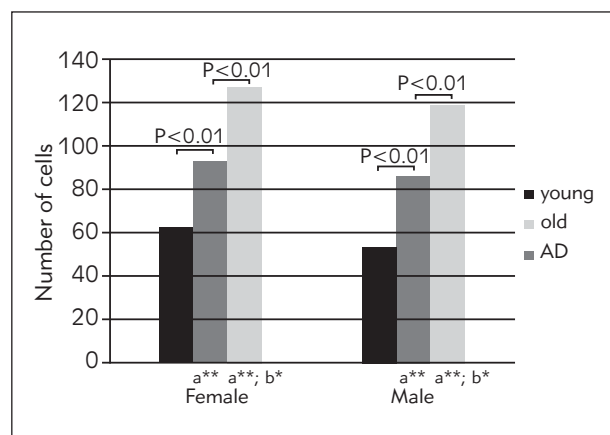


Figure 1 DNA damage of three groups of individuals: AD patients, elderly controls and young controls.

*, $p < 0.05$; **, $p < 0.01$; a vs. young; b vs. old

There were no significant sex-dependent differences in untreated DNA damage in AD patients and control groups. Regarding β -estradiol induced gender dependent DNA damage, there were no significant differences in DNA damage between males and females after β -estradiol treatment in all the examined groups. Therefore, the applied concentration of β -estradiol caused a statistically significant positive response in the Comet assay in all the groups, regardless of gender.

On the other hand, *Figure 1* shows a statistically significant difference in the frequency of DNA damage was observed between the AD group and elderly controls. The frequencies of DNA damage were significantly increased in male patients with AD ($p < 0.05$) when compared to those of the corresponding controls. Likewise, females diagnosed with AD also displayed a significant and marked increase in the frequency of DNA damage ($p < 0.05$) in comparison to control group values. Comparisons between young individuals and the elderly group showed significant differences ($p = 0.51$) among the two groups, displaying higher percentages of DNA damage ($p < 0.01$) in the elderly males and females.

Discussion

Lymphocytes are among the most frequently chosen biological material to study harmful effects resulting from metabolic changes, induced by tested compounds in different concentrations (17). Using the alkaline version of the Comet assay, we have assessed DNA damage by using various therapeutic concentrations of β -estradiol in the peripheral blood lymphocytes of Alzheimer's disease, elderly and young donors.

The results of these investigations demonstrate that there is a clear enhancement in the level of DNA

damage in the peripheral blood lymphocytes of both genders in all the abovementioned treatment groups, treated with different concentrations of β -estradiol. In this study, it was confirmed that cells were sensitive to 30 min treatment with β -estradiol at tested concentrations (50, 100 and 250 $\mu\text{mol/L}$). It was also noticed, from the number of comet-like nuclei, that there is a clear dose-dependent increase in DNA damage in all the groups.

Though Alzheimer's disease is considered a neurodegenerative disorder, investigation in non-neuronal cells is equally important, not only for shedding light on the etiology of the disease, but also for possible diagnostic purposes and monitoring the progress of the disease. For the evaluation of frequency and the level of DNA damage among the three treatment groups used in this study, the Comet assay was used as an invaluable method for exploring fundamental aspects of DNA damage and how the cell consequently responds to stress (18). The results of this work demonstrate the presence of genomic instability in peripheral blood lymphocytes, both in AD patients and healthy subjects of the same age category in relation to the young. Based on the number of cells with DNA damage, the expression of genetic instability is more prominent in AD subjects compared to age-matched controls (19–21). It is accepted that oxidative stress plays a major role in the pathology of Alzheimer's disease (22–24). Oxidative stress represents the loss of balance between the processes of the creation of free radicals and those that are responsible for the removal of oxidized macromolecules (antioxidative cascade) which again leads to cellular dysfunction (changes in cellular homeostasis) and results in increased genomic instability and eventually in cell death (25). Most likely, the oxidative stress is the essence of a greater DNA damage compared to adequate control (18, 26, 27) as one of the initial factors that lead to further genomic instability (28–30). There is substantial evidence supporting the fact that genetic instability is increased in AD lymphocytes compared to corresponding controls. An important aspect of genetic research related to AD is the analysis of lymphocytes chromosome instability which includes: aneuploidies of different chromosomes, telomere shortening and the phenomenon of premature centomere division (31–33). Also, an impaired DNA repair mechanism, particularly the base excision repair pathway, might play an essential role in Alzheimer's disease (34).

Because of their lipophilic nature, steroid hormones can freely and quickly cross the cell membranes (35). Since lymphocytes express receptors for estrogens as well, the steroid hormone has the ability to cross cellular membranes and allows binding to nuclear receptors exhibiting genomic action by altering gene expression and changing DNA (36).

Recent research has shown that estrogens can be involved in redox cycling because of the presence of a phenolic group in the molecule which can be converted to catechol estrogens (37). Free radical generation by redox cycling of catechol estrogens and catechol estrogens-derived quinones are known to produce DNA adducts (38) such as 8-hydroxydeoxyguanosine (39). Chemically modified bases can be further turned into DNA breakage due to the incomplete repair of damaged bases. The deleterious prooxidant effects of catechol estrogens are displayed by the presence of redox-active metal ions in high tissue concentrations. The effects of oxygen free radical-mediated toxicity become evident through formation of single-strand DNA breaks (40). In agreement with the previous findings, this work confirms that high experimental concentrations of β -estradiol create oxidative stress and oxidative radical DNA damage. Experimental findings support this assumption, showing that the antioxidant enzyme catalase successfully attenuates DNA damaging effects induced by estradiol in the Comet assay, both in human lymphocytes (41, 42) and sperm (13). Thus, if tested concentrations of β -estradiol influence the extent of endogenous oxidative DNA damage in lymphocytes, similar differences should be visible in the level of DNA damage in the examined cells from tested groups.

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- Our study demonstrates that β -estradiol within the range of investigated concentrations does not exhibit protective and antioxidant effects on lymphocytes in AD patients. In elderly and Alzheimer's disease subjects β -estradiol causes an additional increase of basal DNA damage. Investigated concentrations of β -estradiol enhance oxidative processes during ageing and neurodegenerative processes characteristic for AD. Our results favor the group of researchers who believe that estrogen therapy does not lead to cognitive improvement. It would be useful to examine the influences of such estrogen concentrations *in vivo*, that is, in patients on adequate estrogen therapy.
- Therefore, it can be concluded that lymphocytes are sensitive to test concentrations of β -estradiol in the Comet assay regardless to gender, age and the health condition of examined subjects. The role of β -estradiol in cellular DNA damage has been confirmed.
- Acknowledgements.** The work was supported by the Serbian Ministry of Education, Science and Technological Development (grant # 173034).
- Conflict of interest statement**
- The authors stated that there are no conflicts of interest regarding the publication of this article.
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