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Dynamics of estrogen-induced oxidative stress

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The objective of this study was to assess the dynamics of oxidative damage to cellular macromolecules such as proteins, lipids and DNA under conditions of oxidative stress triggering early stages of estrogen-dependent carcinogenesis. A rodent model of carcinogenesis was used. Syrian hamsters were sacrificed after 1, 3, 5 h and one month from the initial implantation of estradiol. Matching control groups were used. Kidneys as target organs for estradiol-mediated oxidative stress were excised and homogenized for biochemical assays. Subcellular fractions were isolated. Carbonyl groups (as a marker of protein oxidation) and lipid hydroxyperoxides were assessed. DNA was isolated and 8-oxodGuo was assessed. Electron paramagnetic resonance spectroscopy was used to confirm the results for lipid peroxidation. Exposition to estradiol in the rodent model leads to damage of macromolecules of the cell, including proteins and DNA, but not lipids. Proteins appear to be the primary target of the damage but are closely followed by DNA. It has previously been speculated that protein peroxides can increase DNA modifications. This time sequence was observed in our study. Nevertheless, the direct relation between protein and DNA damage still remains unsolved.

Keywords: protein oxidation, lipid peroxidation, DNA damage, 8-oxodGuo, estradiol, carcinogenesis

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women worldwide (American Cancer Society, 2001; Krajowy Rejestr Nowotworów, 2003). The role of estrogens in the development of estrogen-dependent human breast cancer has been previously underlined but has not been precisely settled so far (Mobley & Brueggemeier, 2002). Although not conclusive, ample biochemical evidence exists to suggest that estradiol (E) and/or estrogen metabolites play a role in breast cancer initiation and progression. Supposedly, estrogen receptor (ER) interactions and/or catechol estrogen (CE) formation involves an oxidative stress-mediated pathway (Mobley & Brueggemeier, 2002). Under the condition of oxidative stress various biomolecules are damaged. Those include proteins and lipids and most interestingly DNA. Oxidative damage to DNA is of particular importance since, contrary to proteins and lipids, entirely new molecules cannot be synthesized to replace the damaged ones. Although background levels of oxidatively damaged DNA exist, oxidative stress can lead to damage increase, which has been

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Abbreviations: CE, catechol estrogens; 5-DSA, 5-doxylstearic acid; ER, estrogen receptors; GSH, reduced glutathione; HPLC-EC, high-performance liquid chromatography with electrochemical detection; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; S, short series experimental group; U, ultra short series experimental group.

described in various pathological conditions, such as carcinogenesis, chronic inflammation/infection, ageing, neurodegenerative and cardiovascular disease (Cooke *et al.*, 2006; Olinski *et al.*, 2007). In the case of estrogens the DNA damage is being potentiated by proliferation stimulation, and that is why even quantitatively insignificant DNA damage may result in tumorigenesis (Malins *et al.*, 1993). Molecularly, the steroid-receptor complex interacts with sequence-specific estrogen response elements in target cell chromatin to induce gene expression and to promote growth of target cells such as breast epithelial cells and estrogen-dependent mammary carcinoma cells (Bowcock, 1999).

Identification of the biological targets of oxidative stress and the initial time sequence is considered as being crucial for a rational control of its deleterious effects (Du & Gebicki, 2002). Although it is commonly believed that lipid peroxidation is primary to protein oxidation, there is increasing evidence reporting proteins to be the early and, in some conditions, the only extranuclear target of oxidative stress (Du & Gebicki, 2002; Kobiela *et al.*, 2002). Du and Gebicki documented no lipid peroxidation at the time of formation of measurable amounts of protein hydroperoxides in cell lines exposed to radiation-generated hydroxyl free radicals. However, DNA oxidation was detected with a 5-h delay as compared to protein in that study (Du & Gebicki, 2002). They concluded that DNA, though an ultimate but not the first target of the randomly-generated hydroxyl radicals, may gradually become degraded by agent(s) generated within the cells in response to direct action of the radicals. Among the various types of oxidative damage to cellular macromolecules, damage to DNA is particularly hazardous as it usually alters the genetic information both in the nuclear and mitochondrial DNA (Friedberg *et al.*, 1995). Damage to the genomic DNA often leads to cell death and the development of degenerative diseases, while nonlethal mutations result in neoplasms and hereditary diseases (Friedberg *et al.*, 1995). Experimental data indicate that CE formed during exposition of susceptible tissues to estradiol are capable of damaging DNA under acute conditions in cell culture, this is even more severe in ER positive cells (Chen *et al.*, 2000; Mobley & Brueggemeier, 2002). Schnurr *et al.* (1996) pointed to a possible specific interaction of the semiquinone radical with membrane proteins. Whether the DNA damage is a direct result of radical action, indirect *via* oxidized proteins or both is still not known. Although the protein oxidation, contrary to lipid peroxidation, is not considered a chain reaction (Renke *et al.*, 2000), its ability to damage DNA has been proved (Gebicki & Gebicki, 1999; Luxford *et al.*, 1999). On the other hand, the mechanism of estrogen influence may also involve second messenger systems activated by receptors situated in the cell membrane (Wellejus & Loft, 2002).

The experimental model used in this study is a reliable rodent model of human estrogen-dependent breast carcinoma (Roy & Liehr, 1999). In this model of carcinogenesis chronic exposition of male Syrian hamsters to estradiol produces kidney tumors with incidence reaching 100% within 12 months of the experiment. The carcinogenetic potential is due to the activity of P450 CYP1B1 that hydroxylates estradiol at the C-4 position (Spink *et al.*, 1992; 1994; 1998; Hayes *et al.*, 1996; Jefcoate *et al.*, 2000). This leads to further spontaneous cyclic generation of catechol estrogens and other primary free radicals (Stefaniak *et al.*, 2002). Such redox cycling should contribute to the increased oxidative DNA damage that has also been detected in human breast cancer tissue (Malins & Haimanot, 1991; Malins *et al.*, 1993; Musarrat *et al.*, 1996; Matsui *et al.*, 2000). Our previous studies in this experimental model proved selective protein (and not lipid) oxidation of the kidneys as target organs during a 4-week exposure of Syrian hamsters to estradiol (Kobiela *et al.*, 2002). It is our belief that a deeper insight on very early changes, namely at 1-, 3- and 5-h intervals after the exposure to the hormone, would help to understand the pathomechanism of estrogen-induced carcinogenesis. It was well established that lipid hydroperoxides (LOOH) as an initial product of lipid peroxidation can damage the proteins both *in vitro* and *in vivo* (Kim *et al.*, 1997; Tsai *et al.*, 1998; Refsgaard *et al.*, 2000). It has also been proved that some proteins localized inside the lipid matrix can separately react with free radicals irrespective of unsaturated lipids at an unusually high rate comparable with classical membrane antioxidant like α-tocopherol (Hashimoto *et al.*, 2000). Considering early occurrence of these changes, ultra short experiments (1, 3, 5 h) were used in addition to 1 month exposition, to enable the detection of oxidation products at very early stages.

The objective of this study was to assess the dynamics of oxidative damage to cellular macromolecules such as proteins and lipids under conditions of oxidative stress triggering early stages of estrogen-dependent carcinogenesis. Furthermore, the time sequence obtained would be used for possible explanation of interactions between the oxidized macromolecules i.e. proteins, leading to DNA oxidation.

MATERIALS AND METHODS

Chemicals. All reagents were of the highest grade commercially available and were used without further purification. 17-β-Estradiol was purchased from Sigma-Aldrich Co. Hormone pellets and suspensions were prepared instantly before administra-

tion to the animals as described before (Kobiela *et al.*, 2002).

Animals. Sixty male Syrian hamsters aged 4 weeks, weighing approx. 80 g each were used in this study. After arrival, the animals were acclimatized for 1 week. Then the animals were divided into four treatment groups: 15 hamsters (A) were implanted subcutaneously with 25 mg of 17-β-estradiol, 30 hamsters (B) were intraperitoneally injected with 75 mg of 17-β-estradiol (in 1 ml of 0.5% carboxymethylcellulose saline solution). Another 15 animals (C) were intraperitoneally injected with the vehicle alone.

All the animals were kept in the animal facility at room temperature, standard humidity and 12 h day/night circadian cycle. They were fed with standard chow and were given water *ad libitum*.

All the procedures concerning animals were approved by the Local Ethical Committee (LEC) and performed according to the instructions authorized by LEC.

Experimental design. The experiments were performed in two series: S (short) and U (ultra short). Animals of the S series (25 mg of estradiol and controls) were sacrificed after one month from the initial implantation of estradiol. The same number of animals of the U series (75 mg of estradiol and controls) were sacrificed after 1, 3 and 5 h of the experiment. The kidneys as target organs for estradiol-mediated oxidative stress were excised for further biochemical assays. The excised organs were immediately placed on ice.

Preparation of homogenates. The kidneys from animals of each treatment were combined and homogenized in 150 mM KCl, 10 mM Tris/HCl, pH 7.4, to form a 10% (for carbonyl groups and lipid hydroperoxide assessments) or 20% (for subcellular fraction isolation) suspension. For DNA extraction, the kidneys were homogenized in 1 ml ice-cold buffer (0.1 M NaCl, 10 mM EDTA, 10 mM 2-mercaptoethanol and 0.5% Triton X-100, pH 8.0).

Isolation of subcellular fractions. A 20% homogenate was prepared from the excised kidneys in 150 mM KCl, 10 mM Tris/HCl, pH 7.4, in a Potter-Elvehjem Teflon homogenizer by 50 gentle up-and-down strokes. Nuclei and cell debris were removed by centrifugation (600×*g*, 10 min), the supernatant was collected and centrifuged again (10000×*g*, 90 s). The pellet (mitochondrial fraction) and supernatant were separated. The supernatant was recentrifuged (15000×*g*, 30 min) in order to remove the light mitochondrial fraction. The obtained supernatant (cytosolic fraction) was centrifuged for 60 min at 100000×*g* in order to obtain the microsomal fraction.

Carbonyl group assessment. This was performed as described by Oliver *et al*. (1987). Each sample to be measured was prepared by combining 100 μl of the homogenate with 100 μl of 20 mM solution of 2,4-dinitrophenylhydrazine — DNPH (Sigma-Aldrich Co.), whereas the respective control sample was made of the equivalent amount of the homogenate and 100 μl 2 M HCl (Sigma-Aldrich Co.). All the samples were incubated at room temperature for 60 min and vigorously shaken every 10 min. The reaction was stopped by an addition of 500 μl of 20% trichloroacetic acid solution (Sigma-Aldrich Co.). The sample was centrifuged at 1000×*g* for 7 min. The supernatant was discarded and the pellet was washed twice with 1 ml and then with 2 ml of 1:1 (v/v) ethanol/ethyl acetate (POCh S.A., Poland; Sigma-Aldrich Co.). Each washing step was followed by centrifugation at 1000×*g* for 7 min and disposal of the supernatant. After the final centrifugation the pellet was suspended in a solution of 6 M guanidine (Sigma-Aldrich Co.), pH 6.5 and then incubated at 50°C with continuous shaking till the pellet dissolved. The colorimetry was performed at the wavelength of 360 nm and the carbonyl group level was quantified per mg of protein *versus* the respective control sample.

Lipid hydroxyperoxide assessment. Lipid hydroperoxides (LOOH) from samples of Syrian hamster kidneys were determined according to Thomas and Poznansky (1990). This highly sensitive method permits to measure lipid peroxides at a level as low as 2 nmol per 1 mg of protein. The assay mixture contained 50 mM sodium acetate (pH 4 for lipid peroxides and pH 4.5 for standard cumene hydroperoxide), 0.1% Triton X-100, 100 μl of 1.6 mM *N,N,N,N*-tetramethylbenzidine in dimethylformamide, hemoglobin (100 μg for lipid peroxides, and 300 μg for cumene hydroperoxide) and 5–50 nmol of peroxide in a total volume of 2 ml. The reaction was started by adding pure hemoglobin and the tubes were immediately transferred to an ice bath, kept closed with a lid in order to protect lipid peroxides against light. After 5 min of incubation in the ice bath, tubes were taken one at a time, 100 μl of 1 M HCl was added and mixed and absorbance at 466 nm was measured immediately against a blank.

DNA isolation and 8-oxodGuo assessment. DNA was isolated using the method described earlier (Gackowski *et al.*, 2002). Determination of 8-oxodGuo by means of HPLC-EC technique was described previously (Foksinski *et al.*, 1999). Briefly, the homogenized kidneys were centrifuged at 4°C for 10 min at 1000 x *g*, and the resulting pellets were resuspended in 0.5 ml lysis solution (120 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.5% SDS, pH 8.0). RNA and protein were digested by incubation with RNase or proteinase K at 55°C for 30 or 60 min, respectively. After extraction, by successive mixing with saturated phenol, a mixture of phenol/chloroform/isoamyl alcohol (25:24:1), and then a mixture of chloroform/

isoamyl alcohol, DNA was precipitated by the addition of five volumes of ethanol (–20°C). The precipitate was removed with a plastic spatula, washed with 70% ethanol, and after centrifugation dissolved in nuclease P1 buffer (40 mM sodium acetate, 0.1 mM $ZnCl_2$, pH 5.1).

DNA samples in nuclease P1 buffer (200 μl) were mixed with nuclease P1 solution (20 μg protein). Samples were incubated for 1 h at 37°C. Thereafter, 30 μl of 1 M Tris/HCl, pH 8.5, and 5 μl of alkaline phosphatase solution containing 1.5 units of the enzyme were added to each sample following 1 h incubation at 37°C. All DNA hydrolysates were ultrafiltered using cut off 5000 Da filter units. 8-OxodGuo and dG in hydrolysates were determined using HPLC with electrochemical and UV detector. The HPLC system (Dionex Corporation, Sunnyvale, CA, USA) consisted of a M480 pump, Gina 50 autosampler, 250 × 4.6 mm LC18S column (5 μm grain) equipped with 20 mm precolumn, and two detectors working in series: UV-VIS (UVD 340S) and Coulochem II 5200A electrochemical detector (ESA, Inc, Chelmsford, MA, USA). DNA hydrolysates were chromatographed isocratically using 25 mM sodium acetate, 12.5 mM citrate, pH 5.0, buffer/methanol (89:11). Detection of dGuo was performed at 254 nm. 8-OxodGuo was determined by the electrochemical detector: guard cell: +750 mV, detector 1: +130 mV (as a screening electrode), detector 2: +450 mV (as a measuring electrode set to sensitivity of 50 nA/V). Acquisition and quantitative analysis of the chromatograms were carried out using Chromeleon 4.3 software (Dionex Corporation). The amount of 8-oxodGuo in DNA was calculated as the number of 8-oxodGuo molecules per 10^6 unmodified dGuo molecules.

Electron paramagnetic resonance spectroscopy and spin labeling techniques. Characteristics of microsomal membrane polarity can be examined by 5-doxylstearic acid (5-DSA), which enables the assessment of the arrangement of the lipids. It is expressed in values between 0 and 1 where 0 represents isotropy, while 1 represents complete anisotropy. 5-DSA dissolved in chloroform was evaporated under argon. Freshly prepared microsomal fractions (10 mg of protein) were incubated in spin label-coated tubes with gentle agitation for 30 min at 20°C. Microsomal membrane lipid peroxidation was assessed by EPR spectroscopy using a paramagnetic reporter group incorporated into membranes which give a characteristic spectrum upon excitation with microwaves in a magnetic field. In brief, the increased production of reactive oxygen species can result in significant accumulation of more polar hydroperoxides. The amphipathic spin probe 5-DSA is thought to intercalate within both halves of a lipid bilayer so that its long alkyl chain is roughly parallel to the alkyl chain of membrane lipids, and its paramagnetic nitroxide moiety near the lipid–water interface. Rapid anisotropic motion occurs about the long axis of the spin probe providing opportunity to detect polarity changes at the relevant EPR parameter of +1 low-field line. A reliable parameter of the environment of 5-DSA calculated from direct measurements of the parallel and perpendicular components of the hyperfine tensor of the spin label, is the order parameter S (Schreier *et al*., 1978). The value of the order parameter S is from zero for totally anisotropic motion of the label molecule to one – when its motion is ideally anisotropic.

Statistical analysis. Each assessment was performed three times and as the data were not significantly different, an average of the three measurements was calculated. The analysis was performed using Student's *t*-test with Bonferroni's correction.

RESULTS

The weight of the testes was used as a marker of estrogenization efficiency in series S and proved to be at least 12-fold lower in each of the animals treated with the estrogen as compared to the control.

Assessment of lipid hydroperoxides and EPR spin labeling with 5-doxylstearic acid as lipid peroxidation induced perturbances of ordering of membrane phospholipids did not reveal statistically significant changes at any point of the experiment comparing to the controls (Figs. 1 and 2).

Protein oxidation presented significant changes when compared to the controls. Levels of carbonyl groups increased as soon as after 1 h exposition to the hormone and progressed further after 3 h to decrease substantially after 5 h. However, the increase remained almost 2 fold one month after the exposition (Fig. 3).

DNA oxidative damage measured as the level of 8-oxodGuo was unchanged after 1 and 3 h of the experiment. A significant 2-fold increase was

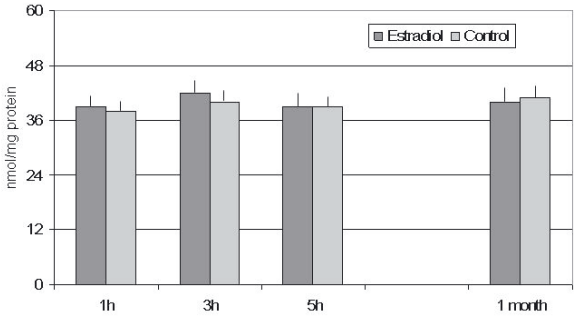

Figure 1. Level of lipid hydroperoxides as markers of lipid peroxidation in hamster kidney homogenates.

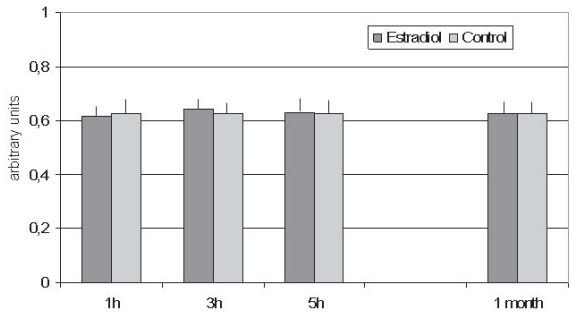

Figure 2. Order parameter S in microsomal fraction of hamster kidney homogenates as marker of lipid arrangement.

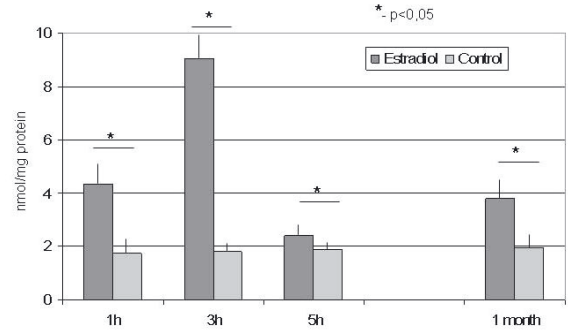

Figure 3. Level of carbonyl groups as markers of protein oxidation in hamster kidney homogenates.

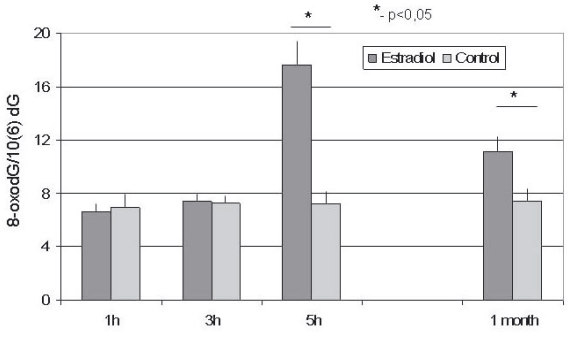

Figure 4. 8-OxodGuo levels as markers of DNA oxidation in hamster kidney homogenates.

observed after 5 h and a 1.5-fold increase remained after 1 month (Fig. 4).

DISCUSSION

Recent studies of hormonal carcinogenesis have tried to establish an approach that could give maximal benefit from supplementary estradiol therapy without the risk of carcinogenesis (Aggarwal & Ichikawa, 2005). Thus both genetic and molecular factors were identified to anticipate the possibility of cancer formation and the possibility to prevent it. In this study, the aim was to discover the molecular background of the changes as early as at the initial stage of the carcinogenesis. Most interestingly, membrane proteins appeared to be the early target of free radicals generated in the redox cycling of CE, what was proved by the results of carbonyl group analysis. Moreover, the lipid peroxidation was observed neither at the ultra early nor the early stage of the carcinogenesis. No lipid peroxidation was also reported by other authors working on a different model of nephrocarcinogenesis, when lower but still carcinogenic doses of ferric nitrilotriacetate Fe-NTA were used (Gautier *et al.*, 2001). However, our results do not completely exclude lipid peroxidation in this model. The earliest reaction was observed in carbonyl groups as markers of protein oxidation. Moreover, our group has shown previously that the extent of the damage was the highest in the microsomal compartment of the cell which seems to confirm the crucial importance of P450 CYP1B1 activity in this model (Kobiela *et al.*, 2002). The membrane protein damage could also simplify the p53 protein redistribution outside the nucleus as it was presented in Molinari's study concerning estradiol influence on an estradiol-dependent lung cell line (Molinari *et al.*, 2000). In this view, the DNA damage that appeared in a delayed mode similarly to results reported by Du and Gebicki (2002) may be an effect of either prolonged impact of the primary and secondary free radicals or "peroxidation transmission" from oxidized proteins. Such transmission was demonstrated by Luxford *et al.* (1999). Nevertheless, this latter hypothesis should be considered preliminary and needs further investigations.

Wellejus and Loft suggested that oxidative effect could be receptor mediated. It could be that catechol estrogen bound to the estrogen receptor is transported to estrogen-sensitive genes in the nucleus, where redox cycling may take place (Wellejus & Loft, 2002). The limited change in oxidized and total glutathione levels would be consistent with localized redox cycling of CE in the nucleus, where the pool of glutathione is considered more resistant to depletion than the cytoplasmic pool (Bellomo *et al.*, 1992).

In our study, during the acute exposition to estradiol there was a remarkable time-dependent limitation of both protein and DNA oxidative damage. These could be due to counteracting activity of antioxidants such as GSH present in the cells, that would be exploited to a certain point, but the severity of the oxidative stress exceeded the protective capacity of intracellular or easily accessible extracellular antioxidants. Moreover, there are several antioxidative defense and repair enzymes, just to mention glutathione-*S*-transferase, catalase, superoxide dismutase (Li *et al.*, 1995; Lebovitz *et al.*, 1996) or cat-

echol *O*-methyl transferase (Mobley & Brueggemeier, 2002), whose level was reported to be increased (due to activation or induction) under the condition of oxidative stress and in several neoplasms.

When considered in terms of cell survival, DNA would be the ultimate target of oxidative stress and that implies two possible pathways depending on the severity of the damage and the energetic status of the cell. Those two pathways would be cell death *via* apoptosis/necrosis or replication of the changes that would lead to carcinogenesis/mutagenesis. It should be remembered that the DNA damage also includes mitochondrial DNA (mtDNA).

In our experiment, the activity of DNA repair enzymes seemed effective enough to ensure the cell survival, however, it was insufficient to prevent DNA damage under condition of protein oxidation. It should be added that 8-oxodGuo is not the only possible DNA alteration, as there are several different base modifications as well as strand breaks, apurinic sites and others, that are more or less susceptible to repair mechanisms (Shen *et al*., 1997; Chen *et al*., 1998; Zhang *et al*., 1999). The lifelong, free-radical derived accumulation of alterations in human tissue during aging is implicated both in degenerative and proliferative diseases. And thus, it may well be the major cause of these diseases, including hormonal carcinogenesis (Friedberg *et al*., 1995).

To conclude, exposition to estradiol in a rodent model mimicking human breast carcinoma leads to formation of CE, which *via* redox cycling damage the macromolecules of the cell, including proteins and DNA, but not lipids. Proteins appear to be the primary target of the damage but are closely followed by DNA. It could also be concluded that the protective and repairing DNA mechanisms, though initially active, are no longer efficient as the exposition to the carcinogen prolongates. On the other hand, it has been established that protein peroxides can increase DNA modifications (Gebicki & Gebicki, 1999). This time sequence was observed in our study. Nevertheless, the direct relation between protein and DNA damage still remains unsolved. In practice, better understanding of this process could contribute to better molecular modeling of antioxidants aiming at selectively blocking the protein peroxidation. Such molecules could be used in additional antioxidative therapy, parallel to hormonal substitution or contraception to prevent cancer induction.

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