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Oestradiol Protects Against the Harmful Effects of Fluoride More by Increasing Thiol Group Levels than Scavenging Hydroxyl Radicals

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Abstract: The aim of the study was to investigate the role of oestrogens in free radical detoxication upon exposure to fluoride. Interactions between xenobiotics and oestrogens need to be investigated, especially as many chemicals interact with the oestrogen receptor. It is still unknown whether free radical-generating xenobiotics can influence the antioxidative ability of oestradiol (E_2). In an *in vitro* examination of human placental mitochondria, thiobarbituric active reagent species (TBARS), hydroxyl radical ($^{\bullet}$OH) generation and protein thiol (–SH) groups were detected. 17β-E_2 was examined in physiological (0.15–0.73 nM) and experimental (1–10 μM) concentrations and sodium fluoride (NaF) in concentrations of 6–24 μM. E_2 in all the concentrations significantly decreased lipid peroxidation measured as the TBARS level, in contrast to NaF, which increased lipid peroxidation. Lipid peroxidation induced by NaF was decreased by E_2. The influence of E_2 on $^{\bullet}$OH generation was not very significant and depended on the E_2 concentration. The main mechanism of E_2 protection in NaF exposure appeared to be connected with the influence of E_2 on thiol group levels, not $^{\bullet}$OH scavenging ability. The E_2 in concentrations 0.44–0.73 nM and 1–10 μM significantly increased the levels of –SH groups, in contrast to NaF, which significantly decreased them. E_2 at every concentration reversed the harmful effects of NaF on –SH group levels. No unfavourable interactions in the influence of E_2 and NaF on TBARS production, $^{\bullet}$OH generation, or –SH group levels were observed. The results suggest that postmenopausal women could be more sensitive to NaF-initiated oxidative stress.

Many studies have reported that free radicals play a significant role in the pathogeneses of certain human diseases. Chemicals can also have toxic effects by forming free radicals (e.g. CCl_4) and reactive oxygen species (e.g. doxorubicin). Recently, the ability of oestrogens as reactive oxygen species scavengers *in vitro* and *in vivo* has been well-documented. However, it is known that oestradiol (E_2) can also act as a pro-oxidant [1,2]. Oestrogens can protect low-density lipoprotein from *in vitro* oxidation induced by different pro-oxidant systems and prevent lipid peroxidation in microsomes [3–5] and liposomes. Inhibition of the oxidation of low density lipoprotein has been observed in postmenopausal women after administration of E_2 [6,7]. It is still unknown whether these functions can be disrupted by free radical-generating xenobiotics, which are present in the environment and can accumulate in the body. Interactions between xenobiotics and oestrogens need to be investigated, especially as many of the chemicals interact with oestrogen receptors (xenoestrogens). Many chemicals influence the reactive oxygen species generation and can also interact with oestrogen along this pathway.

Fluorine is very common in nature. Fluorine-containing compounds can be consumed with naturally or artificially fluorinated water, in food or as pills (for dental caries, osteoporosis) or by environmental pollution. Although we know enough about the toxicity of fluoride, there is still little knowledge about its influence on free radical processes in human cells. Our previous studies showed that fluorides are capable of free radical generation *in vitro*. Recent reports have also demonstrated *in vivo* and *in vitro* that fluoride increased lipid peroxidation in human erythrocytes and tissues of experimental animals [8,9].

The aim of the present study was to investigate the role of hormones in the free radical detoxication process upon exposure to fluoride. In this article, the influence of E_2 on lipid peroxidation, hydroxyl radical ($^{\bullet}$OH) generation and the levels of thiol groups (–SH) in oxidative stress caused by sodium fluoride (NaF) was examined. The antioxidative ability of oestrogen was studied on the tert-butyl hydroperoxide (t-BOOH) and Fe/ascorbate models. The question was whether E_2 can protect cell membranes against lipid peroxidation initiated by NaF. It was interesting to discover whether E_2 is able to prevent the harmful effects of NaF on lipid peroxidation or instead stimulates the fluoride effect by interaction, causing higher sensitivity to exposure in women than in men. The results could provide some information on whether postmenopausal women are more vulnerable or more resistant to free-radical processes of fluoride or fluoride therapy. Measurement of OH$^{\bullet}$ and protein thiol groups could also help to understand the mechanism of action and the possible interactions on free radical processes. E_2 was examined in physiological (nM) and experimental (μM) concentrations. The first part of the experiment evaluated

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the influence of fluoride in the pre- and postmenopausal period and the second during simultaneous hormone replacement therapy and fluoride supplementation. The interaction of ecotoxins with drugs needs to be investigated especially because hormonal replacement therapy is widely used.

Materials and Methods

The study was conducted in an *in vitro* model. Mitochondria were isolated from human placenta from natural deliveries using the methods described by Dlugosz *et al.* [10] and stored at –80° for no longer than three months until use [11]. Mitochondrial proteins were measured by Lowry's method [12]. The protein concentration in the mitochondria used in the tests was 1 mg/ml. The concentration of thiobarbituric active reagent species (TBARS) was determined by the method with thiobarbituric acid (TBA) [13]. The degree of protein damage was measured by determination of the level of protein thiol groups with Ellman's reagent [14]. The generation of •OH was investigated by measuring the degree of deoxyribose degradation using Rice-Evan's method [14]. The concentrations of TBARS, •OH, and –SH groups were expressed relative to the amount of mitochondrial protein.

17β-E_2, Fe(II)sulfate heptahydrate ($FeSO_4$), 2-TBA, tert-butyl hydroperoxide (t-BOOH) (70% aqueous solution), ascorbic acid, 5,5′-dithiobis-(2-nitrobenzoic acid), lauryl dodecyl sulfate, butylated hydroxytoluene, and deoxyribose were purchased from Sigma and NaF from POCh Gliwice. All chemicals were of analytical grade. E_2 was dissolved in ethanol to be dispersed in mitochondrial rinsing buffer (pH 7.4). Final concentrations of E_2 of 0.15, 0.29, 0.44, 0.59, and 0.73 nM and 1, 2, 5, and 10 μM were obtained. NaF was dissolved in water and used at concentrations of 3, 6, 12, and 24 μM.

Determination of TBARS concentration

Evaluation of the influence of E_2 or NaF on malondialdehyde concentration in human placental mitochondria. The mitochondrial suspension was pre-incubated at 37° for 15 min. with various concentrations of E_2. Control experiments were done using the same amount of solvent without E_2 (K1 or K2). Lipid peroxidation was initiated using the systems (i) Fe/ascorbate: 20 μM $FeSO_4$ and 100 μM ascorbate (final concentrations) or (ii) 30 μl 1% t-BOOH. Incubation was carried out at 37° for 30 min. and stopped by adding 0.5 ml of 20% TCA. Then, 30 μl of a 1% ethanol solution of butylated hydroxytoluene and 1.5 ml of a 0.67% TBA solution were added. The mixture was heated at 95° for 15 min. The cooled mixture was centrifuged to pellet the protein. The absorbance of the sample was measured spectrophotometrically at 535 nm. Microsomal lipid peroxidation was expressed as nanomoles of TBARS per milligram of protein. Malondialdehyde values were calculated using a molar extinction coefficient of 156 mM^{-1} /cm at 535 nm.

When NaF was examined, lipid peroxidation in the mitochondrial suspension was first initiated using 20 μM $FeSO_4$ and 100 μM ascorbate and the reaction mixture was incubated at 37° for 30 min. Then, the mitochondrial suspension was incubated at 37° for 30 min. with various concentrations of NaF. Control experiments were done using the same amount of solvent without NaF and chemicals (K3). The TBARS concentration was measured by the methods described above.

Evaluation of E_2 interactions with NaF on TBARS concentration in human placental mitochondria. Evaluation of the interactions was done in the following concentrations:

1. E_2: 0.29, 0.44, 0.59, and 0.73 nM and 1 and 10 μM (final concentrations)
2. NaF: 6, 12, and 24 μM (final concentrations)

The mitochondrial suspension was pre-incubated at 37° for 15 min. with E_2. Lipid peroxidation was initiated using the systems (i) Fe/ascorbate: 20 μM $FeSO_4$ and 100 μM ascorbate (final concentrations) or (ii) 30 μl of 1% t-BOOH. The incubation was continued at 37° for 30 min. Then, NaF was added and incubation was carried out at 37° for 30 min. Control experiments were done using the same amount of solvent without E_2 and NaF (K4). The TBARS concentration was measured by the methods described above.

Determination of •OH generation

Evaluation of the influence of E_2, NaF, and E_2 after exposure to NaF on OH generation in mitochondria. The mitochondrial suspension in a quantity of 0.5 ml was incubated at 37° for 15 min. with 15 μl of 1% t-BOOH, 0.5 ml of 20 mM deoxyribose, and E_2, NaF, or E_2-NaF mixtures at the proper concentrations. After incubation, the samples were centrifuged and 0.8 ml of the supernatant was collected. To the supernatant were added 0.5 ml of 2.8% TCA and 0.5 ml of 1% TBA in 0.1 M NaOH. The samples were incubated at 100° for 20 min. Then, the samples were cooled and centrifuged. The level of •OH generation was measured spectrophotometrically at 532 nm and calculated using the molar coefficient of absorption 1.56×10^5 M^{-1} cm^{-1}. The results were compared with control samples prepared in the same way but with solvents instead of E_2, NaF, or E_2–NaF mixtures.

Determination of the level of protein thiol groups

Evaluation of the influence of E_2, NaF, and E_2 after exposure to NaF on protein thiol group level in human placental mitochondria. Mitochondrial suspension (200 μl) was incubated at 37° for 15 min. with E_2 or NaF at the proper concentration. When the influence of E_2 on exposure to NaF was studied, the mitochondrial suspension was pre-incubated with E_2 (15 min. at 37°), then NaF was added and the incubation was continued at 37° for 15 min. Then, 200 μl of dodecyl sulphate and 1.6 ml of 10 mM sodium phosphate buffer (pH 8.0) were added. The absorbance was read at a wavelength of 412 nm in relation to blind tests (A_0). Afterwards, 200 μl of Ellman's reagent was added to the samples. The samples were incubated at 37° for 1 hr. The absorbance was measured at 412 nm (A_1). Simultaneously, control trials were prepared with the same composition as the investigated tests but instead of Ellman's reagent, 200 μl of 10 mM sodium phosphate buffer (pH 8.0) was added. The difference in absorbance, A_1–A_0 (after subtracting the values obtained analogously in control trials), was a measure of the –SH group content in the test samples. The concentration of the –SH groups in the samples was calculated using a molar absorption coefficient of 13.6 l $mmol^{-1}$ cm^{-1}. The results were compared with the control samples prepared in the same way but using solvents instead of E_2, NaF, or E_2–NaF mixtures.

Statistical analysis

Results are expressed as means ± S.E.M. using the statistical programme Statistica PL 7.1. The normality of the distribution of the results was analysed using the Shapiro-Wilk test. Significance of the differences in the examined variables with a normal distribution was evaluated by Student's t-test or ANOVA. When the data did not follow the Gaussian distribution, the Mann–Whitney *U* nonparametric test or nonparametric multiple comparison (Kruskal–Wallis ANOVA) was used. A value of $P < 0.05$ was considered statistically significant.

Results

It was found that E_2 in concentrations of 0.29–0.73 nM caused statistically significant ($P < 0.001$ to $P < 0.007$) decreases in lipid peroxidation measured as TBARS concentration compared with the control (K1) in both models (fig. 1a). Protective properties of E_2 in 1–10 μM concentrations against iron-induced lipid peroxidation were also noted. E_2 statistically significantly ($P < 0.009$ to $P < 0.04$) decreased TBARS levels in the mitochondria in all the concentrations used compared with the control (K2, fig. 1b).

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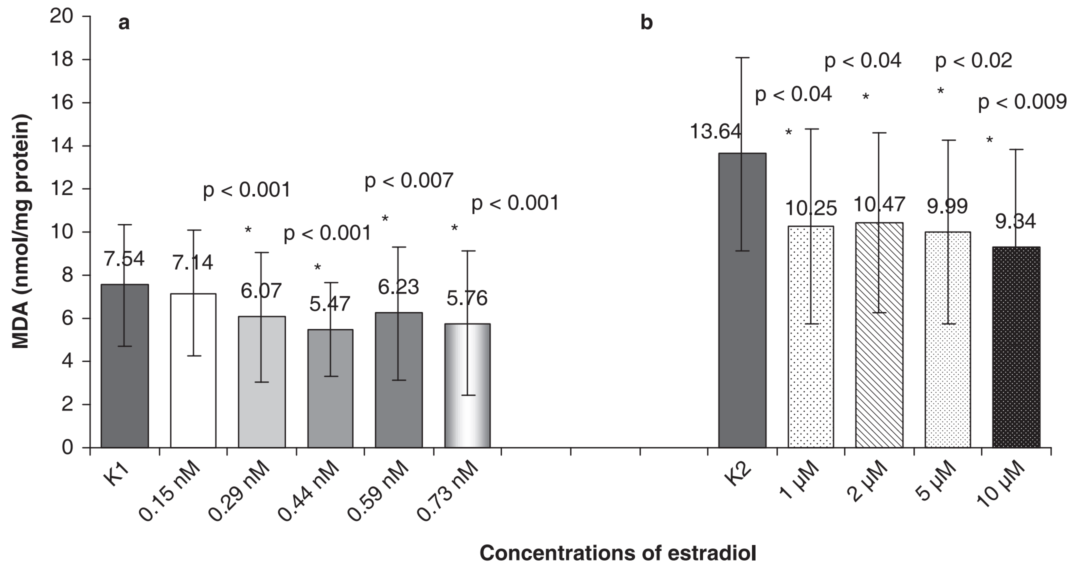

Fig. 1. The influence of oestradiol on TBARS concentration in the t-BOOH (a) and Fe/ascorbate (b) models in mitochondria.

NaF in concentrations of 6–24 μM increased TBARS generation in iron-induced lipid peroxidation. The changes were statistically significant ($P < 0.001$) compared with the control (fig. 2). A previous study with t-BOOH-induced lipid peroxidation showed a similar effect [8].

No statistically significant changes in TBARS level compared with controls were observed for E_2 (0.29–0.73 nM) and all the concentrations of NaF (fig. 3). However, it should be remembered that NaF itself produced an increase of lipid peroxidation, so when final results are compared with the action of NaF, a statistically significant decrease in lipid peroxidation caused by E_2 was noted. Full antiradical effect of E_2 at all the concentrations used (0.29–0.73 nM) of high significance ($P < 0.001$) was observed on lipid peroxidation caused by NaF at concentrations of 6 and 12 μM when compared with the action of NaF (fig. 3). The effect of E_2 on 24 μM NaF was definitely the weakest ($P < 0.02$ to $P < 0.04$). In any event, a protective effect of E_2 (E_2 was given prior to NaF) on the free radical action of NaF was observed.

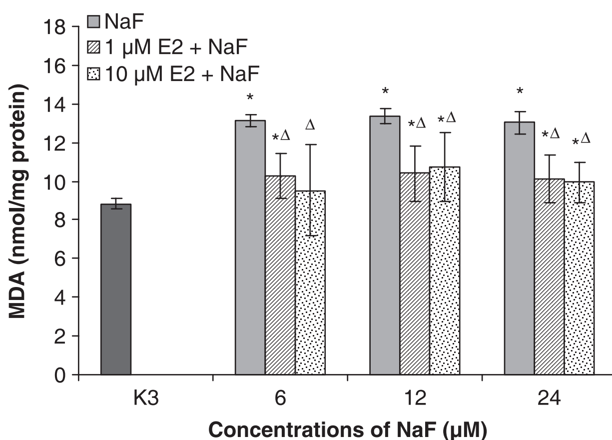

Fig. 2. The influence of sodium fluoride and the effect of oestradiol (μM) after exposure to NaF on TBARS concentration in the Fe/ascorbate model in mitochondria.

In the next step of the study, the effect of concentrations of E_2 higher than physiological (1 and 10 μM) on the action of NaF were examined. Statistically significant ($P < 0.03$ to $P < 0.05$) increases in TBARS concentration compared with the control were observed in the mitochondria treated with 1 μM E_2 and all the concentrations of NaF used (fig. 2). The results indicated that E_2 at a concentration of 1 μM did not totally reduce lipid peroxidation caused by NaF to physiological levels because the combined effect was still higher than the control, but statistically significantly lower than the effect of fluoride.

A fully protective effect of 10 μM E_2 was observed towards lipid peroxidation caused by NaF at the lowest concentration (6 μM). Mitochondrial membranes exposed to 6 μM of NaF and 10 μM of E_2 did not produce increased TBARS levels compared with the controls (fig. 2). The results indicate that E_2 completely inhibits lipid peroxidation compared with the control in only one case (lowest dose of NaF and highest dose of E_2). A protective effect of E_2 against lipid peroxidation caused by fluoride can be concluded, but the inhibition was not complete because the peroxidation was still higher than that of the control.

It was observed that E_2 at concentrations of 0.44 and 0.73 nM caused statistically significant decreases in $^{\bullet}OH$ generation ($P < 0.03$ and $P < 0.001$, respectively, fig. 4). No influence of other E_2 concentrations (μM) on $^{\bullet}OH$ generation was observed (lack of significance compared with the control) (date not shown). The results indicate that E_2 at physiological concentrations partly inhibits $OH^{\bullet}$ generation and that the mechanism of action of E_2 in free radical

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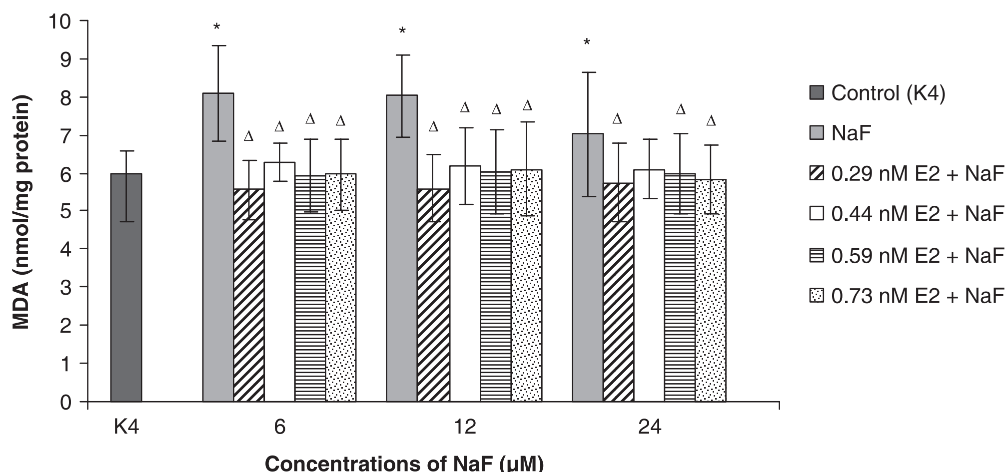

Fig. 3. The influence of sodium fluoride and the effect of oestradiol (nM) after exposure to NaF on TBARS concentration in the t-BOOH model in mitochondria.

processes depends on its concentration. At physiological concentrations of E_2 (nM), the pathway connected with $^{\bullet}$OH scavenging is more pronounced than at higher concentrations (μM).

Our previous study on the influence of NaF on $^{\bullet}$OH generation showed that NaF at the concentrations used (3–24 μM) did not cause significant increases in $OH^{\bullet}$ generation [8].

Studies on the joint influence of E_2 and NaF on $^{\bullet}$OH showed that the activity of E_2 (nM) towards NaF in all cases did not influence $OH^{\bullet}$ generation (data not shown). These results may indicate that the influence of E_2 and NaF on free radical processes engages a mechanism that is not dependent on $OH^{\bullet}$ radicals. The experiments also show that there was no adverse interaction between E_2 and NaF in $^{\bullet}$OH generation processes.

E_2 at concentrations of 0.44–0.73 nM caused statistically significant increases ($P < 0.002$ to $P < 0.04$) in protein thiol group levels in mitochondrial suspensions compared with the control. A similar effect was obtained with the higher range of E_2 concentrations (μM) ($P < 0.001$ to $P < 0.002$, fig. 5). The experiments demonstrate that E_2 has a protective effect on protein thiol groups.

NaF showed a quite opposite effect. NaF statistically significantly decreased –SH levels in mitochondria at concentrations of 6 μM ($P < 0.04$) and 12 μM ($P < 0.006$) compared with the control (fig. 6).

The protective effect of the lowest E_2 concentration (0.29 nM) on –SH groups was observed only towards the lowest exposure of NaF (6 μM). The higher range of physiological concentrations (0.44–0.73 nM) was more effective. A significant increase in –SH group levels ($P < 0.001$ to $P < 0.04$) after exposure to 6 μM of NaF was observed compared with the control (fig. 6). The higher concentration of E_2 (1 μM) also increased the –SH group levels, which was lowered by NaF. The effect was observed for every NaF concentration and with high significance ($P < 0.001$) in comparison with the control (fig. 6). Similarly, 10 μM of E_2 statistically significantly increased –SH levels in mitochondria exposed to 6 μM ($P < 0.004$) and 24 μM of NaF ($P < 0.001$) compared with the control (fig. 6). In general, it could be concluded that E_2 at every concentration (even physiological) reverses the harmful effects of low-dose NaF exposure (6 μM) on the thiol group level. The 1-μM concentration of E_2 was able to reverse the harmful effects of every examined dose of

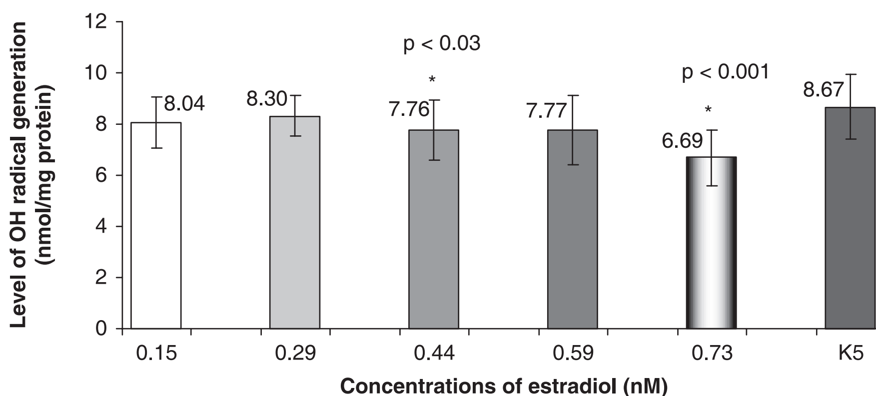

Fig. 4. The influence of oestradiol in nanomoles on hydroxyl radical generation in mitochondria.

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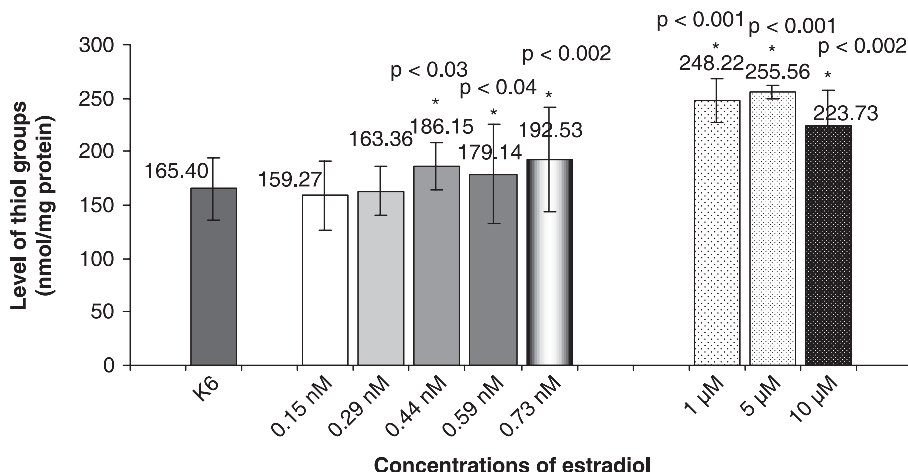

Fig. 5. The influence of oestradiol in nanomoles (a) and micromoles (b) on –SH level in mitochondria.

NaF (6–24 μM) on the thiol group level in mitochondria. The linear correlation between nM E_2 influence on TBARS concentration and its influence on the thiol group level (r = –0.44; P < 0.001) was noted.

Discussion

Disturbance of the natural equilibrium of free radical generation causes dysfunction and promotes oxidative damage to tissues. It is well-known that reactive oxygen species plays a significant role in the pathologies of over one hundred diseases. Many reports describe the antioxidative properties of E_2 [4,6,15]. Its pro-oxidant activity is also known [1,2,16]. It has also been demonstrated that environmental toxins can disturb the oxidative balance in the body, increasing the level of free radicals [17,18]. There is no knowledge about how they interact with hormones in free radical processes. Recent reports stating that exposure to xenobiotics can even cancel the effect of hormonal replacement therapy (e.g. smoking) [19] show that the problem of hormone–xenobiotic interaction is very important and should be a subject of research. NaF is an environmental toxin, appearing, for example, in fluorinated water, toothpaste, and drugs.

It is of interest to know whether fluorine can influence hormonal antioxidative properties and whether E_2 can protect against the harmful effects of fluorine connected with the influence on free radical processes and oxidative stress in the body. The gap in our understanding of possible E_2–fluorine interactions should be filled, especially with respect to hormonal replacement therapy and the sensitivity of postmenopausal women to environmental toxins, such as fluorine. The purpose of this study was to examine the influence of E_2 on lipid peroxidation caused by NaF, that is, whether it produces a harmful interaction or a protective effect. The mechanism of activity was investigated by measuring $^{\bullet}$OH generation and the level of thiol groups. The results could enhance those of our preliminary study on the role of oestrogens in exposure to toxins with regard to the sensitivity of women to fluoride, especially postmenopausal women.

In many *in vitro* studies evaluating oxidative stress, the final products of lipid peroxidation, malondialdehyde, have been frequently used as markers of free radical generation

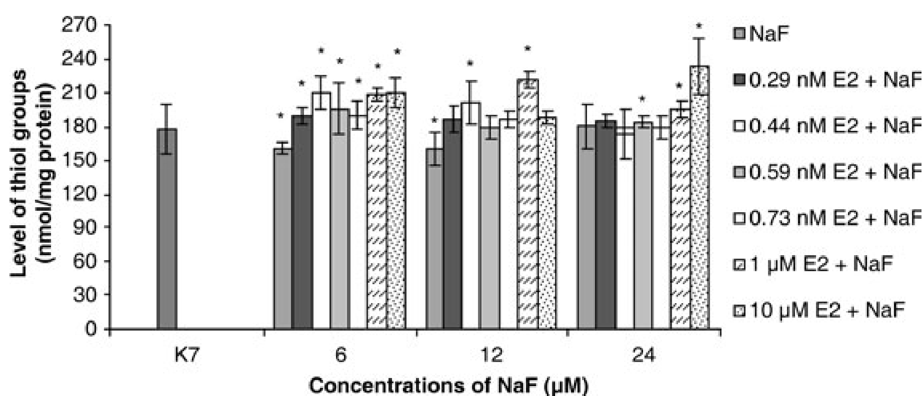

Fig. 6. The influence of NaF and the combined action of sodium fluoride (at three doses: 6, 12, and 24 μM) with oestradiol in low (nM) and high (μM) concentrations on –SH level in mitochondria.

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[3,20,21]. It reacts with TBA and could be expressed as TBARS, however, many other compounds react with TBA. In order to make the TBARS method more specific for malondialdehyde measurement, the inhibitor of spontaneous peroxidation 3,5-diisobutyl-4-hydroxytoluene was added to the samples. In the present study, E_2 at all the concentrations used, both high (μM) and physiological (nM), decreased malondialdehyde level and demonstrated a preventive effect on mitochondrial membranes. This effect was obtained in two lipid peroxidation-induced systems, that is, t-BOOH and Fe/ascorbate. In many other *in vitro* studies with the Fe/ascorbate, Fe/ADP, or t-BOOH models, an antioxidant effect of E_2 was also noted [3–5,16,21]. The reports showed that oestrogens are especially effective in iron-induced peroxidation. Their metabolites are able to reduce Fe (III) and inhibit Fe auto-oxidation. They can also chelate iron ions and affect iron level [4,7,15,21]. It was found that oestrogens at concentrations higher than physiological are effective inhibitors of *in vitro* peroxidation under a variety of conditions. They interact with metal-derived species and protect high density lipoprotein levels [5,18]. An antioxidant effect of 17β-E_2 was observed in postmenopausal women after E_2 infusion [7] and transdermal administration [6].

The *in vitro* studies demonstrated that E_2 and 2-hydroxyestradiol had strong inhibitory activities only when oestrogens were added to the NADPH- and ADP-Fe^{+3}-dependent system before the start of lipid peroxidation. The reason is that oestrogens inhibit the initial stage of lipid peroxidation. In contrast, 2-OH–E_2 showed inhibitory effect even when added during lipid peroxidation induced by t-butyl hydroperoxide-Fe^{+2}. This suggests that it interacts more with alkoxyl than with peroxyl radicals. The antioxidant effect of E_2 depends on the presence of a phenol hydroxyl group, which can donate hydrogen atoms to a lipid-derived radical. The oestrogen phenoxyl radical formed after hydrogen donation is stabilized by the internal delocalization of electrons in the aromatic ring. The scavenging activity of E_2 increases after 4-hydroxylation. 4-OH–E_2 is 15 times more potent against $^{\bullet}$OH radical than E_2 [3].

Some investigations of fluoride compounds on free radical processes and lipid peroxidation were recently reported. A positive correlation between a high concentration of fluoride and increased malondialdehyde concentration in tissues was found [8,22–24]. When HL-60 cells were exposed to NaF, the malondialdehyde and 4-hydroxynonenal levels increased in the cells and a loss of mitochondrial membrane potential was observed [24]. Studies carried out on rats treated with NaF showed that an abundance of NaF (25–100 ppm) can induce lipid peroxidation [9,23].

Previous reports described lipid peroxidation stimulation by fluoride in a mitochondrial/t-BOOH model [11]. Similar results were obtained in the present study with the Fe/ascorbate model. NaF at concentrations above 6 μM significantly increased TBARS concentration in mitochondrial suspensions.

Our study on the influence of fluoride on the antioxidant properties of E_2 indicates that E_2 has a moderate effectiveness in inhibiting the lipid peroxidation caused by NaF. Statistically significant results were obtained for physiological (0.29–0.73 nM) and experimental (1 and 10 μM) concentrations of E_2 in comparison with the action of NaF, but not with the controls. β-E_2 inhibits lipid peroxidation induced by NaF, but it does not restore the physiological state. Although oestrogens are important scavengers both in the aqueous and lipophilic phases, their antioxidative ability is limited by the hydrophilic or lipophilic nature of the scavenged radical [15]. This can probably influence the preventive effect of E_2 on lipid peroxidation during exposure to NaF. In *in vivo* studies, it was shown that physiologically low doses of oestrogen have regulatory effects on the activity of the natural enzymatic antioxidant system [6,25]. However, there is no *in vitro* study which examined the effects of physiological doses of E_2 on antioxidant enzymes. There is also a lack of information about interactions between E_2 and NaF. Our results indicate no harmful joint effect of E_2 and NaF. No additive or synergistic interaction in the influence on lipid peroxidation was found.

Studies on the mechanism of E_2 on lipid peroxidation and DNA damage inhibition showed that E_2 causes a significant reduction in the production of superoxide radical in bovine heart endothelial cell cultures, but has no effect on the formation of neither hydrogen peroxide nor $^{\bullet}$OH [26]. E_2 counteracts the accumulation of peroxides in the intercellular compartment but it does not effectively inhibit $^{\bullet}$OH radical generation [15]. A recent investigation of the neuroprotective effect of E_2 points to a role of quinol, which can be formed directly from E_2 and $^{\bullet}$OH without the participation of metabolic enzymes. E_2 produces by $^{\bullet}$OH-binding a non-phenolic quinol with no affinity to the oestrogen receptors. The quinol is then rapidly converted back to the parent oestrogen via enzyme-catalyzed reduction using NAD(P)H as a coenzyme (reductant) and, unlike the redox cycling of catechol oestrogens, without the production of reactive oxygen species. [27]. Little is known about the influence of physiological concentrations of E_2 on free radical processes. In our *in vitro* studies, it was observed that E_2 at concentrations of 0.44 and 0.73 nM caused significant decreases in $^{\bullet}$OH generation compared with the control, but higher concentrations of E_2 (μM) did not cause changes in $^{\bullet}$OH generation. Thus, the influence of E_2 on OH radical generation seems to be a complex and unexplained problem.

According to our knowledge, there are no reports on the combined effects of E_2 and NaF on $^{\bullet}$OH generation. Our study showed that the inhibitive effect of E_2 (nM) on lipid peroxidation caused by NaF is not connected with $^{\bullet}$OH scavenging ability. An adverse interaction between E_2 and NaF in $^{\bullet}$OH generation processes was also not observed. The level of lipid peroxidation also depends on enzyme activity (SOD, GPx, or catalase) and superoxide anion radical and H_2O_2 generation. A previous study showed that the effect of NaF is probably connected with superoxide anion generation [8]. An *in vitro* study on isolated rat mastocytes incubated with fluoride indicated that the cells released superoxide anion radical [28].

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Proteins are important cell membrane components with sensitive –SH groups. Direct oxidation of protein thiol groups caused by reactive oxygen species takes place under conditions of oxidative stress. Superoxide radicals especially cause oxidation of –SH groups, leading to membrane disintegration and permeability. Thiolate radicals ($S^{\bullet}$) and disulfides are the main products of oxidation. It has been demonstrated that superoxides are removed by glutathione peroxidase and thiolate radicals by glutathione, but disulfides are reduced by thioredoxin [29].

In the present study, E_2 in a wide range of concentrations (0.44–0.73 nM and 1–10 μM) caused significant increases of –SH level in mitochondria. This shows that E_2 has a protective influence on –SH groups in mitochondrial suspensions. Other *in vitro* studies have demonstrated that exogenous 17β-E_2 used during oxidative stress in rat osteoblasts caused significant increases in glutathione concentration and thioredoxin. These compounds play significant roles as thiol antioxidants [30]. Increased intracellular glutathione has also been observed in the blood of women treated with hormone replacement therapy. It has been suggested that thiol groups play a significant role in the protective effect of hormone replacement therapy [31]. Our results show that this mechanism is involved in the hormonal protection against fluoride. The thiol groups are protected by the reduced glutathione increased by E_2. It has been demonstrated that E_2 also increased the level of thioredoxin, which plays a main role in the reduction of disulfide back to thiols [32,33]. E_2 induction of glutathione peroxidase is also reported [6,25]. This seems to have an important impact of the antioxidant E_2 on fluoride toxicity.

There are no known studies which investigated the levels of thiol groups in mitochondrial membrane when oestrogens or NaF are used. Our investigation showed that NaF at concentrations of 6 and 12 μM significantly decreased –SH level in mitochondrial suspensions compared with controls. The oxidation of thiol groups and decreased –SH level are certainly involved in the mechanism of oxidative stress induced by fluoride. There are also some reports about a similar effect of fluoride [9,24]. In mitochondrial suspensions exposed to NaF, it was observed that E_2 at concentrations of 0.29–0.73 nM and 1 and 10 μM with almost all the NaF concentrations protected –SH groups against the oxidative effects of NaF. The linear correlation between nM E_2 influence on malondialdehyde concentration and its influence on the thiol group level ($r = -0.44$; $P < 0.001$) confirms the conclusion. In summary, a mechanism connected with protecting protein thiol groups seems to have greater importance in the influence of oestrogens on the oxidative stress induced by NaF than a mechanism connected with $^{\bullet}$OH scavenging.

Conclusions

1. E_2 at all the concentrations used decreased lipid peroxidation stimulated by t-BOOH or Fe/ascorbate measured as the TBARS level, in contrast to NaF, which increased lipid peroxidation.
2. E_2 at nM and μM concentrations decreased lipid peroxidation caused by NaF.
3. The influence of E_2 on $^{\bullet}$OH generation depends on the E_2 concentration.
4. E_2 at concentrations of 0.44–0.73 nM and 1–10 μM significantly increased protein thiol group levels, in contrast to NaF, which significantly decreased them.
5. E_2 at every concentrations used, reversed the harmful effects of NaF on thiol group levels.
6. It can be concluded that the protective effect of E_2 against the effect of NaF on lipid peroxidation is more connected with its protection of thiol groups than on $^{\bullet}$OH generation.
7. No unfavourable interactions in the influence of E_2 and NaF on lipid peroxidation, $^{\bullet}$OH generation, or protein thiol group levels were observed.

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