Cyclosporine A (CsA) is a cyclic hydrophobic polypeptide isolated from fungus Tolypocladium inflatum. It has immunosuppressive, anti-inflammatory and antiallergic properties which are connected with the inhibition of calcineurin (1). Nowadays CsA is widely used in transplantology as a strong immunosuppressive drug. It is also active in a treatment of many autoimmunological diseases, for example: rheumatoid arthritis, glomerulonephritis, Crohn's disease or allergic diseases such as atopic asthma, atopic dermatitis, and allergic rhinitis (2). There are many reports that CsA may induce generation of reactive oxygen species (ROS). People are exposed to a lot of various ecotoxins and one of them is fluorine. The compounds of fluorine are necessary to correct tooth formation and prevent of cariogenesis. However, compounds of fluorine are toxic and may cause a lot of deleterious effects so their level in environment requires constant monitoring (3). The mechanism of toxic effects of fluorine is not completely explained. It is not clear whether it may be connected with reactive oxygen species generation and influence on the oxidative-reductive balance of organism. There are many researches on animals confirming the ability of fluorine compounds to the generation of free radicals. There are also reports that CsA may induce generation of reactive oxygen species (ROS).

The aim of presented work was to examine the joint effect of CsA and NaF on oxidative stress in human cells in vitro. The oxidative stress level was determined by measurement of malondialdehyde (MDA) concentration, generation of hydroxyl radical (\(^{\cdot}\)OH) and sulfhydryl groups of proteins content.

EXPERIMENTAL

The study was performed on an in vitro model using mitochondria isolated from human placenta obtained after physiological deliveries from Medical University Obstetric-Gynecological Clinic. Mitochondria were isolated by homogenization of the material in 5 mM Tris-HCl buffer, pH 7.4 folding from 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA and 0.2% bovine serum albumin (BSA). The homogenate was centrifuged at 3000 ◊ g at the temperature of 4°C and then recentrifuged under the same conditions at 14,000 ◊ g. Mitochondrial supernatant was collected and washed three times in 50 mM Tris-HCl buffer, pH 7.4, containing 0.575% KCl. The mitochondria obtained were stored at -80°C for no longer than 3 months (4, 5). The proteins in mitochondria were measured by Lowry method (6).

Sodium fluoride (NaF) (M: 41.99 g/mol, POCh Gliwice) was used in aqueous solutions and a concentration of: 3, 6, 12, and 24 µmol/mL; cyclosporine A (Sandimmun, cyclosporinum, Novartis) in aqueous solutions and concentrations as follows: 75, 150 and 300 ng/mL.
The degree of lipid peroxidation was measured by MDA concentration with thiobarbituric acid method (7). The generation of hydroxyl radical was investigated by measurement of deoxyribose degradation degree (8). The proteins damage was estimated by the content of sulfhydryl groups with the Ellman’s method. The concentration of MDA, \( \cdot \mathrm{OH} \) and SH groups was expressed on mitochondrial protein amount.

The evaluation of the cyclosporine A and sodium fluoride mixtures influence on the MDA concentration

The mitochondrial suspension in the quantity of 1 mL was stimulated by 30 min incubation with 30 \( \mu \)L 1% of \( \cdot \)BOOH at 37°C. Then the samples were incubated at 37°C for 30 minutes with 60 \( \mu \)L of mixtures of CsA and NaF in proper concentrations. After that to the samples of 0.5 mL of 20% trichloroacetic acid (TCA), 30 \( \mu \)L 1% butylhydroxytoluene (BHT) and 1.5 mL of 0.67% thiobarbituric acid (TBA) were added and incubation was carried out at 85°C for 15 min. MDA concentration was measured spectrophotometrically at 535 nm. The results were calculated using a molar absorption coefficient of \( 1.56 \times 10^5 \) M\(^{-1}\)cm\(^{-1}\) and expressed as nmoles per mg of mitochondrial protein.

The results were compared to the control samples prepared in the same way, but without mixtures of CsA and NaF.

The evaluation of the CsA and NaF mixtures effect on hydroxyl radical generation in mitochondria

The mitochondrial suspension in quantity of 0.5 mL was incubated at 37°C for 15 min with 0.5 mL of 20 mmol/L deoxyribose, 15 \( \mu \)L of 1% \( \cdot \)BOOH and 30 \( \mu \)L mixtures of CsA and NaF in proper concentration. After incubation, samples were centrifuged and 0.8 mL of supernatant was collected. 0.5 mL of 2.8% TCA, and 0.5 mL of 1% TBA in 0.1 M NaOH were added to the supernatant and the samples were incubated at 85°C for 15 min. Then the samples were cooled and centrifuged. The level of hydroxyl radical was measured spectrophotometrically at 532 nm and calculated using the molar coefficient of absorption of \( 1.56 \times 10^5 \) M\(^{-1}\)cm\(^{-1}\). The results were compared with control samples prepared in the same way, but without the mixtures of CsA and NaF.

The evaluation of the influence of CsA and NaF mixtures on level of proteins sulfhydryl groups

To 200 mL of the mitochondrial suspension was added 30 \( \mu \)L of the mixture CsA and NaF in the suitable concentration and the sample was incubated at 37°C for 15 min. Then, 200 \( \mu \)L of sodium dodecyl sulfate (SDS) was added and the samples were mixed, then 1.6 mL of the 10 mmol buffer of sodium phosphate, pH = 8 was added. The absorbance was read at 412 nm in relation to blind tests (A0). Afterwards, 200 \( \mu \)L of the Ellman’s reagent was added to the samples, and they were incubated at 37°C for 1 h, and the absorbance was measured at 412 nm (A1). Simultaneously, control trials were prepared at the same composition as investigated tests but instead of the Ellman’s reagent 200 \( \mu \)L of 10 mmol of buffer of sodium phosphate pH 8 was added. The difference of the absorbances A1-A0 (after subtracting the analogously received values for control trials) was a measure of the content of SH groups in the test samples. The concentration of -SH groups in the samples was calculated on the basis of standard curve for the glutathione with the regard of the millimolar absorption coefficient of 13.6 mmol\(^{-1}\) L cm\(^{-1}\).

Statistical analysis

The results were rated statistically with the program Statistica PL 6.0. The variation of the distribution was checked by the Lillefors test. Significance of differences of examined variables with normal distribution was evaluated with the Student t-test. Differences at \( p < 0.05 \) were accepted as statistically significant.

RESULTS

Our previous in vitro study in Toxicology Department showed that both CsA (in concentrations >150 ng/mL) and NaF caused an increase of lipid peroxidation measured by MDA level. There are not any researches on joint effect of CsA and NaF on lipid peroxidation and possible interactions between these compounds. In this study the influence of mixture CsA and NaF on free radicals processes in vitro was investigated.

In the first instance, the combined influence of CsA and NaF on lipid peroxidation was evaluated. It was noticed that every mixture except one caused statistically significant increase in MDA level in comparison to the control (Figure 1). Only the mixture of CsA in concentration of 75 ng/mL with 3 \( \mu \)mol/L NaF did not cause statistically significant increase of MDA level in comparison to the control samples (a lack of significant changes). The other investigated mixtures caused statistically significant (\( p < 0.05 \)) increase of lipid peroxidation, measured as concentration of MDA, in relation to the control...
Interactions in free radicals processes between cyclosporide A and sodium fluoride

Figure 1. The influence of NaF and mixtures CsA with NaF on MDA level in mitochondrial suspension; * statistically significant samples in relation to the control (K), (p = 0.000001-0.002929), ∆ statistically significant samples in relation to the NaF samples, (p = 0.000004-0.011007).

Figure 2. The influence of NaF and mixtures CsA with NaF on sulfhydryl group content in mitochondrial suspension; * statistically significant samples in relation to the control (K), (p = 0.001857-0.031779).

Figure 3. The influence of NaF and mixtures CsA with NaF on hydroxyl radical concentration in mitochondrial suspension; * statistically significant samples in relation to the control (K), (p = 0.004834-0.017992).

samples (Figure 1). In order to evaluate the effect of CsA towards peroxidation caused by sodium fluoride the results were compared with single NaF action. In that case it was observed that only the mixture with highest CsA concentration (300 ng/mL) plus NaF in all used concentrations caused statistically significant increase of MDA level in relation to the samples with NaF alone (Figure 1). So it may be concluded that CsA in concentrations 75 and 150 ng/mL did not give any harmful interactions with NaF. However, it
seems that CsA in concentration of 300 ng/mL can interact with NaF in the additional way, not toxicological one (no synergic effect).

In the next stage of experiment the combined influence of CsA and NaF on sulfhydryl groups content in mitochondrial suspension was estimated. In previous study it was observed that NaF caused statistically significant decrease of sulfhydryl groups in comparison to the control (9). It was noticed that CsA in concentration of 75 ng/mL and NaF in all used concentrations (3, 6, 12, and 24 µmol/L) did not cause statistically significant changes in sulfhydryl groups content in relation to the control samples. Similar test data were obtained in case of other used concentrations of CsA and NaF (Figure 2). These results may indicate that simultaneous exposure of CsA and NaF did not cause adverse changes on sulfhydryl group content.

It was interesting to know in which way mixtures of CsA and NaF influence on lipid peroxidation. So the next step of the study was the estimation of joint effect of CsA and NaF on hydroxyl radical generation. In our investigation all used mixtures of CsA (75, 150, and 300 ng/mL) and NaF (3, 6, 12, and 24 µmol/L) did not cause any significant changes in hydroxyl radical level in relation to the control sample (Figure 3). Previous study performed in Toxicology Department demonstrated that NaF by itself did not cause \*OH generation similarly like CsA in concentrations 30 – 600 ng/mL. These results may indicate that there is not any adverse interaction between CsA and NaF in \*OH generation process.

**DISCUSSION**

Free radicals reactions are one of the natural processes which take place in human organism. In physiological conditions a balance between reactive oxygen species (ROS) formation and antioxidant defense exists. Various chemicals from environment can often change this balance towards an increase of ROS formation and similarly, some drugs may act. There was a research showed that cyclosporine A is one of such drugs which increases MDA concentration what means a stimulant influence on lipid peroxidation processes. This could be connected with reactive oxygen species generation by cyclosporine A (10). Usually cyclosporine therapy is a long-lasting process so the problem of interaction with different xenobiotics should be considered, for example, environmental pollutants. In view of increasing environmental pollution, the supply of fluoride compounds to human organism increases. In 1942, total amount of fluorides received by people during 24 h period was approximately about 0.45 mg, but in the seventies – 4.4 mg (11). In regions of controlled level of sodium fluoride in water the content of fluoride in blood plasma oscillates between 0.5 and 1.5 µmol/L (12). It is not possible to foresee precisely the concentration of fluorides because significant amount of them is contained in volcanic soils in endemic areas, for example China or Mongolia (3).

In these regions substantial amounts of fluorides can occur in drinking water and vegetables or animal food. Sodium fluoride is commonly present in human environment because it is used as addiction to toothpastes, gels, foams, mouthwashes and also in oral supplementation (for example pills with fluorides or drinking water fluorination) as dental caries prevention.

There is known a lot about interactions between cyclosporine and other drugs, but almost unknown are the interactions between CsA and ecotoxins. It seems to be interesting, if fluoride (e.g. from fluorinated water) can influence on cyclosporine metabolism in the organism. In the present study cyclosporine and sodium fluoride interactions and its influence on free radicals processes was investigated. In the first stage of experiment the joint effect of CsA and NaF on lipid peroxidation was evaluated. For this purpose the measurement of malondialdehyde (MDA) was performed. All used mixtures of CsA and NaF (excepting CsA in the concentration of 75 ng/mL and NaF in the concentration of 3 µmol/L) caused statistically significant increase of MDA level in relation to the control samples. NaF alone also caused statistically significant increase of MDA level. The obtained results were different depending on concentrations of CsA. CsA in the concentration of 75 or 150 ng/mL and NaF (3, 6, 12, and 24 µmol/L) did not cause any statistically significant changes in MDA concentration in comparison to single NaF effect. These results showed that in low concentrations (75, 150 ng/mL) CsA did not give any harmful interactions in lipid peroxidation level with NaF. The combined exposition on CsA in the concentration of 300 ng/mL and NaF caused an increase of MDA level in relation to exposition to NaF alone. This increase is statistically significant (p = 0,000004 – 0,011), however, it is smaller than the total sum of action of single compounds, because CsA and NaF used separately also increased MDA concentration. Not only the joint effect of CsA and NaF seems to be depended on the concentration of compounds. There are some reports that CsA action in free radicals processes may depend on its concentration. Sigh et al. reported that low doses of CsA (3 mg/kg
i.v.) protected from lipid peroxidation induced by ischemic reperfusion in rat kidneys (13). CsA concentrations in plasma of 70-120 ng/mL did not cause statistically significant changes of MDA level in children after transplantation (14). On the other hand, Chen et al. observed that CsA in therapeutic concentrations (500 ng/mL) caused an increase of lipid peroxidation in human spleen cells in vitro (15). It follows from these investigations that the low concentrations of CsA did not induce lipid peroxidation but high CsA concentrations stimulate it.

Both in vitro and in vivo study reported that fluorides caused the lipid peroxidation increase (16). Guana et al. observed that fluorides increased the level of oxidative stress in brains of animals exposed to compounds of fluorine. In animals brains the level of antioxidative enzymes decreased and the level of lipid peroxidation increased. It was observed that these adverse effects can be reduced by application of antioxidative vitamins C or E. Additionally, high concentration of fluorides can inhibit superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) (17). Similar study on in vitro model (hepatocytes of human fetus) confirms that exposition to NaF in doses of 40-160 µg/mL causes an increase of lipid products of peroxidation (LPO), a decrease of reduced glutathione level, DNA damage and apoptosis (18).

An in vitro study on apoptosis induced by NaF in HL-60 cells showed that exposition to NaF increases MDA and 4-hydroxynonenal (4-HNE) concentration. It decreases of mitochondrial membrane capability and increases of cytochrome c in cytosole. Antioxidants as N-acetylcysteine (NAC) or glutathione (GSH) protected cells from the change of membrane capability and cytochrome c release, which suggests that antioxidants can prevent apoptosis induced by NaF (19).

The study of CsA neuroprotective mechanism in excitotoxic neurodegeneration showed that CsA prevented mitochondrial swelling and cytochrome c release. This can be associated with CsA-evoked indirect inhibition of neuronal NOS connected with calcineurin inhibition (20).

Similarly as in the case of CsA, there are some reports that lipid peroxidation induced by NaF depends on its concentration. Such dependence was noticed in in vivo study performed by Shivar-jashankara et al. Wistar rats were administered with fluorides in doses 30 or 100 ppm. It was observed that 30 ppm of fluorides did not cause any significant changes in MDA level in red blood cells (RBC) but 100 ppm caused an increase of MDA concentration. In both groups the glutathione peroxidase activity increased but particularly in the groups exposed to 100 ppm. Superoxide dismutase activity in RBC decreased significantly after high doses of fluoride, but the ratio of reduced glutathione to total glutathione and uric acid level in RBC decreased in both groups (21).

The mechanism of interactions between CsA and NaF is not known. One of the mechanisms of xenobiotics toxic action is the release of free radicals through mobilization of toxic oxygen metabolite chain or biotransformation of xenobiotics to free radicals. Xenobiotics may be able to take single electron from cytochrome P-450 reductase. Then they form free radical which has high ability to transfer electron on molecular oxygen and to transform oxygen into superoxide anion radical. The radical form of xenobiotics are regenerated to parent form. In this way, as the result of alternate oxidation and reduction, from one molecule of xenobiotic a large amount of superoxide anion radicals can be formed and they are quickly transformed into hydroxyl radicals (22).

It is very possible that both CsA and NaF in specific concentrations caused the formation of ROS. CsA probably induces superoxide radical formation, which can undergo deoxygenation into *OH radical. By the action of NaF probably the *OH and **O radicals are formed (23, 24). To this conclusion lead the in vitro research on mastocytes isolated from rats incubated with fluoride. It was shown that as the result of an exposition on fluorides the mastocytes released histamine and generated superoxide radicals (24).

The mechanism of peroxidation can be connected with xenobiotic influence on initiation phase. It is usually happened by stimulation of *OH radical generation. It can be also caused by inhibition of enzymes (SOD, GPx, CAT) activities which result in formation of superoxide radical and hydrogen peroxide.

In order to explain the mechanism of joint action of CsA and NaF the influence on hydroxyl radical (°OH) generation was examined. The analysis of results indicates that all used mixtures of CsA and NaF did not cause statistically significant increase of the hydroxyl radical concentration. It was quite predictable result because CsA and NaF used separately did not stimulate °OH generation too. However, the interaction should not be excluded before an experiment.

The results received indicate that only in the case of usage in therapy of small CsA doses (75 and 150 ng/mL), NaF did not cause statistically significant increase of MDA production.
However, combined usage of CsA in a dose of 300 ng/mL and NaF increased the MDA concentration, which indicates the stimulation of free radicals processes.

It can be that in the case of lower concentrations of CsA the amount of formed \( \cdot \text{O}_2 \) radical is more or less the same like \( \cdot \text{O}_2 \) radical produced by NaF stimulation.

The next stage of experiment was the influence of CsA and NaF mixtures on sulfhydryl groups estimation. It was noticed that the tested mixtures did not influence on the concentration of sulfhydryl groups. Our previous investigation showed that NaF caused a decrease of sulfhydryl groups concentration in mitochondrial suspension.

The results indicate that there are not any adverse interactions in the influence on sulfhydryl groups of proteins between CsA and NaF. So the mechanism of MDA increase caused by a mixture of CsA and NaF stay still unclear and requires further research.

**CONCLUSIONS**

1. Any toxicological interactions in investigated parameters between CsA and NaF were not observed.
2. The presence of sodium fluoride causes that cyclosporine even in concentration below 150 ng/mL increases lipid peroxidation in comparison to the control, what seems to be the result of NaF action.
3. The combined exposure on CsA and NaF did not cause hydroxyl radical generation.
4. The mixtures of CsA and NaF did not cause any changes in sulfhydryl groups of proteins content.

**REFERENCES**


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