124 Research report
Fluoride 53(1 Pt 2):124-135
January 2020

A STUDY ON THE CYTOTOXIC EFFECT OF FLUORIDE ON THE HUMAN FIBROBLAST CELL LINE HS27

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ABSTRACT: Bibliographic data indicates that different types of cell cultures exhibit a detrimental effect upon fluoride (F) exposure, which in high concentrations inhibits enzyme activity and damages protein synthesis. Furthermore, there are data showing that F^- influences proliferation and apoptosis in healthy cells. The long ongoing discussion on either the beneficial or the harmful effects of fluoridation of water and dental products shows the need for further study on the subject. The aim of this study was to determine the concentrations and incubation times of F^- ions which would induce a cytotoxic effect on a normal, human fibroblast cell line (Hs27). Experiments were performed on an *in vitro* cellular model. The chosen cell line was selected due to the important role of fibroblasts in connective tissue. Changes in the cell membrane integrity were tested using the lactate dehydrogenase (LDH) cytotoxicity protocol. The determination of the mRNA level of human metalloproteinase-9 (hMMP9), collagen, and 3-mercapto-pyruvate sulfurtransferase (MPST), later extended by thiosulfate sulfurtransferase (TST), and cystathionine γ-lyase (CTH) was performed by RT-PCR. At the protein level, the amount of the chosen proteins was measured by the Western Blot technique. The changes in cell viability were visualized by crystal violet staining. A cytotoxic effect on fibroblasts was shown at concentrations of F^- ions equal to or higher than 0.012% NaF (3 mM), after 48 hr incubation. A reduction in cell numbers was confirmed after incubation with selected concentrations of the tested agent. No changes in the expression of hMMP9 and collagen were observed for the range of concentrations used. The changes in the mRNA levels of MPST, TST, and CTH were insignificant, even after incubation with the highest concentration of F, and were not confirmed at the protein level. The studies show that F^- ion accumulation may affect proliferation and cytotoxicity but no significant changes in the expression of the enyzmes tested have been established.

Keywords: 3-Mercaptopyruvate sulfurtransferase; Cystathionine γ-lyase; Fibroblasts; Sodium fluoride; Thiosulfate sulfurtransferase.

INTRODUCTION

Fluoride ions (F^-) can penetrate the cell membranes of both hard and soft tissues. Many negative effects of F^- have been reported on different types of cells, tissues, and living organisms.[1-3] In the metabolism of osteoblasts and osteoclasts, changes after exposure to F^- occurred with abnormal mineralization of bone tissue and the inhibition of oxidative pathways, as well as in the induction and the release of reactive oxygen species (ROS).[4-6] The most recent studies provide clear and detailed examples of the negative effect of F^-. Gutowska et al. showed that μmolar levels of F^- may activate apoptosis of the liver cell line HepG2, reducing their vitality after 48 hr incubation. This effect intensified when an increased concentration of NaF solution was added to the cell culture, up to 10 μM.[7] Gu et al. studied the osteoblastic cell line MC3T3-E1 after exposure to NaF at concentrations ranging from 1 to 5 mM for a period of 24 hr. The results showed that after exposure to NaF there was a promotion in the apoptosis rate and the intracellular ROS levels, and a blockage of the S phase of the cell cycle.[8] In a

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study by Chen et al. Neuro-2A cells were investigated for the effects of F^- on the cytoskeleton.[9] The Neuro-2A cells were exposed to 1, 2, 4, and 6 mM NaF for 24 hr. F^- reduced the cell viability, disrupted cellular membrane integrity, and produced high levels of lactate dehydrogenase (LDH). NaF suppressed the release of neurotransmitters, thus effecting neuronal function.[9] The effect of F^- from drinking water on major organs was tested in an *in vivo* Wistar rat model by Pereta et al.[10] There were histopathological changes in the liver with increased hepatocellular necrosis and portal inflammation which varied with the concentration of F^-. The possible induction of renal damage by long-term, high F^- exposure was also discussed.[10] The current literature describes various pathological changes in different species, with exposure to high dosages of F in drinking water, food, and the atmosphere, in the liver, kidney, heart, thyroid, nervous system, and reproductive abilities.[11] The risk of excessive F^- exposure and the ability of F^- to accumulate in the tissues has led to increased awareness of the presence of the addition of F^- to water and dental products. In contrast to the situation in the European Union where there is little water fluoridation, in certain countries, e.g. Canada, obligatory artificial fluoridation of water is more common and raises many questions.[12-14] Due to the ongoing discussions on the harmfulness of F^-, it was decided to conduct the present research to examine certain crucial aspects of F^- toxicity in a normal, human fibroblast cell line (Hs27).

MATERIALS AND METHODS

Cell line: The normal fibroblast cell line Hs27 from human neonatal foreskin was derived from ATCC (USA). Cells were propagated in standard conditions (37ºC, 5% CO_2) in DMEM High Glucose medium with the presence of 100 U/mL penicillin and 100 µg/mL streptomycin solution (HyClone) supplemented with 10% fetal bovine serum (FBS, Biowest). After 24 hr, the medium was changed and the cells were incubated in the absence or presence of the tested concentrations of F^-. The pilot experiments, to determine the appropriate concentrations, had been performed and the set of various concentrations had been tested in the Hs27 cell line starting from 1 mM and increasing to 8 mM. The selected panel of concentrations of F^- after the pilot experiments was: 1, 3, and 6 mM. The highest chosen concentration was the first in the panel showing a cytotoxic effect on fibroblast cells. To support our choice, we conducted a literature search regarding the effect of different concentrations of F^- that were used in cellular models in the existing research. In the studies of Szczepański et al. covering the apoptosis and activity of human umbilical vein endothelial cells, the concentrations of F used were listed as 5.0 and 7.5 mM and delivered in the form of NaF in the culture medium.[15] A study by Slompo et al. evaluated the influence of 1 mM F^- in culture medium on cell viability and the activity of matrix metalloproteinases (MMP) 2 and 9 secreted by preosteoblasts (MC3T3-E1 murine cell line) after 24, 48, 72, and 96 hr incubation.[16] In the Ren et al. studies, primary cultured mouse osteoblasts where tested in the presence of 1 and 10 mM NaF in the medium and the effect on proliferation and apoptosis after 48 hr was assessed.[17]

Evaluation of cytotoxicity and quantitation of viable cells by crystal violet staining:

The cells were seeded and grown on 96-well plates until they were 90% confluent. The medium was changed, the respective concentrations of F^- were added. Incubation was continued for 24 and 48 hr. The medium was collected and analyzed for LDH activity using the Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific), according to the producer's manual. The remaining cells were treated with 0.2% crystal violet solution by modified Gillies et al. method.[18]

Expression of beta-actin, hMMP-9, collagen, MPST, CTH, and TST:

a) Isolation of RNA: Total RNA was extracted with Tri-Reagent (Lab Empire) following the method described by Chomczyński and Sacchi.[19]

b) Reverse transcription: 3 µg of isolated RNA was reverse transcribed with Reverse Transcriptase in 5 X Reaction buffer (GoScriptTM Promega) with $MgCl_2$, RNAse Inhibitor, and dNTP mix (Thermo Scientific) in 10 µL of final volume of reaction mixture following the Promega manufacturer's protocol.

c) Polymerase chain reaction (PCR): PCR was performed using a mixture of: cDNA, adequate reverse (R) and forward (F) primers, DNA polymerase in Tris-HCl pH 8.8 with $MgCl_2$, KCl, Triton X-100, dNTP mix (Thermo Scientific), and H_2O-DEPC in a total reaction volume of 12.5 µL. The PCR products were analyzed in 2.0% agarose gels during electrophoresis and imaged with UVI-KS 4000i/ImagePC (Syngen Biotech).

Table 1. PCR conditions

Gene	Initiation	Denaturation	Amplification	Elongation	Termination
MPST	5 min at 95°C	30 sec at 95°C	30 sec at 55°C	2 min at 72°C for 29 cycles	72°C for 8 min
CTH	5 min at 95°C	30 sec at 95°C	1 min at 51°C	8 min at 72°C for 30 cycles	72°C for 8 min
TST	5 min at 95°C	30 sec at 95°C	30 sec at 54.5°C	2 min at 72°C for 34 cycles	72°C for 8 min
β-actin	5 min at 95°C	30 sec at 95°C	30 sec at 55°C	2 min at 72°C for 30 cycles	72°C for 8 min

d) Densitometric evaluation of PCR: Photos saved in the jpeg format (Bio-Rad ChemiDoc MP Imaging System) were used for the densitometry analysis. The densitometry data for band intensities in the different experiments were generated by analyzing the gel images on the Gene Tools Software (Syngene).

Western blotting: The protein load for electrophoresis was 20 µg per well. SDS-PAGE electrophoresis and electrotransfer on PVDF membrane were conducted according to the Bio-Rad protocol. The relative amounts of CTH, MPST, and TST were determined using the appropriate antibody (Abnova, GeneTex, ProteinTech; 1:1000). Anti-β-actin and anti-α-tubulin antibodies were used to check for loading (Sigma, ProteinTech; 1:5000). Proteins of interest were incubated with alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG antibody (ProteinTech, 1:2000). Proteins were visualized by immunohistochemical detection with NBT/BCIP (Roche) staining solution.

Statistical analysis: Standard deviation was used for the error bars and for the statistical analysis the Mann-Whitney U test was used.

RESULTS

The cytotoxic effect of a wide range of F^- concentrations in the culture medium was measured by the LDH cytotoxicity test. As shown in Table 2, the H27 fibroblast cell membranes were treated with three concentrations of F^-: 0.004% NaF (1 mM), 0.012% NaF (3 mM), and 0.024% NaF (6 mM), and a control group was left untreated (non-treated cells, 0% NaF, 0 mM).

Table 2. Changes in the integrity of the Hs27 fibroblast cell membranes after 24 and 48 hr incubation with F^- measured by the LDH cytotoxicity kit

Fluoride concentration in cell culture medium	% of cytotoxicity	Incubation time
0.004% (1 mM)	0	24 hr
0.012% (3 mM)	0	
0.024% (6 mM)	2	
0.004% (1 mM)	0	48 hr
0.012% (3 mM)	10.5	
0.024% (6 mM)	16.4	

Cytotoxic effects of F^- on fibroblast cells are shown for concentrations ³. 0.012% NaF (3mM) after 48 hr incubation. The tested concentrations of F as listed: 0.004%, 0.012%, and 0.024%. The amount of LDH present in the medium is expressed as a percentage of the total (0% in untreated cells; 100% in cells lysed with provided lysis buffer solution). The data refers to the values obtained for the control (non-treated) cells. The numbers listed in the table represent the results from at least 3 experiments done in 6 repetitions.

A cytotoxic effect was demonstrated for concentrations equal to or higher than 0.012% after the 48 hr incubation time. Next, for the same F^- concentrations, the proliferation protocol was introduced. The number of cells was determined with crystal violet staining. A reduced number of cells was registered for the two tested concentrations equal to and above 0.012% NaF. The amount of living fibroblast cells

decreased down to 70% in comparison to the control (listed as 100%), for both incubation times (Figure 1).

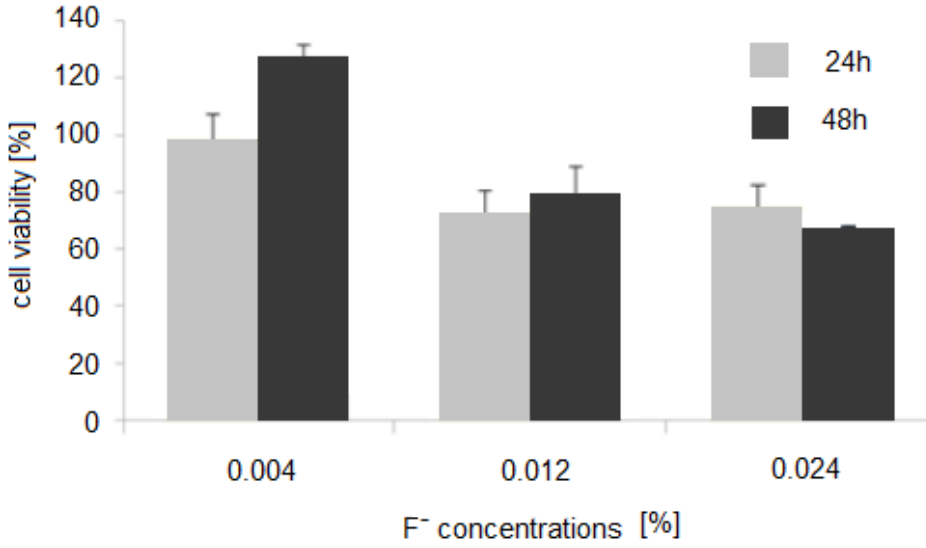


Figure 1. Cell viability in Hs27 cell line after the 24 and 48 hr incubation with F^- measured by crystal violet staining. The concentrations of F^- tested in the medium were: 0.004%, 0.012%, and 0.024%. The data refers to values obtained for the control (non-treated) cells normalized and labeled as 100%. The bars represent the results from at least 3 experiments done in 6 repetitions. The error bars represent the standard deviation.

The following experiments included testing the expression of the chosen genes under the same conditions. The tests were conducted using the RT-PCR and the Western Blot. No changes in the mRNA levels for hMMP-9 and collagen were observed and therefore no confirmation was done at the protein level. Some changes in the expression in mRNA were observed in the MPST gene bars as shown with the RT-PCR method. The expression of this gene seemed to increase after incubation with increased concentrations of F^- with the most intense band appearing for 0.024% NaF after 24 hr (in comparison to the control). A range of experiments were performed to create the densitometric analysis for the MPST mRNA levels. No statistically significant changes were observed (Figure 2A). The results were confirmed at the protein level in Western Blot analysis for the human MPST antibody, where no significant changes in the level of protein were observed after the incubation with F^- (Figure 3C).

The MPST changed the expression in the RT-PCR and the anticipated role in the anti-oxidative response urged us to check the levels of other enzymes participating in the metabolism of sulfur-containing compounds: CTH and TST. No statistically significant changes in the mRNA levels for the TST gene in the RT-PCR method were observed, as shown in the graph representing densitometric analysis of the bars from multiple PCR tests (Figure 2B). Also, there were no changes in the protein levels for the assumed range of factor concentrations in the Western Blot analysis (Figure 3A). Furthermore, no statistically significant changes in the mRNA levels for the CTH gene in the RT-PCR method were observed as shown on the graph representing the densitometric analysis of the bars from multiple PCR tests (Figure

2C). Also, there were no changes in the protein level for the assumed range of factor concentrations in the Western Blot analysis (Figure 3B).

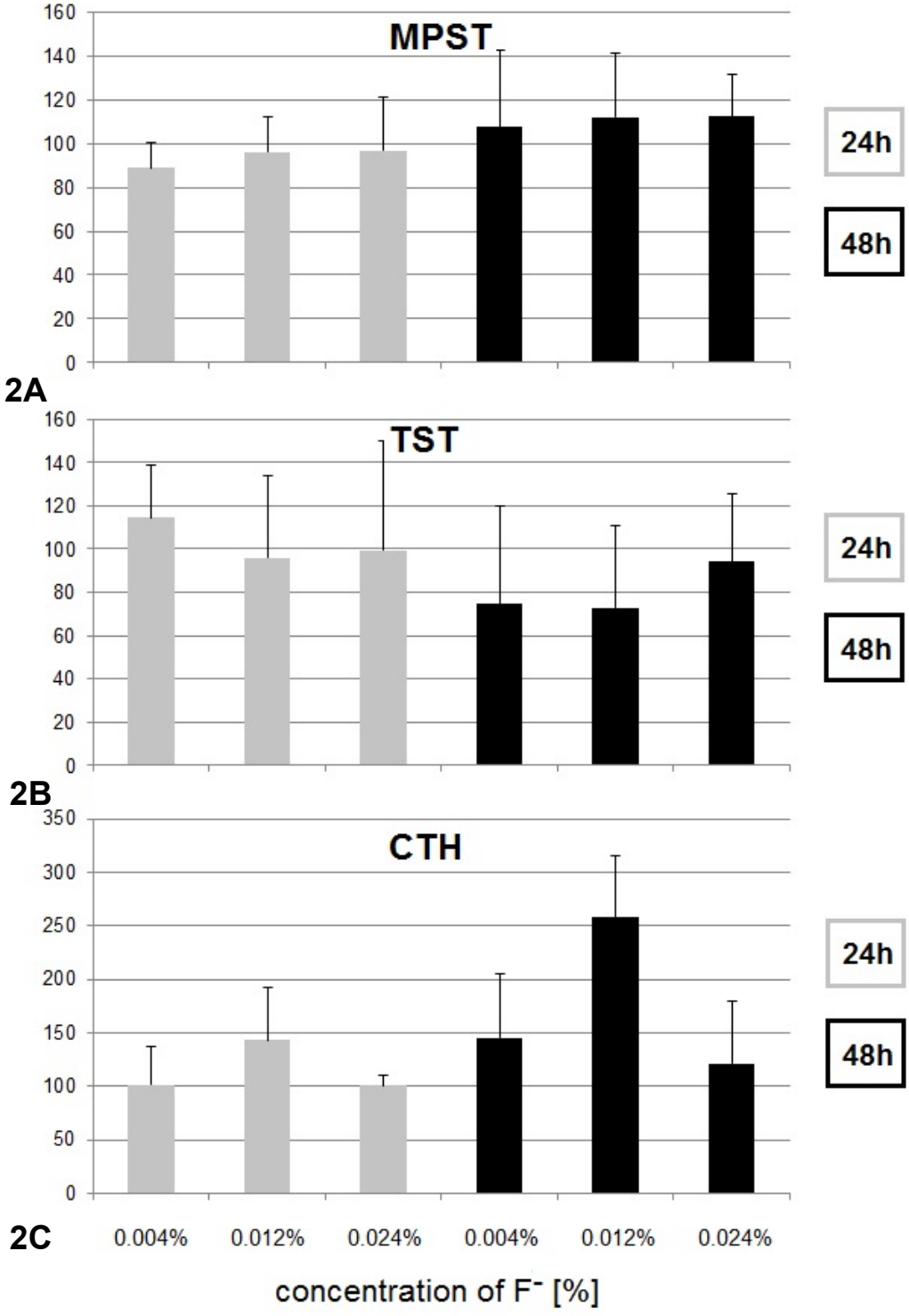
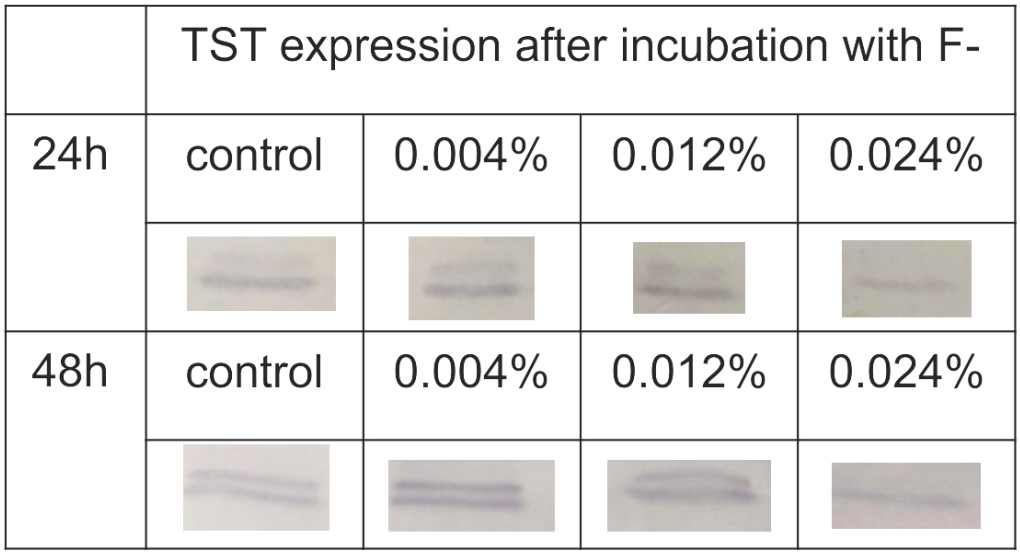
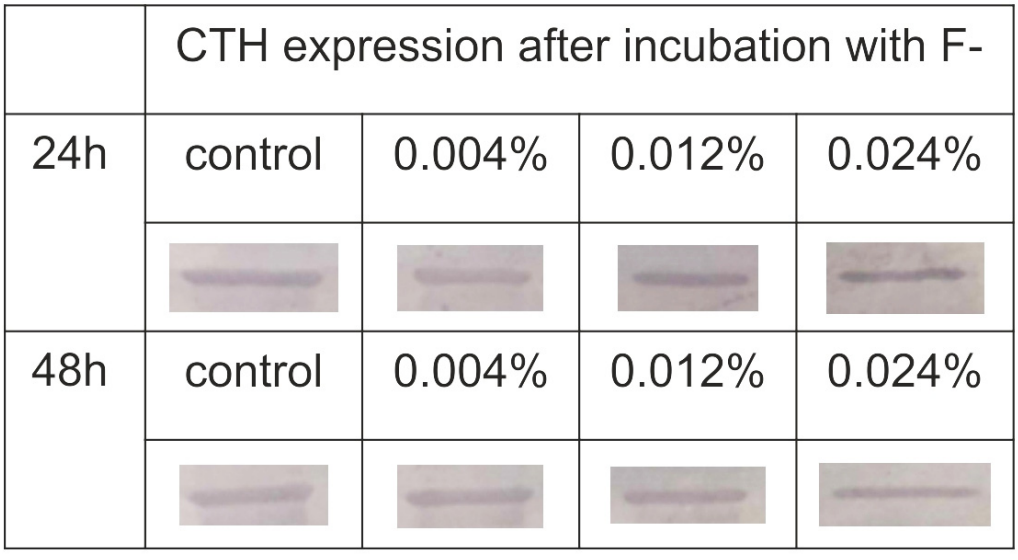


Figure 2. Densitometric visualization of the expression of 2A: MPST, 2B: TST, and 2C: CTH in Hs27 fibroblast cells after a 24 and 48 hr incubation with F^- with the RT-PCR method. The tested concentrations of F^- in the culture medium were: 0.004%, 0.012%, and 0.024%. The experiment was performed in triplicate, and the PCR was run at least three times for the samples from each experiment. The error bars represent the standard deviation.

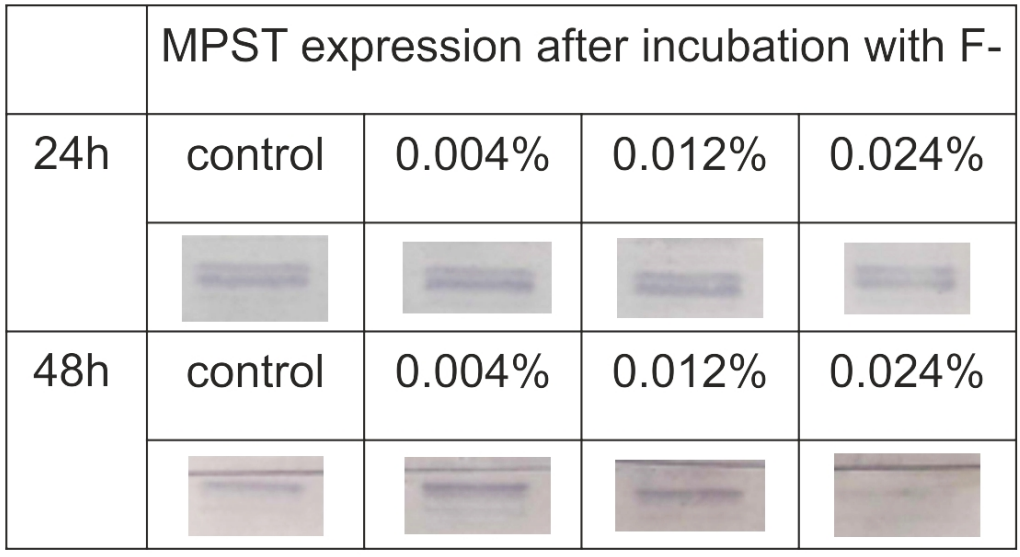


3A

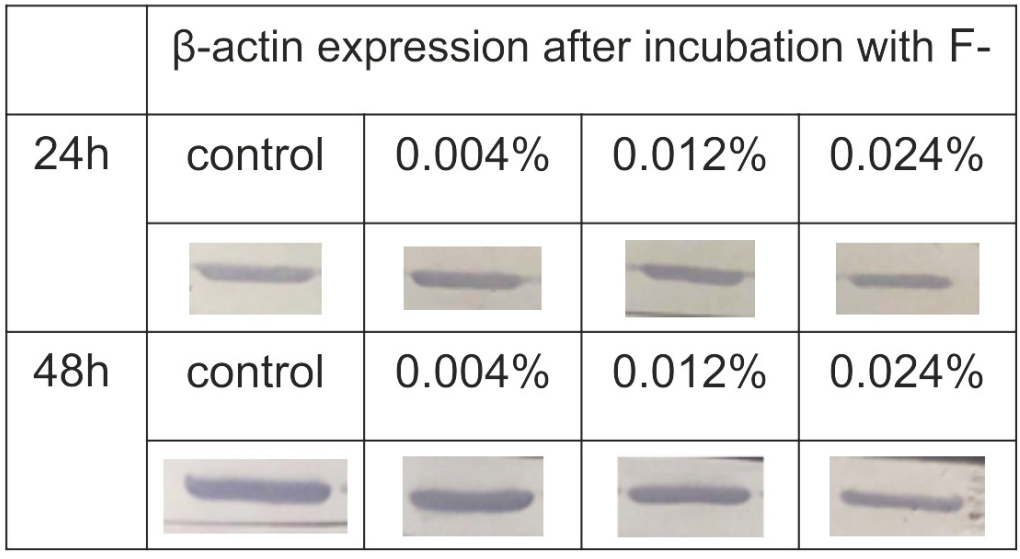


3B

Figures 3A and 3B. Expression of 3A: TST and 3B: CTH in Hs27 fibroblast cells after a 24 and 48 hr incubation with F^- measured by the Western Blot. The tested concentrations of F^- in the culture medium were: 0.004%, 0.012%, and 0.024%. The experiment was performed at least in duplicate and the Western Blot was run two times for the samples from each experiment with similar results. A representative result is shown.



3C



3D

Figures 3C and 3D. Expression of 3C: MPST and 3D: β-actin in Hs27 fibroblast cells after a 24 and 48 hr incubation with F^- measured by the Western Blot. The tested concentrations of F^- in the culture medium were: 0.004%, 0.012%, and 0.024%. The experiment was performed at least in duplicate and the Western Blot was run two times for the samples from each experiment with similar results. A representative result is shown.

DISCUSSION

Fibroblasts play a crucial role in the process that allows tissue reparation to occur after tissue damage. Connective tissue cells are able to migrate to the damaged part of the skin and proliferate to repair the injury.[20,21] With there being a considerable literature on the toxic and harmful effects of toxic substances on different types of tissue and living organisms, the decision was made to examine fluoride toxicity using the normal fibroblast cell line Hs27. The effects of fluoride on cytotoxicity and the ability of cells to proliferate were examined by incubating cell cultures with fluoride in concentrations ranging from 1 mM to 6 mM NaF. The F^- concentrations were selected on the basis of a literature search on *in vitro* tests where cytotoxic effects were induced with incubation times of between 24 and 48 hr.[22-24] The proven ability of fluoride to inhibit certain groups of enzymes like phosphatases was also taken into consideration.[25,26] In the present study, we found a fluoride-induced cytotoxic effect on fibroblasts was shown at concentrations of F^- equal to or higher than 0.012% NaF (3 mM) but only after the longer 48 hr incubation time. A reduction of the percentage of viable cells was demonstrated after incubation with 0.012% NaF for both incubation times of 24 and 48 hr. The results confirmed the cytotoxic effect of fluoride and the ability of fluoride to affect cell viability with exposure to F in millimolar concentrations. Similar results have been described by Jeng et al. for another fibroblast cell line from human oral mucosa.[27] To examine the condition of fibroblasts and how NaF may affect this important connective tissue element, two collagen genes and hMMP9 have been studied. Yan et al. found evidence of a damaging effect of long-term F exposure on collagen 1A1 and 1A2 expression in rat hard tissue.[28] Brackett et al. reported reduced activity of rccombinant human MMP9 protein (rhMMP9) in dentin matrices and, similarly, Kato et al. found that NaF exposure resulted in MMP9 inhibition in human saliva.[29,30] Accordingly, in our study, we measured the mRNA levels for both of these genes. However, in our study, there was no visible change in the collagen and hMMP9 expression after 24 and 48 hr incubation as measured by the RT-PCR method, even for the highest cytotoxic concentration of NaF. These results suggest that, for this type of cell, the millimolar concentration range, up to 6 mM, has no effect on the mRNA level of expression of collagen and hMMP9 and higher concentrations or longer incubation times should be considered for future tests. These results confirm the many reports in the literature that there is a non-specific response to the same levels of F^- in different types of cells.[31]

It is widely described in the literature that certain concentrations of F^- in various cell types are able to cause oxidative stress.[32-34] Toxic amounts of F^- take part in the processes of generating free radicals, such as $OH\cdot$ and $\cdot O_2^-$, and have the ability to lower the enzymatic activity of antioxidant proteins that play a crucial role in coping with free radicals in the main oxidative systems such as SOD.[35,36] Recent literature data show that the group of sulfur-containing enzymes such as MPST, TST, and CTH may play a role in the responses to oxidative stress. Recent studies have confirmed the anti-oxidative effect of H_2S metabolites produced by these enzymes. In addition, sulfurtransferases may also show a local antioxidant activity due to the presence of free SH groups.[37-39] The long-term response to the cytotoxic effect of F may be linked to the level of sulfur enzyme expression. Thus, the enzymes involved in the metabolism of sulfur-containing compounds were chosen for testing to examine the

relationship between the cytotoxicity of different concentrations of F^- and the anti-oxidative response. Although there were some promising changes in the first experiments for the MPST enzyme after 48 hr incubation with the highest cytotoxic concentration of F^-, the further extended tests did not confirm the changes in the expression of this enzyme. The other enzymes studied also did not show any statistically significant changes in their expression after exposure to the tested range of NaF concentrations, as was confirmed by the Mann-Whitney U test. Some of the reports show a lack of any connection between the presence of F^- and NaF-induced oxidative stress. Moreover, in the research conducted by Lee et al., ROS production was shown in NaF-treated human gingival fibroblasts cells.[40] It seems that, in the case of the human fibroblast Hs27 cell line, the cytotoxic effect is not connected with the changes in the sulfur-containing enzymes tested, elevated levels of which could possibly show an antioxidant response to the cytotoxic effect of F^-.

In the present study, the cytotoxic effect of fluoride and the influence of fluoride on the proliferation rate in the fibroblast cell line Hs27 were confirmed. Exposure to F^- did not result in any changes in the expression of collagen, hMMP9, MPST, CTH, and TST. There is no study in the literature on the effect of fluoride on the expression of the aforementioned sulfur-containing enzymes, so the findings of the present study are an addition to the knowledge on the effect of fluoride on the enzymatic activity profile of this normal Hs27 cell line.

ACKNOWLEDGEMENTS

This work was financially supported by the Polish Ministry of Science and Higher Education, and grant K/DSC/003573 of the Jagiellonian University Medical College.

CONFLICT OF INTEREST

The authors declare no conflict of interests in any relationships or with the financial support received. The authors alone are responsible for the content and writing of this paper.

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