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ORIGINAL ARTICLE

Investigation of the genotoxic effects of fluoride on a bone tissue model

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Abstract

Fluorides are thought to be a major cause of osteocarcinogenesis, due to their widespread industrial use, ability to accumulate in bone tissue, and genotoxic and probable carcinogenic properties. In vitro experiments investigating the genotoxic potential of fluorides in bone tissue models can provide valuable indirect information on their involvement in osteocarcinogenesis. Here, we investigated whether sodium fluoride (NaF) has the ability to induce DNA damage and chromosomal abnormalities in human osteosarcoma cells after 48 and 72 h of exposure. The cell cultures were treated with NaF in concentrations of 0, 20, 100 and 200 µg/ml. The level of DNA damage was assessed by the comet assay, and the frequency of chromosomal abnormalities by a micronucleus test. A significant increase in DNA damage indicators was noted in the samples treated with fluoride concentrations of 100 and 200 µg/ml, after 48 and 72 h of exposure. The micronucleus test revealed a dose-dependent increase in cells with micronuclei, nucleoplasmic bridges and nuclear protrusions. Increasing the concentration of NaF led to an increase in the prevalence of cytogenetic indicators after both treatment durations. This demonstrated ability of fluorine to exert genotoxic effects on bone cells indirectly indicates the possible importance of fluoride in the aetiology of osteosarcoma.

Keywords Genotoxicity · NaF · In vitro · DNA comets · Micronucleus test

Introduction

Despite their relative rarity, primary oncological diseases of bone tissue pose a significant problem for modern biomedicine. Along with these cancers being therapeutically challenging, the causes of pathology also remain uncertain. Determining the aetiology of this type of disease is thus an important issue, as knowing its cause(s) will allow us to achieve significant progress in prevention and treatment. The main factor in the development of osteocarcinogenesis is believed to be environmental exposure. Fluorides are thought to be the environmental factors, due to their widespread industrial use, ability to accumulate in bone tissue [1], and genotoxic [2] and probable carcinogenic properties [3, 4]. This assumption is based on previous ecological-epidemiological studies [5] and studies of the correlation of fluoride in sera with the frequency of oncogenic mutations of the p53 gene in human tumour tissues [6]. However, some publications have presented evidence against the involvement of fluoride in osteocarcinogenesis [7, 8]. To test the fluoride hypothesis, an experimental assessment of the genotoxic effects of fluoride-ion exposure on human osteoblast cells is necessary. To date, a limited number of experiments on non-human bone cell cultures have been conducted. When cultured with sodium fluoride (NaF), an increase in the frequency of chromosomal aberrations in the primary culture of rat vertebral cells was observed [9], as was an increase in DNA damage in the UMR 106 rat osteosarcoma [10]. Surprisingly, though, no studies on human bone models have been conducted.

In vitro experiments investigating the genotoxic potential of fluorides in bone tissue models can provide valuable indirect information on the involvement of these species in osteocarcinogenesis. Hence, the purpose of this work was to determine the genotoxicity of NaF to human bone cells.

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Materials and methods

Cell culture

The human osteosarcoma cell line (HOS) was used as a model of bone tissue, and was obtained from the collection of the State Research Center of Virology and Biotechnology VECTOR (Novosibirsk, Russia). Removal of culture from cryopreservation was performed by rapid warming in a water bath (37 °C), with further washing of the cryoprotectant by centrifugation (1000 × *g*), followed by replacing the environment and seeding the cells in a culture flask. The seeding concentration was 75,000–100,000 cells per ml. The culture environment consisted of 90% Dulbecco's modified Eagle's medium (DMEM) as nutrient (containing L-glutamine and an antibiotic Penicillin–Streptomycin (Biolot, St. Petersburg, Russia)) and 10% blood serum of cow embryo (HyClone, Logan, United States). The cells were incubated at 37 °C and in a humidified atmosphere of 5% CO_2. Passaging was carried out every 3–5 days. The cells were removed from the monolayer using trypsin–Versen solution (1:1) (Biolot, St. Petersburg, Russia). After the accumulation of cell mass, they were seeded in culture plates. The maximum exposure concentration was determined by a preliminary assessment of substance cytotoxicity in different concentrations. An experiment was conducted with final concentrations of NaF of 50, 100, 200, 300 and 400 µg/ml. The cytokinesis-block proliferation index (CBPI) was evaluated and the permissible concentrations of the test substance were determined. Subsequently, an experiment was conducted with NaF concentrations causing cytotoxicity of less than 55% (CBPI > 1.55).

$$\mathrm{CBPI} = \frac{((\text{no. of mononucleate cells}) + (2 \times \text{no. of binucleate cells}) + (3 \times \text{no. of multinucleate cells}))}{(\text{Total number of cells}).}$$

The process of cell growth and plating in culture plates was repeated. Next, 24 h after passage, the samples were treated with PBS (Eco-service, St. Petersburg. Russia) (the control group) or NaF (Vecton, St. Petersburg, Russia) to a final concentration of 0, 20, 100 and 200 µg/ml (the same concentrations that were used in similar studies on other types of cell culture. Additionally, part of the samples was cultured as a positive control with mitomycin C (Sigma-Aldrich) at a final concentration of 12.5 ng/ml for the micronucleus test and with hydrogen peroxide for the comet assay (100 µM for 2 min, with further cultivation for 48 or 72 h). Subsequently, the cells were cultured for 48 or 72 h. Six samples were tested for each combination of a concentration of NaF and duration of exposure. The samples intended for the micronucleus test with cytochalasin blocking were treated 24 h before the end of cultivation of with cytochalasin B (Pan Eco, Moscow, Russia), to a final concentration of 6 µg/ml. After the end of cultivation, the cells were transferred to suspension and subjected to sample preparation for the micronucleus test and the comet assay. The experiment was repeated 3 times.

Micronucleus test

The micronucleus test was performed in accordance with the recommendations of Fenech [11]. After cultivation, the preparations were treated with a hypotonic solution of KCl (Vecton, St. Petersburg, Russia) and fixed using Carnoy's fixative. Then, staining was performed using a 2% solution of Giemsa stain (Pan Eco, Moscow, Russia). The resulting preparations were analysed using a Nikon Eclipse 80i microscope at 1000 × magnification. The selection criteria for the cells to be included in the analysis, and the criteria for the recording of cytogenetic damage, were consistent with generally accepted recommendations [12]. In each preparation, the nuclei of 1000 binuclear cells were analysed, in which micronuclei (Fig. 1), nucleoplasmic bridges (Fig. 2) and nuclear protrusions (Fig. 3) were observed.

Comet assay

The comet assay was performed in an alkaline modification developed by Singh and colleagues [13]. The fragmentation parameters were estimated by photomicrographing preparations stained with SYBR Green using a Zeiss Axio Imager 2 fluorescence microscope. A total of 200 randomly selected comets from each sample were photographed with a magnification of 200 × (Fig. 4). The subsequent processing of

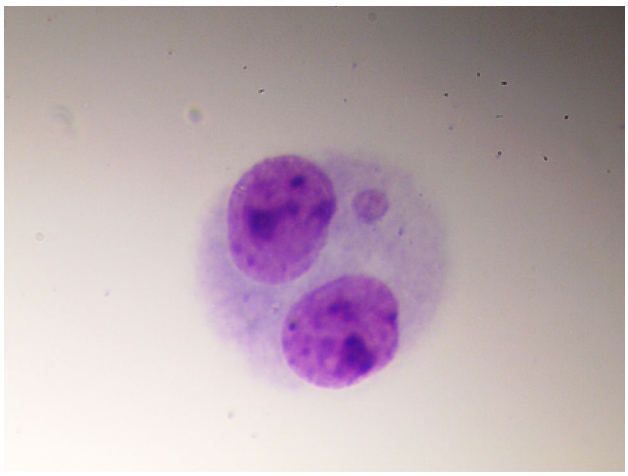

Fig. 1 MNBN in HOS

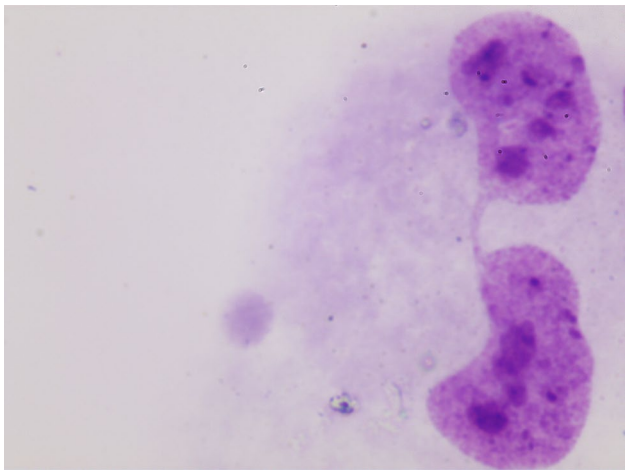

Fig. 2 Binucleated cell with nucleoplasmic bridge

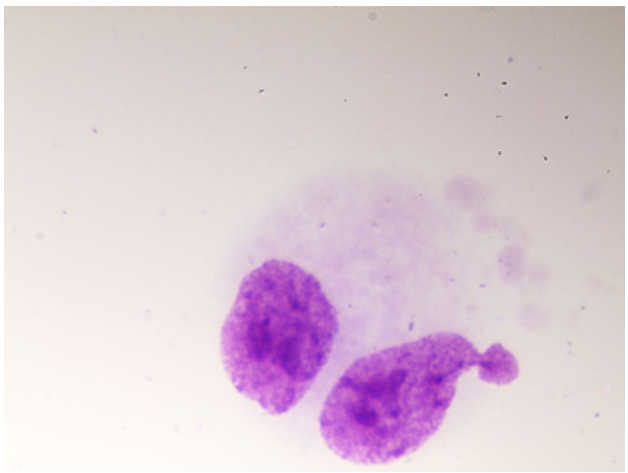

Fig. 3 Binucleated cell with nuclear protrusion

the photographs was carried out using the CASP software package (Krzysztof Konca, http://casplab.com). Two parameters, namely the percentage of DNA in the comet's tail and the DNA-comet index, were calculated as follows: index of DNA comet (IDC) = $(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4)/\Sigma$, where n_0–n_4 are the numbers of comets of each type, Σ is the sum of the calculated DNA-comets.

Statistical analysis

Statistical analysis of the data was carried out using the Statistica 10.0 package (StatSoft, TIBCO Software). The average values and the limit of the 95% confidence interval (CI 95) were calculated for the two quantitative indicators. Group comparisons were performed using the Mann–Whitney U-test.

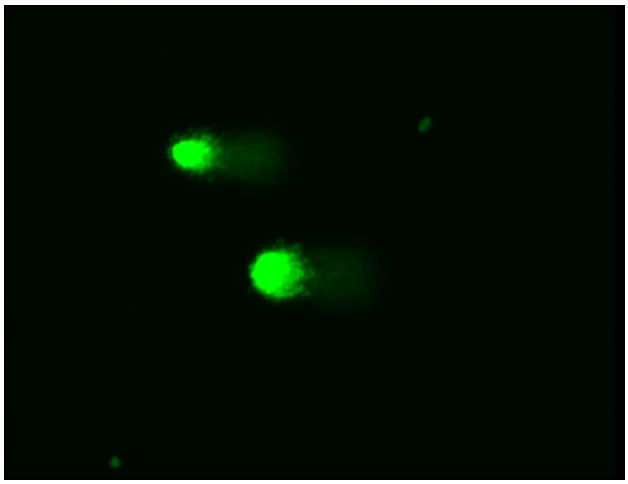

Fig. 4 DNA-Comets in HOS

Results

Comet assay

The highest average value of percentage of DNA in the comet's tail was observed in the samples cultured for 48 h in 200 µg/ml NaF (Table 1).

A significant increase in the percentage of DNA in the comet's tail was observed in samples with a NaF concentration of 20, 100 and 200 µg/ml with 48 h exposure ($p < 0.01$), and in those with concentrations of 100 and 200 µg/ml with 72 h exposure ($p < 0.01$), compared with the control samples (0 µg/ml). In addition, a significant increase in the percentage of DNA in the comet's tail was observed with an increase in the NaF concentration from 20 to 200 µg/ml and after 48 h of exposure.

Similar to the trend observed with the percentage of DNA in the comet's tail, the highest value of the DNA-comet index was observed in samples exposed for 48 h to a NaF concentration of 200 µg/ml (Table 2). Compared with the control, significant increases in the DNA-comet index were observed in samples with a NaF concentration of 20, 100 and 200 µg/ml with 48 h of exposure ($p < 0.01$), and in the samples with 100 and 200 µg/ml NaF with 72 h of exposure ($p < 0.01$). In addition, a significant increase in this index was observed with an increase in the NaF concentration from 20 µg/ml to 200 µg/ml with 48 h of exposure ($p < 0.05$). Additionally, samples exposed to 100 and 200 µg/ml NaF for 72 h showed a significant increase in the DNA-comet index compared with those exposed to only 20 µg/ml NaF for the same duration ($p < 0.05$). There was also a significant decrease in the DNA-comet index in the positive control samples with an exposure time of 72 h compared with those exposed for 48 h ($p < 0.01$).

Micronucleus test

The results of the micronucleus test are presented in Table 3. Compared with the control, the concentration of NaF of 20 µg/ml in the culture medium did not generate a statistically significant increase in the frequency of binucleated cells with a micronucleus (MNBN), nucleoplasmic bridges or nuclear protrusion at either exposure time. In the case of a NaF concentration of 100 µg/ml, compared with the control, a statistically significant increase was observed in the numbers of MNBNs ($p < 0.05$ with 48 h exposure and $p < 0.01$ with 72 h exposure), binucleated cells with nucleoplasmic bridges ($p < 0.01$ at 48 exposures and $p < 0.05$ at 72 h exposure) and binucleated cells with nuclear protrusions at 72 h exposure ($p < 0.05$). In the case of a NaF concentration of 200 µg/ml in the culture medium, compared with the control there was a statistically significant increase in the number of MNBNs ($p < 0.01$ at 48 h exposure and $p < 0.01$ at 72 h exposure), binucleated cells with nucleoplasmic bridges ($p < 0.05$ at 48 h exposure and $p < 0.01$ at 72 h exposure) and binucleated cells with nuclear protrusions ($p < 0.05$ at 48 h exposure and $p < 0.05$ at 72 h exposure). In addition, a statistically significant increase in the number of MNBNs was observed with an increase in exposure time from 48 to 72 h in samples with a NaF concentration of 200 µg/ml ($p < 0.01$).

Table 1 The percentage of DNA in the comet's tail, in the studied samples, means [95%, CI], %

Cultivation time	The percentage of DNA in the comet's tail, in the studied samples (%)				
	0 µg/ml NaF	20 µg/ml NaF	100 µg/ml NaF	200 µg/ml NaF	H_2O_2 100 µM for 2 min
48 h	1.38 [1.24–1.52]	3.79 [3.46–4.13]**	4.09 [3.86–4.33]**	4.47 [4.20–4.76]**,##	4.89 [4.65–5.13]
72 h	1.18 [1.01–1.36]	1.15 [1.01–1.30]	2.16 [1.89–2.43]**,##	2.76 [2.30–3.21]**,##	3.42 [3.05–3.80]

**$p < 0.01$, Significant differences against samples treated with 0 µg/ml of sodium fluoride; ##$p < 0.01$, Significant differences against samples treated with 20 µg/ml of sodium fluoride

Table 2 Index of the DNA-Comets, means [95%, CI]

Cultivation time	Index of the DNA-Comets				
	0 µg/ml NaF	20 µg/ml NaF	100 µg/ml NaF	200 µg/ml NaF	H_2O_2 100 µM for 2 min
48 h	0.31 [0.28–0.42]	0.88 [0.80–0.95]**	0.97 [0.92–1.03]**	1.07 [1.00–1.14]**,#	1.17 [1.11–1.23]
72 h	0.24 [0.21–0.27]	0.26 [0.23–0.29]	0.54 [0.47–0.61]**,#	0.72 [0.60–0.84]**,#	0.90 [0.79–1.01]

**$p < 0.01$, Significant differences against samples treated with 0 µg/ml of sodium fluoride; #$p < 0.05$, Significant differences against samples treated with 20 µg/ml of sodium fluoride

Table 3 The results of the micronucleus test, means [95%, CI] (‰)

Cultivation time	The frequency of micronucleus test's indicators (‰)				
	0 µg/ml NaF	20 µg/ml NaF	100 µg/ml NaF	200 µg/ml NaF	12.5 ng/ml Mitomycin C
MNBN					
48 h	32.33 [25.16–9.50]	42.00 [24.90–9.09]	60.67 [51.26–70.07]*	69.11 [62.08–76.13]**	88.78 [82.57–94.98]
72 h	35.00 [17.09–2.91]	32.00 [25.42–8.57]	73,91 [61.34–86.48]**	90.21 [88.82–91.60]**	100.07 [76.42–123.71]
Binucleated cells with nucleoplasmic bridges					
48 h	7.67 [0–15.41]	9.33 [1.74–16.92]	23.66 [19.87–27.46]**	21.82 [19.22–24.42]*	33.98 [28.05–39.90]
72 h	7.66 [1.93–13.40]	12.67 [6.63–18.71]	17.32 [9.52–25.13]*	24.54 [20.44–28.64]**	38.09 [29.29–46.89]
Binucleated cells with nuclear protrusion					
48 h	11.67 [7.87–15.46]	6.67 [0–13.84]	18.67 [7.46–29.87]	33.43 [25.03–41.83]*	27.02 [19.5–34.54]
72 h	7.33 [5.90–8.77]	7.67 [4.80–10.53]	22.73 [18.61–26.85]*	23.41 [21.16–25.67]*	29.19 [22.36–36.01]

*$p < 0.05$, Significant differences against samples treated with 0 µg/ml of sodium fluoride; **$p < 0.01$, Significant differences against samples treated with 0 µg/ml of sodium fluoride

Discussion

The results of this study showed that NaF was able to induce damage to the DNA of osteoblast cells. In the comet assay, both the percentage of DNA in the comet's tail and the DNA-comet index increased with the concentration of fluorine in the growth medium. In the case of 48 h of exposure, both parameters differed significantly in samples with NaF concentrations of 20 or 100 µg/ml, compared with the control sample (0 µg/ml). In samples with a concentration of 200 µg/ml, a further significant increase was noted relative to the cell cultures exposed to only 20 µg/ml. In the case of 72 h of exposure the level of DNA damage was lower, possibly because of the activation of DNA repair or apoptotic programs in cells with most DNA damage.

Nonetheless, the average extent of genotoxic effects increased with the concentration of NaF in the growth environment, as in the case of 48 h exposure. In the positive control, the DNA-comet index decreased in the case of 72-h cultivation compared with 48-h cultivation, in agreement with the abovementioned conclusions. The extent of DNA damage in samples with an NaF concentration of 100 µg/ml and 200 µg/ml was far greater than that in samples with concentrations of 0 and 20 µg/ml. The obtained data are consistent with the results from similar studies conducted on mammalian cells. Specifically, several authors have studied the ability of NaF in concentrations from 0 to 100 mM to cause DNA damage, using methods for assessing the extent of DNA fragmentation. It has been determined that exposure to fluoride ions can lead to dose-dependent DNA damage in rat osteosarcoma cell lines [10], primary mouse hepatocytes [14, 15] and primary rat kidney cells [16].

When assessing the extent of chromosomal damage with a micronucleus test, a dose-dependent increase in cells with micronuclei, nucleoplasmic bridges and nuclear protrusions was noted. An increase in the concentration of NaF for both exposure durations led to an increase in the numbers of these cytogenetic indicators. Notably, an increase in the exposure time did not lead to a decrease in the number of cytogenetic markers, in contrast to the increased extent of DNA damage. On the contrary, a significant increase in the number of MNBN cells was observed at 72 h of exposure, as opposed to 48 h.

The obtained results are consistent with data from other authors. Cultivation of cells in the presence of fluoride ions led to an increase in chromosome aberration and sister chromatid exchanges in cell cultures of the Syrian hamster embryo [17, 18], Indian muntzhak [19] and the red bone marrow, hippocampal neurons and tracheal epithelium [20–22] of rats. In vitro experiments on human cell lines showed that fluoride was significantly genotoxicity at low concentrations. The ability of fluoride ions to induce DNA damage and increase the frequency of clastogenic effects in human leukocytes has been noted [23–25] for buccal epithelial cells [26], fibroblasts [27], JHU-1 foreskin fibroblasts [28], HL-60 human leukaemia cell lines [29] and human primary hepatocytes [30]. The results of these studies, as well as the present work, are in conflict with a number of other works in which the genotoxic potential of fluoride ions was not confirmed in experiments on primary cells of mice, sheep, cows [31, 32] and human leukocytes [33].

Taken together, the published data strongly suggest that genotoxicity is connected to the ability of fluoride ions to induce mitochondrial damage and oxidative stress. Such events usually culminate in either apoptosis of cells, subsequent to activation of proapoptotic caspases (caspase 3, 9 and others), or necrosis. Some authors have demonstrated that fluoride in low concentrations induces oxidative stress, leading to apoptosis of human lymphocytes in vitro [34]. Similar observations have been made in rat foetal hepatocytes [30]. According to other research, the influence of fluoride contributes to the synthesis of active forms of oxygen with the induction of SIRT1/autophagy, via signalling of the c-Jun N-terminal kinase in ameloblasts. Other observed effects of fluoride are its triggering of the release of cytochrome C, weakening of ATP synthesis and phosphorylation of γH2AX [35]. Simultaneous treatment of a cell line with fluoride ions and catalase greatly reduced the apoptosis of cells, suggesting that the reactive oxygen species (ROS)-mediated genotoxic mechanism of fluoride is a primary mechanism underpinning its activity [36]. There is also evidence of fluoride ions' ability to inhibit the active state of enzymes involved in the biotransformation of phase I and II xenobiotics, as well as enzyme markers of oxidative stress [37]. Moreover, it has been noted that low concentrations of fluoride ions affected the progression of the cell cycle [34] and, that they lead to DNA fragmentation through activation of caspase 3 [38].

In this study it has been shown that NaF influenced the genomic integrity of human bone cells. It was noted that short-term cultivation of tumour osteoblasts in the presence of NaF increased the extent of DNA damage and chromosomal aberrations. The results indirectly testify to the possible roles of fluorides in the aetiology of osteosarcoma.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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