

## FLUORIDE AND GENERATION OF PRO-INFLAMMATORY FACTORS IN HUMAN MACROPHAGES

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**SUMMARY:** Fluorosis from excessive exposure to fluoride can result in inflammatory reactions involving macrophages and their differentiation, a process that is rapidly followed by generation of prostanoids—products of arachidonic acid metabolism, including the pro-inflammatory factors prostaglandin PGE<sub>2</sub> and thromboxane TXA<sub>2</sub>, which are implicated in atherogenesis and rapidly increase during acute inflammation. This paper examines the effect of fluoride at concentrations found in the blood of individuals environmentally exposed to fluorine compounds as the production of PGE<sub>2</sub> and TXA<sub>2</sub> is affected. Peripheral blood mononuclear cells (PBMCs) used in the present study were isolated from the blood of donors and incubated with 1, 3, 6, and 10 μM NaF. Secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>) activity and the concentrations of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and thromboxane A<sub>2</sub> (TXA<sub>2</sub>) were measured by enzyme immunoassay kits, arachidonic acid (AA) release by gas chromatography, and apoptosis by flow cytometry. Incubation of macrophages with NaF caused increased concentrations of PGE<sub>2</sub>, TXA<sub>2</sub>, sPLA<sub>2</sub>, and AA in the cells and increased the number of macrophages in early-stage apoptosis in a dose-dependent manner. The results indicate that NaF, even in small concentrations, may induce an inflammatory process and an apoptotic effect in macrophages through the stimulation of the metabolism of prostanoids and increased synthesis of PGE<sub>2</sub> and TXA<sub>2</sub>.

Keywords: Fluoride and macrophages; Inflammation; Human macrophages; prostaglandin E<sub>2</sub>; thromboxane A<sub>2</sub>.

### INTRODUCTION

During the last decade, interest in adverse health effects of fluoride (F) has resurfaced owing to increased awareness that F interacts with cellular systems even at comparatively low doses or concentrations.<sup>1</sup> Exposure of humans to F results primarily from its presence in the air, water, and food.<sup>2</sup> Long-term exposure to F may lead to fluorosis and changes in the amount and catalytic properties of many enzymes<sup>3,4</sup> taking part in inflammatory reactions.<sup>5</sup> Inflammatory processes underlie the pathogenesis of the atherosclerotic process,<sup>6</sup> in which a significant role is played by macrophages, cells that participate in the formation of atherosclerotic plaques.<sup>7</sup>

The first step in atherogenesis is a recruitment of monocytes from the peripheral blood to the intima of the vessel wall and differentiation into the macrophages, which is rapidly followed by the generation of prostanoids, cyclooxygenase-dependent products of arachidonic acid (*n*-6, 20:3 and 20:4) (AA) metabolism, including prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and thromboxane A<sub>2</sub> (TXA<sub>2</sub>).<sup>8</sup> Prostanoids exert a variety of actions in various tissues and cells, and although prostanoid levels are generally very low in noninflamed tissues, they immediately increase during acute inflammation.<sup>9</sup>

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Eicosanoid synthesis in macrophages is controlled by the availability of free AA,<sup>10</sup> and the activation of the phospholipase A<sub>2</sub> (PLA<sub>2</sub>) is an important mechanism leading to increased eicosanoid production.<sup>11</sup> Phospholipases participate in the regulation of physiological and pathological processes in the cell, including the release of pro-inflammatory mediators and stimulation of inflammatory processes.<sup>12,13</sup> Secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>) is associated with the development of the atherosclerotic process, and expression of sPLA<sub>2</sub> increases dramatically during inflammation<sup>14,15</sup> as seen with the high levels of this enzyme found in human atherosclerotic, macrophage-rich arterial walls.<sup>16</sup>

In this paper we have examined the effect of F at concentrations found in the blood of people environmentally exposed to fluorine compounds as they affect the production of PGE<sub>2</sub> and TXB<sub>2</sub>, the pro-inflammatory factors that are implicated in atherogenesis.

### MATERIALS AND METHODS

*Cell culture preparation:* Blood for experiments was taken from 14 healthy male donors, age 25 to 35. Exclusions from the experimental group were individuals with diabetes mellitus, atherosclerosis, high blood pressure, and other chronic diseases. Peripheral blood mononuclear cells (PBMCs) were isolated from anti-coagulated blood by lymphocyte separations media density gradient and monocytes were separated by adherence to plates (2 hr at 37°C, 5% CO<sub>2</sub>). Cells ( $2.5 \times 10^6$ ) were cultured for 7 days with 10% autologous serum and next for 48 hr (37°C, 5% CO<sub>2</sub>) with NaF at final concentrations of 1, 3, 6, and 10 μM. NaF solutions were selected on the basis of the F concentrations found in human serum.<sup>17-19</sup> Cell viability was examined using a trypan blue dye exclusion method. The cell count was determined with a Bright Line Hemacytometer (Sigma-Aldrich, Poznań, Poland). Protein concentration was measured by the Bradford method.<sup>20</sup>

The Pomeranian Medical University local ethics committee approved the design and conduct of this study.

*Measurement of macrophage sPLA<sub>2</sub> activity:* After incubation with NaF, the PBMCs were scraped from the plate and centrifuged (800 × g/10 min/4°C). After discarding the cell pellet the activity of sPLA<sub>2</sub> in the culture supernatants was measured spectrophotometrically (ELISA) using the sPLA<sub>2</sub> enzyme immunoassay kit (Cayman Chemical) according to the manufacturer's protocol. The activity of sPLA<sub>2</sub> in the sample was compared with sPLA<sub>2</sub> activity of the standard by comparison of the yellow color generated. The results are expressed as enzyme content in pg per mg of protein.

*Measurement of AA concentration in cells:* PBMCs ( $2.5 \times 10^6$ ) were incubated with 1, 3, 6, and 10 μM NaF for 48 hr and then 1 hr (37°C) with 5 μM ionophore (A 23187) with gentle agitation. After incubation the cells were scraped and collected with the medium. Folch mixture (2:1 v/v chloroform/methanol) containing 0.01% (w/v) butylated hydroxytoluene as antioxidant<sup>21</sup> was used for total lipid extraction in the probes. The extracts were evaporated until dry under nitrogen flow, suspended in 100 μL of n-hexane and applied on the thin layer

chromatography plates, which were developed with petroleum ether/diethylether/acetic acid mixture 90/10/1 (v/v/v).<sup>22</sup> The fraction of free fatty acids was scraped off the plate, extracted with Folch mixture, methylated with 20% (w/v) boron trifluoride-methanol, and extracted using n-hexane.

The influence of F on AA concentration was determined by gas chromatography using a Perkin Elmer AutoSystem XL chromatograph equipped with a flame-ionization detector (FID) and Elite 5 (60-m × 0.25-mm × 0.25µm) column. The analysis parameters were: split injection ratio 1:10; nitrogen as carrier gas at a flow of 1.1 cm<sup>3</sup>/min; the oven temperature from 170°C (16 min) to 210°C (30 min), 1°C/min. AA was identified by comparison of its retention time with a pure standard (Sigma-Aldrich, Poznań, Poland). Fatty acids content in cells is expressed as µg per mg of protein.

*Measurement of PGE<sub>2</sub> concentration in macrophages:* PBMCs were incubated for 48 hr with NaF solutions as described above. PGE<sub>2</sub> was extracted from the cells with the use of Bakerbond columns as described in manufacturer's instructions. The resulting concentrations of PGE<sub>2</sub> were measured spectrophotometrically in the culture supernatants by using the PGE<sub>2</sub> enzyme immunoassay kit (Cayman Chemical) and ELISA equipment.

*Measurement of TXA<sub>2</sub> concentration in cells:* PBMCs were incubated with NaF solutions as described above for PGE<sub>2</sub> release, and TXA<sub>2</sub> was assayed after neutrophil removal by centrifugation. Bakerbond columns were used for TXA<sub>2</sub> extracted from the cells. As TXA<sub>2</sub> has a short half-life (37 sec) and is rapidly hydrolyzed nonenzymatically to its stable derivative TXB<sub>2</sub>, the thromboxane B<sub>2</sub> enzyme immunoassay kit (Cayman Chemical) and ELISA were used to measure free TXA<sub>2</sub> indirectly.

*Measurement of early-stage apoptosis:* After NaF treatment, 5 × 10<sup>5</sup> PBMCs were collected, washed twice with PBS (phosphate buffered saline), suspended in Annexin V Binding Buffer (0.1 M HEPES/NaOH (pH = 7.4), 1.4 M NaCl, 25 mM CaCl<sub>2</sub>) and stained with 1 ng/mL annexin V-FITC and 5 ng/mL propidium iodide for 30 min in the dark. The externalization of phosphatidylserine as a marker of early-stage apoptosis was detected by the annexin V protein conjugated to FITC (fluorescein isothiocyanate).<sup>23</sup> Cells were analyzed by flow cytometry using FACScan cytofluorometry. Measurement of apoptosis was performed with a Becton-Dickinson annexin V/FITC (Oxford, UK) apoptosis detection kit according to the manufacturer's instructions. For each sample, the results are expressed as percentages of the 5 × 10<sup>5</sup> cells in early-stage apoptosis.

*Measurement of F concentration in donors' serum:* Blood samples obtained from the donors were centrifuged (800 × g/4°C/10 min). Afterward the serum was separated and kept frozen at -80°C until analysis. TISAB III buffer solution (0.5 mL) was added to 0.5 mL of serum sample and the F content was determined with the potentiometric method.

*Statistical analysis:* Each investigated parameter was analyzed statistically and characterized using an arithmetical mean and standard deviation (SD). Because most of the distributions deviated from the normal (Gauss) distribution (Shapiro-

Wilk test), further analysis involved the non-parametric tests. In order to find statistically significant differences in the concentration levels of the examined parameters in macrophages the Wilcoxon test was used. The level of significance was assumed to be  $p = 0.05$ .

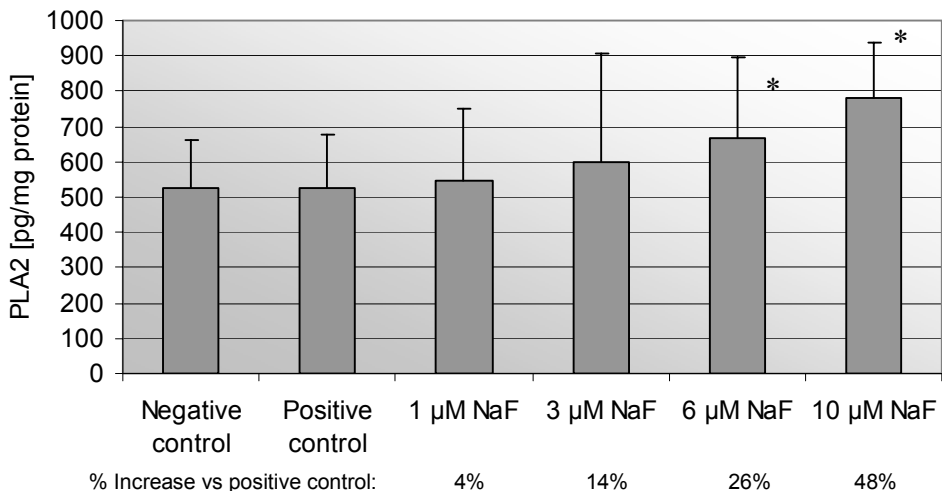
### RESULTS

The F content of the serum of the donors qualified for the experiment was at the level reported by other authors<sup>17</sup> for healthy persons without fluorosis (Table 1).

**Table 1.** Concentration of F in the serum of PBMCs donors

No. of donor	Age (yr)	F (mg/L)
1	34	0.0404
2	35	0.0212
3	34	0.0312
4	33	0.0301
5	25	0.0215
6	35	0.0241
7	34	0.0271
8	35	0.0196
9	31	0.0226
10	25	0.0246
11	32	0.0565
12	35	0.0294
13	28	0.0256
14	35	0.0232

The increase in NaF concentration added to the PBMC culture was followed by an increase in sPLA<sub>2</sub> activity in the supernatant of the macrophages: a significant increase was observed for 6 and 10  $\mu\text{M}$  NaF ( $p = 0.013$ , 26%;  $p = 0.012$ , 48%, respectively; Figure 1).



**Figure 1.** Effect of F on sPLA<sub>2</sub> activity in the supernatants from macrophage cultures incubated with increased concentration of NaF.

Positive control - H<sub>2</sub>O. Monocytes/macrophages were cultured with NaF for 48 hr. After incubation, cells were scraped, and the activity of sPLA<sub>2</sub> was measured spectrophotometrically in supernatant by using the sPLA<sub>2</sub> enzyme immunoassay kit. The results obtained from 5 separate experiments were normalized to protein levels.

\* $p < 0.02$  - significant difference vs. positive control.

As seen in Table 2, addition of ionophor A23187 to the PBMC culture with NaF present caused an increase of intracellular AA concentration. A statistically significant increase for all F concentrations ( $p < 0.03$ ) and in a dosage-dependent manner.

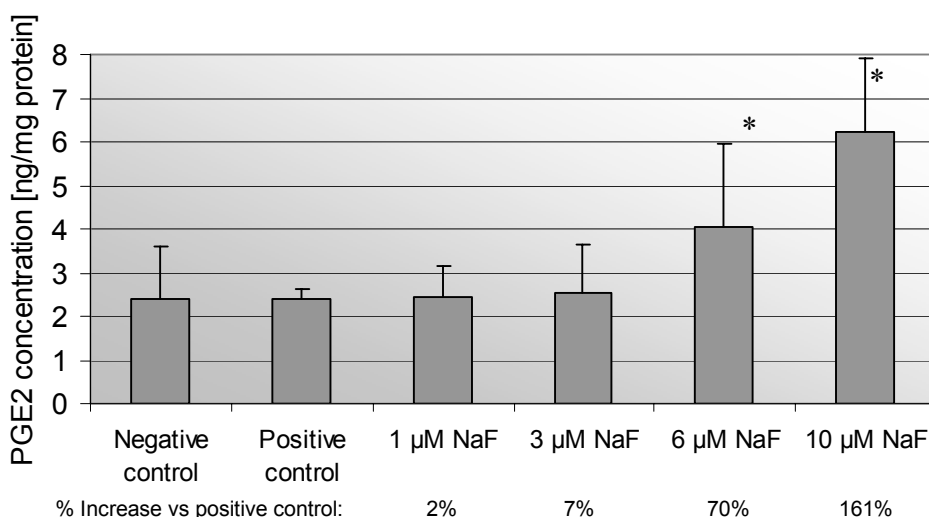
**Table 2.** Effect of F on arachidonic acid concentration in macrophages

Experimental condition	Concentration of arachidonic acid [ug/mg protein]	Increase vs positive control
Positive control	110.76 ± 7.32	
1 μM NaF	134.02 ± 20.11 *	21%
3 μM NaF	162.53 ± 53.63 *	47%
6 μM NaF	166.23 ± 39.74 *	50%
10 μM NaF	182.83 ± 40.18 *	65%
Negative control	113.64 ± 24.99	

Positive control - H<sub>2</sub>O. Monocytes/macrophages were cultured for 48 hr with NaF and for 1 hr with ionophor A23187. AA concentration was measured by using the GC method. The results obtained from 6 separate experiments were normalized to protein levels.

\* $p < 0.03$  - significant difference vs. positive control.

As seen in Figure 2, incubation of the PBMCs with increasing concentrations of NaF caused a significant increase in the PGE<sub>2</sub> concentration in macrophages in a dosage-dependent manner. NaF at concentrations of 6 μM and 10 μM significantly increased the prostaglandin PGE<sub>2</sub> concentration by about 70% ( $p = 0.043$ ) and 161% ( $p = 0.043$ ) respectively. At 1 μM and 3 μM concentrations, NaF added to the culture caused only an insignificant increase in PGE<sub>2</sub> value in macrophages.

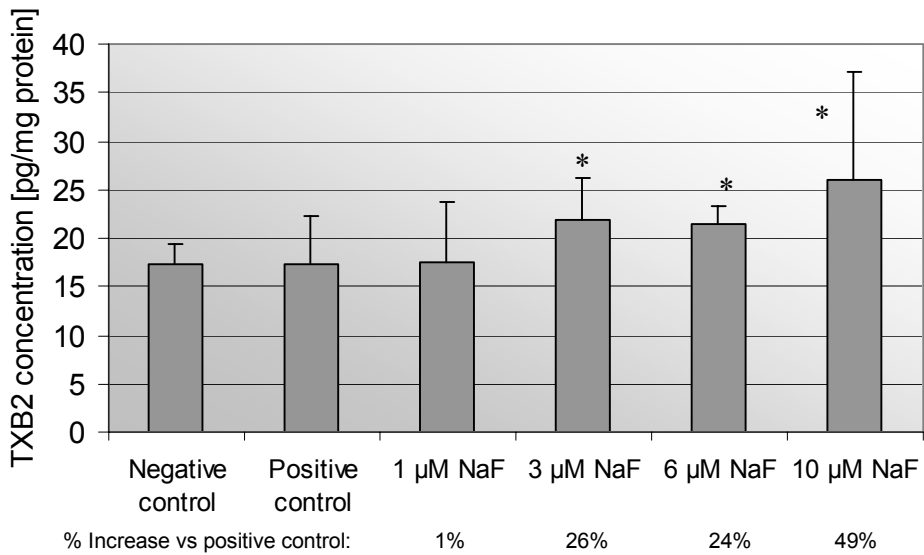


**Figure 2.** Mean concentration of PGE<sub>2</sub> in the supernatants from macrophage cultures incubated with increased concentration of NaF.

Positive control - H<sub>2</sub>O. After incubation, cells were scraped and the PGE<sub>2</sub> concentration was measured in supernatant by using the PGE<sub>2</sub> enzyme immunoassay kit and ELISA equipment. The results obtained from 5 separate experiments were normalized to protein levels.

\* $p < 0.05$  - significant difference vs. positive control.

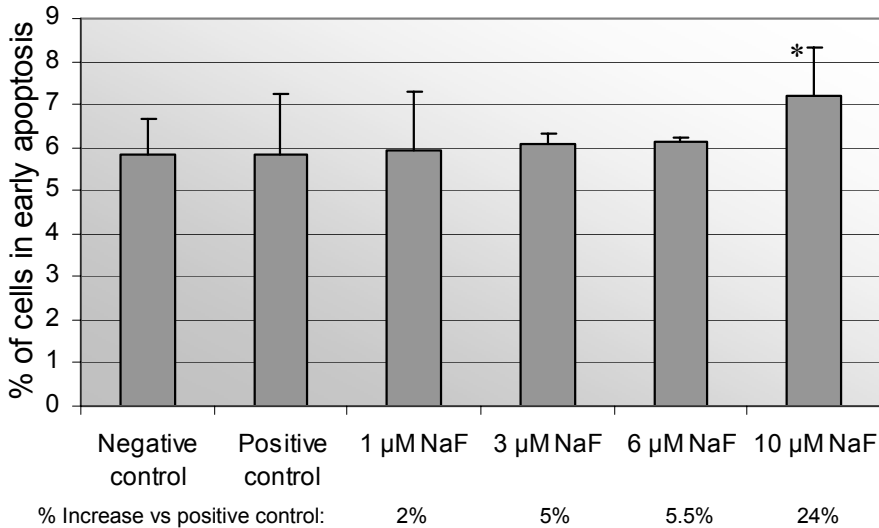
Figure 3 presents mean values of TXB<sub>2</sub> produced by PBMC differentiated monocytes/macrophages after addition of increasing concentrations of NaF to the incubation mixture. We observed increase in the TXB<sub>2</sub> concentration in a dosage-dependent manner, but statistically significant only for 3 μM (p = 0.027), 6 μM (p = 0.027), and 10 μM (p = 0.027) concentration of NaF.



**Figure 3.** Mean concentration of TXB<sub>2</sub> in the supernatants from macrophage cultures incubated with increased concentration of NaF. Positive control - H<sub>2</sub>O. After incubation, cells were scraped and the TXB<sub>2</sub> concentration was measured in supernatant by using the TXB<sub>2</sub> enzyme immunoassay kit and ELISA equipment. The results obtained from 5 separate experiments were normalized to protein levels.

\*p<0.03 - significant difference vs. positive control.

Finally, addition of NaF to the PBMC culture caused an early-stage apoptosis in macrophages in a dosage-dependent manner (only for 10  $\mu\text{M}$  NaF solution  $p = 0.07$ , Figure 4).



**Figure 4.** Effect of fluoride on early-stage apoptosis in PBMC macrophages cultured with NaF.

Positive control -  $\text{H}_2\text{O}$ . Cells were cultured with NaF for 48 hr and next with Annexin V-FITC (1 ng/mL) and propidium iodide (5 ng/mL) for 30 min in the dark. Results are expressed in percentage of cells in early-stage of apoptosis from 3 separate experiments.

\* $p = 0.07$  - difference vs positive control.

## DISCUSSION

Lipid mediators are involved in many physiological processes, and their deregulation has often been linked to various diseases such as inflammation and atherosclerosis. The evolving inflammatory reaction is instrumental in the initiation of atherosclerotic plaques, and the macrophages are the main effector cells in this process.<sup>24</sup> The eicosanoids,  $\text{PGE}_2$  and  $\text{TXA}_2$  produced by macrophages, are involved in inflammatory events.<sup>25</sup>

In our work here we demonstrated that F as NaF increased the concentration of  $\text{PGE}_2$  and  $\text{TXB}_2$  (stable derivative of  $\text{TXA}_2$ ) in PBM macrophages in a dosage-dependent manner. The results are in line with data reported by other authors, who also observed F-induced generation of  $\text{PGE}_2$  and  $\text{TXA}_2$ .<sup>26,27</sup> These eicosanoids formed during inflammatory reactions contribute to and aggravate atherosclerotic lesions.<sup>28</sup> They sustain homeostatic functions and mediate pathogenic mechanisms, including the inflammatory response.<sup>29</sup> They can alter the response of both the host tissue and the recruited inflammatory cells.<sup>9</sup> Belton et al.,<sup>30</sup> noted markedly enhanced formation of  $\text{PGE}_2$  and  $\text{TXB}_2$  in patients with atherosclerosis. In addition, alterations in the profile of prostanoid synthesis can occur upon cellular activation. While resting macrophages produce  $\text{TXA}_2$  in excess of  $\text{PGE}_2$ , this ratio changes to favor  $\text{PGE}_2$  production after macrophage activation.<sup>9</sup>

Phospholipases participate in the regulation of physiological and pathological processes in the cell, including the release of pro-inflammatory mediators and stimulation of inflammatory processes.<sup>13</sup> Macrophages are cells that can secrete the enzyme sPLA<sub>2</sub>,<sup>1,7,31</sup> which is an acute-phase protein participating in the release of AA from membrane phospholipids and is expressed in response to various pro-inflammatory stimuli.<sup>16</sup> As shown in the present study, F may be one of them.

Activation of sPLA<sub>2</sub> is also an important mechanism leading to increased eicosanoid synthesis.<sup>10</sup> Our results demonstrate that incubation of macrophages by F caused an increase in sPLA<sub>2</sub> activity within the cells in a dosage-dependent manner. Although statistical significance was noted only for 6 μM and 10 μM NaF, which is comparable with the serum F concentrations in humans with fluorosis,<sup>18,19</sup> the increase was also observed for concentrations found in healthy persons,<sup>17</sup> thereby suggesting that even low doses of F may have a significant impact.

Since AA released by PLA<sub>2</sub> is known to play an important role as a precursor of inflammatory lipid mediators, eicosanoid synthesis in macrophages is controlled by the availability of free AA.<sup>10</sup> It has been proved in this study that application of NaF to PBM macrophages at a very low dosage significantly increased the amount of released AA (Table 2) for all concentrations of NaF. The same results were obtained by Dodam and Olson,<sup>32</sup> but they used 30 mM NaF, a much higher concentration than in our experiment. Our data suggest that F may not only increase the activity of sPLA<sub>2</sub> in macrophages (Figure 1) but also that the activity of the enzyme causes the increase in AA release (Table 2). Probably F acts by stimulation of the production of reactive oxygen species (ROS) associated with protein tyrosyl residue phosphorylation and PLA<sub>2</sub> activation.<sup>33,34</sup> This hypothesis is supported by our previous study, in which we have shown that incubation of monocytes/macrophages with NaF caused a significant increase in ROS synthesis in cells.<sup>35</sup>

The pro-apoptotic properties of F and the mechanism of apoptosis induction by the addition of F to cell cultures have been well described.<sup>36,37</sup> Increased inflammatory process participates in apoptotic stimulation,<sup>38</sup> and, as shown here, F may also increase the number of macrophages in early-stage apoptosis (Figure 4), probably through the stimulation of oxidative stress<sup>1</sup> and synthesis of pro-inflammatory products. The results obtained in this study for apoptosis were not as spectacular as in other reports,<sup>36,37</sup> which used mM rather than μM concentrations of NaF. We believe, however, that our findings with low concentrations of NaF to PBM cell cultures do, in fact, indicate the possibility of clinically-detectable arthritic-type injury.

In conclusion, the present results suggest that NaF, even in small concentrations, may induce an inflammatory process and apoptotic effect in PBM macrophages through the stimulation of prostanoids metabolism and increased synthesis of PGE<sub>2</sub> and TXA<sub>2</sub>.



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