

## EFFECT OF FLUORIDE INGESTED BY LACTATING MICE ON THE THYROID FUNCTION AND BONE MATURATION OF THEIR SUCKLING PUPS

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**SUMMARY:** Our aim was to study the effects on thyroid function and bone maturation in suckling Wistar mice resulting from ingestion by their mothers of 500 ppm NaF in their drinking water from the 15<sup>th</sup> day of pregnancy until the 14<sup>th</sup> day after delivery, at which time the pups were sacrificed. Compared to a control group, the suckling mice from the NaF-treated mothers showed a 15% decrease in body weight and a reduction in plasma free T<sub>4</sub> and T<sub>3</sub> hormones by 15% and 6%, respectively. Compared to the control group, a 10% and 3% increase was observed in the fluoride content of bone and urine, but no significant change was found in the plasma. These changes were confirmed by bioanalytical data concerning the bone mineral content. Calcium and phosphate levels of bone decreased by 30% and 27%, respectively. The calcium concentration in plasma increased by 34%, and the phosphorus concentration in plasma decreased by 26%, while urinary levels of calcium decreased by 25% and those of phosphate increased by 28%. These results suggest that fluoride accelerated bone resorption activity. In fact, biochemical markers such as total tartrate-resistant acid phosphatase (ACP) and total alkaline phosphatase (ALP) increased significantly by 46% and 35%, respectively.

Keywords: Bone biomarkers; Calcium-phosphate homeostasis; Fluoride-exposed mice; Suckling mice; Thyroid function.

### INTRODUCTION

Excessive exposure to fluoride causes toxicity and chronic metabolic disorders in animals and humans.<sup>1,2</sup> Prolonged intake of elevated levels of fluoride may result in skeletal fluorosis.<sup>1</sup> Many factors can adversely affect bone formation. These include defective bone growth affecting both height and weight<sup>3</sup> and abnormal calcium-phosphate homeostasis.<sup>4,5</sup> Bone remodelling and skeleton consolidation result from a complex sequence of hormonal changes in interaction with nutritional factors.<sup>6</sup> Thyroid hormones are necessary particularly for skeleton maturation in the postnatal animal.<sup>7,8</sup> They stimulate bone growth directly and indirectly by increasing growth hormone secretion.<sup>9</sup> Increased dietary fluoride can result in thyroid enlargement,<sup>10</sup>

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reduced thyroid adenylcyclase, and decreased blood thyroxine ( $T_4$ ) and triiodothyronine ( $T_3$ ). Hypothyroidism occurs not only with antithyroid medications<sup>11,12,13</sup> but also with fluoride.<sup>14</sup> Numerous studies concerning the effects of fluoride on bone maturation have been carried out on humans, chickens, and adult rats. But reports on the effects of fluoride effects on bone remodelling remain scarce for adult mice and appear to be lacking for newborn mice.

The purpose of this study was to investigate the exposure effect of NaF in lactating mice on the thyroid function and bone maturation of their pups during the suckling period.

### MATERIAL AND METHODS

The same protocol was followed as in our previous paper.<sup>15</sup> Adult Wistar mice (Central Pharmacy, Tunisia), weighing about 30 g, were housed at  $22\pm 3^\circ\text{C}$  with light-dark periods of 12 hr and a minimum relative humidity of 40%. They had free access to water and commercial diet (SICO, Sfax, Tunisia). The standard diet contained  $0.992\pm 0.014$   $\mu\text{g}$  of iodine by gram of diet (iodine level was determined in diet after acid mineralization using the catalytic method<sup>16</sup>). After acclimatization to the laboratory conditions for one week, female mice were caged overnight with males and the presence of spermatozoa in the vaginal smear was taken as an indicator of day 0 of pregnancy. Twenty pregnant mice were divided into two groups of 10. The first group represented control animals. The second group was given 500 ppm of NaF in their drinking water from day 15 of their pregnancy until the 14th day after delivery. Daily fluoride intake and iodine quantities ingested by lactating mice were calculated after measuring drinking water and food consumption, respectively.

Pregnant female mice were allowed to deliver spontaneously three weeks after coitus. At birth, litters were reduced to eight pups each, and the day of birth was considered as postnatal day 0. All pups ( $n = 160$ ) were sacrificed on postnatal day 14. After anaesthesia with chloral hydrate intra-abdominally, body weights were measured. Femurs were dissected out, their length and weight were measured, urine was taken from bladder, and blood samples were collected from the brachial artery and centrifuged at 2200 g. Plasma samples were kept at  $-20^\circ\text{C}$  until free  $T_4$  and  $T_3$  were measured by radioimmunoassay (Immunotech, ref: 1363; 1579, respectively). Total alkaline phosphatase and total tartrate resistant acid phosphatase levels were determined by a colorimetric method (Elitech diagnostics, ref: 02 – 0345; Biomerieux, ref: 746419901, respectively). Fluoride, calcium, and phosphorus levels were determined in femurs after acid mineralization and in plasma and urine using,

respectively, a fluoride ion specific electrode,<sup>17</sup> an automatic colorimetric method by Technicon RA-XT,<sup>TM</sup> 18,19 and spectrophotometric methods.<sup>20</sup> Comparisons of mean values between treated and control animals were made using Student's t test.<sup>21</sup> Statistical significance was defined as a P value of less than 0.05.

## RESULTS

Compared with the control group, the NaF-treated mothers (estimated F intake  $5.251 \pm 0.171$  mg/day), had a decrease in consumption of drinking water (31%), in food consumption (11%), and in ingested iodine (11%) (Table 1).

**Table 1.** Daily food, water, fluoride, and iodine quantities ingested by lactating mice

	Controls (n = 10) (mean $\pm$ SEM)	NaF group (n = 10) (mean $\pm$ SEM)
Food consumption (g/day)	11.566 $\pm$ 1.041	10.324 $\pm$ 1.271*
Water (mL/day)	15.285 $\pm$ 1.736	10.502 $\pm$ 0.342*
Ingested Fluoride quantities (mg/day)	—	5.251 $\pm$ 0.171
Ingested Iodine ( $\mu$ g/day)	11.473 $\pm$ 1.032.	10.241 $\pm$ 1.261*

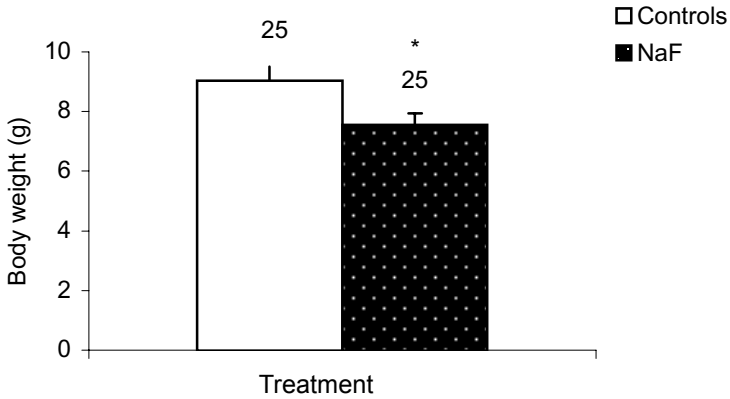
Significant differences: NaF vs Controls: \*  $p \leq 0.001$ .

Their pups showed a 15% decrease in body weight ( $p \leq 0.001$ ) (Figure 1), a 14% decrease in femur length, and an 18% decrease in femur weight ( $p \leq 0.001$ ) (Table 2).

**Table 2.** Effect on femur lengths and weights of 14-day-old mice administered NaF in the drinking water (0.5 g/L) of the mother from the 15th day of pregnancy until sacrifice of the pups on the 14th day after birth

	Controls (n = 57) (mean $\pm$ SEM)	NaF group (n = 58) (mean $\pm$ SEM)
Femur lengths (mm)	9.79 $\pm$ 0.25	8.43 $\pm$ 0.45*
Femur weights (mg)	30.37 $\pm$ 1.01	24.85 $\pm$ 2.01*

Significant differences: NaF vs Controls: \*  $p \leq 0.001$ .



**Figure 1.** Effect on the body weight of 14-day-old mice administered NaF in the drinking water (0.5 g/L) of the mother from the 15th day of pregnancy until sacrifice of the pups on the 14th day after their birth.

Significant differences: Fluoride vs Controls: \*p<0.001.

In these pups, thyroid hormone levels (plasma free T<sub>4</sub> and T<sub>3</sub>) were reduced by 15% (p<0.05) and 6%, respectively (Figure 2). A 10% and 3% increase in femur (p<0.001) and urine (p<0.05) fluoride was also observed, but no significant change in the plasma fluoride level (Table 3).

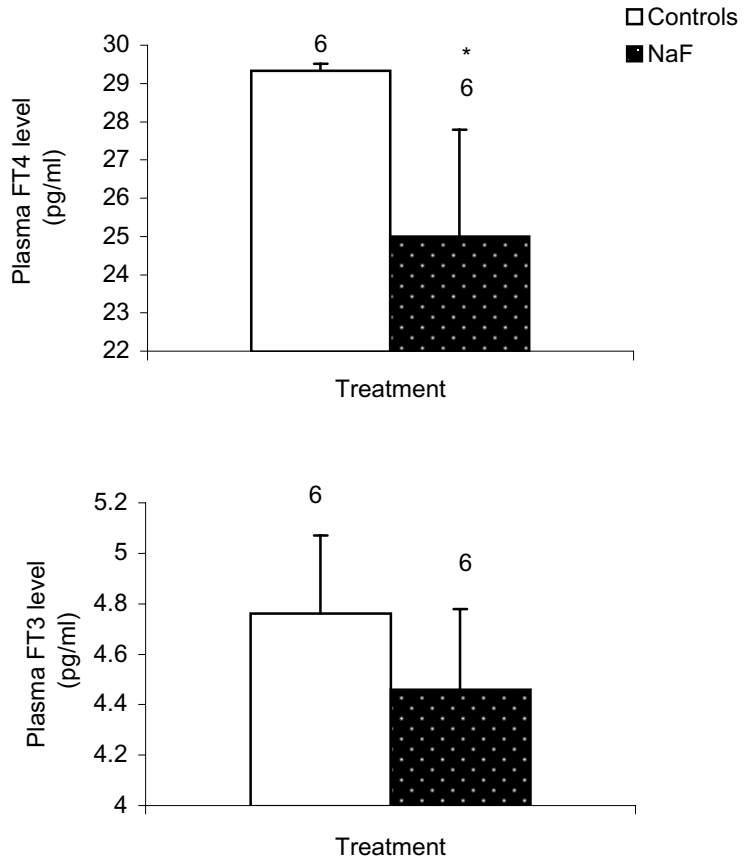
**Table 3.** Fluoride concentrations in bone, plasma, and urine of 14-day-old mice administered NaF in the drinking water (0.5 g/L) of the mother from the 15th day of pregnancy until sacrifice of the pups on the 14th day after birth

	Fluoride content (ppm, means±SEM) of :		
	Bone	Plasma	Urine
Controls (n = 6)	109.7 ± 4.2	0.087 ± 0.008	0.258 ± 0.005
NaF group (n = 6)	120.1 ± 1.4 <sup>†</sup>	0.077 ± 0.011	0.265 ± 0.000*

Significant differences: NaF vs Controls: \* p<0.05; † p<0.001

Exposure of the mothers to 500 ppm NaF also altered bone mineral composition in their pups, especially calcium and phosphorus levels, which decreased by 30% (p<0.001) and 27% (p<0.001), respectively, while the calcium concentration in plasma increased by 34% (p<0.001) and the phosphorus concentration in plasma decreased by 26% (p<0.01) compared to the control group. Compared to the controls, the urinary calcium level

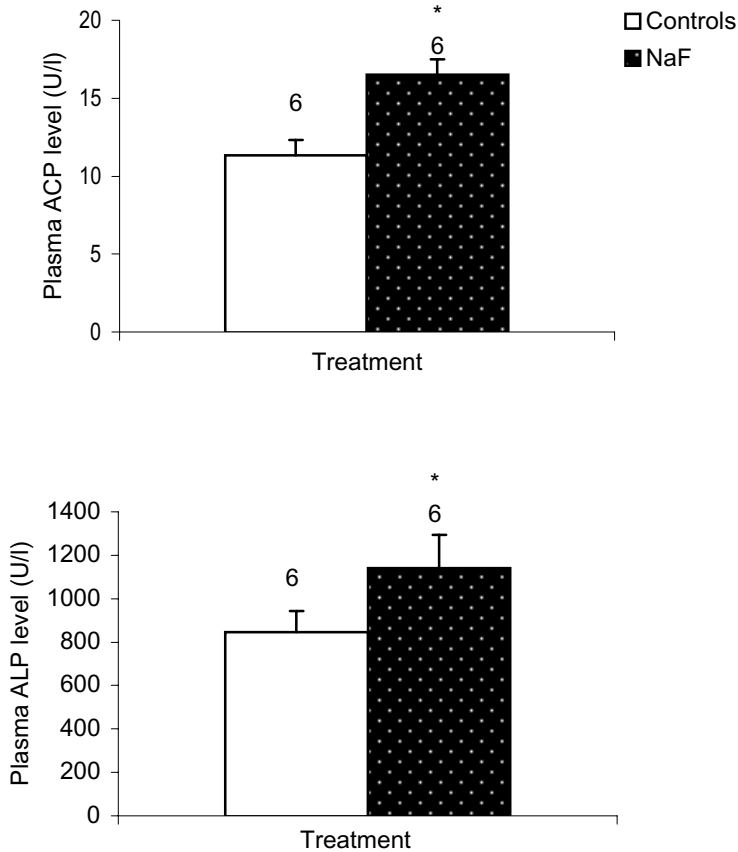
decreased by 25% ( $p \leq 0.001$ ), and the phosphate urinary level increased by 28% ( $p \leq 0.01$ ) (Table 4).



**Figure 2.** Effect on the plasma thyroid hormones level (FT4 and FT3) of 14-day-old mice administered NaF in the drinking water (0.5 g/L) of the mother from the 15th day of pregnancy until sacrifice of the pups on the 14th day after their birth.

Significant differences: Fluoride vs Controls: \*  $p \leq 0.05$ .

Biochemical markers such as total resistant acid phosphatase (ACP) level, which reflected bone resorption, increased by 46% ( $p \leq 0.001$ ), while total alkaline phosphatase (ALP) level, which reflected bone formation, increased by 35% ( $p \leq 0.001$ ) (Figure 3).



**Figure 3.** Effect on the plasma level of total tartrate-resistant acid phosphatase (ACP) and total alkaline phosphatase (ALP) of 14-day-old mice administered NaF in the drinking water (0.5 g/L) of the mother from the 15th day of pregnancy until sacrifice of the pups on the 14th day after their birth.

Significant differences: Fluoride vs Controls: \*  $p \leq 0.001$

**Table 4.** Effect on bone, plasma, and urinary calcium and phosphorus concentrations of 14-day-old mice administered NaF in the drinking water (0.5 g/L) of the mother from the 15th day of pregnancy until sacrifice of the pups on the 14th day after birth

	Controls (n = 6) (mean±SEM)	NaF group (n = 6) (mean ± SEM)
Bone levels (mg/g)		
Calcium	102.15 ± 3.85	71.64 ± 2.58 <sup>†</sup>
Phosphorus	64.46 ± 1.48	47.25 ± 1.26 <sup>†</sup>
Plasma levels (mg/L)		
Calcium	84.51 ± 1.81	113.73 ± 5.09 <sup>†</sup>
Phosphorus	33.86 ± 4.8	24.95 ± 1.85 <sup>*</sup>
Urinary levels (mg/L)		
Calcium	53.62 ± 4.21	40.17 ± 2.28 <sup>†</sup>
Phosphorus	242.05 ± 21.43	310.59 ± 33.23 <sup>*</sup>

Significant differences: NaF vs Controls: \* $p < 0.01$ ; <sup>†</sup> $p < 0.001$

## DISCUSSION

In this study 500 ppm NaF in the drinking water of the maternal mice decreased the body weight of their offspring in the suckling period. This perturbation was probably primarily due to a reduction in hormone production by the thyroid gland. In fact, a decrease in thyroid hormones in plasma (FT<sub>4</sub> and FT<sub>3</sub>) was observed. These results confirm previous data from our laboratory, where, in a similar experiment, a 75% decrease was recorded in the plasma thyroxine level of suckling mice, which was five times what we found in the present study.<sup>15</sup> This difference may be possibly due to a higher ingestion in that study of NaF (6.112±0.197 instead of 5.251±0.171 mg F/day), lower food consumption (8.059±0.429 instead of 10.324±1.271 g/day), and lower amounts of iodine ingested by the lactating mice (5.802±0.308 instead of 10.241±1.261 µg I/day). Decreases in T<sub>3</sub> and T<sub>4</sub> were found after the administration of fluoride to animals,<sup>22,23</sup> as also in a study conducted by Yu,<sup>24</sup> where 50 ppm of fluoride in the drinking water of rats reduced the serum T<sub>3</sub> and T<sub>4</sub>, and in a study on lambs by Vashishth *et al.*<sup>25</sup>

In the present work, fluoride exposure of the mothers delayed bone growth in their nursing pups, reflected by a sharp decrease in femur length and weight, thus verifying that thyroid hormones are crucial for optimal bone growth in humans and rats.<sup>9</sup> Other findings demonstrated that thyroid hormones stimulated bone growth indirectly by increasing GH secretion<sup>26</sup> and

directly by independent mechanisms as shown by stimulating longitudinal bone growth in hypophysectomized rats.<sup>27</sup>

On the other hand, our results indicated that plasma and urine fluoride levels in the nursing pups did not differ between the test and control groups. These data could be explained by the finding that limited transfer of fluoride from plasma to breast milk occurred when 1–5 mg of fluoride was given orally to lactating women.<sup>28</sup> After the intake of higher doses of fluoride by lactating mothers (25 mg of NaF/day), a slight increase in fluoride concentration in breast milk was observed.<sup>29</sup> Nevertheless, we found that the pups of the NaF-treated mice had a significant increase in their bone fluoride compared to the control group. This result may be explained by transplacental transfer of fluoride from the mother to its fetus. Our results also confirm earlier observations of Shen and Taves, who found that fluoride was readily taken up by calcifying fetal bones and teeth during the period of gestation, reflecting that the placenta is not a barrier to the passage of fluoride to the fetus.<sup>30</sup>

The mineral fraction of bone is largely composed of calcium and phosphate in the form of hydroxyapatite, which constitutes about one quarter of bone volume and one half of bone mass.<sup>31</sup> In this structure, fluoride ion ( $F^-$ ) substitutes for the hydroxyl ion ( $OH^-$ ), giving rise to fluoroapatite.<sup>32,33</sup> Exposure of lactating female mice to NaF in our experiment alters the bone mineral composition of their pups, especially the calcium and phosphorus content. These data concur with those showing that fluoride alters the chemical composition of bone mineral.<sup>32,34</sup> Consequently, we observed an increase in plasma calcium and urine phosphorus levels and a decrease in urine calcium and plasma phosphorus levels, which could be explained by parathyroid hormone (PTH) action. This hormone mobilizes calcium from bone and increases kidney calcium reabsorption from the glomerular filtrate.

Bone mass, normally maintained by close matching of bone formation to bone resorption, could be reduced when these two opposing influences are uncoupled.<sup>35</sup> Fluoride increases bone turnover with increased bone destruction and bone formation. Activation of osteoclasts and osteoblasts were confirmed respectively by biochemical markers such as total tartrate-resistant acid phosphatase (ACP) and total alkaline phosphatase (ALP). In fact, these biomarkers significantly increased in the pups of the treated mice compared to the control group. Our results thus confirm previous findings of Ando and coworkers<sup>36</sup> and those of Okazaki,<sup>37</sup> who reported that fluoride stimulates both bone resorption and formation, especially in patients with skeletal fluorosis. Other data confirm that fluoride increases both osteoblast<sup>32,38</sup> and osteoclast<sup>39</sup> activities.



In conclusion, we found that fluoride ingested by lactating mice is retained in the bones of their suckling pups, leading to alteration of calcium-phosphate homeostasis and provoking perturbations in bone mineral formation.

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