FETOTOXICITY OF FLUORIDE IN RATS AND THE PROTECTIVE ACTION OF SOME ANTIOXIDANTS

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SUMMARY: The aim of this study was to assess the efficacy of antioxidants to prevent or alleviate fluoride (F) toxicity in pregnant female Wistar rats and their fetuses. Groups of pregnant rats (10 in each group) were treated by oral intubation with F (40 mg F^-/kg bw/day from NaF), antioxidants (a mixture of vitamins A, C, and E and selenium) (25 mg/kg bw/day), and a combination of F and antioxidants at the same dosage levels, respectively, from the 8th to the 19th day of gestation. A control group received tap water. No significant change occurred among treated groups in outcome of pregnancy (no. of resorbed, dead, live normal, and abnormal fetuses/ litter) on gestational day 20. However, fetuses of F-treated rats exhibited significant reduction of body weight and length concomitant with a significant decrease of total protein content in liver tissue. On the other hand, the F concentration in serum and amniotic fluid of F-treated rats was significantly higher than all other groups. Pregnant rats with higher serum F concentration had a significantly lower serum Ca level and a higher serum P concentration with a non-significant reduction of serum total protein in comparison with the control. Administration of the antioxidants reduced the F-induced changes. Less fetal growth retardation occurred in the rats treated with both F and the antioxidants than in those treated with F alone. Moreover, antioxidant treatment resulted in some recovery of serum Ca and P levels in the F-treated group. Antioxidants were therefore found to protect against or ameliorate F-induced toxicity in pregnant rats and their fetuses.

Keywords: Amniotic fluid; Antioxidants (Vitamins A, C, and E, and Se): Fetal resorption; Fetotoxicity; Fluoride and pregnancy; Liver tissue protein; Rat fetotoxicity; Serum calcium; Serum phosphorus; Skeletal retardation.

INTRODUCTION

In many parts of the world, toxic effects of fluoride (F) are a major public health problem resulting mainly from long-term consumption of water with high F levels.[1] Egypt is one of about 21 developed and developing nations that have problems with endemic fluorosis,[2] where the main pathway of F exposure is the ingestion of tap water from contaminated ground water sources.[3] The F concentration in industrial waste water samples collected from Abu Zabaal and Ahlia areas around Cairo during six months vary from 1.13 to 7.10 mg/L,[4] significantly exceeding the World Health Organization recommended maximum 1 mg F/L.[5]

Earlier studies indicate that the incidence and severity of chronic F intoxication are greatly influenced by socio-economic, climatic, and nutritional status and conditions.[6] Various injurious effects are associated with chronic exposure to 1.5 mg F/L or more in drinking water, including dental fluorosis, skeletal fluorosis, neurological damage, and reproductive disorders.[3] Many studies on laboratory animals over a range of F concentrations (0–250 mg/L in drinking water) indicate that adverse reproductive and developmental outcomes occur at high F concentrations.[7] Occupational exposure to organofluorine compounds has been

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reported to induce abnormal menstruation and increased miscarriages and pregnancy complications among female workers in fluorine factories.[8] Epidemiological studies have also shown decreasing total fertility rate with increasing fluoride levels in drinking water.[9] In addition, the possibility that F may be a carcinogen is strengthened by recent findings of genotoxicity (increased sister-chromatid exchange) in humans consuming elevated levels of F in drinking water.[10]

On the other hand, antioxidants including vitamins A, C, and E and selenium have been found to protect against the toxicity of different toxicological agents, diseases, and age-related phenomena.[11] The present study was conducted to determine the effect of F (from NaF) on pregnant rats (from the 8th to the 19th day of gestation, a critical period of organogenesis) and their fetuses and to investigate the potential protective role of certain antioxidants (a mixture of antioxidants vitamin A, C, and E and selenium) against such toxic effects of F during pregnancy.

MATERIALS AND METHODS

Animals: Forty mature female Wistar rats weighing about 200–220 g (100–110 days old) were used in the present work. The animals were obtained from the Animal Farm of the Egyptian Organization for Vaccine and Biological Preparation at Helwan. The rats were housed in well-ventilated cages under a controlled temperature of 25±1.0ºC on a 12-hr light/dark cycle. The animals received a standard diet and water *ad libitum.* Both diet and water had low levels of F, which were considered negligible, and our calculations of F intake are therefore based on the amount of sodium fluoride administered orally to the rats. Pregnancy was established by housing females in the pro-estrous stage with sexually potent males overnight. The next morning, females with positive vaginal smears were considered pregnant, and the day of detection was defined as the first day of pregnancy.

Applied chemical compounds: Sodium fluoride (NaF) was obtained from Sigma Chemical Co., St. Louis, Mo., USA. It was dissolved in tap water and administered orally to pregnant rats daily from the 8th to the 19th day of gestation at a dosage of 40 mg F^{-}/kg bw according to Verma and Sherlin.[12] In addition, a mixture of antioxidants (Antox) composed of vitamin A (0.554 mg), vitamin C (100 mg), vitamin E (30 mg), and Selenium (50 µg), obtained as a tablet from Arab Company for Pharmaceuticals and Medicinal plants MEPACO-Egypt, was dissolved in tap water and given orally at a dose (25 mg/kg bw), equivalent to the human therapeutic dose.

*Animal grouping***:** Pregnant rats were divided into four equal groups of ten rats each as follows: Group (1) control group received 0.5 mL of tap water by gavage; group (2) rats administered Antox in 0.5 mL of water by gavage at 25 mg/kg bw/day; group (3) rats administered NaF in 0.5 mL of water by gavage at 40 mg F^{-}/kg bw/day; group (4) rats administered both NaF and Antox together in the same manner and dosages.

Sampling and tissue extraction: Pregnant rats of all groups were weighed on the 1st, 7th, 13th, and 20th day of gestation, and the average body weight of mothers

was recorded. On the 20th day of gestation, the pregnant rats were lethally anesthetized by ether inhalation, blood was collected from the heart ventricle, centrifuged, and the supernatant fraction (pure serum with no haemolysis) was used for determination of F, total protein, and calcium and phosphorus levels. At the same time, amniotic fluid (1–2 mL) was collected by syringe from the uteri for determination of the amniotic F level. The uteri were then searched for total implantation sites, number of resorptions, and living and dead fetuses. The body weight of fetuses was recorded, the fetuses were then examined for any morphological abnormalities, after which the crown-to-rump length was measured. Two or three living fetuses from each mother were sacrificed and liver tissues were removed, washed, weighed, homogenized, and centrifuged. The supernatant was then used for determining the total protein content. Finally, the 20-day-old fetuses were preserved in 95% ethanol. For examination of any skeletal abnormalities, they were stained with the Alcian blue/Alizarin red-S technique according to the method of McLeod.[13] Total protein content in serum and liver tissue was determined by the method of Gornall.[14] Calcium ion concentrations and inorganic phosphorus were measured as described by Corns and Ludman[15] and by Young,[16] respectively. Determination of F in both serum and amniotic fluid was performed with a fluoride ion electrode according to the method of Hall et al.[17] at the central laboratory of Ain Shams University.

Data are expressed as the mean ± standard error (SE). The significance of the difference between means was analyzed using Student's t test. The percentage change was taken as the difference between the experimental and control value, divided by the control value, calculated in percent.

RESULTS

In the present study, data for all parameters showed non-significant differences between the control group and antioxidants (Antox) treated group.

Effect on maternal body weight: Oral administration of NaF (40 mg F^-/kg bw/day) alone or in combination with Antox showed a significant decrease in the average maternal body weight from the 13th to the 20th day of gestation compared to the control (Table 1).

Table 1. Average increase of maternal body weight (g) in different rat groups

Group	7th day of gestation	13th day of gestation	20th day of gestation
Control	13.33 ± 4.21	33.33 ± 3.33	59.16 ± 2.71
Antox	15.71 ± 2.02	31.42 ± 1.42	58.33 ± 4.01
NaF	12.00 ± 1.33	21.25 ± 2.26†	30.00 ± 3.89‡
NaF + Antox	13.57 ± 1.42	22.50 ± 2.50*	30.83 ± 3.74‡

Values are mean ± SE. Compared with the control group: *p<0.05; †p<0.01; ‡p<0.001.

Outcome of pregnancy on gestational day 20: The mean number of implantations per litter was similar in all groups. Although the frequency of resorption, dead (resorbed fetuses and dead fetuses at birth), and external

abnormalities per litter in the NaF group were about two times higher than those of the control (Table 2), the differences were not statistically significant.

Table 2. Outcome of pregnancy on gestational day 20

Group	No. implantations /litter	No. resorbed fetuses/litter	No. dead fetuses/litter	No. Abnormal fetuses/litter
Control	8.33 ± 0.40	0.22 ± 0.14	0.22 ± 0.14	0.77 ± 0.36
Antox	8.28 ± 0.64	0.28 ± 0.18	0.28 ± 0.18	0.71 ± 0.18
NaF	8.30 ± 0.93	0.54 ± 0.45	0.63 ± 0.45	1.72 ± 0.33
NaF + Antox	8.42 ± 0.36	0.25 ± 0.16	0.25 ± 0.16	0.87 ± 0.35

Values are mean ± SE.

External abnormalities were restricted to dwarf fetuses in addition to hemorrhage and hematoma at different regions in the body (Figure 1). Treatment with Antox reduced such defects to the control level.

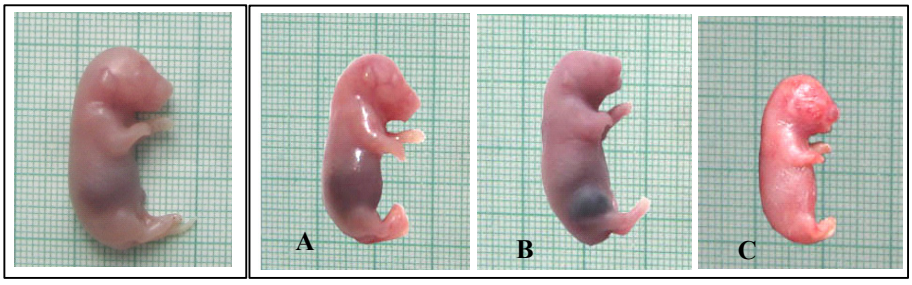

Figure 1. Fetuses of control and NaF treated rats on the 20th day of gestation: A) abdominal hemorrhage, B) hematoma, and C) dwarf fetus**.** (3.6X 12 cm)

Fetal growth on gestational day 20: Fetuses from NaF treated rats exhibited a significant decrease of body weight ($p<0.01$) and length ($p<0.001$) compared to the control. Antioxidants treatment improved fetal body weight to the normal level and significantly ameliorated fetal body length ($p<0.01$) compared with the NaF group (Table 3).

Table 3. Fetal body weight and length on gestational day 20

Group	Fetal weight (g)	Fetal length (cm.)
Control	2.21 ± 0.04	5.00 ± 0.06
Antox	2.15 ± 0.07	4.92 ± 0.07
NaF	1.99 ± 0.04†	4.50 ± 0.05‡
NaF + Antox	2.12 ± 0.06	4.83 ± 0.04*§

Values are mean ± SE.

Compared with the control group: $^{*}p<0.05$; $^{\dagger}p<0.01$; $^{\ddagger}p<0.001$.

Compared with the NaF group: $^{\S}p<0.01$.

Skeletal condition of fetuses: Administration of NaF to pregnant rats caused general retardation of skeletal development of their fetuses characterized by delayed ossification and shortening of bone elements compared with the control. On the other hand, treatment with Antox reduced such defects in the skeletal development of their fetuses (Figures 2 and 3).

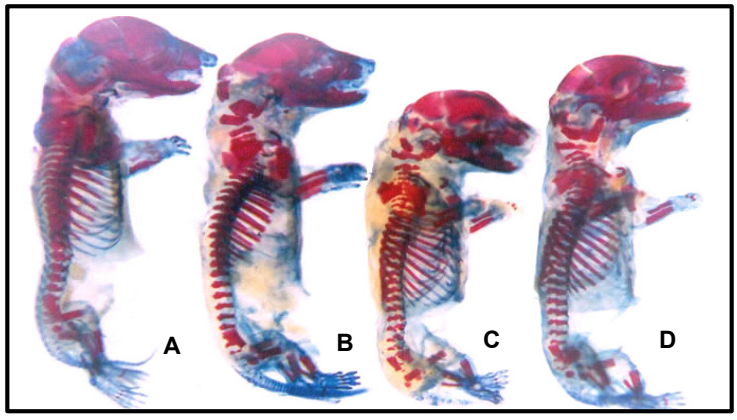

Figure 2. Lateral view photographs of rat fetal skeletons on the 20th day of gestation: A) control fetus; B) Fetus of Antox treated dam showing normal skeletal development; C) Fetus of NaF treated dam showing skeletal growth retardation; D) Fetus of dam treated with NaF + Antox showing improvement in skeletal development. (5.2 X 9.5 cm)

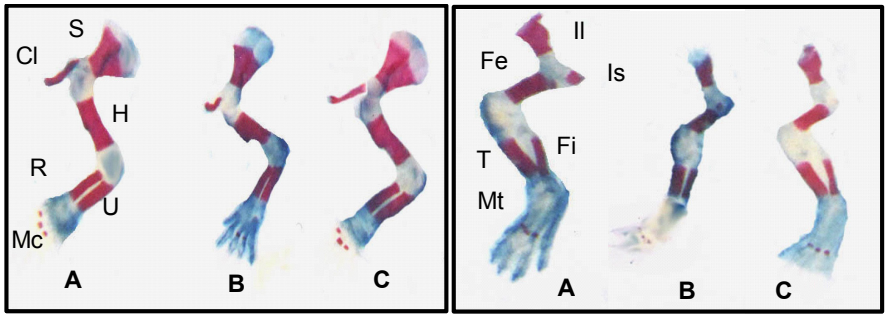

Figure 3. Fore and hind limb photographs of rat fetuses on the 20th day of gestation: A) limbs of control fetus; B) limbs of fetus of NaF treated dam showing less ossification and shortening of bones; C) limbs of fetus of NaF + Antox treated dams showing improvement of skeletal defects induced by fluoride.
Abbreviations: S, scapula; Cl, clavicle; H, humerus; R, radius; U, ulna; Mc, metacarpals; Il, ilium; Is, ischium; Fe, femur; T, tibia; Fi, fibula; Mt, metatarsals. (4X6 cm)

Fluoride content in maternal serum and amniotic fluid: The fluoride content in both maternal serum and amniotic fluid increased significantly ($p<0.001$) after NaF administration when compared to the control (about three and two times higher, respectively). It also recovered significantly ($p<0.001$) in maternal serum after treatment with the antioxidants but not in the amniotic fluid (Table 4).

Table 4. Fluoride content in maternal serum and amniotic fluid (ppm) in the different rat groups

Group	Maternal serum level	Amniotic fluid level
Control	0.08 ± 0.009	0.06 ± 0.001
Antox	0.09 ± 0.004	0.05 ± 0.001
NaF	0.28 ± 0.014‡	0.15 ± 0.007‡
NaF + Antox	0.16 ± 0.007‡§	0.14 ± 0.004‡

Values are mean ± SE.

Compared with the control group: ‡p<0.001.

Compared with the NaF group: §p<0.001.

Total protein, calcium, and phosphorus content: Although serum total protein showed a non-significant 8.08% decrease in rats treated with NaF, the protein level in fetal liver tissue decreased significantly ($p<0.05$) by 24.39% (Table 5). Moreover, the level of calcium in serum was found to decrease significantly ($p<0.01$) by 12.51% after treatment with NaF. On the other hand, serum phosphorus increased significantly ($p<0.01$) by 21.57% the NaF treatment. After administration of Antox the values of all parameters were comparable to control values and recovered very close to the control levels.

Table 5. Total protein, calcium, and phosphorus levels of the different rat groups.

Group	Total protein		Serum calcium (g/dl)	Serum phosphorus (g/dl)
	Maternal serum (g/dl)	Fetal liver tissue (mg/100mg tissue)		
Control	9.78 ± 0.54	20.5 ± 2.0	12.87 ± 0.18	7.51± 0.49
Antox	9.84 ± 0.71	20.5 ± 0.5	12.63 ± 0.79	6.38 ± 0.17
NaF	8.99 ± 0.43	15.5 ± 1.5*	11.26 ± 0.46†	9.13 ± 0.34†
NaF + Antox	9.58 ± 0.53	19.9 ± 1.5	11.72 ± 0.41*	7.88 ± 0.37

Values are mean ± SE.
Compared with the control group: *p<0.05; †p<0.01.

DISCUSSION

Sodium fluoride treatment:

In the present study NaF was administered through days 8–19 of gestation. During this period organogenesis of the embryo is greatly accelerated, and each developing organ is susceptible to teratogenesis at a critical period during which its development can be affected.[18,19]

We found that exposing pregnant female rats to NaF during the period of organogenesis had significant effect on average maternal and fetal body weight and fetal body length as well as causing fetal growth retardation in the general

skeleton. Earlier reports on NaF treatment of pregnant rats[20] and the weight of their pups[21,22] corroborate results of our study. Fetal growth retardation may be considered secondary to maternal weight loss. Also, according to our results, significant reduction of total protein of fetal liver tissue and the calcium level of maternal serum may be an important cause of the reduction of fetal growth rate and skeletal development. A significant elevation of F in serum and amniotic fluid was accompanied by a decrease in the level of total protein in maternal serum and fetal liver tissue, in agreement with earlier studies.[1,23] This decline with NaF treatment might be related to impairment of protein synthesis by F ions.[24,25]

Many studies have revealed there is wide variation with some correlation between F concentration in maternal serum and cord blood, thereby indicating that F readily crosses the placenta.[7] In the present work, NaF treatment revealed a significant decline in serum calcium level concomitant with a significant elevation of serum phosphorus level compared with the control. In line with these results, Grucka-Mamczar et al.[23] observed significant reduction in serum calcium of male rats treated with a single dose of NaF (35 mg/kg bw).

The significant increase in maternal serum phosphorus in the present work may be due to impairments in liver function and liver cell damage[1,24,25] and/or disturbance in kidney function and renal cell damage.[26,27] Furthermore, as noted by Guminska,[28] kidney lesions can occur when plasma F approaches 90 μmole/L (1.7 ppm F). Such lesions may reduce the formation of the active form of vitamin D (calcitriol) in the kidneys, thereby resulting in inefficient calcium absorption in the gut and the secondary release of parathyroid hormone that acts on bone in an attempt to increase extracellular fluid Ca.

Antioxidants treatment:

Numerous studies have been conducted on the relationships between F and free radical reactions. The results indicate that free radicals may play an important role in the pathogenesis of fluorosis. On the other hand, experimental studies have shown that the dietary factors such as vitamins can mitigate the toxic effects of F.[12,29]

In the present study, administering a mixture of vitamins A, C, and E and selenium to NaF-treated pregnant rats brought about recovery almost to control levels in most of the parameters studied. Earlier work showed that oral administration of vitamin C and/or vitamin E ameliorates NaF-induced toxicity in adult mice[29] and also mitigates F-induced reduction of body weight and embryotoxicity in rats.[12] The beneficial effects of vitamins A, C, and E and selenium might be due to their antioxidant and detoxification properties in suppressing F toxicity. Furthermore, these antioxidants may act synergistically just as vitamin C works synergistically with vitamin E and can regenerate vitamin E as it becomes depleted in its fight against free radicals. Tocopherols (vitamin E) and ascorbic acid (vitamin C) can therefore mutually reinforce one another by a mechanism in which one reducing agent acts as a regenerator for the oxidized form of the other.[30] Moreover, as is well known, selenium also enhances immune function, and that effect is improved by vitamin E.[31]

In conclusion, the present study revealed that NaF fluoride has deleterious effects on pregnant rats and their fetuses. Supplementation with an antioxidant mixture containing vitamins A, C, and E and selenium proved to be useful in preventing or alleviating some of these toxic effects of F.

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