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Research Article

The Effect of Acute Fluoride Poisoning on Nitric Oxide and Methemoglobin Formation in the Guinea pig

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Abstract: To study the effect of acute fluoride poisoning on nitric oxide and methemoglobin formation, 250 mg/kg bw sodium fluoride was applied alone and verapamil was applied together with fluoride. Blood nitric oxide (Griess reaction) and calcium levels; hemoglobin, methemoglobin and hematocrit values; and erythrocyte counts were determined and compared with those of the controls. After the fluoride application it was found that there was a relative relationship between the increase in nitric oxide and methemoglobin levels and the decrease in calcium, hemoglobin and hematocrit levels and erythrocyte count. It was concluded that the increase seen in blood nitric oxide levels as a result of the ionophore effect of fluoride could come from cNOS, as that increase is related to the decrease in calcium amount.

Key Words: Fluoride poisoning, nitric oxide, methemoglobin, guinea pig.

Kobaylarda Akut Flor Zehirlenmesinin Nitrik Oksit ve Methemoglobin Oluşumu Üzerine Etkisi

Özet: Araştırmada, akut flor zehirlenmesinin nitrik oksit ve methemoglobin oluşumu üzerine etkisinin belirlenmesi amacıyla, 250 mg/kg dozunda sodyum florür yalnız ve flor varlığında verapamil uygulandı ve kontrol grubuna göre kan nitrik oksit (Griess reaksiyonu), kalsiyum düzeyleri ve hemoglobin, methemoglobin, hematokrit değerleri ile alyuvar sayıları belirlendi. Florun uygulanması sonrasında kan nitrik oksit ve methemoglobin düzeyindeki yükselme ile rölatif ilişkili olarak kalsiyum, hemoglobin, hematokrit ve alyuvar değerlerinde azalma belirlendi. Akut flor zehirlenmesinde florun iyonafor etkisi ile kan nitrik oksit düzeyinde şekillenen yükselmenin kalsiyum miktarındaki düşüşle ilişkili olmasından dolayı cNOS kaynaklı olabileceği kanısına varılmıştır.

Anahtar Sözcükler: Flor zehirlenmesi, nitrik oksit, methemoglobin, kobay

Introduction

Sodium fluoride is currently used in many areas, like as an insecticide and in anti-helmintic drugs, and most commonly in rodenticide drugs. In smaller amounts it prevents cavities in teeth, and it is used in the treatment of osteoporosis in humans (1). Cases of poisoning with organic and inorganic fluorides, which are definitely considered poisonous in high dosages, are seen rather frequently these days.

Fluorine is an anion with a rather small molecular weight, and it shows its effect on the organism by combining with calcium (Ca^{+2}) and causing a severe hypocalcemia picture. On the other hand, it is named a calcium ionophore, as it enhances the transport of Ca^{+2} into cells. In studies on fluoride poisoning, it is generally mentioned that there are increases in calcium affinity and also enzyme level changes tending to increase the calcium metabolism, and that hypoxia forms following the inhibition of the enzyme system in general (2).

It has been reported that histamine is released following the stimulation of isolated rat mast cells and hypoxia is formed. Related to this, there are reports that the formation of nitric oxide (NO), which is considered a free radical, is stimulated by uncontrolled increases in histamine in the body (3), and the effect of NO increases the permeability of the blood-brain barrier (4). On the other hand, hypoxia that forms in conditions resembling the effects of fluoride poisoning and increasing NO levels due to hypoxia in the circulation, and several internal and external factors that enhance calcium ion transport and thus result in intracellular Ca^{+2} increase are also mentioned (5).

NO is a molecule that easily passes through the cell membrane, and that rapidly reacts with the ferro part of the proteins containing heme and also reacts with oxygen, and it is synthesized from the amino-acid L-arginine in many cells of the body. Nitric oxide synthase (NOS) is involved in the formation of NO, and it is a de-oxygenase that is dependent on nicotine amide dinucletide phosphate (NADPH) (6,7). NOS has 2 isomers, 1 inducible (iNOS) and the other constitutional (cNOS) (8,9). In a study in which the mechanism of the fluorine effect was examined the activation of NADPH required in NO formation (10) was mentioned, and, on the other hand, it was reported that extra cellular calcium was needed for the effects of fluorine to take place (11).

In this study, we took into consideration the previous reports stating that the substances known as calcium ionophores increase NO levels, that hypoxia causes NO production, and thus the body develops a defense mechanism, and we studied the possible effects of acute fluoride poisoning on NO levels, and also methemoglobin formation and anemia that is thought to be related to the hypoxic picture following the poisoning.

Materials and Methods

Laboratory animals

Thirty albino male guinea pigs were used in this study, the weights of which differed between 280 and 320 g. One week was allowed for the guinea pigs to get familiar with the environment, and during this period they were fed ad libitum.

Fluoride and verapamil exposure

The animals were divided into 3 groups: 1 control (group I), and 2 experimental groups (groups II and III). For the first experimental group (group II) 250 mg/kg (live weight) sodium fluoride was applied subcutaneously to cause acute fluoride poisoning (12) and blood samples were collected from the heart 8 h later. To the other experimental group (group III), 7 h following the application of sodium fluoride in the same dosage, intramuscular verapamil (isoptin), which is a calcium channel blocker, was applied at a dosage of 0.8 mg/kg (live weight), and blood samples from the heart were collected after 1 h (13,14).

Analysis of calcium and nitric oxide

Serum from blood samples that were taken in experimental tubes not containing anti-coagulants were stored at –18 ºC. In serum samples, calorimetry was used to determine calcium levels using SIGMA-diagnostic kits (587-A), while diazotization (Griess reaction) was used when measuring nitric oxide amounts in serum samples. According to the procedure, sulfanilamide was solved in H_3PO_4 to the concentration 1%, and N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) was solved in the same solvent to the concentration 1%. On the other hand, solutions of sodium nitrite were prepared with concentrations between 0.25 and 0.50 µM and calibration curves were determined. Then 1.5 ml serum samples from each of the 3 groups were taken, and 0.75 ml of sulfanilamide solution and 0.75 ml of NEDD solution were added to these samples. After waiting for 15 min at room temperature, absorption of the samples at wavelength 550 nm was measured by spectrophotometer (15).

Other laboratory analyses

Erythrocyte counts (16) and hematocrit (17), hemoglobin (18), and methemoglobin (19) values were determined in blood samples collected from all 3 groups and taken into test tubes containing EDTA.

Statistical analysis

Statistical significance was assessed using one-way ANOVA followed by the Newman-Keuls multiple comparison test ($P < 0.05$) (20).

Results

The statistical comparison (Table) between the control and the experimental groups showed that there were significant decreases in erythrocyte counts and hemoglobin and hematocrit values of the experimental groups compared to those of the control group ($P < 0.05$). The decreases in erythrocyte counts were about 50% for both experimental groups. The mean value of methemoglobin increased in the fluoride and verapamil combined group. This was statistically significant ($P < 0.05$) compared to the values of the control and only fluoride applied groups, although the increase in the

fluoride applied group was about 3-fold that in the control group, which was not statistically significant due to the high error values. On the other hand, there was a statistically significant decrease ($P < 0.05$) in the level of calcium after applying fluoride alone (Table and Figure 1). Using fluoride with verapamil returned the value near to that of the control group, which was not obviously significant. In contrast, applying fluoride alone caused a significant increase ($P < 0.05$) in the level of nitric oxide at a rate of 125% (Table and Figure 2) and this value statistically decreased ($P < 0.05$) at a rate of 73% when using fluoride with verapamil, which was statistically lower than the value of the control group too ($P < 0.05$).

Discussion

This study aimed to determine how acute fluoride poisoning occurs and the effects of fluoride on the formation of NO. With this purpose, the dosage of sodium fluoride was chosen as 250 mg/kg, and the waiting period after the dosage was 8 h.

The NO level in animals after the application of sodium fluoride was higher than that obtained before ($P < 0.05$). This finding apparently supports the studies that report histamine release following fluoride treatment (3). Likewise, histamine is mentioned in the literature among the factors that enhance NO formation (11,21-23). Authors state that hypoxia also affects NO formation (24), and, likewise, hypoxia takes place as a result of NO poisoning (5). This hypoxic picture shows itself with low iron levels, and can be explained with the inactivation of hemoglobin and other iron components (25), and there are reports on anemia resulting from excess NO levels (26). Although the stimulation of NO production as a defense mechanism of the body, following the decrease in oxygen content of blood indirectly to below-normal levels in the anemic hypoxia picture seen when hemoglobin levels are below normal can be considered an expected

Table. Hematological values of control and experimental groups (fluoride and fluoride + verapamil groups). Mean values were given with ± standard errors (n = 10).

	Control X ± Sx	Fluoride X ± Sx	Fluoride+Verapamil X ± Sx
Erythrocyte (mm^3)	5,054,500 ± 306,461.7[a]	2,640,300 ± 121,343.77[b]	2,553,000 ± 137,097.28[b]
Hemoglobin (g/100 ml)	12.52 ± 0.51[a]	8.06 ± 0.54[b]	7.11 ± 0.48[b]
Hematocrit (%)	37.10 ± 1.36[a]	24.00 ± 1.14[b]	27.20 ± 1.40[b]
Methemoglobin (%)	0.85 ± 0.17[a]	3.01 ± 1.73[a]	8.07 ± 1.23[b]
Calcium µmol/l	10.22 ± 0.30[a]	7.98 ± 0.19[b]	9.67 ± 0.23[a]
Nitric oxide µmol/l	3.45 ± 0.27[a]	7.76 ± 0.38[b]	2.09 ± 0.09[c]

a, b: Different letters in the line indicate significant difference ($P < 0.05$).

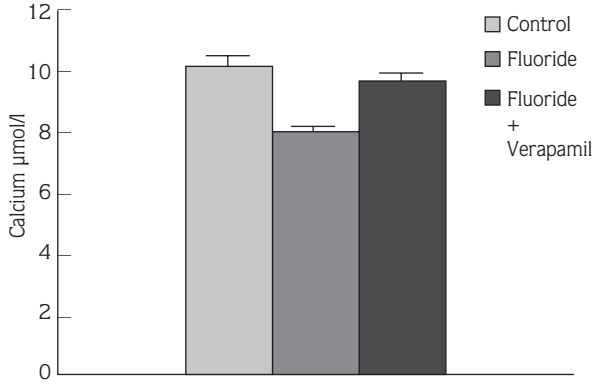

Figure 1. Blood calcium levels of control and experimental groups (fluoride and fluoride + verapamil).

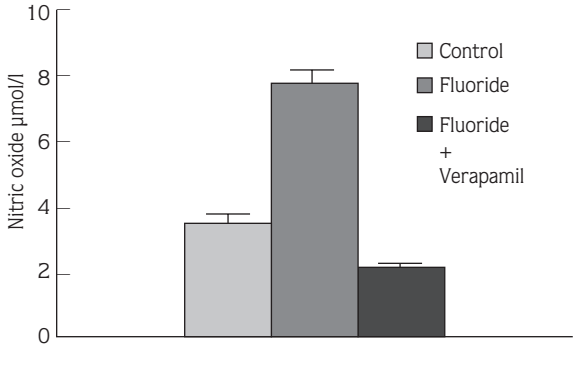

Figure 2. Blood nitric oxide levels of control and experimental groups (fluoride and fluoride + verapamil).

effect, findings in studies on fluoride poisoning are considered results of hypoxia in the circulation (5). However, in this study, significant decreases in hemoglobin and hematocrit values and erythrocyte counts seen in experimental groups relative to the control group after sodium fluoride application, and the relative increase in methemoglobin values indicate an anemic hypoxia picture. Likewise, the findings obtained with the measurements based on milliliters are findings of anemic hypoxia. Of course, the uncontrolled stagnation in circulation, slowing down of blood in veins, and hypoxia due to circulatory reasons are the inevitable final findings of poisoning after NO formation. In this context, after respiratory distress, cardiac failure and myocardial infarction arising from an increase in the work load of the heart are among the last findings of fluoride poisonings reported in humans (1,27,28).

In this study, the hypocalcemia formed after the application of sodium fluoride is explained by the calcium ionophore property of fluorine, and the calcium-dependent increase in cNOS levels can be interpreted as an expected effect of the increase in intracellular calcium levels. Again, the increase in intracellular levels appears to cause an inevitable increase in NO production originating from cNOS. Likewise, increases in blood NO levels are found in the group II along with hypocalcemia after fluoride application.

The data obtained from the third group, to which the calcium channel blocker verapamil was applied after sodium fluoride application, support the findings of group II with decreases in blood NO levels and increases in calcium levels.

It has been reported in studies on the mechanisms of the effects of fluorine that intracellular calcium is required for it to increase cyclic guanosine monophosphate (cGMP) levels at the cellular level, and NO has the same effect, and that intracellular calcium is required for the effects of both to take place. Furthermore, the necessity of activation of NADPH for the effects of NO to take place is stated (10), and it is interesting that NO formation is NADPH-dependent.

Super oxide dismutase (SOD) is among the protective enzymes against the toxic effects of free radicals (29). Rzeuski et al. (10) reported that SOD is inhibited in higher concentrations of fluorine, and also reported that the said enzyme is found in low concentrations in people living in areas of endemic fluorosis. Researchers have stated that the formation of methemoglobin in guinea pigs occurs at high levels compared to the low activity of super oxide dismutase, which is an enzyme that transforms the excess NO to nitroxyl ions (30). The high level of methemoglobin in group II compared to the control group ($P < 0.05$) in our study can be explained by the increase originating from the suppression of SOD with high concentrations of fluorine. However, verapamil application in the third group after sodium fluoride was insufficient to prevent methemoglobin formation. Likewise, Raikhlin-Eisenkraft et al. (27) reported that verapamil therapy was not successful in fluoride poisonings. Fahey and Isaacson (31) found that calcium channel blockers given 2 h before poisoning could result in a decrease in methemoglobin formation. In our study, the higher levels of methemoglobin in the third group as compared to the control group in a statistically significant manner can be explained by the application of verapamil in the last hour, and by the failure of the reductive system to operate. On the other hand, while the normal levels of NO can be explained by the sufficient levels of Ca^{+2} channel blockers that prevent the transport of calcium, the high levels of methemoglobin can be explained by the insufficiency of the preventive factors effective in the transformation of methemoglobin back to oxy-hemoglobin following intoxication. Likewise, the high levels of methemoglobin after verapamil application, and the low levels of blood hemoglobin and hematocrit, and erythrocyte counts in the same group in a similar and related fashion ($P < 0.05$) appear to be findings supporting each other.

In conclusions, an increase in NO levels with the ionophore effect of fluorine in acute fluorine intoxication is observed in this study.

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