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Research Article

# Effect of Combined Nitrate-Fluoride Intoxication on the Function of No-Synthases and Arginases in the Gastric Mucosa, Blood and Heart of Rats

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**Abstract**

The focus of this article is on the functioning of NO-synthases and arginases in the gastric mucosa, blood and heart of white rats under excessive intake of sodium fluoride and sodium nitrate during 30 days. Excessive intake of sodium nitrate was modeled by infusion of aqueous solution into stomach through special catheter at a dose of 500 mg / kg. Excessive intake of sodium fluoride was modeled by infusion of aqueous solution into stomach through special catheter at a dose of 10 mg / kg. The activity of these enzymes was measured by spectrophotometric methods.

Analysis of the influence produced by combined nitrate-fluoride intoxication on total NOS activity in the gastric mucosa, heart and blood showed the presence of antagonistic effect of nitrates and fluorides. Nitrates act as NOS inhibitors, whereas the fluorides are inducers of NOS. Under their combined action in the organism, fluoride-induced elevation prevails over nitrate-induced inhibition, but only in tissue homogenates. Such effect is absent in blood.

The inductive effect of combined nitrate-fluoride intoxication on functioning of arginases is mediated by nitrate release of L-arginin from NOS. Combined intoxication showed prevalence of inducing influence of nitrates over inhibiting effect of fluorides.

## INTRODUCTION

Fluorides are salts containing one of the most active halogens. Fluoride can be naturally presented in ground water and food. Fluorides are also ingested with toothpastes and medicines. Fluoridation of water can result in excessive fluoride intake. Most of the European Union (EU) abandoned the practice of fluoridation of drinking water, but in some EU countries this practice still persists. Republic of Ireland is the only EU country, where water fluoridation is mandatory [1]. In Ukraine, water fluoridation at the nationwide level is not carried out, but there are regions with natural high fluorine content in the soil and ground water. Poltava region is one of them. Fluoride in excessive quantities can cause changes in the functioning of NO-synthases (NOS, E.C. 1.14.13.39), leading to excessive activation of the inducible isoform of NOS and inactivation of endothelial isoform of NOS [2]. In addition to effects on NO-synthases, the literature describes evidences of the inhibitory effect of fluoride ions on arginases function (Arginase, E.C. 3.5.3.1.) [3].

Nitrates can enter the body through food, water and medicines. Nitrates are exogenous donators of nitric oxide (NO), and recently their ability to generate NO through nitrate reduction-pathway is urging researchers to study their metabolism. Exogenous nitrates showed effectiveness in preventing the development of gastric ulcerative lesions, caused by non-steroidal anti-inflammatory drugs [4]. In the case of excessive NO generation by nitrate-nitrite reductive pathway, reciprocal NOS inhibition becomes possible, the excessive amount of nitric oxide may inhibit the activity of arginases. However, reduction in NOS activity may result in the increase of free reaction substrate for arginases. Increased quantity of L-arginine is able to enhance the activity of arginases.

Thus, simultaneous effect of two factors that can affect the functional activity of NOS is possible. The aim of this study was

to determine the effect produced by combined nitrate-fluoride intoxication on function of arginases and NOS in the gastric mucosa, blood and heart.

## MATERIALS AND METHODS

The experiment was conducted on 52 white Wistar rats. Chronic excessive intake of nitrate, fluoride and combined intoxication was modeled by infusion of sodium nitrate trough special catheter at a dose 500 mg / kg, sodium fluoride at a dose 10 mg / kg. Intoxication continued for 30 days. All manipulations were carried out according to the "European Convention for the Protection of Vertebrate Animals used for research and other scientific purposes." The animals were divided into four groups: first group included intact animals (n = 10); the second group was exposed to fluoride intoxication (n = 13); third consisted from animals, which received nitrate intoxication (n = 14); fourth group received combination of sodium nitrate and fluoride (n = 15). Animals were sacrificed after the experiment under thiopental anesthesia. Biochemical study of blood, 10% of gastric mucosa homogenate and 10% heart homogenate was then performed.

Total activity of NO-synthases (NOS) was evaluated by increase in $NO_2^-$ concentration after incubation homogenated tissue samples for 30 minutes in the incubation solution (2.5 ml 0.1 M Tris-buffer, 0.3 ml 320 mM L-ARG water solution and 0.1 ml 1mM NADPH solution). 0.2 ml of 10% homogenate was taken for the analysis. Immediately after mixing homogenate with the incubation solution, we took 0.2 ml of it to evaluate the initial nitrite concentration. 1% sulfanilic acid in 30% acetic acid and 0,1% 1-naphtylamine in the same solvent were chosen as nitrite specific reactants. Then we added 1.8 ml of distilled water to 0.2 ml of solution taken for initial nitrite assessment. Following this we added 0.2 ml of 1% sulfanilamide acid and then in 10 minutes 0.2 ml of 0,1% 1-naphtylamine were added as well. The amount of nitrites was measured by spectrophotometer Ulab-101 (540 nm in cuvette with optical path length of 5 mm). Concentration was calculated as C = 0.30104·Absorbtion (μmol/g).

Following 30-min. incubation the reaction was stopped by adding 0.02% of sodium aside in a dose of 0.02 ml. Then 0.2 ml was taken from to assess final nitrite concentration. Total NOS activity was calculated as NOS = $(A_2-A_1)\cdot 2057/N$ (min·g of protein), where $A_2$ means absorbance of solution taken for final nitrite measurement, $A_1$ stands for absorbance of solution taken for initial nitrite measurement, N is the concentration of protein calculated by Biurette method g/L.

Total activity of arginases was assessed by using the following technique. First, 0.1 ml of homogenate was taken to estimate initial level of L-ornithine. Then we used the modified Chinard's reactive. 0.5 ml 0.2 M phosphate buffer (pH = 7,0) and 0.1 ml of 2.5% ninhydrin on acidic mixture (2:3 60% orthophosphoric and ice acetic acids and 6:4 with water) and 1.0 ml of ice acetic acid was added to 0.1 ml of homogenate. The solution was being boiled for 40 min. to achieve maximal color yield. Then 1 ml of 20% trichloracetic acid was added to precipitate proteins and after centrifugation (1000 g) for 30 min. the absorbance of 1 ml of supernantate was measured ($A_1$) (10 mm cuvette against water on 515 nm wavelength).

The activation of agrinase-dependent L-ARG metabolism can be designed under the following conditions: 20 hrs incubation in 0.5 ml of 0.2 M phosphate buffer (pH = 7.0) and adding 0.2 ml of 24 mM solution of L-ARG under t = 37°C. Then we carried out the procedure of L-ornithine assessment as described above ($A_2$). Arginase activity was calculated by formula: V = $(232\cdot A_2-216\cdot A_1)$ / $(1,2\cdot N)$ μmol/min g of protein [5].

All spectrophotometrical studies were performed by a spectrophotometer Ulab 101. Data yielded were statistically analyzed by ANOVA, followed by analysis by Heims-Howell (normal distribution), or by Kruskal-Wallis ANOVA, followed by analysis by Leffe (if distribution was different from the normal). Statistical processing was performed using Microsoft Excel with the help of extension Realstatistics. Data are presented as means and standard errors of means.

## RESULTS AND DISCUSSION

Fluoride intoxication increases NOS activity in gastric mucosa by 121% when compared to the intact animals while reducing total arginases activity by 40%. Nitrite concentration increases by 69.5%. Similar picture can be observed in the blood of rats exposed to fluoride intoxication. NOS activity increases by 58.8%, arginases reduce their activity by 69%, the nitrite concentration increases by 137%. The NOS activity of heart tissue increases by 33.4%. Arginases reduce their total activity by 24%. Nitrite concentration drops by 35%. These changes can be explained by the activation under fluoride influence of inducible NOS, which competes with arginases for the substrate, thus inhibiting the activity of the latter. Km for arginine of arginases is 2-20 μM while Km for arginine of various NOS isoforms 2-20 mM. However substrate competition is still possible because $V_{max}$ of arginases 1000 times higher than that of NOS [6].

In condition of sodium nitrate intoxication total NOS activity reduces in the gastric mucosa by 35% in the blood by 29% and in heart by 32%. Reduced NOS activity may be explained by an increase in NO production from exogenous precursor by nitrate reductase. Total arginases activity in the gastric mucosa and blood tends to increase. Arginases demonstrate increase by 82% in the gastric mucosa and by 41% in the blood. In the heart no significant differences in arginases activity were found. Excessive activation of arginases is possible due to the increase in L-arginin concentration. NOS-dependent NO generation pathway leaves L-arginin for arginases in the presence of exogenous NO donator. Nitrite concentration in the gastric mucosa lowers by 20%, but elevates in the blood nearly six-fold, and decreases by 20% in the heart. The increase in nitrite concentration in the blood is explicable taking into account nitrate-reducing effect mediated by red blood cells [7].

Combined nitrate-fluoride intoxication elevates total NOS activity in the gastric mucosa by 19% and in the cardiac tissue by 23%. Total NOS activity in blood decreases by 46%. Reduction in total NOS activity in blood can be explained by the presence of large amounts of nitrate reductases and nitrite reductases that potentiate the inhibitory effect of nitrates, the ten-fold growth of nitrite in blood can serve as indirect evidence of this effect under combined intoxication. When compared with the fluoride intoxication, NOS activity tends to decrease in all the

**Table 1:** Changes of functioning of NOS and arginases in the gastric mucosa, blood and heart of rats under nitrate-fluoride intoxication (M±m).

| | Intact rats, n=10 | Fluoride intoxication, n=13 | Nitrate intoxication, n=14 | Combined nitrate-fluoride intoxication, n=15 |
|---|---|---|---|---|
| Gastric mucosa | | | | |
| Total NOS activity, μmol/min·g of protein | 6,51±0,41 | 14.37±0.82* | 4.23±0.36*/** | 7,74±0,27*/**/*** |
| Total arginase activity, μmol/min·g of protein | 2,07±0,08 | 1.24±0.11* | 3.77±0.38*/** | 3,34±0,08*/** |
| Concentration of nitrite ($NO_2$), nmol/g of tissue | 11,56±0,51 | 19.59±0.46* | 9.25±0.44*/** | 18,9±0,8*/**/*** |
| Blood | | | | |
| Total NOS activity, μmol/min | 11,63±2,0 | 18,47±1,07* | 8,3±1,13*/** | 6,25±0,85*/** |
| Total arginase activity, μmol/min | 1,64±0,32 | 0,51±0,09* | 0,72±0,09* | 1,32±0,21**/*** |
| Concentration of nitrite ($NO_2$), nmol/L | 3,91±0,47 | 9,26±1,05* | 22,9±0,89*/** | 36,69±0,96*/**/*** |
| Heart | | | | |
| Total NOS activity, μmol/min·g of protein | 1,68±0,07 | 2,57±0,15* | 1,14±0,04*/** | 2,07±0,05*/**/*** |
| Total arginase activity, μmol/min·g of protein | 2,24±0,08 | 1,71±0,09* | 2,39±0,1** | 2,07±0,13 |
| Concentration of nitrite ($NO_2$), nmol/g of tissue | 5,78±0,26 | 3,77±0,23* | 4,62±0,22* | 3,43±0,4* |

* - difference is significant compared to intact group with p<0,05
** - difference is significant compared to fluoride intoxication group with p <0,05
*** - difference is significant compared to nitrate intoxication group with p <0,05
**Abbreviations:** NOS: Nitric Oxide (NO) Synthase.

studied tissues. Total NOS activity shows tendency towards the decrease, comparing to the combined intoxication with the nitrate intoxication, in the gastric mucosa and in the heart, this is irrelevant for the blood. Thus, we can suggest that there is some antagonism between nitrate and fluoride taking into account their effects on total NOS activity. Under excessive intake of sodium nitrate and fluoride, total arginases activity increases only in the gastric mucosa, showing no statistically significant changes in blood and heart. Despite the fact that blood arginases do not show significant changes compared with intact animals; however they elevate by 159% and 83%, when compared with the isolated intoxication by fluorides and nitrates respectively. The amount of nitrite reduces by 41% in the heart, but increases by 63.5% in the gastric mucosa and ten-fold in blood.

## CONCLUSION

The analysis of the effect produced by combined nitrate-fluoride intoxication on total NOS activity in the gastric mucosa, heart and blood showed the presence of antagonistic interaction between nitrates and fluorides. Nitrates act as NOS inhibitors, whereas the fluorides are NOS inducers. During their combined presence in the organism, fluoride-induced elevation prevails over nitrate-induced inhibition, but only in tissue homogenates. In blood such effect is absent.

The inductive effect of combined nitrate-fluoride intoxication on function of arginases is mediated by nitrate release of L-arginin from NOS. Combined intoxication showed prevalence of inducing influence of nitrates over inhibiting effect of fluorides.

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