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ORIGINAL PAPER

INFLUENCE OF COMBINED NITRATE AND FLUORIDE INTOXICATION ON CONNECTIVE TISSUE DISORDERS IN RATS GASTRIC MUCOSA

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Abstract

Introduction. Certain regions in Europe and Ukraine have high concentration of fluorides in drinking waters. Highly developed agriculture can result in increased concentration of nitrates in ground and drinking waters. Therefore, their combined excessive intake with food and water is not excluded.
The objective of the study was to determine the influence of combined nitrate-fluoride intoxication on concentration of different fractions of glycosaminoglycans (GAG) and L-hydroxyproline concentration in gastric mucosa of rats.
Materials and methods. Chronic intoxications were induced by intragastric injection of sodium fluoride in a dose of 10 mg/kg, sodium nitrate in a dose of 500 mg/kg and their combination during 30 days. Concentration of GAG was determined by carbazole method. To evaluate concentrations of different fractions of GAG we used the method of differential precipitation with ethanol. For L-Hydroxyproline concentration estimation, we used Hydroxyproline Colorimetric Assay Kit (BioVision K555-100).
Results. The total concentration of GAG decreases during chronic fluoride and nitrate intoxications, but increases during combined intoxication. Concentration of Heparin/Heparan GAG fraction and Keratan/Dermatan GAG fraction drops during

Résumé

Influence de l'intoxication combinée nitrate-fluorure sur les troubles tissulaires conjonctifs dans la muqueuse gastrique des rats

Introduction. Certaines régions d'Europe et d'Ukraine présentent une concentration élevée de fluorures dans les eaux potables. Une agriculture très développée peut entraîner une concentration accrue de nitrates dans les eaux souterraines et les eaux potables. Par conséquent, leur consommation excessive combinée avec de la nourriture et de l'eau n'est pas exclue.
Le but de l'étude est de déterminer l'influence de l'intoxication combinée au fluorure de nitrate sur la concentration de différentes fractions de glycosaminoglycanes (GAG) et la concentration de L-hydroxyproline dans la muqueuse gastrique de rats.
Matériaux et méthodes. Les intoxications chroniques ont été induites par une injection intragastrique de fluorure de sodium à une dose de 10 mg / kg, de nitrate de sodium à une dose de 500 mg / kg et de leur association pendant 30 jours. La concentration en GAG a été déterminée par la méthode au carbazole. Pour évaluer les concentrations de différentes fractions de GAG, nous avons utilisé une méthode de précipitation différentielle à l'éthanol. Pour l'estimation de la concentration en L-hydroxyproline, nous avons

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nitrate and fluoride intoxications, but increases during combined intoxication. Chondroitin GAG fraction shows no statistically significant changes during fluoride intoxication, but increases during combined and nitrate intoxications. Concentration of L-hydroxyproline increases in all studied groups.

Conclusions. We established that chronic excessive fluoride or nitrate intake can cause oxidative and nitrosative stress-depended collagen degradation, simultaneously decreasing the concentration of anti-inflammatory GAG. Combined intoxication leads to even more severe connective tissue degradation, but subsequently activates compensatory mechanisms through increase in concentration of anti-inflammatory GAG.

Keywords: glycosaminoglycans, L- hydroxyproline, fluoride intoxication, connective tissue, gastric mucosa.

Abbreviations

GAG – Glycosaminoglycans
sGAG – sulphated Glycosaminoglycans
ROS – reactive oxygen species
HSPG – Heparan sulphate proteoglycans
DSPG – Dermatan sulphate proteoglycans
KSPG – Keratan sulphate proteoglycans
CSPG – Chondroitin sulphate proteoglycans

utilisé le kit de dosage colorimétrique Hydroxyproline (BioVision K555-100).

Résultats. La concentration totale de GAG diminue pendant les intoxications chroniques au fluorure et au nitrate, mais augmente pendant l'intoxication combinée. La concentration des fractions GAG héparine / héparine et GAG kératane / dermatane diminue pendant les intoxications au nitrate et au fluorure, mais augmente au cours de l'intoxication combinée. La fraction GAG de la chondroïtine ne présente pas de changements statistiquement significatifs au cours de l'intoxication au fluorure, mais augmente au cours des intoxications combinées et d'intoxication aux nitrates. La concentration de L-hydroxyproline augmente dans tous les groupes d'étude.

Conclusions. En conclusion, nous pouvons supposer qu'une consommation excessive chronique de fluor ou de nitrates peut provoquer une dégradation du collagène liée au stress oxydant et nitrique, tout en diminuant simultanément la concentration de GAG anti-inflammatoire. L'intoxication combinée entraîne une dégradation encore plus grave du tissu conjonctif, mais active par la suite des mécanismes compensatoires par une augmentation de la concentration en GAG anti-inflammatoire.

Mots-clés: glycosaminoglycanes, L-hydroxyproline, intoxication au fluorure, tissu conjonctif, muqueuse gastrique

Introduction

Certain regions of Europe and Ukraine have high concentration of fluoride ions in ground water. Therefore, excessive intake of fluorides with drinking water is quite possible. For example, we can name Poltava region in Ukraine, North Bohemia (Czech Republic)[1] and Southern Saxony (Germany)[2].

Fluorine is one of the most active elements of periodic system. In low concentrations, fluorine is beneficial to dental health[3,4] and can be used in caries prevention strategy. However, high exposure to fluoride ions can be harmful to human and animal health[5, 6].

Fluoride ions can cause increase in production of ROS with subsequent oxidative stress development and lipid peroxidation[7,8]. There are evidences that fluoride can alter state of connective tissue in periodontium[9]. Several studies have shown that fluoride intoxication can lead to increase in concentration of L-hydroxyproline[10,11], which is the marker of collagen breakdown.

Nitrates have controversial effects on human and animal health. There are evidences in scientific literature that dietary nitrates can prevent ischemia-reperfusion injury by controlling reactive oxygen and nitrogen species production[12]. However, in higher dosage, nitrates can increase oxidative and nitrosative damage to tissues and organs[13]. Nitrate and fluoride interaction in human organism and its effects on functions of different organs and systems are still not enough revealed in scientific literature.

During excessive intake of fluoride and nitrate ions with drinking water, one of the first organs to suffer from adverse effects of fluorides is stomach.

The objective of the study was to evaluate the influence of chronic combined nitrate-fluoride intoxication on concentration of different GAG fractions and L-hydroxyproline concentration in rat gastric mucosa.

Materials and methods

The experiment involved 52 mature female and male rats, with a mean body weight of 180-220 g. The experiment was carried out in accordance with Ethical Principles and Guidelines for Experiments on Animals. Animals were divided into 4 groups. The

first group (Control group) received 1 ml 0.9% sodium chloride solution via feeding probe for 30 days. On animals of second group we performed induction of chronic fluoride intoxication. Animals of the third group received excessive amounts of nitrates. The fourth group was group of combined intoxication.

Excessive fluoride intake was modeled by administration of water solution of sodium fluoride intragastric, in a dose of 10 mg/kg via feeding probe, once a day, before feeding, for 30 days. Excessive nitrate intake was modeled by administration of water solution of sodium nitrate intragastric, in a dose of 500 mg/kg, via feeding probe, once a day, before feeding, for 30 days. Excessive combined fluoride and nitrate intake was modeled by administration of water solution of sodium fluoride intragastric, in a dose of 10 mg/kg, and sodium nitrate in a dose of 500 mg/kg, via feeding probe, once a day, before feeding, for 30 days.

Maximal volume of infusion was no more than 1 mL per day to avoid stomach overstretching.

The animals were euthanized by thiopental overdosage (45 mg/kg). The rat gastric mucosa was carefully rinsed by 0.9% sodium chloride solution, then it was removed from underlying tissues and taken for biochemical analysis. Before performing biochemical studies we homogenized gastric mucosa in tissue homogenizer to achieve 10% tissue homogenate.

To estimate the sGAG concentration, we used carbazole method and procedure described by Frazier[14]. To define the concentration of different fractions of sGAG in tissue homogenate, we used method of sequential precipitation proposed by Volpi[15]. After sequential precipitation, we used carbazole method to determine concentrations of different fractions.

For L-Hydroxyproline concentration estimation, we used Hydroxyproline Colorimetric Assay Kit (BioVision K555-100). Protein concentration was determined by Biurette method. All spectrophotometric studies were performed using Ulab 101 spectrophotometer.

Data obtained from experiment was analyzed with RealStatistics extension for Microsoft Excel. To assess statistical significance of differences between groups we used Mann-Whitney test. Differences were deemed statistically significant if p was lower than 0.05.

Results

Total concentration of GAG decreases by 35.35% during chronic fluoride intoxication (Table 1). Concentration of Heparin/Heparan GAG fraction decreases by 64.1%; Keratan/Dermatan GAG fraction drops by 43.33%. Chondroitin GAG fraction shows no statistically significant changes. Concentration of L-hydroxyproline increases by 7.06 times.

Chronic nitrate intoxication decreases total concentration of GAG by 30.3%. Concentration of Heparin/Heparan GAG fraction decreases by 64.1%; Keratan/Dermatan GAG fraction drops by 43.33%. Concentration of Chondroitin GAG

Table 1. Changes in connective tissue metabolism under chronic combined nitrate-fluoride intoxication (M±m).

Parameter	*Groups*			
	Control group, n=10	*Fluoride intoxication group, n=13*	*Nitrate intoxication group, n=14*	*Combined intoxication group, n=15*
Total GAG concentration, μmol of Uronic acid/L of tissue homogenate	0.99±0.07	0.64±0.04*	0.69±0.01*	1.61±0.03*/**/***
Heparin/Heparan GAG fraction concentration, μmol of Uronic acid/L of tissue homogenate	0.39±0.04	0.14±0.01*	0.14±0.003*	0.54±0.01*/**/***
Keratan/Dermatan GAG fraction concentration, μmol of Uronic acid/L of tissue homogenate	0.3±0.03	0.17±0.01*	0.17+0.003*	0.66+0.02*/**/***
Chondroitin GAG fraction concentration, μmol of Uronic acid/L of tissue homogenate	0.3±0.03	0.34±0.02	0.38±0.01*	0.42±0.01*/**/***
L-hydroxyproline concentration, μmol/g of tissue	0.116±0.003	0.819±0.035*	0.512±0.05*/**	0.865±0.006*/***

* – differences are statistically significant compared to control group
** – differences are statistically significant compared to fluoride intoxication group
*** – differences are statistically significant compared to nitrate intoxication group

fraction increases by 26.7%. Concentration of L-hydroxyproline increases by 4.41 times.

Combination of nitrates and fluorides elevates total GAG concentration by 1.63 times. Concentration of Heparin/Heparan GAG fraction increases by 38.4%; Keratan/Dermatan GAG fraction elevates by 120%. Concentration of Chondroitin GAG fraction increases by 40%. Concentration of L-hydroxyproline increases by 7.46 times.

Comparisons of intoxications between themselves shows that combined intoxication increases concentration of Heparin/Heparan GAGs by 3.86 times compared to both single nitrate and fluoride exposure. Content of Keratan/Dermatan fraction increases by 3.88 times compared to both nitrate and fluoride intoxications. Concentration of Chondroitin GAG fraction increases by 23.5% compared to fluoride intoxication and by 10.5% compared to nitrate intoxication. Combined intoxication does not change concentration of L-hydroxyproline compared to fluoride intoxication, but increases concentration of L-hydroxyproline by 1.69 times compared to nitrate intoxication.

Discussion

The decrease in Heparin/Heparan GAG fraction concentration may impair binding of growth factors, cytokines, chemokines, enzymes, enzyme inhibitors, and extracellular matrix proteins to cells of gastric mucosa[16]. For instance, binding of FGFs to basal membrane fibroblasts may be disrupted, which in turn may lead to basal membrane destruction and lesion formation.

HSPG contribute to cell differentiation by controlling „stem cell niche". This effect was shown by Oikari at al in their work[17], where heparan sulphate proteoglycans clearly contributed to astrocyte cell differentiation. It is still unclear if HSPG are main factors determining stem cell fate or they are only a part of a complex interaction between different factors necessary for correct differentiation. We can assume that the decrease in their concentration can disrupt cell differentiation in gastric mucosa.

Heparin can be used as alternative approach in gastric ulcer treatment[18]. Since excessive fluoride intake can cause gastric mucosa lesions[19,20], the decrease in heparin concentration may be one of possible mechanisms of fluoride-induced gastric ulcers. However, more studies are necessary to estimate the possible connection between fluoride ions and heparin in rat gastric mucosa.

DSPG play a key role in cell to cell interaction during immune response. For instance, DSPG is the main binding agent to IFN-γ[21]. DSPG are required for wound healing[22], a decrease in their concentration will impair physiological injury/repair processes in gastric mucosa.

KSPG has the ability to decrease the inflammatory response in a murine model of chronic obstructive pulmonary disease[23]. Fluoride ions can cause inflammation in many tissues and organs[24,25]. A decrease in KSGP may contribute to the progression of fluoride-induced inflammation. The flaw of our study model is that we did not determine the separate concentrations of DSGP and KSGP. However, since the entire Keratan/Dermatan GAG fraction concentration was lowered by fluoride intoxication, we can assume that both DSGP and KSGP concentrations were decreased.

Our study has not shown any changes in CSPG concentration during fluoride intoxication. CSPG can limit inflammation, by inhibition of the expression of pro-inflammatory markers in bone-derived macrophages[26]. This may explain the lower collagen destruction in nitrate intoxication group.

The increase in L-hydroxyproline concentration shows progressive collagen destruction in gastric mucosa. Our previous work showed that during fluoride intoxication there is a hyperproduction of nitric oxide (NO) from NO-synthases and nitrite reductases[27]. We also observed development of oxidative stress in gastric mucosa under same conditions[28]. Therefore, development of oxidative and nitrosative stress may be the reason of progressive collagen degradation.

Conclusions

In conclusion, we can assume that chronic excessive fluoride or nitrate intake can cause oxidative and nitrosative stress depended collagen degradation, simultaneously decreasing concentration of anti-inflammatory GAG.

Combined excessive intake of nitrates and fluorides to even more severe connective tissue degradation, but subsequently activates compensatory mechanisms through increase in concentration of anti-inflammatory GAG.

Compliance with Ethics Requirements:

„The authors declare no conflict of interest regarding this article"

„The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law."

„All institutional and national guidelines for the care and use of laboratory animals were followed"

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