INFLUENCE OF DICLOFENAC SODIUM ON BIOCHEMICAL INDICATORS OF STOMACH MUCOSA CONDITIONS AGAINST THE BACKGROUND OF EXCESS AND DEFICIENCY OF HYDROGEN SULFIDE IN RATS

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Abstract. In this article the effect of diclofenac sodium on biochemical parameters of gastric mucosa against the background of deficit and excess of hydrogen sulfide in rats was investigated. It turned out that such NSAIDs increased the process of peroxidation of lipid and protein and caused an imbalance in the system of antioxidant, disturbed the phospholipid spectrum and reduced mucus production of glycosaminoglycans in stomach. Administration of diclofenac sodium against background of H$_2$S deficit is accompanied by an increasing of range of these changes in metabolic processes in gastric mucosa, while its use with H$_2$S donor normalizes the course of biochemical processes in gastric mucosa. Keywords: gastrototoxicity, diclofenac sodium, hydrogen sulfide, glycosaminoglycans, phospholipid spectrum, peroxidation processes of lipids and proteins.

Introduction
Nonsteroidal anti-inflammatory drugs (NSAIDs) are the large class of drugs that occupies the leading place in the pharmacotherapy of pathological processes characterized by pain, inflammation and fever. The uncontrolled use of these drugs is accompanied by the development of adverse and undesirable effects, the most common of them is gastropathy (erosion and peptic stomach ulcer). Effect of NSAIDs on gastric mucosa is realized through a variety of molecular mechanisms. Drugs in this group disturb “pre-epitelial” protective barrier of the gastric mucosa due to destruction of mucous-bicarbonate- phospholipid barrier of epithelium cell surface of stomach. NSAIDs also alter the epithelial component of gastric mucosa protection, which is determined by the resistance of epithelial cells and cell-cell contacts to reverse diffusion of hydrogen ions, hydrophobic properties of the mucous membrane that promote “repulsion” of hydrochloric acid, as well as very high ability of epithelial cells to proliferation. Local toxic effects of NSAIDs may be associated with their ability to induce processes of oxidative stress, affect the intracellular calcium concentration, reduce the formation of glutathione, disconnect tissue respiration and oxidative phosphorylation, enhance chemotaxis of neutrophils in gastric mucosa (Laine et al., 2008; Bindu et al., 2011; Takeuchi, 2012).

Recently the attention of scientists was attracted to new gaseous molecule - hydrogen sulfide. It has been shown to act as a new gaseous transmitter in mammalian tissues. The relevant literatures have documented the physiological role of H$_2$S including vasodilation, neuromodulation and smooth muscle relaxation, cytoprotection, regulation of inflammation and apoptosis (Kimura et al., 2012; Stein and Bailey, 2013). It was shown that violations in H$_2$S content in tissues are associated with various pathological conditions. Reduced basal H$_2$S content in plasma observed in patients with hypertension, coronary heart disease, deep vein thrombosis, Alzheimer's disease, hyperhomocysteinemia. Increased H$_2$S level detected in patients with Down syndrome, liver cirrhosis, sepsis, ischemic stroke, chronic obstructive pulmonary diseases (Lowicka and Beltowski, 2007; Caliendo et al., 2010). These conditions as a significant number of others may be accompanied by administrations of NSAIDs. It has been shown that the injection of NSAIDs has inhibiting influence on the formation of hydrogen sulfide in rat’s gastric mucosa, which is one of the possible factors of gastric toxicity of this group of drugs (Fiorucci et al., 2005; Voloshchuk, 2010).
different saturation levels of rats’ organisms with hydrogen sulfide on NSAID-induced changes in biochemical parameters of gastric mucosa remains unresolved.

The aim of the research: to evaluate the effect of diclofenac sodium on biochemical parameters of gastric mucosa against the background of deficiency and excess of hydrogen sulfide in rats.

Method

Male Wistar rats (180–210 g, 2–3 months) were maintained under standard laboratory conditions (12h light/dark cycle, temperature of 22±5°C) with free access to standard food and water. Use of animals for all experimental procedures was conducted in accordance with the guidelines of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986). The study protocol was approved by the Ethical Committee of Vinnitsa National Pirogov Medical University.

The experimental animals were divided into several groups: group I was administered solvents and served as normal control. Group II received diclofenac sodium (“Voltaren”, Novartis, 8 mg/kg per 1% starch gels), groups III and IV were administrated diclofenac sodium according to the abovementioned regimen on the background of deficit and excess of hydrogen sulfide, respectively.

Excess and deficiency of hydrogen sulfide created by the intragastric administration of hydrogen sulfide donor; NaHS (Sigma, USA) at a dose of 4 mg/kg on phosphate buffer (pH=7.4) and specific inhibitor of H\(_2\)S synthesis – propargylglycine (PPG) (Sigma, USA) at a dose of 50 mg/kg for 5 days, respectively. Euthanasia of animals was done by dislocation of the cervical vertebrae according to the requirements of bioethics.

For biochemical studies gastric mucosa was isolated, perfused with cold 1.15% solution of potassium chloride and homogenized at 3000 rev/min (Teflon-glass) in 1:3 potassium chloride medium in 1:3 ratio. Homogenates were centrifuged for 30 min at 600 g, aliquots of postnuclear supernatant were selected in Microtubes Eppendorf and stored at 20°C. Protein content was determined by microbiuret method (Kochetov, 1980). Content of malonic dialdehyde (MDA) was estimated by the reaction with thiobarbituric acid (Vladimirov and Archakov, 1972). Amount of carbonyl groups of proteins (CGP) was estimated by the reaction with 2,4-dinitrophenylhydrazine (Shevchuk et al., 2003). The activity of NADPH-oxidase was determined by the absorbance of NADPH and read at 340 nm using the blank (Fukui et al., 1997). Activity of superoxide dismutase (SOD) was determined by the ability to inhibit oxidation of quercetin (Kostyuk et al., 1990). The level of glucosaminoglycans (GAG) was assessed by the content of hexosamines by reaction with para-dimethylbensaldehyde (Ludowieg and Benmaman, 1967). Fractions of phospholipids were determined by chromatography on silicagel L5/40 (Chemapol, Czech Republic) using the chloroform-methanol-water solvent system at a ratio of 65:30:5 (by volume). The identifications of phospholipids-phosphatidylcholine fractions (FH), lisophosphatidylholine (LFH), phosphatidylethanolamine (FEA) were performed using qualitative reactions and the values of Rf, and quantitative assessment was performed after chromatographic reaction with phosphorus-vanillynic reagent (Kejts, 1975).

Statistical analyses of received data were performed by software «STATISTIK 5.5». Mean (M) and standard error (m) were calculated, percentile analyses were carried out, probability of differences (P) was evaluated. The results were considered statistically significant if the P <0.05.

Results

The introduction of diclofenac sodium is accompanied by activation of free radical oxidation of lipids and proteins (Table 1), which conclusively demonstrates a significant increase in the content of malonic dialdehyde (23.3%) and carbonyl groups of proteins (20.7%). Different levels of hydrogen sulfide in the organism change the severity of NSAID-induced activation of oxidative modification of lipids and proteins processes. It was established that the use of diclofenac sodium against the background of deficit of hydrogen sulfide level (induced PPG pretreatment) significantly increased the free radical oxidation: the level of MDA and CGP in the gastric mucosa significantly (by 42.4% and 40.1%, respectively) exceeded the indicators in the control group. Instead, the pretreatment with diclofenac sodium (exogenous hydrogen sulfide donor) normalized lipid and protein peroxidation processes according to the absence of statistically significant differences in MDA and CGP levels in gastric mucosa in comparison with the control group of animals.

The use of NSAIDs is accompanied by the formation of an imbalance in pro- and antioxidant system (Table 2). The significant increasing (+20.6%) of oxidative enzyme (NADPH oxidase) activity and decreasing of antioxidant enzyme superoxide dismutase activity (-17.6%) in gastric mucosa in comparison with the control group were recorded against the background of diclofenac sodium introduction. The use of NSAIDs
against the backdrop of hydrogen sulfide deficiency increases the magnitude of perturbations in the antioxidant enzymes system: under these conditions NADPH oxidase activity increased (+45.8%) and the superoxide dismutase activity decreased (-31.9%). Being introduced together with hydrogen sulfide donor, diclofenac sodium didn’t cause statistical differences in NADPH oxidase and superoxide dismutase activities in comparison with the control group of animals.

Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>MDA, nmol/mg of protein</th>
<th>CGP, nmol/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>6.62±0.27</td>
<td>2.32±0.09</td>
</tr>
<tr>
<td>II</td>
<td>Diclophenac sodium (D)</td>
<td>8.16±0.29*</td>
<td>2.80±0.10*</td>
</tr>
<tr>
<td>III</td>
<td>D+NaHS</td>
<td>6.82±0.31#</td>
<td>2.42±0.12#</td>
</tr>
<tr>
<td>IV</td>
<td>D+PPG</td>
<td>9.43±0.33*#</td>
<td>3.25±0.13*#</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (n = 10).
* Indicates significance at p < 0.05 from the control group.
# Indicates significance at p < 0.05 from the diclofenac-treated group.
MDA = malonic dialdehyde; CGP = carbonyl groups of proteins; PPG – propargylglycine.

Effect of diclofenac sodium on pro-and antioxidant balance of gastric mucosa according to the varying saturation of rats with hydrogen sulfide

Table 2

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>NADPH-oxidase, nmol/min per 1 mg of protein</th>
<th>SOD, standart unites/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>1.07±0.06</td>
<td>1.82±0.06</td>
</tr>
<tr>
<td>II</td>
<td>Diclophenac sodium (D)</td>
<td>1.29±0.08*</td>
<td>1.50±0.04*</td>
</tr>
<tr>
<td>III</td>
<td>D+NaHS</td>
<td>1.09±0.05#</td>
<td>1.75±0.06#</td>
</tr>
<tr>
<td>IV</td>
<td>D+PPG</td>
<td>1.56±0.07*#</td>
<td>1.24±0.07*#</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (n = 10).
* Indicates significance at p < 0.05 from the control group.
# Indicates significance at p < 0.05 from the diclofenac-treated group.
PPG = propargylglycine; SOD = superoxide dismutase.

Effect of diclofenac sodium on gastric mucosa phospholipid spectrum according to the varying saturation of rats with hydrogen sulfide

Table 3

<table>
<thead>
<tr>
<th>No.</th>
<th>Phospholipids, µg/mg of protein</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>Total phospholipids (Ph)</td>
<td>250±6.58</td>
</tr>
<tr>
<td>2</td>
<td>Phosphatidyl choline (PhCh)</td>
<td>113±5.75</td>
</tr>
<tr>
<td>3</td>
<td>Phosphatidyl ethanolamine (PhE)</td>
<td>60.5±4.21</td>
</tr>
<tr>
<td>4</td>
<td>PhCh / PhE</td>
<td>1.93±0.13</td>
</tr>
<tr>
<td>5</td>
<td>Lysophosphatidyl choline (LPh)</td>
<td>16.9±1.12</td>
</tr>
<tr>
<td>6</td>
<td>PhCh / LPh</td>
<td>6.81±0.32</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (n = 10).
* Indicates significance at p < 0.05 from the control group.
# Indicates significance at p < 0.05 from the diclofenac-treated group.
Further we estimated changes in phospholipid spectrum according to diclofenac sodium introduction against the background of different level of hydrogen sulfide in organism (Table 3). The introduction of NSAID is accompanied by the decreasing of total phospholipids (Ph) (-19.2%) and phosphatidyl choline (PhCh) (-25.3%), increasing levels of phosphatidyl ethanolamine (PhE) (+21.9 %) and lysophosphatidyl choline (LPh) (+24.2%) and thus the decreasing in the ratio of PhCh/PhE (-38.8%) and PhCh/LPh (-38.5%). The use of diclofenac sodium against the background of PPG caused deeper changes in the phospholipid spectrum: the reduction the amount of Ph, PhCh, PhCh/PhE and PhCh/LPh (by 30.4%; 39.0%; 58.0% and
and simultaneous increasing of PV and LFH levels (by 45.1% and 48.5%, respectively). At the same time NSAID administration together with donor of hydrogen sulfide didn’t influence on phospholipids in cell membranes of gastric mucosa: statistically phospholipid spectrum didn’t differ significantly from the control group of animals.

Diclofenac sodium affects the gastric mucous barrier according to the statistically significant decreases of glycosaminoglycans content (by 22.5%) compared with control group of animals (Fig. 1). Effect of NSAIDs on the formation of mucus is closely associated with the level of hydrogen sulfide in rats. Introduction of diclofenac sodium together with PPG decreases the level of glycosaminoglycans more expressively (-35.4%) in comparison with appropriate parameter of animals in the control group and in the group of rats that have been treated with diclofenac sodium alone (-16.6%). Statistically the use of NSAIDs together with sodium hydrogen sulfide didn’t cause significant changes in mucus production.

![Fig 1. Effect of diclofenac sodium on the content of glycosaminoglycans (GAG) according to the varying saturation of rats with hydrogen sulfide. Boxes include results from 25 to 75 percentile, vertical lines outside the boxes - minimum and maximum results](image)

**Discussion**

Our studies confirmed some molecular mechanisms of NSAIDs-induced gastrototoxicity. It was shown that the toxic effects of diclofenac on stomach was associated with the activation of oxidative modification of lipids and proteins, formation of imbalance in the system of vasodilators/vasoconstrictor, oxidative damage of membrane phospholipids, phospholipid methylation disturbances and reducing of production of mucus glycosaminoglycans. Modulation of hydrogen sulfide content greatly affected the potential gastrototoxicity of NSAIDs. It was found that the use of diclofenac against the background of hydrogen sulfide deficiency significantly potentiated the negative effects of NSAIDs on the processes of lipid peroxidation and protein production of mucus and phospholipid membranes of gastric mucosa cells. At the same time, the introduction of diclofenac sodium with donor of hydrogen sulfide substantially eliminated the negative effect of NSAIDs on biochemical processes in gastric mucosa.

The ability of hydrogen sulfide to modulate the gastrototoxic potential of NSAIDs substantially associated with its antioxidant, cytoprotective properties and the ability to regulate the processes of proliferation and apoptosis. Our results are also confirmed by other studies, where it was shown that exogenous H$_2$S protects the gastric mucosa from different models of experimentally induced gastric ulcer in rats (Wallace et al., 2007; Mard et al., 2012).

**Conclusion**

Diclofenac sodium caused the increasing in the content of the products of lipid and protein peroxidation (by 21-23%), the imbalance in the system of antioxidants (NADPH oxidase activity increased by 21% and SOD activity decreased by 17.6%), the impairments in the phospholipid spectrum (the content of oxidized forms of phospholipids (lisophosphatidyl choline) increased by 24.3%) and the reducing of glycosaminoglycan levels (by 22.5%). Administration of diclofenac sodium against the background of H$_2$S deficit (induced PPG) was accompanied with the increasing of negative effect on the process of lipid
peroxidation and protein balance in the antioxidant system, phospholipid spectrum and production of glycosaminoglycans in gastric mucous, whereas the use of NSAIDs with H2S donor practically normalized the course of biochemical processes in gastric mucous.

References


Fukui, T. et al. (1997) p22phox mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats. *Circ. Res.*, 80(1), pp. 45-51. Available at: http://dx.doi.org/10.1161/01.RES.80.1.45


