INTRODUCTION
NSAIDs are widely used in medical therapeutics: rheumatic, cardiovascular, gastroenterological diseases and not only. Acute inflammation produced in these sufferings leads to the release of specific mediators of local origin and plasma cell. Among the newly-formed mediators, following tissue damage, arachidonic acid is released from the membrane phospholipids under the effect of phospholipase A2. Cyclooxygenase and lipoxygenase act on arachidonic acid to form prostaglandins, prostacyclins, leukotrienes, and thromboxanes. Constant concern to the mechanism of producing gastroduodenal ulcers under the effect of continued therapy with NSAIDs has revealed the intimate mechanism agreed almost unanimously: inhibition of prostaglandin synthesis.

NSAIDs (derivatives of acetic acid, propionic acid, arilantranilic acid, oxicyclic type butilpirazolidinic type) in ulcerogenesis involve the possible imbalance of arachidonic acid derivatives production and the synthesis proportionally higher of leukotriene LTB4, which activate substances of carbonic anhydrase (CA) and produce microvascular and gastric acid secretion changes.(1)

Thus, Indomethacin produces digestive lesions and by vascular affectionation, it reduces circulatory flow through the gastric mucosa making the prostaglandina E1 (PGE1) effect inefficient.(2) Studies show that in vivo inhibition of cyclooxygenase produced by NSAIDs occurs via CA (3), and the activator effect of red blood cells CA and gastric mucosa CA is greatly increased in vivo versus in vitro data.(4) In vivo studies have shown that Aspirin, cyclooxygenase inhibitor, is a direct activator of carbonic anhydrase from gastric mucosa.(5) It was also proved that the cytoprotective effect of Acetazolamide (6), the first sulphonamide inhibitor used for treating peptic ulcers, is antagonized by Indomethacin through the direct action mechanism.(7)

Puscas et al. (8) have continued their predecessor’s trials, H. Davenport (1939), who posits that the H+ ion of gastric acid secretion results from the catalytic hydration of CO2 and Maren (9) who publishes the first monography of the CA and its inhibitors in 1968. Since 1968, studies made by the team in Șimleu Silvaniei have been highlighting: the direct activation that histamine exerts on gastric acid secretion (1978) and also that the Histamine, Gastrin and Acetylcholine are potent activators of red blood cells CA II and CA IV of gastric mucosa, the role of Acetazolamide in inhibition of CA IV and the decreasing gastric acid secretion (1968), with direct application in the treatment of peptic ulcers by approving the preparation Ulcosilvanil (acetazolamide), continues to set high with the establishment of the role of various inhibitors of gastric secretion, Calcitonin, Somatostatin, derivatives benzimidazole, vasodilatory prostaglandins that inhibit CA and vasoconstrictor prostaglandins and leukotrienes that grow CA IV gastric enzyme activity.

Ulcers associated with chronic NSAID therapy continue to raise medical interest for establishing optimal healing therapy. It was established that Helicobacter pylori infection is an additional risk factor in the patients treated with NSAIDs gastroduodenal ulcerative lesions and the presence of infection also increases the risk of ulcer complications: bleeding and perforation.

Yeomans et al., together with Hawkey et al. (10,11) have compared the effectiveness of Omeprazole versus Ranitidine or Misoprostol in therapy of ulcerative lesions associated with NSAIDs. Omeprazole is more effective as Ranitidine or Misoprostol in therapy of NSAID induced ulcers. In 1991, Campbell and Yamada (12) conducted in vitro studies on canine gastric parietal cells pretreated with Omeprazole and assessed gene expression of two enzymes involved in gastric acid secretion: H (+) - K (+) - ATP -ase and CA II. Stimulation with Carbachol on these cells demonstrated that induction CA II mRNA is not dependent of activation of H (+) - K (+) - ATP -ase, and the expression of mRNA species is much larger than the samples hybridized with H (+) - K (+) - ATP -ase.

In vitro studies that track the effect of stimulation by Carbachol (stimulant of gastric acid secretion) on gastric parietal cells demonstrate that the interaction between Omeprazole and the inhibitory effect of gastric acid secretion is located on gastric...
CA.

In vitro studies (13) on the CA coming from bovine stomach proved the CA activity inhibition after taking Omeprazole, Ranitidine and Famotidine, and red blood cells (RBC) CA activity was significantly inhibited by the these drugs to 3 hours after administration.

**PURPOSE**

The objective of the work was to study the effect of Indomethacin on CA I and CA II purified and the CA IV separated from parietal cells of the gastric mucosa of pigs. Following, the effect of equimolecular association of these NSAIDs with gastric antisecretory was investigated, namely Ranitidine and Omeprazole on the same CA isozymes. The determinations were made after dose-response relationship at concentrations between $10^{-8}$ and $10^{-4}$ M.

**MATERIALS AND METHODS**

In vitro study - Determination of carbonic anhydrase activity

For in vitro studies, Carbonic Anhydrase I (CA I) from human erythrocytes (Sigma, CAS Number: 9001-03-0) and Carbonic Anhydrase Isozyme II (CA II) from bovine erythrocytes were used (Sigma, CAS Number: 9001-03-0). CA isozyme IV was separated from the parietal cells of the gastric mucosa of pigs. The separation method is performed in succession after videendoscopy when taken fragments from gastric mucosa of the gastric bottom zone in order to determine the CA activity. The fragments are harvested in 0.15 M NaCl solution and heparin 1% for washing and removing of red blood cells and mucus. After washing, biopic fragments are dried on filter paper and weighed and after that, the technique of isolation of parietal cells is performed, (14) technique that uses collagenase and pronase.

After isolation, parietal cells are homogenized and suspended in bidistilled water, making a stock solution of 1 mg / ml, which is used to determine enzyme activity.

CA activity in this case will be calculated as specific activity.

\[
A = \frac{T0 - T}{T} \times 100 \% / \text{mg sample (U.E.)}
\]

The specific activity is $T0 - T / T x 1 / \text{mg sample (U.E. / mg)}$

The carbonic anhydrase activity was assessed using the stopped-flow method –Khalifah.(15) This method consists in measuring the enzymatic activity of CO$_2$ hydration and is based on the colorimetric determination of the rate of pH change. The time in which the pH of the reagent mixture decreases from its initial value of 7.5 to its final value of 6.5 is measured. The follow-up of the reaction is achieved spectrophotometrically at 400 nm wavelength, using a rapid kinetic spectrophotometer HI-TECH SF-51MX (England), equipped with a mixing unit and a system of two syringes which supply the reagents. The signal transmitted by the photomultiplier from the mixing chamber is received and visualized by a computer equipped with a mathematical coprocessor and a kinetic software package RKBIN IS1.

Enzymatic activity of carbonic anhydrase was obtained by the formula:

\[
A = \frac{(T0 - T)}{T} [\text{EU/ml}]
\]

Where,

$T0$ represents the uncatalyzed reaction time, and $T$ represents the catalyzed reaction time (in the presence of CA I, CA II gastric mucosa CA IV).

In the first phase, enzymatic activity of isozymes CA (I, II and IV) in the presence of Indomethacin (NSAID) was determined. In the second phase the effect of equimolar association of NSAIDs with gastric antisecretory (Ranitidine and Omeprazole) on the enzymatic activity of CA isozyme was observed. The determinations were made after dose-response relationship at concentrations between $10^{-8}$ and $10^{-4}$ M.

The results were expressed as a percentage (increase / decrease) from enzymatic activity reported for the enzyme only in the presence of the substrate, which was considered 100 %.

In vivo study - Determination of carbonic anhydrase activity

The study was conducted in accordance with the Declaration of Helsinki amended by Resolution of the 21st World Meeting in Venice, Italy in 1983, and then by Resolution 41 of the World Meeting in Hong Kong in 1989.

We selected 100 volunteers (male and female) in good general health, aged between 38-59 years old (55% male and 45% female), which were randomly divided into five groups (1-5). All subjects were screened before participation to this study for physical examination, routine laboratory analysis, electrocardiogram and their medical history. They did not have any gastroduodenal or rheumatic diseases and they did not take any medication two weeks before the beginning of the study.

Written informed consent was obtained from all volunteers, prior to enrolment and the study was approved by the Ethics Committee of the “Prof. Dr. Ioan Pușcaș” Șimleu Silvaniei Municipal Hospital Sălaj, Romania, where the experiment took place between January 2010 and June 2010. The study focused on dose-response studies. The drug treatment of each group is shown in the table no. 1.

Table no. 1. The drug treatment of each group involved in the study

<table>
<thead>
<tr>
<th>Group(n=20)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Indomethacin per os. 3 mg/kg body</td>
</tr>
<tr>
<td>2</td>
<td>Ranitidine, p.o., 300 mg/day</td>
</tr>
<tr>
<td>3</td>
<td>Omeprazol, p.o., 40 mg/day</td>
</tr>
<tr>
<td>4</td>
<td>Associated treatment Indomethacin+ranitidine</td>
</tr>
<tr>
<td>5</td>
<td>Associated treatment Indomethacin+ ranitidine</td>
</tr>
</tbody>
</table>

For patients in all 5 groups, the enzyme activity of CA I and RBC CA II according to the protocol described above were determined. Results are expressed in EU/ml. In parallel to the determination of enzyme activity, gastric acid secretion in the 5 groups was also determined.

Red cell CA II activity was separated from red cell CA I according to the test with nicotinates.(16) This test relies on the selective inhibition of CA I activity. In the first step, we assayed the total carbonic anhydrase activity. Methylnicotinate added in the concentration of 5x$10^{-4}$ M completely inhibited CA I activity. The remaining carbonic anhydrase activity in red blood cells represents CA II activity.

Statistical analysis

The data were expressed as average ± SD. P-values < 0.05 were considered as significant. Unpaired t test was performed using GraphPad Prism version 5.00 for Windows. GraphPad Software, San Diego California USA. www.graphpad.com”.

**RESULTS**

In vitro study

In this study, in the first phase, the influence of Indomethacin, Ranitidine and Omeprazole, at 3 different concentrations ($10^{-8}$, $10^{-5}$, $10^{-4}$ M) on the enzymatic activity of various CA isozymes (I, II, IV) were observed. The results showed the activator effect of Indomethacin at all three concentrations studied, the highest value being registered at $10^{-4}$ M (34 % for CA I, 42 % for CA II and 47% for CA IV) (figure no. 1). On the other hand, in case of Ranitidine at concentration of $10^{-8}$ M, there has been no activator effect on the 3 isozymes, in contrast to a weak decrease of antioxidant activity, which was recorded at concentration of $10^{-4}$ M for all three isozymes (-1%...
for CA I -3 % for CA II and -26 % for CA IV) (figure no. 1). In the case of Omeprazole, the inhibitory effect on the enzymatic activity of isozymes CA is more pronounced compared to the Ranitidine effect, being dependent to the concentration of the used drug. Thus, at the concentration of $10^{-4}$ M, the inhibitory effect reported for the isozymes was -48% for CA I, -70% for CA II and -65 % for CA IV (figure no. 1). In the second part of the in vitro study, we followed the influence of equimolar association of Indomethacin with Ranitidine and Omeprazole. The results showed that after these associations (Indomethacin+Ranitidine) enzyme activity is increasing in all three isozymes and the values obtained for the $10^{-4}$ M concentration being of 9.15 %, 7.14 % and 12% for CA I, CA II, respectively CA IV. The combination of Indomethacin with Omeprazole had as effect the inhibition of CA isoenzymes, but it was weaker compared with Omeprazole alone. The results obtained were in the case of the concentration of $10^{-4}$ M, like this: -4 %,-15.3 % and -20 % for CA I, CA II, respectively CA IV (figure no. 1).

Figure no. 1. The Indomethacin and the antisecretory effect ($10^{-4}$ M) on CA isozymes

In vivo studies, the patients (volunteers) were grouped in 5 groups, each group having its own treatment (table no. 1). Before and after treatment, the enzymatic activity of AC I and II of red blood cells was determined. In the group I, treated with Indomethacin orally 3 mg / kg-body, the results reveal a significant increase in enzyme activity both for CA I and CA II (0.25 EU / ml to 1.5 EU / ml) ($P < 0.001$) (figure no. 2A). In the case of Group 2 after treatment with Ranitidine p.o., 300 mg / day, there has been observed a significant decrease in CA II enzyme activity, from 1.35 EU/ml to 0.85 EU/ml ($P < 0.001$), instead, enzymatic activity of enzyme CA I remained unchanged (figure no. 2B).

In the case of group 3, after treatment with Omeprazole po 40 mg/day, there was a significant reduction ($P < 0.001$) of the enzymatic activity of the isoenzyme CA II, from the 1.5 EU/ml to the 0.5 EU/ml (figure no. 2 C).

Figure no. 2. The modification of enzyme activity in vivo, of CA I and CA II isoenzyme after treatment with Indomethacin (A), Ranitidine (B) and Omeprazole (C)

The association of the treatment involving the combination of Indomethacin and Ranitidine (Group IV) did not lead to any significant changes in the values of the enzymatic activity of isozymes CA I and CA II (0.25 EU/ml, respectively 0.26 EU/ml for CA II and 1.55 EU/ml respectively 1.65 EU/ml for CA I) (figure no. 3 A).

Similar results were obtained by combining the treatment of Indomethacin and Omeprazole (group 5), where the enzymatic activity of CA I did not change significantly (0.25 EU/ml and 0.27 EU/ml for basal respectively after treatment), while CA II enzymatic activity values decreased significantly after treatment (1.85 EU/ml to 1.35 EU/ml) ($P = 0.0795$) (figure no. 3B).

Figure no. 3. Change in vivo enzymatic activity, of the CA I and CA II isoenzymes after Indomethacin therapy with Ranitidine (A) and Indomethacin with Omeprazole (B)
The values of gastric acid secretion (BAO) slightly increase after administration of Indomethacin and in patients treated with Ranitidine or Omeprazole, there was a decrease of these values. The association of Indomethacin to Ranitidine, respectively to Omeprazole, antagonizes the antisecretory effect of those substances (table no. 2).

Table no. 2. Modification of gastric acid secretion (BAO)

<table>
<thead>
<tr>
<th>Groups</th>
<th>BAO (mEq/h) before treatment</th>
<th>BAO (mEq/h) after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>2.40 ± 1.01</td>
<td>8.79 ± 1.86</td>
</tr>
<tr>
<td>Group 2</td>
<td>4.98 ± 1.16</td>
<td>1.63 ± 0.81</td>
</tr>
<tr>
<td>Group 3</td>
<td>5.43 ± 1.28</td>
<td>1.44 ± 0.69</td>
</tr>
<tr>
<td>Group 4</td>
<td>5.22 ± 0.95</td>
<td>2.79 ± 0.98</td>
</tr>
<tr>
<td>Group 5</td>
<td>3.26 ± 1.08</td>
<td>3.02 ± 1.01</td>
</tr>
</tbody>
</table>

DISCUSSIONS

In vitro study, Indomethacin activates CA I, CA II and CA IV after dose-response relationship. The activator effect of NSAIDs is present at concentrations of 10^{-4}M and at 10^{-3}M is the maximum. Ranitidine does not significantly modify the basal AC isoenzymes values and Omeprazole decreases the activity of isoenzymes depending on the dose. The equimolecular association of NSAID with antisecretory medication reduces their inhibitory effect on isozymes I, II, IV of CA. The studied NSAIDs increased the activity of red blood cells CA I and CA II both in vitro and in vivo, and also the CA IV gastric activity. The studied antisecretory medications produce a slight inhibition of CA I and more evident CA II and gastric CA IV, both in vitro and in vivo.(17)

In vitro, the association of the NSAIDs to antisecretory medication, at concentrations between 10^{-8} and 10^{-4}M, and also their association in usual therapeutic doses in vivo antagonize their inhibitory effect on isozymes of CA and on the gastric acid secretion, too.

The data suggest that carbonic anhydrase is the site of interaction between NSAIDs and gastric antisycretory and, by this the enzyme is involved in the mechanism by which NSAIDs antagonize their effects of lowering gastric acid secretion. Moreover, the activation of CA I, isoenzyme involved in vascular mechanisms, NSAIDs produce gastroduodenal lesion by reducing gastric microcirculation.

This way, the antagonizing of the effects of Ranitidine and Omeprazole on CA I could explain gastric lesions and ulcerations after long treatment with NSAIDs, even if they are associated with antisecretory therapy. Reference data confirm the fact that comes out of our research as well: under the effect of administering NSAIDs in combination with Ranitidine or Omeprazole, namely that in patients taking NSAIDs regularly, preventing ulcers can be made more effective with Omeprazole than with Ranitidine.(18)

Activation of CA produced by NSAIDs could be considered responsible for increasing gastric secretion, the role of CA in ulcerogenenesis caused by NSAIDs is achieved by the following general mechanism: once fixed in the active site of the enzyme, NSAIDs activates CA and lower pH, which could influence the conformation structural protein, making the protein Gi act on adenylate-cyclase with production of vasoconstriction (at the level of micirculation smooth muscle cell). In the cytosol, activation of CA II ensure increased production of H^+, which will be expelled by luminal K^+ -H^+ dependency ATPase, achieving a gastric hypersecretion.

The team led by Puscas et al. (19) propose a dual mechanism of action for NSAIDs, both directly on the CA (with the effects described above) and on cyclooxygenase resulting in reduced production of prostaglandins (PG). Between the two enzymes there is a close interdependence, enzymes can be linked, and in addition we consider that changes in pH produced by activation of CA would compete in a high affinity of NSAIDs for cyclooxygenase. In addition, NSAIDs cancel the activation of CA through phospholipase A_2.

Using combination of NSAIDs with specific inhibitor of CA, Acetazolamide (known as being with cytoprotective properties) Puscas et al. (20) observed that Indomethacin gradually reduces the CA inhibition caused by Acetazolamide until its cancellation. This phenomenon is because of the fact that the inhibitor effect of Indomethacin on CA installs faster than that of Acetazolamide. Indomethacin proves in this sense to be the most powerful antagonist of Acetazolamide known until now. These results demonstrate that the center of the interaction between Indomethacin (a well known ulcerigen drug) and Acetazolamide (a drug with cytoprotective properties) is on the carbonic anhydrase, enzyme that we believe to be involved in cytoprotective (by modulating vascular processes in gastric microcirculation) and gastric acid secretion.(21)

Recent research has shown that all the pharmacological agents of NSAIDs type also interact with CA activity, kinetic studies showing an activation mechanism of the non competitive type with the CO_2 substrate, so the binding site of the enzyme is different from the hydrophobic pocket in which CO_2 binds.

By forming enzyme-activator (NSAIDs) complex particularly stable, e.g. CA-IMC, CA-ASA(22), the additional generating of hydrogen H\(^+\) is lasting, what explains the phenomenon of prolonged activation of CA (5-7 days after discontinuation) maintaining low intracellular pH. In addition, endogenous inhibitors (Calcitonin, Somatostatin, Prostaglandins) and exogenous (Acetazolamide) of the CA can not antagonize the activation with Indomethacin, probably due to the higher thermodynamic stability of the complex enzyme-activator, enzyme - inhibitor complex compared. This phenomenon does not increase intracellular pH. The molecular basis of this phenomenon is probably the very favourable interaction of activating molecules and amino acid residues in the active site of the enzyme (by saline interactions, hydrophobic, Van der Waals).

Since most potent cyclooxygenase inhibitors are potent activators of CA, forming lasting enzyme-activator complex, it is possible that this mechanism also controls the processes of inhibition of cyclooxygenase by NSAIDs.(23) Low levels of pH due to activation of CA by NSAIDs may boost certain conformations of cyclooxygenase as well as its affinity for its inhibitory substrates. Particularly high stability of the enzyme-activator complex called NSAIDs has as consequence the impossibility of antagonism, activation through the inhibition (exo- or endogenous) or its potentiatio by exo- or endogenous activators. The result is to maintain a high intracellular acidity, constant and prolonged. In conclusion, CA is a modulator of cyclooxygenase activity by intra- and extracellular pH changes which it produces, with the biosynthesis of prostaglandins consequences.

NSAIDs are known as decreasing cyclooxygenase activity and consequently prostaglandin synthesis, affecting the gastric and duodenal mucosa and antagonize the cerebral vascular response to prostacyclin. The results suggest that between the carbonic anhydrase and cyclooxygenase there is an inverse relationship, namely: activation CA is accompanied by Cox inhibition and vice versa, and the correlation between the two enzymes would be achieved through changes in pH induced by activation or inhibition of CA produced NSAIDs.

CONCLUSIONS

The results of our study suggest that carbonic
anhydrase is the site of action of NSAIDs and gastric antisecretory, and by this, the enzyme is involved in the mechanism by which NSAIDs antagonize their effects of lowering gastric acid secretion. Moreover, the activation of the AC I, isoenzyme involved in the vascular mechanisms, NSAIDs produce gastroduodenal lesion by reducing gastric microcirculation. This way, the antagonizing of the effects of Ranitidine and Omeprazole on CA I could explain lesions and gastric ulcerations occurrence after long treatment with NSAIDs, even if they are associated with antisecretory therapy.

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