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Research Article

Role of Vitamin D3 and Alginates in Prevention of NSAID-Dependent Cellular Injury

Claudio Molinari[1], Vera Morsanuto[1], Sara Ruga[1], Ian Stoppa[1], Felice Notte[1], Mahitab Farghali[1], Chiarella Bozzo[2] and Francesca Uberti[1]*

[1]*Laboratory of Physiology, Department of Translational Medicine, University of Piemonte Orientale, Novara, Italy*

[2]*Laboratory of Biology, Department of Translational Medicine, University of Piemonte Orientale, Novara, Italy*

***Corresponding Author:** Francesca Uberti, Laboratory of Physiology, Department of Translational Medicine, University of Piemonte Orientale, Novara, Italy.

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Abstract

About 30 million people use nonsteroidal anti-inflammatory drugs. Therefore, they have a high risk of developing gastropathic and enteropathic damages. These patients receive anti-acid treatment, but a number of clinical studies provided evidence of the ineffectiveness of proton-pump inhibitors. Vitamin D, on the other hand, appears to have high preventive and therapeutic potential. Recently, it has been introduced a product that, in addition to anti-acid properties of alginates, claims to possess gastroprotective properties deriving from vitamin D3 and from plant extracts.

This study was planned to verify the effectiveness of vitamin D3 combined with alginates to prevent the damage induced in cultured gastric cells by diclofenac during acidic or hyperacidic exposition measuring cell viability, radical oxygen species production along with apoptotic and survival pathways.

Findings show that this combination is more potent to counteract the negative effects of diclofenac and hyperacidic conditions than some other gastroprotective agents on epithelial gastric cells. This was confirmed by the maintenance of p53 expression at physiological level. In addition, when added before diclofenac, it can exert beneficial effects counteracting the negative effect of diclofenac alone. These data were similar to the sample treated with pantoprazole, supporting the hypothesis that the combination could act as a gastroprotector to prevent cell loss.

These results have pointed out the gastroprotective effect of the combination when compared to other commercial natural extracts, this effect is obtained via antioxidant pathway, inhibiting apoptosis, enhancing cell viability and activating survival kinases.

Keywords: *Alginates; Gastric Cells; Gastroprotection Mechanism; Hyperacidity; Vitamin D3*

Abbreviations

NSAID: Nonsteroidal Anti-Inflammatory Drugs; PPI: Proton-Pump Inhibitor; HCl: Hydrochloric Acid; Aq: Aquilea Reflux®; DMEM: Dulbecco's Modification of Eagle Medium; FBS: Foetal Bovine Serum; Neo: Neobianacid®; D: Diclofenac; P: Pantoprazole; Ham's F12: F-12 Nutrient Medium; ATP: Adenosine Triphosphate; NO: Nitric Oxide; ROS: Radical Oxygen Species; H_2O_2: Hydrogen Peroxide; PMSF: Phenylmethanesulfonyl Fluoride; PVDF: Polyvinylidene Fluoride

Introduction

Around 30 million people consume nonsteroidal anti-inflammatory drugs (NSAID) globally every day [1] since they are widely prescribed because of their efficacy in the management of pain, inflammation, and fever [2]. Generally, the action mechanism of these drugs consists in the inhibition of the biosynthesis of prostaglandins, the inactivation of cyclooxygenase, and an increase in leukotrienes production [3]. Adverse events associated with NSAID, such as alterations in renal function, effects on blood pressure, hepatic injury, and platelet inhibition, are a challenge in clinical treatment optimization [4]. However, severe gastrointestinal disorder accompanied by gastric mucosal perforation and bleeding is a major concern as well as the worst outcome of prolonged NSAID-therapy [5]: indeed, they induce gastric mucosal lesions because of their acidic properties [3]. Gastric mucosal erosions, ulceration, bleeding, and perforation, as well as an increased risk of bleeding from pre-existing peptic ulcers are major causes of gastrointestinal iatrogenic diseases [6]. The mechanism behind gastric damage involves a highly acidic gastric environment that favours the migration of nonionized lipophilic NSAID into the

epithelial cells, and at the cell surface these are dissociated into ions, trapping hydrogen ions and inducing mucosal injury. This action is further enhanced by the decrease mucosal blood flow, secretion of mucous and bicarbonates, and the defensive factors of the gastric layer [3,7]. Indeed, these side effects lead to reduced intestinal mucus formation, disturbed micro-circulation causing increased intestinal motility, and increased mucosal permeability to many inflammatory mediators including neutrophils and cytokines [8]. The consequences can range from dyspepsia to severe peptic ulcer and bleeding; endoscopically the range from subepithelial haemorrhages and erosions to total destruction of epithelial membrane and full thickness ulcer [9]. Diclofenac, an anthranilic acid derivative with pKa of 4.0 [1], is the most widely prescribed NSAID for treating several forms of pain and inflammation, such as rheumatic and non-rheumatic diseases, pain, fever, chronic inflammation, dysmenorrhea and several forms of cancers [10-12]. The main clinical problem of diclofenac is toxicity induced by oxidative tissue injury in the intra-mitochondrial environment, which appears to play a prominent role in the pathophysiology of digestive ulceration [1,13,14]. Indeed, NSAID uncouple mitochondrial oxidative phosphorylation, which causes loss of intercellular integrity, with increased intestinal permeability and subsequent mucosal damages [15,16]. For these reasons, NSAID users are subjected, often empirically by doctors, to anti-acid treatments such as the proton-pump inhibitors (PPI) [17], thus, not surprisingly, NSAID and PPI are among the most frequently co-prescribed drugs worldwide [18]. The potent anti-secretory effects of PPI are claimed to account for their efficacy in various acid-related diseases; however, the anatomical and functional integrity of gastric mucosa rely on the balance between aggressive and defensive mechanisms [2]. In addition, the success of pharmacological treatments in either the prevention or healing of ulcerative lesions may depend not only on the blockade of acid secretion, but also on the enhancement of mucosal protective factors [2,19]. Moreover, a number of clinical studies have provided the support for the ineffectiveness of PPI in preventing or reducing the NSAID-damages [20-22]. Indeed, current evidence suggests that PPI are also associated with numerous side effects such as hypergastrinemia [23] enteric infections [24], adverse cardiovascular events [25] and increased mortality rates [26]. The need for NSAID clinical use despite their side effects encourages continuous research to create novel agents able to counteract their adverse effects with better safety profile, in particular using natural compounds. Recently, a crucial role for vitamin D in digestive system health has been described [27]. In gastric mucosa, vitamin D appears to have high preventive and therapeutic potentials [28] and is able to regulate endocrine and paracrine secretion of gastrin with secondary effects, for instance, on parietal cell HCl and pepsinogen secreting chief cells [29]. Recently, a product has been introduced that, in addition to having the antacid properties of alginates, claims to possess gastroprotective properties deriving from vitamin D3 and from plant extracts. It has been tested alone and combined with diclofenac in order to provide an effective and safer strategy for the management of NSAID-induced gastroenteric lesions. Hence, the primary objective of this work was to evaluate whether it is possible to improve the protection of the gastric mucosa during therapy with diclofenac. Observations have been compared to a PPI and to another commercial product in oxidative or hyperacidic conditions. Thus, the present study was designed to examine the protective effects of alginates combined with vitamin D3 assessing its effects on factors related to gastric protection such as viability, radical productions and intracellular mechanism involved. Moreover the aim was to examine whether this combination has direct beneficial effects through activation of survival pathways.

Materials and Methods

Agents preparation

Alginates combined with vitamin D3 was used to prepare a commercial product (named Aquilea Reflux®, Laborest Italia srl, Milan, Italy; Aq) used as a dietary supplement indicated to counteract high acidic conditions thanks to its specific composition [27]. Aq is a dietary supplement combining the properties of calcium alginate with a tyndalized probiotic (Pylopass®) and an extract of prickly pear and olive leaves (Mucosave®) in a buffer solution. It is composed of Mucosave® (0.83 mg/ml), calcium alginate (1.66 mg/ml), magnesium hydroxide (2.66 mg/ml), potassium citrate (4.66 mg/ml), Pylopass® (0.66 mg/ml) and vitamin D3 (0.000083 mg/ml). It was dissolved according to solubility information reported in manufacturer's instructions, directly in the DMEM without red phenol and FBS but supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (white medium).

A mixture of polysaccharides and flavonoids present as a commercial product (Neobianacid®, Aboca, Italy; Neo), is a product that improves the protection of the stomach and the esophagus thanks to the presence of Poliprotect® and a flavonoid fraction (*Matricaria recutita* and *Glycyrrhiza glabra*). This product was dissolved following the manufacturer's instructions directly in white medium and added without dilution to the cells. Diclofenac (D) and Pantoprazole (P) were prepared directly in white medium and added to the cells at final concentrations of 250 µM and 10 µM respectively, as reported in literature [2,30] before or after Aq or Neo in presence or absence of hyperacidic condition. HCl was used to prepare an acidified medium (pH 4) that was added to the cells without dilution. To create a hyperacidic condition, before stimulation 1:172 (%v/v) of HCl were also administered to the samples.

Primary cell culture

Primary epithelial gastric cells were obtained from the stomach of anaesthetized prepubescent pigs as previously described [27]. Briefly, the gastric cells were isolated using enzymatic solution in agitation (collagenase/dispase solution, Sigma-Aldrich, Milan, Italy) for 60 minutes at 37°C, centrifuged at 1500 rpm for 5 minutes at 4°C and then the pellet resuspended in complete medium (Ham's F12 supplemented with 10% FBS, Sigma-Aldrich, Milan, Italy) on collagen coated dishes. The cells used for the experiments were obtained from passage 3 to passage 5. The cells were used to perform different experiments; to study cell viability, ATP level and NO production 1 x 10^4 cells were plated on 96 well-plates; to study radical oxygen species (ROS) production 1 x 10^5 cells were plated on 24 well-plates; to study the intracellular pathways by Western blot and to analyse the activities by kit ELISA the cells were plated on 60 mm dishes until confluence. To synchronize the cells, before stimulations they were maintained in DMEM without red phenol and FBS and supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate in an incubator at 37°C, 5% CO2 and 95% humidity for 18h and then to create a similar condition of human stomach, the cells were maintained for 2h on acidified medium (HCl was added at 5 DMEM without red phenol and FBS and supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate to obtain a medium with pH 4) before the stimulation. This acidified medium was maintained during the successive stimulations.

Experimental protocol

The cells were used to analyse the effect of Aq and Neo in preventing or reverting the damage caused by anti-inflammatory drugs (in particular Diclofenac) during oxidative stress or hyperacidic condition. Aq and Neo were tested in preliminary experiments before or after 200 µM H_2O_2 or 1:172 (%v/v) HCl to determine the effects (prevention or repair, respectively) during oxidative stress or gastric hyperacidity. The time of stimulation was 24h, in order to observe cell viability, ATP consumption, ROS production and intracellular pathways activations. Since the greatest effects were observed during hyperacidic condition, this state was maintained during successive stimulations with anti-inflammatory drugs. In particular the protective effects on gastric epithelial cells of Aq and Neo were examined during pre and post stimulations with 250 µM Diclofenac (D). The time of stimulation was 24h to study the effects on cell viability, ROS production, and the intracellular pathways involved. To confirm the potential use of Aq and Neo as gastroprotective agents, some data were compared to those obtained from experiments with 10 µM pantoprazole (P), added before diclofenac.

Cell viability

MTT-based *In Vitro* Toxicology Assay Kit (Sigma-Aldrich, Milan, Italy) was used to determine cell viability, as previously described [31]. Briefly, at the end of each stimulation, the cells were incubated with 1% MTT dye for 2h - 3h at 37°C in incubator, until the purple crystals were dissolved in equal volume of MTT Solubilization Solution. The relative viability (%) was based on absorbance measuring through a spectrometer (VICTORX4 Multilabel Plate Reader) at 570 nm with correction at 690 nm. Finally, viability was calculated comparing results to control cells (taken as 100% viable).

Radical oxygen species production

The rate of superoxide anion release was measured using a standard protocol [27,31] analyzing the superoxide dismutase-inhibitable reduction of cytochrome C. Briefly, on both treated and untreated cells, 100 µL of cytochrome C were added, and in another sample, 100 µL of superoxide dismutase were also added for 30 minutes in incubator (all substances from Sigma-Aldrich, Milan, Italy). The O2 was determined by measuring the absorbance at 550 nm by a spectrometer (VICTORX4 Multilabel Plate Reader) and expressed as means ± SD% of nanomoles per reduced cytochrome C per microgram of protein compared to control [32].

ATP assay

At the end of each stimulation the medium was removed and the cells were immediately treated with the components of the ATP assay kit (nucleotide releasing buffer, ATP monitoring enzyme, enzyme reconstitution buffer, ATP), following the manufacturer's instructions. Luminescence was measured 1 min after the addition of ATP monitoring enzyme in a VICTORX4 multilabel plate reader (PerkinElmer Waltham, Massachusetts, U.S.A.), and luminescence was expressed as means ± SD% of µmol of ATP/g protein [33].

p53 activity

p53 activity was measured by specific ELISA kit (p53 transcription factor assay kit, Cayman Chemical, Ann Arbor, Michigan, USA), examining the nuclear extracts obtained at the end of each stimulation following the manufacturer's instructions. The nuclear extraction

was obtained by classical technique using a complete buffer present in the kit. Briefly, the cells were lysed with ice-cold 1X Complete Hypotonic Buffer, supplemented with NP-40 and then centrifuged at 12,000g at 4°C for 10 minutes. The pellet was solubilized with ice-cold Complete Nuclear Extraction Buffer 1x supplemented with protease and phosphatase inhibitors, and then centrifuged at 12,000g for 15 minutes at 4°C; the supernatant was examined to analyse the activity of p53 related to the protein quantification through BCA assay (Thermo Fisher).

ERK and Akt activation assay

ERK/MAPK and PI3K/akt activities were measured by the InstantOne™ ELISA (Thermo-Scientific) on cell lysates following the manufacturer's instructions, as previously described [34]. Briefly, at the end of treatments cells were lysed with 100 µL Cell Lysis Buffer Mix and 50 µL/well of each sample were tested in InstantOne ELISA microplate strips. Moreover, the Antibody Cocktail was added to each well and incubated for 1h at room temperature on a microplate shaker. At the end, the Detection Reagent was added to each well and after 20 minutes the reaction was stopped adding to each Stop Solution. The strips were measured by a spectrometer (VICTOR X4 multilabel plate reader) at 450 nm. The results were expressed as mean Absorbance (%) compared to control.

Western blot

After each stimulation, the cells were washed with iced PBS 1X supplemented with 2 mM sodium orthovanadate (Sigma-Aldrich, Milan, Italy) and lysed in iced Ripa Buffer (10 mM Na2HPO4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulphate, 1% sodium deoxycholate, 50 mM sodium fluoride; Sigma-Aldrich, Milan, Italy) supplemented with 2 mM sodium orthovanadate (Sigma-Aldrich, Milan, Italy), 1:1000 phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich, Milan, Italy) and 1:100 protease inhibitors cocktail (Sigma-Aldrich, Milan, Italy). 35 µg of proteins were resolved on 10% SDS-PAGE gels (SDS-PAGE; Bio-Rad Laboratories, Hercules, CA, USA), transferred to polyvinylidene fluoride membranes (PVDF, GE Healthcare, Milan, Italy) anti-VDR (1:250, Santa-Cruz) which were incubated overnight at 4°C with specific antibodies: anti-p53 (1:400, Santa-Cruz), anti-Annexin V (1:1000, Sigma-Aldrich, Milan, Italy), and anti-Ki67 (1:800,Santa-Cruz), The protein expressions were normalized and verified through β-actin detection (1:5000; Sigma-Aldrich, Milan, Italy).

Statistical analysis

Results are expressed as means ± SD of at least 5 biological replicates for each experimental protocol and each replicate was reproduced 3 times. Statistical comparisons between groups were performed by one-way ANOVA with Bonferroni's post hoc test or the Mann-Whitney U test, to compare percentages of responses, using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Analysis of the effects of Aq and Neo on epithelial gastric cells under oxidative or acidic conditions

Both products act as a physical barrier for gastric epithelial cells and exert protection against hyperacidity, but the different compositions can determine different beneficial effects. The first set of experiments analysed the ability of act before or after the damage caused by oxidative stress and acidity of Aq and Neo after 24h of stimulation. As reported in figure 1, the effects on cell viability and ROS production were different between Aq and Neo alone. Aq induced a significant increase in cell viability compared to Neo (about 215%) and to control ($p < 0.05$, see figure 1a); Aq also induced a significant reduction in ROS release (about 256%) compared to Neo (see figure 1b). These preliminary data confirmed the hypothesis that the composition can influence the efficiency during gastroprotection. In order to clarify the efficacy of Aq and Neo in gastroprotection, all the following experiments were performed in presence of pre-treatment or post-treatment for 30 minutes with 200 µM H_2O_2 or 1:172 (%v/v) HCl. As reported in figure 1 the pre-treatment with H_2O_2 or HCl caused a significant reduction ($p < 0.05$) of cell viability and an increase of ROS production ($p < 0.05$) compared to control. Aq was able to counteract the negative effect exerted by H_2O_2 or HCl on cell viability ($p < 0.05$) both before and after the damage; in particular, the most evident beneficial effects were observed in reversing the influence exerted by HCl both on cell viability and ROS production. However, regarding cell viability (see figure 1a) Aq seems to be more effective when it is added after pre-treatment (about 100% in oxidative and about 63% in hyperacidic conditions). On the other hand, Neo seems to have a beneficial effect only when added after the injury, reported in instructions. Between oxidative and hyperacidic pre-treatment conditions, Neo was able to have a greater effect on cell viability when administered in presence of oxidative conditions (about 70% compared to hyperacidic conditions). As regards ROS production, that is an

important parameter to evaluate cellular damage, Aq and Neo had a different effect under oxidative and hyperacidic conditions, confirming the differences observed in cell viability (see figure 1b). Indeed, both Aq and Neo had better results when added after pre-treatment, but Aq had a greater beneficial effect on hyperacidic conditions and Neo during oxidative stress. These data confirmed a different effect on cell viability and ROS production of Aq compared to Neo and suggest the existence of a different mechanism of protection.

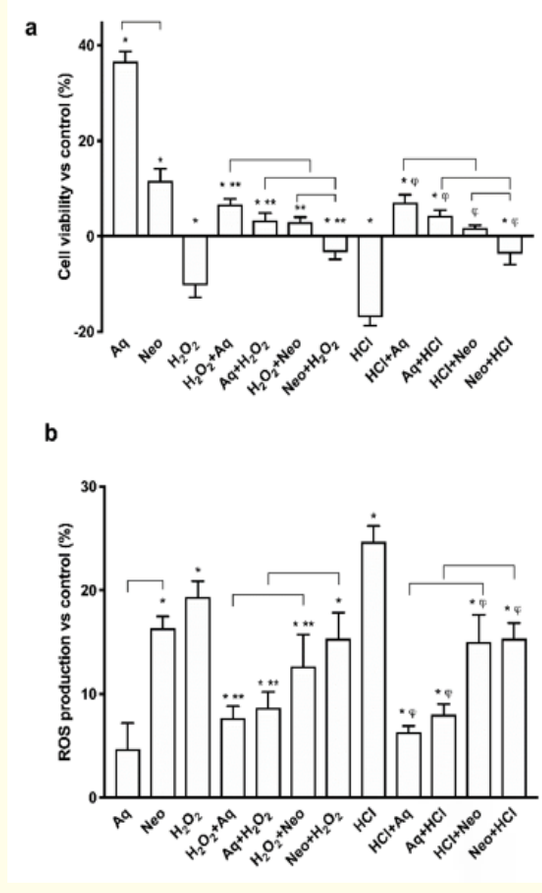

Figure 1: *Effects of Aq and Neo during acidic and oxidative stress. In panel (a) cell viability and in panel (b) ROS production measured in gastric epithelial cells treated with Aq and Neo for 24h. Aq= combination between alginates and vitamin D3, Neo= mixture of polysaccharides and flavonoids. Data are expressed as means ± SD (%) of five independent experiments normalized to control values (0% line). *p < 0.05 vs control; ** p < 0.05 vs H2O2; ϕp < 0.05 vs HCl; arrows indicate p < 0.05 between different groups.*

Analysis of the effects of Aq and Neo to prevent cell death

Cell death is an energy-dependent process that requires ATP: typically, apoptotic cells exhibit a significant decrease in ATP level. This parameter is important in evaluating the effectiveness of Aq and Neo to prevent loss of gastric epithelial cells caused by oxidative stress or hyperacidity. As reported in figure 2a, ATP level was higher in presence of Aq than Neo in basal conditions ($p < 0.05$), confirming data observed on cell viability. In addition, treatment with 200 µM H2O2 or 1:172 (%v/v) HCl caused a significant reduction ($p < 0.05$) of ATP indicating cell death. Aq was able to counteract the negative effects exerted by H2O2 or HCl on ATP production ($p < 0.05$) both before and after the damage, but the best result was observed in reversing the influence exerted by pre-treatment with HCl compared to control ($p < 0.05$). Neo was able to exert beneficial effects when added after the injury and confirmed a greater effect on ATP production when in presence of oxidative conditions compared to control ($p < 0.05$). These data support the hypothesis of a different protection mechanism between Aq and Neo. Since p53 is a key component for cellular-induced apoptosis through mitochondrial stress markers, additional experiments were performed under the same conditions as reported before. As reported in figure 2b, the activity of p53 induced by Aq and

Neo alone was at physiological level supported the beneficial effects observed above; moreover, in presence of H_2O_2 and HCl alone, p53 activity significantly increased compared to control, indicating a significant mitochondrial stress and a potential activation of the pathway leading to cell death. The ability to prevent and restore the damage caused by H_2O_2 and HCl of Aq ($p < 0.05$) was confirmed: indeed, no significant differences were observed between pre and post treatments. Contrary to what observed for Aq, Neo was able to prevent and restore the damage caused by H_2O_2 ($p < 0.05$), but a little activation was observed with HCl: this data confirms the ability of Neo to better counteract the negative effects of oxidative stress during hyperacidic conditions. All these results support the hypothesis that Aq and Neo act differently to prevent epithelial stomach cell death. Finally, since the greater effects of Aq were observed in pre-treatment conditions with HCl, in all successive experiments only these conditions were maintained.

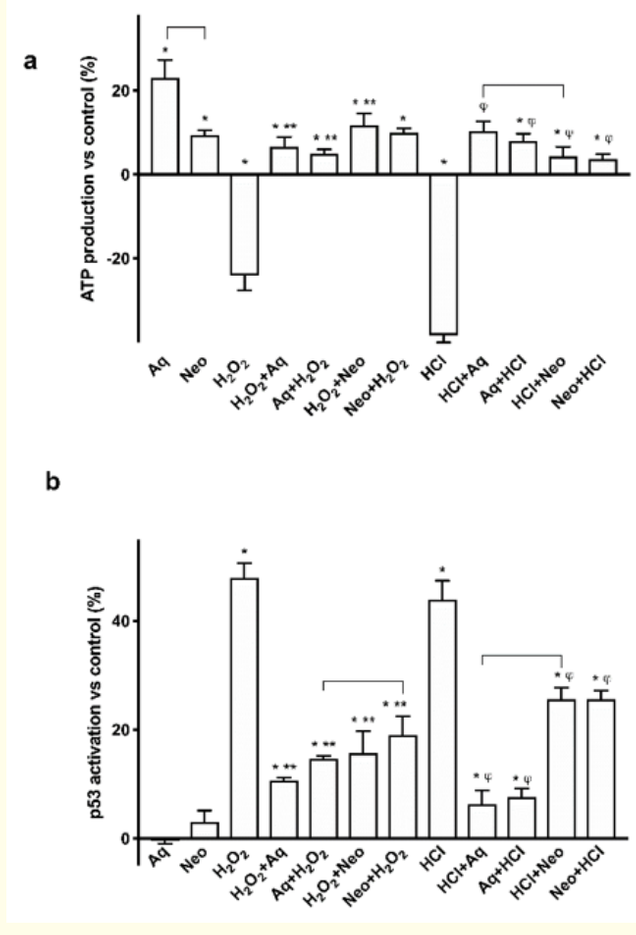

Figure 2: *Effects of Aq and Neo on apoptotic mechanism. In panel (a) ATP production and in panel (b) p53 activation in gastric epithelial cells treated with Aq and Neo for 24h during acidic condition or oxidative stress. The abbreviations are the same reported in figure 1. Data are expressed as means ± SD (%) of five independent experiments normalized to control values. *p < 0.05 vs control; **p < 0.05 vs H2O2; ϕp < 0.05 vs HCl; arrows indicate p < 0.05 between different groups*

Aq and Neo as gastroprotective agents

Since the chronic use of NSAID can cause gastric ulcers, gastroprotective agents are frequently used in combination with anti-acid treatments. However, these agents often do not remove the causes of hyperacidity or oxidative stress created by NSAID in the stomach. So today scientific research is trying to find new resources of natural origin, especially derived from plants, able to reduce gastric cells damage. In this context the effects of Aq and Neo were tested in presence of diclofenac; in particular Aq and Neo were added before and after the stimulation with diclofenac under hyperacidic conditions. As shown in figure 3a, in presence of diclofenac alone, cell viability

was reduced compared to control ($p < 0.05$), indicating a lower tolerability of the cells, and the pre-and post-stimulations with Aq and Neo were able to reduce this negative effect. In particular, the greatest effect was obtained using Aq compared to Neo (about 64%) when added before diclofenac. On the contrary, during the stimulation after diclofenac, the greatest effect was obtained with Neo compared to Aq (about 40%). These data suggest the possible ability of Aq and Neo to be used as gastroprotective agents under hyperacidic conditions in a different manner; in particular the best result was observed using Aq compared to Neo. This hypothesis was confirmed by comparing data obtained with Aq and Neo on cell viability with pantoprazole experiments (P), where similar effect of Aq and Neo preventing the loss of cell caused by diclofenac were observed. It is noteworthy that Aq and Neo added before diclofenac seemed to be more effective than P ($p < 0.05$), indicating Aq and Neo as a new possible therapeutic approach. Finally, all these results were confirmed under acidic conditions, in which Aq and Neo counteracted the negative effects of diclofenac better than P ($p < 0.05$). In particular, pre-treatment with HCl caused a significant reduction ($p < 0.05$ vs control) in cell viability induced by diclofenac and only Aq was able to prevent (pre-stimulation) and restore (post-stimulation) the damage caused by diclofenac under these conditions ($p < 0.05$ vs control and vs diclofenac), indicating a greater effectiveness of Aq than Neo as a gastro-protector agent. Since a consequence of hyperacidity is the production of oxidative stress, additional experiments were carried out to investigate the ability of Aq and Neo to reverse the damage acting on radical oxygen species (ROS) release. As reported in figure 3b, diclofenac induced a significant increase (about 30%) in ROS production compared to control, confirming its negative effect on cell proliferation. Pre-stimulation with Aq was able to significantly reduce the ROS release caused by diclofenac compared to the post-stimulation (about 70% vs post-treatment). Moreover, Neo seemed to have greater effects when added after diclofenac to reduce ROS production ($p < 0.05$, about 33% compared to pre-treatment). These data confirm a different ability of Aq and Neo to maintain a gastric protection. In addition, comparing the effects of Aq and Neo to P, in presence of diclofenac, the gastro-protection mechanism was confirmed: indeed, Aq and Neo had more effectiveness than P ($p < 0.05$). Similar data were observed under acidic conditions, in which diclofenac improved its negative effects and Aq and Neo counteract it ($p < 0.05$) exerting greater effects compared to P (0 < 0.05). As before, Aq had higher effects when added before diclofenac compared to post-stimulation ($p < 0.05$), supporting the idea that it can be used as a gastroprotective agent.

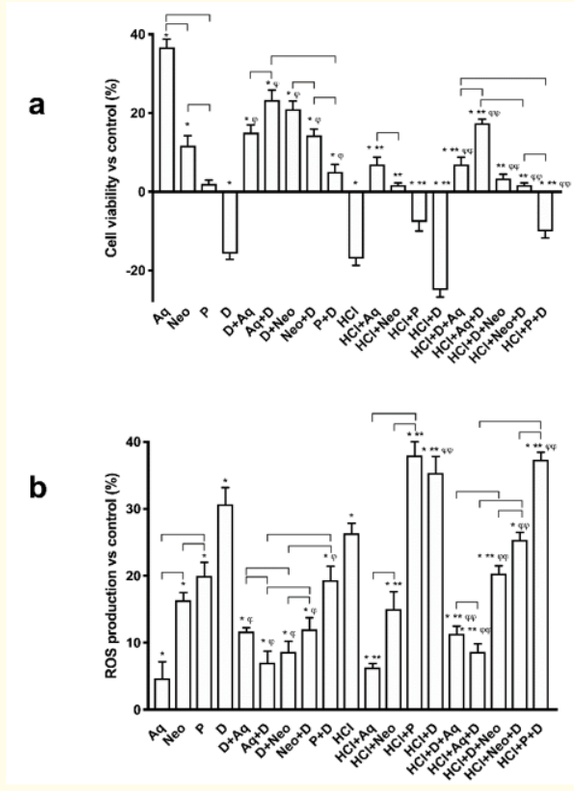

Figure 3: *Effects of Aq and Neo alone and combined with diclofenac during acidic and hyperacidic conditions. In panel (a) cell viability and in panel (b) ROS production observed in gastric epithelial cells treated for 24h. P= pantoprazole, D= diclofenac, the other abbreviations are the same reported in figure 1. Data are expressed as means ± SD (%) of five independent experiments normalized to control values. *p < 0.05 vs control; **p < 0.05 vs HCl; ϕp < 0.05 vs diclofenac; ϕϕp < 0.05 vs HCl+diclofenac; arrows indicate p < 0.05 between different groups.*

Analysis of intracellular pathways activated by Aq and Neo during gastro-protection

Since the most critical consequence for taking NSAID is hyperacidity, the latter has only been further investigated; in order to verify the gastroprotection induced by Aq and Neo, some additional experiments were performed to analyse annexin V, p53, Ki67, PI3K/Akt expressions and ERK/MAPK activity. As reported in figure 4a, annexin V, a marker of nuclear integrity, is important to exclude the activation of cell death pathway. Hyperacidic conditions confirmed their negative effects ($p < 0.05$ vs control) and Aq and Neo alone, significantly reduced the annexin V expression compared to HCl alone ($p < 0.05$). In addition, they were also able to counteract the expression of annexin V in presence of diclofenac: in particular Aq had a better result when added before diclofenac (about 25% vs post-stimulation) and Neo showed its best effect when added after diclofenac (about 23% vs pre-stimulation). In order to confirm the protection exerted by Aq and Neo and to exclude a damage leading to cell death, p53 expression was evaluated under the same conditions (see figure 4b). Also in this case results confirmed the negative effects on p53 expression of both hyperacidity and diclofenac, with an increase of about 36% and 32% respectively, compared to control. The positive mechanism activated by Aq on hyperacidic condition was able to reduce the activation of p53 caused by hyperacidity and diclofenac ($p < 0.05$): in particular the greater effect was observed when added before diclofenac (about 59% vs diclofenac). On the contrary, Neo was able to induce a beneficial effect when it was added after diclofenac (about 25% vs diclofenac), confirming data observed previously. Since an important factor for protection against damage, in addition to the maintenance of vitality, is the induction of cell proliferation, the Ki67 marker was also studied. The expression of Ki67 (see figure 4c) in presence of hyperacidic condition or diclofenac were significantly reduced compared to control ($p < 0.05$) and the pre- and post-stimulation with Aq reverted these conditions ($p < 0.05$); in particular a greater effect was observed when it was added before diclofenac (about 44% vs post-stimulation), supporting previous data about cell viability. Neo was also able to induce an increase in cell proliferation, but the best result was observed when it was administered after diclofenac (about 50% vs pre-stimulation), indicating a different protective mechanism compared to Aq.

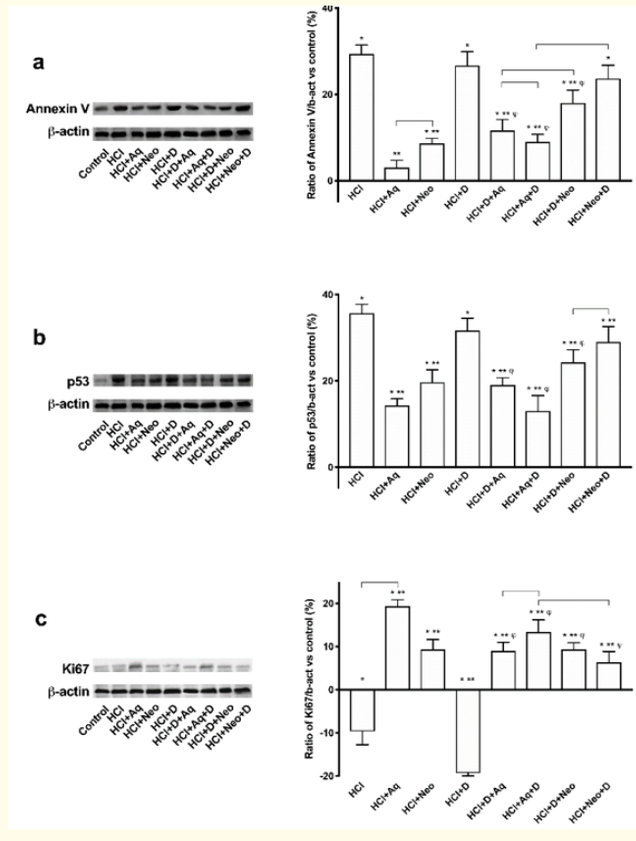

Figure 4: *Western blot and densitometric analysis of apoptotic and proliferation pathways during hyperacidic condition. In (a) Annexin V, in (b) p53 and in (c) Ki67 expressions analysed through Western blot (an example on the left) and densitometric analysis (on the right) of gastric epithelial cells treated for 24h. The abbreviations are the same used in figure 3. All results are expressed as means ± SD (%) normalized to control values of five independent experiments. *p < 0.05 vs control; **p < 0.05 vs HCl; ϕ p < 0.05 vs HCl+diclofenac; arrows indicate p < 0.05 between different groups.*

Furthermore, cell survival is an important parameter, useful to better understand the mechanism that supports gastroprotection. In this context ERK/MAPK and PI3K/Akt were also analysed by ELISA test on hyperacidic condition. As reported in figure 5, diclofenac alone induced a significant reduction on ERK and Akt activities, indicating a negative influence on cell survival ($p < 0.05$ vs control). The pre- and post-stimulations with Aq were able to counteract these negative effects: indeed, ERK ($p < 0.05$) and Akt activities ($p < 0.05$) were significantly increased compared to diclofenac, supporting previous data about the ability of Aq to act both before and after the damage. In addition, the major effects were observed when it was added before diclofenac (about 156% and 55% respectively, compared to post-stimulation) supporting the hypothesized mechanism of action. On the other hand, Neo exerted a beneficial effect on ERK and Akt marker, but a greater result was observed when it was added after diclofenac (about 18% and 100%, respectively, vs pre-stimulation). All these results are important to confirm the different potential gastroprotective activity of Aq and Neo.

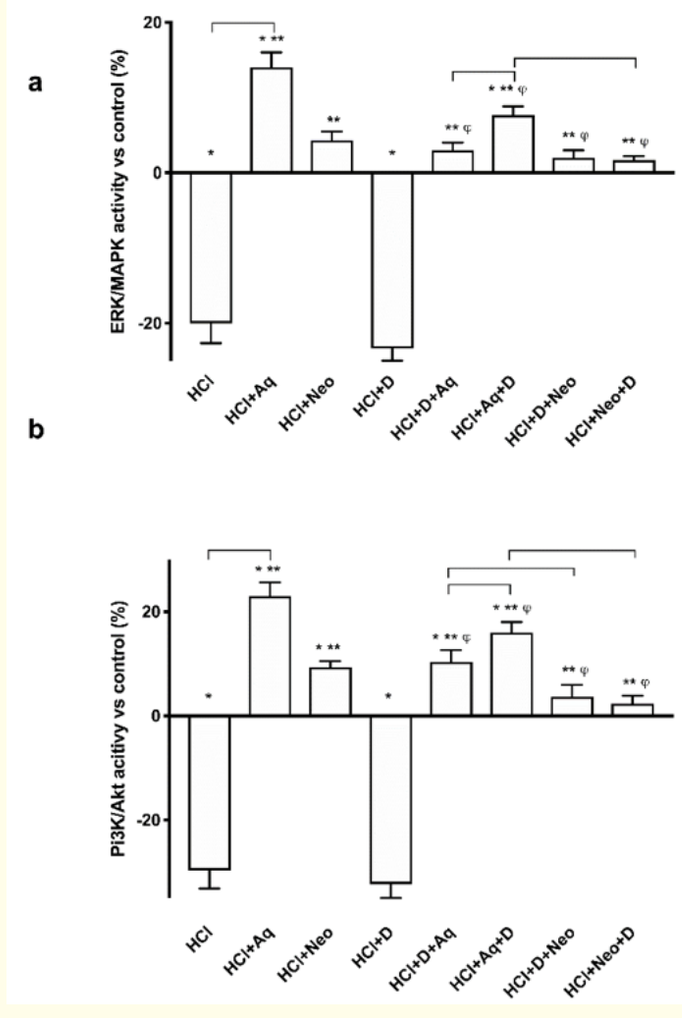

Figure 5: *Analysis of survival kinases activity on hyperacid condition. In panel (a) ERK/MAPK and in (b) PI3K/Akt activities measured by ELISA test in gastric epithelial cells treated for 24h. The abbreviations are the same used on figure 3. All the results are expressed as means ± SD (%) normalized to control values of five independent experiments. *p < 0.05 vs control; **p < 0.05 vs HCl; ϕ p < 0.05 vs HCl+diclofenac; arrows indicate p < 0.05 between different groups.*

Discussion

Recent clinical investigations reveal that NSAID, widely used to alleviate inflammation, fever, and pain in clinical practice, have an equal risk to develop gastropathic and enteropathic damage [5,35] and then the relative gastric toxicity is a major consideration [3,36]. NSAID-

induced gastroenteropathy has emerged as an important socioeconomic problem because there are no approved therapeutic strategies/interventions to prevent/treat NSAID-induced enteropathic damage [37]. Despite recent medical advances, the management of peptic ulcers and their complications remains a challenge, involving high morbidity and death rates for the disease [38]. NSAID can be divided into COX-2 selective inhibitors and non- selective COX inhibitors (COX1 and COX2 inhibitors) [39] which have a higher risk of ulcers in the GI tract [40]. The most important non-selective COX inhibitors include aspirin, diclofenac, indomethacin, piroxicam, naproxen, and ketoprofen [41]. Diclofenac is extensively used worldwide in several formulations due to its analgesic, anti-inflammatory and antipyretic activities [12]. The extensive usage of diclofenac has been further evident since in 2012, a significantly high amount of prescriptions in USA included this drug. Presently, it is the most commonly used over-the-counter NSAID [1]. Nevertheless, several side effects are consequences of their administration, especially with chronic use, including cardiovascular problems, renal function disorders, hepatic injuries, alterations in gastrointestinal mucosal integrity [8,42] and electrolyte disturbances [43,44]. A classical gastrointestinal consequence is a peptic ulcer which is a multifaceted process that includes the generation of ROS, inducing inflammatory molecules of the COX-2, cytosolic phospholipases A2, leukotriene B4, 5-lipoxygenase, PGE2, TNF-alfa, IL-1beta, and IL-6 production [45,46], increasing LPO, xanthine oxidase, and DNA damage [47,48] and the inhibition of antioxidant enzymes including glutathione peroxidase, glutathione reductase, glutathione S-transferase, catalase, superoxide dismutase, and heme oxygenase-1 as well as glutathione (GSH)/oxidized-GSH ratio in vivo [49]. The toxic effects of diclofenac on stomach mitochondria is well known [1]. Human gastric epithelial cells treated with diclofenac exhibited significant O2 production. The generated O_2 subsequently resulted in the production of several other reactive prooxidants. Ultimately these ROS collectively affected the viability of the cells by striking at the basis of cellular powerhouses or mitochondria via mitochondrial depolarization [1]. Therefore, ROS may also cause oxidative damage to biological macromolecules and react with proteins, lipids, and mitochondrial DNA, leading to cell death by affecting various apoptotic pathways in the gastrointestinal tract [47-49]. Furthermore, NSAID also down-regulates angiogenesis, inhibits mucosal cellular restitution, and promotes extracellular matrix degradation [50]. Thus, it is important to regulate the levels of ROS in the gastrointestinal tract. In addition, another important proinflammatory effect of HCl, consistent with the existing literature, is its action on synthesis [51]. PPI are currently used for the treatment and prevention of NSAID-induced gastroduodenal lesions [52]. The efficacy of PPI depends on their ability to inhibit gastric acid secretion [6]; however, in recent years, it has been proposed that acid-independent mechanisms may also contribute to the antiulcer actions of PPI [2]. Additionally, there are some reports that showing how PPI may produce gastric glands toxicity [53] and this is associated with an appreciable amount of systemic adverse effects in humans [54,55]. Thus, the identification of more effective and safer therapies for the treatment of NSAID-induced gastroenteropathic lesions remains an urgent priority. For this purpose, researchers are still investigating new molecules to find an ideal agent with a better safety profile. In the current study, the effects of a new compound named Aq on an *in vitro* experimental model of gastric cells have been observed. This work demonstrates that Aq can exert protective effects on gastric cells in terms of viability, ROS and NO production, thanks to vitamin D3, which is able to improve the beneficial effects induced by alginates, supporting existing data about their mechanism of gastroprotection [27,56].

Moreover, further *in vitro* studies demonstrate that VDR activation enhances intracellular junctions and promotes mucosal wound repair through vitamin D3 activity [57]. There is evidence that oxidative stress plays an important role in the pathogenesis of gastric injury [27]. It is noteworthy that experimental evidence shows how ROS production or superoxide ($O-_2$) are induced both by cellular exposure to an alkaline environment and/or by acidic injury [58]. It is well known that vitamin D3 may prevent cell death inhibiting superoxide anion generation, maintaining mitochondria function and cell viability, and activating survival kinases [31]. Our results show that Aq alone, during H_2O_2 or HCl exposure is able to improve cell viability compared to control and to other commercial products such as Neo, composed of different natural extracts. In addition, Aq significantly reduced ROS production and decreased cell viability loss when added after the injury, suggesting that cell damage and cytotoxicity can be prevented. These results suggest that Aq may exert a better gastroprotective effect through an antioxidant pathway, inhibiting apoptosis and activating survival kinases. Such effect was stronger in preventing epithelial damage than what observed using other gastroprotective agents such as Neo. Since the gastroprotection is more important during the use of diclofenac, further experiments were carried out to validate the observed results. The results obtained from this new series of experiments have allowed us to significantly confirm beneficial effects of Aq to prevent injury caused by diclofenac under both acidic and hyperacidic conditions. Indeed, the gastroprotection exerted by Aq was similar to effect of pantoprazole indicating its effectiveness on cell viability, on reducing ROS production and on apoptotic mechanism, increasing the activation of survival kinases and cell proliferation.

Conclusion

In conclusion, this work demonstrates for the first time that Aq has a beneficial effect on gastric epithelial cells under both acidic and hyperacidic conditions, joining the effects of a mechanical barrier with the modulation of intracellular pathways in order to maintain or restore the integrity of gastric epithelium. This mechanism is important to prevent the damage induced by NSAID, such as diclofenac. However, further *in vivo* or research will be necessary in order to assess, with histological and immunocytochemical methods, the efficacy of Aq on gastric mucosa treated with diclofenac.

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Conflict of Interest

The authors declare no competing financial interests.

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