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Research Paper

Oxidative stress, nitric oxide and prostaglandin E_2 levels in the gastrointestinal tract of aging rats

Frederic Mármol[a], Juan Sánchez[a], Diego López[b], Nuria Martínez[b], Maria Teresa Mitjavila[b] and Pere Puig-Parellada[a]

[a]Unitat de Farmacologia, Facultat de Medicina, IDIBAPS and [b]Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain

Abstract

Objectives To evaluate the presence of oxidative stress and alterations in the levels of two cytoprotective agents, prostaglandin E_2 and nitric oxide, in the gastrointestinal tract of aging rats.

Methods The production of superoxide anion, lipid peroxides, levels of superoxide dismutase and catalase, and production of prostaglandin E_2 and nitric oxide in the stomach and duodenum of rats were determined at 1.5, 3, 12, 18 and 24 months of age.

Key findings Oxidative stress was present in the stomach of the old rats (24 months), whereas prostaglandin E_2 and nitric oxide production remained stable at 18 and 24 months. In the duodenum, no oxidative stress was observed at 24 months, but at 18 months, an increase in superoxide anion levels was detected. Prostaglandin E_2 remained constant in the aged rats but nitric oxide decreased significantly at 24 months.

Conclusions The absence of macroscopic gastric injury throughout the gastrointestinal tract indicates that the oxidative stress in the stomach and the significant decrease of nitric oxide in the duodenum in the old rats are not sufficient to disrupt the mucosal defence network. The results support the notion that the disruption of the mucosal network is essentially regulated by the cytoprotective agents prostaglandin E_2 and nitric oxide, and that injury appears only when both substances are concurrently reduced.

Keywords aging; gastrointestinal tract; nitric oxide; oxidative stress; prostaglandin E_2

Introduction

The generation of reactive oxygen species (ROS) during oxidative metabolism in aerobic organisms has long been believed to play a role in aging. Indeed, the free radical theory of aging suggests that damage produced by the interaction of such free radicals with cellular macromolecules results in cellular senescence and aging.[1] One of the major free radical species produced during oxidative metabolism is the superoxide anion (O_2^-), formed when oxygen acts as the final electron acceptor of the electron transport chain. This O_2^- anion can act as both an electron donor and an electron acceptor, which makes it particularly reactive.

An antioxidant defence pathway removes these anions from the cellular environment. This two-step enzymatic pathway involves superoxide dismutase (SOD), which removes an electron from O_2^-, producing H_2O_2. The second step is the reduction of H_2O_2 to H_2O by either glutathione peroxidase or catalase.[2]

Lipid peroxidation reactions on the cell membrane may contribute to free radical mediated injury because polyunsaturated fatty acids in the cellular membrane are degraded by lipid peroxidation, with the consequent disruption of membrane integrity.[3]

The gastrointestinal mucosa also generates two products that protect against mucosal defence disruption: prostaglandin E_2 (PGE_2) and nitric oxide (NO). PGE_2 plays a role in the maintenance of mucosal integrity, stimulating virtually every component of the mucosal defence network.[4,5] It also inhibits the formation of gastric erosions and ulcers induced by a wide variety of experimental techniques, including pyloric ligation, the administration of non-steroidal anti-inflammatory drugs (NSAIDs) and stress.[4,6] Protection against damage by several prostanoids has been confirmed in humans by direct endoscopic observation of the gastric mucosa following challenge with NSAIDs.[7]

Endogenous NO is generated by the constitutive forms of nitric oxide synthase that produce small amounts of NO.[8–11] The NO generated is involved in the modulation of

Correspondence: P. Puig Parellada, Unitat de Farmacologia, Facultat de Medicina Casanova, 143, E-08036 Barcelona, Spain.
E-mail: ppuigp@ub.edu

mucosal blood flow under physiological and pathological conditions and has a key interactive role in the regulation of vascular integrity.[12–14] Support for this conclusion is found in experiments using low doses of nitrovasodilators that spontaneously release NO or L-arginine, which protect against mucosal injury.[15–19] However, high doses of these agents can lead to mucosal injury.[16,20] Significantly, NO accelerates the healing of chronic gastric ulcerations,[9,11,17] inhibits gastric acid secretion,[8,21] inhibits NADPH oxidase activity from neutrophils and hence O_2^- production, reduces leucocyte adhesion and is also an antioxidant.[5,22]

Most of the studies of the gastrointestinal tract focus on the injury induced by NSAIDs because of the clinical importance of this pathology in the aging process. Several research groups have proposed that ROS produced by various mechanisms contribute to NSAID-induced gastropathy, mainly by inducing the microvascular disturbances that precede gastric mucosal injury.[3,4,23–30] However, aging itself is associated with significant alterations in various gastric mucosal defence mechanisms and diminished responsiveness to injury.[31]

This study is original in focusing on the presence of oxidative stress and alterations in the levels of two cytoprotective agents in the aging process, which are necessary preconditions for the presence of macroscopic gastrointestinal injury.[14] We designed an experimental approach using rats at 1.5, 3, 12, 18 and 24 months of age, in which we evaluated the production of O_2^- and two antioxidant enzymes: SOD and catalase. We also studied malondialdehyde (MDA) levels as an indicator of lipid peroxidation, which is considered a parameter of oxidative stress. The production of two gastrointestinal cytoprotective agents, PGE_2 and NO, was also assayed. All these parameters were studied in stomach and duodenum. The rats did not undergo any manipulation as age was the only parameter to be considered.

Materials and Methods

Animals and sample preparation

Male Wistar rats (Harlan Interfauna Iberica, Barcelona, Spain), 1.5, 3, 12, 18 and 24 months old, were used. The animals had free access to a standard laboratory chow and water. Animals were fasted for 24 h before the experiments.

Experiments were conducted in accordance with the Guide for the Care of Laboratory Animals (EEC Council Directive 86/609/EEC). Approval of the Research and Development and Animal Care Committee of the University of Barcelona was obtained for all studies.

The rats were anaesthetised with sodium pentobarbital and killed by exsanguination. The stomach was cleaned with saline, extended on a board and 4-mm fragments were obtained from the mucosal part of the stomach with an appropriate punch, frozen in liquid nitrogen and kept at −80°C. The first part of the duodenum was cleaned with saline and a 5-cm piece was obtained from the area 2 cm below the pylorus. This piece was cut into 2-cm fragments, frozen in liquid nitrogen and kept at −80°C.

Superoxide anion generation

The technique developed by Pagano *et al*.[32] was used. Stomach or intestine samples from each rat were defrosted by immersion in a Tyrode solution (in mM: NaCl 137, KCl 3, $CaC1_2$ 1.36, $MgSO_4$ 1.2, NaH_2PO_4 0.5, $NaHCO_3$ 12, glucose 5.5) at 4°C. Each experiment was performed in duplicate. The fragments were incubated in 1 ml Tyrode solution for 30 min at 37°C with continuous air bubbling, rinsed with Tyrode solution and then placed in 1 ml of the same solution in a luminescence cuvette, containing a non-redox cycling concentration of lucigenine (5 μM). The cuvettes were then placed in a luminometer (Bio-Orbit 1251; Bio-Orbit, Turku, Finland) and maintained at 37°C. The luminometer was set to report arbitrary units of emitted light, and integrated over a 30-s interval for 3 min. A blank Tyrode solution gave low and constant readings that were comparable with those obtained from a blank lucigenine solution. The results are expressed as luminescence units/min per g tissue. We also assayed the possible differences in the luminescence response to fresh or frozen samples. The behaviour of both types of sample was similar (data not shown).

Lipid peroxidation

MDA as thiobarbituric acid reactive substances (TBARS) was evaluated in samples of stomach and intestine after homogenisation using a Kinematica Polytron homogeniser (Kinematica AG, Littau, Switzerland) with 120 mM KCl (1 ml/50 mg wet tissue) and 50 μl of 0.5% butylated hydroxytoluene. After centrifugation at 1000*g* for 10 min, 100 μl of supernatant was removed. Then, 100 μl trichloroacetic acid 40% (w/v), 400 μl of thiobarbituric acid 0.67% (w/v) in 0.05 M NaOH were added to the supernatant. The mixture was then heated in a water bath at 97°C for 10 min using a glass ball as a condenser. After cooling, 200 μl of glacial acetic acid and 400 μl of chloroform were added and shaken with a vortex. After centrifugation at 1700*g* for 30 min, the organic layer was used for spectrophotometric measurements at 530 nm. The results are expressed as ng TBARS/mg protein. A standard curve was made by preparing an MDA solution (10 mM) by hydrolysis of MDA tetraethylacetate with hydrochloric acid. Protein quantification was performed following the method of Lowry *et al*.[33] using serum albumin as standard.

Antioxidant enzyme assay

Stomach and intestine samples were homogenised in two 20-s steps at 37°C using a Kinematica Polytron homogeniser in 2 ml of phosphate buffer (50 mM, pH 7.0) containing 10 mM ethylenediaminetetraacetic acid, 0.13 mM 3,5-di-*tert*-4-butylhydroxytoluene, and 0.13 mM desferrioxamine (iron chelator), to minimise oxidation during the homogenisation procedure. The homogenates were centrifuged at 300*g* for 10 min at 4°C to remove cellular debris. The supernatant was then collected to analyse SOD and catalase antioxidant enzyme activity.

SOD activity was assayed by the inhibition of pyrogallol autooxidation.[34] Then, 1.2 ml of ethanol : chloroform (5 : 3 v/v) was added to 750 μl of supernatant. After centrifugation at 3400*g* for 5 min at 4°C, different volumes of the supernatant were added to 25 μl of Tris HCl. Finally, 20 μl of pyrogallol were added to start the reaction. Results are expressed as units/g tissue (1 unit induces an inhibition of 50% pyrogallol autooxidation).

Catalase activity was assayed by H_2O_2 consumption following the method of Aebi[35] as modified by Pieper *et al.*[36] Briefly, ethanol was added (1 : 100 v/v) to the supernatants and incubated for 30 min in an ice bath. Triton X-100 1% (1 : 10 v/v) was then added to the homogenates. This solution was placed for an additional 15 min in the ice bath. Then, 500 μl of this solution was introduced in a glass cuvette and maintained at 25°C, followed by the addition of 250 μl of 30 mM H_2O_2 in a phosphate buffer to start the reaction. After a 15-s interval, the absorbance at 240 nm was read every 15 s for 45 s. The first-order reaction rate (k) of H_2O_2 consumption by catalase was calculated and results are expressed as k/g tissue.

Prostaglandin E_2 assay

Stomach and intestine samples were incubated for 30 min at 37°C. After centrifugation, the supernatants were stored at –80°C until the day of the analysis. The production of PGE_2 was determined with a PGE_2 monoclonal antibody (Prostaglandin E_2 EIA kit; SPI BIO, Cayman Chemical European Platform, Montigny le Bretonneux, France) according to the manufacturer's protocol. The results are expressed as pg/mg tissue.

Nitric oxide assay

The detection of NO took place as described by Vanin.[37] Stomach and intestine samples were preincubated at 37°C for 20 min in Ringer solution (pH 7.4) and exposed to the spin trapping agents diethyldithiocarbamic acid (DETC; 5 nmol/l) and $FeSO_4.7H_2O$ (50 μmol/l) for 30 min. The samples were finally weighed, frozen in liquid nitrogen and stored at –80°C for posterior electron spin resonance (ESR) analysis. The ESR-detectable paramagnetic complex was evaluated in a Bruker 300E spectrometer (Bruker Instruments, Billerica, MA, US). The signal due to the complex corresponded to the difference in intensity between a maximum at 3440 [G] and a minimum at 3470 [G]. A standard curve of Fe-NO-DETC was generated by diethylamine NONOate (Cayman Chemical Co.) (10 nmol/l to 100 μmol/l). This curve was used to extrapolate the Fe-NO-DETC signal and also to estimate the linearity of the assay. Results are expressed as relative intensity units/mg tissue.

Gastrointestinal damage

The presence of macroscopic damage in the mucosal part of the stomach and in the fragment of the selected duodenum was evaluated with an appropriate image analysis program.

Statistical analysis

One-way analysis of variance was performed and Dunnett's post-test was used to compare all the groups versus the 3 month old group. Statistical analyses were performed using the InStat 3.0 program (GraphPad Softwere Inc., La Jolla, CA, US). The results are expressed as mean ± SE. Differences of $P < 0.05$ were considered significant. A linear correlation test (Pearson's test) was also performed.

Results

Stomach

One-way analysis of variance revealed a significant effect of age on O_2^- ($P < 0.001$) and PGE_2 ($P < 0.001$) production and on the levels of SOD ($P < 0.05$), catalase ($P < 0.01$) and TBARS ($P < 0.001$), but no significant influence was detected for NO. We selected 3 month old rats as the control group for statistical analysis.

Superoxide anion (O_2^-) was the first free radical generated in the univalent reduction of oxygen and showed a 30% increase in production at 24 months ($P < 0.01$) in comparison with the control group (Figure 1). SOD, the enzyme that eliminates O_2^-, showed a trend towards a bell-shaped distribution. SOD levels fell significantly (47% decrease, $P < 0.05$) at 24 months (Figure 2). Catalase presented a good negative correlation with age ($r = –0.997$, $P < 0.001$); comparing the results obtained at 24 months of age with the control group, catalase levels had fallen significantly (46% decrease, $P < 0.01$; Figure 3). Only in 24 month old rats were SOD and catalase levels significantly reduced in relation to the control group. As shown in Figure 4, lipid peroxidation (TBARS) increased significantly at 18 and 24 months (114 and 52%, respectively, compared with the control group, $P < 0.01$). In the next step, we studied the levels of NO from 3 to 24 months of age, observing a significant negative correlation ($r = –0.984$, $P < 0.05$) between NO concentrations and age, although the statistical analysis did not show any significant

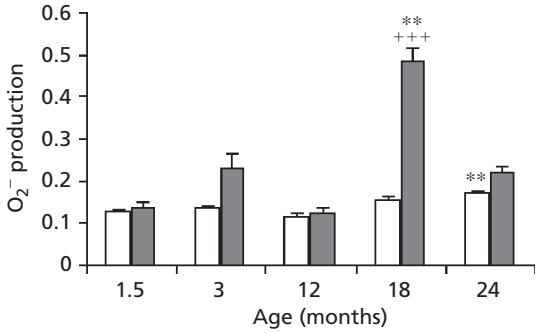

Figure 1 Effects of aging on superoxide anion production. Effects of aging (1.5–24 months) on superoxide anion (O_2^-) production in rat stomach (□) and duodenum (■). Results are expressed as luminescence units/min per g tissue. Values are mean ± SEM ($n = 6$ per group). $^{**}P < 0.01$, significant difference compared with the 3 months old group. $^{+++}P < 0.001$, significant difference between the 18 and 12 month old groups.

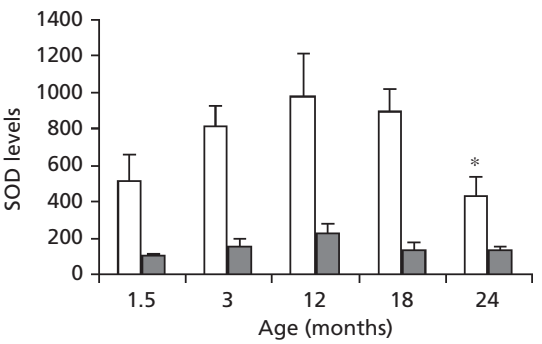

Figure 2 Effects of aging on superoxide dismutase levels. Effects of aging (1.5–24 months) on superoxide dismutase (SOD) levels in rat stomach (□) and duodenum (■). Results are expressed as units/g tissue. Values are mean ± SEM ($n = 6$ per group). $^{*}P < 0.05$, significant difference compared with the 3 months old group.

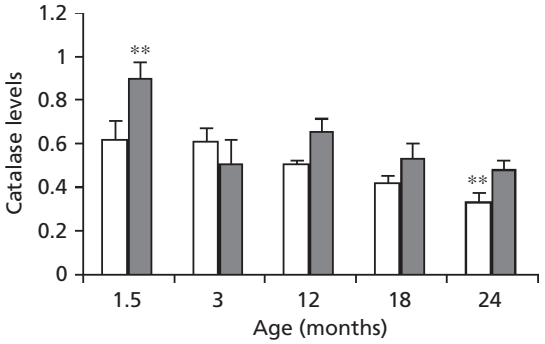

Figure 3 Effects of aging on catalase levels. Effects of aging (1.5–24 months) on catalase levels in rat stomach (□) and duodenum (■). Results are expressed as k/g tissue. Values are mean ± SEM (n = 6 per group). $^{**}P < 0.01$, significant difference compared with the 3 months old group.

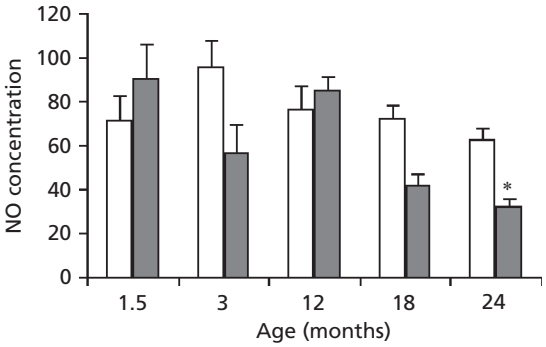

Figure 5 Effects of aging on nitric oxide concentration. Effects of aging (1.5–24 months) on nitric oxide (NO) concentrations in rat stomach (□) and duodenum (■). Results are expressed as relative intensity units/mg tissue (U/mg tissue). Values are shown as mean ± SEM (n = 6 per group). $^{*}P < 0.05$, significant difference compared with the 3 months old group.

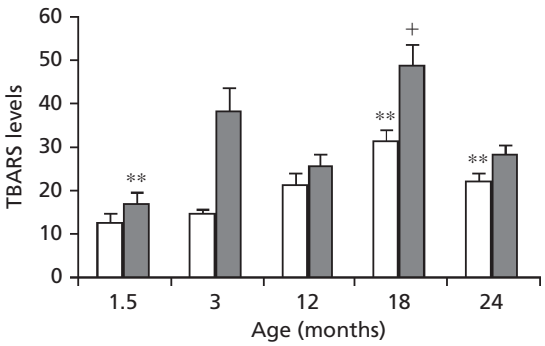

Figure 4 Effects of aging on levels of thiobarbituric acid reactive substances. Effects of aging (1.5–24 months) on levels of thiobarbituric acid reactive substances (TBARS) levels in rat stomach (□) and duodenum (■). Results are expressed as ng TBARS/mg protein. Values are mean ± SEM (n = 6 per group). $^{**}P < 0.01$, significant difference compared with the 3 months old group. $^{+}P < 0.05$, significant difference between the 18 and 12 month old groups.

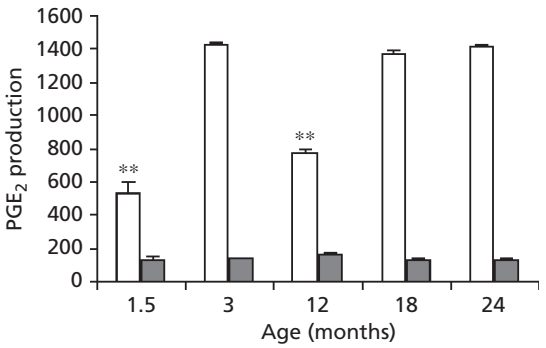

Figure 6 Effects of aging on prostaglandin E_2 production. Effects of aging (1.5–24 months) on prostaglandin E_2 (PGE_2) production in rat stomach (□) and duodenum (■). Results are expressed as pg/mg tissue. Values are mean ± SEM (n = 6 per group). $^{**}P < 0.01$, significant difference compared with the 3 months old group.

differences between the age groups and the control group (Figure 5). For PGE_2, an arachidonic acid metabolite, we observed a significant decrease in the production at 1.5 and 12 months of age (falls of 62% and 46%, respectively, $P < 0.01$; Figure 6).

Duodenum

In the duodenum, one-way analysis of variance revealed a significant effect of age on the production of O_2^- ($P < 0.001$), NO ($P < 0.001$), catalase ($P < 0.001$) and TBARS levels ($P < 0.001$), but no significant differences for SOD and PGE_2 were observed.

O_2^- generation increased significantly (110%) at 18 months compared with the control group ($P < 0.01$). The difference was even greater between 12 and 18 month old animals (295%, $P < 0.001$) (Figure 1). No alteration in the SOD levels was observed during the aging process (Figure 2). Catalase levels were significantly higher (77%, $P < 0.01$) at 1.5 months of age than in the control group (Figure 3); catalase showed a slight downward trend with aging, although the decrease did not reach statistical significance. Levels of lipid peroxides in the duodenum only decreased significantly in relation to the control group at 1.5 months of age (55%, $P < 0.01$; Figure 4); no other ages presented significant differences. Lipid peroxides also rose by 89% between 12 and 18 months of age ($P < 0.05$, Figure 4). The NO concentration fell significantly at 24 months of age (44%, $P < 0.05$). The other age groups did not show significant differences with respect to controls (Figure 5). No variations were observed in PGE_2 production in any of the age groups (Figure 6).

Significant differences between stomach and duodenum were observed in some of the parameters, particularly SOD and PGE_2. Duodenum SOD levels were 5-times lower than gastric SOD, and duodenum PGE_2 levels were on average 10-times lower.

As regards gastric damage, no macroscopic ulcers were observed in the stomach or the duodenum in any rats at any age.

Discussion

Oxidative stress was observed in the stomach of the oldest group of rats (24 months), as evidenced by the increase of O_2^- and lipid peroxides, together with a simultaneous decrease in the activity of enzymatic antioxidants SOD and

catalase. Similar results were reported in the gastric mucosa in which damage had been induced by chemicals (NSAIDs).[3,4,23–30] At 18 months of age, we observed an increase in lipid peroxides that was not related either to the levels of the enzymes assayed or to O_2^- production. In these circumstances, the increase in lipid peroxides may be a consequence of the ROS generated by the synthesis of eicosanoids, which are believed to be among the major sources of ROS in the aging process.[38,39]

Levels of lipid peroxides in the stomach at 24 months were lower than at 18 months. This may be because this group was more susceptible to oxidative stress than the older rats. Furthermore, immunological parameters in very old mice are reported to be more similar to those in adult mice rather than old mice. This is explained in terms of better adaptation in the modulation of the immune response in the very old animals.[40] Similar results have been reported in human centenarians,[41] in rat aorta,[42] and in rat brain.[43] Ehrenbrink *et al.*[43] explain these effects in terms of hormonal alterations induced by andropause.

In the duodenum, no oxidative stress was observed at 24 months of age. At 18 months, the increase in O_2^- production was independent of the levels of SOD and catalase and was related to the increase in lipid peroxides. This conclusion emerges when we compare the values at 12 and 18 months of age for superoxide and lipid peroxides. The low levels of lipid peroxides in the duodenum at 1.5 months could be attributed to the increase observed in catalase levels at this age.

Our results corroborate those of several studies that found that SOD levels and lipid peroxidation in the rat duodenum are not modified in the aging process.[44,45] In contrast, other authors, using mice, reported that both parameters increased.[2] The use of different animal species may explain these contradictory results.

NO production decreased significantly at 24 months of age in the duodenum. This reduction in NO generating capacity may be due to changes in the NO biosynthesis pathway, such as the reduced abundance of NO biosynthetic enzymes.[46] In this context, the contribution of NO to the electrical field stimulation induced non-adrenergic non-cholinergic relaxation in various intestinal regions of rats at different ages indicates that NO mediates this effect in young animals but its action gradually decreases with age.[47]

We observed stable levels of PGE_2 production in the stomach at 3, 18 and 24 months of age, consistent with the cytoprotective effects of this eicosanoid.[6] In human gastric mucosa, no significant differences in prostaglandin levels were observed in subjects under 70 years old.[48] In the gastrointestinal tract of rats, Uchida *et al.* [49] observed that PGI_2 levels were altered only in age periods of exacerbation, but remained constant between such periods. These findings, together with environmental conditions and genetic factors of the rat strain, could help explain the maintenance of PGE_2 levels in aged rats. The levels in PGE_2 production observed at 1.5 and 12 months of age are difficult to explain. The levels at 1.5 months may be due to the immaturity of this group of rats, whereas the decrease in the PGE_2 production observed at 12 months of age corroborates the results obtained in the mouse immune system (macrophages).[50] PGE_2 levels in the duodenum remain constant throughout the aging process and are one order of magnitude lower than in the stomach. This indicates that the cytoprotective activity is greater in the stomach than in the duodenum, possibly due to the inhibition of gastric acid secretion in the stomach.[6,8]

Conclusions

The absence of macroscopic gastric injury throughout the gastrointestinal tract indicates that the oxidative stress in the stomach and the significant decrease of NO in the duodenum observed in the old rats are not sufficient to disrupt the mucosal defence network. The results support the notion that the disruption of the mucosal network is essentially regulated by the cytoprotective agents PGE_2 and NO, and that injury appears only when both substances are concurrently reduced.[14,51,52] Our findings corroborate the contribution of prostaglandins in the regulation of mucosal integrity in the gastrointestinal tract, which is particularly important under conditions in which the synthesis or release of NO is reduced, as observed in the duodenum of the oldest group of rats.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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