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**POINT OF VIEW**

# *H. pylori* and mitochondrial changes in epithelial cells. The role of oxidative stress

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## ABSTRACT

Infection with *H. pylori* plays a role in the pathogenesis of gastritis, peptic ulcer, gastric carcinoma, and gastric lymphoma, but mechanisms leading to the various clinical manifestations remain obscure and are the primary focus of research in this field.

Proliferation and apoptosis are essential in the maintenance of gastric tissue homeostasis, and changes seen in their balance may condition gastric mucosal changes during infection. Thus, excessive apoptosis or proliferation inhibition will result in cell mass loss, which is observed in gastric ulcers. On the other hand, accelerated epithelial cell turnover is characteristic of carcinogenic mucosas.

There is also scientific evidence that demonstrates an association between *H. pylori* infection and exacerbated synthesis of free radicals, the latter being well known as a primary cause of cell death.

A thorough review of the literature and the results of our experimental research lead to conclude that *H. pylori*-induced oxidative stress activates the intrinsic pathway of apoptosis. Structural and functional changes caused by this process on mitochondrial organelles lie at the origin of gastric mucosal toxicity, and lead to the development of the various manifestations associated with this infection. Based on these data we suggest that therapy with antioxidants should prove beneficial for the clinical management of patients with *H. pylori* infection.

***Key words:*** *Helicobacter pylori.* Oxidative stress. Apoptosis. Mitochondria.

## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a gram-negative, extracellular, microaerophilic bacterium that selectively colonizes the human stomach with a prevalence of up to 90% in developing populations, and is the second most common pathogen for human beings. In all infectes subjects this bacterium induces chronic gastritis of varying severity, which in around 10-15% of cases progresses to peptic ulcer (infection is associated with 90% of gastric and duodenal peptic diseases), and in 1-2% of subjects ultimately results in MALT lymphoma or gastric adenocarcinoma (1-3) (Fig. 1). Despite this being the most common origin of peptic ulcer and gastric adenocarcinoma, the latter two conditions have different clinical courses and rarely develop concomitantly in the same mucosa.

The mechanisms through which *H. pylori* damages the gastric mucosa, and which determine the various clinical presentations, are not well understood; whether such mechanisms are exclusively dependant on the characteristics of the organism, the host, or both has not been established. In fact, while obvious toxicity differences are seen between strains (4,5) and 70% of peptic ulcers exhibit cagA+ organisms (6,7), the percentage of ulcers is lower than estimated according to the high prevalence of this bacterial genotype (60-80%, depending on populations) (8); on the other hand, despite the high percentage of infected subjects among those who develop gastric carcinoma, a direct mutagenic effect has not been established for these bacteria as yet.

According to theory by Correa et al. (9) three types of bacterium-host interaction exist that may explain gastric mucosal changes:

—*The bacterium alters the mucus layer and thus decreases mucin secretion:* this renders mucus ineffective

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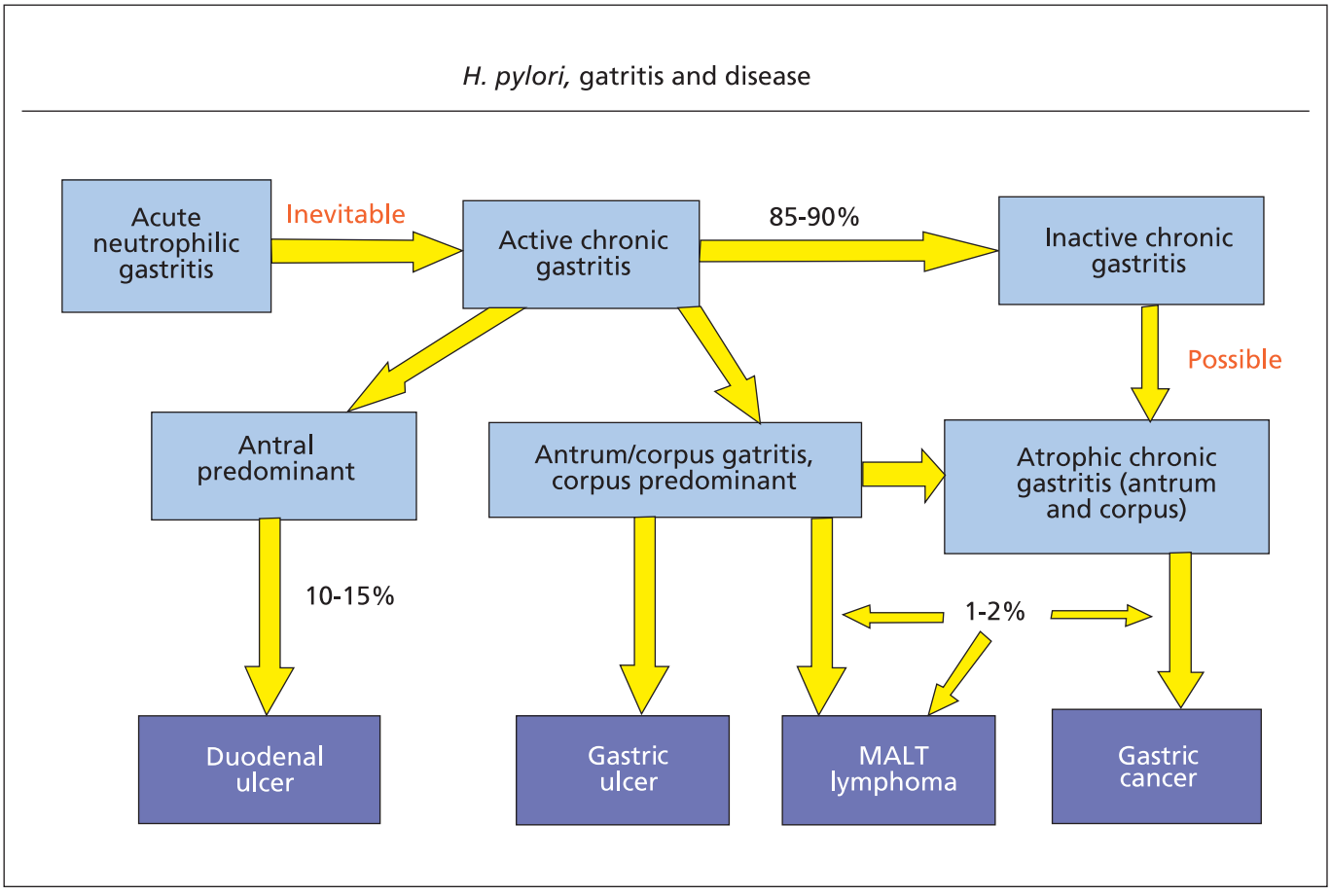


Fig. 1. *H. pylori and disease.* During infection with *H. pylori* a number of inflammation patterns are associated with different disease stages.

as a barrier defending the gastric epithelium from acid secretions and luminal toxins. This varies from one subject to the next both according to the infecting strain (cagA+ strains are more toxic) and host characteristics (blood group 0, non-secretor Lewis antigen, is associated with higher susceptibility to infection and increased risk for gastro-duodenal disease) (10).

—*The bacterium causes a migration of inflammatory cells to the gastric mucosa:* in some patients this results in nonatrophic, predominantly antral, gastritis associated with the development of duodenal ulcer (11) with no risk for progression to carcinoma (12). However, in other patients this associated gastritis does result in glandular loss, which triggers multifocal atrophic gastritis and intestinal metaplasia (13); these patients exhibit a high risk of progression to gastric carcinoma (14). Determinants and mechanisms involved in the occurrence of such divergent pathways are unknown and represent the primary focus of research.

—*H. pylori alters cell turnover rates:* these bacteria stimulate both cell proliferation (15) and apoptosis (16) in gastric epithelial cells. These changes seem to play a key role in the outcome of infection, including the risk for gastric carcinoma (17,18).

## *H. PYLORI* AND CELL TURNOVER

Proliferation and apoptosis are essential for turnover in the gastric tissue (19) and rate changes in the latter process seem associated with carcinoma development predisposition, and are considered a risk marker for progression (20).

In the mucosa of infected subjects with gastritis (17,21) or adenocarcinoma (18) increased epithelial proliferation has been found, and while accelerated proliferation is not carcinogenic in itself, highly proliferating cells are more sensitive to mutagenic factors (22), which may represent an association between infection and carcinoma (23).

On the other hand, there is also evidence relating infection to increased apoptosis (24-26). In the non-infected mucosa apoptotic cells are rare (fewer than 3%) and are situated in superficial areas in gastric glands (17). These cells are found in higher numbers deep in gastric glands in infected tissues, and significantly decrease after eradication.

The apparent conflict entailed by concomitant increases in proliferation and apoptosis may be interpreted from two distinct viewpoints (3):

—*The bacterium induces epithelial cell death,* which activates cell proliferation as a mechanism to compensate for cell loss, or

—*The bacterium changes the growth rate and causes hyperproliferation,* and apoptotic mechanisms are activated to compensate for increased cells.

Evidence is not enough to resolve this circular argument, but the potential fact that *H. pylori* would trigger a change cascade leading to cell self-elimination is most widely endorsed. In this respect, *in vitro* research has irrefutably proven that infection suffices to significantly increase apoptosis (27-29), hence this is likely the underlying cause of proliferation, thus aimed at restoring homeostatic balance (17).

However, infection chronification with alternating hyperproliferation and apoptosis stages represents a risk factor for balance disruption, either as a result of sustained bacterium-inherent toxicity or because of some cause triggering an exacerbated immune response that ultimately damages epithelial tissues. Thus, excessive apoptosis or proliferation inhibition will result in cell loss, which is observed in gastric ulcers (17). When disbalance occurs towards accelerated epithelial cellular turnover eventual failures in repair mechanisms are propitiated; the mucosa may become infiltrated by fibrotic tissue and/or epithelial replacement by little-differentiated cells may ensue. In fact, glandular tissue atrophy with or without fibrosis is commonplace in association with chronic infection. On the other hand, chronic inflammation and sustained stimulation of epithelial turnover are factors that contribute to the development of intestinal metaplasia. Regulatory mechanisms in these processes are poorly defined, but their significance is highlighted by the association of these morphological changes with sequential progression to gastric carcinoma (9).

In summary, bacterial persistence within the mucosa (either through direct action or because of a subsequent inflammatory response) sequentially induces damage-repair processes that may significantly alter the epithelium (Fig. 2).

## *H. PYLORI* AND APOPTOSIS

Programmed cell death or apoptosis is an evolutionarily preserved mechanism that uses a complex system for cell self-elimination. It plays a key role in multiple physiological processes (30): immune system and central nervous system functional organization, morphogenetic changes during embryonary development, tissue homeostasis, and clearance of superfluous, ectopic, aging, damaged, mutated, and infected cells (31). In addition, apoptotic processes are involved in the pathogenesis of various disorders including cancer, immune changes, cardiovascular disease, and degenerative conditions (32). Some of these changes are associated with insufficient apoptosis (lymphoma, cancer) whereas others are accompanied by excessive apoptosis (AIDS, amyotrophic lateral sclerosis, ischemia-reperfusion lesions) (33).

This type of cell death is structurally unlike necrosis, and was first described based on characteristic morphology changes. Apoptosis-inducing stimuli may be physiological (lack of growth factors, hormonal environment changes, etc.) or stress-related (UV light, radiation, viral or bacterial infection, etc.), and activate different pathways according to involved molecules and/or organelles, and the morphological and functional changes they induce in cells. Most physiological stimuli initiate apoptosis by activating surface receptors and the so-called extrinsic pathway. In contrast, the origin of stress-induced apoptosis is poorly understood, but mitochondrial in-

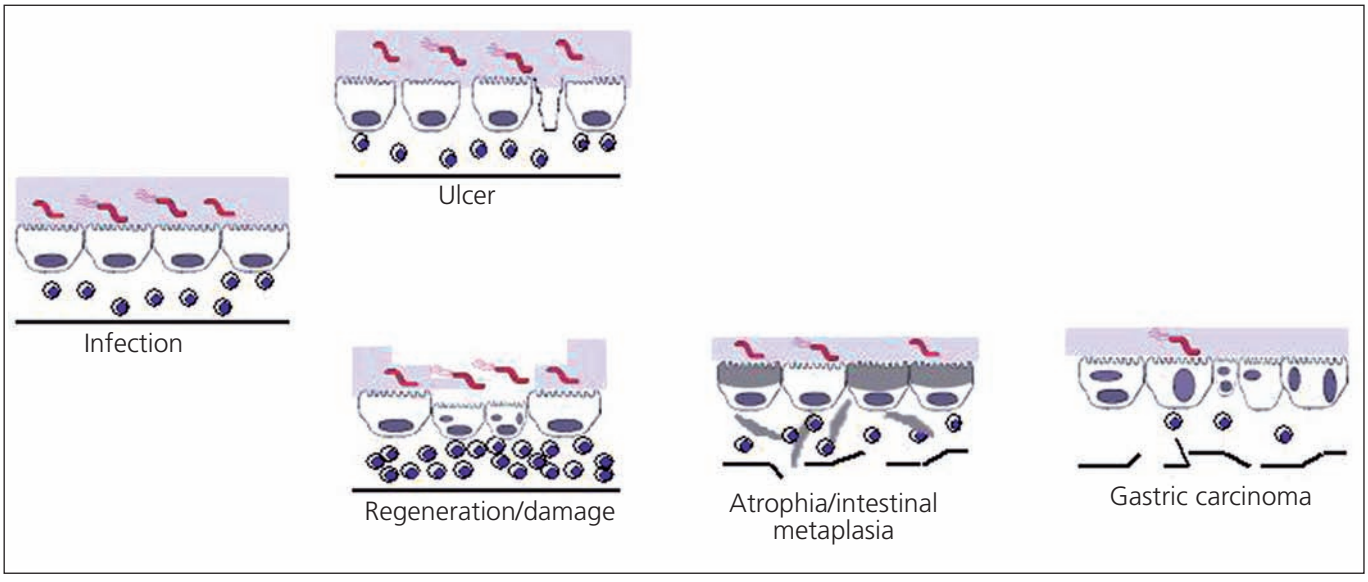


Fig. 2. *Mucosal changes following infection with H. pylori.* Long-term infection with *H. pylori* may induce ulceration or repeat "cell damage-repair" cycles, which gradually lead to gastric atrophy, intestinal metaplasia, and ultimately adenocarcinoma.

volvement early in the intracellular signaling cascade making up the intrinsic pathway is now established (35).

## Apoptosis mechanisms

—*"Extrinsic or death receptor pathway":* following an activation of "death receptors" (molecules in the TNF receptor superfamily, including TNF-R1, CD95 (Fas) and TRAIL-R1 and -R2), caspase-8 is activated after being recruited into the DISC complex through FADD *(Fas-associated death domain)* (36) (Fig. 3). The cascade then activates caspase-3 and eventually induces apoptosis (37).

A number of reported studies implicate death receptors in *H. pylori*-induced apoptosis (16,38,39), as strains or coculture supernatants induce Fas/FasL overexpression in epithelial cells. However, whether these receptors represent the primary apoptosis route as induced by these bacteria remains unknown.

—*"Intrinsic or mitochondrial pathway":* various signals may converge at the mitochondrial level to induce a translocation of mitochondrial cytochrome *c* (cyt *c*) into the cytosol. The binding of cyt *c* to the Apaf-1 *(Apoptosis protease-activating factor-1)* complex recruits procaspase-9, which following hydrolization to caspase-9 activates procaspase-3 (40) (Fig. 3). Proteins in the Bcl-2 *(B-cell leukemia/lymphoma 2)* family represent the primary regulators in this pathway: antiapoptotic members (Bcl-2, Bcl-$X_L$, Bcl-W, Bfl-1 and Mcl-1) act as inhibitors while proapoptotic members (Bax, Bak, Bad, Bcl-$X_S$, Bid, Bik, Bim and Hrk) serve as promoters (41,42) by blocking or enhancing, respectively, the release of cyt *c* into the cytosol.

While the origin and evolution of the extrinsic and intrinsic pathways differ, both apoptotic cascades converge at caspase-3; after caspasa's activation, the changes experienced by cells are identical regardless of the initial pathway (Fig. 3).

Therefore, following exposure to stress-related stimuli, cells enter a highly regulated, controlled process leading to self-elimination. In this process biochemical and morphological changes occur, that involve all cellular compartments and include cellular and nuclear contraction, translocation of phosphatidilserine on the outer leaflet of the lipid bilayer, caspase activation, membrane "blebbing", chromatine condensation, apoptotic bodies formation, DNA fragmentation (43), and specifically when apoptosis results from the intrinsic pathway, various mitochondrial changes (Fig. 3).

Mitochondrial changes during infection with *H. pylori* have been scarcely researched even when some intrinsic pathway components have been known to be activated by

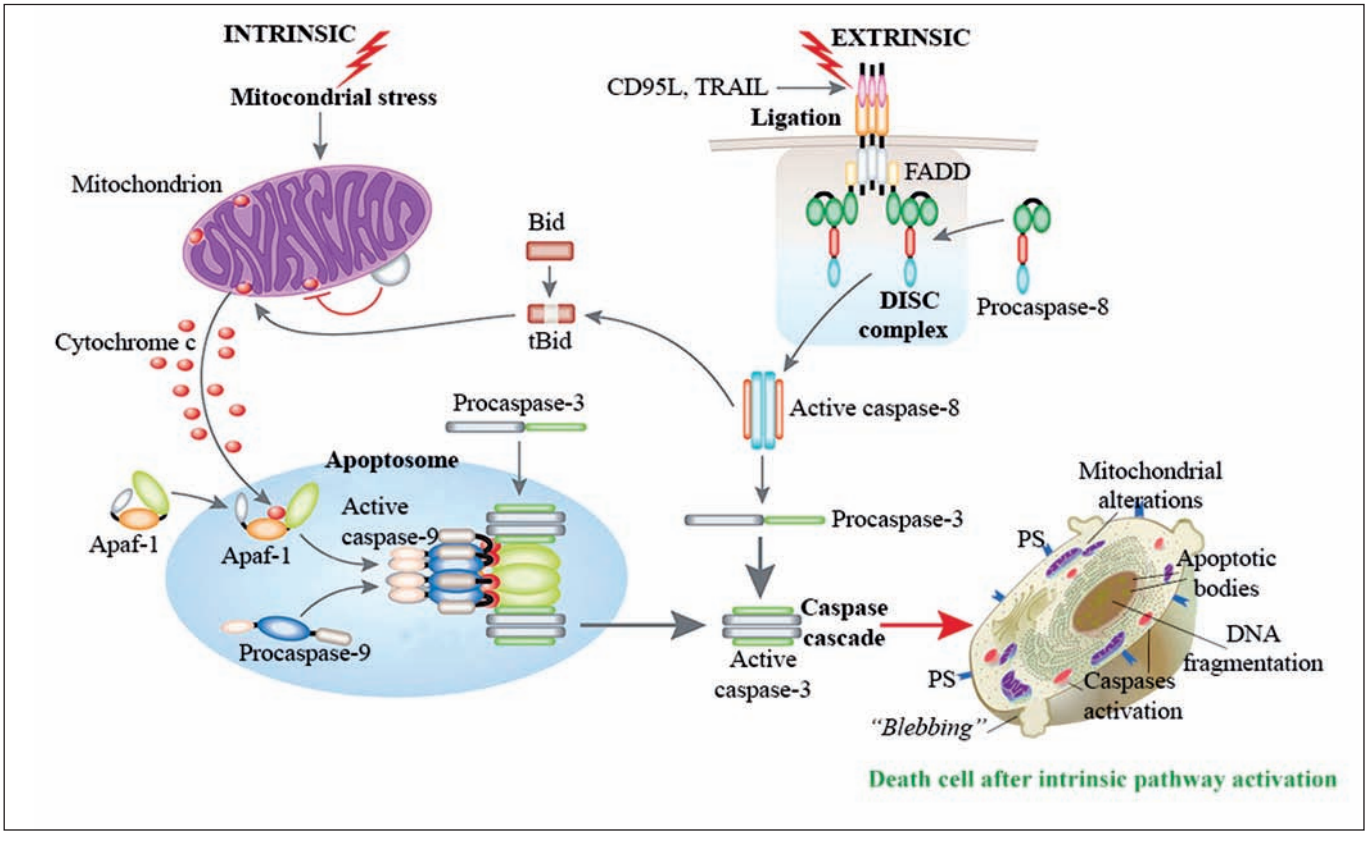


Fig. 3. *Apoptotic pathways:* the extrinsic pathway involves so-called death receptors (CD95, TRAIL); the intrinsic one involves mitochondrial granules. Both pathways converge at caspase-3 activation, where classic biochemical and morphological changes in association with the apoptotic phenotype are originated.

this bacterium for years. Thus, some authors point out an association with Bcl-2 family members (44-46), and the organism has been shown to increase the expression of proapoptotic protein Bax in the gastric mucosa. Zhang et al. (47) demonstrated in a gastric adenocarcinoma cell line cultivated with *H. pylori* that, in parallel with apoptosis induction, there was an increase in bax, bid and bcl-2 expression, as well as caspase 3 and 9 activation; they established a relationship between both facts by using caspase inhibitors, which decreased apoptosis. The involvement of the Bcl-2 family suggests that the mitochondrial pathway also plays a role in apoptosis as induced by *H. pylori*. Along this same line Kim et al. recently reported data pointing to bacterial protein gamma-glutamyltranspeptidase as an activator of this pathway (48).

## *H. PYLORI* AND APOPTOSIS-RELATED MITOCHONDRIAL CHANGES

While stimuli capable of triggering cell death through apoptosis are many, the changes they induce all converge at mitochondria. The role of this organelle as an apoptosis regulator (49) specifically consists of proteins release from the intermembrane space into the cytosol, which requires a number of functionality-conditioning morphological changes.

The most relevant mitochondrial function in eukaryotic cells is energy production in the form of ATP molecules from oxygen and metabolic by products derived from beta-fatty acid oxidation, urea cycle, and respiratory chain (50). Their structure and compartmentalization are highly related to a perfect performance of these functions. Every mitochondrion has a double lipid envelope (Fig. 4A) delimiting the matrix, located within the inner mitochondrial membrane (IMM), and the intermembrane space, located between the IMM and the outer mitochondrial membrane (OMM). IMM cristae or invaginations increase the area where specific mitochondrial processes develop (electron transport and oxidative phosphorylation) (Fig. 4B). The efficacy of these processes greatly depends on the bilayer's appropriate composition and structure, which relies on the role of the phospholipid cardiolipin. CL is a specific component of IMM and the most abundant at that; protein complexes (complexes I-IV) in the respiratory chain that carry $e^-$ from NAD(P)H and $FADH_2$ molecules to oxygen molecules to result in reduction to $H_2O$ (Fig. 4B) are anchored on CL.

In addition to these complexes other proteins are embedded on the IMM, with cyt *c* being most significant amongst them. For years, e-transportation from complex III to complex IV in the respiratory chain was deemed to be their only function, until 1996 that Wang et al. (51) revealed their role in apoptotic processes after the addition of dATP to normal-growth cells to induce apoptosis, their presence was detected in the cytosol. Various data have since corroborated their rapid release into the cytosol (52,53) following some apoptotic stimuli.

Particularly relevant is the electrostatic bond between CL and cyt *c* in the IMM (54) (Fig. 4B) as this implies the phospholipid is involved in processes where cyt *c* translocation to the cytosol is unnecessary (51). The oxidation of CL by free radicals (55) has been shown to alter such bond, to substantially modify IMM structure, and eventually to induce malfunctioning in the electron transport chain, which translates into decreased transmembrane potential.

Once in the intermembrane space cyt *c* must cross the OMM to enter the cytosol. Petit et al. (56) explain this

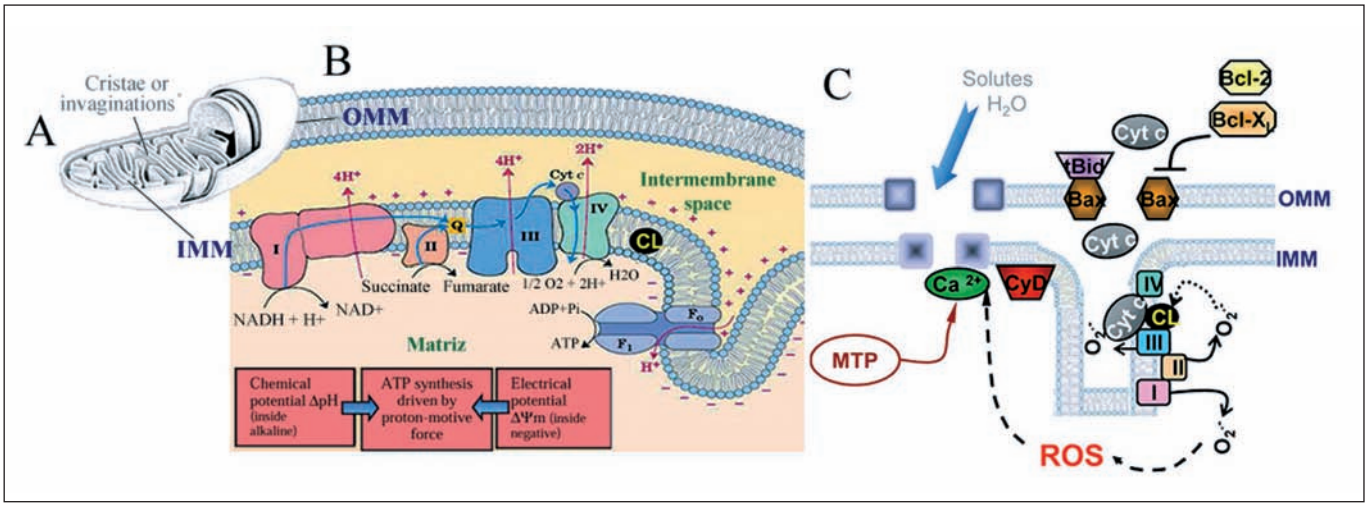


Fig. 4. *Mitochondria: structure and function.* A. Mitochondrial structure. B. Mitochondrial membranes: relationship between electron transport chain complexes, cytochrome c, and the phospholipid cardiolipin where they are inserted. C. Mitochondrial transition pore formation: ruptured links between protein complexes and cardiolipin, and passage of cytochrome c into the cytosol (78). OMM: external mitochondrial membrane; IMM: inner mitochondrial membrane; CL: cardiolipin; cit c: cytochrome c; MTP: mitochondrial transition pores.

stage by means of mitochondrial transition pores (MTPs) (Fig. 4C). These pore result from the opening of a conductance channel in the IMM because of electrochemical gradient dissipation and osmotic swelling arising from high salt contents in the matrix, which ultimately breaks the OMM. A second model supports the presence of a specific channel (57,58) in which Bax, Bid and CL play a role. Bax is present in free monomeric form in the cytosol or weakly bound to the OMM until its binding of Bid, which determines a complete insertion into the membrane where at least 4 tetrameres result, which causes permeabilization. Bid action on Bax is regulated by the rest of molecules in the Bcl-2 family: antiapoptotic molecules Bcl-2 and Bcl-$X_L$ inhibit this whereas proapoptotic Bad and Bik inhibit the antiapoptotic molecules function. CL is therefore insufficient but necessary for OMM permeabilization. Another possibility, which is consistent with the above, is that Bax when inserted in the mitochondrion physically and functionally interacts with some protein in the MTP complex, this being the ultimate cause of permeabilization.

Whatever the route taken by cyt *c* to translocate into the cytosol, a caspase activation cascade is initiated that represents the point of no return in the pathway to cell elimination.

## *H. PYLORI* AND ROS

There is scientific evidence that reactive oxygen species (ROS) play a relevant role in the pathogenesis of inflammation in the gastroduodenal mucosa, peptic ulcer disease, and – likely – gastric cancer (59). While an association has also been established between *H. pylori* infection and exacerbated free radical synthesis (60,61), many clinical data suggest that other factors inherent to host conditions (stress, diet, tobacco, hygiene, genetics...) contribute to the pathogenesis of this infection (62). It should be noted that some of these factors, including ingested food and tobacco smoke (63), directly influence mucosal oxidative status, as they expose the gastric epithelium to the ROS they generate within the gastric lumen in a sustained manner.

Besides these ROS other potential sources associated with infection include:

1. Inflammatory cells (neutrophils, macrophages) infiltrating the mucosa (64).
2. Gastric epithelial cells (61).
3. The bacterium itself, which generates a great amount of superoxide anion ($O_2^-$) to inhibit the bactericidal effects of nitric oxide as synthesized by inflammatory cells (65).

Zhang et al. (66) demonstrated that increased ROS in the mucosa of subjects with *H. pylori*-associated gastritis can be correlated with bacterial load.

From a physiological standpoint cells protect themselves from oxidative stress by activating antioxidant defense mechanisms involving oxygen scavenger enzymes such as superoxide dismutase, catalase and glutathione peroxidase (67), as well as vitamins (Vit) E and C.

The mechanisms by which *H. pylori* products (and the ensuing inflammation) affect the ability of gastric cells to protect themselves from ROS are unknown (63). *H. pylori* is known to release catalase and superoxide dismutase but in amounts likely insufficient to clear excess extracellular oxidants (67), as these enzymes primarily play a role in the elimination of ROS generated by the bacterium itself. Furthermore, in subjects with predominantly antral infection Vit E levels are known to decrease in the gastric body, which may reflect a mobilization of antioxidant defense mechanisms to maximally inflamed sites (68). On the other hand, infected subjects have significantly lower Vit E and Vit C levels (69).

In short, antioxidant systems seem also involved in infection to counteract increased ROS (61), hence their impairment or deficiency would notably reduce the ability of cells to tolerate an environment rich in free radicals. A study in rats with aspirin-induced gastric lesions showed that Vit E deficiency facilitates peptic ulcer, and that Vit E supplementation has protective actions, possibly because of its capacity to limit lipid peroxidation brought about by acetylsalicylic acid (70). Another study in Mongolian gerbils concludes that Vit C or Vit E supplementation protects from *H. pylori*-induced gastritis in the short term, its effects seemingly declining during persistent infection (71). Experiments in rats with other antioxidants, including a sunflower oil compound (72) and a flavonoid derivative (73), have shown an absence of gastric mucosal damage following exposure to indomethacin or ethanol, respectively.

Thus, when excessive ROS generated by bacteria on the epithelium is augmented by reduce antioxidant defense effectiveness, the risk of toxicity from oxidation and DNA damage is potentially increased (63). An impaired "oxidant-antioxidant" balance may then result in cell death, which would alter cell proliferation rate and/or facilitate the development of mutations leading to increased oncogene expression, hence the association between infection and gastric cancer (74).

## *H. PYLORI,* APOPTOSIS, INTRINSIC PATHWAY, ROS, AND ANTIOXIDANTS

As seen above, the intrinsic pathway has been recently involved in the development of apoptosis in the *H. pylori*-infected gastric mucosa (47). The relationship between apoptosis during infection and increased oxidative stress is also well known (75,76), but no data associate bacteria with free radicals and apoptosis through the mitochondrial pathway.

Our team has studied bacteria-induced changes in mitochondria (27) using the AGS gastric epithelial cell line to demonstrate that bacteria activate intrinsic pathway apoptosis. Furthermore, these same experiments performed in cells previously supplemented with Vit E show the indisputable involvement of ROS in these changes. Our results allow to develop a hypothesis suggesting the mechanisms for these changes (Fig. 5):

To demonstrate oxidative stress we assessed the synthesis of free radicals and the status of antioxidant defenses both at cell and mitochondrial level. We measured "reactive oxygen species" (ROS) and superoxide anion levels (1a), the latter being the primary, specific mitochondrial free radical. In parallel to these molecules' induction we saw an impairment of antioxidant defenses in terms of reduced glutathione (GSH) and NADPH contents (1b). GSH contents is a reflection of the highest physiological antioxidant reserve whereas NADPH reflects electron transport chain functionality, which is impaired by oxidative processes.

Once an impaired oxidant/antioxidant balance was demonstrated we studied whether oxidative stress also affected mitochondria by oxidating mitochondrial components. To this end, given that lipids are the most susceptible molecules to oxidation, we analyzed CL (2), the primary phospholipidic constituent of the IMM. Its obvious oxidation led us to consider that cardiolipin degradation (3) must affect the electron transport chain (4), whose components are inserted in CL, which in turn must result in low function and consequently in membrane potential loss (5).

CL oxidation and membrane potential loss are theoretically associated with increased mitochondrial membrane permeability as a result of MTP opening (6) by any of the above mechanisms, which will facilitate substance exchange between mitochondria and cytosol. In our experiments we detected that MTPs were indeed opened, and that cyt c levels increased in the cytosol as a result of its release from mitochondria (7). The release of cyt c into the cytosol is also related to changes in family Bcl-2 component levels (8), with balance shifting towards proapoptotic members. We detected an increased expression of proapoptotic genes (bax and bid) concomitant with a decreased expression of the antiapoptotic gene bcl-2, which had already been found in previous experiments by other research teams (44,47,77). Cytosolic cyt c activates the caspase cascade (9), which showed in our experiments; both the initiating caspase 2 and effector caspases 6 (spe-

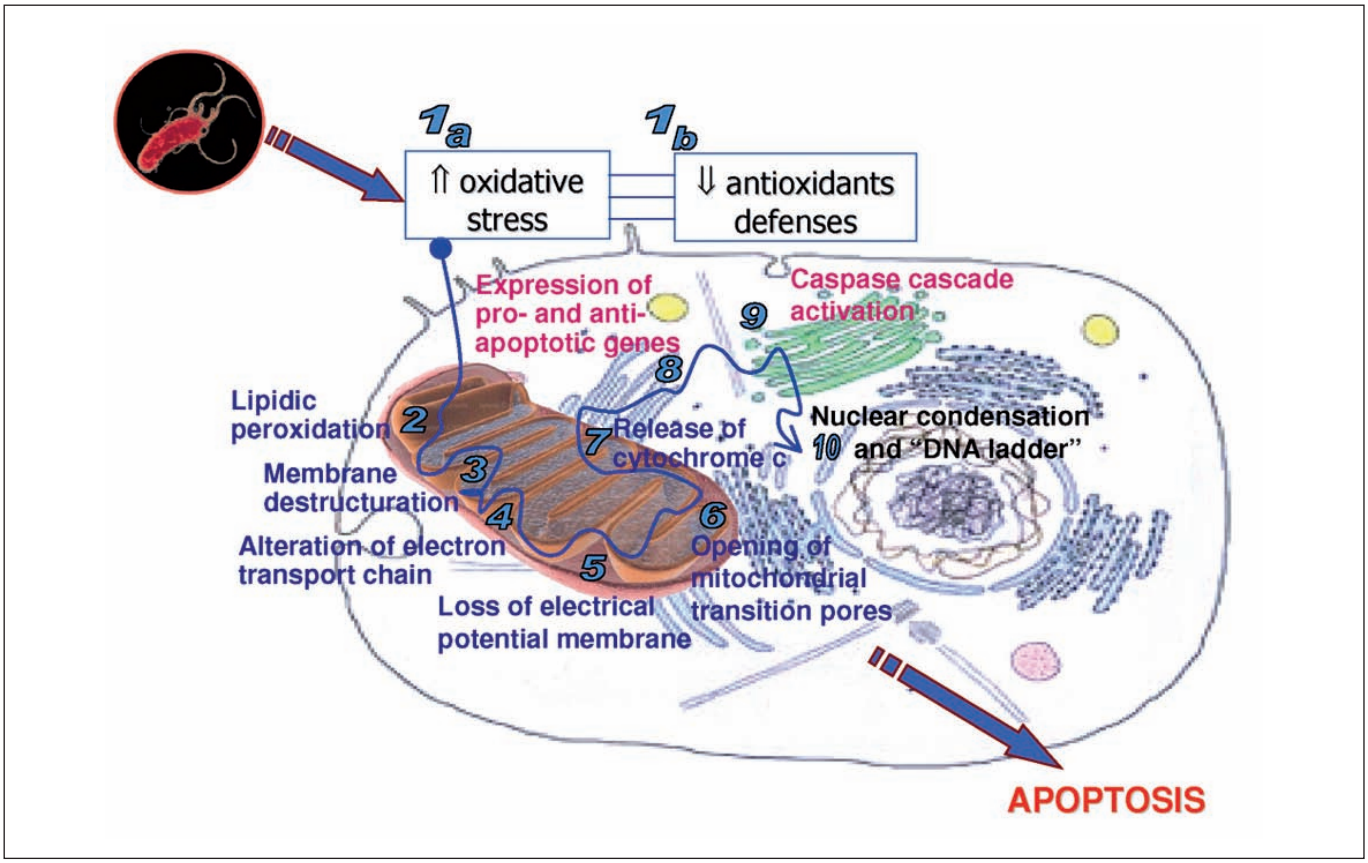


Fig. 5. *A theoretical model of H. pylori toxicity on gastric mucosal cells.* Oxidative stress as induced by *H. pylori* initiates a change cascade that leads gastric cells to apoptotic death by mitochondrial pathway.

cific to the mitochondrial apoptotic pathway) and 3 (molecule where the intrinsic and extrinsic pathways converge) were overexpressed.

For the cell to initiate apoptosis all these mitochondrial and cytosolic changes must translate in the nucleus into chromatin condensation, apoptotic body development, and DNA fragmentation (10), facts that we showed in our cells by visualizing nuclei with specific markers and identifying typical "DNA ladder" patterns in agarose gel.

If oxidative stress is at the origin of all these changes, therapy with antioxidants would prevent them as well as their ultimate consequence: apoptosis. In our experiments the addition of Vit E at $10^{-4}$ M concentration restored oxidative status and prevented or reduced all these alterations, with a statistically significant decrease of apoptosis in our cultures.

Experiments were performed using confocal microscopy, flow cytometry, Western blot, real-time PCR, and ELISA. Mitochondrial structure and function analyses were carried out with the following specific markers:

—MitoSOX Red to establish mitochondrial $O_2^{\cdot-}$ contents.

—JC-1 and MitoTrackers (Orange and Green) to analyze membrane potential.

—Calcein-AM and $CoCl_2$ to determine MTP aperture.

—10-N-nonyl acridine orange to assess cardiolipin oxidation.

## CONCLUSIONS

Based on these results and on evidences reported before our data were published, we show the likely beneficial effect of therapy with antioxidants in patients with *H. pylori* infection. Antioxidants would reduce the impact of exacerbated oxidative stress on the mucosa, and block the ensuing damage-regeneration processes that seem to arise with long-term infection, thus allowing to prevent progression to severe diseases such as ulcer or carcinoma.

Furthermore, we suggest this would be a highly useful preventive method for societies where eradicating therapy is less common and indiscriminate when compared to ours.

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