Pattern of gastritis as manipulated by current state of *Helicobacter pylori* infection

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Abstract: - Helicobacter pylori (H. pylori) infection prevails from 60-80% in patients with gastric ulcer and 90-100% in those having duodenal ulcer**.** Patients with such type of chronic infection are at increased risk to develop peptic ulcers or gastric adenocarcinomas. The present work aims mainly to identify the pattern of chronic gastritis and potential effect of H. pylori infection using certain biomarkers, histological and immunochemical tests.
Fifty eight individuals, clinically diagnosed as having chronic gastritis, were participated in the present study. They were categorized into 2 groups, the first one (31%) demonstrated positive reaction to IgM antibodies of Helicobacter pylori (H. pylori) (>40u/ml) and the second group (69%) demonstrated negative reaction. Blood and antral biopsy samples were collected, directed to determination of serum gastrin, pepsinogen I (PgI), pepsinogen II (PgII), prostaglandin E_2 (PGE_2) and interlukin-6 (IL-6). Immunohistochemistry technique was also done in antral biopsy to demonstrate the expression of inducible nitric oxide synthase (iNOS), nitrotyrosine, DNA fragmentation, myeloperoxidase and histopathological examination.
Serum gastrin, PgI, PgII, PGE_2, IL-6 demonstrated significant increase in gastritis patients as compared to normal group. PgI, PgII showed significant increase joined with slight increase of IL-6 in IgM positive group as compared to negative one. Immunostaining testes in antral biopsy showed strong positive reactions for the above mentioned markers as compared to IgM negative group (mild positive reaction).
In conclusion, gastritis patients who express IgM antibodies for H. pylori infection showed higher gastrinaemia and more pronounced atrophic, inflammatory and apoptotic damage than those not expressing IgM antibodies.

Key-Words: - H. pylori gastritis; gastrin; pepsinogen; prostaglandin E2; iNOS; DNA fragmentation; myeloperoxidase; IgM

1 Introduction

Gastritis represents an inflammatory state of the stomach lining in response to injury which may be either acute or chronic and has many underlying causes [1]. Chronic type can be sub-classified as non-atrophic and special types (chemical, radiation, lymphocytic, non infectious, eosinophilic and others) [2]. Helicobacter pylori (H. pylori) may cause an acute or chronic gastritis and is associated with peptic ulcers, but the relationship to erosions is uncertain.
Normal gastric epithelial cells that line the stomach are necessary for H.pylori persistence and not in any atrophied metaplastic epithelium .
However some cases have been reported describing significant morphological changes to the gastric mucosa, ranging from mild inflammation to ulceration [3]. H. pylori infection is ubiquitous; its prevalence ranges from 60-80% in patients with gastric ulcer and 90-100% in those having duodenal ulcer [4]. Most patients with peptic ulcers are infected with this organism mainly in the antrum, but the entire stomach may be involved [5]. The capacity of H. pylori to colonize the human stomach can be attributed to the production of specific bacterial products [6], urease here r epresents an essential virulence factor, potent antigen leading to immunoglobulin production (IgG and IgM).
Attachments of H. pylori to gastric epithelial cells results in activation of numerous signaling pathways and permits efficient delivery of toxin or other effectors molecules into the cells [7], where adherence is mainly through adhesions and receptions [8]. Bacterial adherence to host cell receptors triggers certain cellular changes (signal transduction cascades), leading to infiltration of

inflammatory cells (neutrophils and monocytes), indeed persistence of the microorganism [7,8].
Another important virulence factor in H. pylori is the cytotoxin-associated protein (Cag A) identified as immune dominant antigen, located on the bacterial surface [9]. CagA protein is frequently co-expressed with vacuolat cytotoxin VacA, expressed as cytotoxin associated protein [10]. People with cytotoxin positive infection have endoscopically proven inflammation that is more pronounced than having cytotoxin negative one. VacA is responsible for the *in vivo* form of vacuoles in gastric epithelial cells [11]. It has direct cell damaging effects *in vitro*, inducing cytoskeletal changes and apoptosis by forming pores in the mitochondrial membranes [11].
Although H. pylori infection is highly associated with chronic gastritis, studies however, revealed that not all people exposed become infected and children may be able to spontaneously clear an acute infection [12].
Patients have H. pylori infection demonstrate increased oxidative damage due to high level of reactive oxygen species (ROS) and increased apoptosis level in human gastric mucosa [14,15]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are normally generated by tightly regulated enzymes such as Nitric oxide synthase (NOS) and NAD(P)H oxidase isoforms respectively . Over production of ROS (arising from mitochondrial electron transport chain or excessive stimulation of NADPH may initiate oxidative stress , acting as mediator to damage cell structures , including lipids , membranes ,proteins and DNA[16] .
Additionally infection also leads to expression of inducible nitric oxide synthase (iNOS) in host macrophage and polymorphonuclear leukocytes . Nitric oxide (NO) produced by these cells, infiltrating the gastric mucosa may damage DNA [[17]Interaction between NO and Superoxide anion can form peroxynitrite , potent nitrating and oxidizing agents leading to apoptosis in a variety of cell types [18] . On the other hand ,over production of RNS is called Nitrosative stress [19] . This occurs when the generation of RNS in a system exceeds the system's ability to neutralize and eliminate them.
PG , the precursor of pepsin exists as 2 main types (PG1 and PG11) ,both of which are produced by the chief and mucus cells in the gastric fundus [20].
In the serum of healthy person the pepsinogen 1 concentration is approximately 6 times that of PG11 level [21] . In atrophic gastritis of the corpus area the serum PG1 concentration decreases whereas PG11 concentration remains at the previous level . Thus the serum PG1 concentration fairly well reflects the number of pepsinogen secreting cells in the corpus area of the stomach and their conditions . Gastrin is the principal hormonal inducer of gastric acid flow . It is l secreted from the G cells , located in antrum and duodenum directly to the gastrointestinal tract in at least 3 different forms . Gastrin subtypes are the so called minigastrin (G-14) , little gastrin (G-17) and the big gastrin [22]
The most important physiological form are gastrin-17 and gastrin-34 [23] .
Helicobacter pylori infection raises basal and meal stimulated serum gastrin concentration . Infection represents the main cause of non autoimmune chronic gastritis which increases gastrin secretion .
Consequently correlation between gastrinemia and severely form of Gastritis has been identified [24

Prostaglandins (PGS) are lipid mediators of the inflammatory immune response which are derived from oxidative metabolism of arachidonic acid.
These lipids are synthesized in large quantities by inflammatory cells in response to both acute and inflammatory stimuli [25] .
The primary enzyme responsible for prostaglandin synthesis is cyclo-oxygenase , exists in at least two isoforms . cyclo-oxygenase-1 (cox-1) is constitutively expressed in gastro intestinal tract and most other tissues usually at sites of inflammation .
Endogenous prostaglandins are involved in adaptive gastric protection against acute injury .

For such reason prostaglandins especially prostaglandin E_2 (PGE_2) in the stomach may play an important role in maintenance of gastric mucosal integrity via several mechanisms including regulation of gastric mucosal blood flow, kinetics of epithelial cells, synthesis of mucus and inhibition of gastric acid secretion [26], referring to its protective potential to gastric mucosa.
H pylori infection is associated with specific local and systemic immune responses. Early after 18 days of H. pylori infection, IgM response is detectable, whilst IgG and IgA response occur later after 60 days of infection, at which time IgM titers decline [27]. IgG and IgA serology is widely used as an accurate test for the diagnosis of H. pylori infections, but these two immunoglobulins remain detectable even after eradication of H. pylori and do not demonstrate the status of infection (acute, chronic, or previously treated infections) [28]. The use of IgM test would allow for direct screening of specimens and serve as a diagnostic tool for establishing active or recent infection. We used IgM serology in patients with H. pylori infection to study the differences in some gastric, inflammatory and oxidative biomarkers between those having positive IgM (active or recent infection) and those with negative IgM (chronic infection).
The present study aims mainly to determine i) the pattern of chronic gastritis, whether either H. pylori infection or not has any potential effect (current state

of infection) using certain biomarkers, e.g. gastrin, pepsinogen I (PgI), pepsinogen II (PgII), PGE_2 and interleukin-6 (IL-6). ii) mucosal immunostaining test for nitrotyrosine, iNOS, myeloperoxidase and DNA fragmentation in antral biopsies isolated from individuals having positive IgM and those with negative IgM, followed by histopathological examination of the tissues.

2 Materials and methods

2.1 Subjects

Age and BMI-matched 58 patients (49 male and 9 females) were recruited from those attending gastroenterology department, Ain Shams university hospital, Cairo, Egypt, for esophagogastroduodenoscopy and diagnosed as having H. pylori infection. All these patients are newly diagnosed and none of them had previously undergone anti-*H. pylori* treatment or had received antibiotics within the previous 2 months. Further 20 healthy volunteers (17 male and 3 females) participated in the current study. Patients received consents before going through the endoscopy procedures. Both patients and healthy volunteers received consent for the study which was approved by local ethical committee.

2.2 Sampling and biopsy

Before going through endoscopy, blood was collected from all patients and kept at 4ºC. Blood was centrifuged at 3300 xg for 15 minutes to separate serum, divided into aliquots and stored at -20ºC to be used later for immunoassays. During endoscopy, gastric tissue, antral biopsy, was obtained by means of routine biopsy forceps. The biopsy collected from each patient was kept in 10 % formalin to be processed later for histological and immunohistochemical staining.

2.3 Immunoassay serum measurements

The level of IgM antibody was measured in the serum of all patients using AccuBind ELISA Kits (Monobind Inc, Lake Forest, CA,USA) and hence these patients were then categorized into IgM positive [IgM(+)] and IgM negative [IgM(-)] groups. Gastrin-17, PgI and PgII were measured in serum using sandwich enzyme immunoassay (ELISA) kits provided from Biohit Pic, Helsinki, Finland following manufacturer instructions. IL-6 was measured using a sandwich ELISA kit supplied from DRG international, Inc, Mountainside, USA. Serum PGE_2 were determined by enzyme immunoassay kits supplied from R&D Systems, Inc., Minneapolis, USA following the instructions of manufacturer.

2.4 Histopathological and immunostaining of antral biopsy

Standard histological technique was employed using Haematoxylin and eosin staining. Immunohistochemical staining iNOS was determined using polyclonal antibody kit, highly purified from rabbit antiserum by peptide affinity chromatography, supplied from Zymed Lab, Inc, San Francisco, CA, USA. Nitrotyrosine, a stable marker of peroxynitrite formation, was done immunohistochemically to evaluate nitrosative stress involved in H. pylori gastritis using monoclonal mouse anti-nitrotyrosine kit supplied from Zymed Lab Inc, CA, USA. Immunostaining for DNA fragmentation, a marker for apoptosis, was determined using polyclonal antibody kit (DFF45), supplied from Lab Vision Corp, Fermont, CA, USA.

2.5 Statistical analysis

Statistical analyses of data were done by the Statistical Package for Social Sciences software (SPSS, Illinois, USA). Results were expressed as mean ± SD. Student t test was used to study any statistical differences between gastritis groups and healthy volunteers taking $P< 0.05$ as statistically significant.

3 Results and Discussion

H. pylori infection represents an etiological agent, acting as inducer for active chronic gastritis, reaching 70-95% [29]. Although it is highly associated with chronic gastritis, studies revealed that not all peoples exposed become infected and children may be able to spontaneously clear an acute infection [30]. Present work has been focused on well diagnosed chronic gastritis cases. The 58 patients diagnosed as having H. pylori infection were predominantly male (84%). This was in consistent with other studies, including a Meta analysis, showing the male predominance of *H. pylori* infection in adults [13]. Due to different factors, 31% (15 male, 3 females) of those H. pylori infected individuals (43 ± 7 years old) demonstrated positive results for IgM antibodies in their sera, while the remaining 69% (45 ± 6 years old) revealed IgM negative reaction of whom only 6 were female (Table 1). Detection of anti-H. Pylori IgM is highly specific, western blot analysis revealed a variable IgM response to H. pylori antigens among patients with the most reactive antigenic fractions being in the range of 55-100 kDa [31].

Table (1) demonstrated significant gastremia in both patient groups (IgM+ and IgM- groups, $P<0.001$),

which is mostly attributed to intragastric increase of H. pylori inducing corpus atrophy and G cells damage in the antrum part. It may be also depends on alkalinization in G cells environment caused by H. pylori urease and in agreement with previous reports [32-34)

Infection represents the main cause of non autoimmune chronic gastritis which increases gastrin secretion . Consequently correlation between gastrinemia and severity of gastritis have been identified .

Gastrin receptors have been observed in several leukocytes , additionally gastrin and cholecystokinin (CCK) have been shown to include changes in leukocyte functions such as chemotaxis , adherence or phagocytosis in vitro

[35] . Gastrin releasing peptide (GRP) can stimulate gastric acid secretion which is particularly valuable in detecting disturbances of gastric secretory function of patients with duodenal ulcer and H. pylori infection [36] .

Serum pepsinogen (I&II) are higher in patients with H. pylori infection than in normal controls (P<0.001). The IgM+ group of patients demonstrated significantly higher levels of serum pepsinogen (I&II) than the IgM- group (P<0.05) as illustrated in Table 1. This may be attributed to a polypeptide secreted by H. pylori during earlier infection which stimulates chief cells directly and promotes pepsinogen synthesis and secretion, specifically PgII. This is mainly through intracellular mechanisms of increasing Ca^{++}, cyclic adenosine monophosphate (cAMP) and phosphoinositide.

As mentioned before serum of healthy person demonstrate pepsinogen 1 concentration approximately six times that of PG11 level [21].

In atrophic gastritis PG1 decreases where PG11 concentration remains at the previous level.

The more series the atrophic gastritis of the corpus area of the stomach is the lower serum PG1 concentration [37] . Additionally Low level of PG1 concentrations in cases of atrophic corpus gastritis may reflects a sensitivity of over 90 percent and a specificity of almost 100 percent(38) .

As the severity of atrophy advances , chief cells are replaced by pyloric gland and PG11 remains high while PG1 level decreases, the ratio in turn between them is greatly reduced . Thus serum PG concentration can reflects the morphological and functional status of the gastric mucosa[20]

Helicobacter pylori – related gastric atrophy and its subsequent changes may start at the antrum , antrum-corpus junction , antral lesser curvature and finally the proximal corpus(40) . In this way serum PG1 & PG11 concentrations and the ratio between PG1 and PG11 may be related to the histological and functional status of the gastric mucosa [39,40]

Certain reported studies concluded similar findings, referred to higher PgI and lower ratio of PgI/PgII in IgM(+) group than in IgM(-) group [35,36]. Recent study has been expressed pepsinogen as a biomarker of gastric mucosal status including atrophic change and inflammation i.e as the fundic mucosa reduces, serum pepsinogen level gradually decreases. This can detect extensive atrophic gastritis regardless of their H. pylori status [37]

Table 1: Gastrin-17 (pmol/l), pepsinogen I (PgI; µg/l) pepsinogen II (PgII; µg/l), prostaglandin E_2 (PGE_2; pg/ml) and interleukin-6 (IL-6; pg/ml) measured in serum from patients having H. pylori either with IgM(+) or IgM(-) and healthy volunteers.

	Mean (SD)		
	Normal controls	IgM (+)	IgM (-)
N	20	18	40
(M/F)	(17/3)	(15/3)	(34/6)
Age (year)	47 (6)	43 (7)	45 (6)
Serum gastrin-17 (pmol/l)	31.28 (2.78)	56.79* (6.3)	53.17* (5.28)
Serum PgI (µg/l)	90.8 (25.6)	154.6* (41.9)	132.68* (30.1)
Serum PgII (µg/l)	10.26 (1.47)	20.45* (8.7)	14.3* (4.6)
PgI/PgII	8.78 (2)	8.58 (3.45)	9.78 (2.61)
Serum PGE2 (pg/ml)	308.3 (22.38)	388* (26.6)	392* (23.36)
Serum IL-6 (pg/ml)	81.8 (9.55)	121.76* (34.91)	107.9* (34.77)

Results were expressed as mean (SD), Significantly different from healthy volunteers group* P< 0.001.

Chronic gastritis can be turned to atrophy, intestinal metaplasia and dysplasia which are precancerous [40,41]. Accordingly histological and physiological improvements through treatment of patients having positive atrophic gastritis are promising in prevention of gastric cancer [40 ,41 ,42] . Those authors have considered serum pepsinogen level as a non endoscopic blood test in the diagnosis of atrophic gastritis, H. pylori eradication and a screening tool for high risk subjects having atrophic gastritis rather than a test for cancer itself.

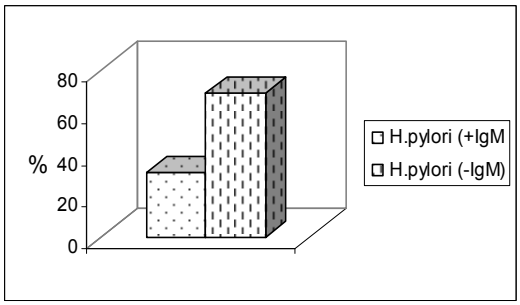

Fig (14): Percent of H.pylori (+ ve IgM) and (- ve IgM)

Vaananen et al (2003) [43] concluded that diagnosis of atrophic gastritis using test panel of serum gastrin-17, PgI and H. pylori antibodies were in good agreement with the endoscopic and biopsy findings, considering such panel a non endoscopic diagnostic and screening tool.

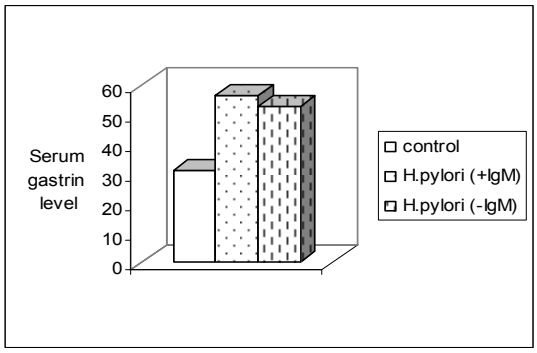

Fig (15 a): Sera mean levels of gastrin in different groups

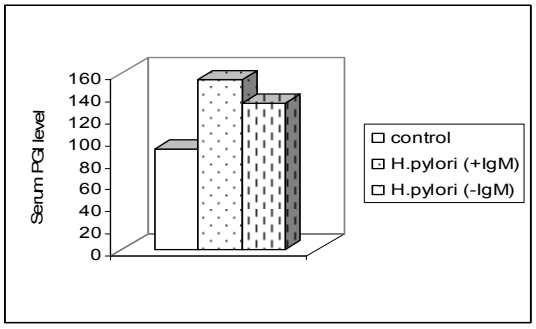

Fig (15b): Sera mean levels of PGI in different group

Few years ago certain study hypothesized that IL-6 expression in macrophage is mainly heat shock protein-60 (HSP60) dependent [44].

Its release is likely to occur in vivo and may be capable of reaching mucosal macrophages within the gastric epithelium .

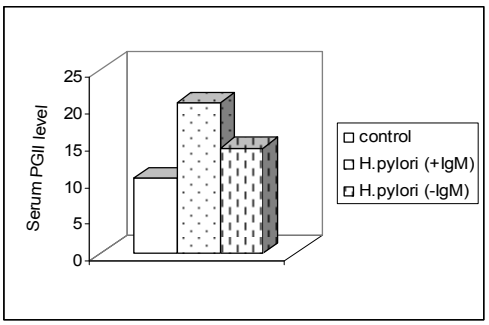

Fig (15c): Sera mean levels of PGII in different group

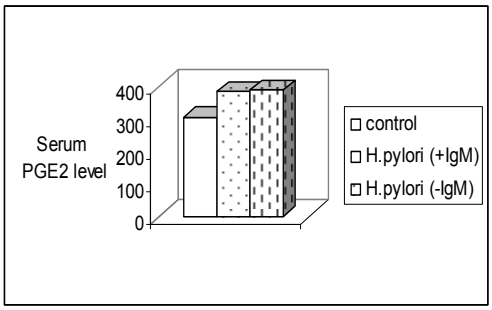

Fig (16 a): Sera mean levels of PGE2 in different groups

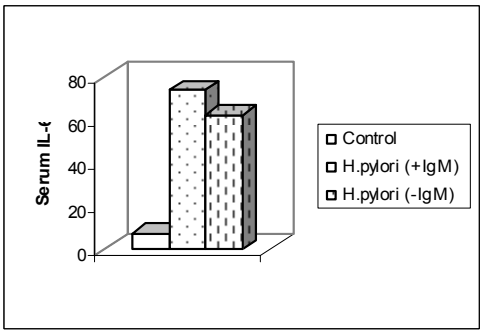

Fig (16b): Sera mean levels of IL-6 in different groups

Serum IL-6 level showed also significant increase in both patient groups as compared to control, mostly marked in IgM(+) group only although non significant (Table 1). H. pylori infection can induce IL-6 in monocyte/macrophages and in chronically inflamed tissues, leading to the development of gastritis. However, activated macrophages represent the main sources [45 ,46].

Oxidative stress can also influence the expression of multiple genes in monocytes and other cells including signal molecules such as protein kinase C (PKC), nuclear factor kabba (NF-kB) [45] and overexpression of these genes stimulates the secretion of pro-inflammatory cytokines [47].

Serum PGE_2 showed also significant increase as shown in Table 1. This may be attributed to increased cyclooxygenase-2 (COX-2) expression in subsequent to leukocytes infiltration [47].

Haematoxyline and eosin staining for IgM(-) category showed irregular lumen (LU), damaged epithelium(E), irregular shape of fundic gland (FG) and multiple inflammatory cells (Fig.1a and Fig.1b).

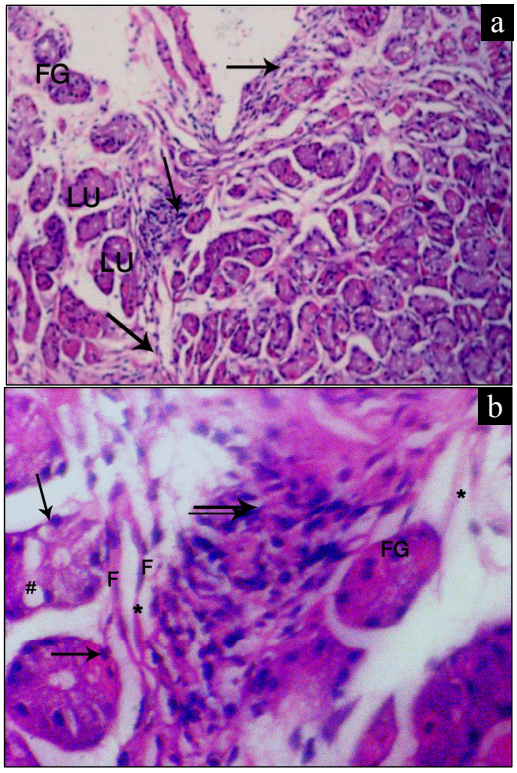

Fig.1: Histological section of human fundic gland of patient suffering from gastritis with anti H. pylori IgM negative (a) x100 showing irregular lumen (LU), damaged epithelium (E), irregular shape of fundic gland (FG) and multiple inflammatory cells (arrows) between them. (b) x400 showing irregular shaped fundic gland (FG) with multiple pyknotic nuclei (arrows) in addition to fatty infiltrations (#) of their cells. Multiple inflammatory cells (double arrows), fibroblast (F) and collagen fibers (*) are filling lamina propria (LP).

The immunostaining toward nitrotyrosine and myeloperoxidase showed negative reaction in the epithelial (E) lining FG and inflammatory cells filling LP (Fig.2a and Fig.2b). Immunostaining for iNOS revealed a moderate positive reaction in the cells of the FG (Fig.2c) as well as mild positive reaction for DFF in the epithelial cells lining FG and cells filling LP (Fig.2d).

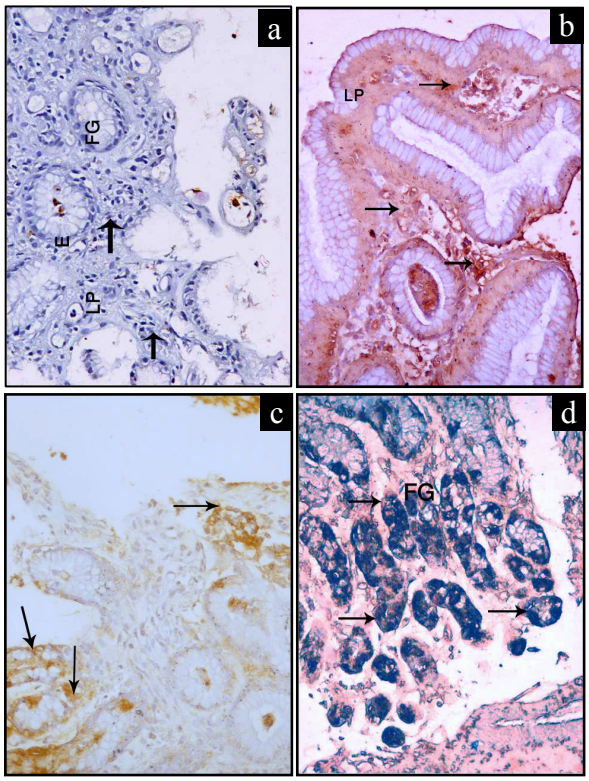

Fig.2: Immunostaining section of human fundic gland from gastritis patients, with anti-H. pylori IgM(-) showing for (a) nitrotyrosine negative reaction in the epithelial (E) lining fundic gland (FG) and inflammatory cells (arrows) filling lamina propria (LP), (b) myeloperoxidase showing strong positive reaction in the inflammatory cells (arrows) infiltrating lamina propria (LP) (c) iNOS showing moderate positive reaction in the cells of the fundic gland (arrows), (d) DNA fragmentation factor (DFF) showing mild positive reaction in the epithelial cell (arrows) lining fundic gland (FG) (x200).

Histological examination of IgM(+) category demonstrated irregular short fundic gland (FG), wide gastric pit (GP), multiple inflammatory cells and blood vessels filling lamina propria (LP) as shown in Fig.3a and Fig.3b. Strong positive reaction for nitrotyrosine in the epithelial (E) lining FG and inflammatory cells filling LP (Figure 4a) as well as for MPO in the surface columnar epithelial cells (E) and other cells lining fundic gland (Fig.4b). Immunostaining for iNOS showed positive reactions in the inflammatory cells filling LP (Fig.4c). Furthermore, a strong positive reaction for DNA fragmentation factor (DFF) in the epithelial lining FG and inflammatory cells filling LP was also observed (Fig.4d).

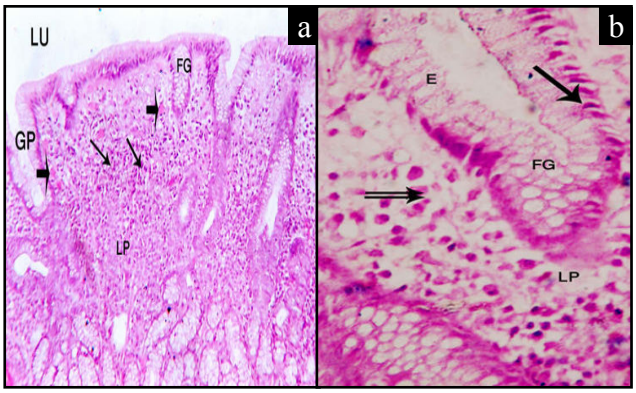

Fig.3: Histological section of human fundic gland of patient suffering from gastritis with anti H. pylori IgM positive group showing (a) x100 irregular short fundic gland (FG), wide gastric pit (GP), multiple inflammatory cells (arrows) and blood vessels (double arrows) filling lamina propria (LP), (b) x400 showing irregular simple columnar epithelium (E), small pyknotic nuclei (arrows) of cells lyning fundic gland (FG) and multiple inflammatory cells (double arrows) filling lamina propria (LP).

Inflammatory cells such as polymorphonuclear cells and macrophages can express iNOS in mammals [48,49,] mostly associated with nitrotyrosine production additionally can induce apoptosis (DNA fragmentation) as mentioned before [49,50]. Ding and colleagues demonstrated that H. pylori can induce programmed cell death in cultured gastric epithelial cells as do pro-inflammatory cytokines which released during infection [50,51]. Others concluded that hypergastrinaemia can render epithelial cells within corpus tissues much more susceptible to apoptosis either by radiation or H. pylori infection [34].

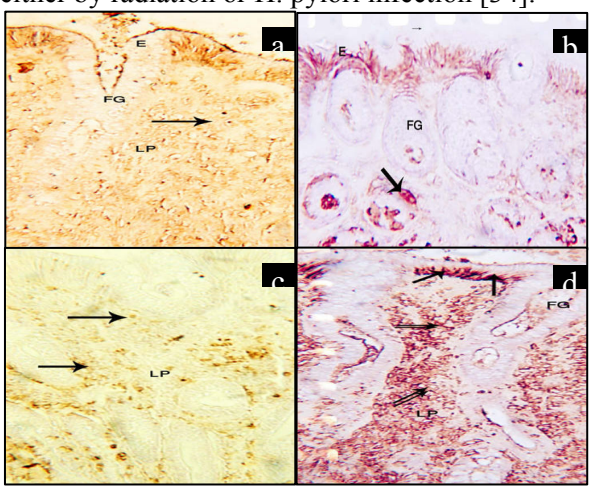

Fig.4: Immunostaining section of Gastritis patients IgM(+) category for (a) nitrotyrosine showing strong positive reaction in the epithelial (E) lining fundic gland (FG) and inflammatory cells (arrows) filling lamina propria (LP), (b) myeloperoxidase showing strong positive reaction in the surface columnar epithelial cells (E) and other cells (arrows) lining fundic gland (FG) (c) iNOS showing strong positive reaction in the inflammatory cells (arrows) filling lamina propria (LP), (d) DNA fragmentation factor (DFF) showing strong positive reaction in the epithelial (arrows) lining fundic gland (FG) and inflammatory cells (double arrows) fill lamina propria (LP) (x200).

4 Conclusion

Individuals demonstrating IgM positive reactions recorded significant increase in pepsinogen I, II joined with slight increase in IL-6 level as compared to IgM negative group. Their fundic gland showed strong positive reactions for nitrotyrosine, iNOS, DNA fragmentation and myeloperoxidase as compared to IgM negative group.

Infiltration of neutrophils into tissues which is commonly assessed by changes in MPO activity represents a causative agent.

Activated neutrophils located in the inflammatory foci and secreting MPO into the extracellular space can convert hydroperoxides into free radicals indeed cellular damage.

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