Gastroprotective Effects of Montelukast and Nigella Sativa Oil against Corticosteroids-Induced Gastric Damage: Much More Than Antiasthmatic Drugs

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<td>Rizk, Fatma; Faculty of medicine, Tanta university, Physiology Ibrahim, Marwa; Faculty of Medicine, Tanta University, Histology Abd-Elsalam, Marwa; Faculty of Medicine, Kafr-Elsheikh University, Histology Soliman, Nema; Faculty of Medicine, Tanta University, Biochemistry Abd-Elsalam, Sherief; Faculty of Medicine, Tanta University, Tropical medicine</td>
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Gastroprotective Effects of Montelukast and Nigella Sativa Oil against Corticosteroids-Induced Gastric Damage: Much More Than Antiasthmatic Drugs

Fatma H. Rizk\textsuperscript{a,*}, Marwa A.A. Ibrahim\textsuperscript{b}, Marwa M. Abd-Elsalam \textsuperscript{c}, Nema A. Soliman\textsuperscript{d}, Sherief M. Abd-Elsalam \textsuperscript{e}

Departments of \textsuperscript{a}Physiology, \textsuperscript{b}Histology, \textsuperscript{d}Biochemistry, and \textsuperscript{e}Tropical medicine Faculty of Medicine, Tanta University, Egypt.

\textsuperscript{c}Histology Department Faculty of Medicine, Kafr-Elsheikh University, Egypt.

\textsuperscript{*}Corresponding author, Egypt, +2-01064122556, (e-mail: Fatma.rezk@med.tanta.edu.eg)
Abstract

Corticosteroids are used to treat variety of diseases like bronchial asthma. However, long term corticosteroids have a gastric ulcerogenic potential. Montelukast (MTK) and nigella sativa oil (NSO) are used in treatment of bronchial asthma. Previous studies showed that MTK and NSO had gastroprotective effects in other models of gastric ulcer. The present study assesses synergistic gastroprotective effects of both drugs in dexamethasone (DXM)-induced gastric damage. 50 male rats were divided into 5 groups; normal control (I), DXM group (II), MTK+DXM group (III), NSO+DXM group (IV), MTK+NSO+DXM group (V). After 7 days, stomachs were removed for biochemical analysis and histological examinations. Significant increases in MDA level, SOD activity, MPO activity, and proliferating cell nuclear antigen (PCNA) positive cells, with significant decreases in mucus secretion were detected in DXM-treated group compared with group I. While, significant decreases of MDA level, MPO activity, and PCNA positive cells and significant increases in mucus secretion were detected in treated groups compared with group I and II. SOD activity significantly decreased in group V compared with group II only. We could conclude that administration of either MTK or NSO or both with DXM counteracts DXM induced-gastric lesions.
**Key words:** Montelukast, Nigella sativa, Dexamethasone, Gastric damage.
Introduction

Corticosteroids are used to treat variety of health problems (Lu and Cidlowski 2004). Bronchial asthma is one of the diseases which needs long term treatment with corticosteroids (Ukena et al. 2008). Corticosteroids have a gastric ulcerogenic potential (Tripathi 2004). The mechanisms of this damaging action are not completely clear but previous studies suggested that corticosteroids causes gastric erosions by inhibiting two important gastroprotective enzymes, prostaglandin synthetase and peroxidase, so it blocks the gastroprotective action of prostaglandin and increases the endogenous H2O2 level which generates more reactive hydroxyl radical. Also, it decreases the levels of nitric oxide (Swamy et al. 2011), and inhibits the angiogenesis (Luo et al. 2004).

Montelukast (MTK) is a selective reversible cysteinyI leukotriene D4 (LTD4)- receptor antagonist that’s used in the treatment of allergic rhinitis and bronchial asthma. It is well known that leukotrienes (LTS) are secreted by eosinophils, mast cells, monocytes, and macrophages, are important mediators of asthma as LTS play a crucial role in inflammation, bronchoconstriction, and airway remodeling of asthmatics, thus MTK is helpful in the treatment of this illness by reducing airway eosinophilic inflammation in asthmatic patient (Suddek 2014) with subsequent reducing
the dose of inhaled corticosteroids needed to control the bronchial asthma (Ducharme 2002). In addition to the role of LTS in bronchial asthma, they are implicated in gastric mucosal injury induced by non-steroidal anti-inflammatory drugs (NSAID) (Ewadh et al. 2015), and ethanol (El-Maraghy et al. 2015). Therefore, MTK can be used as a gastroprotective drug in these experimental models as it has anti-inflammatory, antioxidant (Cuciureanu et al. 2008) and antiapoptotic effects (Muthuraman and Sood 2010).

In addition to these synthetic drugs which are used in the treatment of bronchial asthma, some herbal drugs were proved to have prophylactic and curative effects in management of asthmatic patients without the side effects of synthetic drugs. Among these herbs, nigella sativa oil (NSO) which was proved to be an excellent prophylactic agent for these patients due to its anti-histaminic effect (Salama 2008). Also, it had gastroprotective effects against NSAID (Rajkapoor et al. 2002) and ethanol - induced gastric damage (Kanter et al. 2006). The gastroprotective effects in these models could be due to its antisecretory (Rajkapoor et al. 2002), antioxidant and antihistaminic effects (Kanter et al. 2006). Thus we hypothesized that the combination between MTK and NSO might have synergistic gastroprotective effects in asthmatic patients treated with corticosteroids. So
the aim of this study was to evaluate the effect of leukotriene receptor antagonist MTK along with NSO on dexamethasone (DXM) - induced gastric damage in rats and identify some of the possible mechanisms involved in corticosteroids induced gastric damage which could be antagonized by this combination.
Material and methods

Drugs

Dexamethasone was purchased from Kahira pharmaceuticals and chemical industries company, Egypt. Montelukast was purchased from Global Napi pharmaceuticals, Egypt. Both drugs were freshly prepared by suspending in distilled water. Nigella sativa oil was obtained from Pharco Company, Egypt, in the form of capsules then the oil was drained using a syringe. All Chemicals used unless otherwise described were purchased from Sigma (Sigma, St Louis, USA) and were of high analytical grade.

Animals

Fifty male wistar albino rats weighing 170 – 200gm were housed in clean cages, 5 rats in each cage, in Physiology department, Faculty of Medicine, Tanta University. Rats were acclimatized to standard laboratory conditions (12: 12 light – dark cycle, environmental temperature 25±2°C and free access to water and food) for 7 days before start of experiment. All experiments were performed during the same time of the day between 9 a.m and 12 p.m to avoid variations due to diurnal rhythms (Shimizu et al. 2000). All animals were cared in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA). The experimental
protocol was reviewed and approved by the animal care review committee at the faculty of medicine, Tanta University.

The rats were randomly divided into 5 groups 10 rats each.

Group I: normal control (distilled water 1ml / rat/ day).

Group II: DXM group (5mg/ kg/ day) (Swamy et al. 2011).

Group III: DXM (5mg/ kg/ day) + MTK (10 mg/kg/ day) (Dengiz et al. 2007)

Group IV: DXM (5mg/ kg/ day) + NSO (500 mg /kg/ day) (Kanter et al. 2006).

Group V: DXM (5mg/ kg/ day) + MTK (10 mg/kg/ day) + NSO (500 mg /kg/ day).

All drugs were given by oral gavage once daily for 7 days.

After completing the experimental regimen, the rats were sacrificed by decapitation under general anesthesia by an intraperitoneal injection of phenobarbital sodium 30 mg/kg (Ramírez et al. 2009) and the stomach was dissected, removed, and then opened along the greater curvature, washed by 0.9% NaCl solution, to clean it, then divided into two parts for biochemical analysis and histological examination.

**Biochemical analysis of stomach tissue**

One part of each stomach sample was used for biochemical analysis as it
was cut into small pieces and underwent homogenization in Tris buffer (10 mM, pH 7.4) at a concentration of 10% (w/v), and centrifuged at 11,000 × g for 15 min at 4°C. The clear supernatant was used for assessment of lipid peroxidation malondialdehyde (MDA) content using commercial kits (Biodiagnostic, Giza, Egypt) and its level was expressed as nmol/g tissue, endogenous antioxidant enzymes (Cu/Zn) Superoxide dismutase (SOD) activity and myeloperoxidase (MPO) activity according to Sun et al. (1988) and Krawisz et al. (1984) respectively. Both enzymes were expressed as U/mg protein. Tissue protein was calculated according to Lowry et al. (1951).

**Histological examination**

The other parts of glandular stomach specimens were immediately fixed in 10% neutral buffered formalin, washed, dehydrated, cleared and embedded in paraffin. Sections of 5µm thickness were stained with haematoxylin and eosin (H&E) for the study of general histological features and Periodic Acid Schiff reagent (PAS) for detection of neutral mucopolysaccharide (Gamble 2008).

**Immunohistochemistry**

Paraffin sections of 5µm thickness were dewaxed, rehydrated, and washed with phosphate buffered saline (PBS) and then incubated with PBS containing 10% normal goat serum. Sections were incubated with the mouse
monoclonal antibody PC10 against proliferating cell nuclear antigen (PCNA) (sc-56, Santa Cruz Biotech, Santa Cruz, USA) (1:100) overnight in a humid chamber at 4°C. Section were then rinsed in PBS and incubated with biotinylated rabbit anti-mouse Ig (1:200) for 60 min at room temperature. Sections were incubated with a streptavidin–biotin–horseradish peroxidase complex (1:100) prepared 30 min in advance and mixed shortly before use with an equal volume of PBS. The immunoreactivity was visualized using 3,3′-diaminobenzidine (DAB) hydrogen peroxide as a chromogen and sections were counterstained with Mayer’s haematoxylin. The negative control sections were prepared by excluding the primary antibodies (Ramos-Vara et al. 2008).

Morphometric analysis:-

The images were acquired using an Olympus light microscope BX50 (Tokyo, Japan) coupled to an Olympus digital camera (Tokyo, Japan) and the software “ImageJ” (National Institute of Health, Bethesda, Maryland, USA) was used for image analysis. Ten non-overlapping fields in slides of each group were examined at magnification of 200-fold. Images were analyzed for: 1) Mean color intensity of PAS reaction: expressed as a magenta red cytoplasmic coloration in the epithelial cells of the gastric mucosa. 2) Mean percentage of PCNA-immunopositive cells (in DAB
stained sections): expressed as a percentage of the total number of cells counted (number of brown labeled nuclei x100/total cell number).

Statistical analysis

The data were expressed as the mean ± SD. Statistical comparison between different groups was carried out using one-way analysis of variance (ANOVA) followed by LSD test (multiple comparison). Statistical tests were performed with SPSS (Version 23). P-values <0.05 were considered statistically significant.
Results

Effect of dexamethasone, montelukast and nigella sativa oil on the gastric oxidative stress parameters

Administration of DXM for 7 days significantly increased the gastric MDA level compared with normal control group. While, administration of either MTK or NSO or both with DXM for 7 days significantly decreased the gastric MDA level compared with DXM group (11.33%, 28.57%, and 28.57% respectively). Gastric SOD activity significantly increased in DXM group compared with normal control group. Administration of MTK along with NSO in group V significantly decreased the gastric SOD activity compared with DXM. While, administration of MTK or NSO separately led to non-significant changes in the gastric SOD activity compared with DXM group (Table 1).

Effect of dexamethasone, montelukast and nigella sativa oil on the gastric MPO activity

DXM administration for 7 days significantly increased the gastric MPO activity as compared with normal control group. While, administration of either MTK or NSO or both with DXM for 7 days significantly decreased the gastric MPO activity compared with DXM group (31.98%, 14.72%, and 34.52% respectively) (Table 1).
**Histological examinations**

Haematoxylin and eosin-stained sections from the normal control group showed the normal architecture of the fundic mucosa with intact epithelial lining and gastric pits (Fig. 1a). Sections from group II revealed a distorted glandular pattern with focal exfoliation of the superficial epithelium and gastric pits with occasionally observed areas of hemorrhage. Surface epithelial cells appeared vacuolated with abnormal nuclei together with marked widening and separation in particular at the base of the gastric glands. Mononuclear cells were frequently observed (Figs. 1b, c). Sections from group III showed an apparently normal architecture of the gastric mucosa, yet some surface epithelial cells appeared pale and vacuolated. Moreover, evident spacing was observed between the glands in addition to some mononuclear cells mainly at the base of the gastric glands (Fig. 1d). Sections from group IV also showed an almost normal architecture of the gastric mucosa, only few surface epithelial cells appeared vacuolated, yet a degree of widening between the glands was observed along with many mononuclear cells at the base of the gastric glands (Fig. 1e). Sections from group V showed a near normal architecture of the gastric mucosa with intact gastric glands and minimal cellular alterations (Fig. 1f).
PAS staining

PAS-stained sections from the normal control group revealed the characteristic strong magenta red color of PAS-positive film of mucus on the surface epithelium and gastric pits (Fig. 2a). Sections from group II revealed a diffusely weak PAS reaction in the surface epithelium and gastric pits (Fig. 2b). Sections from group III showed that only some gastric pits revealed moderate PAS reaction (Fig. 2c). Similarly, sections from group IV showed that only the most apical part of the gland expressed strong PAS reaction (Fig. 2d). Sections from group V showed that the apical part of the gland revealed strong PAS reaction (Fig. 2e). Morphometrical analysis of the PAS reaction mean color intensity confirmed a significant decrease in PAS reaction in groups II, III, IV and V compared with normal control group, while a significant increase was detected in groups III, IV and V compared with group II (Fig. 2f).

Proliferating cell nuclear antigen (PCNA) immunostaining

PCNA-immunostained sections from the normal control group showed minimal number of PCNA-positive cells in the gastric mucosa (Fig. 3a). While sections from group II revealed numerous PCNA-positive cells in the gastric mucosa (Fig. 3b). Sections from groups III showed some PCNA-positive cells mainly in the apical aspect of gastric glands (Fig. 3c).
Moreover, group IV showed many PCNA-positive cells but in a more scattered pattern throughout the gastric glands (Fig. 3d). On the other hand, sections from group V showed only a minimal number of PCNA-positive cells in the gastric mucosa (Fig. 3e). Morphometrical analysis of the mean percentage of PCNA positive cells confirmed a highly significant increase in group II compared to the control, while both groups III and IV showed a significant decrease compared to group II. On the other hand, group V expressed a non-significant change from the normal control group (Fig. 3f).
Discussion

Corticosteroids are an essential therapy for many diseases (Lu and Cidlowski 2004). Dexamethasone is one of the corticosteroids which induce gastric damage (Swamy et al. 2011). Up to date, according to our knowledge, there is no previous research studied the combined effects of MTK and NSO on gastric damage induced by DXM. So, this is the first study which evaluates the possible protective effects of concomitant administration of MTK along with NSO on DXM - induced gastric damage with investigation of some possible mechanisms involved in such effect. This combination was chosen because both MTK and NSO are used in the treatment of bronchial asthma.

The results of the present work showed that DXM administration for 7 days induced gastric damage as proved by biochemical analysis and histological examinations of gastric mucosa. The mechanism of DXM- induced gastric damage is multifactorial as evidenced by the current study. Results of the present work showed that DXM led to a significant increase in MDA level in gastric tissue compared with normal control group, suggesting that lipid peroxidation and free radicals generation were involved in the pathogenesis of DXM-induced gastric mucosal damage (Manjari and Das 2000).

It is well known that antioxidant enzymes scavenge free radicals but
excessive generation of reactive oxygen species depletes these enzymes (Swamy et al. 2011). Surprisingly, SOD activity in this experiment was significantly increased in DXM treated group compared with normal control group. In contrast to our results, Swamy et al. (2011) showed that SOD activity significantly decreased in DXM treated groups. This controversy might be attributed to the difference in the duration of both experiments as the duration of this experiment was 7 days while, that of Swamy et al. was 10 days. SO the effect of DXM on SOD activity might be initially increased as a compensatory mechanism then it decreased with the progress of oxidative stress damage.

Administration of either MTK, or NSO, or both with DXM decreased lipid peroxidation as they significantly decreased MDA level in treated groups compared with DXM group. Also, the combination of MTK and NSO in group V restored the normal activity of SOD enzyme. The reduction in MDA levels and restoration of normal SOD enzyme activity in this experiment might be due to decrease lipid peroxidation and strongly suggested the antioxidant activity of MTK and NSO. This antioxidant activities of MTK and NSO were previously studied by Al-bayati et al. (2015) and Abdel-Satar (2009) respectively in different animal models of gastric mucosal damage.
In addition to oxidative stress, DXM-induced gastric lesions through induction of inflammatory reactions which evidenced by biochemical assay of gastric MPO activity and histological examination in the present study. Gastric MPO activity showed a significant increase in DXM-treated groups compared with normal control group. The MPO activity is used as an index of neutrophils infiltration in gastric injuries (Bayir et al. 2006). Neutrophils are important source of leukotrienes which are considered as a possible cause of gastric damage due to its vasoconstrictive (But et al. 2003) and inflammatory effects (Gandhi et al. 2012). It’s well known that activated neutrophils cause tissue damage through the production of reactive oxygen metabolites and cytotoxic proteins (e.g. MPO, proteases, and lactoferrin) into the extracellular fluid (Sullivan et al. 2000).

Moreover, histological examination of the gastric epithelium in this experiment showed vacuolated surface epithelial cells with mononuclear cells infiltration and hemorrhagic areas. Marked widening and separation of gastric mucosa indicated presence of edema (Fiorucci et al. 2001).

On the other hand, treatment with either MTK, NSO, or both with DXM antagonized inflammation induced by DXM, especially group V, as proved by significantly decreased gastric MPO activity compared with DXM group as well as by the histological examinations of gastric epithelium. The
decreased MPO activities by MTK and NSO might be attributed to their abilities to decrease neutrophil infiltration. Also, the anti-inflammatory effects of nigella sativa could be due to its ability to inhibit leukotrienes release (Mansour 2000), neutrophil elastase activity (Kacem and Meraihi 2006) and cyclooxygenase (COX) enzymes activity (Marsik et al. 2005). Future studies are needed to study the effect of DXM on leukotrienes and to determine the leukotrienes affected by MTK and NSO in DXM induced gastric damage.

The anti-inflammatory effects of MTK and nigella sativa were previously reported by Özbakifi-Dengiz et al. (2013) and Abdelwahab et al. (2013). Moreover, DXM administration for 7 days in this study significantly decreased mucus on the surface epithelium and gastric pits compared with normal control group as shown in morphometrical analysis of PAS reaction. The decreased mucus secretion by DXM might be attributed to the decrease in prostaglandins (PGS) synthesis as DXM inhibits PGS synthetase enzyme (Bandyopadhyay et al. 1999). The PGS especially PGE2 and PGI2 play an important role in enhancing mucus secretion and gastric mucosal blood flow (Wallace 2008).

While, treatment with either MTK, or NSO, or both with DXM significantly increased mucus on the surface epithelium and gastric pits especially in
group V compared with DXM group. The ability of NSO to increase mucus production could be due to Thymoquinone which is an important constituent of nigella sativa \textit{(Magdy et al. 2012)}. Thymoquinone increase the biosynthesis of PGS in the stomach \textit{(Tsuji et al. 1990)}. While, the mechanism of MTK- induced gastric mucus secretion are still unclear and need further studies to elucidate it.

PCNA-immunostaining in the present work showed a significant increase in the mean percentage of PCNA-positive cells in the gastric mucosa in DXM group compared with normal control group. PCNA is a proliferation marker, where increased number of PCNA-positive cells indicates the excessive cell proliferation to overcome an extensive damage of epithelial cells \textit{(Romarheim et al. 2011)}. DXM-induced damage of gastric epithelium was proven in this study by light microscopy and this might be due to oxidative stress, inflammation and decreased mucus production. These results were in accordance with \textit{Klein et al. (2016)} who showed that glucocorticoid-induced proliferation in Acute Myeloid leukemia. In contrast to our results, \textit{Luo et al. (2007)} reported that DXM inhibits gastric epithelial cell proliferation.

On the other hand, administration of either MTK, or NSO, or both with DXM significantly decreased the mean percentage of PCNA-positive cells in
the gastric mucosa compared with DXM group. Interestingly, group V showed non-significant changes from normal control group. This result indicates that MTK and NSO could successfully prevent excessive gastric damage induced by DXM probably by antagonizing DXM-induced oxidative stress and inflammation in gastric mucosal wall with stimulation of mucus secretion. This was in accordance with a previous study which argued that MTK had an antiproliferative capacity especially on inflammatory cells (Spinozzi et al. 2004). Moreover, the antiproliferative activity of nigella sativa was previously studied in lung tissue (Rahayu et al. 2012).

Conclusion
We could conclude that dexamethasone induces gastric damage in rat due to promotion of oxidative stress, inflammation, altered proliferation and decrease mucus secretion in rat stomach. While administration of either MTK or NSO or both with DXM counteracts DXM- induced gastric lesions by their antioxidant, anti-inflammatory, and antiproliferative effects with stimulation of mucus secretion in gastric mucosa. The gastroprotective effects of MTK administration along with NSO were better than using each drug separately thus suggesting synergistic gastroprotective effects. Therefore, it is suspected that combined treatment of MTK and NSO with
corticosteroids in asthmatic patients will provide a greater efficacy, protect
against steroids related gastric damage and reduce the cost of treatment of
bronchial asthma that requires chronic drugs treatment. Future studies are
needed to replicate this work on asthmatic patients and evaluate the side
effects of steroids in patient taking (or not) MTK and NSO.

Conflict of interest

The authors declare that there is no conflict of interest associated with this
work.
References


Bandyopadhyay, U., Biswas, K., Bandyopadhyay, D., Ganguly, C.K., and Banerjee, R.K. 1999. Dexamethasone makes the gastric mucosa susceptible to ulceration by inhibiting prostaglandin synthetase and


Table 1. The levels of studied biochemical parameters in different experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA level (nmol/g tissue)</th>
<th>SOD activity (U/mg protein)</th>
<th>MPO activity (U/mg protein)</th>
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<td>Group I</td>
<td>1.22 ± 0.03</td>
<td>1.95 ± 0.03</td>
<td>0.95 ± 0.04</td>
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<td>Group II</td>
<td>2.03 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.39 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.97 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Group III</td>
<td>1.80 ± 0.06&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.36 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.34 ± 0.16&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<tr>
<td>Group IV</td>
<td>1.45 ± 0.04&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.40 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.68 ± 0.05&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group V</td>
<td>1.45 ± 0.04&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.10 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.29 ± 0.06&lt;sup&gt;a,b&lt;/sup&gt;</td>
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Note: Data are given as mean ± SD. <sup>a</sup>p<0.05 vs group I, <sup>b</sup>p<0.05 vs group II.
Fig. 1: H&E staining: a) Normal control group showing normal architecture with intact epithelial lining (notched arrow) and gastric pits (curved arrow). b, c) Group II showing focal exfoliation of the superficial epithelium (asterisk) with an area of hemorrhage (wavy arrow). Vacuolated epithelial cells (thin arrow) and pyknotic nuclei (arrow head) are observed together with marked widening and separation. Notice some mononuclear cells (thick arrows). d) Group III showing pale and vacuolated cells (thin arrows) with evident spacing observed between the glands. Notice some mononuclear cells mainly at the base of the gastric glands (thick arrows). e) Group IV showing some spacing between the glands. Notice many mononuclear cells mainly at the base of the gastric glands (thick arrows). f) Group V showing almost normal gastric mucosa with intact epithelial lining (notched arrow). (Magnification X200, scale bar=100 µm, inset magnification X400, scale bar=50 µm)

Fig. 2: PAS staining: a) Normal control group showing characteristic strong magenta red color of PAS-positive film of mucus on the surface epithelium and gastric pits (arrows). b) Group II showing diffuse weak PAS reaction. c) Group III showing moderate PAS reaction in only some gastric pits. d) Group IV showing strong PAS reaction in the most apical part of the gland. e) Group V showing strong PAS reaction in the apical part of the gland. f)
Morphometrical analysis of PAS reaction mean color intensity, *indicates significance compared with normal control group, Δ indicates significance compared with group II. (Magnification X200, scale bar=100µm)

Fig. 3: PCNA immunostaining: a) Normal control group showing minimal number of PCNA positive cells (arrow), b) Group II showing numerous PCNA positive cells, c) Group III showing some PCNA positive cells, d) Group IV showing many PCNA positive cells, e) Group V showing minimal number of PCNA positive cells, f) Morphometrical analysis of the mean percentage of PCNA positive cells, *indicates significance compared with normal control group, Δ indicates significance compared with group II. (Magnification X200, scale bar=100µm)