

# Histological study of the effect of leflunomide on fundic mucosa of Adult male albino rat and the possible alleviating effect of L-arginine

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Article

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## ABSTRACT

Leflunomide is a disease-modifying anti-rheumatic drug used in the treatment of rheumatoid arthritis due to its anti-inflammatory and immunosuppressive effects. This study aimed to investigate the histological effect of leflunomide on the fundic gastric mucosa of adult male albino rat and the possible ameliorative role of the non-essential amino acid L-arginine. Fifty adult male albino rats were divided randomly into four main groups, group I (control group), group II treated with leflunomide, group III received the L-arginine and group IV received the leflunomide and the L-arginine concomitantly. The four groups were subdivided into two subgroups; the first received the drugs for two weeks and the second received the drugs for four weeks. At the end of the experiment, all rats were sacrificed; the fundic mucosa was dissected out and processed for light and electron microscopic study. Paraffin sections were stained with hematoxylin and eosin, periodic acid Schiff (PAS), Mallory trichrome and immunohistochemically for PCNA antibody. Leflunomide treated (group II) showed sloughing and desquamation of epithelial cells, superficial ulceration, and inflammatory cellular infiltration. Electron microscopic examination revealed absence of apical microvilli and coalescence of secretory granules. L-arginine ameliorated to some extent the histological changes occurred by leflunomide. It can be concluded that leflunomide exerts some histological changes on fundic gastric mucosa and co-administration of L-arginine had ameliorating effects on the histological changes.

**Key Words:** Fundic, L-arginine, leflunomide, mucosa, rat

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## INTRODUCTION

The gastric mucosa is continuously under stress by exposure to various physical and chemical injuries during the digestive process, the dietary contents or even abuse drug intake. The gastric mucosal barrier protects the mucosa from being digested by HCL, enzymes and erosive substances in gastric secretion. This mucosal barrier includes the compact epithelial lining of the stomach and their tight junctions, the mucus layer covering the surface epithelium, bicarbonate ions and the blood supply to the mucosa (Kossi *et al.*, 2001 & Cesar *et al.*, 2006).

Leflunomide is a disease-modifying anti-rheumatic drug (DMARD) used in the treatment of active rheumatoid arthritis. It improves physical function and delays structural damage associated with the disease owing to its anti-inflammatory and immunosuppressive effects. It exerts its role by inhibiting the cell division. After ingestion of leflunomide; it is metabolized to teriflunomide named A771726, which has many side effects on different systems in the body especially the gastrointestinal tract causing gastritis that was aggravated to ulceration at high dose (Rossi, 2013).

The mechanism by which leflunomide induces gastritis and gastric ulcer depends on an interaction between the defense mechanisms of the stomach, gastric acid output and mucosal blood flow (Derosa *et al.*, 2008). The gastric mucosal cells are in a process of continuous proliferation and apoptosis. Both these processes are in balance but if this balance is disturbed either due to increase apoptosis and/or decrease proliferation, mucosal injury will occur. Proliferating Cell Nuclear Antigen (PCNA) as a marker for cell proliferation that reaches its maximum level in the nucleus during late G1/S phase of cell cycle and this indicates the onset of DNA synthesis (Tousson *et al.* 2014).

L-arginine is non-essential amino acid that has multiple biological properties e.g; the ability to stimulate anabolic hormone secretion, improving nitrogen balance, accelerating wound healing and increasing immunity (Cynober, 1994). Nitric oxide (NO) is produced from L-arginine by endothelial NO synthase and plays an important role in regulating endothelium-dependent vasodilatation, preventing the adhesion of blood cells and platelets along the lining endothelial cells and has scavenging effects against oxygen radical species (Morita *et al.*, 2014). Many researchers demonstrate the benefits of

L-arginine in alleviation of gastric ulcer induced by non-steroidal anti-inflammatory drugs as indomethacin and ibuprofen (Khattab *et al.*, 2001, Sanchez-Fidalgo *et al.* 2005 and Ajiboye *et al.* 2014). The aim of the present work was to study the histological and immunohistochemical effect of leflunomide on the fundic mucosa of adult male albino rat and the possible ameliorative role of L-arginine.

## PATIENTS AND METHODS

### **Drugs:**

Leflunomide was obtained in the form of vials, each contained 1gm of the powder. The experimental dose of leflunomide was 10mg/kg/day dissolved in distilled water, considering that each 1ml of distilled water contains 1.5 mg/kg of leflunomide was given to the animal by gastric gavage once daily (Hagras *et al.*, 2014).

L-arginine was in the form of white powder. The received dose of L-arginine was 200mg/kg/day dissolved in 1 ml of distilled water and was given orally by gastric gavage (Brzozowski *et al.*, 1997 & Kandeel *et al.*, 2013). Both drugs were obtained from Sigma Chemical Company, Cairo, Egypt.

### **Animals:**

The present study was carried out on fifty adult male albino rats, with an average weight of 120-150gm each. All the animals were housed in suitable clean properly ventilated cages under similar conditions in a 12h light/dark cycle and were fed on a similar commercial laboratory diet and water ad libitum. They were acclimatized to their environment for one week before starting the experiment that was approved by the local Ethical Committee of Faculty of Medicine, Tanta University (Nehru *et al.*, 2007). All the medications were given orally prior to feeding in the morning.

### **Experimental protocol**

#### **The animals were divided into four main groups:**

Group I (control group): consists of 20 rats subdivided equally into two subgroups;

Subgroup Ia kept without treatment to study the normal histological structure of fundic mucosa. Subgroup Ib received 1 ml distilled water daily and orally.

Group II (leflunomide-treated group): included 10 rats that received leflunomide in a dose of 1.5.mg orally and daily.

Group III (L-arginine-treated group): included 10 rats that received L-arginine in a dose of 200mg/kg orally and daily.

Group IV (Leflunomide and L-arginine-treated group): included 10 rats that received l-arginine and leflunomide at the same time with the same previous doses daily and orally. All animals were subdivided equally into two subgroup (A) and subgroup (B). Half of animals of the four groups were sacrificed after 2 weeks and the other half after 4 weeks.

After 24 hours from the last administration, the animals were fasted overnight and were anesthetized with intra peritoneal sodium thiopental injection as general anesthesia. The stomach was dissected out, opened at the greater curvature and washed with saline (Karthikeyan *et al.*, 2009). The specimens were taken immediately from the glandular stomach below the limiting ridge and processed for histological, immunohistochemical examination and electron microscopic study.

### **Light microscopic examination:**

Specimens from the fundus of stomach were immediately fixed in 10% neutral formol saline, dehydrated, cleared and embedded in paraffin. Sections of 5µm thickness were stained with haematoxylin and eosin (H&E) for routine histological examination, Periodic Acid Schiff for detection of mucopolysaccharides and Mallory trichrome stain for detection of collagen fibers in the lamina propria and submucosa (Culling *et al.*, 1985, Kiernan, 2008 and Suvarna *et al.*, 2013).

### **Immunohistochemical examination:**

It was carried by using avidin-biotin peroxidase technique for localization of proliferating cell nuclear antigen (PCNA) by using primary antibodies (anti-PCNA monoclonal antibody, dilution 1: 400-800, obtained from Lab Vision Company). Slides were counterstained with hematoxylin before mounting. The negative control sections were prepared by excluding the primary antibodies (Bancroft & Gamble, 2008 and Sharma & Gandhi, 2012).

### **Electron microscopic examination:**

The specimens were fixed in 2.5% phosphate buffered glutaraldehyde, processed and embedded in epoxy resin by routine protocol. Semithin sections (1 µm thick) were obtained and stained with 5% toluidine blue and examined by light microscope. Ultrathin sections (80-100nm) were stained with uranyl acetate and lead citrate to be examined by (JEOL-JEM-100 SX electron microscope, Japan) electron microscope in E.M unit, Tanta, Faculty of Medicine, Tanta University (Bozzola & Russell, 1999).

### **Morphometric study:**

The image analysis software program (Image j. 1.46version) was used to examine sections from the fundic mucosa in all groups. The optical density of PAS stain was

measured in 5 randomly different microscopic fields for each specimen at a magnification power of (x400). The area percentage of collagen fibers with Mallory trichrome stain was measured in 5 randomly different microscopic fields for each specimen at a magnification power of (x400). The number of PCNA positive cells of the fundic glands were counted in 10 randomly selected different microscopic fields for each specimen at a magnification power of (x400).

#### **Statistical analysis:**

The data obtained were analyzed using SPSS software version 13 (SPSS Inc., Chicago, IL, USA), then compared by one way analysis of variance (ANOVA) test followed by Tukey's test to compare different groups with the control group. The results were expressed as mean  $\pm$  standard deviation (SD). The differences were considered statistically significant if probability value  $P < 0.05$  and highly significant if  $P < 0.001$  and non-significant if  $p > 0.05$  (Davies & Crombie, 2009).

## **RESULTS**

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### ***H & E-stained sections:***

Group I (control group): The fundus of the stomach of control group showed thick mucosa separated from the underlying submucosa by muscularis mucosa. The lamina propria is occupied by tall straight densely packed gastric glands. Each one consists of isthmus, neck and base with narrow gastric pits. The gastric glands were lined by tall columnar mucous secreting cells having basal oval nuclei, low columnar mucous neck cells with basal flattened nuclei and large rounded parietal cells with acidophilic cytoplasm and central rounded nuclei (Fig.1).

### ***Group II (leflunomide-treated group):***

subgroup (IIA) showed separation of gastric glands, shortening of gastric pits, desquamation and sloughing of surface epithelial cells into the lumen. Congested blood vessels and inflammatory cellular infiltration were noticed in the submucosa (Fig.2a). Some parietal cells appeared with vacuolated cytoplasm while others with deep acidophilic cytoplasm and dark stained nuclei (Fig.2b). while subgroup (IIB) revealed affection of gastric mucosa in the form of focal areas of ulceration reaching the muscularis mucosa with detaching of fundic mucosa from the underlying muscularis mucosa, also there was wide separation between the fundic glands and widening of gastric pits (Fig.3a). Many parietal cells appeared ballooned and vacuolated with eccentric pyknotic nuclei (Fig.3b).

Group III (L-arginine-treated group): Examined sections of the fundus of the stomach of both subgroups

IIIA & IIIB showed more or less the same histological structure of the fundus as that of the control group (Fig.4).

Group IV (L-arginine and leflunomide-treated group): Subgroup (IVA) revealed some improvement in the ultrastructural changes in the fundic mucosa than group II. It showed thick mucosa with tall, straight and packed gastric glands with variable gastric pits. Most of the lining cells appeared normal except few vacuolated parietal cells with mononuclear inflammatory cells were aggregated at the base of the glands in lamina propria (Fig.5a). Subgroup (IVB) showed thick mucosa containing straight packed glands with variable gastric pits and nearly normal appearance of their lining cells except presence of few ballooned vacuolated parietal cells (Fig.5b).

### ***Periodic Acid Schiff (PAS) stained sections:***

Examination of PAS stained sections obtained from the fundic mucosa of both group (I) and group (III) revealed PAS positive reaction covering the surface of gastric mucosa and extending downwards to fill the gastric pits & isthmus of the glands while weak PAS positive reaction was observed at the base of the glands (Fig.6a). In subgroup (IIA) there was localized decrease of PAS reaction of the surface mucous coat, in the isthmus & neck of the glands (Fig.6b), while sections of subgroup (IIB) showed marked decrease of the PAS reaction with areas of focal loss (Fig.6c). Sections from the fundic mucosa of subgroups (IVA & IVB) revealed PAS positive reaction covering the intact surface epithelium and extending to fill the gastric pits (Figs.6d&6e).

### ***Mallory trichrome stained sections:***

Examination of Mallory trichrome stained sections obtained from the fundic mucosa of both group I and group (III) revealed few collagen fibers between the fundic glands and in the submucosa (Fig.7a). Moderate increase in collagen fibers between the fundic glands, in the lamina propria especially at the base of the gland and in the submucosa was observed in subgroup (IIA) (Fig.7b), while sections of subgroup (IIB) showed marked deposition of collagen fibers inbetween the fundic glands, in the lamina propria at the base of glands and in the submucosa (Fig.7c). Sections from subgroups (IVA & IVB) revealed few collagen fibers between fundic glands more or less similar to normal (Figs.7d&7e).

### ***Immunohistochemical results:***

Examination of PCNA immunostained sections obtained from the fundic mucosa of both group (I) and group (III) revealed strong positive PCNA immune reaction in many nuclei of cells lining the isthmus and the

neck of gastric glands (Fig.8a). Weak immune reaction in few nuclei of cells lining the isthmus and neck of the glands was observed in subgroup (IIA) (Fig.8b), whereas subgroup (IIB) revealed very weak PCNA reaction (Fig.8c). Sections of subgroup (IVA) showed strong PCNA immune reaction in many nuclei of cells of the isthmus and neck regions of gastric glands (Fig.8d), while subgroup (IVB) showed moderate increase in PCNA immune reaction (Fig.8e).

### ***Electron microscopic results:***

Group I (control group): Examination of ultrathin sections of the mucosa of fundic glands revealed surface columnar cells which were high columnar having apical microvilli, apical mucinogen granules, and basal oval nuclei with prominent nucleoli (Fig.9). Mucous neck cells appeared low columnar with apical microvilli, numerous apical secretory granules, basal rER and basal nuclei with prominent nucleoli (Fig.10). Parietal cells were large pyramidal containing intracellular canaliculi lined with long microvilli, numerous mitochondria having regular prominent cristae with rER in-between, Golgi cisternae and central spherical nucleus with normal chromatin distribution (Fig.11).

Group II (leflunomide-treated group): Examination of the surface columnar cells of subgroup (IIA) showed sparse apical microvilli and decreased amount of apical secretory granules, indentation of the nucleus, nucleolar margination and wide intercellular spaces (Fig.12). Mucous neck cells revealed coalescence of apical secretory granules and dilated rER (Fig.13). Parietal cells showed cytoplasmic vacuoles, normal mitochondria and shrunken indented nucleus with dilated perinuclear space (Fig.14). By TEM, subgroup (IIB) showed surface columnar cells with few apical microvilli, few apical secretory granules, cytoplasmic vacuoles and indented nucleus with nucleolar margination and wide perinuclear space (Fig.15). Mucous neck cells showed absence of apical microvilli, coalescence of mucous secretory granules, normal mitochondria, markedly dilated rER and perinuclear vacuolation (Fig.16). Parietal cells revealed flattening in its shape, dilatation of intracellular canaliculi, normal mitochondria, cytoplasmic vacuoles and basal nucleus with nucleolar margination (Fig.17).

Group III (L-arginine-treated group): Subgroups IIIA and IIIB revealed normal ultrastructural features of gastric mucosa.

Group IV (L-arginine and leflunomide treated group): Subgroup (IVA) revealed some improvement in the ultrastructural changes in the fundic mucosa. Most of surface columnar cells and mucous neck cells appeared normal with apical microvilli, apical mucous granules, basal rER and normal basal oval nucleus (Figs.18&19). Most of parietal cells looked normal except for slight nuclear indentation (Fig.20). Electron microscopic examination of surface columnar cells, mucous neck cells and parietal cells of subgroup (IVB) revealed marked improvement (Fig. 21, 22 & 23).

### ***Morphometric results and statistical analysis:***

1. Statistical results for the optical density of PAS stain:

The mean optical density of PAS stain in group (III) revealed non-significant change when compared with control group (I). The leflunomide treated subgroups IIA & IIB revealed statistically highly significant decrease when compared with control group, while the L-arginine & leflunomide treated subgroup (IVA) showed non-significant change from control group and subgroup (IVB) showed highly significant decrease compared with the control group (Table 1 & Histogram 1).

2. Statistical results for the area percentage of collagen fibers with Mallory trichrome stain:

The mean area percentage of collagen in the L-arginine treated group (III) revealed non-significant change compared with control group (I). Subgroup (IIA) revealed non-significant increase compared with control group while subgroup (IIB) showed highly significant increase. Subgroups IVA & IVB showed non-significant change compared with control group (Table 2 & Histogram 2).

3. Statistical results for the PCNA immunoreactivity:

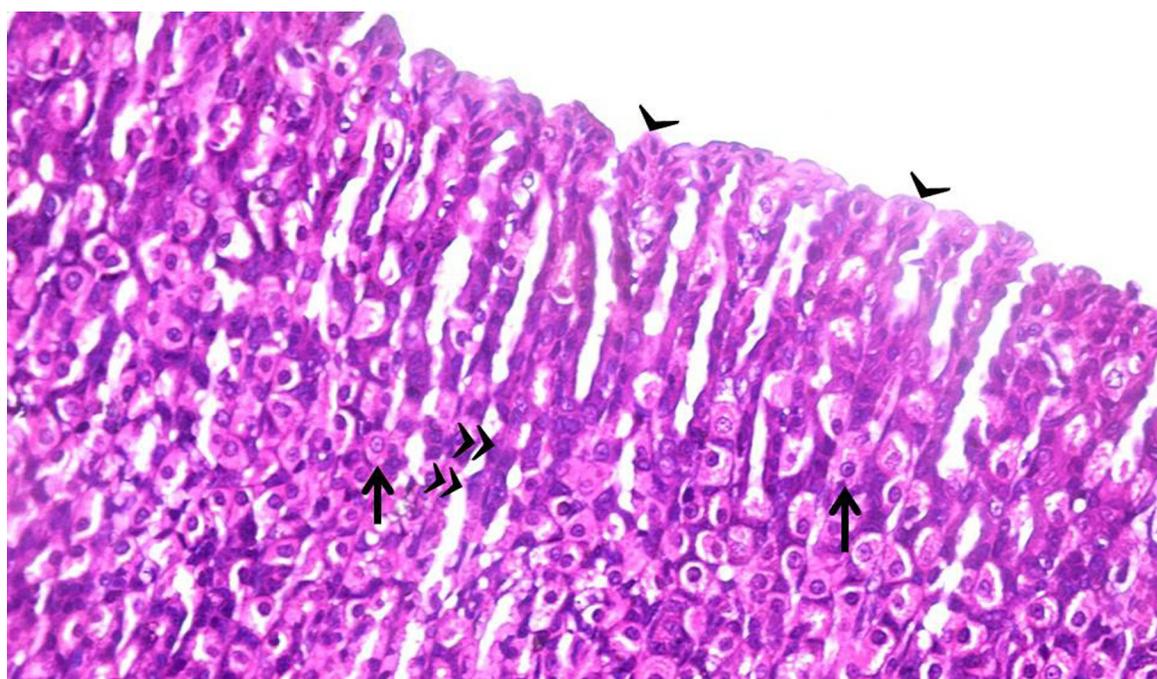
The mean number of PCNA positive cells in the fundic glands in the L-arginine treated group (III) revealed non-significant change compared with control (group I). Subgroups IIA & IIB revealed highly significant decrease compared with control group. Subgroups IVA & IVB showed significant increase compared with control group (Table 3 & Histogram 3).

Table (1): The mean optical density of PAS stain in different studied groups.

Groups	Optical density of PAS					ANOVA	
	Range		Mean	±	SD	F	P-value
Group I	53.39 -		57.24	55.522 ±	1.543	69.261	<0.001**
Group IIA	35.62 -		46.73	41.538 ±	4.731		
Group IIB	23.7 -		31.38	28.244 ±	3.187		
Group III	52.67 -		56.74	54.784 ±	1.661		
Group IVA	51.58 -		55.47	53.352 ±	1.413		
Group IVB	43.18 -		49.83	46.758 ±	2.894		
Tukey's Test							
	Group I (control)	Group IIA	Group IIB	Group III	Group IVA		
Group IIA	<0.001**						
Group IIB	<0.001**	<0.001**					
Group III	0.998 NS	<0.001**	<0.001**				
Group IV A	0.827 NS	<0.001**	<0.001**	0.965 NS			
Group IV B	0.001**	0.072	<0.001**	0.002**	0.013*		

SD: standard deviation, P value > 0.05: non-significant, P value < 0.05: significant, P value < 0.001(\*): highly significant.

**Histogram (1)** : The mean optical density of PAS in different studied groups.



**Table (2) :** The mean area percentage of collagen with Mallory trichrome stain in different studied groups.

Groups	Area% of collagen in the treated group				ANOVA			
	Range	Mean	±	SD	F	P-value		
Group I	14.47 - 23.68	19.072	±	3.467				
Group IIA	20.22 - 26.58	23.600	±	2.581				
Group IIB	28.81 - 35.14	30.722	±	2.706	8.403	<0.001**		
Group III	13.19 - 26.25	19.500	±	5.927				
Group IVA	18.16 - 22.53	19.786	±	1.818				
Group IVB	18.42 - 24.01	21.390	±	2.355				
Tukey's Test								
	Group I (control)	Group IIA	Group IIB	Group III			Group IVA	
Group IIA	0.322 NS							
Group IIB	<0.001**	0.032*						
Group III	1.000 NS	0.427 NS	<0.001**					
Group IV A	0.999 NS	0.505 NS	<0.001**	1.000 NS				
Group IV B	0.887 NS	0.906 NS	0.003**	0.949 NS	0.974 NS			

SD : standard deviation, P value > 0.05: non-significant, P value < 0.05: significant, P value < 0.001(\*): highly significant.

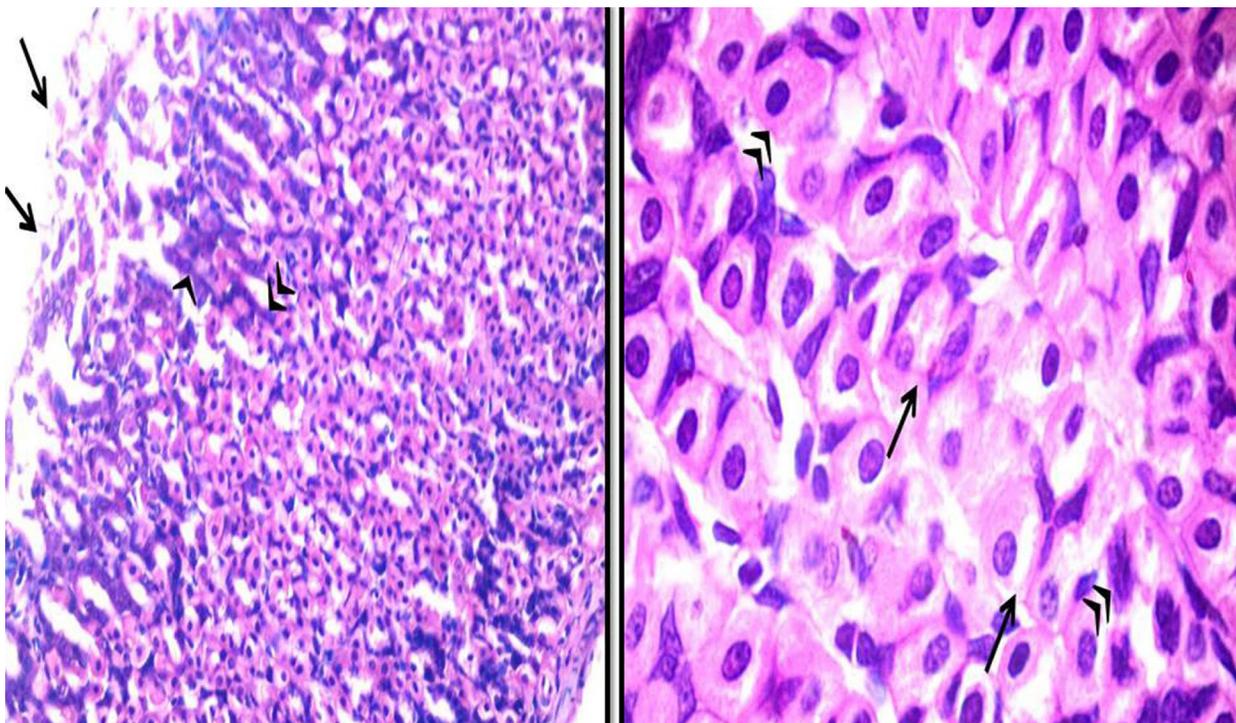
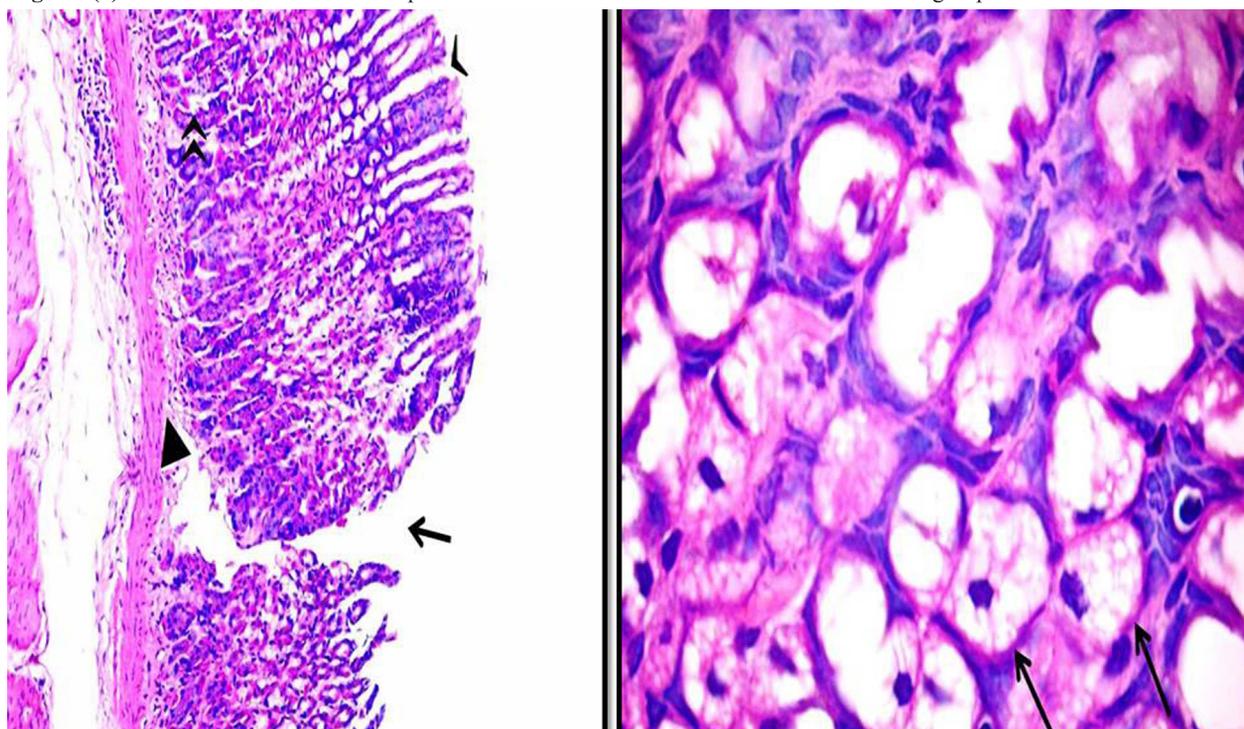
**Histogram (2):** The mean area percentage of collagen with Mallory trichrome stain in different studied groups.

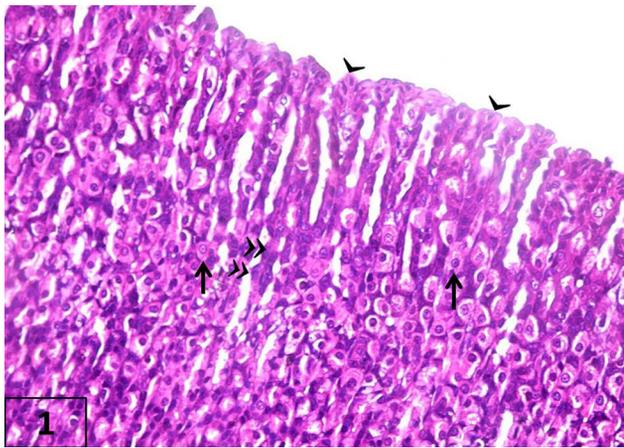
Table (3): The mean number of PCNA positive cells of the fundic mucosa in different studied groups.

Groups	Number of PCNA Positive cells					ANOVA	
	Range		Mean	±	SD	F	P-value
Group I	45	-	52	47.900 ±	2.923		
Group IIA	22	-	48	36.700 ±	8.744		
Group IIB	22	-	33	28.500 ±	3.808	67.409	<0.001**
Group III	40	-	55	46.200 ±	4.590		
Group IVA	53	-	55	54.200 ±	0.789		
Group IVB	59	-	63	61.100 ±	1.370		
Tukey's Test							
	Group I (control)	Group IIA	Group IIB	Group III	Group IVA		
Group IIA	<0.001**						
Group IIB	<0.001**	0.002**					
Group III	0.959 NS	<0.001**	<0.001**				
Group IV A	0.034*	<0.001**	<0.001**	0.003**			
Group IV B	<0.001**	<0.001**	<0.001**	<0.001**	0.015*		

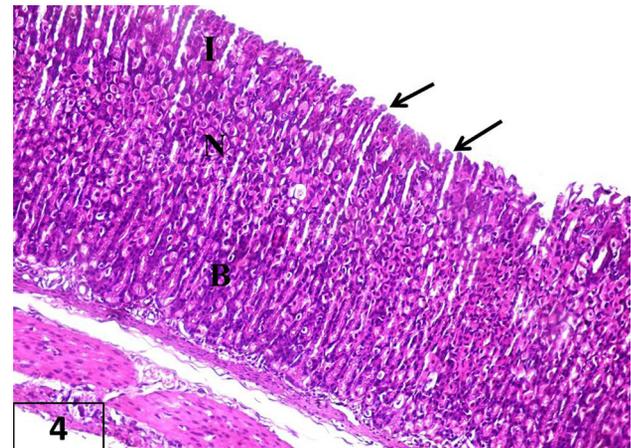
SD: standard deviation, P value > 0.05: non-significant, P value < 0.05: significant, P value < 0.001(\*): highly significant.

**Histogram (3):** The mean number of PCNA positive cells of the fundic mucosa in different studied groups.

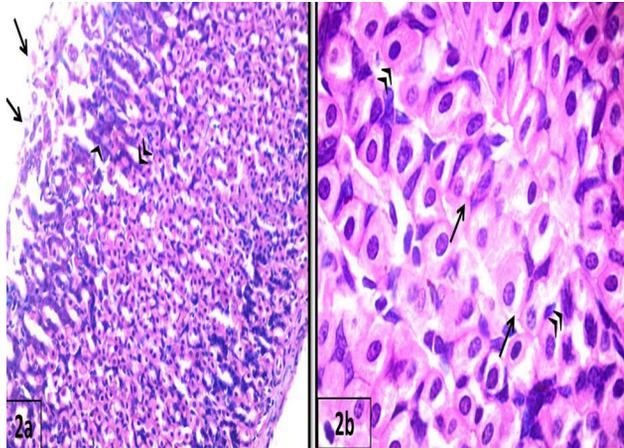




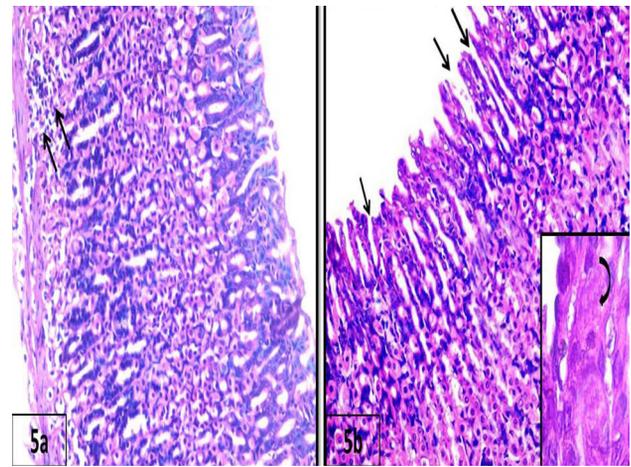
**Fig. (1):** A photomicrograph of the fundic gastric mucosa of the control group, showing long, straight, packed gastric glands lined with normal surface columnar cells with basal oval nuclei (◁), mucous neck cells with basal flattened nuclei (◁◁) and parietal cells with central rounded nuclei (→) (H&E x 400).



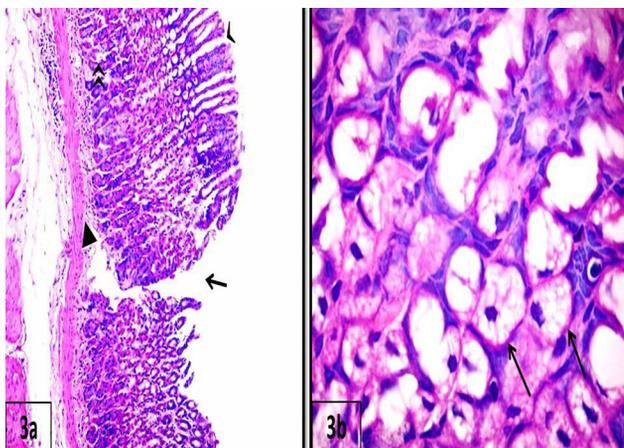
**Fig. (4):** A photomicrograph of subgroup IIIB, showing apparently normal mucosa (→) consisting of packed glands formed of isthmus (I), neck (N) and base (B) (H&E x 200).



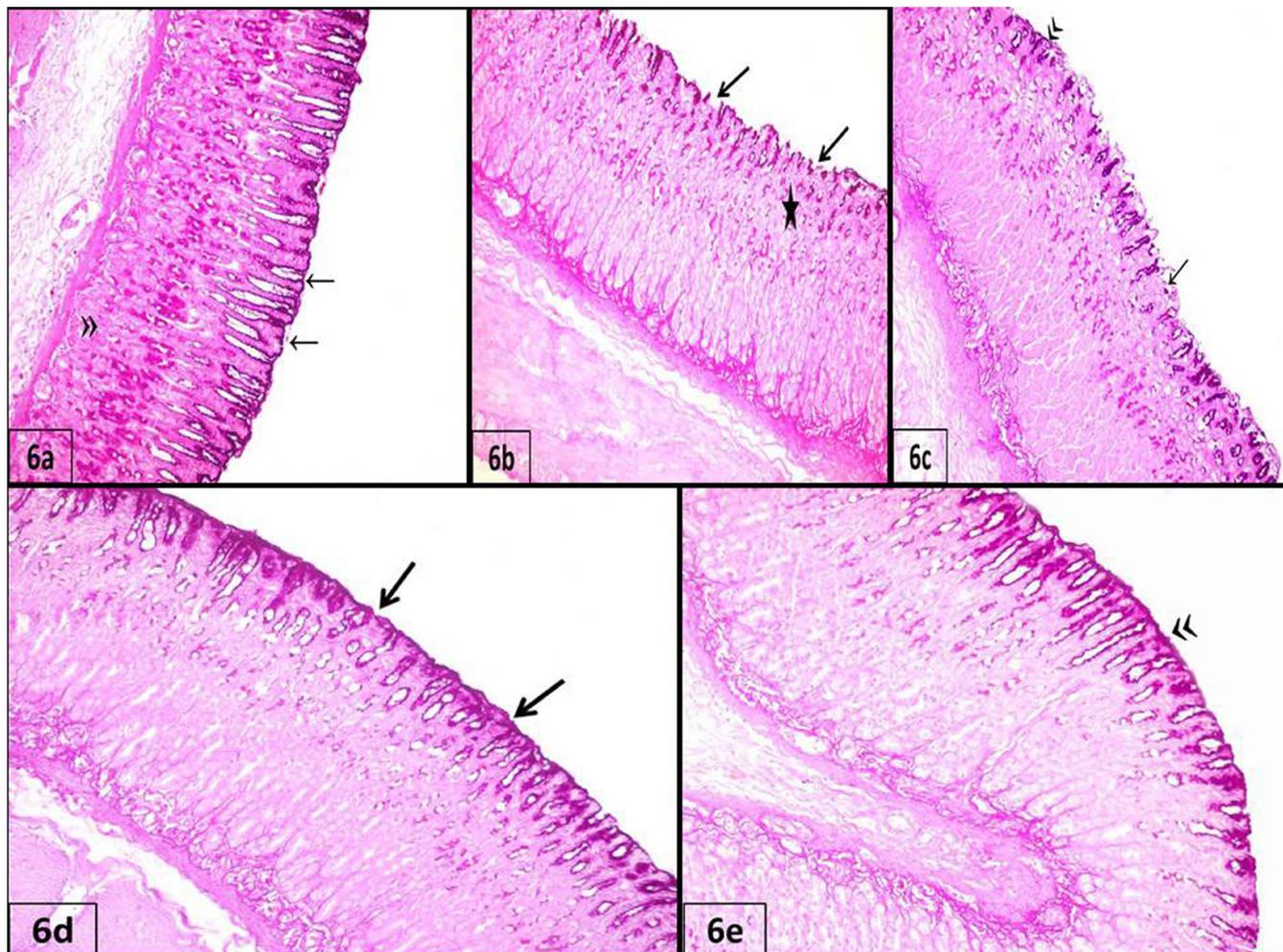
**Fig. (2):** A photomicrograph of the fundic gastric mucosa of subgroup IIA; (2a) showing separation in the gastric glands (▶), short gastric pits (\*), sloughing and desquamation of surface epithelial cells into the lumen (→) while (2b) showing parietal cells with vacuolated cytoplasm (→), others with deeply acidophilic cytoplasm and deeply stained nuclei (◁◁) (H&E 2a x 400 & 2b x 1000).



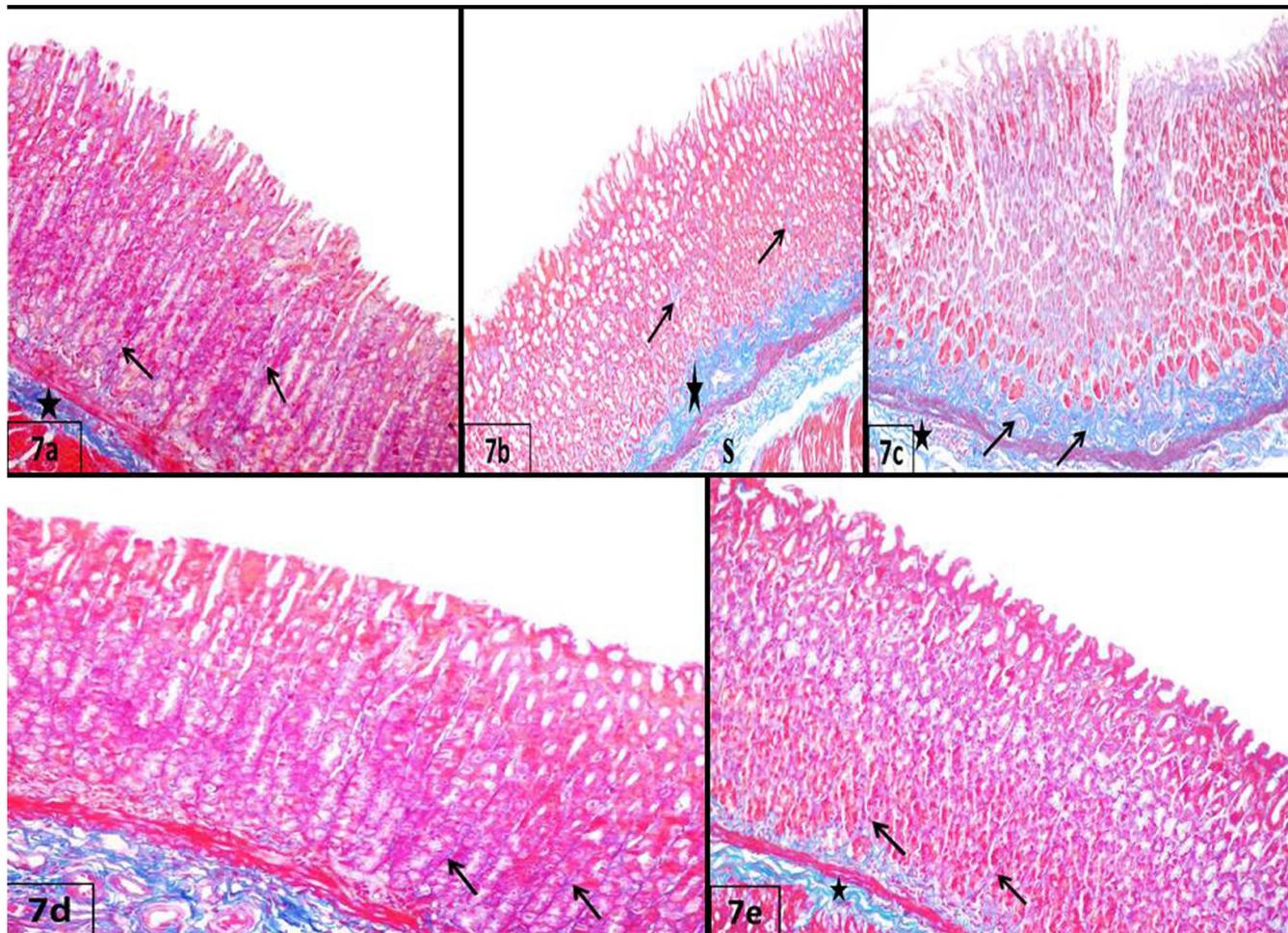
**Fig (5):** A photomicrograph of subgroup IVA; (5a) showing thick mucosa with variable gastric pits, some parietal cells appeared with vacuolated cytoplasm ( ) and aggregated inflammatory cells are noticed in the base of lamina propria (→) while (5b) showing straight packed, parallel gastric glands with variable gastric pits (→). The inset shows ballooned parietal cells (curved arrow) (H&E 5a & 5b x 400. (Inset x 1000)).



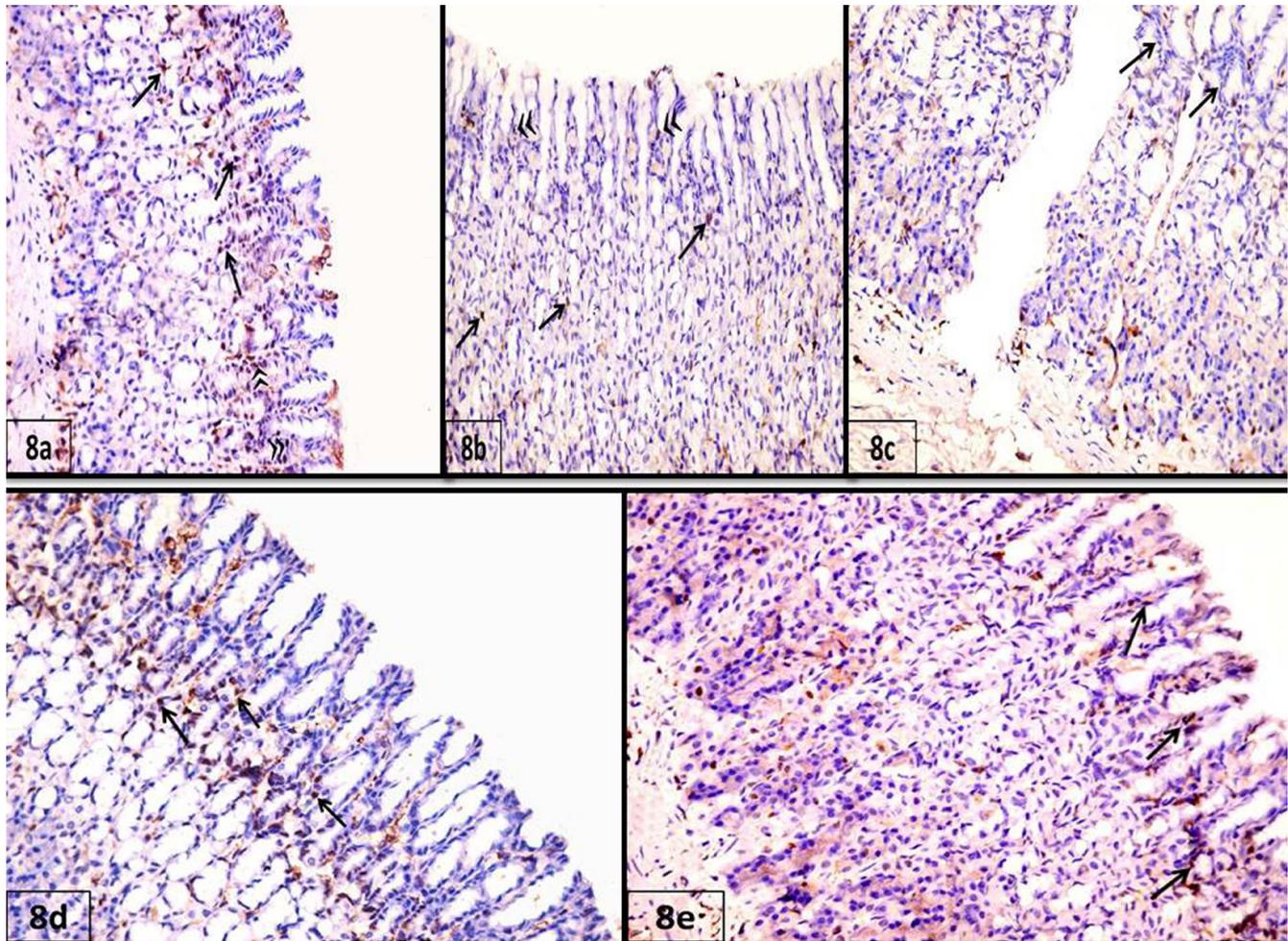
**Fig. (3):** A photomicrograph of subgroup IIB; (3a) showing deep ulceration (→) reaching the muscularis mucosa with detaching of fundic mucosa from the underlying muscularis mucosa (▶). Notice: widening of gastric pits (◁) and separation between fundic glands (◁◁) while (3b) showing marked ballooning & vacuolations of parietal cells with eccentric pyknotic nuclei (→) (H&E 3a x 200 & 3b x 1000).



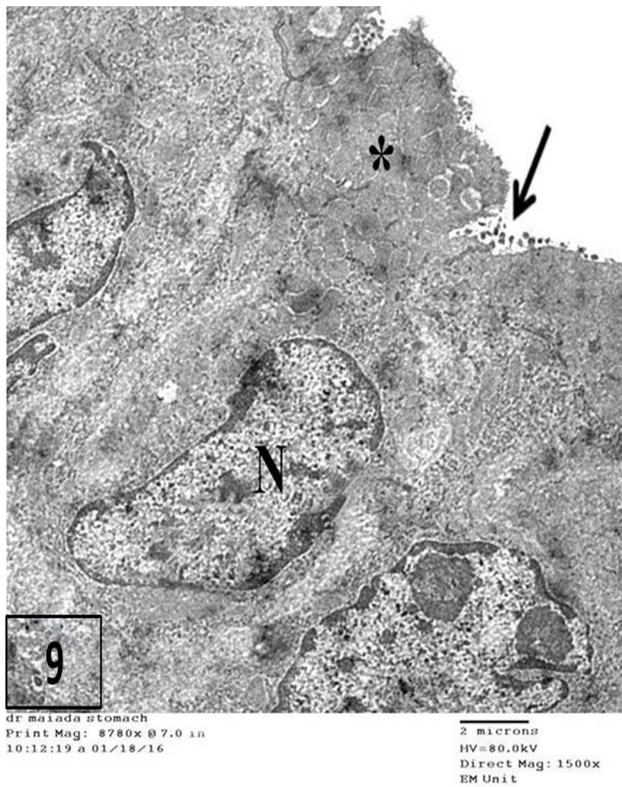
**Fig. (6):** A photomicrograph of PAS stained fundic gastric mucosa of different studied groups: (6a control group) showing strong PAS positive material (→) covering the surface of gastric mucosa and extending downwards to fill the gastric pits & isthmus of the glands and weak reaction at the base of the glands (»). (6b subgroup IIA) exhibiting localized decrease of PAS reaction of surface mucous coat (→) and in the isthmus and neck of the glands (\*). (6c subgroup IIB) displaying marked decrease of PAS reaction of the surface mucous coat (») with areas of focal loss (→). (6d subgroup IVA) showing PAS positive reaction covering the mucosal surface (→) and extending to line the gastric pits. (6e subgroup IVB) depicting PAS positive material covering the surface columnar cells of (») extending to fill the pits (PAS X 200)



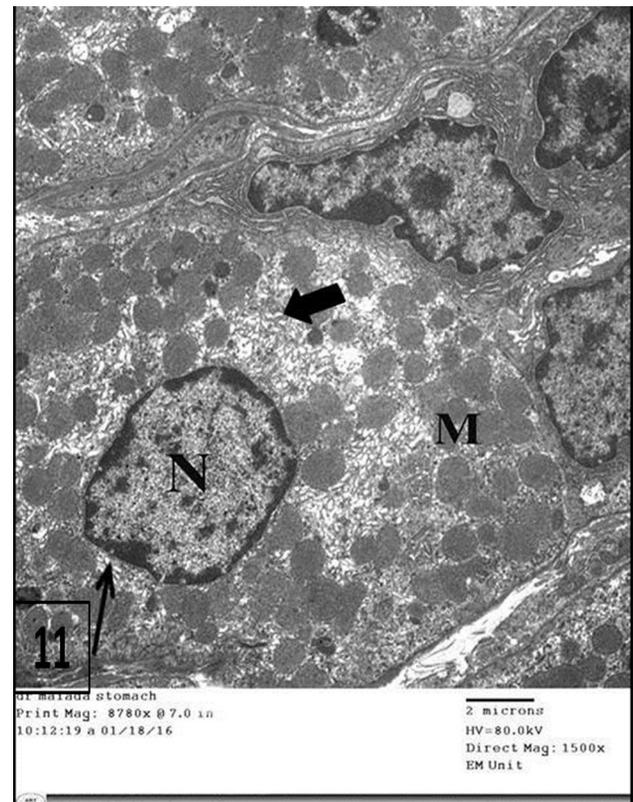
**Fig. (7):** A photomicrograph of Mallory trichrome stained fundic gastric mucosa of different studied groups; (7a control group) showing few amount of collagen fibers between the fundic glands (→) and mild in the submucosa (\*). (7b subgroup IIA) showing moderate increase in collagen fibers between the fundic glands (→), in the lamina propria especially at the base of the glands (\*) and the submucosa (S). (7c subgroup IIB) showing marked deposition of collagen fibers in between fundic glands, in the lamina propria (→) and in the submucosa (\*). (7d subgroup IVA) showing few collagen fibers between fundic glands (→). (7e subgroup IVB) showing few collagenous fibers between the fundic glands (→) but great in the submucosa (\*) (Mallory trichrome X 200).



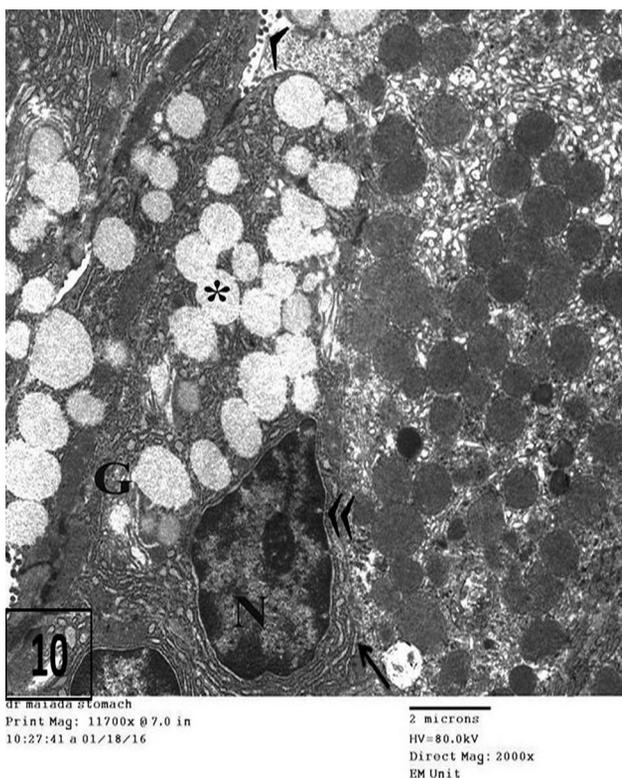
**Fig. (8):** A photomicrograph of PCNA immunostained fundic gastric mucosa of different studied groups. (8a control group) showing strong positive brown PCNA immunoreactivity in many nuclei of cells lining the isthmus (») & neck of the gastric glands (→). (8b subgroup IIA) revealing weak immunoreaction in few nuclei of cells lining the isthmus (») & neck of gastric glands of (→). (8c subgroup IIB) showing very weak PCNA immunoreactivity in the nuclei of cells lining the neck of gastric glands (→). (8d subgroup IVA) showing strong PCNA immunoreactivity in many nuclei of cells lining the isthmus & neck of gastric glands of (→). (8e subgroup IVB), showing moderate PCNA immunoreactivity in the nuclei of cells lining the isthmus of gastric glands (→) ( PCNA immunostaining, with counter stain Haematoxylin x 400).



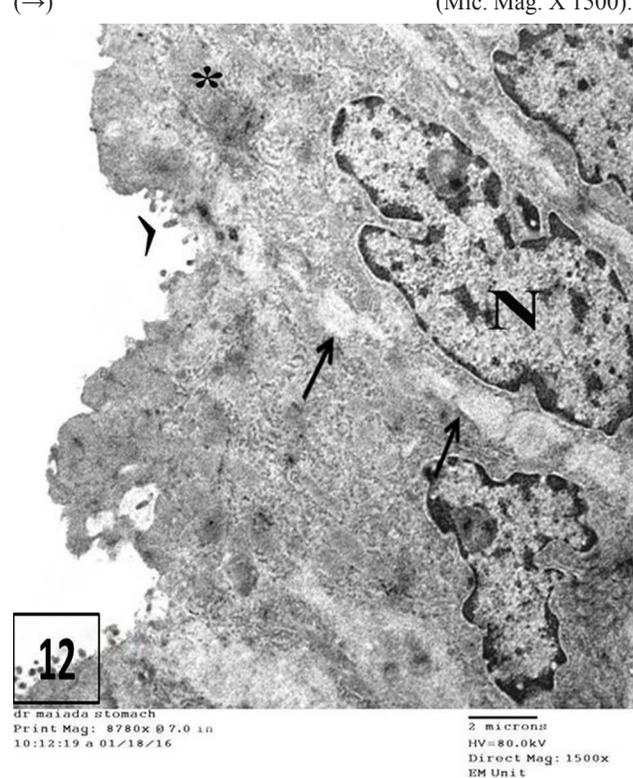
**Fig. (9):** An electron micrograph of surface columnar cell of the control group appeared tall columnar cell having apical microvilli (→), apical mucinogen granules (\*) and basal oval nucleus (N) with prominent nucleolus (Mic. Mag. X 1500).



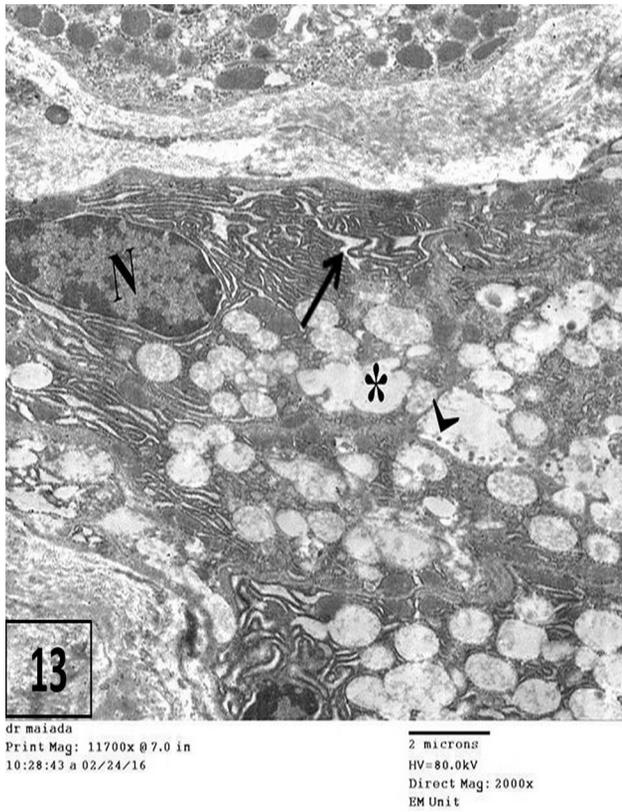
**Fig. (11):** An electron micrograph of parietal cell of the fundic gastric mucosa of the control group showing, large pyramidal cell having; intracellular canaliculi (→), numerous mitochondria (M) and central spherical nucleus (N) with prominent nuclear pores (→) (Mic. Mag. X 1500).



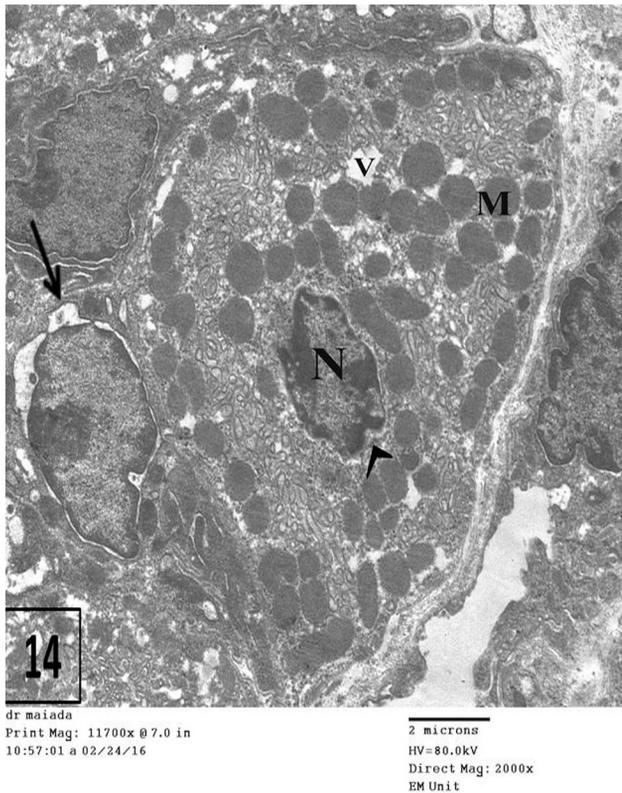
**Fig. (10):** An electron micrograph of mucous neck cell of the control group showing, apical microvilli (→), apical numerous secretory granules (\*), Golgi apparatus (G), basal rER (→) and basal nucleus (N) with prominent nucleolus and nuclear pores (⇨) (Mic. Mag. X 2000).



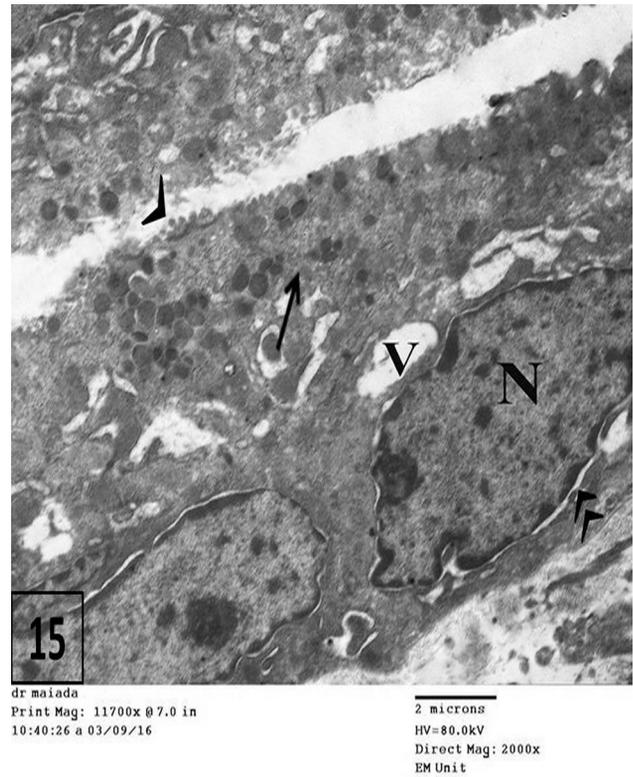
**Fig. (12):** An electron micrograph of surface columnar cell of subgroup IIA showing sparse apical microvilli (→), little amount of secretory granules (\*), wide intercellular space (→) and the nucleus appeared indented (N) and (Mic. Mag. X 1500).



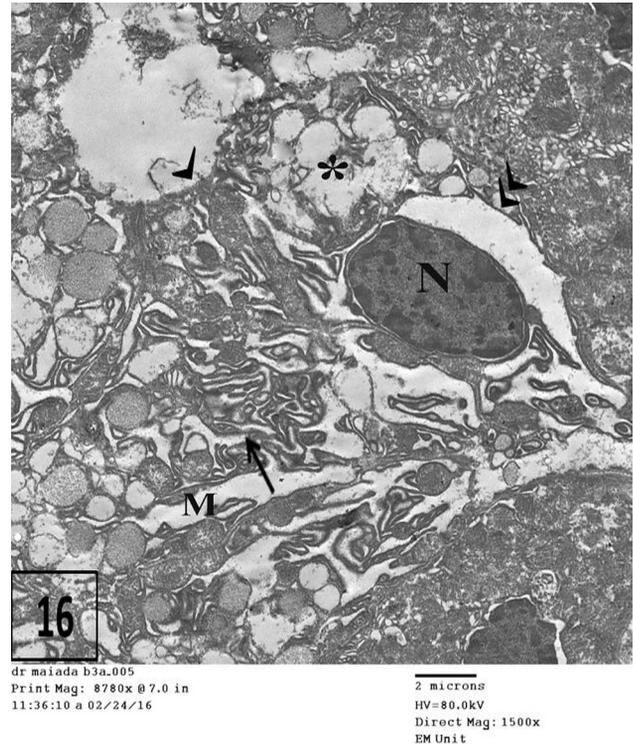
**Fig. (13):** An electron micrograph of mucous neck cell of subgroup IIA showing few microvilli (◁), coalescence of secretory granules (\*) and dilated rER (→) (Mic. Mag. X 2000).



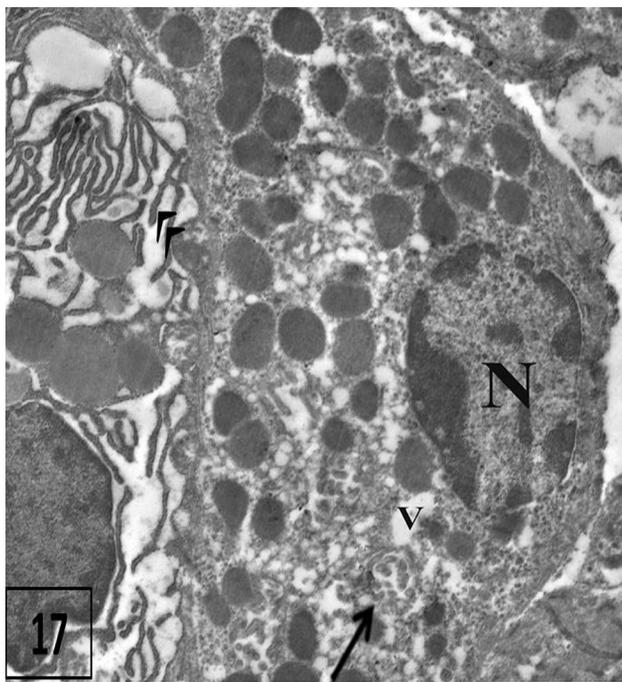
**Fig. (14):** An electron micrograph of parietal cell of subgroup IIA showing; few cytoplasmic vacuoles (V), normal mitochondria (M), indented shrunken nucleus (N) (◁) and dilated perinuclear space (→) (Mic. Mag. X 2000).



**Fig. (15):** An electron micrograph of surface columnar cells of subgroup IIB showing few apical microvilli (◁), few apical mucinogen granules (→), cytoplasmic vacuoles (V), basal indented nuclei (N), nucleolar margination and wide perinuclear spaces (») (Mic. Mag. X 2000).



**Fig. (16):** An electron micrograph of mucous neck cell of subgroup IIB showing, absence of apical microvilli (◁), coalescence of secretory granules (\*), markedly dilated rER (→), normal mitochondria (M) and perinuclear vacuolation (») (Mic. Mag. X 1500).



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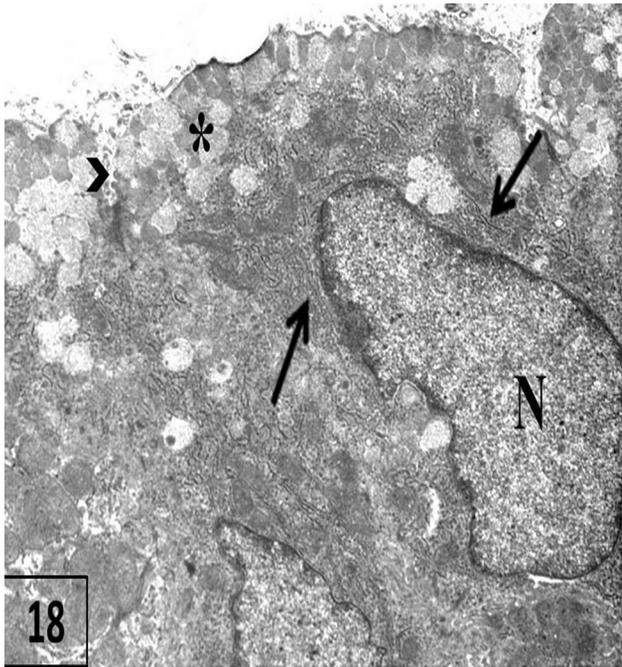
**Fig. (17):** An electron micrograph of parietal cell of subgroup IIB showing flattening of the cell (>), cytoplasmic vacuoles (V), normal mitochondria (M), dilated intracellular canaliculi (->), dilated rER (>>) and basal nucleus (N) (Mic. Mag. X 2000).



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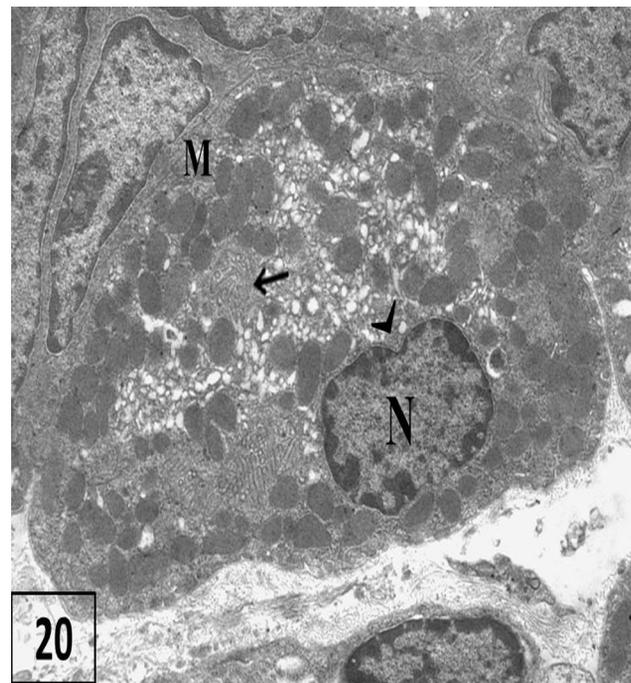
**Fig. (19):** An electron micrograph of two mucous neck cells of subgroup IVA showing few apical microvilli (>), presence of apical secretory granules (\*), basal rER (->) and basal oval nuclei (N) with prominent nucleoli and nuclear pores (>>) (Mic. Mag. X 2000).



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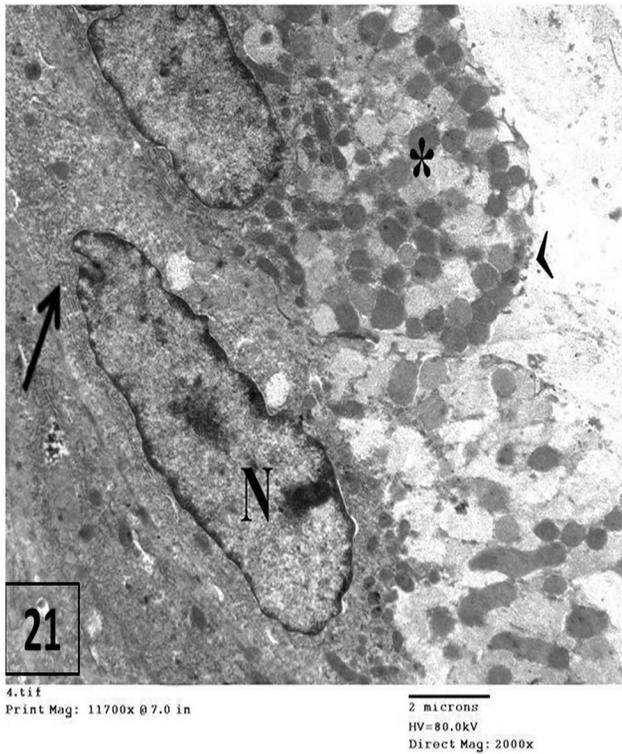
**Fig. (18):** An electron micrograph of surface mucous cell of the fundic gastric mucosa of subgroup IVA showing, columnar cell having normal apical microvilli within the gastric lumen (>), apical secretory granules (\*), basal rER (->) and basal oval nucleus (N) with regular nuclear membrane and extended chromatin (Mic. Mag. X 2000).



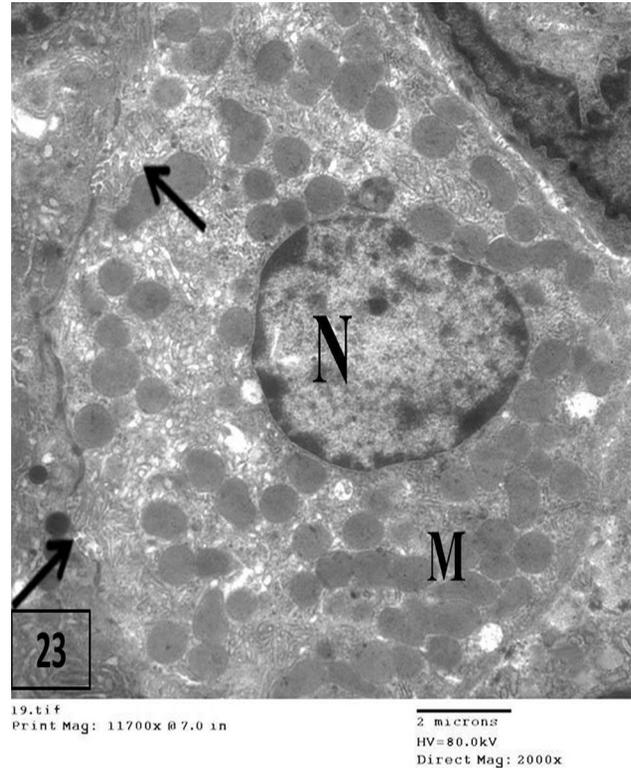
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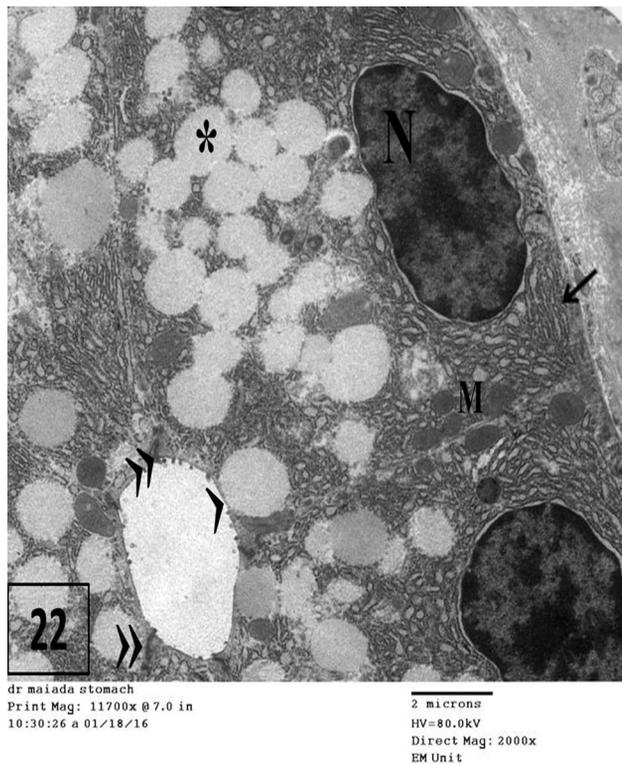
**Fig. (20):** An electron micrograph of parietal cell of subgroup IVA showing, more or less normal parietal cell having normal intracellular canaliculi (>), numerous mitochondria (M) and slight indentation of the nuclear membrane (->) (Mic. Mag. X 2000).



**Fig. (21):** An electron micrograph of surface columnar cell of subgroup IVB showing, apical microvilli (◁), apical numerous secretory granules (\*), basal rER (→) and basal oval nucleus (N) with prominent nucleolus (Mic. Mag. X 2000).



**Fig. (23):** An electron micrograph of parietal cell of subgroup IVB showing, pyramidal cell with intracanalicular lined with microvilli (→), rounded to oval normal mitochondria (M) and nearly central spherical nucleus (N) (Mic. Mag. X 2000).



**Fig. (22):** An electron micrograph of mucous neck cell of subgroup IVB showing, few apical microvilli (▷), normal intercellular junctions & intercellular spaces (»), coalescence of apical secretory granules (\*), basal rER (→), mitochondria (M) and basal flattened nucleus (N) with prominent nucleolus and normal chromatin distribution (Mic. Mag. X 2000).

## DISCUSSION

The present work was designed to investigate the effect of leflunomide on the histological structure of the fundus of the stomach of adult male albino rats and to evaluate the possible ameliorative role of L-arginine. The light microscopic examination of this current study revealed that leflunomide induced focal changes on the fundic gastric mucosa in the form of epithelial discontinuity, erosions, sloughing & desquamation of surface epithelial cells two weeks after drug administration and aggravated after four weeks causing deep ulceration reaching the muscularis mucosa. These degenerative changes were observed by Duncan & Grant (2003), Youmba *et al.*, (2012), Vivar & Van Vollenhoven (2014) and Hagraas *et al.*, (2014) and they referred these changes to the affection of tight junction proteins as claudin-1, occluding and zonula occludens-1; resulting in, disruption of cell to cell interaction, inhibition of mitosis and decrease of epithelial integrity give rise to sloughing and desquamation. Moreover, leflunomide inhibits DNA synthesis and cell proliferation resulting in impaired epithelial regeneration (Mossalam & Yousuf, 2013).

Shortening and widening of gastric pits observed in group II in this work could be attributed to the excess mucus secretion as an attempt by the body to safe

guard against more damage to the surface coat of the gland (Hagras *et al.*, 2014). In addition, the separated gastric glands could be due to inflammatory cellular infiltration that was formed of different kinds of cells mainly mast cells which release histamine. The latter led to dilatation of blood capillaries, increase their permeability and their filtration in the interstitial spaces and subsequently; edema of interstitial spaces (Chandrasoma & Taylor, 2008). The inflammatory infiltration might occurred from the imbalance between the gastrototoxic agents and the protective mechanisms of gastric mucosa stimulating release of interleukin-1 and tumor necrosis factor alpha leading to infiltration of gastric mucosa with inflammatory cells (Konturek *et al.*, 2000). Also, it could be due to affection of the integrity of intercellular junction between surface columnar cells as the mucosa became more exposed to the effect of acid and proteolytic enzymes with subsequent bacterial invasion which might be chemotactic for neutrophils, lymphocytes and macrophages (Ribet & Cossart, 2015).

The vacuolated parietal cells and their darkly stained nuclei were observed with Vassilev *et al.* (2004) and Hail *et al.* (2010). They reported that leflunomide promotes apoptotic effect on epithelial cells. These degenerative changes could be explained on the basis of oxidative stress induced by leflunomide especially on long duration as it promotes mitochondrial damage and release of cytochrome-c into cytosol. (McInnes *et al.*, 2011).

In this research, there was reduction of PAS reaction of the surface mucous coat and in the isthmus & neck of the glands with appearance of areas of focal loss of PAS over the sites of erosions and ulceration. These results could be due to depletion of PAS stained mucous secretory granules of surface columnar cells as confirmed by electron microscopic findings. Hamilton *et al.* (1999) also who reported that leflunomide via its active metabolite A771726 decreases the accumulation of prostaglandins through inhibiting the cyclooxygenase (COX) enzymes and so the mucus secretion and the PAS reaction decreased.

The increase in the deposition of collagen fibers observed between fundic glands in the lamina propria and in the submucosa after two weeks and became more evident after 4 weeks of leflunomide administration could be explained by the ability of A771726 to induce pulmonary fibrosis through activation of myofibroblast which started deposition of collagen and extracellular matrix components (Namba *et al.*, 2010).

The immunohistochemical results of this study revealed statistically highly significant decrease in the mean number of PCNA positive cells in the isthmus and neck of gastric glands after two and four weeks of

leflunomide intake compared with the control group. This finding might be due to the ability of A771726 to inhibit the proliferation of cells stimulated by platelets derived growth factor (PDGF) through suppressing tyrosine phosphorylation of PDGF receptors, so leflunomide has anti-proliferative and anti-angiogenic effect. It also inhibits the proliferation of stem cells in the isthmus of gastric glands as an attempt of healing because the gastric stem cells are under the control of PDGF (Kawai & Rokutan, 1995 and Nguyen *et al.*, 2007).

It was observed during this research by TEM loss of apical microvilli of mucous cells, cytoplasmic vacuoles, dilated rough endoplasmic reticulum and presence of perinuclear space in most cells of the gastric mucosa in group II. These findings could be attributed to lipid peroxidation of biological membranes which increases its permeability to ions causing disruption of membrane structure. Moreover, Moriel *et al.*, (2000) referred it to the blunt effect of leflunomide on the mitochondrial dihydroorotate dehydrogenase, leading to formation of high conductance channel in the mitochondrial membrane, loss of membrane potential, ATP depletion and activation of phospholipase enzyme leading to breakdown of phospholipids and their depletion from the cell membrane. Dilatation of rER were referred by Fawcett & Jensch, (2002) as a trial of the cell to increase its surface area which is a compensatory mechanism bearing the function of severely damaged cells. Other researchers added that deleterious agents might interfere with ATP-dependent Na<sup>+</sup>/K<sup>+</sup> pump by increasing the permeability of cell membrane to sodium, thereby exceeding the capacity of the pump to extrude sodium, or damaging the pump itself. Accumulation of sodium in the cell leads to increase in water content and cell swelling, a condition called hydropic degeneration (Rubin & Strayer, 2012).

Coalescence of mucous secretory granules observed in mucous neck cells and apparent decrease in the amount of secretory granules were also observed in surface columnar cells two and four weeks after leflunomide treatment. The coalescence of secretory granules was attributed to the increase in the mucus secreted by gastric mucous cells due to accelerated exocytosis as a response to cell membrane disruption. This disruption-induced exocytosis acts as protective mechanism depends on entrance of extracellular Ca<sup>++</sup> and liberating mucus at sites of injury (Miyake *et al.*, 2006). The apparent decrease in secretory granules was also observed by Caselli *et al.* (1995) and Crivellato *et al.* (2002).

Dilatation of intracellular canaliculi of parietal cell might be due to the stimulation of the cell by histamine leading to re-arrangement of cytoskeleton and recruitment of (H<sup>+</sup>/K<sup>+</sup>-ATPase) rich tubulo-

vesicles to the apical membrane expanding the intracellular canaliculi with loss of luminal microvilli (Ogata & Yamasaki, 2000). This dilatation occurs in the active phase of secretion. The initiation of these morphological changes is induced by the cytoskeletal actin and phosphorylated ezrin which were essential for fusion of membranes of tubulo-vesicular system with the canaliculi causing its expansion and activation of H<sup>+</sup>/K<sup>+</sup>-ATPase with subsequent gastric acid secretion (Wakamatsu *et al.*, 2005).

Nuclear changes in the form of nuclear membrane irregularities, abnormal distribution of chromatin and even shrunken hyperchromatic nuclei with nucleolar margination might be a step towards cell apoptosis. These results were in harmony with Shi *et al.*, (2011) who suggested that leflunomide and its active metabolite A771726 have cytotoxic effect on cytochrome P450 enzymes leading to its inhibition. Accordingly, Mossalam & Yousuf (2013) attributed these ultrastructure findings to over-expression of Bcl-2 immunolocalization in both hepatocytes and endothelial sinusoids.

In the present study, when L-arginine was taken with leflunomide concomitantly either for two or four weeks the histological changes occurred in the cells lining the fundic gastric mucosa became improved and appeared more or less near normal architecture. The gastric glands were lined with nearly normal cells. The mononuclear inflammatory cells and congested blood vessels in the lamina propria were markedly reduced.

Many experimental studies revealed that L-arginine has beneficial effect on the healing of gastric ulcer in rats and referred it to the release of nitric oxide which plays an important role in gastric mucosal defense mechanisms though maintaining mucosal integrity, controlling gastric motility and secretion, enhancing mucosal blood flow and wound healing (Elliot *et al.*, 1995, Khattab *et al.*, 2001, Nishio *et al.*, 2006, Ajiboye *et al.*, 2014 and Usman *et al.*, 2014). NO has the ability to activate COX-1 and COX-2 enzymes responsible for gastric defense via the action of prostaglandins E and F<sub>2</sub>α. The latter stimulate epithelial cells to release more bicarbonate and mucus, inhibit gastric secretion, increase mucosal blood flow, reduce the release of a number of the inflammatory mediators and accelerate ulcer healing (Wallace, 2008).

The released NO improves microcirculation around the ulcer by enhancing angiogenesis through stimulating vascular endothelial growth factor (VEGF) synthesis. These new blood vessels would deliver more blood and transport oxygen and nutrients to the site of inflammation enhancing healing, as well as it delivers bicarbonates to the surface epithelium to neutralize the acid secretion (Chatterjee *et al.*, 2012

and Zandifar *et al.*, 2012). The apparent decrease of mononuclear cellular infiltration in the current work could be referred to the anti-inflammatory activity of NO (Lefer *et al.*, 1999). In addition, L-arginine itself has an antioxidant activity by which it gets rid of superoxide anions and hydrogen peroxide (Khattab *et al.*, 2001 & Lundberg *et al.*, 2008).

Subgroup IVA revealed non-significant change in mean optical density of PAS reaction covering the gastric mucosa when compared to control. This was in parallel with Ito *et al.* (2008) who mentioned that NO has gastro-protective mechanism by increasing the thickness of gastric mucus gel through stimulating the secretion of mucus by gastric epithelial cells regaining the thick mucus coat. Few collagen fibers were observed in-between the gastric glands and in the submucosa by Mallory trichrome stain and this was attributed to the anti-inflammatory effect of NO and L-arginine. This result was in agreement with Svegliati-Baroni *et al.* (2001) who reported that NO attenuate the proliferation of hepatic stellate cells induced by reactive oxygen species leading to decrease the amount of collagen deposition.

Subgroup IVA also showed significant increase in number of PCNA immunostained cells and highly significant increase in cells in subgroup IVB after four weeks. This might be attributed by Wong & Fiscus (2011) to nitric oxide which in low concentration induces cell proliferation.

## CONCLUSION

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Based on the current study and from all previously mentioned results; it was found that leflunomide had deleterious effects on the fundic gastric mucosa and L-arginine was used to protect against this damage as a precursor of nitric oxide which plays an important roles in the stomach.

## CONFLICT OF INTEREST

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There is no conflict of interest to declare.

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