Effect of Meloxicam on Lipoxygenase Activity in Trachea, Skin, Stomach and Serum of Rabbits (*in Vivo* and *in Vitro*)

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Abstract: The effect of meloxicam (MLX: 4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide.) on lipoxygenase enzyme (LOX) in different tissues of rabbits *in vitro* and *in vivo* were studied. MLX (117 nM) inhibited trachea, skin, stomach and serum LOX enzyme activities *in vitro* with varying inhibitory degrees. The administration of MLX (0.214 mg/kg body mass/day for 7days) inhibited trachea, skin and stomach LOX activities. However, MLX had no effect on serum LOX activity. Linweaver-Burk plots showed that MLX inhibited LOX enzyme *in vitro* and *in vivo* by competitive inhibition manner. The values of Km and Vmax in the absence and presence of MLX were determined.
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Key Words: Lipoxygenase, Meloxicam, Arachidonic acid, Rabbit, Inhibition.

1. Introduction

Lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) is a non-haeme iron dioxygenase enzyme that catalyzes the hydroperoxidation of polyunsaturated fatty acids having a cis-cis 1,4 pentadiene moiety such as linoleic acid and arachidonic acid to form the conjugated cis, trans dienehydroperoxidesaccording to the following reaction(1).
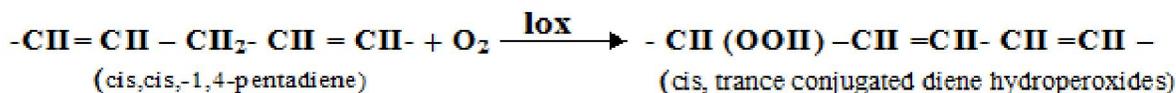
This enzyme is present in mammalian tissues such as skin (2), stomach(3), trachea (1), and blood leukocytes (4) such as oesinophils (5), polymorphonuclear leukocytes (6), neurtophil and blood platelets (7).

Arachidonic acid metabolism diverges down two main pathways, thecyclooxygenase (COX) and the lipoxygenase (LOX) pathways. The COXpathway leads to prostaglandins and thromboxanes production and the LOXpathway leads to the leukotrienes (LTs) and hydroxyeicosatetraenoic acid, HETEs, (8). Hydroperoxides, LTs and HETEs as well as prostaglandins have various physiological and pharmacological activities (2) and play a role as proinflammatory molecules (8, 9). These activities of inflammatory mediators are related to COX and LOX activities(10).

Nonsteroidal anti-inflammatory drugs (NSAIDs) play a major role in the management of inflammation and pain (11). However, they are also associated with high incidence of gastrointestinal (GI) ulceration and bleeding (12), hepatocellular dysfunction and organ failure (13) and skin reactions (14). Both beneficial and harmful effects of NSAIDs result from the inhibition of the COX enzyme responsible for prostaglandin synthesis (15). The GI side effects of NSAIDs are thought to be due to inhibition of COX-1 (16), which is constitutive COX isoform and has cytoprotective prostaglandin synthesis (17). However, the inducible COX-2 isoform is responsible for some aspects of pain and inflammation (18, 19). Therefore, the selective NSAIDs provide excellent pain and relief and less GI toxicity than nonselective NSAIDs do (12, 20).The older NSAIDs like aspirin and indomethacin are nonselective inhibitors of COX activity and therefore in addition to the inhibition of COX-2 activity inhibit the formation of prostaglandins, by COX-1. They are associated with the complications associated with GI ulcers, such as perforation and bleeding (11).

The NSAID meloxicam,MLX, (Fig. 1) has shown preferential activity in its ability to inhibit COX-2 compared to COX-1. It is tenfolds more potent against COX-2 than COX-1(18), i.e. MLX is a highlyselective inhibitor of COX-2 (21,22). MLX has shown potent anti-inflammatory and analgesic activity with improved tolerability in animals models (24), together with lower GI and renal toxicity

(24,25) than currently available NSAIDs (26). MLX reduces the incidence of GI mucosal abnormalities (27), but increases the intestinal permeability (28) in short term studies. The benefits of COX-2 inhibition may be reduced by aspirin use (29). Long term administration of MLX produced significant anti-edemetous effect but did not show any apparent lesions in the gastric mucosa (30).

In contrast to prostaglandin biosynthesis, only few compounds are known to inhibit the LOX pathway of the arachidonic acid cascade (31,32). In general, most inhibitors of the COX-2 are not active when tested against LOX. Yet the development of compounds or drugs with inhibitory effect against COX and LOX pathways could have additional beneficial activities for the inflammatory processes.

Many studies indicated that NSAID MLX has shown a high selective inhibitory effect on COX-2 (19, 20). Therefore, the present study was undertaken to investigate the effect of MLX on rabbit trachea, skin, stomach and serum LOX enzyme activities *in vitro* and *in vivo* to show its potential role as anti-inflammatory.

2.Materials and methods
1.1. Chemicals:
Highly purified arachidonic acid and Trishydroxymethyl-metylamine were obtained from Sigma Chem. Co, USA.Miloxicam(MLX): 4-hydroxy-2-methyl-N-(5-methyl-2-thiazoly)-2H-l,2-benzothiazine-3-carboxamide-1,1-dioxide was obtained from the Egyptian Co. for Chemicals and Pharmaceuticals.
1.2. Animals:
14 Male rabbits (Balady), average weight one kg, were obtained from local market of Alexandria, Egypt. They were kept in special cages,four rabbits each, and given standard diet and water for two weeks prior to handling. After acclimatization, rabbits were divided into two groups, seven rabbits each (n=7). Group I, control rabbits (untreated), Group II were injected intramuscularly with MLX in a daily therapeutic dose of 0.214 mg/kg body weight/day (31). After 7 days rabbits wereanesthetized using petroleum ether and then decapitated and allowed to bleed. Blood samples were collected immediately in clean centrifuge cups and left in ice for 15-20 min. The blood samples were centrifuged at 3,000 rpm for 20 min at 4°C. Serum samples were collected and kept frozen for latter assaying of lipoxygenase.

Stomach, trachea and skin (after removing hair) were removed quickly as soon as possible and from each organ small part taken and put in 10 % formalin for histopathological examination. The residual parts from each organ were blotted on filter paper, weighed and rinsed thoroughly with ice cold saline (0.9%

NaCl). All subsequent procedures were performed at 0-4°C.
1.3. Preparation of Stomach, trachea or skin extracts
Untreated or treated Stomach, trachea or skin were homogenized, in four volumes (w/v) of ice cold 0.05M Tris-HCl buffer (pH 7.8) containing 100 mM EDTA (disodium salt) and 0.1 mM KC1. The homogenates were centrifuged at 10,000 rpm for 30 min at 4°C and the supernatants were stored at -30 ^{o}C till used for lipoxygenase assay (33).
1.4. Enzyme assay
Substrate: 10mM sodium arachidonate: It is prepared by using 85 mg of arachidonic acid with an equal weight of tween-20, both were added to 02-free water. The mixture was homogenized avoiding air bubbles, then measured and equivalent amount of 0.5N NaOH was added and the total volume, made up to 25 ml in distilled water. The total volume was divided into 1-2 ml portions in small screw-cap vials, flushed with nitrogen before closing and were kept frozen until used.

Lipoxygenase activity was determined spectrophotometrically (Ultrospec 1000, Phamacia, Biotech, England). The reaction was started by adding substrate (sodium arachidonate). The Increase In absorbance at 234 nm was followed at 25°C on spectrophotometer. The extinction coefficient for diene is taken to be 2.5×10^4 $M^{-1}cm^{-1}$ for the arachidonic acid product at 234 nm.One unit lipoxygenase enzyme is defined as the quantity of the enzyme catalyzing the formation of one μmol of hydroperoxyarachidonate (HPA) per min/mg protein under the assay conditions (34).
1.5. Protein assay:
Protein content in the stomach, trachea, skin or serum of untreated rats. was determined by the method of Ohnishi & Barr (35) using bovine serum albumin as a standard.(36).
1.6. Effect of MLX on LOX activity *in vitro*
LOX assays in the following section were carried out on stomach, trachea, skin extracts or serum of untreated rats (control).
1.6.1. Effect of MLX on LOX activities of studied organs:
In this experiment, LOX (360 μg protein/assay) was incubated with 0.12 μMLX for 10 min. before enzymeassay.
1.6.2. Preincubation studies:
To establish the time-dependent mechanism of inhibition, skin LOX (360 μg rabbit skin protein/assay) and 0.12μM of MLX (i.e. inhibitor) were incubated for various periods (0 – 70 min.). After the preincubation period, the substrate (40.6μM of sodium arachidonate) was added and LOX activity

was determined. The remaining LOX activity wasexpressed as percentages of control basal activity.

1.6.3.   Effect of MLX on kinetic parameters of LOX:

The effect of different substrate concentrations (from 0 to 120 μM of sodium arachidonate) on the initial velocity of LOX activity in the absence and in the presence of 0.12 μM of MLX was determined. Then Lineweaver – Burk plot (37) was used to calculate the values of steady state kinetic constants (Michaelis constant, K_m, maximum velocity, V_m, and K_i.

1.7.   Effect of MLX onLOX activity *in vivo*:

LOX activities were measured in the extracts of stomach, trachea, skin or serum of untreated and treated rats with MLX.

1.8.   Statistical analysis:

Each parameter was tested in triplicate. Conventional statistical methods were used to calculate the means, standard deviations and (ANOVA) differences (*P*< 0.05). To ascertain significant differences between the levels of the main factor, Dunnett's multiple comparison tests were applied between the means. Statistical data analyses were undertaken using the statistical software Prism, version 3.

1.9.   Histopathological Study:

Sections ofstomach, skin and trachea which were put in 10% neutral formalin were fixed by immersion, processed, embedded in paraffin and 5 micron sections stained with hemotoxylin and eosin.

Results and Discussion

1.10.   InhibitionofLOX by MLX*in vitro*:

The inflammatory process involves a series of events can be elicited by numerous stimuli such as infectious agents, ischaemia, antigen-antibody interaction and thermal or physical injury(38). Inflammation is usually associated with pain as a secondary process resulting from the release of algesic mediators (38).Therapy of inflammatory diseases is usually directed at the inflammatory process. Through years of ingenious syntheses and structural modifications, which usually accompany design and development of new drug substances, many non-steroidal ant-inflammatory agents NSAIDS have been of immense help in the management of various inflammatory conditions like rheumatism, arthritis and pain. However, most drugs are known to provoke gastrointestinal irritation.

The present study showed that 0.117 μM of MLX caused significant inhibition of trachea, skin, stomach and serum LOX activities by about 73, 55, 50 and 18 %, respectively as compared to the controls (Fig. 2). As shown from the results in Fig. 2, LOX activity of trachea was more affected by MLX then skin, stomach and serum LOX activities.

1.11.   Inhibition of LOX by MLX is time dependent:

Inhibition of LOX activity was found to be affected by varying the preincubation time (0-70 min) with MLX (Fig. 3). The maximal inhibition after 70 min of preincubation indicating that, the inhibition of LOX by MLX could be due to complex formation rather than the enzyme oxidation.The increase in the inhibitory potency after preincubation is consistant with earlier results which suggested a non covalent interaction between the enzyme and MLX (39, 40).

1.12.   Inhibition type:

Skin LOX activities were measured within a range of 10-121 μM substrate concentration in the absence and presence MLX. The relationshipbetween LOXactivities against substrate concentrations gave rectangular hyperbola (Fig. 4 A). The Lineweaver-Burk plot (1/ (v) versus 1/ [S]); is a characteristic pattern of competitive type inhibition, Fig. 4 B. The apparent kinetic constants were calculated from a double reciprocal plot. The $_{Km}$ values in the absence and presence of MLX were 38.25 and 77.88 μM respectively. While $_{Vmax}$ values were fixed (equal to 0.01μmol HPA/mg protein/min). In general, the $_{Km}$ value in the presence of MLX washigher than that of the control. This indicates that MLX combines with the free enzyme in a manner that prevents substrate binding, since, the inhibitor (MLX) and the substrate (arachidonic acid) are mutually exclusive, often because of true competition for the same site. Otherwise Ki value was equal to 0.113 μM.

1.13.   Lipoxygenase inhibition *in vivo*

The results of the present study showed that the administration of MLX caused a significant inhibition of trachea, skin and stomach LOX activities by 82, 78, and 71%, respectively, (Fig. 5). While serum LOX activity has shown a nonsignificant inhibition as compared with control and this may be related that, 99% of MLX is bound to plasma proteins (41). Binding is generally a reversible process and, therefore, arrives at an equilibrium in which only the unbound (free) fraction of the drug (about 1%) is available to act on the LOX enzyme and to be biotransformed and excreted. While the bound fraction function as depot from which the drug is released as the equilibrium is reestablished after removal of the free fraction (41).

The relationship between different substrate concentrations and the LOX activities of skin rats which treated with MLX (0.214 mg /kg / day for seven days) gave rectangular hyperbola (Fig. 6 A). The data showed that the type of inhibition is competitive (Fig. 4 B) since the values of Km in nontreated (control) and treated rabbits were equal to

38.25 and 76.61 µM respectively. However MLX had no effect on the values of V_{max} which equal to 0.010 µmol HPA/mg protein/ min. The determination of Ki value *in vivo* study is very difficult since the true MLX concentration is unknown. The competitive mechanism *in vitro* or *in vivo* has suggested that MLX was bound at the substrate binding site. The previous study suggested a simple mechanism of LOX involving formation of a biradical from arachidonate and oxygen on the surface of the enzyme. The biradical (free radical of arachidonate and $^{\bullet}$OOH radical) reacts to give conjugated peroxide. Thus, the inactivation of lipoxygenase enzyme by meloxicam may be attributed to the block of arachidonic acid oxidation. The free radical of arachidonate may accept hydrogen to become arachidonate again or the free radical ($^{\bullet}$00H) may accept hydrogen from this drug and forming H_2O_2(42) which is degraded by catalase or glutathione peroxidase (43, 44).Otherwise, among many publications, the one describing the drug ebselen (45), is an excellent example of a mechanism of lipoxygenase inhibition. It illustrates that the same inhibitor could bind in a competitive or noncompetitive manner depending on the ionization state of iron. In the presence of the fatty acid substrate, it is competitive, while acting on a ferrous, silent ground-state enzyme it binds covalently, causing irreversible inhibition. In both cases the enzyme's performance can be illustrated by a classic Lineweaver-Burk plot.So from these mechanisms we concluded that, MLX prevent oxidation of arachidonate to form free radicals which induce inflammation.

Histological studies:
The histological studies were performed to detect the side effects of MLX when administered for short time (one week). Microscopic examination of control groups showed that stomach fundus with well oriented normal gastric glands lined by normal surface epithelium with gastric pits in between the glands tightly packed and the muscularis mucosa was intact (Fig. 7a). However,

after MLX treatment it showed that stomach fundus with thickening of the lining gastric glands but they were lined by normal surface epithelium. Laaminapropria and muscularis mucosa were normal (Fig. 7b).

Control skin revealed that skin lined by stratified squamous epithelium was covered by horney layer. The subepithelial connective tissue was compact with the presence of hair follicles (Fig. 7 c). While, after treatment with MLX skin showed little thickening of stratified squamous epithelium compared to the control group (Fig. 7d). Also oedematous and loose subepithelial layer were seen, but no pathologic change could be detected (Fig. 7 e).Control trachea revealed pseudostratified ciliated columnar epithelium and normal hyaline cartilage (Fig. 7 f). However, trachea of treated rabbits with MLX revealed dilated blood vessel congestion, whereas the lining epithelium and the cartilage were normal (Fig. 7 g).

The present results about inhibition of rabbit LOX by MLX are in agreement with the previous studies which reported that MLX inhibited leukotriene B4 (LTB4) production significantly in the gastric fluid (46, 47).

Otherwise,LOX expression levels increase with age, suggesting that it may be involved in their diseases of aging. Therefore, inhibition of lipoxygenase could participate in the anti-inflammatory action (48).Several NSAID inhibit COX-2 only 1(14, 15), whereas a few drugs inhibit both LOX and COX-2 activities (49). The inhibition of COX-2 and LOX pathways could have additional beneficial activities for the inflammatory process.

In conclusion, MLX is a new dual inhibitor of both LOX and COX-2 which can decrease the inflammation resulted from the metabolites of these enzymes. In addition,treatment with MLX for a short period was safe since the histological studies showed minor changes compared to control since MLX treatment for a long period causes inhibition of ovulation in rabbits (31).
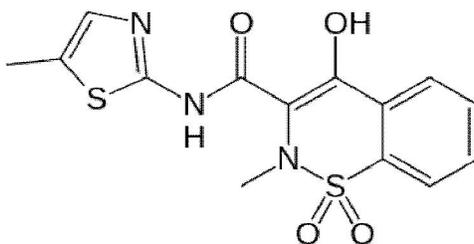

Fig.1: Meloxicam (MLX: 4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide.)
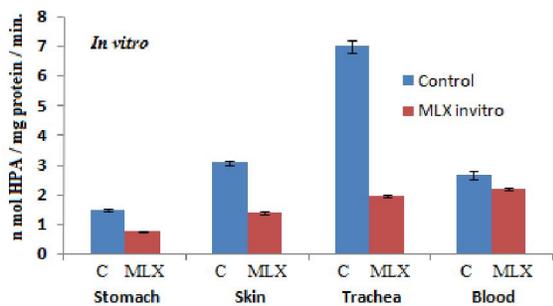
Fig. 2: Effect of meloxicam on rabbit trachea, skin, stomach, and serum lipoxygenase activities *in vitro*.
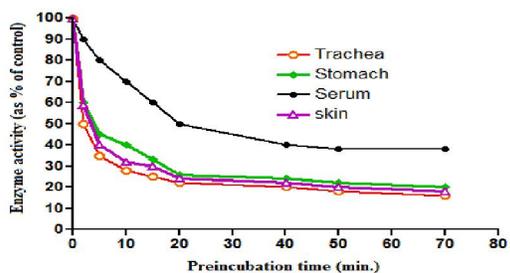

Fig. 3: Effect of preincubation time upon inhibition of lipoxygenase activity by MLX. Enzyme (360 μg rabbit skin, trachea, stomach and serum protein) was incubated with 10 – 121 μM of MLX for different periods. After the preincubation period, lipoxygenase activity was determined as described in section materials and methods using 40.6 μM arachidonic acid as specific substrate and compared to the control sample that had been incubated for the same time in the absence of inhibitor. Each point represents the mean of triplicate experiments.
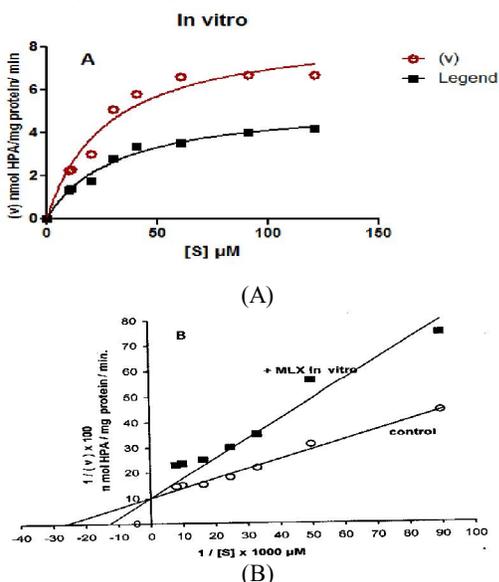

(A)

(B)

Fig. 4: Effect of different substrate concentrations on rabbit skin lipoxygenase in the absence and presence of MLX *in vitro*. (a) v against [S] and (b) Lineweaver-Burk plot analysis. Enzyme (360 μg protein) was preincubated with 117 nM of MLX for 5 min. at 30 oC prior to the addition of arachidonate (the concentration ranging from 10 - 121 μM). Each point represents the mean of triplicate experiments. (v) = nmol HPA / mg protein / min.).
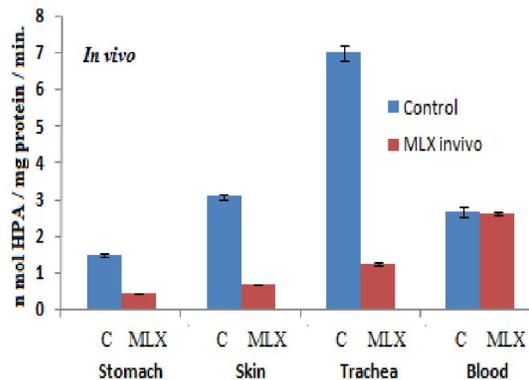

Fig. 5: Effect of meloxicam on rabbit trachea, skin, stomach, and serum lipoxygenase activities *in vivo*.
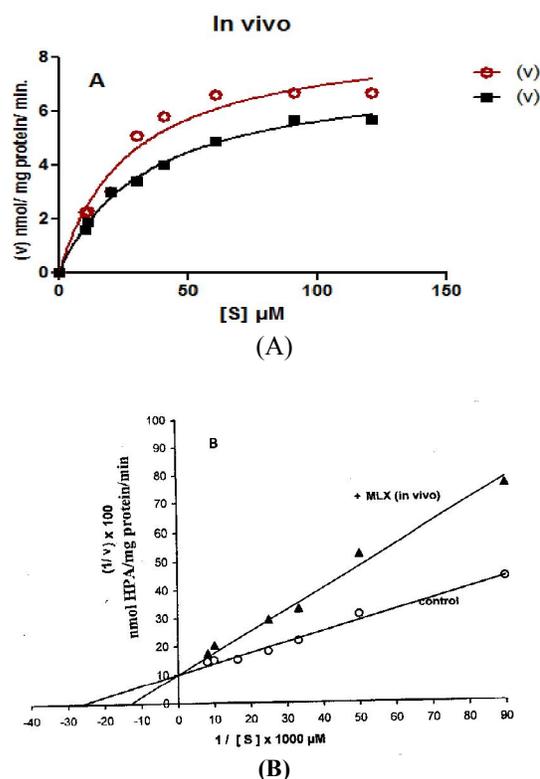

(A)

(B)

Fig. 6: Effect of different substrate concentrations on skin lipoxygenase of untreated or treated rabbit with MLX *in vivo*. (a) v against [S] and (b) Lineweaver-Burk plot analysis. Since the control = untreated rabbit, while MLX = the rabbit were treated with 0.214 mg of MLX / kg /day for 7 days. Enzyme (360 μg protein) and different concentrations of arachidonate (10 - 121μM) were used in enzyme assay. Each point represents the mean of triplicate experiments. (v) = nmol HPA / mg protein / min.).
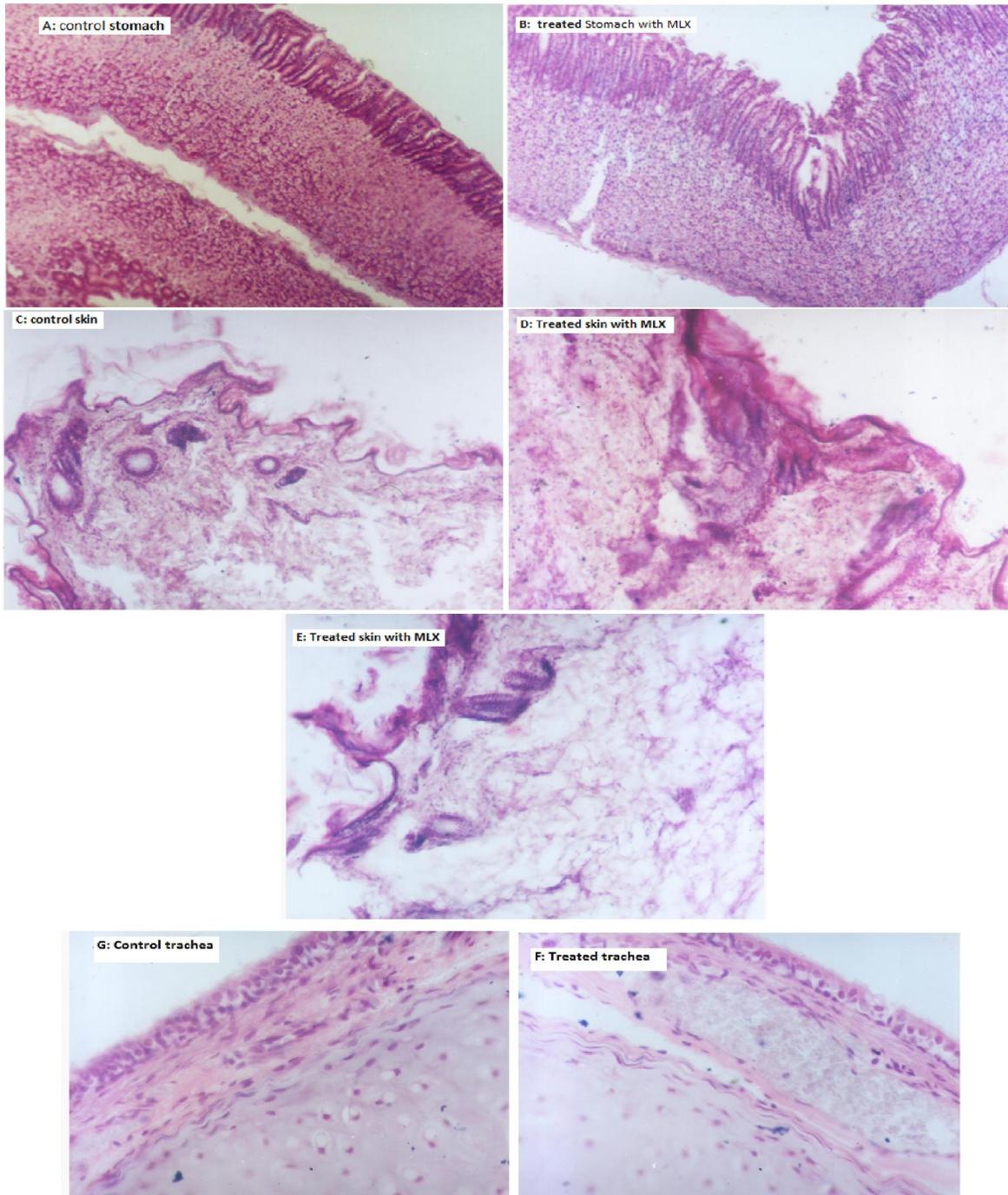
Fig. 7: Histological examination of different studied organs of untreated and treated rabbits. (a) Control stomach fundus: showing normal gastric glands lined by normal surface epithelium with gastric pits in between. The lamina propria in between is loose. The muscularis mucosa is intake (H &E stain x100). (b) Treated stomach fundus: showing thickening epithelial glandular layer with normal cellular lining, normal lamina propria and muscularis mucosa. (H & E stain x 100); (c) Skin: showing thin stratified squamous epithelium covered by borney layer. The sub epithelial connective tissue is compact with presence of hair follicles (H & E stain x 100); (d) skin of treated rats: showing little thickening of stratified squamous epithelium compared to the control (H & E stain x 100); (e) skin of treated rats: showing oedematous, loose sub epithelium layer (H & E stain x 100); (f) Control trachea: it lined by pseudo stratified ciliated columnar epithelium and showinghyaline cartilage (H & E stain x 400) and (g) trachea of treated with MLX: showing congested dilated blood vessel. The lining epithelium and hyaline cartilage are almost normal (H & E stain x 400).

Acknowledgement

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