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Research Article
Protective Effect of Carob Pods Extract on Esomeprazole-Induced Changes on the Renal Cortex of Rats: Histological, Immunohistochemical and Statistical Study

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Abstract

Background and Objective: The kidney is an important organ highly susceptible to the toxic effects of different toxic substances. Esomeprazole is one of the proton pump inhibitors used in the treatment of gastrointestinal disorders but with many side effects. Carob is used in many Arab countries as a popular drink. Carob pod extract has an antioxidant and anti-inflammatory effect. This work aimed to investigate the possible protective effect of carob pod extract (*Ceratonia siliqua*) on Esomeprazole-induced changes in the renal cortex of adult male albino rats using histological and immunohistochemical studies. **Materials and Methods:** A total of 40 rats (100-150 g) were divided into four groups (10 rats each), control, carob group: Rats received 200 mg/kg/day carob pods extract, esomeprazole group: Rats received esomeprazole 20 mg/kg/day orally and carob and esomeprazole group: Rats received 200 mg/kg/day carob pods extract 1 hr before 20 mg/kg/day esomeprazole orally by intragastric tube. The collected data from the different experimental groups were analyzed by student's t-test after evaluation of the F-test. **Results:** Esomeprazole group showed shrunken glomeruli, dilated Bowman's space, dark tubular cell nuclei, cytoplasmic vacuolations and intraluminal cellular debris in toluidine blue stained sections, a significant decrease in the mean optical density for PAS, significant decreased iNOS immunoreaction and significant EM changes. Esomeprazole and carob groups showed improvement in the previous changes. **Conclusion:** Esomeprazole-induced structural changes in the renal cortex of rats and carob pod extract could improve such changes.

Key words: Esomeprazole, renal cortex, carob pods, histopathology, immunohistochemistry, electron microscopy

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Esomeprazole is one of several most recent proton pump inhibitors (PPIs) used in the treatment of different gastrointestinal disorders like gastroesophageal reflux (GERD), peptic ulcer, dyspepsia, *H. pylori* eradication and other pathological hyper-secretory conditions as Zollinger-Ellison syndrome[1,2].

Despite the safety of PPIs, different side effects can be noticed especially if used for a long period such as hip fracture, community-acquired pneumonia and *Clostridium difficile* infection. Additionally, about 80% of esomeprazole is eliminated mainly through the kidney so, deleterious side effects on the kidney may be predicted like acute interstitial nephritis (AKI) that may proceed to chronic kidney diseases if recurrent AKI occurs[3].

Ceratonia siliqua is the carob tree, also called algarroba, which is an evergreen tree that grows throughout the Mediterranean area, contains leaves, leaflets and fruit pods (carob pods)[4]. Carob is used in many Arab countries as a popular drink, consumed mainly in the month of Ramadan and also in the USA and other western countries in health food stores as a substitute for cocoa as it has no caffeine or thiobromine[5]. Additionally, carob pods are used in the synthesis of mucilaginous gum (tragasol), which is used in a wide range of commercial products as a thickener, stabilizer, binder and gelling or dispersion agent. Moreover, due to its sweet taste, it can be used in powdered, chip, or syrup form as an ingredient in cakes as well as cookies[6].

Carob pods have anti-inflammatory and antioxidant properties due to the presence of polyphenols especially tannins, minerals, vitamins and sterols so, could protect the kidney from the effect of different noxious substances and drugs[7].

So, the present study aimed to investigate the possible protective effect of carob pod extract on esomeprazole-induced structural changes in the renal cortex of rats by using histological, immunohistochemical and statistical studies.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Histology and Cell Biology, Faculty of Medicine, Tanta University, Egypt by the year (2018).

Preparation of carob pods aqueous extract: Carob pods were obtained from the local market, seeds were removed and the carob was ground to particles of $\leq$1 mm, then ground until a fine powder was obtained, then the dried and ground carob pod powder was extracted with cold water (100 mL water for each 1 gm carob pod powder) and allowed to stand for 12 hrs at 4°C. The extract was then filtered to remove sugars present in carob pods. This extraction procedure was performed twice. Then the residue of the carob pods was extracted with water at room temperature and boiled for 10 min with stirring. The sample was allowed to stand for 12 hrs at 25°C. After filtration, the filtrate was concentrated to give carob pod polyphenol (CPP)[8].

Experimental animals and groups: A total of 40 male Wistar rats (100-150 g). The animals were housed in clean properly ventilated cages and fed on a similar commercial laboratory diet and water *ad libitum*. The experiment was done following the guidelines as well as the care for the animals guided by the University's ethical committee.

Rats were randomly divided into four groups each including 10 rats, the control group: Rats kept without any treatments and then sacrificed at the same time as their corresponding experimental groups, the carob group: Rats received an oral dose of 200 mg/kg/day carob pods extract by an intragastric tube for 4 weeks, esomeprazole group: Rats received esomeprazole (AstraZeneca, Egypt) 20 mg/kg/day orally by an intragastric tube for 4 weeks and carob and esomeprazole group: Rats received 200 mg/kg/day carob pods extract one hour before esomeprazole 20 mg/kg/day orally once daily by an intragastric tube for 4 weeks.

On the last day of the experiment, all animals were sacrificed by intraperitoneal injection of sodium thiopental (40 mg kg^{-1}). After that, specimens from the rats' renal cortex were obtained and processed for histological, immunohistochemical and statistical study.

Haematoxylin and Eosin (H&E) stain: The renal cortical specimens were immediately fixed in 10% formol saline for 24 hrs. Then dehydration followed by clearance in two changes of xylol. After that, impregnation in pure soft paraffin for 2 hrs followed by embedding in hard paraffin. Finally, sections of 5 um were obtained. Later, sections were de-waxed, hydrated and stained with Harris' hematoxylin for 2-5 min. Then, staining with 1% eosin for 1-3 min. Lastly, dehydration, clearance and mounting with Canada balsam. The nucleus appeared blue while the cytoplasm appeared pink[9].

Periodic acid Schiff's (PAS) stain: Sections were deparaffinized, hydrated and immersed in 1% periodic acid for 8 min then placed in Schiff's reagent for 15 min. After that,

sections were dehydrated, cleared and mounted in Canada balsam. Deep red or magenta staining of mucopolysaccharides appeared while nuclei stained blue[10].

As regards the statistical analysis of the optical density of PAS stain, the software (Image J) (National Institute of Health, Maryland, USA) used by which 10 images stained with PAS at a magnification ×400 from each experimental group were used.

Immunohistochemistry for Inducible Nitric Oxide Synthase (iNOS)[11]: Sections were deparaffinized and rehydrated, then incubated in hydrogen peroxide (10%) for 10-15 min. Antigen retrieval was obtained by immersing the sections in a citrate buffer solution and then heat in a microwave for 10-20 min. Sections were then left to cool for 20 min at room temperature after that, iNOS primary antibody (Santa Cruz Biotechnology) 1:200 was applied to the slides overnight at 4°C. Later, the secondary antibody (Thermo Fisher Scientific, UK) was applied. Finally, diaminobenzidine (DAB) was applied and incubated until the desired reaction was achieved followed by counterstaining with Mayer's haematoxylin then examined using a digital camera (Olympus, Japan) connected to a light microscope (Leica, Olympus, Japan). The brownish cytoplasmic colouration of the renal cortical cells is seen as a positive reaction. While a negative control was obtained through the application of PBS instead of the primary antibody. Also, the lung is considered to be a positive control.

For estimating the optical density of the iNOS immunohistochemical reaction, 10 images from each experimental group stained with iNOS (X 400) were evaluated by the ImageJ program (NIH, USA).

Preparation of specimens for electron microscopic (EM) study[12]: Specimens fixed in 2.5% phosphate-buffered glutaraldehyde, processed and embedded in epoxy resin. Then, semithin sections with 1 µm thick were obtained and stained with toluidine blue to choose the suitable sections for ultrathin parts. For the ultrathin sections, 75 nm thick sections were obtained then picked up on 200 mesh uncoated copper grids and stained with uranyl acetate and lead citrate. At last, sections were examined by an EM (JEOL-JEM-100, Japan) at The Electron Microscopic Unit, Faculty of Medicine, Tanta University, Egypt.

As regards the morphometry of the Glomerular Basement Membrane (GBM), 10 dissimilar EM images (×4000 magnification) from each experimental group were used by the software (ImageJ) (NIH, Bethesda, USA) to determine the mean thickness of GBM. The measurements estimated between the cytoplasmic membrane of the endothelial cells and the outer lining of the lamina rara externa beneath the cytoplasmic membrane of podocytes' foot processes (excluding the areas of tortuosity to avoid tangential plane of sections).

Statistical analysis: The collected data from the different experimental groups were analyzed by student's t-test after evaluation of the F-test. Then expressed as (Mean±SD). The significance of the data was established when $p<0.05$.

RESULTS

H&E results: Control and carob groups of H&E stained sections revealed renal corpuscles (Malpighian corpuscles) consisted of a tuft of capillaries (glomerulus) enveloped by a regular continuous Bowman's capsule. Bowman's capsule consisted of a parietal layer lined by simple squamous epithelial cells. While its vascular pole showed a darkly stained nucleated area corresponding to the juxtaglomerular apparatus consisting of densely packed epithelial cells of macula densa and nearby juxtaglomerular cells. Proximal convoluted tubule cells (PCTs) showed narrow lumen lined with 4-6 large cubical or low columnar cells, with deeply acidophilic cytoplasm and rounded nearly basal or centrally situated nuclei. In addition to poorly distinct cell boundaries and striated luminal borders. Whereas, the distal convoluted tubule cells (DCTs) revealed a wide lumen lined with 5-8 low cubical cells with less acidophilic cytoplasm and euchromatic nuclei, besides distinct cell boundaries without a brush border (Fig. 1a-d).

Considering the esomeprazole group, it showed shrunken glomeruli with the widening of the Bowman's space, besides vacuolated glomerular cells. As for the cortical tubular cells, some showed vacuolated cytoplasm and darkly stained nuclei and others with nuclear extrusion into the tubular lumen. These were associated with congested intertubular capillaries and interstitial mononuclear cellular infiltrations. On the other hand, the esomeprazole and carob group exhibited a nearly normal picture (Fig. 1).

PAS results: The basement membrane of both the parietal layer of Bowman's capsule as well as the basement membrane of the cortical renal tubules besides PCTs' brush borders exposed PAS +ve reaction in the control and carob groups. The esomeprazole group showed a strong PAS +ve reaction of the basement membrane of both the parietal layer of Bowman's capsule and the cortical renal tubules and lost

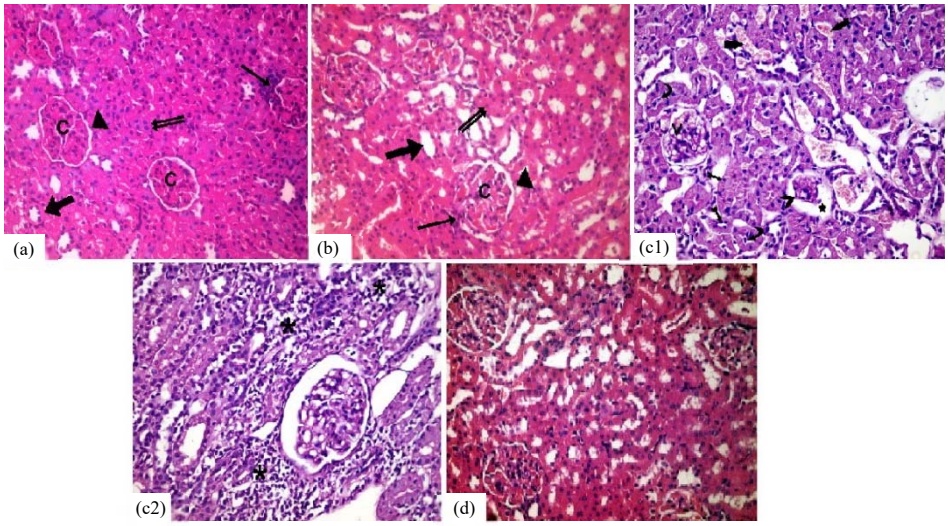

Fig. 1(a-d): Effect of carob pod extract on esomeprazole-induced changes on the renal cortex of rats using H&E (×400)

(a,b) Control and carob: Capillary tufts (C), simple squamous cells (▸), macula densa and juxtaglomerular cells (thin arrow), cubical cells, rounded central nuclei and brush border of PCTs (double arrow), cubical cells, euchromatic nuclei of DCTs (thick arrow), (c1-c2) Esomeprazole: Shrunken glomeruli (▸), wide Bowman's space (*), vacuolated glomeruli (V), vacuolated tubules, dark nuclei, nuclear extrusion (curved arrow), congested capillaries (thick arrow) and cellular infiltrations (*) and (d) Esomeprazole and carob: Nearly normal renal cortical structures

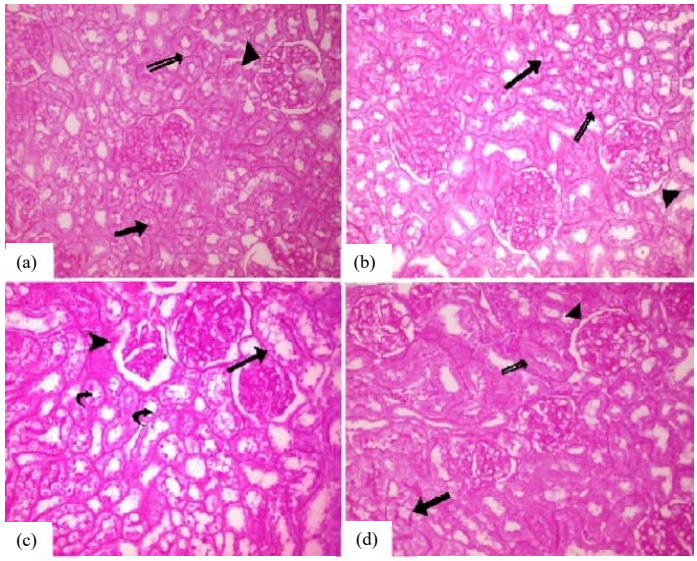

Fig. 2(a-d): Effect of carob pod extract on esomeprazole-induced changes on the renal cortex of rats using PAS (X400)

(a,b) Control and carob groups: PAS +ve reaction of the basement membrane of both the parietal layer of Bowman's capsule (▸) and the basement membrane of the cortical renal tubules (double arrow) and PCTs' brush borders (→), (c) Esomeprazole group: Strong PAS +ve reaction of the basement membrane of both the parietal layer of Bowman's capsule (▸) and the cortical renal tubules (→) and lost PAS reaction in the brush border of the PCTs (curved arrow) and(d) Esomeprazole and carob group: Nearly normal PAS staining of the basement membrane of both the parietal layer of Bowman's capsule (▸) and the basement membrane of the cortical renal tubules (double arrow) and PCTs' brush borders (→)

PAS reaction in the brush border of the PCTs. While the examined sections of the esomeprazole and carob group showed a nearly normal appearance (Fig. 2a-d).

The mean optical density of PAS-stained sections revealed a significant increase when compared with the control group. However, the esomeprazole and carob groups discovered a significant decrease in comparison to the esomeprazole group (Fig. 3).

Immunohistochemical results of iNOS: Control and carob groups expressed strong iNOS immunohistochemical reaction in the cytoplasm of the cortical renal tubular cells

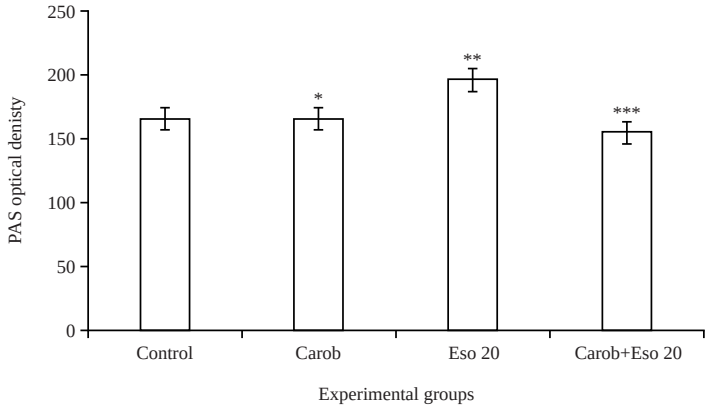

Fig. 3: Mean optical density of PAS in the different experimental groups expressed as mean±SD
*p>0.05 non-significant relative to the control group, **p<0.05 significant increase relative to the control and ***p<0.05 significant decrease relative to the esomeprazole group

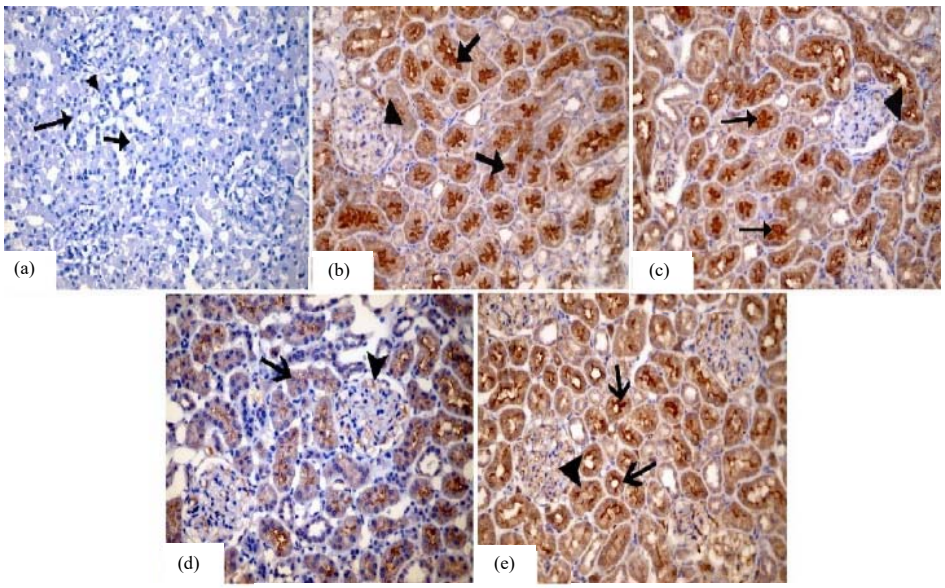

Fig. 4(a-e): Effect of carob pod extract on esomeprazole-induced changes on the renal cortex of rats using iNOS immunohistochemistry (×400)
(a) Negative showed no iNOS immunoreaction of renal glomeruli (▸) and cortical tubules (→), (b, c) Control and Carob groups: Strong iNOS immunohistochemical reaction in the cytoplasm of the cortical tubular cells (→) and the mild immune reaction of the glomerular cells (▸), (d) Esomeprazole group: Decreased iNOS immunohistochemical reaction of the cortical tubular cells (→) and glomerular cells (▸) and (e) Esomeprazole and carob pods group: Strong iNOS immunohistochemical reaction of cortical tubular cells (→) and the mild immune reaction of the glomerular cells (▸)

and a mild reaction in the glomerular cells, while the esomeprazole group showed decreased iNOS immunohistochemical reaction. On the other hand, the esomeprazole and carob group displayed a nearly normal expression of iNOS (Fig. 4a-e).

Regarding the optical density of iNOS immunohistochemical reaction, it showed a significant decrease in the esomeprazole group as compared to the control group, while a significant increase in the esomeprazole and carob group when compared to the esomeprazole group (Fig. 5).

Electron microscopic results: Electron microscopic (EM) results of the glomerular basement membrane and podocytes of the control and carob groups showed a homogenous and trilamellar glomerular basement membrane, formed of outer

and inner electron-lucent layers and a middle electron-dense layer. The podocytes' cytoplasm contained a euchromatic nucleus and had a cell body from which cytoplasmic extensions arise forming primary (major) processes. From these primary processes, secondary (minor) processes or pedicles arise and are terminated by feet-like expansions on the basal lamina of the capillary wall. The Esomeprazole group revealed focal thickening of the glomerular basement membrane besides effacement of the secondary foot processes and fusion of some of them. Moreover, podocytes showed dilated RER, cytoplasmic vacuolations and areas of cytoplasmic rarefaction. As for the esomeprazole and carob group, it showed a nearly normal picture (Fig. 6a-d).

The mean thickness of the GBM exposed a significant increase in the esomeprazole group as compared to the control group, while significantly decreased in the esomeprazole and carob group (Fig. 7).

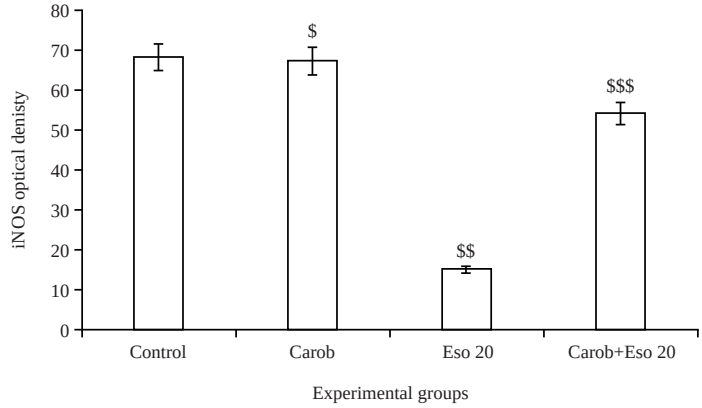

Fig. 5: Optical density of iNOS immunohistochemical reaction

Data expressed as mean±SD, $^{\$}p>0.05$ non-significant relative to control, $^{\$\$}p<0.05$ significant relative to control and $^{\$\$\$}p<0.05$ significant relative to the esomeprazole group

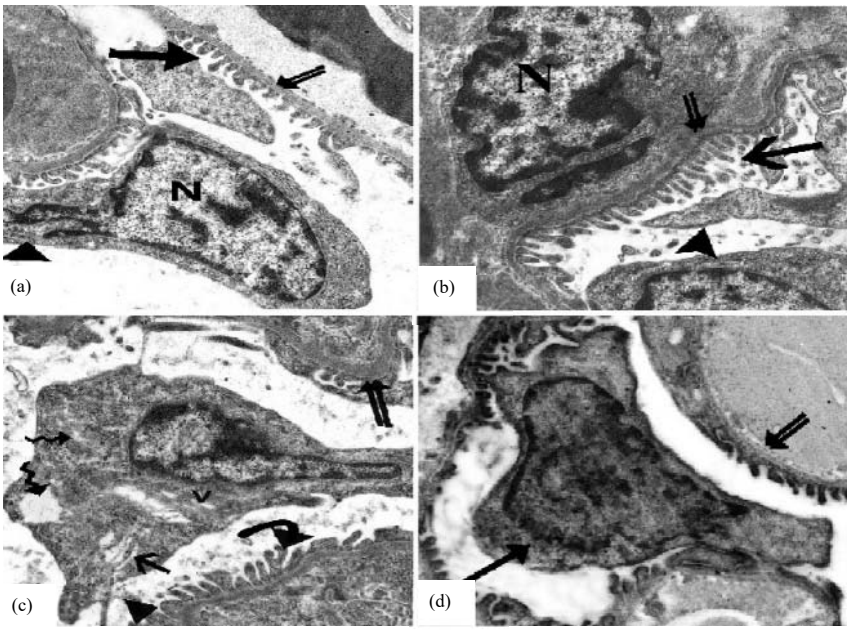

Fig. 6(a-d): Effect of carob pod extract on esomeprazole-induced changes on podocytes and GBM of rats using EM (×4000)

(a, b) Control and carob groups: Trilamellar GBM with outer and inner electron-lucent layers and middle electron-dense layer (double arrow), podocytes with euchromatic nucleus (N), primary processes (▸), secondary (minor) processes (→), (c) Esomeprazole group: Focal thickening of GBM (double arrow), secondary processes effacement (▸) and fusion (curved arrow), podocytes with dilated RER (→), vacuolations (V) and areas of cytoplasmic rarefaction (wavy arrow) and (d) Esomeprazole and carob group: Nearly normal trilamellar GBM (double arrow) and podocytes (→)

As regards EM examination of PCTs of the control and carob groups, they showed cells resting on a thin basement membrane with numerous thin, long apical microvilli forming its characteristic brush border. Each cell of the PCTs contained a single nearly central large spherical euchromatic nucleus with clumped chromatin. Additionally, there was basal plasma membrane enfolding and numerous elongated mitochondria arranged in the basal half of the cell parallel to its long axis (palisade arrangement). For the esomeprazole group, it showed cells with lost brush border, besides cytoplasmic vacuoles, swollen degenerated mitochondria and hyperchromatic irregular nucleus. While in the esomeprazole and carob group, a nearly normal EM picture was seen (Fig. 8a-d).

Considering the EM examination of the DCTs of the control and carob groups, they exhibited cubical cells with few short scattered microvilli on their luminal surfaces. Each cell contained a rounded or ovoid nucleus with less extended chromatin in addition to large numbers of mitochondria in its basal part. In the esomeprazole group, there were many cytoplasmic vacuoles and areas of cytoplasmic rarefaction. Conversely, the esomeprazole and carob group indicated a nearly normal EM image (Fig. 9a-d).

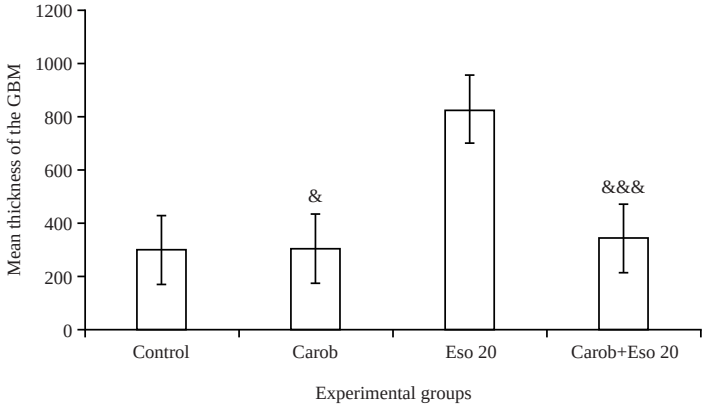

Fig. 7: Mean thickness of the glomerular basement membrane

Data expressed as mean±SD, $^{\&}p>0.05$ non-significant relative to control, $^{\&\&}p<0.05$ significant relative to control and $^{\&\&\&}p<0.05$ significant relative to the esomeprazole group

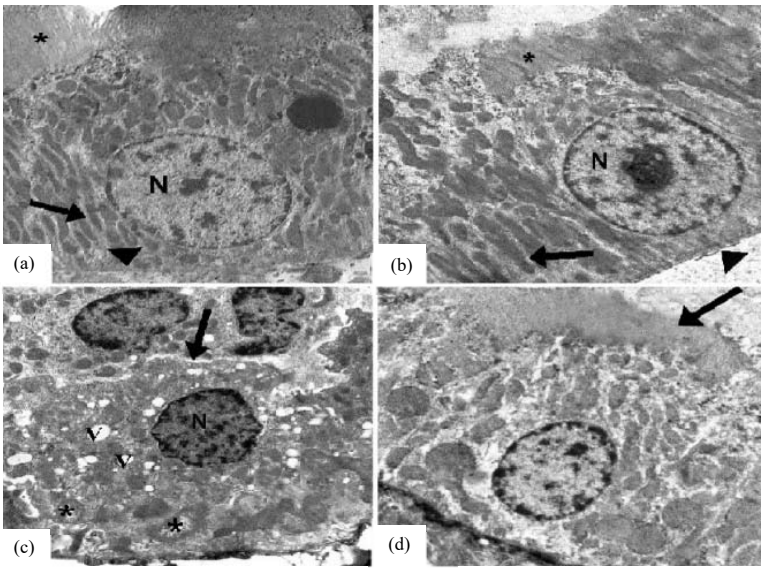

Fig. 8(a-d): Effect of carob pod extract on esomeprazole-induced changes on PCTs of rats using EM (×2000)

(a, b) Control and carob groups: Cells resting on a thin basement membrane (▸) with numerous thin, long apical microvilli (*), a single central spherical euchromatic nucleus with clumped chromatin (N), basal plasma membrane enfolding with numerous elongated mitochondria have palisade arrangement (→), (c) Esomeprazole group: Lost brush border (→), cytoplasmic vacuoles (V), swollen degenerated mitochondria (*) and hyperchromatic irregular nucleus (N) and (d) Esomeprazole and carob group: Nearly normal PCT cells (→)

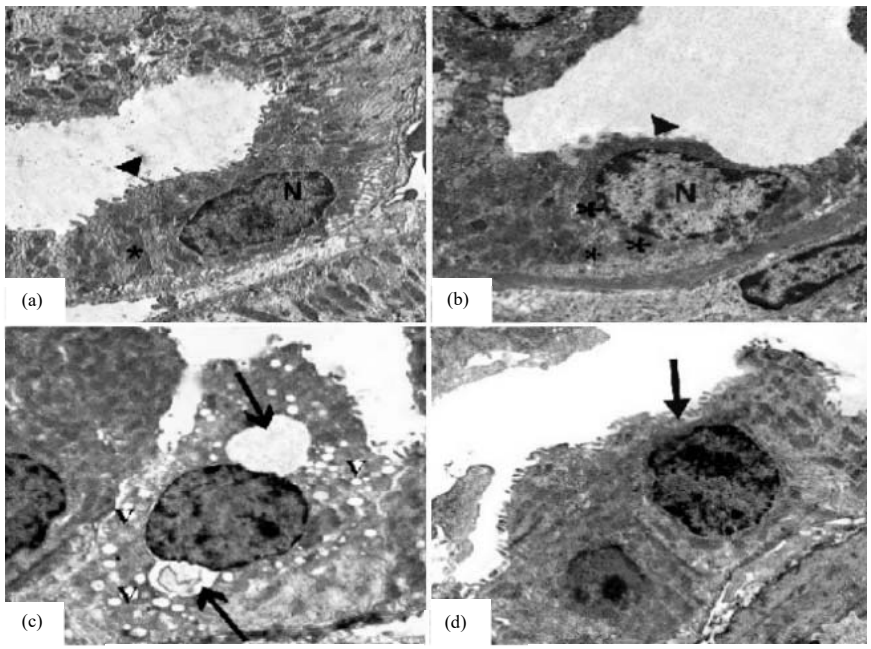

Fig. 9(a-d): Effect of carob pod extract on esomeprazole-induced changes on DCTs of rats using EM (×2000)

(a,b) Control and carob groups showed cubical cells with few short scattered microvilli on their luminal surfaces (▸), rounded or ovoid nuclei with less extended chromatin, (N) and mitochondria (*), (c) Esomeprazole group showed many cytoplasmic vacuoles (V) and areas of cytoplasmic rarefaction (→) and (d) Esomeprazole and carob group showed nearly normal DCT cells (→)

DISCUSSION

The kidney has unique anatomical and physiological features. About 20% of the resting cardiac output is perceived through kidneys consequently, any chemicals situated in the systemic circulation will be delivered in high amounts to it. Additionally, urine concentration leads to the accumulation of toxic substances in the renal tubular cells as well as their lumen. So, kidneys will be highly susceptible to the toxic effects of such substances[13].

Esomeprazole is one of the proton pump inhibitors (PPIs) widely prescribed for the treatment of different gastroesophageal disorders. While they are generally safe, several side effects arise especially when taken for a long time[14].

The present work revealed significant light microscopic structural changes in the renal cortex of the esomeprazole group. These were in the form of shrunken glomeruli with the widening of the Bowman's space, vacuolated glomerular cells, cortical tubules with vacuolated cytoplasm and darkly stained nuclei. These were associated with congested intertubular capillaries, interstitial mononuclear cellular infiltrations and a significant increase of the mean optical density of PAS with lost PAS reaction of the brush border of some PCTs. There was also a significant decrease in the optical density of the iNOS immunohistochemical reaction, besides significant EM structural changes.

It was recorded that long-term exposure to esomeprazole impairs the lysosomal enzyme activity consequently, an accumulation of protein aggregates and an increase in the generation of reactive oxygen species, with the impairment of the NO synthase pathway, could be seen[15].

Shrunken glomeruli with the widening of the Bowman's space in the present work could be attributed to the vasoconstriction of the renal glomerular blood capillaries in response to long exposure to esomeprazole that leads to ROS generation and NO release[16].

The congested intertubular capillaries seen can be attributed to interstitial oedema which is common in acute interstitial nephritis also, might be due to the dilatation and congestion of the vasa recta of the renal outer medulla[17]. While the interstitial cellular infiltration can be explained by the fact that PPI-induced acute interstitial nephritis is triggered by a hypersensitive immune reaction to the drug or one of its metabolites[18].

The cortical tubular cytoplasmic vacuolations and darkly stained nuclei as well as the lost brush border of the PCTs seen in the esomeprazole group due to esomeprazole-induced cell injury with subsequent disturbed cellular membrane permeability to Ca^{+2}. Consequently, an increase in intracellular

oxygen uptake and an imbalance between the production of free radicals and the antioxidant defense system. This also could lead to the release of different cytotoxic lysosomal enzymes to the cytosol and excess calcium influx with the activation of calcium-dependent phospholipases[19].

The present significantly increased mean optical density of PAS in the present research was due to glomerulonephritis associated with esomeprazole treatment which lead to the thickening of the parietal layer proper or deposition of amorphous electron-dense proteins on the endothelial or epithelial side of the parietal layer or within the membrane itself[18]. Also, the focal loss of PAS reaction in the brush border of some PCTs cells may be a result of the progressive acute cell swelling with the disintegration of the actin core of microfilaments and the linker proteins which connected them to the cell membrane[20].

In the present study, a significant decrease in iNOS immunohistochemical reaction is attributed to the increased generation of reactive oxygen species with the impairment of the nitric oxide (NO) synthase pathway. Also, the endothelial dysfunction induced by esomeprazole leads to an increase in the generation of superoxide anion and a decrease in nitric oxide (NO) levels[16].

In addition to the reported light microscopic findings of the present research, EM findings were established to confirm the previous one. These changes were related to the effect of esomeprazole on mitochondria with defective ATP production with the release of ROS. This lead to lipid peroxidation together with protein oxidation, as well as mitochondrial DNA transmutations and damage. Consequently, membrane and cellular organelles degenerative changes. Lenaz[21] and Lee *et al.*[22] also added that the decreased mitochondrial function is associated with oxidative stress which in turn results in post-translational alterations of proteins with the accumulation of protein aggregates ending with autophagic cell stress with different degenerative changes among membranes and organelles.

The protective effect of carob pods might be due to their antioxidant and anti-inflammatory effects. This is because of its polyphenols content which is the most important Phyto-constituents that act against drug-nephrotoxicity[23]. Moreover, Stavrou *et al.*[24] confirmed that carob powder polyphenolic extracts showed better antioxidant potency four folds than that of many well-known potent antioxidants free radical scavenging activity and inhibition of lipid peroxidation. Additionally, flavonoids inhibit enzymes such as xanthine oxidase and cyclooxygenase and also the production of cytokines[25].

The anti-inflammatory effect of carob pods is mediated through the inhibition of the release of different inflammatory mediators such as serotonin, histamine, cyclooxygenase, prostaglandin and cytokines[25]. In addition, the polyphenolic compounds of carob pods inhibit the expression of NF-kappa B-regulated pro-inflammatory genes like TNF-alpha and IL-beta1[26].

Based on the results obtained from the current study, it could be concluded that esomeprazole induced structural changes in the renal cortex in rats and these changes can be ameliorated by carob pod extract due to its antioxidant and anti-inflammatory properties. Also, it is recommended to limit the use of esomeprazole to the therapeutic indication beside it needs more studies on the effect of esomeprazole on the renal medulla and the possibility to use of other protectives for esomeprazole-induced renal injury.

SIGNIFICANCE STATEMENT

This work aimed to investigate the possible protective effect of carob pod extract on esomeprazole-induced changes in the renal cortex of adult male albino rats. In addition, esomeprazole showed structural changes in the renal cortex of rats and carob pod extract could improve such changes through its antioxidant and anti-inflammatory properties.

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