Original Article

Histological and Immunohisto-chemical Study of Toxic Effect of Gibberellic Acid Postnatally on Renal Cortex of Albino Rats

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ABSTRACT

Background: Gibberellic acid (GA3) is one of naturally occurring plant growth regulator, it is one of the family of "biocides" that are phyto-hormones with insecticidal effects. Most of nitrogenous wastes are eliminated through the two kidneys that in addition maintains blood volume, composition and pressure and keeps bone density.

Aim: To evaluate the histopathological effect GA3 on rat's renal cortex postnatally in different ages (PND1, PND 7 and PND 21).

Material and Methods: Pregnant female albino rats (n=20) had been distributed into: control group and treated group, GA3 was administered from the 14th day of pregnancy to the 21st postnatal day at a dose of 55 mg/kg of body weight /day. After giving birth, the male pups of each group were randomly divided into three subgroups and were sacrificed at the (1 day), (7 days) and at (21 days). Animals were anaesthetized and sacrificed. Kidneys of pups born to control and treated mothers were obtained and processed for examination of oxidative stress marker, histological and immunohistochemical examination.

Results: Pups of GA3 treated mothers showed increase in urea, creatinine serum level and MDA level and decrease in CAT and GPX content. Moreover, histological examination of the kidneys of these pups revealed multiple atrophied immature forms of glomeruli, multiple congested blood capillaries, some renal tubules showed loss of some cells lining them and others with vacuolated cytoplasm. Statistically, GA3–treated groups at all age groups displayed a highly significant decrease in the mean cortical thickness than control groups. Moreover, the area percentage of collagen and of renal PCNA expression showed a significant increase with GA3 treatment of mothers.

Conclusion: Exposure of pregnant rats to GA3 in late pregnancy and during lactation induced oxidative stress and histopathological alterations in renal cortex of suckling pups.

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Key Words: Albino rats, gibberellic acid, renal cortex, Oxidative stress and Microanatomy.

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INTRODUCTION

The World Health Organization (1990) considered GA3 as one of plant growth regulators (PGRs) that are widely used in agricultural field in many countries including Egypt[1]. Additionally, the American Society of Agricultural Science considered Gibberellins a main plant growth regulators. Few people knew about its harmful effects on human health[2]. These regulators may induce dangerous effects more than those of insecticides[3]. (GA3) acts by influencing many plant growth mechanisms as elongation of the stem through motivating the cellular division and increase in length, fruit growth and seed sprouting[4]. GA3 increases the growth of dwarf peas, beans or maize if applied to them. But it was proved that, the use of (GA3) for long time induced many hazardous effects as oxidative stress that causes formation of free radicals that cause cell damage, tumors in different organs as hepatic, breast and lung carcinomas[5,6]. People can be exposed to Gibberellic acid by utilizing fresh fruits and vegetables[7]. Moreover, occupational exposure could occur through inhalation of the powder and skin contact giving an acute toxicity picture[8] The kidney is the most liable organ for being affected by toxic substances as its function is to extract and concentrate toxic elements from the blood as it has large blood flow. In postnatal kidney the appearance of nephrogenic zones and the peripheral cortical tubules in the twelfth day and twenty eighth day postnatally correspondingly are the first indicators of renal function. The authors reported that postnatal morphogenesis is an undeniable element in the kidney of rodents[9]. Although many studies investigated effect of exposure to gibberellic acid (GA3) on various organs, few of them paid attention to its effect on off springs of exposed mothers. Current study aimed to investigate the effect of exposure to GA3 transplacental and through lactation on developmental morphogenesis of renal cortex of albino rat.

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MATERIAL AND METHODS

2.1. Chemicals

2.1.1. Gibberellic acid (GA3)

(99% pure) Gibberellic Acid (2,4a, 7-Trihydroxy-1-methyl-8-Methylenegibb-3-ene-1, 10 - dicarboxylic acid 1,4a-lactone) in the form of white crystalline powder, was from Sigma -Aldrich chemical Co., Germany.

2.2. Experimental animals

A total of 10 Wister male and 20 female rats weighing 180-200 g, aging 3 months were purchased from Zagazig University Animal house. They were kept under controlled conditions of temperature (23±2°C) and humidity (40%) with a 12-h light/dark cycle. All experimental procedures were performed in accordance with the guidelines of the institutional Animal Care and Requirements of the Ethical Committee of faculty of Medicine, Zagazig University, with ZU-IACUC committee approval with number of (ZU-IACUC/3/F/66/2019)[10]. All animals were feed with ad libitum water and commercial rodent diet. one week after acclimatization, virgin female and male rats were housed in plastic cages overnight; detection of sperms in the vaginal smears were considered as an indicator day 0 of pregnancy. Twenty pregnant female albino rats were appropriated into two equivalent groups: group I (control) and group II (treated). Control group (n=10) received 2 ml of distilled water by gastric gavage from the fourteenth day of getting pregnant till 21st day after delivery. In the treated group (n=10), GA3 was managed at a dose of 55 mg/kg of body weight/day from the fourteenth day of intrauterine life to the 21st day postnatally.

GA3 was liquefied in distilled water and taken orally. Each animal administrated 11mg/200mg body weight dissolved in 2ml of distilled water by gastric gavage. After conceiving an offspring, the male newborn of both control and treated moms were classified into three subgroups and were sacrificed at the (1 day), (7 days) and at (21 days). The given dose represented 1/100 of LD50[11]. The birthday was considered as postnatal day 0.

2.2. Methods

2.2.1. Experimental Methodology

At the end of the experiment, the animals were anaesthetized with intraperitoneal injection of Thiopental (50mg/kg)[12], its heart was perfused by saline solution through the left ventricle until the coming out fluid, from the right atrium after being opened, was blood-free. After sacrifice, laparotomy was done and kidneys were excised out and processed for histological examination. From each animal right kidney was homogenized by immersion in ice-cold 50 mM sodium phosphate buffer (pH 7.4) has 0.1 mM ethylene diamine tetra acetic acid (EDTA). The left kidney was fixed in 10%formol fixative for histopathological examination.

2.2.2. Biochemical analysis

Blood samples were withdrawn from tail vein from all rats at time of sacrifice; each sample was kept to clot and later centrifugation was done at 1000 xg for 20 min. The sera were used for the estimation of serum level of urea and creatinine by using kit of biomerieux, France.

2.2.3. Lipid peroxidation estimation and oxidative enzyme assay

The resultant supernatant of homogenized kidney was centrifuged at 1000 g for 20 min at 4 C to be separated. The supernatant was analyzed for MDA, GPxand CAT

- Lipid peroxidation estimated by measuring malondialdehyde (MDA). It was measured colorimetrically in renal homogenate with the use of a commercially available kit (Biodiagnostic, Cairo, Egypt) according to Lapenna *et al*.[13].
- Glutathione peroxidase (GPx) and Catalase (CAT) were measured in renal homogenate according to Arenas-Ríos *et al*.[14] with the use of a commercially available kit (Biodiagnostic, Cairo, Egypt).

2.2.4. Light microscopic examination

The fixed samples in 10% formol saline fixative were processed and embedded in paraffin wax. Sections of 5-µm thickness were obtained and prepared from the renal tissue for the following stains:

- Hematoxylin (H) and Eosin (E) and Masson's trichrome stains according to method of Bancroft and Layton[15].
- Immunostaining for Anti-Proliferating cell nuclear antigen (PCNA)

Proliferating cell nuclear antigen (PCNA) its expression is indicator to cell proliferation. Immunohistochemical staining was processed by using primary antiserum to PCNA (Clone PC 10, DAKO A/S Denmark), which diluted in Trisbufferd saline (1:50), as mentioned by the data sheet. Later, incubation of the specimens with the primary antibody overnight at + 4°C. Enough Biotinylated secondary antibodies were applied to cover specimen then the binding of the primary antibody was noticed by using a commercial avidinbiotin-peroxidase detection system (DAKO, Carpenteria, USA). A mouse monoclonal antibody was applied in place of the primary antibody to act as a negative control. Small intestine sections were used as a positive control for (PCNA). Then the slides were stained with diaminobenzene (DAB) as the chromogen and counter stained with hematoxylin then slide dehydrated in 95% ethanol, cleared in xylene then cover slips were mounted using two drops of DPX mounting medium[16].

2.2.5. Morphometric analysis

For morphometrical study, the image analyzer (the Image J software plugin) in Anatomy department, Zagazig University, Egypt was used as follow:

- H and E stained Sections were analyzed morphometrically by the image analyzer computer system (Leica Qwin 500, Engl.) in the image analysis unit in Human Anatomy and Embryology Department, Faculty of Medicine, and Zagazig University. Renal cortical thickness was assessed in H and E slides under 100 high power fields in random microscopic areas. A mean of 15 readings was measured from 5 serial sections from slides of each rat in each group.
- In addition, the area of collagen fibers was measured in Masson's trichrome stained sections under 400 high power fields.
- Perspective Anti-Proliferating cell nuclear antigen (PCNA): immunohistochemical stained slides of each group were analyzed to estimate the area percentage of positive immune reaction for PCNA which was done after image splitting. Images were split into RGB stacks then red stack was adjusted to threshold to mark it with a binary mask. Then the percent area in relative to the field was calculated at the objective lens of 40x.

2.2.6. Statistical analysis

The data was computerized and statistically analyzed using SPSS version 25. Quantitative data was expressed as mean and SD. Independent t test was used to compare between the two studied groups. Repeated measure ANOVA was used to compare between different age in the same experimental group. P value of less than 0.05 consider to be significant and less than 0.01 highly significant.

RESULTS

3.1. Biochemical results

Urea and creatinine level

There was a highly statistically significant difference between the control and treated groups in all age groups for both urea and creatinine serum level. No difference was found between 1st day, 7th day and 21st day in both urea and creatinine level in control group. But a statistically significant increase between first day, seventh day and twenty first day in urea level in treated groups was found. In addition, there was statistical significance increase between 1st day and 7th day and between 1st day and 21st day in creatinine level in treated group (Table 1, Figures 1,2).

Assay of lipid peroxidation marker (MDA) and anti-oxidant activity markers (CAT and GpX)

Level of MDA, CAT and GPX in all age group of follow up there was highly statistical significance difference between the control group and treated group. Regarding differences between different times in each group no difference was found between 1st day, 7th day and 21st day in all MDA, CAT or GPx level in control group. But there were statistical significance differences between 1st day, 7th day and 21st day in all MDA, CAT and GPX level in treated groups as there was significant increase in MDA level and significant decrease in CAT and GPx levels in rats born to GA3mothers in comparison to control groups (Table 2, Figures 3,4,5).

3.2. Histo-pathological Results

3.2.1. H and E stain

At the first post-natal day (PND1)

Control group: (Figures 6 a,b,c)

The cortex of kidney in a control (PND1) was composed of the subcapsular nephrogenic zone contained immature forms of glomeruli and a juxtamedullary zone contained more mature nephrons. The intercortical zone showed rays of tubules (Figure 6a). Some immature renal glomeruli appeared as comma-shaped bodies placed in the superficial part of cortex, well developed glomeruli appeared at deeper cortex and Bowman's capsule and space with distinct parietal layer also seen. Proximal convoluted tubules appeared with acidophilic granular cytoplasm and basal nuclei. The lumen of the distal convoluted tubules was dilated. The intercortical zone formed rays of tubules that correspond to the medullary rays in adult straight ureteric bud was observed as straight tubules and was lined with cuboidal cells with centrally placed nuclei (Figure 6b). S shaped body appeared to have an upper limb, middle segment and lower limb with vascular cleft contained capillary loops lined by endothelial cells (Figure 6c).

GA-3 treated group: (Figures 7 a,b,c)

The renal cortex of a Gibberellic acid (GA-3)-treated (PND1) rat was composed of the subcapsular nephrogenic zone with multiple shrunk immature forms of glomeruli (comma and renal vesicle). A juxtamedullary zone was containing multiple atrophied nephrons. The intercortical zone formed dilated rays of tubules that correspond to the medullary rays in adult (Figure 7a). Deep in the cortex, glomeruli and some tubular cells revealed darkly stained nuclei, other tubules revealed loss of their cell lining and vacuolated cytoplasm. Multiple congested blood capillaries were found (Figure 7b). Immature forms of renal glomeruli appeared as comma-shaped bodies with flat cells and dilated capsular space. Acidophilic exudates were present intertubular and some proximal tubules showed loss of cell lining and distal tubules show some dark stained nuclei (Figure 7c).

At the seventh post-natal day (PND7)

Control group: (Figures 8 a,b,c)

The renal cortex of a control (PND7) rat at the sub capsular zone had immature forms of renal glomeruli. Medullary rays remained prominent in the cortex and loose connective tissue in the outer region of the medulla was decreased (Figure 8a). At the deeper cortex, more developed renal glomeruli appeared. Cells of proximal convoluted tubule had vesicular rounded basal nuclei and acidophilic granular cytoplasm. Cells of distal convoluted tubule exhibited less acidophilic

cytoplasm (Figure 8b). The renal cortex showed oval maturing glomeruli with few capillary loops. The cells of the parietal layer appeared flattened (Figure 8c).

GA-3 treated group: (Figures 9 a,b,c)

The renal cortex of a Gibberellic acid (GA-3)-treated (PND7) rat was near maturation with multiple degenerating renal corpuscles and well distinguished from medulla with dilated tubules (Figure 9a). At the deeper cortex, some glomeruli with darkly stained cells and others were atrophied. There were multiple congested capillaries. Partial loss of cell lining the distal convoluted tubules and vacuolated cytoplasm of some cells lining proximal tubules were detected (Figure 9b). The cells of the parietal layer of glomerular covering appeared flattened with darkly stained nuclei and loss of cell continuity in some areas. Proximal convoluted tubules were with vacuolated cell lining and distal convoluted tubules were with degenerated and lost cell lining (Figure 9c).

At the twentieth first post-natal day (PND21)

Control group: (Figures 10 a,b,c)

The renal cortex of a control (PND21) rat was near maturation and well distinguished from medulla (Figure 10a). In the cortex, there were well-defined glomeruli and Bowman's capsule looked with its visceral and parietal layers and distinctive capsular space with few capillary loops. There was flattening of cells of the parietal layer, while those lining the proximal convoluted tubule showed basal vesicular rounded nuclei and acidophilic granular cytoplasm. But, the cytoplasm of the cells lining the distal convoluted tubule was less acidophilic (Figure 10b). Moreover, peritubular capillaries and rounded mature glomeruli with few capillary loops were detected. The cells of the parietal layer appeared flattened while the cells of the visceral layer were separated and surround the capillary loops (Figure 10c).

GA-3 treated group: (Figures 11 a,b,c)

The renal cortex of a Gibberellic acid (GA-3)-treated (PND21) rat was near maturation with multiple degenerating renal corpuscles and well distinguished from medulla with dilated necrotic tubules (Figure 11a). Well-developed glomeruli appeared at the deeper cortex and some glomeruli showed hyper cellularity. The epithelial lining of some renal tubules lining cells showed cytoplasmic vacuolation (Figure 11b). There was also necrotic glomerulus with very few capillary loops. The cells of the parietal layer were necrotic and others were showing ballooning with lost cell lining in other areaswith dilated Bowman's space. Necrotic proximal and distal convoluted tubules were also detected with necrotic lining cells (Figure 11c).

3.2.2. Special Stain (Masson Trichrome)

Masson's trichrome stained specimens showed very little collagen fiber in the renal interstitium around renal corpuscles and tubules (Figures 12a,13a,14a). However, in GA 3 treated groups, there was noticeable increase in collagen fiber deposition in the renal interstitium around renal corpuscles and tubules (Figures 12b,13b,14b).

3.2.3. Immune-histochemical stains

Anti-Proliferating cell nuclear antigen (PCNA) and Hematoxylin as a counter stain:

PCNA-positive cells were detected in the renal cortical tissues of the control rats of all age groups (Figures 15a,16a,17a). However, the signal density of positive cells was higher in the GA3 received groups with apparent increase in positive cell number (Figures 15b,16b,17b).

3.3. Statistical Results

Regarding cortical thickness, there was statistical significance difference in 1st day and highly statistical significance difference in 7th day and 21st day between the control group and treated group. Additionally, there was significant decrease in cortical thickness of treated rats in comparison with control in each age group. (Table 3, Figure 18)

While in area percentage of collagen in Masson Trichrome stain, there was highly statistical significance difference between the control group and treated group in all times. Regarding differences between different times in each group there were statistically significant increase in 1st day, 7th day and 21st day in treated groups only with no significant difference between control groups in all age groups. (Table 4, Figure 19)

While in PCNA there were highly statistical significance difference between the control group and treated group in all times. Regarding differences between different times in each group there were statistically significant increase in 1st day, 7th day and 21st day in PNCA in both control and treated groups (Table 5, Figure 20).

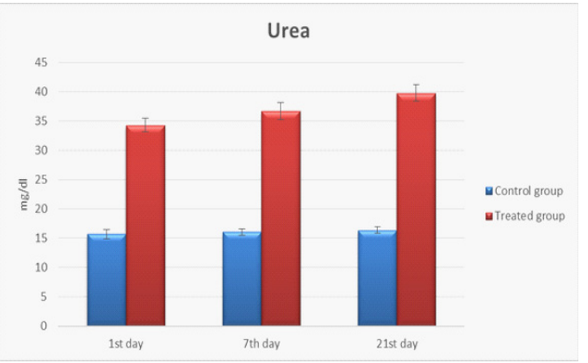

Fig. 1: A bar chart shows serum urea level in both control and GA-3 treated groups in all age groups (mg/dl).

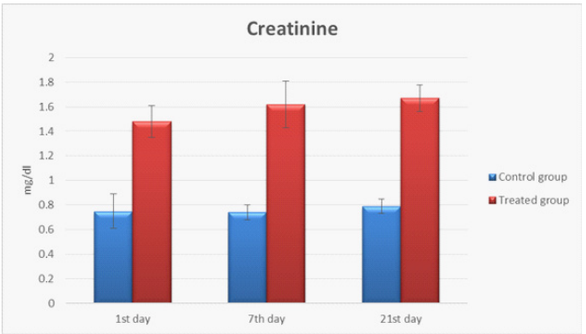

Fig. 2: A Bar chart shows serum creatinine level in both control and GA-3 treated groups in all age groups (mg/dl).

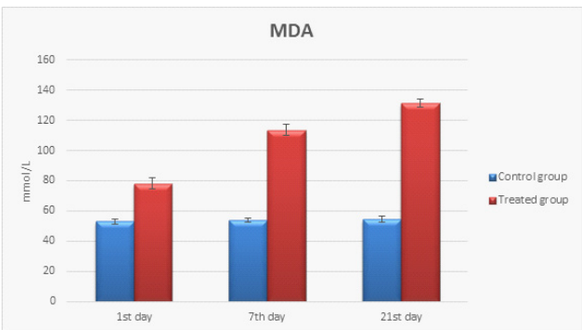

Fig. 3: A bar chart shows MDA level (mmol/L) in both control and GA-3 treated groups in all age groups.

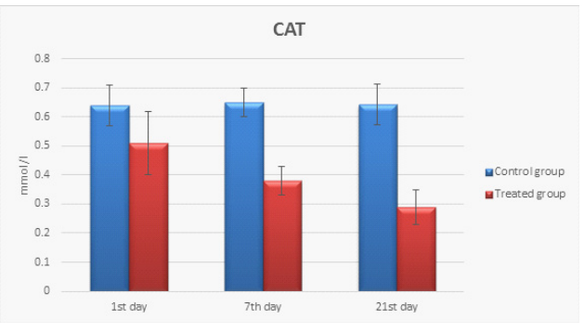

Fig. 4: A bar chart shows CAT level (mmol/L) in both control and GA-3treated groups in all age groups.

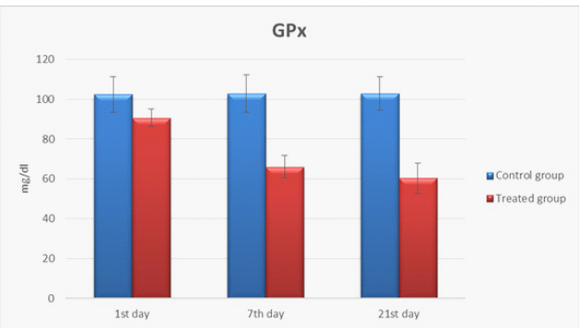

Fig. 5: A bar chart shows GpX level (mg/dl) in both control and GA-3 treated groups in all age groups.

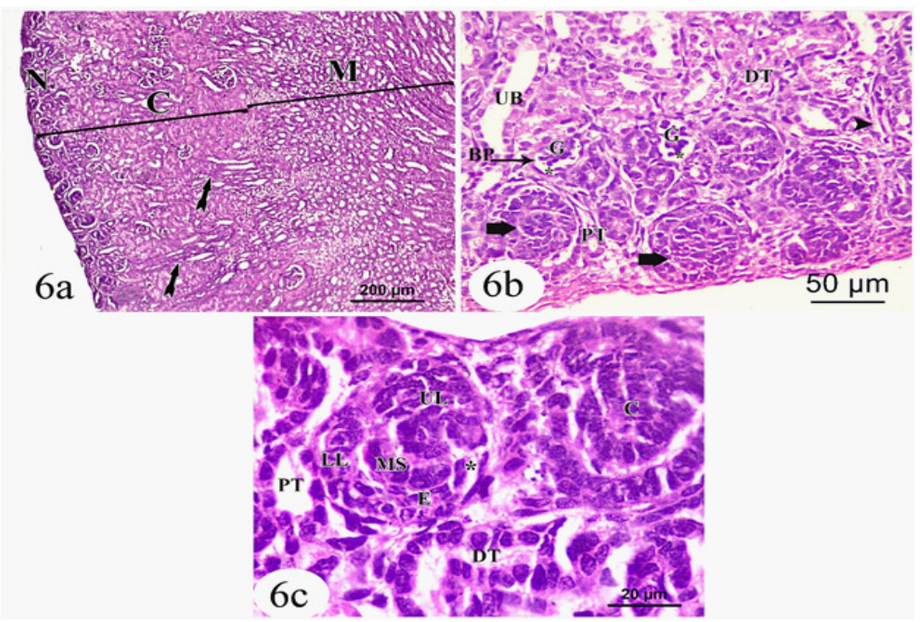

Fig. 6: (6a) A photomicrograph of kidney section of a control (PND1) rat shows renal cortex (C) which is composed of subcapsular nephrogenic zone (N) containing immature forms of glomeruli and a juxtamedullary zone containing more mature nephrons. The intercortical zone forms rays of tubules (thick tailed arrows), note the medulla(M). (H&E× 100). (6b) A magnified photomicrograph of kidney section shows some immature renal glomeruli, such as comma-shaped (thick arrow) bodies placed in the outer cortex. Mature glomeruli (G) at deeper cortex and Bowman's capsule with distinct parietal layer (BP). Note the distinct Bowman's space (*). Some PCT(PT) are detected. Distal convoluted tubules show dilated lumen (DT). Note medullary rays (arrow head) extending between the outer and deeper parts of the cortex. Straight ureteric bud (UB) are seen.(H&E× 400). (6c) A highly magnified photomicrograph of kidney cortical section shows some immature renal glomeruli, such as comma-shaped (C), S shaped body having an upper limb (UL), middle segment (MS) and lower limb (LL). A vascular cleft (asterisk) is seen containing capillary loops lined by endothelial cells (E). Some proximal convoluted tubules (PT) and dilated distal convoluted tubules (DT) are present. (H&E × 1000)

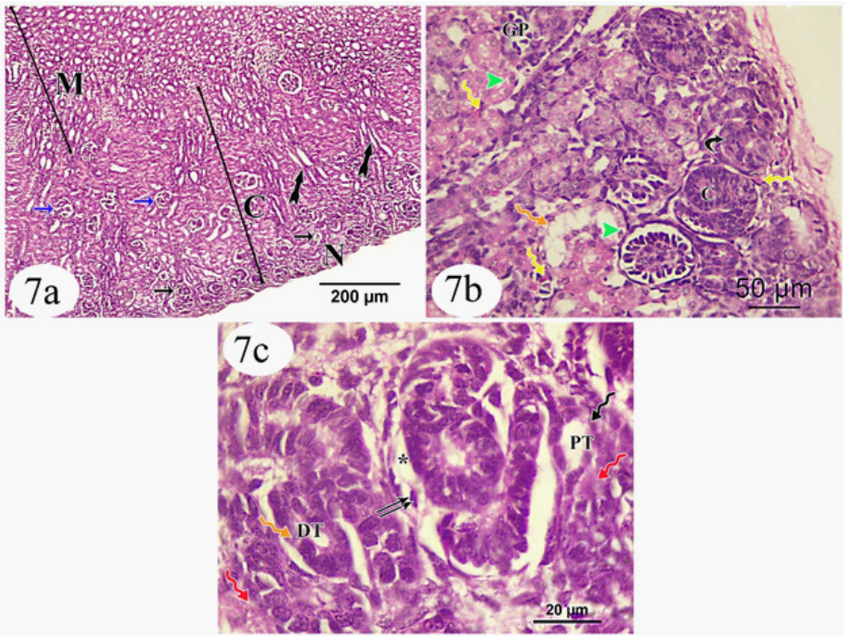

Fig. 7: (7a) A photomicrograph of kidney section of a Gibberellic acid (GA-3)-treated (PND1) rat shows renal cortex (C) which is composed of the subcapsular nephrogenic zone (N) with multiple shrunken glomeruli (thin arrows) and a juxtamedullary zone containing multiple atrophied nephrons (thin blue arrows). The intercortical zone forms dilated rays of tubules (tailed arrows) that correspond to the medullary rays in adult, note the medulla (M). (H&E× 100). (7b)A magnified photomicrograph of kidney cortical section shows immature forms of renal glomeruli in the form of renal vesicle (curved arrow) and comma-shaped (C) glomerulus. Deep in the cortex, glomeruli are with apoptotic cells (GP). Multiple congested blood capillaries (zigzag yellow arrows) and some renal tubules show loss of some cells lining them (zigzag orange arrow) and others with vacuolated cytoplasm (green arrow heads) are observed(H&E × 400). (7c) A highly magnified photomicrograph of kidney cortical section shows some immature forms of renal glomeruli, such as comma-shaped bodies with flat cells (double arrows) and dilated capsular space (Asterisk).Acidophilic exudates (zigzag red arrows) and some PCT(PT) show loss of cell lining (Zigzag black arrow) and DCT(DT) show some apoptotic cells (zigzag orange arrow)(H&E × 1000)

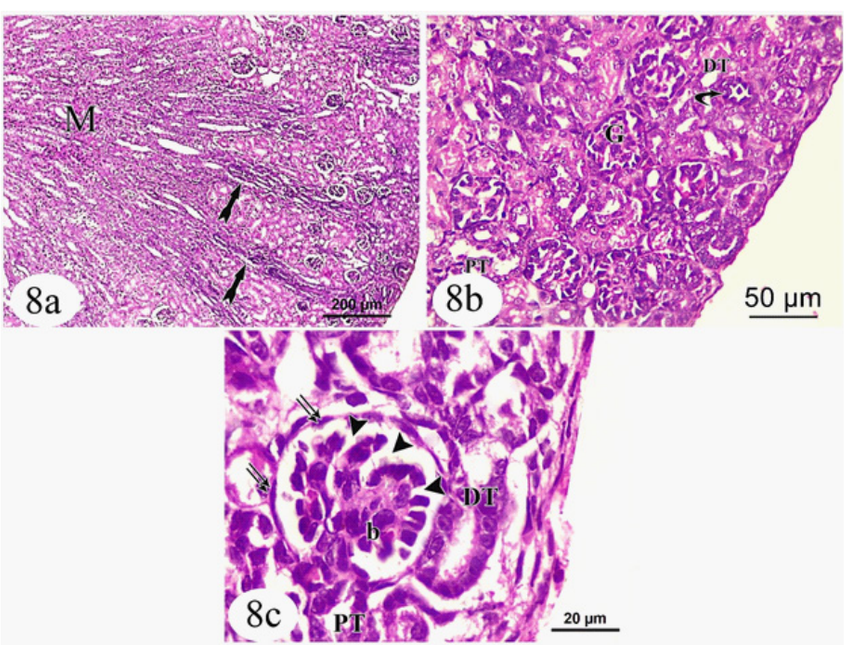

Fig. 8: (8a) A photomicrograph of a section of kidney cortex of a control (PND7) rat at the sub capsular zone shows immature forms of renal glomeruli occasionally, medullary rays (tailed arrows) are prominent in the cortex and the outer medulla(M) has less loose connective tissue. (H & E × 100). (8b) A magnified photomicrograph of a section of kidney cortex shows undifferentiated solid masses of cells (curved arrows). The deep cortex shows more developed renal glomeruli (G). Proximal convoluted tubule (PT) cells show vesicular adjusted basal cores with acidophilic granular cytoplasm. Distal convoluted tubule (DT) cells show less acidophilic cytoplasm. (H&E × 400). (8c)A highly magnified photomicrograph renal cortex shows oval maturing glomeruli with few capillary loops (b).The cells of the parietal layer appear flattened (double arrow) while the cells of the visceral layer are separated (arrow heads) and surround the capillary loops. Proximal (PT) and distal (DT) convoluted tubules are also seen. (H&E × 1000)

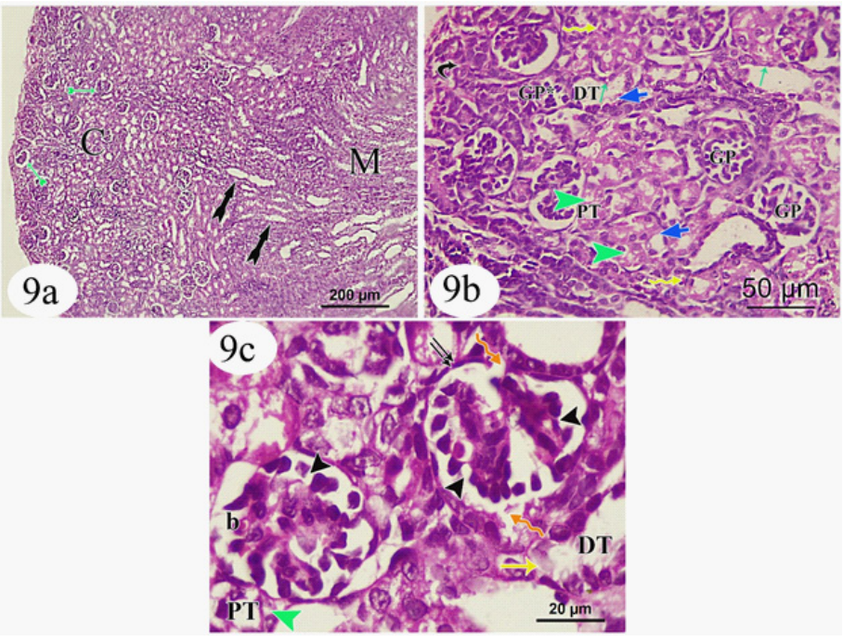

Fig. 9: (9a) A photomicrograph of a section of kidney of a Gibberellic acid (GA-3)-treated(PND7) rat at the sub capsular zone with the cortex (C) near maturation with multiple degenerating renal corpuscles (thin tailed green arrows) and well distinguished from medulla (M) with dilated necrotic tubules (thick tailed arrows)(H & E × 100). (9b) A magnified photomicrograph of kidney cortex shows undifferentiated solid masses of cells (curved arrows). The deep cortex shows renal glomeruli and some glomeruli with apoptotic cells (GP) and others are atrophied (GP*). Partial loss of the distal (DT) convoluted tubular brush border (thin green arrows) and loss of some cells lining (Blue short arrows). Vacuolated cytoplasm of some cells lining proximal (PT) tubules (green arrow heads). There are multiple congested capillaries (zigzag yellow arrows). (H&E × 400) (9c)A highly magnified photomicrograph of section in renal cortex shows oval maturing glomeruli with few capillary loops (b).The cells of the parietal layer appear flattened with darkly stained nuclei (double arrow) and loss of cell continuity in some areas (zigzag orange arrows) while the cells of the visceral layer are apoptotic and separated (arrow heads) and surround the capillary loops. Proximal (PT) convoluted tubules are with apoptotic vacuolated cell lining (green arrow head) and distal (DT) convoluted tubules are with degenerated brush border and lost cell lining (yellow arrow)(H&E × 1000)

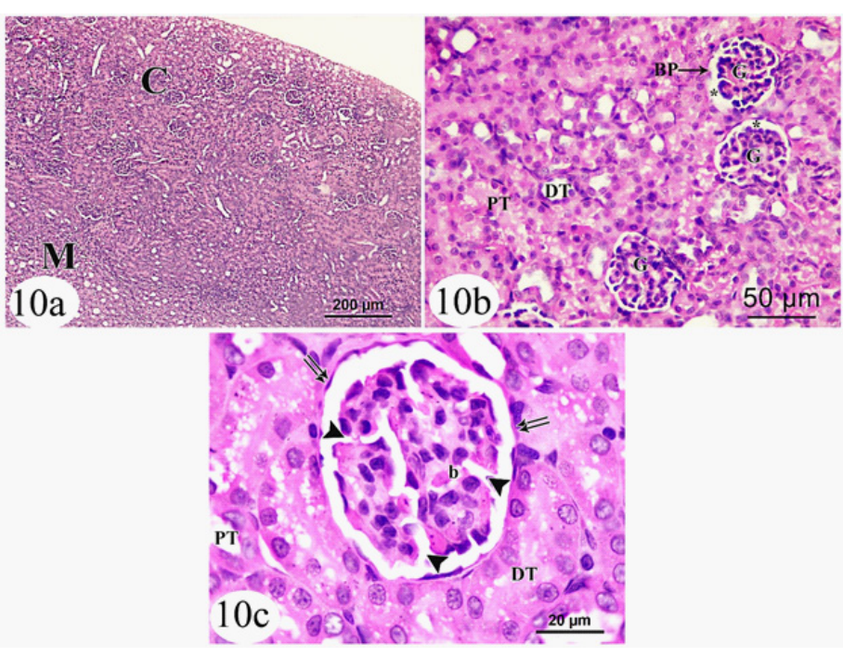

Fig. 10: (10a) A photomicrograph of a section of kidney cortex of a control (PND21) rat at the sub capsular zone with the cortex (C) near maturation and well distinguished from medulla(M). (H & E × 100). (10b) A magnified photomicrograph of kidney cortex shows fully mature glomeruli (G) with distinct Bowman's capsule with parietal (BP) layers and distinct capsular space (*). Proximal convoluted tubule (PT) cells with vesicular rounded basally situated nuclei and acidophilic granular cytoplasm. Distal convoluted tubule (DT) cells show less acidophilic cytoplasm. (H&E × 400) (10c) A highly magnified photomicrograph of kidney cortex shows rounded mature glomeruli with few capillary loops (b). The cells of the parietal layer appear flattened (double arrow) while the cells of the visceral layer are separated (arrow heads) and surround the capillary loops. Proximal (PT) and distal (DT) convoluted tubules are also seen. (H&E × 1000).

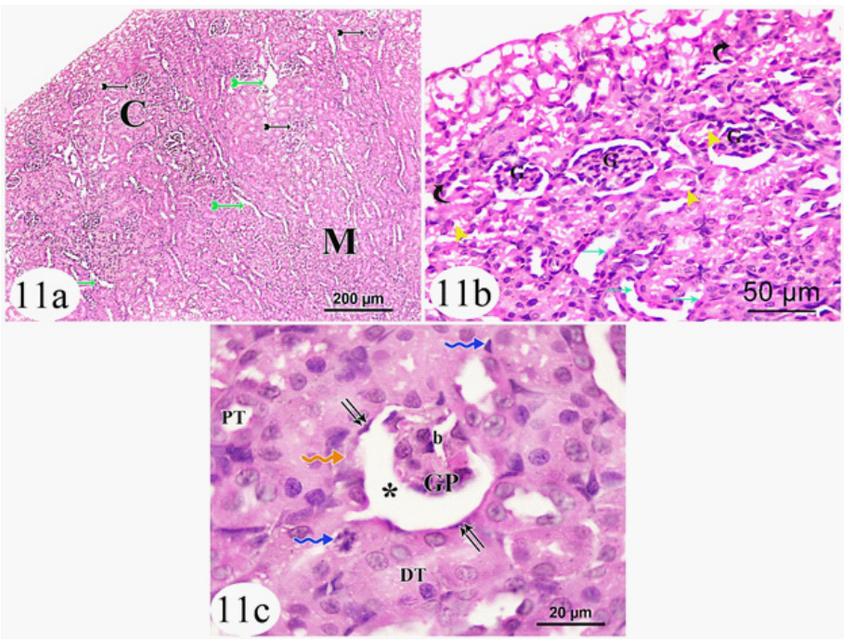

Fig. 11: (11a) A photomicrograph of a section of kidney cortex of a Gibberellic acid (GA-3)-treated (PND21) rat at the sub capsular zone with the cortex (C) near maturation with multiple degenerating renal corpuscles (thin tailed arrows) and well distinguished from medulla (M) with dilated necrotic tubules (thin tailed green arrows). (H & E × 100). (11b) A magnified photomicrograph of kidney cortex shows undifferentiated solid masses of cells (curved arrows). The deep cortex shows fully mature glomeruli and some glomeruli (G) with hyper cellularity. Note the cytoplasmic vacuolation in the epithelial lining of the renal tubules (Yellow arrow heads). Partial loss of the brush border and cell lining(thin green arrow). (H&E × 400) (11c) A highly magnified photomicrograph of kidney cortex shows necrotic glomerulus (GP) with very few capillary loops (b). The cells of the parietal layer are necrotic (double arrow) and others are showing ballooning (orange zigzag arrow) with lost cell lining in other areas with dilated Bowman's space (Asterisk). Necrotic proximal (PT) and dilated distal (DT) convoluted tubules are also seen with necrotic lining cells (blue zigzag arrow) (H&E × 1000).

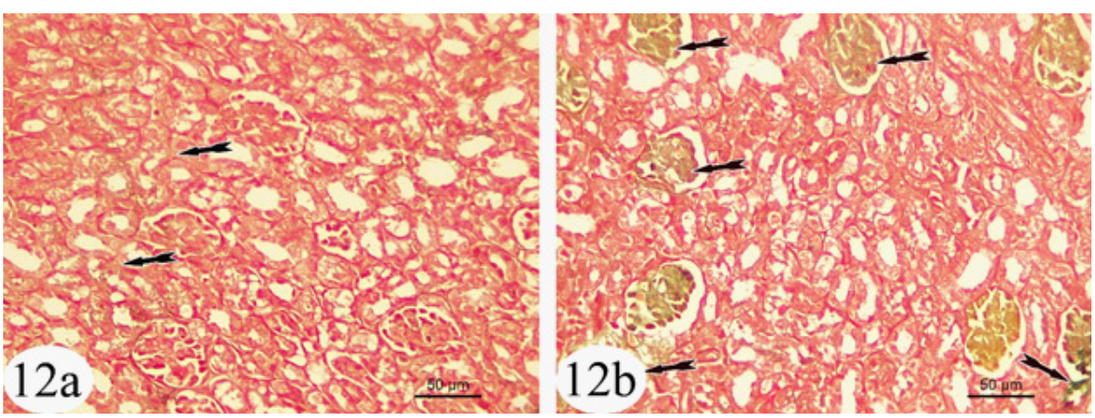

Fig. 12: (a) A photomicrograph of a section in kidney cortex of control (PND 1) rat shows little amount of the collagen fiber (tailed arrows). (b) A photomicrograph of a section of the renal cortex of a Gibberellic acid (GA-3)-treated (PND 1) rat shows large amount of the collagen fiber is distributed mainly around the renal tubules and within the glomeruli (tailed arrows) (Masson Trichrome × 400).

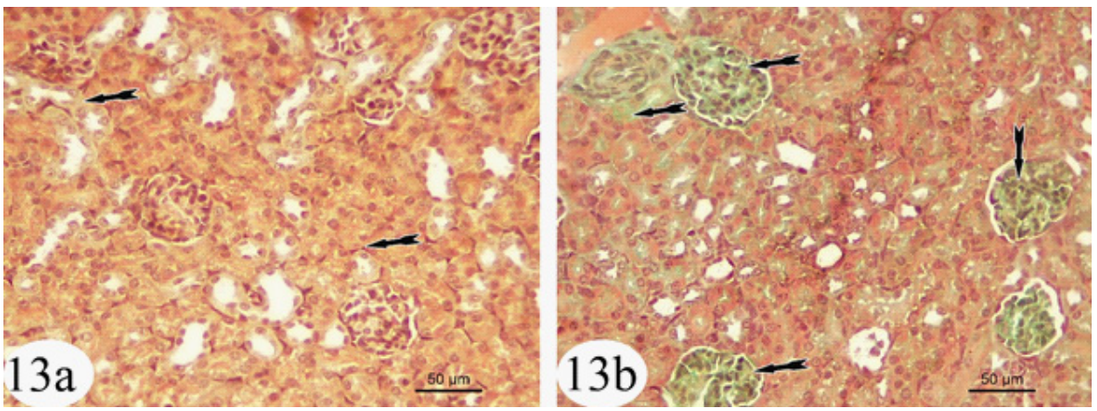

Fig. 13: (a) A photomicrograph of a section in kidney cortex of control (PND 7) rat shows little amount of the collagen fiber (tailed arrows). (b) A photomicrograph of a section of the renal cortex of a Gibberellic acid (GA-3)-treated (PND 7) rat shows large amount of the collagen fiber is distributed mainly around the renal tubules and within the glomeruli (tailed arrows) (Masson Trichrome × 400).

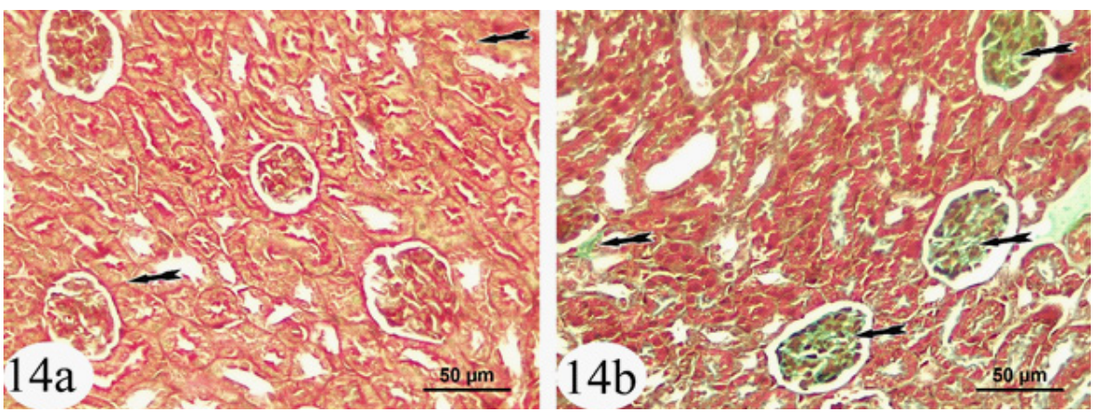

Fig. 14: (a) A photomicrograph of a section kidney cortex of control (PND 21) rat shows little amount of the collagen fiber (tailed arrows). (Masson Trichrome × 400). (b) A photomicrograph of a section of the renal cortex of a Gibberellic acid (GA-3)-treated (PND 21) rat shows large amount of the collagen fibers distributed mainly around the renal tubules and within the glomeruli (tailed arrows) (Masson Trichrome × 400).

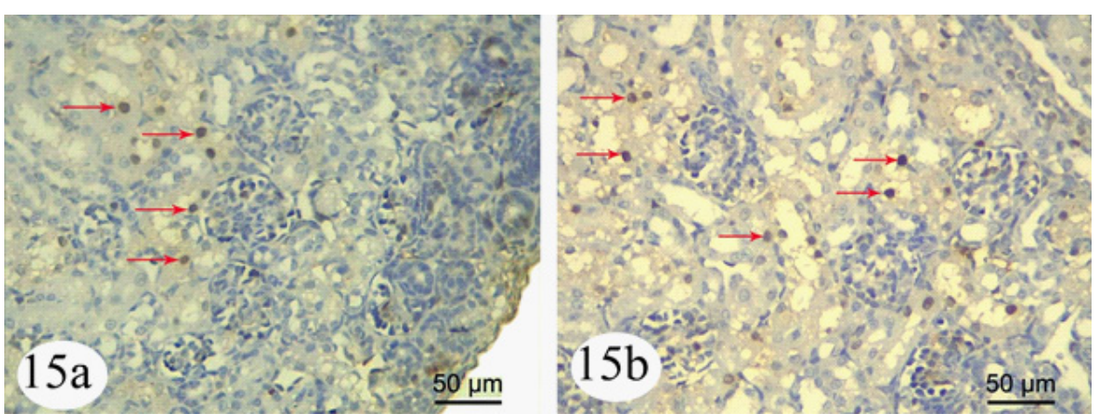

Fig. 15: (a) A photomicrograph of a section in kidney cortex of a control (PND 1) rat shows a few numbers of positive PCNA immunoreactivity in renal tubular cells and around renal glomeruli (Red arrows). (Immunostaining for PCNA and H as a counter stain × 400). (b) A photomicrograph of a section of the renal cortex of a Gibberellic acid (GA-3)-treated (PND 1) rat shows apparent increase positive PCNA immunoreactivity in renal tubular cells and around renal glomeruli (Red arrows) (Immunostaining for PCNA and H as a counter stain × 400).

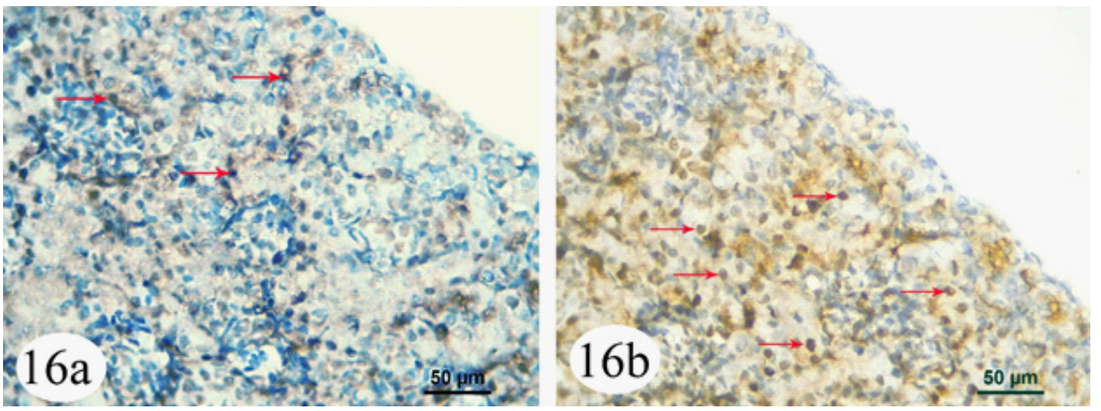

Fig. 16: (a) A photomicrograph of a section of kidney cortex in a control (PND 7) rat shows a few numbers of positive PCNA immunoreactivity in renal tubular cells and around renal glomeruli (Red arrows). (b) A photomicrograph of a section of the renal cortex of a Gibberellic acid (GA-3)-treated (PND 7) rat shows apparent increase positive PCNA immunoreactivity in renal tubular cells and around renal glomeruli (Red arrows) (Immunostaining for PCNA and H as a counter stain × 400).

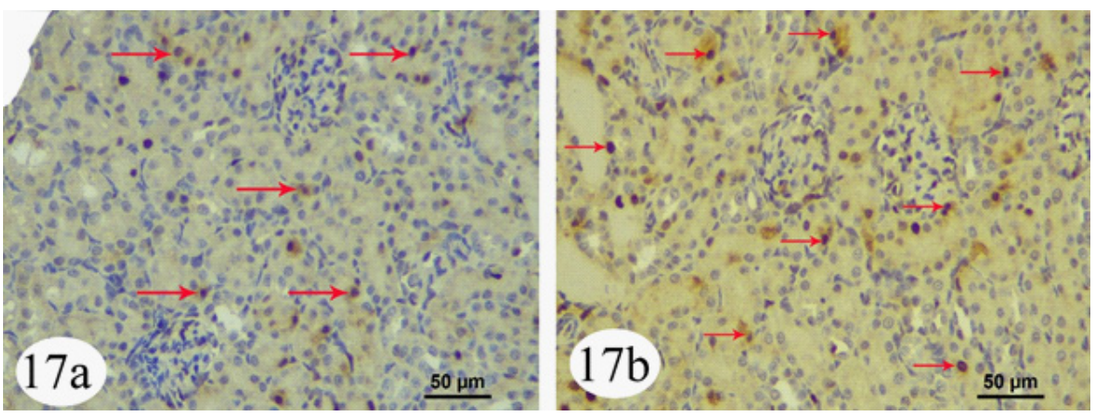

Fig. 17: (a) A photomicrograph of a section of kidney cortex in a control (PND 21) rat shows a few numbers of positive PCNA immunoreactivity in renal tubular cells and around renal glomeruli (Red arrows).(b)A photomicrograph of a section of the renal cortex of a Gibberellic acid (GA-3)-treated (PND 21) rat shows apparent increase positive PCNA immunoreactivity in renal tubular cells and around renal glomeruli (Red arrows) (Immunostaining for PCNA and H as a counter stain × 400).

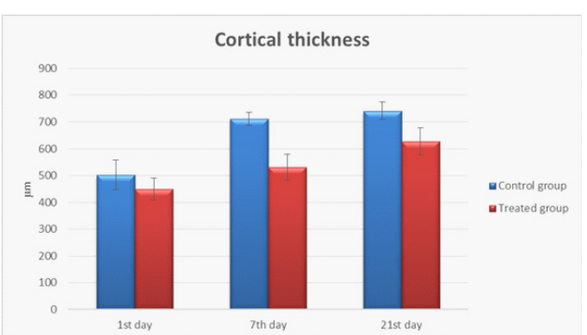

Fig. 18: A bar chart shows cortical thickness (um) in both control and GA-3 treated groups in all age groups.

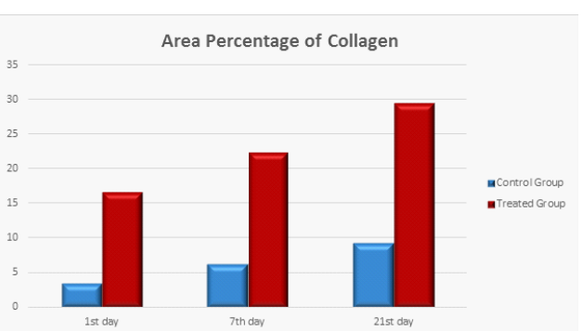

Fig. 19: A bar chart shows area percentage of Collagen in Masson Trichrome Stain in both control and GA-3 treated groups in all age groups.

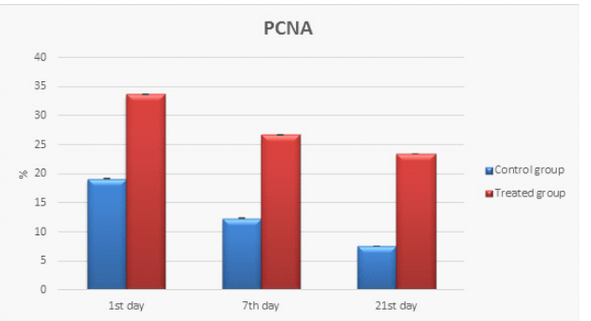

Fig. 20: A bar chart shows area percentage of PCNA immunoreactivity in both control and GA-3 treated groups in all age groups.

Table 1: Comparison between the control and treated groups in mean urea & creatinine serum level in all age groups

Variable	Groups \ Days	1st day	7th day	21st day	(F)	P
		Mean ± SD	Mean ± SD	Mean ± SD		
Urea (mg/dl)	Control	15.7 ± 0.79[a]	16.06 ± 0.52[a]	16.39 ± 0.55[a]	2.63	0.14[NS]
	Treated	34.30 ± 1.18[a]	36.72 ± 1.44[b]	39.77 ± 1.39[c]	46.60	<0.001[**]
t		39.76	40.6	49.82		
P		<0.001[**]	<0.001[**]	<0.001[**]		
Creatinine (mg/dl)	Control	0.75 ± 0.14[a]	0.74 ± 0.06[a]	0.79 ± 0.06[a]	4.26	0.06[NS]
	Treated	1.48 ± 0.13[a]	1.62 ± 0.19[b]	1.67 ± 0.11[b]	11.92	0.004[**]
t		11.65	13.53	21.76		
P		<0.001[**]	<0.001[**]	<0.001[**]		

SD: Standard deviation. t: Independent t test F: Repeated measure ANOVA test NS: Non significant (P>0.05)
**: Highly significant (P<0.01) LSD for repeated measure F: Groups with different letters are statistically significant (P<0.05)

Table 2: Comparison between the control and treated groups in mean MDA, CAT and GPx tissue levels in all age groups

Variable	Groups \ Days	1st day	7th day	21st day	(F)	P
		Mean ± SD	Mean ± SD	Mean ± SD		
MDA (mmol/L)	Control	52.90±1.42[a]	54.01±1.44[a]	54.28±1.97[a]	2.66	0.14[NS]
	Treated	78.18±3.58[a]	113.57±3.59[b]	131.38±2.98[c]	7.41	<0.001[**]
t		19.74	46.41	69.97		
P		<0.001[**]	<0.001[**]	<0.001[**]		
CAT (mmol/l)	Control	0.64±0.07[a]	0.65±0.05[a]	0.642±0.07[a]	1.58	0.27[NS]
	Treated	0.51±0.11[a]	0.38±0.05[b]	0.29±0.06[c]	116.0	<0.001[**]
t		3.04	10.94	12.47		
P		0.007[**]	<0.001[**]	<0.001[**]		
GPx	Control	102.38±8.99[a]	102.77±9.28[a]	102.95±8.42[a]	1.23	0.35[NS]
	Treated	90.64±4.34[a]	66.06±5.54[b]	60.32±7.57[c]	4.90	<0.001[**]
t		3.69	10.60	12.50		
P		0.002[**]	<0.001[**]	<0.001[**]		

SD: Standard deviation. t: Independent t test F: Repeated measure ANOVA test NS: Non significant (P>0.05)
**: Highly significant (P<0.01) LSD for repeated measure F: Groups with different letters are statistically significant (P<0.05)

Table 3: Comparison between the two studied groups in mean cortical thickness at different age groups

Variable	Groups \ Days	1st day	7th day	21st day	(F)	P
		Mean ± SD	Mean ± SD	Mean ± SD		
Cortical thickness	Control	502.29±55.40^{a}	711.03±23.8^{b}	741.26±32.65^{c}	61.59	<0.001**
	Treated	449.63±40.5^{a}	532.25±47.93^{b}	627.35±50.85^{c}	24.69	<0.001**
t		2.38	10.10	6.10		
P		0.03*	<0.001**	<0.001**		

SD: Standard deviation. t: Independent t test F: Repeated measure ANOVA test NS: Non significant (P>0.05)
**: Highly significant (P<0.01) LSD for repeated measure F: Groups with different letters are statistically significant (P<0.05)

Table 4: Comparison between the two studied groups in mean Area Percentage of Collagen in Masson Trichrome stain at different study times

Variable	Groups \ Days	1st day	7th day	21st day	(F)	P
		Mean ± SD	Mean ± SD	Mean ± SD		
Area Percentage of Collagen in Masson Trichrome stain	Control	3.45 ± 0.25^{a}	6.23 ± 0.98^{a}	9.22 ± 0.21^{a}	1.48	0.35ns
	Treated	16.65 ± 0.27^{a}	22.43 ± 0.30^{b}	29.40 ± 0.38^{c}	37.8	<0.001**
t		106.25	150.376	146.663		
P		<0.001**	<0.001**	<0.001**		

SD: Standard deviation. t: Independent t test F: Repeated measure ANOVA test NS: Non significant (P>0.05)
**: Highly significant (P<0.01) LSD for repeated measure F: Groups with different letters are statistically significant (P<0.05)

Table 5: Comparison between the two studied groups in mean area percentage of PCNA immunoreactivity at different age groups

Variable	Groups \ Days	1st day	7th day	21st day	(F)	P
		Mean ± SD	Mean ± SD	Mean ± SD		
PCNA	Control	19.16 ± 0.06^{a}	12.32 ± 0.06^{b}	7.54 ± 0.03^{c}	291.94	<0.001**
	Treated	33.71 ± 0.05^{a}	26.70 ± 0.03^{b}	23.42 ± 0.04^{c}	107.37	<0.001**
t		55.97	67.01	97.56		
P		<0.001**	<0.001**	<0.001**		

SD: Standard deviation. t: Independent t test F: Repeated measure ANOVA test NS: Non significant (P>0.05)
**: Highly significant (P<0.01) LSD for repeated measure F: Groups with different letters are statistically significant (P<0.05)

DISCUSSION

Many chemicals result in contamination of the environment and food as plant growth regulators with many hazards on health especially bone mineral composition, development in human and rats[17]. A plant growth regulator induced reactive oxygen species damage in bone[11].

Although GA3 is extensively used in Egypt, little is known about its potential hazardous effects on the human healt[h][18]. So, the current research was performed to identify the toxic effect of GA3 on the cortex of a developing kidney. Light microscopic, immunohistochemical studies in addition to morphometrical and statistical analysis were used to achieve this goal. Three postnatal ages of the rat were used in the present study (newborns, 7 days old and 21 days old) to detect postnatal changes in the kidney tissue in both the control and treated animals.

Current study revealed renal injury of pups of GA3 treated mothers. As there was significant increase of urea and creatinine serum level of rats born to mothers administrated GA3. In accordance, Troudi *et al*.[19] reported that, levels of creatinine and urea in treated off springs were higher in plasma and lower in urine than those of the control. Moreover, Erin *et al*.[20] explained the increased urea level due to either the Ammonia increased protein catabolism or the transformation of ammonia to urea. This was in the same line of Sakr *et al*.[21].

Renal injury may be caused by either disturbed activities of antioxidant enzyme or suppression of their expressions in the rats' kidneys. This study reported statistical significant increase in MDA level and decline of GPx and CAT in pups born to treated mothers than control ones. In accordance, Troudi *et al*.[19] detected that, GA3 induced a state of oxidative stress in mothers and their siblings, which in turn confirmed the release of GA3 through milk. This study supported previous study of Celik and Tulice[22] who detected reduction in the actions of SOD, CAT and GPx in adult rats' kidneys which administrated 75 ppm of GA3. Stimulation of oxidative stress could be due to inflammation induced by gibberellic acid, as it increased mast cell degranulation and

substance P levels[23]. Moreover, gibberellic acid increased MDA content of kidney, the end product of lipid peroxidation, which ensures renal free radicals formation[19].

In the present work, examination of newborn control rats detected immature forms of renal developmental stages in the subcapsular zone with more mature renal corpuscles in the juxtamedullary zone. Abdel-Aziz and Mohamed[24]; Brown *et al*.[25] and El-gammal *et al*.[26] observed the same findings. Moreover, S shaped body had an upper limb, middle segment and lower limb. A vascular cleft was seen containing capillary loops lined by endothelial cells. Brown *et al*.[25] supposed that, the different parts of S-shaped body would give rise to future tubule, vascular pole and glomerulus.

By light microscopy, PCT showed an acidophilic granular cytoplasm and basally situated nuclei while, DCT revealed a dilated lumen. These results support the findings of the studies done by Hassan *et al*.[27]. In addition, Brown *et al*.,[25] reported that, distal and proximal convoluted tubules were well distinguished in juxtamedullary cortex.

At (PND 7), interruption of the nephrogenic zone by the growing glomeruli with occasional immature developmental forms of renal glomeruli and tubules were observed. The superficial glomeruli were compact and small while the juxtamedullary glomeruli appeared more developed with the parietal layer cells appeared flattened, while the cells of the visceral layer were separated and surrounded the capillary loops, medullary rays remain prominent in the cortex and loose connective tissue in the outer region of the medulla was decreased. Marquez *et al*.[28] reported that, in rats as well as other mammals, nephrogenesis in the metanephros proceeds centrifugally. Thus, a higher degree of nephrological maturation was observed in the juxtamedullary zone, where nephrons were more differentiated than the subcapsular ones. Histological study of Balbi *et al*.[29] displayed glomeruli in different stages of renal cortical development from 1- and 7-day-old animals, on contrary, Cunha *et al*.[30] mentioned that, kidneys of the age 7 days pups of mice showed only mature (vascularized) glomeruli. Medullary rays remain prominent in the cortex and loose connective tissue in the outer region of the medulla is decreased in accordance with Brown *et al*.[25].

The biochemical alterations which were detected in siblings of treated mothers could be correlated with histopathological modifications found in the kidney of GA3-treated rats. In the present study, the effect of GA3 treatment of mothers on the architecture of the developing renal cortex of the off spring as there were immature arrangements of renal glomeruli frequently in the GA3-treated newborn and 7 days old rats which indicated a late growth of the glomeruli. Also, the appearance of homogeneous dense masses of cells was additional proof of retarded renal development of rats treated with GA3. The present findings were in line with those of other investigators who detected delayed development of the renal cortex of the rats whose mothers were treated with monosodium glutamate[24].

Current study revealed loss of some cells lining some renal tubules and others with vacuolated cytoplasm and acidophilic exudates were observed intertubular in pups of treated mothers which may be caused by the intertubular edema. In accordance, Hassan *et al*.[27] observed acidophilic exudates and intertubular extravasation of red blood cells in newborn pups of GA3 treated mothers.

Light microscopic examination of the renal cortex of the control (PND21) rats in this work revealed the normal histological structure, as the cortical glomeruli acquired full maturation and increased in size, became no longer compact and acquired lobulations. Additionally, Well-developed glomeruli with Bowman's capsule had visceral and parietal layers with distinct capsular space and few capillary loops. The cells of the parietal layer appeared flattened, while the cells of the visceral layer were separated and surround the capillary loops. These findings were in association of Sabry *et al*.[31] who observed Bowman's capsule consisted of an outer parietal layer and an inner visceral one, which were separated by a capsular space; the urinary space. The glomerulus was formed of capillary loops of the afferent and efferent arterioles, supported by the mesengial cells.

Proximal convoluted tubular cells exhibited vesicular rounded basal nuclei with acidophilic granular cytoplasm while, those of the distal ones showed less cytoplasmic acidophilia. The current findings were similar to those of other authors[32]. Additionally, Abdel-Aziz and Mohamed[24] reported that, renal cortices of 21-day-old control albino rat showed mature renal corpuscles at the subcapsular with distinct Bowman's space. Additionally, Moawad *et al*.[33] mentioned that, the outer parietal layer of Bowman's capsule was formed of simple squamous epithelium. The podocytes of the visceral layer had pale nuclei and cytoplasm and invested the glomerular capillaries.

The observed damage that occurred in the renal corpuscles in the studied age groups of the treated rats could be attributed to peritubular fibrosis and it was in agreement with that seen by other researchers[32]. Some investigators referred this lesion as 'cystic glomerular atrophy,' based on the small size of some glomeruli within the dilated Bowman's space[33]. Other authors attributed the pathogenesis of this lesion to periglomerular fibrosis and stated that thickening of glomerular basement membrane resulted in disturbance of glomerular outflow that led to cystic changes in Bowman's space[34]. But, in the current work, some renal cortical glomeruli of GA-3 treated rats exhibited more cellularity. Some authors attributed this due to compensation for the decrease in developing glomeruli with increase in vascular glomeruli firmly filling Bowman capsule[35]. In addition, GA3 increases the mitotic division so has growth promoting effects in animal tissues[11].

In the present work, renal tubules lining cells in the studied age groups of the treated rats showed vacuolations and pyknotic nuclei. These results were the same as those of other investigators[32,36]. The cytoplasmic vacuolization was one of the main early replies to variable forms of cellular

damage. It might occur due to increased permeability of cell membranes, this in turn causes increase intracellular water leading to vacuolization[37].

In the present work, morphometrical and statistical analysis of the renal cortical thickness showed significant increase of cortical thickness in control pups with advance of age. These findings in the same line with findings of Hassan *et al*.[27] and Moawad *et al*.[33].

In addition, current research detected a decrease in the cortical thickness in the off springs of treated mothers when compared with the control ones. These findings could be attributed to atrophy of the glomeruli and degeneration of the renal tubules that occurred after GA3 treatment[27]. Thinning of the renal cortex is likely the result of tubular and glomerular atrophy[38].

This study revealed little collagen fibers in the renal cortical interstitium around the renal corpuscles and tubules in Masson's trichrome stained specimens of control groups in different age groups. The results agreed with Ahmed *et al*.[39]. Collagen fibers showed marked increase in renal sections of GA3 exposed groups. These findings confirmed by morphometric results of area percent of collagen. This is in accordance with Javaid *et al*.[40] who reported that interstitial fibrosis positively correlated with the extent of glomerular involvement and loss. Moreover, Pawlina and Ross[41] explained the marked fibrosis by lipid peroxidation caused by GA3 administration, as it led to protein and nucleic acid damage. These reactions resulted in collagen formation.

Regarding PCNA stained sections, there were marked increase in its expression in newborn and 7-day old pups than in 21-day rats. These findings confirmed by morphometric analysis that PCNA expression was significantly more in newborn than in 7-day old pups. Balbi *et al*.[29] agreed with these findings and reported that the number of glomerular PCNA-positive cells was higher in 1- and 7-day-old control rats than in 30-day-old ones. The expression of PCNA was significantly higher in renal sections of rats born to treated mothers with GA3. In accordance, Cunha *et al*.[30] reported that the PCNA expression in cortical nuclei of kidney in STZ-treated pups of mice was significantly higher than control. This proved retardation in the glomerulo-genesis, leading to fewer glomeruli at weaning. There was increased PCNA expression in the tubular nuclei of STZ 7- and 21-days pups, indicating glomerular endothelial dysfunction. On contrary, Balbi *et al*.[29] found that, increased sodium intake during pregnancy resulted in reductions of renal cortical PCNA of neonatal rats, causing disturbance of renal development and functions during adult life.

CONCLUSION

Rats exposed to GA3 during gestation and lactation periods exhibit nephrotoxic manifestations. Additionally, the use of GA3 may be hazardous on non-target organisms, including humans. So that, it should be used carefully, with monitoring its levels in agricultural products.

ABBREVIATIONS

Gibberellic acid **(GA3)**, Hematoxylin **(H)**, Eosin **(E)**, ANOVA **(analysis of variance)**, Plant growth regulators **(PGRs)**.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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الملخص العربى

دراسة نسيجية كيميائية مناعية للتأثير السام لحمض الجبريليك بعد الولادة على القشرة الكلوية للجرذان البيضاء

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المقدمة: حمض الجبريليك (GA٣) هو منظم لنمو النبات بشكل طبيعي ، وهو واحد من عائلة "المبيدات الحيوية" التي هي هرمونات نباتية لها آثار المبيدات الحشرية. يتم التخلص من معظم النفايات النيتروجينية من خلال الكليتين التي تحافظ بالإضافة إلى ذلك على حجم الدم وتكوينه وضغطه ويحافظ على كثافة العظام.

الهدف من البحث: ويهدف هذا البحث الى تقييم التأثير الهستوباثولوجي لحمض الجبريليك على قشرة الفئران الكلوية بعد الولادة في مختلف الأعمار (اليوم الاول ، السابع و الحادى والعشرين بعد الولادة

المواد و طرق البحث: تم تقسيم عشرين جرذا من الجرذان البيضاء الحامل إلى مجموعتين متساويتين: المجموعة الأولى (المجموعة الضابطة) والمجموعة الثانية (مجموعة حمض الجبريليك). في المجموعة المعالجة (العدد = ١٠) ، تم إعطاء حمض الجبريليك بجرعة ٥٥ مغ / كغ من وزن الجسم / يوم من اليوم الرابع عشر من الحمل إلى اليوم الحادي والعشرين بعد الولادة. بعد الولادة ، تم تقسيم الذرية من الذكور للمجموعة الضابطة والمعالجة إلى ثلاث مجموعات فرعية وتم التضحية بهم فى (اليومالاول) ، (٧ أيام) وفي (٢١ يومًا). تم استخراج الكليتين وتم تشريح كلتيهما. تمت معالجة الكلى من كافة المجموعات قيد الدراسة لفحص علامة الإجهاد التأكسدي والفحص النسيجي والمناعي

النتائج: كان هناك فرق ذو دلالة إحصائية عالية بين المجموعة الضابطة والمجموعة المعالجة بحمض الجبريليك في جميع الفئات العمرية لكل من مستوى اليوريا والكرياتينين فى الدم وكذلك في مستوى CAT ، MDA و GPX.. كشف الفحص النسيجي الجرذان المعالجة بـحمض الجبريليك عن وجود أشكال متعددة غير ناضجة من الكبيبات ، والشعيرات الدموية المتعددة المزدحمة ، وأظهرت بعض الأنابيب الكلوية خسائر في بعض الخلايا التي تبطنها وغيرها مع السيتوبلازم المفرغ. إحصائيا ، أظهرت المجموعات التي عولجت بحمض الجبريليك في جميع الفئات العمرية انخفاض كبير للغاية في متوسط سمك القشرة بالمقارنة مع مجموعات الضبط الخاصة بهم. علاوة على ذلك ، أظهرت النسبة المئوية للمساحة من ألياف الكولاجين فيمقاطع مصبوغة بـMasson Trichrome زيادة كبيرة مع استخدام حمض الجبريليك ، وكذلك تم توثيقه في النسبة المئوية للمساحة للتعبير الكلوي PCNA.

الخلاصة: واستخلص البحث الى ان تعرض الجرذان الحامل إلى حمض الجبريليك في أواخر الحمل وخلال الرضاعة ينجم عنه إجهاد تأكسدي وتغيراتمرضية في القشرة الكلوية للجرذان الرضيعة.