Life Sciences 239 (2019) 117082

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

The protective role of estrogen and its receptors in gentamicin-induced acute kidney injury in rats

Sayed M. Abd El-Lateef, El-Sayed M. El-Sayed*, Ahmed M. Mansour, Salama A. Salama

Department of Pharmacology & Toxicology, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt

ARTICLE INFO

Keywords:
Gentamicin
Nephrotoxicity
Megalin
Estrogen/estrogen receptors

ABSTRACT

Aim: Investigating the impact of 17β-Estradiol/estrogen receptors in gentamicin-induced nephrotoxicity.
Main methods: Three weeks post-ovariectomy or sham surgery for the Wistar albino female rats, thirty sham rats were randomly grouped ($n = 6$), received either vehicle or gentamicin; the estrogen receptors down regulator (fulvestrant); gentamicin plus fulvestrant; gentamicin plus the phytoestrogen (genistein). Forty–eight ovariectomized rats were randomly grouped ($n = 6$), treated with either vehicle or gentamicin; fulvestrant; gentamicin plus fulvestrant; genistein; gentamicin plus genistein; estradiol benzoate; gentamicin plus estradiol benzoate. Just post-treatment termination, the traditional kidney injury biomarkers (serum creatinine and blood urea nitrogen) and novel biomarkers (serum Kidney injury molecule −1, cystatin C, lactate dehydrogenase and, gamma-glutamyl transferase) were determined. Bovine serum albumin labeled with fluorescence isothiocyanate assessed megalin expression/endocytic functionality in the proximal tubules epithelial cells (PTECs). The immunohistochemical investigation for the same-sectioned slides of PTECs assessed the correlation between estrogen receptors α and megalin receptors expression. Histopathological examination of PTECs and subjective scoring system graded the damage markers.
Key findings: Estrogen receptor α expression was markedly dimensioned post-ovariectomy, co-localized and inversely correlated to megalin expression. Serum levels of the novel biomarkers were directly proportional to megalin expression in the PTECs and inversely correlated with estrogen receptor α expression. The injury was exaggerated in ovariectomized and intact rats received fulvestrant. Supplementation with estrogen or genistein ameliorated this injury.
Significance: Estrogen/estrogen receptors have a protective impact on gentamicin-induced acute kidney injury. Estrogen receptors antagonist exacerbate the injury, and oppositely, estrogens or phytoestrogens improve it.

1. Introduction

Gentamicin, one of the aminoglycoside antibiotics is highly effective in the treatment of severe gram-negative infections which are resistant to other antibiotics [1]. Single daily dose of 160 mg of gentamicin is effective in the treatment of urinary tract infections [2]. Unfortunately, the clinical use of this antibiotic is limited due to its nephrotoxicity mostly on the epithelial cells of the proximal tubules [3].

Megalin, a multi-ligand endocytic receptor, belonging to the low-density lipoprotein receptor family [4], is widely expressed in epithelial cells of the proximal tubules [5]. The uptake of aminoglycosides into the kidney in mice lacking megalin is eliminated and directly correlates with renal megalin expression/functionality; providing unequivocal evidence, that megalin is the only major pathway responsible for the renal accumulation of aminoglycosides and representing a unique target to prevent aminoglycoside-induced nephrotoxicity [6]. Given the indispensable endocytic and renal uptake role of megalin to aminoglycosides, it is rational to anticipate that modulation of this receptor expression/endocytic functionality in the epithelial cells of the proximal tubules could modulate gentamicin-induced AKI.

The kidney is a non-reproductive estrogenic organ with specific renal estrogen-induced gene activation. The estrogen receptor α

Abbreviations: E2, 17β-Estradiol; ERs, estrogen receptors; AKI, acute kidney injury; OVX, ovariectomized; Kim-1, kidney injury molecule-1; LDH, lactate dehydrogenase; GGT, gamma-glutamyl transferase; FITC-BSA, bovine serum albumin labeled with fluorescence isothiocyanate; IHC, immunohistochemical; PTECs, proximal tubular epithelial cells; LDL, low-density lipoprotein; ERE, estrogen response element; DR, direct repeat; SP1, stimulatory protein; AP-1, activator protein-1; TRE, TPA responsive element; LRP-2, megalin; BUN, blood urea nitrogen; GPR30, G protein-coupled estrogen receptor 1; AF1, activation function 1; AF2, activation function 2; ChIP-seq, chromatin immunoprecipitation sequencing

* Corresponding author.
E-mail address: elsayed200_1956@hotmail.com (E.-S.M. El-Sayed).

https://doi.org/10.1016/j.lfs.2019.117082
Received 22 September 2019; Received in revised form 5 November 2019; Accepted 13 November 2019
Available online 19 November 2019
0024-3205/

expression in the epithelial cells of the proximal tubules is more pronounced than estrogen receptor – β subtype [7], however, both subtypes are co-expressed with megalin receptor [8,]. The co-localization of estrogen and megalin receptors, suggests that estrogen receptors, which are activated by ligand with 17-β estradiol are involved in regulating megalin expression in the epithelial cells of the proximal tubules. Besides, substantial evidence demonstrated that aminoglycosides exhibit sex-dependent nephrotoxicity, however, the exact molecular mechanisms whereby 17-β estradiol/estrogen receptors exert their effects are still unknown [10–12].

Serum creatinine is considered as delayed and unreliable indicator of acute kidney injury because it is influenced by multiple non-renal factors, such as age, gender, muscle mass, muscle metabolism, diet, and hydration status; it takes several days to reach a new steady-state and not rise until more than half of the kidney function is lost [13]. The blood urea nitrogen is relatively insensitive and unreliable to early kidney deterioration; its level is affected by high catabolic states and high protein diets. Besides, damage to 75% of nephrons is required before increase in its level [14].

Given the limitations of the traditional biomarkers (serum creatinine and blood urea nitrogen) as imprecise markers for acute kidney injury, they are substituted by the more sensitive and reliable biomarkers (kidney injury molecule-1, cystatin C, gamma-glutamyl transferase and lactate dehydrogenase).

Regarding the extremely efficacious effect of gentamicin in critical infections, it is imperative to achieve optimal renoprotective therapy against its nephrotoxicity. Our study aimed to estimate the role of 17-β estradiol/estrogen receptors, estrogen receptor down-regulator (fulvestrant), the phytoestrogen (genistein) and external supplementation of estrogen (estradiol benzoate) in megalin expression/endocytic functionality and consequently their roles in exaggerating or mitigating the gentamicin-induced acute injury.

2. Materials and methods

2.1. Animal care and ethical approval

Adult female Wistar albino rats (2 months old, weighing approximately 180–200 g) were purchased from the experimental animal facility at the Nile for Pharmaceuticals & Chemical Industries (Cairo, Egypt). Rats were kept two per cage for two weeks for acclimatization in controlled housing conditions (room temperature 25 ± 2 °C, humidity (50–70%) and 12/12 h dark-light cycles) and kept free on a standard diet. Rats were given ad libitum access to food and water. The study was performed under the approval of the Institutional Animal Ethics Committee of Faculty of Pharmacy, Al-Azhar University, and strictly followed the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Chemicals and reagents

Gentamicin sulfate salt powder (CAS Number: 1405-41-0, Schering-Plough Cor. (New Jersey, USA)), a kind gift from Memphis Pharmaceutical Company (Cairo, Egypt), was prepared in isotonic saline. Thiopental sodium was purchased from Egyptian International Pharmaceutical Industries Company (EPICO) (Cairo, Egypt). The pure estrogen receptors antagonist fulvestrant (ICI 182 780) (I4409, CAS Number 129453-61-8), the isoflavone phytoestrogen genistein (G6649, CAS Number 446-72-0), and FITC-BSA (A9771, MDL number MFCD00282182), were purchased from Sigma-Aldrich Company (Darmstadt, Germany). Fulvestrant and genistein solutions were prepared by dissolving the powder in ethanol and then diluted with isotonic saline. FITC-BSA was dissolved in 10 mM Tris, pH 7.0 (10 mg/ml). Estradiol benzoate (Folone 5 mg/ml ampoule), obtained from Misr Company for Pharmaceutical Industries (Cairo, Egypt), was prepared in sesame oil. Isotonic saline was purchased from El-Nasr Pharmaceutical Chemicals Company (Abou-Zaabal, Egypt). Povidone-iodine was purchased from the Nile for Pharmaceuticals & Chemical Industries Company (Cairo, Egypt). Pentobarbital sodium (Nembutal injection 50 mg/ml, Akorn Pharmaceuticals, USA) was purchased from Buy Goofballs online (Egypt). ELISA kits Kim-1 (Rat Kidney injury molecule 1, Kim-1 ELISA Kit MBS265649) and cystatin C (Rat Cystatin C ELISA Kit MBS 733592), were purchased from My Biosource Company (California, San Diego, USA). LDH, GGT, creatinine and BUN kits were purchased from Bio-Med Company (Cairo, Egypt). Primary antibodies (mouse monoclonal megalin antibody (H-10, sc-515772) and mouse monoclonal ERα antibody (D-12, sc-8005) were purchased from Santa Cruz Biotechnology Company (SCBT) (Texas, USA). All of the other chemicals and reagents were of the highest commercially available grade.

Table 1
Effect of Gentamicin, ovariectomy and/or Estradiol benzoate, Fulvestrant and Genistein on serum creatinine and BUN of female albino rats.

Groups	Creatinine (mg/dl)	BUN (mg/dl)
Control (n = 6)	0.43 ± 0.07	32.5 ± 3.73
Sham (n = 6)	0.38 ± 0.04	30.7 ± 3.88
Sham + Gentamicin (n = 6)	0.30 ± 0.06	30.67 ± 2.94
Sham + Gentamicin + Fulvestrant (n = 5)	0.28 ± 0.15	32.60 ± 3.58
Sham + Gentamicin + Genistein (n = 6)	0.43 ± 0.06	33.00 ± 1.55
Sham + Fulvestrant (n = 6)	0.43 ± 0.06	31.7 ± 3.44
OVX (n = 6)	0.33 ± 0.04	33.3 ± 2.16
OVX + Gentamicin (n = 6)	0.34 ± 0.08	30.33 ± 3.20
OVX + Fulvestrant (n = 6)	0.30 ± 0.05	33.3 ± 2.88
OVX + Gentamicin + Fulvestrant (n = 6)	0.30 ± 0.02	31.7 ± 4.22
OVX + Genistein (n = 6)	0.35 ± 0.03	36.5 ± 3.62
OVX + Gentamicin + Genistein (n = 5)	0.37 ± 0.21	37.0 ± 3.54
OVX + Estradiol benzoate (n = 6)	0.39 ± 0.20	35.0 ± 5.24
OVX + Estradiol benzoate + Gentamicin (n = 6)	0.44 ± 0.05	37.33 ± 2.503

Data were presented as means ± SD and statistically analyzed by one-way (ANOVA) test, followed by post hoc Tukey-Kramer test for multiple comparison between groups. No statistically significant difference was seen between means of all groups at P ≤ 0.05.

2.3. Ovariectomy and sham surgery in rats

Animals were fasted overnight, they were subjected to either bilateral ovariectomy (OVX) or bilateral sham surgery (Sham) post-anesthesia by thiopental (50 mg/kg, i.p) as previously described by Lasota and Danowska-Klonowski [15]. After the removal of bilateral ovaries, the skin and muscle incisions were closed with sterile nylon sutures. For the female Sham operation, rats received a similar surgery except the ovaries were not removed. After surgery, rats were allowed to recover for three weeks before starting the designated treatment [16,17].

2.4. Experimental design and treatment protocol

The experiment lasted for 17 days. Gentamicin, fulvestrant, genistein, and estradiol benzoate treatment regimens were chosen according to the previous studies and were further confirmed based on our preliminary pilot trials. Three weeks post-ovariectomy (OVX) or sham

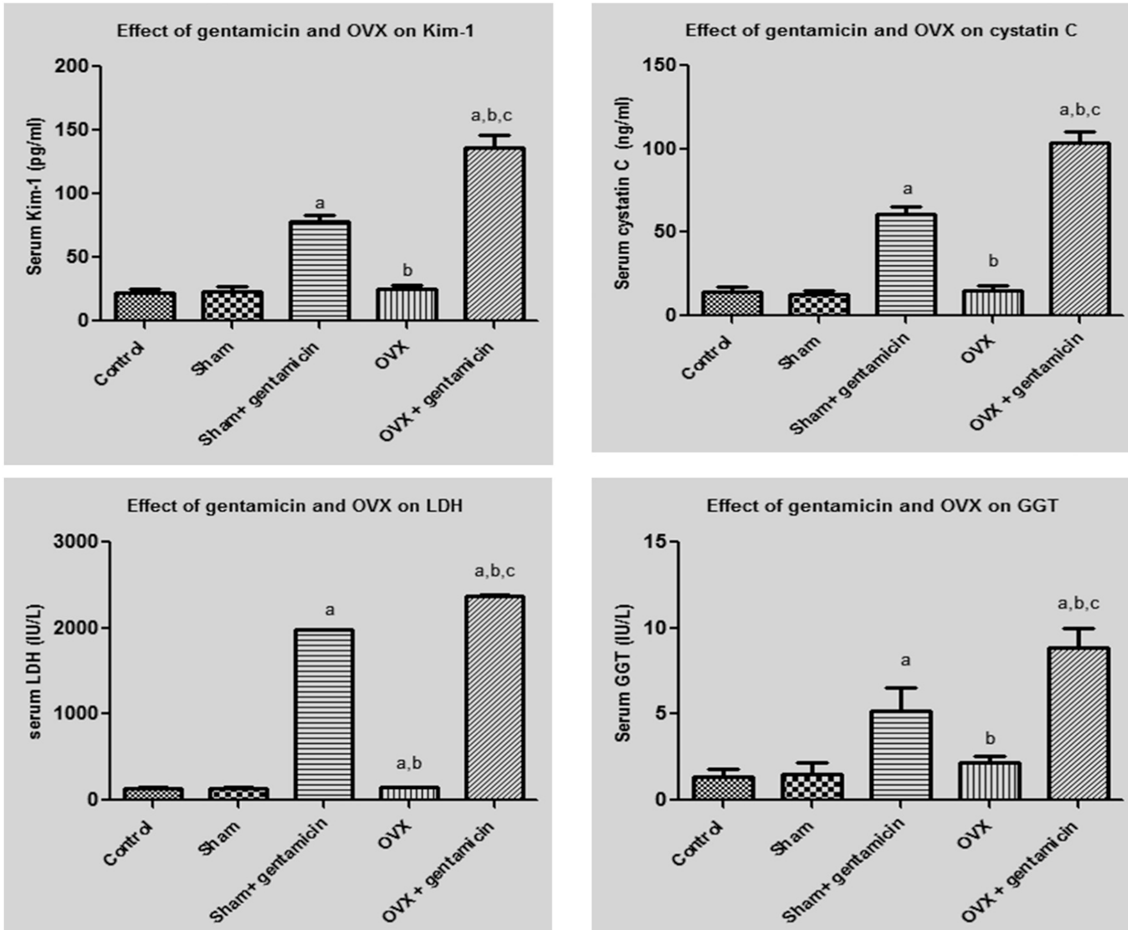


Fig. 1. Effect of Gentamicin and/or ovariectomy on serum Kim-1, cystatin C, LDH and GGT of female albino rats. Data were presented as means ± SD (n = 6) and statistically analyzed by one-way (ANOVA) test followed by post hoc Tukey-Kramer test. a: significantly different from Sham; b: significantly different from Sham + Gentamicin; c: significantly different from OVX at P ≤ 0.05.

surgery (Sham), a total of Eighty–four (6 intact control rats; 30 shams; and 48 OVX rats) were randomly divided to fourteen groups (n = 6) as following:

1) Control: intact rats were kept without receiving any treatment throughout the experiment.
2) Sham: Sham-operated, treated with vehicle (0.2 ml; i.p) once daily over the experiment.
3) Sham + gentamicin: Sham-operated, received gentamicin (70 mg/kg, i.p) once daily for concessive 10 days starting on the eighth day of the experiment [18].
4) Sham + fulvestrant: Sham-operated, injected with fulvestrant (0.25 mg/kg, i.p) once daily, one week before synchronization to the gentamicin treatment period [19].
5) Sham + gentamicin + fulvestrant: Sham-operated, received the intended treatment regimen for gentamicin and fulvestrant.
6) Sham + gentamicin + genistein: Sham-operated, injected with the scheduled treatment regimen for gentamicin, plus genistein (10 mg/kg, i.p) once daily, one week before coincidence to gentamicin treatment period [20].
7) OVX: OVX treated with vehicle (0.2 ml; i.p) once daily, during the experiment.
8) OVX + gentamicin: OVX received a particular treatment regimen for gentamicin.
9) OVX + fulvestrant: OVX received the destined treatment regimen for fulvestrant.
10) OVX + gentamicin + fulvestrant: OVX injected with the specified treatment regimen for gentamicin and fulvestrant.
11) OVX + genistein: OVX treated by the designed treatment regimen for genistein, one week before concurrence to the gentamicin treatment period.
12) OVX + gentamicin + genistein: OVX handled with the intended treatment regimen for gentamicin and genistein.
13) OVX + estradiol benzoate: OVX injected with estradiol benzoate (30 µg/kg, s.c) once daily [21], one week before coincidence to the gentamicin treatment period.
14) OVX + gentamicin + estradiol benzoate: OVX received the purposed treatment regimen for gentamicin and estradiol benzoate.

2.5. Blood sampling and rat's euthanizing

At the end of the treatment period, just before rat's euthanizing and after general anesthesia with pentobarbital (50 mg/kg, i.p), the blood samples from each rat (2 ml) were withdrawn via retro-orbital plexus bleeding technique. Before and during the procedure, the rat's respiratory rate, response to noxious stimulus and spontaneous

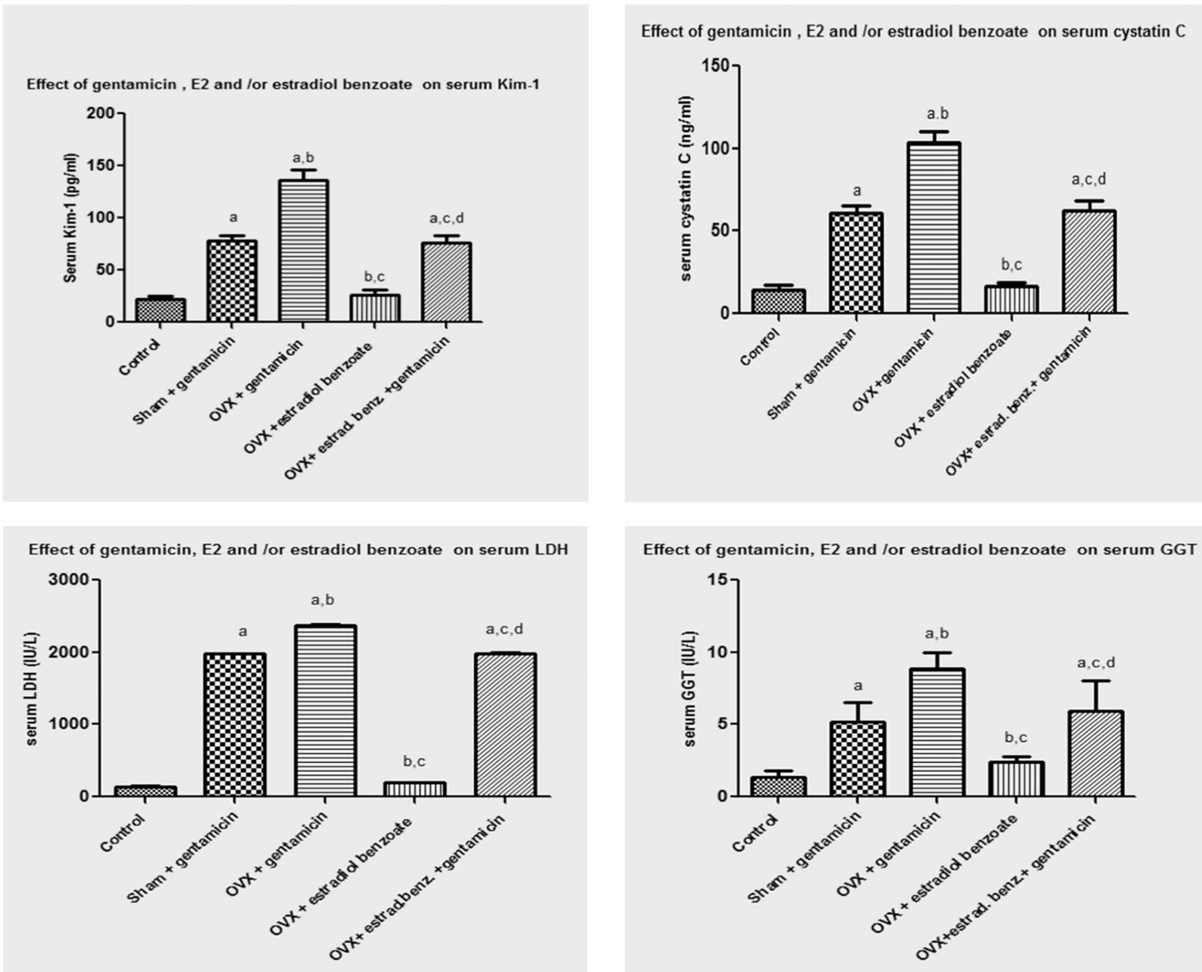


Fig. 2. Impact of Gentamicin, ovariectomy and/or estradiol benzoate on serum Kim-1, cystatin C, LDH and GGT of female albino rats. Data were presented as means ± SD (n = 6) and statistically analyzed by one-way (ANOVA) test followed by post hoc Tukey-Kramer. a: significantly different from control; b: significantly different from Sham + Gentamicin; c: significantly different from OVX + Gentamicin; d: significantly different from OVX + estradiol benzoate at P ≤ 0.05.

movement was monitored. The rats were covered well. During recovery from anesthesia, the respiratory rate, movement and ability to maintain sternal recumbency were monitored at 5-min intervals until the animal was ambulatory [22]. Thereafter rats were euthanized by cervical dislocation technique according to standard animal euthanasia method guidelines developed by the Canadian Council on Animal Care (CCAC), 2010. The kidneys were rapidly removed, preserved in 10% buffered formalin solution and processed for immunohistochemical (IHC) and histopathological assessment. The blood samples were allowed to clot at room temperature for 15–30 min. The sera were separated by centrifugation at 1500 ×*g* for 10 min in a refrigerated centrifuge. The separated sera were stored at −80 °C until used for biochemical analysis.

2.6. Megalin endocytic functionality assessment

FITC-BSA was injected i.p at a dose of 20 mg/kg [23] into Sham and OVX groups. Twenty min later, kidneys from the intact control female rats, which did not receive FITC-BSA, Sham, and OVX groups, were harvested and fixed in a 10%, buffered formalin solution, sectioned as for IHC and slides were counterstained with alcian blue [24]. Six Images for each slide were taken using a Nikon 55i fluorescence microscope equipped with DS-Fi1 5-Meg Color C digital camera (Nikon, Melville, NY). The intensity of the blue fluorescence in the PTECs Images for each group was analyzed and expressed in pixel by Image J version 1.46 software.

2.7. Immunohistochemical assessment of the of megalin and ERα expression in the PTECs

Formalin-fixed, paraffin-embedded tissue sections from PTECs had been treated by heat-induced epitope retrieval technique (temperature 50 °C for up to 60 min). Concentrated primary antibodies (megalin and ERα) thereafter were diluted, incubated and auto stained with high-sensitivity visualization system (Envision™ FLEX, High pH, and Link system) in the autostainer Link 48 in which the software had been pre-programmed according to manufacturer's recommendations provided in the package insert of the concentrated primary antibodies. The sectioned slides were examined using a light microscope to assess the expression of both megalin and ERα in all groups. Six images for each slid were obtained (×400). Immunoreactivity was evaluated by estimating the area percentage of the positive brown immunostained cells, using computed image analysis (Leica qwin software 500, Germany).

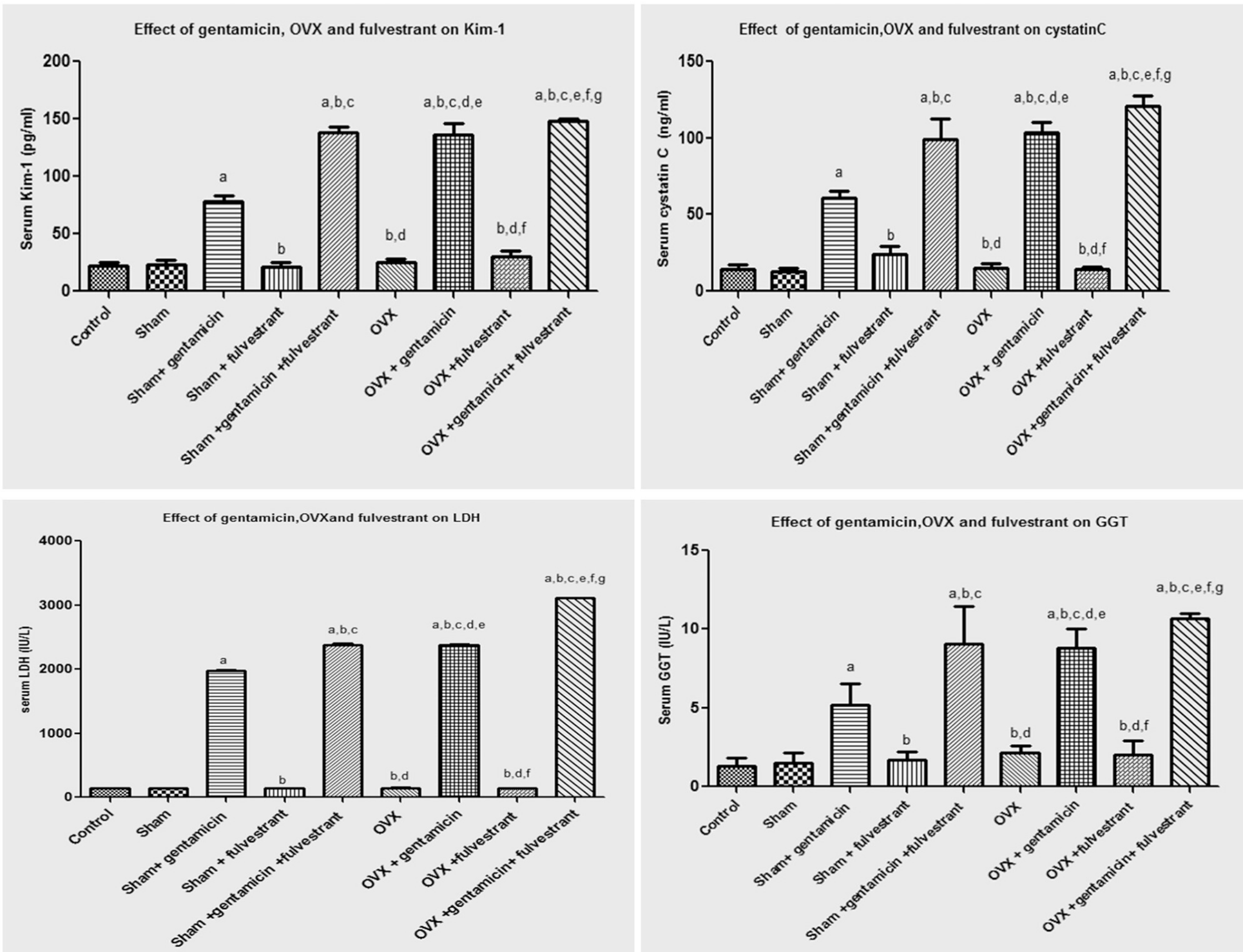


Fig. 3. Effect of Gentamicin, ovariectomy and Fulvestrant on serum Kim-1, cystatin C, LDH and GGT of female albino rats. Data are presented as means ± SD and statistically analyzed by one-way (ANOVA) test followed by post hoc Tukey-Kramer test. a: significantly different from Sham; b: significantly different from Sham + Gentamicin; c: significantly different from Sham + fulvestrant; d: significantly different from Sham + Fulvestrant + Gentamicin; e: significantly different from OVX; f: significantly different from OVX + Gentamicin; g: significantly different from OVX + Fulvestrant at $P \leq 0.05$.

2.8. Assessment of kidney tissues damage biomarkers

Serum Kim-1 levels were detected according to Kim-1 enzyme-linked immunosorbent assay (ELISA) kit manufacturer's instructions according to Prozialeck and Jin technique [25,26]. Cystatin C ELISA estimation was performed as described elsewhere [27–29]. Automated clinical chemistry analyzer Spinlab photometer was used after standardization for the quantitative colorimetric assay for LDH, creatinine, BUN and kinetic estimation for GGT according to the standard manufacturer's instructions.

2.9. Histopathological assessment of kidney tissues

Kidney tissues from different treatment groups were fixed in 10% buffered formalin solution for two days, dehydrated in ascending grades of ethanol, cleared in xylene, embedded in paraffin and sectioned at a thickness 4–5 μm. Sections were stained by a combination of hematoxylin and eosin (H&E) according to the previously described method [30].

2.10. Statistical analysis

The collected data were represented as means ± standard deviations (SD). Data were statistically analyzed using Prism 5 version 5.01, Graph Pad software (USA). Statistical significance was set at $P \leq 0.05$. Parametric data of serum levels of gentamicin-induced AKI biomarkers and megalin fluorescence intensity were analyzed and statistically compared by the ANOVA test followed by the Tukey-Kramer test. Correlation analysis was performed to reveal the correlation between ERα and megalin expression in PTECs, in addition to the correlation between both of megalin and ERα expression to serum levels of the gentamicin-induced AKI biomarkers.

3. Results

3.1. Serum creatinine and BUN assessment

No statistically significant difference was noticed in serum levels of creatinine and BUN in Sham and OVX groups either received or did not receive the scheduled treatment regimen of gentamicin. At the proposed treatment dose level and duration of fulvestrant, genistein and

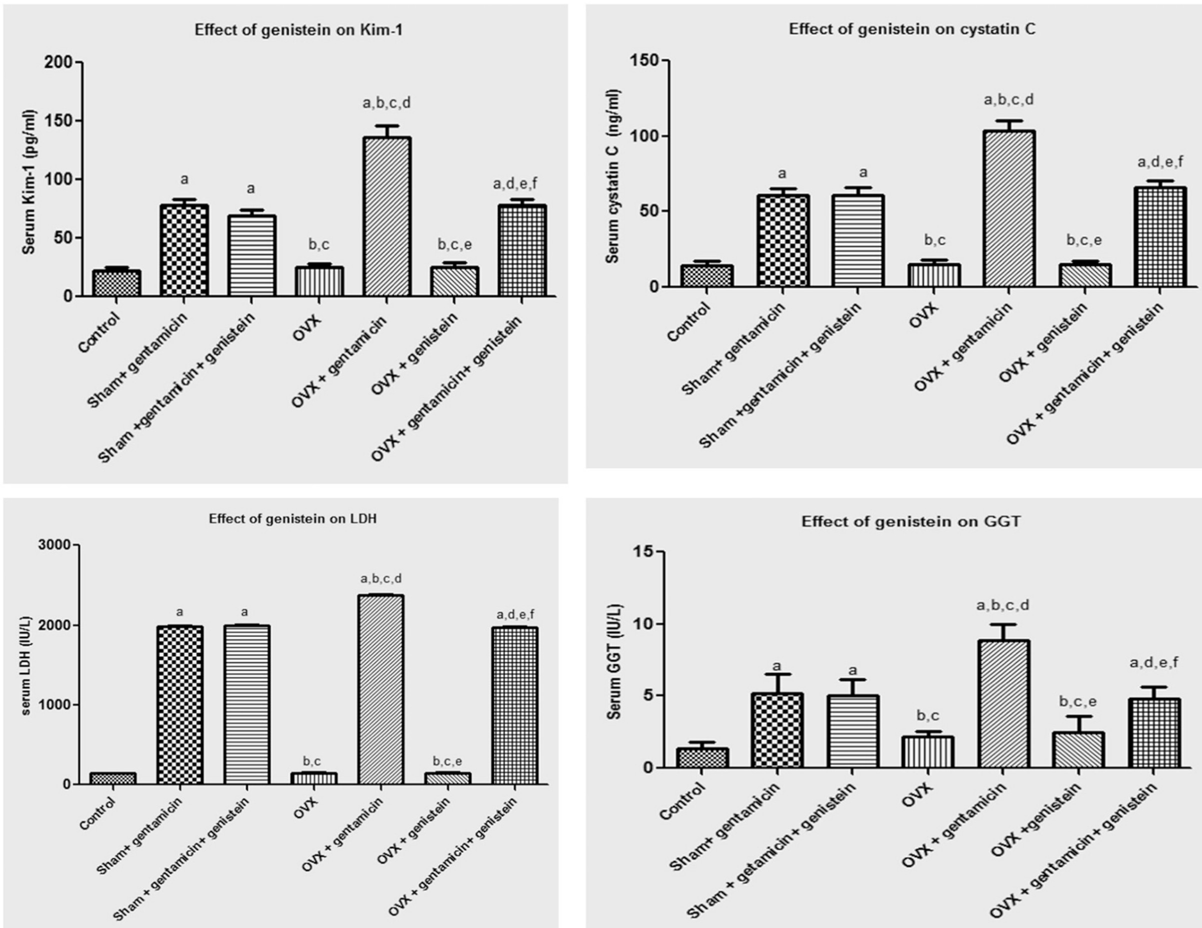


Fig. 4. Effect of Gentamicin, ovariectomy and genistein on serum Kim-1, cystatin C, LDH and GGT of female albino rats Data were presented as means ± SD and statistically analyzed by one-way (ANOVA) test followed by post hoc Tukey-Kramer test. a: significantly different from control; b: significantly different from Sham + Gentamicin; c: significantly different from Sham + Gentamicin + Genistein; d: significantly different from OVX; e: significantly different from OVX + Gentamicin; f: significantly different from OVX + Genistein at P ≤ 0.05.

estradiol benzoate, no significant difference in serum levels of creatinine and BUN was estimated between Sham and OVX groups either received or did not receive gentamicin compared to those received fulvestrant, genistein and estradiol benzoate either plus or without gentamicin treatment (Table 1).

3.2. Kim-1, cystatin C, LDH and GGT estimation

3.2.1. Effectiveness of the designed gentamicin schedule and impact of OVX on gentamicin-AKI induction

Data analysis for serum levels of Kim-1, cystatin C, LDH and GGT in control, Sham, Sham + gentamicin, OVX, and OVX + gentamicin groups, were performed to detect the capability of the designed gentamicin treatment schedule to induce AKI and to study the impact of OVX on this injury.

Comparing serum levels of Kim-1, cystatin C, LDH and GGT biomarkers for both of Sham and OVX groups to control one, we revealed the absence of statistically significant difference; these data rejected the possibility that ovariectomy or sham operation effect alone could induce AKI and interfere with gentamicin-induced AKI.

The ability of the scheduled gentamicin treatment regimen to induce the AKI was evident through the estimated remarkable increase in the serum levels of Kim-1, cystatin C, LDH, and GGT of Sham group received gentamicin compared to Sham one, amounting to 238%, 369%, 1276%, and 247%, respectively. Besides, the revealed marked increase in Kim-1, cystatin C, LDH, and GGT serum levels for the OVX group that received gentamicin in contrast to the OVX group, reaching about 437%, 582%, 1512%, and 305%, respectively.

The marked increase in Kim-1, cystatin C, LDH, and GGT serum levels in OVX group treated with gentamicin compared to Sham group which received the same schedule of gentamicin treatment, tantamount to 74%, 69%, 20%, and 69%, respectively, proved the overdone impact of OVX on the gentamicin-induced AKI (Fig. 1).

3.2.2. Impact of estrogen supplementation on gentamicin-AKI induction

To investigate the protective effect of estrogen supplementation treatment against gentamicin-AKI induction, we analyzed the Kim-1, cystatin C, LDH and GGT serum levels of control, Sham + gentamicin, OVX + gentamicin, OVX + estradiol benzoate, and OVX + estradiol benzoate + gentamicin groups.

We detected non-statistically significant difference in Kim-1, cystatin C, LDH, and GGT serum levels of OVX group treated with estradiol benzoate alone compared to control one; however, there was statistically significant remarkable increase in Kim-1, cystatin C, LDH,

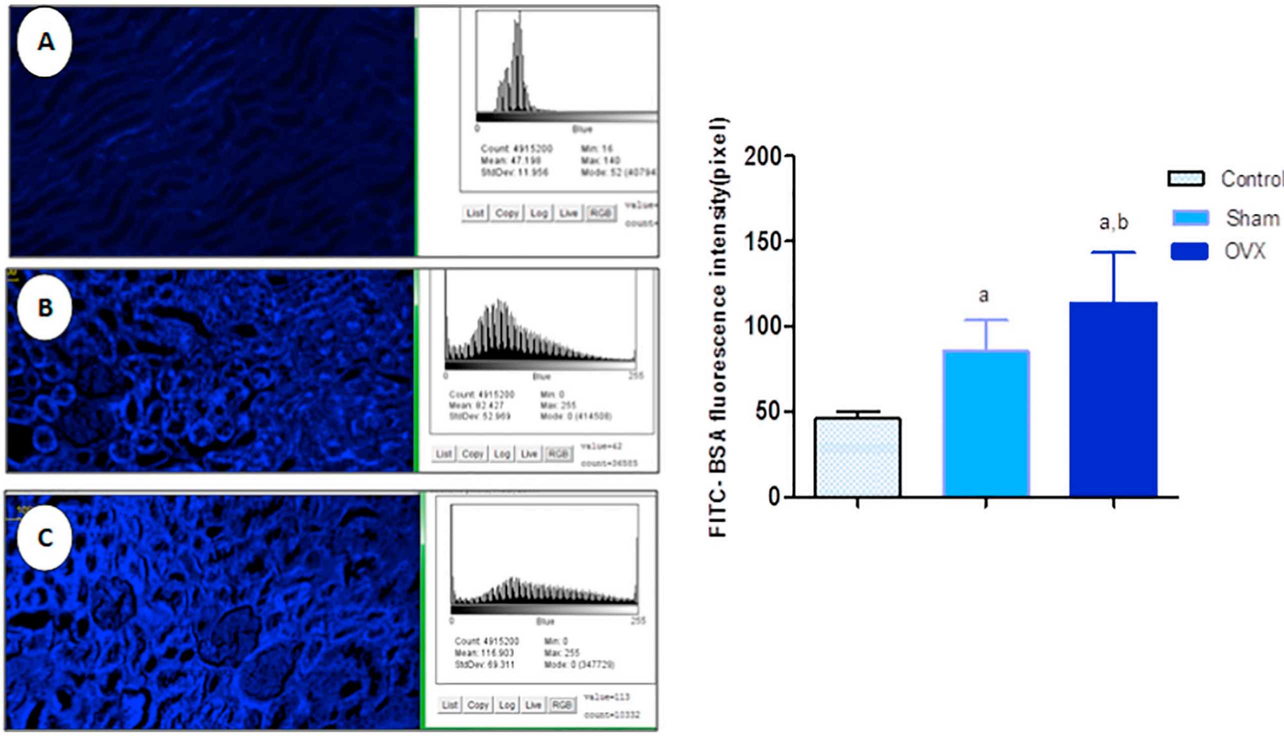


Fig. 5. Assessment of megalin endocytic functionality in samples of kidney epithelial cells of control (A), Sham (B), and OVX(C) female albino rats. The data of the blue fluorescence intensity post FITC-BSA infusion are expressed as mean ± SD (n = 6). a: significantly different from control; b: significantly different from Sham using one–way ANOVA test followed by post hoc Tukey-Kramer test at P ≤ 0.05.

and GGT serum levels in OVX group treated with gentamicin compared to the control group; these data eliminated the probability that estradiol benzoate treatment solo could induce AKI and embarrass with the gentamicin-AKI induction.

The protective effect of external supplementation of estrogen (estradiol benzoate) toward gentamicin–AKI induction was estimated by the significantly remarkable lower Kim-1, cystatin C, LDH, and GGT serum levels in OVX group treated with gentamicin plus estradiol benzoate compared to OVX group received the same gentamicin treatment without estradiol benzoate, equal to −78%, −65%, −20%, and −49%, respectively. Besides, there was no statistically significant difference in Kim-1, cystatin C, LDH, and GGT serum levels for the Sham group treated with gentamicin versus OVX group treated with gentamicin plus estradiol benzoate (Fig. 2).

3.2.3. *Influence of the ERs antagonist (fulvestrant) treatment on gentamicin-AKI induction*

Statistical analysis to the serum Kim-1, cystatin C, LDH, and GGT collected data of Sham, Sham + gentamicin, Sham + fulvestrant, Sham + gentamicin + fulvestrant, OVX, OVX + gentamicin, OVX + fulvestrant and OVX + gentamicin + fulvestrant groups, clarified the leverage of ERs on gentamicin-AKI induction.

Comparing Kim-1, cystatin C, LDH, and GGT serum levels in both Sham and OVX groups received fulvestrant to control one, revealed no statistically significant difference; these results excluded the probability that fulvestrant treatment alone could induce AKI and confuse with gentamicin-AKI induction.

The exaggerating effect of blocking ERs by fulvestrant on gentamicin – induced AKI was obvious through the estimated statistically significant elevated serum levels of Kim-1, cystatin C, LDH, and GGT in Sham group treated with gentamicin plus fulvestrant compared to Sham group that received the same gentamicin treatment without fulvestrant, equal to 76%, 62%, 20%, and 75%, respectively; these data also illustrated the independent protective effect of ERs detached from the impact of E2 concerning the gentamicin-induced AKI.

Data analysis for Kim-1, cystatin C, LDH, and GGT serum levels in Sham group received gentamicin plus fulvestrant versus OVX treated with gentamicin alone reported no statically significant difference between the two groups, revealing that E2 depletion effect alone post OVX, approximately matched the ERs blocking effect alone by fulvestrant on gentamicin-induced AKI (Fig. 3).

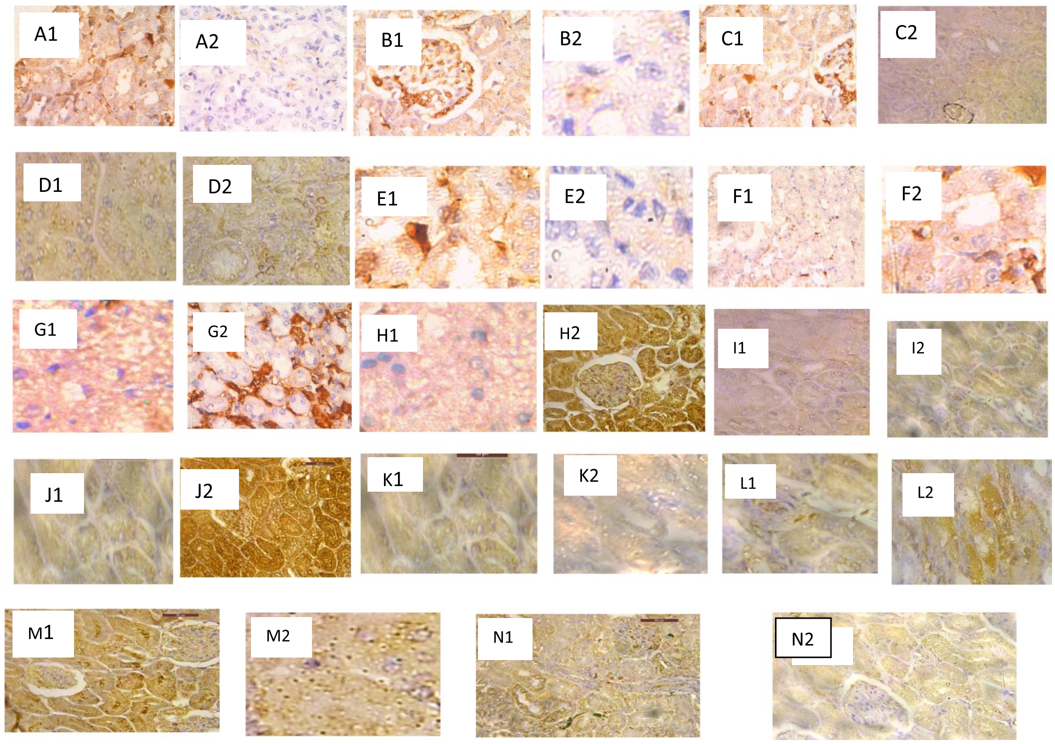
3.2.4. *Effect of genistein treatment on gentamicin-AKI induction*

The Kim-1, cystatin C, LDH, and GGT serum levels in Sham + gentamicin, Sham + gentamicin + genistein, OVX, OVX + gentamicin, OVX + genistein, and OVX + gentamicin + genistein groups were analyzed to track the effect of genistein treatment on gentamicin-AKI induction.

The approximate equivalence of Kim-1, cystatin C, LDH, and GGT serum levels in the control group in contrast to OVX one received genistein neglected the prospect that genistein treatment could on his own induce AKI and confuse with gentamicin-AKI induction.

Data analysis showed significantly detectable decrease in serum levels of Kim-1, cystatin C, LDH, and GGT in OVX group treated with gentamicin plus genistein compared to those in OVX group that received the same regimen of gentamicin treatment without genistein, mounting to −74%, −58%, −20%, and −84%, respectively.

The fade of the estrogenic effect of genistein in high levels of E2 was apparent in the detectable absence of the difference in serum levels of Kim-1, cystatin C, LDH, and GGT on comparing Sham group, which received gentamicin plus genistein to Sham one treated with gentamicin plus genistein (Fig. 4).



Group	ERα area % expression(mean±SD)		Megalin area % expression(mean±SD)	
Control (n=6)	A1	22.0±5.33	A2	2.37±1.22
Sham (n=6)	B1	22.8±4.47	B2	3.13±2.20
Sham+ Gentamicin (n=6)	C1	19.0±5.23	C2	7.69±2.08
Sham + Gentamicin+ Fulvestrant (n=5)	D1	6.46±1.66	D2	18.4±4.30
Sham + Gentamicin + Genistein (n=6)	E1	22.1±5.18	E2	4.09±3.47
Sham + Fulvestrant (n=6)	F1	3.89±1.62	F2	20.2±5.37
OVX (n=6)	G1	2.80±1.66	G2	20.5±5.37
OVX + Gentamicin (n=6)	H1	2.74±1.36	H2	57.2±3.77
OVX + Fulvestrant (n=6)	I1	3.83±2.12	I2	12.9±1.57
OVX + Gentamicin + Fulvestrant (n=6)	J1	6.55±0.912	J2	79.5±4.61
OVX + Genistein (n=6)	K1	14.6±2.13	K2	14.3±1.44
OVX + Gentamicin + Genistein (n=5)	L1	13.2±2.52	L2	20.6±3.91
OVX + Estradiol benzoate (n=6)	M1	26.2±2.39	M2	6.35±0.748
OVX+ Estradiol benzoate + Gentamicin (n=6)	N1	22.7±2.94	N2	14.6±1.93

Fig. 6. Immunohistochemical assessments of ERα and megalin co-localization and expression in samples of PTECs of Sham and OVX female albino rats treated with Gentamicin, Genistein, Fulvestrant, and Estradiol benzoate. The ERα and megalin expressions are represented as mean ± SD of the area % of the positively immunostained cells. Six images were captured for each group (×400).

3.3. Effect of OVX on megalin expression/endocytic functionality in PTECs

To illustrate the impact of E2 depletion post OVX on megalin expression/endocytic functionality in PTECs, we analyzed the data of the blue fluorescence intensity disclosed post-FITC-BSA infusion, expressed in pixel for control, Sham, and OVX groups.

We detected a statistically significant increase in the blue fluorescence intensity in the Sham group compared to control one amounting to 86%.

The comparative analysis reported a statistically significant increase in the blue fluorescence intensity of the OVX group compared to both Sham and controls ones, reaching about 33% and 147%, respectively (Fig. 5).

3.4. Immunohistochemical investigation

Immunoreactivity represented as the area percentage of the positively immunostained cells of megalin and ERα antibodies of the same sectioned slides of PTECs proceeded for IHC assay detected the co-localization of megalin and ERα in PTECs (Fig. 6).

Correlation statistical analysis for ERα and megalin expression in PTECs in all groups revealed a statistically significant inverse correlation between ERα and megalin (Fig. 7).

Correlation analysis for data of megalin expression in PTECs and serum levels of Kim-1, cystatin C, LDH, and GGT demonstrated the statistically significant directly proportional correlation between megalin expression and serum levels of Kim-1, cystatin C, LDH, and GGT in all groups. In contrast, data analysis pointed to the inverse correlation between ERα expression and serum levels of these markers

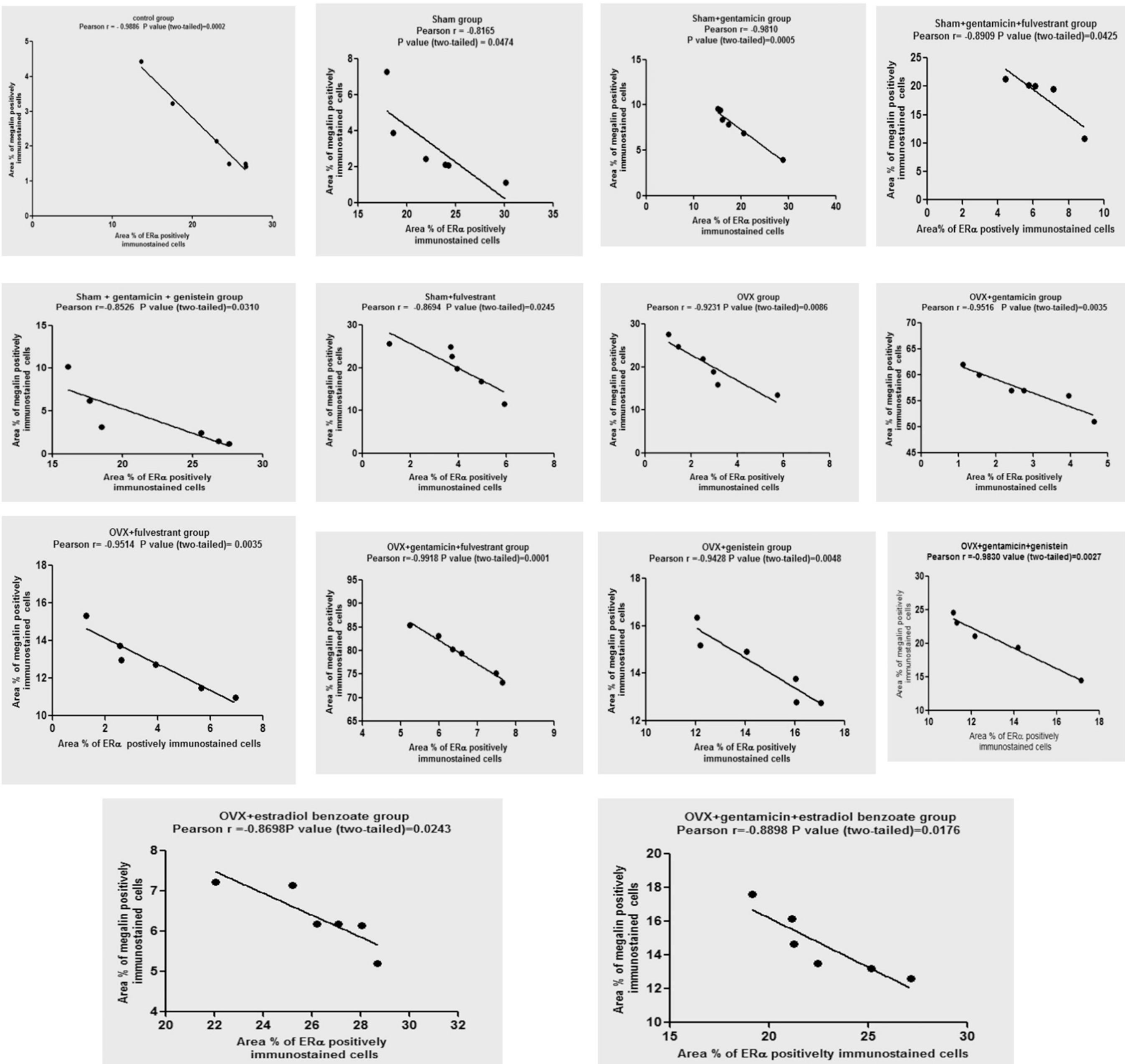


Fig. 7. Correlation analysis for ERα and megalin expression represented as area % of the positively immunostained cells in samples of same sectioned slides PTECs of control, Sham, and OVX female albino rats treated with gentamicin, fulvestrant, genistein and estradiol benzoate.

(Fig. 8A, B).

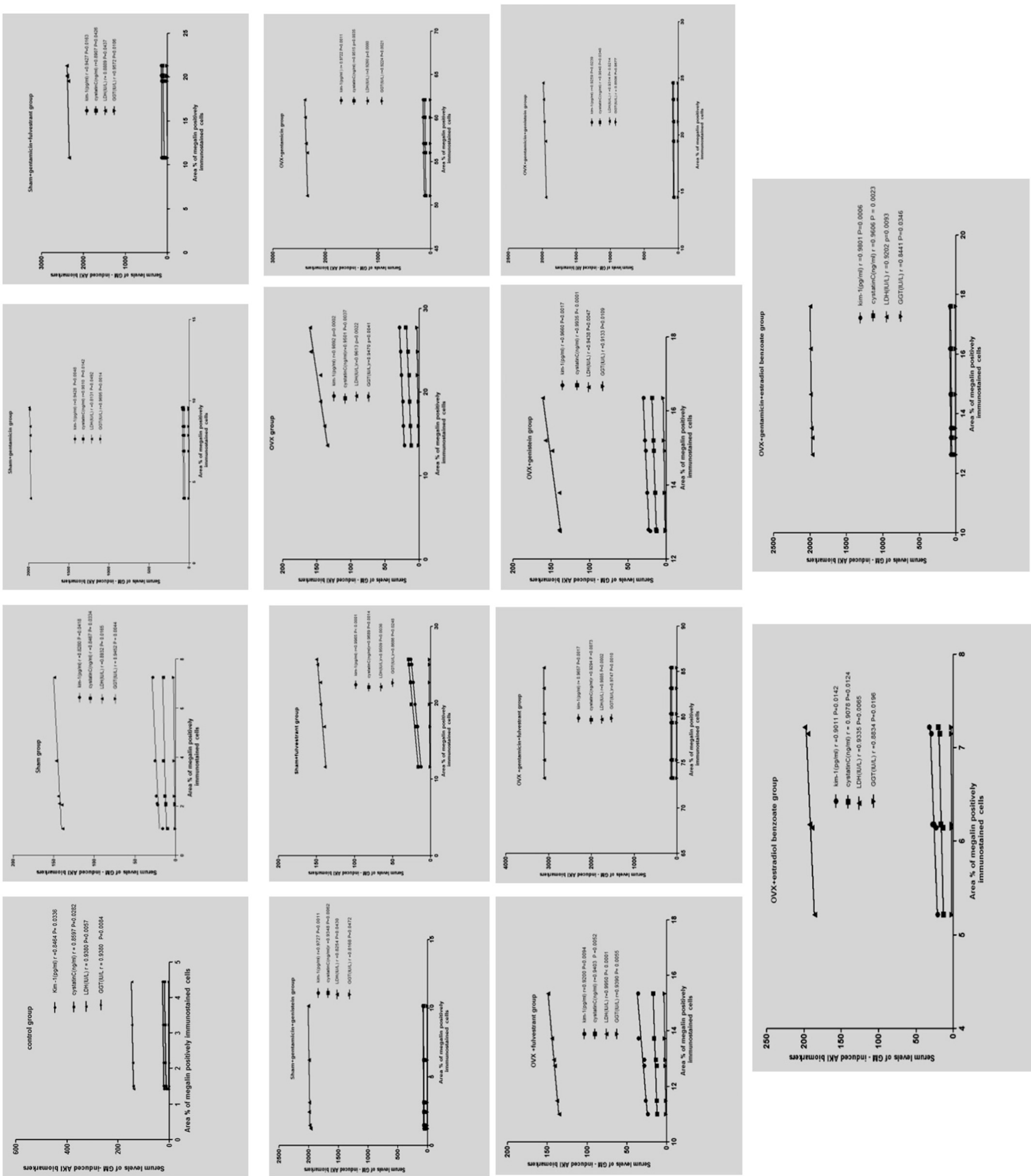
3.5. Histopathological assessment

The histopathological examination and subjective scoring system of the PTECs damage markers of control, Sham, OVX, and other groups that did not receive gentamicin treatment regimen, revealed normal kidney tissue denoted by unchanged nephrons (glomeruli and tubules), Bowman's spaces and interstitium (Table 2, Fig. 10A).

The surgical stress response post-OVX or sham surgery, represented as dilated congested blood vessels was observed clearly in OVX groups that received a vehicle, fulvestrant, genistein, estradiol benzoate, and Sham group received fulvestrant alone compared to control one (Fig. 9A–E).

The histopathological examination of Sham and OVX groups which received gentamicin compared to those that did not receive it, pointed to the clear gentamicin tissue deteriorating effect indicated by few scattered small-sized glomeruli with widened Bowman's spaces, few proximal tubules with partial loss of brush borders, and renal medulla showed collecting tubules with edematous epithelial lining (Fig. 10B).

Sham group received the designed gentamicin plus fulvestrant treatment regimen showed more exaggerated gentamicin tissue deteriorating effect compared to those that received gentamicin alone; manifested by an increase in glomeruli size, necrotic/atrophied tubular



(caption on next page)

Fig. 8. A: Correlation analysis for megalin expression represented as area % of the positively immunostained cells in samples of PTECs and serum levels of gentamicin-induced AKI of Sham and OVX female albino rats treated with gentamicin, fulvestrant, genistein and estradiol benzoate. B: Correlation analysis for ER α expression represented as area % of the positively immunostained cells in samples PTECs and serum levels of gentamicin-induced AKI of Sham and OVX female albino rats treated with gentamicin, fulvestrant, genistein and estradiol benzoate.

lining, complete loss of brush border, markedly dilated blood vessels/edema in the Interstitium, Intra-tubular casts, and interstitial hemorrhage associated with inflammatory infiltrates in the medulla (Fig. 10C).

The overdone gentamicin-tissue deteriorating effect post--ovariectomy in OVX rats received gentamicin compared to Sham one received gentamicin, was unclouded; expressed as scattered atrophied glomeruli, more widened Bowman's spaces, partially necrotic proximal tubules with apoptotic epithelial lining, intra-tubular casts, partial loss of brush borders, interstitial and perivascular edema with area of inflammatory infiltrate, and renal medulla showed collecting tubules with intra-tubular debris and interstitial edema (Fig. 10D).

In harmonization with the identical impact of both of E2 and ERs on gentamicin-induced AKI revealed from the chemical data analysis, histopathological examination of Sham rats that received gentamicin plus ERs blocker (fulvestrant) showed almost the same grade of gentamicin tissue deteriorating markers in OVX rats received gentamicin alone, in addition to markedly dilated congested blood vessels (Fig. 10C, D).

The conservative impact of genistein on gentamicin-induced AKI was clarified by estimating less tissue deterioration markers in OVX rats received the proposed gentamicin treatment regimen plus genistein, represented by fewer glomeruli size, less Bowman's spaces, less edematous/apoptotic tubular limning, preserved brush border, free tubular lumen, and average medulla (Fig. 10E).

The alleviated effect of estrogen supplementation on gentamicin tissue deteriorating effect was obvious in OVX rats treated with gentamicin treatment regimen and estradiol benzoate, manifested by the observed average glomeruli, renal capsule, Bowman's spaces, less edematous/apoptotic tubular limning, preserved brush border and free tubular lumen (Fig. 10F).

4. Discussion

It is believed that gender disparity effects on nephrotoxic drugs could be attributed to the differences in the expression of drug transporters; thus, this sex-related difference in the expression of drug transporters could alter the renal uptake, accumulation, and nephrotoxicant-induced renal injury [31].

Megalin role in gentamicin endocytosis has received much attention. The estimated directly proportional correlation between megalin expression and the serum levels of the novel gentamicin-induced AKI tissue damage biomarkers in our study supported the rationality to anticipate that modulation of megalin expression/endocytic functionality in PTECs could modulate gentamicin-induced AKI.

High doses (40 mg/kg or more) for gentamicin are necessary for animals to rapidly induce extended cortical necrosis and overt renal dysfunction. At this stage, a large number of toxic structural, metabolic, and functional alterations were observed in tubular cells, and several of these alterations have been claimed to be responsible for cell death or dysfunction [35].

Our histopathological investigation disclosed prominent tissue deterioration markers after gentamicin treatment; this investigation concurred with the recorded remarkable higher levels of AKI biomarkers (Kim-1, cystatin C, GGT and LDH) detected in groups that received gentamicin treatment compared to those which did not receive it; these results pointed to the actuality that our experimentally designed gentamicin treatment regimen has already induced the intended AKI.

Serum creatinine and BUN are not rigorous biomarkers for AKI. In a gentamicin dose-response, time course study in rats, Kim-1 was elevated within 24 h, in cases where acute drug exposure caused necrosis of around half of all proximal tubules, serum Kim-1 levels increased but serum urea and creatinine did not differ from controls, indicating that these were too insensitive to detect acute tubular injury [36]. Cross-sectional study (based on a single point in time) suggested that serum level of cystatin C is a more precise test of kidney function than serum creatinine levels [37]. Following gentamicin treatment in rats, increased levels of LDH and GGT at 4, 7, 10, or 14 days of treatment reflected the loss of brush border integrity as momentary marker for acute injury [38].

In our study, the disappearance of the statistically significant difference in the conventional biomarkers (serum creatinine and BUN) between groups, contrary to the estimated difference in the novel biomarkers, was in harmonization with other previous studies, confirming the inaccuracy of creatinine and BUN in estimating acute injury.

E2 is the fundamental type of circulating estrogens. The effects of E2 on cells are mediated by ligand with ERs (ERα, ERβ and GPR30) that are encoded by distinct genes [39]. It appears that ERα predominates in the kidney, however, even within a single tissue; the expression pattern of each subtype is cell type-specific [40–42]. Long-term exposure to E2 regulates steady-state ERα levels, and the clearest evidence for this type of control stems from investigations of nonproductive estrogen target tissues including kidney, liver, brain, and peripheral nervous tissue; most studies showed that E2 maintains these tissues in an estrogen-responsive condition by up-regulating steady-state ERα expression levels. This view is supported by the presence of estrogen response elements in many of the ERα gene promoter regions [43]. This positive relation between E2 and ERs was supported by our estimated higher ERα expression in the PTECs in the Sham group compared to E2 depleted OVX one.

The ERE, a short sequence of DNA within the promoter of a gene, can bind E2/ERs complex, up or down-regulating this gene transcription. The ERE are permutations of the 5′-GGTCAnnnTGACC-3′ DNA palindrome, wherein ‘n’ denotes a non-specific three-nucleotide spacer, located at various distances from the transcription start site and/or within a gene locus [44,45]. The nuclear ERs interact with chromatin target sites through two distinct modes; ERE-dependent and ERE-independent pathways. The regulation of gene expression by the binding of E2/ERs complex to the ERE is referred to as the “ERE-dependent signaling pathway”. On the other hand, the transcriptional modulation of target genes through the interactions of E2–ERα with transcription factors, exemplified by SP1 and AP-1, bound to their cognate regulatory elements on DNA, denotes the ERE-independent signaling pathway [46–51].

Previous DNA binding experiments have demonstrated that ERα binds direct repeat (DR) of the ERE half-site 5′-AGGTCA-3′, as well as ERE palindromes [52–54].

The AP-1 proteins generally bind DNA forming a tight complex that binds specifically to the AP1 recognition sequence at the promoter and enhancer regions of target genes; a palindromic DNA element referred

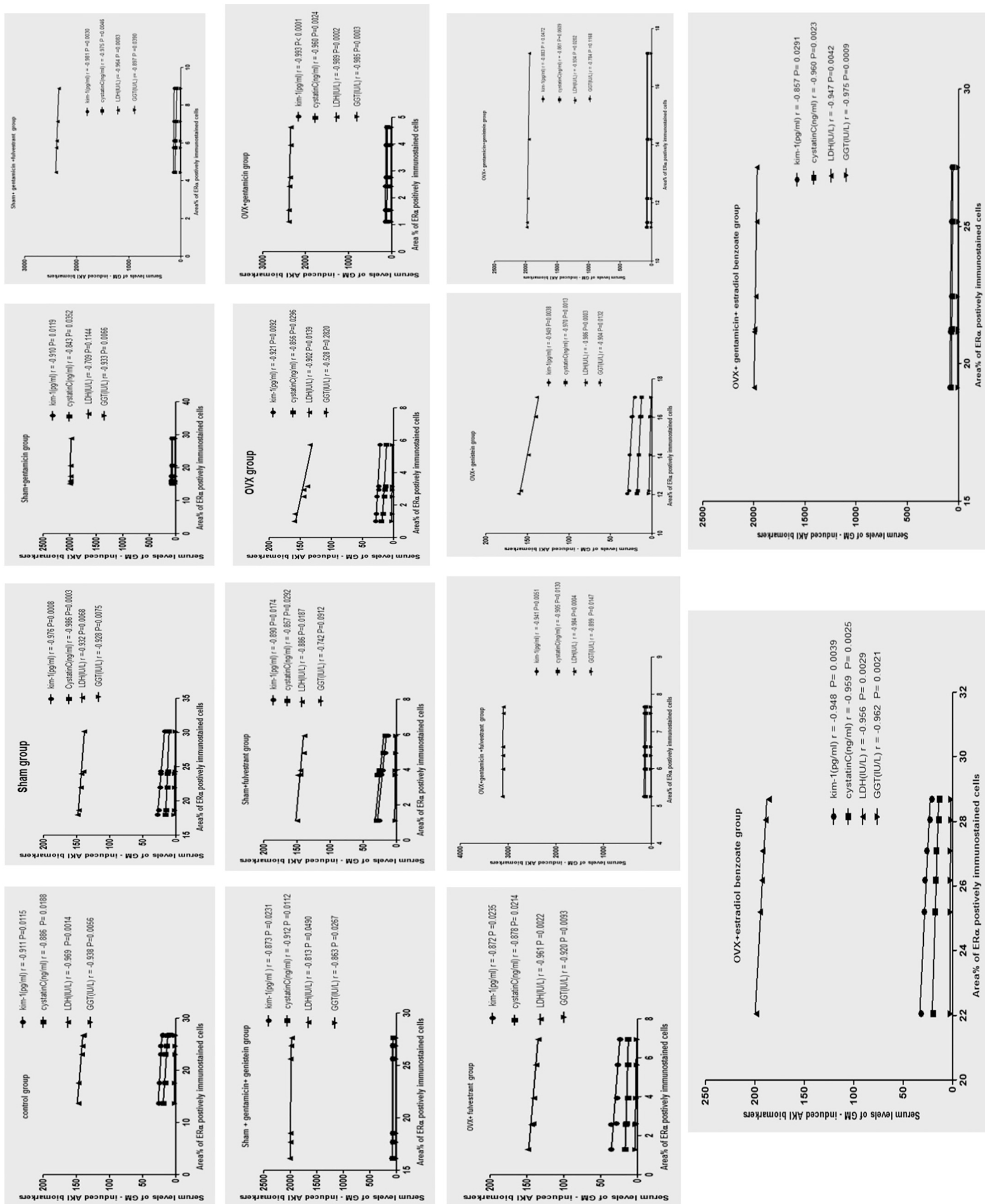


Fig. 8. (*continued*)

Table 2
Descriptive scoring system for PTECs damage markers of Sham and OVX female albino rats treated with Gentamicin, Fulvestrant, Genistein, and Estradiol benzoate.

Group	Marker							
	Capsule	Glomeruli	BS	Tubular lining	Brush border	Tubular lumen	Interstitium	Medulla
Control (n = 6)	0	0	0	0	0	0	0	0
Sham (n = 6)	0	0	0	0	0	0	0	0
Sham + Gentamicin (n = 6)	0	+	+	0	+	0	0	+
Sham + Gentamicin + Fulvestrant(n = 5)	0	+ +	+	+ +	+ +	+ +	+	+ +
Sham + Gentamicin + Genistein (n = 6)	+	+	+	+	+	0	+ +	0
Sham + Fulvestrant (n = 6)	0	0	0	0	+	0	+ +	+
OVX (n = 6)	0	0	0	0	+	0	+ +	+
OVX + Gentamicin (n = 6)	0	+ +	+	+ +	+ +	+ +	+ +	+
OVX + Fulvestrant (n = 6)	0	0	0	0	0	0	+	+
OVX + Gentamicin + Fulvestrant (n = 6)	0	+	+	+	0	0	+	+
OVX + Genistein (n = 6)	0	0	0	+	0	0	+	+
OVX + Gentamicin + Genistein (n = 5)	+	+	+	+	0	0	+ +	0
OVX + Estradiol benzoate(n = 6)	0	0	0	0	0	0	0	0
OVX + Gentamicin + Estradiol benzoate (n = 6)	0	0	0	+	0	0	+ +	+

Renal capsule: 0: Average, +: Detached.
Glomeruli (G): 0: Average, +: Small-sized, + +: Atrophied.
Bowman's spaces (BS): 0: Average, +: Widened/dilated.
Tubular lining: 0: Average, +: Edematous/apoptotic, + +: Necrotic/atrophied.
Brush border: 0: Preserved, +: Partial loss, + +: Complete loss.
Tubular lumen: 0: Free, +: Intra-tubular debris, + +: Intra-tubular casts.
#Interstitium: 0: Average, +: Dilated blood vessels/edema, + +: Interstitial hemorrhage/inflammatory infiltrate.
Medulla: 0: Average, +: Dilated blood vessels/edema, + +: Interstitial hemorrhage/inflammatory infiltrate.

to TRE, TGA C TCA or TGA gentamicin TCA. Several AP-1 sites variants have been reported to function as AP-1 binding sites and some differ from the consensus TRE sequence by two or more bases i.e. Imperfect TPA responsive element [55].

The SP1 transcription factor contains a zinc Finger protein motif, by which it binds directly to DNA and enhances gene transcription. Its zinc Fingers bind the consensus sequence 5′-(gentamicin/T) GGGCGG (gentamicin/A) (gentamicin/A) (C/T)-3′ (GC box element). This is a distinct pattern of nucleotides found in the promoter region of some eukaryotic genes. The GC box has a consensus sequence GGGCGG. The GC elements are bound by transcription factor SP1 and have similar functions to enhancers [56].

In silico analysis of megalin (LRP-2) DNA gene promoters' sequence demonstrated the presence of half the palindromic repeat of ERE (GGTCA), the cognate regulatory elements of SP1 (GC box element GGGCGG), and the imperfect TPA (TGACT) cognate regulatory element for activator protein AP-1 [57]. This analysis supports the assumption that restoration or depletion of E2/ERs could up or down-regulate megalin expression/endocytic functionality.

Co-localization detects a close spatial expression between two targets, is important for investigating metabolism, signaling events, and transcriptional control [32]. Co-localization can be split into two different phenomena, co-occurrence, which refers to the presence of two (possibly unrelated) targets of interest, and correlation, a much more significant statistical relationship between the two targets indicative of a biological interaction [33]. Our IHC assessment for ERα and megalin expression in the same PTECs sectioned slides in the kidney revealed the co-localization of megalin and ERα. Our finding was in agreement with Lindsey and coworkers, who revealed that ERs are highly expressed in the brush border of the proximal tubules and co-localized with megalin receptors [34]. The estimated values of Pearson's Correlation Coefficient (< -1) in our study, reflected the perfect negative correlation between ERα and megalin, supporting the potential genetic down-regulating role of ERα to megalin. Indeed, this correlation in PTECs was linked directly to the E2 level. The observed expression/endocytic functionality, linked inversely to E2 level post-FITC-BSA infusion, added another support to the assumed reciprocal relationship. However, special experiments such as ChIP-seq are necessary to verify the cross talk between the two receptors. In addition, our data highlight the need for publication of more studies to fully explore this correlation with other ERs subtypes (ERβ and GPR30).

Estradiol benzoate is metabolized through hydrolysis by esterase enzyme to estradiol and benzoic acid; therefore, it is considered as a prodrug and bioidentical forms of E2 have the same downstream effects within the body through binding to the ERs, which are located in various tissues [58,59]. Supplementation with estradiol benzoate in OVX rats minified serum Kim-1, cystatin C, GGT and LDH; this estrogenic protective effect was unclouded upon histopathological examination of PTECs.

Genistein is an isoflavonoid phytoestrogen derived from soy products. The chemical structures of isoflavones are similar to that of E2, and these compounds appear to exert an estrogenic or antiestrogenic effect depending on the circulating estrogen level, i.e. they exert an antiestrogenic when the circulating estrogen level is high, but when the estrogen level is low, their effects become more estrogenic [60].

Our data analysis revealed the protective estrogenic effect of genistein expressed by: (1) Lower Kim-1, cystatin C, LDH and GGT markers of OVX groups received gentamicin plus genistein in contrast to those treated with gentamicin alone, (2) Almost convergence of the serum levels of these markers in the Sham rats treated with gentamicin and genistein compared to the OVX one treated with gentamicin plus genistein, (3) Histopathological examination pointed to the ameliorated gentamicin-tissue deteriorating effect in OVX rats treated with gentamicin plus genistein compared to those received gentamicin alone. The fading of genistein estrogenic protective effect in high levels of the circulating E2 was illustrated by vanishing these differences upon comparing Sham rats treated with gentamicin to Sham one received gentamicin plus genistein.

Fulvestrant is receptor degrader, blocks the estrogen receptor-mediated transcription; upon binding with ERs monomers, it inhibits ERs dimerization, inactivates AF1 and AF2, reduces translocation of ERs to the nucleus, accelerating their degradation [61]. The degrading effect of fulvestrant on ERs clarified the protective impact of ERs on gentamicin-induced injury, manifested by estimating higher serum levels of Kim-1, cystatin C, LDH and GGT in Sham rats injected with gentamicin plus fulvestrant corresponding to Sham rats similarly received gentamicin alone. Our histopathological examination was following these results, it revealed exaggerated markers of gentamicin

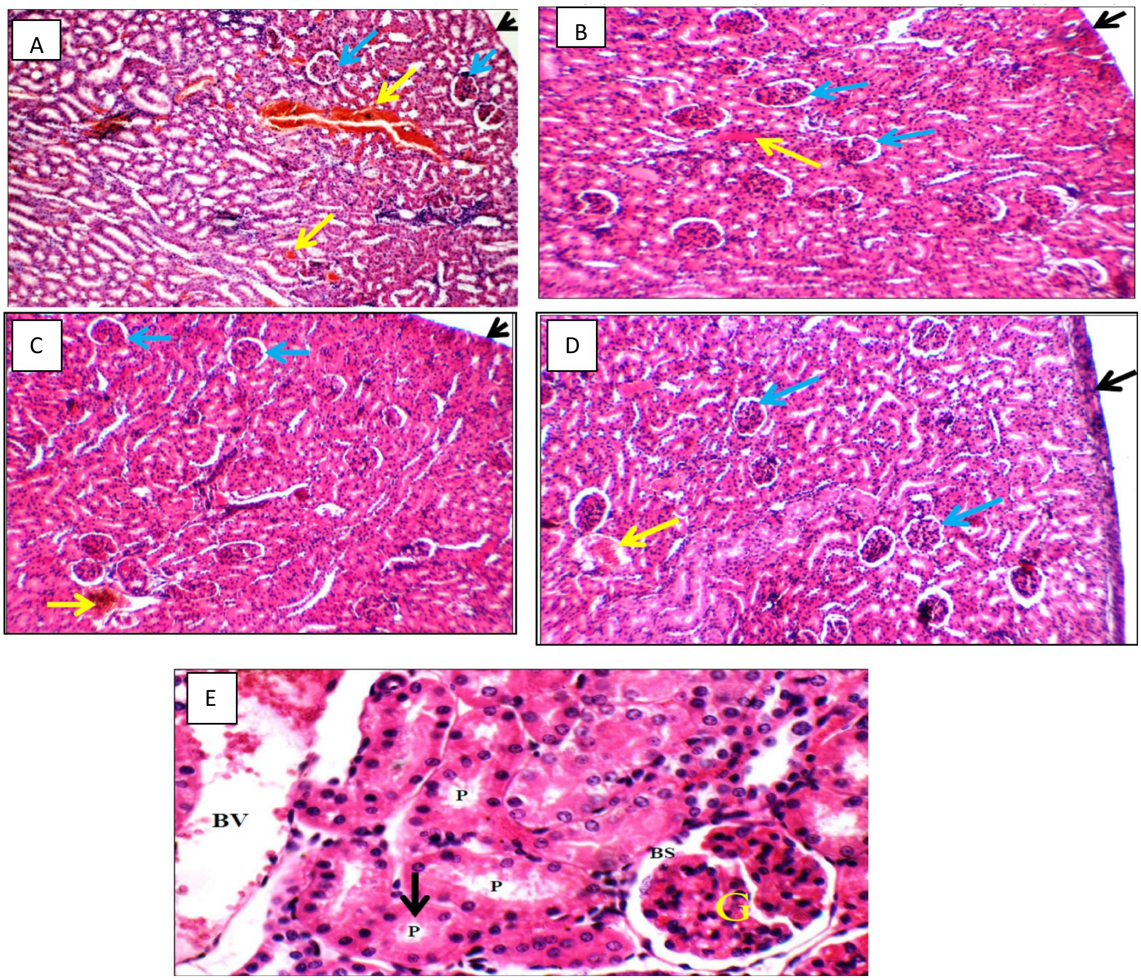


Fig. 9. Histopathological examination of samples from PTECs for OVX and Sham female rats treated with Fulvestrant, Genistein and Estradiol benzoate.
A (OVX + Vehicle): kidney showing average renal capsule (black arrow), average glomeruli (blue arrows), and dilated congested blood vessels (yellow arrows) (H&E X 200).
B (OVX + Fulvestrant): kidney showing average renal capsule (black arrow), average glomeruli (blue arrows), and dilated congested blood vessels (yellow arrow) (H &E X 200).
C (OVX + Genistein): kidney showing average renal capsule (black arrow), average glomeruli (blue arrows), and dilated congested blood vessels (yellow arrow) (H& E X 200).
D (OVX + Estradiol benzoate): kidney showing average renal capsule (black arrow), average glomeruli (blue arrows), and dilated congested blood vessel (yellow arrow) (H&E X 200).
E (Sham + Fulvestrant); Kidney showing average glomerulus (G) with average Bowman's space (BS), average proximal tubules (P) with preserved brush borders (black arrow), and markedly dilated congested blood vessel (BV) (H&E X 400). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tissue deteriorating effect in PTECs of Sham animals received gentamicin plus fulvestrant compared to those treated with gentamicin alone.

5. Conclusion

The estimated directly proportional relationship between megalin expression and the serum levels of the novel AKI biomarkers, sharply confirmed that megalin is implicated in gentamicin-induced AKI. The protective effect of E2/ERs against gentamicin-induced AKI was clarified by our finding that E2 depletion post-ovariectomy and ERs blocking with fulvestrant elevates renal tissues megalin expression, linked directly with the apparent exaggerated gentamicin-induced AKI. The gentamicin-induced AKI was ameliorated by treatment with ERs agonist (estradiol benzoate and genistein). We revealed in our study the inverse correlation between ERα and megalin expression/functionality supporting the possibility of down regulating impact of ERα towered megalin transcription.

Declaration of competing interest

The authors declare that there was no conflict of interest.

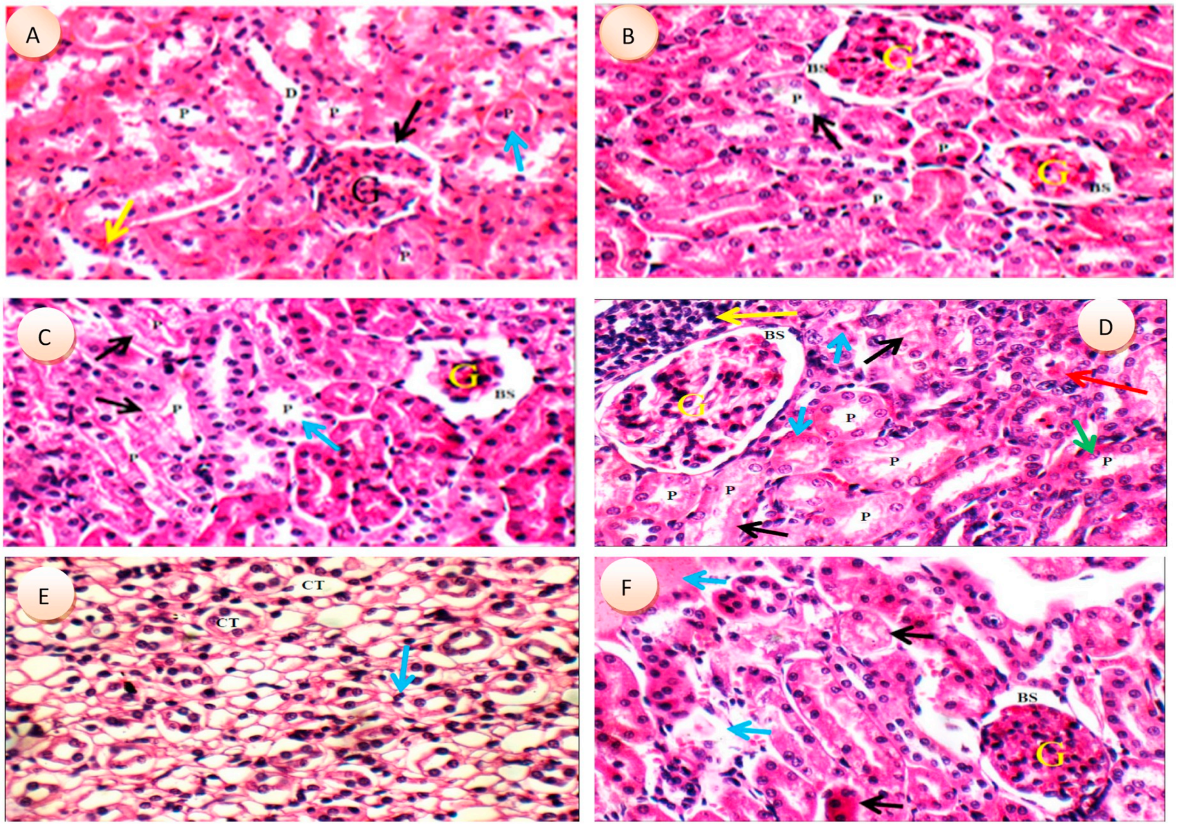


Fig. 10. Histopathological examination of samples from PTECs for Sham and OVX female rats treated with Gentamicin, Fulvestrant, and Genistein and Estradiol benzoate. High power (H&E X 400) view.
A (Sham): showed average glomerulus (G) with average Bowman's space (black arrow), average proximal tubules (P) with preserved brush borders (blue arrow), average distal tubules (D), and average interstitium (yellow arrow).
B (Sham + Gentamicin): showed small-sized glomerulus (G) with widened Bowman's spaces (BS), and few proximal tubules (P) showing partial loss of brush borders (black arrow).
C (Sham + Gentamicin + Fulvestrant): showed atrophied glomerulus (G) with widened Bowman's space (BS), scattered partially necrotic proximal tubules (black arrows) and others showing loss of brush borders (blue arrow) and markedly dilated congested blood vessel (BV).
D (OVX + Gentamicin): showed average glomerulus (G) with average Bowman's space (BS), partially necrotic proximal tubules (black arrows) with apoptotic epithelial lining (blue arrows), intra-tubular casts (red arrow) and partial loss of brush borders (green arrow), and area of inflammatory infiltrate (yellow arrow).
E (OVX + Gentamicin + Genistein): showed renal medulla showing average collecting tubules (CT) with average interstitium (blue arrow).
F (OVX + Estradiol benzoate + Gentamicin): showed average glomerulus (G) with average Bowman's space (BS), proximal tubules (P) with apoptotic epithelial lining (black arrow), and interstitial edema (blue arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Acknowledgments

The authors are greatly thankful to Dr. Wagdy Khalil, Professor of Molecular Genetics, Cell Biology Department, National Research Centre (NRC), Dokki, Giza, Egypt for his kind help in ELIZA assay and Dr. Sayed Abdel Raheem, Assistant professor of pathology, Al-Azhar University, Faculty of Medicine, Cairo for his kind help in histopathological investigation.

References

[1] P. Pillans, J. Iedema, P. Donovan, R. Newbery, V. Whitehead, C. Halliday, R. Sheehy, A. Springford, T. Patterson, Outcomes in patients with gram-negative sepsis treated with gentamicin, Ther. Adv. Drug Saf. 3 (2012) 109–113.
[2] E. Labovitz, M.E. Levison, D. Kaye, Single-dose daily gentamicin therapy in urinary tract infection, Antimicrob. Agents Chemother. 6 (1974) 465–470.
[3] P. Randjelovic, S. Veljkovic, N. Stojiljkovic, D. Sokolovic, I. Ilic, Gentamicin nephrotoxicity in animals: current knowledge and future perspectives, EXCLI J. 16 (2017) 388–399.
[4] Y. Quiros, L. Vicente-Vicente, A. Morales, J. López-Novoa, F. López-Hernández, An integrative overview on the mechanisms underlying the renal tubular cytotoxicity of gentamicin, Toxicol. Sci. 119 (2011) 245–256.
[5] P. May, E. Woldt, R.L. Matz, P. Boucher, The LDL receptor related protein (LRP) family: an old family of proteins with new physiological functions, Ann. Med. 3 (2007) 219–228.
[6] C. Schmitz, J. Hilpert, C. Jacobsen, C. Boensch, E.I. Christensen, F.C. Luft, T.E. Willnow, Megalin deficiency offers protection from renal aminoglycoside accumulation, J. Biol. Chem. 277 (2002) 618–622.
[7] P.H. Lane, Estrogen receptors in the kidney: lessons from genetically altered mice, Gend. Med. 5 (A) (2008) S11–S18.
[8] O. König, L. Rüttiger, M. Müller, U. Zimmermann, B. Erdmann, H. Kalbacher, M. Gross, M. Knipper, Estrogen and the inner ear: megalin knockout mice suffer progressive hearing loss, FASEB J. 22 (2008) 410–417.
[10] J. Neugarten, L. Golestaneh, N. Kolhe, Sex differences in acute kidney injury requiring dialysis, BMC Nephrol. 19 (2018) 131.
[11] J. Valdivielso, C. Jacobs-Cachá, M. Soler, Sex hormones and their influence on chronic kidney disease, Curr. Opin. Nephrol. Hypertens. 28 (2019) 1–9.
[12] M. Cheema, D. Irsik, Y. Wang, W. Miller-Little, K. Hyndman, E. Marks, J. Frøkiær, E. Boesen, R. Norregaard, Estradiol regulates AQP2 expression in the collecting duct: a novel inhibitory role for estrogen receptor α, Am. J. Physiol. Renal Physiol. 4 (2015) F305–F317.
[13] J. Mårtensson, C.R. Martling, M. Bell, Novel biomarkers of acute kidney injury and failure: clinical applicability, Br. J. Anaesth. 109 (2012) 843–850.
[14] C.L. Edelstein, Biomarkers in acute kidney injury, in: Biomarkers of Kidney Disease, 2nd ed., Elsevier Health Science (2017), chap. 6: pp: 241–315.
[15] A. Lasota, D. Danowska-Klonowska, Experimental osteoporosis-different methods of ovariectomy in female white rats, Rocz. Akad. Med. Bialymst. 49 (2004) 129–131.
[16] M.G. Latour, M. Shinoda, J. Lavoie, Metabolic effects of physical training in ovariectomized and hyperestrogenic rats, J. Appl. Physiol. 90 (2001) 235–241.
[17] V.R. Souza, E. Mendes, M. Casaro ATFB. Antiorio, F.A. Oliveira, C.M. Ferreira, Description of ovariectomy protocol in mice, Methods Mol Biol. 1916 (2019) 303–309.

[18] W.J. Al-Bazii, Estimation of some oxidative stress parameters in the serum and cerebellum of ovariectomized rats, J. Kerbala Univ. 12 (2014) 87–94.
[19] R. Boddu, C. Fan, S. Rangarajan, B. Sunil, S. Bolisetty, L.M. Curtis, Unique sex- and age-dependent effects in protective pathways in acute kidney injury, Am. J. Physiol. Renal Physiol. 313 (2017) F740–F755.
[20] L.L. Yacovino, L.M. Aleksunes, Endocrine and metabolic regulation of renal drug transporters, J. Biochem. Mol. Toxicol. 26 (2012) 407–421.
[21] F. Eshraghi-Jazi, A. Talebi, F.S. Mirsaeedi, S. Ahmadian, F. Moslemi, M. Nematbamikacinhsh, Gentamicin induced nephrotoxicity: the role of sex hormones in gonadectomized male and female rats, Scientifica (Cairo) 7 (2016) 502–509.
[22] A. Sharma, B.L. Fish, J.E. Moulder, M. Medhora, J.E. Baker, M. Mader, E.P. Cohen, Safety and blood sample volume and quality of a refined retro-orbital bleeding technique in rats using a lateral approach, Lab Anim. 43 (2014) 63–66.
[23] Z. Jia, A.C. Johnson, X. Wang, Z. Guo, A.W. Dreisbach, J.R. Lewin, P.B. Kyle, M.R. Garrett, Allelic variants in Arhgef11 via the rho-rock pathway are linked to epithelial-mesenchymal transition and contributes to kidney injury in the dahl salt-sensitive rat, PLoS One 10 (2015) e0132553.
[24] J.D. Bancroft, A. Stevens, The Theory and Practice of Histological Techniques, 2nd Edition, Longman Group Limited, Churchill Livingstone, Edinburgh, 1982.
[25] W.C. Prozialeck, V.S. Vaidya, J. Liu, M.P. Waalkes, J.R. Edwards, P.C. Lamar, A.M. Bernard, X. Dumont, J.V. Bonventre, Kidney injury molecule-1 is an early biomarker of cadmium nephrotoxicity, Kidney Int. 72 (2007) 985–993.
[26] J. Jin, H. Zhang, L. Kong, Gentamicin Gao, J. Luo, Plant TFDB 3.0: a portal for the functional and evolutionary study of plant transcription factors, Nucleic Acids Res. (42 (2014 D1182–1187.
[27] H. Ishiguro, I. Ohkubo, M. Mizokami, K. Titani, M. Sasaki, The use of monoclonal antibodies to define levels of cystatin C in normal human serum, Hybridoma 8 (1989) 303–313.
[28] U. Skaleric, J. Babnik, V. Curin, T. Lah, V. Turk, Immunochemical quantitation of cysteine proteinase inhibitor cystatin C in inflamed human gingival, Arch. Oral Biol. 34 (1998) 301–305.
[29] K. Shimode, S. Fujihara, M. Nakamura, S. Kobayashi, T.T. Sunematsu, Diagnosis of cerebral amyloid angiopathy by enzyme-linked immunosorbent assay of cystatin C in cerebral fluid, Stroke 22 (1991) 860–866.
[30] H.M. Carleton, R.A.B. Drury, E.A. Wallington, Carleton's Histological Technique E-Book, 5th edition, Oxford University Press, Oxford, New York, 1980.
[31] H. Si, R.S. Banga, P. Kapitsinou, M. Ramaiah, J. Lawrence, Gentamicin Kambhampati, A. Gruenwald, E. Bottinger, D. Glicklich, V. Tellis, S. Greenstein, D.B. Thomas, J. Pullman, M. Fazzari, K. Susztak, Human and murine kidneys show gender- and species-specific gene expression differences in response to injury, PLoS One 4 (2009) e4802.
[32] V. Zinchuk, O. Grossenbacher-Zinchuk, Recent advances in quantitative colocalization analysis: focus on neuroscience, Prog. Histochem. Cytochem. 44 (2009) 125–172.
[33] J. Adler, S.N. Pagakis, I. Parmryd, Replicate-based noise corrected correlation for accurate measurements of colocalization, J. Microsc. 230 (2008) 121–133.
[34] S.H. Lindsey, L.M. Yamaleyeva, K.B. Brosnihan, P.E. Gallagher, M.C. Chappell, Estrogen receptor GPR30 reduces oxidative stress and proteinuria in the salt-sensitive female mRen2.Lewis rat, Hypertension 58 (2011) 665–671.
[35] K. Miura, D.A. Pasino, R.S. Goldstein, J.B. Hook, Effects of gentamicin on renal function in isolated perfused kidneys from male and female rats, Toxicol. Lett. 26 (1985) 15–18.
[36] Y. Zhou, V.S. Vaidya, R.P. Brown, J. Zhang, B.A. Rosenzweig, K.L. Thompson, T.J. Miller, J.V. Bonventre, P.L. Goering, Comparison of kidney injury molecule-1 and other nephrotoxicity biomarkers in urine and kidney following acute exposure to gentamicin, mercury, and chromium, Toxicol. Sci. 101 (2008) 159–170.
[37] V.R. Dharnidharka, C. Kwon, G. Stevens, Serum cystatin C is superior to serum creatinine as a marker of kidney function: a meta-analysis, Am. J. Kidney Dis. 40 (2002) 221–226.
[38] I. Lippi, F. Perondi, V. Meucci, B. Bruno, V. Gazzano, G. Guidi, Clinical utility of urine kidney injury molecule-1 (Kim-1) and gamma-glutamyl transferase (GGT) in the diagnosis of canine acute kidney injury, Vet. Res. Commun. 42 (2018) 95–100.
[39] P. Yaşar, G.M. Ayaz, S.D. User, G. Güpür, M. Muyan, Molecular mechanism of estrogen-estrogen receptor signaling, Reprod. Med. Biol. 16 (2016) 4–20.
[40] H.A. Harris, Estrogen receptor-beta: recent lessons from *in vivo* studies, Mol. Endocrinol. 21 (2007) 1–13.
[41] K.J. Hamilton, Y. Arao, K.S. Korach, Estrogen hormone physiology: reproductive findings from estrogen receptor mutant mice, Reprod. Biol. 14 (2014) 3–8.
[42] S.C. Hewitt, W. Winuthayanon, K.S. Korach, What's new in estrogen receptor action in the female reproductive tract, J. Mol. Endocrinol. 56 (2016) R55–R71.
[43] C. Donaghue, B.R. Westley, F.E.B. May, Selective promoter usage of the human estrogen receptor alpha gene and its regulation by estrogen, Mol. Endocrinol. 13 (1999) 1934–1950.
[44] C.M. Klinge, Estrogen receptor interaction with estrogen response elements, Nucleic Acids Res. 29 (2001) 2905–2919.
[45] M.J. Meyers, J. Sun, K.E. Carlson, G.A. Marriner, B.S. Katzenellenbogen, J.A. Katzenellenbogen, Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues, J. Med. Chem. 44 (2001) 4230–4251.
[46] P.J. Kushner, D.A. Agard, Gentamicin L. Greene, T.S. Scanlan, A.K. Shiau, R.M. Uht, P. Webb, Estrogen receptor pathways to AP-1, J. Steroid Biochem. Mol. Biol. 74 (2000) 311–317.
[47] J.M. Hall, J. Fulvestrant Couse, K.S. Korach, The multifaceted mechanisms of estradiol and estrogen receptor signaling, J. Biol. Chem. 276 (2001) 36869–36872.
[48] S. Safe, Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions, Vitam. Horm. 62 (2001) 231–252.
[49] L. Björnström, M. Sjöberg, Mutations in the estrogen receptor DNA-binding domain discriminate between the classical mechanism of action and cross-talk with Stat5b and activating protein 1 (AP-1), J. Biol. Chem. 277 (2002) 48479–48483.
[50] J. Huang, X. Li, R. Hilf, R.A. Bambara, M. Muyan, Molecular basis of therapeutic strategies for breast cancer, Curr. Drug Targets Immune Endocr. Metabol. Disord. 5 (2005) 379–396.
[51] E. Cheung, M.L. Acevedo, P.A. Cole, W.L. Kraus, Altered pharmacology and distinct coactivator usage for estrogen receptor-dependent transcription through activating protein-1, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 559–564.
[52] S. Kato, L. Tora, J. Yamauchi, S. Masushige, M. Bellard, P. Chambon, A far upstream estrogen response element of the ovalbumin gene contains several half-palindromic 5′-TGACC-3′ motifs acting synergistically, Cell 68 (1992) 731–742.
[53] S. Mader, P. Leroy, J.Y. Chen, P. Chambon, Multiple parameters control the selectivity of nuclear receptors for their response elements, selectivity and promiscuity in response element recognition by retinoic acid receptors and retinoid X receptors, J. Biol. Chem. 268 (1993) 591–600.
[54] S. Kato, J.H. Sasaki, M. Suzawa, S. Masushige, L. Tora, P. Chambon, H. Gronemeyer, Widely spaced, directly repeated PuGGTCA elements act as promiscuous enhancers for different classes of nuclear receptors, Mol. Cell. Biol. 15 (1995) 5858–5867.
[55] H. Zhou, T. Zarubin, Z. Ji, Z. Min, W. Zhu, J.S. Downey, S. Lin, J. Han, Frequency and distribution of AP-1 sites in the human genome, DNA Res. 12 (2005) 139–150.
[56] W.S. Klug, M.R. Cummings, C.A. Spencer, M.A. Palladina, Concepts of Genetics, Ninth ed., Pearson Benjamin Cummings, San Francisco, 2009, pp. 463–464.
[57] https://switchdb.switchgeargenomics.com/productinfo/id_712992/.
[58] T. Yamamoto, H. Honjo, T. Yamamoto, K. Oshima, K. Otsubo, H. Okada, K. Shibata, The metabolic fate of estradiol benzoate in female dog, J. Steroid Biochem. 11 (1979) 1287–1294.
[59] M.B. O'Connell, Pharmacokinetic and pharmacologic variation between different estrogen products, J. Clin. Pharmacol. 35 (1995) 18S–24S.
[60] S. Bedell, M. Nachtigal, Fulvestrant Naftolin, The pros and cons of plant estrogens for menopause, J. Steroid Biochem. Mol. Biol. 139 (2014) 225–236.
[61] D.P. McDonnell, S.E. Wardell, J.D. Norris, Oral selective estrogen receptor down-regulators (SERDs), a breakthrough endocrine therapy for breast cancer, J. Med. Chem. 58 (2015) 4883–4887.