Development of a new *in ovo* model for the assessment of nephrotoxicity and its comparison with an existing *in vivo* model

Joyani Das[1], Dinakar Sasmal[1], Papiya Mitra Mazumder* and Kaushal Kumar Singh[2]

[1]Department of Pharmaceutical Sciences and Technology, Birla Institute of Technology, Mesra, Ranchi 835 215, India
[2]Department of Veterinary Pathology, Ranchi Veterinary College, Birsa Agricultural University, Kanke, Ranchi 834 006, India

In this study, the chick embryo model has been used as an alternative to the mammalian model for assessing nephrotoxicity. Resemblance of the chick embryo to mammals in having metanephric kidney has prompted us to explore the possibility of using this system for the assessment of kidney toxicity caused mainly by nephrotoxic drugs, namely gentamicin, cisplatin and doxorubicin. Fertilized hens' eggs incubated at $38° \pm 2°C$ and 58%–60% relative humidity, were injected with the above-mentioned drugs on embryonic day 11. A significant decrease in the uric acid level of amniotic fluid was shown by embryos of cisplatin and doxorubicin-treated groups when compared to control. Embryos of the gentamicin-treated group showed significant increase in urea concentration. Alterations in uric acid, urea and creatinine levels were found in all groups. Histopathological study showed nephron degeneration. Similar results were found in a mouse model.

Keywords: Chick embryo, kidney cells, mammalian model, nephrotoxic drugs.

ANY substance that may cause an injury to the kidney cells is termed as nephrotoxic. Drug-induced nephrotoxicity is an important area of concern as the kidneys form the major organs of excretion. The renal vascular bed is exposed to a quarter of the resting cardiac output which makes the renal cells encounter a high concentration of drugs and their metabolites, resulting in an increased vulnerability of these cells to toxicity. Initial damage generally goes undetected due to the huge functional capacity of the renal system, and becomes evident only when there are overt changes in the serum or urine biochemical parameters such as urea nitrogen, creatinine, etc. Most episodes of drug-induced renal damage are reversible with renal function returning to normal after discontinuation of the concerned drug[1]. Some examples of drug-induced nephrotoxicity include acute tubular necrosis (aminoglycosides, cephalosporins, NSAIDs), acute interstitial nephritis (methicillin, ampicillin, sulphonamides, thiazides), chronic glomerulopathy (heroine), chronic tubulointerstitial disease (NSAIDs, thiazides, lithium), etc.[2].

Gentamicin is an aminoglycoside antibiotic that mainly causes proximal tubular injury. The cationic aminoglycoside binds to negatively charged membrane phospholipids in the proximal tubule and also binds to receptor megalin. After lysosomal internalization phospholipidosis occurs, which results in the formation of myeloid bodies. The result is disruption of many intercellular processes causing cell necrosis[3].

The antineoplastic agent, cisplatin is seen to be actively accumulated by the renal parenchymal cells where it is metabolized to a more potent toxin. A cascade of conjugation reactions leads to the formation of highly reactive thiols which are responsible for oxidative stress, DNA damage and hence apoptosis and necrosis[4].

The likely mechanisms for renal toxicity of yet another anticancer drug, doxorubicin, are: formation of semiquinone free radicals by NADPH-dependent reductase and non-enzymatic reaction which involves reaction of DOX with iron. The semiquinone free radical further gives rise to various other free radicals and non-free radicals which include reactive oxygen species (ROS) like superoxide anion (O^{2-}), hydroxyl radical (OH^-), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), which lead to oxidative stress. Enhanced generation of ROS is responsible for causing damage to biomolecules[5].

Toxicity studies in biomedical research are recently facing ethical hindrances as they involve the termination of maximum number of animals at preclinical level of research. The concepts of reduction, refinement and replacement given by Russell and Burch[6] have urged scientists to search for new domains in toxicity studies. Various methods involving cell and tissue cultures, use of lower vertebrates or invertebrates, and *in silico* processes have emerged. Here the chick embryo model has been used for assessment of nephrotoxicity. Chick embryo has been used for biological experimentation earlier, but its full potential is yet to be exploited. The advantage of the model is that it presents all the benefits of an alternative

to a mammalian model, but does not require any sophisticated instrumentation, and is quick and easy[7]. Chick embryos may be used whole (*in ovo*), *ex ovo* or in *in vitro* cell cultures. Here we have used the *in ovo* process. At about 11 days of incubation the metanephros become the functional units of the embryonic kidneys and the major excretory product becomes uric acid (birds are uricotelic). The avian kidneys are compressed in the dorsoventral aspect of the body and typically have three divisions – anterior, middle and posterior[8]. So the chick embryonic kidney may be used to screen drugs that might be nephrotoxic quite accurately as the hen is found to possess about the same number of genes as humans, but is extremely compact and with a notable level of conserved synteny with mammals[9].

Materials and methods

In ovo model

Fertilized eggs (within 24 h of laying) of DBN (Dehlem red X BN cross) breed of hens were purchased from Ranchi College of Veterinary Sciences and Animal Husbandry, Kanke, Ranchi, Jharkhand, India. Eggs were incubated at 37–38°C and relative humidity (RH) of 58–60%, and were turned thrice daily for 17 days. The eggs need to be turned along their horizontal axis to prevent sticking of the embryo to the shell. The turning should be done odd number of times so that the embryo does not stay at the same position for a long duration (overnight) every day[10].

Gentamicin used was procured as the formulation, genticin injection (containing gentamicin sulphate equivalent to 80 mg/2 ml of gentamicin; marketed by Abott, Cisplatin) was obtained as a gift sample from the Indian Institute of Chemical Biology (IICB), Kolkata, and doxorubicin was obtained as a gift sample from Biochem Pvt Ltd, Bengaluru.

Induction of nephrotoxicity in embryos

The fertilized eggs were divided into two groups (G1, G2) consisting of 40 eggs each.
G1: Embryos to be sacrificed on embryonic day (ED) 17.

In group G1, eggs were subdivided into four groups (G1 a–d) of 10 eggs each.
G1a: Control – treated with 0.1 ml water for injection.
G1b: Treated with 0.1 ml gentamicin solution (0.2 mg/ml).
G1c: Treated with 0.1 ml cisplatin solution (0.3 mcg/ embryo).
G1d: Treated with 0.1 ml doxorubicin solution (70 mcg/ embryo).

In group G2 eggs were subdivided into four groups (G2 a–d) of 10 eggs each and subgrouped similarly as G1.

Injection of drugs into eggs

Each egg was candled on ED 5 and those found to be unfertilized were discarded. The process of injection was carried out under aseptic conditions maintained by laminar air flow (HEPA filter) on a working bench provided with a UV light. The surface of each egg was wiped with 70% v/v ethanol for sterilization to minimize any type of microbial contamination that might lead to damage of the growing embryo. An orifice was made at the narrow end of the egg. Through this orifice 0.1 ml drug solution (or water for injection in case of control group eggs) was injected using a 26 gauge needle attached to a 1 ml tuberculin syringe and the orifice was immediately sealed with molten paraffin wax. A similar orifice was drilled at the broad end of the egg to release the pressure developed inside due to increase in fluid volume and then it was sealed with molten paraffin wax. According this procedure the eggs were treated in accordance with their grouping on ED 11 (ref. 11).

Procedure after completion of incubation period

Embryos of group G1 were sacrificed on ED 17 by carefully breaking open the eggs. The contents were collected in a petri dish. The membrane covering the embryo was identified as the amniotic sac, into which a needle was gently inserted to collect the amniotic fluid using a tuberculin syringe. For each of the subgroups, six samples of amniotic fluid were separately collected and stored in microfuge tubes for carrying out various tests.

Embryos of group G2 were sacrificed on ED 19. The embryos were dissected, and the kidneys were carefully removed and weighed individually. A minimum of six embryos per subgroup were dissected to obtain six pairs of kidneys, from which two pairs were stored in 10% v/v formalin solution and four pairs were collected in phosphate buffered saline (PBS), pH 7.4 at 4°C temperature.

Biochemical tests

Tests were performed using Mindrey BA-88A Semi Auto Chemistry Analyser.

Amniotic fluid samples: The amniotic fluid samples collected from 17-day-old chick embryos were centrifuged at 2000 rpm for 20 min, and the separated supernatant from each was subjected to biochemical tests to estimate the levels of urea[12], creatinine[13] and uric acid[14]. Commercially available kits were used for this purpose.

Kidney tissue samples: Nineteen-day-old chick embryos from each group were sacrificed by decapitation and their kidneys were removed. The organs were washed with ice

cold saline (0.9% sodium chloride solution) to remove blood and immediately weighed. A 5% homogenate for the kidney samples was prepared with PBS, pH 7.4 (8.01 g sodium chloride, 0.2 g potassium chloride, 0.27 g potassium dihydrogen phosphate and 1.78 g disodium hydrogen phosphate in 1000 ml solution in distilled water) in ice. The homogenates were then centrifuged at 10,000 rpm for 20 min at 4°C, and the supernatants so obtained were subjected to biochemical studies mentioned above.

The kidney tissue homogenates were additionally subjected to tests for oxidative stress.

Reduced glutathione: The tissue supernatant was deproteinized with 10% trichloroacetic acid (TCA) solution (supernatant : TCA 1 : 1), then centrifuged to remove the precipitated protein, and then assayed for reduced glutathione (GSH). The supernatant (0.5 ml) was mixed with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (0.06% solution in 1% sodium citrate solution; 4 ml) and phosphate buffer (0.2 M pH 8, 1.5 ml) and estimated at 412 nm. Concentration was calculated from the equation: $y = 0.0003x + 0.0235$, where y is the absorbance and x is the activity of GSH (μmol/mg of protein)[15].

Catalase: Here, 0.1 ml of kidney tissue homogenate was mixed with 1.8 ml of phosphate buffer (50 mM, pH 7) and 1 ml hydrogen peroxide (0.6 ml 30% H_2O_2 dissolved in 99.4 ml phosphate buffer). Blank was similarly prepared, except that it contained 0.1 ml distilled water instead of the sample. All the assay samples were mixed properly and change in absorbance was measured at 240 nm for 3 min at intervals of 30 sec against blank. Concentration was calculated from the equation: $y = 0.075x - 0.09$, where y is the absorbance and x is the catalase activity expressed in unit/mg of protein[16].

Superoxide dismutase: Here, 0.1 ml of the tissue homogenate was mixed with 1.11 ml phosphate buffer (50 mM), 0.075 ml L-methionine (20 mM in dilute acid or alkali), 0.04 ml Triton X-100 (1% v/v), 0.075 ml hydroxlamine hydrochloride (10 mM), 0.1 ml EDTA (50 μM) and 0.08 ml Riboflavin (50 μM in dilute alkali). Blank was similarly prepared using 0.1 ml distilled water instead of the sample. Then 1.4 ml aliquots from the reaction mixture were taken in test tubes and 100 μl of the test sample was added to all tubes followed by pre-incubation at 37°C for 5 min. Next, 80 μl riboflavin was added to all tubes. The tubes were exposed for 10 min to two 20 W, fluorescent lamps (Philips) fitted parallel to each other in aluminium foil-coated wooden box. Test and blank test tubes were run together. At the end of the exposure time, 1 ml of Greiss reagent (one part of 0.1% naphthyl ethylenediamine dihydrochloride in distilled water mixed with one part 1% sulphanilamide in 5% concentrated phosphoric acid) was added to each tube, and the absorbance of the colour formed was measured at 543 nm. One unit of superoxide dismutase (SOD) activity is defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay conditions. The enzyme activity was calculated using the following formula: Enzyme activity = $\{(V_0/V) - 1\}$, where V_0 and V are the absorbance of the control (blank) and test respectively. The estimated values are expressed in unit/mg of protein[17].

Total protein: To 100 μl of the tissue homogenate, 0.1 ml phosphate buffer (50 mM, pH 7.4) and 5 ml Bradford reagent were added. All the assay samples were mixed well and incubated for 5 min at 37°C. The absorbance of all samples was then measured against blank. The equation $y = 159.5x + 238.9$ was used to determine the protein content (x) in unknown samples[18] (Table 1).

Histopathological evaluations of kidney

The chick embryo kidneys were fixed in fixative solution (10% v/v formalin) for 24 h. They were then dehydrated in increasing concentration of isopropyl alcohol (70%, 80%, 90%, 100% v/v IPA respectively). After impregnating the tissues in molten paraffin, they were embedded in paraffin wax to obtain blocks. They were then cut into 5 μm sections using microtome. Hematoxylene and eosin (H&E) staining was performed and after mounting the tissue sections in DPX, the slides were observed using compound microscope sequentially under 100 and 450X magnifications[19].

In vivo model

Swiss albino mice of either sex having body weight between 25 and 30 g were used for the study. Animals were procured from CPCSEA-approved Institutional Animal House of Birla Institute of Technology, Mesra, Ranchi. All animals were kept in polyacrylic cages and maintained under standard housing conditions (22–25°C and RH 60–65% with 12 : 12 light : dark cycles). Food was provided in the form of dry pellets (FDA) and water ad-libitum. The animals were allowed to acclimatize to the laboratory conditions for 7 days before commencement of the experiment. The three drugs used for induction of nephrotoxicity in mice were gentamicin, cisplatin and doxorubicin.

The mice were divided into four groups of six mice each.

Group 1: Control, injected with water for injection.
Group 2: Gentamicin-induced nephrotoxicity[20].
Gentamicin at a dose of 100 mg/kg body wt was administered i.p. for 25 days.
Group 3: Cisplatin-induced nephrotoxicity[21].

Table 1. Estimation of total protein in kidney tissues of treated embryonic day (ED) 19 chick embryos

Tissue total protein (mg/g tissue)	Group G2a (control)	Group G2b (gentamicin-treated)	Group G2c (cisplatin-treated)	Group G2d (doxorubicin-treated)
Kidney	262.51 ± 0.26	261.11 ± 0.16	260.51 ± 0.31	262.81 ± 0.069

Cisplatin at a single dose of 20 mg/kg body wt was administered i.p. and sample processing was done after 72 h.

Group 4: Doxorubicin-induced nephrotoxicity[22].

Doxorubicin at a single dose of 20 mg/kg body wt was administered i.p. and sample processing was done after 10 days.

Sample processing: Blood was collected by cardiac puncture after anaesthetizing the animals using diethyl ether. Animals were sacrificed at the end of experiment and dissected to collect the kidneys, which were then stored in 10% v/v formalin.

Biochemical tests on serum samples and histopathological evaluation of kidney tissues were similar to those in the *in ovo* model.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's test using Graph pad prism 5: Statistical software (trial version).

Results

Biochemical tests on amniotic fluid samples of 17-day-old chick embryos

Treatment with gentamicin (0.2 mg/embryo) produced a significant ($P < 0.001$) elevation in uric acid concentration of the aminiotic fluid of 17-day-old chick embryos and a non-significant increase in urea and creatinine concentration when compared to control group. On the other hand, a significant ($P < 0.001$) decrease in urea and uric acid concentration and a significant ($P < 0.001$) increase in creatinine concentration were observed in the amniotic fluids of cisplatin (0.3 mcg/embryo)-treated embryos. As for doxorubicin (70 mcg/embryo)-treated group, a significant ($P < 0.001$) decrease in uric acid concentration and a significant ($P < 0.001$) increase in urea and creatinine concentration were observed. Table 2 provides a summary of the results.

Assessment of biochemical parameters for oxidative stress in kidney tissues of 19-day-old chick embryos

GSH and catalase activities decreased significantly in the kidney tissue homogenates from all the groups, viz. 0.2

mg/embryo gentamicin-treated ($P < 0.001$), 0.3 mcg/embryo cisplatin-treated ($P < 0.001$) and 70 mcg/embryo doxorubicin-treated ($P < 0.01$) 19-day-old chick embryos (Table 3). SOD activity was found to decrease significantly ($P < 0.05$) in the 0.2 mg/embryo, gentamicin-treated and 0.3 mcg/embryo cisplatin-treated groups, but no significant decrease was observed in the 70 mcg/embryo doxorubicin-treated group. All comparisons were made with respect to the control group (Table 3).

Histopathological studies on kidney tissue of 19-day-old chick embryos treated with nephrotoxic drugs

Microscopic section of the kidneys treated with nephrotoxic drugs, namely gentamicin, cisplatin and doxorubicin revealed degenerative changes varying from irreversible to reversible type of injury in the lining epithelial cells of well-differentiated tubules. Reversible changes were characterized by cellular swelling in association with changes such as granular and vascular degeneration.

Irreversibly injured cells showed changes of coagulative necrosis characterized by picnosis, karyorrhexis, karyolysis and cytoplasmolysis along with few cells showing apoptotic changes. Moreover, less differentiated cells showed changes dominated by apoptosis as well as necrosis. Undifferentiated mass of cells also showed such types of degenerative changes.

On the contrary, in the kidneys of control embryos, well-differentiated tubules did not show any change of pathological significance. Likewise undifferentiated cellular mass showed only apoptotic changes (Figure 1).

Cellular injury in gentamicin-treated group revealed congestive, haemorrhagic and infiltrative changes (Figure 2). A few hyaline droplets were present in lining epithelial cells of renal tubules, though the number of cells showing such changes varied from tubule to tubule. Cells showing changes of irreversible injury, revealing features of coagulative necrosis have been found. They were characterized by nuclear changes, including picnosis, karyorrhexis, karyolysis and anucleosis along with cytoplasmic changes like eosinophilia and cytoplasmolysis. The number of cells showing irreversible changes was also variable from tubule to tubule as well as from area to area. In some areas, significant number of cells in adjacent tissues was undergoing coagulative necrosis revealing the features of focal and tubular necrosis. Glomeruli also appeared hypercellular with proteinaceous deposition along the

**Table 2.** Effect of nephrotoxic drugs on kidney parameters in amniotic fluid of chick embryos

Group	Urea (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)
Group G1a (control)	23.36 ± 0.7406	0.5017 ± 0.02088	14.75 ± 0.3038
Group G1b (gentamicin-treated)	24.07 ± 0.5034	0.5100 ± 0.02113	24.72 ± 0.1412***
Group G1c (cisplatin-treated)	3.017 ± 0.1046***	0.6733 ± 0.01801***	1.587 ± 0.0451***
Group G1d (doxorubicin-treated)	35.59 ± 0.4202***	0.7767 ± 0.03461***	2.882 ± 0.1274***

All values are mean ± SEM for $n = 6$. ***indicates $P < 0.001$ when compared to control group.

Table 3. Effect of nephrotoxic drugs on reduced glutathione, catalase and superoxide dismutase activities in kidney of chick embryos

Group	GSH (µmol/mg protein)	Catalase (U/mg protein)	SOD (U/mg protein)
Group G2a (control)	5.595 ± 0.2963	32.36 ± 0.8553	3.751 ± 0.2436
Group G2b (gentamicin-treated)	2.802 ± 0.1294***	27.19 ± 0.8137***	2.684 ± 0.838*
Group G2c (cisplatin-treated)	2.553 ± 0.1800***	7.746 ± 0.5751***	2.186 ± 0.1200**
Group G2d (doxorubicin-treated)	4.215 ± 0.3519**	27.66 ± 0.7010**	2.906 ± 0.2655

All values are mean ± SEM for $n = 6$. *,** and ***indicate $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively, when compared to control group.
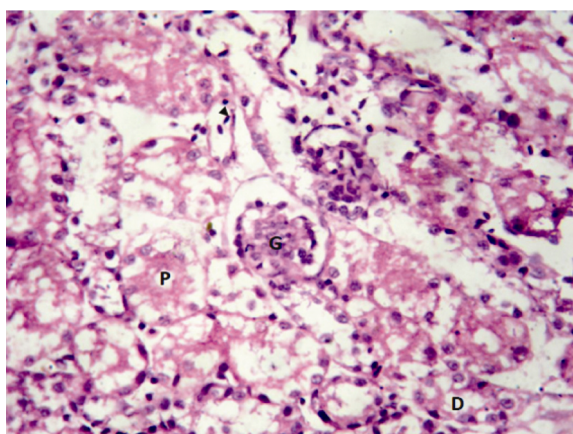
Figurer 1. Histology of renal cortex of a control embryonic day (ED) 19 chick embryo showing the glomeruli (G), proximal convoluted tubules (P) and distal convoluted tubules (D; Hematoxylin and Eosin stain (H&E) 400×).
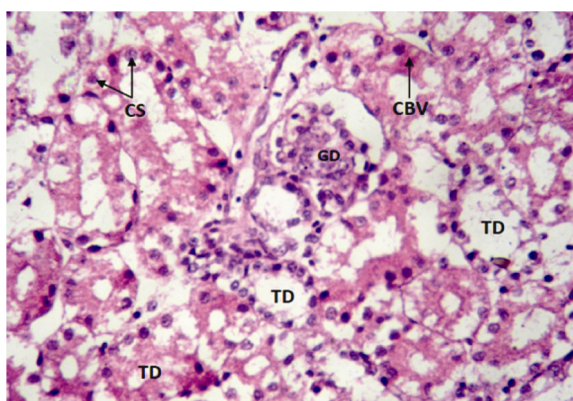
Figure 2. Histology of renal cortex of a gentamicin-treated ED 19 chick embryo showing, cellular swelling (CS), glomerular degeneration (GD), tubular degeneration (TD) and congested blood vessels (CBV; H&E 400×).

basement membrane. In the interstitium, there was infiltration of less mature mononuclear cells.

However, microscopic section of kidneys from doxorubicin-treated chick embryos revealed comparatively more severe degenerative, vascular and infiltrative changes compared to those found in the case of gentamicin treatment (Figure 3). Cells showed more severe and widespread degenerative changes compared to gentamicin-treated animals. Majority of cells showed granular vacuolar and hyaline changes. Increase in size and number of vacuoles and degree of cytcytolysis, cytoplasmolysis and desquamation proved presence of coagulative necrosis.

The kidneys of cisplatin-treated chick embryos showed even more severity in lesions compared to gentamicin and doxorubicin-treated groups in terms of granular, vacuolar and necrotic changes (Figures 4 and 5).

Estimation of kidney parameters in serum samples of mice

Table 4 summarizes the results of various biochemical tests regarding kidney parameters of mice. Concentration of urea, creatinine and uric acid was found to increase in all the groups of mice, but the level of significance varied from group to group as follows. There was a significant ($P < 0.001$) increase in serum urea levels in group 2 (100 mg/kg body wt gentamicin-treated) and group 3 (20 mg/kg body wt cisplatin-treated) mice, but no significant increase in group 4 (20 mg/kg body wt doxorubicin-treated) mice was noticed to control group. A significant increase in serum creatinine level was observed in group 3 ($P < 0.001$) and group 4 ($P < 0.01$) animals, whereas a significant increase in serum uric acid level was found in group 2 ($P < 0.05$) and group 3 ($P < 0.001$) animals.

**Table 4.** Effect of nephrotoxic drugs on kidney parameters in serum samples of mice

| Groups	Urea (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)
Group 1 (control)	37.1 ± 0.8489	0.406 ± 0.0056	1.431 ± 0.0432
Group 2 (gentamicin-treated)	62.11 ± 0.7831***	0.6410 ± 0.0161	2.906 ± 0.1081***
Group 3 (cisplatin-treated)	332.5 ± 4.058***	2.454 ± 0.2058***	3.302 ± 0.3396***
Group 4 (doxorubicin-treated)	43.58 ± 2.015	0.9840 ± 0.0594**	2.022 ± 0.0520

All values are mean ± SEM for $n = 6$. ** and ***indicate $P < 0.01$ and $P < 0.001$ respectively, when compared to control group.
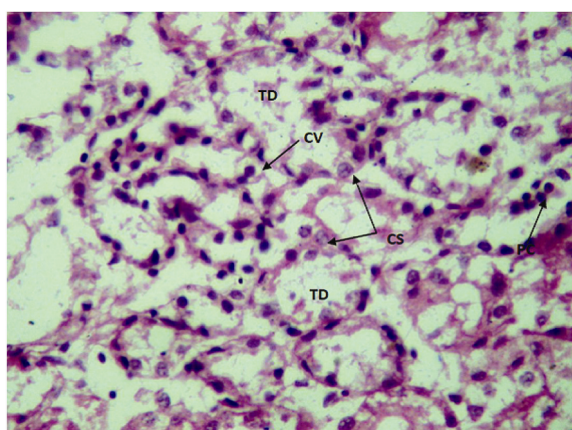

Figure 3. Histology of kidneys of doxorubicin-treated, ED 19 chick embryo showing cytoplasmic vacuolation (CV), tubular degeneration (TD), cellular swelling (CS) and pycnotic cells (PC, H&E 400×).
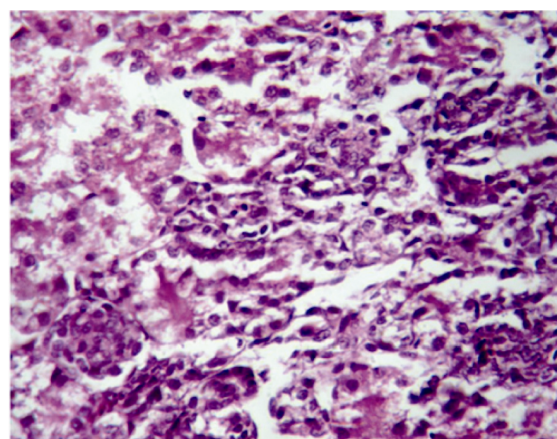
Figure 5. Area of extensive tubular degeneration in kidney of cisplatin-treated chick embryos (H&E 400×).
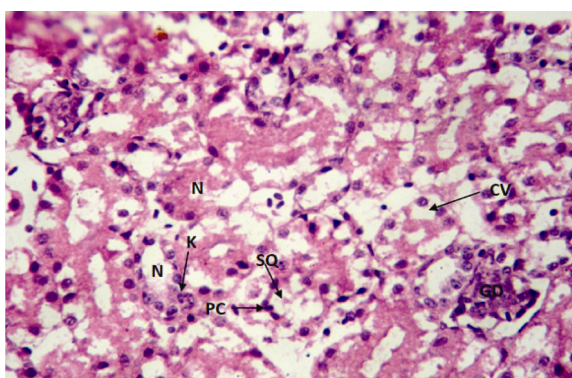
Figure 4. Histology of renal cortex of a cisplatin-treated ED 19 chick embryo showing cellular vacuolation (CV), glomerular degeneration (GD), necrotic changes in tubules (N), pycnotic cells (PC), desquamated epithelial cells (SQ) and karyorhhectic cell (K; H&E 400×).
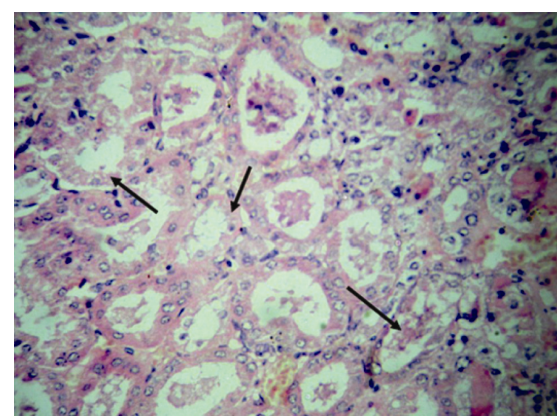
Figure 6. Histological study of kidney from gentamicin treated mice showing marked tubular degeneration (H&E, 400×).

No significant change in serum creatinine level was found in group 2 animals.

Histopathological studies on kidney tissue of mice treated with nephrotoxic drugs

Degenerative changes observed in kidneys of mice treated with nephrotoxic drugs, namely gentamicin (Figure 6), cisplatin (Figure 7) and doxorubicin (Figure 8) were found to be similar to those of the chick embryos.

Irreversibly injured cells showed changes of coagulative necrosis characterized by picnosis, karyorrhexis, karyolysis and cytoplasmolysis, whereas reversible changes were characterized by cellular swelling in association with changes such as granular and vascular degeneration when compared to histology of normal control kidneys (Figure 9).

Discussion

The stepwise concentration process of urine as it moves along the various segments of the nephron increases the concentration of toxins as well, and as the proximal tubule

presents a large area for binding and transport into the renal epithelium, it is the worst affected by nephrotoxins[23]. The levels of urea, uric acid and creatinine were estimated in the aminotic fluids of 17-day-old chick embryos. A significant decrease ($P < 0.001$) in the uric acid level of amniotic fluid was shown by embryos of cisplatin- and doxorubicin-treated groups when compared to control. Birds being uricotelic, uric acid forms the major nitrogenous waste product[8]. Cytotoxic drugs are reported to decrease the excretion of uric acid, thus causing hyperuricaemia[24]. This condition can be expected if the glomerular filtration rate (gfr) has been reduced more than 70–80%. Approximately 90% of blood uric acid is eliminated by secretion into lumen of the tubules. Gentamicin-treated embryos showed a significant increase ($P < 0.001$) in urea concentration consistent with the fact that direct tubular toxicity is associated with aminoglycosides[25].

Birds produce little creatinine from its precursor, creatine. Creatinine is eliminated by tubular secretion, but clearance is variable and not of much clinical significance[26]. The present study shows a rise in creatinine level in the amniotic fluid in all the three groups with significant ($P < 0.001$) change in the cisplatin- and doxorubicin-treated groups. Unlike mammals, urea in birds is produced only in small amounts (by renal mitochondrial breakdown of arginine) and does not serve as the end-product of protein metabolism. Urea was found to increase in gentamicin and doxorubicin-treated (significant with $P < 0.001$) groups[23], but cisplatin-treatment group showed a drastic fall in urea concentration in amniotic fluid possibly due to low gfr as stated above and higher retention of urea in the blood[27]. Hence it may be concluded that significant nephrotoxicity has been induced in each group of treated embryos.

The homogenates from the kidneys were analysed for possible changes in oxidative stress markers, namely GSH, catalase and SOD, in which significant ($P < 0.05$) decrease in the activities of these markers was found in all three groups (except that decrease in SOD was non-significant in doxorubicin-treated group) compared to control group[28]. So a possible mechanism for the induction of nephrotoxicity was found to be oxidative stress for all the three drugs.

Histopathological studies on the kidney tissues of 19-day-old chick embryos showed marked tubular and glomerular degeneration. A graded degeneration in the tubular lining epithelial cells of the nephrons and glomerulus was observed, with cisplatin producing most severe apoptotic and necrotic changes in the nephrons followed by doxorubicin and gentamicin when compared to normal control kidneys. The *in vivo* nephrotoxicity models using Swiss albino mice showed increase in serum biochemical parameters, namely urea, creatinine and uric acid as reported in the literature[20,29]. The histopathological observations showed an increase in the severity of nephron degeneration with a minimum in gentamicin-treated mice, followed by doxorubicin-treated group and a maximum in cisplatin-treated animals. The study showed
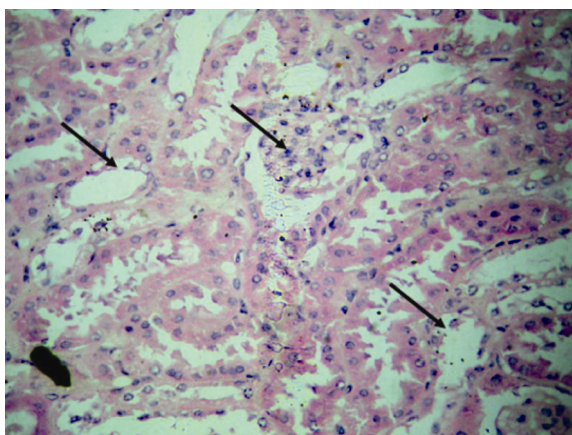
Figure 7. Histological study of kidney from cisplatin-treated mice showing marked tubular and glomerular degeneration (H&E, 400×).
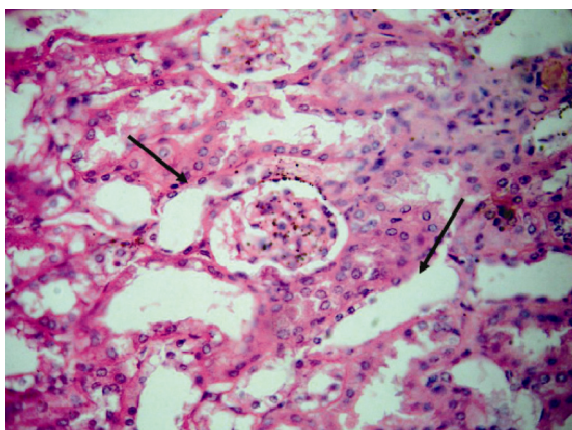
Figure 8. Histological study of kidney from doxorubicin-treated mice showing marked tubular and glomerular degeneration (H&E, 400×).
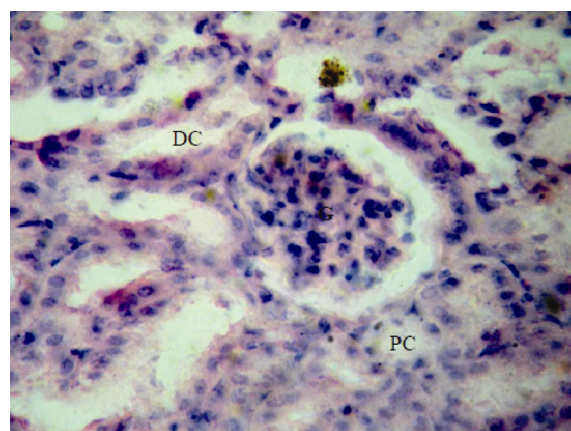
Figure 9. Histology of kidney of control group mice showing glomerulus (G), proximal convoluted tubule (PC) lined with cuboidal epithelium and distal convoluted tubule (DC; H&E, 400×).

that probably cisplatin was the best inducer of nephrotoxicity.

The outcome of this study supports the use of chick embryo as a model for evaluating nephrotoxic potential of substances for all the three proposed models, viz. gentamicin-induced, cisplatin-induced and doxorubicin-induced, with the best results obtained using the cisplatin-treated group in terms of changes in biochemical parameters as well as histopathological studies when compared to normal control group.

1. Choudhury, D. and Ahmed, Z., Drug-associated renal dysfunction and injury. *Nature Clin. Pract. Nephrol.*, 2006, **2**(2), 80–81.
2. Singh, N. P., Ganguli, A. and Prakash, A., Drug induced kidney diseases. *J. Assoc. Phys.*, 2003, **51**, 971.
3. Sandhu, J. S., Sehgal, A., Gupta, O. and Singh, A., Aminoglycoside nephrotoxicity revisited. *JIACM*, 2007, **8**(4), 330–332.
4. Miller, R. P., Tadagavadi, R. K., Ramesh, G. and Reeves, W. B., Mechanisms of cisplatin nephrotoxicity. *Toxins*, 2010, **2**, 2492–2497.
5. El-Moselhy, M. A. and El-Sheikh, A. A. K., Protective mechanisms of atorvastatin against doxorubicin-induced hepato-renal toxicity. *Biomed. Pharmacother.*, 2014, **68**, 101–110.
6. Russell, W. M. S. and Burch, R. L., *The Principles of Humane Experimental Technique*, Methuen & Co, London, 1959, pp. 69–154.
7. Tuan, R. S. and Lo, C. W., *Developmental Biology Protocols 1. Methods in Developmental Biology*, Humana Press, Totowa, New Jersey, 1999, p. 25.
8. Braun, E. J., Osmoregulatory system in birds. In *Sturkie's Avian Physiology*. Academic Press, Wisconsin, United States, 2000, 6th edn, pp. 286–288, 755–756.
9. Stern, C. D., The chick embryo – past, present and future as a model system in developmental biology. *Mech. Dev.*, 2004, **121**, 1011–1013.
10. New, D. A. T., A critical period for the turning of hens' eggs. *J. Embryol. Exp. Morphol.*, 1957, **5**(3), 293–299.
11. Sinroha, R., Srivastava, S. K. and Chhikara, P., Effect of gentamicin on kidney in developing chicks. *Eur. J. Anat.*, 2012, **16**(2), 119–126.
12. Fawcett, J. K. and Scott, J. E., A rapid and precise method for the determination of urea. *J. Clin. Pathol.*, 1960, **13**, 156–159.
13. Bowers, L. D. and Wong, E. T., Kinetic serum creatinine assays. II. A critical evaluation and review. *Clinol. Chem.*, 1980, **26**(5), 555–561.
14. Dubois, H., Delcoux, B., Ehrhardt, V. and Greiling, H., An enzymatic assay for uric acid in serum and urine compared with HPLC. *J. Clin. Chem. Clin. Biochem.*, 1989, **27**, 151–156.
15. Moron, M. S. and Depierre, J. W., Levels of glutathione, glutathione reductase and glutathione S transferase activities in rat lung and liver. *Biochim. Biophys. Acta*, 1979, **582**, 67–78.
16. Luck, H., Catalase. In *Methods of Enzymatic Analysis* (ed. Bergmeyer, H. U.), Academic Press, New York, 1971, pp. 885–893.
17. Das, K., Samanta, L. and Chainy, G. B. N., A modified spectrophotometric assay of SOD using nitrate formation by superoxide radicals. *Indian J. Biochem. Biophys.*, 2000, **37**, 201–204.
18. Bradford, M. M., A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 1976, **72**, 248–254.
19. Qadir, M. I., Tahir, M., Lone, K. P., Munir, B. and Sami, W., Protective role of ginseng against gentamicin induced changes in kidney of albino mice. *J. Ayub Med. Coll. Abbottabad*, 2011, **23**(4), 53–57.
20. Eslami, S. H., Ebrahimzadeh, M. A., Moghaddam, A. H., Nabavi, S. F., Jafari, N. and Nabavi, S. M., Renoprotective effect of *Eryngium caucasicum* in gentamicin-induced nephrotoxic mice. *Arch. Biol. Sci.*, 2011, **63**(1), 157–160.
21. Pan, H. *et al.*, Cannabidiol attenuates cisplatin induced nephrotoxicity by decreasing oxidative/nitrosative stress, inflammation and cell death. *J. Pharm. Exp. Ther.*, 2009, **328**(3), 708–714.
22. Hassan, M. H., Ghobara, M. and Abd-Allah, G. M., Modulator effects of meloxicam against doxorubicin induced nephrotoxicity in mice. *J. Biochem. Mol. Toxicol.*, 2014, **28**(8), 337–347.
23. Shingadia, H. U. and Dalvie, V., Embryo protective effect of leaf extract of *Vitex negundo* Linn. in adriamycin induced toxicity. *J. Med. Sci. Clin. Res.*, 2014, **2**(4), 730–740.
24. Nanji, A. A., Stewart, D. J. and Michael, N. Z., Hyperuricemia and hyperalbuminamia predispose to cisplatin induced nephrotoxicity. *Cancer Chemother. Pharmacol.*, 1986, **17**(13), 274–176.
25. Choudhury, D. and Ahmed, Z., Drug induced nephrotoxicity. *Med. Clin. North Am.*, 1997, **81**(3), 705–718.
26. Fudge, A. M., Avian clinical pathology – hematology and chemistry. In *Avian Medicine and Surgery* (ed. Altman, R. B. *et al.*), WB Saunders, Philadelphia, 1997, pp. 142–157.
27. Dogukan, A. *et al.*, A tomato lycopene complex protects the kidney from cisplatin-induced injury via affecting oxidative stress as well as Bax, Bcl-2, and HSPs expression. *Nutr. Cancer*, 2011, **63**(3), 427–434.
28. Raheem, I. T. A., Ghany, A. A. A. and Mohamed, G. A., Protective effect of quercetin against gentamicin induced nephrotoxicity in rats. *Biol. Pharm. Bull.*, 2009, **32**(1), 2009, 61–67.
29. Mansour, M. A., Kashef, H. A. and Al Shabanah, O. A., Effect of captopril on doxorubicin induced nephrotoxicity in normal rats. *Pharmacol. Res.*, 1999, **39**(3), 233–238.