Ginger extract as a potential therapeutic intervention for gabapentin-induced renal toxicity in chick embryos

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ABSTRACT

Gabapentin (GBP) is an antiepileptic drug designed to treat partial seizures and other neuropathic diseases; however, it has raised many concerns about its toxic effects and interactions. The current study investigated the potential protective effect of ginger extract against kidney damage in chick embryos exposed to GBP during organogenesis. White Leghorn eggs were divided into five groups, namely 1) Control; with no injection, 2) Sham, with distilled water injection, 3) Ginger, with 4.5mg/egg ginger injection, 4) GBP, with 5.4 mg/egg GBP injection and 5) Ginger+GBP, with combined injection. Chick embryos injected with GBP exhibited severe kidney abnormalities, including tubular damage, glomerular atrophy, and increased Caspase-3 and decreased Bcl-2 expression as indicators of apoptosis, as well as swollen and ruptured mitochondria, degenerated nuclei and other ultrastructural damage. Ginger extract, when administered alongside GBP, significantly ameliorated these defects at the histological, immunohistochemical, and ultrastructural levels. These findings suggest that caution should be implied when using GBP during pregnancy and highlight ginger as a promising therapeutic option to mitigate its detrimental effects on fetal kidney development.

Keywords: Chick Embryo; Gabapentin; Kidney; Antiepileptic drugs; Embryo

1. Introduction

Management of epileptic seizures often involves continuous exposure to medication throughout life, even during pregnancy. Moreover, the physiological changes during pregnancy may alter the pharmacokinetics of antiepileptic drugs (AEDs) (Avachat et al., 2022). Approximately, 12.5 million women of childbearing age around the globe are affected by epilepsy (Fiest et al., 2017). During pregnancy, the developing fetus of epileptic mothers is exposed to AEDs in utero via the placenta. Although taking AEDs is necessary to the mother, AEDs may induce teratogenic effects, including major congenital malformations which affects the functions of the developing embryo, including congenital heart disease, cleft lip/palate, urogenital defects, and neural tube defects (Pennell, 2016).

Several AEDs have been obligatory used to cure epilepsy, though their deleterious effect on embryogenesis and growth (Singh and Mishra, 2005). Overall, no one drug can be specifically recommended; but monotherapy has been reported to have a satisfactory outcome unlike polytherapy which should be avoided as it is associated with greater incidence of congenital malformations. Control of maternal epilepsy, including drug selection and dose adjustment, must be balanced with the fetal and neonatal risks associated with anticonvulsant drugs as well as the clinical status of the patient (Lowe, 2001). AEDs should be chosen to be effective, safe and free from fetal toxicity as possible as it can (Nasar et al., 2014).
Gabapentin (GBP) is a 3rd generation AED and is most commonly prescribed to treat neuropathic pain (Alsanie et al., 2022). The mechanism of action of GBP is not clear; however, it has been reported that it may decrease the release of pain-related peptides and reduce opioid-induced hyperalgesia (Compton et al., 2010). Additionally, GBP can also bind to protein subunits of the voltage-dependent Ca++ channel complex (Gee et al., 1996). Gabapentin was first approved as an AED in 1993 (Mack, 2003). The ability of gabapentin to cross the placental barrier has raised concerns regarding its safety on child-bearing women and their babies (Patorno et al., 2020).

Clinically, GBP has been reported to be effective pain status, including reflex sympathetic dystrophy (Mellick et al., 1995), trigeminal neuralgia (Sist et al., 1997), and postherpetic neuralgia (Gilron et al., 2005) as well as acute pain in herpes zoster infection (Berry and Petersen, 2005). GBP is also effective in the postoperative pain management in different surgeries such as breast (Fassoulaki et al., 2002), hysterectomy (Rorarius et al., 2004), cholecystectomy (Pandey et al., 2004), spinal (Turun et al., 2004), and knee surgeries (Menigaux et al., 2005).

GBP is a structural analog of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Smith et al., 2022). It is very water soluble and its half-life is 5 to 7 hours in humans (Afshar and Golalipoor, 2008). GBP is categorized as ‘C’ class considering its possible teratogenic risk by FDA (Singh et al., 2014). GBP has a good oral absorption, doesn’t bind to plasma proteins, and is excreted mainly unchanged through the kidneys (Welson et al., 2020). Due to its lack of appreciable metabolism, no drug interaction in the body, rapid glomerular filtration rate, and good tolerance of this drug, it is extensively used (Tatum et al., 2000).

Ginger is the rhizome of the plant Zingiber officinale Roscoe, member of the family of Zingiberaceae. It is widely used worldwide as powder or as the whole fresh root (Zahedi et al., 2012). Ginger contains several compounds such as gingerol, shogaol, volatile oils and vitamins C and A (Johari et al., 2013). Ginger possesses antioxidant activity (Nanjundaiah et al., 2011), neuroprotective effect (Sharma et al., 2024), and anxiolytic effect (Hasenohrl et al., 1996). Furthermore, ginger has anti-cancer, anti-inflammatory properties as well as anti-nausea/vomiting properties (Portnoi et al., 2003; Young et al., 2005). Bryer (2005) advised using ginger as an effective treatment for pregnancy sickness. The effect of ginger on renal damage was studied by some investigators. Sabraz et al. (2013) reported that the aqueous extract of ginger showed an ameliorative effect against cadmium bromide induced nephrotoxicity.

Crushed ginger and celery seeds have been used as nutrient supplements in broiler breeder diets. It has been mentioned that a lot of active materials exists in the crushed ginger and celery seeds which improved the hatching rate (Saeid, 2012), since the ginger contains components like flavonoids, limoin and vitamins E and C (Shalaby and Zorba, 2010), as well as a great amount of feed elements, minerals and vitamins that are considered important in the growth of the embryos (Abbas et al., 2014). The aim of the current study is to investigate the possible renal toxic side effects of in ovo injection of GBP during the organogenesis phase of the embryonic development of chick embryo. In addition, we aim to assess the possible alleviative effects of ginger in the GBP-induced renal toxicity.

2. Material and methods

2.1. Chemicals

2.1.1. GBP administration

GBP, with the trade name Gaptin, (Delta Pharma Company, Egypt) was employed for the study. GBP in a dose of 5.4 mg/egg equivalent to the therapeutic human dose 1800 mg/kg. The single dose was calculated according to Guvec et al. (2013). Gaptin capsules of 300 mg/kg concentration were used. The capsules were emptied, and the powder was weighed and dissolved in 1 ml distilled water and injected in ovo as a single dose during the 6th day of incubation.

2.1.2. Preparation of ginger extract

Fresh rhizomes of ginger (Zingiber officinale) were purchased from a local market at Shebeen El-Koom, Menoufia, Egypt. They were shade dried at room temperature and then crushed to powder. 125 g of the powder were macerated in 1000 ml of distilled water for 12 h at room temperature and filtered through a 5 µm filter paper to obtain the final aqueous extract. Accordingly, the concentration of the obtained extract was 24 mg/ml and equal to 120 mg/kg (Kamtchouing et al., 2002). Ginger extract was injected in ovo as a single dose of 4.5 mg/egg in the 6th day of incubation (Hajati et al., 2014).

2.2. Animals and grouping

Principles of animal care and use were carefully followed during conducting the present study according to the guide for the care and use of laboratory animals approved by Faculty of Science, Menoufia University, Egypt (Approval No. MNSE2180), and according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Normal fertilized hen eggs (Gallus domesticus) were...
obtained from a local breeder at Shebeen El-Koom, Menoufia governorate. Before incubation at 37°C in an artificial incubator, eggs were cleaned with distilled water followed by 70% ethanol weighed (50 ± 5 g) and then labeled on the outer shell. To ensure the relevant humidity (65%), an open 1-liter container filled with distilled water was placed at the bottom of the incubator. The eggs were put horizontally and turned over, at least, three times a day. On the 6th day of incubation, the eggs were divided into five groups (15 eggs each).

1- Group A: the eggs were not subjected to any injection (Control group).
2- Group B: the eggs were injected in ovo with 0.2 ml of distilled water (Sham group).
3- Group C: the eggs were injected in ovo with 0.2 ml of GBP (Guvenc et al., 2013).
4- Group D: the eggs were administered 0.2 ml of ginger extract (Hajati et al., 2014).
5- Group E: the eggs were given 0.2 ml of 1:1 mixture of GBP (5.4 mg/egg) and ginger extract (4.5 mg/egg).

On the 6th day of incubation, a hole was made on the blunt end of the egg with a sharp and thin needle under septic conditions. Using a sterile syringe, 0.2 ml of fluid was injected into the air sac. The holes were carefully sealed with molten paraffin wax after the single dose injection in all experimental groups. The egg was returned to the incubator again for further development. All eggs were opened on the 20th day of incubation. The embryos were carefully freed from the eggshell. The embryos were dissected, and the kidney was removed and fixed for subsequent analysis.

2.3. Histological examination

For light microscopical examination, the kidney of chick embryos of the different groups was fixed by immersion in 10% neutral formalin for 24 hours at room temperature. Fixation was followed by washing the specimens under running tap water for 12 hours. All specimens were transferred to 70% ethanol and then dehydrated in an ascending series of ethanol, cleared in xylol, infiltrated and embedded in paraffin wax. The specimens were then oriented and blocked out in fresh paraffin wax. Five µm thick transverse sections were produced using a rotary microtome (Leica, Model Rm 2125, Germany). Sections were mounted on positive charged slides. Histological staining was performed with Ehrlich’s hematoxylin and counterstained with aqueous eosin (Humason, 1979). Histological sections were subjected to microscopical examination and photographed using Olympus microscope (BX41).

2.4. Immuno-histochemical investigation

The anti-apoptotic mediator Bcl-2 (Rabbit Polyclonal Bcl-2 antibody, 1:1000, ab194583, Abcam, Cambridge, UK) and pro-apoptotic antigen Caspase-3 (Anti-Caspase-3 antibody [EPR18297], 1:1000, ab184787, Abcam, Cambridge, UK) were checked by immuno-histochemical technique in the kidney of chick embryos of different groups. Expression was detected using Avidin biotin complex (ABC) immuno-histochemical method (Sternberger, 2006). The criterion for a positive reaction confirming the presence of Bcl-2 and Caspase-3 proteins was a dark, brownish, intracytoplasmic precipitate. For the negative control, the primary antibody was omitted to guard against any false positive results that might develop from a non-specific reaction. All stained slides were viewed using Olympus microscope and images were captured by a digital camera (Canon Power Shot A620). Brightness and contrast of the images were adjusted using Adobe Photoshop software (Adobe Systems, San Jose, CA).

2.5. Image analysis

Digital images were analyzed by a semi-quantitative scoring system (Figi-Image J software, Java based application for analyzing images). The brown stained immuno-histochemical expressions of Bcl-2 and Caspase 3 were analyzed; the percentage-colored stained area (area fraction) per field area was determined by measuring six randomly photographed high-power fields (X400 magnifications) (Schindelin et al., 2012).

2.6. Ultrastructural investigation

For ultrastructural investigation, which has been done using the transmission electron microscope, kidney of chick embryo of different groups was separated and immediately fixed rapidly for 4 h at room temperature in glutaraldehyde, post fixed in buffered solution of 1% osmium tetro-oxide, washed in phosphate buffer, dehydrated in ascending grades of ethanol (30% up to absolute) and cleared in propylene oxide. The samples were then infiltrated and embedded in the epoxy resins using beam capsules and blocks were prepared. Ultra-thin (50 nm) sections were cut, mounted on formvar-coated grids and stained with uranyl acetate and lead citrate. Examination of grids was done by using JEOL electron microscope, Electron Microscope Unit, Mansoura University (Karnovsky, 1965).

2.7. Data evaluation and statistical analysis

All data sets were expressed as mean ± standard error of the mean (SEM). The data were analyzed
statistically for normal distribution (student’s T test) and homogeneity of variances (Levene test) using statistical program of social sciences (IBM SPSS) statistics software for Windows, Version 22 (IBM Corp., Armonk, NY, USA). Differences were considered insignificant whenever P>0.05. The significances of the obtained data were classified into three categories, i.e. P<0.0001, P<0.001 and P<0.05 according to the obtained P values.

3. Results
3.1. GBP altered the histological architecture of the kidney

Histological analysis of kidneys of 20-day-old chick embryos of control group showed that kidney was a flattened organ embedded in the ventral surface of synsacrum bone and incompletely divided into three lobes; cranial, middle and caudal lobes and enclosed in a capsule composed of fine collagen and reticular fibers. Each lobe was divided into lobules composed of 2 zones, the cortex and medulla or medullary cone. The cortex made up the majority of the kidney, while the medulla formed only a small portion of the organ. The cortex was composed of two kinds of nephrons or renal corpuscles, numerous reptilian or cortical type, small sized with no loops of Henle and located mainly in the cortex, and a less frequent mammalian or medullary type, large or intermediate sized with long or intermediate length loops and located mainly near the medulla. The renal corpuscle consisted of an outer Bowman’s capsule separated by Bowman’s space from a centrally located glomerulus. The glomeruli consisted of tightly packed central core of mesangial cells, surrounded by capillary loops. The proximal convoluted tubules were lined with darkly stained simple cuboidal epithelium with well demarcated brush border and narrow lumen. The distal convoluted tubules were lined with simple cuboidal epithelium with a more clearly defined wide lumen. Collecting tubules occurred in the peripheral part of the cortex and were lined with pale cuboidal cells. Medulla of the kidney was composed of thin and thick segments of Henle’s loop and separated by collecting ducts. Thick segments were restricted to the periphery of the medullary cones and surrounded by a ring of collecting ducts. Both segments of Henle’s loop consisted of simple cuboidal epithelium, while the medullary collecting ducts consisted of a columnar epithelium and continued into a distal papillary duct (Fig. 1 A&B). Chick embryos of both sham and ginger groups showed similar histological structure compared with control group (Fig. 1 C&D).

Close examination of kidneys of 20-day-old chick embryos injected in ovo with GBP on the 6th day of incubation showed prominent histopathological features. Tubular cell degeneration, necrosis and edema of interstitial tissue were evident. Proximal and distal convoluted tubules were dilated with vacuolar degeneration of the lining epithelia with pyknotic or karyolytic nuclei (Fig. 1 E-H). The proximal convoluted lining cells appeared swollen with close lumen, while the distal convoluted tubules appeared distended with dilated lumen (Fig. 1 E-H). Some of the renal corpuscles showed widened Bowman’s space and atrophied glomeruli, also there was a complete loss of glomeruli in the Bowman’s capsule (edematous corpuscle) at some places (Fig. 1 E, F & H). Necrotic areas were seen in some renal tubular epithelial cells (Fig. 1 E). The necrotic cells appeared shrunken and degenerated with dark dyeing pyknotic nuclei and loss of architecture (tubular necrosis). Congestion and hemorrhage of the parenchyma between the tubules was a consistent feature (Fig. 1 E&F).

Chick embryos of the combined GBP and ginger group showed better histological structure of the kidney compared with the GBP group, however in some cases there were atrophied and degenerated glomeruli (Fig. 1 I, J & L). Few epithelial cells lining the renal tubules showed vacuolar degeneration (Fig. 1 I-L). Few hemorrhagic foci were seen between the tubules (Fig. 1 K).

3.2. GBP induced apoptosis in the developing chick embryo kidney

Investigations of immunohistochemically stained kidney sections revealed that Bcl-2 was detected strongly in the renal tubules of the control chick embryos, with weak or almost no expression in the Malpighian corpuscles cells (Fig. 2 A). The concentration and distribution of Bcl-2 protein in the cytoplasm of renal tubule cells was profoundly increased in chick embryos from sham and ginger groups (Fig. 2 B&C). Injection of GBP resulted in a profound decrease in the expression and immunoreaction to Bcl-2 antibodies where almost no or few epithelial cells of renal tubules exhibited mild expression of Bcl-2 protein (Fig. 2 D&E). More pronounced immunoreactivity of Bcl-2 expression was evident in the renal tubules and few glomeruli cells in chick embryos injected with both ginger and GBP (Fig. 2 F).
Figure 1. Photomicrographs of transverse sections in the kidney of 20-day-old chick embryos from control (A&B), sham (C), ginger (D), GBP (E-H) and GBP+ginger (I-L) groups showing that the kidney is composed of large cortex (Ctx) and small medulla (Md). Three types of nephrons are recognized, reptilian (red arrow), intermediate mammalian (green arrow) and mammalian glomerulus (black arrow). Proximal convoluted (PT) tubules with narrow lumen and distal convoluted tubules (DT) with (wide lumen) as well as collecting tubules (CT) can be seen in the cortex. Tubular necrosis (*) with loss of architecture and pyknotic nuclei of the renal tubules. Vacuolar degeneration with karyolytic nuclei of tubules (arrowhead). Disrupted glomeruli with widened urinary space of the glomeruli (arrow) and hemorrhagic foci between the tubules (wavy arrow). Empty or atrophied renal corpuscles (arrow), hemorrhage (wavy arrow) and vacuolated and degenerated convoluted tubules with karyolysis (arrowhead). There are few empty and degenerated renal corpuscles (thick arrow) (I&L) and hemorrhagic areas (h) (J). Scale bar A&I= 0.06 mm; B-H, K-L= 0.03 mm.

Diminished activity of Caspase-3 expression with decreased number of immuno-stained cells and lower intensity of immunoreactivity was observed in the renal cortex of control group (Fig. 2 G). A loss of Caspase-3 immunoreactivity was also observed within the renal cortex of sham and ginger groups (Fig. 2 H&I). Conversely, there was increased expression of Caspase-3 in the renal tubule epithelium, although the Malpighian corpuscles showed faint and weak expression of the antigen (Fig. 2 J&K). Moderate levels of expression were found in the combined ginger and GBP group (Fig. 2 L).

The mean area percentage of Bcl-2 and Caspase-3 positive cells in the kidney of chick embryos was shown in Table (1) and Figure (3). It was evident that there was no significant difference in the expression percentage among the control, sham and ginger groups (35.6±1.41 %; 34.47±0.96 %; 30.68 ± 0.86%, respectively). Administration of GBP resulted in severe reduction in the expression percentage and showed a high significant decrease when compared with control group (4.33±0.39 %; 35.6±1.41 %, respectively). Meanwhile, it was found that co-administration of ginger after GBP led to significant increase in the mean area expression percentage of Bcl-2 positive renal tubule cells when compared with GBP group only, however it showed low significance decrease when compared with control group (18.55±0.82 %; 4.33±0.39 %; 35.6±1.41 %, for the three groups, respectively). Caspase-3 mean area percentage showed conversed results. The least group that expressed the antigen was the control group, followed by sham, then ginger group (7.59±0.27 %; 7.87±0.18 %; 8.34±0.34 %, for the three groups respectively). The level of expression showed high significant increase in the GBP group compared with control group and about 33.79±0.58 % of the renal cells expressed the antigen. Co-administration of ginger with GBP led to significant reduction in mean area percentage expression of Caspase-3 compared with GBP and consequently only 14.81±0.71 % of the cells were immuno-reactive, though these levels showed low significant increase in comparison with the control group.
Figure 2. Photomicrographs showing immuno-histochemical localization of Bcl-2 (A-F) and Caspase-3 (G-L) antigens in transverse sections of kidney of 20-day-old chick embryos of different groups. Massive Bcl-2 expression, especially in the cytoplasm of the renal tubule cells of control, sham and ginger groups, respectively (A, B & C). Weak or absent expression of Bcl-2 protein in the kidney of chick embryos from the GBP group. Few renal tubules expressed the Bcl-2 protein (D&E). Increase in the intensity and distribution of Bcl-2 expression in the cytoplasm of renal tubule cells in the ginger and GBP group compared with the GBP group with overall moderate immunoreaction (F). Diminished Caspase-3 expression in the cytoplasm of the renal tubule cells of control, sham and ginger groups, respectively (G, H & I). Massive expression of Caspase-3 protein in the kidney of chick embryos from the GBP group, especially in the renal tubular epithelium. Few cells within the glomeruli expressed the Caspase-3 protein (J&K). Decreased expression of Caspase-3 in the cytoplasm of renal tubule cells in the ginger + GBP group compared with the GBP group with overall moderate immunoreaction (L). Scale bar = 0.03 mm.

Table (1): The mean area percentage, SEM of Bcl-2 and Caspase-3 expression in the kidney of chick embryos of different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Sham</th>
<th>Ginger</th>
<th>GBP</th>
<th>GBP + Ginger</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>35.6 ± 1.41</td>
<td>34.47 ± 0.96</td>
<td>30.68 ± 0.86</td>
<td>4.33 ± 0.39***</td>
<td>18.55± 0.82* b</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>7.59 ± 0.27</td>
<td>7.87 ± 0.18</td>
<td>8.34 ± 0.34</td>
<td>33.79 ± 0.58***</td>
<td>14.81 ± 0.71* b</td>
</tr>
</tbody>
</table>

Data are represented as mean area% ± SEM.
Asterisks (* - ***) refer to the P value compared with the control group.
b=significant (P<0.001) compared with GBP group. * P<0.05,   *** P< 0.0001
3.2.3. GBP altered the ultrastructure of the kidney of chick embryos

Ultrastructural observations of the renal tissues of the control chick embryos disclosed a healthy picture of the Bowman’s capsule and tubular system. The podocytes were irregular shaped cells resting on a thin basal lamina. These cells were found to have given off thin and short cytoplasmic processes (pedicels) perpendicularly attached to the basement membrane of the capillary and were especially numerous in the places in which the inner layer of the corpuscle contacted the capillary branch. At origin, they had a conic aspect, which gradually became thinner. Small spaces separated each pedicel from its neighbor (filtration slit) and in most cases these slits were closed by a very thin membrane. The lumen of the capillary loop occasionally contained erythrocytes. The glomerular basement membrane was uniform in thickness and was consisted of three layers (trilaminar in structure) with less electron dense central zone surrounded on either side by more electron dense layers. The podocytes exhibited indented nucleus and electron dense cytoplasm. Its cytoplasm contained elongated and rounded mitochondria and rER (Fig. 4 A).

The cells of the proximal tubules had conspicuous apical microvilli projecting within the tubular lumen forming a brush border. Electron dense cytoplasm was observed containing large, rounded nucleus with a distinct peripheral nucleolus and surrounded by nuclear membrane and was situated in the lower third of the cell. Numerous rounded and elongated mitochondria were seen dispersed in the supranuclear and basal cytoplasm, while the rER was less developed than in the hepatocytes. Few apical endocytic vacuoles were present in the apical cytoplasm. The basal surfaces lacked the deep enfolding of the membrane seen in tubules from mammalian kidneys (Fig. 4 B). The cells of the distal convoluted tubules exhibited basal spherical euchromatic nucleus, extensive round or elongated mitochondria, parallel stacks of rER and several lysosomes. They had regular basement membrane, sharp luminal outline, and wide lumen with scarce microvilli (Fig. 4 C). The ultrastructure of kidneys of chick embryos from the sham and ginger groups appeared normal with similar glomerular and tubular ultrastructure to that of the control group (Fig. 4 D-I).

The ultrastructure of the kidney of chick embryos injected with GBP showed dramatic changes of the components of the cortex. The glomerular capillaries were dilated, congested, and engorged with blood. The irregularities of the glomerular basal lamina and deletion of the foot processes were observed. The Glomerular basement membrane showed local thickening in some areas and local corrugations or irregularities in other parts and lost its three-layered structure. The nucleus of the podocytes appeared irregular with dark nuclear condensation adjacent to the nuclear envelop. The podocyte processes were almost obliterated and seen broadened increasing the interstitial space between the capillary loops. On the other hand, from outside the glomerular capillary wall, the podocytes were severely injured, losing most of their cytoplasmic organelles. Multiple electron lucent vacuoles of different sizes and shape were seen within and in-between the podocytes. The basal cytoplasm of the podocytes and their primary processes fused with their foot processes forming a continuous band of cytoplasm and became adherent to the thick glomerular basement membrane. This appearance denoted the effacement or spreading out of the foot processes, where the foot processes were no longer seen (Fig. 5 A).
The epithelial cells of the proximal tubules showed drastic alterations. The lumen of the tubules was greatly narrowed (Fig. 5 B&C). The microvilli of the apical brush border were severely degenerated, either shortened, dilated (Fig. 5 C) or sloughed and detached in the lumen (Fig. 5 D). Some cells showed fragmented microvilli (Fig. 5 E&G). Most of the nuclei appeared shrunken, pyknotic with clumping of the chromatin (Fig. 5 B, D, F-H) and the nuclear envelope was irregular in some nuclei (Fig. 5 B, D-F) and showed widening of the perinuclear space (Fig. 5 I).

![Figure 4](image-url)

Figure 4. TEM of sections of in the kidney of 20-day-old chick embryos from control (A-C), sham (D-F) and ginger (G-I) groups. The glomerular basal membrane (GBL) shows its dark-light-dark laminar structure. Podocytes (Po) containing normal mitochondria (M) and rER and gave rise to thin and short cytoplasmic processes perpendicularly attached to the basement membrane of the capillary (tailed arrow). The capillary lumen contained erythrocytes (Er) (A, D & G). Proximal convoluted tubule cells with normal apical brush border (Bb), rounded euchromatic nuclei (Nu) with regular nuclear envelope (Ne) and prominent nucleolus (n), numerous mitochondria (M) with clear cristae, well defined Golgi (G) and apical vacuoles (Av) and lysosomes (Ly) and rER (B, E & H). Distal convoluted tubule cells with rounded euchromatic nuclei (Nu) surrounded by numerous rounded and elongated mitochondria (M), parallel stacks of rER and plenty of lysosomes (Ly) and a wide lumen (L) devoid of cell debris (D, F & I). Scale bar A, D, F-I = 2 µm; B & E = 500 nm; C = 200 nm.

The cytoplasm showed wide spread of apical vacuoles (Figs. 5 D&F). In some cases, the cytoplasm showed scanty organelles and appeared electron lucent and revealed lysis of most of its organelles (Fig. 5 G-I). The mitochondria were swollen with ruptured outer and inner membrane and scarce or almost absent cristae with loss of integrity (Fig. 5 I). The sER was dilated and enlarged with intracytoplasmic vacuoles and dilated rER was evident (Fig. 5 G-I). At the same
time, lysosomal bodies were numerous and of normal structure (Fig. 5 H). The basal laminae showed irregularities in some cells (Fig. 5 I).

The lining epithelial cells of the distal convoluted tubules showed pyknotic shrunken nuclei with chromatin condensation. The lumen of the tubules contained destroyed or damaged epithelium and a small amount of homogenous substance. In some tubules “empty” epithelial cells, with “washed out” cytoplasm and nuclei with circumferential chromatin condensation were observed (Fig. 6 A). Some cells had greatly narrowed or even diminished lumen with loss of luminal outlines (Fig. 6 B&C). Hemorrhage was evident as some erythrocytes were seen between the tubules (Fig. 6 D).

The ultrastructure of kidney of 20-day-old chick embryos injected with GBP and ginger displayed an evident amelioration. The glomerular basal lamina regained its somewhat normal three-layered structure in some parts, while it was thickened in other parts, although the foot processes and infiltration slits were not clearly observed (Fig. 6 E). The proximal and distal convoluted tubular cells revealed marked amelioration compared with those of the GBP group, however the microvilli of some cells showed a slight dilatation at their bases. The cytoplasm in both types of cells was electron dense and studded with organelles (Fig. 6 F-H).

4. Discussion

It has been accepted that when embryos or fetuses become subjected to a potentially teratogenic agent during development, developmental alteration occurs due to their interaction. The result of this interaction can be morphological or functional abnormalities, delay in intrauterine growth and development, fetal death of the conceptus (García-Peláez et al., 2010). Susceptibility of teratogenicity in an organism towards any teratogen depends on many factors such as genotype of an organism including species as well as strain differences, critical developmental stage at which the organisms are exposed, the teratogen nature, the conceptus capacity to respond before the teratogenic insult, the dose and route of teratogen and also on the types of initiating mechanism of teratogenesis (García-Peláez et al., 2010; Bhashkar et al., 2014). It has been known that administration of higher dosages of AEDs, higher concentrations of these drugs in the blood, and polytherapy are all associated with higher risks for both anatomical and behavioral teratogenesis in the embryo (Meador et al., 2009). We have recently showed that GBP induced morphological and skeletal abnormalities in the developing chick embryo (Badawy et al., 2023). Therefore, the present study was designed to address the potential toxic effects of the AED, GBP on the kidney development in chick embryos.

The present study showed that GBP induced histopathological alterations in the kidney of chick embryos evidenced by tubular cell degeneration, dilated renal tubules with vacuolar degeneration of the lining epithelia, widened Bowman’s space and atrophied glomeruli, as well as edematous corpuscles. Necrotic areas were seen in some renal tubular epithelial cells along with congestion and hemorrhage of the parenchyma between the tubules. Similar results were shown under the effect of phenytoin injection as it has been reported that injection of phenytoin in ovo on the 4th day of incubation resulted in widespread glomerular degeneration with marked clumping of glomeruli (Singh et al., 2000). Additionally, Wilson et al. (2020) reported that GBP induced histopathological changes in the kidney of male rats, such as renal tubular epithelial degeneration, hemorrhage, and glomerular atrophy, as well as inducing oxidative stress and increasing the levels of urea and creatinine, (Torregrosa-de Juan et al., 2012; Badawy et al., 2019).

These results come in line with other studies reporting that renal failure can occur due to GBP long term use (Grunze et al., 1998; El-Demerdash et al., 2013). Avian kidney also showed similar results under the effect of arsenic (Khan et al., 2013). Chicks fed aflatoxin B1 showed significant renal pathology with thickening of the glomerular capillary basement membrane and mild leukocytic infiltration of the glomeruli (Valchev et al., 2014; Doaa and Ghada, 2015; Gholami-Ahangaran et al., 2016).

Co-administration of ginger with GBP resulted in profound amelioration of the kidney structure of chick embryos to be quite similar to that of the control group. The results of the present study were similar to that of Doaa and Ghada (2015) who investigated the effect of Curcuma longa and ginger to ameliorate the effect of aflatoxin B1 in broiler chicken kidney. The authors showed that groups treated with Curcuma longa and/or ginger showed improvement in histopathological effects of aflatoxin B1. Turmeric also was found to moderate the histopathological alterations in the kidney of chick broilers fed aflatoxins (Gholami-Ahangaran et al., 2016). In addition, vitamin C had been reported to diminish the deleterious histopathological effect of arsenic in the kidney of broiler chicks (Khan et al., 2013). The nephroprotection of ginger extract have also been reported in other induced kidney injuries (Shanmugam et al., 2010; Ramudu et al., 2011; Hamed et al., 2012). Moreover, Elshafae et al. (2023) stated that ginger extract ameliorated the nephrotoxic effect in rat model of diazinon-induced renal damage and that ginger improved the histological structure and biochemical profile of the kidney.
Figure 5. TEM of sections in the kidney of 20-day-old chick embryos injected with GBP. The podocytes (Po) of the renal corpuscle had a heterochromatic nucleus with condensed chromatin and a vacuolated cytoplasm (V) and with almost absent processes, except for slight projection that hardly could be seen (tailed arrow). The glomerular basal membrane (GBL) was thickened and lost its three-laminar structure. Note erythrocytes (Er) in the capillary lumen (A). Proximal convoluted tubule with extremely narrowed lumen (short arrow) and well-developed brush border (Bb). The nuclei (Nu) were shrunken and pyknotic. Mitochondria (M) and apical vacuoles (Av) can be seen (B). Proximal convoluted tubules with almost diminished lumen, degenerated and sloughed microvilli (double arrow) (C-G). Partial degeneration of brush border (Bb) (G) and its absence from parts of the cell (short arrow) (H). Rarefied cytoplasm (*) with vesiculated sER accompanied by intracytoplasmic inclusion bodies (thick arrow). The mitochondria were swollen and ruptured (arrowhead) and the basal lamina (BL) was irregular at some places. The widening of the perinuclear space (wavy arrow) was evident (I). Scale bar = A-D, F & H= 2 µm; E= 500 nm; G&I= 200 nm

Immuno-histochemical investigation showed that Bcl-2 expression in the kidney of control chick embryos occurred mainly in the convoluted tubule cells, while it rarely in the glomerulus. On the other hand, Bcl-2 expression in GBP group was minimal. Meanwhile, conversed expression of Caspase-3 was noticed.

Studies in fetal kidney have shown that Bcl-2 oncoprotein is mainly expressed in the induced metanephrogenic mesenchymal cells that differentiate into renal vesicles and nephrons, while it is not
detected in uninduced mesenchyme, renal ampullae, and collecting system (Chen et al., 2006). Concomitantly, similar immune-expression patterns were observed in studies addressing apoptosis in the avian kidney. Cadmium administration significantly increased the expression levels of the pro-apoptosis gene Caspase-3, and the rate of apoptosis, while it markedly decreased the anti-apoptosis gene Bcl-2 expression levels (Liu et al., 2015). Aflatoxin B1 administered in the diet of broilers also induced increased number of apoptotic renal cells evidenced by increased expression of Bax and Caspase-3 mRNA and decreased expression of Bcl-2 (Yu et al., 2015). The study of Welson et al. (2020) indicated that GBP could induce apoptosis in the kidney through a mechanism mostly related to the upregulation of Bax and p53 protein.

Figure 6. TEM of sections in the kidney of 20-day-old chick embryos from GBP (A-D) and GBP + ginger (E-H) groups. Distal tubule cells having pyknotic nuclei (Nu), one of which had rarefied cytoplasm (*) with few organelles. The cell debris and homogenous substances in the lumen (L) can be noted. The lumen was almost obliterated in some cells (short arrow) (A-C). Hemorrhage evidenced by the presence of erythrocytes (Er) between the tubules was shown. Some of the tubule cells had a somewhat rarefied cytoplasm (*) (D). The glomerular basal lamina approximately regained its normal three-layered structure in some parts. The foot processes and infiltration slits were not clearly observed, except for a dilated one (tailed arrow). There was no vacuolation in the neighboring podocytes (Po) (E). Proximal convoluted tubule cells with euchromatic nucleus (Nu), well preserved elongated mitochondria (M) and intact brush border (Bb) (F), however some cells showed somewhat dilated brush border (Bb) (G), as well as a number of lysosomes (Ly) and well defined rER. Few apical vacuoles (Av) were seen within the electron dense cytoplasm. A distal convoluted tubule cell with euchromatic nucleus (Nu), elongated mitochondria (M) and profiles of rER. The widening of the lumen which had no cell debris was evident (H). Scale bar A, B, E, F & G= 2 µm; C&H= 200 nm, D= 500 nm.

The immuno-histochemical investigation of the present study revealed that ginger suppressed apoptosis evidenced by increased expression of Bcl-2 in the combined ginger and GBP group and decreased Caspase-3 expression compared to the GBP only. We have previously shown that maternal administration of ginger decreased apoptosis in rat fetuses whose mothers were injected with GBP during pregnancy (Badawy et al., 2019). Ginger extract has been shown to exert significant protective effects on the kidney against alloxan-induced diabetes mellitus evidenced by better histopathological structure and decreasing the expression of Bax (El-Kott et al., 2010). Welson et al. (2020) reported that Vitamin C, which is a component of ginger, decreased apoptosis and Bax expression in the kidney of GBP-treated rats. Baiomy and Mansour (2016) also showed that cadmium increased the expression of Caspase-3, while ginger reduced its expression in the kidney tubular epithelium of rabbit. Additionally, Elshafae et al. (2023) showed that ginger decreased the expression of cleaved Caspase-3 in the kidney of rats treated with diazinon. The authors attributed this mitigating effect by ginger extract and decrease of cleaved caspase-3 expression in the renal tissue to its enhancement of anti-oxidants enzymes in kidneys and to the anti-apoptotic properties of ginger extract.

In the present study, the ultrastructural effects of GBP were seen in both the podocytes and tubule cells of the chick embryo kidney which included obliterated foot processes, pyknotic nuclei, thickened basement
membrane, as well as partial destruction of brush border and degenerated organelles, especially mitochondria. Aktaş, et al. (2010) and El-Sayyad et al., (2013) reported that valproate and lamotrigine induced similar results in epileptic rat mothers which illustrate the impairment of renal function as a result of disease and drug severe toxicity. It is noteworthy that these changes were similar to those induced in aflatoxin treated birds (Kumar and Balachandran, 2014; Saleemi et al., 2015).

The structures located in the basal region of the proximal convoluted tubules are important for the function of the sodium pump; thus, morphological changes that occur in this region and in the glomeruli appear to be significant (Yang et al., 2005). Podocytes are the most critical components in the kidney that maintain glomerular structure in the kidney. They control the bulk flow of filtrate through the intracellular spaces and are situated at the basement of glomeruli as the terminal element in ultrafiltration barrier (Fries et al., 1989). Injury of podocytes causes focal segmental glomerulosclerosis and chronic renal diseases, in addition to proteinuria (Kriz et al., 1998). Podocytes are generally attached to several capillaries by way of their foot and primary processes to increase the mechanical resistance of cells (Omary et al., 2004).

Meanwhile, the present ultrastructural investigation showed ameliorated effect of ginger on kidney ultrastructure of chick embryos, most importantly restored the typical shape of brush border of the proximal convoluted tubules and foot processes of the glomeruli as well as normal mitochondria. Similar results concerning the nephroprotective effect of ginger had been reported by Ali et al. (2015) who showed that glomeruli had improved foot processes, while the basal lamina tended to be less thick than those of cisplatin treated rats, in addition to restoration of the normal brush border and mitochondria of the proximal convoluted tubules. Curcumin or turmeric have shown similar ameliorative effect on the kidney ultrastructure (Ramya et al., 2013).

The present study indicated that most of the mitochondria of the renal cells were either swollen, ruptured or vacuolated with distorted cristae. The nuclei showed apoptotic characteristics, this was accompanied by decreased Bcl-2 expression, which is mainly located on the surface of the mitochondria and can protect the cell from apoptosis via preventing cytochrome c from being released into plasma (Peng et al., 2016). Concomitantly with the mitochondrial dysfunction, massive fragmentation of rER and detachment of its ribosomes was evident in the renal cells from GBP group. The study of Chen et al. (2010) and Liang et al. (2011) found that Bcl-2 mRNA and protein expressions were decreased in lung epithelial cells or selenium deficient chicken liver, respectively, during the process of apoptosis induced by ER stress. Therefore, GBP may be thought to induce apoptosis via the rER pathway along with the mitochondrial pathway.

The antioxidant value of ginger is thought to be due to its ability to scavenge a number of free radicals and protect cell membrane lipids from oxidation and inhibiting lipid peroxidation, leading to regeneration of damaged tissues and cells in a dose dependent manner (Ebrahimnezhad et al., 2014; El-Kordy and Makhlouf, 2014). Gingerols and shogaols are the major bioactive flavonoids present in ginger, they suppress the accumulation of reactive oxygen and/or nitrogen species in the cells (Dugasani et al., 2010). Anti-inflammatory activity of ginger is due to the presence of gingerols which have the ability to inhibit prostaglandins and leukotriene synthesis (Nurtjahja et al., 2003).

5. Conclusion

The current study showed that GBP injection during organogenesis period of chick embryo development induced renal apoptosis, histopathologic lesions in the kidney, altered the ultrastructure of the renal cells possibly via the mitochondrial and rER pathways. Interestingly, administration of ginger extract attenuated these nephrotoxic effects of GBP and improved the histological and ultrastructure of the chick embryo kidney, as well as decreased apoptosis. These effects could be attributed to the free radical scavenging and antioxidant properties of ginger. Further studies are needed to specify the mechanisms of GBP-induced nephrotoxicity.

Declarations
Author contribution statement: BG, Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data and revision of the manuscript. AM, Performed the experiments; Analyzed and interpreted the data; Wrote the paper. SS, Contributed reagents, materials, analysis tools or data. Funding: This research received no external funding. Ethical Approval Statement: The animal study protocol was approved by the Ethics Committee of the Faculty of Science - Menoufia University - Egypt (Approval No. MNSE2180). Informed Consent Statement: Not applicable. Data Availability Statement: All data supporting this work are available upon reasonable request. Conflicts of Interest: The authors declare no conflicts of interest.

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