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EFFICACY OF CURCUMIN ON SUNSET YELLOW AND TARTRAZINE INDUCED HEPATOTOXICITY AND NEPHROTOXICITY IN THE CHICK EMBRYO *Gallus domesticus*.

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ABSTRACT

Coloring agents have been reported to exert deleterious effects on human health and oxidative stress plays a key role in synthetic color induced hepatotoxicity and nephrotoxicity. The present study focused on evaluating histopathological and immune-histochemical effects of two of the most widely used synthetic food coloring agents and the ameliorative role of curcumin (Cur) against this effects. Fertilized eggs were administrated sunset yellow (SY) (1.575mg/egg) and tartrazine (Tz) (0.375mg/egg) with or without Cur (3mg/egg) during organogenesis of developing chick embryo at a dose equivalent to 14 times the acceptable daily intake (ADI) for synthetic colorants SY and Tz. At the histological level, the liver of embryos of SY and Tz groups displayed diffuse vacuolar degeneration in hepatic parenchyma and the portal area showed sever changes. The kidney showed degenerated tubules at cortex with leucocytes inflammatory cells infiltration. In addition, perivascular edema and hyalinized cell lining the proximal tubules were seen. In regarding, the immune-histochemical level expression of Caspase-3 in cytoplasm of hepatocytes and renal convoluted tubules increased in both groups SY and Tz. Co-administration of Cur, as an antioxidant agent, with SY and Tz showed ameliorative effect in all investigated parameters.

KEYWORDS: Chick embryo, Sunset yellow, Tartrazine, Curcumin, Liver, Kidney, Apoptosis.

INTRODUCTION

Food additives are substances added to food to preserve flavor or enhance its taste, appearance, or other qualities.[1] Each additive is assigned a unique number called an (E number) which is used in Europe and other countries for all approved additives[2][3][4] but some countries use number without prefix "E".[5] The individual response to these chemicals varies according to dose, age, gender, nutritional status and genetic factor.[1][3] Prolonged use of food additives can cause hazard effects in all body organs.[6] Some food additives have nephrotoxic potential as Monosodium glutamate (MSG) which is widely added to food. Recent studies reveal involvement of MSG in distortion of renal cytoarchitecture that is, cellular proliferation of mesangial cells and infiltration of inflammatory cells.[7] The use of melamine as an additive in children milk, results in acute renal failure.[8][9] Other food additives such as potassium bromate have been shown to cause renal damage accompanied by hazard changes in many brush border enzymes.[10] Moreover, some food additives have hepatotoxic effects as they cause injury of hepatocyte membranes, cytoplasmic vacuolization, blood clot in central veins, disorganized hepatocyte cords, lymphocyte and neutrophil infiltration.[11][12][13][14]

Coloring agents are used in the processing of food additive, drug and cosmetics and are regulated by U.S. Food and Drug Administration (FDA) to ensure safety.[15] They are classified into two classes; the certified (synthetic) color additives, and the natural color additives (biocolorant).[6][16]

Cur (E100) is Dicinnamoylmethane dye authorized as a food additive. It is the effective compound of Turmeric (*curcuma longa)*. It is used as a spice and for curing many disease including real disorder and hepatic disorder. It is widely used as food coloring pigment and preservative. The (JECFA) allocated an ADI of Cur is 0-3 mg/kg b.wt/day.[17][18] FDA have been proved curcuminoids as" Generally Recognized as Safe"(GRAS).[19][20] Cur has several therapeutic properties such as antioxidant.[21] *In vivo* and *in vitro* studies reported that Cur has a protective effect against oxidative stress.[22] It has the ability of scavenging different forms of free radicals. Also, Cur's ability of capturing hydrogen peroxide is higher than that of the commercial antioxidants at the same concentration.[20][23][24] Cur has a protective effect against kidney and liver toxicity caused by certain medications.[25][26][27] Moreover, it has been reported that Cur has ameliorative effect on adverse changes in liver and kidney of mothers and their fetuses induced by oral

administration of betamethasone.[28][29] Also, Cur administration has been reported to have a protective effect on renal toxicity caused by exposure to heavy metals.[26][27] Cur is also able to increase the activity of xenobiotic and detoxifying enzymes both in liver and kidney and has a protective ability against carcinogenesis processes and also reduces the iron induced hepatic damage by lowering lipid peroxidation.[30] Turmeric has been used during pregnancy in a variety of traditional medical systems for thousand years and no Western authority recommends against its use during pregnancy.[31]

SY (E110) is a synthetic coal tar and mono azo yellow dye derived from petroleum aromatic hydrocarbons, commonly used in food industry especially fermented foods which must be heat treated[32][33] also, in cosmetics and pharmaceutical field.[34] It's alternative to natural color known as carminic acid.[3][35][36][37] Although using SY in food industry, it has adverse effect on human health due to the presence of aromatic ring and azo function group which has toxic derivatives formed during the dye degradation process.[3][38] Prolonged use of SY as a synthetic dye may cause allergic reactions such as abdominal pain, nasal congestion, broncho-constriction, kidney tumors, chromosomal damage, hyperactivity, urticaria and distaste for food. It has been reported that SY is carcinogenic when fed to animals.[1][13][39] Ching *et al*.[40] and EFSA.[41] reported that oral administration of synthetic SY to rats after three days at does 500, 1000 and 2000 mg/kg b.wt/day revealed a number of dose dependent degenerative, inflammatory and proliferative lesions, especially in the liver, kidney and spleen. In addition, Ismail&Sakr[23] and Hashem *et al*.[42] found that the dose of 47 mg/kg b.wt of food coloring amaranth and SY could impair hepatic function and consequently should be avoided during pregnancy.

Tz (E102) is an artificial lemon yellow azo dye derived from coal tar. It is water - soluble mono azo color which used in human food, pharmaceutical products and cosmetics. Many countries are using Tz as saffron substitute.[13][43][44] The JECFA evaluated ADI to be 0-7.5 mg/kg/day.[13][43][45] The metabolites generate ROS which cause oxidative stress.[43][44][46] Also, it promoting lipid peroxidation and inhibiting endogenous antioxidant defense enzymes which, in turn, accelerate oxidative stress and damage most cellular components leading finally to cell death.[47][48] It has been reported that certain doses of Tz induce hepatic and kidney pathological changes.[13][44] The current study was carried out to investigate the effect of some food coloring agent (natural and synthetic) administration on the liver and kidney during the development of chick embryo by using both histological and immune-histochemical methods.

MATERIALS AND METHODS

Egg incubation and grouping

Fresh fertilized chicken eggs (*Gallus domesticus*) were obtained from a local hatchery at Menouf, Menoufia governorate. Before incubation at 37°C in an artificial incubator, eggs were cleaned with distilled water followed by 70% ethanol weighted (50 ± 5 g) and then labeled. 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. Eggs were candled before treatment and the unfertilized eggs were excluded and the remaining eggs were divided into seven groups (40 eggs each) and injected at the six day of incubation with a single dose equivalent to 14 times ADI for synthetic colorants SY and Tz. A total of 280 fertilized eggs were included.

1- Group A was not subjected to any injection (Control group).
2- Group B was injected in *ovo* with 0.2 ml of sterile water (Sham group).
3- Group C was injected in *ovo* with 0.2 ml of Cur extract at a dose of 3mg/egg.[18]
4- Group D was injected in *ovo* with 0.2 ml of SY at a dose of 1.575 mg/egg.[42]
5- Group E was injected in *ovo* with 0.2 ml of Tz at a dose of 0.375 mg/egg.[45]
6- Group F was injected in *ovo* with 0.2 ml of 1:1 mixture of Cur extract and SY at the same doses for both.
7- Group G was injected in *ovo* with 0.2 ml of 1:1 mixture of Cur extract and Tz at the same doses for both.

Coloring agents administration: Cur, as a natural food coloring agent was obtained from a local herbal store at Shebeen El-Koom, Menoufia while, SY FCF(E110) and Tz FD&C yellow n°5(E102) were obtained in a pure powder form, from Kamina Co. (Cairo, Egypt). All coloring agents were dissolved (SY and Tz) or suspended (Cur) in sterile water and injected in *ovo* in 0.2 ml / egg.

Water extraction of Cur: Dry rhizomes of the plant (*Curcuma longa*) were crushed into powder. 125g of the powder were macerated in 1000 ml of sterile water for 12 h at room temperature and filtered through a 5 µm filter paper. The concentration of obtained extract was 24 mg/ml.[49] Cur extract was applied at a dose of 3 mg/egg.[18] At the 6th day of incubation and using a sterile syringe, 0.2 ml of fluid was injected as single dose into the air sac. The holes were carefully sealed with molten paraffin wax.

Embryo collection: At the 20th day of incubation, the egg shells were broken with a scalpel and embryos were carefully freed from the egg shell. The living embryos were anaesthetized by ether and dissected to remove liver and kidney.

Investigated parameters

A-liver and kidney weight (gm)

The liver and kidney were removed from embryo of all groups aseptically and weighted.

B-Histological investigation

A portion of liver and kidney tissue from all groups were collected immediately and the specimens were labeled and fixed in 10% neutral buffered formalin solution overnight. The fixed samples were washed and then preserved in 70% ethanol, dehydrated in ascending grades of ethyl alcohol, cleared in xylene, and embedded in paraffin wax. Sections of 5 µm in thickness were obtained by rotatory microtome, deparaffinized, and stained with hematoxylin and eosin (H&E) stain for examination under the light microscope.[50]

C- Immuno-histochemical investigation

Avidin-biotin peroxidase method was used for the immune-histochemical demonstration of the proapoptotic antigen Caspase-3 as an indicator for presence of apoptosis.[51] The standard for positive reaction was that the cells had dark brown granules in their cytoplasm. For negative control, the primary antibody was omitted to guard against any false positive results that might develop from a non-specific reaction. Negative control sections were produced by substituting the primary antibodies of Caspase3 by normal goat serum. All stained slides were examined by light microscope and representative sections were photographed. Digital images were analyzed by a semi-quantitative scoring system (Figi-Image J software, Java based application for analyzing images).[52]

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 package of social sciences (IBM SPSS) statistics software for windows, Version22 (IBM corp., Armonk, NY,USA). Differences were considered insignificant whenever $P>0.05$. The significances of the obtained data were classified into three categories according to the obtained P values, i.e. $P<0.0001$, $P<0.001$ and $P<0.05$.

RESULTS

Morphometric analysis

Embryo-toxicity data from control, sham, Cur, SY, Tz, Sy+Cur and Tz+Cur injected groups are presented in Fig. (1&2). The results reflected the embryo-toxicity of synthetic color on liver and kidney in terms of weight. On the other hand, administration of Cur with SY and Tz led to significant ameliorations in both growth parameters.

Liver weight

Fig. (1) Display changes in liver weight of the embryos of different groups. The liver weight of the embryos in the sham and Cur injected groups were slightly increased compared with control (0.62±0.013; 0.62±0.013 & 0.54±0.035 of the three groups respectively). There was a moderate significant increase in liver weight of SY and Tz injected groups compared with control (0.71±0.019; 0.74±0.035 & 0.54±0.035, for the three groups respectively). On the other hand, embryos injected with SY+Cur and Tz +Cur showed significant amelioration in the liver weight compared with SY and Tz injected groups (0.62±0.003; 0.70±0.048; 0.71±0.019; 0.74±0.035, respectively) and slightly significant difference with SY and moderate significant with Tz when compared with control group.

Kidney weight

Fig. (2) illustrates the changes in kidney weight of embryos of different groups. The kidney weight of the embryos in the sham and Cur injected groups were slightly decreased compared with control (0.133±0.014; 0.138±0.002; 0.147±0.012, for the three groups respectively). SY administration caused a highly significant increase in kidney weight compared with the control group (0.250±0.004; 0.147±0.012, for the two groups respectively). Also, embryos injected with Tz showed moderate significant increase in the weight of kidney compared with control group (0.193±0.033; 0.147±0.012, for the two groups respectively). Whereas, the co-administration of Cur with SY or Tz led to marked amelioration in weight of kidney compared with SY and Tz injected groups (0.130±0.004; 0.165±0.015; 0.250±0.004; 0.193±0.033, for the four groups respectively) and there was insignificant difference when compared with control group.

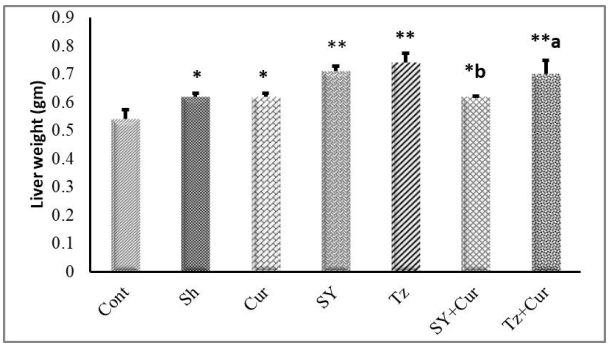


Figure 1: Graph showing liver weight of 20-days old chick embryos of different groups.

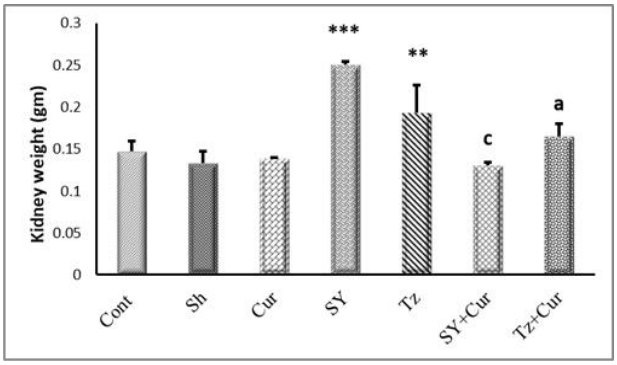


Figure 2: Graph showing kidney weight of 20-days old chick embryos of different groups.

Histological investigation

1- Liver

Control group

Light microscopic observation of control liver of 20-day-old chick embryos showed normal structure. The liver was divided into right and left lobes whereas the left lobe was smaller than the right, joined together at the midline and covered by mesothelium called glisson's capsule. The parenchymal cells were consisted of polygonal hepatocytes arranged radially around central vein without any signs of congestion as hepatic cords. The hepatocytes had specific cell membrane, acidophilic granulated cytoplasm and circular one or more nuclei. The hepatic cords were separated and surrounded by fenestrated and partially discontinuous intercellular space called sinusoids. The sinusoids had variable width and lined by endothelial cells which were flattened in shape and Kupffer cells which had large nucleus and prominent phagocytic activity. Bile ductile appeared rounded in shape and lined by cuboidal epithelial cells (Fig. 3A&B).

Sham and Curcumin groups

Comparing the histological structure of liver of chick embryos injected with single dose of distal water and Cur extract on the 6th days of incubation revealed the same pattern as in the control embryos. The hepatocytes were radically arranged around the central vein in cords as the sinusoids interspersed in them with irregular edge lined with endothelial cells and Kupffer cells. Also, a small sized hepatic artery and branch of bile ductile were seen (Fig. 3C&D).

Coloring groups

In the chick embryos injected with single dose of SY and Tz the liver showed different degenerative changes accompanied with loss of its normal shape compared with control group. Tissues sections of liver from SY group showed severe degenerative changes in numerous hepatocytes. The hepatocytes were enlarged, had light and foamy cytoplasm with hemorrhagic condition (Fig. 4A-D) and the cytoplasm filled with fatty vacuoles of variable size (Fig. 4B-D). The area around central vein had hepatocytes with highly eosinophilic cytoplasm and inflammatory infiltration (Fig. 4A&B). There was also, dilation and congestion in the central vein which was filled with erythrocytes (Fig. 4B-D). The hepatocytes showed evident necrotic changes with small pyknotic nuclei and condensed chromatin (Fig. 4C&D). Moreover, slight collagenous connective tissue fiber proliferation was showed around central vein (Fig. 4B&D) with hyalinization of hepatocyte (Fig. 4B).

The tissue section of liver from Tz group revealed diffuse vacuolar degeneration in hepatocytes (Fig. 4C-D; Fig. 5A-B). There was also dilation and congestion in central veins (Fig. 4E-F; Fig. 5A-B). Furthermore, Tz induced slight fibrous connective proliferation around central vein and hepatic artery (Fig. 4F; Fig. 5A-B). Hepatocytes with pyknotic nuclei, necrosis and complete loss of architectural details of hepatic parenchyma were evident (Fig. 4E&F; Fig. 5B). Leukocytic infiltration and microgranuloma were also seen (Fig. 4E ; Fig. 5B).

Coloring agents+curcumin groups

Histological structure of liver of both combined groups showed more the less similar structure of hepatocytes compared with control group. In addition central vein showed normal appearance but was slightly dilated than in the control group (Fig.5C&D).

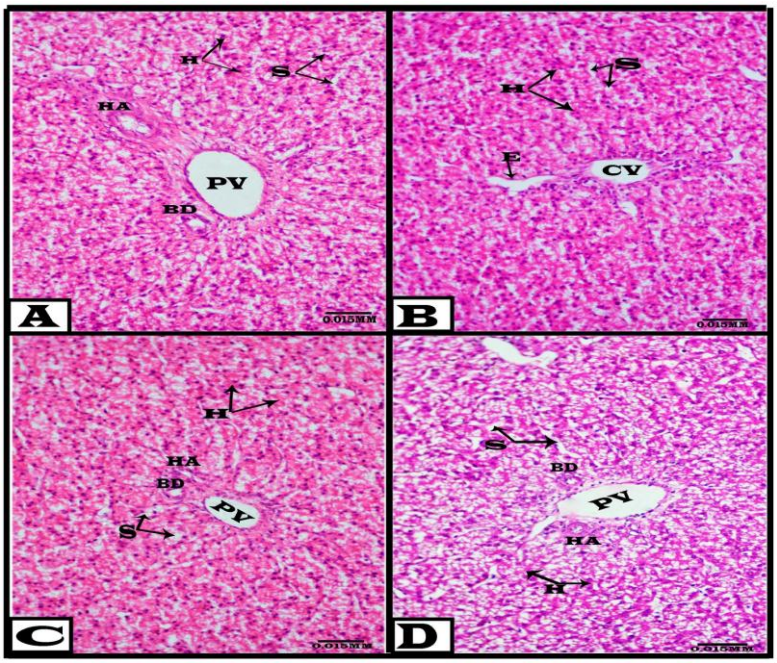


Figure 3: Photomicrographs of transverse liver sections in 20-old-chick embryos. (A&B) Control, (C) Sham, (D) Cur groups. Hepatocytes (H), Portal vein (PV), Central vein (CV), Blood sinusoids (S), Endothelial cell (E), Hepatic artery (HA), Bile ductule (BD). Scale bare = 1cm.

2- Kidney

Control group

Light microscopic examination of kidney tissue of 20-day-old chick embryos of control group showed normal histological structure. The kidney was flattened organ extend from ventral aspect of the lung to the synsacral as the dorsal half embedded deeply in the synsacral fossa and their color was brown and each kidney consists three lobs; cranial, middle and caudal. Each lobe was divided into lobules. Each lobule had cortex outer area which made the majority of the kidney with only a small portion medulla or medullary cone inner area. The cortex composed of two types of nephrons or renal corpuscles, reptilian small in size, few in number without loop of henle and most of this type located in sub capsular region of each lobule and mammalian or medullary type two sizes large and intermediate with loop of henle. The renal corpuscle had spherical structure which composed of 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 those surrounded by podocytes.

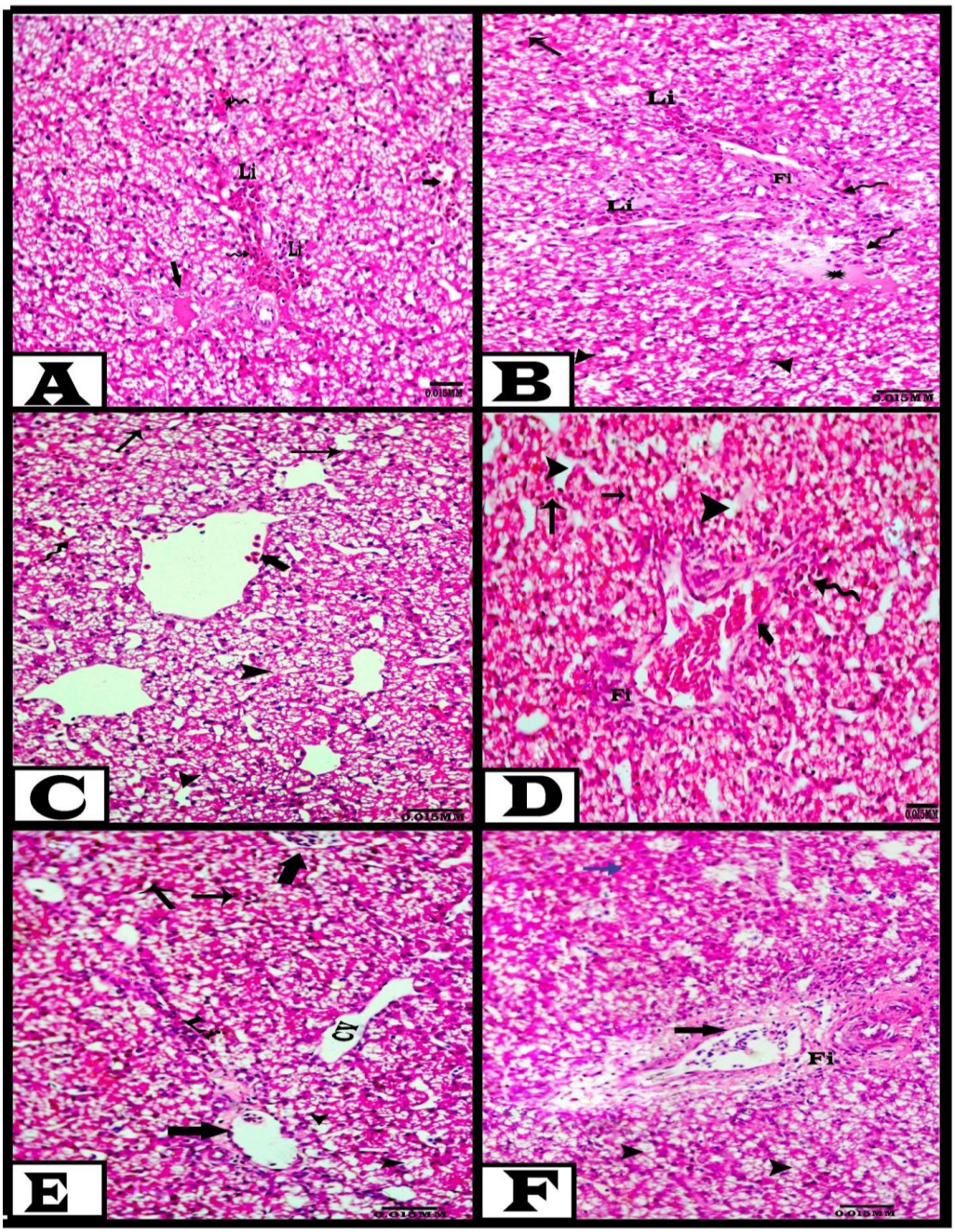


Figure 4: Photomicrographs of transverse liver sections in 20-days-old chick embryos. (A-D) SY, (E&F) Tz groups. Congested central vein (thick arrow), leukocytic infiltration (Li), hemorrhagic hepatocytes (wavy arrow), fatty infiltration hepatocytes (black head arrow), pyknotic nuclei (arrow), hyalinization of hepatocytes (black star), slightly fibrosis (Fi), necrotic hepatocytes (blue arrow) can be seen. Scale bare = 1cm.

The proximal convoluted tubules were lined by simple low cuboidal epithelium which showed brush border and narrow lumen. The distal convoluted tubules were also lined by simple cuboidal epithelium with wide lumen and smooth apical surface. Collecting tubules intermingled between thin and thick limbs of loop of Henel and were lined by columnar epithelium and occurred in the peripheral part. The two segments of Henel's loop consisted of simple cuboidal epithelium (Fig. 6A).

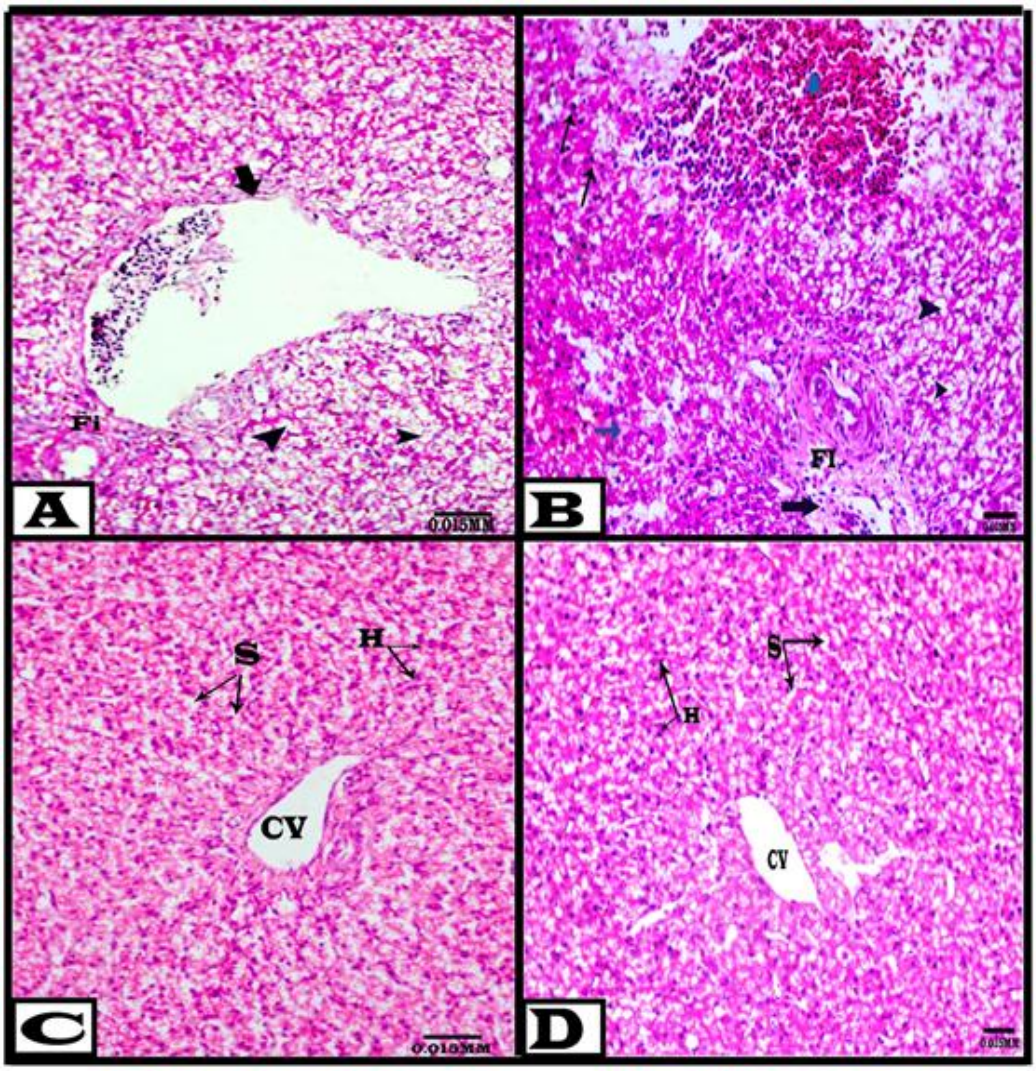


Figure 5: Photomicrographs of transverse liver sections in 20-days-old chick embryos. (A-B) Tz, (C) SY+Cur, (D) Tz +Cur groups. Dilated, curved and congested portal vein (thick arrow), fibrosis (Fi), fatty infiltration hepatocytes (black head arrow), microgranuloma (blue star), pyknotic nuclei (arrow), necrotic hepatocytes (blue arrow) can be seen. Hepatocytes (H), Blood sinusoids (S), Central vein (CV). Scale bare = 1cm.

Sham and curcumin groups

The histological analysis of kidney of chick embryos injected with single dose of distal water and Cur extract on the 6th days of incubation showed the same pattern as in the control embryos (Fig. 6B&C).

Coloring groups

The structure of the kidney of embryos injected in *ovo* with single dose of SY and Tz in the 6th day of incubation showed pathological form compering with control group. Transverse sections of kidney from SY group revealed hemorrhage of the parenchyma between tubules, vascular degeneration in proximal and distal convoluted tubules (Fig. 6D-F) and tubular cell degeneration (Fig. 6D; Fig. 7A). Also, the convoluted tubules appeared with highly eosinophilic cytoplasm and inflammatory infiltration (Fig. 6E). Degenerated tubules with dark pyknotic nuclei and oedema between tubules were also seen (Fig. 6F; Fig. 7A).

The kidney of the chick embryo injected in *ovo* with Tz showed vascular degeneration in both proximal and distal convoluted tubules (Fig. 7B-D), hemorrhage, hyalinization of tubules, hypertrophy of renal glomeruli and tubular cell degeneration (Fig. 7C-D). Collagenous connective tissue fiber proliferation was seen (Fig. 7B).

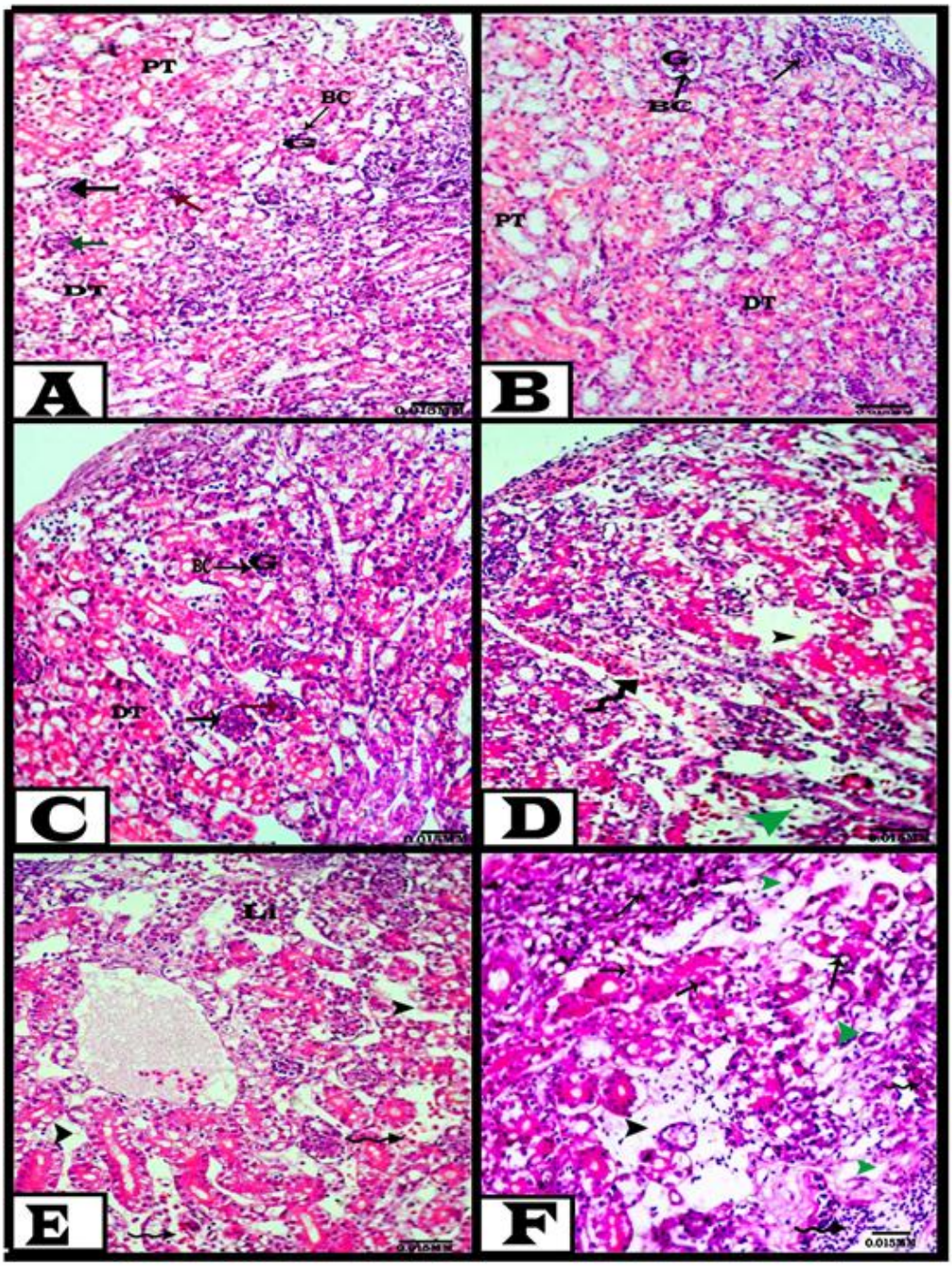
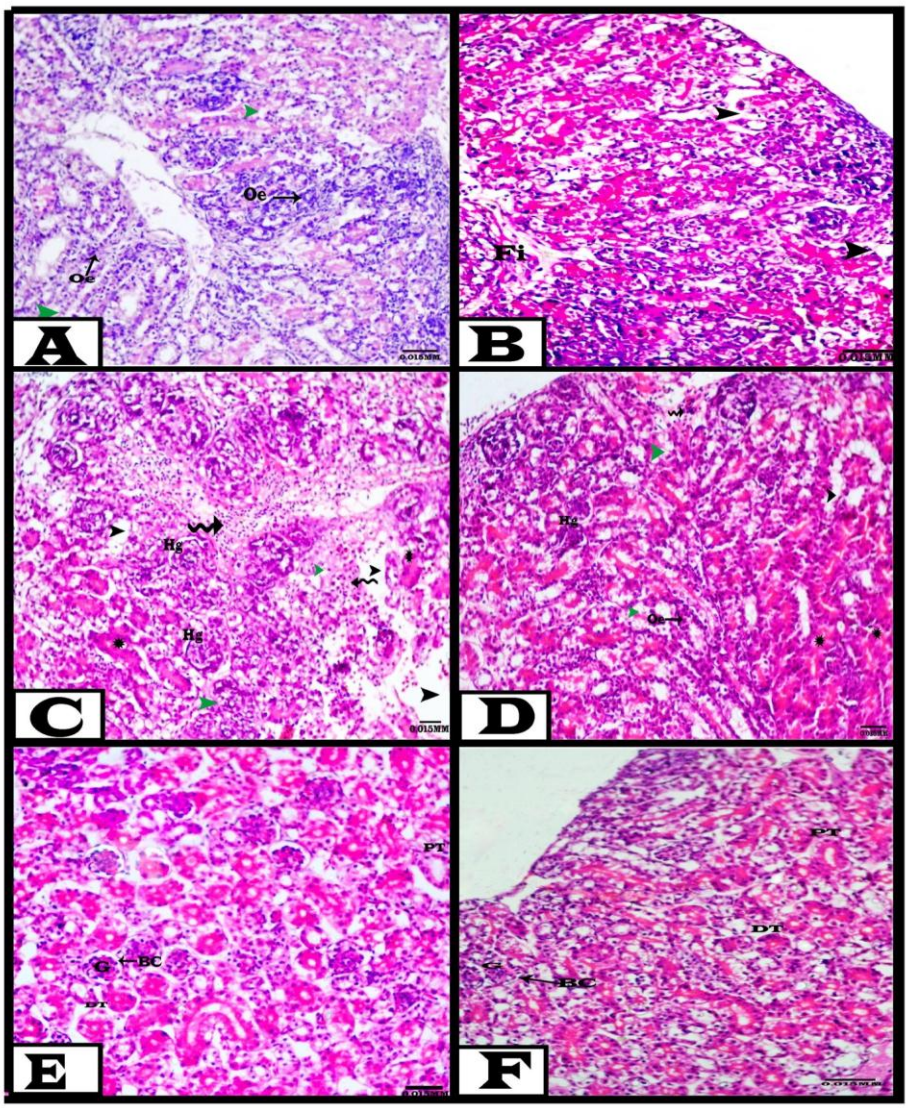


Figure 6: Photomicrographs of transverse kidney sections in 20-days-old chick embryos. (A) Control, (B) Sham, (C) Cur, (D-F) SY groups. Reptilian glomerulus (green arrow), Intermediate mammalian glomerulus (red arrow), Mammalian glomerulus (black arrow), Glomerulus (G), Bowman's space (BC), Proximal convoluted tubules (PT), Distal convoluted tubules (DT), Degenerated convoluted tubules (green head arrow), Hemorrhagic foci between tubules (wavy arrow), Vacuolated convoluted tubules (black head arrow), leukocytic infiltration (Li), Pyknotic nuclie (arrow). Scale bare = 1cm.



Figure(7): Photomicrographs of transverse kidney sections in 20-days-old chick embryos. (A) SY, (B-D) Tz, (E) SY+Cur, (F) Tz +Cur groups. Degenerated tubules (green head arrow), ooedema (Oe), Vacuolated tubules (black head arrow), fibrosis (Fi), Hemorrhage (wavy arrow), Hypertrophy of the renal glomeruli (Hg), Hyalinization of convoluted tubules (black star), Glomerulus (G), Bowman's space (BC), Proximal convoluted tubules (PT), Distal convoluted tubules (DT). Scale bare = 1cm.

Coloring agents+curcumin groups

Embryos of SY+ Cur and Tz+ Cur groups revealed better histological structure of the kidney compared with coloring groups. The organization was similar to control with some cellular vaculation (Fig. 7E) and few hemorrhage (Fig. 7F).

Immune-histochemical investigation

The expression of the pro-apoptotic protein marker Caspase -3 in liver and kidney tissue were adopted for detection of apoptosis.

1- Liver

The control group showed visible reduction in the expression of Caspase -3 protein which was located in cytoplasmic component of few hepatocytes (Fig. 8A). Also, the hepatocytes of embryos from sham and curcumin groups showed the same results with subdued caspase-3 positive immune-staining in few cells (Fig. 8B&C). On the other hand, there was increase of caspase-3 immunoreactivity in hepatocytes of the liver of embryos treated with SY and Tz (Fig. 8D; Fig. 9A; Fig: 9B-D respectively). But, administration of curcumin after SY and Tz reduced the caspase-3 immunoreactivity in hepatocytes (Fig. 9E; Fig. 9F respectively), none the less, the expression was still more declared than that of the control group.

Fig. (10) Summed up image analysis of Caspase-3 reactions in the liver which was evaluated by calculating area with a positive reaction expressed in percentage area. The results showed slightly variations between control, sham and curcumin in immune-reactive hepatocytes (10.25±0.048%; 11.82±0.267%; 13.41±0.088% respectively). On the other hand, SY and Tz groups showed highly significant increase in percentage compared with control (35.59±0.304%;

44.49±0.437 respectively). SY+Cur and Tz+Cur groups displayed a significant decrease of Caspase-3 expression percentage when compared with SY and Tz groups (30.69±0.199; 32.06±0.322 respectively) but there was a significant increase when compared with control group.

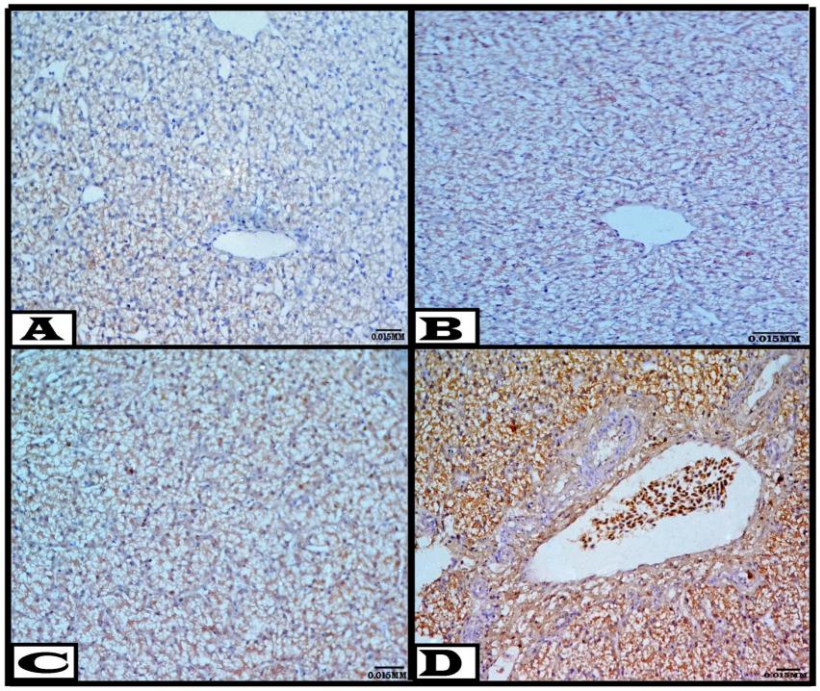


Figure 8: Photomicrographs of transverse liver sections in 20-days-old chick embryos showing immune-histochemical Caspase-3 of different groups. (A) Control, (B) Sham, (C) Cur, (D) SY. Scale bare = 1cm.

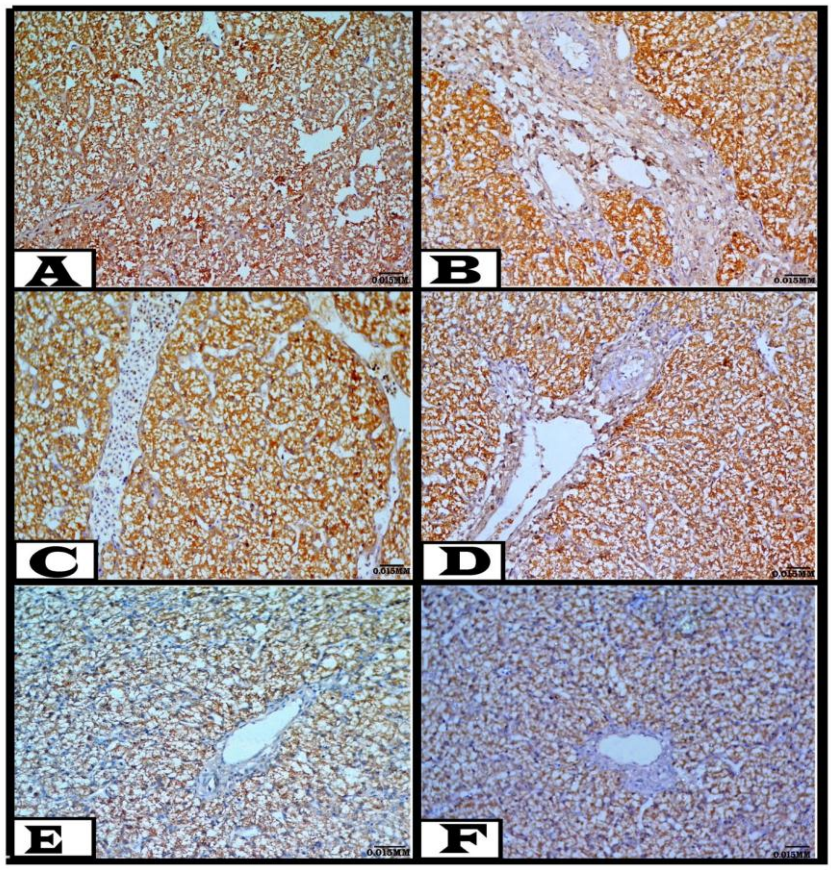


Figure 9: Photomicrographs of transverse liver sections in 20-days-old chick embryos showing immune-histochemical Caspase-3 of different groups. (A) SY, (B-D) Tz, (E) SY+Cur, (F) Tz +Cur. Scale bare = 1cm.

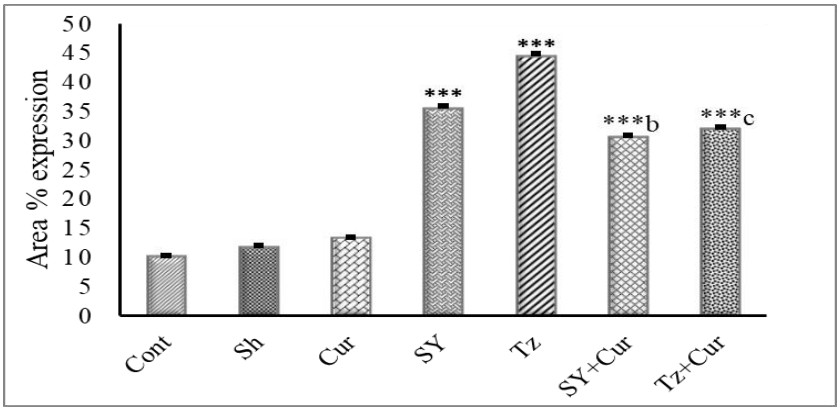


Figure 10: Graph showing the mean area %, SEM of Caspase-3 expression in the liver of 20-days old chick embryos of different groups.

2- Kidney

Reduction in the level of Caspase-3 expression with decreased number of immune-stained cells and lower intensity of immunoreactivity was observed in the renal cortex of control group (Fig. 11A). In addition, immunoreactivity was also restricted to the renal cortex of sham and curcumin groups (Fig. 11B&C). On contrast kidney of embryos injected with SY and Tz showed increasing in expression of Caspase-3 in the renal tubule epithelium, although the Malpighian corpuscles showed faint and weak expression (Fig.11D; Fig. 12A-D). When curcumin administrated with SY and Tz the level of expression of Caspase-3 was decreased compared with the coloring groups (Fig.12E; Fig. 12F).

The mean area percentage of Caspase-3 positive cell of kidney was shown in Fig. (12). There was slightly difference in the expression percentage between control, sham and curcumin (17.15±0.032%; 17.36±0.039%; 20.88±0.278% respectively). But, kidney of embryos administrated with SY and Tz showed highly significant increase in the expression percentage when compared with control (38.02±0.239%; 36.49±0.491% respectively). While mean, co-administration of curcumin after SY and Tz led to significant decrease in the percentage of area when compared with the coloring agent and significant increase when compared with control (29.06±0.205%; 26.05±0.311% respectively).

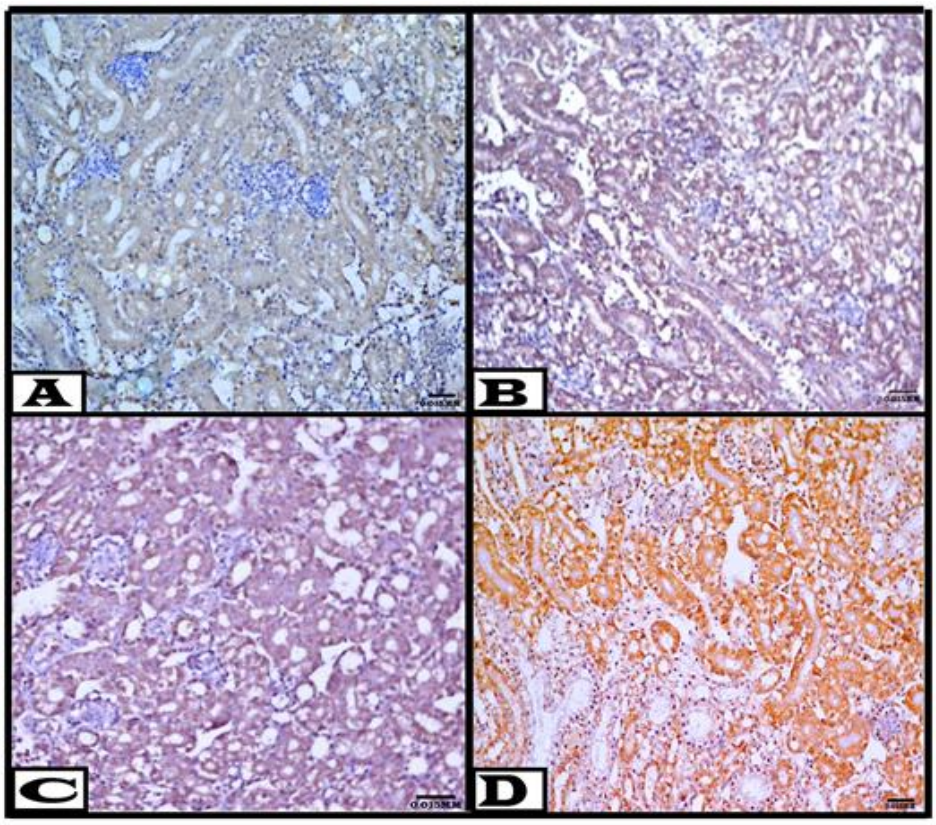


Figure 11: Photomicrographs of transverse kidney sections in 20-days-old chick embryos showing immune-histochemical Caspase-3 of different groups. (A) Control, (B) Sham, (C) Cur, (D) SY. Scale bare = 1cm.

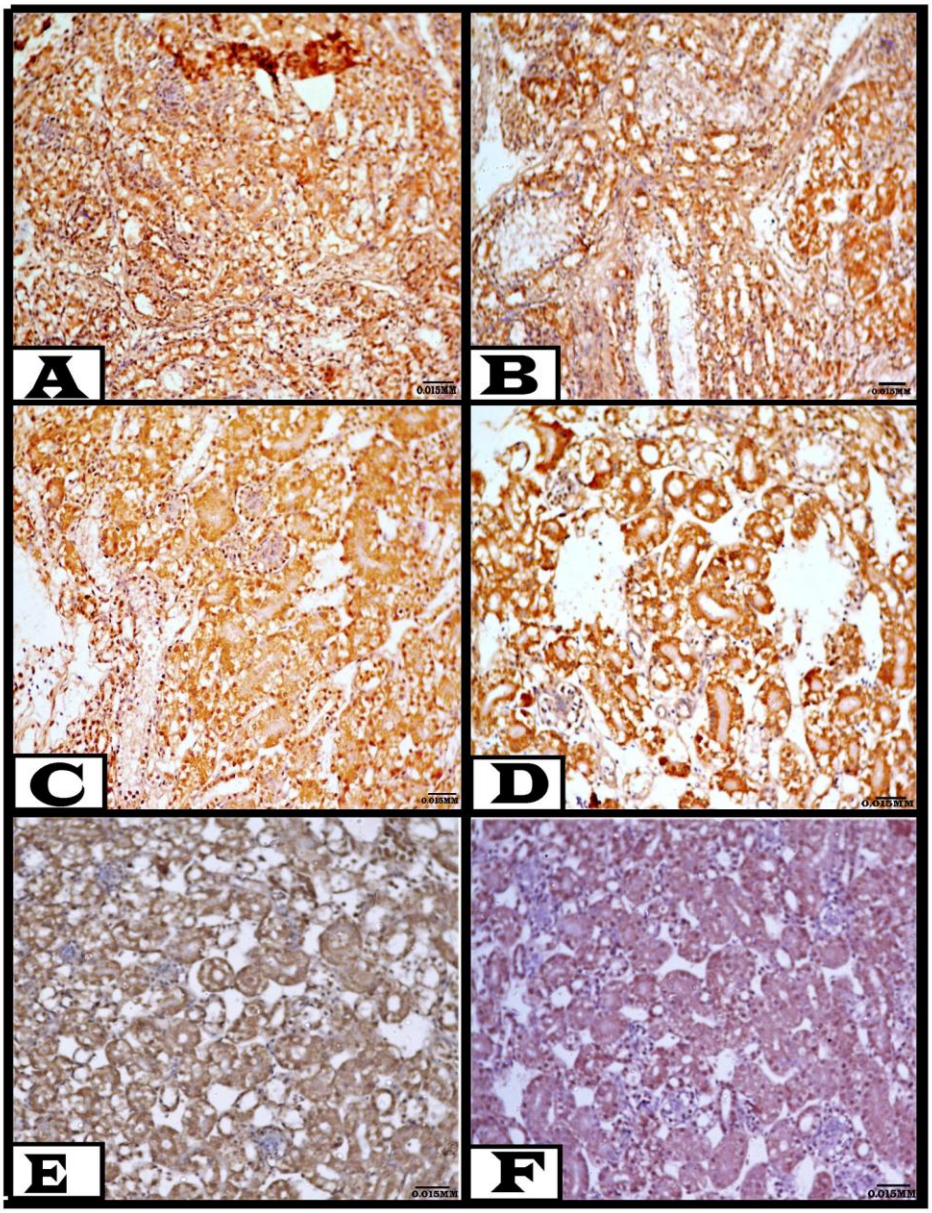


Figure 12: Photomicrographs of transverse kidney sections in 20-days-old chick embryos showing immune-histochemical Caspase-3 of different groups. (A) SY, (B-D) Tz, (E) SY+Cur, (F) Tz +Cur. Scale bare = 1cm.

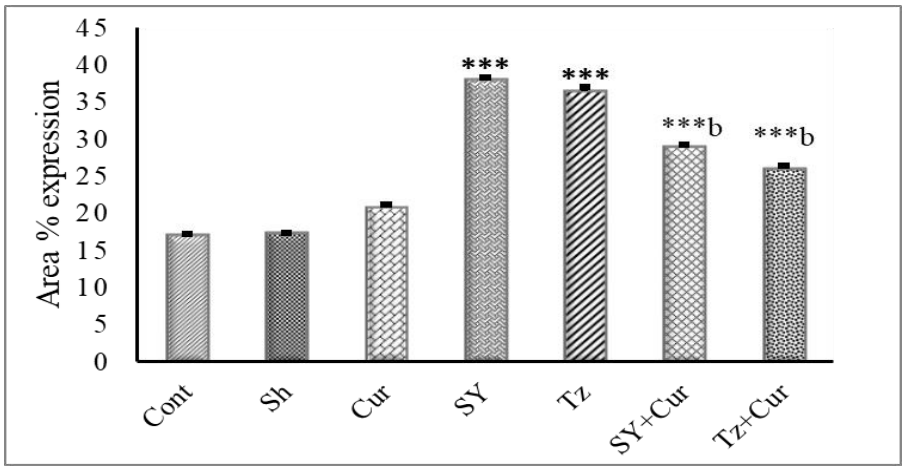


Figure 13: Graph showing the mean area %, SEM of Caspase-3 expression in the kidney of 20-days old chick embryos of different groups.

DISCUSSION

The present study was carried out to investigate the possible embryo-toxic effects of three of the widely used food coloring agents (Cur, SY and Tz) to clarify the side pathological effect of their use. The data obtained from the injection of colorants on liver and kidney weight revealed no change in Cur group as a natural color while, injection of SY and Tz showed statistically significant increase the weight of liver and kidney when compared with control in this study. This increase may be due to the presence of ROS which initiate oxidative damage and consequently impairment of membrane function of liver and kidney.[1] These results were in agreement with Dafallah *et al*.[16] who demonstrated that oral administration of SY and Tz at dose (10 mg/kg, 0.1 g/km b.wt for two groups respectively) to rats for 12 weeks as one dose every two days caused significant increase in the weight of liver and kidney but, when he used Cur as natural color at a dose of 10 mg/kg the weight of the two organs didn't affected. Also, Kushwaha &Bharti[32] examined the effect of different doses of SY (100, 200, 400 mg/kg b.wt) and recorded the result on 7th, 14th,21th and 28th day. He reported increase in liver weight of treated rats compering with control. Arefin *et al*.[53] showed that mice treated with different doses of Tz (200, 400 mg/kg b.wt) revealed decreased in weight liver especially in large dose while weight of kidney increased comparing with control. On the other hand, Himri *et al*.[43] showed that rats treated with different doses of Tz (5, 7.5, 10 mg/kg b.wt) for 13 week there was a significant decrease in weight of kidney and significant increase in weight of liver. Also, El-desoky *et al*.[54] revealed that the daily administration of Tz at dose 7.5 mg/kg for 90 days to male rats had decrease in liver weight was observed. Moreover**,** Abd Elhalem *et al*.[36] fed rats different doses of SY (161.4, 80.7, 40.35 mg/kg b.wt) at the end of experiment, significant increase in organ weight were observed.

The administration of Cur with SY and Tz during organogenesis of embryonic development improved weight of organ when compared with the SY and Tz alone. It is probably that Cur improved the metabolism as it acting as a scavenger of free radical and increasing the ability of antioxidant. Simillary, El-desoky *et al*.[54] found that when rats fed daily for 90 days Tz at dose 7.5 mg/kg plus different doses of Cur 1 gm/kg, 2 gm/kg and 4 gm/kg showed that relative masses of livers of rats fed mixtures of Cur and Tz were greater than those of rats fed only Tz. Also, Dafallah *et al*.[16] reported that a mixture of equal amount of SY and Cur at dose 20 mg/kg b.wt for 12 weeks as the dose of SY was 10 mg/kg b.wt and Cur dose was also 10 mg/kg b.wt to rats revealed that using of Cur with SY decrease the ratio weight of liver of mixture when compared with SY (121%, 137% respectively) also the ratio of kidney decrease when compared with SY (134%, 161% for two groups respectively). In addition, El-Zawahry&Abu El Kheir[55] showed that Cur administration with injection of gentamicin in rats could normalize the increase in kidney weight which caused by gentamicin.

In this study the histological findings revealed no pathological effect on liver and kidney in the group in *ovo* injected with Cur. While, the liver of embryos injected with SY showed vacuolation of hepatocytes owing to the fact that their lipid content was dissolved in cytoplasm. The hepatocytes became swollen and a single large vacuole took up their entire cytoplasm, pushing aside the nucleus and making the hepatocyte impress-ring shaped. In addition, the inflammation between hepatocytes increased and accelerated the production of WBC which caused leukocytic infiltration. Also, the hepatocellular damage appeared in distortion of the hepatic architecture, necrosis, pyknosis and formation of fibrous bridge especially around blood vessels. Furthermore, dilated and congested central veins and sinusoids were seen and some hepatocytes showed hyalinization with hemorrhagic condition. This is in agreement with Abd Elhalem *et al*.[36] who found that the liver of rats treated with different doses of SY at doses 161.4, and 80.7 mg/kg b.wt have many histopathological alternations as interlobular space with a dilated blood vessel, inflammation areas, edema, fibrosis and focal necrotic areas. But, at a dose of 40.35 mg/kg, the liver showed normal parenchymal architecture almost similar to control. Moreover, degenerated hepatocytes with pyknotic and apoptotic nuclei, fatty degeneration, dilation of the central vein and sinusoids, hypertrophy and Kupffer cell proliferation were observed in liver of rats fed SY at dose of 10 mg/kg/90 days. Also, bile ducts with aggregations of inflammatory cells were evidently seen.[56]

Al-Dahhan *et al*.[57] revealed that injection of rats with SY at a dose of 2 g/kg/b.wt for 45 days is associated with liver fatty degeneration. Also, El-Malky *et al*.[58] showed that when rats fed SY at a dose 1.57mg/kg for 8 weeks, the liver reflected some pathological effects such as dilatation and congestion in the central veins. According to Khayyat *et al*.[38] the destructive effects of oral administration of SY at a dose of 2.5 mg/kg/b.wt for 30 days in the liver were the disorganization of hepatic strands, as well necrotic and hydropic degeneration of hepatic cells. Many hepatocytes were filled with vacuoles of variable size while others appeared with irregular-shape or pyknotic nuclei with condensed chromatin. Also, marked damage in the central veins, congestion of the blood sinusoids, remarkable leukocytes infiltration and increased number of Kupffer cells were recorded.

The present study showed that the microscopic examination of liver of embryos in *ovo* injected with Tz displayed dilation and congestion of hepatic blood vessels with erythrocytes. Loss of hepatic architecture and vacuolation of hepatocytes which appeared swollen with central and sometimes peripheral nuclei. Moreover, slight collagenous connective tissue fiber proliferation

around the blood vessels and bile ductile, necrosis, pyknosis, microgranuloma between hepatocytes and massive aggregative of inflammatory cells inflammation were seen. This result correlated with Himri *et al*.[43] who found that, the microscopic examination of liver of rats orally injected with different doses of Tz (5, 7.5, 10 mg/kg) for 90 days showed brown pigment deposition in the Kupffer cells and fatty degeneration of the hepatocytes. Meanwhile, Ghonimi and Elbaz[59] reported that rats fed (500 mg/kg b.wt) Tz for 30 days showed degenerative changes in the liver and vacuolated hepatocytes. Necrotic changes with small pyknotic cellular nuclei and condensed chromatin, remarkable leukocytes infiltration, congested portal veins, accumulation of dense collagenous connective tissue fiber around blood vessels and bile duct were also recorded. When rats orally administrated Tz at a dose of 200 mg/kg for 60 days, liver showed many alterations such as vascular degeneration in hepatic parenchyma, congestion in portal blood vessel, mild mononuclear leukocytes inflammatory cells infiltration and necrotic hepatocytes. Moreover, Hashem *et al*.[60] showed that pregnant rats fed Tz in two different doses (0.45, 4.5 mg/kg) from 6 days to 15 days of gestation induce hepatic damage and parenchymal necrosis in rat fetal liver. As well, El-Desoky *et al*.[54] found that rats fed Tz at a dose of 7.5 mg/kg for 90 days, the liver showed several degenerative signs including dilatation of blood sinusoids and central veins, hemorrhage and necrotic hepatocytes.

Also, Al-Qudsi and Al-Jahdali[61] found that the liver of chick embryos injected before incubation with 0.75 mg MSG/egg showed less condensed hepatic tissue in all studied developmental stages with more dilation in blood sinusoids and veins. Meanwhile, Al-Ghamdi[62] showed that injection of chick embryo with MSG as a food additive caused pathological examination in liver tissue including pyknosis and apoptotic changes in some hepatic cells. Also, the shape of some of sinuses was disrupted and many cells are submerged and fall into the sinusoids with presence of RBCs and the remains of necrotic cells. Fibrosis and damage of the hepatic cells around portal vein were evident. Furthermore, Karakahya and Koca[14] showed that examination of liver of chick embryos inject with different doses of sodium benzoate as food additive (250, 500 and 1000 mg/kg) after five days of injection induced degeneration of the hepatocyte sequences on liver periphery with necrotic appearance, sinusoidal dilation, edema in some veins and congestion especially at high dose. SY and Tz are azo dyes and the result of their metabolism were generation of ROS which is essential causative factor for histopathological alternations. Because it generates oxidative stress which leads to peroxidation of lipid in cell membrane. Meanwhile, the biological membrane sensitive to the effect of ROS and peroxidation of lipids which leads to decrease the fluidity and disruption of membrane integrity and function which is implicated in serious pathologies.[54]

Concerning the renal histopathological alternations of embryos injected in *ovo* with SY showed perivascular edema, diffuse hemorrhage, degeneration and vacuolization of renal tubules, focal inflammatory cell infiltrations and pyknotic nuclei of the proximal and distal convoluted tubule cells. Other researchers demonstrated similar histopathological effects of SY in the kidney.[38][56] A study by Al-Dahhan *et al*.[57] revealed that rats administrated SY at a dose of 2 g/kg b.wt for 45 day showed pathological changes in the kidney. Inflammation, necrosis and vacuolisation of the tubular epithelium occurred, in addition to the destruction of glomeruli with thickening of Bowman's capsule in the kidney of rats treated with SY at a dose of 2.5 mg/kg b.wt for 4 weeks.[38] In addition, SY given to rats at a dose of 1mg/kg/b.wt/day for one month causes renal cellular infiltrations.[63]

As regarded to the microscopic examination of the kidney of embryos in *ovo* injected with Tz, vacuolation and sloughing of tubular epithelium were observed. In addition, hyalinization in the wall of renal tubules with perivascular edema and hypertrophy of the renal glomeruli were also detected. As well as, some places of the medullary part of the kidney showed fibrous tissue proliferation and peritubular hemorrhage. This result was also in coincidence with Himri *et al*.[43] who found that the kidney of rats fed a diet containing 5, 7.5 or 10 mg/kg b.wt of Tz showed tubular degeneration, tubular dilatation, intercapillary sclerosis and atrophy of glomerulus. Also, Ali *et al*.[13] revealed focal mononuclear cells infiltration, congestion in renal blood vessels with necrotic changes, hyalinization of renal tubules, hypertrophy of renal glomeruli and degenerated renal tubules in the kidney of rats orally administrated Tz at a dose of 200 mg/kg b.wt for 60 day. Moreover, Hashem *et al*.[60] revealed that the pathological examination of the kidney of fetal rat were focal fibrosis in the destructed and necrotic renal tubules when mother rats fed Tz in two doses (0.45, 4.5 mg/kg) from 6 days to 15 days of gestation. Similar observation were previously reported in rats treated with Tz.[59] Meanwhile, El-Sakhawy *et al*.[64] indicated that the kidney obtained from rats treated with different doses of Tz (7.5, 15, 100 mg/kg b.wt) showed ill-defined cell boundaries of the renal tubules and enlarged glomerulus with dilated glomerular capillaries that overfilled with blood. Furthermore, wide intercapillary spaces were noticed. Some of the proximal convoluted tubules showed large and pyknotic nuclei with ill-defined brush borders. Some of distal convoluted tubules showed sloughing of the cytoplasm and nuclei into tubular lumen. Renal medulla revealed severe desquamation of the lining epithelial cells of the collecting tubules with vacuolation of the cytoplasm. Renal medulla had hyalinized portions of some collecting tubules with noticeable interstitial hemorrhage.

The histological examination of the present study demonstrated that Cur administration ameliorated the

histopathological hepatic changes induced by SY and Tz. It decreased hemorrhage and dilatation of blood vessels. Also, it recovered the cellular vacuolization and restored normal architecture of the hepatocytes. The curative effect of Cur against oxidative damage induced by synthetic color was probably due to its powerful antioxidant property whereby scavenges oxygen free radical and its ability to increase intercellular GSH levels which leads to the efficient control of lipid peroxidation levels and decrease the pathologies of tissues.[65] This is in agreement with El-Desoky *et al*.[54] who provided evidence for Cur potential as natural coloring agent to minimize the histopathological effect in liver of rats fed with Tz as less necrosis and less degeneration of hepatocytes. Also, Hashish and Elgaml[27] mentioned that Cur exhibited ameliorating effects against $CuSO_4$ induced pathological effect in liver as mild vacuolar and hydropic degenerations of hepatocytes adjacent to the central vein besides mild hypertrophy of Kupffer cells. Previous study by Abd-Allah *et al*.[66] demonstrated that Cur protects the liver of rats against CCl_4 induced injury by suppressing hepatic inflammation and reduce hepatic oxidative stress. Cur also improved the histopathological changes in hepatic tissue induced by $AlCl_3$.[67] Gad El-Hak and Mobarak[68] showed that Cur improved the histopathological and cellular damage in liver induced by copper oxychloride.

On contrary, slight ameliorative effect could be detected in the liver tissue of female rats and their fetuses fed on diet containing fried bread simultaneously with Cur.[69] However, Woo *et al*.[70] demonstrated that high doses of Cur can cause cell damage and induced cytotoxicity.

The histological results of kidney of the present study revealed that embryos injected with Cur after SY or Tz improved the necrotic, inflammatory changes and fibrosis, despite the presence of mild tubular vacuolization in the urinary tubules and almost normal renal corpuscle. Many studies confirmed the ameliorative effect of Cur against nephrotoxicity. Cur treatment ameliorated the lithium induced histopathological renal changes in rats.[71] Furthermore, Ghoniem *et al*.[72] found that treatment with Cur caused protective effect against histopathological changes in kidney of female rats treated with lead acetate. Also, Badawy *et al*.[29] confirmed the curative potential of Cur against the histopathological effect of betamethasone in maternal and fetal kidneys of rats.

Immuno-histochemical investigations in the present study revealed that Caspase-3 expression determined cell death as it was key inducer of apoptosis and activation of it destroyed numerous cellular structures leading to cell death. Activation of caspase-3 is the key event in the course of intrinsic apoptosis. It is well known that mitochondria are responsible for the intrinsic apoptosis pathway. This is based on the fact that it control apoptosis by maintenance of ATP production and mitochondrial membrane potential and permeability for the release of certain apoptogenic factors from the interspace into cytosol .[73]

Immunostaining of both liver and kidney of embryos treated with SY and Tz showed high caspase-3 expression brown stain filled evenly whole cytoplasm of hepatocytes and renal cells as indicator to apoptosis. This is supported by Hashem *et al*.[60] who revealed that synesthetic food coloring agent as Tz inhibit mitochondrial respiration in rat liver and kidney and deactivate the integrity of mitochondrial membrane as it is vital in maintaining mitochondrial function and inducing cellular apoptosis. So, Tz induced apoptosis in rat embryo. Also, Khayyat *et al*.[38] showed decrease in the expression of BCl_2 as indicator to anti apoptotic in kidney section of rats treated with SY that's mean SY had apoptotic effect in renal cells of rats. Also, Khayal *et al*.[74] showed that the administration of MSG as food additive to rat for 8 weeks induced strong expression of Caspase-3 in hepatic tissue.

The present study revealed moderate Caspase-3 positive reaction indicating improvement in Cur administration concomitantly with SY and Tz. This result is in harmony with Gad El-Hak&Mobarak[68] who showed that treating rats with copper oxychloride and Cur improved and ameliorated reaction of Caspase-3 in hepatocytes and moderate intensity of color compared with control. Meanwhile, Abo El-Noor *et al*.[75] showed mild Caspase-3 staining indicated improvement to administration of Cur with herbicide Atrazin by reducing renal cell apoptosis of rat. Furthermore, Abd El Fadil *et al*.[76] evoked the best effect of co-administration of Cur with gentamicin and cefotaxime showed mild positive immuno-histochemical reaction in hepatocytes using Caspase-3 antibody.

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