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Protective effect of morin against cadmium induced toxicity in the developing chicken embryo

Chinmaya Bhusan Mishra, Jeevan Ranjan Dash, Ritun Patra*, Uma Kanta Mishra*, Subash Chandra Parija and Santwana Palai

Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, OUAT, Bhubaneswar-751003, Odisha, India

** Department of Veterinary Anatomy and Histology, College of Veterinary Science and Animal Husbandry, OUAT, Bhubaneswar-751003, Odisha, India*

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Abstract

Cadmium is a metal that is frequently utilised in industry and released by fossil fuels. Free radical production by cadmium results in adverse effects damaging blood vessels, liver, heart and kidney. Morin is a secondary plant metabolite that acts as a free radical scavenger. This study looks into the effects of morin on cadmium-induced toxicity in developing chicken embryos by evaluating gross and histological changes. Forty-eight numbers of fertilised chicken eggs were divided into four groups, each containing twelve eggs. Eggs of group-I were taken as control, group-II were challenged with cadmium (3 µg/egg), group-III were treated with cadmium (3 µg/egg) and morin (5 µg/egg), and group-IV were treated with cadmium (3 µg/egg) and morin (10 µg/egg) *in ovo* inoculation on day 1 of incubation. All of the eggs were incubated at 37°C with a relative humidity of 65-75%. Six eggs from each group were carefully opened in a Petri dish on the 7th day to determine the degree of vascularization. The histology of the liver, heart, and kidney of chicken embryos was performed on the 14th day. Cadmium suppressed vascularization in 7th day chicken embryo. Enriching effect of morin was clearly visible with increased vascularization and arbourization of major and minor blood vessels. In 14th day chicken embryo, group-II showed severe necrosis, congestion, haemorrhage and moderate fibrosis in the hepatic, cardiac and renal tissues. The improved histology results justified that morin alleviated the tissue injuries. Thus, morin has the potency to minimize the cadmium-induced toxicity in chicken embryo.

1. Introduction

Heavy metals like cadmium are found in the earth's crust naturally with various industrial and commercial applications. Accumulation of cadmium can be detrimental to the body, producing acute and chronic toxicities in humans (Engwa *et al*., 2019) due to closeness of residential areas to industrial corridors. Cigarette smoke is the most well-known source of cadmium. Cadmium enters the body by ingestion or inhalation. Because of its lengthy half-life of 15 to 30 years after getting entry in the body, it accumulates in kidney, liver, testis, and bone, causing functional and structural damage (Ma *et al*., 2017). Cadmium increases reactive oxygen species (ROS) production and simultaneously depletes antioxidant levels. Antioxidants either naturally created in the body or externally supplemented, helps body to combat oxidative stress and repair damage caused by ROS and reactive nitrogen species (RNS) which results in improvement of immunity (Pham-Huy *et al*., 2008).

Natural products with antioxidant and anti-inflammatory action are becoming popular since they interact with different pro-inflammatory mediators causing oxidative stress (Forni *et al*., 2019). Morin chemically 3,5,7, 22, 42-pentahydroxyflavone is a flavonoid naturally found in plants like *Malus pumila* (apple), *Psidium guajava* (guava), *Moringa olifera* (moringa), *Ficus carica* (figure), *Morus alba* (mulberry), *Camellia sinensis* (tea), *Allium cepa* (onion), *Prunus dulcis* (almond) *etc*. (Figure 1).

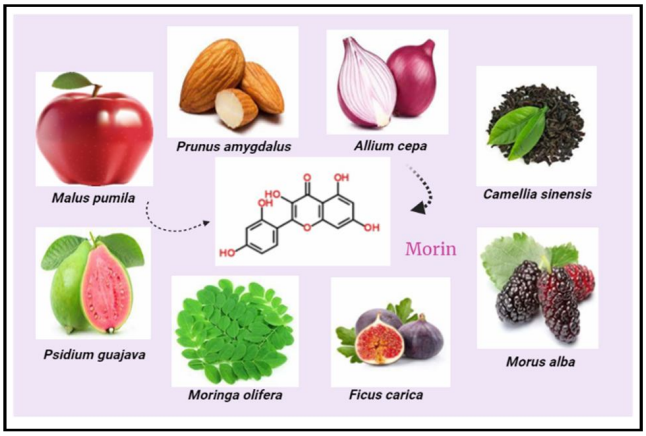

Figure 1: Sources of morin

It has antioxidant (Kim *et al*., 2010), neuroprotective (Ola *et al*., 2014) and anti-inflammatory effects (Yong and Ahn, 2018). Morin

Corresponding author: Dr. Santwana Palai

Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, OUAT, Bhubaneswar-751003, Odisha, India

E-mail: palaisantwana@gmail.com

Tel.: +91-9438298384

exerts free radical scavenging activity (Choudhury *et al*., 2017) and hepatoprotective activity due to increase in the endogenous antioxidant activities (Khanam and Firdous, 2020). It also prevents acute liver damage caused by carbon tetrachloride by inhibiting IL-6, IL-1beta, TNF-alpha and iNOS production (Lee *et al*., 2017). Inhibition of hepatic antioxidant enzymes by cadmium is well protected by morin (Ola *et al*., 2014). In the acrylamide-induced toxicity, morin reversed the alterations in levels of apoptotic inflammatory markers such as caspase-3, cytochrome c, IL-1, IL-6, TNF-α, and COX-2 (Kandemir *et al*., 2020).

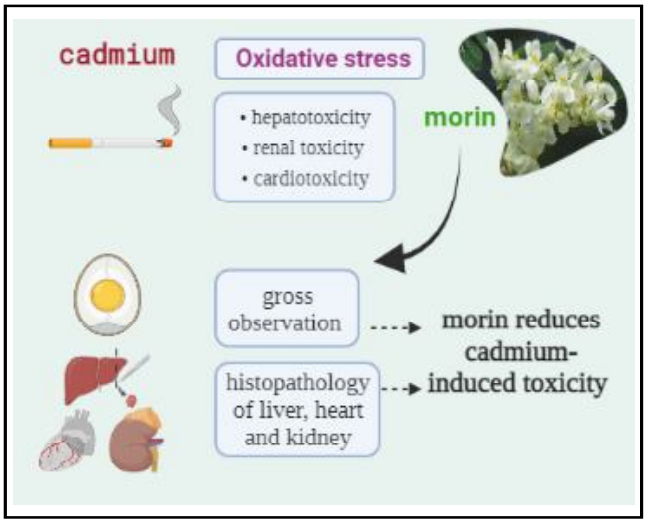

Figure 2: A proposed overview on the protective effect of morin in cadmium-induced toxicity.

Pollutants including heavy metals can impact eggs severely causing high embryo mortality or abnormal embryonic development due to the accumulation of pollutants as reported in case of reptile eggs (Díaz-Paniagua *et al*., 2002). The direct toxic effect of cadmium (Figure 2) on embryonic eggs and growing chicks could affect adversely to the poultry industry in long run. Considering the potential of morin as antioxidant, we investigated the protective effect of morin on cadmium-induced reduced neo-vascularisation and organo-toxicity in pre-hatched chicks.

2. Materials and Methods

2.1 Chemicals

Cadmium was used @ 3 µg/egg which is equal to the lethal dose (LD_{50}) as per Khandia *et al*. (2017). The used chemicals like cadmium, morin were procured from MP Biomedicals, France and were stored at temperature of 2-8ºC. The stock solutions of both cadmium and morin were prepared in water and serial dilution were done up to desired dose before inoculation in liquid form.

2.2 Fertilised chicken eggs

Forty-eight numbers of fertilised eggs of chicken, weighing about 55.0 ± 2.0 g and 0th day of age were purchased from Central Poultry Development Organization, Bhubaneswar, Odisha. Candling was done for identification of fertilised eggs. The research was carried out at the College of Veterinary Science and Animal Husbandry, OUAT, Bhubaneswar-751003, India. According to the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), the experiments in the avian embryos/chick CAM model do not require official permission (Fauzia *et al*., 2018) because the chick embryo does not experience pain until 14th day due to incomplete neuronal differentiation and lack of nociception (Ribatti, 2016; Buhr *et al*., 2020; Kundeková *et al*., 2021). However, all precautions were taken for ethical conduct of the experiment.

2.3 Experimental protocol

Prior to the experiment, a pilot study was done to ascertain the sublethal dose of cadmium where different doses of cadmium were inoculated to observe the embryonic death percentage. Among these doses, 3 µg/egg was found to be the subtoxic dose which caused toxicity affecting vascularisation and histoarchitecture of organs with no embryonic mortality. Thus, this dose was used to induce cadmium toxicity in the developing chicken embryo.

The entire number of eggs was divided into four groups, each of which included 12 eggs. On day 1 of incubation, eggs of group-I were set aside as control, group-II were challenged with cadmium (3 µg/egg), group-III were treated with cadmium (3 µg/egg) and morin (5 µg/egg), and group-IV were treated with cadmium (3 µg/egg) and morin (10 µg/egg) *in ovo* inoculation. The eggs were sprayed with 70% ethanol and a small hole was drilled into each egg on the side opposite the air sac, *i.e*., into egg albumin, using a sterilised dental drill as per Dzugan *et al*. (2011). After chemical injections using sterilised insulin syringe, the holes of the eggs were plugged with help of parafilm. All these methods were done under laminar flow. Then, incubation was done at 37°C with relative humidity of 65-75%.

2.4 Gross examination

On the 7th day (after completion of angiogenesis), 6 eggs from each group were carefully opened in a Petri dish to determine the degree of vascularisation and embryonic malformation. On macroscopic level, the vascularisation, angiogenesis in chorioallantoic membrane of treated eggs and embryonic malformation were graded as per Khandia *et al*. (2017).

2.5 Histological evaluation

On 14th day (after completion of organogenesis), 6 eggs from each treatment group were opened to collect liver, heart, and kidney tissue samples in 10% BNF. After 72 hours of fixation, the tissues were processed routinely to obtain 6 µm thick serial paraffin sections. As per Bancroft and Stevens (1996), Hematoxylin and Eosin were used to stain the tissue slices for histoarchitecture and with Masson's trichrome stain for demonstration of collagen fibers. A trinocular research microscope was used to record histopathological changes on each tissue slide (Leica, DM 2500, Digital camera system DFC 290).

3. Results

3.1 Gross examination

The four groups of resealed fertilised chicken egg were unfastened and examined at 7th day of incubation after treatment (Figure 3). Table 1 shows the degree of changes on the process of vascularisation, angiogenesis and embryonic malformation due to cadmium and morin through gross changes visualised in chorioallantoic membrane.

Table 1: Gross pathological changes of fertilised chicken egg at 7th day of incubation period

Group/parameters	Vascularisation (Number of primary blood vessels, their organization and network formation)	Angiogenesis (Number of secondary and tertiary blood vessels)	Embryonic malformation (Disorganization of the embryo)
I	+++++	+++++	-
II	±	±	++++
III	+++	++	+
IV	++++	+++	±

Note:(+++++) = Strong, (+++) = Moderate, (++) = Fair, (+) = Mild, (±) = Feeble, (-) = Negative

Cadmium @ 3 µg/egg caused subdued vascularisation in 7th day fertilised chicken egg (Figure 4). Our results showed that cadmium acts directly on the endothelial cells angiostatically. The number of secondary and tertiary blood vessels is reduced. Morin @ 5 µg was not able to recover the cadmium toxicity but morin @ 10 µg nearly inverted the cadmium-induced altered vascularisation, angiogenesis and embryonic malformation (Table 1) which are indicators of growth and development of fertilised chicken egg. The reduced neo-vascularisation by cadmium and its reversal by morin were clearly observed from the gross appearance of major and minor blood vessels in the images depicted at Figures 5 and 6.

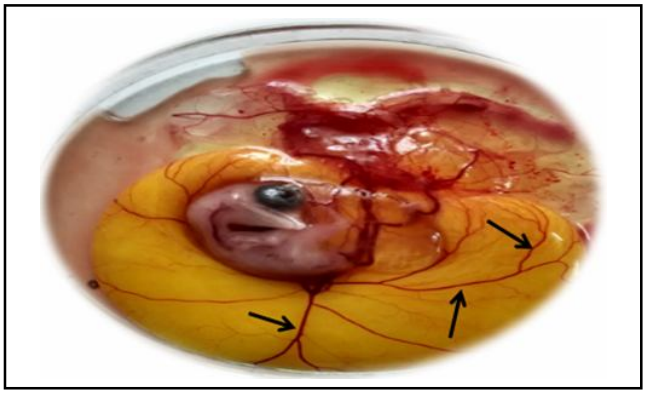

Figure 3: Gross changes in neovascularisation in chorioallantoic membrane of fertilised chicken embryo at 7th day of incubation period showing the normal formation of embryo. The normal vascularisation and angiogenesis (arrow) are noted.

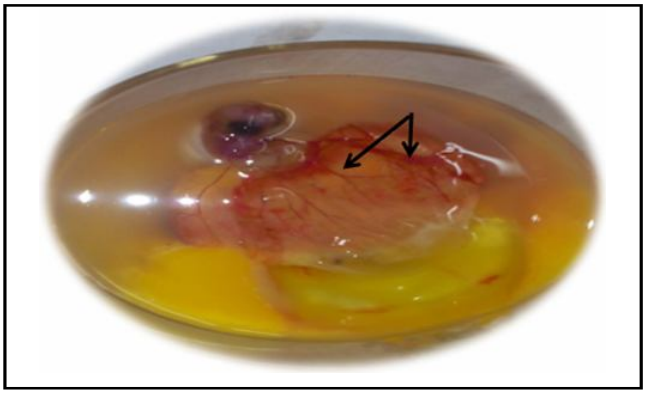

Figure 5: Gross changes in neovascularisation in chorioallantoic membrane of fertilised chicken embryo at 7th day of incubation period treated with cadmium (3 µg) + morin (5 µg) showing decreased embryonic malformation and proliferation of the blood vessels (arrow).

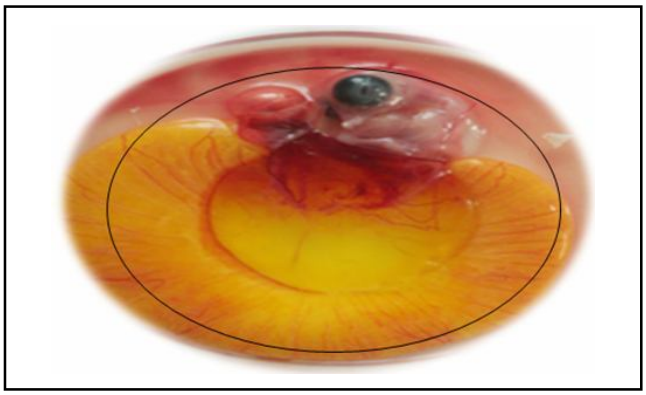

Figure 4: Gross changes in neovascularisation in chorioallantoic membrane of fertilised chicken embryo at 7th day of incubation period challenged with cadmium @ 3 µg/egg showing embryonic malformation and subdued vascularisation (circle) are noted.

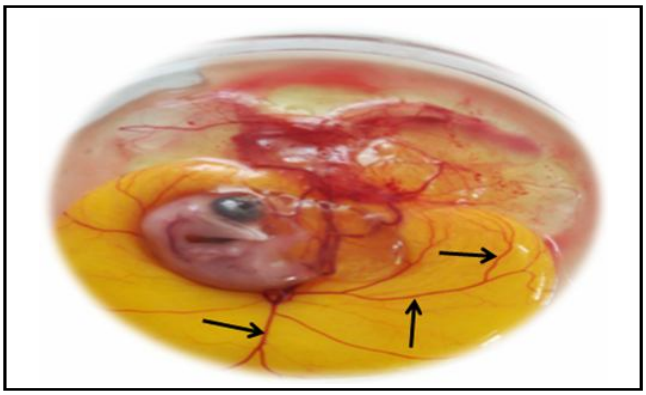

Figure 6: Gross changes in neovascularisation in chorioallantoic membrane of fertilised chicken embryo at 7th day of incubation period treated with cadmium (3 µg) + morin (10 µg) showing absence of embryonic malformation. Vascularisation and angiogenesis (arrow) are nearly restored.

3.2 Histological examination

At 14th day of incubation, samples from the liver, heart, and kidney of chicken embryos were taken and analysed for histopathological alterations from the remaining 6 fertilised chicken egg of each group. The tissue slides of these three organs from group-I revealed normal histoarchitecture without any pathological changes.

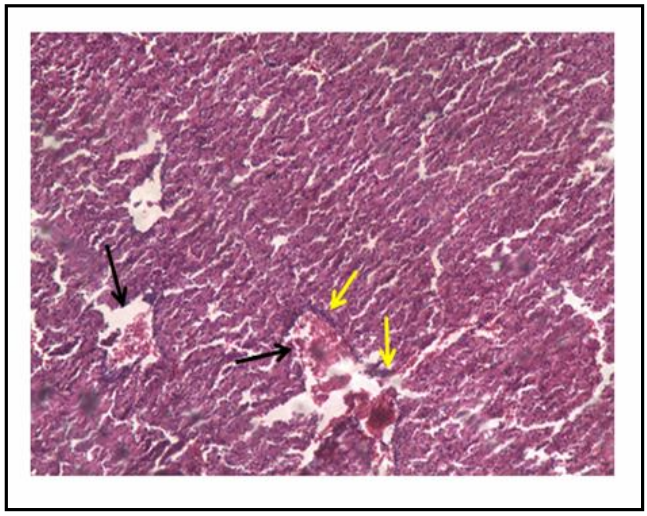

Figure 7: Photomicrograph of liver of chick embryo at 14th day of incubation period treated with cadmium (3 µg) showing hypoplasia of blood vessels (black arrow) in the parenchyma and mild localized inflammatory cell infiltration (yellow arrow) in the perivascular space. (H and E × 100).

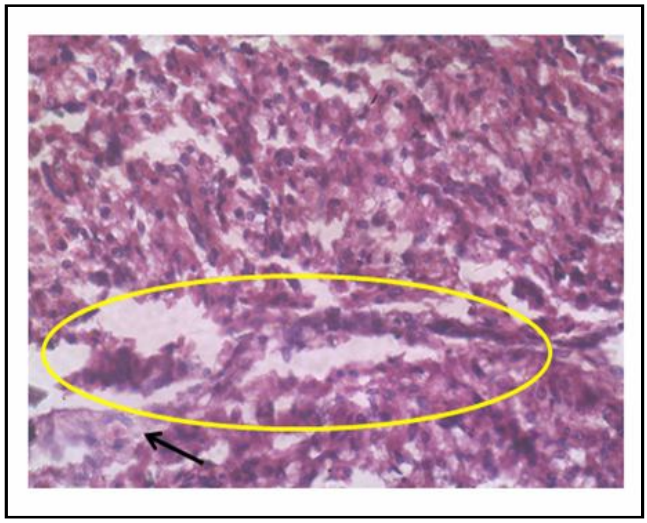

Figure 8: Photomicrograph of heart of chick embryo at 14th day of incubation period treated with cadmium (3 µg) showing cytoplasmic hyalinization (arrow) of myocardiocytes. Note the formation of syncytium (circle) due to detachment of myofibres from each other. (H and E × 400).

The liver of group-II revealed hypertrophied hepatocytes, pycnotic nuclei, cytoplasmic vacuolation, enlarged hepatic sinusoids, fibrosis, necrosis and degenerative changes. There was also significant reduction in the frequency of haemopoietic foci (Figure 7), moderate hyperplasia and hypertrophy of kupffer cells, moderate degree of infiltration of mononuclear cells (Figure 7) as perivascular cuff, severe congestion and haemorrhage. The heart of fertilised chicken embryo from group-II showed acute interstitial myocarditis, irregularly arranged myofibers in the configuration of a syncytium (Figure 8), loss of typical cross-striation pattern, moderate vacuolation in cytoplasm, scattered accumulation of inflammatory

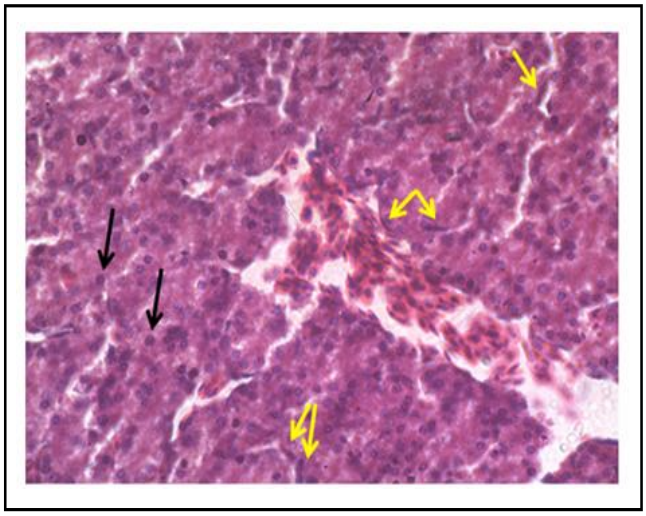

Figure 9: Photomicrograph of liver of chick embryo at 14th day of incubation period treated with cadmium (3 µg) + morin (5 µg) showing the predominant euchromatic nuclei (black arrow) granular, moderately eosino-philic and vacuolated cytoplasm of the hepatocytes. The kupffer cells (yellow arrow) are mildly hyper-plastic. (H and E × 400).

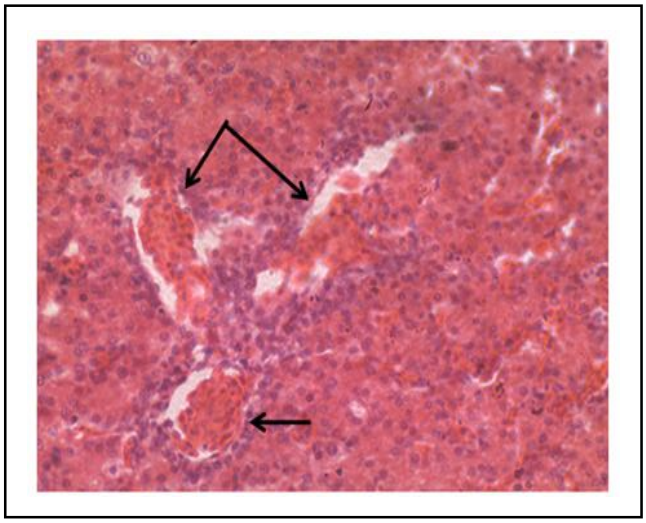

Figure 10: Photomicrograph of liver of chick embryo at 14th day of incubation period treated with cadmium (3 µg) + morin (10 µg) showing the extensive proli-feration of blood vessels (arrow) in the hepatic parenchyma. (H & E × 400).

cells between the myofibers, endothelial necrosis and desquamation and moderate congestion. Both the ventricular walls were thickened. The renal tissue from group-II revealed extensive dilatation of renal tubules, hypertrophy of tubular epithelial cells, loss of apical brush border, cellular degeneration, congestion, and inter-tubular

haemorrhage, moderate degree of fibrosis and profuse permeation of mononuclear cells.

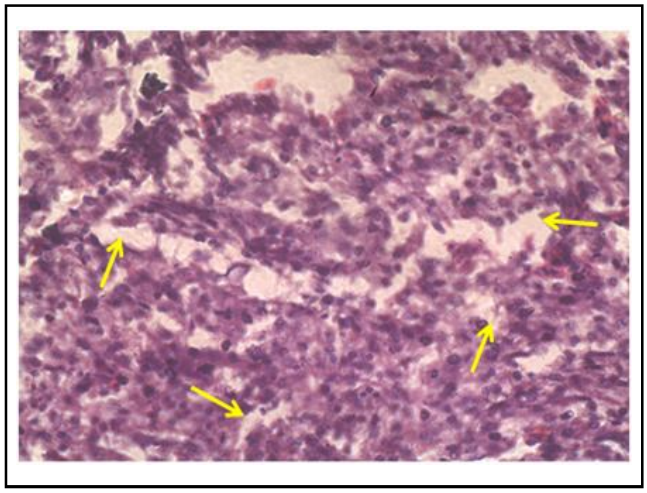

Figure 11: Photomicrograph of myocardium of chick embryo at 14th day of incubation period treated with cadmium (3 µg) + morin (5 µg) showing the heterochromatic nuclei of a major population of myocardiocytes. Note the extensive vacuolation between myocardiocytes (arrow) and pycnotic nuclei. (H and E × 400).

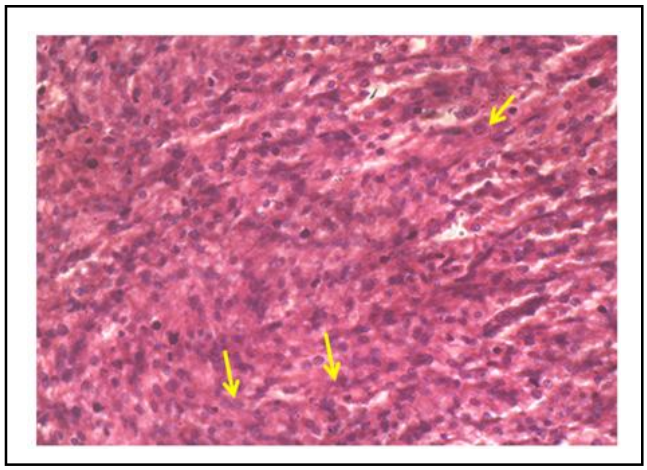

Figure 12: Photomicrograph of myocardium of chick embryo at 14th day of incubation period treated with cadmium (3µg) + morin (10 µg) showing normal circular euchromatic nuclei (arrow) in most of the myocardiocytes. Some of the myocardiocytes carry an atrophied dense nucleus and the vacuolation is less apparent. (H and E × 400).

In group-III, the liver parenchyma revealed slightly better histo-architecture than the cadmium treated group. Most of the hepatocytes were normal except few cells with mild vacuolation, haematopoietic foci were comparatively more than group-II (Figure 9). There was slight sinusoidal dilatation and inflammatory cell infiltration at few locations. The liver tissue from group-IV showed paranormal histomorphology. The hepatocytes discerned normal configuration with euchromatic central nucleus, frequent hematopoietic foci, normal sized sinusoids and kupffer cells, and mild infiltration of mononuclear cell (Figure 10). The heart tissue from group-III exhibited diffuse cross striation pattern of myocardial

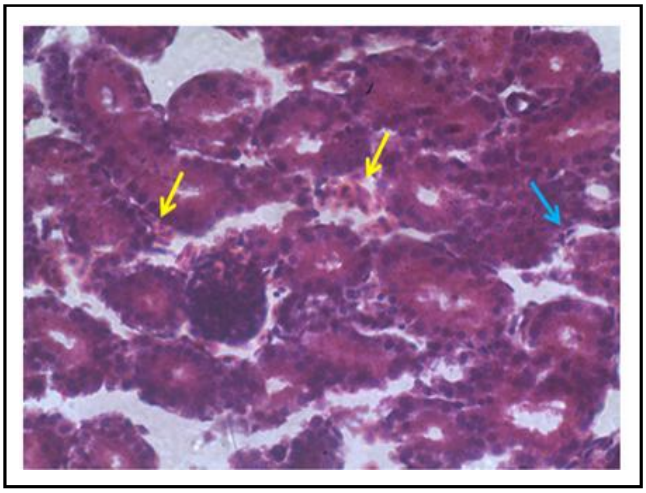

Figure 13: Photomicrograph of kidney of chick embryo at 14th day of incubation period treated with cadmium (3 µg) + morin (5 µg) showing focal intertubular haemorrhages (yellow arrow) along with mild degree of infiltration of mononuclear cells (blue arrow) in the kidney parenchyma. (H & E × 400)

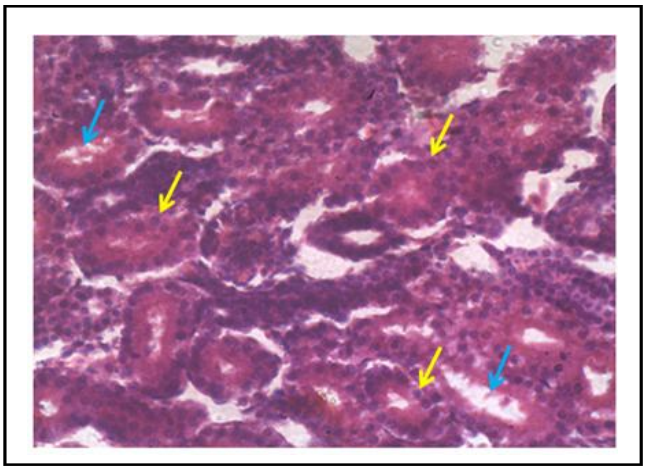

Figure 14: Photomicrograph of kidney of chick embryo at 14th day of incubation period treated with cadmium (3 µg) + morin (10 µg) showing the nuclear condensation in majority of the cells of the renal tubules. Note that a sparse number of cells carrying the euchromatic nuclei (yellow arrow) and the brush border (blue arrow) of tubular cells was apparent. (H and E × 400).

fibers, mild interstitial myocarditis, vacuolation (Figure 11) and congestion, occasional infiltration of mononuclear cells and intact endocardium. Most of the myocardiocytes had heterochromatic (Figure 11) and pycnotic nuclei. The heart tissue from group-IV revealed myocardial fibers with prominent cross-striation pattern, no myocardial inflammation and congestion, intact endocardium. The myocytes had euchromatic nucleus, eosinophilic cytoplasm with less apparent vacuolation (Figure 12). In group-III kidney, there was moderate tubular dilatation but had normal lining cells

with indistinct brush border, sparse mononuclear cell infiltration and focal inter-tubular haemorrhages (Figure 13). The kidney of fertilised chicken embryo of group-IV discerned normal arrangement of tubular epithelial cells with apparent brush border. There was absence of fibrosis, haemorrhage, inflammatory cell infiltration approaching near normal kidney histomorphology (Figure 14).

4. Discussion

The chorioallantoic membrane of fertilised chicken eggs is an extremely vascularized extra-embryonic membrane linked to the embryo *via* circulatory system. The fertilised chicken eggs can be an ideal model system for xenotoxicity testing (Meena Bai *et al*., 2014), biomedical research (Winter *et al*., 2020) and angiogenic or anti-angiogenic molecules efficacy assessment because metabolic activation and deactivation of xenobiotics occurs in chorioallantoic membrane (Ribatti, 2016). The *in ovo* imaging of the chick embryo *via* chorioallantoic membrane has gained interest over costly and less efficient rodent models. Also, researchers are attracted to the CAM test because chick embryos lack pain awareness and senescence (Marshall *et al*., 2020).

Our findings using fertilised chicken eggs as a model showed that exposure to cadmium at an early embryonic stage inhibits vasculogenesis and angiogenesis, resulting in delayed growth. The reduced number of secondary and tertiary blood vessels occurs as a result of the development of a strong cadmium complex, which may be limiting nitric oxide generation in endothelial cells, resulting in anti-angiogenesis (Majumder *et al*., 2008). Bhuvaneswari *et al*. (2019) have reported retarded growth and congenital malformations after cadmium exposure in avian embryos and our observations are in accordance to their findings. Similarly, Branca *et al*. (2020) have proven that cadmium is a powerful cell toxin that causes oxidative stress by increasing lipid peroxidation and/or altering intracellular glutathione levels.

The cellular mechanisms involved in reappearance of vascular architecture by morin in cadmium-treated chick embryos though unclear but could be supported by several findings reported on morin's effect such as: (i) antioxidant levels increased through inherent free radical scavenging capacity and stimulation of the nuclear factor erythroid 2-related factor-2/heme oxygenase-1 pathway, which protects cells from death (Lee *et al*., 2017; Alberdi *et al*. 2018; Verma *et al*., 2019), (ii) vascular endothelium protection in cardiovascular-related endothelial disorders (Madkhali, 2020), (iii) cell protection in oral epithelial cells against smokeless tobacco extract-induced cytotoxicity (Ganguli *et al*., 2016).

The histopathological findings revealed that administration of morin initiated repair of the damaged cytoarchitecture of liver which was more prominent in morin @10 µg. As observed in the current study, Al-Baqami and Hamza (2021) also observed severe congestion and haemorrhage of liver due to cadmium toxicity which was significantly reversed by using resveratrol in male rats. Pawlak *et al*. (2013) found similar histological alterations in the cardiac wall, but thickening was observed exclusively in the right ventricle wall. This approach towards normal histomorphology of heart was also observed by Yanchun *et al*. (2021) as marked improvement in cardiac histomorphology with *Ipomoea staphylina* extract against cadmium-induced toxicity.

Riaz *et al*. (2020) also observed tubular degeneration, fibrosis, hemorrhage and vacuolation in kidney of rats due to heavy metal toxicity. These findings authenticate the nephrotoxicity character of cadmium as reported by Abnosi and Golami (2017). These findings of chicken embryo kidney are in accordance with the findings of Jihen *et al*. (2009) who reported supportive outcome of selenium and zinc against cadmium intoxicated rat kidney.

Cadmium-treated chicken embryos' hepatic, cardiac, and renal histoarchitecture showed vacuolization, degeneration, significant necrotic alterations and inflammatory cell infiltration in this study. The histological changes in morin treated groups clearly justify its ability to alleviate damaging effects of cadmium which is more pronounced @10 µg. As per histopathological analysis, morin treatment resulted in improvements in liver, cardiac and renal tissues. It extended protection against tissue damage in the liver, heart and kidney tissues which may be due to its: (i) ability to decreased free radical production (Devakumar *et al*., 2011), (ii) regulating influence on TNF-α, IL-1, and NF-κB levels, and (iii) capability to decrease Bcl-2, AQP-2 and nephrin expression (Kuzu *et al*., 2019).

The gross outcomes match our histological findings, which revealed high level of damaging effect of cadmium toxicity. Our findings show that giving morin to cadmium-intoxicated embryos prevented the oxidative hepatic, cardiac and renal dysfunction caused by cadmium. Morin treatment significantly decreased the aberrant alterations caused by cadmium and changed the hepatotoxicity, cardiotoxicity and renal toxicity at tissue levels to nearly normal levels.

Morin's antioxidant and metal-chelating efficiency dramatically decreased oxidative stress, resulting in decreased histopathological changes and the restoration of usual bodily condition. This ameliorating effect of morin observed in the present gross and histological study is supported by the result of: (i) Bhuvaneswari *et al*. (2019) revealing recovering effect of beetroot juice on fetal growth retardation and congenital malformations with cadmium exposure *via* its vasodilatory effect promoting embryonic angiogenesis, (ii) Vasiljeva *et al*. (2018) showing protective effect of *Beta vulgaris* juice fraction against cadmium-induced oxidative damage in cadmium-treated chicken providing immunomodulating effect, (iii) Miltonprabu and Manoharan (2016) stating that grape seed proanthocyanidins had a hepatoprotective impact due to their free radical scavenging, antioxidant and metal chelating properties, which help to protect rats' liver from cadmium-induced mitochondrial damage, and (iv) Dkhil *et al*. (2020) reporting *Pleurotus ostreatus* extracts reduced hepatic cadmium poisoning by improving histopathological deficits and decreasing accumulated cadmium in liver tissue of cadmium-intoxicated rats through reduced oxidative stress and decreased Nrf2 expression.

5. Conclusion

Cadmium potentially arrests neovascularisation in chorioallantoic membrane of chicken embryo and also impairs the cellular architecture of liver, kidney and heart of fertilised chicken embryo. Morin has shown protective effect: (i) to the growing chicken embryo by ameliorating the decreased neovascularisation in chorioallantoic membrane, and (ii) on the normal cellular architecture by interfering the toxico-pathological changes in liver, kidney and

heart of growing chicken embryo treated with cadmium. This improvement in the histological alterations caused by cadmium further confirmed the hepatoprotective, cardioprotective and renal protective nature of morin against cadmium. In conclusion, it seems possible that morin can act as an antioxidant, scavenger of free radicals to improve the unfavourable state of liver, heart and kidney revealing its potential in attenuation of cadmium-induced toxicity.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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