Growth and cardiovascular development are repressed by florfenicol exposure in early chicken embryos

Dongfang Hu,[*,†,a] Fanliang Meng,[‡,a] Yunli Cui,[*,a] Mei Yin,[*] Hongmei Ning,[*] Zhihong Yin,[*,†] Lingli Chen,[*] Yaming Ge,[*,1] and Sidang Liu[‡,1]

[*]*College of Animal Science and Technology, Henan Institute of Science and Technology, Xinxiang 453003, China;* [†]*Postdoctoral Research and Development Base, Henan Institute of Science and Technology, Xinxiang 453003, China; and* [‡]*College of Animal Science and Technology, Shandong Agricultural University, Tai'an 271018, China*

ABSTRACT Florfenicol (**FLO**) is one of the most popular antibacterial drugs used in veterinary clinics and aquaculture. The drug was found to decrease the hatchability of eggs laid by treated hens in veterinary clinics and research work. However, the pathological changes in developing embryos and their cardiovascular system and the mechanism underlying FLO-induced embryonic death remain unclear. In the present study, fertilized eggs laid by hens treated with a therapeutic dose of FLO were collected and incubated. Results showed that FLO exposure repressed embryonic development and induced early embryonic death. As a result, FLO decreased the hatchability and increased the proportion of weak chicks. Moreover, FLO exposure led to embryonic lethality and inhibited the development of chick embryos as characterized by decreased weights, lagging distribution of Hamburger–Hamilton stages, and dysplastic eyes. Pathological examination indicated that FLO exposure affected the normal development of the heart in 4.5-day-old chick embryos, as characterized by shorter transverse cardiac diameter, disordered arrangement of trabecular muscles in ventricles, and reduced thickness of ventricular walls. Furthermore, FLO decreased blood vascular densities and down-regulated the expression levels of key angiogenesis-related genes, including the vascular endothelial growth factor and fibroblast growth factor, in the yolk sac membrane. These findings indicated that FLO exposure restricted vascular development during early embryonic development. In summary, our data suggest that the restricted growth and abnormal cardiovascular development may be responsible for FLO-induced early embryonic death. Thus, these findings can be useful for guiding the proper use of FLO and in laying a foundation for further studies on the mechanism of FLO-induced embryonic toxicity.

Key words: florfenicol, chick embryo, embryonic toxicity, embryonic development

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INTRODUCTION

Florfenicol (**FLO**) is an antibacterial drug used in veterinary clinics and aquaculture. The drug, together with chloramphenicol (**CAP**), belongs to amphenicols, which are effective broad-spectrum antibiotics that can bind to the ribosomes and subsequently inhibit microbial protein synthesis in most Gram-positive and Gram-negative bacteria (Cannon et al., 1990). The use of CAP has been banned in food animal production around the world owing to its severe side effects including aplastic anemia and leukemia (Brauer and Dameshek, 1967; Cohen and Creger, 1967; Xiao et al., 2015). Florfenicol is a fluorinated derivative of CAP and was originally used for the treatment of bacterial diseases of the fish, shrimp, crab, and shellfish (Gaikowski et al., 2013; Gaunt et al., 2015; Esmaili et al., 2017). The drug was quickly approved in many countries for the prevention and control of bacterial diseases in pigs, poultry, cattle, and sheep (Ueda et al., 1995; Shin et al., 2005; Budd et al., 2017). In some countries, it is also permitted to be added to livestock diets to improve feed conversion and growth. Moreover, FLO can inhibit the production of various inflammatory factors and the inflammatory response of the body (Zhang et al., 2008; Xinxin et al., 2011; Zhang et al., 2011), indicating its potential application value in the treatment of septic shock and respiratory inflammation.

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[a]These authors contributed equally to the work.

[1]Corresponding authors: ndgeyaming@126.com (YG); liusidang@126.com (SL)

With the wide application of FLO, an increasing number of studies have reported its toxic side effects. So far, FLO-induced immunotoxicity, reproductive toxicity, and embryonic toxicity have been reported by other researchers and our team (Guan et al., 2011; Hu et al., 2014; Al-Shahrani and Naidoo, 2015; Hu et al., 2016). The administration of FLO (12 mg/kg/day for 90 D) to rats could reduce epididymal weights, decrease pup survival, and reduce milk production. The administration of FLO (400 mg/kg by gavage) to pregnant mice could induce embryonic lethality. Moreover, treatment of hens at doses of 60 and 90 mg/kg resulted in decreased hatchability of 0% in comparison with 70% of the control as early as 24 h after treatment. The toxic effects of FLO are completely reversible with comparable hatchability being present by day 4 after treatment withdrawal (Al-Shahrani and Naidoo, 2015). Florfenicol has been widely used in the breeding of cattle, pigs, and poultry in some provinces of China, which is accompanied by increasing reports focused on high levels of FLO residue in animal foods and environmental pollution caused by FLO in breeding waste (Zong et al., 2010; Guo et al., 2016; Wei et al., 2016). Some farmers often use FLO in laying hens for the treatment of bacterial disease. Florfenicol residue in eggs not only poses potential public health problems but also decreases the economic benefit of breeding by decreasing egg hatchability.

The cardiovascular system is the first system to emerge during embryonic development and plays critical roles in the distribution of blood to various tissues to fulfill oxygen and nutrient requirements. This system is composed of the heart and the vascular system, which are derived from the embryonic mesoderm and extraembryonic yolk sac (Wang et al., 2015a). The formation of the heart is a complex morphogenetic process that depends on the spatiotemporally regulated contribution of cardiac progenitor cells (Vincent and Buckingham, 2010). The heart initially forms in an embryonic disc as a simple paired heart tube, followed by the formation of a straight heart tube and the right looping of the heart tube and septation during early embryonic development (Linask, 2003). Vascular development occurs in many regions as the mesoderm differentiates into blood islands. These blood islands then participate in the generation of blood vessel epithelium and fetal red blood cells. Eventually, the primary blood plexus generated from the joining of blood islands is formed and connects with the forming heart tube (Bikfalvi, 2017). Many reproductive toxins, including 2, 5-hexanedione (Cheng et al., 2015), aluminum (ElMazoudy and Bekhet, 2016), ethanol (Wang et al., 2016), high salt (Wang et al., 2015a), and high glucose (Jin et al., 2013), have been reported to induce embryonic lethality and developmental deficiency through inhibiting angiogenesis and cardiovascular development.

After an extensive literature review, we found that no pathological study on embryos exposed to FLO and the development of their heart and vascular system has been conducted. Therefore, the influence of FLO on the development of embryos and their cardiovascular system should be deeply and more thoroughly understood. The main objective of this study is to establish the toxic effects of FLO on chicken embryos, clarify the pathological changes in FLO-exposed embryos and the cardiovascular system, and lay a foundation for further studies on the mechanism of FLO-induced embryonic toxicity.

MATERIALS AND METHODS

Hens and Experimental Design

Two hundred eighty laying Hy-Line hens were selected from a commercial breeder farm and were already vaccinated for local conditions. The birds were equally divided into 2 groups and housed in cages in naturally ventilated rooms. The birds had access to a standard diet and potable municipal water ad libitum. Research was approved by the Animal Use and Care Committee of Shandong Agricultural University. Florfenicol was purchased from Guobang Pharmacy (Zhejiang, China) and mixed with the fodder at the concentration of 50 mg/kg. Treatment was administrated to the hens in the FLO group once daily for 5 D. Before treatment, the hens were all deemed to be clinically healthy, within normal weight limits, with all treatment groups having equivalent egg hatchability to the control.

Monitoring of Hens and Chick Embryos

The hens were monitored daily for clinical signs of toxicity. The fertilized eggs that were collected at day 6 were selected for incubation (an egg incubator maintained at 37°C to 37.5°C and 50% relative humidity, which was automatically turned on every hour) to determine fertility by candling, egg breakouts, or hatchings. In total, 100 eggs were incubated in each group. Ten incubated eggs were randomly selected and subjected to breakouts at 3, 8, 11, 14, and 18 D after incubation in each group. The mortalities were determined as the percentages of dead embryos at the indicated time points. The hatched chicks were housed in standard conditions and immunized with combined Newcastle disease and avian influenza (subtype H9) inactivated vaccine at 10 D after hatching. The chicks were weighed at 9, 15, and 22 D after hatching. Peripheral serum was collected, and the specific antibody levels against avian influenza virus and Newcastle disease virus were determined by using the hemagglutination inhibition test. Five chicks in each group were sacrificed for general necropsy and determining organ indices at 15 and 22 D and hatching, respectively.

Egg Injection and Sample Collection

Fertilized eggs (55 ± 5 g) were obtained from a commercial breeder farm and incubated at 37°C to 37.5°C at 50% relative humidity. After 24 h of incubation, the chick embryos were injected with FLO to mimic an

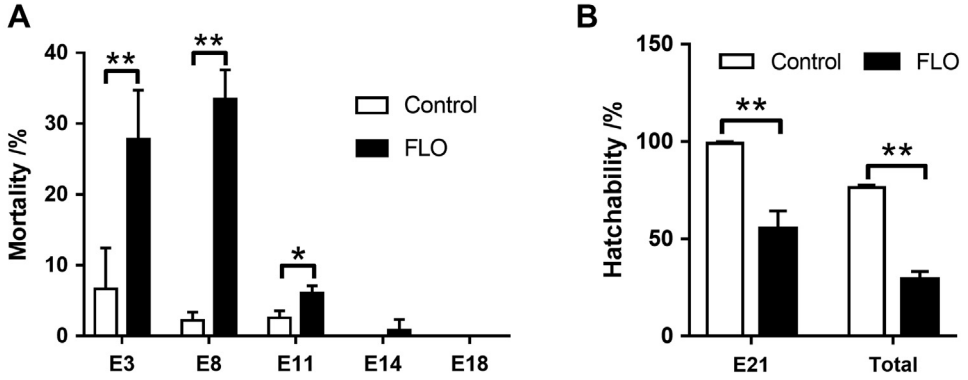

Figure 1. Embryonic mortality and hatchability of eggs collected from treated hens. (A) The mortalities of 3-, 8-, 11-, 14- and 18-day-old embryos. Ten eggs were broken, and the mortality was obtained by determining the ratio of dead embryos number to the total number at each time point. (B) The hatchability of chicks counted at day 21 after incubation. The hatchability of 21-day-old embryos and the hatchability obtained by comparing the number of hatched chicks to the total number of incubated eggs are shown. $^{*}P < 0.05$, $^{**}P < 0.01$.

embryonic exposure environment of FLO. Florfenicol was dissolved in a nontoxic solvent (propanediol: dimethylformamide: glycerin [19:6:5]) (Hu et al., 2016) to prepare 3.2 and 1.6 mg/mL solutions. The fertilized eggs, treated using routine disinfection with 75% alcohol, were punched with a sterile needle at the blunt air chamber and injected with freshly prepared FLO solutions at 1 μL/g. Thus, the final concentration of FLO surrounding the developing embryos was 3.2 μg/g (Al-Shahrani and Naidoo, 2015) in the high-FLO group (n = 30) and 1.6 μg/g in the low-FLO group (n = 30).

The treated embryos were incubated for several days as designed and were subjected to breakouts to determine the developmental stages as per the standard charts (Hamburger and Hamilton, 1951). Survival rate from the breakouts is presented as the percentage of eggs with viable fetuses. All the harvested embryos were photographed using a stereomicroscope (Olympus MVX10, Tokyo, Japan) before they were fixed in 4% paraformaldehyde for histological analysis. Only live embryos were used for further research. The morphology of the vessel plexus were photographed under the stereomicroscope. The areas occupied by the blood vessel plexus were quantified using an IPP 5.0 image analysis program. The blood vessel density was expressed as the percentage of area occupied by the blood vessels over the whole area under the microscopic field. The yolk sac vasculatures were then collected and stored at −80°C for further analysis.

Histopathological Examination

For histopathological examination, the eyes and hearts of the treated embryos were dehydrated, embedded in paraffin wax, and serially sectioned at 5 μm using a microtome (Leica RM2125RTS, Bensheim, Germany). The sections were dewaxed in xylene, rehydrated, and stained with the hematoxylin and eosin dye. The sections were photographed using a digital camera system (CX41, Olympus).

RNA Isolation and Quantitative Polymerase Chain Reaction

Total RNA was isolated from the yolk sac vasculatures of the treated chick embryos using RNAiso Plus (Takara Biomedical Technology Co., Ltd. Beijing, China). The first-strand cDNAs were synthesized and quantitative measurements were performed with Fast qPCR Mix (Takara, RR430). Normalized expression levels were then calculated using the starting levels of peptidylprolyl isomerase A in each sample to normalize for differences in total RNA content in individual samples. The sets of primers used for quantitative PCR were designed as per a previous report (Wang et al., 2016).

Data Analysis

Data are shown as the mean ± SD. Data were analyzed using a one-way ANOVA followed by a least significant difference multiple comparison or independent sample *t*-test by SPSS 20.0 software (SPSS, Chicago, IL). *P* value less than 0.05 was considered statistically significant.

RESULTS

Florfenicol Induces Early Embryonic Death and Decreases the Hatchability of Eggs Collected From the Treated Hens

The administration of FLO to laying hens in the veterinary clinic caused chicken embryonic lethality. The embryonic survival and hatchability of the fertilized eggs collected from untreated (n = 140) and FLO-treated (n = 140) hens were carefully evaluated to highlight the effect of FLO on chicken embryo. Florfenicol was mixed in fodder at a dose of 50 mg/kg for 5 D. Eggs were collected for incubation to determine fertility by candling, egg breakouts, or hatchings. As shown in Figure 1A, the administration of FLO to laying hens mainly induced early embryonic death. The mortalities of chicken embryo at embryonic day 3 (E3), 8 (E8),

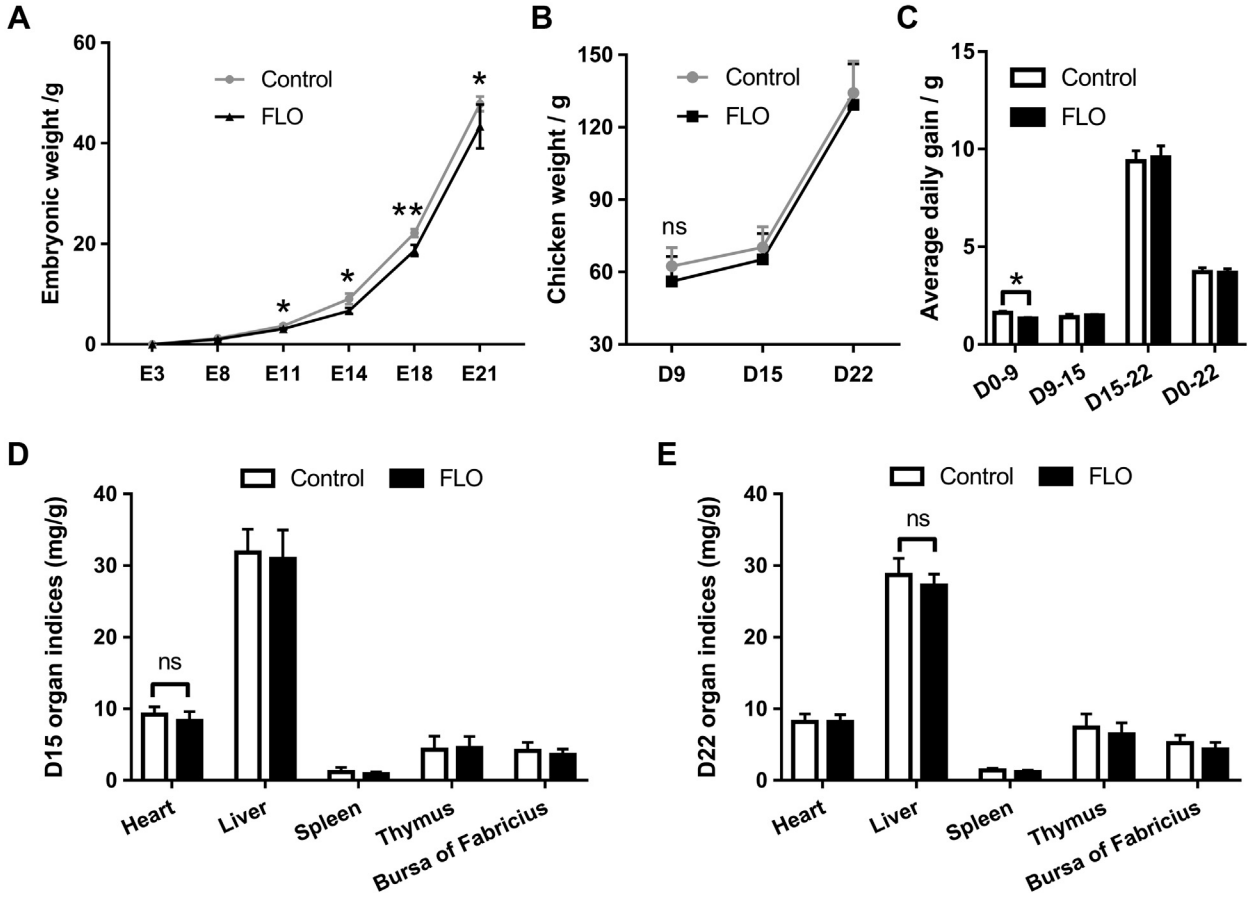

Figure 2. Embryonic development was repressed by FLO in eggs collected from treated hens. (A) Embryonic weights at the indicated time points. (B) Chicken weights obtained at 9, 15, and 22 D after hatch. (C) Average daily gains of the hatched chickens. Organ indices of the indicated organs obtained at 15 D (D) and 22 D (E) after hatch. Abbreviation: FLO, florfenicol.

and 11 (E11) were 27.78, 33.47, and 6.07%, respectively. The mortalities of embryos significantly decreased since E14 to hatching. The rate of hatchability in the FLO-treated group was 55.13% at E21, which was significantly lower than that in the untreated group (98.92%) (Figure 1B). The fertility of the untreated flock was generally excellent, and of the whole incubation process, the incubation rate was 76.25%, which was comparable with the results of a previous report (Al-Shahrani and Naidoo, 2015). The fertility of FLO-treated flock (29.17%) was significantly lower than that of the untreated group. These results indicated that FLO induced early embryonic death and decreased the hatchability of eggs collected from the treated hens.

Florfenicol Represses Embryonic Development of Eggs Collected From Treated Hens before Hatching

We examined the differences in embryonic development and chicken growth between the FLO-treated and untreated groups. Chicken embryos were weighed at E3, E8, E11, E14, E18, and E21. Significant differences in embryonic weight were observed since E11 (Figure 2A), indicating that FLO exposure in laying hens restricted the development of embryos. We further evaluated the differences in growth rate and organ development of chickens at 9, 15, and 22 D after hatching between the 2 groups. Surprisingly, no significant differences in the chicken weight were observed between the 2 groups after hatching ($P > 0.05$) (Figure 2B), even though FLO significantly decreased the embryonic weight at E21 (Figure 2A). No significant differences in average daily gain were observed between the 2 groups (Figure 2C). Moreover, some chickens at 15 and 22 D of age were removed from the pen and weighed. The chickens were then subjected to necropsy. The heart, liver, spleen, thymus, and bursa of Fabricius were removed and weighed to calculate the organ indices. As shown in Figure 2D and 2E, FLO exposure in laying hens did not restrict the development of the selected organs after hatching.

Our previous experiments showed that the administration of FLO in mice and piglets decreased their humoral and cellular immune responses (Hu et al., 2014; Hu et al., 2016). To study whether FLO exposure in laying hens could impact the immune response of the hatched chickens, the chickens were immunized with combined Newcastle disease and avian influenza (subtype H9) inactivated vaccine at 10 D after hatching, and the peripheral serum antibody levels were detected. The

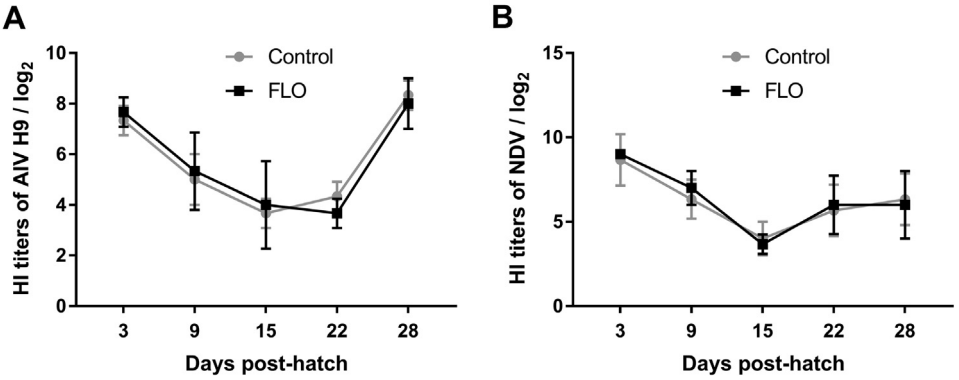

Figure 3. The serum antibody levels of the hatched chickens. The specific antibody levels against avian influenza virus (subtype H9) (A) and Newcastle disease virus (B) were determined by using the hemagglutination inhibition (HI) test at the indicated time points.

antibody levels gradually decreased after hatching until the immunization and increased after immunization (Figure 3A and 3B). However, no significant differences in the specific antibody levels were observed between the 2 groups at the selected time points, which agreed with the results of immune system organ indices (Figure 2D and 2E). Taken together, FLO exposure in laying hens repressed embryonic development before hatching and did not influence the growth and organ development of chickens after hatching.

Florfenicol Exposure Leads to Embryonic Lethality and Inhibits the Development of E7 Chick Embryos

To understand the possible mechanism underlying the embryonic lethality, an egg injection model of FLO was established to mimic an embryonic exposure environment of FLO. Florfenicol was dissolved (Hu et al., 2016) to prepare 3.2 and 1.6 mg/mL solutions and was injected into E1 fertilized eggs (55 ± 5 g) through the eggshell window (1 μL/g). Thus, the final concentrations of FLO surrounding the developing embryos were 3.2 μg/g (Al-Shahrani and Naidoo, 2015) in the high-FLO group (n = 30) and 1.6 μg/g in the low-FLO group (n = 30). The treated embryos were incubated for 6 D. After 6 D, 22.63 ± 1.95 and 12.11 ± 2.91 of 30 embryos were dead after exposure to high- and low-FLO treatments, resulting in E7 survival rates of 24.58 and 59.63%, respectively (Figure 4A).

All the embryos in each group were removed and weighed. As shown in Figure 4B, the embryos from the control group (solvent injected) showed normal morphology (Hamburger and Hamilton, 1951). The 3 major segments of the wing and leg were clearly demarcated. The gap between the mandible and the beak narrowed to a small notch. Eye pigmentation was distinct, and one or more scleral papillae were observed on either side of the choroid fissure. The embryos collected from the FLO-exposed group showed abnormal development as characterized by barely recognizable beak, not yet demarcated digits in the wing and toes, indistinct elbow and knee joints, and faint eye-pigmentation (Figure 4B). These conditions were more severe in the high-FLO treated group. Florfenicol treatment also significantly decreased the E7 embryonic weight. The weight of embryos in the control group was 4.22-fold (0.696/0.165) and 1.67-fold (0.696/0.418) higher than that in the high- and low-FLO treated groups, respectively (Figure 4C). Moreover, we counted the distribution of the Hamburger–Hamilton (**H&H**) stages of embryos from each group by checking the morphology of the embryos (Hamburger and Hamilton, 1951). As shown in Figure 4D, most of the embryos in the control group developed H&H stage 29 (E6.5), whereas those in the high- and low-FLO treated groups had mean H&H stages of 21 (E3.5) and 25 (E4.5), respectively.

As mentioned previously, FLO exposure restricted the development and pigmentation of the eyes in E7 embryos (Figure 4B). To further check the influence of FLO on early development of the eyes, histopathological examination was performed in E4.5 embryos, which were exposed to 1.6 μg/g of FLO. Florfenicol exposure in E4.5 embryos induced more embryos with deficient eyes. Embryos with smaller or no eyes were observed, and eye pigmentation was faint (Figure 4E). By viewing the transverse sections of these deficient eyes, we observed swollen and structurally unclear retinal progenitor cells. Taken together, FLO exposure led to embryonic lethality and inhibited the development of early chick embryos.

Florfenicol Exposure Affects the Normal Development of the Heart in E4.5 Chick Embryos

The cardiovascular system is the first organ system to develop, as subsequent embryonic development relies on its physiological function. Therefore, severe embryonic heart defects can cause embryonic death. We compared the developing hearts of the control and FLO-exposed (1.6 μg/g) live E4.5 embryos. Well-developing hearts were observed in the control group, whereas poorly developed hearts, which were characterized by the

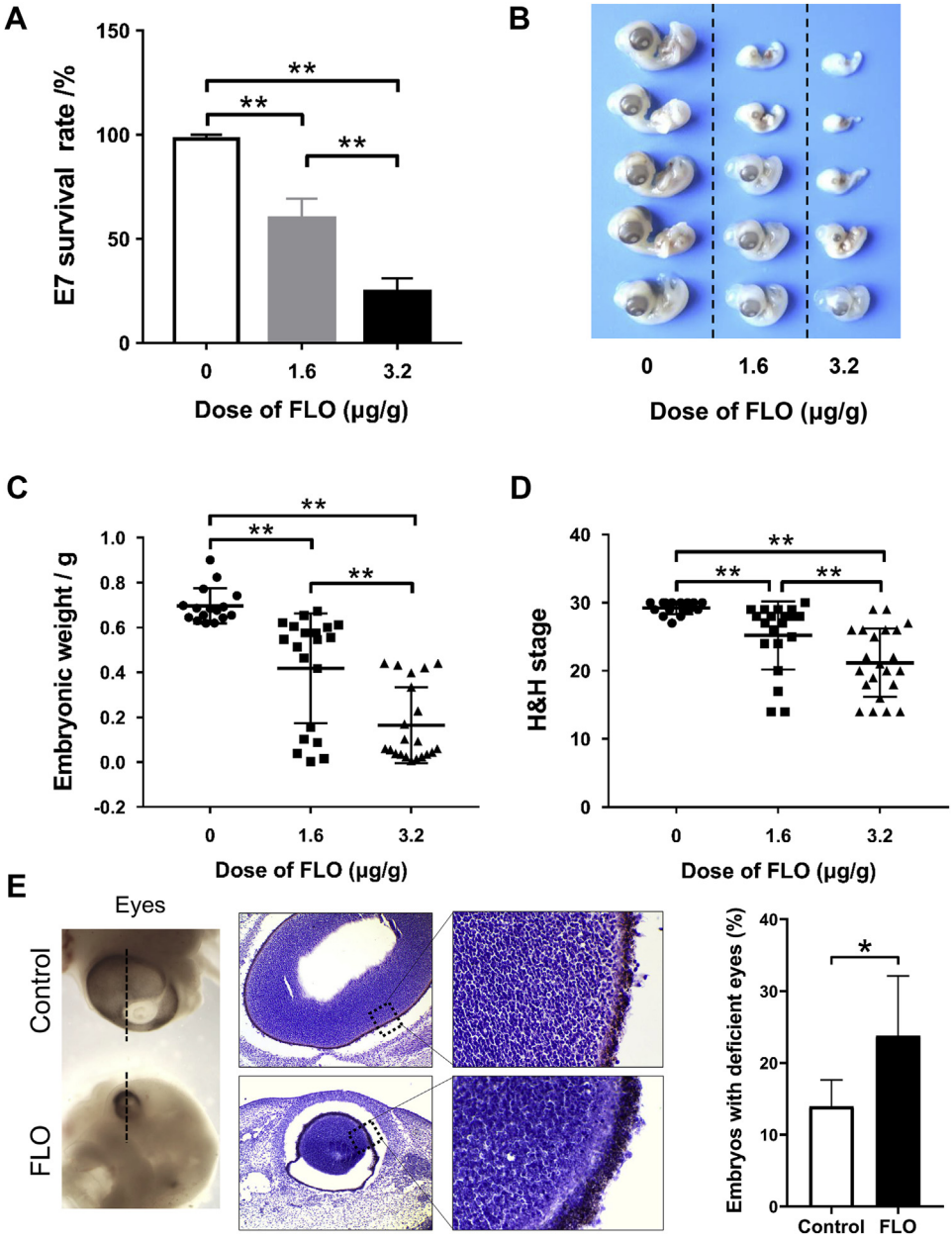

Figure 4. The survival and development of early chick embryos treated with FLO by egg injection. (A) The survival rates were determined by comparing the number of living embryos with the total number of incubated eggs at the seventh day after incubation. (B) Representative images of the 7-day-old chick embryos that were treated with different doses of FLO. Embryonic weights (C) and the distribution of H&H stages (D) of the 7-day-old embryos. (E) Transverse sections of eyes in 4.5-day-old embryos were taken at the sites indicated by dashed lines. The proportions of embryos with deficient eyes in the FLO-treated (1.6 μg/g) and the control group are shown in the bar graph. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: FLO, florfenicol; H&H, Hamburger–Hamilton.

shorter transverse cardiac diameter, occurred in the FLO-treated group (Figure 5A). Histopathological examination revealed some differences in the structures of ventricle and cardiomyocyte morphology. A disordered arrangement of trabecular muscles in the left and right ventricles was observed in the FLO-exposed embryos. The trabecular muscles were distributed in a reticular pattern without an obvious beam structure (Figure 5B), and some cardiomyocytes were observed with condensed nucleus and reduced cytoplasm. Moreover, FLO exposure to embryos significantly reduced the thickness of the left (control: 60.33 ± 7.84, n = 6; FLO-treated: 46.83 ± 5.91, n = 6) (Figure 5B and 5C) and right (control: 44.80 ± 5.26, n = 6; FLO-treated: 22.40 ± 5.77, n = 6) ventricular walls (Figure 5B and 5D). These results strongly suggested that FLO restricted normal heart development in early embryos, which may play an important role in FLO-induced embryonic death.

Florfenicol Restricts Embryonic and Vascular Development of E3 Chick Embryos

Vasculogenesis and angiogenesis play critical roles in embryonic development. Thus, we determined

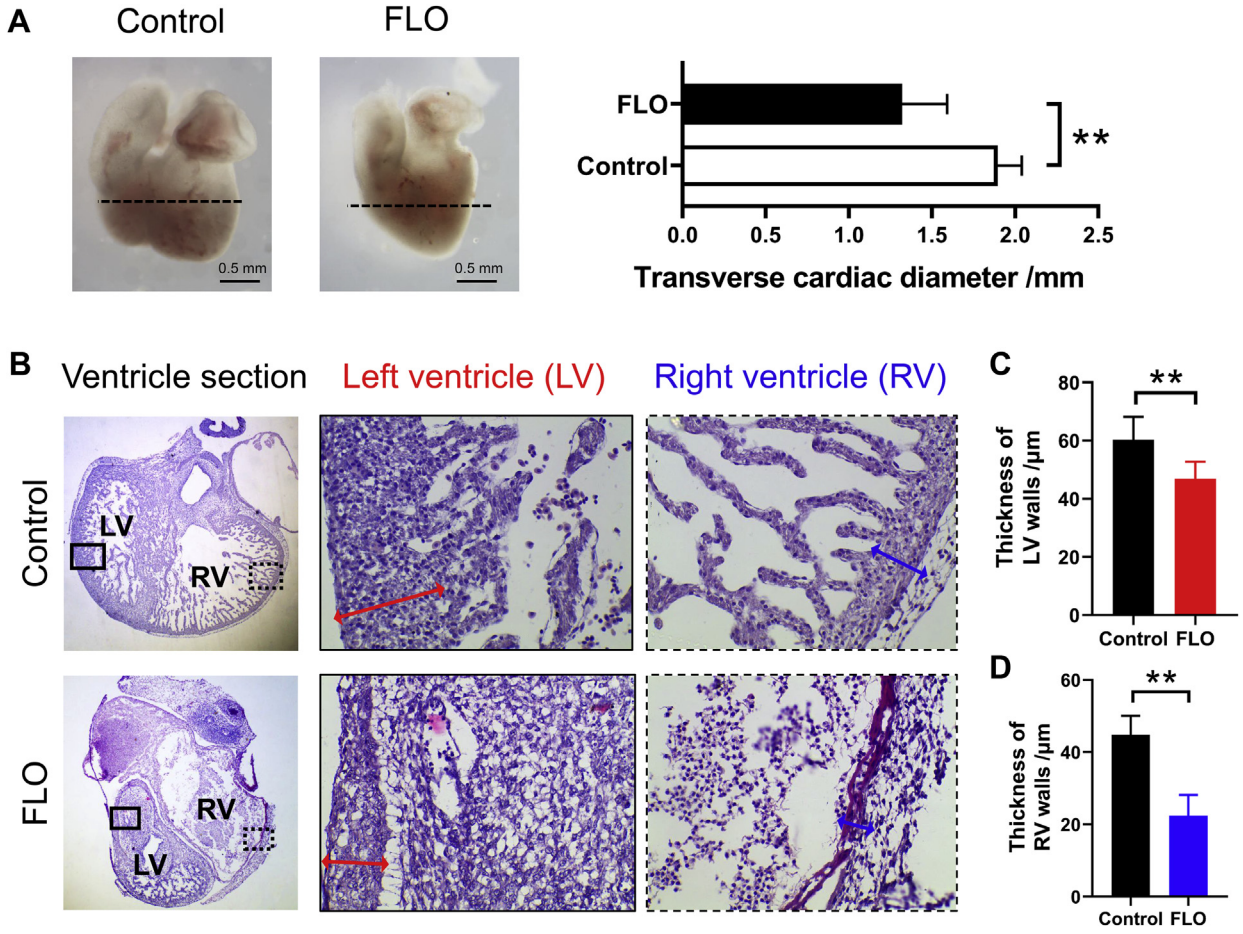

Figure 5. Morphological alteration of the hearts taken from 4.5-day-old embryos induced by FLO exposure. (A) The representative hearts of embryos in the FLO group (1.6 μg/g) and the control group. Bar graph is showing the differences in transverse cardiac diameter between the FLO group and the control group. (B) Transverse sections of hearts were taken at the sites indicated by dashed lines in (A). The ventricle sections were stained by H&E and the thickness of the left and the right ventricular walls are shown in (C) and (D), respectively. $^{**}P < 0.01$. Abbreviations: FLO, florfenicol; H&H, Hamburger–Hamilton.

whether angiogenesis was normal in FLO-exposed early embryos. FLO was administered to the embryos, and the chick yolk sac membrane (**YSM**) model in the extraembryonic area opaca was studied as it is the earliest region of angiogenesis and can be easily manipulated. We examined the development of blood plexuses at the same location in the YSM in the control and FLO-exposed embryos (Figure 6A). The blood vascular densities of the left and right omphalomesenteric vessels and posterior vitelline veins were lower in the presence of FLO (Figure 6C), indicating that FLO exposure inhibited angiogenesis in the chick yolk sac. Moreover, as mentioned previously, FLO also restricted the development of E3 embryos, which was reflected by the decreased length of FLO-exposed embryos (Figure 6B). We further detected the expressions of key angiogenesis-related genes in the YSM after FLO exposure in E3 embryos (Figure 7). Florfenicol treatment downregulated the expression levels of vascular endothelial growth factor and fibroblast growth factor 2 in the YSM. These results indicated that FLO exposure in E3 chick embryos restricted embryonic and vascular development.

DISCUSSION

Florfenicol is an intensively used antibiotic that was developed to replace CAP and thiamphenicol to eliminate their toxic side effects on humans and animals. However, with the use of FLO, its hematopoietic toxicity, immunotoxicity, genetic toxicity, and embryonic toxicity have attracted increasing attention (Guan et al., 2011; Hu et al., 2014; Al-Shahrani and Naidoo, 2015; Hu et al., 2016; Ren, et al., 2017). Previous reports have showed that the exposure of FLO to laying hens resulted in a reversible reduced hatchability of 70 to 80% because of the inhibition of hatching (Al-Shahrani and Naidoo, 2015). However, the mechanism underlying FLO-induced embryonic toxicity and the pathological changes in FLO-exposed embryos remain unclear. In this study, we administered FLO to laying hens in a controlled manner to mimic the clinical application of FLO and tracked the survival and hatchability of the exposed embryos. The growth performance and immune function of the hatched chickens until 28 D after hatching were also monitored. Then, we established an egg injection model of FLO based on the chicken embryo test and evaluated the influence of

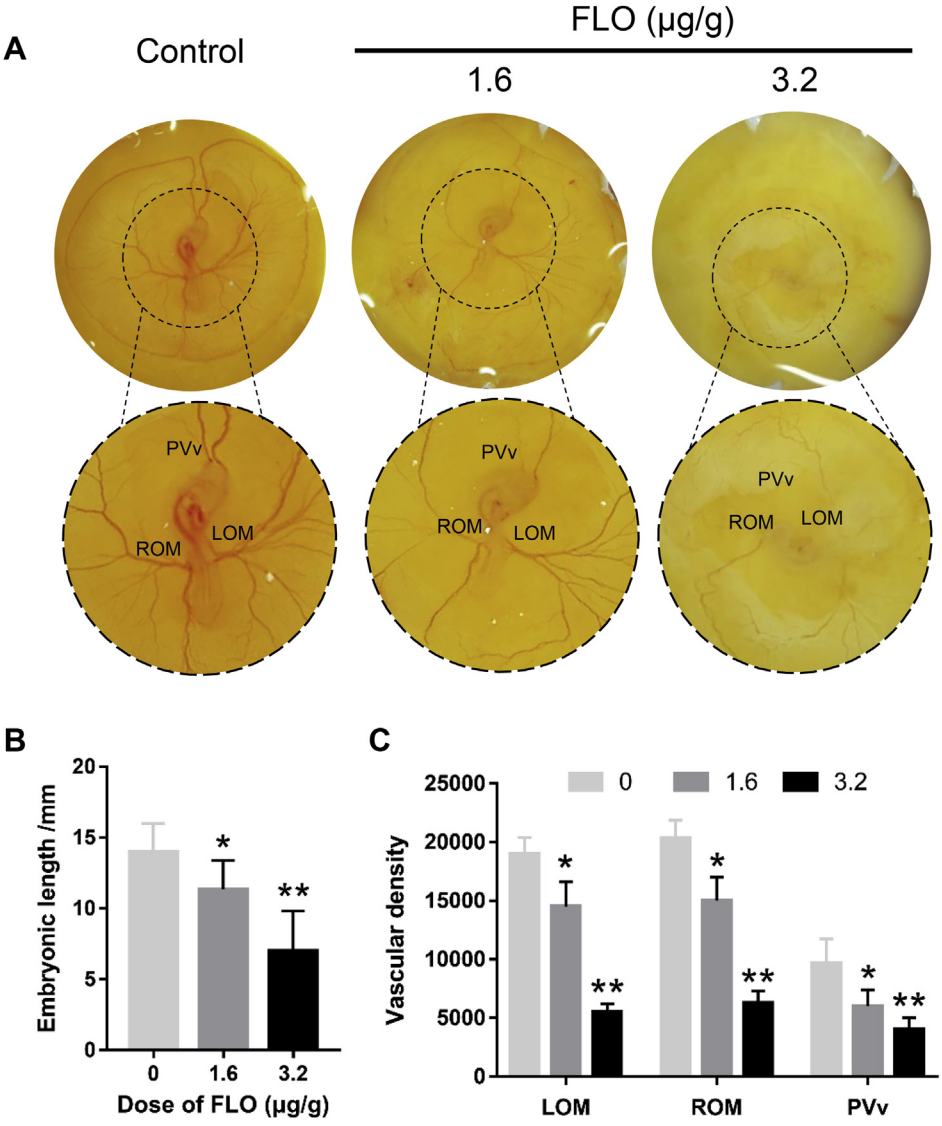

Figure 6. FLO reduces the density of the vascular plexus in the yolk sac membrane of the embryos that were exposed to FLO for 3 D. (A) Representative images of the developing embryos and the yolk sac vasculature. Three major blood vessels are indicated in each image—LOM, left omphalomesenteric vessel; ROM, right omphalomesenteric vessel; PVv, posterior vitelline veins. (B) The length of 3-day-old embryos in each group. (C) Quantification of the density of blood vessels of the yolk sac vasculature analyzed by using an Image-Pro plus 6.0 program. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviation: FLO, florfenicol.

FLO on the growth and development of embryos and on the development of cardiovascular system. To the best of our knowledge, this is the first report regarding the developmental toxicity and pathological changes associated with the use of FLO in breeding hens and in developing chick embryos.

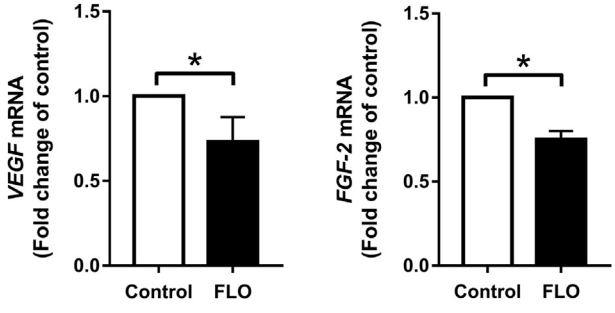

Figure 7. FLO exposure inhibits expression of angiogenesis-related genes. Quantitative RT-PCR analysis showing the effect of FLO (1.6 μg/g) on the mRNA expression levels of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-2 genes in the yolk sac vasculature of 3-day-old embryos. $^{*}P < 0.05$. Abbreviation: FLO, florfenicol.

Laying hens administered with FLO at a dose of 50 mg/kg for 5 D did not induce any overt sign of toxicity or pathological changes. Egg breakouts showed that FLO-induced embryonic death mainly occurred at approximately day 3 to day 8 of development (Figure 1A) and a hatchability of 29.17% was counted (Figure 1B). These results were consistent with those of previous reports (Al-Shahrani and Naidoo, 2015). Moreover, during hatching, some chicks could not drill the shell successfully, indicating that FLO increased the number of weak chicks. By checking the weights of embryos by breaking out and the growth of hatched chicks, the results revealed that FLO exposure in laying hens only restricted the development of the embryos (Figure 2). Moreover, we examined the development of immune system organs and detected the specific

antibody levels in hatched chickens that were generated by FLO-exposed hens because the immunotoxicity of FLO had been highlighted (Guan et al., 2011; Hu et al., 2014). We previously found that FLO exposure in piglets and mice decreased the organ indices, damaged the structure, and induced the death of immunocytes in the spleen and thymus (Hu et al., 2014; Hu et al., 2016). Here, we surprisingly found that FLO exposure in laying hens did not restrict the development of immune system organs (Figure 2) and did not affect immunity (Figure 3) of hatched chickens, which suggested that the immunotoxicity of FLO might be caused by the direct damage of immune system organs and immunocytes but not developmental toxicity.

For this study, we established an egg injection model of FLO to mimic an embryonic exposure environment of FLO. Florfenicol exposure (1.6 μg/g) to developing embryos significantly increased mortality, as demonstrated by a survival rate of 59.63% in E7 embryos (Figure 4A). Morphological examination showed that FLO exposure induced abnormal development as characterized by the barely recognizable beak, not yet demarcated digits in the wing and toes, indistinct elbow and knee joints, and faint eye pigmentation (Figure 4B). Meanwhile, FLO restricted the development of E7 embryos when counting the developmental stages according to the H&H method (Hamburger and Hamilton, 1951). Moreover, we found that the development of eyes was restricted as characterized by faint pigmentation and the observation of swollen and structurally unclear retinal progenitor cells via histopathological examination (Figure 4E). These results suggested that the model, which was established by injecting FLO to fertilized eggs at a dose higher than 1.6 μg/g based on the corresponding concentration of FLO in the eggs generated by treated hens, could successfully mimic the embryonic exposure condition, which was achieved by treating laying hens with FLO. Certainly, the egg injection model established in this study can be further used to investigate the mechanism of FLO-induced embryonic toxicity in ova.

The cardiovascular system, which is composed of the heart and the vascular system, is the first system to emerge during embryonic development and plays critical roles in subsequent embryonic development. Thus, severe embryonic heart defects and angiogenesis disorder could cause embryonic death. Several compounds that cause embryonic toxicity, such as 2, 5-hexanedione (Cheng et al., 2015), aluminum (ElMazoudy and Bekhet, 2016), ethanol (Wang et al., 2016), high salt (Wang et al., 2015a), and high glucose (Jin et al., 2013), have been shown to induce heart defects and angiogenesis disorder. Our results, which demonstrated that FLO inhibited heart development and angiogenesis, were consistent with a previous report showing that CAP-induced mitochondrial protein synthesis reduced the development of blood islands and vessels in the area vasculosa (Billett et al., 1965). However, the mechanism underlying the FLO-induced restriction of heart and vascular development needs to be further investigated.

Since the 1940s, the discovery and use of antibiotics have made great contributions in the prevention and control of human and animal diseases. So far, more than 40% of antibiotics work by interfering with the bacteria's protein-making machinery, namely, the ribosomes. There are 2 kinds of ribosomes in animal cells, namely, cytoplasmic and mitochondrial ribosomes. Mitochondrial ribosomes share more structural similarities and chemical properties with bacterial ribosomes than with eukaryotic cytoplasmic ribosomes because the mitochondria originate from the endosymbiosis of α-proteobacteria (O'Brien, 2002). Because of the similarities between mitochondrial and bacterial ribosomes, the antibiotics developed to inhibit bacterial ribosomes inevitably inhibit mitochondrial ribosomes, leading to mitochondrial damage (Wang et al., 2015b). Our previous reports showed that FLO inhibits the expression of mitochondrial DNA-encoded proteins, resulting in mitochondrial damage and dysfunction (Hu et al., 2017). Mitochondria are eukaryotic cellular organelles specialized for energy conversion and ATP production. Recently, the mitochondrial roles in regulating cell division and proliferation, cell death, pluripotency and differentiation potential of stem cells, and other complex biological processes have been clarified (Zhong, et al., 2019). A previous report, which aimed to study the effects of D- and L-threo-CAP on the early development of chick embryo, showed that D-threo-CAP produced marked abnormalities, whereas L-threo-CAP almost did not show any effect (Billett et al., 1965). The researchers concluded that the failure of the L-isomer to give rise to the abnormalities caused by the D-isomer was because of their differences in inhibiting mitochondrial protein synthesis. Based on the reports and our previous research, we speculated that the reason of embryonic toxicity caused by FLO is also related to the inhibition of mitochondrial protein synthesis, which needs to be verified by further experiments. Moreover, to reveal the mechanism of embryo toxicity caused by FLO at the cellular level, subsequent studies on the toxic effects, including proliferation inhibition and differentiation disorder of embryonic stem cells, will become our research work.

In summary, our present study provides critical information about the lethality and abnormality in FLO-exposed chick embryos and clarifies the pathological changes in FLO-exposed embryos and their cardiovascular system. Our study may be useful for guiding the proper use of FLO and lay a foundation for further studies on the mechanism of FLO-induced embryonic toxicity.

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