Egypt. J. Vet. Sci. **Vol. 54,** No. 2, pp. 245-252 (2023)

Egyptian Journal of Veterinary Sciences
https://ejvs.journals.ekb.eg/

Florfenicol Induces Proliferation of Splenic Germinal Centers in Chick Embryos Model

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BACKGROUND: Florfenicol (Flo) is one of the widely used antibacterial in food-producing animals. There is controversy about the histological changes in splenic immunocyte induced by Flo. **Objective:** The proliferation of splenic germinal centers (SGCs) in poultry is considered a marker of an immune response.Therefore, chick embryos were used as a model in our study, as they have a high cellular proliferation rate. **Methods:** Thirty fertilized eggs were divided into three groups (10 eggs/group), C as control, F1 and F2 treated on day 12 of incubation with distal water, 75 and 150 mg/kg of Florfenicol in air cell, respectively. Then, on day 18 of incubation, chick embryos were sacrificed for spleen harvesting to examine the histological changes using TUNEL assay (Terminal deoxynucleotidyl transferase dUTP nick end labeling) to detect DNA fragmentation (apoptosis) and hematoxylin and eosin stain to count blood vessels (BVs) and splenic germinal center (SGCs) and their diameter. Chick embryos exposed to Flo led to splenic histological changes in a dose-dependent manner, represented by a significant increase in the number of both BVs and SGCs but the diameter of these SGCs decreased significantly due to the occurrence of apoptosis in the F2 group compared with control and F1 groups. **Conclusion:** We concluded that Flo induced proliferation in the SGCs of chick embryos, which is considered as an indicator of the immune response that depended in its intensity on the dose and to regulate and modify this response by apoptosis, which is recorded for the first time.

Keywords: Florfenicol, Splenic germinal centers, Chick embryos, Proliferation, Apoptosis.

Introduction

Florfenicol (Flo) is one of the widely used antibacterial in the field of veterinary medicine in treatment of food-producing animals (cattle, sheep, and poultry) [1]. Thus, it is important to study its effects, including the histological changes caused by this antibacterial in the spleen, which is responsible for humoral and cellular immunity [2] in order to ensure the safety of exposed consumers for residues of this drug in animal products (meat, milk, eggs) [3-6].

The spleen in poultry is one of the largest secondary lymphoid immune organs and has the characteristic that when antigenic stimulation occurs, it will stimulate the formation of splenic germinal centers (SGCs) located in the white pulp zone [7, 8]. Thus, it is possible to take advantage of this point to determine the histological changes in the immunocyte of the white pulp zone of the spleen induced by Flo. To achieve this goal, the chick embryos were selected as a model to study the histological changes in the spleen because the

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(*Received* 05/10/2022; *accepted* 04/11/2022)
DOI. 10.21608/EJVS.2022.167115.1403
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embryos have a high rate of cellular proliferation and differentiation, therefore this model will be highly sensitive to histological changes and its biology and physiology are well-known, gives faster results with simplicity and a lower cost [9,10]. The question that arises is whether Flo will stimulate or inhibit the immune response in the white pulp of the spleen in chick embryos.

Material and Methods

Thirty (30) fertilized eggs were divided into three groups (10 eggs/group), C as control, F1 and F2 treated on day 12 of incubation [10] with Flo (AD FLORICOL-20P - 20% (water soluble)- Advance Aqua Bio Technologies-India) dosed (0 (distal water), 75 and 150 mg/kg, air cell) respectively. Then, on day 18 of incubation, chick embryos were sacrificed for spleen harvesting.

Florfenicol doses (75 and 150 mg/kg) were prepared by dissolving in distilled water (D.W) with volume of injection (0.2 ml/egg). The doses of Flo were calculated in mg/kg per egg weight on basis of the average egg weight of (50±1 g). It is worth noting that the selection of Flo doses (75 and 150 mg/kg) based on preliminary experiments.

The incubation starts at d 0 so that d1 is considered as an incubation period of 24 h. Eggs that did not display a vascular system that normally develop during embryonic development were discarded. On the day 12 of incubation, the eggshell was opened at the blunt end to obtain access to the air cell, where the respective D.W (C) or Flo (F1and F2) in (0.2 ml) was pipetted directly onto air cell. Covering the hole by a piece of adhesive tape ensured the embryo's vitality until spleen harvesting from chick embryos on day 18 of incubation [9].

The spleen collected for each of the chick embryos on day 18 of incubation was fixed in 10% buffered formalin and embedded in paraffin [11]. Thin sections of the fixed tissues were stained with hematoxylin and eosin [12] and examined microscopically for the presence of any tissue changes in the white pulp zone of the spleen.

Imaging the histological sections and count the number of each of the splenic germinal centers [13] and blood vessels of developing chick embryos exposure to Flo using a microscope camera (OMAX 18MP-China) equipped with Toup View software program, the camera was fixed on a microscope (Olympus CX31-Japan) using light microscope under low (×40) and high (×400) magnifications.

In order to measure the diameter of the splenic germ centers, the camera program was calibrated on the four objective lenses of the dissection microscope (Japanese Olympus VMT 1x-2x Inspection Stereo Microscope) using the stage micrometer ruler, and the calibration information was fixed on the computer.

TUNEL reaction For in situ detection of DNA fragmentation (apoptosis) in paraffin embedded issue sections, the TUNEL method was performed using the TUNEL Apoptosis Assay Kit (HRP – DAB) (ab206386- Elabscience company, USA) following the manufacturer's instructions. TUNEL stain was used to detect apoptosis in spleen cells [14].

Statistical analysis

Data are shown as mean + SEM. Statistical analysis was carried out using a one-way analysis of variance and then subjected to LSD test. $P<0.05$ was deemed significant.

Results

The exposure of chick embryo to Flo at a dose (0 (C), 75 (F1) and 150(F2) mg/kg egg weight into air cell) on day 12 of incubation for one time resulted in histological changes in the immunocyte located in the white pulp of the spleen on day 18 of incubation in a dose-dependent manner, it was represented by a significant increase in the number of splenic germinal centers (SGCs) from 42.4 ± 2.13 in control group to 83.4 ± 2.5 and 133.2 ± 2.46 in F1 and F2 groups respectively (Fig. 1,2).

It is important to mention that this increase in the number of SGCs in F2 group was accompanied by a significant decrease in the diameter of these SGCs from 70.5 ± 1.18 and 97.13 ± 2.18 μm in C and F1 groups respectively to 46.92 ± 3.44 μm in F2 group (Fig. 3).

The group (F1) also showed a significant increase in the number of SGCs (83.4 ± 2.5) compared to the control group C (42.4 ± 2.13), (Figure. 2). It is worth noting that the significant increase in these centers in this group was accompanied by a significant increase in their diameters (97.13 ± 2.18) compared to control group (70.5 ± 1.18), (Fig. 3).

The TUNEL assay revealed the occurrence of programmed cell death (apoptosis) in the immunocyte forming the SGCs in chick embryos exposed to Flo. The severity of apoptosis between groups (F1 and F2) was different. So

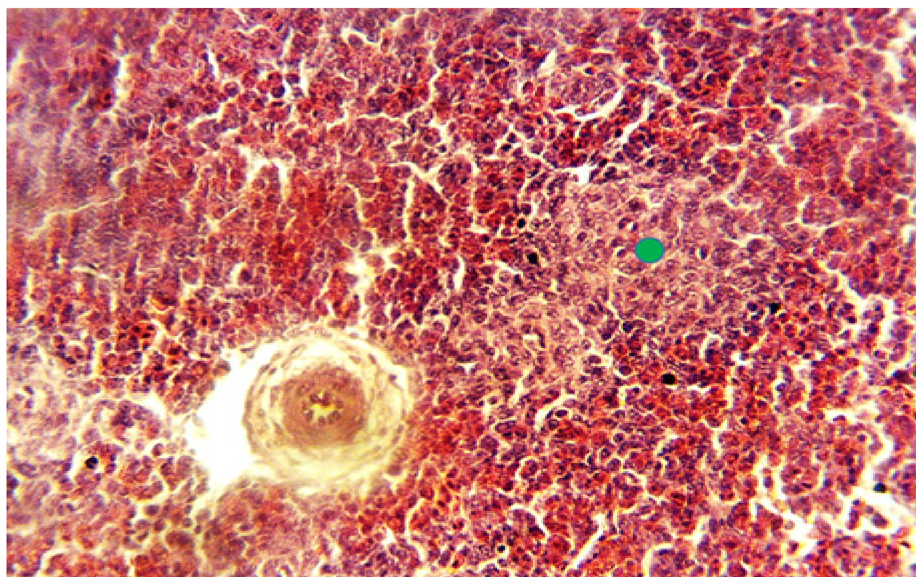

Fig. 1. Splenic germinal center (SGC) of chick embryos on day 18 of incubation exposed to florfenicol at a dose of (150 mg/kg egg weight in the air cells) for only one time on day 12 of incubation . SGC have a pale central zone and completely lack of blood vessels (●). (Hematoxylin and Eosin staining, 400x).

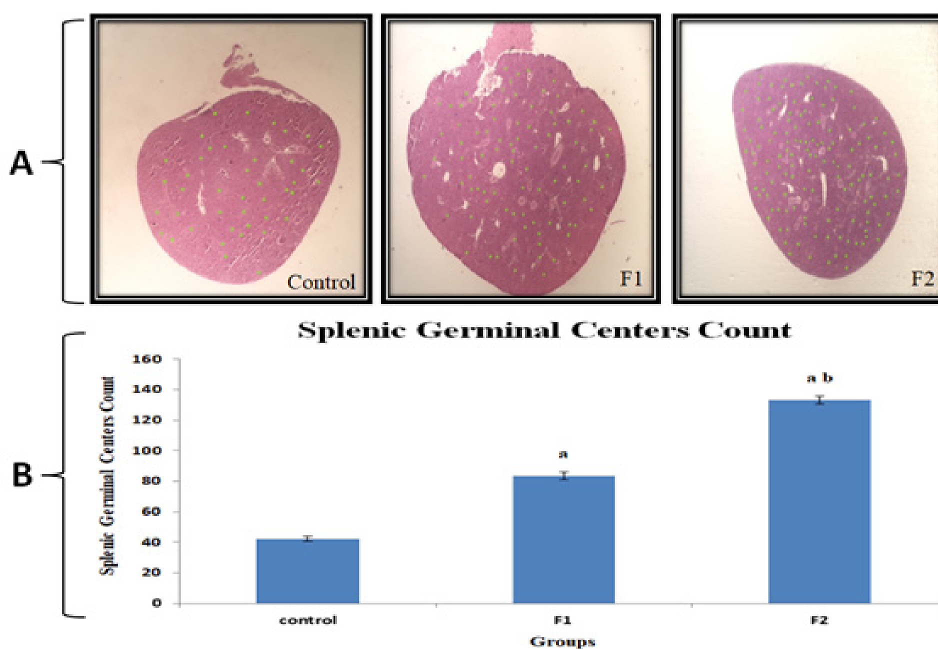

Fig. 2. A-The histological changes in the spleen of chick embryos on day 18 of incubation, exposed to florfenicol at a dose of 0 (C), 75 (F1) and 150 (F2) mg/kg egg weight in the air cells) for only one time on day 12 of incubation. F1 and F2: Shows the induction of florfenicol on the proliferation of splenic germinal centers (green dots) in a dose-dependent manner. Where is C in the Fig. showed normal histological structure of spleen. (Hematoxylin and Eosin staining, 40x)
(green dots): Represents the splenic germinal centers

B- Florfenicol significantly increased the number of splenic germinal centers (means ± standard error, n = 10). a-Significant difference from the control group at $p < 0.05$ level. b-Significant difference from the group treated with Florfenicol (75 mg/kg egg weight) at $p < 0.05$ level.

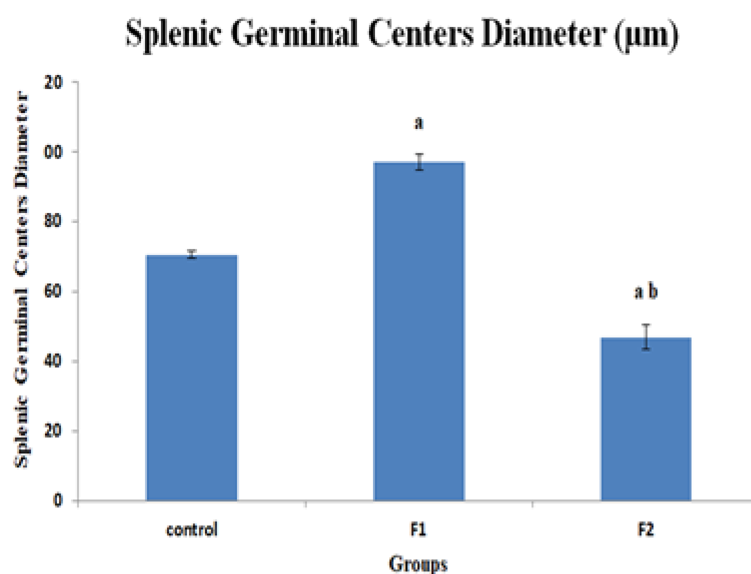

Fig. 3. Shows the effect exposure of chick embryos to florfenicol at a dose of (0 (C), 75(F1) and 150(F2) mg/kg egg weight in the air cells) for only one time on day 12 of incubation on the diameters of the splenic germinal centers on day 18 of incubation .F1: Significant increase in diameter of SGCs. F2:Significant decrease in diameter of the SGCs.

(means ± standard error, n = 10). a-Significant difference from the control group at p < 0.05 level. b-Significant difference from the group treated with Florfenicol (75 mg/kg egg weight) at p < 0.05 level.

that the apoptosis in the F2 group included most of the immunocyte that forming SGCs, while the apoptosis in the F1 group was limited to the immune cells located at the marginal of SGCs (Fig. 4).

With regard to the effect of exposure of chick embryos to Flo on the density of splenic blood vessels (SBVs), the density of these vessels increased in the groups in a dose-dependent manner represented by a significant increase in the density of SBVs from (22.2 ± 1.16 and 33 ± 1.52) in the groups C and F1 to (92.6 ± 1.36) in the group F2 (Fig. 5).

Discussion

The exposure of chick embryos to florfenicol (Flo) led to a significant increase in the number of splenic germinal centers (SGCs). This increase in these centers may be attributed to the fact that Flo or its metabolites led to antigenic stimulation, which supports this explanation to the findings of Yasuda et al. [7] indicated that the increase in the number of SGCs in poultry is considered as an indicator of antigenic stimulation.

The increase in the number of these SGCs was in a manner dependent on the dose of Flo, as the increase in the dose from 75 mg/kg egg weight in (F1 group) to 150 mg/kg egg weight in (F2 group) led to severe antigenic stimulation, which resulted in a massive increase in the number of these SGCs .

It is important to mention that there is a direct relationship between the immune response (increase in the number of SGCs) and apoptosis in the immunocytes of these centers. The evidence for this is that apoptosis in the group treated with a dose of 150 mg/kg egg weight was included most of the immunocyte that forming SGCs because the immune response in this group is more severe compared to the group treated with 75 mg/kg egg weight. While the apoptosis in the group treated with 75 mg/kg egg weight was limited to the cells located at the marginal of these centers, because the immune response in this group is less severe compared to the group treated with 150 mg/kg egg weight.

Scientific references indicate that regulated apoptosis of SGCs cells is important for appropriate SGCs formation and optimal (limits excessive)

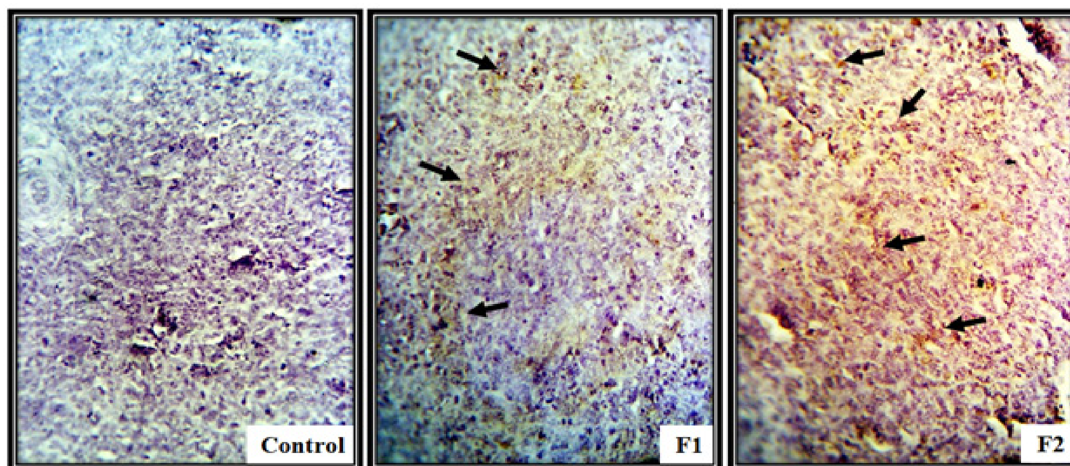

Fig. 4. The TUNEL assay revealed the occurrence of programmed cell death (apoptosis) (➔) in the immunocyte forming the SGCs in the white pulp of chick embryos on day 18 of incubation, exposed to florfenicol at a dose of (0 (C) , 75 (F1) , 150 (F2) mg/kg egg weight in the air cell) for only one time on day 12 of incubation .The severity of apoptosis between groups (F1 and F2) was different. So that the apoptosis in the (F2) group included most of the immunocyte that forming SGCs , while the apoptosis in (F1) group was limited to the immunocyte located at the marginal of SGCs.

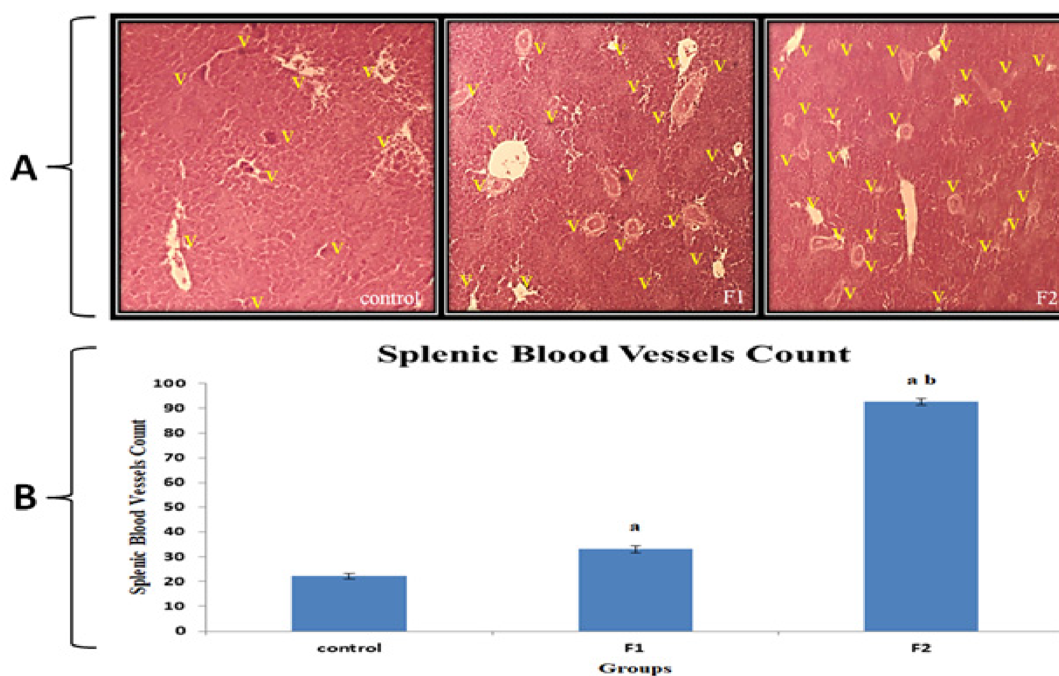

Fig. 5. A-The histological changes in the spleen of chick embryos on day 18 of incubation, exposed to florfenicol at a dose of (0 (C), 75(F1) and 150(F2) mg/kg egg weight in the air cells) for only one time on day 12 of incubation. F1 and F2: shows an increase in SBVs density in groups treated with florfenicol in a dose-dependent manner. Where is C in the Fig. showed the normal density of the splenic blood vessels. (Hematoxylin and Eosin staining, 40x).
(V): Represents the splenic blood vessels

B- Florfenicol significantly increased the number of splenic blood vessels (means ± standard error, n = 10). a-Significant difference from the control group at $p < 0.05$ level. b-Significant difference from the group treated with Florfenicol (75 mg/kg) at $p < 0.05$ level.

humoral immune responses and elimination of self-reactive GC B cells (autoimmune response) [15, 16]. Based on what was mentioned above, the results of our current study can be interpreted about the significant increase in the number of SGCs accompanied by apoptosis of the immunocyte that forming these centers in a Flo dose-dependent manner which is the exposure of chick embryos to Flo (150 mg/kg) induced an excessive immune response, which was expressed by a significant increase in the number of SGCs and in order to regulate the immune response and prevent the production of autoimmune bodies, apoptosis was stimulated. The proof of this interpretation is what the researcher reached [17] about the existence of a factor named ELL-associated factor 2 (EAF2) regulates transcription elongation as an apoptosis inducer for SGCs and in the absence of this factor (EAF2) resulted in excessive humoral immune responses and autoantibody production, this researcher [17] reached these conclusions through their experiments on mice deficient in this factor (EAF2) and immunized with type II collagen a significant increase in the production of autoantibodies accompanied by severe arthritis.

It is worth noting that the results of our current study on the increase in the immune response represented by an increase in the number of SGCs in the embryos of chicks exposed to Flo were in agreement with the findings of Hassanin et al. [18] about a dose dependent increase in the humoral immune responses of broiler chickens treated with florfenicol (30 and 60 mg/kg, orally) for 5 consecutive days .It can be concluded from the study [18] that the developing chicken embryo model used in our current study was more sensitive than the adult chicken, where the immune response (increase in the number of SGCs) was obtained during the treatment of embryos only one time on a day 12 of the incubation while [18] needed to treat adult chickens for five days to elicit an immune response. The reason for the high sensitivity of the developing chick embryo model may be due to its high degree of proliferation and cellular differentiation [9].

With regard to the increase in the density of splenic blood vessels to (92.6 ± 1.36) in (F2 group), this increase may be related to the huge increase in the number of SGCs, which reached (133.2 ± 2.46) in this group compared to the control group (42.4 ± 2.13) where the higher rate of proliferation in the SGCs requires a blood supply. While the F1 group did not show a significant increase in SBVs (33 ± 1.52) compared to the control group (22.2 ± 1.16), because this dose (75 mg/kg) did not induce a significant increase in the number of SGCs (83.4 ± 2.5) compared to the F2 group (133.2 ± 2.46), so the vascular density was consistent with the number of centers.

The results of our current study showed that the chick embryo model is more sensitive than the standard animal model (mice) to the occurrence of apoptosis in the immunocyte located in the white pulp region of the spleen induced with Flo. This is evidenced by the occurrence of apoptosis in the currently study in the splenic immunocytes of chick embryos treated with Flo (75 mg/kg egg weight) only one time on day 12 of incubation while [19] needed to treat mice with Flo (1500 mg/kg, orally) for 7 consecutive days to induce apoptosis in splenic immunocyte.

We conclude from our current study that Flo induced proliferation in the SGCs of chick embryos, which is considered as an indicator of the immune response that depended in its intensity on the dose and to regulate and modify this response by apoptosis, which is recorded for the first time.

Acknowledgement

We thank College of Veterinary Medicine, University of Mosul for permission to conduct this research.

Conflicts of Interest

The authors declare there is no conflict of interest.

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الفلورفينكول يحفز تكاثر المراكز الانتاشية الطحالية في نموذج اجنة افراخ الدجاج

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الخلفية العلمية: يعتبر الفلورفنيكول هو أحد مضادات الجراثيم المستخدمة على نطاق واسع لعلاج الحيوانات المنتجة للغذاء. هناك جدل حول التغيرات النسيجية في الخلايا المناعية الطحالية التي يسببها الفلورفنيكول.

الهدف: يعتبر تكاثر المراكز الانتاشية الطحالية في الدواجن علامة على الاستجابة المناعية ، لذلك تم استخدام أجنة افراخ الدجاج كنموذج في دراستنا ، حيث تتميز بانها تمتلك معدل تكاثر خلوي عالي.

طرائق العمل: تم تقسيم ٣٠ بيضة مخصبة إلى ثلاث مجموعات (مجموعة السيطرة ، اف١ و اف٢) بواقع (١٠ بيضات / مجموعة) تمت معاملتها في اليوم ١٢ من الحضانة باستخدام الفلورفنيكول (٠ (ماء مقطر) ، ٧٥ و ١٥٠ ملغم / كغم ، الغرفة الهوائية) على التوالي. بعد ذلك ، في اليوم الثامن عشر من الحضانة ، تم التضحية بأجنة افراخ الدجاج من أجل جمع الطحال لفحص التغيرات النسيجية فيه باستخدام تقنية TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) للكشف عن تجزئة الحمض النووي (موت الخلايا المبرمج) وصبغة الهيماتوكسيلين والأيوزين لعد الأوعية الدموية والمراكز الانتاشية الطحالية وقطرها. أدى تعرض اجنة افراخ الدجاج الفلورفينكول الى تغيرات نسيجية في الطحال بطريقة تعتمد على الجرعة ، ممثلة بزيادة كبيرة في عدد كل من الاوعية الدموية والمراكز الانتاشية لكن قطر هذه المراكز انخفض بشكل معنوي بسبب حدوث موت الخلايا المبرمج في المجموعة (اف٢) مقارنة مع المجموعات (اف١ والسيطرة).

الاستنتاج: نستنتج من دراستنا الحالية أن الفلورفينكول يحفز تكاثر المراكز الانتاشية الطحالية لأجنة افراخ الدجاج ، والذي يعتبر مؤشرا على الاستجابة المناعية والتي تعتمد في شدتها على الجرعة وتم تنظيم وتعديل هذه الاستجابة عن طريق موت الخلايا المبرمج ، والتي يتم تسجيلها لأول مرة.

الكلمات المفتاحية: الفلورفينكول ، المراكز الانتاشية الطحالية ، اجنة افراخ الدجاج ، التكاثر ، موت الخلايا المبرمج .