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In ovo given sunset yellow adversely affects embryonic development of chick thymus and bursa Fabricii as evidenced by histological and enzyme histochemical findings

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Abstract

Background Sunset yellow (E110) has largely been used as food coloring agent. Complaints accumulated on E110 for possible detrimental effects on the immune functions such as allergenicity in the children. In this study, the effects of different doses of E110 on embryonic development of chicken primary lymphoid organs, thymus and bursa of Fabricius were determined by means of histological, histomorphometrical and histochemical methods. A total of 250 fertilized eggs from the Ross 508 line were used in the study. The eggs were divided into 5 groups as non-treated, sham-exposed and 100 ng/egg, 500 ng/egg, 1000 ng/egg E110-injected groups, each having 50 eggs.

Results In the 1.000 ng/egg E110-administered group, embryonic development of chicken thymus and bursa of Fabricius were retarded.

Conclusions E110 given in ovo before incubation retarded the embryonic development of the avian thymus and bursa of Fabricii. The effect is more pronounced in the 1.000 ng/egg group.

Keywords Bursa of Fabricius, E110, Avian thymus, Acid phosphatase, Esterase

1 Background

Sunset yellow (Chemical Abstracts Service (CAS) No. 2783-94-0), also known as orange yellow S, food Yellow 3, color index (CI) No. 15985, registered as (E110) is a water-soluble powder food coloring. Structurally, it is an azo dye having a rather complex molecular structure and its chemical name is disodium salt of 6-hydroxy-5-(4-sulfophenyl) azo-2-naphthalene sulfonic acid. Azo dye compounds are generally dark colored since they contain two aromatic rings bridged with an azo nuclear group (–N=N–). The water solubility of E 110 is relatively dependent on the ambient temperature [1] and its biodegradability is low [2].

The low acute oral toxicity of E110 reflected by half lethal dose (LD_{50}) values has been reported to be > 10.000 mg/kg in rats and > 6.000 mg/kg in mice [3]. European Food Safety Authority (EFSA) established a new daily intake (ADI) for sunset yellow of 4 mg/kg body weight/day (BW/D) [4]. Although this level might be relatively reasonable, high-level exposed children receive 0.2–5.8 mg/kg body weight/d (BW/D), which is highly higher than ADI level.

Recommended E110 doses have been concerned as safe and did not cause mutagenicity in the following generations in the rat and dog, even given 1X, 10X, 30X ve 100X of the recommended doses. Nevertheless, 1.000 mg/kg/daily dose increased the incidence of partially joined twins in rabbits [3].

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In the experiments on the genetic effects of E110, sister chromatid exchange (SCE) assays showed controversial results up to 5.000 µg/ml doses [5]. Micronucleus (MN) test revealed that sunset yellow and tartrazine did not induce a genotoxic effect in the MN gut assay in mice at doses up to 2000 mg/kg BW/D [6]. Carcinogenicity of E110 has not been observed in both male and female rats in 103-week feeding experiment at 12.500, 50.000 ppm doses [7]. Embryotoxic and adverse reproductive effects of 100, 300, or 1.000 mg/kg E110-administered by gastric gauge were not found in Charles River CD rats [8].

E110 is mainly metabolized via the bacterial azo reduction in the alimentary tract, and the major metabolites of the reaction are sulfanilic acid and amino-2-naphthol-6-sulfonic acid [9]. About 3.6% of orally given E110 is absorbed and 0.8% is substantially excreted via feces [10], urine excretion is also another clearance mechanism of E110 from the blood serum [11].

In an experimental study [12], E110 given at 250 and 1.500 mg daily doses for 90 days adversely affected rat testes, and the 250 mg dose was regarded as the lowest observed adverse effect level (LOAEL). Oestrogenic effects of E110 were not observed in the H295R cell culture [13]. Reactions to food colorings triggered by immune and non-immune mechanisms are assumed to be infrequent in the population [3]. Hypersensitivity reactions did not develop against E110 in Guinea pigs [14]. However, skin tests revealed that hypersensitive people to p-phenylenediamine cross-reacted with E110 [15].

Mortality and tumor formation rates did not change in a long-term exposure experiment, in which 1 mg daily dose of E110 was administered to hamsters either subcutaneously or intraperitoneally for 330 days [3]. Orally given 4% E110 for 18 months also did not cause tumoral changes although the coloration of epithelial cells of the gastric glands increased [16].

Acid phosphatase (ACPase) is a lysosomal enzyme, which is mainly found in myelocytes, polymorphonuclear leucocytes (PMNLs), lymphocytes, plasma cells, megakaryocytes, blood platelets and mononuclear phagocytes [20]. The ACPase positivity has been suggested to be specific for T-lymphocytes in mammals [21], whereas avian B-lymphocytes specifically give a positive reaction to the enzyme [22]. α-Naphthyl acetate esterase (ANAE) is another lysosomal enzyme [23] that T-lymphocytes gain ANAE positivity during the late thymocyte maturation in the thymus [24]. ANAE positivity is peculiar to mature T-lymphocytes in both peripheral blood smears and tissue sections of the chickens [25]. T-lymphocytes give localized granular (dot-like) ANAE positivity [23], and ANAE positivity pattern of the null cells is fine granular [26]. Monocytes and macrophages display a strong and diffuse ANAE positivity [26].

The avian thymus and bursa of Fabricius play significant roles as central lymphoid organs by producing immuno-competent T-lymphocytes and B-lymphocytes, respectively [17]. Their embryonic development process is elucidated in detail [18, 19]. Thus, any harmful agent disturbing the normal embryonic development of the thymus and bursa of Fabricius might give valuable information about the immunotoxicity of any substance given before incubation.

Although there are detailed data evidencing negative effects of E110 on the various cells and organs, we have limited information on the effects of this food coloring on the embryonic development of immune system organs. In the present study, we aimed to determine adverse effects of in ovo-administered E110 prior to incubation on the embryonic development of the bursa of Fabricius and thymus by histological, histomorphological and enzyme histochemical methods.

2 Methods

2.1 Egg material and groups

All experimental procedures applied in this experiment were approved by the Ethical Committee of the Veterinary Faculty of Selçuk University (2013/044), Konya, Türkiye. A total of 250 fertilized eggs from the Ross 508 line were used in the study. Prior to incubation, the eggs were disinfected by fumigating with 130 ml of formaldehyde 37% and 80 g of potassium permanganate mixture per cubic meter.

Crystallized E110 (90% purity Sunset yellow, 465,224-25G, Sigma-Aldrich, USA) was used as test material in the study. The eggs were divided into non-treated (control-I), sham control (control-II), 100 ng/egg E110-injected (experiment-I), 500 ng/egg E110-injected (experiment-II) and 1000 ng/egg E110-injected (experiment-III) groups, each having 50 eggs (Table 1). Sterile, 20 µl of test solutions was injected into the air sac of the eggs through drilled blunt ends. Following injection,

Table 1 Experimental groups and procedures

Groups N = 50	Procedures
Control-I	Non-treated
Sham control	20 µl of sterilized distilled water was injected
Experiment-I	20 µl of 100 ng/egg E110 was injected
Experiment-II	20 µl of 500 ng/egg E110 was injected
Experiment-III	20 µl of 1000 ng/egg E110 was injected

the holes were sealed with liquid paraffin and incubated in the incubator (Veyisoğlu, İstanbul, Türkiye), at 37.8 °C and 65% relative humidity.

2.2 Tissue samples

On the 11th, 15th, 18th and 21st days of incubation, randomly selected 5 eggs from each of the control-I, sham control groups and 10 eggs from each of the experimental groups were opened. The thymus and bursa of Fabricius were dissected out, and tissue samples were taken for Crossmon's trichrome and Papenheim's panoptic stains. Tissue samples were also taken for ANAE and ACPase histochemistry at the same time. Crossmon's trichrome and Papenheim's panoptic stains were performed in paraffin sections from the tissue samples fixed in 10% buffered formal saline (0.1 M, pH 7.4). Enzyme histochemical reactions were performed in the frozen sections. For ANAE demonstration, tissue samples were fixed in a formal-sucrose solution at + 4 °C [27]. ACPase was ascertained in the formal-calcium fixed tissue samples [28]. ANAE and ACPase were demonstrated by using specific substrates and chromogens for each enzyme by the methods of previous researchers [28, 29].

3 Results

3.1 Macroscopic findings

Embryos in the control-I, sham control, 100 ng and 500 ng/egg E110-administered groups displayed a normal development, which is similar to the Hamburger-Hamilton scale [30]. In the 1.000 ng/egg E110 given group, some embryos were developmentally retarded and ectopia viscera were common in these embryos.

3.2 Embryonic development of the bursa of Fabricii

In the control-I, sham control, 100 ng/egg and 500 ng/egg E110 given animals, embryonic development of the bursa of Fabricii followed its normal course in all embryonic periods (Figs. 1A, 2A, 3A, 4A). In the 1.000 ng/egg E110-administered groups, embryonic development of the bursa of Fabricius retarded as early as the 11th day of incubation. Plicae were less in number, flattened and smooth. First morphological step of lymphoid follicle development, epithelial bud formation was delayed in the 500 ng/egg and 1.000 ng/egg E110 given experimental groups. In later stages, the difference between the controls and the experimental became more evident. Plicae were blunt and shorter, lymphoid follicle development retarded, and ACPase-positive lymphocytes in follicle

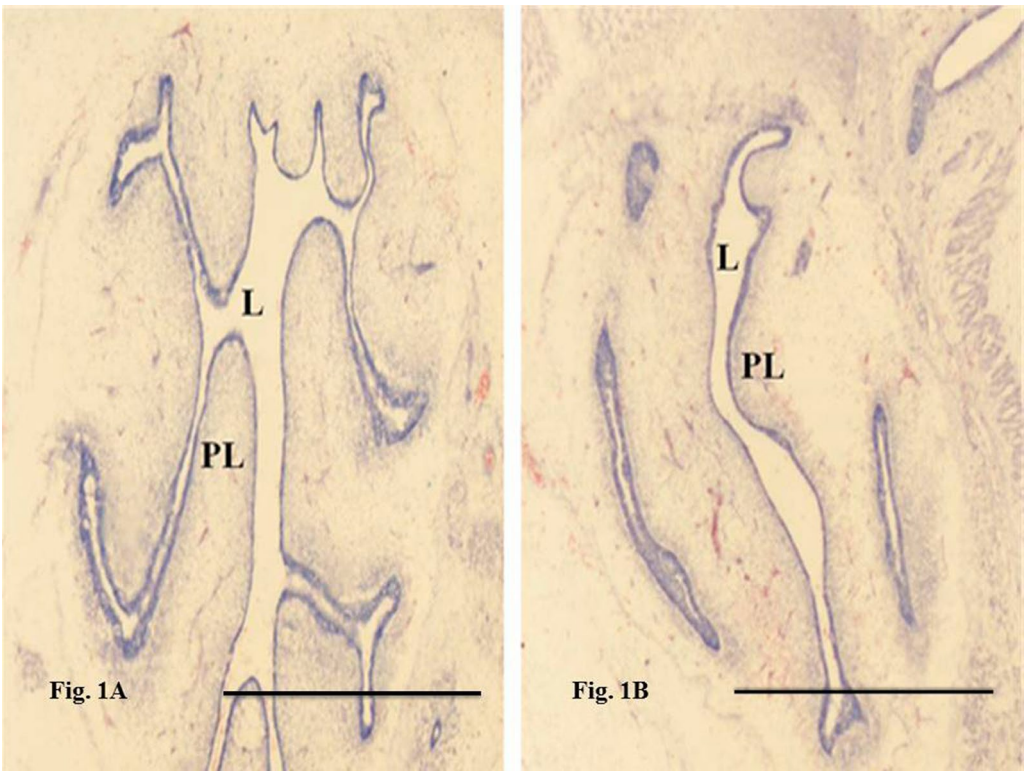

Fig. 1 Sections of the bursa of Fabricius from the control-I group (**A**) and 1.000 ng/egg group (**B**) on the 11th day of the incubation. L) Lumen, PL) plica. Plicae in the 1.000 ng/egg group are flattened and smooth. Pappenheim's panoptic stain. Magnification bar: 100 µm

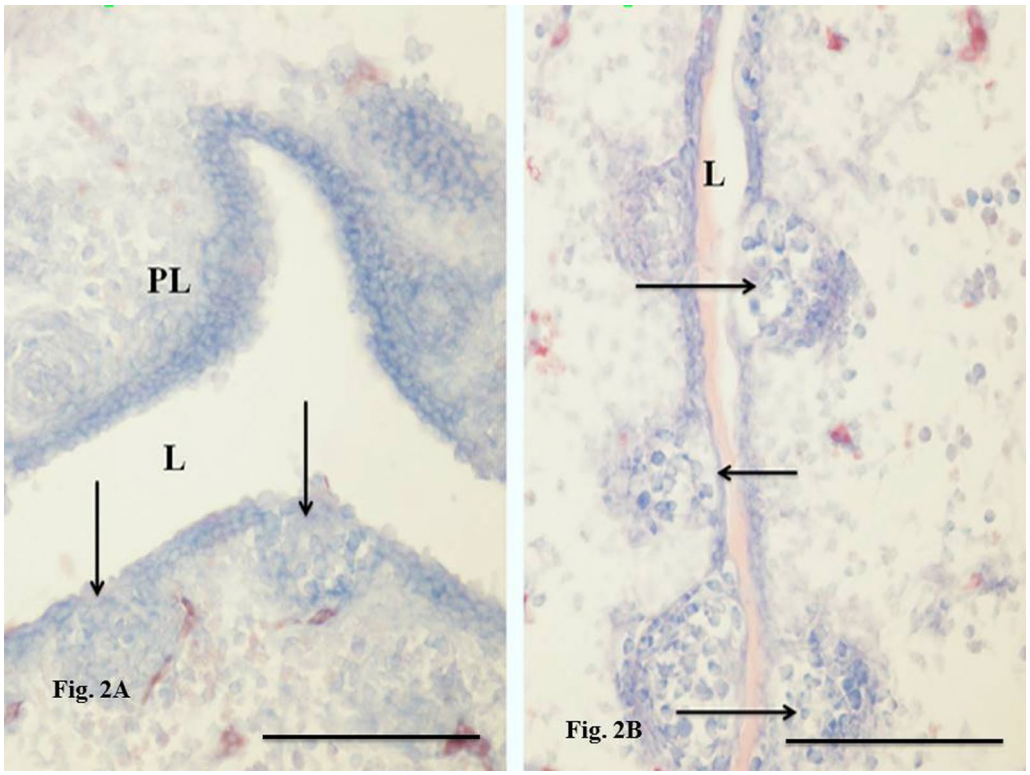

Fig. 2 Sections from the bursa of Fabricii from the control-I group (**A**) and 1.000 ng/egg E110-treated group (**B**) on the 15th day of the incubation. L) Lumen, PL) plica, Arrows) developing epithelial buds. Epithelial bud formation in the 1.000 ng/egg E110 given group is retarded. Pappenheim's panoptic stain. Magnification bar: 100 µm

centers were very scarce in the E110-administered groups (Figs. 1B, 2B, 3B, 4B).

3.3 Embryonic development of the thymus

The thymus followed its normal development (Figs. 5A, 6A, 7A, 8A) in the control and low dose (100 ng/egg and 500 ng/egg) E110-injected groups at day 11th, 15th, 18th and 21st days of the incubation. Lobe formation retarded in the 1.000 ng/egg E110 groups, and the developing organ was relatively smaller in all of the embryonic developmental stages. The centrally located vascular area was weaker and the cells with lymphocyte morphology were less in number. ANAE-positive cells with lymphocyte morphology were seen in developing thymic lobes in the control, 100 and 500 ng/egg injected experimental groups; however, the 1.000 ng/egg E110-injected group was lack of these cells on the 11th day. Retardation of embryonic thymus development continued in later stages (Figs. 5B, 6B, 7B, 8B).

4 Discussion

Although food colorings have been concerned as safe when not exceeding specified limits, in practice, especially children might often exceed these limits. E110 and tartrazine are the most important food colorings consumers encountered in daily life. Because that E110 is a sulfonated version of Sudan-I, which is a possible carcinogen, a certain amount of Sudan-I might be in the end product of E110. E110 is also suspected to be responsible for the allergic reactions observed in aspirin-intolerant people. Among the commonly observed allergic reactions are diarrhea, vomiting, urticaria, angioedema of the skin, rarely anaphylactic shock and headache. These reactions are common symptoms of azo group food colorings, including E110. Other symptoms include asthma, rhinitis, vasculitis, inhibition of thromboxane synthesis, purpura, other gastrointestinal disorders and rarely anaphylactic shock. In clinical surveys, some of the patients with urticaria, and angioedema patients are related to hypersensitivity to Allura Red AC, amaranth, E110, ponceau 4R and tartrazine [31]. Four weeks of orally received E110 provoked urticaria and/or angioneurotic edema in 17% of 56 patients suffering from these chronically [31].

It has been suggested that E110 might be associated with childhood hyperactivity [32]. It might increase allergic reactions and trigger aggression in children. Indeed, when artificial colorants, such as tartrazine, azorubine, E110 and ponceau-4R are removed from the

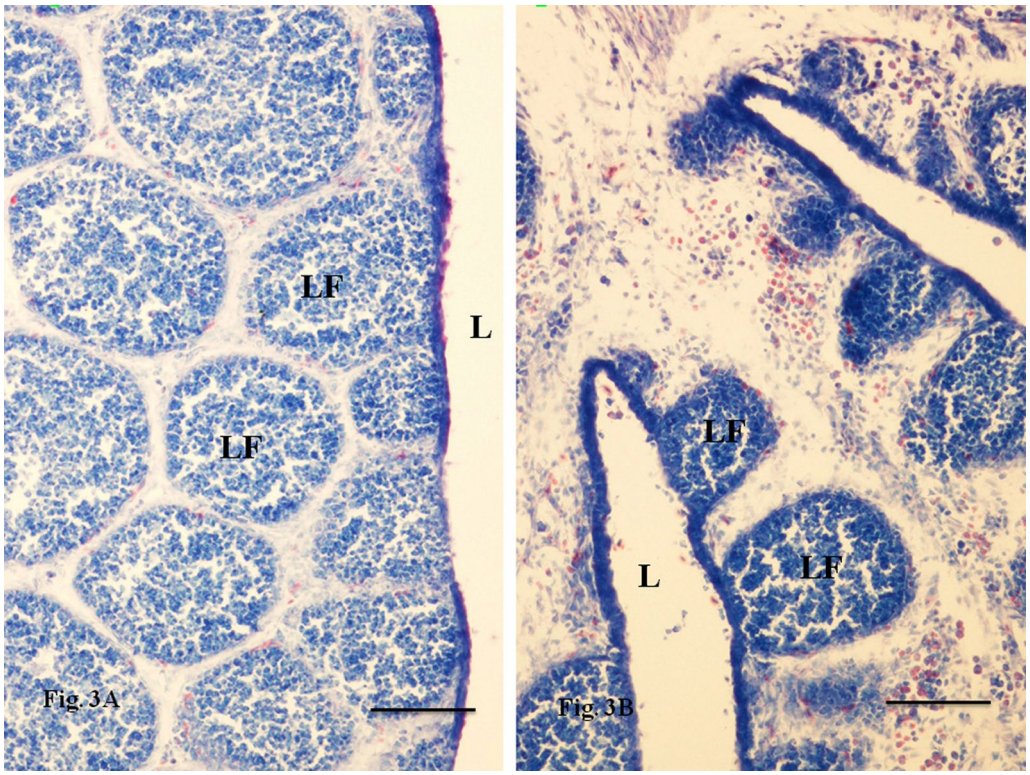

Fig. 3 Sections from the bursa of Fabricii from the control-I group (**A**) and 1.000 ng/egg E110 given group (**B**) on the 18th day of the incubation. L) Lumen, LF) lymphoid follicles. Retardation of lymphoid follicle development in the 1.000 ng/egg E110 given group is seen. Pappenheim's panoptic stain. Magnification bar: 100 µm

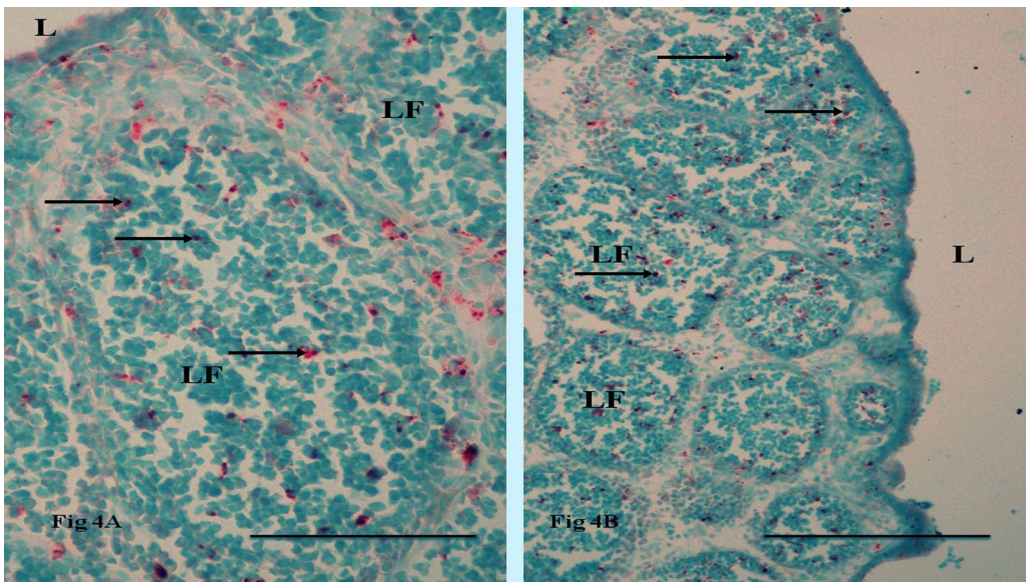

Fig. 4 Sections of the bursa of Fabricii from the sham control group (**A**) and 1.000 ng/egg E110 given group (**B**) on the day of hatch. L) Lumen, LF) lymphoid follicles. Arrows) ACPase-positive lymphocytes. ACPase-positive lymphocytes are quite sparse in the 1.000 ng/egg E110 given animal. ACPase demonstration. Magnification bar: 100 µm

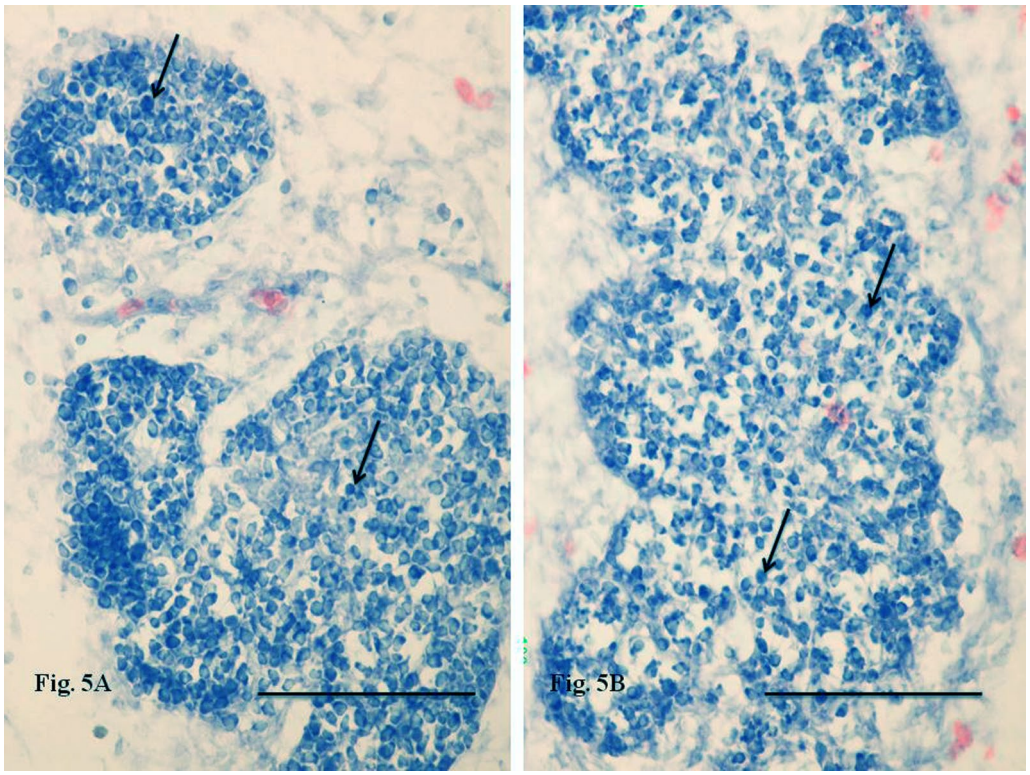

Fig. 5 Sections of the thymus from the sham control group (**A**) and 1.000 ng/egg E110 given group (**B**) on the 11th day of the experiment. Arrows) cells with lymphocyte morphology. Developing thymic lobes are sparsely populated with lymphoid cells in the 1.000 ng/egg E1100 given group. Pappenheim's panoptic stain. Magnification bar: 100 µm

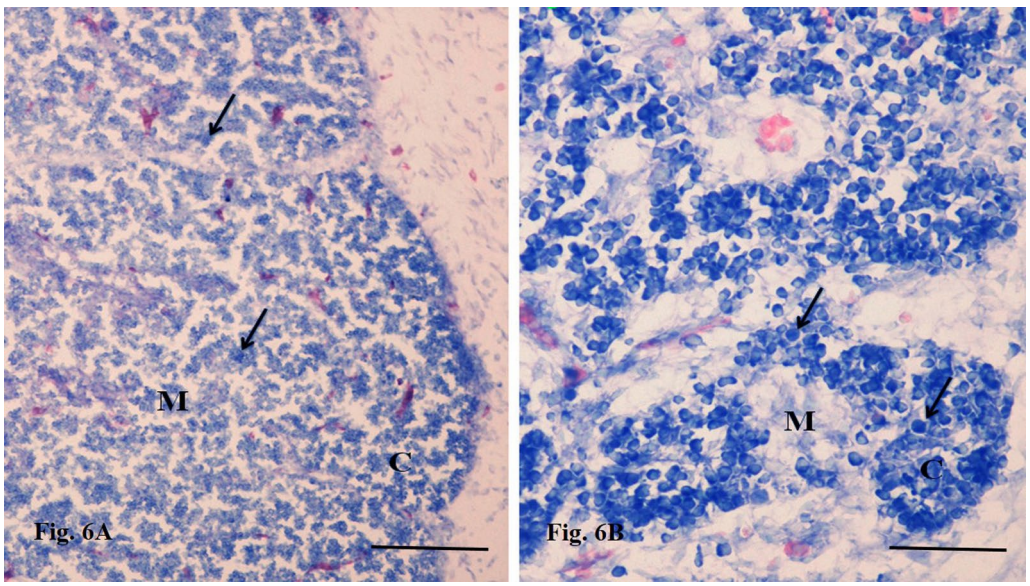

Fig. 6 Sections of the thymus from the sham control (**A**) and 1.000 ng/egg E110 given group (**B**) on the 15th day of the experiment. Arrows) cells with lymphocyte morphology. C) Cortex, M) medulla of the lobe. In the 1.000 ng/egg E110 given group, the medullae of smaller thymic lobes are almost empty. Pappenheim's panoptic stain. Magnification bar: 100 µm

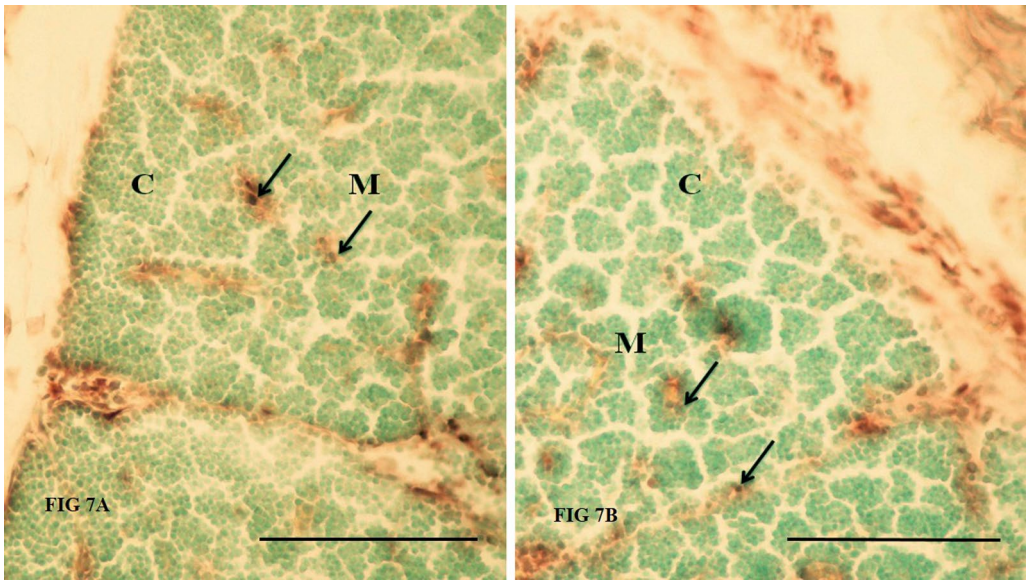

Fig. 7 Sections of the thymus from the control-I group (**A**) and 1.000 ng/egg E110 given group (**B**) on the 18th day of the experiment. Arrows) ANAE-positive lymphocytes, C) cortex, M) medulla. Arrows) ANAE-positive lymphocytes. ANAE-positive cells are lesser in the E110-given animal. ANAE demonstration. Magnification bar: 100 µm

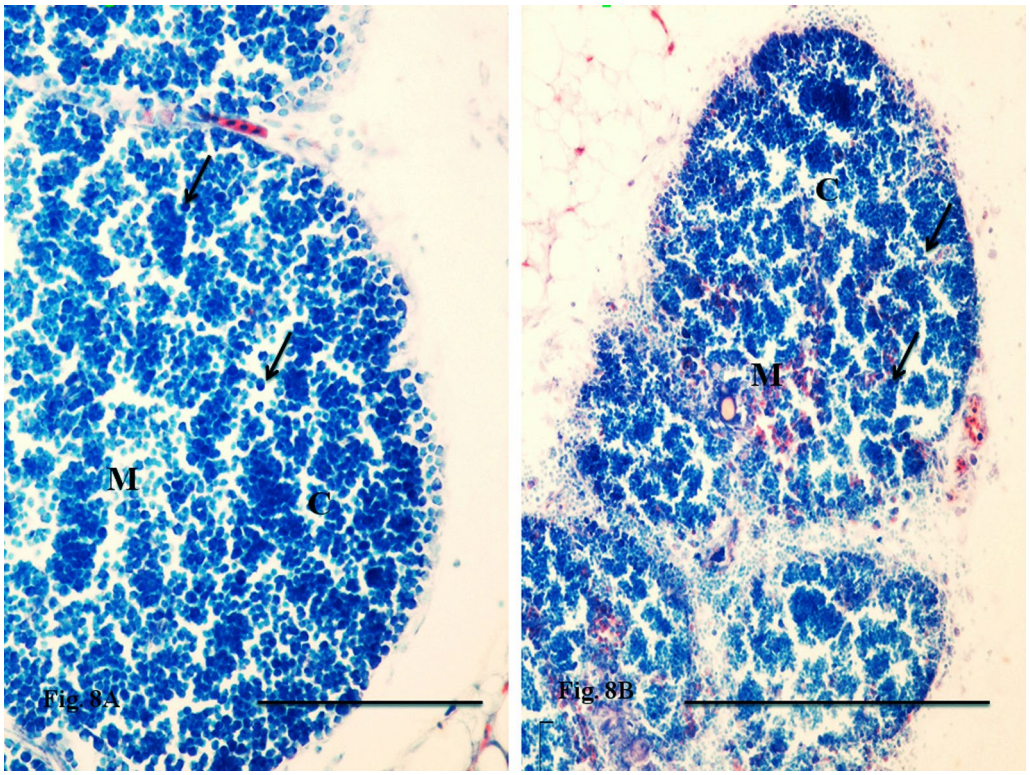

Fig. 8 Sections of the thymus of a newly hatched chick from the control-I group (**A**) and 500 ng/egg E110 given group (**B**). C) Lobular cortex, M) lobular medulla. Thymic lobes are relatively smaller and have low cellularity in the 500 ng/egg E110 given group. Pappenheim's panoptic stain. Magnification bar: 100 µm

beverages of hyperactive children, they return to normal, but behavioral disorders reappear by reintroducing the drinks containing these substances. Similarly, in 4% of patients with chronic or relapsed urticaria, intolerance has been identified against benzoates and sorbic acid. Incidence of instant or delayed hypersensitivity reactions in European societies ranged between 0.14 and 2% [32–34]. Mixed consumption of food colorings and food preservatives, such as sodium benzoate, might increase hyperactivity in children aged 3–9 years [32]. Intolerance against benzoates, sorbic acid, and E110 has been shown in 4% of the patients with chronic recurrent urticaria [35]. A small patient group with atopic dermatitis developed hypersensitivity reactions against food additives including E110 [36]. In a double-blind experimental study, 0.1 mg E110 was given in opaque capsules to 36 of 43 children with angioedema and/or chronic urticaria, and 10 of them developed hypersensitivity to E110 [31, 40]. Hypersensitivity reactions against to tartrazine/E110 combination have been observed in one of 13 children at an 8.5 mg/250 ml dose [37]. Cross-hypersensitivity reaction developed in 2 patients with eczema who are receiving colored antihistamine preparation [38]. It is interesting that side effects such as vascular and urticaria reactions to E110 mostly occur when the food coloring is used together with other synthetic colorings [39]. Such diminished immune reactions might have arisen from damaged immune system organs or diminished cell functions in young people.

Previous studies on food colorings are mainly focused on field surveys, clinical observations and biochemical and physiological studies [3, 10, 40, 41]. In the European countries, the daily intake of E110 in children (1–10 ages) was calculated as 0.3–6.7 mg/kg BW/D. The value was reported as 0.5–1.1 mg/kg BWD for adults. Although these values were lower than the temporary acceptable ADI, which is 1 mg/kg BW/D, the daily intake of high-level exposed children is between 0.2 and 5.8 mg/kg BW/D, and the dose is higher than the ADI level. It has been suggested that levels of contaminating sulfonated dyes such as orange II and Sudan I, colorless sodium chloride and sodium sulfate should be taken into account while determining the high-level limits of E110 [3]. E110 might contain < 5% other food colorings, < 0.5% 4-aminonaphthelene-1-sulfonic acid, 7-hidroxynaphthelene-1, 3-disulfonic acid, 3-hydroxynaphthelene-2, 7-disulfonic acid, 6-hydroxynaphthelene-2-sulfonic acid, 4.4′-diazoaminodibenzene sulfonic acid and 6.6′-oxydinaphthelene-1.3-disulfonic acid. Although the amount depends on the producer, the orange II content of E110 sold in the market is about 2%. According to Food and Agriculture Organization (FAO) and World Health Organization (WHO) regulations, the upper limit of orange-II is 2% [42].

In Ireland, the E110 level is higher than 0.1 mg/L limit of detection (LOD) in 61 beverages; the high limit was exceeded in 3 products and ranged between 1 and 61 mg/L [3]. In European countries, E110 limit was determined as 50 mg/L by Union of European Soft Drinks Associations (UNESDA) in 2005 [43]. Nevertheless, the limits were revised as 1–48 mg/L [4].

Theoretical calculations showed that a 60-kg European person consuming 1.5 L of 200 mg/L E110 containing beverage and 375 g of food containing 500 mg/kg E110 might take 8.1 mg/kg BWD E110 [4]. Although the same value was found for children (15 kg for 3 years of age), the level per kg of body weight (BW) might be higher [3].

In mammals, the deleterious effects of food colorings on the histology and embryonic development of immune system organs have not been elucidated yet. The reasons for the lack of information might be the very low level of food colorings, the metabolism of food colorings in the intestines and liver, and insufficient information on placental transmission. However, 100, 300 and 1.000 mg/kg BW/D E110 given nasogastric gavage at 6–15th days of gestation caused the low birth weight of newborns. Neither gender ratio deviation nor congenital malformation has not been observed [8, 16].

Fertilized chicken egg is one of the most preferred test materials for testing embryotoxic, genotoxic, teratogenic and immunotoxic effects of physical and chemical agents because those chickens have no placental barrier, embryonic developmental stages elucidated in detail and also low problems concerning ethics and animal rights [44–48]. Test results might also be extrapolated to the mammals by multiplying the test result by 10^4. The result is the toxic dose level per kg BW of pregnant mammals [45]. In order to reduction, refinement and replacement of animal experimentation, it is very important to note that the hen's egg test (HET) is a borderline case between in vivo and in vitro systems, and the material also does not conflict with ethical and legal considerations, especially animal protection laws. There is a very high correlation between findings in mammalian systems and the hen's egg test. The HET is a rapid, sensitive and inexpensive toxicity test, giving information on the embryotoxicity, teratogenicity, systemic and immunopathological effects and metabolic pathways of chemical substances. This is true for a broad range of parameters examined, including histopathology. Toxicity testing by the HET should not and could not replace totally the currently used toxicological tests in mammals in these fields, but it can reduce the number of investigations in mammals and also minimize or eliminate pain and damage in animal experiments. In addition HET makes possible the identification

of toxicity categories and target organs of toxicity [46–51]. However, the method has some disadvantages such as being samples mammalian origin and the difficulty in directly extrapolating results to humans. Moreover, serious objections were raised against use of chick embryo in teratological testing, namely the absence of mammalian maternal fetal relations, pharmacokinetic dissimilarities inherent in the closed character of the avian egg and high non-specific sensitivity resulting in an unjustifiable number of false-positive results. The difficulty of extrapolation is, however, inherent also to all other tests using non-human mammals, because interspecies variability in metabolism [47, 48].

The avian immune system is an excellent experimental material since its embryonic development is well defined, there is no placental barrier, and both the thymus and bursa of Fabricius in which T and B lymphocytes mature, respectively, are distinct organs [49–51]. In this study, the effects of E110 on the embryonic development of the thymus and bursa of Fabricius were determined by chick embryotoxicity screening test (CHEST). In the study, 3 of the E110 doses, 100 ng, 500 ng and 1.000 ng/egg, were used. Embryos in the control groups and 100 and 500 ng/egg E110-injected experimental groups developed in the normal course according to the Hamburger-Hamilton scale [30]. Nevertheless, malformations, such as retarded development and ectopic viscera, were observed in some embryos of the 1.000 ng/egg E110-injected group.

Results of a previous study [52] showed that 0.5 g E110/100 mL was not genotoxic to Escherichia coli. E110 also has no mutagenicity in Salmonella typhimurium [53] and in Saccharomyces cerevisiae [54]. In vivo test results have revealed that 2000 mg/kg BW orally given E110 did not cause mutagenic effects on bone marrow cells by micronucleus test [6]. In the mice, orally given 0.17 or 1.7 mg/kg BW E110 did not increase the number of cells with chromosome damage [55]. These results evidenced that within normal limits E110 had no genotoxicity. However, it has been suggested that high concentrations of food colorings had cytotoxicity potential because that brilliant blue and E110 increased MN incidence and decreased mitotic index and replication frequency.

In this study, 1.000 ng/egg E110 depressed normal embryonic development of the bursa of Fabricius. In the affected animals, organ-specific lymphoid follicles were smaller and their cell population intensity was lower. ACPase-positive lymphoid cells were seen infrequently. These morphological findings might imply that the organ functions might be diminished and possibly humoral immune deficiency occurs after hatching.

In the control groups, embryonic development of the thymus was similar to previous researchers [30, 47]. In all the experimental groups, the thymus displayed developmental retardation; it was more evident in the 1.000 ng/egg group. In this group, ANAE-positive lymphoid cells were rarely seen in the thymic medulla. These findings show that these animals might be born with a functionally impaired and deficient cellular immune system. In a previous experiment [56], E110 increased the degranulation of mast cells, which play key roles in allergic reactions. This finding shows that E110 might cause allergic reactions.

5 Conclusion

Based on the results of the present study, it was concluded that 1.000 ng/egg E110 given in ovo before incubation retarded the embryonic development of the avian thymus and bursa of Fabricii. Although these morphological findings need to be confirmed by functional tests, this developmental retardation possibly will be reflected as an immune deficiency or impaired immune functions in the postnatal life of the animal. When regarding a striking increase in childhood allergic reactions in recent years, doubts focused on the possible harmful effects of food additives, especially food colorings. The researchers of this study strongly emphasize that detailed experiments including mammalian animal species are necessary for clarification of the subject.

Abbreviations

ACPase	Acid phosphatase
ADI	Acceptable daily intake
ANAE	Alpha-naphthyl acetate esterase
BW	Body weight
BW/D	Body weight/day
CAS	Chemical Abstracts Service
CHEST	Chick embryotoxicity screening test
CI	Color index
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
LD50	Half lethal dose
HET	Hen's egg test
LOAEL	Lowest observed adverse effect level
LOD	Limit of detection
MN	Micronucleus
PMNLs	Polymorphonuclear leucocytes
SCE	Sister chromatid exchange
UNESDA	Union of European Soft Drinks Associations
WHO	World Health Organization

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Not applicable.

Author contributions

İÇ contributed to conception and design. All authors contributed to administrative support, provision of study materials and final approval of the manuscript. EB and SÇ contributed to collection and assembly of data, data analysis and interpretation and manuscript writing. All authors read and approved the final manuscript.

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yellow FCF (E110) on the embryonic development of chicken thymus and bursa Fabricii by means of histological and enzyme histochemical methods").

Availability of data and materials
All data generated and analyzed in the experiment are included in this published article.

Declarations

Ethics approval and consent to participate
All experimental procedures applied in this experiment were approved by the Ethical Committee of the Veterinary Faculty of Selçuk University (2013/044), Konya, Türkiye.

Consent for publication
Not applicable.

Competing interests
The authors have no competing interests.

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