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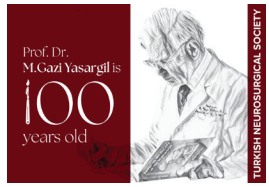

Embryotoxic Effects of Sunset Yellow in Congenital Neural Tube Defect Formation in Early-Stage Chick Embryos: A Histopathological Study

Fatih TOPRAK[1], Dila SENER AKCORA[2], Dilek AKAKIN[2], Ezgi AKAR[3], Selin TURAL EMON[3]

[1]Merzifon Kara Mustafa Pasha State Hospital, Department of Neurosurgery, Amasya, Türkiye
[2]Marmara University School of Medicine, Department of Histology and Embryology, Istanbul, Türkiye
[3]Health Sciences University Haydarpaşa Numune Training and Research Hospital, Department of Neurosurgery, Istanbul, Türkiye

Corresponding author: Fatih TOPRAK ✉ drfatihtoprak@gmail.com

ABSTRACT

AIM: To investigate the embryotoxic effects of Sunset Yellow (SY) exposure on neural tube development in an experimental model of chicken embryos.

MATERIAL and METHODS: Sixty fertilized special pathogen-free (SPF) Leghorn genus chick embryos were used. Three experimental groups were determined, comprising the control group (0.1 ml saline), daily intake dose [2.5 mg/kg Sunset Yellow FCF E110 (SY)], and high dose [5 mg/kg SY] groups (n=20). SPF status was determined on day 0 when eggs were placed in the incubator. Eggs were injected at the 30th hour of incubation, and incubated for the following 72 hours.

RESULTS: Upon light microscope examination, in the control group the surface ectoderm was intact, the neural tube was closed, and the neuroepithelium, basement membrane surrounding the neuroepithelium, notochord, and somites were all normal. In the daily intake dose SY group, four examples of neural tube defects (NTDs) were observed. Six instances of NTDs were observed in the high-dose SY group. The high-dose group had a statistically significant increase in the number of embryos with NTDs compared to other groups (p=0.0004).

CONCLUSION: These results suggest that SY consumption can cause irregular neural tube development. SY should not be ingested in high doses for extended periods of time, should be regulated even when used as an additive, and should be avoided during pregnancy. Further studies are needed in a wider range of dose groups to observe the embryotoxic effects of SY on neuronal development.

KEYWORDS: Food coloring agents, Spinal dysraphism, Neural tube defects, Sunset yellow, Chick embryo

ABBREVIATIONS: FA: Food additives, **FAO:** Food and Agriculture Organization of the United Nations, **H&E:** Hematoxylin and eosin, **HH:** Hamburger-Hamilton, **NaB:** Sodium benzoate, **NT:** Neural tube, **NTD:** Neural tube defect, **SPF:** Special pathogen-free, **SY:** Sunset Yellow FCF E110, **WHO:** World Health Organization

Fatih TOPRAK : 0000-0002-5232-6286
Dila SENER AKCORA : 0000-0001-9496-7324
Dilek AKAKIN : 0000-0002-1781-3708
Ezgi AKAR : 0000-0002-8582-2948
Selin TURAL EMON : 0000-0002-7150-1684

INTRODUCTION

The use of various substances as food additives (FA) is common to color products, prevent their deterioration, and boost their nutritional value (29). Food dyes are used to enhance the appeal of foods and beverages by altering their appearance and aroma. With the increased consumption of certain products, an individual's exposure to processed food dyes increases daily. Certain additives used in the food industry, such as Sunset Yellow FCF E110 (SY), tartrazine, azorubin, and ponceau-4R, are known to have negative effects on human health (2,3). SY is a synthetic azo dye used in the food, pharmaceutical, and cosmetic industries. It is widely utilized in the food industry for sweet and savory products, frozen foods, alcoholic and non-alcoholic beverages, cosmetics, and pharmaceutical food supplements (11,22). Some azo dyes have been linked to allergic reactions such as contact dermatitis, angioneurotic edema, asthma, rhinitis, anaphylaxis, and immunosuppression. In sensitive individuals, this can result in immune responses such as allergies and hypersensitivity. SY has been reported to cause bronchial contractions in patients with chronic asthma, food intolerance and behavioral hyperactivity disorder in children, cancer, birth defects, genetic disorders, and brain damage (2,7,22).

Neural tube defects (NTDs), caused by aberrant tissue movement during neural crest cell migration and neurulation, are the second most common birth defect after congenital cardiac disease and represent a significant public health issue from both a sociological and economic perspective (9,10). The first three weeks of pregnancy are essential for the embryo's neural development. Many chemicals can be transmitted from mother to infant through the placenta. Exposure to synthetic chemicals, even in low amounts, can have a negative impact on embryonic development and, by extension, the newborn. The neural development of a human embryo during its first month is similar to that of a chicken embryo during its first seventy-two hours. Moreover, the low cost and replicability of the chicken embryo experimental model are important advantages for neural development studies (20,21,23). While the in ovo toxic effects of various compounds on neural tube development have been studied (8,12,24,27), an evaluation of the link between SY and NTDs in a chicken embryo model has not been reported (12,21).

In this study, we aimed to investigate the histopathological and embryotoxic effects of SY exposure on neural tube development in a chicken embryo model.

MATERIAL and METHODS

Experimental Embryos and Study Design

Sixty, 65 ± 2 g, Leghorn genus fertilized special pathogen-free (SPF) chicken embryos were used from eggs obtained from The Ministry of Agriculture and Rural Affairs, Bornova Veterinary Control Institute, Izmir, Turkey. Animal experiments in this study were performed with ethical approval from the Local Animal Experiments Ethics Council of Marmara University (approval number and date: 83.2021mar, 09/11/2021). Sample numbers and doses were determined according to data obtained from previous studies and considering the 0–2.5 mg/kg dose recommended by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) as a daily allowable maximum. The SPF eggs were randomly divided into three groups of twenty eggs each. Groups were determined as control (0.1 ml saline), daily intake dose (2.5 mg/kg SY), and high dose (5 mg/kg SY) (2,7,8,28). Due to potential losses from physical damage and infertile eggs during the experiment, each group utilized forty extra SPF eggs. Fertilized SPF eggs were placed in a CT60S incubator (Cimuka, Turkey) with their pointed edges pointing downward (Figure 1). The day on which the eggs were placed in the incubator was recorded as day 0. The eggs were kept at 37.2 ± 0.1°C and 60–70% humidity for 30 hours in an incubation system with an automated air cycle feature and were rotated every two hours to vary their positions. Stage 9 of the Hamburger-Hamilton (HH) staging series (HH 9; 30th hour of incubation) coincides with the development of the neural plate (24).

Preparation of Sunset Yellow FCF E110

Pure SY (Cas No. 2783-94-0; 90% purity, IFC, Turkey) in powdered form was dissolved in 0.9% isotonic sodium chloride. A main stock was prepared for each group. Powdered SY was weighed at 12.8 mg for a daily dose and 26.4 mg for a high dose. The main stocks were prepared using 8 ml of physiological serum (Polifarma, Turkey) in 15 ml Falcon containers. The main stocks were wrapped in foil and stored in the refrigerator at 4°C for later use. The solutions were vortexed (BioCote, United Kingdom) before application. A 0.22 µm filter (Minisart nonpyrogenic hydrophilic filter unit, USA) was used to prepare solutions for use.

In Ovo Administration, Evaluation and Sample Collection

At HH 9, infertile and fertile eggs were separated according to their injection status. Fertilized SPF eggs were removed from the incubator and sterilized with 70% ethyl alcohol. To expose the ring-shaped embryonic discs, apertures of 0.5–1 cm in diameter were cut into the eggshell membranes. This process was performed using a new technique on all eggs used in the experiment (8,12,24). The SY solution was carefully injected subblastodermically into the chicken embryonic disc using an insulin injector attached to a 26 G 1/2 needle. Since excessive injection could damage the embryonic disk, the amount of substance did not exceed 0.1 ml. The openings in the eggs were then covered with a band, and the eggs placed in the incubator for 72 hours (Figures 2A–C). At the end of the incubation period, the SPF eggs were removed from the incubator, the egg windows were expanded, and the egg yolk was placed in a glass container with serum physiologic water. Afterward, the embryonic membranes and vitellin membranes were cut with surgical scissors over the yolk sac (Figures 3A–D). Both the vitellin membrane and the membrane-attached blastoderm were dissected from the yolk sac. The samples were then placed in an embryo container with 10% neutral buffered formalin for histological analysis.

Histopathological Analysis

Embryos from all groups were fixed in a 10% neutral buffered formalin solution for 72 hours at ambient temperature for light

Figure 1: The arrangement of special pathogen-free (SPF) eggs within the incubator. The pointed edges of SPF eggs project downwards.

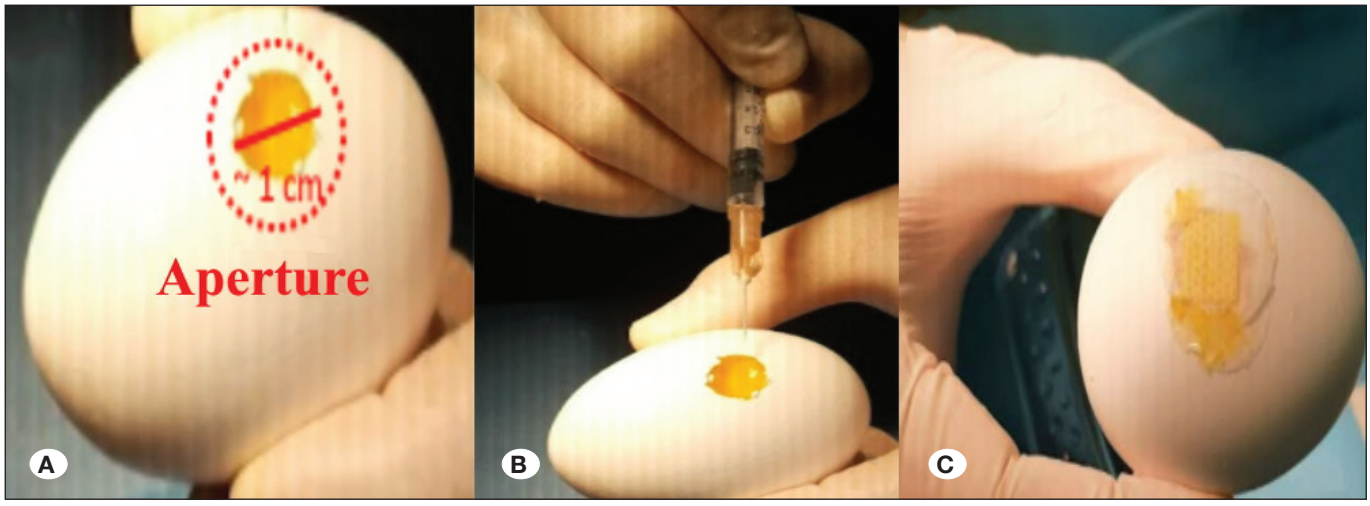

Figure 2: Demonstration of the injection technique in chicken embryos. **A)** The aperture opens to a diameter of about 0.5–1 cm. **B)** Subblastomeric injection is performed through a small aperture. **C)** The openings in the special pathogen-free eggs are covered with point injection bands.

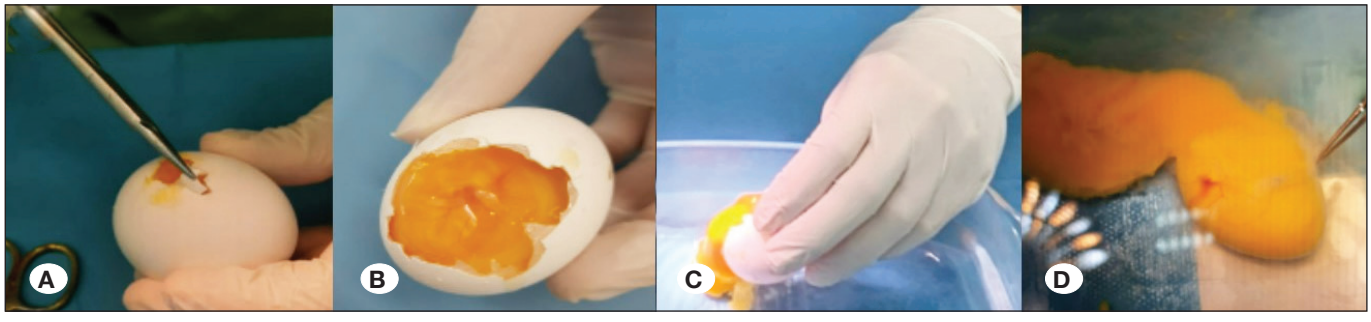

Figure 3: Chicken embryo collection from special pathogen-free eggs**. A)** Picture demonstrating the expansion of the previously opened aperture. **B)** Early chicken embryo from the expanded window at 72 hours. **C)** The placement of the chicken embryo in the glass container with serum physiological water. **D)** Cutting the embryonic membrane, vitellin membrane, and yolk sac using surgical scissors.

microscopic examination. The embryos were dehydrated using an increasing series of ethyl alcohol (70%, 80%, 90%, and 96%). Samples were then cleared with xylene and embedded in paraffin blocks. The blocks were cut into 4 µm thick sections with a rotary microtome (Medite M530, Germany), placed on the glass sides, and deparaffinized in an incubator. The sections were stained with hematoxylin and eosin (H&E) for histomorphological evaluation and covered with mounting medium (Entellan). Morphological changes in the neural tube, notochord, and neuroepithelium were examined and photographed under a computer-equipped CCD camera (Olympus DP 72, Tokyo, Japan) and attached photo-light microscope (Olympus BX51, Tokyo, Japan).

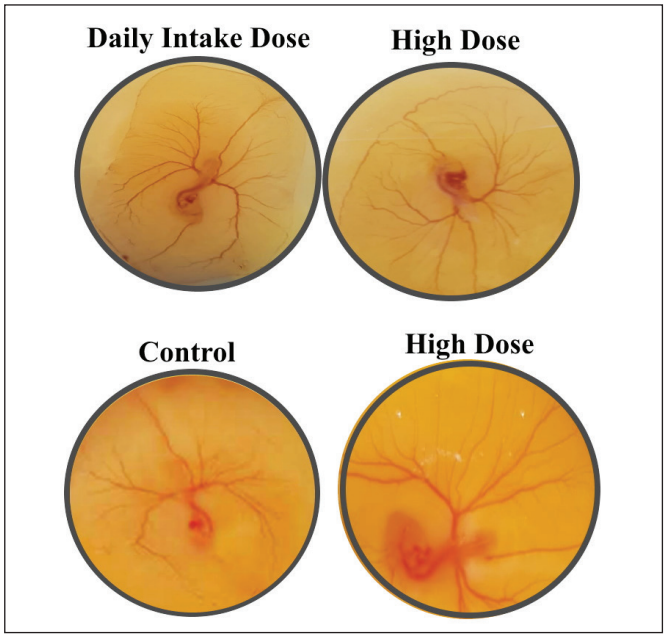

Figure 4: Macroscopic images of the samples after 72 hours of incubation**.** The embryonic disc and vascular structure of the yolk sac are more advanced in the control group, but the embryos in the daily and high-dose groups have weaker vascular structures.

Statistical Analysis

Data were analyzed with the chi-squared test using Graphpad Prism 9. Probability values of $p<0.05$ were considered statistically significant. The independent Student's t-test was used to determine whether changes were random in comparisons between the experimental groups.

RESULTS

Macroscopic Analysis

Following the completion of the injection procedures for the experimental groups, the number of dead and viable embryos was recorded in the 72nd hour of incubation. In the control group, 90% of embryos were alive and 10% were dead; in the daily intake group, 89% of embryos were alive and 11% were dead; and in the high dose group, 81% of embryos were alive and 19% were dead. The ratio of dead samples in the was significantly higher in the groups administered SY than in the control group, and was higher in the high-dose compared to the daily dose group ($p=0.006$). Compared to the SY groups, the vascular structure of the embryonic disc and yolk sac was observed to be more prominent in the control group (Figure 4). At the 72nd hour, all experimental groups were found to be compatible with HH 19.

Histopathological Analysis

The neural tube and notochord were observed in the transverse and sagittal planes in all groups. In the control group, the surface ectoderm was intact, the neural tube was closed, and the notochord, somites, neuroepithelium, and basal membrane surrounding the neuroepithelium were normal (Figure 5A). NTDs were detected in four embryos in the daily dose SY group (Figure 5B). In one additional sample from the same group, the neural tube was intact, but the width of the lumen was remarkable. In another embryo, neuroepithelial tissue was observed to have thickened, but the neural tube and notochord were intact. In addition, the caudal region of the neural tube was enlarged, and the lumen was not observable. In other embryos from the same group, the neural tube

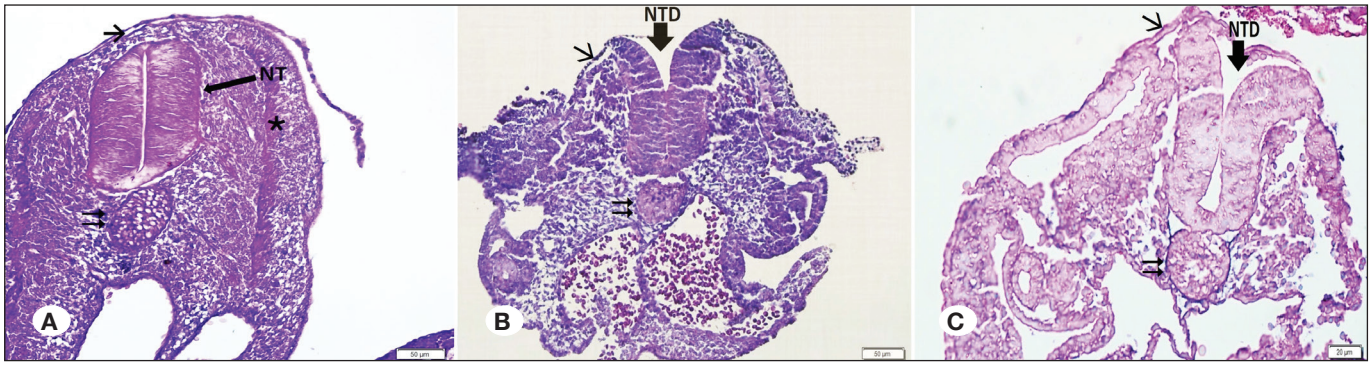

Figure 5: Hematoxylin and eosin-stained light micrographs showing the neural tube, notochord, and surface ectoderm in the transverse plane in all groups. **A)** Neural tube (NT), notochord (⇉), somite (*), and surface ectoderm (→) are intact in the control group (Scale bar: 50 µm). **B)** Neural tube defect (NTD) as observed in the 2.5 mg/kg Sunset Yellow FCF E110 (SY) group. **C)** NTD as observed in the 5 mg/kg SY group (Scale bars: B: 50 µm, C: 20 µm).

structure was similar to that of the control group. In the high-dose SY group, NTD was observed in six embryos (Figure 5C). In addition to NTD cases, extensive vacuoles were observed in the notochord of another embryo, and abnormal neural tube boundaries and neuroepithelial tissue were observed in a further sample. No evidence of NTD was observed in the remaining embryos of this group. 75% of embryos developed normally in the daily SY dose group, with 25% developing with NTD. In the high-dose SY group, 45% of embryos developed healthy, while 55% of embryos developed NTDs. As expected, a higher proportion of embryos with NTD was observed in the high-dose group compared to the other groups. The chi-squared test for independence yielded a p-value of 0.0004. The experimental groups were compared using the Student's t-test, indicating a statistically significant ($p<0.05$) relationship between the variables (Figures 6A–C) (Table I).

DISCUSSION

In humans, the development of the neural tube coincides with the third week of gestation; this period is particularly sensitive to external factors (3,21). Determining the effects of short- and long-term consumption of FA and adjusting the dosages of these substances accordingly is crucial (15,17,18). FA are commonly used chemicals present in our daily lives to extend the shelf life of foods, satisfy nutritional requirements, and improve the flavor, appearance, and quality of nutrients (6,16). SY is a synthetic azo dye used in numerous industries, particularly the food industry (15,22,28).

The first 72 hours of neural and spinal development in the chicken embryo are very similar to the first month of the human embryo (5,8,23). The minimal cost and repeatability of the chicken embryo experimental model are significant benefits. The use of numerous chicken embryos provides a statistical advantage for the evaluation of toxicity when compared with mammalian species studies. For this reason, we utilized chicken embryos in our study.

It has previously been reported that no adverse effects are observed when food coloring limits are not exceeded. However, in certain developing countries, the average consumption of SY exceeds the permissible daily ingestion

Table I: Dispersion of Neural Tube Defect Development and Statistical Evaluation in Chicken Embryos in All Groups

Groups	Sunset Yellow FCF E110 (SY)		p-value ($p<0.05$)
	Intact	NTD	
Control Group, (0.1 ml saline) (n=20)	20	0	**0.0004**[1]
Daily Intake Dose Group (2.5 mg/kg SY) (n=20)	16	4	Control vs Daily Intake dose groups **0.002**[2]
High Dose Group (5 mg/kg SY) (n=20)	14	6	Control vs High dose groups **0.0002**[2]

[1]Chi-square test; [2]Student's t-test.

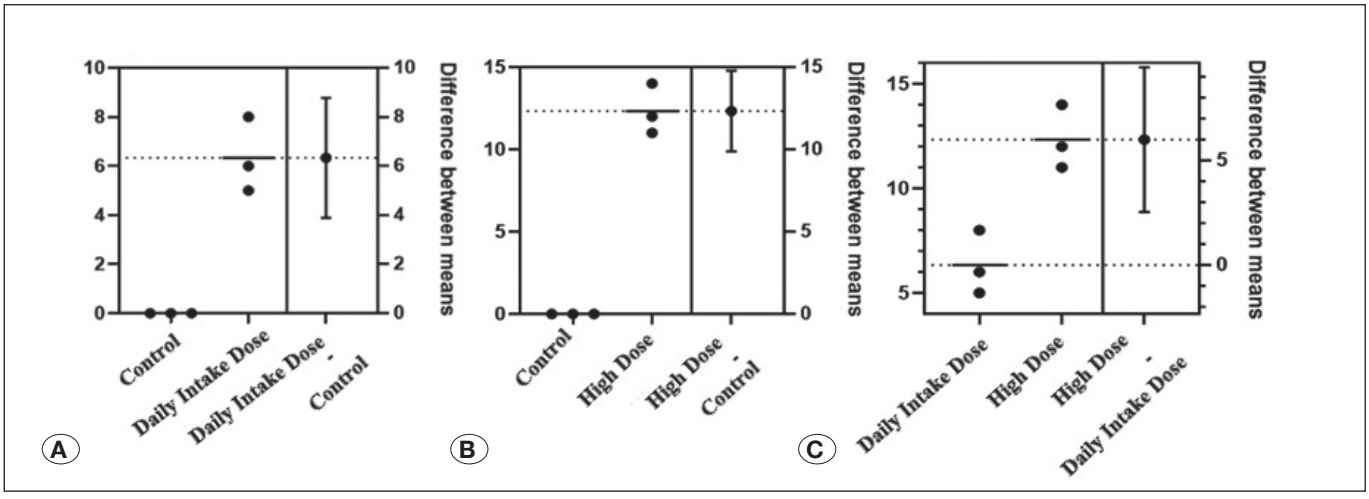

Figure 6: Graphs showing student t-test results of experimental groups. **A)** Comparison between the control and daily intake dose groups (p=0.002); **B)** Comparison between the control and high dose groups (p=0.0002); **C)** Comparison between the daily and high dose groups (p=0.0086).

value by 88%, whereas in Europe and the United States, this value varies between <0.2 and 12% (6,16,25,29). In nearly all relevant age groups, the consumption of food dyes exceeds the recommended daily allowance. Considering the body mass of children, this calculated value is disproportionately high. This is a factor that should raise alarms and concerns in terms of public health in many developing countries. Amplifying these concerns, we found NTDs in every group to which SY was applied in our study.

Previous studies have suggested that consuming SY in food can result in various adverse effects. However, it is unknown whether this was a result of the toxic effects of SY or a dose-related effect. The findings of this study contribute to the overall understanding of these circumstances. Changes in developmental rate, morphological abnormalities, other induced deformities, and altered survival/mortality rates of embryos in experimental groups have been previously observed (1,2,25,28). In one study, pregnant rats were exposed to food dyes including SY; learning and memory difficulties were observed in the newborn rats, reportedly due to changes in expression of nicotinic acetylcholine receptors (1,2). In our study, the majority of embryonic deaths during the incubation period occurred in the SY groups, particularly the high-dose group. During microscopic examinations of high-dose group embryos, neural tube fragility was prominent, its boundaries were often abnormal, and the neuroectoderm was generally thin. These findings suggest that food dye affected the development of the embryos.

SY has also been associated with certain behavioral changes, with reports of hyperactive behavior disorder in children, certain allergies, and side effects caused by effects on the immune system (18). Sweeney et al. demonstrated that azo dyes, including SY, could directly initiate oxidative genotoxicity (26). In a study with chicken embryos, necrosis developed in the hepatocyte and renal tubule cells of all SY-treated groups as a result of structural alterations to organelles. Histopathological alterations, such as hydropic degeneration and renal tubules in hepatocytes, indicated that intracellular edema developed as a consequence of toxicity or immune response. It was concluded that SY had a significant cytotoxic effect on cells (8,22,25). In our study, the 2.5 mg/kg daily dose SY group exhibited vacuolization at the rostral end of the caudal neural tube region. In addition, the neuroepithelial tissue was intertwined, the neural tube exhibited hydropic alterations, and the lumen was inaccessible due to edema. In the 5 mg/kg SY group, the notochord was severely vacuolated and adhered to the neural tube, and the cell nuclei were heterochromatic. In addition, neural tube boundaries were irregular, and neuroepithelial tissue was abnormal. Ali et al. observed that in vivo combinations of SY and sodium benzoate (NaB) in rats caused structural abnormalities associated with genotoxicity through DNA damage and abnormal serum protein distribution (1). McCann et al. suggested that dietary food colorings such as SY, food preservatives such as sodium benzoate, or combinations of the two, caused increased hyperactivity in 3-year-old and 8/9-year-old children (18). Studies have shown that tartrazine administration in rats causes neuronal loss, vacuolar degeneration, and several other histopathological changes in the cellular layers of both the cerebellar and cerebral cortex (4,13,14,19). These findings suggest that it can induce neuro-degenerative changes, chromatolysis, pyknosis, and apoptotic cell death in the rat brain. These changes were attributed to aromatic amines that increase reactive oxygen species production (17,22,26,29). In our study, in the histopathologic evaluation of both SY groups, the notochord was densely vacuolated and swollen in the caudal region of the neural tube, and the cell nuclei had a heterochromatic appearance. In addition, the neural tube borders were irregular, and abnormal neuroepithelial tissue was present. Since both food dyes belong to the azo group, they share final degradation products. Therefore, the histopathologic results of the two studies are thought to be parallel to each other.

We believe that SY may cause malformations in spinal cord development in human embryos due to its negative effects on neural tube development in chicken embryos. While the data obtained from studies using the chicken embryo model cannot be directly translated to human embryo developmental stages, they may indicate future evaluations to be made in this field.

CONCLUSION

In conclusion, although SY was applied at dosages within the upper limits of the daily recommended intake dose, we have demonstrated its degenerative effects on the neural tube development of chicken embryos. In our study, we detected statistically significant incidence of NTD in all groups in which SY was administered. Based on these data, the reference values for food dyes could be reevaluated. We believe that SY should not be ingested in high doses for an extended period of time, and its use should be controlled, particularly during pregnancy. Further studies are needed to investigate the NTD-inducing potential of SY in chicken embryos through specific molecular pathways, and using a broader range of groups and doses.

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This study was produced from the first author's speciality thesis.

Declarations

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Availability of data and materials: The datasets generated and/or analyzed during the current study are available from the corresponding author by reasonable request.

Disclosure: The authors declare no competing interests.

AUTHORSHIP CONTRIBUTION

Study conception and design: STE, FT, DA
Data collection: FT, DSA
Analysis and interpretation of results: FT, DA, DSA
Draft manuscript preparation: FT, EA
Critical revision of the article: FT, STE, EA
Other (study supervision, fundings, materials, etc...): FT, STE, EA
All authors (FT, DSA, DA, EA, STE) reviewed the results and approved the final version of the manuscript.

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