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Congenital Malformations in Chicken Embryos After Oxybenzone Exposure

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ABSTRACT

Topical use of oxybenzone, commonly found in sunscreens, can be absorbed by the skin, and long-term use may cause endocrine disruption, cancer, and teratogenic effects. However, its potential teratogenic effects on embryonic development have not been well-studied. This study aims to determine the impact of oxybenzone exposure on the early stage of embryonic development. Chicken embryos aged 72 hours (20 Hamburger-Hamilton/HH stage) were exposed to a pure oxybenzone for 24 hours at varying concentrations (0, 1, 5, 10, and 20 ppm), each group consisting of 3 embryos. Embryo preparations were made using the wholemount method. Morphological abnormalities were observed with a stereo microscope, and descriptively morphometric measurements were analyzed using ImageJ software. Statistical analysis used One-way ANOVA and Tukey's test for normally distributed data, while Kruskal-Wallis H and Mann-Whitney U test for non-normally distributed data. This study found that oxybenzone significantly enlarged the embryo, telencephalon, and eye. Several abnormalities were observed in the embryos exposed to oxybenzone, including incomplete closure of the anterior neuropore, concavity in the anterior and lateral of the mesencephalon, and depressions in the tail bud. This study concludes that oxybenzone acts as a teratogen, causing abnormalities in embryonic development, particularly in the central nervous system.

1. Introduction

Adverse manifestations of sun exposure on the skin can be reduced using sunscreens (Benson 2017). Based on their protection method, sunscreens are divided into chemical and physical sunscreens (Draelos and Thaman 2005). Physical sunscreens reflect and scatter UV radiation by forming a layer over the skin like clothing that acts as a mechanical barrier. The reflectance properties will determine the sunscreen's effectiveness, including refractive index, particle size, layer thickness, and base dispersion (Siller *et al.* 2018; Gabros *et al.* 2022). Physical sunscreens usually contain zinc oxide (ZnO) and titanium oxide (TiO_2) (Tampucci *et al.* 2018).

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Chemical sunscreens are known as organic sunscreens. The structure of the aromatic component conjugated with carbonyl groups causes UV light with high energy to be absorbed and causes the molecule to reach an excitation state. When the molecule returns to the ground state, it will release lower energy with a longer wavelength. Chemical sunscreens consist of UVA and UVB blockers. UVB filters absorb the entire spectrum of UVB radiation (290-320 nm), for example, amino benzoate compounds, salicylates, cinnamates, camphor derivatives, and ensulizole. Meanwhile, UVA filters absorb part of the spectrum, namely 340-400 nm and 320-340 nm, for example, benzophenone, anthranilate, avobenzone, and ecamsule (Gabros *et al.* 2022). Chemical sunscreens are generally combined because no active agent at the concentration levels currently permitted by law can provide adequate UV protection (Díaz-Cruz *et al.* 2008).

Oxybenzone (2-hydroxy-4-methoxy-benzophenone) is one of the compounds belonging to the benzophenone group that is widely used in chemical sunscreens (Corrêa *et al.* 2012). Oxybenzone has a molecular weight of 228.24 g/mol. It is a white to pale yellow powder or crystal with a melting point of 62-65°C, insoluble in water, and easily soluble in ethanol (95%) and isopropyl alcohol (De Gennaro *et al.* 1995; Baughman *et al.* 2009; American Chemical Society 2018). The chemical structure of oxybenzone can be seen in Figure 1. This compound can absorb UVA 2 and UVB at 290-340 nm wavelengths as an antidote to solar radiation.

Currently, oxybenzone is limited to a maximum concentration of 6% in preparations due to its allergic and photoallergic potential. In addition, systemic absorption is possible in long-term use because it can penetrate the stratum corneum due to its lipophilic nature, low molecular weight, and high log P. Drugs must circulate systemically in the body, and oxybenzone that circulates systemically has been investigated for its endocrine and carcinogenic effects (Krause *et al.* 2012).

The chicken embryo (*Gallus gallus gallus*) is a beneficial model for developmental biology, experimental embryology, and teratology. Complete descriptions of chicken development are widely available (Patten *et al.* 1948; Lillie 1952). Importantly, developmental genes identified in human and rodent studies have expression patterns and functions identical to those of chickens. In addition, chicken genome sequences show strong similarities with other vertebrates and allow the use of molecular biology techniques such as in ovo siRNA electroporation and gene transfection with viral vectors (Fekete and Cepko 1993; Hermann and Logan 2003; Nakamura *et al.* 2004; Rao *et al.* 2004). A significant advantage of using avian models in teratology and experimental biology is that researchers can open the eggshells, examine the embryos, and precisely target exposure to specific developmental stages. Agents such as growth and teratogenic factors can be targeted using implants or grafts (Tickle *et al.* 1982; Eichele *et al.* 1984) to particular tissues. Contributions from maternal metabolism are absent in the in-ovo system, which makes it possible to isolate teratogenic effects directly. In summary, the chick embryo model provides an easy and inexpensive system to apply modern experimental tools to ascertain how teratogens interfere with specific mechanisms underlying organogenesis and morphogenesis (Drake *et al.* 2006).

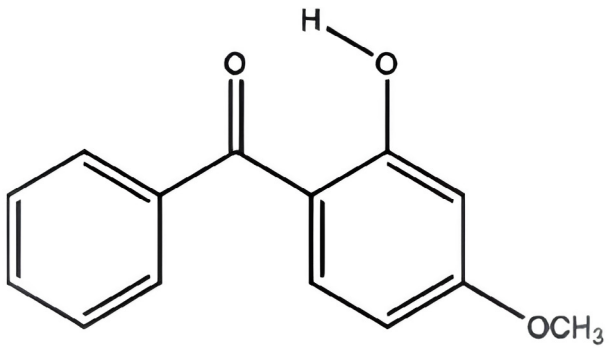


Figure 1. Oxybenzone structure (Baughman *et al.* 2009)

Chicken embryos have been used to study suspected teratogens such as thalidomide, phenytoin, ethanol, and the Zika virus. The abnormal phenotype produced by the chicken embryo model can accurately mimic the phenotype formed in humans. Understanding the mechanism of teratogenesis of a factor can provide insight into the compound's biological action and how it causes congenital anomalies, which can lead to potential ways to make safer drugs. In addition, the more we understand how congenital anomalies occur, the more prevention strategies can be determined (Jurand 1966; Therapontos *et al.* 2009; Beedie *et al.* 2016a, 2016b; Knobloch *et al.,* 2017; Vargesson dan Hootnick 2017; Davey *et al.* 2018; Wachholz *et al.* 2021).

Research on the teratogenic effects of oxybenzone on the developmental stages of chicken embryos has not been widely conducted. Therefore, this research aims to study the teratological effects caused by oxybenzone, which is often found in sunscreen products and cosmetics, using chicken embryos as an experimental model. It is important to evaluate the potential risks associated with exposure to this substance at an early stage of embryonic development. This research is also expected to contribute to teratology by identifying and understanding the causative factors of congenital anomalies induced by certain chemical substances such as oxybenzone.

2. Materials and Methods

2.1. Ethical Statement

This study was performed in accordance with several ethical references, including the Institutional Animal Care and Use Committee Policies and Procedures of University of Louisville (2010); ACUC Guidelines The Use and Euthanasia Procedures of Chicken/Avian Embryos (2012); & Ribatti and Annese (2023), which states that ethical review is generally not required for

research involving chick embryos up to 13-17 days of incubation (E13-17). These guidelines recognize that embryos at these stages have limited sentience and a reduced capacity for pain perception. This study strictly adhered to these principles, ensuring that procedures involved minimal handling and did not cause significant pain or distress to the embryos.

2.2. Preparations of Oxybenzone Solution

Pure oxybenzone powder from the Syah-house brand was prepared for weighing using ACIS digital analytical balance. The solvent used to dissolve oxybenzone was olive oil. A 1 ppm concentration solution is prepared by dissolving 1 mg of solute in 1,000 ml of solvent (Katoch 2011). In this study, oxybenzone solution was made with four different concentrations, 1, 5, 10, and 20 ppm, each as much as 100 ml. The oxybenzone solution of 1 ppm concentration was prepared by dissolving 0.1 mg of pure oxybenzone powder in 100 ml of olive oil. An oxybenzone solution of 5 ppm concentration was made by dissolving 0.5 mg of pure oxybenzone powder in 100 ml of olive oil. The oxybenzone solution of concentration 10 ppm was prepared by dissolving 1 mg of pure oxybenzone powder in 100 ml of olive oil. An oxybenzone solution of 20 ppm concentration was made by dissolving 2 mg of pure oxybenzone powder in 100 ml of olive oil.

2.3. Egg Incubation and Oxybenzone Exposure

The treatments consisted of 5 treatment groups with three fertile kampong chicken eggs (*Gallus gallus gallus*) each, so the total fertile eggs incubated using an incubator at 37°C for 72 hours were 15 eggs. After incubation, candling was carried out using a light bulb in a dark room to observe the eggs. The egg is positioned above the bulb to determine the embryo's presence, characterized by blood vessels. Eggs containing embryos are marked using a pencil. The eggs were transferred individually to the Biobase laminar airflow (LAF) workbench for the injection process. The egg is hollowed out at the pointed part using scissors. Syah-house's oxybenzone solution was taken using a micropipette as much as 100 µL and then dripped into the egg through the hole that had been made previously. Eggs in the control group were dripped with 100 µL of 0.9% NaCl solution. The egg hole is then covered using Hypafix Leukoplast. After being treated with oxybenzone and NaCl, the eggs were incubated again in the incubator for 24 hours. This method is based on research by Henshel *et al.* (2002) & Wachholz *et al.* (2021) with modifications.

2.4. Embryo Collection and Making Whole Mount Preparations

Eggs that have been incubated for 96 hours are removed from the incubator. The eggshell was opened in a container containing a warm 0.9% NaCl solution at a temperature of ±40°C. The membrane around the embryo is cut to collect the embryo. The embryos were transferred to a watch glass and washed with 0.9% NaCl solution. A hole is made in the filter paper and attached to the embryo, which is positioned in the middle of the hole that has been made. Embryos were fixed for 24 hours using Bouin's solution. After 24 hours, the embryos were washed using 70% alcohol until the yellow color of Bouin's solution disappeared. Embryos were stained using Eosin Y for 5 seconds and then washed using 70% alcohol. The embryos were soaked in graded alcohol for 5 minutes each. Clearing is done by soaking the embryos in toluene for 10 minutes and then in xylol for 30 minutes. This method is based on research by Chuncharunee *et al.* (2003) with modifications.

2.5. Embryo Morphological Observation and Measurement

Observations and photography were carried out using a stereo microscope. Millimeter blocks are used to calibrate sizes and create scales. Photographic and morphometric analysis of embryos using the ImageJ software (Abràmoff *et al.* 2004). The method for measuring body area, mesencephalon, telencephalon, and eyes is based on research by Wachholz *et al.* (2021), shown in Figure 2.

2.6. Data Analysis

Descriptive analysis is used to describe the morphological abnormalities that appear in the embryo. Data was analyzed using SPSS 26.0 software (SPSS Inc. Chicago, USA). The data were tested for distribution patterns and homogeneity using the Shapiro-Wilk test. Data that follows a normal and homogeneous distribution pattern will be tested using the One-way ANOVA test to determine differences between treatment groups, then continued using the Tukey test to determine the significance level between treatment and control. Data that do not follow a normal and homogeneous distribution pattern will be tested

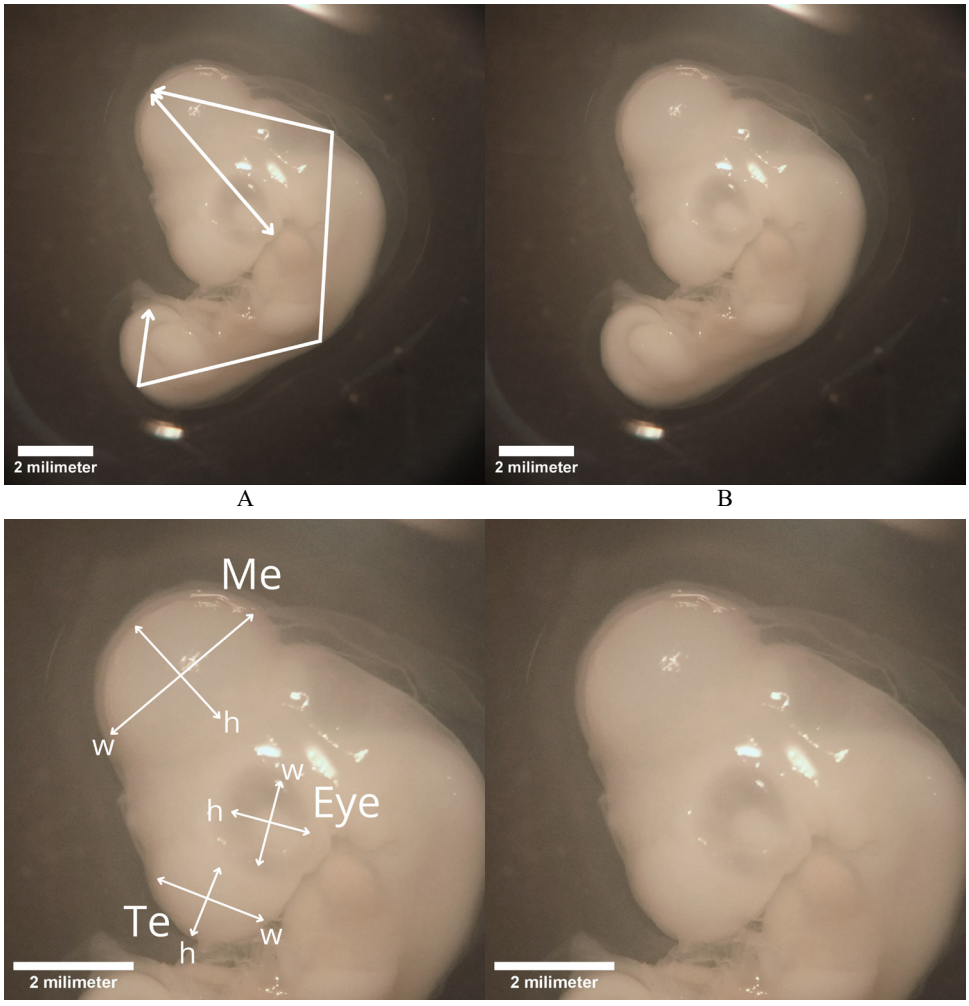


Figure 2. Morphometric measurements and documentation of chicken embryos using ImageJ software. (A) Measurement method of the body area of intact embryos, (B) How to document intact embryos, (C) Measurement method of encephalon vesicles (Me and Te) and eyes, (D) How to document encephalon vesicles (Me and Te) and eyes. Me: mesencephalon; Te: telencephalon; h: height; w: width. Scale bar: 2 mm

using the Kruskal-Wallis H test to compare the means of more than two groups. Then, it will continue using the Mann-Whitney U test to compare the means of the two groups (Utomo 2021).

3. Results

The results of this study are presented in Table 1 and Figure 3. Chicken embryos exposed to oxybenzone for 24 hours show that the largest area of the body, mesencephalon, and eyes was found at an exposure to a concentration of 1 ppm, while the largest area of the telencephalon was at an exposure to a concentration of 10 ppm when compared with controls. Even though there was an increase in mean size, exposure to a concentration of 5 ppm showed a smaller body area than controls. The smallest mesencephalon area was found at a concentration of 20 ppm, the smallest telencephalon area was found at 1 and 5 ppm, and the eye area was the smallest at 1 and 20 ppm when compared with controls. The significance of the size differences can be seen in Table 1. The wide decrease in random concentration indicates that the effect caused by oxybenzone does not depend on increasing concentration (concentration-dependent).

Figure 4 shows that embryos exposed to oxybenzone for 24 hours with a concentration of 1 ppm in replication 1 have tail buds that appear concave and are not fully formed. The tail bud is an aggregate of mesenchymal cells located at the caudal end of the vertebrate embryo. These mesenchymal cells represent

Table 1. Body area, mesencephalon, telencephalon, and eyes (mm^2) in 96-hour-old chicken embryos after exposure to oxybenzone for 24 hours

Parameters	Control	1 ppm	5 ppm	10 ppm	20 ppm
	Mean ± standard deviation (SD)				
Body area (mm^2)	20.99±0.49[a]	23.92±1.58[b]	17.96±1.14[a]	21.98±1.72[b]	21.82±1.59[b]
Mesencephalon area (mm^2)	2.50±0.24[a]	3.23±0.56[a]	2.52±0.12[a]	2.52±0.35[a]	2.41±0.21[a]
Telencephalon area (mm^2)	1.83±1.11[ab]	1.51±0.25[a]	1.80±0.19[ab]	2.05±0.24[b]	1.93±0.05[ab]
Eye area (mm^2)	1.54±0.20[a]	2.12±0.21[c]	1.27±0.26[a]	1.83±0.20[b]	1.47±0.12[a]

Numbers in the same row with different superscripts indicate significant differences ($p \leq 0.05$)

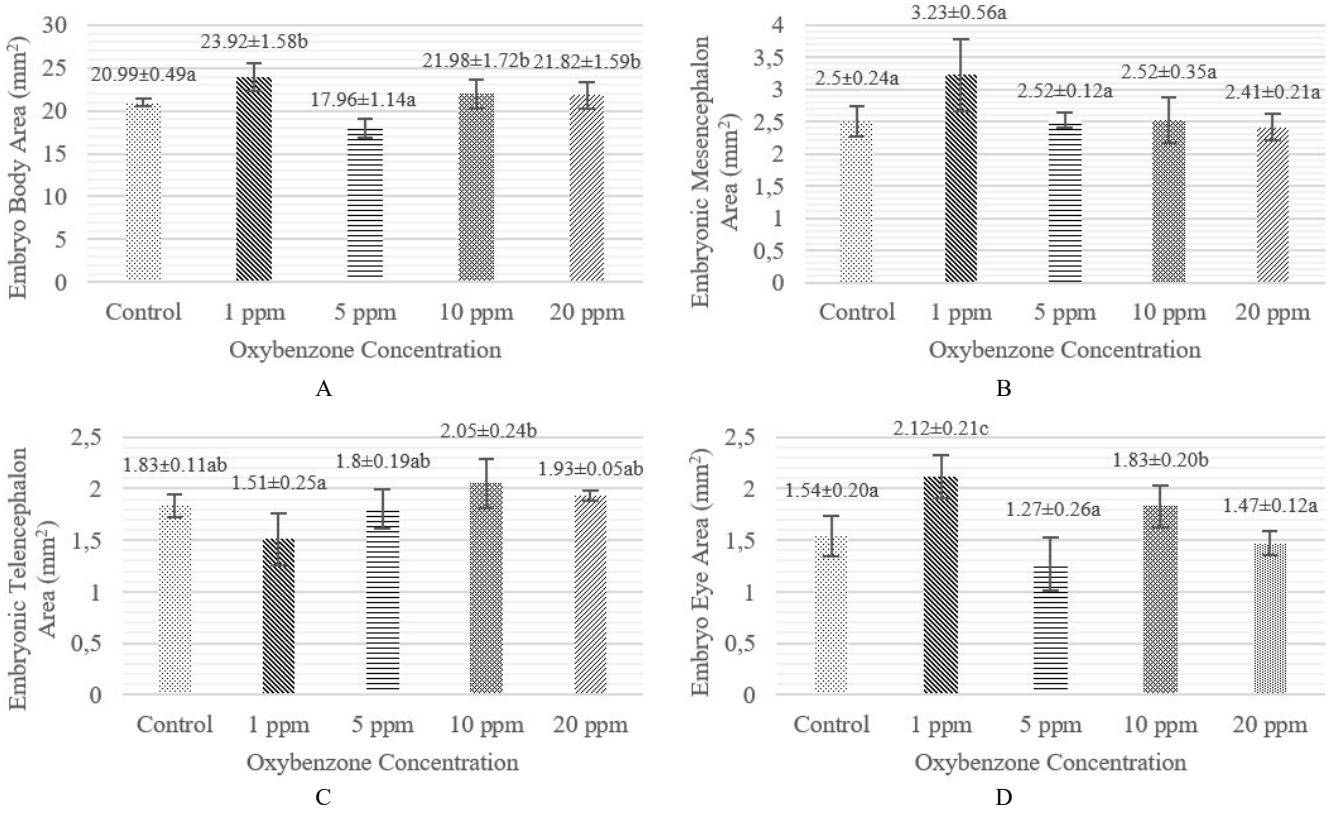


Figure 3. (A) Body area, (B) mesencephalon area, (C) telencephalon area, and (D) eye area (mm^2) exposed to oxybenzone at various concentrations (ppm) were compared between treatment groups. Mean ± standard deviation (SD). Letters a, b, and c indicate significant differences ($p \leq 0.05$). The histogram shows that the effect of oxybenzone does not depend on concentration on chicken embryo growth

the fusion of Hensen's node with the remainder of the primitive streak.

In the second replicate embryos exposed to oxybenzone at 10 ppm, the body appeared shorter, and the wing buds were not yet visible. Meanwhile, in the third replicate embryo, the body appeared abnormally longer. Furthermore, morphological abnormalities could be observed in embryos exposed to oxybenzone at concentrations of 1, 10, and 20 ppm, namely that the anterior neuropore did not appear to be closed entirely and was flatter and more concave in the anterior part. In the first and third replicate embryos exposed to oxybenzone at a concentration of 10 ppm, the lateral side of the mesencephalon appeared concave. Meanwhile, there were no abnormalities in the eye morphology of embryos exposed to oxybenzone at concentrations of 1, 5, 10, and 20 ppm.

4. Discussion

The chicken embryo is a highly relevant model because its brain development and patterns are similar to those of humans (Stern 2004). Therefore, the chicken embryo model can mimic human phenotypes after teratogen exposure, as seen in recent Zika cases (Wachholz *et al.* 2021). Based on other researchers' compilation of

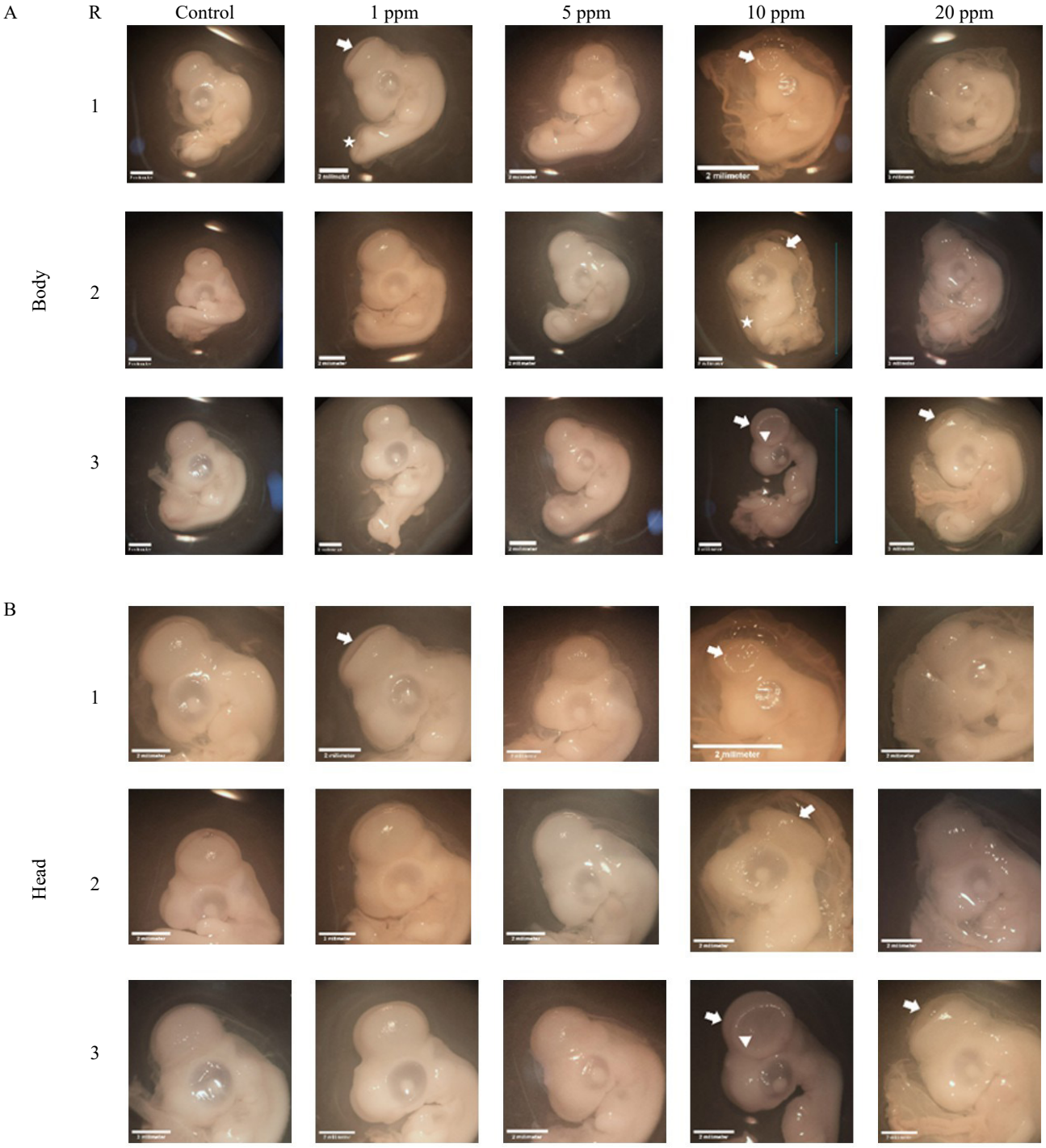
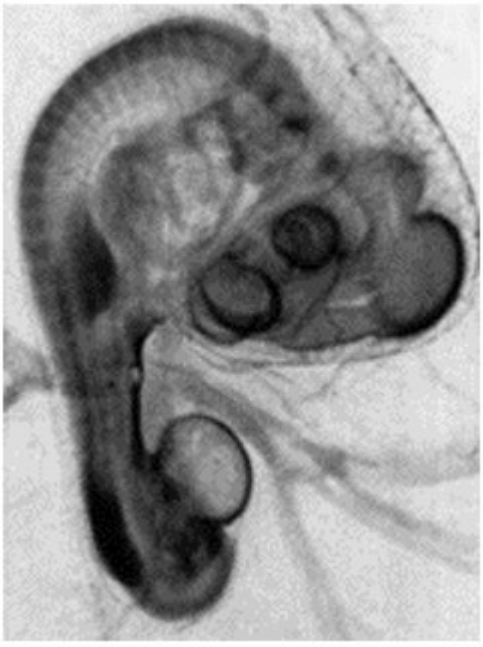


Figure 4. (A) Whole chicken embryo body, (B) chicken embryo head. Exposure to oxybenzone with concentrations of 1, 5, 10, and 20 ppm for 24 hours caused body area, telencephalon, and eyes to be larger than controls. Morphological abnormalities in the form of open anterior neuropores (arrow), incompletely formed tail buds (asterisk), and depressions on the lateral side of the mesencephalon (triangle). Scale bar: 2 mm

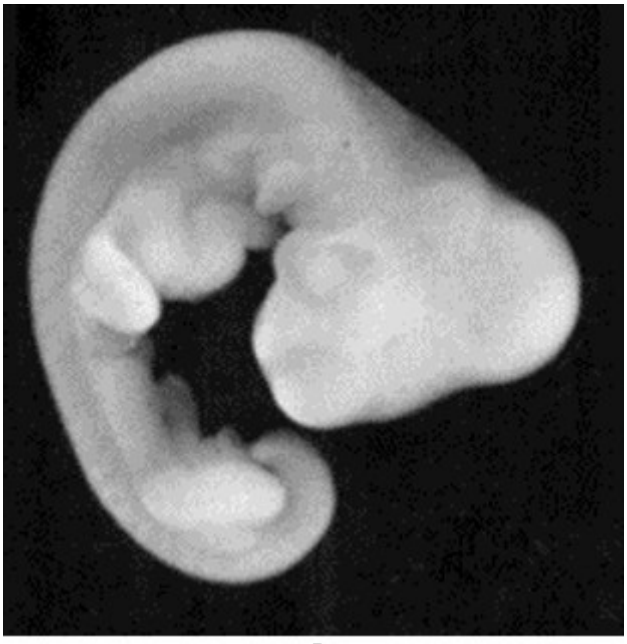
images and photographs, the Hamburger-Hamilton (HH) stages are proposed by Hamburger and Hamilton (1951) to describe embryonic stages. These stages are divided based on the number of somites formed, with each stage having an interval of 3 somites (Doty 2011).

This research utilized chicken embryos as an oxybenzone experimental model to identify morphological abnormalities following exposure to varying concentrations of oxybenzone. The results show several issues in 72-hour-old chicken embryos (20 HH stage) exposed to oxybenzone for 24 hours, including significant differences in body area, telencephalon, eye size, and morphological abnormalities in the mesencephalon. The mesencephalon (midbrain) is formed as early as stage 10 HH (33-38 hours), while the telencephalon forms due to the division of the prosencephalon into the telencephalon and diencephalon, starting at stages 12-13 HH (45-52 hours). The eyes begin to form, with the first sign of eye development being bulging on the lateral sides of the prosencephalon at stage 9 HH (29-33 hours) (Bellairs and Osmond 2014). The chicken embryo experimental model allows researchers to observe the precise day when major morphological defects begin to occur (Hill 2020).

Based on Bellairs and Osmond (2014), in 72-hour-old chicken embryos (20 HH stage) with normal development, the wing buds are symmetrical about the anteroposterior axis and are five-somite wide. The proximo-distal length of the wing buds increases. The leg buds still appear asymmetrical. There are 40-43 pairs of somites, and the embryo has finished rotating. The visceral (pharyngeal) arch indicates the maxilla is equal to or exceeds the length of the mandible-the second arch (hyoid) projects on the surface. The fourth arch is smaller than the third arch. The size of the allantoic vesicle varies. The eye pigment appears to be a faint gray color. The pulmonary arch and ductus arteriosus form, and the embryo is surrounded by the amnion. In 96-hour-old chicken embryos (24 HH stage), the length of the wing and leg buds appears to be greater than their width. The finger plates on the wings have not yet formed. There is a slight indication of two bulges in the mandibular formation process and three bulges in the second arch formation process. The second arch is longer ventrally and wider than the mandible. The third curve gets smaller, and the fourth curve gets bigger. These two curves begin to sink deeper into the surface of the embryo. The third visceral fissure is a longitudinal groove. The fourth visceral fissure is reduced to a small hole (Bellairs and Osmond 2014). The development of chicken embryos aged 72 and 96 hours is shown in Figure 5.



A



B

Figure 5. Chicken embryo development age (A) 70-72 hours (stage 20 HH); (B) 96 hours (stage 24 HH) (Hamburger and Hamilton 1951; Bellairs and Osmond 2014)

The method of oxybenzone exposure in this study involved dripping an oxybenzone solution onto 72-hour-old embryos. This approach is possibly less invasive than previous studies on chicken embryos (Thawani *et al.* 2018). However, other methods, such as direct injection into the embryonic blood vessels, are pretty challenging as they require specialized skills. This method

was selected to minimize bias associated with excessive embryo manipulation. Oxybenzone (benzophenone-3) is a compound that can easily cross the placenta, thus increasing the risk of exposure during the prenatal period (Wnuk and Kajta 2021). Several studies have detected oxybenzone in amniotic fluid, placental tissue, umbilical cord, and fetal blood (Wnuk and Kajta 2021). Therefore, the dripping method chosen in this study is appropriate to illustrate how oxybenzone penetrates.

In this study, phenotypes in chicken embryos resulting from oxybenzone solution exposure were similar to phenotypes observed in infant patients with hydrocephalus, such as an increased head circumference (Toma 2015), indicating brain developmental disorders. This study also found that oxybenzone exhibits teratogenic effects that are not dependent on increasing concentrations. Still, the abnormalities caused by oxybenzone in chicken embryos were consistent across all treatment groups, namely increased body area, telencephalon, and eye size, as well as morphological abnormalities in the mesencephalon. The mechanism of disturbance caused by oxybenzone is suspected to involve disrupted endocrine regulation, increased neurotoxicity, and apoptosis.

Oxybenzone is an endocrine-disrupting chemical that interferes with hormone regulation in developing embryos. Hormones such as estrogen and thyroid are crucial for controlling cell growth and differentiation, and disruptions to these hormones can lead to abnormal development (Talsness *et al.* 2009). Oxybenzone can mimic or block natural hormones in the body, affecting processes regulated by the endocrine system. In fish, oxybenzone has been associated with estrogenic, anti-estrogenic, or anti-androgenic activities, which alter vitellogenin levels and lead to decreased hatching rates. The disruption of hormone regulation can have significant developmental effects, as hormones play a major role in regulating growth and differentiation during embryogenesis (Kunz *et al.* 2006; Coronado *et al.* 2008). Oxybenzone exposure can act like an Endocrine Disrupting Chemical (EDC) by affecting normal hormone signaling pathways during the development of chicken embryos. This can lead to developmental abnormalities, including body size, organ development, and sex differentiation (Shulhai *et al.* 2023).

Oxybenzone has been found to influence embryonic neuronal cells and the developing brains of mammals by triggering neurotoxic effects and apoptosis and interfering with autophagy (Wnuk *et al.* 2018; Wnuk *et al.* 2019; Wnuk and Kajta 2021). Abnormalities in apoptosis and autophagy can lead to neurodevelopmental disorders, including autism and schizophrenia (Rudin and Thompson 1997 ; Jarskog *et al.* 2005). Abnormalities in the tail bud of embryos exposed to 1 ppm oxybenzone are suspected to occur through this mechanism. A typically formed tail bud in chick embryos starts at the 11 HH stage. It should continue to elongate until stage 25 HH with a shape that becomes increasingly conical towards the caudal end before undergoing remodeling, which includes differential growth between the tip of the tail bud and the anterior region, fusion of the anterior region of the tail bud with the caudal body, and cell death starting at the tip of the tail bud (Griffith *et al.* 1992). Morphological abnormalities showing a depression in the tail bud may indicate earlier apoptosis or cell death.

Exposure to oxybenzone in zebrafish larvae (*Danio rerio*) 24 hours after fertilization increases cell apoptosis in the head region. Morpholino knockdown experiments of rxrgb can restore the effects of oxybenzone, such as inhibited axonal growth, cell proliferation, and cell apoptosis. This suggests that the rxrgb receptor might mediate the teratogenic effects of oxybenzone (Tao *et al.* 2020). Additionally, Huo *et al.* (2016) it found that oxybenzone was present in the urine of mothers with children who had Hirschsprung's disease, a type of enteric neuropathy. Hirschsprung's disease can occur due to the failure of enteric neural crest cell migration to specific colon segments during weeks 5 to 12 of human embryogenesis (Nishiyama *et al.* 2012).

Exposure to oxybenzone in chicken embryos significantly increased the size of the body, telencephalon, and eyes compared to the control. Oxybenzone also caused morphological abnormalities and malformations in the head area, resembling hydrocephalus, incomplete closure of the anterior neuropore, concave in the anterior and lateral mesencephalon, and depression in the tail bud. This study concluded that oxybenzone may exert concentration-independent teratogenic effects on chick embryos. Based on this preclinical study, pregnant women may need to reduce or limit the use of this compound during the critical period of embryonic development. This study's limitations are still based on morphological and morphometric data and have not yet reached the molecular-level mechanism. Further analysis could be conducted to understand the underlying teratogenic mechanism of oxybenzone and to identify potential drugs that could repair the damage caused by oxybenzone exposure.

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