INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 24, No. 2, p. 133-145, 2024

RESEARCH PAPER OPEN ACCESS

In ovo exposure to commercial artificial sweeteners causes congenital malformations in chick embryos- A morphological study

Eman Al-Hazmi, Aisha Alrofaidi, Fatma Al-Qudsi*

Biology Department, King Abdulaziz University, Jeddah, Saudi Arabia

Key words: Chick embryo, Commercial artificial sweeteners, Growth retardation, Congenital malformations

http://dx.doi.org/10.12692/ijb/24.2.133-145 Article published on February 08, 2024

Abstract

Manufactured dietary sweeteners are spreading worldwide replacing the use of sucrose in nutrition. Artificial sweeteners are also used to manage weight, reduce calories and avoid dental problems. This study investigated the potential teratogenic effects that may arise from the consumption of a specific commercial artificial sweetener on chick embryo development on specific selected embryonic days 7, 10, 14, and 18. The fertilized chicken eggs were divided into 3 main groups control, vehicle control, and treated. The used sweetener contained (sorbitol, acesulfame K, and sucralose). Before incubation, the artificial sweetener solution (40 mg/kg body weight) was injected into the air sac of the treated group, while the vehicle control was injected with saline solution. Then embryos were incubated and sacrificed on the selected days. The results showed body growth alteration in the selected embryonic days. In addition, several congenital malformations were seen in these embryonic days such as subcutaneous bleeding, brain deformation, feather absence, abdominal hernia, and limb deformation. In conclusion, this study highlights some of the adverse effects of a selected commercial artificial sweetener used by many consumers and shows that the congenital malformations that resulted were caused by the combined effect of the selected sweetener components (sorbitol, acesulfame K and sucralose).

* **Corresponding Author:** Fatma Al-Qudsi ✉ falqudsi@kau.edu.sa

Introduction

Excessive sugar consumption is harmful to both our oral health and overall body weight. It also contributes to the development of various degenerative diseases. In recent years, there has been a growing emphasis on health, and fitness. Nowadays, a common view to managing weight involves substituting table sugar (sucrose) with low-calorie sweeteners (Bigos, 2012), leading people to shift to sugar-free or low-sugar alternatives present in products such as drink powders, jams, candies, jellies, carbonated beverages, dairy items, and canned food (Alsoufi *et al.*, 2017). These sugar substitutes can be either natural or synthetic, often referred to as artificial sweeteners (AS) (Tandel, 2011).

Artificial sweeteners give a sugar taste with reduced calories (Whitehouse *et al.*, 2008). They are widely used in the industry as they are added to highly processed items, such as flavored yogurts, low-sugar snacks, and convenient pre-made meals; they are also used as table sweeteners (Debras *et al.*, 2022). Also, many studies stated that farmers are introducing AS in the feed of cattle and chicken (Jiang *et al.*, 2020) This might lead later to the accumulation of these AS or their by-products in the milk or meat of cattle or chicken.

Depending on the source of calories, artificial sweeteners are classified as nutritive and non-nutritive sweeteners, also called non-caloric artificial sweeteners (NAS). The nutritive sweeteners are approximately equivalent to sucrose in sweetness and include the monosaccharide polyols (e.g., sorbitol, mannitol, and xylitol) and the disaccharide polyols (e.g., maltitol and lactitol). The non-caloric artificial sweeteners are from several different chemical classes that interact with taste receptors on the tongue, and typically exceed the sweetness of sucrose by a factor of 30 to 13,000 times (Whitehouse *et al.*, 2008).

NAS consumption is considered safe and beneficial, yet, supporting scientific data remains sparse and controversial (Suez *et al.*, 2014). According to the American Diabetes Association, there is still insufficient data to determine the role played by artificial sweeteners in the regulation of energy balance, appetite, body weight, and their influence as cardiometabolic risk factors (Bigos, 2012). Nowadays, many types of sweeteners are available in the market, such as aspartame, cyclamate, acesulfame K, sucralose, saccharin, and neotame (Alsoufi *et al.*, 2017).

Numerous studies have highlighted the minimal impact of artificial sweeteners on glycemic responses, indicating potential advantages in their consumption. However, some research has also pointed to connections between the intake of NAS and unfavorable outcomes such as weight gain and the elevated risk of developing type 2 diabetes (Suez *et al.*, 2014).

Artificial sweeteners have possible potential effects on the occurrence of cancer, migraine, preterm delivery and thrombocytopenia in humans in general, additionally; they have possible health benefits during use in weight control and diabetes (Alsoufi *et al.*, 2017). It was found that NAS can contribute to impaired glucose regulation in rodent models and in humans by altering the gut microbiota (Swithers, 2015). Three potential mechanisms have been discussed to explain the role of NAS in metabolic syndrome: the first one suggests that NAS interacts with sweet taste receptors in the oral cavity and throughout the human body, while the second one proposes that NAS causes the acceleration of glucose absorption due to the relationship between sweetness and calorie ingestion; therefore promoting adipogenesis and alteration of the gut microbiota, while the third proposed mechanism suggests that NAS interferes with learned responses to sweetness (Liauchonak *et al.*, 2019; Sylvetsky, 2018).

In this study, the selected commercial artificial sweetener contained sorbitol, acesulfame K, and sucralose in its ingredients. Sorbitol referred to as D-glucitol occurs naturally in berries, and fruits. It was first discovered in 1872 as a linear sugar alcohol ($C_6H_{14}O_6$). Commercially sorbitol was produced by hydrogenating d-glucose as a solution or crystalline.

Sorbitol is about one-third less calories (2.6 calories per gram) and 60% as sweet as sucrose. It is useful for people with diabetes and is safely used in processed foods, cosmetics and the pharmaceutical industry (Nezzal *et al.*, 2009; Silveira and Jonas, 2002). The common symptom that might be caused by sorbitol is chronic diarrhea, which results from excessive chewing gum consumption (Liauw and Saibil, no date). The acceptable daily intake (ADI) of sorbitol is (2.6 mg/kg/d) (Chung *et al.*, 2005).

Acesulfame potassium or (acesulfame K) is a quick perceptible non-caloric sweetener, suitable for numerous products, and stably soluble under high temperatures. Acesulfame K was approved in 1998 by the US Food and Drug Administration (FDA) for use in liquid non-alcoholic beverages and in 2003. It was approved to be safe by the Joint Expert Committee on Food Additives (Whitehouse *et al.*, 2008; Tandel, 2011). The ADI of Acesulfame K is (15 mg/kg/d) (Dwyer *et al.*, 2000).

In 1976, British researchers from Tate and Lyle discovered sucralose. It is 600 times as sweet as sugar, non-caloric (zero-calories) sweetener sugar substitute made from sucrose. It is used in chewing gum, baked goods, food and beverages, and frozen desserts. Most of the sucralose is excreted out of the body without any change, therefore; it is minimally absorbed by the body. Sucralose belongs to a class of chemicals called organic chlorides, some members of this class are carcinogenic or toxic. Sucralose does not accumulate in fat because it is fat-insoluble. The maximum estimated daily intake of sucralose does not show any effect on glucose levels (Tandel, 2011). Sucralose acceptable daily intake in the US is 5 mg/kg body weight/day (Chattopadhyay, 2014; de la Sucralosa en Humanos *et al.*, 2009)

Many studies were performed to assess the impact of aspartame on embryonic development (Al-Rashdi and Al-Qudsi, 2020) reported abnormal brain and eye development in chick embryos in response to treatment with some commercial artificial sweeteners containing aspartame. In a study on zebrafish embryos, some abnormalities were found after treatment with aspartame, and these abnormalities included a lack of tail and eye (Weerasooriyagedara, 2018). In utero exposure to AS containing aspartame caused defects in mice mammary glands (Fatma, 2019). An increase in the rate of mortality and retardation in brain formation, brain flexure, microphthalmia, and no branchial arch was seen in chick embryos exposed to low concentrations of aspartame. While anencephaly, anophthalmi, incomplete fusion of otic placode, abnormal heart looping, tail degeneration, lack of limb buds and somite retardation appeared in embryos exposed to high concentrations of aspartame (Kormsing *et al.*, 2020). One of the most important issues about artificial sweeteners was the accumulative precipitation of these sweeteners in the environment. Artificial sweeteners have been found in wastewater, groundwater, tap water and even in the air dust that consequently polluted plants and therefore increased the artificial sweeteners dosage for people consuming these plants (Gan *et al.*, 2013).

Historically, a large body of information known about human development came from the oldest discoveries from chick embryological studies; since chick embryo was the oldest vertebrate-developmental model system (Kain *et al.*, 2014).

Commercial artificial sweeteners (CAS) present in the market are a combination of different (AS). The teratological effect of each AS is different from their combined effect. Pregnant females consume CAS directly as a sachet added to coffee or tea or indirectly when consuming products containing CAS. This might unintentionally lead to exceeding the permitted daily dose of any of these AS such as sucralose, acesulfame-K, aspartame, and cyclamate.

As a result of the intensive research performed on the teratogenic effect of aspartame on embryos, it was noticed that many manufacturers excluded it from the commercial artificial sweeteners found in the markets (according to the content's label present on the products).

Therefore, this research aimed to evaluate the potential teratogenicity effects associated with the *in Ovo* consumption of aspartame-free commercial AS containing sorbitol, sucralose and acesulfame K using chick embryo as an experimental model.

Material and methods

All experimental procedures were done according to the ethical approval of the Unit of Biomedical Research Ethics Committee (REC) NCBE Registration No: (HA-02-J-008) from King Abdulaziz University (Reference No 459-22) Intervention (Animal Study).

Animals and materials

Fertilized eggs of chicken (*Gallus domesticus*) were obtained from Abdullateef Al-Katheeri farm in the third industrial zone on Alleeth road. The mean range of egg weight of the different batches throughout the experiment was 39.42 g.

The selected commercial artificial sweetener (CAS) was bought from local supermarkets. According to the manufacturer, each sachet (2 grams) was composed of the following 26 mg Sorbitol (E420), 0.134 mg sucralose (E955), 0.106 mg Acesulfame-K (E950), and 1973.76 mg corn powder and chromium picolinate. The dose used was 40 mg artificial sweetener/kg body weight (Al-Qudsi, Al-ahmadi and Ganash, 2021)

Experimental design

For each batch of eggs, the eggs were weighed before the beginning of the experiment to calculate the mean weight and form homogenate experimental groups. Then eggs were divided into 3 experimental groups: The control group (C), the vehicle control group (VC), which was injected with 0.1 ml saline solution in the air chamber by making two holes with a fine dissecting needle, and the treated group (T), which was injected in the air chamber by the same way with 40 mg/kg body weight of the CAS solution. Each group was then divided into four groups according to the designated sacrifice day 7 days, 10 days, 14 days and 18 days of incubation. All groups were incubated under identical standard conditions; temperature 37.5 C° and humidity 80%.

Sampling

The eggs were cracked at the side by scissors. Then embryos were extracted from the egg and released from the embryonic membranes into a Petri dish. Embryos were washed with saline solution and then dried with a tissue. Embryos then were weighed and photographed.

Photography

Whole Embryos were photographed at 2x magnification by using a mobile ultra-wide camera 8MP (F2.2) version 11.0.15.83 of Galaxy Samsung A22. All photos were taken from a fixed distance of 17 cm; a ruler was put near each embryo to be used as a scale.

Image analysis and morphometry

All photos were analyzed by using the Image J program. The whole-body length was measured from the whole embryo photos, taken from at least 16 embryos per age-treatment group.

Morphological description

Control embryos were staged and described as in (Hamburger and Hamilton, 1992) chick embryo stage (30-31), 10-day chick embryo stage (36), 14-day chick embryo stage (40) and 18-day chick embryo stage (44). While the other experimental groups were described by comparing them to the controls.

The abnormal morphological features of the embryos were recorded, and the percentage of malformations was calculated according to the following formula.

(Number of embryos with congenital malformations ÷ Total number of embryos in the group) × 100

Calculating growth rate

Growth rate (G) was calculated according to the formula $G= (W2 – W1)/(t2 – t1)$, where W1 and W2 are the weights of the embryo at times t1 and t2 (Björnsson and Steinarsson, 2002).

Statistical analysis

All the experimental data was transferred to the IBM SPSS statistic 22 program. The differences between the experimental groups were determined by using

the test of homogeneity and one-way ANOVA multiple comparison with post-hoc test Student-Newman-Keuls and Tukey HSD for treatments against controls. When data showed significant differences in the homogeneity, a nonparametric 2 independent sample Mann-Whitney test was used to compare between each two experimental groups. *P-values* less than 0.05 were determined to be statistically significant ($p<0.05$).

Results

Morphological description of 7-day chick embryo

Controls

The following features were seen in control 7-day embryos. The brain was subdivided into three segments; prosencephalon, mesencephalon, and rhombencephalon. Big dark pigmented eyes located at both sides of the head were observed and separated by the beak and the prosencephalon Fig. 1. The vertebral column extended to the end of the tail bud. The heart was clearly observed in the middle of the chest by its dark coloration and its beating. The beaks were slightly visible. The fore limb and hind limb were clearly visible and were segmented but still in paddle shape without any digits, see Fig. (1-A).

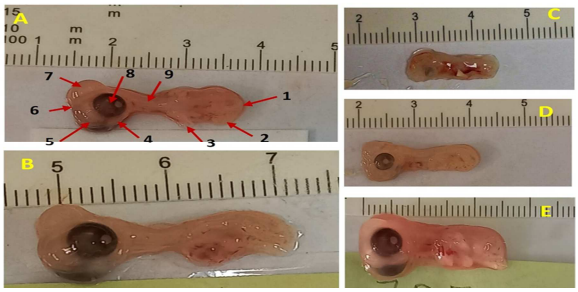

Fig. 1. Photographs showing 7-day chick embryo. a) Control, (B) Vehicle control, (C_E) treated. Embryonic parts seen in (A) 1-Tail bud; 2- leg bud; 3-wing bud; 4- beak; 5-Prosencephalon; 6- Mesencephalon; 7- Rhombencephalon; 8- Eye and 9- Neck. Vehicle control embryo in (B) seemed smaller compared to the controls in (A). Note the eye retardation, fragile body and limbs absence, growth retardation in treated chick embryo and abnormal head and neck size in the treated embryos (C-E). Photographs were taken immediately after extracting embryos, using a mobile phone camera (see methodology).

Vehicle control

Embryos showed slight growth retardation compared to the control group as shown in Fig (1-B).

Treated

The embryos in the treated group showed growth retardations compared to the controls and vehicle control groups figure (1-C and D). In other embryos, the head size seemed to be a bit bigger than the body and the brain divisions were hardly noticed, in figure (1-E). The wing and leg buds were observed in some embryos and the eye pigmentation was clearly seen. The beaks were so small and could hardly be seen, in figure (1-D). Some embryos had internal bleeding over some parts of their bodies (back of the head, on the spine, around the eye and on legs) Fig. (1- C, D and E).

Morphological description of 10 day chick embryo

Control

The body color became darker. The eyes were massive compared to the head size. The fore and hind limb segments were long and claws were observed. The claws were visible at the ends of the toes. Along the dorsal midline of the beak, the comb was visible. The beak tooth was clearly visible as it became whiter compared to 7-day control embryos and the nostrils were observed. Feather germs became visible on the thighs, fibula, spine, wing, and some parts of the head between the upper eyelid and the dorsal midline, in Fig. (2-A).

Vehicle control

Most of the vehicle control 10-day embryos were similar to the controls. However, some congenital malformations were seen, such as growth retardation, small beak, fragile tissue, pale skin color, and subcutaneous bleeding as seen in Fig. (2-B).

Treated

Embryos of treated groups showed more severe retardation compared to the vehicle controls. The congenital malformations seen were subcutaneous bleeding on the top back of the head between the eyes, a big abnormal head compared to the body without neck segment. Others had fragile and paler skin and, unclear limbs and beak absence Fig. (2 C, D and E).

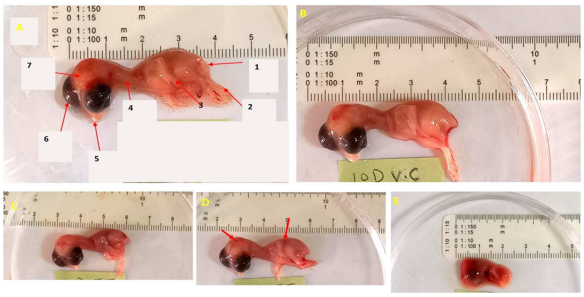

Fig. 2. Photographs showing 10- day chick embryos (a) control showing 1-Tail; 2- leg; 3-wing; 4-Neck;5-beak; 6- Eye and 7- Head. b) Vehicle control (C-E) Treated. Note that the vehicle control embryo in (B) seemed smaller compared to the control. Also note the growth retarded embryo in (c); the subcutaneous bleeding in (d) (red arrows) and the Fully malformed embryo with retarded limbs, no beak and abnormal head in (E). Photographs were taken immediately after extracting embryos, using a mobile phone camera (see methodology).

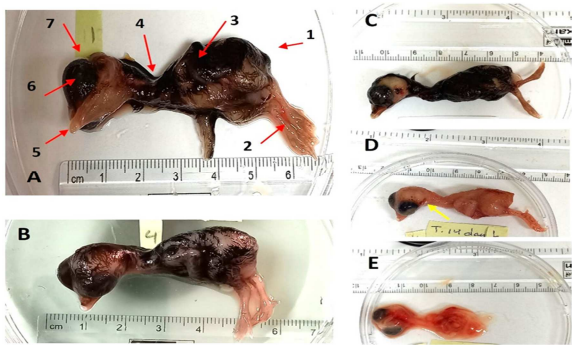

Fig. 3. Photograph showing 14-day chick embryo: a) control showing 1-Tail; 2- leg; 3-wing; 4-Neak;5-beak; 6- Eye and 7- Head. b) Vehicle control; (C_E) treated. Note the complete partial and lack of feathers in (C - E); also note the subcutaneous bleeding around the eye (yellow arrow) in (d) and the malformed fragile embryo in (E) . Photographs were taken immediately after extracting embryos, using a mobile phone camera (see methodology).

Morphological description of 14-day chick embryo:
Control

Embryos appeared bigger and longer compared to the 10-day embryos Fig. (3-A). The anterior Limb became longer also. The soft and fine feather covered almost all the body parts. The beak increased in size and the beak tooth seemed more defined. The eyes were covered with eyelids. The body was completely covered with feathers.

Vehicle control

Embryos were mainly similar to the controls. However, some revealed growth retardations, hernia and fragile brownish body Fig. (3- B).

Treated

Most of the embryos did not have feathers. Other embryos suffered from growth retardation, subcutaneous bleeding, hernia, fragile body, and highly retarded legs and wings, Fig. (3- C, D and E).

Morphological description of 18-day chick embryo
Control

Control embryos of 18 days were fully formed and covered with feathers. They were similar to stage Hamburger and Hamilton stage (44) (see Fig. 4 A)

Vehicle control

Embryos in the vehicle group were very similar to the controls, however, they seemed smaller compared to the controls. All the external general features were observed (see Fig. 4 B)

Treated

Embryos in the treated group showed a dramatic decrease in the body weight and the body whole length when compared to C and VC group. A percentage of 35.3 of the treated embryos showed an intense decrease in growth, fragile body, featherless, and had a hernia in the abdominal region. Also, 5.8% of the treated embryos showed abnormal crooked toes (see figure 4 C-E). Fig. 5 shows The Percentage of malformations seen in the treated groups.

Morphometric studies
Effect of artificial sweeteners on whole body weight

The mean whole-body weight of chick embryo control group (C) in this study was (0.5 1g), (1.48 g), (3.7 1g), and (14.97 g) of embryonic day (E); E7, E10, E14, and E18 respectively. Compared to the control, there was a non-significant decrease in the body weight in VC, T of E7, E18, and in T of E10. On the other hand, a non-significant increase was seen in VC of E10, E 14, and T of E14 compared to control. T group showed a non-significant increase in E7 and E14, on the other hand, it showed a non-significant decrease in E10 and E18 compared to the VC group see Fig. 6.

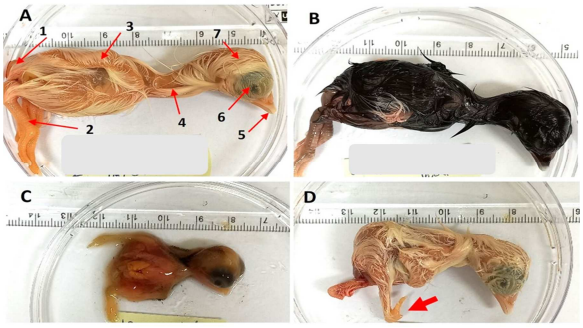

Fig. 4. Photograph showing 18-day chick embryo: a) control showing 1-Tail; 2- leg; 3-wing; 4-Neak;5-beak; 6- Eye and 7- Head. (b) Vehicle control (C & D) treated. Note the growth retardation, opened abdomen, and fragile body in the treated chick embryo in (C) and the crooked toes in (D).

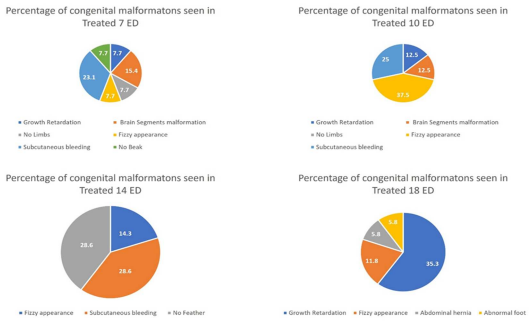

Fig. 5. The percentage of the congenital malformations seen in each experimental day in the treated group. Data was taken from 16 samples from each age group.

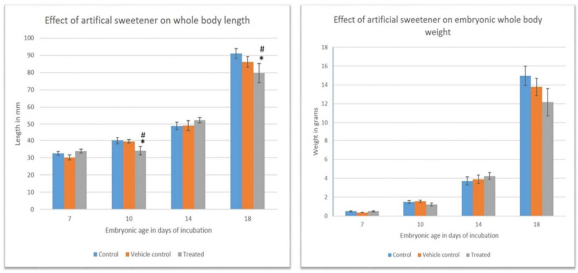

Fig. 6. The effect of commercial artificial sweeteners on chick embryo's whole body length and whole body weight, Values are mean ± SE taken from 16 samples for each group age treatment. (*) p< 0.05 compared to the controls, (#) p< 0.05 compared to the vehicle control group.

Effect of artificial sweetener on whole body length

The mean of the whole-body length of the control groups was (32.56 mm), (40.12 mm), (49.09 mm) and (91.13 mm) for E7, E10, E14 and E18, respectively. (VC) of E7, E10 and E18 showed a non-significant decrease compared to the controls, while VC of E14 showed a non-significant increase compared to controls. While T of E10 and E18 showed a significant decrease p=0.026, p=0.020, respectively compared to the controls. T of E14 showed a non-significant increase compared to the control. By comparing VC and T groups, the T group showed a non-significant increase in body weight in E7 and E14 while it showed a non-significant decrease in E10. However, the decrease was significant in E18 p=0.033, see Fig. (6).

Effect of (CAS) on growth rate

Between 7-10 days, the mean control growth rate was 0.38 g/day. Compared to the controls, the VC group had a non-significant increase in growth rate, while the T group had a significant decrease in growth rate p= (0.018). The T group had also a significant decrease in the growth rate p= (0.015) compared to the VC group. For the period between 10 and 14 days, the mean control growth rate was 0.6 g/day. Both VC and T groups showed a non-significant increase in growth rate in this period. The T group also showed a non-significant increase compared to the VC group. For the period between 14 and 18 days, the mean control growth rate was 2.8 g/day. Both VC and T groups showed a non-significant decrease in growth rate in this period. The T group also showed a non-significant increase compared to the VC group (see Fig. 7).

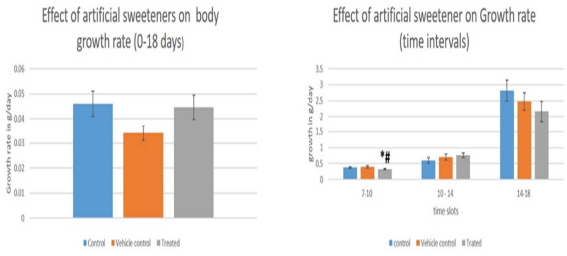

Fig. 7. The effect of commercial artificial sweeteners on chick embryo growth rate. Growth rate for each time interval and growth rate for the entire experimental time. Values are mean ± SE taken from 16 samples for each group age treatment. (*) p< 0.05 compared to the controls. (#) p< 0.05 compared to the vehicle control group.

Discussion

The strong tendency of many people to replace regular sweeteners in their diet and change them with artificial sweeteners, in order to lose weight and reduce calories (Kormsing *et al.*, 2020), prompted this study to investigate the effect of these sweeteners on chick embryos.

A number of side effects can be caused by the excessive consumption of AS. They can be considered safe products, however, there were some doubts about consuming some of them. A recommended daily safety value should be taken into consideration (Ižaković, 2021).

The health effects of non-caloric artificial sweeteners (NAS). have many questions that must be resolved before the dietary substitution for sucrose-sweetened foods to prevent obesity and overweight. Also, the long-term effects of NAS such as the impact on sweetness perception, appetite control, and weight status should be understood (Wilk *et al.*, 2022).

The present study used a commercial artificial sweetener to identify its effects on the embryological development of chick embryos. The commercial artificial sweetener components were: Sorbitol (E420), sucralose (E955) /kg, Acesulfame K (E950) /kg, corn powder and chromium picolinate.

Several congenital malformations were seen in the results of this study, such as growth retardation, subcutaneous bleeding, abnormal brain formation, hernia in the abdominal region, featherless embryos, and abnormal fore and hind limbs including crooked toes.

Growth retardation was the most common congenital malformation seen in all experimental ages in this study. All growth parameters such as whole-body weight, whole-body length, and growth rate showed a decrease that was significant in some experimental days.

Growth retardation is reported in many human infants all around the world. It has many causes, one of which is mother nutrition or chemicals in the mother's diet. Growth retardation is the cause of many mortalities and morbidity. It also might cause several aspects during early life stages such as low IQ (Sharma *et al.*, 2016). Studies on humans showed that many AS such as sucralose, acesulfame K, cyclamate, and saccharine cross the placenta (Aguayo-Guerrero *et al.*, 2023; Leth-Møller *et al.*, 2023).

In the present study, the treated (T) group of E7, E 10 and E18 embryos showed a non-significant decrease in body weight compared to the control (C) group. This result is in alignment with a study conducted by (Al-Qudsi, 2019) that revealed that compared to controls, there was a non-significant decrease in the body weight of 18-day fetuses and 4 weeks old mice neonates whose mothers had a daily dose of CAS during pregnancy and 3 weeks of nursing. Another study showed that maternal consumption of acesulfame K during pregnancy showed a significant reduction in fasting fetal glycemia and fetal weight (Plows *et al.*, 2020).

After feeding sucralose to rats, some reduction in food consumption and weight was found. On the other hand, there was an alteration in relative organ weights such as caecal enlargement at doses of 3–8% sucralose in the diet (Grice and Goldsmith, 2000).

The whole-body length of (T) of E10 and E18 in this study showed a significant decrease compared to the controls, which agreed with (Al-Qudsi, 2019) that found a significant decrease in the whole-body length of four-week-old mice of treated mothers with AS compared to controls.

In a recent study, it was found that depending on dose and time, adding sorbitol to preimplantation embryo media leads to stress that can cause a decrease in three biological outcomes: apoptosis, cell number accumulation, and embryo growth. The added sorbitol induces a proportion level of phosphorylated stress-activated protein kinase (SAPK) that leads to a reduction in embryo cavitation, cell accumulation and increased apoptosis (Xie *et al.*, 2007).

That might explain the growth retardation that was found in the length and weight of embryos in this study as sorbitol was one of the used artificial ingredients.

The influence of acesulfame K and sucralose was investigated on the gut microbe *Escherichia coli* (*E. coli*) K-12 growth and metabolism. The growth of *E. coli* was accelerated by acesulfame K during the incubation period. On the other hand, sucralose had a less prominent effect on *E. coli* growth. The consumption of acesulfame K was found to be associated with adipose tissue dysfunction and glucose intolerance as it may play a strong role in the uptake of intestinal glucose (Shahriar *et al.*, 2020).

Moreover, it was found that a low dosage of sucralose can significantly alter the gut microbiome in mice without having any effect on the mice body weight. Sucralose can alter the microbiome of the mice gut by (0.3 mg/mL) concentration level although humans should administrate lower than this concentration (Zheng *et al.*, 2022).

When comparing the growth rate during the experimental period from 7 to 18 days in both CV and T groups, a non-significant decrease was shown. The results show that the control growth rate increased by about 7 folds from 7 to 18 days. The effect of AS was very clear at the beginning of development between 7 and 10 days of incubation as it significantly reduced the growth rate, and at the end of the period, where the treated group growth rate was non-significantly reduced. It can be assumed that the sorbitol effect is mainly exerted on embryo growth, that the sucralose and acesulfame K effects are more concerned with the gut microbiome, or that all of their combined effects might have caused the growth retardation seen in this study.

The results of this study showed that 28.6% of E 14 treated embryos were featherless. This was not seen in any other experimental groups. Feathering rate, feather color and structure can be influenced by nutritional and environmental status.

The amino acids and hormones can also influence young birds feathering. Hormonal output affects feather development directly by thyroxine and oestrogen and indirectly by testosterone (Leeson and Walsh, 2004). Sucralose can impact the thyroid axis activity by diminishing the synthesis of thyroid hormones as thyroid peroxidase (TPO) activity and lowering the concentration of thyroxine (T4), and triiodothyronine (T3) in the plasma. Metabolic disorders might be exacerbated by the adverse effect of sucralose on thyroid hormone metabolism (Pałkowska-Goździk, 2018). Therefore, the result seen in this study (featherless embryos) might have been caused by the sucralose present in the used AS, and as T3 and T4 play a vital role in metabolism, they might have a role in the growth retardation as well. More studies should be done to investigate the mode of action of sucralose on presenting feathering in E14 embryos. It should be mentioned that treated E18 embryos were covered with feathers similar to the controls, which proves that the treated embryonic body was able to overcome the disturbance that was caused by sucralose concerning feather development. However, feather density and morphometry were not investigated in this study.

This study showed some brain deformities and abnormal brain development, which might have been caused by AS. This finding agreed with (Al-Rashdi and Al-Qudsi, 2020) that showed brain abnormalities. Recently (Rizas, 2023) found that the use of AS could promote blood vessel obstruction as it affects the platelets physiology. Endothelial cells can produce nitric oxide (NO) by endothelial nitric oxide synthase (eNOS), which is involved in a variety of physiological functions in the brain. Morphological defects can be caused by the absence or excess of NO levels. In research on the effect of NO on chick embryo during organogenesis, it was found that these defects were represented as an alteration in the normal axial development of somites and neural tube (Alexander, 2007). The deficiency of eNOS or NO can affect the healthy brain at an early age (George *et al.*, 2022).

In this study, 5.8% of treated E18 chick embryos showed crooked toes. This incidence can be caused during the last eight days of incubation or any time prior to somatic maturity. It is a morphological defect in the plantar digits of the feet that has a genetic characteristic and it is a polygenically determined trait but it may also be influenced by environmental conditions. Crooked toes can occur between days 13-21 of incubation and hatched chicks (Hollands, 1956). (Taha, 1979) Suggested that this incidence may be due to inheritable factors in the chicks or to deficiency of some unknown essential nutrients in the diet. Some toe deformation can be observed in chicks that are incubated in machines that require elevated temperatures. Leg health parameters such as crooked toes were affected by incubation conditions (Oviedo-Rondón *et al.*, 2009). In the present study, as the temperature factor was constant, the phenomena (crooked toe) which was seen only in the treated group, which appeared in a percentage of 5.8, might have been caused by the used AS components that might have blocked one of the essential factors that have a role in the toe formation.

The excess consumption of AS (sucralose, aspartame and acesulfame K has a direct association with the risk of acceleration of cardiovascular disease (Debras *et al.*, 2022). In this study, most of the treated embryos had subcutaneous bleeding. This may have resulted from the AS according to the finding of (Hoffmann, 2018) who suggested that the high intake of AS and sugar can impair the vascular system and lead to homeostatic alterations. Elevated levels of NO induce endothelial barrier dysfunction by altering the proteins of the endothelial cell cytoskeleton. (Knepler Jr *et al.*, 2001). Rho-associated kinase (ROCK) can be involved in the pathogenesis of atherosclerosis; as it can control cell apoptosis, contraction, migration, proliferation and adhesion. ROCK activity can be inhibited by the overexpression of NO (Maruhashi *et al.*, 2014).

Some 18-day treated embryos in this study suffered from abdominal hernia (5.8 %) which according to (Duess, 2016) suggested that ventral body wall defect can be caused by ROCK signals that control the cell contractility and the assembly of actin-cytoskeleton filament during embryogenesis. The failure of embryonic abdominal wall formation seen in this study might have been attributed to the decontrolled migration and adhesion activity of embryonic cells during embryogenesis. The disorganization of actin filament at the umbilical ring was observed in ROCK-knockout mice (Maruhashi *et al.*, 2014).

Conclusion

This study sheds light on some of the general adverse effects of a selected commonly used artificial sweetener. Consumers use sweeteners without paying attention to the cumulative daily permitted dose. By only using the dose written on the commercial artificial sweetener box on chick embryos, many abnormalities occurred. These abnormalities were exhibited as growth retardation, subcutaneous bleeding over some body parts, abdominal hernia, and crocked toe in some embryos. What distinguishes this study from other studies is that the used commercial sweetener does not contain aspartame, which was proven by many researchers to induce embryonic abnormalities. Also in this study, the researcher used the manufacturer-recommended dose. Most of the previous studies on artificial sweeteners used aspartame, a single AS, or a combination made of AS in the lab, whereas this study used the actual commercial product used by consumers. The combined effect of the selected sweetener components (sorbitol, acesulfame K and sucralose) was responsible for the congenital malformations seen in this study.

Recommendation

Pregnant women should reduce the consumption of products containing artificial sweeteners.

Acknowledgments

The Authors would like to thank Mrs. Amna Al-Biladi for her help in sample collection and assembly of data.

References

Aguayo-Guerrero JA, Méndez-García LA, Manjarrez-Reyna AN, Esquivel-Velázquez M, León-Cabrera S, Meléndez G, Zambrano E, Ramos-Martínez E, Fragoso JM, Briones-Garduño JC. 2023. Newborns from Mothers Who Intensely Consumed Sucralose during Pregnancy Are Heavier and Exhibit Markers of Metabolic Alteration and Low-Grade Systemic Inflammation: A Cross-Sectional, Prospective Study. Biomedicines **11**(3), 650. https://doi.org/10.3390/biomedicines11030650

Alexander PG, Chau L, Tuan RS. 2007. Role of nitric oxide in chick embryonic organogenesis and dysmorphogenesis. Birth Defects Research Part A - Clinical and Molecular Teratology **79**(8), 581–594. https://doi.org/10.1002/bdra.20386.

Al-Qudsi FM, Al-ahmadi AZ, Ganash M. 2021. Commercial Artificial Sweeteners Affect Mice Limb Development: A Morphology and Morphometric Study. International Journal of Pharma and Bio sciences **11**, 200–211.

Al-Qudsi, Fatma M, Al-Hasan MM. 2019. In utero exposure to commercial artificial sweeteners affects mice development and mammary gland structure. Environmental Science and Pollution Research **26**(5), 5054–5064. https://doi.org/10.1007/s11356-018-3935-5.

Al-Rashdi A, Al-Qudsi F. 2020. Commercial artificial sweeteners caused abnormal development in chick embryo, a morphological study. Bioscience Research, **17**(1), 371–382. http://cme.msu.edu/cmeias/.

Alsoufi M, Aziz R, Hussein Z. 2017. Effect of some artificial sweeteners consumption in Biochemical Parameters of Rats **5,** 1095-1099.

Bigos A, Palkowska E, Rosołowska-Huszcz D. 2012 Effect of artificial and natural sweeteners on glucose and insulin in plasma of rats. Journal of Pre-Clinical and Clinical Research **6**(2), 93-97.

Björnsson B, Steinarsson A. 2002. The food-unlimited growth rate of Atlantic cod (Gadus morhua). Canadian Journal of Fisheries and Aquatic Sciences **59**(3), 494–502.

Chattopadhyay S, Raychaudhuri U, Chakraborty R. 2014. Artificial sweeteners- A review. Journal of Food Science and Technology, Springer, 611–621. https://doi.org/10.1007/s13197-011-0571-1.

Chung MS, Suh HJ, Yoo W, Choi SH, Cho YJ, Cho YH, Kim CJ. 2005. Daily intake assessment of saccharin, stevioside, D-sorbitol and aspartame from various processed foods in Korea. Food Additives and Contaminants **22**(11), 1087–1097. https://doi.org/10.1080/02652030500202092.

Debras C, Chazelas E, Sellem L, Porcher R, Druesne-Pecollo N, Esseddik Y. 2022. Artificial sweeteners and risk of cardiovascular diseases: Results from the prospective NutriNet-Santé cohort. Bmj **378**, e071204.

Duess JW, Puri P, Thompson J. 2016. Impaired cytoskeletal arrangements and failure of ventral body wall closure in chick embryos treated with rock inhibitor (Y-27632). Pediatric Surgery International **32**, 45–58.

Dwyer JT, Melanson KJ, Sriprachy-anunt U, Cross P, Wilson M. 2000. Dietary Treatment of Obesity. MDText.com, Inc., South Dartmouth (MA).

Gan Z. Sun H, Feng B, Wang R, Zhang Y. 2013. Occurrence of seven artificial sweeteners in the aquatic environment and precipitation of Tianjin, China. Water Research **47**(14), 4928–4937. https://doi.org/https://doi.org/10.1016/j.watres.2013.05.038.

George H, Mercer GV, Stapleton D, Dawson L, MacCallum PE, Spring S, Sled JG, Blundell J, Cahill LS. 2022. Structural brain abnormalities in endothelial nitric oxide synthase-deficient mice revealed by high-resolution magnetic resonance imaging. Brain and Behavior **12**(11), e2801.

Grice HC, Goldsmith LA. 2000. Sucralose- An overview of the toxicity data. Food and Chemical Toxicology **38**, 1–6.

Hamburger V, Hamilton HL. 1992. A series of normal stages in the development of the chick embryo. 1951. Dev Dyn. **195**(4), 231-72.

Hoffmann BR, Ronan G, Haspula D. 2018. The Influence of Sugar and Artificial Sweeteners on Vascular Health during the Onset and Progression of Diabetes. The FASEB Journal **32**, 603–620.

Hollands KG. 1956. A genetic analysis of the crooked toes defect in chickens (T). University of British Columbia. Retrieved from https://open.library.ubc.ca/collections/ubctheses/831/items/1.0106328

Ižaković M, Ačkar Đ, Šubarić D. 2021 'Commonly used artificial sweeteners in Europe. Hrana u zdravlju i bolesti: znanstveno-stručni časopis za nutricionizam i dijetetiku **10**(1), 24–34.

Jiang J, Liu S, Jamal T, Ding T, Qi L, Lv Z, Yu D, Shi F. 2020. Effects of dietary sweeteners supplementation on growth performance, serum biochemicals, and jejunal physiological functions of broiler chickens. Poultry Science **99**(8), 3948–3958. https://doi.org/10.1016/j.psj.2020.03.057.

Kain KH, Miller JWI, Jones-Paris CR, Thomason RT, Lewis JD, Bader DM, Barnett JV, Zijlstra A. 2014. The chick embryo as an expanding experimental model for cancer and cardiovascular research. Developmental Dynamics **243**(2), 216–228. https://doi.org/10.1002/dvdy.24093.

Knepler Jr. JL, Taher LN, Gupta MP, Patterson C, Pavalko F, Ober MD, Hart CM. 2001. Peroxynitrite causes endothelial cell monolayer barrier dysfunction. American Journal of Physiology-Cell Physiology **281**(3), C1064–C1075.

Kormsing N, Viravud Y, Roongruangchai J. 2020. Teratogenic effects of aspartame exposure of chick embryonic development. In Rangsit Graduate Research Conference: RGRC, 2731–2736p.

Rodero Ab, de Souza Rodero L, Azoube R. 2009. Toxicity of sucralose in humans: a review. Int. J. Morphol. **27**(1), 239-244.

Leeson S, Walsh T. 2004. Feathering in commercial poultry II. Factors influencing feather growth and feather loss. World's Poultry Science Journal **60**(1), 52–63.

Leth-Møller M, Duvald CS, Stampe S, Greibe E, Hoffmann-Lücke E, Pedersen M, Ovesen PG. 2023. Transplacental Transport of Artificial Sweeteners. Nutrients **15**(9), 2063. https://doi.org/10.3390/nu15092063.

Liauchonak I, Qorri B, Dawoud F, Riat Y, Szewczuk MR. 2019. Non-Nutritive Sweeteners and Their Implications on the Development of Metabolic Syndrome. Nutrients **11**(3), 644. https://doi.org/10.3390/nu11030644.

Liauw S, Saibil F. 2019. Sorbitol Often forgotten cause of osmotic diarrhea. Can Fam Physician **65**(8), 557-558.

Maruhashi T, Noma K, Iwamoto Y, Iwamoto A, Oda N. 2014. Critical Role of Exogenous Nitric Oxide in ROCK Activity in Vascular Smooth Muscle Cells. PLOS ONE **9**(10), e109017. https://doi.org/10.1371/journal.pone.0109017

Nezzal A,, Aerts L, Verspaille M, Henderickx G, Redl A. 2009. Polymorphism of sorbitol. Journal of Crystal Growth **311**(15), 3863–3870. https://doi.org/10.1016/J.JCRYSGRO.2009.06.003.

Oviedo-Rondón EO, Wineland MJ, Funderburk S, Small J, Cutchin H, Mann M. 2009. Incubation conditions affect leg health in large, high-yield broilers. Journal of Applied Poultry Research **18**(3), 640–646.

Pałkowska-Goździk E, Bigos A, Rosołowska-Huszcz, D. 2018. Type of sweet flavour carrier affects thyroid axis activity in male rats. European Journal of Nutrition **57**, 773–782.

Plows JF, Morton-Jones J, Bridge-Comer PE, Ponnampalam A, Stanley JL, Vickers MH, Reynolds CM. 2020. Consumption of the artificial sweetener acesulfame potassium throughout pregnancy induces glucose intolerance and adipose tissue dysfunction in mice. The Journal of Nutrition 150(7), 1773–1781.

Rizas KD, Sams LE, Massberg S. 2023. Non-nutritional sweeteners and cardiovascular risk. Nature Medicine **29**(3), 539–540.

Shahriar S,Ahsan T,Khan A, Akhteruzzaman S, Shehreen S, Sajiba AA. 2020. Aspartame, acesulfame K and sucralose-influence on the metabolism of *Escherichia coli*. Metabolism Open **8**, 100072.

Sharma D, Shastri S, Farahbakhsh N, Sharma P. 2016. Intrauterine growth restriction–part 1. The Journal of Maternal-Fetal & Neonatal Medicine **29**(24), 3977–3987.

Silveira M, Jonas R. 2002. The biotechnological production of sorbitol. Applied Microbiology and Biotechnology **59**(4), 400–408. https://doi.org/10.1007/s00253-002-1046-0.

Suez J, Korem T, Zeevi D. 2014. Artificial sweeteners induce glucose intolerance by altering the gut microbiota. Nature **514**(7521), 181–186. https://doi.org/10.1038/nature13793.

Swithers SE. 2015. Artificial sweeteners are not the answer to childhood obesity. Appetite **93**, 85–90. https://doi.org/10.1016/J.APPET.2015.03.027.

Sylvetsky AC. 2018. Metabolic Effects of Low-Calorie Sweeteners: A Brief Review. Obesity 26, S25–S31. https://doi.org/10.1002/oby.22252.

Taha, NT. 1979. A genetic study of crooked toes and crooked keels in chickens. PhD diss., University of Tennessee. https://trace.tennessee.edu/utk_graddiss/7880.

Tandel KR. 2011. Sugar substitutes: Health controversy over perceived benefits. Journal of Pharmacology and Pharmacotherapeutics **2**(4), 236–243. https://doi.org/10.4103/0976-500X.85936.

Weerasooriyagedara MS. 2018. Toxicity effects of aspartame on embryonic development of Zebrafish (*Danio rerio*). International Journal of Engineering and Management Research (IJEMR) **8**(1), 183–188.

Whitehouse CR, Boullata J, McCauley LA. 2008. The Potential Toxicity of Artificial Sweeteners. AAOHN J. **56**(6), 251-9.

Wilk K, Korytek W, Pelczyńska M, Moszak M, Bogdański P. 2022. The Effect of Artificial Sweeteners Use on Sweet Taste Perception and Weight Loss Efficacy: A Review. Nutrients **14**(6), 1261. https://doi.org/10.3390/nu14061261

Xie Y, Zhong W, Wang Y, Trostinskaia A, Wang F, Puscheck EE, Rappolee DA. 2007. Using hyperosmolar stress to measure biologic and stress-activated protein kinase responses in preimplantation embryos. Molecular Human Reproduction **13**(7), 473–481. https://doi.org/10.1093/molehr/gam027.

Zheng Z, Xiao Y, Ma L, Lyu W, Peng H, Wang X, Ren Y, Li J. 2022. Low dose of sucralose alter gut microbiome in mice. Frontiers in Nutrition **9**, 848392.