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Commercial artificial sweeteners caused abnormal development in chick embryo, a morphological study.

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Artificial sweeteners have become an important part of everyday food consumption. Pregnant women consume these substances with lack of awareness about their impact on embryo development. Nutrition during intrauterine life plays a crucial role in the congenital malformations. The aim of this study was to study the effect of commercial artificial sweeteners on the early development of chick embryo. The chick embryo provides a suitable model for *in vivo* evaluation of the toxicity. Two doses were used in this study 50mg/kg and 100mg/ kg body weight. Fertile chicken eggs were injected once in the air chamber before incubation. Eggs were then incubated under normal incubation conditions. Embryos were extracted examined and photographed on day 2, 3, 4, and 5 of incubation. Growth retardation, abnormal brain and eye development were seen treated embryos. The heart rate of treated embryos of 4 day significantly decreased compared to the control. This study concluded that commercial artificial sweeteners present in the market had an association with abnormal development in chick embryos.

Keywords: Chick embryo, eye malformation, artificial sweeteners, brain malformation, morphometry, morphology.

INTRODUCTION

One of the human traits is the desire for sweet taste. The sweet taste of the food was regarded by primitive people as a sign of food safety while the bitter taste was a sign of food toxicity. In the past people just received their sweetener needs from natural sources like honey(Sardesai and Waldshan, 1991). Honey is considered as one of the oldest sweeteners used by mankind. After a period of time honey was replaced by common sugar (sucrose). Which was extracted from sugar cane. During the World Wars, the sucrose was derived from the sugar-beets (Weihrauch and Diehl, 2004). Now the advances in food technology have allowed many types of sweeteners to be produced from different sources.

Based on their source, sweeteners are divided into two types ;(a) natural sweeteners such as honey;(b) synthetic sweeteners (Sardesai and Waldshan, 1991). Sweeteners can also be devided into two classes based on whether they are a source of calories, nutritive sweeteners (caloric sweeteners), and non- nutritive sweeteners (non-caloric), often referred to as Artificial sweeteners. Artificial sweeteners are food additives, with low calories or without them (Whitehouse et al. 2008). Food and Drug Administration (FDA) has approved certain types of artificial sweeteners depending on the acceptable daily intake of ADI (specific amount of artificial sweeteners that do not cause health risk with daily exposure). US- FDA has approved five types of artificial sweeteners (saccharine, aspartame, sucralose, neotame, and acesulfame-k).

Artificial sweeteners are commonly used in beverages, nutritional products, drugs and mouthwashes. Use of artificial sweeteners is still controversial in general (Sharma et al. 2016).

Artificial sweeteners have become an important part of everyday food consumption. Pregnant women consume these substances with lack of awareness about their impact on embryo development. Nutrition differences during intrauterine life plays a crucial role in the congenital malformations. The present study used chick embryo as a model due to the similarity to the mammalian embryo in the embryonic development and morphological complexity, and has more accessibility than mammalian embryo (Wolpert et al. 2002). All chick embryo stages were well studied by Hamburger and Hamiliton (Kotwani,1998).

A lot of people believe that artificial sweeteners are healthy, but many epidemiological studies have shown the opposite (Pepino, 2015). The studies on the impact of these artificial sweeteners on the embryonic development are not sufficient therefore, the purpose of the current research was to study the effect of commercial artificial sweeteners on the early development of chick embryo.

MATERIALS AND METHODS

All experimental procedures were approved by the Biology Department at King Abdul-Aziz University. Fertilized chicken eggs were obtained from private farm chicken in Dhaban north of Jeddah. The artificial sweetener used in this study contained 0.617mg of acesulfame K, 0.546mg of aspartame, and silicon dioxide. It was purchased from local supermarkets.

Experimental design

Fertilized chicken eggs were divided into four groups Control, sham, treated low, and treated high. The eggs were weighed, and the average weight calculated. Based on the average weight of the eggs, the dose of artificial sweetener was calculated.

Dose calculation and Preparation

The dose of artificial sweeteners (Ac) used in this study was 50 mg\Kg according to (Al-qudsi and Al-Hasan, 2019). Mean of the egg weight (*x*) was 44.8 g. the doses were calculated as follows

Low dose = $\frac{Ac*x}{1Kg} = \frac{50mg*44.8g}{1000g}$ = 2.24 mg.

High dose = low dose * 2 = 2.24* 2= 4.48 mg

The experimental solution of the low dose was prepared by dissolving 11.2mg of artificial sweetener into 500 ml of distilled water (40°C). While the solution of high dose prepared by dissolving 22.4 mg into 500 ml of distilled water (40°C). To reach the amount of 2.24 mg artificial sweetener in 0.1 ml (low dose), and 4.48 mg artificial sweetener in 0.1 ml (high dose).The eggshell was sterilized at the blunt end with 70 % ethanol. At the air chamber two holes were made by a sharp needle, the solution was entered from a hole into air chamber, where the air came out from the next hole.

The sham group was injected with 0.1 ml of distilled water, while the low treated group was injected by 0.1 ml of solution containing 2.24 mg of artificial sweetener, and the high treated group injected with solution containing 4.48 mg of artificial sweetener. After injection, the holes were sealed with tape. All groups were incubated under identical standard conditions; temperature 37.5 C˚ and humidity 80%.

Sample collection

Embryos were extracted from all groups on the following incubation days 2, 3, 4, and 5, a batch was left until hatching. The egg was opened by knocking the eggshell at the blunt end by scissors, and then the top of the eggshell was removed. The embryos were extracted by a soft brush and placed it in a Petri Dish, then washed in warm saline solution, then photographed (see figure 1) The embryos were then fixed in 70% ethanol.

Morphological studies

To perform morphological studies, we compared our control embryos to the normal stages of chick embryo in Hamburger and Hamilton (1951). Control day 2 with stage 13, control day 3 with stage 19, control day 4 with stage 23 and control day 5 with stage 27.

Photographing

Each embryo was photographed using an iPhone 6 Plus camera 12 Megapixel the iPhone was attached to a tripod. A ruler was put near the egg to be used as a scale when preforming morphometry using the photos. After extracting the embryo from the egg, each embryo was photographed again by a mobile phone attached to a Dissecting Microscope (Leica WILD TYP 308700) to highlight any malformation. (See figure 1-A)

Morphometric method

All eggs were weighted before and after incubation. The whole body length of the embryos (see figure 1-B) and the space between tail and mid-brain were measured as shown in (figure 1-C).

The eggshell thickness was measured by digital Vernier caliper 150 mm GH-720 (see figure 1-D).

The measurement of the number of heartbeats per minute was taken from video photographing of the embryo for a minute then counting the heartbeats. The eyes diameter see figure 3-15 and the whole body weight were measured in 5-day embryos only. The measurements were taken from the photos taken by the iPhone camera using a free computer software called (Image tool) downloaded from (http://cme.msu.edu/cmeias/).

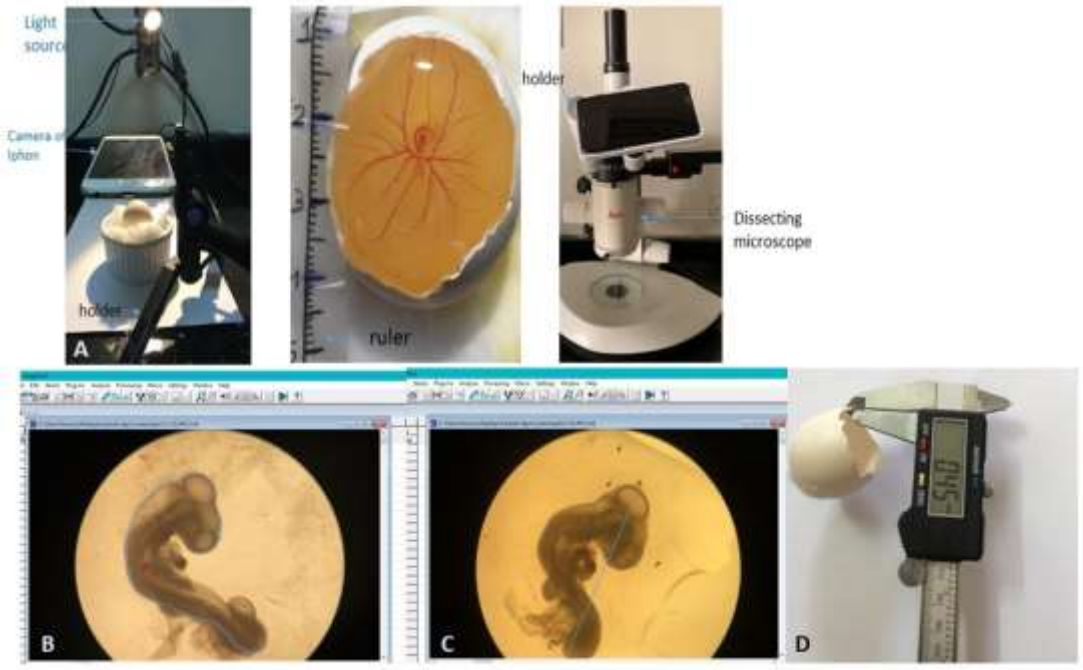

Figure 1 : Methods of photography and morphometry

Statistical analysis

Data was analyzed using SPSS 24. The test used with normal distribution was Anova, S-N-K, and Tukey test. In case of abnormal distribution, Mann-Whitney *U* test was used from the non-parametric test. Significance (*****) was at $p < 0.05$**.**

RESULTS

Morphological study

The control chick embryos on the second day of development had a head that was turned onto the left side, and had three cerebral lobes (forebrain, midbrain, and hindbrain). Small eyes were seen near the forebrain. The heart had a U shape tube. Two lateral folds could be seen on either side of the embryos. At the posterior end of the embryos, the tail fold began to form (figure 2-A). The 2-day sham embryos seemed similar to the control embryos (figure2-B). In low treated group of 2-day embryos, the eyes seemed larger than the control (figure2-C). The high treated 2 day embryos had similar malformations to the low treated group moreover the embryos were curved (figure2-D).

In the control 3 day embryos the processes of flexion and torsion seemed to initiate. The brain seemed to be further developed, as it was composed of five brain vesicles (telencephalon, diencephalon, metencephalon, mesencephalon, and myelencephalon). The eyes were large and unpigmented. The heart was located inside the anterior curve of the body. The somites extended into the tail, but the end of the tail was unsegmented. The fore limb-buds were enlarged, and symmetrical. The hind-limb buds seemed slightly larger than the fore limb-buds. The tail-bud was curved and its tip pointing forward.

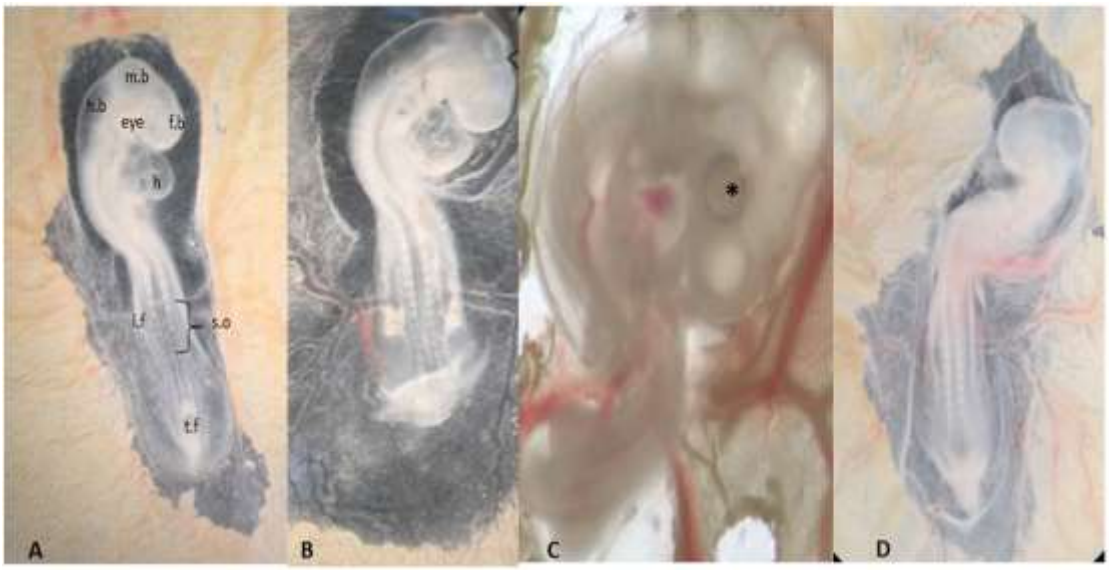

Figure 2:Congenital malformations seen in 2 day chick embryos.(A) control, (B)sham, (C)low treated, (D) high treated. (C) * indicate to larger eyes compared with control (A) and sham (B). (D) The high treated embryos seemed curved. (A) h.b hind-brain, m.b mid-brain, f.b fore-brain, h heart, l.f lateral fold, s.o somites, t.f tail fold. Scale bar = 1 cm. A,B,C,D (16X).

The allantois appeared as a small pocket near the tail bud. The amnion surrounded the embryo. (figure 3-A).

The sham embryos seemed similar to the control embryos (figure 3-B).The defects seen in low and high treated groups were abnormal brain development, and enlarged heart compared to the control (figure 3-C, 3-D).

The control chick embryos on the 4th day of development seemed to have a normal body size and head size. The brain was divided into five parts, and the eyes appeared large and pigmented. The heart was not yet enclosed by the body. The embryo's head and tail have come closer together, forming a distinct C shape, due to embryo bending, growth and increase in size (figure 4-A). The 4 day sham embryos seemed similar to the control embryo with a slight decrease in body size (figure 4-B).

The defects seen in 4-day low- treated embryos was that the embryos seemed smaller in size compared to the control group. The heart seemed enlarged in some embryos. The eyes seemed less pigmented compared to the control group. One embryo had an abnormal brain development (figure 4-C). The high dose of artificial sweetener seemed to cause growth retardation, abnormal brain development, and the heart seemed enlarged in some embryos compared to the controls (figure 4-D).

Control 5-day embryos had limbs that seemed considerably lengthened compared to control day 4 embryos. The somites extended until the tip of the tail. The eyes were large and located on either side of the head separated by the forebrain (figure 5-A). The 5-day sham embryos were similar to the control embryos but seemed smaller in size (figure 5-B). The defects seen in 5 day low and high treated embryos were hemorrhage, abnormal brain development, growth retardation, and abnormal eye development (figure 5-C, 5-D).

The main congenital malformations and their percentage are shown in table 1.

Hatchability

Chicken embryo groups started hatching on day 21-22 for control, sham, and low treated group. Only one egg hatched on day 27 in high treated group and died immediately without external deformities (see figure 6-A). In the low treated group two embryos hatched on day 22 with Omphalocele (see figure 7), and one embryo died after hatch (see figure 6-B). Hatching rate in control was 90% two eggs were unfertilized, sham 100%, low treated 45%, and high treated 5%.

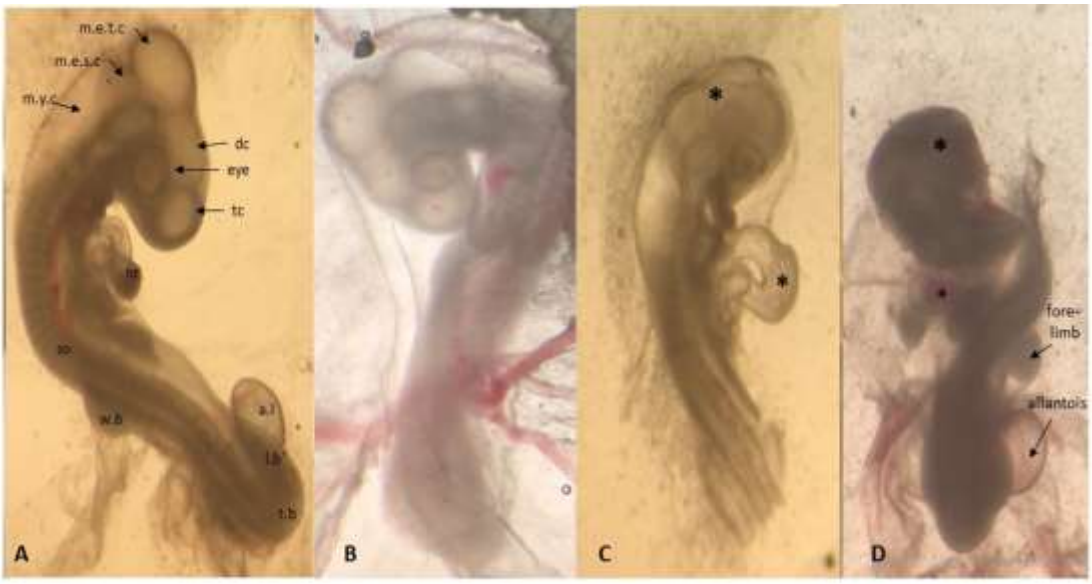

Figure 3:Congenital malformations seen in 3 day old chick embryos. (A) control, (B) sham, (C) treated low, (D) treated high. Note the major growth retardation in low treated embryo* (C) and high treated embryos (D) compared to normal development in control (A) and sham (B) embryo, enlarged heart and abnormal brain development in (C) and in (D). Control (A) (tc) telencephalon, (dc) diencephalon, (m.e.t.c) metencephalon, (m.e.s.c) mesencephalon, and (m.y.c) myelencephalon, (hr) heart, (so) somite, (w.b) wing bud, (l.b) leg bud, (t.b) tail bud, (a.l) allantois. Scale bar = 1cm. A-2,B-2,C-2,D-2 (16X).

Table 1: Table showing the summary of the main congenital malformations seen in this study. *No number of samples*

Embryo age	Groups	Congenital malformations					
		Growth retardation		Abnormal brain development		Abnormal eyes development	
		No	%	No	%	No	%
2 days	control	-	-	-	-	-	-
	sham	-	-	-	-	-	-
	Treated low	-	-	-	-	-	-
	Treated high	-	-	-	-	-	-
3 days	control	-	-	-	-	-	-
	sham	-	-	-	-	-	-
	Treated low	1	10%	1	10%	1	10%
	Treated high	1	10%	1	10%	1	10%
4 days	control	-	-	-	-	-	-
	sham	-	-	-	-	-	-
	Treated low	-	-	1	10%	1	10%
	Treated high	1	10%	2	20%	1	10%
5 days	control	-	-	-	-	-	-
	sham	-	-	-	-	-	-
	Treated low	2	20%	3	30%	2	20%
	Treated high	-	-	3	30%	1	10%

Figure 4:Showing 4 day chick embryos. Figures were taken immediately after extracting embryos of eggs. (A) control, (B) sham, (C-1, C-2) low treated embryos, (D-1, D-2) high treated embryos. Sham seemed slightly decreased in body size compared with control. The embryo (C-1) seemed smaller compared to the control group (A). Note in (C-2) * abnormal brain development, eyes seemed less pigmented compared to the control group, # the heart seemed enlarged compared with control embryo. Note abnormal brain development and bleeding * in (D-2).Note growth retardation GMR, and abnormal brain in (D-1). (fb) forebrain, (mb) midbrain, (hb) hindbrain, (hr) heart, (fl) forelimbs, (hl) hindlimbs. Scale bar = 1cm. A, B, C-1, C-2, D-1,D-2(12.5X).

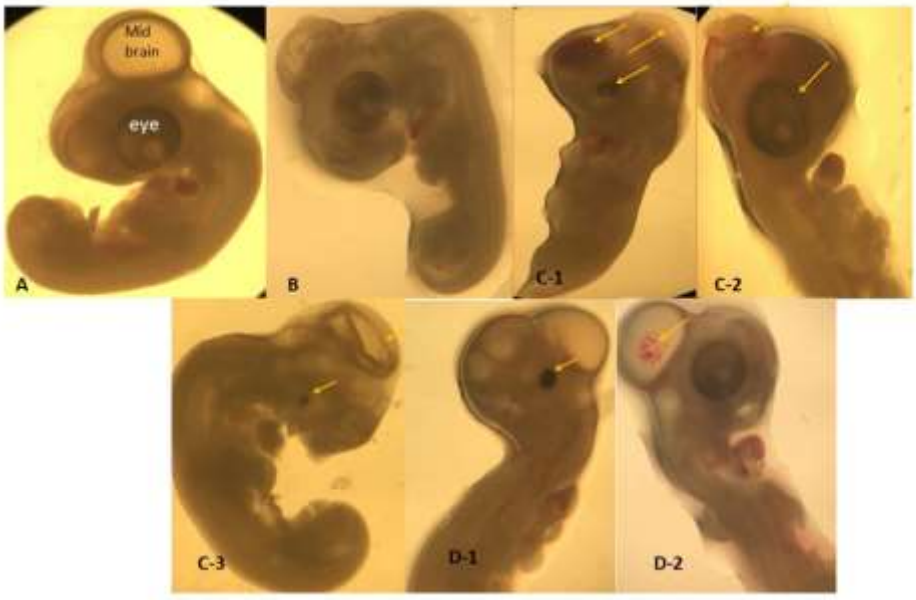

Figure 5:Showing 5-day chick embryos. Figures were taken immediately after extracting embryos of eggs. (A) Normal control, (B) sham.(C1-C2-C3) low treated embryos, (D1-D2) high treated embryos. Sham seem smaller than control embryos. In (C1) the arrows indicate to abnormal brain development, abnormal eye, and bleeding, in (C2) show abnormal brain and eye development, also in(C-3) abnormal eye . Note (D2) hemorrhage in the mid- brain , (D1) abnormal brain and eye development, in (D1) abnormal eye. Scale bar = 1cm. A, B,C-1,C-2, C-3, D-1,D-2 (10X).

Figure 6:(A) Showing the high treated embryo after hatching (27 day of incubation) without external deformities, it immediately died.
(B) Showing the low treated embryo after hatching (22 day of incubation) without external deformities, it immediately died.

Morphometric studies

Effect of artificial sweeteners on the weight of the eggs after incubation

The mean of control egg weight after incubation in this study was 39.2 g for 2 days, 40.6 g for 3 days, 41.8 g for 4 days, and 43.06 g for 5 days. There was no significant difference in the eggs weight between the treated groups, sham group, and control group in 2, 3, 4, and 5 days of incubation. However, the egg weight of the treated groups of 2, 3, and day 5 of incubation was slightly decreased compared to the control.

Effect of artificial sweeteners on the distance between tail and mid-brain of chick embryo

The mean of distance between tail and mid-brain of control chick embryo in this study was 4.6 mm for 2 days, 6.01 mm for 3 days, 8.03 mm for 4 days, and 13.7 mm for 5 days of incubation. There was no significant difference in the distance between tail and mid-brain between the treated groups, sham group, and control group in 3, 4, and 5-day embryos. While in 2 day embryos there was a significant increase in the distance between tail and mid-brain of the low treated group compared to the control (P= 0.006). The high treated group showed a non-significant increase in space between tail and mid-brain compared to the control. It was noticed that there was a significant difference between the two treated groups (P= 0.032) as the low treated embryos had longer distance between the tail and mid brain compared with high treated ones. (See figure 8-A).

Effect of artificial sweeteners on the chick embryo heart rate for 3 and 4-day embryos

The mean of control heart rate/minute of chick embryo in this study was 90 beat/minute for 3 days, and 70.7 beat/minute for 4 days. There was no significant difference in the heart rate between the two treated groups, sham group, and control group in 3-day embryos, however the heart rate of treated groups and sham group were slightly decreased compared to the control. In 4 day embryos the low and high treated groups has significantly decreased heart rate compared to the control (P=0.029) and (P=0.03), while the high treated was also significantly decreased compared to the sham (P=0.004). (See figure 8-B).

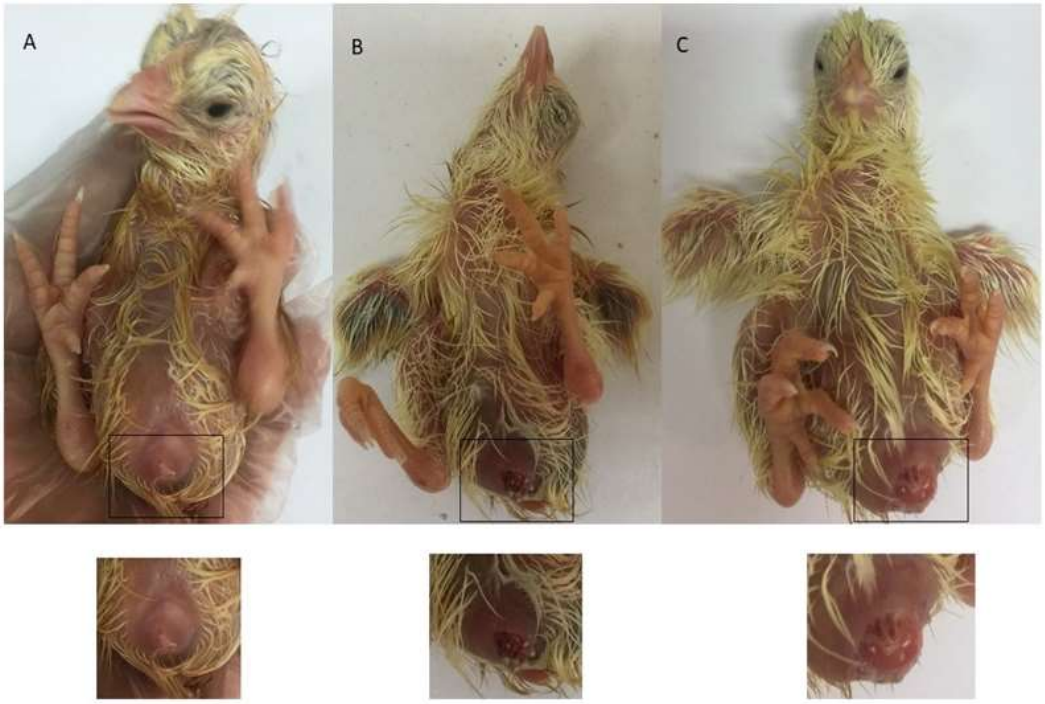

Figure 7:Showing the low treated group (B-C) two embryos hatched on day 22 with Omphalocele compared with normal control (A) the insert magnifies the malformation.

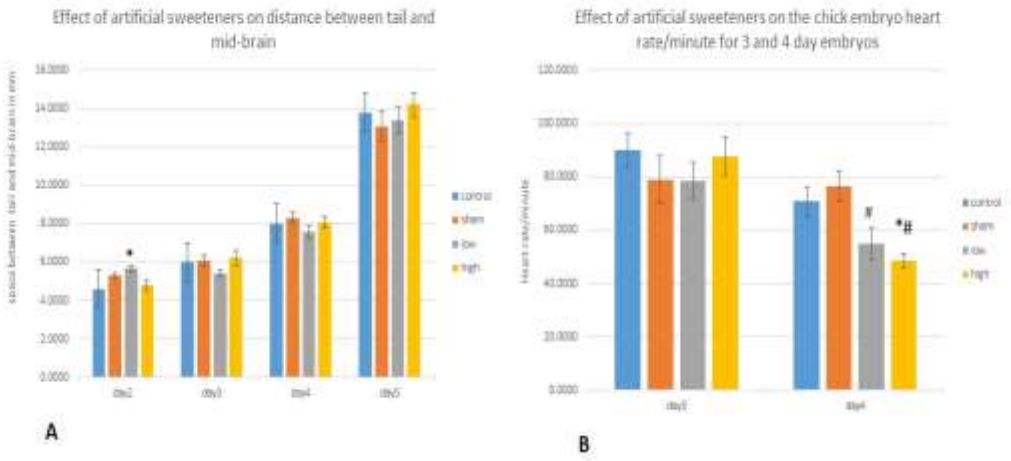

Figure 8:(A) Graph showing the effect of artificial sweeteners on the distance between tail and mid-brain of chick embryo, Values are mean ± SE taken from 10 samples for each group age treatment. (*) p< 0.05 compared to the control.
(B) Graph showing the effect of artificial sweeteners on the chick embryo heart rate/minute for 3 and 4 day embryos. Values are mean ± SE taken from 10 samples for each group age treatment. (*) p< 0.05 compared to the control, (#) p< 0.05 compared to the sham.

Table 2; Showing the hatchability rate percentage.

Group	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27
control	50%	40%	-	-	-	-	-
sham	80%	20%	-	-	-	-	-
Low treated	10%	35%	-	-	-	-	-
High treated	-	-	-	-	-	-	5%

Effect of artificial sweeteners on the whole body weight of 5- day embryos

The mean of control whole body weight in this study was 0.23 g for 5-day embryos of incubation. There was a non-significant increase in the treated groups, and sham group compared to the control group. Also the treated groups slightly increased compared to the sham group.

Effect of artificial sweeteners on the eggshell thickness

The mean of control eggshell thickness in this study was 0.5 mm for 2 days, 0.43 mm for 3 days, 0.43 mm for 4 days, and 0.40 mm for 5 days. In 2-day embryos there was a non- significant decrease in the low and high treated group compared to the control. Also there was a non- significant decrease in sham group compared to the control group. In 3-day embryos, there was a non-significant decrease in high and low treated groups compared to the control. In 4-day embryos, there was slight decrease in eggshell thickness in treated groups and sham group compared to the control. While 5-day embryos, had a slight increase in sham and low groups compared to the control group.

Effect of artificial sweeteners on the whole body length

The mean of control whole body length in this study was 12.13 mm for 2 days, 16.96 mm for 3 days, 30.5 mm for 4 days, and 31.55 mm for 5 days. In day 2 the low treated group slightly increased compared to the control group, while the high treated group slightly decreased compared to the control group. The treated groups non-significantly decreased compared to the sham group. In day 3 the treated groups and sham group non- significantly increased compared to the control group, also the treated groups slightly increased compared to the sham group. In day 4 the treated groups and sham group non-significantly decreased compared to the control, the low treated group slightly increased compared to the sham group, while the high treated group slightly decreased compared to the sham group. In day 5 the treated groups and sham group non-significantly decreased compared to the control group, also the treated groups slightly decreased compared to the sham group.

Effect of artificial sweeteners on the eyes diameter of 5-day embryos

The mean of control eye diameter in this study was 3.02 mm for 5-day embryos. The low and high treated embryos were slightly decreased in the eye diameter compared to control.

DISCUSSION

Artificial sweeteners have become more popular in recent years, and are being used in most of the foods and beverages on the market. Women consumed artificial sweeteners as a means of avoiding weight gain or diabetes (Al-qudsi and Al-hasan, 2019). In this study the chick embryo was used as a model to detect the effect of artificial sweeteners on the chick embryo development. The morphology of control chick embryos was similar to Hamburger and Hamilton stages (1993). In the current study the control group had growth parameters such as whole body length, whole body weight for 5 day embryos, and distance between tail and mid-brain similar to the growth parameters seen in other studies (Rahman et al. 2014). In the present study a growth retardation was seen in the low and high treated chick embryos. The distance between tail and mid-brain was significantly increased in the low treated group compared to the control group in day 2 embryos, also the high treated groups of day 3, 4, and 5 were increased compared to the control. The short distance between tail and mid-brain indicate more growth, due to curvature the embryo during the growth. The whole body length of the treated groups were reduced in 4 and 5 days embryos.

In a previous study, where pregnant mice were treated with commercial artificial sweetener 50mg / kg body weight. The dose was given on day 1 of pregnancy until 3-weeks nursing. The weight and

length of the18-day embryos of treated mothers was significantly reduced compared to the controls (Al-qudsi and Al-hasan, 2019). The methanol resulting from aspartame metabolism contributed to alcohol accumulation in the placenta and induced disruption in nutrient transfer between mother and fetus. In a study to investigate the impact of aspartame on Zebrafish's embryonic development. Different concentrations of aspartame were used as a test solution 100, 250, 500, 1000, 2000, 5000, 10000, 15000, and 20000 mg/L. The results clearly demonstrated that the rise in aspartame concentrations of 10,000-15,000-20000 mg / L resulted in many fetal abnormalities in zebrafish embryos such as retardation of development, loss of pigmentation and tail deformities. (Weerasooriyagedara, 2018).

In the present study, the artificial sweeteners caused significantly reduced heart rate of treated groups on day4 of incubation. While in another study when assessing the developmental toxicity of caffeine and artificial sweeteners (aspartame and saccharin) as mixtures in medaka, the treated groups had significantly higher heart rates than the control (Lee and Wang, 2015). The low heart rate in our study might be due to the mixture of artificial sweeteners or silicon dioxide. Du et al. (2019) noted that silica nanoparticles (silicon dioxide) induce cardiomyocyte apoptosis via the mitochondrial pathway in rats following intratracheal instillation. Reducing the heart rate may lead to pumping less blood and thus leads to a decrease in the amount of oxygen and nutrients that the fetus needs to grow, this may explain the growth retardation.

In this study commercial artificial sweetener caused abnormal brain development to chick embryos, clearly seen on day 3, 4, and 5 of incubation. Early brain development might be affected by aspartame consumption. Phenylalanine (which is a product of aspartame metabolite) has the ability to cross the blood- brain barrier and cause severe changes in the production of very important neurotransmitters. The metabolites of aspartame disturbs the metabolism of amino acid and protein (Humphries et al. 2008). In a study, long-term rat consumption of 40mg/kg of aspartame, led to alteration of the brain function and activated apoptosis in brain. This study concluded that methanol metabolism released formate, which induced oxidative stress and neurodegeneration in brain regions (Ashok et al. 2015). Similar congenital malformation was caused by high-glucose dose in chick embryo. It was concluded that the high concentration of glucose was associated with alterations in oxidative stress. (Tan et al. 2015). Another study concluded that aspartame ingestion for 4 and 6 weeks induced oxidative stress in the rat cerebral cortex (Mourad and Noor, 2011). In a study that used high concentration of aspartame on zebrafish 20000 mg/l concentration level showed no head formation (Weerasooriyagedara, 2018). In a study long term Acesulfame k (40 weeks) could affect cognitive functions, potentially via altering neuro-metabolic functions (abnormalities of enzymes, the metabolic consequences of which affect the development of nervous system) in male C57BL/6J mice (Cong et al. 2013). It might be that, the brain abnormalities seen in this study might have been caused by the artificial sweeteners or their metabolites, that caused disturbance to the neurotransmitters or transcription factors that were vital for brain development therefore inducing brain malformations.

In this study, the commercial artificial sweetener caused eyes abnormalities to some chick embryos. The eye is an extension of the brain. It is built by neurons and axons. several neurodegenerative conditions that affect the brain have an expression in the eye (London et al. 2012). In this study it seems that, the eye abnormalities were caused by brain disorder. In a study using medaka embryos as a model to investigate the developmental toxicity of caffeine and sweeteners, the eye width and length of the embryos treated with aspartame were significantly smaller than the control, also the mid brain width was significantly shorter than the control (Lee and Wang, 2015). Another study that investigated the toxicity effects of Aspartame on embryonic development of Zebrafish, at high concentrations of aspartame no eye and head formation occurred (Weerasooriyagedara, 2018).

In the present study, the artificial sweeteners caused a significant increase in the mortality of the treated groups, especially the high treated group. Only one embryo of the high treated group hatched at day 27 of incubation and died immediately, while other embryos died at early embryonic stages. In a previous study the physiological effect of artificial sweeteners was determined in the presence of hyperlipidemia, zebrafish fed with aspartame and cholesterol exhibited significant increase in mortality with acute swimming abnormalities, indicating that there might be damage in the brain and neurons (Kim et al. 2011). Another study found that, saccharin at 55Mm induced 92% mortality within 24h post fertilization in zebrafish embryos (Selderslaghs et al. 2009).

As the artificial sweeteners used in this study was bought from the market, and the low dose (50mg/kg) used in this study was the normal permissible dose and it caused multiple malformations in the chick embryo. Add to this that the high dose is easily reached in daily life as a person could have a cup of coffee with 50mg/kg artificial sweeteners, then have a snack containing artificial sweeteners or have a medicine containing artificial sweeteners, and this person might be a pregnant mother. Therefore, the awareness on the possible teratogenicity of artificial sweeteners should be raised through multiple studies.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

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AUTHOR CONTRIBUTIONS

A.A. and F.A. designed experiments and reviewed the manuscript. All authors read and approved the final version.

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