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Research Article

Biology

Artificial Sweeteners Alter Early Vitelline Vessel Development in Chick Embryo

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Abstract: During early embryonic development, nutrients are transferred to the embryo via vitelline vessels. Disorders that affect vitelline vessel development lead to embryonic lethality or vascular diseases. In recent years, the use of artificial sweeteners has increased and become more widespread, however the potential effects of these compounds on embryonic development remain poorly understood and controversial. This study aimed to establish the effect of commercial artificial sweeteners on early vitelline vessel development in chick embryos. The commercial artificial sweetener used in this study was a mixture of aspartame and acesulfame k. Fertile chicken eggs were divided into four groups: control, sham, and two treated groups that were injected in the air chamber before incubation with 50mg/kg or 100mg/ kg sweetener by body weight. The eggs were then incubated under normal incubation conditions. Embryos were extracted and photographed on days 3 and 4 of incubation, then vitelline vessel measurements were taken (length and width of the vitelline vessels, right and left angles, and number of branches). VEGF concentration within the area vasculosa of 3-day embryos was measured by ELISA. Low- and high-treated embryos showed abnormal vitelline vessel development at 3 and 4 days post-incubation. Some vessels were enlarged while others displayed atrophy. The pattern of branching was also altered compared to controls, and some vessels had hemorrhage. The concentration of VEGF was lower in the treated groups compared to that observed in the control group. This study revealed an association between artificial sweeteners and abnormal vitelline vessel development. These findings could have broad potential implications for human health, as this study used commercial artificial sweeteners that are commonly available in consumer markets.

Keywords: Chick embryo, vitelline vessels, artificial sweeteners, angiogenesis, vascular endothelial growth factor VEGF, vasculogenesis.

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1. INTRODUCTION

While the addition of artificial sweeteners to a variety of foods, drinks, and pharmaceutical products has increased in recent years, relatively little is known about how these products affect cell growth and development [1]. A limited number of studies have illustrated a relationship between the sweetener aspartame and angiogenesis *in vivo* [2] and *in vitro* [3]. Similarly, the artificial sweetener acesulfame k has been shown to inhibit angiogenesis, [4] the process whereby new blood vessels are formed from pre-existing vasculature [5]. Vascular endothelial growth factor (VEGF) plays a pivotal role in angiogenesis. Specific populations of endothelial cells in existing blood vessels respond to this growth factor and subsequently form new blood vessels. These VEGF-responsive cells are called tip cells [6]. The chick embryo is a common model animal in developmental biology, primarily due to ease of experimental manipulation. At the early stages of chick embryo development, the yolk sac serves as the primary source of nourishment, decreasing in size as its resources are consumed by the embryo. It is rich with blood vessels, which are responsible for vitelline circulation. The mammalian embryo also depends on vitelline circulation in early stages, eventually atrophying and disappearing as placental circulation becomes the dominant mode of embryonic blood flow [7]. The vitelline vessels are generated from blood islands, which are formed in the yolk sac by vasculogenesis. The hemangioblasts of the blood islands in the yolk sac generate the angioblasts of blood vessels and hematopoietic cells of blood [8]. Malfunction during vitelline vessel development can lead to congenital malformations [9]. Artificial sweeteners are consumed directly (table artificial sweeteners) or indirectly (chewing gums, medicines, yogurt) by many individuals including pregnant mothers and small children. Vitelline vessels are essential in the establishment of nourishment flow for the embryo, and malformation of these structures might impede nutrient delivery to the developing embryo, leading in turn to malformations of the embryo itself. The aim of this research, therefore, was to understand the effects of commercial artificial sweeteners on the development of vitelline vessels in chick embryos.

2. MATERIALS & METHODS

All experimental procedures were approved by the Biology Department at King Abdul-Aziz University. Fertilized chicken eggs were obtained from private farm chickens in Dhaban north of Jeddah. The artificial sweetener used in this study was purchased from local supermarkets as 50 mg sachets. Each sachet contained 0.617mg of acesulfame K and 0.546mg of aspartame as non-nutritive sweeteners. The remaining 48.8 mg consisted of Dextrose, Maltodextrin and silicon dioxide.

2.1 *Experimental Design*

Fertilized chicken eggs were divided into four groups: Control, sham, treated low, and treated high. The mean egg weight in this study was 44.8 g. Two doses of sweetener were used in this study. The low dose of artificial sweeteners was 50 mg/Kg, as established in previous work [10]. The high dose was the double amount of the low dose. The low dose group was injected with 2.24 mg artificial sweetener in 0.1 ml distilled water, while the high dose group was injected with 4.48 mg artificial sweetener in 0.1 ml distilled water. The sham group was injected with 0.1 ml of distilled water. All groups were injected once before incubation. Eggs were then incubated at 37.5 °C and 80% humidity.

2.2 *Sample Collection*

Embryos were extracted from all groups on the 3rd and 4th days after incubation. The egg was opened by knocking the eggshell at the blunt end with scissors and removing the top of the eggshell. The area vasculosa of each embryo was photographed immediately after removing the blunt end of the eggshell. Four area vasculosa of each experimental group of 3-day embryos were frozen for further analysis by ELISA.

2.3 *Photography and Morphometry*

Each area vasculosa was photographed using an iPhone 6 Plus 12 Megapixel camera. The iPhone was attached to a tripod. A ruler was put near the egg for scale when performing morphometry using the photos. The measurements were taken on days 3 and 4, and embryos were assessed for the length and width of the vitelline vessels, right and left angle of vitelline vessels, and number of vessel branches to determine the relative progression of vitelline vessel morphogenesis. Measurements were taken from the photos taken with the iPhone camera and quantified using the free computer software (Image tool) downloaded from (http://cme.msu.edu/cmeias/). All images were obtained and analyzed under identical conditions without any alteration. Figure 1 shows all vitelline vessels measured in this study.

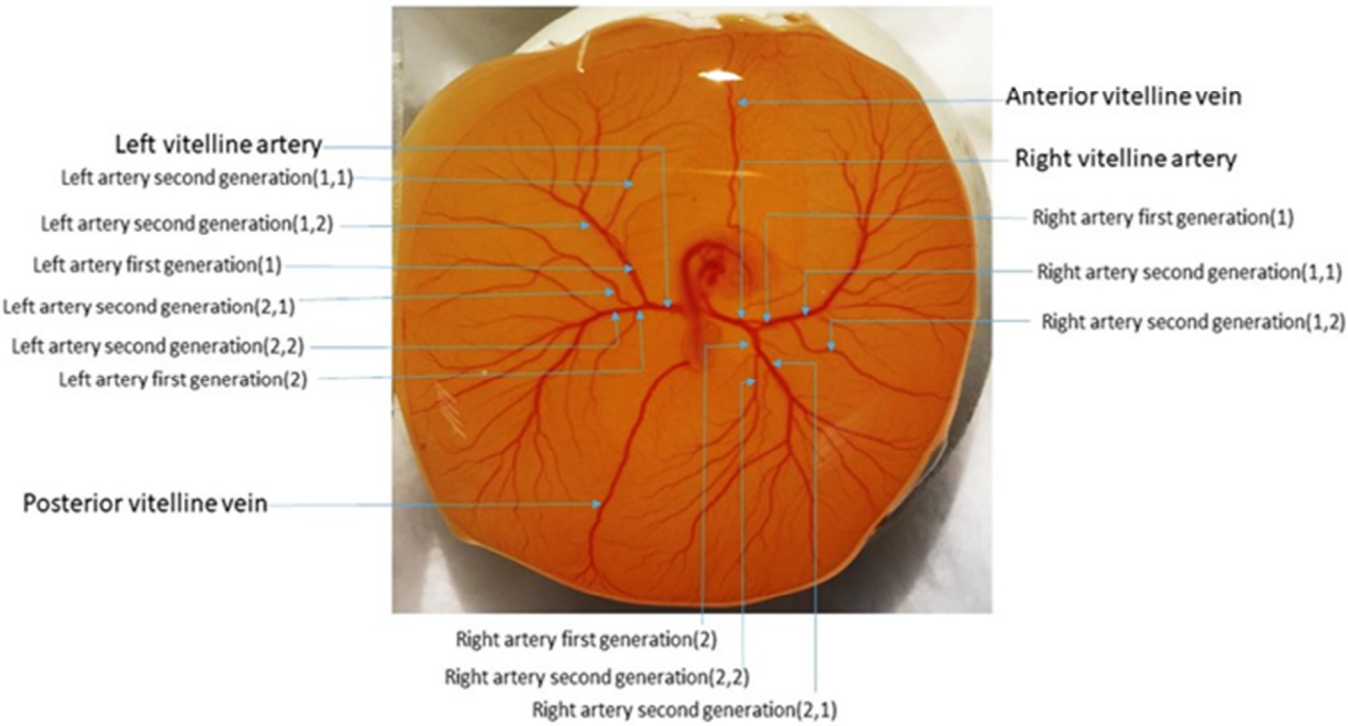


Fig 1: Vitelline vessels measured in this study

2.4 *ELISA for Vascular endothelial growth factor-A (VEGF-A)*

2.4.1 *Tissue homogenization*

The area vasculosa was dissected from the embryos and weighted inside Eppendorf tubes. An appropriate amount of phosphate buffered saline was added to the tissues. The samples were ground using TissueLyser II solution (QIAGEN). The samples were subjected to two freeze-thaw cycles to further break the cell membranes, then centrifuged for 5 minutes at 5000×g. The supernatants were collected and saved in other tubes at -80 °C.

2.4.2 *ELISA*

The samples were assayed using a gallinaceous VEGF-A Enzyme Linked Immunosorbent Assay (ELISA) kit purchased from (Cloud-Clone Corp). This assay employs the quantitative sandwich enzyme immunoassay technique. Briefly, stock solution was diluted to 1,000pg/mL, and the diluted standard served as the highest standard (1,000pg/mL). Next, 7 tubes were prepared containing 0.5mL of Standard Diluent. The diluted standard was used to produce a double dilution series. Each tube was mixed thoroughly before subsequent transfer. Seven concentrations of diluted standard were set up: 1,000pg/mL, 500pg/mL, 250pg/mL, 125 pg/ml, 62.5pg/mL, 31.2pg/mL, and 15.6pg/ml. The last tube with Standard Diluent served as the blank, with a concentration of 0 pg/ml. The Detection Reagent A and Detection Reagent B were centrifuged, then diluted to the working concentration with Assay Diluent A and B, respectively (1:100). The wells were counted and determined as follows: 7 wells were used for the diluted standard, 1 for the blank, and 16 wells were used for the samples. Each well received 100µL of dilution standard, blank, or samples. The wells were covered with plate sealer and incubated for 2 hours at 37 °C. The liquids were removed, and 100µL of biotin antibody (Detection reagent A) was added to each well and incubated for 1 hour at 37°C. The wells were washed 3 times by microplate washer, then 100µL of conjugated antibody (Detection reagent B) was added to each well and incubated for 30 minutes at 37 °C. Wells were washed an additional 3 times by microplate washer, then 90µL of substrate solution was added to each well and the wells were incubated for 20 minutes at 37 °C. When the color of the wells changed to blue, 50µL of stop solution was added to each well. The color change was measured at 450 nm using a microplate reader.

2.4.3 *Quantification of the total protein*

The quantity of protein was determined by BCA protein assay based on bicinchoninic acid (BCA). The kit was purchased from (Thermo scientific). First, standard and working reagent were prepared. The standard bovine serum albumin (BSA) underwent serial dilution. The working reagent was prepared by mixing 50ml of BCA reagent A with 1ml of reagent B. Each microplate well received 25µL of standard or sample. Then, 200µL of working reagent was added to each well and the plate was mixed thoroughly. The plate was then covered and incubated at 37 °C for 30 minutes. Finally, the absorbance was measured at 560 nm on a microplate reader.

3. STATISTICAL ANALYSIS

Data was analyzed using SPSS 24. The tests used with normal distribution were Anova, S-N-K, and Tukey tests. In cases of abnormal distribution the Mann-Whitney U test was used. Significance was set at $p < 0.05$.

4. RESULTS

4.1 *Morphology*

As can be seen in Figure 2A, vitelline vessels extended from the embryo and were beginning to branch in 3-day controls.

The sham embryos appeared similar to the control embryos, however the vitelline membranes of the sham embryos seemed more fragile, demonstrating a high tendency to break down, as shown in figure 2B . In low and high treated groups, bleeding was observed, and the vitelline vessels seemed to have fewer branches compared to the control (figure 2C, 2D).

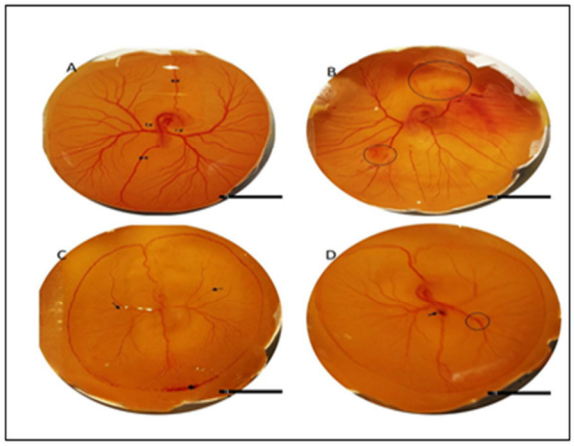


Fig 2: Congenital malformations seen in 3 day old chick embryos. (A) Control, (B) sham, (C) treated low, (D) treated high. Note the fragile vitelline membrane of sham (B) compared to the control. Note the bleeding in the high treated embryo (D). Vitelline vessels of the two treated groups (C) and (D) had abnormal branching compared to the control. (a.v: anterior vein; p.v: posterior vein; r.v: right vessel; l.v: left vessel.)

In the control 4-day chick embryo, the area vasculosa almost covered the yolk sac with radially growing vitelline vessels (see figure 3A). The sham embryos appeared similar to the control embryos (see figure 3B). The defects seen in 4 day low-treated embryos were vitelline vessel atrophy (diminution of size), and fragile vitelline membrane, as the vitelline membrane was easily ruptured in these embryos (see figure 3C). The high dose of artificial sweetener seemed to cause vessel atrophy. Vessel width also appeared larger compared to the control and sham groups, and vitelline membrane fragility was also observed (see figure 4 D1, D2, D3).

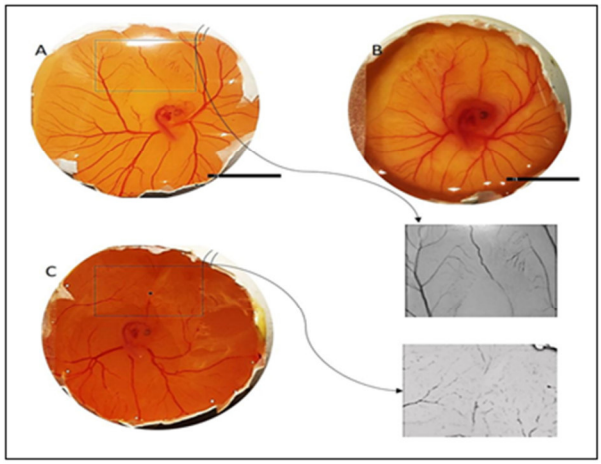


Fig 3: Congenital malformations seen in 4-day chick embryos. (A) normal control, (B) sham, (C) low-treated embryos. Part of (C) was enlarged to show vitelline vessel atrophy compared with normal vitelline vessels of control embryos.

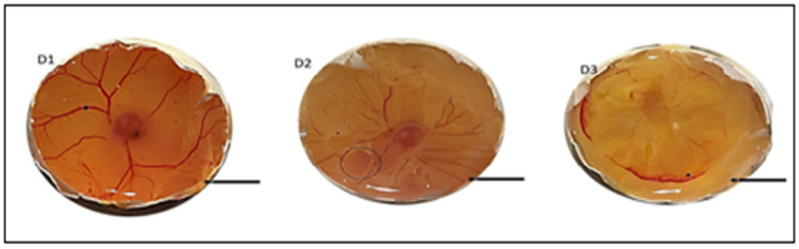


*Fig 4: Congenital malformations seen in 4-day chick embryos treated with a high dose of artificial sweetener (D1, D2, D3). Note large vessels in (D1) * vessel atrophy in (D2, D3), and hemorrhage * in (D2).*

4.2 *Morphometry Results*

4.2.1 Branching

Mean vitelline vessel branching of control chick embryos in this study was 39 branches at 3 days and 37 branches at 4 days. There was a non- significant decrease in the vitelline

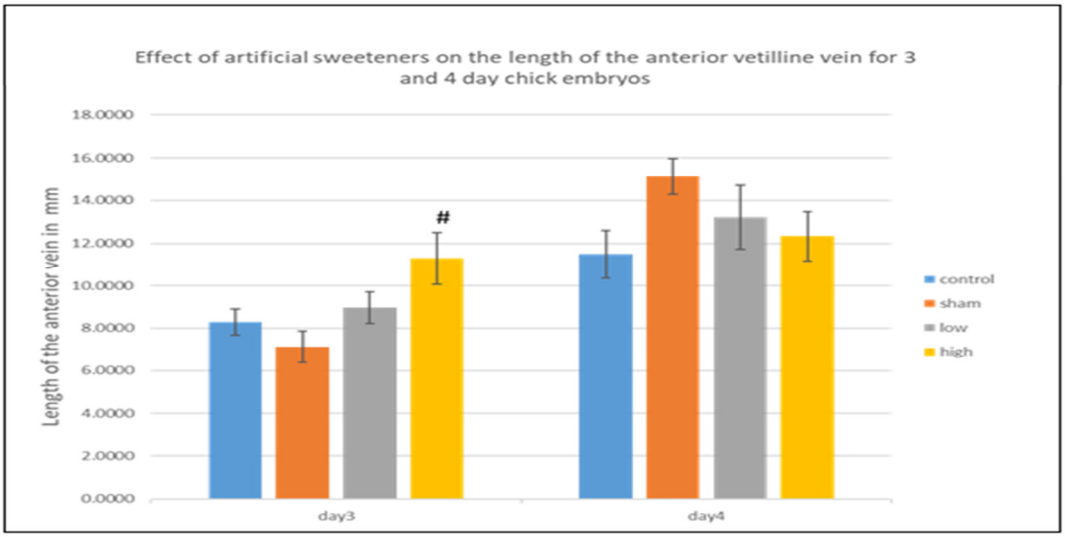
vessel branching of the two treated groups and sham groups compared to the control group in 3- and 4-day embryos.

4.2.2 *Length of the anterior vitelline vein for 3- and 4-day embryos*

The mean anterior vitelline vein length in this study was 8.29 mm at 3 days post-incubation, and 11.49 mm at 4 days after incubation in control embryos. There was a non-significant increase in anterior vitelline vein length in the treated groups compared to the control group. The mean anterior vitelline vein length in the low-treated group increased non-significantly compared to the sham group, while vein length in the high-treated group significantly increased compared to the sham group in 3-day embryos (P= 0.008). In 4-day embryos, there was a non- significant increase in the length of the anterior vitelline vein in the high- and low-treated groups and sham group compared to the control group. However, there was a non-significant decrease in anterior vitelline vein length in the treated groups compared to the sham group (see figure 5).

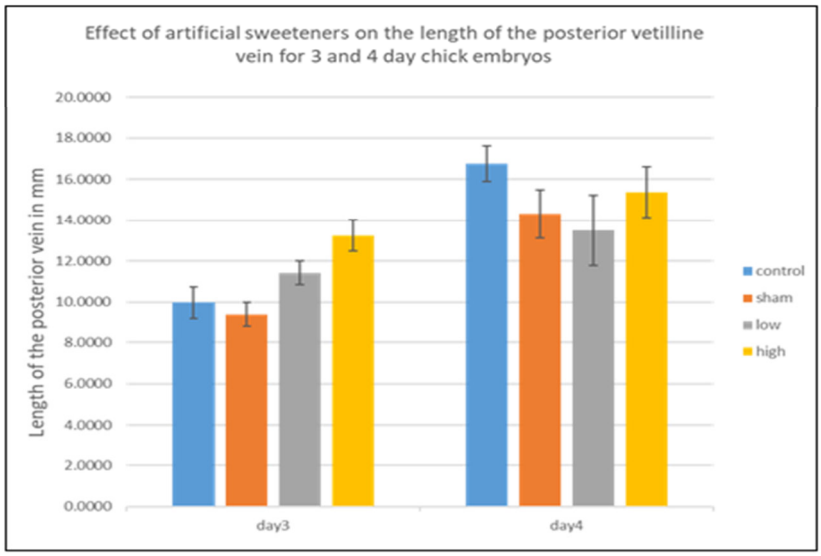
4.2.3 *Length of the posterior vitelline vein for 3- and 4-day embryos*

We observed a mean posterior vitelline vein length of 9.97 mm at 3 days, and 16.76 mm at 4 days post-incubation in control embryos. In 3-day embryos, there was a non-significant increase in posterior vitelline vein length in the low-treated group compared to the control group, and there was a significant increase in the length of the posterior vein of the high-treated group compared to the control group (P= 0.01). Posterior vein length increased non-significantly in the low-treated group compared to the sham group, while the high-treated group showed a significant increase in the length of the vein compared to the sham group (P= 0.002). In 4-day embryos, there was a non- significant decrease in the length of the posterior vitelline vein of the treated groups compared to the control group. Posterior vein length in the low-treated group was slightly shorter compared to the sham group, while the vein length in the high-treated group was slightly increased compared to the sham group (see figure 6).



Values are mean ± SE taken from 10 samples for each group age treatment. (#) p< 0.05 compared to the sham.

Fig 5: Effect of artificial sweeteners on the length of the anterior vitelline vein for 3- and 4-day embryos.



Values are mean ± SE taken from 10 samples for each treatment group by age. (#) p< 0.05 compared to the sham, (*) p< 0.05 compared to the control.

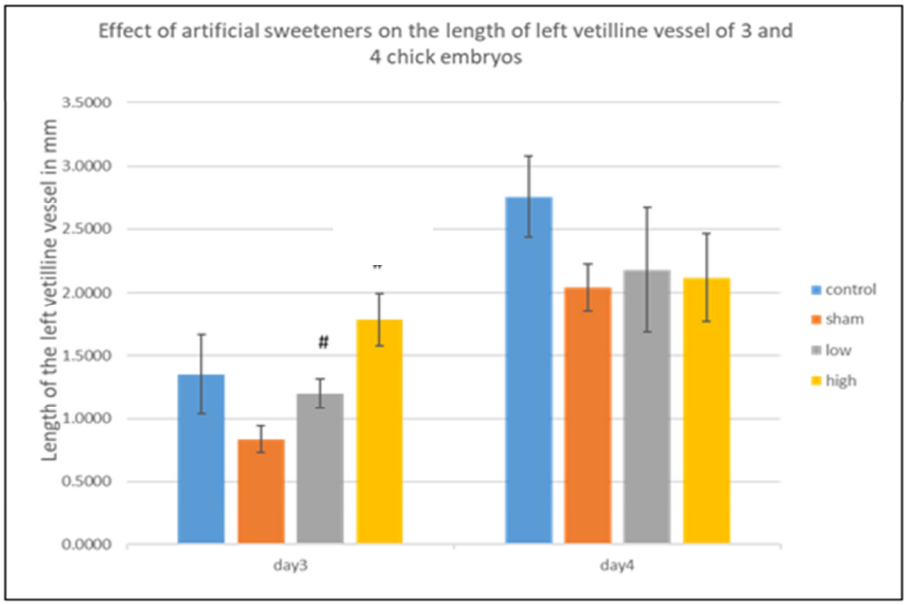
Fig 6: Effect of artificial sweeteners on the length of the posterior vitelline vein for 3- and 4-day chick embryos.

4.2.4 *Length of the right vitelline artery (major) for 3- and 4-day embryos*

The mean control length of the right vitelline artery in this study was 1.7 mm at 3 days, and 2.04 mm at 4 days after incubation in control embryos. There was a slight decrease in this average in the 3-day low-treated group compared to the controls. Right vitelline artery length increased marginally but non-significantly in the high-treated group relative to controls. In the sham group, right vitelline artery length was shorter than in all other groups. In 4-day embryos, a non-significant decrease in right vitelline artery length was observed in the low-treated group compared to the control group, while a slight increase was observed in the high-treated group. Unlike sham-treated 3-day embryos, the sham 4-day embryos had longer right vitelline arteries than all groups.

4.2.5 *Length of the left vitelline artery (major) for 3- and 4-day embryos*

The mean length of the left vitelline artery was 1.35 mm for 3-day embryos, and 2.57 mm for 4-day embryos in the control group. In 3-day embryos, mean left vitelline artery length in the low-treated group was slightly decreased compared to controls, while artery length in the high-treated group was marginally but non-significantly longer than in the control group. Left vitelline artery length was significantly increased in the low-treated group compared to the sham group (P=0.052). There was also a significant increase in artery length in the high-treated group compared to the sham group (P= 0.001). In 4-day embryos, there was a slight decrease in artery length in the treated groups and sham group compared to the control group. Left vitelline arteries were shorter in the sham group compared to all groups (see figure 7).



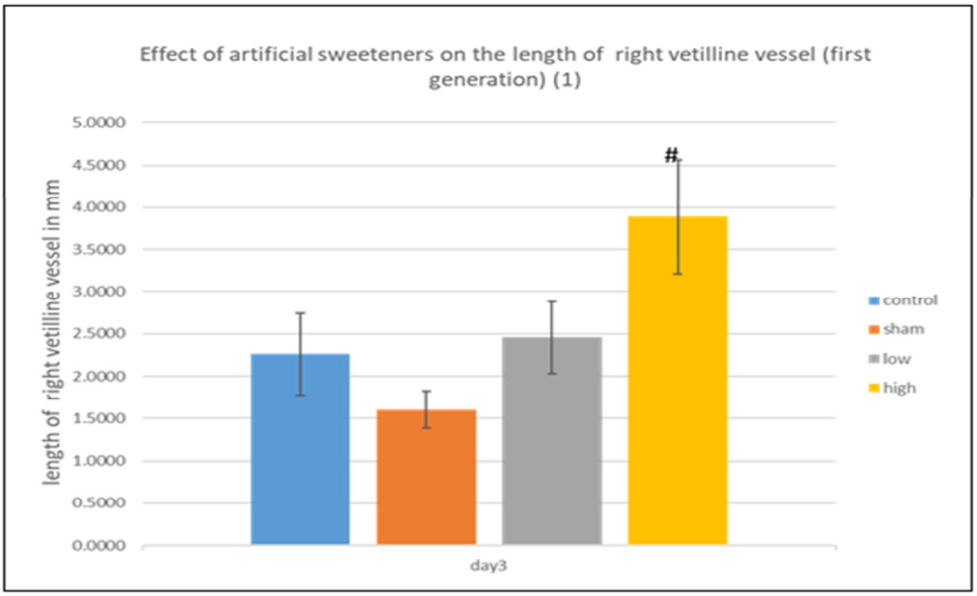
Values are mean ± SE taken from 10 samples for each age group and treatment condition. (#) p< 0.05 compared to the sham group. Measurements of first and second generation of vitelline vessels were not applicable for day 4 treated embryos because of atrophy of the vitelline vessels.

Fig 7: Effect of artificial sweeteners on the length of the left vitelline arteries of 3- and 4-day chick embryos.

4.2.6 *Effect of artificial sweeteners on the length of the first generation of vitelline vessels of 3-day embryos*

4.2.6.1 *Right vessels*

The mean control length of first generation right vessels (1) in this study was 2.26 mm. There was a non- significant increase in vessel length in the treated groups compared to the control group. Vessel length in the low-treated group was non- significantly increased compared to the sham group. Vessel length in the high-treated group was significantly increased compared to the sham group (P= 0.004). The high-treated group had the longest veins of all treatment groups (see figure 8).



Values are mean ± SE taken from 10 samples for each group age treatment. (#) p< 0.05 compared to the sham.

Fig 8: Effect of artificial sweeteners on the length of the right vitelline vessel (first generation) (1) of 3-day chick embryos.

The mean control length of first generation right vessels (2) in this study was 2.27 mm 3 days post-incubation. There was a non-significant increase in right vessel length in the low-treated group compared to the control group, while in the high-treated group this value slightly decreased compared to controls. Right vessel length was shorter in the sham group than in all other groups.

4.2.6.2 *Left vessels*

The mean control length of first generation left vessels (1) was 2.16 mm at 3 days post-incubation. There was a non-significant decrease in left vessel length in the low-treated group compared to the control group, while in the high-treated group vessel length was slightly increased compared to controls. Left vessels were slightly shorter in the low-treated group compared to the sham group, while in the high-treated group left vessel length was non-significantly increased compared to the sham group. The mean control length of first generation left vessels (2) in this study was 1.75 mm at 3 days post-incubation. There was a non- significant decrease in vessel length in the treated groups compared to the control group. Left vessels in all treated groups were slightly shorter than in the sham group.

4.2.7 *Effect of artificial sweeteners on the length of the second generation of vitelline vessels of 3-day embryos*

4.2.7.1 *Right vessels*

The mean control lengths of second-generation vessels in this study was 2.63 mm for (1,1) vessels, 3.87 mm for (1,2) vessels, 2.03 mm for (2,1) vessels, and 1.67 mm for (2,2) vessels 3 days after incubation. There was a non-significant increase in vessel length in the low-treated group of (1,1) vitelline vessels compared to the control group, while in the high-treated group these values were slightly decreased compared to the control. Mean vessel lengths in the sham group were decreased compared to those measured in all groups. Mean lengths in the treated groups and the sham group of (1,2) vitelline vessels were non-significantly decreased compared to the control group. Vessel lengths in the treated groups were non- significantly increased compared to the sham group. In the treated groups and sham group of (2,1) vitelline vessels, lengths were non-significantly increased compared to those measured in the control group. Vessel lengths in the treated groups were slightly decreased when compared to the sham group. In the treated groups and sham group of (2,2) vitelline vessels, lengths were non-significantly increased compared to controls. The vessel lengths measured in the treated groups were slightly longer compared to sham group vessels. The vitelline vessel lengths of the high-treated group were increased compared to all groups.

4.2.7.2 *Left vessels*

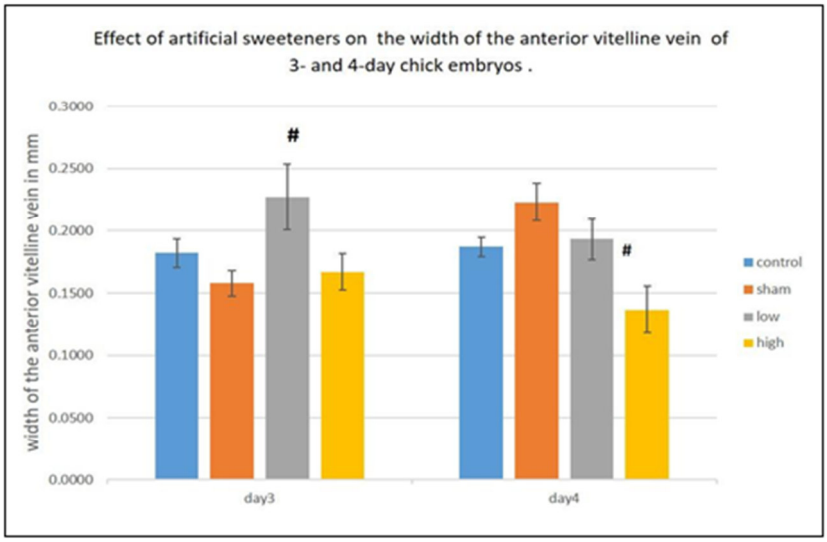
The mean control length of left second-generation vessels in this study was 2.22 mm for (1,1) vessels, 2.27 mm for (1,2) vessels, 2.8 mm for (2,1) vessels, and 3.34 mm for (2,2) vessels at 3 days post-incubation. There was a non-significant increase in second-generation vessel length in the treated

groups and sham groups of (1,1) vessels compared to the control group. Vessel length in the treated groups was slightly increased compared to the sham group. In the treated groups and sham group of (1,2), vessel lengths were slightly increased compared to the control group. A similar trend was observed in the treated groups compared to the sham group. Vessel length in the low-treated group was increased compared to all groups. In (2,1) vessels, lengths increased non-significantly in the treated groups and sham group compared to controls. These values in the low-treated group were slightly decreased compared to the sham group, while in the high-treated group they increased non-significantly compared to the sham group. Vessel lengths in the treated groups and sham group of (2,2) were non-significantly decreased compared to controls, while values in the sham group were decreased compared to all groups.

4.2.8 Effect of artificial sweeteners on the width of the major vitelline vessels (anterior, posterior, right, and left) of 3- and 4-day chick embryos

4.2.8.1 Width of the anterior vitelline vein for 3- and 4-day embryos

The mean control width of the anterior vitelline vein was 0.18 mm in 3-day embryos and 0.18 mm in 4-day embryos. In 3-day embryos there was a non- significant increase in vein width in the low-treated group compared to controls. In the high-treated group, vein width was slightly decreased compared to the control group. There was a significant increase in the width of the anterior vitelline vein in the low - treated group compared with the sham group (P= 0.03). In the high-treated group, vein width was non-significantly increased compared to the sham group. In 4-day embryos, vein width in the low-treated group slightly greater than in control embryos. In the high-treated group, vein width was non-significantly decreased compared to the control group. The low-treated group exhibited slightly narrower veins compared to the sham group, and the width of the anterior vein in the high-treated group was significantly more narrow than in the sham group (P= 0.001) (see figure 9)

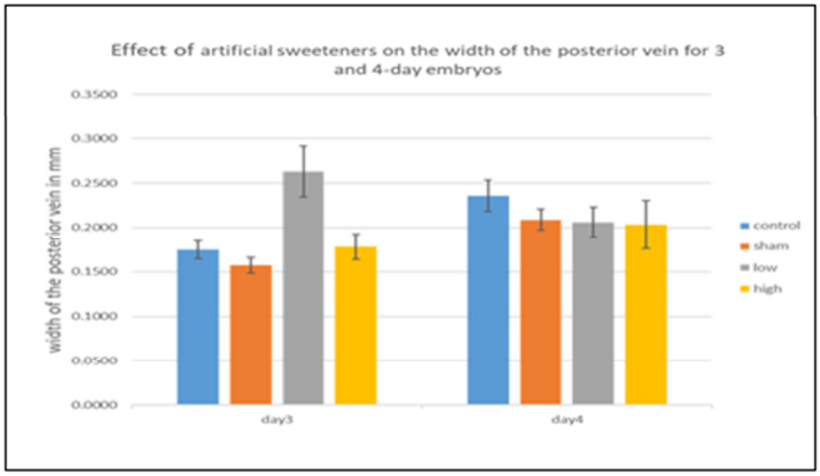


Values are mean ± SE taken from 10 samples for each group age treatment. (#) p< 0.05 compared to the sham

Fig 9: Effect of artificial sweeteners on the width of the anterior vitelline vein of 3- and 4-day chick embryos.

4.2.8.2 Width of the posterior vitelline vein for 3- and 4-day embryos

The mean control width of the posterior vitelline vein in this study was 0.175 mm at 3 days, and 0.23 mm at 4 days post-incubation. The widths of the posterior veins of the low-treated 3-day embryos were significantly increased compared to the controls (P=0.009), while vein width in the high-treated group non-significantly increased compared to the control group. Vein width in the low-treated group was significantly increased compared to the sham group (P=0.003). Vein width in the high-treated group was non-significantly increased compared to the sham group. In 4-day embryos there was a non-significant decrease in vein width in the treated groups and sham groups compared to the control group. Vein widths in the low-treated group were slightly increased compared to the sham group, while these values were slightly decreased in the high-treated group compared to the sham group (see figure 10).



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

Fig 10: Effect of artificial sweeteners on the width of the posterior vein of 3- and 4-day chick embryos.

4.2.8.3 Width of the right vitelline artery for 3 and 4-day embryos

The mean control width of the right vitelline artery was 0.26 mm for 3-day embryos, and 0.41 mm for 4- day embryos. There was a significant increase in the width of the right vitelline artery of 3-day embryos in the low-treated group compared to control (P= 0.04), and also in the high- treated group compared to the control (P=0.03). Vein widths in the treated groups were non-significantly increased compared to the sham group. In 4-day embryos there was a slight increase in vein width in the low-treated group compared to the control group, whereas in the high treated group vein width was non- significantly decreased compared to the control group. Vein widths in the treated groups were increased non- significantly compared to the sham group (see figure 11)

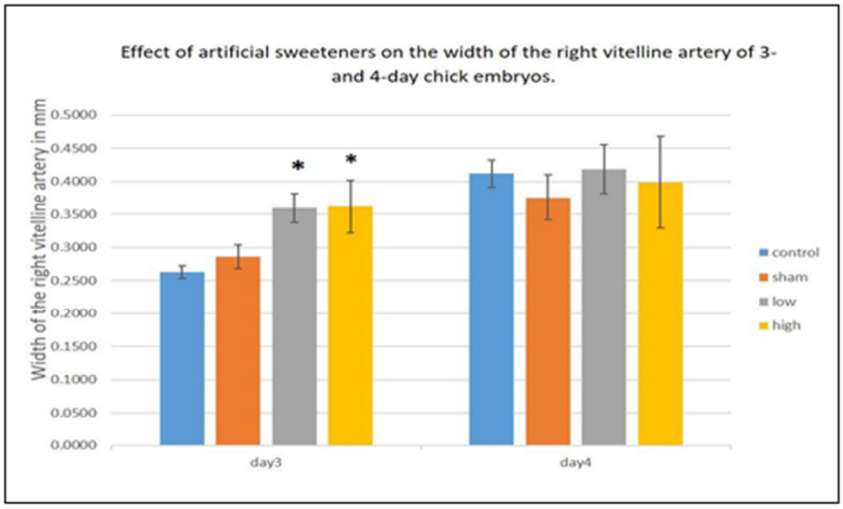


Fig 11: Effect of artificial sweeteners on the width of the right vitelline artery of 3- and 4-day chick embryos.

Values are mean ± SE taken from 10 samples for each group age treatment. (*) p< 0.05 compared to the control.

4.2.8.4 Width of the left vitelline artery for 3 and 4-day embryos

The mean control width of the left vitelline artery in this study was 0.30 mm for 3-day embryos and 0.36 mm for 4-day embryos. There was a non-significant increase in artery width in the treated groups compared to the control group, and a similar trend in the treated groups compared to the sham group. Artery width in the sham group was decreased compared to widths measured in all other groups. In 4-day embryos, arteries were slightly wider in the treated groups and sham group compared to the control group, although this difference was not statistically significant. Similarly, artery widths in the treated group were non- significantly increased compared to the sham group.

4.2.9 Effect of artificial sweeteners on the right and left angle of vitelline vessels in 3- and 4-day embryos

The mean of control right angle in this study was 〖101.58〗^° for 3-day embryos and 〖91.22〗^° for 4-day embryos. In 3-day embryos, there was a non-significant decrease in vessel angle in the treated groups and sham group compared to the control group, while a non-significant increase in vessel angle was observed in the treated groups compared to the sham group. In 4-day embryos, slightly smaller vessel angles were recorded in the low- treated group compared to the control group, while in the high-treated group vessel angles were slightly increased compared to the control group. Vessel angles in the treated groups were non- significantly decreased compared to the sham group The mean control left angle was 〖99.06〗^° for 3 days post-incubation and

79.84〗 ^° for 4 days. In 3-day embryos, there was a non-significant decrease in vessel angle in the treated groups compared to the control group, and this trend was also observed in the treated groups when compared to the sham group. In 4-day embryos, vessel angles in the treated groups and sham group were slightly increased compared to the control, and this trend persisted when the treated groups were compared to the sham group.

4.2.10 Effect of artificial sweeteners on the concentration of VEGF-A protein

VEGF-A concentration in the area vasculosa of 3-day embryos following artificial sweeteners exposure was slightly but non-significantly lower in the high- and low- treated groups compared to the sham and control groups. An average value of 336 pg/mg of total protein was recorded in the control group, while the average concentration in the sham group was 327.49 pg/mg of protein. Average VEGF-A concentration in the low-treated group was 205.69 pg/mg of total protein, while the average value in the high treated group was 295.7 pg/mg of protein.

5. DISCUSSION

In this study, commercial artificial sweeteners caused vessel hemorrhage and alteration of vascular branching patterns in low- and high-treated embryos. Some vessels were enlarged while others were atrophied. VEGF-A is a glycoprotein that plays an important role in the regulation of blood vessel growth. Early in embryonic development, VEGF-A is highly expressed in the yolk sac and in embryonic sites of vessel formation to support the synthesis of the cardiovascular system [11]. Baseline levels of VEGF-A measured in chick embryos in this study were similar to levels reported in other studies [12]. Here, artificial sweeteners were found to reduce levels of VEGF-A in the area vasculosa of the developing chick embryo. The commercial artificial sweetener used in this study contained a mixture of aspartame and acesulfame k. Several studies have illustrated the relationship between aspartame and angiogenesis *in vitro* and *in vivo* [2-3]. Further, the artificial sweetener sucralose has been shown to reduce VEGF-A-induced vasculogenesis in retinal microvascular endothelial cells [13]. Other work revealed that reduced VEGF levels may lead to neurodegeneration. Thus, dysregulation of VEGF is an important contributing factor to the pathogenesis of several disorders [14]. In the present study, an association between the reduction of VEGF expression and alteration of vascular branching patterns in the area vasculosa of exposed chick embryos was clearly seen. A previous study that evaluated the effect of cadmium on the area vasculosa of the chick embryo found decreased expression of VEGF that was proposed to lead to endothelial cell disruption, contributing to reduction in vascular branching patterning [12]. The reduction of capillary density seen in this study may lead to hypertension, as has been suggested elsewhere [15], and the resultant increase in blood pressure might rupture the blood vessels and cause hemorrhage [16], as was seen in the sweetener-treated groups in this study. Artificial sweeteners also caused a reduction in vitelline vessel branching, while most of the vitelline vessels of the treated 3-day embryos were longer and wider compared to the control embryos. The increase in the length and width of the vessels observed here might be due to decreased branching. Kloosterman, [17] noted that decreased vessel length could be attributed to longer vessels branching into multiple smaller ones. The width of vitelline vessels decreases gradually with each branch, with the diameter of the parent vessel equaling the sum of the diameters of the new branches [18]. A previous study showed that the heparin-binding isoform of VEGF-A plays an essential role in vessel branching. Transgenic mice that solely expressed a VEGF-A isoform which lacks heparin binding capability (VEGF 120/120) had vessels with larger diameters and more endothelial cells, but less vessel branching relative to wild-type littermates. Endothelial cells were integrated within existing vessels, which increased vessel diameter rather than contributing to the formation of new branches [19]. The results of this study suggest that artificial sweeteners may cause a decrease in VEGF-A levels, combined with a decrease in branching and increase in length and width of vitelline vessels. It is well known that acesulfame k inhibits angiogenesis [4], while aspartame promotes angiogenesis, [2] and that both sweeteners may exert other damaging effects.[20] The application of a mixture of these two sweeteners to embryos in this study (0.546mg for aspartame and 0.617mg for acesulfame k) affected vitelline vessel development at two discrete time points during embryonic development, resulting in reduced branching of some vessels and atrophy of others. This study was unique in that it used a common, commercially available product in concentrations routinely consumed by normal individuals on a daily basis, therefore highlighting the potential deleterious consequences for embryonic development of the regular use of these food additives.

6. CONCLUSION

Artificial sweeteners are widely available in commercial markets and are regularly used by many individuals, including pregnant women. Because they are common in so many kinds of food, it is easy to unintentionally consume these sweeteners at concentrations that constitute a daily overdose. The causes of many congenital malformations seen all over the world remain to be established, which merits an investigation of substances that are ubiquitous in global food supplies, such as artificial sweeteners. The results of this study revealed that low and high doses of common commercial artificial sweeteners altered the formation of vitelline vessels during day 3 and 4 of chick development and decreased the expression of VEGF-A, implying that these artificial sweeteners may be capable of altering the molecular microenvironment of endothelial cells. Further research will be needed to dissect the molecular mechanisms responsible for these alterations using the combinations of chemicals present in sweetener mixtures sold in stores and incorporated into prepared foods.

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8. AUTHOR CONTRIBUTION STATEMENT

Fatma Al-Qudsi and Amna Al-Rashdi contributed to the design and implementation of the research, to the analysis of the results, and to the writing of the manuscript.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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