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Research Article

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Commercial Artificial Sweeteners Affect Mice Limb Development: A Morphology and Morphometric Study

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Abstract: Artificial sweeteners are food additives that provide a sweet taste like that of sugar while containing significantly fewer or no calories. The aim of this study was to evaluate the effect of commercial artificial sweeteners on mouse limb development. Adult female mice were divided into three groups: control group, vehicle control group and treated group. The treated group received orally 40 mg/kg body weight artificial sweetener solution, while the vehicle control group received distilled water. The groups were left to mate with normal untreated male mice. On confirmed conception, pregnant females were separated from males. The dose was orally given to the test group before mating, throughout the gestational period till three weeks after giving birth. Foetuses E14.5 and E19.5, were extracted, and newborn and 3 weeks old offspring were examined, and their growth parameters were measured. Congenital malformations were seen in the treated group, such as cerebellar hypertrophy, an increase in the number of forelimbs, atrophy in the lower limb. In some embryos, the amniotic membrane was adherent to the embryo with a very low amount of amniotic fluid. Bone malformation was clearly seen in 1-week offspring, as basic forelimb and hind limb bones were missing. The results of this study showed that there was significant growth retardation in E14.5 and newborns. While in E19.5- and week 3 offspring, there was a significant growth increase (increase in weight, body length tail length and tail length to body length ratio). It was concluded that commercial artificial sweeteners altered growth rate during embryonic development and produced bone and limb congenital malformations in mice.

Keywords: Morphology, teratogenicity, commercial artificial sweeteners, congenital malformation, limb malformation.

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1. INTRODUCTION

Artificial sweeteners are being considered an alternative to ordinary sugar [1,2]. Artificial sweeteners are chemical compounds that are used on a large scale added to powdered soft drinks, food, and beverages [2]. There are many artificial sweeteners, however, five only have been tested and approved by the U.S. Food and Drug Administration (FDA), these are acesulfame potassium (also called acesulfame K), aspartame, saccharin, sucralose and neotame [3]. A study on *Xenopus laevis* showed that after ingestion of 10 µg/ml of sucralose, saccharine, stevia, and aspartame the offspring grew with ri curved and underdeveloped tails in presence of aspartame only, but not the other artificial sweeteners [4]. Long-term consumption of artificial sweeteners, particularly aspartame, during the peri gestational period may produce obesity and metabolic syndrome in the offspring later in life. Metabolic changes that took place due to an increase in intestinal glucose absorption, alterations in intestinal microbiota, induction of oxidative stress, a dysregulation of appetite and reward response [5]. However, in another study it was found that an administration of sodium cyclamate 10000 mg/kg, saccharin sodium, 10000 mg/kg, cyclohexylamine sulfate 150 mg/kg, ethanol 5 ml/kg, in the pro-estrus cycle of female mice had no effect on the offspring [6]. A study showed that maternal fructose intake during fetal and neonatal development caused the impairment of hepatic and immune systems [7]. The limbs raise from both sides of the embryo in the form of buds. The buds consist of a nucleus of mesenchymal cells that is formed from the somatic lateral plate mesoderm. The apical ridges of the epiblast extend along the edge of the developing limb on the border between dorsal and ventral ectoderm. The bud grows and acquires additional cells by the migration of myoblasts from the myotome of the adjacent somites. There are 3 regions at the limbs, the first region is the humerus, femur (stylopod), the middle region is, ulna; tibia, fibula (zeugopod), and the distal region is carpus, metacarpus (autopod). The limbs ossify in order of sequence, except for the wrist bones they are ossified after birth. In the autopod, the metacarpals, ossify before the bones of the fingers. They have 5 digits (fingers), the numbering starts with the thumb. for the short gestation (19–20 days) like in mice, ossification is not apparent until the postnatal period in the thumb [8]. In Saudi communities' artificial sweeteners which are intended to replace sugar with lower-calorie substitutes are considered the fastest moving diet items, available diet foods and drinks are labelled in various phrases such as diet, light, low calorie, no sugar, sugar-free, and zero calorie [9]. The mouse is a good model for use in biological experiments, as it is easy to handle and have a short gestation period. Also, mice are inexpensive with high reproductive performance [10].The aim of this study is to find out the potential teratogenicity of commercial artificial sweeteners (present in the market) which are usually a mixture of several types of artificial sweeteners using the normal permitted dose on embryonic mouse development, therefore, mimicking the situation occurring in human beings, when pregnant females use these sweeteners several times a week.

2. METHODOLOGY

2.1 Animals

All experimental work was approved by the ethics committee of (KFMRC) Animal care and use committee (ACUC) (ACUC-170314). The animals were purchased from the animal house unit at King Fahd Medical Research Centre at King Abdul Aziz University. Forty-five Swiss White Rodeless mice (SWR) (30 females and 15 males) were used, with bodyweight between 25 to 30 grams. They were placed in plastic cages, the temperature was 22± 2 °C. with light and dark cycle 12: 12 hours They were fed corn cob pellets with a water bottle according to normal feeding instruction at KFMRC.

2.2 Chemical material

The commercial artificial sweetener used in this study was purchased from a local supermarket. Its constituents were (Sorbitol 1.98 g, sucralose 9.8 mg, Acesulfame potassium 8 mg) in each 2 g sachet.

2.3 Dose Administration

In this research, one dose was used. The dose was 40 mg/kg body weight of artificial sweeteners. Estimation of the dose was according to the information written on the package from the manufacturer of artificial sweeteners. The dose of 1.17 mg artificial sweetener in 1ml of distilled water was given using gavage needles size (24 G), five days per week to each female mouse in the treated group two weeks before mating till three weeks after birth. The dose was calculated according to the mean weight of the experimental mice.

2.4 Experimental design

Thirty female mice were divided into 3 groups (n=10 each group): control group (C), vehicle control group (V) and treated group (T). Mice were acclimated for one week. After that, the dose was given to female mice for two weeks before mating. In vehicle control group (V), mice were orally given 1ml of distilled water at 40 °C. The treated group (T) was given (1ml of the experimental solution) at a temperature of 40 °C. Water was heated to 40 °C to mimic hot drinks according to the study of [11]. The female mice were given the dose orally, before and after mating.

2.5 Determination of estruses stage and mating

The estruses stage was determined according to the method described in [12]. For mating, male, and female (in estruses stage) were placed in one cage for 24 hours for mating. The next day, the female vagina was examined for the presence of a vaginal plug as a sign of fertilization. This was considered to be day one of pregnancy.

2.6 Sample collection

The samples were collected from all groups on day 14, 19 of gestation and day 7, 21 after birth. Then 1 and 3-week offspring were washed in distilled water and weighed samples were then preserved in formalin 10%.

2.7 Skeleton Staining

Bone and cartilage staining was done on control and treated groups of offspring of 1 and 3 weeks old only. For one week old the numbers were (3 control, and 10 treated) while 10 samples from each group were used for 3 weeks old. The method of staining was done according to [13] with some modifications. The first step, skin was removed, and the

thorax and abdomen cavities were emptied. The sample was left in absolute ethanol for seven days at least.

2.8 Cartilage staining

The samples were completely immersed in a solution of 0.01% Alcian blue which was prepared 20 mg for Alcian blue, ethanol 95% for 120 ml and glacial acetic acid 8 ml for 24 hours. Next day, samples were placed in ethyl alcohol from 95%,95%, 75%, 40%15% for an hour for each concentration for rehydration. Then the samples were immersed and rinsed with distilled water for 2 hours. The samples were then left in 1% potassium hydroxide (100 ml distilled water and 1 g KOH) for 24 hours [13] .

2.9 Bone staining

The samples were wholly immersed in Alizarin red for 3 days. To prepare the alizarin red solution: in a clean jar 100 ml of the 0.5% KOH solution was poured. Then 10 mg of Alizarin red powder was added, and the solution was mixed until it looked uniform. After that, samples were immersed and rinsed in 1% potassium hydroxide for 24 hours. Then samples were transferred to 50% glycerol, (1%) potassium hydroxide, and kept in it for 2 days. For storage, the samples were placed in pure glycerin for long term storage. After staining the bone should be coloured red and the cartilage should be coloured blue.

2.10 Photography

Fresh samples were photographed during and immediately after collection using (iPhone 5s ,8 megapixels) and dissection microscope camera (Olympus DP72)). A ruler was put near the samples during photo taking as scale. Bone photos were taken under a dissecting microscope (Nikon SMZ1500) and a (Nikon DS-Fi1) camera, at the embryonic stem cells research unit at KFMRC.

2.11 Morphological studies

Morphological examination of embryos was done during sample collection and by using the photos taken by iPhone and under the dissecting microscope. The control samples were compared to other studies to confirm their normality [14]. While treated samples were compared to controls and vehicle control groups to detect any congenital malformation.

2.12 Morphometric studies

Samples photos were used to collect morphometric measurements, which were whole body length, tail length, fore and hind limb length. The software image tool program." downloaded from https://cmeias.software.informer.com/download/. (2017) was used. (Figure 1A, B). The number of samples data was taken from the following groups: control group (E14.5)14, (E19.5)20, (week 1)13 and (week 3) 18, vehicle control group (14.5)22, (19.5) 24, (week 1)15 and (week 3)11 and treated group (E14.5) 9, (E19.5)27, (week 1)19 and (week 3)19. Body Mass Index (BMI) for each mouse was computed as follows: BMI=(body weight (g))/(Body length(cm))^2 [15].

3. STATISTICAL ANALYSIS

Data was collected into excel, then transferred to SPSS 25. The tests used with normal distribution were ANOVA and Student-Newman Keuls test. In case of abnormal distribution, Mann-Whitney U test was used from the non-parametric tests. Percentage significance was analysed using chi-square test from the non-parametric tests. *P* values <0.05 were considered statistically significant.

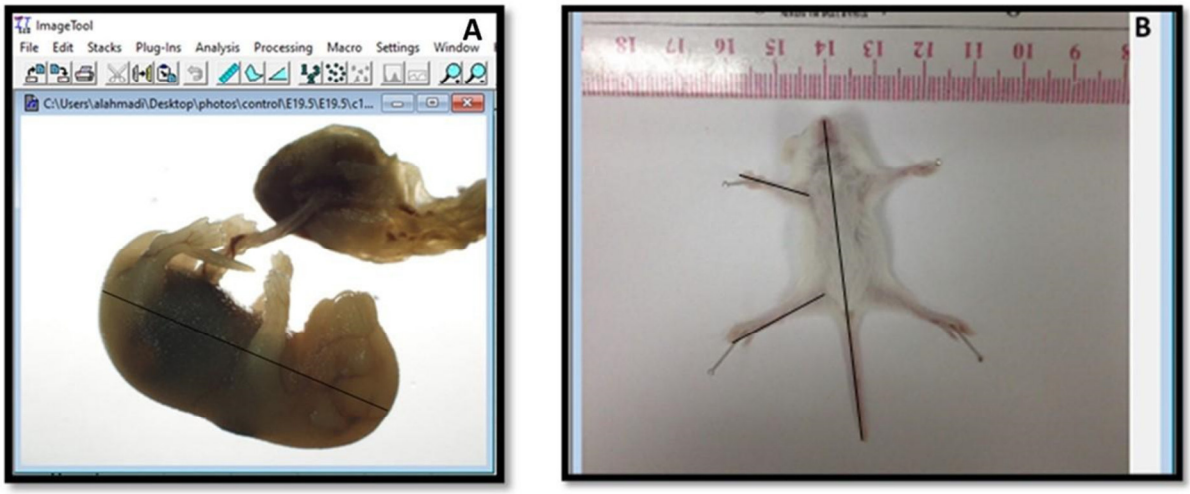
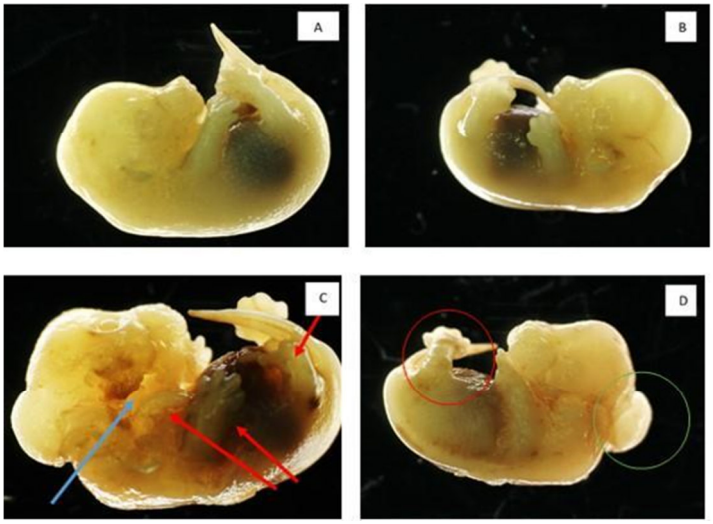


Fig 1: Showing the method of taking the measurements of whole body length, fore and hind limb length and tail length (A) 3 week old offspring (B) E14.5 foetus.

4. RESULTS

4.1 Morphological results Fourteen-day mice foetus (E14.5)

The control mice on day 14.5 of development had separate digits distally developed. The vehicle control group embryo seemed like the control embryo with a slight decrease in body and limbs size. On day 14.5 there were some differences in the treated group compared to the control group. Cerebellar hypertrophy was observed in some embryos and an increase in the size of hind limbs (Figure 2 D). Also, atrophy appeared in the lower limb in one side, while the other lower limb seemed to be longer. One foetus had three forelimbs and incomplete face formation (Figure 2C).

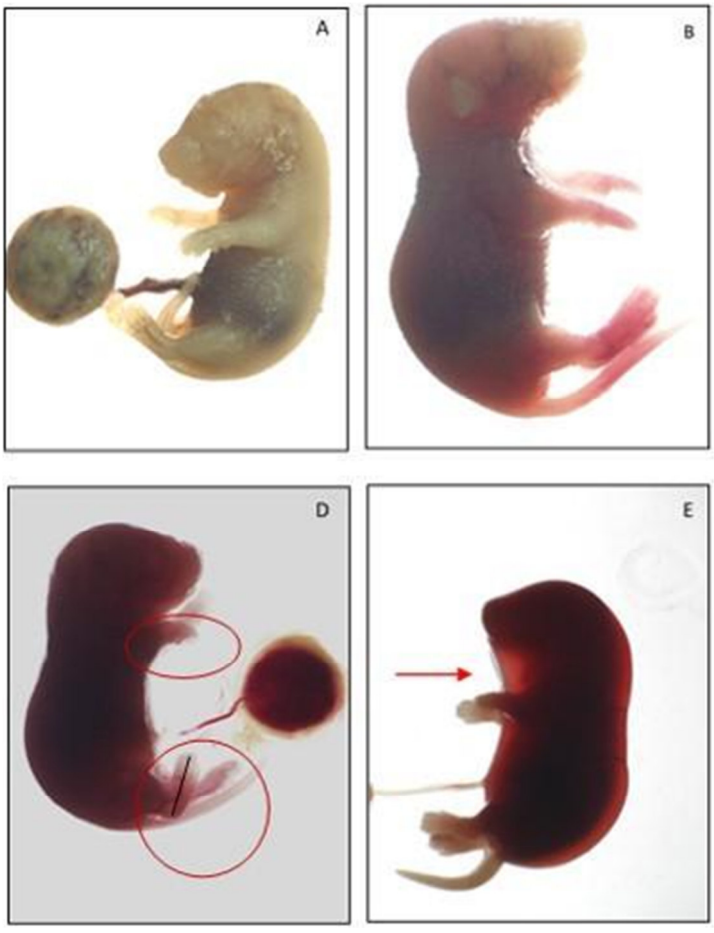


(A) Control, (B) Vehicle control, (C,D) Treated. Note the congenital malformation in the treated group, seen as malformation of fore (blue arrow) and hind limbs (red arrow) in (C), cerebellar hypertrophy in embryo shown in green circle, and hind limb malformation (red circle) in (D).

Fig 2: showing embryos at day 14.5.

4.2 Nineteen-day mice foetus (E19.5)

The control mice on day 19.5 had thick skin that formed wrinkles. The eyes were closed, and barely visible through the closed eyelids. The ears were absent (Figure 3A). (Note: some of the control pregnant mice had delivered on day 19.5). The vehicle control group 19.5 foetuses seemed similar to the control embryos. However, vehicle control foetuses seemed bigger than the control group (note: all vehicle control pregnant mice delivered on day 19.5) (Figure 3B). 19.5-day old mice foetuses of the treated mothers were similar to the control and vehicle control group. However, the fore and hind limbs had some differences in size and shape. In two foetuses the amniotic membrane adhesiveness was increased compared to the control, as it was difficult to separate the foetus from the amniotic membrane, which contained reduced amounts of amniotic fluid compared to the controls. It should be noted that all treated mothers did not give birth on day 19.5. (Figure 3C, and Figure 3D).

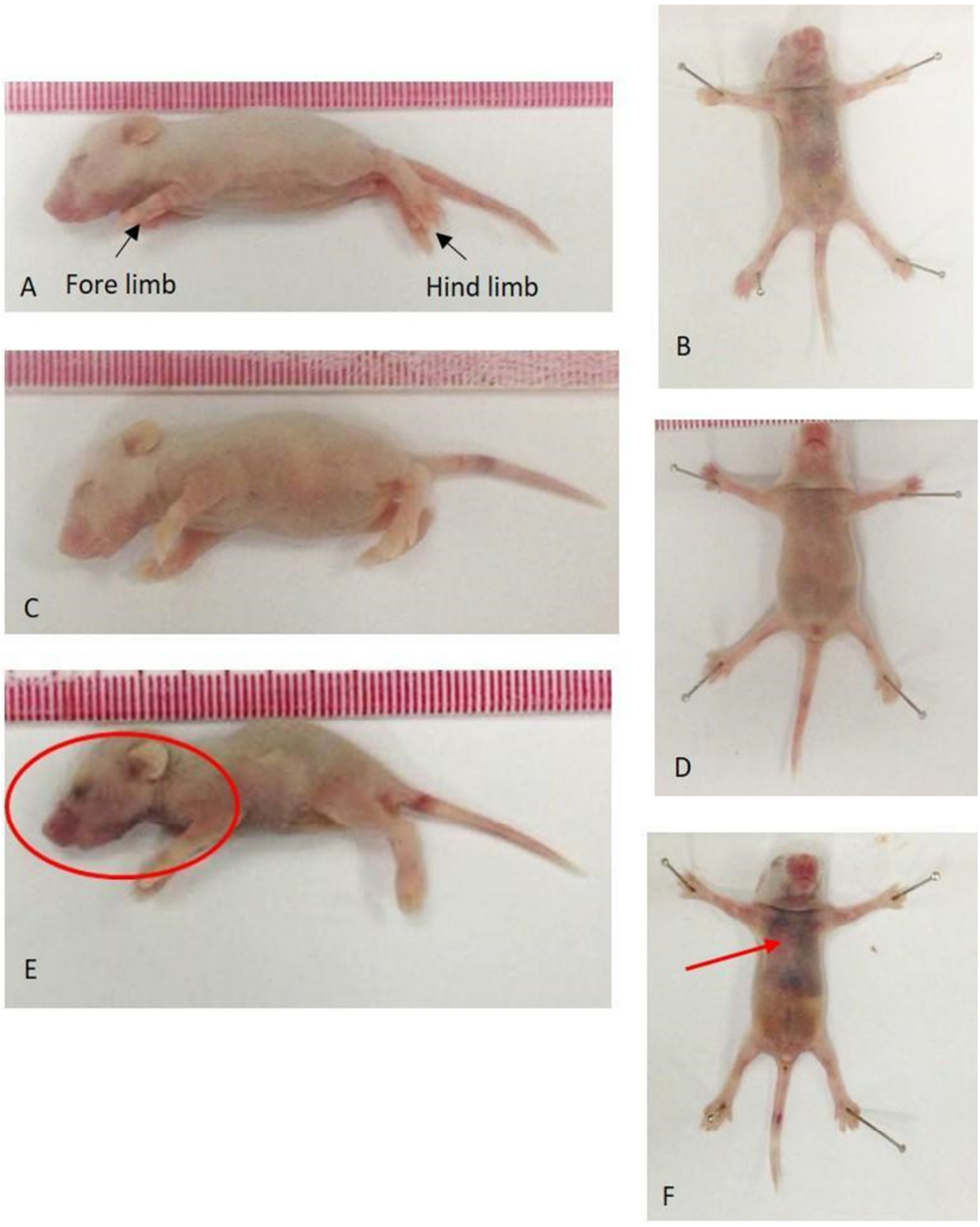


(A, B) Control, (D, E) Treated. the Photo showing the congenital malformation in mice embryos in day 19.5, malformation of fore and hind limbs (red circle), amniotic membrane (red arrowhead). Note that the Control Group was saved in Formalin 10% .

Fig 3: showing embryos at day 19.5.

4.3 Week one mice offspring

The control mice in the age of one week after birth had normal sizes compared to other studies. Hair began to appear, and Ears were open, the eyes were not open yet. the hind and fore limbs had five digits. The vehicle control group had the same external features but had increased weight compared to the control group. One-week old mice of treated mothers were similar to the controls and vehicle control group. However, they seemed thinner than the control and vehicle control group and hematoma was significantly seen in the chest and head area especially around the nose and the eyes (Figure 4).

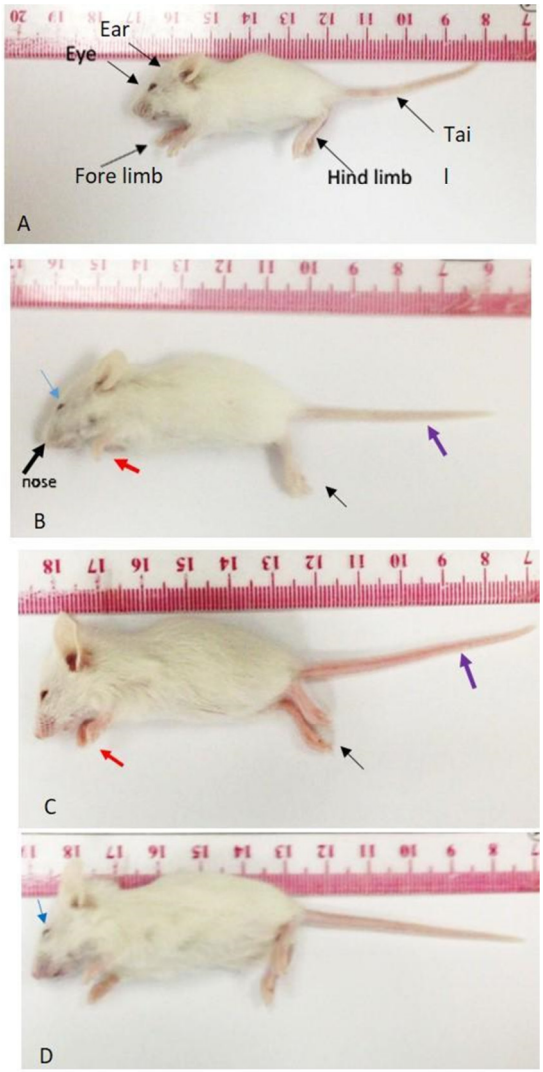


(A, B)Controls, (C, D) Vehicle control, (E, F) Treatment. The red circle and red arrow show hematoma in figure (E, F).

Fig 4: Showing embryos at day 7 neonate.

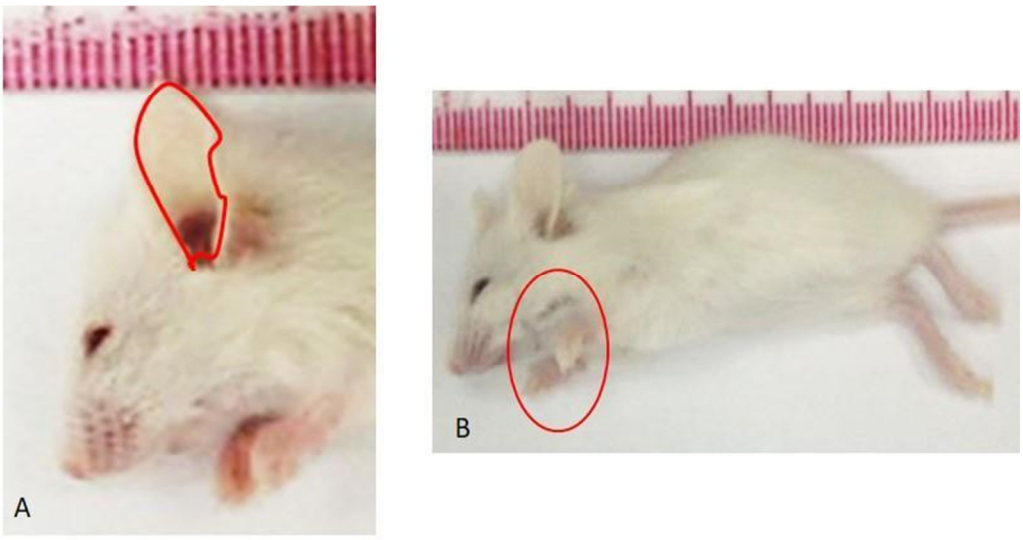
4.4 The three-week mice offspring

The control mice on week 3 after birth had a normal body size and head size. It had short hair, a long naked tail, ears were opened and had a round shape. The eyes were open and were located on each side of the head. Also, it had long mouth and tentacle hair around the ears and nose. The vehicle control embryo seemed similar to the control embryo. The malformations seen in week 3 vehicle control group embryos were in limbs, tail, eyes, and ears. The fore and hind limb seemed to be of unequal length compared to the control. In the head, there were some differences between control and vehicle control group in the eye. The eyes were closed in the vehicle control group (figure 5 D) and the ears seemed to be bigger (figure 6). Vehicle control group seemed bigger compared to the controls. Three-week-old mice offspring of treated mothers were similar to the controls and vehicle control group. However, some congenital malformations in the fore and hind limbs were seen (Figure 6). The fore limbs seemed nearer to the head compared to the controls (figure 6). While the tail seemed longer than the control group. The ears had a difference in size and eyes were closed. One of the treated groups had an abnormal ear shape compared to the control (Figure 6). The frequency of congenital malformations is shown in table1.



(A) Controls, (B) Vehicle control, (C-D) Treated. Note the congenital malformations such as the tail in B,C (purple arrow) , fore limb (red arrow) in B-C, Hind limb (black arrow) in B-C. Also note the closed eyes (blue arrow) in D.

Fig 5: Showing day 21 old offspring.



(A) note the outer ear with a sharp angle while the controls and sham had a smooth oral shape. (B) The forelimbs are near the head.

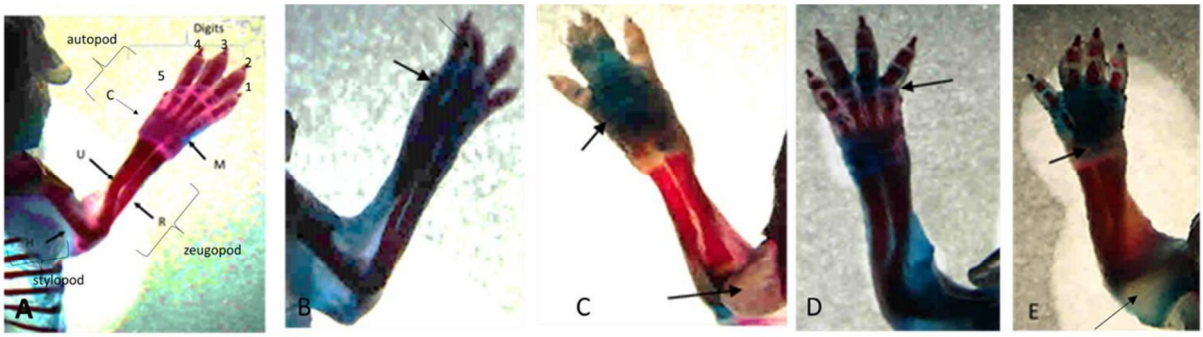
Fig 6: Congenital malformation in week 3 treated offspring

Table 1 : Study of percentage and types of malformation								
Embryonic age		Congenital Malformation						
		H.L	T.L	F.L	S.E	SH.E	hematoma	head malformation
Day14.5	control	-	5%	-	5%	-	-	-
	vehicle control	36%	36%	63%	18%	18%	-	-
	treated	22%	11%	33%	31.5%	21%	-	11%
	control	-	5%	-	5%	-	-	-
Day 19.5	vehicle control	36%	36%	63%	18%	18%	-	-
	treated	-	-	-	-	-	-	-
	control	-	-	-	-	-	-	-
Week 1	vehicle control	-	-	-	-	-	-	-
	treated	-	-	-	-	-	57%	-
	control	-	5%	-	5%	-	-	-
Week 3	vehicle control	36%	36%	63%	18%	18%	-	-
	treated	42%	10%	15.7%	31.5%	21%	-	-

H.L: Hind limb malformation , T.L: Tail length, F.L: fore limb malformation , S.E; eye malformation , SH.E: Shape of Ear. Only the hematoma and head malformation were statistically significant (p=0.007).

4.5 Bone malformations

This study concentrated on the malformations seen in the fore and hind limbs only. On examining the control samples of one week and three-week-old offspring, the following features were seen. The forelimb had: the humerus (H) seen as a long bone, articulated with the radius (R) and ulna (U), metacarpus (Mc), and the bones of the autopod. In the hind limb the femur (F), tibia (T), fibula (Fi), and the patella (Pa) were clearly seen. In one-week, old offspring treated group, some congenital malformations were seen in the forelimb, such as the disappearance of digit 5 in one specimen and the abnormality in digit 3 in the right forelimb of another specimen see figure (7: B). Also the disappearance of metacarpal and humerus was seen in other samples see figure (7: D,E). Feingold syndrome was seen clearly in digits (2,3 and 5) in the left forelimb of a treated sample, see figure (7: C).



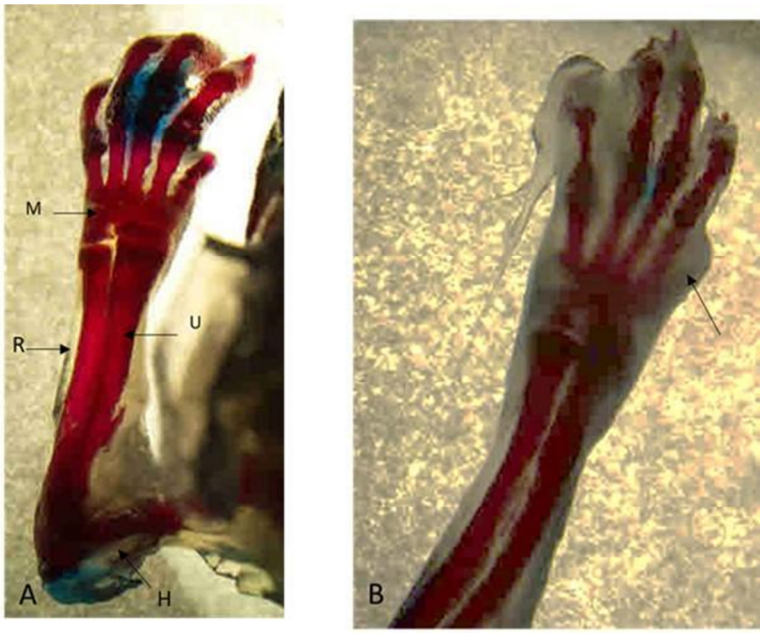
(A)control group, the forelimb consisted of: stylopod - Humerus (H), zeugopod, Ulna (U), Radius (R), autopod (carpals (C), Metacarpal (M)), 5 digits. The treated groups (B, C, D, E). Note in (B) the disappearance of digit 5 and abnormality in digit 3, (C) The disappearance of metacarpal and humerus and appearance of Feingold syndrome in digits (2,3 and 5). (D) Disappearance of some metacarpal bones. (E) Disappearance of humerus, carpals bone, and digit 5 (Mag. 0.75).

Fig 7: The forelimb in one-week old offspring

In three week treated offspring digit number 5 was not present in one of the sample's forelimb in the right forelimb Figure (8: B). In the hindlimb some congenital malformations were seen such as the protrusion in the femur see figure (9: B), disconnection of femur with small size compared to controls, see figure (8: C), disappearance of patella and thinning of fibula and tibia, see figure (9:D). increased space between femur bone and tibia. Missing of coccygeal vertebrae in some mice, while they were very thin in other treated specimens see figure (9: E). The cartilage in the treated group seemed to be more compared to the control group. In some samples it was noticed that the patella remained as cartilage.

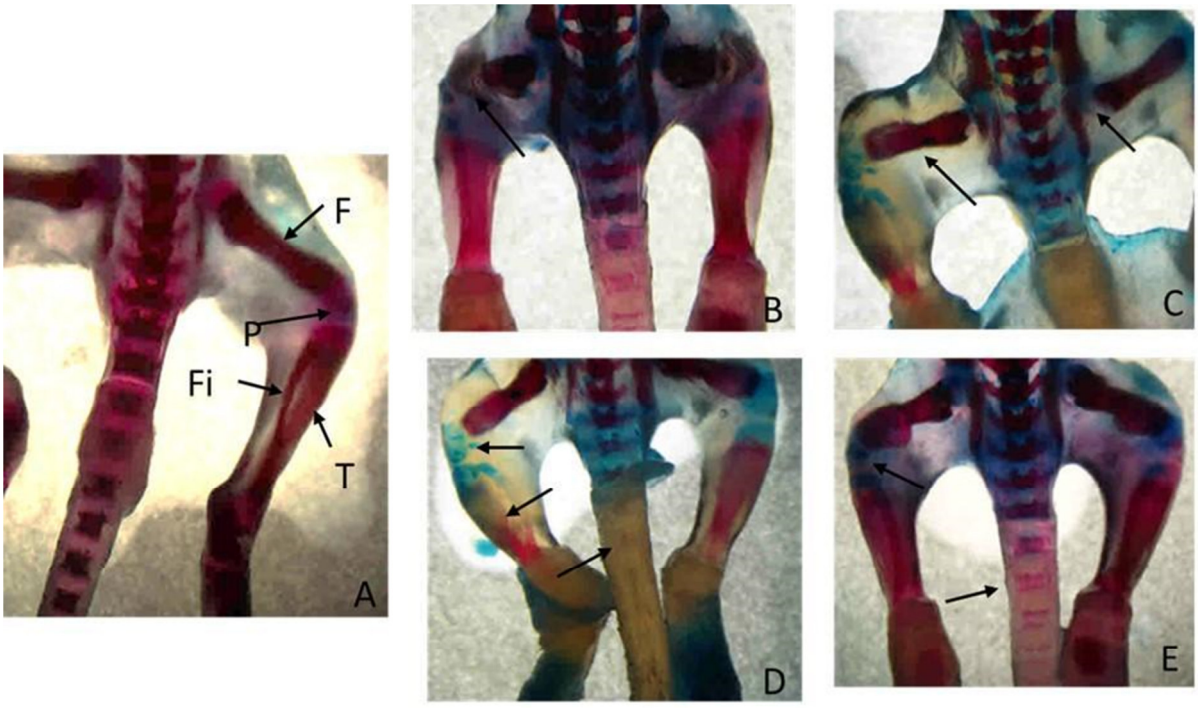
4.6 Morphometric studies

All morphometric results are detailed in table 2, 3, 4 and 5. Growth parameters were decreased in all treated ages except 19.5 E where it increased significantly compared to controls. Limb and tail length were Significantly decreased in treated groups compared to controls.



(A)control group, the forelimb consists of 5 digits: Ulna (U), Radius (R), Metacarpal (M), Humerus (H), the treated group (B)treated sample showing the disappearance of digit 5 (Mag. 0.75).

Fig 8: The forelimb in week 3 old offspring



(A) Control group, The hindlimb consists of the femur (F), tibia (T), fibula (Fi), patella (Pa), the treated group (B, C, D, E). Note in (B) the protrusion in the femur,(black arrow) (C) the femur were small and were not connected with bone ,(black arrows) (D) the patella has disappeared and fibula and tibia were thin. large space between femur bone and tibia. Coccygeal vertebrae disappeared in some mice., (black arrows) (E) Coccygeal vertebrae were light and thin compared to controls, (Mag. 0.75).

Fig 9: The hindlimb in one-week old offspring

Table 2: Effect of Artificial Sweeteners on mouse development in E14.5.

	Groups	N.	Mean	Std. Deviation	P value
	Control group	14	0.0937	0.08484	
BMI	Vehicle control group	22	0.0882	0.14679	0.296
	Treated group	9	0.0830	0.10479	0.829
	Control group	14	2.4086	0.12253	
whole body length in cm	Vehicle control group	22	2.2832	0.26904	0.102
	Treated group	9	2.4878	0.06180	0.159
	Control group	14	0.5964	0.44750	

Foetus weight in gram	Vehicle control group	22	0.1935	0.06855	0.001*
	Treated group	9	0.3678	0.21908	1.000
	Control group	14	0.7557	0.08724	
Hindlimb length in cm	Vehicle control group	22	0.6745	0.16604	0.227
	Treated group	9	0.6422	0.06200	0.003*
	Control group	14	0.7507	0.07549	
Forelimb length in cm	Vehicle control group	22	0.6268	0.18222	0.071
	Treated group	9	0.7322	0.13581	0.439
	Control group	14	1.2271	0.26175	
Tail length in cm	Vehicle control group	22	0.2491	0.22052	0.000*
	Treated group	9	0.5644	0.49890	0.009*
	Control group	14	0.4841	0.07174	
Tail/whole body	Vehicle control group	22	0.1080	0.08674	0.000*
	Treated group	9	0.2263	0.20222	0.005*

*The values are expressed as mean ± SD, N = number of mice. * P<0.05 compared to the control group.*

Table 3: Effect of Artificial Sweeteners on mouse development in E19.5.

	Groups	N.	Mean	Std. Deviation	P value
	Control group	20	0.2066	0.07958	
BMI	Vehicle control group	24	0.1963	0.05973	0.932
	Treated group	27	0.2827	0.06677	0.002*
	Control group	20	2.3315	0.4264	
Whole body length in cm	Vehicle control group	24	2.7061	0.37092	0.009
	Treated group	27	2.4654	0.33661	0.790
	Control group	20	1.166	0.41409	
Foetus weight in gram	Vehicle control group	24	1.4496	0.08093	0.154
	Treated group	27	1.5369	0.21912	0.008*
	Control group	20	0.7855	0.0979	
Hindlimb length in cm	Vehicle control group	24	0.8074	0.13329	0.679
	Treated group	27	0.6927	0.11155	0.019
	Control group	20	0.7635	0.06442	
Forelimb length in cm	Vehicle control group	24	0.8065	0.11056	0.056
	Treated group	27	0.5992	0.1256	0.000*
	Control group	20	1.2885	0.1324	
Tail length in cm	Vehicle control group	24	1.3265	0.22811	0.761
	Treated group	27	2.0569	0.76321	0.000*
	Control group	20	0.5388	0.07174	
Tail/whole body	Vehicle control group	24	0.4928	0.09291	0.060
	Treated group	27	1.0633	0.39047	0.000*

*The values are expressed as mean ± SD, N = number of mice. * P<0.05 compared to the control group.*

Table 4: Effect of Artificial Sweeteners on the mouse development in week1 offspring.

	Groups	N	Mean	Std. Deviation	P value
	Control group	13	0.1343	0.01590	
BMI	Vehicle control group	15	0.1356	0.04175	0.981
	Treated group	19	0.1174	0.02114	0.007*
	Control group	13	5.4592	0.33240	
Whole body length in cm	Vehicle control group	15	5.9050	0.55098	0.003*
	Treated group	19	5.6332	0.41091	0.362
	Control group	13	3.9831	0.32247	
Offspring weight in gram	Vehicle control group	15	4.5621	0.86926	0.280
	Treated group	19	3.7347	0.34521	0.041
	Control group	13	1.5454	0.11508	
Hindlimb length in cm	Vehicle control group	15	1.6379	0.15744	0.094
	Treated group	19	1.6274	0.23096	0.099
	Control group	13	1.2415	0.08009	
Forelimb length in cm	Vehicle control group	15	1.3157	0.14324	0.155

	Treated group	19	1.3113	0.10363	0.059
	Control group	13	3.9831	0.32247	
Tail length in cm	Vehicle control group	15	4.5621	0.86926	0.068
	Treated group	19	3.7347	0.34521	0.570
	Control group	13	2.0023	0.19387	
Tail/whole body length	Vehicle control group	15	2.2250	0.25410	0.185
	Treated group	19	2.0632	0.21891	0.970

The values are expressed as mean ± SD, N = number of mice. * P<0.05 compared to the control group.

Table 5: Effect of Artificial Sweeteners on the mouse development in week3 offspring

	Groups	N.	Mean	Std. Deviation	P value
	Control group	18	0.1250	0.03148	
BMI	Vehicle control group	11	0.1157	0.04771	0.276
	Treated group	19	0.1031	0.01343	0.019
	Control group	18	7.1483	0.84246	
Whole body length in cm	Vehicle control group	11	9.0000	1.08224	0.000*
	Treated group	19	9.6168	0.43771	0.000*
	Control group	18	6.1661	0.78293	
Offspring weight in gram	Vehicle control group	11	9.2100	2.72285	0.003*
	Treated group	19	9.4753	0.83066	0.000*
	Control group	18	1.8483	0.21330	
Hindlimb length in cm	Vehicle control group	11	2.3236	0.18864	0.000*
	Treated group	19	2.4753	0.20419	0.000*
	Control group	18	1.4700	0.12843	
Forelimb length in cm	Vehicle control group	11	1.9900	0.33009	0.000*
	Treated group	19	1.9889	0.21794	0.000*
	Control group	18	3.1028	0.42671	
Tail length in cm	Vehicle control group	11	3.9900	0.43790	0.000*
	Treated group	19	4.2337	0.46090	0.000*
	Control group	18	0.4336	0.02495	
Tail/whole body length	Vehicle control group	11	0.4441	0.01430	0.276
	Treated group	19	0.4403	0.04385	0.126

The values are expressed as mean ± SD, N = number of mice. * P<0.05 compared to the control group.

5 DISCUSSION

Nowadays, artificial sweeteners became more popular and are used in soft drinks and food to help reduce weight [16]. This study showed that artificial sweeteners caused a fluctuation in foetal weight, whole-body length, and tail length from day 14.5 of pregnancy until week 1 after birth and an increase in neonate weight in week 3 after birth. Sugar intake during pregnancy may affect neonatal metabolism and might cause obesity risk [17]. Using aspartame during pregnancy might reduce foetal weight [18, 19]. Studies showed that daily consumption of artificially sweetened beverages during pregnancy may increase the risk of an infant being overweight at 1 year of age [20]. The results of this study showed that 3-week offspring had increased growth compared to the controls. Studies showed that there is a negative impact of consuming artificial sweeteners beverages on weight gain, metabolic syndrome, and cardiovascular disease[21,22]. The consumption of acesulfame- k may increase body weight and induce different gut bacterial composition changes[22]. One of the main factors that contribute to the development of the nervous system function is gut bacteria[23]. Higher doses of sorbitol may lead to substantial weight loss [24]. Also, studies in rat showed that consumption of sugar alcohol during gestation caused a significant increase in crown-rump length and somite numbers [25]. The sorbitol and acesulfame- k present in the artificial sweeteners used in this study might have affected the mother microbiome and affected foetus metabolism, and consequently affecting body weight and length of the foetuses. The result of this study for the tail length to body length ratio was decreased in E14.5 and increased in week 1, 3 offspring in the treated group compared to the control group. The tail plays the important role in balance during movement. Therefore, decreased in tail length led to an effect on spontaneous movement patterns [26, 27]. In this study there were some foetuses who had cerebellar hypertrophy in the treated group in E19.5. Increasing the percentage of sorbitol causes defects in eye tissues, causes cataract formation and increases activity in the brain (cerebellum) [28]. Long-term uses of artificial sweeteners may affect cognition in adult rats [29]. A study found that consumption of the acesulfame K could affect cognitive, metabolic and brain functions [30]. The increase of aspartame concentrations resulted in observable deformities in embryo such as growth retardation, lack of pigmentation and tail deformities [31]. This study found closed eyes mice in week 3 in the vehicle control group and treated group. The high levels of sorbitol in the eye lens in rats led to cataract eye [32]. The percentage of sorbitol present in the artificial sweeteners used for the experiment might have led to the cataracts of

mice, which made the eyes close. A study showed that consumption of artificial sweeteners affects eye development density and decreased body length [33]. This study found that there was atrophy in the lower limb in the foetus. Study found that dietary sorbitol supplementation retards bone resorption in rats [34]. The presence of sorbitol in the experimental artificial sweeteners in this study might have affected limb formation. In this study, the artificial sweeteners caused a decrease in limb length in the vehicle control group and treated group. In the treated group in week 3, the forelimbs were near to the head and there was an increase in the size of limbs. The skeleton photos endorsed these results by showing a series of bone malformations in the fore and hind limbs of the treated offspring with reduced ossification in some places. This was seen in other studies that showed that forelimb had decreased in size compared to body size [35, 36]. Artificial sweeteners have a genotoxic effect [37]Sorbitol consumption during and after pregnancy, caused a decrease in offspring length, and increase in sorbitol levels in milk and caused genotoxic effects in offspring bone marrow [38]. It was seen that there are some similarities in the results between the treated group and the vehicle control group, showing that there is a possibility that the artificial sweeteners given to mothers were not the cause of all congenital malformations seen, but that there are other factors that could have an impact such as distilled water and stress. Therefore, more studies should be done to investigate these effects.

6 CONCLUSION

The uniqueness of this study is that the artificial sweeteners used in this study were bought from the market, available for normal people including pregnant women and the dose used in this study is a normal dose and yet it caused significant congenital malformations in mice foetus and offspring, such as hematoma and decreased growth. Also, the significant decrease in fore and hind limb length and the malformation in fore limbs, where several digits were not present while several bones were malformed or had no ossification in the hind limb. Therefore, it is clear from these results that the used commercial artificial sweetener significantly affected limb development in foetuses and offspring of treated mothers. Also, the female mice were given the dose before mating which in our knowledge have never been studied before. Most of the studies done on artificial sweeteners only included aspartame or several types of artificial sweeteners mixed with aspartame. While this study used a commercial artificial sweetener, which did not contain aspartame and consisted of a mixture of several types of sweeteners sold in one sachet to the customer. More molecular investigations should be done to understand the mechanism used by these sweeteners to affect limb development.

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8 AUTHOR CONTRIBUTION STATEMENT

Fatma Al-Qudsi, Abrar Al-Ahmadi and Magdah Ganash 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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