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# The detrimental effects of sweet stevia on the growth of swiss white mouse embryos

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**Abstract**

The current study's objective is to determine the effects of stevia sweetener on the development of albino mice embryos on D12, D15, and D18. One of the sweeteners that has become very popular in recent years as an alternative to conventional sugar is stevia. 48 pregnant female mice were used, divided into four groups. The first group was the control group, which received distilled water, while the other three groups received a stevia-aqueous solution from the first to the last day of pregnancy. The embryos were extracted from the uterine horns on special days, and the kidneys were then taken from these embryos. The kidneys were then examined for morphological defects and histological abnormalities. In addition to studying the biochemical indicators (urea and creatinine) in pregnant mothers' serum, embryonic kidney tissue and MDA lipid peroxidation were investigated. The study found a variety of morphological abnormalities in mouse embryos on all study days compared to the control group, including contortion of limbs, meningomyelocele, and encephalomenigocele. In terms of histological alterations, the study discovered pathological changes in the kidney tissue, such as necrosis, degeneration, congestion, and hemorrhage, and the study found significant changes at a significant level about lipid peroxidation MDA in the fetal kidney tissue and a substantial increase in the ranks of urea and creatinine in the serum of pregnant mothers. The study revealed that Stevia is unsafe for pregnant women and requires further studies.

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## Introduction

In recent years, there has been a significant rise in interest in low-calorie sweeteners that can replace sugar (1). However, it is still uncertain how these sweeteners affect pregnancy outcomes and fetal health (2). Artificial sweeteners such as aspartame, acesulfame K, saccharinate, and cyclamate; semi-industrial sweeteners include lactitol, maltitol, and xylitol as well as natural sweeteners such as thaumatin, and steviol glycosides are among the several types of sweeteners (3). Individuals use these sweeteners to limit their intake of common sugars. The most popular of these sweeteners are stevia and xylitol, and the effort to find sugar replacements continues (4). Stevia is extracted from the leaves of the *Stevia rebaudiana* Bertoni plant (5) In many regions of the world, it is widely farmed and mainly used as a sweetener (6). Stevia leaves contain a complex mixture of sweet diterpene glycosides such as stevioside and *rebaudioside A.* (7). *S. rebaudiana* and *S. grosvenorii* are the most common species. The most important are steviol glycosides (*S. rebaudiana*); the European Food Safety Authority approved its usage in food in 2011 (8). Stevia leaves contain around 65% stevioside, about 25% *rebaudioside A*, and minor amounts of rebaudioside B, C, D, E, F, glucoside A, and steviolbioside (9) The sweetest of these with a good flavor and the least bitter or sour is rebaudioside A (10) While stevioside is 200-450 times sweeter than sugar and is responsible for the bitter taste and scent when combined in equal parts with *rebaudioside A*, the bitter flavor alters and becomes less noticeable (11). This is

why the most frequent steviol glycosides in the food sector are stevioside and rebaudioside A. Their appeal stems not just from their sweetness but also from their lack of bitterness, as opposed to Rebaudioside M., which has substantial bitterness (12). When stevia is consumed, it is broken down in the intestines to a diterpenoid aglycone known as steviol and absorbed into the circulation. The liver then transports the steviol to the kidneys for excretion (13). Stevia is classified as a food additive in the European Union under the number E960. The name Bertoni is derived from the scientist Moises Santiago Bertoni, who was the first to formally characterize these plants in Paraguay in 1899 and worked on classifying and defining them in terms of shape and characteristics (14). Stevia became available for purchase in Japan for the first time in 1970 (1,15). Numerous products on the market include stevia, and the European Commission approved a regulation in 2011 authorizing the use of steviol glycosides in 31 different products, including drinks, juices, and sweets (16). Commercial sweeteners are frequently made with natural and artificial sweeteners; however, certain people, notably pregnant and lactating, should avoid these artificial sweeteners (17).

The purpose of this study was to determine if the commercial product Stevia could have negative impacts on the fetuses of pregnant albino mice given an aqueous solution of stevia in terms of weight gain, morphological and histopathological abnormalities, urea, creatinine in pregnant serum, and antioxidant MDA concentrations in fetal kidney tissues.

## Materials and methods

### Ethical approval

The Scientific Committee for the Department of Biology, College of Education for Pure Sciences, University of Mosul approved this research on 22/6/2022 that it did not violate animal laws. Euthanasia was performed on experimental animals by its guidelines.

### Chemical

Stevia sweetener from the Magnesia company was used. It was in the shape of a sweet-tasting powder mixed in distilled water to produce an aqueous solution.

### Study animals

In this investigation, 48 pregnant female mice were mated with 24 male mice. The pregnant mice were housed in proper cages at the animal house of the College of Veterinary Medicine, University of Mosul under consistent environmental conditions regarding temperature and lighting, and they were provided water and food.

### Dosage

The dosing concentrations were established based on the median fatal dosage LD50 orally of 15 g/kg body weight (18,19), which was 13 g/kg body weight; pregnant mice were given 0.7 ml of an aqueous solution of stevia via oral dosing by gavage needle from the first day of pregnancy, at a rate of 8.4 ml to D12 of pregnancy, and 10.5 and 12.6 ml to D15 and D18, respectively.

### Experimental design

Four experimental groups were formed up of 14 pregnant mice. The first group, the control group, received distilled water and was sacrificed on D12, D15, and D18 of pregnancy. In contrast, the second, third, and fourth group received an aqueous stevia solution and was sacrificed on D12, D15, and D18 of pregnancy.

### Morphological and histopathological analysis

Once pregnant females were dissected, fetuses were removed by separating the fetal membranes (20). They were placed in a dish containing physiological buffer (0.9 NaCl), then embryos and kidneys of embryos were obtained and fixed in 10% formalin neutral buffer, then the kidneys were treated with paraffin, and 5-µm-thick sections were stained (21) with hematoxylin and eosin (22,23) The number of living and dead fetuses was recorded, and each fetus was weighed. The fetuses were then closely scrutinized to note any morphological anomalies.

### Sample collection

Serum was obtained by centrifugation of a maternal blood sample from the abdominal aorta at 3500 rpm at 4 °C for 10 min; the supernatant was collected and stored at -80°C until it was used (24); serum urea levels were estimated based on (25) using the urease enzyme and determination of serum creatinine level without protein precipitation (26).

### Lipid peroxidation in fetal kidney tissues

Lipid peroxide levels were evaluated by adding 3 ml of 1.15% KCl solution to the 1 gm kidney cell emulsion and used for MDA calibration (27) kidney homogenates were produced and centrifuged in phosphate buffer, the supernatant was then diluted with 2 ml of TBA indicator solution which included 15% (w/v) TCA, 0.375% (w/v) TBA, and 0.25N HCL in sealed tubes following shaking, closed tubes were immersed in boiling water for 15 minutes before cooling at room temperature for 10 minutes before centrifugation at 10000g, at 535nm, supernatant absorption was observed (28).

### Statistical analysis

The current study experiments were statistically analyzed using one-way analysis of variance (ANOVA) at a significant level of ($P<0.05$); the least significant differences were used to identify differences (LSD) (29).

## Results

### Live and dead fetal

The current study was designed to assess the adverse effects of an aqueous stevia solution on the fetuses of pregnant albino mice. The study included four experimental groups, each with 14 pregnant mice. The control group was the first, and on D12, D15, and D18 it was given distilled water and the number of embryos for these (95,100,112) and the second group was given an aqueous solution of stevia and explained on the D12, D15, and D18 of pregnancy was found that there were several dead fetuses which were on days 12, 15, and 18 (61, 37, 16) respectively, while the number of live fetuses was (79, 63, 51) respectively, compared to the control group (Table 1).

Table 1: Shows fetuses details included in current study

| Group | Fetus | | |
|---|---|---|---|
| | Whole (n) | Live (%) | Dead (%) |
| Control | 112 | 100 | - |
| D13 | 79 | 70.5 | 16.9 |
| D15 | 63 | 56.2 | 37 |
| D18 | 51 | 45.5 | 54.5 |

### Fetal weight

The study found changes in fetal weight gain as treatment with an aqueous stevia solution resulted in a significant decrease in fetus weights compared to the control group in D12 and D18. There was a significant increase over the control group at D15 (Figure 1).

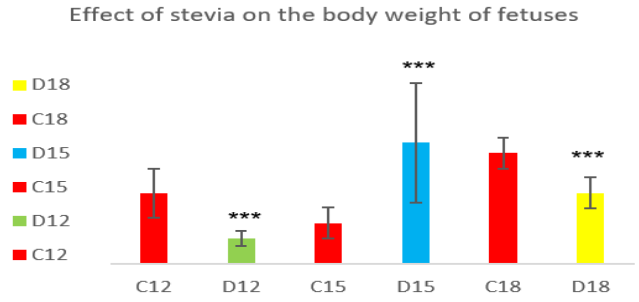


Figure 1: It shows the changes in the weight of the fetuses in (D12, D15, and D18), where a significant decrease is noted in D12 D18, while there was a significant increase in D15. Data represented as Mean± STD, n = 25, *** P<0.001.

### Morphological anomalies

According to the percentages shown in table 2, the current study finds several congenital malformations in the fetuses under investigation, including system and neural tube malformations such as head malformations, Encephalomenigocele and Meningomylocele and skeletal system malformations such as skeletal deformities and contortion limbs (Figure 2).

Table 2: Shows the percentages of congenital malformations in fetuses

| Deformity | D12 | D15 | D18 |
|---|---|---|---|
| Contortion of limbs | 25 | 34.1 | 46.9 |
| Hydrocephalus | 0 | 29.5 | 40.6 |
| Deformity of skeleton | 18.3 | 25 | 34.4 |
| Sept optical dysplasia | 16.7 | 22.7 | 31.3 |
| Corpus callosum agenesis | 16 | 17.5 | 28 |
| Absence of eyes | 0 | 20.1 | 28.1 |
| Hemimegalencephaly | 14.1 | 19.3 | 26.6 |
| Meningomylocele | 12.5 | 17 | 23.4 |
| Megalencephaly | 11.7 | 15.9 | 21.9 |
| Microcephaly | 11.7 | 0 | 21.9 |
| Arrhinencephalia | 8.3 | 11.4 | 15.6 |
| Wrinkles | 0 | 10.2 | 14 |
| Encephalomenigocele | 6.7 | 9.1 | 12.5 |
| Cleft lips | 0 | 0 | 9.4 |
| Spine bifida | 0 | 5.7 | 0 |
| Tail kink | 0 | 4.5 | 6.3 |
| Encephalophyma | 0 | 0 | 2 |

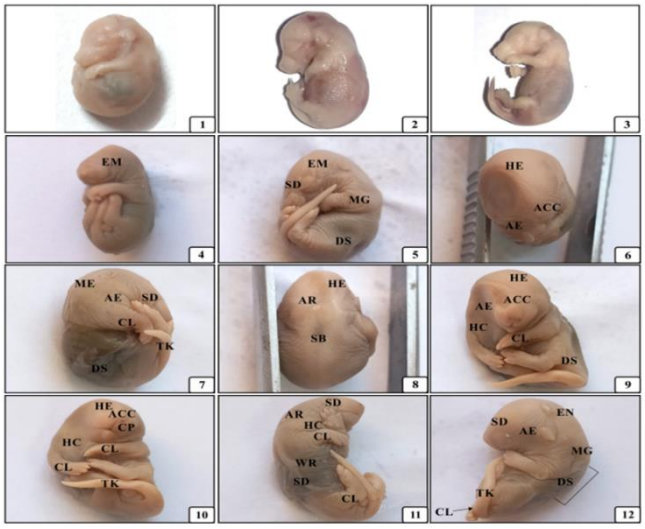


Figure 2: Picture 1, 2, and 3 showing control embryos in D12, D15, D18. Picture 4 Fetus of mice in D12 whose mothers were dosed with stevia aqueous solution showing Encephalomenigocele (EM), P. (5) Fetus in D12 explain Sept optical Dysplasia (SD), Megalencephaly (MG), (EM), P.(6) Fetus in D12 show Hemimegalencephaly (HE), Agenesis of Corpus Callosum (ACC), Absence of Eyes (AE), Picture (7) Fetus in D15, Contortion of Limbs (CL), Tail kink (TK), (AE), (SD) P.(8) Fetus in D15 show Spine bifida (SB), Arrhinencephalia (AR), (HE), Picture (9) of fetuses in D18 explain Hydrocephalus (HC), (ACC), (AE),(CL),(DS), P.(10) fetus in D18 show Cleft Lips (CP), (HE),(ACC),(TK),(HC), P. (11) fetus in D18 showing Wrinkles (WR), (HC),(CL),(AR),(SD), P.(12) fetus in D18 explain Encephalophyma (EN),(SD), (DS),(MG).

**Urea and creatinine test**

The stevia dosage significantly increased urea levels on days D12, D15, and D18 compared to the control group. However, the study found no significant variations in urea levels between days D15, and D18, creatinine concentrations on all study days the study found significant differences between the experimental and control groups but not between the two groups D12 and D15 (Figures 3 and 4).

**Malondialdehyde (MDA)**

The statistical analysis results revealed a significant increase in the levels of MDA in the fetus's kidney tissue between the experimental and control groups. At the same time, there were significant differences between the experimental groups at a significant level (Figure 5).

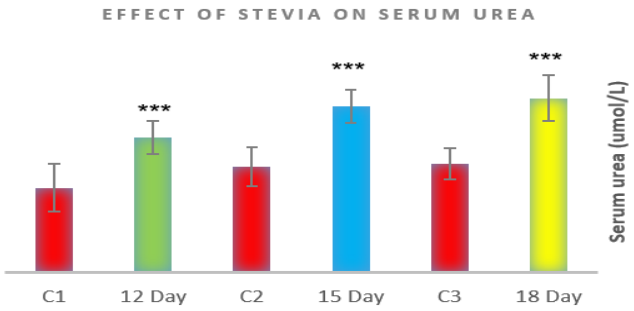


Figure 3: showing the level of urea in serum, all study days have increased significantly at a significant level P<0.001 Data represented as Mean ± STD, n = 25, *P<0.05, *** P<0.001.

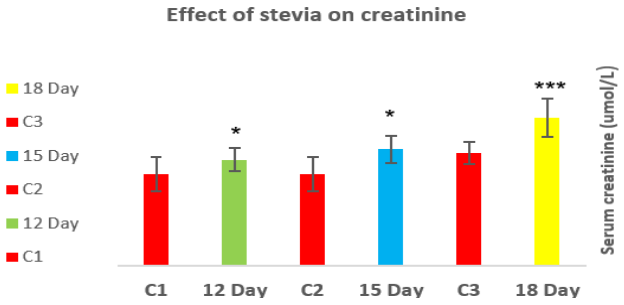


Figure 4: showing creatinine level in serum, there is a significant increase in D12 D15 at a significant level of P<0.05, while the rise was at a significant level of P<0.001. Data represented as Mean± STD, n = 25, *** P<0.001

**Kidney histopathology**

The findings of our study also revealed that the kidney tissue in mice had many histopathological abnormalities compared to the control group. The severity of the abnormalities was high even on the D12, as evidenced by deformities of the glomeruli and tubules, as well as the occurrence of degeneration, necrosis, congestion, and hemorrhage (Figure 6).

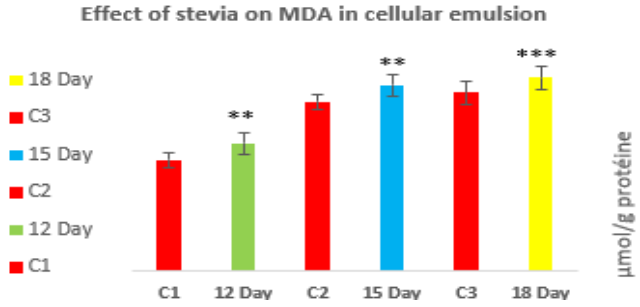


Figure 5: showing the level of MDA in renal tissue it indicates the significant increase in MDA levels that occurred at the level of P<0.01 in D12, D15, and at P<0.001 in D18. Data represented as Mean ± STD, n=42, ** P<0.01 *** P<0.001.

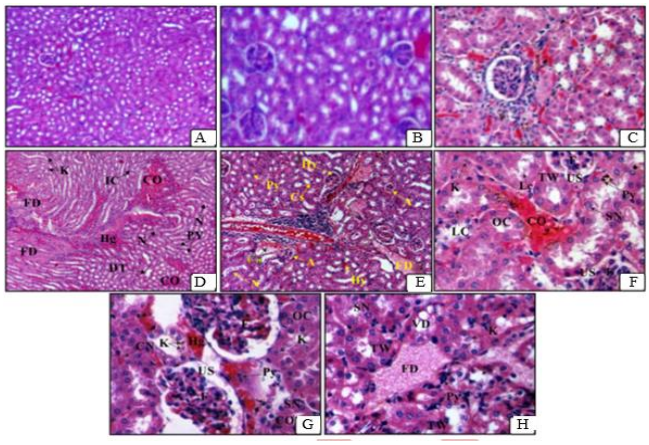


Figure 6 : Picture A, B, and C A cross-sectional in kidney of control fetuses CD12, CD15, CD18 A cross-sectional in kidney of control fetuses, showing the Glomeruli (G) and Urinary tubules (UT), Picture (D): A cross-sectional section of the kidneys of mice fetuses in D12 The kidney of mice embryos whose mothers were dosed with Stevia aqueous solution showing Congestion (CO ), Karyolysis (K), Pyknosis (PY ), Inflammatory Cell Infiltration (IC), Fibrin Deposition (FD), Picture (E): A cross-sectional section of the kidneys of mice fetuses in D15 explain, Hypertrophy in glomeruli (PY), Atrophy of some glomeruli (A), Hemorrhage (HG), Necrosis (N),Cell Swelling in epithelial layer (CS), (FD), (IC). Picture (F) Cross section in kidney of fetuses D15 showing Breath of urinary space (US), Laceration Cytoplasm (LC), Occlumency in urinary tubules, Picture (G) A cross-sectional section of the kidneys of mice fetuses in D18 The kidney of mice embryos whose mothers were dosed with Stevia aqueous solution explain Fragmentation of the tufts some of glomeruli (F), Coagulative Necrosis (CN), Swelling Nucleus (SN) (OC), (HG), (K), (PY). Picture (H) Cross section in the kidney of fetuses D18, expansion of urinary tubules (arrow), Thickening of the vessel wall (TW), Vacuolated Degeneration (VD) (FD), (SN), (K), (PY).

**Discussion**

The current study investigated the harmful effects of stevia sweeteners on embryos of pregnant albino mice. *Mus musculus*, the research showed numerous dead fetuses on days 12, 15, and 18 was 61, 37, and 16 fetuses, respectively, whereas the number of living fetuses was 79, 63 and 51. when compared to the control group, the live fetuses were distinguished from the dead embryos when dissected. The living embryos were red or pink, while the dead fetuses were green or black; the reason may be an increase in the incidence of malformations of the nervous system, leading to fetuses' death inside the uterus (30). The results also found embryo resorption in the late stages; the number of embryos on D13 was subtracted from the number of embryos on D18 to calculate embryo resorption (31). Concerning the weight of the fetuses, the study found significant reductions in fetal weights in D12 and D18 compared to the control group, while there was a significant increase at D15.

There are conflicting studies regarding the effect of stevia on weight. Low fetal weight may indicate less food intake by mothers during dosing periods; this, in turn, led to a decrease in her weight on days D12 and D18, whereas a study by Jiang (32) indicated that the weight decreased on the D18 of pregnancy due to *rebaudioside A* in rats. A study AboElnga (33) conducted on rats' body weight suggests that it may have decreased due to a lack of fast glucose-releasing sources or a reduced calorie intake. While the results of this research showed that weight increase at D15 is in agreement with the results of the study by Gómez (34), which suggested that mice given sweeteners increased in embryo weight, The present study identifies several congenital abnormalities in the fetuses under investigation, including system and neural tube malformations, as well as skeletal system malformations, it is safe to consume artificial sweeteners in moderation during pregnancy. Yet, other research suggests mothers' sweetener use harms pregnancy (35).

Also, during periods of fast differentiation, the fetus' organs are more susceptible to external influences, which results in abnormal growth (36). When it comes to the nervous system, problems often arise as a result of the neural tube's inability to close on its own (37). Anencephaly, encephalocele, Meningomyelocele, and spine bifida are defects of the nervous system but covered by the skin (38). The meninges and brain matter are contained within the encephalomenigocele, which looks like a solid, saccular mass that herniates through the skull defects (39), The meningomyelocele is a protrusion of the meninges caused by a malformation in the skull or spine, an absence of brain tissue separates it from the encephalomenigocele (37). It is caused by incomplete fusion of the neural tube (40). The study also discovered that administering stevia in an aqueous solution caused the Agenesis of Corpus Callosum (ACC) to deform; it is conceivable that stevia had an impact on the receptors of the DCC Netrin 1 gene, which is important for the development of this body part (41), the investigation also finds the presence of sept optical dysplasia, which has been linked to mutations in the SOX2, SOX3, or OXT2, and HESX1 genes (42). Megalencephaly was discovered as a result of aqueous stevia consumption, which is a deformity caused by mutations in genes that govern critical biological processes, such as phosphatidylinositol 3-kinase (43,44). This study also discovered a hemiplegalencephaly deformity. According to research, this deformity is caused by gene mutations controlling the main pathways phosphatidylinositol 3-kinase (PI3K / AKT) -mTOR (45).

The current study referred to the defects of the skeletal system, which is an important indicator of embryonic development, and changes in its growth reflect changes in the environment of the mother and fetus (46). The influence of bone development on the dosage of the aqueous stevia solution might explain the incidence of skeletal system abnormalities. Studies have shown environmental factors to hurt fetal skeletal growth (47). Due to the fetus's insufficient protection against free radicals throughout the phases of embryonic development, the stevia dosage may have produced oxidative stress, which has a detrimental influence on the ossification process (48). reduced oxidative stress in the uterus and placenta, which explains why birth abnormalities emerge during embryonic development (49,50), where the blastocyst is especially sensitive to damages (51), according to one study, Burton (52) a lack of oxygen in placental tissues increases exposure such as stressing the endoplasmic reticulum and stimulating the formation of inappropriate proteins in the cell, which causes stimulation of the response of other proteins to try to restore the endoplasmic reticulum's balance. To avoid tissue damage that leads to embryo death or the incapacity of uterine tissues to accept a fertilized egg in the first place (53), studies have found that the abnormal development of the tail in mice may be due to the abnormal position of the fetus inside the uterine (48).

Although the study found no significant variations in urea levels between days D15 and D18, the stevia dosage caused a significant increase in urea levels on days D12, D15, and D18 compared to the control group, while the creatinine concentrations on all study days showed significant differences between the experimental and control groups but not between the two groups D12 and D15, so the administration of a stevia aqueous solution increased urea and creatinine levels. These findings are consistent with the findings of another study by Chaitanya (54) that found an increase in creatinine levels as a result of stevia dose, and this is due to a decrease in glomerular filtration as a result of nephron damage, which leads to a decrease in their number and thus a decrease in glomerular filtration rate and renal secretion. In the case of hazardous chemicals (55), cases of high creatinine only occur when at least half of the kidney nephrons are damaged (56), and the findings of our investigation were compatible with the findings of another

study Farid (57), which found that the dosage of stevia caused high levels of urea and creatinine. Urea and creatinine are by-products of the blood metabolic process that are transported throughout the body and eliminated from the blood by the kidneys (58).

The study's findings agree with Al-Qazzaz (59), who indicated that stevia influences urea levels, leading them to rise and that a high level indicates impaired renal function since they are health indicators. Thus, a high level signifies kidney disease and damage (60). NO and reduced SOD levels, which are enzymes critical in renal function and urea and creatinine levels (61), indicated an instance of oxidative stress leading to compromised kidney functioning, as seen by the renal tubule's failure to maintain normal levels of plasma urea and creatinine (62). The study showed a significant increase in MDA levels in the kidney tissue of the fetuses between the experimental groups and the control group. Still, there were significant differences between the experimental groups. Oxidative stress is caused by an imbalance between antioxidants and oxidants called Free radicals that cause DNA, protein, and lipid destruction. In contrast, antioxidants neutralize free radicals and convert them into stable molecules.

Lipid peroxidation is one of the most prominent markers of oxidative stress, and its ultimate result is MDA; biochemical changes enhance oxidative stress, which causes programmed cell death and inhibits the Pax3 gene (63,64). Our findings also revealed that compared to the control group, the kidney tissue in mice had many histopathological abnormalities. The severity of the abnormalities was high even on the D12, as evidenced by deformities of the glomeruli and tubules, as well as the occurrence of degeneration, necrosis, congestion, and hemorrhage; the findings of our study did not agree with Rizwan (65) who conducted experiments on rats and agree with Al-Thanoon (66) in a study on the effect of food additives in rats steviol (a poisonous metabolite of sativoside) is absorbed from the gut into the circulatory system and accumulates in the kidneys to be eliminated in the urine, which in turn affects the kidney tissues in a negative way, according to studies Farid (57).

Stevia administration induces a rise in the amount of pro-inflammatory cytokines in the kidneys of mice, which causes damage to high markers of renal function and increases oxidative stress. Degeneration of another organ, such as the kidneys (67), when heart failure (myocardial infarction) has a substantial impact on renal tissues (68). Cardio-nephrotic syndrome is the heart and kidney relationship (69). Degeneration is a generic phenomenon resulting from various causes that disrupt cell function and is frequently an early sign of necrosis. In rare circumstances, vacuolation precedes degeneration. Degeneration is generally distinguished by morphologic and variable cell characteristics, such as cell enlargement with or without cytoplasmic vacuolation and fragmented cytoplasm. Apoptosis, the natural process of cell turnover in the kidney, must be separated from degeneration. Cell swelling, nuclear pyknosis karyorrhexis, and cellular sloughing are all necrosis symptoms; degeneration can be reversed or irreversible (70).

## Conclusion

Stevia is one of the sweeteners that is commonly used as a sugar substitute nowadays. In the current study, stevia caused morphological and histological deformities, significant changes in MDA levels in fetal kidney tissue, and significant changes in urea and creatinine levels in pregnant mice serum. So, stevia is unsafe for pregnant women and will need additional research. The report advises pregnant women to use extreme caution while using these sweeteners**.**

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## Conflict of interest

The authors explain that there are no conflicts of interest

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# الآثار الضارة لمحلي ستيفيا على نمو أجنة الفئران البيض السويسرية

**أروى إدريس النعيمي و هاني مال الله حمودي**

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**الخلاصة**

الهدف من الدراسة الحالية هو معرفة التأثيرات الضارة التي يسببها محلي ستيفيا على نمو أجنة الفئران البيض في الأيام ١٢ و ١٥ و ١٨، يعد محلي ستيفيا احد المحليات التي انتشرت في الوقت الحاضر بشكل كبير كبديل عن السكر الاعتيادي، استخدمت في هذه الدراسة ٤٨ أنثى فأر حامل قسمت الى اربع مجاميع المجموعة الأولى كانت المجموعة الضابطة وأعطيت الماء المقطر فيما أعطيت المجاميع الثلاث الأخرى المحلول المائي للستيفيا من اليوم الأول للحمل الى اليوم الثامن عشر منه واختيرت تراكيز الجرع اعتماداً على الجرعة القاتلة الوسطية التي تم تحديدها في دراسات سابقة، شرحت الفئران في الأيام المحددة وتم استخراج الأجنة من القرون الرحمية وثم استخراج الكلى من هذه الأجنة حيث تم دراسة التشوهات المظهرية للأجنة والتغيرات النسيجية المرضية وبيروكسيد الدهون والمالونداليهايد لنسيج كلى الأجنة إضافة لدراسة المؤشرات البيو كيميائية (اليوريا والكريتينين) في مصل الأمهات الحوامل ، كشفت الدراسة وجود عدد من التشوهات المظهرية في أجنة الفئران وفي كل أيام الدراسة مقارنة بالمجموعة الضابطة منها التواء الأطراف، القيلة النخاعية السحائية، القيلة الدماغية السحائية. أما بالنسبة للتغيرات النسيجية فقد وجدت الدراسة تغيرات مرضية على نسيج الكلى منها النخر والتنكس والاحتقان والنزف. كما وجدت الدراسة تغيرات ذات دلالة معنوية عند مستوى معنوية بالنسبة بيروكسيد الدهون والمالوندديهايد في نسيج كلى الأجنة، وارتفاعاً ذات دلالة معنوية في مستويات اليوريا والكريتينين في مصل الأمهات الحوامل، كشفت الدراسة أن ستيفيا لم يكن أمناً بشكل تام بالنسبة للحوامل ويتطلب إجراء مزيدا من الدراسات.