



The Role of Aspartame sweetner in Instant Beverages in Inducing Gross Malformations and Histopathological Lesions in White Mice Liver *Mus musculus* and Their Embryos

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Abstract

The present study was made to investigate the role of high doses of aspartame used as sweeteners of instant beverages (Tropicana Slim) for inducing gross malformations and histopathological lesions in the liver of white mice and their embryos, furthermore malformations in the embryos of these mice. For this purpose (25) pregnant mice were used which were divided into five equal groups (one control and four experimental groups). Aspartame was given to the mice as single daily dose in a concentration of (0,1500, 2500, 3500, 5000) mg/Kg of body weight (b. w.). At the concentration 1500 mg/Kg, there were closure of left eye and death of some of the pregnant mice. In the dose 3500 (b.w.), abortion was seen in 20% of the pregnant mice as well as vaginal bleeding at the 10th day of pregnancy. The liver of pregnant mice was pale, greenish with the appearance of fatty substance appeared. Microscopically, there were dilatation of the sinusoids, degenerative and necrotic change of hepatocysts, fibrin deposition, apoptosis, Kupffer cell hypertrophy, and malformations occurred at rate of 84% and the main congenital defects induced curved and hyperatrophied embryos, hypertrophy of the brain, rounded head with bird peak-like appearance, and a groove separate the small head from body, meningocoele, spina bifida, shortening and curvature of the extremities, tail that looks like question mark. The histological lesions in the liver of the embryos were similar to these seen in the liver of their mothers

Introduction

The sweetener aspartame was discovered by James Schlatter in 1965 by chance during the production of medicine for stomach ulcer [1]. It was about 180-200 times sweeter than sucrose in the each one gram contained of it 17 KJ or 4 calories, therefore the small amount is required to produce the sweetness in addition to it calories[2]. The aspartame is widely used in over than 90 countries and it is involved production of more than 6000 food products and 500 pharmaceuticals [3,4]. Therefore, it is used by diabetes, athletes and people looking for slimness taken it as well as hundreds of millions around the world particularly children and women in the reproductive period [5]. The aspartame consists from two amino acids, phenylalanine 50%, aspartic acid 40% and methanol 10%, These substances are dangerous when combined together and they had cumulative effect, because their absorption is rapid

but their elimination is slow [6]. The aspartame performed to alter a group of biological processes in the body including metabolism, amino acid building, proteins manufacture, DNA safety, nerve cells function and endocrine gland balance [7]. For these reasons, many researchers have studied the effect of aspartame on the body organs and tissue especially the liver [8,9,10,11]. But at the embryonic level there are very few studies observed as in study [12] which indicated the negative effect of aspartame on the liver of white rats [13]. Which studied the cytotoxic effect of aspartame on the histological and genetic structures of female albino rats and their offspring and due to the lack of studies and conflicting views on the safety of aspartame the current study was designed to determine the role of aspartame in the fast soluble beverage sweeteners in the induction of

congenital malformations and pathological lesions in white mice liver and their embryos.

Materials and Methods

Twenty five white pregnant Mice *Mus musculus* were used for the current study, and were placed under same laboratory conditions in terms of ventilation, humidity, tap water and unified light cycle [14]. For fertilization, two females were placed with one male: in single cage, the vaginal plug was observed in the next day which indicated that fertilization happened and the next day after observing the vaginal plug considered as the first day of pregnancy [15].

In this study local industrial aspartame kind Tropicana Slim (made in Indonesia) was used. The ratio of aspartame in the sweetener it measured by High Performance Liquid Chromatography (HPLC) equipment [16]. The percentage of aspartame in the sweetener was calculated using liquid solution of aspartame by dissolving (1.5 - 5 gm) of the aspartame in distilled water.

The experiments were designed using five groups each contained five pregnant females. The control group, administered with distilled water and the four experimental groups have water solution of aspartame

orally administered. The oral administration of aspartame was started at the 7th day of pregnancy on the concentrations of (1500, 2500, 3500, 5000 mg/Kg of body weight was based on the medium lethal dose (LD50) [17].

The pregnant females were dissected at 18th day of pregnancy and examined grossly and the required and measurements were recorded. The fixation and preparation of microscopic slides were made [18]. The sections were stained with hematoxylin and eosin, loading with DPX and examined under light microscope. The histological sections were photographed using light microscope with digital camera.

Results

Estimation of Aspartame in the Instant Beverage Sweeteners by HPLC

The amount of aspartame in the sweeteners has been estimated by comparing the peak of the aspartame in the sweetener with peak of standard aspartame, and the retention time of the sweetener aspartame was identical to the peak of the standard aspartame ($t \sim 3.1$), Thus the concentration of aspartame sweetener was 436 mg/Kg (Figs 1,2).

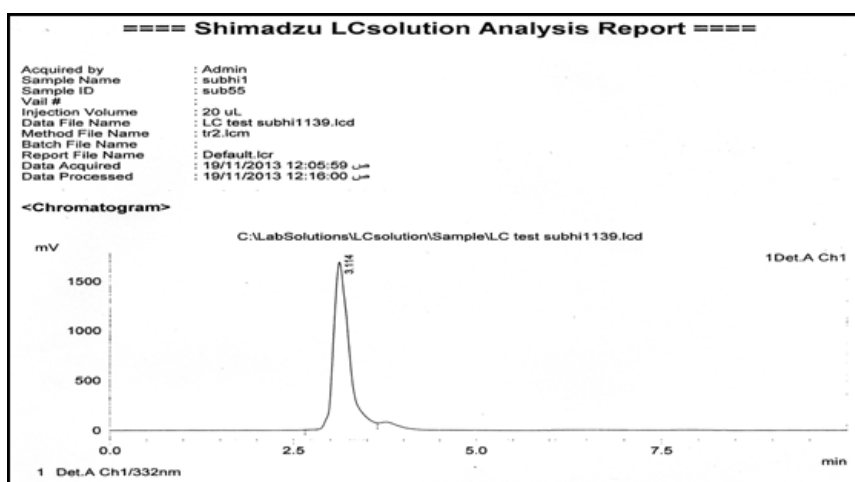


Fig (1): The chromatography curve of standard aspartame

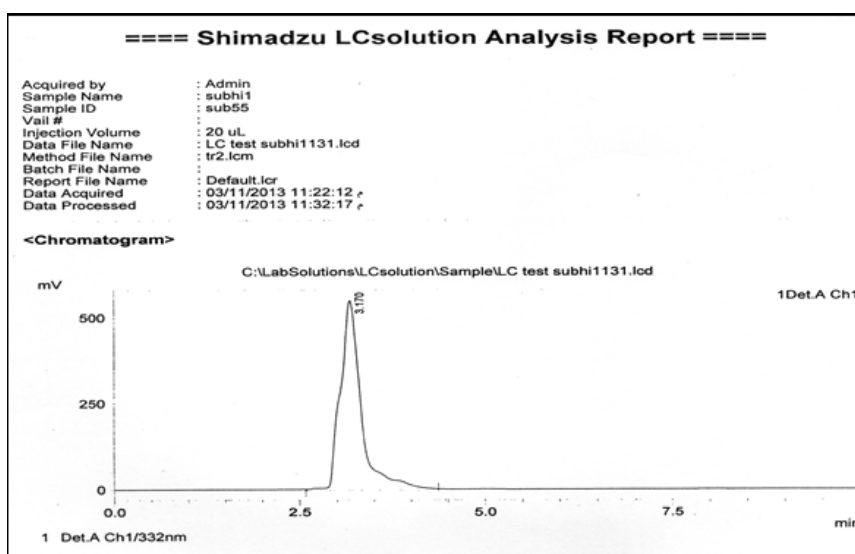


Fig (2): The chromatography curve of instant sweetener aspartame

Control Groups with Treatment with Distilled Water

I. The Gross and Histological Descriptions of Pregnant Female Mice Liver:

The gross examination of liver in the control group showed that the liver was normal, had reddish brown color and consists from six intact lobes (Fig 3). Whereas the histological examination showed regularity of hepatic cells in cords around the central vein separated by a number of sinusoids (Fig 4).



Fig (3): The liver of pregnant female in control groups (c.g.) show the normal liver and its six lobes

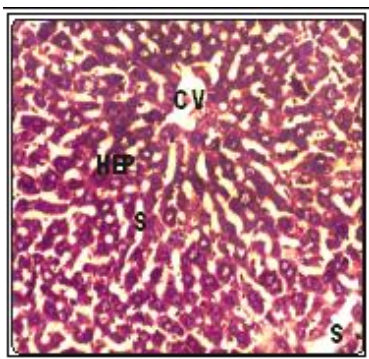


Fig (4): liver of pregnant female (c.g.) show the hepatocytes (HEP), Central Vein (CV), and Sinusoids (S), (100 X)

II. Gross and Histological Descriptions of White Mice Embryo:

The embryos had average weight (0.5 ± 0.9 gm) and average length (0.9 ± 20 mm) and had pink color with clear head area, ere pinna, closed eyes, integrity of trunk, differentiation of the extremities, thick skin and differentiation of the tail and it was coiling into abdominal area (Fig 5). While the histological examination of the liver showed the differentiation of central vein surrounded by hepatocytes separated by sinusoids (Fig 6).



Fig (5): Lateral view of white mice embryo of (C.G.)

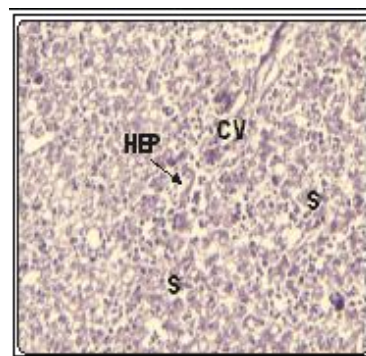


Fig (6): liver of (c.g.) to white mice embryo (18th day of pregnancy) show (CV), (HEP), and (S). (100X).

Experimental Groups Treated with Aspartame Sweeteners Instant Beverages Concentration 1500 mg/kg of Body Weight (Day Anatomy)

I. Gross and Histological Descriptions of the Liver in Pregnant Female Mice.

Gross morphology included congested lobes, large white scars appearance, accumulation of fatty substance (Fig 7). Histologically there were inflammatory cell infiltrates, dilated sinusoids, kupffer cells hypertrophy and focal necrosis (Fig 8). Also seen hemolysis, swelling of hepatocytes, pyknosis and congestion of central vein in some cases (Fig 9).

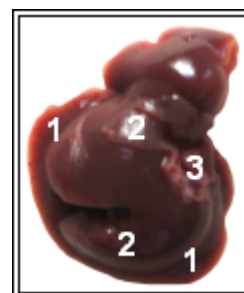


Fig (7): The liver of pregnant female show the congested lobes (1), large white scars (2), accumulation of fatty substance (3).

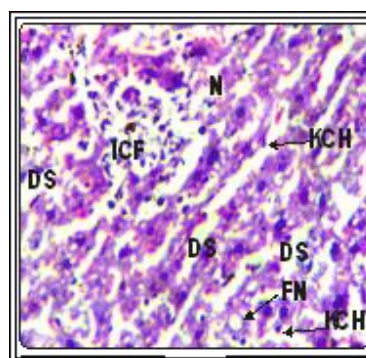


Fig (8): liver of pregnant female show Dilated Sinusoids (DS), Infammatory Cell Infiltrate (ICF), Kupffer Cell Hypertrophy (KCH), Focal Necrosis (FN), and Necrosis (N). (400X).

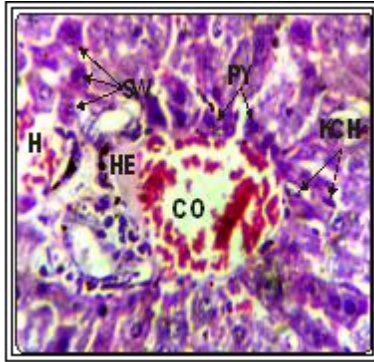


Fig (9): liver of pregnant female show Hemolysis (HE), Swelling (SW) of (HEP), Pyknosis (PY), (KCH), Congestion (CO) of (CV), (400X).

II. Gross and Histological Descriptions of Embryo White Mice.

The ratio of malformed embryos was 55% average weight (0.4 ± 0.7) gm, and average length (15 ± 19) mm. The most common malformation was larger head size, brain hypertrophy and down curvature, flattening telencephal, hemorrhage between the brain, exophthalmia of eyes, larger ear pinna, spina bifida, bloating of anticus limbs and cleft the fingers, and thick tall tail reach to head area (Figs 10,11). Histologically there were hemorrhage vacuolar degeneration, megakaryocytes, pyknosis of some nuclei and increased density of others, and Kupffer cells hypertrophy (Fig 12).

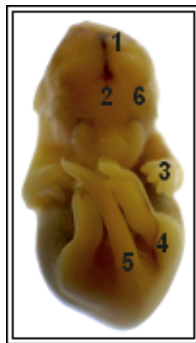


Fig (10): Anterior view of embryo show malformation in skull, and flatting telencephal (1), hemorrhage between of hemisphere (2), bloating of anticus limbs (3), curvature posticus limbs (4), tall thick tail aquiline (5), exophthalmia (6).

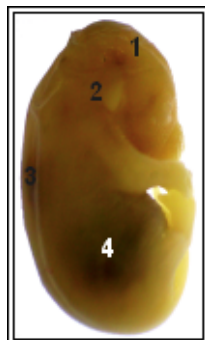


Fig (11): Lateral view of embryo show head hypertrophy and curvature to down (1), large ear pinna (2), spinal bifida in dorsal area (3), congestion of stomach (4).

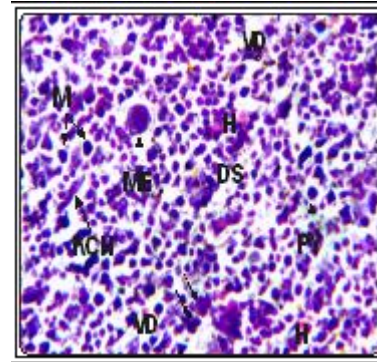


Fig (12): liver embryo show Hemorrhage (H), Vacuolar Degeneration (VD), Macrophages (M), Megakaryocytes (ME), (PY), density of some others nucleus (vectors), (KCH) and (DS), (400X).

Experimental groups Treated with Aspartame Sweeteners Instants Beverages Concetration 2500 mg/kg of Body Weight.

I. Gross and Histological Descriptions of the Liver in Pregnant Female Mice.

Gross lesions included congestion of liver lobes was observed, damage of hepatocyt and appearance. Greenish substance between the lobes (Fig. 13). Histologically there where hemolysis inside the blood vessel, and vacuolar degeneration of hepatocytes around the blood vessel (Fig.14). Also fibrin deposition, degeneration of hepatocytes and swelling of others were observed (Fig. 15).

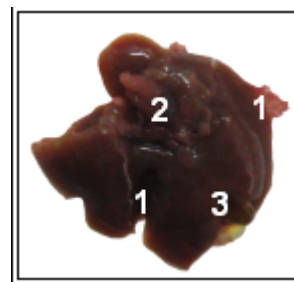


Fig (13): The liver of pregnant female show increase of congestion in liver lobes (1), damage in liver tissue (2), greenish substance appearance (3).

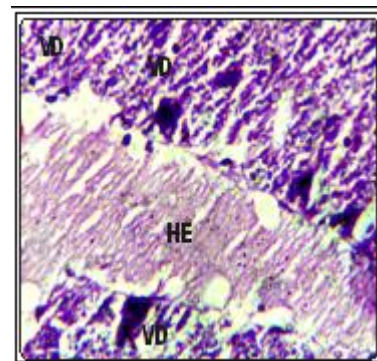


Fig (14): liver of pregnant female show (HE) insaid Blood Vessel (BV), and (VD) to (HEP) around (BV), (400X).

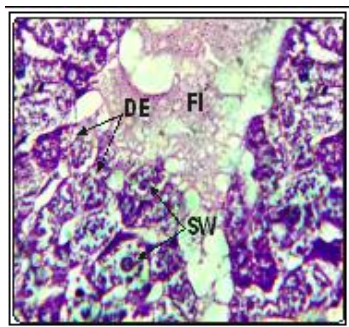


Fig (15): liver of pregnant female show Fibrin Deposition (FI), (SW) of (HEP), and Degeneration (DE), (400X).

II. Gross and Histological Descriptions of Embryo White Mice.

Malformations occurred at arte of it 67.5% average weight (0.6 ± 0.7) mg and average length (18 ± 24) mm. The embryos looked with round head like bird peak, arcua trunk and bloating of the trunk, lack of neck, sunken eyes, nose hypertrophy, dermatocoelc, congestion of ventrodorsal area and its bloating, micromelia anticus and posticus limbs, and curevature and adhesion of fingers, and aquiline thick tail reach the head area (Figs 16, 17). Histologically assimilation in disorder of natural arrangement of the hepatocytes, increase of megakaryocyte number, coagulative necrosis, deep staining cytoplasm, inflammatory cells infiltrate look like lymphocyte nodule (Fig. 18).

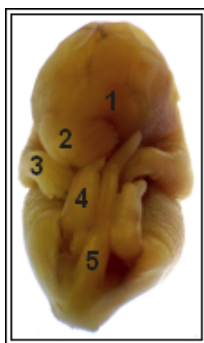


Fig (16): anticus view of embryo show sunken eyes (1), hypertrophy of nose (2), curvature of anticus limbs and adhesion of fingers (3), micromelia posticus limbs and adhesion of fingers (4), thick taill aquiline (4).

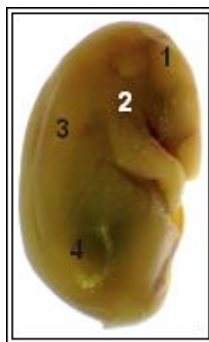


Fig (17): Lateral view of embryo show cycle head suck bird peak (1), lack of neck (2), trunk arcuation (3), congestion of ventrodorsal area and bloating of the trunk (4).

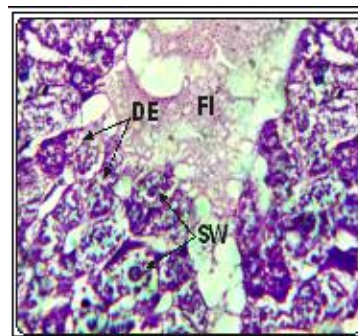


Fig (18): liver of embryo show increase of (ME), Coagulative Necrosis (CN), Deep Staining Cytoplasm (DSC), and (ICF) such lymphoid nodule, (400X).

Experimental Groups Treated with Aspartame Sweeteners Instant beverages Concetration 3500 mg/kg of Body Weight .

I. Gross and Histological Descriptions of the Liver of Pregnant Female Mice.

The liver of pregnant mice showed dis-arrangement of lobes, white scars, damage and partial accumulation of fatty substance (Fig.20). Histologically showed increase inflammatory cells inside blood vessel, vacuolar hepatocytes, pyknosis of hepatocytes (Fig. 22).

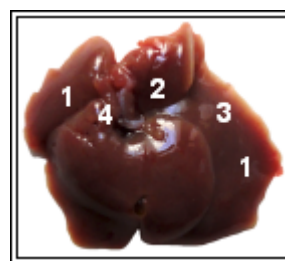


Fig (19): The liver of pregnant female show dis-arrangement of lobes (1), damage (2), partial accumulation to fatty substance (3).

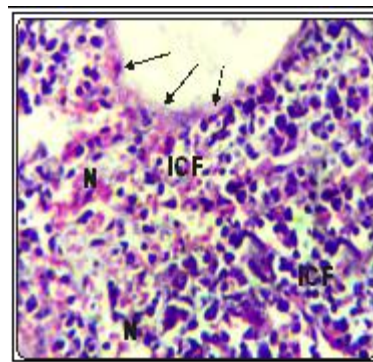


Fig (20): liver of pregnant female show (ICF), (N) in (HEP), and thickening of blood vessel (vectors), (400X).

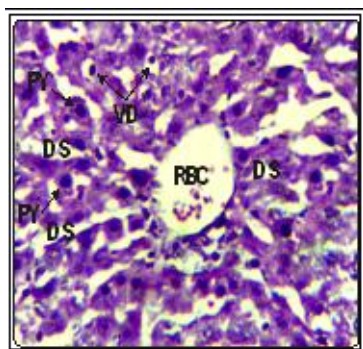


Fig (21): liver of pregnant female show (DS), spread of Red Blood Cells (RBC), (PY), and (VD), (400X).

II. Gross and Histological Descriptions of Embryo White Mice: The percentage of malformation embryos was 72%, average weight (0.3 ± 0.5) gm and average length was (14 ± 18) mm. The most prominent malformation was head hypertrophy and distinct hemiencephalon under the thin skin, and look like fornix skull and around the eyes. Encephalocele appeared, arcuation embryo look like C letter, sunken eyes, shorter of anticus limbs and hypertrophy of fingers and cleft of it, arthrotropia insteps postcus limbs and adhesion of fingers, and thick straight caudate tail (Figs. 23, 24). Histologically congestion of central vein and shape change, wide and disorder of hepatocyte disarrangement, Kupffer cells hypertrophy, necrosis and degeneration of hepatocytes (Fig 25).

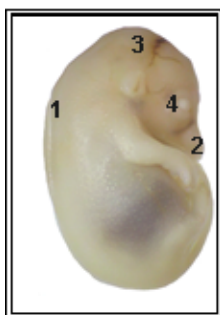


Fig (22): Lateral view of embryo show curvature of embryo (1), head look such duck head (2), hemorrhage in foramen skull and around the eyes (3), sunken eyes (4).

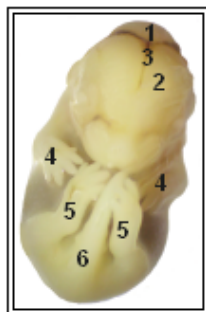


Fig (23): Anticus view of embryo show hypertrophy of head and meningocele (1), encephalocele (2), hemorrhage in foramen skull (3), micromelia of anticus limbs and cleft the fingers (4), arcuation of instep postcus limbs (5), thick straight caudate (6).

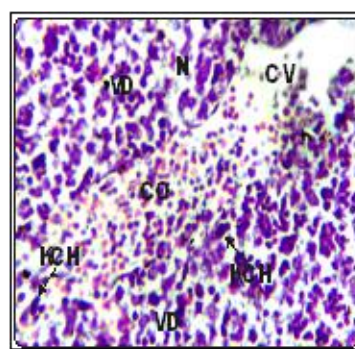


Fig (24): liver embryo show change of (CV), (CO), (KCH), (N) in liver tissue. (400X).

Experimental Groups Treated with Aspartame Sweeteners Beverages Concentration 5000 mg/kg of body weight.

I. Gross and Histological Descriptions of the Liver in Pregnant Female Mice.

Grossly, there were change in liver shape and paleness of some liver lobes, as well as the presence of white scar (Fig 25). Histologically there were disorder of hepatocytes arrangement, apoptosis, karyolysis (Fig 26) also infiltrates of inflammatory cells, kupffer cells hypertrophy, necrosis of hepatocytes and shrinkages, and vacuolar degeneration (Fig. 27).

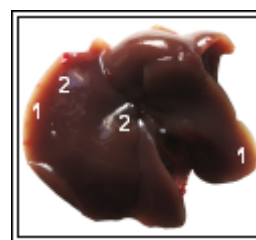


Fig (25): The liver of pregnant female show paleness in some liver border lobes (1), accumulation of white scars on surface lobes (2).

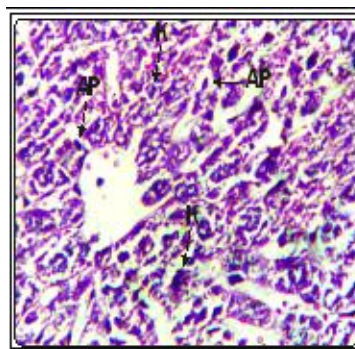


Fig (26): liver of pregnant female show Apoptosis (AP) of cell, and Karyolysis (K), (400X).

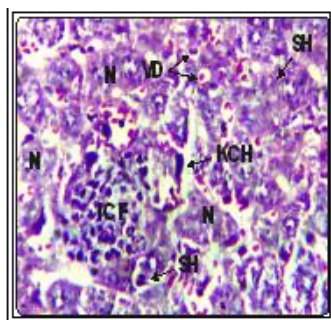


Fig (27): liver of pregnant female show (ICF) to forming such as lymphoid nodule, (N), (VD), (KCH), and Shrinkage (SH), (400X).

II. Grossing and Histological Descriptions of White Mice Embryo.

The ratio of malformed embryos was 84% average weight (0.7 ± 1.1) gm and average length average was (23 ± 27) mm, the most important malformations were macrogenesis embryos and cyanosis of skin, circular head and triaglim head, valleula separ the small head from the body in clearly shape malformation of nasofacial cast and face shape, elongation of nose area and occurrence to pro the face. Sunken or loss of eyes, malformation of ear pinna and change of its place, meningmyelocele in the back area of brain bloating of abdomen area, micromelia of anterior limbs and instep rhombuses, hypertrophy of posterior and adhesion of fingers, tail look like question mark (Figs 28,29, 30). Histological examination pointed to severally congested of central vein, focal pyknosis of nuclei (Fig. 31), also pointed to severe hemorrhage, dilate sinusoids, vacuolar degeneration, and wide necrosis between elements of embryo tissue (Fig 32).



Fig (28): Laterale view of embryo show macrogenesis and cyanosis skin (1), valleula sparte the small head from the body (2), arcuation the trunk and dorsal area (3), suncken eyes (4), (400X).

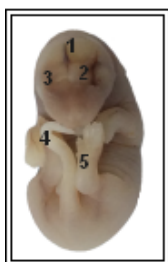


Fig (29): Anticus view of embryo show cycle head (1), hemorrhage between hemiencephalon and eyes area and face chape (2), loss the eyes (3), tail look like question mark (4), hypertrophy of posticus limbs and instep rhaebosis (5).

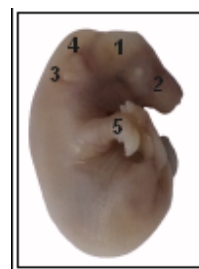


Fig (30): Latrale view of embryo show triangular head (1), elongation of nosily area (2), malformation of ear pinna (3), meningmyelocele (4). Micromelia of anticus limbs and arcuation of instep it (5).

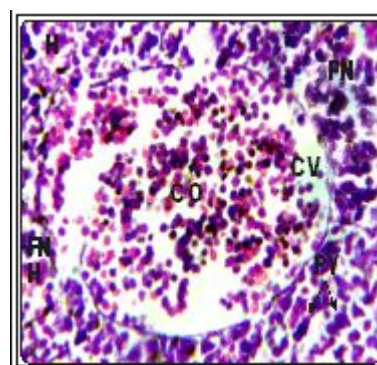


Fig (31): liver embryo show (CO) in (CV), (FN) in liver tissue, (PY) of (HEP), (400X).

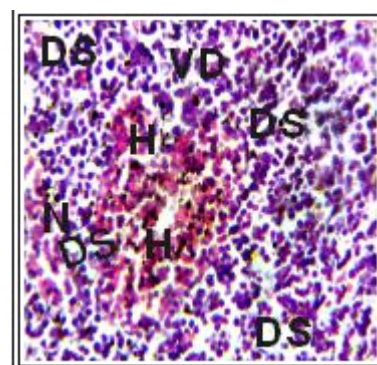


Fig (32): liver embryo show severely (H), (DS), (VD), (N) between the elements of liver tissue. (400X).

Discussion

In the current study, hepatic lesions represented by vacuolar changes (congestion or palenss), presence of white scars in the inner surface, partial accumulation of fat substances and appearance of greenish bile among the lobes. These changes may be created by the hepatotoxic effect of the aspartame that recorded by other studies [12]. Also the pathologic lesions in the pregnant mice included infiltration of inflammatory monocytes, degeneration (vacuolar degeneration) and necrosis changes in the hepatocytes, blood vascular changes (congestion and hemorrhage), disorder in the normal arrangement of hepatic cord, dilated sinsoids, hypertrophy of hepatocytes and Kupffer cells and apoptosis of the cells, these changes, also recorded by other researchers [8, 10, 11, 13]. These studies referred to that, these changes caused by releasing of the free radices after production of the methanol and aspartic

acid after taking and metabolism of aspartame [3]. This suggestion has been ascertained by other researchers, and they found that disorder of secretion and composition of coagulation factor VII and fibrinogen that created by aspartame preformed the elongation of hepatitis [19]. Other researchers suggested that, the damage of hepatocytes may be a secondary activation of Kuppfer cells which secreted tumor necrosis factor alpha and interleukins, reactive oxygen, nitrogen species, proteases and prostaglandins. These intermediates can affect directly the nuclei and caused cell death [11].

The results of this study also referred to the presence of deforming embryos at ratio 42%, 55%, 67.5% and 84% in the experimental groups and the embryos were small and had a little weight at the concentration (1500, 2500, 3500) mg/kg and these results agreed with [20], and the reason of this was due to malnutrition of the pregnant mice in the last days of pregnancy when the embryo required pillars of growth and energy production such as the glucose which is the main source of energy needed in the growth and metabolism, where these substance are needed by the fetus provided by the mothers blood to the fetus [21,22]. Also, the replacement of the sucrose by the industrial sweeteners such as aspartame preformed to decrease the calories which led to reduce body weight, along with that, the consumption of the aspartame coincided in the longterm in maintaining the weight [23]. At the dose (5000 mg/kg), the embryos appeared hypertrophied compared with the control group and this was similar with the results of other study [24]. Which referred that the people who drink diet soda are exposed to increase in the weight reach to 41% per pack or bottle per day and this is possibly is due to the high doses of aspartame that led to increase the density of muscular structure and hypertrophy in the skeleton as a reaction to the adaptation of the body and to compensation by increase of hyperplasia because this will make following the cell division impossible [25].

The results showed hypertrophy, inflammation and swelling in the brain similar to the study of Ganong (1998) on the effect of aspartame [26], Other studies showed meningocele and decline in some areas of skull when coke cole containing aspartame was taken [27], And small head size of relative to the body [28], and triangle head separated from the body by clear groove, when inhaled cigarette smoke riched with aspartame and methanol [29]. The cause of the distortions in the current study and the excitotoxic of the placental blood with consumption of aspartame that can lead to damage or impairment of the development of fetal nervous system, cerebral paralysis that included all developmental disorders was also mentioned [8,30].

This study also reveled changes in the embryos eyes represented by ophthalmocoele and it's invagination, in addition to its loss in the high concentration, these results were similar to other studies, which referred to

Invagination of the eye and lost when injecting the methanol at concentration 4300 mg/kg in the peritoneal membrane of pregnant mice, these changes, may be, happen due to oxidation of methanol to formic acid, which selectively inhibits nitrous oxide, which lead to the accumulation of formate, which coincided with the development of metabolic acidosis and visual toxicity [31,32].

The results of the study also, resemble other researchers notes in the deformation of the nasal facial features and clearness, cracked face, stem curvature and it's curve to lower, convexity of trunk region, lack of neck and C appearance of embryo [31,32]. The reason of these disorders were due to the methanol effect wich increased the cervical ribs and increase ossification in the lateral side of 7th cervical vertebrae [33]. Also the results were similar to the studies of Smithells (1981) and Laurence (1981) [34, 35]. in the presence of the spina bifida and it's direct relation with the lack of folic acid and present of congestion in different areas of body was similar to other studies[36]. As a result of treatment of pregnant mice with ethambutol concentration 25 mg/kg, the effect was due to the methanol in the aspartame causes congestion in the skin [37].

The aspartame in the sweetener also, caused appearance of the syringocoele as cystic tumor in the dorsal region, this may be due to the malnutrition of the pregnant mice in the last period of pregnancy, which led to lack of folic acid and erythrocytes and these related directly to increase the abnormal closure of neural tube during pregnancy [38].

The results showed deformity in the integumentary system in the embryos represented by Crispation, Malacia and Dermatolysis as in Ghaseminezhad and Hejazi (2015), and this is perhaps happened because the aspartame cross the placenta and penetrates the embryonic tissues in the early days of organization and this caused distortions in the skin through the processes of embryonic development. The result of this study showed swelling, shortening and detening of fingers, of the anterior extremities, as that appeared in Ghaseminezhad and Hejazi (2015) and Al-Brawary (2013) [39,40].The curvatures of anterior and posterior limbs identical to that indicated by [36] as well as the shortening and malformations of the posterior limbs and adhesion of from limb finger also indicated by[36], those changes may be due to the interaction changing for each ectoderm and mesoderm that creates the cartilage forming the bones, also the occurrence of the apoptosis to the ectoderm, cells because of the toxic cellular factor [41].

Whereas the adhesion of the fingers due to absence of apoptosis of the cells between the membranes founded between the fingers [42]. Also, the results showed caudal distortions as convolute, short and straight as is was mentioned by Saft *et al* (2014) [43]. that study the forms of caude associated with neurological damage and its association with autism,

also, the appearance of tail similar to question mark and the long tail that reached to head was similar to Al-Noaemi (2012) [27]. The different forms of caud part was due to the position of fetus in the uterus or because the androgen hormone and its control of the length of fingers [44].

The histological results of current study included vacuolar changes (congestion and hemorrhage), degeneration change (acute swelling) disarrangement of hepatocytes, apoptosis dilated sinusoids, Kupper cell hypertrophy, and hepatomegalocytes, these results were similar to those of Portela *et al* (2007) and Abd Elfatah *et al* (2012) [12,13], whom recorded

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دور اسبارتام محليات المشروبات سريعة الذوبان في احداث التشوهات العيانية والآفات النسجية في

كبد الفئران البيض *Mus musculus* واجنتها

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الملخص

أجريت هذه الدراسة للتعرف على دور الجرعات العالية من الاسبارتام والمستخدم في المشروبات سريعة الذوبان (تروبيكانا سلم) في إحداث الآفات النسجية في كبد الفئران البيض الحوامل وأجنتها فضلا عن التشوهات العيانية في أجنة هذه الفئران، واستخدمت لهذا الغرض (25) أنثى فأر حامل وزعت على خمس مجموعات متساوية (سيطرة وأربع مجموعات تجريبية) وبمعدل (5) فئران لكل مجموعة. تم إعطاؤها الاسبارتام على شكل جرعة فموية مفردة يوميا بالتراكيز (0، 1500، 2500، 3500، 5000) ملغم/كغم من وزن الجسم. سجلت الدراسة تغيرات مظهرية لإثاث الفئران الحوامل عند الجرعات المختلفة، فعند التركيز 1500 ملغم/كغم من وزن الجسم لوحظ انسداد في العين اليسرى ومن ثم موتها، كما لوحظت حالات الإجهاض بنسبة 20% ونزف دموي خلال اليوم العاشر من الحمل في التركيز 3500 ملغم/كغم من وزن الجسم. أوضح الفحص المظهري لكبد الفئران الحوامل ضرر في النسيج الكبدي، وشحوب في بعض حوافها، وتجمع للمواد الشحمية، وظهور مادة صفراء مخضرة بين فصوصه. وحدوث التشوهات المظهرية للأجنة بنسب تصل إلى 84% وشملت ابرز التشوهات أجنة مقوسة ومتضخمة، وكبر حجم الرأس وتضخم الدماغ، ورأس مستدير يشبه منقار الطير، ورأس شبيه بمنقار البط، وظهور أخدود يفصل الرأس الصغير عن الجسم، وتمايز القيلة السحائية الدماغية والقيلة النخاعينية والشوكة المشقوقة، وقصر واعوجاج الأطراف الأمامية والخلفية ، وذنب ذو نهاية شبيهة بعلامة الاستقهام. شملت الآفات النسجية التي تم تسجيلها في كبد الفئران الحوامل توسع الجيبانيات، وتضخم خلايا كوففر، وتتكس ونخر الخلايا الكبدية، وترسب الليفيين، والموت المبرمج، وارتشاح الخلايا الالتهابية أحادية النواة، وكانت التغيرات النسجية الكبدية الجنينية مماثلة تقريبا مع التي لوحظت مع أمهاتها.

الكلمات المفتاحية: اسبارتام، محليات المشروبات سريعة الذوبان، تشوهات جنينية، فحص نسجي.