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Role of Astaxanthin in Improving the Physiological and Teratolgical Changes of Aspartame in the Pregnant Albino rats and Their Fetuses

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ABSTRACT

Aim of the work: the present work was done to investigate the role of astaxanthin in ameliorating the physiological and teratological effects of aspartame on the pregnant rats and their fetuses.

Materials and methods: in this study 70 virgin mature female albino rats (*Wistar wistar*) were used and 35 males (for mating); pregnancy was ascertained by vaginal smearing. The pregnant rats were injected on days7, 9, 11 and 13 of gestation (organogenesis period). The animals were divided into the following groups: animals of the first group which were intraperitoneally injected with 0.5ml saline (solvent of aspartame); animals of the second were intraperitoneally injected with 0.5 ml olive oil (solvent of astaxanthin). The third group was divided into 2 subgroups, the pregnant rats of the first subgroup were intraperitoneally injected with aspartame (20mg./kg. body weight), the pregnant rats of the second subgroup were intraperitoneally injected with aspartame (40 mg./kg. body weight). Pregnant rats of the 4th group were intraperitoneally injected with astaxanthin (50mg.\kg. body weight) 2 hours after aspartame injection by its 2 doses.

Results: aspartame at the dose of 40 mg./kg.body weight induced pre- term (early delivery); however the 2 doses of aspartame resulted in very highly significant decrease in the mean maternal body weight , unequal horns of the uteri and unequal distribution of the fetuses between them , abnormal amount of fats surrounding the 2 horns of the uterus, regions of hemorrhage were present on the external membrane of the uteri ,very highly significant uterine weight decrease , cases of abortion were noticed as well as highly significant increased resorption of the fetuses. Intraperitoneal injection with aspartame at the 2 doses resulted in fetal mortality, very highly significant fetal body weight and length decrease with a significant decrease in fetal tail length. Moreover, aspartame induced fetal morphological changes such as body diminution, exencephaly, cognition of the blood vessels in the head region and cleft lips. The skin seemed thin and fragile in the head region as well as the fore and hind regions; hematoma was obvious beside edema, clubbed fore and hind limbs, kyphosis was obvious beside hernias on some regions of the skin with hypertrophy of some fetuses and protrusion of the viscera outside the body. Aspartame exposure at the 2 doses induced highly significant increase in mean of maternal serum AST activity, very highly significant increase in mean serum GGT and serum creatinine activity.

Conclusion-Treatment with astaxanthin induced obvious improvement in all of the physiological and morphological changes caused by aspartame in the pregnant rats and their fetuses.

Key words: Aspartame- Astaxanthin - Pregnant albino rats - Fetuses -Physiology -Teratology.

INTRODUCTION

Aspartame (APM) is an artificial sweetener widespread in the world; its brand name is NutraSweet. Millions of people use aspartame; which is used in many food products like chewing gum, desserts, yoghurts, vitamins, medicines and particularly in diet beverages as well as people trying to lose weight or patients with diabetes, including children, frequently use these products. [1]It was found that aspartame is present in 68% of comestible assortment among sugar-free products in Poland.[2] It is applied by diabetics as tablets instead of sugar (NutraSweet, Equal, Sugar Free, Canderel).The sweetener was accidentally discovered by James Schlatter during his attempts to obtain a gastric ulcer drug in 1965.[3]This white, crystalline and odorless powder is 180-200 times sweeter than sucrose. The intense sensation of sweetness allows the use of such small doses so that the product is almost non-caloric although its energy value is 4 kcal/g of aspartame. The sweetener is a chemical agent, N-L-Alpha-Aspartyl-L-phenylalanine methyl ester ($C_{14}H_{18}N_2O_5$).[4] This substance is hydrolyzed completely in the intestinal lumen during metabolism of the body to the three main breakdown products: phenylalanine (50%),

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aspartic acid (40%) and methanol (10%). The use of aspartame as a sweetening agent has been controversial for years due to the harmful effects of high concentrations of its metabolites. It was cited that the excess of phenylalanine blocks the transport of important amino acids to the brain which leads to reduced levels of dopamine and serotonin.[5] Moreover, aspartic acid at high concentrations is a toxin that causes hyperexcitability of neurons and is also a precursor of other excitatory amino acid glutamates.[6]

Hence the European Food Safety Authority established an acceptable daily intake of 40 mg/kg b.w./day.[7]Aspartame was approved for dry goods in 1981 and for carbonated beverages in 1983. It was originally approved for dry goods on 1974.[3]

Aspartame accounts for over 75 percent of the adverse reactions to food additives reported by FDA. Many of these reactions are very serious including seizures and death. A few of 90 different documented symptoms listed in the report as being caused by aspartame include: headaches/migraines, dizziness, seizures, nausea, numbness, muscle spasms, weight gain, rashes, depression, fatigue, irritability, tachycardia, insomnia, vision problems, hearing loss, heart palpitations, breathing difficulties, anxiety attacks, slurred speech, loss of taste, tinnitus, vertigo, memory loss and joint pain. [5,8&9]

It can be concluded from the observations of [10]that long term consumption of aspartame leads to hepatocellular injury and alterations in liver antioxidant status mainly through glutathione dependent system. The associations between consumption of sugar-sweetened and possibilities of chronic kidney disease was studied [11], they found statistically significant increased risks of chronic kidney disease in patients consuming sugar-sweetened soda. Moreover, it was being concluded that soft drink intake is associated with higher risk of ischemic stroke for women.[12&13]

Studies on consumption of sugar-sweetened during pregnancy revealed that daily intake of artificially sweetened soft drinks may increase the risk of preterm delivery. [14]The nutritional environment during embryonic, fetal and neonatal development plays a crucial role in the offspring's risk of developing diseases later in life. Although non-nutritive sweeteners (NNS) provide sweet taste without contributing to energy intake; animal studies showed that long-term consumption of NSS, particularly aspartame, starting during the per gestational period may predispose the offspring to develop obesity and metabolic syndrome later in life.[15,16&17]

On the other hand, astaxanthin(ATX), xanthophyll carotenoid, it is a dietary carotenoid of crustaceans and fish that contributes to their coloration. [18] Dietary ATX is important for development and survival of salmonids and crustaceans and has been shown to reduce cardiac ischemic injury in rodents and it is considered as a powerful antioxidant, anti-inflammatory, anticancer agent and it also shows neuroprotective property, protects from hepatotoxicity, lipid peroxidation and renal injury.[19-21]

MATERIALS AND METHODS

The experimental animals

70 virgin mature female albino rats (*Wistar wistar*) were used and 35 males (for mating). The rats were obtained from King Faisal Specialist Hospital Research Centre. The animals were kept under adequate dietary, ventilation and humidity conditions. Pregnancy was ascertained by daily examinations of vaginal smears.[22]

Experimental design:

The animals were divided into the following groups(n=10).

I -The experimental control groups:

1-Pregnant rats were intraperitoneally injected with 0.5ml saline (solvent of aspartame).

2-Pregnant rats were intraperitoneally injected with 0.5 ml olive oil (solvent of astaxanthin).

II - The treated groups:

1-Aspartame treated group: this group was divided into 2 subgroups, the pregnant rats of the first subgroup were intraperitoneally injected with aspartame (20mg./kg. body weight), the pregnant rats of the second subgroup were intraperitoneally injected with aspartame (40 mg./kg. body weight).

2- The astaxanthin group: the pregnant rats were intraperitoneally injected with astaxanthin (50mg./kg.body weight).

3 -The treatment groups: this group was divided into 2 subgroups in the first subgroup the pregnant rats were intraperitoneally injected with astaxanthin (50mg./kg. body weight) 2 hours after aspartame injection (20mg./kg. body weight), in the second subgroup the pregnant rats were intraperitoneally injected with astaxanthin

(50mg./kg. body weight) 2 hours after aspartame injection (40 mg./kg. body weight).

In all the above mentioned groups the pregnant rats were injected on days7, 9, 11 and 13 of gestation (organogenesis period).

I- Maternal Investigations:

1- The female rats were weighed pre-gestation with Sartorius 1104.

2-Pregnant rats were weighed on days 7, 10 and 13 of gestation to evaluate the increase in the body weight due to pregnancy.

3- Observing of blood from the vaginal opening, of the pregnant female was taken as an indication of abortion.

4- The mortality rate in the different groups was illustrated.

5- On day 20 of gestation the pregnant females of all groups were sacrificed for estimation of serum aspartate amino acid transaminase (SAST), Serum Gamma-Glutamyl Transpeptidase(SGGT) and Serum Creatinine(SCr). Blood was taken from the heart of pregnant rats in clean, sterile test tubes. The tubes were centrifuged at 3000 r.p.m. then the sera were obtained and kept in deep freezer under -20^0.

Determination of the Activity of Serum AST:

serum transaminase (SAST) activity in U/l was determined using an assay kit BioMerieux Sa, USA.[23]

Determination of the Activity of Serum GGT:

Serum Gamma-glutamyl Transpeptidase (SGGT) activity in U/l was determined using an assay kit.[24]

Determination of the Activity of Serum Creatinine:

Serum creatinine (Scr) levels in U/l were determined using the Kodak 2000MM System, Eastman Koda**k** Company, Rochester, New York, USA.[25]

II- Embryological Investigations:

The embryos were examined carefully for the following studies:

1-The mean number of alive, dead and malformed fetuses for each group.

2- The mean body weight of live fetuses using Metller, AC100(0.01-100g.)

3- The mean body length of live fetuses was measured in cm. using a compass filament.

4-The malformed embryos were examined carefully and photographed by digital camera.

RESULTS

Results of the pregnant rats:

The uteri of the pregnant rats of the control groups and the group injected with astaxanthin are illustrated in figs.1-3.

Aspartame at the dose of 40 mg./kg.body weight induced pre- term (early delivery), however the 2 doses of aspartame resulted in very highly significant decrease $P<0.001$ in the mean maternal body weight change(Table 1) , unequal horns of the uteri and unequal distribution of the fetuses between them , abnormal amount of fats surrounded the 2 horns of the uterus, regions of hemorrhage were present on the external membrane of the uterus(Figs.4&5) ,very highly significant uterine weight decrease $P<0.001$ (Table1) , cases of abortion were noticed as well as highly significant $P<0.001$ increase in resorption of the fetuses (Table 1). Treatment with astaxanthin post injections by the 2 doses of aspartame induced obvious improvement of these induced changes (Figs 6&7).

Results of the fetuses:

Intraperitoneally injection with aspartame at the 2 doses resulted in fetal mortality, very highly significant $P<0.001$ fetal body weight and length decrease, significant decrease $P<0.05$ in fetal tail length comparing with the normal control experimental groups, astaxanthin treatment improved these changes (Table2).

External morphology of fetuses of the experimental control groups and the experimental group injected with astaxanthin are illustrated in figs.8-10. Aspartame induced fetal morphological changes such as body diminution, exencephaly, exophthalmus, cognition of the blood vessels in the head region and cleft lips. The skin seemed thin and fragile in the head region as well as the fore and hind regions. Hematoma was obvious beside edema in the head region, clubbed fore and hind limbs and kyphosis were obvious beside hernias on some regions of the skin, hypertrophy in some regions and protrusion of the viscera (evisceration) (Figs11-14). Treatment with astaxanthin showed improvement of the fetuses (Figs15&16).

Physiological studies:

Aspartame exposure at the 2 doses induced highly significant increase($P<0.001$) in maternal serum AST activity, very highly significant increase($P<0.001$) in serum GGT and creatinine

activity(SCr). Treatment with astaxanthin improved the mentioned physiological parameters (Table 3).

DISCUSSION

Aspartame is a synthetic sweetener that has been used safely in food for more than 30 years. Its safety has been evaluated by various regulatory agencies in accordance with procedures internationally recognized and decisions have been revised and updated regularly. The most relevant conclusions of epidemiological studies concerning the use of low-calorie sweeteners (mainly aspartame), published between January 1990 and November 2012 were studied by **Marinovich *et al*.**[26]

Several studies on laboratory animals have been made to verify aspartame's toxicity. **Soffritti *et al*.** [27] reported that aspartame is a multipotential carcinogenic agent when given at a daily dose of 20 mg/kg body weight, an amount well below the acceptable daily dose of 40mg/kg body weight

The present study revealed that intraperitoneally injection of pregnant rats with aspartame by 2doses (20mg./kg. and 40 mg./kg. body weight) on days 7, 9, 11 and 13 of gestation (organogenesis period) induced a very highly significant decrease in the mean maternal body weights. These results are in agreement with other researchers. [28&29] Moreover, a very highly significant decrease in the number of live fetuses, fetal body weights and lengths were obvious, these results are in agreement with other authors [29,30&31] who observed morphological alterations which may indicate that aspartame crossed the placenta causing a reduction of fetal weight, following the administration. However, a lot of congenital anomalies were evident such as subdermal blood bleeding, evisceration, anomalies of the fore and hind limbs as well as kyphosis of the body. These results agree with those of **Araújo *et al*.**[15]who stated that administered 20mg aspartame /kg body weight daily from the 10^{th} to the 14^{th}day of pregnancy caused delayed fetal development. The nutritional environment during embryonic, fetal and neonatal development plays a crucial role in the offspring's risk of developing diseases later in life as cited by [15] these toxicological changes were directly proportional to the duration of its administration. People regularly consume low doses of methanol in fruits, vegetables and fermented beverages as well as soft drinks and foods sweetened with aspartame (which breaks down to methanol in the gastrointestinal tract) and this may be highly toxic if sufficient quantities are consumed. [28] Methanol consumption may result in metabolic acidosis, blindness, and even death. Although the body has the capacity to metabolize the low doses of methanol to which people are regularly exposed, it cannot handle high doses because too much methanol reduces the body's ability to remove a toxic metabolite (formate),when formate accumulates, methanol poisoning occurs; these observations are in agreement with those of **Magnuson**[32]who cited that methanol breaks down into formic acid and formaldehyde in the body which is a deadly neurotoxin; the methanol metabolites cause CNS depression, vision disorders and other symptoms leading ultimately to metabolic acidosis and coma. One factor that regulates the rate at which formate is removed is the liver level of a derivative of the folic acid. People who are deficient in folic acid (including 15% to 30% of pregnant women) may be particularly susceptible to the toxic effects of methanol. Methanol is not classified as a human reproductive toxicant. However, fetal toxicity may arise secondary to maternal toxicity. Methanol is rapidly transferred to the fetus and cumulates in fetal circulation. It is unlikely that exposure to low concentrations of methanol would result in adverse effects in the fetus. [33]

The physiological results of the present study revealed very highly significant increase($P<0.001$) in of activities of aspartate aminotransferase (SAST), γ-glutamyl transferase (SGGT) and creatinine in the serum of the pregnant rats with aspartame by 2doses (20mg./kg. and 40 mg./kg. body weight) on days 7, 9, 11 and 13 of gestation compared with those of the control group these results agree with Abhilash *et al*.[10]who observed that aspartame induced a significant increase in activities of serum aspartate aminotransferase (SAST) and serum γ-glutamyl transferase (SGGT) and they attributed these results to that long term consumption of aspartame may lead to injury and alterations of antioxidant status , however these observations agree with the observations of [34&35]that methanol and its metabolites may be responsible for the generation of oxidative stress. According to **Hansen and Harris** [36]the oxidative stress leading to dysmorphogenesis.

On the other hand, intraperitoneally injection with astaxanthin (ATX)to the pregnant rats ((50mg./kg. body weight) 2 hours after aspartame injection by its 2 doses resulted in improvement of the morphometric, morphological and physiological changes caused by aspartame injection by its 2 doses, these results are in agreement with those who cited that astaxanthin is a powerful antioxidant which can support anti-oxidative defense mechanisms, anti-inflammatory and anticancer agent also it shows neuroprotective property, protects hepatotoxicity, lipid peroxidation and renal injury as well as it prevents oxidative stress on human endothelial cells without inducing any toxicity.[19, 20,21,37, 38 ,39,40,41&42]

CONCLUSION

According to the results obtained in the present study intraperitoneally injection with Astaxanthin after aspartame injection at the 2 doses provides obvious improvement of aspartame induced physiological and teratological effects in the pregnant albino rats and their fetuses.

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Tables

Table 1-: Morphometric studies on the pregnant rats of the different groups.

Groups / Parameters	saline	Olive oil	Asp. 20mg/kg.	Asp. 40mg/kg	AXT	AXT+Asp 20mg/kg	AXT+Asp 40mg/kg
Mean Increasing Maternal B. Wt./g.	42	47.33	32.33	23.66	57.33	42.5	35
Standard Error	±1	±2.02 ***	±0.66 ***	±2.33 ***	±3.84 ***	±1.84	±1.46 ***
Mean uterus Wt./g.	38.65	39.47	33.12	23.08	48.54	37.76	32.48
Standard Error	±0.39	±0.7	±2.18 ***	±3.89 ***	±1.9 ***	±1.05	±0.74 ***
% of abortion	-	-	30	50	-	-	-
Mean fetal absorption	0	0	3	5.8	0	1.4	2.2
Standard Error	0	0	±1.09	±1.06	0	±0.4	±0.37

* Significant at P<0.05 **Highly significant at P<0.01 *** Very highly significant at P<0.001

Table 2- Morphometric studies on the fetuses of the different groups.

Groups / Parameters	saline	Olive oil	Asp. 20mg/kg.	Asp. 40mg/kg	AXT	AXT+Asp 20mg/kg	AXT+Asp 40mg/kg
Mean number of live embryos	10.3	10.6	5.1	0.1	11.2	8.6	5.1
Standard Error	±0.59	±0.71	±0.27 ***	±0.1 ***	±0.32	±0.36 **	±0.27 ***
Mean number of Dead embryos	0	0	2.3	5	0	0.9	3.1
Standard Error	0	0	±0.21	±0.25	0	±0.27	±0.1
Mean Fetal B. wt/g.	4.17	4.39	2.53	2.09	4.63	4.14	3.29
Standard Error	±0.02	±0.03 **	±0.16 ***	±0.1 ***	±0.03 ***	±0.06	±0.07 ***
Mean Fetal B. lengths/cm.	4.1	4.09	3.15	2.74	4.2	3.75	3.22
Standard Error	±0.02	±0.01	±0.04 ***	±0.1 ***	±0.01	±0.07 ***	±0.13 ***
Mean Fetal Tail Lengths/cm.	1.52	1.49	1.29	1.1	1.5	1.35	1.19
Standard Error	±0.01	±0.01	±0.34	±0.004 **	±0.001	±0.01	±0.005 *

* Significant at P<0.05 **Highly significant at P<0.01 *** Very highly significant at P<0.001

Table 3- Physiological studies on the pregnant rats of the different groups.

Groups / Parameters	saline	Olive oil	Asp. 20mg/kg.	Asp. 40mg/kg	AXT	AXT+Asp 20mg/kg	AXT+Asp 40mg/kg
Mean SAST Activity in Pregnant Rats U/l	7.48	7.41	10.87	12.59	7.31	9.26	9.78
Standard Error	±0.77	±0.75	±3 ***	±1.23 ***	±0.77	±0.69 ***	±0.74 ***
Mean SGGT Activity in Pregnant Rats U/l	16.25	15.75	55.5	81	14.5	27.75	36.66
Standard Error	±1.11	±1.31	±5.98 ***	±4.08 ***	±1.91	±1.11 *	±2.96 ***
Serum Creatinine/U/l	0.3	0.3	0.43	0.45	0.3	0.3	0.32
Standard Error	0	0	±0.03 ***	±0.02 ***	0	0	±0.02

* Significant at P<0.05 **Highly significant at P<0.01 *** Very highly significant at P<0.001

Figures

Fig 1- A uterus on day 20 of gestation of saline injected group showing two equal horns.

Fig 2-A uterus on day 20 of gestation of olive oil injected group showing that the two horns are equal.

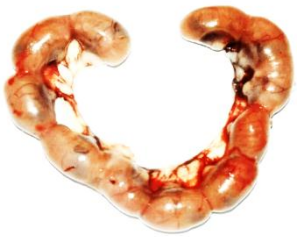

Fig 3- A uterus on day 20 of gestation of astaxanthin injected group showing that the two horns are equal.

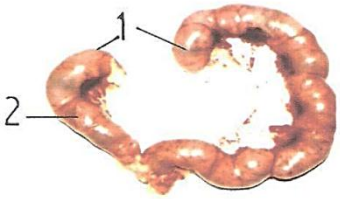

Fig 4- A uterus on day 20 of gestation of aspartame injected subgroup (20mg./kg b. wt. showing that the two horns are unequal (1) and the fetuses were unequally distributed between them (2).

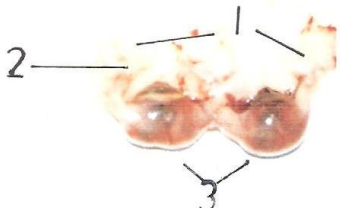

Fig 5- A uterus on day 20 of gestation of aspartame injected sub group (40mg./kg b. wt) showing shortness of the 2 horns (1), lot of fats around them (2) as well as obvious signs of abortion of some embryos (3).

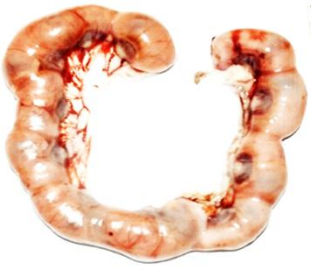

Fig 6-A uterus on day 20 of gestation of astaxanthin injected sub group after aspartame injection (20mg./kg b. wt) showing improvement of the uterus.

Fig 7-A uterus on day 20 of gestation of astaxanthin injected sub group after aspartame injection (40mg./kg b. wt) showing improvement of the uterus.

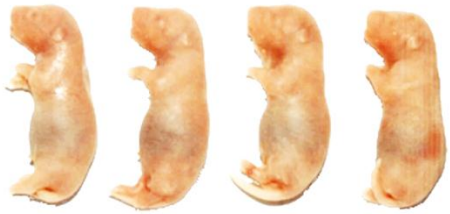

Fig 8-Fetuseson day 20 of gestation of the saline injected group showing normal external morphology.

Fig 9-Fetuses on day 20 of gestation of the olive oil injected group showing normal external morphology.

Fig 10-Fetuses on day 20 of gestation of the astaxanthin injected group showing normal external morphology.

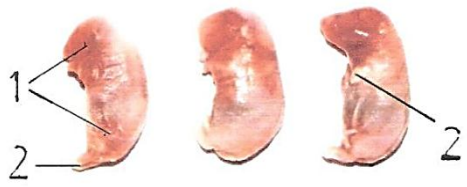

Fig 11-Fetuses on day 20 of gestation of aspartame injected sub group (20mg./kg b. wt). showing hematoma in the head and hind regions (1) with malformed fore and hind limbs (2).

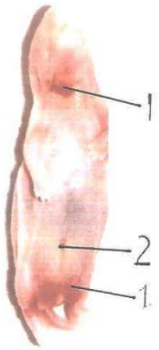

Fig 12- A fetus on day 20 of gestation of aspartame injected sub group (20mg./kg b. wt) showing hematoma in the neck and hind regions (1) with hernias of the skin on some regions (2).

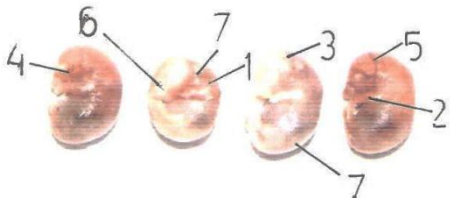

Fig 13-Fetuses on day 20 of gestation of aspartame injected sub group (40mg./kg b. wt) showing kyphosis (1), clubbed fore and hind limbs (2), exencephaly (3), exophthalmos (4), cognition of the blood vessels in the head region (5), cleft lips appeared (6), the skin seemed thin and fragile in the head region as well as the fore and hind regions (7).

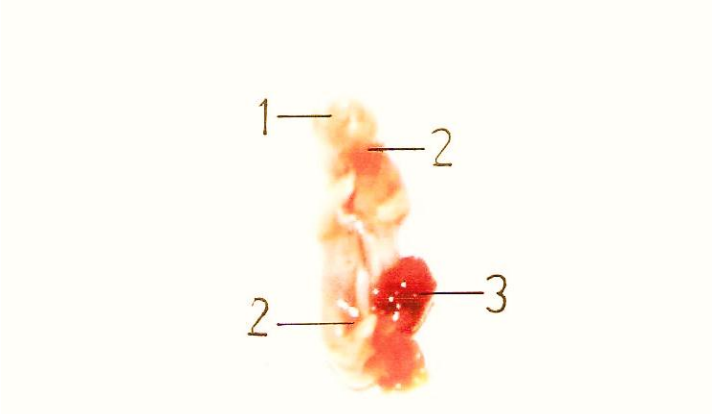

Fig 14- A fetus on day 20 of gestation of aspartame injected sub group (40 mg./kg b. wt) showing malformed head (1), hematoma on the neck and hind regions (2) with protrusion of the viscera (evisceration).

Fig 15-Fetuses on day 20 of gestation of astaxanthin injected sub group after aspartame injection (20mg./kg b. wt) on day 20 of gestation showing improvement of the fetuses.

Fig 16- Fetuses on day 20 of gestation of astaxanthin injected sub group after aspartame injection (40mg./kg b. wt) on day 20 of gestation showing improvements of the fetuses.