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RESEARCH ARTICLE

Effect of aspartame on the placenta of adult albino rat. A histological and immunohistochemical study

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ABSTRACT

Aspartame is an artificial sweetener usually consumed by hundreds of millions of persons all over the world. Its metabolites can be toxic to many organs and there are only a few studies on the use of aspartame during gestation. The present study was designed to fully evaluate the effect of aspartame on the histological structure of the placenta in the adult albino rat. Twenty pregnant female rats were equally divided into group I that served as control, and group II that received aspartame at a dose 14 mg/kg by gavage on the 9th, 10th and 11th day of pregnancy. Placental specimens were processed for histological and immunohistochemical staining against vascular endothelial growth factor (VEGF). Aspartame induced a significant decrease in the mean placental weight and the mean thickness of both labyrinth and basal zones. Damage in the placenta was detected in the form of rupture of the interhemal membrane, lysis of glycogen trophoblast cells, spongiotrophoblast cells with vacuolated cytoplasm and darkly stained nuclei. A significant increase in vascular endothelial growth factor expression in both labyrinth and basal zones was detected. Ultrastructural examination showed fetal capillaries with condensed nuclei of endothelial cells, cytotrophoblasts with condensed fragmented nuclei and vacuolated cytoplasm, and syncytiotrophoblasts with irregular condensed fragmented nuclei. It could be concluded that aspartame has deeply impacted the normal structure and presumably the function of the placenta, therefore, restrictions are to be imposed on the consumption of aspartame especially during pregnancy.

1. Introduction

The placenta is a transient structure and an essential organ for fetal development. It has many important functions such as exchange of various materials between the maternal and fetal circulatory systems, hormonal production, as well as its immunological functions that are necessary for the maintenance of a proper pregnancy (Carter, 2012; Boyd, 2013; Malek, 2013). Moreover, it acts as a protective barrier against xenobiotics. Therefore, drug or chemical-induced placental dysfunction or injury usually interferes with the maintenance of pregnancy and fetal growth and development (Furukawa et al., 2011).

Generally, rat placental models have been useful for evaluating the effect of drugs or chemicals on human reproductive development, owing to the several similarities between rat and human in early placental development (Pijnenborg et al., 1981). However, there are some differences between rats and humans (Maranghi et al., 1998; Georgiades et al., 2002), such as the embryo/fetal period ratio, implantation type, function of the yolk sac placenta, placental structure, and endocrine synthesis (Nakanishi, 2007). Thus, extrapolating data from rat to human in drug- or chemical-induced developmental toxicity are usually done based on fully understanding the differences and similarities between the rat and human placenta.

Aspartame is an artificial sweetener commonly consumed by hundreds of millions of persons all over the world (Rycerz and Jaworska-Adamu, 2013). It has many commercial names as Nutra Sweet, diet sweet and others. It is present in about 6000 food products like chewing gum, tabletop sweeteners, candies, soft drinks and medicines (Butchko et al., 2002). Aspartame is almost non-caloric and is 200 times sweeter than sucrose. This allows the use of even small doses to give the same level of sweetness (Rowe et al., 2009), therefore, diabetic patients including children and individuals desiring weight loss use these products frequently (Humphries et al., 2008).

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Aspartame is metabolized in the gastrointestinal tract into phenylalanine, methanol and aspartic acid, and further into formaldehyde and formic acid in many tissues (Butchko et al., 2002). Human studies (Sturtevant, 1985) reported that the metabolic products of aspartame, namely phenylalanine cross the placenta, even if they argued that this would not interfere with fetal development.

Soffritti et al. (2005) stated that aspartame is considered a multipotential carcinogenic agent based on increases in malignant tumor-bearing animals after a long-term administration of aspartame at different doses as low as 4 and 20 mg/kg in rats. Administration of aspartame during pregnancy was reported to induce a decrease in the placental and fetal weights as well as the length of umbilical cord in rats at a dose of 14 mg/kg (De Matos et al., 2013). Hence, the present study was designed to evaluate histological alteration in the placenta of rats submitted to the administration of aspartame at a dose 14 mg/kg according to the previous study done by Portela et al. (2007) during the gestation period extending from ninth to eleventh day of pregnancy, which coincided with the biggest stage of embryogenesis. Moreover, it is the time when the largest number the decidual cells are present as they predominate in the placenta on day six of pregnancy in rats and increase in number until the tenth day and decrease on the fourteenth day (De Matos et al., 2013).

Many earlier studies utilized the rat placental weight as a placental toxicity index (Khera, 1992; Akay and Kockaya, 2005) which is not enough to evaluate the histopathological changes that differentially occur in the placenta depending on the causing toxicant and the period of exposure, in order to properly understand the underlying mechanism, teratogenicity and developmental toxicity (Furukawa et al., 2011). Therefore, the present study was designed to evaluate the effect of aspartame on the histological structure of placenta in adult female albino rats.

2. Materials and methods

2.1. Animals and experimental design

Virgin adult female albino rats weighing between 180 and 200 g were used in this study. They were maintained in clean ventilated cages under controlled conditions of temperature (22 ± 2 °C), humidity (50 ± 10%), and a 12-h light/dark cycle with free access to a standard commercial rodent diet and tap water. All animal work was conducted in accordance with the guidelines for the use of animals in research established by the local ethical committee of the Faculty of Medicine, Zagazig University, Egypt (Approval number: ZU-IACUC/3/F/86/2018). These guidelines comply with the international guidelines set by national institutes of health guide for the care and use of laboratory animals.

Following one week of acclimatization to the laboratory environment, the female rats were mated with males with a ratio (1:1). Finding the sperm in the vaginal smear was designated as day 1 of pregnancy. Twenty pregnant female rats were included in this research and were randomly allocated into two equal groups: **Group I (Control group):** that was further subdivided into two equal subgroups: subgroup (i) that was kept without any treatment throughout the experiment and subgroup (ii) which received 0.5 ml of water (the diluting vehicle for aspartame) via oral gavage on the 9th, 10th and 11th days of pregnancy. **Group II (Aspartame group):** that received aspartame at a dose 14 mg/kg by gavage on the 9th, 10th and 11th days of pregnancy (De Matos et al., 2013; Portela et al., 2007). Observation of any vaginal bleeding of the pregnant female was considered as an indicator of abortion (Abu Gabal and Al Waely, 2016). Aspartame was purchased from Al- Ameriya Pharma Company, Egypt in the form of tablets, each containing 20 mg of aspartame. The tablets were dissolved in tap water and given to pregnant rats in the previously mentioned dose.

On the 19th day of gestation, the pregnant animals were anesthetized using ethyl ether (Ha and Kim, 2013). A lower midline abdominal incision was done, and the gravid uterine horn was exposed and held out with forceps. The uterus was excised and washed in phosphate buffered saline (PBS) (Šerman et al., 2015). The uterine horns were dissected under the binocular dissecting microscope, the fetal viability and the condition of the placenta, fetal membranes, and fluids were examined in situ. At that time, the number and distribution of implantation sites were recorded and classified as either live fetuses or dead implantations. Any dead implantations were then subclassified into early resorptions or late resorptions, with visible dead fetal tissue. Only all placentas with living fetuses were isolated and weighed then they were cut in two equal parts, one half was processed for light microscopy and the other for transmission electron microscopy.

2.2. Histological analysis

2.2.1. Light microscopic studies

For light microscopy; the placentas were cut into 5 mm^3 pieces, fixed in 10% buffered formaldehyde solution, dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax (Chandler and Roberson, 2009). Serial sections of 5 μm thickness were cut and subjected to the following techniques:

- Hematoxylin and Eosin staining (Gamble, 2008): to study the general histological structure of rat placenta.
- Immunohistochemical staining using the streptavidin-biotin-peroxidase technique for vascular endothelial growth factor (VEGF).

The immunohistochemistry was done according to (Shalaby and Bahey, 2018). Briefly, sections were treated with 3% H_2O_2 in PBS for 30 min to block endogenous peroxidase activity. Each of the successive steps was followed by a thorough rinse with PBS. All steps were performed in a humid chamber under care to avoid dehydration of the sections. Antigenic retrieval was performed by immersing the slide in citrate sodium solution (10 mM, pH 6.0) for 15 min at 95 °C. Non-specific staining was blocked by incubating the slide with 10% normal goat serum in PBS for 1 h at room temperature. Then the sections were incubated overnight at 4 °C in the diluted primary antibody; rabbit monoclonal vascular endothelial growth factor (VEGF) (GENNOVA, cat. #AP10527C). After rinsing in PBS, sections were incubated with biotin-conjugated goat anti-rabbit IgG (Rockland, Gilbertsville, PA, USA), diluted 1:1000 in PBS for 2 h at room temperature, followed by washing in PBS. The avidin-biotin complex was added to the sections for 20 min. The sections were then incubated with diaminobenzidine (DAB) as chromogen followed by staining with Mayer's hematoxylin as a counterstain. Finally, the slides were dehydrated, cleared and mounted with DPX.

2.3. Electron microscopic studies

Processing the placental samples for transmission electron microscope was done according to Woods and Stirling (2008). To summarize, very small pieces of placental tissue (1 mm^3) were fixed in 2.5% phosphate-buffered glutaraldehyde for two hours at 4 °C. After rinsing in PBS, the specimens were then post-fixed in prepared 1% phosphate buffer osmium tetroxide for one hour at 4 °C. After that, the specimens were dehydrated in ascending grades of alcohol at 4 °C. Sections were then immersed in propylene oxide and embedded in epoxy resin mixture. Semithin sections (0.5–1 μm thick) were cut by the automatic LKB ultramicrotome, mounted on glass slides, stained with toluidine blue and examined under

Table 1
Morphometrical and statistical analysis of the different study groups.

Parameters			Control group	Aspartame-treated group
Mean placental weight (g)			0.603 ± 0.05	0.428 ± 0.04[b]
Mean thickness (μm)	Labyrinth zone		450.33 ± 32.11	279.39 ± 20.44[b]
	Basal zone		127.48 ± 16.72	95.09 ± 7.88[b]
VEGF immunopositive reaction	Labyrinth zone	Mean area percentage %	43.29 ± 3.82	48.64 ± 7.99[b]
		Mean color intensity	25.49 ± 3.56	36.77 ± 6.27[b]
	Basal zone	Mean area percentage %	8.01 ± 2.73	11.78 ± 3.96[a]
		Mean color intensity	19.33 ± 2.98	26.84 ± 4.61[a]

Data is expressed as mean ± standard deviation.
[a] Indicates significance vs control.
[b] Indicates high significance vs control.

the light microscope to select the suitable areas. Ultrathin sections (80–100 nm thick) were cut and contrasted with uranyl acetate and lead citrate for examination under JEOL-JEM-100 transmission electron microscope (Japan). Electron microscopy sample processing, examination and photographing were done in the electron microscopy unit, Faculty of Medicine, Tanta University, Egypt.

2.4. Morphometric analysis

A Leica light microscope (DM500, Switzerland) coupled to a Leica digital camera (ICC50, Switzerland) was used for image acquisition at the histology department, Faculty of Medicine, Tanta University. The software "ImageJ" (version 1.48v National Institute of Health, Bethesda, Maryland, USA) was used for image analysis. Ten different non-overlapping randomly selected fields from each slide were examined to quantitatively evaluate:

1) The mean thickness of labyrinth and basal zones in μm [in H&E stained sections at a magnification of 100].
2) The mean area percentage and color intensity of VEGF positive immunohistochemical reaction in the labyrinth and basal zones [in DAB-stained sections at a magnification of 400].

2.5. Statistical analysis

The data were analyzed by using Student T-test for comparison between the two groups using statistical package for social sciences statistical analysis software (IBM SPSS Statistics for Windows, IBM Corp, version 22.0. Armonk, NY, USA). All values were expressed as mean ± standard deviation. Differences were regarded as significant if probability value $p < 0.05$ and highly significant if $p < 0.001$ (Dawson-Saunders and Trapp, 2001).

3. Results

During the study period, no morbidity or mortality or vaginal bleeding was recorded in the experimental animals. Late fetal resorptions, characterized macroscopically by the presence of placental membranes and embryo-fetal tissue at different degrees of autolysis, were recorded in the aspartame group (≤2/dam). Upon examination of placentas in each animal of each control subgroup, no apparent or evident histological differences were noted between the two subgroups and thus were represented collectively as a single control group.

3.1. Placental weight measurements

Rats treated with aspartame showed a highly significant decrease in the mean placental weight (0.428 ± 0.04 g) as compared to the control group (0.603 ± 0.05 g) (Table 1) (Histogram 1A).

3.2. Placental thickness measurements

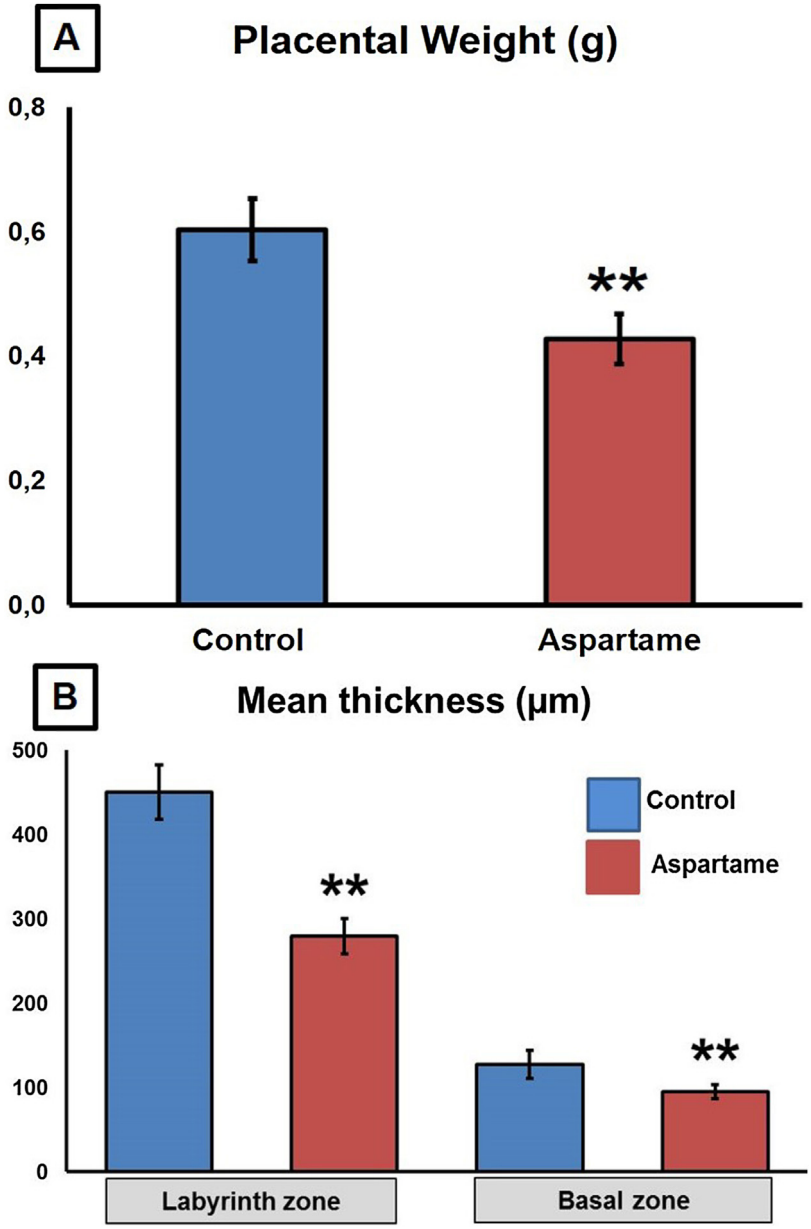
Placentas of rats treated with aspartame showed a highly significant decrease in the mean thickness of both labyrinth and basal zones (279.39 ± 20.44 μm, 95.09 ± 7.88 μm respectively) as compared to the control group (450.33 ± 32.11 μm, 127.48 ± 16.72 μm respectively) (Table 1) (Histogram 1B).

3.3. Placenta histopathology

The hematoxylin and eosin-stained placental sections of the control group showed the normal architecture of the mature rat placenta on GD 19. It showed three characteristic morphological zones, the labyrinth, basal and decidua (Fig. 1A). The labyrinth zone was composed of a web-like vascular system of sinuses for maternal blood and capillaries for fetal blood circulation. Both circulations were strongly interdigitated with each other but were compartmentalized by the interhemal membrane. This membrane was composed of cytotrophoblast cells and syncytiotrophoblast cells with their elongated nuclei. Fetal capillaries were lined by endothelial nuclei and had a smaller luminal diameter in comparison to the maternal sinuses (Fig. 1B). The basal zone consisted of the main population of spongiotrophoblast cells with basophilic cytoplasm intermingled with glycogen trophoblast cells that were arranged in clusters with a clear and vacuolated aspect. A layer of giant trophoblast cells with large euchromatic nuclei formed the border between the basal zone and the decidua (Fig. 1C). On the other hand, placental sections of the aspartame-treated group showed an apparent decrease in the thickness of labyrinth and basal zones (Fig. 1D). The labyrinth zone showed rupture of the interhemal membrane with loss of blood compartmentalization and the cytotrophoblasts had darkly stained nuclei (Fig. 1E). Moreover, glycogen trophoblast cells underwent lysis leaving areas filled with eosinophilic fluid (Fig. 1F). Additionally, spongiotrophoblast cells showed vacuolated cytoplasm and darkly stained nuclei. The giant trophoblast cells had shrunken dark nuclei, and caused nuclear vacuolation as well as disruption of their plasma membrane (Fig. 1G).

3.4. Immunohistochemistry of VEGF

The placental sections were immunolabelled with an antibody against VEGF, a specific marker for angiogenesis. The placental sections of the control group showed few areas with a weak VEGF expression in the trophoblasts in the labyrinth zone and in the cytoplasm of the spongiotrophoblasts and giant cells in the basal zones (Fig. 2A and B). While the aspartame-treated group showed extended areas with a strong VEGF expression in the trophoblasts in the labyrinth and in the cytoplasm of the spongiotrophoblasts and giant cells basal zones (Fig. 2C and D). The morphometrical and statistical analysis of the mean area percentage and color intensity of VEGF immunoexpression in the labyrinth zone in aspartame-treated group revealed a highly significant increase



Histogram 1. Morphometrical and statistical analysis of [A] Placental weight and [B] Mean thickness of labyrinth and basal zones. * indicates significance vs control, ** indicates high significance vs control.

(48.64 ± 7.99, 36.77 ± 6.27 respectively) as compared to the control group (43.29 ± 3.82, 25.49 ± 3.56 respectively). Additionally, the mean area percentage and color intensity of VEGF immunoexpression in the basal zone in aspartame-treated group revealed a significant increase (11.78 ± 3.96, 26.84 ± 4.61 respectively) as compared to the control group (8.01 ± 2.73, 19.33 ± 2.98 respectively) (Table 1) (Histogram 2).

3.5. Placenta ultrastructure

The transmission electron microscopic analysis of the placenta ultrathin sections of the control group revealed the normal ultrastructure of the placenta. The labyrinth zone showed fetal capillaries and maternal blood spaces separated by interhemal membrane containing cytotrophoblast cells with euchromatic nuclei (Fig. 3A). The blood in fetal capillaries was separated from maternal blood by blood placental-barrier which consisted of the endothelial cell lining fetal capillary, the basement membrane of the fetal capillary and syncytiotrophoblast cells which possessed numerous microvilli projecting into maternal blood space (Fig. 3B and C). While the basal zone showed spongiotrophoblast cells having rounded euchromatic nuclei, rough endoplasmic reticulum, and mitochondria (Fig. 3D).

On the other hand, placental ultrathin sections of the aspartame-treated group showed degenerative changes in the labyrinth zone in the form of thinning and destruction of interhemal membrane (Fig. 3E). Some fetal capillaries were lined by endothelial cells having shrunken condensed nuclei and were surrounded by a trophoblastic layer containing swollen mitochondria with complete loss of their cristae (Fig. 3F). In addition to the presence of syncytiotrophoblast cells with irregular nuclei and few microvilli projecting into maternal blood spaces, others appeared with irregular condensed fragmented nuclei (Fig. 3G). Collagen fibers appeared beneath syncytiotrophoblast cells (Fig. 3H). Moreover, the cytotrophoblast cells showed fragmentation of their condensed chromatin and vacuolation of their cytoplasm (Fig. 3I). As regards spongiotro-

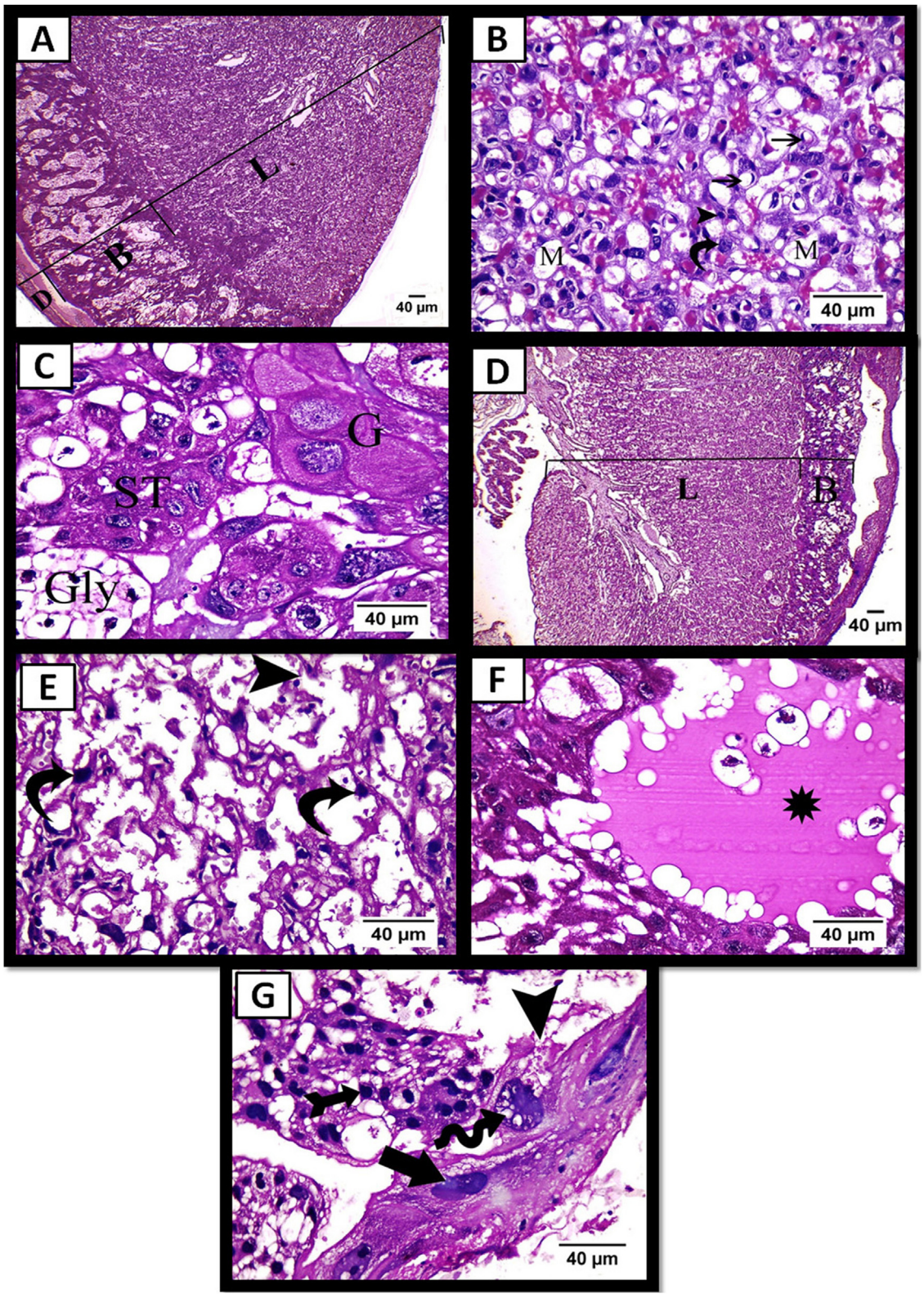


Fig. 1. Representative photomicrographs of hematoxylin and eosin-stained placental sections of the experimental groups showing; **[A]** Normal architecture of rat placenta consisting of labyrinth zone (L), basal zone (B) and decidual zone (D). **[B]** The normal labyrinth is composed of a web-like vascular system of maternal blood spaces (M) and fetal capillaries (thin arrows) separated by interhemal membrane containing cytotrophoblast (curved arrow) and syncytiotrophoblast (arrowhead) cells. **[C]** The normal basal zone consisting of spongiotrophoblast cells (ST) with basophilic cytoplasm, glycogen cells (Gly) with clear vacuolated aspect and giant trophoblast cells (G) with large euchromatic nuclei. Sections of the aspartame-treated group showing; **[D]** Apparent decrease in the thickness of labyrinth (L) and basal (B) zones. **[E]** Rupture of the interhemal membrane (arrowhead) with loss of blood compartmentalization. Notice cytotrophoblasts (curved arrows) with darkly stained nuclei. **[F]** Lysis of glycogen trophoblast cells leaving areas filled with eosinophilic fluid (asterisk). **[G]** Spongiotrophoblast cells with highly vacuolated cytoplasm and darkly stained nuclei (bifid arrow). The giant trophoblast cells have shrunken dark nuclei (thick arrow), nuclear vacuolation (wavy arrow) and disruption of the plasma membrane (arrowhead).

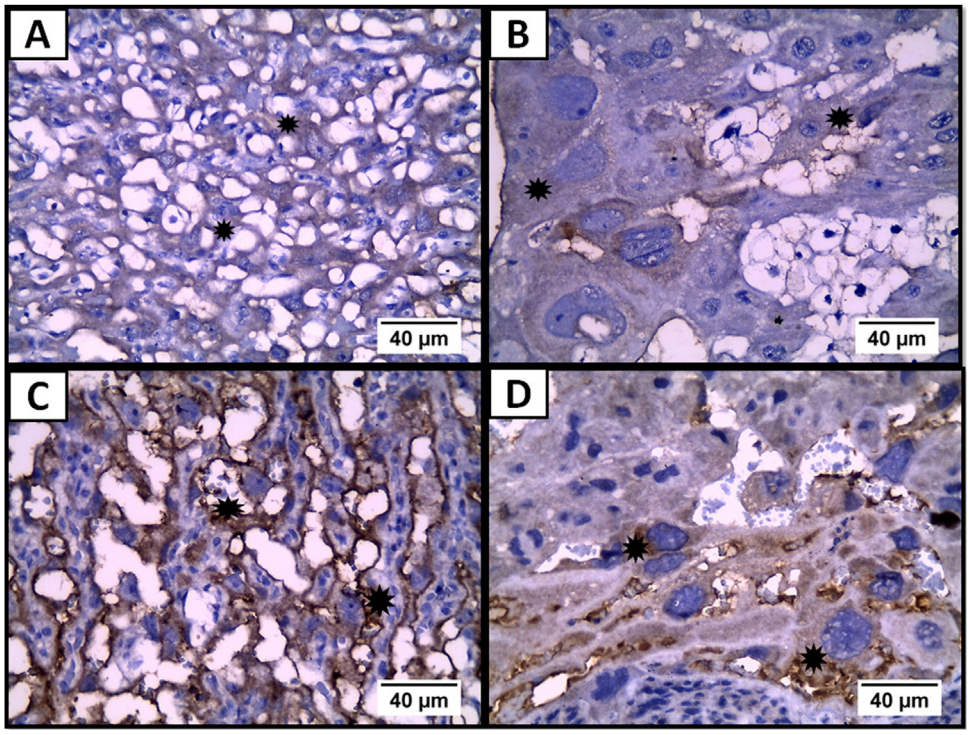
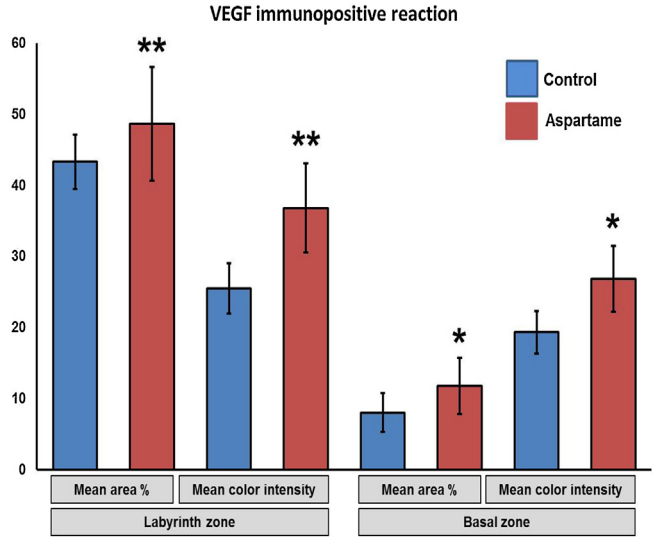


Fig. 2. Representative photomicrographs of immunohistochemical stained placental sections with the anti-VEGF antibody; **[A]** and **[B]** The control group shows few areas with weak VEGF expression in the trophoblasts in the labyrinth and in the cytoplasm of the spongiotrophoblasts and giant cells of the basal zone respectively (asterisks). **[C]** and **[D]** Aspartame-treated group shows extended areas with strong VEGF expression in the trophoblasts in the labyrinth and in the cytoplasm of the spongiotrophoblasts and giant cells of the basal zone respectively (asterisks).



Histogram 2. Morphometrical and statistical analysis of VEGF immunopositive reaction. * indicates significance vs control, ** indicates high significance vs control.

phoblast cells in the basal zone, some showed irregular nuclear outlines with peripheral condensation of their chromatin and vacuolation of their cytoplasm (Fig. 3J). Others had shrunken nuclei (Fig. 3K).

4. Discussion

Aspartame is an artificial sweetener with a carcinogenic potentiality particularly in female rats even at low daily doses down to 20 mg/kg/day (Soffritti et al., 2005). The present study could show a significant decrease in the placental weight associating with a significant thinning of both labyrinth and basal zones, this resembles the effect of gestational protein restriction in rats (Rebelato et al., 2016). This could be attributed to the mitotic inhibition, apoptosis, degeneration of trophoblasts (Furukawa et al., 2011).

Light microscopic results could reveal rupture of the interhemal membrane with loss of blood compartmentalization in the labyrinth zone, this might be attributed to the lipid peroxidation action of aspartame reported in rats at a dose of 40 mg/kg for up to six weeks (Mourad, 2011). Such degenerative changes may be due to the direct toxic effect of aspartame on placenta or as a consequence of the release of free radicals and production of aspartic acid and methanol (Maaruf et al., 2017). These signs of degeneration correspond to a moderate placental degeneration of grade 3 (Rey Moreno et al., 2013), they suggested that the damage of the interhemal membrane was not only a mechanical damage by the cystic dilation of the maternal sinuses but also by the changes in the cellular detail including karyolysis, cytoplasmic vacuolization, and eosinophilic cytoplasmic change.

Moreover, sections from the basal zone of the placenta revealed lysis of glycogen trophoblast cells, which might be attributed to the failure of their designated regression that should have been almost complete toward the end of pregnancy (Furukawa et al., 2011). Additionally, the spongiotrophoblast and giant trophoblast cells revealed several cytoplasmic and nuclear alterations. As regards the nuclear disruption, Elfatah et al. (2012) reported massive structural and numerical chromosomal abnormalities along with a highly significant increase in the percentage of DNA fragmentation in pregnant female rats and their offspring upon maternal exposure to aspartame at a dose of about 50 mg/day. They attributed these findings to the accumulation of the aspartame-derived methanol and its metabolite formaldehyde adducts that exerted their cytotoxicity through the functional alteration of proteins and DNA mutations, thus leading to cell death or a malignant transformation. Additionally, the nuclear damage could be induced by oxidative stress

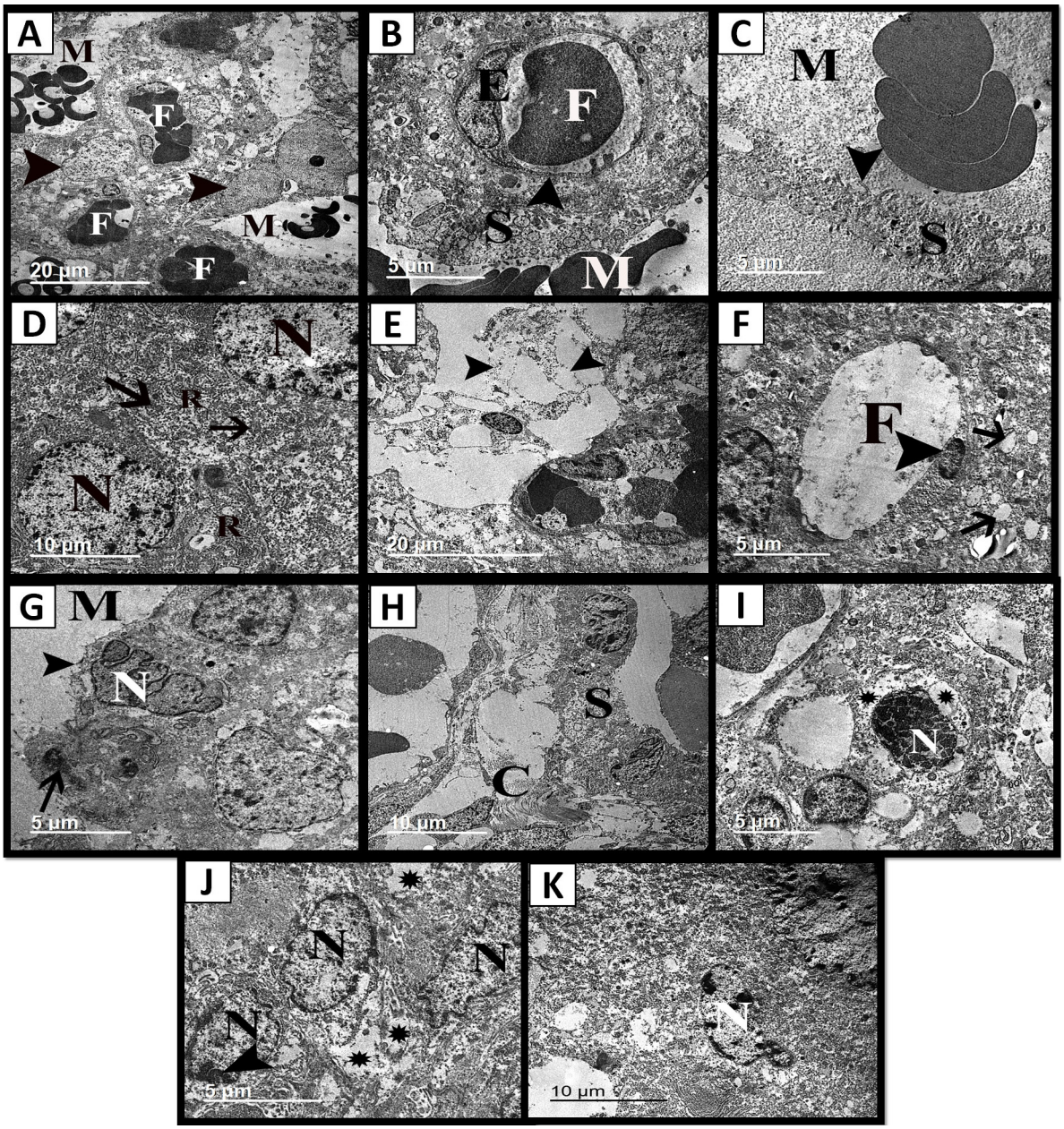


Fig. 3. Representative transmission electron photomicrographs of placenta ultrathin sections of the experimental groups showing **[A]** the normal labyrinth zone with fetal capillaries (F), maternal blood spaces (M) and cytotrophoblasts with euchromatic nuclei (arrowheads) in the interhemal membrane. **[B]** A fetal capillary (F) and maternal blood space (M) in the labyrinth zone separated by a thin placental barrier formed of endothelial cell of the fetal capillary (E), the basement membrane of the fetal capillary (arrowhead), and syncytiotrophoblast cell (S). **[C]** Syncytiotrophoblast cell (S) with numerous microvilli (arrowhead) projecting into maternal blood space (M) in the labyrinth zone. **[D]** Two spongiotrophoblast cells in the basal zone with rounded euchromatic nuclei (N), Rough endoplasmic reticulum (R) and mitochondria (arrows). Sections of the aspartame-treated group showing; **[E]** The labyrinth zone with thinning and destruction of the interhemal membrane (arrowheads). **[F]** A fetal capillary (F) lined with an endothelial cell having a shrunken condensed nucleus (arrowhead) and surrounded by trophoblast layer containing swollen mitochondria with complete loss of their cristae (arrows). **[G]** Syncytiotrophoblast cells with irregular nucleus (N), others with irregular condensed fragmented nuclei (arrow), few and short microvilli (arrowhead) projecting into maternal blood space (M). **[H]** Collagen fibers (C) beneath syncytiotrophoblast cells (S). **[I]** A cytotrophoblast cell with a condensed fragmented nucleus (N) and vacuolated cytoplasm (asterisks). [J] Spongiotrophblast cells having irregular nuclei (N) with peripheral condensation of chromatin (arrowhead) and vacuolated cytoplasm (asterisks). **[K]** Spongiotrophoblast cell with a shrunken nucleus (N).

propagated by the methanol metabolite of aspartame as previously reported in rats consuming up to 1000 mg/kg/day (Abhilash et al., 2011). Moreover, De Matos et al. (2013) reported a significant alteration in the karyometric parameters related to the nuclear shape and size of the placenta upon aspartame ingestion from 10th to the 14th day of pregnancy.

The methanol metabolite could be also responsible for the cell membrane damage after aspartame administration due to the increased mitochondrial production of superoxide anion and hydrogen peroxide. On the other hand, cytoplasmic vacuolation is most likely induced by inhibition of intralysosomal proteolysis (Rogers et al., 1985). Additionally, trophoblastic giant cells have been reported as the precursor cells of more differentiated trophoblasts. Their degeneration could finally cause diminishment of trophoblasts in general (Kosif et al., 2008).

In the present work, immunohistochemical staining against VEGF revealed a significantly increased expression in the labyrinth zone rather than the basal zone. It was reported that the placenta

secretes several growth factors including placenta growth factor that promotes proliferation of placental vasculature with an affinity to VEGF receptors (Hauser and Weich, 1993). Expression of VEGF proved an important role in promoting normal placental angiogenesis, which is crucial for gestational success, this process involves proliferation, migration, and maturation of both maternal and fetal endothelial cells, the process is dynamic towards the end of pregnancy (Pietro et al., 2010). Aspartame is presumed to have the ability to induce oxidative stress (Prokić et al., 2015). Additionally, Alleva et al. (2011) suggested that aspartame is a potential angiogenic agent that induces radical oxygen species (ROS) production and oxidative stress, thus stimulating a number of cytokines and growth factors, namely the VEGF and its soluble receptors release from the endothelial cells. The VEGF would then induce vascular smooth muscle cell proliferation especially in hypoxic cells (Herrera et al., 2014).

On the other hand, Farias et al. (2014) suggested that hypoxia was responsible for the increased VEGF expression, yet they reported an increased VEGF expression in the basal zone rather than the labyrinth zone while studying the effect of diabetes on the placenta (Zorn et al., 2011).

Placental ultrastructural examination of the aspartame-treated group in this work depicted several degenerative signs. Similar degenerative changes have been reported in pre-eclampsia and hypertensive diseases of pregnancy studies, suggesting that these cells are most likely to be affected by placental injury. The decreased microvillus density observed in the current study has been reported with other complications such as pre-eclampsia (Selim et al., 2013) and intrauterine growth retardation placentae (Battistelli et al., 2004). Nevertheless, cytotrophoblasts continuously differentiate into syncytiotrophoblasts during villous formation and development and are thus important in the invasion of blood vessels and remodeling in the early stages of implantation. Their degeneration could undermine placental function thus contributing to a poor outcome (Awad et al., 2017).

Although most evidence suggests that methanol is the key metabolite responsible for aspartame deleterious effect, The EFSA Panel on Food Additives and Nutrient Sources added to Food (2013) has attributed most of the pathological outcome of the aspartame over intake to the accumulation of its metabolite; L-phenylalanine.

The deposition of collagen fibers beneath syncytiotrophoblast cells in the labyrinth zone might be attributed to the accumulation of the aspartame metabolites on the cell proteins causing oxidative damage to the proteins resulting in an increased collagen and ground substance formation (Elfatah et al., 2012).

5. Conclusion

Taken altogether, it could be concluded from the current work that aspartame has deeply impacted the normal structure and presumably the function of the placenta, this effect was most probably mediated through its methanol metabolite. More safety evaluation studies should be performed and restrictions are to be imposed on the consumption of aspartame especially during pregnancy.

Ethical statement

All animal work was conducted in accordance with the guidelines for the use of animals in research established by the local ethical committee of the Faculty of Medicine, Zagazig University, Egypt (Approval number: ZU-IACUC/3/F/86/2018). These guidelines comply with the international guidelines set by national institutes of health guide for the care and use of laboratory animals.

References

Abhilash, M., Paul, M.S., Varghese, M.V., Nair, R.H., 2011. Effect of long term intake of aspartame on antioxidant defense status in liver. Food Chem. Toxicol. 49, 1203–1207.

Abu Gabal, H., Al Waely, M., 2016. Role of astaxanthin in improving the physiological and teratolgical changes of aspartame in the pregnant albino rats and their fetuses. Egypt. J. Hosp. Med. 31, 1–12.

Akay, M.T., Kockaya, E.A., 2005. The effects of alcohol on rat placenta. Cell Biochem. Funct. 23, 435–445.

Alleva, R., Borghi, B., Santarelli, L., Strafella, E., Carbonari, D., Bracci, M., Tomasetti, M., 2011. In vitro effect of aspartame in angiogenesis induction. Toxicol. In Vitro 25, 286–293.

Awad, O., Ochieng, S.J., Malek, A., Ogeng'o, J., 2017. Chronic anaemia causes degenerative changes in trophoblast cells of the rat placenta. Anatomy 11, 72–78.

Battistelli, M., Burattini, S., Pomini, F., Scavo, M., Caruso, A., Falcieri, E., 2004. Ultrastructural study on human placenta from intrauterine growth retardation cases. Microsc. Res. Tech. 65, 150–158.

Boyd, C., 2013. Epithelial aspects of human placental trophoblast. Placenta 34, S24–S26.

Butchko, H.H., Stargel, W.W., Comer, C.P., Mayhew, D.A., Benninger, C., Blackburn, G.L., de Sonneville, L.M., Geha, R.S., Hertelendy, Z., Koestner, A., 2002. Aspartame: review of safety. Regul. Toxicol. Pharmacol. 35, S1–S93.

Carter, A.M., 2012. Evolution of placental function in mammals: the molecular basis of gas and nutrient transfer, hormone secretion, and immune responses. Physiol. Rev. 92, 1543–1576.

Chandler, D.E., Roberson, R.W., 2009. Bioimaging: Current Concepts in Light & Electron Microscopy. Jones & Bartlett Publishers, pp. 29.

Dawson-Saunders, B., Trapp, R., 2001. Section 5.6: Proportions When the Same Group is Measured Twice. Basic & Clinical Biostatistics: Lange Medical Book, 3rd ed. McGraw-Hill Book Co, New York, Montreal, pp. 115–118.

De Matos, M.A., Martins, A.T., Azoubel, R., 2013. Effects of sodium cyclamate and aspartame on the rat placenta a morphometric study. Int. J. Morphol. 24, 137–142.

EFSA Panel on Food Additives and Nutrient Sources added to Food, 2013. Scientific Opinion on the re-evaluation of aspartame (E 951) as a food additive. EFSA J. 11, 3496.

Elfatah, A.A., Ghaly, I.S., Hanafy, S.M., 2012. Cytotoxic effect of aspartame (diet sweet) on the histological and genetic structures of female albino rats and their offspring. Pak. J. Biol. Sci. 15, 904–918.

Farias, P.S., Souza, K.D., Marçal, A.C., Fioretto, E.T., Aires, M.B., 2014. Altered placental morphology and VEGF expression in diabetic rats. Microscopy: advances in scientific research and education. Formatex, 285–290.

Furukawa, S., Hayashi, S., Usuda, K., Abe, M., Hagio, S., Ogawa, I., 2011. Toxicological pathology in the rat placenta. J. Toxicol. Pathol. 24, 95–111.

Gamble, M., 2008. The hematoxylins and eosin. In: Bancroft, J.D., Gamble, M. (Eds.), Theory and Practice of Histological Techniques. , 6th ed. Churchill Livingstone, London, pp. 121–135.

Georgiades, P., Ferguson-Smith, A.C., Burton, G.J., 2002. Comparative developmental anatomy of the murine and human definitive placenta. Placenta 23, 3–19.

Ha, A.W., Kim, W.K., 2013. The effect of fucoxanthin rich power on the lipid metabolism in rats with a high fat diet. Nutr. Res. Pract. 7, 287–293.

Hauser, S., Weich, H.A., 1993. A heparin-binding form of placenta growth factor (PlGF-2) is expressed in human umbilical vein endothelial cells and in placenta. Growth Factors 9, 259–268.

Herrera, E.A., Krause, B., Ebensperger, G., Reyes, R.V., Casanello, P., Parra-Cordero, M., Llanos, A.J., 2014. The placental pursuit for an adequate oxidant balance between the mother and the fetus. Front. Pharmacol. 5, 149.

Humphries, P., Pretorius, E., Naude, H., 2008. Direct and indirect cellular effects of aspartame on the brain. Eur. J. Clin. Nutr. 62, 451.

Khera, K.S., 1992. Valproic acid-induced placental and teratogenic effects in rats. Teratology 45, 603–610.

Maaruf, N.A.A.-Q., Mahmood, Z.M., Abdulhameed, T.T., 2017. Effect of aspartame on the liver of male albino rats: a histopathological and immunohistochemical study. Med. J. Babylon 14, 558–566.

Malek, A., 2013. Role of IgG antibodies in association with placental function and immunologic diseases in human pregnancy. Expert Rev. Clin. Immunol. 9, 235–249.

Maranghi, F., Macri, C., Ricciardi, C., Stazi, A.V., Mantovani, A., 1998. Evaluation of the placenta: suggestions for a greater role in developmental toxicology. Adv. Exp. Med. Biol. 444 (129), 136.

Mourad, M., 2011. Effect of aspartame on some oxidative stress parameters in liver and kidney of rats. Afr. J. Pharm. Pharmacol. 5, 678–862.

Nakanishi, T., 2007. The problem of species comparison of developmental toxicity: can we extrapolate human developmental toxicity induced by environmental chemicals from the data of rodent. Yakugaku Zasshi 127, 491–500.

Kosif, R., Akta, G., Öztekin, A., 2008. Microscopic examination of placenta of rats prenatally exposed to aloe barbadensis: a preliminary study. Int. J. Morphol. 26, 275–281.

Pietro, L., Daher, S., Rudge, M.V.C., Calderon, I.D.M.P., Damasceno, D.C., Sinzato, Y.K., Bandeira, C., Bevilacqua, E., 2010. Vascular endothelial growth factor (VEGF) and VEGF-receptor expression in placenta of hyperglycemic pregnant women. Placenta 31, 770–780.

Pijnenborg, R., Robertson, W.B., Brosens, I., Dixon, G., 1981. Review article: trophoblast invasion and the establishment of haemochorial placentation in man and laboratory animals. Placenta 2, 71–91.

Portela, G.S., Azoubel, R., Batigália, F., 2007. Effects of aspartame on maternal-fetal and placental weights, length of umbilical cord and fetal liver: a kariometric experimental study. Int. J. Morphol. 25, 549–554.
Prokić, M.D., Paunović, M.G., Matić, M.M., Đorđević, N.Z., Ognjanović, B.I., Štajn, A.Š., Saičić, Z.S., 2015. Effect of aspartame on biochemical and oxidative stress parameters in rat blood. Arch. Biol. Sci. 67, 535–545.
Rebelato, H.J., Esquisatto, M.A.M., de Sousa Righi, E.F., Catisti, R., 2016. Gestational protein restriction alters cell proliferation in rat placenta. J. Mol. Histol. 47, 203–211.
Rey Moreno, M.C., Fussell, K.C., Gröters, S., Schneider, S., Strauss, V., Stinchcombe, S., Fegert, I., Veras, M., van Ravenzwaay, B., 2013. Epoxiconazole-induced degeneration in rat placenta and the effects of estradiol supplementation. Birth Defects Res. B Dev. Reprod. Toxicol. 98, 208–221.
Rogers, J.M., Daston, G.P., Ebron, M.T., Carver, B., Stefanadis, J.G., Grabowski, C.T., 1985. Studies on the mechanism of trypan blue teratogenicity in the rat developing in vivo and in vitro. Teratology 31, 389–399.
Rowe, R., Sheskey, P., Quinn, M., 2009. Handbook of Pharmaceutical Excipients. Lexi-Comp: American Pharmaceutical Association. Inc.
Rycerz, K., Jaworska-Adamu, J.E., 2013. Effects of aspartame metabolites on astrocytes and neurons. Folia Neuropathol. 51, 10–17.
Selim, M., Elshmry, N., Hamidi, E., Rashed, A., 2013. Electron and scanning microscopic observations on the syncytiotrophoblast microvillous membrane contribution to preeclampsia in early placental rats. J. Blood Disorders Transf. 4, 2.
Šerman, L., Žunić, I., Vrsaljko, N., Grbeša, Đ., Gjurčević, E., Matašin, Ž., Martić, T.N., Jakuš, F.B., Gajger, I.T., Šerman, A., 2015. Structural changes in the rat placenta during the last third of gestation discovered by stereology. Bosn. J. Basic Med. Sci. 15, 21–25.
Shalaby, A.M., Bahey, N.G., 2018. Reversal of the hepatic damage induced by the supraphysiological dose of nandrolone decanoate after its withdrawal in the adult male rat. Tissue Cell 53, 44–52.
Soffritti, M., Belpoggi, F., Esposti, D.D., Lambertini, L., Tibaldi, E., Rigano, A., 2005. First experimental demonstration of the multipotential carcinogenic effects of aspartame administered in the feed to Sprague-Dawley rats. Environ. Health Perspect. 114, 379–385.
Sturtevant, F.M., 1985. Use of aspartame in pregnancy. Int. J. Fertil. 30, 85–87.
Woods, A., Stirling, J., 2008. Electron microscope. In: Bancroft, J., Gamble, M. (Eds.), Theory and Practice of Histological Techniques. Churchill Livingstone/Elsevier, Philadelphia, PA, pp. 600.
Zorn, T., Zuniga, M., Madrid, E., Tostes, R., Fortes, Z., Giachini, F., Martin, S.S., 2011. Maternal diabetes affects cell proliferation in developing rat placenta. Histol. Histopathol. 26, 1049–1056.