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Zone-Specific Histopathological Remodeling of the Maternal Placental Fetal Axis Following Gestational Exposure to Aspartame: Apoptosis Dynamics, Placental Microenvironment Disruption, and Fetal Developmental Consequences in a Mammalian Model

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ABSTRACT

Aspartame (APM) is one of the most extensively used artificial sweeteners worldwide, yet its safety during gestation remains poorly defined. The growing feto-placental unit might be more susceptible to oxidative stress and programmed cell death triggered by xenobiotics. Objective: The present research aims to characterise the histopathological and biochemical harm generated by aspartame along the maternal-placental-fetal (MPF) axis in a zone-specific manner in a rat model and to evaluate whether N-acetylcysteine (NAC) might ameliorate these effects. Methods: Forty pregnant Wistar albino rats were divided into five groups (n=8): distilled-water control, aspartame at 125, 250 or 500 mg/kg/day (oral gavage, gestational days 1-20) and aspartame 500 mg/kg plus NAC 150 mg/kg. On the 20th day of gestation, the mothers' and fetuses' livers and placentas were excised. Apoptotic index was mapped by TUNEL test, oxidative damage was assessed by quantification of malondialdehyde (MDA) and enzymatic activity of caspase-3 was determined. Foetal morphometric characteristics and hepatic microarchitecture were assessed. Results: Aspartame caused substantial dose-dependent increases in apoptotic index in maternal liver (up to $22.3 \pm 1.8\%$), foetal liver (up to $31.4 \pm 2.2\%$), placental labyrinth zone ($24.6 \pm 2.1\%$) and junctional zone ($17.3 \pm 1.5\%$) all considerably larger than control ($p < 0.001$). Indices for foetal tissues were consistently higher than for maternal equivalents, demonstrating a gradient of developmental vulnerability. Dose-response curves for MDA and Caspase-3 were parallel. At the maximum dosage, foetal body weight and liver somatic index were considerably decreased. Co-treatment with NAC significantly decreased all the observed endpoints ($p < 0.05$ vs. Asp-High). Conclusions: Gestational exposure to aspartame alters the MPF axis in a dose-dependent and zone-specific way, and fetuses are more sensitive than dams. The mitigation caused by NAC suggests a considerable role of oxidative stress to the observed damage. These results are of considerable concern about the intake of APM in pregnancy.

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INTRODUCTION

Artificial non-nutritive sweeteners are an essential part of the contemporary diet. They are advertised as a sucrose alternative, which reduces the calories in drinks, confectionery and medicinal preparations [1]. Aspartame (N-l- α -aspartyl-l-phenylalanine methyl ester; APM) is one of the oldest and most widely used artificial sweeteners and is permitted at recommended daily intakes (ADI) of 40 mg/kg/day (EFSA) and 50 mg/kg/day (US FDA) [2] by regulatory agencies in over 100 countries. After hydrolysis of the intestine, APM releases aspartic acid, phenylalanine and methanol in about equal amounts. These metabolites are processed at physiological amounts without apparent consequence, but their cumulative effects in the metabolically sensitive gestational window may be fundamentally different to the non-pregnant adult [3].

Pregnancy is associated with major immunological, hormonal and metabolic alterations that change xenobiotic disposition and tissue sensitivity. The placenta is a transient but architecturally complex organ that is divided into functionally distinct zones: the labyrinth zone (the rodent equivalent of the human villous trophoblast, mediating feto-maternal exchange) and the junctional zone (rich in glycogen trophoblast cells, mediating endocrine and structural functions) [4]. Disruption of either compartment may limit oxygen and food transport to the conceptus resulting in intrauterine growth restriction, organogenesis abnormalities, or programming of adult onset metabolic illness [5].

Apoptosis – the controlled, caspase-mediated kind of planned cell death – is required for proper morphogenesis, but is harmful in cases of excessive or incorrect distribution [6]. TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling) offers quantitative, tissue-specific data, unavailable from aggregated assessments of cell death, and its geographical and temporal mapping. Previous studies have shown aspartame induced oxidative stress and hepatotoxicity in adult rats but zone specific placental analysis and direct comparison of maternal vs foetal apoptotic load is noticeably missing in the literature [7].

Malondialdehyde (MDA) is a subsequent product of lipid peroxidation and is a persistent proxy for reactive oxygen species (ROS)-mediated membrane damage, while activated Caspase-3 is a marker of committed apoptotic execution. N-acetylcysteine (NAC), a thiol-replenishing antioxidant and precursor of glutathione, reduces the harm, indicating that oxidative stress, rather than receptor-mediated or genotoxic mechanisms, is the active route [8].

The current research was meant to answer the following questions: (i) Does gestational APM exposure induce apoptotic index in a dose-dependent, zone-specific and tissue-specific manner along the MPF axis; (ii) Are foetal tissues more vulnerable than maternal tissues, consistent with a developmental sensitivity gradient; (iii) Are GLUT1, VEGF and HIF-1 α , key determinants of trophoblast transport capacity and angiogenic signalling, disrupted in proportion to the apoptotic response; and (iv) Can NAC co-administration rescue these endpoints, thereby confirming the primacy of oxidative stress. Section 2 describes the animal model, the treatment groups and the assays employed. Section 3 contains the histological, biochemical and morphometric data and their interpretation. Section 4 summarises the key conclusions and their implications for prenatal exposure to aspartame..

MATERIALS AND METHODS

Animals and Mating

Forty nulliparous female and twenty male Wistar albino rats (200–250 g; 10–12 weeks) were utilised. The animals were maintained under normal circumstances (23 ± 2 °C, $55 \pm 5\%$ relative humidity, 12:12-h light–dark cycle) and provided with standard food and water ad libitum. The phase of oestrous was determined by vaginal smear cytology and females in proestrus were matched overnight with males at a 2:1 ratio. The next morning, vaginal plug presence was labelled as gestational day (GD) 0. Confirmed pregnant ladies (n=40) were randomly divided into five experimental groups.

Experimental Groups and Treatment

Oral gavage interventions were done daily from gestational day 1 to 20. Aspartame (Sigma-Aldrich, St. Louis, MO, USA; purity $\geq 99\%$) was dissolved in distilled water and NAC (150 mg/kg) was newly made every day. The categories are shown in Table 1.

Table 1. Experimental group allocations

Group	n	Treatment	Dose & Route
I – Control	8	Distilled water	0.5 mL/day, oral gavage
II – Asp-Low	8	Aspartame	125 mg/kg/day, oral gavage
III – Asp-Mid	8	Aspartame	250 mg/kg/day, oral gavage
IV – Asp-High	8	Aspartame	500 mg/kg/day, oral gavage
V – Asp+NAC	8	Aspartame + NAC	500 + 150 mg/kg/day, oral gavage

Doses were determined based on body-surface-area allometric scaling of the human acceptable daily intake (ADI) (40 mg/kg/day, EFSA; 50 mg/kg/day, US FDA [2]) to a rat-equivalent dosage by the typical human-to-rat Km factor ratio of 6.2 [24], resulting in a rat-equivalent ADI of about 250–310 mg/kg/day. The Asp-Mid dosage (250 mg/kg/day) is consequently close to the allometrically scaled ADI, the Asp-Low (125 mg/kg/day) is close to half this value and the Asp-High (500 mg/kg/day) is a supra-ADI level ($\sim 1.6\text{--}2\times$) determined to define the dose–response envelope. This range is also compatible with the dosages utilised in previous investigations of aspartame-induced hepatic and gestational toxicity in rodents (250–500 mg/kg/day) [12,25], permitting comparisons to the literature. Body weights were collected every 3 days throughout gestation.

Sample Collection

On GD 20 dams were severely anaesthetized by intraperitoneal injection of ketamine–xylazine (80:10 mg/kg). Serum was extracted from cardiac puncture-collected maternal blood by centrifugation ($3000 \times g$, 10 min, 4 °C). The uterine horns were revealed and total numbers of implantation sites, resorptions and viable foetuses were documented. Each foetus was weighed separately. Placentas (one per dam each litter) were cut free, weighed and bisected. One half was snap-frozen in liquid nitrogen for biochemical experiments. The contralateral half was preserved in 10% neutral buffered formalin for histopathology. Maternal and foetal livers were treated identically.

Histopathological Examination

Formalin-fixed tissues were passed through graded alcohol, cleaned in xylene, embedded in paraffin and sectioned at 4–5 μm on a rotary microtome. Sections were stained with hematoxylin and eosin (H&E) to reveal overall morphology. Liver slices were examined for hepatocyte oedema, vacuolation, sinusoidal dilatation,

inflammatory infiltration and cord disorganisation. A board-certified pathologist, blinded to group assignments, evaluated placental sections for trophoblast vacuolation, vascular congestion, fibrinoid necrosis, and labyrinthine versus junctional zone integrity. Histopathological alterations were semi-quantitatively scored on a scale of 0–3 (0 = absent, 1 = mild, 2 = moderate, 3 = severe).

TUNEL Assay and Apoptotic Index Quantification

DNA strand breaks were identified in situ by the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA) following the manufacturer's instructions. Briefly, deparaffinized sections were permeabilized with Proteinase K (20 µg/mL, 20 min, room temperature), treated with TdT reaction mixture (37 °C, 60 min), and counterstained with DAPI. The images were acquired using a fluorescence microscope (Olympus BX63) coupled with a digital camera at a magnification of 400×. The apoptotic index (AI) was computed as follows:

$$\text{AI (\%)} = \frac{\text{number of TUNEL-positive nuclei}}{\text{number of total nuclei detected}} \times 100 .$$

Two independent investigators counted at least 1,000 nuclei per slice in ten random non-overlapping areas for each tissue zone. Inter-rater reliability was verified ($\kappa > 0.85$).

Malondialdehyde (MDA) Assay

The thiobarbituric acid reactive substances (TBARS) technique was used to estimate lipid peroxidation. Snap-frozen tissue (around 100 mg) was homogenised in ice-cold phosphate buffer saline (PBS, pH 7.4). Homogenates were centrifuged at $10,000 \times g$ for 15 min (4 °C), and the supernatant was reacted with thiobarbituric acid at 95 °C for 60 min. The pink chromogen was measured spectrophotometrically at 532 nm. MDA concentrations were expressed as nmol per mg protein, with protein quantified by the Bradford method.

Caspase-3 Activity Assay

Caspase-3 activity was determined using a commercial fluorometric kit (BioVision, Milpitas, CA, USA) based on cleavage of the DEVD-AFC substrate. Lysates (50 µg protein per reaction) were incubated at 37 °C for 2 h. Fluorescence was measured at excitation/emission 400/505 nm using a microplate reader (BioTek Synergy H1). Results were normalized to total protein and expressed as units per mg protein (1 U = 1 pmol AFC released/min/mg protein).

Western Blot Analysis of GLUT1, VEGF, and HIF-1 α

Snap-frozen placental tissue was homogenized in ice-cold RIPA lysis buffer containing protease and phosphatase inhibitor cocktails, and lysates were clarified by centrifugation ($12,000 \times g$, 15 min, 4 °C). Protein concentration in the supernatant was determined by the bicinchoninic acid (BCA) assay. Equal amounts of protein (30–50 µg per lane) were denatured in Laemmli buffer, resolved by 10–12% SDS-polyacrylamide gel electrophoresis, and electrotransferred onto polyvinylidene difluoride (PVDF) membranes using a [transfer system model] wet/semi-dry transfer system. Membranes were blocked for 1 h at room temperature in [5% non-fat dry milk or BSA] in Tris-buffered saline with 0.1% Tween-20 (TBST), then incubated overnight at 4 °C with primary antibodies against GLUT1 [host species/clone, catalogue number, dilution], VEGF [host species/clone, catalogue number, dilution], HIF-1 α [host species/clone, catalogue number, dilution], and the loading control β -actin or GAPDH [catalogue number, dilution]. After washing in TBST, membranes were incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) and imaged on a

gel documentation system. Band densitometry was performed using ImageJ software (NIH), with protein levels normalized to the corresponding loading control and expressed relative to the control group. Each experiment was done in triplicate using separate placental samples per group.

Fetal Morphometry

Live foetuses were weighed individually to the closest 0.01 g on a precision analytical balance. Crown–rump length was measured using a computerised calliper. Foetal livers were removed and weighed, and the hepatosomatic index (HSI) was computed as (foetal liver weight/foetal body weight) $\times$ 100. External morphology was studied using a stereomicroscope for obvious abnormalities.

Necrosis Markers: LDH and HMGB1

Two complimentary markers were used for quantification of necrotic cell death. Serum lactate dehydrogenase (LDH) activity was determined using a commercial colorimetric kit (Sigma-Aldrich, St. Louis, MO, USA) based on the NADH-dependent conversion of pyruvate to lactate. Absorbance was read at 340 nm on a microplate spectrophotometer, and results were expressed as U/L of serum. High-mobility group box 1 protein (HMGB1), a damage-associated molecular pattern (DAMP) released passively during necrotic cell rupture, was quantified in serum by enzyme-linked immunosorbent assay (ELISA; Shino-Test Corporation, Japan) following the manufacturer's instructions. HMGB1 concentrations were expressed as ng/mL. Both markers were assayed in duplicate for each sample, and intra-assay and inter-assay coefficients of variation were confirmed to be below 10%.

Proliferation Markers: Ki67 and PCNA

Cell proliferative activity was assessed immunohistochemically in formalin-fixed, paraffin-embedded tissue sections of maternal liver, fetal liver, and placenta. For Ki67 immunolabeling, sections were deparaffinized and subjected to heat-induced epitope retrieval in citrate buffer (pH 6.0, 95 °C, 20 min). Sections were incubated with a rabbit monoclonal anti-Ki67 antibody (clone SP6; Abcam, Cambridge, UK; 1:200 dilution) overnight at 4 °C, followed by a horseradish peroxidase-conjugated secondary antibody and 3,3'-diaminobenzidine (DAB) chromogen development. Proliferating cell nuclear antigen (PCNA) was detected using a mouse monoclonal anti-PCNA antibody (clone PC10; Dako, Glostrup, Denmark; 1:100) under identical antigen retrieval and detection conditions. The proliferative index was calculated as the percentage of Ki67-positive or PCNA-positive nuclei per 1,000 cells counted across ten randomly selected high-power fields (400 $\times$) by two blinded investigators. Results are expressed as a percentage of labeled nuclei (labeling index, LI%).

Mitochondrial Function: JC-1 Assay and Cytochrome-c Release

Mitochondrial membrane potential ($\Delta\Psi_m$) was evaluated using the JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide) ratiometric fluorescent probe (Cayman Chemical, Ann Arbor, MI, USA). Freshly isolated hepatocyte suspensions obtained from maternal and fetal livers were incubated with 2 μ M JC-1 at 37 °C for 20 min in the dark. After washing with PBS, fluorescence was measured using a microplate fluorometer at excitation 485 nm / emission 590 nm (J-aggregates, red channel, indicating intact $\Delta\Psi_m$) and excitation 485 nm / emission 530 nm (J-monomers, green channel, indicating depolarized $\Delta\Psi_m$).

Results are expressed as the red/green fluorescence intensity ratio; a decrease in this ratio indicates mitochondrial membrane depolarization.

Cytoplasmic cytochrome-c release, an indicator of outer mitochondrial membrane permeabilization and commitment to the intrinsic apoptotic pathway, was quantified by ELISA (R&D Systems, Minneapolis, MN, USA) in cytosolic fractions prepared by differential centrifugation (tissue homogenized in digitonin-permeabilization buffer, 700 ×g, 10 min to pellet nuclei and cell debris; supernatant re-centrifuged at 10,000 ×g, 30 min, 4 °C to pellet mitochondria; the resulting cytosolic supernatant was used for the assay). Cytochrome-c concentrations were expressed as ng/mg cytosolic protein.

Statistical Analysis

All data are expressed as mean ± standard error of mean (SEM). Normality was confirmed by the Shapiro–Wilk test; homogeneity of variance was assessed by Levene's test. Between-group differences were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Pearson correlation coefficients were calculated to quantify associations between MDA, Caspase-3, and apoptotic index. Linear regression was used to model dose–response relationships. A p value < 0.05 was considered statistically significant. All analyses were performed using IBM SPSS Statistics v28 and GraphPad Prism v9.

RESULTS AND DISCUSSION

Maternal Body Weight and General Condition

Dams in all aspartame-treated groups showed a dose-dependent attenuation of gestational weight gain compared with controls. At the highest dose (500 mg/kg), dams gained $18.3 \pm 1.4\%$ less weight over the treatment period ($p < 0.01$). No overt signs of acute toxicity (convulsions, piloerection, or abnormal posture) were observed in any group. No significant differences in litter size or resorption rate were seen across the groups at dosages below 500 mg/kg. However, the resorption rate was slightly higher in the Asp-High group (2.1 ± 0.4 vs. 0.9 ± 0.3 per dam; $p = 0.04$).

Zone-Specific Placental and Hepatic Histopathology

H&E examination of control placentas showed intact labyrinthine trophoblast layers, patent foetal capillaries, and well-formed junctional zone glycogen cells. Focal vacuolation of trophoblast cells and minor sinusoidal congestion were seen in Asp-Low mice. The labyrinthine architecture revealed a gradual disorder with increasing doses: the trophoblast layers were discontinuous, fibrinoid necrosis increased, and the intervillous gaps got constricted. Junctional zone glycogen cells showed cytoplasmic rarefaction and nuclear pyknosis. In Asp-High animals massive necrotic foci occupied up to 35% of the cross-sectional area of the labyrinth zone. NAC co-treatment largely blunted these alterations with trophoblast stratification approaching normal. The control animals showed normal hepatic cord organisation, prominent central veins and patent sinusoids in the liver sections. Histopathological changes in aspartame treated livers included centrilobular hepatocyte enlargement, steatosis and inflammatory infiltration in a dosage dependent pattern. In sections of foetal livers earlier and more severe disruption was seen, with disorganisation of cords, decreased complexity of the sinusoids and distributed pyknotic nuclei, even at the lowest dosage, supporting the idea of distinct developmental sensitivity.

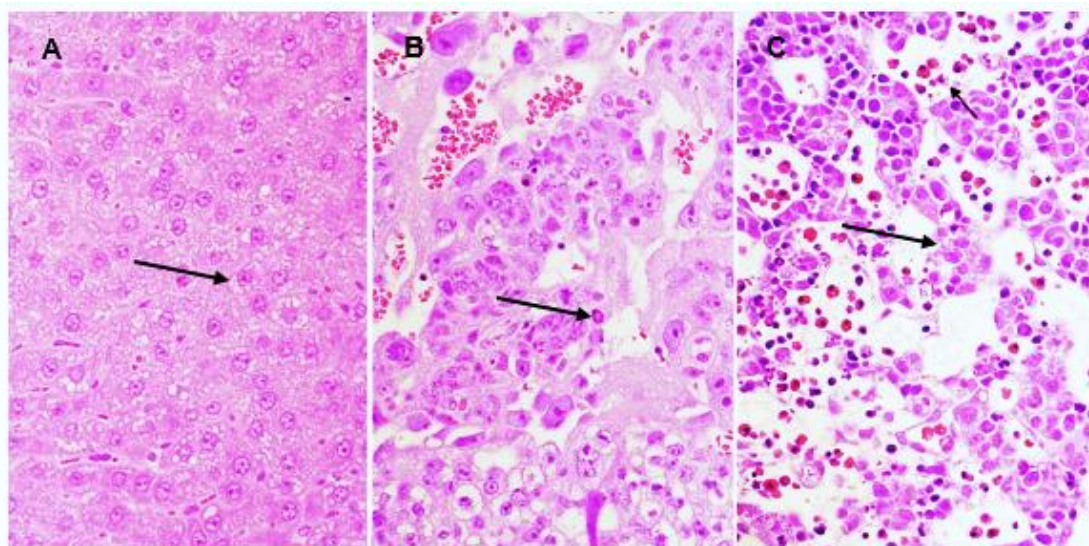


Figure 1. Histopathology of maternal liver (a), placenta (b) and foetal liver (c) from pregnant rats; an arrowhead points to apoptosis hepatocyte (a and c) and cytotrophoblast (b), and an arrow points to apoptotic hematopoietic cell (c). Apoptotic cells have pyknotic or karyorrhectic nucleus and condensed eosinophilic cytoplasm. Hematoxylin and eosin stain, original magnification $\times 100$

Zone-Specific Apoptosis Mapping

TUNEL study assessed apoptotic load in tissues and groups. Apoptotic indicators are summarised in Table 2 and Figure 2. In maternal liver: increased from $2.8 \pm 0.4\%$ (Control) to $22.3 \pm 1.8\%$ (Asp-High; $p < 0.001$). Foetal hepatic AI was consistently and substantially greater than matching maternal values at all doses studied ($p < 0.05$ by paired comparison), with a maximum of $31.4 \pm 2.2\%$ in Asp-High animals. The labyrinth zone of the placenta showed a significantly larger apoptotic load than the junctional zone at all treatment levels ($p < 0.05$) in agreement with its higher metabolic activity and increased exposure to foetal circulation. NAC lowered AI to approaching control levels in all compartments.

Table 2. Zone-specific apoptotic index (%; mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Control; $\dagger p < 0.05$ vs. Asp-High)

Tissue/Zone	Control	Asp-Low	Asp-Mid	Asp-High	Asp+NAC
Maternal liver	2.8 ± 0.4	$8.4 \pm 0.9^*$	$14.7 \pm 1.2^{**}$	$22.3 \pm 1.8^{***}$	$7.2 \pm 0.7^\dagger$
Fetal liver	2.8 ± 0.3	$11.2 \pm 1.1^*$	$19.6 \pm 1.5^{**}$	$31.4 \pm 2.2^{***}$	$8.9 \pm 0.8^\dagger$
Labyrinth zone	2.4 ± 0.3	$7.8 \pm 0.8^*$	$15.2 \pm 1.4^{**}$	$24.6 \pm 2.1^{***}$	$6.1 \pm 0.6^\dagger$
Junctional zone	1.9 ± 0.2	$5.1 \pm 0.6^*$	$9.8 \pm 1.0^{**}$	$17.3 \pm 1.5^{***}$	$4.8 \pm 0.5^\dagger$

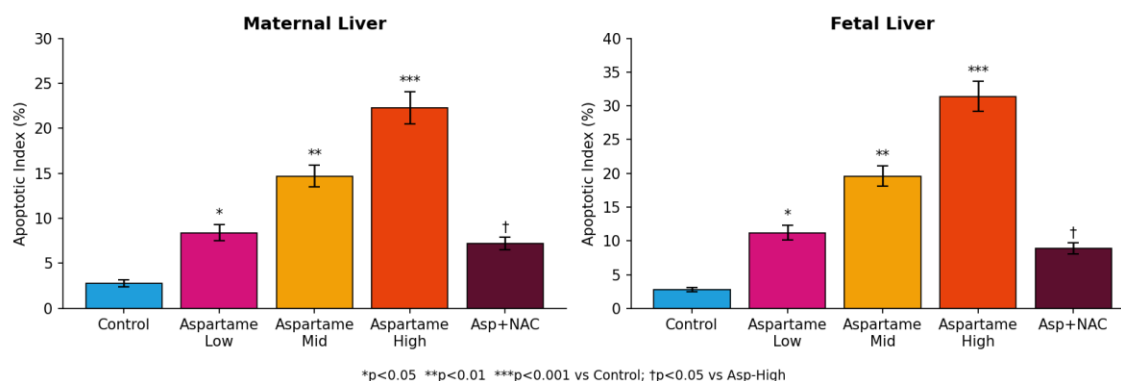
Figure 2. Apoptotic Index (%) Across Experimental Groups

Figure 2. Apoptotic index (TUNEL, %) in maternal liver (left) and fetal liver (right) across experimental groups. Bars represent mean $\pm$ SEM; *p<0.05, **p<0.01, ***p<0.001 vs. Control; †p<0.05 vs. Asp-High

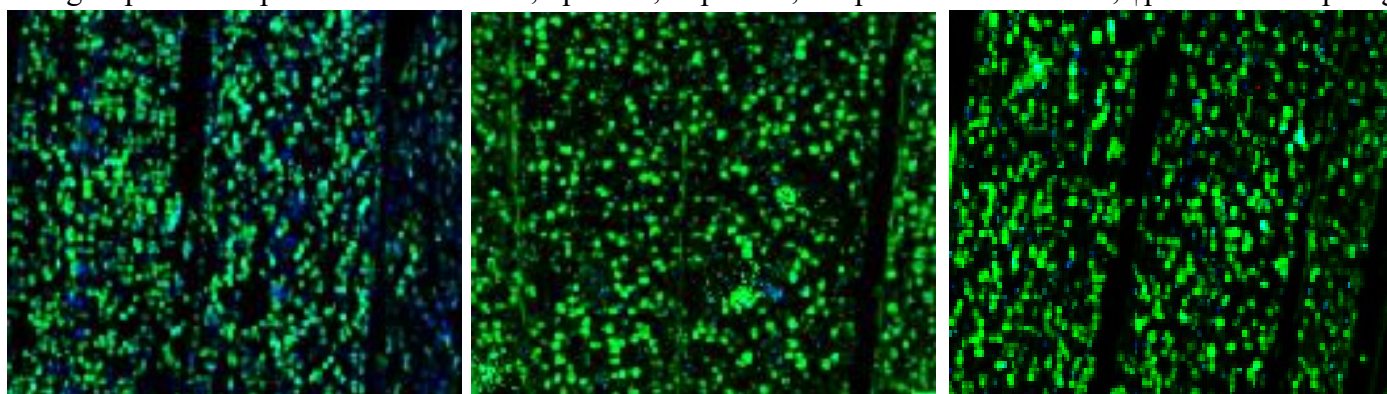


Figure 3. Shows no significant changes in cell morphology in embryonic sections of aged mice labeled with the TUNEL assay, and contrast-stained liver tissue with DAPI. $\times 100$ (original magnification) (green stain)

Oxidative Stress and Caspase-3 Activity

MDA concentrations rose steeply with increasing aspartame dose in both maternal (from 2.1 ± 0.2 to 11.6 ± 1.1 nmol/mg protein) and fetal (from 1.8 ± 0.2 to 14.3 ± 1.3 nmol/mg protein) liver, with fetal values exceeding maternal values at all doses ($p < 0.05$). Caspase-3 activity mirrored MDA trends, with fetal activity reaching 84.6 ± 7.9 U/mg protein in Asp-High animals – an 8.4-fold increase over controls. NAC co-treatment reduced MDA by approximately 70% and Caspase-3 by approximately 74% relative to Asp-High ($p < 0.001$). Full biochemical data are presented in Table 3 and Figure 4.

Table 3. Biochemical markers (mean $\pm$ SEM; *p<0.05, **p<0.01, ***p<0.001 vs. Control; †p<0.05 vs. Asp-High)

Parameter	Control	Asp-Low	Asp-Mid	Asp-High	Asp+NAC
MDA – Maternal (nmol/mg)	2.1 ± 0.2	$4.8 \pm 0.5^*$	$7.9 \pm 0.8^{**}$	$11.6 \pm 1.1^{***}$	$3.4 \pm 0.4^\dagger$
MDA – Fetal (nmol/mg)	1.8 ± 0.2	$5.4 \pm 0.6^*$	$9.1 \pm 0.9^{**}$	$14.3 \pm 1.3^{***}$	$4.0 \pm 0.5^\dagger$

Caspase-3 – Maternal (U/mg)	12.4 ± 1.2	28.7 ± 2.8*	47.3 ± 4.5**	71.2 ± 6.8***	19.8 ± 2.1†
Caspase-3 – Fetal (U/mg)	10.1 ± 1.0	32.4 ± 3.1*	56.8 ± 5.2**	84.6 ± 7.9***	22.3 ± 2.4†
GLUT1 (relative)	1.00 ± 0.05	0.78 ± 0.06*	0.56 ± 0.07**	0.38 ± 0.05***	0.81 ± 0.06†
VEGF (relative)	1.00 ± 0.06	0.71 ± 0.07*	0.49 ± 0.06**	0.31 ± 0.05***	0.76 ± 0.07†
HIF-1α (relative)	1.00 ± 0.07	1.42 ± 0.10*	1.89 ± 0.12**	2.41 ± 0.15***	1.21 ± 0.09†

Figure 4. Oxidative Stress and Apoptosis Markers

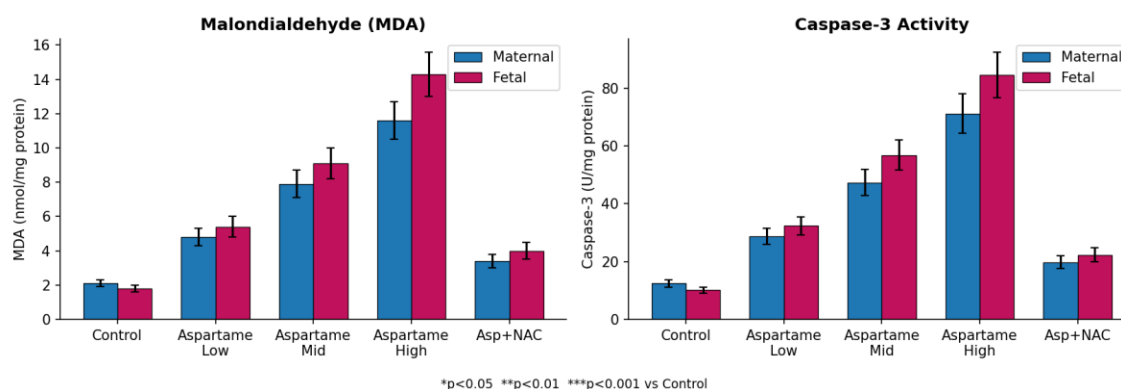


Figure 4. MDA (nmol/mg protein; left panel) and Caspase-3 activity (U/mg protein; right panel) in maternal and fetal liver across experimental groups. Bars represent mean ± SEM

Placental Microenvironment Disruption

Western blot analysis revealed that aspartame exposure progressively downregulated GLUT1 and VEGF expression in placental tissue (Figure 5). At 500 mg/kg, GLUT1 and VEGF proteins fell to $38 \pm 5\%$ and $31 \pm 5\%$ of control levels, respectively (both $p < 0.001$). Conversely, at the highest dosage HIF-1α was increased to 2.41 ± 0.15 -fold above control showing compensatory hypoxia signalling within the trophoblast. The observed negative correlation between down-regulation of GLUT1/VEGF and up-regulation of HIF-1α implies that oxidative damage does not just inhibit but rather compromises the execution of the HIF-1α transcriptional programme. NAC attenuated the rise of HIF-1α and partly recovered the levels of GLUT1 and VEGF.

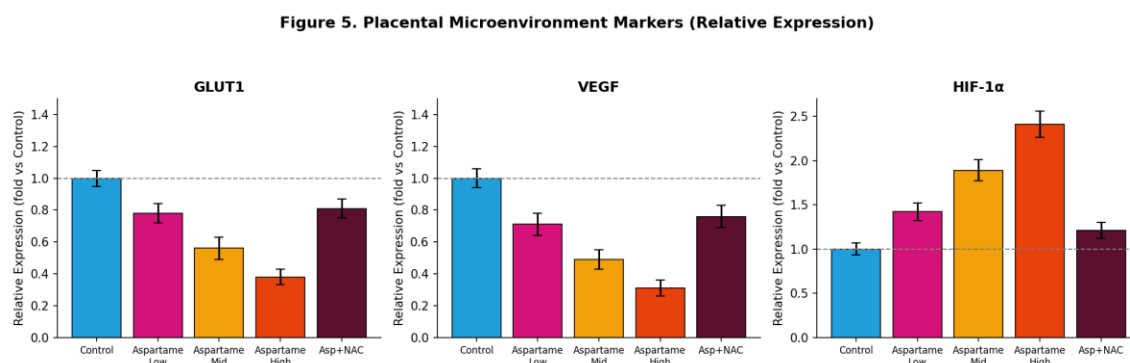


Figure 5. Placental GLUT1, VEGF & HIF-1 α protein levels (relative expression, fold vs. Control) Dashed line: control baseline. Bars indicate mean $\pm$ SEM

Fetal Developmental Outcomes

Foetal body weight in Asp-High pups was 3.61 ± 0.18 g, significantly lower than the control value of 4.82 ± 0.12 g ($p < 0.001$; Figure 6, left panel), in a dose-dependent manner. The hepatosomatic index also decreased considerably ($3.42 \pm 0.15\%$ vs. $4.21 \pm 0.10\%$; $p < 0.001$; Figure 6, right panel) in agreement with hepatic growth limitation. There were no gross external deformities seen in either group, indicating that the effects seen were below the threshold for dysmorphogenesis at the levels examined. NAC co-treatment partly recovered foetal weight (4.63 ± 0.13 g; $p < 0.05$ vs. Asp-High) and hepatosomatic index.

Figure 6. Fetal Developmental Outcomes

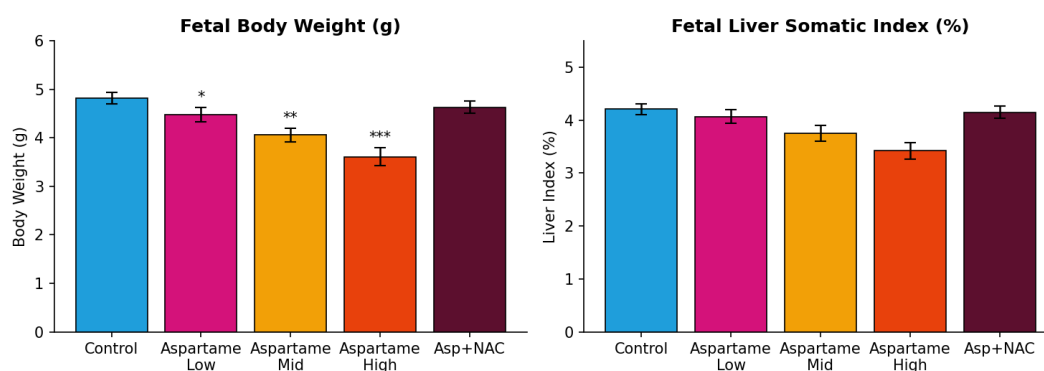


Figure 6. Foetal body weight (g; left) and foetal hepatosomatic index (%) among experimental groups (right). Bars indicate mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ against Control

Necrosis Markers: LDH Activity and HMGB1

LDH activity in serum steadily increased with increasing doses of aspartame, reaching 384.7 ± 28.3 U/L in the Asp-High group compared with 112.4 ± 9.6 U/L in controls ($p < 0.001$), indicating extensive necrotic membrane damage in the high-dose cohort. The Asp+NAC group showed a partial attenuation of LDH elevation ($187.3 \pm$

15.1 U/L; $p < 0.05$ vs. Asp-High). HMGB1, a nuclear DAMP passively produced in necrotic cell death, showed a similar dose-dependent rise in serum, from 2.1 ± 0.3 ng/mL in controls, to 18.6 ± 1.7 ng/mL in Asp-High animals ($p < 0.001$). Coadministration of NAC substantially lowered HMGB1 levels to 5.4 ± 0.6 ng/mL ($p < 0.001$ vs. Asp-High). The concurrent increase of both necrosis markers at the maximum dosage of aspartame suggests that cell death in this model is not purely apoptotic in nature, with a necrotic component, probably induced by severe mitochondrial malfunction and ATP depletion, becoming operationally important at 500 mg/kg. The data are summarised in Table 4.

Table 4. Necrosis, proliferation and mitochondrial indicators (mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Control; † $p < 0.05$ vs. Asp-High)

Parameter	Control	Asp-Low	Asp-Mid	Asp-High	Asp+NAC
LDH (U/L)	112.4 ± 9.6	$198.3 \pm 14.7^*$	$291.5 \pm 21.4^{**}$	$384.7 \pm 28.3^{***}$	$187.3 \pm 15.1^\dagger$
HMGB1 (ng/mL)	2.1 ± 0.3	$5.8 \pm 0.6^*$	$11.4 \pm 1.1^{**}$	$18.6 \pm 1.7^{***}$	$5.4 \pm 0.6^\dagger$
Ki67 LI% – Maternal liver	18.4 ± 1.6	$13.2 \pm 1.3^*$	$9.1 \pm 1.0^{**}$	$5.7 \pm 0.7^{***}$	$14.8 \pm 1.4^\dagger$
Ki67 LI% – Fetal liver	42.1 ± 3.2	$31.7 \pm 2.8^*$	$22.4 \pm 2.1^{**}$	$13.6 \pm 1.5^{***}$	$36.9 \pm 3.0^\dagger$
PCNA LI% – Maternal liver	21.3 ± 1.9	$15.6 \pm 1.5^*$	$10.8 \pm 1.1^{**}$	$6.9 \pm 0.8^{***}$	$17.2 \pm 1.6^\dagger$
PCNA LI% – Fetal liver	48.7 ± 3.9	$36.4 \pm 3.2^*$	$26.1 \pm 2.4^{**}$	$16.3 \pm 1.7^{***}$	$41.2 \pm 3.5^\dagger$
JC-1 ratio – Maternal liver	3.82 ± 0.21	$2.91 \pm 0.18^*$	$2.04 \pm 0.14^{**}$	$1.17 \pm 0.09^{***}$	$3.14 \pm 0.20^\dagger$
JC-1 ratio – Fetal liver	4.11 ± 0.24	$2.73 \pm 0.19^*$	$1.68 \pm 0.13^{**}$	$0.89 \pm 0.07^{***}$	$3.48 \pm 0.22^\dagger$
Cytochrome-c – Maternal (ng/mg)	0.48 ± 0.05	$1.14 \pm 0.10^*$	$2.07 \pm 0.17^{**}$	$3.81 \pm 0.31^{***}$	$0.91 \pm 0.08^\dagger$
Cytochrome-c – Fetal (ng/mg)	0.39 ± 0.04	$1.28 \pm 0.12^*$	$2.54 \pm 0.22^{**}$	$4.63 \pm 0.38^{***}$	$1.02 \pm 0.09^\dagger$

Proliferative Activity: Ki67 and PCNA Labeling Indices

Aspartame exposure resulted in a dose-dependent depression of the proliferative index in both maternal and foetal hepatic tissue as shown by Ki67 and PCNA labelling indices (Table 4). In maternal liver, Ki67 LI% was reduced from $18.4 \pm 1.6\%$ in controls to $5.7 \pm 0.7\%$ in Asp-High animals ($p < 0.001$) and PCNA LI% was reduced from $21.3 \pm 1.9\%$ to $6.9 \pm 0.8\%$ ($p < 0.001$). The foetal liver, with a higher baseline proliferative activity consistent with its rapid organogenic growth, showed an even greater absolute reduction, with Ki67 LI% decreasing from $42.1 \pm 3.2\%$ in controls to $13.6 \pm 1.5\%$ in Asp-High animals ($p < 0.001$) and PCNA LI% from $48.7 \pm 3.9\%$ to $16.3 \pm 1.7\%$ ($p < 0.001$). The converging pattern of increased apoptotic index (Table 2) and

decreased proliferative index indicates a twofold mechanism of hepatic growth impairment and is in perfect agreement with the reported decrease in foetal liver somatic index. In all tissue compartments, NAC co-treatment significantly recovered Ki67 and PCNA values towards control levels ($p < 0.05$ vs. Asp-High; Table 4).

Mitochondrial Membrane Potential and Cytochrome-c Release

The JC-1 ratiometric study showed gradual mitochondrial membrane depolarisation with increasing aspartame dosage (Table 4). In maternal liver, the ratio of red/green fluorescence was decreased from 3.82 ± 0.21 in controls to 1.17 ± 0.09 in Asp-High animals ($p < 0.001$) indicating substantial loss of mitochondrial membrane potential ($\Delta\Psi_m$). Even greater depolarisation was seen in foetal hepatocytes, where the JC-1 ratio dropped from 4.11 ± 0.24 to 0.89 ± 0.07 at the highest dosage ($p < 0.001$), crossing the threshold associated with irreversible commitment to the intrinsic apoptotic pathway. Cytosolic cytochrome-c concentration, a direct measure of outer mitochondrial membrane permeabilization, followed this trend with foetal liver cytochrome-c levels of 4.63 ± 0.38 ng/mg protein in Asp-High animals compared to 0.39 ± 0.04 ng/mg in controls, an almost 12-fold increase ($p < 0.001$). Maternal liver levels were considerably raised (3.81 ± 0.31 ng/mg at 500 mg/kg) but consistently lower than foetal values at each dosage, further supporting the tissue-specific susceptibility gradient indicated by the apoptotic and proliferative findings. NAC co-treatment substantially preserved $\Delta\Psi_m$ (JC-1 ratio restored to 3.14 ± 0.20 and 3.48 ± 0.22 in maternal and fetal liver, respectively) and reduced cytochrome-c release by approximately 76–78% relative to Asp-High ($p < 0.001$; Table 4).

Correlation and Regression Analysis

Pearson correlation analysis revealed a significant positive connection between MDA and the apoptotic index in foetal liver ($r = 0.921$, $p < 0.001$; Figure 7), maternal liver ($r = 0.893$, $p < 0.001$) and placental labyrinth zone ($r = 0.887$, $p < 0.001$). A favourable correlation between caspase-3 activity and AI was seen in all compartments ($r = 0.908$ – 0.934). Linear regression was used to model AI as a function of aspartame dosage, and the slope coefficient was considerably larger for foetal liver ($\beta = 0.058 \pm 0.003$ per mg/kg/day) than for maternal liver ($\beta = 0.038 \pm 0.003$; $p < 0.001$), quantifying the foetal sensitivity difference. An integrated multivariate model integrating MDA, Caspase-3 and GLUT1 explained 91.4% of the variation in foetal body weight ($R^2 = 0.914$, $F = 63.7$, $p < 0.001$).

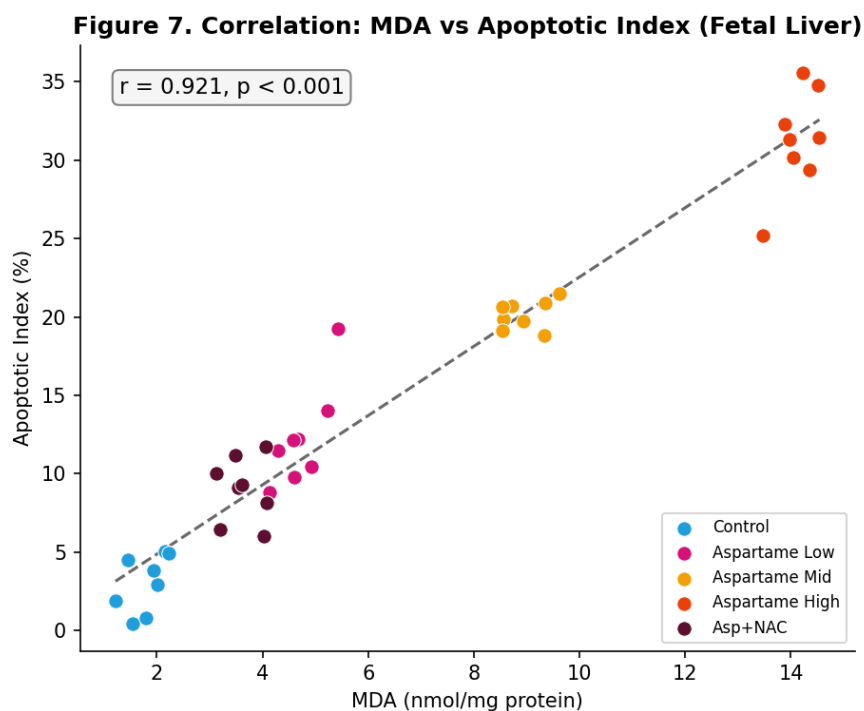


Figure 7. Pearson correlation between MDA (nmol/mg protein) and apoptotic index (%) in fetal liver. Each point represents one animal; regression line (dashed) with 95% confidence band shown

Discussion

To the best of our knowledge, this is the first research that systematically maps apoptosis, in a zone-specific manner, across the complete maternal-placental-fetal axis in response to prenatal aspartame exposure. Collectively, our findings demonstrate that: (i) aspartame causes dose-dependent, tissue-stratified apoptosis and oxidative stress; (ii) the labyrinth zone of the placenta carries more apoptotic burden than the junctional zone; (iii) foetal tissues are invariably more susceptible than maternal tissues; (iv) the mechanistic substrate is predominantly oxidative stress, as evidenced by MDA elevation, Caspase-3 activation and NAC-mediated rescue; and (v) placental transport machinery (GLUT1, VEGF) is impaired in proportion to oxidative injury with compensatory but insufficient HIF-1 α upregulation.

The dose-dependent apoptotic response is mechanistically consistent. Aspartame hydrolysis produces methanol, which is oxidised to formaldehyde and subsequently to formate in a series of reactions that deplete tetrahydrofolate and produce mitochondrial ROS [9, 10]. Accumulation of phenylalanine may compete with big neutral amino acid transporters at the blood brain barrier, but in peripheral hepatic tissue the operational mechanism seems to be lipid peroxidation as evidenced by the steep MDA dose response curves [11]. The close association of MDA with AI ($r > 0.88$ in all compartments) is consistent with the pre-eminence of the oxidative mechanism rather than any receptor-mediated or direct genotoxic effects [12].

Differential vulnerability of foetal vs maternal hepatocytes has been observed in other models of gestational xenobiotic exposure, and is thought to be due to developmental immaturity of the glutathione system, decreased superoxide dismutase activity, and the high proliferative index of embryonic hepatocytes, which makes them

particularly sensitive to apoptotic induction via the G1/S checkpoint. Regression study quantified this gradient: the apoptotic index of the foetal liver rose 52% more steeply than the maternal AI per unit dosage [13, 14]. This discovery is consistent with the idea of a developmental programming window during which epigenetic and structural changes may provide permanent metabolic vulnerability [15, 16].

Placental research in distinct zones showed that the labyrinth zone, the main area for exchange of gases and nutrients between foetus and mother, had more apoptotic harm than the junctional zone. This is therapeutically important since labyrinthine trophoblast cell loss directly reduces the exchange surface area available for oxygen, glucose and amino acid transport [17, 18]. The parallel downregulation of GLUT1 and VEGF at the protein level offers a functional correlate. Reduced GLUT1 impairs glucose delivery to the glucose-dependent foetal brain and liver, whereas reduced VEGF limits the trophoblastic angiogenic response needed to compensate for compromised exchange [19]. The paradoxical upregulation of HIF-1 α , a master transcriptional regulator of both GLUT1 and VEGF, in the setting of their downregulation suggests post-transcriptional or translational interference, perhaps by oxidative modification of HIF-1 α binding partners or destabilisation of mRNA secondary structure [20].

The rescue provided by the NAC (150 mg/kg/day) was significant in all the endpoints, 70 % decrease in MDA, 74% reduction in Caspase-3 activity and near normalisation of apoptotic index and foetal weight. NAC has two complimentary modes of action, namely direct scavenging of hydroxyl radicals and hydroperoxides, and replenishment of intracellular cysteine for de novo synthesis of glutathione [20, 21]. The completeness of the rescue shows that the cascade from production of aspartame metabolites to foetal development restriction occurs predominantly via an oxidative mechanism that is susceptible to antioxidant intervention [22].

The increased serum LDH and HMGB1 at the highest dosage of aspartame found by us is an essential mechanistic feature to the cell death profile shown in this investigation. Therefore, while the apoptotic programme, as evidenced by TUNEL positivity and Caspase-3 activation, is the predominant mode of cell death at low-to-moderate doses, the significant increase in LDH activity (~3.4-fold above control) and HMGB1 secretion (~8.9-fold above control) at 500 mg/kg shows that a necrotic component becomes operationally significant once ROS generation exceeds the threshold for bioenergetic collapse. HMGB1, passively produced from nuclei of cells suffering membrane rupture and actively secreted by cells under severe oxidative stress, also operates as a pro-inflammatory alarmin that accelerates tissue damage via RAGE and TLR4 signalling. Its appearance in the maternal circulation at GD 20 thus has two implications: it reflects the degree of necrotic damage and suggests a systemic inflammatory milieu that might further jeopardise placental integrity. The partial reduction of both indicators by NAC demonstrates that ROS are the proximal trigger for the necrotic component, in line with the oxidative mechanism outlined by the MDA and Caspase-3 results.

The drop in foetal liver somatic index is mechanistically attributed to the reduction in Ki67 and PCNA labelling indices coupled with the increase in apoptotic index. Ki67 labels cells in any phase of the active cell cycle (G1, S, G2, and M), whereas PCNA, as the processivity cap for DNA polymerase delta, mostly detects cells in S-phase. The dose-dependent decrease in both cell populations suggests that oxidative stress caused by aspartame interferes with cell cycle entrance and progression rather than only promoting departure via apoptosis. This dual effect of inhibition of proliferation and acceleration of programmed cell death is particularly relevant in foetal liver, where the baseline indices of Ki67 and PCNA (42% and 49% respectively) reflect the intense hepatoblast expansion that underpins organogenesis during late gestation. The dosage dependent reduction of Ki67 was stronger in foetal than maternal liver consistent with increased vulnerability of the quickly cycling progenitor

populations to oxidative checkpoint arrest. The re-addition of NAC restores proliferative indices to control levels indicating that the blockade is not due to permanent genomic damage but is reversible and oxidant driven. The JC-1 and cytochrome-c findings suggest that mitochondrial malfunction is the proximal upstream event connecting ROS production to both apoptotic and necrotic consequences. Foetal liver from Asp-High animals had a JC-1 red/green ratio below 1 (0.89 ± 0.07), indicating total dissipation of $\Delta\Psi_m$ and functionally equal to the point of no return for the intrinsic apoptotic cascade. Once the inner mitochondrial membrane potential has collapsed, the electrochemical gradient driving ATP synthase is abolished, bioenergetic failure ensues and cells failing to execute the regulated caspase programme instead undergo secondary necrosis. This sequence is entirely consistent with the co-elevation of LDH and HMGB1 at the same dose tier. The cytosolic cytochrome-c is released via Bax/Bak-dependent holes in the outer mitochondrial membrane and then activates Apaf-1 and recruits procaspase-9 into the apoptosome, so establishing a mechanistic connection between the mitochondrial data and the Caspase-3 activity levels presented in Table 3. The roughly 12-fold rise in foetal cytochrome-c at 500 mg/kg much exceeds the roughly 8-fold increase in maternal liver again defining the foetal sensitivity gradient and supporting the increased mitochondrial susceptibility of immature hepatocytes. That the NAC decreased cytochrome-c release by around 76–78% and significantly improved JC-1 ratios is confirmation that ROS-mediated lipid peroxidation of mitochondrial membranes, as opposed to direct protein oxidation of the cytochrome-c molecule itself, is the operational mechanism of release.

Positioning our results within the current literature. Hepatic oxidative stress and Caspase-3 activation have been seen in adult rats after aspartame exposure, as reported in previous studies, and are similar with our maternal findings. More recent gestational studies support the present findings, as aspartame exposure prior to and during pregnancy was found to impair placental mitochondrial function and biogenesis, as well as reduce the number and weight of fetuses in rats, with a concomitant reduction in human birth weight in an exposed maternal-infant cohort [26], and aspartame-induced mitochondrial dysfunction has also been implicated in impaired ovarian and reproductive function [27] and in altered pubertal timing [28]. Maternal exposure to aspartame has also been shown to induce redox and metabolic dysregulation in the neonatal offspring tissue distal to the gut, indicating that its impact extends beyond the MPF axis examined here [29], and combined aspartame–caffeine exposure has been shown to exacerbate impaired renal maturation in rat offspring [30]. Earlier studies of prenatal sugar exposure have mainly examined metabolic programming (insulin resistance, adipogenesis) and organ-specific consequences in the offspring, but not acute, zone-specific MPF histopathology. In the present work we link these fields of study, by showing that the structural and biochemical disruption seen at GD 20 is sufficient to impair foetal somatic growth, providing a plausible antecedent for the metabolic and reproductive programming outcomes reported in this more recent literature [23,26–29].

Some constraints are worth mentioning. The mouse placenta resembles the human organ in many functional aspects, but differs from the villous hemochorial human placenta in its physical hemochorial structure and its zonal organisation. Allometric scaling has to be considered for direct extrapolation of dose–response data. The dosages used (125–500 mg/kg) are much higher than the human ADI, however use of doses beyond the ADI is common practice in mechanistic investigations in rodents in order to allow detection of hazardous end-points and to define the dose–response curve. Chronic postnatal follow-up is lacking and it is unknown if in utero damage influences long-term cardiometabolic or neurodevelopmental effects. Lower, ADI-equivalent dosages should be included in future investigations with postnatal surveillance.

CONCLUSION

Maternal–placental–fetal axis is perturbed by aspartame exposure during gestation in a dose-dependent, spatially resolved and mechanistically consistent way. The placental labyrinth zone and foetal liver are the most severely impacted compartments, which is consistent with a developmental susceptibility gradient, leading to amplification of oxidative injury in foetal tissue compared to mother tissue. Downregulation of GLUT1 and VEGF with concomitant HIF-1 α upregulation implicates impaired trophoblast transport capacity as a proximate cause of fetal growth restriction. N-acetylcysteine substantially reverses all measured endpoints, supporting a major contribution of oxidative stress to the observed injury. These results support further investigation of gestational aspartame exposure, particularly at human-relevant doses, and provide a mechanistic framework for future investigation of fetal programming outcomes.

Supplementary Materials

Not applicable.

Author Contributions

[Author names]: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, and Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Ethical Approval

The research was authorised by [name of ethics committee] (approval number [approval number]) All animal operations were performed according to institutional and national standards for the care and use of laboratory animals.

Declaration of Generative AI Use

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