

## Abnormal Retinal Structure and Function of Mother Wistar Rats Supplemented Aspartame, Glutamate and Galactose

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### Abstract

Three dietary supplements are investigated in the present study. Aspartame is a low calorie sweetener utilized instead of sugar for diabetic patients. Monosodium Glutamate (MSG) is used in varieties of food stuffs to enhance food flavor. Meanwhile galactose is presents in many of food items such as milk, dairy products, fruits and vegetables and overloads enhanced aging like process and altered body function. The present work aimed to illustrate the effect of dietary supplements of aspartame, glutamate and galactose in retina of mother Wistar rats. Virgin Wistar rats were mated with fertile male and zero date of pregnancy was determined. Pregnant rats were orally administered aspartame (100 mg/kg), monosodium glutamate (500 mg/kg) and galactose (1 g/kg body weight) from 6th day of gestation until parturition and 21 days post-partum. Four main groups of pregnant rats were used; control, aspartame, glutamate and galactose treatment. Each animal group was composed of ten individuals. Mother rats were sacrificed at 21 days post-partum and their retinas were dissected and processed for histological, and transmission electron microscopic investigation. Biochemical assessments of vascular endothelial growth factor (VEGF), endothelin-1 (ET-1), adhesion molecules (ICAM-1 & VCAM-1), 8-hydroxy-deoxyguanosine (8-hdG), iron, and zinc content were done. The present findings revealed that the used dietary supplements induced retinal damage assessed by degeneration of ganglion cells, inner and outer nuclear layer and widespread necrotic patches of photoreceptor outer segment. A striking missing of outer nuclear and photoreceptor outer segments was detected post-glutamate treatment. Glutamate and galactose treatment showed apparent increase of pleomorphic and pyknotic nuclei of inner and outer nuclear cells. The retinal thickness was markedly decreased in experimental groups especially in those of glutamate treatment. Retinal serotonin, dopamine and zinc and iron contents were markedly depleted, however, VEGF and ICAM-1& VCAM-1 and 8-hydroxy-guanosine were overexpressed in experimentally-treated groups compared to the control. It can be concluded that the applied dietary supplements affected the retinal structure and function of mother rats.

**Keywords:** Aspartame; Glutamate; Galactose; Mother rats; Retina; Light and transmission electron microscopy; Biochemistry

### Introduction

Aspartame (L-aspartyl-L-phenylalanine methyl ester) is a low calorie sweetener attained 200 times greater more than sugar. It is absorbed immediately via the intestinal lumen and metabolized to phenylalanine (50%), aspartate (40%) and methanol (10%) [1]. Aspartame was found to develop neurological and behavioral disorders and development of seizures attack in mice [2]. Also, cognition [3], headaches [4], alterations in mood and depression [5], migraines, multiple sclerosis and blurred vision [6] were reported post-increased supplementation of aspartame. Increase levels of phenylalanine and tyrosine and aspartic acid in the brain, led to a decrease in brain dopamine [7] and development of phenylketonuria disorder [8], the main cause of demyelination and depletion of dopamine levels in brain and retina of infants [9].

Monosodium Glutamate (MSG) is excitotoxic material widely used as food enhancer in meats, poultry, seafood, snacks, and soups and stews [10]. It is induced a visceral sensation from the stomach, intestine and portal vein outside the sense organs region which is described as “umami” taste. The average glutamate consumption has

increased dramatically in recent years [11] reaching up to 0.3-0.5 g per day in European countries and 1.2-1.7 g per day in Asia [12].

Glutamate is metabolized by glutamine synthetase into nontoxic glutamine following uptake by glutamate transporter, GLAST, into Müller cells [13]. In the retina, glutamic acid is responsible for the synaptic transmission between photoreceptor cells, bipolar cells and ganglion cells and its overload is responsible for neuronal cell death [14] such as retinal ganglion cells [15].

Galactose is presents in milk, dairy products, fruits and vegetables [16]. Its absorption occurs across the brush border membrane of the proximal jejunum and renal epithelium via the Na<sup>+</sup>-glucose co-transporters SGLT1 and SGLT2 [17]. Abnormal galactose metabolism occurred through impairment of galactokinase, galactose-1-phosphate uridylyltransferase, and UDP-galactose-4-epimerase, the enzymes involved of its metabolism [18] leading to galactosaemia. Chronic exposure of mice to D-galactose (100 mg/kg, S.C., 7 weeks) induced neuronal damage of hippocampus and sub-granular zone in the dentate and granular layer [19] leading to cognitive function and Alzheimer's disease [20].

The present study aimed to illustrate the detailed retinal structures and function through assessments histo-cytological structures and

biochemical markers post-supplementation of aspartame, monosodium glutamate and galactose.

## Materials and Methods

### Chemicals

All of the chemicals used were of highest purity. Aspartame, monosodium glutamate and galactose were obtained from Sigma-Aldrich Company. All of the used nutrients were dissolved in water and orally administered daily at doses of 100 mg aspartame/kg [21], 500 mg MSG/kg [22] and 1 g galactose/kg [23] throughout gestation and lactation period.

### Experimental work

Virgin female and male Wistar albino rats (*Rattus norvegicus*) were obtained from Laboratory Farm, Ministry of Health, and Cairo, Egypt. They were acclimatized for 15 days before experimentation. Pregnancy occurred after mating virgin female rat with fertile male by placing them in plastic cages. In the next morning, zero date of gestation was determined by observing sperm in vaginal smear and pregnant were separated. They were kept in well aerated condition with 12 h light and dark cycle. Free excess of diet and water were allowed *ad-libitum*. The pregnant rats were divided into four groups; control, aspartame, glutamate and galactose treated groups. Each group was composed of 10 individuals.

At 21 days post-partum, mother rats of both control and experimentally treatment were euthanized by diethyl ether and sacrificed. Ocular regions were dissected and their retinas were separated and subjected to the following investigations:

- Transmission Electron Microscopy (TEM)

The specimens were fixed in 2.5% buffered glutaraldehyde, followed on 1% osmium tetroxide, dehydrated in ascending concentration of ethyl alcohol, cleared in propylene oxide and embedded in epoxy-resin. Semithin sections were obtained and stained with toluidine blue. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate, and examined under a Joel 100CX transmission electron microscope.

- Biochemical investigation

The retinal specimens were homogenized in 10% ice-cold 2.5 mM-tris buffer (pH 7.5) and centrifuged at 14000 × g for 15 min at 4°C and the supernatant was kept in deep freeze. Determination of serotonin and dopamine were carried out as described to Schlumpf et al. [24].

Vascular Endothelial Growth Factor (VEGF) was determined by R&D ELISA kit (Minneapolis, MN, USA). (HRP) and TMB substrate were added to wells containing retinal samples and VEGF antibodies. The color density was measured spectrophotometrically at 450 nm.

Intracellular Adhesion Molecule (ICAM)-1 and Vascular Adhesion Molecule (VCAM)-1 were assayed using ELISA kit (R&D Systems; Minneapolis, MN). Amount of 100 µL antibodies against recombinant human rat ICAM-1 and VCAM-1 conjugated to horseradish peroxidase were added to each well. After incubation, 100 µL of tetramethylbenzidine was added for color development and optical density was determined at wavelengths of 450 & 620 nm.

Endothelin-1 (EDN1) was assayed using ELISA Kit (USCN Life Science Inc. Avidin (Biotin separated from raw chicken egg albumen) conjugated with horseradish peroxidase was added and the amount of bounded HRP was proportional to the amount of EDN1 at 450 nm.

Concerning 8-hydroxy-2-deoxy guanosine (8-OH-dG), its amount was determined by the Bioxytech-ELISA Kit (OXIS Health Products, Portland, OR, USA). A volume of 50 µL of sample or standards and 50 µL of primary antibody were added to the specimen of 8-OHdG-coated microtiter plates and incubated at 37°C for 1 h. Thereafter, treatment with horseradish peroxidase-conjugated secondary antibody was done and it was followed by the addition of tetramethylbenzidine to visualize the color which was measured at a wavelength of 450 nm [25].

Iron and zinc were determined in dried tissues after lipid extraction was performed by a mixture of chloroform and methyl alcohol at a ratio of 2:1. Known weights of the dried samples were digested with nitric acid of highest purity (1 mL/sample), diluted with 4 mL bi-distilled water, and measured by atomic absorption spectrophotometer [26].

- Statistical analysis

Data were recorded as mean ± SE and analyzed utilizing SPSS software (Version 13) by one way post-hoc Analysis of Variance (ANOVA) between studied groups and the level of statistical significance was set at P<0.05.

## Results

### Biochemical observations

Table 1 illustrates the biochemical markers of retinal cell function of mother rats treated with either aspartame or glutamate or galactose. There was a detected depletion of retinal serotonin, dopamine and zinc and iron contents and increase of VEGF and ICAM-1 & VCAM-1 and 8-hydroxy-guanosine in aspartame, glutamate and galactose treatment compared to the control.

### Morphometric assessments

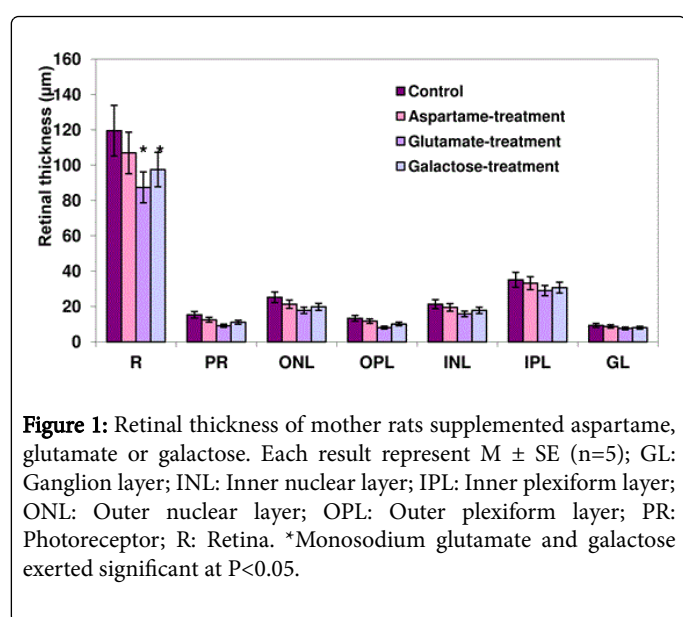
Figure 1 illustrates the retinal thickness of aspartame, glutamate and galactose treatment. The retinal thickness markedly decreased in glutamate compared to galactose and aspartame treatment.

### Aspartame

Compared to the control (Figures 2A-2E), mother rats that received aspartame treatment during gestation and lactation period revealed apparent alterations of histological structures such as comparative reduction of ganglion cells, spongy-appearance of inner plexiform layer, thinning of inner and outer nuclear layer and vacuolation of photoreceptor outer segment. At ultrastructural level, the Retinal Pigment Epithelium (RPE) possessed karyorrhexis of their nuclei. The apical cytoplasmic microvillus structures were distorted. The photoreceptor outer segment possessed numerous degenerated spots of their stacked membranes. Damage of inner and outer nuclear cells and neovascularization in ganglion and inner plexiform layer were detected. Vacuolar degeneration of the ganglion cells was observed (Figures 3A-3E).

	DA (ng/mg)	5-HT (ng/mg)	VEGF (Pg/100 mg)	8-HDG (ng/100 mg)	ICAM-1 (ng/100 mg)	VCAM-1 (ng/100 mg)	Minerals	
							Fe (µg/g dwt)	Zn (µg/g dwt)
Control	35.29 ± 1.84	133.03 ± 2.56	140.35 ± 1.93	1.23 ± 0.07	2.05 ± 0.06	1.29 ± 0.07	98.64 ± 2.28	29.17 ± 1.14
Aspartame	32.39 ± 1.84	121.47 ± 1.97	149.8 ± 2.49	*1.572 ± 0.07	2.17 ± 0.08	1.34 ± 0.04	91.94 ± 1.63	21.46 ± 2.00
Glutamate	*18.00 ± 2.28	*120.85 ± 2.12	*156.86 ± 1.58	*1.33 ± 0.05	2.27 ± 0.05*	1.36 ± 0.037	*79.9 ± 2.28	*26.45 ± 2.28
Galactose	30.549 ± 2.28	*130.81 ± 2.06	*167.39 ± 1.84	*1.64 ± 0.07	2.39 ± 0.11	*1.42 ± 0.05	*127.09 ± 1.84	25.11 ± 1.70

**Table 1:** Biochemical markers of retinal cell function of mother rats treated with either aspartame or glutamate or galactose. Each result represent M ± SE (n=5); C: Control; DA: Dopamine; ICAM-1: Intercellular adhesion molecule; Gl: Glutamate; VCAM-1: Vascular cell adhesion molecule; 5-HT: Serotonin; VEGF: Vascular endothelial growth factor; 8-HDG, 8-hydroxyguanosine. \*Significant at P<0.05.



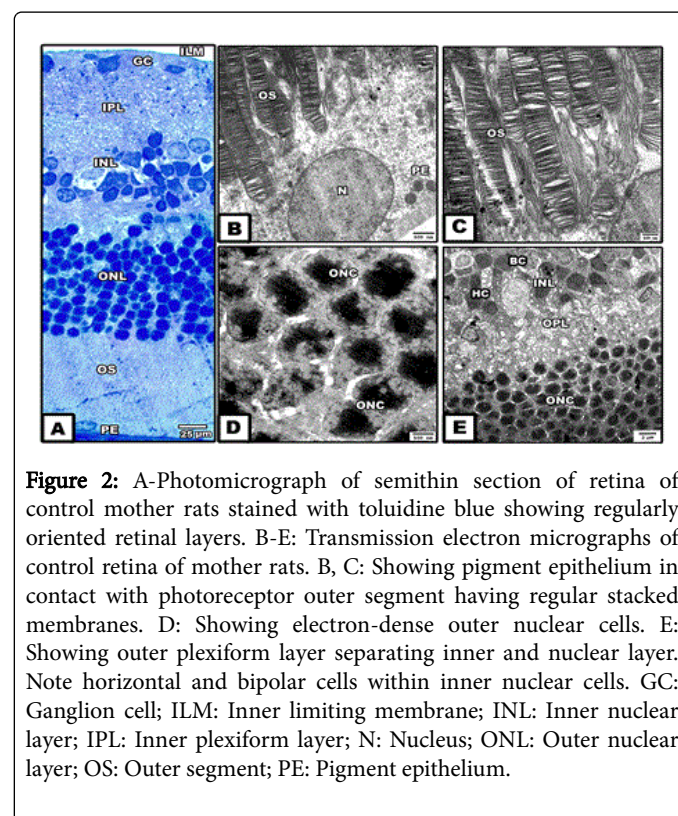
### Monosodium glutamate

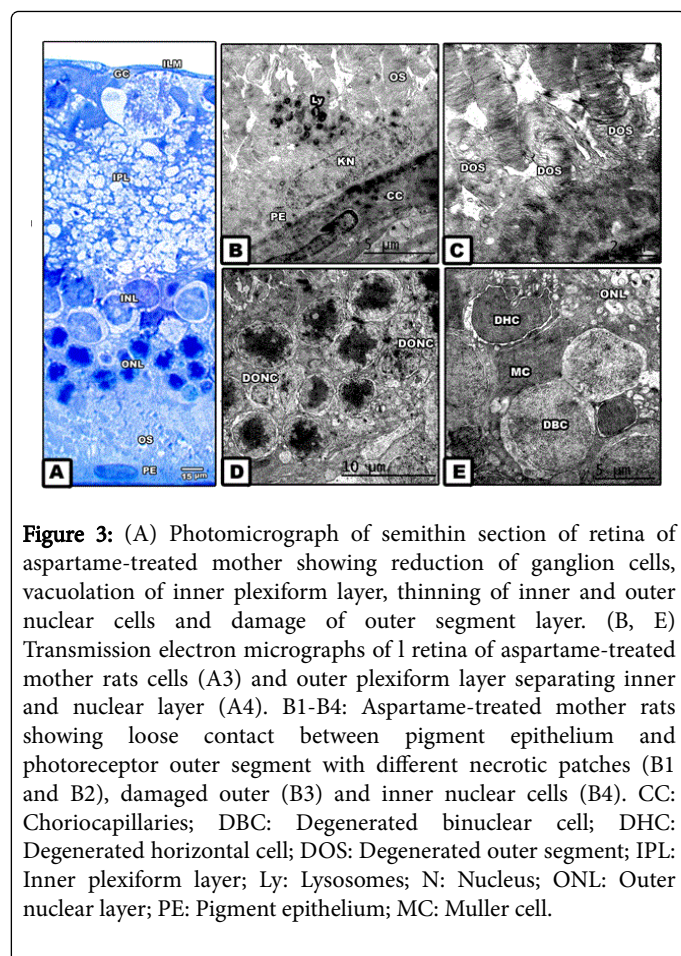
Compared to the control, semi thin sections of glutamate-treated mother rats revealed massive degeneration of ganglion cells, spongy-appearance of inner plexiform layer, thinning of inner nuclear layer and striking missing of outer nuclear and photoreceptor layer in 8 of 20 mother retina. There was a comparative decrease in the number of pigment epithelium (Figure 4A). At ultrastructural level, the RPE possessed karyorrhexis of their nuclei and disintegration of their apical cytoplasm microvillus structures. Many of the inner nuclear cells possessed karyorrhexis of their nuclei. There were a detected malformed photoreceptor nuclei and photoreceptors. Dense neovascularization was detected in inner and outer plexiform layer (Figures 4B-4E).

### Galactose

Compared to the control, semi thin sections of galactose-treated mother rats revealed massive degeneration of ganglion cells, spongy-appearance of inner plexiform layer, thinning of inner nuclear layer and different foci of degenerated nuclear cell. There was a comparative decrease in the number of pigment epithelium (Figure 5A). At ultrastructural level, the ganglion cells were markedly disintegrated

and possessed neovascularization. Many of the inner nuclear and outer nuclear cells become pleomorphic and showed electron-dense chromatin material. Numerous blood vessels were detected at the outer plexiform layer, at the interphase between inner and outer nuclear layer. The photoreceptor outer segment revealed different necrotic patches of their stacked membranes (Figures 5B-5E).





**Figure 3:** (A) Photomicrograph of semithin section of retina of aspartame-treated mother showing reduction of ganglion cells, vacuolation of inner plexiform layer, thinning of inner and outer nuclear cells and damage of outer segment layer. (B, E) Transmission electron micrographs of retina of aspartame-treated mother rats cells (A3) and outer plexiform layer separating inner and nuclear layer (A4). B1-B4: Aspartame-treated mother rats showing loose contact between pigment epithelium and photoreceptor outer segment with different necrotic patches (B1 and B2), damaged outer (B3) and inner nuclear cells (B4). CC: Choriocapillaries; DBC: Degenerated binuclear cell; DHC: Degenerated horizontal cell; DOS: Degenerated outer segment; IPL: Inner plexiform layer; Ly: Lysosomes; N: Nucleus; ONL: Outer nuclear layer; PE: Pigment epithelium; MC: Muller cell.

## Discussion

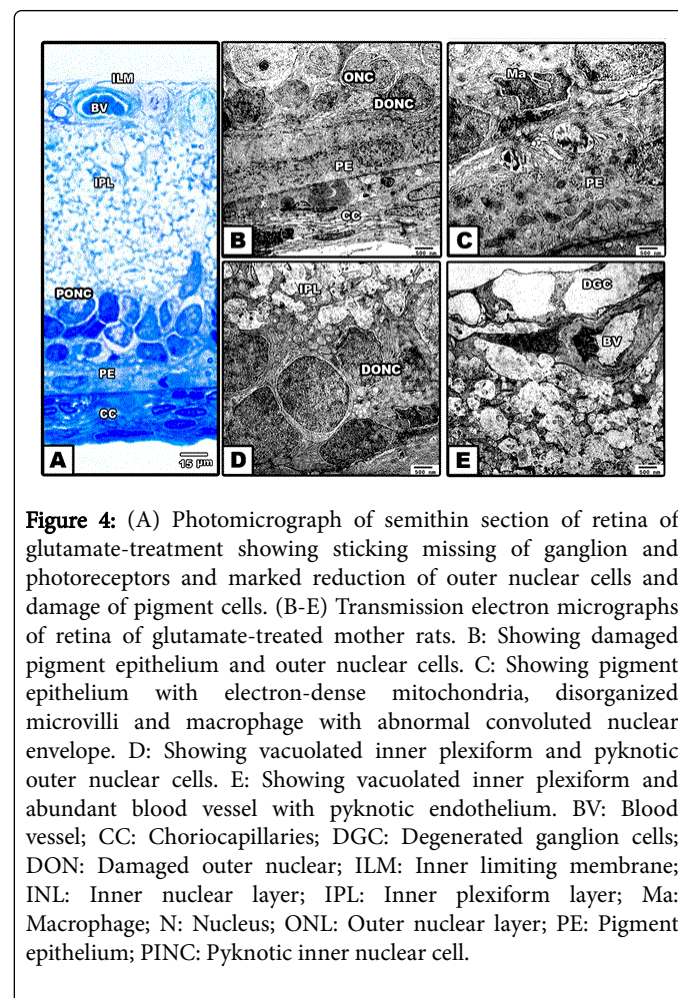
The observed findings revealed that supplementation of aspartame (100 mg/kg), MSG (500 mg/kg) and galactose (1 g/kg) exhibited marked reduction of retinal thickness, degeneration of ganglion cells and development of spongiosis of inner plexiform layer. Glutamate exhibited apparent reduction compared to the other treatments.

Similar atrophy of retinal thickness was reported in glutamate treatment [27]. The decreased retinal thickness appeared resulted from the missing outer nuclear cells and photoreceptors in mother rats supplemented monosodium glutamate.

Similar findings of apoptic retinal ganglion cells were reported in mice [27-29] (and rabbits [30] subjected to N-methyl-D-aspartate. Administration of aspartame (200 mg/kg) to rats led to two fold increase of phenylalanine and its product tyrosine in brain and plasma level [31] and contributed to decrease the ganglion cells [32-35]. Monosodium glutamate exhibited similar reduction of ganglion cells in rat [36] and rabbits [27].

Galactose associated damage retinal ganglion cells were reported in experimentally induced diabetes of rats [37], dogs [38] and db/db Mice [39]. D-galactose treatment represents a model of ageing-related through increased production of *galactitol* which accumulated in cells and reacts with amines group of to form advanced glycation end products, and intern increase oxidative stress and cellular damage [23,40]. The contribution of galactose in development of diabetes

resulted from its influences in increased accumulation of glycogen phosphorylase and phosphorylated glycogen synthase, the promoter of diabetes [41].



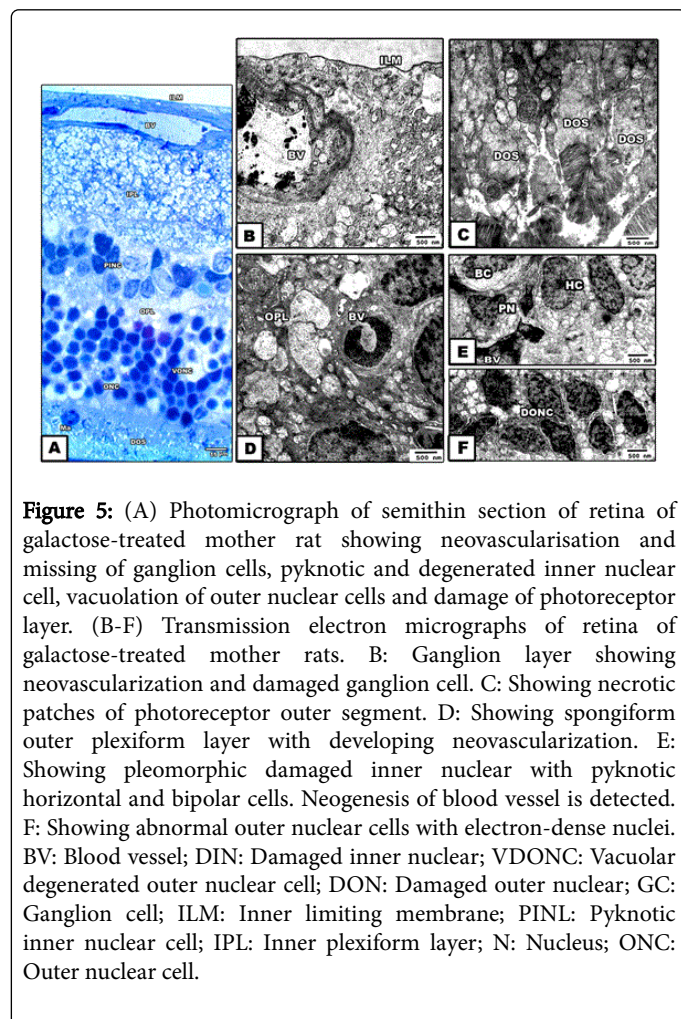
**Figure 4:** (A) Photomicrograph of semithin section of retina of glutamate-treatment showing missing of ganglion and photoreceptors and marked reduction of outer nuclear cells and damage of pigment cells. (B-E) Transmission electron micrographs of retina of glutamate-treated mother rats. B: Showing damaged pigment epithelium and outer nuclear cells. C: Showing pigment epithelium with electron-dense mitochondria, disorganized microvilli and macrophage with abnormal convoluted nuclear envelope. D: Showing vacuolated inner plexiform and pyknotic outer nuclear cells. E: Showing vacuolated inner plexiform and abundant blood vessel with pyknotic endothelium. BV: Blood vessel; CC: Choriocapillaries; DGC: Degenerated ganglion cells; DON: Damaged outer nuclear; ILM: Inner limiting membrane; INL: Inner nuclear layer; IPL: Inner plexiform layer; Ma: Macrophage; N: Nucleus; ONL: Outer nuclear layer; PE: Pigment epithelium; PINC: Pyknotic inner nuclear cell.

Also, aspartame, monosodium glutamate and galactose induced damage of the retinal pigment epithelium characterized by disorganization of apical microvillus structures, pyknosis or karyolysed nuclei, electron-dense mitochondria, and abundant lysosomes. Defects of pigment epithelium may be involved in decrease nutrient transport and impair phagocytosis of the terminal end of photoreceptor outer segment leading to the development of necrotic patches of their stacked membranes. Monosodium glutamate exerted a striking missing of both outer nuclear cells and photoreceptor outer segment in 8/20 mother rats. The observed retinal damage was supported by the overexpression of 8-hydroxydeoxyguanosine, the marker of cell damage.

Similar findings of increased 8-hydroxy-deoxyguanosine-positive cells associated with retinal ganglion cell loss were observed after intravitreal administration of NMDA (200 nmol/eye) [35].

It is know that RPE promoted nutrient transport from the choriocapillaris to the photoreceptors [42] and continuous renewal of their apical parts and phagocytosis [43] which maintains the function of photoreceptors outer segment [44]. Tezel et al. [45] detected expression of hemoglobin in RPE which exceeded that in human

erythroblast. The photoreceptors are the dominant oxygen consumers when compared to the inner retina [46].



**Figure 5:** (A) Photomicrograph of semithin section of retina of galactose-treated mother rat showing neovascularisation and missing of ganglion cells, pyknotic and degenerated inner nuclear cell, vacuolation of outer nuclear cells and damage of photoreceptor layer. (B-F) Transmission electron micrographs of retina of galactose-treated mother rats. B: Ganglion layer showing neovascularization and damaged ganglion cell. C: Showing necrotic patches of photoreceptor outer segment. D: Showing spongiform outer plexiform layer with developing neovascularization. E: Showing pleomorphic damaged inner nuclear with pyknotic horizontal and bipolar cells. Neogenesis of blood vessel is detected. F: Showing abnormal outer nuclear cells with electron-dense nuclei. BV: Blood vessel; DIN: Damaged inner nuclear; VDONC: Vacuolar degenerated outer nuclear cell; DON: Damaged outer nuclear; GC: Ganglion cell; ILM: Inner limiting membrane; PINL: Pyknotic inner nuclear cell; IPL: Inner plexiform layer; N: Nucleus; ONC: Outer nuclear cell.

Furthermore, the damage of RPE leads to altered RPE-photoreceptor function causing retinal ischemia and enhanced the development of neovascularization in ganglion and nerve fiber layer as well as in the outer plexiform layer.

Retinal neovascularization were also reported in dog fed on 30% galactose for 28-41 months [47,48]. Pericytes are mural or vascular smooth muscle cells containing contractile fibers and unsheathed the endothelial cells. There is a gap junction between the cytoplasm of pericytes and endothelial cells, which allow diffusions of ions and small molecules. Diabetes was found to increase of mitochondrial damage leading to increase liberation of free radicals. This led to the production of glycation end product facilitated in thickening of the basement membrane, loss of inter-endothelial tight junctions and pericyte apoptosis [49].

These are supported by the observed increase of retinal level of ET-1, VEGF and adhesion molecules (ICAM-1 & VCAM-1). Similar findings of retinal ischemia was induced in mice by NMDA and expressed by increase interleukin-1 $\beta$  and TNF-alpha, and endothelial adhesion molecules (ICAM-1) and leukocyte accumulation in the retinal vessels [50].

Endothelin 1 (ET-1) is contributed to the development of microangiopathy in patients with type-2 diabetes [51] and VEGF is associated with retinal vascular leakage, neovascularization in Kimba mouse eyes [52] and increased the expression of adhesion molecules in the retinal vasculature [53].

The damage of retinal structures were supported by the observed contribution of the applied nutrients to the depletion of retinal dopamine and serotonin levels; the main neurotransmitters that coordinate retinal function [54] and produced by ganglion cells and their axons [55]. Depletion of the dopamine system during retinal damage may lead to retinal ischemia [56] which is represented as arterial thrombosis in atherosclerotic patient [57].

Also, there is a detected marked depletion of retinal contents of zinc and iron of mother rats supplemented aspartame, glutamate and galactose. The retina demonstrated high amounts of zinc especially in the photoreceptors which varies in dark and light, indicating a role for zinc in a light-regulated process. Zinc deficiency in humans may induce abnormal dark adaptation and/or age-related macular degeneration [58]. Retinal zinc deficiencies were reported in spontaneous diabetes mellitus [59]. Patients with leukocyte zinc-deficiency had impaired photoreceptor function [60]. In pigmented rats, zinc deficiency led to accumulation of lipofuscin in the RPE and reduction at Bruch's membrane [61]. Also, iron is important in many metabolic processes and its abnormal shift may contribute to the development of diseases. In mice, mutation of the iron exporter ceruloplasmin led to age-dependent retinal iron overload and increase retinal degeneration characteristic of age-related macular degeneration [62]. Anemic children exhibited thinning peripapillary retinal nerve fiber layer [63].

Finally, it can be concluded that aspartame, glutamate and galactose supplementation are contributed to altered their retinal structure and function. These were carried out via different pathways such as cytological structure, neurotransmitters, VEGF, ET-1, adhesion molecules and zinc and iron elements.

## Conflict of Interest

The author declares there is no conflict of interest.

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