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Ameliorative effect of *Morus alba* leaves extract against developmental retinopathy in pups of diabetic and aluminum intoxicated pregnant albino rats

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PEER REVIEW

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Comments

This is an interesting work that showed the ameliorative effect of *M. alba* leaves extract developmental retinotoxicity in pups of diabetic and aluminum intoxicated pregnant albino rats. The scientific part of the manuscript is good and sound and so is the methodology. Results obtained are interesting and encouraging.
Details on Page 307

ABSTRACT

Objective: To investigate the possible ameliorative effect of crude water extract of *Morus alba* (*M. alba*) leaves on retinopathy of rat pups maternally subjected to diabetes and/or Al intoxication.

Methods: Both control and experimental groups were subjected to certain integrated approaches, namely, biochemical assessments, light microscopic investigation, transmission electron microscopic investigation, single cell gel electrophoresis (comet assay) and determination of DNA fragmentation.

Results: The retina of pups of diabetic and/or Al-intoxicated mothers exhibited abnormal alterations in retinal cell layers including retinal pigmented epithelium, photoreceptor inner segment and ganglion cells. Increased incidence of DNA fragmentation and apoptosis were evident in pups of diabetic and/or Al-intoxicated mothers. However, retina of pups maternally received *M. alba* extract plus diabetes or Al-intoxicated alone or in combination showed marked amelioration. Less degree of ameliorations was seen in retina of pups maternally subjected to combined treatment. Furthermore, application of crude water extract of *M. alba* resulted in amelioration of the alterations of maternal serum glucose as well as Al concentration.

Conclusions: Based on the results of the present study, *M. alba* extract is effective against experimentally diabetic and Al-induced developmental retinopathy.

KEYWORDS
Morus alba, Retinopathy, Aluminum, Diabetes, TEM, Comet assay

1. Introduction

Diabetes and aluminum (Al) intoxication possess the major health problems. Al intoxication comes from different sources such as cooking utensils, food additives, medicines such as antacids or deodorants, *etc*.[1], drinking water[2], vaccines, inhaled fumes and particles from occupational exposures[3]. Corn, yellow cheese, salt, herbs, spices, tea, cosmetics were found to have increased amounts of Al[4]. Diabetes mellitus is a heterogeneous metabolic disorder characterized by hyperglycemia. The disease is worldwide increasing and affecting children and adolescents in industrialized as well as in developing countries, posing a major challenge to global human health[5] and is now considered as one of the main threats to human health in the 21st century[6].

Several authors who investigated streptozotocin (STZ)-diabetes described segmental demyelination and remyelination as well as abnormalities of the paranodal myelin at a similar rate with the control animals[7], suggesting these alterations to be more related to aging[8,9]. Thomas *et al*.[10] described a reduction in the myelin thickness in chronic STZ-diabetic rats especially if the induction of diabetes was in the early stages of life. More recently, it has been reported that chronic STZ-diabetes was able to cause demyelination,

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especially on the small fibers either on the aortic depressor nerve or the phrenic nerve[11].

The presence of retinopathy, even in its early stages has also been associated with cerebral white matter lesions[12]. In otherwise healthy diabetic adults, a recent exploratory analysis has suggested that increasing severity of retinopathy is related to reductions in cortical grey matter density[13]. Laboratory studies on rats revealed that diabetes of 8 months' duration increased the release of cytochrome c into the cytosol and Bax protein into the mitochondria prepared from the retina, and this phenomenon was not observed in 2 months of diabetes[14]. Diabetes increases oxidative stress, which plays an important role in the development of diabetic complications[15]. Oxidative stress is increased in retina of diabetes and in isolated retinal capillary cells incubated in high-glucose medium. The antioxidant defense system is impaired in the retina of diabetes, glutathione levels are decreased and superoxide production is increased[15,16].

Research works concerning Al retinopathy are evidently scarce[17]. Fry *et al.*[18] intoxicated rabbit with Al and reported neurofibrillary tangles in a subpopulation of retinal ganglion cells (GCs), located primarily in the peripheral retina. The distribution of affected cells suggested a differential susceptibility of GCs to Al intoxication. Following daily injection of 0.3 mL of 4% $AlCl_3$ to 4-week-old Wistar Kyoto rats, Lu *et al.*[19] reported that thin retinal pigmented epithelium (PE) and disappearance of the photoreceptor (Phs) outer and inner segments were the most evident observations.

There is a growing tendency towards using phototherapy owing to the general belief that it has no side effects compared to chemotherapy. Several studies have indicated that hyperglycemia can be controlled via different sorts of medicinal plants[20-24]. Singab *et al.*[25] studied the hypoglycemic activity of the flavonoids rich fraction of 70% alcohol extract of Egyptian *Morus alba* (*M. alba*) root bark in STZ-induced diabetic rats. The authors found that administration of Egyptian *M. alba* root bark for 10 days (600 mg/kg) significantly reduced the amount of glucose from control level to a lower level and significantly increased the insulin level from the control to a high level.

It has been reported that consumption of *M. alba* extract with 75 g of sucrose significantly attenuated the increase in blood glucose concentration in non-diabetic and Type 2 diabetic individuals[26]. *M. alba* leaf contains active compounds that can inhibit galactosidases, such as 1-deoxynojirimycin[27], and this effect may help suppress postprandial hyperglycemia by reducing the rate of digestion and absorption of carbohydrates from the intestine. However, intraperitoneal administration of *M. alba* leaf extract has a hypoglycemic effect in experimentally-induced diabetic mice[28].

The present study dealt with investigating the developmental neurotoxicity of retina of pups of mothers subjected to aluminum chloride ($AlCl_3$) intoxication and/or diabetes during perinatal life. Treatment with *M. alba* leaves extract were carried out to examine its possible ameliorative effect upon the developmental retinopathy. The study involved several integrated parameters conducted on both control and experimental groups: (1) determination of glucose level in the mother's serum; (2) determination of Al concentration in the mother's serum; (3) light and transmission electron microscopic investigation for the development and differentiation of retinal neuronal cells; (4) comet assay; (5) assessment of DNA damage.

2. Materials and methods

2.1. Animals and grouping

Principles of animal care and use were followed during the conducting of the present study. One hundred and forty fertile male and virgin female albino rats (*Rattus norvegius*) weighing (180 ± 20) g were purchased from Hellwan Breeding Farm, Ministry of Health, Cairo, Egypt and used for the experimentation. Rats were housed in individual cages and maintained in a room with good ventilation at 23 ˚C. They were fed on standard diet free from excess fats and free access of food and water was allowed *ad libitum* throughout the experimental period. Females were mated in a special cage (1 male/2 females) during overnight and gestation was determined in the next morning by the presence of sperm in a native vaginal smear. The pregnant rats were arranged into seven groups (15 individuals in each group); control, experimental diabetic, diabetic and *M. alba*, Al-intoxicated, Al intoxicated and *M. alba*, experimental diabetic and Al intoxicated, diabetic and Al intoxicated group plus *M. alba*. The control group was subdivided into two subgroups, the first as control (C) and the second as *M. alba* (M) group. At the end of the experimental period *i.e.* after 7 and 14 days from parturition, mothers and pups of both control and experimental groups were anesthetized by an intraperitoneal injection of sodium pentobarbital solution (50 mg/kg body weight), sacrificed, dissected and eye was separated and processed differently according to the required investigations.

2.2. Water extraction of M. alba leaves

Mulberry leaves were washed and dried in a hot air oven at 50 ˚C for 6-8 h. The dried material was ground to a fine powder and kept in an airtight container at 4 ˚C until further use. Four grams dried *M. alba* leaves were powered and extracted with 50 × (w/v) of hot water (85 ˚C) for 3 h. The extract was filtered with Whatman No.1 filter paper and concentrated to a volume of 1/20 of the initial solution volume by heating at a no boiling temperature near 100 ˚C, and then dried completely under vacuum at 25 ˚C. The dried extract (w/w = 0.5 g, yield = 15%) was used during experimentation. The applied dose of *M. alba* extract was 100 mg/kg body weight[29], and the extract was orally administered after the induction of diabetes every other day till the end of experimentation.

2.3. Induction of diabetes

Diabetes mellitus was induced experimentally in all rat groups except the control and Al intoxicated alone by a single intraperitoneal injection of STZ (60 mg/kg) in citrate buffer (0.05 mol/L, pH 4.5) at 5th day of gestation for two consecutive days and injected within 10 min of dissolution[30]. Control animals were treated with physiological saline as a vehicle. Maternal hyperglycemia was verified by measuring the blood glucose level. Rats with a level of more than 300 mg/dL were selected for this study.

2.4. Induction of Al-toxicity

For the present study, $AlCl_3$ of highest purity was purchased from El-Nasr Pharmaceutical Chemicals, Menoufiya, Egypt. It was dissolved in physiological saline solution and intragastrically administered by stomach intubation every other day from the 6th day of gestation till the end of the experiments. The applied dose was 80 mg/kg body weight[31].

2.5. Biochemical assessments

At the end of each experiment *i.e.* after 7 and 14 days from parturition, overnight food was removed; the mothers and their pups of both control and experimental groups were anesthetized, sacrificed and blood was collected from heart of mothers in non-heparinized tube and centrifuged at 3000 r/min for 30 min and serum was collected and diluted with bi-distilled water. Al concentration was determined in the serum by using Perkin Elmer Model 5000 spectrophotometer[32].

2.6. Light microscopic investigation

Eye specimens from both control and experimental groups were fixed in 10% phosphate-buffered formalin for one day, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and mounted in molten Paraplast at 58-62 ℃ and processed to generate 5 μm thick paraffin sections. The obtained sections were stained with hematoxylin and eosin and subjected to microscopic examination.

2.7. Transmission electron microscopic investigation

Eye specimens from both control and experimental pups aged 7 and 14 days were separated and immediately fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L cacodylate buffer (pH 7.4). After rinsing in 0.1 mol/L cacodylate buffer, they were post fixed in a buffered solution of 1% osmium tetra oxide at 4 ℃ for 1.5 h and dehydrated in ascending grades of ethyl alcohol and embedded in epoxy-resin. Ultrathin sections were cut with a diamond knife on an LKB microtome and mounted on grids, stained with uranyl acetate and lead citrate and examined with a Joel transmission electron microscope (TEM).

2.8. Single cell gel electrophoresis (comet assay)

For comet assay, eye specimens from both control and experimental pups were homogenized in chilled homogenizer buffer, pH 7.5, containing 75 mmol/L NaCl and 24 mmol/L Na_2EDTA to obtain a 10% tissue solution. A Potter-type homogenizer was used and eye samples were kept on ice during and after homogenization. Six microlitres of eye homogenate was suspended on 0.5% low melting agarose and sandwiched between a layer of 0.6% normal-melting agarose and a top layer of 0.5% low melting agarose on fully frosted slides. The slides were kept on ice during the polymerization of each gel layer. After the solidification of the 0.6% agarose layer, the slides were immersed in a lysis solution (1% sodium sarcosinate, 2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris-HCl, 1% TritonX-100 and 10% dimethylsulfoxide) at 4 ℃. After 1 h, the slides were placed in electrophoresis buffer (0.3 mol/L NaOH, 1 mmol/L Na_2EDTA, pH 13) for 10 min at 4 ℃ to allow DNA to unwind. Electrophoresis was performed for 10 min at 300 mA and 1 V/cm. The slides were neutralized with Tris-buffer, pH 7.5, and stained with 20 μg/mL ethidium-bromide for 10 min. Each slide was analyzed using the Leitz Orthoplan epifluorescence microscope (Wetzlar, Germany). One hundred cells were analyzed on each slide using the comet assay II automatic digital analysis system. Perspective tail length (μm) is the distance of DNA migration from the center of the body of the nuclear core and is used to evaluate the of DNA damage. The tail moment is defined as the product of the tail length and the fraction of the total DNA in the tail (Tail moment=tail length × % of DNA in the tail). Both tail length and tail intensity were measured automatically by image analysis software[33].

2.9. Determination of DNA fragmentation

Extraction of DNA was done according to the method of Aljanabi and Martinez and Hassab El-Nabi[34,35]. Briefly, biopsies of freshly eye specimens weighing 10 mg were squeezed in Eppendorf tubes, lysed with 600 μL buffer (50 mmol/L NaCl, 1 mmol/L Na_2EDTA, 0.5% sodium dodecyle sulphate, pH 8.3) and shacked gently. The mixture was incubated overnight at 37 ℃. For protein precipitation, an amount of 200 μL of saturated NaCl was added to the samples, shacked gently and centrifuged at 12000 r/min for 10 min. The supernatant was transferred to new Eppendorf tube and the DNA was precipitated by 600 μL cold iso-propanol. The mixture was inverted several times till fine fibers of nucleic acids appeared, at which time the mixture centrifuged for 5 min at 12000 r/min. The supernatant was then removed and the pellets (DNA and RNA) were washed with 500 μL 70% ethanol and centrifuged at 12000 r/min for 5 min. The supernatant was decanted and the tubes were plotted on Whatman paper to dry for 10 min. The pellets were re-suspended in 50 μL of Tris-EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8). The re-suspended DNA was incubated for 30-60 min with loading mix (Rnase + loading buffer) and then added into the agarose gel wells. A gel was prepared with 2% electrophoretic grade agarose containing 0.1% ethidium bromide (200 μg/mL). The DNA samples were mixed with loading buffer (0.25% bromophenol blue, 0.25% xylene cyanole FF and 30% glycerol) and loaded into the wells (2 μg of DNA/lane) with a standard molecular-sized ladder marker (Pharmacia Biotech., USA). The gel was electrophoresed at a current of mA for 2.5 h using the submarines gel electrophoresis machine. The DNA was visualized and photographed with illumination under ultraviolet light using a photo-documentation hood (Fisher Scientific, Pittsburgh PA, USA) equipped with a Polaroid 667 film with an orange filter (Kodak, Rochester, NY, USA). The ultraviolet reacts with the ethidium bromide to show the DNA fragments. Apoptotic bands appeared and located at 200 bp and its multiples.

2.10. Statistical analysis

All data sets were expressed as mean ± SEM. The data were

analyzed statistically for normal distribution (student's *t* test) to find out the significant difference[36] and homogeneity of variance using SPSS software for Windows, version 11. Values where $P<0.05$ were considered statistically significant.

3. Results

3.1. Glucose levels in rats

Glucose level (mg/dL) reached the highest level in either diabetes alone or in combination with $AlCl_3$. However, Al intoxicated mothers possessed no effect on glucose level compared with the other treatments. Treatment with *M. alba* leaves extract reduced glucose levels but it was still above the normal level in diabetes alone or with Al (Figure 1).

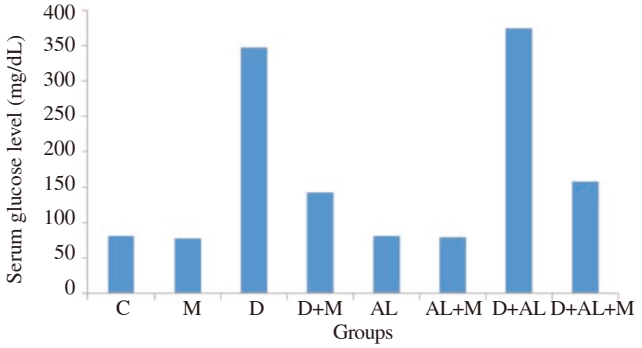

Figure 1. Biochemical changes of serum glucose level in mothers of control and experimental groups.
C: Control group; D: Experimental diabetic group; D + M: Diabetic and *M. alba* treated group; AL: Al-intoxicated; AL + M: Al intoxicated and *M. alba* treated group; D + AL: Experimental diabetic and Al intoxicated; D + AL + M: Diabetic and Al intoxicated plus *M. alba* treated group.

3.2. Al levels in rats

Al level (μg/L), on the other hand, reached the highest level in either Al intoxicated mother alone or in combination with diabetes. However, diabetic mother possessed moderate effect on Al level compared with the other groups. Treatment with *M. alba* leaves extract reduced Al levels but it was still above the normal level in Al alone and/or with diabetes (Figure 2).

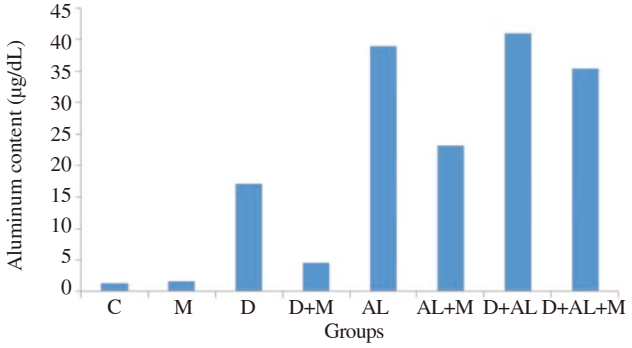

Figure 2. Biochemical changes of serum aluminum content in mothers of control and experimental groups.
C: Control group; D: Experimental diabetic group; D + M: Diabetic and *M. alba* treated group; AL: Al-intoxicated; AL + M: Al intoxicated and *M. alba* treated group; D + AL: Experimental diabetic and Al intoxicated; D + AL + M: Diabetic and Al intoxicated plus *M. alba* treated group.

3.3. Microscopic observations of the retina seven-day-old rats

Light microscopic observations of control group revealed that the retina of seven-day old rat was composed of six cell layers arranged from outer into PE, outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) and nerve fiber layer (NFL). The OPL is thinner comparing with the IPL. Internally adjacent to the vitreous humour, the NFL attained highly organization and possessed newly formed blood capillaries. Numerous oval GCs were detected lying adjacent with each other (Figure 3A). In the corresponding age of diabetic mother, abnormal alterations in retinal cell layers were detected. The NFL has become vacuolated with massive degenerative lesions forming reticular structural pattern. Few numbers of GCs were detected and sparsely distributed at the periphery of the IPL comparing with an abundant arrangement in control. The IPL attained considerable thinning and possessed massive hyaline degeneration. The OPL lacked differentiation and nuclear cell layer was organized in only one layer. Sprouts of degenerated and disorganized nuclear cells were detected. In other specimens, massive degenerative lesions were detected at the periphery of the nuclear cell layer. Eosinophilic necrotic foci were observed in the distal cell layer adjacent to the PE. Degenerative lesions including pyknosis, vacuolar degeneration and karyolysis were detected. The OPL attained a considerable thinning and degeneration in the majorities of specimens (Figure 3B).

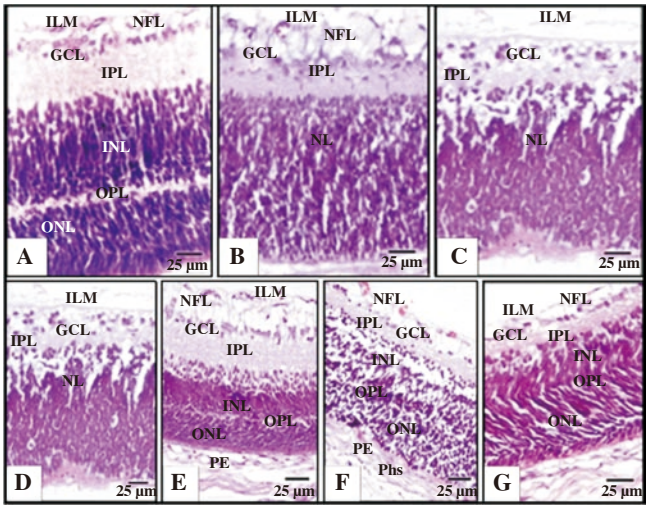

Figure 3. Photomicrographs of transverse histological sections of retina of 7 days old pups of both control and experimental groups (H & E).
A: Control showing regular arrangements of retinal cell layers including pigmented epithelium, inner segment and ganglion cell layer; B: Maternally diabetic showing degeneration of nerve fiber, ganglion cells and nuclear cells; C: Maternally diabetic and received *M. alba* extract showing marked amelioration of retinal layers; D: Maternally intoxicated with Al showing degeneration of ganglion and nuclear cells; E: Maternally intoxicated with Al and received *M. alba* extract showing restoration of almost normal pattern structure; F: Maternally diabetic, Al intoxicated showing degeneration of nerve fiber, ganglion layers and nuclear cells; G: Maternally diabetic, Al intoxication and received *M. alba* extract showing amelioration of histological picture but still not matched with the control.

In pups of mother received either Al intoxication alone or in combination with diabetes, the retinal cell layer showed

massive deterioration. Both the granular layer and nuclear layer possessed increased cell loss and lacked regular structural pattern. Pups of diabetic and Al–intoxicated mothers were highly susceptible of retinal cell damage (Figures 3D and F). However, the retina of pups maternally subjected to *M. alba* extract beside either diabetes or Al-toxication alone or in combination showed evident amelioration. Lesser degree of ameliorations was seen in retina of pups maternally subjected to combined treatment and received *M. alba* extract (Figsures 3C, E and G).

At the TEM level, the control pups exhibited normal PE with underlying choriocapillaris (Figure 4 A1). However, pups of diabetic mother possessed a considerable atrophy of PE with increased clumping of heterochromatin, vacuolated cytoplasm and swollen blood vessel (Figure 4 A2). Maternally diabetic and protected pups showed the cytoplasm of PE with mitochondria, rough endoplasmic reticulum and cytoplasmic vesicles (Figure 4 A3). Maternally intoxicated pups exhibited karyolysis of PE (Figure 4 A4). Maternally intoxicated and treated pups displayed normal PE with differentiated apical part characterized by radically arranged microvilli adjacent to macrophages and newly formed inner segment of photoreceptors (Figure 4 A5). Maternally diabetic and intoxicated pups had PE with karyolysis chromatin material and abnormal cytoplasmic organelles (Figure 4 A6). Maternally diabetic, intoxicated and treated pups displayed partial amelioration and had PE with almost normal chromatin and nucleoli (Figure 4 A7).

The control pups showed a normal inner segment (Figure 4 B1). Maternally diabetic pups showed degeneration of inner segment and their inclusions of mitochondria (Figure 4 B2). Maternally diabetic and protected pups showed moderate amelioration of cytological structure of the inner segment of photoreceptors. The cytoplasm of inner segment was rich in electron-dense mitochondria (Figure 4 B3). Maternally intoxicated pups exhibited distorted inner segment of photoreceptor (Figure 4 B4). Maternally intoxicated and treated pups showed macrophages and disorganized inner segment (Figure 4 B5). Maternally diabetic and intoxicated pups exhibited deformed inner segment with underlying macrophages in contact with their free ends (Figure 4 B6). Maternally diabetic, intoxicated and protected pups showed abundant macrophages adjacent to the inner segment (Figure 4 B7). Control pups showed normal ganglion cell layer (Figure 4 C1). Maternally diabetic pups exhibited pycknotic ganglion cells and vacuolated nerve fiber (Figure 4 C2). Maternally diabetic and protected pups showed moderate amelioration of ganglion cells (Figure 4 C3). Maternally intoxicated pups showed massive degeneration of ganglion cells (Figure 4 C4). Maternally intoxicated and treated pups exhibited moderate amelioration of ganglion cells (Figure 4 C5). Maternally diabetic and intoxicated pups showed pyknotic ganglion cells with increased incidence of cell death (Figure 4 C6). Maternally diabetic, intoxicated and treated pups had ameliorated ganglion cells, but still lacked normal arrangement of chromatin (Figure 4 C7).

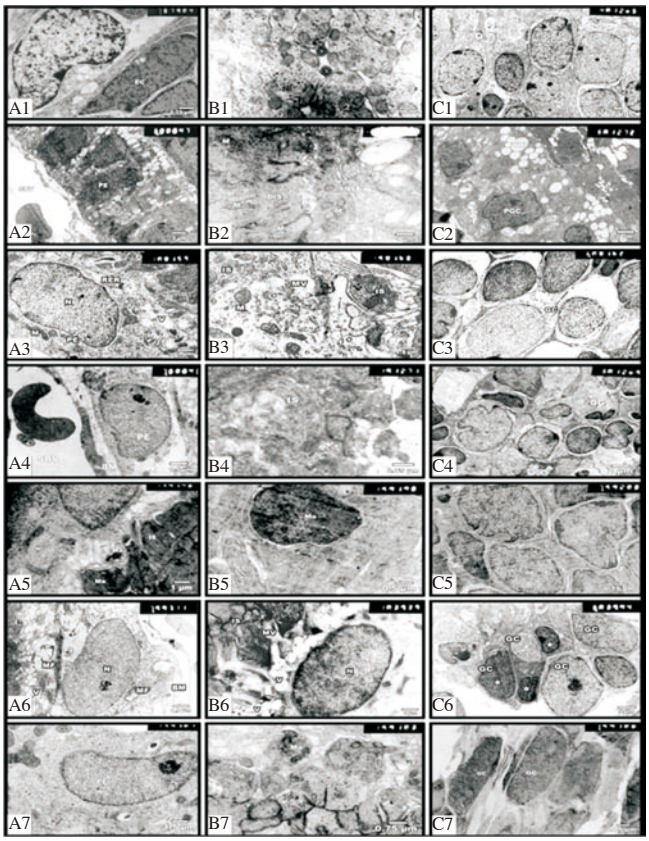

Figure 4. TEM of seven days old pup rats' retinal PE, retinal inner segment and retinal ganglion cells (uranyl acetate & lead citrate).
A1-A7: Retinal PE; B1-B7: Retinal inner segment; C1-C7: Retinal GCs. Numbers from 1 to 7 denote different groups *i.e.* control, maternally diabetic, maternally diabetic and protected with *M. alba*, maternally intoxicated with Al, maternally intoxicated with Al and received *M. alba* extract, maternally diabetic and intoxicated with Al, maternally diabetic, intoxicated with aluminum and received *M. alba* extract.

3.4. Microscopic observations of the retina of fourteen-day-old rats

Light microscopic examination of control pups revealed that it is composed of regular arranged retinal cell layers composed of eight cell layers and two limiting membranes arranged from the choroidal to the vitreal side as follows: cuboidal PE, rod and cone cell layers, ONL, OPL, INL, IPL, GCL, NFL and inner limiting membrane (ILM) (Figure 5A). Maternally diabetic pups showed massive loss of ganglion and nuclear cells (Figure 5B). The NFLs showed widespread vacuoles causing obliteration of GCs. There was a massive loss of GCs (Figure 5B). Maternally diabetic and treated pups showed marked improvements of histological picture (Figure 5C). Maternally Al intoxicated pups showed vacuolation of nerve fibers and massive degeneration of both ganglion and nuclear cells (Figure 5D). Maternally intoxicated and treated pups showed partial amelioration of affected layers (Figure 5E). Maternally diabetic and intoxicated pups showed massive damage of nerve fiber layer, ganglion and nuclear cells (Figure 5F). Maternally diabetic, intoxicated and treated pups exhibited marked amelioration (Figure 5G).

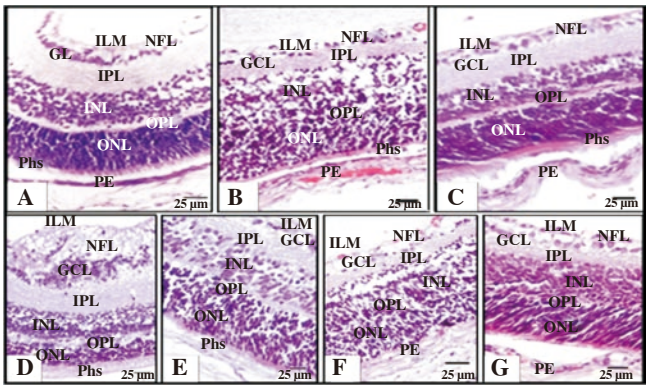

Figure 5. Photomicrographs of transverse histological sections of retina of 14 days old pups of both control and experimental groups (H & E).
A: Control showing regular arrangements of retinal cell layers; B: Maternally diabetic showing massive loss of ganglion and nuclear cells; C: Maternally diabetic and received *M. alba* extract showing marked improvements of histological structure; D: Maternally intoxicated with Al showing vacuolation of nerve fibers and massive degeneration of both ganglion and nuclear cells; E: Maternally intoxicated with Al and received *M. alba* extract showing partial amelioration of affected layers; F: Maternally diabetic, Al intoxicated showing massive damage of nerve fiber layer, ganglion and nuclear cells; G: Maternally diabetic, Al intoxicated and received *M. alba* extract showing amelioration of histological structures.

At the TEM level, control pups possessed PE with cytoplasm rich in rough endoplasmic reticulum and mitochondria and underlying thin basal membrane choriocapillaries (Figure 6 A1). Maternally diabetic pups showed pyknotic nuclei of PE. Their cytoplasm was enclosed by numerous vacuoles. The underlying choriocapillaries were swollen (Figure 6 A2). Maternally diabetic and treated pups showed PE with normal nuclei and abnormal vacuolation of cytoplasm still existed. Mitochondria and rough endoplasmic reticulum restored most of their structure. Outer segment of photoreceptors showed slight degeneration (Figure 6 A3). Pups maternally intoxicated with Al showed PE with vacuolated cytoplasm, swollen mitochondria and abnormal choriocapillaries (Figure 6 A4). Maternally intoxicated and treated pups showed pigmented epithelial cells having cytoplasm rich in mitochondria. The base showed well development basement membrane (Figure 6 A5). Maternally diabetic and Al intoxicated pups showed pyknotic nuclei of PE with vacuolated cytoplasm, degenerated mitochondria and vesiculated rough endoplasmic reticulum (Figure 6 A6). Maternally diabetic, intoxicated and treated pups exhibited pigment epithelial cells with moderate amelioration. The cytoplasm was enclosed by vesiculated rough endoplasmic reticulum and almost intact mitochondria (Figure 6 A7).

Control pups showed outer segment of photoreceptors with regular arrangement of stacked membranes (Figure 6 B1). Maternally diabetic pups showed degeneration of outer segment of photoreceptors (Figure 6 B2). Maternally diabetic and treated pups had outer segment of photoreceptors with regular arrangement of stacked membranes (Figure 6 B3). Maternally intoxicated pups showed disintegration of stacked lamellar membrane of outer segment (Figure 6 B4). Maternally intoxicated and treated pups showed outer segment of photoreceptors with almost regular stacked membranes (Figure 6 B5). Maternally diabetic and Al intoxicated pups showed degenerated outer segment of photoreceptors with lack of stacked membranes (Figure 6 B6). Maternally diabetic, intoxicated with Al and treated pups had outer segment of photoreceptors arranged with peculiar rearrangement of their stacked membranes (Figure 6 B7). Control pups showed normal GCs (Figure 6 C1). Maternally diabetic pups had damaged GCs (Figure 6 C2). Maternally diabetic and treated pups had normal pattern structure of GCs (Figure 6 C3). Maternally Al intoxicated pups showed degenerated GCs (Figure 6 C4). Maternally Al intoxicated and treated pups exhibited ameliorated outer nuclear cells (Figure 6 C5). Maternally diabetic and Al intoxicated pups showed vacuolar degeneration of pleomorphic GCs (Figure 6 C6). Maternally diabetic plus Al intoxicated and received *M. alba* extract rats showed moderate amelioration of GCs enclosed by numerous vacuoles (Figure 6 C7).

Figure 6. TEM of 14 days old pup rats' retinal PE, retinal inner segment and retinal ganglion cells (uranyl acetate & lead citrate).
A1-A7: Retinal PE; B1-B7: Retinal inner segment; C1-C7: Retinal GCs. Numbers from 1 to 7 denote different groups *i.e.* control, maternally diabetic, maternally diabetic and protected with *M. alba*, maternally intoxicated with Al, maternally intoxicated with Al and received *M. alba* extract, maternally diabetic and intoxicated with Al, maternally diabetic, intoxicated with Al and received *M. alba* extract.

3.5. Comet assay

Retinal neuronal cells of 14-day-old pups of both diabetic and/or Al intoxicated mothers possessed increased DNA concentrations (Figure 7) and tail length (Figure 8). Massive detachment of DNA damage was detected in combined treatment. On the

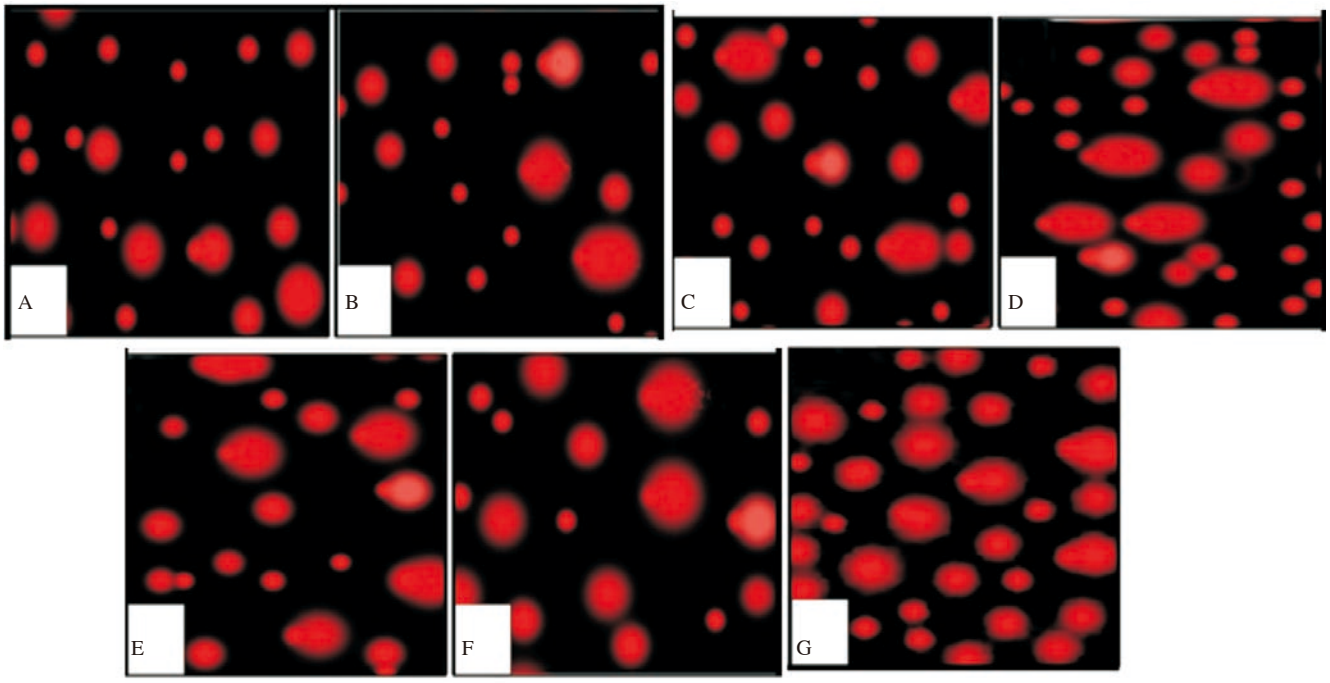

Figure 7. Photographs of retinal cells of 14 days-old pups analyzed by comet assay exhibits increased stretching apoptotic cells in experimental diseased groups.

The "dark/red" round spot represents the intact DNA without migration. The less dark "comet shaped" area adjacent to the nucleus represents DNA breaks that are small enough to move in the gel. A: Control; B: Maternally diabetic showing detached retinal cells; C: Maternally diabetic and received *M. alba* extract showing less damaged DNA; D: Maternally intoxicated with Al showing numerous detached cells; E: Maternally intoxicated with Al and received *M; alba* extract showing decreased DNA damage; F: Maternally diabetic, Al intoxicated showing increased DNA damage; G: Maternally diabetic, Al intoxicated and received *M. alba* extract showing reduction of DNA damage.

other hand, pups of treated mother besides Al intoxication and/or diabetes revealed reduction of neuronal cells with DNA damage.

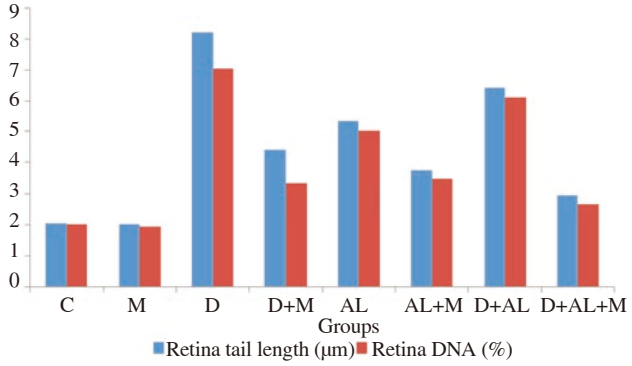

Figure 8. Tail length (μm) and DNA concentration of retina of pups maternally diabetic and/or intoxicated with Al and treated with *M. alba* leaves extract at the end of the experiment.

3.4. DNA fragmentation

Retinal neuronal cells of 7 and 14 days old pups of both diabetic and/or intoxicated mothers possessed genomic DNA fragmentation. Highest incidence of genomic DNA fragmentation was markedly increased in pups of Al intoxicated mother alone or in combination with diabetes. On the other hand, pups of treated mother besides intoxication and/or diabetes revealed resolution of DNA damage especially in diabetic or intoxicated groups (Figure 9).

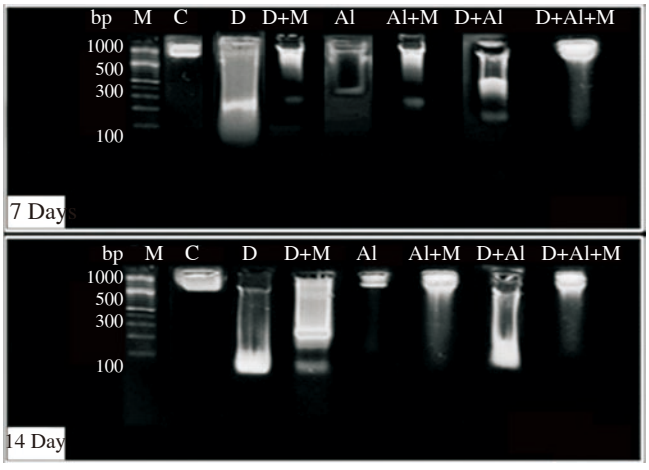

Figure 9. DNA fragmentation of retina of 7 and 14 days old pups.

C: Control; D: Diabetic showing detached retinal cells; D + M: Diabetic and *M. alba* treated showing less damaged DNA; Al: Al intoxicated showing numerous detached cells; Al + M: Al intoxicated and *M. alba* showing decreased DNA damage; D + Al: Diabetic and intoxicated with Al (D+Al) showing increased DNA damage; D + Al + M: Diabetic, intoxicated with Al and received *M. alba* treatment showing reduction of DNA damage.

4. Discussion

Diabetes represents a critical problem especially during the first trimester which is the critical period for organ development and differentiation[37]. Al is a neurotoxicant and it is known that accumulation of Al leads to a large number of neurological

disorders[17]. Although retinopathy is a major complication, yet only few works on the cyto-ultrastructural of the neuronal retina on pups maternally subjected to diabetes and/or Al intoxication are available. The present findings revealed that maternal intoxication with Al and/or diabetes led to marked disruption of retinal cell layers including massive thinning and degeneration of GCs, hyaline necrosis of inner plexiform and numerical reduction of nuclear layer plus neovascularization and partial loss of pigmented and photoreceptor layer. When compared with their control counterpart, the differentiation of retinal cell layers of the experimental groups were delayed with the advancement of growth of the developing 7 and 14 days-old pups.

Various mechanisms have been proposed for Al-induced neurotoxicity, including free radical damage via enhanced lipid peroxidation, impaired glucose metabolism, effects on signal transduction and protein modification, alterations on the axonal transport, and alteration of phosphorylation levels of neurofilaments[38], as well as induced chromosomal aberrations, micronuclei and sister-chromatid exchanges in human lymphocytes[39]. Furthermore, Al ion was found to induce cell apoptosis *in vivo* and cause neuronal death[17,40]. Elevated Al levels in the brain of rabbit offspring after subcutaneous injection of large amounts of Al into pregnant rabbits has been recently reviewed[41]. Following daily injection of 0.3 mL of 4% $AlCl_3$ to 4-week-old rats, Lu *et al*.[19] reported that thin retinal PE and disappearance of the photoreceptor outer and inner segments are the most evident observations. There were also high density irregular granules in the OPL and IPL inner and in the INL. The obtained findings agree with that of Weberg *et al*.[42] who revealed high concentration of Al in the plasma of mothers consumed high dose Al containing antacids during pregnancy. Similar findings were reported by Sharma *et al*.[43] on Wistar rats exposed to $AlCl_3$.

At the ultrastructural level, the choriocapillaries appeared swollen with marked degeneration of their endothelial lining cells. These results agree with experimental diabetic studies of Schröder *et al*.[44] and the findings of Bandello *et al*.[45] on humans. Both studies reported disruption of retinal microcirculation which has a major role in the retinopathy of diabetic rats and humans. Disturbance of microvasculature of the retina is resulted from bombarded retina by high glucose, and this insult led to many metabolic, structural and functional changes[46]. Increase in leukocyte adhesion might be a critical factor in the early retinopathy through decrease in retinal flow and increases in cytokine expression and vascular endothelial growth factor[47]. Extracellular superoxide dismutase was found to increase together with vascular endothelial growth factor in the vitreous body[48], as well as the potential antioxidant, lipid peroxide of -nepsilon-hexanoyl-lysine-[49] in proliferated diabetic retinopathy patients. Exposure of retinal endothelial cells to high glucose was found to increase mtDNA damage and compromised the DNA repair machinery[50].

Many of the PE cells showed apparent cell death with highest incidence in the combined maternal diabetes and Al intoxication. Clumping of nuclear chromatin, damage of mitochondria and vesiculation of rough endoplasmic reticulum were the major findings. The apparent damage of PE may disturb the retinal circuit function of renewal of the differentiation and active function of outer segment of photoreceptors, the main integral part of vision. These may impair transmission of nutrient and gases to the retinal cells causing marked cytotoxicity especially the adjacent one in the pigmented cell layer which concomitantly possessed dramatic cytological changes. Similar findings of retinal damage was reported in pups maternally diabetic[29] or adult rat received Al intoxication[19]. In addition, there was a marked increased incidence of nuclear cell death of maternally diabetic pups retina. However pups maternally intoxicated with Al alone or in combination with diabetes exhibited abnormal nuclear pleomorphism.

Furthermore, pups maternally diabetic and/or intoxicated with Al exhibited striking thinning of nerve fiber layers besides massive loss of GCs. Al exerted the highest degenerative damage in nerve fibers and GCs. In experimental rabbits intoxicated with Al[18] similar findings of degenerated nerve fiber layer were detected in diabetic patients[51]. The latter study demonstrated the presence of spot of neurofibrillary tangles in a subpopulation of retinal GCs of Al intoxicated rabbits. More recently, Yuki *et al*.[52] reported a reduction of both retinal GCs and nerve fiber layer in mice. The observed dramatic alterations may be attributed to either microvascular dysfunction[53] or generations of advanced glycated end products, through a nonenzymatic reaction of glucose with cellular proteins, lipids, and nucleic acids, and forming key intermediates such as methylglyoxal, which increased oxidative stress and elaborated proinflammatory and prosclerotic cytokines[54], and liberation of reactive oxygen species is both a direct consequence of hyperglycemia and an indirect consequence through mediators of glucotoxicity such as cytokines and growth factors[55,56].

M. alba has been reported to have antioxidant, antidiabetic, antiplatelet and antithrombotic activity[6,57-59]. A striking amelioration with *M. alba* leaves extract in experimental diseased groups was shown by potential hypoglycaemic effects as shown in serum of diabetic and/or Al intoxicated mother in previous researches[28,59] and the present study. In addition, there were a marked protection of histo- & cytogenesis retinal neuronal cells to some extent as well as of reduction of DNA fragmentation of pups maternally diabetic and/or Al intoxicated and received *M. alba* leaves extract. In conclusion, the results of our study prove that *M. alba* extract is effective against experimentally diabetic and Al-induced developmental retinopathy. Other possible neurotoxicity which may affect both cerebellum and cervical spinal cord during development is currently under investigation in our lab.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The manuscript describes work dealing with the ameliorative effect of *M. alba* leaves extract against developmental retinotoxicity in pups

of diabetic and aluminum intoxicated pregnant albino rats.

Research frontiers

Authors investigated the effect of the *M. alba* leaves extract against developmental retinotoxicity in rats using different techniques that included biochemical, spectroscopic, as well as transmission electron microscopy. The experiments were detailed and thorough.

Related reports

Diabetes was induced in investigated rats through the use of STZ which is a known technique for this kind of work. In addition, induction of aluminum intoxication was done by intragastrically administered method according to literature procedures.

Innovations and breakthroughs

In this paper, the authors have demonstrated that leaf extract of *M. alba* has marked antidiabetic effect in rats in addition to having remarkable protection of histo- & cytogenesis retinal neuronal cells.

Applications

This extract could be further studied to include cytotoxicity, safety, and other factors before jumping to conclusions.

Peer review

This is an interesting work that showed the ameliorative effect of *M. alba* leaves extract developmental retinotoxicity in pups of diabetic and aluminum intoxicated pregnant albino rats. The scientific part of the manuscript is good and sound and so is the methodology. Results obtained are interesting and encouraging.

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