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Role of Pomegranate Juice and Atorvastatin in Ameliorating Spinal Neurotoxicity of Wistar Rats Maternally Fed on Hypercholesterolemic Diet

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Abstract

Background: The contribution of high dietary cholesterol to encephalomyelitis and retinal diseases give more attention to overcome its neurotoxicity. These led physician and scientists of using anti-lepidemic statin drugs, however their long-term treatment may develop complication. Today many views recommended supplements of natural products especially fruit to get demand of needed antioxidant to scavenge bad free radicals liberated from inflammation. Pomegranate juice supplementation is used in the present study in combination with atorvastatin drug hoping to give synergistic effects and improve neurotoxicity.

Material and methods: Eighty pregnant Wistar albino rats weighing 180 g to 200 g body weight were used. They were arranged into eight groups (n=8) such as control, atorvastatin (1 mg/kg), pomegranate (0.5 ml 50%/rat), atorvastatin and pomegranate, hyper ccholesterolemic group (3% cholesterol diet for 6 weeks before onset of gestation), hypercholesterolemia and atorvastatin and/or pomegranate. All treatments were maintained throughout gestation and lactation period till 2 and 3 week-old. Offspring were sacrificed by diethyl ether and their cervical spinal cord were dissected and separated. The spinal cord was subjected for histological and transmission electron microscopical investigations, biochemical assays of neurotransmitters (dopamine, serotonin and γ-Aminobutyric acid), vascular endothelial growth factors, 8-hydroxydeoxyguanosine, caspase 3 and caspase 7 as well as comet assay).

Results: The present findings revealed massive cell death and necrosis within ependymal canal as well as pyknosis and edematous lesions of neuronal cells in offspring of hypercholesterolemic mothers. At ultrastructural level, there is a detected demyelination and vacuolar degeneration of myelinated axons. Many of sensory and motor neuronal cells exhibited compacted chromatin materials of their nuclei. These were associated with depletion of assayed neurotransmitters (DA and 5-HT), overexpression of vascular endothelial growth factors, caspases 3 and 7 and 8-hydroxydeoxyguanosine. Stretched DNA damage (comet assay) with obvious head and tail region was detected in neuronal cells of offspring of hypercholesterolemic mothers and decreased post-pomegranate-supplementation. Atorvastatin and pomegranate supplementation exhibited apparent improvement comparing with single atorvastatin or pomegranate manifesting highly synergistic effects.

Conclusion: Feeding pregnant on hypercholesterolemic diet induced spinal cord injury of their offspring and these can be improved by pomegranate juice supplementation in combination with atorvastatin- treatment.

Keywords: Cholesterol; Hypercholesterolemic diet; Oligodendrocytes; 24S-Hydroxycholesterol; γ-Aminobutyric acid

Introduction

Cholesterol represents the main integral elements in myelin synthesis. About 25% of the total amount of the cholesterol involved in de novo synthesis of myelin which ensheaths and insulates the axons of the nervous system [1]. Oligodendrocytes and glia cells are the main cells required cholesterol for myelin formation through PI3K/Akt/mTOR signaling pathway [2]. Under pathological condition, release of the cytochrome P-450-generated oxysterol 24S-hydroxycholesterol which led to the neurodegenerative disorder [1]. Mutant mice with cholesterol-deficient oligodendrocytes possessed ataxia and tremors [3].

Hypercholesterolemia is a metabolic disease and recently its average markedly increased to about 34 $\times 10^6$ in the United States [4]. Abnormal expression of cholesterol hydroxylases led to over expression of oxysterols in the brain tissues [5] which increase the production of β amyloid, the marker of Alzheimer's disease [6]. Hypercholesterolemia and diabetes are interrelated with each other via altering lipid metabolism and increase cholesterol, LDL and triglycerides [7] and consequently altered blood brain barriers integrity [8]. Experimental studies revealed neurodegenerative disorder and amyloid deposition in hippocampus of LDLr knockout and APPswe/PS1dE9 mice [9] as well as of mice post-feeding on a diet composed of 20% fat and 1.25% cholesterol for 8-weeks [10]. Hypercholesterolemic old patients revealed apparent accumulation of A-beta (Abeta) peptides, the precursor of amyloid plaques in Alzheimer's disease (AD) and in the Niemann-Pick disease [11,12].

Furthermore, atorvastatin is an HMG-CoA reductase inhibitor of cholesterol biosynthesis through mevalonate pathway [13] and

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represent good tools [14] for treatment of neurological diseases such as cerebrovascular disease, multiple sclerosis and Parkinson's and Alzheimer's disease [11,12,15,16]. Experimental rats subjected for atorvastatin-treatment (10 mg/kg b. wt, OA) revealed improvement of brain cholesterol synthesis [17], decrease apoptosis and improve and locomotion after spinal cord injury [18,19]. Hyperlipidemic patients possessed a neurological disability of the lumbar spinal cord and improved only after low dose statin drug treatment [20].

Pomegranate juice (*Punica granatum* L.) is rich in antioxidants such as (nica flavonoids, ellagitannins, ellagic acid and 3-glucosides/3,5-diglucosides of the cyanidin, anthocyanins delphinidin and pelargonidin [21-24] and vitamin A, C and E, which exert anti-inflammatory and anti-oxidant activities *in vitro* and *in vivo* [25]. Pomegranate juice has the protective effects in a transgenic model of Alzheimer's disease [26]. Ellagic acid improved the right medial forebrain bundle-lesioned rats through reducing the neuroinflammatory biomarkers TNF-α and IL-1β [27]. Also, MPTP-intoxicated fetal brain cells were improved through maintaining its antioxidative defense, LDH and intracellular NAD^+ and ATP levels in *in vitro* culture [28]. Dietary administration of green *Gingko biloba* (0.022% or 0.045%) [29], with anoside IV (10 micro mol/kg/day for 12 days) and 20 mg/kg *Angelica sinensis* [30] improved animal models of spinal cord damage including locomotion and axonal density.

There is available little clinical examination in patients of spinal cord injury with either obesity or hypercholesterolemia and yet no available experimental work outlined the detailed pathological and physiological alterations as well as illustrating the amelioration of phytotherapy. The present work aimed to illustrate the histo-cytological structure and function of offspring maternally fed on hypercholesterolemic diet treated with atorvastatin and or/pomegranate juice.

Material and Methods

The used experimental rats were carried out according to the general ethical guidelines and care of it. Eighty pregnant Wistar albino rats weighing approximately 210 g to 240 g. and maintained in good aerated room with standard 25°C to 28°C and 12 hour light and dark cycle. Free access of standard diet and water *ad libitum*. The experiments were performed on 35 female Wistar rats weighing 210 g to 240 g.

Chemicals

All of the used chemicals are of highest purity. Cholesterol, cholic acid, thiouracil and diethyl ether were supplied from Sigma-Aldrich Company, England.

Induction of hypercholesterolemia: This was carried out by feeding rats on high fat diet (15% butter) containing 3% cholesterol, 0.2% cholic acid and 0.2% thiouracil for 6 weeks [31] prior to conception as well as throughout gestation and lactation period till 2 and 3 weeks. The control group was fed on a standard diet free from cholesterol and fat diet.

Atorvastatin-treatment: Atorvastatin calcium is drug is produced by Pfizer pharmaceutical company, Egypt. It is [R- (R*, R*)]-2-(4-fluorophenyl)-ß, δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1H-pyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate. The drug is suspended in saline solution and dosed orally at 2 mg/kg body weight every other day from the 6^{th} day of gestation until 14 and 21 days postnatal.

Pomegranate juice supplementation: Pomegranate fruits were purchased from the market, cut transversely and squeezing by doing mechanical equipment for obtaining juice free from seeds. The juice was daily freshly prepared and mixed with water at 50%. Mother rats received oral doses of about 0.5 ml/rat every other day.

Experimental Work

Eighty virgin female and twenty fertile male Wistar albino rats weighing approximately 210 g to 240 g were obtained from Breading Farm, Ministry of Health, and Giza, Egypt. They were acclimatized for two weeks prior to experimentation. Mating was carried out at ratio of 2 female/one male during overnight and zero date of gestation was determined in the next morning after observing sperm in vaginal smear. Pregnant were arranged into 8 groups (N=10), such as control, pomegranate-supplementation (0.5 ml 50%), atorvastatin (2 mg/kg body weight), atorvastatin and pomegranate, hypercholesterolemic-group, hypercholesterolemic and pomegranate-, hypercholesterolemic and atorvastatin, combined hypercholesterolemic and atorvastatin and pomegranate. At 2 and 3 weeks post-partum, twenty offspring per each group were anesthetized by diethyl ether and sacrificed. Their cervical spinal cord was separated. Part of the specimens were kept in refrigerator at -70°C for biochemical and DNA investigations and the other ones processed immediately for histological and transmission electron microscopy as follows:

Histological investigation

Cervical spinal cord was separated immediately and fixed in 10% phosphate buffered formalin (pH 7.4) for 12 hours. Then, they were dehydrated in ascending grades of ethyl alcohol, cleared in xylene and mounted in molten paraplast at 58°C to 62°C. Five µm histological sections were cut, stained with hematoxylin and eosin and investigated under a bright field Leitz microscope.

Transmission electron microscopy

The specimens were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), fixed in 1% osmium tetraoxide at 4°C for 1.5 h, dehydrated in ascending percentages of ethyl alcohol, and embedded in epoxy-resin. Ultrathin sections were cut with a diamond knife on a LKB Ultratome IV (LKB Instruments, Bromma, Sweden), mounted on grids, stained with uranyl acetate and lead citrate, and examined under a Joel 100 CX transmission electron microscope (Musashino 3-chome, Akishima, Tokyo 196-8558, Japan).

Biochemical investigations

The 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 (5-HT), dopamine (DA) and γ-aminobutyric acid (GABA)

Serotonin was estimated according to Schlumpf et al. [32] by adding of 0.02 ml O-pthaldialdehyde (OPT) to 0.02 ml of the HCl extract and the colour was developed after heating for 10 min. The intensity of reaction was determined at 360 nm to 470 nm. For DA, 0.02 ml of HCl phase, 0.05 ml 0.4 M and 0.01 ml EDTA/Sodium acetate buffer (pH 6.9) were added to the specimen. Oxidation was carried out by adding of 0.01 mL iodine, followed by addition of 0.01 mL Na_2SO_3 in 5 m NaOH. Heating facilitated color development and the intensity of reaction was measured at 330nm to 375 nm [32].

Determination of GABA was carried out using high performance liquid chromatography (HPLC) having the precolumn phenyl

		5-HT (ng/g)	DA (ng/g)	GABA (ng/g)	VEGF (Pg/100 mg)	8-OHdG (ng/100 mg)	Casp3 (ng/100 mg)	Casp7 (ng/100 mg)
P3W	Control	2.8±0.3	14.2±1.3	35±3.2	345.4±13.4	0.36±0.05	0.440.01	3.29±0.31
	P	2.9±0.3	14.6±1.3	36±3.2	347.4±13.4	0.37±0.05	0.45±0.01	3.39±0.31
	A (1 mg/kg)	2.1±0.2	12.1±1.4	27±3.4	376. 5±7.8	0.56±0.07	0.65±0.03	4.38±0.50
	A and P	2.3±0.3	13.2±1.2	29±3.0	365.8±10.5	0.47±0.03	0.52±0.02	4.04±0. 60
	H	1.6 ± 0.2*	9.4±1.0*	22±2.7*	398.8±15.8*	0.69±0.08*	0.76±0.04*	5.470.73*
	H and A	2.1±0.3	10.7±1.1	25±2.3	374.2±11.7	0.48±0.08	0.56±0.03	4.86±0.43
	H and P	2.3±0.4	12.8±1.4	28±2.8	367.1±17.3	0.46±0.02	0.53±0.02	4.54±0.58
	H and A and P	2.5±0.3	13.3±1.7	31±3.2	355 .3±14.5	0.41±0.02	0.48±0.03	4.14±0.49
P3W	Control	3.1±0.4	14.8±1.4	37±3.5	350. 9±11.8	0.42±0.03	0.49±0.02	3 .43±0 .38
	P	3.4±0.5	14.9±1.6	38±3.7	351.9±12'8	0.43±0.05	0.51±0.03	3.51±0.42
	A (1 mg/kg)	2.3±0.2	13.4±1.5	28±2.7	370.6±10.3	0.56±0.04	0.62±0.03	4.41±0.47
	A and P	2.5±0.3	13.7±1.2	30±3.1	368. 5±14.7	0.45±0.02	0.54±0.02	4.17±0.42
	H	1.6 ± 0.3	9.7±1.1*	22±2.3*	401.8±14.9*	0.75±0.05*	0.83±0.05*	5.57±0.58*
	H and A	2.4±0.3	10.9±1.2	26±2.4	382.9±12.7	0.62±0.03*	0.61±0.04*	5.12±0.71*
	H and P	2.6±0.3	12.6±1.5	28±2.2	375 .2±11 '9	0.56±0.02	0.58±0.04	4.46±0.81
	H and A and P	2.7±0.3	13.5±1.7	33±3.1	362.7±15.4	0.47±0.03	0.53±0.03	4 .06±0 .41

*Significant at P < 0.05; C: Control; Casp3: caspase 3; casp 7: caspase 7; H: hypercholesterolemia; HA: hypercholesterolemia and atorvastatin; HP: hypercholesterolemia and pomegranate; HLP: hypercholesterolemia and atorvastatin and pomegranate; DA: dopamine; 5-HT: serotonin; GABA; gama amino-butyric acid; VEGF: vascular endothelial growth factor; 8-HDG: 8- hydroxy guanosine

Table 1: Biochemical markers of spinal cord function of rat offspring maternally fed on hypercholesterolemic diet treated with atorvastatin and or/pomegranate juice. Each result represents M ± SE (n=10).

thiocarbamyl (PTC) derivatization technique according to the method of Heinrikson and Meredith [33].

Vascular endothelial growth factor (VEGF)

This was carried out by R and D ELISA Kit (Minneapolis, MN, USA). The samples were incubated for 2 hours, followed by adding of the peroxidase linked polyclonal antibody containing VEGF. After incubation and washing times, a substrate solution was added that react with the enzyme-antibody complex. The intensity of reaction was measured at 450 nm and 540 nm by UV-Spectrophotometer (Perkin Elmer Victor 3™) and is directly proportional to the concentration of the specimens. reaction.

8-hydroxy-2-deoxy guanosine (8-OH-dG)

Estimation was done using the Bioxytech 8-OHdG-ELISA Kit (OXIS Health Products, Portland, OR, USA). A 50 µL sample or standards and 50 µL of the primary antibody were added to each well of 8-OHdG-coated microtitre plates and incubated at 37°C for 1 hour. Horseradish peroxidase-conjugated secondary antibody and a substrate containing 3, 30, 5, 50-tetramethylbenzidine were added. The intensity of color was measured at 450 nm and concentration of 8-OHdG was expressed as ng/ml [34].

Caspases 3 and 7

These are determined by using a Stressgen colorimetric Kit (USCN Life Science Inc., Wuhan, China; Cat. No. 907-013 for Caspase 3 and Cat. No. E0449Ra for caspase 7). The spinal tissues were lysed to collect their intracellular contents, and tested for protease activity by conjugating a caspase-specific peptide to the p-nitroaniline (pNA) and the colour intensities measured at a wavelength of 405 nm for caspase 3 and wavelength of 450 nm for caspase 7.

Comet assay

The specimens were homogenized in phosphate buffered solution at pH 7.5. Six µL of homogenate was suspended on 0.5% low melting agarose and placed in between a layer of 0.5% normal-and low melting agarose on frosted slides. Lysis was carried out and electrophoresis was run for unwinding of DNA for 10 min at 300 mA and 1 V/cm. The slides were stained with 20 mg/ml ethidium bromide and each one was analyzed using a Leitz Orthoplan (Wetzlar, Germany) epifluorescence microscope. Fifty cells/each was analyzed using the comet assay automatic digital analysis system. Perspective tail length (mm) (DNA migration from the center of the body of the nuclear core) was used to determine DNA damage [35].

Statistical analysis

Data were presented as mean ± standard error. The statistical analysis was carried out by one way of variance (MANOVA, version 13) between the control and studied groups with Tukey's *post hoc* test. P<0.05 was considered statistically significant.

Results

Biochemical observations

Table 1 illustrates the assayed biochemical changes in hypercholesterolemic groups in relation to different experimental treatments including serotonin (5-HT) and dopamine (DA), γ-Aminobutyric acid (GABA), vascular endothelial growth factors (VEGF), 8-hydroxy-deoxyguanosine and caspases 3 and 7 of 2 and 3 week-old rats. There was a marked depletion of 5-HT, DA and GABA and increase of VEGF, 8-hydroxy-guanosine and caspases 3 and 7 in spinal cord tissues of offspring of hypercholesterolemic mother. Pomegranate juice supplementation improved the levels of neurotransmitters and decreased the average of single strand DNA damage and apoptosis compared to atorvastatin-treatment. Highest degree of improvement was reported in experimental group received atorvastatin and pomegranate-treatment (Table 1).

Light and transmission electron microscopic observations

Experimental group 3 week-old offspring maternally treated with atorvastatin, exhibited shriked ependymal canal but with normal ependymal lining cells. The neuronal cells are abundant and less differentiated (Figure 1 [B-B2]). Similar normal pattern structures of ependymal canal and neuronal cells were reported in atorvastatin and pomegranate-treated group (Figure 2 [A-A2]). Offspring maternally fed on hypercholesterolemic diet revealed the presence of necrotic patches in the ventral margin of ependymal canal associated with

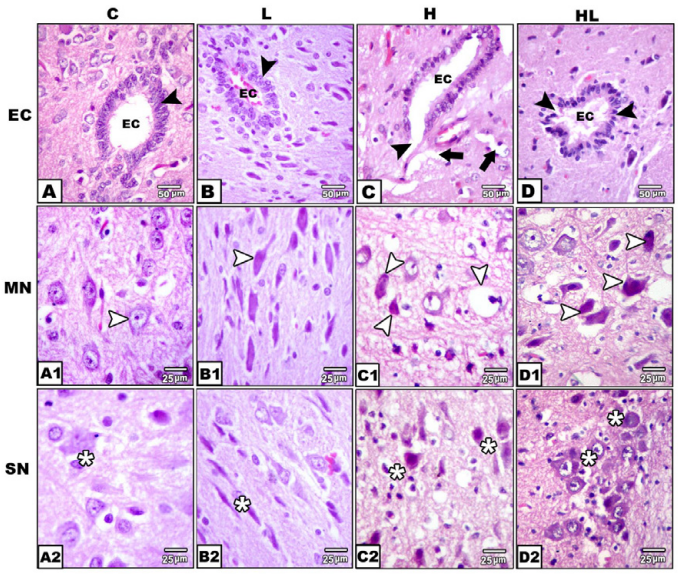

Figure 1: Photomicrographs of transverse histological sections of spinal cord of 3 week-old offspring or rat. A-A2. Control spinal cord showing normal ependymal canal lining with cubical cell (A, arrow head) and different pattern of multipolar (A1, arrow head) and sensory (A2, star) neuronal cells. B-B2. Offspring maternally-treated with l atorvastatin showing normal ependymal canal lining with cubical cell (A, arrow head) and different pattern of multipolar (A1, arrow head) and sensory (A2, star) neuronal cells. C-C2. Offspring maternally fed on hypercholesterolemic diet showing necrotic patches of ependymal canal lining with pyknotic cells (A, arrow head) and either pyknotic or vacuolar degenerated multipolar (A1, arrow head) and sensory (A2, star) neuronal cells associated with edematous lesions. D-D2. A-A2. Offspring maternally-fed on hypercholesterolemic diet and treated with atorvastatin showing improved ependymal canal (D), moderate improvement of multipolar neurons but still possess some pyknotic cell (D1) as well as sensory neurons (D2). Abbreviations; C: Control; EC: ependymal canal; H: hypercholesterolemia; HL: hypercholesterolemia and lepitor; MN: motor neurons; SN: sensory neurons. White arrow indicates motor multipolar neurons. Black arrow head indicate ependymal lining cells. Star indicated sensory neurons.

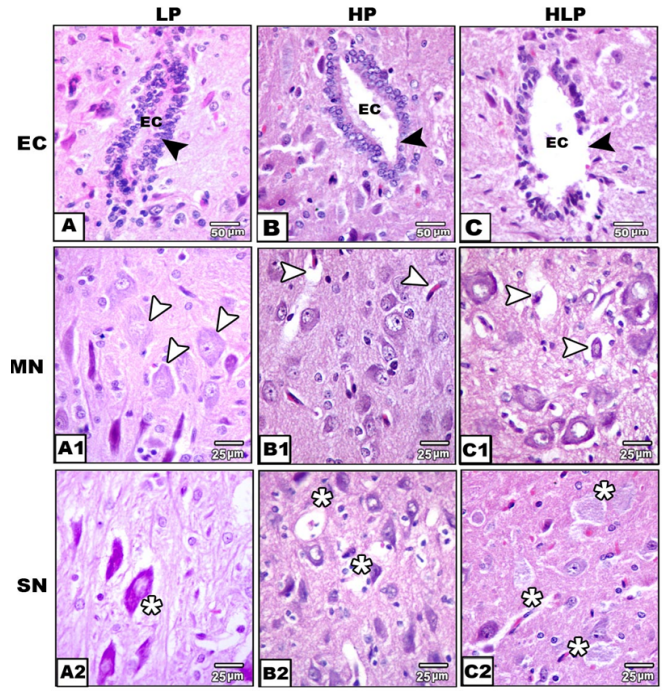

Figure 2: Photomicrographs of transverse histological sections of spinal cord of 3 week-old offspring or rat. A-A2. Offspring maternally-treated with atorvastatin and pomegranate juice showing normal ependymal canal lining with cubical cell (A, arrow head) and different pattern of multipolar (A1, arrow head) and sensory (A2, star) neuronal cells. B-B2. Offspring maternally fed on hypercholesterolemic diet and supplemented pomegranate juice showing improved of ependymal canal (A, arrow head) and multipolar (B1, arrow head) and sensory (B2, star) neuronal cells. C-C2. Offspring maternally-fed on hypercholesterolemic diet and received atorvastatin and pomegranate juice showing improved ependymal canal (C), moderate improvement of multipolar neurons but still possess slight cytological alterations (C1) as well as sensory neurons (C2). C: Control; EC: ependymal canal; HP: hypercholesterolemia and pomegranate; HLP: hypercholesterolemia and lepitor and pomegranate; MN: motor neurons; SN: sensory neurons. White arrow indicates motor multipolar neurons. Black arrow head indicate ependymal lining cells. Star indicated sensory neurons.

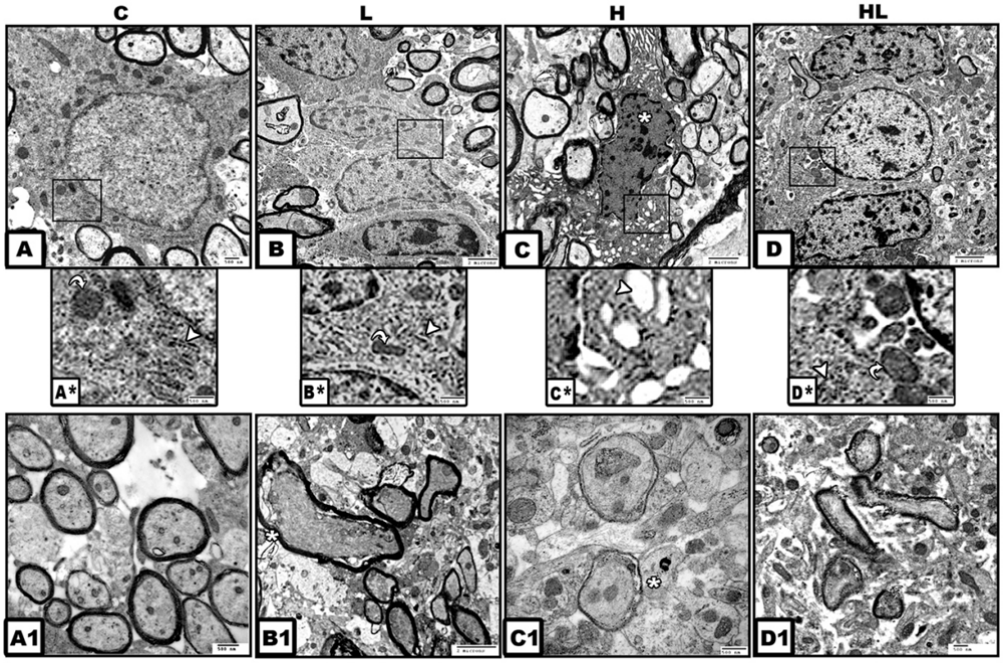

Figure 3: Transmission electron microscopy of spinal cord of 3 week-old offspring. A-A1. Control showing normal neuronal cell having cytoplasm rich in mitochondria (A), and rough endoplasmic reticulum (A*) and myelinated axons (A1). B-B1. Atorvastatin -treatment showing intact neuronal cell (B) with peculiar mitochondria and rough endoplasmic reticulum (B*), and myelinated axons (B1). C-C1. Maternally fed on hypercholesterolemic diet showing pyknotic nuclei (C) with vesicuolated rough endoplasmic reticulum and degenerated mitochondria (C*) and demyelinated axons (C1, star). D-D1. Maternally fed on hypercholesterolemic diet and treated with atorvastatin-treatment showing improved neuronal cells (D), intact mitochondria and less improved rough endoplasmic reticulum (D*) and intact myelinated axons (D1).

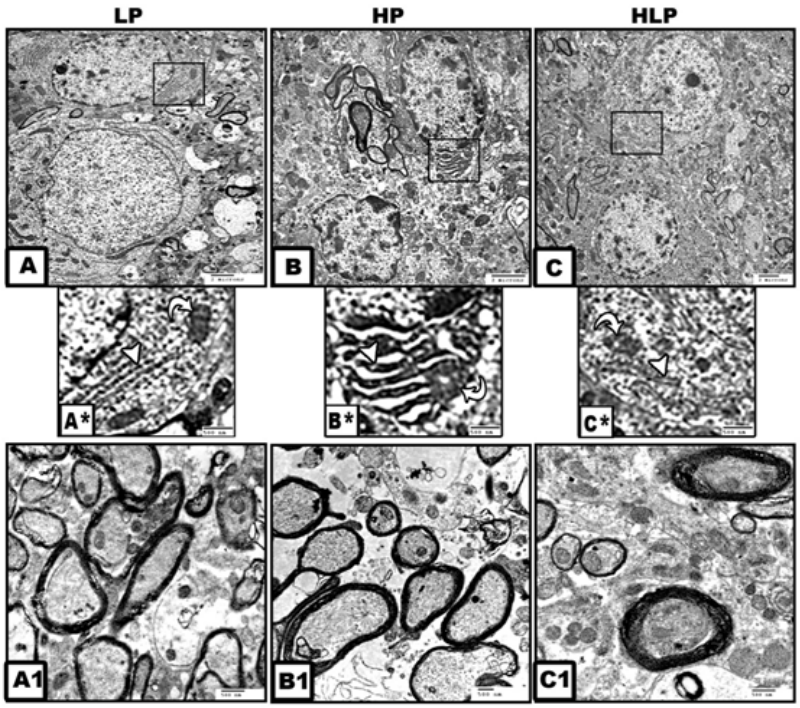

Figure 4: Transmission electron microscopy of spinal cord of 3 week-old offspring. A-A1. Maternally treated with atorvastatin and pomegranated juice showing normal neuronal cell having cytoplasm rich in mitochondria (A), and rough endoplasmic reticulum (A*) and myelinated axons (A1). B-B1. Maternally fed on hypercholesterolemic diet and supplemented pomegranate juice showing normal neuronal cell (B) with peculiar mitochondria and rough endoplasmic reticulum (B*), and myelinated axons (B1). C-C1. Maternally fed on hypercholesterolemic diet and treated with pomegranate juice and atorvastatin showing improved nuclei (C) with less altered rough endoplasmic reticulum and mitochondria (C*) and improved demyelinated axons (C1, star). D-D1. Maternally fed on hypercholesterolemic diet and treated with atorvastatin and pomegranate juice showing improved neuronal cells (D), intact mitochondria and less improved rough endoplasmic reticulum (D*) and intact myelinated axons (D1).

increased average of pyknotic nuclei throughout the ependymal canal characterized by clumping of nuclear chromatin. The grey matter possessed gliosis of nerve axons and widespread of edematous lesions. Many of the multipolar neuronal cells are eosinophilic with characteristic sign of cell death (Figure 1[C-C2]). Regard the control which shows similar characteristic pattern structures of the experimental group received pomegranate juice. The ependymal canal is lined by a single layer of pseudostratified cuboidal cell with characteristic nuclei. The multipolar neurons are normally distributed in both dorsal and ventral column within the grey matter (Figure 1 [A-A2]).

Offspring of hypercholesterolemic mother and treated with atorvastatin showed improved ependymal lining cells, however, slight numbers of mutipolar neurons of dorsal column were still exhibiting edematous lesions and cell death comparing with less changes in the ventral one (Figure 1 [D-D2]). However, marked improvement of ependymal lining cells and multipolar neurons of both dorsal and ventral column were observed in offspring maternal fed on hypercholesterolemic diet and supplemented pomegranate juice. A slight edematous lesion was still appeared (Figure 2 [B-B2]). Experimental hypercholesterolemic group treated with atorvastatin and supplement pomegranate exhibited almost normal pattern structure of multipolar neuronal cells, however a slight patches of edematous lesions were remarked (Figure 2 [C-C2]).

In offspring maternally treated with atorvastatin, the nuclei of multipolar neurons possessed a slight aggregation of their heterochromatin. Vesicuolated rough endoplasmic reticulum and electron-dense mitochondria were observed within cytoplasm. In white matter, the nerve axons appeared intact (Figure 3 [B, B* and B1]).

In offspring maternally fed on hypercholesterolemic diet, pyknotic neurons having electron-dense nuclear chromatin and vesicuolated rough endoplasmic reticulum and atrophied mitochondria were detected. Many of the nerve axons appeared either necrotic or demyelinated (Figure 3 [C-C1]).

A marked improvement of neuronal cells and myelinated axons were observed in offspring maternally fed on hypercholesterolemic diet and treated with atorvastatin. However, few numbers of neurons showed moderate heterochromatin condensation in their nuclei (Figure 3 [D, D* and D1]). Experimental hypercholesterolemic group received the drug treatment and supplemented pomegranate showed marked improvement (Figure 4 [A, A* and A1, B* and B1, C, C* and C1]). Regard the control showing normal pattern structure of nuclei and regular arrangement of rough endoplasmic reticulum and mitochondria and peculiar structure of myelinated axons (Figure 3 [A, A* and A2]).

Genomic single DNA fragmentation (Comet assay)

There was a detected increase of stretched neuronal cells and increased tail length in offspring of hypercholesterolemic mother and markedly improved post-supplementation with pomegranate and/or atorvastatin-treatment. Atrovastatin-treatment showed marked increase of tail length and highly detected in those of mother fed on hypecholesterolemic diet. However, offspring maternally fed on hypercholesterolemic and received atrovastatin and or/pomegranate supplement showed marked improvement but still above normal value (Figures 5 and 6).

Discussion

The observed findings revealed depletion of γ-aminobutyric acid (GABA), serotonin (5HT) and dopamine (DA) in spinal cord of 2 and 3 week-old offspring maternally fed on hypercholesterolemic diet. Similar depletion of extracellular GABA was significantly decreased in cerebrum [36] and olfactory region of diabetic rats [37] and ocular region of hypercholesterolemic rats [38].

It is known that γ-aminobutyric acid is an inhibitory neurotransmitter amino acids of spinal interneurons involving both pre- and post-synaptically. GABA neurotransmission depletion may contribute to the neuronal death and impairing the spinal cord function [39]. Depletion dopamine D2 receptor was also reported in obese individuals parallel with their reduction of their motivation and reward circuits [40]. Experimental diabetic rat impairment of brain function related to a depletion of serum dopamine [41]. It is known that DA overexpressed glutamatergic transmission onto motoneurons and stabilizes the output of interneurons [42] and modulates the spinal bladder reflex [43].

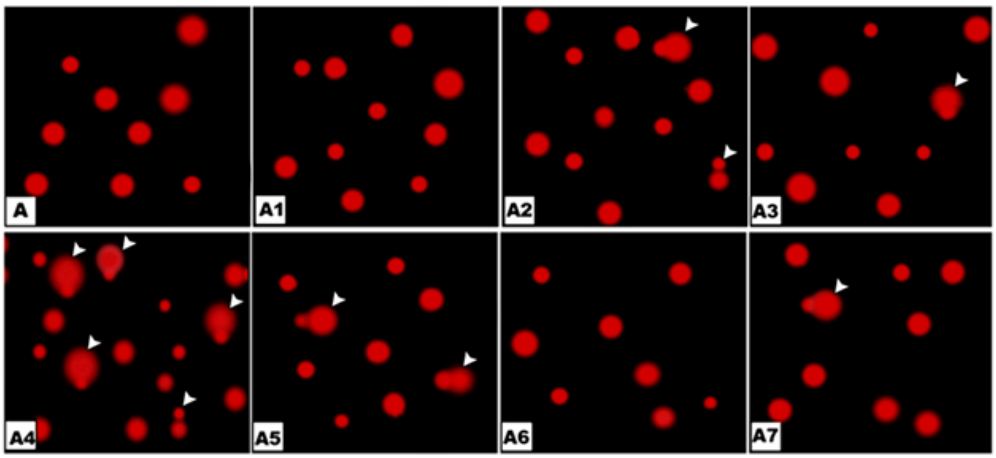

Figure 5: Comet assay of spinal cord cells of 3 week-old offspring. A. Control. A1. pomegranate juice supplementation. A3. Atorvastatin -treatment showing stretched neuronal cell. A3. Atorvastatin and pomegranate-treatment showing intact neuronal cells. A4. Hypercholesterolemic showing apoptic cells characterized by stretched nuclei. A5. Hypercholesterolemic and atorvastatin -treatment showing few stretched nuclei of neuronal cells. A6. Hypercholesterolemic and pomegranate showing improved neuronal cells. A7. Hypercholesterolemia plus atorvastatin and pomegranate showing a slight number of neurons with stretched nuclei. Arrow head indicate stretched pyknotic neuronal cells. X1000. Arrow heads indicate stretched nuclei of neurons.

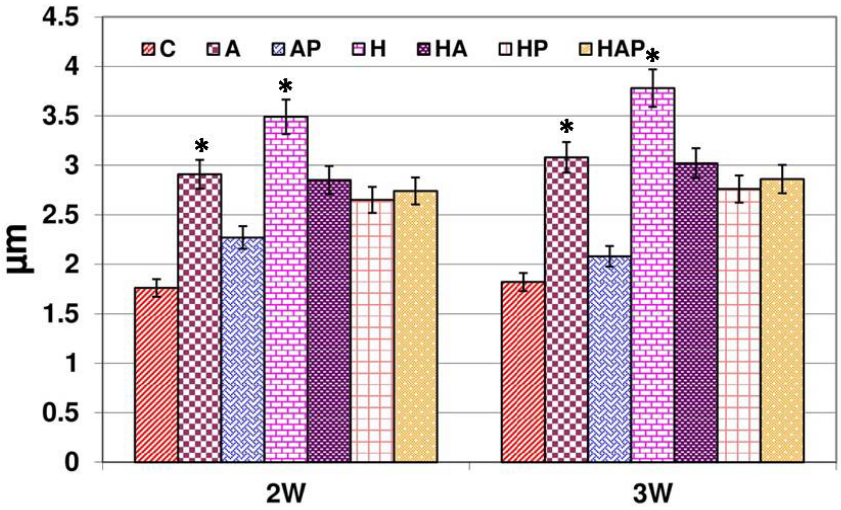

Figure 6: Tail length chart of neuronal cells of Comet of spinal cord of 2 and 3 week-old offspring of different group showing increased tail length of hypercholesterolemic group and improved by varying degree after treatment with atorvastatin and/or pomegranate juice supplementation. Significance only in atorvastatin or hypercholesterolemic group at P<0.05. N=50 cells. C: Control; H: hypercholesterolemia; HL: hypercholesterolemia and Atorvastatin; HP: hypercholesterolemia and pomegranate; HAP: hypercholesterolemia and atorvastatin and pomegranate.

Serotonin is responsible for modulating the input-output gain of motoneurons in spinal cord [44]. Its apparent depletion in spinal cord of offspring of hypercholesterolemic rat supported the findings of Henley **and** Bellush [45] whom reported reduced serotonin turnover in brain stem of diabetic rats.

Furthermore, pomegranate juice supplementation and or/ atorvastatin to offspring of hypercholesterolemic mother improved the levels of GABA,5HT and DA. The present finding contradicted with the work of Tapias et al. [26] whom reported that pomegranate juice failed to improved rotenone induced experimental Parkinson's disease as well as exacerbates DA neuron loss, inflammation, and caspase activation, leading to neurodegeneration. These findings may be related to massive neurodegeneration of the rotenone. In vitro studies of human brain neurons intoxicated with 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine carried out by Braidy et al. [28], supplementation of malasi pomegranate juice improved the neurotoxicity and brain antioxidative enzymes.

The observed findings of decreased levels of neurotransmitters reflected the dramatic neuronal cell damage and disruption of ependymal lining cells. These were parallel with demyelination of nerve axons, increased average of pyknosis, vesicuolation of rough endoplasmic reticulum and atrophy of mitochondria. The present findings support the work of Raddatz et al. [46] whom mentioned that the depletion of cholesterol biosynthesis Theiler's murine encephalomyelitis is an important marker for demyelination Similar findings were reported in diabetes mellitus related demyelinating polyradiculoneuropathy [47] taking in consideration the interrelationship between diabetes and hypercholesterolemia [38].

There is a great association between spinal cord damage and type 2 diabetes [48]. Of 1127 diabetic patients, 189 (16.8%) had various neurologic disorders, including 32 patients (16.9%) with chronic inflammatory demyelinating polyneuropathy [49].

Furthermore, spinal cord of offspring of atorvastatin showed a moderate degree of amelioration, except a slight accumulation of heterochromatin within neuronal cells.

Similar findings were reported by Chung et al. [20] in patients with disability of the lumbar spinal cord and received low dose statin drug treatment.

Also, the observed damage demyelination and neuronal cell death reflected by upregulation of 8-doxyhydroxyguanosine, caspase 3, caspase 7 and increase of single strand DNA damage in neonates of hypercholesterolemic mother. Similar findings were reported in neuronal cells of diabetic retinopathy [50].

It is known that caspases are cysteine proteases that attack and degrade the C-terminal of an aspartic acid amino acid and cause apoptotic cell death in neuronal cells [51].

Also, 8-hydroxy-deoxyguanosine (8-OH-dG) is a main product of oxidatively damaged DNA [52]. Twelve months-treatment diabetic Sprague Dawley rats exhibited similar neuronal cell death of root ganglion [53].

On the other hand, offspring of hypercholesterolemic mother treated with pomegranate alone and or/atorvastatin showed marked degree of improvement. Synergistic effects of combined atorvastatin and pomegranate-treatment were highly detected.

The amelioration of pomegranate juice come from its high content of antioxidants such as nica flavonoids, ellagitannins, ellagic acid and 3-glucosides/3,5-diglucosides of the cyanidin, anthocyanins delphinidin and pelargonidin [21-24] and vitamin A, C and E, that exert anti-inflammatory and anti-oxidant activities *in vitro* and *in vivo* [25]. These phytochemical components serve as free radicals scavenging which reduced inflammation and consequently decreased cell death.

Similar findings of amelioration of transgenic model of Alzheimer's disease was reported after pomegranate juice-supplementation [26].

Ellagic acid one of the pomegranate juice was found to improve the right medial forebrain bundle-lesioned rats through reducing the neuroinflammatory biomarkers TNF-α and IL-1β [27].

The author finally concluded that pomegranate juice improved the hypercholesterolemia related damage of spinal cord assessed by histo-cytological structure, neurotransmitters, VEGF and apoptic markers caspases and 8-hydroxydeoxy guanosine.

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