Malted Barley Ameliorated Spinal Cord Injuries of Offspring of Hypercholesterolemic Mother Rats

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Abstract

The prevalence of obesity and neurodegenerative diseases is high due to the consumption of diet rich in cholesterol. However, there was no data available about the injuries of spinal cord of breast feeding young. The current research work has demonstrated the ameliorative effect of malted barley feeding on the injuries of spinal cord of offspring maternally ingested on a high cholesterol diet.

Sixty virgin female and twenty adult male albino rats (Rattus norvegicus) weighing 100 gm body weight. A diet consisting of 3 percent cholesterol was consumed by virgin females for 4 months before conception and during gestation and lactation period. Virgin rats were made pregnant by mating with healthy males and onset of gestation was determined by observing sperm within the vaginal smears in the next morning. The pregnant were divided into 4 groups (n = 15); control (C), hypercholesterolemic (H) (3%) with or without malted barley (20%) feeding (H+B) group. Offsprings of the studied groups were sacrificed at 21 days-old. Their cervical spinal cord was removed and investigated histologically, immunohistochemically of glial fibrillary acidic protein (GFAP) and proliferating cell nuclear antigen (PCNA) and transmission electron microscopy. Also, biochemical assessments of glutathione-s-transferase and superoxide dismutase, malondialdehyde, caspase-3, 8-hdG, neurotransmitters (DA, 5-HT and GABA) and cytokines (IL6& 10) were carried out. The present results showed that ingestion of malted barley to mothers fed on a high cholesterol diet restored the ependymal canal epithelium, vesicular structure of neuronal cells and myelination of the nerve axons. They also, observed increased immunohistochemistry of PCNA and decreased immunostaining of GFAP. The assayed antioxidant enzymes and neurotransmitters contents of the spinal cord were increased. However, the apoptic markers caspase-3, 8-hdG and cytokines expressing inflammation were decreased.

The author concluded that barley supplementation in combination with high cholesterol diet ameliorated the spinal injuries due to its high contents of antioxidants which scavenges free radicals.

Keywords: Spinal Cord; Histology; Transmission Electron Microscopy; Antioxidants; Neurotransmitters

Introduction

Although cholesterol is a major component of the neuronal tissues, its deficiency has led to brain malformations, multiple congenital defects, microcephalus, autism and other behavioral disorders [1]. Meanwhile, feeding on a cholesterol rich diet increased accumulation of fat in the vertebral body and interfered with decreased blood supply and damaging of spinal tissues [2].
High-fat diet associated obesity has been found to cause abnormal changes in cholesterol biosynthesis that lead to spine injury [3]. Increase of high cholesterol level in maternal environment reprogramed the fetal tissues and promoting the formation and progressive atherosclerosis in human aortic fatty streak formation [4-6] and experimental animals [7-9]. This was also led to increased 27-hydroxysterol in the brain [10] and consequently cerebral amyloid angiopathy [11].

Barley grains are rich of a number of vital components including antioxidants and phenolic compounds [12,13]. β-glucan is a functional component of barley grain and is active in improving diabetes and high cholesterol levels [14] especially of cholesterol and triglyceride levels in animal model [15] and human [16-18]. Dietary consumption of hull-less barley β-glucan was carried out by regulating the activities of HMG-CoA reductase and CYP7A1 in hypercholesterolemic hamsters [19,20]. Many epidemiological studies have been clarified decreased incidence of mortalities and improvement of coronary heart disease, diabetes and cancer following dietary intake of barley grains [21,22].

Aim of the Study

The present work aimed to demonstrate the therapeutic potential of malted barley on the spinal cord structure and function of offspring of mother ingested diet rich in cholesterol.

Materials and Methods

Preparation of a hypercholesterol diet

The diet consists of 3% cholesterol plus 7% animal fat plus 1% cholic acid and 1% thiouracil in combination with normal standard dietary components [23]. Feeding was carried out for 4 months before to conception and during the time of pregnancy and lactation.

Diets containing malted barley

Freshly malted barley was mixed with either normal or hypercholesterolemic diet at a ratio of 20 percent. Both control and hypercholesterolemic mothers were fed on this dietary regimen throughout gestation and lactation period.

Experimental animal

This laboratory work was carried out according to the requirements of the National Institute of Health and the Egyptian Committee of Bioethics and use of Laboratory Animals.

Sixty virgin female and twenty adult male albino rats (Rattus norvegicus) weighing approximately 100 gm body weight were brought from Helwan Breading Farm, Ministry of Health, Egypt. They were acclimatized and housed in good aerated lab with 12 hours light and dark period. Free access of food and water were provided ad libitum. Mating was carried out and pregnancy was determined by observing sperm in vaginal smears in the next morning and zero date of gestation was recorded. The pregnant were divided into 4 groups (n = 15); control (C), hypercholesterolemic (H) (3%) and/or malted barley (20%) feeding (H+B) group. At 3 week-old, the offsprings were sacrificed and their cervical spinal cord were dissected and investigated as follows.

Histological investigation

The specimens were fixed in 10% phosphate buffered formalin (pH 7.4), dehydrated in increased sequences of ethyl alcohol, cleared in toluene and mounted in melted periplasm at 5-6ºC. Five μm histological sections were cut, stained with hematoxylin and eosin [24] and viewed under a bright field of light microscope.

Transmission electron microscopy (TEM)

This was achieved by fixation in 2.5 percent phosphate buffered glutaraldehyde (pH 7.4), followed by post-fixing in 1 percent osmium tetroxide at 4ºC, dehydration in upgraded concentrations of ethyl alcohol, clearing in acetone and embedding in epoxy-resin. Ultrathin
sections were cut on an LKB Ultratome IV (LKB Instruments, Bromma, Sweden), mounted on grids, stained with uranyl acetate and lead citrate, and investigated at a Joel 100CX transmission electron microscope at Mansoura University Lab, Egypt.

**Immunohistochemistry for PCNA and GFAP**

Five μm histological sections of formalin-fixed, paraffin-embedded tissue were placed on coated glass slides. Dewaxed in toluene and rehydration in degraded ethyl alcohol was carried out. Endogenous peroxidase activity was extracted by incubation in 3 percent hydrogen peroxide for 10 minutes at room temperature. The spinal sections were placed in media containing 0.05% trypsin (pH 7.8) for 15 minutes at 37°C and incubated with the primary l mouse antibody against glial fibrillary acidic protein (GFAP, DAKO, clone MIB5, 1:50, mouse) and primary antibody against proliferating cell nuclear antigen PCNA (DAKO, clone MIB5, 1:50, mouse) for overnight at 4°C. The slides were incubated with a secondary biotin linked anti-mouse antibody and with the streptavidin-peroxidase complex. Washing was carried out per every step. Incubation with diaminobenzidine-hydrogen peroxides (DAKO) and counterstained with hematoxylin. The reaction was visualized after counterstained with hematoxylin and appeared dark-brown in nuclear region for PCNA and in axons for GFAP. Negative control was carried out by 1% non-immune serum phosphate buffer solution (PBS) solution. The immune reaction was investigated under bright field of light Olympus microscope with a digital canon camera.

**Biochemical assays**

The spinal tissue was homogenized in tris buffer at pH 7.5, centrifuged and their supernatant were separated and kept in a refrigerator.

**Determination of superoxide dismutase and glutathione-S-transferase activities**

Superoxide dismutase (SOD) was determined by reduction of the superoxides with nitro blue tetrazolium to a blue color of formazan and measured at 560 nm [25]. Meanwhile, glutathione S-transferases (GST) is measured by precipitation of protein using tungstate-sulfuric acid solution followed by reaction with 5,5’ dithiobis- 25-nitrobenzoic acid (DNTB) and formation of the yellow color and read at 412 nm [26].

**Determination of lipid peroxidation end product malondialdehyde**

It is determined by addition of 500 µl of thiobarbituric acid reagent to the supernatant of the sample and boiling in water bath followed by coaling and developing reddish-pink color was measured at 534 nm peroxidation and expressed as nmol/mg protein [27].

**Determination of 8-hydroxy-2-deoxy guanosine (8-hdG)**

It is determined according to the manufacturer’s instructions using the Bioxytech-ELISA Kit (OXIS Health Products, Portland, OR, USA, Catalog. No. KOG-200S/E) and the reaction was measured at 450 nm [28].

**Assessments of caspase-3**

It is determined according to the manufacture by using a Stressgen colorimetric kit (catalog No. 907-013). The cleavage of the peptide was measured spectrophotometrically at a wavelength of 405 nm.

**Determination of dopamine (DA), serotonin (5-HT) and γ-aminobutyric acid (GABA) neurotransmitters**

High-performance liquid chromatography (HPLC) with the precolumn PTC derivatization technique was used to measure γ-aminobutyric acid, dopamine and serotonin [29]. The assay conditions were carried out at 46°C, 254 nm and flow rate: 1 ml/min. Dopamine and serotonin were assayed on bases of removal of trace element and lipid from the tested samples by solid phase extraction CHROMABOND column NH₂ phase Cat. No. 730031 and injected directly into an AQUA column 150 54.6 mm (Phenomenex, USA) [30].

Determination of interleukin 6 and 10 contents

It was by Enzyme-linked Immunosorbent Assay Kit of Cloud-Clone Corp. catalogue no. SEA079Ra for IL6 and catalogue No. SEA056Ra for IL10.

Statistical analysis

The data of the study was statistically analyzed using one-way ANOVA post-hoc analysis of variance between control and experimental test. All results were expressed as mean ± standard error (SE) and significance was observed at p < 0.05.

Results

Light and ultra-structural observations

Microscopically, compared to the control (Figure 1B and B1), the cervical spinal cord of offspring maternally ingested hypercholesterolemic diet displayed deformed ependymal canal with massive degeneration of epithelial lining cells. The multipolar neuronal cells are enclosed within defined necrosis within a halo spaces. The grey matter is fragile (Figure 1 and A1). On the other side, offspring of mother ingested malted barley plus hypercholesterolemic diet showed improved spinal tissues characterized by normal epithelium lining the ependymal canal. The multipolar neuronal cells appeared with vesicular nuclei and homogenous ground grey elements (Figure 1C and C1).

Figure 1: Photomicrographs of histological cross section of spinal cord of 21 day old offspring. A&A1. Control showing ependymal canal (EC) with normal lining epithelial cells (ELC). The grey matter showing motor neuronal cells (MNC) with normal nuclei and vesiculated nucleoli. B and B1. Offspring of hypercholesterolemic mother showing injured ependymal lining cells (DELC) and massive damage of motor neuronal cells (DMNC) within the grey matter. C and C1. Offspring of mother ingested malted barley plus a hypercholesterolemic diet showing improved ependymal canal lining cells plus normal picture of the motor neuronal cells.
Ultrastructurally, compared with the control (Figure 2A3-2C3), the offspring’s spinal cord of mother ingested a hypercholesterolemic diet, possessed karyolysed nuclei of many of the neuronal cells. Fragmented rough endoplasmic reticulum and shrieked mitochondria with ill-differentiated cristae were also observed within their cytoplasm. Demyelinated nerve axons were observed (Figure 2A1-2C1). However, offspring of mother fed on barley plus hypercholesterolemic diet showed a comparative increase of vesicular nuclei within the neuronal cells. Cytoplasmic organelles such as lamellar rough endoplasmic reticulum and mitochondria with in-folded cristae were identified. There was a detected myelinated of neuronal axons and abundant mitochondria in their neuropil (Figure 2A2-2C2).

**Immunohistochemistry**

There was a detected decrease of PCNA immunohistochemical reaction in neuronal cells of offspring of mothers ingested hypercholesterolemic diet (Figure 3C) compared to the control (Figure 3A). However, there was a moderated reaction of PCNA immunohistochemistry in those of mothers supplemented barley plus a high cholesterol diet (Figure 3B). Examination of the immunohistochemical picture showed that the PCNA immunohistochemical reaction was significantly reduced in spinal neurons of those maternally ingested a hypercholesterolemic diet compared to the other studied groups (Figure 3D).

Also, offspring of mothers ingested a hypercholesterolemic diet possessed over-expression of the GFAP immune reaction in the neuronal cells (Figure 3B1) compared to the control (Figure 3A1). However, there was a detected decrease of the immune reaction in those of mothers supplemented barley plus a hypercholesterolemic diet (Figure 3C1). Analysis of the immune picture showed the highest increase of the immunostaining in offspring of mothers ingested a hypercholesterolemic diet compared with observed reduction of that in those of mothers supplemented barley plus the rich cholesterol diet (Figure 3D).

**Figure 3:** Photomicrographs of formalin-fixed, paraffin-embedded cervical spinal cord of rat offspring 21-day old. A-C. PCNA immunostaining showing decreased immune reaction in spinal tissues of offspring of mother fed on a hypercholesterolemic diet (B) compared to control (A) and improved in those of mothers ingested barley plus a hypercholesterolemic diet (C). A1-C1. Spinal cord of offspring immunohistochemically stained with GFAP-antibody. Note decreased immune reaction in offspring of mother ingested a hypercholesterolemic diet (B1) compared to the control (A1) and improved in those of mother ingested barley plus a hypercholesterolemic diet (C1). Arrow head indicates the increased immunohistochemical reaction. D. Chart illustrating analysis of the immunohistochemical picture of PCNA and GFAP of the different studied group. Abbreviations; B, barley supplemented mother; C, control mother; H, mother fed on a high cholesterol diet; HB, mother fed on a high cholesterol diet containing barley. Star indicates a marked depletion of the immunoreaction.

**Biochemical observations**

Offspring maternally consumed a hypercholesterolemic diet showed significantly decreased the activities of both SOD and GST. This was followed by significant increase of the MDA, 8-HdG and Casp-3. The assayed antioxidant enzymes were increased coincides with de-
creased lipid peroxidation MDA and apoptic markers 8-HdG and Casp-3 in those of mothers ingested barley plus a hypercholesterolemic diet compared to the control (Table 1).

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<td>4.1 ± 0.2**</td>
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<td>8-HdG (ng/mg. protein)</td>
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<td>5.8 ± 0.29</td>
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<td>3w</td>
<td>5.4 ± 0.27</td>
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<td>5.9 ± 0.29</td>
<td>5.7 ± 0.28</td>
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Table 1: Cervical spinal cord contents of antioxidant enzymes, lipid peroxidation and apoptic markers of offspring of mother ingested hypercholesterolemic diet and/or barley-supplementation.

Each result represent the mean ± SE of n = 5. One star means significant at P < 0.05; double star mean highly significant at P < 0.01. Abbreviations: B: Barley; C: Control; Casp 3: Caspase 3; GST: Glutathione-S-Reductase; H: Hypercholesterolemic Diet; HB: Hypercholesterolemic Diet Containing Barley; 8-Hdg: 8-Hydroxydeoxyguanosine; MDA: Malondialdehyde; SOD: Superoxide Dismutase.

Also, there was a decrease of the spinal contents of 5-HT, DA and γ-ABA in offspring maternally consumed a hypercholesterolemic diet. These were reflected by depletion of the spine contents of interleukin 10 and increased contents of inflammatory interleukin 6. However, in conjunction with hypercholesterolemic diet, offspring of mother ingested barley showed a vice versa significant increase of interleukin 10 and depletion of inflammatory marker interleukin 6 correlated with increased the assayed neurotransmitters compared to the control (Table 2).

Discussion
From the present results, offspring of mothers intake of a hypercholesterolemic diet showed injured spinal cord located primarily in epithelial cells lining the ependymal canal and pyknotic damage nuclei of sensory or multipolar neuronal cells enclosing in a halo spaces.

These results were consistent with Elsayyad, et al. [31] in offspring of mothers ingested a hypercholesterolemic diet and treated with pomegranate juice and/or atorvastatin. It is reported that the cholesterol is transmitted to the fetus via placenta and enters the fetal circulation [32], de novo synthesis of neuronal cells and brain development [33]. Abnormal enzymes in the sterol biosynthesis pathway such as 3β-hydroxyosterol-Δ7-reductase gene [34], may alter gene expression and disrupted cholesterol metabolism and development of

neurodegenerative disorders of the spinal cord. Goharkhay, et al. [35] reported significant transcriptional of genes involved in the endogenous cholesterol synthesis, as well as LDLR, in the liver of mice born to hypercholesterolemic dams.

It is known that increase of high cholesterol level in maternal environment reprogramming the fetal tissues and facilitated the aortic fatty streak formation and development of atherosclerosis in human [4-6] and experimental animals [7-9] and consequently narrowing the blood vessels supplied the nutrients and oxygen required for cell differentiation leading to cell growth defects.

Also, there was a sharp rise of the inflammatory marker of interleukin 6 and caspase-3 in spinal tissues of offspring of a hypercholesterolemic dam. These results supported the research of Rahman, et al. [36] (2005) who reported increased neuro-inflammation, assessed by upregulation of interleukin 6 and neuronal damage by overexpression of caspase 1 in the brain of apolipoprotein E knockout and wild type mice. Neuronal cell death has been associated with neuronal apoptosis mediated caspase activation [37,38].

In addition, the increased lipid peroxidation of MDA is known to responsible for the pathogenesis of the spinal tissues demonstrated by increased level of 8-hydroxyguanosine and MDA contents parallel with decreased activities of both superoxide dismutase and glutathione s-transferase. These findings supported the work of Aytan, et al. [39] whom mentioned that feeding of hypercholesterolemic diet increased the malondialdehyde predicting oxidative stress in the hippocampus and developed memory loss [40] and Alzheimer’s disease [41]. It is also, associated with endothelial dysfunction in cerebral arterioles [42] and axonal degeneration and demyelination [43,44].

Table 2: Cervical spinal cord neurotransmitters and interleukin contents of offspring ingested hypercholesterolemic diet and/or barley-supplementation.

| Table 2: Cervical spinal cord neurotransmitters and interleukin contents of offspring ingested hypercholesterolemic diet and/or barley-supplementation. |

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<td><strong>DA (ng/g. protein)</strong></td>
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<tr>
<td>1w</td>
<td>56.1 ± 2.8</td>
<td>57.4 ± 3.1</td>
<td>45.7 ± 2.3**</td>
<td>48.8 ± 2.4</td>
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<tr>
<td>2w</td>
<td>56.4 ± 2.8</td>
<td>58.0 ± 3.2</td>
<td>47.5 ± 2.4**</td>
<td>49 ± 2.4</td>
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<td>3w</td>
<td>56.9 ± 2.9</td>
<td>57.8 ± 3.1</td>
<td>50.7 ± 2.5**</td>
<td>52.5 ± 2.6</td>
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| **5-HT (ng/g. protein)** |         |         |         |         |
| 1w             | 64.1 ± 3.2 | 64.6 ± 3.2 | 51.9 ± 2.6** | 55.4 ± 2.7* |
| 2w             | 64.7 ± 3.2 | 64.9 ± 3.2 | 53.9 ± 2.7** | 55.7 ± 2.8* |
| 3w             | 65.9 ± 3.3 | 67.1 ± 3.3 | 57.6 ± 2.9** | 59.6 ± 3.1 |

| **GABA (ng/g. protein)** |         |         |         |         |
| 1w             | 81.9 ± 4.1 | 82.1 ± 4.1 | 65.9 ± 3.3** | 68.4 ± 3.4* |
| 2w             | 82.2 ± 4.1 | 82.5 ± 4.1 | 68.5 ± 3.4** | 70.7 ± 3.5  |
| 3w             | 83.8 ± 4.2 | 85.2 ± 4.3 | 73.1 ± 3.6** | 75.6 ± 3.8* |

| **IL6 (pg/mg. protein)** |         |         |         |         |
| 1w             | 137.4 ± 6.9 | 136.5 ± 6.8 | 154.1 ± 7.7** | 140.4 ± 7.1 |
| 2w             | 143.9 ± 7.2 | 140.3 ± 7.1 | 161.3 ± 8.2** | 151.7 ± 7.6 |
| 3w             | 148.6 ± 7.4 | 145.6 ± 7.3 | 170.3 ± 8.5** | 154.6 ± 7.7 |

| **IL10 (pg/mg. protein)** |         |         |         |         |
| 1w             | 7.6 ± 0.39 | 8.2 ± 0.41 | 3.7 ± 0.18** | 5.1 ± 0.25 |
| 2w             | 8.4 ± 0.42 | 8.9 ± 0.44 | 4.1 ± 0.21** | 5.2 ± 0.26* |
| 3w             | 8.7 ± 0.43 | 9.6 ± 0.48 | 4.6 ± 0.23** | 5.4 ± 0.27* |

Additionally, elevated dietary cholesterol contributed to a pronounced loss in brain function determined by reduction of the neurotransmitters dopamine, serotonin and γ-aminobutyric acid. The results presented are consistent with Paul, et al. [45] who reported significant depletion of dopamine in striatum and serotonin in cortex of hypercholesterolemic mice. This depletion of neurotransmitters is responsible for impairing brain function.

Dopamine and serotonin has been found to control the sensory, motor and autonomic functions of the spinal cord [46], enhanced the human spinal reflex [47] and axon healing in invertebrates, low vertebrates and vertebrate animals [38]. High fat diet was associated with impairment of the prefrontal-dependent cognition disorder associated with severe changes at the cellular and synaptic scales within the medial prefrontal cortex adolescence [10].

The present research also demonstrated a decrease in the immunohistochemical reaction for PCNA and increased in GFAP immunohistochemical reaction in hypercholesterolemic rats suggesting neurodegenerative states and metabolic abnormalities. These were consistent with the study of Kalayci, et al. [49] which indicated that high cholesterol diet may interfere the integrity of blood-brain barrier (BBB) by overexpressing of the tight junction proteins and glial fibrillary acidic protein (GFAP). This was accompanied by increased the vascular endothelial growth factor (VEGF) in hypertensive conditions via increased nitric oxide, TNF-α and catalase in hypertensive conditions.

Beside the mentioned, barley supplementation to mother fed on a hypercholesterolemic diet decreased MDA involved in lipid peroxidation with a concomitant reduction apoptic markers of casp-3 and 8-Hdg and decreased the assayed inflammatory markers. Barley showed varieties of antioxidants and phenolic compounds [12,13], that are potentially useful for decreasing free radicals and improvements [50].

Barley β-glucan has antioxidant activity on the brain and sciatic nerve of the streptozotocin-induced diabetic rat [51] and an antioxidant, anti-inflammatory and immunomodulatory effect toward cisplatin-induced neurotoxicity [52]. Barley seed contains ferulic acid and coumaric acids which are of high antioxidant activity [53].

Daily oral administration of talbina to male albino rats for 1, 2, 3 and 4 weeks resulted in significant increases in the dopamine (DA), serotonin (5-HT) and gamma-aminobutyric acid (GABA) contents in different brain regions (Cerebellum, striatum, cerebral cortex, hypothalamus, brain steam and hippocampus) [54]. Additionally, tocotrienol (vitamin E derivative) - a drug was medication has been found to reduce the oxidative stress in Alzheimer disease by scavenging free-radicals and facilitating mitochondrial function and cellular repair. This has also reduced neurotoxicity caused by the release of glutamate into the neuronal cells [55].

Conclusion
Finally, the current research concluded that maternal fed on hypercholesterolemic diet is associated with increased lipid peroxidation and oxidative stress associated neurodegeneration of offsprings spinal tissues. The decrease of inflammatory and apoptic markers achieved by increased the antioxidant enzymes and neurotransmitters was observed after consumption of barley and hypercholesterolemic diet. This was attributed by highest antioxidant activity of barley which scavenge liberated free radicals and improved the injuries of spinal neuronal cells.

Conflict of Interest
The authors declare that there is no conflict of interest.

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