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Gero-Protective Impact of Astaxanthin on Experimental Model of Brain Aging: Biochemical and Molecular Study

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Abstract

Brain activity and multiple organ functions declined with aging. Although the complexity of brain aging process, releasing free radicals and inflammation were strongly linked to the disorder. Astaxanthin is a dietary carotenoid which exerts antioxidant and anti-inflammatory activities. We intended to estimate the protective impact of Astaxanthin on aging caused by D-galactose. Forty male Wister rats were separated into 4 groups as follow: G1: (control) group:G2: (D-Gal) rats received D-Gal (300mg/kg,ip) ;G3 (D-Gal+Met) 4 h after D-Gal administration, rats received metformin (300 mg/kg, orally) , as reference drug group;G4 : (D-Gal+Asta) group: 4h after D-Gal treatment, rats received Astaxanthin (25 mg/kg , orally). Results showed that administering D-Gal caused brain aging manifested by significant ($p<0.05$) disturbance of antioxidant defense system, inflammatory biomarkers. Moreover, results of gene expression and histological assessment ensured biochemical findings. Astaxanthin supplementation promoted a remarkable significant ($p<0.05$) normalization in all parameters by triggering different brain mechanisms. In conclusion these results suggested that Astaxanthin modulates D-Gal induced brain-abnormalities associated with aging.

Key words: Astaxanthin , Brain Aging , D-Galactose, Gero-protective ,Metformin

1. Introduction

Elderly is a complex biological process related to the functional decrease in different tissues, that lead to various chronic conditions such as diabetes, atherosclerosis, and neurodegenerative diseases [1]. Aging causes a segmental and progressive deficiency includes different physiological tasks and physical ability during long time periods, thus leading to comparative physiological weakness and loss of resilience [2]. Meanwhile, brain activity and multiple organ functions declined with aging [3]. Furthermore, cognitive deficiency and dementia related to brain elderly were identified as the directing sources of debility and dependency among worldwide elder people in the absence of effective therapeutic approaches [4].

2. While the complexity of brain aging pathogenesis, oxidative stress and inflammation were strongly linked to many disorders. Due to its high metabolism and low antioxidant enzyme action, the brain is mainly vulnerable to free radical damage. Neurons accumulate oxidatively damaged molecules as they age, and an excess of reactive oxygen species (ROS) leads to nerve cell aging and death [5]. Additionally, chronic inflammation was perceived in the aged brain. Microglia can become more sensitive to inflammatory stimuli as they age, and higher amounts of inflammatory cytokines causes dementia. As a result,

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controlling oxidative stress and inflammation should be a helpful remedy for treating the aged brain [6].
Metformin (Met), a frequently used anti-hyperglycaemic drug, quickly goes through the blood-brain barrier in circulation and builds up in various brain regions, such as the pituitary gland, olfactory bulb, and hippocampus. It is now considered as a pharmacotherapeutic mediator for central nervous system damage as it promotes adult hippocampal dentate gyrus neurogenesis and offers potent anti-inflammatory characteristics [7]. Certainly, numerous reports explain the neuroprotective impacts of metformin in many CNS injury models [8].
There is an increased interest in inducing aging in animal models using different compounds, among them is D-galactose. D-galactose-injected rodent models show brain aging features and aid in studying brain ageing mechanisms [9]. D-galactose (D-Gal) is a reducing sugar, long-term use of it disturbs ROS balance and antioxidant enzyme activity and boost building up of advanced glycation end products (AGEs), thereby boosting aging -related disorders like learning and cognitive deficits [10]. Each country's aging population grows progressively, and the aging concern has gained worldwide interest. As a result, it is critical to investigate safe and effective methods of delaying or ameliorating brain aging [11]. Polyphenols, dietary antioxidants, were demonstrated to be useful against the production of oxidative stress in a variety of aging-related disorders [12].

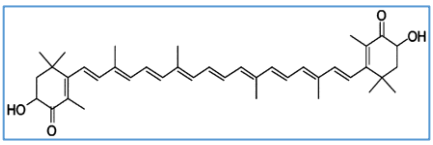


Fig. 1: Chemical structure of Astaxanthin [13] .

Astaxanthin (3,3′ -dihydroxy-β,β-carotene-4,4′ -dione) (Figure 1), a dietary carotenoid, related to the family of xanthophylls, the oxygenated derivatives of carotenoids [14] . Additionally, is a natural compound in microalgae and seafood, such as salmon, trout, and shrimp [15] with a molecular formula C40H52O4 and melting point 224 ◦C. Asta is soluble in fat and organic solvents [16].
For now, Astaxanthin efficiently inhibits oxidative stress in obesity, osteoarthritis, ethanol-induced liver injury, brain tissue damage, and cardiovascular disease [17]. Also, it has essential metabolic roles in animals, involving alteration to vitamin A, improvement of immune system, and defense against illnesses such as cancer by scavenging oxygen radicals, and frequently applied nutritional supplements [18]. Astaxanthin has great brain tissue transferability that keeps brain vasculature from cerebral ischemia and subarachnoid hemorrhage [16]. The antioxidant property of Astaxanthin was greater than that of vitamin C and around ten times stronger than that of other tested carotenoids, including zeaxanthin, lutein, canthaxanthin, and h-carotene, and 100 times more than that of a-tocopherol. Astaxanthin's conjugated double bonds and terminal ring moieties were demonstrated to capture radicals on cell membranes and interior membranes [19].
This work aimed to assess the protective effect of Astaxanthin on aging induced by D-galactose, and also investigate potential biochemical and molecular mechanisms.

2. Materials and methods

2.1. Reagents and preparations

Astaxanthin (Asta) and D- galactose (D- Gal) were obtained from Sigma –Aldrich (CA, USA). Asta was prepared by dissolving in 0.2% sodium carboxymethyl cellulose to form homogeneous suspension and administered by oral gavage. D-Gal was dissolved in saline and injected intraperitoneally. Metformin (Met) was obtained from Chemi-pharm Pharmaceutical Company, Egypt, and was dissolved similarly as Astaxanthin.

2.2 Animals

Forty adult male Wister rats weighing 180–220 obtained from the Animal House Colony of the National Research Centre (Cairo, Egypt), and kept in special cages at 20-22°C with 12h light/dark cycle and humidity (60%). Rats were given one week for acclimatization and gave free access to standard rodent chow and water. A commercial balanced diet and tap water were given throughout the experimental period *ad libitum*.

2.3. Aging induction

Brain aging was induced in rats according to the procedure described by Liu et al [20] Briefly, rats administered D-galactose orally (300 mg/kg) daily for 8 consecutive weeks.

2.3.1 Animal treatments

Forty male rats were divided into four groups (10 rats /each)
Group 1: (Control) group: rats got saline solution (0.9% Sodium Chloride solution) intraperitoneally.
Group 2: (D-Gal) group: rats received D-galactose (300mg/Kg, intraperitoneally)
Group 3: (D-Gal+Met) group: after D-galactose administration, rats received Metformin (300 mg/Kg,

orally), according to Wang et al [21] served as reference drug group.

Group 4 : (D-Gal+Asta) group: after D-galactose treatment, rats received Astaxanthin (25 mg/kg body weight , orally by gavage) according to Xue et al [22].

At the end of the experimental period (eight consecutive weeks), rats were sacrificed, brain of each rat was dissected, weighed, part of the harvested brains were kept in 10% formalin –saline for histological examination, while the other part was homogenized in 0.1 M phosphate buffer and kept at -80 C for further biochemical and molecular analyses.

2.4. Biochemical analysis

2.4.1. Preparation of brain homogenate for determination of oxidative stress

Brains were sonicated in 10% (w/v) RIPA buffer, then treated with a protease inhibitor cocktail. Homogenates were incubated on ice for 30 minutes before being centrifuged at 600g for 10 min. Supernatants were kept at 80 °C until they were analyzed. According to previous work [23], brain tissue lipid peroxidation (MDA) and total antioxidant capacity (TAC) were calorimetrically assessed using test kits (Bio-diagnostic, Egypt).

2.4.2. Evaluation of neurological deficits

While, 8-hydroxy-2-deoxyguanosine (8-OHdG), Nuclear factor erythroid 2- related factor (Nrf2) , Nuclear Factor-κB (NF-kB), Brain-derived neurotrophic factor (BDNF) and Caspase -3 (Cas-3) were determined by enzyme linked immunosorbent assay (ELISA) by commercial kits.

2.4.3. Real-Time Polymerase Chain Reaction and Gene expression analysis

Total RNA of brain tissues was isolated with Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. PCR amplification and quantification were performed in a real-time PCR system (ABI-7300, Foster City, CA, USA). Relative quantification of gene expression of Forkhead boxO3 gene (FOXO3 Accession No NM_001415145.1 product size 237) , sirtuin 1 (Sirt1 Accession No JQ768366.1 product size 174), B-galactosidase -1 (Glb1 Accession No KR709981.1 product size 165) and KLOTHO (KL Accession No AB005142.1 product size 204) to the reference (β-actin) was analyzed from the measured threshold cycles (Ct) by using the 2–ΔΔCt method in the experiment.

Table 1. Sequences of specific primer of genes

Gene	Forward	Reverse
Forkhead boxO3 gene (FOXO3)	TCT ACG AGT GGA TGG-TGC GTT G	CTC TTG CCA GTT CCC TCA TTC TG
Sirtuin1 (Sirt1),	TCC TCA CTA ATG GCT TTC ATT CCT G	GTG CCA ATC ATG AGA TGT TGC TG
B-galactosidase -1 (Glb1)	TGA TCG GGC TTA CGT CTC TGT G	CTG TCA TGA GGT TTC GAT CAA GGA
KLOTHO (KL)	CGC AAA GTG CTC AAC TGG CTA A	AAG GTT GAT GTC GTC CAA CAC GTA

2.5. Histopathological assessment

Brain tissue samples were fixed in natural buffered formalin (10%) for 12 h, and then embedded in paraffin. After that, brain tissues were cut into approximately 5 μm thick sections and stained with hematoxylin and eosin (H&E), then examined by Light microscopy.

2.6. Statistical analysis

Data was displayed as mean ± SEM. Results were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical analysis was performed by GraphPad Prism© software (version 8.01; Graph Pad Software, California, USA). For all the statistical tests, the level of significance was fixed at $P < 0.05$.

3.Results

Results obtained revealed that administration of 300 mg/kg D-gal induced significant elevation in brain level of MDA an index of lipid peroxide (164 .7%), 8-OHdG (180%) (Fig.2A) , NF-kB (83%) and cas-3 (169.9%) (Fig.2B) , while significantly attenuated the brain levels of TAC (-51.7%), Nrf2 (-65%) and BNDF (-54.3%) (Fig.2C) as compared to the control group . While, treating senescent induced rats with 25mg/Kg Asta showed neuroprotective result against D-Gal induced brain aging evidenced in significant attenuation in brain levels of MDA, 8-OHdG , NF-kB and Cas-3, while significantly elevated TAC , Nrf2 and BDNF comparable with D-gal group . Current results indicated that the effect of Asta treatment surpassed that of Met (standard drug) and insignificant

differences were detected between Asta and control groups for all studied biochemical parameters.

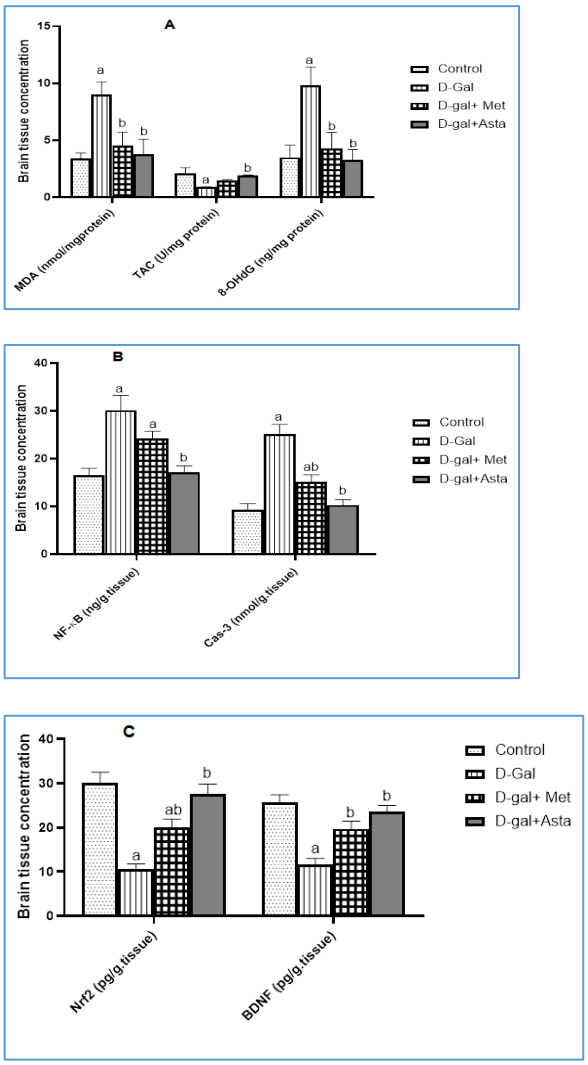


Fig. 2: Effect of treatments on (2A) Brain tissue malondialdehyde (MDA), total antioxidant capacity (TAC) and 8-hydroxy-2-deoxyguanosine (8-OHdG) in D-galactose–brain aging rats (2B) Brain tissue (NF-κB) and Caspase -3 (Cas-3) in D-galactose –brain aging rats (2C) Brain tissue (Nrf2) and Brain-derived neurotrophic factor (BDNF) in D-galactose –brain aging rats. (n=10, X± SEM). ap<0.05 vs. control group, bp<0.05 vs. D-gal group. D-gal :D-galactose (300 mg/Kg), Met: Metformin (300 mg/Kg) , Asta :Astaxanthin (25 mg/Kg).

To better understand the consequence of Asta in lessening the harshness on aging rats, we investigated treatment with Asta on expression of Forkhead box O3 gene, sirtuin 1 (Sirt1), B-galactosidase -1 (Glb1) and Klotho (KL) in D-galactose –brain aging rats (Figures 3A, 3B ,3C and 3D) respectively. D-Gal treated rats presented significant downregulation in FOXO3 , Sirt1 and KL genes expression to -50 , -55 and -39.7% respectively (Figs 3A,3B,3D),while, significantly unregulated Glb 1 gene (300%) (Fig. 3C) as compared to control rats. While treatment of D-gal senescent rats with Asta modulated the expression of the genes to standard levels compared to D-gal group. Treatment by Asta for these genes' expression were positively good in contrast with Met in this work study, these findings indicated Asta possible neuroprotective attributes.

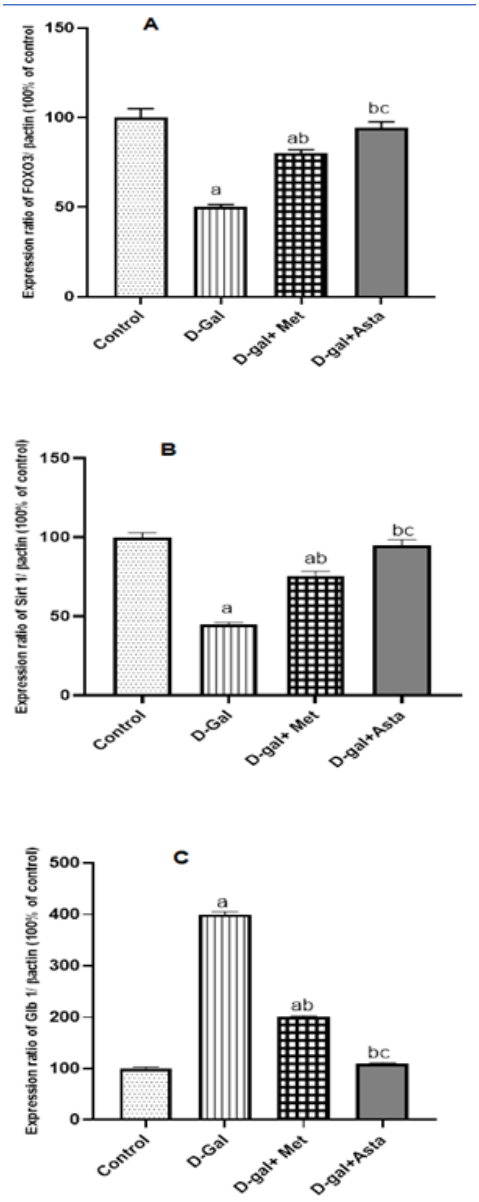
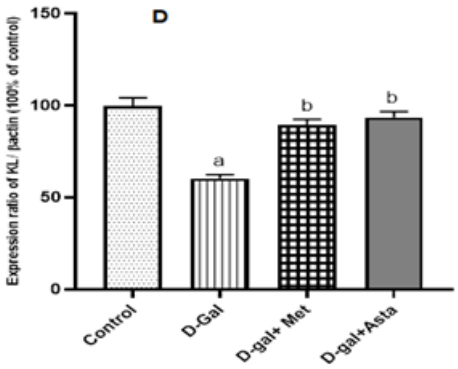


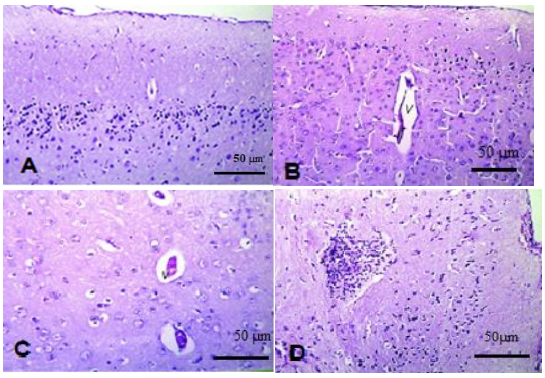
Fig.3: Effect of different treatments on brain tissue gene expression of :(3A) Forkhead box O3 gene (FOXO3) in D-galactose –brain aging rats. (3B) sirtuin 1 (Sirt1), in D-galactose –brain aging rats. (3C): B-galactosidase -1 (Glb1) in D-galactose –brain aging rats. (3D): KLOTHO (KL) in D-galactose –brain aging rats (n=10, X± SEM). [a]p<0.05 vs control group, [b]p<0.05 vs D-gal group, [c]p< 0.05 vs D-gal+Met. D-gal :D-galactose (300 mg/kg), Met: Metformin (300 mg/kg) , Asta :Astaxanthin (25 mg/kg).



3.1. Histological assessment

Fig. 4: Effect of Astaxanthin on cerebral cortex of brain (H&E staining magnification (40x) (5A)Control group showed normal histological structure with no evident pathological changes; (4B) D-Gal model group ; showed disorganized nerve fibers with irregular neurons, marked congestion in cerebral blood vessels and necrosis (4C) D-Gal+ Met; mild improvement in brain injury was indicated, moderate cerebral blood vessels congestion and increased round shaped neurons (4D) D-Gal +Asta. revealed marked improvement in morphological structure V indicates blood vessels.

The brain section photomicrograph of the control group showed normal histological structure with no evident pathological changes. In D-Gal (4B) treated group, significant brain injury was observed, including disorganized nerve fibers with irregular neurons, marked congestion in cerebral blood vessels and necrosis. However, in (D-Gal+Met) (4C) group, mild improvement in brain injury was indicated, moderate cerebral blood vessels congestion and increased round shaped neurons. However, intervention with Asta (4D) revealed marked improvement in morphological structure which appeared like control group (4A).



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4.Discussion

The aging process can damage a variety of brain processes. Reports have found a decline in brain volume in older people when compared to the younger adults, particularly in cognition related brain regions. Even though the processes of aging were complicated, general theories, including free radical theory, particularly neuroinflammation can describe it [24]. While Xue et al [22] revealed that Asta improved learning and memory deficiencies after cerebral damage by Infra-Red.

Usually, aging models can be categorized into two categories: naturally aging model and enhanced aging models which are commonly used [25]. D-Gal is a reducing sugar that is commonly used for induction in brain aging changed to aldose then hydroperoxide by galactose oxidase, causing ROS releasing and establishment of advanced glycation end products (AGEs) [26].

In this study, administering D-Galactose orally daily for 8 consecutive weeks induced noticeably aging-related changes that manifested by disturbance in oxidative and inflammatory biomarkers, and disorders in brain gene expression. Similar results were obtained by Chen et al [27].

Meanwhile, Epidemiological and animal studies by Wang et al [28] indicated apoptosis in the liver and brain in D-Gal-induced aging mice. However, the

MDA formation of MDA triggered nerve molecules inflammations and oxidative damage of brain cells [29]. Excessive ROS buildup was able to injure biological components thus increasing inflammation and damaging nervous tissue [30].

Astaxanthin is a valuable natural antioxidant of great biological properties. It could cross the blood-brain and retinal barriers [31]. Previous works proved that Asta molecular structure provides it the strong antioxidant activity. Asta scavenges oxygen free radicals, block fatty acid chain reactions, and provide strong antioxidant activity [32].

The study results revealed an improvement in these parameters after treatment either by using reference drug Metformin or Astaxanthin. Dietary astaxanthin accumulates in the hippocampus and may exert neuroprotective effects. The dose of Asta that was used in the study is (25 mg/kg body weight). It was represented that administering Asta at doses of 25 mg/kg, 75 mg/kg, 30 mg/kg, 50 mg/kg showed neuroprotective activity [33].

Our result came in line with earlier study by Fakhri et al [34], which revealed that Astaxanthin offers neuroprotection against multiple sclerosis, cerebral ischemia, and Parkinson's disease. A study by Manabe et al [13] supports using astaxanthin in or ameliorating cognitive damage related to normal aging. Moreover, Asta prevented neural death in cell models associated with Parkinson by inhibiting mitochondrial apoptotic cascade. Similar results were obtained previously [35] Another study [36] evaluated the neuroprotective role of Asta on brain damage.

While Ji et al [25] showed the importance of Astaxanthin in protecting against various models of disease via anti-inflammation, anti-oxidation. While previous study [17] found that administering Asta in low doses diminishes MDA blood levels and boosts the brain’s defense system in treated rats by reducing neutrophils infiltration and the pro-inflammatory cytokines also indicated that Asta protects heart from alcoholic cardiomyopathy partially by attenuating endoplasmic reticulum stress in rats.

Meanwhile, Maoka et al [37] discussed the importance of the conjugated double bond of Asta in exhibiting strong antioxidant activities. A study by Zhang et al [38] proved that Asta could protect against neurotoxicity both in vivo and vitro studies. While Wu et al [39] showed that Asta protects against ischemic brain injury in neural cells. In addition, different studies have demonstrated the anti-apoptotic effects of Asta in myocardial damage [40]. Previous work [32] Astaxanthin effect on increasing mitochondrial biogenesis, thus maintaining the skeletal muscle against the oxidative damage. Similarly, Asta therapy prevents the progress of colonic mucosal ulcers and adenocarcinoma by decreasing the production of inflammatory-related cytokines [41]. As a result, by reducing oxidative damage, Asta protects against epilepsy-induced neuronal loss, lipid peroxidation and inhibits the intrinsic apoptotic pathway in the rat hippocampus. Previous in vivo work by Liu et al [20] proved the Asta antioxidant activity.

The study findings suggested that Asta restrained excessive inflammation, as Kurihara et al [42] proved its strong pharmacological effects against inflammation and its role in modulating excessive inflammatory response. Furthermore, astaxanthin controls neuroinflammation by lowering oxidative stress, decreasing neuroinflammatory factor production, regulating peripheral inflammation, and preserving the blood-brain barrier's integrity. In addition, it was reported that oxidative stress increases the expression levels of the proinflammatory cytokines. Nrf2 is a cytoprotective factor controlling the expression of genes coding for antioxidant, anti-inflammatory, and detoxifying proteins [43]. When subjected to oxidative stress, Nrf2 migrates to the nucleus and attaches to antioxidant response elements (AREs), activating target genes and lowering oxidative stress [44]. Reducing Nrf2 expression boosted the vulnerability to oxidative damage, particularly in aging and neurodegenerative disorders. In one study, Asta protects against cardiac injury by activating the Nrf2/ARE signaling pathway and modulating mitochondrial-mediated apoptosis pathway [45].

Nuclear factor-kappa B (NF-κB) prompts expression of several pro- and antiapoptotic genes, including BDNF. Our observations agree with the findings of a previous research [46] , which found that the expression of NF-κB was lowered in Asta-treated mice with UV-induced photokeratitis. Similarly, Asta has exhibited effectiveness against cardiovascular complications through NF-κB pathway [47]. Similarly, Asta diminishes pro-inflammatory cytokines levels by lowering NF-κB expression and in activates Ikappa B kinase, thus plays a critical role in controlling of NF-κB. Likewise, Asta mitigates hepatotoxicity after exposure to arsenic by slowing cytokine-mediated cell-cell connections [48]. In addition, administering 8 mg Asta potentiated the immune system by decreasing plasma levels of 8-OHdG.

Caspases are a group of proteases involved in different cellular functions, such as differentiation, remodeling, and death. While Tong et al [49] found that caspases activity increased by aging process. Also, activation of Caspase-3 shows considerable role in apoptosis and considered the terminal event preceding cell death. A study by Lee et al [50] showed the effect of Asta in

inhibiting the activation of caspase-3 in a Parkinson disease mouse model.

Neurotrophic factor generated from the brain BDNF is among neurotrophins that is involved in plasticity, neuronal survival, the development of new synapses, dendritic branching, and the regulation of excitatory and inhibitory neurotransmitter profiles in the brain. Although the impact is more obvious in the younger population, it also benefits the elderly, particularly those who are cognitively, physically, and socially active, lowering the risk of age-related comorbidities [51]. BDNF is also released in a variety of peripheral tissues, including platelets, lymphocytes, skeletal and smooth muscle cells [52]. In line with this notion, decreased BDNF expression has been linked to neuronal shrinkage and neurological diseases [53]. Aging is correlated with decreased neurotrophins expression under resting conditions and impaired the function of BDNF. In animal models of acute cerebral infarction (ACI), Asta supplementation reduced BDNF expression [29]. Asta slows brain aging by increasing levels of BDNF in the rat hippocampus [39] . Similar results were obtained by Sharma et al [54].

Results revealed that treatment of D-Gal senescent rats with Asta modulated the expression of the genes (FOXO3, Sirt1, Glb1 and KL) to regular levels as compared to D-Gal treated group. expression of D–Galactose –brain aging rats. The role of FOXO3 response to an external stimulus is important in the treatment of aging and age-related illnesses [50].One study represented the role of Asta in upregulating FOXO3 expression in the renal tubular epithelium [55].While Audesse et al [56] showed the relation between dietary administering of Asta and the activating FOXO3 gene in rat heart tissue and blood also established the effect of FOXO3 in keeping healthful mammalian stem cell pool to increase lifelong neurogenesis.

B-galactosidase -1 (Glb1) is a major hallmark of aging that is chronically accumulated in cellular senescence. Cellular senescence weakens the capability of tissue regeneration and leads to chronic low-grade inflammation, that aggravates the aging development [57]. Silent mating type information regulation 2 homolog (SIRT)1 and KL (Klotho) are two longevity genes that delay cellular senescence by regulating multiple anti-aging cellular procedures. Similar results were obtained previously [58].

Sirtuin1 considered as enzyme which deacetylates proteins that promote reaction to stressors and anti-aging mechanisms [59]. While Klotho was implicated in several human and animals' biological pathways, thus affects longevity, memory, and cognition. In a study by Yook et al [60], Asta was found to enhance cardiac function and diminished fibrosis and SIRT1 expression. Additionally, when the impact of Asta on sirtuin1 and klotho was evaluated in D-Galactose brain aged models, Asta was discovered to reverse the decreased expression pattern of sirtuin1 and Klotho and to have strong anti-aging capabilities.

In our study, histopathological analysis instructed that Asta lessened D-Gal-induced brain damage and reduced neuronal loss. Similar results were obtained in previous work [61] This effect may be due to the brain penetrating power of Asta and its role in quenching of singlet oxygen also improved brain damage associated with aging [62].

Conclusion

Astaxanthin is a potent antioxidant that mitigates the effects of oxidative stress without causing any negative side effects. It also reduces ROS production and lowers pro-inflammatory mediators. Dietary administration of Astaxanthin promotes gero-protective properties as it can modulate various signal pathways related to longevity.

Ethical Approval

Experiments were conducted in accordance with the rules of the Ethical Committee of National Research Centre, Egypt (Publication No. 85-23, revised 1996).

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Neveen A. Salem and Rasha H. Hussein were responsible for the conceptualization, Hanadi M. Baeissa and Aminah A. Barqawi were responsible for the data analysis, Manal A. Tashkandi and Leena S. Alqahtani were responsible for the investigation Reem Al-azragi, Neveen A. Salem were responsible for the methodology responsible for the and supervision, Neveen A. Salem and Rasha H. Hussein were responsible for writing the manuscript. Hanadi M. Baeissa, Aminah A. Barqawi, Manal A. Tashkandi and Leena S. Alqahtani contributed equally to this work.

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