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Potential Effect of Nattokinase on Brain Growth Factors in Experimental Model of Alzheimer's Disease

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Abstract: Alzheimer's disease (AD) is a progressive disorder in which brain cells (neurons) deteriorate, resulting in the loss of cognitive functions, primary memory, judgment, reasoning, movement coordination and pattern recognition. This study was constructed to investigate the effect of nattokinase (Natto) on growth factors in the brain of Alzheimer's disease induced in rats. *S*ixty adult female Sprague Dawley rats were divided into four groups, (1) normal control group (con group), (2) group underwent surgery to remove ovaries (Ovx control group), (3) ovariectomized group received aluminum chloride in a dose of 17 mg/kg daily, orally for 2 months to induce AD (AD group) (4) AD group treated with nattokinase at dose of 72 U/rat/day orally for three months (Natto group). Transforming growth factor-β (TGF-β), brain derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) levels were determined in the brain of rats in the different studied groups. Also, histological examination of the brain tissue was carried out on the all groups under study. In comparison with the normal control group, the Ovx group recorded significant increase in the brain level of TGF-β, while brain levels of BDNF and VEGF were significantly decreased. Similarly, AD group recorded significant increase in the brain of TGF-β level accompanied with significant decrease in brain BDNF and VEGF levels as compared to Ovx group. However, treatment of AD group with nattokinase resulted in remarkable improvement in the studied biochemical parameters which reflected by decreased brain TGF-β level accompanied with significant increase in the brain BDNF and VEGF levels, compared to AD group. Histological investigation of brain tissue of Ovx group showed that many neurons in the cortex area are in the degenerative form. Microscopic examination of brain tissue of Ovx group administered with aluminum showed AD plaques indicating the validation of AD model. While, AD group treated with nattokinase showed great improvement in the brain morphological structure and disappearance of most of amyloid plaques. This study provided clear evidence that nattokinase represents a novel therapeutic approach for Alzheimer's disease *via* its degrading ability for amyloid β in the brain and amelioration of brain growth factors levels.

Key words: Alzheimer's disease · Nattokinase · Growth factors · Proteolytic activity · Rats

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in elderly people worldwide. It is reported that the number of affected people is expected to double within the next 20 years and 1 in 85 people will possibly be affected by 2050 [1]. Alzheimer's disease is characterized mainly by progressive neuronal cell dysfunction and the formation of amyloid plaques in the brain. The major constituent of AD plaques is the amyloid β-peptide (Aβ), which is a product cleaved from the membrane-bound amyloid precursor protein (APP) by β- and γ-secretases [2]. Although the development of AD is incompletely understood, amyloid-β (Aβ), a 39-43 amino acid peptide, is thought by many, though not all, to play a major role in disease pathogenesis. The neurotoxicity of Aβ may result from the formation of protease-resistant oligomeric and fibrillar forms of Aβ and thus, the blocking of Aβ aggregation may provide a valuable therapeutic approach [3]. The main risk factors for AD are age, age-related diseases such as cardiovascular disease, diabetes and obesity, low educational levels, head trauma

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and exposure to aluminum and heavy metals such as cupper, iron and zinc [4-6]. During the last decade, an abundance of research has continued to link aluminum (Al) with AD [7]. Aluminum (Al) is a common metal in the environment and one of the most abundant in the terrestrial crust. Al is liberated in the environment by natural processes of soil erosion, volcanic eruptions and anthropogenic actions [8]. Aluminum utensils are widely used in the world, especially in the developing countries. This element appears mainly in food products and in drinking water derived from both natural sources and treatment methods [9]. The sources of Al are specially corn, yellow cheese, salt, herbs, spices, tea, cosmetics, aluminum ware and containers. Also, aluminum is widely used in food additives and toothpaste [10]. Aluminum compounds are widely used in medical products such as antacids, phosphate binders, buffered aspirins, vaccines and allergen injections. Environmental pollution with the different aluminum-containing compounds, especially those in industrial waste water, exposes people to higher than normal levels of Al. Excessive Al intake leads to memory impairments [11], deposition of amyloid protein in central nerve cells and overexpression of APP [12]. Researches show that Al-induced neurotoxicity is associated with apoptosis and oxidative stress [13]. Growth factors naturally occurring proteins, are capable of stimulating cellular growth, proliferation and cellular differentiation. Growth factors are important for regulating a variety of cellular processes. Growth factors typically act as signaling molecules between cells such as cytokines and hormones that bind to specific receptors on the surface of their target cells [14]. Transforming growth factor beta 1 (TGF-β1) plays a central role in response of the brain to injury and the increased TGF-β has been found in the central nervous system of patients with Alzheimer's disease [15].The involvement of BDNF in the pathogenesis of AD has been reported [16]. Peng *et al.* [17] demonstrated the downregulation of brain-derived neurotrophic factor (BDNF) in the cortex area that occurs early in the progression of Alzheimer's disease (AD). It has been stated that there is a severe reduction in VEGF secretions in AD patients compared to healthy elderly subjects [18]. Proteolytic enzymes refer to a group of enzymes that break down long molecules of proteins into shorter pieces that eventually become amino acids.

These enzymes work to aid the body in digesting proteins. Proteolytic enzymes are produced naturally in the pancreas but may also be found in certain foods. Supplements containing these enzymes may be used to address a variety of health concerns [19]. Nattokinase is a profibrinolytic serine protease originally extracted and purified from natto, a traditional fermented soybean food popular in Japan. Nattokinase may support healthy coagulation of blood within normal levels and may be useful in the maintenance and enhancement of normal healthy endogenous fibrinolysis, dissolution of the essential portion of the blood clot or thrombus [20]. Maintenance of optimum functioning of the body's fibrinolytic/thrombolytic mechanisms may benefit the function of many bodily systems, in particular the cardiovascular system and the brain [20]. Nattokinase has greater thrombolytic activity than plasmin [20], a natural thrombolytic protease in blood and increases the production of plasmin from plasminogen due to its action on plasminogen activator. It not only degrades fibrin in thrombi [21] but also cleaves plasminogen activator inhibitor type I [22]. Thus, it has a potent fibrinolytic activity [22]. The ability of nattokinase to be absorbed across the intestinal tract after oral administration and induce fibrinolysis makes nattokinase a potential clot-dissolving agent for the treatment of cardiovascular disease [21]. Therefore, nattokinase is currently used as a nutrient supplement to improve circulation in the body [23]. Although much research has been carried out on nattokinase, there has been no interest in whether it can degrade amyloids which are also highly insoluble and protease-resistant. Hsu *et al.* [24] demonstrated that nattokinase has a high amyloid-degrading ability and this suggests that it may be useful in the treatment of amyloid-related diseases.

This study was constructed to evaluate the efficacy of nattokinase in modulation of brain growth factors in experimental model of Alzheimer's disease.

MATERIALS AND METHODS

Experimental Animals: Sixty adult female Sprague Dawley rats (130±10 g) were obtained from the Animal House Colony of the National Research Centre. They were kept in plypropylene cages at room temperature (25±2°C) and humidity (55%) under 12 hrs dark-light cycle. All animals were accommodated with laboratory conditions for at least two weeks before starting the experiment and they were maintained under the same conditions allover the experiment. Diet and water were allowed *ad libitum*. All animals received human care in compliance with the guidelines of the Ethical Committee of Medical Research of the National Research Centre, Giza, Egypt.

Experimental Design: Animals were randomly divided into four groups (15 rats for each). The first gonad intact group received saline solution orally daily for two months and served as normal control group (con group). The second group was subjected to surgical operation to remove ovaries and left for three months for complete depletion of estrogen and this group served as ovariectomized control group (Ovx control group). The third and fourth ovariectomized groups received aluminum chloride ($AlCl_3$) in a dose of 17 mg/kg b.wt. [25] orally daily for two months to induce AD and the third group served as Alzheimer's disease group (AD group). The fourth group was treated orally with nattokinase in a dose of 72 U/rat/day, which is equivalent to the recommended human dose [26] for three months and this group served as nattokinase-treated group (Natto group).

Samples Collection: At the end of the experimental period, the animals were sacrificed and the whole brain of each animal was rapidly removed, thoroughly washed with isotonic saline and dried on filter paper. The whole brain from each animal was sagitally divided into two halves. One half of each brain was weighed and then homogenized immediately to give 10% (w/v) homogenate in ice-cold medium contained 50 mM Tris-HCl (pH 7.4) and 300 mM sucrose [27]. The homogenate was centrifuged at 1800 xg for 10 min at 4°C. The supernatant (10%) was separated for biochemical analysis. The second half of each brain was fixed in 10% buffered formalin and embedded into paraffin blocks. Histological examination was carried out on 5μ m-thick, hematoxylin-eosin (HandE) stained brain sections [28].

Biochemical Analysis: Quantitative estimation of total protein concentration in the brain homogenate was carried out according to the method of Lowry *et al.* [29] to express the concentration of each studied growth factor per mg protein [30]. Brain transforming growth factor -beta (TGF-β) level was determined by ELISA technique according to the method of Kropf *et al.* [31] using kit purchased from Drug-Diagnostics Co., Germany. Brain derived neurotrophic factor (BDNF) was estimated by ELISA technique [32] using kit purchased from Ray Biotech Co., USA. Brain vascular endothelial growth factor (VEGF) was assayed by ELISA procedure according to the method described by He *et al.* [33] using kit purchased from Invitrogen Co., Camarillo and Ventura, California, USA.

Statistical Analysis: In the present study, all results were expressed as mean ±S.E of the mean. Statistical Package for the Social Sciences (SPSS) program, version 11.0 was used to compare significance between each two groups. Difference was considered significant when $P \leq 0.05$. Percentage difference representing the percent of variation with respect to the corresponding control group was calculated using the following formula:

$$\% \text{ difference} = \frac{\text{Treated value} - \text{Control value}}{\text{Control value}} \times 100$$

RESULTS

The results in Table 1 showed the effect of nattokinase on brain growth factors of AD-induced rats. In comparison with the normal control group, there was significant increase ($P<0.05$) in the brain TGF-β level (25.96%) accompanied with significant decreases ($P<0.05$) in BDNF (-31.25%) and VEGF (-19.96%) levels in the Ovx group. Similarly, AD-induced group recorded significant elevation ($P<0.05$) in the brain TGF-β_1 level (9.99%), while it recorded significant decrease ($P<0.05$) in the brain BDNF (-18.18%) and VEGF levels (-37.78%) as compared to the Ovx control group. On the other hand, treatment of AD group with nattokinase caused significant reduction ($P<0.05$) in the level of TGF-β (-24.90%) accompanied with significant elevation ($P<0.05$) in the brain BDNF (38.89%) and VEGF levels (36.77%) when compared with the untreated AD group.

Histological Investigation: Histological examination of brain tissue section of normal control rat showed highly active nerve cells in the cortex area that having huge nuclei with relatively pale stain. The nuclear chromatin and prominent nucleoli disappeared. The surrounding support cells have small nuclei with dense stain, condensed chromatin with no visible nucleoli (Fig. 1). Microscopic investigation of brain tissue section of ovariectomized rat showed dark neurons with corkscrew dendrites and many other neurons of the cortex area appeared in a degenerative form (Fig. 2). Histological examination of brain tissue section of $AlCl_3$ administered rat (AD group) showed the formation of various sizes of amyloid plaques in the cerebral cortex and hippocampus (Fig. 3). Microscopic examination of brain tissue section of AD-induced rat treated with nattokinase showed focal gliosis in the cerebral cortex and hippocampus associated with disappearance of the most amyloid plaques (Fig. 4).

Table 1: Effect of nattokinase on brain growth factors levels in AD-induced rats

Parameters ----------			
Groups	TGF-β Pg/mg protein	BDNF ng/mg protein	VEGF Pg/mg protein
Normal control group	779.20±1.25	0.32±0.008	3593.11±39.50
Ovx control group	981.51±1.49[a]	0.22±0.003[a]	2876.95±101.26[a]
	D (25.96%)	D (-31.25%)	D (-19.96%)
AD group	1079.55±2.17[b]	0.18±0.003[b]	1790.18±244.01[b]
	E (9.99%)	E (-18.18%)	E (-37.78%)
Natto group	810.73±2.05[c]	0.25±0.003[c]	2448.36±55.69[c]
	F (-24.90%)	F (38.89%)	F (36.77%)

The mean difference is significant at P ≤ 0.05.

a =compared to the normal control group. b =compared to Ovx control group.

c = compared to AD group. D%=Percent difference from the normal control.

E%= Percent difference from Ovx control group. F%= Percent difference from AD group.

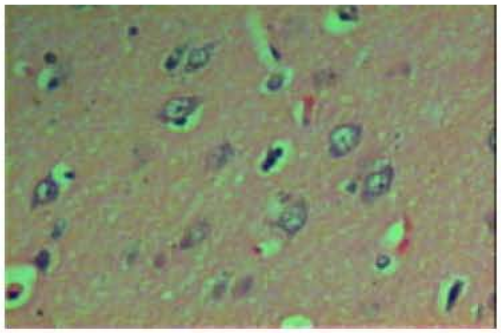

Fig. 1: Micrograph of brain section of normal control rat showing the highly active nerve cells, in the cortex area, that having huge nuclei with relatively pale stain. The nuclear chromatin and prominent nucleoli disappeared. The surrounding support cells have small nuclei with dense stain, condensed chromatin and no visible nucleoli (Hand E X 400)

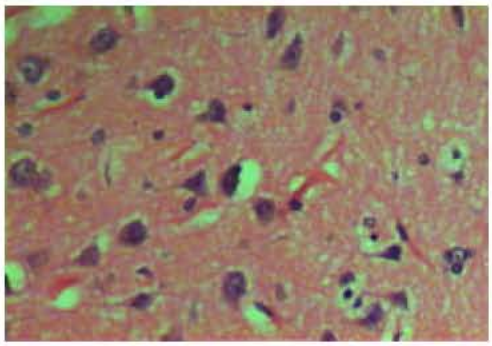

Fig. 2: Micrograph of brain section of ovariectomized rat showing dark neurons with corkscrew dendrites and many other neurons of the cortex area appeared in a degenerative form (H and E X 400)

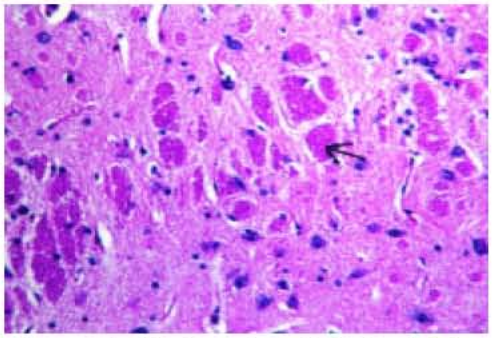

Fig. 3: Micrograph of brain section of AlCl3 administered rat AD group) showing formation of various sizes of amyloid plaques (arrow) in the cerebral cortex and hippocampus (Hand E X 40).

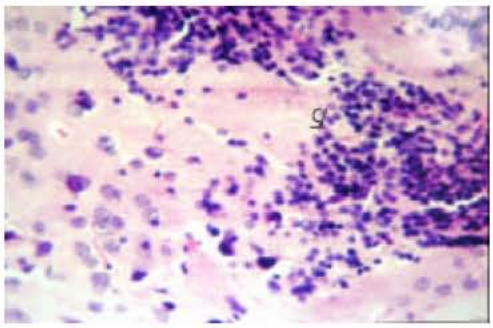

Fig. 4: Micrograph of brain section of AD-induced rat treated with nattokinase showing focal gliosis (g) in the cerebral cortex and hippocampus associated with disappearance of the most amyloid plaques (Hand E X40).

DISCUSSION

Alzheimer's disease (AD) is an age-dependent neurodegenerative disease characterized by progressive

loss of cognitive functions and pathological changes in the brain, such as extracellular aggregates of beta-amyloid and hyperphosphorylation of tau proteins. Cholinergic neurons have been found to degenerate in AD and the lack of cortical acetylcholine correlates with the marked cognitive decline observed in AD [34]. TGF-β levels appear to increase in response to several factors, including high glucose, oxidative stress, advanced glycation endproduct and high lipid levels [35]. TGF-β1 is a multifunctional cytokine with a pivotal role in tissue injury and repair, regulating the growth and survival of neurons [36]. The results of the present research showed a significant increase in the brain TGF-β level in Ovx group versus the normal control group. This effect is in agreement with Choi and Song [37] who stated that ovariectomy-induced estrogen deficiency results in significant increase in the expression level of TGF-β.

Data of the current study also revealed that $AlCl_3$ administration in rats caused significant elevation in the brain TGF-β level as compared to the Ovx control group. This result is in agreement with the observation of Tarkowski *et al.* [38] who showed an increase in TGF-β and TGF-β receptor immunoreactivity in senile plaques, neurites, neuronal neurofibrillary tangles, microglia, astrocytes and macrophages in the brains of AD patients. Those authors mentioned that TGF-β is a pleiotropic cytokine, whose cellular site of synthesis and targets are widely distributed throughout the body, including the central nervous system (CNS) [38]. Also, Motta *et al.* [36] suggested that the deposition of Aβ in the brain tissue seems to induce a remarkable inflammatory cascade, which in turn activates the immune system leading to inflammatory cytokines production. The present data are in consistent with other reports that showed elevations of TGF-β1 expression and immunoreactivity in AD patients in comparison to non-demented elderly controls [39]. The other possible explanation for the increasing TGF-β in the brain of AD group in the present study was suggested by Zetterberg *et al.* [40]. TGF-β1 may increase clearance of Aβ from the brain and cerebrospinal fluid (CSF) by activating microglia cells. Hence, the increased levels of TGF-β in the CNS of AD patients may actually reflect a defense mechanism against further accumulation of Aβ in the brain parenchyma, implying a dual effect of TGF-β1 in amyloid plaque metabolism and AD pathogenesis [40]. On the other hand, treatment of AD-induced group with nattokinase recorded significant decrease in the brain TGF-β level. Herein, the decreased brain TGF-β level was proposed to be as a result of the role of nattokinase in the lysis of Aβ protein [24] which forms plaques in the brain tissue due to aluminum administration, thereby, preventing the inflammatory cascade and production of inflammatory cytokines. Since its discovery over 20 years ago, BDNF has been shown to play a key role in neuronal survival, in promoting neuronal regeneration following injury, regulating transmitter systems and attenuating neural-immune responses [41].

Data in the present study revealed that the Ovx group recorded significant decrease in the brain BDNF level as compared to normal control group. This result is in agreement with Franklin and Perrot-Sinal [42] who suggested that estrogen has a neuroprotective effect *via* the regulation of BDNF. BDNF has been reported to be linked to neurogenesis and the low levels of BDNF in ovariectomized rats, as demonstrated in the study, indicate the decreased basal neurogenesis. The present study recorded significant decrease in the brain BDNF level in AD group in comparison to the Ovx control group. This decrease of BDNF was guessed to be as a result of the injuries of the nerve and glial cells in the brain and this coincides with the reports of Gotohda *et al.* [43]. These injuries may be due to the neurotoxicity and oxidative stress induced by aluminum administration [44]. Furukawa [45] reported that BDNF is not increased in the surroundings of the damaged area of the brain. Also, downregulation of proBDNF was suggested by Furukawa [45] to be an event underlying early Alzheimer's disease. Moreover, Komulainen *et al.* [46] reported that both the intensity of BDNF immunopositive neuronal cell bodies in hippocampus and in temporal cortex appear to be decreased in AD. On the other hand, the brain BDNF level was significantly increased after treatment of AD-induced group with nattokinase. This finding could be attributed to the antioxidant capacity of this proteolytic enzyme [47]. The free radical scavenging ability and the promotion of the antioxidant defense system in the brain induced by nattokinase allowing the damaging area of the brain to be regenerated. This means the restoring of neurogenesis and the correction of brain BDNF level. Vascular endothelial growth factor (VEGF) is a critical angiogenic factor known to be required for the normal development of the vasculature as well as for pathologic angiogenesis. VEGF exerts its effects on the vascular endothelium through binding to 2 high-affinity receptors, R1 (fms-related tryosine kinase [Flt-1]) and R2 (kinase insert domain-containing receptor/fetal liver kinase [KDR/Flk-1]). This binding, in turn, activates the intrinsic tyrosine kinase activity of their cytodomains, initiating intracellular signaling [48].

The present results recorded significant decrease in the brain VEGF level of Ovx group as compared with that in the normal control group. These results agree with the results of Mekraldi *et al.* [49] who suggested that estrogen stimulates endothelial cell proliferation in a paracrine manner and this might help in maintaining vessel number. The significant decrease in VEGF level in Ovx group may be due to a significant decrease in vessel number due to estrogen depletion. Moreover, Yu *et al.* [48] found that the expression of VEGF was extremely lower after ovariectomy. AD group recorded significant decrease in the brain VEGF level compared to the Ovx group. VEGF is a homodimeric glycoprotein which acts as a highly specific mitogen for vascular endothelial cells, being capable of inducing angiogenesis. In addition, it is a potent inducer of vascular permeability and it acts as a survival factor for the newly-formed blood vessels [50]. Aluminum has been found to cause brain damage due to its role in induction of oxidative stress and production of reactive oxygen species (ROS) [13]. So, aluminum might destruct the vasculature of the brain while inducing AD model. This suggestion is in agreement with the post-mortem examination of the brain of AD patients which displayed a cerebrovascular pathology concomitant to AD pathology [38]. Also, the electron microscopy study showed that alterations of the capillaries are a common finding in AD [38]. On the other side, treatment of AD-induced group with nattokinase caused significant elevation in the brain VEGF level. This might be attributed to: (1) the ability of nattokinase to decompose the proteinous plaques [24] accumulated in the brain due to aluminum administration and (2) the free radical scavenging property and the antioxidant capacity of nattokinase [47] which could restore the normal brain vasculature and in turn brain VEGF level.

Microscopic examination of the brain tissue of ovariectomized rats showed that ovariectomy didn't produce any histological changes in the hippocampus and this finding is in agreement with Van Groen and Kadish [51] who demonstrated that estrogen depletion by ovariectomy did not produce amyloid-beta deposition in hippocampus. However, histological examination of cortex area of the brain of ovariectomized rats showed dark neurons with corkscrew dendrites and many of these neurons appeared in a degenerative form. This finding is in agreement with that of Hua *et al.* [52] who observed neuronal loss and synaptic degeneration in the cerebral cortex of ovariectomized rats which were not observed in gonad intact control rats. The microscopic examination of brain tissue of $AlCl_3$ administered rat revealed the formation of various sizes of amyloid plaques in the cerebral cortex and hippocampus. These results are merging with previous studies [53, 54, 55] which provide a direct evidence to support the viewpoint that Al may be a potential contributing factor in the formation of neurofibrillary tangles and cognitive deficits in Alzheimer's disease.

In conclusion, nattokinase represents a novel therapeutic modality for Alzheimer's disease (AD) *via* its capability to digest and target amyloid beta (Aβ) protein beside its powerness in modulating the essential growth factors in the brain. Therefore, nattokinase may be a promising new drug candidate for management of Alzheimer's disease.

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