Glycogen synthase kinase-3β may contribute to neuroprotective effects of Sargassum oligocystum against amyloid-beta in neuronal SH-SY5Y cells

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Please cite this article as:

ABSTRACT
Glycogen synthase kinase (GSK)-3β mediates amyloid-beta (Aβ) and oxidative stress-induced neurotoxicity in neurodegenerative disorders. Natural products with antioxidant activity, such as Sargassum (S.) oligocystum may modulate GSK-3β enzyme and protect against Aβ-induced neurotoxicity. Therefore, we aimed to assess the neuroprotective effects of a methanolic extract of S. oligocystum against Aβ-induced neurotoxicity in the SH-SY5Y cells and the contribution of GSK-3β inhibition to the neuroprotective effects of the S. oligocystum extract. SH-SY5Y neuroblastoma cells were seeded in 96 well plates and incubated with Aβ (20µM) and the methanolic extract of S. oligocystum (40, 50, and 70µg/ml) for 24h. We measured cell viability using the MTT assay. Western blot method was used to measure the expression of the GSK-3β and phosphorylated (p)-GSK-3β protein levels. The data were analyzed using one-way analysis of variance (ANOVA) followed by the LSD test. Amyloid-beta (20µM) reduced neuronal cell viability compared with the control group. Addition of S. oligocystum extract at concentrations of 40, 50 and 70µg/ml decreased the neurotoxic effects of Aβ. The extract of S. oligocystum at a concentration of 70µg/ml also decreased the effects of Aβ on the GSK-3β protein level. The pGSK-3β protein levels in the S. oligocystum groups (40 and 70µg/ml) plus Aβ were lower than the Aβ-treated group. The methanolic extract of S. oligocystum protected SH-SY5Y cells from Aβ-induced neurotoxicity. The attenuation of the GSK-3β protein level may contribute to the neuroprotective effects of S. oligocystum extract.

Conflicts of Interest: Declared None
Funding: Shiraz University of Medical Sciences

INTRODUCTION
Alzheimer’s disease (AD) is a progressive neurological disorder and the leading cause of dementia in the old ages. The deposition of intra-neuronal fibrillary tangles and extraneuronal amyloid-beta (Aβ) aggregates in the brain are the hallmarks of AD [1]. Accordingly, Aβ aggregation activates neuronal signaling systems and induces an inflammatory response, oxidative stress, and apoptosis in the brain regions responsible for cognitive functions [2]. Glycogen synthase kinase-3β (GSK-3β) is an enzyme with essential functions in inflammation, oxidative stress, and neuronal apoptosis. This enzyme substantially contributes to the Aβ-induced neurotoxicity [3]. Embi and his colleagues discovered the GSK-3 protein as an enzyme responsible for glycogen metabolism [4]. Cur-
currently, it has been considered as a regulator of several cellular functions, including cellular metabolism and apoptosis [5]. GSK-3 has two isoforms, including GSK-3α and GSK-3β, though GSK-3β is the enzyme primarily involved in the neurodegenerative disorders [6]. GSK-3β phosphorylates the tau protein and links the Aβ-induced tau phosphorylation and neurodegeneration [7]. This enzyme enhances pro-apoptotic protein expression and inhibits the anti-apoptotic proteins [8]. GSK-3β mediates the Aβ and oxidative stress-induced neurotoxicity in the process of neurodegenerative disorders [9]. 

Considering GSK-3β roles in the AD, many groups tried to find GSK-3β inhibitors as the potential treatment for neurodegenerative disorders [10]. Some studies have reported that GSK-3β inhibitor VIII and lithium chloride suppresses Aβ-induced neuronal death [8, 10]. Moreover, GSK-3β inhibitors II or VIII have reduced oxidative stress neuronal apoptosis in differentiated PC12 cells [9]. Therefore, the inhibitory effects of the antioxidant agents on the GSK-3β may ameliorate Aβ-induced neuronal cell death.

Sargassacea is a family of brown algae with broad distribution in tropical and subtropical seas [11]. Several reports have documented a potent antioxidant activity of Sargassaceae species in the peripheral tissues [12, 13]. The natural habitat of Sargassum oligocystum (S. oligocystum), a member of Sargassaceae family, is the coastal waters of the Persian Gulf [14]. Previous studies have shown that S. oligocystum has antibacterial, anticancer and anticonvulsant activity [14-16]. Importantly, in a study documented in 2017, we showed that S. oligocystum exerted potent antioxidant activity in the mouse brain [15]. Some evidence has shown the beneficial effects of Sargassum family on the neuronal cells. Therefore, in this study, we aimed to explore the role of GSK-3β inhibition on the neuroprotective effects of the S. oligocystum extract.

**Materials and Methods**

**Materials and reagents**

Human SH-SY5Y neuroblastoma cells (Pasteur Institute, Iran) were used in this study. Dulbecco's Modified Eagle's Medium and Ham's Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum (FBS), and Penicillin-Streptomycin from Gibco® life technologies™ (USA) were used for cell culture. Amyloid-β25-35 (Sigma-Aldrich, USA), the anti-actin, anti-GSK-3β, and anti-phosphorylated (p)-GSK-3β antibodies (Cell Signaling Technology®, USA) were prepared.

**Neuronal Cell Culture**

The cells were seeded at a density of 1x10⁵ cells/well in the 96-well plates and maintained in a mixture of (1:1) DMEM/F-12, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The plates were kept in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

**Preparation of methanolic extract of S. Oligocystum**

The S. oligocystum is a native alga of the Persian Gulf. After the alga was collected, it was washed with distilled water and dried at room temperature. Then, the powdered alga was soaked in methanol in a ratio of 1:10 and homogenized. We separated the supernatant using a Whatman filter paper No. 1. After centrifugation for 10 min at 4000 rpm and 4°C, a rotary evaporator removed the solvent. The methanolic extract was maintained in the refrigerator (4°C).

**Amyloid-β25-35 Preparation**

Amyloid-β25-35 was dissolved in sterile distilled water at a concentration of 2µg/µl and maintained at −70°C until use. After the preparation of 20µM solution, Aβ25-35 was incubated for 4 days at 37°C to induce the aggregation process.

**Treatment**

We dissolved the methanolic extract of S. oligocystum in the phosphate-buffered saline (PBS). An appropriate concentration of Aβ and S. oligocystum were determined in a pilot study. On the day of treatment, the culture media were replaced with the serum-free media, and then Aβ23-35 (20µM), the S. oligocystum extract (40, 50 and 70µg/ml), or both of them were added.

**Cell Viability Assay**

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reagent at a concentration of 5mg/ml was added to the cell culture media at 24h after the treatments. The culture media were removed after 4h and the precipitate was dissolved in 100µl of dimethyl sulfoxide (DMSO). The absorbance at a 570 nm was measured using a microplate reader (Synergy HT, Biotek®) to determine the cell viability.

**Total protein determination**

The SH-SY5Y cells were cultured in the 6-well plates at a density of 10⁶ cells/ml to analyze the protein content. Two ml of cell suspension was added to each plate. After incubation with S. oligocystum extract and Aβ, the cells were harvested by centrifuging at 14000 xg for 5 min. The cells lysed using the radio-immunoprecipitation assay lysis (RIPA) buffer containing protease and phosphatase inhibitor cocktail. The cell lysates were centrifuged at 13000 rpm for 25 min at 4°C to remove the insoluble debris. The supernatant was used to determine the total protein level. Total protein was measured by Lowry method.

**Western blot analysis**

Equal amounts of proteins were separated by sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE) and transblotted onto a polyvinylidene fluoride (PVDF) membrane. After transfer, the membrane was blocked with 5% bovine serum albumin (BSA) for 1h at the room temperature. The membrane was incubated overnight with the GSK-3β (27C10) rabbit monoclonal antibody, p-
GSK-3β (Ser9) antibody, and actin antibody. The membrane was washed with PBS and incubated with the Anti-rabbit IgG (HRP- linked antibody, 1:2500) (7074s, cell-signaling) at 37°C for 1h. Then the membrane was incubated in enhanced chemiluminescence (ECL) Western Blotting Substrate (enhanced chemiluminescence kit, GE Healthcare, Amersham) for 1 min at the room temperature. Finally, the membrane was scanned using the ChemiDoc™ XRS+ imaging System. The bands were analyzed by the image-J® software (version 1.8.0, Bethesda, MD, USA).

**Statistical analysis**

We analyzed the variables using the one-way ANOVA followed by the LSD test. P<0.05 was considered statistically significant. All analyses were performed using SPSS software version 23 (SPSS, Inc.).

**RESULTS**

**Neuroprotective effects of S. oligocystum against Aβ toxicity**

The present study showed that the cell viability in various groups was different (F(df)= 15.299(4), P<0.001) (Fig. 1). The pairwise comparison showed that Aβ (20µM) reduced the neuronal cell viability compared with the control group (P<0.001) (Fig. 1). Moreover, the addition of S. oligocystum extract at concentrations of 40µg/ml (P=0.002), 50µg/ml (P=0.001) and 70µg/ml (P<0.001) to the neurons treated with Aβ diminished the neurotoxic effects of Aβ (Fig. 1).

**Glycogen synthase kinase-3β contribution to the neuroprotective effects of S. oligocystum against Aβ toxicity**

The expression of GSK-3β and pGSK-3β were different in the studied groups (F(df)=1961.09 (5), P<0.001, and F(df)= 99.99 (5), P<0.001), respectively) (Fig. 2). The GSK-3β protein level in the Aβ-treated group was lower than the control group (P<0.001) (Fig. 2). The GSK-3β protein level in the S. oligocystum (40 and 70µg/ml) plus Aβ groups was lower compared to the Aβ-treated group (P=0.001) (Figure2). Moreover, the GSK-3β protein levels in the S. oligocystum (40 µg/ml) (P<0.001) and (70 µg/ml) (P<0.001) groups were lower than the control group (Fig. 2).

Treatment of neuronal cells with Aβ deceased the pGSK-3β protein level compared to the control group (P<0.001) (Fig. 2). However, the pGSK-3β protein level in the groups of S. oligocystum (40µg/ml) plus Aβ was higher than the Aβ-treated group (P<0.011) (Fig. 2). In contrast, the pGSK-3β in the S. oligocystum (70µg/ml) plus Aβ group was lower than the Aβ-treated group (P<0.014) (Fig. 2).

Figure 1. Neuronal viability of SH-SY5Y neuroblastoma cells treated with beta amyloid (Aβ) (20µM) and Sargassum oligocystum methanolic extract (40, 50, and 70µg/ml) for 24h. The data indicated 4 independent experiment. The cell viability was measured by the MTT test. Data were analyzed using one-way analysis of variance (ANOVA) followed by the LSD test. Data were expressed as mean ± standard error of mean. P<0.05 was considered statistically significant. † † † †: P<0.001 compared to the control group, * and ***: P<0.05 and P<0.001 compared to the Aβ-treated group, respectively. Aβ: beta amyloid, Sar40: sargassum oligocystum methanolic extract 40µg/ml, Sar50: sargassum oligocystum methanolic extract 50µg/ml, Sar70: sargassum oligocystum methanolic extract 70µg/ml.
DISCUSSION

This study showed that the S. oligocystum extract prevented the Aβ-induced neurotoxicity in the SH-SY5Y cell lines. Some studies have shown the neuro- and glioprotective effects of Sargassum family or their derivatives. Yang et al. [17] showed that 3 extracts of S. crassifolium possessed a potent antioxidant effect and neuroprotective activity in PC-12 cells. Moreover, the methanolic extract of S. muticum produced the neuroprotective effects against 6-hydroxydopamine (6-OHDA) neurotoxicity in the SH-SY5Y cells [18]. An in vivo study in a scopalamine-induced amnesia model showed that a Sargassum species protected cerebral cortex neurons of mice and enhanced the animal’s memory [19]. Furthermore, the crude extract of S. fusiforme enhanced memory, but it decreased the Aβ load in an animal model [20]. Except for the crude extract of Sargassum family, some studies have reported the neuroprotective and neuromodulatory effects of Sargassum derivatives. Jin and his colleagues [21] showed that the heteropolysaccharides from Sargassum species attenuated 6-OHDA-induced neurotoxicity in MES 23.5 cells. Moreover, sargachromenol, a compound extracted from S. macrocarpum, exerted the neuroprotective effects and promoted neurogenesis in PC-12 cells [22]. Fucosterol, another brown alga component, decreased the intracellular level of Aβ and protected SH-SY5Y cells against Aβ [20]. Pheophytin-a produced a nerve growth factor (NGF)-like activity in neurons [23]. Sargaquinic acid, another substance extracted from S. macrocarpum, promoted neurite outgrowth by the mediation of TrkA-dependent MAP kinases pathway [24]. Thus, the Sargassum species especially S. oligocystum are potential targets to produce new neuroprotective agents in the treatment of AD.

Exact mechanism of action of S. oligocystum is not completely clear. However, some studies have shown that the antioxidant activity and the manipulation of signaling systems involved in neuronal apoptosis may be relevant [21, 25]. We previously showed that a S. oligocystum extract produced an antioxidant activity in the mouse brain [15]. Furthermore, the methanolic extract of S. muticum protected SH-SY5Y cells by decreasing the peroxide free radicals and protecting mitochondria by reducing Caspase-3 activity [18]. In addition, Huang et al [26] have shown that fucooidan extracts of S. hemiphyllum exerted antioxidant and neuroprotective activity against 6-OHDA-induced apoptosis. Moreover, Sargassum species protected the neurons via reducing hydrogen peroxide production, repairing the impaired mitochondrial membrane’s potential and the amelioration of...
Caspase-3 activity [18]. Thus, the neuroprotective effects of *S. oligocystum* might be related to the antioxidant activity of this alga.

Ongoing rigorous investigations try to elucidate the pathophysiology of AD. However, the Aβ aggregation may start a process that changes the central nervous system (CNS) homeostasis and causes neuronal death in the brain regions responsible for memory [8]. Glycogen synthase kinase-3β may be involved in the apoptotic mechanism of Aβ [3]. Thus, GSK-3β regulation is an attractive target for the treatment of neurodegenerative disorders [9]. The GSK-3β pathway is among intracellular pathways that have close convergence with oxidative-stress-induced apoptotic death in neurons [9]. Furthermore, some GSK-3β inhibitors suppressed oxidative-stress-induced neurotoxicity by the modulation of GSK-3β activity [9]. The *S. oligocystum* extract produced strong antioxidant effects in the CNS [15]. Therefore, the regulation of GSK-3β activity may contribute to the neuroprotective effects of *S. oligocystum*. In the present study, the methanolic extract of *S. oligocystum* protected the neuronal cells by decreasing of GSK-3β. It is possible to suggest that the attenuation of oxidative stress by *S. oligocystum* may suppress GSK-3β activity in the SH-SY5Y cells. Another possibility may be the notion that *S. oligocystum* derivatives have a direct GSK-3β inhibiting activity. Therefore, future studies by extracting specific fractions or compounds from *S. oligocystum* may help to produce a new GSK-3β inhibitor.

Our study also showed that Aβ reduced the phosphorylated (p)-GSK-3β level in the SH-SY5Y cells. Similarly, Crouch et al. [27] showed that Aβ decreases the p-GSK-3β level in the SH-SY5Y cells. The interaction of Aβ with insulin, Wnt signaling or N-Methyl-D-Aspartate (NMDA) receptors elevated the GSK-3β activity in the brain of patients with AD and mouse model of AD [28]. In the CNS, Akt is a downstream cascade of these signaling systems that is responsible for the GSK-3β phosphorylation and inactivation [29]. Amyloid-β oligomers exert an antagonizing activity on insulin receptors in neurons and prevent the activation of PI3 kinase/Akt [30]. Moreover, Aβ effects on Wnt signaling prevents the inactivation of GSK-3β[31]. The hydroalcoholic extract of *S. oligocystum* exerted beneficial effects on diabetics mellitus in an animal model [32]. The finding of our study showed that methanolic extract of *S. oligocystum* increased the p-GSK-3β protein level after treatment with Aβ.

The main limitation of this study may be the use of the crude extract of *S. oligocystum*. The extraction of the specific fractions of Sargassum may result in more favorable results. Moreover, the present study was conducted in the cell line and future studies in the animal models of AD seems necessary.

**CONCLUSION**

The methanolic extract of *S. oligocystum* protected SH-SY5Y cells from Aβ-induced neurotoxicity. The suppression of oxidative stress and the attenuation of GSK-3β protein level were the possible mechanisms of action of *S. oligocystum* extract. Future studies on specific derivatives of this alga may help to find new treatments for AD.

**ACKNOWLEDGMENT**

The authors express their gratitude to the Vice-chancellor for research affairs of Shiraz University of Medical Sciences for financial support of the study. We also gratefully thank Dr. Nabipour for providing *S. oligocystum* extract.

**CONFLICTS OF INTEREST**

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

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