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Original Article

SERICIN AS A CHOLINERGIC MODULATOR IN ALZEIMER'S DISEASE INDUCED RAT

KUTAGOLLA PEERA*, KUNA YELLAMMA

DBT-BIF Centre & Division of Neurobiology, Department of Zoology, Sri Venkateswara University, Tirupati 517502, India
Email: drkutagollapeera2014@gmail.com

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ABSTRACT

Objective: Alzheimer's disease (AD), characterized by formation of Amyloid plaques, neurofibrillary tangles and loss of neurons in the cerebral cortex and certain sub-cortical regions eventually results in gross atrophy of the affected regions, including degeneration in cholinergic regions. According to Cholinergic hypothesis, AD is caused by reduced synthesis of the neurotransmitter ACh, wherein the AChE levels were increased which causes damage to the cholinergic neurons finally leading to cognitive impairments. Today most of the drugs available in the market are Cholinesterase inhibitors to treat AD, these drugs temporarily de accelerate the progressive cognitive decline in some AD cases but not all other forms of dementia. Research on Alzheimer's proven the importance of the Antioxidants and AChEI to treat AD. To cure AD effectively it is necessary to identify a natural product with Antioxidant and AChEI activity. The Silk Protein, Sericin a natural protein has wide applications in the Pharma industry.

Methods: The present study was aimed to evaluate potential "AChE inhibitor" activity of Sericin in AD-induced rat model by conducting experiments mainly on the Cholinergic system and also on the Morphometric and Cognitive aspects in control and experimental rats.\

Results: The results of the present study demonstrated that Sericin could effectively counteract the AChE activity in AD-induced rat and retains the ACh levels in the brain cholinergic regions. In the end of the experiment AD-induced rat showed recovery tendency in Morphometric and Cognitive aspects are the passive evidence for decline AD-induced AChE levels.

Conclusion: From this study, it may suggest that Sericin act as a potential Cholinesterase inhibitor in AD.

Keywords: Silk protein, Sericin, Alzheimer's Disease, Morphometric aspects, Cognitive aspects, Cholinergic system.

INTRODUCTION

Alzheimer's disease (AD), an incurable, neurodegenerative and terminal disease was first discovered by the German psychiatrist and neuro pathologist, Alois Alzheimer in 1906 and was named after him [1]. The incidence of AD rises from 2.8% per every 1000 persons in the age group of 65-69 to 56.1 % per every 1000 persons in people older than 90 years. Approximately 450 million people are suffering from them worldwide with a mortality rate of 14.7%. They are usually more common in older people aged above 60 years.

According to the Cholinergic hypothesis, the oldest one on which most currently available drug therapies are based highlights that Alzheimer's disease, is caused by reduced synthesis of the neurotransmitter, Acetylcholine. The cholinergic hypothesis has not maintained widespread support, largely because medications intended to treat Acetylcholine deficiency have not been very effective. Other cholinergic effects have also been proposed, for example, large scale aggregation of amyloid [2] leading to generalized neuroinflamation [3]. Therefore the treatment strategies have been focused on elevating the Acetylcholine by cholinesterase inhibitors such as Tacrine, Donepezil, Rivastigmine and Galantamine but they have cholinergic side effects such as nausea, anorexia, vomiting, and diarrhoea [4, 5]. AD therapy is largely based on compounds to increase ACh concentration, including AChE inhibition [6, 7]. Therefore, it is worthwhile to explore the alternative source for a suitable medicine for treatment of Alzheimer's disease.

Now-a-days the majority of clinical drugs available in the market are synthetic in origin and they have side effects [4, 5]. From 19th century onwards, extensive research work is going on to identify the medicinally useful compounds from natural Plant and Biological sources. It is interesting to note that 6 out of the top 20 pharmaceutical prescription drugs dispensed in 1996 were natural products and that over, 50% of the top 20 drugs could be linked to natural product research [8]. It is estimated that 60% of tumor and anti-infectious drugs already in the market or under clinical trials are of natural origin [9]. In addition to above mentioned facts, the extensive practice of traditional medicine in developing countries and the rapidly growing demand for alternative and basic therapeutic means in industrialized countries constitute the international relevancy of research and development in the field of traditional drugs.

While manufacturing Silk, Sericin is removed as waste during the degumming process. It is estimated that out of about 1 million tons (fresh weight) of cocoons produced worldwide approximately, 4, 00, 000 tons of dry cocoon are generated, that have 50, 000 tons of recoverable Sericin. Indian production of 1, 600 tons of silk can be the source of about 250 to 300 tons of Sericin per year [10]. If this sericin protein is recovered, recycled and utilized to developed value-added products from the wastes, it would be a significant economic and social benefit [11]. The Silk cocoon produced from the silkworm, Bombyx mori consists of two major proteins, Fibroin and Sericin. Sericin contributes about 20-30% of total cocoon weight and remaining fibroin. There are 18 kinds of amino acids present in Sericin, among these 8 amino acids are essential for human, which plays a key role in different metabolic pathways. In Pharma industry, it has wide applications viz., Anti-bacterial, Antioxidant, Wound healing, Cell proliferation, Anti-tumor activity, UV protection, prevent colon cancers, medical composites of Sericin, additives to rice cooking, fabric care compositions, light and sunscreen compositions, foam-forming aerosol shaving gels, Sericin-coated powders for cosmetics, as dermatitis inhibitor, nail cosmetics, and chewing gums etc., [10, 11]. Sericin is a complete protein and the amino acids compositions are with appropriate proportions in line with FAO/WHO standards. Nutritional values of Sericin are 2 times higher than the Pork and 3 times higher than the Meat. Due to all these properties sericin acts as a value added product in Pharma, food and Cosmetic industries.

In this connection, in our study, Silk Protein, Sericin was taken as a new kind of valuable natural protein source to evaluate its neuroprotective effect on the cholinergic system and also on the Morphometric and cognitive aspects in AD-induced rat model.

MATERIALS AND METHODS

Procurement and maintenance of experimental animals

Healthy Wistar strain Albino rats, *Rattus norvegicus* of the same age group of 3 months, weighing 160±20 grams, obtained from Sri

Venkateswara enterprises, Bangalore were used as the experimental model. Prior to experimentation, the rats were acclimatized according to the instructions given by [12]. They were housed in polypropylene cages under the controlled conditions of 28±2°C temperature with photoperiod of 12 hours light and 12 hours dark and 75% relative humidity maintained in the animal house of the Department Zoology, according to the ethical guidelines for animal protection and welfare bearing the Resolution No. 04/(i)/ a/CPCSEA/IAEC/SVU/ KY-KPR / Dt. 28-03-2011. The rats were fed with standard pellet diet supplied by Sri Venkateswara Enterprises, Bangalore and water *ad libitum* throughout the period of experimentation.

Induction of AD

Rat aging models induced by Aluminium trichloride and D-Galactose have been widely used for studying mechanism of Alzheimer's disease and for screening the drugs for many years, they eventually cause similar pathological changes in mouse brains and lead to enhance Amyloid-β peptide (Aβ) deposition [13-15] which are the key pathological features of AD [16-18]. Previous studies showed that D-Gal administration significantly accelerates inflammatory brain injury through activation and degeneration of astrocytes and causes cognitive deficits by activating AChE [19, 20]. D-Galactose is a physiological nutrient, but over supply of D-Galactose will result in an abnormality of metabolism. In addition, the non-enzymatic glycation is another pathway that can enhance oxidative lesions in ageing and age-associated disease such as Alzheimer's Disease [21]**.** Thus, long-term intra peritoneal injection of D-Galactose induces AD characteristics symptoms in normal rat [22, 23] and has been gradually accepted to establish an aging model for brain aging or anti-aging pharmacological research to induce AD [24, 25]. Accordingly, in the present study, D-Gal was used as a model compound for facilitated AD animal model.

Collection of silk cocoons and extraction of Sericin

Extraction of sericin

Raw Silk cocoons, purchased from the local market of Chittoor were boiled in water for 1hour and the resulting solution was cooled and filtered. After repeating this process for 3 times, the filtrate was concentrated by using Hahnvapor Rotary Evaporator (HS-2005V). 95% ethanol was added to the extract to precipitate Sericin, collected by filtration, dried at 40 ^{o}C, powdered and finally preserved in the clean container for further use.8% SDS PAGE was done to confirm the presence of Sericin, based on the molecular weight.

Administration of sericin

Silk Protein, Sericin **(SP-S)** extract (200 mg/kg body weight) was dissolved in distilled water and given to the rat [26]. A gavage tube was used to deliver the substance by oral route, which is clinically expected route for administration. The volume of Sericin administered was kept at 1 ml to the animal.

Grouping of animals

After the rats were acclimated to laboratory conditions for 10 days before the experimentation, they were randomly divided into four groups. Each main group was again divided in to 2 sub groups of six each and was housed in separate cages. These different groups of rats except control were treated with selected doses of Sericin and D-Gal as given below. Keeping in view the altered activity of rats during the nights compared to day time, all doses were given once in the morning hours in between 8 A. M. to 9 A. M.

Group-I	Control Rat
Group-II (SP-S)	Rat, orally administered with Sericin (200 mg/kg body weight) up to 60 days (31st day to 90th day) continuously once in a day [26].
Group-III (AD)	Rat, Intraperitoneally (IP) administered with D-Gal (120 mg/kg body weight) up to end of the experiment (1st day to 90th day) [27, 28].
Group-IV (AD+SP-S)	Rat, Intraperitoneally injected with D-Gal (120 mg/kg body weight) once daily for first 30 days. From 31stday onwards rats were administered with Sericin (orally; 200 mg/kg body weight) along with D-Gal (IP) up to 90th day.

In the present study, duration of the experimental selected was 90 days. D-Gal was given for first 30 days period to observe AD symptoms with the assessment of cognitive skills in rats (AD group). Further AD induced rats were again treated with D-Gal as well as Sericin simultaneously. To observe the cognitive skills, the rats were subjected to behavioral studies on selected days ie. on 15th, 30th, 45th, 60th, 75th and 90th day of the experimentation.

Table 1: Differences in the total body weights (Grams) of control and different groups of Experimental rats on selected days of experimentation

Group	Days					
	15th day	**30th day**	**45th day**	**60th day**	**75th day**	**90th day**
Control	138.00±4.25	171.83±6.74	193.83±3.19	207.33±4.40	219.33±4.46	243.83
SP-S Treated	153.50±2.70 (-3.58)	184.00±3.43 (-7.08)	207.83±2.94 (-9.32)	226.66±5.21 (-10.86)	260.33±6.20 (-16.67)	284.50 (-18.69)
AD-induced	129.50±1.83 (6.15)	145.83±3.51 (15.13)	148.16±4.94 (25.85)	138.83±2.24 (33.03)	125.83±1.79 (42.62)	112.16 (54.00)
AD+SP-S Treated	148.16±4.29 (-7.36) (-14.72)*	157.50±1.78 (8.33) (-8.00)*	181.83±5.51 (9.423) (-22.72)*	199.16±2.12 (3.94) (-44.31)*	205.83±5.48 (6.15) (-63.57)*	228.00 (6.49) (-103.28)*

Values are Mean±SEM of six observations each from tissues pooled from 6 rats, Values in parentheses are percent changes from control (Except *), *Values in parentheses are percent changes from AD-induced rats, Values are significantly different from control at $p<0.01$

Morphometric aspects

The total body weights of control and experimental rats were recorded at selected time intervals by using a digital balance.

Cognitive aspects

Experiments related to evaluation of the cognitive functions were performed by using the water maze setup [29]. A great deal of knowledge has been obtained on the Neurochemical, Neuroanatomical and Neurophysiological basis for the behavior associated with this paradigm. The time spent by the animal to reach the hidden platform in the tank was called as the Escape Latency and used as the index of memory.

Cholinergic system

ACh content and AChE levels were determined for the all the above mentioned four groups of rats were sacrificed on selected days ie., on 60th day and 90th day by cervical dislocation and the brain was isolated

immediately and placed on a chilled glass plate. Cerebral Cortex and Hippocampus were separated by following standard anatomical marks [30], frozen in liquid nitrogen and stored at-80°C until further use. At the time of biochemical analysis, the tissues were thawed and used. Acetylcholine (ACh) was estimated by the method of Metcalf (1957) as given by Augustinsson (1957) and Acetylcholinesterase (AChE) by the method of Ellman *et al.,* (1961) [31-33].

The results obtained were analyzed statistically and the values of the measured in different parameters were expressed as Mean±SEM. One way ANOVA was used to test the significance of difference among the four different groups with Dunnett's post-hoc test for multiple comparisons using standard statistical software, SPSS (Version-16).

RESULTS

Morphometric aspects

Analysis of the results revealed that the control rats have registered a gradual gain in their body weights from 15th day to 90th day. In case of experimental rats treated with Sericin, the gain was more (16.67%) than the controls. Contrary to this, AD-induced rats lost their body weights (54.00%) and become very weak when compared to control while the AD-induced rats simultaneously treated with Sericin restored their body weights almost to control levels.

Cognitive aspects

The control and experimental rats were subjected to Morris water maze task to measure cognitive skills viz., the spatial learning and memory ability. The results revealed that, Sericin treated rats have showed equal escape latency as that of the control one. In contrast to this, AD-induced rats showed a significant increase in the escape latency time from 15th day to 90th day with maximum latency on 90th day (872.00%). However, AD-induced rats treated with Sericin showed significant recovery tendency from 30th day (3.57%) to 90th day (76.13%) when compared to AD-induced rats thus demonstrating the positive effects of Sericin on cognitive skills in AD induced rats.

Table 2: Differences in escape latency (Seconds) of control and different groups of experimental rats on selected days of experimentation

Group	Days					
	15th day	**30th day**	**45th day**	**60th day**	**75th day**	**90th day**
Control	15.16±0.31	13.66±0.67	13.33±0.33	13.00±0.57	14.00±0.57	12.50
SP-S Treated	14.16±0.60	14.00±0.58	13.50±0.56	12.50±0.67	11.50±0.22	11.00
	(6.59)	(-2.48)	(-1.27)	(3.84)	(17.85)	(12.00)
AD-induced	15.83±0.60	37.50±0.43	61.16±0.87	74.66±0.88	109.00±2.28	121.50
	(-4.41)	(-174.52)	(-358.81)	(-474.30)	(-678.57)	(-872.00)
AD+SP-S Treated	14.66±0.33	36.16±0.65	55.16±0.60	63.83±1.53	42.00±0.36	29.00
	(3.29)	(-164.71)	(-313.80)	(-391.00)	(-200.00)	(-132.00)
	(7.39) *	(3.57)*	(9.81)*	(10.83)*	(61.46)*	(76.13)*

Cholinergic system

Acetylcholine content (ACh) & Acetylcholinesterase (AChE)

The results of the present study on the Cholinergic system revealed that ACh content was significantly elevated in both selected brain regions of rat brain treated with Sericin alone when compared with AD-induced and Control groups. Maximum increase (26.89%) in ACh content was found in HC region on 90th day. However, in case of AD-induced rats, simultaneously treated with Sericin, the ACh content almost reached the normal control levels. Contrary to Ach content, the AChE activity levels were significantly inhibited in CC and HC brain regions of Sericin alone treated rats. Whereas the AChE activity levels were increased significantly in selected AD-induced rat brain regions with a maximum elevation in CC region on 90th day (73.91%). AD-induced rats treated with Sericin showed significant inhibition in the AChE activity levels (maximum inhibition was in HC (11.89%) on 90th day) and reached to the control levels.

Table 3: Differences in ACh content of selected regions of control and experimental groups of rats on selected days of experimentation

Name of the group	60th day		90th day	
	CC	**HC**	**CC**	**HC**
Control	32.08±0.24	32.77±0.34	33.87±0.21	34.09±0.29
SP-S Treated	36.05±0.42	37.68±0.58	41.74±0.37	43.26±0.53
	(-12.37)	(-14.98)	(-23.23)	(-26.89)
AD-induced	26.09±0.24	24.56±0.68	22.82±0.22	20.43±0.41
	(18.67)	(25.05)	(32.62)	(40.07)
AD+SP-S Treated	28.29±0.34	29.81±0.31	30.32±0.37	31.00±0.27
	(11.81)	(9.03)	(10.48)	(9.06)
	(-8.43)*	(-10.68)*	(-32.86)*	(-51.73)*

Table 4: Differences in AChE activity (µ moles of ACh hydrolyzed/mg protein/h) of selected regions of control and experimental groups of rats on selected days of experimentation

Groups	60th day		90th day	
	CC	**HC**	**CC**	**HC**
Control	6.56±0.14	7.35±0.22	6.71±0.16	7.82±0.15
SP-S Treated	5.39±0.13	5.61±0.15	5.01±0.17	5.29±0.10
	(17.83)	(23.67)	(25.33)	(32.35)
AD-induced	10.25±0.19	10.46±0.29	11.67±0.16	11.91±0.14
	(-56.25)	(-42.31)	(-73.91)	(-52.30)
AD+SP-S Treated	9.07±0.21	9.42±0.15	8.40±0.22	8.75±0.13
	(-38.26)	(-28.16)	(-25.18)	(-11.89)
	(11.51)*	(9.94)*	(28.02)*	(26.53)*

DISCUSSION

The results of the present study clearly indicated oral administration of Sericin significantly reversed the weight loss and memory impairments noticed in AD-induced rats, indicating that Sericin has the potential nature to enhance the cognitive skills. In rodents, spatial learning and memory are closely related to the function of the dorsal hippocampus [34] to which cholinergic neurotransmission contributes significantly [35]. Although especially prominent in AD, cholinergic deficits in the cortex and hippocampus occur during normal human ageing [36] and atrophy of surviving cholinergic neurons in the basal forebrain were shown in aged animals with impaired learning and memory [37].

Our present findings derive strong support from similar observations of Dongsun *et al.,* (2011) [38] who reported that the rats showed high levels of cognitive impairment when treated with D-Gal in passive avoidance and Morris water maze tests while silk peptides could increase the retention and retrieval of learned tasks in rats. Not only on cognitive functions, but also in diabetes-induced rat model, consumption of silkworms and Silk Proteins demonstrated their protective effects against tissue injury [39, 40]. Similarly, Silk Amino Acids (SAA) exerted neuroprotective effects on 6-hydroxydopamine-induced dopaminergic neurotoxicity and thereby improved movement functions of PD-induced animals [41].

It was obvious that Sericin exerted Anti-Cholinesterase properties in AD-induced rats by elevating the levels of ACh and inhibiting the AChE activity in selected regions of brain. AChE is an important regulatory enzyme that controls the transmission of nerve impulses across cholinergic synapses by hydrolyzing the excitatory transmitter, ACh [42]. It was assumed that cholinergic deficit in AD was responsible for much of the short-term memory deficit since Acetylcholine (ACh) is required for short-term memory. Markers for cholinergic neurons such as Choline Acetyltransferase and Acetylcholinesterase, which are enzymes responsible for synthesis and degradation of ACh, respectively are decreased in the cortex and hippocampus areas of the brain involved in cognition and memory [43]. The earliest loss of neurons in AD patients occurs in the nucleus basalis and the entorhinal cortex, where cholinergic neurons are preferentially affected. Perry *et al.,* (1991) postulates that many of the cognitive, functional and behavioral symptoms experienced by patients with AD results from a deficiency in neurotransmitter, Ach [44].

Chronic IP injection of D-Gal to the rat induced a significant decrease of ACh content and increase of AChE activity in CC and HC regions of brain, indicating that there was oxidative damage in the brain. On the other, Sericin has significantly elevated the ACh levels and lowered the levels of AChE activity in AD-induced animals indicating the counteracting action of Sericin on cholinergic system. In support of the present study, Dongsun Park *et al.,* (2011) reported that silk peptides (SP) acts as cognitive enhancers in aging rat model and also stated that the D-Gal (150mg/Kg) activated hippocampal astrocytes upto 1.7 folds (a marker of Brain injury and aging) and decreased AChE concentration upto 45-50%. Oral treatment with SP preparations showed recovery in the concentration levels of ACh. In the present study, sericin treatment might be an evidence that the Silk peptide can improve damaged brain function, especially by suppressing the AChE activity levels and thus preventing the brain damage by inhibiting neuronal apoptosis or the activity of choline acetyltransferase and muscarinic receptor binding of Acetylcholine might have been improved [45, 46].

Observations on the morphometric and cognitive aspects were well correlated with those of the cholinergic changes in forebrain throughout the duration of the experiment reiterating the fact that loss of cholinergic function in these areas is associated with a decline in learning capacity and memory. The resultant decrease in ACh-dependent neurotransmission is thought to lead to the functional deficits of Alzheimer's disease. Interest on the function of the basal forebrain cholinergic system greatly increased with the neuropathological demonstration that cholinergic markers in the hippocampus and cerebral cortex are changed in AD [47, 48] and that these changes have been linked to its pathology and degree of cognitive impairment [47]. Therefore, much of the research on cognitive decline has been focused on the cholinergic system [49].

CONCLUSION

In general, the present investigation on the Silk Protein, Sericin in AD-induced rat has shown its neuroprotective effect on cholinergic system based on which sericin may be suggested as a cognitive enhancer with reference to Alzheimer's Disease

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CONFLICT OF INTERESTS

Declared None

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