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Original Article HISTOLOGY & EMBRYOLOGY

Urtica dioica can regulate autophagy pathway in the rat hippocampal tissue after STZ-induced neurodegeneration

Sule Ayla,[1] Halil Ibrahim Saygi,[1] Merve Sahin,[1] Ebru Ciftkaya,[2] Aysu Kilic,[3] Sadrettin Pence,[4] Fatemeh Bahadori,[5] Elif Gelenli Dolanbay,[1] Birsen Elibol[6]

[1]Department of Histology and Embryology, Istanbul Medeniyet University Faculty of Medicine, Istanbul, Turkiye
[2]Department of Internal Medicine, Marmara University, Pendik Training and Research Hospital, Istanbul, Turkiye
[3]Department of Physiology, Bezmialem Vakif University Faculty of Medicine, Istanbul, Turkiye
[4]Department of Physiology, Istanbul Medeniyet University Faculty of Medicine, Istanbul, Turkiye
[5]Department of Basic Pharmaceutical Sciences, Istanbul University-Cerrahpasa, Faculty of Pharmacy, Istanbul, Turkiye
[6]Department of Medical Biology, Istanbul Medeniyet University Faculty of Medicine, Istanbul, Turkiye

ABSTRACT

OBJECTIVE: Autophagy plays a crucial role in neuroprotection by helping to clear toxic substances, like misfolded proteins. In neurodegeneration, autophagy is impaired leading to the accumulation of harmful proteins that disrupt neuronal function, promote inflammation, and contribute to the degeneration of brain cells. Therefore, because of its anti-inflammatory and anti-oxidative actions, the effects of Urtica dioica (UD) on the proteins of autophagy signaling pathways was studied in the hippocampus of rats with streptozotocin-(STZ) induced neurodegeneration.

METHODS: Neurodegeneration model of rats was induced by intracerebroventricular injection of STZ (3 mg/kg) to observe both cognitive deficits and autophagic dysfunction. Then, the rats in the treatment group were consumed UD at the dose of 50 mg/kg/day for 4 weeks. At the end of 4 weeks, passive avoidance test was applied for cognitive functions and hippocampal tissue of rats were investigated to determine the changes in the proteins related to autophagy by western blotting and immunofluoresecence.

RESULTS: UD treatment slightly attenuated the STZ-induced memory deficiencies in the rats. In addition, an increase in the autophagy was noted by increasing the expression of Beclin, ATG5, and LC3β proteins in the STZ-UD group compared to the STZ group.

CONCLUSION: In summary, UD may be a candidate molecule as a therapeutic strategy to protect neurons in neurodegeneration through increasing autophagy to reduce toxic protein accumulation.

Keywords: Alzheimer's disease; autophagy; rat; streptozotocin; urtica dioica.

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Alzheimer's Disease (AD) represents a gradual and persistent neurodegenerative illness that leads to serious cognitive decline, memory disturbances, and noticeable behavioral changes [1]. Being a multifactorial disease, AD is mainly affected by genetic risk factors, aging, and oxidative stress [2]. The disease is

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Correspondence: Birsen ELIBOL, PhD. Istanbul Medeniyet Universitesi Tip Fakultesi, Tibbi Biyoloji Anabilim Dali, Istanbul, Turkiye.
Tel: +90 533 231 07 23 e-mail: birsen.elibol@medeniyet.edu.tr

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thought to begin with neuronal degeneration in layer II of the entorhinal cortex, progressively extending to the hippocampus, temporal and frontoparietal cortices, and ultimately reaching the subcortical nuclei [3]. It is thought that oxidative stress has been strongly linked to cognitive decline and the progression of AD. Increased oxidative stress contributes to the accumulation of senile plaques and synaptic loss, ultimately leading to neurodegeneration [2].

Autophagy is a tightly regulated process that adapts to oxidative stress by breaking down cytoplasmic macromolecules and damaged organelles within lysosomes, thereby promoting cellular growth, development, and homeostasis. In addition to oxidative stress, autophagy can be triggered by cellular stress factors such as nutrient deprivation, energy shortages, hypoxia, toxins, radiation, DNA damage, and intracellular pathogens. This makes it a promising research target for preventing the progression of neurodegenerative diseases like AD [4]. Previous studies have shown that neuronal cell autophagy is markedly reduced in the onset of AD, and that sustaining autophagy in neuronal cells may help mitigate the progression of AD [5, 6]. Moreover, impaired autophagic flux has been associated with the accumulation of amyloid-beta (Aβ) peptides and hyperphosphorylated tau, which are hallmark pathological features of AD [7, 8]. Dysfunctional autophagy leads to the formation of autophagic vesicles that fail to mature and fuse with lysosomes, resulting in intracellular build-up of toxic protein aggregates [9]. Several key regulators of autophagy, including Beclin-1 and the mTOR signaling pathway, have been found to be dysregulated in AD brains, further implicating autophagic failure in the disease pathology [5, 10]. As such, enhancing autophagy through pharmacological or genetic approaches is being actively investigated as a potential therapeutic strategy in AD.

Urtica dioica (UD) is a plant which could have anti-inflammatory and anti-oxidative properties because it is rich in minerals and vitamins, such as pro-vitamin A and vitamin C. UD has been extensively studied as an antioxidant in protecting against hyperglycemia and diabetes [11]. Additionally, previous studies have demonstrated that this herbal extract treatment significantly improves learning and memory in an aged mouse model [2]. Furthermore, it has been shown that UD has potential therapeutic impacts against neuroinflammatory conditions due to its antioxidative effect [12]. Some of the constituents of UD, such as carvacrol, scopoletin, and rosiglitazone, have shown a neuroprotective effect against neurological impairments [13]. In one of the previous studies, it was shown that UD has an ameliorative effect on the midbrain dopaminergic neurons of animals with Parkinson's disease by restoring the autophagic machinery as a one of the therapeutic strategies [14]. Therefore, this study aimed to examine the effects of UD on the autophagic process in the hippocampus of rats with neurodegeneration, considering its potential therapeutic role in treating neurodegenerative diseases. In the present study, we employed an AD-like model induced by intracerebroventricular (i.c.v) injection of streptozotocin (STZ). This model closely mimics several pathological features of sporadic AD, including cognitive impairment, insulin resistance, oxidative stress, and neurodegeneration. Importantly, STZ-induced neurodegeneration models have also been shown to exhibit impaired autophagic flux, characterized by the accumulation of autophagic vesicles and altered expression of key autophagy-related proteins in the hippocampus [15, 16]. These features make it a suitable model for investigating autophagy-related mechanisms in the context of AD progression.

Highlight key points

- *Urtica dioica* slightly attenuates memory deficiencies.
- Neurodegeneration decreases autophagy in the hippocampus of rats.
- Urtica dioica increases the levels of Beclin, ATG5, and LC3β proteins to activate autophagy in neurodegeneration model.

MATERIALS AND METHODS

Experimental Subjects

Long-Evans Hooded rats (female, 6-month-old, weighing 350–400 g) taken from Bezmialem Vakif University Animal Research Center were used in the present study. Experimental procedures were followed in line with NIH guidelines (NIH publication No. 85-23, revised 1996) and ethical principles of the Declaration of Helsinki. Rats were free to food and water with housing in 12-hour light/dark cycles at 22°C temperature and 60% humidity. All procedures were authorized by the Animal Research Ethics Committee at Bezmialem Vakıf University (2019/266) and conducted in accordance with ARRIVE guidelines to minimize animal suffering.

Neurodegeneration Model by Streptozotocin (STZ) Injection and *Urtica Dioica* (UD) Treatment

At the start of the study, in a random manner, rats were divided to four experimental groups: the control group (without any experimental procedures, n=5), the Sham control group (injection of artificial cerebrospinal fluid (aCSF) via i.c.v., n=6), the STZ group (i.c.v. STZ injection, n=6), and the STZ+UD group (STZ injection via i.c.v. followed by UD treatment, n=5). The numbers of animals were determined according to a priori sample size calculation. To induce neurodegeneration, i.c.v. injections of STZ were administered following a protocol adapted from previous studies [17–19]. STZ (Sigma, St. Louis, MO) was dissolved in aCSF and injected bilaterally into the rats (coordinates: 0.8 mm anteroposterior, 1.5 mm mediolateral, 3.5 mm dorsoventral) for 48 hours apart at a total dose of 3 mg/kg per animal. For the sham control group, aCSF was injected (20 µl) similar to the STZ group.

After the second STZ injection, the rats stayed in their cages for 6-month to develop most of the AD-like phenotype as suggested in a previous study [20]. Subsequently, the STZ+UD group received UD treatment (50 mg/kg/day) via intragastric intubation for 4 weeks [21, 22]. The dose of UD used in our study (human equivalent dose was 10 mg/kg) falls within a range that could reasonably be expected to be achievable in human populations. This dose was calculated based on the literature [23] and the typical consumption levels of commercially available as herbal supplement capsules (500 mg/capsule) for humans [22].

UD was collected from a local Istanbul-Turkiye market and identified by Assoc. Prof. Dr. Cagla Kizilaslan Hancer, from the Department of Pharmaceutical Botany- Faculty of Pharmacy- Bezmialem Vakif University. The plants were cleaned and stripped of leaves in the laboratory to obtain healthy, uniformly colored, and sized leaves, with their stems and petioles removed. The nettle leaves were initially dried in a cool, dry, and shaded environment. Following the drying process, the leaves were manually ground into a fine powder using a pestle and mortar. For ultrasound-assisted extraction, 1 gram of the dried nettle powder was measured and placed into a screw-cap tube containing 15 mL of preboiled double-deionized water. The extraction was conducted in an ultrasonic bath at 65 °C for one hour, based on parameters established in previous studies [24, 25]. This temperature was also chosen to mimic the typical preparation method of nettle leaves, reflecting how they are usually consumed. After extraction, the mixture was filtered using Whatman No.1 filter paper to separate the solid residues from the liquid extract. The final extracts were stored in the dark at 5 °C until further analysis [26].

Passive Avoidance Test

This task was conducted at the day following the last UD treatment. The apparatus used for this task consisted of two compartments: one illuminated and the other dark. The apparatus comprised two chambers separated by a guillotine door. The test included an acquisition and a retention session. During acquisition, each rat was placed in the illuminated compartment for 20 seconds, after which the door opened automatically. Upon entry into the dark compartment, a 1 mA scrambled foot shock was delivered for 2 seconds. Animals were then returned to their cages. In the retention session, rats were reintroduced to the illuminated compartment; after 20 seconds, the door was opened, and step-through latency to the dark compartment was recorded. The maximum cutoff time for this session was 300 seconds.

Molecular Studies

Western Blotting

After the behavioral test, rats were decapitated using guillotine under anesthesia via ketamine and xylazine. Half of the brain (left) was dissected and quickly frozen for immunofluorescence studies. The right hippocampal tissue were dissected from each rat and was homogenized in RIPA lysis buffer containing metallic beads in an homogenizator after treated with 0.1 M phosphate-buffered saline (PBS). Then, homogenized tissues were centrifuged at 9000 x g for 15 min at 4 °C. After determination of protein concentration with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham), equal amounts of protein (40 µg/µl) were diluted in Laemmli sample buffer (Bio-Rad Laboratories, Inc. U.S.A), boiled for denaturation of proteins and loaded onto 4–20% gradient of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). At the end of the SDS-PAGE, the PVDF membranes were used to transfer proteins and the membranes were treated with blocking solution (5% milk powder in Tris-buffered saline-containing Tween 20 (TBST)). After an overnight incubation with primary antibodies including ATG5, Beclin, MAP LC3β (1:1000; Santa Cruz, USA), and NeuN (1:1000; Cell Signaling, Danvers, USA),

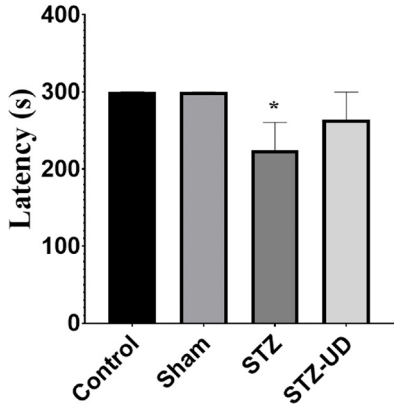

Figure 1. *Urtica dioica* (UD) treatment ameliorated the STZ-induced memory loss. The time to the entrance to the dark side of passive avoidance task for rats in the control (n=5), sham control (n=6), STZ (n=6) and STZ-UD (n=5) groups. Error bars denote SEM (standard error of mean). The degree of significance was denoted as * for p≤0.05.

membranes were incubated with secondary antibodies (1:1000; Cell Signaling, Danvers, USA). Luminol substrate (Advansta, San Francisco, USA) were used for determination of signal with Azure Western Blot Imaging System (Azure Biosystems, USA) containing CCD camera. Protein loading was normalized by β-actin (1: 1000; Cell Signaling, Danvers, USA). ImageJ analysis system (NIH; Washington, USA) were used to quantify the immunoreactive protein densitometrically.

Immunofluorescence Staining

Immunoflourescent analysis of rat brain tissue was done on 4 μm fresh frozen sections. The sections were fixed by 4% paraformaldheyhde (PFA) for 10 minutes at 4 °C. The permabilization was done by 10-min incubation with Triton X-100 (0.3%) at room temperature (RT). The blocking of sections were performed with 1% BSA in TBST for 1h at RT. The antibodies against NeuN (NBP1-92716, Novus) and Beclin (AC-0276RUO, Epitomics) were applied overnight at 4 °C. Negative controls were achieved by using T-TBS instead of the primary antibodies. The secondary antibody applied was Alexa Fluor 488 (ab150077) with 2 μg/ml concentration for 30-min at RT. The slides were covered by using Fluoroshield Mounting Medium with DAPI (ab104139, Abcam).

The sections were then examined under Zeiss Vert. A1 microscope and photographed by ZEN 2.6 application using Axiocam 503 color camera. The images taken was evaluated by QuPath [27]. In hippocampal CA1-CA3 regions, positive cells were counted for NeuN evaluation and H-scores were calculated for Beclin evaluation. The evaluations were done over the images taken three different sites of specified regions.

Statistical Analysis

The mean values and standard error of means were calculated for behavioral and molecular data. One-way ANOVA with Fisher's Least Significant Difference (LSD) test for pairwise comparisons was applied for behavioral and western blotting data to calculate the differences between groups. For analysis of data and representing the results as a graph, the Graphpad Prism 8 (GraphPad Software, Boston, USA) was used. The statistical significance value was accepted as p≤0.05.

RESULTS

Effect of UD Treatment on Cognitive Performance in STZ-Induced Neurodegeneration

During the retention session of the passive avoidance test, a shorter step-through latency were observed in the STZ group compared to both control groups, indicating impaired memory performance. This reduction was statistically significant only relative to the sham group (p=0.042). Conversely, administration of UD to STZ-exposed animals led to a noticeable improvement in retention performance, with latency values approaching those observed in the control groups (Fig. 1).

Effect of UD Treatment on Autophagy Protein Expression of STZ-Induced Neurodegeneration

The effect of STZ-injection and UD administration on the autophagy proteins including Beclin, ATG5, and LC3β were demonstrated in the hippocampal tissue (Fig. 2). Injection of STZ significantly declined the expressions of Beclin protein in the rats (Fig. 2A, p=0.0021), ATG5 (Fig. 2B, p=0.0078), and LC3β (Fig. 2C, p=0.0003) compared to that of the sham control group. In addition, an increase in the expression of these autophagy markers were noted in the sham control group compared to the control group (p<0.05). On the other hand, UD administration significantly increased the concentration of Beclin (p=0.0421) and LC3β (p=0.0038) compared to the STZ group approaching to the levels in the control groups. In contrast, the protein expression of ATG5 was not significantly changed due to UD treatment compared to the STZ group (Fig. 2B).

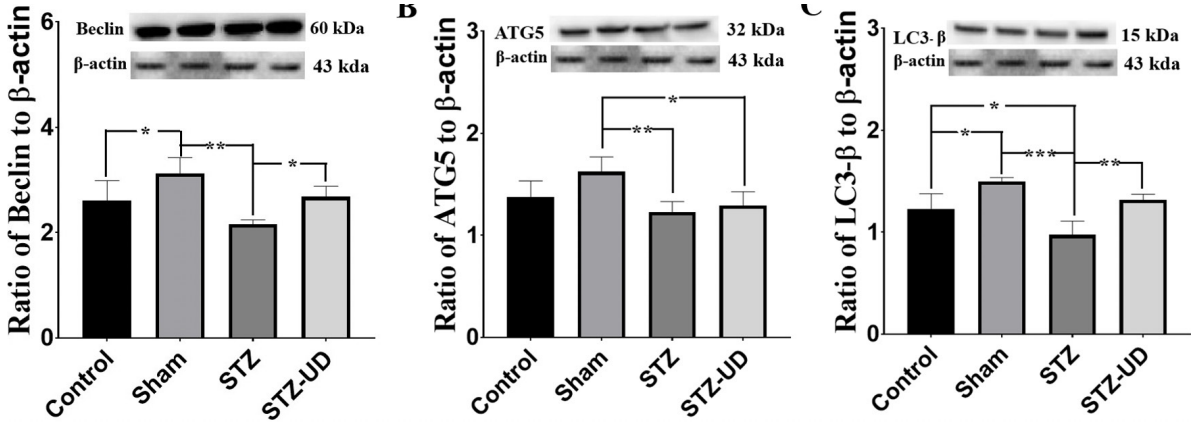

FIGURE 2. Representative pictures and relative amounts of (A) Beclin (B) ATG5, and (C) LC3β to β-actin which were analyzed by western blotting for control (n=5), sham control (n=6), STZ (n=6) and STZ-UD (n=5) groups. Error bars indicate SEM (standard error of mean). The degree of significance was denoted as * for p≤0.05, ** for p≤0.01, *** for p≤0.001. Lines show differences between two represented groups.

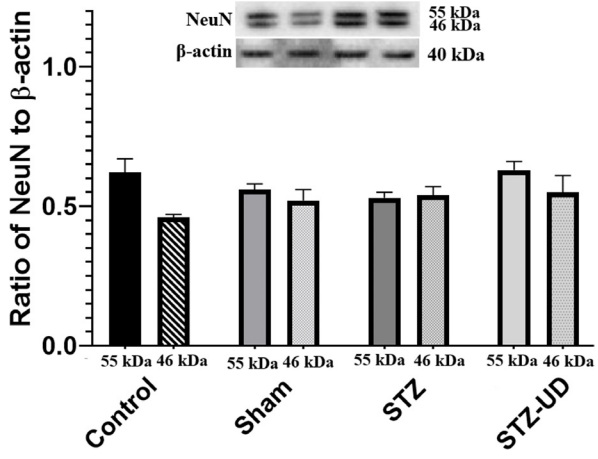

FIGURE 3. Representative pictures and relative amounts of NeuN protein to the to β-actin protein which were analyzed by western blotting for control (n=5), sham control (n=6), STZ (n=6) and STZ-UD (n=5) groups. Error bars indicate SEM (standard error of mean).

To observe effects of UD and the autophagy on the neuronal levels, we measured the expression of NeuN in the hippocampus of all studied groups (Fig. 3). Moreover, both STZ injection and UD treatment did not have any significant effect on the expression of NeuN protein compared to control animals (Fig. 3).

The Effect of UD Treatment on the Histological Parameters

To further detect and quantify NeuN positive cells over hippocampal CA1-CA3 subregions, immunofluorescence labeling was done (Fig. 4). In both CA1 and CA3 regions, the number of NeuN positive cells significantly decreased in the STZ group compared to the both control groups (Fig. 5, $p<0.01$). The UD-treatment group showed a statistically significant increase in both regions of the hippocampus compared to that of the STZ group ($p<0.05$).

The autophagy marker Beclin was also evaluated with immunofluorescence labeling for validation of western blotting data (Fig. 4). The STZ group had a significantly low Beclin H-Score compared to the both control and sham groups in hippocampal CA1-CA3 regions (Fig. 5, $p<0.001$). The UD treatment group, however, showed significantly high Beclin H-Score in both regions compared to the STZ group ($p<0.01$).

DISCUSSION

Neurodegenerative disorders are also defined as proteinopathies due to the accumulation of aggregates of insoluble proteins. Multiple risk genes related to neurodegenerative disorders, including *PICALM*, *PSEN1*, *TREM2*, *CLU*, etc. have been modulate autophagic flux [28]. Therefore, promoting autophagy to improve the removal of misfolded proteins is suggested as a potential therapeutic approach. Strategies targeting autophagy could offer a novel method for developing medications to combat neurodegenerative disorders, particularly phytotherapeutics [29]. Herbal drugs are considered as probable sources of antioxidants [30]. The most regular used forms of the herbal drugs are

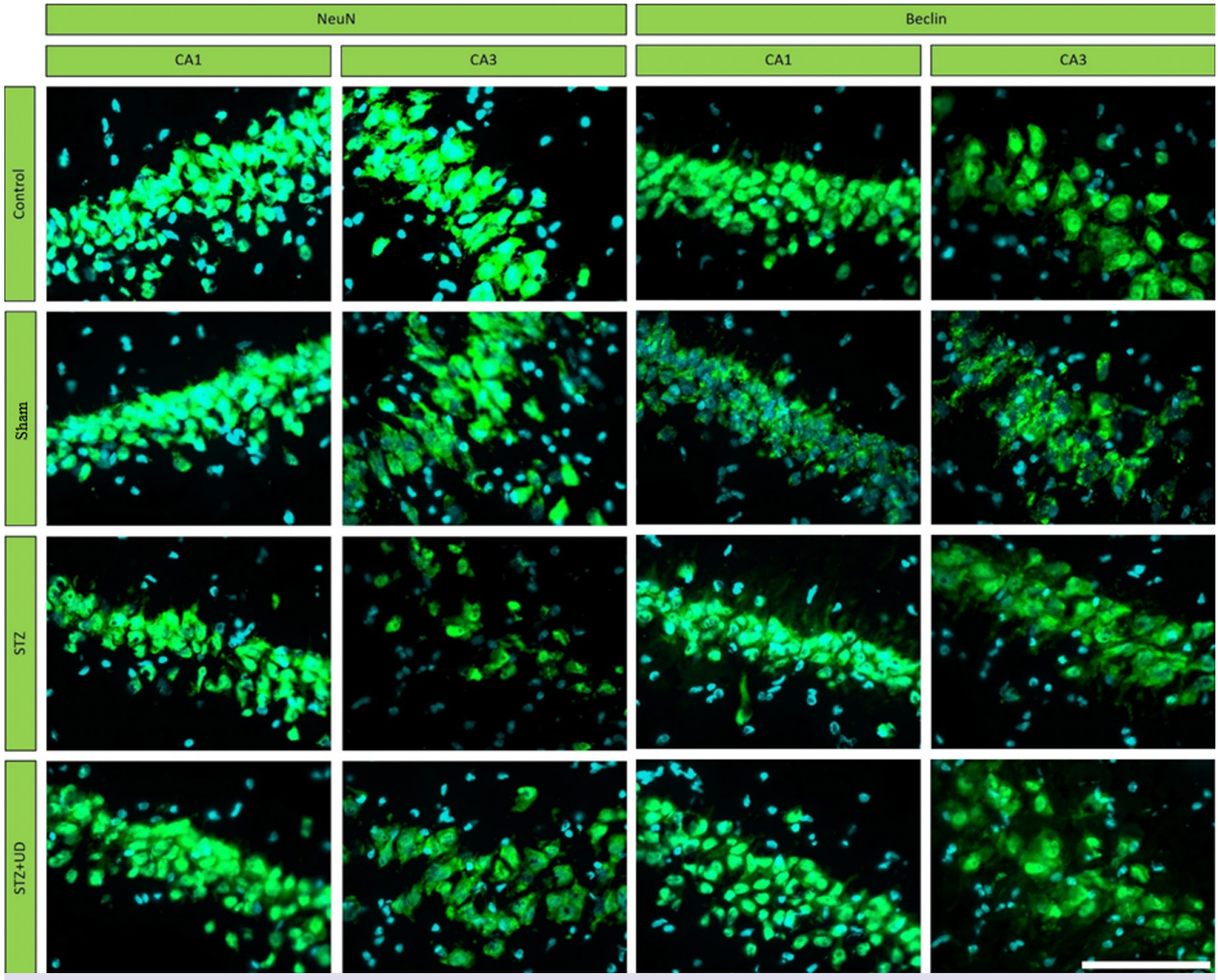

Figure 4. Immunoflourescence images from hippocampal CA1-CA3 subregions stained with NeuN and Beclin antibodies (Scale Bar:100 µm) X63.

plant extracts [31]. UD is a plant which has anti-infammatory and anti-oxidation properties with great promises for chronic inflammatory diseases [32, 33]. In the present study, we investigated the correlation between UD's protective role on the hippocampal neurons in regards to autophagy which remains to be elucidated for STZ-induced neurodegeneration.

In the present study, we observed the ameliorative role of UD on the STZ-induced cognitive impairment as previously noted in the rats with diabetic neuropathy [34, 35]. In addition to memory dysfunction in the diabetic animals, the healing effects of UD on memory function was also recorded in brain lesions, aging, and experimental memory deficiency models [12, 36–38]. In a previous study, when an herbal extract including UD was given to the rats with sporadic AD, an improvement in the spatial learning and memory was considered that this herbal extracts might have anti-dementia properties [2]. In these previous studies, the underlying molecular mechanism of this memory enhancing effect of UD might be related to the its antioxidative effects to increase some proteins that are crucial for memory formation and synaptic plasticity such as muscarinic cholinergic system, BDNF, NGF, and synaptophysin [2, 12, 36, 39].

On the other hand, the molecular parameters that activate cell survival and death in the hippocampal region are important for the alterations in the learning and memory. The two important physiological cell

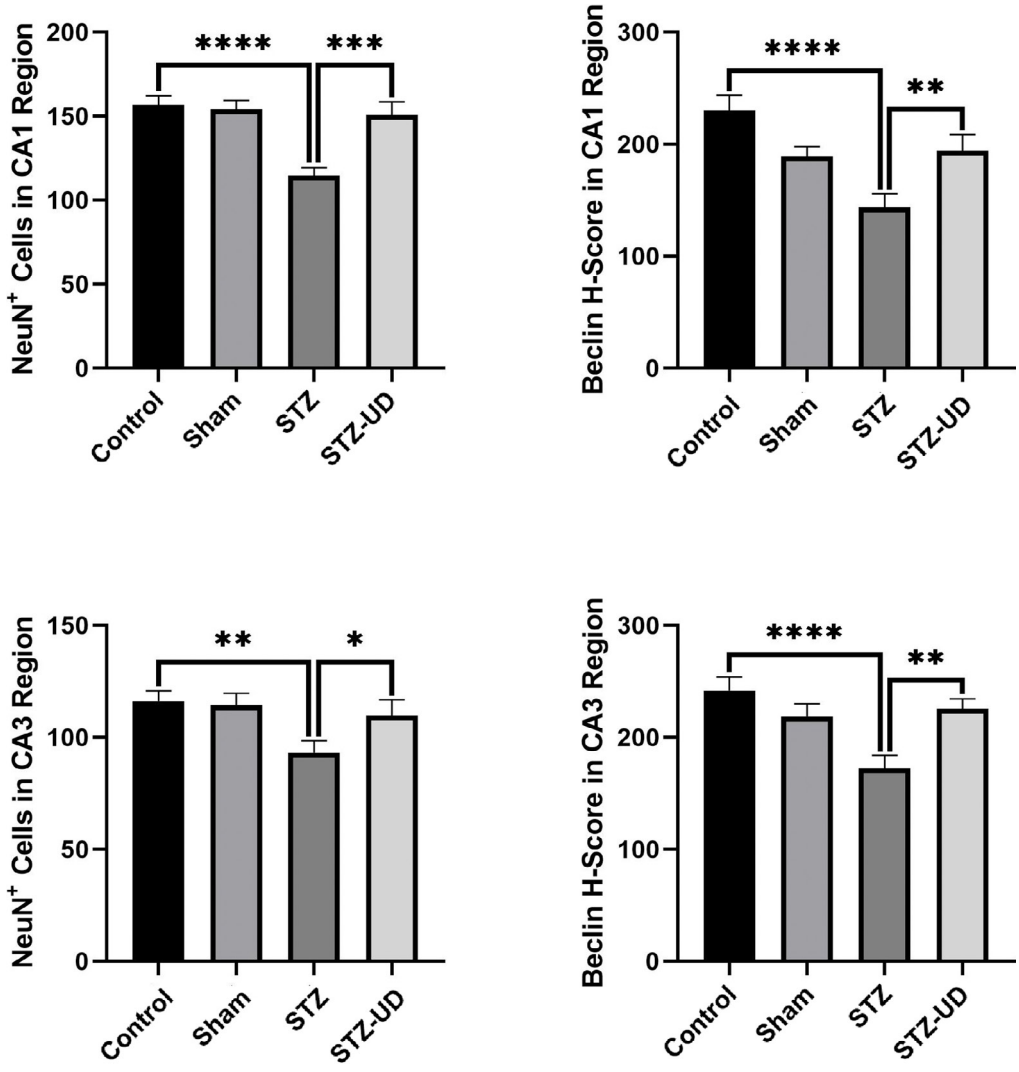

FIGURE 5. The levels of immunofluorescence of NeuN and Beclin from hippocampal CA1-CA3 subregions. The positive cells were counted for NeuN evaluation and H-scores were calculated for Beclin evaluation in the control (n=5), sham control (n=6), STZ (n=6) and STZ-UD (n=5) groups. Error bars indicate SEM (standard error of mean). The degree of significance was denoted as * for $p \leq 0.05$.

death mechanisms, autophagy and apoptosis, and their crosstalk between each other influence homeostasis of the cell, the clearance of cell debris, in addition to the action of therapeutics. Depending on the context, autophagy can either inhibit apoptosis or function in concert with it [40]. In our study, we investigated the alterations in the expression of proteins related to the autophagy to understand the neuroprotective effect of the UD on the STZ-induced neurodegeneration and its memory enhancing activity. As previously mentioned, we observed a significant decrease in the autophagy parallel to memory decline in the STZ-induced neurodegeneration [1, 4, 41, 42]. Impaired autophagy is recognized as a key contributor to cellular damage and neuronal death in neurodegenerative disorders, leading to abnormal protein aggregation, mitochondrial dysfunction, oxidative stress, and apoptosis [43, 44]. Therefore, the activation of autophagy has been suggested as a potential therapeutic approach for AD because of increasing evidence of it in maintaining neuronal function [45]. In our study, we observed a significant effect of UD on the autophagy by increasing the expression of Beclin, a crucial marker in the early stages of autophagy which is essential for autophagosome formation, and LC3β, a marker of the later stages of autophagy which is a membrane-bound protein asso-

ciated with autophagosomes, in the hippocampal tissue [46], while no variations were noted in autophagy levels of some other tissues such as ovarian cancer cells [47]. In addition, as seen in our study, ATG5 expression did not change due to UD supplementation in diabetic mice hippocampi [47]. As a validation of western blotting results, a significant increment in the immunofluorescence of Beclin protein were noted in the both CA1 and CA3 regions of hippocampus.

The downregulation of autophagy indices in the STZ-induced neurodegeneration has been associated with increased apoptosis in hippocampus suggesting a neuronal loss and cognitive impairment [35]. In the current study, while there was a slight decline in the NeuN protein expression of the hippocampal tissue which was determined by western blotting, the decrease in the NeuN positive cells in the both CA1 and CA3 regions was significant in the STZ-induced neurodegeneration model suggesting neuronal death in these parts of the hippocampus. Furthermore, parallel to the increase in the Beclin H-score, an UD-dependent increase in the NeuN positive cells of the both regions suggest the anti-apoptotic role of the UD through upregulating autophagy pathways in the hippocampus [48].

Conclusion

In the current study, we noted that UD may improve cognitive dysfunction by altering autophagy-related indexes in STZ-induced neurodegeneration model rats. This study aligns with previous research, further supporting the critical role of autophagy in the pathology of AD. Additionally, it suggests that autophagy may serve as a potential therapeutic target for AD, providing a foundation for future treatments with phytotherapeutics, like UD, that modulate autophagy as a strategic intervention offering new perspectives for AD treatment.

Ethics Committee Approval: The Bezmialem Vakif University Animal Experiments Ethics Committee granted approval for this study (date: 06.12.2019, number: 2019/266). Conducted in accordance with ARRIVE guidelines to minimize animal suffering.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Use of AI for Writing Assistance: The authors declared that artificial intelligence-supported technologies (such as Large Language Models [LLMs], chatbots or image generators, ChatGPT) were not used in the production of the study.

Authorship Contributions: Concept – SA, EC, BE; Design – SA, FB, BE; Supervision – SA, BE; Fundings – SA, SP, BE; Materials – BE, FB, EGD; Data collection and/or processing – SA, HIS, MS, AK, EGD, BE; Analysis and/or interpretation – SA, SP, BE; Literature review – SA, MS, AK, BE; Writing – SA, HIS, EC, BE; Critical review – SA, HIS, MS, AK, EC, EGD, FB, SP, BE.

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