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Neuropathological study

Melatonin affects the release of exosomes and tau-content in in vitro amyloid-beta toxicity model

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ABSTRACT

Background: Recent studies have been revealed that oxidative damage is the main cause of aging and age-related neurodegenerative diseases like Alzheimer's disease (AD). Melatonin is secreted from the pineal gland and its secretion has been found to be altered in AD. In the last decade the role of exosomes in spreading toxic proteins and inducing the propagation of diseases like AD has been discussed. However, it is not known how melatonin affects the amount of exosomes released from the cells and the content of the exosomes.
Objective: Herein, we investigated the possible role of melatonin treatment in the releasing of exosomes and exosomal tau content in an in vitro Aβ toxicity model.
Method: SH-SY5Y cell line was used. The optimum concentration of Aβ was determined by cell viability and cell proliferation tests. Melatonin (100 μM) was applied before and after Aβ application. Total exosomes isolated from cell culture media were immunoprecipitated. The amount of released exosomes and their tau content were analyzed by Western blots.
Results: Our data demonstrated for the first time that melatonin treatment clearly affected the amount of released exosomes. It would decrease the amyloid beta load and toxicity by inhibiting exosome release. We also demonstated that melatonin also affected the level of tau carried by exosomes depending on whether melatonin was applied before or after Aβ application.
Conclusion: It is considered that the effect of melatonin in the release of exosomes and exosomal tau content would contribute the development of therapeutic strategies in AD and related disorders.

1. Introduction

Melatonin and its metabolites have been shown to enforce the antioxidant system by scavenging free radicals [1–6]. Melatonin stimulates synthesis of antioxidant enzymes and protects antioxidative enzymes from oxidative damage [7–9]. Furthermore, melatonin regulates cellular signaling pathways through receptor-dependent and independent mechanisms [10–16].

Alzheimer's Disease (AD) is the most common, progressive neurodegenerative disorder affecting people over 65. Neuropathologically, AD is characterized by the presence of intracytoplasmic protein inclusions, called neurofibrillary tangles (NFTs) and extacellular senile plaques composed mainly of amyloid beta (Aβ) protein [17–19]. NFTs primarily contain a hyperphosphorylated form of microtubule-associated protein tau in the form of paired helical filaments (PHFs) [17,19,20]. The PHFs progressively accumulate in the soma of diseased neurons, dystrophic neurites, and neuropil threads. Postmortem analyses of AD brain samples show that this progressive spread of tau deposits usually starts from

Abbreviations: Aβ, Amyloid beta; AD, Alzheimer's disease; ANOVA, Analysis of variance; APP, Amyloid precursor protein; DMEM, Dulbecco's Modified Eagles Medium; LDH, Lactate dehydrogenase; LSD, Least significant difference; 100 μM melatonin, Mel; NFTs, Neurofibrillary tangles; O.C, Only cells; PHFs, Paired helical filaments; PVDF, Polyvinylidene fluoride membranes.

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transentorhinal cortex to the hippocampus and eventually to nearly all cortical regions. NFT deposition leads to loss of synaptic function and neuronal death, and this process amplifies itself due to the release of tau fibrils into the extracellular environment [20,21].

Aβ found mainly in senile plaques is generated by the sequential cleavage of amyloid precursor protein (APP) through the action of secretases, and resulting Aβ fragments (39–42 amino acids in length) are secreted. Normally this APP processing is kept in a steady state, but disruption of the metabolic balance of Aβ causes the formation of toxic aggregates which are linked to AD pathogenesis. Studies also show that Aβ aggregates trigger the deposition of tau and contribute to the formation of NFTs [17,19–21].

Exosomes are spherical membranous vesicles, 30–100 nm in diameter, and they are generated by the invagination of the limiting membrane of the late endosome [22–24]. Through this inward budding, exosomes encapsulate proteins, miRNAs and mRNAs from the cytoplasm in a non-random way [25–28]. Different cell types release exosomes with various functions including platelet activation, regulation of immune response and intercellular communication. It was previously reported that exosomes were associated with APP, Aβ and tau. It has also been found that exosomes carry cytoplasmic and hyperphosphorylated form of tau and through this function, they may be contributed to the spread of pathogenesis in AD [29–35]. The effects of melatonin as a powerful free radical scavenger is already known from several studies and it has also been found that melatonin has anti-amyloidogenic functions [36–38]. The one of the pathological hallmarks of AD is tau hyperphosphorylation seen in neurons and melatonin inhibits tau hyperphosphorylation both in vitro and in vivo [37]. The effects of melatonin on amyloid beta toxicity are different. Although melatonin seems to have no or limited effect to inhibit amyloid plaque formation in transgenic mice, it has an anti-amyloidogenic effect on wild-type mice. It seems that the key point lies on the timing of melatonin application; if melatonin application starts before amyloid plaque formation, it exhibits its anti-amyloidogenic effect. If it is applied after the induction of amyloid plaque formation, it has little or no effect [37].

It is not known how melatonin affects the amount of exosomes and the content of the exosomes. This study was designed to investigate the effect of melatonin in the releasing of exosomes and exosomal tau content in an in vitro Aβ toxicity model.

2. Materials and methods

2.1. Establishing in vitro $A\beta_{1-42}$ toxicity model, cell viability and proliferation assays

SH-SY5Y human neuroblastoma cell line was purchased (ATCC, USA) and they were propagated and subcultured with Dulbecco's Modified Eagles Medium (DMEM) containing 10% (v/v) heat-inactivated foetal bovine serum (Gibco, USA) and 100U penicilin-streptomycin (Gibco, USA).

Cells were seeded in a 96-well plate at a density of 10,000 cells/well and after 24 h four different $A\beta_{1-42}$ (Abcam, Cat No:120301, UK) concentrations (1.25 μM, 2.5 μM, 5 μM and 10 μM) were applied in order to find the effective toxic dose. $A\beta_{1-42}$ has been dissolved in 1% (v/v) NH_4OH [39]. Lactate dehydrogenase (LDH) assay (Roche, Cat No:11644793001, Germany) was performed for cell viability measurements. Briefly, the working solution of the LDH assay was prepared according to manufacturer's instructions, and was incubated in complete darkness for 15 min at room temperature. The medium in which our cells were cultured was taken into a new 96-well plate in exactly the same order of the original plate. 100 μl of LDH working solution was added to each well containing the cell culture medium to a final volume of 200 μl. At 492 nm, absorbance values in each well were measured in a microplate reader (Chromate Manager 4300, Palm City/USA). To investigate the effect of melatonin and $A\beta_{1-42}$ in cell proliferation WST-1 assay (Roche, Cat No: 05015944001, Germany) was applied. Briefly, 10 μl of WST-1 reagent was added to each well of the 96-well plate in which SH-SY5Y cells were cultured and $A\beta_{1-42}$ and melatonin were applied. After adding the WST-1 reagent, cells were incubated at 37 °C for 4 h. Absorbances were detected at 450 nm by a microplate reader (Chromate Manager 4300, Palm City/USA).

2.2. Immunocytochemistry

SH-SY5Y cells were seeded into petri dishes at a density of 50,000. After 24 h, $A\beta_{1-42}$ (10 μM) was applied and cells were incubated for 48 h. Using primary $A\beta_{1-42}$ antibody (Santa Cruz, Cat No: sc-28365, USA), amyloid beta aggreates were visualized fluorescently by using laser confocal microscopy. Cell nuclei were visualized with DAPI. Petri dishes in which SH-SY5Y cells were cultured without $A\beta_{1-42}$ were used as negative controls. All microscopy experiments have been conducted at least twice with different Aβ preparations.

2.3. Exosome isolation

This experiment consisted of six groups: (1) only Cell; (2) only Aβ; (3) only melatonin; (4) melatonin + Aβ; (5) Aβ + melatonin; (6) melatonin + Aβ + melatonin. Herein, we examined the effect of melatonin on exosome quantity and exosomes' molecular contents. For exosome isolation SH-SY5Y cells were seeded in T-25 cell culture flasks (Corning, USA). After 24 h incubation the cell culture medium was replaced with a culture medium containing exosome-depleted FBS (Gibco, Cat No: A2720801, USA). Melatonin (Sigma, Cat No: M5250, USA) was dissolved in ethanol as recommended by the manufacturer and because it is already known that up to 20 mM of ethanol has no toxic effect on SH-SY5Y cells, it is reasonable to consider that the amount of ethanol used here as a solvent has no toxic effect [40]. Melatonin (Sigma, Cat No: M5250, USA) was applied in 100 μM before and after $A\beta_{1-42}$ addition for 8-hour periods repeatedly in order to find out pre- and post-treatment effect of melatonin on $A\beta_{1-42}$ toxicity. After 48 h of incubation, cell culture medium was obtained and exosome isolation was performed by using Total Exosome Isolation Kit (Invitrogen, Cat No: 4478359, USA). Briefly, cell culture medium was centrifuged at 2000×g for 30 min at 4 °C and supernatant was taken. Total exosome isolation reagent was added and the supernatant was incubated at 4 °C overnight. Then, another centrifugation step was applied at 10,000×g for 1 h at 4 °C in order to pellet the exosomes and the pellet was resuspended by using 1X phosphate-buffered saline (1XPBS) (Gibco, USA) for further use.

2.4. Exosome immunoprecipitation

For immunoprecipitating the exosomes, PureProteome Protein G Magnetic Beads (Millipore, Cat No: LSKMAGG02, USA) was used. Magnetic beads were washed initially with 1XPBS containing 0.1% Tween-20 and resuspended in the same buffer. Anti-Alix antibody (Cell Signaling, Cat No: 2171S, USA) was used as a capture antibody. Capture antibody and resuspended exosomes were added to the beads and the sample was incubated overnight at 4 °C while rotating.

2.5. Western Blotting

Immunoprecipitated exosome samples were denatured and the protein concentrations were measured spectrophotometrically (Implen, Germany). 40 μg of each protein sample from exosomes were separated by 4–12% NuPAGE electrophoresis system and samples were transferred to polyvinylidene fluoride membranes (PVDF) using iBlot Dry Blotting System (Invitrogen, USA). Membranes were blocked in 5% non-fat milk in 50 mM Tris-buffered saline containing 0.1% Tween for 1 h at room temperature, washed in Tris-buffered saline containing 0.1% Tween and incubated overnight with membranes were anti-Tsg101 antibody (SantaCruz, Cat No: sc-7964, USA), anti-phospho tau antibody (Cell Signaling, Cat No: 12885S, USA), anti-total tau primary antibody (Cell Signaling, Cat No: 4019S, USA), diluted 1:1000 in Tris-buffered saline containing 0.1% Tween. On the second day, the membranes were washed and further incubated in blocking solution with peroxidase-conjugated –secondary antibody (Cell Signaling, Cat No: 7074S, USA) for 1 h at room temperature.

All blots were performed at least three times and revealed using a ECL-Advanced Western Blotting Detection Kit according to the manufacturer's protocol. (Amersham, Cat No: RPN2232, UK). Proteins were visualized by Bio-Rad ChemiDoc XRS System (Bio-Rad Laboratories Inc., USA) and analyzed densitometrically with ImageJ software.

2.6. Statistical analysis

Data were statistically evaluated with one-way ANOVA. A p value of less than 0.05 was regarded as being statistically significant. For statistical data comparisons, a standard software package (SPSS 18 for Windows; SPSS Inc., Chicago, IL) was used. Data were statistically analyzed by using repeated-measures analysis of variance (ANOVA), followed by the post-hoc least significant difference (LSD) test. Values are given as mean standard error of the mean (SEM).

3. Results

3.1. Cell viability and proliferation assays

In order to establish the Aβ toxicity model on SH-SY5Y cells, four different $A\beta_{1\text{-}42}$ concentrations (1.25 μM, 2.5 μM, 5 μM and10 μM) were tested. The cell viability (LDH) and cell proliferation (WST-1) assays were performed. WST-1 analysis showed no significant difference among the $A\beta_{1\text{-}42}$ concentrations, whereas LDH data exhibited that 10 μM $A\beta_{1\text{-}42}$ concentration was the most effective dose for cell toxicity (Fig. 1a and b).

To elucidate the effect of melatonin on the Aβ toxicity model, 100 μM melatonin was applied before and after $A\beta_{1\text{-}42}$ addition for 8-hour periods repeatedly and its effect was measured by using LDH analysis (Fig. 1c). The cell viability data indicated that pre-, post- and pre-/post- combined melatonin treatment significantly reduced the Aβ toxicity; the most dramatic decrease was observed in combined (pre- and post-treatment) melatonin application (85.63%, $p < 0.05$, Fig. 1c). It was also observed that the LDH analysis gave higher absorbance value for the control sample than that of rest. The most probable reason is that because the cells of the control sample was let to proliferate in the absence of $A\beta_{1\text{-}42}$, at the end of the experiment they already started to dye due to the contact inhibition.

3.2. Immunocytochemistry for $A\beta_{1\text{-}42}$

To visualize the internalization of applied $A\beta_{1\text{-}42}$, immunocytochemical method was preferred. SH-S5Y5 cells were incubated with $A\beta_{1\text{-}42}$ for 48 h, and fixed by 4% paraformaldeyde. After permeabilized, anti-Aβ antibody was applied. Petri dishes in which SH-SY5Y cells cultured without $A\beta_{1\text{-}42}$ were used as negative control for immunocytochemistry (Fig. 2b). Confocal microscopy clearly exhibited that $A\beta_{1\text{-}42}$ was internalized by the cells and it formed intracellular aggregates (Fig. 2d).

3.3. Immunoprecipitation and Western Blotting analyses

Here the immunoprecipitated exosomes by using anti-Alix antibody as a capture antibody, were investigated in their phospho- and total-tau content. In western blot analyses we used a different antibody against another exosome marker protein, Tsg-101. By using two different antibodies against two different exosome marker proteins, the possibility of accidental inclusion of other extracellular vesicles in these analyses was excluded. Our results showed that Aβ application alone and Melatonin application alone (14.49%; 73.58% respectively $p < 0.05$, Fig. 3) led to a significant decrease in exosome release as compared to the control group (only cell; OC). Melatonin pre-treatment reduced the exosome quantity by 36.23% (Fig. 3). Post-treatment of melatonin and combined melatonin treatment (pre- and post-treatment together) showed no statistically significant changes as compared to the OC.

In the present study, total and phosphorylated tau content of immunoprecipitated exosomes were examined. Although cell lysates contain phosphorylated tau, exosomes extracted from cell culture media did not carry phoshorylated tau (Fig. 4a). Western blot membranes, which were used to visualize phosphorylated tau, were stripped and incubated with total tau antibody. The total tau content seemed to be affected by whether melatonin was applied before or after $A\beta_{1\text{-}42}$ addition (Fig. 4b). In the melatonin pretreatment group, exosomal tau content remained unaltered, whereas, melatonin post-treatment after $A\beta_{1\text{-}42}$ addition reduced the exosomal total tau content significantly (94.14%) (Fig. 4b). When melatonin was used pre- and post-treatment in a combined fashion, tau content of exosomes showed no significant difference as compared to control group (O.C.). In addition, the experimental groups were compared with each other, and we found that the exosomal tau content of the group where only melatonin was applied, decreased significantly. When melatonin post-treatment group was compared with the other experimental groups, exosomal tau content was also found to be reduced in a statistically significant way (Fig. 4b).

Our results showed that the application of Aβ and melatonin separately affect the amount of exosomes released from the cells, and exosomal tau content. Pre-treatment of melatonin reduced the exosome quantity, whereas post-treatment of melatonin reduced the exosomal total tau content significantly.

4. Discussion

Alzheimer's disease (AD) is characterized with severe neuronal loss in the brain, and it is identified by a variety of molecular pathologies leading cortical dementia with a prominent memory deficit [41–44]. Distribution of pathologies is highly correlated with the clinical symptoms. Accumulating evidence for AD and also other neurodegenerative diseases has begun to show that spreading of neuropathology in the central nervous system is mediated by exosomes containing different forms of tau. The phosphorylated form of tau protein carried by exosomes is one of the likely candidates for spreading neurodegeneration in different disease settings [45–51].

In the present study, we made use of an in vitro amyloid beta toxicity model in a human SH-S5Y5 cell line to investigate the role of melatonin [52–56]. It is well known that melatonin has a neuroprotective effect. Thus, we herein used a single melatonin concentration (100 μM), which corresponds to the most effective dose (4 mg/kg) established in vivo mice models by Kilic et al. [12,13,57]. Data clearly showed that 10 μM amyloid beta is the

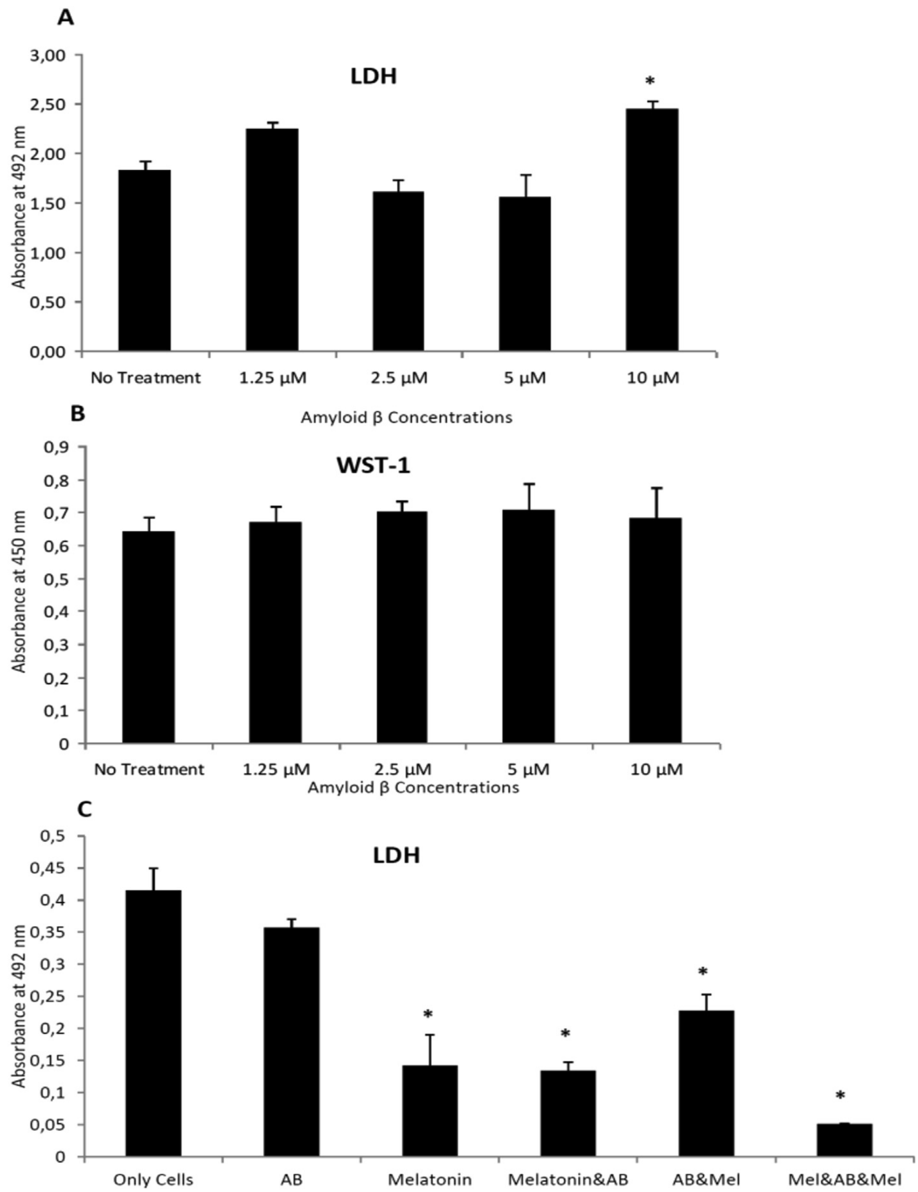

Fig. 1. Lactate Dehydrogenase (LDH) and WST-1 analyses. (A) shows cell viability data indicating the most effective toxic dose of Aβ, which was 10 μM among the four different concentrations, (B) cell proliferation data from the WST-1 analysis showed that the chosen concentrations of Aβ did not have any significant effect on cell proliferation, (C) effect of melatonin (100 μM) on cell viability in the context of Aβ toxicity (10 μM). The LDH data exhibited that the use of melatonin alone or in three different combinations (pre-, post- and pre-/post-treatment of Aβ) had a significant reducing effect on cytotoxicity. (*) symbol indicates the statistical significance where $p < 0.05$.

most effective dose in our toxicity model, and none of the concentrations used in our in vitro setting seemed to alter the cell proliferation rate (Fig. 1b). In agreement with in vivo studies found in the relevant literature, application of melatonin ameliorates the toxic effects of amyloid beta in all experimental groups significantly (Fig. 1c). We found that melatonin alone led to cells (Fig. 3). When $A\beta_{1\text{-}42}$ was applied alone, exosomal release was still found to be reduced. It could be deduced from these findings that presence of amyloid beta triggers the exosome release from the SH-SY5Y cells. Melatonin pre-treatment before $A\beta_{1\text{-}42}$ also decreased releasing exosomes significantly. On the other hand, neither post-treatment of melatonin nor combined melatonin treatment (pre- and post-) altered the exosomal release (Fig. 3). These data demonstrated that both amyloid beta and melatonin applications have direct effects on the exosomal release in our experimental model separately, and melatonin pre-treatment also reduced the exosomal release with respect to both control and Aβ groups (Fig. 3). This may imply that the effect of melatonin would be based on the cellular mechanisms regulating the release of exosomes in this model. In fact Dinkins et al. [58] demonstrated in 5XFAD mice that amyloid beta plaque formation and its load have been reduced when exosome release was inhibited. In our in vitro model, melatonin alone inhibited the exosome release from SH-SY5Y cells and in addition to that in the presence of amyloid beta melatonin still reduced the exosome release. Our findings which are consistent with the literature would provide support for studies to inhibit amyloid beta load by decreasing the exosome release.

The effect of melatonin on the total and phospho-tau levels in exosomes seems to be different. Our data indicate that exosomes did not contain the phosphorylated form of tau in our experimental setting (Fig. 4a). Although this could be considered to be specific to our in vitro model, recent studies present the similar findings.

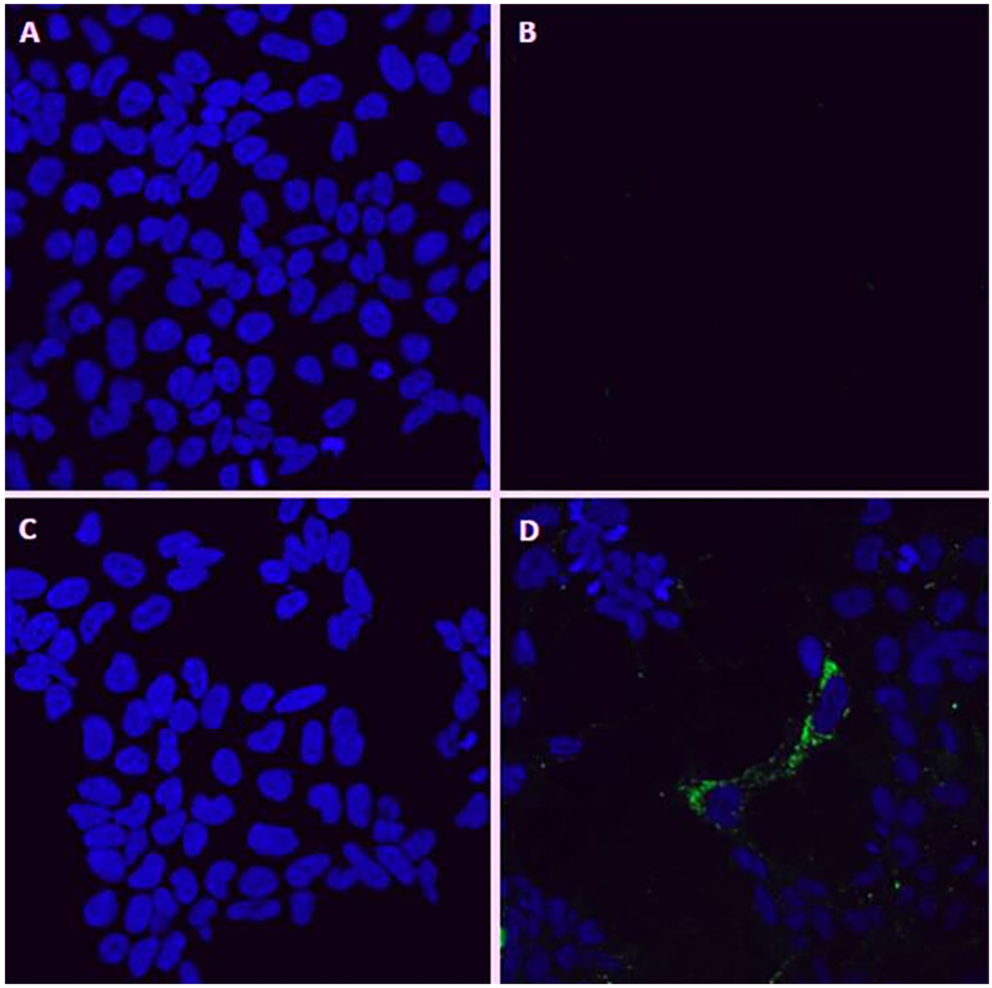

Fig. 2. Immunocytochemistry. (A) shows the cell nuclei of the control sample without Aβ; (B) exhibits the control sample without Aβ incubated with anti-Aβ antibody; our antibody for Aβ did not give any background florescence as expected (C) shows the cell nuclei of the sample where 10 μM Aβ has been applied; (D) the sample where 10 μM Aβ has been applied and incubated with anti-Aβ antibody. The green fluorescence shows the amyloid beta aggregates inside the cells as expected.

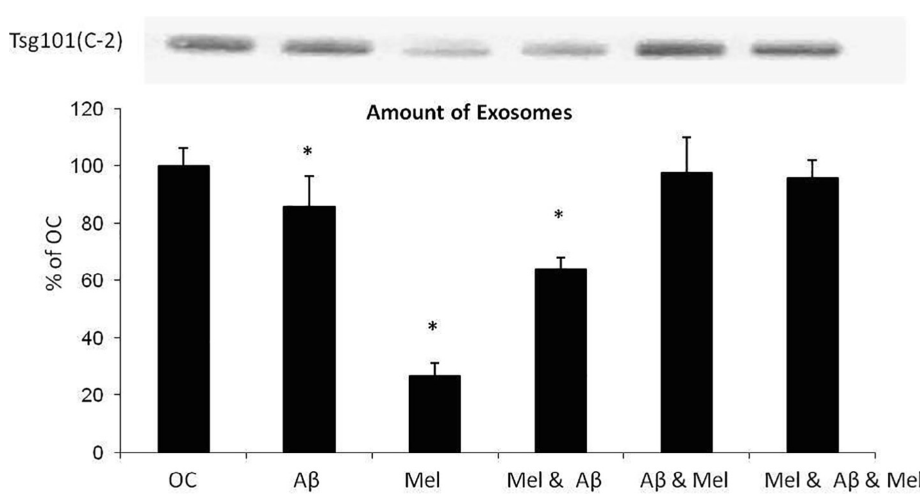

Fig. 3. Effect of melatonin on the amount of exosomes released by the SH-SY5Y cells. (O.C.: Only cells), Aβ: 10 μM amyloid beta 1–42; Mel: 100 μM melatonin Mel&Aβ: 100 μM melatonin pre-treatment before 10 μM Aβ application; Aβ&Mel: 100 μM melatonin post-treatment after 10 μM Aβ application; Mel& Aβ&Mel: 100 μM melatonin pre- and post-treatment with 10 μM Aβ application. The amount of exosomes released was quantified by the amount of Tsg-101 (an exosome marker protein) and it was significantly reduced in Aβ, Mel and Mel&Aβ groups. (*) shows the comparison with the O.C. group. $p < 0.05$ for all comparisons.

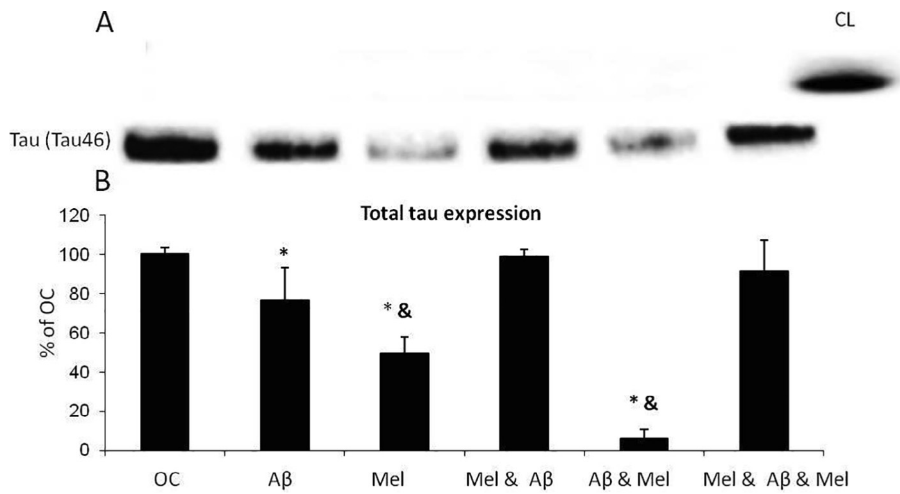

Fig. 4. (A). Phosphorylated tau content of immunoprecipitated exosomes. O.C.: Only cells, Aβ: 10 μM amyloid beta 1–42; Mel: 100 μM melatonin Mel&Aβ : 100 μM melatonin pre-treatment before 10 μM Aβ application; Aβ&Mel: 100 μM melatonin post-treatment after 10 μM Aβ application; Mel& Aβ&Mel: 100 μM melatonin pre- and post-treatment with 10 μM Aβ application; CL: Cell Lysate. The isolated exosomes did not contain phosphorylated tau as expected from the literature (B) Total tau content of immunoprecipitated exosomes. O.C.: Only cells, Aβ: 10 μM amyloid beta 1–42; Mel: 100 μM melatonin Mel&Aβ : 100 μM melatonin pre-treatment before 10 μM Aβ application; Aβ&Mel: 100 μM melatonin post-treatment after 10 μM Aβ application; Mel& Aβ&Mel: 100 μM melatonin pre- and post-treatment with 10 μM Aβ application. In Aβ, Mel and Aβ&Mel groups exosomal total tau content was significantly reduced according to the O.C. The exosomal total tau was also found to be lowered in Mel and Aβ&Mel groups according to the Aβ group. (*) shows the comparison with the O.C. Group, (&) shows the comparison with the Aβ group. $p < 0.05$ for all comparisons.

When the total tau content of exosomes was investigated, amyloid beta application alone not only reduced the amount of exosome release (Fig. 3), but it also decreased the exosomal total tau content (Fig. 4b). The very similar trend would be observed in melatonin application alone, namely, melatonin use reduces both exosome release and total tau content of released exosomes (Fig. 4b).

On the other hand, melatonin pre-treatment before amyloid beta application did not affect the exosomal total tau content while reducing the amount of exosomes (Figs. 3 and 4b). Another important point is that although melatonin post-treatment did not alter the the amount of exosomes, exosomal tau content was significantly decreased (Figs. 3 and 4b).

The effect of melatonin in the context of neurodegeneration is usually two fold. First melatonin due to its anti-oxidant activity, reduces the oxidative stres in the neurons and supports the neuronal survival. The second aspect of the effect of melatonin is its anti-amyloidogenic activity. Studies about this aspect emphasize the timing of melatonin application; if melatonin is applied before the generation of amyloid plaques, it shows its anti-amyloidogenic activity. On the other hand, if melatonin is used after the formation of amyloid plaques, its effect remain very limited. In addition to these effects, melatonin also lowers the amount of hyperphosphorylated tau, an intracellular indicator for AD, both in in vitro and in vivo studies. All these findings clearly exhibit the role of melatonin in neurodegenerative processes [36–38].

Overall, our findings indicated that both amyloid beta application and melatonin treatment directly affected the amount of exosome released from SH-SY5Y cells. Melatonin pre-treatment did not change the amount of total tau in the exosomes, whereas reducing the amount of exosomes.

From the clinical point of view, our preclinical findings are particularly interesting regarding rapidly increasing evidence showing the role of exosomes in spreading of neurodegeneration and degeneration related toxicities [58–63]. It is well established the role of tau protein and its post-translational modifications in the pathogenesis of AD and other neurodegenerative disorders [64–69]. Additionally, several studies have shown that melatonin levels are significantly decreased in both AD patients and healthy aged individuals [61,62,70–81]. The important point of our study in the context of translational neuroscience is that besides melatonin's anti-oxidant, anti-amyloidogenic and lowering effects of hyperphosphorylated tau, it also has an effect on exosome release and exosomal tau content. This can provide a new starting point for developing novel strategies for treating AD knowing that one of the spreading ways of neurodegeneration in AD is through exosomes [82–85]. Taken together our study suggested the importance of the effects of amyloid beta and melatonin in the mechanisms of controlling the release of exosomes and the findings also imply their effects on exosomal tau content with respect to the 8-hour periodicity of application.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] Gitto E, Tan DX, Reiter RJ, Karbownik M, Manchester LC, Cuzzocrea S, et al. Individual and synergistic antioxidative actions of melatonin: studies with vitamin E, vitamin C, glutathione and desferrioxamine (desferoxamine) in rat liver homogenates. J Pharm Pharmacol 2001;53:1393–401. https://doi.org/10.1211/0022357011777747.

[2] Hardeland R, Backhaus C, Fadavi A. Reactions of the NO redox forms NO+, *NO and HNO (protonated NO) with the melatonin metabolite N1-acetyl-5-methoxykynuramine. J Pineal Res 2007;43:382–8. https://doi.org/10.1111/j.1600-079X.2007.00489.x.

[3] Reiter RJ, Tan DX, Osuna C, Gitto E. Actions of melatonin in the reduction of oxidative stress. A review. J Biomed Sci 2000;7:444–58. https://doi.org/10.1007/BF02253360.

[4] Reiter RJ, Tan DX, Qi WB, Manchester LC, Karbownik M, Calvo JR. Pharmacology and physiology of melatonin in the reduction of oxidative stress in vivo. Biol Signals Recept 2000;9:160–71. https://doi.org/10.1159/000014636.

[5] Tan X, Manchester LC, Terron MP, Flores LJ, Reiter RJ. One molecule, many derivatives: A never-ending interaction of melatonin with reactive oxygen and nitrogen species? J Pineal Res 2007;42:28–42. https://doi.org/10.1111/j.1600-079X.2006.00407.x.

[6] Tan DX, Reiter RJ, Manchester LC, Yan MT, El-Sawi M, Sainz RM, et al. Chemical and physical properties and potential mechanisms: Melatonin as a broad spectrum antioxidant and free radical scavenger. Curr Top Med Chem 2002;2:181–97. https://doi.org/10.2174/1568026023394443.

[7] Hardeland R. Recent Findings in Melatonin Research and Their Relevance to the CNS. Cent Nerv Syst Agents Med Chem 2018;18(2):102–14. https://doi.org/10.2174/1871524918666180531083944.

[8] Hardeland R. Melatonin: Signaling mechanisms of a pleiotropic agent. BioFactors 2009;35:183–92. https://doi.org/10.1002/biof.23.
[9] Mayo JC, Tan DX, Sainz RM, Lopez-Burillo S, Reiter RJ. Oxidative damage to catalase induced by peroxyl radicals: functional protection by melatonin and other antioxidants. Free Radical Res 2003;37:543–53. https://doi.org/10.1080/1071576031000083206.
[10] Brunner P, Sözer-Topcular N, Jockers R, Ravid R, Angeloni D, Fraschini F, et al. Pineal and cortical melatonin receptors MT1 and MT2 are decreased in Alzheimer's disease. Eur J Histochem 2006;50:311–6. https://doi.org/10.5167/uzh-49561.
[11] Hardeland R, Cardinali DP, Srinivasan V, Spence DW, Brown GM, Pandi-Perumal SR. Melatonin—a pleiotropic, orchestrating regulator molecule. Prog Neurobiol 2011;93:350–84. https://doi.org/10.1016/j.pneurobio.2010.12.004.
[12] Kilic U, Caglayan AB, Beker MC, Gunal MY, Caglayan B, Yalcin E, et al. Particular phosphorylation of PI3K/Akt on Thr308 via PDK-1 and PTEN mediates melatonin's neuroprotective activity after focal cerebral ischemia in mice. Redox Biol 2017;12:657–65. https://doi.org/10.1016/j.redox.2017.04.006.
[13] Kilic U, Yilmaz B, Ugur M, Yuksel A, Reiter RJ, Hermann DM, et al. Evidence that membrane-bound G protein-coupled melatonin receptors MT1 and MT2 are not involved in the neuroprotective effects of melatonin in focal cerebral ischemia. J Pineal Res 2012;52:228–35. https://doi.org/10.1111/j.1600-079X.2011.00932.x.
[14] Selkoe DJ, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Mol Med 2016;8(6):595–608. https://doi.org/10.15252/emmm.201606210.
[15] Witt-Enderby PA, Bennett J, Jarzynka MJ, Firestine S, Melan MA. Melatonin receptors and their regulation: biochemical and structural mechanisms. Life Sci 2003;72:2183–98. https://doi.org/10.1016/S0024-3205(03)00098-5.
[16] Zhang Y, Cook A, Kim J, Baranov SV, Jiang J, Smith K, et al. Melatonin inhibits the caspase-1/cytochrome c/caspase-3 cell death pathway, inhibits MT1 receptor loss and delays disease progression in a mouse model of amyotrophic lateral sclerosis. Neurobiol Dis 2013;55:26–35. https://doi.org/10.1016/j.nbd.2013.03.008.
[17] Blennow K, de Leon MJ, Zetterberg H. Alzheimer's disease. Lancet 2006;368(9533):387–403. https://doi.org/10.1016/S0140-6736(06)69113-7.
[18] Musiek ES, Holtzman DM. Three dimensions of the amyloid hypothesis: time, space and 'wingmen'. Nat Neurosci 2015;18:800–6. https://doi.org/10.1038/nn.4018.
[19] Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, et al. Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. Nat Med 2008;14(8):837–42. https://doi.org/10.1038/nm1782.
[20] Ittner LM, Götz J. Amyloid-β and tau–a toxic pas de deux in Alzheimer's disease. Nat Rev Neurosci 2011;12(2):65–72. https://doi.org/10.1038/nrn2967.
[21] Götz J, Lim YA, Ke YD, Eckert A, Ittner LM. Dissecting toxicity of tau and beta-amyloid. Neurodegener Dis 2010;7(1–3):10–2. https://doi.org/10.1159/000283475.
[22] Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol 2014;30:255–89. https://doi.org/10.1146/annurev-cellbio-101512-122326.
[23] Lo Cicero A, Stahl PD, Raposo G. Extracellular vesicles shuffling intercellular messages: for good or for bad. Curr Opin Cell Biol 2015;35:69–77. https://doi.org/10.1016/j.ceb.2015.04.013.
[24] Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 2013;200:373–83. https://doi.org/10.1083/jcb.201211138.
[25] Rajendran L, Honsho M, Zahn TR, Keller P, Geiger KD, Verkade P, et al. Alzheimer's disease β-amyloid peptides are released in association with exosomes. Proc Natl Acad Sci USA 2006;103:11172–7. https://doi.org/10.1073/pnas.0603838103.
[26] Sharples RA, Vella LJ, Nisbet RM, Naylor R, Perez K, Barnham KJ, et al. Inhibition of γ-secretase causes increased secretion of amyloid precursor protein C-terminal fragments in association with exosomes. FASEB J 2008;22:1469–78. https://doi.org/10.1096/fj.07-9357com.
[27] Tsilioni I, Panagiotidou S, Theoharides TC. Exosomes in neurologic and psychiatric disorders. Clin Ther 2014;36(6):882–8. https://doi.org/10.1016/j.clinthera.2014.05.005.
[28] Yuyama K, Sun H, Usuki S, Sakai S, Hanamatsu H, Mioka T, et al. A potential function for neuronal exosomes: sequestering intracerebral amyloid-β peptide. FEBS Lett 2015;589:84–8. https://doi.org/10.1016/j.febslet.2014.11.027.
[29] Fiandaca MS, Kapogiannis D, Mapstone M, Boxer A, Eitan E, Schwartz JB, et al. Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study. Alzheimers Dement 2015;11(6):600–7. https://doi.org/10.1016/j.jalz.2014.06.008.
[30] Goetzl EJ, Kapogiannis D, Schwartz JB, Lobach IV, Goetzl L, Abner EL, et al. Decreased synaptic proteins in neuronal exosomes of frontotemporal dementia and Alzheimer's disease. FASEB J 2016;30(12):4141–8. https://doi.org/10.1096/fj.201600816R.
[31] Saman S, Lee NC, Inoyo I, Jin J, Li Z, Doyle T, et al. Proteins recruited to exosomes by tau overexpression implicate novel cellular mechanisms linking tau secretion with Alzheimer's disease. J Alzheimers Dis 2014;40:S47–70. https://doi.org/10.3233/JAD-132135.
[32] Saman S, Kim W, Raya M, Visnick Y, Miro S, Saman S, et al. Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease. J Biol Chem 2012;287:3842–9. https://doi.org/10.1074/jbc.M111.277061.
[33] Vella LJ, Hill AF, Cheng L. Focus on Extracellular Vesicles: Exosomes and Their Role in Protein Trafficking and Biomarker Potential in Alzheimer's and Parkinson's Disease. Int J Mol Sci 2016;17(2):173. https://doi.org/10.3390/ijms17020173.
[34] Yuyama K, Sun H, Sakai S, Mitsutake S, Okada M, Tahara H, et al. Decreased amyloid-β pathologies by intracerebral loading of glycosphingolipid-enriched exosomes in Alzheimer model mice. J Biol Chem 2014;289:24488–98. https://doi.org/10.1074/jbc.M114.577213.
[35] Yuyama K, Sun H, Mitsutake S, Igarashi Y. Sphingolipid-modulated exosome secretion promotes clearance of amyloid-β by microglia. J Biol Chem 2012;287(14):10977–89. https://doi.org/10.1074/jbc.M111.324616.
[36] Galano A, Tan DX, Reiter RJ. Melatonin as a natural ally against oxidative stress: a physicochemical examination. J Pineal Res 2011;51(1):1–16. https://doi.org/10.1111/j.1600-079X.2011.00916.x.
[37] Lin L, Huang QX, Yang SS, Chu J, Wang JZ, Tian Q. Melatonin in Alzheimer's disease. Int J Mol Sci 2013;14(7):14575–93. https://doi.org/10.3390/ijms140714575.
[38] Rosales-Corral SA, Acuña-Castroviejo D, Coto-Montes A, Boga JA, Manchester LC, Fuentes-Broto L, et al. Alzheimer's disease: pathological mechanisms and the beneficial role of melatonin. J Pineal Res 2012;52(2):167–202. https://doi.org/10.1111/j.1600-079X.2011.00937.x.
[39] BioTek Instruments USA, 2018. Technical Resources https://www.biotek.com/assets/tech_resources/Alzheimer's%20App%20Note_FINAL.pdf. (Accessed 20 March 2018).
[40] Manavalan S, Getachew B, Manaye KF, Khundmiri SJ, Csoka AB, McKinley R, et al. PACAP Protects Against Ethanol and Nicotine Toxicity in SH-SY5Y Cells: Implications for Drinking-Smoking Co-morbidity. Neurotox Res 2017;32(1):8–13. https://doi.org/10.1007/s12640-017-9727-8.
[41] Benilova I, Karran E, De Strooper B. The toxic Abeta oligomer and Alzheimer's disease: an emperor in need of clothes. Nat Neurosci 2012;15:349–57. https://doi.org/10.1038/nn.3028.
[42] Bennett DA, Schneider JA, Wilson RS, Bienias JL, Arnold SE. Neurofibrillary tangles mediate the association of amyloid load with clinical Alzheimer disease and level of cognitive function. Arch Neurol 2004;61:378–84. https://doi.org/10.1001/archneur.61.3.378.
[43] Kang JH. Cerebrospinal Fluid Amyloid β1-42, Tau, and Alpha-Synuclein Predict the Heterogeneous Progression of Cognitive Dysfunction in Parkinson's Disease. J Mov Disord 2016;9(2):89–96. https://doi.org/10.14802/jmd.16017.
[44] Lanctôt PV, Rajaram RD, Herrmann N. Therapy for Alzheimer's Disease: How Effective are Current Treatments? Ther Adv Neurol Disord 2009;2(3):163–80. https://doi.org/10.1177/1756285609102724.
[45] Arbor SC, LaFontaine M, Cumbay M. Amyloid-beta Alzheimer targets – protein processing, lipid rafts, and amyloid-beta pores. Yale J Biol Med 2016;89:5–21 [PMID: 27505013] https://www.ncbi.nlm.nih.gov/pubmed/27505013.
[46] Buée L, Bussière T, Buée-Scherrer V, Delacourte A, Hof PR. Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. Brain Res Rev 2000;33:95–130. https://doi.org/10.1016/S0165-0173(00)00019-9.
[47] Hasegawa M. Molecular Mechanisms in the Pathogenesis of Alzheimer's disease and Tauopathies-Prion-Like Seeded Aggregation and Phosphorylation. Biomolecules 2016;6(2):24. https://doi.org/10.3390/biom6020024.
[48] Khan SS, Bloom GS. Tau: The Center of a Signaling Nexus in Alzheimer's Disease. Front Neurosci 2016;9:31. https://doi.org/10.3389/fnins.2016.00031.
[49] Martin L, Latypova X, Terro F. Post-translational modifications of tau protein: implications for Alzheimer's disease. Neurochem Int 2011;58:458–71. https://doi.org/10.1016/j.neuint.2010.12.023.
[50] Medina M, Hernández F, Avila J. New Features about Tau Function and Dysfunction. Biomolecules 2016;6(2):21. https://doi.org/10.3390/biom6020021.
[51] Šimić G, Babić LM, Wray S, Harrington C, Delalle I, Jovanov-Milosevic N, et al. Tau Protein Hyperphosphorylation and Aggregation in Alzheimer's Disease and Other Tauopathies, and Possible Neuroprotective Strategies. Biomolecules 2016;6:6. https://doi.org/10.3390/biom6010006.
[52] Liu J, Su H, Qu QM. Carnosic Acid Prevents Beta-Amyloid-Induced Injury in Human Neuroblastoma SH-SY5Y Cells via the Induction of Autophagy. Neurochem Res 2016;41:2311–23. https://doi.org/10.1007/s11064-016-1945-6.
[53] Sadigh-Eteghad S, Sabermarouf B, Majdi A, Talebi M, Farhoudi M, Mahmoudi J. Amyloid-beta: a crucial factor in Alzheimer's disease. Med Princ Pract 2015;24:1–10. https://doi.org/10.1159/000369101.
[54] Sengupta U, Nilson AN, Kayed R. The Role of Amyloid-β Oligomers in Toxicity, Propagation, and Immunotherapy. EBioMedicine 2016;6:42–9. https://doi.org/10.1016/j.ebiom.2016.03.035.
[55] Tang Z, Ioja E, Bereczki E, Hultenby K, Li C, Guan Z, et al. mTor mediates tau localization and secretion: implication for Alzheimer's disease. Biochim Biophys Acta 2015;1853:1646–57. https://doi.org/10.1016/j.bbamcr.2015.03.003.
[56] Yang Y, Keene CD, Peskind ER, Galasko DR, Hu SC, Cudaback E, et al. Cerebrospinal Fluid Particles in Alzheimer Disease and Parkinson Disease. J Neuropathol Exp Neurol 2015;74(7):672–87. https://doi.org/10.1097/NEN.0000000000000207.
[57] Beker MC, Caglayan AB, Kelestemur T, Caglayan B, Yalcin E, Yulug B, et al. Effects of normobaric oxygen and melatonin on reperfusion injury: role of cerebral microcirculation. Oncotarget 2015;6:30604–14. , https://doi.org/10.18632/oncotarget.5773.

[58] Dinkins MB, Dasgupta S, Wang G, Zhu G, Bieberich E. Exosome reduction in vivo is associated with lower amyloid plaque load in the 5XFAD mouse model of Alzheimer's disease. Neurobiol Aging 2014;35:1792–800. https://doi.org/10.1016/j.neurobiolaging.2014.02.012.
[59] Hogan MV, El-Sherif Y, Wieraszko A. The modulation of neuronal activity by melatonin: in vitro studies on mouse hippocampal slices. J Pineal Res 2001;30:87–96. https://doi.org/10.1034/j.1600-079X.2001.300204.x.
[60] Huang YN, Lin CI, Liao H, Liu CY, Chen YH, Chiu WC, et al. Cholesterol overload induces apoptosis in SH-SY5Y human neuroblastoma cells through the up regulation of flotillin-2 in the lipid raft and the activation of BDNF/Trkb signaling. Neuroscience 2016;328:201–9. https://doi.org/10.1016/j.neuroscience.2016.04.043.
[61] Kilic U, Kilic E, Reiter RJ, Bassetti CL, Hermann DM. Signal transduction pathways involved in melatonin-induced neuroprotection after focal cerebral ischemia in mice. J Pineal Res 2005;38:67–71. https://doi.org/10.1111/j.1600-079X.2004.00178.x.
[62] Musshoff U, Riewenherm D, Berger E, Fauteck JD, Speckmann EJ. Melatonin receptors in rat hippocampus: molecular and functional investigations. Hippocampus 2002;12:165–73. https://doi.org/10.1002/hipo.1105.
[63] Ranade DS, Bapat AM, Ramteke SN, Joshi BN, Roussel P, Tomas A, et al. Thiosemicarbazone modification of 3-acetyl coumarin inhibits Aβ peptide aggregation and protect against Aβ-induced cytotoxicity. Eur J Med Chem 2016;121:803–9. https://doi.org/10.1016/j.ejmech.2015.07.028.
[64] Asai H, Ikezu S, Tsunoda S, Medalla M, Luebke J, Haydar T, et al. Depletion of microglia and inhibition of exosome synthesis halt tau propagation. Nat Neurosci 2015;18:1584–93. https://doi.org/10.1038/nn.4132.
[65] Chai X, Dage JL, Citron M. Constitutive secretion of tau protein by an unconventional mechanism. Neurobiol Dis 2012;48:356–66. https://doi.org/10.1016/j.nbd.2012.05.021.
[66] Gui Y, Liu H, Zhang L, Lv W, Hu X. Altered microRNA profiles in cerebrospinal fluid exosome in Parkinson disease and Alzheimer disease. Oncotarget 2015;6(35):37043–53. https://doi.org/10.18632/oncotarget.6158.
[67] Howitt J, Hill AD. Exosomes in the Pathology of Neurodegenerative Diseases. J Biol Chem 2016;291(52):26589–97. https://doi.org/10.1074/jbc.R116.757955.
[68] Lee CH, Yoo KY, Choi JH, Park OK, Hwang IK, Kwon YG, et al. Melatonin's protective action against ischemic neuronal damage is associated with up-regulation of the MT2 melatonin receptor. J Neurosci Res 2010;88:2630–40. https://doi.org/10.1002/jnr.22430.
[69] Van Niel G. Study of Exosomes Shed New Light on Physiology of Amyloidogenesis. Cell Mol Neurobiol 2016;36(3):327–42. https://doi.org/10.1007/s10571-016-0357-0.
[70] An K, Klyubin I, Kim Y, Jung JH, Mably AJ, O'Dowd ST, et al. Exosomes neutralize synaptic-plasticity-disrupting activity of Aβ assemblies in vivo. Mol Brain 2013;6:47. https://doi.org/10.1186/1756-6606-6-47.
[71] Avila J, Simón D, Díaz-Hernández M, Pintor J, Hernández F. Sources of extracellular tau and its signaling. J Alzheimers Dis 2014;40:S7–S15. https://doi.org/10.3233/JAD-131832.
[72] Cardinali DP, Furio AM, Brusco LI. Clinical aspects of melatonin intervention in Alzheimer's disease progression. Curr Neuropharmacol 2010;8:218–27. https://doi.org/10.2174/157015910792246209.
[73] Herholz K. Tau PET and tauopathies. Eur J Nucl Med Mol Imaging 2016;43:1684–5. https://doi.org/10.1007/s00259-016-3406-5.
[74] Joshi P, Benussi L, Furlan R, Ghidoni R, Verderio C. Extracellular vesicles in Alzheimer's disease: friends or foes? Focus on Aβ-vesicle interaction. Int J Mol Sci 2015;16:4800–48013. https://doi.org/10.3390/ijms16034800.
[75] Liu RY, Zhou JN, van Heerikhuize J, Hofman MA, Swaab DF. Decreased Melatonin Levels in Postmortem Cerebrospinal Fluid in Relation to Aging, Alzheimer's Disease, and Apolipoprotein E-Epsilon4/4 Genotype. J Clin Endocrinol Metab 1999;84:323–7. https://doi.org/10.1210/jcem.84.1.5394.
[76] Mahlberg R, Walther S, Kalus P, Bohner G, Haedel S, Reischies FM, et al. Pineal Calcification in Alzheimer's Disease: an in vivo Study Using Computed Tomography. Neurobiol Aging 2008;29:203–9. https://doi.org/10.1016/j.neurobiolaging.2006.10.003.
[77] Mishima K, Tozawa T, Satoh K, Matsumoto Y, Hishikawa Y, Okawa M. Melatonin Secretion Rhythm Disorders in Patients With Senile Dementia of Alzheimer's Type With Disturbed Sleep-Waking. Biol Psychiatry 1999;45:417–21. https://doi.org/10.1016/S0006-3223(97)00510-6.
[78] Ohashi Y, Okamoto N, Uchida K, Iyo M, Mori N, Morita Y. Daily Rhythm of Serum Melatonin Levels and Effect of Light Exposure in Patients with Dementia of the Alzheimer's Type. Biol Psychiatry 1999;45:1646–52. https://doi.org/10.1016/S0006-3223(98)00255-8.
[79] Perez-Gonzales R, Gauthier SA, Kumar A, Levy E. The exosome secretory pathway transports amyloid precursor protein carboxyl-terminal fragments from the cell into the brain extracellular space. J Biol Chem 2012;287(51):43108–15. https://doi.org/10.1074/jbc.M112.404467.
[80] Skene DJ, Vivien-Roels B, Sparks DL, Hunsaker JC, Pevet P, Ravid D, et al. Daily Variation in the Concentration of Melatonin and 5- Methoxytryptophol in the Human Pineal Gland: Effect of Age and Alzheimer's Disease. Brain Res 1990;528:170–4. https://doi.org/10.1016/0006-8993(90)90214-V.
[81] Uchida K, Okamoto N, Ohara K, Morita Y. Daily Rhythm of Serum Melatonin in Patients With Dementia of the Degenerate Type. Brain Res 1996;717:154–9. https://doi.org/10.1016/0006-8993(96)00086-8.
[82] Bondy SC, Yang YE, Walsh TJ, Gie YW, Lahiri DK. Dietary modulation of age-related changes in cerebral pro-oxidant status. Neurochem Int 2002;40(2):123–30. https://doi.org/10.1016/S0197-0186(01)00084-5.
[83] Lahiri DK, Chen D, Ge YW, Bondy SC. Sharman EH. Dietary supplementation with melatonin reduces levels of amyloid beta-peptides in the murine cerebral cortex. J Pineal Res 2004;36(4):224–31. https://doi.org/10.1111/j.1600-079X.2004.00121.x.
[84] Lahiri DK, Ge YW, Sharman EH, Bondy SC. Age-related changes in serum melatonin in mice: higher levels of combined melatonin and 6-hydroxymelatonin sulfate in the cerebral cortex than serum, heart, liver and kidney tissues. J Pineal Res 2004;36(4):217–23. https://doi.org/10.1111/j.1600-079X.2004.00120.x.
[85] Savaskan E, Jockers R, Ayoub M, Angeloni D, Fraschini F, Flammer J, et al. The MT2 melatonin receptor subtype is present in human retina and decreases in Alzheimer's disease. Curr Alzheimer Res 2007;4(1):47–51. https://doi.org/10.2174/156720507779939823.