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The effects of lack of melatonin in experimental rat model of Alzheimer's Disease: relationship with FEZ1 gene expression

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Abstract
Alzheimer's Disease (AD), which is the most common reason for dementia, is an irreversible neurodegenerative brain disease. FEZ1 is a protein expressed in the brain. High expressions of FEZ1 mRNA have been interpreted as an indicator of high neural plasticity for memory and learning. This study was planned to determine the effect of melatonin deficiency (pinealectomy; PnX) on AD and to reveal its association with FEZ1. 30 male rats were used in the study. The rats were divided into three as SHAM, PnX+streptozotocin (STZ) and PnX+STZ+melatonin (MLT) groups. The pineal glands of rats were surgically removed (except SHAM group) and STZ was intracerebroventricularly (icv) administrated at the first and third days. MLT (10 mg/kg/day) was intraperitoneally (ip) injected 1 h before the first STZ application and it was continued for 14 days. STZ and MLT solvents were applied to the rats in the SHAM group. At the end of the applications, Morris water maze (MWM) test was carried out the rats. At the end of MWM tests, the rats were sacrificed and their blood and hippocampus tissues were taken. FEZ1 gene expression and protein levels were determined from hippocampus tissue, while serum nonadrenaline, dopamine and serotonin levels were detected from blood samples. FEZ1 protein levels of PnX+STZ+MLT group were found to be statistically significantly lower than those of SHAM and PnX+STZ groups ($p<0.05$). While noradrenaline levels were found to be lower in both groups when compared with the SHAM group ($p<0.05$), no difference was found between the dopamine and serotonin levels of groups. Our results showed that in AD made up in the deficiency of melatonin, FEZ1 levels got higher and the increases were revealed to return to normal levels with exogenous melatonin application.

Keywords: FEZ1, Alzheimer's disease, pinealectomy, melatonin

Introductıon

Alzheimer's Disease (AH), which is a progressive form of dementia, is a neurodegenerative disease which causes disorders in memory, judgment, decision making and adapting to physical environment and it is characterized by irreversible neural loss especially in the hippocampus and cortex [1,2]. The number of people suffering from this disease is expected to reach 65.7 million by 2030 and 115.4 million by 2050 [3]. AD is characterized by two histopathological lesions called neurofibrillary tangle (NFT) and amyloidal plaque (AP) [4,5]. AP is formed as a result of the accumulation of extracellular amyloid (Aβ) peptides, while NFT is formed as a result of the accumulation of intracellular hyperphosphorylated tau proteins [6]. Amyloid precursor protein (APP) has a great importance in the pathophysiology of AD caused by Aβ peptide production through ordered proteolytic split [7]. APs are formed by the extracellular accumulation of 40-42 amino acid long Aβ protein [8] which occurs as a result of APP's proteolytic splits [9]. APP is a membrane protein cut by two different proteolytic enzyme called α and β secretase. This cutting process by α and β secretase is followed by the cutting procedure by γ-secretase [10,11]. If this cutting occurs by β-secretase, it turns into a form called Aβ; if it occurs by α-secretase, it turns into a form called P3 which is accepted as non-amyloidogenic [12,13]. The fact that AD is a well-defined model that begins from the neurons in the transentorhinal region of the brain and spreads to hippocampus and finally to the cortex. Thus, brain areas that show tau accumulation reflect the advance of clinical symptoms from mild cognitive disorder to intense dementia [14]. In AD, hyperphosphorylated tau proteins accumulate in the axoplasm and eventually they aggregate in matched spiral and plain filaments and bond to form tangles [15]. FEZ1 is a brain-specific coiled-coil protein, it is expressed in neurons and it contains 392-amino acid residues [16]. FEZ1 is expressed in all developmental stages of the central nervous system in rats

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and also FEZ1 has been reported to be necessary for neural development [17], neural polarization and transport processes [18]. With the help of proteomic approaches, FEZ1 has been found to have a role in the transport of loads, cell cycle regulation and retrovirus spread and to be in interaction with various functional proteins [19]. Studies have shown that a great number of neurological diseases result from functional disorders in neocortex and that these diseases can be associated with FEZ1 expressed in the deep parts of neocortex [20]. Axonal mitochondrial transport is very important for the axon structure and the defects in this transport can cause neurodegenerative diseases [21]. Aβ is suggested to have harmful effects on the mitochondrial function of the brain in AD [22]. In addition, FEZ1 is stated to be required for axonal mitochondrial movement in hippocampal neurons [18].

Melatonin, which is released by pineal gland has been reported to act as a cytoskeleton modulator and have the ability to influence microfilament and microtubule [23], and to have a role in the neuroprotective mechanism in AD by having an effect on mitochondrial functions [24]. Studies have shown melatonin to be effective in stopping neurodegenerative cases seen in ischemic stroke, Parkinsonism and AD experimental models [25]. The fact that FEZ1 regulates mitochondrial transport and shows high neural plasticity for long term memory and learning [16], brings to mind that there may be a possible relationship between melatonin and FEZ1. This study was planned to find out how AD formation is influenced especially in the deficiency of MLT and to present the relationship between this state and FEZ1.

Materıal and Method

In this study, 30 male rats with a weight of between 220-280 gr taken from Inonu University Experimental Animals Research and Application Center were used. The rats were divided into three groups as SHAM, pinealectomy (PnX)+streptozotocin (STZ) and PnX+STZ+melatonin (MLT) (10 rats in each group). The rats were kept in an environment with a temperature of 21±1ºC and a period of 12 h of light/dark. They were given normal tap water and fed *ad libitum* with standard rat feed. All the applications in the study were performed as stated in Inonu University Experimental Animals Ethical Board protocol (Protocol # 2014/A-50).

Pinealectomy: The rats, which were anesthetized with 70 mg/kg ketamine (Richter Pharma AG, Australia) and 8 mg/kg xylazine (Bioveta PLC, Czech Republic), were placed in stereotaxic device (Small Animal Stereotaxic System, ASI Instruments, USA). A cut was made on the skull skin and subcutaneous tissue and a circular cut with a diameter of 3 mm was made on the upper parts of the skull according to Lambda reference point by using micro cutter drill (Proxxon MİCROMOT 50/E, Germany) and the pineal gland under venous sinus was taken out in one piece with the help of a fine-tipped forceps.

Intracerebroventricular STZ injections:
Intracerebroventricular (icv) STZ induced-cognitive impairment has been commonly used as an experimental model of Alzheimer's disease [26]. STZ administered rats are proposed as a probable experimental model of sporadic Alzheimer's disease. Recent studies suggest that central STZ administration induces brain pathology and behavioural alterations resembling those in sporadic Alzheimer's disease patients [27]. STZ produces similar characteristic pathology such as altered glucose metabolism, insulin signaling, synaptic dysfunction, protein kinases such as tau hyperphosphorylation, Aβ deposition, and neuronal apoptosis. In this study, STZ was administered (icv) bilaterally 3 mg/kg in rats [28]. For the first STZ injection to pinealectomised rats, The coordinates of the right and left lateral ventricle were found by taking the bregma point as reference (0.8 mm posterior from Bregma, 1.4 mm lateral from sagittal suture and 4.8 mm vertical from skull bone) for [29] 3 mg/kg STZ solubilized in sterile artificial brain-cerebrospinal fluid (aCSF) was injected into both lateral ventricles of the rats with the help of Hamilton injector, 10 µl in each ventricle; the injection was repeated 48 h later [30].

Intraperitoneal MLT injections:
MLT prepared with a final concentration of 10 mg/kg was solubilized in ethanol and suitable concentration was adjusted with physiological saline solution. While this solution was prepared, care was taken not to make the final concentration of ethanol more than 0.5% [31, 32]. 10 mg/kg/day ip melatonin injection was started 1 h before the first dose STZ application and it was continued for 14 days [33].

Morris Water Maze Test
MWM tank was used to test the animals' spatial learning and memories [34]. MWM is a 30 cm high, 150 cm in diameter, made from stainless steel, black and big circular pool. The temperature of the water is automatically kept at the level of 23±1°C [35]. A black platform in 8 cm diameter was placed 1 cm under the water levels. The water in the tank was colored with a non-toxic black dye (Mixol concentrate colorizer 20 ml No:1 black) in order to hide the platform. The data were obtained with the help of a camera attached to the video monitoring system fixed on the roof of the pool's center which was hypothetically divided into four quarters (north, south, east, west) [36]. Before MWM test the rats in the all groups were made to swim free without platform to get used to the environment. MWM test started 14 days after applications and operations and they were repeated four times a day for five days [37]. Each rat was allowed for 90 seconds to look for the platform. The rats which found the platform within this time were allowed for 30 seconds to stay on the platform. The rats which could not find the platform in 90 seconds

were left on the platform and they were made to stay on the platform for 30 seconds. The time to reach the platform, the distance swam to find the platform and the period of time on quadrant were measured and recorded [38]. The scores were analyzed with EthoVision XT10 Image Analysis (Noldus Information Tech.) software program.

Taking blood and tissue

Following the behavioral tests, the animals were sacrificed and their hippocampus tissues were separated from brain tissue on dry ice and kept in -80°C until FEZ1 gene expression and protein amount analyses. The serums obtained from blood samples taken from animals were also kept in -80°C until dopamine, serotonin and noradrenaline (NA) analyses.

Real Time-PCR

Brain tissue samples taken from groups were put in a RNA later solution to determine levels of β-actin and FEZ1 mRNA and total RNA distillation was made from these tissues by using High Pure RNA Tissue kit (Roche, USA; lot no: 11596700, ref no: 12033674001). The samples were found to have pure RNA in consequence of the spectrophotometric analyses of RNA samples. The amount of RNAs was measured at the spectrum of 260 and 280 nM UV by using Gen5 programs and spectrophotometer (BioTek, USA) and RNA amount was calculated in ng/µL. Transcriptor First strand cDNA Synthesis kit (Lot no:10842322, Ref no: 04 896 866 001, Roche, Switzerland) was used for cDNA synthesis and conducted on real time-PCR (RT-PCR) device (Roche Light Cycler, , Roche, Switzerland) by using hydrolysis probed primers. cDNAs obtained from RNAs distilled from each sample, β-Actin was reproduced with RT-PCR by using primers specific to FEZ1 genes (Table 1) and the change in gene expression were determined in proportion to β-actin gene.

Table 1. Primers of β-Actin and FEZ1 genes

Gene	Primers sequence(Forward ve Reverse)	Accession number	Size (bp)
β-Actin	F: 5'CTAAGGCCAACCGTGAAAAG 3' R: 5'GCCTGGATGGCTACGTACA 3'	NM_031144.3	79 bp
FEZ1	F: 5'CTCCAGTGAAGAACCAGTTGC 3' R: 5'GTCAGAGCATCCCAAACCTC 3'	NM_031066.1	76 bp

Western Blot Analysis

Samples which contained 50 µg total protein from each tissue lysate were performed at 90V with sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis at a stage of 6-12% and transferred to nitrocellulose membrane. After the transfer procedure, membrane was blotted for 1 h in 5% dried milk solution which prepared with Tris-Buffer-Salin (TBS) including tween 20. Tween 20 including TBS (TBS/T) and it was washed three times with TBS/T with time intervals of 10 min. It was incubated for a night at +4°C with monoclonal FEZ1 antibody diluted at a rate of 1:1000 in 2.5% dried milk. After this, it was washed three times with TBS/T with time intervals of 10 min and it was treated for an hour in room temperature with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody prepared in 2.5% dried milk solution. After being washed, the membranes were treated with Luminol and peroxide mixed 1:1 and they were monitored with UVP ChemiDoc-It2 chemoiuminescence. B-actin antibody was used as loading control and as a result of being treated as secondary antibody, the images were taken. Band volumes were assessed found by using ImageJ.

Statistical Analysis

IBM SPSS Statistics version 22.0 for Windows package program was used for the statistical analysis of data. Shapiro Wilk test was used to find out whether the data were normally distributed. Kruskal-Wallis test or one way Anova was used for comparisons between groups. In multiple comparisons, after one way Anova analysis, Tamhane test was used for variances that were not homogenous, while Conover test was used after Kruskal-Wallis test. The data were expressed in Mean±SD. $p<0.05$ was accepted as statistically significant.

Results

MWM Test Results

Swimming Distance: Average swimming distances in PnX+STZ were found to be significantly higher than SHAM and PnX+STZ+MLT groups ($p<0.05$) (Figure 1A). No statistically significant difference was found between SHAM and PnX+STZ+MLT groups on the first and fifth days.

Swimming Times: When the post-operative fifth day swimming times were compared, average swimming times in PnX+STZ were found to be significantly higher than SHAM and PnX+STZ+MLT groups ($p<0.05$). In swimming times of group PnX+STZ+MLT, a statistically significant decrease was found when compared with other groups ($p<0.05$) (Figure 1B).

Time to stay in the quadrant: When the times for staying in the quadrant for post-op first day were compared between groups, it was found that there was a significant decrease in PnX+STZ's time for staying in the quadrant when compared with SHAM and PnX+STZ+MLT ($p<0.05$). When the times for staying in the quadrant for post-op fifth day was compared between groups, it was found that the times for staying in the quadrant was statistically higher than PnX+STZ group ($p<0.05$) (Figure 1C).

Percentage of time to stay in the quadrant: When the percentages of staying in the quadrant on the first postop 1st day were compared within groups, a statistically significant decrease was found in group PnX+STZ's percentage of staying in the quadrant when compared with groups SHAM and PnX+STZ+MLT ($p<0.05$). When the percentages of staying in the quadrant on the fifth postop day were compared between groups PnX+STZ, it was found that SHAM and PnX+STZ+MLT groups' percentages of staying in the quadrant were statistically higher than PnX+STZ ($p<0.05$) (Figure 1D).

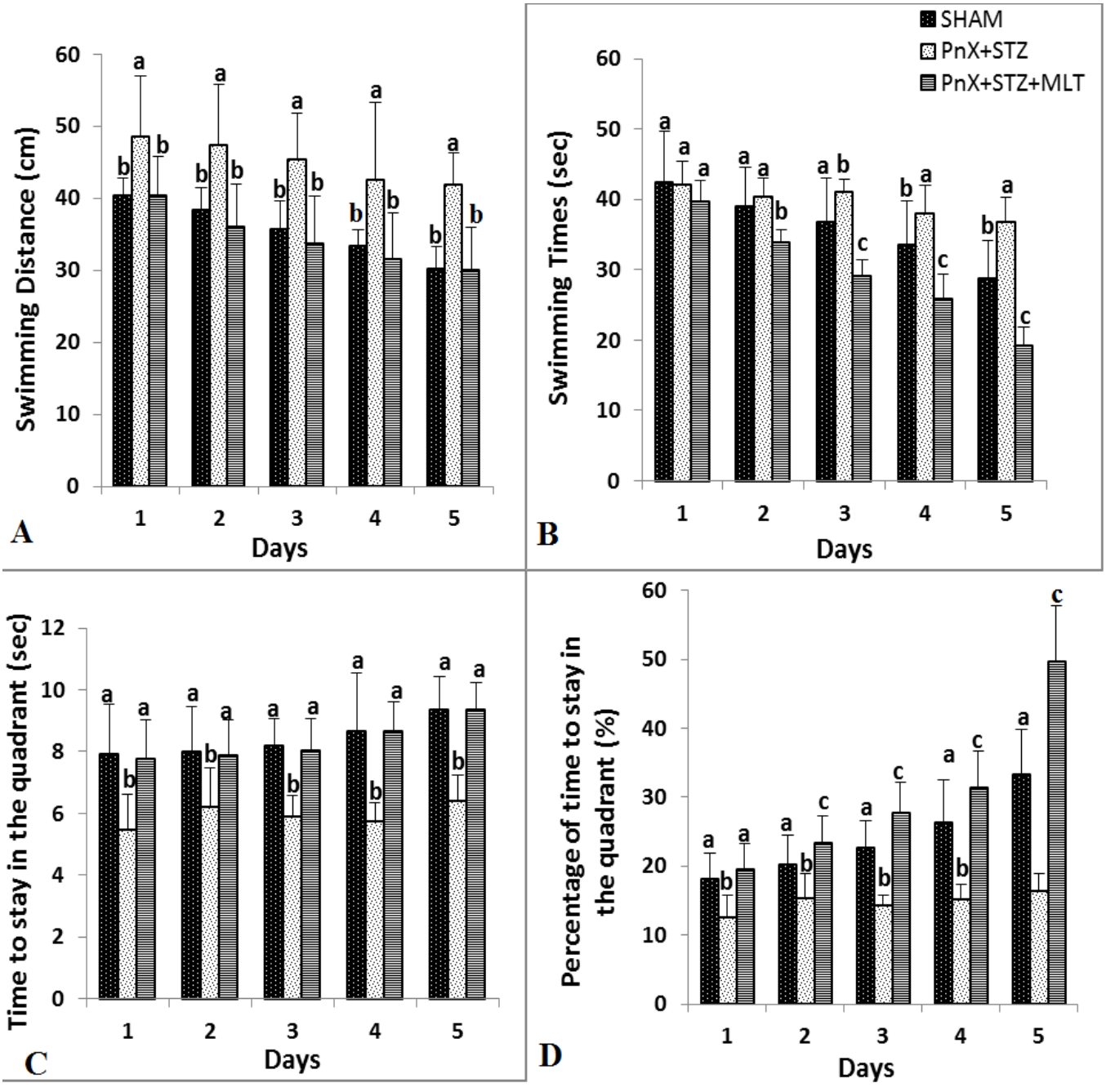

Figure 1. Average swimming scores of animals in 5-day-long swimming tests. Swimming distance (A), swimming times (B), time to stay in the quadrant (C) ve percentage of time to stay in the quadrant (D).

(In the graph, the groups were compared within each other every day; but no comparisons were made between days. The groups which carry different letters are statistically different from each other $p<0.05$. Vertical bars show the standard deviation of the average.(The groups which carry different letters in columns are statistically significant to each other)

RT-PCR Results

FEZ1/β-actin mRNA rate of PnX+STZ was found to be statistically significantly low when compared with PnX+STZ+MLT group ($p<0.05$) (Figure 2).

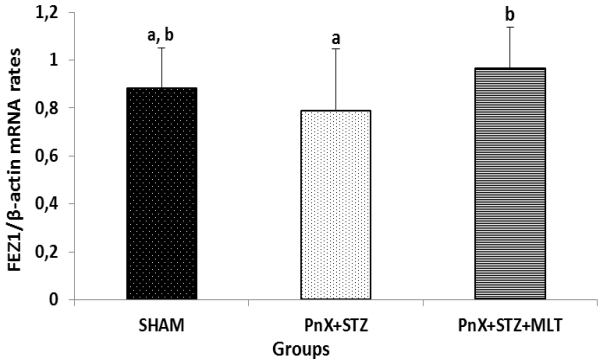

Figure 2. FEZ1/β-actin mRNA rates of groups. (The groups which carry different letters in columns are statistically significant to each other) ($p<0.05$).

Western Blot Analysis Results

When the hippocampus Fez1/β-actin protein rates of rats in all groups were compared, it was found to be significantly low in PnX+STZ+MLT group when compared with SHAM and PnX+STZ groups ($p<0.05$) (Figure3).

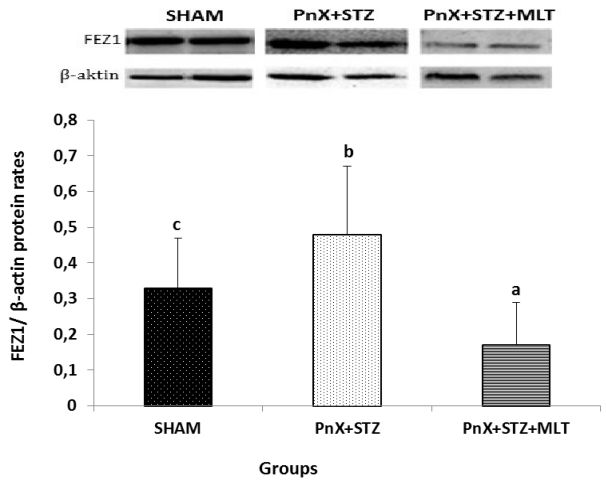

Figure 3. FEZ1/ β-actin protein rates of groups. (The groups which carry different letters in columns are statistically significant to each other) ($p<0.05$).

Serum Dopamine, Serotonin ve NA Results

Table 2 shows serum NA, dopamine and serotonin levels of the animals. In comparisons between groups, serum NA levels of both PnX+STZ and PnX+STZ+MLT groups were found to be statistically significantly lower when compared with SHAM group ($p<0.05$). No statistical difference was found between the groups in terms of serum dopamine and serotonin levels.

Tablo 2. Serum NA, dopamine and serotonin levels of groups.

GROUPS	n	Noradrenaline (pg/ml)	Dopamine (ng/ml)	Serotonin (ng/ml)
SHAM	10	1339.59±633.9 [a]	0.15±0.05 [a]	28.69±12.28 [a]
PnX+STZ	10	630.69±316.38 [b]	0.16±0.05 [a]	34.20±20.72 [a]
X+ STZ+MLT	10	515.88±222.5 [b]	0.15±0.05 [a]	30.63±22.10 [a]

The data were expressed in Mean±SD. $p<0.05$ was accepted as statistically significant. The groups which carry different letters in columns are statistically significant to each other ($p<0.05$))

Discussion

While it was reported that decreased levels of melatonin and melatonin rhythm loss can cause disorder of noradrenergic innervations [39], it is also suggested that melatonin can increase activities about learning and memory, decrease Aβ loads, and become a treatment approach in AD like neurodegeneration [40]. In addition, it has also been reported that melatonin has an ability to influence microfilament and microtubule [23], and also neural plasticity loss caused by aging can be prevented with melatonin [41, 42].

Melatonin has been reported to influence mitochondrial functions and thus play a role in the neuroprotective mechanism of AD [24], and it is believed that this protective effect is partly mitochondria mediated [43]. It is suggested that the decrease in melatonin production can be effective in the development of neurodegenerative diseases such as AD in later ages [25]. In a study which started chronic melatonin treatment to transgenic rats (Tg2576 rats) which carried amyloid plaque previously (4 months later) no statistical difference was found between mice which could see and which could not [44]. In another study, it was stated that melatonin could be a therapeutic candidate as antioxidant therapy only at this stage of disease and under oxidative stress seen in the early pathogenesis of AD [45]. In our study, before PnX procedure and icv STZ application, ip melatonin injection was started to rats and these injections were continued for 14 days. The statistical difference between PnX+STZ and PnX+STZ+MLT in MWM tests is a proof that the melatonin application started before application and injections may have a protective/preventive function. It is suggested that melatonin protects neural cells from Aβ mediated toxicity effectively by inhibiting Aβ production and preventing the formation of amyloid fibrils. Recent studies suggest that melatonin decreases Alzheimer like tau hyperphosphorylation and plays a role in the protection of

neurons [46]. In a study conducted with experimental Alzheimer models, it was stated that it had effects such as healing anti-oxidative damage, anti-apoptosis and cognitive functions [47]. In a study which was conducted with transgenic rats which were given exogenous melatonin injections and those which were not given exogenous melatonin injections, Aβ increase was partly inhibited and transgenic rats which had injection had more chances to survive [32].

Transport of synaptic vesicules through axonal transport is necessary for neurons to communicate. Mitochondrial movement in axons occurs through two motor proteins called dynein and kinesine which have a duty in the transport of intercellular cargo along the microtubule which includes synaptic vesicules [48-50]. In the absence of this cargo, kinesin-1 autoinhibiton mechanism steps in order not to spend energy in vain and to ensure the control of motor activity [21]. In a study, it was suggested that JNK interacting Protein 1 (JIP1) cargo protein was not sufficient for microtubule binding and activating motility motor and that FEZ1 protein had to be bound from one area of kinesin-1 to another. [51]. FEZ1 has been shown to interact with tubulin and kinesine motor proteins and it has been reported to control the movement of mitochondria the growing neuritis of PC12 cells stimulated by nerve growth factors [52]. Melatonin has also been reported to play a role in neuroprotective mechanism of AD by influencing mitochondrial factors [24]. Although there are a great number of studies about AD and melatonin (humans and animals) in literature, most of these studies question the protective mechanism as a result of the determination of melatonin levels and the application of exogenous melatonin.

In our study, PnX was performed, AD model was formed in the absence of MLT and the effect of melatonin (MLT absence) in the pathogenesis of the disease was presented more clearly. The increase of FEZ1 protein levels in PnX+STZ group is important in that it shows the presence of a relationship between AD and FEZ1. In addition, return to control levels of FEZ1 levels with MLT application supports this idea that the known positive effect of MLT on AD can be associated with FEZ1. Future studies on the mechanism will be important in terms of understanding this subject better.

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