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OBESITY-DERIVED DYSREGULATION OF NEUROSTEROIDS AND ITS IMPACT ON NEUROPROTEINS CONTENT IN BLOOD SERUM OF CHILDREN

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Abstract
Childhood obesity is a serious medical condition that affects physical health, social and emotional well-being, and self confidence in more than 15 % of children. Obese children suffer from different degrees of neurological complexity with unknown origin. Therefore, this study aimed to clarify some ambiguities in obesity-derived neuropathology by evaluation of the neurosteroids (NS) and neuromodulatory proteins. For this purpose, gas chromatography was used to detect and evaluate NS in blood serum samples. The brain-derived neurotrophic factor (BDNF) and Neuropeptide Y (NPY) were measured by using specific antibody as vital biomarkers of neurodevelopment and acetylcholinesterase (AChE) and tau proteins were assessed as risk factors for neural damages. Results showed cholic acid, deoxycholic acid and lithocholic acid increased in obese samples in comparison with healthy-weight children. While tetrahydrodeoxycorticosterone decreased and tau protein increased in circulation of obese children that represented neural damages. Decreased amounts of BDNF and NPY also could be refer to neural development disruption. The results elucidated NS biological functions in direct modulation of molecular events and also indirectly by gene expression regulation. Our results confirmed involvement of NS in obesity but it's not clear that NS dysregulation is cause or consequence of overeating behavior. Overall, obesity-derived molecular events are associated with dementia pathophysiology that warns about the chance of dementia later in older age.

Keyword: Obesity; Risk of dementia; High-fat pathophysiology; Neurodevelopment; Tau protein

Introduction
Obesity is known as a main public health in both developed and developing countries that is a result of overnutrition, high-fat foods, less physical activity and genetic background (Lin and Li 2021). It could be a serious situation that cause cardiovascular disease (CVD), hyperlipidemia and diabetes (Lin and Li 2021). Therefore, obesity reduces life quality and life span and also enhances human death rate due to diabetes complication and side effects of myocardial infarction (Lin and Li 2021). Accumulation of ectopic lipids in adipose tissue is accompanied by raised fatty acid (FA) content in blood serum that leads to increased lipids in non-adipose tissue and applies metabolic abnormalities in cardiac, hepatic, vasculature, brain and etc (Uganami et al. 2021). Previous studies reported some neurological complications in fat people which affects central nervous system (CNS) and peripheral

nervous system (PNS) (O'Brien et al. 2017). Both of the CNS and PNS are susceptible to obesity-mediated dysregulations that could be caused by direct accumulation of lipids and metabolic dysregulation or indirectly by insulin resistance and increased body mass index (BMI) (Uganami et al. 2021; Hölscher 2020). The recent meta-analysis manifested a positive correlation between obesity and neurological disorders such as Alzheimer's (AD) and Parkinson's disease (PD) and other types of dementia (Chen et al. 2014). High amounts of amyloid β (Aβ), β-amyloid precursor protein (APP) and tau contents that are essential risk factor for dementia related disease significantly increase in hippocampus tissue of obese patients (Mrak 2009). While in children, increased BMI causes abnormalities in neurological function as a result of structural changes in the brain and diminished cerebral integrity, especially in the hippocampus which play role in learning, memory and thinking ability (Alosco et al. 2014). Increased BMI also accompanied with reduced volume of frontal and limbic cerebral gray matter regions and also decreased hippocampal volume in obese children (Yau et al. 2012). The mentioned high-grade of destruction was certainly caused by brain related metabolites and proteins. According to the previous studies neurosteroids (NS), endogenous or exogenous steroids that modulate neural function by rapid non-genomic actions, play crucial role in CNS (Frye 2009; Reddy 2010). Some of the neurotransmitters and neurohormones exerts their role by mediation of the NS (Do Rego et al. 2012). Neuroactive steroids abnormal circular content causes pathological conditions in CNS and they have high potential to be used as sedative drugs (Eddy 2003). According to our knowledge NS impact on obesity have not been studied yet. They also regulate gene expression and influence the neuroactive peptides and proteins expression so are involved in feeding regulation, circadian rhythm and cognition (Do Rego et al. 2012; Ubuka and Tsutsui 2022). Multiple studies have demonstrated that cholic acid (CA) and deoxycholic acid (DCA) regulated cholinergic function in animal model of AD through inhibition of the acetylcholinesterase (AChE) enzyme (Sadeghi et al. 2020). Our recent study also approved Tetra hydro deoxycorticosterone (THDOC) could inhibit catalytic and non-catalytic function of AChE and reduce plague formation in AD (Saleh and Sadeghi 2019). While different types of NS could affect Aβ expression in hippocampal tissue (Sadeghi et al. 2020; Akwa 2020). Therefore, absence or decreased level of NS during child's development and in adults may be accompanied with neurological, cognitive, or behavioral abnormalities (Frye 2009). Therefore, this study aimed to evaluate the possible effects of obesity on circular NS level that seems be a reason for cognitive and learning dysfunction in overweight children (Ratner et al. 2019). For this purpose, CA, DCA, THDOC, lithocholic acid (LCA) and allopregnanolone (AP) were measured in blood sample of obese children with an increased BMI compared with normal-weight control. We also tried to evaluate the obesity-mediated dysregulation in neurodevelopment through measurement of brain-derived neurotrophic factor (BDNF), Neuropeptide Y (NPY), AChE and tau proteins as essential risk factors for neural damages. Actually, we want to study the possible cross talk between NS and neurodevelopment-related proteins in childhood obesity. This area of research could increase our basic knowledge about the molecular mechanism of NS function and helps to choose more effective targets to drug design in cognitive and dementia related disease.

Material and methods

Experimental design and samples

This is an experimental study was done on 40 obese children and 40 normal-weight and age-matched control were referred to the Clinical laboratory of Imam Reza Hospital, Mashhad, Iran. Ethical approve and patients consent statement were obtained from all of the participants and their parents. Inclusion criteria for patients with obesity are age between 6 and 13 years old and BMI at or above the 95th percentile on the CDC growth charts (Oliveira et al. 2022). Patients with liver deficiency, kidney disorder, thyroid disorder, acute coronary syndromes, different cancers, patients taking vitamin supplements and people with family history of dementia were excluded from the study. Control group was selected among age- and sex-matched normal weight children with BMI between the 5th and 85th percentile that had not been diagnosed with diabetes or prediabetes and don't suffer from heart

disease, thyroid disorder and familial history of AD or other neurological disorders. The number of girls and boys in two experimental groups is similar and statistical analysis could not detect any significant difference. General characteristics and circular triglyceride (TG) and thyroid hormones level related to the patients and control cases were joined this study are shown in table 1.

About 7 mL of venous blood were collected in an anti-coagulated tubes containing sodium EDTA and the second plain tube for serum will be taken from all participant children and blood serum will be separated by centrifugation at 1500 g for 10 min at 4°C, and separated to small aliquots, and stored at -20 °C until its use for the experimental tests.

Gas chromatography/mass spectrometry (GC/MS) analysis of NS in serum samples

The measurement was performed with three sample groups: standard solution, blood serum related to normal-weight control and serum samples of over-weight children. The multiple standard solution contains 1 µg/ml CA, 10 µg/ml DCA, 100 µg/ml LCA and 50 µg/ml THDOC in phosphate buffer (10 mM, pH 7.2) which has BSA 1%. The first step is extraction of NS from each sample according to previous study (Valverde-Som et al. 2018). 200 µL of each sample were added into a glass tube along with 40 µL botulin as internal standard and 0.5 mL of ethyl acetate (EA). The tube was mixed in 1 min and sonicated for 15 min, then centrifuged for 5 min at 10,000 rpm to separate organic phase that contains NS and should be stored in the dark place at -20 °C until the analysis was completed.

GC/MS analysis was performed using an Agilent 6890 gas chromatograph equipped with an Agilent 5973 mass spectrometer (Agilent Technologies, Palo Alto, USA) operating in electron impact ionisation (EI) mode at 70 eV. The system was fitted with a 30 m × 0.25 mm HP-5MS capillary column saturated with a 5 % diphenyl-95 % dimethyl polysiloxane as stationary phase. Helium was used as the carrier gas. Selective ion monitoring (SIM) mode was used to monitor different ions in each part. Peak identification was based on the comparison of retention times (RT) and MS signals with peaks resulted from multiple standard solution analysis.

Western blot

The content of BDNF, AChE and tau were measured by western-blot analysis due to antibodies availability. Anti-BDNF antibody (ab226843) and Anti-Acetylcholinesterase antibody (ab31276) were prepared from Abcam Company, USA and Anti-tau antibody (RTM38) was purchased from FUJIFILM Wako Pure Chemical Corporation, USA. Western blotting was carried out according to our previous work (Sadeghi et al. 2017). At first whole proteome of the plasma samples were separated by using SDS-PAGE and then actively transferred onto PVDF membrane in the transfer buffer at 140 V for 1.5–2 h. After transfer process, the membrane was incubated in the presence of primary specific antibodies (anti-BDNF, anti-AChE and anti-tau in 1:1500 dilution) after washing with TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and blocking with 5% BSA in TBST. The membrane was washed four times again and incubated in secondary HRP-conjugated antibody. The bands which have our goal protein were imagined using an ECL detection system according to the guide. Density of each band was quantified by using ImageJ 1.46r; Java 1.6.0_20 software. β-actin (1:1000) (Cell Signaling Technology) was used as a housekeeping protein to control protein concentration in lanes.

Measurement of NPY in plasma samples

Plasma samples were collected in EDTA container tubes and were immediately centrifuged for 10 min at 3000 rpm. After centrifugation, each sample was labeled and stored at -20 °C until use. Quantification of NPY in plasma was performed using a specific ELISA kit (Human Neuropeptide Y (NPY) ELISA | EZHNPY-25K, from Sigma-Aldrich company). All assays were performed according to the manufacturer's protocol and reagents were kept at room temperature (20–25 °C) at least 30 min before use.

Statistical analysis

Statistical evaluation was done by using the One-Way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test to determine statistical differences between results related to obesity patients and normal-weight children as control. Tukey's multiple comparison test was applied to compare two experimental groups with each other and significant differences were showed in each plot by indication of star symbol according to the P value. All statistical analyses were performed with the GraphPad Prism software (version 9.5, GraphPad Software, Inc. San Diego, CA, USA).

Results

By considering altered mood, cognition and learning ability in obese children, this study aimed to investigate the possible mechanism in the levels of neuro-active steroids and neuroproteins to illuminate neurological complexity that can be observed in overweight children. For this resolve, blood samples related to 40 obese children and 40 healthy-weight children were prepared and samples categorized based on CDC growth chart (Oliveira et al. 2022). Some important biochemical parameters were measured in each group. TG content was evaluated to be 154.11±9.63 and 185.72±21.73 mg/dl in control and obesity groups respectively that shows significant difference based on statistical analysis ($P<0.05$). Total cholesterol in obesity group (182.21±11.52 mg/dl) is remarkably ($P<0.05$) more than healthy-weight control (165.27±8.95 mg/dl). Thyroid stimulating hormone (TSH) and thyroid hormones did not show significant changes between experimental groups ($P>0.05$). The average age of samples in both groups is 9±4 years. Sphingomyelin content was also measured to be 24.62±5.26 mg/dl that significantly reduced in obese children (18.14±6.12 mg/dl) ($P<0.05$).

Obesity causes dysregulation of NS in children

NS are specific metabolites that mainly form by neural cells and also release to the blood therefore, its concentration in nervous tissue is more than blood serum (Reddy 2010). But measurement of NS in serum is useful to evaluate them level in CNS. They could regulate some important events in CNS such as proliferation, development and death of neural and non-neural cells while their specific function in peripheral tissues is not examined exactly (Frye 2009; Mellon 2007). This study used the GC/MS as an analytical platform to detect and quantify NS in plasma samples according to the previous reports (Valverde-Som et al. 2018). Each peak identified according to retention time (RT) that was compared with standard solution and extra peaks that could not be identified removed in each chromatogram. After biological and technical repeat, software calculated area of each peak that represented concentration of NS in serum samples. Fig 1 represents chromatograms related to standard solution, normal-weight and over-weight groups that quantified as Table 2 that shows RT, molecular weight (Mw), area of peaks for each NS. We also calculated fold change of each NS that refer to difference of NS concentration in blood serum of obesity in comparison with control. According to the results CA, DCA and LCA increased in obesity samples. CA showed 40 % raise and DCA manifested 22.68 % increase while LCA represented near to the 50 % increase rather than healthy-weight serum samples. Unlikely, THDOC significantly reduced in obesity serum samples and this reduction calculated to be 36.16 % (near to the half).

AP is the other endogenous NS was examined here and we used Elisa kit to evaluate AP concentration in experimental samples due to availability, high accuracy and validity. After normalization and comparing with the standard solutions according to the guideline, AP concentration calculated to be 0.51±0.05 nM in healthy control while its content determined as 0.85±0.18 nM in obesity samples. The results confirmed increased concentration of AP as a consequence of obesity.

Obesity changes expression of the neuroproteins and neuropeptides in children

By considering obesity-induced changes in neuro-active steroids content, it seems neuroproteins and other neuromodulators affected by over-weighting. Based on previous reports high fat diet feeding in animal models causes a reduction in markers of neurogenesis, synaptic plasticity, and neuronal

growth, including brain-derived neurotrophic factors (Arnold et al. 2014). Therefore, we aimed to measure BDNF in blood serum of the overweight children as a likely response to NS dysregulation. Immunoblotting results showed more strong bands in samples related to control. Quantification of bands after normalization confirmed BDNF content decreased in serum sample more that 3-fold as a consequence of obesity (Fig 2).

As our previous study, AChE enzyme is important to keep brain homeostasis and some of neurological diseases cause its up-regulation in brain tissue and also serum samples (Walczak-Nowicka and Herbet 2021). This multifunctional protein regulates cholinergic functions through digestion of the acetylcholine neurotransmitter in neuromuscular junction and also facilitates fibrillation process (Sadeghi et al. 2020). Our results showed no significant difference between AChE content in obese and control samples.

As regulatory function of tau protein in CNS that maintain the stability of microtubules in axons, it could be considered as axonal damages and neurodegeneration biomarker in biological fluids including the blood-based liquids (Chang et al. 2021). Recently it was considered as an effective biomarker in AD and PD (Ossenkoppele et al. 2022). Our results manifested increased expression of the tau protein in obesity. Circular content of tau in obese patients is increased 1.5-fold in comparison with healthy control, statistical analysis revealed this amount of difference is significant ($P<0.05$).

We also measured NPY content in circulation of the obese and control children due to its importance in feeding behavior regulation (Czerwińska et al. 2021). NPY is a hypothalamic peptide was regulated by leptin hormone and bind to its receptor in hippocampus, hypothalamus, skeletal muscle, coronary artery, pancreas, kidney and lung to coordinate parasympathetic and sympathetic neurons (Zhang et al. 2021). According to our results NPY measured to be 26.83±4.73 and 16.50±4.87 pg/ml in blood serum of normal-weight and overweight patients respectively. Analysis of variance that followed by Duncan's Multiple Range Test confirmed difference between both experimental groups is significant ($P<0.05$).

Discussion

NS are neuroactive metabolites were mainly produced by neural cells and regulate CNS and PNS homeostasis through binding to the gamma amino butyric acid (GABA), sigma receptor complexes and progesterone intracellular receptor which cause early phase modulation (Do Rego et al. 2012). They could also regulate gene expression as late phase modulation (Ubuka and Tsutsui 2022). Therefore, they control neural growth, development, signaling function and death (Frye 2009; Reddy 2010). The involvement of NS in neurological abnormalities and also their therapeutic functions were reported previously (Eddy 2003). Biosynthesis and metabolism rate of NS changed during different pathophysiological conditions (Sadeghi et al. 2020; Saleh and Sadeghi 2019). For example, AP biosynthesis increased during obesity and stimulates food intake and weight gain by mediation of the GABA-A receptor (Holmberg et al. 2018). Increased BMI is an effective risk factor for cardiovascular, endocrine system and neurological disease in adults (Lin and Li 2021). While obesity of children induced shrinkage of the brain gray matter and hippocampal tissue which could lead learning and thinking ability deficits and also poor neurocognitive functions (Alosco et al. 2014; Yau et al. 2012). The molecular mechanism of the mentioned neural complications has not been investigated yet. Our previous works revealed that CA and DCA could inhibit catalytic activity of AChE through blocking the active site and non-catalytic function by attenuation of the fibrillation process (Sadeghi et al. 2020). The other neuroprotective functions of NS evaluated to be attenuate the excitotoxicity, brain edema, inflammatory processes, oxidative stress, and neural degeneration (Borowicz et al. 2011). Our results revealed circular content of CA, DCA and LCA increased during the obesity in comparison with normal weight control. By considering multiple function of them specially antioxidant and neuroprotection, high concentration of NS could mediate the wide range of neural abnormalities in

overweight children such as neural proteins or peptides dysregulation. Changed amounts of NS also make gene expression alteration (Ubuka and Tsutsui 2022). Fig 2 showed increased amounts of NS accompanied by BDNF reduction. BDNF supports differentiation, maturation, and survival of neurons in the CNS (Waterhouse et al. 2012). This neurotrophic factor has curative potential for helping axonal renewal, preserving synaptic strength, avoiding neuron loss and encouraging neuronic redifferentiation in severe CNS damages (Waterhouse et al. 2012). In addition to neuroprotective effects, BDNF plays a major role in energy homeostasis due to role in energy intake suppression that leads to BMI alteration (Podyma et al. 2021). Diminished neuroprotective agents that studies here could cause increased risk factor of cholinergic irregularities and microglial-proinflammatory activation. The results demonstrated that concentration of AChE protein in blood serum related to obese children is similar to its content in control serum but it's possible AChE expression in CNS is different between two groups that need to further investigation. Because, previously reported insulin resistance that are one of the main reasons for obesity could cause CNS damages by mediation cholinergic activity (Hölscher 2020). Obesity-induced cholinergic dysfunction and resulted neural damages up-regulate tau protein in CNS and blood sample that was observed in obese children. Total content of tau protein in blood serum is validated risk factor and effective biomarker of neural damages and cognitive decline (Ossenkoppele et al. 2022). Therefore, obesity associated with tau pathology was often assumed to be linked dysregulation of CNS (Chang et al. 2021; Ossenkoppele et al. 2022). Fig 3 revealed up-regulation of tau protein in circulation of over-weight children.

Our results demonstrated content of THDOC in blood samples related to obesity is significantly less than control samples. THDOC has been showed to improve special learning ability in animal model of AD (Saleh and Sadeghi 2019). It could also reduce the expression of APP and Aβ in hippocampal tissue that led to decreased amyloid plaque deposition in brain tissue of AD model according to our previous results (Saleh and Sadeghi 2019). THDOC also showed anti-convulsant properties against seizures induced by the GABA receptor antagonists, like picrotoxin, pentylenetetrazole and bicuculline (Borowicz et al. 2011). Therefore, decreased concentration of THDOC which represents its less content in CNS is an effective risk factor to cognitive impairment which observed in obese children and high-fat animal models (Borowicz et al. 2011). Of course, reduced neuroprotection in high BMI condition could also be caused by sphingomyelin reduction. Plasma sphingolipids could increase risk of neural damages indirectly through their increased risk of vascular diseases and insulin resistance (Mielke and Haughey 2012) but its molecular mechanism in unknown yet.

Conclusion:

Overall, this study tried to clarify some ambiguities in obesity-derived neuropathology. Based on neuromodulatory properties of NS, this study manifested raised amounts of CA, DCA and LCA and lower THDOC level in obesity that refer to dysregulation of biosynthesis, degradation and active content of NS in brain. The risk factors were studied here are closely associated with neurodevelopment such as BDNF and NPY that decreased in obese children and warn about high-fat diet. Overweight children also manifested high amount of tau protein in blood serum that refer to neural damages. According to the results, the molecular events that take place in obesity and its associated diseases are linked with initiation and development of AD so, childhood obesity could lead to dementia later in older age. Our results also could be used to improve neurological side effects of obesity in children after additional investigation.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Tables

Table 1. Demographic characteristics of the recruited overweight and normal-weight children. Star symbols manifested significant differences in comparison with control (P<0.05).

Blood parameters	**Normal-weight children**	**Obese children**
Age (year)	9±4	9±4
Triglyceride (mg/dl)	154.11±9.63	185.72±21.73*
Total cholesterol (mg/dl)	165.27±8.95	182.21±11.52*
Triiodothyronine (T3) (pg/ml)	3.03±0.88	4.64±0.83*
Thyroxine (T4) (pg/ml)	14.75±2.80	14.16±2.78
thyroid stimulating hormone (TSH) (mIU/ml)	2.06±0.82	3.72±1.74*
Allopregnanolone (nM)	0.51±0.05	0.85±0.18*
Sphingomyelin (mg/dl)	24.62±5.26	18.14±6.12*

Table 2. Quantification of GC/MS analysis. RT, Mw and area of peak values for each identified peak related to NS.

Neurosteroid	Retention time (min)	Molecular weight (Da)	Area GC/MS%±SD		Fold Change (Obesity/Control)	Fold Change (%)
			Control	Obesity		

Cholic acid (CA)	18.7	408.6	15.38±1.25	21.61±1.53	1.40	40.50
Deoxycholic acid (DCA)	13.5	392.6	9.17±0.81	11.25±0.86	1.22	22.68
Lithocholic acid (LCA)	6.9	376.6	5.21±0.36	7.72±0.64	1.48	48.17
Tetrahydrodeoxycorticosterone (THDOC)	8.9	334.5	4.48±0.31	2.86±0.21	0.64	-36.16

Figure legends

Fig 1. Chromatogram related to experimental samples which have 4 peaks related to NS. (A) represented the multi-standard solution chromatogram which have different concentrations of CA, DCA, LCA, THDOC and BSA. The chromatogram showed 4 main peaks that refer to LCA (RT=6.9 min), THDOC (RT=8.9 min), DCA (RT=13.5 min) and CA (RT=18.7). Chromatogram of serum sample related to normal-weight and obese children represented as (B) and (C). The numbers used to refer to RT in each detected peak.

Fig 2. Evaluation of Neuroproteins in circulation of obese children and healthy weight control. Results showed significant reduction of BDNF and raise amount of tau protein in samples related to obesity group in comparison with control. Two experimental groups are similar to each other in term of circular concentration of AChE. Intensity of bands quantified by using ImageJ software in the right plot. Data were expressed as mean ± SD. Significant differences indicated by star symbol (P<0.05).

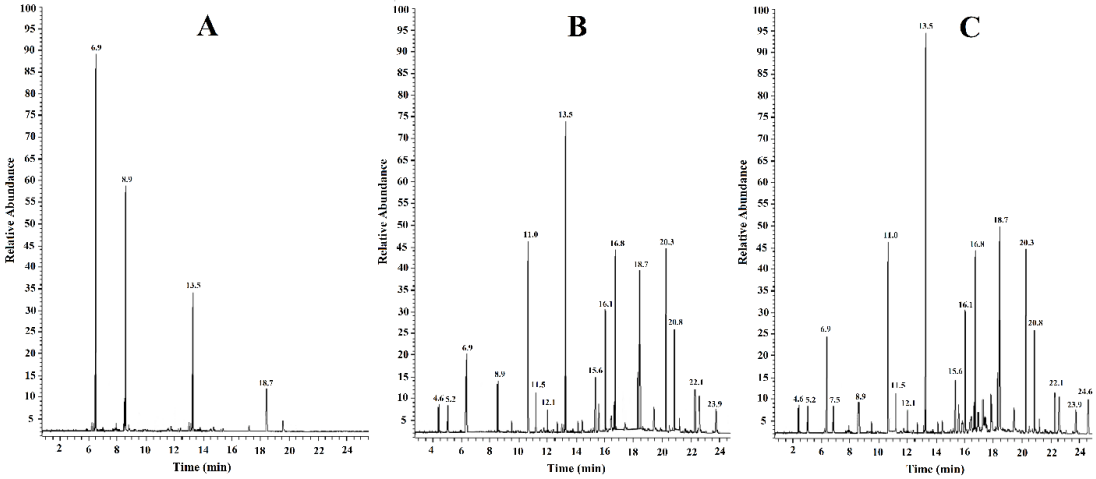

Fig. 1

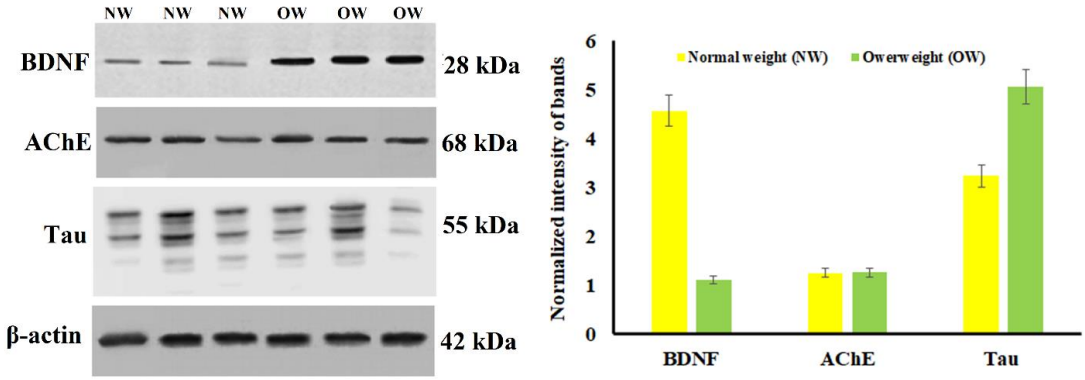

Fig.2