Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v30i1.2620

INVESTIGATION OF NEUROLOGICAL ABNORMALITIES IN AZOOSPERMIA PATIENTS: NEUROACTIVE PROTEINS AND NEUROENDOCRINE ASSESSMENT

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Abstract

Aim: Close relationship between neuroendocrine and reproductive systems causes neural hormones play crucial role in fertility and reciprocally Gonadotropin hormone-releasing hormone (Gn RH) dysregulation makes aging related neural damages. This study aimed to investigate the possible changes of neuroactive proteins, peptides and hormones in azoospermia patients. Azoospermia is one of the main cause of men infertility and known by reduction of GnRH production and release that accompanied by several neurological complications.

Methods: This study was performed on 33 azoospermia patients that half of them were smokers and 30 healthy control that have two or more child and half of them were smokers. The level of neurohormones and biochemical parameters were evaluated by specific ELISA kit in blood serum samples. Neuroactive proteins including neuropeptide Y (NPY), Brain-derived neurotrophic factor (BDNF), soluble amyloid precursor protein-alpha (sAPPα) and β-secretase (BACE1) circular concentrations were estimated by using specific ELISA kit according to antigen-antibody interaction.

Results and discussion: Results revealed between measured neurohormones, serotonin changed significantly as a result of azoospermia. While BDNF and NPY increased significantly in azoospermia patients in comparison with control and smoker patients showed more remarkable changes. This study could not detect significant alteration in sAPPα between four experimental groups. The circular concentration of BACE1 increased more than 1.5 folds in azoospermia patients, this increase was improved slightly in smoker patients. Our results highlighted participation of BACE1 in reproductive system as a multifunction protein. It seems decreased level of serotonin could motivate the further events so hormone replacement therapy seems to be helpful for limiting neurological side effects.

Keywords: Reproductive system; Infertility; Neurological abnormalities; β secretase enzyme; BDNF; serotonin

1. Introduction

According to the WHO report, nearly 20–30 % of about 70 million infertility cases worldwide are related to male factors (1). The most severe type of male infertility was identified as azoospermia that is a medical condition was characterized by the nonattendance of sperm in the ejaculate (semen) (2). The pathophysiological properties of azoospermia are not fully understood yet but according to the previous studies it can be due to pre-testicular, testicular, and post-testicular causes. It is also subdivided into obstructive azoospermia (OA) or non-obstructive azoospermia (NOA) (2). Statistical reports revealed that azoospermia could affect approximately 1 % of the male population and around 10 %-15 % of all males suffering from fertility problems (3). Healthy function of the reproductive system is dependent on neuroendocrine system which produce neuro active hormones could regulate gonad and sex hormones release including estrogen, progesterone and testosterone (4). Hypothalamic-pituitary-gonadal (HPG) axis belongs to neuroendocrine system and hypothesized to have important role in human fertility and pathophysiology of azoospermia disease (5). HPG axis could induce spermatogenesis and androgen biosynthesis mainly through gonadotropin-releasing hormone (GnRH) is secreted by hypothalamus (6). GnRH induces pituitary gland to produce follicle stimulating hormone (FSH) and the luteinizing hormone (LH), two gonadotropin hormones release to the blood circulation and reach to the gonads and facilitate gametogenesis (6).

According to the previous experiments GnRH is an effective element in spermatogenesis process and daily subcutaneous injection of Nal-Glu as antagonist of the hormone could inhibit mature sperm production in healthy men (7). However, the inhibition of testosterone could not cause the same effects on spermatogenesis process that suggest the more critical role of GnRH in azoospermia disease (7). In addition to the mentioned traditional knowledge about the GnRH role in reproductive system, its functions in central nervous system (CNS) are beginning to be revealed. Recent studies reported that GnRH level decreased during the human aging that are accompanied by cognitive decline (8). While administration of exogenous GnRH could slow down aging process and increase lifespan of mice, suggesting that hypothalamic GnRH neuron death and dysfunction of GnRH over time may be a serious event during aging or some age-related disease (9). Raised levels of LH and FSH that regulate reproductive functions and were controlled by GnRH could trigger age-related neurodegenerative disease also (10). The upregulated levels of LH and FSH have been reported previously in circulation of patients suffering from Alzheimer's disease (AD) (11).

While hippocampal neurons with LH receptor follow a degenerative pattern in LH increased condition after menopause (12). Additionally, decreased content of LH in serum is accompanied with improved cognitive abilities in AD patients possibly due to its toxic effects in motivation of neural cell death (12). Mentioned evidences suggests there are a near correlation between neural abnormalities and azoospermia disease. Therefore, this study was designed to evaluate the possible mechanism of neurological complications in azoospermia patients through analyzing the circular concentration of some neuroproteins and neurohormones that known to play critical role in neurodegeneration process. For this purpose, the circular level of LH, FSH, thyroid hormones, cortisol, dopamine, gamma-aminobutyric acid (GABA), acetylcholine and serotonin were measured and compared with control individuals. By considering the significant accompaniment of azoospermia with neurodegeneration that was reported latterly (2, 13), we also evaluated soluble amyloid precursor protein-alpha (sAPPα), beta secretase (BACE1) and brain-derived neurotrophic factor (BDNF) in blood samples related to azoospermia patients. The sAPPα is neuroprotective and antioxidant protein which could be cleaved by BACE1 enzyme and produce Amyloid β (Aβ) peptide that has more propensity to fibrillation (14). BDNF is also characterized to have critical role in neural growth and survival that involved in neural plasticity through neurotransmitter regulation (15). Therefore, analyzing of them could help to clarify some of the ambiguities in azoospermia induced neural abnormalities and also elucidate molecular events cause more lability of infertile men to manifest dementia related signs.

2. Material and methods

2.1. Experimental design and samples

This study aimed to compare the neurodegeneration-related proteins expression and also neurohormones concentration in blood serum of the patients were diagnosed with azoospermia and healthy control individuals that were referred to the Maternity and children teaching hospital in Al Diwaniyah and the Specialized Center for Endocrinology and Diabetes in Baghdad (Iraq) between 1 October to 15 December 2022. There is good evidence that cigarette smoking is negatively associated with fertility and semen quality (16) therefore, we checked the hormones and proteins in four groups as follow: 30 non-smoker controls, 30 controls who smokes regularly, 30 non-smoker patients suffering from azoospermia and 30 azoospermia patient who smokes regularly. In this study smoker individuals selected between the men that smokes regularly more than 5 years and control individuals means they have two or more children and did not suffer infertility problems yet. Informed and written consent is obtained from patients. Semen quality of all participants was checked by a specialist that confirmed all of the smoker and non-smoker controls have from 15 million sperm to more than 200 million sperm per milliliter (mL) of semen and azoospermia patients don't have any motile and developed sperm in their ejaculate.

Patients with liver deficiency, kidney disorder, thyroid disorder, acute coronary syndromes, Alzheimer disease, 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 healthy peoples that had two or more children and did not suffer from infertility problems yet. After characterizing the participants position in each group, 5 ml of blood samples were taken from the participants and centrifuged at 10000 rpm for 5 min to obtain blood serum samples and then stored at -20°C temperature until experiments. The study was approved by the health and ethics committee of the health center, and all the participants gave their informed consent in accordance with the Declaration of Helsinki. Relevant sociodemographic, clinical and laboratory data were obtained from the medical records of the patients including: age, body mass index (BMI), thyroid stimulating hormone (TSH), T3, T4, Testosterone, prolactin, FSH and LH. This information was recorded on the data sheet. Related data was manifested in Table 1.

2.2. Measurement of circular contents of neurohormones

The levels of the following neurohormones were determined by ELISA Kit. Human ELISA kits were used for determination of GABA (Abcam, ab287792), Serotonin (Abcam, ab133053) and dopamine (Abcam, ab285238) according to the manufacture guideline in each case. All of the reagents and solution were placed on bench 30 min before test.

2.3. Evaluation of neuroproteins and neuropeptides in blood samples

By considering the high accuracy and availability of ELISA kits to measure the desired proteins and peptides, this study was used from the commercial kits that measure one specific protein or peptide by using specific antigen-antibody interactions. For this purpose, human soluble amyloid precursor protein α (sAPPα) ELISA kit (orb406714) was purchased from Biorbyt Company human NPY ELISA kit (E-EL-H1893) and human BDNF ELISA kit were prepared from Elabscience Company. Circular content of BACE1 also was measured by using human Beta- Secretase 1 (BACE1) ELISA kit from Abexa company (abx150780).

2.4. Statistical Analysis

All of the experiments repeated at least three times independently and the data were reported as the mean ± standard deviation (SD) using the Graph Pad in Stat version 10.0.2 program (Graph Pad Software, San Diego, CA). Analysis of variance (ANOVA) and multiple comparison test was used to determine statistical differences between results related to four experimental groups.

3. Results

There is additional evidence that azoospermia patients possibly suffer from other systemic disease and therefore an exact evaluation of these patients and corresponding pathophysiological signs are important. Men with a history of azoospermia showed increased risk of cancer developing or neurological abnormalities according to the previous reports (17). Therefore, this study aimed to assess the possible alterations of neuropeptides and neuroproteins concentration in blood serum.

3.4. Azoospermia causes circular changes of neurohormones remarkably

Neurohormones are involved in cognition, thinking ability and also homeostatic regulation of energy balance in body that could be affected by HPG axis and also gonadal hormones (18) thus, hypothesized to be involved in azoospermia condition. Here we compare some of important neurohormones between four experimental groups such as dopamine, serotonin, GABA and cortisol. Results revealed (Fig 1A) circular content of dopamine was evaluated to be 33.71±2.91 ng/L in non-smoker control and 33.53±3.55 ng/L in smoker control. While its content in patient groups was estimated as 34.16±1.65 ng/L and 35.30±2.04 ng/L in non-smoker and smoker azoospermia groups respectively. Statistical analysis could not detect any significant changes between four groups. Whereas, concentration of serotonin is significantly different between control and patient groups as showed in Fig 1B. According to the results serotonin contents are 18.81±2.97 ng/ml and 18.56±2.27 ng/ml in non-smoker and smoker control groups, that don't show significant different with each other. While circular level of serotonin in non-smoker patients and smoker patients measured to be 15.81±0.63 and 15.93±1.03 ng/ml. According to multiple comparison analysis of ANOVA, serotonin reduced in blood serum of azoospermia patients significantly (** $P<0.01$, *** $P<0.001$). GABA content was also measured to be 81.41±4.21, 80.38±6.32 nmol/dL in both of the control groups and this parameter in non-smoker and smoker patients was evaluated as 81.22±7.99 and 83.45±5.16 nmol/dL respectively (Fig 1C). These changes are not significant according to multiple comparison of one-way ANOVA statistical analysis (ns). According to the results (Fig 1D), cortisol concentration in blood samples of control was estimated as 237.03±35.04 nmol/L while this parameter in azoospermia subjects was 187.67±32.05 nmol/L. Statistical analysis showed there is not significant difference in cortisol content ($P>0.05$). our results showed smoking also could not change cortisol level of blood in control and patient men.

3.5. Azoospermia could affect circular contents of NPY and BDNF

Our results revealed NPY content of blood serum related to azoospermia patients increased significantly in both of the smoker and non-smoker groups (Fig 2A). NPY content of non-smoker control blood serum was measured as 24.88±5.49 pg/ml while smoker control showed its concentration is 23.42±5.40 pg/ml. Statistical analysis revealed that smoking could not affect the circular content of NPY. Whereas, azoospermia patients showed 42.00±10.62 and 49.80±11.55 pg/ml concentration of NPY in non-smoker and smoker groups respectively. Totally, both of the smoker and non-smoker patient groups showed significant changes of NPY content in comparison with corresponding control group.

According to Fig 2B, circular content of BDNF showed significant changes between control and azoospermia groups. BDNF concentration was measured as 118.50±10.56 and 119.25±12.59 pg/ml in non-smoker and smoker control participants respectively, both of the controls are similar to each other without any significant changes (ns, $P>0.05$). while azoospermia induced significant increase in circular level of BDNF that non-smoker patients showed 192.42±10.79 pg/ml and smoker patients manifested 208.30±19.66 pg/ml. Statistical analysis showed P value is less than 0.0001 so difference is significant.

3.6. Azoospermia changes amyloid precursor and amyloid processing proteins

Amyloid precursor protein (sAPP-α) is a proteolytic fragment of amyloid precursor protein (APP) was produced by non-amyloidogenic process (14). sAPP-α is known by antioxidant and

neuroprotective properties and its increased amounts means reduced production of amyloid β (Aβ) that is prone to aggregation (14). According to our results (Fig 3A), circular content of sAPP-α ranged between 33.04 ng/ml and 66.07 ng/ml in all of the participants. Its concentration was evaluated to be 47.25±6.82 and 45.42±11.51 ng/ml in non-smoker and smoker control groups. While non-smoker and smoker patients showed 47.25±9.12 and 38.40±6.26 ng/ml respectively. Results revealed slightly reduction of sAPP-α in azoospermia patient but this alteration is not significant (P=0.43). While smoking causes relatively reduced amount of sAPP-α in control and patient participants, these differences are not significant.

The proteolytic cleavage of APP by β-secretase enzyme (also called BACE1) is a critical step in Aβ production and aggregation (14). Therefore, circular content of BACE1 is important biomarker of neurodegenerative disease while it works originally in central nervous system (CNS). Our results also showed increased concentration of this enzyme in Azoospermia blood samples. Amount of β-secretase in blood serum related to non-smoker and smoker controls is evaluated to be 27.13±6.33 and 28.17±8.86 pg/ml respectively, both of the control groups are similar to each other statistically. While smoking could not change BACE1 concentration in blood serum of azoospermia patients. Fig 3B revealed significant changes between control and patient groups and BACE1 increased about 40 % in patient groups (non-smoker control versus non-smoker patients ****, $P<0.0001$; smoker control versus smoker patients *, $P<0.05$).

4. Discussion

By considering the important role of neurohormones and CNS in fertility (18), this study aimed to evaluate the possible CNS-related abnormalities in azoospermia patients. The most type of azoospermia especially non-obstructive type have unknown origin that possibly are related to nervous system specially HTG axis. According to our results serotonin reduced significantly in azoospermia patients in comparison with control while other neurohormones did not changed significantly. Literature confirmed that Serotonin and serotonin receptors play crucial role in all of the brain function and serotonergic system disfunction causes many psychiatric and neurological disease (19).

Today its clear that serotonin has critically important functions in many human organ systems outside the CNS, including the regulation of energy balance and food intake, endocrine system, cardiovascular and pulmonary physiology and reproduction (20). It seems circular serotonin are effective in fertility in men and women, previously reported that ovarian hyperstimulation are accompanied with increased secretion of serotonin to the blood (21). It has been confirmed that regulation of fertility done by serotonin by mediation of the hypothalamo–hypophysial system that increase gonadotrophin output finally (21). But it's not obvious that reduced serotonin in patient groups is cause or consequence of azoospermia. Our results revealed a negative correlation between serotonin and BDNF content that make a significant raise in circular level of BDNF. Previously approved that balanced level of BDNF is important not only or neural development and cognitive function but also for reproductive system of men (22, 23). BDNF has important role in sperm mobility and viability, it also involved in lipid peroxidation and oxidative stress that are damaging factor in fertility system (23). In addition, this factor improves mitochondrial function and motivates insulin and leptin production and secretion so could regulate metabolism rate, these factors affect reproductive system function indirectly (24). This negative correlation also was observed in patients that received serotonin reuptake inhibitors as antidepressant drug, inhibition of serotonin reuptake upregulates BDNF gene (25). Therefore, mentioned synergism between the two systems in affective behaviors and genetic epitasis between BDNF and the serotonin transporter genes was interrupted in azoospermia patients (26).

NPY hypothesized to be involved in the hypothalamic regulation of reproduction and energy homeostasis by mediation of galanin and opioid network (27). Our results showed significant

dysregulation of NPY in non-smoker and smoker patient groups. Previous experiments showed that hypothalamic concentration of NPY negatively regulate production and release of serotonin hormone in rats (28), our results also showed this negative relationship because decreased serotonin is accompanied with increased NPY in azoospermia patients. The observed hypothalamic abnormalities in patient groups possibly are related to HPG axis and GnRH dysregulation that is one of the primary events in non-obstructive azoospermia patients (29). Fig 3 showed sAPPα has not been changed in both of the patient groups. While our results manifested increased concentration of BACE1 in patients' circulation as a result of azoospermia. This enzyme is one of the effective risk factors that its upregulation was reported in Alzheimer disease (AD) in CNS tissue and in blood samples (14). Although brain is chief tissue to BACE1 enzyme, its related mRNA and protein is also detected in different type of cells in human body such as liver cells, adipocytes, pancreatic β cells, vascular tissue (30). Increased BACE1 activity and expression is observed during the development of a number of diseases like diabetes and cardiovascular disease, in addition to AD, it increased level was also reported in azoospermia disease here (30). Therefore, it seems this multifunctional enzyme could be used as potential prognostic and/or therapeutic biological marker for azoospermia.

Conclusion

Overall, this study tried to clarify some ambiguities in azoospermia-related neuropathy. According to important role of neuromodulators in reproductive system function it can be concluded that nervous system and reproductive system have undeniable relationship with each other. According to our results NPY, BDNF and BACE1 increased and serotonin decreased as consequence of azoospermia disease. Our results also confirmed smoking worsens detected abnormalities. Involvement of BACE1 was detected in different type of neurological and non-neurological disease and we propose a new role in reproductive system. By considering significant changes in neuroactive proteins and peptides, azoospermia could be considered as an effective risk factor in initiation and development of AD or dementia-related disease later in older age. It seems hormone replacement therapy and also BACE1 inhibitors could be used to limit the neurological side effects of azoospermia and other types of infertilities.

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

Parameters	Non-smoker control	Smoker control	Non-smoker patients	Smoker patients
Age (year)	32.37±7.37	29.46±5.16	31.06±7.42	30.67±6.23
BMI (kg/m^2)	26.09±3.15	27.48±3.87	28.14±3.61	26.20±4.46
TSH (mIU/ml)	2.00±0.55	2.33±1.09	1.81±1.03	2.05±0.68
T3 (nmol/L)	2.35±0.58	1.84±0.35	1.79±0.41 *	1.90±0.44
T4 (nmol/L)	98.48±17.48	102.65±26.09	95.66±21.47	101.19±15.91
Testosterone (ng/ml)	6.14±1.41	5.49±1.62	4.27±1.38 *	4.00±1.96
Prolactin (ng/ml)	6.81±1.24	7.44±1.38	10.55±6.12	12.25±5.68 #
FSH (mIU/ml)	5.94±2.00	6.07±1.61	6.29±8.17	10.91±7.91
LH (mIU/ml)	4.16±1.37	3.80±0.82	4.07±3.24	5.23±3.46

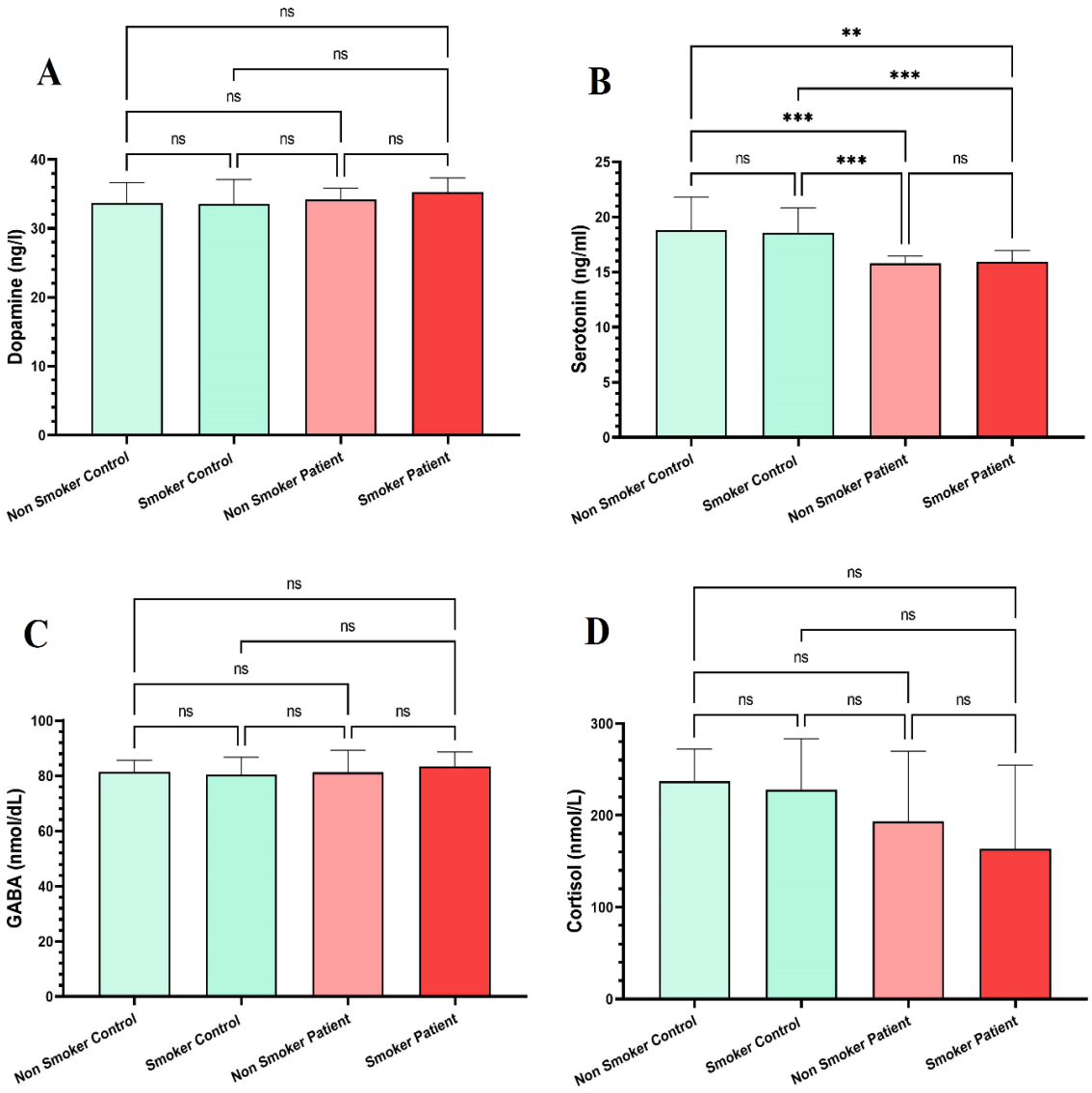
Shows significant difference in comparison of the smoker control and patient groups
* Shows significant difference in comparison of the non-smoker control and patient groups

Figure Legends:

Fig.1 Circular concentration of neurohormones in azoospermia patients and healthy controls. (A) Dopamine concentration of blood serum increased in azoospermia rather than control. (B) Serotonin concentration in blood samples related to patient groups remarkably reduced rather than control groups. (C) GABA and (D) Cortisol is the same between four experimental groups. All data were expressed as mean ± SD. Significant differences were indicated by star symbol (ns means no significant; ** P<0.01; *** P<0.001).

Fig 2. Evaluation of Neuroproteins in circulation of azoospermia patients and control. (A) Results showed significant increase of NPY in smoker and non-smoker patient groups. (B) azoospermia patients manifested raised level of BDNF in azoospermia patients. Data were expressed as mean ± SD. Significant differences indicated by star symbol (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

Fig 3. Measurement of Alzheimer related biomarkers in circulation of azoospermia patients in comparison with control. (A) Results showed no significant change of sAPPα and (B) raise amount of BACE1 protein in blood samples related to azoospermia patients and control. Data were expressed as mean ± SD. Significant differences indicated by star symbol (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).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Smoker Patient

Fig.1

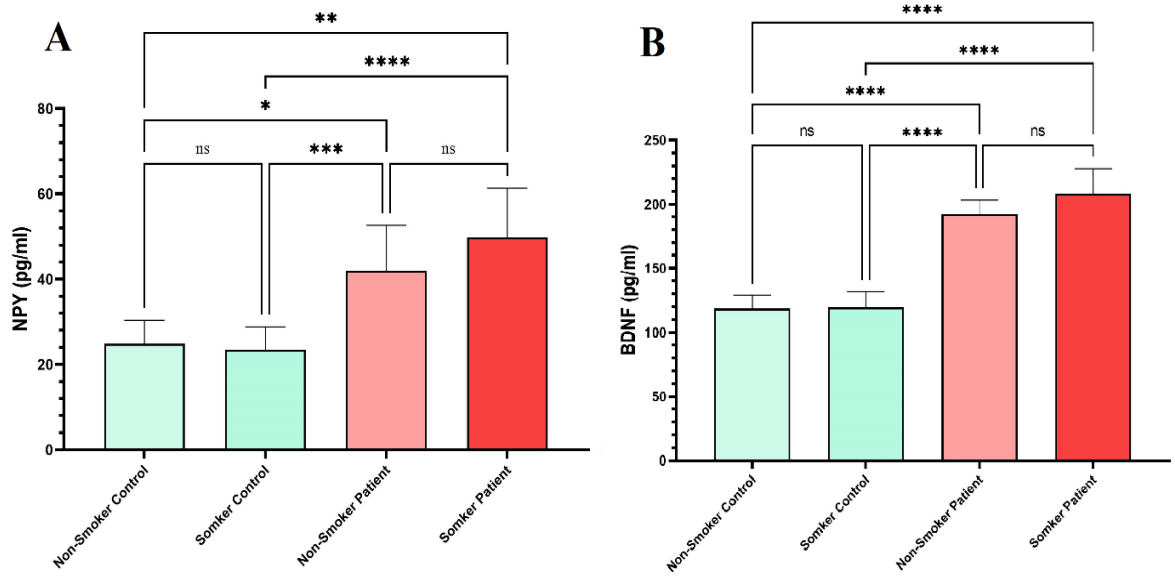
A
NPY (pg/ml)
**
****
*
ns
***
B
BDNF (pg/ml)
Non-Smoker Control
Somker Control
Non-Smoker Patient
Somker Patient

Fig. 2

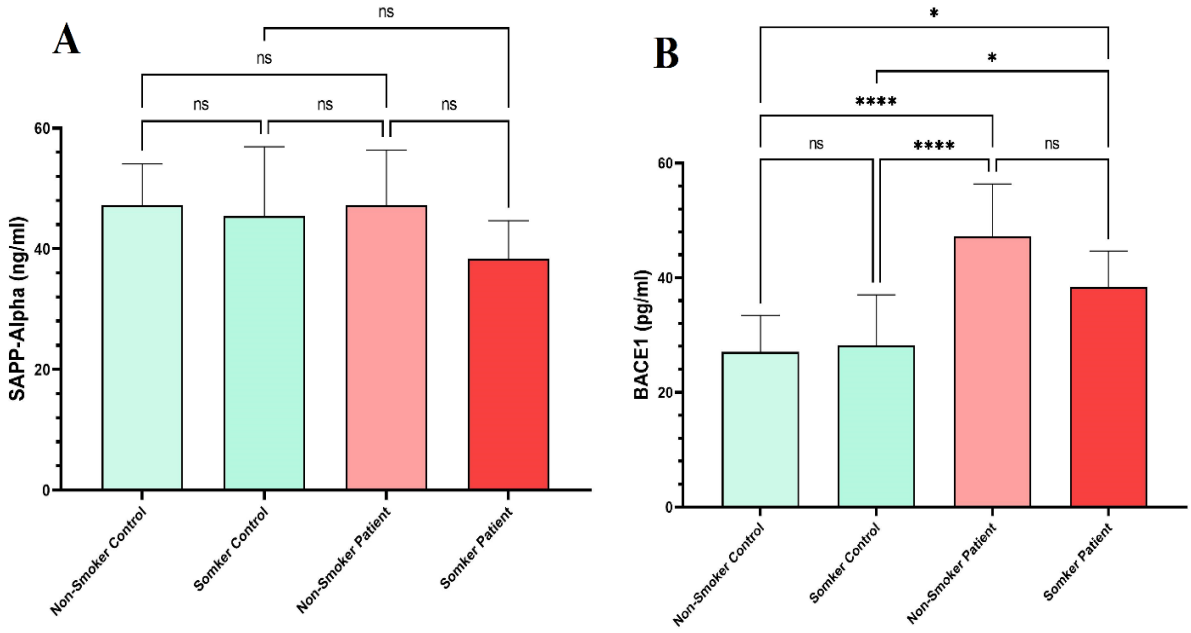
A
ns
ns
ns
ns
ns
SAPP-Alpha (ng/ml)
60
40
20
0
Non-Smoker Control
Somker Control
Non-Smoker Patient
Somker Patient
B
*
*
****
ns
****
ns
BACE1 (pg/ml)
60
40
20
0
Non-Smoker Control
Somker Control
Non-Smoker Patient
Somker Patient

Fig. 3