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The journal covers all areas of neuroscience with an emphasis on psychiatry and psychology as long as the target is to describe the neural mechanisms underlying normal or pathological behavior. Pre-clinical and clinical studies are equally acceptable for publication. In this context; the articles and treatment results of computational modeling methods of psychiatric and neurological disorders are also covered by the journal.

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DRUG INTERACTIONS BETWEEN COVID-19 AND PSYCHIATRIC MEDICATIONS: A MINI REVIEW

Filiz İzci[1], Filiz Kulacaoglu*[1]

Ethics committee approval: There is no need for ethics committee approval.

Abstract

Coronavirus disease 2019 (COVID-19) has become a pandemic with 8.708.000 confirmed cases and, 461.715 death in the world until 21 June 2020. The SARS-CoV-2 virus which is an enveloped and single-stranded RNA virus belonging to Coronaviridea family causes multiple organ systems diseases such as respiratory, gastrointestinal, hepatic and neurological tracts in humans and, animals. With the rapid and global spread of COVID-19, the scientific authorities have developed treatment algorithms for COVID-19. Besides the supportive care, chloroquine, hydroxychloroquine, lopinavir/ritonavir, favipiravir, tocilizumab, azithromycin, vitamin C, Convalescent plasma therapy are frequently used off-label to treat COVID-19. For severe COVID-19 cases, US Food and Drug Administration (FDA) has issued an Emergency Use Authorization (EUA) Remdesivir, which is the first drug to earn the title. COVID-19 treatment may cause many side effects and drug interactions. Psychiatric diseases are also chronic diseases and psychiatric drugs may have severe interactions with COVID-19 medications. Drug interactions with psychiatric medication and neuropsychiatric side effects should be considered within the action mechanism of COVID-19 treatments. Thus, In this review, we aimed to elucidate the interactions of COVID-19 medications with the antidepressants, antipsychotics and, the other psychotropic drugs that have been frequently used in psychiatric diseases. Chloroquine, hydroxychloroquine, azithromycin, ribavirin, lopinavir/ritonavir have interactions with psychiatric medications such as antidepressants, antipsychotics, anticonvulsants and, anxiolytics in terms of QTc interval prolongation, hepatotoxicity, myelosuppression, increased or decreased concentration of medications. Remdesivir has lower interactions with most of the psychiatric drugs except St John's Wort and some anticonvulsants. However, favipiravir is considered as the COVID-19 medication which has lower interaction and side effects in combination with psychiatric medication. The use of tocilizumab is also safe with most of the psychiatric drugs and has a positive effect on depressive symptoms, although there is a risk of hepatotoxicity.

Keywords: COVID-19, treatment, pschotropic drugs, drug interactions, psychiatric diseasest

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1. Introduction

In December 2019, Coronavirus Disease-2019 (COVID-19) was first identified in Wuhan city, China. This disease was caused by a novel coronavirus was named with Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). This virus had not been previously identified in humans before. Since December 2019, COVID-19 has become a severe risk factor for global health. In March 2020, due to a rapid increase in the number of cases outside China WHO declared COVID-19 a pandemic (Li et al., 2020).

The SARS-CoV-2 virus which is an enveloped and single-stranded RNA virus belonging to Coronaviridea family causes multiple organ systems diseases such as respiratory, gastrointestinal, hepatic and neurological tracts in humans and, animals. The symptoms of COVID-19 can range from asymptomatic clinical features to severe symptoms (Agrawal et al., 2020).

With the rapid and global spread of COVID-19, the scientific authorities have developed treatment algorithms for COVID-19. The treatment algorithms of COVID-19 are composed of the medications that have been used in previous pandemics and the medications that have been thought potentially effective for COVID-19. For severe COVID-19 cases, US Food and Drug Administration (FDA) has issued an Emergency Use Authorization (EUA) Remdesivir, which is the first drug to earn the title. However, numerous medication like chloroquine, hydroxychloroquine, lopinavir/ritonavir, favipiravir, tocilizumab, azithromycin, vitamin C, Convalescent plasma therapy are frequently used off-label to treat COVID-19 (Kalil, 2020).

Multiple organ systems can be affected by COVID-19 and, it also causes psychiatric conditions. It has been reported that COVID-19 causes psychiatric disorders such as panic attacks, anxiety, depression, insomnia in especially patients and health care profesionals (Agrawal et al., 2020); Nguyen et al., 2020); Li et al., 2020). Since COVID-19 medications have possible side effects and affects multiple organs, psychiatrists should be aware of the mechanism of action of COVID-19 treatments, neuropsychiatric side effects and potential drug interactions with psychiatric drugs. In this review, we aimed to elucidate the interactions of COVID-19 medications with the antidepressants, antipsychotics and the other psychotropic drugs that have been frequently used in psychiatric diseases.

2. Chloroquine and Hydroxychloroquine

Chloroquine and Hydroxychloroquine are antimalarial drugs and have also been used for various rheumatic diseases such as systemic lupus erythematosus and, rheumatoid arthritis. Besides the antimalarial effects, these two drugs have immune-modulating effects as well (Schrezenmeier & Dörner, 2020).

A synthetic form of quinine, Chloroquine is used for the treatment and prophylaxis of malaria, inhibits glutamate-induced death of a neuronal cell by reducing reactive oxygen species through the sigma-1 receptor. Thus, using chloroquine as a neuroprotector against oxidative stress has been suggested (Hirata et al., 2011). The hydroxychloroquine is frequently used in inflammatory disorders. Both chloroquine and hydroxychloroquine have been suggested as a possible treatment for COVID-19 due to their immune-modulating and anti-inflammatory effects, antiviral effects by interference with virus-receptor binding (Colson et al., 2020).

Antidepressants, antihistamines, antipsychotics and, verapamil were used to investigate the increasing in vitro susceptibility of chloroquine in several studies (Gerena et al., 1992);(Safa, 1988);(Basco & Le Bras, 1992). In a study that investigated the role of antidepressants in reversing chloroquine resistance, Citalopram which is a selective serotonin reuptake blocker, was found the most effective to reversing the chloroquine resistance among amitriptyline, oxaprotiline and, nomifensine (Taylor et al., 2000). However, in another study that the pharmacokinetic interaction between chloroquine and imipramine was investigated, no significant change was found in plasma concentrations of chloroquine and its pharmacokinetic parameters when imipramine was coadministered with chloroquine (Onyeji et al., 1993).

Similarly, in another study chloroquine and chlorpromazine were found to inhibit Crimean-Congo Hemorrhagic Fever virus (CCHFV) replication in vitro. Moreover, it was reported that a synergistic effect was observed when ribavirin was added to those two molecules combination (Ferraris et al., 2015). In sum, the synergistic effect of psychiatric drugs such as citalopram, chlorpromazine may cause increasing in levels of chloroquine should be taken into account during the combination usage of these drugs.

Hydroxychloroquine which is a less toxic derivate of chloroquine, is also effective in inhibiting SARS-CoV-2 infection in vitro (Liu et al., 2020). In a recent observational study, 80 relatively mildly infected inpatients treated with a combination of hydroxychloroquine and azithromycin and a rapid fall of nasopharyngeal viral load was reported at the end of the day 7 (Gautret et al., 2020). However, it has been shown that the body temperature recovery time, the cough remission time was shortened and pneumonia was improved in patients with hydroxychloroquine treatment (Chen et al., 2020). Both hydroxychloroquine and chloroquine have neuropsychiatric side effects from mild to (mood swings, agitation, nervousness, sleep disturbances) to severe (psychosis, delirium, suicidality). Risk factors for induced neuropsychiatric effects caused by hydroxychloroquine and chloroquine include family history of psychiatric disease, alcohol usage, using of CYP3A4 inhibitors, glucocorticoids, and female gender (Good & Shader, 1977);(Mascolo et al., 2018).

3. Psychiatric drug interaction with Hydroxychloroquine and Chloroquine:

CYP3A4 enzyme metabolizes both hydroxychloroquine and chloroquine. Therefore, CYP3A4 inhibitors may cause an increase in the plasma levels and increase the possible effects of hydroxychloroquine and chloroquine whereas CYP3A4 inducers could decrease the plasma levels so that hydroxychloroquine and chloroquine could be less effective with the combination of CYP3A4 inducers (Browning, 2014). The cardiogenic side effects of hydroxychloroquine and chloroquine are; QT interval prolongation, AV block (McGhie et al., 2018). Usage outside of the hospital setting is not recommended, and the cardiogenic side effects

should be taken into account when used in combination with QT-prolonging antipsychotics.

Combination with Anticonvulsants:

The anticonvulsants that have the effects of mood-stabilizing, anxiolytic and sedation are using in psychiatric treatments.

- Because hydroxychloroquine and chloroquine are metabolized by CYP3A4, CYP3A4 inducers such as carbamazepine, oxcarbazepine may cause lower levels of hydroxychloroquine or chloroquine. Thus, a combination of these drugs is not suggested (Mascolo et al., 2018).

- No interaction has been reported with lamotrigine, valproic acid, clonazepam.

- Lithium, as a mood stabilizer, should be used cautiously, with chloroquine or hydroxychloroquine due to the QT interval prolongation effect of these drugs.

Combination with antidepressants:

-Antidepressants that have fewer interactions with hydroxychloroquine and chloroquine are sertraline, reboxetine, milnacipran, agomelatine and, bupropion.

-Amitriptyline, clomipramine, citalopram, escitalopram, trazodone should be used carefully due to the QT interval prolongation effect.

- Paroxetine, fluoxetine, amitriptyline, fluvoxamine, venlafaxine, mirtazapine may increase the plasma levels of chloroquine and hydroxychloroquine

-A combination with St John's Wort Oil is not recommended due to decreasing plasma levels of chloroquine and hydroxychloroquine.

Combination with Antipsychotics:

-Aripiprazole, amisulpride, asenapine, olanzapine, paliperidone, perphenazine are the antipsychotics that have lower side effects with the combination of chloroquine and hydroxychloroquine.

-Chlorpromazine, clozapine, haloperidol, pimozide, quetiapine, zuclopenthixol should be used carefully due to their potential QT interval prolongation effect.

-A combination with ziprasidone is contradicted due to higher risk for QTc interval prolongation.

- Due to clozapine is associated with myelosuppression risk, monitoring hematological parameters is essential during the combination with clozapine.

Combination with anxiolytics:

- Although the combination with anxiolytics is safe, hydroxyzine combination should be taken into account for prolonged QTc (http://Www.Covid19-Druginteractions.Org, n.d.).

Azithromycin:

Azithromycin is an antibacterial agent and used in the treatments of respiratory tracts, skin and soft tissues infections and urethritis/cervicitis (Peters et al., 1992). Besides the antibacterial activity, azithromycin has also antiviral and anti-inflammatory activities that may help the treatment of COVID-19 if used in combination with chloroquine and hydroxychloroquine. In a recent study, azithromycin and hydroxychloroquine combination was found higly more effective against the virus, when compared to hydroxychloroquine treatment alone (Gautret et al., 2020). Controversially, in another study, no clear benefit was found with the combination of hydroxychloroquine and azithromycin in patients with serious COVID-19 infection (Molina et al., 2020). However, the risk of QTc prolongation, cardiogenic comorbidities should be considered when using a combination of both hydoxychloroquine and azithromycin.

Psychiatric side effects of azithromycin are listed as; catatonia, delirium, agitation, anxiety, psychotic depression, and somnolence (Bilbul et al., 2020).

Psychiatric drug interaction with Azithromycin:

Combination with Anticonvulsants:

-Lithium has a moderate risk due to the risk of QTc prolongation.

Combination with antidepressants:

- Citalopram, escitalopram have a severe risk for QTc prolongation.

- Amitriptyline, clomipramine, trazodone, imipramine have a moderate risk for QTc prolongation

Combination with Antipsychotics:

-Amisulpride, ziprasidone, clozapine, pimozide, haloperidol, thioridazine have a severe risk for QTc prolongation.

-Aripiprazole, chlorpromazine, olanzapine, paliperidone, perphenazine, quetiapine, risperidone have a moderate risk for cardiac side effects.

Combination with anxiolytics:

-Due to the risk of QTc prolongation, combination with Hydroxyzine is not suggested (Https://Www.Drugs.Com/Drug-Interactions/Azithromycin-Index.Html, n.d.).

Remdesivir:

Remdesivir is an antiviral whcih acts by inhibiting RNA polymerase, hence lowering viral RNA levels. It has been shown that remdesivir has activity against SARS-CoV-2. Apparently, it has a clinical safety profile. The most common side effects are hypotension, sweating and, shivering. No neuropsychiatric side effect has been reported so far. However, on May 1, 2020, US Food and Drug Administration (FDA approved to use Remdesivir for treatment of suspected or confirmed severe COVID-19 (Https://Www.Fda.Gov/Media/137566/Download, n.d.).

Psychiatric drug interaction with Remdesivir:

Combination with Anticonvulsants:

- Due to carbamazepine is decreasing the plasma levels of remdesivir, combination with carbamazepine is not recommended

-No interaction has been reported with lamotrigine, valproic acid, topiramate and, lithium.

Combination with Antidepressants:

-No interaction has been reported between most of the antidepressants. But, as St John's Wort may decrease the plasma levels of Remdesivir, a combination of those two drugs is not recommended.

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Combination with Antipsychotics:

- No interaction has been reported with antipsychotics

Combination with Anxiolytics:

- No interaction has been reported with anxiolytics.

Lopinavir/Ritonavir

Lopinavir/ritonavir combination is an antiviral medication. Lopinavir is a protease inhibitor and ritonavir inhibits the metabolism of lopinavir and leads to increasing the plasma levels of lopinavir. This combination is used in treatment of HIV-1 infection and it was used to treat MERS as well. Recently, this combination is used to treat COVID-19 infection. But, according to a recent randomized, controlled, open-label trial involving hospitalized adult patients with COVID-19 in Wuhan China, no significant benefit was observed with Lopinavir/ritonavir combination. The most common neuropsychiatric effects of Lopinavir/Ritonavir are reported as agitation, anxiety, confusions, emotional instability (Cao et al., 2020).

Psychiatric drug interaction with Lopinavir/Ritonavir:

Combination with Anticonvulsants:

-A combination with carbamazepine may decrease the plasma levels of Lopinavir/Ritonavir so that the single dose of carbamazepine per day is recommended.

-Lopinavir/ritonavir lowers the concentration of lamotrigine. The increased dose is required.

-Lopinavir/ritonavir increases the plasma levels of Valproic acid. Thus, the dose requirement is needed.

- No change in plasma levels of lithium when it is combined with Lopinavir/ritonavir. It should be taken into account for QTc interval prolongation

Combination with Antidepressants

-Lowering the doses of trazodone is recommended due to side effects such as dizziness, nausea.

-The risk of QTc prolongation increases due to the plasma levels of clomipramine, citalopram, escitalopram, desipramine, imipramine, amitriptyline increase.

-The concentration of Bupropion decreases. Thus, the dose requirement is needed.

-A combination with St John's Wort is not recommended due to it decreases the concentration of antiviral medication.

Combination with Antipsychotics

-A combination with lurasidone ve pimozide is contraindicated due to lopinavir/ritonavir increases drug levels and side effects.

-A combination with quetiapine is contraindicated due to the side effect of a coma.

-No interaction has been shown with amisulpride.

-It is not recommended to use with clozapine due to myelosuppression side effect.

Combination with Anxiolytics:

- A combination with midazolam and triazolam is contraindicated due to increased drug levels.

- Combination with anxiolytics which do not depend on CYP metabolism is suggested, such as lorazepam. (Http://Www.Covid19-Druginteractions.Org, n.d.).

Favipiravir:

On 15 February 2020, China has approved Favipiravir for treatment of influenza, which is an antiviral that act as a RNA dependant RNA polymerase inhibitor. It is also used in clinical trials for COVID-19 treatment (Baranovich et al., 2013). In a recent review that 29 studies were identified, it has been reported that favipiravir was tolerable and showed a safety but more evidence was needed to assess the long-term side effects such as hyperuricemia, teratogenicity and QTc prolongation (Pilkington et al., 2020). However, no neuropsychiatric side effect has been reported so far.

Psychiatric drug interaction with Favipiravir:

-No interaction with anticonvulsants, antidepressants, antipsychotics, anxiolytics has been reported with the combined use of Favipiravir so far (Http://Www.Covid19-Druginteractions.Org, n.d.).

Tocilizumab:

Cytokine storm has been observed in patients with SARS-CoV-2 and it is caused by elevated Interleukin-6 (IL-6) (Luo et al., 2020). Tocilizumab is a monoclonal antibody and acts as an IL-6 receptor antagonist. Recently, it has been used as an alternative treatment for COVID-19 for lessening cytokine storm (Zhang et al., 2020). In a recent study that investigates the efficacy and safety of tocilizumab in patients with COVID-19, no obvious side effect was reported and the rate of secondary infection was not so high. It was reported as an effective treatment to reduce mortality (Xu et al., 2020).

According to the literature, the medications that have immune-modulating effects such as adalimumab, etanercept, infliximab, tocilizumab, have positive effects on depressive symptoms (Kappelmann et al., 2018). Similarly, it has been also indicated that inflammation and IL-6 activity may be related with clinical features and pathophysiology of schizophrenia (Girgis et al., 2018).

Psychiatric drug interaction with Tocilizumab

Combination with Anticonvulsants:

-No major interactions reported with anticonvulsants

Combination with Antidepressants:

- No major interactions reported with antidepressants.

Combination with Antipsychotics:

- No major interactions reported with antipsychotics. But risky used with clozapine is reported for potentially hepatotoxic side effects

Combination with Anxiolytics:

-No major interactions reported with anxiolytic (Http://Www.Covid19-Druginteractions.Org, n.d.).

4. Conclusion

Since COVID-19 impacts multi organs systems, the infection and its treatment may cause many side effects and drug interactions as well. Psychiatric diseases are also chronic diseases and psychiatric drugs may have severe interactions with COVID-19 medications. Thus, drug interactions should be known for providing the best treatment with lower side effects for psychiatric patients with COVID-19.

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It has been reported that chloroquine, hydroxychloroquine, azithromycin, ribavirin, lopinavir/ritonavir have interactions with psychiatric medications such as antidepressants, antipsychotics, anticonvulsants and, anxiolytics in terms of QTc interval prolongation, hepatotoxicity, myelosuppression, increased or decreased concentration of medications. Remdesivir has lower interactions with most of the psychiatric drugs except St John's Wort and some anticonvulsants. However, favipiravir is considered as the COVID-19 medication which has lower interaction and side effects in combination with psychiatric medication. The use of tocilizumab is also safe with most of the psychiatric drugs and has a positive effect on depressive symptoms, although there is a risk of hepatotoxicity.

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EVALUATION OF THE EFFECTS OF LACTATIONAL EXPOSURE TO HYOSCYAMINE FRACTION OF *DATURA STRAMONIUM* L. SEEDS ON LEARNING AND MEMORY IN WISTAR RATS (*RATTUS NORVEGICUS*)

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Ethics committee approval: The ethics committee approval has been obtained from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC/2018/042).

Abstract

The study was designed to determine whether lactational exposure to hyoscyamine fraction of Datura stramonium L. (D. stramonium) seeds affect the cognitive, spatial learning and memory functions of the hippocampus in Wistar rats at adulthood. Fresh seeds of D. stramonium L. were procured, macerated and fractionated using high-performance liquid chromatography (HPLC). Eight (8) Wistar rats weighed 150-250 grams of equal gender were used for the study. The rats were mated and divided into control and treatment groups. Equivalent body weight of normal saline and 400 mg/kgbwt of hyoscyamine fraction were orally administered to the breastfeeding rats respectively on lactational days (LD) 1-21. At adulthood, the rats were subjected to neurobehavioural tests using novel objects recognition (NORT) and Morris water maze (MWM) respectively. The data obtained were expressed as mean ± SEM, independent two samples t-test and General Linear Model (GLM) repeated-measures ANOVA with Fisher's multiple comparisons post-hoc tests were used to show the mean differences using Minitab 17 (LLC., U.K.) statistical package software. $P < 0.05$ was considered statistically significant. A significant increase in the meantime of exploration between the groups ($p = 0.049$) was observed during the NORT. No statistically significant increase ($p = 0.626$) in the meantime to locate the escape platform between the groups during the MWM test. The CA1 region of the treated group showed mild nuclear hyperchromasia, cytoplasmic vacuolations and pyknosis. In conclusion, exposure to hyoscyamine fraction of D. stramonium L. seeds at lactation caused histologic changes in the CA1 region, loss in short-term memory but not spatial learning and memory functions of the hippocampus of Wistar rats at adulthood.

Keywords: D. stramonium, hippocampus, histology, hyoscyamine, lactation

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1. Introduction

Breastfeeding has an advantage for both infants and mothers. It provides the nutritional requirement of developing of infants (WHO, 2013). It gives optimal nutrition and improved mental performance and neurological development (Onyiapat *et al*., 2011). It also enhances immunity (Okoronkwo *et al*., 2014; Bodeker *et al*., 2002) of the developing infants. It decreases the chances of an unexpected death of infants, allergic diseases, and development of Type-1 and Type-2 diabetes mellitus (Zhang *et al*., 2012; Achigbu & Achigbu, 2014; Okoronkwo *et al*., 2014; Alwhaibi and Sambamoorthi, 2016) when compared to infant formula. Breastfeeding reduces postpartum depression, bleeding, and improves weight control (Kaadaaga *et al*., 2014). Transfer of medical substances by the breastfeeding women (Zhang *et al*., 2012; Kaadaaga *et al*., 2014) to their babies is a matter of concern. In humans, the medicines that circulate in the maternal bloodstream can be transferred to their babies through lactation, hence, exposing the infants to such medicines which may potentially be harmful (Zhang *et al*., 2012; Kaadaaga *et al*., 2014). Some of the conventional medicines indicated to have compromised milk production include cabergoline (Tsai *et al*., 2012), bromocriptine (Chien *et al*., 2006), ergotamine (Ebrahim *et al*., 2012), pseudoephedrine (Gatti, 2008), and oestrogens (Ebrahim *et al*., 2012; Jackson, 2010). Nowadays, the patronage of traditional herbs is on the rise globally. Many developed countries across the globe including the United States (Sibeko *et al*., 2005; Kimani-Murage *et al*., 2015), Canada (Ranasinghe *et al*., 2015), the United Kingdom (Diaz *et al*., 2013; James *et al*., 2018), United Arab Emirates (James *et al*., 2018) and Australia (Nordeng *et al*., 2013; Macfoy, 2013; Mothupi, 2014; Pieterse and Lodge, 2015; James and Bah, 2016 and 2018; Witter *et al*., 2016), have reported significant patronage of traditional herbs amongst the general population. The trend is similar in Africa such that either modern health care and medicine is often available only to a limited number of people, the facilities are too expensive or too few to carter the needs of too many people (Ahmed *et al*., 2018).

Datura stramonium, a family member of Solanaceae, an annual plant that possesses phytoconstituents, with alkaloids possessing strong anticholinergic properties (Berger & Ashkenazi, 2003; Nuhu & Ghani, 2002, Oberndorfer *et al*., 2002; Ruhwald, 2005). They competitively antagonize acetylcholine at peripheral and central muscarinic receptors for the common binding site (Friedman and Levin, 1989; Ruhwald, 2005). Its leaves are used to bust-up sagged breast among Pakistan women, while seeds are taken in a cup of green tea to relieve headache (Hussain *et al*., 2006).

In Nigeria, the juice of *Datura* leaves mix with warm milk is used to expel intestinal worms (Egharevba and Ikhatua, 2008), while the seeds in palm oils are used for external treatment of insect bites and stings (Rajbhandari, 2001). Both seeds and leaves of *Datura* are consumed by the youths as a part of the local beverage (Zobo) or in porridge for recreation at ceremonies and public stroke-beating (Shadi/Sharo) in the suitors of Fulani tribe. This marks a sign of courage and responsibility to the suitress and its family. Pieces of literature have reported that *D. stramonium* decreases the production of breast milk (Barrager *et al*., 2002), however, little was reported as to whether it is safe or harmful to the nursing infant (Mills, 2006) if consumed at lactation.

The current study aimed to find out whether maternal ingestion of hyoscyamine fraction of *D. stramonium* L. seeds at lactation affects the cognitive function of the hippocampus in Wistar rats at adulthood. The study may provide awareness on the risk associated with the ingestion of psychoactive ethnomedicinal plants such as *D. stramonium* L. seeds during lactation on the memory functions of the hippocampus.

2. Materials and Methods

The Ethics committee approval: The study was approved by the ethics committee of Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC/2018/042) which is in line with the Declaration of Helsinki.

2.1. Plant materials

Fresh *D. stramonium* seeds were procured from Sharada residential area of Kano Municipal Local Government, Kano State, Nigeria. The seeds were identified and a voucher number (VN108) was issued at the herbarium of the Botany Department, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Kaduna state, Nigeria. The seeds were separated from the pods, washed thoroughly with clean tap water and air-dried under shade. Two thousand grams of the dried seeds were weighed using a digital weighing machine, grounded to a pulp using an electronic blender. The powdered sample was collected into a sterile cellophane bag and kept in a cool dry place for extraction.

2.2. Ethanol extraction of crude D. stramonium seeds

Extraction was carried out using cold maceration according to Djilani *et al*., 2006. The 200g of pulverized seeds were soaked in 1, 500 ml of 70% (v/v) ethanol at room temperature and allowed to macerate for 72 hrs. The extract was filtered and the solvent was evaporated in a water-bath at 40 °C. The residue, dissolved in 250 ml H_2O and acidified with few drops H_2SO_4 to pH 3-4, extracted with petroleum ether and diethyl ether to remove lipophilic, acidic and neutral material, and basified with the aqueous solution of NH_4OH (0.25M) at pH 9-10. The extract was washed with distilled water to neutral pH, dried with Na_2SO_4 and concentrated to dryness under reduced pressure to obtain crude alkaloids.

2.3. Fractionation of hyoscyamine

The fractionation was carried out according to Salamah and Ningsih, 2017. Five grams of the viscous extract was dissolved in 10 ml of water. The solution was then poured into a separating funnel, added with 10 ml of chloroform, and shaken to solve with two phases, namely water and chloroform. These two phases were separated and collected. This was repeated until the chloroform phase had the same colour as the chloroform solvent. The chloroform was then evaporated and recrystallized to obtain the hyoscyamine fraction. The alkaloid was analyzed with UV-Vis spectrophotometric method. The extraction and fractionation were carried out at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria.

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2.4. Quantification of hyoscyamine fraction

HPLC Condition

A reversed-phase Techsphere 50DS C18 HPLC column (25 cm × 4.6 mm i.d.) particle size 5 µm, Supelco, Bellefonte, PA, USA) with oven temperature, 40°C in conjunction with UV adsorption detector operating at 270 nm was employed. The mobile phase was a mixture of 20% acetonitrile, and 45 % methanol, 35 % water (H_2O) and 0.1 mol/L phosphoric acids which adjusted the pH to 7.0 and flow rate of 1 ml/min. A calibration curve for l-hyoscyamine was plotted to determine the amount of the hyoscyamine in the sample fraction. All analyses were carried out at the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria.

2.5. Experimental animals

Eight (8) healthy Wistar rats comprised of equal numbers of adult males and virgin females were procured from the Animal House of the Anatomy Department, Faculty of Basic Medical Sciences, Bayero University Kano, Nigeria. The animals were transported to the Animal House of the Pharmacology Department, Faculty of Pharmaceutical Sciences, Ahmadu Bello University (ABU) Zaria, Kaduna State, Nigeria. The males were separated from the females, housed and allowed to acclimatize for two weeks at ambient temperature, with alternate day and night cycles at the natural condition. Rat chow (Vital feeds®) and tap water were made available to the animal's *ad libitum*. The median lethal dose (LD_{50}) of hyoscyamine fraction was determined using Lorke's (1983) method. Neurotoxicity symptoms were observed and the animals were allowed for twenty-four hours to be observed for mortality.

2.6. Synchronization and phase determination of the oestrus cycle and mating

The female rats were injected with an equivalent bodyweight of Zoladex® (3.6 mg AstraZeneca) intraperitoneally to synchronize their oestrus cycle. Twenty-four after the synchronization, vaginal smears were collected by vaginal lavage (Michelle, 2015) using a 1 ml plastic pipette filled with 10 µL of normal saline (NaCl 0.9%). The tip of the pipette was gently but superficially inserted into the rat vagina (Marcondes *et al*., 2002). The vaginal fluid was carefully aspirated and placed on a cleaned glass slide. A different glass slide was used for each rat, and unstained material was viewed under a light microscope, without the use of the condenser lens, at 10 x objective lenses. The proportion of the round-nucleated cells, cornified cells, the leukocytes among them was used to determine phases of the oestrous cycle (Marcondes *et al*., 2002).

2.7. Experimental design

The animals were randomly selected and divided into two (2) groups; control and treatment. Each group contained a total of eight (8) rats in the ratio of 1:1 adult male to virgin female. Animals in each group were allowed to mate freely and evidence of mating was established by the presence of sperms tails in the vaginal smears collected and viewed under a light microscope after 24 hrs (figure 1b). Abdominal palpation was carried out to avoid error due to pseudopregnancy. The pregnant dams detected, were isolated and transferred to maternity cages. Animal from the same group was kept closely but separately together in different cages. A total of forty pups (40) were obtained at after parturition. The control and treatment groups received an equivalent bodyweight of a single dose of normal saline and 400 mg/kgbwt of hyoscyamine fraction of *D. stramonium* seeds respectively orally daily for three weeks, from the lactational day (LD) 1-21. After treatment, the animals were nurtured to adulthood (PND 75).

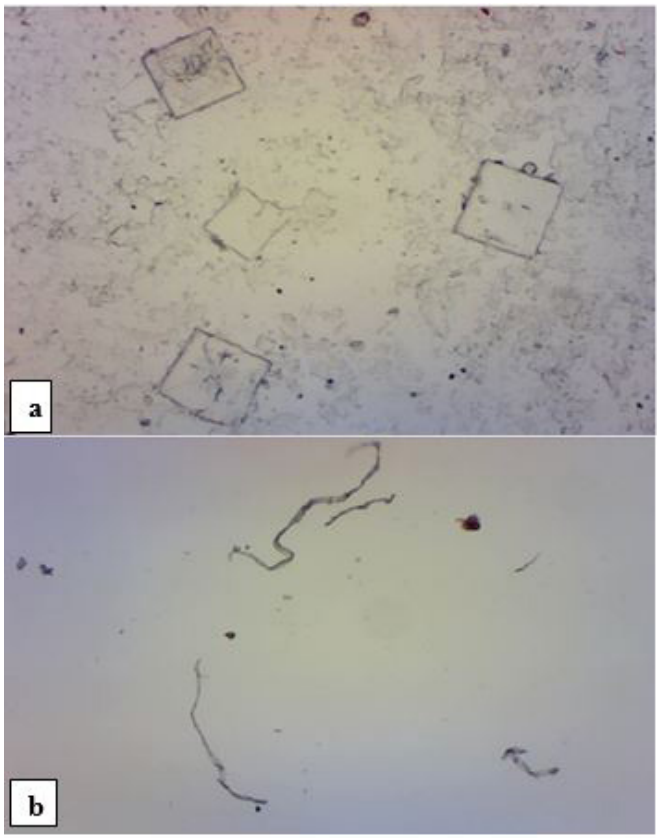

Figure I a&b. Proestrous phase and sperm tails in vaginal smears of Wistar rats showing nucleated leukocytes and sperm tails 24 hrs after mating (x 10 magnification).

2.8. Novel object recognition test

This was conducted at adulthood (PND 60 -75). The aim was to test short-term memory according to Gaskin *et al*., 2010. The test consisted of three phases i.e. (habituation, sampling and test) which was completed in two days. For each phase of this test, the open field arena was thoroughly cleaned with an unscented bleach germicidal wipe, 70% Ethanol followed by distilled water before initial use. A day before object exposure, the rats were habituated to the open field arena in a 50 x 50 cm wooden box. Before the habituation session, a digital and a video camera (Model DCR – PJ5E, SONY®) was used for proper video coverage of the rats' activity in the maze. A rat at a time was gently removed from the home cage and placed in the centre of the arena. The video covering system was turned on and the rat was allowed to freely explore the arena for 10 minutes. At the end of every session, the arena thoroughly sanitized before the next session began.

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This was repeated for all the rats until all got habituated the arena. The same protocol was observed during the sampling and test phases only that, two identical objects (A1 and A2) and two unidentical objects (A and B) objects for were used 15 minutes respectively. The object bias score was calculated according to Ennaceur and Delacour (1988).

2.9. Morris water maze

This was carried out also at adulthood (PND 60 – 75) for six consecutive days using Morris (1984) protocol to test spatial learning and memory. The apparatus consisted of a circular Aluminium tank of 100 cm diameter and 60 cm depth with an escape platform of 20 cm long and 12 cm diameter, filled with a pool of clean water to about the two-third depth of the tank at 22 – 25°C, deep enough to expose 2.54 cm (1 inch) of the platform above the water surface. A digital video device (Model DCR – PJ5E, SONY®) was suspended directly over the pool to capture the entire setup. The rats were trained for 5 days with methylene-blue stained water that submerged the platform 1 inch beneath except for the pretraining day1, where the platform was 1 inch above the clean and clear water. A latency period of 60 sec was allowed for each rat to find the platform. This was repeated for all the rats at five different locations by changing the positions of the platform in the pool within the N, E, S, and W directions following Qing *et al*. (2008) protocol. On day 6, the test day, the setup was maintained as the previous days except that 30 seconds per trial with no escape platform was observed. The time taken for each rat to identify the usual position of the platform was recorded and all videos recorded for the trials were analyzed for the escape latency.

2.10. Animal sacrifice and histological methodology

The animals were euthanized using 75% Ketamine (10 mg/ml USP) anaesthesia, the brains were dissected, removed and preserved in Bouin's fluid for histological procedures. The tissues were processed in the Department of Pathology, Ahmadu Bello University Teaching Hospital (ABUTH), Shika, Zaria, Kaduna state, Nigeria. The brain tissues were dehydrated (graded alcohol) and cleared (in xylene) using an automatic processing machine (Shandon Southern Duplex Processor). The tissues were embedded in paraffin wax and blocked in the coronal plane. Serial sections of the blocks were taken at 8 µm with a microtome (Leitz Wetzlar), mounted on glass slides and allowed to dry overnight. The staining technique employed was hematoxylin and eosin in paraffin sections (Lillie and Fullmer, 1976). Sections were viewed under a light Olympus Binocular Microscope (Ch-20i, Uttar Pradesh, India) high magnifications (x 40) and micrographs were taken with the help of Celestron® eyepiece digital camera (EC 3.0 MP, China). The sections of the hippocampus were observed in the treated rats and compared to the controls.

2.11. Statistical Analyses

The data obtained were expressed as mean ± SEM. Independent samples – t-test and pairwise General Linear Model (GLM) repeated measures ANOVA followed with Fisher's multiple comparisons post-hoc was carried out to find the mean differences in the escape latency, exploration, discrimination and novelty preference time between groups using Minitab 17 (LLC., U.K.) statistical package software. $P < 0.05$ was considered statistically significant. All figures and charts were constructed using GraphPad Prism 8.

3. Results

No mortality was observed in the first phase when the animals received 10, 100 and 1,000 mg/kgbwt. However, toxicity symptoms characterized by restlessness (hyperactivity), laboured breathing, piloerection, abdominal cramps, stooling (diarrhoea), and urination, were observed especially at the 1,000 mg/kgbwt. The symptoms later disappeared and the animals became calm, weak and quiet. During the second phase, the symptoms persisted with high intensity in all the groups treated with 1,600, 2,900 and 5,000mg/kgbwt. Neurotoxicity symptoms were observed but, no mortality was recorded even at the highest dose. The *D. stramonium* fraction was therefore considered safe in Wistar rats and 5000 mg/kgbwt was taken as the LD50.

Figure 2 shows percentage exploration time in the sampling phase of cognitive function test using novel objects recognition test (NORT) using two identical objects (A1 and A2) between the control, and adult Wistar rats treated with 400 mg/kgbwt hyoscyamine treated groups. There was no statistically significant difference ($p > 0.05$) in the exploration time for objects exploration. Any rat that scored less than or above 20 or 80% respectively was excluded for the test-phase of the experiment.

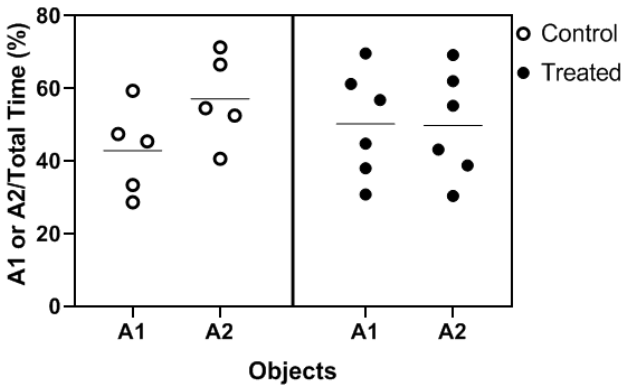

Figure 2. Sampling phase of Novel object recognition in Wistar rats exposed to an equivalent bodyweight of normal saline and 400 mg/kgbwt hyoscyamine fraction of *D. stramonium* seeds at lactational day (LD)1-21.

The result of comparisons of the novel object recognition test in the test-phase between the control and treated groups was shown in figure 3. The time spent to explore the novel object (B) decrease significantly ($p = 0.049$) compared to the familiar (A). Although, there was an increase in the time taken to explore novel object (B) in the treated group but was not statistically significant ($p = 0.238$).

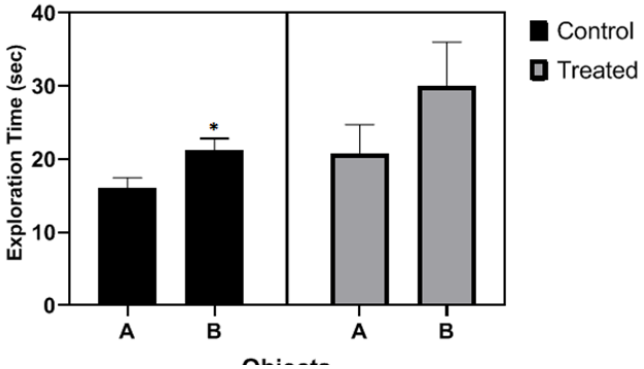

Figure 3. Test-phase in the novel object recognition test in Wistar rats exposed to an equivalent bodyweight of normal saline and 400 mg/kgbwt hyoscyamine fraction of D. stramonium seeds at lactational day (LD)1-21. *p = 0.049.

Figure 4 comparison of discrimination index in novel object between the control and treated groups. No statistically significant difference (p >0.05) in the discrimination ability between the groups.

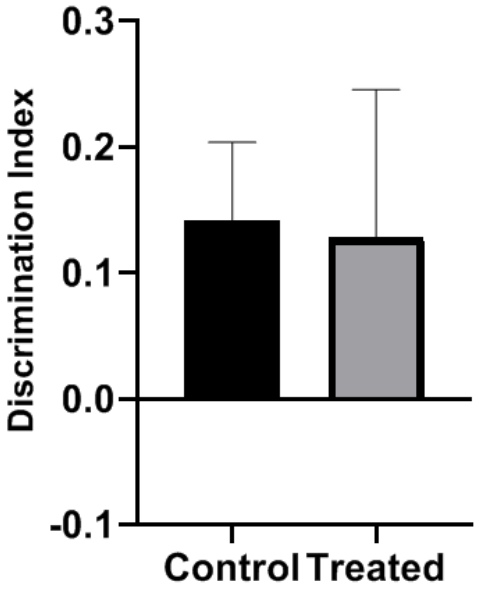

Figure 4. Discrimination index of novel object recognition test in Wistar rats exposed to an equivalent bodyweight of normal saline and 400 mg/kgbwt hyoscyamine fraction of *D. stramonium* seeds at lactational day (LD)1-21.

In figure 5 independent two samples t-test of novelty preference between the groups. The time taken to explore the novel object between the groups was not statistically significant (p = 0.411). Thus, the control and the treated had an equal preference for the novel object.

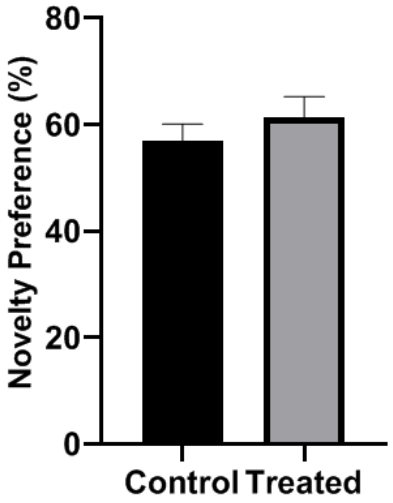

Figure 5. Novelty preference in Wistar rats exposed to an equivalent bodyweight of normal saline and 400 mg/kgbwt hyoscyamine fraction of *D. stramonium* seeds at lactational day (LD)1-21

Figure 6 shows a comparison of the spatial learning and memory test. No significant differences in the time taken to locate the escape platform between groups [F (1, 54) = 0.02, p = 0.875] or between groups and days [F (5, 54) = 0.64, p = 0.670]. The post-hoc test further showed that during the first and third training days the treated groups showed lesser escape latencies when compared to the controls, there was however no statistically significant (p > 0.05) differences in the spatial learning was observed when compared to the controls. Similar observations were made in the control group on the second and fifth training days of the training (p > 0.05). On the fourth training day, the two groups located the escape platform at an equal time with no statistically significant (p = 0.799) difference in the escape latencies. On the sixth day (probe), the escape latency time was shorter in the treated compared to the control group but not significant statistically (p = 0.626).

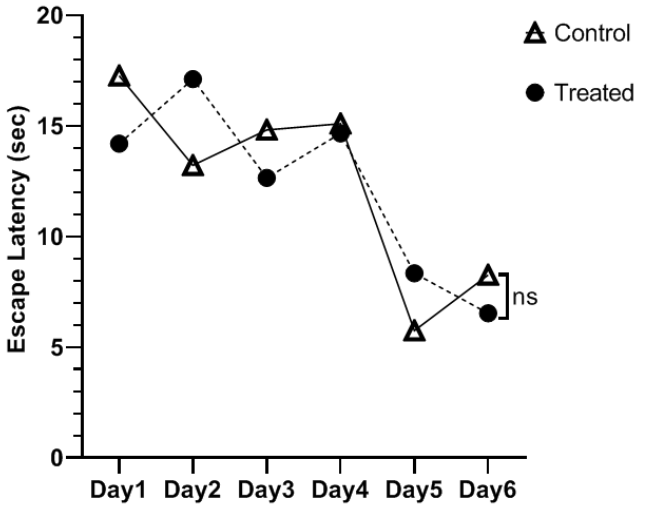

Figure 6. Morris water maze test in Wistar rats exposed to an equivalent bodyweight of normal saline and 400 mg/kgbwt hyoscyamine fraction of D. stramonium seeds at lactational day (LD)1-21. p = 0.626.

Figure 7 a&b, H&E photomicrographs of CA1 regions in adult Wistar rats at 12th weeks after being treated with normal saline and hyoscyamine fraction of *D. stramonium* L. seeds from the lactational day (LD) 1 – 21 as control and treated groups respectively. The control group showed normal histology of the SO, SR and PCL with slight patches of oligodendrocytes as compared to the treated group were, slight patches of smaller but mild nuclear hyperchromasia nuclei (black arrows), mild cytoplasmic vacuolations (black asterisk) and mild pyknotic cells (white arrowhead).

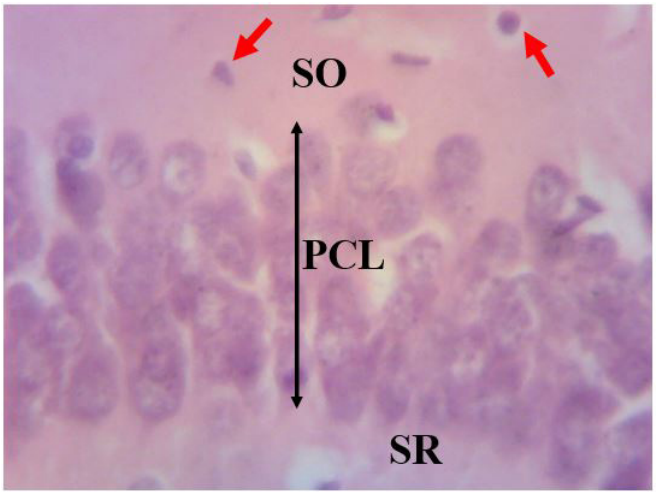

Figure 7a. Cornu Ammonis (CA1) region of the hippocampus in Wistar rats exposed to an equivalent bodyweight of normal saline at lactational day (LD)1-21. (H&E, x 400). PCL = Pyramidal Cell Layer, SO = stratum oriens, SR = stratum radiatum, △ = pyknotic pyramidal cells, ◀ = hyperchromic granular cells → = oligodendrocytes.

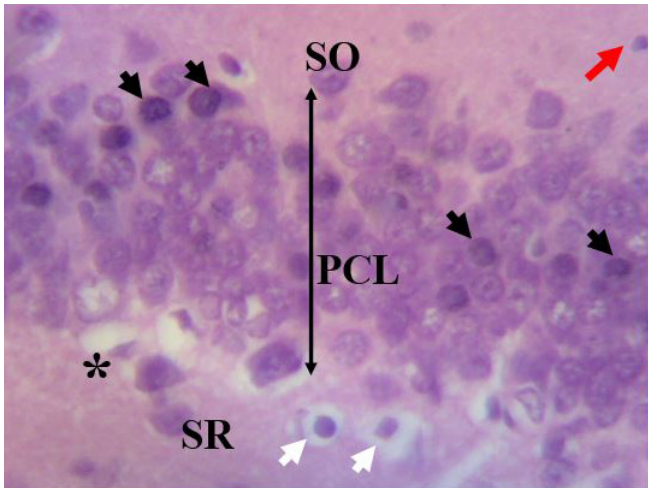

Figure 7b. Cornu Ammonis (CA1) region of hippocampus in Wistar rats exposed 400 mg/kgbwt hyoscyamine fraction of *D. stramonium* seeds at lactational day (LD)1-21. (H&E, x 400). PCL = Pyramidal Cell Layer, SO = stratum oriens, SR = stratum radiatum, △ = pyknotic pyramidal cells, ◀ = hyperchromic granular cells → = oligodendrocytes, * = cytoplasmic vacuolations.

4. Discussion

Ingestion of hyoscyamine fraction resulted in both central and peripheral neurotoxicity symptoms in the rats fed with graded doses of the fraction. However, no mortality was recorded as a result of ingestion both during toxicity testing and experiment itself. In a related study by Babalola *et al*. (2015) reported that the median toxic dose of *D. stramonium* fed orally in dogs was at the safety margin as considered Centre for Disease Control (CDC) the United State of America, states. However, no published literature works available to make a comparison of the current study in Wistar rats. Considering the foregoing it could be assumed that oral ingestion of *D. stramonium* seeds might have a high safety margin in Wistar rats. The clinical symptoms observed might probably result from the anticholinergic properties of tropane alkaloids which competes and irreversibly inhibits acetylcholine on muscarinic receptors, thereby causing both central and peripheral nervous system manifestations (Hanna *et al*., 1992). The central nervous system features include restlessness (hyperactivity), laboured breathing and delirium, while the peripheral symptoms observed include breathing, piloerection, abdominal cramps, stooling (diarrhoea), and urination. Similar observations were reported in patients involved in *D. stramonium* poisoning (Ramirez *et al*., 1999).

Hyoscyamine accounts for 66% of the total tropane alkaloid content (El Bazaoui, 2011) with about 99% of the analyzed *Datura* seeds as (-)-hyoscyamine (Marín-Sáez *et al*., 2016). The antimuscarinic activity of hyoscyamine is stereospecifically caused by the (-)-hyoscyamine enantiomer which was estimated to be more potent than the (+)-enantiomer (FAO/WHO, 2020). Acetylcholine (ACh) is one among the main neurotransmitters that cause changes in the brain memory function. It has numerous receptors that are found in various tissue involved in learning and memory (VanPatten & Al-Abed, 2016). Hyoscyamine competes for acetylcholine by binding to muscarinic receptors the nervous systems. The ability of tropane alkaloids to cause a change in neurogenesis have been reported with unclear mechanisms of action (Joels *et al*., 2004; Joosen, 2009). The current study did not observe a statistically significant increase in the novel object recognition test (NORT) during the novel object discrimination or novelty preference between the groups except for the exploration time where the significant increase was observed in the control group, thus more curiosity towards the novel object. There was also, no statistically increase in the time taken by the treated rats to locate the escape platform during spatial learning and memory test of the Morris water maze. To the best our knowledge, this probably is the first report that evaluates the effects of exposure to hyoscyamine fraction of *D. stramonium* seeds on Wistar rats treated at the lactation stage, for neurobehavioral impairments. The lack of cognitive deficit observed in the adulthood perhaps probably indicates that hippocampus exposure to hyoscyamine fraction of *D. stramonium* seeds does not affect the memory functions of the hippocampus at adulthood in Wistar rats. In a similar study, however, adolescent Wistar rats exposed to atropine were reported to suffer a deficit in hippocampus cognitive function at adulthood (Olawepo *et al*. (2017). This discrepancy might be attributed to the route of exposure and enantiomerization during the extraction processes, as enantiomerisation of hyoscyamine from (-) to (+)-hyoscyamine is possible under aqueous alkaline

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solution and elevated temperature conditions. Although, it was reported that insignificant quantity is obtainable in breast milk (Alexander *et al*., 2008), stage of development of the Wistar rats may also be considered as the possible reason of the discrepancy, as adolescence is considered as a critical period of neuronal plasticity, hence easily susceptible to neurotoxic tendencies. Studies have also found that exposure to tropane alkaloids to influence the quality (unpleasant taste) and quantity (yield) of milk from lactating animals (Alexander *et al*., 2008), and that passage of substances through breastmilk of the lactating mothers to their newborn babies rely on different various factors, which include; physical and chemical properties of the substances, maternal physiology and molecular velocity of substances (Clewell & Gearhart, 2002). By extension, the pups probably did not receive enough dose of the hyoscyamine fraction to cause damage in the hippocampal neurogenesis that could alter the memory. Another reason attributable to this might probably cause by the short exposure duration and rapid metabolization of the alkaloids in the biological system, as it was reported that oral absorption of the anticholinergic agents, such as atropine and glycopyrrolate was poor as a trace or no amount was reported to be found in breast milk (Hale, 1999).

Hippocampus locates under the cerebral cortex and plays an important role in memory formation (Káli & Dayan, 2004) and navigation (Koene *et al*., 2003). It subdivides into narrow areas with distinguished parts, known as Cornu Ammonis (CA) areas. The cornu ammonis (CA1) neurons are important processing episodic memory in the rats (Bartsch *et al*., 2011). The current study did not observe serious changes in the histology of CA1 regions of the hippocampus between the groups. However, a slight patch of smaller but hyperchromic nuclei, mild cytoplasmic vacuolations and pyknotic cells were observed in the treated group. These observations indicate changes in the histology of the CA1 region caused by the fraction probably due to the release of generated neurotoxic elements such as reactive oxygen species resulted from the action of the fraction. Studies have confirmed that release of nitric oxide, p53, residual oxygen species and cytokines cause excitotoxicity, which could lead to cells loss in the hippocampus (Coyle and Puttfarcken, 1993; Epstein *et al*., 1994; Ankarcrona *et al*., 1995; Morrison *et al*., 1996). To our knowledge, no similar works of literature offer data about histopathological effects of hyoscyamine fraction of *D. stramonium* seeds on pups via lactation, however, Ekanem *et al*. (2016) reported cytoplasmic vacuolation, cellular necrosis in adult Wistar rats treated with ethanol extract of *D. stramonium* seeds intraperitoneally. Also, Bihaqi *et al*. (2012) reported a neuronal lesion characterized by necrosis, ghost cells, haemorrhage and cytoplasmic vacuolations in rats that received intraperitoneal treatment of scopolamine. All tropane alkaloids of *D. stramonium* Linn species parts have central anticholinergic symptoms as it can cross the blood-brain barrier and cause long-lasting effects (Bania *et al*., 2004). It induces hypnosis and neuronal degeneration (Hughes & Clark, 1939).

5. Conclusion

In conclusion, exposure of Wistar pups to hyoscyamine fraction of *D. stramonium* L. seeds at lactation causes mild changes in the histoarchitecture of the CA1 region, a loss in short-term but not enough to impair spatial learning and memory functions of the hippocampus of Wistar rats at adulthood.

Patient informed consent: There is no need for patient informed consent.
Ethics committee approval: The ethics committee approval has been obtained from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC/2018/042).
Conflict of interest: There is no conflict of interest to declare.
Financial support and sponsorship: No funding was received.
Author contribution subject and rate:
Idris Abdu Tela (50%): Design the research, data collection and analyses and wrote the whole manuscript. Sunday Abraham Musa (20%): Organized the research and supervised the article write-up. Ibrahim Abdullahi Iliya (15%): Contributed with comments on research design and slides interpretation.
James Oliver Nzalak (15%): Contributed with comments on manuscript organization and write-up.

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COMPUTATIONAL MODELLING OF TRANS-ZEATIN AS A NOVEL TARGET OF ADENOSINE A2A RECEPTOR: INSIGHTS INTO MOLECULAR INTERACTIONS

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Abstract

Adenosine A2A receptor (A2AR) is a G-protein coupled receptor that is involved in various physiological functions. Zeatin, a plant cytokinin and a derivative of adenine, is recently identified as new ligand of A2AR. However, the ligand-receptor interaction mechanism is not fully revealed. Here, we report a model structure of A2AR in complex with zeatin for the first time, to provide a better understanding of this interaction mechanism. A model structure of A2AR in complex with caffeine used as a positive control. As a result, zeatin displayed the ability to stay more stable at the binding pocket compared with caffeine and the residues involved in the interaction are identified. We propose that zeatin is indeed a novel and promising target for A2AR

Keywords: adenosine A2A receptor, binding pocket, caffeine, protein-ligand interaction, zeatin

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1. Introduction

Adenosine receptors (A1, A2A, A2B, A3) are transmembrane G-protein coupled receptors (GPCRs) that can either stimulate or inhibit adenylyl cyclase (AC) by their Gi and Gs subunits (Lazarus et al., 2011). Adenosine A1 receptor (A1R) and adenosine A3 receptor (A3R) inhibit the AC via Gi subunit,whereas adenosine A2A receptor (A2AR) and adenosine A2B receptor (A2BR) stimulate the activity of AC via Gs subunit, catalyze the production of cyclic adenosine monophosphate (cAMP) (Thiel., 2003). A2ARs are expressed in higher density in the basal ganglia of brain and in lower density in the cardiovascular, and immune system (Schiffmann et al., 2007; Doré et al., 2011). A2AR consists of seven transmembrane domains with an extracellular amino terminus and a cytosolic carboxy terminus as a common feature with all GPCRs. The connection between transmembrane domains consist of between three extracellular and three cytoplasmic loops (Ijzerman et al., 1994). The binding of a ligand occurs on the extracellular side, leading to the conformational changes in the heptahelical transmembrane helix network of the receptor (Jaakola et al., 2010).

Caffeine is a plant-derived methylxanthine and a well known nonselective antagonist of A2AR (Fredholm et al., 2005). A2AR has well defined binding pocket and previous studies showed that caffeine interacts with the residues PHE 168, ILE 274, LEU 249, MET 270, ASN 253, TRP 246, and VAL 84 with an additional polar contact to HIS 278 in the hydrophobic pocket (Dore et al., 2011; Carpenter et al., 2017). Caffeine blocks the activity of A2AR and therefore, it is considered as an effective and widely consumed psychoactive drug and stimulant (Fredholm et al., 1995). Caffeine can display neuroprotective effects by preventing the β-amyloid-induced neurotoxicity or promote wakefulness. (Lazarus et al., 2011; Dall'Igna et al., 2003; Dall'Igna et al., 2007).

Zeatin,a plant phytohormone, promotes plant growth and development, was recently shown to interacts with A2AR, however the binding mechanism has not been discovered yet (Lee et al., 2012). Plant cytokinins are adenine derivatives substituted at the N6-position with either an isoprenoid or aromatic side chain, and cis- and trans-zeatin includes a substitiution of isoprenoid side chain (Lee et al., 2012). Cytokinins have important antioxidative and protective effects in animals at molecular, cellular, tissue and organismal levels (Voller et al., 2017). Zeatin showed antioxidative and cell protective effects against β-amyloid-induced neurotoxicity, similar to caffeine (Choi et al., 2009). Most recently, the possible anti-depressant effect of zeatin on female and male rats was shown, together with the interaction of zeatin with A2A receptor on the same binding site with caffeine (Öz et al., 2020). These findings further suggest that zeatin exerts its effects via A2A-R-mediated downstream pathways. Zeatin can be converted to zeatin riboside by adenosine phosphorylase and zeatin riboside was shown to prevent the serum deprivation-induced apoptosis (Dall'Igna et al., 2007). Previously, it has been known that A2ARs regulates CD4+ T lymphocyte, even suppressing the activation-induced cell death (AICD) of peripheral T cells (Himer et al., 2010). In addition to, zeatin riboside treatment promotes the production of cAMP in T lymphocytes and inhibits the production in CD3+CD4+ T cells of interferons (Lappas et al., 2015). These findings clearly indicate an interaction between zeatin and A2AR, and better understanding of this interaction can be achieved by structural modelling.

2. Materials and Methods

The authors declare that ethics commitee approval does not required for this study, as the study involves only computational work.

2.1. Structure preparation and docking

The crystal structure of A2AR (PDB entry: 5NLX) was used for docking of the ligands: trans-zeatin and caffeine. All other ligands found in the crystal structure of A2AR were removed in order to exert protein for docking step via PyMol (DeLano et al., 2002). Trans-Zeatin and caffeine were used as the ligand while A2A was kept as the receptor. Structures of ligands were obtained from RCSB PDB database and its parameters were determined using SwissParam (Zoete et al., 2011). The crystal structure of A2AR that contains caffeine as a ligand (PDB entry: 5MZP) was used as a control during docking to detect possible binding pocket and connecting residues based on previously published alignments (Carpenter et al., 2017; Cheng et al., 2017). The docking position of caffeine and trans-zeatin were detected in the same binding pocket and docked structure of A2AR complex with caffeine used as positive control during the study. Docking was performed with AutoDock (Goodsell et al., 1996). Final docking poses were selected based on Autodock binding scores that gives best down binding energy and inhibition constant.The binding interactions that were performed during docking were analyzed by Arpeggio (Jubb et al., 2017) and PyMOL(DeLano et al., 2002).

2.2. Designing of the membrane bound protein

Membrane protein tutorial (Aksimentiev et al., 2009) was performed to docking structures A2AR in complex with zeatin (5NLX/ZEA) and A2AR in complex with caffeine (5NLX/CFF) ,were produced previously via AutoDock (Goodsell et al., 1996), by using Visual Molecular Dynamics (VMD) (Humphrey et al., 1996). To provide interaction between membrane and protein, the following steps were adapted from membrane protein tutorial (Aksimentiev et al., 2009): generating PSF and PDB files for builting whole structure, building membrane patch for preparing complete membrane involving water around it, alignment of membrane and protein, combination of membrane and protein for avoiding overlap between protein and lipid molecules, solvation and ionizaiton with 100mM NaCI as the ionic concentration of the system. To perform molecular dynamic simulations, the structures were fixed according membrane protein tutorial (Aksimentiev et al., 2009).

2.3. Molecular dynamics simulations

Molecular dynamics (MD) simulations were performed with the structures that were obtained as a result of the previously described in the stage of desingning of membrane bound protein. The 5NLX/ZEA and 5NLX/CFF complex, composed of 78892 atoms and 78757 atoms were placed in water boxes with dimensions of 83x94x120 Å3 and neutralized with NaCl. The resulting systems for 5NLX/ZEA and 5NLX/CFF were used in MD simulations by

using the NAMD Phillips et al. (2005) with the CHARMM22 MacKerell et al. (1998); Brooks et al. (2009) parameters included correction map (CMAP) for backbone atoms (Feig et al., 2003; MacKerell et al., 2004). TIP3P model Jorgensen et al. (1983) was used for water molecules within the system during the simulation. An NpT ensemble was used in MD simulations with periodic boundary conditions to maintain pressure and temperature while the long-range Coulomb interactions were computed using the particle-mesh Ewald algorithm. Within 50 ns of MD simulation, the pressure was maintained at 1 atm and temperature was maintained at 310 K using the Langevin pressure and temperature coupling. The time step was determined as 1 fs in all MD simulations. For providing the removal of high energy contacts between atoms and highly repulsive orientations of the initial simulated systems, the systems were fully energy minimized in 50,000,000 steps and each system was heated slowly from 0 K to 310 K in 5 ps. Then, the systems were equilibrated under constant temperature and volume for 0.5 ns before production runs. The production runs were completed for 50 ns as it is a large complex and repeated twice. In total, 300 ns MD simulation was performed during the study.

2.4. Detection of interacting residues with ligands: zeatin and caffeine

The MD simulations which were repeated twice as previously described by using the NAMD Phillips et al. (2005) were analyzed to detect interacting residues between protein (5NLX) and ligands (zeatin and caffeine) by using VMD (Humphrey et al., 1996). Hydrogen bonds, Hydrophobic contacts and salt bridges were analyzed in VMD (Humphrey et al., 1996) for the detection of interacting residues during the simulations. Then, these interactions were plotted by using GraphPad Prism (Miller et al.,2003). For the comparison of detected residues with published alignments, a known A2AR with caffeine (PDB entry:5MZP) was used and the residues of 5MZP were renumbered as protein sequence was numbered as 1-305 in 5MZP while protein sequence was numbered as 9-409 in our model.

2.5. Analysis of the molecular dyanmics simulations

VMD (Humphrey et al., 1996) was used for the analysis of trajectories and the visualization of structures. Root mean square displacements (RMSDs) for the backbone atoms of each protein were analyzed for the stability in 50 ns MD simulations. Residue-specific distance between ligand (zeatin and caffeine) and protein was analyzed within 50 ns. In addition, Distance between the interacting atom of residue and the interacting atom of ligand (zeatin and caffeine) was analyzed. Residue-wise root mean square fluctuations (RMSFs) of ligands with protein were measured for the flexibility analysis of the ligand and t-test was applied to determine the significant difference between zeatin and caffeine values based on p-value of <0.05. The numerical data was expressed as mean ± SEM in the graph. RMSFs of the detected residues of the protein were also analyzed within 20-50 ns based on the result of distance analysis because the time that ligands are unstable was detected by interpreting distance and RMSF was calculated within this range. Also, Radius of gyration was analysed for both 5NLX/ZEA and 5NLX/CFF complex. Distance, Radius of Gyration and RMSF graphs were plotted by taking the average of 3 production runs for both 5NLX/ZEA and 5NLX/CFF complex via GraphPad Prism Jorgensen et al. (1983) while RMSD graphs were plotted for each production run respectively via GraphPad Prism (Miller et al.,2003).

3. Results

Trans-zeatin and caffeine were docked to the binding pocket of A2AR (PDB entry:5NLX) as described (Figure 1A) and the docking structure 5NLX/CFF was used as the positive control during this research to understand this targeting mechanism.

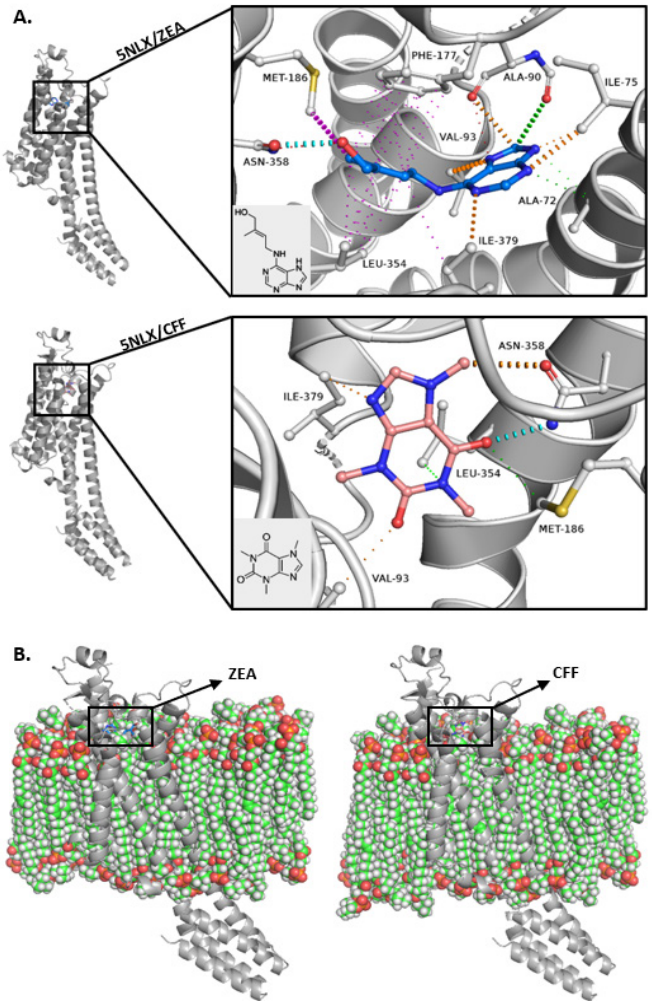

Figure 1: Detection of protein-ligand interaction and membrane alignment before the MD simulations.

A. Interaction of A2AR with ligands (zeatin and caffein) after docking were detected using Arpeggio (Jubb et al., 2017) and PyMOL (DeLano et al., 2002) was used in order to visualize the interactions. 7 weak polar conctacts (orange), 3 polar conctact (red), 2 hydrogen bond (cyan), 3 weak hydrogen bonds (green), 21 hydrophobic contact (magnetta) were found for 5NLX/ZEA. For 5NLX/CFF complex, 5 weak polar contact (orange), 1 polar contact (red), 1 hydrogen bond (cyan), 2 weak hydrogen bonds (green) were found. The other interactions that not mentioned here include aromatic contacts, carbonyl interactions or other type of interactions. **B.** Both 5NLX/ZEA and 5NLX/CFF complex were placed into membrane via membrane protein tutorial (Aksimentiev et al., 2009). Zeatin: ZEA, Caffeine: CFF

Based on crystal structure of A2AR (PDB entry:5MZP), caffeine was previously reported to interact with residues ILE 66, VAL 84, PHE 168, GLU 169, MET 177, LEU 249, ASN 253, MET 270 and ILE 274 (Carpenter et al., 2017; Cheng et al., 2017). The residues of the structure of A2AR (PDB Entry:5MZP) was renumbered be able to compare with the structure of A2AR (PDB Entry:5NLX) used in our model. The corresponding residues are detected as ILE 75, VAL 93, PHE 177, GLU 178, MET 186, LEU 354, ASN 358, MET 375 and ILE 379, respectively. As the result of docking of ligands to receptor, it was determined that caffeine interacts with residues ILE 379, ASN 358, LEU 354, MET 186, VAL 93 while zeatin interacts with residues MET 186, PHE 177, VAL 93, ALA 90, ILE 75, ALA 72, ILE 379, LEU 354, ASN 358 based on our model. This finding showed that zeatin have ability to form more interaction than caffeine in the same binding pocket. 181 contacts in total were detected for 5NLX/ZEA complex while 156 contacts were detected for 5NLX/CFF complex. 7 weak polar conctacts, 3 polar conctact, 2 hydrogen bond, 3 weak hydrogen bonds, 21 hydrophobic contacts were found for 5NLX/ZEA. The hydrogen bonds were formed with the aminoacid residues ASN 358, ALA 90 and ALA 72; polar contacts were formed with ALA 90, ILE 379, ILE 75 and VAL 93 and hydrophobic contacts were formed with MET 186, PHE 177 and LEU 354. For 5NLX/CFF complex, 5 weak polar contact, 1 polar contact, 1 hydrogen bond, 2 weak hydrogen bonds were found. The hydrogen bond were formed with MET 186, ASN 358 and LEU 354; polar contacts were formed ILE 379 and VAL 93. As A2AR is a transmembrane protein, both 5NLX/CFF and 5NLX/ZEA complexes were aligned into membrane (Figure 1B) for the MD simulations.

The hydrophobic pocket residues of A2AR were simulated for 50 ns to compare the adjacency of zeatin and caffeine. RMSD of protein backbone atoms of 5NLX/ZEA and 5NLX/CFF complexes for 3 production runs reached to a steady plateau after 10 ns, indicating stable simulations for analysis (Figure 2A). To explore whether if caffeine and zeatin were held in close proximity to the hydrophobic pockets, the fluctuations of the atoms of caffeine and zeatin were analyzed and the average of 3 production runs was taken for both 5NLX/ZEA and 5NLX/CFF complex. Zeatin showed lower flexibility than caffeine with A2AR based on RMSF as an expected result (Figure 2C) because zeatin has the ability to form more interaction than caffeine, which reduces the flexibility of zeatin.

The interactions between ligands (zeatin and caffeine) and A2AR are given (Table 1) during 50 ns MD simulation. While hydrogen bonds between A2AR and zeatin were mostly formed with the residues MET 375 and HIS 383, hydrogen bonds between A2AR and caffeine were mostly

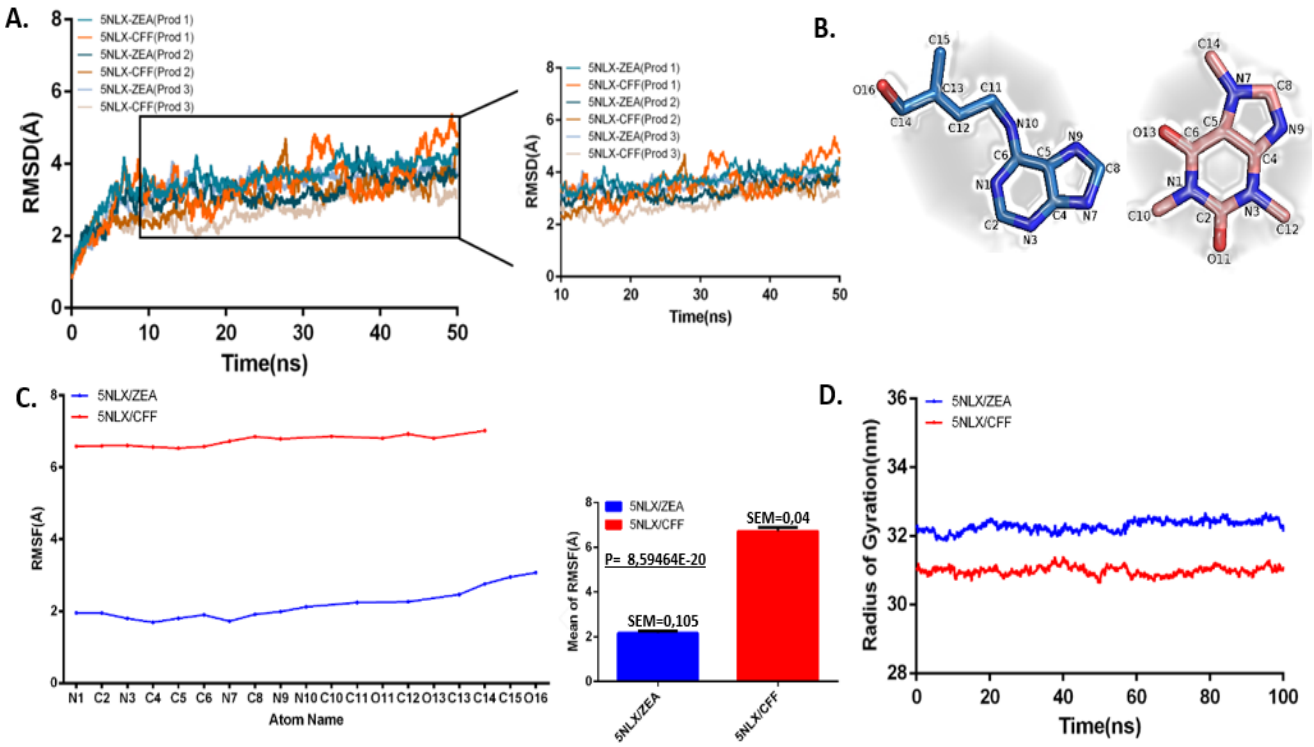

Figure 2: *Identification of 5NLX/ZEA and 5NLX/CFF complexes during MD simulations.*

A. RMSD of backbone atoms of 5NLX/ZEA and 5NLX/CFF complexes was plotted for 3 production run. RMSD of backbone atoms for both complex and for each run was calculated in 3 Å in order to detect the similarity between structures and production runs. The required stability was reached after 10 ns. **B.** The atoms of ligands (zeatin and caffeine) were described in the image. **C.** RMSF of the atoms of zeatin for 5NLX/ZEA (red) complex versus RMSF of the atoms of caffeine for 5NLX/CFF (blue) complex were analyzed in order to compare two structure in 50 ns MD simulation. It is determined that zeatin (blue) showed lower flexibility than caffeine (red). **D.** Radius of gyration which means the distrubition of molecules of all compound was measured for both 5NLX/ZEA (red) and 5NLX/CFF (blue) complex and it showed that structure shape stay stable during 50 ns MD simulation. Å: Angstrom, C: Carbon, O: Oxygen, N: Nitrogen

formed with residues ASN 358. Furthermore, the number of hydrogen bonds on frames are also given (Figure 3). Hydrophobic interactions between A2AR and zeatin were mostly formed with the residues ILE 379, LEU 354, ALA 382, PHE 177, TYR 376, HIS 383, ILE 75 while hydrophobic interctions between A2AR and caffeine were mostly formed with the residues PHE 177, GLU 178, ILE 379, HIS 369. Salt bridges were only observed between A2AR and caffeine with the residues GLU 178. These residues were detected based on higher occupancy (>50%) between 999 frames during 50 ns MD simulations. It was determined that most of the residues interact with both zeatin and caffeine but the occupancy between the frames is higher for zeatin and the interactions with the occupancy under 50% for both zeatin and caffeine were not analyzed in this study. For instance, Interaction between zeatin and ILE 379 was observed with the percentage of 86.4% between frames for 5NLX/ZEA çomplex while it was observed with the percentage of 62.8% for 5NLX/CFF complex. Salt bridges were not detected for 5NLX/ZEA complex during the analysis. These findings indicates that zeatin and caffeine display common residue interactions the same binding pocket.

Table 1: The summary of interactions between A2AR with ligands (zeatin and caffeine).

5NLX/ZEA				
Interactions after docking			Interactions during Simulation	
Hydrogen bonds	Polar Bonds	Hydrophobic Bonds	Hydrogen Bonds	Hydrophobic Bonds
ALA 72	ALA 90	MET 186	MET 375	ILE 379
ASN 358	ILE 75	LEU 354	HIS 383	LEU 354
ALA 90	VAL 93	PHE 177		ALA 382
	ILE 379			PHE 177
				TYR 376
				HIS 383
				ILE 75

5NLX/CFF				
Interactions after docking		Interactions during Simulation		
Hydrogen bonds	Polar Bonds	Hydrogen Bonds	Hydrophobic Bonds	Salt Bridges
LEU 354	ILE 379	ASN 358	PHE 177	GLU 178
MET 186	VAL 93		GLU 178	
ASN 358			ILE 379	
			HIS 369	

After the docking of both ligands (zeatin and caffeine) to A2AR (PDB entry:5NLX), interacting residues were observed via Arpeggio (Jubb et al., 2017) as in the table. For MD simulations, interacting residues were detecting between the frames by using VMD Humphrey et al. (1996) and the residues that have higher occupancy (>50%) between 999 frames were choosen during the analysis of hydrogen bonds,hydrophobic bonds and salt bridges.

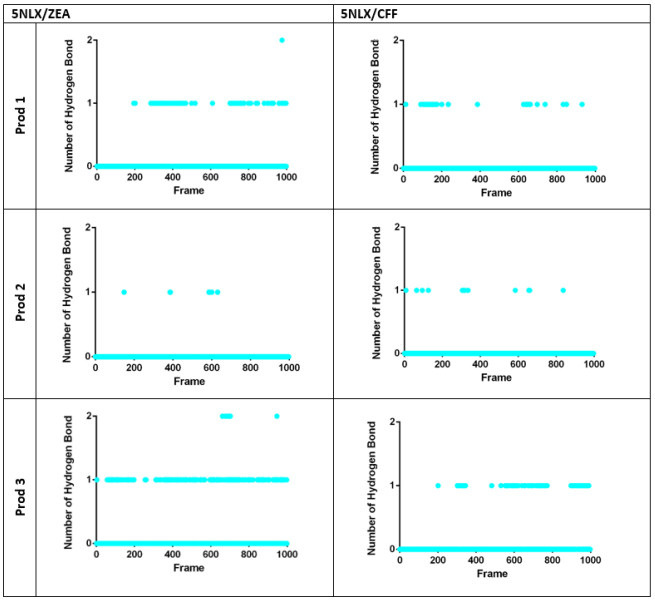

Figure 3: The plots of hydrogen bonds for 5NLX/ZEA and 5NLX/CFF complex.

Hydrogen bond anlaysis was performed in VMD Humphrey et al. (1996) for 3 productions run based on frames in 3 Å. 5NLX/ZEA complex contains more hydrogen bonds than 5NLX/CFF complex for each production run in 50 ns MD simulation.

After the detection of critical residues for both 5NLX/ZEA and 5NLX/CFF complex, distance for 50 ns and RMSF for 20-50 ns were analyzed based on the critical residues. In distance analysis, zeatin stayed stable with the residues TYR 376 (Supplementary Figure 1), LEU 354 (Supplementary Figure 2), ILE 379 (Supplementary Figure 3), MET 375 (Figure 4), PHE 177 (Figure 5), ALA 382 (Figure 7), ILE 75 (Supplementary Figure 4), HIS 383 (Supplementary Figure 6) during 50 ns while caffeine is fluctuated after 20 ns. Caffeine and zeatin get closer during interaction with the residue GLU 178 (Figure 6) between 20-50 ns. For the residue ASN 358 (Supplementary Figure 5), zeatin and caffeine get closer between 20-30 ns and then caffeine moves away from the residue ASN 358. In addition, caffeine and zeatin get closer to HIS 369 (Supplementary Figure 7) between 30-50 ns. Therefore, It was expected that RMSF of this residues would be lower for 5NLX/ZEA complex than 5NLX/CFF complex because zeatin stays more stable during the interaction with these residues in 50 ns MD simulation. RMSF values based on each reasidue was calculated between 20-50 ns but RMSF of these residues were found as similar for both 5NLX/ZEA and 5NLX/CFF complex in our model.

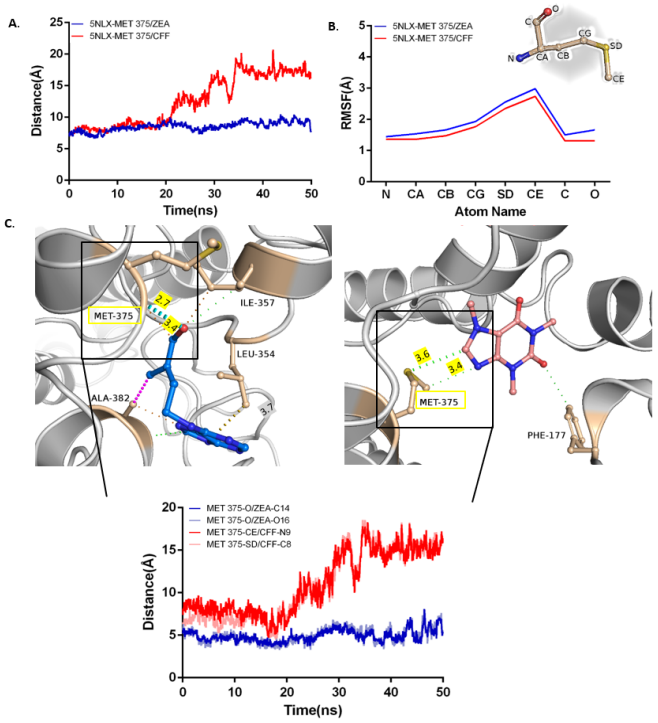

Figure 4: The comparison of interactions with the residue MET 375 for 5NLX/ZEA and 5NLX/CFF complex via analysis of distance and RMSF.

A. Distance of zeatin (red) and caffeine (blue) to the residues MET 375 of A2AR receptor were measured via VMD (Humphrey et al., 1996) . While zeatin stays stable in ~5 Å distance during 50 ns MD simulation, caffeine moves away after 20 ns, from ~5 Å to ~15-20 Å. **B.** Based on RMSF of backbone atoms of the residue MET 375 for both 5NLX/ZEA (red) and 5NLX/CFF (blue) complexes, the flexibility of the residue MET 375 was observed as similar for both 5NLX/CFF and 5NLX/ZEA complexes. Distance analysis provide better understanding for the comparison of the effect of caffeine and zeatin on A2AR, shows that zeatin stays longer with the residues caffeine interacts normally. **C.** The image that was obtained from 422. frame (21,1. Ns) of first production run for 5NLX/ZEA and 327. frame (16,35. Ns) for 5NLX/CFF specifically show the interaction with the residue MET 375 and zeatin. The interaction was highlighted as polar interaction (orange) with the distance 3.4 Å and hydrogen bond (cyan) with the distance 2.7 Å for 5NLX/ZEA, weak hydrogen bonds (green) with distance 3.6 and 3.4 Å for 5NLX/CFF via PyMol (Himer et al., 2010). Distance was also calculated in order to observe the interaction in all 50 ns MD simulation as atom-atom. It was determined from selected images that the interaction performs between the O atoms of residue MET 375 and the C17, O16 atoms of zeatin in one hand while the interaction performs between the CE, SD atom of residue MET 375 and the C8, N9 atoms of caffeine in other hand. The atom-atom distance graph showed that interaction stays stable in 5 Å distance for 5NLX/ZEA complex but distance was increasing after 20 ns for 5NLX/CFF complex. This result indicates that when zea interacts with the residue MET 375, CFF moves away from the interaction point. Zeatin: ZEA, Caffeine: CFF, Å: Angstrom, C: Carbon, CA (Cα): alpha carbon, CB (Cβ): Beta carbon, CG (Cγ): Gamma carbon, CE (Cε): Epsilon carbon O: Oxygen, SD (Sδ): Delta sülfür, N: Nitrogen

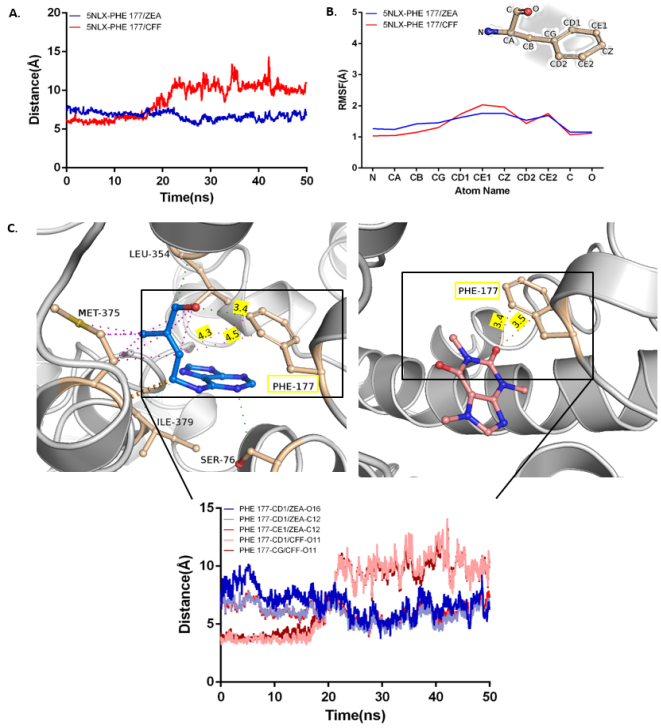

Figure 5: The comparison of interactions with the residue PHE 177 for 5NLX/ZEA and 5NLX/CFF complex via analysis of distance and RMSF.

A. Distance of zeatin (red) and caffeine (blue) to the residues PHE 177 of A2AR were measured via VMD (Humphrey et al., 1996). While zeatin stays stable in ~5-10 Å distance during 50 ns MD simulation, caffeine moves away after 20 ns FROM 5 Å to ~10-15 Å. **B.** Based on RMSF of backbone atoms of the residue PHE 177 for both 5NLX/ZEA (red) and 5NLX/CFF (blue) complexes, the flexibility of the residue PHE 177 was observed as almost similar for both 5NLX/CFF and 5NLX/ZEA complex. Distance analysis provide better understanding for the comparison of the effect of caffeine and zeatin on A2AR, shows that zeatin stays longer with the residues caffeine interacts normally. **C.** The image that was obtained from 554. frame (27.7. ns) of first production run for 5NLX/ZEA and 544. frame (27,2. Ns) for 5NLX/CFF specifically shows the interaction with the residue PHE 177 and zeatin. The interaction was highlighted as hydrophobic interaction (purple) with the distance 4.3 and 4.5 Å and weak hydrogen bond (green) with the distance 3.4 Å for 5NLX/ZEA, polar contacts (orange) with distance 3.4 and 3.5 Å for 5NLX/CFF via PyMol (Himer et al., 2010). Distance was also calculated in order to observe the interaction in all 50 ns MD simulation as atom-atom. It was determined from selected images that the interaction performs between the CD1, CE2 atoms of residue PHE 177 and the C12, O16 atoms of zeatin in one hand while the interaction performs between the CD1, CG atom of residue PHE 177 and the O11 atoms of caffeine in other hand. The atom-atom distance graph showed that interaction stays stable in 5-10 Å for 5NLX/ZEA complex but distance was increasing after 20 ns for 5NLX/CFF complex. This result indicates that when zeatin interacts with the residue, caffeine moves away from the interaction point. Zeatin: ZEA, Caffeine: CFF, Å: Angstrom, C: Carbon, CA (Cα): alpha carbon, CB (Cβ): Beta carbon, CG (Cγ): Gamma carbon, CD (Cδ): Delta carbon, CE (Cε): Epsilon carbon, CZ (Cζ): Zeta carbon, O: Oxygen, N: Nitrogen

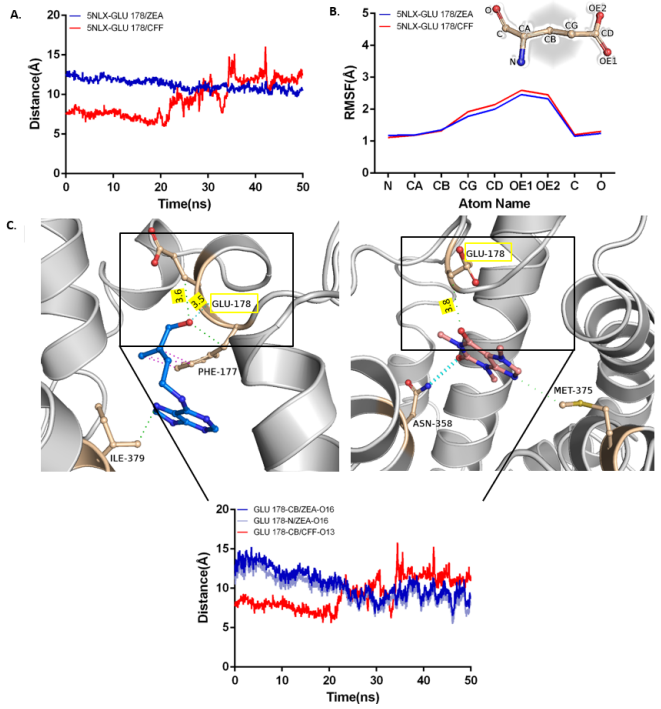

Figure 6: The comparison of interactions with the residue GLU 178 for 5NLX/ZEA and 5NLX/CFF complex via analysis of distance and RMSF.

A. Distance of zeatin (red) and caffeine (blue) to the residues GLU 178 of A2AR measured via VMD (Humphrey et al., 1996). While zeatin stays stable in ~10-15 Å distance during 50 ns MD simulation, caffeine stays stable until 20 ns then, it gets closer with GLU 178 between 20-50 ns, reaching ~15 Å. This data also indicates that caffeine and zeatin stays close position to each others after 20 ns. **B.** Based on RMSF of backbone atoms of the residue GLU 178, both 5NLX/ZEA (red) and 5NLX/CFF (blue) complexes showed that flexibility of the residue GLU 178 was observed as similar for both 5NLX/CFF and 5NLX/ZEA complexes. The possible interaction was formed with another residue can reduce the the flexibility of GLU 178 for 5NLX/CFF complex **C.** The image that was obtained from 924. frame (46,2. ns) of first production run for 5NLX/ZEA and 707. frame (35,35. ns) for 5NLX/CFF specifically show the interaction with the residue GLU 178 and ZEA. The interaction was highlighted as weak hydrogen bond (green) with the distance 3.6 and 3.5 Å for 5NLX/ZEA and 3.8 Å for 5NLX/CFF via PyMol (Himer et al., 2010). Distance was also calculated in order to observe the interaction in all 50 ns MD simulation as atom-atom. It was determined from selected images that the interaction performs between the CB, N atoms of residue E178 and the O16 atom of zeatin in one hand while the interaction performs between the CB atom of residue GLU 178 and the O13 atom of caffeine in other hand. The atom-atom distance graph showed that interaction stays stable in 10-15 Å for 5NLX/ZEA complex but distance was increasing after 20 ns for 5NLX/CFF complex and caffeine gets closer position with the residue and zeatin. This result indicates differently from other residues that both zeatin and caffeine stay stable with the residue GLU 178 after 20 ns which supports the previous RMSF results. GLU 178 can play critical role in stability of caffeine as a salt bridge. Zeatin: ZEA, Caffeine: CFF, Å: Angstrom, C: Carbon, CA (Cα): alpha carbon, CB (Cβ): Beta carbon, CG (Cγ): Gamma carbon, CD (Cδ): Delta carbon, O: Oxygen, OE (Oε): Epsilon oxygen, N: Nitrogen

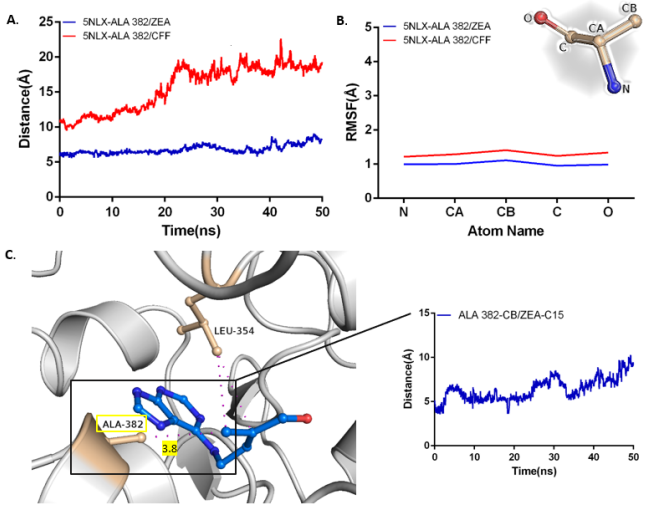

Figure 7: The comparison of interactions with the residue ALA 382 for 5NLX/ZEA and 5NLX/CFF complex via analysis of distance and RMSF.

A. Distance of zatin (red) and caffeine (blue) to the residues ALA 382 of A2AR were measured via VMD (Aksimentiev et al., 2009). While zeatin stays stable in ~5-10 Å distance during 50 ns MD simulation, caffeine moves away after 20 ns from 10 Å to ~20 Å. **B.** Based on RMSF of backbone atoms of the residue ALA 382, both 5NLX/ZEA (red) and 5NLX/CFF (blue) complexes showed that flexibility of the residue ALA 382 is lower for 5NLX/ZEA as expected and this residue may play important role the stability of zeatin during the interaction with A2AR. **C.** The image that was obtained from 457. frame (22,85. ns) of first production run for 5NLX/ZEA specifically shows the interaction with the residue ALA 382 and zeatin. The interaction was highlighted as hydrophobic interaction (purple) with the distance 3.8 Å for 5NLX/ZEA via PyMol (Himer et al., 2010). Distance was also calculated in order to observe the interaction in all 50 ns MD simulation as atom-atom. It was determined from selected images that the interaction performs between the CB atom of residue ALA 382 and the C15 atom of zeatin. No interaction was detected between caffeine and the residue ALA 382 during analysis with the occupancy >50%. The atom-atom distance graph showed that interaction stays stable in 5 Å until ~25 ns and after, distance reachs 10 Å. Zeatin: ZEA, Caffeine: CFF, Å: Angstrom, C: Carbon, CA (Cα): alpha carbon, CB (Cβ): Beta carbon, O: Oxygen, N: Nitrogen

The movement of ligands (zeatin and caffeine) was analysed in each production run and it was detected that caffeine moves outward direction while zeatin moves inward direction during 50 ns MD simulation (Figure 8). The reason of this movement may be the formed new interactions with other residues in 5NLX/CFF complex during the simulation, leading the reduction of flexibility of the residues but these all findings proof that zeatin has ability to stay more stable during interaction with A2AR and interacts with either the same or different residues in order to stay longer in the binding pocket compared to caffeine. Zeatin may have potential role as ligand on A2AR and promising a new target for further studies.

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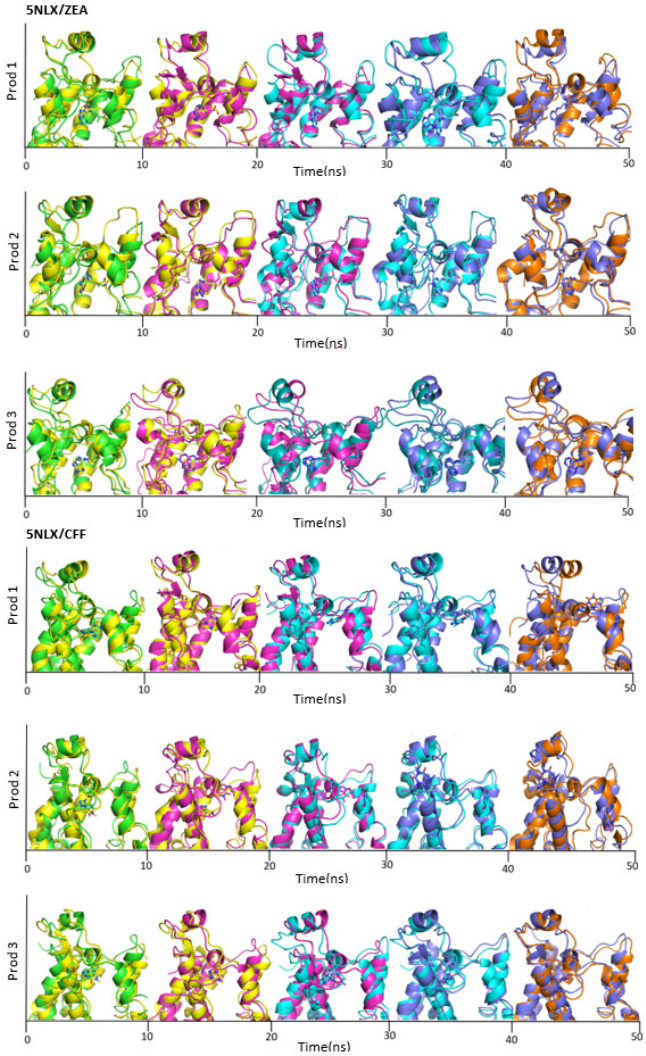

Figure 8: The movement of ligands (zeatin and caffeine) complex within 50 ns MD simulation for 3 production runs.

In 5NLX/ZEA complex, zeatin moves inward direction while caffeine moved outward direction in 5NLX/CFF complex during 50 ns and the most possible reason for the movement of caffeine is the formed new interactions between the different residues of protein and caffeine. Each color represents the structure in certain time. Structure in 0th ns: Green, Structure in 10th ns: Yellow, Structure in 20th ns: pink, Structure in 30th ns: cyan, Structure in 40th ns: purple, Structure in 50th ns: Orange

4. Discussion

A2ARs play a key role in regulating transmembrane signaling pathways in response to specific ligand and caffeine is known as one of the nonselective A2AR antagonist as well as is the most consumed psychostimulant in the World (Stevens et al., 2013; Boia et al., 2016). The main action of caffeine involves the blockade of A2AR on a various of physiological process.[35] Despite of harmful effect of caffeine when it is overtaken, the main reason for consumption of caffeine is to in cognitive performance and mood (Smith et al., 2013; Pasman et al., 2017). However, it enhances performance more in fatigued than well-rested subjects (Lorist et al., 1994; Childs et al., 2008). It has recently been demonstrated that zeatin which is one of the plant hormones activate the mammalian A2AR, playing a role in the regulation of cells involved in both innate and adaptive immunity as well as protect cognitive dysfunction such as improves memory impairment or mood disorders (Lappas et al., 2015; Kim et al., 2008). The interaction mechanism of zeatin on A2AR has not been demonstrated clearly yet. The binding of ligand on A2AR causes the conformational changes on the receptor and leads the activation of signalling pathways (Carpenter et al., 2017; Dalton et al., 2015). The model structure of A2AR in complex with zeatin and caffeine (as positive control) were constructed for this study and analyzed within 50 ns MD simulation with the repeated 3 production runs. Because of the size of system, the simulation time was kept as 50 ns for each production run but it can be extended for future studies to provide better estimation. The results indicate that both zeatin and caffeine have interaction in the same hydrophobic pocket. The residues TYR 376, LEU 354, ILE 379, MET 375, PHE 177, ALA 382, ILE 75, ASN 358, GLU178, HIS 383, HIS 369 were determined as critical for the interaction between A2AR and both ligands. Residue-specific distance showed that zeatin stays more stable than caffeine during 50 ns MD simulation, supporting zeatin as a new target. Despite of the distance analysis, RMSF of each residue showed close results for both 5NLX/ZEA and 5NLX/CFF complex. This finding provides a new question for the further studies, how the flexibility of the protein residues in 5NLX/CFF complex based on residue-specific RMSF analysis can be observed almost the same with in 5NLX/ZEA complex although zeatin is determined as more stable based on distance analysis? In addition to, zeatin was also determined as less flexible during RMSF analysis for ligands (Figure 2). The most possible reason for it can be because of new performed interactions occuring on the specific residue with other residues of protein during 50 ns MD simulation. As the residues are allowed to interact for a period of time to observe dynamic evolution of the system, the flexibility of the specific residue can be observed as less for both models. The combination of study with distance analysis provided more accurate and sensitive results to detect the stability of zeatin on A2AR. Although, zeatin was detected as stable in most of the choosen residues, the residue ALA 382 may plays important role in stabilization of zeatin in complex because the interaction with the residue ALA 382 only was performed in 5NLX/ZEA complex and the flexibility of the residue was lower for 5NLX/ZEA complex, indicating longer interaction during 50 ns MD simulation. During the study, local rigidity was considered to understand the stability of binding by looking RMSD, RMSF and distance analysis but these considerations were not considered enough to understand exactly stability of binding and binding affinity. For further studies, binding energy estimation is required to detect the effect of zeatin on A2AR and the strength of the binding interaction. By this study, zeatin is identified as potential ligand of A2AR based on computational model for the first time and the interacting resdiues was analysed in order to provide better understanding of the binding mechanism based on positive control described as A2AR in complex with caffeine.

5. Conclusion

Zeatin, a plant hormone, has recently been detected as potential target for A2AR but protein-ligand interaction mechanism has not been clarified yet. Based on structural modelling, zeatin was identified as a ligand of A2AR for the first time and interacting resdiues were analyzed based on A2AR in complex with caffeine as positive control to reveal binding mechanism.

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Author contribution subject and rate:
Ebru Destan (%34): Collected data, performed analysis and wrote the paper.
Ahmet Can Timuçi (%33): Conceived and designed the analysis, collected data and wrote the paper.
Pınar Öz (%33): Conceived and designed the analysis, collected data and wrote the paper. .

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THE RELATIONSHIP BETWEEN THE VISUAL-SPATIAL PERCEPTIONS AND FACE RECOGNITION SKILLS IN PARENTS OF THE CHILDREN WITH AUTISM SPECTRUM DISORDER

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Ethics committee approval: An approval was granted for the study by the local ethics committee in Bursa Yuksek Ihtisas Training and Research Hospital (Date: 17.1.2015, No: ETK/2015/1232) and all study procedures were conducted in accordance with the Declarations of Helsinki and local laws and regulations.

Abstract

The aim of this study is to examine the correlation between the autism symptoms of children with Autism Spectrum Disorder and the visual-spatial perceptions and face recognition skills of the parents of these children. In the study, the parents of 30 children (30 mothers, 30 fathers) diagnosed with Intellectual Developmental Disorder and the parents of 30 children (30 mothers, 30 fathers) without any psychiatric disorder, totally 180 people, were assessed in order to compare the data of the parents of 30 children (30 mothers, 30 fathers) diagnosed with Autism Spectrum Disorder. Autism Behavior Checklist (ABC) was used in order to assess the symptoms of the children diagnosed with Autism Spectrum Disorder. Benton's Judgment of Line Orientation Test (BJLO), Benton Facial Recognition Test (BFRT), and Symptom Checklist (SCL-90) were used in order to assess the parents in three groups. It was found that there was no correlation between the autism symptoms of the children diagnosed with Autism Spectrum Disorder and the visual-spatial perceptions and face recognition skills of their parents. The spatial perception skills of the fathers in the Autism Spectrum Disorder group were higher compared to the mothers in this group ($p<0.001$), it was found that the 14.4% ($p=0.022$) of the BFRT scores of the fathers in the Autism Spectrum Disorder and 11.8% ($p=0.035$) of the BFRT scores of the fathers in the Intellectual Developmental Disorder group were explained by the BJLO scores. It may be useful to focus on the gender, face recognition skill and the visual-spatial perception performance when researching the genetical aspects of the face processing disorder seen as consistent information in the Autism Spectrum Disorder phenotype.

Keywords: visual spatial perception, face recognition skill, autism, gender, neuropsychology

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1. Introduction

It has been found that the individuals with ASD observe the featureless parts of a face more frequently and they observe the featured parts of faces (namely eyes, nose, and mouth) significantly less compared to the healthy individuals (Pelphrey et al., 2002). It has been assessed that the children with ASD were less successful in most parts of the face configuration function (emotion, gaze direction, gender and lip reading) compared to the healthy individuals, however, it was also assessed that the children with ASD configured faces in the high spatial frequency field (local facial features) better compared to the healthy individuals (Deruelle et al., 2004).

Face recognition is an important dimension of face processing through which the identity-related information is obtained (Mukerji et al., 2013). It has been assessed that the deterioration in the face processing observed in ASD is accompanied by the deterioration in the face recognition skills and the face recognition anomalies in ASD is not only explained by the deterioration in face processing (Joseph & Tanaka, 2003). It has been reported that the face recognition difficulties observed in ASD are more specific to the disorder at early ages compared to the healthy individuals and the difference in face recognition skills starts to decrease in the increasing ages (Fedor et al., 2018). However, it has been stated that there is a significant inconsistency between eye tracking and the neurobiological studies and there are both qualitative and quantitative differences between the individuals with and without ASD in face recognition (Tang et al., 2015).

The visual-spatial perception includes the perception of the space, visualization, and orientation, visual-spatial scanning, response speed, impulsivity, focused or continuous attention (Kurt, 2002). It has been revealed that children with ASD are better in some visual-cognitive tasks compared to normal children (Amudha et al., 2015). A study in which children with Asperger's syndrome, high functioning autism, and healthy individuals were compared, revealed that the individuals with Asperger's syndrome exhibited a higher performance for the thin detailed figures in terms of perception and memory (Zachi & Ventura, 2014). It was stated that although individuals with ASD displayed high performance in the visual-spatial tasks emphasizing local data processing, they cannot display the same performance related to the hierarchic stimuli (Guy et al., 2016). Some researchers have suggested that the hypothesis suggesting that the performance of the people with ASD in terms of visual-spatial tasks is neither better nor worse is not true compared to the healthy individuals (Falkmer et al., 2016).

It has been stated that advanced visual skills and atypical face processing may be the phenotype of ASD and the impairment in the face processing skill observed in the individuals with ASD may be inherited from generation to generation (Samson et al., 2012; Fiorentini et al., 2012). Visual-spatial perception is a cognitive process associated with visual skills (Kurt, 2002) and face recognition skill is a significant part of face processing performance (Joseph & Tanaka, 2003). Despite this, the correlation between the visual-spatial perception, face recognition skill and ASD symptoms in the parents of the children with ASD, has not been examined enough. Therefore, in the present study, it was first aimed to examine the correlation between face recognition and visual-spatial perception skills of the parents and autism symptoms of the children. Besides, it has been stated in the literature that gender has a confounding effect on spatial perception (Vecchi & Girelli, 1998) and face recognition (Lui et al., 2009) performance and ASD is observed in men 3 times more compared to women (Xu et al., 2018). For this purpose, the second aim of the present study is to compare the face recognition and visual-spatial perception skills among the mothers and fathers of the children with ASD. The third aim of the study is to compare face recognition and visual-spatial perception skills of parents in three groups (parents of children with ASD via healthy control and intellectual disabled children). Lastly, it was investigated whether the facial recognition performance of the parents in three groups were explained by visual-spatial perception.

2. Materials and Methods

An approval was granted for the study by the local ethics committee in Bursa Yuksek Ihtisas Training and Research Hospital (Date: 17.1.2015, No: ETK/2015/1232) and all study procedures were conducted in accordance with the Declarations of Helsinki and local laws and regulations.

2.1. Participants

Group 1: The children aged between 4-10 years, who were followed up in Bursa Yuksek Ihtisas Training and Research Hospital child and adolescent psychiatry outpatient clinic between 2016 and 2017 due to diagnosis of ASD and their parents were included in the study. The children were diagnosed with ASD by a child and adolescent psychiatrist based on the DSM-V criteria. All the children in Group 1 were getting special education and attending schools for autistics. A total of 68 parents including 34 mothers and 34 fathers who were voluntary to participate in the study and 34 children with ASD were assessed in terms of eligibility for the study. The inclusion criteria for the children with ASD were determined as follows; being diagnosed with ASD, being over the age of 4, and living with their family. The exclusion criteria for children with ASD were determined as follows; having additional diseases such as Down Syndrome, Fragile X syndrome, and Rubinstein-Taybi; having hearing and visual impairment; being severely physically disabled; having a specific birth injury and/or having a neurological disease (i.e. epilepsy).

The inclusion criteria for the parents of the children with ASD were determined as being over the age of 18, being at least primary school graduate, the mother and the father' living with the autistic child, and being alive mother and father. The exclusion criteria for the parents of the children with ASD were determined as follows; having any of the neuro-developmental and neuro-cognitive disorders, schizophrenia spectrum and other psychotic disorders, bipolar and related disorders, and drug and alcohol dependence disorders. Due to the inclusion and exclusion criteria, one couple was excluded as they got divorced, one couple was excluded as the father was diagnosed with deficits in intellectual functioning, 2 people were excluded with their spouses as they were not primary school graduate. As a result of this, a total

of 60 parents (30 mothers, 30 fathers; Group 1), who were voluntary to participate in the study and met the inclusion and exclusion criteria and 30 children with ASD were included in the study.

When it came to compare the parents of children with ASD, two other groups were comprised.

Group 2: 30 children, who were diagnosed with Intellectual Developmental Disorder (IDD) and aged between 4-10 years, and their parents (30 fathers, 30 mothers; Group 2) were invited to the study. The children in Group 2 were getting special education and attending schools for intellectual disabled children. The inclusion and exclusion criteria for the children in Group 2 were determined as Group 1. The parents in Group 2 were included in the study as they were exposed to the stress (due to negative effect caused by the treatment, care, education and behavioral problems of the children) similar with the parents in Group 1.

Group 3: In addition, the parents of the children being aged between 4-10 years and having no psychiatric disease (30 fathers, 30 mothers; Group 3) were invited to the study as the control group. The inclusion and exclusion criteria for the parents in Group 2 and 3 were the same as the criteria for the parents in the Group 1. The children in Group 3 were attending general primary schools.

In three groups included in the study, a total of 180 parents including 90 males and 90 females aged between 25-51 years, were assessed.

2.2. Measures

Before starting the study, the file records were examined and the participants followed up with the diagnosis of ASD and IDD were determined. The parents of the children who met the inclusion and exclusion criteria based on the file records were informed about the study and the parents and the children were invited to the study. Firstly, a child and adolescent psychiatrist reassessed the children based on DSM-V (American Psychological Association, 2013) diagnosis criteria, the eligibility of the children in three groups for the study were reviewed. Then, the parents in per group were assessed by the same interviewer based on the psychiatric interview and DSM-V diagnosis criteria in terms of eligibility for the study.

In order to assess autism symptoms of the children with ASD, the child and adolescent psychiatrist applied the Autism Behavior Checklist (ABC) for the children in Group 1 (The other two group did not take ABC since they were not autistic).

The parents who met the inclusion criteria were assessed by the experts (two psychologists who have the competence to apply neuro-psychological tests) in an environment suitable to take neuro-psychological measurements. The assessment order of the parents was determined randomly. In the study, Benton's Judgment of Line Orientation Test was used to measure the visual spatial perception skill, Benton Facial Recognition Test was used to measure the face recognition skills, and Symptom Checklist was used to measure the psychopathological characteristics. The order of application of the neurological tests was determined randomly for each participant.

2.3. The assessment of the child with ASD

Autism Behavior Checklist (ABC)

The ABC test is a measurement instrument with 57 items used to screen the autism symptoms of individuals (Krug et al., 1980). The scale assesses the autism symptoms in five subcategories: sensory, relating (social skills), body and object use, language, social and self-help. The Cronbach's α value of the scale was found as 0.92 in the validity and reliability study conducted in Turkey (Yilmaz-Irmak et al., 2007). A high score in the scale indicates that the autism symptoms of an individual are high.

2.4. The assessment of the parents in three groups

Symptom Checklist-90 (SCL-90)

It is an assessment instrument developed to assess the psychological problems and including nine subscales (Derogatis, 1983). There are subscales of the checklist as Somatization, Obsessive-Compulsive, Interpersonal sensitivity, Depression, Anxiety, Anger-hostility, Phobic Anxiety, Paranoid ideation, Psychoticism, and Additional Scale (Dağ, 1991). In addition, there is The Global Severity Index (GSI) to evaluate general psychopathology. GSI used for assessing the mental health of people is calculated by using all the items of the checklist. A high score obtained in GSI and its subscales indicates an increase in the mental problems.

Benton's judgment of line orientation test (BJLO)

It measures the visual-spatial perception and the other characteristics related to spatial perception (Benton et al., 1978). In BJLO, it is aimed to estimate accurately the spatial location of two lines. It is a measurement instrument sensitive to the damage of the right parietal hemisphere. The standardization of BJLO was performed in Turkey and its norm valueswere calculated (Karakas et al., 2004). A high score indicates that spatial perception performance increased the detection. It is a measurement instrument without time limitation and its application lasts for averagely 20 minutes.

Benton Facial Recognition Test (BFRT)

It was developed to determine the people's capacity of distinguishing and identifying the images of the faces of the unknown people (Benton, 1994). Norms are available up to the age of 74 years. The reliability and validity study of the test was performed in Turkey and their norm values were calculated (Keskinkılıç, 2008).

2.5. Ethical Considerations

An approval was granted for the study by the local ethics committee in Bursa Yuksek Ihtisas Training and Research Hospital (Date: 17.1.2015, No: ETK/2015/1232) and all study procedures were conducted in accordance with the Declarations of Helsinki and local laws and regulations. Parental informed consent and verbal assent of children (in verbal children) were required for study participation.

2.6. Statistics

In order to assess the sociodemographic characteristics of the participants, descriptive statistical methods such as percentage, mean, and standard deviation were used. Pearson Correlation Analysis was used in order to measure the correlation between ABC subscale scores of the

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children with ASD and the BJLO, BFRT, GSI mean scores of the parents. Independent Samples t-test was used in order to compare the age, years of education, BJLO, BFRT, SCL-90 mean scores between the mothers and fathers. One-way ANOVA Analysis was used to compare the age, year of education, BJLO, BFRT, and SCL-90 mean scores among three groups. In this analysis, Tukey Test was used in order to determine the differences between the groups. Linear Regression Analysis was used in order to assess the part of the BFRT scores of the mothers and fathers explained by BJLO. Normal distribution hypothesis was met for Pearson Correlation Analysis, Independent Samples t-test, one-way ANOVA Analysis and Linear Regression Analysis. The level of significance for all the statistical analysis was $p<0.05$ and IBM SPSS statistics 22.0 software was used in performing the analyses.

3. Results

The average age of the children in three groups assessed in the study was 6.67±1.88, 7.17±1.70, and 6.13±1.87, respectively, the average ages among the three groups became different (F=2.5, p=0.082). 4 of the children in Group1 were girls (13.3%), 26 of them were boys (86.7%); 14 of the children in Group 2 were girls (46.7%), 16 of them were boys (53.3%); 16 of the Group 3 were girls (43.3%) and 14 of them were boys (46.7%) (Table 1).

Table 1. The comparison of the sociodemographic characteristics of the participants.

Autism Parent		n	Mean/sd	t	p
Age	Female	30	32.43±4.68	-3,29	0.002
	Male	30	36.63±5.20		
Year of education	Female	30	8.93±3.05	-1.36	0.180
	Male	30	9.93±2.63		
IDD Parent					
Age	Female	30	34.77±5.71	-3.15	0.003
	Male	30	39.10±4.90		
Year of education	Female	30	7.20±3.01	-0.42	0.674
	Male	30	7.53±3.09		
Control Group Parent					
Age	Female	30	33.93±4.40	-2.09	0.041
	Male	30	36.27±4.26		
Year of education	Female	30	9.13±3.43	-0.28	0.784
	Male	30	9.37±3.11		
All the mothers				**F**	**p**
Age	Autism	30	32.43±4.68	1.70	0.188
	IDD	30	34.77±5.71		
	CG	30	33.93±4.40		
All the fathers					
Age	Autism	30	36.63±5.20	3.08c	0.051
	IDD	30	39.10±4.90		
	CG	30	36.27±4.26		
All mothers					
Year of education	Autism	30	8.93±3.05	3.37b	0.039
	IDD	30	7.20±3.01		
	CG	30	9.13±3.43		
All the fathers					
Year of education	Autism	30	9.93±2.63	5.40a	0.006
	IDD	30	7.53±3.09		
	CG	30	9.37±3.11		

a, b, and c are Tukey test results. a= IDD<ASD,CONTROL. b= IDD, ASD, CONTROL. c= IDD>ASD,CONTROL.

Based on the Pearson's correlation analysis, it was assessed that there was no correlation between the autism symptoms of the children in Group 1 and the BJLO and BFRT scores of their parents. It was found that GSI scores of the fathers of the children in Group 1 had a positive correlation with the ABC social-self-help subscale scores of the children (r=0.40, p<0.05). It was found that BJLO and BFRT scores of the fathers of the children with ASD were negatively correlated (r=-0.41, p<0.05) (Table 2).

Table 2. The correlation between ABC subscale scores of the 30 children with ASD (Group 1) and BJLO, BFRT, and GSI scores of their parents.

		1	2	3	4	5	6a	6b	6c	7a	7b
1	Sensory	-									
2	Relating	0.63**									
3	Body and object use	0.68**	0.47**								
4	Language skills	0.34	0.38*	0.49**							
5	Social and self-help	0.39*	0.43*	0.35	0.44*						
6-Female (n=30)	6a-BJLO	0.01	-0.05	0.04	0.04	0.28					
	6b-BFRT	-0.03	-0.08	-0.24	0.03	0.01	-0.05				
	6c-GSI	-0.08	-0.09	-0.09	0.03	0.02	0.35	-0.19			
7-Male (n=30)	7a-BJLO	-0.13	0.11	0.13	0.19	0.19	0.08	0.05	0.06		
	7b-BFRT	0.27	0.00	-0.07	-0.20	-0.01	-0.21	0.09	0.08	-0.41*	
	7c-GSI	0.18	0.17	0.20	0.35	0.40*	0.08	0.10	0.14	-0.07	-0.05

*<0,05, **<0,01

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Table 3. 2-way ANOVA analysis results related to interaction between groups and gender.

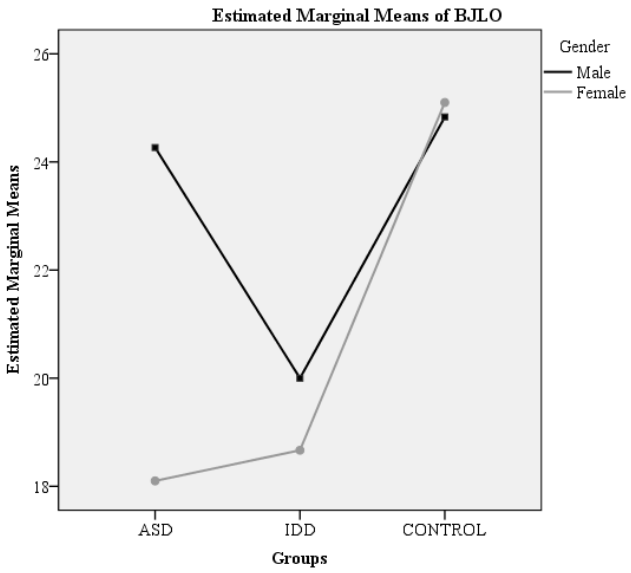
	Female	Male		Tukey HSD							
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	df	F	Group 1-2	Group 1-3	Group 2-3
Somatization	1.08±0.64	0.94±0.84	0.58±0.55	0.75±0.69	0.62±0.64	0.38±0.35	2, 174	0,08 a	NS	0,001	NS
Obsessive-Compulsive	1.21±0.73	0.92±0.70	0.70±0.59	1.04±0.56	0.92±0.84	0.55±0.50	2, 174	0,26 a	NS	<0,001	NS
Interpersonal Sensitivity	1.17±0.72	0.92±0.79	0.57±0.57	0.90±0.71	0.78±0.77	0.38±0.42	2, 174	0,47 a	NS	<0,001	0,008
Depression	1.26±0.74	1.20±1.00	0.58±0.66	0.74±0.67	0.70±0.69	0.44±0.41	2, 174	1,34 a	NS	0,001	0,003
Anxiety	0.87±0.69	0.72±0.65	0.42±0.49	0.59±0.51	0.53±0.68	0.27±0.29	2, 174	0,23 a	NS	0,001	NS
Anger-Hostility	0.86±0.72	0.84±0.81	0.55±0.66	0.81±0.65	0.67±0.74	0.47±0.61	2, 174	0,11 a	NS	NS	NS
Phobic Anxiety	0.60±0.60	0.36±0.47	0.20±0.25	0.35±0.45	0.24±0.44	0.10±0.17	2, 174	0,65 a	NS	<0,001	NS
Paranoid Ideation	0.82±0.73	0.93±0.84	0.73±0.62	0.88±0.57	0.72±0.62	0.61±0.60	2,174	0,60 a	NS	NS	NS
Psychoticism	0.75±1.08	0.44±0.44	0.17±0.28	0.51±0.49	0.39±0.63	0.17±0.25	2, 174	0,66 a	NS	<0,001	NS
Additional Scale	1.00±0.67	0.80±0.69	0.51±0.49	0.90±0.69	0.78±0.68	0.44±0.50	2, 174	0,06 a	NS	<0,001	NS
GSI	0.98±0.63	0.83±0.65	0.50±0.46	0.74±0.53	0.64±0.61	0.38±0.33	2, 174	0,17 a	NS	<0,001	NS

a>0,01, NS=Not Significant

The suitability of SCL-90 subscale scores for 2-way ANOVA analysis was checked with Levene Test. As a result, Levene Test was found to be significant (p <0.05). Therefore, it was decided that the significance level for this analysis would be p <0.01. According to 2-way ANOVA analysis performed between three groups according to gender, SCL-90 subscale score averages were not statistically different (p> 0.01) (Table 3).

The suitability of BJLO scores for 2-way ANOVA analysis was checked with Levene Test. As a result, Levene Test was found to be significant (p = 0.001). Therefore, it was decided that the significance level for this analysis would be p <0.01. According to the results of 2-way ANOVA analysis, the interaction between gender and groups was statistically significant [F (2, 174) = 5.84, p = 0.004]. However, the effect size (Partial Eta Squared = 0.063) was found to be small. In post-hoc comparisons using Tukey HSD, the BJLO scores from Group 1 and 2 were not statistically different (p = 0.145), in addition, the mean scores of BJLO of the parents in Group 3 were found to be statistically significantly different from the mean scores of the parents in Group 1 (p <0.001, 99% CI: LL = 0.89-UL = 6.68) and Group 2 (p <0.001, 99% CI: LL = 2.74-UL = 8.53) (Figure 1).

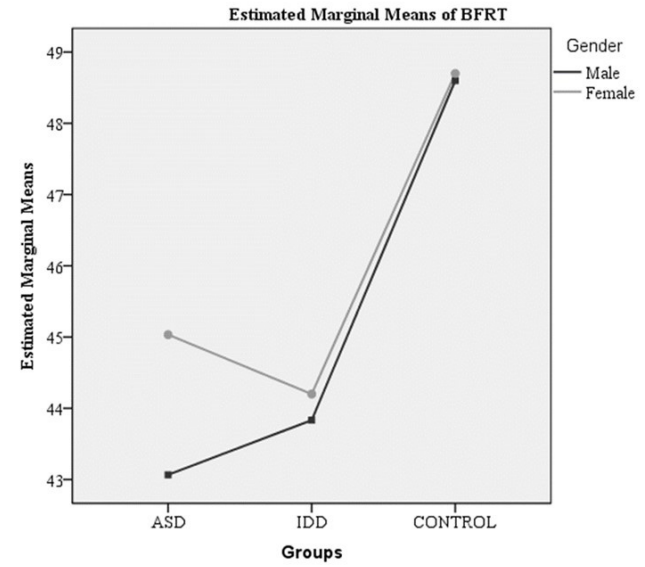
Figure 1: Estimated Marginal Means of BJLO



The suitability of BFRT scores for 2-way ANOVA analysis was checked with Levene Test. As a result, Levene Test was found to be significant (p = 0.014). Therefore, it was decided that the significance level for this analysis would

be p <0.01. According to the results of 2-way ANOVA analysis, the interaction between gender and groups was not statistically significant [F (2, 174) = 0.68, p = 0.507]. In post-hoc comparisons using Tukey HSD, the BFRT scores from Group 1 and 2 were not statistically different (p = 0.009), in addition, the mean scores of BJLO of Group 3 parents were found to be statistically significantly different from the mean scores of the parents was Group 1 (p <0.001, 99% CI: LL = 2.05-UL = 7.15) and Group 2 (p <0.001, 99% CI: LL = 2.08-7.19) (Figure 2).

Figure 2: Estimated Marginal Means of BFRT



Based on the linear regression analysis, it was found that 44% of BFRT scores of the mothers in Group 2 were explained by BJLO test (p<0.001). It was found that 14.4% (p=0.022) and 11.8% (p=0.035) of BFRT scores of the fathers in Group 1 and 3 of were explained by BJLO scores (Table 4).

Table 4. The efficiency of BJLO scores to account for BFRT scores.

Females	R2	Adjusted R2	B	SE	B	p
Group 1	0.003	-0.032	-0.064	0.215	-0.056	0.769
Group 2	0.460	0.440	0.587	0.120	0.678	<0.001
Group 3	0.112	0.080	0.296	0.157	0.335	0.071
Males	**R2**	**Adjusted R2**	**B**	**SE**	**B**	**p**
Group 1	0.173	0.144	-0.450	0.186	-0.416	0.022
Group 2	0.149	0.118	-0.242	0.109	-0.385	0.035
Group 3	0.001	-0.035	0.027	0.194	0.27	0.889

4. Discussion

As a result of the study, it was found that the visual-spatial skills of the mothers of the children with ASD was lower compared to the fathers of the children with ASD and the mothers of the healthy children. In addition, it was found that the face recognition skills of the parents of the children with ASD were similar and the face recognition skills of the parents of the children with ASD were lower compared to the parents of the healthy children. These results may indicate that the significant decrease of the face recognition skills of the parents of the children with ASD was similar in the parents and the decrease in the visual-spatial perception performance may differ based on gender. In the studies in the literature, it has been reported that the face recognition disorders in the mothers and fathers of the children with ASD are significant (Adolphs et al., 2008; Fiorentini et al., 2012) and the parents of the children with ASD has a relative weakness in the face memory skill and a relative strength in the visual recognition skill, similar to their children (Kuusikko-Gauffin et al., 2011).

It was found that the face recognition and visual-spatial perception skills of the mothers of the children with ASD and the mothers of the children with IDD were similar and the face recognition skill of fathers of the children with ASD was similar to the performance of the fathers of the children with IDD. The ASD and IDD diagnoses are evaluated in the category of neurodevelopmental disorders and it is stated that these two diseases have similarities and dissimilarities (Matson & Shoemaker, 2009). Additionally, it is reported that the apparent deficiencies in the face recognition field in the people diagnosed with ASD may be observed in the people diagnosed with IDD (Zaja & Rojahn, 2008). However, in the literature, there is no study examining the face recognition and visual-spatial perception skills of the parents of the children with IDD. In addition, it was assessed in the study that the spatial perception performance of the fathers of the children with IDD was lower compared to the fathers in the ASD and control groups. This may be associated with the fact that the ages of the fathers assessed in the IDD group were higher and their educational levels were lower compared to the other two groups in the present study. Because it has been revealed that the visual-spatial perception performances of the individuals may change according to educational period and age (Karakas et al., 2004).

It has been reported that the children with ASD aged between 4-18 years have more psychopathological characteristics compared to the children with IDD and the children with ASD have more emotional and behavioral problems (Brereton et al., 2006). The fact that the parents of the children with ASD have more mental problems compared to the parents of the healthy children in the present study may be associated with this situation. In a study comparing the parents of the children with ASD and IDD and the healthy children, it was assessed that the mothers of the children with ASD had higher depressive symptoms compared to the mothers of the children with IDD and the healthy children, which was compatible with the results obtained in the present study (Olsson & Hwang, 2001).

In the study by Wilson (2010), it was assessed that the face memory performance of the parents of the children with ASD, especially their fathers, was lower compared to the parents of healthy children. It was found that 14.4% of the face recognition skills of the fathers in the ASD group were explained by the visual-spatial perception skill. It has been stated that ASD is observed 3 times more among boys compared to girls (Xu et al., 2018).

Therefore, it has been discussed that some characteristics of male gender may cause ASD. According to the linear regression analysis, it was found that 44% and 11.8% of the face recognition skills of parents in IDD group were explained by the spatial perception skill. It has been stated that BJLO and BFRT may be used to assess the damages in the similar brain regions and both tests are sensitive to the functions of the right hemisphere and occipital cortex (Tranel et al., 2009). For this reason, the interaction between the face recognition and the spatial perception skills of the parents of the children with IDD and ASD may be associated with the anomalies in their parietal and occipital cortices. It was found that there was a negative correlation between the face recognition and spatial perception skills of the fathers in IDD group. When it is considered that the spatial perception and face recognition skills are the characteristics they affect each other positively, this result may be evaluated as unexpected data. However, some recent studies have revealed that the face recognition skills in the adults continue to develop as in the children and the space recognition skill slows down in the adulthood (Gomez et al., 2017; Grill-Spector et al., 2017).

The eligibility of the parents assessed in the study was assessed based on DSM-V. In addition, the IQ performance of the parents of the children with ASD and IDD was not investigated in the study. This may be evaluated as a significant limitation of the study. Another limitation of the study is that the diagnosis was not established for the cases using a structured interview chart and the diagnoses were established by a child and adolescent psychiatrist using the DSM-V diagnostic criteria. The other limitation of this study are that the number of participants assessed in the study was limited and the psychopathological characteristics were assessed with a psychometric measurement instrument.

Another limitation of the study is that the average age and gender of children in three groups could not be matched. Since the diagnoses of autism and mental retardation are different from each other and they are seen at different frequencies between genders, it was not possible to make matches. Also, the age and education levels of the parents in all three groups could not be matched exactly. In the study, the psychopathologies of the parents were evaluated using SCL-90, but there was no assessment of the parent-child interaction. This is another limitation of the research.

Patient informed consent: Informed consent was obtained.
Ethics committee approval: An approval was granted for the study by the local ethics committee in Bursa Yuksek Ihtisas Training and Research Hospital (Date: 17.1.2015, No: ETK/2015/1232) and all study procedures were conducted in accordance with the Declarations of Helsinki and local laws and regulations. Parental informed consent and verbal assent of children (in verbal children) were required for study participation.
Conflict of interest: There is no conflict of interest to declare.
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Author contribution subject and rate:
Emel Sari Gokten (%40): Evaluation of psychiatric diagnoses, hypothesis develop, data analyze, wrote manuscript, revising it critically for important intellectual content.
Burcu Uckun (%20): Neuropsychological assessment, wrote manuscript.
Ersin Budak (%20): Hypothesis develop, neuropsychological assessment, data analyze, wrote manuscript.
Ali Evren Tufan (%20): Data analyze, wrote manuscript.

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DETERMINATION OF THE ANXIETY – RELATED SLC6A4 GENE PROMOTER "S" AND "L" ALLELES IN FOOTBALL PLAYERS

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Ethics committee approval: The protocol used in the present study was approved by the Üsküdar University Ethics Committee and was performed in accordance with the principles of the Declaration of Helsinki II. All participants signed consent forms containing all the information such as the study protocol, results and evaluation of the results.

Abstract

The anxiety level of an athlete is important. The serotonin transporter protein is encoded by the SLC6A4 gene. Serotonin is one of the most important members of anxiety metabolism. Serotonin transporter protein plays a role in bringing serotonin back from the synaptic space to the presynaptic neuron. We aimed to determine the genotype and allele distributions of the SLC6A4 promoter L/S polymorphism in football players and compare it with the control group. For this purpose, 20 football players participated in our study. Genotyping was performed by PCR after DNAs were isolated from buccal epithelial cells. When we examined the genotype distributions, the LL, LS and SS were found as 4 (20%), 8 (40%) and 8 (40%), respectively. In the control group, the same genotype number and percentages were determined as 10 (23%), 25 (57%) and 9 (20%), respectively. In terms of genotype distribution, no statistically significant significance was found between the two groups (p = 0.2493). When allelic distributions were examined in the athlete group, the L allele was counted as 16 (40%) and the S allele as 24 (60%). In the control group, the L allele 45 (51%) and the S allele were counted as 43 (49%). When the two groups were compared statistically in terms of allelic distribution, no statistically significant difference was found (p = 0.2423). As a result, LS and SS genotype were co-dominant and at the same time higher than LL genotype. At the same time, when we compared the S allele with the L allele, it was found that the S allele was more in percentage. Studies have found that the S allele is associated with anxiety. Sports psychologist can support athletes with S allele who are prone to anxiety problems. This is the first study to report that S allele is more common than L allele in Turkish football players.

Keywords: sports, genetics, polymorphism, SLC6A4, football

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1. Introduction

Athletic performance is the combination of an individual's innate genetic abilities as well as abilities acquired under the influence of environmental factors (Ulucan et al., 2014). Sports genetic was accepted as a new branch of science that studies the functioning and regulation of genes that affect athletic performance (Ulucan, 2016). Studies in the field of sports genetics also include analysis of genetic factors that determine psychological mood, such as serotoenergic and dopaminergic systems, which affect sports activity. Psychological characteristics of athletes negatively affect their athletic performance (Yiğit et al., 2020). Psychological parameters such as stress, anxiety, and aggression are effective in determining the performance of athletes (Eken et al., 2018). Serotonin is one of the endogenous molecules affecting human psychology. The serotonin neurotransmitter has been shown to play a role in symptoms and syndromes such as depression, anxiety, stress, and addiction (Ateş et al., 2017).

Serotonin transporter protein (5-HTT or SERT) is one of the most important members of serotonin metabolism and is encoded by the SLC6A4 gene. Serotonin is stored in vesicles within the neurons after synthesized. Serotonin is released into the synaptic space between two neurons to stimulate receptors on other neurons when the cell is stimulated. Excess serotonin released between neurons is taken back to the neuron where it was released. The serotonin transporter protein synthesized by the SLC6A4 gene on chromosome 17 performs this process (Ulucan et al., 2014). There are 14 repeat short (S) and 16 repeat long (L) alleles in the promoter region of the SLC6A4 gene, depending on the deletion / insertion of a 44 bp repeat sequence (Heils et al., 1996). The L allele increases the transcriptional efficiency of the SLC6A4 gene and accordingly increases the rate of serotonin. The S allele has the opposite effect (Collier et al., 1996). Studies conducted to date are associated with the "S" allele from some personality traits associated with anxiety, hostility and depression (Lesch and Merschdorf, 2000). Previous studies have shown that the distribution of SLC6A4 polymorphism among athletes (synchronized swimming, football, and hockey) is different from non-athletes (Sysoeva et al., 2009). In another study, it was reported that students who were aggressive and used drugs had more SS genotypes compared to students who did not use drugs (Gerra et al., 2005). On the other hand, individuals with LL genotype and L allele have been reported to be more resistant to stress and trauma (Caspi et al., 2006).

In this study, we aimed to examine the distribution of "S" and "L" alleles of the promoter region of the SLC6A4 gene in professional Turkish football players and compared the results with sedentary individuals.

2. Materials and Methods

The protocol used in the present study was approved by the Üsküdar University Ethics Committee and was performed in accordance with the principles of the Declaration of Helsinki II. All participants signed consent forms containing all the information such as the study protocol, results and evaluation of the results.

2.1. Study group

20 professional football players were participated in our study. As a control group, 44 individuals who did not exercise regularly participated in our study. The study protocol was prepared in accordance with the Helsinki Declaration-2 (2015) guidelines and was approved by the Üsküdar University Non-Interventional Ethics Committee. Before the study, all participants signed consent forms containing all the information such as the study protocol, results and evaluation of the results.

2.2. Genotyping

DNA isolations from buccal epithelial cells of the athletes participating in our study were performed with the commercially obtained PureLink DNA isolation kit (Invitrogen, Van Allen Way, Carlsbad, CA, USA) and the polymerase chain reaction (PCR) technique was used for genotyping. Briefly, a total volume of 50 µL of 100 ng DNA, 0.5 mM dNTP, 10 pmol primers and 2U Taq polymerase was performed (Fermantas, Vilnus, Lithuanian). PCR conditions 95 ° C 3 min pre-denaturation, 35 cycles in total, 95 ° C 30 s, 53 ° 45 s, 72 ° C 1 min and after the last cycle at 72 ° C 10 min final elongation It was applied in the form. The amplicons obtained were imaged under Ultraviolet (UV) light in 2.5% agarose gel electrophoresis with ethidium bromide.

2.3. Statistical analysis

Genotype distribution and allele frequencies between groups of athletes and controls were then compared by χ^2 testing using the SPSS (version 18.0 for Windows, SPSS, Chicago, IL, USA) program in the statistical analysis of the obtained results. P <0.05 value was accepted as statistically significant.

3. Results

In the SLC6A4 analysis, it was determined that 4 (20%) of 20 players had LL, 8 (40%) of them LS and 8 (40%) of them had SS genotype. When allele distributions were examined, it was observed that the percentage of L allele was 40% and the S allele was 60%. In the control group (n = 44), 10 individuals had LL, 25 individuals LS and 9 individuals had SS genotype. L allele was counted as 45 (51%) and S allele as 43 (49%). When the two groups were compared statistically In terms of genotype distribution, no statistically significant significance was found between the two groups (p = 0.2493) and allelic distribution, no statistically significant difference was found (p = 0.2423). The genotype and allele number distributions of the athletes are summarized in Table 1.

Table 1. Genotype and Allelic Distribution of players for the SLC6A4 Gene Region

	Genotype			p Value	Allelic Frequency		p Value
	LL	LS	SS		L	S	
Athlete (21)	4	8	8	0,2493	16	24	0,2423
Percentage	20%	40%	40%		40%	60%	
Control (44)	10	25	9		45	43	
Percentage	23%	57%	20%		51%	49%	

Significance was assessed at least at the p <0.05 level. Comparison with the control group was made using the x2 test.

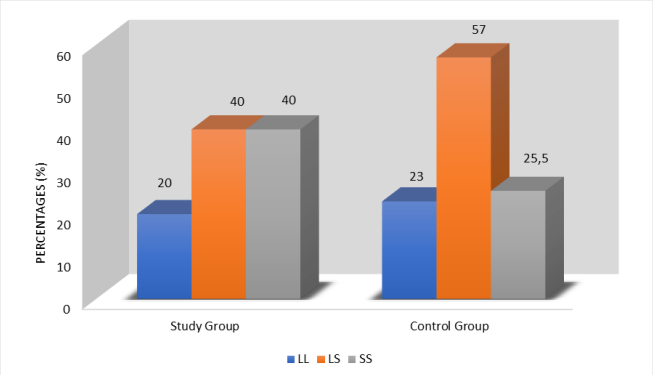

Figure 1: Genotypic distributions of *SLC6A4* polymorphism in our cohort

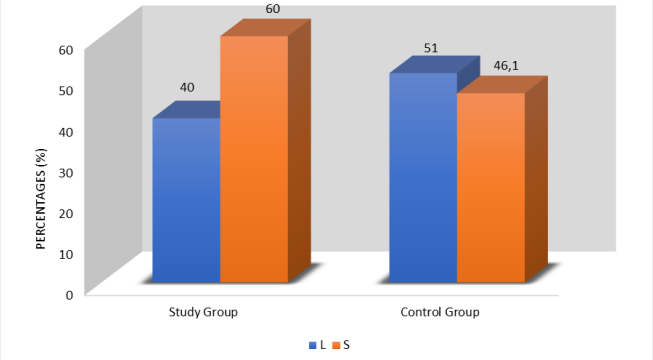

Figure 2: Allelic distributions of *SLC6A4* polymorphism in our cohort

4. Discussion and Conclusion

Sport is associated with a high emotional and psychological pressure on the players. Not only training and nutrition balance, but also avoiding feelings of depression and anxiety are important for players or athletes during sports activity or in daily life.

Serotonin transporter protein, encoded by the SLC6A4, takes the released serotonin back from the synaptic cavity to the presynaptic neuron. The S allele of SLC6A4 L/S promoter polymorphism is thought to be responsible for some personality traits associated with anxiety, hostility and depression (Ulucan et al., 2014). In our cohort, LL, LS and SS genotypes were found as 4 (20%), 8 (40%) and 8 (40%), respectively. L and S allele number percentages were counted as 16 (40%) and 24 (60%), respectively.

There are limited number of studies investigating the relationship between SLC6A4 promoter polymorphism and sports performance. Ateş et al. (2017) investigated the SLC6A4 polymorphism in Turkish young football players and found that the LS genotype was higher than the LL and SS genotype, and the L allele was greater than the S allele. Ulucan et al. (2014) examined a total of 24 young basketball players for the SLC6A4 promoter polymorphism and accepted LL genotype and L allele as active allele. They revealed that the SS genotype and the S allele are insufficient expression alleles. Sysoeva et al. (2009) examined aggression and SLC6A4 polymorphism in female swimmers and found that the LL genotype was higher compared to the LS and SS genotype. Corak et al. (2017), in a similar study they conducted in 26 healthy Turkish athletes, found that the LL genotype was higher than the LS and SS genotypes.

In our study, LS and SS genotype were co-dominant and at the same time higher than LL genotype. At the same time, when we compared the S allele with the L allele, it was found that the S allele was more in percentage. Our findings differ from those of previous studies. The L allele is high expression allele and is associated with resistance to stress and trauma. The S allele is associated with anxiety. This study is the first study investigating the relationship between the SLC6A4 gene and anxiety in Turkish athletes and finding a higher S allele. Thus, our work will contribute to the genetic information pool and support other studies in this field. Our study can help to take early precautions for anxiety disorders encountered in athletes.

Patient informed consent: Informed consent was obtained.
Ethics committee approval: The protocol used in the present study was approved by the Üsküdar University Ethics Committee and was performed in accordance with the principles of the Declaration of Helsinki II. All participants signed consent forms containing all the information such as the study protocol, results and evaluation of the results.
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Özlem Özge Yılmaz (%40): : data collection, formation of the article
Tolga Polat (10%): data collection, statistics
Çisem Şılar (10%): laboratory assistance
İpek Yüksek Gözler (10%): laboratory assistance
Sezgin Kapıcı (10%): laboratory assistance
Canan Sercan Doğan(10%): data collection
Başak Eken (5%): data collection
Korkut Ulucan (5%): formation of the article

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DEVELOPMENT OF THE COVID-19 PHOBIA SCALE: VALIDITY AND RELIABILITY STUDY

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Abstract

It is suggested that the psychological and emotional reactions that occurred during the COVID-19 pandemic may lead to serious psychiatric disorders in the long run. This study aims to establish the validity and reliability of the COVID-19 Phobia Scale developed for measuring emotions and behaviours related to the COVID-19 pandemic. The sociodemographic form, COVID-19 Phobia Scale developed by the researchers, Hospital Anxiety and Depression Scale, and Health Anxiety Scale were applied in the study conducted through a web-based survey method. An item-total analysis was performed as the first step of the study that was conducted with 1243 participants. The exploratory and confirmatory factor analyses revealed the final version of the COVID-19 Phobia Scale to comprise four subscales made up of 22 items, where the subscales were termed as Worry, Mood, Reassurance Seeking/Precaution and Avoidance. The internal consistency of the COVID-19 Phobia Scale measured by the Cronbach alpha coefficient was 0.84. A statistically significant positive correlation was found between the COVID-19 Phobia Scale and Hospital Anxiety and Depression Scale, Health Anxiety Scale. The COVID-19 Phobia Scale is a valid and reliable scale that can be used to measure emotions and related behavioural changes.

Keywords: COVID-19, scale, worry, phobia

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1. Introduction

From the beginning of 2020, COVID-19 cases caused by 2019-nCoV—a new type of Coronavirus that was never detected in humans before—were observed with symptoms such as respiratory distress, fever, joint pain and a high risk of mortality. Following the announcement of rapidly increasing incidents of cases and fatalities from numerous countries, the World Health Organization (WHO) announced a global pandemic by specifying the personal protective measures that are to be taken. Globally, there have been 40 million confirmed cases of COVID-19, including 1 million deaths, reported to WHO; while the number of detected cases in Turkey was 340.000 with 9300 deaths (World Health Organization, 2020).

The behavioural or emotional reactions demonstrated by society during the pandemic period, which affects many people in the world and has different psychological effects on every individual, determine the progress of the pandemic (Arden & Chilcot, 2020; Riva, Benedetti, & Cesana, 2014). These emotional reactions can vary from fear and paranoia to indifference (Taylor, 2019; Wheaton, Abramowitz, Berman, Fabricant, & Olatunji, 2012). Moderate levels of fear and anxiety are known to motivate individuals to cope with threats to health while severe levels of anxiety might affect them more negatively. As a result of a chain of psychological reactions, some individuals experience severe anxiety accompanied by symptoms such as distress, avoidance and decreased functionality that may require treatment (Wheaton et al., 2012). Just like the concept of SARS phobia (Cheng, 2004) was coined during the SARS epidemic, the term Coronaphobia, which defined unreasonable fear of the Coronavirus transmission, started to be used in many countries during this period (Asmundson & Taylor, 2020a; Zarghami, 2020). It is speculated that as the virus was a novel discovery, the obscurity of negative scenarios it might lead to and the information pollution in media led to the emergence of the Corona phobia concept; although the number of cases and fatalities resulting from seasonal influenza infections was found to be much higher than that of COVID-19 (Asmundson & Taylor, 2020a). Individuals with severe anxiety and fear of being infected with the virus might demonstrate behaviours that are not recommended by experts, such as withdrawal and taking extreme precautions to protect oneself from the risks (Shigemura, Ursano, Morganstein, Kurosawa, & Benedek, 2020). Although the impact of COVID-19 on mental health is yet not fully known, research on previous epidemics and pandemics suggest that individual trait differences such as intolerance towards uncertainty, perceived vulnerability to disease and tendency towards anxiety might be significant contributing factors to the emergence of serious psychiatric disorders, such as depression, post-traumatic stress disorder and alcohol/substance abuse (Shigemura et al., 2020; Taylor, 2019; Usher, Bhullar, & Jackson, 2020). Individuals with high levels of health anxiety start thinking that they are infected with the disease by misinterpreting their physical sensations, which are physiological. These misinterpretations consequently might lead to certain negative behaviours that can even lead to self-harm by disrupting healthy decision-making abilities due to increased levels of anxiety (Asmundson & Taylor, 2020b; Goyal, Chauhan, Chhikara, Gupta, & Singh, 2020). Moreover, some individuals with high levels of health anxiety might demonstrate unhealthy behaviour due to their anxiety of getting infected, such as avoiding consulting health institutions and refraining from availing health services that they need. Furthermore, some individuals tend to show maladaptive behaviours, such as overstocking food, excessive hand-washing, locking themselves in their house and avoiding any kind of physical contact, as a result of the severe anxiety, which can be termed as fear, due to the informational convergence resulting from their constant struggle to research and obtain the right information. Conversely, other individuals with low levels of health anxiety tend to disobey the recommended procedures with the mindset that nothing will happen to them, and thus pose a considerable risk in terms of infectiousness (Asmundson, Taylor, Carleton, Weeks, & Hadjstavropoulos, 2012). Psychoneuroimmunology studies demonstrate that adverse emotions and stressful incidents in life might increase the susceptibility to infection by adversely affecting the immune system, suggesting that anxiety is a psychological symptom that should carefully be considered during the pandemic period (Irwin & Slavich, 2017). Only a limited number of structured psychometric measurement tools that facilitate evaluating emotions and behaviours observed during infections or epidemics exist. It is noteworthy that in former times during pandemics, research was conducted with scales constructed by researchers that were not specific to the pandemic; however, a 7-question COVID-19 fear scale (Ahorsu et al., 2020) was constructed by Iranian researchers in March 2020 (Cheng, 2004; Kanadiya & Sallar, 2011; Rajkumar, 2020; Rubin, Amlot, Page, & Wessely, 2009; van der Velden, Marchand, Cuelenaere, & Das, 2020). Studies generally focus on diagnosis and treatment during pandemics, while research on the psychological effects of pandemics is usually conducted towards the end of the pandemic or once it is over. Simple and rapid psychometric measurement tools that facilitate the evaluation of general status in terms of facilitating the determination of risky individuals for the psychological effects of pandemics are thus needed. Research conducted suggests the significance of investigating the symptoms of anxiety and depression with scales at the initial stage (Taylor, 2019). Evaluation of the rapid change in human emotions and behaviours throughout the pandemic, so that the long term psychiatric outcomes could be foreseen and relevant precautions could be taken, would make it easier for us to prepare for new pandemics or disasters that may occur in the years to come. The aim of this study, in this sense, was to demonstrate the validity and reliability of the COVID-19 Phobia Scale constructed for prioritization of symptoms such as worry, avoidance, precaution and mood changes caused by the Coronavirus pandemic in Turkey.

2. Materials and Methods

The ethics committee approval has been obtained from Üsküdar University Non-Interventional Clinical Research Ethics Committee (61351342/2020-227). The current study carried out via internet survey. The link for the survey form prepared through SurveyMonkey was sent to the participants through various social media and e-mail groups. After confirming their participation in the current

study from the first page of the survey that was displayed right after clicking the electronic link, the participants proceeded to the survey questions and completed the study.

2.1. Measurement Tools

2.1.1. COVID-19 Phobia Scale

Development of the COVID-19 Phobia Scale comprised 3 stages. A 60-item question pool was compiled by the researchers during the first stage, mainly by using past research that related to psychological disorders that arise during pandemics and the researchers' experience on the subject matter. During the second stage, six different experts working in the field of mental health (psychiatrists, psychologists, and academics) evaluated the questions in terms of parameters that they wanted to measure, namely, comprehensibility and quality. Seventeen items were deleted from the draft form concerning scores assigned to items by the experts, and research continued with the remaining 43-item scale. At the third stage, selected items were restructured for a 5-point Likert scale and applied to a group of 15 people to be tested for question clarity. Finally, the COVID-19 Phobia Scale was applied to the specified sample group for further validity and reliability studies.

2. Sociodemographic Information Form: This form was prepared by researchers based on information obtained from the literature. The form included questions on sociodemographic information of participants, such as gender, age, educational status, current job description, health problems, and attitudes related to Coronavirus.

3. Hospital Anxiety and Depression Scale: The Turkish adaptation of the scale developed in 1983 by Aydemir et al. in 1997 was used. The 4-point Likert type scale with 0–3 scoring comprised a total of 14 items, where seven questions were related to anxiety and the remaining seven with depression. The higher scores pointed out the greater severity of anxiety and depression (Aydemir, 1997).

4. Health Anxiety Scale: The Turkish adaptation of the 18-item self-report type Health Anxiety Scale developed by Salkovskis et al. ((Salkovskis, Rimes, Warwick, & Clark, 2002) was conducted by Aydemir et al (Aydemir, Kirpinar, Sati, Uykur, & Cengisiz, 2013). The internal consistency in terms of Cronbach alpha was found to be 0.918 in reliability analysis. The score range of items was 0–3, and higher scores indicated higher levels of health anxiety.

2.1.2. Data Analysis

Data collected in the study were statistically analyzed by using SPSS and SPSS Amos v.21 program. Correlation coefficients between total scores of the scale and items of the scale were calculated before proceeding to the validity analysis of the COVID-19 Phobia Scale, and items with $r < .20$ were deleted from the scale. Following the item analysis, the lowest factor load was taken as .300 during the exploratory factor analysis, and the remaining items were subjected to obliquely rotated principal components analysis. Items obtained as a result of five-step obliquely rotated principal components analysis were then evaluated in terms of their logical features and certain items were distributed to other factors. Cronbach alpha reliability coefficients were calculated for the scale and subscales obtained as a result of the analysis and items decreasing the reliability were deleted from the scale at this point. Model-data fit was then analyzed for the obtained model by conducting first- and second-order confirmatory factor analysis. The lower and upper group averages of 27% were compared with independent samples t-test for calculating the scale's discriminant validity.

3. Results

3.1. Participants

The study population comprised N =1243 participants with ages ranging from 20 to 78 years (Age ave. = 44.74; SD =13.061), where 738 were female (Age ave. = 42.94; SD = 12.824), 497 were male (Age Ave. = 47.51; SD = 12.977) and eight refraining from providing their gender information (Age ave. = 37.49; SD = 8.655). Participants joined the study voluntarily and were randomly selected. The sociodemographic characteristics of participants can be found in Table 1.

Table 1. Frequency Distributions and Percentages of Participants' Sociodemographic Properties

Sociodemographic Variable	Category	n	%
Gender	Female	738	59.4
	Male	497	40.0
	I prefer not to respond	8	.6
	Total	1243	100.0
Marital Status	Married	784	63.5
	Single	309	25.0
	Widow (divorced)	116	9.4
	Widow (lost his wife)	19	1.5
	Separated	7	.6
	Total	1235	100.0
Educational Status	Literate	4	.3
	Primary school	3	.2
	Junior high school	5	.4
	High school	90	7.2
	University degree	627	50.4
	Master's degree	325	26.1
	Doctoral degree	137	11.0
	Associate professor	52	4.2
	Total	1243	100.0
Income Status	Low	48	3.9
	Middle	750	60.3
	High	423	34.0
	Very high	22	1.8
	Total	1243	100.0

3.2. Item Analysis

Before proceeding to reliability and validity studies, item analysis for the 43-item COVID-19 Phobia Scale was conducted in terms of correlation of each item in the scale with the total score of the scale. As a result of the

Table 2. Findings of Spearman Correlation Analysis for Item-total Scores of 39 Items and Individual Items

I3	I27	I24	I35	I34	I39	I29	I40	I36	I8
.234**	.241**	.261**	.278**	.295**	.315**	.330**	.363**	.374**	.379**
I30	**I9**	**I14**	**I41**	**I17**	**I43**	**I33**	**I38**	**I4**	**I18**
.387**	.389**	.409**	.414**	.422**	.426**	.441**	.493**	.506**	.517**
I6	**I5**	**I12**	**I42**	**I7**	**I32**	**I19**	**I25**	**I13**	**I26**
.528**	.531**	.536**	.551**	.559**	.588**	.630**	.638**	.640**	.645**
I21	**I16**	**I10**	**I1**	**I23**	**I15**	**I11**	**I37**	**I20**	
.653**	.659**	.660**	.662**	.678**	.692**	.701**	.708**	.711**	

**p < .01

Table 3. Final Obliquely Rotated Key Components Analysis Applied to COVID-19 Phobia Scale

Item		F1	F2	F3	F4	F5	F6
	Eigenvalue: 8.682; Explained Variance: 31,006						
I21	I have doubts about the symptoms of the disease that I had after I learned about coronavirus	.801					
I20	I became more concerned with the any disease symptoms I had after I learned about coronaviruss	.791					
I15	I am afraid of diseases after I learned about coronavirus	.779					
I19	I thought getting infected with a contagious disease was more likely for me after I learned about coronavirus	.778					
I23	Having my mind occupied with thought of what would happen to me in case I get sick after I learned about the Coronavirus is keeping my mind occupied	.767					
I26	I started to think that I had a high risk of COVİD-19 disease after I learned about coronavirus	.765					
I16	Compared with other people, I became more worried about my health after I learned about coronavirus	.758					
I25	I was curious about the changes in my body after I learned about coronavirus	.757					
I11	I became more worried about my health after I learned about coronavirus	.732					
I13	My preoccupation to deal with what's going on in my body after I learned about the Coronavirus	.689					
I1	I became more worried after I learned about coronavirus	.636					
I32	Once I learned about the Coronavirus, I had a worry that a severe illness will affect all areas of my life	.632					
I18	Once I learned about Coronavirus, I am always preoccupied with a feeling that I might be sick, even though my test results are negative	.631					
	Eigenvalue: 1,850; Explained Variance: 6.606%						
I33	My joy of life after I learned about the coronavirus*		-.836				
I34	My energy to focus on the meaning of my life after learning about the coronavirus*		-.808				
I29	After I learned about the coronavirus, I still think I'm going to continue to have enjoy in my life Even if I have a severe illness *		-.633				
	Eigenvalue: 1.464; Explained Variance: 5.227%						
I7	I became more willing to wash food after learning about coronavirus			.748			
I5	I became more willing to wash my hands frequently after learning about coronavirus			.727			
I6	I became more interested in following the news after learning about coronavirus			.714			
I4	I became more irritated with people coughing and sneezing after learning about coronavirus			.567			
I8	I am concerning to order takeaway food after learning about coronavirus			.474			
	Eigenvalue: 1.432; Explained Variance: 5.114%						
I35	I became more willing to avoid crowds after learning about coronavirus				.836		
I40	After I learned about the Coronavirus, I don't want to be indoors				.815		
	Eigenvalue: 1.125; Explained Variance: 4.016%						
I24	After learning about coronavirus, I want to get away from conversations where the subject is the disease					.776	
I36	I care to pay attention to healthy eating after learning about coronavirus					.521	
	Eigenvalue: 1,063; Explained Variance: 3.797%						
I30	Once I learned about the Coronavirus, I have no difficulty focusing on my tasks						.668
I39	I need to talk to others after learning about coronavirus						.609
I9	I'm having trouble with my sleep pattern after learning about coronavirus						.585
	*Reverse Items						

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first round of Spearman correlation analysis conducted during item analysis, items 2, 22, 28 and 31 were found to correlate .20 with the item-total score and thus were deleted from the scale. Correlations of the remaining 39 items with the total item score were found to range between .234 (item 3) and .711 (item 20), according to the correlation analysis results obtained with the new total score after deletion of these items from the scale. Factor analysis was then performed as the correlations of the remaining 39 items with the total score were found to be r > .20. Item-total scores and correlation coefficients for 39 items are provided in Table 2.

3.3. Factor Analysis

The exploratory factor analysis procedure was conducted with obliquely rotated principal components analysis (KMO = .948; Bartlett Test (741) = 17745,116; p < .001) for all 39 items that remained after item analysis of the COVID-19 Phobia Scale. As a result of the analysis, an 8-factor structure with 39 items having an eigenvalue above 1 was obtained, explaining 53.81% of the variance. Considering the items with factor loads above .30, I37, I10, I38 and I42 were observed to have factor loads on multiple factors; however, the difference between these loads was less than .10. These items, therefore, were eliminated from the scale, and a second factor analysis was then applied. All 35 remaining items were subjected to obliquely rotated basic components analysis (KMO = .939; Bartlett Test (595) =14726.081; p < .001). As a result of the analysis, a 7-factor structure with 35 items having an eigenvalue above 1 was obtained, explaining 51.75% of the variance. Considering the items with factor loads above .30, I17, I3 and I14 were observed to have factor loads on multiple factors; however, the difference between these loads was less than .10. Therefore, these statements were eliminated from the scale and a third factor analysis was applied. All 32 remaining items were subjected to obliquely rotated basic components analysis (KMO = .938; Bartlett Test (496) = 13946.204; p < .001). As a result of the analysis, a 7-factor structure with 32 items having an eigenvalue above 1 was obtained, explaining 54.59% of the variance. Considering the items with factor loads above .30; it was found that I41, I43 and I12 were observed to have factor loads on multiple factors, however the difference between these loads was less than .10. These items were thus removed from the scale and a fourth factor analysis was conducted. All 29 remaining items were subjected to obliquely rotated basic components analysis (KMO = .936; Bartlett Test (406) = 13026.053, p < .001). As a result of the analysis, a 6-factor structure with 29 items having an eigenvalue above 1 was obtained, explaining 54.42% of variance. Considering the items with factor loads above .30, it was found that I27 received a load on more than one factor, but the difference between these loads was less than .10. This item was thus deleted from the scale and a fifth factor analysis was performed. All 28 remaining items were subjected to obliquely rotated basic components analysis (KMO = .937; Bartlett Test (378) =12825; p < .001). As a result of the analysis, a 6-factor structure explaining 55.77% of the variance, comprising 28 items with eigenvalues above 1 was obtained. The final results of obliquely rotated basic components analysis performed for the COVID-19 Phobia Scale can be found in Table 3. Logical investigation of item distributions to factors was performed following the obliquely rotated basic components analysis, and I13, I18, and I32 belonging to first factor; I30 and I9 from the sixth factor were decided to be eliminated from the scale. Moreover, I24 with its loading on fifth factor was found to be compatible with the statements of fourth factor; I39 with its loading on sixth factor along with I36 with its loading on fifth factor were found to be compatible with the statements of the third factor. These items were thus allocated to related factors for confirmatory factor analysis.

3.4. Internal Consistency

The internal consistency coefficients of the 23 items remaining after exploratory factor analysis based on their final factor distributions are provided in Table 4.

The internal coefficients of the scale were found to be cra = .84 for items in total; cra = .92 for F1 (Worry); cra = .67 for F3 (Reassurance Seeking/Precaution) and cra = .60 and F4 (Avoidance). It was observed that the internal consistency coefficient decreased with the presence of I24 in the avoidance subscale, and, thus, this item was eliminated from the scale before proceeding to confirmatory factor analysis for the remaining 22 items and four subscales. After the elimination of I24 from the scale, the internal consistency coefficients were found to be cra = .84 for the items in total; and cra = .60 for subscale F4 (Avoidance).

Table 4. Internal Consistency Analyses Findings for COVID-19 Phobia Scale

Factor	Factor Name	Item Numbers	Number of Items	Cronbach's Alpha	After Deletion of Item
F1	Worry	1, 11, 15, 16, 19, 20, 21, 23, 25, 26	10	.918	.918
F2	Mood	29*, 33*, 34*	3	.673	.673
F3	Reassuarance Seeking/ Precaution	4, 5, 6, 7, 8, 36, 39	7	.601	.601
F4	Avoidance	24**, 35, 40	3 (2)	.441	.601
Total			23 (22)	.838	.840

*Reverse Items **Deleted item

3.5. Criterion Validity

Spearman correlation analysis was conducted for the total scale scores and the scores of Hospital Anxiety and Depression Scale and Health Anxiety Scale for the determination of scale's criterion validity. Results of analysis are provided in Table 5.

Table 5. Spearman Correlation Analyses Findings between the Scores of COVID-19 Phobia Scale and the Scores from the Hospital Anxiety and Depression Scale and Health Anxiety Scale

	HAD Anxiety	HAD Depression	Health Anxiety Total	SA Physical Sensitivity	SA Negative Outcomes
Covid Total	.464**	.371**	.471**	.457**	.340**
Covid Worry	.568**	.434**	.531**	.509**	.394**
Covid Mood	-.529**	-.585**	-.363**	-.325**	-.356**
Covid Reassurance Seeking/ Precaution	.371**	.409**	.416**	.390**	.338**
Covid Avoidance	.283**	.287**	.232**	.200**	.254**

*p <. *5; **p < .01

A significant positive correlation was found to exist between the total COVID-19 Phobia Scale score and anxiety (r = .464; p = .000); depression (r = .371; p = .000); total health anxiety score (r = .471; p = .000); health anxiety subscales "oversensitivity to physical symptoms" and "anxiety" (r = .457; p = .000) and "negative outcomes" of health anxiety subscale (r = .340; p = .000).

A significant positive correlation was found to exist between COVID-19 Phobia Scale Worry Subscale and anxiety (r = .568; p = .000); depression (r = .434; p = .000); total health anxiety score (r = .531; p = .000); health anxiety subscale (r = .531; p = .000); health anxiety subscales "oversensitivity to physical symptoms" and "anxiety" (r = .509; p = .000) and "negative outcomes" of health anxiety subscale (r = .394; p = .000).

A significant negative correlation was found to exist between COVID-19 Phobia Mood subscale and anxiety (r = −.529; p = .000); depression (r = −.585; p = .000); total health anxiety score (r = −.363; p = .000); health anxiety subscale (r= −.363; p = .000); health anxiety subscales "oversensitivity to physical symptoms" and "anxiety" (r = −.325; p = .000) and "negative outcomes" of health anxiety subscale (r = −.356; p = .000).

A significant positive correlation was found to exist between COVID-19 Phobia Scale Reassurance Seeking/ Precaution subscale and Anxiety (r = .371; p = .000); depression (r = .409; p = .000); total health anxiety score (r = .416; p = .000); health anxiety subscale (r = .416; p = .000); health anxiety subscales "oversensitivity to physical symptoms" and "anxiety" (r = .390; p = .000) and "negative outcomes" of health anxiety subscale (r = .338; p = .000).

A significant positive correlation was found to exist between COVID-19 Phobia Scale Avoidance subscale and anxiety (r = .283; p=.000); depression (r = .287; p = .000); total health anxiety score (r = .232; p = .002); health anxiety subscales "oversensitivity to physical symptoms" and "anxiety" (r = .200; p = .008) and "negative outcomes" of health anxiety subscale (r = .254; p = .001).

Depending on the results of correlation analyses for criterion validity, it can be concluded that the COVID-19 Phobia Scale proved for criterion validity.

3.6. Confirmatory Factor Analysis

Investigation of correlation findings revealed a significant positive relationship between COVID-19 Phobia Scale total score and the scores of subscales. Correlation coefficients between the subscales of COVID-19 Phobia Scale are provided in Table 6.

Table 6. Correlation Coefficients between the Subscales of the COVID-19 Phobia Scale

		1	2	3	4	5
1. Worry	r	1				
	p					
2. Mood	r	.346**	1			
	p	.000				
3. Reassurance Seeking/ Precaution	r	.527**	.128**	1		
	p	.000	.000			
4. Avoidance	r	.097**	-.029	.176**	1	
	p	.001	.313	.000		
5. COVID-19 Phobia Total	r	.888**	.457**	.756**	.372**	1
	p	.000	.000	.000	.000	
**p < .01						

The result of confirmatory factor analysis conducted for identifying the measurability of implicit structures of anxiety, mood, reassurance-seeking/precaution and avoidance parameters used for predicting COVID-19 phobia is depicted in Figure 1.

The most commonly used statistical measures for calculating the model-data fit with Confirmatory Factor Analysis are the Chi-Square (χ2), χ2/sd, RMSEA, RMR, GFI and CFI. GFI values less than 0.85 and RMR and RMSEA values less than 0.10 are nevertheless considered as the acceptable lower limits for model-data fit (Cole, 1987; James C. Anderson & David W. Gerbing, 1984). A model-data fit is said to exist whenever the calculated χ2/df ratio is less than 5, the GFI and CFI values are higher than 0.90 and RMR and RMSEA values lower than 0.05 (Jöreskog KG, 1993). Goodness of fit results based on the first-order confirmatory structure tested with four latent and 22 indicator variables was found as χ2/sd = 3.899, RMSEA = .048, RMR = .033, GFI = .943, CFI = .942 (Table 7).

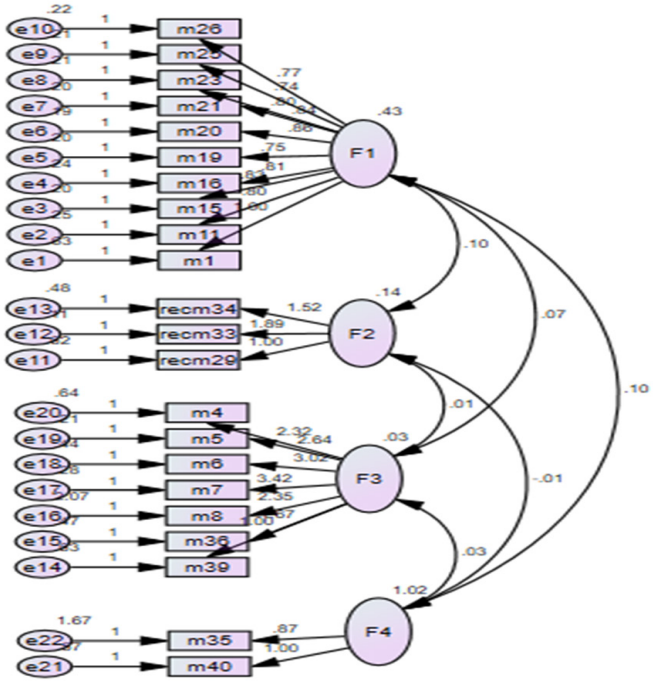

Figure 1. Four-factor implicit structure established with CFA

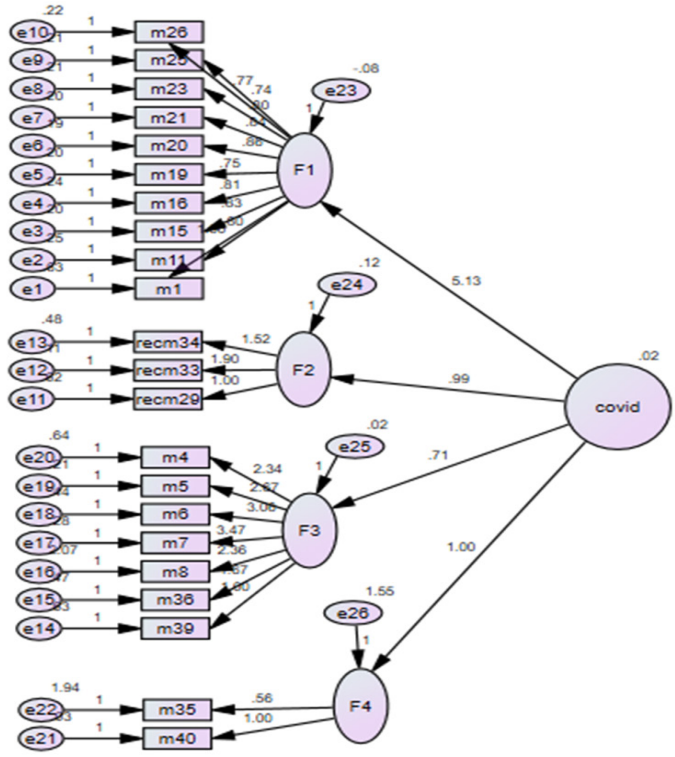

Figure 2. Second-order CFA

Table 7. Findings of First-order Confirmatory Factor Analysis for COVID-19 Phobia Scale

Measurement Tool	χ 2	df	χ 2/df	RMR	CFI	GFI	RMSEA
COVID-19 Phobia Scale	791.542	203	3.899	.033	.942	.943	.048

Comparing the results of CFA with the acceptable fit indices reported in literature, it was concluded that the model constructed for the theoretical structure of COVID-19 Phobia Scale complied with the fit indices.

A second-order confirmatory analysis indicating the structural relations of worry, mood, reassurance-seeking/precaution and avoidance dimensions with the upper dimension of COVID-19 phobia was constructed and depicted in Figure 2 to prove that these four dimensions obtained by the first-order confirmatory factor analysis for COVID-19 Phobia Scale represented the theoretically suggested COVID-19 phobia.

Goodness of fit results based on testing the second-order factor model constructed with the addition of COVID-19 latent variable to the first-order confirmatory structure tested with four latent and 22 indicator variables were found to be χ2/sd = 3.939, RMSEA = .049, RMR = .037, GFI = .942 and CFI = .940 (Table 8).

Table 8. Findings of Second-order Confirmatory Factor Analysis for COVID-19 Phobia Scale

Measurement Tool	χ 2	df	χ 2/df	RMR	CFI	GFI	RMSEA
COVID-19 Phobia Scale	807.538	205	3.939	.037	.940	.942	.049

Reviewing the results of first-order and second-order factor analyses, it can be stated that the COVID-19 Phobia Scale can be used as a measurement tool to predict COVID-19 phobia, worry, mood, reassurance-seeking/precaution and avoidance levels.

3.7. Discriminative feature of the scale

A 27% upper-lower subgroup comparison was performed to determine the distinctiveness of the COVID-19 Phobia Scale. Independent samples t-test was utilized to determine whether there exists a significant difference between the averages of 27% upper subgroups and 27% lower subgroups. The results are provided in Table 9.

Table 9. COVID-19 Phobia Scale Lower–Upper Group Means, Standard Deviations and Independent Samples t-test Findings

Groups	N	AVE	SD	t	p
27% Lower Group	335	70.30	4.857	-60.484	.000
27% Upper Group	335	93.27	4.972		

Results of the t-test revealed a significant difference between the averages of 27% upper subgroups and 27% lower subgroups of the COVID-19 Phobia Scale (t = −60.484; p = .000). This finding suggested that those who scored higher on the COVID-19 Phobia Scale could be differentiated from those who scored low.

4. Discussion

As negative emotions such as anxiety, phobia and fear that arise during a pandemic period adversely affect the behaviour of individuals, relevant psychometric

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measurement tools are needed for determining the risk factors to guide both the individual and social preventive actions during the pandemic. This study aimed to reveal whether the COVID-19 Phobia Scale, which was constructed to evaluate the changes in emotions and behaviours that arise during the COVID-19 pandemic, was a valid and reliable tool. Exploratory and confirmatory factor analyses of the COVID-19 Phobia Scale demonstrated the scale to be valid and reliable, along with the correlation analyses providing the same result. The COVID-19 Phobia Scale, comprising 22 items and four subscales to measure the emotions and behavioural changes observed during the COVID-19 pandemic, was thus shown to be a valid and reliable measurement tool.

The main factor structure was first determined through the utilization of exploratory factor analysis, where the items having lower reliability coefficients were eliminated from scale and the analysis was repeated to construct the final version of the scale. The lower acceptable limit for the Cronbach alpha coefficient, indicating the reliability of scales, is accepted to be 0.70 in the literature. The Cronbach alpha coefficient of the COVID-19 Phobia Scale was found to be 0.84. The scale proved to have a good factor structure concerning total and individual subscales.

A 4-factor scale comprising Worry, Mood, Reassurance-Seeking/Precaution and Avoidance subscales was obtained as a result of the factor analyses performed with the scale items. The Cronbach alpha coefficient of the first factor, which was coined as "Worry subscale" that comprised 10 questions including feelings and thoughts, such as thoughts about the disease that arose with the outbreak of the pandemic, worry to be infected with the disease, health and future concerns, was found to be 0.92. The Cronbach alpha coefficient value of the first factor, which indicated the severity of the initial emotional reactions that arise following the outbreak and constitute almost half the total number of questions on the scale, is the highest among all factors. The second factor coined as the "Mood subscale" evaluates the depressive mood, comprised three questions on the joy of life, enjoying life and meaning of life. Research conducted during pandemic suggests that depressive symptoms were the second most observed group of symptoms following the first group of symptoms being anxiety (Brooks et al., 2020; Rajkumar, 2020). We believe that the inclusion of depressive symptoms, unlike other scales used for COVID-19 and its psychological effects, would add to the authenticity of our COVID-19 Phobia Scale. The "Reassurance Seeking/Precaution" determined as the third factor comprised seven questions that involved individual measures taken to protect oneself from infection, acquiring information about infection and talking to others to feel safe and comfortable. The "Avoidance subscale," determined as the last factor, comprised two questions involving the avoidance to enter crowded and closed environments. "Avoidance in talking about COVID-19" was also considered to be included in this subscale during the item preparation stage; however, it was observed through analyses that elimination of this item increased the validity of the total and the existing factorial structure. This might be related to the level of trauma experienced by individuals during the process. While some individuals experiencing traumatic symptoms might refrain from talking about COVID-19, other individuals might feel the urge to constantly talk about the subject for alleviating their anxiety levels. This ambivalent situation might have resulted in the increase of the internal consistency coefficient as the 24th item was eliminated from the scale.

Hospital Anxiety and Depression Scale and Health Anxiety Scale were used for determining the criterion validity of the COVID-19 Phobia Scale. Health Anxiety Scale was used for this purpose as the health anxiety was considered to be one of the main factors that determine human behaviour during pandemic periods (Asmundson & Taylor, 2020b). Because the COVID-19 Phobia Scale measures both anxiety and depression symptoms, the Hospital Anxiety and Depression Scale, where anxiety and depression are evaluated together, was chosen to be another scale to test for criterion validity. Correlation analyses revealed that the total and subscales of the COVID-19 Phobia Scale correlated highly with other scales and that the criterion validity was high.

In conclusion, the COVID-19 Phobia Scale is found to be a valid and reliable tool that can be used to detect mood changes, such as anxiety and depression in individuals, during the pandemic and the behavioural changes that arise to cope with worry. Our scale is constructed such that it can be used to establish health policies against possible outbreaks in the coming years, and is relatively short and accessible online can be counted as one of its advantages.

4.1. Limitations

Despite the high number of participants in the study, one of its limitations was the online introduction method of scales to the participants due to the circumstances of the pandemic. Face-to-face interviews constitute a crucial stage in clinical research, and this could not be performed for this study. Although online face-to-face interviews with participants were considered to be conducted early during the pandemic, it was predicted that the possible biasing effects of online meetings might fail to be controlled. The fact that a re-test could not be conducted due to the pandemic circumstances was yet another limitation of the research. Conversely, likely, re-test results of the participants who adapted more easily to the process might change in the opposite direction as no research on coping with this process has yet been conducted. The lack of a re-test was thus found reasonable, while it also was a limitation of the study. It is noteworthy that the majority of participants were females and had an educational status of an undergraduate degree or above. It was concluded that the online conduction of research facilitated access to individuals with higher educational statuses rather than individuals with lower educational statuses. It might be of use to reassess the scale with participants that have an educational qualification of high school and below.

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Ethics committee approval: The ethics committee approval has been obtained from Üsküdar University Non-Interventional Clinical Research Ethics Committee (61351342/2020-227)

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Nesrin Dilbaz (%40): Conception/design of the work, data acquisition, drafting and its critical revision for important intellectual content.
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THE DISTRIBUTION OF BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) RS6265 POLYMORPHISM IN TURKISH VOLLEYBALL PLAYERS

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Ethics committee approval: The protocol used in the present study was approved by the Üsküdar University Ethics Committee and was performed in accordance with the principles of the Declaration of Helsinki II. All participants signed consent forms containing all the information such as the study protocol, results and evaluation of the results.

Abstract

Athletic performance is associated with many environmental factors such as inborn genetic factors, nutrition, psychological factors, and education. We aimed to analyze the brain derived neurotrophic factor (BDNF) rs6265 polymorphism, which is an important genetic marker related to psychological factors, in 21 professional female volleyball players and compare it with the control group. Genotyping was assessed by Real-time PCR technique. BDNF rs6265 polymorphism genotypes were calculated as 72% and 28% for GG and GA in volleyball players, respectively. No AA genotype was detected. In the control group, the GG, GA and AA genotype percentages were calculated as 57.8, 36.3 and 5.9, respectively. In the allelic distribution, the percentages in the athlete group were calculated as 86% (36) for the G allele and 14% (6) for the A allele, respectively. In the control group, for the G and A alleles, respectively; it was 76.25% and 23.75%. No statistical significance was found in terms of both genotype distribution (p = 0.407) and allelic frequency distribution (p = 0.218). Our results were in line with data indicating the stress and anxiety-related nature of professional volleyball players. More studies with more athletes and more groups of athletes are needed to understand the effect of these parameters on volleyball players.

Keywords: BDNF, sport genetics, volleyball, brain, polymorphism

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1. Introduction

Environmental factors such as nutrition of athletes, training, coaching, family support, proper training conditions have important effects on the formation and development of athletic performance in sports. In addition to these effects, athletes' response to appropriate training models, susceptibility to appropriate sports branch, resistance to stress and ability to control the emotional and psychological pressure on them are very important. All these traits include, in part of genetic factors. The studies in the field of sports genetics include the analysis of genetic factors that affect sports activity, which are related to sports nutrition, and which determine our psychological emotional state such as serotonergic and dopaminergic. Studies in this area have begun in a large cohort, including single and double twins, and have continued to this day rapidly (Eken et al., 2018).

Among the sports genetics studies, athletic performance, as well as the determination of genes that affect mental performance, determine the mechanisms of these genes are involved. These studies have an important role not only in individual sports but also in the provision of psychological, mental programs suitable for genetic predispositions in team sports.

Volleyball, an advanced form of team play, needs a long-term low-density aerobic exercise. It requires special physical structure and motor characteristics with individual and team play types. Volleyball players, like other athletes, require a high degree of physical and physiological parameters to achieve high performance (Ulucan et al., 2017; Yüksel et al., 2017).

BDNF is a neurotrophin that affects the survival, growth, and function of neurons in the central and peripheral nervous system stabilizes synapses, regulates synaptic function, axon, and dendrite branches (Ulucan, 2016). This protein bolsters the survival of nerve cells (neurons) by playing a role in the growth, maturation (differentiation), and maintenance of these cells. In the brain, the BDNF protein is active at the connections between nerve cells (synapses), where cell-to-cell communication happens. The synapses can change and adapt over time in reply to experience, a characteristic called synaptic plasticity. In recent studies, it has been observed that, contrary to the above studies, exercise increases the serum level of BDNF, and even different types of exercise can affect the level of this increase. Studies investigating the effect of different types and loads of exercises on BDNF levels in sedentary individuals were examined (Bulğay et al., 2020). The BDNF protein helps regulate synaptic plasticity, which is important for learning and memory (Sears et al., 2011). The BDNF gene is localized at 11p13. BDNF, which is the neurotrophin in the brain, is synthesized as proBDNF because of progenitor cell proliferation and differentiation. A functional single nucleotide polymorphism rs6265 found in this gene causes the Val66Met transformation (Eken et al., 2018). According to studies in the literature, the G allele codes for Val, while the A allele codes for Met.

This transformation in BDNF polymorphism affects the motivation by causing stress during exercise and causes psychological problems in individuals (Hashimoto, 2007). In the present study, we aimed to determine the effects of BDNF polymorphisms on volleyball players.

2. Materials and Methods

The protocol used in the present study was approved by the Üsküdar University Ethics Committee and was performed in accordance with the principles of the Declaration of Helsinki II. All participants signed consent forms containing all the information such as the study protocol, results and evaluation of the results.

2.1. Study subjects:

21 volleyball players volunteered to support our study. 26 sedentary individuals also participated as a control group. All the volunteers had no transmitted genetic anomalies. The study protocol was approved by Uskudar University Ethical Committee and was in line with the principles of the Declaration of Helsinki II. All the bodybuilder was informed by showing the experimental steps and ethical results and the consent form was signed.

2.2. Genotyping: DNA isolation from buccal cells was performed using a commercially available DNA Isolation kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A mean total of 20 ng of the DNA was isolated from each sample, and the purity of the isolates were assessed based on the OD260/280 spectrophotometric ratio (Implen NanoPhotometer, München, Germany).

Genotyping of the BDNF rs6265 polymorphism was performed using real time-quantitative PCR on a StepOnePlus (Thermo Fisher Scientific, Inc.) using a TaqMan Genotyping assay according to the manufacturer's protocol (cat. no. 4362691; Thermo Fisher Scientific, Inc.). PCR conditions were 60 °C for 30 s and 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C for and 1 min at 60 °C. Finally, 60 °C for 30 s was applied for postPCR reading. The fluorescent signal was detected at the prePCR, amplification at the end of each cycle, and postPCR reading steps. G and A alleles were determined using VIC and FAM primers, respectively (Fig. 1). The sequences of the TaqMan Probe used for genotyping are listed in Table 1.

2.3. Statistical analysis: Genotype distribution and allele frequencies between groups of athletes and controls were then compared by χ^2 testing using the GraphPad InStat statistical package. p values of $p<0.05$ were considered statistically significant

Table 1. Sequences of the TaqMan probe used for genotyping BDNF rs6265 polymorphism

qPCR	Sequence, 5'-3'
VIC/FAM	TCCTCATCCAACAGCTCTTCTATCA**[C/T]**GTGTTCGAAAGTG TCAGCCAATGAT

3. Results

BDNF was examined, it was found that 6 of 21 volleyball players (28%) were GA and 15 (72%) were GG genotypes. For BDNF there were no volleyball players in the AA genotype. Allele distributions are 14% A, 86% G. Table 2 lists the genotype and allelic frequencies of the BDNF rs6265 polymorphism in volleyball players. Also, Table 3 list the genotype and allelic frequencies of the BDNF rs6265 polymorphism in control group.

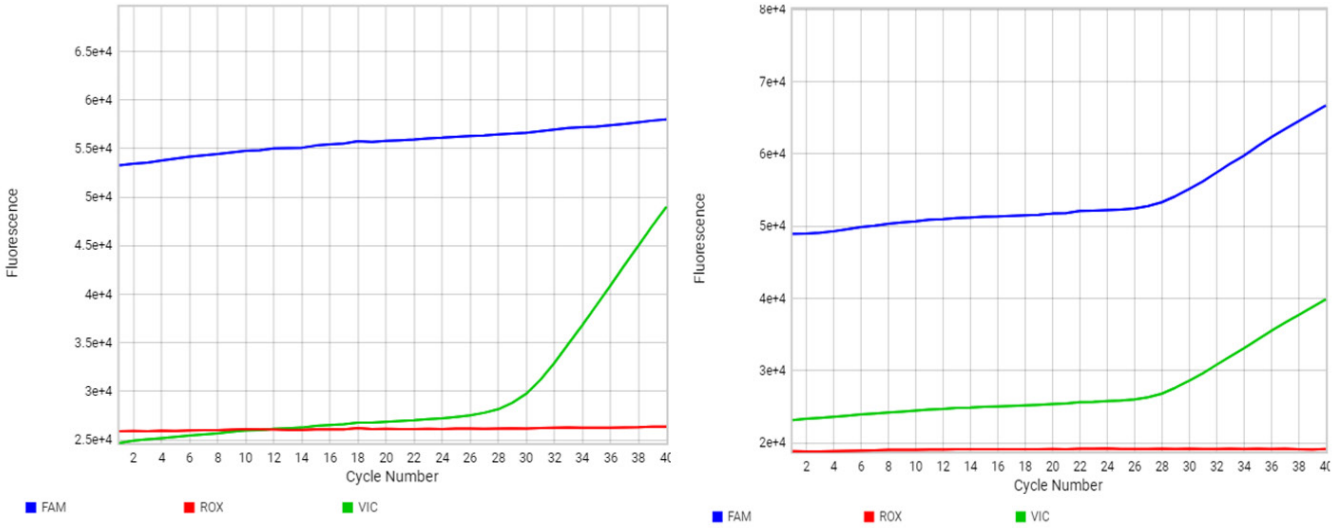

Figure 1. Quantitative PCR amplification of the GG genotype (on the left) and GA genotype (on the right) of BDNF rs6265 polymorphism. FAM indicates the A allele, whereas VIC indicates the G allele). The single blue curve indicates the homozygous genotype of GG on the left, whereas the blue and green curves indicate the heterozygous genotype of GA (CT), on the right.

Table 2. Genotypic and allelic distribution of the BDNF rs6265 polymorphism in volleyball players.

		GENOTYPES			p Value	ALLELES		p Value
		GG	GA	AA		G	A	
BDNF (n=21)	Number	15	6	-	0.407	36	6	0.218
	Percentage (%)	72	28	-		86	14	

* p,0.05, statistically significant differences. Comparison with controls using x2 test.

Table 3. Genotypic and allelic distribution of the *BDNF* rs6265 polymorphism in the control group.

		GENOTYPES			ALLELES	
		GG	GA	AA	G	A
BDNF (n=40)	Number	23	15	2	61	19
	Percentage (%)	57.8	36.3	5.9	76.25	23.75

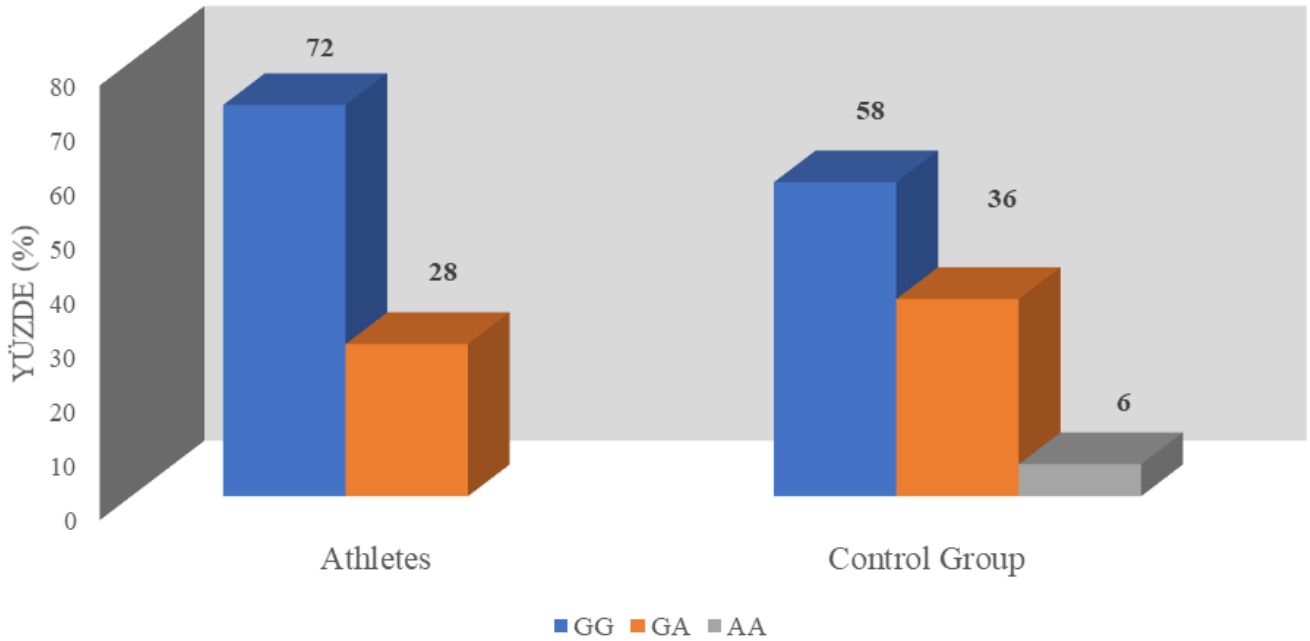

Figure 2. BDNF rs6265 genotype distributions by groups.

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4. Discussion

Stress-related depressive and anxious behaviors have been found to be associated with decreasing BDNF levels, especially in the hippocampus (Duman & Monteggia,2006). It is reported that BDNF, one of the neurotrophic proteins in the brain, prevents cognitive diseases (such as Alzheimer's and Parkinson's) during physical exercise (Podewils et al., 2005). Since BDNF comes along to be related in activity-dependent synaptic plasticity, there is great interest in its activities in learning and memory (Yamada & Nabeshima, 2003). These characteristics are important factors that affect players activities.

Recent studies have shown that some genes are effective in determining the performance of athletes both physiologically and psychologically. Genetic models can be developed and used to find the optimal genetic hardware of a volleyball player to help build up scientists or to find their predisposition. These results will be very useful for sports genetics also athletic performance. This study researches the association between the BDNF rs6265 polymorphism psychological and learning mechanism properties in 21 volleyball players. In our study cohort, the GG genotype for BDNF genotype was higher than GA. The G allele considered to be the wild-type allele of the gene and associated with protective allele. The association of G alleles with neurodegenerative diseases was less than A allele.

Figueiredo et al. (2019) examined, in the long term, the modulation of BDNF and HDL-c concentrations may be a determining factor for protection against neurological and cardiovascular diseases. Physical activity was conceptualized as a protective factor; the interaction between BDNF and physical activity was hypothesized to be associated with lower levels of depressive symptoms. Central nervous system and peripheral nervous system BDNF levels have been reported to play a role in the pathophysiology of mood disorders. A negative correlation was found between the severity of depression and BDNF levels. Major depression is characterized by low serum BDNF concentration, which supports the hypothesis that BDNF plays a role in mood disorders (Kazak & Yarim, 2015).

Another study shown like that, a consistent finding from previous research is that higher lifetime educational attainment is associated with a lower risk of dementia, an effect often explained through the heightened resilience of brain networks indicative of higher cognitive reserve (Ward et al., 2020).

Thomas Seifert et al., (2010) showed that endurance training increased the expression of BDNF mRNA in mice hippocampus rather than in the cortex and the BDNF release from the human brain. Long-term regular exercise on BDNF values a significant difference was found in the direction of rising depending on ($P < 0.05$).

In one study, individuals who were in high daily physical activity were followed for 9 years. While cognitive decline was slower and gradual in individuals with the Val/Val (GG) genotype, this beneficial decline was not seen in individuals carrying the Met (A) allele (Thibeau et al., 2016)

5. Conclusion

In this study, we examined the distribution of BDNF polymorphisms in volleyball players for the first time. BDNF GG genotype and G allele dominated our cohort. In our study, the high ratio of G allele in the volleyball players group comes across with the studies in the literature. These results indicate that BDNF rs6265 polymorphism may be one of the determining factors of psychological susceptibility in sports. In our cohort, volleyball players, the G allele was expected to be high because the psychological study data gave us this information. These polymorphisms are well known, but few studies have been studied in different populations and in sports. According to our results, psychological and plasticity of volleyball players were parallel to cognitive tests. This polymorphism, alone or in combination with additional polymorphisms, should be considered when determining a genomic score profile for sport success. In order to make clearer interpretations, it is necessary to increase the studies in this direction and to analyze in larger groups of athletes. Since there are few and limited number of athletes, it is important to continue to examine how the BDNF rs6265 polymorphism affects the effects of regular exercise on attention and information processing speed. Such studies will be an important data source to make sense of sports genetics and BDNF rs6265 region.

Patient informed consent: Informed consent was obtained.
Ethics committee approval: The protocol used in the present study was approved by the Üsküdar University Ethics Committee and was performed in accordance with the principles of the Declaration of Helsinki II. All participants signed consent forms containing all the information such as the study protocol, results and evaluation of the results.
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Author contribution subject and rate:
Tolga Polat (40%): Data collection, formation of the article
Canan Sercan Doğan (10%): Data collection
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THE DEVELOPMENT OF A FUZZY LOGIC MODEL-BASED SUICIDE RISK ASSESSMENT TOOL

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Abstract

The purpose of the research was to develop a fuzzy logic model-based risk assessment tool and to determine the views of health professionals working in community mental health centers concerning this. A methodological method was employed in developing the fuzzy logic model-based risk assessment tool, and the qualitative research method was used to elicit attitudes toward it. In the first stage, the suicide risk assessment tool was developed. In second stage the health professionals used the suicide risk assessment tool for four weeks. Feedback regarding health professionals' opinions of the suicide risk assessment tool was then elicited handling a semi-structured reportage form, and data were investigate handling descriptive analysis. Health professionals reported powerful aspects making the suicide risk assessment tool a functional, practical, comprehensive, and highly applicable guideline. Participants also recommended the addition to the results screen of a chart showing the course of the suicide risk assessment, and that the reliability and validity of the tool be confirmed. In conclusion, health professionals expressed positive opinions regarding the scope of the suicide risk assessment tool and its measurement of that risk. Following confirmation of its reliability and validity, it may be useful for the fuzzy logic-based suicide risk assessment form to be integrated into and applied in the community health centers system.

Keywords: community mental health, fuzzy logic, suicide risk assessment, suicide

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1. Introduction

Around 800,000 people global lose their lives because of suicide every year (WHO, 2018). In 2018, 3161 individuals in Turkey died from suicide (TUIK, 2018). The World Health Organization 2013-2020 Mental Health Action Plan, adopted by the World Health Assembly in 2013, targeted a 10% decrease in national suicide levels by 2020 (WHO, 2013).

Retrospective and psychologic autopsy researches have shown that a detectable psychological disease is available in at least 90% of all completed suicides (Conwell et al., 1996). Half to two-thirds of psychiatric diagnoses involve mood disorders (Herrera, 2018). Other research findings concerning the relationship between suicide and mental disorders show that the highest suicide-related mortality rates occur in individuals diagnosed with substance misuse and eating disorders, and indicate moderate suicide levels for mood and personality disorders, and relatively low proportions for anxiety disorders (Harris & Barraclough, 1998). The risk of suicide also increases with the use of more than one substance (Borges et al., 2000), and in case of depression (Malone et al., 2000), eating disorders (Harris & Barraclough, 1997), and personality disorder (Soloff et al., 1994). The groups with the highest risk of suicide are those with borderline personality disorder (Black et al., 2004; Söderberg, 2001) and antisocial personality disorder (Black et al., 2004).

Community Mental Health Centers (CMHCs) in Turkey provide psychosocial support services, follow-up and treatment within the framework of a community-based mental health model for individuals with chronic mental disorders such as schizophrenia, other psychotic disorders and bipolar disorder (Turkish Ministry of Health, 2011). Twenty to fifty percent of schizophrenia patients attempt suicide (Lu et al., 2019). Individuals with bipolar disorder also frequently attempt suicide during depressive attacks (APA, 2003), and the risk of suicide increases still further in case of substance use disorder, anxiety disorder, eating disorder, and personality disorder accompanying bipolar disorder (Hansson et al., 2018). Due to their characteristics, it is particularly important to assess the suicide risks of the individuals being followed-up by CMHCs.

There is no method capable of completely accurately showing the risk of suicide. However, the probability of risk can be predicted by means of risk assessment. Health professionals should trust their own clinical experience in risk assessment, but must also confirm their assessments on the basis of evidence-based findings (Fawcett, 2006). Important factors determining suicidal behavior are interactions between suicide risk and protective factors (Goldsmith et al., 2002). Factors protecting against suicide include a sense of belonging (Joiner et al., 2006), a good cognitive level (Malone et al., 2000), useful and good developed coping capabilities and the fact that religious faiths disapprove of suicide (APA, 2003), being married and having children (Nock et al., 2008), powerful family bonds, and positive supportive relations between the patient and clinician (Samra, 2007).

Factors reported to involve a high risk of suicide include suicidal ideations, preparatory actions, stressful life events and cognitive/emotional conditions (Rudd et al., 2006), extensive suicidal behaviors and objective signs of suicidal attainment (such as specific planning, access to lethal means, and the occasion to set these into action) (Walsh et al., 2015), stressful life events (Konkan et al., 2014), admission to and discharge from hospital (Goldacre et al., 1993; Harris & Barraclough, 1997), important physical diseases (Erlangsen et al., 2005), chronic painful diseases impairing functioning and external appearance, diseases rendering the individual dependent on others or affecting the ability to see and hear, and chronic diseases (Chehil & Kutcher, 2012), hopelessness (Qiu et al., 2017), and social isolation.

The risk of suicide is generally assessed using scales, but these may be limited in terms of the extent to which the assessment logic actually reflects human ways of thinking, the inclusion in the assessment process of health professionals' knowledge and experience and these being supported with proven data, and taking into account factors protecting the individual against suicide. The use of existing suicide risk assessment scales is for this reason insufficient in terms of health professionals confirming their own clinical experience with evidence-based information. A single numerical value obtained from scales may also result in important risk-related situations being missed. A risk evaluation and suicide risk assessment appears in the official CMHC directive, but no structured objective measurement tool is employed, and risk assessment relies more on the individual's worker's own knowledge and experience.

One of the most appropriate procedures for assessing non-linear, multi-dimensional, complex, and imprecise subjects such as suicide is fuzzy logic (Chattopadhyay et al., 2010). This was invented in 1965 by Lotfi A. Zadeh under the name of fuzzy logic or the fuzzy setting theory (Zadeh, 1965). The greatest advantage of fuzzy logic is that it very closely matches human reasoning. In the classic set theory, also known as binary logic, an element either belongs to a set, or else does not. However, there are several conditions in which an element's membership of a set is uncertain or indefinite. These are frequently seen in the sphere of health (Phuong & Kreinovich, 2001). When classic logic is used in suicide risk assessment, binary results are elicited for the risk of suicide, in the form of low or high, or present or absent. The production of the absolute results regarding the risk of suicide can result in health professionals missing risky or borderline situations. In fuzzy logic theory, an element may have various degrees of membership or may belong to more than one set. The degree of membership of elements in a fuzzy set ranges between 0 and 1, and these values show partial membership of a set (Zadeh, 1965). For instance, when an individual's suicide risk is assessed using fuzzy logic, risk status may be present in a low risk group with one particular degree of membership and at the same time in a high risk group with another particular degree of membership. In this way, health professionals can see the extent of the individual's membership of different groups, and can at the same time include their own knowledge and experimentation in the suicide risk assessment process. There is no tool and/or scale assessing the risk of suicide using the fuzzy logic method in Turkey. In international terms, the UK Galatean Risk and Safety Tool (GRIST)

program is based on the fuzzy logic model (Buckingham, 2002).

If effective suicide risk assessment is not performed, the individual may experience various risks, ranging from mild damage to potentially fatal situations. This makes accurate risk assessment essential. The fuzzy logic-based suicide risk assessment tool developed in the scope of the present research is practical and user-friendly and may be expected to contribute to suicide risk assessment depending on health professionals' knowledge and experience. The aim of this study to develop a fuzzy logic model-based suicide risk assessment tool and to establish the views of health professionals working in CMHCs regarding that tool.

2. Materials and Methods

Approval for the research was granted by the Üsküdar University Non-Interventional Research Ethical Committee (No. B.08.6.YÖK.2.ÜS.0.05.0.06/2017/327).

Type of research: A methodological design was employed in the development of a fuzzy logic model-based suicide risk assessment tool, and a qualitative method was used to collect opinions concerning the tool developed.

Application: Application was performed in two phases. In the first phase, a fuzzy logic model-based suicide risk assessment tool was developed, while in the second, user views concerning the tool were elicited.

Stage 1: The development of a fuzzy logic model-based suicide risk assessment tool

The procedures performed during the development of the fuzzy logic model-based suicide risk assessment tool are listed, in order, below.

1. Determination of suicide risk factors: Evidence-based study findings in systematic reviews and meta-analyses were examined in order to identify factors increasing and protecting against the risk of suicide. Fourteen risk areas and eight protective factors were identified (Franklin, 2017; Harris & Barraclough, 1997; Ribeiro et al., 2016; Runeson et al., 2017).

2. The collection of specialist opinions to determine the effect levels of risk factors: Specialist opinions were elicited in order to determine the effect levels of factors increasing or protecting against the risk of suicide. Views were collected from 19 specialists working in psychiatric clinics (nine physicians, four nurses, two social services experts, and four psychologists). These specialists had been working in psychiatric clinics for between 10 and 42 years. They were asked to score each factor reducing or increasing the risk of suicide between '0 and 1' with 1% sensitivity. These numerical values were transferred onto Excel, and mean values were determined (Table 1).

Table 1. Mean scores awarded by specialists for suicide risk factors

Suicide risk indicators	Mean value
History of suicide attempts	
History of more than one suicide attempts	0.88
History of one suicide attempt	0.65
No history of attempted suicide	0.12
Current thinking regarding suicide	
Presence of clear and constant intent to commit suicide	0.81
Occasional suicidal ideation	0.64
No suicidal ideation	0.10
Suicide plan	
Presence of a suicide plan	0.87
No suicide plan	0.20
Impairment of thought content	
Presence of intensive delusions/hallucinations (containing the instruction to commit suicide) in the current period	0.85
Absence of intensive delusions/hallucinations (containing the instruction to commit suicide) in the current period	0.19
Other diagnoses accompanying schizophrenia and bipolar disorder	
Schizoaffective disorder	0.55
Substance abuse	0.61
Depression	0.64
Personality disorders	
Cluster A disorders (Paranoid personality disorder, schizotypal personality disorder, schizoid personality disorder)	0.10
Cluster B disorders (Borderline personality disorder, antisocial personality disorder)	0.70
Anxiety disorders	0.31
Eating disorders	0.28
Postpartum psychosis	0.63
Life-threatening chronic disease causing pain and function impairment	0.50
Drug therapy adherence	
No drug therapy adherence	0.61
Occasional drug therapy adherence	0.46
Adheres to drug therapy	0.20
Time since discharge from the psychiatric ward	
Between one day and one month after discharge	0.46
After the first month following discharge	0.37
No condition requiring admission to the psychiatric ward for an extended period	0.14
Stressful life events (the 'stressful life events' list can be used if the specialist so requires)	
Presence of perceived highly stressful life event/events/ in the previous month	0.64
Presence of perceived moderately stressful life event/events/ in the previous month	0.42
Presence of perceived mildly stressful life event/events/ in the previous month	0.20
Family history of suicide	
Death of a member of the family due to suicide	0.80

Hopelessness (the 'hopelessness scale' can be used if the specialist so requires)	
Feelings of hopelessness, helplessness and weakness, a pessimist aspect perspective toward life	0.70
Uncertain, negative future plans	0.54
Having some plans and hopes for the future	0.16
Beck Depression Inventory score	
Scoring 17 or more on the Beck Depression Inventory	0.61
Scoring under 17 on the Beck Depression Inventory	0.24
Emotional state	
Emotional turmoil (anxiety, agitation, anger).	0.53
Emotional fluctuations	0.50
Low level anxiety	0.18
Distress level	
Scoring 0-3 on the distress thermometer	0.17
Scoring 4-6 on the distress thermometer	0.34
Scoring 7-10 on the distress thermometer	0.56
Psychological health	
Scoring above average on the brief resilience scale (12 or more)	0.16
Scoring below average on the brief resilience scale (less than 12)	0.39
Protective factors	
Being married (with a positive relationship)	0.30
Having children	0.31
Having a good cognitive level	0.26
Holding spiritual/religious beliefs (disapproving of suicide)	0.31
Having strong family ties	0.26
Having strong ties with friends or neighbors	0.26
Having a job or occupation	0.26
A positive relationship between the patient and counselor	0.29

4. Calculating the risk of suicide with a fuzzy logic model: An Excel file based on probability density function was prepared to calculate Gaussian distribution for the calculation of suicide risk with a fuzzy logic model. The model functions through the formula:

When the risk assessment tool items increasing the risk of suicide are completed, the maximum possible score is 9.41 and the minimum score is 2.16. Risk levels are assessed as low, moderate or high. The high risk rate rises the closer scores approach to 9.41, the moderate risk rate the closer they approach to 5.78, and the low risk rate the closer they approach to 2.16. Factors protecting the individual against suicide on the risk assessment tool are evaluated separately from the fuzzy logic model. The software produces a result by adding the effect levels of each protective factor. The maximum possible score from the factors protecting the individual against suicide is 2.25, and the minimum possible score is 0.26. Higher scores indicate a greater level of protection against suicide. After determining an individual's suicide risk level, health professionals can form an opinion concerning suicide risk status in the light of their clinical knowledge and experience and of the score obtained from protective factors.

5. Conversion of the fuzzy logic model-based suicide risk assessment tool into an online format: The suicide risk assessment tool was converted into an online format in order to enhance various facilitating features, such as ease of access, maintenance of confidentiality, and remote access (www.ufalt.net).

Stage 2: Determination of the views of health professionals working in CMHCs concerning the fuzzy logic model-based suicide risk assessment tool

Sample: The research sample consisted of health professionals working in two CMHCs affiliated to the Istanbul Provincial Health Directorate and one affiliated to the Karaman Provincial Health Directorate. A social services specialist, two nurses and two psychologists were working in the first CMHC, and a psychiatrist, two nurses, a psychologist, a social services specialist and an ergotherapist in the second, and a psychiatrist, two nurses, a psychologist, and a social services specialist in the third. Fifteen health professionals in CMHCs agreeing to take part in the study were enrolled.

Date Collection Tool: Health professionals' opinions were elicited using a semi-structured reportage form developed by the authors. This consisted of two parts. The first section contained eight questions designed to elicit characteristics such as health professionals' age, sex, work experience, and receipt of occupational and risk assessment training. The second section contained one question regarding how health professionals' use of the suicide risk assessment tool affected their evaluations, and two others concerning the powerful aspects of the tool and those requiring further development.

Application: Health professionals in the three CMHCs were informed about the suicide risk assessment tool developed, which was made available for their use. They used the suicide risk assessment tool for four weeks. Feedback concerning their opinions of the tool was obtained through a semi-structured reportage form. Interviews were held face to face with each health professional, in their own offices, and lasted between 15 and 35 min. The interviews were also recorded.

Data Reliability and Validity: The principles of credibility, transmissibility, consistency and confirmability in qualitative inquiry were employed in the establishment of data reliability and validity (Yıldırım & Şimşek, 2013).

Data Evaluation: The study data were subjected to descriptive evaluation. This involves data being summarized and interpreted according to previously determined themes (Yıldırım & Şimşek, 2013).

3. Results

Fifteen health professionals were interviewed in the scope of the research. The nurses participating in the research were numbered from N1 to N6, the psychiatric specialists were numbered PS1 and PS2, the psychologists were numbered from P1 to P3, the social services specialists were numbered from SS1 to SS3, and the ergotherapist

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was coded ERG. The health professionals enrolled in the research and some of their characteristics are shown in Table 2.

Table 2. Characteristics of the health professionals who provided views concerning the suicide risk assessment tool

Participants	Age	Sex	Occupation	Education level	Length of time worked in the CMHC	Length of time worked in other psychiatri	Receipt of risk assessment training
PS1	38	Male	Psychiatrist	Postgraduate	3 Years	13 Years	No
PS2	31	Male	Psychiatrist	Postgraduate	1 Year	10 Years	No
N1	36	Female	Nurse	Postgraduate	6 Years	4 Years	Yes
N2	39	Female	Nurse	Postgraduate	2 Years	11 Years	Yes
N3	35	Female	Nurse		6 Years	5 Years	Yes
N4	45	Female	Nurse	Bachelor's	6 Years	8 Years	No
N5	40	Female	Nurse	Bachelor's	5 Years	5 Years	Yes
N6	38	Female	Nurse	Postgraduate	5 Years	5 Years	Yes
P1	26	Female	Psychologist	Postgraduate	1 Year	10 Years	No
P2	27	Female	Psychologist	Postgraduate	1 Year	11 Years	No
P3	33	Male	Psychologist	Bachelor's	5 Months	11 Years	Yes
SSS1	25	Female	Social services specialist	Bachelor's	4 Months	12 Years	No
SSS2	28	Male	Social services specialist	Bachelor's	2 Years	12 Years	Yes
SSS3	26	Male	Social services specialist	Bachelor's	1 Year	15 Years	No
ERG	25	Female	Ergotherapist	Bachelor's	1 Year	9 Years	No

The data obtained from the interviews were subjected to descriptive analysis, and themes and subthemes were identified. Themes refer to powerful aspects of the tool and those requiring development, while subthemes refer to scope, applicability, and suicide risk measurement.

Theme 1. Powerful aspects: The health professionals participating in the research reported powerful aspects concerning the scope of the fuzzy logic model-based suicide risk assessment tool, its applicability, and suicide risk assessment status.

Subtheme 1. Scope: The participants declared that the number and content of the questions in the tool were sufficient to prevent questions being missed in the assessment and for the consideration of protective factors: "PS 1: In my opinion the scope is sufficient… I consider it ideal in terms of the number of questions… We must obtain maximum benefit with a minimum number of questions, particularly when interviewing cases of this type …". "N5: I think that the questions fully meet requirements…"

Subtheme 2. Applicability: Some health professionals reported that the applicability of the suicide risk assessment took was practical and facilitated suicide risk evaluation. The participants who described the developed suicide risk assessment tool as practical ascribed this to its not being time-consuming in nature, its being simple and very easy to apply, its being based on information elicited when speaking to the patient, its being capable of application within the interview, its containing few areas requiring interpretation, and its being useful in case of uncertain assessments and highly practical. The fact that it could be easily completed by anyone with psychiatric training was also described as a powerful aspect of the tool:"P 1: …It is very simple. I mean it is very simple to apply. It is easy to apply it verbally… It did not take much time, and represents an excellent conversation in suicide assessment. It is something I can apply within the interview …" "SSS 4: …Our having such a form available may be something that will enhance the quality of our interviews with patients. I therefore consider it functional…"

The way the tool facilitated suicide risk assessment was an element emphasized by almost all participants: "PS1: It facilitates our work because the questions involve are basic parameters we need to know about the individual being assessed, whom we think may be a suicide risk. You have in all likelihood selected the most rational or valuable items from among these, and this facilitates our work in that sense. In other words it prevents us skipping any question and also considers protective factors right from the beginning, and that makes our job easier. It serves us as a guide." "SSS 1: The questions are definitely comprehensible. Patients definitely respond to these questions, because there is nothing that any patient cannot understand."

Other powerful aspects cited by health professionals include the easy accessibility of the suicide assessment risk tool due to its availability in an electronic environment, the fact it provides a numerical figure at the end of the assessment, that it permits more professional assessment, its enhancement of the task being performed, the fact it provides a common language, and the way it permits official documentation: "PS 1: It made things easier. I can make a more professional assessment. In addition, it also enhanced the quality of my assessments…" "P1:… The risk assessment tool provides results along the lines of a 10% low risk, a 50% moderate risk, and a 20% high risk. This enables us to say something about the risk and contributes to the analysis." "N5: I think this should be applied to all patients. Then we can proceed based on the results... There will still be situations that are missed. But at least it will have been applied. Then we will have documentation to say that 'We applied it, and there was no such ideation then.'

Subtheme 3. Suicide Risk Measurement Status: Health professionals reported that the assessment result they obtained using the suicide risk assessment tool was similar to that they estimated themselves, and that the tool provided clarity in case of uncertainty: "N4: The assessments are exactly what we think ourselves. For example, it provides immediate clarity about things we are uncertain of…"

One opinion to the effect that the suicide risk assessment tool is consistent and suited to evidential study was expressed as follows: "P2: … In my view, the system

is consistent internally and with its results. Of course, something more empirical would be needed to determine how useful this is. But I think the results are consistent and suitable for use …"

Theme 2: Aspects Requiring Development

Subtheme 1. Applicability: In terms of the applicability of the suicide risk assessment scale, some participants stated that the application of the tool could be enhanced if it were completed by a counselor well acquainted with the patient, if it were integrated into the system employed in CMHCs, and if graphics were added to the result screen: "P2: The risk assessment could easily be completed if the person applying the test is sufficiently acquainted with the patient..." "PS1: …if it were integrated into systems used in our daily procedures, for example, there is a program on the web containing the forms used in our CMHCs, and if it were integrated with that, then we could apply it very quickly…Graphics could be added to the results screen, and every graphic we produce could be given separately when we enter the patient's file. Let us assume that we apply this scale every two weeks, if there were a graphic showing the course, then that would be very useful very quickly."

4. Discussion

The views of health professionals in CMHCs regarding the fuzzy logic model-based suicide risk assessment tool are discussed below under the themes identified.

Theme 1. Powerful aspects

Participants stated that the items in the suicide risk assessment tool can ensure that various questions that should be asked during assessment are not overlooked. They also considered that it contains questions that assess existing emotions, ideas and behaviors that can affect the individual's suicide risk while considering dynamic or state changes, that the tool can provide a common language. Participants also felt that the calculation of a number at the end of the analysis permits a more professional assessment, that the tool professionalizes application, is very simple and functional, and also permits official documentation. Reasons cited in studies for health professionals expressing positive views of risk assessment tools include the presence of questions that are very useful in evaluating previous psychological history and that might not otherwise come to mind (Buckingham, 2002), their facilitation of discussions about risk on the part of care providers (Muir-Cochrane et al., 2011), and their facilitation of professional decision-making (Holley et al., 2016). Causes for criticism of risk assessment tools include lack of attention to dynamic or state changes since they tend to focus on previous (static) risk factors, and their inability to consider individual factors concerning the patient (Doyle & Dolan, 2002), their being highly mechanical and behaviorally reductive, dehumanizing, and their failure to include professional intuition in the assessment process (Godin, 2004). We attribute the generally positive opinions of our suicide risk assessment tool on the part of participants to the questions evaluating both the individual's previous (static) and variable (dynamic) characteristics. We also think that participants approved the tool due to its providing information about risk group membership percentages as a result of the fuzzy logic model employed, rather than providing definite values as in classic logic, thus including health professionals' views and experience in the decision-making process.

In terms of the applicability of the suicide risk assessment tool, the participants stated that it contained questions requiring consideration and professionalized application, was highly functional and easy to administer, and permitted official documentation. The provision of official documentation is also cited as the most important reason for positive attitudes toward the using of risk assessment tools on the part of health professionals in previous similar research (Godin, 2004;Muir-Cochrane et al., 2011;Woods, 2013). Some researches of attitudes toward risk assessment tools have reported that health professionals are undecided regarding the use of such tools, and that they may regard them as bureaucratic instruments of no value or purpose or else as psychiatric technology developed in order to reduce the role of clinical expertise (Gerace et al., 2013;Muir-Cochrane et al., 2011). Studies have also suggested that health professionals consider them to be useful guidelines capable of informing and guiding less able or less experienced applicators (Godin, 2004), and that they would employ a risk assessment tool if it was appropriate and user-friendly (Woods, 2013). We attribute the positive approach to the application of the risk assessment tool in the present study to the fuzzy logic model, an artificial intelligence program, very closely reflecting human thinking, and to participants being aware of the need for a suicide risk assessment tool.

While the result yielded by the tool was consistent with health professionals' own estimations of the individual's risk status, they also reported positive views concerning the fact that it additionally provided clarity in uncertain situations. More than one in four of the nurses participating in Holley, Chambers and Gillard's (2016) research believed that risk assessment tools were capable of predicting the probability of risk, but they also did not object to the statement that risk could not be predicted.

Theme 2. Aspects requiring development

The health professionals participating in this research also recommended that the suicide risk assessment tool be integrated with the CMHC system, and suggested that a graphic demonstrating the route of the patient's suicide risk assessment on the outcome monitor would also be useful. This finding shows that the participants in our study held positive opinions regarding the routine use of the suicide risk assessment tool. In contrast to these findings, one study involving community mental health nurses reported that some participants felt that the using of a standard risk assessment tool who a totally unnecessary bureaucratic imposition, while the majority felt that imposing the use of a standardized risk assessment tool would act as an obstacle to creativity in their work (Godin 2004). We think that the integration of our suicide assessment risk tool into the CMHC system will not create an additional workload since it will become part of existing practices. The addition to the results screen of a graphic demonstrating the route of the patient's suicide risk assessment will also be useful in terms of evaluating change in the patient's suicide risk.

Longitudinal researches are needed to define the effectiveness of the suicide risk assessment tool developed in this study in measuring suicide.The lack of data regarding the validity of the scale is the limitation of the study.

5. Conclusion

In conclusion, the health professionals in this research held positive views concerning the scope of the suicide risk assessment tool we developed. In the light of health professionals' views regarding the questions in the suicide risk assessment tool and their suggestions for how it might be improved, we may conclude that some aspects require further development.

Patient informed consent: Informed consent was obtained.
Ethics committee approval: Approval for the research was granted by the Üsküdar University Non-Interventional Research Ethical Committee (No. B.08.6. YÖK.2.ÜS.0.05.0.06/2017/327).
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Author contribution subject and rate:
Fatma AYHAN.(%45), Besti ÜSTÜN (%35) and Türker Tekin ERGÜZEL (%20) contributed to the conception and design of this study.
Fatma AYHAN.(%45) and Besti ÜSTÜN (%35) performed the descriptive analysis and drafted the manuscript.
Türker Tekin ERGÜZEL (%20) prepared the fuzzy logic software and critically reviewed the manuscript and supervised the whole study process. All authors read and approved the final manuscript.

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VITAMIN B12 AND FOLIC ACID IN DEPRESSION AND ANXIETY: A PILOT STUDY

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Ethics committee approval: The non-interventional research ethics committee approved this study at Uskudar University (Code: 61351342-/2019-80; date: 27.02.2019).

Abstract

The association between nutrition and mental diseases has been extensively studied. Vitamin B12 and folic acid are the main vitamins in the development of the central nervous system. This study aimed to investigate the relationship between vitamin B12 and folic acid levels with depression and anxiety.

A total of 150 patients (77 with anxiety and 73 with depression) between 18 and 79 were enrolled. The initial mood state was evaluated using Beck's Depression Inventory-II (BDI-II) and Beck's Anxiety Inventory (BAI) for the presence of depression and anxiety, respectively. Venous blood samples were collected after overnight fasting and vitamin B12 and folic acid levels were measured.

Serum concentrations of folic acid were significantly lower in patients with depression than those with anxiety (p=0.04). Substantially higher vitamin B12 levels were described in men than females counterparts with severe depression (p=0.049). Additionally, there was a significant inverse correlation between folic acid levels and Beck's inventory score among patients with severe depression (r=-0.250, p=0.049).

Serum levels of vitamin B12 and folic acid could be used as markers that assess anxiety and depression's therapeutic efficiency.

Keywords: mood disorders; beck's depression inventory score, vitamin b12; folic acid, anxiety, depression

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1. Introduction

Depression and anxiety are considered the most prevalent mental diseases, resulting in the loss of the affected individuals' socioeconomic productivity. Therefore, the effective treatment of these disorders should be included as coherent chronic disease management (Chisholm et al., 2016). During the past decade, various epidemiological studies have been conducted to investigate the relationship between nutritional patterns and individuals' mental status so that dietary habits have been suggested as an underlying cause to onset the psychiatric symptoms (Owen & Corfe, 2017). In this regard, "nutritional psychology" mainly focuses on nutrition or a single nutrient on mood disorders, including depression and anxiety (Jacka, 2017). The most frequent nutritional-related deficiencies in mental diseases include ω-3 fatty acids, vitamin B family, mineral elements, and amino acids, which are the neurotransmitters precursors in the nervous system (Coppen & Bolander-Gouaille, 2005).

Vitamin B12 and folic acid as the main water-soluble vitamins play a crucial role in the central nervous system (CNS) development and health (Reynolds, 2006). Vitamin B12 and folic acid have a shared metabolism so that the deficiency in one vitamin impairs the other vitamin function (Black, 2008). There are two main mechanisms proposed for the role of vitamin B12 and folic acid in CNS development: i) The role of these vitamins in the myelination process and ii) methionine synthesis from homocysteine (h-Cys) as a crucial amino acid in neural tube formation and development (Black, 2008). Moreover, folate deficiency increases h-Cys and decrease S-adenosylmethionine (SAMet), causing methylation capacity reduction and finally alters neurotransmitter synthesis (Morris, Trivedi, & Rush, 2008).

However, the role of these vitamins in depression and anxiety management remains controversial. It has been reported that patients with low folate levels show a mean longer time for depression improvement compared to individuals with normal folate levels. Additionally, the relapse rate was also higher in patients with low folate levels (Papakostas et al., 2004). Furthermore, increases in the blood folate levels during antidepressive treatment significantly improved the Hamilton depression scale score. A higher blood folate concentration was also observed after the treatment in "responding" than that of "nonresponding" patients (Martínez-Cengotitabengoa & González-Pinto, 2017). Sepehrmanesh et al. (Sepehrmanesh, Omidi, & Gholampoor, 2017) reported that folic acid supplementation in major depressive disorder improves the antidepressant response in these patients be considered as a complementary treatment. However, Walker et al. showed no significant improvement in depressive symptoms following folic acid administration (Walker et al., 2010).

On the other hand, recent literature has also indicated the link between vitamin B12 deficiency and depression (Syed, Wasay, & Awan, 2013). High serum vitamin B12 levels have reported being associated with satisfactory treatment outcomes; however, high h-Cys levels, which is usually observed in folate/vitamin B12 deficiency in depressive patients, are typically associated with inadequate response to antidepressant therapy (Hintikka, Tolmunen, Tanskanen, & Viinamäki, 2003; Sachdev et al., 2005). Syed et al. (Syed et al., 2013) reported a significant improvement in depressive symptoms after vitamin B12 supplementation combined with antidepressants. In contrast, in a two-year vitamin B12 supplementation study, no significant improvement in depressive symptoms was observed in adult patients. However, a beneficial effect on health-related quality of life (HR-QoL) was reported in this research (De Koning et al., 2016). Vitamin B12 and folic acid supplementation also have improved the Hamilton depression and anxiety scores in human immunodeficiency virus (HIV)-positive patients (Adhikari et al., 2016).

According to a recent report, the vitamin B12, not the folic acid concentrations, were inversely correlated with the severity of depression (Skarupski et al., 2010). This study examined the association between the serum concentrations of these two nutrients and the severity of mood disorders observed in individuals of both sexes over a wide range of ages.

2. Materials and Methods

The non-interventional research ethics committee approved this study at Uskudar University (Code: 61351342-/2019-80; date: 27.02.2019).

2.1. Patients

Seventy-seven patients with anxiety and 73 patients with depression referred to the Neuropsychiatry Clinic at Uskudar University were screened and enrolled in this study from March to December 2018. The patients with overlapping characteristics of depression and anxiety were excluded. History of serum vitamin B12 and folic acid altering diseases such as hepatic dysfunction, renal disorders, diabetes mellitus, or current use of the mentioned vitamins supplements were also considered as exclusion criteria.

2.2. Clinical assessments

The psychiatric examination of the patients was done using Beck's Depression Inventory-II (BDI-II) and Beck's Anxiety Inventory (BAI) for the presence of depression and anxiety, respectively, and the scales were validated in Turkey (Mustafa, Sahin Nesrin, & Husnu, 1998; Yildirim & Ilhan, 2010). These tests contained 21 multiple choice questions with a maximum score of 3 for each item. The patients performed the tests under the supervision of a psychologist. BDI-II scores ≤9 was considered as no depression. Depression severity was also categorized as mild (BDI-II 10–15), moderate (BDI-II 16–23), and severe (BDI-II ≥24). BAI scores of <15, was also considered as unfavorable for anxiety; mild (BAI scores 16–22), moderate (BAI scores 23–42), and severe (BAI scores ≥43). Patients with BDI-II ≤9 and BAI<15 scores were excluded from the study.

2.3. Sampling and biochemical assay

Serum folic acid and vitamin B12 were measured after overnight fasting in all subjects using a Roche Cobas e411 (Mannheim, Germany) instrument. Low serum vitamin B12 (<200 pg/ml) and folic acid (<2.2 ng/ml) levels were considered as deficiency.

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2.4. Sample size determination and Statistical analysis

The sample size was determined to establish a power of 80% and a confidence interval of 95%, using the following formula: sample size=Z1-α/22*P(1-P) /d2. Data were analyzed using the statistical package for social sciences (SPSS® version 23, IBM Inc., Chicago, IL). The numerical variables were expressed as mean ± standard deviation (SD). If a normal distribution was not detected with the Kolmogorov-Smirnov test, the variables were analyzed with a non-parametric Kruskal Wallis test to compare the mean differences between groups. Additionally, bivariate correlation analysis was performed to assess the association between clinical variables and vitamin B12 or folic acid levels. P values <0.05 were considered as statistically significant.

3. Results

3.1. Demographics and general characteristics of participants:

There were 77 patients with anxiety and 73 patients diagnosed with depression. The median, range, and proportion of vitamin B12 and folic acid deficient individuals are presented in Table 1. Vitamin B12 and folic acid concentrations were significantly lower in subjects with depression than those with anxiety (p=0.04). However, there were no differences in serum concentrations of vitamin B12 and folic acid in patients with anxiety or depression when the subjects grouped according to their age (Table 2). An analysis of gender subgroups revealed that vitamin B12 and folic acid were similar between the patients of both sexes. However, men with depression had marginally higher levels of vitamin B12 than female patients with depression (p=0.049) (Table 3)

Table 1. General characteristics of individuals

Parameters		Anxiety (n=77)	Depression (n=73)
Age (year)	18-40	47	51
	41-60	26	21
	61-79	4	1
Vit B12 (pg/ml)	Median	210	267
	Range	33 -1399	112 -1451
	Deficient patients	17	25
Folic acid (ng/ml)	Median	6.1	3.9
	Range	1.5 - 16.9	1 - 15.5
	Deficient patients	5	16
Beck.Depression	Median	15	31
	Range	2 - 46	12 - 46
Beck.Anxiety	Median	28	18
	Range	10 - 43	6 - 60

Table 2. Comparison of biochemical indices between anxiety and depression groups and sub-groups

Parameters	Anxiety (n=77)	Depression (n=73)	p
Vit B12 (pg/ml)	356 ± 212	321 ± 210	0.112
Folic acid (ng/ml)	6.3 ± 3.2	5.4 ± 3.5	0.040*
Anxiety (n=77)			
	Mild anxiety (n=18)	Moderate anxiety (n=59)	P
Vit B12 (pg/ml)	314 ± 137	370 ± 229	0.890
Folic Acid (ng/ml)	6.3 ± 3.8	6.3 ± 3.0	0.584
Depression (n=73)			
	Moderate depression (n=12)	Severe depression (n=61)	P
Vit B12 (pg/ml)	381 ± 172	313 ± 216	0.081
Folic acid (ng/ml)	6.8 ± 3.1	5.2 ± 3.6	0.178

Data are presented as mean±SD. p<0.05 was considered as statistically significant.

Table 3. Comparison of biochemical indices between male and female individual through anxiety and depression sub-groups

Parameters	Anxiety sub-groups (n=77)	Male	Female	p
Vit B12 (pg/ml)	Mild anxiety (n=18)	356 ± 129	260 ± 135	0.131
	Moderate anxiety (n=59)	349 ± 233	396 ± 227	0.410
Folic acid (ng/ml)	Mild anxiety (n=18)	5.8 ± 3.0	7.0 ± 4.8	0.790
	Moderate anxiety (n=59)	6.0 ± 2.6	6.7 ± 3.5	0.521
	Depression sub-groups (n=73)	Male	Female	p
Vit B12 (pg/ml)	Moderate depression (n=12)	356 ± 192	396 ± 174	0.705
	Severe depression (n=61)	347 ± 166	297 ± 237	0.049*
Folic Acid (ng/ml)	Moderate depression (n=12)	5.4 ± 3.2	7.5 ± 2.9	0.257
	Severe depression (n=61)	5.1 ± 3.5	5.2 ± 3.6	0.939

Data are presented as mean±SD. P <0.05 was considered as statistically significant.

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Table 4. Correlation of biochemical parameters with anxiety and depression beck degree

Parameters	Anxiety sub-groups (n=77)	Beck.Anxiety r	Beck.Anxiety p
Vit B12 (pg/ml)	Mild Anxiety (n=18)	0.403	0.093
	Moderate Anxiety (n=59)	0.105	0.427
Folic Acid (ng/ml)	Mild Anxiety (n=18)	-0.144	0.57
	Moderate Anxiety (n=59)	0.215	0.102
Depression sub-groups (n=73)		**Beck.Depression r**	**Beck.Depression p**
Vit B12 (pg/ml)	Moderate depression (n=12)	0.056	0.871
	Severe depression (n=61)	0.106	0.418
Folic Acid (ng/ml	Moderate depression (n=12)	-0.322	0.332
	Severe depression (n=61)	-0.250	0.049*

r: Correlation coefficient

3.2. Correlation of vitamin B12 and folic acid concentration with Beck's depression/anxiety inventory scores

As it is presented, a significant negative correlation was observed between folic acid levels and BDI-II in severely depressed patients (r=-0.250, p=0.049). Univariate association and correlation between vitamin B12 and folic acid levels and Beck's inventory scores for depression and anxiety are presented in Table 4.

4. Discussion

The study aim was to evaluate the association of anxiety and depression with the levels of vitamin B12 and folic acid. We described higher levels of vitamin B12 in depressed males than female subjects with the same mood disorder. A lower level of serum folic acid in the depression group was observed compared to the anxiety group. Moreover, correlation analysis showed a significant negative correlation between folic acid levels and Beck's inventory score in severely depressive patients. This finding is similar to the results of a study conducted by Sanchez-Villegas et al. (Sánchez-Villegas et al., 2009).

Vitamin B12 and folic acid are critical for the development and the proper function of the nervous system in humans (Calderón-Ospina & Nava-Mesa, 2020). Conducted studies during the last decades have indicated an association of these vitamins' deficiencies with psychological symptoms, such as depression and cognitive impairment (Bottiglieri et al., 2000; Ford et al., 2008).

In an experimental study, Lerner et al. compared the serum concentations of vitamin B12 and folic acid in 224 newly diagnosed psychiatric patients and healthy controls in an Israelian population (Lerner et al., 2006). The results showed no significant differences between vitamin B12 levels within groups. In contrast, folate serum level was significantly higher in controls compared to patients with mood disorders. Moreover, a significant positive correlation was also observed between low folic acid levels and depression, consistent with our data. In a recent cross-sectional large cohort study on 7963 healthy Israelian individuals aged 18-65 years, Margalit et al. observed a significantly lower level of serum vitamin B12 in men compared to the women (Margalit, Cohen, Goldberg, & Krause, 2018). On the other hand, most of the examined population belonged to a higher socioeconomic class and may affect the external validity of the study by Margalit et al. Additionally, vitamin B12 deficiency prevalence in men was also higher. Nutritional, hormonal and social factors may partially clarify the obseved differences between the two genders.

Single nucleotide polymorphism (SNP) in the gene fucosyltransferase two have been associated with vitamin B12 absorption and serum level (Tanaka et al., 2009). Among the healthy population, men are susceptible to vitamin B12 deficiency. This can be explained by neither diet habits nor estrogen effects. Genetic variations involved in vitamin B12 homeostasis are therefore hypothesized to play a role. The lower serum vitamin B12 level in female severe depressive individuals in our study may explain the importance of this vitamin in mood disorders. Although the serum levels of vitamin B12 levels were low, they did not reach a "deficient" state in studied sub-groups.

Based on the previous studies reports, folic acid's role in mood disorders, including depression, seems to share a higher degree of importance. In a cross-sectional analysis was carried out by the National Health and Nutrition Examination Survey (NHANES) data, a non-causal association was observed between various serum vitamins (including vitamin B12 and folate) concentrations and depression in 2,791 individual (Huang et al., 2018) s. In this study, folate and vitamin B12 concentrations were also identified to be correlated with depression in the females.

In a double-blind, randomized controlled trial, depression scores were significantly reduced following combined treatment with 20 mg citalopram plus 2.5 mg folate when compared to controls (Sepehrmanesh et al., 2017). Furthermore, 73% of the patients receiving folate supplement had favorable response to citalopram, while only 40% of the patients in the control group demonstrated a favorable outcome to the treatment. The lower levels of folate in depressed patients and the negative association between folate and severe depression in our study may also confirm this vitamin's importance in mood disorders, especially depression.

Passeri et al. also compared the effects of 5'-methyltetrahydrofolate (50 mg/day) and trazodone (100 mg/day) in addition to standard psychotropic medication on depression and cognitive status in a double-blind design for eight weeks in an Italian population (Passen et al., 1993). In this study, a significant decrease in the Hamilton depression rating scale was observed

in patients supplemented with folate than the baseline, and the trazodone treated group after 4 and 8 weeks of intervention.

The association of low folate levels and poor response to selective serotonin reuptake inhibitors (SSRIs) has also been previously described in significant depression (Alpert et al., 2002; Papakostas et al., 2012). Papakostas et al. reported a higher efficacy of adjunctive 15 mg/day L-methyl-folate in combination with SSRI therapy compared to the placebo group for 60 days in both primary and secondary depression symptoms improvement. Similar results have also been reported from the United States (Alpert et al., 2002).

The limitations of our study include i) relatively small study population. A higher population may exert more confident results, particularly in anxiety patients. ii) Lack of a healthy control group and iii) the cross-sectional data. Other factors, including the duration of the vitamin B12 and folic acid deficiency, duration of psychiatric symptoms, lifestyle, and genetic variations, may also explain these relationships. Thus, further studies are still needed to examine the causal pathways between these vitamin deficiencies and mood disorders.

5. Conclusion

Based on our findings, Vitamin B12 and folic acid measurement may help the physicians for efficient anxiety and depression management. In this regard, folic acid seems to be more critical in mood disorders improvement outcomes and is suggested to be evaluated more carefully. Moreover, regarding the fact that the mean level of Vit B12 and folic acid were within typical values in this study, the need for treatment when no deficiency exists may be beneficial for better treatment outcomes.

Patient informed consent: Informed consent was obtained.
Ethics committee approval: This study was approved by the non-interventional research ethics committee at Uskudar University (Code: 61351342-/2019-80; date: 27.02.2019).
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Habib Erensoy (%100): prepared the abstract, statistical analysis, and discussion.

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DISTRIBUTION OF DOPAMINE RECEPTOR 2 (DRD2) RS1800497 POLYMORPHISMS IN PROFESSIONAL CYCLIST

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Ethics committee approval: The protocol used in the present study was approved by the Üsküdar University Ethics Committee and was performed in accordance with the principles of the Declaration of Helsinki II. All participants signed consent forms containing all the information such as the study protocol, results and evaluation of the results.

Abstract

Genetic and environmental factors are important determinants of the athletic performance. Sports genetic determines certain the alleles for the identification of the genes that affect athletic performance. Comprehensive researches, including the biology of mental properties are accumulating due to the improvement of the information of molecular biology. Dopamine is an important neurotransmitter of the dopaminergic system that affects the athlete mentally and psychologically. In this study, our goal is to determine the genotype and allele distributions of the DRD2 rs180047 polymorphism in the cyclists. 19 cyclists and 52 sedentary individuals (controls) participated in our study. Genotyping was carried out by real time PCR (rt-PCR) after DNA was isolated from buccal epithelial cells. In our cohort, AG and GG genotypes were detected as 6 (32%) and 13 (68%), respectively. In the control group, the respective AA, AG and GG genotypes were detected as 9 (17%), 18 (35%) and 25 (48%). No statistically significant difference was detected in terms of genotype distribution between the two groups (p= 0,1107). When allelic distributions were examined, in athlete cohort. A and G allele numbers were counted as 6 (16%) and 32 (84%), respectively. In the control group, same alleles were count as 36 (35%) and 68 (65%). There was no significant difference in the terms of alleles in our study cohort (p=0,0295). In our cohort, GG genotype and the G allele of the DRD2 rs1800497 polymorphism were dominant. Recent studies showed the association of the A allele with addiction. Therefore we hypothesized the association of the related allele and sucess in cyclists. Although we were unable to find statistically significant difference, we suggest to analyse the same polymorphism in athletes with different sport branches to fulfill the role of the given polymorphism.

Keywords: sports, genetics, polymorphism, DRD2, cyclist

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1. Introduction

The need for research in athletic performance is increasing due to the development of technology and science in sports. There are many factors determining success in sports. Training types and diversity, genetic factors, epigenetics, diet patterns, motivation, equipment and other environmental factors are some of them. Mental and physical factors make up the overall athletic performance and increases with the progress in environmental factors such as training and nutrition. Having information about the genetic structure in athletes is important to have certain functions and personal training programs in both individual and team sports.

Cycling activities that requires long-term endurance and strength. Muscle volume and muscle fiber types affect many biomechanical variables like pedal force in cyclists. Therefore, it effects the cycling performance. There are also many different types of cycling and the performance in these types varies accordingly.

Dopamine is an important neurotransmitter that significantly effects the dopaminergic system and physical activities that we face in daily routine. Apart from these activities, it affects exercise and athletic performance to a great extent. It provides regulation of neurological functions and communication between neurons, that are crucial in motor activities. Factors affecting the central nervous system are directly related to psychology. Therefore, dopamine affects mentally athletic performance (Ulucan et al.,2014).

There are five different types of dopamine receptors; DRD1, DRD2, DRD3, DRD4 and DRD5 (Gingrich et al., 1993). DRD2 gene that encoding dopamine metabolism is localized at 11q22-q23. Some polymorphisms have been identified in the gene, one of which is rs1800497 (G/A transition). The G allele in the DRD2 rs1800497 polymorphism is considered wild type and related with the high numbers of the receptor moleculed on the cell membrane. The A allele is considered to be polymorphic allele and studies to date have linked the A allele with lower receptor numbers and lower dopamine levels (Turner et al., 1992). Dopaminergic neurons and regions in the brain also appear to play a role in addiction and with some neuropsychiatric disorders (Pohjalainen et al., 1998).

In the present study, we aimed to examine the distribution of receptor 2 (DRD2) rs1800497 in cyclists, and comparee the results with the sedentary individuals. This report, according to the best of our knowledge, is the first which is carried out in cyclists.

2. Materials and Methods

The protocol used in the present study was approved by the Üsküdar University Ethics Committee and was performed in accordance with the principles of the Declaration of Helsinki II. All participants signed consent forms containing all the information such as the study protocol, results and evaluation of the results.

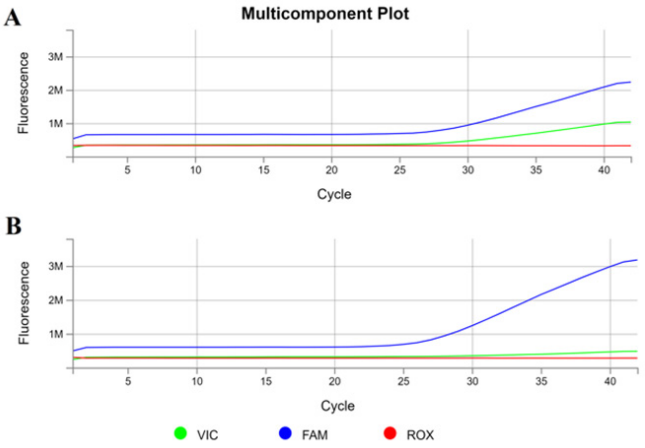
2.1. Study subjects

19 cyclist were participated in our study. 52 sedentary individuals also participated as a control group. All the volunteers had no transmitted genetic anomalies. The study protocol was approved by Üsküdar University Ethical Committee and was in line with the principles of the Declaration of Helsinki II. Before the study, all participants signed consent forms containing all the information such as the study protocol, results and evaluation of the results.

2.2. Genotyping:

DNA isolations from buccal epithelial cells of the athletes were carried by the commercially obtained PureLink DNA isolation kit (Invitrogen, Van Allen Way, Carlsbad, CA, USA). Genotyping of the DRD2 rs1800497 polymorphism was performed using quantitative real- time PCR (StepOnePlus, Thermo Fisher Scientific, Inc.), using a TaqMan Genotyping assay (cat. no. 4362691; Thermo Fisher Scientific, Inc.). Manufacturer's protocols were followed for the genotyping processes. PCR conditions were 60 °C for 30 s and 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C for and 1 min at 60 °C. Finally, 60 °C for 30 s was applied for post PCR reading. The fluorescent signal was detected at the prePCR, amplification at the end of each cycle, and postPCR reading steps. G and A alleles were determined using VIC and FAM primers, respectively (Fig. 1). The sequences of the TaqMan Probe used for genotyping are listed in Table 1.

Figure 1. Quantitative PCR amplification of the AG genotype and GG genotype of *DRD2* rs1800497 polymorphism. FAM indicates the G allele (blue curve), whereas VIC (green curve) indicates the A allele. The blue and green curves indicate the heterozygous genotype of AG (A) whereas the single blue curve indicates the homozygous genotype of GG (B).



2.3. Statistical analysis

Genotype distribution and allele frequencies between groups of athletes and controls were compared by χ^2 test, using the SPSS (version 18.0 for Windows, SPSS, Chicago, IL, USA) program. $p<0.05$ value was accepted as statistically significant.

Figure 2. Sequences of the TaqMan probe used for genotyping DRD2 rs1800497 polymorphism.

qPCR	Sequence, 5'-3'
VIC/FAM	CACAGCCATCCTCAAAGTGCTGGTC**[A/G]**AGGCAGGCGCC-CAGCTGGACGTCCA

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3. Results

In our cohort, 13 (68%) of 19 players had GG and 6 (32%) of them had AG genotypes. No AA genotype was detected. When allele distributions were examined, it was observed that the percentage of A allele was 16% and the G allele was 84%. In the control group (n = 52), 9 individuals had AA, 18 individuals had AG and 25 individuals had GG genotype. A allele was counted as 36 (35%) and G allele as 68 (35%). The genotype and allele number distributions of the athletes are summarized in Table 1.

Table 1. Genotypic and allelic distribution of the DRD2 rs1800497 polymorphism in the study cohort.

	Genotype			p Value	Allelic Frequency		p Value
	AA	AG	GG		A	G	
Cyclist (19)	-	6	13	0,1107	6	32	0,0295
Percentage	0%	32%	68%		16%	84%	
Control (52)	9	18	25		36	68	
Percentage	17%	35%	48%		35%	65%	

Significance was assessed at the $p < 0.05$ level. Comparison with the control group was made using the x^2 test.

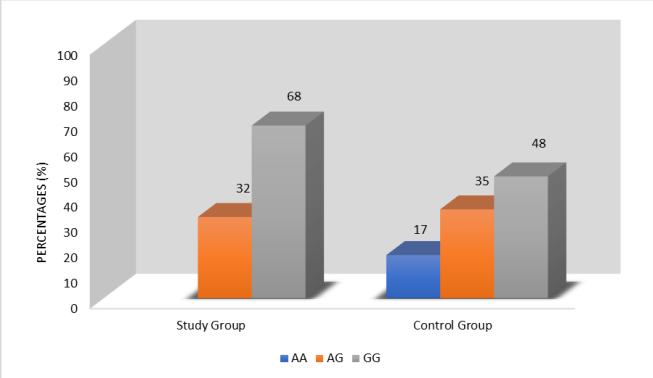

Figure 3. Percentage of the genotype distributions of DRD2 rs1800497 polymorphism.

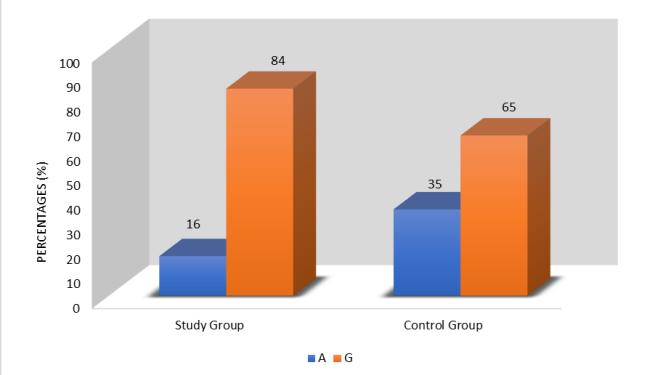

Figure 4. Percentage of the allelic distributions of DRD2 rs1800497 polymorphism.

4. Discussion and Conclusion

Variations in the DRD2 gene can affect athletic performance. Low dopamine level due to DRD2 polymorphisms may be a parameter that prevents success in sports. Low dopamine levels are associated with neurological diseases and hyperactivity as well as sports performance. Apart from that, high dopamine levels are also associated with abnormal brain function (Ndamanisha et al., 2009). There are studies in which polymorphism is associated with sports addiction (Munafo et al., 2007).

Dopamine shows its biological effects by binding to its receptors (DRDs). DRD1, DRD2, DRD3, DRD4 and DRD5 are the known five different dopamine receptors. Studies shows that DRD2 receptors in neuronal membranes are higher in numbers who have the GG genotypes than in the AA genotypes (Grandy et al. 1989).

In our cohort, AG and GG genotypes were detected as 6 (32%) and 13 (68%), respectively. A and G allele numbers and percentages were respectively 6 (16%) and 32 (84%). There were no statistically significant difference between athletes and controls.

There are few studies investigating the relationship between the DRD2 rs1800497 polymorphism and sports performance. Yüksel et al. (2017) investigated the rs1800497 polymorphism in volleyball players. In their study, all the genotypes of all players were found as GG. Before, a allele has been poorly associated with addiction in sports. Özcan et al. (2018) anaylsed DRD2 rs1800497 polymorphism in sprinter and endurance athletes and reported that GG genotype and G allele were superior in the study cohort. Abe et al. (2017) examined the COMT, DRD2 and DRD3 polymorphisms which have effect on dopaminergic nerve functions, and reported that AA genotype of DRD2 rs1800497 polymorphism was lower when compared to AG and GG genotypes.

In our study with 19 cyclists, the GG genotype was found higher than the AG genotype. There were no athletes with the AA genotype. At the same time, when we compare the G allele with the A allele, G allele is higher than the A allele. The results of our study were in agreement with the previous studies. Our results are similar to the findings of previous studies. The DRD2 A Allele has been found to be associated with addiction. Studies have shown that the same allele is associated with sports addiction and athletic performance. Our study will contribute to the literature and support other studies in this field. It can help prevent early psychological disorders encountered in athletes.

Patient informed consent: Informed consent was obtained.

Ethics committee approval: The protocol used in the present study was approved by the Üsküdar University Ethics Committee and was performed in accordance with the principles of the Declaration of Helsinki II. All participants signed consent forms containing all the information such as the study protocol, results and evaluation of the results.

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Çisem Şılar (20%): data collection, formation of the article
Esra Karagöz (20%): data collection, formation of the article
Tolga Polat (10%): data collection, statistics 10%
Özlem Özge Yılmaz (10%): laboratory assistance
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Beste Tacal Aslan(10%): laboratory assistance
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Tuğba Kaman(5%): data collection

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