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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Hydrocortisone and Vitamin B12 Protect SHSY-5Y Cells Against Glutamate Excitotoxicity by Altering VIP And GAL Levels [*]

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Abstract: Prolonged elevation of extracellular glutamate levels triggers intracellular events, increases glutamate excitotoxicity, and activates apoptotic pathways, causing Alzheimer's disease (AD). The literature has reported that vitamin B12 exhibits anti-inflammatory and anti-apoptotic activities in various diseases. Hydrocortisone (HC) therapy also substantially inhibits microglia and astrocyte hyperactivation, minimizing pro-inflammatory cytokines and reducing neuroinflammation. That is why, our study aimed to evaluate the therapeutic effects of HC and B12 combination on oxidative stress and VIP and GAL levels in an in vitro Alzheimer's model. To create the Alzheimer's model, the neuroblastoma cell line (SH-SY5Y) was cultured. Then, all cells except the control group were treated with different doses of HC and B12 combination for 24 hours by applying Glutamate (10^{-5} mM) to create excitotoxicity. The results were evaluated using MTT and ELISA tests. When the results were examined, it was determined that exceptionally high-dose combination groups showed protective activity against glutamate excitotoxicity. HC+B12 25 µg/ml groups observed the most statistically significant results. According to our results, oxidative stress decreased in the HC+B12 25 µg/ml group, and cell viability increased. Important changes were also observed in Vasoactive Peptide (VIP) and Galanin (GAL) levels in correlation with other analyses obtained. This study is the first to report the potential of vitamin B12 combined with hydrocortisone to prevent oxidative stress and glutamate excitotoxicity in SHSY-5Y cells. It provides a basis for further investigating its clinical application in neurodegenerative diseases.

Keywords: Excitotoxicity, GAL, glutamate, VIP.

Hidrokortizon ve B12 Vitamini VIP ve GAL Düzeylerini Değiştirerek SHSY-5Y Hücrelerini Glutamat Eksotoksisitesine Karşı Korur

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Öz: Hücre dışı glutamat seviyelerinin uzun süreli yükselmesi hücre içi olayları tetikler, glutamat ekzositozunu artırır ve apoptotik yolları aktive ederek Alzheimer hastalığına neden olur. Literatürde B12 vitamininin çeşitli hastalıklarda anti-inflamatuar ve anti-apoptotik aktiviteler gösterdiği bildirilmiştir. Hidrokortizon tedavisi ayrıca mikroglia ve astrosit hiperaktivasyonunu önemli ölçüde inhibe ederek pro-inflamatuar sitokinleri en aza indirir ve nöroinflamasyonu azaltır. Bu nedenle, çalışmamız, hidrokortizon ve B12 kombinasyonunun in vitro Alzheimer modelinde oksidatif stres ve vazoaktif intestinal peptit ve galanin seviyeleri üzerindeki terapötik etkilerini değerlendirmeyi amaçlamıştır. Alzheimer modelini oluşturmak için nöroblastoma hücre hattı (SH-SY5Y) kültürlendi. Daha sonra, kontrol grubu hariç tüm hücreler, ekzotoksisite oluşturmak için Glutamat (10-5 mM) uygulanarak 24 saat boyunca farklı dozlarda HC ve B12 kombinasyonu ile tedavi edildi. Sonuçlar MTT ve ELISA testleri kullanılarak değerlendirildi. Sonuçlar incelendiğinde, istisnai olarak yüksek doz kombinasyon gruplarının glutamat ekzotoksisitesine karşı koruyucu aktivite gösterdiği belirlendi. HC+B12 25 µg/ml grupları istatistiksel olarak en anlamlı sonuç olarak gözlemledi. Sonuçlarımıza göre, HC+B12 25 µg/ml grubunda oksidatif stres azaldı ve hücre canlılığı arttı. Elde edilen diğer analizlerle korelasyon içinde vazoaktif intestinal peptit ve galanin seviyelerinde de önemli değişiklikler gözlendi. Bu çalışma, SHSY-5Y hücrelerinde oksidatif stresi ve glutamat eksitotoksisitesini önlemek için hidrokortizonla kombine B12 vitamininin potansiyelini bildiren ilk çalışmadır. Nörodejeneratif hastalıklarda klinik uygulamasının daha fazla araştırılması için bir temel sağlar.

Anahtar kelimeler: Eksitotoksisite, GAL, glutamat, VIP.

INTRODUCTION

Alzheimer's disease (AD) is defined as a fatal degenerative dementia that initially presents mild memory impairment and then progresses to a complete loss of mental and physical abilities (Smith, 1998). AD is the most common form of dementia and currently affects more than 35 million people worldwide. Age is the greatest risk factor for AD and, as global life expectancy increases, the number of people living with AD is projected to reach 87 million by 2050 (Tzioras et al., 2023). While β-amyloid plays a role in the etiology of AD, the mechanisms responsible for the regional specificity of neuronal loss have not yet been resolved. Disorders in the neurotransmitter system, called endogenous chemicals, are thought to play a role in the pathogenesis of these neurodegenerative diseases (Cicek et al., 2021). It is known that neurotransmitters have essential roles in psychological functions such as learning, memory, neuronal growth, survival, and plasticity (Hyman, 2005; Kanazawa, 1984; Shen, 2010). Neurotransmitters are critical for cognitive functions such as learning, memory, and neuronal survival. Among these neurotransmitters, glutamate is the most prominent one in the brain. Glutamate is necessary for both healthy brain development and function. Destruction of glutamate neurotransmission, nonetheless, has serious consequences. Prolonged elevation of extracellular glutamate levels triggers intracellular events, increasing glutamate exocytosis and activating apoptotic pathways. Glutamate excitotoxicity is, therefore, implicated in several brain disorders, including epilepsy, amyotrophic lateral sclerosis, Huntington's disease, Alzheimer's disease, ischemia, and trauma (Andersen et al., 2021; Walton & Dodd, 2007).

Methylcobalamin, a form of vitamin B12, is thought to exhibit anti-inflammatory and anti-apoptotic activities in various diseases. It improves neuronal transmission by supporting axonal regeneration and myelin formation and repairing damaged nerve tissue (Akbay, 2019; Hisatake et al., 2007). It demonstrates a neuroprotective effect via regulating the detoxification of reactive oxygen compounds, particularly superoxide, and modulating cytokines and growth factors (Okamoto et al., 2014). Various studies have found that it prevents oxidative damage and protects retinal neurons against glutamate excitotoxicity (Akaike et al., 1993; Kikuchi et al., 1997; van de Lagemaat et al., 2019).

The adrenal glands produce hydrocortisone (HC), a crucial glucocorticoid hormone essential for regulating homeostasis, the circadian rhythm, and the stress mechanism. It is generally controlled via the hypothalamic-pituitary-adrenal (HPA) axis. So chronic stress and aging can disrupt this regulation. Animal models have investigated stress's physiological and neuroendocrine effects, revealing social factors' role in HC dynamics. Furthermore, chronic stress has been linked to the progression of neurodegenerative diseases such as Alzheimer's and Parkinson's, major depressive disorder, and chronic pain (Knezevic, Nenic, Milanovic, & Knezevic, 2023). Studies show that vitamin B12 increases the anti-inflammatory activity of hydrocortisone (Hadnagy, Horváth, Elekes, Puia, & Nicoara, 1964; Hashemi, Abbasi, & Masoudpour, 2023). However, the underlying mechanism has not been fully explained. We believe that vitamin B-12, which has been found to increase the anti-inflammatory activity of hydrocortisone in the literature, will also be effective by eliminating inflammation in neuronal cells. Based on the current literature, we hypothesize that the combination of HC and vitamin B12 would have a neuroprotective effect by attenuating glutamate excitotoxicity and reducing oxidative stress.

Therefore, our study created an Alzheimer's model by exposing SH-SY5Y cells to glutamate excitotoxicity and aimed to investigate the neuroprotective effects of HC and Vitamin B12. For this purpose, after Glutamate (10^{-5} mM) exposure, cells were treated with different doses of HC and vitamin B12. The treatment efficacy was investigated by applying MTT (2.5-diphenyltetrazolium bromide), TAS (Total antioxidant level), TOS (total oxidant level), GR (glutathione reductase), GAL (galanin), and VIP (vasoactive intestinal peptide) tests.

MATERIAL AND METHOD

Cell Culture: The SH-SY5Y (ATCC: CRL-2266) cell line was acquired from the Department of Medical Pharmacology at Bilecik Şeyh Edebali University (Bilecik, Turkey). Cells were grown and developed in Dulbecco's Modified Eagle Medium, which was enhanced with F12 medium (Euroclone, Milan, Italy) containing 0.1% Penicillin/Streptomycin and 10% Fetal Bovine Serum (Euroclone, Milan, Italy). The cells were cultured at 37 °C in a 5% CO_2 environment (Nalci, Nadaroglu, Genc, Hacimuftuoglu, & Alayli, 2020)

Cellular Therapy: Afterward, with 85% confluency, the cells underwent passage and were seeded into 96-well plates. Initially, investigations were performed across a broad dosage spectrum (1-500 µg/ml) to ascertain the effective dose, and our investigation was designed subsequent to this determination.They were then subjected to glutamate (10^{-5} mM) (Avci et al., 2022). After exposure, the cells were placed in the incubator, and the experimental groups were determined as follows;

1. Negative control (SH-SY5Y)
2. Positive control (SH-SY5Y+ Glutamate)
3. B12 (10, 12.5, and 25 µg/ml)
4. HC (12.5, 25, and 50 µg/ml)
5. HC+B12 (12.5 and 25 µg/ml doses)

MTT Assay: A commercial kit from Sigma-Aldrich (USA) was used to perform the MTT test. In conclusion, 10 µL of MTT (5 mg/ml) was added to each well, and the mixture was incubated for 4 hours at 37 °C with 5% CO2. 100 µL DMSO was then applied to each well to dissolve the formazan crystals. The optical density (OD), measured at 570 nm with a spectrophotometer, was used for determining the percentage of viable cells. The OD of the control group was set as 100, and the viability rate of the other groups was calculated using the formula below (Genc et al., 2023; Taghizadehghalehjoughi, Sezen, Hacimuftuoglu, & Güllüce, 2019).

Viability (%) = (group OD/control OD)×100

Total Antioxidant Capacity (TAC) Assay: The TAS value was measured calorimetrically using the Total Antioxidant Status Kit as described in our previous studies (RL0017, Rel Assay Diagnostics kit). The TAC value obtained was calculated as mmol Trolox Equiv./L according to the formula below (Nalci et al., 2020).

A2-A1= ΔAbsorbance (standard, sample or H_2O)

$$TAC = \frac{H2O\Delta Abs - Sample\Delta Abs}{H2O\Delta Abs - Standard\Delta Abs}$$

Total Oxidant Level (TOS) Assay: The TOS assay was evaluated calorimetrically using the Total Oxidant Status Kit as described in our previous studies (RL0024, Rel Assay Diagnostics kit). The TOS value obtained was calculated as mmol H_2O_2 Equiv./L according to the formula below (Taghizadehghalehjoughi et al., 2019).

A2–A1 = ΔAbsorbance (standard or sample).2–A1 = ΔAbsorbans

$$TOS = \frac{Sample\ \Delta Abs}{Standard\ \Delta Abs} \times 10$$

Biochemical Analysis: VIP (BT Lab, Shanghai, China), NO (BT Lab, Shanghai, China), GR (BT Lab, Shanghai, China) and Galanin (BT Lab, Shanghai, China) levels were evaluated using an ELISA kit. The optical densities of each sample were measured at 450 nm.

Statistical Analysis: Statistical assessments throughout groups were calculated using the one-way ANOVA method. For statistical analysis, SPSS 20 software was used for all computations, and a difference that was determined as of statistical importance in all tests was $p < 0.05$. The standard deviation and mean of the results (mean ± SD) are reported.

RESULTS

MTT Assay Results: The neuroprotective effects of HC (12.5, 25, 50 µg/ml) and B12 (10, 12.5, 25 µg/ml) on cells exposed to Glutamate 10^{-5} mM were determined after 24 hours using the MTT method (Figure 1). A control group was also used to compare with the glutamate control group. After 24 hours of exposure, the group to which glutamate was applied at a toxic dose (Glutamate 10^{-5} mM) showed significantly reduced cell viability compared to the control group ($p<0.01$). On the other hand, the treatment groups (B12 25 µg/ml, HC 12.5 + B12 10 µg/ml, HC 12.5 + B12 25 µg/ml) provided a significant increase in viability compared to the glutamate group ($p<0.05$). HC 25 + B12 10 µg/ml and HC 25+ B12 25 µg/ml groups almost approached the control group in terms of viability ($p<0.01$).

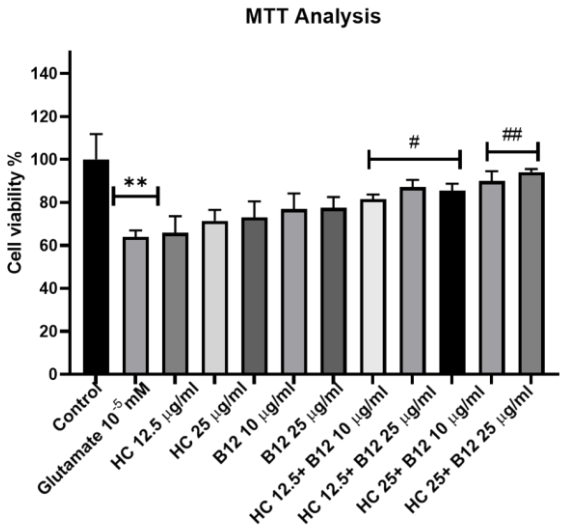

Figure 1. Cell viability of SH-SY5Y cells at 24 hours. When the glutamate group was compared with the control group, the significance level was determined as ** ($P < 0.01$). When the B12 25 µg/ml, HC 12.5 + B12 10 µg/ml, HC 12.5 + B12 25 µg/ml groups were compared with the glutamate group, the significance level was determined as * ($P < 0.05$). When the HC 25 + B12 10 µg/ml, HC 25+ B12 25 µg/ml groups were compared with the glutamate group, the significance level was determined as ** ($p < 0.01$).

TAC Results: TAC levels after 24h incubation are shown in Figure 2. TAC level in the glutamate group decreased significantly (approximately 28%) compared to the control group ($p<0.01$). When the treatment groups (B12 25 µg/ml, HC 12.5 + B12 10 µg/ml, HC 12.5 + B12 25 µg/ml) were compared with the glutamate group, TAC level increased by approximately 10% ($P <0.05$). HC 25 + B12 10 µg/ml, HC 25+ B12 25 µg/ml groups, when compared with the glutamate group, TAC level increased by approximately 25% and approached the control ($p<0.01$).

TOS Results: TOS levels after 24 h of incubation are shown in Figure 3. TOS level in the glutamate group increased significantly (approximately 70%) compared to the control group ($p<0.001$). When the treatment groups (B12 25 µg/ml, HC 12.5 + B12 10 µg/ml, HC 12.5 + B12 25 µg/ml) were compared with the glutamate group, TOS level decreased by approximately 30% ($P < 0.05$). When the HC 12.5 + B12 10 µg/ml, HC 12.5+ B12 25 µg/ml groups were compared with the glutamate group, TOS level decreased by approximately 50% and approached the control ($p<0.01$).

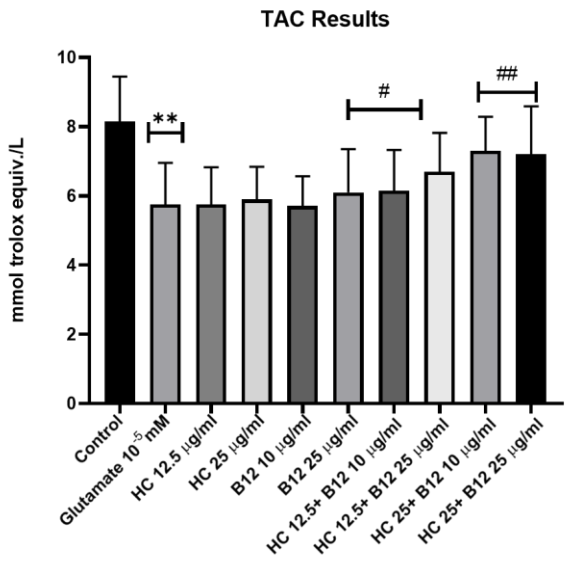

Figure 2. TAC levels of control, glutamate, and treatment groups. The Glutamate group is significant compared to the control group, and treatment groups are significant compared to the glutamate group (** p<0.01, # p<0.05, ## p<0.01).

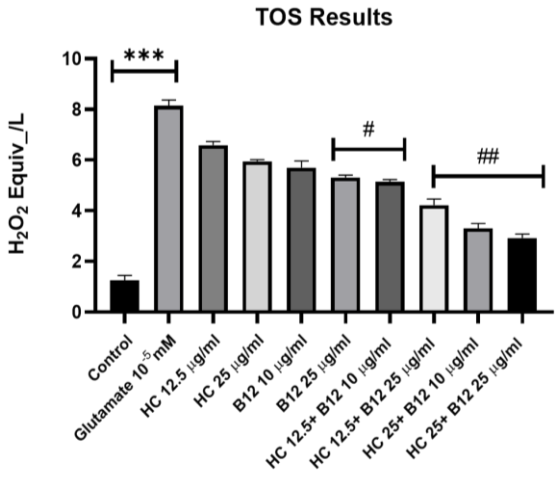

Figure 3. TOS analyses of control, glutamate and treatment groups. Glutamate group is significant compared to control group, and treatment groups are significant compared to glutamate group (*** p<0.001, # p<0.05, ## p<0.01).

ELISA Results: After 24 hours of incubation, VIP, GR and GAL tests were applied to the cell groups. In all tests, the glutamate group showed a significant decrease compared to the control group (**p<0.01). In VIP and GR measurements, the treatment group (HC 12.5 + B12 10 µg/ml, HC 12.5 + B12 25 µg/ml) increased compared to the glutamate group (# p<0.05). HC 12.5 + B12 10 µg/ml, HC 12.5+ B12 25 µg/ml groups showed a significant increase compared to the glutamate group (## p<0.01). In GAL results, the treatment group (B12 25 µg/ml, HC 12.5 + B12 10 µg/ml) increased compared to the glutamate group (# p<0.05). HC 12.5 + B12 10 µg/ml and HC 12.5+ B12 25 µg/ml groups showed a significant increase compared to the glutamate group (## p<0.01).

DISCUSSION

In neurodegenerative disease groups, the human neuroblastoma cell line SH-SY5Y is frequently preferred due to its human origin, catecholaminergic neuronal properties, and ease of maintenance (Xicoy et al., 2017). Applying certain chemical substances to the SH-SY5Y cell line induces different neuronal phenotypes and biochemical changes, which provides more potential for studying neurotoxicity and neuroprotection on the cells (Arık, 2022; Xie et al., 2010). We hypothesize that the combination of HC and vitamin B12 will reduce oxidative stress, protect against glutamate excitotoxicity, and regulate neuropeptide levels (VIP and GAL) in SH-SY5Y cells exposed to glutamate. For this reason, MTT, TAS, TOS, VIP,GAL and GR analyses were carried out in order to determine the elimination of oxidative damage that will occur in our study.

Glutamate is a primary excitatory neurotransmitter. It plays an essential physiological role in neurotransmission, differentiation, and synaptic plasticity (Andersen et al., 2021). Glutamate is a vital neurotoxin and one of the most important excitatory neurotransmitters in the central nervous system (Özgür et al., 2024). The neurotoxic effect of glutamate has been associated with many neurological and psychiatric diseases. Under normal conditions, glutamate is responsible for cell survival, migration, and differentiation during brain development, while excessive glutamate leads to neuronal cell death via oxidative stress or excitotoxicity (Choi, 1988; Kostic et al., 2017)

Excessive glutamate release, which results from prolonged activation of glutamate receptors, leads to calcium overload, which plays a vital role in neurodegeneration (Jia et al., 2016). In this study, excitotoxicity was induced in the Alzheimer's disease model by applying high concentrations of glutamate (10–5 mM). After creating toxicity, B12 (10 µg/ml and 25 µg/ml) and HC (12.5 µg/ml and 25 µg/ml) were applied in combination, and cytotoxicity tests were performed. The results given in the MTT test determined that cell viability decreased in cells exposed to glutamate, and this situation showed that excitotoxicity occurred. With the application of the B12 and HC combination, there were increases in the viability level, and the cell viability level in the combination group was found to be close to the control group. These findings show that the B12 and HC combination influences increase the chance of cell survival by reducing the toxic effects of glutamate applied at high concentrations. B12 and HC exhibit a protective effect against glutamate-induced excitotoxicity, perhaps due to their antioxidant and anti-inflammatory effects.

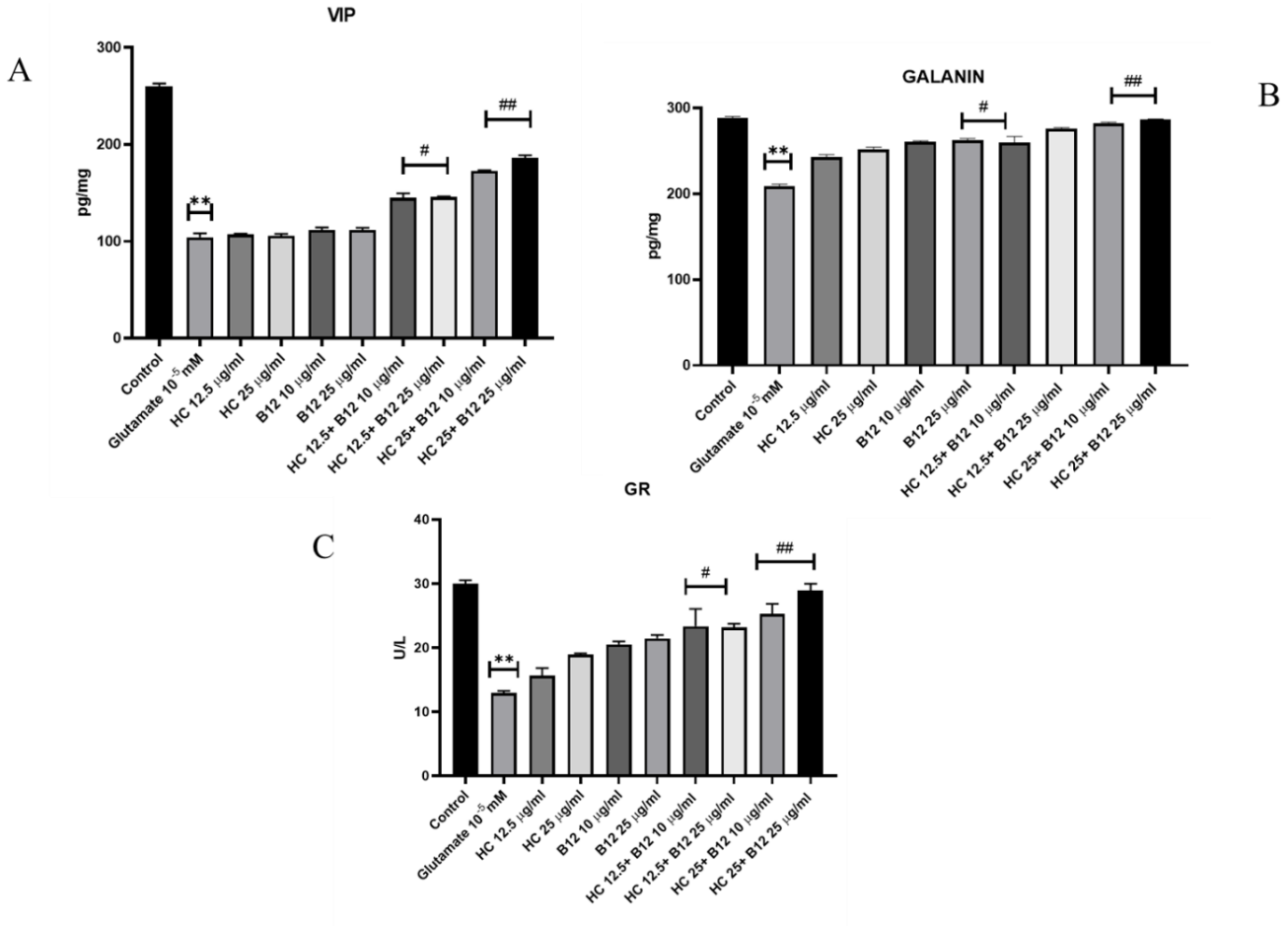

Figure 4. ELISA test results. A) VIP analysis results, B) GAL analysis results, C) GR analysis results.

Glucocorticoids (GCs) are extensively utilized as anti-inflammatory medicines, and their combination with other pharmaceuticals has shown potential benefits and neuroprotective effects. Furthermore, they have demonstrated significant efficacy in alleviating the degenerative characteristics of Alzheimer's disease and performing protective and regulatory functions (Bracken et al., 1990; Vandewalle et at., 2018). Hydrocortisone (HC) is notable among natural and endogenous glucocorticoids for its powerful anti-inflammatory properties, providing distinct pharmacological advantages in autoimmune diseases, septic shock, and allergy disorders (Hui et al., 2020). HC provides neuroprotection by inhibiting cytotoxic agents generated by active microglia and suppressing astrocyte growth.

Further, B12 may indirectly enhance ROS scavenging through the maintenance of glutathione, possibly involving a complex network of processes that remains incompletely understood (Manzanares & Hardy, 2010). Vitamin B12 may also influence immunological responses: a correlational investigation in Alzheimer's patients revealed significantly elevated basal interleukin-6 production in those with deficient B12 levels compared to those with adequate B12 levels (Politis et al., 2010). Studies with B12-deficient rats and severely B12-deficient patients also showed increased tumour necrosis factor alpha and decreased epidermal growth factor levels compared to controls (Birch et al., 2009) The results indicate that B12 may safeguard against oxidative stress generated by low-grade inflammation through the modulation of cytokine and growth factor production (Birch et al., 2009). B12 is hypothesized to accomplish this by altering the activity of transcription factors, including nuclear factor-κB (Birch et al., 2009; Politis et al., 2010). The accumulation of reactive oxygen species, which increases with glutamate and causes toxicity in the cell, triggers oxidative stress, mitochondrial damage, decreased energy production, and inhibition of other metabolic functions, leading to cell death. In our study, while TAS levels decreased and TOS increased in the group treated with high glutamate concentrations, TAS levels increased and TOS decreased, approaching the control group when combined drugs were administered at therapeutic doses. These results show the effectiveness of combined drug treatment in reducing glutamate-induced oxidative damage in the cell.

GR maintains the reduced glutathione/oxidized glutathione ratio in the cell environment by reducing

glutathione (Temel et al., 2017). Oxidized glutathione is reduced by glutathione reductase, ensuring the continuity of the antioxidant effect. The ratio of the reduced form to the oxidized form of glutathione (GSH/GSSG), widely found in all compartments in the cell, is an essential parameter for reflecting the redox status (Masella et al., 2005). In a study conducted by Yerer et al., blood samples were taken from a healthy control group, and individuals with different AD and erythrocyte deformability were determined. Catalase, glutathione peroxidase, and plasma nitric oxide levels were determined spectrophotically. As a result of the experiment, plasma nitric oxide and catalase activities were significantly higher, and glutathione peroxidase activity was significantly lower in patients with severe AD compared to the control group (Yerer et al., 2012). Targeting the studies in the literature, we aimed to measure toxicity and evaluate the effectiveness of the treatment groups by looking at the GR enzyme level in our experiment. GR increased almost 2-fold in cells exposed to glutamate excitotoxicity compared to the control group, indicating that the cell increased its GR requirement for high glutamate concentration. In the treatment groups, this rate approaching control suggests that the treatment inhibits glutamate.

Vasoactive intestinal peptide (VIP) is one of the essential regulatory peptides in the mammalian brain. Studies have shown that it exhibits hormone-like functions in the central and peripheral nervous systems, neurotransmitter, and endocrine cells. Studies have pointed out the importance of VIP, which acts as a mediator or regulator of some essential functions within the cell. VIP is an important factor in brain activity and neuroendocrine functions. VIP fulfills its function through receptor-mediated systems and activates signal transduction pathways, including cAMP. It can act as a neurotransmitter, neuromodulator, and secretagogue (Gozes & Brenneman, 1989). Our study examined VIP receptor activity using the ELISA test, and the results obtained were compared statistically. While a low amount of degeneration was observed in VIP receptors with glutamate, this value approached the control group in the treatment groups.

Galanin (GAL) and its GAL receptors (GALR) are overexpressed in AD's limbic brain regions associated with cognition. Since GAL blocks cholinergic transmission and restricts long-term potentiation in the hippocampus, GAL overexpression may worsen the clinical features of AD. In contrast, GAL expression increases in response to neuronal injury, and galaninergic hyperinnervation prevents the decreased production of protein phosphatase one subtype mRNAs in cholinergic basal forebrain neurons in AD (Counts et al., 2003). In an experiment, the group aimed to investigate their potential effects on AD and examined several peptides, including the galanin peptide, in samples taken from 90 individuals, 45 AD, and 45 healthy individuals. The experiment results showed that galanin was at higher levels in the AD group compared to the healthy group (Gul et al., 2012). In some animal studies, it has been reported that over expression of galanin prevents the secretion of acetylcholine (ACh), which is necessary for cholinergic transmission in some brain regions associated with AD and restricts long-term potentiation (Collard et al., 2022; Gul et al., 2021). In our study, which is consistent with the literature, glutamate excitotoxicity decreased galanin levels in cells. With the treatments we applied, there was a change in galanin levels, and they approached the control group.

CONCLUSION

In our study, HC 12.5 + B12 10 µg/ml, HC 12.5 + B12 25 µg/ml treatment doses showed a therapeutic efficacy against glutamate toxicity induced in SH-SY5Y cell line. In conclusion, in addition to the separate use of HC and B12, their combinations can also be used for therapeutic purposes in glutamate toxicity-induced AD models.

Limitations of In Vitro Models: Although the SH-SY5Y cell line is a recognized model for Alzheimer's disease research, it may not adequately represent the intricacies of neurodegeneration seen in human brains, especially with glial cell participation and the blood-brain barrier.

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