Nanosilibinin ameliorates anxiety, learning impairment and Wnt-β catenin related genes expression deficits in zebrafish model of Autism Spectrum Disorder

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Abstract

purpose

The present study evaluated the effect of Nanosilibinin (as an inhibitor of Wnt signaling pathway), on neurobehavioral and molecular deficits in Valproic acid zebrafish model of Autism Spectrum Disorder.

Methods

Zebrafish embryos were exposed to Valproic acid (1μM) and Nanosilibinin (100,200 and 500 μM) for 96 h, then survival rate, inattentive and anxiety behavior and the expression of CHD8, CTNNB, GSK3β, LRP6, TNFα, IL1β and BDNF genes were assessed 7 days post fertilization (dpf).

Results

The results indicated that except 500 μM, other concentrations of Nanosilibinin didn't have any adverse effect on survival, hatching and morphological development when were used with Valproic acid at the same time. In addition, 100 and 200 μM of Nanosilibinin could ameliorate the anxiety and learning deficit in zebrafish larvae. Real-time analysis revealed that Nanosilibinin prevented raising the expression of a number of genes associated with autism such as CHD8, CTNNB, GSK3 β, LRP6, TNFα, IL1β and BDNF after exposure to Valproic acid.

Conclusion

In conclusion, Nanosilibinin treatment for the first 96 h of life showed therapeutic effect on an ASD-like phenotype by decreasing anxiety and learning deficits and reduction in expression of number ASD related genes.

Introduction

Autism Spectrum Disorder (ASD) is a group of neurodevelopmental disabilities that has an early onset. The core symptoms of ASD include impairment of social interaction, repetitive behaviors and restricted interest. To date, no effective pharmacological treatment is known to cure the core symptoms of this disorder (Belmonte et al., 2004; Frith & Happé, 2005; Lord, Elsabbagh, Baird, & Veenstra-Vanderweele, 2018; Maenner, Shaw, & Baio, 2020).

Disturbance in the β-catenin wnt (wingless) signaling pathway is considered as one of the mechanisms included in pathology of ASD. Wnt signaling pathway is highly conserved from lower to higher organisms and plays important and various roles in neurodevelopment. Because of the critical role of wnt signaling pathway in synaptogenesis and neurodevelopment, disruption in this pathway has been reported in ASD. Genetic evidence suggests that mutation in the gene of the main components or modulators of canonical β- catenin signaling pathway may involve in ASD pathogenesis (Mulligan & Cheyette, 2016; Okerlund & Cheyette, 2011; Qin, Dai, & Yin, 2016).

Previous studies reported that Valproic acid (VPA) is an environmental factor for ASD. This drug is used in treatment of epilepsy, bipolar disorder and migraine headaches (Horder et al., 2018; Jentink et al., 2010; José, Emilio, Maria, & Glauce, 2012). Prenatal exposure to VPA may increase the risk of autism. Several studies using different animal models have been shown that embryonic exposure to VPA can increase the risk factor for ASD (Chen et al., 2018; Kim et al., 2014; Mabunga, Gonzales, Kim, Kim, & Shin, 2015; Moore et al., 2000; Narita et al., 2010; Nicolini & Fahnestock, 2018; Piazza et al., 2003). Studies have shown that VPA can lead to overactivation of the canonical β- catenin signaling pathway in VPA-induced ASD animal models. It has been reported that Inhibitor of canonical β- catenin signaling pathway can improve the symptoms of ASD in these animal models (Qin & Dai, 2015; Qin et al., 2016; Zhang et al., 2012; Zhang et al., 2015).

Silibinin is a natural flavonoid and main component of silimaryin that is isolated from silybum marianum. Silibinin is widely used, as a traditional herbal, for its strong antihepatotoxic activity against any kind of human liver disorder. It also has anti-cancer, anti-inflammatory and anti-oxidant properties(Deep & Agarwal, 2010; Wing Ying Cheung, Gibbons, Wayne Johnson, & Lawrence Nicol, 2010). Recent studies have shown that chemotherapeutic effects of silibinin is associated with inhibition of canonical β- catenin signaling pathway (Lu et al., 2012; Ramakrishnan et al., 2009; Vaid, Prasad, Sun, & Katiyar, 2011).

Recently, both zebrafish larvae and adult are used as a powerful animal model of ASD in research studies. Zebrafish are genetically tractable, transparent during development and amenable to high-throughput phenotyping, making it a valuable experimental model. Zebrafish has also high physiological and genetical similarities with human (Gerlai, 2010; Hill, Teraoka, Heideman, & Peterson, 2005; Kalueff et al., 2013; Kalueff, Stewart, & Gerlai, 2014; Stewart et al., 2013; Stewart, Nguyen, Wong, Poudel, & Kalueff, 2014).

In the present study, VPA was used to induce autism in zebrafish larvae. Then, the effect of nanosilibinin (NS), (as an inhibitor of Wnt signaling pathway), on neurobehavioral parameters was evaluated. In addition, the expression of ASD related genes in the brain of zebrafish larvae was investigated.

Materials And Methods

Fish husbandry and embryo collection:

Adult wild-type zebrafish (Danio rerio) were raised and maintained at standard laboratory conditions of 28±1 on a 12:12 (dark:light) cycle. Zebrafish embryo were collected by natural spawning. Mature male and female zebrafishe with a sex ratio of 2:1 were placed in reproductive tank and they were separated by a transparent barrier overnight. In the following morning, the barrier was removed for breeding. Embryos were collected in 1 hours of spawning and were incubated at 28±1 in blue egg medium (water+methylene blue). Fertilized embryo with the same developmental stage were utilized for research. All animal care and experimental procedures were done observing the National Institute of Health

guidelines on animal care and use and were approved by Institutional Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1399-209).

Drug:

VPA was purchased from Sigma-Aldrich. This drug was diluted in system water to prepare 1µM concentration. The VPA solution was freshly provided before the zebrafish embryo were exposed to it. Silibinin was purchased from Sigma-Aldrich.

Synthesis and characterization of Nanosilibinin:

Due to the low solubility of silibinin, Nanosilibinin(NS) was synthesized with antisolvent crystallization method. 5 mg of silibinin was dissolved in 1 ml ethanol. 0.2 gr PVP was dissolved in 100 ml distilled water. The solution of silibinin in ethanol was added dropwise in to the solution of PVP while stirred at 800 rpm. In order to remove the remaining solvent, the obtained suspension was placed on stir for at least 6 hours. After solvent removal, the silibinin nanosuspention was centrifuged at 10000 rpm and the precipitated was redispersed in PVP solution. The suspension was lyophilized and the obtained powder was used for further experiments. The size and morphology of the nanoparticle was characterized using scanning electron microscopy (TESCAN VEGA3).

Experimental Design:

The embryos were randomly allocated in to five groups including (control, VPA, and VPA +1001µM NS, VPA+200µM NS and VPA+500µM NS). Zebrafish embryos at 4 hours post fertilization (hpf) were exposed for 4 days to 1 µM VPA and different concentrations of NS in plates (100 embryos/group). During the exposure, old blue egg medium was replaced with new one daily and embryos were observed every 24 h to assess survival. The behavioral tests (openfield, inattentive behavior) were performed in 7 dpf between 10am to 2pm, followed by molecular study (Figure,1).

Behavioral assessment:

In this study, we used the population approach to evaluate the behavioral parameters. In population approach, group, s behavior was studied instead of single larvae(Dwivedi et al., 2019). All behavioral assessments were carried out in a soundproof room. The medium of larvae was changed 1 hour before behavioral tests and the larvae were transferred to behavioral room. All behavioral evaluations were done using the Ariya Shide Botiya software.

Open field Test:

Open field test was used to measure anxiety. In zebrafish larvae, this test was carried out in a plate with diameter of 90mm and height of 15 mm. 10 larvae were located in the center of the plate and their behavior were recorded for 30 minutes by a camera located above of the plate. The number of larvae on the plate margin was counted per minute to evaluate thigmotaxis behavior of larvae (Figure,2A).

Inattentive Behavioral Test:

To evaluate the larvae response to an aversive stimulus, inattentive behavioral test was performed. For this a rectangle Plexiglas plate with dimensions 90 mm to 40 mm was used. 10 larvea were located in the plate. At first blank white background was displayed for 30 minutes beneath the plate using a 10 inches screen. Then a moving red bar (aversive stimulus) was showed on the upper half of the plate for 30 minutes. Acclimatization and aversive stimulus phase were recorded by a camera located above the plate. The number of larvae was counted at the upper and lower half of the plate every two minute. The number of the larvae at the upper half of the plate showed the inattentive behavior of larvae (Dwivedi et al., 2019)(Figure,2B).

%Larvae in upper over acclimatization = (Aversive stimulus - Acclimatization)/ Acclimatization*100

Real-Time PCR study:

The expression of Wnt signaling –related genes (CHD8, CTNNB, GSK3 beta, LRP6), pro-inflammatory cytokins(TNFα, IL1β) and neurotropic factor (BDNF) in the brain of zebrafish larvae was measured in 7 dpf using real-time PCR. Total RNA was extracted from whole brain of zebrafish larvae exposed to VPA treated with or with out different concentrations of NS using TRIZOL reagent. RNA was isolated utilizing chloroform and precipitated using isopropyl alcohol. The amount of RNA (µg/ml) was analyzed using mass spectrometer. The RNA was converted in to cDNA by exploitation YTA cDNA synthesis kit as per manufactures instructions. All primers were designed by Primer-BLAST (NCBI) and their sequences were mentioned in table 1. The results are expressed relative to B2M, that is employed as an internal control.

Results

SEM image:

Scanning electron microscopy (SEM) was used to confirm formation of nanoparticles. The SEM images of NS was depicted in Figure 3. The SEM images demonstrated formation of particles with spherical like morphology. The number of at least 50 particles were analyzed using Image software to obtain size and size distribution. The diameter of the particles were in range of 50-110 nm with average of 78±14nm(mean±SD).

The results of survival study showed that all zebrafish embryos were exposed to 1µM VPA, 1µM VPA+100 and 200 µM NS survived until 7 dpf while The survival rate of embyros exposed to 500 µM NS was 90% at 1 dpf, but this index decreased to 50% at 2 dpf and none of them was alive at 3dpf (Table2). Consequently, 100 and 200 µM concentrations of NS were chosen to continue the neurobehavioral and molecular studies.

Thigmotaxis behavior:

Open field test was carried out to assess the thigmotaxis behavior. The results of this test revealed that the average number of VPA treated larvae in the margin of the plate was significantly more than that of the control group. Thigmotaxis behavior in the VPA+ 100 μM NS group didn't differ from the VPA group. Although, the average number of VPA+ 200 μM NS treated larvae in the margin of the plate was significantly decreased compared to that of the VPA group (Figure,4A).

Inattentive behavior :

The results of the inattentive behavior test showed that animals in the control group, VPA treated group and VPA+(100 or 200μM) NS groups moved randomly in upper and lower half of the plate during acclimatization phase. During aversive stimulus phase, the VPA treated larvae presented in two half of the plate but the control group and VPA+ 100 and 200 μM NS treated groups significantly shifted to the lower half of the plate While increased of Inattentive behavior in both VPA+ 100 and 200 μM NS treated groups to control group was not significant (Figure,4B).

Real-Time PCR analysis:

Expression levels of CHD8, CTNNB, GSK3β, LRP6, BDNF, IL1β, TNFα are altered in experimental groups:

RT-PCR results showed that the expression of Wnt signaling –related genes (CHD8, CTNNB, GSK3β and LRP6) mRNA were significantly increased in VPA treated larvae compared to control group. the expression of these genes were significantly decreased in VPA+100 μM NS group compared to VPA treated larvae. the expression of CHD8, CTNNB and LRP6 genes were significantly increased in VPA+200 μM NS group compared to control, VPA treated and VPA+100 μM NS groups. The expression of GSK3β mRNA in VPA+200 μM nanosilibinin group was significantly decreased compared to VPA treated larvae while this expression was significantly increased compared to VPA+100 μM NS group (Figure, 5A,B,C,D). The expression of BDNF, IL1β and TNFα genes were significantly increased in VPA treated larvae compared to control group. The expression of these genes were significantly decreased in VPA+100 μM and VPA+200 μM NS group compared to VPA treated larvae (Figure, 6A,B,C). The results of Real-Time PCR analysis are shown in table3.

Discussion

Maternal exposure to VPA (which is used clinically to treatment epilepsy, migraine headache and bipolar disorder) during pregnancy has been associated with appearance of ASD in the offspring (Bromley et al., 2013; Christensen et al., 2013; Dean et al., 2002; Meador et al., 2009; Ornoy, Weinstein-Fudim, & Ergaz, 2016; Williams et al., 2001). The exact molecular mechanism of VPA to induce ASD is not known. This antiepileptic drug affects the glutamatergic and GABAergic system and it also as a strong inhibitor of histone deacetylases (HAD) that leads to change in gene expression(Barrett et al., 2017; Gottfried et al., 2013; Phiel et al., 2001; Sun et al., 2016). It has been reported that VPA can lead to overactivation of the canonical β- catenin signaling pathway in VPA-induced ASD animal models (Qin & Dai, 2015; Qin et al., 2016).

Considering the association of wnt signaling pathway with autism spectrum disorder, in this study we investigated whether the symptoms of VPA induced ASD in zebrafish model could be ameliorated by using an inhibitor of this pathway. Therefore we used NS as an inhibitor of wnt signaling pathway and studied the effect of NS on behavioral and molecular deficits in autism zebrafish model induced by VPA.

We started our investigation by examining the effect of different concentrations of NS (100, 200, 500 μM) on survival of zebrafish embryos. Our results showed that 100 and 200 μM concentration of NS did not have any adverse effect on survival of zebrafish embryos while all zebrafish embryos were exposed to 500 μM died after 3dpf. This finding can indicate that the efficacy of NS on survival of zebrafish embryo is concentration-dependent and high concentration of NS has a toxic effect. consequently, we proceeded with 100 and 200 μM concentration of NS for studying the effect of NS on behavioral and molecular deficits in 1μM VPA based autism model in zebrafish larvae. our results showed, increased of thigmotaxis behavior, anxiety index in VPA-treated animals. Our results are in agreement with previous studies showed that VPA exposure led to increase anxiety in zebrafish larvae (Dwivedi et al., 2019; Joseph et al., 2021; Liu et al., 2016; Robea et al., 2021; Zimmermann, Gaspary, Leite, Cognato, & Bonan, 2015). Interestingly, NS could reduce this anxiety-like behavior. moreover, inattentive behavior test showed that VPA-treated larvae were indifferent to the aversive stimulus. Our data is in line with previous study showed that the behavior of inattentiveness in 7 dpf zebrafish larvae treated with VPA (Dwivedi et al., 2019). This test was performed to evaluate a passive avoidance learning in zebrafish larvae. The lack of comprehension of aversive stimulus by VPA treated group may be indicate their learning impairment.

Inattentive behavior in the NS treated groups showed a significant increase compared to VPA group. Our finding showed that NS has been able to reduce learning impairment in VPA + NS treated groups.

We analyzed the expression of several autism related genes in the brains of VPA-exposed zebrafish larvae treated with or with out NS at 7 dpf. Our results showed that VPA exposure for 96 h increased the expression of CHD8, CTNNB1, LRP6, GSK3β, TNFα, IL1β, BDNF genes. Our results are in agreement with previous study showed that prenatal exposure to VPA increased TNFα and IL1β in the brain of rat (Win-Shwe et al., 2018). On the other hand, we observed that in comparison with the VPA-exposed group, VPA-NS exposure for 96 h at the same time can result to decreased the expression of the mentioned genes.

Our results are in line with studies have shown that VPA can lead to overactivation of the canonical β-catenin signaling pathway and inhibition of this pathway can improve the symptoms of ASD in VPA-induced ASD model in animals(Qin & Dai, 2015; Qin et al., 2016; Zhang et al., 2012; Zhang et al., 2015). In this study, 100 and 200 μM NS could reduce TNFα, IL1β and BDNF genes expression in VPA-NS treated group while 200μM NS led to increase the expression of wnt-β catenin signaling pathway related genes (CHD8, CTNNB1, LRP6, GSK3β). This result indicates the concentration- dependent effect of NS on wnt-β catenin signaling pathway related genes expression. In conclusion, the concurrency of NS with VPA exposure in early stages of zebrafish development prevented neurobehavioral deficits probably via down regulation of wnt-β catenin signaling pathway related genes.

Declarations

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Author Contributions: ZK and HA conceived and designed research. ZK, HA, AZ, MD, EM and MD conducted experiments. ZK and HA analyzed data and prepared figures 1-6. ZK and HA wrote manuscript. All authors reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Ethics approval: All animal care and experimental procedures were done observing the National Institute of Health guidelines on animal care and use and were approved by Institutional Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1399-209).

Author Contributions

ZK and HA conceived and designed research. ZK, HA, AZ, MD, EM and MD conducted experiments. ZK and HA analyzed data and prepared figures 1-6. ZK and HA wrote manuscript. All authors reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Tables

Tables 1 to 3 are available in the Supplementary Files section

Figures

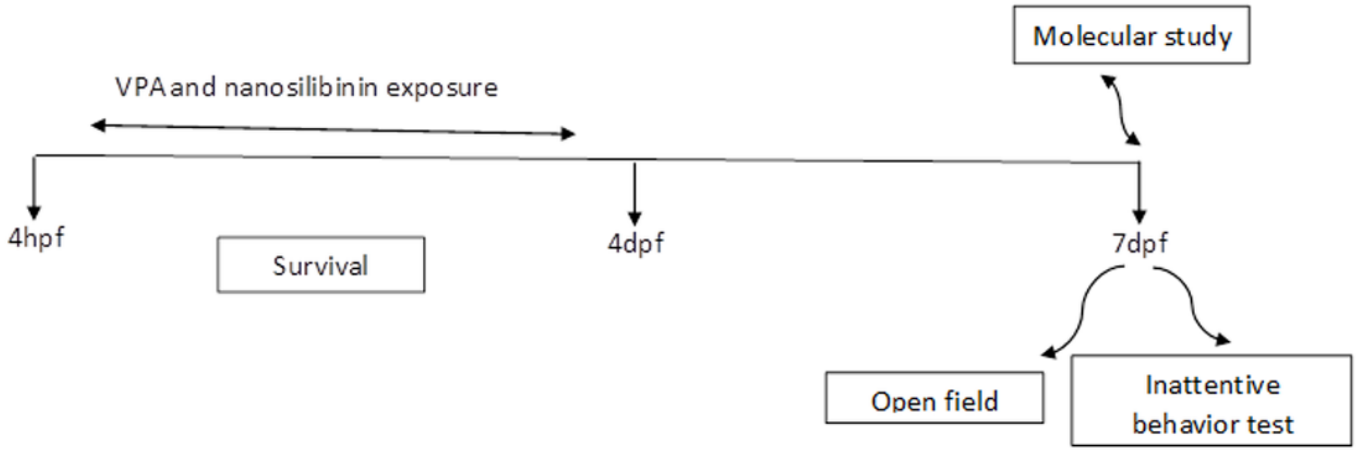

Figure 1

Study design. Treatments were performed from 4 hours post fertilization(4hpf) to 4 days post fertilization(4dpf) survival rate was assessed from the beginning of the study followed by molecular and behavioral evaluations at 7dpf.

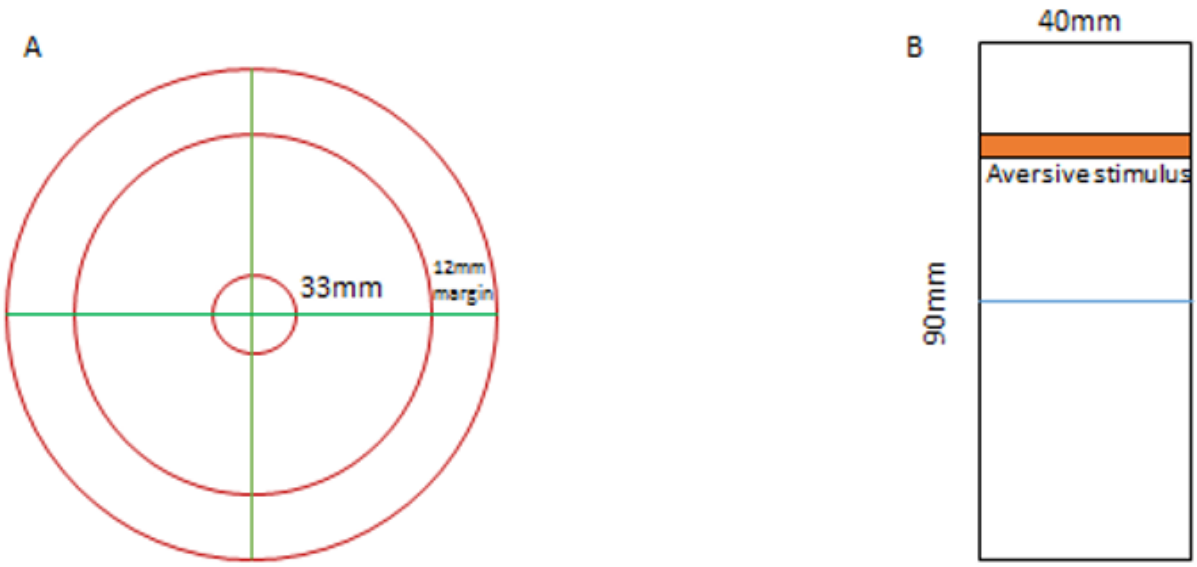

Figure 2

Behavioral assessments. A) Open field test: This test was performed in a petri dish with diameter of 90mm and height of 15 mm. Ten embryos used per petri dish. B) **Inattentive behavior test:** This test was carried out in a rectangle Plexiglas plate with dimensions of 90mm to 40mm. PowerPoint presentation was use to perform the test. At first, a blank white background was displayed for 30 minutes and then a moving red bar (aversive stimulus) was showed on the upper half of the plate for 30 minute. Acclimatization and aversive stimulus phase were recorded by a camera.

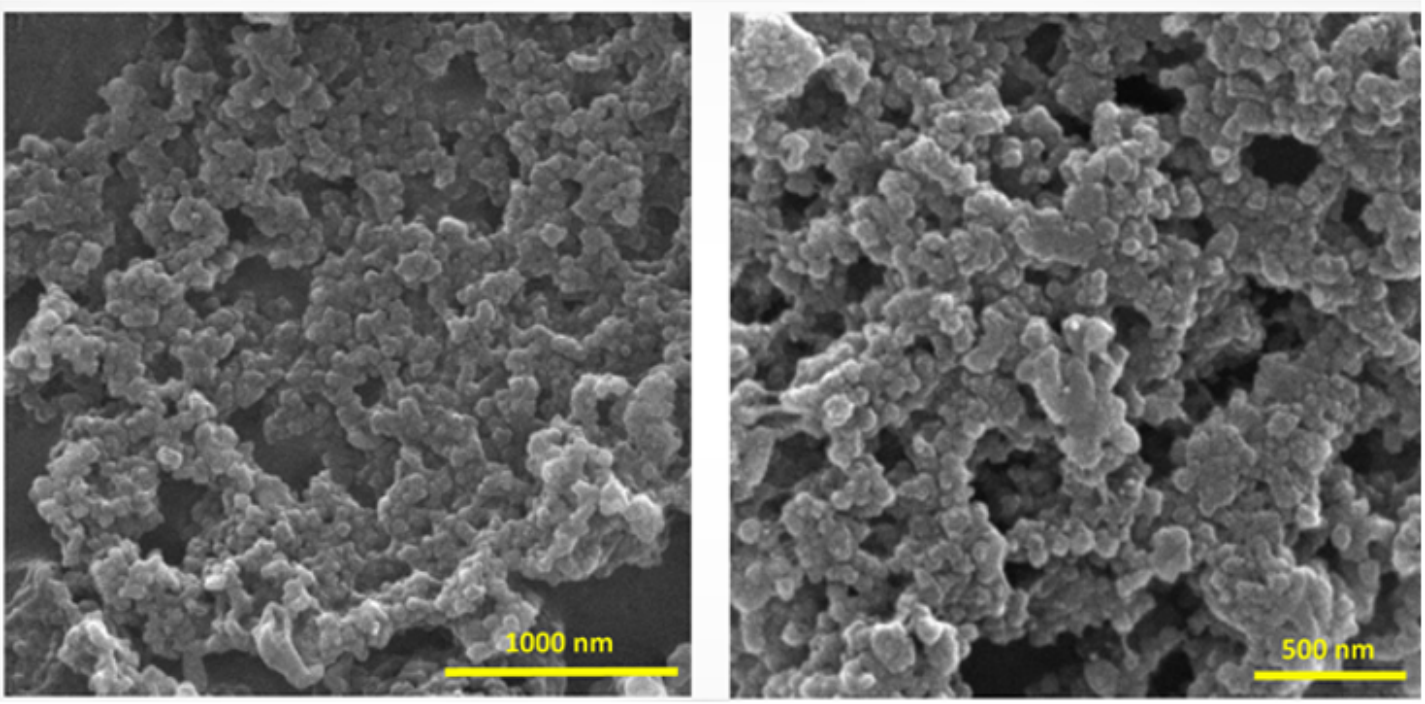

Figure 3

The SEM image of nanoparticle (NS) at two magnefications: left image 50kx and right image 70kx.

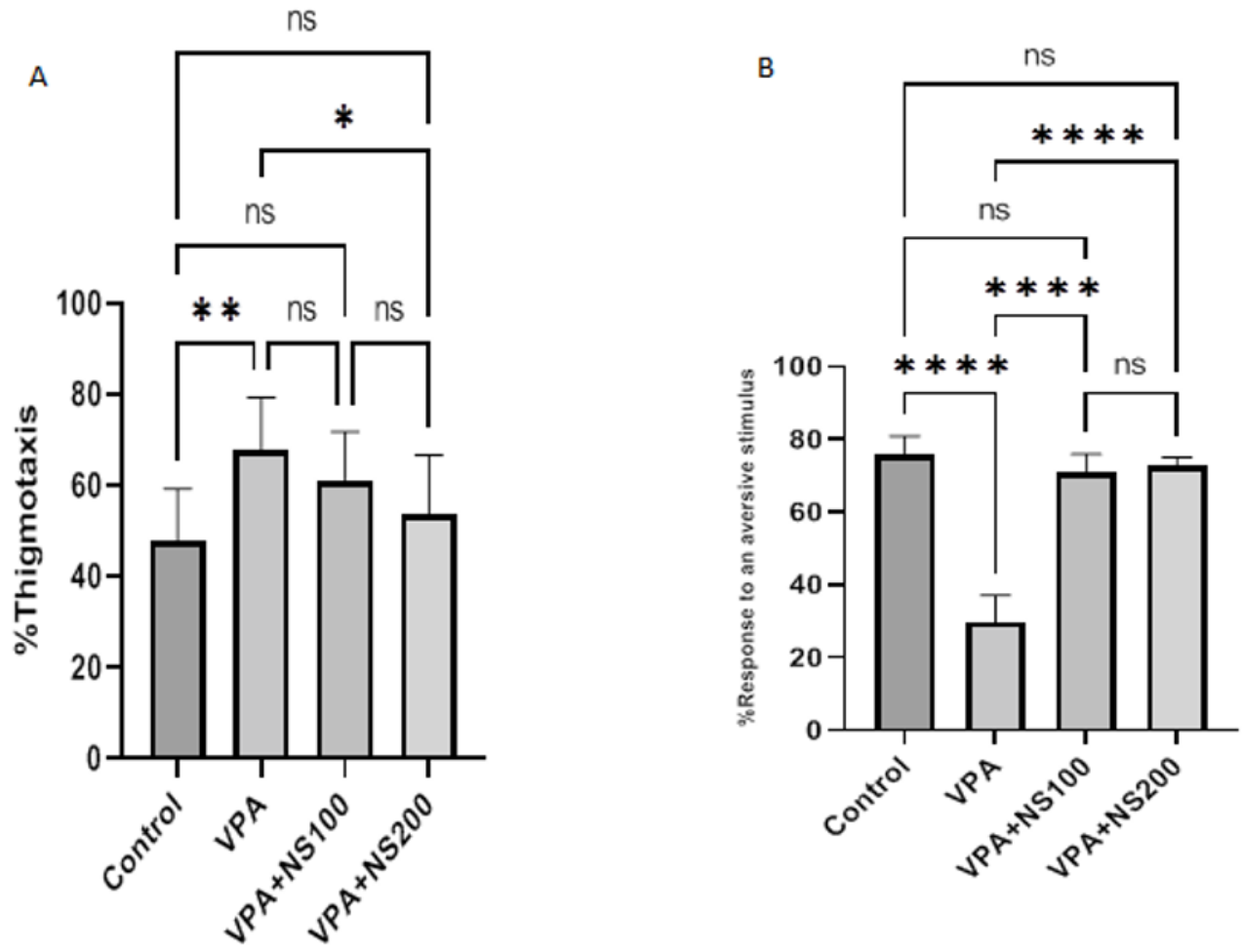

Figure 4

Effect of nanosilibinin on thigmotaxis and inattentive behavior. Analysis of thigmotaxis behavior (A) and inattentive behavior (B) in different experimental groups. All results are reported as mean±SEM and statistical significance was ascertained using ONE-WAY ANOVA test in GraphPad prism software, where ****p<0.0001, **p<0.01, *p<0.05

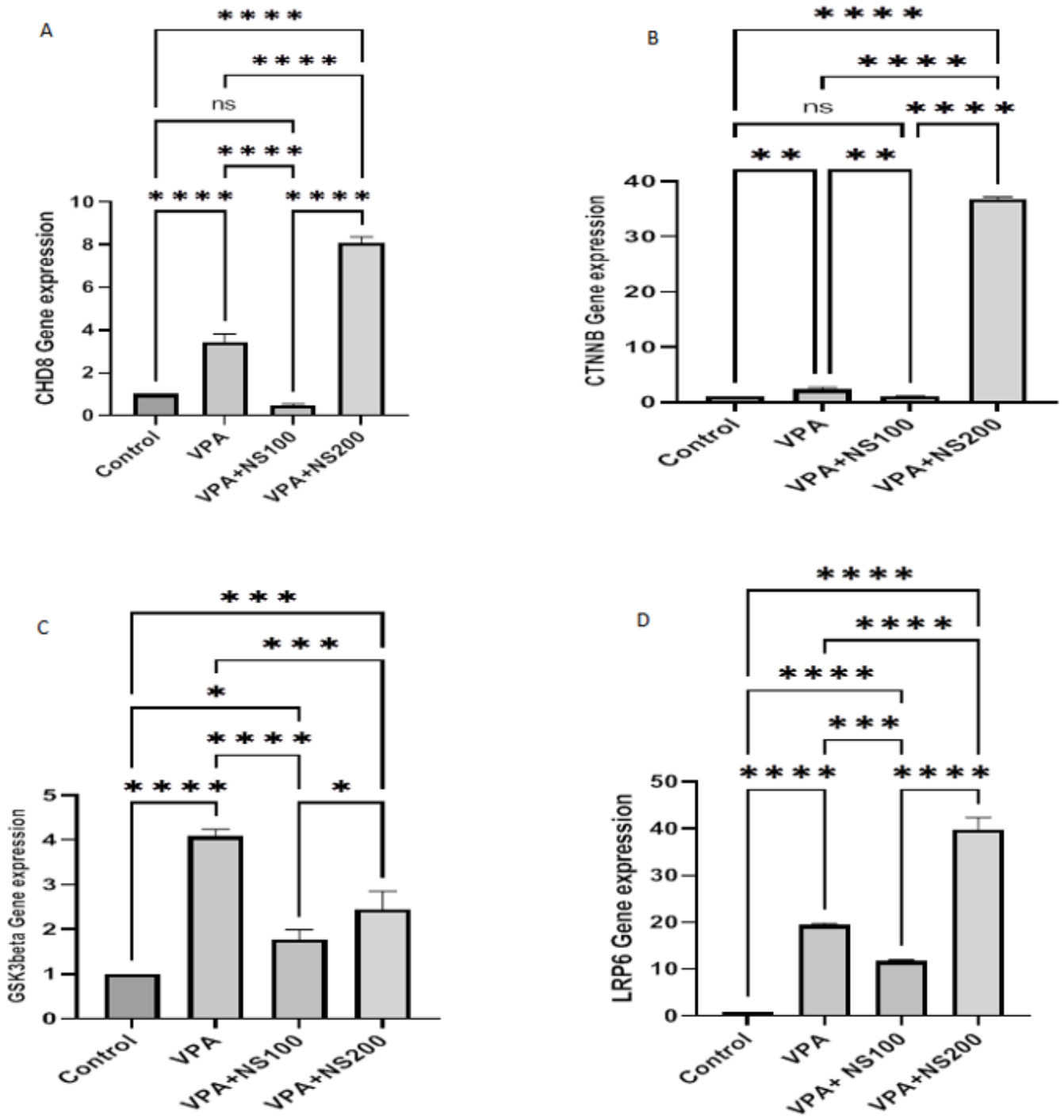

Figure 5

Effect of NS on Wnt- βcatenin pathway related genes. The expression level of CHD8 (A), CTNNB(B), GSK3 beta(C) and LRP6(D) in the brain of zebrafish larvae were significantly altered in different expremintal groups compared to control group. All results are reported as mean±S.E.M and statistical significance was ascertained using ONE-WAY ANOVA test in GraphPad prism software, where *p<0.05,**p<0.01, **p<0.001, ****p<0.0001 as compared to control group.

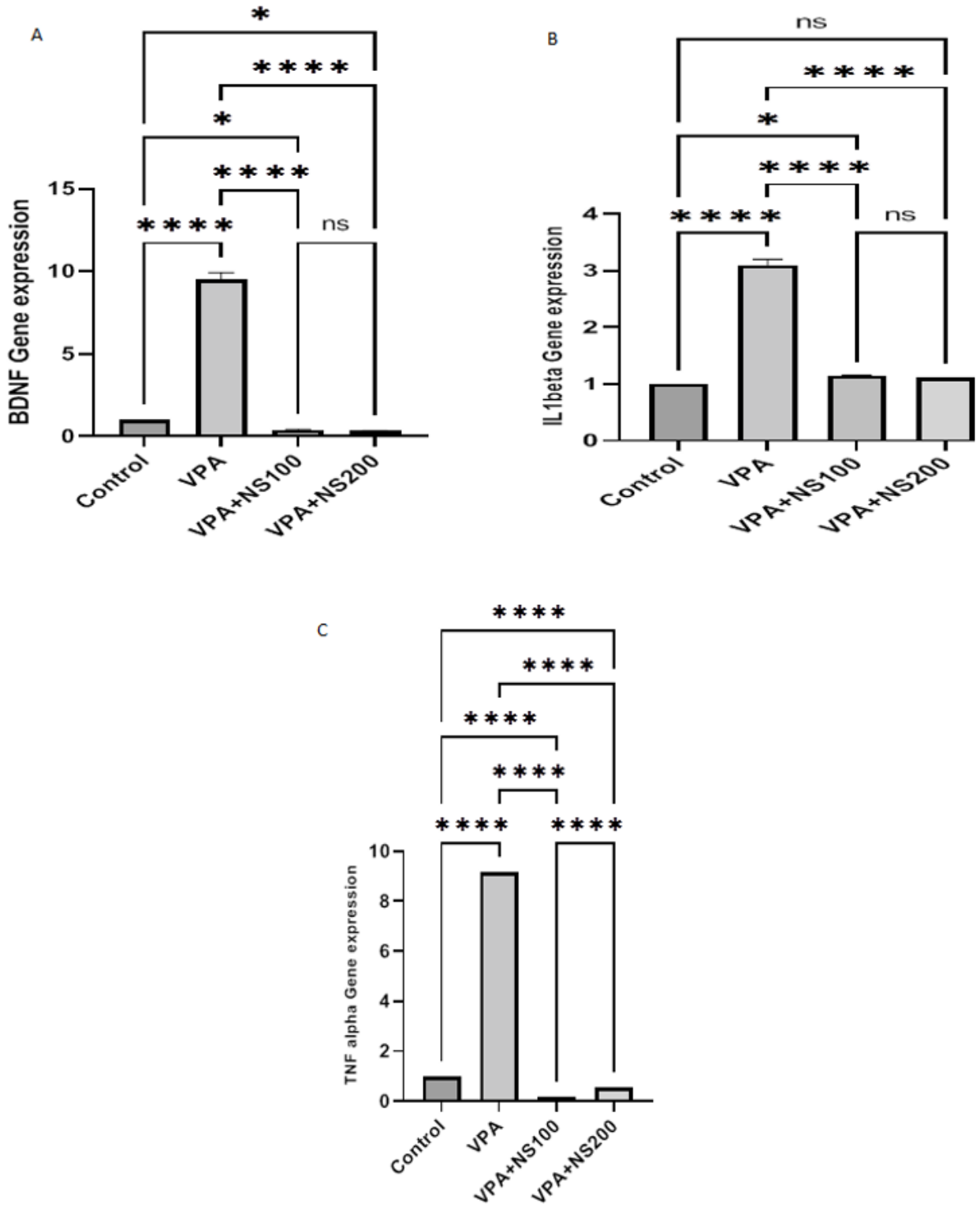

Figure 6

Effect of NS on the expression of ASD-related genes. The expression level of BDNF(A), IL1 beta(B) and TNF alpha(C) in the brain of zebrafish larvae were significantly altered in different experimental groups compared to control group. All results are reported as mean±S.E.M and statistical significance

was ascertained using ONE-WAY ANOVA test in GraphPad prism software, where *p<0.05,**p<0.01, **p<0.001, ****p<0.0001 as compared to control group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Tables.docx