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Vitexin inhibits acrylamide-induced neuroinflammation and improves behavioral changes in zebrafish larvae

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ABSTRACT

Neuroinflammation is crucial for the pathophysiological hallmarks of many neurodegenerative disorders. Hyperactivated microglia has long been implicated as a detrimental player in regulating unresolvable inflammatory insults which cause damage to neurons. In the context of acrylamide (ACR) neurotoxicity, microglia activation is documented to correlate with ACR-adduct formation in the presynaptic neurons. Thus, inhibition of inflammatory mediators through vital candidate is greatly warranted to retard the disease progression. In the present study, we investigated, whether vitexin, a C-glycosylated flavone, with anti-inflammatory activity, could inhibit ACR-induced neuroinflammation-like behavior in zebrafish larvae. ACR was exposed at a dose 1 mM to 3 days post fertilization (dpf) zebrafish larvae for 3 days, whereas vitexin (10 μM) was treated for 24 h. After vitexin treatment, a series of histopathology, behavioral tests and molecular analyses were measured. Our data show that ACR larvae exhibited abnormal morphologies in brain cartilage and histological patterns. At behavioral levels, motor function was altered while the expression of pro-inflammatory mediator levels was markedly up-regulated in ACR larvae. Further, we validated the enhanced CDK5 activity is known to trigger microglia activation, also we found reduced expressions of neuroplasticity (CREB1 and ATF1) and antioxidant response makers (Nrf2, SOD-1 and CAT) in ACR intoxicated larvae. Interestingly, vitexin treatment markedly alleviated ACR-induced histological and behavioral changes in zebrafish larvae. Moreover, vitexin effectively inhibited CDK5 expression, and also hampered the release of pro-inflammatory mediators in ACR larvae. Finally, vitexin treatment rescued the loss of neuroplasticity markers along with enhanced antioxidant markers in ACR larvae. Taken together, results in the present study showed the possibility of vitexin as a potential therapeutic drug in the suppression of neuroinflammation.

1. Introduction

Neuroinflammation plays an active role in the pathophysiology of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and autoimmune diseases (Ransohoff, 2016; Molteni and Rossetti, 2017). It represents the coordinated cellular response to tissue damage and leads to classical motor symptoms including bradykinesia, rigidity and body tremors. Several experimental and post-mortem studies suggest an intimate relationship between microglial activation and neuroinflammation in the brains of neurodegenerative diseases (Bartels and Leenders, 2007). Other studies have previously reported that activated glial cells release the glial maturation factor (GMF) that acts on resting microglia and induces neurodegeneration by releasing inflammatory metabolites in the brain (Kempuraj et al., 2013). In recent years, the role of nitric oxide (NO) in neuroinflammation has been validated in rodent models, characterized by glutamate accumulation and microglial activation (Yuste et al., 2015).

Acrylamide (ACR) is a classic neurotoxin, formed in food during heat processing, especially in deep fried and oven-baked foods (Tareke et al., 2002; Sirot et al., 2012). ACR neurotoxic effect can mimic the common pathological signatures observed in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Erkekoglu and Baydar, 2014). A wealth of evidence suggested that ACR-neurotoxicity was associated with oxidative stress, which can mediate the activation of glial cells (Pawate et al., 2004; Solleiro-Villavicencio and Rivas-Arancibia, 2018). Typically, glial cells such as microglia and astrocyte play a crucial role to induce significant innate and adaptive immune responses in the central nervous system (CNS) (Anderson and Swanson, 2000; Yang et al., 2010). Moreover, the gain of reactive astrocytes and activated microglia are widely seen in neuroinflammation and correlates with cognitive impairment in AD (Liddelow et al., 2017). Upon infection, injury or neurotoxins, glial cells

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are actively involved in the release of distress signals such as reactive oxygen species (ROS), noxious factors (NO) and other pro-inflammatory cytokines (TNF-α, IL-1β, COX-2, and inducible nitric oxide synthase (iNOS)), all of which can lead to neuronal cell death (Moss and Bates, 2001; Sloan and Barres, 2014; LoPachin and Gavin, 2012; Hansen et al., 2018).

Also, neurotoxins released from glial cells can inversely alter transcription factors involved in neurogenesis. One major transcription factor affected by immune response is cAMP-responsive element-binding protein (CREB), which belongs to a subfamily of the basic leucine zipper transcription factors (Frank and Greenberg, 1994). CREB is widely expressed in all cells of the brain and actively contributes to neuronal plasticity, learning and memory (Carlezon Jr et al., 2005). Studies linked that CREB function in memory regulation particularly in adult hippocampus neurogenesis (AHN) (Ortega-Martínez, 2015). Noticeably, several antidepressant therapeutic approaches increase hippocampal CREB activity under mood disorders, suggest that CREB function in the modulation of depression-related behaviors (Vogt et al., 2014). Among the CNS functions, CREB has a selective role in immune responses including NFκB inhibition, enhancing macrophage survival and regulation of T and B lymphocytes, thereby limiting pro-inflammatory signaling (Wen et al., 2010).

Cyclin-dependent kinase 5 (CDK5), is a member of serine/threonine kinases works with its activator p39/p35 for the normal function of neural development (Dhariwala and Rajadhyaksha, 2008). However, proteolytic cleavage of p35 and p39 via calcium-dependent calpains induce an active form of CDK5/p25 complex, can able to promote elevated tau phosphorylation and neurodegeneration (Patrick et al., 1999; Camins et al., 2007). CDK5 dysfunction has been shown to augment hippocampal degeneration-related cognitive deficits (Gutiérrez-Vargas et al., 2015). Recently, CDK5 inhibitor increases CREB activity in the hippocampal region implicates the crosstalk between CDK5 and CREB in cognitive regulation (Bettayeb et al., 2008; Liu et al., 2017). These findings represent the direct role of chronic inflammation in the progression of neurodegeneration and interventions that inhibit the features of neuroinflammation may also counteract Alzheimer's disease in human.

Vitexin (Fig. 1) is a naturally derived bioactive flavonoid of *Crataegus pinnatifida* (hawthorn) and other medicinal herbs (Choo et al., 2012; Wu et al., 2014). It possesses a wide range of biochemical functions including anti-oxidative, anti-inflammatory, neuroprotective and anticancer properties. In addition, it also exhibits antinociceptive activities against noxious thermal stimuli induced rodent models (An et al., 2012; Rosa et al., 2016; Liu et al., 2016; Bhardwaj et al., 2018). However, it remains vague whether vitexin also induces an anti-neuroinflammatory response in ACR model of neurotoxicity. Accordingly, the present study aimed to investigate the potential effect of vitexin on ACR-induced neurotoxicity in zebrafish larvae with particular emphasis on biomarkers of neuroinflammation and neurodegeneration.

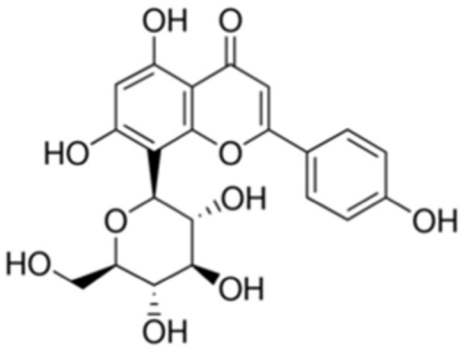

Fig. 1. Structure of Vitexin.

2. Materials and methods

2.1. Chemicals and reagents

Vitexin, acrylamide, tricaine mesylate, dichloro-dihydro-fluorescein diacetate (DCFH-DA), Alcian blue, radioimmunoprecipitation assay buffer (RIPA), and hematoxylin and eosin (H&E) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies toward iNOS, nNOS, COX-2, p-p38, Nrf2, superoxide dismutase-1(SOD-1), catalase (CAT), CDK5, CREB1, p-CREB1, ATF1, β-actin, and lamin B1 was purchased from Abcam, Cell Signaling, and Santa-Cruz Biotechnology. The secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from Santa-Cruz Biotechnology. The specificity of all the antibodies used in this study toward zebrafish was examined by western blot analysis in our laboratory, as previously described (Kim et al., 2017; Krishnan et al., 2019). All other reagents and chemicals used were of high analytical grade.

2.2. Maintenance of zebrafish

Adult zebrafish AB stains (*Danio rerio*) were purchased from a local aquarium (Daegu Aquarium, South Korea) and maintained in fresh water at 28 °C, on a 14:10 h light-dark cycle under standard conditions. Fish were fed commercial food thrice a day (TABIA, TOPMEAL, and South Korea). Embryos were obtained by natural spawning and were maintained in a petri dish containing the embryo (E3) medium (0.2 mM Ca $(NO_3)_2$, 0.13 mM $MgSO_4$, 19.3 mM NaCl, 0.23 mM KCl, and 1.67 mM HEPES) at 28 °C (Kimmel et al., 1995). For live imaging acquisitions, the larvae were anaesthetized with 0.168 mg/ml tricaine prior to use (Yousfi et al., 2015). All experiments were performed according to the guidelines for animal care and handling norms approved by the Institutional Animal Care and Use Committee of Daegu University (Kyoungbook, South Korea).

2.3. Experimental design

A preliminary study was conducted to determine the dose-dependent effect of vitexin on ACR intoxicated zebrafish. Three-day post fertilized zebrafish larvae were challenged to 1 mM ACR (Prats et al., 2017) for 3 days, and then treated with vitexin (2, 10 and 20 μM) for 24 h. After treatment, larvae were subject to histological and biochemical analyses. We observed that (Supplementary Figs. S1 and S2) vitexin exhibits, dose-dependent protection against ACR induced cartilage damage, NO and TNF-α levels in zebrafish larvae. Although 10 and 20 μM vitexin shows potential effect but not significant with each other, therefore, we selected 10 μM vitexin dose for further research.

In the second line of a protective model of neuroinflammation (Fig. 2), 3 dpf zebrafish larvae were divided into four treatment groups (30 larvae per group in triplicate). The larvae maintained in the E3 medium served as normal control (group I) while larvae in group II served as a negative control was exposed daily to 1 mM ACR for 3 days. Larvae in group 3 received ACR as like group 2 and on day 4, the larvae were treated with vitexin (10 μM) for 24 h. Group 4 larvae were exposed to 10 μM vitexin alone for 24 h. After the treatment period (Day 5), larvae from all groups were assessed for behavioral phenotypes and then processed for histological and biochemical analyses.

2.4. Behavioral analysis

The rate of swimming behavior including total distance and average speed of zebrafish larvae were used to evaluate the effect of vitexin on ACR-induced locomotion impairments. Larval behavior was monitored at 8 dpf after treatment with vitexin (Day 5), using Logitech C310 camera facing down on the plates. The videos were taken at 30 frames per second and recorded within at 1 min at 28 °C. Briefly, larvae of each group (15 larvae per group, sorted into 5 larvae/well) were placed in a

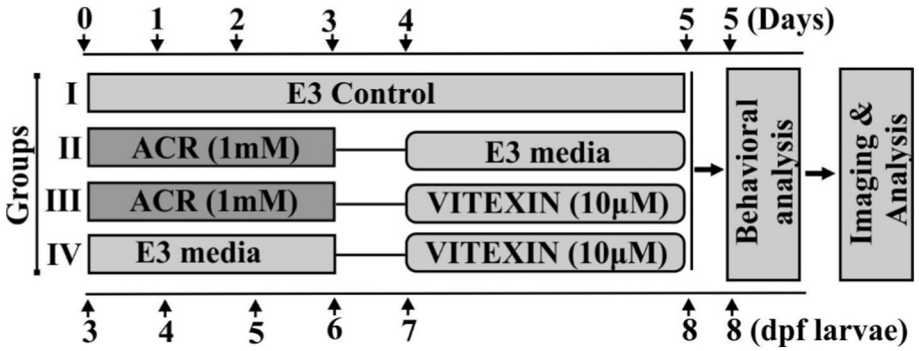

Fig. 2. The schematic illustration represents the experimental protocol. Zebrafish larvae (3 dpf) were divided into four groups (30 larvae per group): group I, normal control maintained in E3 medium; group II, negative control exposed to ACR (1 mM) for 3 days; group III, larvae exposed to ACR as like group II followed by treatment with vitexin for 24 h; group IV, drug control treated with vitexin for 24 h. After the treatment period (5th day), behavioral, imaging and immunodetection studies were conducted to detect the neuroprotective effect of vitexin against ACR toxicity.

white ice cube tray (3 × 3 cm) filled with E3 medium (2.0 ml). The advantages of the white plate include preventing visual contact of larvae acting from adjacent wells, increase the contrast between background and the larvae. After 10 min of acclimatization under noise-free conditions, behavioral tests were performed and data acquired from the recorded sequences were analyzed using ToxTrac software-v2.61 (Rodriguez et al., 2018).

2.5. Cartilage staining

The changes of cartilaginous phenotypes in zebrafish larvae were performed according to the described method with some modifications (Neuhauss et al., 1996). Larvae at 8 dpf were anaesthetized with tricaine, after which they were fixed overnight with 4% (*w/v*) paraformaldehyde (PFA) at 4 °C. The fixed larvae were washed with phosphate buffered saline (PBS) and incubated with a bleaching agent (1:1 ratio of 3% (v/v) hydrogen peroxide (H_2O_2), 2% (w/v) potassium hydroxide (KOH)) for 1 h. After incubation, the larvae were stained overnight in 0.1% (v/v) Alcian blue pre-dissolved in acidic ethanol (70% (v/v) ethanol, 5% (v/v) concentrated hydrochloric acid). The larvae were washed extensively with acidic ethanol, dehydrated, and stored in 80% (v/v) glycerol. Ventral view of the stained larval sections was visualized for cartilage changes under a light microscope (Nikon Eclipse TS100, Japan).

2.6. Histological analysis

The head portion of all experimental larvae were examined for brain anatomical changes, according to the optimized protocol (Copper et al., 2018). The head portions were fixed with 10% buffered formalin solution and then embedded in paraffin. Six-micrometre-thick sections were prepared and stained with hematoxylin and eosin. The histopathological changes were examined under light microscopy (Nikon Eclipse TS100, Japan). We utilized the web-based bio-atlas tool (http://bio-atlas.psu.edu) to examine the brain micro-anatomical changes between the control and treated animals.

2.7. Determination of nitrite levels in zebrafish

The level of nitric oxide was determined by the Griess method, with some modifications (Pekarova et al., 2009). Briefly, zebrafish larvae were anaesthetized with tricaine, and the head portion (8 larvae per group) without the eye and yolk sac regions were homogenized with ice-cold phosphate buffer (0.1 M, pH 7.4); the resultant suspension was subjected to centrifugation (12,000 xg) at 4 °C for 20 min, and the lysates were collected for further analysis. Total protein levels were quantified by Bradford's method (Kruger, 1994). Tissue lysate (100 µL) was mixed with 100 µL of Griess reagent and incubated for 15 min at room temperature, after which the absorbance was read at 540 nm using an ELISA reader. NO concentrations (µM) in samples were determined using sodium nitrite as a standard.

2.8. Evaluation of cytokines

The concentration of tumor necrosis factor α (TNF-α) in the larval head supernatant was quantified using mouse TNF-α Detection Kit (Invitrogen, USA), in accordance with the manufacturer's protocols. The quantities of TNF-α were evaluated by measuring the absorbance at 450 nm in an ELISA reader.

2.9. Western blot analysis

Protein contents were extracted from pools of 8 larvae of all groups (8/replicate in three replicates) using RIPA buffer containing protease-phosphatase inhibitor, followed by centrifugation (12,000 xg) at 4 °C for 20 min; the supernatant was collected and stored at −20 °C till further use. Nuclear fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific), according to the manufacturer's protocol. Proteins (50 µg (v/v)) were electrophoresed by SDS-PAGE using a 12% (*w/v*) polyacrylamide resolving gel and electroblotted to PVDF membrane, after which they were blocked using 5% (w/v) non-fat milk for 1 h at room temperature (RT). The membranes were subsequently incubated with primary antibodies at 4 °C overnight, followed by incubation with their respective secondary antibody for 1 h at RT. The protein-antibody complexes were visualized using an enhanced ECL system (LuBioScience, GmbH, Switzerland). Densitometry analysis of signals was quantified using the Image J (NIH, USA) software.

2.10. Data analyses

All statistical analyses were performed using the GraphPad Prism Software (Version 5.0, San Diego, California, USA). The data are presented as the mean ± S.E.M. of three independent experiments. The differences in behavioral assessment were evaluated by one-way ANOVA (non-parametric) followed by Kruskal-Wallis tests (with Dunn's multiple comparisons posthoc test). The statistical analysis for biochemical and protein expressions was performed with one-way ANOVA and Tukey's honestly significant difference (HSD) posthoc test and $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Vitexin alters swimming impairments induced by ACR in zebrafish larvae

It is well-documented that ACR neurotoxicity has been implicated in uncoordinated behavioral responses through a direct inhibitory effect on presynaptic functions (LoPachin and Gavin, 2012). Primarily, we analyzed the rate of locomotion in larvae by tracking swimming behaviors of all experimental larvae. As shown in Fig. 3A, larvae exposed to ACR displayed significantly ($P < 0.05$) reduced average speed (12.19 ± 0.88 mm/s) and distance travelled (916.4 ± 35.32 mm/s) at

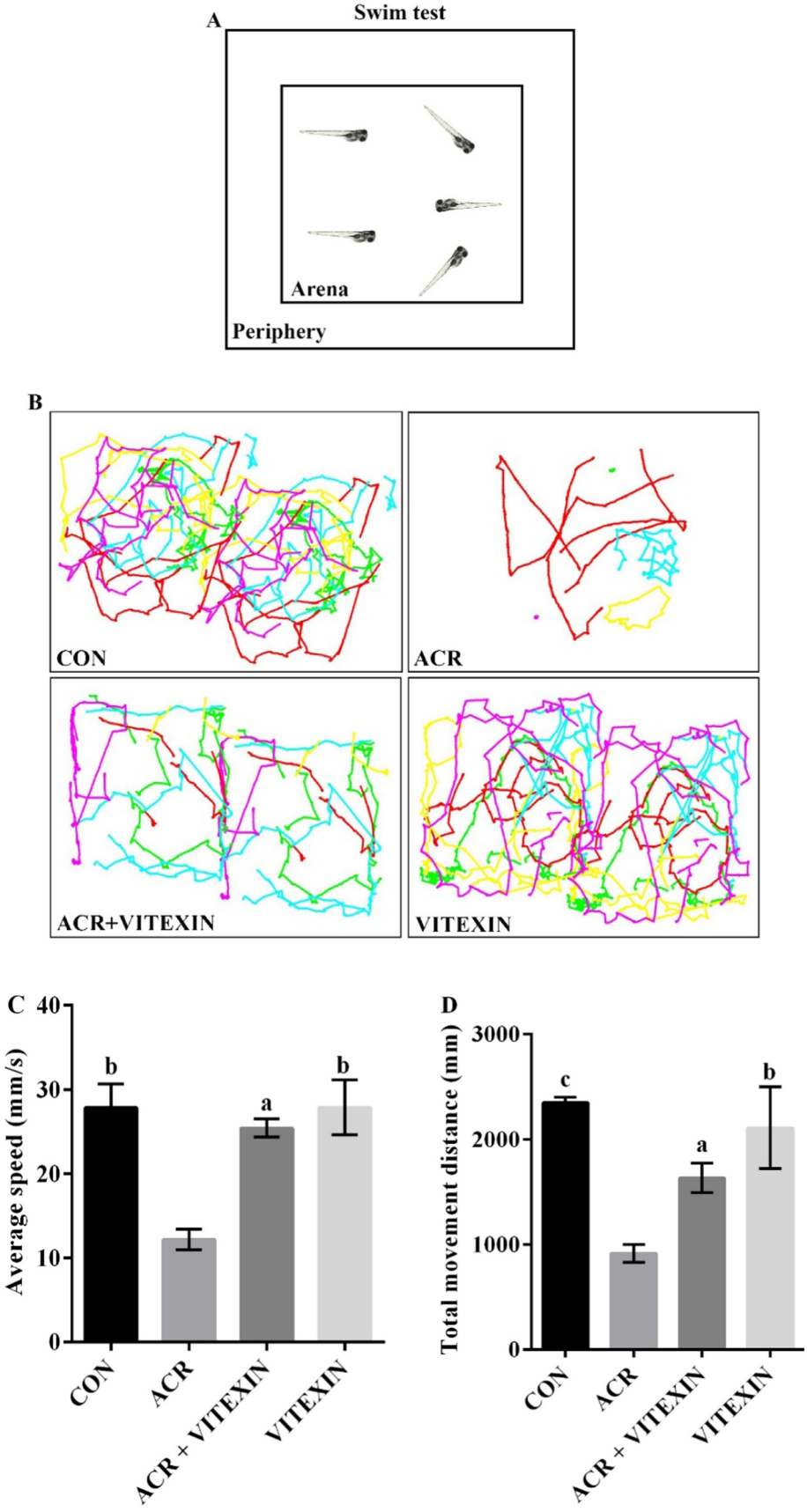

Fig. 3. Vitexin recovered the locomotor defect in ACR exposed larvae. (A) Schematic diagram of the swim test (15 larvae per group, sorted into 5 larvae/well), (B) Each color track symbolizes an individual zebrafish larval behavior in the swim test (C) Average speed and (D) Total distance travelled by an individual larval over 1 min interval in the arena zone. Values are expressed as mean ± S.E.M and are statistically significant by Kruskal Wallis test with Dunn's multiple comparison post hoc test. Comparisons: $^{a}P < 0.05$, $^{b,c}P < 0.0001$ relative with ACR.

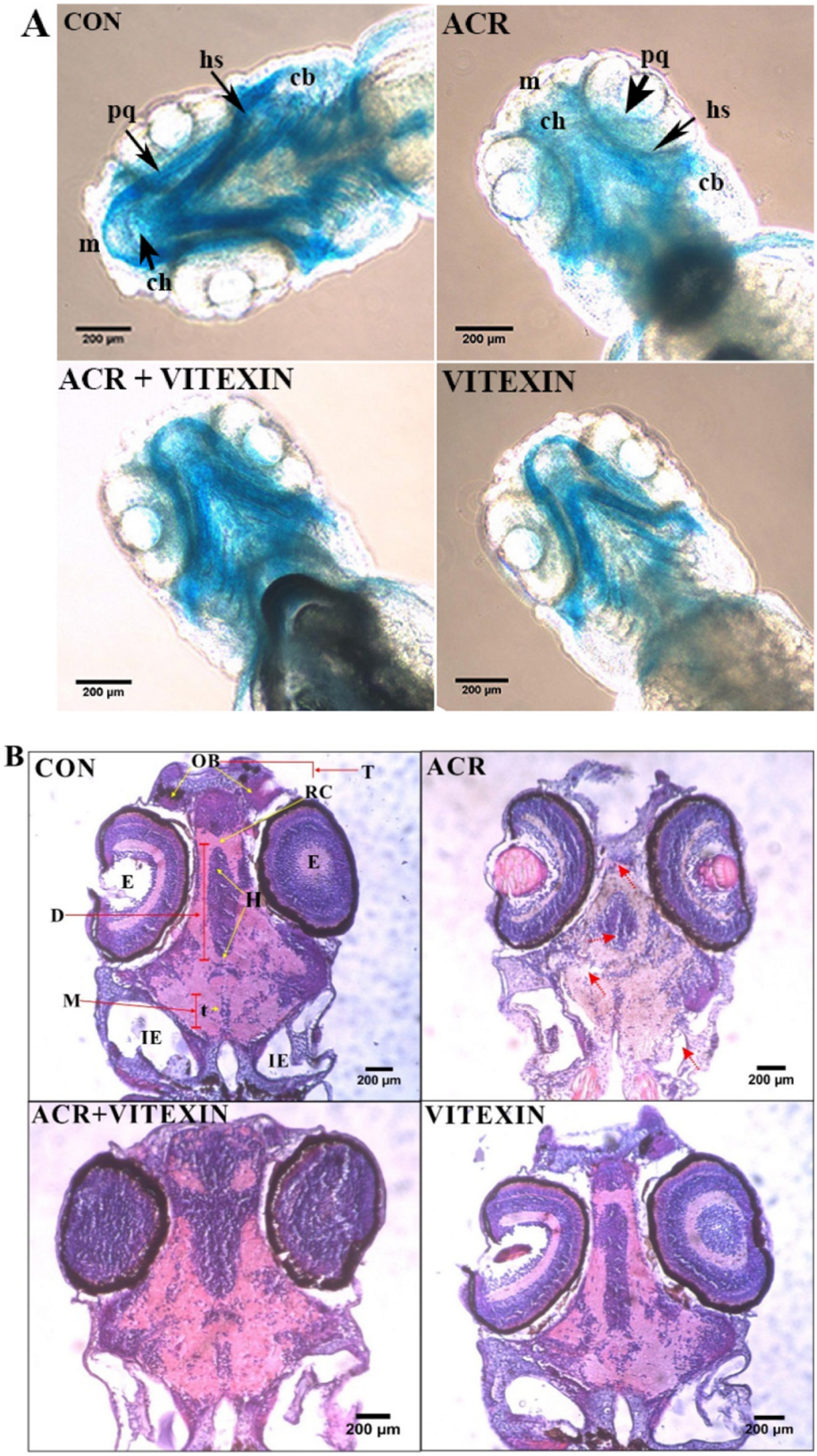

Fig. 4. Vitexin rescues the histological phenotype of ACR toxicity in larval brain sections. (A) Ventral view of 8 dpf larvae stained for cartilage with Alcian blue. Arrow indicates the normal arrangement of craniofacial cartilage elements including Meckel's cartilage (m), palatoquadrate (pq), ceratohyal (ch), hyosymplectic (hs) and ceratobranchials 3-7 (cb). (B) Larval head sections stained with hematoxylin and eosin show the protective effect of vitexin against ACR-induced pathological alterations including telencephalon (T), diencephalon (D), mesencephalon (M), olfactory bulb (OB), hypothalamus (H), rostral commissure (RC), tuberculum (t), eye (E), and inner ear (IE). Magnification ×10 and scale bar 200 µm.

8 dpf when compare to the average speed and distance travelled measures of control larvae (28.62 ± 2.9 mm/s and 2348 ± 20.85 mm/s, respectively). However, vitexin treatment significantly ($P < 0.05$) rescued the uncoordinated swimming patterns induced by ACR at 8 dpf based on the increase in average speed (24.55 ± 0.92 mm/s) and distance travelled (1634 ± 94.69 mm/s). This result suggests that vitexin could prevent the deficit in motor-neuron performance.

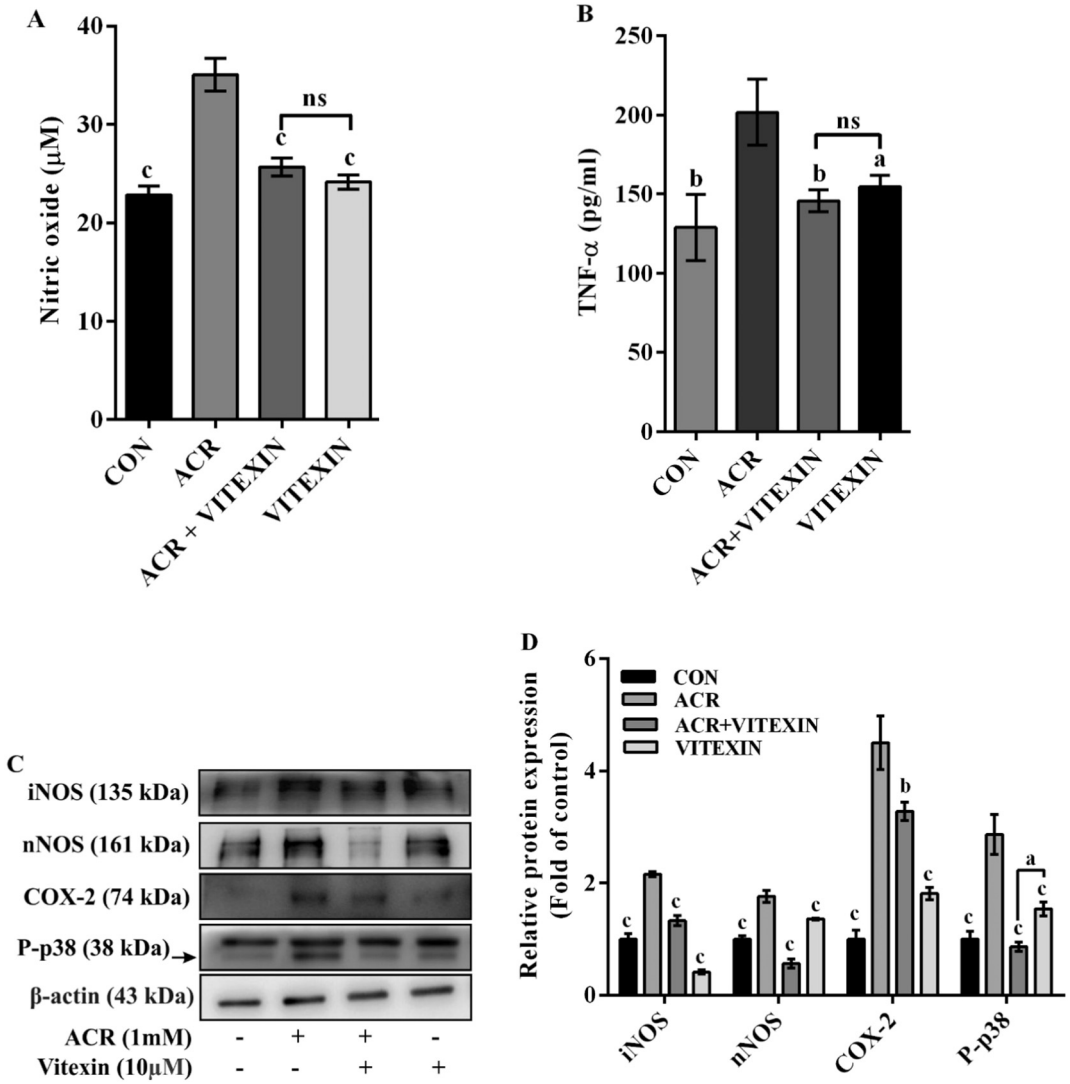

Fig. 5. Vitexin affects ACR-mediated inflammation in larval zebrafish (A). Nitric oxide levels, (B) TNF-α, (C) Western blot expression of major inflammatory mediators (iNOS, nNOS, COX-2 and P-p38). Beta-actin was used as the loading control. (D) Fold change of relative protein levels were quantified by densitometry (mean ± S.E.M, $n = 3$). Significant differences compared with ACR and vitexin alone groups were denoted by $^{a}P < 0.05$, $^{b}P < 0.001$, $^{c}P < 0.0001$, ^{ns}non-significant (Tukey's posthoc test).

3.2. Vitexin rescued zebrafish larvae from ACR-induced cartilage damage

Neural crest cells serve as a matrix for most craniofacial cartilages and connective tissues of the vertebrate head. Since ACR is a potent neurotoxin, we sought to identify whether vitexin exposure protects the craniofacial variability observed after ACR exposure. As shown in the Fig. 4A and Supplementary Fig. S1, Alcian blue stained controls and vitexin alone larvae revealed regular cartilage phenotypes, including the first pharyngeal arch (Meckel's cartilage and palatoquadrate), second or hyoid arch derivatives (ceratohyal, hyosymplectic), and the posterior pharyngeal arches (ceratobranchials 3–7). In contrast, larvae exposed to ACR shows impaired cartilage elements such as damaged curvature of the first pharyngeal arches (Meckel's and palatoquadrate) and absence of posterior pharyngeal arches (ceratobranchials 3–7) (Fig. 4A). Compared with ACR larvae, vitexin treatment markedly recovered the cartilage damage in zebrafish larvae, indicating the potential effect of vitexin in the improvement of craniofacial phenotypes.

3.3. Vitexin protects ACR-induced alterations in the larval brain

Since, cartilage act as a backbone matrix for brain organization, we next examined whole brain histological changes of all experimental group of larvae by H&E staining (Fig. 4B). Micro-anatomical changes in the whole brain region of control and vitexin alone treated larvae were restricted and showed normal architecture in major brain subdivisions that include the telencephalon (T), diencephalon (D), mesencephalon (M), olfactory bulb (OB), hypothalamus (H), rostral commissure (RC), tuberculum (t), eye (E), and inner ear (IE). Conversely, ACR larvae exhibited severe histological alterations in the above-mentioned brain subdivisions. Whereas, treatment with vitexin notably recovered the aberrant changes induced by ACR in zebrafish larvae, indicating the neuroprotective effect of vitexin against functional ACR toxicity.

3.4. Vitexin inhibits the release of pro-inflammatory mediators in ACR larvae

We reasoned that hyperactive NO can influence microglial inflammatory response following neuronal damage in CNS (Olivera et al., 2016). We next quantified NO levels by Griess method, as shown in Fig. 5A and Supplementary Fig. S2A, ACR exposed larvae exhibited significantly increased NO levels ($P < 0.0001$) when compared to control. However, vitexin treatment significantly ($P < 0.0001$)

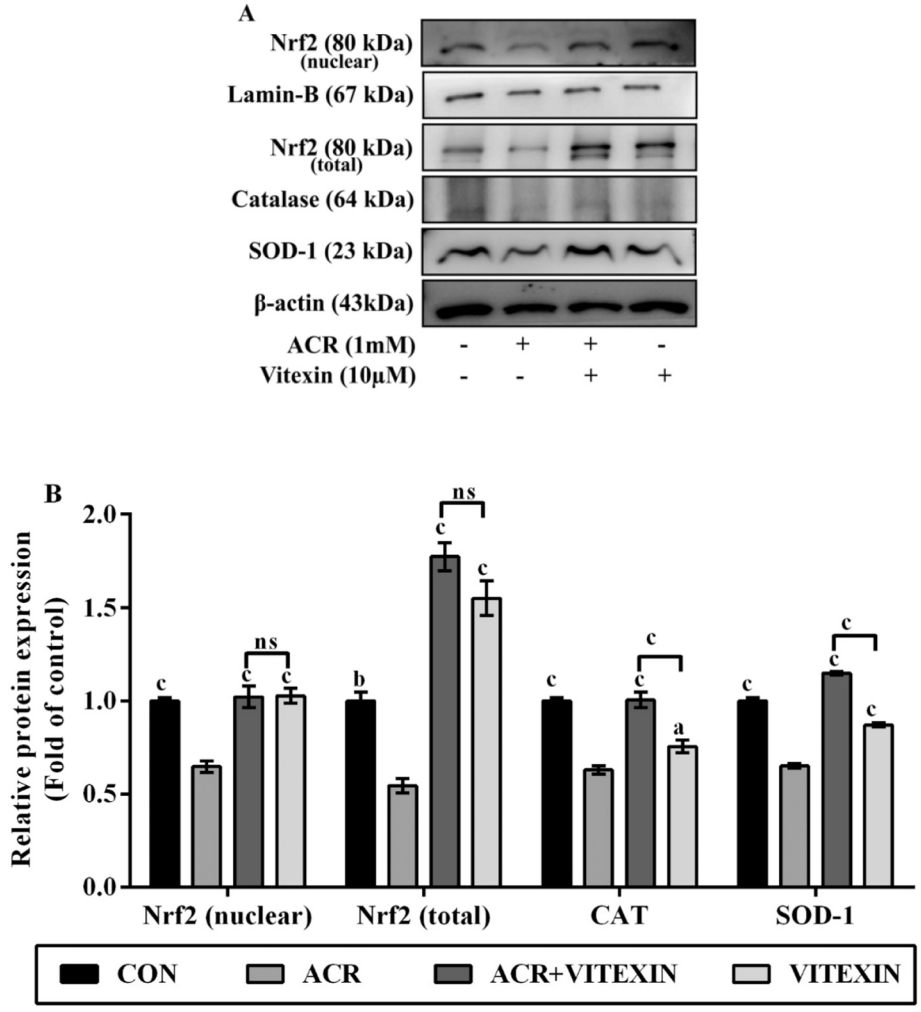

Fig. 6. Vitexin stimulates the expression of antioxidant response element via Nrf2 signaling in ACR larvae. (A) Protein levels of SOD-1, CAT and Nrf2 of all experimental group of larvae. Beta-actin and lamin-B was used as the loading controls. The relative intensity of corresponding protein levels was performed by ImageJ. Data are represented as mean ± S.E.M. of three independent experiments and the significant differences compared with ACR and vitexin alone group are denoted by $^{a}P < 0.05$, $^{b}P < 0.001$, $^{c}P < 0.0001$, ^{ns}non-significant (Tukey's posthoc test).

reduced the NO level in ACR larvae. This result suggests that NO has the capacity in provoking other inflammatory conditions, while vitexin abolished the toxic effect of NO in ACR larvae.

In addition, we quantified the level of TNF-α by ELISA and the results in Fig. 5B and Supplementary Fig. S2B showed that ACR larvae exhibited significantly ($P < 0.001$) increased TNF-α levels when compared to control. Whereas, vitexin treatment markedly reduced TNF-α levels in the larvae exposed with ACR. Additionally, we validated the expressions of pro-inflammatory cytokines by immunoblotting (Fig. 5C and D). We observed a significantly ($P < 0.05$) increased the expression of iNOS, nNOS, COX-2, and p-P38 in ACR larvae relative to control. However, 24 h of vitexin treatment significantly ($P < 0.05$) lowered the levels of inflammatory cytokines (iNOS, nNOS, COX-2, and p-P38) in ACR exposed larvae. These data provided the evidence for a detrimental and immunomodulatory effect of vitexin in microglial homeostasis.

3.5. Stimulation of Nrf2 expression by vitexin in ACR treated larvae

We then next evaluated the effect of vitexin on antioxidant proteins in control and treatment groups. Results from immunoblot analyses (Fig. 6) revealed the activation of oxidative stress in response to ACR treatment, as evinced by the impaired Nrf2 expression and its downstream pathway proteins (SOD-1 and CAT). Transcriptional activation of an antioxidant signaling pathway is strongly dependent on Nrf2 translocation, our results showed an increased expression of Nrf2 in the nuclear region associated with up-regulated SOD-1 and CAT levels upon treatment with vitexin. This result further suggests that the neuroprotective effect of vitexin might be involved in the regulation of Nrf2 signaling pathway.

3.6. Vitexin attenuates CDK5 and rescued the loss of neuroplasticity proteins in ACR larvae

Aberrant CDK5 expression is a sign of neural inflammation and a major criterion for AD pathogenesis (Cheung and Ip, 2012). Thus, we validated whether vitexin regulates CDK5 expression in ACR-induced larvae. In Fig. 7A and B, ACR larvae exhibited significantly ($P < 0.05$) elevated CDK5 expressions when compared to control. Whereas, vitexin treatment significantly ($P < 0.05$) inhibited the expression of CDK5 induced by ACR in zebrafish larvae. Thus inhibition of CDK-expression in ACR larvae suggests the protective effect of vitexin from neuronal injury signals. Studies also linked that hyperactivated CDK5 causes attenuation of neuroplasticity markers in the CNS such as CREB1 and ATF1 (Takahashi et al., 2010). Finally, we investigated the expression of above-mentioned protein makers by immunoblotting. As shown in Fig. 7A, larvae exposed to ACR for 3 days exhibit a significantly ($P < 0.0001$) declined expressions of pCREB1, CREB1, and ATF1 when

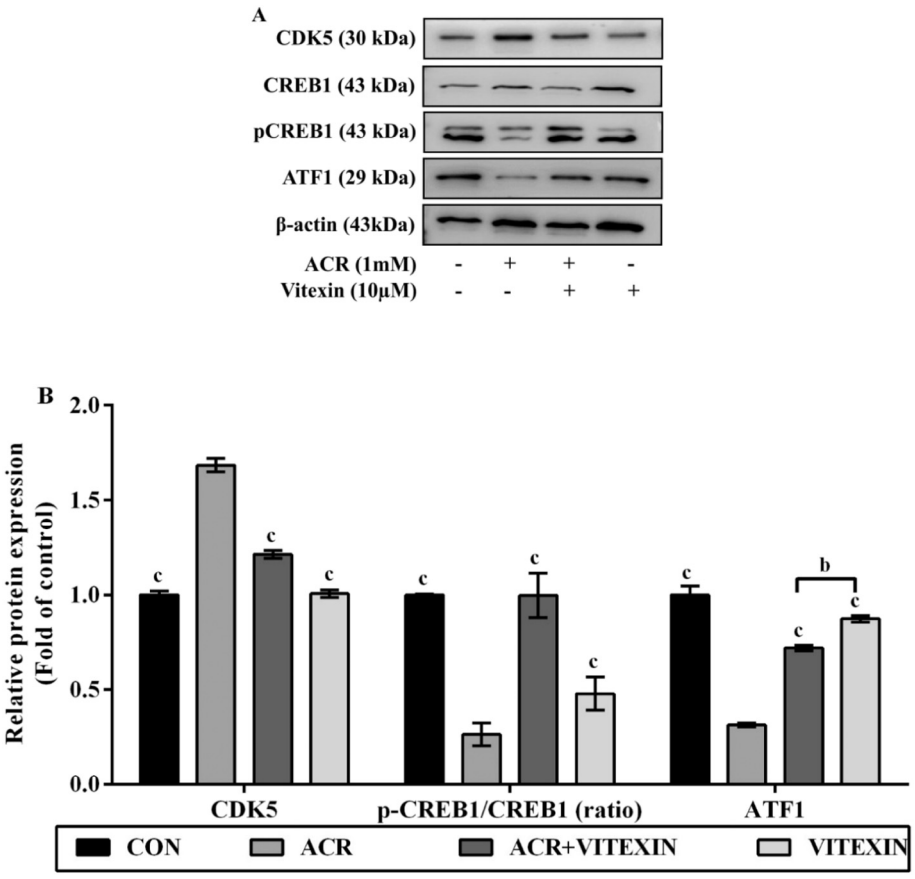

Fig. 7. Vitexin inhibits Cdk5 initiation and promotes CREB signaling in ACR larvae. (A) Immunoblot expressional analysis of CDK5, p-CREB1, CREB1, and ATF1 from ACR larvae treated with or without vitexin (B) Quantitative data representing the relative fold change of corresponding protein levels were performed using densitometry. Each bar represents the mean ± S.E.M. of three independent experiments and the values are statistically significant by Tukey's posthoc test. Comparisons: $^{a}P < 0.05$, $^{b}P < 0.001$, $^{c}P < 0.0001$ relative to ACR and vitexin alone larvae.

compared to control. Whereas, treatment with vitexin significantly rescued the loss of neuroplasticity marker protein in ACR-intoxicated larvae. Taken together our result suggests that a positive association of CREB1/ATF1 phosphorylation to vitexin, as well as its repressive inhibitory effect on CDK5, may contribute to attenuation of inflammatory responses in ACR exposed zebrafish larvae.

4. Discussion

Acrylamide (ACR) is a well-recognized neurotoxin, which can mimic the mechanistic features of the common neurological disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) (LoPachin and Gavin, 2012). Typically, microglia plays an important role in the maintenance of neurogenesis and neuroplasticity. This specialized macrophage cells constitute about 20% of the neuroglial cells and also act as a gatekeeper in regulating CNS innate immune systems. Microglia dysfunction on toxic insults can be enrolled in ROS activation followed by the release of several pro-inflammatory cytokines in the injured region and ultimately causes neuronal damage (Block and Hong, 2005). Numerous rodent models have already been addressing the involvement of microglia in the pathogenesis of neurodegenerative diseases (Dansokho and Heneka, 2018; Kaminska et al., 2016).

Considering all the above unresolvable factors, researchers have made a considerable effort in identifying plant-derived antioxidant compounds with potent neuroprotective properties for the treatment of neural disorders (Kelsey et al., 2010; Craggs and Kalaria, 2011). This study therefore undertook to investigate the neuroprotective effect of vitexin against ACR-induced neuronal inflammation in zebrafish larvae. In particular, we observed that vitexin induced an adverse outcome like Nrf2/CREB1 activation and CDK5 down-regulation, which provides a more profound protective approach against ACR toxicity.

Behavioral responses are validated as an indicator of motor neuron functions of an organism (Wu et al., 2016). Moreover, numerous reports related to motor neuron disorders have been extensively studied when exposure to neurotoxic compounds in zebrafish model systems (Altenhofen et al., 2019; Andersen et al., 2000). Zebrafish become a mature swimmer as early as 6dpf, therefore swimming behaviors can be used to study motor neuron patterns (Drapeau et al., 2002). We observed that ACR exposure reduced larval swimming behavior and the condition not rescued after being removed from exposure condition at 8 dpf, representing that these larvae had uncoordinated movement similar to those associated with Alzheimer-related symptoms (Faria et al., 2018). Recently Li et al. (2016) have hypothesized ACR exposure mediates severe locomotor deficits along with loss of dopaminergic neurons and α-synuclein aggregation in *C. elegans*. Interestingly, vitexin treatment rescued the uncoordinated swimming behavioral induced by ACR in zebrafish larvae. Our result suggests that the modulatory effect induced by vitexin on behavioral patterns might be the result of changes in the neurotransmission and neuronal plasticity defects.

Apart from the physiological role of NO in brain development, an intimate relationship between microglia activation and NO signaling in the neuroinflammation is well documented (Yuste et al., 2015). Studies have confirmed that MPO act as a mediator among inflammation and oxidative stress in the human brain which can be evoked by the action of NO pathway (Anatoliotakis et al., 2013). Once NO is altered by MPO under toxic insults, transcriptional activation of inflammatory mediators (iNOS, nNOS, COX-2, TNF-α, and P-p38) are up-regulated, leading to dopaminergic neuronal loss through an inflammatory action (Brown and Bal-Price, 2003; Anandhan et al., 2013). In this study, an increase in NO levels following ACR exposure is confirmed by enhanced expressions of iNOS, nNOS, COX-2, TNF-α, and P-p38 levels. Notably, these changes were diminished when larvae were treated with vitexin, thereby indicating that vitexin is capable of neutralizing neuronal inflammation induced by ACR. Our results strongly correlate with the neuroprotective effects of minocycline on MPTP-induced PD mouse model, exerted by suppressing the iNOS expression and possibly by

preventing the phosphorylation of p38 (Du et al., 2001).

It is well-documented that ACR results in oxidative stress and consequently unbalances the homeostasis of cellular antioxidant levels (SOD and CAT) by modulating the Nrf2 signaling in CNS. This change in Nrf2 signaling potentially leads to increased mitochondrial-associated susceptibility and neuronal death (Pan et al., 2017). Under oxidative stress, Nrf2 is rapidly dissociated from Keap1 complex and then translocated to the nucleus where it binds to an antioxidant response element (ARE) region, activating the release of scavenger metabolites in the cells (Kaspar et al., 2009). Our current study showed that ACR exposure decreased levels of SOD and CAT by inhibiting Nrf2 nuclear translocation in zebrafish larvae, whereas vitexin treatment markedly up-regulated nuclear translocation of Nrf2-mediated expressions of SOD and CAT. These results are in agreement with reported research (Pan et al., 2018), and imply that activation of the Nrf2 pathway can act as a central protective mechanism to reduce ACR-induced oxidative damage.

Cyclin-dependent kinase 5(CDK5) is a pleiotropic protein kinase molecule which plays a critical role in the number of beneficial and detrimental processes in the brain. The physiological process of CDK5 in the maintenance of neuronal plasticity and neurotransmission is tightly regulated by p35 or p39 activators. However, its pathological process is controlled by p25 or p29 activators (Camins et al., 2007). Recently research stressed that proteolytic removal of amyloid beta (Aβ) stimulates cleavage of p25 or p29 thereby prolonging CDK5 activation and increased amyloidogenesis (Wilkaniec et al., 2018). ACR exposure to zebrafish larvae induces significant up-regulation of CDK5 in the larval head region. The induction of CDK5 presumably correlates to the Aβ peptide-induced neuroinflammation. Several reports stressed that CDK5 inhibition activates the CREB signaling which has a beneficial role in regulating neurogenesis and plasticity (Gutiérrez-Vargas et al., 2015). Our analysis revealed that vitexin treatment stimulates the phosphorylation and activation of CREB1 and ATF1 in zebrafish larvae, indicating that inhibition of CDK5 by vitexin might unlock the CREB1/ATF1 signaling, resulting in improved neuronal survival.

5. Conclusion

By using acrylamide model of neurotoxicity in zebrafish larvae, we have validated the evidence that vitexin has the potentials on the recovery of behavioral phenotypes by suppressing oxidative stress and pro-inflammatory cytokines. Furthermore, vitexin add-on the neuroprotective effect in this study may be mediated through the inhibition of CDK5 release and rescuing the loss of neuroplasticity markers in zebrafish larvae. Further investigations are in progress to demonstrate the mechanism through which vitexin exerts its neuroprotection against AD disease model.

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Competing interests

The authors declare that they have no competing interests.

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