Fluoride – influenced presynaptic and MAPK – mediated TrkB signaling alterations, induced neurotoxicity

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Abstract

Fluoride (F), an inorganic substance, is everywhere in the environment. Fluoride exposure is caused mainly by geogenic sources, such as drinking wells and deep soil water. Excess fluoride-containing water usage for many years may cause neurotoxic damage. The mechanism underlying the neurotoxicology of endemic fluorosis remains obscure. Fluoride crosses the blood-brain barrier and accumulates within neurons. In our study, chronic exposure to fluoride-induced epileptogenesis in neuroblastoma (SK-N-SH) cells was followed with tools like Brain-derived neurotrophic factor (BDNF) and Synapsin 1 (SYN 1) protein expression. SK-N-SH cells were incubated with fluoride water (1 mg/L and 6 mg/L) for 24 h in-vitro. Prolonged ingestion of fluoride causes chronic fluorosis. Fluoride exposure reduced cell viability gradually from 48 to 96 h. The standard structure of axon spines and dendritic outgrowth in high fluoride water vanished. TrkB activated MAPK/ERK downstream signaling pathway is triggered by increased BDNF protein expression and decreased SYN 1 protein levels observed in fluoride water incubation. Further 96 hours incubation with fluoride-cleared water increased viability of SK-N-SH cells and a normal expression of BDNF and SYN 1 protein levels, suggests that fluoride-blocked BDNF-TrkB pathway might have been replaced by high-levels expression of BDNF protein where incubation medium contains concomitant lowered levels of fluoride. Hypothesis is that scaffold proteins regained normal expression might have influenced conducted normal excitation and transmission could reduce neurotoxic effect or even might control seizures.

1. Introduction

One of the essential resources and necessities of every human being is access to safe drinking water. Shallow reservoirs become contaminated as groundwater percolates via a range of geological formations. Fluoride is the most hazardous ion for people to drink from the water, and it consequently exists in the environment as fluoride. There are two sources of fluoride in water: anthropogenic and geological (Amit Saini & Pinki Rani Agarwal, 2021). Fluoride is a water pollutant that is frequently present in the environment, and it forms fluoride compounds and mineral components in deep soil and rock combined with other elements. Globally, endemic fluorosis is common and poses a severe health risk to humans (John J Warren & Steven M Levj, 2003).

Endemic fluorosis, a condition caused by continuous fluoride exposure, was recognized as a severe public health concern in many developing countries where widespread fluoride pollution of drinking water has been observed (Grandjean P & Landrigan PJ, 2014). According to research, the most severely affected countries in the world, China and India, have groundwater fluoride concentrations that reach 48 mg/L (Feng F et al., 2020; Mridha D et al., 2021). Fluoride ions (F) are found in groundwater in India in concentrations ranging from 0.01 mg/L to 48 mg/L. According to estimations, more than 200 million people drink water with fluoride levels higher than the WHO-recommended limit of 1.5 mg/L (Kut KMK et al., 2016).

According to Yan et al.(2016), fluoride can accumulate in the brain after crossing the blood-brain barrier and harming neurological functions. Epidemiological studies suggest that high fluoride levels in drinking water have been related to adverse effects on children's neurodevelopment, including problems with IQ, memory, and cognitive performance (Guth S et al., 2020). Additionally, studies on animals have demonstrated that long-term exposure to fluoride causes seizures, anxiety-depressive symptoms, memory, cognitive and concentration problems, autism spectrum disorder, and sensory-motor gating (Flace p et al., 2020; Strunecka A & Strunecky O, 2019; Adkins EA & Brunst KJ, 2019). According to this study, F was among the most environmental and developmental neurotoxicants (Grandjean Philippe & Landrigan J Philipp, 2014).

The environmental toxicity of F for the brain has been proven in invitro using cells with a neuronal origin and in vivo using laboratory animals. However, scientists and clinicians have disagreed over the issue of whether F is harmful to neurological and cognitive functioning for a few decades. Even though reports of the detrimental effects of F consumption on IQ and mental sharpness continue to be made, these assertions are still refuted (Dec K et al., 2017).

Research on environmental risk factors for autism spectrum disorders (ASD) has increased significantly. According to recent studies, new ecotoxicological factors could explain up to 40 to 50% of the variation in the risk of ASD (Almandil NB et al., 2019). The possible consequences of this toxicant exposure on ASD remain little understood. Based on a UNICEF assessment released in December 1999, fluorosis is prevalent in at least 25 countries, including China and India, Indonesia, South Africa, Iran, and other areas (UNICEF, 1999). There is a connection between ASD and this developmental disorder that encompasses social, verbal, cognitive, and behavioral issues, and recurrent seizures are a recognized indicator of epilepsy. About 0.1% of children globally have ASD. In the US, 1.5% of children are thought to have ASD. Children with autism are more likely to have epilepsy. Seizures are the most common neurologic impact in ASD (Frank MC Besag, 2018).

Even though fluoride has long been thought to be a developmental neurotoxin, the mechanisms behind fluoride's adverse effects on the developing brain are unknown. Hundreds of billions of synapses of individual brain neurons are connected to create precise neural circuits that support cognitive and peak performance (Wu Y et al., 2020). Cognitive difficulties and mental retardation are neurological developmental problems caused by inadequate synapse formation or function throughout infancy (Lepeta K et al., 2016).

It has been shown that in the rat hippocampus, severe perinatal fluoride exposure decreases PSD 95 and SYN abundance, widens synaptic clefts, and reduces PSD thickness (Chen J et al., 2018). The effects of prenatal fluoride exposure on the development and proliferation of synapses and dendritic spines, as well as the underlying mechanisms, are yet unknown. It is well known that BDNF plays a crucial role in controlling cognitive function. BDNF must interact with the TrkB receptor for neurons to survive, differentiate, generate dendritic spines, and mature their synapses (Miranda M et al., 2019). During synaptic development and maturation, the BDNF-TrkB pathway and its intracellular signaling pathways

targets, including PSD 95 and SYN, are crucial for synapse formation and maintenance (Yoshi A & Constantine Paton M, 2010). Cognitive deficit problems and anxiety have been linked to abnormalities in the BDNF-TrkB pathway, as have treatment outcomes (Qiu LL et al., 2020).

According to Chen J et al. (2018), the study investigated the role of BDNF-TrkB signaling in the effects of fluoride exposure on synaptogenesis. We demonstrated that fluoride decreased the dendritic spine density and the synaptophysin (SYN) expression and postsynaptic density protein-95 (PSD-95) synapse proteins in the hippocampus, suggesting that fluoride-induced cognitive deficits are associated with synaptic damage. In human neuroblastoma SH-SY5Y cells, fluoride treatment decreased dendritic outgrowth and expression of SYN and PSD95. These findings imply that fluoride's developmental neurotoxicity is linked to synaptogenesis dysfunction, which is brought on by disruption of the BDNF-TrkB signaling pathway, which is mediated by MAPK/ERK1/2.

It is unclear whether BDNF-TrkB signaling plays a role in the synaptic dysfunction brought on by fluoride. According to epidemiological research, fluoride works as a possible neurotoxic in utero, and higher fluoride exposure during pregnancy is linked to lower IQ (intelligence quotient) in children, particularly in boys (Green R et al., 2019). Fluoride-induced excitotoxicity that harms the central nervous system is currently a significant concern. The mechanism underlying the associations between fluoride exposure and anxiety and depression, however, is still unknown. Therefore, in the current study, we established the mouse model exposed to NaF for 90, 120, and 150 days, respectively, to investigate the effect of fluoride on depression, seizures, and anxiety. Various methods were then used to evaluate behavioral models of anxiety and depression, such as elevated levels of BDNF (Blaylock RL, 2004).

According to Almeida et al. (2005), the MAPK signaling pathway may be the most important in BDNF-related neuroprotection. The high-affinity receptor TrkB binding to BDNF is crucial for the plasticity, survival, and learning memories of neurons (Leal G et al., 2015; Park H & Poo MM, 2013). BDNF and its imitators have been shown to offer therapeutic promise in various neurological disorders, including traumatic brain injury (TBI). People often get epilepsy after a head injury, with seizures happening in the months or years after the injury (Lin TW et al., 2020). BDNF enhances neuronal excitability and is concentrated and increased in regions linked to epileptogenesis. BDNF protein levels rise during seizures, and studies have shown that blocking BDNF signal transduction stops seizures from starting and that BDNF promotes epileptogenesis (Binder KD et al., 2001).

In this study, the impact of fluoride-content water exposure on epileptic impairments and cellular, synaptic changes was examined using a matured neural cell model using human neuroblastoma (SK-N-SH cell) to discuss the neurotoxic effects of fluoride (F⁻), as well as the underlying mechanisms of subsequent neuronal repair. We also examined how BDNF-TrkB affects fluoride-induced neurotoxicity and the signaling pathways involved.

2. Materials And Methods

Chemicals and reagents

Antibodies specific to (MAPK) p-ERK1/2 (#9101), TrkB (#4607), Synapsin 1 (#5297), and PSD 95 (#3409) were purchased from Cell Signaling Technology (Beverly, MA, USA). BDNF (SAB4200744), -actin (A2228), p-MEK 1 inhibitor (PD98059), and all other general chemicals were analytical grade and purchased from Sigma-Aldrich, USA.

Selection of Sample site

The criteria for selecting sample sites are based on the population density and area of industrial or anthropogenic such as minerals and mining activities. The sample collection spotted dense fluoride deposited areas in the Dharmapuri district, and the sample types were deep soil water (bore well). In the sample studied, fluoride levels were found to be higher than the WHO cutoff level of 1.5 mg/L in drinking water samples at Pappireddipatti village – zone of Manjavadi (F concentration range at 6 mg/L) during winter in the year 2020-21.

Experimental design for the In vitro method

This study aims to use human neuroblastoma cells (SK-N-SH) that were exposed to 1 to 6 mg/L of fluoride concentration for 24 hours. It was observed that fluoride exposure reduced cell viability for 48–96 hours.

Group I: Control (Normal cells)

Group II: Human neuroblastoma (SK-N-SH) cells were administered with an untreated water sample (6 mg/L of fluoride) for four continuous days. Fluoride water: Fluoride content of groundwater at a concentration of 6 mg/L for 48 to 96 hours (Fluoride contaminated water).

Group III: Human neuroblastoma (SK-N-SH) cells were administered a fluoride-removed water sample (1 mg/L of fluoride) for four continuous days. Fluoride removed water: *Strychnos potatorum* seeds imbibed water for 48 to 96 hours. *Strychnos potatorum* seeds (clearing nut) were at 2 g/L filled with water sample suspension, and filtrates were separated through a filter system design. The dosage of *Strychnos potatorum* seeds was chosen based on an adsorbent dosage study (Jayanthi G & Akshaya V, 2022).

Groups II & III were subdivided into groups (48 h, 72 h, and 96 h) varied by incubation periods. It was observed daily, and then cell viability was examined to identify cell morphological changes in prolonged ingestion of F-for periods of 48–96 hours.

Cell culture and treatment

The National Centre for Cell Science provided human neuroblastoma SK-N-SH cells (NCCS Complex, Pune, India). Cells were grown in Dulbecco's Modified Eagle Medium/F-12 Ham's (Gibco, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) supplemented with 10% fetal bovine serum in an incubator

at 37°C with a humidified environment that contained 5% CO_2 (Gibco, Thermo Fisher Scientific Inc.). Exposure to fluoride concentrations of 6 mg/L in untreated water and 1 mg/L in treated water was done on exponentially developing cells for 24 to 96 hours. Non-exposed cells were used as a control (normal cells).

Cell viability assays

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) by the manufacturer's instructions after cells were seeded onto 96-well plates at a density of 6000 cells per well. After exposure, 10l of CCK-8 solution was added to each well, and the plates were then incubated at 37°C for an additional 2 h. A multi-detection microplate reader (Model, Synergy 2; BioTek Instruments Inc., Winooski, VT, USA) was used to measure the optical density at 450 nm. Cells were pretreated for 1 hour with the 10M MEK inhibitor PD98059 to inhibit the phosphorylation of ERK.

Immunological staining for morphological analysis

In 24-well plates, the SK-N-SH cells were seeded onto sterile coverslips. The cells were exposed to the untreated water sample (6 mg/L of fluoride) for 48–96 hours. The coverslips were washed three times with phosphate-buffered saline (PBS), fixed for 30 minutes in 4 percent paraformaldehyde (PFA), and permeabilized for 30 minutes with 0.1 percent Triton X-100. Coverslips were stained with an immunological (DAPI) staining solution for 15 minutes at room temperature to see the nuclei. They were then rinsed with PBS in the dark while examining the slides under a fluorescence microscope.

Western blotting examination

After treatment, the SK-N-SH cells were centrifuged at 12,000 rpm at 4°C for 15 minutes after being homogenized in RIPA buffer (Radiant research center, Bangaluru). The bicinchoninic acid (BCA) protein assay kit collected the supernatant and determined its protein level (Radiant research center, Bangaluru). After equalizing the concentration of the samples with a loading buffer, the mixture was heated for 10 minutes. 50 g of proteins were loaded, separated by 12% SDS-PAGE, transferred to PVDF membranes (Roche, Inc., USA), and blocked at room temperature for 2 hours in 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST). Primary antibodies against Synapsin 1 (1:5000), BDNF (1:5000), p-ERK1/2 (1:5000), TrkB (1:5000), PSD 95 (1:5000), and -actin (1:5000) were then treated with the membranes overnight at 4°C (1:5000). All primary antibodies were diluted to a concentration of 1:1500 and secondary antibodies to a concentration of 1:5000. After that, the membranes were treated with goat anti-rabbit IgG that had been HRP-conjugated for 1.5 hours. The enhanced chemiluminescence (ECL) plus substrate (Solarbio, Beijing) was utilized to identify the protein bands, which were then used to expose and observe on X-ray film. Using Image J, the generated protein bands were examined (National Institutes of Health, USA).

Statistical analysis

Each experiment was performed in triplicate (n = 3). Results from the data were presented as mean and standard deviation (SD). One-way ANOVA was used to examine differences between various groups, and $P < 0.05$ values were considered significant. MS Excel Autosum ($\sum$) was used to do the statistical analysis, and MS Excel was used to plot the bar graphs.

3. Results

Fluoride toxicity and cell viability of the SK-N-SH cell line

SK-N-SH cells are prone to toxicity and stress when exposed to 6 mg/L fluorides in natural deep soil water (untreated) and 1 mg/L fluoride concentration (treated) water for 48–96 hours. The percentage of cell viability was then observed and displayed in Table 1. When cells were exposed to 6 mg/L of fluoride-contaminated water for 48 hours, 72 hours, and 96 hours in triplicates, their viabilities were substantially decreased from 29.40%, 26.45%, and 20.54%, respectively. Reduction in viable cells compared to control cells, significantly different from triplicates discovered at level P < 0.05 (Fig. 1).When fluoride-removed water was exposed for 48–96 hours, cell viability was raised considerably at 73.45%, 72.47%, and 72.34%, respectively. In the present study, deep soil natural water containing 6 mg/L of fluoride content was exposed to SK-N-SH cells for 96 hours. A maximum percentage of viable cells (98.9 ± 30.12) was noted. In most cases, fluoride activity elevation significantly differed from the control (Table 1).

Table 1
presents cell viability in untreated & treated ground water contamination of fluoride toxicity on human neuroblastoma SK-N-SH cell line

S.No	Sample	Concentration	% Cell viability
1	Untreated water (Group II)	6 mg/L	
	48 h		29.40 ± 0.66
	72 h		26.45 ± 0.79
	96 h		20.54 ± 0.61
2	Treated water (Group III)	1 mg/L	
	48 h		73.45 ± 0.50
	72 h		72.47 ± 0.86
	96 h		72.34 ± 0.63

Units: Table values are expressed as average concentration of Mean ± SD in each group. All units are expressed in mg/L. Water sample collected in Dharmapuri block: Sample – Pappireddipatti (Avarankaatur). Group II & III were subdivided into three groups (48 h, 72 h, & 96 h) varied by incubation periods.

Morphological changes

Fluoride content & removed water causes synaptic morphology to change on a cellular level and reduce synaptic protein production in SK-N-SH cells.

Synaptic morphological and structural abnormalities and synaptic protein level expression were applied in the present study to understand further the mechanisms underlying fluoride neurotoxicity. SK-N-SH cells underwent several alterations after exposure to fluoride-contaminated and treated water. In contrast, cells induced with fluoride-contaminated water samples developed smaller, spherical bodies that were loosely adherent, and their cytoplasm contained a dark substance. Control and treated cells appeared highly dendritic, were firmly attached to the culture vessels, and were reflective to white light, according to morphological examination (Fig. 2). Several in vitro tests were also performed to evaluate fluoride's effects on synapses. The length and quantity of dendritic branches in neuroblastoma SK-N-SH cells were decreased by fluoride-contaminated water, according to the results of immunofluorescent staining (Fig. 2). to assist in the morphological examination of the cells, SK-N-SH cells were stained with DAPI. It was found that axon spines vanished and lost their typical shapes and diameters after exposure to fluoride-contaminated water—these alterations developed in a dose-dependent manner.

These findings imply that fluoride exposure during development results in morphological changes to neurons' dendrites and synapses, which is closely related to cognitive impairment. In SK-N-SH cells, fluoride toxicity was noted from fluoride content water and removed the water, and the morphological alteration in synapses was also noted. When compared to control and fluoride-removed water, the expression of the presynaptic marker SYN 1 (Synapsin 1) and the postsynaptic marker PSD 95 protein was decreased after exposure to fluoride in fluoride-contaminated water at 48 and 72 hours (Fig. 2a**&b**; P < 0.05). Similar to the findings of the in vitro studies, the expression levels of SYN 1 and PSD-95 at 72 hours in the group exposed to 6 mg/L fluorides are significantly lower than those in the control and fluoride-removed groups. Furthermore, exposure to fluoride-containing water reduced the protein marker of PSD 95 expression, particularly in the synapse.

These findings suggest that fluoride-contaminated water disrupts dendritic development and the preservation of effective synapses in vitro.

Western blot examination

BDNF-TrkB protein expression in the SK-N-SH cell line

BDNF is widely expressed in the brain and regulates neurotransmitter release, survival, synapse formation, and activity-dependent synaptic shape and function changes. As shown in Fig. 3a **and b**, TrkB levels were significantly lower. In contrast, BDNF levels were significantly higher in the group of water contaminated with 6 mg/L fluorides for 72 hours, indicating that the BDNF-TrkB signaling pathway had been disrupted compared to the control fluoride-removed water (P < 0.05). According to our findings, seizures might cause modifications and abnormalities in the expression of neurotrophins and their receptor TrkB with the support of various scientific study references.

Epileptogenic defects in SK-N-SH cells: influence of fluoride on p-ERK1/2 protein levels

We also looked for evidence of ERK1/2 in aberrant alterations of the BDNF–TrkB pathway and synapse (Fig. 4). p-ERK1/2 (MAPK) protein levels were significantly higher in the 6 mg/L fluoride-containing contaminated (toxic) group at 48 and 72 hours compared to control and fluoride-removed water (P < 0.05). PD98059, a phosphorylation inhibitor for the activation of ERK1/2, reversed the abnormal synapse structure and its expression, BDNF signaling, and synapsin 1 & TrkB expression caused by fluoride contamination (Fig. 4a**&b**). These findings imply that p-ERK1/2 protein plays a significant role in the dysregulation or malfunction of ERK1/2 signaling fluoride, which has been linked to influencing stroke and causing Alzheimer's disease.

SK-N-SH cells in the expression of Synapsin 1 protein under the influence of fluoride in water

Synapsins are a class of proteins found in synaptic vesicles at presynaptic terminals, where they play a role in neurotransmitter release controlled by phosphorylation. When compared to the control and fluoride-removed water, fluoride-contaminated water resulted in lower levels of expression of the presynaptic marker SYN 1 (Synapsin 1) protein in 48 and 72 hours of incubation (Fig. 5a**&b**; P < 0.05). In SK-N-SH cells incubated for 48 & 72 hours with fluoride-removed water, SYN 1 protein expression levels were near normal compared to the control. Reduced SYN1 protein expression and synaptic density were discovered, as well as a decline in synaptogenesis linked to neuronal degeneration such as epilepsy and behavioral disorders. From the results and figures from our findings, excessive exposure to fluoride-contaminated water impairs synaptogenesis in neurons and might increase excitability in neurons, which would have increased epilepsy susceptibility.

The influence of fluoride in water on the epileptogenic status and synaptic plasticity of SK-N-SH cells in the expression of PSD 95 protein

PSD 95 is an essential scaffolding protein that regulates many receptors, channels, and signaling molecules in the postsynaptic density of excitatory synapses. The level of expression of PSD 95 (postsynaptic density 95) was assessed. High fluoride water exposure at 48 and 72 hours resulted in lower levels of expression of the postsynaptic density (PSD) 95 protein at 48 and 72 hours compared to control and fluoride-removed water (Fig. 6a**&b**; P 0.05). The cerebral cortex, hippocampus, and striatum have high PSD 95. Decreased PSD 95 has been associated with cognitive and learning difficulties in neurodevelopmental diseases. The scaffold protein is necessary for preserving the intricate network of protein connections in the postsynaptic density (PSD) of excitatory neurons, which is crucial for neuronal survival and function.

4. Discussion

In many emerging nations, fluorine pollution in drinking water was found to be high (up to 24.17 mg/L). It is now known that endemism fluorosis, an endemic disease brought on by persistent fluoride exposure, poses a severe threat to public health (Yadav KK et al., 2017). Fluoride levels in underground water can

reach up to 48 mg/L, with 35 and 26 million people affected in China and India, respectively (Murntaz N et al., 2015; Jadhav SV et a., 2015). In a previous study, elevated fluoride has been found to diminish the survival of SH-SY5Y cells after 48 hours of exposure (Liu YJ et al., 2011), with cell survival loss ascribed to a decrease in SIRT1 expression. Although there is consensus on the capacity of NaF to produce neurodegenerative disorders, the mechanism of neurotoxicity remains unknown. According to the findings of the MTT and LDH assays in this research (Gu X et al., 2016), fluoride may drastically affect regular cellular functioning and membrane integrity in a dose-dependent manner.

Drinking ground and deep soil water is humans' most common source of fluoride contamination. Maximum fluoride concentrations in groundwater have been reported to reach 30–50 mg/L (Barbier O et al., 2010). Furthermore (Wu J et al., 2015), NaF doses of 20, 40, and 80 mg/L have been utilized to treat a variety of cell lines in the past, including primary rat hippocampus neurons, neuron-like rat pheochromocytoma (PC12) cells handled by Zhang M et al. 2007, and SH-SY5Y cells handled by Xu B et al. (2011). SK-N-SH cells were given and incubated for the following times: 48 hours, 72 hours, and 96 hours.

According to Song C et al. (2013), 2 ppm fluoride treatment significantly time-dependent cell proliferation in TM3 Leydig cells to 73.5% at 24 hours, 65.6% at 48 hours, and 62.3% at 72 hours, whereas with 16 ppm concentration, the time-dependent activity of viable cells was 65.7% at 24 hours, 54.3% at 48 hours, and 50.1% at 72 hours. These findings demonstrate that NaF reduced the quantity of DNA generated in Leydig cell types, corresponding to a reduction in cell proliferation.

The cell viability data demonstrated that F exposure reduced cell viability, similar to He P et al. (2006) findings of F-induced damage in primary cultured rat hippocampus cells. Furthermore, a linear association was discovered between cell activity decrease and increased exposure period, which was similar to the results of Kubota et al. (2005). Cell viability demonstrated a dose-effect response at 12 and 24 hours after F exposure, with "proliferation in low concentration and inhibition in high concentration." Low F concentrations encouraged cell survival, but high F concentrations reduced it. Low amounts of F, as with many toxins, may cause (Duan WX & Wang GF, 2012).

Fluoride treatment has been shown to reduce cell survival in dosage and temporal-dependent ways in various cells, including Sertoli (Yang Y et al., 2015) and primary hippocampal neural cells (Zhang M et al., 2007). Furthermore, several investigations found that following NaF treatment, the number of germinal and interstitial cells dramatically decreased (Bataineh NH & Nusier MK, 2006). In Leydig cells, 2 ppm NaF exposure reduced cell viability by 90% after 24 hours, 89% after 48 hours, and 88% after 72 hours, whereas 16 ppm sodium fluoride exposure reduced cell viability by 86% after 24 hours, 83% after 48 hours, and 81% after 72 hours, indicating that NaF reduces cell viability dose and time-dependently.

Fluoride is toxic to the brain, and long-term exposure results in abnormalities in the structure of the brain's cells. The morphological changes in the various brain areas and subregions in fluoride-treated mice have been attempted to quantify. In a study carried out by our team, rats were subjected to maternal exposure to drinking water containing 30 or 100 ppm fluoride from when they were fetuses until they were ten

weeks old. The brain histology of young rats exposed to 30 ppm fluoride did not exhibit any noticeable abnormalities, while animals subjected to 100 ppm fluoride displayed severe neurodegenerative changes in the hippocampus, amygdala, motor cortex, and cerebellum. The alterations included shrinking Purkinje cells in the cerebellum, shrinking neurons in all brain parts, and evidence of chromatolysis and gliosis in the motor cortex (Shivarajashankara YM & Shivashankara AR, 2012).

According to Shivarajashankara YM & Shivashankara AR (2012), in addition to the loss of molecular layer and glial cells layer in the brain tissue chromatolysis and ballooned Purkinje neurons, vacuolization in the perikaryon, and the presence of spheroid bodies in the neuroplastic were also significantly observed in 5–50 mg/ml/kg/day sodium fluoride injected (15 weeks) rabbits.

Fluoride ingestion at 100 ppm for 1 month in rats and various changes in neural cells was demonstrated by Guinea et al. (2012). Like Purkinje cells, signs of chromatolysis, gliosis in the motor cortex, hippocampus region, neuron size in all areas, the neurons were swollen in the cerebrum, pyknosis, irregular staining of Nissl substance, hyperchromatic nuclei, and irregularities in nuclear shape were also observed. Subcellular damage like mitochondrial swelling, cellular edema, necrosis, and vacuolization was similar. The disappearance of an axon, nuclear dissolution, and a gradual reduction in fluorescence intensity of F-actin

Rico PE et al. (2022) study results suggest that agrochemicals and drinking water contain fluoride, an essential component. Neurological and toxicological disturbances are linked to excessive fluoride exposure. Long-term exposure to sodium fluoride in the Zebrafish brain affects the cholinergic and glutamate systems and oxidative stress balance. The sodium fluoride-prompted neurodegenerative processes in the Zebrafish brain may result from the inhibitory influence on the cholinergic and glutamatergic signaling mechanism.

Neuronal plasticity is aided by the protein brain-derived neurotrophic factor (BDNF). A growing body of data shows that psychological stress reduces BDNF expression and that a lack of neurotrophic support leads to depression. Sleep problems lower BDNF levels, and a lack of sleep makes you more vulnerable to stress, which leads to a drop in BDNF levels. Stress is a strong risk factor for depression and has been linked to lower levels of BDNF in animal studies (Torregrossa MM et al., 2006). According to various studies, long-lasting seizures like status epilepticus (SE) change the expression of NT, pro-NT, and their receptors (Montroull L et al., 2019). According to a recent study by Usain et al. (2008), inhibiting both matured BDNF and pro-BDNF signal lowers neural cell death after SE in vivo.

BDNF is a chemical that regulates activity-dependent changes in synapse shape and function and controls synapse development. The Ras/ERK pathway is activated by BDNF activation, which enhances synaptic spine density. Dysfunction or a reduction in BDNF causes synaptic plasticity to fail, as well as a drop in excitatory neurons and glutamate, which may contribute to depression (Bathina S & Das UN, 2015). Through TrkB BDNF, TrkB signaling plays a pathophysiological role in TBI and SE-induced epilepsies. In axonal transection models, the contribution of BDNF/TrkB signaling to epileptogenesis is

the opposite in neocortex and hippocampal lesions. The BDNF–TrkB axis was identified (Lin TW et al., 2020).

According to Greenwood et al. (2018) findings, seizures cause alterations in the expression of neurotrophins, proneurotrophins, and their receptors. Pilocarpine causes a protracted seizure state termed status epilepticus (SE) in rats previously treated with lithium, which results in apoptosis in various parts of the brain (Fujikawa DG, 1996). SE promotes the expression of matBDNF and pro-BDNF proteins (Unsain et al., 2008; Rudge JS et al., 1998), and matured BDNF's high production and release causes TrkB to be rapidly downregulated (Unsain et al., 2009). It has previously been demonstrated that endogenous matBDNF and proBDNF have a role in SE-induced neuronal injury (Unsain et al., 2009). However, the methods used in that study were not good enough to tell what role each type of BDNF played in neuronal death after SE.

BDNF stimulates dendritic and axonal growth and synaptogenesis during brain growth and maturity (Benarroch EE, 2015). In vitro research has revealed elevated BDNF levels, consistent with our findings (Jiang C et al., 2014). Evidence suggests that, in vivo and in vitro, chemical stimulation greatly raises levels of BDNF in neurons (Donnerer J & Liebmann J, 2017; Zhang B et al., 2017). Children's blood levels of BDNF increased, and their olfactory memory decreased in some areas, whereas increased levels from recycling e-waste and dismantling sites were present (Zhang B et al., 2017). These findings demonstrate that BDNF is overexpressed in limbic seizure-prone brain regions and that BDNF-TrkB signaling disruption is associated with fluoride-induced developmental neurotoxicity. It has been proven to be the root cause of both brain cell destruction and seizures.

The Ras/ERK pathway is activated by BDNF activation, which enhances synaptic spine density. Dysfunction of BDNF–TrkB causes synaptic plasticity to malfunction and reduces excitatory neurons, which may contribute to depression (Yang T et al., 2020).

The above findings infer that high levels of BDNF enhance cell excitability and vulnerability to epilepsy, resulting in neuronal damage and perhaps promoting epileptogenesis. The findings of our study suggest that molecular strategies to regulate TrkB signaling include activation for neuroprotection or psychiatric issues and suppression of cell proliferation and epileptogenesis. According to the underlying premise, BDNF/TrkB signaling changes cause or promote several illnesses. Alteration in TrkB signaling in Alzheimer's disease, Rett syndrome, and traumatic brain damage such as status epilepticus have been linked (SE) (Wang X et al., 2021).

Signaling pathways regulate several physiological and pathological processes, and abnormal signaling pathway activation leads to the progression of many disorders. The mitogen-activated protein kinase family includes ERK1/2, which impacts several processes, including metabolism, motility, and inflammation, as well as cell death and survival. In the neurological system, ERK1/2 is a potent modulator of neuronal CNS diseases (Jing sun & Guanxian Nan, 2017). According to Alam R and Gorska MM (2011), ERK1/2 signaling disruption has been connected to a variety of human diseases, including cancer, asthma, stroke, and Alzheimer's disease.

Ratto and Pizzorusso (2011) demonstrated that ERK1/2 controls synaptic plasticity in the visual cortex in both vivo and in vitro experiments. Inhibition of ERK1/2 in the hippocampus and amygdala may inhibit the formation of different types of long-term potentiation (LTP) and long-term depression (LTD). According to this research, many kinds of synaptic plasticity need ERK1/2 activation, although the particular targets of ERK1/2 may vary.

Fluoride is a neurotoxin that has a significant adverse effect on the developing brain; impaired mental abilities were seen in children belonging to endemic fluorosis areas and in fluoride-induced neurotoxicity in experimental animals. Fluoride generates free radicals to relax, thereby increasing lipid peroxidation, depleting antioxidants, inhibiting critical enzymes in metabolic pathways, reducing energy production, and decreasing protein synthesis. Animal trials on chronic fluoride toxicity have shown a wide range of results, which could be owing to changes in the fluoride administration, dose, duration made of animal species employed, or organ-specific metabolic reactions. Excitotoxicity, which has been suggested as the leading cause of fluoride's neurotoxic effects, needs to be studied more through mechanistic studies, and there needs to be more research into how to treat brain problems caused by fluoride (Shivarajashankara YM & Shivashankara AR, 2012).

Karube et al. (2009) reported that fluoride could impact a signaling cascade that involves MAP kinase, a stress-induced response. According to O. Barbier et al. (2010), the Ras/MEKK/MEK pathway activates extracellular signal-regulated protein kinase (ERK). A plausible mechanism for NaF-induced ERK activation in Ras, a direct target for fluoride. Changes in tyrosine phosphorylation and the PKC-dependent pathway were observed in NaF-induced MAPK activation.

Synapsin 1 regulates synapse formation (synaptogenesis) throughout neuronal development and synaptic function in the adult brain. SYN1 is a gene-coded protein associated with X-linked illnesses with primary neuronal degeneration, such as Rett syndrome, epilepsy, learning difficulties, and behavioral abnormalities (Guamieri FC et al., 2017).

Additionally, Alabi AAR & Tsien WR (2011) research indicates that the dynamic reconfiguration of synaptic vesicle pools is problematic after extended stimulation of synaptic transmission. Take into account the following to clarify this distinction: We investigated whether synapsin phosphorylation known to influence synaptic plasticity, is associated with the epileptic activity. We discovered a fluoride-induced decrease in synapsin phosphorylation using a western blot at MAPK/ERK sites. These two proteins are essential for synaptic modulation.

Genetic and functional research have linked synapsin genes to a variety of neurological illnesses, including schizophrenia, bipolar disorder (BD), Alzheimer's disease (AD), Multiple Sclerosis (MS), Huntington's disease (HD), and epilepsy. In all the above diseases, the expression of synapsin genes and proteins was linked to neurological abnormalities, piquing researchers' interest in learning more about the regulatory mechanisms that underpin these alterations (Fatima Javed Mirza & Saadia Zahid, 2018).

The levels of the synaptic proteins glutamate receptor 2, postsynaptic density protein (PSD) 95, and synapsin 1 significantly decreased after endoplasmic reticulum (ER) stress was induced, offering new information about the cognitive deficits and compromised neurotransmission in the hippocampus of Alzheimer's patients. Also, a significant loss of the postsynaptic markers PSD 95 and synapse-associated protein 97, as well as the presynaptic markers synapsin 1 and synaptophysin, in people with mild cognitive impairment raises the possibility that synaptic loss in the posterior cingulate gyrus is linked to a significant loss of synaptic function in both the early and late stages of Alzheimer's disease (Fatima Javed Mirza & Saadia Zahid, 2018).

SYN 1 gene expression abnormalities have been associated with severe neuropsychiatric diseases like epilepsy and autism. Neuronal plasticity abnormalities in SYN 1 are linked to epileptic seizures and behavioral problems. Also, a nonsense mutation in the SYN 1 open reading frame (ORF) has recently been found to cause epilepsy and autism. This mutation causes problems with neurite outgrowth and phosphorylation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), which leads to poor synaptic function (Francesco Paonessa et al., 2013).

Evidence suggests that epileptic status may be due to defective synapsin protein expression or function. Increased fluoride content in water-incubated cells expressing down-regulated synapsin vividly explains fluoride interruption in synapsin protein expression and the occurrence of epilepsy or seizures in our findings.

Furthermore, our findings suggest that the expression of PSD 95 was progressive reduction as fluoride concentration increased and lowered significantly in the moderate fluoride concentration group compared to that of the control group. It infers that a drop in PSD 95 expression could be the molecular cause of central nervous system damage caused by fluoride (Zhu W et al., 2011).

Samuels et al. (2009) suggest that brain development and functions are affected by ERK1/2 molecules. Deletion or mutation of components in ERK1/2 molecules may result in developmental brain disorders like cognitive function. Inhibition of ERK is linked to dendritic spine loss and reduced SYN, PSD 95, and BDNF expression in the hippocampal region (Li Y et al., 2017). Most of the time, phosphorylated ERK1/2 encourages the development of dendritic spines (Ohta KI et al., 2017). Upregulation of ERK1/2 signaling, however, reduces neurogenesis, synapse formation, and dendritic spine growth in an in vitro investigation into the onset of autism. Additionally, in rat sympathetic neurons, inhibiting ERK1/2 activity promotes dendritic development and synapse formation (Yang K et al., 2013). In our study, similar to that, NaF treatment of SH-SY5Y cells resulted in a notable rise in p-ERK1/2 expression, repeatedly shown in the brains of rats with chronic fluorosis (Liu YJ et al., 2010; Chen J et al., 2018).

The current study suggests that the MAPK/ERK pathway is involved in PSD-95 synaptic expression, as the inhibitor PD98059 drastically reduces PSD-95 soma and dendrite intensities. This is in line with a prior study that found MAPK/ERK1/2 involved in the BDNF-induced increase in dendritic spine density (Alonso M et al., 2004). Increased phosphorylation of eukaryote function factor ribosomal protein S6

levels will concomitantly influence MAPK/ERK regulating protein-synthesis dependent plasticity (Kelleher RJ et al., 2004; Klann E & Dever TE, 2004).

Ugalde Trivino Lola & Margarita Diaz Guerra (2021), and this study's findings suggest PSD 95 can be useful for stroke neuroprotection through methods that combat excitotoxicity, a fundamental cause of neuronal death following ischemic stroke. Moreover, fluoride decreased the expression of the presynapse protein SYN and the postsynapse protein PSD 95 in both vitro and vivo. The PSD's most prevalent protein, PSD 95, is crucial for neurotransmission, synaptic plasticity, and the creation of dendritic spines throughout neurodevelopment (Coley AA & Gao WJ, 2018). Upon neuroplasticity induction, PSD 95 developed a PSD protein marker outside the activated spinal cord, causing spine development to be initiated and then stopped (Steiner P et al., 2008). These results suggest that fluoride-induced downregulation of PSD-95 protein expression in synapse impulses conduction pathway might get misled for their target, which could hurt spine development and maintenance.

5. Conclusion

In the synapse, synaptic plasticity is expected by the scaffolding proteins, two such proteins, synapsin 1 and PSD 95 identified for the appropriate conduction of nerve impulses. In brain cells, an apparent effective is transmission of signal is the TrkB signaling pathway, which is significantly promoted by the PSD 95 protein. These proteins' contributive molecular function is unclear, but their presence promotes concomitant neurotransmission successfully. This was similar in our in vitro study with SK-N-SH cells for the proteins, BDNF, and PSD 95 levels. They were found to be altered from regular expression in fluoride-contaminated water-incubated cells. A prolonged period and continuous ingestion of fluoride exposure cause synapse proteins excitation function increased by contributed in brain cells. Meanwhile, fluoride toxicity was removed by incubating cell lines using a natural biocoagulant, seeds from *Strychnos potatorum* tree. A significant increase in BDNF and decreased synapsin protein levels were observed in expression. MAPK - ERK1/2 channel might have opened for conductivity of synaptic transmission of signal to control/inhibit excitation, thus may be epilepsy or seizures reduced/controlled (Fig. 7). Here, F-anion binding to TrkB receptor signal pathway induces uncontrolled excitation of neuronal cells, which may be due to depletion of BDNF or modification of BDNF configuration at TrkB receptor. In our future study a decline PSD 95 protein, cause of uncontrolled excitation could be ruled out by exploring TrkB and the MAPK−ERK1/2 signaling pathway.

Declarations

Ethics Approval Not Applicable

Competing interest On behalf of all authors, the corresponding authors states that there is no competing of interest.

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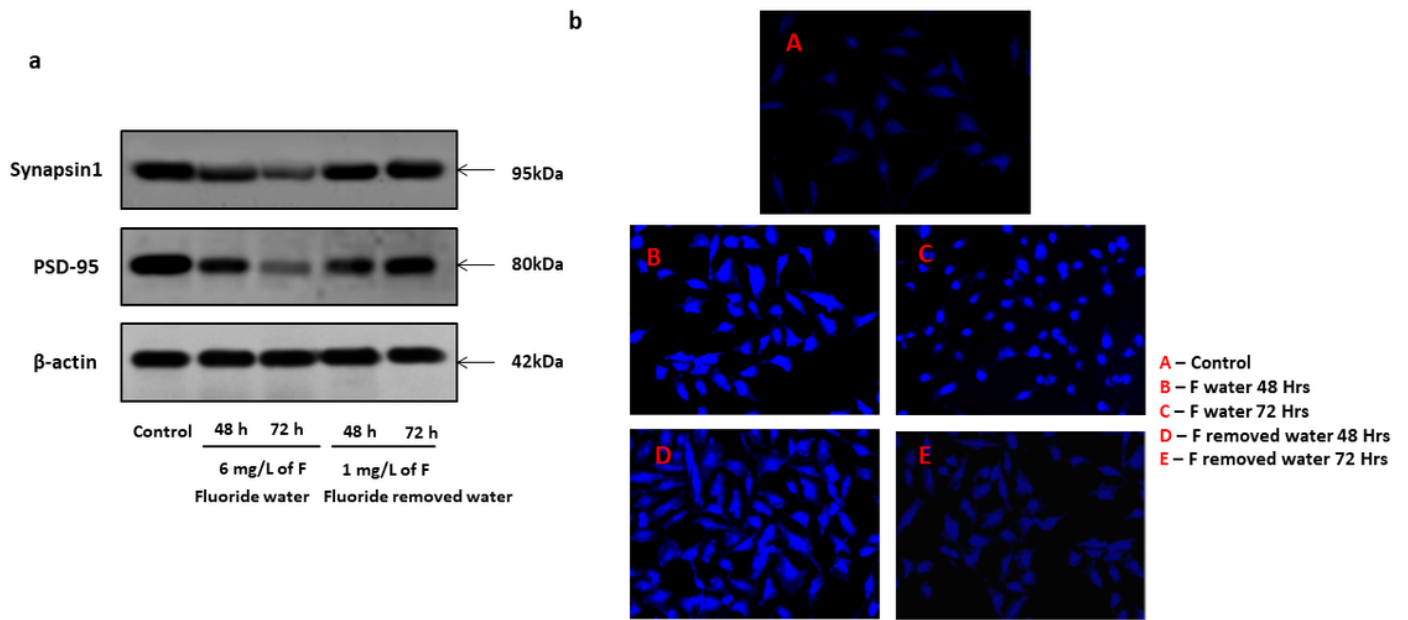
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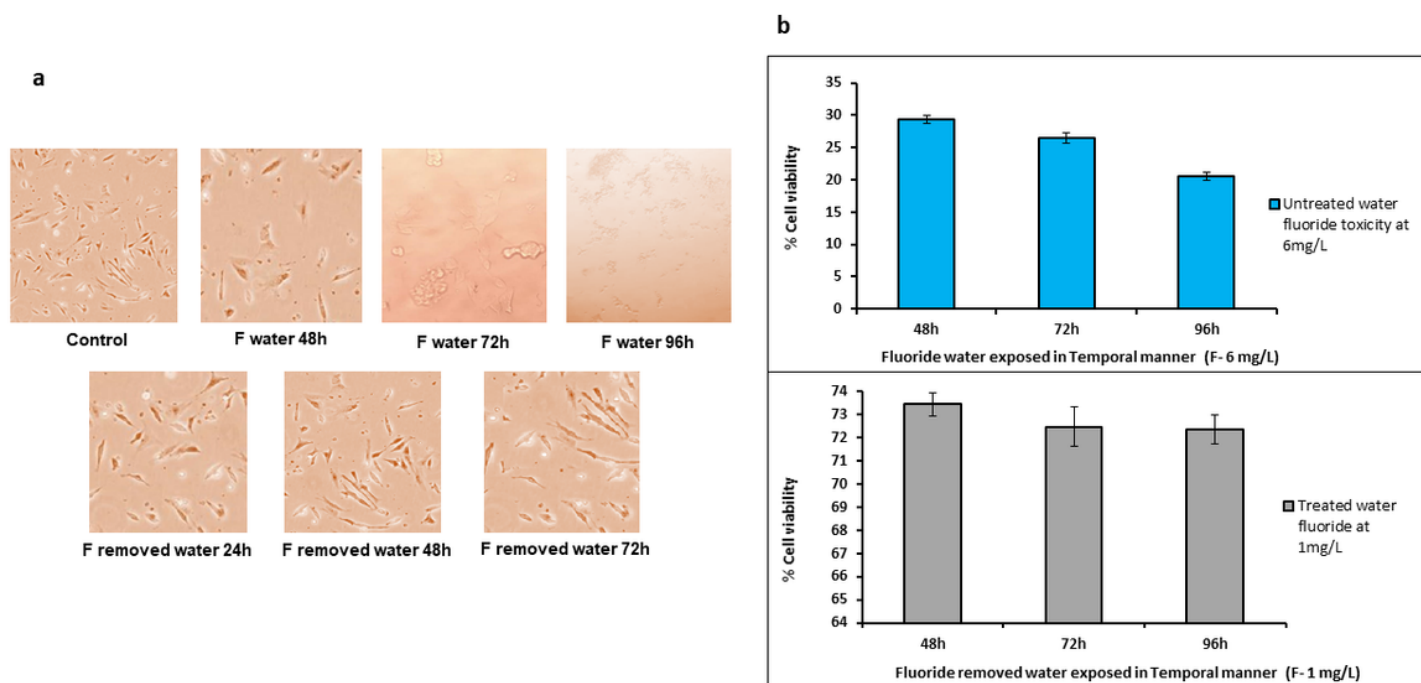
Figure 1

Fluoride content (Untreated) removed (Treated) water induces changes in SK-N-SH cells and the percentage of cell survival.

The cells were incubated with fluoride content & removed from the water for 48, 72, and 96 hours. a) Images of cell survival at a microscopic scale b) On SK-N-SH cells, respective percentage of cell survival in untreated and treated groundwater fluoride toxicity. Results are expressed as mean ± SD (n=3), indicating a significant difference at $P<0.05$ between the other samples and control concentrations.

Figure 2

Morphological and synaptic changes caused by fluoride content & removed water induced in SK-N-SH cells.

The cells were incubated with fluoride content & removed from the water for 48, 72, and 96 hours. a) Representative confocal images of SK-N-SH cells morphology with immunofluorescent stained b) On SK-N-SH cells, representative images of western blotting for Synapsin 1, PSD 95 synaptic protein & β-actin control for comparing morphological & synaptic changes caused by fluoride water.
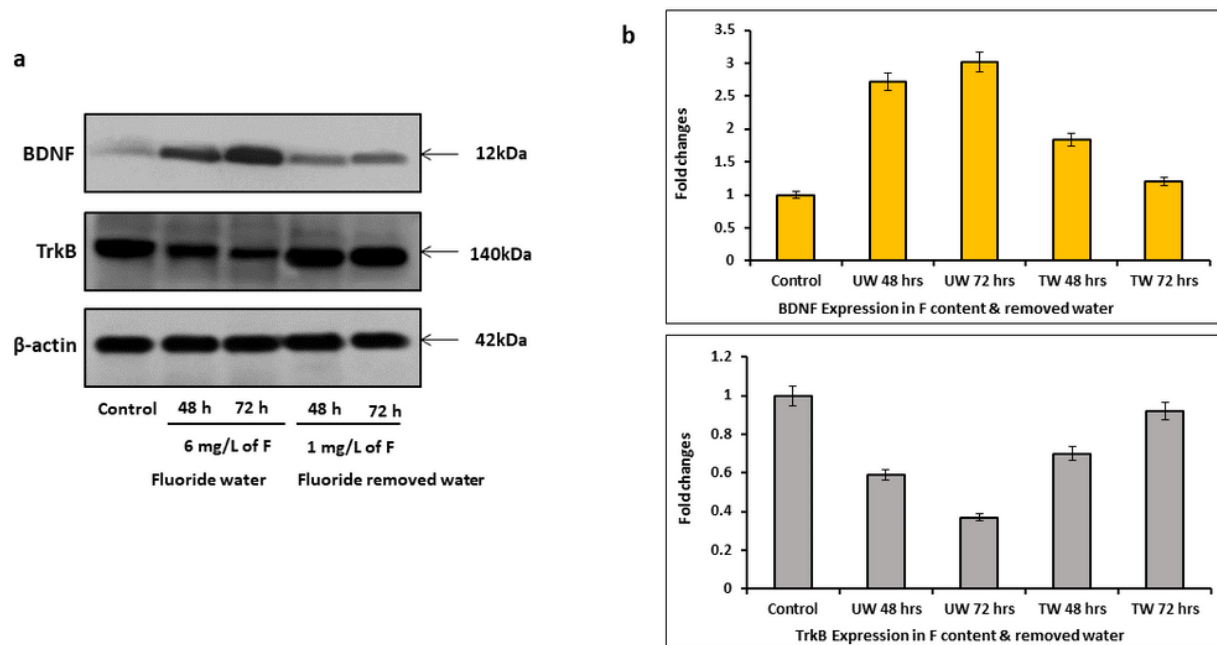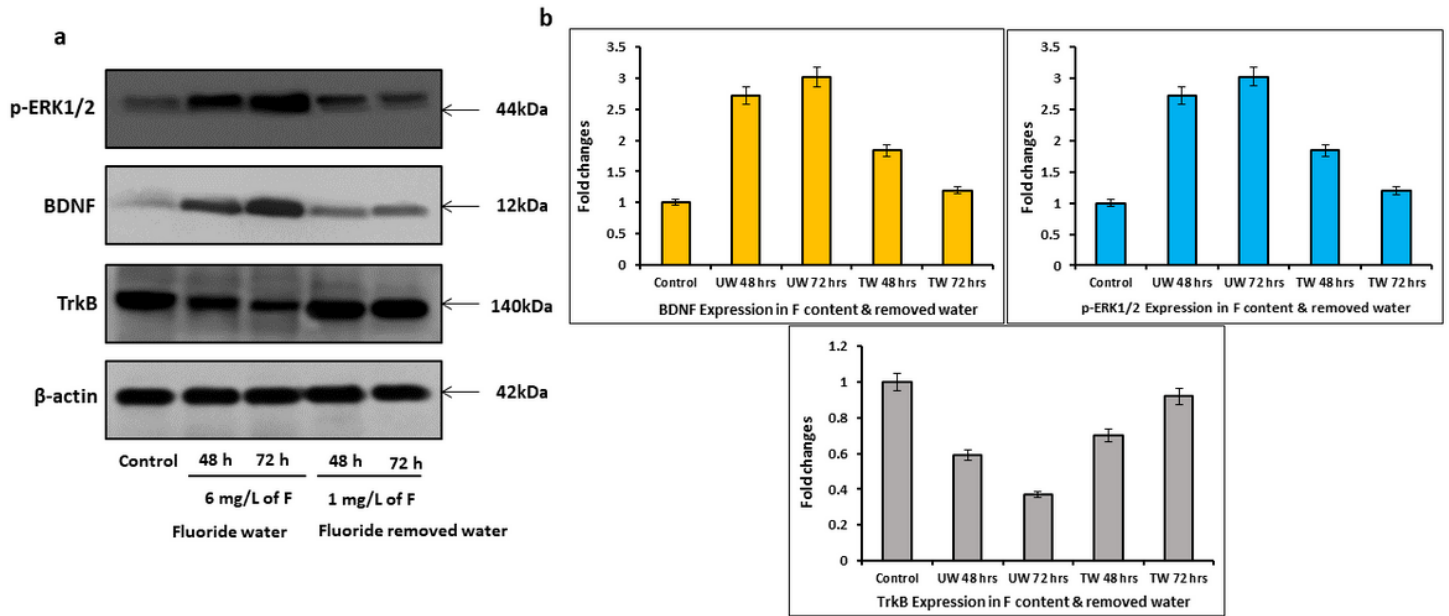
Figure 3

Expression of BDNF – TrkB protein level & signaling axis disruption caused by fluoride content and removed water induced in SK-N-SH cells

The cells were incubated with fluoride contaminated and treated water for 48 & 72 hours a) On SK-N-SH cells, representative images of western blotting for BDNF – TrkB & β-actin control b) Quantitative analyses of BDNF, TrkB expression level normalized to the internal control β-actin. Results are expressed as the mean ± SD. Quantified band intensities are presented as fold of control, indicating significant differences at $P<0.05$ between the different concentrations of the diverse sample vs. the control group.
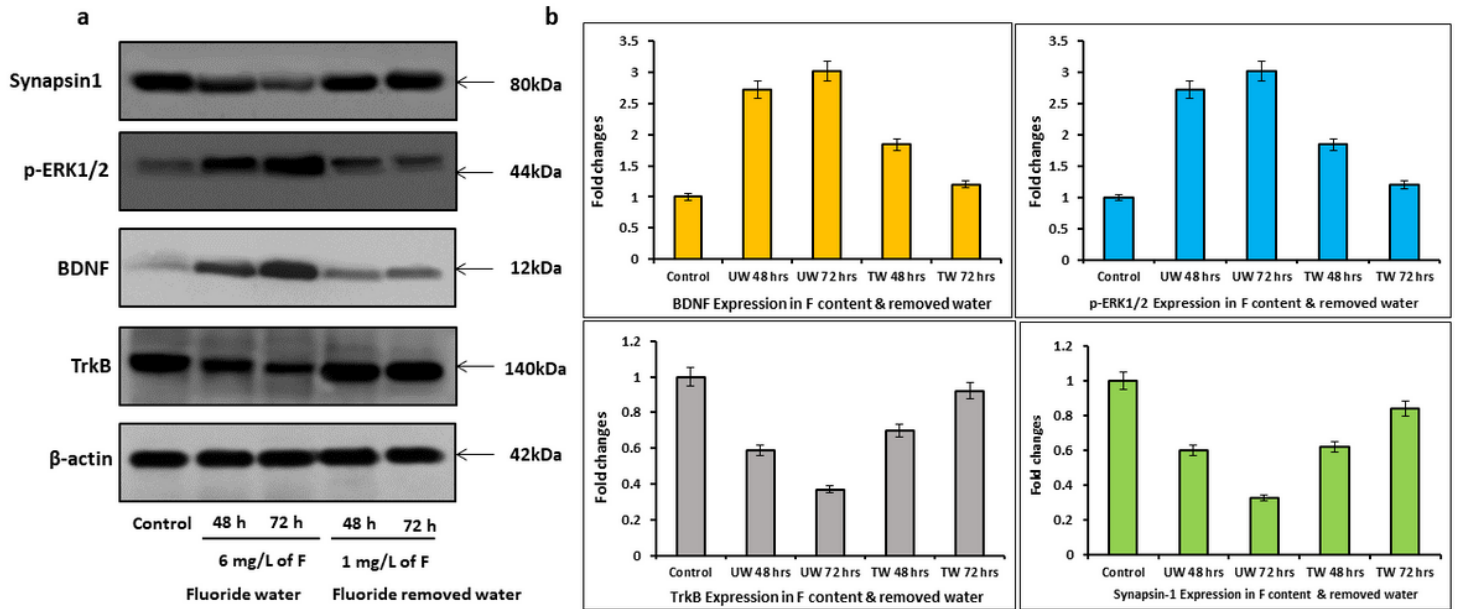
Figure 4

Expression of p-ERK1/2 protein level plays an important role in fluoride content & removed water changes alterations of BDNF – TrkB signaling and epileptogenic deficits in SK-N-SH cells.
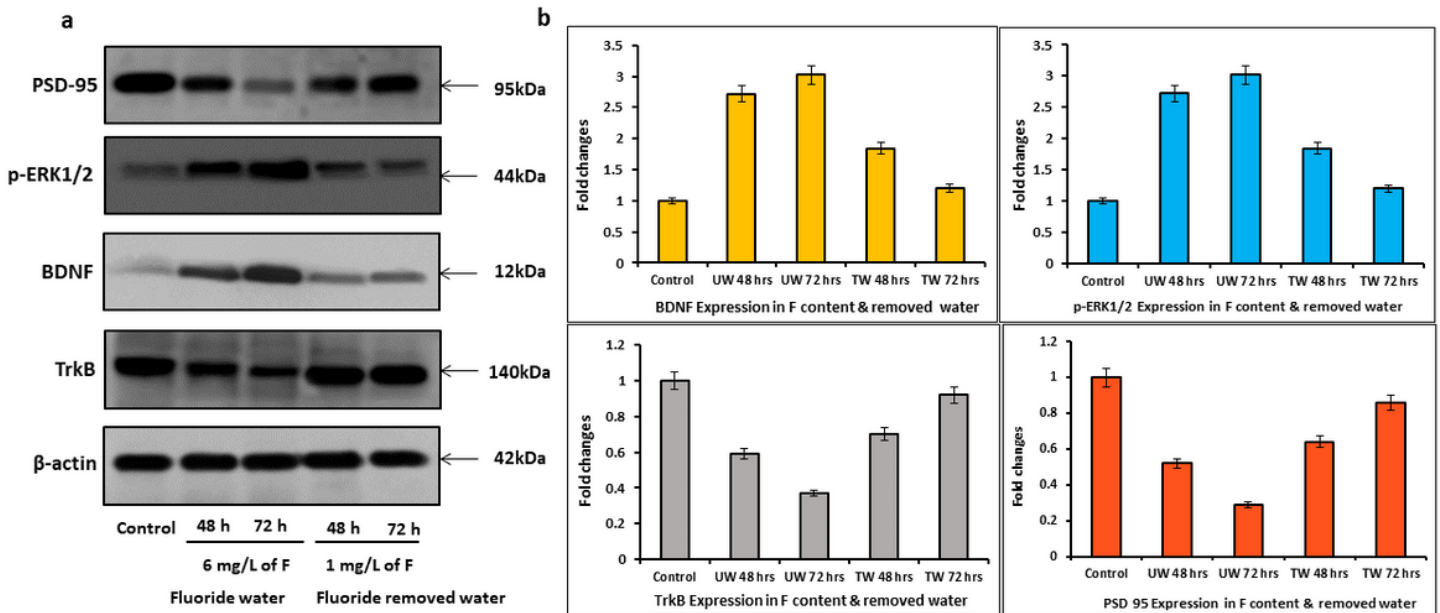
The cells were pretreated with MEK inhibitor PD98059 (10µM) for 1 hour. Then incubated with fluoride content (6 mg/L) & removed (1 mg/L) water for 48 h & 72 h; a) On SK-N-SH cells, representative images of western blotting for p-ERK1/2, BDNF, TrkB, and β-actin control b) Quantitative analyses of p-ERK1/2, BDNF, TrkB expression level normalized to the internal control β-actin. Results are expressed as the mean ± SD. Quantified band intensities are presented as fold of control; it indicates significant differences at $P<0.05$ between the different concentrations of the diverse sample vs. the control group.

Figure 5

Expression of Synapsin 1 protein level & its role in fluoride content water disrupts BDNF – TrkB receptor on the p-ERK1/2 (MAPK) mediated epileptogenic deficits in SK-N-SH cells.

The cells were incubated with fluoride content (6 mg/L) & removed (1 mg/L) water for 48 h & 72 h; a) On SK-N-SH cells, representative images of western blotting for SYN 1, BDNF, TrkB, p-ERK1/2, and β-actin control b) Quantitative analyses of SYN 1, BDNF, TrkB, p-ERK1/2 expression level normalized to the internal control β-actin. Results are expressed as the mean ± SD. Quantified band intensities are presented as fold of control; it indicates significant differences at $P<0.05$ between the different concentrations of the diverse sample vs. the control group.

Figure 6

Expression of PSD 95 protein level & its role in fluoride content water disrupts BDNF – TrkB receptor on the p-ERK1/2 (MAPK) mediated cognitive & learning memory disorders in SK-N-SH cells.

The cells were incubated with fluoride content (6 mg/L) & removed (1 mg/L) water for 48 h & 72 h; a) On SK-N-SH cells, representative images of western blotting for PSD 95, BDNF, TrkB, p-ERK1/2, and β-actin control b) Quantitative analyses of PSD 95, BDNF, TrkB, p-ERK1/2 expression level normalized to the internal control β-actin. Results are expressed as the mean ± SD. Quantified band intensities are presented as fold of control; it indicates significant differences at P<0.05 between the different concentrations of the diverse sample vs. the control group.
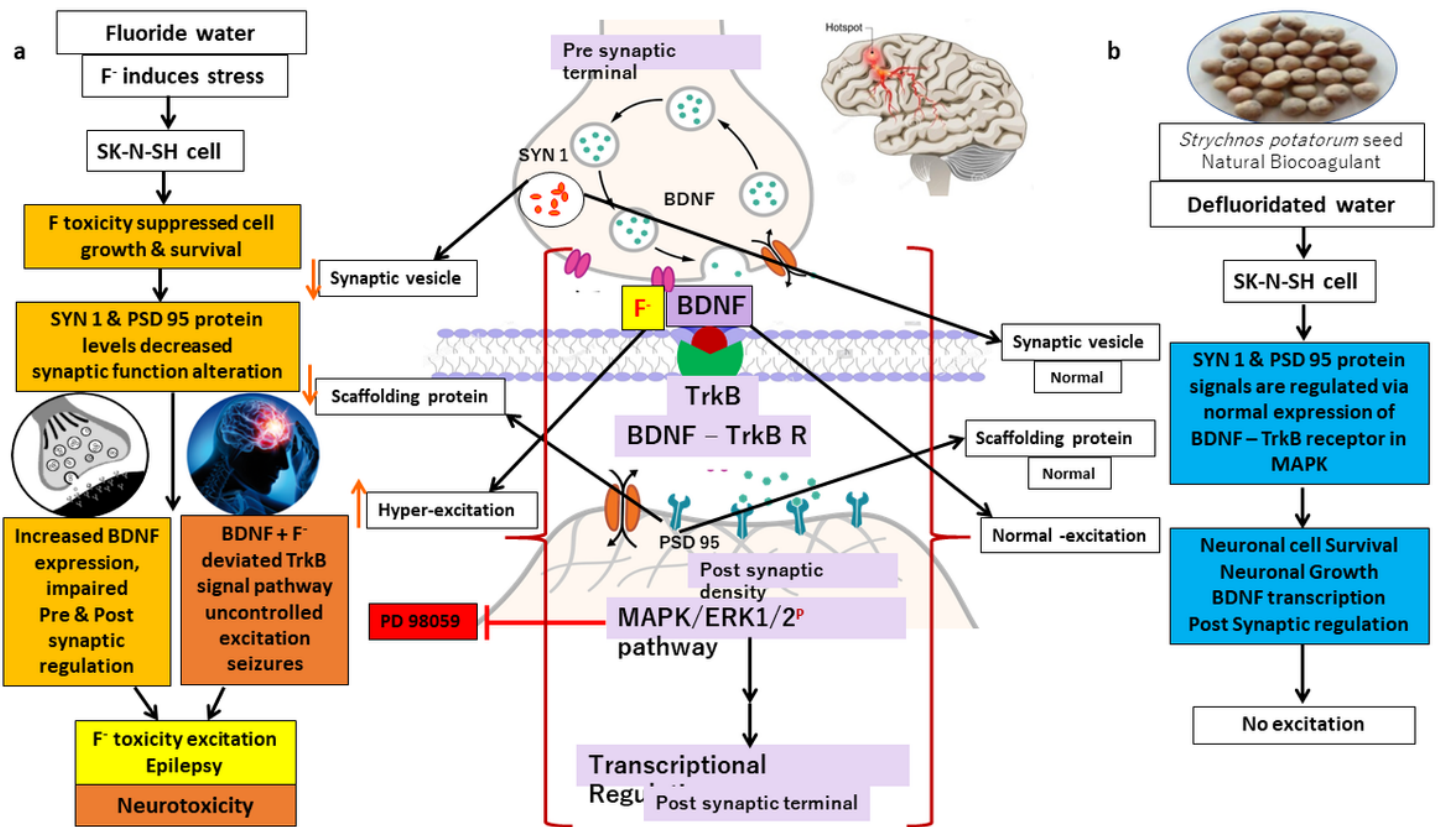
Figure 7

Diagramatic narration on the influence of fluoride content and fluoride-free water treatment against SK-N-SH cells

Synapse plasticity circumference by entangles scaffold proteins like PSD 95 and synapse impulse conductivity gateway through BDNF expression opens TrkB signal pathway progresses to conductivity, and MAPK/ERK1/2 pathways express pre-signaling route for postsynaptic transmission. Epilepsy occurring due to excitation at the presynaptic site may be due to higher levels of BDNF and lower levels of scaffold proteins. a) Fluoride toxicity corrupts BDNF binding site and might be complex with BDNF. It alters configuration that leads to altered TrkB signaling to excitation or BDNF anion bonded with F⁻ might bind with TrkB receptor, may induce uncontrolled excitation at presynaptic cell. b) Incubation of SK-N-SH cells in fluoride-removed treated water, BDNF upregulation (normal expression) with adequate scaffolding proteins generates normal conductivity of impulse transmission between presynaptic and postsynaptic cell, no excitation occurs devoid of epilepsy.