2-BFI enhances the proliferation of SH-SY5Y cells with fluorosis by stabilizing endoplasmic reticulum–mitochondria contact sites and inhibiting activation of NLRP3 inflammasomes

Rui Chen
The Second People's Hospital of Huai’an and The Affiliated Huai’an Hospital of Xuzhou Medical University

Wan Xu
Xuzhou Medical University

Yifu Sun
The Second People's Hospital of Huai’an and The Affiliated Huai’an Hospital of Xuzhou Medical University

Rongrong Zhi
Lianshui County People's Hospital Affiliated to Kangda College of Nanjing Medical University

Peng Xie
Huai’an Hospital of Xuzhou Medical University

Zhongwen Zhi
The Second People's Hospital of Huai’an and The Affiliated Huai’an Hospital of Xuzhou Medical University

Xiaohong Tang
Hongze Huai’an District People's Hospital

Caiyi Zhang (amanzcy@live.cn)
Xuzhou Medical University Affiliated Xuzhou Oriental Hospital

Research Article

Keywords: 2-BFI, proliferation, endoplasmic reticulum–mitochondria contact sites, NLRP3 inflammasomes, neuroinflammation, NaF.

Posted Date: May 9th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1624745/v1

License: ☕️ This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

2-(2-benzofu-ranyl)-2-imidazoline (2-BFI) is a drug that has attracted much attention in recent years. It has a therapeutic effect on brain diseases such as Alzheimer's disease and cerebral infarction. However, whether 2-BFI affects the proliferation of cells exposed to fluoride is not known. We investigated the proliferation and apoptosis of SH-SY5Y cells exposed to fluoride, and 2-BFI was used to protect SH-SY5Y cells against the effects of fluoride. We found that 2-BFI could enhance the proliferation of SH-SY5Y cells upon fluorosis by maintaining the stability of endoplasmic reticulum–mitochondria contact sites and inhibiting activation of NLR family pyrin domain containing 3 (NLRP3) inflammasomes. This study may provide a new method for protecting against the inhibition of cell proliferation induced by fluoride.

1. Introduction

Fluoride is an inorganic, monatomic anion of fluorine, with the chemical formula $F^-$. It is found commonly in drinking water, tea, meat, beverage, and air. Fluoride can prevent tooth decay, so it is often added to toothpaste and other oral cavity-related products [1].

However, excessive intake of fluoride can cause chronic fluorosis. Chronic fluorosis can cause skeletal fluorosis, dental fluorosis, and other damage. However, fluoride can also enter brain tissue through the blood–brain barrier and cause neurological dysfunction [2]. Spittle reported that chronic fluorosis can produce neurological symptoms such as sleepiness, memory/attention impairment, and difficulty in thinking [3, 4]. In areas of high fluoride content in drinking water, the intelligence quotient (IQ) of children has been shown to be significantly lower than that for children living in areas in which the fluoride level in drinking is lower [5].

The mechanism underlying fluoride-induced cognitive impairment is incompletely understood. Previously, we demonstrated that neuroinflammation is a possible mechanism of cognitive dysfunction caused by fluoride exposure [6]. Some research teams have reported that neuroinflammation is characterized by increased intracellular levels of reactive oxygen species (ROS) and intracellular calcium ion ($Ca^{2+}$) overload in brain tissue [7].

In recent years, some scholars have found that neuroinflammation is related to dysfunctional endoplasmic reticulum–mitochondria contact sites (ERMCS). The latter can promote the transfer of $Ca^{2+}$ in endoplasmic reticula (ERs) to mitochondria and, eventually, cause mitochondrial $Ca^{2+}$ overload which, in turn, generates many ROS [8]. ROS can promote excessive activation of NLR family pyrin domain containing 3 (NLRP3) inflammasomes and cause nerve-cell apoptosis [9]. Garrido-Maraver and colleagues illustrated that expressing a linker that can force contacts between mitochondria and ERs suppressed motor impairment and extended the lifespan in a Drosophila model of Alzheimer's disease (AD) [10]. That study demonstrated that dysfunctional ERMCS are important links in the mechanism of cognitive impairment. Therefore, we speculate that fluoride affects cognitive function by causing EMRCS disorders.
At present, there is no “ideal” treatment for neuronal apoptosis after injury. Therefore, elucidating the mechanism of neuronal apoptosis after fluorosis and finding new drug targets are rational approaches for the development of drugs for treatment of cognitive dysfunction.

2-(2-benzofuran-2-yl)-2-imidazoline (2-BFI) is a drug that has attracted much attention in recent years. It has a therapeutic effect on brain diseases such as AD and cerebral infarction [11, 12]. 2-BFI is a new type of imidazoline I2 receptor ligand (I2RL) and has high affinity with I2R. Studies have demonstrated that the I2R has an important regulatory role in multiple functions in the brain, and has been identified as a potential drug target for several neurological diseases [13]. A combination of 2-BFI and the I2R could have a protective effect on nerves and brain cells.

Previously, we found that 2-BFI application could improve the cognitive impairment induced by AD [11]. The mechanism of this protective effect involves inhibition of neuron apoptosis and protection of mitochondrial function as determined in in vivo and in vitro models [11, 13].

To ascertain if 2-BFI has a protective effect on neuron apoptosis and mitochondrial function, we investigated its effect on EMRCS disorders and the effect of its downstream products, NLRP3 inflammasomes, in SH-SY5Y cells exposed to fluoride and 2-BFI. Our aim was to understand the effect of 2-BFI on stabilizing EMRCS and reducing neuroinflammation during fluorosis, and provide a new explanation of its mechanism of action.

2. Materials And Methods

2.1. Materials

SH-SY5Y cells were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Dulbecco’s modified Eagle’s medium-high glucose (DMEM-HG) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, BY, USA). NaF was obtained from Aladdin (Beijing, China). 2-BFI was from Tocris Bioscience (Bristol, UK).

2.2. Cell culture

SH-SY5Y cells were cultured in DMEM-HG containing 10% FBS and 1% penicillin–streptomycin, and maintained in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed every 2 days, and cells were sub-cultured when they grew to 80–90% confluence.

2.3. Viability and proliferation of cells

Cell viability was detected via the Cell Counting Kit (CCK)-8 (Vicmed, Xuzhou, China). Cells were seeded into 96-well plates at 10,000 cells/100 µL. As soon as they became stable (~ 12-h later), cells were exposed to NaF and 2-BFI. After treatment, cells were washed twice with phosphate-buffered saline (PBS). Then, 10% CCK-8 was added to the medium, and plates were incubated for 30 min. We measured
the absorbance at 450 nm with a microplate reader (BioTek, Winooski, VT, USA). Survival was calculated using the following equation:

\[
\text{Survival} = \frac{(\text{Absorbance in experimental hole} - \text{absorbance in blank hole})}{(\text{Absorbance in control hole} - \text{absorbance in blank hole})} \times 100\%
\]

2.4. Staining

Cells were cultured on 48-well plates at 30,000 cells/well. When the cells stabilized (~12-h later), they were treated with different concentrations of NaF and 2-BFI for 24 h. After treatment, cells were washed twice with PBS and fixed with binding buffer. Then, cells were stained with Annexin propidium iodide/fluorescein isothiocyanate (PI/FITC; Unitech, New Delhi, India) for 10 min in the dark at room temperature. Images were viewed with an inverted fluorescence microscope (Olympus, Tokyo, Japan). The experiment was repeated thrice, and percent apoptosis calculated.

2.5. Real-time reverse transcription-quantitative polymerase chain reaction (qRT-PCR)

SH-SY5Y cells were divided randomly into four groups: control, NaF, 2-BFI, and NaF + 2-BFI. The total RNA of SH-SY5Y cells was extracted using TRIzol® Reagent (VicMed, Beijing, China). The RNA concentration was measured by an ultra-micro ultraviolet-visible spectrophotometer (One Drop, Beijing, China). A reverse transcription kit (Vazyme, Beijing, China) was used to reverse-transcribe 1 ng of RNA into complementary-DNA. We configured the reaction system and PCR program according to manufacturer instructions, and detection was undertaken on the LightCycler® 480 thermal cycler (Roche, Basel, Switzerland). Using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference, the relative expression of mRNA of each gene was calculated by the \(2^{-\Delta\Delta Ct}\) method. The primers we used are shown in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ip3r</td>
<td>ATCTGCAAACACTTCCCTT</td>
<td>CCACCTTCTTTTAGCCTCCTT</td>
</tr>
<tr>
<td>Grp75</td>
<td>TGCTACCAAGCGTCTCATTG</td>
<td>TGCCCCAAGTAATTTTCTGC</td>
</tr>
<tr>
<td>Vdac1</td>
<td>CCCGCAGTCTTAGTGGTTTG</td>
<td>CCAAGGGTGTCAGGGTTTCT</td>
</tr>
<tr>
<td>Gapdh</td>
<td>ACTTTGGTATCGTGGAAGGACTCAT</td>
<td>GTTTTTCTAGACGCGCAGGTCAGG</td>
</tr>
</tbody>
</table>

2.6. Western blotting

SH-SY5Y cells were exposed to NaF and 2-BFI for 24 h. They were washed twice in ice-cold PBS and lysed in RIPA lysis buffer for 30 min on ice. Lysates were centrifuged at 15294 × g for 20 min at 4°C. Supernatants were collected and used for evaluation of protein expression. The protein concentration in each sample was measured using a bicinchoninic acid protein assay kit (BioSharp, Beijing, China). Western blotting was undertaken according to standard protocols. Rabbit oligoclonal antibodies were
used in the experiment, including inositol 1,4,5-trisphosphat receptor (IP3R), glucose-regulated protein 75 (GRP75), voltage-dependent anion channel (VDAC), GAPDH, and β-actin antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and NLRP3, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC)-1, and caspase-1 antibodies purchased from Abcam (Cambridge, UK). The antibodies stated were diluted at 1:1000 and used for experiments. The secondary antibody, IgG (Santa Cruz Biotechnology), was diluted 1:200 and used for experiments.

2.7. Measurement of mean fluorescence intensity

Cells were cultured in a 96-well plate. After treatment, the medium was removed, and 100 µL of a green fluorescent calcium-binding dye (Fluo-8® AM; 4 µmol/L) was added. Cells were allowed to incubate in an atmosphere of 5% CO₂ for 40 min at 37°C. Then, cells were washed twice with PBS. Next, the fluorescence intensity (excitation wavelength = 490 nm; emission wavelength = 514 nm) was measured with a multifunctional microplate reader (Biotek). Images were recorded after viewing an inverted fluorescence microscope.

2.8. Measurement of the mitochondrial membrane potential (MMP)

Tetramethylrhodamine methyl ester (TMRM; Sigma–Aldrich, Saint Louis, MO, USA) was used to measure the MMP. SH-SY5Y cells were washed twice with PBS, and treated with TMRM (500 nM), and incubated in a light- protected humidified chamber for 20 min at 37°C. DMEM/F12 medium with TMRM was removed. Cells were washed twice with PBS and photographed by a fluorescence microscope (Olympus) with 200 µL of PBS per well.

2.9. Measurement of mitochondrial ROS

The oxidative fluorescent dye dihydroethidine was used to detect ROS. SH-SY5Y cells were treated with dihydroethidine (50 µM; Keygen Biotech, Nanjing, China) and incubated in a light-protected humidified chamber for 30 min at 37°C. DMEM/F12 medium with DHE was removed. Cells were washed twice with PBS and photographed by a fluorescence microscope with 200 µL PBS per well.

2.10. Enzyme-linked immunosorbent assays (ELISAs)

Cells were seeded into 96-well plates and treated with NaF and 2-BFI for 24 h. IL-1β levels in the culture medium were measured with an ELISA kit (KeyGen Biotech, Nanjing, China) according to manufacturer instructions.

2.11. Statistical analyses

Statistical analyses were undertaken using SPSS 16.0 (IBM, Armonk, NY, USA). Data are the mean ± standard deviation. One-way analysis of variance and Bonferroni post hoc tests were carried out to analyze data. P< 0.05 was considered significant.
3. Results

3.1. NaF reduces the viability of SH-SY5Y cells and causes apoptosis

The viability of SH-SY5Y cells was evaluated with the CCK-8 assay and apoptosis was detected by Annexin PI/FITC staining. To exclude the possibility that NaF is directly toxic to SH-SY5Y cells, NaF (0–4 mmol/L) was added to SH-SY5Y cells, and cell viability evaluated by the CCK-8 assay at 24 h. The viability of SH-SY5Y cells decreased gradually as the concentration of NaF increased ($P < 0.05$) (Fig. 1A, B). After treatment with NaF (0–4 mmol/L), Annexin PI/FITC staining was used to observe apoptosis. Percent apoptosis of SH-SY5Y cells increased gradually as the concentration of NaF increased ($P < 0.05$) (Fig. 1C, D). When NaF at 2 mmol/L interfered with cells, the number of surviving cells was relatively moderate, which was convenient for collection and observation of cells. Therefore, a NaF concentration of 2 mmol/L was chosen for subsequent experiments.

3.2. 2-BFI protected SH-SY5Y cells against NaF-induced damage

SH-SY5Y cells were pretreated with 2 mmol/L NaF for 24 h, and then incubated with 2-BFI (2.5, 5, 10, 25 and 50 µmol/L) for 24 h. Cell viability was evaluated with the CCK-8 assay and apoptosis was detected by Annexin PI/FITC staining. The number of SH-SY5Y cells decreased in the NaF group compared with that in the control group ($P < 0.05$) (Fig. 2A, B). 2-BFI improved the morphology and increased the number of SH-SY5Y cells compared with that in the NaF group ($P < 0.05$). The percentage of apoptotic cells increased in the NaF group compared with that in the control group ($P < 0.05$) (Fig. 2C, D). 2-BFI decreased the percentage of apoptotic cells compared with that in the NaF group. These results showed that 2-BFI at 10 µmol/L inhibited apoptosis as well as the anti-proliferative effects induced by fluoride in SH-SY5Y cells.

3.3. 2-BFI protected SH-SY5Y cells against NaF-induced damage by stabilizing ERMCS

ERs and mitochondria are closely linked through the dynamic stable structure of ERMCS. Dysfunctional ERMCS can cause mitochondrial Ca$^{2+}$ overload and inflammation. The basic components of ERMCS include Ca$^{2+}$ channels located on the ER or outer mitochondrial membrane, such as IP3R and VDAC, as well as various molecular moieties [14].

We carried out experiments to reveal ERMCS damage by NaF and the protective effect of 2-BFI on ERMCS by RT-qPCR. mRNA expression of Ip3r, Grp75, and Vdac was significantly higher in the NaF group than that in the control group ($P < 0.05$) (Fig. 3A–C). 2-BFI could effectively reduce mRNA expression of Ip3r, Grp75, and Vdac compared with that in the NaF group. To ascertain if NaF-induced neural damage
changed the levels of proteins in ERMCS, western blotting was used to measure expression of IP3R, GRP75, and VDAC. Protein expression of IP3R, GRP75, and VDAC was higher in the NaF group than that in the control group (Fig. 4). 2-BFI could effectively reduce protein expression of IP3R, GRP75, and VDAC compared with that in the NaF group. These results indicated that 2-BFI could stabilize ERMCS in SH-SY5Y cells from fluoride damage.

### 3.4. 2-BFI reduces the damage in mitochondrial function induced by NaF

Studies have shown that NaF can destroy EMRCS, and cause dysfunctional expression of the proteins and genes of IP3R, GRP75, and VDAC. The dysfunctionalities of IP3R, GRP75, and VDAC, which are responsible for Ca\textsuperscript{2+} transport, inevitably cause dysfunction of Ca\textsuperscript{2+} balance and disorders of Ca\textsuperscript{2+} distribution. These actions lead to an increased intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}), that is, Ca\textsuperscript{2+} overload. The latter can cause obstacles to mitochondrial oxidative phosphorylation, reduce the MMP, lead to a decrease in tissue content of adenosine triphosphate, activate cytoplasmic levels of phospholipase and proteases, release ROS and, finally, cause irreversible cell damage [15].

The [Ca\textsuperscript{2+}]\textsubscript{i} was detected by staining using a calcium-ion probe (Fluo-8 AM). The [Ca\textsuperscript{2+}]\textsubscript{i} was significantly higher in the NaF group than that in the control group (Fig. 5A, D). 2-BFI could effectively reduce the [Ca\textsuperscript{2+}]\textsubscript{i} compared with that in the NaF group. TMRM staining was used to measure the MMP. The MMP was decreased significantly in the NaF group compared with that in the control group (Fig. 5B, E). 2-BFI could effectively increase the MMP compared with that in the NaF group. The oxidative fluorescent dye dihydroethidine was used to detect ROS. The intracellular ROS level was significantly higher in the NaF group than that in the control group (Fig. 5C, F). 2-BFI could effectively reduce the intracellular ROS level compared with that in the NaF group. These results indicated that 2-BFI had a protective role against fluoride-induced neuronal mitochondrial damage.

### 3.5. 2-BFI inhibits the formation and activation of NLRP3 inflammasomes induced by NaF

In recent years, some scholars have found that mitochondrial Ca\textsuperscript{2+} overload can produce excessive ROS, which causes activation of many NLRP3 inflammasomes and promotes the release of proinflammatory factors [9]. The NLRP3 receptor protein is linked to an effector protein by the receptor protein ASC-1. A combination of NLRP3 and ASC-1 promotes the cleavage and activation of caspase-1. Cleaved caspase-1 can promote the maturation of interleukins (ILs) and other cytokines [16]. IL release plays an important part in the inflammatory response of cells [17]. IL maturation also has a role in cell death [16].

Expression of NLRP3, ASC-1, and cleaved caspase-1 increased significantly in the NaF group compared with that in the control group (Fig. 6A–D). 2-BFI could effectively reduce expression of NLRP3, ASC-1, and cleaved caspase-1 compared with that in the NaF group. ELISAs were used to measure IL-1\textbeta expression...
in cell cultures (Fig. 6E). NaF could promote release of the proinflammatory factor IL-1β, but 2-BFI could effectively alleviate this inflammatory response. These results indicated that 2-BFI could inhibit the formation and activation of NLRP3 inflammasomes induced by NaF, thereby reducing the release of proinflammatory factors and blocking apoptosis initiation.

3.6. How 2-BFI may protect against fluoride-induced toxicity

We postulated a mechanism by which 2-BFI may protect SH-SY5Y cells exposed to NaF. Upon NaF exposure, SH-SY5Y cells would increase expression of IP3R, GRP75, and VDAC, which are components of ERMCS. Increased expression of the proteins of IP3R, GRP75, and VDAC would promote Ca²⁺ transport within ERs into mitochondria, which would lead to Ca²⁺ overload. The latter would lead to ROS release, promote the binding of NLRP3, ASC-1, and pro-caspase-1, activate NLRP3 inflammasomes, and induce the cleavage and activation of caspase-1. Activated caspase-1 not only promotes maturation of the proinflammatory cytokine IL-1β, it also has an important effect on apoptosis. 2-BFI addition could inhibit the neuroinflammation and apoptosis caused by the structural disorder of ERMCS induced by NaF. Hence, 2-BFI could enhance the proliferation of SH-SY5Y cells upon fluorosis by stabilizing ERMCS and inhibiting activation of NLRP3 inflammasomes (Fig. 7).

4. Discussion

According to the World Health Organization, the fluoride content in drinking water should be < 1.5 mg/L. A safe amount of fluoride not only prevents caries, it also affects bone metabolism, hematopoietic function, growth, the immune system, and the nervous system [18]. However, a safe range of fluoride in the human body is narrower than that of other trace elements, and excessive intake can cause fluorosis.

Fluorosis can cause: (i) memory loss; (ii) behavioral manifestations of nervous-system damage (e.g., decreased IQ and cognitive dysfunction) in children [19]. The mechanisms of damage to the nervous system caused by fluorosis are not clear. Previously, we found that the mechanism of cognitive impairment induced by fluorosis was apoptosis and oxidative damage of neurons in the hippocampus [20]. We also found that 2-BFI application could improve cognitive impairment by inhibiting neuron apoptosis and protecting mitochondrial function in AD rats [11]. Some reports have suggested that dysfunctional ERMCS is a cause of apoptosis, emergency oxidative responses, and neuroinflammation [21]. Therefore, we suspect that the nerve damage caused by fluorosis is related to dysfunctional ERMCS.

We explored the relationship between dysfunctional EMRCS and nerve damage induced by fluoride, and investigated the protective effects of 2-BFI on SH-SY5Y cells treated with fluoride.

NaF (0–4 mmol/L) was used to treat SH-SY5Y cells for 24 h, and cell viability was evaluated by the CCK-8 assay and apoptosis was detected by Annexin PI/FITC staining. The cytotoxic effect of fluoride increased with increasing concentrations of NaF (Fig. 1), and NaF at 2 mmol/L was used for subsequent experiments.
Several studies have shown that the 12R is present widely in the central nervous system (CNS), can participate in regulation of various brain functions, and is related to the occurrence of several diseases [22]. 2-BFI is a selective and high-affinity 12R agonist first reported by Lione and colleagues in 1996 [23]. Previously, we showed that 2-BFI can alleviate oxidative stress, inhibit mitochondrial apoptosis, and improve cognitive impairment in AD rats [11]. However, whether 2-BFI can improve the nerve damage induced by fluoride has not been reported.

We used 2-BFI (2.5, 5, 10, 25, 50 µmol/L) to treat SH-SY5Y cells pretreated with NaF (2 mmol/L) for 24 h. If the 2-BFI concentration was too low or too high, it had a toxic effect on SH-SY5Y cells (Fig. 2). However, we also found that 2-BIF at 10 µmol/L could significantly increase the viability and decrease percent apoptosis of SH-SY5Y cells exposed to NaF compared with that in the NaF group. This result suggested that 2-BFI could alleviate fluoride-induced nerve damage and inhibit apoptosis.

Neuroinflammation seems to be an important mediator of the effects of fluoride [6]. Neuroinflammation contributes to most pathologic neurologic processes, including CNS infections, ischemic stroke, neurodegenerative disease, and anesthesia-induced neurotoxicity [24, 25]. Neuroinflammation is characterized by ROS release, Ca\(^{2+}\) overload, and MMP changes [13]. ERs and mitochondria are closely connected through EMRCS. Dysfunctional EMRCS can cause mitochondrial Ca\(^{2+}\) overload [14]. Therefore, we investigated the basic composition of EMRCS in SH-SY5Y cells exposed to NaF.

ERMCS are dynamic structures that connect ERs and mitochondria. They are composed of Ca\(^{2+}\) channels (IP3R) located on the ER or the outer mitochondrial membrane, VDAC, various molecular chaperone proteins (e.g., GPR75), enzymes associated with lipid biosynthetic pathways, lipid-transfer proteins, calnexin, and sigma-1 receptors [14]. Protein and mRNA expression of IP3R, GRP75, and VDAC were significantly higher in the NaF group than those in the control group (Fig. 3, Fig. 4). 2-BFI could effectively reduce protein and mRNA expression of IP3R, GRP75, and VDAC compared with that in the NaF group. These results indicated that 2-BFI could maintain the structural stability of EMRCS. A stable EMRCS structure could have a neuroprotective effect against fluorosis, which is reflected by reducing ROS release, alleviating Ca\(^{2+}\) overload, and regulating the MMP (Fig. 5).

In recent years, some scholars have discovered that mitochondrial Ca\(^{2+}\) overload can produce excessive ROS, which causes activation of many NLRP3 inflammasomes and promotes release of proinflammatory factors [26]. In the inactive state, NLRP3 is located mainly on the ER membrane and cytoplasm. However, in the activated state, NLRP3 and apoptosis-related punctate proteins relocate to ERMCS to form NLRP3 inflammasomes to promote the release of ROS that damage mitochondria [27, 28]. The NLRP3 inflammasome (which is composed of NLRP3 and ASC-1) can trigger caspase-1 activation, promote the secretion of IL-6 and IL-1\(\beta\), and induce neuronal apoptosis [16]. We found that NaF could increase expression of NLRP3 and ASC-1 and caspase-1 (Fig. 6A–D). Activated NLRP3 inflammasomes further promoted IL-1\(\beta\) release (Fig. 6E). However, 2-BFI could inhibit activation of NLRP3 inflammasomes and the initiation of neuroinflammation and apoptosis, and reduce the release of proinflammatory factors.
5. Conclusions

We demonstrated that fluoride inhibits the proliferation of SH-SY5Y cells and that 2-BFI reduces the anti-proliferative activity of fluoride. 2-BFI may enhance the proliferation of SH-SY5Y cells upon fluorosis by maintaining the structural stability of ERMCS and inhibiting activation of NLRP3 inflammasomes. These data may provide a new method for fluorosis treatment.

Declarations

Author contributions

Rui Chen: investigation, and writing of the original draft. Wan Xu and Yifu Sun: investigation and writing (review and editing). Rongrong Zhi and Zhongwen Zhi: data analyses. Peng Xie: data analyses, resource acquisition, experimental validation, software accrual. Caiyi Zhang and Xiaohong Tang: conceptualization and supervision of the study.

Funding

This study was supported (HAB201932) by the Natural Science Foundation of Huai’an City, Jiangsu Province, China. and (XWKYHT20200064) Medical Science and Technology Innovation Project of Xu’zhou Health Commission for Young Scholars.

Conflict of interest

There is no conflict of interest to disclose.

Acknowledgment

We are thankful for the technical support provided by Professor Ruiqin Yao (Xuzhou Medical University).

Compliance with Ethical Standards

Not applicable.

References


Figures
Figure 1

Effect of NaF on the viability of SH-SY5Y cells. (A) Change in cell morphology after 24 h of treatment with different concentrations of NaF, scale bar = 20 μm. (B) Measurement of cell viability using the CCK-8 assay (n = 4). (C) Annexin V-FITC/PI staining to detect apoptosis, scale bar = 50 μm. (D) Annexin V-FITC/PI staining showing percent apoptosis (n = 3). **P < 0.01 and *** P < 0.001 versus control group (one-way analysis of variance).
Figure 2

2-BFI has a protective effect on NaF-induced cell damage. (A) SH-SY5Y cells were treated with NaF (2 mmol/L) for 24 h and incubated with different concentrations of 2-BFI for 24 h to observe changes in cell morphology. Scale bar = 20 μm. (B) Cell viability was measured using the CCK-8 assay (n = 4). (C) Annexin V-FITC/PI staining to detect apoptosis, scale bar = 50 μm. (D) Annexin V-FITC/PI staining showing percent apoptosis (n = 3). **P < 0.01 and ***P < 0.001 versus control group, ##P < 0.01 and ###P < 0.001 versus NaF group (one-way analysis of variance).
Figure 3

Use of RT-qPCR to measure mRNA expression of *Ip3r*, *Grp75*, and *Vdac*. (a) *Ip3r*. (b) *Grp75*. (c) *Vdac*. **$P < 0.01$** and ***$P < 0.001$** versus control group (n = 4), ##$P < 0.01$ and ###$P < 0.001$ versus NaF group (n = 4).
Figure 4

Protein expression of IP3R, GRP75, and VDAC by western blotting. (A) Protein bands of IP3R, GRP75, VDAC, and GAPDH. (B–D) Quantitative analysis of protein expression of IP3R, GRP75, and VDAC. *P < 0.05 and **P < 0.01 versus control group, #P < 0.05 versus NaF group (one-way analysis of variance).
Figure 5

2-BFI reduces NaF-induced damage to mitochondrial function. (A) Fluo-8 staining to detect the intracellular Ca\(^{2+}\) concentration scale bar = 50 μm. (B) TMRM staining to measure the mitochondrial membrane potential. scale bar = 50 μm. (C) Use of dihydroethidine to measure ROS levels in living cells. scale bar = 50 μm. (D–F) Quantitative analysis of levels of intracellular Ca\(^{2+}\), TMRM, and ROS. **P < 0.01 versus control group, #P < 0.05 and ##P < 0.01 versus NaF group (one-way analysis of variance).
Figure 6

2-BFI inhibits NaF-induced inflammasome activation. (A) Measurement of expression of the inflammasome-related proteins NLRP3, ASC-1, and cleaved caspase-1 by western blotting. (B–D) Quantitative analysis of NLRP3, ASC-1, and cleaved caspase-1. (E) Use of ELISAs to measure IL-1β expression in cell cultures. *P < 0.05, **P < 0.01 and ***P < 0.001 versus control group, #P < 0.05, ##P < 0.01 and ###P < 0.001 versus NaF group (analysis of single-factor variance).
Figure 7

How 2-BFI may protect against fluoride-induced toxicity.