FLUORIDE

Quarterly reports

Influence of Fluorosis on Wnt/β-Catenin Pathway and Relating Proteins with Apoptosis in Central Nervous System and Neuroprotective Effect of Wnt Agonist SKL2001

Unique digital address (Digital object identifier [DOI] equivalent): https://www.fluorideresearch.online/epub/files/269.pdf

Xian Li[1], Jie Deng[2,3], Yan-Lin Ma[1], Ting Zhang[2,3], Yang-Ting Dong[2,3], Kai-Lin Zhang[1,2,3]*

[1]Department of Biochemistry and Molecular Biology, School of Basic Medicine, Guizhou Medical University, Guiyang 550004, Guizhou, P. R. of China
[2]Key Laboratory of Endemic and Ethnic Diseases (Guizhou Medical University) of the Ministry of Education of P. R. China
[3]Guizhou Provincial Key Laboratory of Medical Molecular Biology, Guiyang 550004, Guizhou, P. R. of China

*Corresponding author:
Kai-Lin Zhang, Department of Biochemistry and Molecular Biology, Guizhou Medical University, Guiyang 550004, Guizhou, P. R. China.
Phone: (+86) 13984102293
E-mail: 476477406@qq.com.

Accepted: 2024 Apr 18
Published as e269: 2024 Apr 22

ABSTRACT

Purpose: Aim of the study is to investigate whether Wnt/β-catenin pathway plays a correlating effect in the damages of central nervous system (CNS) induced by chronic fluorosis and SKL2001, an agonist of Wnt pathway, attenuates the neurotoxicity resulted from high dose of fluoride.

Methods: Sprague-Dawley rats were divided randomly into the control group, in which the rats were fed with normal diet and drinking water, and the fluoride exposure group, treated with 50 ppm fluoride in their drinking water for 6 months; primary rat hippocampal neurons were divided into three groups, i.e., the control (untreated), the fluoride exposure (1 mM fluoride in cultured medium), and the fluoride plus SKL2001 (30 M) treatments. The cultured neurons were incubated for 24, 48 and 72 hrs. Expressions of Wnt/β-catenin pathway and apoptosis-related proteins were detected using Western blotting and immunofluorescence assays. Apoptosis levels were detected by TUNEL staining and Flow cytometry.

Results: The results showed that as compared to controls, the protein levels of Wnt1, β-catenin, cyclin D1 and c-Myc were lower in the hippocampus of rat brains with fluoride exposure and while the levels of phospho-β-catenin, glycogen synthase kinase-3β and apoptosis higher. Moreover, the similar biochemical changes as the rat brans with chronic fluorosis were found in primary neurons exposed to fluoride. In addition, the B-cell lymphoma-2 (Bcl2) level was decreased, and Bcl2 associated X protein (Bax) and caspase-3 were increased in the primary neurons with fluoride exposure. Interestingly, SKL2001 treatment attenuated the changes of Wit/β-catenin pathway and the relating proteins with apoptosis induced by fluorosis.

Conclusion: The results indicate that the expression of Wnt/β-catenin pathway was inhibited by fluorosis in CNS, which was correlated with the enhanced apoptosis. Importantly, the agonist of Wnt pathway, SKL2001, can attenuate the neurotoxicity of fluoride.

Key-words: Fluoride; Wnt/β-catenin pathway; Apoptosis; Rat brains; Primary neurons

INTRODUCTION

Fluoride can cross the blood-brain barrier, enter the central nervous system (CNS), and cause direct injury of brain.[1] High level of fluoride accumulated in brains results in neurotoxic effects on morphology, metabolism and function, including abnormal signal transduction pathways, declines of cognition and ability of learning and memory and increased level of apoptosis.[2,3] However, the molecular mechanism in the damages of CNS by fluorosis has not yet been clarified.[4]

Wnt/β-catenin signaling pathway is a conserved one that plays an important role in regulating various cellular processes, including differentiation, proliferation, and apoptosis.[5] In the canonical pathway, the Frizzled receptor and the lipoprotein receptor-related proteins 5/6 create a seven-pass transmembrane receptor complex to which the Wnt ligands bind.[6] When Wnt binds to its transmembrane receptor, β-catenin accumulates in the cell and is translocated to the nucleus, where it interacts with the nuclear transcription factor such as T-cell factor /lymphoid enhancer factor 1 to activate downstream target genes such as cyclin, c-Myc and survivin.[7]

Studies have shown that defective Wnt/β-catenin signaling is found to be involved with the occurrence and development of CNS diseases, such as Alzheimer's disease, Parkinson's disease, and depression.[8,9] According to the available research, restoring Wnt/β-catenin signaling in the brains of patients with neurodegenerative disorders would improve their conditions.

Interestingly, it has been reported that the Wnt/β-catenin pathway plays a crucial role in fluoride-induced damages of CNS.[10,11] However, the relationship between the changed Wnt/β-catenin pathway and neuronal apoptosis is unclear. Therefore, this study investigated the involvement of Wnt/β-catenin pathway with fluorosis in rat brains and primary neurons and the protective effect of a Wnt agonist on neuronal apoptosis induced by fluorosis.

MATERIAL AND METHODS

Materials: Sodium fluoride (Sigma Aldrich, St. Louis, MO, USA); polylysine (PLOY-Lys), glutamine (Gln), and bicinchoninic acid (BCA) (Solarbio Life Science, China); Dulbecco's modified eagle medium (DMEM/F12), 0.25% trypsin solution, NurobasalTM-A medium, B-27, and fetal bovine serum (FBS) (Gibco, USA); anti-neuron specific nuclear protein (NeuN), -glial fibrillary acidic protein (GFAP), -glycogen synthase kinase-3β (GSK-3β), -c-Myc, and -cyclin D1 antibodies (CST, USA); anti-Wnt1 antibody (Proteintech, China); anti-β-catenin antibody (Absin, China); anti-phosphorylated β-catenin (Ser33/Ser37/Thr41) antibody (Affinity, China); anti-B-cell lymphoma-2 (Bcl-2), -Bcl-2-associated X protein (Bax), and -cysteinyl aspartate specific proteinase-3 (caspase-3) antibodies (MCE, USA); anti-β-tubulin labelled with horseradish peroxidase antibody (Proteintech, USA); polyethylene difluoride membranes and enhanced chemiluminescence (ECL) (Sigma Aldrich, USA); Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences, USA); one-step terminal deoxynucleotidyl transferase-mediated FITC-dUTP Nick end labeling (TUNEL) flow cytometry apoptosis kit (Elabscience, China); and all other general chemicals (Sigma Aldrich, USA) were purchased from the sources indicated.

Experimental animals: Forty Sprague–Dawley (SD) rats, weighing 100–120 g (one month old, half male and half female), were obtained from the Experimental Animal Center of Guizhou Medical University, China. The animals were qualified for use in the study (certificate number: SCXK 2012-0001), and the study protocol was pre-approved by the Animal Ethics Committee of Guizhou Medical University, China (Approval No. 2000868).

After 1 week of adaptive feeding, the rats were divided randomly into two groups: the control group (CR), in which the rats were given free access to normal drinking tap water (<1 ppm fluoride), and the fluoride-treated group (FR) in their drinking water containing 50 ppm fluoride. The experimental period was 6 months.

Cell extraction and identification: One day in advance, the petri-dish was coated with diluted 1X PLOY-Lys, dried and placed in the incubator overnight. The neonatal SD rats within 24 hr after birth were soaked in 75% ethanol.[2] After disinfection, the brains of the rats were directly collected and placed in the DMEM/F12 culture medium on ice bath to peel off the

hippocampal tissues. The tissues were digested with 0.25% trypsin, and then 1 ml DMEM/F12 medium containing 10% FBS was added to stop the digestion. The cells of brain tissues were fully blown on ice, and the filtered cells were collected. The medium was replaced with the maintenance medium containing Nurobasal, B-27 and Gln to continue incubation. The cytarabine inhibitor (0.5 µl) was added 48 hr later. The medium was changed after 24 hr.

The primary cell suspension was inoculated on the climbing tablets treated with PLOY-Lys, and the cell density was adjusted to $1x10^5$. When the cells grew from day 7 to day 10 and the shape was intact, the culture medium was completely absorbed. Each climbing tablet was dripped with anti-NeuN antibody (1:500) (for labeled neurons) and anti-GFAP antibody (1:200) (labeled astrocytes), put in a wet box, and incubated overnight at 4°C. DAPI was used to label the nucleus. A drop (10 µl) of anti-fluorescence quenching liquid seal was placed on the center of clean slides. The images were observed and collected under fluorescence microscope.

Primary hippocampal neurons selected were further cultured for 7-10 days. Then, these cells were divided into three groups: the control group (C) (untreated), the fluoride group (F) treated with 1 mM fluoride, and the fluoride+SKL2001 group (FS) treated with both fluoride (1 mM) and the Wnt agonist SKL2001 (30 µM).[12] The cells were analyzed after 24, 48 and 72 hrs of treatment.

Wnt/β-catenin pathway- and apoptosis-related proteins detected by immunofluorescence and Western blotting assays: For the animal experiments, paraffin-embedded tissue sections were heated at 60°C for 20 min until the paraffin was completely melted, and the remaining paraffin was removed by incubation in xylene I (20 min) and xylene II (20 min). After that the tissue section was rehydrated in a graded series of alcohol and then in 1X phosphate buffered saline, boiled in citric acid buffer (0.01 mol/l, pH 6.0) and finally blocked by incubation with 5% BSA at room temperature for 1 hr. The blocked sections were incubated with the primary antibodies, i.e., anti-Wnt1 (1:200), -GSK-3β (1:200), -β-catenin (1:100), -c-Myc (1:200), -cyclin D1 (1:500), -phospho-β-catenin (1:200), -Bcl-2 (1:1000), -Bax (1:1000) and -caspase-3 (1:1000) antibodies at 4°C overnight and then with the fluorescent secondary antibody (1:2000) at room temperature for 1 hr. DAPI was used to label the nucleus. The sections were finally sealed and examined using laser confocal microscopy (Olympus).

Total proteins were extracted from the primary neurons, quantified using the BCA kit, and subsequently separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins in the gels were transferred to membranes, then the membranes incubated overnight at 4°C with the following antibodies, respectively: anti-Wnt1 (1:1000), -GSK-3β (1:1000), -β-catenin (1:1000), -c-Myc (1:1000), -cyclin D1 (1:1000), -phospho-β-catenin (1:1000), -Bcl-2 (1:1000), -Bax (1:1000), -caspase-3 (1:1000) and -β-tubulin (1:5000) antibodies. Thereafter, the blots were allowed to react with ECL reagent for 5 min and then exposed to film, which was later developed. The bands were quantified using an image analyzer.

Apoptosis detection: Apoptosis in primary neurons was measured using FITC/PI cell apoptosis detection kit and analyzed using Flow Cytometry (BD Biosciences). The apoptosis rates were evaluated by TUNEL staining, which detects DNA fragments in the nucleus that are generated during apoptotic cell death.

Statistical analysis: Data were analyzed using SPSS 22.0 and GraphPad Prism 5.0 software. The values are expressed as mean ± standard deviation (SD). Data were first tested for normal distribution before analysis using one-way analysis of variance (ANOVA). When comparing two groups, the homogeneity of variance was tested using the least significant difference method. If the variance was uneven, Dunnett's ST3 method was used. Statistical significance was set at $P < 0.05$.

RESULTS

Altered expressions of Wnt/β-catenin pathway and the related proteins in rat brains with fluoride exposure: In the animal experiment as compared to controls, immunofluorescence analysis showed that the immunoreactivities of Wnt1, β-catenin, cyclin D1, and c-Myc were markedly lower in the hippocampus of rat brains with high fluoride exposure, whereas GSK-3β and phospho-β-catenin higher (Fig. 1).

Changed apoptosis level and the expressions of its related proteins in rat brains with fluoride exposure: The number of TUNEL-positive neurons in the hippocampus of rats with fluorosis was significantly increased compared to controls (Fig. 2). In addition, immunofluorescence analysis exhibited a significant decrease of Bcl-2, but the obvious raises in the immunoreactivities of Bax and caspase-3 in the hippocampus of rats with fluoride exposure (Fig. 3).

Altered expressions of Wnt/β-catenin pathway, and level of apoptosis and their related proteins in primary neurons exposed to fluoride as well as the interference of Wnt agonist SKL2001: As shown in Fig. 4, the expressions of Wnt1, β-catenin, cyclin D1 and c-Myc were significant lower in the neurons exposed to fluoride than those of controls; whereas, GSK-3β and phospho-β-catenin higher (Fig. 4). Furthermore, exposure of fluoride induced the increased level of apoptosis in the neurons (Fig. 5). The raised expression of Bax (Fig. 6A), declined Bcl2 (Fig. 6B) and increased caspase-3 (Fig. 6C) were observed in the cultured neurons exposed to fluoride. All of these modifications with apoptosis related proteins influenced by fluoride exposure present a time-dependent manner (Fig. 4-6).

Obviously, the treatment of SKL2001 attenuated the changes resulted from fluoride exposure, including Wnt/β-catenin pathway, apoptosis and the related proteins (Fig. 4-6). Furthermore, the decreased levels of β-catenin were significantly correlated with the increased rates of apoptosis (Fig. 6D).

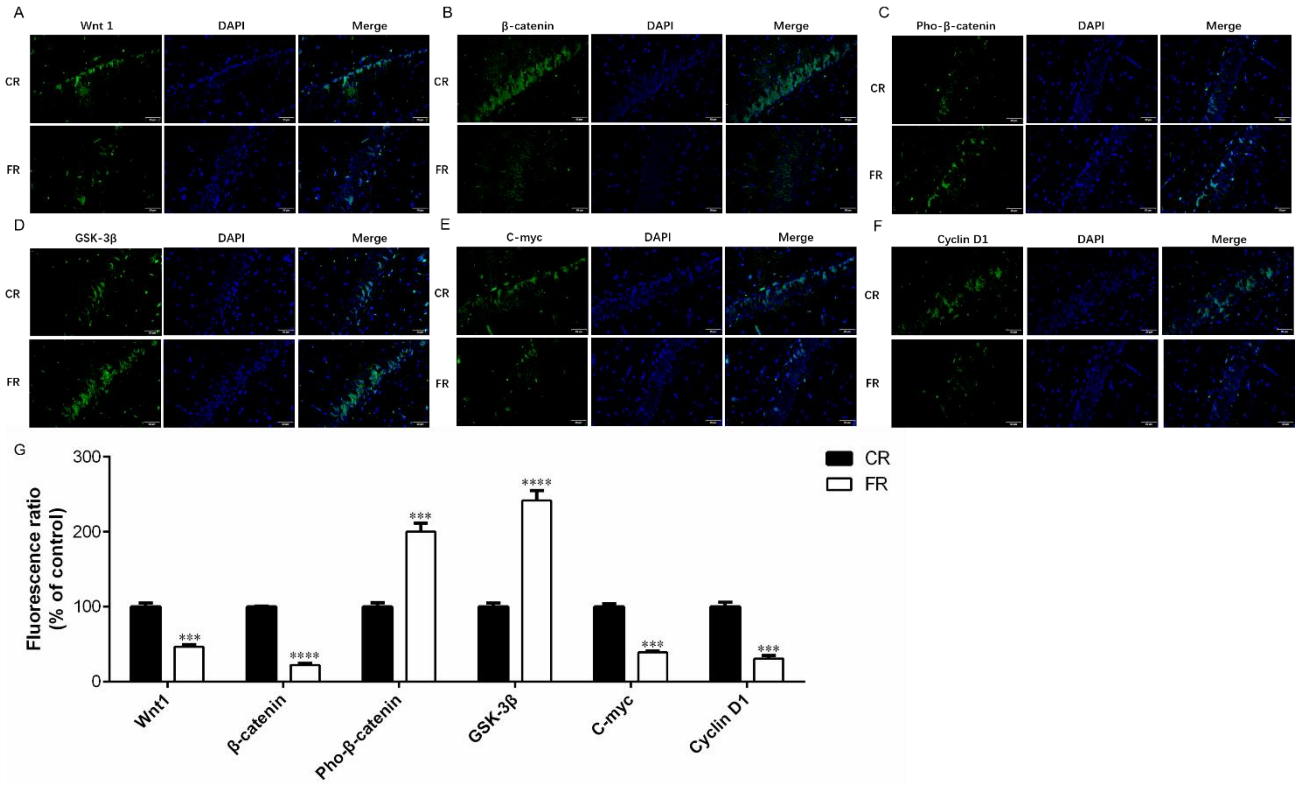

Fig. 1. Expressions of Wnt/β-catenin pathway and related proteins in the hippocampus of rat brains with fluoride exposure. Representative immunofluorescence images (X 400) of Wnt1 (A), β-catenin (B), phospho-β-catenin (C), GSK-3β (D), c-Myc (E), and cyclin D1 (F), and relative quantitative graphs (G). DAPI was used to label nucleus. CR, control group; FR, fluoride exposed group. The data shown are means ± standard deviations (SD). **P < 0.01 in comparison to controls as determined by analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test.

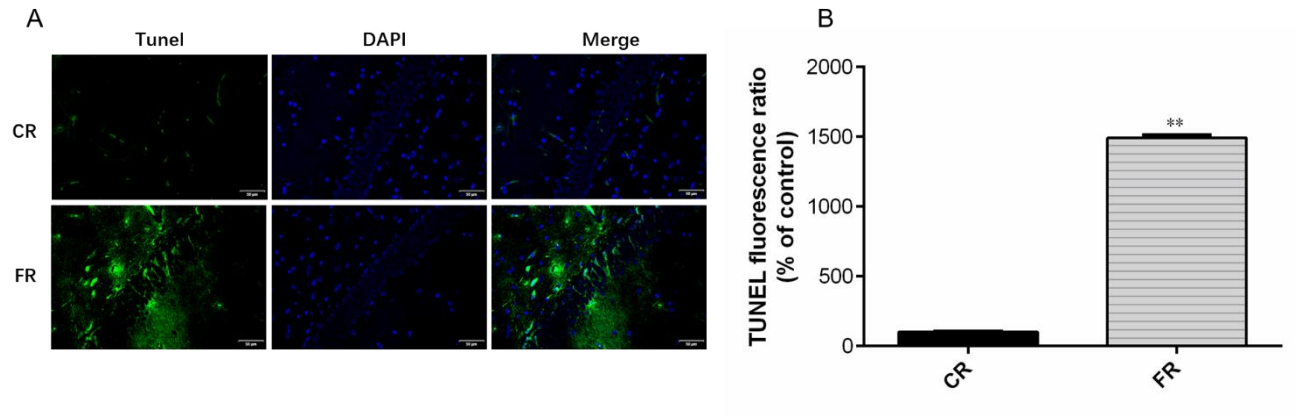

Fig. 2. Apoptosis in the hippocampus of rat brains with fluoride exposure. Representative immunofluorescence images (X 400) of TUNEL staining (A) and relative quantitative graphs (B). DAPI was used to label nucleus. CR, control group; FR, fluoride exposed group. The data shown are means ± SD. **P < 0.01 in comparison to controls as determined by analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test.

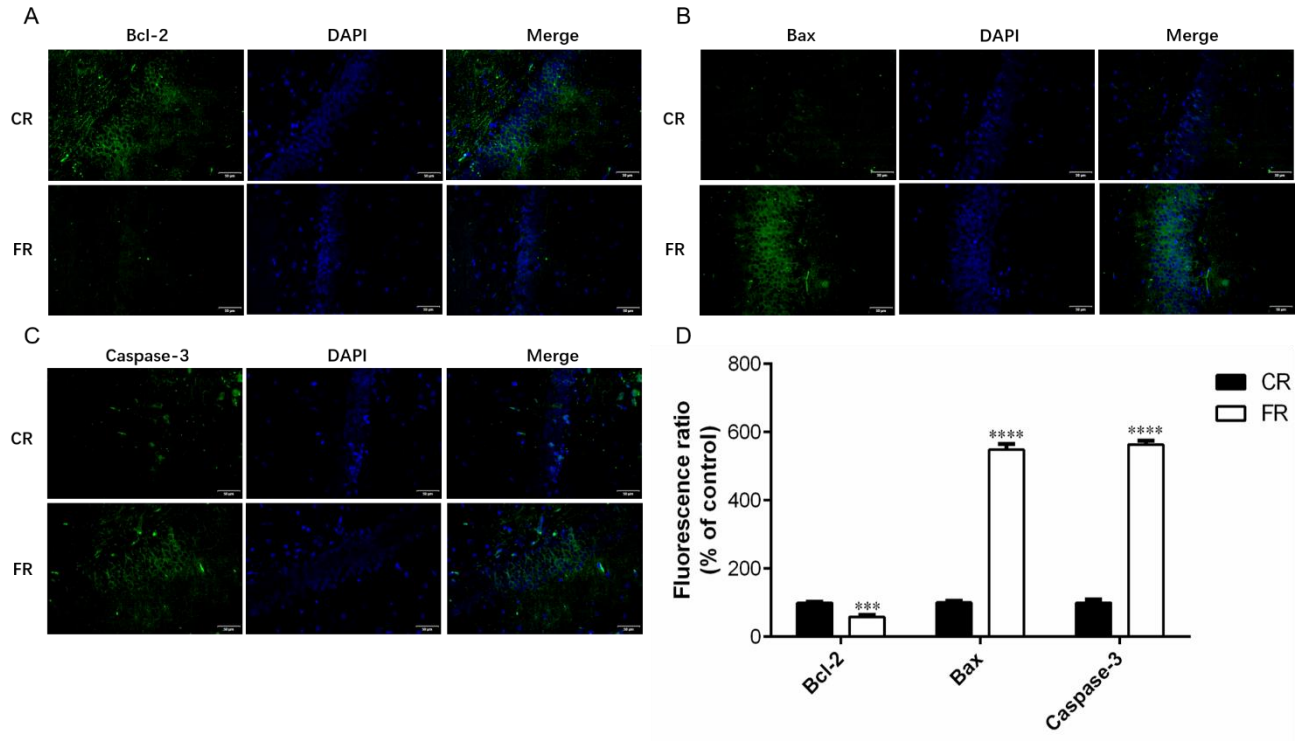

Fig. 3. Expressions of apoptosis related proteins in the hippocampus of rat brains with fluoride exposure. Representative immunofluorescence images (X 400) of Bcl-2 (A), Bax (B) and caspase-3 (C), and relative quantitative graphs (D). DAPI was used to label nucleus. CR, control group; FR, fluoride exposed group. The data shown are means ± SD. ***P < 0.001 and ****P < 0.0001 in comparison to controls as determined by analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test.

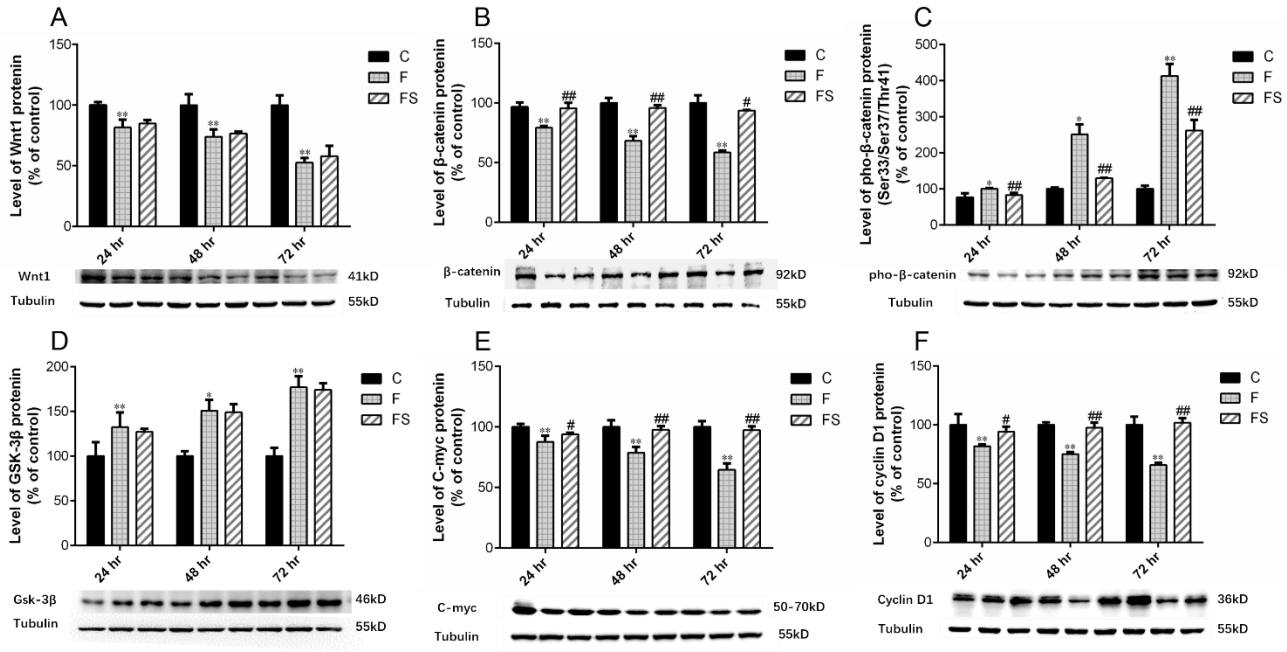

Fig. 4. Expressions of Wnt/β-catenin pathway and related proteins in primary hippocampal neurons exposed to fluoride. The protein expressions were determined by Western blotting method. Wnt1 (A), β-catenin (B), phospho-β-catenin (C), GSK-3β (D), c-Myc (E), and cyclin D1 (F). C, control group; F, fluoride exposed group; FS, fluoride+SKL2001 group. The data shown are means ± SD. *P < 0.05 and **P < 0.01 in comparison to controls; $^{\#}$P < 0.05 and $^{\#\#}$P < 0.01 in comparison to the fluoride group as determined by analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test.

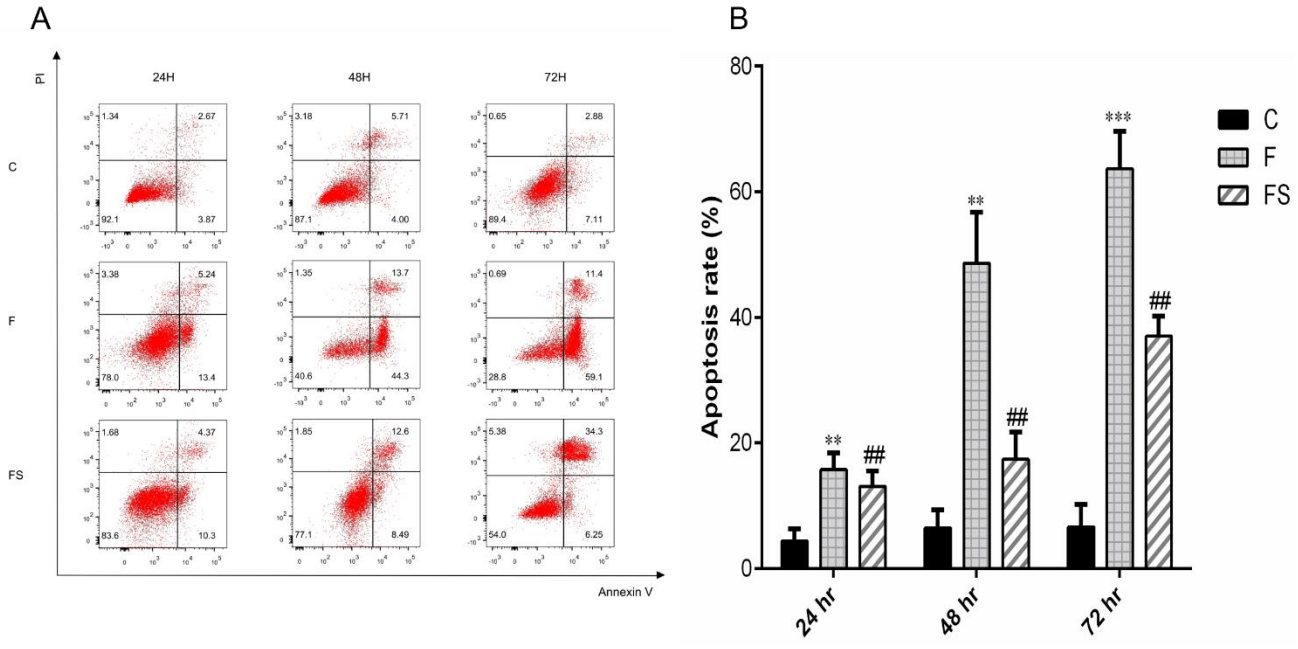

Fig. 5. Apoptosis in primary hippocampal neurons exposed to fluoride. Graphs of Flow Cytometry (A) and their statistical charts (B). C, control group; F, fluoride exposed group; FS, fluoride+SKL2001 group. The data shown are means ± SD. **P < 0.01 and ***P < 0.001 in comparison to controls; $^{\#\#}$P < 0.01 in comparison to the fluoride group as determined by analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test.

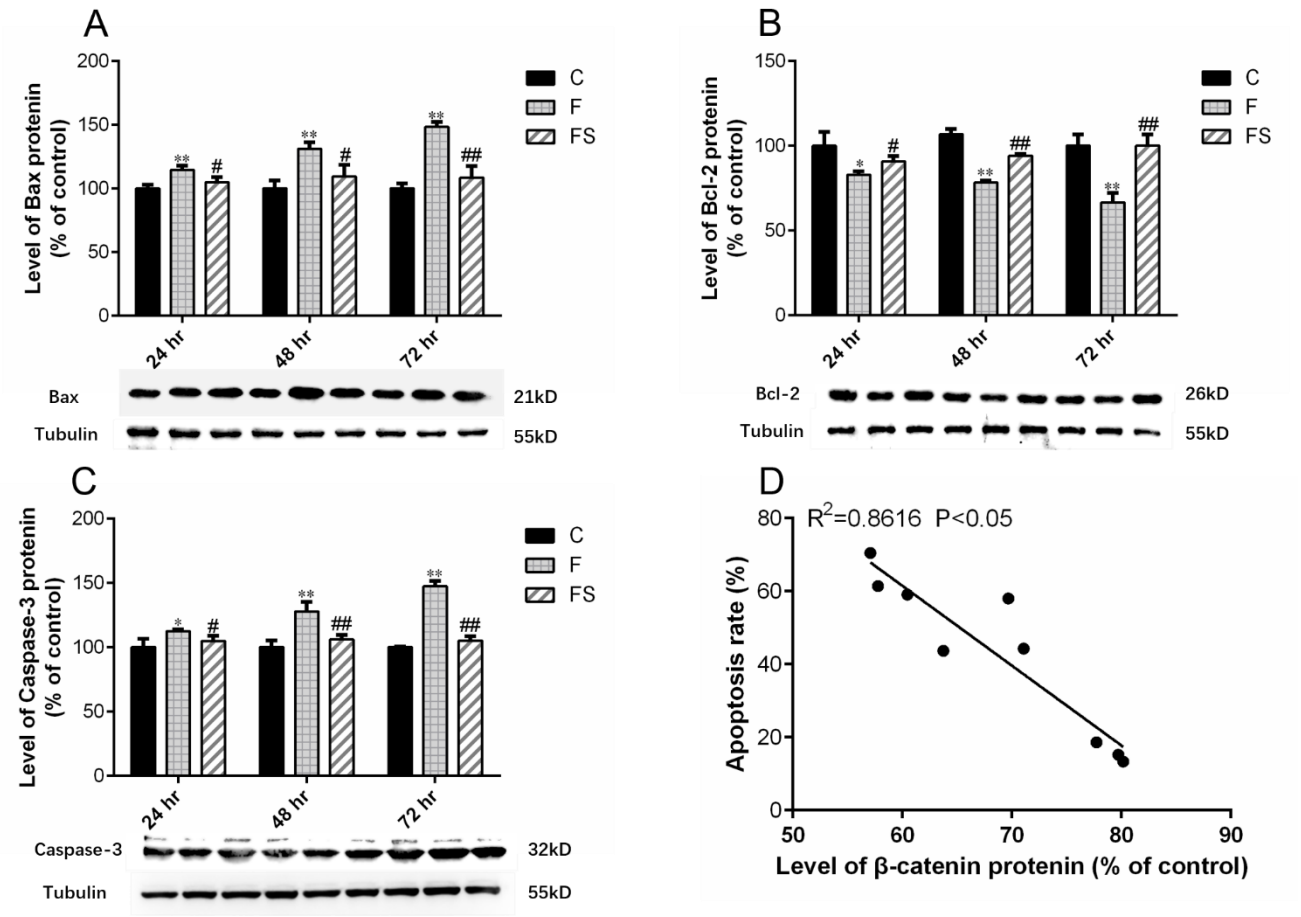

Fig. 6. Expression of apoptosis related proteins in primary neurons exposed to fluoride. The protein expressions were determined by Western blotting method. Bax (A), Bcl-2 (B), caspase-3 (C), and the correlation between the levels of apoptosis and β-catenin (D). C, control group; F, fluoride exposed group; FS, fluoride+SKL2001 group. The data shown are means ± SD. *P < 0.05 and **P < 0.01 in comparison to controls; #P < 0.05 and ##P < 0.01 in comparison to the fluoride group as determined by analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test.

DISCUSSION

It has been indicated that excessive fluoride induces significantly neuropathological changes in CNS.[13] Earlier studies have documented direct toxic effects on brains of experimental animals exposed to high levels of fluoride, including enhanced oxidative stress, reduced levels of nicotinic and muscarinic acetylcholine receptors, and mitochondrial abnormalities, along with impaired learning and memory as well as apoptosis.[14,15]

Signal transduction refers to the process that cell respond accordingly to extracellular signals, the binding between signal molecule in extracellular environment (primary messenger) and target cell receptor is then turned into perceptible signal (secondary messenger) via signal conversion.[16] In recent years, the multiple signal pathways correlating to exposure of fluoride have aroused more interest, such as Wnt/β-catenin, G-protein coupled receptor, cAMP, protein kinase C, mitogen-activated protein kinases, oxidative stress, death receptor/mitochondrion/endoplasmic reticulum stress mediated apoptosis, phosphatidylinositol 3-kinase/protein kinase B, nuclear factor-κB, transforming growth factor-β/Smad, Hedgehog, nuclear factor erythroid 2-related factor 2 and autophagy, in which all involved in physiological and pathological effect of fluoride.[16,17]

Among these signal translations, Wnt/β-catenin pathway has been extensively investigated. Defective Wnt signaling is found to be associated with various neurodegenerative diseases. It is important to restore Wnt/β-catenin signaling in the brains of patients with neurodegenerative disorders, particularly Alzheimer's and Parkinson's diseases, would improve their conditions involving brain function and metabolism.[18] In our early study, the rats fed with high dose of fluoride for six months showed neurodegenerative changes, which exhibited a significantly decreased ability of learning and memory and neuropathology.[19] Obviously, the declined protein levels of Wnt1 and total β-catenin, but an increased phosphor-β-catenin were determined in the hippocampus of rats and the primary neurons exposed to high doses of fluoride in our present study. To date, eight canonical gene elements associated with Wnt/β-catenin signaling have been described as Wnt1/2/3/3a/8a/8b/10a/10b.[20] The Wnt/β-catenin pathway primarily consists of transduction of Wnt signaling across the cell membrane, stabilization and regulation of β-catenin in the cytoplasm, and induction of transcription factor activation in the nucleus.[21] The β-catenin can inhibit action of GSK-3 to increase in phosphorylation of β-catenin but degradation of β-catenin. Therefore, the decreased Wnt1 in our results might induce more production of phosphor-β-catenin and less degradation of the molecular.

A study indicated that fluoride inhibited the activity of Wnt signaling to alter the inflammatory status and oxidative stress in V2 microglial cells, which provides a valid basis for the fluoride-induced neuroinflammation injury theory.[22] In fluoride-treated mouse odontoblasts, the decreased nuclear translocation of β-catenin, downregulated mRNAs for β-catenin and Wnt10b, reduced runt-related transcription factor 2 (RUNX2), and the transcription regulator of dentin sialophosphoprotein (DSPP) were observed, indicating that systemic exposure to excess fluoride resulted in reduced Wnt/β-catenin signaling in differentiating odontoblasts to downregulate DSPP production via RUNX2. [23]

However, the investigations relating skeletal fluorosis suggest that fluoride can enhance the hypoxia inducible factor-1α signaling, which in turn triggered autophagy and canonical Wnt/β-catenin signaling activation, ultimately leading to osteosclerosis in the rats.[24] Fluoride activated Wnt/β-catenin pathway and changed the related gene expression and β-catenin protein location in primary cultured mouse osteoblasts, promoting cell proliferation.[25] Wnt is a cytokine involved in various biological processes. It is indicated that the canonical Wnt/β-catenin pathway plays a crucial role in regulating osteoblast differentiation, osteogenic matrix formation, and bone homeostasis.[26]

On the other hand, Wnt/β-catenin pathway has been shown to be one of the most crucial morphogens in development and during the maturation of CNS, which is relevant during the establishment and maintenance of synaptic structure and neuronal function.[27] In addition, overexpression of Wnt is sufficient to increase neurogenesis from adult hippocampal stem/progenitor cells *in vitro* and *in vivo*; by contrast, blockade of Wnt signaling reduces neurogenesis.[28] A sustained exposure to high fluoride dose-dependently induced neuronal loss and apoptosis, weakened neurogenesis, decreased the protein levels of synaptophysin (one synaptic marker) and post-synaptic density 95 in the hippocampus of rats.[29] Therefore, these contrary results suggest that the role of the Wnt/β-catenin pathway may be related to the reactivity and metabolic requirement of different organs.

GSK 3, cyclin D1 and c-Myc are core effecting factors of the Wnt/β-catenin pathways.[30] GSK-3, an element of the Wnt/β-catenin pathway, plays a key role in numerous cellular processes including cell proliferation, embryonic development, and neuronal functions, and regulates glycogen and lipid synthesis.[31] GSK-3 modulates apoptosis and production of pro-inflammatory cytokines and interleukins, allowing adaptive changes in events such as cellular proliferation, migration, inflammation, and immunity.[32] The c-Myc is involved in the regulation of cell proliferation, differentiation, growth, apoptosis, cell cycle progression and metabolism of intracellular biomacromolecules, as well as malignant transformation of cells.[33] Cyclin D1, as a regulator of the cyclin-dependent kinase, enters the S phase in G1 phase, promotes DNA synthesis and accelerates cell proliferation through activation of CdK4 and CdK6.[34] In this study, excessive fluoride exposure reduced the levels of Wnt1 and β-catenin, leading to the high expression of GSK-3, and the inhibited cyclin D1 and c-

Myc, which might be one of the reasons of brain damages and apoptosis.

Wnt/β-catenin pathway is a multi-faceted regulator in cell apoptosis by mediating activation of the apoptotic regulators, including Bax/Bcl-2 (for mitochondrial pathways) and the family of caspase (for both the initiation and progression of apoptosis).[35] The intrinsic apoptosis pathway is addressed by pro-apoptotic-associated proteins (e.g., Bid, Bak, and Bax) and anti-apoptosis-related members of the Bcl-2 family (e.g., Bcl-2 and Bcl-XL).[36] In the study here, exposure to fluoride resulted in significant apoptosis, exhibiting increased protein levels of Bax and caspase-3 and decreased Bcl-2 in the hippocampus of rats and primary neurons, which could be resulted from the inhibited Wnt/β-catenin pathway. Whereas, activating the Wnt/β-catenin pathway inhibited expressions of pro-inflammatory factors and apoptotic proteins (caspase-3 and Bax/Bcl-2).[37]

SKL2001, an effective agonist of Wnt/β-catenin pathway, disrupts the Axin/β-catenin interaction, inhibits the phosphorylation of β-catenin at residues Ser33/37/Thr41 and Ser45, and upregulated protein level of β-catenin.[38] Interestingly, our results showed that SKL2001 apparently attenuated the neurotoxicity of fluoride on brains and primary neurons exposed to fluoride by activating Wnt/β-catenin pathway. In addition, a correlation between the declined levels of β-catenin protein and the raising levels of apoptosis in primary neurons exposed to fluoride was observed in the study. The obvious effects of SKL 2001 on attenuating the pathological changes induced by exposure of fluoride provide strong evidence to support the view that fluorosis causes decreased expression of Wnt/β-catenin pathway in CNS.

CONCLUSION

The models of rats with chronic fluorosis and the primary hippocampal neurons exposed to high fluoride were successfully established. The expression of Wnt/β-catenin pathway was inhibited in both rat brains and cultured neurons with fluorosis, which resulted in activated GSK-3β and declined cyclin D1 and c-Myc and subsequently led apoptosis exhibited with decreased Bcl2, and enhanced Bax and caspase-3. Interestingly, Wnt/β-catenin activator SKL2001 alleviated these neurotoxic damages caused by excessive fluoride. The results suggest that excessive fluoride induces inhibited expression of Wnt/β-catenin pathway in CNS, which results in apoptosis.

FUNDING

This work was financed by the National Natural Science Foundation of China (81860562, U1812403 and 82260668); the Foundation of Guizhou Province of China (2017-1142, WT2017-15, 2019-1276, ZK2021-490, ZK2022-342 and ZK2022-401).

CONFLICT OF INTERESTS

None.

REFERENCES

[1] Ren C, Li HH, Zhang CY, Song XC. Effects of chronic fluorosis on the brain. Ecotox Environ Safe 2022; 244:114021.

[2] Wei N, Dong YT, Deng J, Wang Y, Qi XL, Yu WF, et al. Changed expressions of N-methyl-d-aspartate receptors in the brains of rats and primary neurons exposed to high level of fluoride. J Trace Elem Med Bio 2018; 45:31-40.

[3] Ran LY, Xiang J, Zeng XX, He WW, Dong YT, Yu WF, et al. The influence of NQO2 on the dysfunctional autophagy and oxidative stress induced in the hippocampus of rats and in SH-SY5Y cells by fluoride. CNS Neurosci Ther 2023; 29:1129-1141.

[4] Grandjean P. Developmental fluoride neurotoxicity: an updated review. Environ Health-Glob 2019; 18:110.

[5] Oliva CA, Montecinos-Oliva C, Inestrosa NC. Wnt signaling in the central nervous system: new insights in health and disease. Prog Mol Biol Transl Sci 201; 153:81-130.

[6] Song Y, Lee S, Kim JR, Jho EH. Pja2 inhibits Wnt/β-catenin signaling by reducing the level of TCF/LEF1. Int J Stem Cells 2018; 11:242-247.

[7] Gehrke I, Gandhirajan RK, Kreuzer KA. Targeting the WNT/beta-catenin/TCF/LEF1 axis in solid

and haematological cancers: multiplicity of therapeutic options. Eur J Cancer 2009; 45:2759-67.

[8] Wang Q, Huang X, Su Y, Yin G, Wang S, Yu B, et al. Activation of Wnt/beta-catenin pathway mitigates blood-brain barrier dysfunction in Alzheimer's disease. Brain 2022; 145:4474-4488.

[9] Tao W, Ruan J, Wu R, Zhao M, Zhao T, Qi M, et al. A natural carotenoid crocin exerts antidepressant action by promoting adult hippocampal neurogenesis through Wnt/beta-catenin signaling. J Adv Res 2023; 43:219-231.

[10] Chen R, Zhao LD, Liu H, Li HH, Ren C, Zhang P, et al. Fluoride induces neuroinflammation and alters Wnt signaling pathway in BV2 microglial cells. Inflammation 2017; 40:1123-1130.

[11] Zhang CY, Chen R, Wang F, Ren C, Zhang P, Li Q, et al. EGb-761 attenuates the anti-proliferative activity of fluoride via DDK1 in PC-12 cells. Neurochem Res 2017; 42:606-614.

[12] Fan Q, Xi P, Tian D, Jia L, Cao Y, Zhan K, et al. Ginsenoside Rb1 facilitates browning by repressing Wnt/beta-catenin signaling in 3T3-L1 adipocytes. Med Sci Monit 2021; 27:e928619.

[13] Cao K, Xiang J, Dong YT, Xu Y, Li Y, Song H, et al. Exposure to fluoride aggravates the impairment in learning and memory and neuropathological lesions in mice carrying the APP/PS1 double-transgenic mutation. Alzheimers Res Ther 2019; 11:35.

[14] Dong YT, Wang Y, Wei N, Zhang QF, Guan ZZ. Deficit in learning and memory of rats with chronic fluorosis correlates with the decreased expressions of M1 and M3 muscarinic acetylcholine receptors. Arch Toxicol 2015; 89:1981-1991.

[15] Zhang KL, Lou DD, Guan ZZ. Activation of the AGE/RAGE system in the brains of rats and in SH-SY5Y cells exposed to high level of fluoride might connect to oxidative stress. Neurotoxicol Teratol 2015; 48:49-55.

[16] Qi X, Effect of fluoride on signal transduction pathways. In: Guan ZZ (Ed). Coal-burning Type of Endemic Fluorosis – Pathophysiology and Clinical Treatments. Publisher: Springer, Singapore, 2021, p:225-249.

[17] Qiao L, Liu X, He Y, Zhang J, Huang H, Bian W, et al. Progress of signaling pathways, stress pathways and epigenetics in the pathogenesis of skeletal fluorosis. Int J Mol Sci 2021; 22:11932.

[18] Anand AA, Khan M, VM, Kar D. The molecular basis of Wnt/β-catenin signaling pathways in neurodegenerative diseases. Int J Cell Biol 2023; 2023:9296092.

[19] Ma YL, Deng J, Zhang T, Li HM, Liang QZ, Zhang KL. Enhanced expression of RAGE/NADPH oxidase signaling pathway and increased level of oxidative stress in brains of rats with chronic fluorosis and the protective effects of blockers. J Trace Elem Med Bio 2023; 80:127288.

[20] Gajos-Michniewicz A, Czyz M. WNT signaling in melanoma. Int J Mol Sci 2020; 21:4852.

[21] Ma Q, Yu J, Zhang X, Wu X, Deng G. Wnt/β-catenin signaling pathway-a versatile player in apoptosis and autophagy. Biochimie 2023; 211:57-67.

[22] Chen R, Zhao LD, Liu H, Li HH, Ren C, Zhang P, et al. Fluoride induces neuroinflammation and alters Wnt signaling pathway in BV2 microglial cells. Inflammation 2017; 40:1123-1130.

[23] Kao YH, Igarashi N, Abduweli Uyghurturk D, Li Z, Zhang Y, Ohshima H, et al. Fluoride alters signaling pathways associated with the initiation of dentin mineralization in enamel fluorosis susceptible mice. Biol Trace Elem Res 2021; 199:3021-3034.

[24] Zhu S, Liu J, Zhao J, Zhou B, Zhang Y, Wang H. HIF-1α-mediated autophagy and canonical Wnt/β-catenin signalling activation are involved in fluoride-induced osteosclerosis in rats. Environ Pollut 2022; 315:120396.

[25] Wang J, Yang J, Cheng X, Yin F, Zhao Y, Zhu Y, et al, Influence of calcium supplementation against fluoride-mediated osteoblast impairment in vitro: involvement of the canonical Wnt/β-catenin signaling pathway. J Agric Food Chem 2019; 67:10285-10295.

[26] Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. Nat Med 2013; 19:179-192.

[27] Oliva CA, Montecinos-Oliva C, Inestrosa NC. Wnt signaling in the central nervous system: new insights in health and disease. Prog Mol Biol Transl Sci 2018;1 53:81-130.

[28] Lie DC, Colamarino SA, Song HJ, Désiré L, Mira H, Consiglio A, et al. Wnt signalling regulates adult hippocampal neurogenesis. Nature 2005; 437:1370-1375.

[29] Jiang P, Li G, Zhou X, Wang C, Qiao Y, Liao D, et al. Chronic fluoride exposure induces neuronal apoptosis and impairs neurogenesis and synaptic plasticity: Role of GSK-3β/β-catenin pathway. Chemosphere 2019; 214:430-435.

[30] Jia XX, Zhu TT, Huang Y, Zeng XX, Zhang H, Zhang WX. Wnt/β-catenin signaling pathway regulates asthma airway remodeling by influencing the expression of c-Myc and cyclin D1 via the p38 MAPK-dependent pathway. Exp Ther Med 2019; 18:3431-3438.

[31] Primot A, Baratte B, Gompel M, Borgne A, Liabeuf S, Romette JL, et al. Purification of GSK-3 by affinity chromatography on immobilized axin. Protein Expr Purif 2000; 20:394-404.

[32] Noori T, Dehpour AR, Sureda A, Fakhri S, Sobarzo-Sanchez E, Farzaei MH, et al. The role of glycogen synthase kinase 3 beta in multiple sclerosis. Biomed Pharmacother 2020; 132:110874.

[33] Aguirre M, Escobar M, Forero Amézquita S, Cubillos D, Rincón C, Vanegas P, et al. Application of the yamanaka transcription factors Oct4, Sox2, Klf4, and c-Myc from the laboratory to the clinic. Genes (Basel) 2023; 14:1697.

[34] Chen Z, Duan RS, Zhu Y, Folkesson R, Albanese C, Winblad B, Zhu J. Increased cyclin E expression may obviate the role of cyclin D1 during brain development in cyclin D1 knockout mice. J Neurochem 2005; 92:1281-1284.

[35] Verbrugge I, Johnstone RW, Smyth MJ. SnapShot: Extrinsic apoptosis pathways. Cell 2010; 143:1192, 1192.e1-2.

[36] Davidovich P, Kearney CJ, Martin SJ. Inflammatory outcomes of apoptosis, necrosis and necroptosis. Biol Chem 2014; 395:1163-1171.

[37] Su X, Jing X, Jiang W, Li M, Liu K, Teng M, et al. Curcumin-containing polyphosphazene nanodrug for anti-Inflammation and nerve regeneration to improve functional recovery after spinal cord injury. Int J Pharm 2023; 642:123197.

[38] Gwak J, Hwang SG, Park HS, Choi SR, Park SH, Kim H, et al. Small molecule-based disruption of the Axin/β-catenin protein complex regulates mesenchymal stem cell differentiation. Cell Res 2012; 22:237-247.