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Extract of *Ginkgo biloba* leaves attenuates neurotoxic damages in rats and SH-SY5Y cells exposed to a high level of fluoride

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ABSTRACT

Background: Potential protection against the neurotoxic damages of high levels of fluoride on rats and SH-SY5Y cells by extract of *Ginkgo biloba* leaves, as well as underlying mechanisms, were examined.
Methods: The rats were divided randomly into 4 groups, i.e., control, treatment with the extract (100 mg/kg body weight, gavage once daily), treatment with fluoride (50 ppm F^- in drinking water) and combined treatment with both; SH-SY5Y cells exposed to fluoride and fluoride in combination with the extract or 4-Amino-1,8-naphthalimide (4-ANI), an inhibitor of poly (ADP-ribose) polymerase-1 (PARP-1). Spatial learning and memory in the rats were assessed employing Morris water maze test; the contents of fluoride in brains and urine by fluoride ion-selective electrode; cytotoxicity of fluoride was by CCK-8 kit; the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and the content of malondialdehyde (MDA) by appropriate kits; the level of 8-hydroxydeoxyguanosine (8-OHdG) was by ELISA; the content of ROS and frequency of apoptosis by flow cytometry; the expressions of phospho-histone H2A.$X_{(Ser139)}$, PARP-1, poly (ADP-ribose) (PAR) and Sirtuin-1 (SIRT1) by Western blotting or immunofluorescence.
Results: The rats with prolong treatment of fluoride exhibited dental fluorosis, the increased contents of fluoride in brains and urine and the declined ability of learning and memory. In the hippocampus of the rats and SH-SY5Y cells exposed to fluoride, the levels of ROS, MDA, apoptosis, 8-OHdG and the protein expressions of histone H2A.$X_{(Ser139)}$, PARP-1 and PAR were all elevated; the activities of SOD and GSH-Px and the protein expression of SIRT1 reduced. Interestingly, the treatment of *Ginkgo biloba* extract attenuated these neurotoxic effects on rats and SH-SY5Y cells exposed to fluoride and the treatment of 4-ANI produced a neuroprotective effect against fluoride exposure.
Conclusion: Ginkgo biloba extract attenuated neurotoxic damages induced by fluoride exposure to rats and SH-SY5Y cells and the underlying mechanism might involve the inhibition of PARP-1 and the promotion of SIRT1.

1. Introduction

Excessive deposition of fluoride in the central nervous system (CNS) can cause cognitive dysfunction in adult patients and lower intelligence quotient of children living in the area of endemic fluorosis [1,2]. At the same time, there is considerable evidence that long-term exposure to fluoride impairs learning and memory of experimental animals, and leads to pathological changes in their brains [3]. Importantly, fluoride can cross the blood-brain barrier into brain tissue and cause significant neuropathological damage [4].

The pathogenesis of chronic fluorosis is quite complex. Accumulating and widely accepted evidence indicates that enhanced oxidative stress is

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an important cause of the systemic damage induced by endemic (chronic) fluorosis [5]. Oxidative stress is commonly proposed to be a key factor in connection with the histopathological damage to CNS caused by chronic fluorosis [6]. It has been indicated that excessive fluoride triggers oxidative stress by elevating the level of reactive oxygen species (ROS) and/or sabotaging intracellular anti-oxidative defense systems [7], which may give rise to permanent brain damage. ROS can attack organelles and cellular components, particularly compromising the integrity of DNA [8]. Within a few minutes after the formation of double-strand breaks in DNA, H2A.X, a member of the histone H2A family, can become extensively phosphorylated at Ser139 [9], a phenomenon commonly utilized as an indicator of DNA damage. In a study concerning dental fluorosis, ameloblasts exposed to fluoride exhibited an enhanced phosphorylation of this histone [10].

Poly (ADP-ribose) polymerase-1 (PARP-1), a nicotinamide adenine dinucleotide (NAD)-dependent enzyme, plays a key role in the detection and repair of DNA breaks caused by oxidative stress [11]. Activation of PARP-1 increases cellular level of its product, poly (ADP-ribose) (PAR), which may be beneficial to maintain DNA integrity. At the same time, catalytic formation of PAR consumes considerable amounts of NAD^+ and may lower the level of this cofactor to an extent that is detrimental to sirtuin-1 (SIRT1), an acetylase that also requires NAD^+ and plays an important antioxidant role [12,13].

We reported previously that exposure of rats or SH-SY5Y cells to high level of fluoride decreased the expression of SIRT1 in the rat brain and this cell line with the enhanced oxidative stress [14]. Whereas, the up-regulation of SIRT1 reversed the oxidative damage caused by fluorosis [14]. Therefore, reducing the activity of PARP-1 and elevating the expression of SIRT1 might help protect against the oxidative damage to DNA resulted from fluorosis.

At present, there are still no effective drugs for the therapy of chronic fluorosis. Since the theory of oxidative stress has been widely recognized in the pathogenesis of systemic damages caused by chronic fluorosis, the development of antioxidant drugs has become a very importantly development direction in recent years [15]. The significantly protective and therapeutic results of vitamin E and C treatments were obtained, in which the damage of DNA oxidation induced by experiment chronic fluorosis was attenuated by the antioxidants [16].

In recent years, Chinese herbal medicine for the treatment of endemic fluorosis has been advocated [17]. Interestingly, the neuroprotective effect of extract prepared from *Ginkgo biloba* leaves has been receiving more and more attention [18]. *Ginkgo biloba* extract can exert anti-oxidant, -inflammatory, -apoptotic and -genotoxic effects [19]. In addition, the treatment by *Ginkgo biloba* extract in coronary artery disease leads to an increase of blood flow of distal left anterior descending coronary artery [20] and ocular blood flow velocity [21], and attenuates the increase in intracranial pressure and the reduction in cerebral blood perfusion after subarachnoid hemorrhage [22]. Furthermore, *Gingko biloba* extract can attenuate the expression of inducible nitric oxide synthase mediated by advanced glycation end products in murine microglia [23] and displays anti-neuroinflammatory activity in lipopolysaccharide-activated primary microglia cells [24]. It has been indicated that the treatment with *Ginkgo biloba* extract significantly improved the cognitive function of the TgCRND8 mice with the pathology of Alzheimer's disease (AD), in which the mechanism involves its inhibited microglial inflammatory activation in the brain [25]. *Ginkgo biloba* extract was reported to attenuate the hepatotoxicity induced by combined exposure to cadmium and fluoride by reducing the redox imbalance and modulating the Bax/Bcl-2 and NF-κB signaling pathways [26]. Chronic exposure to high level of fluoride causes severe impairment in the spatial learning and memory, these deficits can be ameliorated with the vitamin C and *Gingko biloba* [27]. However, the mechanism of *Ginkgo biloba* extract against chronic fluorosis, especially its effect on brain oxidative damage caused by fluorosis, remains unclear.

Here, in the investigation the neurotoxicity of rats and SH-SY5Y cells exposed to high level of fluoride was examined and *Ginkgo biloba* extract used to treat the animals and the cultured cells in order to reveal whether the herb medicine could play a potential therapeutic effect against fluorosis and thereafter to elucidate the underlying mechanism.

2. Materials and methods

2.1. Materials

Sodium fluoride and 4-Amino-1,8-naphthalimide (4-ANI) (Sigma-Aldrich Inc., USA); *Ginkgo biloba* extract (Dr. Willmar Schwabe, GmbH & Co. KG, Germany); anti-GAPDH and -PARP-1 antibodies (GeneTex Inc., USA); anti-histone H2A.X(Ser139) antibody (Biorbyt Inc., England); anti-PAR antibody (Novusbio Inc., USA); anti -SIRT1 antibody (Abcam Inc., USA); horseradish peroxidase (HRP)-conjugated anti-rabbit and -mouse IgG (Cell Signaling Technology, USA); CY-3 or 488-labeled anti-mouse or -rabbit IgG (Thermo Scientific Inc., USA); the BCA protein assay kit (Thermofisher Inc., USA); cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Japan); DNA extraction kit (Tiangen Inc., China); DNA oxidative damage high sensitivity detection kit (Cayman Inc., USA); Annexin V and PI double staining kit and ROS detection kit (Solarbio Inc., China); enhanced chemiluminescence kit (Minipore Inc., USA); blocking medium (Dako Inc., Denmark); and the remaining chemicals (Sigma-Aldrich, USA) were obtained from the sources indicated.

2.2. Experimental animals

Twenty-four adult Sprague-Dawley rats (4 weeks of old with weighing 80–100 g) were purchased from the Animal Experiment Center of Guizhou Medical University, China. These animals were housed in cages in a temperature (20–25 °C) and humidity controlled (40–60 %) environment, with a 12-h light/dark cycle.

Following acclimation for one week, the animals were divided randomly into four groups of 6 animals (half male and half female) each for the following six-month treatments. In the control group, the rats received clean tap water containing less than 0.5 ppm fluoride and received gavage normal saline of 1 ml once per day; in the ginkgo group, the rats, drinking the same tap water indicated above, were treated with *Ginkgo biloba* extract (100 mg/kg) once daily by intragastric administration [28,29]; in the fluoride group, the rats were given drinking tap water containing 50 ppm fluoride and received gavage normal saline [30]; in the fluoride+ginkgo group, the rats were given drinking water containing 50 ppm fluoride and were also treated with *Ginkgo biloba* extract (100 mg/kg) once daily by intragastric administration. The experiment period was 6 months. At the end of the experiment, dental changes and fluoride contents of the brain and urine were examined.

The tablets of *Ginkgo biloba* extract obtained from Dr. Willmar Schwabe GmbH & Co. KG are commercially used for clinical treatment and basic research, and contain 40 mg *Ginkgo biloba* per one. The tablets were ground into powder with a grinder and dissolved in normal saline used for intragastric administration to rats (100 mg/kg) and in cell culture medium for exposure of the cultured SH-SY5Y cells (100 mg/l).

The protocol of the animal study was pre-approved by the Institutional Animal Care and Ethics Committee of Guizhou Medical University, China.

2.3. Fluoride contents in brains and urine

At the end of the experiment, the rats were placed in metabolic cages and deprived food or water, and their 24-h urine volumes, respectively, were collected. In addition, after the rats were sacrificed, brain tissues taken were placed in a high-temperature ash furnace (SX-2.5–12 Box Type Resistance Furnace, Nanchang Jiedao Scientific Instrument Co., Ltd., China), and then calcined into powder at 650 °C for 1 h, and thereafter 0.1 g of the ash sample was dissolved in 5 ml of hydrochloric

acid (0.25 mol/l) [31].

The fluoride contents in rat urine and brain tissue were determined by the analysis of fluoride ion-selective electrode as referring these methods [32,33]. In brief, the working standard solutions of fluoride with 1, 2, 5, 10 and 100 μg/ml, respectively, were prepared. Meanwhile, total ionic strength adjustment buffer solution (TISAB) was prepared by using trisodium citrate, sodium chloride and glacial acetic acid, and pH was adjusted to 5.2 by sodium hydroxide solution (5 mol/l). Perchloric acid (30 %) is used to adjust the pH of these samples. Finally, the detecting solutions of fluoride with 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 μg/ml were prepared by the working standard solution of fluoride and TISAB. The mixture of brain sample solution or urine with TISAB by a ratio of 1:1 was put in a test-tube with a stirring rod on the air-driven magnetic stirrer. The fluoride ion electrode (Changsa Analysis Instrumentation, China) was rinsed with the acetate buffer until the potential was greater than 380 mV. At the same time, saturated calomel electrode was put in the solution. When the ion meter displayed "stable", the corresponding potential values of the samples were read. A standard curve was drawn according to the fluoride standard sample concentrations and potential values, and the fluoride contents of the corresponding samples was obtained by checking the standard curve.

2.4. Cell cultures

SH-SY5Y cell line is originated from human neuroblastoma cells. The cells have these expression characteristics peculiar as nerve cells, such as cholinergic acetylcholine nicotinic and muscarinic receptors, N-methyl-D-aspartic acid receptor, catecholamine neuron specific tyrosine hydroxylase, dopamine β hydroxylase and dopamine transporter, etc., which is widely used in vitro study in many nervous system diseases including fluorosis [34,35]. The concentration of fluoride chosen for treatment of the SH-SY5Y cells was determined by exposing these cells to 0–240 ppm fluoride for 48 h, detecting cell survival rates with the CCK8 assay, in which 160 ppm was selected as the concentration of fluoride used to the exposure to the cultured cells. The experimental groups are as follows. In the control group, cells were received no special treatment.; in the ginkgo group, cells with *Ginkgo biloba* extract (100 mg/l) for 48 h [36]; in the treatment of 4-ANI (a specific inhibitor of PARP-1), cells with 10 μM 4-ANI for 24 h [37]; in fluoride group, cells with 160 ppm fluoride for 48 h [38]; in the fluoride+ginkgo group, cells with 160 ppm fluoride and *Ginkgo biloba* extract (100 mg/l) for 48 h; in fluoride + 4-ANI group, cells first incubated with 10 μM 4-ANI for 24 h and thereafter with 160 ppm fluoride for 48 h.

2.5. The Morris water maze test

Following the 6 months of treatment, the 6 rats in each group each performed the Morris water maze test of spatial learning and memory, in randomized order [39]. This test requires the rat to locate an escape platform located beneath the surface of the water, while the movement of the animal is monitored with the Videotrack software (Viewpoint). Each rat performed this test 4 times daily for 4 days, with 5–7 min intervals of rest between trials The time required to locate the escape platform (escape latency) was recorded, while rats who failed to locate this platform within a min were guided to it and their time recorded as 60 s. After finding this platform, the rat was allowed to rest there for 2 s. The average time required to find the platform on each of the four days was subjected to further analysis. In addition, after removing the platform on the 5th day, the time that elapsed before the rat first crossed the previous location of the platform and the number of times it crossed this location were recorded. All behavioral tests were performed in a quiet environment with subdued lighting.

2.6. Quantification of ROS in rat brain tissues and SH-SY5Y cells using 2′− 7′-dichlorofluorescin diacetate (DCFH-DA) as a fluorescent probe

Immediately after removal of the hippocampus, 0.05 g of this tissue was diced and rinsed twice with pre-cooled PBS to remove blood. Subsequently, the tissue was cut into 1-mm^3 pieces with ophthalmic scissors, an appropriate amount of trypsin solution containing phenol red added, and the sample incubated at 37 °C in a water bath for 20 min, during which time the tissue was gently disrupted by sucking up and down through a straw. Digestion of the tissue was terminated by addition of PBS and the single-cell suspension obtained passed through a 40 μM filter and the filtrate collected into a fresh centrifuge tube. The unicell in the brain tissue were extracted from the hippocampus of the brain tissue, which is mainly composed of neurons after the connective tissue is separated and removed.

Next, the cells were washed twice with PBS and incubated with 10 mM DCFH-DA at 37 °C for 30 min in accordance with the manufacturer's instructions. Intracellularly, DCFH-DA is deacetylated by a non-specific esterase and the deacetylated form oxidized by ROS to give the fluorescent compound 2,7-dichlorofluorescein (DCF) [40]. DCF fluorescence was monitored in a flow cytometer (Becton Dickinson Inc., USA) with excitation and emission wavelengths of 488 nm and 525 nm, respectively and the median fluorescence intensity determined using the FlowJo program.

2.7. DNA oxidative damage product 8-hydroxydeoxyguanosine (8-OHdG) was detected by ELISA kit

The level of 8-OHdG was detected by ELISA kit [41]. In detail, the 0.2 g brain tissue was taken out from − 80 °C and put into a 2 ml EPP centrifuge tube. After 1 ml of 1 × PBS and the enzyme-free grinding beads were added, it was ground in the animal tissue grinder at low temperature of 60 Hz for 1 min. The purified DNA was obtained according to the DNA extraction kit; one unit of nuclease P1 and alkaline phosphatase was added successively to every 100 μg of DNA. Then, it was incubated in a 37 °C for 30 min, boiled at 100 °C for 10 min and stored at 4 °C for use. After that, the ELISA buffer, gradient standards of 8-OHdG, samples and acetylcholine esterase tracer were added into related wells, respectively, and then 50 μl ELISA monoclonal antibody was added into each well covered with plastic film and the mixture incubated at 4 °C for 18 h. After emptying the incubation plate by cleaning with washing buffer, the wells were added with Ellman's reagent or tracer, covered with the plastic film and shaken away from light for 2 h. Finally, the plate was read at a wavelength of 410 nm. The corresponding contents of 8-OHdG in these samples were calculated through the standard curve.

2.8. Assaying the activities of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) and the content of malondialdehyde (MDA) in brain tissues of rats

After homogenizing the hippocampal tissue in ice-cold PBS and centrifuging at 5000 rpm for 10 min at 4 °C, the activities of SOD and GSH-Px, as well as the content of MDA, in the supernatants were determined in accordance with the instructions of the manufacturers of the biochemical assay kits employed (Nanjing Jiancheng Inc., China). Calculation of amounts from the OD values obtained was based on standard values supplied by the instruction manuals.

2.9. Determination of apoptosis in SH-SY5Y cells by staining with Annexin-V in combination with PI

After the treatment period, cells that still adhered were harvested and transferred into sterile centrifuge tubes, followed by centrifugation at 1000 rpm for 5 min at room temperature (RT). The supernatant was aspirated and the cell pellet resuspended in 200 μl of binding buffer.

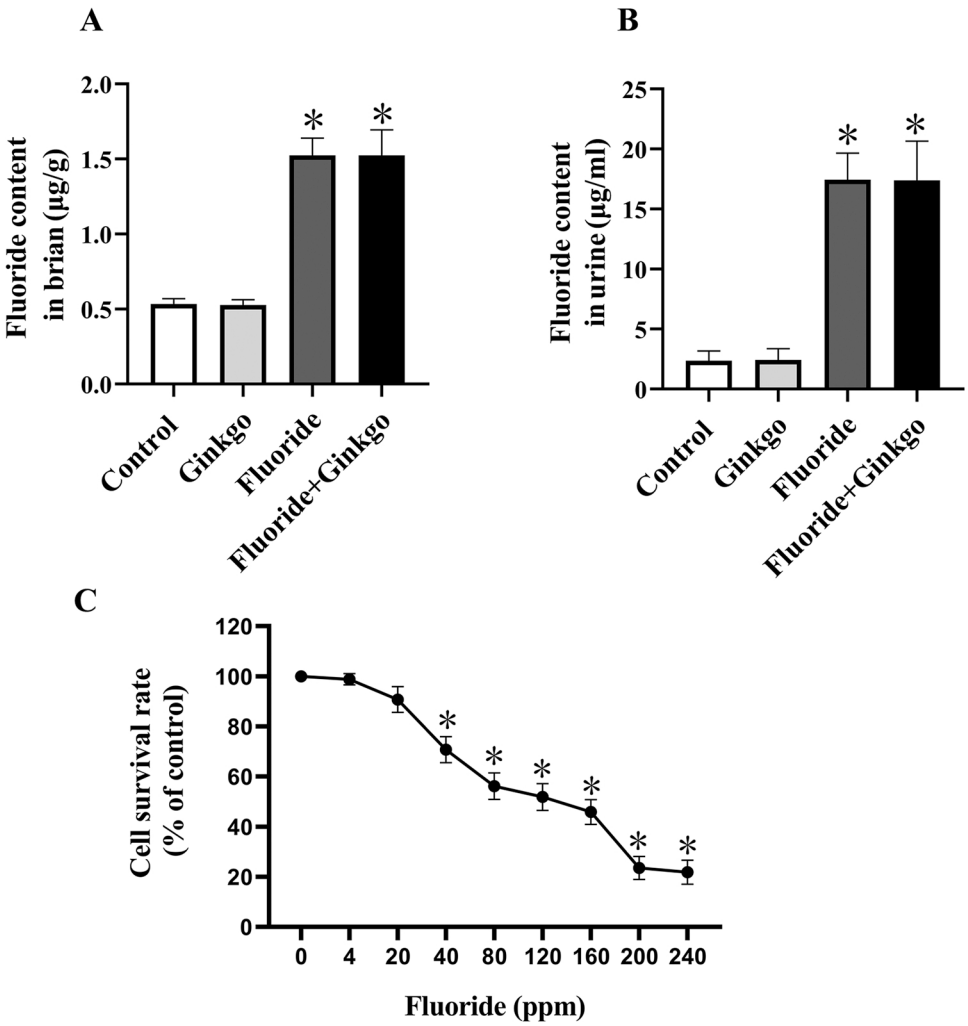

Fig. 1. Confirmation of the successful establishment of the animal model of chronic fluorosis and determination of an appropriate concentration of fluoride for treatment of SH-SY5Y cells. A: content of fluoride in the brains of the rats treated in different ways. B: content of fluoride ion in the urine of these animals. C: the survival rate of SH-SY5Y cells exposed to increasing concentrations of fluoride. Control: drinking water containing less than 0.5 ppm fluoride (F^-); ginkgo: drinking water without fluoride and administration of *Ginkgo biloba* extract (100 mg/kg/day, gavage once daily); fluoride: drinking water containing 50 ppm fluoride; fluoride+ginkgo: drinking water containing 50 ppm fluoride and administration of *Ginkgo biloba* extract. The values presented are means ± SDs (n = 6). $^*P < 0.05$ compared to the control group; $^{\#}P < 0.05$ compared to the fluoride group.

Then, 10 μl Annexin-V and 10 μl PI were added. and incubated with the cells for 5 min at RT in the dark. Within 30 min of this staining, the samples were characterized by flow cytometryand the results used to quantify apoptosis among the SH-SY5Y cells with the FlowJo software [42].

2.10. Examination of the levels of H2A.X$_{(Ser139)}$, PARP-1 and SIRT1 by immunofluorescent staining

Blocks of fresh tissue were first fixed in 4 % paraformaldehyde for 24 h, then washed, dehydrated, rendered transparent, dipped in wax and embedded, and, finally, sliced (in approximately 5-μm sections) for storage. Prior to immunofluorescent staining, these sections were deparaffinized and dehydrated, next subjected to a 20-min treatment in a microwave oven in citric acid buffer (0.01 M, pH 6.0) for antigen retrieval, followed by a 30-min incubation in blocking medium (Dako Inc., Denmark) at RT [43].

Subsequently, the sections were incubated overnight at 4 °C with anti-histone H2A.X$_{(Ser139)}$, anti-PARP-1 or anti -SIRT1 antibodies. The next day, each section was incubated further with anti-rabbit goat IgG or anti-mouse goat IgG antibody labeled with fluorescein isothiocyanate for 1 h at RT. Thereafter, the sections were rinsed 3 times with PBS and then covered with Vectashield (Vector Laboratories., USA).

For immunofluorescent staining of histone H2A.X$_{(Ser139)}$ in SH-SY5Y cells, the cells were first seeded onto a confocal dish, followed by 3 washes with PBS and fixation in paraformaldehyde (4 %). Then, these samples were incubated first for 30 min with goat serum at RT and, subsequently, overnight with antibody against H2A.X$_{(Ser139)}$ at 4 °C. The following day, the cells were incubated with fluorescein isothiocyanate-labeled anti-rabbit goat IgG for 1 h at RT and then rinsed 3 times with PBS.

Employing the Image Pro Plus software (USA), PAR and SIRT1 were quantified on the basis of the integral optical density (IOD) of their immunofluorescent staining and histone H2A.X$_{(Ser139)}$ on the basis of the fraction of the area of visual fields selected stochastically that was stained, with 400-fold magnification.

2.11. Determination of the levels of H2A.X$_{(Ser139)}$, PARP-1, PAR and SIRT1 by Western blotting

Brain tissues or cultured cells were placed in lysis buffer containing a mixture of protease inhibitors and disrupted in a glass homogenizer. The resulting homogenate was centrifuged for 20 min at 12,000 rpm and 4 °C. After assaying the protein concentrations in the supernatants with the BCA kit [43], proteins were isolated by 10% SDS-PAGE and a transfer unit (Bio-Rad Inc., USA) utilized to blot the proteins isolated onto polyvinylidene difluoride films.

For relative quantification of individual proteins, these membranes were incubated with antibodies directed against H2A.X, PARP-1, PAR, SIRT1 or β-actin overnight at 4 °C. Next, they were rinsed and incubated for 60 min with secondary antibody conjugated with HRP. Subsequently, an enhanced chemiluminescence kit was employed to detect the protein bands; the resulting signals visualized by exposure for 30 s–3 min to chemiluminescence film (hyper performance); and signal

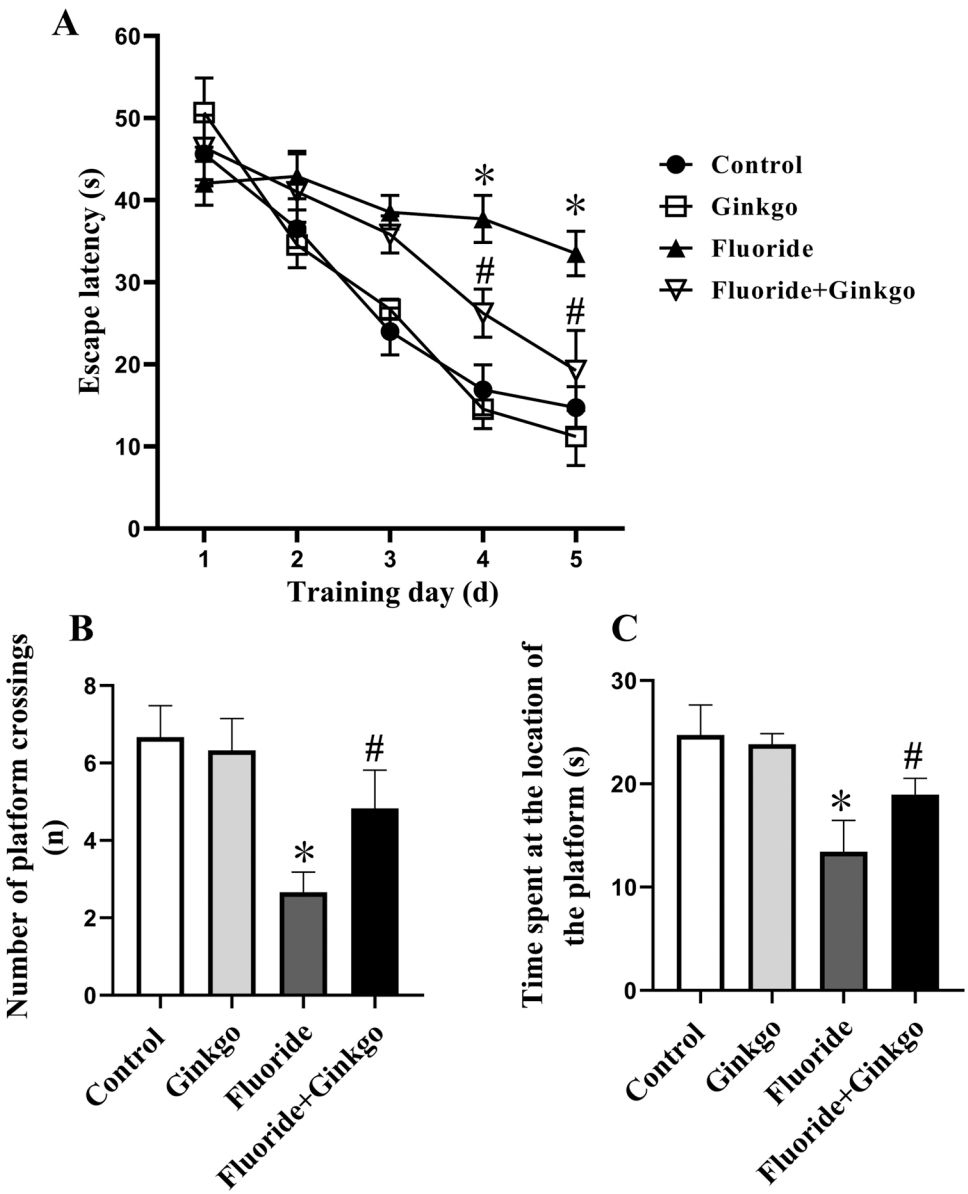

Fig. 2. Assessment of learning and memory in rats treated with fluoride and/or the *Ginkgo biloba* extract using the Morris water maze test. Control: drinking water containing less than 0.5 ppm fluoride; ginkgo: drinking water without fluoride and administration of *Ginkgo biloba* extract (100 mg/kg/day); fluoride: drinking water containing 50 ppm fluoride; fluoride+ginkgo: drinking water containing 50 ppm fluoride and administration of *Ginkgo biloba* extract. The values presented are means ± SDs (n = 6). *P < 0.05 compared to the control group; #P < 0.05 compared to the fluoride group.

Table 1
Dental fluorosis in the different groups of rats.

Group	N	Degree of dental fluorosis		
		I°	II°	III°
Control	6	0	0	0
Ginkgo	6	0	0	0
Fluoride	6	2	3	1
Fluoride+ginkgo	6	2	4	0

Dental fluorosis grading: I°: white or pigmented bands; II°: gray enamel; III°: loss of tooth structure.

Table 2
The activities of SOD and GSH-Px and level of MDA in the brains of rats exposed to different conditions.

Group	n	MDA (nmol/mg pro)	SOD (U/mg pro)	GSH-Px (kU/L)
Control	6	3.90 ± 0.34	380.96 ± 19.76	228.84 ± 19.23
Ginkgo	6	3.65 ± 0.33#	423.67 ± 22.99*#	259.15 ± 24.46*#
Fluoride	6	7.78 ± 0.83*	259.25 ± 25.27*	140.41 ± 12.34*
Fluoride+Ginkgo	6	4.77 ± 0.53*#	314.02 ± 7.54*#	186.88 ± 12.01*#

MDA: malonaldehyde; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; Pro: protein. *P < 0.05 in comparison to the control group; #P < 0.05 in comparison to the fluoride group.

intensity quantified with the Image J software.

2.12. Statistical analysis

For the different groups of rats (n = 6) and SH-SY5Y cells treated in different ways (3 independent experiments), values are presented as means±SDs and compared by analysis of variance (ANOVA) and thereafter a post-hoc test of least significant difference. The SPSS 22.0

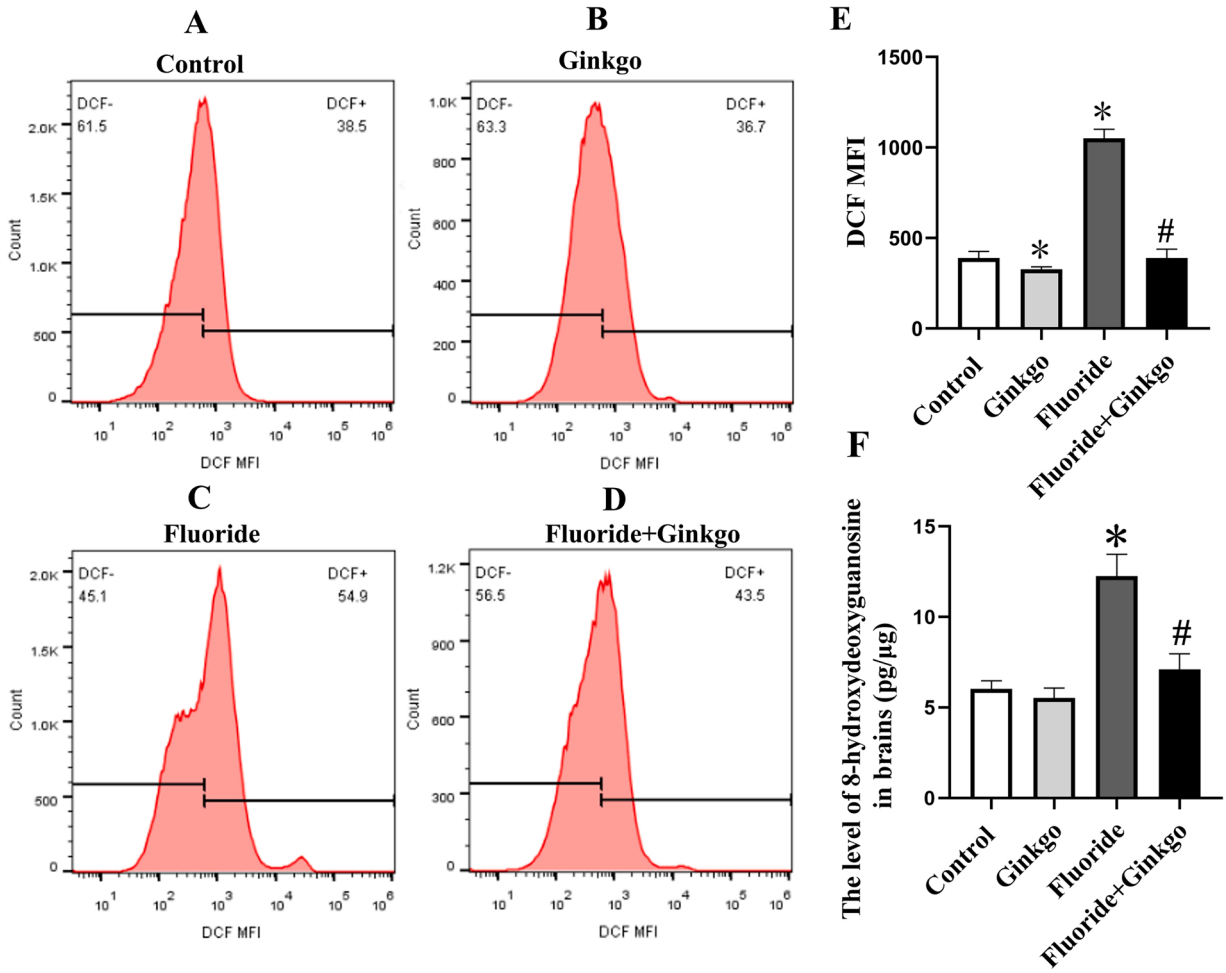

Fig. 3. Flow cytometric determination of the ROS content of single cells isolated from the hippocampus of rats exposed to different conditions. Control: drinking water containing less than 0.5 ppm fluoride; ginkgo: drinking water without fluoride and administration of *Ginkgo biloba* extract (100 mg/kg/day); fluoride: drinking water containing 50 ppm fluoride; fluoride+ginkgo: drinking water containing 50 ppm fluoride and administration of *Ginkgo biloba* extract. DCF MFI: mean fluorescence intensity of dichlorofluorescein. E: quantification and statistical comparison of the results for the four different groups. F: the level of the 8-hydroxydeoxyguanosine in brains for the four different groups. The values presented are means ± SDs (n = 6). *P < 0.05 compared to the control group; #P < 0.05 compared to the fluoride group.

software (SPSS Inc., USA) was used to carry out all of these analyses and *p* values of < 0.05 were regarded as significantly different.

3. Result

3.1. Confirmation of chronic fluorosis in the rats and survival rate of the SH-SY5Y cells exposed to fluoride

Determination of fluoride by ion selective electrode method (Fig. 1A and B) confirmed that the fluoride contents of the brains and urine of rats exposed to fluoride were elevated. In addition, these animals exhibited dental fluorosis (Table 1). Furthermore, the survival of SH-SY5Y cells decreased as the concentration of fluoride in the medium was increased (Fig. 1 C). These observations show that our animal model of chronic fluorosis was established successfully and appropriate conditions for exposure of cultured cells to fluoride chosen.

3.2. Spatial learning and memory in rats exposed to the different conditions (Fig. 2)

In the rats with chronic fluorosis, the escape latency in the Morris water maze was enhanced, while both the number of times for the original position of the platform that was crossed and the time spent at this location were reduced. These changes confirmed the impairment of learning and memory expected to be caused by chronic fluorosis. At the same time, treatment with *Ginkgo biloba* extract for 6 months attenuated this impairment.

3.3. The content of ROS and 8-OHdG in the hippocampus of rats and in SH-SY5Y cells exposed to the different conditions

As can be seen from Fig. 3, the fluorescence intensity of DCF in single cells isolated from the hippocampus of rats treated with fluoride for 6 months was significantly higher than the corresponding value for the control group. At the same time, *Ginkgo biloba* extract attenuated this increase in ROS induced by fluoride. In addition, the level of ROS in the hippocampus of rats receiving *Ginkgo biloba* extract only was lower than in the control group. 8-OHdG, the sensitive index of DNA oxidative damage, showed positive correlation with ROS (Fig. 3F).

As shown in Fig. 4, exposure of SH-SY5Y cells to fluoride elevated their content of ROS, while simultaneous incubation with either *Ginkgo biloba* extract or 4-ANI attenuated this effect. In addition, treatment of SH-SY5Y cells with the extract alone lowered their level of ROS. The level of 8-OHdG showed positive correlation with ROS (Fig. 4H).

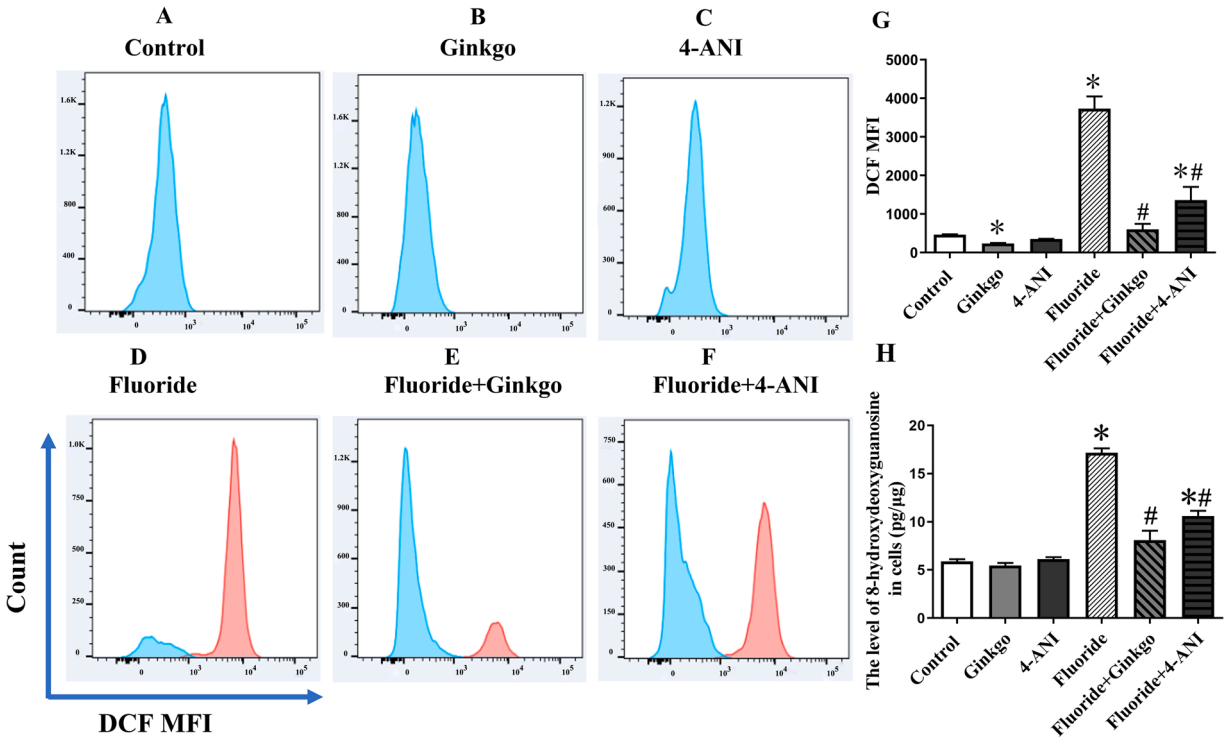

Fig. 4. Flow cytometric determination of the level of ROS in SH-SY5Y cells treated with fluoride, *Ginkgo biloba* extract and/or 4-ANI. Control: untreated cells; ginkgo: cells incubated with *Ginkgo biloba* extract (100 mg/l) for 48 h; 4-ANI: cells incubated with 10 μM 4-ANI for 24 h; fluoride: cells incubated with 160 ppm fluoride for 48 h; fluoride+ginkgo group: cells incubated with 160 ppm fluoride and *Ginkgo biloba* extract (100 mg/l) for 48 h; fluoride+ 4-ANI: cells incubated with 10 μM 4-ANI for 24 h and thereafter with 160 ppm fluoride for 48 h. DCF MFI: mean fluorescence intensity of dichlorofluorescein. G: quantification and statistical analysis of the values for the different groups. H: the level of the 8-hydroxydeoxyguanosine in cells for the different groups. The values presented are the means ± SDs for 3 independent experiments. *$P < 0.05$ compared to the control group; $^{\#}P < 0.05$ compared to the fluoride group.

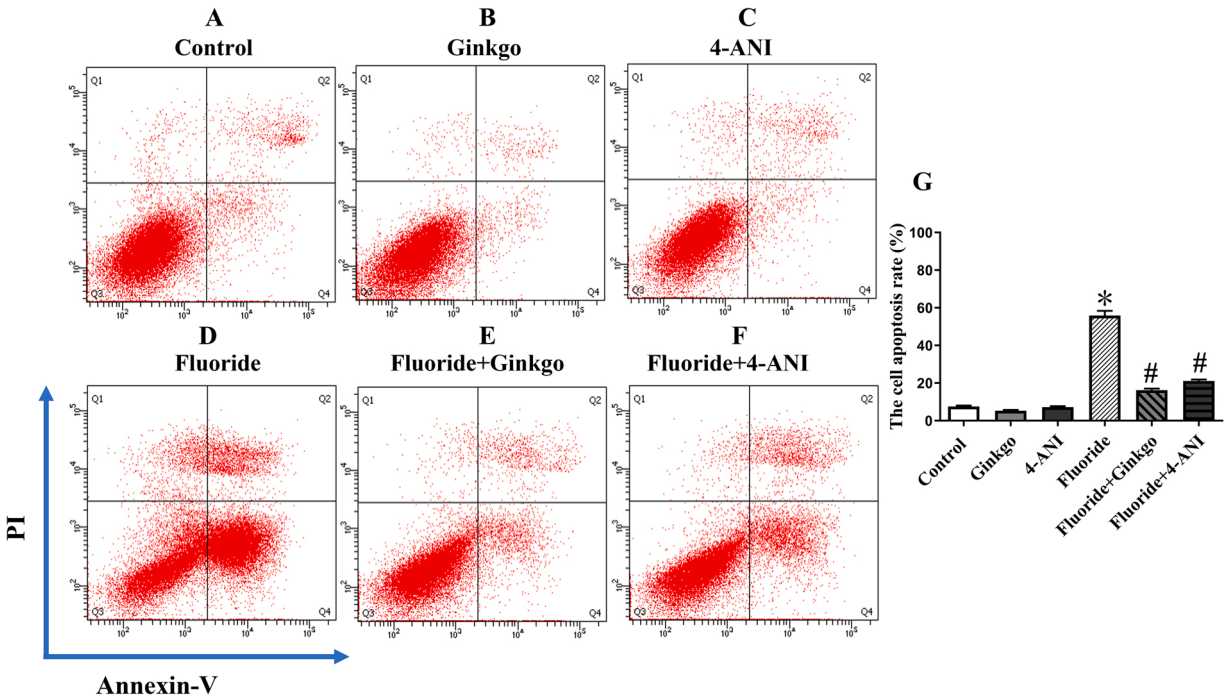

Fig. 5. Assessment of the frequency of apoptosis among SH-SY5Y cells exposed to fluoride, the *Ginkgo biloba* extract and/or 4-ANI by double staining with Annexin/PI. Control: untreated cells; ginkgo: cells incubated with the *Ginkgo biloba* extract (100 mg/l) for 48 h; 4-ANI: cells incubated with 10 μM 4-ANI for 24 h; fluoride: cells incubated with 160 ppm fluoride for 48 h; fluoride+ginkgo group: cells incubated with 160 ppm fluoride and *Ginkgo biloba* extract (100 mg/l) for 48 h; fluoride+ 4-ANI group: cells incubated with 10 μM 4-ANI for 24 h and thereafter with 160 ppm fluoride for 48 h. G: quantification and statistical analysis of the values for the different groups. The values presented are the means ± SDs from 3 independent experiments. *$P < 0.05$ compared to the control group; $^{\#}P < 0.05$ compared to the fluoride group.

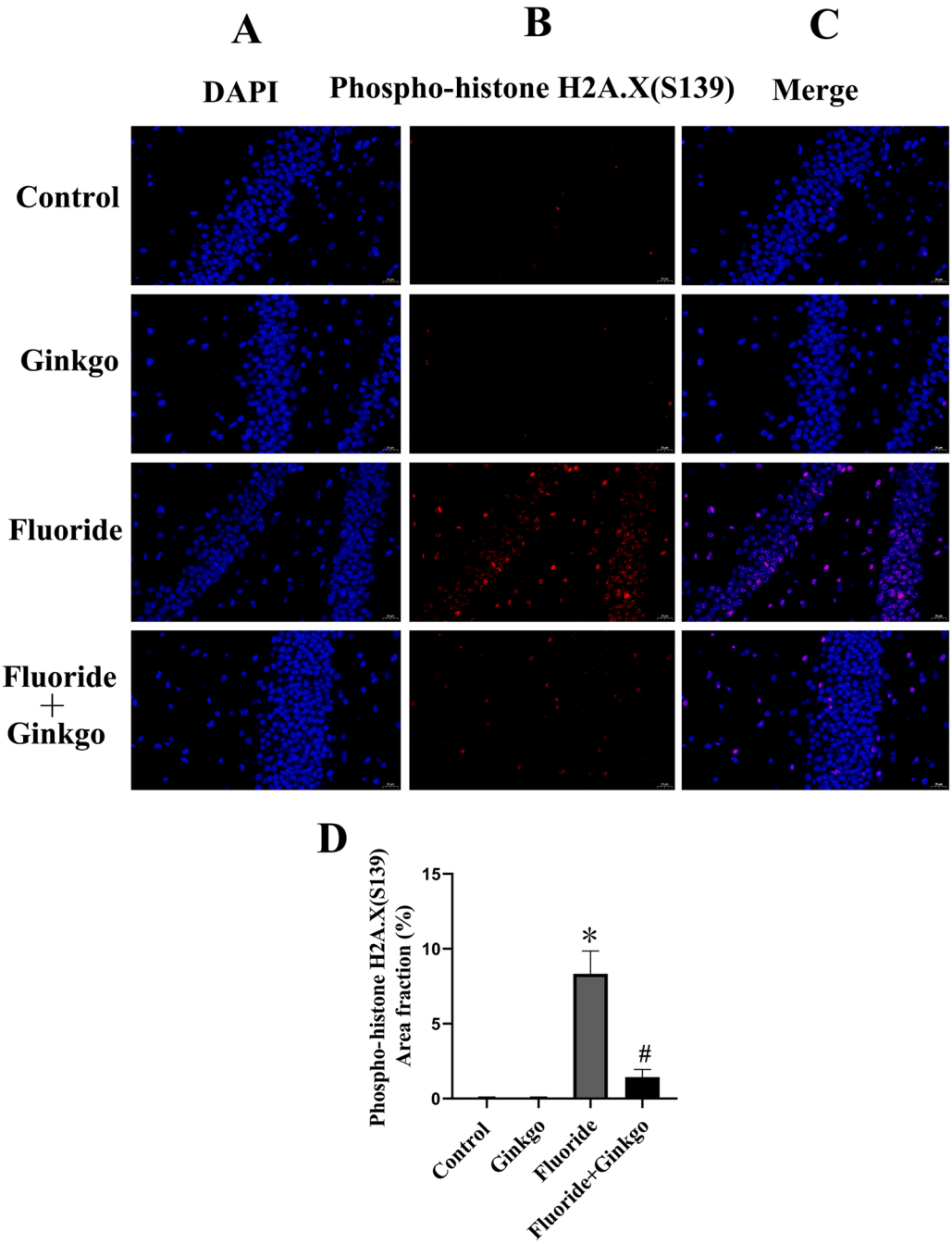

Fig. 6. The level of phospho-histone H2A.X$_{(Ser139)}$ in the hippocampus of rats exposed to the different conditions, as determined by immunofluorescent staining. Control: drinking water containing less than 0.5 ppm fluoride; ginkgo: drinking water without fluoride and administration of the *Ginkgo biloba* extract (100 mg/kg); fluoride: drinking water containing 50 ppm fluoride; fluoride+ginkgo: drinking water containing 50 ppm fluoride and administration of the *Ginkgo biloba* extract. A: staining of nuclei (blue); B: immunostaining of phospho-histone H2A.X$_{(Ser139)}$-positive neurons (red); C: merging of A and B; D: statistical analysis of the fraction of the total area examined in the different groups that contained neurons staining positively for phospho-histone H2A.X$_{(Ser139)}$. Magnification: 400 X, scale bar = 20 μm. The values present are means ± SDs (n = 6). *P < 0.05 compared to the control group; #P < 0.05 compared to the fluoride group.

3.4. The activities of SOD and GSH-Px and content of MDA in the brain of rats exposed to the different conditions (Table 2)

Exposure of rats to 50 ppm fluoride in their drinking water for 6 months lowered the activities of SOD and GSH-Px, while raising the content of MDA in their brains. Simultaneous treatment with *Ginkgo biloba* extract attenuated all of these effects of fluoride. Moreover, treatment of rats with the extract alone elevated the activities of SOD and GSH-Px and decreased the content of MDA in their brains.

3.5. The extent of apoptosis among SH-SY5Y cells exposed to the different conditions (Fig. 5)

As assessed by staining with Annexin-V/PI, exposure to fluoride enhanced the frequency of apoptosis among SH-SY5Y cells, while the *Ginkgo biloba* extract and 4-ANI attenuated this effect. The frequency of apoptosis among SH-SY5Y cells treated with *Ginkgo biloba* extract or 4-ANI alone was unaltered.

3.6. Determination of the levels of phospho-histone H2A.X$_{(Ser139)}$, PARP-1, PAR and SIRT1 in the brains of rats and in SH-SY5Y cells exposed to the different conditions

As depicted in Fig. 6, immunostaining revealed that exposure of rats to fluoride increased their hippocampal levels of phospho-histone H2A.X$_{(Ser139)}$ (although not of total histone H2A). Again, treatment with *Ginkgo biloba* extract attenuated this change.

As detected immunohistochemically, exposure of SH-SY5Y cells to fluoride elevated their level of phospho-histone H2A.X$_{(Ser139)}$ and this effect was reversed by exposure to either the *Ginkgo biloba* extract or 4-

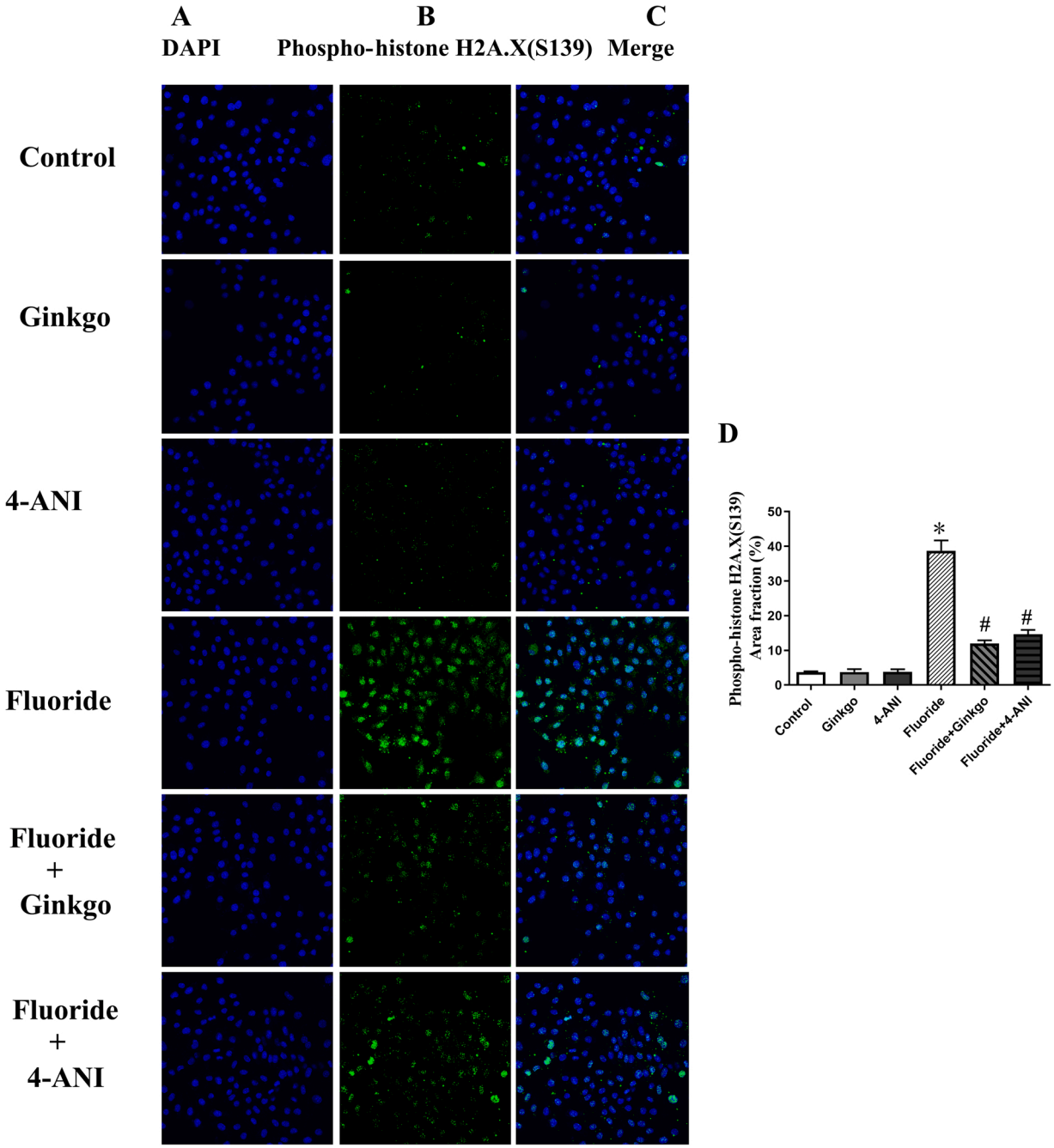

Fig. 7. Immunofluorescent assessment of the level of phospho-histone H2A.X$_{(Ser139)}$ in SH-SY5Y cells treated with fluoride, the *Ginkgo biloba* extract and/or 4-ANI. Control: untreated cells; ginkgo: cells incubated with *Ginkgo biloba* extract (100 mg/l) for 48 h; 4-ANI: cells incubated with 10 μM 4-ANI for 24 h; fluoride: cells incubated with 160 ppm fluoride for 48 h; fluoride+ginkgo: cells incubated with 160 ppm fluoride and *Ginkgo biloba* extract (100 mg/l) for 48 h; fluoride+ 4-ANI: cells incubated with 10 μM 4-ANI for 24 h and thereafter with 160 ppm fluoride for 48 h. A: staining of nuclei (blue); B: immunostaining of phospho-histone H2A.X$_{(Ser139)}$-positive neurons (green); C: merging of A and B; D: statistical analysis of the fraction of the area examined occupied by cells staining positively for phospho-histone H2A.X$_{(Ser139)}$. Magnification: 400 X, scale bar = 20 μm. The values presented are means ± SDs (n = 6) from 3 independent experiments. *P < 0.05 compared to the control group; $^{\#}$P < 0.05 compared to the fluoride group.

ANI (Fig. 7). Neither this extract nor 4-ANI alone altered the level of this phosphoprotein.

In addition, as depicted in Fig. 8, immunostaining revealed that exposure of rats to fluoride increased their hippocampal levels of PAR, and reduced the level of SIRT1. Again, treatment with *Ginkgo biloba* extract attenuated all of these changes.

Application of Western blotting also revealed that the levels of phospho-histone H2A.X$_{(Ser139)}$ (but not of total histone H2A) (Fig. 9A), PARP-1 (Fig. 9B) and PAR (Fig. 9C) in the hippocampus of rats with chronic fluorosis were elevated, while that of SIRT1 (Fig. 9D) was reduced. Again, combined treatment with *Ginkgo biloba* extract reversed these changes (Fig. 9A-D), while this extract alone decreased the levels of PARP-1 and PAR while increasing the content of SIRT1 (Fig. 9B-D).

Application of Western blotting to SH-SY5Y cells revealed similar effects of exposure to fluoride, i.e., elevations in the levels of phospho-histone H2A.X$_{(Ser139)}$ (but not of total histone H2A) (Fig. 9E), PARP-1 (Fig. 9F) and PAR (Fig. 9G), with a decrease in SIRT1 (Fig. 9H). Combined treatment with *Ginkgo biloba* extract or 4-ANI reversed all of these

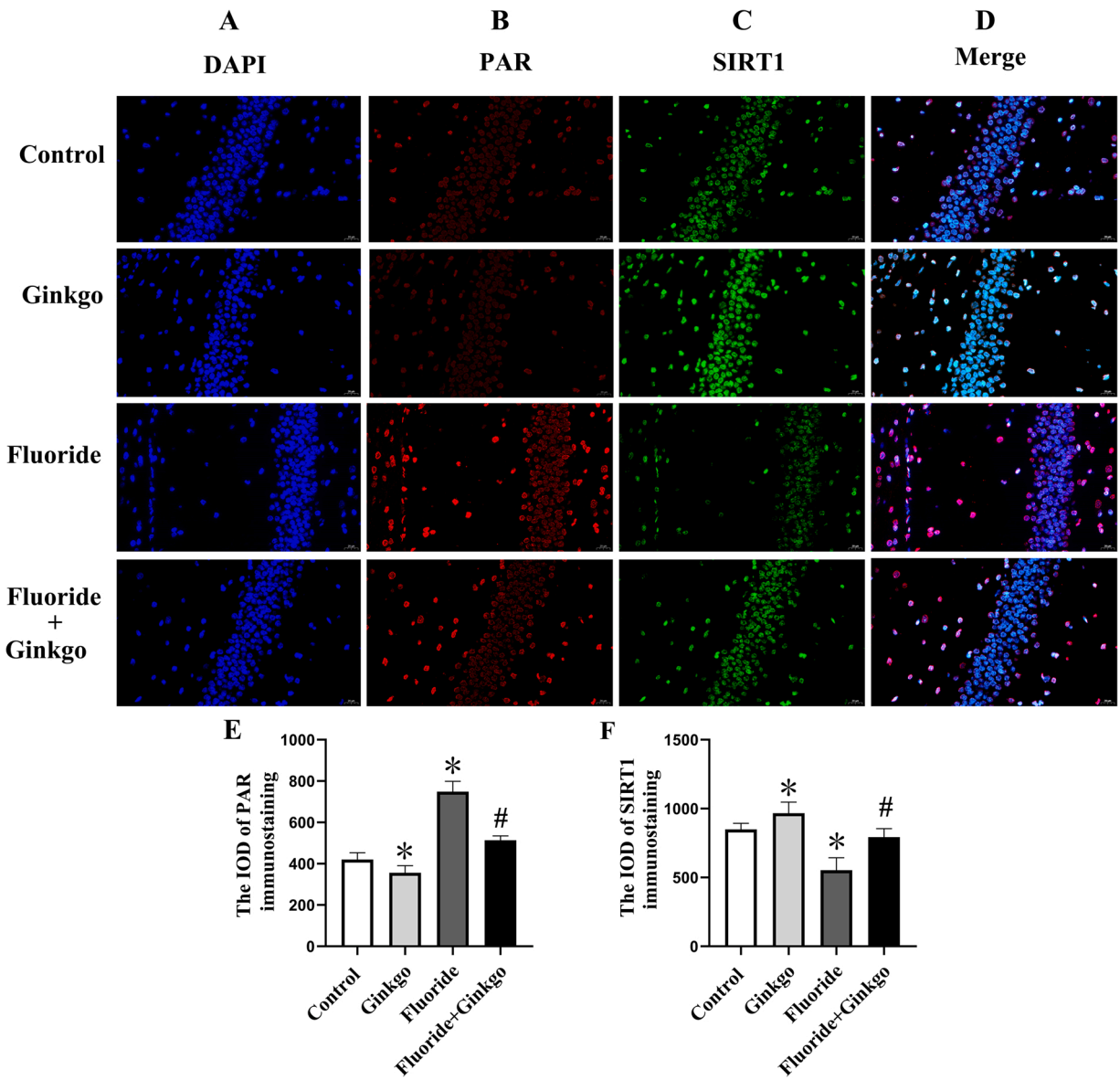

Fig. 8. Immunofluorescent staining of PAR and SIRT1 in the hippocampus of rats exposed to the four different conditions. Control: drinking water containing less than 0.5 ppm fluoride; ginkgo: drinking water without fluoride and administration of the *Ginkgo biloba* extract (100 mg/kg); fluoride: drinking water containing 50 ppm fluoride; fluoride+ginkgo: drinking water containing 50 ppm fluoride and administration of the *Ginkgo biloba* extract. A: staining of nuclei (blue); B: immunostaining for PAR (red); C: immunostaining for SIRT1 (green); D: merging of A, B and C; E and F: semiquantitative analysis of the integrated optical density (IOD) of PAR and SIRT1 staining. Magnification: 400×, scale bar = 20 μm. The values presented are means ± SDs (n = 6). *$P < 0.05$ compared to the control group; $^{\#}P < 0.05$ compared to the fluoride group.

changes (Fig. 9E-H), while either of these preparations alone lowered the levels of PARP-1 and PAR and raised that of SIRT1 (Fig. 9F-H).

4. Discussion

The animal model with chronic fluorosis was established successfully in the study, as confirmed by the elevated levels of fluoride in the brains and urine, as well as the presence of dental fluorosis [3]. Our present findings clearly confirm that fluoride is taken up from the gastrointestinal tract into the blood and can subsequently cross the blood-brain barrier [35] and impair learning and memory [44].

In addition, both biochemical and molecular investigations have revealed close associations between oxidative stress and cognitive dysfunction in connection with aging and associated neuronal diseases [45]. Indeed, the impairment of learning and memory in rats chronically exposed to high level of fluoride appear to be caused by enhanced oxidative stress [35,46]. Previous studies have shown that fluoride is able to induce apoptosis in both intrinsic and extrinsic pathways in certain mammalian cells [47]. The fluoride neurotoxicity including oxidative stress, disorder of synaptic transmission and synaptic plasticity, premature neuronal death, altered activity of components of intracellular signaling cascades, impaired protein synthesis, neurotrophic and loss of transcription factors, metabolic changes, inflammatory processes and apoptosis [48].

In this context, we found here the increased levels of ROS and MDA, but the decreased activities of SOD and GSH-Px in the brains of rats exposed to fluoride, which are consistent with the results of the elevated oxidative stress level caused by fluorosis [49]. In addition, the increases of both ROS and apoptosis in SH-SY5Y cells exposed to high fluoride were detected in the study. It has been reported that fluoride induced neuron apoptosis and expressions of inflammatory factors by activating microglia or JNK phosphorylation [50,51]. Fluoride may induce

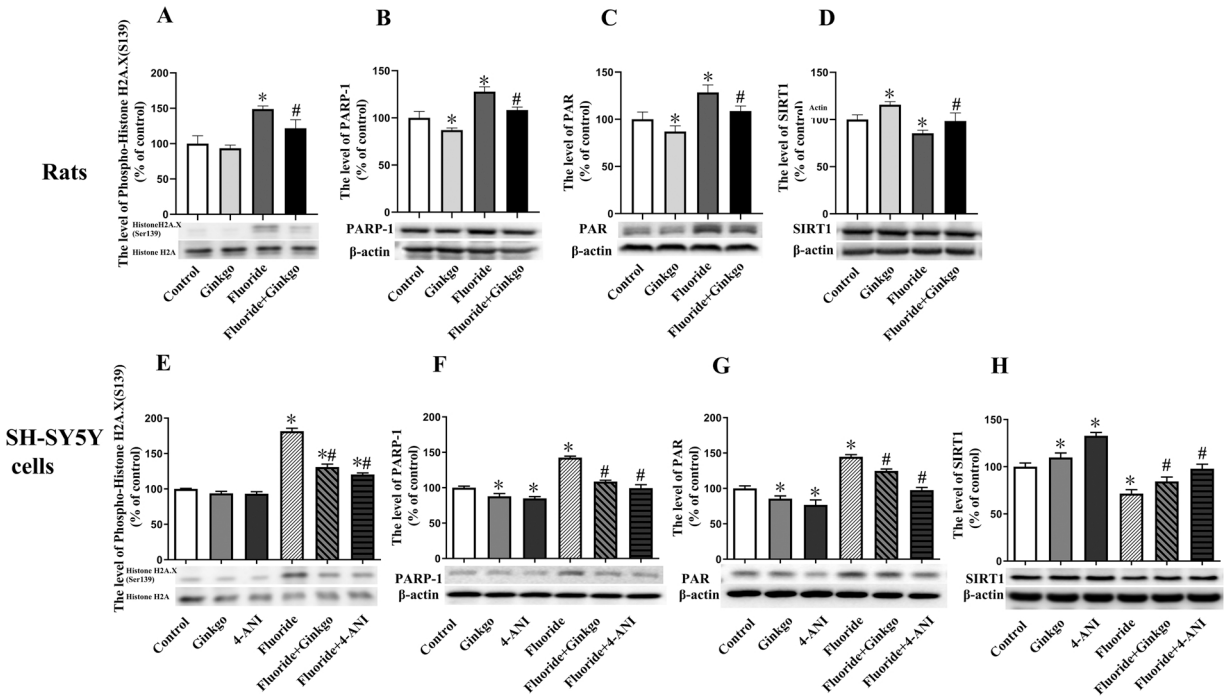

Fig. 9. Western blotting of phospho-histone H2A.X$_{(Ser139)}$, PARP-1, PAR and SIRT1 in the hippocampus of rats and in SH-SY5Y cells exposed to the different conditions. Control: rats receiving drinking water containing less than 0.5 ppm fluoride or untreated SH-SY5Y cells; ginkgo: rats or SH-SY5Y cells exposed to *Ginkgo biloba* extract alone; fluoride: rats receiving drinking water containing 50 ppm F^- or SH-SY5Y cells exposed to 160 ppm F^-; fluoride+ginkgo: rats receiving drinking water containing 50 ppm F^- and administered *Ginkgo biloba* extract, or SH-SY5Y cells exposed to 160 ppm F^- and *Ginkgo biloba* extract; 4-ANI: SH-SY5Y cells exposed to 4-ANI; fluoride + 4-ANI: SH-SY5Y cells exposed to 160 ppm F^- and 4-ANI. A and E: the level of phospho-histone H2A.X$_{(Ser139)}$; B and F: the level of PARP-1; C and G: the level of PAR; D and H: the level of SIRT1. The values presented are means ± SDs from 3 independent experiments. *$P < 0.05$ compared to the control group; $^{\#}P < 0.05$ compared to the fluoride group. Representative immunoblots are shown beneath each graph.

oxidative stress through the activation of mitogen activated protein kinase (MAPK) cascade which can lead to cell apoptosis [15]. Oxidative stress injury of brain tissue or nerve cells caused by fluoride is one of the main mechanisms of promoting neuronal apoptosis [52]. Consistent with the results of present study, neuronal apoptosis induced by fluoride may be in mechanism involved in the increased oxidative damage.

It has been suggested that fluoride-induced ROS generation causes mitochondrial and DNA damages [11]. In our results here, we found the elevated level of 8-HOdG in the hippocampus of rats and enhanced phospho-histone H2A.X $_{(Ser139)}$ in the rat brain and SH-SY5Y cells exposed to high fluoride. Importantly, 8-HOdG is one of the products as a result of oxidative damage to DNA [53]. When DNA double-strand damage occurs, H2A.X $_{(Ser139)}$ is rapidly phosphorylated, thus forming H2AX focal point, which is the gold index for DNA double-strand damage detection [54].

Furthermore, DNA breaks caused by oxidative stress can lead to activate PARP-1 [55] and while pharmacological inhibition of this enzyme activation is likely to delay cell death [56]. Under normal circumstances, PARP-1 is responsible for detecting DNA nicks and monitoring their repair, but stress factors can activate PARP-1 in a manner that actually augments cellular damage and can even result in cell death [57]. In the present case, we observed the activation of PARP-1 and an increase in the level of ADP-ribosylated PAR in the brains of rats exposed to fluoride. Such activation of PARP-1/PAR has also been demonstrated in pyramidal cortical neurons of the brains of patients with AD [58] and Parkinson's disease [59].

In addition, with the elevated PARP-1/PAR in the hippocampus of rats exposed to fluoride, the level of SIRT1 was reduced in our present study, which may be considered as a consequence of activation of PARP-1. It has been demonstrated that fluoride induces apoptosis in SH-SY5Y cells by inhibiting the deacetylase activity of SIRT1 [60], while overexpression of SIRT1 suppresses toxicity caused by fluoride [61]. PARP-1 competes with SIRT1 for the limited cellular supply of NAD^+ [62] and the activation of PARP-1 can reduce the expression of SIRT1 [63]. By the results from our study, we suggest that exposure of fluoride induces high level of oxidative stress and then results in DNA oxidative damage, and thus promotes the activation of PARP-1 and thereafter inhibits the expression of SIRT1.

Interestingly, our current results showed that the treatment with *Ginkgo biloba* extract attenuated the decline in learning and memory of the rats exposed to high level of fluoride in their drinking water for 6 months and the cytotoxic effects in neurons of the hippocampus of such rats. It was indicated that *Ginkgo biloba* extract ameliorated memory deficits of AD mice and increased the number of newborn neurons, dendritic branching and density of dendritic spines in brain of the mice [64].

Ginkgo biloba extract, in our study here, reversed the increased levels of ROS and MDA, and the decreased activities of SOD and GSH-Px in brain of the rats exposed to fluoride, confirming that *Ginkgo biloba* as a potential agent in protecting the neurons suffering from oxidative stress [65]. In addition, *Ginkgo biloba* extract attenuated the raised levels of apoptosis, 8-HOdG and H2A.X$_{(Ser139)}$, which may in mechanism correlated to the inhibited oxidative stress by the extract, resulting in the reduction of oxidative damage. It is indicated that *Ginkgo biloba* extract, acting as intracellular antioxidant, reduces ROS-induced apoptosis in old mice [66], and protects neuroblastoma cells against intrinsic mitochondrial apoptosis [67]. The high glucose increased ROS generation, 8-HOdG content and oxidative DNA damage in human umbilical vein endothelial cells [68]. Obviously, *Ginkgo biloba* suppressed these high glucose-induced oxidative stress in a dose-dependent manner through the improvement of total antioxidant capacity, and thus alleviated endothelial DNA oxidation [68].

In the study, we also found that *Ginkgo biloba* extract reversed these changes in the levels of SIRT1, PARP-1 and PRP in the brain of rats

exposed to fluoride, exhibiting the increased SIRT1 and the declined PARP-1 and PAR. SIRT1, an acetylated protein with neuroprotective property, has been shown to coordinate the DNA damage repair process [69], stimulate mitochondrial biogenesis and oxidative metabolism [70], promote the expression of antioxidant genes [71] and attenuate the inflammatory response by modulating microglia polarization through SIRT1-mediated deacetylation [72]. As a result of this stimulation of antioxidant defenses, the activation of SIRT1 by *Ginkgo biloba* extract in the study may correlate to the attenuation the cognition impairment induced by fluoride [73]. On the other hand, the crucial role of PARP-1 in the response to oxidative stress may present through a mechanism that involves the depletion of NAD^+ [74], and while the inhibition of PARP1 and PAP may bring with a consequent stimulation of SIRT1 by *Ginkgo biloba* extract for competing the supply of NAD^+ [62].

Significantly, these changes in oxidative stress, apoptosis, DNA damages, the enhanced PARP1 and PAR and the inhibited SIRT1 induced by fluoride were also attenuated in SH-SY5Y cells by the treatment of *Ginkgo biloba* extract, which strongly supports the conclusions obtained from the animal experiments in the study. To explore more detail in mechanism of the protective effect of *Ginkgo biloba* extract on fluoride-induced damages, we added 4-ANI to cultured cells and found that this compound specifically inhibited the levels of PARP-1 and PAR, and thereby improved the expression of SIRT1. Moreover, 4-ANI treatment alleviated the uptrend of $H2A.X_{(Ser139)}$, PARP1 and PAR and meanwhile the reduce of SIRT1, showing a neuroprotective effect [75].

5. Conclusion

The rats exposed to fluoride for a long period exhibited dental fluorosis, the increased contents of fluoride in brains and urine and the declined ability of learning and memory. The high level of oxidative stress, apoptosis, DNA damages, the elevated PARP1 and PAR, and decreased SIRT1 were determined in the hippocampus of the rats and in SH-SY5Y cells exposed to fluoride. Interestingly, the treatment with *Ginkgo biloba* extract attenuated these neurotoxic damages induced by fluoride, in which the underlying mechanism might involve the inhibition of PARP-1 and the promotion of SIRT1.

CRediT authorship contribution statement

ZZG planned and designed the study and revised the paper. JX performed the experiment, processed experimental data and drafted the initial manuscript. YLM , JZ, XXZ, XX, YLY, YTD and LYR participated in part of the experiment work. XLQ, WH and YHG discussed the results. All authors have read and approved the final manuscript.

Conflicts of interest

None.

Acknowledgments

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