

Fluoride induced down-regulation of IKBKG Gene expression inhibits hepatocytes senescence



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ABSTRACT

Background: Accumulating evidences have confirmed that liver is one of the more severely damaged organs during chronic fluorosis. However, the detail mechanism is unclear to date. At present, the objective of this study was to investigate the relationship between down-regulation of IKBKG gene expression and hepatocyte senescence induced by sodium fluoride (NaF).

Methods: Chronic fluorosis rats and NaF-exposure human liver L02 cells were reproduced the model of hepatocyte senescence *in vivo* and *in vitro*. The mRNA and protein levels of p16, p21 and IKBKG, the IL-8 level were determined. The role of IKBKG in fluoride-induced senescence of hepatocytes was explored by knock down in hepatocytes *in vivo* and *in vitro*.

Results: The number of senescence-positive cells in rat liver tissues was increased as well as the level of IL-8 and the expression levels of p16, p21 and IKBKG in fluoride exposure to rat depending on the fluoride concentration. The similar results were obtained in NaF treated liver L02 cells, and the number of cells that stagnated in the G2 phase increased significantly. Further, our results confirmed that decreasing the expression of IKBKG in hepatocytes could reduce fluoride-induced hepatocyte senescence and the changes of senescence-related indicators both *in vivo* and *in vitro*.

Conclusion: These results indicated that the elevated expression of IKBKG was positive relation with the fluoride-induced senescence in hepatocytes, suggesting the hepatocyte senescence might have a special relationship with fluoride-caused liver damage. Because of the present results limitation, the mechanism of fluoride induced senescence in hepatocytes should be concentrated in the future in detail, especially the novel targets for fluoride induced liver injury.

1. Introduction

Fluoride is a trace element with a very narrow safety threshold. Low-dose fluoride (<1 mg/L) intake is beneficial for normal bone and tooth development. However, prolonged exposure to high fluoride results in chronic fluorosis and damage to multiple tissues of the body [1]. In addition to causing skeletal fluorosis and dental fluorosis, fluoride can also induce extensive damage on soft and mineralized tissues [2,3]. Fluorosis is a major public health issue in numerous areas around the world, including China, India, Bangladesh, South Africa, Mexico, and

Argentina [4]. The pathogenesis of fluorosis is still not well understood, which hinders the prevention and treatment of fluorosis.

Cellular senescence refers to the occurrence of cell growth inhibition and the potential failure of proliferation under the act of certain physical or chemical factors, causing irreversible changes in the phenotype, structure and function of cells [5]. Senescent cells still maintain metabolic activities for a period of time, but lose their response to mitogens and the ability to synthesize DNA [6]. The stagnation of cell cycle, shortening telomeres, invaginated nuclear membranes, deformed organelles, lipofuscin deposits and other morphological changes

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characterize the cellular senescence, together with the expression of high enzyme activity senescence-associated β -Galactosidase (SA- β -Gal) [7]. In addition, senescence-associated secretory phenotypes (SASP) can also be secreted by senescent cells such as IL-6, IL-8, pro-inflammatory microenvironment reactive cytokines and proteases [8]. SASP further deteriorates neighboring cells, causing chronic inflammation, metabolic disorders, and cell dysfunction. The mechanism of cellular senescence are not yet fully understood. Of note, oxidative stress is currently the more widely accepted main external cause of cell senescence [9].

Liver is one of the more severely damaged organs during chronic fluorosis. Epidemiological investigations and *in vivo* studies have shown that fluorosis can interfere with liver function [10]. Many studies have shown that fluoride can induce increased oxidative stress in rat liver cells, increased ROS production, mitochondrial free radical scavenging system obstacles, and significant changes in mitochondrial morphology and function [11,12]. In addition, fluoride can directly covalently bind to DNA and cause liver DNA damage [13]. In NaF treatment of liver L02 cells, the DNA damage rate was positively correlated with the fluoride concentration [14]. The presented results showed that fluoride-induced oxidative stress and DNA damage play an important part in the pathogenesis of fluorosis. These studies suggested that fluorosis might cause liver damage by inducing cell senescence.

Researches have shown that DNA damage can stimulate the secretion of inflammatory mediators and NF- κ B signaling, which consequently regulates several host defense functions related to cellular senescence [15]. Inhibitor of nuclear factor kappa B kinase subunit gamma (IKK γ , IKBKG) protein is the regulatory subunit of I κ B kinase (IKK) complex and thus an essential modulator of NF- κ B signaling. Recent studies have revealed that IKBKG protein has a major role in the activation process of NF- κ B signaling evoked by DNA damage, which indicates that IKBKG may participate in the process of cell senescence [16]. In addition to IKK components, IKBKG can interact with several other proteins associated with NF- κ B signaling, e.g. Ataxia Telangiectasia Mutated (ATM), ELKS, receptor interacting protein1 (RIP1) and TGF β -activated kinase1 (TAK1) [17].

In the present study, chronic fluorosis rats and human hepatic cell lines (L02) treated with sodium fluoride (NaF) were chosen as the research subjects to investigate whether NaF could induce cell senescence of hepatocytes, and the effects of IKBKG on hepatocytic senescence induced by fluoride. It may shed light on the pathogenesis of fluoride-induced liver injury.

2. Materials and methods

2.1. Materials

Mouse anti P16 (sc-377412) and P21 (sc-6246) antibodies were purchased from Santa Cruz Biotechnology Inc, USA; rabbit anti IKBKG antibody (18474-1-AP) was purchased from Proteintech Group, Inc, USA; rabbit anti- β -actin antibody (GTX109639) was purchased from Gene Tex, USA; HRP-labeled anti-mouse secondary antibody (#7076) and anti-rabbit secondary antibody (#7074) were purchased from Cell Signaling Technology, USA.

Other reagents used in the study were as follows: NuPAGE LDS Sample Buffer (4X), NuPAGE Sample Reducing Agent (10X), NuPAGE Novex 4–12 % Bis-Tris Precast, MES SDS Electrophoresis were bought from Life Technologies Corporation, USA; DMEM high glucose medium was purchased from Thermo, USA; primers for amplification of IKBKG, p16, p21 and β -actin mRNA were synthesized by Shanghai Genecore Bio Technologies, Shanghai, China; human cytokine ELISA kit IL-8 was purchased from Novus Biologicals, USA; rat ELISA kit IL-8 was purchased from Solarbio, China. Dimethyl Sulfoxide (DMSO) and other chemicals used were purchased from Sigma. The adeno-associated virus that silences the rat IKBKG gene (named AAV-IKBKG, and the negative AAV named AAV-Neg) and RNA interference plasmid for human IKBKG gene (named plasmid sh-IKBKG and the negative control named Plasmid

negative) were constructed by Shanghai Jikai Gene Company, China. The interference RNA fragment of rat IKBKG was 5' GCCAGCAATGC-TACATCTT 3' and 5'CCAAGAATACGACAACCACAT3' for L02 cell IKBKG RNAi.

2.2. Experimental animals and treatment

SD (Sprague-Dawley) rats (weighing 90–120 g) were purchased from the Experimental Animal Center in Guizhou Medical University, and ethical permission for the experiment was approved by the ethical committee for experimental animal of Guizhou medical University. The humidity ranged from 30 to 55 % and temperature remained between 22 and 25 °C. The rats were acclimatized for one week in a housing facility before treatment.

60 SD rats were randomly divided into 6 groups with 10 rats in each group (equal number in both genders) with body weight as following: NC group (normal control group): drinking water containing less than 0.5 mg/L fluoride, low fluoride group (Low group): drinking water containing 5 mg/L fluoride, medium fluoride group (Medium group): drinking water containing 10 mg/L fluoride and high fluoride group (High group): drinking water containing 50 mg/L fluoride, High fluoride + Neg group (High + Neg group): drinking water containing 50 mg/L fluoride and tail vein injection with AAV-neg, High fluoride + AAV group (High + AAV group): drinking water containing 50 mg/L fluoride and tail vein injection with AAV-IKBKG. For the rats of High + Neg and High + AAV groups were injected with AAV-IKBKG by tail vein after feeding with NaF for 6 months and examined 4 weeks after virus injection.

2.3. L02 cell culture and treatment

L02 cells, a normal human liver cell line purchased from the cell bank of Chinese Academy of Sciences Shanghai Branch, were grown in DMEM medium supplemented with 15 % heat-inactivated fetal bovine serum and 25 units of penicillin-streptomycin/mL, and cultured at 37 °C with 5% CO₂. Concerning the effects of NaF on cell senescence, cells were divided into four groups, e.g. NC group, low NaF group (cells treated with 1 mmol/L NaF for 48 h), medium NaF group (cells treated with 4 mmol/L NaF for 48 h) and high NaF group (cells treated with 7 mmol/L NaF for 48 h).

As for the effects of IKBKG gene knockdown on L02 cell senescence induced by fluoride, the cells were treated with 7 mmol/L NaF for 48 h and then transfected with plasmid sh-IKBKG for 48 h.

2.4. Analyzing the concentrations of fluoride in blood and urine by fluoride ion selective electrode method

After the rats were fed with NaF for 6 months, the urine was collected from each group for 24 h and the rat serum was collected. The fluoride concentrations were determined by an electronic meter (PHSF-4 F) and a fluoride-specific ion electrode [18]. The detection limit of F-selected electrode (PF-202-CF, INESA, Shanghai) was 0.01 mg/L. During the measurement, samples were diluted in TISAB II, a total ionic strength adjustment buffer, to adjust the pH and ionic strength of standards and urine or blood samples. The concentration was quantified against a fluoride calibration curve in a range of 0.1–20 mg/L prepared with a reagent blank. The measurement accuracy of ion selective electrode ranged from 97.3 % to 101.9 % relative to the standard fluoride solution, with the measurement errors less than \pm 5 %, and the recovery rate from 93.4 % to 108.3 %.

2.5. SA- β -gal activity Histochemical Staining to detect the L02 cell senescence and liver cell senescence of chronic fluorosis rats

After treatment, the frozen section of liver and L02 cells were rinsed with PBS and fixed for 5 min in 3 % formaldehyde. SA- β -gal staining was

Table 1

The primers for analyzing the mRNA levels of p16, p21 and β -actin by RT-qPCR.

gene	Primer sequences (5'→3')	Amplicon size (bp)
β -actin (H)	gtgcstatgttgccttagactcg atgccacaggattccatacc	174
β -actin (R)	cttggccacagcatcaca	142
IKBKG (H)	aagecagatttcgttga ggccaaacaggagggtat Ttccttcggcagcttc	153
IKBKG (R)	tgtggatatgagaaagcg gccaatagaaggatgg	82
P16 (H)	gtggcattttctggca tttggttctgcatttgct	132
P16 (R)	gtgggttatttgcgttac gcgttgtccagaagtgaag	175
P21 (H)	atgagttggggaggagca ctggcggggcacaaagg	111
P21 (R)	Cctcccagctccaaactta cgccaggatcagaaacacag	164

Table 2

Fluoride ion content in urine and blood of rats with chronic fluorosis ($\bar{x} \pm s$).

Group	Urine(mg/L)	Blood(mg/L)
WT	1.40±0.21	0.02±0.001
Low	4.10±0.37**	0.05±0.005**
Medium	6.57±0.33**	0.08±0.012**
High	14.26±2.11**	0.13±0.017**

The values were shown as mean \pm SD of each independent experiment ($n = 10$).

** <0.01 as compared to WT group.

determined using a Senescence β -galactosidase staining kit (Cell Signaling Technology, Danvers, MA, USA). 3 slides of liver tissue of each rat were analyzed, and for L02 cell assays, each experiment has three replicates, and repeated three times.

Randomly selected ten 200-fold fields of view for each cell slide, counted the number of blue-stained senescence cells under light microscope. Then the average value was used to calculate the proportion of senescence-positive cells in the total number of cells to yield the percentage of senescence-positive cells. Observing the integrated optical density (IOD) of positive-stained cells in liver tissue according to the kit instructions of SA- β -gal.

2.6. Analyzing the mRNA levels of p16, p21 and IKBKG by RT-qPCR

The mRNA levels of p16, p21 and β -actin were determined by RT-qPCR as described previously [19]. Briefly, 2 μ g of total RNA from liver of each rat were reverse transcribed to cDNA by using oligo-dT strategy. RT-qPCR was conducted on an ABI 7300 Real-time system and analyzed with the Applied Biosystems SDS2.1 software. Amplifications were performed with the universal TaqMan 2 \times PCR SYBR Green I Master mix according to the manufacturer's instruction. Reactions were carried out in 25- μ L volume with 2.5 μ L cDNA and 1.25 μ L each primer to analyze the relative level of each protein (primer sequences were listed in Table 1). SDS2.1 software was used for analyzing the $\Delta\Delta Ct$ and relative quantity (RQ) value ($RQ = 2^{-\Delta\Delta Ct}$) of each gene. The relative levels of p16 and p21 gene mRNA were calculated with β -actin as an internal control.

2.7. Analyzing protein levels of p16, p21 and IKBKG by Western blot

The levels of p16, p21 and IKBKG proteins in liver tissue lysates and treated L02 cells were quantified by Western blot as described

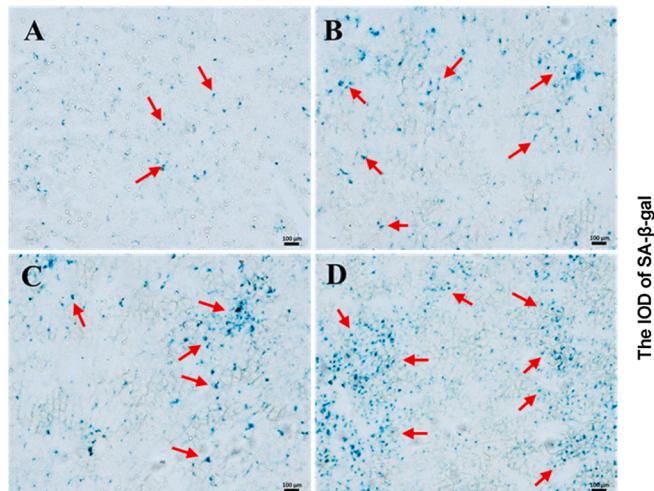


Fig. 1. Results of SA- β -gal staining in liver of fluorosis rats.

A: NC: normal control group, B: Low: low fluoride group, C: Medium: medium fluoride group, D: High: high fluoride group, E: the IOD of SA- β -gal staining. The magnification of the microscope was 100 times, scale bar was indicated in each picture. As compared to NC group (A), the SA- β -gal (blue) positive cells gradually increased significantly with the increase of NaF concentration (B–D). Data were presented as means \pm SD ($n = 10$). ** $P < 0.01$ vs. Control group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

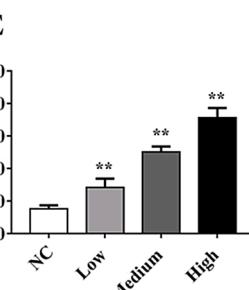


Fig. 2. Results of SA- β -gal staining in liver of fluorosis rats with AAV-IBKKG injection by tail vein.

A: Fluoride: high fluoride group, B: Fluoride + Neg: high fluoride group injected with AAV-Neg by tail vein, C: Fluoride + AAV: high fluoride group injected with AAV-IBKKG, D: the IOD of SA- β -gal staining. The magnification of the microscope was 100 times, scale bar was indicated in each picture. As compared to fluoride group (A), the SA- β -gal (blue) positive cells

were decreased significantly (C). Data were presented as means \pm SD ($n = 10$). ** $P < 0.01$ vs. Fluoride group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

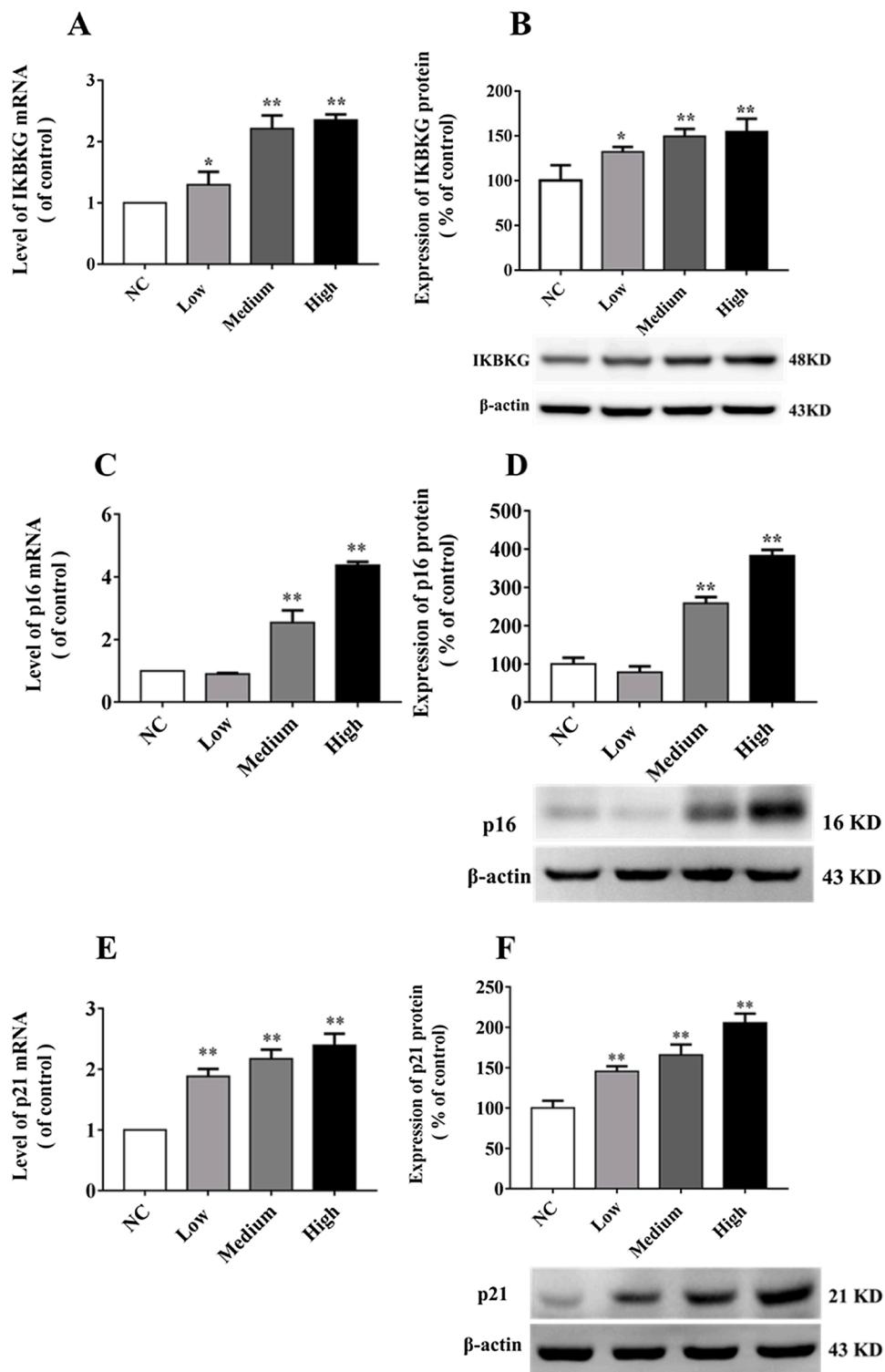


Fig. 3. The mRNA and protein levels of IKBKG (A, B), P16 (D, D) and P21 (E, F) in the liver tissue of fluorosis rats. The mRNA and protein levels, as well as those for β -actin (as an internal standard), were determined by real time RT-PCR and Western blotting respectively, and the values were shown as means \pm SD ($n = 10$). ** $P < 0.01$ in comparison to the corresponding control value.

previously [19]. In sum, proteins extracted from liver tissues or cells were quantitated by BCA Assay. The proteins were separated with 10 % SDS-PAGE electrophoresis, transferred to polyvinylidene difluoride membranes and blocked with 5 % skimmed milk for 2 h at room temperature (RT). Then, the membranes were incubated with primary antibodies (against p16, p21 or IKBKG) overnight at 4 °C. After rinsing, secondary antibody conjugated with HRP was added to the membranes

and incubated for 1 h at RT. Finally, the protein blots were detected with chemiluminescence substrate (ECL) and exposed to film.

After stripping the antibodies off the membranes, they were incubated again with primary mouse antibody against β -actin (1:5000) for 120 min and then incubated with HRP-conjugated anti-mouse IgG for 60 min at RT. ImageJ software was used to analyze the intensity of each protein bands, and β -actin band was used as internal control to calculate

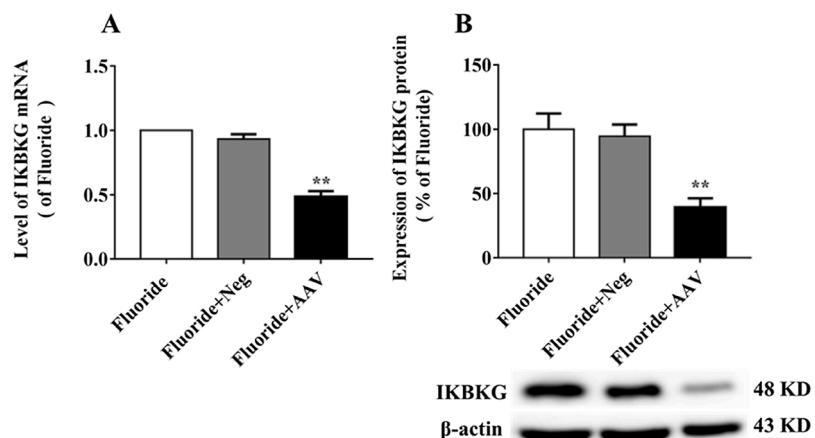


Fig. 4. The mRNA (A) and protein (B) levels of IKBKG in the liver tissue of fluorosis rats injected with AAV-IKBKG. The mRNA and protein levels, as well as those for β -actin (as an internal standard), were determined by real time RT-PCR and Western blotting respectively, and the values were shown as means \pm SD ($n = 10$). ** $P < 0.01$ in comparison to the corresponding control value.

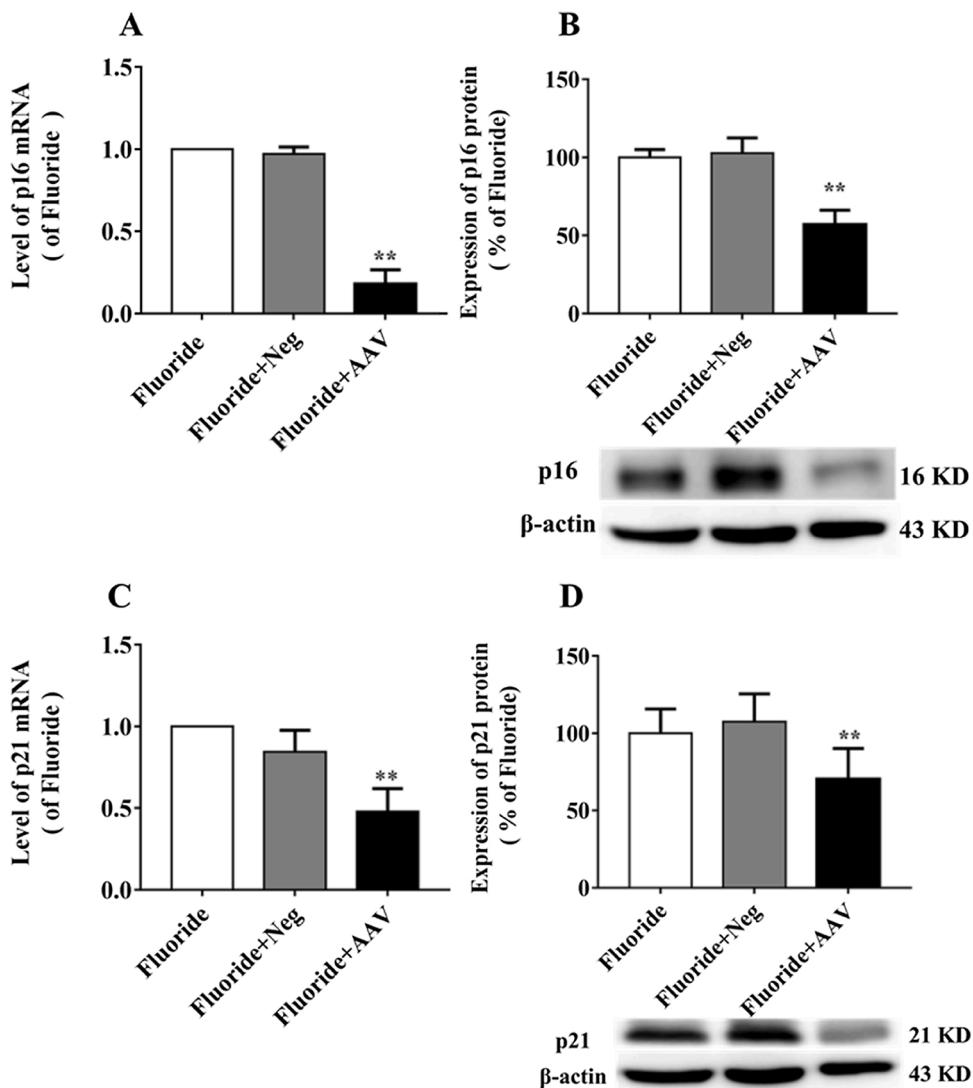


Fig. 5. The mRNA and protein levels of P16 (A, B) and P21 (C, D) in the liver tissue of fluorosis rats injected with AAV-IKBKG. The mRNA and protein levels, as well as those for β -actin (as an internal standard), were determined by real time RT-PCR and Western blotting respectively, and the values were shown as means \pm SD ($n = 10$). ** $P < 0.01$ in comparison to the corresponding control value.

Table 3Expression level of IL-8 in liver homogenate of rats in each group ($\bar{x} \pm s$).

Group	Expression level of IL-8(pg/mL)
NC	717.09±110.36
Low	1142.09±118.08**
Medium	1210.98±177.09**
High	1228.47±112.67**
Fluoride + Neg	1189.25±145.77**
Fluoride + AAV	764.25±123.16##

Data was presented as means \pm SD of each independent experiment (n = 10). **P < 0.01 vs. Normal Control group. ##P < 0.01 vs. High fluoride group.

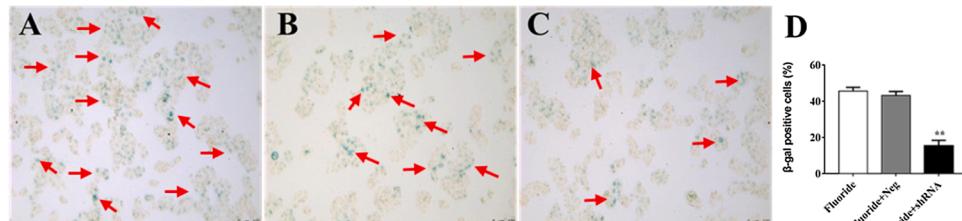
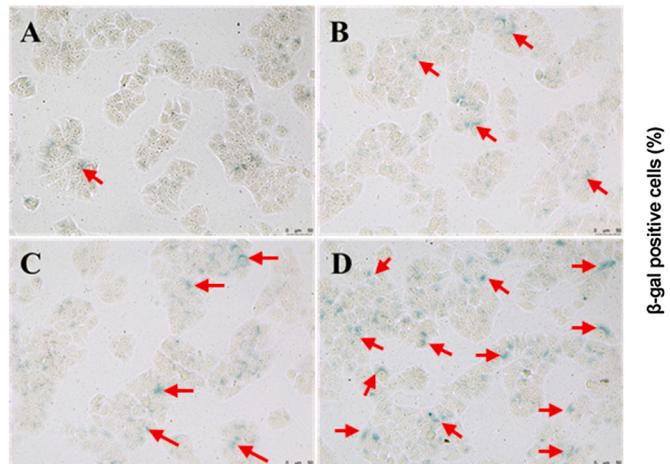
the relative expression levels of the proteins.

2.8. Analyzing the level of IL-8 by ELISA method

The levels of IL-8 in liver tissue lysates and culture media from L02 cells treated with NaF were determined by ELISA according to the protocol. The liver tissue was homogenated with a tissue grinder, centrifuged, and supernatant was collected. The culture media were collected after 48 h of exposure and then centrifuged. All samples were stored at -80°C for subsequent analysis. The OD was measured at 450 nm with a microplate ELISA reader (Bio-Rad).

2.9. Analyzing the cell cycle by flow cytometry

The cells were collected and rinsed with PBS for 3 times, then cells were fixed with pre-chilled 70 % ethanol for at least 2 h at -20°C . Centrifuge fixed cells was decanted with supernatant (ethanol) and rinsed once with 1x PBS to remove ethanol and then 300 μL of PI staining solution per sample was added (composed of 140 μM PI, 38 mM



presented as the means \pm SD (n = 9). ** P < 0.01 vs. Fluoride group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

sodium citrate, 10 $\mu\text{g}/\text{mL}$ RNase A and 0.01 %Triton X-100), samples were mixed and incubated for 30 min in darkness at 37°C . Then the cell cycle was measured by flow cytometry.

2.10. Statistical analysis

All data was presented as means \pm SD. The values were examined using two-tail Student's t-test, and one-way ANOVA with SPSS 22 software (USA), differences between means were considered statistically significant when $p < 0.05$.

3. Results

3.1. Occurrence of dental fluorosis in rats with chronic fluorosis

After feeding water to the rats with different concentrations of NaF for 6 months, it was found that the teeth of control group were normal; in the low-fluoride group, the incidence of mild dental fluorosis was 30 %, and the moderate dental fluorosis rate was 20 %; as for rats in the medium fluoride group, the incidence of mild dental fluorosis was 40 %, and the moderate dental fluorosis rate was 60 %; as for rats in the high fluoride group, the incidence of mild dental fluorosis was 10 %, the moderate dental fluorosis rate was 50 %, and the severe dental fluorosis rate was 30 %.

3.2. Fluoride ion content in urine and blood of rats with chronic fluorosis

The results showed that the fluoride ion content in urine and blood of rats respectively fed with 5 mg/L, 20 mg/L, and 50 mg/L fluoride water increased gradually. Compared with the normal control group, the difference was statistically significant. (Table 2).

Fig. 6. SA- β -gal positive cells in L02 cells treated with NaF.

A. NC: normal control group, B. 1 mmol/L: L02 cells treated with 1 mmol/L NaF, C. 4 mmol/L: L02 cells treated with 4 mmol/L NaF, D. 7 mmol/L: L02 cells treated with 7 mmol/L NaF, E. β -gal positive cells in each group. The magnification of the microscope was 200 times, scale bar was indicated in each picture. As compared to NC group (A), the β -gal positive cells were gradually increased significantly with the increase of NaF treated (B-D). Data was presented as means \pm SD (n = 9). **P < 0.01 vs. NC group.

Fig. 7. Results of SA- β -gal staining in NaF treated L02 cells transfected with sh-IKBKG.

A. Fluoride: L02 cells treated with 7 mmol/L NaF, B. Fluoride + Neg: L02 cells treated with 7 mmol/L NaF and transfected with sh-Neg, C. Fluoride + shRNA: L02 cells treated with 7 mmol/L NaF and transfected with sh-IKBKG, D. β -gal positive cells in each group. The magnification of the microscope was 200 times, scale bar was indicated in each picture. As compared to fluoride group (A), the β -gal (blue) positive cells were decreased significantly (C). Data was

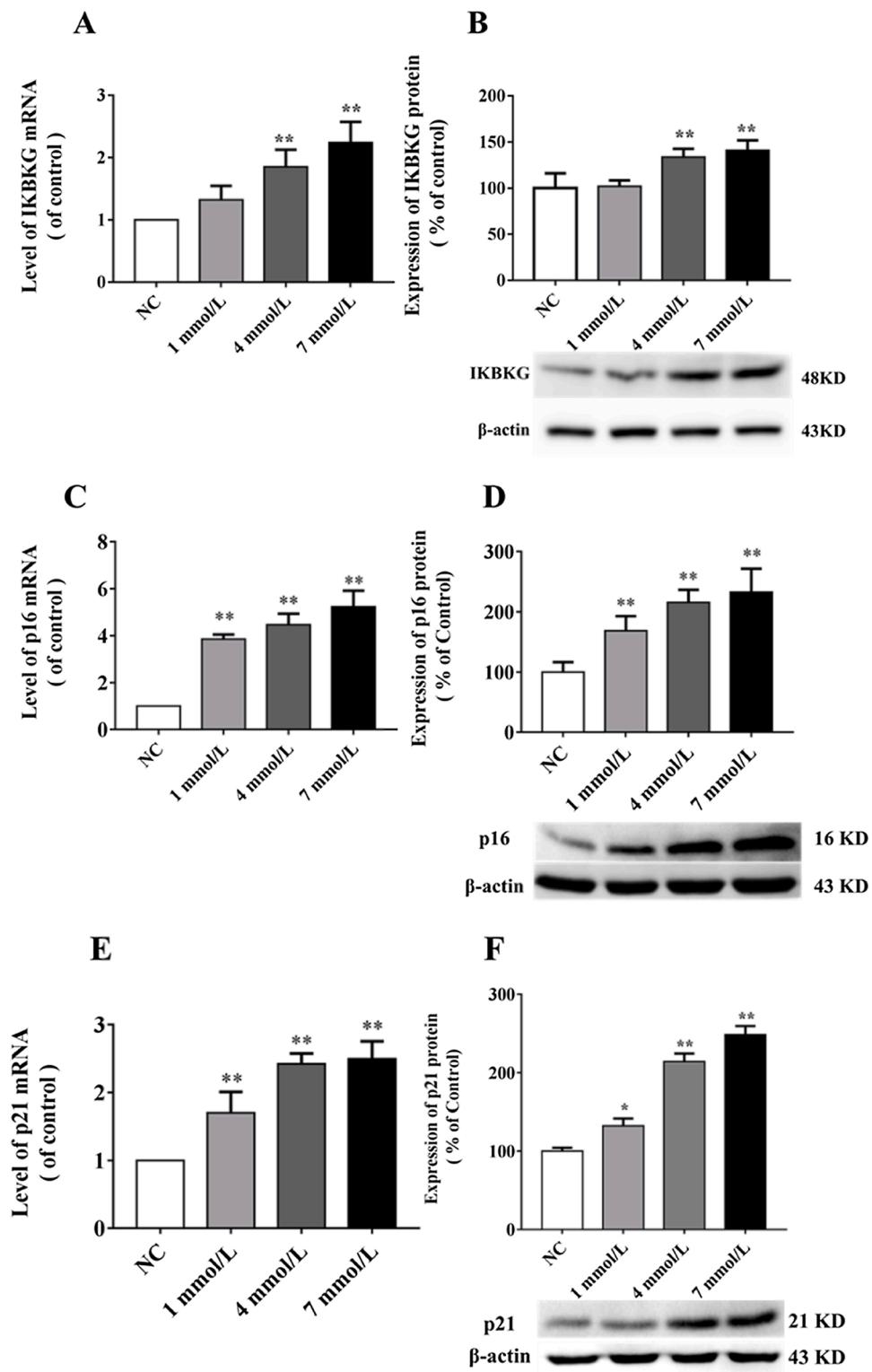


Fig. 8. mRNA and protein levels of IKBKG (A, B), P16 (C, D) and P21 (E, F) in L02 cells treated with NaF. mRNA and protein levels, as well as those for β -actin (as an internal standard), were determined by real time RT-PCR and Western blotting respectively, and the values were shown as means \pm SD ($n = 9$). ** $P < 0.01$ in comparison to the corresponding control value.

3.3. Results of SA- β -gal staining in liver of fluorosis rats

When the cells are senescent, the senescent cells are blue after staining with β -galactosidase. The experimental results showed that a small number of senescent cells in the liver of the normal control group were stained blue (Fig. 1A). With the increase of fluoride concentration,

the area of rat liver tissue stained blue gradually increased (Fig. 1B–D) compared with the normal control group, the difference was statistically significant ($P < 0.01$) (Fig. 1E).

After fluorosis rats were injected with AAV-IKBKG through the tail vein, compared with the high fluorosis group (Fig. 2A), senescence-positive cells in the liver significantly decreased (Fig. 2C), and the

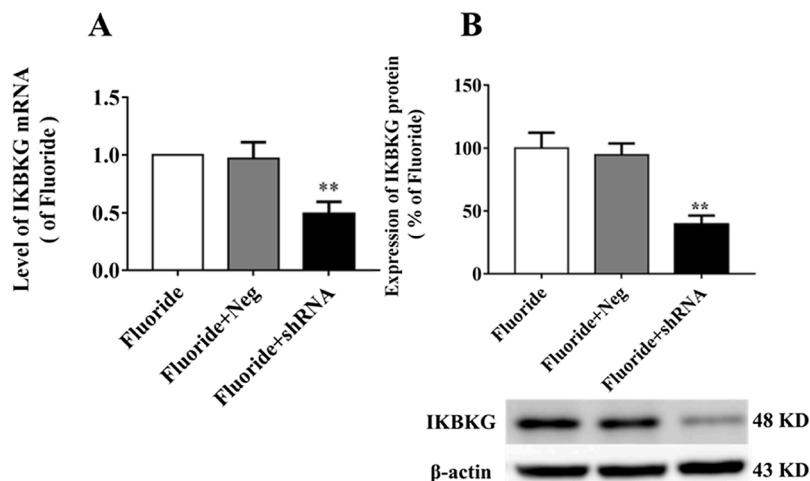


Fig. 9. mRNA and protein levels of IKBKG in L02 cells exposed to 7 mmol/L NaF and transfected with sh-IKBKG. mRNA and protein levels, as well as those for β -actin (as an internal standard), were determined by real time RT-PCR and Western blotting respectively, and the values were shown as means \pm SD ($n = 9$). ** $P < 0.01$ in comparison to the corresponding control value.

difference was statistically significant (Fig. 2D).

3.4. Levels of P16, P21, and IKBKG mRNA and protein in the liver tissue of fluorosis rats

Levels of IKBKG mRNA (Fig. 3A), P16 mRNA (Fig. 3C), P21 mRNA (Fig. 3E) and protein (Fig. 3B, D, F) were increased in the rats of medium and high fluoride groups compared to those in the control group, while there was no difference of P16 mRNA and protein level in the low fluoride group compared to that of control. With the degree of fluorosis increase, the levels of IKBKG, P16 and P21mRNA and protein were increased significantly.

Interestingly, when fluorosis rats were injected with AAV-IKBKG through the tail vein, compared with the high fluorosis group, the mRNA and protein levels of IKBKG (Fig. 4), P16 (Fig. 5A and B) and P21 (Fig. 5C and D) were decreased significantly.

3.5. Level of IL-8 in liver tissue of fluorosis rats

ELISA results showed that with the increase of fluoride concentration, the level of IL-8 gradually increased, and compared with the normal control group, the difference was statistically significant (Table 3). While the IL-8 level decreased significantly in the liver of high fluoride treated rats injected with AAV-IKBKG compared with that of high fluoride group.

3.6. Results of SA- β -gal staining in liver L02 cells treated with NaF

After treating L02 cells with different concentrations of NaF for 48 h, the results of SA- β -gal staining showed that the number of senescence-positive cells increased significantly (blue cells) with the increase of NaF concentration (Fig. 6B–D). Compared with the control group, the difference was statistically significant (Fig. 6E).

When L02 cells were exposed to 7 mmol/L NaF and transfected with IKBKG-shRNA to decrease the expression of IKBKG, the senescence-positive cells in L02 cells were reduced compared with L02 cells treated with 7 mmol/L NaF (Fig. 7).

3.7. mRNA and protein levels of P16, P21, and IKBKG in L02 cells treated with NaF

The levels of IKBKG mRNA (Fig. 8A), P16 mRNA (Fig. 8C), P21 mRNA (Fig. 8E) and protein (Fig. 8B, D, F) levels were increased in L02

cells treated with different concentrations of NaF. As the concentration of sodium fluoride increased, the mRNA and protein levels of IKBKG, P16 and P21 gradually increased in a dose-dependent manner. When L02 cells exposed to 7 mmol/L NaF were transfected with sh-IKBKG, the expression level of IKBKG was decreased (Fig. 9), and compared with the high NaF treated group, the mRNA and protein levels of P16 (Fig. 10A and B) and P21 (Fig. 10C and D) were decreased significantly.

3.8. Level of IL-8 in L02 cells treated with NaF and transfected with sh-IKBKG

ELISA results showed that with the increase of NaF concentration, the level of IL-8 gradually increased; compared with the normal control group, the difference was statistically significant (Table 4). IL-8 level decreased significantly in L02 cells exposed to 7 mmol/L NaF and transfected with sh-IKBKG compared with Fluoride + NC group.

3.9. Cell cycle of L02 cells treated with 7 mmol/L NaF and transfected with sh-IKBKG

Cell cycle detection by flow cytometry showed that compared with normal control group, the cell cycle of L02 treated with different concentrations of NaF mainly stagnated in the G2 phase in a dose-dependent manner (Fig. 11). After transfection of IKBKG-shRNA, the number of L02 cells in the G2 phase was significantly reduced compared with the L02 cell group treated with high fluoride concentration (Fig. 11) (Fig. 12).

4. Discussion

Cellular senescence is a pathophysiological phase in which the cell cycle is arrested and cells stop growing. In recent years, cellular senescence has been widely highlighted as one of the pathogenesis mechanisms that regulate various diseases such as tumors, degenerative diseases, and chronic diseases [20]. The reason and mechanism of cell senescence are not fully understood. Oxidative stress is the main external cause of cell senescence that is more generally accepted. Cells undergoing senescence have characteristic features, displaying a large and flat morphology: an increase in SA- β -gal, DNA damage, and a stable cell cycle arrest executed by interplaying between the RB and p53 tumor suppressor pathways (often accumulate the p16^{INK4a}, p15^{INK4b}, and p21^{Cip1/Waf1} cyclin-dependent kinase inhibitors that engage the RB pathway or mediate p53 effects accordingly) [21,22]. Previous studies have shown that there are senescent cells in the liver of patients with

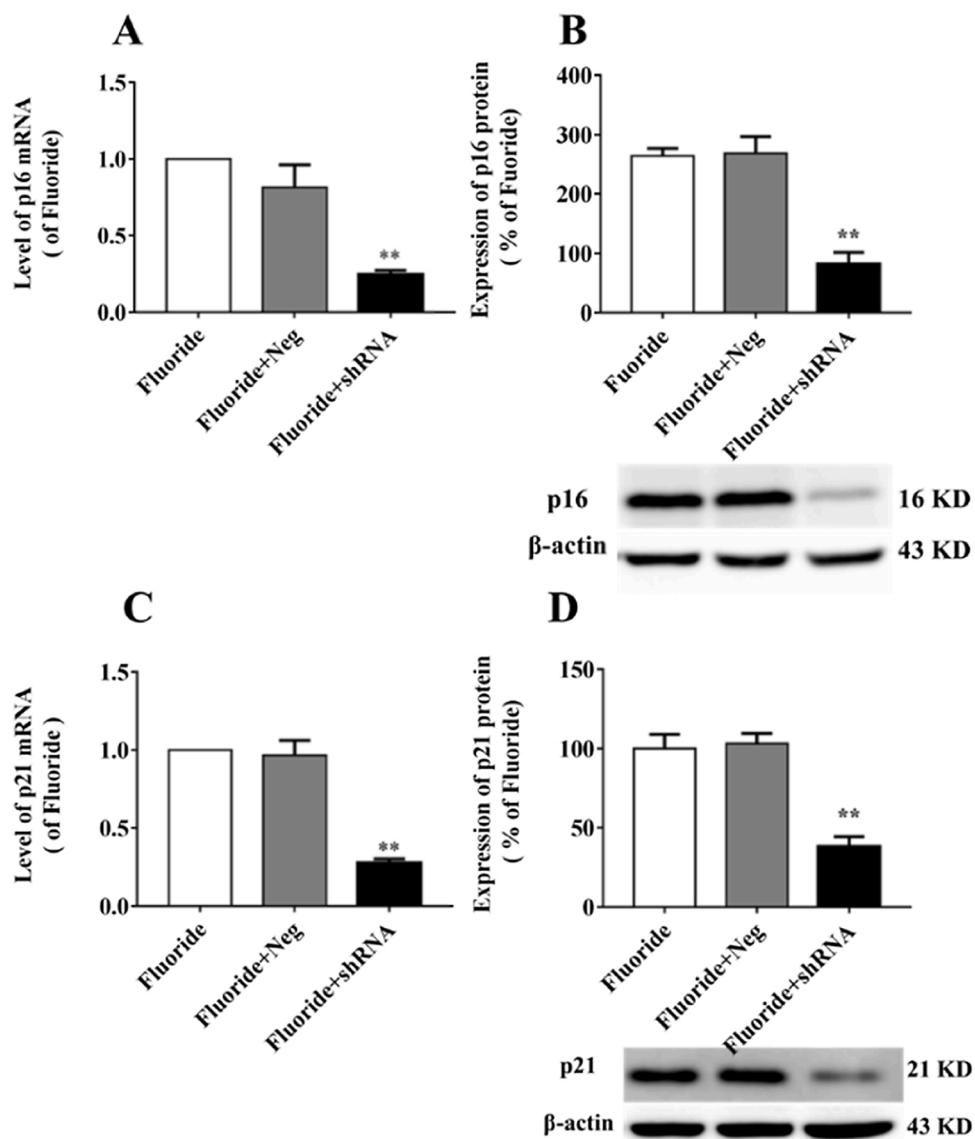


Fig. 10. mRNA and protein levels of P16 (A, B) and P21 (C, D) in L02 cells exposed to 7 mmol/L NaF and transfected with sh-IKBKG. The mRNA and protein levels, as well as those for β -actin (as an internal standard), were determined by real time RT-PCR and Western blotting respectively, and the values were shown as means \pm SD ($n = 9$). ** $P < 0.01$ in comparison to the corresponding control value.

Table 4

Expression level of IL-8 in L02 cells treated with different concentrations of NaF and transfected with sh-IKBKG.

Groups	IL-8(pg/mL)
NC	76.31 \pm 3.77
low NaF group	84.46 \pm 1.73**
middle NaF group	90.75 \pm 6.56**
High NaF group	98.70 \pm 4.50**
Fluoride + NC	509.59 \pm 51.66
Fluoride + shRNA	290.86 \pm 61.86 ##

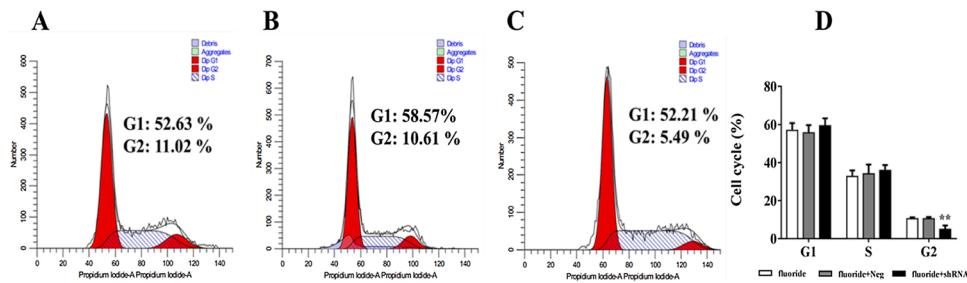
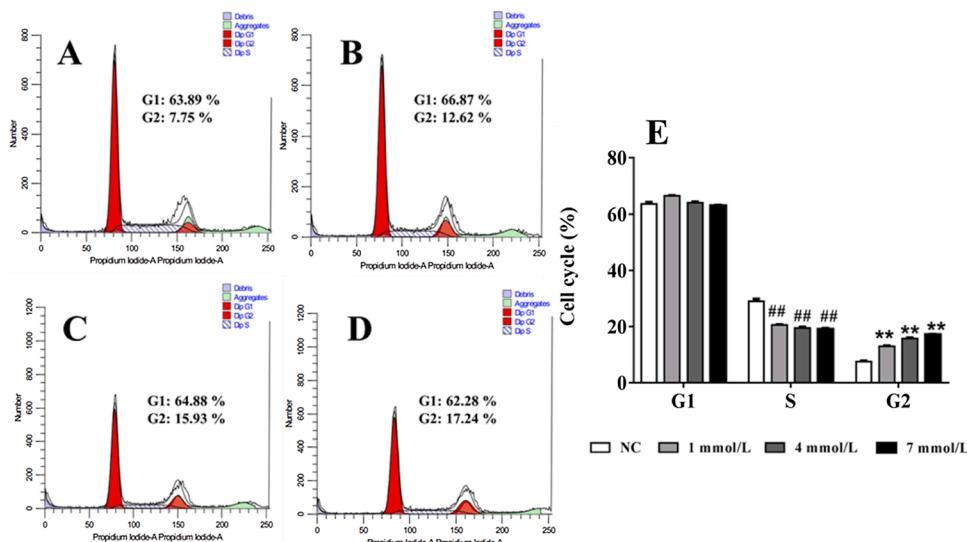
Data was presented as means \pm SD of each independent experiment ($n = 10$). ** $P < 0.01$ vs. Normal Control group. ## $P < 0.01$ vs. Fluoride + NC group.

non-alcoholic fatty liver (NAFLD) and cirrhosis. The increase in the number of senescent cells can promote the occurrence of NAFLD, but the underlying mechanism is not fully elucidated [23,24].

It has been verified that the increased oxidative stress induced by fluoride was associated with DNA fragmentation in rat hepatocytes,

which was one of the main mechanisms leading to liver damage. As oxidative stress is one of the main causes of cell senescence, these studies suggested that fluorosis may cause liver damage by inducing cell senescence. However, there are very few studies on the toxic effects of fluoride by inducing cell senescence. The present study investigated the effects of fluoride on hepatocyte senescence through *in vitro* and *in vivo* experiments.

The results showed that fluoride could induce cell senescence by β -galactosidase staining on frozen sections of liver tissues of chronic fluorosis rats and L02 cells treated with different concentrations of NaF. With the increase of fluoride concentration, the SA- β -gal-positive cells were increased obviously, suggesting that the degree of senescence was positively correlated with the concentration of fluoride. Concerning the levels of p16 and p21, the hallmarks of cell senescence, it was found that the expression levels of p16 and p21 were increased in a concentration-dependent manner in fluorosis rats' liver and NaF treated L02 cells, which further confirmed that fluoride could induce hepatocyte senescence. As p16 was the main tumor suppressor, which could induce cell senescence and cycle arrest. After exposure NaF to L02 cell, the cell cycle was arrested in the G2 phase by flow cytometry. At the same time, the



level of IL-8 in the liver tissue of fluorosis rats and culture medium of fluoride-treated L02 cells were significantly increased. Taken together, our results confirmed that fluoride could induce hepatocyte senescence, which might play an important role in the fluoride induced liver damage.

It is clear now that SASP components actively participate in the senescence process. For example, key SASP factors (IL-6 and IL-8) act in an autocrine feedback loop to reinforce the senescence growth arrest. Nuclear factor- κ B (NF- κ B) is a master regulator of SASP and is used to further probe the impact of SASP biology on the senescence program [25]. A large number of studies suggested that NF- κ B signaling was highly activated in cellular senescence [15]. NF- κ B gene expression was also enhanced in primary rat hippocampal neurons by fluoride treatment in a dose-dependent manner, which meant fluoride might regulate cell senescence by activating NF- κ B signaling [26]. The NF- κ B system is an evolutionarily conserved signaling pathway, which can be triggered by not only immune activation but also diverse external/internal danger signals associated with senescence and even the aging process (oxidative and genotoxic stresses). IKBKG is generally called NEMO, an important regulatory component of IKK complex linked upstream to genotoxic signals and IL-1 and TNF receptor mediated signaling [16]. Recent studies have shown that IKBKG protein plays a major role in the activation of NF- κ B signals caused by DNA damage, which suggests that IKBKG may be involved in the process of cell senescence [17,27]. Studies have found that IKBKG can also regulate liver diseases at different functional levels [28,29]. This study further explored the role of IKBKG in fluoride-induced hepatocyte senescence, hoping to provide a theoretical basis for the pathogenesis of fluorosis.

The results showed that the expression of IKBKG increased in liver tissues of chronic fluorosis rats and liver L02 cells exposed with NaF in a concentration-dependent manner. The results suggested that fluoride

might induce hepatocyte senescence via up-regulating the expression of IKBKG. To further verify the role of IKBKG in fluoride-induced hepatocyte senescence, tail vein injection of AAV-IKBKG and transfected sh-IKBKG to L02 cells were adopted to knock down the level of IKBKG. The results showed that compared with the fluorosis rat or L02 cells treated with fluoride, the SA- β -gal-positive cells were decreased, the expression levels of P16 and P21 decreased, the IL-8 level decreased in rat liver or L02 cells treated with NaF and down-regulation of IKBKG, and the number of L02 cells arrested in the G2 phase was also reduced.

In conclusion, the chronic fluorosis rats and human L02 cells exposed with NaF as to evaluate whether fluoride could induce hepatocyte senescence and its potential regulated mechanism. The results indicated that fluoride might cause liver damage by inducing cell senescence, and its mechanism might be related to the up-regulation of IKBKG expression in hepatocytes. This research might shed more light on the underlying pathogenesis of fluorosis, the senescence is complex pathophysiological process, IKBKG just as a pathological factors and further researches should be designed to fully elucidate the regulation signal pathway of IKBKG in the role of cell senescence in the toxicity of fluoride.

Author contributions

QXL, HXX and WCX conceived and designed the research. WCX, MCY, DJ, ZT performed the experiments. QXL, WCX, ZY, GZZ wrote the manuscript. All authors have read and approved the final manuscript.

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Declaration of Competing Interest

All authors declare that they have no competing interests. All authors have read and approved the final manuscript.

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None

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