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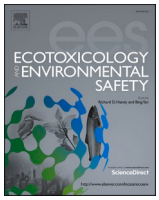

Fluoride caused injury to endothelial cells by disrupting cholesterol synthesis

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ABSTRACT

Fluoride has been identified as an important risk factor for cardiovascular disease, with endothelial cell dysfunction serving as a critical initiating event in its onset and progression. As cholesterol serves as an essential structural component of the endothelial cell membrane, alterations in cholesterol levels can significantly compromise endothelial function. However, the precise mechanisms underlying fluoride-induced cholesterol dysregulation in endothelial cells remain poorly understood. Through a well-established fluorosis mouse model, we observed that fluoride lead to significant detachment and pathological alterations of endothelial cells in mouse aorta. Transcriptomic profiling and enrichment analysis revealed that fluoride disrupted cholesterol biosynthesis pathway in HUVECs, particularly through downregulation of two key enzymes, HMGCR and CYP51A1. Subsequent validation experiments confirmed reductions in TC, HDL-C and LDL-C levels in mouse serum, accompanied by decreased expression of HMGCR and CYP51A1 in both mouse aortic endothelial cells and HUVECs. The reduction of cholesterol synthesis in fluoride induced endothelial injury was regulated by miR-200c-3p, a key regulator involved in fluorosis. To further substantiate the effect of fluoride on cholesterol levels, we conducted an epidemiological investigation and found a significant decrease in serum HDL-C levels and an elevated TG/HDL-C ratio in the population. In conclusion, this study demonstrated that fluoride exposure impaired endothelial cells by disrupting cholesterol synthesis, and fluoride induced dyslipidemia in populations may represent a potential mechanism underlying fluoride associated cardiovascular diseases, which may provide a new perspective on the mechanism of cardiovascular system injury caused by fluoride.

1. Introduction

Fluoride is a naturally occurring element found in food, water, and dental products, leading to widespread and long-term exposure throughout the human lifespan (Aldana et al., 2024). As fluoride accumulates in the body, it can eventually lead to fluorosis. Endemic fluorosis is widespread across 50 countries and regions across Europe, Asia, America, Oceania, and Africa (Liang et al., 2025). Historically, China was among the countries where endemic fluorosis was widespread, severe, and harmful. Except for Shanghai and Hainan Provinces, all other provinces have reported varying degrees of endemic fluorosis prevalence (Wang et al., 2012), affecting nearly 100 million people in endemic areas. Numerous studies have shown that chronic excessive fluoride intake could adversely affect different organ systems. In

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particular, the cardiovascular system is susceptible to disruption by a high concentration of fluoride (Yan et al., 2017). The impact of fluoride on the cardiovascular system has become a significant public health concern.

Endothelial cells, which form the monolayer lining of blood vessels, are a crucial component of the cardiovascular system (Ramnath and Satchell, 2019). As the first to encounter substances in the blood, they are exposed to higher concentrations than other cell types and can respond rapidly to stimulation. The integrity of endothelial cell structure and function plays a significant role in maintaining the normal function of the vessel wall and circulatory system (Sumpio et al., 2002). Previous studies have found that fluoride can cause intimal thickening of the human carotid artery, suggesting its potential to induce endothelial cell injury (Liu et al., 2014). Additionally, fluoride has been found to cause vascular endothelial cell misalignment, detachment, and dysfunction (Wang et al., 2001; Zhang, Wang, et al., 2025). Endothelial cell injury is an early stage in the development of cardiovascular diseases. Therefore, investigating the mechanisms underlying fluoride-induced cardiovascular injury from the perspective of endothelial cell damage is both scientifically justified and critically important. However, the exact mechanism by which fluoride harms endothelial cells remains unclear.

Cholesterol is a key component of the endothelial cell membrane. A reduction in cholesterol content may increase membrane fluidity and permeability (Wang et al., 2013, 2022), altering the structural integrity of the cell membrane. Consequently, cholesterol reduction affects endothelial cell structure and function. The primary source of cholesterol in endothelial cells is de novo synthesis. This process begins with the formation of HMG-CoA from two acetyl-CoA molecules, catalyzed by thiolases and synthases. HMG-CoA is then reduced to mevalonate by HMG-CoA reductase (HMGCR), the rate-limiting enzyme in cholesterol synthesis (Shi et al., 2022). Mevalonate is subsequently converted into lanosterol and further processed into cholesterol by lanosterol-14α-demethylase (CYP51A1) (Cerqueira et al., 2016). The key enzymes HMGCR and CYP51A1 are crucial for cholesterol synthesis. Therefore, investigating the effect of fluoride on these enzymes in the cholesterol biosynthesis pathway could provide insights into fluoride-induced changes in cholesterol levels.

Cholesterol has been widely studied as a risk factor for cardiovascular diseases, but the effect of fluoride on cholesterol levels in blood and tissues remains controversial. Some studies have observed reduced plasma levels of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) among individuals exposed to fluoride (Liu et al., 2014). Conversely, a study has reported a significant elevation in serum levels of TC, TG, and LDL-C in patients with high urinary fluoride (Zhang et al., 2018). Consequently, the potential impact of fluoride exposure on cholesterol metabolism necessitates further investigation. In addition to its free form, cholesterol primarily exists as cholesterol esters, with some molecules binding to lipoproteins to form LDL-C and high-density lipoprotein cholesterol (HDL-C) (Aguilar-Ballester et al., 2020; Luo et al., 2020). TC includes both free cholesterol and cholesteryl esters, and TC, HDL-C, and LDL-C levels serve as key indicators of cholesterol content. An elevated TG/HDL-C ratio is considered a major risk factor for atherosclerosis and is used to assess cardiovascular disease risk in patients (Kosmas et al., 2023). In our study, we initially investigated changes in serum TC, TG, HDL-C, and LDL-C levels in the fluoride-exposed populations in Duerbert Mongolian Autonomous County, China. These findings contribute to a deeper understanding of fluoride's effects on cholesterol levels.

MiRNAs participate in fluoride-induced injury. Several studies have shown that miR-200c-3p, miR-221–3p, and miR-122–5p-378d play regulatory roles in fluorosis (Chen et al., 2021; C. Li et al., 2021; Li et al., 2023). Fluoride has been found to cause skeletal fluorosis by activating the BMP4/Smad pathway through miR-200c-3p, and to induce endothelial cell apoptosis by regulating the miR-200c-3p/Fas axis (Jiang et al., 2018, 2020). Urinary miR-200c-3p expression correlates with fluoride accumulation, dental fluorosis grade, and cartilage damage score, suggesting its potential as a biomarker for fluorosis (Li et al., 2025). MiRNAs also play a role in cholesterol synthesis. They can directly regulate enzymes involved in cholesterol biosynthesis and influence the overall process. Selitsky et al. found that miR-27 inhibits cholesterol synthesis by targeting HMGCR gene transcription in hepatoma cells (Chen et al., 2023; Selitsky et al., 2015). Similarly, miR-223 could inhibit cholesterol biosynthesis through the direct repression of sterol enzymes 3-hydroxy-3-methylglutaryl-CoA synthase 1 and methylsterol monooxygenase 1 in humans (Vickers et al., 2014). As a key molecule in fluorosis, miR-200c-3p has been suggested to regulate HMGCR and CYP51A1 levels (Zhu et al., 2018). Therefore, further research is needed to determine whether fluoride reduces cholesterol content in endothelial cells by affecting cholesterol biosynthesis and whether miR-200c-3p is involved in this process.

In addition, we found that fluoride induced injury to aortic endothelial cells in a mouse model. Transcriptome enrichment analysis revealed that fluoride disrupted cholesterol synthesis in endothelial cells, leading to a decrease in the key enzymes HMGCR and CYP51A1. Subsequent validation confirmed that cholesterol levels, along with HMGCR and CYP51A1 expression, were reduced in mouse serum, the aortic arch, and Human Umbilical Vein Endothelial Cells (HUVECs), with miR-200c-3p playing a regulatory role in this process. Epidemiological findings further indicated that fluoride exposure was associated with decreased HDL-C levels and a reduced TG/HDL-C ratio in human serum. By examining fluoride-induced endothelial cell injury, this study highlights the critical role of cholesterol biosynthesis in endothelial dysfunction and offers a new direction for the prevention and treatment of fluoride-related cardiovascular diseases.

2. Methods

2.1. Animals, treatments, and fluoride determination

Sixty male, 4-week-old specific pathogen-free (SPF) C57BL/6 mice were purchased from Beijing Vital River Laboratories (Beijing, China). The mice were acclimatised for one week under controlled environmental conditions of room temperature 20 ± 2 ℃, humidity 50 %-60 %, and a 12-hour light/dark cycle. At the end of the acclimatisation period, the mice were randomly divided into four groups, each group of in vivo experiments included 5 male mice and the groups were formed using the random number table method: control group (distilled water), 25 mg/L NaF (distilled water containing 25 mg/L sodium fluoride), 50 mg/L NaF (distilled water containing 50 mg/L sodium fluoride), and 100 mg/L NaF (distilled water containing 100 mg/L sodium fluoride). All animals were provided a standard diet and given water ad libitum. After 12 weeks, the occurrence of dental fluorosis was assessed according to established diagnostic criteria. Urine samples were collected using metabolic cages. After euthanasia, blood samples were collected for subsequent serum analysis. The ascending aorta and aortic arch were harvested and fixed with 4 % paraformaldehyde for pathological examination. The pathological assessment was conducted in a blinded manner. All animal procedures were approved by the Animal Care and Use Committee of Harbin Medical University, Harbin, China (No. hrbmuecdc20190303).

2.2. Cells viability

A previous study found that treating HUVECs with NaF at increasing concentrations for 24 h, cell survival was 87.7 %, 67.7 % and 54.8 % for 35, 55 and 75 μmol/L NaF, respectively. Based on these findings, 35, 55, and 75 μmol/L NaF were used in subsequent experiments (Zhang et al., 2023).

2.3. Quantitative real-time PCR

Total mRNA was extracted using TRIzol reagent when the cells

reached 80 % confluence. Quantitative real-time PCR (qRT-PCR) analysis was performed to evaluate RNA expression (n = 3). cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser reverse transcriptase (Takara Bio, Dalian, China). qRT-PCR reactions were conducted using the SYBR Green kit (Takara). Data were normalized to β-actin as the reference gene. The primer sequences utilized were detailed below.

2.4. Western blot

HUVECs were harvested and homogenized in lysis buffer containing protease inhibitors. Total protein was collected and the concentration of total protein was measured by BCA assay kit (Beyotime, P0010). A total of 30 μg of protein was separated by 10 % SDS PAGE and transferred onto 0.45 μm PVDF membranes. After blocking with 5 % skim milk in TBST (Tris buffered saline and 0.05 % TWEEN 20) for two hours, the membrane was incubated with primary antibodies at 4 ℃ overnight. The primary antibodies used were HMGCR (Affinity Biosciences, DF6518), CYP51A1 (Affinity Biosciences, DF3591), and β-actin (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., TA-09) at 1:1000 dilution. The membranes were then incubated with HRP-conjugated secondary antibodies for an hour at room temperature. Finally, protein bands were visualized using enhanced chemiluminescence detection reagents (Biosharp, BL520A) detected with the Tanon imaging system (Shanghai Tanon Technology). Band intensity was quantified using ImageJ software (version 1.8.0.345).

2.5. Immunohistochemical assay

Perform immunohistochemical staining on mouse aortic sections. Primary antibodies to HMGCR (1:100 dilution, bs-5068R) and CYP51A1 (1:100 dilution, bs-15409R) were added to slices and incubated at 4 °C overnight. Subsequently, the sections were washed thoroughly in distilled water and then incubated with HRP-labeled goat anti-rabbit antibody (1:200 dilution, GB23301, Servicebio) for 1 h at room temperature. After washing with distilled water, color was developed with diaminobenzidine chromogenic solution for 1 min at room temperature and washed in tap water to terminate coloration. Then, the slices were stained with hematoxylin. The percentage of positive nuclei was calculated by ImageJ (1.8.0.345) software.

2.6. TC, HDL-C and LDL-C content detection

TC, HDL-C, and LDL-C levels were measured using kits (A111–1–1, A112–1–1, A113–1–1) produced by Nanjing Jiancheng Company. The processing steps are as follows: Serum: direct assay; Culture medium samples: the culture medium is collected and centrifuged at 1000 rpm for 10 min, and the supernatant is used for analysis; Cell samples: centrifuge the prepared cell suspension at 1000 rpm for 10 min, discard the supernatant and keep the cell pellet. Prepare a 2.5 % Triton X-100 lysate in distilled water and add 200 μL to each tube. The cells are lysed for 30 min, after which the lysed cells are directly analyzed without centrifugation. The subsequent experimental procedures were carried out in strict adherence to the manufacturer's protocol.

2.7. Filipin staining

Filipin staining was used to detect cholesterol content on the cell membrane. Cells were seeded on coverslips and incubated with 0.05 mg/L Filipin solution for two hours at room temperature, and then observed and imaged with a laser confocal microscope. The fluorescence intensity of Filipin staining in endothelial cells was analyzed using ImageJ (1.8.0.345) software.

2.8. Gene chip analysis

Affymetrix GeneChip Human Gene 2.1 ST Array Strips (Affymetrix, P/N 902114) were used to establish the mRNA expression profiles of HUVECs in the control and fluoride treatment groups (65 μmol/L NaF for 24 h, n = 3). PARTEK 7.0 software was used to identify differentially expressed mRNAs based on the following criteria: 1) fold change > 1.8 or < -1.8; 2) $P < 0.05$. The differentially expressed mRNAs were then subjected to enrichment analysis.

2.9. Study population and laboratory test

An epidemiological investigation of endemic fluorosis was conducted in Dorbod Mongol Autonomous County, Daqing City, Heilongjiang Province in February 2021 (Environment and Chronic Disease in Rural Areas of Heilongjiang, China (ECDRAHC)) (Jiang et al., 2023). Four natural townships (Bayan Chagan, Yantong Tun, Lianhuanhu, and Keltai) were selected as study sites due to their similar social factors. A total of 755 individuals (291 males and 464 females) were included based on the following criteria: 1) residence in the local area for over five years, 2) use of local water sources as drinking water, 3) ability to communicate normally without severe physical disabilities, and 4) voluntary participation with signed informed consent. Individuals were excluded if they had (1) incomplete personal information, (2) a history of diabetes, stroke, liver disease, kidney disease, respiratory disease, or cancer, or (3) were taking medications that could affect blood pressure. Ethics approval was obtained from the Ethics Committee of Harbin Medical University, and informed consent was obtained from each subject (No. hrbmuecdc20180601). A structured questionnaire was administered to collect demographic information, including age, gender, educational attainment, marital status, as well as smoking and alcohol consumption habits. Venous blood and urine samples were collected after overnight fasting and centrifuged at 3000 rpm for 10 min at 4 °C. Serum TG, TC, HDL-C, and LDL-C (201SJOH201A, 202SJOH202A, 203SJOH203A and 207SJOH207A, Meikang Bio, Ningbo, China) were measured using an automatic biochemical analyzer (Hitachi, Japan). Urinary fluoride concentration was assessed using a fluoride ion selective electrode.

2.10. Statistical analysis

Statistical analysis of the data was performed in Statistical Package for the Social Sciences (SPSS) version 22.0. The basic demographic data of individuals with different urinary fluoride levels were analyzed. Multivariate logistic regression analysis was employed to calculate the odds ratio (OR) and 95 % confidence interval (CI) for the association between urinary fluoride concentration and TG, TC, HDL-C, LDL-C, TG/HDL-C, and non-HDL-C. All tests were two-tailed. One-way analysis of variance (ANOVA) was used for normally distributed data. If the homogeneity of variance is satisfied, the LSD method is used for pairwise comparisons. If the data exhibit a skewed distribution or fail to meet the homogeneity of variance, non-parametric tests should be employed. A significance level of $P < 0.05$, was considered statistically significant. GraphPad Prism 8.4.0 software was used to visualize the results.

3. Results

3.1. Fluoride-induced pathological changes in mice aortic intima

After 12 weeks of feeding, the body weight of mice in the 100 mg/L NaF group showed no significant difference compared with the control group ($P > 0.05$) (Fig. 1A). Fluoride analysis was performed on drinking water from different groups (Fig. 1B). The urinary fluoride content significantly increased in the fluoride-exposed groups ($P < 0.001$) (Fig. 1C), indicating that both external and internal fluoride exposure in mice increased with fluoride dosage. Dental fluorosis, the earliest manifestation of fluorosis, was observed. Compared with the control

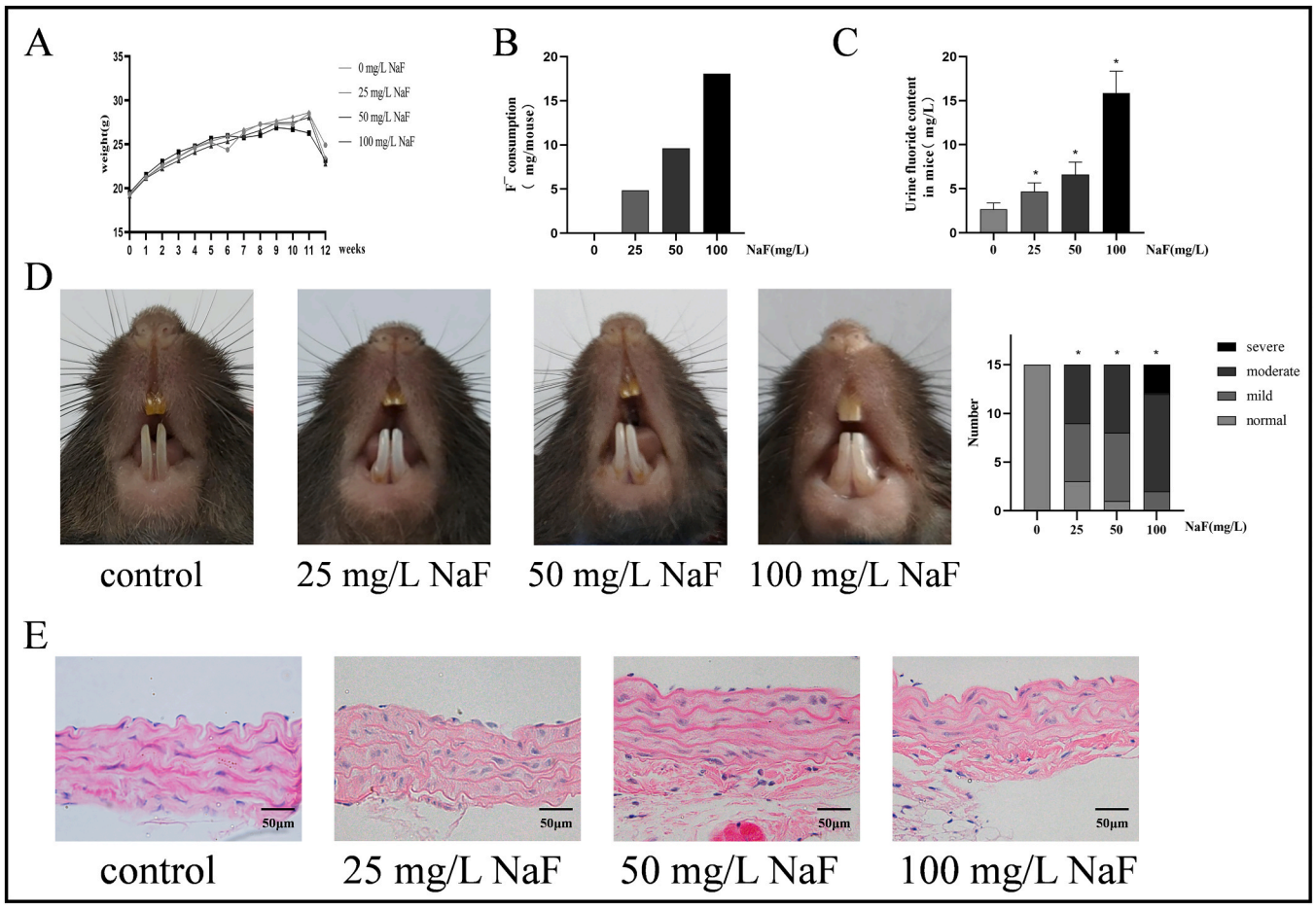

Fig. 1. Fluoride-induced pathological changes in mice. Mice at five weeks of age were treated with fluoride (25, 50, 100 mg/L) in drinking water for three months. A. Changes in body weight in mice during feeding period. B. Estimation of fluoride ion intake in drinking water of mice. C. Urinary fluoride level reflected fluoride exposure in mice. D. Photographs of mouse tooth condition. E. The HE staining of mice aorta (Scale bar = 10 μm)(n = 5). Compared to the control group: * $P < 0.05$, *** $P < 0.001$.

group, 86.7 % of the mice in the 100 mg/L NaF group exhibited varying degrees of dental fluorosis ($P < 0.001$) (Fig. 1D). The fluoride content in drinking water reflects the external fluoride exposure in mice, while the fluoride content in urine reflects the internal fluoride exposure. In conclusion, the mouse model with fluorosis has been successfully established.

In the control group, a well-organized aortic structure was observed, with flat monolayer endothelial cells attached to smooth and continuous intima. As fluoride exposure increased, the intima layer showed a progressive disorder in the arrangement of endothelial cells. Additionally, the smooth muscle cells and elastic fibers in the media were loosely arranged in a wavy disorder (Fig. 1E). In summary, fluoride induced pathological injury of the mice aortic intima.

3.2. Fluoride mainly affected HUVECs cholesterol biosynthesis

Since previous studies have shown that fluoride can induce endothelial cell injury, we performed a mRNA microarray assay to further explore the underlying mechanisms. Gene expression analysis revealed 128 differentially expressed mRNAs between the control and 65 μmol/L NaF group, of which 54 were up-regulated and 74 were down-regulated. Gene Ontology (GO) enrichment analysis of the differential genes indicated that these mRNAs were prominent in several biological processes, including cholesterol biosynthesis, nucleosome assembly, and cell division (Fig. 2A). Meanwhile, steroid biosynthesis pathways were also enriched in the KEGG analysis (Fig. 2B-C). In summary, the transcriptome results suggested that fluoride affected cholesterol biosynthesis, with differentially expressed HMGCR and CYP51A1 as key enzymes in the process. It is speculated that fluoride may influence cholesterol biosynthesis and contribute to endothelial injury by regulating the levels of HMGCR and CYP51A1.

3.3. Fluoride reduced endothelial cholesterol levels

The transcriptome results showed that fluoride affected the biosynthesis of cholesterol. We validated this in animal and cell models and found that TC and LDL-C levels were reduced in the serum of mice in the 100 mg/L NaF group (Fig. 3A). Similarly, compared with the control group, the levels of TC, HDL-C, and LDL-C in the intracellular and supernatant fractions of the 75 μmol/L NaF group were decreased (Fig. 3B-C). With the increase in fluoride concentration, the cholesterol content in the cell membrane decreased (Fig. 3D). The results indicated that fluoride reduced cholesterol content in intracellular, extracellular, endothelial cell membrane, and serum compartments.

3.4. Fluoride reduced the expression of key enzymes in cholesterol biosynthesis

Transcriptome analysis revealed that fluoride regulated the expression of HMGCR and CYP51A1, which are key enzymes in cholesterol synthesis. We then detected the expression of HMGCR and CYP51A1 in mouse aortic endothelial cells. Compared with the control group, the expression of HMGCR and CYP51A1 was significantly decreased in the 100 mg/L NaF group (Fig. 4A-B). After 75 μmol/L NaF treatment, the

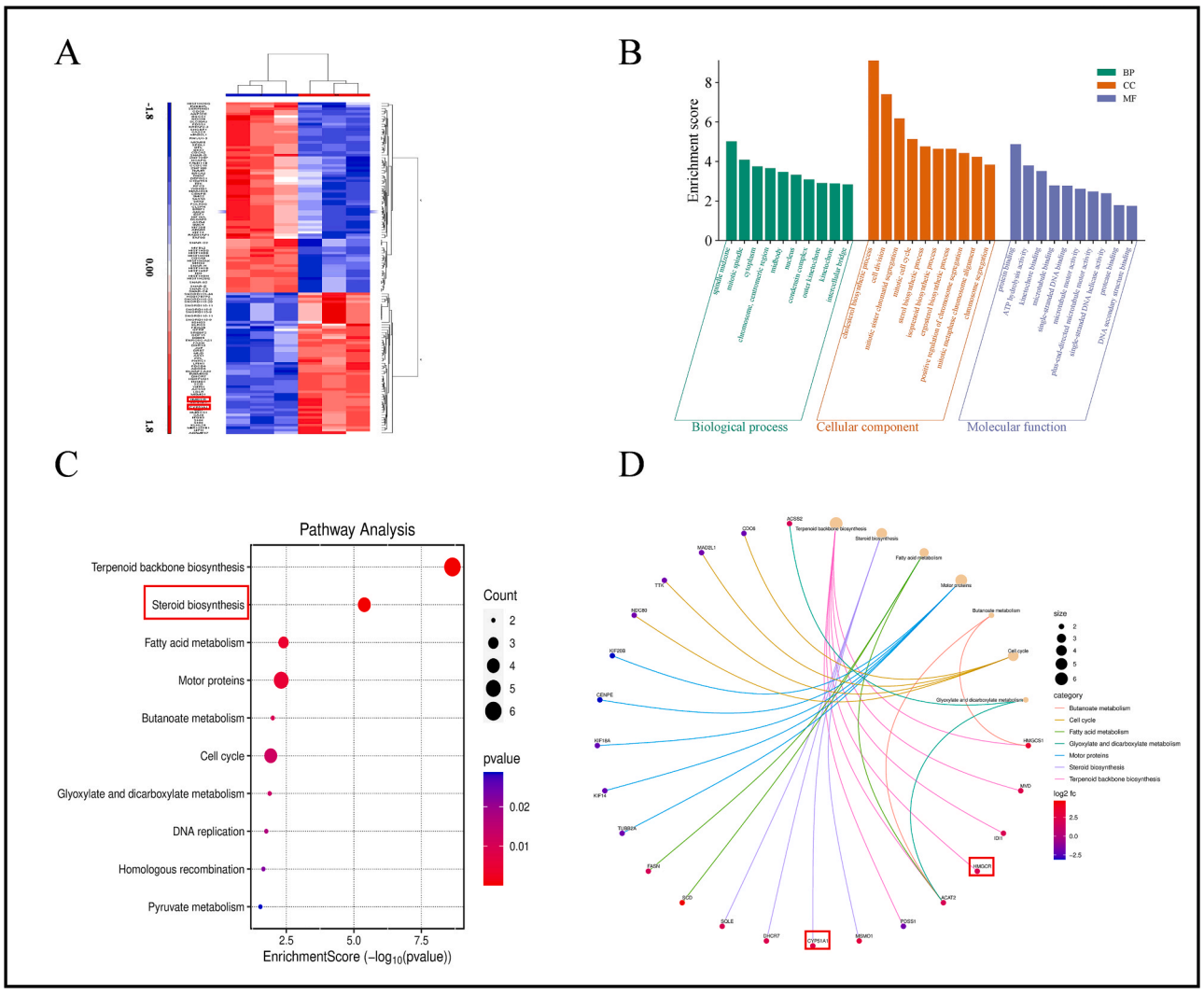

Fig. 2. Transcriptome analysis of HUVECs in control group and 65 μmol/L NaF treatment group. A. Heatmap of the hierarchical clustering of differentially expressed mRNAs. B. Gene Ontology enrichment analysis. C. and D. enrichment analysis of KEGG pathways.

mRNA levels of HMGCR and CYP51A1 in HUVECs increased ($P < 0.05$) [-0.1470, 95 %CI(-0.2317, −0.06225)] [-0.1379, 95 %CI(-0.2424, −0.03343)], but the protein levels decreased ($P < 0.05$) [0.2957, 95 % CI(0.03172, 0.5569)] [0.2459, 95 %CI(0.01567, 0.4762)] (Fig. 4D). These results indicated that fluoride reduced HMGCR and CYP51A1 expression in both mouse aortic endothelial cells and HUVECs.

3.5. *MiR-200c-3p was involved in the effect of fluoride on cholesterol synthesis in endothelial cells*

Previous studies have identified miR-200c-3p as a key molecule in fluoride-induced endothelial injury (Jiang et al., 2020). To investigate the role of miR-200c-3p in the fluoride induced reduction of cholesterol synthesis mediated, we transfected the miR-200c-3p inhibitor. The results showed that the inhibition of miR-200c-3p increased cell membrane cholesterol and intracellular TC, HDL-C and LDL-C levels (Fig. 5A-B), while extracellular TC, HDL-C and LDL-C were not significantly changed (Fig. 5C). Subsequently, we quantified the expression levels of HMGCR and CYP51A1 at both transcriptional and translational levels. Interestingly, transfection with the miR-200c-3p inhibitor resulted in a significant downregulation of HMGCR and CYP51A1 mRNA expression, while their protein levels exhibited a contrasting upregulation (Fig. 5D-E). Through miR-200c-3p inhibition, we observed regulatory effects on HMGCR and CYP51A1 expression, which subsequently reversed the fluoride-mediated suppression of cholesterol biosynthesis in endothelial cells.(Table 1)

3.6. *Serum HDL-C level was decreased in patients with endemic fluorosis*

The cholesterol level in endothelial cells is closely associated with that in the blood, therefore, we further verified serum lipid levels in the population. In accordance with the inclusion and exclusion criteria, a total of 755 subjects, 291 males and 464 females, were enrolled in this study. The subjects were divided into two groups according to their urinary fluoride levels: < 1.6 (normal group) and ≥ 1.6 (high fluoride group) (Gao et al., 2020). The basic demographic information of the survey subjects included gender, age, education level, and marital status as shown in the Table 2. The mean (±SD) age was 58.71(±9.27) years, and 46.2 % of the participants were above 60 years. More females (61.5 %) were included in this study than males (38.5 %). The proportion of participants with a higher educational level was low, with 93.9 % having completed primary school or below. Additionally, 78.1 % of the participants did not smoke, and 76.4 % did not drink alcohol. The median (P25-P75) level of urinary fluoride was 0.75 (0.54, 1.03) mg/L. The mean systolic blood pressure (SBP) was 130.93 (±17.98) mmHg and the mean diastolic blood pressure (DBP) was 81.70 (±9.99) mmHg.

With an increase in urinary fluoride concentration, the level of SBP, DBP, and TG/HDL-C increased, while the level of HDL-C showed a

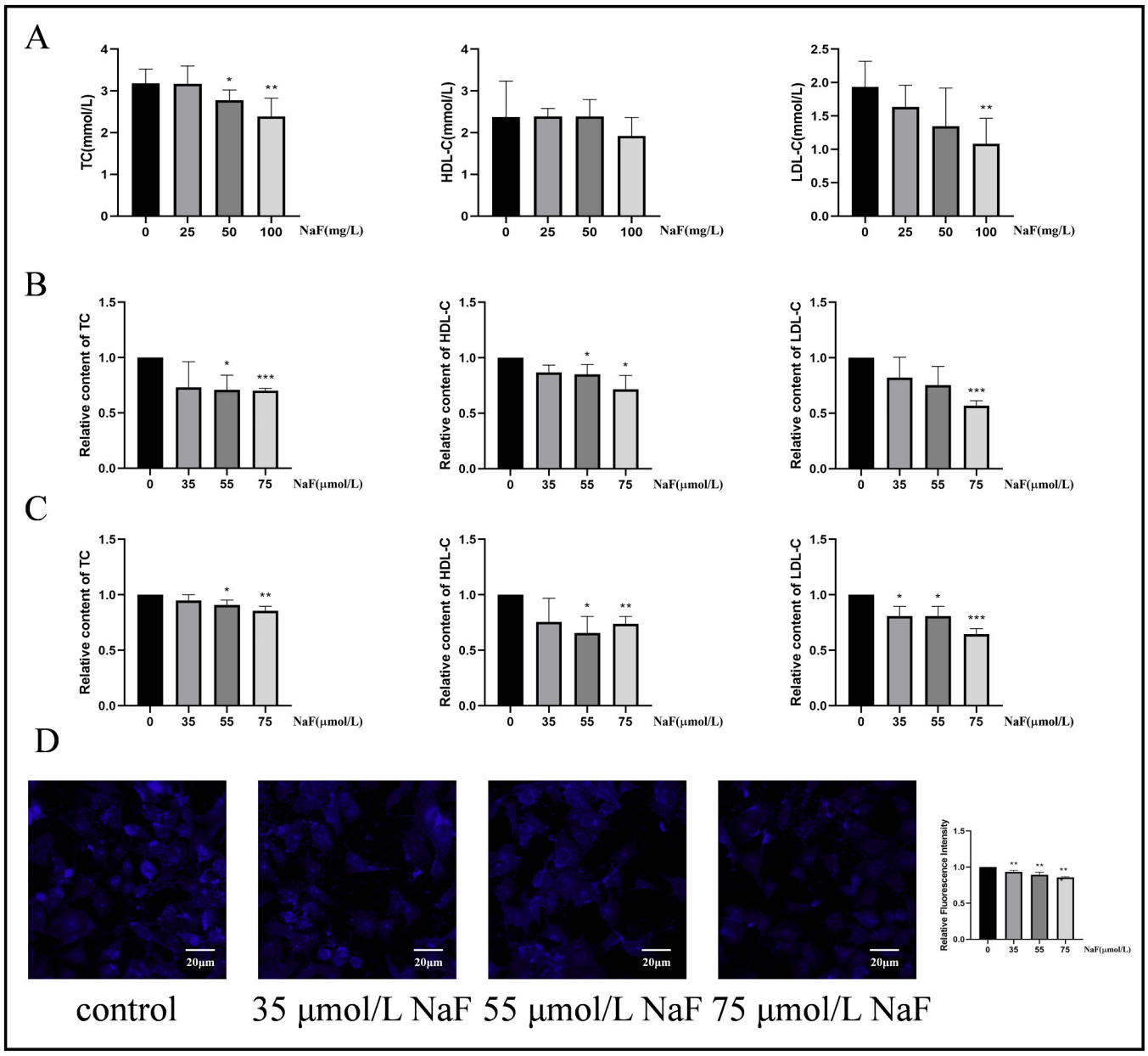

Fig. 3. Cholesterol levels in mice serum and HUVECs. A. TC, HDL-C and LDL-C levels in mice Serum. (n = 5) B. TC, HDL-C and LDL-C levels in HUVECs. C. TC, HDL-C and LDL-C levels in supernatant. D. The cholesterol content within the membrane of HUVECs was quantitatively analyzed by Filipin staining. The results were acquired with a confocal microscope and analyzed with ImageJ. Data are expressed as $\bar{X} \pm SD$. Compared to the control group: * $P < 0.05$, ** $P < 0.01$' *** $P < 0.001$.

downward trend. After adjusting for confounding factors including gender, age, smoking, and alcohol consumption, these effects remained statistically significant (Table 3). Furthermore, participants over 60 years of age showed a decreased level of HDL-C and an increased level of TG/HDL-C (Table 4). Fluoride was found to decrease the level of serum HDL-C in the population.

4. Discussion

Fluorosis is a global public health problem, and its detrimental effects on the cardiovascular system have been widely recognized. Vascular endothelial cell injury is an early event in fluoride-induced cardiovascular disease, yet the underlying mechanism remains unclear. The current study employed in vivo and in vitro experiments to investigate the hazardous effects of fluoride on endothelial cells. Additionally, an epidemiological investigation was conducted to explore the effect of fluoride on serum cholesterol levels within the population. This study provided novel evidence for cholesterol biosynthesis involvement in fluoride-mediated endothelial injury, and explored its population-level effects on cholesterol metabolism. These findings provide a theoretical foundation for understanding the potential mechanisms by which fluoride contributes to cardiovascular diseases.

The safety of fluoride concentration in drinking water has long been a significant global public health concern. Worldwide, some regions exhibit extremely high fluoride concentrations in groundwater due to specific geological conditions, with local levels reaching as high as 20–30 mg/L, leading to severe endemic fluorosis. In response, the World Health Organization (WHO) has established a guideline value of 1.5 mg/L for fluoride in drinking water. Different countries have set their own standards based on this recommendation. For instance, the U.S. Public Health Service recommends an optimal concentration of 0.7 mg/L to maximize dental health benefits, while China's Standards for Drinking

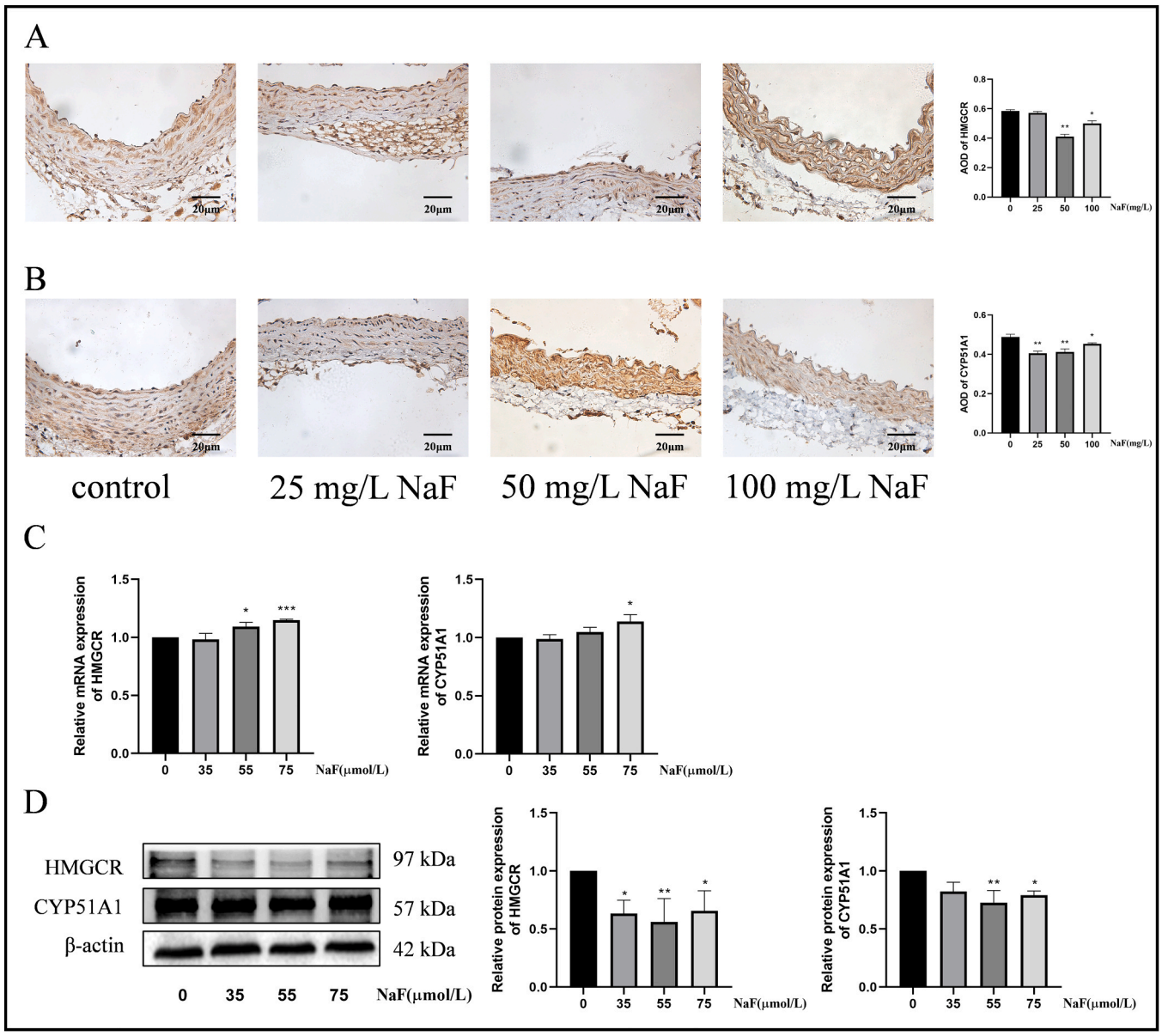

Fig. 4. Fluoride reduced the expression of HMGCR and CYP51A1. A. Relative expression of HMGCR in mouse aortic endothelial cells (Scale bar =20 μm) (n = 5). B. Relative expression of CYP51A1 in mouse aortic endothelial cells (Scale bar =20 μm) (n = 5). C. The mRNA expression of HMGCR and CYP51A1 in HUVECs induced by NaF were validated by qRT-PCR. D. Protein expression levels of HMGCR and CYP51A1in HUVECs detected by Western Blot. The protein expression level of HMGCR and CYP51A1 were evaluated by densitometric analysis and normalized by β-actin protein level. Data are expressed as $\bar{X} \pm$ SD. Compared to the control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Water Quality (GB 5749–2022) set a fluoride limit of 1.0 mg/L. With advancing research on the mechanisms of fluoride-induced physiological damage, scientific understanding has expanded beyond the toxicity of high fluoride exposure. Recent studies have found and suggested that long-term exposure to low concentrations of fluoride (even below 1.0 mg/L) may also cause potential multi-system damage. These effects extend beyond the traditional concerns for teeth and bones, gradually encompassing systems such as the immune system, nervous system, endocrine system, and reproductive development system. This reveals the more complex biological effects of fluoride and its potential health risks, prompting a more cautious global reevaluation of water fluoridation policies and ongoing scientific research.

The primary manifestation of adult fluorosis lies in the skeletal system, cardiovascular and nervous systems, specifically presenting as skeletal fluorosis. Epidemiologic studies have shown that age and living time are risk factors for skeletal fluorosis, the incidence of skeletal fluorosis increased with age (Shi et al., 2025). Similarly, studies have shown that skeletal fluorosis is more common in the age group of 71 years and older (Mohammadi et al., 2017). With the increase of age and residence time, the health risk of fluoride to the population increased significantly. The prevalence of skeletal fluorosis varies among different nationalities in the areas of drinking-water type fluorosis, which may be related to genetic factors such as genes and/or polymorphic loci (Zhang, Lv, et al., 2025; Zhang et al., 2024). Therefore, age, length of residence, and genetic factors are associated with the health risks of fluoride to the population. Children are in a critical period of growth and development, with active metabolism and organs that are more susceptible to environmental toxins. Early-life fluoride exposure (including prenatal, infancy, and childhood) may be associated with disruptions in cholesterol metabolism, endothelial dysfunction, and increased cardiovascular risk later in life (Jiménez-Córdova et al., 2019; Liu et al., 2020). There is substantial evidence that excessive fluoride exposure in childhood is

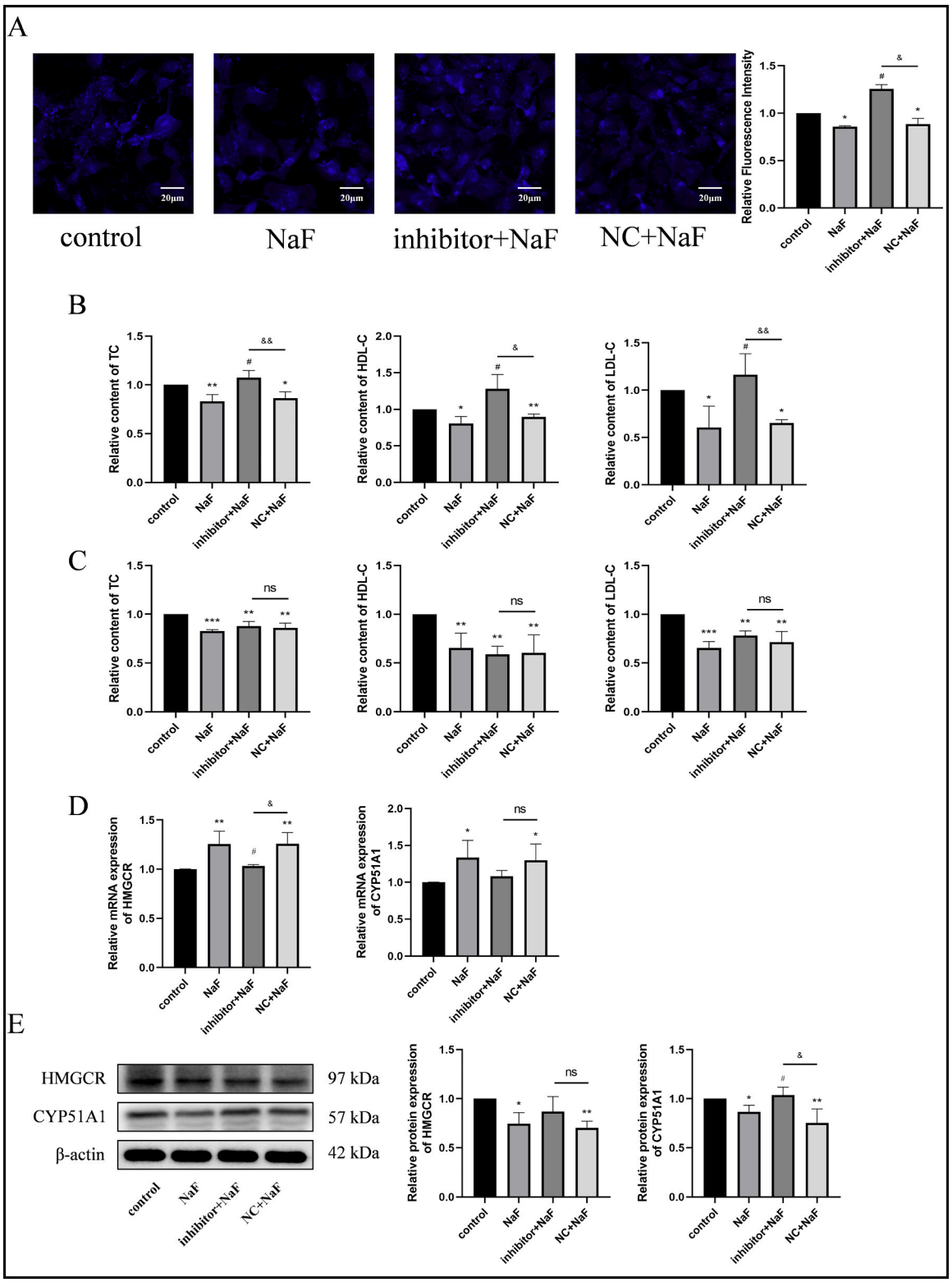

Fig. 5. Changes of cholesterol level and HMGCR and CYP51A1 expression in endothelial cells after miR-200c-3p transfection. A. After inhibiting miR-200c-3p, the cholesterol content within the membrane of HUVECs was quantitatively analyzed by Filipin staining. The results were acquired with a confocal microscope and analyzed with ImageJ. B. TC, HDL-C and LDL-C levels in HUVECs. C. TC, HDL-C and LDL-C levels in supernatant. D. After inhibiting miR-200c-3p, the mRNA expression of HMGCR and CYP51A1 in HUVECs were validated by qRT-PCR. E. After inhibiting miR-200c-3p, protein expression levels of HMGCR and CYP51A1 in HUVECs detected by Western Blot. The protein expression level of HMGCR and CYP51A1 were evaluated by densitometric analysis and normalized by β-actin protein level. Data are expressed as $\bar{X} \pm SD$. Compared to the control group: $^{*}P < 0.05$, $^{**}P < 0.01$· Compared to the NaF group: $^{\#}P < 0.05$. Compared to the NC + NaF group: $^{\&}P < 0.05$, $^{\&,\&}P < 0.01$. Abbreviations: ns, not significant.

Table 1
Primer sequences for qRT-PCR.

Primer	Sequences
HMGCR	Forward: 5′- AGTGAGATCTGGAGGATCCAAGG −3′ Reverse: 5′- TGTCCCCACTATGACTTCCCA −3′
CYP51A1	Forward: 5′- TCTTGTCCATGCTGCTGATCG −3′ Reverse: 5′- CCAAGGAATGGAATTGGGGAGA −3′
β-actin	Forward: 5′- CTCCATCCTGGCCTGGCTGT −3′ Reverse: 5′- GCTGTCACCTTCACCGTTCC −3′

Table 2
Analysis of basic demographic information of study subjects (n = 755).

Characteristics	
Age (y, means± SD)[a]	58.71 ± 9.27
Sex (n)	
Male	291
Female	464
Education (n)	
Junior	709
Senior	35
College	11
Marital status (n)	
Unmarried	8
Married	690
Divorced/widowed	57
Smoke (n)	
Yes	165
No	590
Alcohol (n)	
Yes	178
No	577
BMI (kg/m^2, means± SD)[a]	24.65 ± 3.70
SBP (mmHg, means± SD)[a]	130.93 ± 17.98
DBP (mmHg, means± SD)[a]	81.70 ± 9.99
TG (mmol/L, means± SD)[a]	2.02 ± 1.30
TC (mmol/L, means± SD)[a]	5.91 ± 1.51
HDL-C (mmol/L, means± SD)[a]	1.67 ± 0.57
LDL-C (mmol/L, means± SD)[a]	3.60 ± 1.13
TG/HDL-C[a]	1.46 ± 1.34
non-HDL-C (mmol/L, means± SD)[a]	4.24 ± 1.33
Urinary fluoride (mg/L, median, P25-P75)[b]	0.75 (0.54–1.03)

Abbreviations: BMI, body mass index; SD, standard deviation.
[a] Data were presented as mean ± SD for continuous variables.
[b] Data were presented as median (P25-P75) for continuous variables.

associated with more severe toxic effects, including neurodevelopmental toxicity and dental fluorosis (Pérez-Vázquez et al., 2021). In addition to drinking water, modern children may ingest fluoride in a number of ways: sugar-sweetened beverages, toothpaste and oral care products (Rocha-Amador et al., 2023). There may be a potential interaction between fluoride exposure and childhood obesity. It remains unknown whether fluoride exposure contributes to obesity by perturbing metabolic pathways.

Endothelial cell layer not only serves as a barrier structure but also plays a significant role in regulating vascular function, blood flow stability, and vascular reconstruction (G. Li et al., 2025). Endothelial cell injury is crucial in the progression of cardiovascular diseases and is a key step in initiating vascular injury (Xiang et al., 2023). In a cross-sectional study investigating the association between fluoride exposure and atherosclerosis, results indicated a higher prevalence of atherosclerosis in the fluoride-exposed group compared to the control group. Additionally, plasma levels of intercellular adhesion molecule-1 were significantly elevated in the exposure group (Liu et al., 2014). Further studies revealed that plasma endothelin-1 (ET-1) levels were significantly increased in individuals exposed to high fluoride, with ET-1 being closely associated with hypertension (OR = 2.22) (Sun et al., 2013). High doses of sodium fluoride also increased ET-1, intercellular adhesion molecule-1, and interleukin-6 levels in rats, with vascular endothelial cells exhibiting disorganized arrangements or even exfoliation

Table 3
Association between urinary fluoride and the change in serum lipid levels.

UF (mg/L)	Crude, β(95 %CI)[a]	*P*	Adjusted, β(95 % CI)[b]	*P*
		SBP		
continuous	3.768 (1.463, 6.073)	0.001*	3.342 (1.067, 5.618)	0.004*
		DBP		
continuous	1.355 (0.069, 2.641)	0.039*	1.276 (0.007, 2.545)	0.049*
		TG		
continuous	0.131 (−0.063, 0.325)	0.185	−0.125 (−0.319, 0.068)	0.204
		TC		
continuous	−0.102 (−0.296, 0.092)	0.305	−0.125 (−0.319, 0.068)	0.204
		HDL-C		
continuous	−0.069 (-,0.069, 0.004)	0.062	−0.076 (−0.15, −0.003)	0.043*
		LDL-C		
continuous	−0.034 (−0.179, 0.111)	0.643	−0.058 (−0.203, 0.086)	0.428
		TG/HDL-C		
continuous	0.182 (0.009, 0.355)	0.039*	0.192 (0.018, 0.367)	0.031*
		non-HDL-C		
continuous	−0.024 (−0.197, 0.148)	0.781	−0.045 (−0.216, 0.127)	0.609

β, regression oefficient; CI, confidence interval; UF, urinary fluoride.
* mean p value less than or equal to 0.05
[a] The assessments of OR and 95 % CI for every 1 mg/L increment of urinary fluoride.
[b] Adjustment: Sex, Age, Smoke, Alcohol.

Table 4
Association between urinary fluoride and serum lipid levels in different age groups.

Outcomes	< 60		≥ 60	
	β (95 %CI)	*P*	β(95 %CI)	*P*
SBP	4.544 (0.595, 8.493)	0.024*	2.869 (−0.041, 5.779)	0.053
DBP	1.72 (−0.565, 4.005)	0.14	1.135 (−0.466, 2.735)	0.164
TG	−0.115 (−0.433, 0.203)	0.476	0.131 (−0.063, 0.325)	0.185
TC	−0.206 (−0.571, 0.159)	0.267	−0.074 (−0.301, 0.153)	0.523
HDL-C	−0.017 (−0.153, 0.119)	0.809	−0.095 (−0.181, −0.009)	0.03*
LDL-C	−0.063 (−0.335, 0.209)	0.65	−0.043 (−0.213, 0.127)	0.617
TG/HDL-C	−0.011 (−0.313, 0.291)	0.942	0.262 (0.045, 0.48)	0.018*
non-HDL-C	−0.198 (−0.511, 0.132)	0.247	0.021 (−0.182, 0.224)	0.837

* mean p value less than or equal to 0.05

(Wang et al., 2019; Zhang, Wang, et al., 2025). Additionally, sodium fluoride was found to cause endothelial cell barrier dysfunction through myosin light chain phosphorylation (Wang et al., 2001). Fluoride exposure has been implicated in the progression of cardiovascular diseases, primarily through its detrimental effects on endothelial cell function and integrity. In our present study, after feeding with 25,50, 100 mg/L NaF for three months, experimental animals showed irregular shape and disordered arrangement of endothelial cells. With the increase of NaF concentration, local desquamation of endothelial cells and intimal thickening were observed, and pathological damage of the aortic intima could be observed. This result suggested that fluoride can cause endothelial cell injury, which has profound implications for vascular

health.

The abnormal increase in cholesterol is considered an important risk factor for cardiovascular diseases, but the effect of fluoride on cholesterol levels remains controversial. This study found that serum TC and LDL-C levels were decreased in mice. The levels of TC, LDL-C, HDL-C and cholesterol in endothelial cells decreased with increasing fluoride dosage. The urinary fluoride content of residents in endemic fluorosis areas was negatively correlated with HDL-C levels. These results suggested that fluoride may cause a reduction in cholesterol levels. Li et al. similarly discovered that fluoride exposure significantly decreased serum concentrations of both TC and TG in ducks (Li et al., 2021). The effect of fluoride on cholesterol levels is also influenced by the duration of exposure. Animal experiments found that chronic exposure to elevated fluoride concentrations can lead to significant increases in serum TC, HDL-C and LDL-C levels, ultimately resulting in hypercholesterolemia in rabbit models (Sun et al., 2014).

However, most studies suggest that cholesterol is a risk factor for cardiovascular diseases such as atherosclerosis and hypertension. Correspondingly, the study demonstrated that fluoride exposure significantly elevated serum levels of TC and LDL-C in rats, accompanied by marked pathological alterations in cardiac tissue (Umarani et al., 2015). Studies have shown that TG/HDL-C ratio can predict cardiovascular disease, and an increased TG/HDL-C ratio is associated with a higher CVD risk (Che et al., 2023). An increase in TG/HDL-C was observed in the present study, a result that has not been previously investigated. Non-HDL-C reflects the levels of all atherogenic cholesterol forms in the blood, which may contribute to the development of atherosclerosis (Raja et al., 2023). In the present study, non-HDL-C levels were elevated, but no significant difference was observed. In conclusion, the effect of fluoride on serum cholesterol levels remains controversial. In our present study, fluoride was found to cause a decrease in serum HDL-C levels in humans, as well as an increase in the TG/HDL-C ratio. The levels of TG, LDL-C and non-HDL-C increased, while TC and HDL-C levels decreased, although the differences were not significant. Additionally, a decrease in the serum levels of TC, HDL-C, and LDL-C was observed in mice. Further exploration is required to understand the impact of fluoride on cholesterol levels. Our study is the first to investigate the effect of fluoride on cholesterol levels in endothelial cells.

HMGCR and CYP51A1 are key enzymes in cholesterol biosynthesis, and regulating their expression and activity is crucial for controlling cholesterol synthesis. HMGCR, being the rate-limiting enzyme in cholesterol biosynthesis, plays a critical role in this process. However, the effect of fluoride on the expression of HMGCR has not been previously investigated. In the present study, it was found that the level of HMGCR in the aorta of mice was decreased. Fluoride exposure increased HMGCR mRNA level but decreased its protein level in endothelial cells. HMGCR is tightly regulated by multivalent feedback mechanisms mediated by sterol and nonsterol end products in the mevalonate pathway (Jo and DeBose-Boyd, 2022), including cholesterol intermediates like lanosterol (Chen, Ma, et al., 2019), 24, 25-dihydrolanosterol, cholesterol, its oxosterol derivatives, and the nonsterol isoprene geranyl pyrophosphate. These products reduced HMGCR activity by inhibiting its transcription and translation, accelerating its degradation and phosphorylation. CYP51A1, a member of the cytochrome P450 family (Rozman, 2000), is also a key enzyme in cholesterol biosynthesis in animals (Yin et al., 2023). In the present study, fluoride exposure was found to decrease the level of CYP51A1 in endothelial cells of mice. Fluoride exposure increased the CYP51A1 mRNA evel but decreased CYP51A1 protein levels in endothelial cells. In addition to post-transcriptional regulation mediated by miRNAs, CYP51A1 is regulated through two main pathways: sterol-dependent regulation and cAMP-dependent regulation. The feedback regulation of CYP51A1 by sterols, like other cholesterol-producing genes, is upregulated under sterol limitation and downregulated under cholesterol-rich conditions (Waterman and Lepesheva, 2005). The cAMP-dependent regulation is unique and serves as a vital factor in the germ cell-specific expression of CYP51A1 (Zhao et al., 2020). Therefore, the regulation of HMGCR and CYP1A1 levels may influence cholesterol biosynthesis, although their expression could also be regulated by other pathways. In summary, fluoride exposure may affect cholesterol synthesis in endothelial cells by regulating the levels of key cholesterol enzymes HMGCR and CYP51A1.

MiRNAs are a class of non-coding single-stranded RNAs, typically 21–22 nucleotides in length, that act as important post-transcriptional regulators by directly targeting the 3′ untranslated region of mRNAs and interfering with their translation (Chen, Heikkinen, et al., 2019). In a cross-sectional study, serum miR-200c-3p was found to be highly expressed in patients with fluorosis (Jiang et al., 2020). Focusing on the phenomenon of miR-200c-3p overexpression during the progression of fluorosis, our previous study discovered that fluoride exposure could cause endothelial cell injury by activating the Fas-associated pathway and modulating the miR-200c-3p/Fap-1 axis, which further demonstrated the crucial role of miR-200c-3p in fluorosis. In a study on non-alcoholic liver disease, the expression of miR-200c-3p, HMGCR, and CYP51A1 was found to be significantly different in high-fat diet rats compared with the control group by genome sequencing. Target gene prediction further demonstrated enrichment of miR-200c-3p-regulated genes in cholesterol biosynthesis pathways (Zhu et al., 2018).

The relationship between miR-200c-3p and cholesterol levels requires further investigation. In our present study, miR-200c-3p was found to be involved in fluoride-induced cholesterol reduction in endothelial cells. When miR-200c-3p was inhibited, fluoride-induced reductions in intracellular TC, HDL-C, LDL-C, and cell membrane cholesterol were alleviated, but there was no significant effect on extracellular cholesterol levels. These results imply that miR-200c-3p might primarily be involved in the reduction of endothelial cholesterol synthesis induced by fluoride, rather than cholesterol efflux. To further explore the role of miR-200c-3p in fluoride-induced cholesterol reduction in endothelial cells, miR-200c-3p inhibitor was transfected into fluoride-treated endothelial cells. After knocking down miR-200c-3p, the HMGCR mRNA expression and the protein expression of CYP51A1 were significantly improved. These results indicated that the inhibition of HMGCR and CYP51A1 expression by fluoride could be partially alleviated by reducing the expression of miR-200c-3p. Fluoride exposure inhibited the expression of HMGCR and CYP51A1, reducing cholesterol synthesis in endothelial cells, thereby causing endothelial cell damage. Furthermore, miR-200c-3p may have been involved in this process. However, our study was unable to confirm the interaction between miR-200c-3p and HMGCR/CYP51A1 using RNA immunoprecipitation or luciferase assays. This limitation highlights a key aspect of our research, and while we could only speculate that miR-200c-3p might regulate this process, this hypothesis requires further validation in future studies. Taken together, the present study for the first time indicates that fluoride exposure might cause a disorder of cholesterol biosynthesis in endothelial cells.

5. Conclusion

This study demonstrated that fluoride can reduce the cholesterol levels in both mouse serum and endothelial cells. Fluoride inhibited cholesterol biosynthesis in endothelial cells through the downregulation of key enzymatic regulators, HMGCR and CYP51A1. Our findings demonstrated that miR-200c-3p played a regulatory role in fluoride-induced suppression of cholesterol biosynthesis, although the molecular mechanisms underlying this process remain to be fully elucidated. Furthermore, HDL-C levels were decreased, and the TG/HDL-C ratio was increased in populations living in areas with high historical water fluoride concentrations. This study innovatively explored the effect of fluoride on cholesterol synthesis in endothelial cells and preliminarily examined the controversial issue of fluoride's impact on cholesterol levels in populations. The findings provide new insights and a theoretical basis for further research on fluoride's potential damage to the cardiovascular system.

CRediT authorship contribution statement

Yaoyuan Zhang: Conceptualization, Formal analysis, Writing - original draft. **Wei Huang:** Conceptualization, Validation, Formal analysis. **Fengya Huang:** Conceptualization, Methodology, Formal analysis. **Chao Zhang:** Validation, Methodology. **Yue Wang**: Methodology. **Mingyue Huang:** Formal analysis. **Linet Angwa:** Writing-review. **Wei Zhang:** Investigation, Methodology. **Yuanyuan Li:** Formal analysis, Methodology. **Chang Liu:** Formal analysis, Methodology. **Xiaona Liu:** Investigation, Methodology. **Zhe Jiao:** Investigation, Methodology. **Hongqi Feng:** Resources, Methodology. **Jiayong Liu:** Resources, Methodology. **Yuting Jiang:** Supervision, Project administration, Funding acquisition. **Yanhui Gao:** Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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