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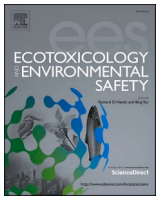

Fluoride increases the susceptibility of developmental dysplasia of the hip via increasing capsular laxity triggered by cell apoptosis and oxidative stress in vivo and in vitro

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ABSTRACT

The etiology of developmental dysplasia of the hip (DDH) is multifactorial, including breech presentation and hip capsular laxity. In particular, hip laxity is the main contributor to DDH by changing the ratio and distribution of collagens. Also, fluoride (F) affects collagens from various tissue besides bone and tooth. To investigate the association of DDH and excessive F intake, we conducted this research in lab on cell and animal model simultaneously. We established animal model of combination of DDH and F toxicity. The incidence of DDH in each group was calculated, and hip capsules were collected for testing histopathological and ultrastructural changes. The primary fibroblasts were further extracted from hip capsule and treated with F. The expression of collagen type I and III was both examined in vivo and in vitro, and the level of oxidative stress and apoptosis was also tested identically. We revealed that the incidence of DDH increased with F concentration. Furthermore, the oxidative stress and apoptosis levels of hip capsules and fibroblasts both increased after F exposure. Therefore, this study shows that excessive F intake increases susceptibility to DDH by altering hip capsular laxity in vivo and in vitro respectively. We believe that F might be a risk factor for DDH by increasing hip laxity induced by triggering fibroblast oxidative stress and apoptosis.

1. Introduction

Developmental dysplasia of the hip (DDH) encompasses a complete spectrum of disorders involving the development of the hip, from mild acetabular dysplasia without dislocation to subluxation to frank dislocation (Zhou et al., 2020). The etiology of DDH is clearly multifactorial, including genetic factors and environmental factors (Zhao et al., 2013). In particular, it is thought that the laxity of the capsule is a major contributory factor (Bakshi et al., 2017; Domb et al., 2013; Larson et al., 2015). However, hip laxity is related to DDH in several ways. It has been hypothesized that nutrition, hormones and mechanical elements could influence joint laxity (Rhodes and Clarke, 2014). Thus, alterations in hip capsule might account for the increased laxity seen in patients with DDH (Guner et al., 2018).

Furthermore, the tensile features of the hip capsule depend on the amount of collagen (Fredensborg and Udén, 1976; Montes, 1996). The fibrillar collagens that are synthesized during normal development of the rabbit hip joint have been determined, especially the ratio of types I to III collagens, which is abnormal in some cases of DDH (Skirving et al., 1984); therefore, the collagens are crucial for the strength of the joint capsule in an effort to maintain the stability of the hip.

It has been noted in a small number of human population that there is an association between DDH and the serum trace element levels, including cuprum (Cu), zinc (Zn), ferrum (Fe), magnesium (Mg) and magnum (Mn) (Guner et al., 2018). Moreover, a number of prior studies have investigated the direct and indirect relationship between the collagen biosynthesis and trace elements (Brodziak-Dopierała et al., 2013; Kaji et al., 1988; Myllylä et al., 1977; Senni et al., 2003).

On the other hand, fluorine belongs to group VII and is the ninth element of the periodic table, and it is also the 13th most abundant element, accounting for 600–700 ppm (0.06–0.09%) of the crust by mass in the Earth's crust (Armienta and Segovia, 2008). Fluoride (F) in

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drinking water has been used for the prevention of dental caries, but it appears to be a two-edged sword for human health, because it has a narrow margin of safety and might cause excessive intake, exerting a threat to the human body, such as dental and skeletal fluorosis (Kao et al., 2021). Fluorosis is endemic in more than 20 developing and developed countries in all the continents including Asia, Africa, Europe, North and South America (Herath et al., 2018). In addition, some researchers have shown that F causes changes in the soft tissue, such as renal, hepatic, neural and endocrine toxicity, which have been both widely and extensively investigated (Li et al., 2020; Lopes et al., 2020; Skórka-Majewicz et al., 2020). Moreover, Susheela proposed that the main target of F toxicity on the non-osseous tissues was the collagen protein in the early 1980 s (Susheela and Sharma, 1982). However, the association of DDH and F has not yet been reported in humans or conducted in the labs. Therefore, taking these studies together, we have hypothesized that F-induced collagen damage leads to further compromise in hip capsules, which influences DDH susceptibility.

2. Results

2.1. Animal experiments

Overall, there were 6 groups, including control group (exposed to 0 mg/L F without swaddling), 50 mg/L group (exposed to 50 mg/L F without swaddling), 100 mg/L group (exposed to 100 mg/L F without swaddling), swaddling control group (exposed to 0 mg/L F with swaddling), swaddling 50 mg/L group (exposed to 50 mg/L F with swaddling) and swaddling 100 mg/L group (exposed to 100 mg/L F with swaddling).

2.1.1. Weight variation among each group

In total, 39 rats of the F1 generation in 3 litters of the control group were collected. The mean weight on 21st day was 40.8 ± 2.8 g (range: 35.0–45.0 g). Regarding 50 mg/L and 100 mg/L groups, 47 rats with an average weight of 35.6 ± 3.4 g (range: 27.0–43.0 g) (P = 0.001) and 42 rats with an average weight of 29.4 ± 2.6 g (range: 21.5–35.0 g) (P = 0.001) were recorded (Fig. 1D and E).

For swaddling groups, the mean weight of 37.7 ± 3.8 g (range: 30.5–45.0 g) was noted in the 39 rats without F intake, whereas 45 rats of 33.5 ± 3.2 g (range: 25.0–39.5 g) (P = 0.001) with 50 mg/L F exposure and 35 rats of 30.9 ± 4.7 g (range: 23.0–39.5 g) (P = 0.001) with 100 mg/L F exposure were also analyzed, regardless of when they were compared with the control or swaddling control groups respectively.

2.1.2. Status of the dental fluorosis

Referring to the modified Dean's classification (Dean, 1942), the dental fluorosis was assessed by the enamel surface of the incisor tooth. Overall, only "questionable" grade was noted (Fig. 1F), and it was obviously that the rate of the dental fluorosis among each group was dramatically different (P = 0.001) (Fig. 1G and Table 1). However, there was no significance as regards the dental fluorosis composition in those groups exposed to F, including 50 mg/L group, 100 mg/L group, swaddling 50 mg/L group and swaddling 100 mg/L groups(P = 0.507) (Table 1).

2.1.3. Status of the hip dislocation

Owing to the absence of proximal femoral ossification on the 21st day (Fu et al., 2014), an accurate ratio of dislocations was obtained through combination of radiographs and anatomic visualization. In those groups without swaddling treatment, no hip dislocation was observed in the offspring rats regardless of exposure to the F (Table 2 and supplementary figure). Nevertheless, the incidence of hip dislocation in swaddling control group was 50.0%, which was significantly lower than in the swaddling 50 mg/L group (70.0%, P = 0.0147) and swaddling 100 mg/L group (78.5%, P = 0.0019) (Table 2 and Fig. 1H). Concurrently, the odds ratio (OR) value of hip dislocation in the swaddling 50 mg/L group was 2.33 (95% CI：1.239–4.392，χ^2 =6.89，P = 0.001) and in the swaddling 100 mg/L group was 3.67 (95% CI: 1.778–7.553, χ^2 =12.401, P = 0.009), indicating a risk of hip dislocation of 2.33 and 3.67 times respectively, compared with the swaddling control group.

2.1.4. The effect of F on the ultrastructure of the surface and cross-/longitudinal-sectioned view of hip joint capsules

Scanning electron microscopy (SEM) graphs (Fig. 2B) showed a dense and homogeneous distribution of pores on the surface of collagen fibers from the hip joint capsule of the control group. The collagen fibers were loosely packed in the F groups with simultaneous malalignment of these collagen fibers.

Under transmission electron microscopy (TEM) (Fig. 2 C), the fibrillar composition in the hip joint capsule from the control group revealed a larger fibril diameter size 58.07 ± 3.11 μm (range: 54.56 ~ 60.52) compared with the 50 mg/L 50.39 ± 6.17 μm (range: 42.91 ~ 55.49) and 100 mg/L groups 44.11 ± 4.34 μm (range: 41.18 ~ 49.10) in both cross-sectioned and longitudinal sectioned view (P = 0.036) (Fig. 2D).

2.1.5. The effect of F on the microstructure of the hip joint capsules

Histopathological changes were determined by Masson trichrome staining in transverse sections of hip joint capsule tissues after exposure to F under a light microscope. Masson trichrome stain (Fig. 2 A) showed a decrease of the thickness of the hip capsule and organization of collagen fibrils (blue regions). Particularly, the disorders of the collagen fibers were also consistent with our TEM results.

2.1.6. The effect of F on the promotion of apoptosis in hip joint capsules

To investigate the promotion of apoptosis after exposure to F, a Hoechst 33258 staining assay kit was applied. In the 50 and 100 mg/L groups, remarkably more apoptotic nuclei were stained with the Hoechst 33258 (Fig. 2F), which suggested that apoptosis occurred more severely than in the control group.

2.1.7. The effect of F on the protein levels of Col1a1, Col3a1, Bax and Bcl2

F affected the collagen composition of the hip joint capsule; in particular, the expression of collagen type I in the F exposed groups was obviously downregulated compared with the control group, whereas the expression of the Col3a1 was lower in control group (Fig. 2E). Regarding the apoptosis index, Bcl2 and Bax showed the same expression trends as the Col1a1 and Col3a1 among each group, which was also consistent with the Hoechst 33258 staining results.

2.2. Cell experiments

2.2.1. Culture and identification of primary rat fibroblasts

Primary fibroblast (PFB) cells adhered to the flasks and showed standard morphological characteristics, including fusiform, trianglular, and multiangular shapes (Fig. 3C). Moreover, the isolated cells were further identified with expression of vimentin by immunofluorescence (Fig. 3A).

2.2.2. Cell viability and morphological changes with graded F concentrations

F inhibited cell viability in a dose- and time-dependent manner. Initially, we observed the cell viability and morphological changes under a light microscope. We found the toxicity of F at the concentrations greater than 2 mM was extremely severe 24 h after F addition to the medium (Fig. 3C). Furthermore, the CCK-8 assay kit was used to quantify and delineate the viability curve at a certain exposure duration. The half inhibitory concentration (IC50) was around the concentration of 1 mM at 24 and 48 h (Fig. 3C). Hence, we adopted 1 mM with 24 h of exposure as the treatment in the cell experiment.

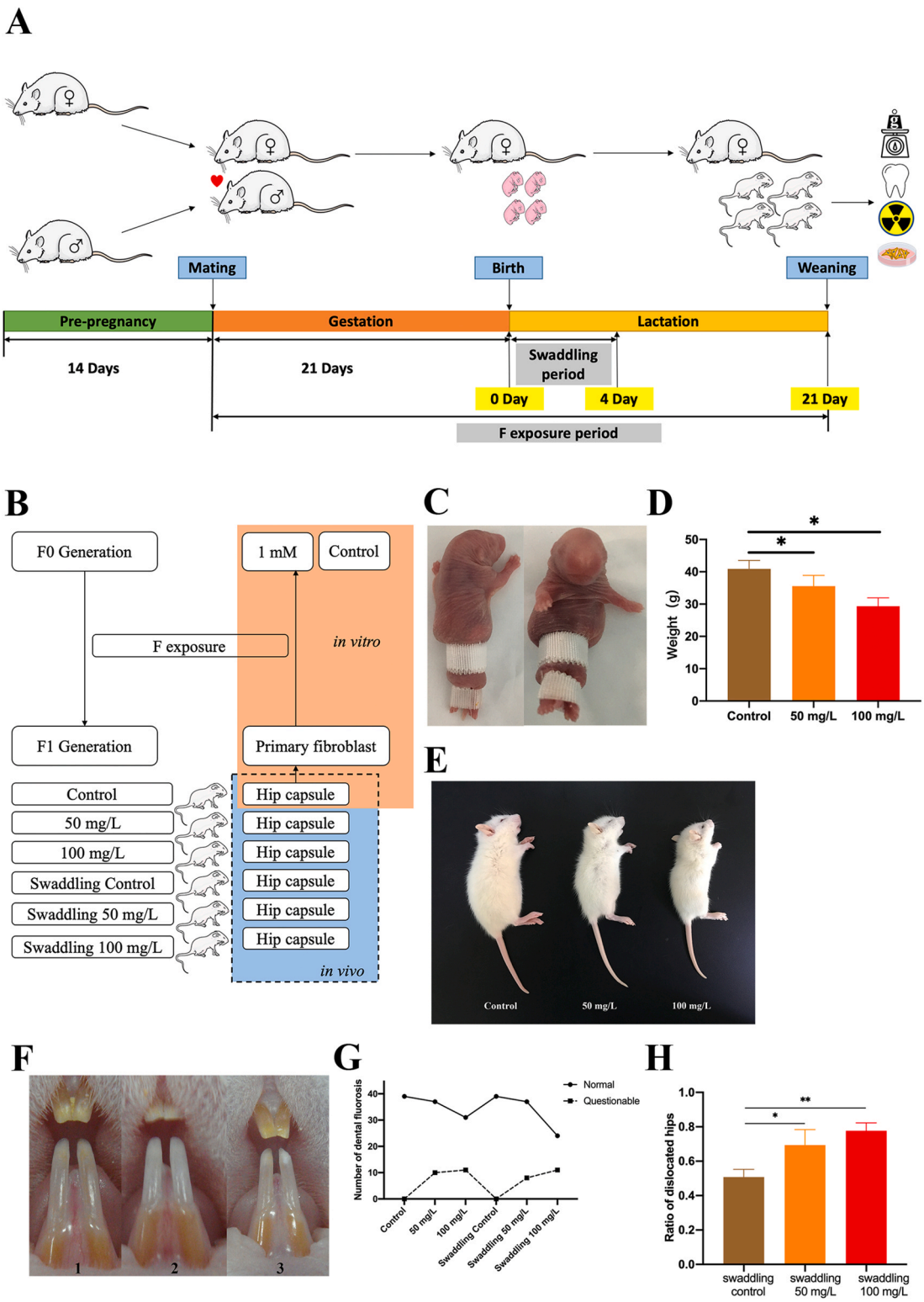

Fig. 1. A. Schedule of rat offspring exposed to F. F was administered to rats via the drinking water at concentrations of 0, 50 and 100 mg/L, and animals were exposed during periods maintained from pregnancy to birth, and ending in weaning. Immediately after treatment, all offspring were euthanized for further testing, such as weight measurement, hip radiographs, dental fluorosis evaluation and hip joint capsule tissues, which were also collected for further testing and primary cell culture. B. Algorithm and origin of samples and cells for experiment design in vivo (blue background) and in vitro (orange background). C. The swaddling treatment on the rats' hips with surgical tape. D and E. The weight variations (D) and appearance (E) of the F1 generation for each litter among the control, 50 mg/L and 100 mg/L groups (* means $P < 0.05$). F. The manifestation of a normal incisor (1) and questionable incisors (2, 3) according to the Dean's classification. G. The number of dental fluorosis among each group. H. Ratio of dislocated hips among subgroups interfered by swaddling. (* indicates significance < 0.05 compared with the swaddling control group; ** indicates significance < 0.005 compared with the swaddling control group.).

Table 1
The numbers of dental fluorosis in each group.

Groups	Normal (n)	Questionable (n)	Total	P value	
Control	39	0	39		
50 mg/L	37	10 (21.3%)	47	0.002*	
100 mg/L	31	11 (26.2%)	42	0.001*	
Swaddling control	39	0	39		0.001
Swaddling 50 mg/L	37	8 (17.8%)	45	0.005*	
Swaddling 100 mg/L	24	11 (31.4%)	35	0.001*	

Note: Chi-square test were applied totally and inter-groups (*); * indicates comparison with the control group.

Table 2
Incidence of hip dislocation in each group.

Groups	Percentage of hip dislocation	Mean	P value
Control group	0% (0/24) 0% (0/28) 0% (0/26)	0	
50 mg/L group	0% (0/30) 0% (0/34) 0% (0/30)	0	
100 mg/L group	0% (0/24) 0% (0/30) 0% (0/30)	0	
Swaddling control group	46.7% (14/30) 55.6% (10/18) 50.0% (15/30)	50% (39/78)	
Swaddling 50 mg/L group	78.1% (25/32) 71.4% (20/28) 60.0% (18/30)	70% (63/90)	0.0147*
Swaddling 100 mg/L group	75% (18/24) 75% (12/16) 83.3% (25/30)	78.5% (55/70)	0.0019*

Note: ANOVA test was applied; * indicates comparison with the swaddling control group.

2.2.3. The effect of F on the fluorescence expression of the Col1a1 and Col3a1

In the group exposed to F at 1 mM for 24 h, the protein level of Col1a1 decreased but Col3a1 was higher (Fig. 3B). These changes in collagen protein expression, such as Col1a1 and Col3a1, were consistent with their ratio trend in the previous western blot results, thus which strengthening the relationship between the animal and cellular experiments.

2.2.4. The effect of F on the ultrastructure of primary fibroblast

Fig. 4 A depicted the changes in the ultrastructure of the PFBs. Clear nuclear membrane and mitochondrial cristae and a normal structure of endoplasmic reticulum were observed in the control group. By contrast, vague nuclear membrane, dilated ER were observed in F-treated cells, especially changes in mitochondria with the breakage of mitochondrial cristae and generation of substantial vacuolated mitochondria.

2.2.5. Evaluation of MMP, intracellular ROS level, CAT, SOD, TAOC and MDA content

Our results showed that treatment with F concentration of 1 mM affect the MMP levels as compared to untreated control (Fig. 4B), indicating that F exposure induced mitochondrial integrity impairment in PFBs. The fluorescence intensity analysis of DCF showed that the level of ROS profoundly increased in the F group compared with the untreated group ($P = 0.016$, Fig. 4D), which was also observed in situ under the fluorescence microscope (Fig. 4C); collectively, these results highlighted that exposure to F induced oxidative stress by impair mitochondrial integrity and ROS generation. Regarding to antioxidants, in comparison with the control group, the activities of CAT, SOD and T-AOC didn't decrease significantly in the cells exposed to F at 1 mmol/L (Fig. 4E to G). However, the MDA content in the F group was 46.47 μM/mg, which was higher than 39.73 μM/mg in the control group ($P = 0.047$, Fig. 4H).

2.2.6. Flow cytometric analysis of the primary fibroblast

After the incubation of primary fibroblast cells with 1 mmol/L of F for 24 h, the percentages of early apoptotic and late apoptotic cells were determined. The apoptotic rates increased significantly in the F group (Fig. 3G).

2.2.7The effect of F on the mRNA level of apoptosis related genes

The expression of apoptosis-related genes, such as Bax and Bcl2, was affected by the F (Fig. 3E). Bcl2 were downregulated significantly in the F-exposed group compared with the control group ($P < 0.01$). Nevertheless, the upregulation was observed in Bax. In addition, the mRNA level of cytochrome-c (Cyt-c) also remarkably increased ($P < 0.05$), but there was no obvious decrease in Caspase-3 ($P = 0.09$).

2.2.8. The effect of F on the protein level of the apoptosis related genes

In accordance with the PCR results, those genes mediating the apoptosis pathways, including Bax, Apaf1, cleaved-caspase-3 and − 9 were highly expressed after exposure to F at 1 mM for 24 h (Fig. 3F), which revealed that the imbalance of pro- and anti-apoptosis and that subsequently activated caspase-3/9 eventually led to the apoptosis of primary fibroblasts.

3. Discussion

The first question in this study was to determine whether excessive F intake influences DDH incidence. Therefore, the establishment of an animal model with a combination of F toxicity and DDH was critical to our experimental design.

To date, the pathogenic mechanism of DDH is complicated, and the exact etiology is not yet sufficiently clarified. It is generally noted a multifactorial and polygenetic disease result from the interaction of genetic, environmental and mechanical factors (Yang et al., 2019).

DDH represents a pathology that is highly affected by the morphology of hip joint, including the creation and differentiation of connective tissue, chondrogenesis and osteogenesis. Aberrations in these pathways lead to abnormalities in the structure of connective tissue, cartilages, bones and joints. Thus, the genetic component of DDH is substantial and consists not only of many associated genes but also presents a significant challenge in the case of systemic genetic aberrations affecting the musculoskeletal system. For instance, CX3CR1, PAPPA2, Gdf5 and TEN3 were all presented as the DDH associated genes in previous studies (Chen et al., 2019; Harsanyi et al., 2020, 2021; Li et al., 2017).

Apart from genetic risk factors, previous studies have suggested that factors such as breech presentation, female gender, firstborn, oligohydramnios, a positive DDH family history and neonatal hip instability may increase DDH risk (Yang et al., 2019).

With regard to breech presentation, the fetal hip joint touches the rear of the maternal pubic symphysis, and in the case of resistance of the birth canal, the lower extremities straighten and stick in front of the chest and abdomen under labor pressure. The femoral head tends to separate from acetabula, and the joint capsule elongates, resulting in dislocation (Oh et al., 2022). The amniotic fluid supports the fetus a certain activity space from the external environment. Once the amniotic fluid volume decreases, the fetus is subject to mechanical pressure from the uterus and the abdominal wall, resulting in hip dislocation. Oligohydramnios and multifetal pregnancies, especially when complicated with other postural malformations (such as torticollis, plantar adduction, and talipes equinovarus), suggests that DDH is related to intrauterine mechanical extrusion (Xu et al., 2022). Moreover, swaddling lifestyle limits hip flexion and abduction movements and has been declared to be a strong correlation between postnatal environmental

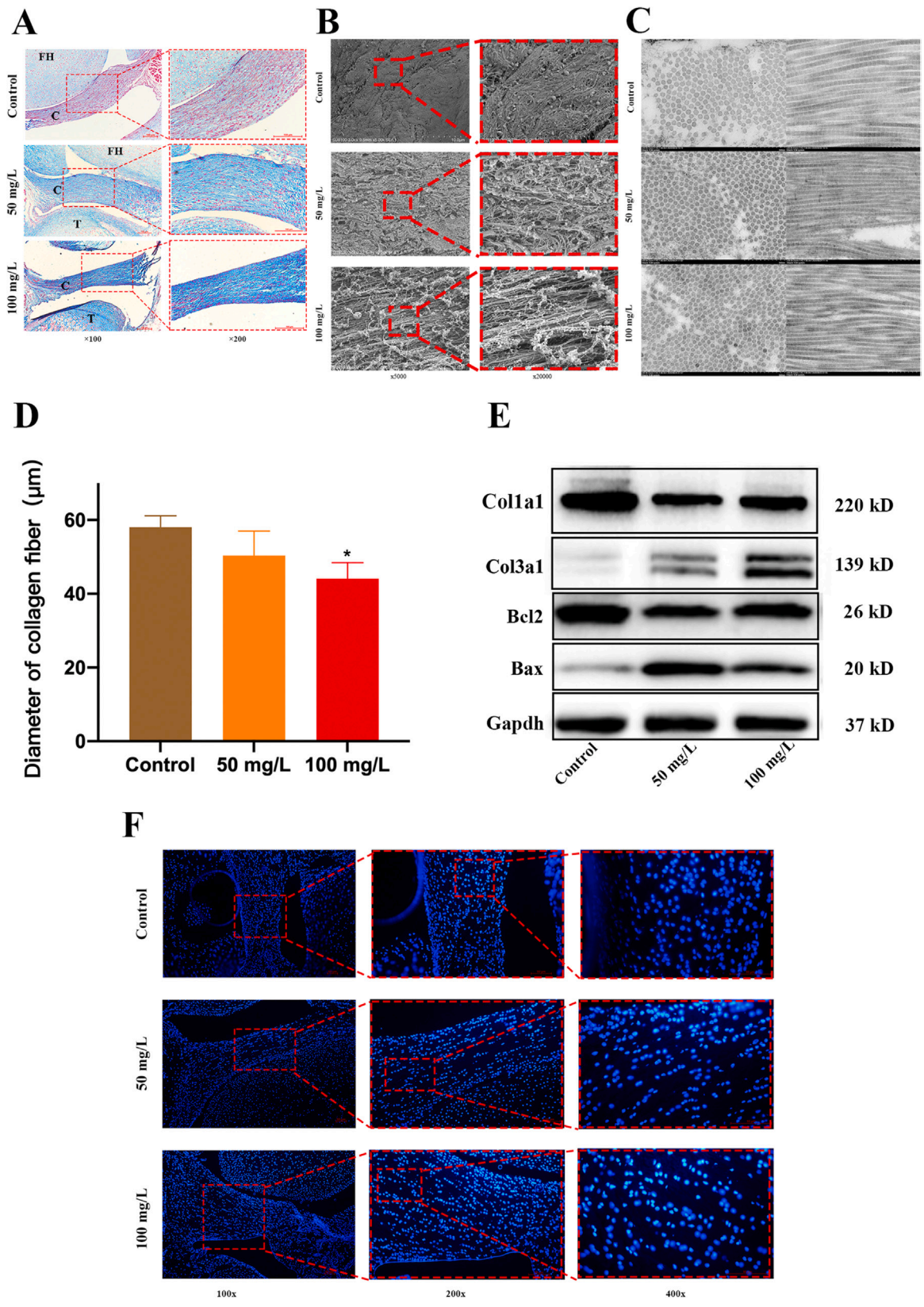

Fig. 2. A. Masson staining showing the hip joint capsule collagen fiber structure features among groups exposed to graded F. FH denotes the femoral head; C denotes the capsule; T denotes the trochanter. Scale bar: 100 μm. B. SEM images of hip joint capsules in rat offspring treated with F at different concentrations. Scale bar: 10 μm. C. TEM images showing the fibril diameter composition in longitudinal and cross-sectioned hip joint capsules among groups exposed to different F concentrations. Scale bar: 500 μm. D. Comparison of fibril diameter from hip capsule among groups, * means $P < 0.05$ when compared with control group. E. Changes in the protein levels of Col1a1, Col3a1, Bax and Bcl2 in the hip joint capsule from the control, 50 mg/L and 100 mg/L groups. F. Hoechst staining revealed more apoptotic nuclei in the 50 mg/L and 100 mg/L groups than the untreated group. Scale bar: 50 μm.

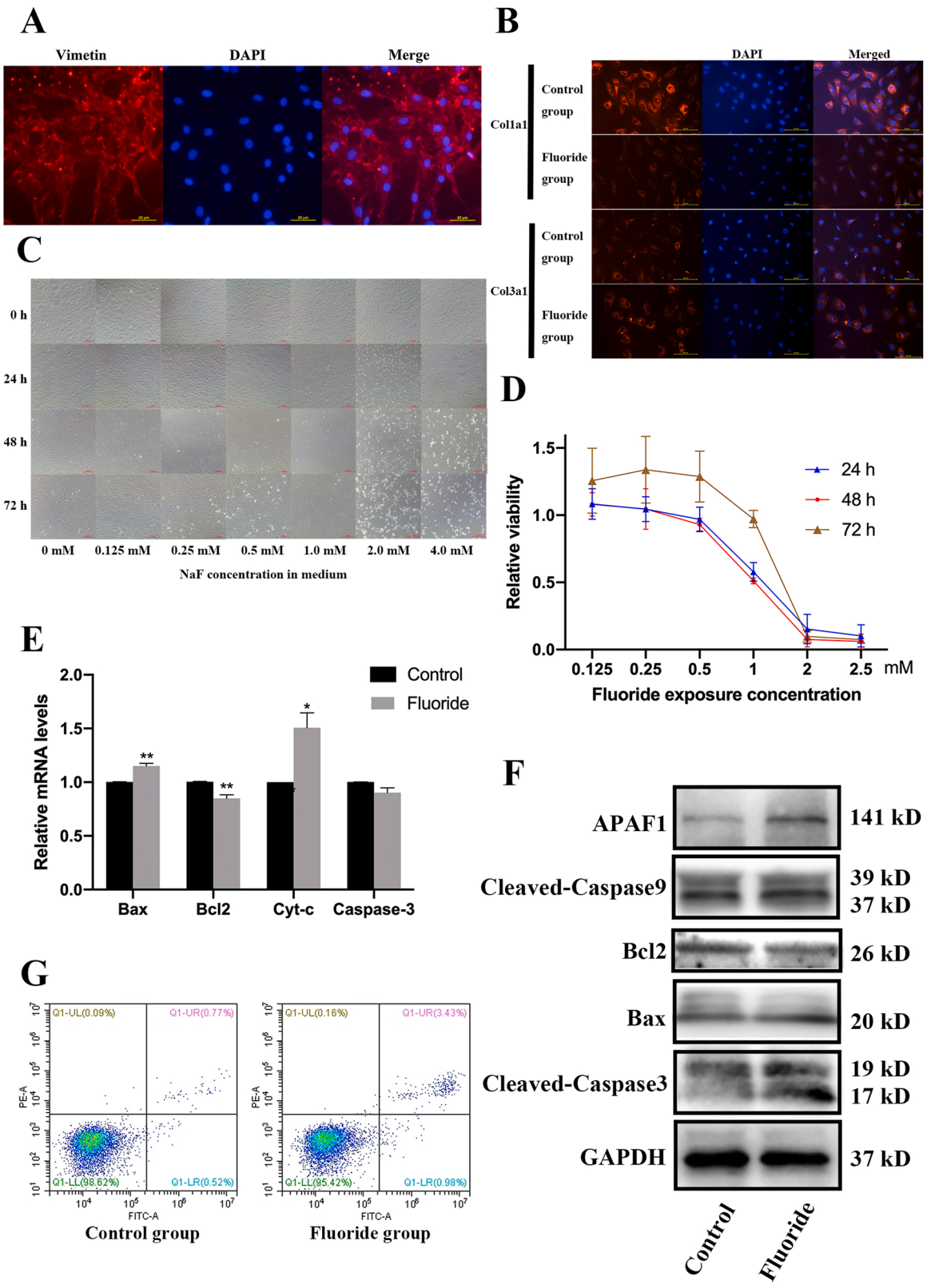

Fig. 3. A. The identification of primary fibroblast by expression of Vimetin. Scale bar: 20 μm. B. The immunofluorescence expression level of Col1a1 and Col3a1 in the primary fibroblasts between the control and F groups. Scale bar: 50 μm. C. The morphological features of primary fibroblasts under a light microscope and their changes and viability after treatment with different F concentrations. Scale bar: 100 μm. D. The relative viability of primary fibroblasts exposed to 0, 0.125, 0.25, 0.5, 1.0, 2.0 and 2.5 mM of F for 24, 48 and 72 h, respectively. E. Changes in the mRNA levels of Bax, Bcl2, Cyt-c and Caspase-3 in the primary fibroblasts after exposure to 1 mM F for 24 h (* means $P < 0.05$, **means $P < 0.01$). F. Changes in the apoptosis-related protein levels in the primary fibroblasts after 24 h of exposure to 1 mM F. G. Flow cytometric analysis of apoptosis via Annexin V-FITC staining between the control and F groups.

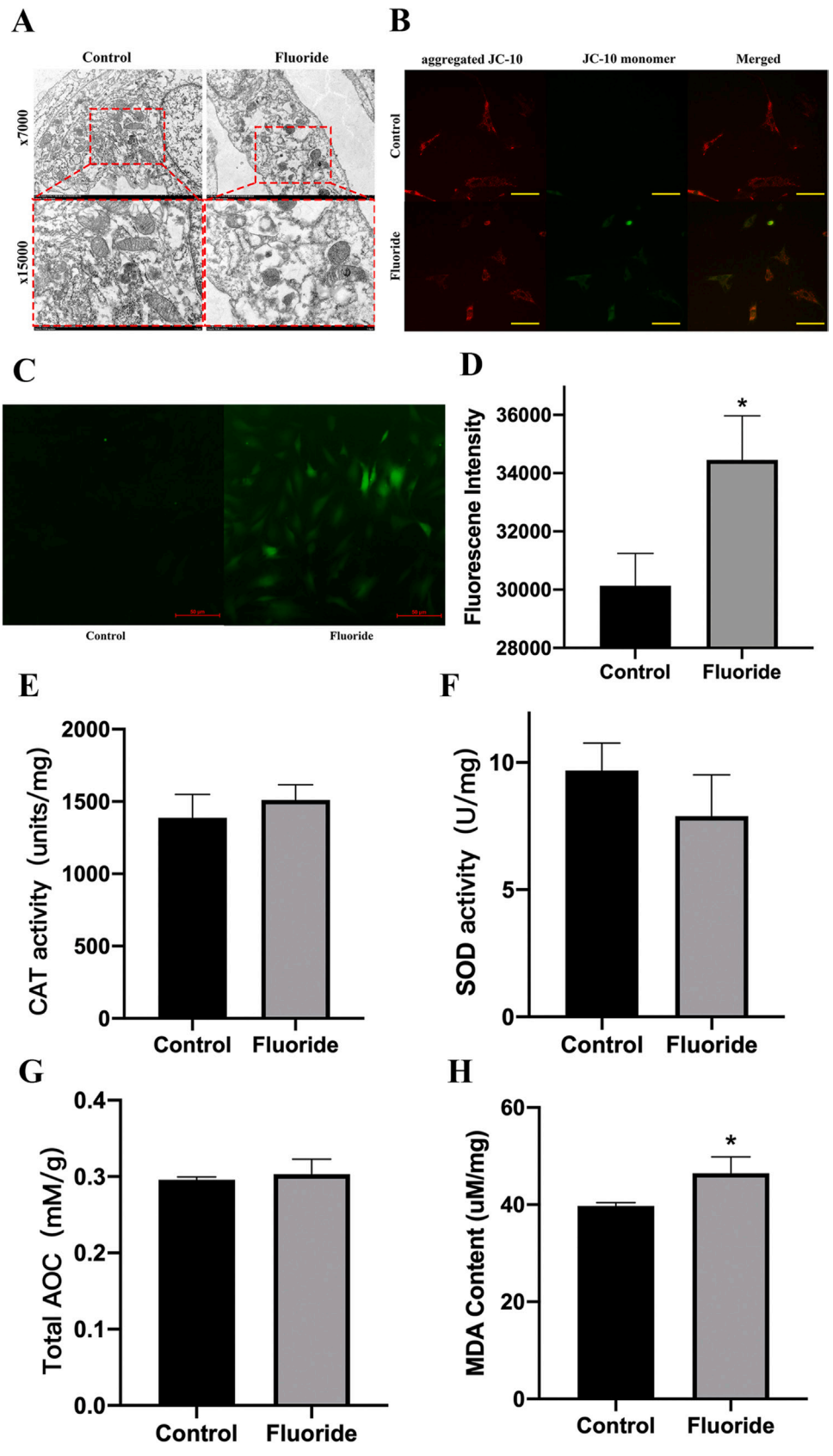

Fig. 4. A. The ultrastructural changes of capsular primary fibroblast between control and F groups. Scale bar: 2 μm. B. The mitochondrial membrane potential changes were detected between control and F groups. Scale bar: 50 μm. C and D. The oxidative stress level evaluated by detecting ROS levels via fluorescence intensity of DCF in situ (C) and under microreader (D) (* means $P < 0.05$). Scale bar: 50 μm. E~G: The antioxidants such as CAT (E), SOD (F) and TAOC (G) between control and F groups was analyzed. H. The oxidative stress level evaluated by detecting MDA content between the control and F groups (* means $P < 0.05$).

factor and DDH (Lee et al., 2022). On the other hand, the most common signs of DDH are the laxity of ligaments and the defective socket of the hip joint. Normal development of the femoral head and acetabulum is codependent; the head must be stable in the hip socket in order to form concentrically and congruently. If either component is deficient, the head becomes loose in the acetabulum, which results in joint instability, subluxation, or even persistent complete dislocation. Besides the bones, the soft-tissue structures, consisting of the labrum, capsule, and muscle, are the most important stabilizers of the hip. Furthermore, articular capsule belongs to connective tissue, which mainly consists of fibroblasts and collagen fibers. In particular, it is clarified DDH is associated with the loss of collagen fibers and fibroblasts, which may cause loose joint capsule formation (Li et al., 2021).

Owing to multiple risk factors, including genes, intrauterine fetal position and postnatal environment contribute to DDH together (Yang et al., 2019), many authors have reported the establishment of a DDH model in different species by various modalities. For example, the shape of the hip joint was profoundly changed in mice, such as the disappearance of the acetabular concavity and irregularity of femoral head, which was performed in the study by Sotiriou et al. through gene modification on *Myf5* and *Myod* (Sotiriou et al., 2019). In addition, Feldman et al. recently found that deletion of CX3CR1 and the mutation of Teneurin 3 in mouse could both cause a delay in acetabular morphology development, which was characterized by an increase in cartilage in the hip (Feldman et al., 2019) and the space between the femoral head and acetabulum (Feldman et al., 2017). Moreover, hip morphology development was also markedly affected in the branchypodism mouse, which harbors a mutation of *Gdf5* inactivation (Kiapour et al., 2018). Regarding to the intrauterine fetal position, Hashimoto et al. performed surgery on the rat fetus at E16.5 with restrained hind limb movement by 9–0 thread, and then the deformity of the hip joint was caused at birth (Hashimoto et al., 2002). On the other hand, the immobilized chick embryo was proposed as a suitable model system for the type of early-onset DDH by injecting fertilized eggs at E4 with 100 μl 0.5% decamethonium bromide, a neuromuscular blocking agent that induces rigid paralysis, to imitate immobilization treatment of the chicken fetus (Nowlan et al., 2014). In contrast to the abovementioned prenatal strategies to establish a DDH animal model, Parker et al. reported that DDH was present in 62% of newborn rats induced by intraperitoneal injection of streptococcal cell walls at dosages of 20–120 mg per body weight in the late 1980 s (Parker et al., 1989). Nonetheless, the most common postnatal method was still conducted by restricting the movement of the hind limb in a physical modality, including cast (Wei et al., 2016), tape (Ji et al., 2020) and internal metal fixator (Dodds et al., 2008). In particular, we demonstrated an increasing incidence of DDH in rats by covering a surgical tape around the hip joint in our previous study (Wang et al., 2012), which is more convenient and economical than the genetic and prenatal methods. Additionally, this modality is more beneficial to observe the effect of F on the incidence of DDH after F uptake.

With respect to F toxicity, it is conventional to establish a chronic fluorosis model by oral administration of F-containing water at different concentrations. However, the concentration of F in drinking water varies according to different agencies or organizations worldwide. For example, the European Council and World Health Organization set the maximum permissible limit of F as 1.5 mg/L, the United States Environmental Protection Agency set this limit as 4.0 mg/L in drinking water (Yadav et al., 2019), and China (Chinese Standard GB 5749–2006, 2007) set it as 1.0 mg/L. Then, based on the local standards of safe and adequate daily intake and acceptable daily intake, 50 mg/L was chosen because it reflected the human environmental exposure in the Dec et al. study on the neurotoxicity of F in the adult rat model (Dec et al., 2020). Similarly, according to the rat metabolism, Ferreira also thought that F concentrations of 10 mg/L and 50 mg/L were equivalent to the consumption of artificially fluoridated water and fluorosis endemic regions, respectively (Ferreira et al., 2021). However, 100 mg/L was adopted in the experimental design of Tian et al. because it was equal to 1/5 of the median lethal dose (Tian et al., 2019). Moreover, several studies have investigated the effect of F on various organs and systems by using F^- ion of 50 mg/L and 100 mg/L to prepare the treatment solutions, which correspond to the 110.5 mg/L and 221.0 mg/L F, respectively (Gao et al., 2020; Susheela and Sharma, 1982). Therefore, the weight and incidence of dental fluorosis were both dramatically influenced in the F groups, which supported the successful establishment of F toxicity. Then, those newborn rats subsequently underwent the straight leg swaddling treatment for the first 4 days, and the incidence of DDH in the F groups was comparably higher than the control group, but no DDH was observed in the group merely drinking F-containing water without swaddling treatment, which indicated that the F would not directly lead to DDH but might be just one of the risk factors increasing its susceptibility. However, the mechanism remains unclear and needs to be clarified in the next step.

The hip capsule functions to constrain translation between the femur and acetabulum (Muratli et al., 2004). Capsular laxity is one of the major contributors to DDH. Subsequently, the harvested hip capsule was further studied to observe its histomorphological and ultrastructural changes. The capsule in F groups under SEM and TEM was thinner and looser, meanwhile, the disorder of the alignment of the collagen fibers could also be noted. The diameter of the capsular collagen fiber from the F group was lower than that from the control group in both the longitudinal section and cross-section views. Moreover, collagen is the major constituent of connective tissue and is responsible for the tensile strength of the ligaments and the joint capsule (Grant and Prockop, 1972). Previously, Ippolito et al. reported a higher proportion of collagen fibrils in children with congenital displacement of the hip, but its diameter was smaller than in age-matched controls (Ipplito et al., 1980). Later, Skirving et al. (1984) also observed that the relative distribution of collagen types was altered in the joint capsule of children suffering congenital dislocations. Therefore, to some extent, the content of collagens indicated the mechanical property of hip joint capsule. In particular, the ratio of collagen type III and type I has been of great importance since Oda et al. found that it was increased in DDH in the 1980 s (Oda et al., 1984). However, there is still no consensus on the change in this ratio. For instance, Skirving et al. found that DDH patients had a ratio of half of the controls within batch comparisons (Skirving et al., 1984), but the collagen III/I ratio in newborns with DDH was higher than in controls in one study conducted by detecting the content and distribution of collagen in umbilical cords (Jensen et al., 1986). Apart from human population samples, the ratio of collagen III/I in canines with hip dysplasia was significantly higher than in controls (Madsen et al., 1994). Recently, several studies were performed to disclose the association of DDH and increased serum prolidase (Soran et al., 2013) or a high rate of estrogen receptors (Desteli et al., 2013) through potentially turning over the collagen III/I ratio. Hence, in the present study, collagen I and III were both estimated in animal and cellular experiments, respectively. The ratio of collagen III/I from the hip joint capsule was increased when compared with the control groups in vivo by western blotting, which was also in accordance with the in vitro immunofluorescence result. Therefore, hip laxity existed in offspring rats exposed to excessive F, which contributed to increasing the susceptibility to DDH.

Finally, we also endeavored to scratch the surface of the potential molecular mechanism regarding the association between F and DDH. It is evident that long-term intake of excessive F can cause damage to various tissues and organs besides teeth and skeleton. In particular, apoptosis induced by triggering oxidative stress has been given more attention with respect to the mechanism of F toxicity.

Apoptosis, which is known as programmed cell death, is a complex and highly regulated physiological process via gene and/or protein expression that is characterized by certain cellular morphological features, including cell shrinkage, chromatin condensation, internucleosomal cleavage of DNA, mitochondrial disintegration, and

formation of apoptotic bodies (Ying et al., 2017). There are 2 pathways induce apoptosis, including the death receptor-mediated pathways (extrinsic) and mitochondria-mediated pathways (intrinsic) (Gupta, 2001). The mitochondrial pathway is stimulated by decreasing MMP, which led to the release of Cyt-c from the mitochondria into the cytosol (Wang et al., 2019). Then, Cyt-c combines with Apaf-1 to form apoptotic bodies, resulting in the activation of caspase-9 and subsequently activation of caspase-3, which leads to the fragmentation of DNA cell apoptosis (Wang et al., 2019). Moreover, proapoptotic Bax and antiapoptotic Bcl-2 are two important proteins that are involved in mediating this signaling pathway (Gao et al., 2021). In this work, compared with the control group, the ratio of Bax/Bcl2 protein expression from the hip capsule was much higher in the groups exposed to F. There were more apoptotic nuclei stained by the Hoechst apoptosis detection assay kit, which also provided strong evidence that excessive F intake increased apoptosis in the rat hip capsule. Similarly, in the cell experiment, the protein expression levels of Apaf-1 and cleaved caspase-3 and − 9 were similarly higher in the F group.

Furthermore, in apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. Annexin V is a calcium-dependent phospholipid-binding protein with a high affinity for PS. Annexin V was conjugated to FITC, which serves as a sensitive probe for flow cytometric analysis of cell apoptosis (Wei et al., 2018). In the current study, the proportion of apoptotic cells was higher in the F group than the control group. Combined with the animal experimental results, apoptosis remarkably occurred in the hip capsule after F over-digestion.

Oxidative stress results from uncontrolled production of ROS beyond proper function of antioxidant systems (Wei et al., 2018). ROS are generated from different sources in biological systems, but the mitochondria are the main site for ROS generation in tissues (Gupta, 2001). The accumulation of ROS can change the permeability of the mitochondrial membrane and activate the intrinsic pathway, eventually causing the activation of the caspase cascade reaction (Ni et al., 2020). Compared with control group, excessive ROS were generated and MMP decreased in F group; but the antioxidants such as CAT, SOD and TAOC didn't change significantly. Despite, MDA level in PFBs exposed to the F was also higher than in the control group, which is believed to be a reliable biomarker for oxidative stress (Ma et al., 2017).

Thus, in the current study, a rat model with F exposure and DDH simultaneously was established through combination of oral administration of F-containing water and swaddling and used to investigate the changes in the capsules of the hip joints under a light and electron microscope and to further disclose the effect and potential mechanism of F on DDH through a combination of in vivo and in vitro experiments.

This study provided an experimental basis for investigating the pathomorphological damage to hip capsules mediated by excess F intake. Those findings enriched a new perspective on the etiology of DDH and provide a new scope for elucidating F toxicity from DDH.

There are several limitations of the current study. Primarily, the lack of clinical or epidemiological evidence to support F exposure as a risk factor for DDH is a major concern for this study. Despite of persistent and widely-extend literature of investigation of F therapeutic and toxic effects on skeleton, F has not been identified as a clinically important risk factor to DDH in geographic areas with high environmental F. Additionally, the results were also independently from the laboratory, it seems a plausible explanation that F has negative effect on strength of hip joint capsule because collagen is protein present in the soft tissue around hip joint. Thereby, continuous study is supposed to determine the relationship between F and the DDH in the population by epidemiologic investigation. Second, it was devoid of the corresponding result for the oxidative stress in the animal because the joint capsule of the rat hip is too small to facilitate the extensive examination; this limitation could be overcome by using larger animals such as rabbits or canines. Next, we were unable to completely and deeply elucidate the underlying pathophysiology of F-induced joint capsule laxity, and it would be better to disclose the change of mechanical strength of capsule tested by some specific sensor after F intake. In addition, F affects the development of bone and cartilage, especially the growth plate (Wang et al., 2021), which are both involved in the pathogenesis of DDH but were not comprehensively investigated in the present experiment.

4. Conclusion

F increases DDH susceptibility by inducing hip laxity, which involves fibroblast apoptosis and oxidative stress. Finally, our study suggested that F might be a risk factor for increasing susceptibility to DDH.

Ethics approval

The study was approved by the Committee on the Ethics of Animal Experiments of Shengjing Hospital, China Medical University, Shenyang, China. The laboratory animal quality certificate code was 2019PS687K.

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Materials and Methods

This section could be found in Supplementary Data.

CRediT authorship contribution statement

Weizheng Zhou: Methodology, Validation, Investigation, Writing – original draft, Visualization. **Wenting Luo:** Methodology. **Dan Liu:** Methodology. **Federico Canavese**: Writing – review & editing. **Lianyong Li**: Conceptualization, Project administration. **Qun Zhao**: Resources, Supervision, 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.

Data Availability

The authors declare that all data are available in the article file or available from the authors upon reasonable request. Source data are provided with this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113408.

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