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JNK/STAT signalling pathway is involved in fluoride-induced follicular developmental dysplasia in female mice

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HIGHLIGHTS

- F induced histological changes in the ovary.
- F induced ultrastructure damage in granulosa cells.
- F inhibited the granulosa cell proliferation.
- F interfered with the expression of JNK/STAT signalling pathway.
- F induced follicular developmental dysplasia.

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ABSTRACT

Excessive fluoride (F) intake decreases the development of potential oocytes by inducing oxidative stress and apoptosis in female mice in our previous study. This study aims to investigate the underlying mechanisms of F-induced follicular developmental dysplasia. Pathomorphological changes in the ovary tissues were observed under light and transmission electron microscopes. DNA damage and proliferation in granulosa cells were analysed by TUNEL staining and BrdU measurement. The protein expression of cell proliferation related regulatory factors including JNK, STAT3, STAT5, CDK2, CDK4, PCNA and Ki67 in the ovary tissues was measured by immunohistochemistry and Western blot analyses. Results indicated that the structure of granulosa cells in the ovary was seriously damaged by excessive F, evident by the swollen endoplasmic reticulum, mitochondria with vacuoles and nucleus shrinkage. F treatment also considerably enhanced the apoptosis and inhibited the proliferation of granulosa cells. The number of granulosa cells around the oocyte decreased after F treatment. The expression levels of STAT3, CDK2, CDK4 and Ki67 in the ovary tissues were up-regulated, and STAT5 and PCNA did not change significantly after F treatment, whereas JNK expression was down-regulated with increasing F dose. In summary, changes in the expression levels of JNK, STAT3, STAT5, CDK2, CDK4, PCNA and Ki67 in the JNK/STAT signalling pathway are involved in F-induced follicular dysplasia in the ovary.

1. Introduction

The ovary is a crucial functional reproductive organ in mammals and contains numerous ovarian follicles. The proliferation and differentiation of granulosa cells play a critical role in the initiation of primordial follicles and the normal development of follicles in the ovary (Lee-Thacker et al., 2018; Schuermann et al., 2018). Cell proliferation and differentiation are dependent on the granulosa cell cycle, and precisely regulating the cell cycle is necessary to ensure normal mitosis (Chen et al., 2017; Gebremedhn et al., 2016; Stratton and Stearns, 2017). Numerous transcriptional regulatory factors, including cell cyclin-dependent kinase (CDK), signal transducer and activator of transcription (STAT), proliferating cell nuclear antigen (PCNA) and Ki67, are involved in regulating the cell cycle to promote cell proliferation and differentiation (Bienaimé et al., 2016; Juríková et al., 2016; Phoomvuthisarn et al., 2018). A series of pathways, such as JNK/STAT signalling pathway, is also involved in regulating cell proliferation and differentiation in the cell cycle (Wang et al., 2018). Both JNK and STAT signalling

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pathways play a key role in transition from secondary to prenatal follicles and growth of prenatal follicles; adding JNK inhibitors can also affect the transformation of granulosa cells from S to G2 and further influence follicle development in the ovary (Hatzirodos et al., 2014; Oktem et al., 2011; Oktay et al., 2008).

STAT3 and STAT5, which are members of STAT families, are downstream regulatory factors of the JNK pathway and involved in regulating cell proliferation (Hatzirodos et al., 2014). STAT3 has been implicated in regulating the self-renewal and differentiation of reproductive stem cells and the control of satellite cell expansion and skeletal muscle repair (Oatley et al., 2010; Tierney et al., 2014; Wei et al., 2018). STAT5 also participates in the formation of corpus luteum and maintenance of luteal function during follicle development in the ovary (Curlewis et al., 2002). Blocking the STAT3 and STAT5 signal transduction pathways down-regulates the expression levels of cyclin-dependent kinase 2 (CDK2), cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6) at varying degrees in Hep-2 cells (Wang et al., 2016). This finding suggests that STAT3 and STAT5 regulated the expression of CDK2 and CDK4, which are the cores of regulation in the cell cycle and play a dual role in initiating DNA replication and inducing mitosis (Korzelius et al., 2011; Lu et al., 2017a; Perrot et al., 2018). In vitro experiments confirmed that the close relationship between cell proliferation and the expression of CDK2 and CDK4; increasing the expression of CDK2 and CDK4 enhances DNA synthesis and promotes cell division and proliferation, whereas decreasing the expression inhibits or reduce cell proliferation (He et al., 2018; Huang et al., 2018). CDK-mediated phosphorylation of CDK2 and CKD4 inactivates ubiquitin ligase activity by reducing affinity to PCNA and then regulates the activity and expression of the antigen (Zhao et al., 2014). CDK regulates the expression of PCNA and Ki67 by inducing DNA synthesis and is implicated in the initiation of cell proliferation. As a marker of cell proliferation, PCNA and Ki67 are usually used to assess cell cycle in hepatocytes and tumour cells (Han et al., 2018; Qiu et al., 2017; Robertson et al., 2018). Meanwhile, BrdU is employed as marker to detect cell proliferation in epithelial cells, keratocytes and endothelial cells in vitro (Shi et al., 2018). Therefore, the protein expression of JNK, STAT3, STAT5, CDK2, CDK4, PCNA and Ki67 in the JNK/STAT signalling pathway is important for the growth and maturation of follicles.

Excessive fluoride (F) intake can induce reproductive toxicity in male and female animals (Cao et al., 2016; Khatun et al., 2017; Wang et al., 2017a). In our previous studies, excessive F intake induced mitochondrial damage and oxidative stress in the ovary decreased the development potential of oocyte and interfered with the expression of MMP-9/TIMP-1 system in the uteri tissues, resulting in embryo implantation (Wang et al, 2017a, 2017b). Ovarian granulosa cells play an important role in follicle development and oocyte growth. Thus far, the involvement of the JNK/STAT pathway in F-induced ovarian follicle dysplasia by inducing the abnormal proliferation of ovarian granulosa cells has not been reported yet. Therefore, in the present study, a model of F-induced ovarian follicle dysplasia in female mice was established. Pathomorphological changes and DNA damage in granulosa cells were observed. BrdU labelled granulosa cells were also analysed. Moreover, the protein expression levels of JNK, STAT3, STAT5, CDK2, CDK4, PCNA and Ki67 in the JNK/STAT signalling pathway were measured to elucidate the molecular mechanisms of F-induced follicular developmental dysplasia in female mice.

2. Materials and methods

2.1. Animals

Forty-eight-3-week-old healthy Kunming female mice (weight 18 ± 0.5 g) were purchased from the Experimental Animal Centre of Zhengzhou University. The mice were kept in a standard animal house with ventilation and hygienic conditions and maintained at 22 °C–25 °C on a 12 h light/dark cycle.

2.2. Experimental design

After 1 week of rearing balance, the experimental mice were divided into four equal groups with 12 mice each. An F-exposed female mice model was designed. Mice in the control group were given distilled water and fed with a standard diet, whereas mice in the F groups were given with drinking water containing 25, 50 and 100 mg/L F and fed with a standard diet. At 90 days after F treatment, the mice were anesthetized with 20% urethane (ethyl carbamate) solution. Fresh ovarian tissues were removed for morphological observation and protein detection and analysis. This study design was approved by the Institutional Care and Use Committee of China (Beijing, China).

2.3. Histopathological examination of ovary tissues

Pathological sections were prepared as described in our previous report (Wang et al., 2017b). The ovary tissues were fixed in 4% paraformaldehyde for 48 h, dehydrated in different ethyl alcohol concentrations, rendered transparent in xylene, and embedded in paraffin. The paraffin samples were sliced into 5 μm and stained with hematoxylin and eosin. Histological changes in the structure of the ovary tissues were observed under a light microscope.

2.4. Ultrastructure observation of ovary tissues

The ultrathin sections of the ovary tissues were prepared using the method described by Wang et al. (2017a). The fresh ovary tissues were fixed in 1% osmic acid for 24 h, dehydrated in different concentrations of ethyl alcohol and embedded in Araldite resin. The ultrathin sections (50 nm) of the ovary tissue were cut and stained with uranyl acetate and lead citrate. Changes in the ultrastructure of the ovary tissues were observed by TEM (H-7500).

2.5. TUNEL staining

DNA damage in the ovary tissues was evaluated by TUNEL staining. Paraffin sections of the tissue were serially cut and stained with a commercially available kit (Roche) according to the manufacturer's instructions. The sections were observed using a fluorescence microscope, and green fluorescence staining in the cells was considered positive for nuclear DNA fragmentation.

2.6. BrdU measurement

BrdU measurement was conducted according to the standard protocol of the manufacturer. At 90 days after fluoride treatment, intraperitoneal injection of 50 mg/kg BrdU (Servicebio) was administered. In brief, 0.3 mL of the BrdU solution (10 mg/mL) was intraperitoneally injected once, every 2 h and four times in total. After the last injection of BrdU for 24 h, the mice were killed and the ovarian tissue was collected for immunohistochemistry assay. Granulosa cells positive for BrdU were observed under the light microscope, and brown particles suggested that cells were in the proliferation period.

2.7. Immunohistochemistry (IHC) staining

The paraffin tissue of the ovary was serially sectioned, dewaxed in xylene and rehydrated in different concentrations of ethyl