Overexpression of FTO Protects Human Granulosa Cells From Cisplatin-Induced Injury

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Research

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Abstract

Background: Premature ovarian failure (POF) is a serious problem for young women who received chemotherapy, and its pathophysiological basis is the dysfunction of granulosa cells. The RNA methylation on the sixth Natom of adenylate (m^6A) plays an important role in epigenetic regulation, and previous studies have demonstrated that the fat mass- and obesity-associated (FTO) was decreased in POF and may be a biomarker for the occurrence of POF. Furthermore, to preserve the fertility of these young females, various approaches have been used to prevent chemotherapy-induced ovarian failure, and menstrual-derived stem cells (MenSCs) have been considered as a promising treatment strategy. Here, we aimed to explore the role of FTO in the MenSCs recovering the function of injured granulosa cells.

Method: First, the cisplatin was used to make a granulosa cell injury model. Then, the menstrual-derived stem cells (MenSCs)-injured granulosa cells co-culture model and POF mouse model were established in this study to explore the role of FTO. Human ovarian granulosa cell lines (KGN) were used to explore the effect of FTO on cell proliferation and apoptosis. Furthermore, gain- and loss-of-function studies, small interfering RNA transfection, and meclofenamic acid (MA), a highly selective inhibitor of FTO, were also conducted to clarified the regulation mechanism of FTO in granulosa.

Results: MenSCs co-culture could improve the function of injured granulosa cells by increasing the expression of FTO. MenSCs transplantation could restore the expression of FTO in the ovary of POF mice. Overexpression of FTO could restore the injured cells' proliferation and decrease its apoptosis through regulating the expression of BNIP3. Down-regulation of FTO got the opposite results.

Conclusion: FTO has a protective effect, which could improve the viability of granulosa cells after cisplatin treatment by decreasing the expression of BNIP3, and it may provide new insight into the therapeutic targets for the cisplatin-induced POF.

1. Background

POF (premature ovarian failure) is defined as cessation of menstruation before 40 years, the potential etiologies including genetic, autoimmune, and iatrogenic categories^[1]. Various studies have indicated that chemotherapy could cause small follicle depletion and even exhaustion of oocytes in the ovaries of young females with cancer, then lead to POF^[2, 3]. Cisplatin (Cis-diamminedichloroplatinum-II (CDDP)) is one of the widely used and effective chemotherapeutic agents for the treatment of cancers including gynecologic malignancies^[4, 5]. However, when used at higher dosages, injury may occur. Previous studies reported that cisplatin caused POF during clinical usage and non-clinical investigation^[6], and exposure granulosa cells to cisplatin caused growth arrest, DNA damage, and increased apoptosis^[7]. To overcome these side effects, many studies and protective adjuvants have been carried out and developed^[8, 9], especially the therapeutic potential of stem cells has attracted more and more attention^[10-12]. Our

previous study has indicated that Menstrual-derived stem cells (MenSCs) have reparative effects on cisplatin-induced premature ovarian failure mice^[2]. However, the regulation mechanism is still unknown.

RNA methylation on the sixth Natom of adenylate (m⁶A), one of the most abundant modifications on RNA, has captured the imagination of researchers^[13]. m⁶A is catalyzed by “writers”, “erasers”, and “readers”. “Writers” refers to RNA methyltransferase, which is composed by METTL3, METTL14, RNA binding motif protein 15 (RBM15), Wilms’ tumor 1-associating protein (WTAP), zinc finger CCH domain-containing protein13 (ZC3H13), vir like m6A methyltransferase associated protein (KIAA1429), and others. “Erasers” is consisted of FTO (fat mass- and obesity-associated) and α -ketoglutarate-dependent dioxygenase AlkB homolog 5 (ALKBH5). “Readers” are serious of YTH-domain-containing proteins, such as YTHDF1, YTHDF2, YTHDF3, YTHDC1^[14, 15]. m⁶A modification is involved in many biological processes, such as gene regulation, translation, alternative splicing, and RNA stability^[16, 17]. Increasing evidence showed that m⁶A is involved in physiological development, and its dysregulation caused infertility. In male germ cells, down-regulation of FTO suppressed spermatogonial proliferation^[14], and YTHDF2 knockout mice are infertile and compared with littermates, YTHDF2 knockout mice have significantly smaller testes^[18]. During the female’s oocyte development, the levels of RNA methylation were decreased^[19], and YTHDF2 deficiency results in female-specific infertility^[20]. Furthermore, a previous study has demonstrated that compared to the control group, the expression level of FTO in POI (premature ovarian insufficiency) patients and mouse models was significantly lower^[21]. Thus, we wonder whether MenSCs can restore the function of the POF ovary by affecting the expression of FTO in granulosa cells, and the underlying molecular mechanism by which the FTO affects the proliferation and apoptosis of granulosa cells.

In this study, we first established the MenSCs-injured granulosa cells co-culture model and examined the expression levels of m6A members. Compared to the controls, only the expression level of FTO was decreased in the cisplatin-treated group and then reversed when co-cultured with the MenSCs. Then, POF mouse models were established, and MenSCs injected via tail vein could restore the expression level of FTO. Furthermore, FTO plasmid and siRNA were applied to explore the role of FTO in the granulosa cells. Finally, we identified that BNIP3, a member of the Bcl-2 family, was a downstream target of FTO in medicating granulosa cell proliferation and apoptosis. Based on the data, we proposed that cisplatin-induced granulosa cell apoptosis via FTO and that BNIP3 might be the downstream factor of FTO in this process.

2. Materials And Methods

2.1 Cell culture

Human ovarian granulosa cell lines KGN (Procell CL-0603) were kindly provided by *Procell Life Science Technology Co., Ltd* (Wuhan, China). Cells were culture in DMEM/F12 with 10% (FBS) in an incubator at 37°C with 5% CO₂. The medium was changed every two days.

2.2 Isolation of menstrual-derived stem cells (MenSCs)

This study was approved in advanced by the Ethical Committee of The First Affiliated Hospital of Xi'an Jiaotong University, and all the participants have written an informed content. MenSCs were isolated and cultured as our previous study described^[2]. Briefly, collecting the menstrual blood from 6 healthy women (about 25-30 years old), then transferring the blood into a 50 ml centrifuge tube, which pre-contained 10 ml of phosphate-buffered saline (PBS), 100 U/ml penicillin, 100 mg/ml streptomycin, amphotericin B (0.25 mg/ml), and ethylenediaminetetraacetic acid (EDTA) (2 mM) (Gibco, Grand Island, NY, USA). Ficoll-Paque Plus (GE Healthcare Amersham, UK) was used to separate and purify the MenSCs. Finally, cells were culture in DMEM/F12 (Hyclone, USA) with 10% fetal bovine serum (FBS) (Sijiqing, China), in a T25 flask (Corning, New York, USA) at 37°C in 5% CO₂.

2.3 establishing granulosa cell injury model and MenSCs-injured granulosa cells co-culture model

For the granulosa cell injury model, KGN was removed from the T25 flasks and seeded in a 96-well plate at a density of 1×10^4 . CDDP (Sigma–Aldrich, St. Louis, MO) was used to make the granulosa cell injury model. After overnight culture, removed the medium and replaced the fresh medium contained CDDP (0-20 μ M), and the 50% inhibitory concentration (IC₅₀) was chosen for the injury model in the later experiments.

For the MenSCs-injured granulosa cells co-culture model, we need to pre-seed the KGN (2×10^5) in a 6-well plate and incubate for 24 h. Then, the CDDP was added for another 48 h. Finally, a 6-well transwell insert (pore size 4 μ m) was applied, and the MenSCs were added in the up chamber (the numbers of MenSCs: KGN were 3:1) for another 48h culturing at 37°C with 5% CO₂.

2.4 Experimental animals, POF model establishment

To establish the POF models, C57BL/6 female mice, aged 6-8weeks, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All experimental procedures were approved by the Ethical Committee and the Institutional Animal Care and Use Committee of Xi'an Jiaotong University. All animals were housed in a relatively stable environment with a cycle of lights on at 8 a.m. and off at 8 p.m., maintained room temperature (21-25°C) with water and food available ad libitum.

To establish the POF model, mice were injected with CDDP (2mg/kg) intraperitoneally for 7 consecutive days, according to our previous study^[2].

2.5 Experimental design

2.5.1 experiment 1

To demonstrated the protective effects of MenSCs transplantation on POF ovarian function, the POF mice were divided into two groups and administered either treatment of MenSC (passage 3-5, 200 μ l cell

suspensions containing 2×10^6) by tail vein injection or an equal volume of the medium. Sacrifice the animals after 7 days of treatment, and collect the serum and ovaries

2.5.2 experiment 2

To explore the effects of FTO inhibitor MA on POF ovarian function, the mice were randomly divided into 4 groups: (1) DMSO group (dimethyl sulfoxide, a solvent of MA); (2) MA group; (3) cisplatin group; (4) cisplatin + MA group. The POF models were established as mentioned earlier. The MA was administered simultaneously with cisplatin injection at the dose of 10mg/kg. After 7 days of treatment, the animals were sacrificed and the serum and ovaries were collected.

2.6 Enzyme Linked Immunosorbent Assay (Elisa)

To assess the ovarian function, we collected the blood samples by eyeball extracting. Then, samples were coagulated for 2 h at room temperature and centrifuged at 3000r/min for 10 minutes at 4 °C to acquire the serum samples. Then, mice Elisa panel kits (Meimian Biotechnology, Jiangsu, China) were used to measure serum oestradiol (E2), FSH, and AMH levels according to the kit instructions.

2.7 Hematoxylin-eosin staining

The ovarian tissues were embedded in paraffin and then cut into 5- μ m serial section. Then the tissue sections were rehydrated by incubating in xylene and subjecting to an alcohol gradient of 100%-70%. After deparaffinization, the section was stained with hematoxylin and eosin (HE).

2.8 Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described^[22]. The ovarian tissues of mice were fixed in 4% formaldehyde and paraffin-embedded using standard procedures. First, consecutive 4-mm sections were cut, deparaffinized, rehydrated, and retrieved the antigen in sodium citrate solution (pH 6.0) for 20 minutes. Followed by 3% hydrogen peroxide and 1% bovine serum albumin blocking for 30 minutes, separately. Then, tissue sections were incubated with anti-FTO (Abcam, Cambridge, USA; 1:150) overnight at 4 °C and a biotinylated secondary antibody (1:1000, Santa, Cruze) for another 30 minutes. Finally, a 3, 3-diaminobenzidine tetrahydrochloride (DAB) (Beyotime, Wuhan, China) substrate kit was applied to detect peroxidase reactivity.

2.9 Plasmids and small interfering RNA transfection

Overexpression and inhibition of FTO were achieved by transfection of pCAG-FTO (Miaoling, Wuhan, China) and FTO small interfering RNAs (siRNAs) (Ribobio, Guangzhou, China) separately. The empty vector for the plasmids and siRNAs were also included. The transient transfection of KGN was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In brief, a total of 2×10^5 cells per well were seeded into a 6-well plate. Until 60-80% confluence, 3.0 μ g vector DNA or 50nM siRNA were transfected by 3.0 μ L Lipofectamine 2000 per well for

6 h, then the medium was switched to fresh DMEM/F12. The overexpression and knockdown efficiency of the target gene was measured by qRT-PCR and western blotting.

2.10 Western blotting analysis

Cells were harvested after 48 h incubation. The total protein was extracted by RIPA buffer, which was pre-added with protease inhibitor cocktail and PMSF. BCA kit (Beyotime, China) was used to detect the protein concentration. 30 µg of protein was subjected to 10% SDS - PAGE gels and then transferred polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in 0.1% TBST (Tris – HCl buffer saline with 0.1% Tween – 20) contained 5% skimmed milk. Then, these membranes were incubated with corresponding primary antibodies in 4°C overnight. The primary antibodies are as follows: FTO (1:1000, ab126605 Abcam, USA), BNIP3(1:1000, ab109362 Abcam, USA), BAX (1:1000, 50599-2-Ig, Proteintech, China), Bcl-2(1:1000, 12789-1-AP, Proteintech, China), β-actin(1:1000, 66099-1-Ig, Proteintech, China). The next day, these membranes were incubated with peroxidase - conjugated secondary antibodies at room temperature for 1 h. Finally, these membranes were visualized by enhanced chemiluminescence (ECL) procedure.

2.11 RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from cells was isolated using RNAiso Plus (Takara, Japan) according to the reagent instruction. Genes primers used in this study were as follows: FTO (forward, 5' – CTTACCAAGGAGACTGCTATTTTC – 3', reverse, 5' – CAAGGTTCTGTTGAGCACTCTG – 3'), METTL3 (forward, 5' – TTGTCTCCAACCTTCCGTAGT – 3' reverse 5' – CCAGATCAGAGAGGTGGTGTAG – 3'), METTL14 (forward, 5' – ACCTTGGAAGAGTGTGTTTACGA – 3', reverse, 5' – TGTGAGCCAGCCTTTGTTCT – 3'), WTAP (forward, 5' – TGTGCTGTGTAAGGGCATTCGTACTCATGC – 3', reverse, 5' – ACTGGGCAAACCTTGGCAGTCATAAACCCAC – 3'), ZC3H13 (5' – AAAGGAGGTTTTACCAGAAGTG – 3', reverse, 5' – CGCTTCGGAGATTTGCTAGAC – 3'), KIAA1429 (5' – AAGTGCCCCTGTTTTTCGATAG – 3', reverse, 5' – ACCAGACCATCAGTATTCACCT – 3'), RBM15 (5' – AGCCGCGAGTATGATACCG – 3', reverse, 5' – GCCCGAAGAATTTTTGGTGCTC – 3'), YTHDF1 (forward, 5' – AACAATGAGGGCGAACCAGT – 3', reverse, 5' – GACACACTGGAGCTGACCAA – 3'), YTHDF2 (forward, 5' – TAGCCAACCTGCGACACATTC – 3', reverse, 5' – CACGACCTTGACGTTTCCTTT – 3'), YTHDF3 (forward, 5' – TGACAACAAACCGGTTACCA – 3', reverse, 5' – TGTTTCTATTTCTCTCCCTACGC – 3'), YTHDC1 (forward, 5' – TCATCTTCCGTTCTGTGCTGT – 3', reverse, 5' – TACAGGGAGCGTGGACCATA – 3'), GAPDH (forward, 5' – AAAATCAAGTGGGGCGATGCT – 3', reverse, 5' – TGGTTCACACCCATGACGAAC), BNIP3 (forward, 5' – TGAGTCTGGACGGAGTAGCTC – 3', reverse, 5' – CCCTGTTGGTATCTTGTGGTGT – 3'), BAX (forward, 5' – AGTGGCAGCTGACATGTTTT – 3', reverse, 5' – GGAGGAAGTCCAATGTCCAG – 3'), Bcl-2 (forward, 5' – TTCCACGCCGAAGGACAGCG – 3', reverse, 5' – GGCACTTGTGGCGGCCTGAT – 3'). Real-time quantitative PCR was performed using SYBR Premix ExTaq™ (Takara) on a StepOne Real – Time PCR System (7300 Real – Time PCR system, Applied Biosystems, USA). The reaction conditions were as follows: 95°C for 30s, 40 cycles at 95°C for 15s, 60°C for 30s, and extension at 72°C for 60s. The target genes relative expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method.

2.12 Cell counting kit-8 (cck-8) assay

The cell proliferation was determined by using the Cell Counting Kit-8 (CCK8) assay. In brief, 1×10^4 HTR8/SVneo cells per well were plated into a 96-well plate. Then, cells were cultured at 37°C with 5% CO₂ for 24 h. Next, 10ul of CCK8 reagent (Dojindo, Kumamoto, Japan) and 90ul culture medium were added to per well and incubated at 37°C for 2 h. Finally, measured the absorbance at 450nm at 24 h, 48h, and 72 h.

2.13 EdU labeling

To assess the KGN proliferation, 5-ethynyl-2-deoxyuridine (EdU) Apollo567 kit (Ribobio Co., Ltd.) was used. First, KGN (1×10^4) were cultured in a 96-well plate and incubated with EdU (1:1000, 50 µmol/L) for 2 h. Then, KGN was fixed with 4% formaldehyde for 30 minutes at room temperature, followed by incubation with glycine (2mg/ml) and PBS for 5 minutes respectively. Next, permeabilized the cells in 0.5% Triton X-100 (100 µL) for 10 minutes and added the Apollo® reaction cocktail (100 µL) for 30 minutes under light-shading conditions at room temperature. Finally, 4',6'-diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei for 30 minutes at room temperature. After 3 times washes with PBS, the images were acquired using fluorescent microscopy (Olympus, Japan).

2.14 Flow cytometry analysis

Annexin V – FITC Apoptosis Detection Kit (BD Biosciences, CA, USA) was used to assess the KGN cell apoptosis. Briefly, KGN was harvested and washed in ice-cold PBS. Then, resuspended the cells in binding buffer (200 µL) and added the Annexin V – FITC (5 µL) and propidium iodide (5 µL) in it at room temperature for 10 minutes (in darkness). Finally, added 300 µL binding buffer to each tube and the percentage of apoptotic KGN cells were analyzed using flow cytometer (FC 500, MCL, CA).

2.15 Statistical analysis

All the experiments were carried out in triplicate. The data were represented as mean ± SEM and statistical analysis was conducted with GraphPad Prism 5. Student's t-test and one-way ANOVA were applied to compare the two experimental groups and multiple groups, respectively. P value < 0.05 was considered statistically significant.

3. Results

3.1 Effects of cisplatin on KGN cells proliferation and apoptosis

To evaluate the effect of cisplatin on KGN cell viability, a cck-8 assay was conducted. The results showed that cisplatin (0-20 µM) induced cell death in a dose-dependent and time-dependent fashion (Fig 1A) and the 50% inhibitory concentration (IC₅₀) was treated the cells with cisplatin 10 µM for 48 h. Then, we detected the mRNA and protein expression levels of BAX and Bcl-2 and found that when the concentration of cisplatin was less than 10 µM, with the increase of cisplatin concentration, the ability of

cell apoptosis was enhanced, while the anti-apoptosis ability was weakened. When the concentration of cisplatin was more than 10 μ M, we got the opposite results (Fig1B-F). We used the KGN cell culture with cisplatin 10 μ M for 48 h as the granulosa cell injury model.

3.2 Treatment with MenSCs or the conditioned medium improved injured granulosa cell function

To examine the effect of MenSCs on injured granulosa, a cell co-culture model was used to mimic the MenSCs-injured granulosa interplay in vitro. Compared with the group that cisplatin treatment only, MenSCs co-culture cell's anti-apoptosis ability enhanced. As shown in fig (2A-E), the mRNA and protein expression levels of Bcl-2 improved, while the BAX decreased. The conditioned medium was collected from the MenSCs, when the MenSCs reached 80% confluence, switched the medium to serum-free medium, and cultured for another 48h. The cck-8 assay showed that the conditioned medium could promote the injured granulosa viability (Fig 2F).

3.3 MenSCs transplantation increased weight and improved injured ovarian function

To assess whether MenSCs transplantation could improve the function of ovarian injured by cisplatin, the evaluation indexes including weight, number of follicles, and serum sex hormone as our previously described^[2]. As shown in fig 3D, the body weight increased significantly in the MenSCs transplantation group than in the POF group. Furthermore, compared with the POF group, the level of E2 and AMH were higher, while the level of FSH was lower in the MenSCs group (Fig 3E-G).

3.4 MenSCs could restore the expression of FTO in injured granulosa and POF mice

We analyzed the expression levels of m6A related element and found that compared with the cisplatin group, the mRNA expression levels of METTL3, METTL14, WTAP, ZC3H13, KIAA1429, RBM15, ALKBH5, YTHDF1, YTHDF2, YTHDF3, YTHDC1 were all decreased in the MenSCs co-culture model group, while the expression of FTO increased (Fig 3A). The protein expression level of FTO was also detected (Fig 3B-C). We further detected the expression levels FTO in animal models and found that, in the POF group, the FTO expression was decreased, and the MenSCs transplantation could reverse it (Fig 3H).

3.5 Overexpression of FTO could reverse the effects of cisplatin on granulosa cells proliferation and apoptosis

First, to explore the effect of FTO on granulosa cells viability, FTO was transient transfection into granulosa cell, resulting in increased protein and transcript level of FTO, when compared to those transfected with negative controls (NC) (fig 4A). Cck-8 and EdU assay showed that overexpression of FTO resulted in a significant increase in the cell proliferation rate (Fig 4L-M). qRT-PCR (Fig 4B-C), western blotting (Fig 4D-G), and flow cytometry – V/PE (Fig 4N-O) staining showed that FTO could decrease granulosa cells apoptosis, resulting in the expression level of BAX decreased, while the repression level of Bcl-2 increased.

Then, we further investigated the effect of FTO on injured granulosa cells. Compared to the cisplatin-treated group, ectopic expression of FTO resulted in its up-regulation and restored the cisplatin-induced down-regulation of FTO in granulosa cells (Fig 4H-K). In addition, cck-8 and EdU assay showed that restoration of FTO expression prevented granulosa cells from their decrease in the proliferation induced by cisplatin treatment (Fig 4L-M). The rate of cell apoptosis was also tested, and the results showed that restoration of FTO decreased cell apoptosis, the expression levels of BAX and Bcl-2 also returned to the level of the NC (Fig 4N-O).

3.6 Down-regulation of FTO aggravate cisplatin induced apoptosis

The effect of FTO knockdown and co-treatment of si-FTO and cisplatin were also examined in this study. Transfection efficiency was confirmed by qRT-PCR. The mRNA level of FTO substantially decreased in the si-FTO group compared to the si-NC group (Fig 5A). After 48 h transfection of si-FTO, the cell proliferation rate was detected by cck-8 and EdU assay. As shown in (Fig 5L-M), the proliferation rate decreased significantly in the si-FTO group. Simultaneously, flow cytometry – V/PE staining showed that silence of FTO in granulosa cells promoted cell apoptosis (Fig 5N-O). FTO, BAX, Bcl-2 expression levels were also detected by both qRT-PCR and western blotting. The expression of FTO and Bcl-2 decreased significantly in the si-FTO group, while the expression of BAX increased (Fig 5A-G).

Furthermore, we investigated the effect of si-FTO on injured granulosa cells. The results showed that the protein expression level of FTO and Bcl-2 in the si-FTO and cisplatin co-treated group were lower than the cisplatin-treated only group, and the expression level of BAX was higher (Fig 5H-K). The cck-8, EdU, and flow cytometry – V/PE staining assay showed the same results (Fig 5L-O). These results demonstrated that down-regulation of FTO aggravated the apoptosis of injured granulosa cells.

3.7 FTO promoted granulosa cells proliferation and attenuated its' apoptosis may by targeting BNIP3

To verify BNIP3 as a downstream target of FTO, the mRNA and protein expression levels were detected in granulosa cells. Compared to the NC group, overexpression of FTO decreased both mRNA and protein levels of BNIP3 in normal granulosa cells (Fig 6A-B). In the injured granulosa cells, we found that up-regulation of FTO could reverse the expression levels of BNIP3 to the NC group, which was increased induced by cisplatin (Fig 6B-C). However, down-regulation of FTO got the opposite results (Fig 6E-G).

3.8 The inhibitor of FTO (MA) promoted cisplatin-induced cytotoxicity in granulosa cells

Granulosa cells were treated with different concentrations of MA (0, 20, 25, 30, 35, 40, 45, 50, 55, 60 μ M) for 48 h to determine the optimum treatment concentration of MA. As shown in the cck-8 assay, we choose the 30 μ M for the following experiment, which was the highest dose showing no significant effect on cell death (Fig 7A).

The cells and animals were divided into 4 groups, including the control group, MA treated group, cisplatin-treated group, and cisplatin + MA treated group, to explore the effect of MA on the cisplatin-induced injury. Results showed that compared to the control group and MA group, the cisplatin-treated group, and

cisplatin + MA treated group cell proliferation rate decreased significantly (Fig 7B). Furthermore, the cisplatin + MA treat group aggravated the cell injury than cisplatin-treated only (Fig 7C-G). H&E staining showed that MA could promote the cisplatin-induced injury in the ovary (Fig 7H).

Discussion

POF is a serious and complicated disease, which incidence reached 1/1000 under 30 years old, 1/250 under the age of 35 years old, and 1/100 under the age of 40 years old in women^[23, 24]. The cellular mechanisms of POF are diverse, and many studies have demonstrated that the granulosa cell apoptosis increase leading to the follicular atresia would be the main cause^[25-27]. Many factors could promote granulosa cell apoptosis, such as chemotherapy^[3, 6], hormones, reactive oxygen species (ROS), growth factors, Bcl-2 family members^[28], and FTO (a member of N6-methyladenosine)^[21, 29]. Moreover, chemotherapy and radiation are the common treatment for cancer today. The side effects are serious, especially for young women, which could cause the reduction of follicle numbers on different levels and lead to a menopausal state with ensuing infertility^[30].

Previous studies before have demonstrated that human menstrual blood stem cells (MenSCs) could recovery the ovary function and decrease the granulosa cell apoptosis induced by chemotherapy agents^[2, 10, 12, 31], however, the regulatory mechanisms are still unknown. In this study, we first established granulosa injured cell model and MenSCs-injured granulosa co-culture model and found that MenSCs could decrease granulosa cell apoptosis induced by cisplatin, and the conditioned medium from MenSCs could promote injured granulosa cell proliferation. The previous study has demonstrated that overexpression of FTO could decrease the cisplatin-induced cell apoptosis in acute kidney injury^[32], and in POI patients and mouse models, the expression level of FTO decreased significantly than in control groups^[21, 29]. So, we wonder whether MenSCs promote injured granulosa cell proliferation and decrease its apoptosis through up-regulation of FTO. The results showed that compared to the control group, the FTO expression decreased in the cisplatin treatment group, while in the MenSCs-injured granulosa co-culture group, the expression level of FTO increased. In addition, we established the POF mouse model and MenSCs transplantation, the results showed that the FTO expression decreased in the POF group, while in the MenSCs transplantation group, the FTO expression increased.

To better understand the role of FTO in granulosa cells' viability, gain- and loss-of-function studies were employed. The flow cytometry assay revealed that ectopic expression of FTO could attenuate cisplatin-induced apoptosis. Cck-8 and EdU assays showed restoration of FTO expression prevented granulosa cells from cisplatin-mediated changes in cell proliferation. While knockdown of FTO in pharmacology (MA) or genetic (siRNA) could promote the cisplatin-induced apoptosis in granulosa cells.

At molecular levels, there are two pathways involved in granulosa cell apoptosis, death receptor, and mitochondrial pathways^[33]. In the mitochondrial pathways, pro-apoptotic protein BAX could initiate apoptosis and accelerate follicular atresia^[34]. While Bcl-2 could act antagonistically on BAX and prevent

cell apoptosis^[35]. Then we further detected the expression levels of BAX and Bcl-2. The qRT-PCR and western blotting showed that in the cisplatin treatment group, the expression level of Bax increased, while the Bcl-2 decreased. Overexpression of FTO in the cisplatin group could recovery the Bax and Bcl-2 to the NC group. However, downregulation of FTO in the cisplatin group could promote the Bax expression and decrease Bcl-2 expression. In summary, molecular and functional analyses confirmed that FTO plays a positive role in cisplatin-induced cell injury.

We further explored the downstream working mechanism of FTO. BNIP3, a member of the Bcl-2 family^[36], was a target of FTO in breast cancer that could mediate cell proliferation and apoptosis^[37]. A previous study has demonstrated that BNIP3 was involved in the cisplatin-induced cell apoptosis^[38]. We wonder whether BNIP3 acts as a target of FTO participated in cisplatin-induced granulosa cells apoptosis. In our study, overexpression of FTO decreased BNIP3 expression both in mRNA and protein levels. And silencing FTO promoted its expression. Furthermore, ectopic expression of FTO in cisplatin injured granulosa cells could attenuate the increase of BNIP3 induced by cisplatin. While silencing the FTO expression in injured granulosa cells got the opposite results.

Previous studies have demonstrated that autophagy is involved in cisplatin-induced apoptosis in various cancers, such as pancreatic cancer^[39], lung cancer^[40]. In hair cell-like HEI-OC1 cell, downregulation of FTO could reduce reactive oxygen species (ROS) accumulation, inhibit apoptosis and the cisplatin-induced excessive autophagy, then protect and improve the viability of the HEI-OC1 cells^[41]. BNIP3, a downstream target of FTO^[37], could act as an autophagy-related protein^[42] and interact with LC-3 through the LC-3-interacting region to promote autophagy^[43]. Such as in ovarian cancer cells, cisplatin-induced cellular autophagy was dependent on BNIP3^[44], and in the lung cancer cell, hypoxia could augment cisplatin-induced autophagy by suppressing the BNIP3 death pathway^[45]. We speculated that, in POF, overexpression of FTO inhibited cisplatin-induced granulosa cell apoptosis through BNIP3 mediated autophagy. We will explore this issue in our future studies.

There are many defects in this study. Admittedly, it would be more convincing if the expression of FTO could be showed in ovary samples of POF patients and normal donate. However, we have no access to collect the ovary samples due to the limitation in clinical treatment and ethics requirements. The previous study has reported that cisplatin-induced injured granulosa cells could promote bone marrow-derived mesenchymal stem cells (BMSCs) migrated to them. And BMSCs could reduce injured cells' apoptosis both in vivo and vitro. However, in vivo, the migrated BMSCs did not locate in the follicles and corpus lutea^[6]. We guess some factors must be secreted from the stem cells to protect the injured granulosa cells. In this study, when the MenSCs co-cultured with injured granulosa cells, the cells apoptosis decreased. Further, we found the expression of FTO increased in the injured cells. However, which factors secreted from the MenSCs promoted the FTO expression is still unknown.

Conclusion

In summary, our studies described that MenSCs could increase the expression of FTO to attenuate the cisplatin-induced granulosa cell apoptosis. And the upregulation of FTO in granulosa cells could decrease cisplatin-induced apoptosis by inhibiting the expression of BNIP3. Inhibition of FTO can further increase granulosa cell apoptosis induced by cisplatin. FTO may play a protective role in cisplatin-induced injured granulosa cell apoptosis.

Abbreviations

POF: Premature ovarian failure

m⁶A: RNA methylation on the sixth Natom of adenylate

FTO: fat mass- and obesity-associated

MenSCs: menstrual-derived stem cells

MA: meclofenamic acid

CDDP: Cis-diamminedichloroplatinum-II

RBM15: RNA binding motif protein 15

WTAP: Wilms' tumor 1-associating protein

ZC3H13: zinc finger CCCH domain-containing protein13

KIAA1429: vir like m6A methyltransferase associated protein

ALKBH5: α-ketoglutarate-dependent dioxygenase AlkB homolog 5

POI: premature ovarian insufficiency

IHC: Immunohistochemistry

PVDF: polyvinylidene difluoride

ECL: enhanced chemiluminescence

IC50: 50% inhibitory concentration

NC: negative controls

ROS: reactive oxygen species

Declarations

Ethical Approval and Consent to participate

This study was approved in advanced by the Ethical Committee of The First Affiliated Hospital of Xi'an Jiaotong University, and all the participants have written an informed content.

All experimental procedures were approved by the Ethical Committee and the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

Consent for publication

Not applicable.

Availability of supporting data

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Xinyuan Yang: conceived the study; Rongli Wang: designed and performed the experiments, collected and analyzed the data, and wrote the manuscript.

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Figures

Figure 1

The effect of cisplatin on granulosa cells proliferation and apoptosis. (A) a cck-8 assay was used to measure the effect of cisplatin on granulosa cell viability. Cisplatin inhibits granulosa cell proliferation rate in a dose-dependent and time-dependent manner. (B-F) qRT-PCR and western blotting showed that when the concentration of cisplatin was lower than 10 μ M, with the increase of cisplatin concentration, the expression of Bax increased while the expression of Bcl-2 decreased. However, when the concentration of cisplatin was higher than 10 μ M, we got the opposite results. Data are shown as the mean $\pm$ SD from three independent experiments. β -actin served as the loading control. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by Student's t test. CCK-8: cell counting kit-8). $n=3$.

Figure 2

Effects of MenSCs and conditional medium (CM) on injured granulosa cells apoptosis and proliferation in vitro. (A-B) qRT-PCR and (C-E) western blotting indicated that MenSCs co-culture with the injured granulosa cells could improve the injured granulosa cells' anti-apoptosis ability by increasing the expression of Bcl-2 and decreasing the expression of BAX at the transcriptional and protein levels. (F) A cck-8 assay was used to measure the effect of condition medium (CM) obtained from the MenSCs on the in injured granulosa cells' proliferation. The results showed that the CM could promote the injured granulosa cell viability significantly. β -actin served as the loading control. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). $n=3$.

Figure 3

MenSCs restored the expression of FTO in injured granulosa cells and POF mice. (A-C) qRT-PCR and western blotting results showed that FTO expression was decreased in injured granulosa cells, and MenSCs co-culture could restore it. (D-G) POF mice models and MenSCs transplantation were established, and changes in the body weight, serum levels of E2, FSH, and AMH in different groups were shown. (H) H&E staining showed the morphological and histopathological change of the ovary after different treatments. IHC results showed the FTO expression was decreased in the POF group compared to the controls, while MenSCs transplantation could restore it. Scale bars in the HE staining represents 500 μ m, while in the IHC staining represents 150 μ m. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). $n=3$.

Figure 4

Overexpression of FTO attenuated cisplatin-induced granulosa cell apoptosis and promoted its proliferation. (A) The transfection efficiency of FTO was measured by qRT-PCR. (B-C) The mRNA expression of Bcl-2 and BAX in transfected granulosa cells was assessed using qRT-PCR and normalized to GAPDH. (D-G) The protein expression of FTO, BAX, and Bcl-2 was determined by western blotting with β -actin as a loading control in different groups. (H-K) Western blotting results showed that overexpression of FTO in injured granulosa cells could restore the cisplatin-induced change of FTO, Bcl-2, and BAX. (L-M) EdU labeling and cck-8 assay indicated that cells viability was inhibited in cisplatin-induced injured cells. However, transfection of FTO in injured cells, the viability was increased. (N-O) Flow cytometry assays were performed to determine the apoptotic rate in different groups. The results showed that cells' apoptotic rate was increased in the cisplatin-induced injured cell, while co-transfection of FTO could restore it. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). $n=3$.

Figure 5

Down-regulation of FTO promoted injured granulosa cells apoptosis. (A) The transfection efficiency of si-FTO was measured by qRT-PCR. (B-C) The mRNA expression of Bcl-2 and BAX in transfected granulosa cells was assessed using qRT-PCR and normalized to GAPDH. (D-G) The protein expression of FTO, BAX, and Bcl-2 was determined by western blotting with β-actin as a loading control in different groups. (H-K) Western blotting results showed that inhibit FTO expression in injured granulosa cells could enhance the cisplatin-induced change of FTO, Bcl-2, and BAX. (L-M) EdU labeling and cck-8 assay indicated that cells viability was inhibited in cisplatin-induced injured cells, and co-transfection of si-FTO in injured cells could promote its effects. (N-O) Flow cytometry assays were performed to determine the apoptotic rate in different groups. The results showed that cells' apoptotic rate was increased in the cisplatin-induced injured cell, and co-transfection of si-FTO could promote it. (*P < 0.05, **P < 0.01, ***P < 0.001). n=3.

Figure 6

Overexpression of FTO attenuated injured granulosa cells' apoptosis by downregulating the expression of BNIP3. (A) Compared to the NC group, the BNIP3 mRNA level was decreased in the FTO group. (B) The western blotting result showed that overexpression of FTO could reverse the expression level of BNIP3 induced by cisplatin. (C) qRT-PCR showed that downregulation of FTO increased the expression level of BNIP3. (D) The western blotting showed that downregulation of FTO promoted the expression level of BNIP3 induced by cisplatin. β -actin served as the loading control. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). $n=3$.

Figure 7

MA promoted cisplatin-induced cytotoxicity in granulosa cells, and cisplatin-induced injury in the ovary. (A) granulosa cells were incubated with different concentrations of MA for 48 h, the cell viability was measured by cck-8 assay. (B) granulosa cells were incubated with selected cisplatin in the presence or absence of MA for 48 h, and the cell viability was measure by cck-8 assay. (C-G) The western blotting showed that MA increased the cisplatin-induced BAX and BNIP3 expression and decreased the cisplatin-induced FTO and Bcl-2 expression. (H) HE staining suggested MA promoted cisplatin-induced injure in the ovary. (*P < 0.05, **P < 0.01, ***P < 0.001). n=3.