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Original Research

miRNA-146 Protects Against Oxidative Stress Induced Ovarian Dysfunction by Suppressing OX-LDL/ROS-Dependent NF-κB Signaling Pathway

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Abstract

The purpose of this study was to determine the role of up-regulation of microRNA (miRNA)-146 expression in alleviating oxidative stress-induced ovarian dysfunction by inhibiting Oxidative Low Density Lipoprotein (OX-LDL)/Reactive Oxygen Species (ROS)- dependent Nuclear Factor (NF)-κB signaling pathway.

In vivo studies, we established mouse models of OX-LDL induced ovarian dysfunction and tested the changes of Follicle-Stimulating Hormone (FSH) and Estradiol (E2) under OX-LDL. Mouse ovarian granulocytes were transfected with miRNA-146 mimics or negative controls or inhibitors, and then treated with OX-LDL. In these studies, miRNA-146 significantly increased granulosa cells viability and decreased granulosa cells apoptosis and ROS levels in OX-LDL treated BALB/c mice.

In the present study, we found that 50-200μg/mL OX-LDL dose-dependently enhanced ROS levels, induced granulocellular apoptosis, and up-regulated the expression of oxidative stress-related genes (TLR-4, IL-6, and TNF-a). Western blotting demonstrated that OX-LDL could not only activate the NF-κB signaling pathway, but also stimulate the expression of TLR-4, IL-6, and TNF-a protein. In addition, OX-LDL significantly increased ROS expression, and miRNA-146 inhibited ROS expression. In addition, up-regulation of miRNA-146 markedly increased the activity of ovarian granulosa cells and decreased the apoptosis of ovarian granulosa cells in OX-LDL treated mice. Meanwhile, up-regulation of miRNA-146 increased estrogen levels and decreased FSH levels in OX-LDL treated mice.

In conclusion, this study revealed that up-regulation of miRNA-146 expression can alleviate ovarian dysfunction by negatively regulating the expression of OX-LDL and ROS, which may shed light on the potential molecular mechanism by which up-regulation of miRNA-146 expression may reverse ovarian dysfunction by inhibiting the OX-LDL/ ROS-dependent NF-κB signaling pathway.

Keywords: Primary ovarian insufficiency; miRNA-146; Oxidized low density lipoprotein; Reactive oxygen species; Nuclear factor-κB

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Introduction

Premature Ovarian Failure (POI) is a common disease that severely affects female reproductive function and even leads to lifelong infertility, affects mental and physical health, and causes great distress to individuals and families. Women under 40 years old account for 1-2%, and young women under 30 years old account for 0.1% [1]. POI usually refers to amenorrhea in women before 40 years of age, with elevated serum Follicle Stimulating Hormone (FSH) and decreased Estrogen (E2) levels [2,3]. Loss of ovarian function leads to amenorrhea and atrophy of sexual organs, and inhibits follicles, growth and development [2]. POI also includes Premature Ovarian Failure (POF), which is the final stage of POI development [3]. The etiology of POI is still unknown, and its pathogenesis and pathogenesis is very complex, which has not been clarified at present. Oxidative stress and Oxidized Low-Density Lipoprotein (OX-LDL) may affect redox status and/or redox sensitive signaling pathways and gene expression, change proteins and DNA, and promote apoptosis [4]. In recent years, results of in vitro, animal models and clinical studies have shown that oxidative stress is also related to human reproductive system diseases [5]. In POI, inflammation reaction and oxidative stress affect the quality of follicles and lead to ovarian dysfunction [6]. Therefore, inhibiting inflammatory response and oxidative stress and protecting ovarian function from damage are effective therapeutic strategies for POI.

MicroRNAs (miRNAs) are single-stranded non-coding RNA molecules composed of 18~22 nucleotides that are involved the occurrence and development of diseases by binding specifically to the target mRNA, it causes the target mRNA to degrade or inhibit its translation process, thus promoting the development of diseases. They are important natural gene regulatory molecules [7]. miRNA-146 has been confirmed participates in regulation of innate immune, including inflammation and oxidative stress[8]. Moreover, Lingbo et al., (2020) demonstrated OX-LDL) and Reactive Oxygen Species (ROS) existence on ovarian granulosa cells from human preovulatory follicles, and the OX-LDL-dependent activation of ROS and NF-κB can induce ovarian granulosa cells death by apoptosis [9]. Furthermore, OX-LDL can act directly on ovaries, including follicular components such as the theca and granulosa cells or oocytes, which may occur in oocytes of follicles under oxidative stress. In the preovulation follicles, oxidative stress appears to be caused by Oxidized Low-Density Lipoprotein (OX-LDL).

Nuclear factor-κB (NF-κB) is an early transcription factor that is ubiquitous in a variety of cytoplasm and participates in the regulation of a variety of inflammatory responses [10]. ROS is a signal transduction molecule produced by cell metabolism or exogenous factors, and can participate in cell apoptosis, gene expression, signal transduction and other physiological functions of the body [11]. It is well known that oxidative stress and inflammation in the development of reproductive system disease is crucial, and the NF-κB signaling pathway is an important chronic inflammatory signaling pathway. OX-LDL is thought to be a key factor in inducing oxidative stress.

It was also found that embryonic, follicular, and ovarian granulosa cells increased ROS expression and oxidative stress induced OX-LDL [12]. In addition, granulosa cells respond to OX-LDL, and the expression levels of interleukin (IL) 6 and TNF-a rare elevated [13]. miRNA-146 can directly regulate TLR4/NF-κB signaling pathway, thereby inhibiting OX-LDL activity and playing anti-inflammatory and antioxidant stress roles [14]. However, the aim of this study was to understand the potential molecular mechanism by which miRNA-146 protects against OX-LDL-induced ovarian dysfunction. We constructed and verified the protective effect of miRNA-146 suppressing OX-LDL/ROS-dependent NF-κB signaling pathways by OX-LDL-induced ovarian dysfunction of POI models. Besides, we also determined whether OX-LDL could directly induce granulose cells apoptosis in BALB/c mice, and detected the changes of FSH and E2 in mice under the effect of OX-LDL. Our goal is to provide a novel therapeutic target for POI.

Materials and Methods

Animals

Female BALB/c mice (8 weeks; 18–22g) were obtained from the Animal Laboratory of Sun Yat sen University. The mice were fed in an environment with temperature of 22±2°C, humidity of 50±5%, light of 12 hours and darkness of 12 hours, and adaptive feeding for one week. All animal experiments were approved by the Animal Experiment Ethics Committee of Sun Yat Sen University (NO.SYSU- IACUC- 2020-B1236).

Establishment of Mouse Premature Ovarian Failure Model

According to references, Katharina et al., (2013) [15] and Ying Liu et al., (2019) [16], POI mouse model was established by injecting OX-LDL (100mg/kg), (n = 20). The establishment of the model was carried out by the Animal Laboratory of Sun Yat sen University. The mice were through the caudal vena cava injection with OX-LDL (100mg/kg) for 7 days. After 21 days, blood samples were collected in dry test tubes without coagulant to obtain the serum. The concentration of Estradiol (E2) and FSH in blood samples was tested by Chemiluminescence.

Induction of POI in mice by OX-LDL-induced and injection of miRNA-146 -mimics Female BALB/c mice (n = 6) aged 8 weeks were purchased from the Animal Laboratory of Sun Yat sen University. The mice were randomly divided into the following two groups (6 mice/group): the miRNA-146 mimics group, which was intravenously administered 100μmol/L through the caudal vena cava injection for 7 days. And the Scramble miRNA group, which intravenously administered 100μmol/L of Scramble miRNA through the caudal vena cava injection for 7 days. After 21 days, blood samples were collected in dry test tubes without coagulant to obtain the serum. The concentration of estradiol (E2) and FSH in blood sampls was tested by chemiluminescence.

Isolation of Mice Granulosa Cells and OX-LDL Treatment

According to references [15,16], mice ovarian granulosa cells were obtained from BALB/c mice (n=6) of the Animal Laboratory of Sun Yat sen University (Guangzhou, China). The female BALB/c mice ovaries were quickly dissected under sterile conditions and put into pre-cooled PBS to remove the surrounding tissues and surface capsule. Under the anatomical microscope, the follicles were punctured with a syringe needle to release the mice ovarian granulosa cells into DMEM-F12 medium, which were blown and dispersed into a single suspended cell in a centrifugal tube. Second, the ovarian granulosacells were incubated at 37°C, 5% CO_2 for 24h, after which 1mg/ml 0.25% trypsin and 0.02% EDTA were added. Subsequently, the ovarian granulosa cells were incubated in an 37°C, 5% CO_2 for another 60 min and filter them with a 200 mesh stainless steel cell sieve. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KgaA), 100U/ml penicillin G (Shanghai, China),

100μg/ml streptomycin (Shanghai, China) and 2mM glutamine (Guangzhou, China). OX-LDL was purchased from Shanghai (Shanghai Bioengineering Co., Ltd, China). Ovarian granulosa cells were cultured in CO_2 incubator (37°C, saturation humidity, 5% CO_2). Under serum-free conditions for 24, the granulosa cells were treated with OX-LDL under different conditions (50, 100, 150, 200μg/mL).

Cell Transfection

miRNA-146 mimics (50nmol/L), miRNA-146 inhibitors (50nmol/L)), negative control (NC), and siRNA NF-κB-specific targeting were all purchased from Shenggong Gene Company (Shanghai, China). Cell transfection was performed with Lipofectamine 3000 transfection reagent in strict according to the manufacturer's instructions (Chagan, Germany). All transfection cells with 50nmol/L of miRNA-146 mimics or 50nmol/L of miRNA-146 inhibitors for 24 hours were collected for further study.

MTT Assay

The cell viability was analyzed by 3 – (4,5-dimethylthiazole-2-yl) – 2,5- diphenyl- 2-tetrazole ammonium bromide (MTT) colorimetry. In short, miR-146 transfected granulosa cells were inoculated at a density of 1×10^3 cells/well, and then the cells were cultured with OX-LDL for 24 hours. After rinsing twice with Phosphate Buffered Saline (PBS), 10μl MTT solutions was added to each well with a final concentration of 5mg/mL. The culture dish was cultured at 37°C for 10 min and 4h, then 150μl of dimethyl sulfoxide was added, and the absorbance was determined after shaking the plate for 10min. Each experiment was repeated three times.

Apoptosis Assay

The apoptosis of ovarian granulosa cells was analyzed using terminal Deoxynuc-Leotidyl Transferase dUTP Nick-End Labeling (TUNEL) assay (Roche, Germany). For each sample, eight visual fields were randomly selected. The apoptotic index was calculated for 100 ovarian granulosa cells by dividing the number of apoptotic ovarian granulosa cells by the total number of ovarian granulosa cells.

ROS Assay

The expression level of ROS in ovarian granulosa cells was detected by using probed with 10μM Dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime Biotechnology, Shanghai, China) dissolved in serum-free medium at 37°C for 30 min after being treated with different concentrations of OX-LDL. The fluorescence was measured at 488 nm excitation/525 nm emission by the laser confocal scanning microscope (LCSM, Leica, Wetzlar, Germany).

ROS in follicular fluid was detected by enzyme-linked immunosorbent assay (Shanghai Enzyme-linked Biotechnology Co., Ltd).

The Expression Levels of miRNA-146 in Mice Blood Samples and Ovarian Granulosa Cells were detected by PCR

After the ovarian tissue was ground with liquid nitrogen, miRNA was reverse- transcribed and amplified, cDNA synthesis kit and SYBR Green Polymerase Chain Reaction (PCR) Master Mix kit were used, and U6 was used as the control of miRNA. All operations were carried out on ice to avoid RNase pollution. The Applied Biosystems ABI 7500 (USA) system was used for real-time fluorescence quantitative PCR. The reaction conditions were as follows: predenaturation at 95°C for 10 min, denaturation at 95°C for 15s, annealing at 55°C for 15s, 35 cycles. The relative expression of miR-146 was calculated by 2-ΔΔ CT. The primer sequence is shown in (Table 1).

Table 1: The sequence of primers.

Primer name	Primer sequence
miR-146-F	5′-ACCAGCAGTCCTCTTGATGC -3′
miR-146 R	5′-GACGAGCTGCTTCAAGTTCC -3′
U6-F	5′-CTCGCTTCGGCAGCACA-3′
U6-R	5′-AACGCTTCACGAATTTGCGT-3'≠

TNF-a, IL-6, TLR4, and NF-κB Protein Expression in Ovarian Granulosa Cells were Detected by Western Blot

Ovarian granulosa cells from ovarian tissues were treated by sonication, the lysates of cells were centrifuged and the proteins were separated by SDS-PAGE and then transferred to Immobilon NC membranes (Millipore, USA). After 2h 5% skim milk blockage with Tris- buffered saline at room temperature, the membrane was incubated with primary antibodies against TLR4, NF-κB, IL- 6, TNF-a, and β-actin overnight at 4°C. Then, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase for 1h at 37°C. Blots were imaged using a Bio-Rad imaging system (Bio-Rad, USA).

Statistics

SPSS 19.0 was used to analyze the data which are presented as the mean ± Standard Error of the Mean (SEM). One-way analysis of variance with a Bonferroni's post hoc test was performed for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The Expression of miRNA-146, E2, FSH and ROS in POI Model

Firstly, in this experiment, the levels of miRNA-146 expression were significantly decreased in POI model, compared with the control group, detected by qRT-PCR analyzed (Table 2). And the concentration of Estradiol (E2) and folicular Stimulating Hormone (FSH) was determined by using Chemiluminescence analyzed, respectively. The concentration of E was significantly reduced and FSH significantly enhanced in POI model (Table 2). Then, the levels expression of ROS in follicular fluid were measured by ELISA assay and significantly increased in POI model (Table 2). The effects of miRNA-146 mimics for 7 days of tail vein injection in mice, and the expression level of FSH, E2 and ROS after 21 days is shown in (Table 3); the concentration of E2 was significantly increased and FSH and ROS expression significantly decreased in POI model.

Table 2: Comparison of miRNA-146, E2, FSH and ROS levels between the two groups in POI model ($\bar{x}\pm s$).

Groups	n	miRNA-146	E2 (pmol/L)	FSH (IU/L)	ROS (ng/ml)
Control	6	3.06±0.32	139.69±25.87	12.76±2.63	12.92±4.72
POI	6	1.66±0.19	63.14±17.18	61.43±19.22	56.67±23.84
t	-	20.127	19.286	17.795	13.669
P	-	0.000	0.000	0.000	0.000

Table 3: Comparison of miRNA-146, E2, FSH and ROS levels between the two groups by injection of miRNA-146 mimics in POI model ($\bar{x}\pm s$).

Groups	n	miRNA-146	E2 (pmol/L)	FSH (IU/L)	ROS (ng/ml)
Control	6	3.06±0.32	137.69±23.91	13.82±2.42	12.92±4.72
POI	6	2.63±0.49	94.72±20.16	26.26±8.15	16.73±6.28
t	-	6.578	11.255	12.786	9.616
P	-	0.09	0.014	0.017	0.015

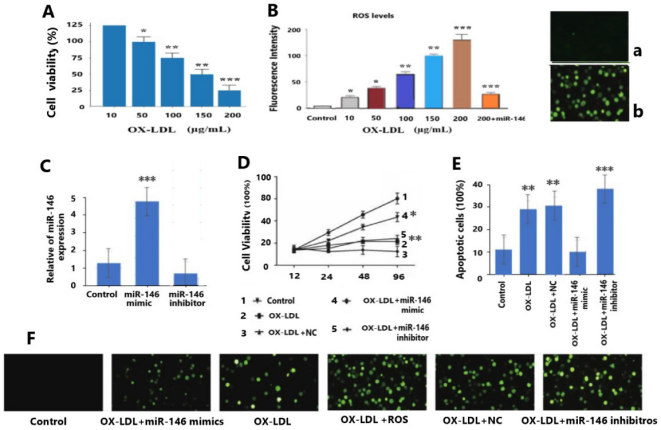

Figure 1: miRNA-146 promoted viability of cells and OX-LDL promoted cells apoptosis.

(A): The cell viability of granulosa cells after treatment with different concentrations (10, 50, 100, 150, and 200μg/mL) of OX-LDL.

(B): OX-LDL treatment led to increased fluorescence intensity of ROS in granulosa cells in a dose-dependent manner, and miRNA-146 treatment decreased OX-LDL induced ROS generation (n=5 per group), ***P<0.001.

(C): miRNA-146 promoted viability of granulosa cells, ***P<0.001.

(D): Granulosa cells were transfected with miRNA-146 mimics (50 nmol/L), miRNA-146 inhibitor (50 nmol/L), or Negative Control group (NC), and then treated with different concentrations of OX-LDL for 12 hours. At 12, 24, 48, and 96 hours after OX-LDL treatment, the viability of granulosa cells was measured by MTT assay. The cell viability of granulosa cells decreased significantly at 96h with 200μg/mL OX-LDL treatments. miRNA-146 significantly increased the viability of OX-LDL treatment granulosa cells *P<0.05; **P<0.01.

(E): The apoptotic cell rate was measured by TUNEL assay. Percentage of apoptotic cells significantly decreased by up-expression of miR-146 compared with the OX-LDL group and OX-LDL+NC group, **P<0.01; ***P<0.001.

(F): Up-regulation of miRNA-146 significantly reduced the apoptosis of OX-LDL treatment granulosa cells, while ROS remarkable enhanced the apoptosis of OX-LDL treatment granulosa cells.

(a and b): ROS level detected by DCFH-DA assay. OX-LDL significantly increased the ROS level. a, Control; b, OX-LDL

Effects of OX-LDL on the Viability of Ovarian Granulosa Cells

To determine the appropriate dosage of OX-LDL used in this study, MTT assay was conducted. The number of ovarian granulosa cells decreased significantly at 96 h with 200μg/mL OX-LDL treatment (P<0.001) (Figure 1A). Therefore, the maximum concentration of OX-LDL used in the subsequent analysis was 200μg/mL OX-LDL.

miRNA146 Down-Regulation in OX-LDL Induces of POI Model

In mouse POI model, firstly we examined the ovarian dysfunction of mouse treated with OX-LDL and the levels of miRNA-146 expression. OX-LDL treatment significantly decreased the cell viability measured by MTT assay. miRNA-146 expression was decreased in POI model by OX-LDL-induced compared with the control group detected by PCR (Tables 2 and 3). In addition, we measured the levels of miRNA-146 expression in ovarian granulosa cells and the significant down-regulation was detected in the OX-LDL group (Figure 1D). Moreover, to explore the role of miRNA-146 protective against in OX-LDL- induced ovarian dysfunction, we increased miRNA-146 expression by miRNA-146 mimics and a Negative Control (NC) in OX-LDL-treated ovarian granulosa cells. Cell viability was significantly increased (Figure 1D), and the ROS were decreased in the miRNA-146 group compared with the OX-LDL group (Figures 1B).

OX-LDL Increased Expression ROS Levels During Granulosa Cells

Our previous research validated that OX-LDL has been proven to be associated with oxidative stress and over expression of ROS during inflammatory state. To investigate whether ROS was involved in OX-LD promoted granulosa cells apoptosis increased, we determined the ROS levels in granulosa cells under OX-LDL. The results showed that OX-LDL-treatment led to increased fluorescence intensity of ROS in granulosa cells in a dose-dependent manner (P<0.01; Figure 1B). However, ROS generation decreased when using miRNA-146 to neutralize intracellular ROS (P<0.001; Figure 1B). These findings suggested that intracellular ROS was activated in OX-LDL- promoted granulosa cells apoptosis (Figure 1F).

Expression of miRNA-146 and Granulosa Cells Viability

To detect the expression of miRNA-146 in granulosa cells and the effect of ovarian granulosa cells viability on OX-LDL-induce ovarian dysfunction and the oxidative status of ovarian granulosa cells. We detected the effect of miRNA-146 and studied the viability of ovarian granulosa cells treated with different concentrations of OX-LDL (10, 50, 100, 150, and 200μg/mL) for 12, 24, 48, and 96 hours by MTT assay (Figure 1A, D). Therefore, select 200 μg/mL was used as OX-LDL stimulant as the following experimental conditions. ROS level was increased in ovarian granulosa cells stimulating used OX-LDL compared with the control group (Figure 1b). We found that the levels of miRNA-146 expression aggrandized cell viability compared with the control group. In contrast, miRNA-146 inhibitors decreased cell viability. These results showed that up-regulation of miRNA-146 significantly enhanced the viability of OX-LDL-treated ovarian granulosa cells.

Up-regulation of miRNA-146 in Granulosa cells and Transfection Efficiency

The transfection efficiency of miRNA-146 was used to evaluate by qRT-PCR in OX-LDL-treated granulosa cells transfected with miRNA-146 mimics, miRNA-146 inhibitors, or Negative Control (NC). As demonstrated in Figure 1C, it was observed that in granulosa cells, miRNA-146 mimics remarkable enhanced the levels of miRNA-146 expression (P<0.01), and miRNA-146 inhibitors remarkable reduced the levels of miRNA-146 expression (P<0.01). These experimental data indicated that the levels of miRNA-146 expression in granulosa cells are successfully up-regulation, promoted cell viability (Figure 1C). The percentage of apoptotic cells by TUNEL detected in granulosa cells was raised in the OX-LDL group compared with the control group, indicating a relative higher apoptosis degree (Figure 1D & F). There was a significant reduction in the miRNA-146 group compared with the OX-LDL group by TUNEL assay, demonstrating anti-apoptotic effect of up-regulation miRNA-146 expres-

sion (Figure 1E & F).

Inhibition of ROS by miRNA-146 Suppressed OX-LDL-Induced NF-κB Activation

So far, we have proven that OX-LDL could induce apoptosis of granulosa cells by activating the NF-κB signaling pathway. We also found that OX-LDL could increase ROS level in granulosa cells. Given that OX-LDL and ROS both activated the NF-κB signaling pathway and induced apoptosis of granulosa cells increased and affect the quality of follicles, ROS might play a crucial role in linking OX-LDL and the NF-κB signaling pathway in apoptosis of granulosa cells. Next, to determine whether OX-LDL-promoted apoptosis of granulosa cells by the NF-κB signaling pathway is ROS-dependent, we added miRNA-146 to determine whether NF-κB activation and apoptosis of granulosa cells with the presence of OX-LDL could be suppressed. The results showed that miRNA-146 pretreatment down-regulated the critical NF-κB signaling expression in granulosa cells treated with 200μg/mL OX-LDL (Figure 2A & 2B), which suggested that ROS was activated before the NF-κB signaling pathway in OX-LDL-promoted poptosis of granulosa cells. We also found that TLR-4 expression of granulosa cells increased less than 200μg/mL OX-LDL and decreased under miRNA-146 with or without OX-LDL (#P<0.01; Figure 2A & 2C). Moreover, the poptosis of granulosa cells related genes IL-6, and TFN-a decreased when treating the poptosis of granulosa cells with miRNA-146 #P<0.01; (Figure 2D & 2E). The protein expression of IL-6 and TNF-a was also promoted under 200μg/mL OX-LDL and decreased with miRNA-146 in OX-LDL-treated granulosa cells. Takern together, these results dermonstrated that OX-LDL could promote in vitro poptosis of granulosa cells through the ROS-depended NF-κB signaling pathway.

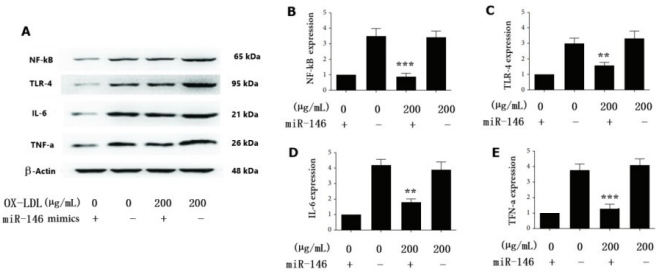

Figure 2: Inhibition of ROS by miRNA-146 suppressed OX-LDL-induced NF-κB activation.

(A,B) The NF-κB, and the protein expression levels of TLR-4, IL-6,TNF-a were promoted under 200μg/mL OX-LDL and suppressed by miRNA-146 with 200μg/mL OX-LDL or without treatment as examined by Western blot (n = 5 per group). (B,C,D,E) granulosa cells were pretreated with 50nmol/L miRNA-146 for 1h before being cultured with or without 200μg/mL OX-LDL.

Discussion

Oxidative stress during ovarian aging is an important pathogenesis [17]. POI is a oxidative stress induced chronic inflammatory reaction of the ovary with functional decline. Low levels of ROS and/or mild oxidative stress are involved in physiological processes, including cell signaling systems, cell growth and differentiation [18]. In addition, ROS levels in specific tissues lead to different processes of cell death. For example, increased ROS levels leads to granulosa cell apoptosis [19]. In humans, granulosa cells exposure to OX-LDL causes autophagy, which further leads to apoptosis and death. According to the study, POI has excess OX-LDL resulting in enhanced granulosa autophagy and cell death. Regression autophagy has been found to be rich in Oxidized Low density lipoprotein (lectin like) Receptor 1 (OLR1) in human ovaries [20]. Thus, the OX-LDL-OLR-ROS pathway induces autophagy in granulosa cells [21]. However, limited information is known about the role of OX-LDL in ovarian dysfunction and the direct induction of granulosa cell apoptosis in POI. In this study, we demonstrated for the first time that OX-LDL induced ovarian dysfunction in mice due to elevated OX-LDL in vivo due to enhanced expression of NF-κB signaling pathway. In addition, we found that OX-LDL directly promotes apoptosis of granulosa cells in vitro through activation of the ROs-dependent NF-κb signaling pathway. These findings have important implications for further understanding the mechanism by which OX-LDL contributes to ovarian dysfunction and granulosa cells apoptosis in POI.

This OX-LDL-induced POI model is different from several traditional animal models of POI, which is characterized by the establishment of humanized immunity, inflammatory response and oxidative stress [22]. In the OX-LDL-induced POI model, we found a meaningful decrease in E2 concentration and a significant increase in FSH concentration compared with the control group. The results of this study displayed the effect of OX-LDL on ovarian hormone [23]. In vivo, after injection of miRNA-146 mimics, FSH concentration, OX-LDL and ROS expression levels were remarkably down-regulated, while E2 concentration was significantly up-regulated. Therefore, our data support that the up-regulation of miRNA-146 expression may regulate ovarian hormones and reverse ovarian function [24]. Furthermore, Marwa et al. (2021) [25] showed that bone marrow mesenchymal stem cells (BM-MSCs) can repair damaged ovaries and protect ovarian function by restoring serum estradiol and follicle-stimulating hormone levels. Thus, they can also save the reproductive results of irradiated rats. To this end, we analyzed in vivo data from OX-LDL-induced POI mouse models and conducted in vitro experiments to verify the effect of our proposed mechanism of miRNA-146 protective against ovarian dysfunction. POI is usually induced by various chemical agents and chemotherapy in animal models. In this study, OX-LDL induced ovarian dysfunction models of POI were established to investigate the role of miRNA-146 in restoring ovarian function in POI. This POI model is significantly different from the animal model established by conventional methods. However, these therapeutic effects of stem cells are still controversial due to differences between model animals and humans [26].

In this study, it showed that OX-LDL could significantly decrease the granulosa cells viability, and the concentration threshold of OX-LDL was set to 200μg/mL in the in vitro studies according to the cell viability test. OX-LDL was witnessed to be a risk factor for various chronic inflammatory diseases. As Karunakaran et al., (2021) reported, OX-LDL could induce coronary heart disease by directly stimulating inflammation in Vascular Smooth Muscle Cells (VSMCs) [27]. Moreover, ovarian dysfunction has the common pathophysiological mechanisms to oxidative stress and chronic inflammation, including OX-LDL and ROS levels [28]. In the current study, we showed that OX-LDL could induce GCs apoptosis in mice. Given that people suffering from ovarian dysfunction tend to face a higher risk of oxidative stress, modulating serum OX-LDL may alleviate oxidative stress in these individuals [29]. The current study may extend the possibility of using OX-LDL as a therapeutic target for POI, especially in patients with ovarian failure and infertility.

The NF-κB signaling pathway regulates many cellular processes including inflammation, immune response, and apoptosis [30]. Yan et al., (2017) study validated that OX-LDL activated NF-κB signals during vascular calcification in Cardio Vascular Disease rats [31]. In our previous study, we have established that the NF-κB signaling pathway is essential for the stimulation of ovarian dysfunction [32]. However, the regulating effect of the NF-κB signaling pathway on OX-LD-induced ovarian dysfunction is still unknown. In the current study, we validated that abnormally increased OX-LD could promote granulosa cells apoptosis by activating the NF-κB signals. Therefore, intervention of NF-κB signals might be promising to attenuate OX-LD-induced granulosa cells apoptosis. In addition, ROS is an upstream signaling molecule to activate NF-κB signals, which is critical for ovarian dysfunction [29]. In the present study, we found that OX-LDL could also induce granulosa cells apoptosis via the stimulation of ROS. Therefore, OX-LDL-induced granulosa cells apoptosis in POI could be caused by an inflammatory process triggered by oxidative stress.

miRNA-146 has been proved to be a negative regulator of the NF-κB activation pathway in innate immune responses and myocardial ischemia/reperfusion injury [33]. And miR-146 can be induced by ROS via NF-κB-dependent pathway [33]. Further identify the protective role and function of miRNA-146, we evaluated the level of NF-κB and TLR4 expression, TLR4 was used for investigation of the relationship between miRNA-146 and TLR4 in OX-LDL induced ovarian dysfunction. In our study, it is shown that TLR4 and NF-κB can up-regulate the expression of OX-LDL, ROS and significantly enlarge the oxidative stress and inflammatory response, which suggested TLR4 and NF-κB are proinflammatory factor [34]. So far, there is no report of OX-LDL/ROS-dependent NF-κB signaling pathway that plays a crucial role in chronic inflammatory failure of POI. NF-κB, as a transcription factor, which is the main feature of chronic inflammatory diseases in cell proliferation, apoptosis, and immune reaction [10]. In current study, we demonstrated that up-regulation of miRNA-146 expression decreased TLR4/ NF-κB, TNF-α and IL-6 expression in mouse model of POI. These results displayed that miRNA-146 significantly depressed OX-LDL/ ROS-dependent NF-κB signaling pathway. It is suggested that the expression of miRNA-146 plays protective against ovarian dysfunction. From the present results, miRNA-146 was significantly decreased with the activation of ROS in OX-LDL-treated granulosa cells and the transfection of miRNA-146 mimics significantly reduced the expression of TLR4 and NF-κB.

These study suggest that up-regulation miRNA146 plays a protective role might be inhibiting TLR4 and NF-κB signaling pathways. TLRs are innate immunetrans membrane receptors and signal transduction receptors found in recent years, which involved in chronic inflammation, oxidative stress and the formation of tumor microenvironment [35]. TLR4 exists in the ovary and reproductive tract, and TLR4 is also involved in inducing apoptosis of ovarian granulosa cells in uterine inflammatory diseases [36].

In summary, in the current study, we have proved that miRNA-146 has anti-antioxidant, anti-inflammatory, and anti-apoptotic effects. Our experimental results are expected to achieve the effect of stem cell therapy, in the future, we will increase clinical case studies to confirm. This study is safe and reliable, sufficient sources and without ethical issues and it will not involve the occurrence of ovarian granulosa cell tumor. Gupta et al., (2018) study shows that autologous stem cell may improve the conditions in patients with POI [37]; however, oocytes extracted from these POI mice after stem cell transplantation were difficult to fertilize with healthy sperm in vitro [38]. However, there are many ethical concerns with the use of these current stem cells are difficult to source. Therefore, the study of stem cells is still at the very early stage, and thus, they are not usually used in clinically [39].

Conclusion

To our knowledge, this study is the first to demonstrate that miRNA-146 may play a protective role in ovarian dysfunction by inhibiting the OX-LDL/ROS-dependent NF-κB signaling pathway. OX-LDL induces ovarian dysfunction by activating the ROs-dependent NF-κB signaling pathway. In addition, OX-LDL could directly induce ovarian granulosa cells apoptosis, and miRNA-146 could inhibit it. Overall, the present findings provide detailed information for understanding the exact mechanism of OX-LDL in ovarian dysfunction and the protection of miRNA-146 against oxidative stress-induced ovarian dysfunction. The present results suggest that miRNA-146 can be used as an immunomodulatory factor and a new target for the treatment of POI.

Disclosure Statement

The authors declared that they do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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