VCD induces premature ovarian insufficiency (POI) in rats by triggering the autophagy of granulosa cells through regulating the AKT/mTOR signal pathway

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Research

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Abstract

Purpose

Investigating the pathological mechanism underlying POI modeling in animals with VCD.

Methods

QRT-PCR was used to detect the expression of miR-144 in the peripheral blood of POI patients, granulosa cells and the ovary tissues. MTT assay was used to evaluate the proliferation and the concentration of E2, FSH, LH, and AMH was determined by ELISA assay. The numbers of autophagosomes were detected by transmission electron microscope and autophagic flux assay. The expression of key proteins in AKT pathway and autophagy proteins was determined by Western blot. HE staining was used to check the state of follicles in the ovary tissues. Immunohistochemistry assay was used to evaluate the expression level of p-AKT, P62, and caspase-3 in the ovary tissues. Finally, the pregnancy rate and embryo resorption rate were calculated.

Results

MiR-144 was down-regulated in the peripheral blood of POI patients. Decreased cell viability, down-regulated miR-144, and increased autophagosomes were observed in VCD treated granulosa cells, which were reversed by the introduction of SC79 or miR-144. The increased concentration of FSH, and LH, decreased concentration of E2 and AMH, increased number of autophagosomes, up-regulated PTEN, and inactivated AKT/m-TOR signal pathway induced by VCD in both granulosa cells and ovary tissues were significantly reversed by SC79 or miR-144. Finally, the decreased follicles and pregnancy rate, as well as the increased embryo resorption rate induced by VCD were greatly reversed by SC79 or miR-144.

Conclusion

VCD induced POI in rats by triggering the autophagy of granulosa cells through regulating the AKT/mTOR signal pathway.

Introduction

Premature ovarian insufficiency (POI) is a disease characterized with premature depletion of follicles and the clinical manifestations of POI include amenorrhea and elevated serum gonadotropin level, which severely influences the fertility ability and mental health of women globally [1, 2]. Statistically, approximately 1% of the total female population have been diagnosed with POI before the age of 40 [3]. And in China, approximately 2.8% females are suffering from POI [4]. As a highly heterogeneous disease, the pathological mechanism of most patients remains unknown. Currently, autophagy of ovarian
granulosa cells is increasingly being paid more attention to be responsible for the pathogenesis of POI [5, 6]. As an important regulatory mechanism of cell homeostasis, autophagy is involved in the mediation of survival ability and homeostasis of ovarian granulosa cells, which further impact the follicular development and ovarian function [7]. It is reported that the regulation and inducement of autophagy are closely related to the apoptosis of granulosa cells and oocytes and mediate the follicular atresia [8, 9]. Reproductive toxic environments, such as smoking [10] and oxidative stress, induce the apoptosis of granulosa cells and oocytes by activating autophagy, which accelerate the follicular atresia and the dysfunction of ovary [11]. Therefore, the autophagy of ovarian granulosa cells is an important point to study the pathogenesis of POI.

Autophagy can be regulated by multiple cellular signal pathways, among which PTEN/AKT is of great significance, especially for linking the correlation between miRNAs and autophagy. Liu [12] reported that miR-181 might regulate cisplatin-resistant non-small cell lung cancer via downregulation of autophagy through the PTEN/AKT pathway. MiR-155 is reported to affect the autophagy of osteosarcoma cells induced by Adriamycin through regulating the PTEN/AKT pathway [13]. Recently, miR-144 has been widely claimed to target PTEN to activate the AKT pathway [14, 15]. In addition, down-regulating miR-144 is reported to induce the development of POI [16]. We suspected that as the regulatory miRNA of PTEN/AKT pathway, miR-144 might be an important mediator for the autophagy of ovarian granulosa cells and thereafter for the pathogenesis of POI.

4-vinylcyclohexene diepoxide (VCD) is a commercial chemical intermediate utilized in animals to simulate the destroy of primordial follicles [17]. Several studies have demonstrated that follicle depletion could be induced by the administration in animals through apoptosis and programmed cell death, as well as speeding up the natural process of atresia [18–20]. Furthermore, the level of 17β-estradiol and estrous cycling could also be altered by VCD [21]. Owning to these pathological changes induced by VCD, it is widely used to induce POI in animals [22]. However, the molecular mechanism remains unknown. In the present study, the pathological mechanism underlying the POI modeling using VCD will be investigated to better understand the pathogenesis of POI on a molecular level.

Materials And Methods

The collection of peripheral blood of POI patients and ovary granulosa cells of rats

The peripheral blood samples were collected from 20 pairs of POI patients and health people from The First Affiliated Hospital of Zhejiang Chinese Medical University (Zhejiang Provincial Hospital of Traditional Chinese Medicine), respectively. Serum was isolated from the peripheral blood to detect the concentration of miR-144. For the cell experiments, 8 female SD rats at the age of 23–25 days were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Certificate No. SCXK(Jing)2016-0006). The rats were kept in cages under the conditions with 12 h light and 12 h dark regime, temperature (22°C), humidity (65%), and fed ad libitum on rat cubes and tap water. The rats were injected subcutaneously with 40 U pregnant mare serum gonadotropins (PMSG). After 48 hours, the
animals were anesthetized with 10% chloral hydrate, followed by stripping the ovarian tissue and puncturing the follicle to isolate the granulosa cells. The cells were cultured with conditional DMEM medium containing 10% FBS. Approximately $5 \times 10^5$ cells/mL were seeded into the 96-well plates, which were identified utilizing the immunofluorescence assay with the antibody against follicle-stimulating hormone receptor (FSH-R) at the logarithmic phase.

**Animals model:** After adapting for a week, another 48 female SD rats at the age of 21 days were divided into 4 groups randomly: control, VCD, VCD + SC79, and VCD + miR-144 groups ($n = 12$ for each group). In the control group, the animals were administered intraperitoneally with 2.5 mL/kg/day sesame oil for consecutive 15 days. In the VCD group, the rats were administered intraperitoneally with 80 mg/kg VCD solution dissolved in 2.5 mL/kg/day sesame oil for consecutive 15 days. For the animals in the VCD + SC79 group, the schedule for the dosing of VCD is consistent with the rats in the VCD group, which was accompanied by subcutaneous injection of 10 mg/day SC79 (activator of AKT signal pathway) for consecutive 15 days. In the VCD + miR-144 groups, the rats were firstly transfected with lentivirus particles containing the miR-144 agomir (Genscript, Nanjing, China) to up-regulate the expression level of miR-144 in the rat, followed by administered intraperitoneally with 80 mg/kg VCD solution dissolved in 2.5 mL/kg/day sesame oil for consecutive 15 days. Part of the animals was anesthetized to collect the experimental tissues approximately 85 days post the treatment of VCD. The blood samples from orbital venousplexus of eight rats in each group were collected on non-estrus period under anesthesia, by intraperitoneally administering 10% chloralhydras (3 ml/kg), as well as oophorectomy. Then, the serum was obtained by centrifugation at 3000 rpm/ min for 15 min and stored at −80°C for future use. Several ovarian tissues were immersed in 10% formaldehyde for histopathology, and the rest were frozen and preserved at-80°C for genes and proteins tests. In addition, the other four rats in each group were raised with the male rats at a ratio of 1:2 for 12h. The mixture of sperm and vaginal smears seen on the next morning indicated the success of pregnancy, and this morning was considered as the 0.5th day of the gestation. The pregnant rats were euthanized on the 15th day of the gestation, and the embryos were isolated. The embryo resorption was defined as no embryo, residual placental tissue or observed clots. Then, the embryo resorption rate was calculated as dividing the total number of embryos with the number of absorbed embryos. Animal experiments were approved by the ethics committee of Zhejiang Chinese Medical University.

**MTT assay**

To select the optimized concentration of VCD in the medium of granulosa cells, the cells were incubated with 0, 0.5, 1, 1.5, 2, and 3 mM, respectively, and the cell viability of each group was determined by the MTT assay. The granulosa cells were seeded on the 96-well plates (3 $\times$ 10^3 cells per well) and incubated for 24 hours, followed by replacing the medium with new fresh medium containing MTT solution. After being incubated for additional 2 h at 37°C, the medium was replaced with DMSO to dissolve the MTT solution for 15 min, followed by observing the absorbance of 490 nm described previously [23].

**ELISA assay**
ELISA commercial kits (Nanjing Jin Yibai Biological Technology Co. Ltd., Nanjing, China) were used to detect the concentration of estradiol (E2), Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), and anti-Mullerian hormone (AMH) in the treated granulosa cells or the serum of each animal, the lysis sample of which was incubated with 5% BSA to remove the non-specific binding proteins for 1 hour at room temperature, followed by being incubated with the primary antibodies for 1 h. Subsequently, the samples were incubated with streptavidin-horseradish peroxidase (HRP) conjugated secondary antibodies for 20 min at room temperature, followed by being read at 450 nm with a microplate spectrophotometer (Thermo Fisher, Massachusetts, USA).

**QRT-PCR**

The total RNA in the treated granulosa cells were extracted using the RNA simple Total RNA kit (Beyotime, Shanghai, China), followed by transforming the RNA into cDNA with the Prime Script RT reagent Kit (Takara, Tokyo, Japan). In the present study, PCR procedure was performed using a SYBR Green PCR Kit (Takara, Tokyo, Japan). The relative expression of related genes was determined using a $2^{-\Delta\Delta Ct}$ methods and GADPH was taken as a negative control. The forward sequence of miR-144 was 5'-GGGAGATCAGAAGGTGATT-3' and the reverse sequence of miR-144 was 5'-GTGCAGGGTCCGAGGT-3'.

**Western blot assay:** The granulosa cells were lysed using a RIPA lysis buffer (Thermo, Mississippi, USA) to obtain the total proteins, the concentration of which was quantified with a BCA protein assay kit (Beyotime, Shanghai, China). Approximately 50 µg proteins for each sample was added and separated by the 12% SDS-PAGE, which were further transferred to the PVDF membrane (Thermo, Mississippi, USA). Subsequently, the membrane was incubated with primary antibodies against AKT (1:1000, Cell Signaling Technology, Boston, USA), p-AKT (1:1000, Cell Signaling Technology, Boston, USA), p-mTOR (1:1000, Cell Signaling Technology, Boston, USA), mTOR (1:1000, Cell Signaling Technology, Boston, USA), PTEN (1:1000, Cell Signaling Technology, Boston, USA), S6K (1:1000, Cell Signaling Technology, Boston, USA), p-S6K (1:1000, Cell Signaling Technology, Boston, USA), ULK1 (1:1000, Cell Signaling Technology, Boston, USA), p-ULK1 (1:1000, Cell Signaling Technology, Boston, USA), Beclin-1 (1:1000, Cell Signaling Technology, Boston, USA), Bcl-2 (1:1000, Cell Signaling Technology, Boston, USA), LC3-II (1:1000, Cell Signaling Technology, Boston, USA), LC3-I (1:1000, Cell Signaling Technology, Boston, USA), P62 (1:1000, Cell Signaling Technology, Boston, USA), Caspase3 (1:1000, Cell Signaling Technology, Boston, USA) or GAPDH (1:1000, Cell Signaling Technology, Boston, USA), followed by incubated with the secondary antibodies for 60 minutes. Afterwards, the membranes were washed and the blots were visualized with an infrared imaging system (Tanon, Shanghai, China), which was quantified using the ImageJ software (Tanon, Shanghai, China).

**Transmission electron microscope**

The granulosa cells in logarithmic growth phase were seeded in 24-well plates at a density of $1 \times 10^5$ cells/well to be incubated for 48 hours. Cells or tissues were digested with trypsin, followed by being collected by centrifugation to remove the supernatant. Subsequently, 3% glutaraldehyde and 1% citric
acid were used to fix the samples, which were gradually dehydrated by acetone and embedded with dipropylene dicarboxylate. The samples were cut into ultrathin sections. A transmission electron microscopy (Olympus, Tokyo, Japan) was used to observe and image the autophagosomes within the cells or the tissues.

**Analysis of autophagic flux**

LC3 was marked and tracked by transfecting the mRFP-GFP-LC3 lentivirus (Hanbio, Shanghai, China) into the granulosa cells. Briefly, tandem fluorescent mRFP-GFP-tagged adenovirus was transfected into the cells for 48 hours according to the instruction of the manufacturer, followed by taking images with the confocal fluorescence microscopy (Olympus, Tokyo, Japan). The early autophagosomes were represented by the yellow puncta (merge of GFP and RFP signal) and the late autophagosomes were represented by the red puncta.

**Hematoxylin and eosin (HE) staining**

The ovarian tissue of each rat was collected and washed over by sterile water for a couple of hours, followed by being dehydrated with 70%, 80% and 90% ethanol solution successively. Subsequently, the samples were incubated with equal quality of ethanol and xylene for 15 min, which were then incubated with equal quality of xylene for another 15 min. The incubation procedure was repeated until the samples looked transparent. Finally, the tissues were embedded in paraffin, followed by being sectioned and stained with hematoxylin and eosin (H&E) staining. Finally, the images selected randomly from 5 fields at 40× and 100× magnification were captured and all phases of follicles and corpora lutea were count by using an inverted microscope (Olympus, Tokyo, Japan).

**Immunohistochemistry:** The ovarian tissues were separated and incubated with pre-dolled saline, which was subsequently embedded in paraffin. After being sectioned, the tissues were incubated with p-AKT, P62, or caspase-3 antibody (1:1000, CST, Cell Signaling Technology, Boston, USA) at 4°C overnight, followed by being washed and incubated with goat anti-rabbit antibody at 37°C for 30 min. DAB agent was used to dye the slides for 5–10 min, which was re-dyed with hematoxylin for 30 min. The images were taken using an inverted microscope (Olympus, Tokyo, Japan).

**The calculation of pregnancy rate and embryo resorption rate**

The pregnancy rate was calculated dividing the total number of rats with the number of pregnant rats. On day 15 of the gestation period, the animals were anesthetized and the embryos were isolated. The surrounding tissues were separated, the embryo resorption was defined as no embryo, residual placental tissue or observed clots. The embryo resorption rate was calculated as dividing the total number of embryos with the number of absorbed embryos.

**Statistical Analysis**
All results are presented as the mean ± SEM and analyzed using Graphpad prism software. Comparisons between two groups were performed using an unpaired Student’s t-test. One-way ANOVA was used for comparisons among three or more groups. A value of P < 0.05 was regarded as statistically significant.

Results

MiR-144 was down-regulated in the POI patients

To check the differentiated expression level of miR-144 in clinical POI patients, peripheral blood from 20 pairs of POI patients was collected. As shown in Fig. 1, compared to control, the expression level of miR-144 was significantly suppressed in the POI patients (**p < 0.01 vs. control).

The inhibited proliferation and promoted excretion of estrogens induced by VCD were reversed by SC79 or miR-144 in the granulosa cells

To further investigate the mechanism underlying the POI modeling using VCD, the granulosa cells were isolated from rats, which were verified by immunofluorescence staining with FSHR antibody shown in Fig. 2A. In addition, to achieve an optimized concentration of VCD for the in-vitro incubation of granulosa cells, the evaluated the cell viability following incubating the cells with different concentrations of VCD. As shown in Fig. 2B, when the concentration of VCD exceeded 1.5 mM, the cell viability decreased greatly (*p < 0.05 vs. 0 mM, **p < 0.01 vs. 0 mM). Therefore, 1.5 mM VCD was used as the incubation concentration of VCD in the subsequent experiments. The granulosa cells were incubated with blank medium, 1.5 mM VCD, 1.5 mM VCD combined with 0.5 µM SC79, and 1.5 mM VCD combined with 50 µM miR-144 mimic. We firstly determined the expression of miR-144 in all the groups. As shown in Fig. 2C, the expression level of miR-144 was significantly suppressed by the stimulation of VCD, which was greatly elevated by introducing the miR-144 mimic into the granulosa cells (**p < 0.01 vs. control, ##p < 0.01 vs. VCD). Additionally, the decreased cell viability induced by VCD was greatly elevated by the treatment of SC79 or miR-144 (Fig. 2D, *p < 0.05 vs. control, #p < 0.05 vs. VCD). Finally, the elevated concentration of FSH and LH induced by the stimulation of VCD was greatly suppressed by the introduction of SC79 or miR-144. On contrary, the decreased concentration E2 and AMH induced by VCD was significantly promoted by the treatment of SC79 or miR-144 (Fig. 2E, **p < 0.01 vs. control, *p < 0.05 vs. control, #p < 0.05 vs. VCD).

The autophagy in granulosa cells was induced by VCD

To explore the effects of VCD on the autophagy in the granule, TEM, Western blot and mRFP-GFP-LC3 assay were performed on the granulosa cells incubated with VCD in the presence of SC79 or miR-144. As shown in Fig. 3A, compared to control, a great deal of autophagosomes (red arrows) were observed in the VCD treated granulosa cells, which were decreased in both the VCD + SC79 and VCD + miR-144 groups. In addition, significant elevated expression level of Beclin-1, LC3-II/LC3-I and P62 (Fig. 3B) was observed in VCD group, which was greatly suppressed by the introduction of SC79 or miR-144 (**p < 0.01 vs. control, #p < 0.05 vs. VCD, ##p < 0.01 vs. VCD). As shown in Fig. 3C, the number of autophagosomes both at early
stage (red puncta) and at advanced stage (yellow puncta) increased significantly in the VCD group, which was greatly decreased in the VCD + SC79 and VCD + miR-144 groups (**p < 0.01 vs. control, #p < 0.05 vs. VCD).

**VCD suppressed the AKT/mTOR signal pathway by up-regulating PTEN**

Autophagy was regulated by the AKT/mTOR signal pathway and contributed to the apoptosis of granulosa cells. We further investigated the effects of VCD on the AKT/mTOR signal pathway. As shown in Fig. 4, PTEN was significantly up-regulated by the stimulation of VCD, which was greatly down-regulated by the introduction of SC79 or miR-144 (**p < 0.01 vs. control, #p < 0.05 vs. VCD). In addition, significant suppressed expression of p-AKT1, AKT1, p-mTOR/mTOR, p-S6K/S6K, and p-ULK1/ULK1 induced by the stimulation of VCD was greatly elevated by the treatment of SC79 or miR-144 (**p < 0.01 vs. control, #p < 0.05 vs. VCD). For the relative expression of anti-apoptotic protein and apoptotic protein, we found that Bcl-2 was extremely down-regulated and caspase-3 was up-regulated by the stimulation of VCD, which was greatly reversed by the introduction of SC79 or miR-144 (**p < 0.01 vs. control, #p < 0.05 vs. VCD).

**VCD induced POI-like symptom in rats**

To verify whether POI model could be induced by VCD in animals, the rats were dosed with VCD for 15 consecutive days. Firstly, as shown in Fig. 5A, the expression level of miR-144 in both the peripheral blood and the ovary tissues was significantly suppressed by the administration of VCD, which was greatly promoted by the transfection of miR-144 mimic (**p < 0.01 vs. control, ##p < 0.01 vs. VCD). As illustrated in Fig. 5B, the pregnancy rate in the control, VCD, VCD + SC79, and VCD + miR-144 was 100%, 25%, 75%, and 50% respectively. While the embryo resorption rate in the control, VCD, VCD + SC79, and VCD + miR-144 was 16.67%, 71.43%, 50%, and 55.56%, respectively. In addition, the elevated concentration of FSH and LH, as well as the suppressed concentration of E2 and AMH (Fig. 5C), induced by VCD was greatly reversed by the co-treatment of SC79 or miR-144 mimic (**p < 0.01 vs. control, *p < 0.05 vs. control, #p < 0.05 vs. VCD). For the pathological changes in the ovary tissues, compared to control, the number of primordial follicles decreased in the VCD group, which was slightly increased in the groups of VCD + SC79 and VCD + miR-144 (Fig. 5D).

**The autophagy in ovary tissues was induced by VCD**

To explore the effects of VCD on autophagy in the ovary tissues, several biological assays were performed on the tissues. As shown in Fig. 6A, more autophagosomes (red arrows) were observed in the VCD treated animals, compared to control, which were decreased in the groups of VCD + SC79 and VCD + miR-144. In addition, the elevated expression level of Beclin-1, LC3-II/LC3-I and P62 (Fig. 6B-C) observed in the VCD group was greatly suppressed by the treatment of SC79 or miR-144 (**p < 0.01 vs. control, #p < 0.05 vs. VCD, ##p < 0.01 vs. VCD). These data indicated that VCD induced significant autophagy in the ovary tissues, which might be related to the inactivation of AKT and the down-regulation of miR-144.
VCD inactivated the AKT/mTOR signal pathway by up-regulating PTEN to induce the POI model: We further verified the effects of VCD on the AKT/mTOR signal pathway in ovary tissues. As shown in Fig. 7A, PTEN was significantly up-regulated in the VCD treated animals, which was down-regulated by the introduction of SC79 or miR-144 (**p < 0.01 vs. control, #p < 0.05 vs. VCD). On the contrary, the expression of p-AKT1AKT1, p-mTOR/mTOR, p-S6K/S6K, and p-ULK1/ULK1 was greatly suppressed by the treatment of VCD, which was significantly elevated by the introduction of SC79 or miR-144 (**p < 0.01 vs. control, #p < 0.05 vs. VCD). In addition, Bcl-2 was extremely down-regulated and caspase-3 was up-regulated in the VCD group, which were greatly reversed by the treatment of SC79 or miR-144 (**p < 0.01 vs. control, #p < 0.05 vs. VCD). The data achieved by Western blot was further confirmed by the immunohistochemical assay (Fig. 7B).

Discussion

VCD is a commonly used chemical reagent to induce POI in the animal model due to its toxicity against ovary [24]. VCD is reported to induce the premature ovarian failure model in mice by suppressing the Rictor/mTORC2 signal pathway, which contributes to the inactivation of AKT and activation of Foxoa. Finally, the expression of pro-apoptotic proteins was induced [25]. In addition, by intraperitoneal injection of 80 mg/kg VCD to mice for a 15-day consecutive dosing, the amounts of primordial follicles and primary follicles decrease greatly in the ovary tissues, accompanied by an inactivation of AKT signal pathway [26]. In the present study, consistent with these reports, decreased primordial follicles, declined pregnancy rate and elevated embryo resorption rate were observed in the VCD treated animals. In addition, both in the VCD treated granulosa cells and VCD treated rats, the expression level of FSH and LH was significantly elevated and the expression level of E2 and AMH was significantly suppressed, which were in accordance with the clinical characteristics observed on POI patients, as well as in other POI animal models [27–29]. These data indicated that the POI symptoms were stimulated by VCD both in vitro and in vivo.

Autophagy is defined as the degradation process of cellular long-lived proteins and damaged organelles induced by lysosome, which is private to the eukaryocytes [30]. Autophagy is involved in multiple biological processes including development, metabolism, immunoregulation and senescence. Additionally, apoptosis can be induced by the excessive self-digestion and degradation of important cellular structures triggered by autophagy [31]. In the ovary tissues of healthy females, as the increase of age, the follicles in the primordial follicle pool gradually diminish. During every menstrual cycle, only 1–2 follicles grow mature to be involved in ovulation, while other follicles will be end up with death due to atresia. Premature atresia of follicles is regarded as the main pathological mechanism of POI [32]. Although follicular atresia occurs periodically according to the menstrual cycle, the mechanism underlying the death of multiple follicles remains unknown. Granulosa cells are located around follicles and involved in the growth, development and mature of follicles. Recently years, the follicular atresia is reported to be related to the autophagy of granulosa cells [33]. In the present study, we investigated the impact of VCD on the autophagy of granulosa cells and ovary tissues. Both in vitro and in vivo data showed that significant autophagy was induced by the treatment of VCD, which was accompanied by the
symptom of POI. These data confirmed that the autophagy of granulosa cells might be an important mediator for the process and development of POI, which could be responsible for the pathological basis of the animal POI modeling by VCD.

AKT/mTOR signal pathway is an important regulatory pathway involved in multiple physiological procedures including proliferation, differentiation, apoptosis, and autophagy [34]. The inactivation of AKT/mTOR signal pathway is reported to contribute to the cellular autophagy through regulating the S6K/ULK1 axis to control the expression of autophagy related proteins, such as LC3-II and P62 [35], which further induces the apoptosis of cells [36], while PTEN is an inhibitory regulatory of AKT/mTOR signal pathway [37, 38]. In the present study, we firstly found that both in granulosa cells and ovary tissues, the expression level of PTEN was significantly elevated by VCD, accompanied by an inactivation of AKT/mTOR signal pathway and activated autophagy. Subsequently, SC79, a widely used activator of AKT signal pathway [39, 40], was used to verify the involvement of AKT/mTOR signal pathway in the regulatory effect of VCD on cellular autophagy and the POI symptom. By the introduction of SC79, we confirmed that the inactivation of AKT/mTOR signal pathway was responsible for the effect of VCD to the autophagy of granulosa cells and the POI symptoms in animals. However, more biological methods will be applied to further verify the correlation in our future work, such as introducing PTRN-knockdown or AKT-overexpressing granulosa cells.

Recently, miRNA has been widely reported to be involved in the pathological process of POI. Dang reported that microRNA-379-5p is associate with biochemical POI through regulating PARP1 and XRCC6 [41]. MiR-15b induces POI in mice via inhibition of α-Klotho expression in ovarian granulosa cells [42]. This year, Yang reported that bone marrow mesenchymal stem cell-derived exosomal miR-144 mediates rat ovarian function after chemotherapy-induced ovarian failure by targeting PTEN [43]. Therefore, we suspected that miR-144 might be an important mediator regulating the pathological process of POI. In the present study, we firstly found that the expression of miR-144 was significantly suppressed in the peripheral blood of POI patients, indicating a possible function of miR-144 to POI. Subsequently, miR-144 mimic was transfected into both VCD treated granulosa cells and VCD treated rats. We found that the POI symptom was significantly improved, the autophagy of granulosa cells was greatly alleviated and the AKT/mTOR signal pathway was activated. These data indicated that VCD might induce the POI symptom by down-regulating miR-144. However, although the correlation between miR-144 and PTEN has been claimed and the expression of PTEN was indeed suppressed by introducing miR-144 into the granulosa cells, the interaction between miR-144 and the gene of PTEN still needs to be verified in our future work, such as by luciferase activity assay. In addition, more investigations will be performed to explore how VCD regulates the expression of miR-144 in our future work, possibly taking long-non coding RNAs or transcriptional factors into considerations.

Taken together, our data showed that VCD induced POI in rats by triggering the autophagy of granulosa cells through regulating the AKT/mTOR signal pathway.

Acknowledgments
Declarations

Acknowledgments

Declaration of Conflict of Interest

Ethics approval and consent to participate: We declare that all animal and human experiments involved in this manuscript were authorized by the ethical committee of The First Affiliated Hospital of Zhejiang Chinese Medical University and carried out according to the guidelines for care and use of laboratory animals and as well as to the principles of laboratory animal care and protection. All patients were aware of the purpose of the samples collected from them and agreed for participation.

Consent for publication: Consent for publication has been achieved from all authors.

Competing interests: The authors declare there is no conflicts of interest regarding the publication of this paper.

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Availability of data and materials

Data will be available from the corresponding author if it is requested by the editor or reader.

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**Figures**
Figure 1

The expression level of miR-144 in the peripheral blood of POI patients and healthy people was determined by qRT-PCR assay (***p<0.01 vs. control).
The proliferation and secretion of hormones in granulosa cells were impacted by VCD through regulating AKP/mTOR signal pathway and miR-144. A. The morphology and identification of isolated granulosa cells. B. The concentration of VCD utilized was determined by MTT assay (*p<0.05 vs. 0 nM, **p<0.01 vs. 0 nM). C. The expression level of miR-144 in the granulosa cells was evaluated by qRT-PCR assay. (**p<0.01 vs. control, ###p<0.01 vs. VCD). D. The cell viability of granulosa cells was determined by MTT assay.

**Figure 2**
assay. (*p<0.05 vs. control, #p<0.05 vs. VCD). E. ELISA was used to detect the concentration of E2, FSH, LH, and AMH in the cells (*p<0.05 vs. control, **p<0.01 vs. control, #p<0.05 vs. VCD).

**Figure 3**

The autophagy of granulosa cells was induced by VCD by regulating AKT/mTOR signal pathway and miR-144. A. The number of autophagosomes was observed by TEM. B. The expression level of Beclin-1,
LC3-I, LC3-II, and P62 was determined by Western blot. The number of autophagosomes was evaluated by autophagic flux assay (**p<0.01 vs. control, #p<0.05 vs. VCD, ##p<0.01 vs. VCD).

Figure 4

AKT/mTOR signal pathway was inactivated and apoptosis was activated by VCD. The expression of AKT, p-AKT, p-mTOR, mTOR, PTEN, S6K, p-S6K, ULK1, p-ULK1, Bcl-2, and Caspase3 in the granulosa cells was determined by Western blot (**p<0.01 vs. control, #p<0.05 vs. VCD).
Figure 5

The POI symptom was simulated in rats by VCD. A. The expression level of miR-144 in the peripheral blood and ovary tissues of animals was determined by qRT-PCR assay. B. The pregnancy rate and embryo resorption rate were calculated. C. ELISA was used to detect the concentration of E2, FSH, LH, and AMH in the serum. D. HE staining was used to check the state of follicles in the ovary tissues (*p<0.05 vs. control, **p<0.01 vs. control, #p<0.05 vs. VCD, ##p<0.01 vs. VCD).
Figure 6

The autophagy in ovary tissues was induced by VCD. A. The number of autophagosomes was observed by TEM. B. The expression level of Beclin-1, LC3-I, LC3-II, and P62 in the ovary tissues was determined by Western blot. C. The expression level of P62 was evaluated by immunohistochemistry assay (**p<0.01 vs. control, #p<0.05 vs. VCD, ##p<0.01 vs. VCD).
Figure 7

VCD inactivated the AKT/mTOR signal pathway by up-regulating PTEN to induce the POI model. A. The expression of AKT, p-AKT, p-mTOR, mTOR, PTEN, S6K, p-S6K, ULK1, p-ULK1, Bcl-2, and Caspase3 in the ovary tissues was determined by Western blot (**p<0.01 vs. control, #p<0.05 vs. VCD). B. The expression level of p-AKT and caspase-3 was evaluated by immunohistochemistry assay.