Long-term intermittent cold exposure affects peri-ovarian adipose tissue and ovarian microenvironment in rats

Li Zhang  
Tianjin Institute of Environmental and Operational Medicine

Gaihong An  
Tianjin Institute Of Environmental and Operational Medicine

Shuai WU  
Tianjin Institute of Environmental and Operational Medicine

Jing Wang  
Tianjin Institute of Environmental and Operational Medicine

Danfeng Yang  
Tianjin Institute of Environmental and Operational Medicine

Yongqiang Zhang  
Tianjin Institute of Environmental and Operational Medicine

Xi Li  
woshiliulangdeyu07@163.com  
Tianjin Institute of Environmental and Operational Medicine

Research

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Abstract

Background: This study aimed to investigate the effects of cold exposure on peri-ovarian adipose tissue (POAT) and the relationship between POAT changes and ovarian function, to gain a deeper understanding of the mechanisms associated with cold exposure-induced changes in female reproductive health.

Methods: Twenty female rats with regular estrus cycles were randomly divided into two groups: (1) the cold exposure group, which was exposed to –10°C, for 4 hours/day, for two weeks, and (2) the control group, which was maintained at 23 ± 1°C.

Results: Cold exposure can increase serum E2 and FSH levels, and reduce AMH hormone levels, and cause the numbers of primary and secondary follicles in the cold-exposed group were significantly higher than those in the control group. Meanwhile, cold-stress-induced browning-related genes, including UCP1, PGC -1a, PRDM16, Fndc5 were upregulated in POAT, and the number of mitochondria in the cold-exposed group was significantly increased. Under cold exposure, the level of adipokines, including APDN, Lep, AMPK were significantly upregulated in POAT. FST was also upregulated in POAT, whereas MST, an AMPK inhibitor, was downregulated. The E2 synthesis rate-limiting-enzymes Cyplla1 and Cyp19 were upregulated in POAT.

Conclusion: Cold exposure caused abnormal follicle development and promoted the browning of POAT. POAT browning was accompanied by the activation of the AMPK-PGC1α-Fndc5 pathway.

Introduction

Cold is a significant environmental stress factor. Studies have shown that exposure to cold environments can cause local or whole-body temperatures to decrease, posing a severe threat to overall health [1, 2, 3]. Cold exposure has adverse effects on the female reproductive system [4, 5, 6, 7], affecting ovarian [8] and uterine [4] functions and hormone secretion [9]. However, the exact mechanisms through which these changes occur have not been well-elucidated.

Estradiol (E2) and follicle-stimulating hormone (FSH) are important steroid-regulating hormones expressed in the ovary. More than 95% of the E2 in circulation is secreted by the ovary, and the growth and development of follicles during each maturation stage require the presence of E2. In addition, E2 can directly promote the development and maturation of eggs and the growth of ovarian granulocytes, in cooperation with FSH [10]. Follistatin (FST) is a non-steroidal ovarian hormone that regulates the secretion and signal transduction of sex hormones and promotes oocyte maturation and embryonic development. FST contents gradually increase as follicles develop. These steroids, non-steroid hormones, and growth factors act as regulatory factors and constitute the microenvironment that determines follicle growth and development. The ovarian microenvironment plays a vital role in ovarian function and follicle development. However, the effects of cold exposure on ovarian function and the ovarian microenvironment have not been well-elucidated.
Studies have shown that the development and maturation of follicles prior to ovulation are primarily regulated by the central neuroendocrine system and growth factors and hormones found in the local ovarian microenvironment [11, 12]. Peri-ovarian adipose tissue (POAT) is a type of white adipose tissue that surrounds the ovaries of rodents. POAT is known to be involved in the development of gonads and germ cells. Unlike brown adipose tissue (BAT), which produces heat, white adipose tissue (WAT) primarily stores energy and secretes biologically active adipokines. Under certain stimuli (cold exposure [13, 14], exercise, etc), WAT will demonstrate characteristics that are typical of BAT, associated with the increased expression levels of BAT marker genes, such as uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), PR-domain-containing 16 (PRDM16), and fibronectin type III domain-containing protein (Fndc5), in a process known as the “browning of WAT.” The body can achieve non-shivering thermogenesis through the browning of WAT [15]. Research on the browning of WAT under cold exposure conditions has primarily focused on subcutaneous adipose tissue (SAT), such as inguinal WAT (iWAT) [16], whereas changes in visceral adipose tissue (VAT), such as POAT, are rarely reported. Determining whether POAT browns after cold exposure and whether POAT browning affects ovarian function is necessary to better understand the mechanisms that influence the reproductive system in cold environments.

In recent years, many studies have shown that adiponectin (ADPN), leptin (Lep), adenylate-activated protein kinase (AMPK), and other adipokines are involved in ovarian function. High levels of ADPN can regulate the production of ovarian steroid regulatory factors, such as E2, FSH, and progesterone (P) [17, 18]. In addition, Lep is expressed in follicular cells and oocytes [19], and its level is positively correlated with E2 and LH levels; levels that are either too high or too low will affect reproductive function [20]. Ovarian microenvironmental regulators, such as FST, also affect adipokines and the browning of WAT. For example, Shane et al. found that FST promotes the browning of WAT, through the AMPK-PGC-1α-Fndc5 axis [21]. These results confirmed the existence of an association between POAT adipokines and ovarian microenvironment regulators. Therefore, whether POAT browns after cold exposure and is regulated by the activation of the AMPK-PGC-1α-Fndc5 pathway remains uncertain. Additionally, the browning of WAT may be regulated by the levels of E2, FSH, and FST in the ovarian microenvironment. Further research on this issue will help us to understand the regulatory mechanisms of ovarian dysfunction that are induced by cold exposure and provide further information regarding the ovarian regulation mechanism.

**Materials And Methods**

**Animal Experiments and Ethical Approval**

Specific pathogen-free, female, Sprague–Dawley (SD) rats, weighing 200 ± 10 g, were obtained from Weitong Lihua Experimental Animal Technology Co., Ltd (Beijing, China) and were housed at 23 ± 1°C and 45–60% humidity, under a 12-h light-dark cycle, with free access to food and water. The animals were fed in their cages for two days, and then vaginal cytological smears were performed, at the same time
every morning and evening. A total of 20 rats with regular estrous cycles were randomly divided into two groups: (1) the control group, in which the rats were maintained at room temperature (23 ± 1°C), for two weeks; and (2) the cold exposure group, in which the rats were maintained at −10°C, for 4 h each day, for two weeks. During cold exposure, all of the rats were fasted and were prohibited from consuming water. Within 8 h after the last cold exposure, a surgical procedure was conducted during the diestrus stage. Blood was sampled from the abdominal aorta, after which, the animals were immediately euthanized. The organs of ovaries and POAT were removed from each rat. The blood samples were centrifuged at 3,000 rpm for 15 min and stored at -80°C.

**Body Weight and Organ Coefficients**

To determine the effects of cold exposure procedures, the body weights and ovary coefficients were measured before and at the end of the experiment in both groups. The ovaries of each rat were dissected and weighed after euthanasia (wet basis). The following formula was used to calculate the organ coefficients:

\[
\text{organ coefficients (\%)} = \frac{\text{Organ weight (g)}}{\text{Body weight (g)}} \times 100\%
\]

**Serum Hormonal Analysis**

The serum levels of FSH, E₂, P, and testosterone (T) were determined using a radioimmunoassay kit (FuRui Runze Biotechnology, Beijing, China), according to the manufacturer's instructions.

**Immunohistochemistry**

Ovary and POAT sections were deparaffinized, rehydrated, and microwaved in 0.01 M citrate buffer (pH 6.0) for antigen retrieval. The sections were then blocked with normal serum and incubated with anti-FSH receptor (FSHR, GB11275-1, Abcam, 1:800) and anti-estrogen receptor β (ERβ) antibodies (GB11268, Abcam, 1:500). Using the Image-Pro Plus 6.0 analysis system (Media Cybernetics, Silver Spring, MD), protein expression was measured, and the regional average optical density value was determined for quantitative analysis.

**Determination of AMH Levels in Serum**

The serum AMH level was determined by enzyme-linked immunosorbent assay (ELISA). The specific experimental steps were performed in strict accordance with the kit instructions [Rat Mueller tube inhibitor/anti-Mueller tube hormone (AMH) ELISA kit (KAMIYA, KT-35857)].

**Ovarian Histology and Antral Follicle Counts**

The ovaries (n = 10) were fixed, processed, stained with eosin and hematoxylin, and examined under a light microscope, to quantify the ovarian follicular reserve. Ten representative sections from each ovary
were selected for follicle counting, with each observed section separated by a distance of over 40 µm. Differential follicle counts were determined by referring to previous literature [22, 23, 24]. The follicles were classified as primitive follicles, primary follicles, secondary follicles, antral follicles, or corpus luteum, based on their structural features [25]. All follicles were counted on every slide from serial sections.

**Transmission Electron Microscopy Analysis**

POAT samples were obtained and fixed in 10% glutaraldehyde, at 4°C for 2 h, and then dehydrated through a graded ethanol series and embedded in embedding agent (Wuhan Service Biotechnology CO., LTD, 90529-77-4). Ultrathin sections, 60–80 nm thick, were cut using an ultrathin microtome (Leica, Leica UC7). A Hitachi transmission electron microscope (HT7700) was used to observe and analyze the images.

**RNA Extraction and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)**

Total RNA was isolated from the ovary and POAT, using RNeasy Mini kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized from 500 ng total RNA, using PrimeScripe RT Master Mix (Perfect Real Time, Takara Bio, Otsu, Japan). Reverse transcription-quantitative real-time-polymerase chain reaction (RT-qPCR) was performed using Step One Plus (Applied Biosystems, Foster City, CA, USA). Template cDNA (5 ng/µl) was mixed with Fast SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), distilled water, and primers (final concentration 500 nM). The reaction was performed at 95°C for 20 s, followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. The primers were designed as follows: FST, F- CAGCGACAATGCCACGTA, R- TGCAACACTGGTGACAGTTTA; Lep, F- CGAGACCTCCTCCATCTGCT, R- CTGCTCAAGGCTCCACCTC; ADPN, F- CTGGGCATCTCTGCCATCA, R- CTGGACAAGGACCTCAGCGATA; AMPK, F- TGAGCTTACAGCTTTACCTGGTG, R- CACTTGACCCAGGCTGTGGA; UCP-1, F- GTACCAGCTGTGCAATGAC, R- GATGACGTCCAGGATCCGA; PGC-1α, F- GCACTGACAGATGGACGCT, R- TCATTGTAGCTGAGCTGTGGA; PRDM16, F- ACAAGGGGAAGCCACGAG, R- GAGGGGGAAGGGAATG; Dpt, F- AGGGCTCAGCAGCAGTGGAACTA, R- ACTGACTCGAAGTAACCGCTTTGG; HOXC9, F- CGGCAGCAAGCACAAAGAG, R- ACCGACGGTGATCCACCCAG; β-actin, F- CCTAAGGCCAACCGTGAAAA, R- CAGAGGCATACAGGGACACAC. The relative expression level of each gene was determined using the ΔΔCt method, normalized against β-actin expression.

**Statistical Analysis**

All values are presented as the mean ± standard error. SPSS 21.0 statistical software was used for statistical analysis. An independent Student’s t-test was performed to determine significant differences
between experimental groups. A repeated-measures analysis of variance (ANOVA), followed by a Tukey's post hoc test, was implemented to compare body weight gains. \( P < 0.05 \) indicated a significant difference.

## Results

### Effect of cold Exposure on body Weight and Organ Coefficient in Female Rats.

Consistent with our previous results, the cold exposure group showed a significantly reduced rate of body weight gain compared with that in the control group (Fig. 1A). The ovary coefficients no significant difference between the two groups (Fig. 1B). The levels of serum \( E_2 \) and FSH in female rats were significantly higher in the cold exposure group than in the control group, whereas the levels of serum \( P \) and \( T \) showed no significant differences between the two groups (Fig. 1C). Compared with those in the control group, the expression levels of \( \text{ERb} \) (Fig. 1D-I and II and E) and \( \text{FSHR} \) (Fig. 1D- and E) were significantly down-regulated in the cold exposure group (\( P < 0.05 \)).

### Cold exposure affects follicular development

The results showed that the serum AMH level decreased significantly after cold exposure for 14 days (Fig. 2A). We also counted the follicles on each slice. Statistical analysis showed that the numbers of primary and secondary follicles in the cold-exposed group were significantly higher than those in the control group (\( P < 0.05 \)), whereas the numbers of antral follicles, corpus lutea, and total follicles showed no significant differences (Fig. 2B).

### Cold exposure promotes POAT browning

After chronic cold exposure, no significant differences in the expression of WAT-related genes, such as \( \text{Dpt} \) and \( \text{HOXC9} \), were observed between the cold exposure group and the control group (Fig. 3A). However, the expression levels of four BAT-related genes, including \( \text{UCP1} \), \( \text{PGC-1a} \), \( \text{Fndc5} \), and \( \text{PRDM16} \), increased significantly in the cold-exposed group compared with those in the control group (\( P < 0.05 \)), as assessed by RT-qPCR on RNA isolated from POAT (Fig. 3C). When comparing the organelles and cell morphologies between the control and cold exposure groups, no obvious lipid droplets were observed in samples from either group, whereas the number of mitochondria in the cold-exposed group was significantly higher than that in the control group. These results showed that cold exposure had a significant effect on the number of mitochondria. These findings confirmed that cold exposure could induce POAT browning (Fig. 3B).

### POAT browning alters the local ovarian microenvironment

Our results showed that the gene expression levels of \( \text{AMPK} \), \( \text{Lep} \), and \( \text{ADPN} \) in the cold exposure group were significantly upregulated compared with those in the control group (Fig. 4A). The \( \text{FST} \) mRNA levels in POAT and ovarian tissue were significantly increased in the cold exposure group compared with those in the control group, whereas the \( \text{MST} \) mRNA level in POAT showed a significant decrease in the cold
exposure group compared with that in the control group (Fig. 4B). The cyb11a1 and cyp19a1 mRNA levels in the ovary showed substantial increases in the cold exposure group compared with those in the control group (Fig. 4B).

**Discussion**

Cold is a necessary environmental stimulus. With rising global temperatures, the probability of working at high altitudes or in cold, polar environments increases significantly [26]. Additionally, cold exposure for losing weight has become a behavioral trend among some female groups [27, 28], which dramatically increases the exposure of females to cold environments. Therefore, studying the changes and mechanisms of female physiological functions that are associated with cold exposure is both necessary and vital.

Our results showed that body weight gains decreased and changes were observed in the levels of serum sex hormones and ovarian hormone receptors, although the ovarian organ index did not change significantly. Additionally, serum AMH levels decreased and the numbers of primary and secondary follicles increased, after two weeks of cold exposure. AMH is one of the most effective and sensitive serological indicators of ovarian function [29], which directly reflects the ovarian reserve function. AMH can inhibit the recruitment of primitive follicles and the early growth of follicles by activating AMH receptor β, preventing premature follicle failure, and AMH concentrations change with age instead of undergoing menstruation-related periodic changes. Follicle counting (FC) is used to examine the different types of follicles during folliculogenesis. Currently, AMH and FC are used to evaluate the development of follicles at different stages under various stimulation conditions [22, 23, 24]. The results of this study and our previous studies [9] have suggested that cold exposure can affect the metabolism of rats, resulting in reductions in weight gain and damage to the ovarian structure and reserve function, which affects follicular development.

POAT is a type of WAT that clings to the ovary. In mammals, adipose tissue exists as BAT, WAT, and beige adipose tissue. BAT produces heat, whereas WAT stores energy and participates in endocrine functions. Studies have shown that to promote bodily adaptations to a cold environment under cold exposure conditions, subcutaneous WAT, such as inguinal fat (iWAT), will brown [6]. However, no research has examined whether gonadal adipose tissue, such as POAT, browns after cold exposure, and some researchers believe that this type of organ-associated adipose tissue does not brown [30]. In this study, our results suggested that after cold exposure, the number of adipocyte mitochondria in POAT increased significantly, and specific genes associated with the browning of WAT, such as UCP1, PGC-1α, PRDM16, and Fndc5, were significantly upregulated. Two weeks of continuous cold exposure was found to induce browning in gonadal adipose tissues, such as POAT.

The changes that occur in the POAT after browning and the effects of these changes on the peri-ovarian microenvironment, the ovarian microenvironment, and ovarian function have not been explored. We found that after POAT browning, the expression levels of adipokines, such as APDN, Lep, and AMPK, were
significantly increased, similar to the effects observed during iWAT browning [4, 31]. Our results suggested that the peri-ovarian microenvironment changes after POAT browning.

Ovarian estrogen, which is regulated by endocrine factors from the central nervous system, can affect the differentiation of female ovarian cells and plays an important role in the regulation of follicular development. E₂ is the steroid hormone with the highest estrogen content and the strongest biological activity. P and T are intermediate products of E₂ synthesis. Studies have shown that E₂ deficiency (such as in an E₂ synthesis rate-limiting enzyme knockout) causes mouse follicle development to stop during the sinusoidal follicle stage, and these symptoms can be relieved by the administration of exogenous E₂ [10]. Simultaneously, other studies have shown that E₂ exerts anti-apoptotic functions and plays roles in cell protection and the regulation of lipid metabolism [32]. FST is a nonsteroidal hormone expressed in the ovary, brain, pituitary, and adrenal gland and acts as an important local regulatory factor for ovarian follicles [33]. FST regulates FSH secretion [34], promotes follicle maturation [35], and promotes embryo development. Jorgez et al. [35] found that mature follicles and oocytes in FST-knockout adult animals presented delayed maturation and development, and ovarian activity was terminated in advance. The administration of exogenous FST to bovine embryos during the cleavage stage could significantly improve the early cleavage rate. Regulatory factors, such as E₂, FSH, and FST, together constitute the ovarian microenvironment. Increasingly, researchers believe that in addition to the central nervous system regulation, ovarian function is also regulated by both the ovarian and peri-ovarian microenvironment. Therefore, we speculate that the ovarian and peri-ovarian microenvironments may also play important roles in the follicular dysplasia induced by cold exposure.

Previous studies have shown that APDN, Lep, AMPK, and other adipokines can exert effects through autocrine, paracrine, and endocrine mechanisms. On the one hand, adipokines affect the development of follicles, and on the other hand, they affect lipid accumulation and adipose tissue metabolism in the ovarian microenvironment. APDN plays an important role in follicular development, by regulating cytochrome P450 cholesterol side-chain lyase (Cyp11a1), acute regulatory protein (STAR), 3β-hydroxysteroid synthase (3β-HSD), 17-α hydroxylase 17, 20-lyase (Cyp17), aromatase (Cyp19) and other rate-limiting enzymes in the E₂ synthesis pathway, to promote E₂ synthesis. In POAT-excised mice, APDN contents decreased, and the expression levels of Cyp11a1, Cyp19, and other genes were suppressed, resulting in decreased E₂ expression in the ovarian microenvironment. Leptin can act on the ovary to promote the sensitivity of granulosa cells to FSH [36]. Wang et al. [12] have demonstrated that POAT excision can cause a decrease in the Lep level of the ovarian microenvironment, which ultimately causes the granulosa cells to become less sensitive to FSH. In addition, in Lep-knockout mice, estrogen synthesis is reduced [37]. These results showed that changes in APDN and Lep levels could affect the expression levels of E₂ and FSH. Therefore, we detected the serum levels E₂, FSH, T, and P, and examined the gene expression levels of ERβ, FSHR, and the rate-limiting enzymes of the E₂ synthesis pathway in rat ovaries after cold exposure. The results showed no differences in serum P and T levels, whereas serum E₂ and FSH levels increased, ovarian ERβ and FSHR expression decreased, and rate-limiting enzymes in the E₂ synthesis pathway, such as Cyp11a1 and Cyp19a1, were upregulated. Ahima et al. [38] also found that Lep
treatment could significantly increase the numbers of primordial follicles, primary follicles, secondary follicles, and mature follicles in obese female mice, suggesting that Lep administration can improve the physiological functions of the ovary, to a certain extent. In this study, Lep upregulation appeared to play a similar role. Although the total numbers of follicles in rats did not change significantly after cold exposure, the numbers of primary and stimulated follicles increased significantly. These results suggested that APDN and Lep may affect follicular development and the ovarian microenvironment after POAT browning.

The development and maturation of follicles require energy, provided by various substrates (glucose, proteins, and lipids). AMPK is involved in energy metabolism, and decreased AMPK expression leads to a decrease in lipid accumulation in the ovarian microenvironment and the compensatory activation of the fatty acid biosynthesis pathway in the ovary [12]. Simultaneously, AMPK participates in the browning of adipose tissue, through the AMPK-PGC1α-Fndc5 pathway. Shan et al. found that the browning of WAT occurred in myostatin (MST)-knockout rats. The deletion of MST led to the increased expression of AMPK protein, and AMPK indirectly activated the expression of PGC-1α and Fndc5. PGC-1α and Fndc5 are related genes that promote the expression of BAT and beige adipose tissue [39, 40]. MST is a negative regulator of skeletal muscle and inhibits AMPK protein expression [41, 42], and MST is inhibited by FST [43]. FST is closely related to the browning of adipose tissue [21, 44]. Singh et al. [44] found that when FST is overexpressed in transgenic mice, the quality of BAT increased and the expression levels of proteins associated with BAT and beige fat increased in WAT [44]. Exogenous FST can promote the expression of Fndc5 in mouse cells [21]. In this study, after the browning of POAT, the expression level of MST was downregulated in POAT, and FST levels in the POAT and ovary were upregulated. These results suggested that FST may promote the browning of WAT by inhibiting MST and activating the AMPK-PGC1α-Fndc5 pathway.

Therefore, we believe that cold exposure causes abnormal follicular development, damages ovarian function, and induces POAT browning. POAT browning relieves the adverse effects associated with cold exposure on ovarian function, to a certain extent. Although we were unable to determine the exact timing of POAT browning and ovarian microenvironment changes, we tend to believe that after cold stimulation, mutual adjustments occur in the levels of adipokines induced by POAT browning and ovarian regulatory factors. This process constitutes one of the body's compensation adjustment mechanisms following cold exposure. In addition, WAT, such as subcutaneous adipose tissue (iWAT) and adipose organ tissue (peridimensional adipose tissue), can directly sense temperature and generate heat, inducing the increased expression of UCP1 and PRDM16 (by 2–3-fold) [45]. We speculate that the effects of POAT browning on the local ovarian microenvironment, due to changes in adipokines, may represent a regulatory mechanism, independent of central regulation. However, this hypothesis requires further verification.

Our study has several limitations. First, the relationship between POAT adipokines and the local ovarian microenvironment has not been fully elucidated and requires further investigation. Second, without dynamic observations, whether POAT browning has a definite protective effect on follicular development cannot be determined conclusively. Third, due to the lack of cold exposure intensity gradient verification,
the potential and limits of POAT browning to provide compensatory protection for ovarian function under cold exposure conditions have not yet been elucidated. Future research remains necessary to clarify these issues. We hope that our research can stimulate interest in this field.

**Conclusions**

In summary, this research showed that cold exposure can cause abnormal follicle development and promote the browning of POAT. POAT browning is accompanied by the activation of the AMPK-PGC1α- Fndc5 pathway. The effects of POAT browning on the local microenvironment of the ovary is hypothesized to represent a mechanism through which the body compensates for the regulation of ovarian function under cold exposure conditions.

**Abbreviations**

3β-HSD: 3β-hydroxysteroid synthase; ADPN: adiponectin; AMH: anti-Mueller tube hormone; AMPK: adenylate-activated protein kinase; BAT: brown adipose tissue; Cyp19: aromatase; Cyplla1: cytochrome P450 cholesterol side chain lyase; Dpt: dermatopontin; E2: estradiol; FC: follicle counting; Fndc5:Fibronectin Type Domain containing Protein5; FSH: follicle-stimulating hormone; FST: follistatin; Hoxc9: homeobox C9; iWAT:inguinal WAT; LEP: leptin; MST:myostatin; P: progesterone; PGC-1α: peroxisome proliferators activated receptor-γ coactivator-1α; POAT: peri-ovarian adipose tissue; PRDM16: PR domain containing 16; STAR: acute regulatory protein; TEM: Transmission electron microscopy; UCP1: uncoupling protein 1; WAT: white adipose tissue

**Declarations**

**Ethics approval and consent to participate**

All procedures relating to animal care and use were approved by the Ethics Review Committee of the Institute of Environmental and Operational Medicine (IACUC of AMMS-04-2020-017 ).

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the publicly available SEER database.

**Competing interests**

The authors declare that they have no competing interests.
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Authors’ Contributions

Li Zhang and Gaihong An conceived and designed the study, performed data analysis and data interpretation, and drafted the manuscript. Li Zhang, Shuai Wu, and Jing Wang performed all the experiments and carried out data analysis. Danfeng Yang, Yongqiang Zhang, and Xi Li were involved in the data interpretation and discussion. All participated in the writing and approved the final version of the manuscript.

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**Figures**
Figure 1

Cold exposure impairs the ovarian function of female rats. A Effects of cold exposure on body weight gain (n = 10). B Effects of cold exposure on the ovary coefficients (n = 10). C Serum levels of E2, FSH, P, and T in control and cold-exposed groups of female rats (n = 10). D and E Expression levels of ERβ and FSHR in ovarian tissue in cold-exposed and control groups of female rats (n = 6). Values are presented as the mean ± standard error. *P < 0.05 and **P < 0.01.
Figure 2

Effect of Cold Exposure on follicular development in female rats. A Serum AMH levels in control and cold-exposed groups of female rats (n = 10). B Effects of cold exposure on ovarian histology and antral follicle counts (n = 10).

Figure 3
Cold exposure promotes POAT browning. A Gene expression levels of Dpt and HOXC9 in cold-exposed and control groups of female rats (n = 6). B Ultrastructural changes in POAT, assessed by TEM. Control group: (I and II). Cold exposure group:(III and IV). C Gene expression levels of UCP1, PGC1α, Fndc5, and PRDM16 in cold-exposed and control groups of female rats (n=6). Values are presented as the mean ± standard error. *P < 0.05 and **P < 0.01.
Effects of cold exposure on the local ovarian microenvironment of female rats. A Gene expression levels of Lep, ADPN, and AMPK in both groups (n = 6). B Gene expression levels of FST in the ovary and POAT of both groups (n = 6). C Gene expression levels of cyb11b2 and cyp19a1 in the ovary of both groups (n = 6). Values are presented as the mean ± standard error. *P < 0.05 and **P < 0.01.

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