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Concentrated ambient fine particles exposure affects ovarian follicle development in mice

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ABSTRACT

Background: Ambient fine particles ($PM_{2.5}$) are known to cause various reproductive and developmental diseases. However, the potential mechanisms of $PM_{2.5}$ exposure induced female reproductive damage remain unclear.

Methods: Four weeks old female C57BL/6 J mice were exposed to filtered air (FA, $n = 10$) or concentrated ambient $PM_{2.5}$ (CAP, $n = 10$) using a versatile aerosol concentration enrichment system. After 9 weeks of the exposure, mice were sacrificed under sevoflurane anesthesia and tissue samples were collected. Immunohistochemical analysis, enzyme-linked immunosorbent assay, quantitative polymerase chain reaction, and RNA-sequencing were performed to analyze the effects of $PM_{2.5}$ exposure on follicle development and elucidate its potential mechanisms.

Results: Chronic $PM_{2.5}$ exposure resulted in follicular dysplasia. Compared to the FA-exposed group, follicular atresia in the CAP-exposed mice were significantly increased. Further studies confirmed that CAP induced apoptosis in granulosa cells, accompanied by a distortion of hormone homeostasis. In addition, RNA-sequencing data demonstrated that CAP exposure induced the alteration of ovarian gene expressions and was associated with inflammatory response.

Conclusions: Chronic exposure to CAP can induce follicular atresia, which was associated with hormone modulation and inflammation.

1. Background

Ambient fine particulate matter ($PM_{2.5}$) composed of solid particles and liquid droplets with an aerodynamic diameter ≤ 2.5 μm (Ma et al., 2017) is one of the most important public health risks in the world. It has been found that the majority of the inhaled $PM_{2.5}$ are deposited in the airway, while some of the $PM_{2.5}$ may enter the circulatory system (Wu et al., 2018). Long-term exposure to $PM_{2.5}$ has been found to be associated with subfertility and reproductive abnormalities, such as increased infertility rate, restricted fetal growth, preterm birth, and neonatal death (Pedersen et al., 2013; Hammoud et al., 2010; Mahalingaiah et al., 2016; Stieb et al., 2016). However, there is little information about the effects and underlying mechanisms of $PM_{2.5}$ exposure on female reproductive health, such as polycystic ovarian syndrome and premature ovarian failure.

The ovarian follicles are the basic structural and functional units of the mammalian ovary and are of great importance to reproductive health. Formed by oocytes, granulosa cells, and theca cells, follicles are places where oocyte maturation is nurtured and regulated by surrounding somatic cells (Honda et al., 2007; Verbraak et al., 2011). During the female fertile lifespan, the majority of the ovarian primordial follicles eventually undergo atresia and only a limited number of primordial follicles develop to the ovulatory stage(Ksiazkiewicz, 2006). Follicular recruitment, selection, ovulation, and atresia are

Abbreviations: AMH, Anti-Müllerian hormone; CAP, concentrated ambient $PM_{2.5}$; DEGs, Differentially expressed genes; E2, estradiol; FA, iltered air; FSH, Follicle-stimulating hormone; GO, gene ontology; HRP, horseradish peroxidase; LH, Luteinizing hormone; P4, progesterone; TMB, Tetramethylbenzidine; VACES, versatile aerosol concentration enrichment system.

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highly-orchestrated, periodic, and gonadotropin-dependent processes in which endocrine hormones and intraovarian regulators play crucial roles (Gebremedhn et al., 2015; Liu et al., 2019). Follicle-stimulating hormone (FSH) stimulates granulosa cell proliferation, steroid hormone production, and follicle development, which are critical for oocyte maturation (Alam and Miyano, 2020; Nowak, 2018). Luteinizing hormone (LH) triggers ovulation and luteinization of the follicle, accompanied by a marked increase in progesterone secretion (Przygrodzka et al., 2021; Elmaoğulları and Aycan, 2018). Granulosa cells are the principal cellular source of estradiol (E2) and progesterone (P4); meanwhile, it also produces chemokines and inflammatory cytokines, which mediate ovarian follicular development (Liu et al., 2021). Follicular atresia is a hormone-controlled apoptotic process that occurs naturally in granulosa cells at every stage of follicular development (Yeung et al., 2017); however, it can be excessively induced by nutrient deprivation, exposure to air pollution, or oxidative stress (He et al., 2016; Shen et al., 2012). Recently, epidemiological studies have found strong associations between ambient $PM_{2.5}$ and menstrual disorders in women. In addition, toxicological studies reported ovarian damage caused by exposure to $PM_{2.5}$ or diesel exhaust particles. However, studies regarding the detailed molecular mechanisms through which ambient $PM_{2.5}$ causes ovarian dysfunction are still limited.

In this study, to determine whether exposure to ambient $PM_{2.5}$ has toxic effects on the female reproductive system, female C57BL/6 J mice were exposed to concentrated ambient $PM_{2.5}$ (CAP) for 9 weeks and their ovarian follicle development was assessed. To better understand the potential underlying mechanisms, the granulosa cell apoptosis, hormone homeostasis, and global gene expression profile of ovaries (using RNA-sequencing) were evaluated.

2. Materials and methods

2.1. Chemicals and reagents

DAPI (4′,6-diamidino-2-phenylindole) staining solution, anti-Ki67 antibody and in situ cell death detection kit were obtained from Beyotime Institute Biotech (Jiangsu, China). Anti-Müllerian hormone (AMH), FSH, and LH enzyme-linked immunosorbent assay (ELISA) kits were provided by Huzhen Biological Technology (Shanghai, China), Liuhe Biological Technology (Wuhan, Hubei, China), and Cusabio LLC (Houston, TX, USA), respectively. P4 and E2 ELISA kits were obtained from EK-Bioscience (Shanghai, China). Interleukin (IL)− 1β, IL-6, and tumor necrotic factor-alpha (TNF-α) ELISA kits were purchased from Dakewe Bio-engineering (Shenzhen, China). TRIzol reagent and Power SYBR Green polymerase chain reaction (PCR) Master Mix were purchased from Thermo Fisher Scientific Inc. (Grand Island, NY, USA). PrimeScript RT Master Mix (Perfect Real-Time) was purchased from Takara Bio Inc. (Shiga, Japan). PCR plastic consumables were purchased from Corning Inc. (Union City, CA, USA). All other reagents and chemicals used in this study were of analytical grade and were obtained from local sources.

2.2. Animals and whole-body inhalation of concentrated ambient $PM_{2.5}$ (CAP)

Four weeks old female C57BL/6J mice were obtained from the Laboratory Animal Center of Fudan University (Shanghai, China). After one week of acclimation, mice were exposed to filtered air (FA, $n = 10$) or concentrated ambient $PM_{2.5}$ (CAP, $n = 10$) using a versatile aerosol concentration enrichment system (VACES) that was modified for long-term whole-body exposures as described previously (Maciejczyk et al., 2005; Geller et al., 2005). The VACES was located on the campus of the School of Public Health, Fudan University (130 Dong'an Rd, Xuhui, Shanghai, China). The exposure was carried out from May to July of 2019. The exposure protocol comprised exposures for eight hours per day and five days per week (there was no exposure during weekends). During the exposure period, mice were housed in standard cages and given access to water and standard food ad libitum under controlled temperature (18–25 ℃) and humidity (40–60%), with a 12:12 h light-dark cycle. In the eighth to the ninth week of the exposure, vaginal smears were performed every morning to record the estrous cycles of the mice. All experimental procedures involving the use of laboratory animals were approved by the Institutional Animal Care and Use Committee of Fudan University, and all the animals were treated humanely and with regard to the alleviation of suffering.

2.3. Mouse euthanization and tissue harvesting

After 9 weeks of exposure to FA or CAP, following the above protocol, mice were sacrificed under sevoflurane anesthesia at similar estrus stages. Blood samples were collected from the orbital venous plexus, set in tubes, and allowed to clot at room temperature for 30 min before centrifugation at 3000 rpm for 15 min at 4 °C. Serum samples were then collected and stored at − 80 °C until further use. Ovaries were harvested and either fixed with neural-buffered formalin or snap-frozen in liquid nitrogen after washing in a physiological solution (0.9% NaCl) and then stored at − 80 °C.

2.4. Ovary histopathology

After fixation, the ovaries were dehydrated in ethanol, cleared in xylene, embedded in paraffin, and serially sliced into 5 μm thick sections. To assess the ovarian follicle development, sections (one in every five sections) were stained with hematoxylin and eosin (H&E) and observed under light microscopy. Follicles were categorized and counted by a pathologist who was blind to the grouping as described previously (Brown et al., 2010; Myers et al., 2004; Xu et al., 2018; Paulose et al., 2012). In brief, the primordial follicle consists of an oocyte surrounded by a monolayer of flattened granulosa cells. The flattened granulosa cells then become squamous, cuboidal, or columnar, thereby forming primary follicles. As the granulosa cells constantly divide to produce multiple layers, the structure is called a secondary follicle. After this phase, the antral follicles are marked by the formation of fluid-filled vesicles among the granulosa cells, with the oocyte enclosed in the cumulus mass. In the Graafian follicle, the antrum is enlarged, with the cumulus oophorous diminished, and the oocyte with corona radiata is free-floated. The atretic follicle is defined when the pyknotic granulosa cells in the antral follicle appear or when the oocyte degenerates.

2.5. In situ DNA labeling by TUNEL assay

The TUNEL assay for apoptosis was performed in ovary sections according to the manufacturer's instructions. In brief, the sections were deparaffinized with xylene, rehydrated, permeabilized with 1% Triton X-100 at 4 °C for 10 min, and incubated with proteinase K at 37 °C for 20 min. Afterward, the sections were rinsed in phosphate-buffered saline (PBS), labeled by incubation with TUNEL reaction mixture for 1 h, and counterstained with DAPI (5 μg/ml) for 15 min at 37 °C in a humidified chamber. After washing with PBS, the sections were observed under a Nikon A1 confocal microscope (Nikon Corp., Japan).

2.6. Immunohistochemical analysis

After deparaffinization and hydration, the tissue sections were heated (in boiling water bath) in antigen retrieval solution for 30 min, followed by cooling to room temperature. The endogen peroxidase activity was inhibited with methanol containing 3% H_2O_2, permeabilized for 10 min with 1% Triton X-100 in PBS, and blocked for 60 min with 5% BSA in PBS at room temperature. All sections were incubated with a primary antibody (1:100) overnight in a humidified chamber at 4 °C, followed by incubation in the biotinylated secondary antibody and streptavidin-peroxidase. Finally, sections were stained with 3,3′-

diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin before viewing under an Olympus BX-43 light microscope (Olympus Corporation, Japan).

2.7. Assessment of serum hormones and cytokines

Serum hormones and cytokines levels were quantified using solid-phase sandwich ELISA kits according to the manufacturers' instructions. In brief, the antigen was captured by a specific antibody that has been pre-coated to the plate and the immobilized antigen can be bonded with horseradish peroxidase (HRP)-conjugated antibody. Tetramethylbenzidine was used as an HRP substrate and the product is in proportion to the amount of antigen. The optical density was quantified using a Multiskan FC microplate photometer (Thermo Fisher Scientific, Inc.) at 450 nm, with a reference wavelength of 620 nm. A standard curve was obtained with a serially diluted standard and hormone level in the serum samples was extrapolated from the standard curve.

2.8. Real-Time quantitative polymerase chain reaction (RT-qPCR) analysis of target gene mRNA expression

Ovarian RNA was extracted using TRIzol reagent and its concentration was determined using an Infinite M200 Pro microplate reader (Tecan Group Ltd.). 500 ng of total RNA was reverse-transcribed using PrimeScript RT Master Mix. The cDNA amplification was performed on Step One Plus Real-Time PCR System (Applied Biosystems) using Power SYBR Green PCR Master mix. The PCR conditions were as follows: an initial denaturation at 95 °C for 10 min, followed by 40 cycles of two-step PCR with denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method(Livak and Schmittgen, 2001) and normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The target gene-specific primers used for the amplification of the different transcripts are listed in Table 1.

2.9. RNA-sequencing

Five ovary samples from each group were collected and used for RNA extraction, followed by mRNA sequencing. The RNA-sequencing libraries were prepared using the MGIEasy RNA Library Prep Set (MGI, Shenzhen, China). For each library, approximately 20 million clean reads were subjected to sequencing using BGISEQ 500 sequencer (BGI, Shenzhen, China). Following the base composition and quality tests, the adapter sequence and low-quality reads were filtered. The filtered reads were then mapped to the reference genes using Bowtie2 and expression levels of each gene were calculated using the RSEM software package. Differentially expressed genes (DEGs) were screened by NOISeq software, with the criteria of $|\log2 \text{ fold change}| \geq 1$ and an adjusted P-value of < 0.05. Heatmaps and volcano plots of the DEGs were established using the online program ImageGP (http://www.ehbio.com). Gene ontology (GO) analysis was performed using the database for annotation, visualization, and integrated discovery (DAVID, https://david.ncifcrf.gov).

Table 1
Primer sequence information.

Primer	Sequence (5′ to 3′)
AMH F	GCAGTTGCTAGTCCTACATC
AMH R	TCATCCGCGTGAAACAGCG
Bcl-2 F	ATGCCTTTGTGGAACTATATGGC
Bcl-2 R	GGTATGCACCCAGAGTGATGC
Bax F	TGAAGACAGGGGCCTTTTTG
Bax R	AATTCGCCGGAGACACTCG
IL-1β F	AGAGCTTCAGGCAGGCAGTA
IL-1β R	AGGTGCTCATGTCCTCATCC
IL-6 F	CCAGTTGCCTTCTTGGGACT
IL-6 R	GGTCTGTTGGGAGTGGTATCC
TNF-α F	ACGGCATGGATCTCAAAGAC
TNF-α R	GTGGGTGAGGAGCACGTAGT
GAPDH F	GGGTGGTCCAGGGTTTCTTACT
GAPDH R	AGGTTGTCTCCTGCGACTTCA

2.10. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) and analyzed by unpaired student's t-test. For all analyses, p values less than 0.05 were considered statistically significant.

3. Results

3.1. CAP exposure caused general toxicity in female mice

Fig. 1**A** illustrates the experimental scheme of the present work. The average concentration of ambient $PM_{2.5}$ during the experimental period was 14.11 ± 4.92 μg/m^3 and the average $PM_{2.5}$ concentrations in FA and CAP chambers were 3.93 ± 2.85 and 128.1 ± 91.16 μg/m^3, respectively. The exposure level in the CAP group was markedly higher than the national ambient air quality standard of China (35 μg/m^3) but was quite common in areas with heavy air pollution. The elemental composition of $PM_{2.5}$ in the CAP chamber was shown in Table 2.

No significant differences were noted in behavior, mental state, and the color and sheen of the fur between the two groups throughout the experiment. We evaluated the effect of $PM_{2.5}$ exposure on ovarian function. As described in Fig. 1**B**, CAP exposure can extend the length of estrous cycles, as the average of days between estrus in the FA-exposed and CAP-exposed groups were 5.40 ± 0.27 and 5.90 ± 0.72, respectively. Based on the cytology of the vaginal smears, CAP exposure can disturb the estrous cycle phases, although this effect was not statistically significant (Fig. 1**C**). CAP-exposed mice exhibited a remarkable increase in body weight but a non-significant increase in the ovary weight and ovarian index (Fig. 1**D**).

3.2. CAP exposure induced follicular atresia in female mice

Follicular atresia is an active process that occurs naturally at all stages of follicular development during the ovarian cycle (Worku et al., 2017). To determine the effects of $PM_{2.5}$ exposure on the follicular atresia, histological analysis was performed on the ovaries of FA-exposed or CAP-exposed mice. As shown in Fig. 2**A**, the ovaries of FA-exposed mice showed normal histological structure with continuous follicular theca and compact granulosa cell layer. After 9 weeks of exposure to CAP, pathological alterations with prominent vacuolization and granulosa cell shrinkage were observed. Afterward, we compared the follicle counts between the two groups and found no significant differences, except for atretic follicles. As demonstrated in Fig. 2**B**, the percentage of atretic follicles was significantly increased ($p < 0.05$) in the CAP-exposed mice ($16.18 \pm 1.93\%$) compared to that in the FA-exposed mice ($8.10 \pm 1.65\%$).

AMH is considered an important clinical marker of ovarian function and ovarian reserve capacity; it is not expressed in atretic follicles (Wędrychowicz et al., 2017). We measured the concentration of serum AMH, as well as the mRNA expression of *AMH* in ovaries. Our results indicated that CAP exposure significantly reduced the level of serum AMH ($p < 0.01$), as well as the mRNA expression of ovarian *AMH* ($p < 0.05$) (Fig. 2**C**).

3.3. CAP exposure promoted granulosa cell apoptosis in the atretic follicles

Granulosa cell apoptosis, which can arrest follicle development and suppress oocyte growth (Regan et al., 2018), has been considered a pivotal mechanism for follicular atresia (Worku et al., 2017). To determine whether CAP exposure induced follicular atresia via promotion of

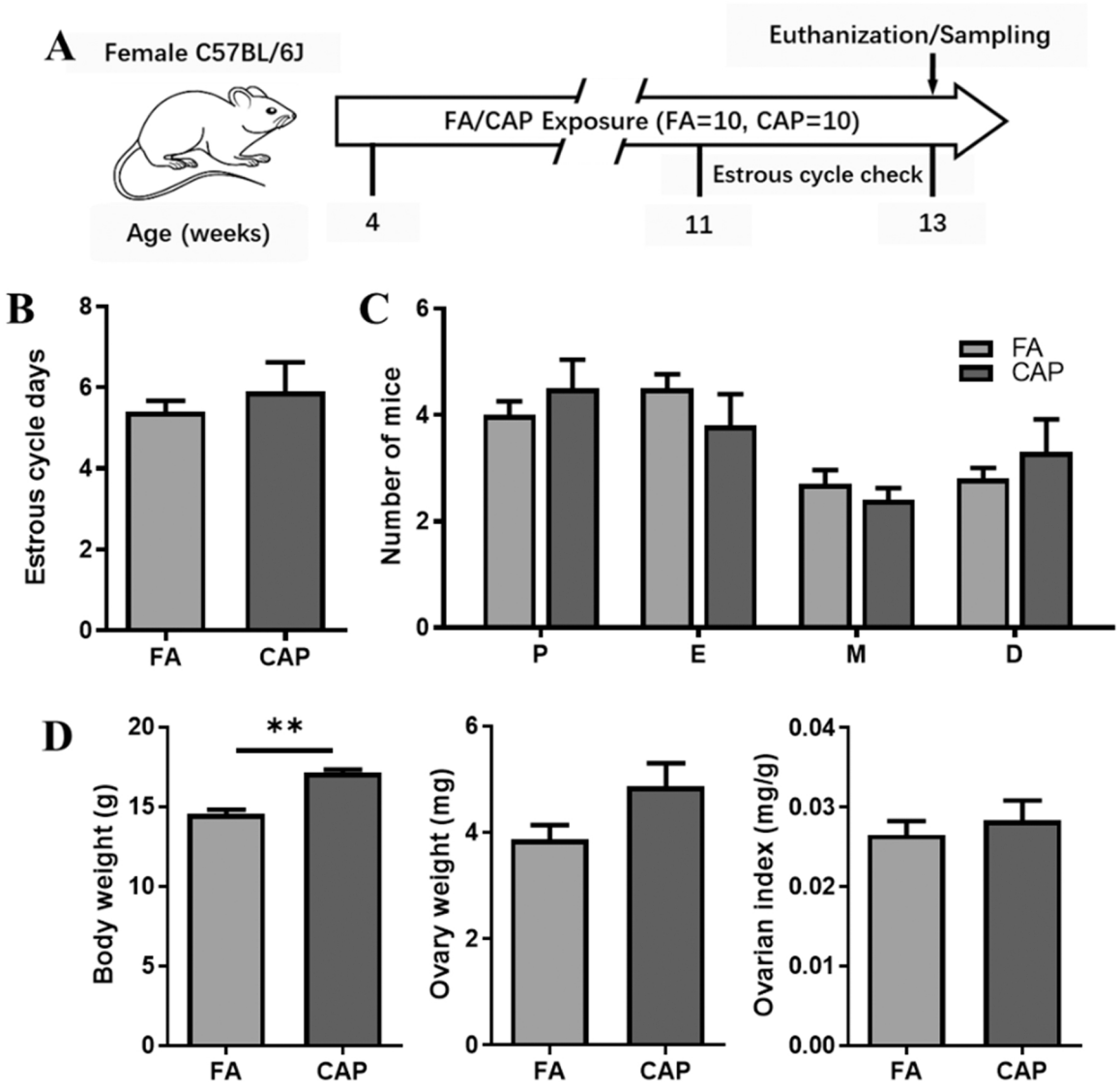

Fig. 1. Chronic exposure to CAP caused female mice general toxicity. A. The experimental scheme. B. Estrous cycle of FA/CAP-exposed mice. C. The number of mice in various stages of estrous cycle (P: proestrus, E: Estrus, M: metestrus, D: diestrus). D. Body weight, ovary weight and ovarian index of mice exposed to FA or CAP. $n = 10$, * $p < 0.05$, * * $p < 0.01$ vs. FA.

granulosa cell apoptosis, the TUNEL assay was performed. Representative images (Fig. 3**A)** demonstrated that few TUNEL-positive cells were detected in the FA-exposed group, while many TUNEL-positive cells were present in the granulosa cell layer of the CAP-exposed group (Fig. 3**B**). Since the balance between pro-apoptotic and anti-apoptotic factors is known to determine the granulosa cell fate and follicle growth (Hannon et al., 2015), we examined the expressions of apoptosis-related Bcl-2 family of genes in the ovaries. Consistent with the TUNEL assay results, the mRNA expression of *Bcl-2* in the CAP-exposed mice was significantly down-regulated ($p < 0.05$), while the mRNA expression of *Bax* was up-regulated ($p < 0.05$) (Fig. 3**C**). These findings indicated that CAP exposure induced apoptosis of granulosa cells in the ovaries.

The effect of $PM_{2.5}$ exposure on the proliferation of granulosa cells was investigated via Ki-67, a well-known indicator of cell proliferation(Salvetti et al., 2009). As illustrated in Fig. 3**D**, intense Ki-67 signals were detected in the granulosa cells and theca layers in the FA-exposed group, whereas this intensity and frequency were markedly decreased in the CAP-exposed group. In addition, the percentage of Ki-67-positive cells was significantly reduced by CAP exposure (Fig. 3**E**). These results revealed that chronic $PM_{2.5}$ exposure disturbed granulosa cell proliferation.

3.4. CAP exposure disturbed hormone homeostasis

Considering that granulosa cells are the major sources of hormones and the main site for hormones action, which are indispensable for the growth and maturation of follicles (Garg et al., 2017), the serum levels of FSH, LH, E2, and P4 were detected by ELISA. As shown in Fig. 4, chronic CAP exposure drastically altered the serum levels of FSH, E2, and P4, while it had almost no effect on LH. Specifically, serum FSH was significantly decreased ($p < 0.05$), while serum E2 ($p < 0.01$) and P4 ($p < 0.05$) levels were significantly elevated in the CAP-exposed mice compared to the FA-exposed mice. These results indicated that CAP exposure impaired the equilibrium of sex hormones in the female mice.

3.5. CAP exposure triggered an inflammatory response in the ovary

To investigate the molecular mechanisms of $PM_{2.5}$-induced ovarian dysfunction, RNA-sequencing analysis was performed. As shown in Fig. 5**A**, expressions of 536 genes (64 up-regulated and 472 down-regulated) were significantly altered by CAP exposure. The heatmap of hierarchical clustering analysis for the DEGs demonstrated an obvious clustering of the identified DEGs between the CAP-exposed mice and the

Table 2
FA/CAP samples were collected weekly, and the elemental composition was determined by X-ray fluorescence analysis (XRF).

	FA (μg/m^3)	CAP (μg/m^3)
Na	*ND*	6.19 ± 7.23
K	1.00 ± 0.32	18.28 ± 12.05
Mg	*ND*	0.36 ± 0.00
Ca	*ND*	6.30 ± 4.90
Ba	*ND*	*ND*
Cd	*ND*	*ND*
Sn	1.82 ± 0.20	2.03 ± 0.30
Ti	*ND*	0.48 ± 0.00
V	*ND*	0.51 ± 0.00
Cr	*ND*	*ND*
Mn	*ND*	1.80 ± 0.17
Fe	*ND*	16.55 ± 0.35
Co	*ND*	*ND*
Ni	*ND*	8.72 ± 8.76
Cu	0.69 ± 0.00	3.94 ± 2.64
Zn	0.22 ± 0.02	8.38 ± 4.36
As	*ND*	0.18 ± 0.00
Se	*ND*	*ND*
Pb	*ND*	1.29 ± 0.18
Al	*ND*	1.55 ± 1.62
S	4.29 ± 0.41	108.26 ± 51.26
Sc	*ND*	*ND*
P	*ND*	0.53 ± 0.51
Si	1.53 ± 0.11	11.69 ± 3.78

FA-exposed mice (Fig. 5**B**). To further investigate the biological relevance of all the DEGs, the DAVID was used to perform the GO functional enrichment analysis. The GO analysis indicated that the biological functions of DEGs focused primarily on the inflammatory response that included interleukin-12 secretion and AP-1 activation (Fig. 5**C**). Consistent with the RNA-sequencing result, the qPCR data revealed that chronic exposure to CAP resulted in a significant increase of IL-6 expression in the ovary ($p < 0.05$), while the mRNA expressions of IL-1β and TNF-α were slightly but non-significantly increased (Fig. 5**D**).

Several studies have demonstrated that inhaled $PM_{2.5}$ causes inflammatory responses in multiple extrapulmonary organs, including the ovary, through the activation of systemic inflammation, followed by pulmonary inflammation or direct penetration into circulation (Riva et al., 2011; Zhang et al., 2016). Therefore, in this study, systemic inflammation was detected by ELISA. As shown in Fig. 5**E**, our result indicated that CAP exposure significantly up-regulated the level of serum IL-6 ($p < 0.05$) and slightly increased the levels of serum IL-1β and TNF-α.

4. Discussion

Due to its large surface area and high adsorption capacity, ambient $PM_{2.5}$ could be a carrier of various hazardous materials, including organic compounds, inorganic elements, and microorganisms (Liang et al., 2016). The toxicity of $PM_{2.5}$ may be due to the combined effect of its particles and adsorbed toxic pollutants (Pardo et al., 2020). Previous epidemiological and toxicological studies have demonstrated that $PM_{2.5}$ exposure may cause injuries to multiple systems, including the cardiovascular, respiratory, reproductive, immune, and central nervous systems(Li et al., 2019). In recent times, the impact of $PM_{2.5}$ exposure on reproductive health has attracted increasing attention. Numerous epidemiological studies have demonstrated that exposure to ambient $PM_{2.5}$ may cause adverse reproductive health, leading to an increased risk of infertility and the impairment of ovarian reserve (Gaskins et al., 2019; Xue and Zhang, 2018; Mahalingaiah et al., 2016). In this study, we found that $PM_{2.5}$ exposure led to follicular atresia and granulosa cell apoptosis in mouse ovary potentially via the induction of inflammatory response, as a mechanism.

Follicular development is a complex process that involves the growth and maturation or atresia of follicles (Weng et al., 2016). Granulosa cells are known to play a critical role in regulating follicular development and

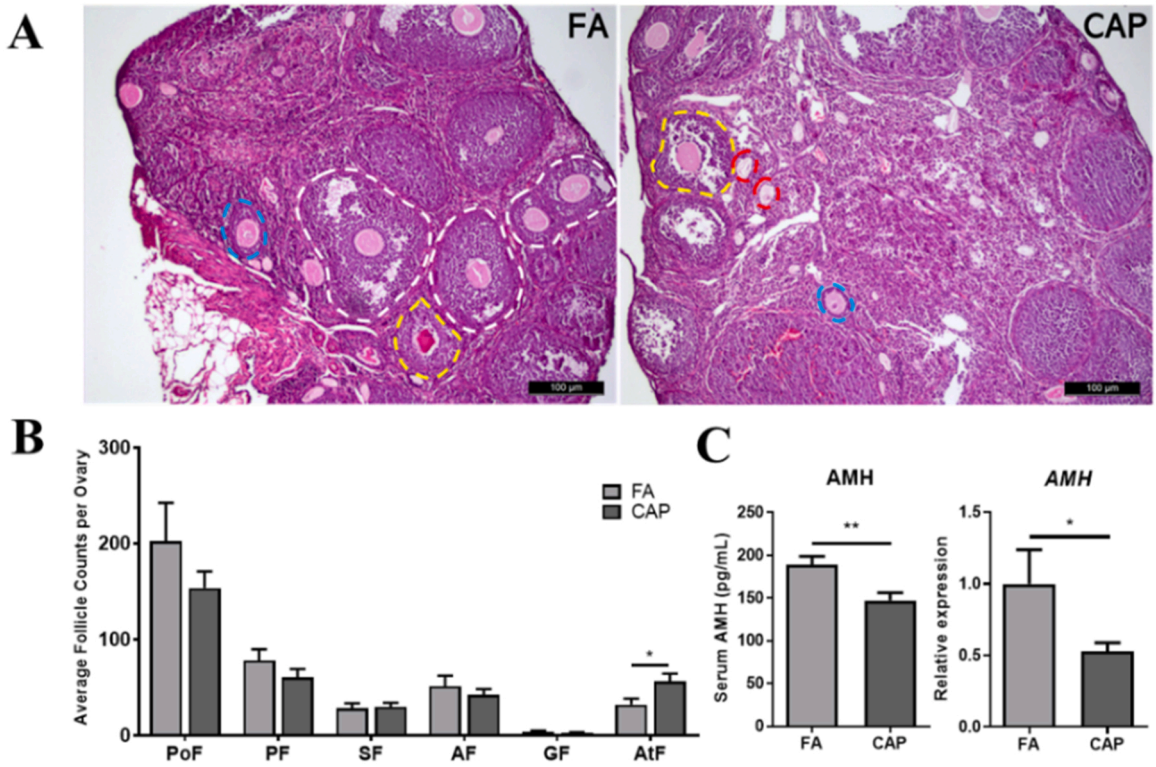

Fig. 2. Chronic exposure to CAP induced follicular atresia. A. Representative H&E staining images of ovarian follicular development in female mice. Red dotted line: Primordial Follicles (PoF); Blue dotted line: Secondary Follicles (SF); White dotted line: Antral Follicles (AF); Yellow dotted line: Atretic Follicles (AtF). B. Proportion of follicles based on H&E images of ovaries. C. The concentration of serum AMH and the mRNA expression of *AMH* in ovaries. $n = 10$, * $p < 0.05$, * * $p < 0.01$ vs. FA.

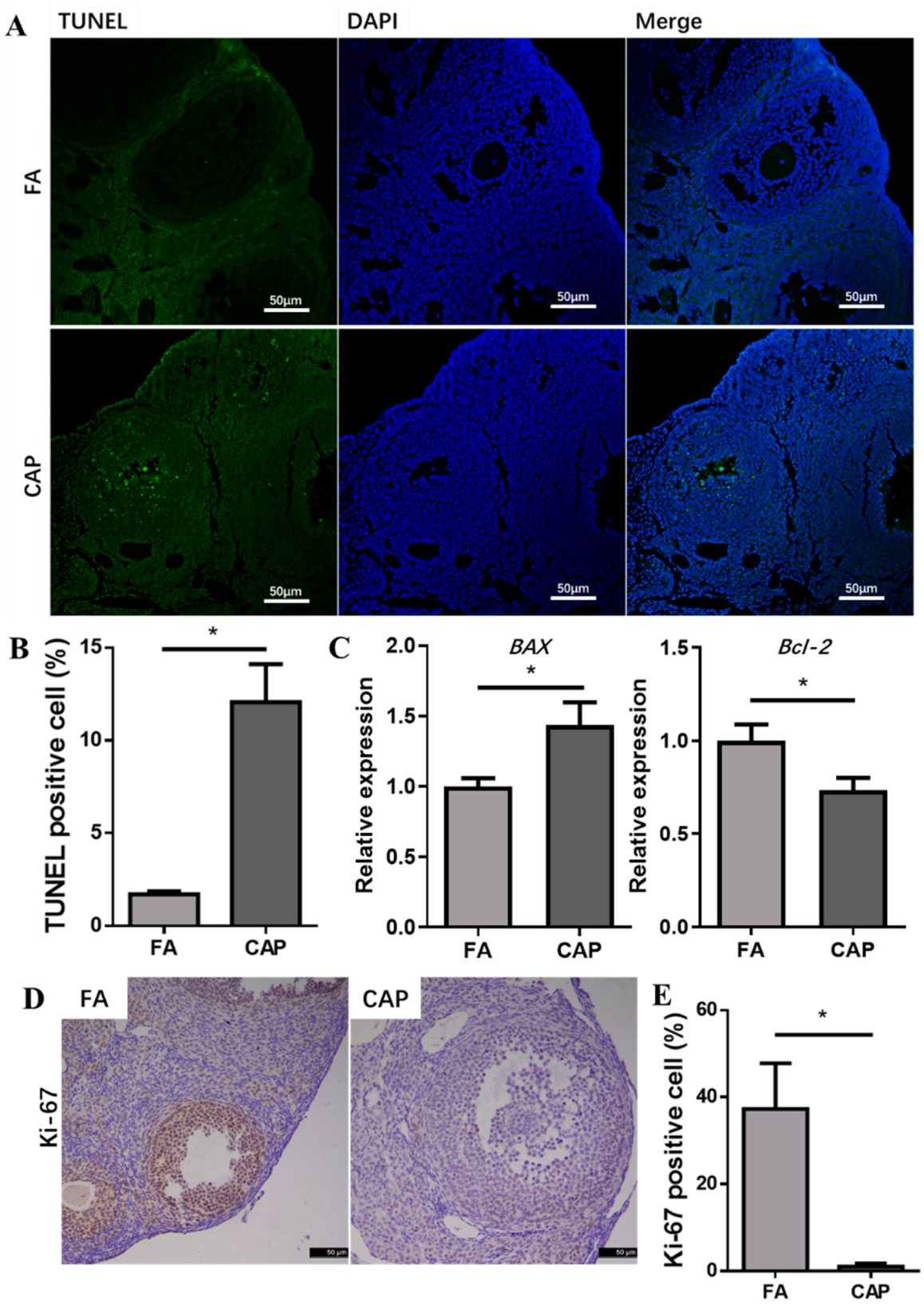

Fig. 3. Chronic exposure to CAP promoted granulosa cell apoptosis. A. TUNEL assay images of granulosa cell apoptosis in mice ovaries. B. Percentage of TUNEL positive cells in ovary. C. The mRNA expressions of apoptosis-related genes in ovaries. D. IHC assay with Ki 67 specific antibody was performed to evaluate the proliferation activity of granulosa cells. E. Percentage of Ki 67 positive cells in ovary. $n = 10$, * $p < 0.05$, * * $p < 0.01$ vs. FA.

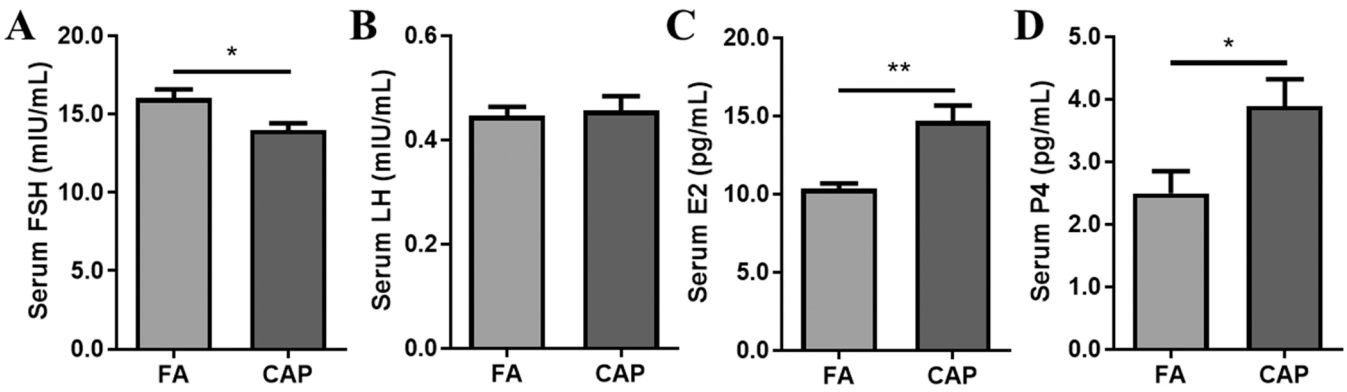

Fig. 4. Chronic exposure to CAP disturbed hormone homeostasis in female mice. The levels of FSH (A), LH (B), E2 (C) and P4 (D) in serum were assessed by ELISA. $n = 10$, * $p < 0.05$, * * $p < 0.01$ vs. FA.

serving intraovarian regulators, including gonadal steroids and growth factors (Matsuda et al., 2012). During follicular atresia, granulosa cells are eliminated via apoptosis, followed by nuclear pyknosis, granulosa cell layer detachment, and basal membrane fragmentation (Liu et al., 2018; Zhang et al., 2019). In a recent study, it was demonstrated that $PM_{2.5}$ induced ovarian dysfunction in C57BL/6J mice through mitochondrial-dependent and NF-κB/IL-6-mediated pathways (Zhou et al., 2020). Consistent with this result, in this study, we observed damage of the ovarian reserve as indicated by a significant increase in the percentage of atretic follicles (Fig. 2) in the $PM_{2.5}$-exposed female mice, which coincided with the induction of granulosa cell apoptosis (Fig. 3**A–C**). In addition, considering that granulosa cells proliferation indicates the growth of normal follicles (Xu et al., 2020), the attenuation of Ki-67 expression (Fig. 3**D, E**) is in agreement with the high occurrence of follicular atresia.

Ovarian follicular development is a complex and coordinated biological process in which hormones play a crucial role. AMH, produced by the granulosa cells of preantral and small antral follicles, can indirectly reflect the number of antral follicles (Wu et al., 2019). Furthermore, there is evidence suggesting that the level of follicular AMH is negatively correlated with granulosa cell apoptosis (Seifer and Merhi, 2014). In the CAP-exposed mice, a significant decrease in serum AMH level and expression of the *AMH* gene (Fig. 2**C**) was observed, which coincided with the elevated granulosa cell apoptosis. In addition, chronic $PM_{2.5}$ exposure also disturbed the homeostasis of other hormones, such as FSH, E2, and P4. FSH acts on the granulosa cells to initiate differentiation and proliferation, which result in the development of follicles, whereas the withdrawal of FSH generally leads to granulosa cell apoptosis and follicular atresia (Kayampilly and Menon, 2012). Our result demonstrates that exposure to $PM_{2.5}$ significantly decreased serum FSH level (Fig. 4), which may be related to follicular atresia. E2 and P4, secreted from granulosa and theca cells of follicles, are known as major regulators of follicular development and atresia. Recent studies have reported that nanoparticles-induced follicular atresia was accompanied by a significantly increased level of serum E2 (Zhao et al., 2013; Yang et al., 2017). Consistent with the findings of these studies, our results also showed that exposure to $PM_{2.5}$ significantly increased the serum concentrations of E2 and P4 (Fig. 4).

Inflammatory cytokines are known as key regulators of ovarian physiology and may trigger granulosa cell apoptosis and follicular atresia (Zhang et al., 2021). Activation of AP-1 leads to increased secretion of pro-inflammatory cytokines (Oh et al., 2016). In this study, $PM_{2.5}$ exposure triggered IL-12 secretion and AP-1 activation (Fig. 5**C**). Our data further revealed that $PM_{2.5}$ exposure induced a significant inflammatory response in the ovaries at both gene and protein levels (Fig. 5**D, E**), which may be caused by systemic inflammation. This result is in agreement with previous epidemiological and toxicological studies, which found that $PM_{2.5}$ exposure can induce toxic effects on extrapulmonary organs through the activation of systemic inflammation (Li et al., 2019; Wu et al., 2020). Inflammation is a complicated biological process that involves diverse immune cells, molecular mediators, and blood vessels. It is thought that after inhalation, the majority of the $PM_{2.5}$ deposit in the airway and induce pulmonary inflammation, while some may enter the circulatory system, which may be both triggered by systemic inflammation. In this study, it is difficult to ascertain the main contributing pathway of the inflammatory response in the ovary. Therefore, further studies are needed in this regard.

5. Conclusion

This study provides evidence that long-term exposure to ambient $PM_{2.5}$ disrupted ovarian follicle development and induced granulosa cell apoptosis through inflammation. The present data not only extend our understanding of the underlying mechanisms of the $PM_{2.5}$ toxicity but call for particular attention to the protection of women in preconception from exposure to ambient $PM_{2.5}$.

Ethics approval and consent to participate

Fudan University is an AAALAC accredited institution. All procedures of this study were approved by the Institutional Animal Care and Use Committee at Fudan, and all the animals were treated humanely and with regard for alleviation of suffering.

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CRediT authorship contribution statement

Mingjun Yang, Fang Tian:Methodology, Formal analysis, Investigation, Data curation, Writing – original draft; **Shimin Tao, Bin Pan, Zhouzhou Li, Renzhen Peng**: Validation, Form analysis; **Minjie Xia, Yuzhu Wang, Jingying Hu**: Methodology, Investigation, Data curation; **Haidong Kan, Yanyi Xu, Weihua Li**: Conceptualization, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Weihua Li reports financial support was provided by Ministry of Science

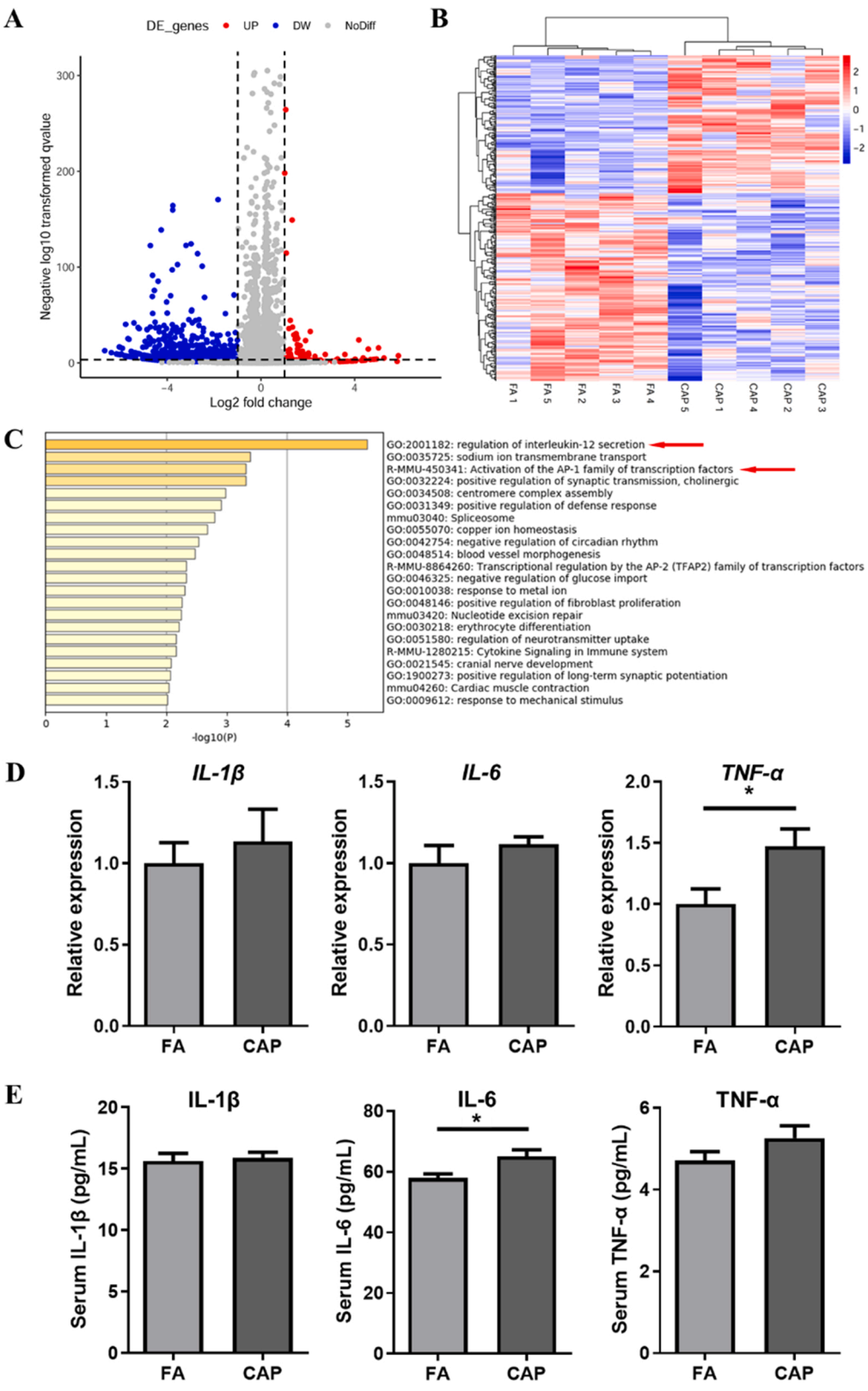

Fig. 5. Chronic exposure to CAP alters the ovarian gene expression profile. A. Volcano plot of the identified ovarian gene expressions. B. Heatmap of differentially expressed genes in the ovaries of FA/CAP-exposed mice. C. Significantly enriched GO terms of the differential genes assessed by GO analysis ($n = 5$). D. The mRNA expression levels of IL-1β, IL-6 and TNF-α in ovaries were detected by real-time PCR ($n = 10$). E. The levels of IL-1β, IL-6 and TNF-α in serum were detected by ELISA ($n = 10$). * $p < 0.05$, * * $p < 0.01$ vs. FA.

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Availability of data and material

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

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Contributions

MY, FT Methodology, Formal analysis, Investigation, Data curation, Writing - original draft; ST, BP, ZL, RP Software, Validation, Form analysis; MX, YW, JH Methodology, Investigation, Data curation; HK, YX, WL, Conceptualization, Writing - review & editing, Project administration, Funding acquisition. All authors read and approved the final manuscript.

Consent for publication

We have obtained consents to publish this paper from all the participants of this study.

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