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REPRODUCTIVE BIOLOGY: ORIGINAL ARTICLE

# Hyperbaric Oxygen Treatment Ameliorates the Decline in Oocyte Quality and Improves the Fertility of Aged Female Mice

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## Abstract

The age-related decay in oocyte quality contributes to the gradual decline in fertility and the final occurrence of natural sterility. In this study, we aimed to investigate the effects of the hyperbaric oxygen treatment (HBOT) on oocyte quality in aging mouse oocyte. Eight- and forty-week-old female C57BL/6 J mice were treated with HBO for 10 days, and the quality of oocytes was analyzed. The results revealed that HBOT improved the age-related serum AMH levels. While compared with untreated aged mice, HBOT showed reduced follicular apoptosis and improved oocyte maturation, fertilization, and blastocyst formation in aged mice. HBO triggered changes in the microRNA expression in the ovaries of aged mice. In this study, 27 DEGs were identified in the HBOT mouse ovarian tissues, of which 9 were upregulated and 18 were downregulated. Notably, KEGG analysis revealed that these genes involved in different biological processes differed significantly in the ovary. Among these, the PI3K-Akt signaling was the most prominent pathway that controlled the recruitment and growth of primordial follicles. The calcium signaling pathway was found to be involved during the peri-implantation period. These results suggest that HBOT can be applied to improve the quality of oocytes, and it could be a potential clinical application to improve the fertility of aged female.

**Keywords** Hyperbaric oxygen · Aged · Female Mice · Ovarian reserve · MicroRNA

## Introduction

It has been well known that with increasing chronological age, female fecundity decreases. In view of the current trend to postpone childbearing in contemporary populations, the age-related decrease of the ability to produce offspring in female has gradually appeared [1]. The mechanisms underlying the observed gradual decline of the follicle pool and the reduced oocyte quality are far from being fully understood. Decreasing numbers of follicles, coinciding with diminished oocyte quality, dictates the gradual changes in menstrual cycle regularity and monthly fecundity [2–4]. Recent knowledge about the age-related female fecundity declines was mainly because the risk of oocyte chromosome abnormalities and with increases adverse pregnancy outcomes [5, 6].

Hyperbaric oxygen treatment (HBOT) is the application of 100% oxygen at environmental pressures > 1 atmosphere [7, 8]. HBOT, generally used as an effective treatment method in ischemia reperfusion injury, anti-inflammatory effects and stimulates the antioxidant system. It has been widely applied to accelerate fracture healing [9], articular cartilage injury [10], type 2 diabetes mellitus [11, 12], and the proliferation of epidermal basal cells [13]. Accumulation of oxides in the ovarian tissues in older women may be one of the reasons for infertility, considering that it reduces the quality of embryos. In male infertility, sperm DNA fragmentation was improved after HBOT [14], and for male recover, erectile function has also been ameliorated [15, 16]. On another hand, HBOT can significantly improve female endometrial receptivity in the cycle for better outcome of pregnancy implantation [17]. Additionally, some research inferred that exposure to mild HBO can increase the oxygen supply to the tissues, thereby enhancing oxidative metabolism [11]. Overall,

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HBOT provides the therapeutical efficacy in many diseases, but it in female ovary influence has rarely known.

In this work, we investigated the effect of HBOT on the ovarian reserve of 40-week-old mice. Moreover, miRNA profiling revealed a possible compensatory mechanism of HBOT in age-related decline in ovarian functions. Our results provided a theoretical basis for the clinical treatment of age-related decline with ovarian function.

## Materials and Methods

### Mice and HBO Therapy

Female C57BL/6 J mice aged 8 and 40 weeks were sourced from the Carvens Animal Laboratory Technology Company [http://www.cavens.com.cn/index.php; animal certificate no. SCXK(SU)2016–0010]. All animal-use experiments were approved by the Animal Care and Use Committee of Nanjing Medical University. The experimental mice were kept in a standard pathogen-free environment under standard housing conditions. The room was maintained under a strictly controlled temperature (22 ± 1 °C) and humidity (52 ± 5%). The mice were provided ad libitum feed with a 12-h light/dark photophase condition. The mice were weighed weekly and their health status was monitored. For the HBOT, the mice were administered 100% oxygen at a pressure of 2.5 ATA in a custom-made mono-chamber intended for small animals for 90 min daily for 10 consecutive days.

### PMSG Injection for Super-ovulated Mice

Female C57BL/6 mice (OC and OC + HBOT groups) were intraperitoneally injected with 5 IU PMSG (Prospec Tany, TechnoGene, Ltd.) to induce super-ovulation. Next, for in vitro fertilization (IVF) and embryo culture, the mice were sacrificed through cervical dislocation 48 h later after the PMSG injection. The IVF and embryo culture were performed as described elsewhere [18].

### Immunofluorescence Staining

The ovarian slides were stained with Alexa Fluor 488 (1:200; Beyotime Biotechnology, China) overnight under 4 °C. The next day, the slides were incubated at room temperature in the dark with Alexa Fluor 594-conjugated goat anti-mouse IgG (Life Technology, Waltham, MA, USA). Hoechst was used to stain the nuclei. Images were collected through fluorescent microscopy (Olympus, U-RFL-T).

### RNA Extraction and Quantitative Real-Time PCR (qPCR)

Total RNA was separated and extracted by the RNeasy Mini Kit according to the manufacturer's protocol (Vazyme Biotech, Nanjing, China). Then, 1.0 μg RNA was used as a template for cDNA synthesis by using the HiScript III 1st Strand cDNA Synthesis Kit (+ gDNA wiper) (Vazyme Biotech). The cDNA samples were analyzed by qPCR using the AceQ qPCR SYBR Green Master Mix (Low ROX Premixed). U6 snRNA was used as an internal control. The experimental data were analyzed by the relative quantification method (2-ΔΔCq) [19]. All experiments were repeated thrice.

### Statistical Analyses

Original data analysis was performed with GraphPad Prism 5.01 (GraphPad Software, Inc.) and ImageJ (National Institutes of Health). Data are presented as the means ± SD ($n = 3$). The differences between the two groups were analyzed by unpaired Student's $t$-test. $P < 0.05$ was considered to indicate statistical significance.

## Result

### HBOT improved age-related decline of the serum AMH and FSH levels.

The bodyweight of the mice in each group was evaluated after 10 cycles of HBOT (Fig. 1A). HBOT showed no significant change in bodyweight (Fig. 1B). However, HBOT mice showed significantly improved serum FSH levels in the OC + HBOT group (Fig. 1C), although the serum LH levels did not change. OC mice (40-week-old) showed lower serum AMH levels, possibly due to decreased ovarian reserve (Fig. 1D). HBOT mice showed significant improvement in their serum AMH levels.

### Treatment with HBO ameliorates the quality of aged oocytes in vivo

In order to explore the effects of HBOT on age-related decline in oocytes in vivo, 3 groups of mice were treated with super-ovulation via hormonal stimulation, and IVF experiments were conducted using super-ovulated oocytes obtained from 3 groups of mice (Fig. 2A). Hence, we assessed the quantity and quality of oocytes. In addition to assessing the morphology of oocytes, the number of oocytes retrieved from the OC group was also significantly lower than that of the YC group, while that of the

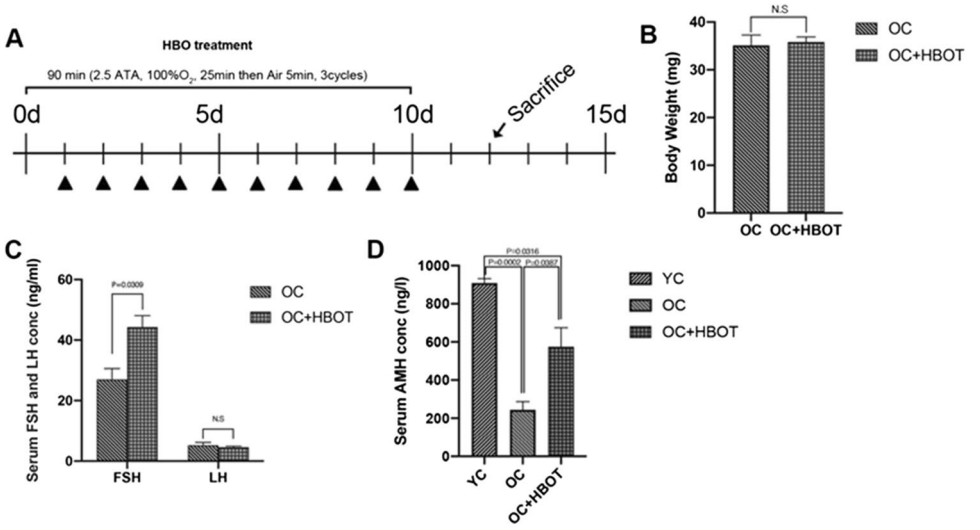


**Fig. 1** Bodyweight variation and serum hormone levels of mice after HBOT. **A** 40-week-old mice in the aging control group ($n = 6$, OC group), and 40-week-old mice were treated with HBO 9 times each day ($n = 6$, OC + HBOT group). Post-HBOT, the changes in the mice bodyweight were measured along with hormonal assessment. **B** Bodyweight variations. **C** Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) serum levels. **D** 8-week-old mice as young controls ($n = 6$, YC group). Anti-Mullerian hormone (AMH) serum levels. Data are presented as mean ±standard error of the mean. Statistical analysis was performed using Student's *t*-test

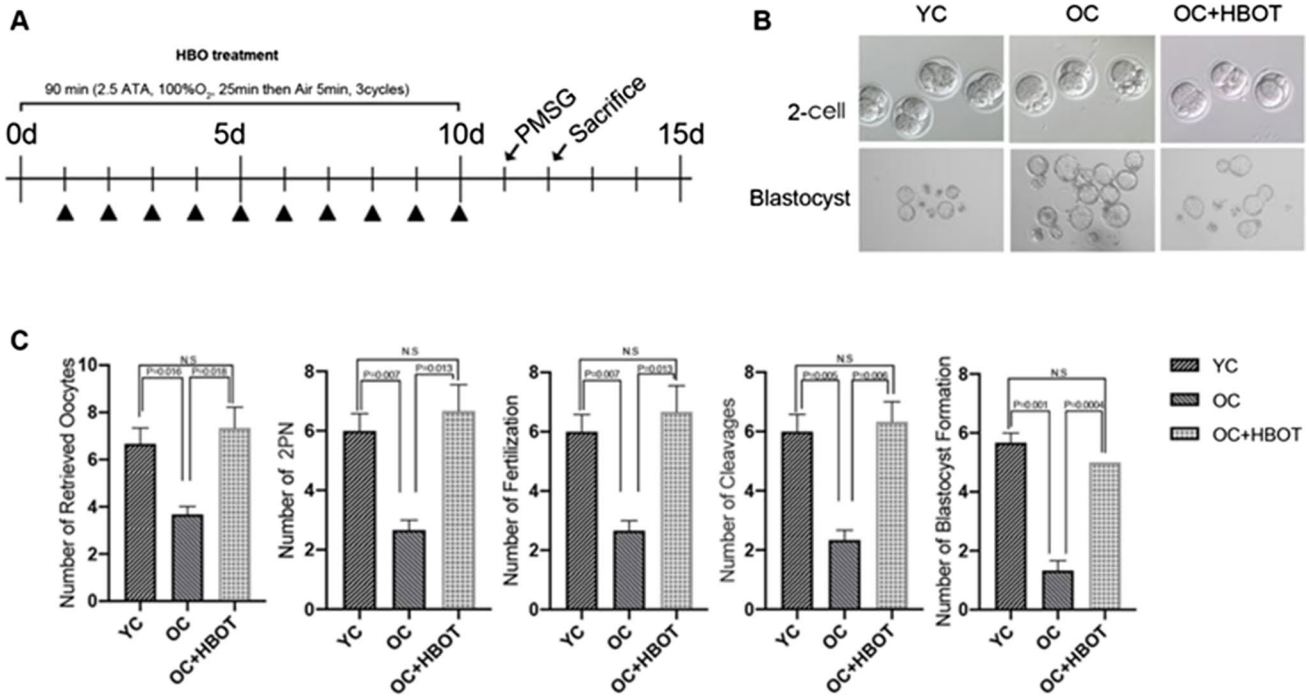


**Fig. 2** Quality and number of mouse oocytes retrieved after HBOT. **A** Eight-week-old mice as young control ($n = 6$, YC group); 40-week-old mice as aging control ($n = 6$, OC group); and 40-week-old mice treated with HBO 9 times each day ($n = 6$, OC+HBOT group). After HBOT, the mice were induced with hormonal stimulation to implement the IVF experiments. **B** Typical images of MII oocytes were collected from YC ($n = 20$), OC ($n = 11$), and OC + HBOT ($n = 22$) mice, and the IVF outcomes from these 3 groups. Scale bar = 100 μm, 400 μm. **C** Oocytes were retrieved from the YC, OC, and OC + HBOT groups after 18 h of PMSG injection. The number of retrieved oocytes and 2-cell embryo (2PN), fertilization, cleavages, and blastocyst formation. Data are presented as the mean ± standard error of the mean. Statistical analysis was performed using Student's *t*-test

OC + HBOT group was closer to that of the YC group in terms of the number of oocytes retrieved ($P < 0.05$; Fig. 2C). The number of fertilizations performed was significantly higher in the OC + HBOT mice than in the OC mice (Fig. 2C). Taken together, these results indicate that exposure to HBO ameliorates the oocyte quality and can hence enhance the quality of fertilization in age-related mice.

### HBOT changes in microRNA expression in aged mouse ovaries

To further explore the potential mechanisms underlying the influence of HBOT, deferentially expressed miRNA profiles in the OC and OC + HBOT groups were compared using RNA sequencing technology. A deep interpretation of the miRNA networks can help elucidate the mechanisms of improvement in the quality of aged oocytes and facilitate the development of new treatment strategies for elderly patients. A total of 27 DEGs were identified in HBOT mice testes by applying the cutoffs of FC > 2 and FDR ≤ 0.05, 9 of which were upregulated and 18 were downregulated (Fig. 3A). Notably, KEGG analyses revealed that these genes involved in different biological processes were remarkably divergent in the ovary (Fig. 3B).

### HBOT reduces follicle apoptosis

The expressions of two DEGs were determined by qPCR. MiR-99b-5p, miR3103-3p, miR3074-1-3p, and miR211-5p were found to have a certain influence in HBOT mice (Fig. 4A). To confirm this effect, DNA fragmentation in the HBOT ovary was stained to assess the quality of aged follicles (Fig. 4B). We have presented a summarized list of known research on miRNAs involved in aged mice after HBOT in Table 1, which includes the combinations of miRNAs and their confirmed target genes. This list is critical to the present research topic.

## Discussion

Currently, delayed childbearing brings forth a common problem in women. Ovarian aging in women correlates with a decrease in the number of ovarian follicles and declining oocyte quality. As a result, reproductive aging in females brings the significant increase in the risk of adverse reproductive outcomes, like higher rates of spontaneous abortion and miscarriage and a significant decline in oocyte quality [19–23]. With most recently research, dehydroepiandrosterone and growth hormone may slightly improve the clinical pregnancy

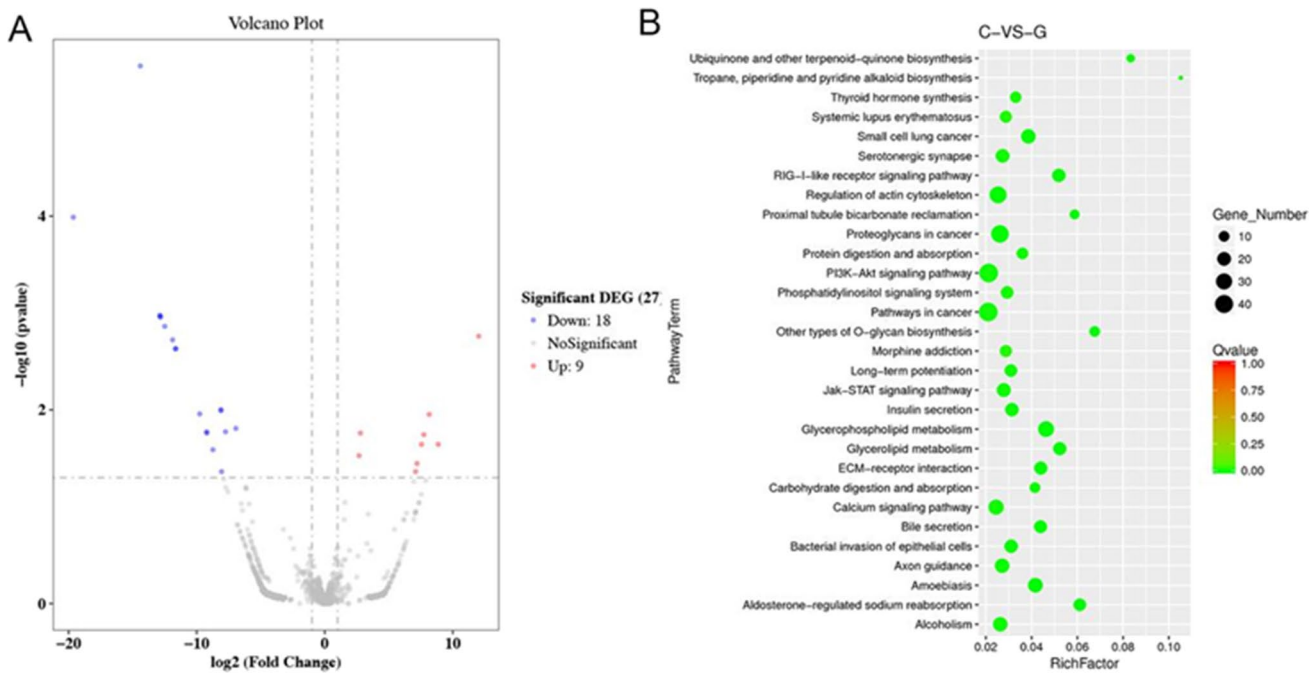


**Fig. 3** Volcano plot analysis of differentially expressed miRNAs and the pathway enrichment scatter plot of differential microRNA target genes. **A** RNA sequencing analysis was performed to compare the gene expressions between the OC ($n = 3$) and OC + HBOT ($n = 3$) groups. We found that 9 DEGs were upregulated and 18 were significantly downregulated. **B** The pathway enrichment scatter plot of differential microRNA target genes. The pathways involved in the differential microRNA target genes. The size of the dots indicates the number of differential microRNA target genes in this pathway, and the colors of the dots indicate different Q values. The greater the rich factor with the better relatives

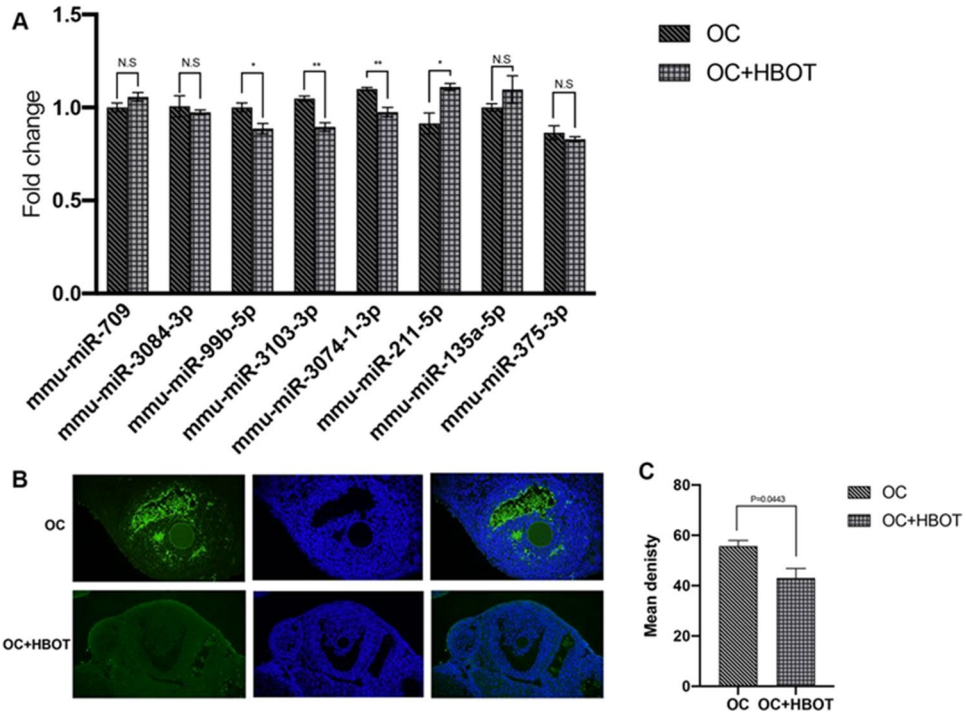


**Fig. 4** qRT-PCR validated differentially expressed micro-RNAs and the number of aged follicles after HBOT. **A** The expression of micro-RNA was measured in the OC and OC + HBOT groups. **B** Follicle quality was assessed by examining the stained nuclei and DNA fragmentation in each mice group. **C** The quantity of aged follicles was assessed from apoptotic nuclei. Data are presented as mean ± standard error of the mean. Statistical analysis was performed using Student's *t*-test

**Table 1** miRNAS target genes and thier functions in HBO treatment mice

| micro_ID | GeneSymbol |
|---|---|
| mmu-miR-99b-5p | *Dip2a*; *Ppp2r5a*; *Gatm*; *Arhgef1*; *Ablim1*; *Polrmt*; *P2rx5* |
| mmu-miR-3103-3p | *Zdbf2*; *Lrrc3*; *Mrtfa*; *Rps6ka3*; *Nfam1*; *ltga6*; *Kcnmb4*; *Spry1*; *Zfp280b*; *Erp27*; *Picxd2*; *Dedd2*; *Spsb2*; *Crem*; *Lemd1*; *Zfp408*; *Dlc1*; *Map4k4*; *Hmx2*; *Orc1*; *Myoz3*; *Ndst1*; *Rbm4b*; *Parqr4*; *Adora1*; *Tctn2*; *Gcn1*; *Slc4a4*; *Klra13-ps*; *Gm18761*; *Gm18337* |
| mmu-miR-3074-1-3p | *Cers3*; *Aspa*; *Angpt4*; *Cntn6*; *Rbfa*; *Med28*; *S1pr2*; *Aopep* |
| mmu-miR-211-5p | *Gm9747*; *Pip4k2b*; *Ccdc50*; *lvl*; *Zfp652*; *Tanc1*; *Socs6*; *Tnpo2*; *Slc9a2*; *Fzd5*; *Nfz1*; *Gm17018*; *Gm9449* |

rate, albeit the data for supporting these therapies are insufficient [24–26]. In this study, we investigated the effects of the hyperbaric oxygen therapy on oocyte quality in aging mouse and found that HBOT could improve the quality of oocytes.

Hyperbaric oxygen therapy (HBOT), a therapeutical method based on exposure to pure concentrations of oxygen ($O_2$), can increasing the concentration of oxygen in the blood and tissues. Thus, HBOT provides multiple effects in the organism, including in the treatment of diabetic foot ulcers [27, 28], sports musculoskeletal injuries [29], novel coronavirus infection [30], and so on. HBOT has been indicated positive value by evidence-based medicine distinctly. The mouse is widely used as a model for human biological aging [31–33]. It is commonly agreed that the absolute age of reproductive biological aging in mice between 9 and 15 months [34, 35]. Accordingly, our research used 40-week-old female C57BL/6 J mice as aging model and revealed that the oocyte quality and fertilization can be improved through exposure to HBO. Moreover, follicle apoptosis was reduced in the HBOT group probably due to the PI3K/Akt or JAK/STAT signaling pathway according to the KEGG enrichment analysis. These data suggest that there is multiple influence for aging follicle development by HBOT.

AMH, which has a stable level in serum, is secreted by granulosa cells in late preantral follicles and small antral follicles, and it is more closely related with the number of primordial follicles [36]. Hence, AMH offers a higher predictive value than FSH and E2 level in clinical ovarian reserve decline patients [37, 38]. More than that, FSH is directly regulate on follicle development [39]. Interestingly, our study observed that HBOT improved the serum AMH and FSH level. And, the slightly increase FSH level may directly stimulate ovarian granulosa cells to promote follicle growth and further development.

Current research has been concentrated upon determining the factors that are most important to ovarian health. One

goal of these studies is to elucidate the molecular mechanisms underlying the changes in ovarian reserve, oocyte viability, and oocyte quality [40]. Insulin-like growth factor (IGF), which regulates the stimulatory effect of FSH on aromatase expression, is the most important local factors in the system [41]. In addition, insulin-like growth factor-1 receptor (IGF1R) is essential for follicle survival and fertility in female mice ovary [42]. In our study, we identified several miRNAs changes by RNA-seq, as expected, miR-99b-5p, an upstream target for IGF1R [43]. In aging oocytes, the raised DNA damage is closely related to apoptosis, poor quality of oocytes, and eventually caused infertility and miscarriage. In the presence of DNA damage, cells activate a coordinated mechanism called the DNA damage response (DDR) to activate different repair processes to correct the damage [40]. There is evidence to support the existence of crosstalk between the PI3K/Akt signaling pathway and the DDR in cells [42, 44]. High PI3K/Akt activity is linked up with a decline in the number of primordial follicles and ovarian aging [45]. Ovarian aging is associated with impaired DDR within oocytes [46]. In our work, KEGG analysis showed that these genes were involved in the PI3K-Akt signaling pathway biological processes. DNA fragmentation stain in the HBOT ovary was showed effectively ameliorated by immunofluorescence. These data together suggest that multiple genes of the primordial follicle recruitment pathway are influenced by HBOT.

In conclusion, HBOT may contribute to the improvement of oocyte quantity in the aged mouse model, although further research and clinical trial are needed to explore and potential therapy for aging female fecundity decreases.

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**Data Availability** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Code Availability** Any software application or custom code described in the manuscript is available for testing by reviewers in a way that preserves their anonymity.

## Declarations

**Ethics Approval** All animal-use experiments were approved by the Animal Care and Use Committee of Nanjing Medical University.

**Consent to Participate** All experiments were conducted according to the National Institute of Health guidelines on the care and use of animals and approved by the Animal Care and Use Committee of Nanjing Medical University.

**Consent for Publication** For the manuscripts that include details, images, or videos relating to animals, written informed consent for the publication of these details was approved by the Animal Care and Use Committee of Nanjing Medical University.

**Conflict of Interest** The authors declare no competing interests.

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