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Effects of in vitro storage temperature and duration time on the state and function of primary granulosa cells after isolated from human follicular fluid

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Abstract

From the collection of human follicular fluid to the isolation of primary granulosa cells, the duration of these two time points is variable. And there is no specific experiment to explore which temperature will make the cell state better in subsequent experiments. In this study, the collected follicular fluid was divided into 7 groups: one fresh control group, three in vitro storage temperatures (4℃, Room Temperature, and 37℃) and two storage duration times (4h and 8h). After isolating the cells, they were cultured in vitro to evaluate their state and function. The results showed that granulosa cells of the fresh control group had better cell viability and steroid hormone secretion ability than the other treatment groups; with the prolongation of in vitro storage time, the cell viability and steroid hormone secretion ability have decreased to varying degrees; low-temperature in vitro storage is conducive to the maintenance of cell survival and steroid hormone secretion ability, which is also confirmed by the detection of ROS, which can reflect the oxidative stress state of cells. This study showed that after obtaining follicular fluid, the in vitro storage time should be shortened as much as possible, and stored at low temperature, which can keep cells maintain good activity and steroid hormone secretion ability, and reduce oxidative stress and aging. It is of great significance for improving the state of human primary granulosa cells after isolation and subsequent experiment, and it also has certain guiding significance for standardization experimental research programs.

Introduction

Follicle is the basic structural and functional unit of the ovary, which is composed of an oocyte and granulosa cells surrounding it [1]. Granulosa cells and oocytes are in close contact through gap junction, and regulate each other to promote the normal growth and development of the follicle [2]. During the formation and development of follicles, granulosa cells change from flat to cubic, and gradually grow from one layer to multiple layers. Granulosa cells secrete a variety of steroid hormones [3], and give nutrition to oocytes through gap junction to promote the development of oocytes [4]. Human primary granulosa cells extracted from follicular fluid are widely used in scientific research and occupy a very important position in scientific research fields of female reproductive system [5]. By using in vitro culture model of granulosa cells, researchers can not only study the effects and mechanisms of affecting the development of ovaries and follicles, but also have certain guiding significance for clinical application of reproduction [6, 7].

There have been previous studies in other medical fields on the effects of different in vitro storage temperatures and duration times on the state of primary cells. Fubo et al. stored periodontal ligament fibroblasts in different storage media and found that the cell survival rate decreased with the increase of storage temperature [8]. The study of fresh osteochondral allografts showed that the cryopreservation at 4℃ has a better cell survival rate than storage at 37℃ [9]. The oral mucosal cells stored at 4℃ are similar with the non-storage fresh control cells in morphology and have the highest survival rate among all groups, while the survival rate of cells stored at 37℃ decreases significantly [10].

However, there are also some studies have different results. Shohei et al. found that retinal pigment epithelial cells preserved at 37℃ will cause hypoxia due to high levels of cell metabolism and cell deposition, which ultimately leads to a significant reduction in cell survival rate; when the cells stored at 4℃, it will be damaged by the fragility of microtubules; but the highest survival rate was obtained when the cells stored at 16℃, and the apoptosis rate was also significantly reduced at this storage temperature [11]. The results in the study of human epidermal cells showed that the cell survival rate was the lowest when the cells stored at 4℃, then gradually increases when they were stored at 8-16℃, and decreases again when they were stored at 24℃ [12]. These results showed that different cell types have different requirements for in vitro storage conditions, which need to be confirmed by experimental analysis.

At present, the research on the extraction of human primary granulosa cells mainly focuses on the study of cell purification during the isolation process [13-16]. There is no research on the effect of in vitro storage temperature and duration time from the collection of follicular fluid to the extraction of primary granulosa cells on cells after isolation. Therefore, in this study, we set up different in vitro storage temperatures and duration times of follicular fluid in order to find the suitable in vitro storage conditions of human primary granulosa cells that are conducive to maintaining the cell viability and the ability of steroid hormone secretion.

Materials And Methods

Cell culture and treatment

The follicular fluid was obtained from patients who undergoing in vitro fertilization-embryo transfer (IVF-ET) in the Center for Reproductive Medicine of the First Affiliated Hospital of Zhengzhou University, and granulosa cells were purified by density gradient centrifugation [17]. Cells were cultured in DMEM/F12 medium (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (Gibco), 100U/mL penicillin and 100 mg/mL streptomycin sulfate (Gibco), and in a humidified incubator at 37℃ with 95% air and 5% CO_2.

β-Galactosidase staining

The β-Galactosidase Staining Kit (Beyotime, Shanghai, China) was used for cell senescence detection. Human primary granulosa cells were seeded in a 24-well plate at a density of 2×10^3 cells/well, and the culture medium was 500 μL/well. After 48 h of culturing, cell culture medium was discarded, and the cells were washed with PBS once, then 300 μL β-Galactosidase staining fixative was added at room temperature for 15 min. After aspirating the fixative, the cells were washed 3 times with PBS for 3 min each time. After adding 300μL of dye working solution to each well, the cells were incubated at 37℃ overnight, then observed and taken photograph under microscope.

ROS staining and ROS level measurement

The oxidative stress state of cells was detected by the reactive oxygen species detection kit (Beyotime). Human primary granulosa cells were cultured in a 24-well plate with a density of 2×10^3 cells/well with culture medium of 500 μL/well. After culturing for 48 h, DCFH-DA was diluted with serum-free culture medium and prepare a working solution with a final concentration of 10 μM. The working solution (300 μL) was added into each well after discarding the culture medium, and the culture plate was incubated in incubator at 37℃ for 20 min. Laser confocal microscope Zeiss LSM 700 (Carl Zeiss, Jena, Germany) was used to observe and taken photograph. In order to detect the level of ROS, the cultured cells were digested with TrypLE™ Express (Gibco), and resuspended in a 96-well plate with PBS at a density of 2×10^3 cells/well. Then Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, USA) was used to detect the DCF fluorescence value (Ex488nm /Em525nm).

Cell viability measurement

Human primary granulosa cells were cultured in a 24-well plate with a density of 2×10^3 cells/well with culture medium of 500 μL/well for 48 h. The CCK solution in Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) was mixed with DMEM/F12 in a ratio of 1:9 to prepare a working solution. After removing the original culture medium, 300 μL working solution was added into each well, and incubated in a 37℃ incubator for 2 h. Then the absorbance at the wavelength of 450 nm with Varioskan Flash microplate reader (Thermo Fisher Scientific) was measured.

Annexin V-FITC/PI apoptosis detection

The Annexin V-FITC/PI double staining cell apoptosis detection kit (KeyGEN, Nanjing, China) was used to detect the cell apoptosis of each group after treatment. Human primary granulosa cells were seeded in a 24-well plate at a density of 2×10^3 cells/well with culture medium of 500 μL/well. After incubated for 48 h, the cells were separated by TrypLE™ Express (Gibco), resuspended with PBS and centrifuged. Then cells were resuspended in Binding Buffer, annexin V-FITC and PI were added subsequently. BD Accuri C6 Plus Flow Cytometers (BD Biosciences, San Jose, USA) were used to detect the cell apoptosis.

Western blotting

After rinsing the granulosa cells with pre-cooled PBS, RIPA lysis buffer was added, and cells were lysed on ice for 20 min. The supernatant was collected after centrifugation, and the protein was quantified with BCA protein quantification kit (Thermo Fisher Science). The protein was isolated by SDS-PAGE, and transferred to PVDF membrane (Bio-Rad Laboratory) by the Bio-Rad Transblot Turbo system (Bio-Rad Laboratory, Hercules, USA). The membrane was blocked with TBS containing 5% skim milk for 1h, and incubated with the primary antibody overnight at 4℃. The next day, after washing the membrane with TBST, the corresponding secondary antibody was added and incubated for 1h. Then the developer (Bio-Rad Laboratories) and ChemiDoc MP Imager (Bio-Rad Laboratories) were used to obtain the blot image. The primary antibodies used were as follows: Bax (1:5000; Proteintech, Chigago, USA), Caspase3 (1:1000; CST, Danvers, USA), Aromatase (1μg/ml; Abcam, Cambridge, UK), β-actin (1:5000; BioWorld, St. Louis,

USA); The secondary antibodies used were Mouse IgG secondary antibody (1:5000; Abcam) and Rabbit IgG secondary antibody (1:5000, Abcam).

Hormone measurement

Human primary granulosa cells were cultured in a 24-well plate with a density of 2×10^3 cells/well, and 500 μL/well of phenol red-free culture medium which was added with 1nM testosterone (Sigma, St. Louis, USA) as a hormone synthesis substrate. After in vitro culture for 48 h, the supernatant was collected. Progesterone ELISA Kit (Elabscience Biotechnology, Wuhan, China) and Human Estrogen ELISA Kit (CUSABIO, Wuhan, China) were used to detect the concentrations of progesterone and estrogen according to the product user instructions. Firstly, diluted the collected culture supernatant to 200 times to determine the concentration of progesterone, and diluted to 400 times to determine the concentration of estrogen, then Varioskan Flash microplate reader (Thermo Fisher Scientific) was used to determine the standard and sample at 450 nm, and a standard curve was drawn to calculate the concentrations of progesterone and estrogen, respectively.

Statistical analysis

The data was analyzed by SPSS (version 19.0, IBM, Armonk, USA). The experimental results were the mean ± standard deviation of at least 3 independent experiments. The two groups of data were compared by t-test, and multiple comparisons were performed by one-way ANOVA or two-way ANOVA. $p<0.05$ was considered statistically significant.

Results

Effects of different in vitro storage temperature and duration time on the cell state after extraction of human granulosa cells

The effects of different in vitro storage temperatures (4°C, RT, and 37°C) and duration times (0 h, 4 h, and 8 h) on the cell state after in vitro culturing of human primary granulosa cells were explored in this study. After 48 h in vitro culturing, the cell status was directly observed under a microscope. The results showed that as in vitro storage temperature increases and the storage duration time increases, granulosa cells gradually shrink and deform, the number of adherent cells gradually decreases, and cell aggregates also gradually disappear (Figure 1a).

The senescence state of cells which cultured for 48 h in vitro was analyzed by β-galactosidase staining. The results showed that the degree of cell staining gradually increased with the extension of in vitro storage time and the increase of storage temperature, which indicated that SA-β- Gal activity level is up-regulated, and the degree of cell senescence gradually increases (Figure 1b).

These above results all showed that the longer time the follicular fluid stored in vitro, the worse the cell state are after extraction; and as the temperature of follicular fluid stored in vitro gradually rises, the cell

state gradually decreases after extraction.

Effects of different in vitro storage temperature and duration time on oxidative stress of human granulosa cells after extraction

Furthermore, ROS staining was used to detect the oxidative stress state of cells after 48 h in vitro culture. The stained cells were observed with a laser confocal microscope. The results showed that, with the increase of in vitro storage temperature and duration time, the ROS staining of cells gradually increased, and ROS staining of granulosa cells extracted after 8 h in vitro storage at 37℃ was the strongest (Figure 2a). The same results were obtained by the fluorescence value of the primary granulosa cells after ROS staining, and the cells stored in vitro for 4 h or 8 h at 37℃ was significantly higher than that of the other groups ($p<0.0001$) (Figure 2b).

Effects of different in vitro storage temperature and duration time on cell survival after extraction of human granulosa cells

Human follicular fluid was obtained and treated according to the experimental requirements. Human primary granulosa cells were extracted from the treated follicular fluid and cultured for 48 h. The effects of different in vitro storage temperatures and duration time on cell viability were measured by CCK-8 method. The results showed that the survival rate of granulosa cells stored in vitro for 4 h at 4℃ was significantly higher than that of both RT and 37℃ treatment groups ($p<0.0001$), while the cell survival rate under RT was significantly higher than that of 37℃ treatment group ($p<0.0001$) (Figure 3a). In addition, the 4℃, RT and 37℃ treatment groups under the condition of 8 h of in vitro storage time are basically consistent with the results of the treatment groups under 4 h storage period (Figure 3a).

The cell proliferation curve of human primary granulosa cells was drawn by detecting cell viability with CCK-8. The results showed that the viability of granulosa cells decreased gradually with the extension of in vitro storage time; and the three temperature groups (4℃, RT, and 37℃) within the same in vitro storage time, the higher in vitro storage temperature, the lower survival rate of cells are after extraction ($p<0.0001$) (Figure 3b).

The same conclusion was also obtained by flow cytometry for the detection of cell apoptosis rate. Although the difference between the results of the 4 h group and the control group was not as obvious as that between the 8 h group and the control group, from the overall trend, the apoptosis rate still increased with the extension of the in vitro storage time and the increase of the in vitro storage temperature (Figure 3c).

In addition, the expression levels of apoptotic proteins (Bax and Caspase3) were also increased with the prolongation of in vitro storage time and the increase of in vitro storage temperature, which is consistent with the results of cell apoptosis detection (Figure 3d).

Effects of different in vitro storage temperature and duration time on the secretion ability of steroid hormones

The expression level of Aromatase, an antibody which related to steroid hormone secretion, was detected by Western Blotting. The results showed that within a certain range, the higher in vitro storage temperature and the longer in vitro storage time is, the more significantly the expression level of Aromatase decreased (Figure 4a). Therefore, we speculate that the increase of in vitro storage temperature and the extension of in vitro storage time will lead to the impaired ability of granulosa cells to secrete steroid hormones.

Subsequently, we used ELISA kit to detect the levels of progesterone and estrogen in culture medium after the granulosa cells cultured for 48 h. The results showed that with the increase of in vitro storage temperature and the extension of in vitro storage time, the level of progesterone secretion gradually decreased (Figure 4b), and the level of estrogen secretion also showed the same trend, and the change was more obvious (Figure 4c).

Discussion

As we know that in the actual research process, from the collection of follicular fluid to the extraction of primary granulosa cells, the follicular fluid may need to be stored in vitro for a certain time due to various reasons, and there is no specific experimental exploration on the temperature at which the follicular fluid should be placed during this period to maintain the cell state for subsequent experiments.

In this study, the collected human follicular fluid was divided into seven groups. In addition to the fresh control group requiring immediate extraction, 3 non-freezing in vitro storage temperatures (4℃, RT, and 37℃) and two storage duration times (4 h and 8 h) were set up to explore the effects of in vitro storage temperature and duration time on the cell state of human primary granulosa cells after extraction. The results showed that the higher in vitro storage temperature and the longer in vitro storage duration time is, the worse the cell state of human primary granulosa cells is after extraction. Within a certain temperature range (4℃, RT, and 37℃), as the in vitro storage temperature rising and the in vitro storage duration time prolonging, the cell survival rate decreased, and the degree of cell aging, the level of reactive oxygen species, the rate of apoptosis and the expression level of apoptotic proteins (Bax and Caspase3) increased. In addition, this study also explored the effects of in vitro storage temperature and duration time on the secretion of steroid hormones. The experimental results showed that the higher in vitro storage temperature and the longer in vitro storage time, the lower the progesterone and estrogen secretion capacity of granulosa cells. In summary, the human primary granulosa cells extracted from follicular fluid should be stored as short as possible and stored at a lower temperature, especially at 4℃.

In this study, as the in vitro storage time prolonging, the cell state getting worse. This result seems to be uncontroversial, because cell growth requires a suitable environment, and short-term in vitro storage did not transfer the cells to the culture medium, which is far from enough for cell growth. Previous studies have shown that when bone marrow mesenchymal stromal cells are stored in physiological saline, regardless of the storage temperature, the cell viability, proliferation and differentiation potential are

significantly reduced after the cells stored in vitro for more than 2 h [18]. Although the storage media are different, the results are still consistent.

Similarly, as the in vitro storage temperature rising, the cell state also getting worse, that may be due to the following reasons. Firstly, human primary granulosa cells are adherent grown cells. Within a certain temperature range, the closer in vitro storage temperature to 37℃, the more adherent grown cells is promoted. When granulosa cells need to be extracted after storage in vitro for a certain time periods, it is necessary to shake the follicular fluid again. At that time, granulosa cells may have begun to grow adherently already. Shaking the follicular fluid will force cells to fall off the culture bottle, which will cause a physical damage to the granulosa cells by external force. Secondly, the granulosa cells did not pass through the seed plate with uniform density, and the cells are deposited together. The higher the temperature, the faster the cell metabolize and the higher the cell sedimentation rate, that resulting in hypoxia of granulosa cells and poor cell state. This is consistent with the results of previous studies reported [11]. The only difference is that in this study, granulosa cells were not damaged due to the brittleness of microtubules under the storage conditions of 4℃. Instead, they were better than RT and 37℃.

Studies of Hideaki et al. (2007) have stored cat ovaries in normal saline at 4℃, 23-25℃ or 38℃ for 24 h to explore the quality of collected oocytes. The results showed that with the increase of ovarian storage temperature, the morphological integrity rate and in vitro maturation rate of oocytes were significantly reduced [19]. Although the research objects are different, the state of granulosa cells can reflect the state of the oocytes, and the state of oocytes are also the reflection of the granulosa cells. We proposed that the results of this experiment are consistent with this study. There was also a study regarding bovine ovaries stored in normal saline at 15℃, 25℃ or 35℃ for 3-4 h. The results showed that the quality and developmental ability of oocytes stored at 15℃ are significantly higher than the other two groups [20]. Although the setting temperature in their experiment is different from this study, the general trend is consistent with it, that is, low temperature is beneficial to cell growth and vitality maintenance.

In addition, there was also a report regarding 4-week-old mice ovary stored at 4℃, 14℃, RT, and 37℃ for 24 h. The results showed that there was no significant histological difference between the 4℃ group and the fresh control group, while the other groups showed varying degrees of interstitial edema and decreased adhesion of granulosa cells [21]. This study more intuitively showed that in vitro low-temperature storage is conducive to maintaining the healthy state of ovaries and granulosa cells.

In fact, the results may be inconsistent due to different cell types. There are also some researchers found that the lower the storage temperature, the worse the cell state, such as human epidermal cells [12], human limbal epithelial cells [22] and human oral keratinocytes [23], which result may have no significance for granulosa cells.

However, this study also had some limitations. First, the room temperature was not constant which floating at 20-25℃, that may affect some experimental results. Second, the different temperature groups were not detailed enough. Considering that the researchers usually put the follicular fluid in a 4℃

refrigerator, room temperature or 37℃, and will not be placed for more than 8 h. Therefore, the experiment did not have too much experimental groups.

In fact, in many studies which using primary cells for experiment, the results may be inconsistent due to different processing methods for primary cells, and sometimes even opposite results. In addition, some primary cells or tissue materials for research are far away from the laboratory. At that time, it is necessary to consider the storage temperature and duration time during in vitro transportation. Therefore, we suggest that these conditions should be specified and recorded in detail in the research to help maintain the stability and repeatability of the experimental results.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Tongwei Zhang performed the experiment and wrote the first draft of the manuscript. Material preparation were helped perform by Huiying Fan and Junya Zhang. Data collection were performed by Jingyi Hu, Guang Yang and Qina He. Yucheng Bai and Jiahuan He helped performed the data analyses, and Ziwen Xu helped perform the analysis with constructive discussions. Guidong Yao contributed significantly to supervision, resources, review and project administration. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

This study was approved by the Biomedical Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All the patients signed informant consent.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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Figures

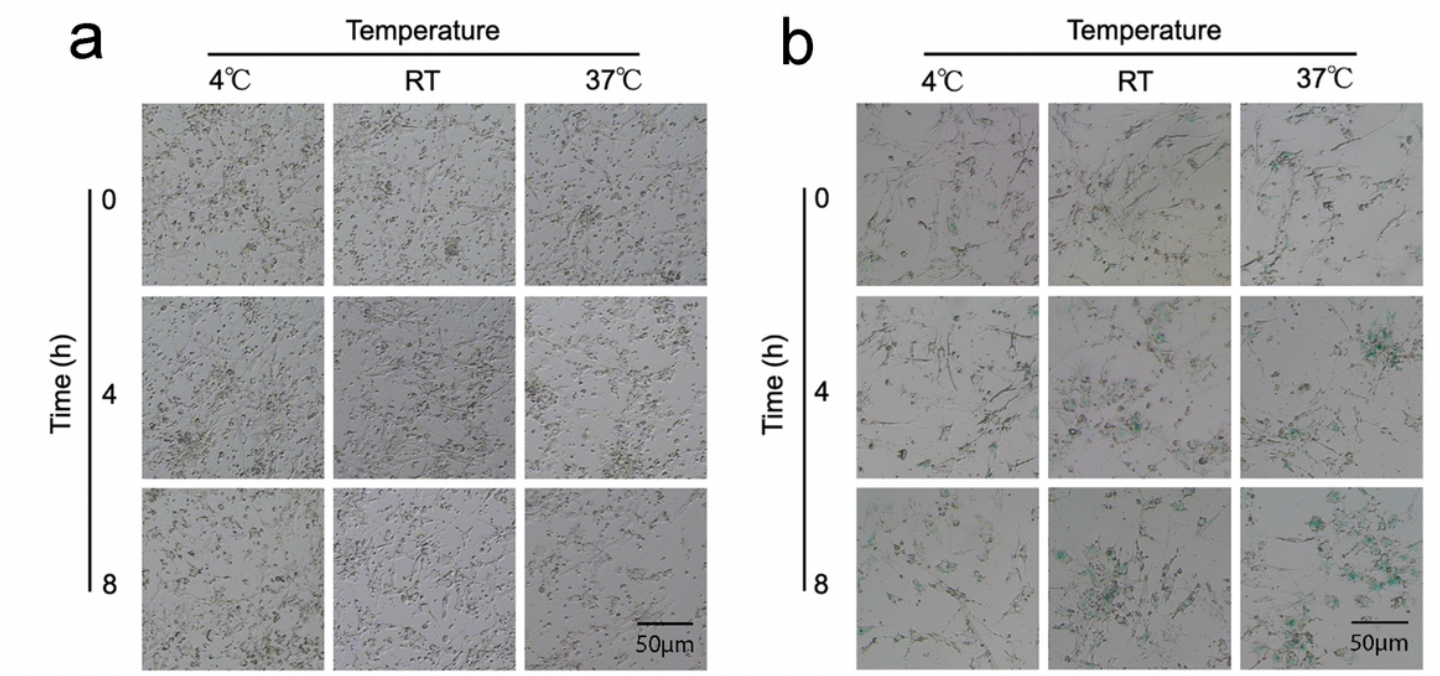

Figure 1

Effects of different in vitro storage temperature and duration time on the cell state after extraction of human granulosa cells

The collected follicular fluid was divided into 7 groups: except the fresh control group, the rests were placed at 4°C, RT and 37°C, respectively; and the primary granulosa cells were extracted after 4 h or 8 h in vitro storage duration time. The extracted human primary granulosa cells were cultured in DMEM/F12 medium containing 10% FBS for 48 h, observed and photographed under an optical microscope (a). The extracted human primary granulosa cells were cultured in DMEM/F12 medium containing 10% FBS for 48 h, stained with β-Galactosidase staining kit, incubated overnight at 37°C, observed and photographed under an optical microscope (b). RT= room temperature. Scale bar=50 μm.

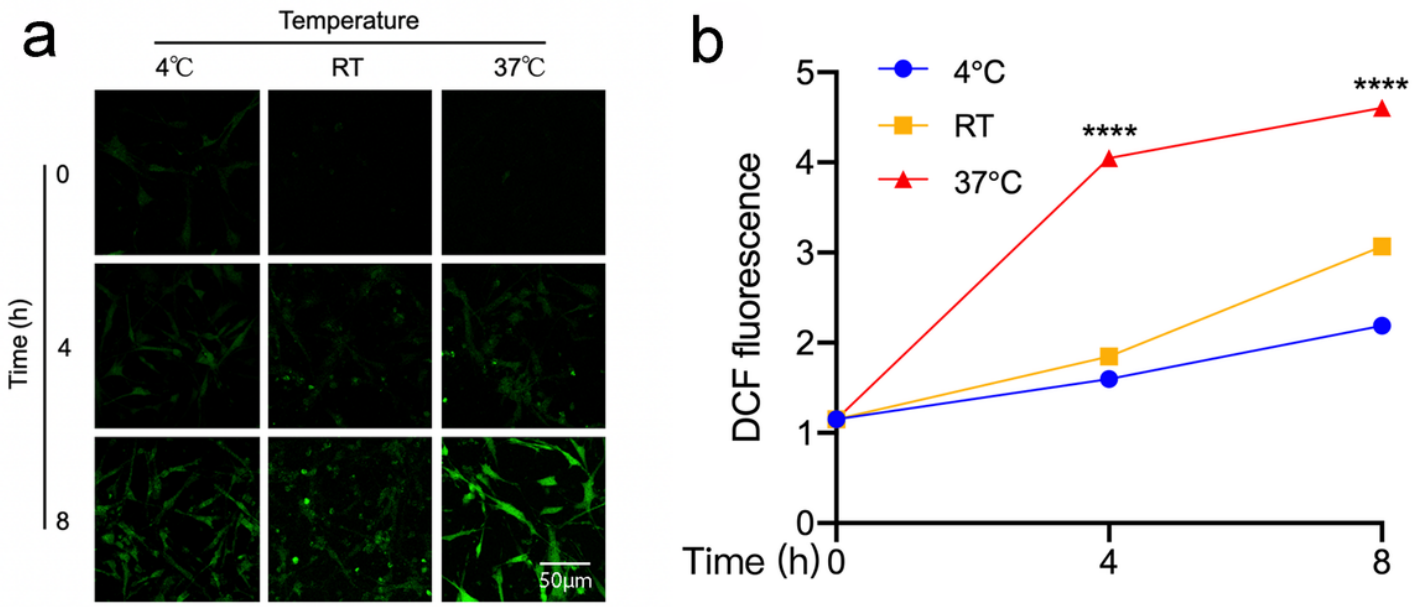

Figure 2

Effects of different in vitro storage temperature and duration time on oxidative stress of human granulosa cells after extraction

The extracted human primary granulosa cells were cultured in DMEM/F12 culture medium containing 10% FBS for 48 h. After loading the probe in situ with Reactive Oxygen Species detection kit, cells were observed and photographed by laser confocal microscope (a), or the cells were collected and detected by Varioskan Flash microplate reader (b). **** indicates $p<0.0001$. Scale bar=50 μm.

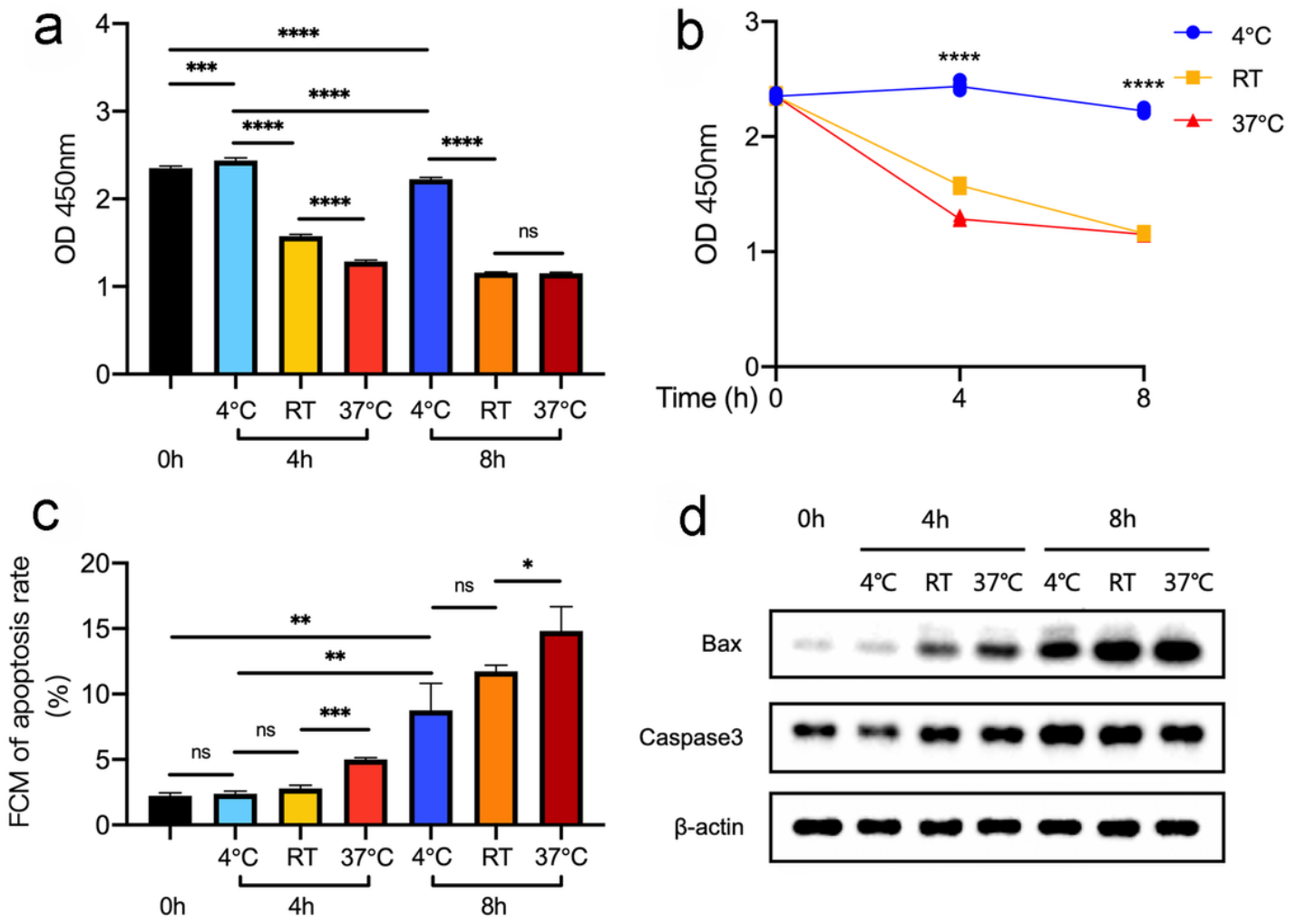

Figure 3

Effects of different in vitro storage temperature and duration time on cell survival after extraction of human granulosa cells

Human primary granulosa cells were cultured in DMEM/F12 medium containing 10% FBS for 48 h, then CCK8 reagent was added and incubated at 37℃ for 2 h. The OD value at 450nm wavelength was measured by Varioskan Flash microplate reader (a), and the cell viability was compared and analyzed in each group (b). The apoptosis rate was determined by flow cytometry using Annexin V-FITC/PI double staining cell apoptosis detection kit (c). ns indicates $p>0.05$, *indicates $p<0.05$, **indicates $p<0.01$, ***indicates $p<0.001$, and **** indicates $p<0.0001$. The extracted human primary granulosa cells were cultured in DMEM/F12 medium containing 10% FBS for 48 h, and then the protein was extracted. The expression levels of Bax and Caspase3 were analyzed by Western Blotting (d). β-actin was used as an internal reference protein.

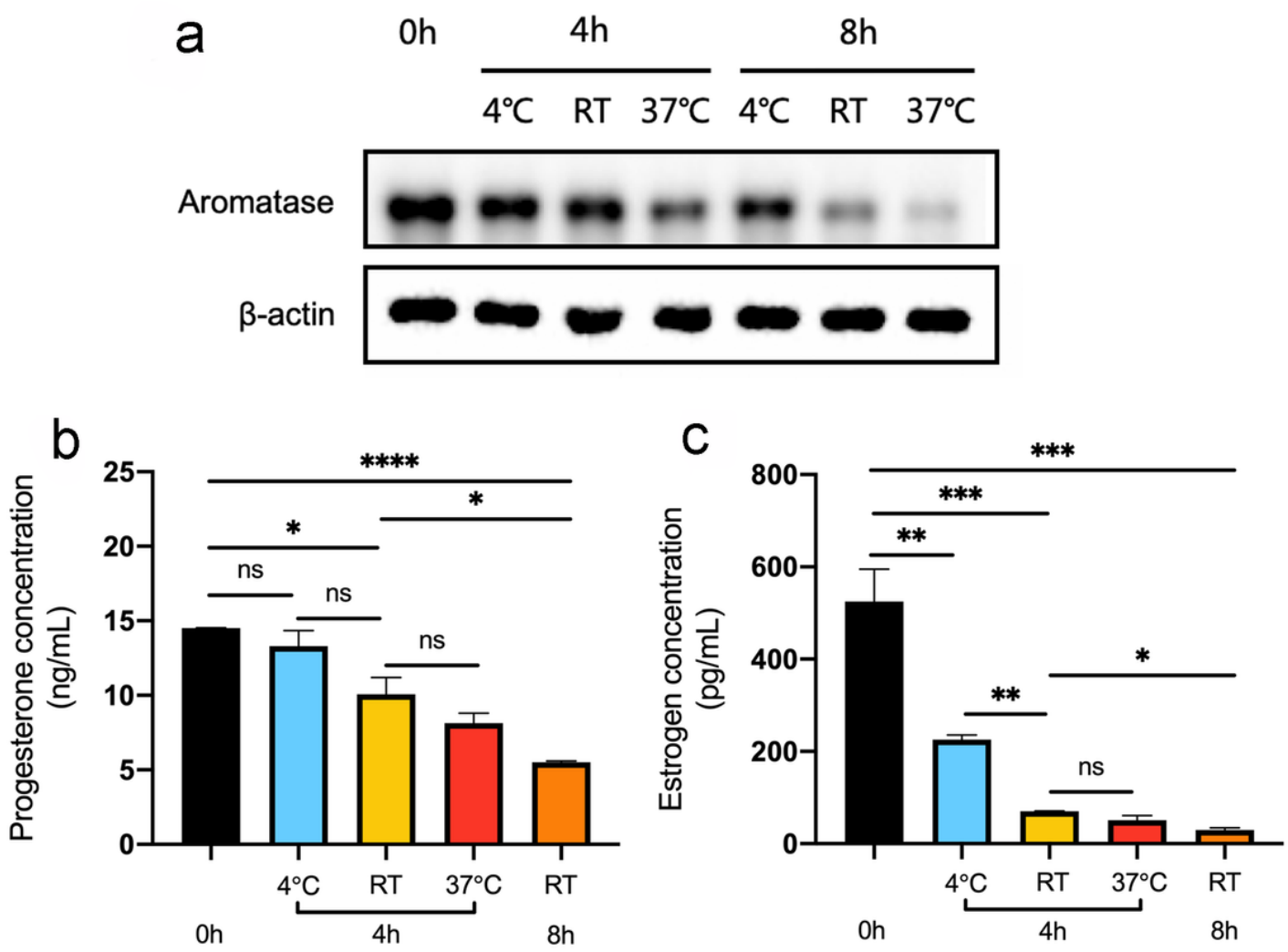

Figure 4

Effects of different in vitro storage temperature and duration time on the secretion of steroid hormones after extraction of human granulosa cells

The extracted human primary granulosa cells were cultured in DMEM/F12 medium containing 10% FBS for 48 h, the protein was extracted, and the expression level of Aromatase was analyzed by Western Blotting (a). β-actin was used as an internal reference protein. The extracted human primary granulosa cells were cultured in phenol red-free DMEM/F12 medium containing 10% FBS for 48 h. The supernatant of the culture medium was collected and diluted to an appropriate concentration, and then the Progesterone ELISA Kit and Estrogen ELISA Kit were used to detect the level of steroid hormones secretion. Varioskan Flash microplate reader was used to measure the level of progesterone and estrogen concentrations (b & c). ns indicates $p>0.05$, *indicates $p<0.05$, **indicates $p<0.01$, and *** indicates $p<0.001$.