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Cryopreservation of ovary tissue using vitrification, research and application on animal models of premature ovarian aging

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Abstract
This study aims to establish a cryopreservation procedure for early aging ovarian tissue using the vitrification method on animals, evaluating the survival rate of follicles after freezing and thawing as the effectiveness criterion. Cryopreservation techniques for ovarian tissue in animals have seen significant advancements in recent years. However, in Vietnam, there has been no research applying the vitrification method to ovarian tissue of prematurely aging animals. The choice of animal models is due to ethical constraints and limited access to human ovarian tissue. Animals share genetic and physiological characteristics with humans, making them an ideal model for studying ovarian aging. This study is expected to successfully establish a cryopreservation procedure for early aging ovarian tissue using the vitrification method on animals and evaluate the survival rate of follicles after freezing and thawing. The study is conducted on pig ovarian tissue using experimental methods. The ovarian tissue is divided into two groups: the control group (without cryopreservation) and the experimental group (cryopreserved using the slow freezing and vitrification methods). After freezing and thawing, the ovarian tissue is evaluated for survival rate, quality, and developmental potential of the follicles.

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Keywords: Cryopreservation, ovarian aging, ovarian tissue, vitrification

1. Introduction

Premature ovarian insufficiency (POI) is a condition in which the number of follicles and the quality of oocytes gradually decrease, determined by the loss of ovarian function before the age of 40. This leads to disturbed estrogen secretion and abnormal follicular development, which can lead to related diseases, premature menopause, sexual dysfunction then becoming an increasingly common cause of infertility, affects the overall quality of life of affected women (Cui & Wang, 2024) [4]. According to the European Society of Human Reproduction and Embryology (ESHRE) Guidelines the incidence of POI varies between ethnic groups around the world and studies show a prevalence ranging from 1% to 5, 5% (Shi *et al*., 2023) [14] and the incidence of POI in women under 40 years old is about 1% (Cui & Wang, 2024) [4].

There is now a need to develop new treatments and create new opportunities (Kristensen & Andersen, 2015) [1]. Since then, cryopreservation of ovarian tissue is one of the techniques to help preserve fertility for organisms with premature ovarian aging. And the verification method is the most advanced technique in the field of ovarian tissue cryopreservation, with a higher survival rate of ovarian follicles than traditional methods. Previous studies have only investigated and successfully frozen and thawed immature ovarian tissue in animals such as mice, sheep, and cows (Campos). *et al*., 2016) [3] with two methods of slow freezing and vitrification. However, there have been no publications on cryopreservation of prematurely aging ovarian tissue applied to singleton animals using the vitrification evaluation method. Therefore, the research project chose to cryopreserve aging animal ovarian tissue. From successes in aging animal models, the project aims to build a model of ovarian tissue freezing process applicable to older people. Due to ethical constraints and limited access to human ovarian tissues, research from

animals that share genetic and physiological characteristics with humans, constitutes an ideal model for Research on ovarian aging.

Previous studies mainly focused on applying the traditional slow freezing method on animals or analyzing the results on humans. Therefore, conducting research on the topic "Ovarian tissue cryopreservation by vitrification method, research and application on animal models of premature ovarian aging" in Vietnam will be an important step forward. It can be said that this is a potential study, showing good results to improve the quality of artificial insemination in animal models, thereby moving to a higher level in the future by applying it on ovarian tissue. Aging eggs in the elderly aims to solve the problem of elderly women having poor fertility rates in today's society. This study will provide valuable and modern information about the effectiveness and potential of vitrification, thereby opening up new opportunities in the field of assisted reproduction and fertility conservation in the country. This study aims to establish a procedure for cryopreservation of prematurely aging ovarian tissue using vitrification in an animal model, and evaluate the survival rate and quality of ovarian follicles after freezing and thawing.

In Vietnam, the number of cases of ovarian tissue cryopreservation reported and researched is very small compared to the world. Up to now, there has been no research on cryopreservation of aging ovarian tissue in animal models. Previous studies mainly focused on applying the traditional slow freezing method on animals or analyzing the results on humans. Therefore, conducting research on "Ovarian tissue cryopreservation by vitrification in an animal model of premature ovarian aging" in Vietnam will be an important and valuable step forward. This study will provide valuable and modern information about the effectiveness and potential of the vitrification method, thereby opening up new opportunities in the field of assisted reproduction and fertility conservation in Vietnam.

2. Materials and methods

Pig ovaries were collected at the slaughterhouse (Ho Chi Minh City). At the time of collection, ovaries were sprayed with 70° alcohol and stored in PBS– solution supplemented with antibiotics (penicillin/streptomycin). Store at 4 °C for a maximum of 12 hours before performing the experiment. Chemicals used in the study: Fetal Bovine Serum (FBS), dimethyl sulfoxide (DMSO), ethylene glycol (EG), phosphate - buffered saline (PBS), mineral oil (Paraffin).

Ovarian treatment

Using a medical surgical knife and scissors set, cut the ovary into small pieces of tissue about 10x10x1 mm in size. Then the tissue pieces were stabilized in DMEM/F12 medium supplemented with 10% FBS and antibiotics (Gentamycin 80mg) in a CO_2 incubator at 37 °C, 5% CO_2 for 30 minutes, and cultured in an incubator. CO_2 temperature 37°C, 5% CO_2 (control) and frozen ovarian tissue (experiment).

2.1. Investigating the effects of foreign preservatives on the freezing process of aging ovarian tissue

Purpose: Select the appropriate concentration of preservative - extracellular CPA (Sucrose) and preservation method for high survival rate of oocytes after thawing.

Ovarian tissue freezing procedure:

Slow-freezing: Pig ovarian tissue after stabilization is transferred to each 1.5 mL cryotube containing 0.8 mL of frozen medium ES1, ES2, ES3, ES4, ES5 with sucrose concentrations equal to respectively: 0 M, 0.05 M, 0.1 M, 0.15 M and 0.2 M, compared with an unrefrigerated control placed in a Coolrack box and slowly cooled from 26 °C to -30 °C (1° speed). C/min) for 55 minutes. Then dip the cryotube directly into liquid nitrogen (-196 °C), and store it frozen for 48 hours.

Vitrification: pig ovarian tissue was transferred into each 2 mL cryotube containing 1 mL of frozen medium EDS1, EDS2, EDS3, EDS4, EDS5 with sucrose concentrations of 0 M, 0.05 M, 0.1 respectively. M, 0.15 M and 0.2 M, compared with the control without refrigeration for 25 minutes. Transfer ovarian tissue to EDS frozen medium for 15 minutes. Then dip the cryotube directly into liquid nitrogen (-196 °C), and store it frozen for 48 hours.

2.2. Investigating the effect of intracellular preservative concentration on the freezing process of aging ovarian tissue

Purpose: Select appropriate intracellular CPA concentration (DMSO, EG) and preservation method for high survival rate of oocytes after thawing.

Ovarian tissue freezing procedure

Slow-freezing: Pig ovarian tissue after stabilization is transferred into each 1.5 mL cryotube containing 0.8 mL of freezing medium containing 0.1 M sucrose: SE1, SE2, SE3, SE4, SE5 with The EG concentrations are: 0.5 M, 1 M, 1.5 M and 2 M, respectively, compared with the unrefrigerated control, placed in a Coolrack box and slowly cooled from 26 °C to -30 °C (fast). Degrees 1°C/min) for 55 minutes. Then dip the cryotube directly into liquid nitrogen (-196°C), and store it frozen for 48 hours.

Vitrification: Pig ovarian tissue was transferred into each 2mL cryotube containing 1mL of EDS freezing medium for 25 minutes. Transfer ovarian tissue to frozen media ED1, ED2, ED3 and ED4 with EG and DMSO ratios of 5%:5%, 7.5%:5%, 5%:7.5%, 7.5%:7.5%, respectively, compared with the unrefrigerated control, for 15 minutes. Then dip the cryotube directly into liquid nitrogen (-196°C), and store it frozen for 48 hours.

2.3. Investigate the influence of environment and preservation method on egg stability after thawing

Purpose: Determine the environmental concentration, preservation method and appropriate time for the process of stabilizing eggs after thawing.

Procedure: Create microdroplets with 150 μl egg culture medium (Global® Collect®) covered with Paraffin liquid mineral oil in a 60 mm culture dish. Observe and use a 10mm micropipette to collect type A and B eggs according to the concentrations transferred to the culture drops, each microdrop contains 5-7 eggs. Stabilize in a CO2 incubator (37C, 5% CO2), monitor and observe egg stability after the 0 hour, 24 hour and 48 hour time points.

Evaluation criteria for studies 1.1, 1.2, 1.3: Observe the morphology of tissue stained with HE before and after storage, monitor whether the tissue is intact, not broken, uniform in size, color, and quality Eggs after thawing are screened for egg groups A and B (the nucleus is uniformly intact without shrinkage or fragmentation, the zona membrane covering the outside of the egg cell shell is not broken, and the cumulus layer surrounding it) egg C (no

surrounding layer of cumulus cells).

2.4. Investigate the effects of environment and preservation methods on the fertilization of post-stabilized eggs

Purpose: Determine the appropriate environment and preservation method for the fertilization process to develop into stable egg embryos.

Evaluation criteria: The egg is fertilized and develops into an embryo from 2 cells to the blastocyst stage.

Data processing

The obtained data were subjected to descriptive statistics and analysis of variance (ANOVA), data were expressed as mean value ± standard deviation, statistical comparison between the control group and the treated experimental group using Statgraphics Centurion 18 software with Tukey HSD method with 95% reliability. A p value < 0.05 was considered to represent a statistically significant difference. Graphs were drawn using Graphpad 9 software.

3. Results and Discussion

3.1. Investigation of the effects of extracellular preservatives on the freezing process of aging ovarian tissue

Calculate sample size:

$$n \geq \left(1+\sqrt{g-1}\right)\frac{\left(Z_{1-\frac{\alpha}{2}}+Z_{1-\beta}\right)^2}{d^2}+\frac{\sqrt{g-1}}{2\left(1+\sqrt{g-1}\right)}$$

Choose effect size d = 1.5 $\frac{Mean}{SD} \approx 1.5$)

From the above formula, the appropriate sample size needed for each group is over 17 ovarian tissue samples and over 102 oocytes needed for survey treatments.

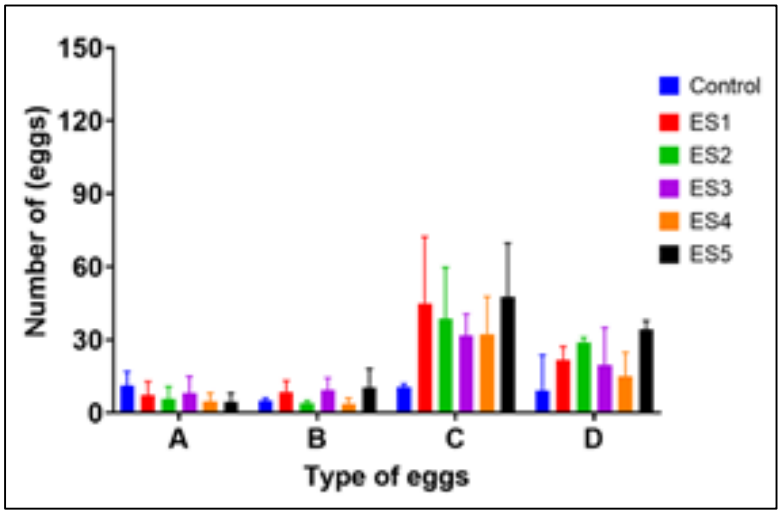

Fig 1: Graph representing the survey results on the influence of extracellular cryoprotectants and slow-freezing methods

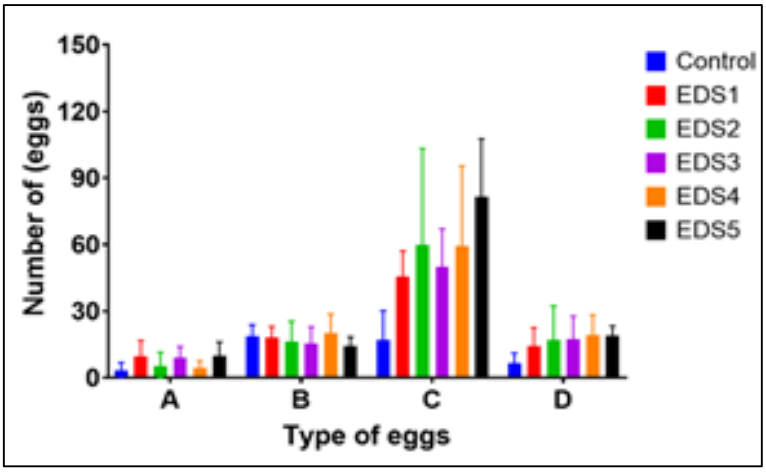

Fig 2: Graph representing the survey results on the influence of extracellular cryoprotectants and vitrification methods

Table 1: Results of the influence of preservation methods on the freezing process

Method	Egg quality			
	A	B	C	D
Control	21,00 ^{b}±15,56	29,00 ^{a}±19,80	40,50 ^{a}±12,02	22,50 ^{a}±6,36
Slow freezing	17,60 ^{a}±3,78	21,60 ^{a}±9,40	115,40 ^{b}±24,05	71,40 ^{c}±23,26
Vitrification	20,83 ^{b}±8,84	49,17 ^{b}±6,74	156,50 ^{c}±64,54	44,67 ^{b}±13,89

(a, b, c, d: Different letters in the same column indicate statistically significant differences at the α=0.05 level; Data are presented as mean ± standard error)

With 81 ovarian tissues, the study obtained 2,863 oocytes of all types, with an average yield of 35 oocytes/tissue. Performing freezing of 81 ovarian tissues, research on collecting type A oocytes using the vitrification method reached a value of 20.83b 8.84 cells, giving better survey results than the slow freezing method reaching 17. 60a 3.87 cells and not different from the control group 21.00 b 15.56 cells. In type B oocytes, the vitrification method resulted in the collection of 49.17 a 6.74 cells, much better than the amount of oocytes collected by the slow freezing method 21.60 a 90.40 cell; and control sample 29.00 a 19.80 (table 3.1).

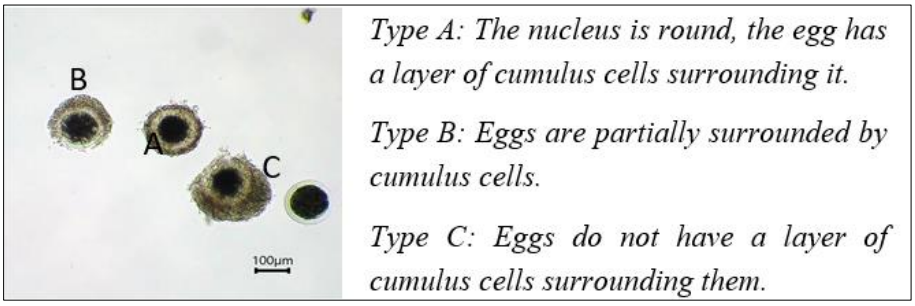

Fig 3: Shape of collected oocytes after thawing

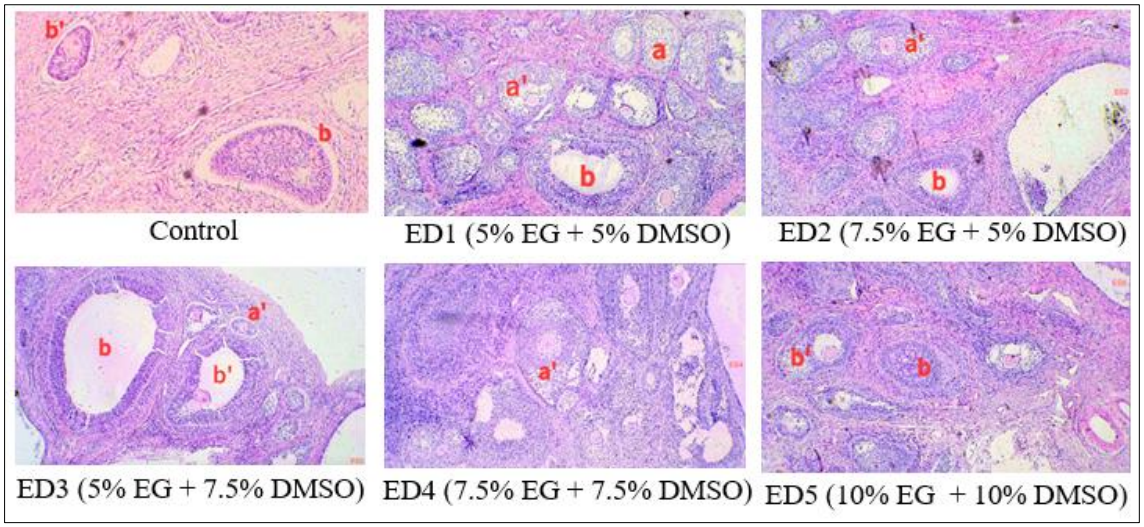

Fig 4: Ovarian tissue structure after thawing stained with HE A: normal tissue without ovules; a': normal tissue with ovules. B: senescent tissue without ovules; b': senescent tissue with ovules

3.2. Investigating the effect of intracellular preservative concentration on the freezing process of aging ovarian tissue

Calculate sample size:

$$n \geq \left(1+\sqrt{g-1}\right)\frac{\left(Z_{1-\frac{\alpha}{2}}+Z_{1-\beta}\right)^2}{d^2}+\frac{\sqrt{g-1}}{2\left(1+\sqrt{g-1}\right)}$$

Effect size d = 1 $\left(\frac{Mean}{SD} \approx 1\right)$

From the above formula, the appropriate sample size needed for each group is over 36 ovarian tissue samples and over 216 ocytes are needed for survey treatments.

Table 2: Results of the number of eggs collected to investigate the influence of intracellular preservatives

Method	Egg quality			
	A	B	C	D
Control	20,50 ^{b}±3,54	59,50 ^{b}±13,44	165,00 ^{ab}±15,56	60,50 ^{a}±36,06
Slow freezing	17,00 ^{a}±4,47	22,20 ^{a}±5,97	234,72 ^{b}±30,72	81,00 ^{b}±8,43
Vitrification	30,20 ^{c}±13,50	43,60 ^{b}±16,09	134,6 ^{a}±55,66	78,6 ^{ab}±12,30

(a, b, c, d: Different letters in the same column indicate statistically significant differences at the α=0.05 level; Data are presented as mean ± standard error)

With 144 ovarian tissues, 3472 oocytes of all types were obtained, with an average yield of 24 oocytes/tissue. Freezing 144 ovarian tissues, research and collection showed that type A oocytes using the vitrification method yielded better results in collecting 30.20^{c}±13.50 cells than the slow freezing method 17, 00^{a}±4.47 cells and control group 20.50^{b}±3.54 cells. In type B oocytes, the vitrification freezing method resulted in 43.69b±16.90 cells, better than the slow freezing method 22.22^{a}±5.97 cells, less than the sample control 59.50^{c}±13.44 cells (table 3.2).

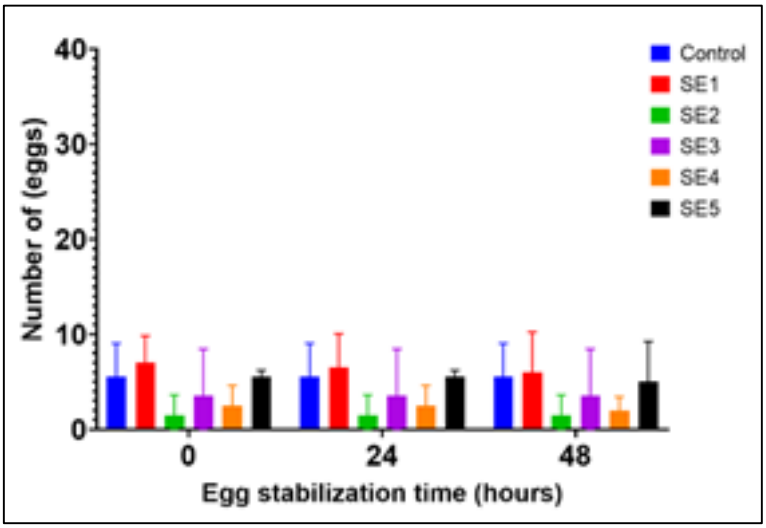

Fig 5: Graph representing the survey results on the influence of intracellular cryoprotectants and slow-freezing methods on the stability of eggs after thawin

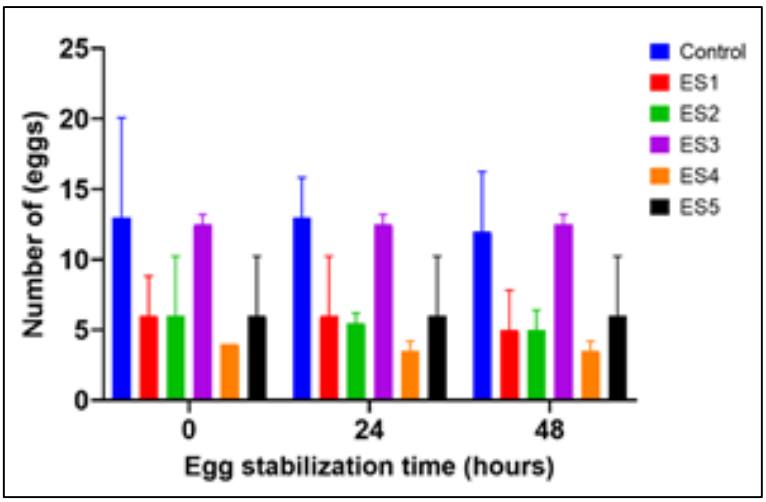

Fig 6: Graph representing the survey results on the influence of extracellular cryoprotectants and slow-freezing methods on the stability of eggs after thawing

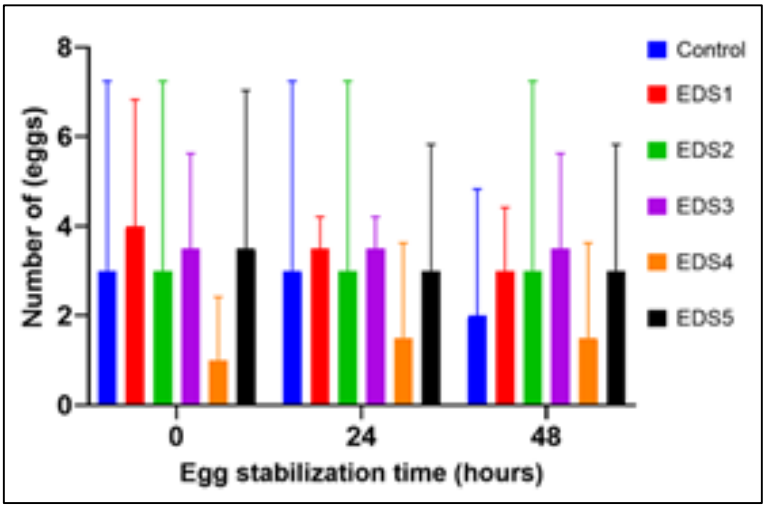

Fig 7: Graph representing the survey results on the influence of extracellular cryoprotectants and vitrification methods on the stability of eggs after thawing

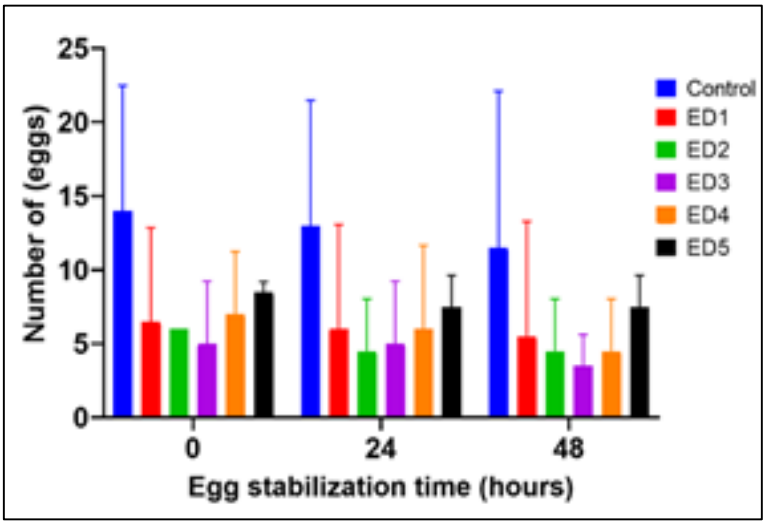

Fig 8: Graph representing the survey results on the influence of intracellular cryoprotectants and vitrification methods on the stability of eggs after thawing

Most of the freezing methods and preservatives, media 1 (ES, SE, ED), 2 (ES, SE, ED), 3 (ES, SE, ED), 4 (ES, SE, ED) is more effective in preserving eggs than the control environment. Environment 1 (ES, SE, ED) has the best egg preservation effect, followed by 2 (ES, SE, ED), 3 (ES, SE, ED), 4 (ES, SE, ED). Environment 5 (ES, SE, ED) (has the lowest egg preservation efficiency. P-value at all times (0h, 24h, 48h) is greater than 0.05. At 0h, the number of surviving cells in all environments is the highest. Environment 1 (ES, SE, ED) has a significantly higher number of surviving cells than other environments (p-value > 0.05).). Environment 2 (ES, SE, ED) and 3 (ES, SE, ED) have similar numbers of viable cells (p-value > 0.05). The number of viable cells is higher than 5 (ES, SE, ED) (P-value > 0.05). At 24 hours, the number of viable cells in all environments decreased compared to 0 hours. Environment 1 (ES, SE, ED) still has the highest number of viable cells, but the difference compared to other environments is no longer too large (p-value > 0.05). SE, ED) and 3 (ES, SE, ED) had similar numbers of viable cells (p-value > 0.05). Media 4 (ES, SE, ED) and 5 (ES, SE, ED) have a similar number of viable cells (P-value > 0.05). At 48 hours, the number of viable cells in all environments decreased sharply compared to 0 hours and 24 hours. P-value at all time points (0h, 24h, 48h) is greater than 0.05. This shows that there is no statistically significant difference between the control environment and environments 1 (ES, SE, ED), 2 (ES, SE, ED), 3 (ES, SE, ED), 4 (ES, SE, ED) for stable number of oocytes (P-value > 0.05).

The results of surveying the effects of intracellular preservatives and slow freezing method on egg stability after thawing in Figures 3.7, 3.8, 3.9, 3.10 show that there is no difference between the treatments compared to the control time points 0h, 24h and 48h. This shows that there is no statistically significant difference between the control environment and the environments in terms of the number of stable oocytes (P-value > 0.05).

3.4. Evaluate the fertilization efficiency of eggs after stabilization

Fig 9: Fertilization results of 2-cell and morula embryo stages

The results in Figure 3.11 show that egg cells can undergo fertilization after tissue preservation by vitrification and develop into embryos at different stages. The results can evaluate the ovarian tissue freezing environment to bring good quality of oocytes after freezing - thawing.

4. Conclusion

The study investigated the effects of intracellular and extracellular preservatives on the process of cryopreservation of prematurely aging ovarian tissue using the vitrification method in animal models, and evaluated the rate of ovarian follicle survival after frozen and thawed. Research conducted on pig ovarian tissue showed good results of cryopreservation using the vitrification method. After freezing and thawing, ovarian tissue was evaluated for survival, quality, and follicular development.

5. Thank you

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