HAYATI
Journal of Biosciences

Vol. 28 No. 4, October 2021 304-311
DOI:10.4308/hjb.28.4.304-311
ISSN: 1978-3019
EISSN: 2086-4094

Effect of Ethylene Glycol on Structural Integrity at Each Stage of Preantral Follicle Development Post Vitrification of Rat Ovary-Histological Analysis

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ARTICLE INFO

Article history:
Received July 19, 2021
Received in revised form August 25, 2021
Accepted September 23, 2021

KEYWORDS:
Cryoinjury,
follicle density,
follicle index,
grading method,
preservation

ABSTRACT

The structure of follicular tissue affects the ability to maintain the structural integrity of follicles against cryoinjury post-vitrification. Histological analysis was conducted on the structural integrity of each stage of preantral follicles post-vitrification using 7.5% and 15.0% doses of ethylene glycol (EG), and ovarian sections with HE staining were observed using an Olympus CX21 microscope connected to Optilab 3.0 lens and Image Raster software. Analysis was conducted on the ovarian cortex in the tracing line area using polygon measure tools to obtain follicle density (follicles/mm^2) and follicle index (%) data. The result showed that the EG group 7.5% (KP1) increased follicle density compared to the vitrified group (KKV) in primordial (15.83±1.77) and primary (22.94±8.51) stages. Meanwhile, KP2 (EG 15%) was in primordial (41.92±6.45), primary (11.69±1.95), secondary (33.48±3.63), and tertiary (5.93±0.69) stages. KP1 increased grade 3 follicle index compared to KKV in primary (27.66±2.34), secondary (32.41±6.99), and tertiary (25.00±5.00) stages. Meanwhile, KP2 was in primary (26.87±6.68) and tertiary (25.00±5.00) stages. Both doses of 7.5% and 15.0% EG were able to maintain structural integrity at certain stages of preantral follicles. Secondary and tertiary follicles are the best stages in maintaining grade 3 follicular integrity with the addition of 7.5% EG.

1. Introduction

Follicle preservation through ovarian vitrification technique aims to produce competent oocytes for *in vitro* culture (Amorim *et al.* 2003). The follicle is the basic structure and functional unit of the ovary that provides the microenvironment needed for oocyte growth and maturation (Collado-Fernandez *et al.* 2012). These oocytes were obtained from preantral follicles, which consist of primordial, primary, and secondary follicles, that make up 90% of the ovarian population (Amorim *et al.* 2003).

The cryoinjury due to the formation and melting of ice crystals during the cooling and thawing process as well as changes in osmotic pressure during the vitrification causes damage and disrupts the fluidity of the follicular cell membrane (Jang *et al.* 2017). This damage causes the release of oocyte granulosa cells when cultured which will affect oocyte maturation (Kim *et al.* 2018). Studies on the structure of the preantral follicle of post-vitrified rat ovaries are limited to the number and viability of follicles that are maintained post-vitrification using the EG at a dose of 3.75-15.0% (Milenkovic *et al.* 2012; Fathi *et al.* 2013). Meanwhile, studies on how the integrity of the follicular structure in each preantral follicles is to be maintained with the correct optimal dose are still limited. Therefore, this study is necessary to identify the potential of primordial, primary, secondary, and tertiary follicles which are to be utilized after preservation (Campos *et al.* 2019).

Ethylene glycol (EG) has a small molecular weight of 62.068 g/mol with a lower level of toxicity compared to dimethyl sulfoxide (DMSO) and propanediol (PROH). Furthermore, the disadvantage when dehydrated, it enters the intracellular fluid during the cooling process, and it still forms an incomplete glass layer that triggers cryoinjury and cell damage (Fathi *et al.* 2013). Therefore, the use of the right type and amount of intracellular cryoprotectant is needed to reduce the potential for cell

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damage during the vitrification. This strategy requires complete information on how to describe the structural integrity of each type of post-vitrified preantral follicle using EG as an intracellular cryoprotectant.

To date, rodents, especially mice, are the only species that undergo a complete *in vitro* culture process from the primordial stage to offspring (Dole *et al.* 2008). Furthermore, this study used rat ovary to identify the structural integrity at each stage of the preantral follicle and to obtain the optimum dose of EG that maintains the integrity at each post-vitrified preantral follicle stage. These data are needed as a basis and information input in the development of methods to maintain ovarian function, especially in high economic value, rare animals, and endangered animals whose populations are decreasing due to inbreeding depression caused by climate change (Ledda *et al.* 2001). Another goal is to maintain ovarian function in cancer patients of reproductive age undergoing chemotherapy, which causes ovarian dysfunction, premature apoptosis, and decreased female reproductive function (Meirow and Nugent 2001).

2. Materials and Methods

This research is experimental, using 24 samples of rat (*Rattus norvegicus*) ovary, which is determined according to the Resource Equation method (Charan and Kantharia 2013). Inclusion criteria: female rats, Sprague-Dawley strain, 7-10 weeks of age, healthy, active, 120-150 g of body weight, obtained from the Indonesian Research Center for Veterinary Sciences, Bogor. Rats were randomly divided into 4 groups, a normal control group without vitrification (KKN), a vitrified control group with 0.9% NaCl (KKV), a vitrified treatment group with 7.5% EG (KP1), a vitrified treatment group with 15% EG (KP2).

2.1. Proestrus Phase Identification

Ovarian isolation was conducted in the proestrus phase by observing vaginal epithelial cytology. Vaginal epithelial isolation procedure refers to the method of Marcondes *et al.* (2002) and Sjahfirdi *et al.* (2013). Observation of the vaginal epithelium was conducted using a light microscope with 100x and 400x magnifications by observing the most representative cells. The proestrus phase was characterized by a moderate amount of SNAC (++), a high number of NAC (+++), a small amount of EAC (++), and a few neutrophils (++) (Cora *et al.* 2015).

2.2. Euthanasia and Ovarian Isolation

Euthanasia of experimental animals was conducted using an intraperitoneal injection of a lethal dose of 148 mg/kg of ketamine, and this procedure has been approved by The Ethics Committee of the Faculty of Medicine, Universitas Indonesia-Ciptomangunkusumo Hospital with protocol number 21-05-0531. The ovaries were then cleaned using 0.9% NaCl solution and immediately immersed in 10% formal saline fixation medium (for KKN) or 0.9% NaCl, 7.5%, and 15.0% EG vitrification medium.

2.3. Vitrification and Thawing

The vitrification medium consisted of EG 7.5% and 15.0%. The cryotubes were then exposed to liquid nitrogen vapor for 10 seconds to allow time for the cryoprotectants to penetrate the cells. After that, the cryotubes were immersed into liquid nitrogen at a temperature of -196°C for 48 hours. Then the thawing process was carried out by immersing the cryotube containing the ovaries into a water bath with a temperature of ±37°C for 3 minutes (Milenkovic *et al.* 2012).

2.4. Preantral Follicle Histological Analysis

Histological staining processes were conducted using the paraffin method and were stained with Hematoxylin-Eosin (HE) at the Histology Laboratory FMUI (Anita 2004). Tissue sectioning was conducted serially with a thickness of 5 µm, and a length of 50 µm interval between serials (Watermann *et al.* 2008). Each type of preantral follicle was observed for its structure using a light microscope (Olympus CX21) connected to Optilab 3.0 lens and Image Raster software. Histological analysis, which includes the identification of the integrity structure and the density of the follicles was conducted.

The parameters of intact follicles are:

- The granulosa cells are dense, regular, without vacuolization, and have intact, dense, regular in shape nucleus (Myers *et al.* 2004)
- The space between the oocyte and the granulosa cells is narrow (Picut *et al.* 2015)
- The oocytes are round, regular, dense, have the appearance of a nucleolus, and surrounded by an intact zona pellucida (Myers *et al.* 2004)

While the structure of the atresia follicle has the following criteria:

- The granulosa cells are loose, not dense, irregular, separated from each other, with vacuolization (Picut *et al.* 2015), and have an irregular shape nucleus (Zhou *et al.* 2010)
- Enlarged space between oocyte and granulosa cells (Wang *et al.* 2016)
- The oocytes are not intact, irregular, dense, shrunken (Myers *et al.* 2004), pyknotic (Wang *et al.* 2016), with non intact zona pelucida

The follicular integrity of each preantral stage was evaluated according to the following criteria (Lee *et al.* 2015):

Primordial/primary follicle:

G1 : Oocytes are deformed, irregular, and pyknotic

G2 : The granulosa cells are not dense, irregular, but the oocyte is round, intact, and dense

G3 : Round follicles with dense and regular granulosa cells, without vacuolization

Secondary/tertiary follicle:

G1 : Irregular follicles, loss of granulosa cells, pyknotic nuclei, and irregular oocytes

G2 : Follicles are not round, granulosa and theca cell between layers are not evenly distributed, loose, irregular but the oocytes are intact, dense, and homogeneous

G3 : Round follicles, granulosa, and theca cells between layers are evenly distributed, oocytes are round, intact, dense, and homogeneous, and the antrum is intact

The characteristics of intact follicles that meet the criteria are in grade 3 (Lee *et al.* 2015).

Follicle density was analyzed by counting each follicle in the ovarian cortex (number of follicles/cortex area (mm^2)) in the tracing line area determined using the polygon measure tool. This technique has been validated and used extensively to estimate cell density in heterogeneous tissues (Courbiere *et al.* 2005).

3. Results

3.1. Preantral Follicle Density

Table 1, Figure 1, and the results of the LSD test ($p \leq 0.05$) showed that vitrification caused a decrease in follicle density (follicles/mm^2) in all stages of preantral follicles with the highest decrease in the tertiary stage (0.73±1.26), and the lowest in the primordial (8.51±3.09) and secondary (12.54±4.40) stages.

The data also showed that the group with 7.5% EG dose (KP1) was able to increase follicle density compared to the vitrified group without cryoprotectant (KKV) in the primordial (15.83±1.77 > 8.51±3.09) and primary (22.94±8.51 > 2.41±1.32) stages. Meanwhile, KP2 (EG 15%) in the primordial (41.92±6.45 > 8.51±3.09), primary (11.69±1.95 > 2.42±1.32) secondary (33.48±3.63 > 12.54±4.40), and tertiary (5.93±0.69 > 0.73±1.26) stages.

The EG dose of 7.5% showed that follicle density was not significantly different ($p > 0.05$) with the control group without vitrification (KKN) in the primary (22.94±8.51 > 16.70±2.43) and tertiary (4.11±1.67 < 4.50±1.83) stages. Meanwhile, the 15% EG dose showed no significant difference in follicular density ($p > 0.05$) with KKN at all stages of preantral follicles; primordial (41.92±6.45 > 37.14±16.05), primary (11.69±1.95 < 16.70±2.43), secondary (33.48±3.63 < 41.47±6.84), and tertiary (5.93±0.69 > 4.50±1.83).

3.2. Follicle Index

The comparison of the histological structure of atretic follicles with intact follicles and between grades can be seen in Figure 2 and 3. The results

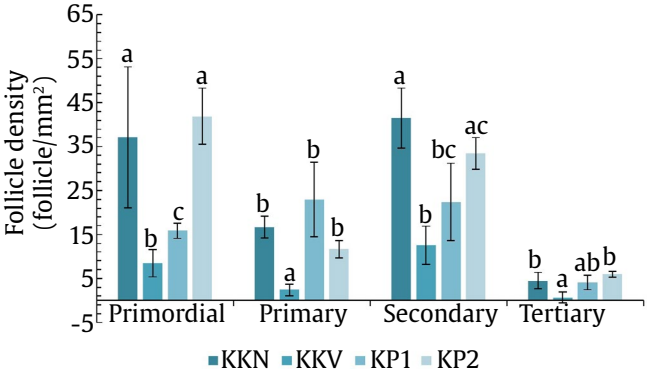

Figure 1. The ovarian follicle density. The bar shows SD; the different letters indicate a significant difference ($p \leq 0.05$)

Table 1. The ovarian follicle density between groups (follicles/mm^2)

Follicle	KKN	KKV	KP1	KP2
Primordial	$37.14±16.05^{a}$	$8.51±3.09^{b}$	$15.83±1.77^{c}$	$41.92±6.45^{a}$
Primary	$16.70±2.43^{b}$	$2.42±1.32^{a}$	$22.94±8.51^{b}$	$11.69±1.95^{b}$
Secondary	$41.47±6.84^{a}$	$12.54±4.40^{b}$	$22.38±8.82^{bc}$	$33.48±3.63^{ac}$
Tertiary	$4.50±1.83^{b}$	$0.73±1.26^{a}$	$4.11±1.67^{ab}$	$5.93±0.69^{b}$
Total follicles	$99.81±19.63^{a}$	$24.19±4.87^{b}$	$65.26±9.51^{c}$	$93.01±7.43^{a}$

Density data are shown as means ± SD

The different letters indicate a significant difference ($p \leq 0.05$)

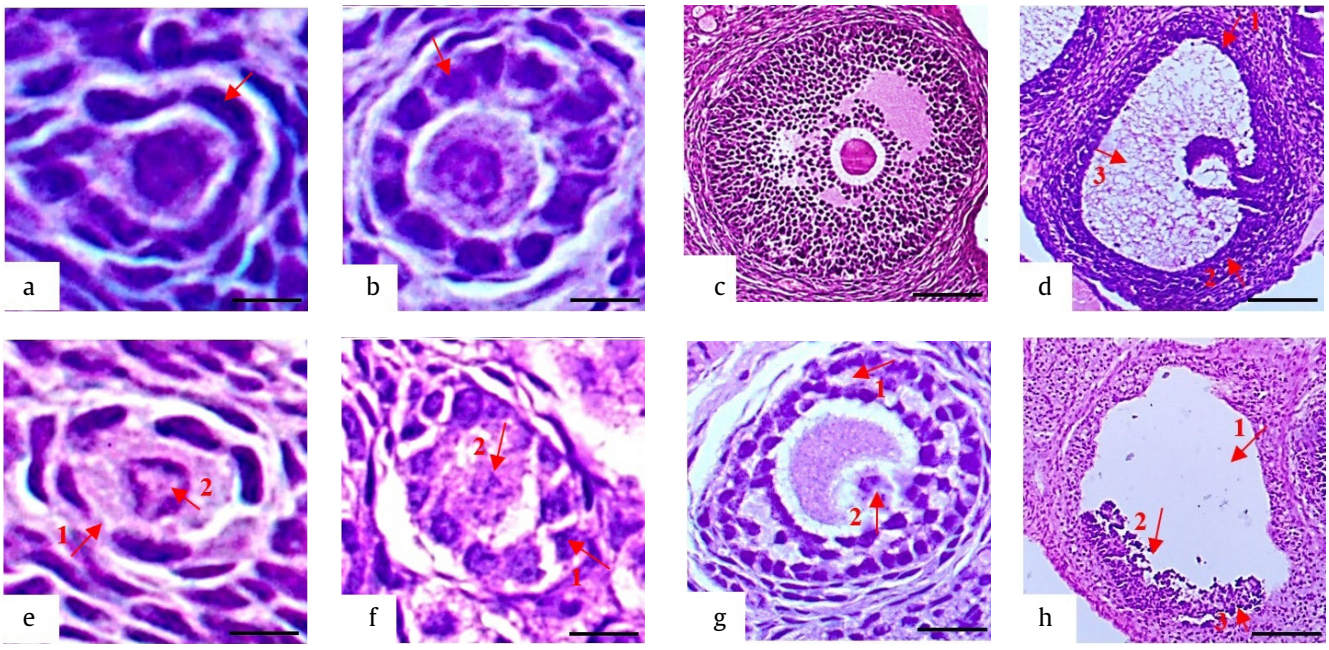

Figure 2. Histological structure of grade 3 intact and atretic preantral follicles. (a) Intact primordial follicle with squamous epithelial cells (red arrow) (400x magnification) (b) intact primary follicle with cuboidal epithelial cells (red arrow) (400x magnification), (c) intact secondary follicle with zona pellucida and intact basement membrane (400x magnification), (d) intact tertiary follicle with dense interlayer granulosa cells (red arrow 1), theca cells (red arrow 2) and antrum cavity clearly visible (red arrow 3) (400x magnification), (e) atretic primordial follicle with vacuolization (red arrow 1), oocyte nucleus fragmentation, and condensation (red arrow 2) (400x magnification), (f) atretic primary follicle with irregular cell arrangement (red arrow 1) and atretic oocytes (red arrow 2) (400x magnification), (g) atretic secondary follicle with vacuolization (red arrow 1), non-intact oocytes (red arrow 2), non-intact zona pellucida, oocyte nucleus fragmentation, and condensation (400x magnification), (h) atretic tertiary follicle with enlarged antrum cavity (red arrow 1), atretic granulosa cells (red arrow 2), non-intact basement membrane (red arrow 3) (400x magnification). (a, e) scale bar = 50 µm, (b, f) scale bar = 100 µm, (c, d, g, h) scale bar = 200 µm

showed that vitrification increased the index of grade 1 follicles which were categorized as atretic follicles in the primordial, primary and tertiary stages. Also, it showed the differences in the ability of follicles to maintain their structural integrity at grade 2 or 3 after the addition of EG, both at a dose of 7.5% and 15.0%. EG dose of 7.5% was able to increase the integrity of grade 2 follicles with the highest was in the primordial stage (47.78±13.47%), as well as the integrity of the grade 3 follicles in the primary (27.66±2.34%), secondary (32.41±6.99%) and tertiary (25.00±5.00%) stages. Meanwhile, EG dose of 15% increased the integrity of grade 2 follicles with the highest was in the primordial stage (50.82±6.94%), as well as the integrity of grade 3 follicles in the primary (26.87±6.68%) and tertiary stages (25.00±5.00%) (Table 2 and Figure 4).

4. Discussion

The decrease in follicular density at all stages of follicles is thought to be related to plasma membrane damage triggered by changes in osmotic pressure during the vitrification. The plasma membrane is the basic structure for connexin proteins (gap junctions). Gap junctions facilitate communication between granulosa cells that promote proliferation to produce various types of follicles. Ackert *et al.* (2001) studies showed that the failure of granulosa cell proliferation was caused by the structural damage of the gap junction (Kim and Lee 2021). This factor is thought to affect the decrease in follicle density at all stages of development, which begins with a decrease in the density of the primordial follicle.

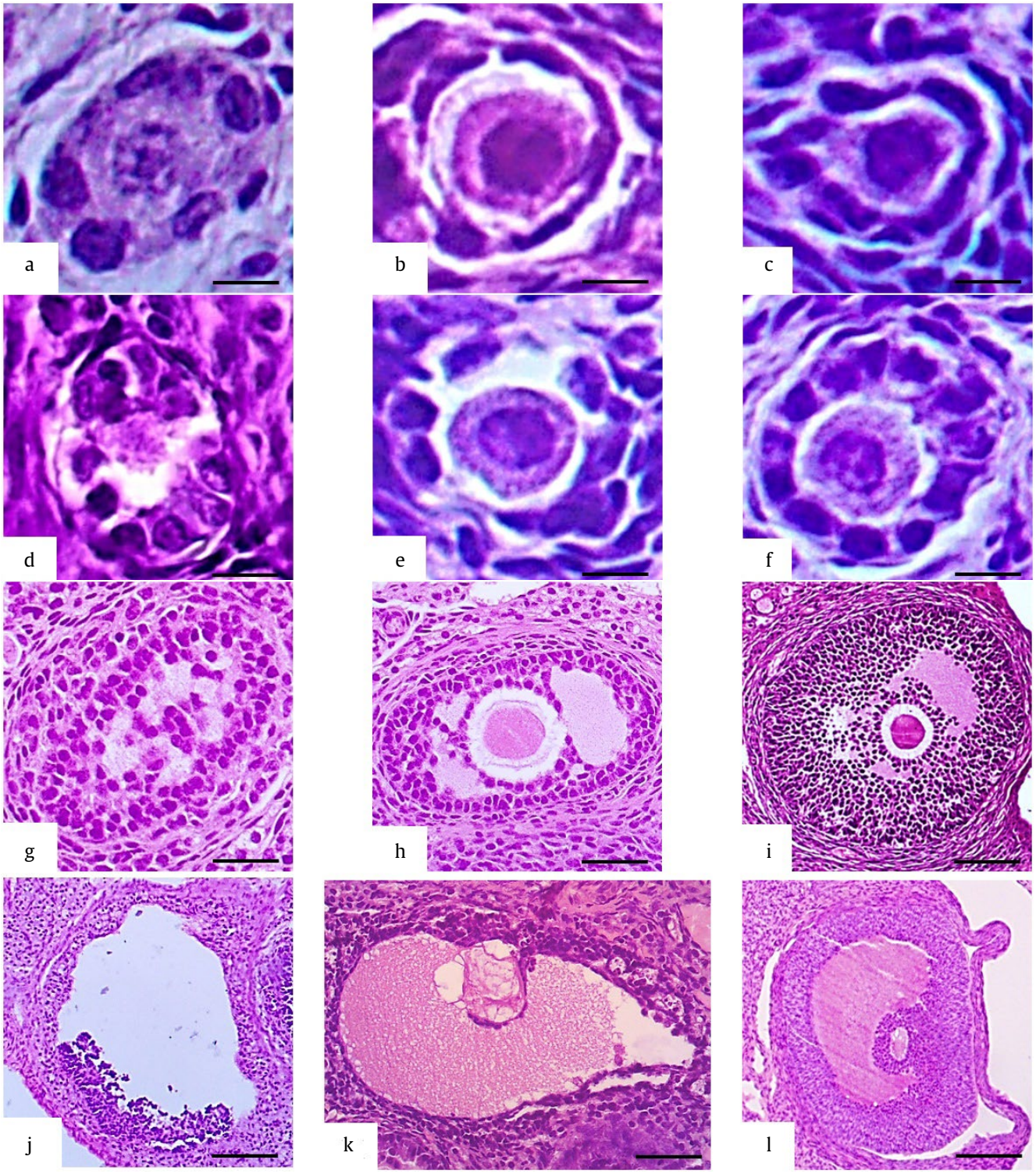

Figure 3. Structural integrity of grade 1, 2, and 3 preantral follicles. (a-c) Grade 1, 2 and 3 primordial follicles, respectively (400x magnification), (d-f) grade 1, 2, and 3 primary follicles, respectively (400x magnification), (g-i) grade 1, 2, and 3 secondary follicles, respectively (400x magnification), (j-l) grade 1, 2, and 3 tertiary follicles, respectively (400x magnification). (a-c) scale bar = 50 µm, (d-f) scale bar = 100 µm, (g-l) scale bar = 200 µm

Table 2. The percentage of grade 1, 2, and 3 follicle index between groups

Grade	Follicle	KKN	KKV	KP1	KP2
1	Primordial	47.62±2.03^{a}	69.87±1.38^{b}	34.44±5.09^{c}	42.10±3.70^{a}
	Primary	27.78±5.09^{a}	99.74±0.35^{b}	46.64±3.59^{c}	39.60±5.39^{c}
	Secondary	60.37±2.44^{a}	56.07±2.10^{b}	50.46±0.61^{c}	60.87±1.42^{a}
	Tertiary	5.56±0.42^{a}	86.07±5.19^{b}	74.94±3.66^{c}	68.11±6.17^{c}
2	Primordial	27.86±2.58^{a}	16.98±2.87^{a}	47.78±13.47^{b}	50.82±6.94^{b}
	Primary	3.70±1.70^{a}	0.09±0.03^{a}	25.70±2.59^{b}	33.54±6.37^{c}
	Secondary	5.69±0.51^{a}	30.89±2.38^{b}	17.13±4.88^{c}	24.38±1.44^{d}
	Tertiary	61.67±7.64^{b}	0.05±0.01^{a}	0.06±0.02^{a}	6.89±1.02^{c}
3	Primordial	24.52±4.31^{a}	13.15±1.95^{bc}	17.78±1.92^{ac}	7.08±1.23^{b}
	Primary	68.52±13.98^{a}	0.17±0.03^{b}	27.66±2.34^{c}	26.87±6.68^{c}
	Secondary	33.95±4.39^{a}	13.04±5.86^{b}	32.41±6.99^{a}	14.75±4.12^{b}
	Tertiary	32.78±2.55^{a}	13.89±3.47^{b}	25.00±5.00^{a}	25.00±5.00^{a}

Grade 1, 2, and 3 follicle index data are shown as means ± SD
The different letters indicate a significant difference ($p \leq 0.05$)

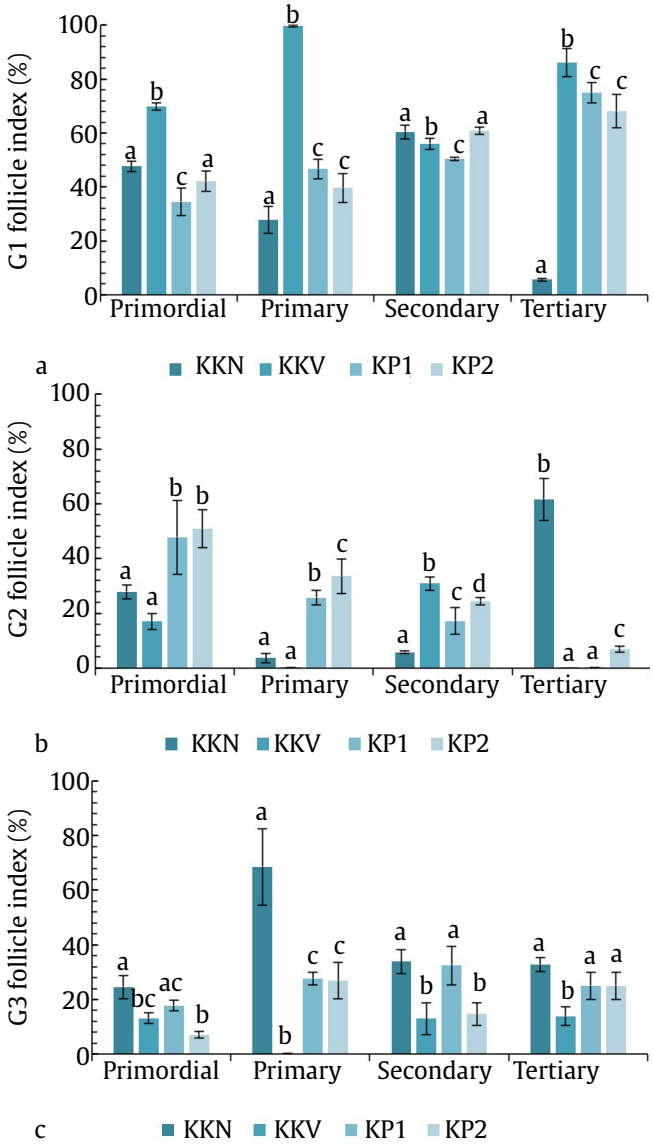

Figure 4. The percentage of ovarian grade 1, 2, and 3 follicle index. (a) Grade 1 follicle index, (b) grade 2 follicle index, (c) grade 3 follicle index. The bar shows SD; the different letters indicate a significant difference ($p \leq 0.05$)

However, according to Webb *et al.* (2004), three phases occur in the folliculogenesis to produce mature oocytes and they include recruitment, selection, and dominance. The recruitment phase begins with the development of primordial follicles derived from the proliferation of "stem cells" which constitute the germinal epithelium of the ovarian cortex, such that the primordial follicles have abundant numbers and better resistance than other types of follicles (Lima *et al.* 2013). The secondary follicles have a rigid basement membrane layer which serves not only to maintain the compactness of the stratification of the granulosa cell layer but is also protected such that the secondary follicle with this structure is better able to maintain its number from post-vitrification injury (Picut *et al.* 2015). Furthermore, secondary follicles undergo a selection process to produce tertiary follicles, naturally, that the density of tertiary follicles is the least when compared to other types of preantral follicles. This small amount is thought to decrease further due to the effect of increasing levels of free radicals caused by the increase in extremely cold temperatures during ovarian vitrification which triggers atretic follicles (Aerts and Bols 2010; Afrin *et al.* 2018).

The addition of EG at a dose of 7.5% and 15.0% was able to increase the density of the post-vitrification follicle, with an increase that results from the addition of 15.% dose EG in the primordial, primary, secondary, and tertiary stages. The average intracellular cryoprotectant concentrations that were used were in the range of 5% to 15% to allow cells to survive after freezing and thawing process (Kar *et al.* 2019).

Secondary and tertiary follicles are early antral follicles that undergo the same phase, namely the selection and dominance phase, and also have a relatively similar structure. Therefore, the addition of EG at a dose of 7.5% or 15.0% has the same effect

on the density of the secondary and tertiary follicles, but descriptively, the follicle density which is close to results in the non-vitrified group is KP2 (dose of 15%). Thus, the effective dose that increases the density of follicles is a dose of 15%.

The results showed that the grade 1 index which was categorized as atretic follicle had the highest value in almost all stages of follicles in the vitrified group without the addition of EG, except for secondary follicles. This proves that vitrification causes a cryoinjury due to the formation and melting of ice crystals in the cooling and thawing process, which causes damage and disruption of the fluidity of the cell membrane, and affects the integrity of the cell structure (Jang *et al.* 2017). Secondary follicles have a stratification of the granulosa cell layer and differentiation of this layer into a special layer, theca cells, that have an affinity for local growth factors and gonadotropins. Therefore, these structures are thought to strengthen the resistance of secondary follicles to post-vitrification cryoinjury (Heiligentag and Eichenlaub-Ritter 2018).

Studies have also shown that the earlier the follicle development stage, the better the ability to maintain its structural integrity. The primordial and primary follicles had the best resistance in grade 2 compared to secondary and tertiary follicles, both with the addition of EG at a dose of 7.5% or 15.0%. This is due to differences in the number and structure of organelles and metabolic activities. Whereas in growing follicles or early antral follicles, the oocyte is actively synthesizing protein and RNA (Hyttel *et al.* 1997). Follicles at this stage are more sensitive to an environment that is poor in nutrients and oxygen (Thompson *et al.* 2015). As a result, the process of cell degeneration will take place more quickly.

The results also showed that EG had a toxic effect, which increased with concentration. Furthermore, EG 7.5% was able to increase the grade 2 follicle index at the primordial and primary stages, due to the maximum penetration capacity of EG through a layer of granulosa cells. However, at the same time, the maximum effect of the toxicity of EG which also triggers cell necrosis, such that with the addition of 15% EG dose, the primordial grade 3 follicle index was decreased. The more complex structure that constitutes the follicular structure at that stage causes a decrease in the maximum capacity of EG penetration into the intracellular space. On the other hand, the maximum toxicity effect was avoided thereby reducing the number of necrotic cells.

Primordial, primary and tertiary stage follicles are stages of preantral follicles that maintains their structural integrity, both in grade 2 and 3, and this shows the potential of these follicles to be used after the preservation process. There is a positive correlation between follicle density and grade 2 and 3 indexes, especially at the secondary follicle stage. Meanwhile, tertiary follicles have the lowest density but have the best ability to maintain structural integrity post-vitrification.

The addition of EG, both 7.5%, and 15.0%, was able to increase the density of follicles post-vitrification. The optimum dose that can increase the density of follicles at all stages of preantral follicles is a dose of 15%. These results showed the different abilities of each follicle stage in maintaining its structural integrity post-vitrification. The addition of a dose of 7.5% was better able to maintain the grade 2 follicle index at the primordial and primary stages, while the grade 3 follicle index was at the secondary and tertiary stages.

Acknowledgements

This study was funded by a Doctoral Dissertation Research Grant year 2021 from the Directorate General of Research Enhancement and Development, Ministry of Research Technology and Higher Education. The authors are grateful to the Head of the Department and lab assistants of Biology and Histology Department Universitas Indonesia that supported this study.

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