Original Article

Bax/Bcl-2 Expression Ratio Analysis of Rat Ovary Vitrified with Date Juice Concentrate as a Natural Extracellular Cryoprotectant

Aisyah Safrina, Nova Anita[1], Ahmad Aulia Jusuf[2], Rahimi Syaidah[2], Huriyah Adani Saoemi[3]

Department of Histology, Faculty of Medicine, University of Medicine, [1]Faculty of Mathematics and Natural Sciences, Universitas Indonesia, [2]Faculty of Medicine, Universitas Indonesia, [3]Department of Anatomy, Universitas Indonesia, Depok, West Java, Indonesia

Abstract

Background: The use of extremely low temperatures in vitrification is known to cause cryoinjury so that it can trigger the activation of the intrinsic apoptotic pathway, which can damage the structural integrity of the pre-antral follicle. Based on that, it is necessary to use an appropriate cryoprotectant to protect the preserved cell. **Aims:** This study aimed to identify the potential use of date juice concentrate (DJC) as a natural extracellular cryoprotectant to suppress the rate of apoptosis after vitrification. **Settings and Design:** This experimental research uses 24 samples of ovarian rats. Rats were fed and drank an *ad libitum.* **Materials and Methods:** Ovaries were isolated in the proestrus phase, then processed into slides for immunohistochemistry (IHC) staining using anti-Bax and anti-Bcl-2 antibodies. IHC results were evaluated for the brown colour using ImageJ IHC Profiler. The results were analysed as an optical density and displayed in the Bax/Bcl-2 ratio. **Statistical Analysis Used:** All data were statistically analysed with either parametric (analysis of various) or non-parametric (Kruskal–Wallis) tests. **Results:** The combination of EG 7.5% + DJC 15% (KP2) showed the lowest Bax/Bcl-2 ratio in primordial and primary follicles. Meanwhile, the lowest Bax/Bcl-2 ratio in secondary follicles is found in KP4 (EG 15% + DJC 15%). The DJC is known to contain a dominant amount of glucose. The DJC shows antioxidant activity and contains antioxidant compounds, phenols and flavonoids. **Conclusion:** The sugar content and antioxidant compounds of DJC can protect against follicle membrane damage, so the rate of intrinsic apoptosis pathway is also suppressed initially with Bax protein suppression in the mitochondrial membrane.

Keywords: *Cryoinjury, date juice concentrate, follicle structure, pre-antral follicle, vitrification*

Introduction

Assisted reproductive technology through *in vitro* fertilisation (IVF) is one of the alternatives to overcome the problem of infertility in women of productive age. The initial stage of IVF is the preservation of fertility, especially with cryopreservation. Preservation of ovarian tissue has its advantages because it contains a large number of follicles, especially follicles of the pre-antral stage.[1,2] Most types of pre-antral follicles consist of primordial follicles that are known to be more resistant to cryopreservation effects than other types of follicles.[1] Oocytes in the pre-antral follicles have smaller sizes with inactive cell metabolism, thus allowing oocytes to be more resistant to vitrification damage.[3]

Vitrification is considered more effective and efficient, but there are still challenges to this method, including the use of extreme temperatures that

Received: 06-03-2023 **Revised:** 16-06-2023
Accepted: 18-06-2023 **Published:** 30-06-2023

Access this article online
Quick Response Code:
Website: www.jhrsonline.org
DOI: 10.4103/jhrs.jhrs_29_23

Address for correspondence: Dr. Nova Anita, Pondok Cina, Beji, Depok City, West Java 16424, Indonesia.
E-mail: nova.arief@gmail.com

How to cite this article: Safrina A, Anita N, Jusuf AA, Syaidah R, Saoemi HA. Bax/Bcl-2 expression ratio analysis of rat ovary vitrified with date juice concentrate as a natural extracellular cryoprotectant. J Hum Reprod Sci 2023;16:106-13.

damages the tissue and constraints on the selection of appropriate cryoprotectants.[4,5] The use of intracellular cryoprotectants such as ethylene glycol (EG) with high concentrations can be toxic, which affects the osmotic balance to cause cell membrane damage, changes in the structure of ovarian tissue and the occurrence of apoptosis.[6,7]

The use of extreme temperatures is also known to trigger an increase in free radical levels in the ovaries, causing oxidative stress.[8] Toxic compounds and free radicals are known to interfere with the potential of mitochondrial membranes so that the release of pro-apoptotic proteins through intrinsic pathway apoptosis. The activation of pro-apoptotic proteins such as Bax will trigger a further cascade of apoptosis to the stage of apoptotic body formation.[9] The effort to overcome this problem is the use of a combination of cryoprotectants.[10] Studies have shown that the addition of extracellular cryoprotectants can overcome the osmotic imbalances to prevent cryoinjury.[6]

In recent times, the use of extracellular cryoprotectants sourced from natural materials has begun to replace synthetic extracellular cryoprotectants and to lower the concentration of intracellular cryoprotectants due to the toxicity effect.[11,12] Date juice concentrate (DJC) is an alternative source of natural sugar that can be used as a natural extracellular cryoprotectant.[12,13] Currently, with the development of processed date palm fruit, the most popular is DJC because it is considered practical in terms of packaging.[14] Dates and their processed products are known to have many benefits in the field of health, especially in the reproductive system.[15]

A few researchers have started using DJC as natural extracellular cryoprotectorates or extenders in fertility cryopreservation, but yet still very limited.[12,16,17] This study was conducted to identify the potential use of DJC as a natural extracellular cryoprotectant in combination with EG to minimise the cryoinjury effect during and after vitrification. The combined use of cryoprotectants with DJC was reviewed for its effect on the incidence of apoptosis through the ratio of Bax/Bcl-2 protein expression in the pre-antral follicle after vitrification.

Subjects and Methods

Ethical clearance

The procedure for maintenance, handling and euthanasia of experimental animals carried out in accordance with applicable ethics and has been approved by The Ethics Committee of the Faculty of Medicine, University of Indonesia - RSCM Hospital, with protocol number 21-05-0531.

Animals and experimental groups

Experimental animals were 24 rats (*Rattus norvegicus*) Sprague–Dawley strain. The inclusion criteria in this study were female rats of the Sprague–Dawley strain, aged 7–13 weeks, with body weight ranging from 120 to 150 g, having a healthy physique and active activity. The study consisted of control group and treatment group. Both groups are subdivided into four subgroups (n = 3 ovaries per subgroup). Control and treatment group describes (KKN, KKV, KKP1 and KKP2) and (KP1, KP2, KP3 and KP4), respectively. The normal control group (KKN) was the group without vitrification, the treatment control group (KKV) ovarian vitrification using NaCl 0.9%, the treatment control group with ovarian vitrification using EG cryoprotectant 7.5% (KKP1), the treatment control group with ovarian vitrification using EG 15% cryoprotectant (KKP2), the ovarian vitrification treatment group using combination cryoprotectant EG 7.5% + DJC 7.5% (KP1), ovarian vitrification using combination cryoprotectant EG 7.5% + DJC 15% (KP2), ovarian vitrification using combination cryoprotectant EG 15% + DJC 7.5% (KP3) and ovarian vitrification using combination cryoprotectant EG 15% + DJC 15% (KP4). Experimental animals that are in the proestrus cycle are euthanised using a lethal dose of ketamine intraperitoneally.[18] The ovarian sample used in the study was the ovary. The ovaries are then inserted into cryotubes with various vitrification mediums, according to the research group.

Date juice concentrate

The DJC used in the study came from semi-dry type dates that have been processed into DJC with trademarks in the market. The storage time of DJC based on the product used is 2 years. The DJC used was tested for sugar content (HPLC) in the form of glucose, sucrose and fructose. Phytochemical tests carried out on the research sample are the antioxidant activity test (DPPH), phenolic total test (Folin–Ciocalteau) and flavonoids (spectrophotometry). All tests are carried out by research institutions such as Centre for Agro Industry (BBIA, Bogor, Indonesia) and Agricultural Post Harvest Research and Development (BB Pascapanen, Bogor, Indonesia) with their laboratory standard procedures.

Vitrification and thawing

Ovaries were exposed to the cryoprotectant for 10–15 min before freezing.[19] Cryotube containing each ovary was put into a liquid nitrogen tank with a temperature of −196°C for 48 h. After 48 h, cryotubes are removed from the liquid nitrogen tank and a warming process is carried out using a water bath with a temperature of ± 37°C for 2–3 min. Then, the ovaries

are transferred into a dark bottle container containing 10% formol saline fixation solution.

Tissue processing and ovarian paraffin blocks

The rat ovary histology sections were carried out following the standard histological procedure. The ovaries are processed into paraffin blocks and then cut by serial methods of ±5 μm each. The ovarian section is placed on a Poly-L-Lysine coated slide for immunohistochemistry (IHC) staining. Ovarian sections are also stained with H and E. H and E staining for follicular morphology has been conducted in our previous studies.[7]

Immunohistochemistry

The IHC staining uses two types of primary antibodies, the Rabbit Anti-Bax polyclonal antibody (No. A00183; Boster Biological Technology) and Rabbit Anti-Bcl-2 polyclonal antibody (No. A00040-2; Boster Biological Technology). Detection systems (secondary antibody) for both primary antibodies using the polymer One Step Neopoly Detection Kit (No. BGNK-0100; Biogear). Incubation of the primary antibody was performed at a dilution of 1:1500 for the Bax protein and 1:500 for the Bcl-2 protein overnight at a temperature of 4°C.

Immunohistochemistry analysis and Bax/Bcl-2 ratio

All ovaries were observed using Olympus CX21 microscope connected to optilab lens. All pre-antral follicles in IHC staining analysed the intensity of brown colour expressed in the cytoplasm of the follicular epithelial cells and the ovum of each follicle in the ovarian cortex.[20] Semi-quantitative analysis of Bax and Bcl-2 protein expression was performed with ImageJ software (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation) in the IHC profiler programme. The analysis will show a percentage value of the contribution of the intensity of expression (negative, low positive, moderate positive and high positive). The value is then used for the calculation of optical density (OD) with the following formula: (Percentage of contribution (high positive × 4) + (moderate positive × 3) + (low positive × 2) + (negative × 1))/100.[21] Protein expressions based on OD values according to the IHC profiler have the following range: Negative: 1–1.5; Low positive: 1.5–2; Medium positive: 2–3 and High positive: >3. The results of the OD calculation value for each antibody were then used to determine the ratio of Bax/Bcl-2.[22]

Statistical analysis

The research data were processed and analysed using the Statistical Package for the Social Sciences (SPSS, IBM, SPSS Inc) software. All statistical data processing uses a 95% confidence level ($P < 0.05$). In all data, normality tests (Shapiro–Wilk) and homogeneity tests (Levene) were carried out. Data showed a normal and homogeneous distribution, then further tests were carried out in the form of parametric tests (analysis of various) and two-way comparisons (Least Significant Differences). Data that did not show a normal and homogeneous distribution then carried out further tests in the form of non-parametric tests (Kruskal–Wallis) and multiple comparisons (Dunnet).

Results

Sugar content and phytochemical tests

The DJC used in the study was known to contain several sugars, such as glucose, sucrose and fructose, 21.9%, 12.3% and 0.37%, respectively. The DJC used has a more dominant amount of glucose than other types of sugar. DJC also shows an antioxidant activity of 13.79%–28.47% (percentage of inhibition) and contains antioxidant compounds, such as phenols and flavonoids. The total phenols and flavonoids in DJC were 1770.61 mgTAE/kg and 30.98 mgQE/100g, respectively.

Analysis of immunohistochemistry

The entire sample of ovarian IHC slide is run along with negative and positive control (rat colon). The expression of Bax and Bcl-2 proteins on the ovaries shows the brown colour present in the cytoplasm of the follicular and ovum epithelial cells of each follicle in the ovarian cortex. Each type of pre-antral follicle (primordial, primary and secondary) is observed the intensity of the brown colour expressed from each protein. The results of the analysis using ImageJ IHC Profiler of all photos of pre-antral follicles showed a percentage score of the contribution of weak positive protein expression (A, D and G); medium (positive) (B, E and H) and the strong (high positive) (C, F and I) of each protein. The results of IHC with Bax protein are shown in Figure 1 and Bcl-2 protein in Figure 2. The Bax/Bcl-2 ratio of pre-antral follicles is shown in Table 1 and Figure 3.

Discussion

The DJC used in the study has been tested on sugar levels. Sugar components can be divided into two types, namely reducing sugars (glucose, fructose, manosa and maltose) and non-reducing sugars (especially sucrose).[23] The results of measuring glucose and fructose levels in DJC used are in the normal range according to Kahina Djaoud *et al.*, namely 16.64%–39.89% and 0.00%–53.06%. The result on sucrose sugar shows low levels when compared to range levels, according to Kahina Djaoud *et al.,* which is 20.67%–49.22%. However,

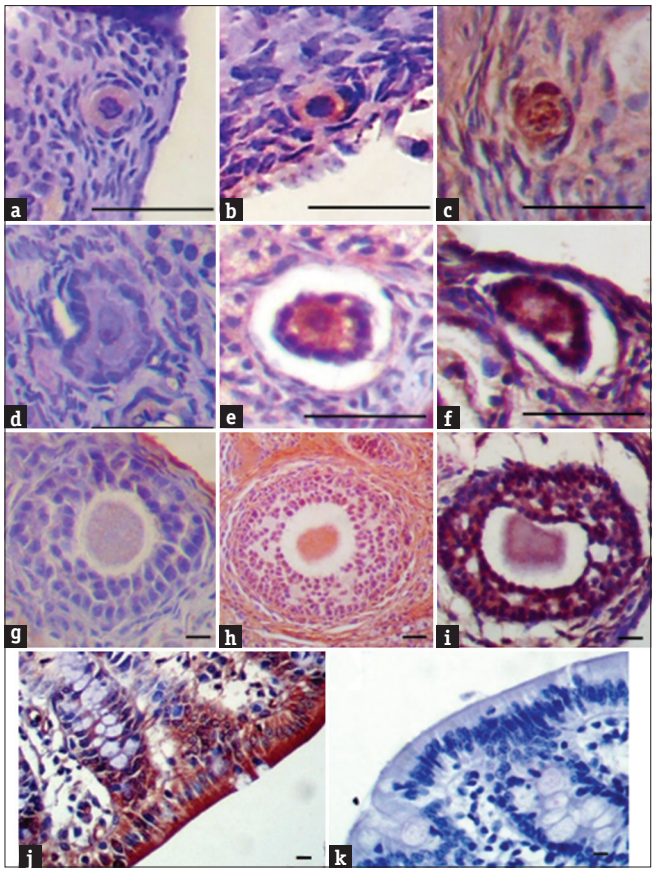

Figure 1: Expression of Bax protein in preantral follicles. The figure a-c shows primordial follicles expressing the Bax protein on the cytoplasm of follicular and ovum epithelial cells with intensity: (a) Low positive with an optical density (OD) value of 1.549, (b) Moderate positive with OD value of 2.384 and (c) High positive with OD value of 3.387. The figure d-f shows the primary follicle expressing the Bax protein on the cytoplasm of follicular and ovum epithelial cells with intensity: (d) Low positive with an OD value of 1.486, (e) Moderate positive with OD value of 2.521 and (f) High positive with OD value of 3.331. The figure g-i shows secondary follicles expressing the Bax protein on the cytoplasm of follicular and ovum epithelial cells with intensity: (g) Low positive with OD value of 1.540, (h) Moderate positive with OD value of 2.404 and (i) High positive with OD value of 3.725. Colon sections are used as controls as in (j) which is a positive control and (k) which is a negative control for the Bax protein (Bar scale = 50 μm)

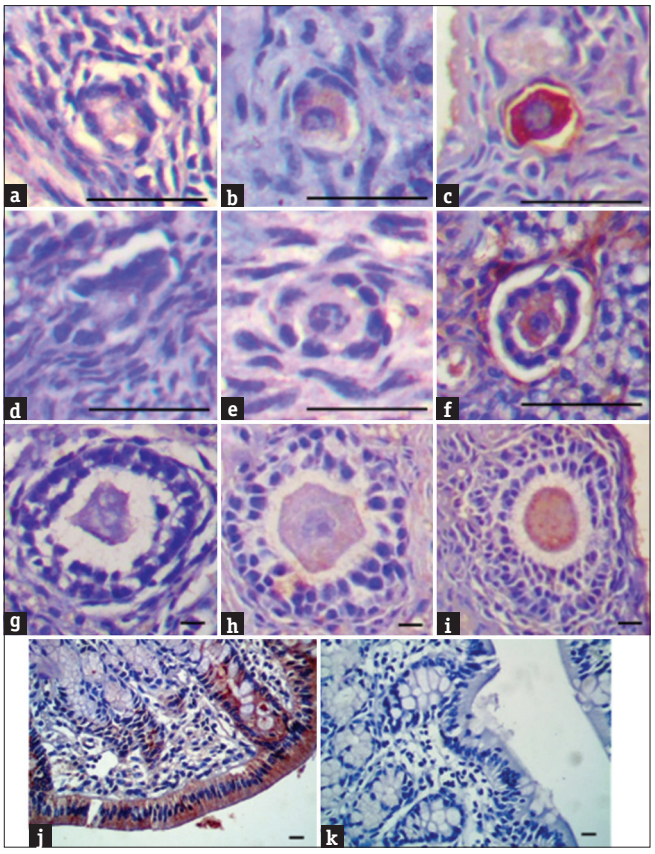

Figure 2: Expression of Bcl-2 protein in the preantral follicles. Figure a-c shows primordial follicles expressing the Bcl-2 protein on the cytoplasm of follicular and ovum epithelial cells with intensity: (a) Low positive with an optical density (OD) value of 1.404, (b) Moderate positive with OD value of 2.585 and (c) High positive with OD value of 3.494. The figure d-f shows the primary follicle expressing the Bcl-2 protein on the cytoplasm of follicular and ovum epithelial cells with intensity: (d) Low positive with an OD value of 1.641, (e) Moderate positive with OD value of 2.767 and (f) High positive with OD value of 3.468. The figure g-i shows secondary follicles expressing the Bcl-2 protein on the cytoplasm of follicular and ovum epithelial cells with intensity: (g) Low positive with an OD value of 1.510, (h) Moderate positive with OD value of 2.419 and (i) High positive with OD value of 3.382. Colon sections are used as controls as in (j) which is a positive control and (k) which is a negative control for the Bcl-2 protein (Bar scale = 50 μm)

these results are in accordance with literacy which states that DJC contains about 70% reducing sugar, namely glucose. This is in accordance with the test results, which show glucose levels are more dominant than other types.[24] The sugar component, especially glucose, is known to be one of the important compounds in the selection of extracellular cryoprotectants.[25] This supports the result that DJC has equally good potential as other extracellular cryoprotectants that have been widely used in vitrification.

The DJC used also has antioxidant activity of 13.79%–28.47% using the DPPH method. The results are in accordance with the range value according to the research of Abbès *et al*. which states that the inhibition value DJC is around 27.97%–76.40%.[26] The per cent value of inhibition states the ability of an extract or ingredient that is suspected to contain antioxidant compounds in warding off free radicals.[27] Based on these results, it shows that DJC has potential antiradical properties that are able to inhibit the formation of free radicals. The DJC is also known to contain antioxidant compounds in the form of phenolics and flavonoids of 1770.61 mgTAE/kg and 30.98 mgQE/100g, respectively. The total phenolic test results are in accordance with the values according to research by Ali *et al*. in the range 1165–1744 mgTAE/100g.[28] Flavonoid test results showed low levels when compared to the range of flavonoid levels according to Al-Mamary *et al*., which is 310–372 mgQE/100g.[29]

Table 1: Bax/Bcl-2 ratio of pre-antral follicles

Group	Mean±SD	Median	Minimum–maximum
Bax/Bcl-2 ratio of primordial follicles			
KKN	0.559±0.081*	0.591	0.467–0.619
KKV	1.052±0.226	0.939	0.904–1.312
KKP1	1.234±0.104	1.213	1.142–1.346
KKP2	1.028±0.077	1.001	0.969–1.115
KP1	0.988±0.110	0.962	0.894–1.108
KP2	0.604±0.163*	0.607	0.440–0.766
KP3	1.049±0.021	1.046	1.029–1.071
KP4	1.083±0.185	1.019	0.938–1.291
Bax/Bcl-2 ratio of primary follicles			
KKN	0.519±0.157*	0.477	0.386–0.693
KKV	0.969±0.093	0.972	0.875–1.060
KKP1	1.040±0.071	1.028	0.976–1.116
KKP2	0.794±0.115	0.770	0.694–0.920
KP1	1.016±0.123	1.023	0.889–1.136
KP2	0.570±0.069*	0.594	0.492–0.623
KP3	0.956±0.107	0.998	0.834–1.035
KP4	1.042±0.104	1.035	0.943–1.149
Bax/Bcl-2 ratio of secondary follicles			
KKN	0.503±0.082*	0.478	0.436–0.594
KKV	1.202±0.420	1.035	0.891–1.680
KKP1	1.287±0.317	1.346	0.944–1.569
KKP2	1.174±0.049	1.170	1.127–1.224
KP1	1.327±0.145	1.397	1.160–1.423
KP2	1.334±0.257	1.239	1.138–1.625
KP3	1.303±0.135	1.274	1.185–1.449
KP4	0.907±0.201	0.900	0.709–1.111

*$P \leq 0.05$. The data show the mean±SD, median, minimum and maximum values. Significance compared with KKV data. KKN=No vitrification, KKV=Vitrification control group (NaCl 0.9%), KKP1=EG 7.5%, KKP2=EG 15%, KP1=EG 7.5% + DJC 7.5%, KP2=EG 7.5% + DJC 15%, KP3=EG 15% + DJC 7.5%, KP4=EG 15% + DJC 15%. SD=Standard deviation, EG=Ethylene glycol, DJC=Date juice concentrate, NaCl=Sodium chloride

Phenolics and flavonoids are natural antioxidants that are found in many natural ingredients, such as dates. Both antioxidants act effectively as scavengers in reducing, counteracting free radicals and dampening the formation of free oxygen.[26] Fresh dates contain phenolic antioxidants and flavonoids that are higher than processed date products. The literacy study stated that the total phenolic levels in fresh dates ranged from 217 to 343 mg/100 g and decreased after processing into DJC, which ranged from 27.2 to 42.6 mg/100 g.[30] Due to the stage of making date palm products into DJC that goes through a heating and enzymatic process, it allows the loss of a number of antioxidant contents such as flavonoids.[31] Whereas, in general, antioxidant activity is known to correlate with the high phenolic content of dates.[23,28] This is in accordance with the results of research on antioxidant and total phenolic tests. Although DJC shows antioxidant activity that is not too high, in DJC there are quite high phenolic compounds. Based on this, it is suspected that the antioxidant activity of DJC is played largely by phenolic compounds.[30,32]

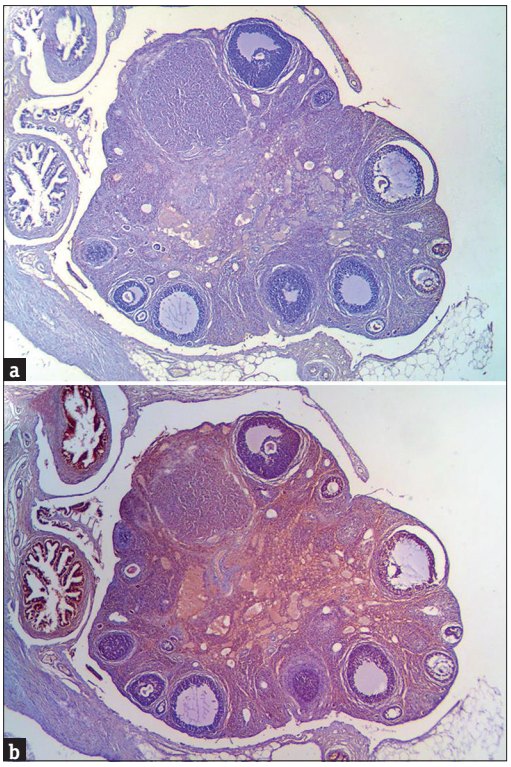

Figure 3: Lower magnification (×40) of ovarian section expressing protein pro-apoptosis Bax (a) and anti-apoptosis (Bcl-2) (b)

The selection of Bax/Bcl-2 is based on the signalling pathway of the apoptosis that is most commonly known. This study is the initiating stage of apoptosis protein markers caused by vitrification. According to Wang *et al*., ovarian vitrification can interfere with mitochondrial membrane potential as well as the regulation of apoptosis-related genes such as Bax/Bcl-2 expression. Therefore, the Bax/Bcl-2 ratio plays an important role in determining the incidence of apoptosis. A high Bax/Bcl-2 ratio will stimulate the effector and executor stages of apoptosis of the intrinsic pathway.[33]

The use of other apoptosis protein markers have not been done by researchers, but it is possible for further test studies to be carried out. According to research, protein markers such as caspase-3 can be used as apoptosis markers to confirm the final pathway of the apoptotic cascade. caspase-3 is also one of the most frequently activated markers during apoptosis.[34] The apoptosis signal has reached the activation point of caspase-3, there will be no cell rescue or called irreversible apoptosis, so the cell undergoes apoptosis.[35]

The lowest pre-antral follicle Bax/Bcl-2 ratio amongst the entire study group was found in KKN. This illustrates that under normal conditions without vitrification, an incidence of apoptosis is by physiological factors during folliculogenesis producing atresia follicles.[36] Meanwhile, KKV showed a higher Bax/Bcl-2 ratio compared to KKN. Therefore, these results suggest that the use of extreme temperatures during vitrification can lead to changes in cell membrane conformation so that membrane fluidity decreases and cell function is disrupted.[37,38] The condition can induce oxidative stress, which has an impact on increasing reactive oxygen species (ROS) and triggering the activation of intrinsic pathway apoptosis.[8,33]

Mitochondria as energy producers for cells, respond to these conditions by actively metabolising to maintain cell survival. The metabolic by-product can produce free oxygen, which if produced continuously, can form several free oxygen radicals. Free oxygen radicals result in too much reactivity in the cell so that the cell structure becomes irregular, disrupting the potential of mitochondrial membranes to damage the mitochondria. The destruction of mitochondria can facilitate the release of cytochrome c proteins that can activate the apoptotic cascade.[10,39] Literature studies state that activation of apoptosis is often induced by cold-induced temperatures, long-term storage of cells, organs or tissues, damage from freezing or damage from cold-struck.[5]

The addition of an EG of 15% is better in preventing or reducing the incidence of apoptosis compared to an EG of 7.5%. Ethylene glycol has been widely used as a cryoprotectant in the vitrification process, but EG is known to have toxic effects at certain concentrations that can cause damage to ovarian tissue. An exact EG concentrations are used, especially in the ovaries, until now it is still being studied.[7,10,40] EG in cryoprotectant medium is known to increase intracellular Ca^{2+} influx. EG will carry in Ca^{2+} extracellularly to intracellular cells through the plasma membrane. Therefore, Ca^{2+} homeostasis is disturbed and can lead to the accumulation of calcium in the oocyte zona pellucida. These conditions can affect the quality and development of oocytes after preservation. The increase in Ca^{2+} concentration also interferes with oxidative phosphorylation in the mitochondria, which can result in the opening of mitochondrial permeability transition pore, resulting in mitochondrial dysfunction producing ROS. The accumulation of ROS can lead to changes in the morphological structure of the follicles and the death of the ovarian follicles by apoptosis of the intrinsic pathway.[41] Based on that, the optimal balance between protection against cryoinjury and toxicity needs to be considered.

The ratio of Bax/Bcl-2 primordial and primary follicles with the lowest value is found in KP2 (EG 7.5% + DJC 15%). The combination of cryoprotectants used in KP2 may be sufficient in protecting small pre-antral follicles with simple structures, such as primordial and primary follicles. Primordial and primary follicles are also located in the peripheral cortex, allowing them to respond better to cryoprotectants than other follicles.[4,42] In particular, primordial follicles are known to have follicular recovery ability after being damaged during vitrification.[43] However, the recovery mechanism cannot be explained definitively.

The lowest secondary follicle Bax/Bcl-2 ratio is KP4 (EG 15% + DJC 15%). This result can be attributed to a more complex and differentiated secondary follicle structure than primordial and primary follicles, so a higher concentration of cryoprotectants is needed to reduce the rate of apoptosis. The group with a combination of cryoprotectants (KP1–KP4) showed a better pre-antral follicle Bax/Bcl-2 ratio than KKP1 and KKP2. The use of EG 15% can be reduced by adding DJC, which also shows a lower pre-antral follicle Bax/Bcl-2 ratio than KKP2. With a combination of cryoprotectants, an EG 7.5% added with DJC 15% can maximally suppress apoptosis in the primordial and primary follicles.

Low temperatures during vitrification can cause phospholipids on the cell membranes to be close to each other, forming irreversible conformation changes so that membrane fluidity decreases.[37,38] The condition has an effect on the movement of molecules, including water between cell compartments related to cell dehydration, allowing the formation of ice crystals in the intracellular and extracellular.[5] Simple sugars derived from DJC are thought to work to stabilise membrane fluidity. Sugars can insert hydrogen bonds with phospholipids from cell membranes forming distances between them, thereby increasing membrane fluidity and the movement of molecules between compartments can be maintained.[12,44]

Membrane stability and fluidity greatly affect the dehydration process during vitrification to prevent the formation of ice crystals that can damage cell components. With the preservation of the integrity of cells and follicles, the homeostasis of cells is also maintained so that mitochondrial function is not disturbed and apoptosis is inhibited. Based on this mechanism, it is suspected that sugar from DJC can play a role in suppressing the incidence of apoptosis due to vitrification.[12,44] DJC is also known to have antioxidant activity and contains antioxidant compounds in the form of phenolics and flavonoids. Both compounds are known to play an important role as scavengers against free radicals. According to the literature, one of the

mechanisms of cell defense by antioxidants against free radicals due to vitrification is to donate one hydrogen atom or one electron to ROS, thereby stabilising reactive species. Antioxidants at the right concentration can significantly reduce damage from ROS, decrease ROS production and reduce oxidative stress, thereby improving vitrification efficiency.[45]

Conclusion

Based on the results and discussion above, the content of sugar and antioxidant compounds in DJC plays an important role in maintaining the integrity of the follicles during vitrification. Date juice concentration has the potential to be a natural extracellular cryoprotectant that can be combined with EG, which is known to have a toxic effect at high concentrations. The combination of EG with DJC 15% (KP2 and KP4) is known to be able to suppress the incidence of apoptosis reviewed through the Bax/Bcl-2 ratio best compared to other groups. In conclusion, the concentration of a DJC 15% combined with EG has the potential to be used as a natural extracellular cryoprotectant due to the content of sugars and antioxidant compounds that can protect the pre-antral follicles in the ovaries after vitrification so that the incidence of apoptosis can be suppressed.

Acknowledgment

This research 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 thankful to the Head of the Department and lab assistants of the Biology and Histology Department University of Indonesia for all the support.

Financial support and sponsorship

This research was funded by a Doctoral Dissertation Research Grant from the Directorate General of Research Enhancement and Development, Ministry of Research Technology and Higher Education (Hibah PDD Kemendikbudristek) in 2021.

Conflicts of interest

There are no conflicts of interest.

Data availability statement

All relevant data that support the findings of this study are including within the paper.

References

1. Wiweko B, Yuningsih T, Affandi B, Ahmad A, Boediono A, Maidarti M, *et al.* Ovarian tissue vitrification as a method for fertility preservation: A study of follicle number and morphology after vitrification. IVF Lite 2014;1:148.
2. de Figueiredo JR, de Lima LF, Silva JR, Santos RR. Control of growth and development of preantral follicle: Insights from *in vitro* culture. Anim Reprod 2018;15:648-59.
3. Rivas Leonel EC, Lucci CM, Amorim CA. Cryopreservation of human ovarian tissue: A review. Transfus Med Hemother 2019;46:173-81.
4. Fathi R, Valojerdi MR, Salehnia M. Effects of different cryoprotectant combinations on primordial follicle survivability and apoptosis incidence after vitrification of whole rat ovary. Cryo Letters 2013;34:228-38.
5. Erdem Öztürk A, Numan Bucak M, Bodu M, Başpınar N, Çelik İ, Shu Z, *et al.* Cryobiology and cryopreservation of sperm. In: Cryopreservation: Current Advances and Evaluations. Turkey: InTechOpen; 2020. p. 1-42.
6. Bhattacharya S. Cryoprotectants and their usage in Cryopreservation Process. In: Cryopreservation Biotechnology in Biomedical and Biological Sciences. Bilakhia College of Pharmacy, Gujarat, India: Intech Open; 2018. p. 13.
7. Anita N, Abinawanto A, Jusuf AA, Bowolaksono A, Saoemi HA. Effect of ethylene glycol on structural integrity at each stage of preantral follicle development post vitrification of rat ovary-histological analysis. HAYATI J Biosci 2021;28:304-11.
8. Len JS, Koh WS, Tan S×. The roles of reactive oxygen species and antioxidants in cryopreservation. Biosci Rep 2019;39:BSR20191601.
9. Yuan J, Lan H, Jiang X, Zeng D, Xiao S. Bcl2 family: Novel insight into individualized therapy for ovarian cancer (review). Int J Mol Med 2020;46:1255-65.
10. Bojic S, Murray A, Bentley BL, Spindler R, Pawlik P, Cordeiro JL, *et al.* Winter is coming: The future of cryopreservation. BMC Biol 2021;19:56.
11. Anita N, Abinawanto EY, Jusuf AA, Bowolaksono A, Saoemi HA, Safrina A. Cryoprotective effects of longan honey on preantral follicle integrity of rat ovary post vitrification. Cryo Letters 2023;44:26-36.
12. Rosvita MA, Anita N, Abinawanto A, Jusuf AA, Awanis A, Muhiardi I, *et al.* The combination effect of ethylene glycol and palm dates juice on ovarian tissue of female Sprague-Dawley rats (*Rattus norvegicus* L.) post-vitrification. In: AIP Conference Proceedings. Indonesia: AIP Publishing; 2019. p. 2168.
13. Malik A. Effects of addition of juice date palm to the extender on the semen qualities of frozen thawed in bull spermatozoa. Global Veterinaria 2016;16:100-4. DOI: 10.5829/idosi.gv.2016.16.01.10274.
14. Elisya Y, Cartika H, Rizkiana A. Antioxidant activity and total phenolic content of date palms syrup (*Phoenix dactylifera* L). Elisya 2017;8:63-71.
15. Shehzad M, Rasheed H, Naqvi SA, Al-Khayri JM, Lorenzo JM, Alaghbari MA, *et al.* Therapeutic potential of date palm against human infertility: A review. Metabolites 2021;11:408.
16. Lestari S, Abinawanto, Bowolaksono A, Gustiano R, Kristanto AH. The percentage of embryo viability after 48h sperm cryopreservation: Effect of various natural cryoprotectant. IOP Conf Ser Earth Environ Sci 2020;441:1-5.
17. Widyaningsih W, Abinawanto A, Lestari R, Bowolaksono A, Zavitri NG. Effect of various concentrations of palm date (*Phoenix dactylifera* L.) on spermatozoa motility of giant grouper (*Epinephelus lanceolatus*). AIP Conf Proc 2019;2168:1-6.
18. Silverman J, Suckow M, Murthy S. Animal Euthanasia Policy. Washington University in St. Louis, United States; 2015. p. 1-4.
19. Balaban B. The alpha consensus meeting on cryopreservation key performance indicators and benchmarks: Proceedings of an expert meeting. Reprod Biomed Online 2012;25:146-67.
20. Zhang G, Wan Y, Zhang Y, Lan S, Jia R, Wang Z, *et al.*

Expression of mitochondria-associated genes (PPARGC1A, NRF-1, BCL-2 and BAX) in follicular development and atresia of goat ovaries. Reprod Domest Anim 2015;50:465-73.
21. Seyed Jafari SM, Hunger RE. IHC optical density score: A new practical method for quantitative immunohistochemistry image analysis. Appl Immunohistochem Mol Morphol 2017;25:e12-3.
22. Li Z, Xiao G, Wang H, He S, Zhu Y. A preparation of *Ginkgo biloba* L. Leaves extract inhibits the apoptosis of hippocampal neurons in post-stroke mice via regulating the expression of Bax/Bcl-2 and caspase-3. J Ethnopharmacol 2021;280:114481.
23. Ashraf Z, Hamidi-Esfahani Z. Date and date processing: A review. Food Rev Int 2011;27:101-33.
24. El Sharnouby GA, Aleid SM. Liquid sugar extraction from date palm (*Phoenix dactylifera* L.) fruits. J Food Process Technol 2014;5:1-5.
25. Alfoteisy AB. Natural Honey as a New Cryoprotectant to Improve Post-Warming Viability of Vitrified Bovine Oocytes. Veterinary Medicine, Canada. 2012.
26. Abbès F, Kchaou W, Blecker C, Ongena M, Lognay G, Attia H, *et al.* Effect of processing conditions on phenolic compounds and antioxidant properties of date syrup. Ind Crops Prod 2013;44:634-42.
27. Farahnaky A, Mardani M, Mesbahi G, Majzoobi M, Golmakani MT. Some physicochemical properties of date syrup, concentrate, and liquid sugar in comparison with sucrose solutions. J Agric Sci Technol 2016;18:657-68.
28. Ali DM, Momen AA, Khalid MA. Antioxidant activity and total phenolic content of date palm (*Phoenix dactylifera* L.) fruits from Taif governorate, Saudi Arabia. Res J Pharm Biol Chem Sci 2016;7:1708-21.
29. Al-Mamary M, Al-Habori M, Al-Zubairi AS. The *in vitro* antioxidant activity of different types of palm dates (*Phoenix dactylifera*) syrups. Arab J Chem 2014;7:964-71.
30. Hamad I, AbdElgawad H, Al Jaouni S, Zinta G, Asard H, Hassan S, *et al.* Metabolic analysis of various date palm fruit (*Phoenix dactylifera* L.) cultivars from Saudi Arabia to assess their nutritional quality. Molecules 2015;20:13620-41.
31. Bouhlali ED, Alem C, Zegzouti YF. Antioxidant and anti-hemolytic activities of phenolic constituents of six Moroccan date fruit (*Phoenix dactylifera* L.) syrups. Btaij (Biotechnology an Indian Journal) 2016;12:045-052.
32. Ghnimi S, Umer S, Karim A, Kamal-Eldin A. Date fruit (*Phoenix dactylifera* L.): An underutilized food seeking industrial valorization. NFS J 2017;6:1-10.
33. Wang Y, Zhang M, Chen ZJ, Du Y. Resveratrol promotes the embryonic development of vitrified mouse oocytes after *in vitro* fertilization. *In Vitro* Cell Dev Biol Anim 2018;54:430-8.
34. Goldblatt ZE, Cirka HA, Billiar KL. Mechanical regulation of apoptosis in the cardiovascular system. Ann Biomed Eng 2021;49:75-97.
35. Puccini J, Kumar S. Caspases. Encyclopedia of Cell Biology: South Australia, Adelaide, SA, Australia: Elsevier Ltd.; 2016.
36. Sirotkin AV. Regulators of Ovarian Functions. New York: Nova Science Publishers, Inc.; 2014.
37. Kusuma GD, Barabadi M, Tan JL, Morton DA, Frith JE, Lim R. To protect and to preserve: Novel preservation strategies for extracellular vesicles. Front Pharmacol 2018;9:1199.
38. Eroschenko VP. Atlas of Histology with Functional Correlations. 13th ed. Philadelphia: Wolters Kluwer; 2017.
39. Jang TH, Park SC, Yang JH, Kim JY, Seok JH, Park US, *et al.* Cryopreservation and its clinical applications. Integr Med Res 2017;6:12-8.
40. Joshi AJ. A review and application of cryoprotectant: The science of cryonics. PharmaTutor 2016;4:12-8.
41. Dos Santos Morais ML, de Brito DC, Pinto Y, Mascena Silva L, Montano Vizcarra D, Silva RF, *et al.* Natural antioxidants in the vitrification solution improve the ovine ovarian tissue preservation. Reprod Biol 2019;19:270-8.
42. Leonel EC, Lucci CM, Leonel EC, Lucci CM, Amorim CA. Cryopreservation of preantral follicles. In: Cryopreservation Biotechnology in Biomedical and Biological Sciences. Nature research, London, England: Intech; 2018. p. 13.
43. Bos-Mikich A, Marques L, Rodrigues JL, Lothhammer N, Frantz N. The use of a metal container for vitrification of mouse ovaries, as a clinical grade model for human ovarian tissue cryopreservation, after different times and temperatures of transport. J Assist Reprod Genet 2012;29:1267-71.
44. Amorim CA, Curaba M, Van Langendonckt A, Dolmans MM, Donnez J. Vitrification as an alternative means of cryopreserving ovarian tissue. Reprod Biomed Online 2011;23:160-86.
45. Liu X, Xu Y, Liu F, Pan Y, Miao L, Zhu Q, *et al.* The feasibility of antioxidants avoiding oxidative damages from reactive oxygen species in cryopreservation. Front Chem 2021;9:648684.