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Potential ability for implantation of mouse embryo post-vitrification based on Igf2, H19 and Bax Gene expression

Noer Muhammad Dliyaul Haq[1], Diah Pristihadi[1], Vista Budiariati[1], Ahmad Furqon[2], Mokhamad Fahrudin[1], Cece Sumantri[2], Arief Boediono[1,*]

[1] Department of Anatomy, Physiology and Pharmacology, Faculty of Veterinary Medicine
[2] Department of Animal Production and Technology, Faculty of Animal Science
[1,2] Bogor Agricultural University, Bogor 16680, Indonesia

Abstract

Vitrification is one of cryopreservation method for freezing cells without ice formation so that biological materials such as sperm, oocyte, or embryo, can be preserved and later can be used for a specific purpose including wildlife conservation efforts. Unfortunately, high concentration of cryoprotectan in vitrification process can cause osmotic stress and has high toxic levels that may affect embryo quality. The purpose of this research is to analyze the quality of post-vitrification embryos at morula and blastocyst stages based on morphometric variable, and Bax gene expression, futhermore potency of implantation of the post-vitrification embryo were also examined based on H19 and Igf2 gene expression. The results showed embryo viability post-vitrification decreased 5.67% and 6.02% in morula and blastocyst. Development ability from morula to blastocyst post-vitrification was also decreased by 10.15%. Morphometry analysis of morula post-vitrification showed decreased values of zona pellucida tickness (ZPT), zona pellucida tickness variation (ZPTV) and blastomeres area, while perivetellin space (PVS) area was increased compared to fresh morula. Blastocyst post-vitrification had increased values of ZPT, ZPTV, and PVS, while in ICM and the blastocoel were also decreased compared to fresh blastocyst. The result of relative levels mRNA of Igf2, H19, and Bax gene show no significant difference gene expression between fresh blastocyst, blastocyst post-vitrification, and morula post-vitrification group.

Keywords

Blastocyst, gene expression, morphometry, morula, implantation, vitrification.

1. Introduction

Cryopreservation is cells freezing method that aims to reduce cell metabolism so that cells can be stored for a long time. Cryopreservation can be used to support the success of conservation by storing source of primary genetic material such as sperm for males and oocytes for females. In addition, storage of genetic material can also be carried out on embryos. The development of cryopreservation method that can be used to store genetic material are slow freezing and vitrification method (Best, 2015). Currently, many researchers prefer to do vitrification than slow freezing for genetic

* Corresponding author. E-mail: ab@apps.ipb.ac.id

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material storage. The advantages of the vitrification method are lower costs and it does not require long time process compared to slow freezing method.

The results of the study by Li et al., (2014) showed that the vitrification method gave a higher post-warming survival rate of the embryos compared to the traditional method of slow freezing, but there are several things that should be considered before decided to use vitrification method. There are the toxicity of cryoprotectants and osmotic stress induced by replacement water content in cells with cryoprotectant. These effects may cause morphological and morphometric changes of mouse embryos post-vitrification (Homayoun et al., 2016). The morphological assessment and morphometric approach of the embryos was carried out to determine the quality of the embryos, including implantation capability and development prediction of embryo after embryo transfer.

In addition, previous study showed that vitrification in pig embryos causes changes of genome imprint expression (Bartolac et al., 2018). Genome imprinting is the expression of genes or parts of chromosomes that are derived by the parental allele. The maternal or the paternal alleles are found but only one allele is active, while the other alleles are silenced or inactive. The influential factor in the process of imprinting is the binding of the methyl group on DNA which causes genes to be inactive (Hitchin and Moore, 2004). The process of epigenetics is caused by three mechanisms, i.e. non-coding RNAs, histone modification and DNA methylation. During development of the pre-implantation embryo, the genome undergoes a demethylation and methylation process, but differentially methylated regions (DMRs) from the imprint gene are maintained. Disorders in gene imprint expression can be caused by exposure to the artificial environment during embryo culture and embryo manipulation that can lead to changes in methylation patterns, such as the development of extra embryonic tissue in parthenogenetic embryos (Marhendra and Boediono, 2010).

There are several imprint genes that influence the embryogenesis process including growth of the embryos, placenta and neonate. Specifically, genes expressed by paternal alleles such as Igf2, Peg1, Peg3, Rasgrf1, and Dlk1 act as growth promoters, while specifically genes expressed by maternal alleles act as growth inhibitor such as Igf2r, Gnas, Cdkn1c, H19, and Grb10. In addition, there are genes that influence neurological such as Nesp, Ube3a, and Kcnq1 (Plasschaert and Bartolomei, 2014). The imprint genes which have a function to maintain the growth and development of fetal and placental is the Igf2 and H19 genes. The aberration of gene imprint expression causes the condition of fetal and placental dysplasia which subsequently causes abnormal embryo development (Park et al., 2011). In addition, Ig2 and H19 gene expression at the stage of the preimplantation embryo were fluctuate. This was indicated by the repressed of H19 gene transcription in early develop embryo to stage 4 cells. The H19 gene was stable expressed at the morula stage and increased at the blastocyst stage. The Igf2 gene transcription was detected in all stages of preimplantation embryo development and increased at the 8 cell stage.

Embryos at blastocyst stage are more preferable to be performed vitrified than at 4-8 cell and morula stage. The results of the Ochota et al. (2014) on cat's embryo vitrification at the stages of 4-8 cells and morula showed that the ability of 4-8 cells and morula embryos to develop into blastocysts are 13% and 27% respetively. Based on those previous studies, the aim of this research was to analyze the effect of vitrifica-

tion in mice embryos at the morula and blastocyst stages based on Igf2, H19 and Bax expression genes.

2. Materials and Methods

2.1 Material and Media

The experimental animals used as sources of embryos are adult female DDY mice at 2 months of age. Mice were kept under controlled temperature of 22-25 ^{o}C and 12 hours light conditions (6:00-18:00). Food and water supplied ad libitum. Experimental procedures of research was approvel by the animal ethics committee Faculty of Veterinary Medicine, Bogor Agricultural University (No: 121/KEH/SKE//IV/2019).

The materials used in this research are pregnant serum gonadotrophin (PMSG) (Folligon, Intervet, Netherland), human chorionic gonadotrophin (hCG) (Chorulon, Intervet, Netherland), GMOP (Vitrolife, Sweden), G2 culture medium (Vitrolife, Sweden). Cryoprotectant used in vitrification-warming process obtained from Sigma-Aldrich (St. Louis, MO, USA) which are ethylene glycol (EG), dimethyl sulfoxide (DMSO) and sucrose.

Molecular analysis were done using the RNeasy mini kit (Qiagen), reverted Ace qPCR master RT mix with gDNA remover (Toyobo), iTaqTM SYBR Green Supermix with Rox PCR core reagent (Bio-Rad).

2.2 Embrio *In Vivo* Collection

The superovulation methods were carried out based on Eckardt and McLaughlin (2009). The embryos were collected on day 3 for the embryo at the morula stage, while the blastocyst stage embryo was collected on day 4. The embryos that have been obtained are transferred in drop of GMOP and the vitrification process was carried out.

2.3 Vitrification and Warming Process

The method and vitrification-warming media in this research were carried out based on the research report of Boediono (2005). The vitrification medium used is (V2) 10% EG, (V3) 15% EG + 15% DMSO + 0.5 M sucrose, while the warming medium is (V4) 0.5 M sucrose, (V5) 0.25 M sucrose and (V6) 0.1 M sucrose. Embryos vitrification further warming at room temperature, followed cultured in G2 medium.

2.4 Viability and Embryo Development Analysis

Analysis of viability of morula and blastocyst embryos were analyzed based on morphology and the ability of the embryo to return to its original shape (re-expansion). Morula embryos were cultured in G2 medium for 42 hours to determine the ability of the embryo to develop to blastocyst, while the blastocyst embryos were cultured in G2 medium for 24 hours to analyze the number of cells.

2.5 Numbers Cells of Blastocyst Embryos Analysis

Analysis of the number of cells in the blastocyst embryo was carried out based on the method of making chromosome preparations by Notosoediro et al. (2001). Staining was done using 5% Giemsa for 20 minutes.

2.6 Morphometric Analysis

Embryo morphometry analysis was carried out with Standard Cellens software. The method of analysis is carried out based on the method Molina et al. (2014) and Sun et al. (2005). Embryos morphometry analysis was carried out based on the parameters of zona pellucida thickness (ZPT), zona pellucida thickness variation (ZPTV), and periviteline space. Blastocyst embryos were analyzed of inner cell mass (ICM) and blastocoel area, while embryos of morula were analyzed for blastomer area.

2.7 Embryo RNA Extraction

Embryo RNA extraction method was carried out based on Bartolac *et al*. (2018). The morula and blastocyst embryos that cultured in G2 medium were collected in 8-10 embryos in one tube and RNA extraction was carried out based on the RNeasy mini kit (Qiagen) protocol. Furthermore, cDNA synthesis was performed based on Ace qPCR reverta protocol mix master RT with gDNA remover (Toyobo). cDNA concentration was measured using the nanodrop method.

2.8 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) Analysis

For the analysis of qRT-PCR, the specific gene primers were manually designed and displayed in Table 1. The initial step was the 96-well microtiter plate filled with each cDNA sample and no-template control. qRT-PCR analysis was carried out with a program of 95 ^{o}C for 1 minute, 95 ^{o}C for 15 seconds, and 58 ^{o}C for 1 minute. The

Table 1. Spesific genes primer.

No.	Access number			Gene name	Product length (bp)
1	BC058615.1	Igf2	Forward	5′-GTCTTCATCCTCTTCCAGCC-3′	195
			Reverse	5′-CGGTCCGAACAGACAAACTG-3′	
2	BC025150.1	H19	Forward	5′-GCAGTCATCCAGCCTTCTTG-3′	190
			Reverse	5′-GAAGTCCCCGGATTCAAAGG-3′	
3	BC018228.2	Bax	Forward	5′-CAAGAAGCTGAGCGAGTGTC-3′	156
			Reverse	5′-CCCCAGTTGAAGTTGCCATC-3′	
4	BC138614.1	Actb	Forward	5′-CTGTATTCCCCTCCATCGTG-3′	181
			Reverse	5′-GTGTGGTGCCAGATCTTCTC-3′	

housekeeping gene used in this research is Actb gene. The 2Delta Ct (ΔCt) method is used to calculate the difference between the target gene and housekeeping genes: (2-ΔΔCt = [(ΔCt target gene) - (ΔCt housekeeping genes) sample] - [(ΔCt target gene) - (Δ Housekeeping CT gen) standard]).

2.9 Statistical Analysis

Statistical analysis in this research was carried out with SPSS software. Gene expression data were analyzed using a one-way ANOVA test, viability of the embryo was analyzed using the Wilcoxon test. Number of cells, ZPT, ICM, blastocoel and blastomer area were analyzed using t-independent test, while ZPTV, perivitelline space, and embryo development were analyzed using the mann-whitney test.

3. Results

Vitrification method widely used for storage of genetic material. In addition, vitrification in human embryos is also used as an alternative step to reduce the incidence of multiple pregnancies that can affect maternal or fetal development. Vitrification can be performed at different embryonic stages. The results showed that viability of morula and blastocyst stage post-vitrification was decreased 6.02% and 5.67% respectively (Table 2), but statistically did not show significant differences ($p>0.05$). This is confirmed by the ability of post-vitrified embryo re-expansion (Figure 1). In addition, Blastocyst rate in post-vitification morula embryos cultured in G2 medium also decreased by 10.15%, similar compared to fresh morula embryos (Table 3).

Post-vitrified embryo morphometry analysis is one of method that can be used to determine the quality of the embryo. Morphometry analysis in post-vitrified morula embryos showed decreased values of zona pellucida thicknes (ZPT), zona pellucida thickness variation (ZPTV) and blastomer area of 2.73 μm, 0.2% and 1504.83 μm^2 respectively, while in variable perivetellin space area showed increased of 2280,195

Table 2. Viability of post-vitrification at morula and blastocysts stage embryos.

Group	N	Viability (%) ± SE
Fresh morula	66	100 ± 0^a
Post-vitrified morula	66	$93,98 \pm 2,72^a$
Fresh blastocyst	54	100 ± 0^a
Post-vitrified blastocyst	54	$94,33 \pm 2,47^a$

Table 3. Ability development of post-vitrification morula embryo.

Group	N	Ability develop to balstocyst (%) ± SE
Fresh morula	70	$94,29 \pm 2,51^a$
Post-vitrified morula	62	$82,14 \pm 5,08^a$

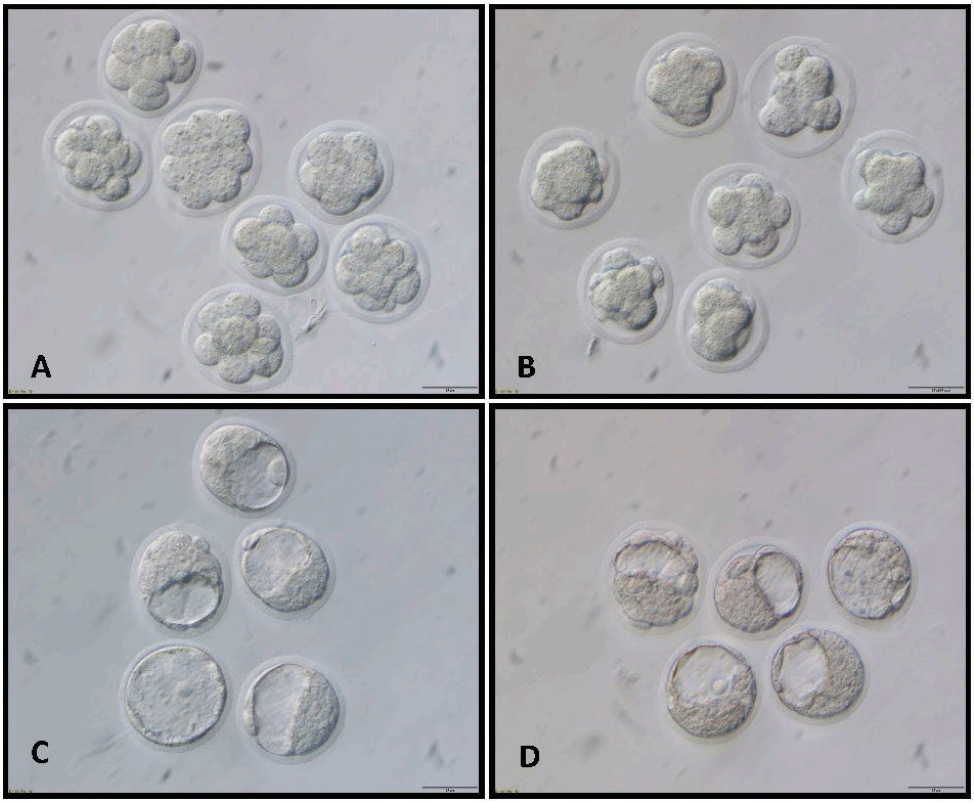

Figure 1. Comparison of embryonic morphology (A) Fresh morula, (B) post-vitrified morula, (C) fresh blastosis, and (D) post-vitrified blastosis. Scale bar = 50 µm.

μm^2 compared to the fresh morula. In post-vitrified blastocyst embryos showed increased values of ZPT, ZPTV, and perivitellin space of 1.27 μm, 0.46% and 265.727 μm^2 repectively, while ICM and blastocoel area were decreased 710.16 μm2 and 84.29 μm^2 respectively compared to fresh blastocysts. Statistically the morphometric analysis result on post-vitrified embryos at the blastocyst stage showed no significant difference ($p>0.05$) compared to fresh blastocyst embryos (Table 4). the morphometric analysis results in post-vitrification embryos at the morula stage showed significant differences ($p<0.05$) in ZPTV, periveteline space, and blastomers variables, while in ZPT did not show significant differences ($p>0.05$) compared to fresh morula embryos (Table 5).

Advanced applications that can be performed on post-vitrified embryos which have good quality morphology and morphometry are embryo transfer (TE). The success of embryo transfer is influenced by several factors, such as genes expression that play an important role in embryogenesis. The results of the analysis of Igf2, H19, and Bax gene expression in post-virification embryos did not show significant differences ($p>0.05$) compared to fresh embryos. It attempted by the relative levels of the mRNA

Table 4. Morphometric analysis of post-vitrified blastocyst embryos.

Group	N	ZPT (μm) ± SE	ZPTV (%) ± SE	PVS (μm²) ± SE	ICM (μm²) ± SE	*Blastocoel* (μm²) ± SE
Fresh blastocyst fresh	45	12,83 ± 0,99 [a]	5,61 ± 0,19 [a]	880,785 ± 98,50 [a]	11613,56 ± 459,88 [a]	13936,69 ± 875,87 [a]
Post-vitrified blastocyst	50	14,10 ± 1,07 [a]	6,07 ± 0,15 [a]	1146,512 ± 135,73 [a]	10903,4 ± 458,26 [a]	13852,4 ± 787,73 [a]

ZPT: zona pellucida thickness; ZPTV: zona pellucida thicknss variation; PVS: perivitellin space; ICM: Inner Cell Mass. (a-b, $p<0.05$).

Table 5. Morphometric analysis of post-vitrified morula embryos.

Group	N	ZPT (μm) ± SE	ZPTV (%) ± SE	PVS (μm²) ± SE	*Blastomer* (μm²) ± SE
Fresh Morula	30	10,23 ± 0,93 [a]	6,91 ± 0,08 [a]	5553,53 ± 423,03[a]	22473,87 ± 389,78 [a]
Post-vitrified morula	28	7,50 ± 0,75 [a]	6,71 ± 0,10 [b]	7833,725 ± 911,4075 [b]	20969,04 ± 367,25[b]

ZPT: zona pellucida thickness; ZPTV: zona pellucida thicknss variation; PVS: perivitellin space; ICM: Inner Cell Mass. (a-b, $p<0.05$).

Table 6. Cells number of post-vitrified blastocyst.

Group	N	Cells number ± SE
Fresh blastocyst	9	63,67 ± 3,09[a]
Post-vitrified blastocyst	9	63,33 ± 3,35[a]

Table 7. Relative levels Igf2, H19, and Bax genes expression.

Group	ΔΔCt *Igf2* ± SE	ΔΔCt *H19* ± SE	ΔΔCt *Bax* ± SE
Fresh Blastocyst	0,75 ± 0,31[a]	1,30 ± 0,61[a]	1,62 ± 0,79[a]
Vitirified Blastocyst	0,76 ± 0,68[a]	0,11 ± 0,04[a]	1,06 ± 1,04[a]
Vitrified Morula	1,24 ± 0,66[a]	1,19 ± 0,54[a]	0,68 ± 0,36[a]

expression in the Igf2 gene of 0.75 ± 0.31, 0.76 ± 0.68, 1.24 ± 0.66 and H19 gene of 1.30 ± 0.61, 0.11 ± 0.04, 1.19 ± 0.54, and Bax gene of 1.62 ± 0.79, 1.06 ± 1.04, 0.68 ± 0.36 in fresh blastocysts, post-vitrified blastocysts and post-vitrified morula respectively (Table 7). In addition, cells number analysis of post-vitrified blastocyst embryos showed a number of cells of 63.67 ± 3.09 and 63.33 ± 3.35 in fresh blastocyst embryos and vitrified blastocysts respectively, statistically not significantly different ($p>0.05$) compared with fresh embryos (Table 6).

4. Discussion

Vitrification method has been widely applied for animals and humans. This is because method has several advantages compared to slowfreezing cryopreservation, such as shorter time and simpler tools. Moreover, viability rate of post-vitrification embryos are higher compared to slow freezing cryopreservation methods (Li et al., 2014). In this research, the viability rate of post-vitrified embryos at the morula and blastocyst was similar compared to fresh embryos. The higher level of viability rate of post-vitrified embryos caused by no formation of ice crystals in cells during the vitrification process.

In general, cell freezing process can impact cell damage through two main pathways, which are mechanical damage that affects cell shape due to the formation of ice crystals and cell damage caused by toxicity from cryoprotectants and osmotic stress that occurs related to cryoprotectant concentration used in the cell cryopreservation process. Cryoprotectants used in the cryopreservation process of this study are dimethyl sulfoxide (DMSO), ethylene glycol (EG), and sucrose. Dimethyl sulfoxide (DMSO) and ethylene glycol (EG) are penetrating cryoprotectant, while sucrose is one of non-penetrating type cryoprotectant. Both types of cryoprotectants have a function to inhibit formation of ice crystals in the vitrification process. The difference of the two cryoprotectants is the size of the molecule. Penetrating cryopotectants are small molecules that able to pass cell membranes, while non-penetrating cryoprotectants are large molecules, so it will not penetrate cell membranes. In addition, non-penetrating cryoprotectants usually have lower toxic properties compared to penetrating cryoprotectant (Bhattacharya and Prajapati 2016).

Combination of penetrating and non-penetrating cryoprotectant on research is expected to reduce toxic and osmotic stress caused by penetring cryoprotectant. In the process of warming, sucrose solution plays a role as an osmotic buffer so that cryoprotectants can be released without spending excessive intracellular water. In addition, multilevel concentrations in sucrose solution can reduce membrane damage due to osmotic pressure (Dattena et al., 2004). Mouttham and Comizzoli (2017) report that sucrose is very effective in maintaining structural integrity and function of membranes. Other factors to the success of vitrification are the small volume of high concentrations and times of cryoprotectant exposure in the vitrification process (Liebermann, 2003).

Cryopreservation of biological materials consist of six important steps, starting with exposure biological material to cryoprotectants, freezing (slow/rapid) to subzero temperatures, storage, thawing/warming, dilution and removal of cryoprotectants and restoring biological material in environmental physiology (Liebermann, 2003). In this research, morula and blastocysts embryos post-vitrified were cultured in G2 medium. Morula stage embryos cultured for 42 hours after warming, whereas blastocyst embryos cultured for 24 hours after warming. At the morula post-vitrified, embryo culture aims to determine the ability of embryo develop to blastocyst. Hence, embryo culture is expected can perform a repairing mechanism. The results showed that post-vitrified morula embryos were able to develop to blastocysts. These results indicate that vitrification does not affect embryo metabolism.

Embryo metabolism is one of the factors that influence the ability of the embryo to develop into the next stage. Morbeck et al. (2014) explained that disorder of meta-

bolic and reductive-oxydative balance can inhibit embryonic development and potentially reduce embryo viability, fetus and postnatal development. Other researchers suggest that amino acid metabolism in embryos can be used as an approach to predict the ability of embryonic development. Post-freezing embryos that are able to develop to blastocyst stage show the same amino acid metabolism as fresh embryos, whereas non-developing embryos show metabolic homeostatic disorders (Stokes et al., 2007). On the other hand, there are other variables that can be used to determine the quality of the embryo, such as morphology and morphometry embryo. Morphology embryo analysis is very subjective and requires high skill to be able to distinguish embryo quality. Because of that, several researchers carried out embryonic morphometry analysis to predict the ability of embryo development and implantation.

Morphometry analysis on the research carried out after the warming process includes zona pellucida thickness (ZPT), zona pellucida thickness variation (ZPTV), perivitelline space, inner cell mass (ICM), and blastocoel area for post-vitrified blastocyst embryos, while morphometric analysis in post-vitrification morula embryos include ZPT, ZPTV, perivetillen space, and blastomeres. Zona pellucida thickness (ZPT) and zone pellucida thickness variation (ZPTV) are variables that can be used to predict the ability of the embryo for hatching and implantation. Zona pellucida serves to maintain the occurrence of poly-sperm fertilization. In addition, at the early stage of embryonic development, zona pellucida function is to maintain the integrity and transportation of the embryo through the fallopian tube. At the stage of the blastula, zona pellucida is depleted in preparation for implantation due to the effects of endometrial lysine and expansion of blastocyst.

The depletion zona pellucida correlated positively with embryonic development, but in some cases zona pellucida fail to rupture resulting in failed hatching of the blastocyst embryo (Sun et al., 2005). It can be caused by impaired secretion of trypsin-like proteases from the tropectoderm (TE) and the phenomenon of zona hardening. Cohen et al., (1989) explained that the thickness of the pellucida zone is not the same, some thinner parts than other parts. It allows the embryo to transport the membrane and secrete the substrate to lyse the local zona. In this research, vitrification of blastocyst embryos showed an increase in the value of ZPT and ZPTV, although statistically it did not show significantly differencent. Whereas the morula embryo showed a decrease in the value of ZPT and ZPTV, but only the value of ZPTV which showed statistically significant differences. Sun et al., (2005) explained that the high value of ZPTV increases the potential of the pregnancy rate in human embryos. Decreasing ZPTV values can be negatively correlated with patient age. In addition, high-grade embryos have higher ZPTV values than low-grade embryos.

The volume analysis of post-vitrified embryonic cells was also carried out in this research. Hnida et al., (2004) explained that the incidence of embryonic cell fragmentation associated with the blastomere volume. It is indicated by an increase in the incidence of fragmentation significantly in the blastomer which has decreased volume. In the results of this research there was a decrease in blastomer volume of post-vitrified morula embryos, ICM and blastocoel volume in blastocyst embryos. Whereas perivetilen space has increased volume in post-vitrified morula and blastocyst embryos. The decrease volume is possible due to substitution process of cryoprotectants with water in cells during warming process. These results were supported by an analysis of the number of cells in the post-vitrified blastocyst embryos that did not

show significant differences. Konc et al., (2014) explained that during the vitrification process, majority of cells undergo dehydration before starting ultrarapid freezing due to high concentrations exposure of cryoprotectant. It is needed for the stages of intracellular and extracellular glass formation in the vitrification process. Nonetheless, failure of vitrification can lead to increased incidence of necrosis and cell apoptosis.

Research result by Baust et al., (2001) showed an increase in apoptotic protease (caspase-3) activity of 3.3 fold after thawing compared to normal cells. Increased apoptotic activity occurred at 12 to 21 hours post-thawing, while increased necrosis activity occurred at 6 hours post-thawing. After that, the activity returned to normal at 48 hours post-thawing. Increased activity can be inhibited by the addition of molecules that can reduce sublethal hypothermic stress that occurs in cells due to the cryopreservation process. Sublethal stress possible associated with oxidative stress, adenylate depletion, production and accumulation of free radicals, cell dehydration, and upregulation of nitric oxide. The accumulation of these things able to initiate the occurrence of the apoptotic mechanism in several cellular systems. The success of vitrification in this research can also be seen from Bax gene expression which statistically did not show significant differences in post-vitrified embryos compared to fresh embryos. Bax is a pro-apoptotic protein and plays an important role in the process of apoptosis. Based on molecular mechanism, Bax and Fas complex receptors in the mitochondrial membrane will be stimulated by the Trp53 protein when the cells have DNA damage. Activation of Bax and Fas complex receptors will cause release of cytochrome c and caspase 3 activation. In some cases, apoptosis and anti-apoptotic genes in apoptotic pathways are stimulated by hyperglycemic stress conditions that cause increased DNA fragmentation (Fabian et al., 2009).

Furthermore, this research analyzed the potency for implantation ability in post-vitrified embryos based on Igf2 and H19 gene expression. The results showed that post-vitrified blastocyst embryos had similar Igf2 gene expression levels and increased expression in post-vitrified morula embryos compared to fresh embryos. Whereas H19 gene expression pattern showed an increased expression in post-vitrified morula and blastocyst embryos compared to fresh embryos. However, statistically it did not show a significant difference in Igf2 and H19 gene expression. This results can be caused by the vitrified close system method used in this research. The close system keeps the embryo indirect contact with LN_2, so that the physical shock effect of the embryo can be reduced. These results were supported by the research result Bartolac et al. (2018) which indicated that pig blastocyst embryos exposed to vitrification and warming cryoprotectants without being inserted into LN2 showed the same transcription level as fresh embryos.

Bartolac et al., (2018) explained that disruption of gene expression can be caused by cellular and molecular changes associated with contact embryo-LN_2. In addition, the vitrification process at room temperature is believed to reduce the toxic effects of cryoprotectants used. The results showed that post-vitrified embryos in this research had potential implantation capability after embryo transfer. The results of this research indicated that post-vitrification embryos was safe and could be applied to support the success of embryo transfer. It was shown by the ability of post-vitrified embryo development to the next stage and genes that influenced on fetal and placental development had a relative level of mRNA similar to fresh embryos. Changes in the transcription levels of Igf2 and H19 genes could be influenced by the embryo

culture system. It was shown by the results of research by Park et al. (2011) which explained that the transcription levels of the Igf2 and H19 genes in pig embryos in the blastocyst stage in vitro were higher than in vivo embryos.

Igf2 is a gene that is specifically expressed by the paternal allele and has a function to regulate the development of the placenta and fetus. Whereas, H19 is a gene that is specifically expressed by the maternal allele and its function is to inhibit the development of the placenta and fetus. Abberant of gene expression cause abnormalities in embryonic development, such as Beckwith-Wiedemann Syndrome (BWS) which causes excessive fetal growth or Silver-Russell Syndrome (SRS) which causes less optimal fetal growth (Plasschaert and Bartolomei, 2014). Sivastava et al., (2000) showed that the H19 promoter is control part of the monoallele expression of the Igf2 and H19 genes in different regulatory pathways. On the maternal allele, the H19 gene promoter is required as an insulator which functions to deactivate the Igf2 gene. Whereas in the paternal allele, the upstream element mediates epigenetic modification of the H19 promoter during development which results in H19 in the paternal allele being inactive.

Igf2 gene in the blastocyst embryo is strongly expressed in the inner cell mass (ICM). The Igf2 gene functions to stimulate the classic Erk1/2 pathway and activation of Akt kinase. Erk 1/2 activation serves as an important proliferative signal of the embryo to continue the cell cycle during embryonic development. Erk1/2 activation plays a role in the proliferation of polar tropectoderm cells (TE) and tropoblast cell formation, while activation of Akt signaling plays an important role in the success of implantation by maintaining cell survival ability, tropoblast invasion and induction of extracellular matrix remodeling (Thieme et al. 2012).

Based on these results, it can be concluded that vitrification in morula and blastocyst stage embryos did not affect embryo quality based on morphometric analysis and Bax gene expression. In addition, post-vitrified embryo still has potential ability for implantation. It is indicated by the levels of Igf2 and H19 gene expression which is almost the same as the fresh embryo. Nevertheless, confirmation of the implantation ability in post-vitrified embryos is needed by embryo transfer.

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