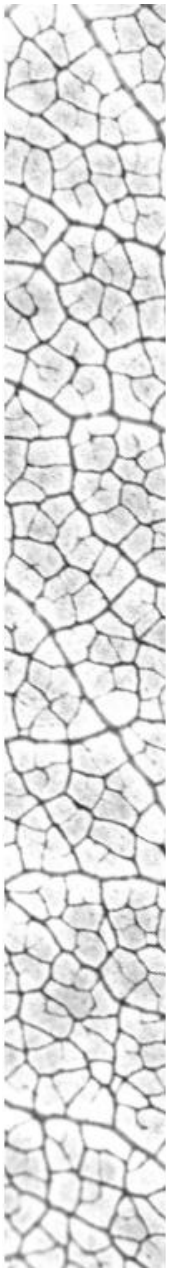

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Effect of vitrification on spermatozoa quality in bull semen

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Abstract
Cryopreservation of spermatozoa is part of the assisted reproductive biotechnology to enhance reproductive capacity in livestock. Conventional cryopreservation applies slow-gradual freezing method permitted ice crystallization and causes cryodamage resulting in poor post-thawed semen quality. Hence, vitrification is introduced by solidifying the solution into glassy state without causing any crystallization in fast and inexpensive manner. This ultra-rapid cooling method requires high concentration of cryoprotectants that potentially toxic the spermatozoa. Therefore, this study was conducted to determine the effect of vitrification on the quality of bull semen. A total of 24 bull semen samples were collected using an electro-ejaculation technique. Tris-based extender was compared with vitrification using solution of different concentration of cryoprotectants at 10% (Vitrification Solution 1; VS-1) and 20% (VS-2) containing dimethyl sulfoxide (DMSO) and ethylene glycol respectively. The result revealed that high mortality and nearly zero motility in all post-warmed vitrified spermatozoa, but the general and progressive motilities parameters in VS-1 at initial evaluation was 22.45% and 24.87% respectively better than Tris-based extender and was statistically significant. In conclusion, vitrification has potential as an alternative for cryopreservation. Therefore, further research on spermatozoa vitrification technique on enhancement in cooling and warming should be conducted and investigated.

Keywords: bull, spermatozoa, cryopreservation, vitrification, electro-ejaculation

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INTRODUCTION

Cryopreservation is the use of very low temperatures to preserve structurally intact living cells which played an important role in livestock reproduction. Assisted reproductive biotechnology (ARB) has been applied in various species of animal to enhance reproductive capacity, improve and preserve livestock genetics, and to develop new animal products. Artificial insemination is the leading ARB whereby various studies and development programs have been conducted to improvise quality of thawed semen (Fleisch et al. 2017, Shahzad et al.2016), Spermatozoa can be cryopreserved via two methods, namely conventional cryopreservation or vitrification. Conventional cryopreservation is a slow-gradual freezing process that described as a slow process of dehydration to reduce intracellular ice crystallization (Amirat-Briand et al 2010). In contrast, vitrification applies ultra-rapid cooling method to solidify liquid into glassy state without ice crystal formation (Isachenko et al. 2004, Magnotti et al. 2018).

Conventional cryopreservation is rather expensive and time-consuming, even though lesser amount of CPA is required. Formation of ice crystal during slow freezing increases cyro-damage of spermatozoa which produces poor to moderate result of viable spermatozoa after thawing. In contrast, vitrification is an inexpensive, ultra-rapid freezing method that preserve cells to sub-zero temperatures whereby fast cooling rates result in solidification of solution into glass-like structure rather than the ice crystals which reduces cryo-damages. Higher CPA concentration is required and it is toxic to the cells, therefore exposure time to perform this technique must be conducted very fast (Lawson et al.

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2011). However, complete understanding and development of vitrification in bovine spermatozoa is yet to be discovered. Thus, the objectives of this study were to determine the suitability of freezing bull semen by vitrification and to evaluate the quality of vitrified bull semen. Therefore, the hypothesis of this project was that the quality of vitrified bull semen is better than conventional cryopreservation.

MATERIALS AND METHODS

Animals

Four sexually matured Brangus bulls belonging to Beef Cattle Unit of University Agricultural Park, Universiti Putra Malaysia were used for semen collection. Convenient sampling method was applied. The age of the bulls was within the range of 3 to 5 years old with body weight between 300 to 550 kg. All the bulls were kept under semi-intensive system, provided with a commercial feed. The bulls were also allowed to graze daily. Commercial mineral block and water were also given ad libitum.

Semen collection

A total of 8 semen samples were collected using convenient sampling method with automatic electro-ejaculator (Baiee et al. 2018) for 2 consecutive weeks. The bulls were physically restrained with head crush. Anal probe of the electro-ejaculator was inserted after evacuation of feces manually and rectally. The ejaculated semen was collected directly into graduated collection centrifuge tube and kept in a 37oC insulated box temporarily. The average time for each collection cycle was 12 min. The bulls were allowed to rest for range of two days for the same week of collection, to four days for different week of collection .

Semen evaluation

The semen samples were evaluated macroscopically and microscopically. Macroscopic evaluations included volume and color, while microscopic evaluation included concentration, motility, morphology and livability of the spermatozoa. The component for motility included total motility and progressive motility (Kaka et al. 2015). The semen sample was diluted with Tris-based extender of ratio 1:40 for initial evaluation and CASA machine was used to assess the first four parameters of microscopic evaluation. The morphology and livability of spermatozoa were evaluated using the Eosin-Nigrosine staining technique (Khumran et al 2015, Tarig et al 2017).

Extenders preparation

Tris-egg yolk glycerol extender (TEYG) was prepared for conventional cryopreservation according to (Baiee et al. 2017, Baiee et al, 2018). Two extenders were prepared: Extender-A without glycerol and Extender-B with glycerol. To prepare this Tris-based extender, 2.42 g of Tris (hydroxymethyl), 1.48 g of citric acid, and 1.0 g of fructose (Mixture-A) were weighed and dissolved in 80mL of distilled water and mixed well manually. Then, 20 mL of freshly separated village chicken egg yolk (EG; 20%) from the egg albumin was added into dissolved Mixture-A. Extender-B was prepared with addition of 12.8 mL of glycerol (12.8%) into dissolved Mixture-A, followed by inserting 20 mL of EG (20%) and distilled water to obtain a 100 mL extender .

Vitrification solution preparation

Two vitrification solutions with different concentrations were prepared. Holding solution (HS) is one of the major components in vitrification that prepared with Hepes-buffered medium 199 and 20% of calf bovine serum. VS-1 contained 10% of DMSO and 10% of EG in HS, while VS-2 contained 20% of DMSO and EG respectively with 0.5M sucrose in HS. 0.5M sucrose was calculated manually with formula of 0.5 mole/L × 342 grams/mole × 1 L whereby 171 g of sucrose was required to produce a 0.5M sucrose in 1 liter of solution or 17.1 g of sucrose in 100 mL of solution or vice versa(Martins et al 2005, Arando et al 2017)

Experimental design

The semen collection and animal handling for this study had been approved by the Institutional Animal Care and Use Committee (IACUC). Each 0.25 mL straw contained targeted final concentration of 20 × 106 spermatozoa (Parthipan et al. 2017) for conventional cryopreservation. Initially, Extender-A was use for both initial evaluation and chilling at 5 oC for 3 hr (**Fig. 1**). Post-chilled suspension will be assessed after adding calculated volume of Extender-B. The extended semen was packed into straws and sealed. Straws were placed horizontally on a cold rack of 5 oC for 5 min. Next, transferred them into LN2 vapor of -50 oC, about 3.5 cm above the surface of LN2 for 5 min, followed by lowering the frozen straws were to -100 oC for 3 min. Next, straws will be plunged into LN2 and stored (Baiee et al 2018). Post-thawed evaluation will be done after 24 hr of storage by placing the straw in 36oC water bath for 45s.

For vitrification, 0.02 mL of fresh semen was added to pre-warmed 0.18 mL of vitrification solution with short exposure time of 35 s for VS-1 and 25 s for VS-2. Next, it was packed and sealed in a 0.25 mL straw, and plunged directly into the LN2. Straws were kept for 24 hr before evaluation. The straw was thawed at 36 oC for 20 s. Immerse the content in 1 mL of pre-warmed 0.25 M sucrose with HS for 5 min at 36oC, followed by transferring the content into 1 mL of pre-warmed 0.15 M sucrose with HS and lastly washed twice with HS for 5 min. Finally, 0.5 mL of HS was added into the washed spermatozoa for post-warming evaluation. The methods above referred to (Hadi et al 2011) with modifications.

Thawing and warming

All sealed straws were stored in LN2 for 24 hr before evaluation. TEYG straws were removed immediately

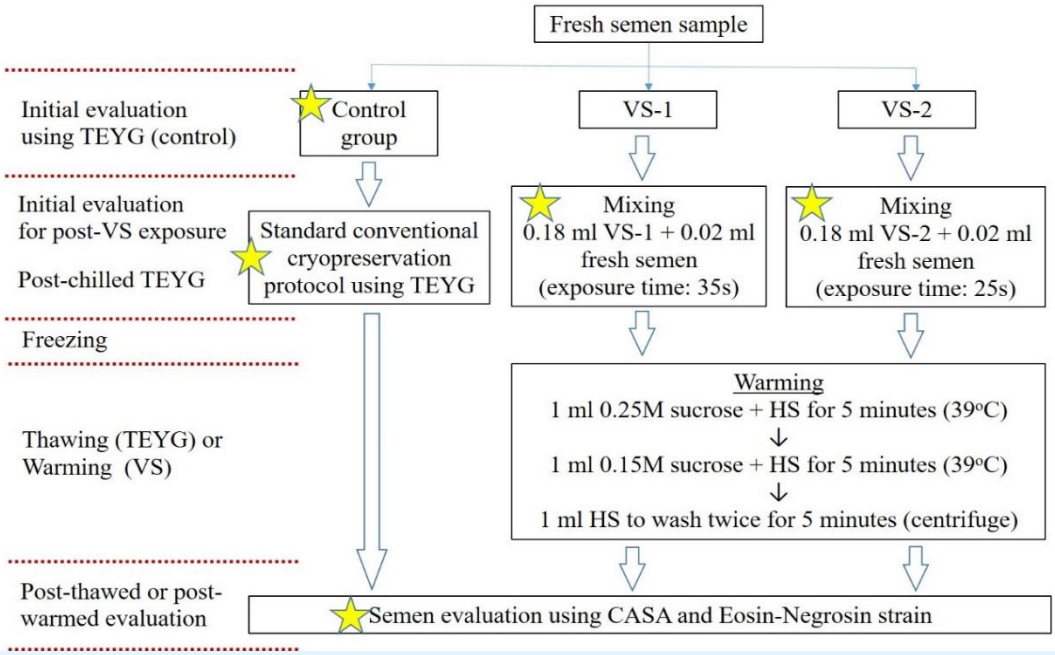

Fig. 1. Experimental design

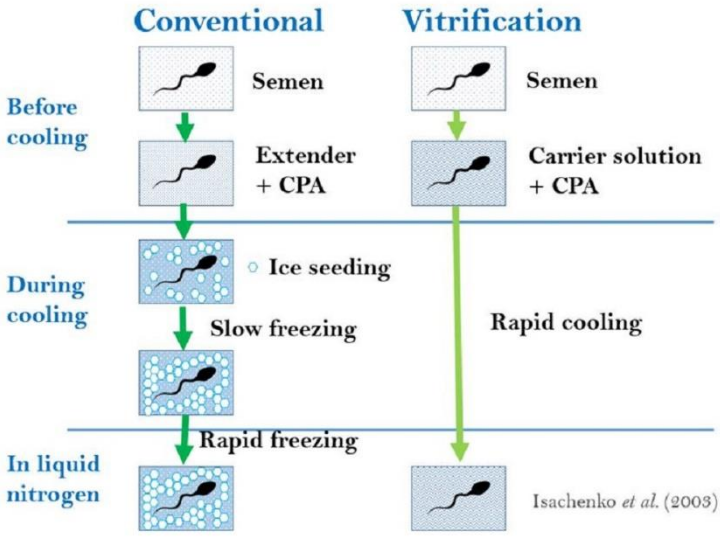

Fig. 2. Differences of Cooling Process and Condition of Extracellular Cryopreservation between Conventional Cryopreservation and Vitrification

from LN2 tank and thawed at 37oC water bath for 30 s (Amirat-Briand et al 2010). The straws were cut and the semen quality was evaluated again using CASA machine and stained with eosin-nigrosin stain. For vitrified straws, the methods for warming were described as above and post-warming semen quality evaluation was similar to conventional cryopreservation.

Statistical Analysis

Data collected including livability, normal morphology, general motility and progressive motility between the control group and new technique with two different concentrations were subjected to statistical analysis using IBM SPSS Statistics 20. Significance between data were evaluated by Kruskal-Wallis H test followed by Mann Whitney U test between the groups. All differences with P value < 0.05 were considered to be statistically significant.

RESULTS

All data obtained were expressed into mean ± standard error mean (SEM) with TEYG as the control. The data obtained were not normally distributed (P < 0.05) whereby non-parametric test of Kruskal-Wallis H (KWH) test and Mann Whitney U (MWU) test were applied. The result revealed both control and vitrification groups did not affect the morphology of spermatozoa

Table 1. Mean ± SEM of semen quality parameters during initial semen evaluation

Parameters (%)	TEYG	VS-1	VS-2
General motility	55.88 ± 9.55a	78.33 ± 10.16a	0 ± 0.00b
Progressive motility	12.13 ± 4.98a	37 ± 11.03b	0 ± 0.00c
Normal morphology	88.75 ± 3.50	83 ± 13.00	84 ± 14.00
Livability	80.75 ± 4.74	80 ± 15.00	4.5 ± 0.50

abc with different superscript across the rows indicate significant differences (P < 0.05)

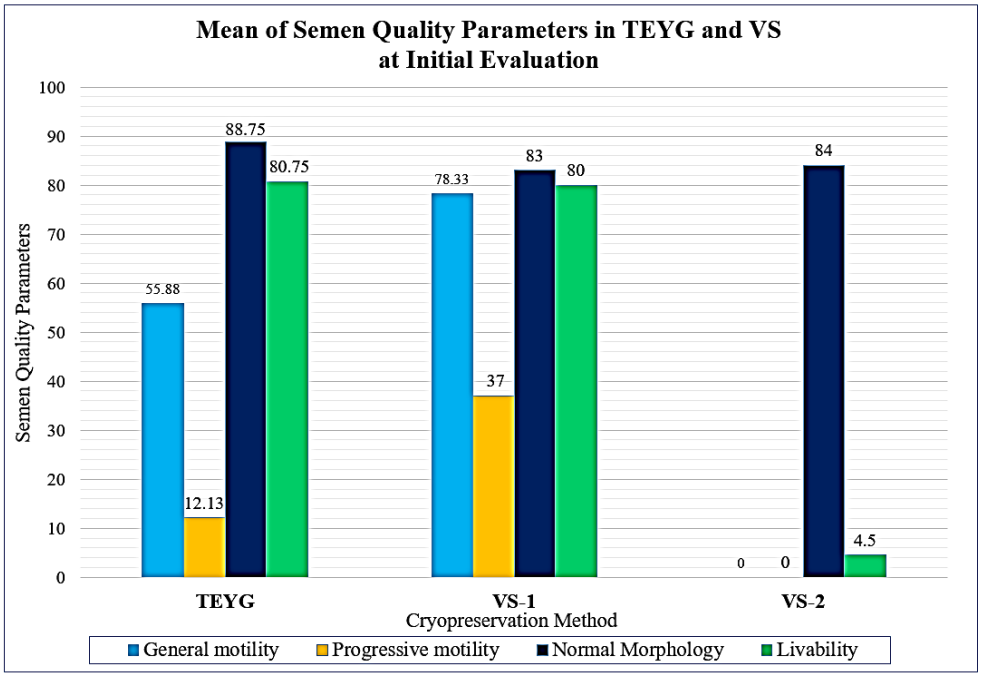

Fig. 3. Mean of semen quality parameters during initial semen evaluation

Table 2. Mean ± SEM of post-cryopreserve semen quality parameters

Parameters (%)	TEYG	VS-1	VS-2
General motility	41.5 ± 8.88a	0.25 ± 0.16b	0 ± 0.00b
Progressive motility	9.5 ± 2.54a	0 ± 0.00b	0 ± 0.00b
Normal morphology	89.25 ± 2.96	86.75 ± 3.85	87.13 ± 3.96
Livability	69 ± 7.04a	0.63 ± 0.32a	0 ± 0.00b

ab with different superscript across the rows indicate significant differences (P < 0.05)

with no significant difference among all the groups initially and post-thawed or post-warmed (P > 0.05).

Table 1 showed the semen quality parameters of initial evaluation with VS-1 had the most superior motility parameters with 22.45% and 24.87% for general and progressive motilities respectively better than TEYG even though it was not statistically significant. VS-2 causes high mortality in spermatozoa and was statistically significant (P < 0.05) for all parameters except for normal morphology when compared to the control. The progressive motility was statistically significant for both KWH and MWU tests (P < 0.05) between all the groups. Livability parameter for initial evaluation was likely to be invalid due to the presence of missing data. The result showed no significant difference between groups for KWH test but was significantly different between control group and VS-2 only for MWU test, even though the livability parameter for both control and VS-1 were similar with 80.75% and 80% respectively. **Fig. 3** depicted the differences of each parameter during initial evaluation whereby VS-1 displayed the most superior semen quality, while VS-2 achieved the poorest parameters.

The semen quality parameters dropped as compared to the initial parameters of control group after cryopreservation. **Table 3** and **Fig. 5** depicted the effect of cryopreservation on the semen quality for this study. The result revealed that general motility and mortality were statistically significant (P < 0.05) but VS-1 and VS-2 were not statistically difference. For progressive motility, KHW test revealed no statistical significance but MWU test showed significant difference between control and VS-1 (P < 0.05) with 2.63% and 12.13% respectively. Vitrified spermatozoa significantly dropped in general motility and has high mortality rate post-warmed up to 55% and 80% respectively. There were no differences between the spermatozoa underwent vitrification on all the parameters.

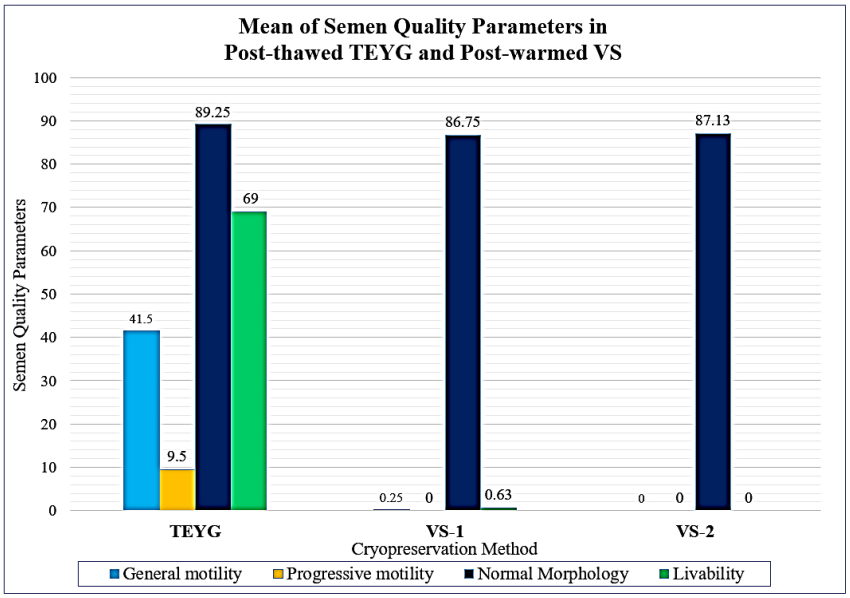

Fig. 4. Mean of post-cryopreserve semen quality parameters

Table 3. Mean ± SEM of differences between initial evaluation of control group and post-cryopreserve evaluation semen quality parameters

Parameters (%)	TEYG	VS-1	VS-2
General motility	14.38 ± 11.31a	55.63 ± 9.50b	55.88 ± 9.55b
Progressive motility	2.63 ± 6.51	12.13 ± 4.98	11.88 ± 4.96
Normal morphology	-0.50 ± 0.98	2 ± 1.98	1.62 ± 2.20
Mortality	11.75 ± 5.11a	80.13 ± 4.91b	80.75 ± 4.74b

ab with different superscript across the rows indicate significant differences (P < 0.05)

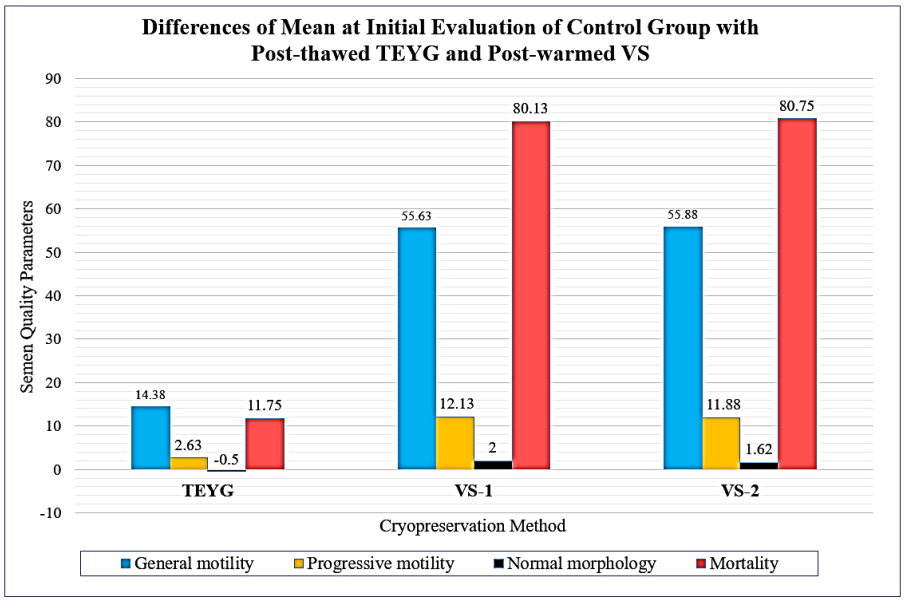

Fig. 5. Differences of mean between initial evaluation of control group and post-cryopreserve evaluation semen quality parameters

DISCUSSION

During cryopreservation, spermatozoa are subjected to deleterious effect as they possess notorious chemical and physical stresses which inevitably reduce the quality of spermatozoa post-cryopreserve (Crespilho et al. 2012, Khoshvaght et al. 2015, Naz et al. 2019) which similar to this study. The main differences between gradual-slow freezing and ultra-rapid cooling are the concentration of CPAs, as well as the cooling and warming rates. The aim of cryopreservation is to prevent osmotic damage and intracellular ice formation that has negative effects on survival. In conventional method,

these harmful effects are avoided by cellular dehydration, while in vitrification, ice formation is avoided by converting the solution into viscous glass state that correspond to a viscosity of 1013 poise (Mazur et al. 2008).

The post-thawed general motility and livability parameters of TEYG deteriorated 14.38% ± 11.31 and 11.75% ± 5.11 respectively as compared to the initial evaluation. Conventional cryopreservation applies slow equilibrium freezing method where straws were chilled and froze gradually from +37oC to +5oC, followed by -50oC to -100oC, and finally -196oC in LN2 had allowed freezing of water and resulted in high concentration of solutes leading to osmotic shock. At the same time, the spermatozoa potentially damaged by the ice crystal formed from freezing of water causes both internally and externally cryodamage. Hence, glycerol was added in the Tris-based extender as CPA to increase the total concentration of solutes in the media and reduce crystallization at any given temperature (Siemeet al 2016) .

In contrast, vitrification requires higher concentration of CPAs and the exposure time is critical to ensure vitrification and maintenance of an ice-free state upon warming. The major problem of vitrification is the need of high concentration of CPAs but the spermatozoa are very sensitive to these agents (Isachenko et al 2003). Various researches had conducted over the year, but whether vitrification is a better alternative to cryopreserve spermatozoa is still uncertain (Agha-Rahimi et al. 2014)

In this study, VS-1 achieves the best motility parameters for initial evaluation. Hence, the concentration of CPAs in VS-1 was biologically acceptable and tolerable by the spermatozoa. For VS-2, the concentration of CPAs is doubled which cannot be tolerated by the spermatozoa and lead to high mortality even at initial stage even though 0.5 M of sucrose was added as non-permeable CPA. Besides that, the experimental design for this project referred to vitrification of bovine oocytes which might be less compatible to spermatozoa as they are more sensitive with the agents. In addition, oocytes are bigger in size whereby its capacity to cope with stresses could be better. Succeeded vitrification of human spermatozoa by Isachenko et al. (Isachenko et al. 2003,2004,2008) applied spermatozoa selection by swim-up technique and used medium HTF-HSA 1% (Human Tubal Fluid – Human Serum Albumin) and 0.5 M sucrose at 37oC, and maintain the suspension in the atmosphere of 5% carbon dioxide for 5 min before vitrification. Swim-up technique was not performed in this experiment where both good and poor quality spermatozoa were included which could affect the post-warmed semen quality. Next, supplementation of protein such as albumin and sucrose can be added as non-permeable CPA. Consideration of including antioxidants may be beneficial to reduce production of excessive free oxygen radicals (Taher-Mofrad et al 2020) that generated during mitochondrial electron transport which decreases the survival and fertility of spermatozoa by the reactive oxygen species formation and membrane lipid peroxidation.

The cooling and warming rates also affect the viability of spermatozoa. Since vitrification for bovine semen is new and yet to be discovered, no specific protocols or publications on this methodology. Thus, the cooling and warming rates for this experiment was referred and modified from previous work conducted by Hadi et al.(Hadi et al, 2011) The result for both post-warmed vitrified spermatozoa had zero motility parameters except for VS-1 with total 2% motility. For cooling, a full-loaded sealed 0.25 mL straw will cause breakage and even unsealed the straw. This phenomenon could be due to sudden change in temperature from +37 oC to -196oC and the straw unable to exert the pressure. Hence, the straws were only filled with 0.2 mL and sealed to reduce the pressure. Apart from that, directly plunging of the straws into LN2 might not cool and vitrified the straws simultaneously. Successful example of human spermatozoa vitrification use cryoloop (Schuster et al 2003), vitrified solid spheres using pipettes and a double-layered straw contained 0.25 mL straw within a 0.5 mL sealed straw (Isachenko et al 2004,2008) that allowed generalize cooling. Besides that, mishandling at any stages involving cooling can affect the post-warmed semen quality .

Warming is another critical factor determining the success of vitrification. Vitrification maintained at -130 oC and below, but any temperature above it may induce fracture of the glass and cleave the cells causing irreversible injury and additional ice nucleation (Kumar et al. 2019). Once the straw was removed from LN2 storage tank, the surrounding temperature begins thawing process and allows crystallization. Hence, rapid warming is essential to avoid injury from devitrification and subsequent recrystallization which determines the success of vitrification. For this experiment, 39 oC was used and it could be one of the factors contributing to the viability of spermatozoa. Warming from -196 oC to 39 oC might not fast enough to prevent crystallization from occurring. Therefore, increasing the warming rate can be considered. Apart from that, warming a straw would be harder as compared to warming a vitrified solid sphere or cryoloop. Vitrified spheres or cryoloop obtained directly from LN2 container and warmed directly and faster as compared to straws contained in goblets stored in a LN2 storage tank**.**

Even though the livability of vitrified spermatozoa achieved nearly zero in this experiment, but there are some motile microorganisms such as protozoa observed in this study that had survived the process of vitrification. This indicated that there are potential and possibilities of

vitrification for cryopreservation. Hence, improvising and enhancement of vitrification techniques potentially allow ultra-rapid cooling as an alternative to cryopreserve spermatozoa of bovine species in an ice-free manner.

CONCLUSION

Based upon the findings of this study, the following conclusions could be made: effect of conventional cryopreservation is better than vitrification of bull semen. Vitrification using VS-1 achieved highest semen quality parameters at initial evaluation with 10% of DMSO and ethylene glycol in HS. Moreover, vitrification has potential to cryopreserve bull spermatozoa. Concentration, types and exposure time of CPAs use in vitrification is critical but yet to be discovered. Supplementation of antioxidants can reduce the production of excessive free oxygen radicals too. All these supplementations are essential to ensure vitrification and maintenance of an ice-free state upon warming. Hence, further research on concentration and types of permeable and non-permeable CPAs should be conducted. Besides that, cooling and warming rates determine the success of vitrification and affects the post-warmed semen quality. Consideration of depositing the aliquots directly and rapidly into LN2 forming solid spheres allowed complete simultaneous cooling. Besides that, these vitrified spheres allows uniform warming and able to determine the suitable warming temperature better. Hence, this can prevent devitrification and recrystallization that deteriorate the semen quality. In addition, warming temperature can be increase to allow rapid warming. However, the temperature should not be too high as it causes high mortality to spermatozoa.

REFERENCES

Agha-Rahimi, A., Khalili, M. A., Nabi, A., & Ashourzadeh, S. (2014). Vitrification is not superior to rapid freezing of normozoospermic spermatozoa: effects on sperm parameters, DNA fragmentation and hyaluronan binding. *Reproductive biomedicine online*, *28*(3), 352-358.

Amirat-Briand, L., Bencharif, D., Vera-Munoz, O., Pineau, S., Thorin, C., Destrumelle, S.,... & Tainturier, D. (2010). In vivo fertility of bull semen following cryopreservation with an LDL (low density lipoprotein) extender: Preliminary results of artificial inseminations. *Animal reproduction science*, *122*(3-4), 282-287.

Arando, A., Gonzalez, A., Delgado, J. V., Arrebola, F. A., & Perez-Marín, C. C. (2017). Storage temperature and sucrose concentrations affect ram sperm quality after vitrification. *Animal reproduction science*, *181*, 175-185.

Baiee, F. H., Wahid, H., Rosnina, Y., Ariff, O. M., Yimer, N., Salman, H.,... & Khumran, A. M. (2017). Hypo-Osmotic Swelling Test Modification to Enhance Cell Membrane Integrity Evaluation in Cryopreserved Bull Semen. *Pertanika Journal of Tropical Agricultural Science*, *40*(2).

Baiee, F. H., Wahid, H., Rosnina, Y., Ariff, O., Yimer, N., Jeber, Z.,... & Harighi, F. (2018). Impact of Eurycoma longifolia extract on DNA integrity, lipid peroxidation, and functional parameters in chilled and cryopreserved bull sperm. *Cryobiology*, *80*, 43-50.

Baiee, F., Haron, W., Yusoff, R., Ariff, O., Yimer, N., Hammadi, S.,... & Kaka, A. (2018). Modification of Electro-Ejaculation Technique to Minimise Discomfort during Semen Collection in Bulls. *Pakistan Journal of Zoology*, *50*(1).

Crespilho, A. M., Sá Filho, M. F., Dell'Aqua Jr, J. A., Nichi, M., Monteiro, G. A., Avanzi, B. R.,... & Papa, F. O. (2012). Comparison of in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or new lecithin based extenders. *Livestock Science*, *149*(1-2), 1-6.

Fleisch, A., Malama, E., Witschi, U., Leiding, C., Siuda, M., Janett, F., & Bollwein, H. (2017). Effects of an extension of the equilibration period up to 96 hours on the characteristics of cryopreserved bull semen. *Theriogenology*, *89*, 255-262.

Hadi, H., Abd, W. H., Rosnina, Y., Daliri, M., Dashtizad, M., Holmes, R., & Abas, M. O. (2011). Nuclear maturation of immature bovine oocytes after vitrification using open pulled straw and cryotop methods. *African Journal of Biotechnology*, *10*(12), 2334-2339.

Isachenko, E., Isachenko, V., Katkov, I. I., Dessole, S., & Nawroth, F. (2003). Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. *Reproductive biomedicine online*, *6*(2), 191-200.

Isachenko, E., Isachenko, V., Weiss, J. M., Kreienberg, R., Katkov, I. I., Schulz, M.,... & Sanchez, R. (2008). Acrosomal status and mitochondrial activity of human spermatozoa vitrified with sucrose. *Reproduction*, *136*(2), 167-174.

Isachenko, V., Isachenko, E., Katkov, I. I., Montag, M., Dessole, S., Nawroth, F., & van der Ven, H. (2004). Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. *Biology of reproduction*, *71*(4), 1167-1173.

Kaka, A., Wahid, H., Rosnina, Y., Yimer, N., Khumran, A. M., Sarsaifi, K.,... & Ebrahimi, M. (2015). α-Linolenic acid supplementation in BioXcell® extender can improve the quality of post-cooling and frozen-thawed bovine sperm. *Animal reproduction science*, *153*, 1-7.

Khoshvaght, A., Towhidi, A., Zare-shahneh, A., Noruozi, M., Zhandi, M., Davachi, N. D., & Karimi, R. (2015). Dietary n-3 PUFAs improve fresh and post-thaw semen quality in Holstein bulls via alteration of sperm fatty acid composition. *Theriogenology*, *85*(5), 807-812.

Khumran, A. M., Yimer, N., Rosnina, Y., Ariff, M. O., Wahid, H., Kaka, A.,... & Sarsaifi, K. (2015). Butylated hydroxytoluene can reduce oxidative stress and improve quality of frozen–thawed bull semen processed in lecithin and egg yolk based extenders. *Animal reproduction science*, *163*, 128-134.

Kumar, A., Prasad, J. K., Srivastava, N., & Ghosh, S. K. (2019). Strategies to minimize various stress-related freeze–thaw damages during conventional cryopreservation of mammalian spermatozoa. *Biopreservation and Biobanking*, *17*(6), 603-612.

Lawson, A., Ahmad, H., & Sambanis, A. (2011). Cytotoxicity effects of cryoprotectants as single-component and cocktail vitrification solutions. *Cryobiology*, *62*(2), 115-122.

Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J. G., Valdebenito, I., & Figueroa, E. (2018). Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. *Reviews in Aquaculture*, *10*(1), 15-25.

Martins, R., et al.(2005)., Effects of vitrification of immature bovine oocytes on in vitro maturation. Animal Reproduction, 2005. 2(2): p. 128-134.

Mazur, P., Leibo, S. P., & Seidel Jr, G. E. (2008). Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. *Biology of reproduction*, *78*(1), 2-12.

Naz, S., Umair, M., & Iqbal, S. (2019). Ostrich egg yolk improves post thaw quality and in vivo fertility of Nili Ravi buffalo (Bubalus bubalis) bull spermatozoa. *Theriogenology*, *126*, 140-144.

Parthipan, S., Selvaraju, S., Somashekar, L., Arangasamy, A., Sivaram, M., & Ravindra, J. P. (2017). Spermatozoal transcripts expression levels are predictive of semen quality and conception rate in bulls (Bos taurus). *Theriogenology*, *98*, 41-49.

Schuster, T. G., Keller, L. M., Dunn, R. L., Ohl, D. A., & Smith, G. D. (2003). Ultra-rapid freezing of very low numbers of sperm using cryoloops. *Human Reproduction*, *18*(4), 788-795.

Shahzad, Q., Mehmood, M. U., Khan, H., ul Husna, A., Qadeer, S., Azam, A.,... & Ahmad, M. (2016). Royal jelly supplementation in semen extender enhances post-thaw quality and fertility of Nili-Ravi buffalo bull sperm. *Animal reproduction science*, *167*, 83-88.

Sieme, H., Oldenhof, H., & Wolkers, W. F. (2016). Mode of action of cryoprotectants for sperm preservation. *Animal reproduction science*, *169*, 2-5.

Taher-Mofrad, S. M. J., Topraggaleh, T. R., Ziarati, N., Bucak, M. N., Nouri, M., Seifi, S.,... & Shahverdi, A. (2020). Knockout serum replacement is an efficient serum substitute for cryopreservation of human spermatozoa. *Cryobiology*.

Tarig, A. A., Wahid, H., Rosnina, Y., Yimer, N., Goh, Y. M., Baiee, F. H.,... & Ebrahimi, M. (2017). Effect of different concentrations of soybean lecithin and virgin coconut oil in Tris-based extender on the quality of chilled and frozen-thawed bull semen. *Veterinary World*, *10*(6), 672.