International Journal of Technology 13(8) 1755-1767 (2022)
Received July 2022 / Revised November 2022 / Accepted December 2022

International Journal of Technology

http://ijtech.eng.ui.ac.id

Can Cryoprotectant's modification in Spermatozoa Cryopreservation be an Alternative to Improve Embryo Quality? A Review

Manggiasih Dwiayu Larasati[1], Silvia Werdhy Lestari[2*], Andon Hestiantoro[3], Mulyoto Pangestu[4]

[1]*Doctoral Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No. 6, Jakarta 10430 Indonesia*
[2]*Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jakarta 10430 Indonesia*
[3]*Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Indonesia, Jakarta 10430 Indonesia*
[4]*Education Program in Reproduction and Development, Department of Obstetrics and Gynecology, School of Clinical Sciences, Monash University, Victoria, Australia*

Abstract. Spermatozoa cryopreservation is an effective method for maintaining male fertility in humans. Nevertheless, there are some limitations of sperm cryopreservation, which is called as cell injury by cryoprotectant, that cannot be avoided. This process will affect embryo quality. Therefore, it is mandatory to modify cryoprotectant in spermatozoa cryopreservation, to improve embryo quality. This review aimed to summarize the modification of cryoprotectant that can damage the cell, thereby improving embryo quality. To this purpose, a computerized search of EMBASE, PubMed, Scopus and Google Scholar databases from 2008 to 2022 were performed on general term such as "sperm cryopreservation", "cryoprotective agent", "modified cryoprotectant", "cell injury". Of these, 1847 publications were screened and 38 articles were obtained and evaluated. Although no formal conclusions can be drawn regarding the cryopreservation of spermatozoa to obtain good embryo quality, our results suggest that modified cryoprotectants can be an alternative cryoprotectant compared to commercial cryoprotectants. In addition, the use of antioxidant in spermatozoa cryopreservation can also prevent cell damage due to the negative effects of cryoprotectants. However, further researches still need to be performed to investigate the cellular mechanisms.

Keywords: Cell injury; Cryoprotectant agent; Modified cryoprotectant; Sperm cryopreservation

1. Introduction

Cryopreservation is needed for medical purposes such as the preservation of organs, tissues and pharmaceutical research (Bojic et al., 2021). To maintain reproductive ability in women, cryopreservation of adult oocytes is required (Jang et al., 2017). Furthermore, the main reasons for cryopreservation of semen and testicular tissue are autoimmune diseases, neoplasia, spinal cord injury, vasectomy, urological pathology, patients undergoing assisted reproductive programs, or due to changes in spermatogenesis(Gandini et al., 2017) . The first successful embryo cryopreservation dates back in 1996, in a woman with breast cancer diagnosis and undergoing natural IVF before chemotherapy.

*Corresponding author's email: finallysilvia@gmail.com, Tel.:+6285959501201
doi: 10.14716/ijtech.v13i8.6129

Overall, cryopreservation is useful for preserving the fertility of men planning to undergo vasectomy or procedures that could compromise their fertility, such as surgery, radiotherapy or chemotherapy (Singer et al., 2019).

Cryopreservation spermatozoa cells are an effort to store spermatozoa cells in a frozen state so that they can be used at any time when needed and used in support application of assisted reproductive technology (Höfner et al., 2020). Cryopreservation is the process of cooling and storing cells in liquid nitrogen at a temperature of -196^{o}C to stop all metabolic processes. There are two major methods of cryopreservation - slow freezing and vitrification (Jang et al., 2017). Slow cooling involves a heating rate of about 1°C/min with a cryoprotectant of less than 1.0 M, using a controlled rate freezer or a benchtop portable freezing container (Jang et al., 2017; Li et al. 2019). Slow freezing is advantageous due to the lower risk of contamination during procedures and requires fewer manipulation skills.

On the other hand, slow freezing has a higher risk of extracellular ice formation that may cause harm to the cells (Agarwal & Tvrda, 2017; Jang et al., 2017). In recent times, vitrification has become a more popular alternative to slow freezing. Vitrification is directly changing the nature of the cell suspension from an aqueous phase to a glassy state with liquid nitrogen. This process lowers the risk of cell injury and increasing cell survival rate dramatically. The vitrification technique, however, carries a higher risk of contamination from pathogenic agents and requires high manipulation skills (Jang et al., 2017). Slow cooling and vitrification techniques aim to protect the cells from temperature-induced damage, intracellular ice formation (Sieme et al., 2015), dehydration, and preventing toxic effects at high and low temperatures (Pegg, 2015). Temperature changes on the process of osmosis results in an increase in water flux. This situation relates to the effect of temperature on density and viscosity (Sutijan et al., 2022).

Cryopreservation involves a decrease in temperature. A further drop in temperature below 0°C causes the water in the extracellular environment to freeze, resulting in the increase of the concentration of solutes. The imbalance in solutes creates osmotic pressure, causing the solvent to flow through the plasma membrane from the inside to the outside of the cell and the abrupt flow of solvent can affect the structure of the sperm plasma membrane (Peris-Frau et al., 2020). The effect on the structural integrity of the membrane threatens the viability of the biological samples and lowers the quality of cryopreservation. To limit the structural damage, cryoprotectants that possesses minimum cytotoxic effects are often utilized (Whaley et al., 2021; Sieme et al., 2016). Unfortunately, the simple cryoprotective agents still have a negative effect to the cell, related to cell injury issues. Cell injury is damage that can result from spermatozoa cryopreservation induced by cellular stress during the cell freezing process. Cell injury can decrease cell viability due to cold shock and crystal ice formation (Morris et al., 2012). Direct damage will affect cellular structure and function, e.g. degradation processes spermatozoa metabolism, whereas damage does not directly difficult to observe and only seen after thawing. The main effect of cold shock on spermatozoa cells is a decrease in motility and vitality, changes in permeability and changes in components lipids in the spermatozoa membrane. Meanwhile, the effect on spermatozoa cells is due to the formation of ice crystals is a decrease in motility and viability spermatozoa, increased release of intracellular enzymes outside the cell, and damage to organelles such as lysosomes and mitochondria. An optimal freezing temperature is required before the cells are stored in liquid nitrogen to avoid cryoinjury. In addition, during cell thawing, cryoprotectants must be removed to prevent toxicity. Therefore, it is really needed to modify the cryoprotective agents, so that cells, particularly sperm cells can be preserved well. This review aimed to summarize the modification of cryoprotectant that can damage cell injury, thereby improving embryo quality.

2. Methods

This review article was conducted using several search engines such as EMBASE, PubMed and Google Scholar. The relevant publications have been located using a boolean search method (AND, OR, NOT) which included the phrases sperm, vitrification, freezing, cryopreservation, fertility preservation, cryoprotectant and cell injury. The search term 'sperm cryopreservation', 'cryoprotectant agent', 'modified cryoprotectant' and 'cell injury' were all included (Figure 1).

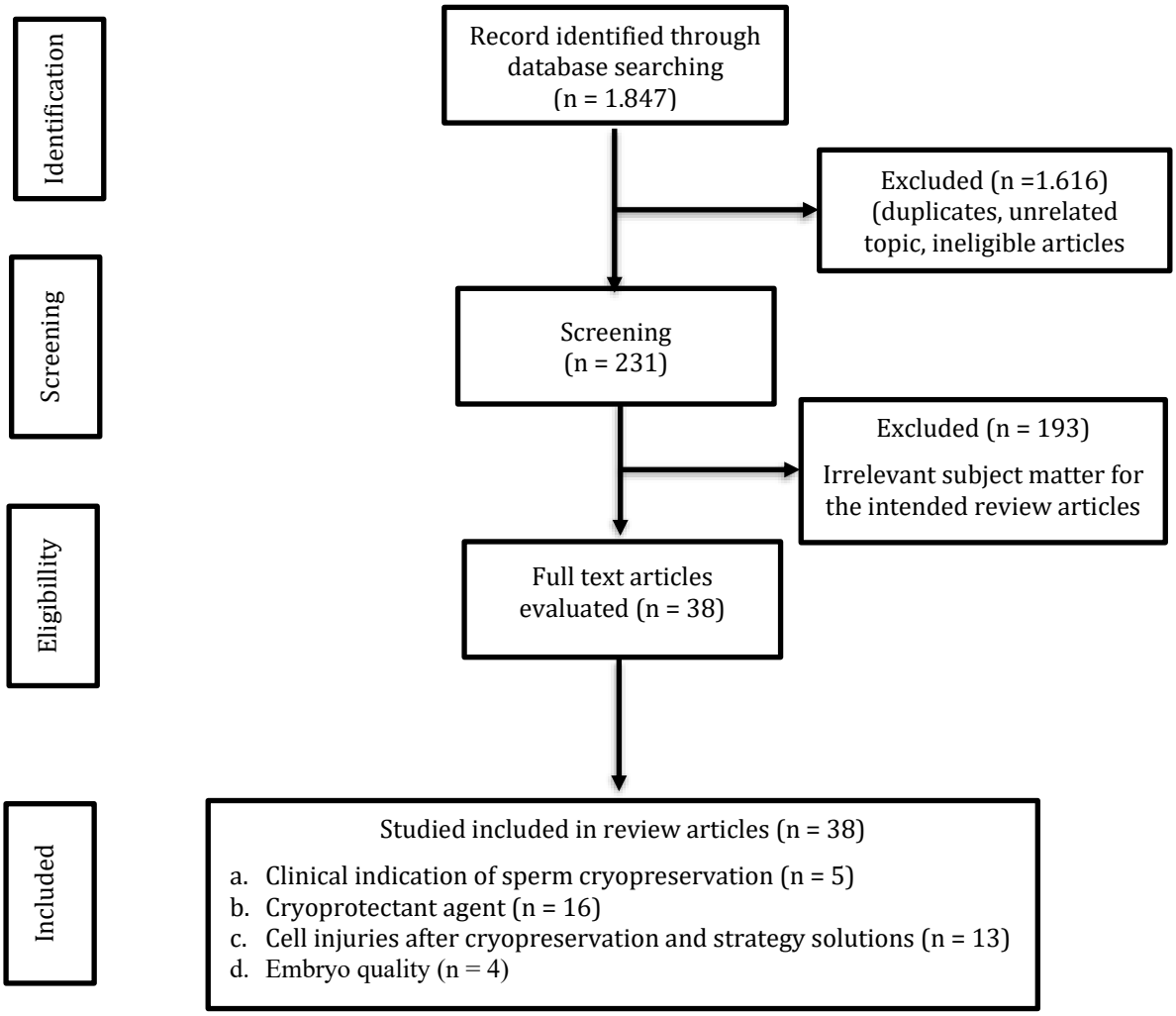

Figure 1 Search strategies for literature review

3. Results and Discussion

3.1. Cryoprotectant Agent

Cryoprotectants are substances nonelectrolyte chemicals that play a role in reducing lethal effect during freezing either form the influence of the solution and the formation of ice crystals so that cell viability can be maintained (Whaley et al., 2021; Fahy & Wowk, 2015). Osmotic stress become dangerous for the cells during frozen storage (Sieme et al., 2015). Cryoprotectant agents (CPAs) are utilized during the freezing process for tissue or other biological samples to reduce osmotic stress. Therefore, it is necessary to identify proper CPAs that are safe for use for biological samples. Table 1 shows some of the concentrations of cryoprotectants commonly used during the freezing process. CPAs can be classified into two major categories: a) cell membrane-permeating cryoprotectants, such as

glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, propylene glycol (Varisli et al., 2009; Sieme et al., 2016) and b) non-membrane permeating cryoprotectants, such as 2-methyl-2,4-pentanediol and polymers such as polyvinyl pyrrolidone, hydroxyethyl starch, and various sugars. (Whaley et al., 2021; Best, 2015; Sztein et al., 2001). Based on the summary of table 1, it is known that glycerol is the best and most commonly used cryoprotectant for spermatozoa cryopreservation. Because of glycerol has hydroxyl groups (Ni'mah et al., 2019) so as to prevent the formation of ice crystals (Hanifah et al., 2020; Hamidi, 2010).

Table 1 A brief overview of the several forms of CPAs, along with their characteristic concentrations and properties

Cryoprotective agent	Commonly used concentrations	Organism and biological samples	Effects and features	References
Glycerol	50% - 70%	Buffalo bull sperm, Human sperm*	Less hazardous at elevated concentrations	(Tariq et al. 2020) (Kitporntheranunt et al., 2017)
DMSO	5% - 10%	Sea bream sperm	Toxic but widely accessible	(Zilli et al. 2014)
Ethylene glycol	60%	Mice sperm Boar sperm	Some toxicity, although the most efficient cryoprotectants	(Critser & Mobraaten 2000) (Guthrie & Welch 2005)
Propylene glycol	5% - 15%	Rat sperm	A synthetic organic substance that it also utilised as antifreeze	.(Varisli et al. 2009)
Trehalose	90%	Ram semen	Contains two glucose molecules and is generated by numerous species	(Pelufo et al. 2015)

*GEYC = glycerol egg yolk citrate.

3.1.1. Glycerol

Glycerol is a typical cryoprotectant that has been used extensively for storing sperm cells (Sztein et al., 2018; Jang et al., 2017) , and there has been a significant amount of research on it. Glycerol aids in the protection of cells by crossing the cell membrane and beginning to influence the water molecules (Best, 2015; Pegg, 2007) . During freezing-induced membrane phase transitions, glycerol can permeate cellular membranes and affect the rate and extent of cellular dehydration (Sieme et al. 2016). Glycerol establishes hydrogen bonds with water molecules, which, when exposed to freezing temperatures, results in the formation of an amorphous solid. This process is also referred to as vitrification. After recovery, cells cryopreserved in glycerol exhibit excellent vitality (Wessel & Ball, 2004). In addition, glycerol also affects the rate of reaction and the balance of chemical reactions (Sulistyo et al., 2020).

3.1.2. Dimethyl sulfoxide (DMSO)

Similar to Glycerol, Dimethyl sulfoxide (DMSO) is a permeating agent. It functions identically to glycerol but is harmful to live cells. Commonly, it is utilised at a concentration of 10%. Today, it is the most common CPA. DNA methylation and histone modification have been associated with a decrease in cell survival and the induction of cell differentiation (Miyagi-Shiohira et al., 2015).

3.1.3. Ethylene glycol

Additionally, ethylene glycol is a regularly used CPA. It was discovered that substituting ethylene glycol for propylene glycol in vitrification solutions lessens the solution's non-specific toxicity. When combined with water, it modifies the hydrogen bonding, and water

forms the same amorphous solid. Ethylene glycol makes weaker hydrogen bonds than propylene glycol, therefore macromolecules are shielded by a greater number of residual water molecules, resulting in increased hydration. (Bojic et al., 2021). However, since it cannot pass through the cell membrane, it is a non-permeable agent that operates on the cell's outside.

3.1.4. Propylene glycol

Propylene glycol is a non-permeable substance that acts on the cell's outside. It showed the highest percentage of motility recovery following freezing and warming of all CPAs examined (about 70%; P <0.05). In addition to that, it is a widely utilized CPA (Varisli et al., 2009)

3.1.5. Trehalose

Trehalose is a kind of sugar that may be produced by various species, including fungi, bacteria, yeast, and even certain insects and plants (Whaley et al., 2021). It aids these organisms in their ability to endure frigid conditions. The optimal method for preparing ram ejaculates for deep freezing appears to be the simultaneous addition of glycerol and disaccharide after cooling to 5 °C, utilising trehalose as the impermeable sugar. Sperm motility after thawing suggested that trehalose possessed a stronger ability for cryopreservation than sucrose. Consequently, it is applied in cryopreservation (Pelufo et al., 2015).

3.2. In Vitro Fertilization and Embryo Quality

Male fertility is influenced by sperm morphology, sperm motility, plasma membrane integrity and acrosomal reactions. Further analysis of other factors is needed to provide a complete picture of the potential for male fertility. In vitro fertilization process is influenced by sperm and oocyte quality. Many factors affect sperm quality in freezing and thawing e.g. the freezing method, temperature control, sperm preparation technique, and type of cryopreservative agent (Kitporntheranunt et al., 2017). Likewise, many factors affect oocyte quality. Ideally, good quality sperm and oocytes will produce good embryos too. But on the contrary, the embryo quality will decrease if the sperm quality is low due to frozen storage. Table 2 describes the research results related to in vitro fertilization using sperm cryopreservation. Embryo quality can be measured through the normal stages of cell division and blastocyst. On day three, embryos of high grade are identified as having 7-8 uniformly sized cells that are not fragmented (Lestari, 2019).

Table 2 Summary table of studies sperm cryopreservation to evaluate the quality embrio before or after in vitro fertilization

Year	Sample size	Cryoprotectant agent	Main outcome	Conclusion	References
2021	There were four main groups base on the origin of oocytes and semen; the FO/FS group (n = 19); th FO/CrS group (n = 14); the CrO/FS group (n = 85); and the CrO/CrS group (n = 34).	Cryopreserved semen: glycerol 12%, egg yolk 20% Vitrification and warming oosit by Kitazato® (Tokyo, Japan	The CrO/CrS group had significantly lower normal day 3 cleavage rates (55.5%), blastocyst development (24%), and implantation rates (14.5%) than the FO/FS group.	In an egg-sharing donation programme, frozen sperm was inserted into vitrified oocytes, embryo developmental competence and implantation potential were diminished.	(Setti et al., 2021)

2019	Chicken sperms 25week old	Sucrose and raffinose (1 mmol, 5 mmol, 10 mmol)	1 mmol sucrose concentration is efficient in increasing sperm plasma membrane integrity/viability, acrosome integrity, and mitochondrial potential	Sucrose is more efficient than raffinose in chicken sperm	(Thananurak et al., 2019)
2010	The sperm of C57BL/6J mice. Female and male donors were 8-to-10 weeks old and a 12-to-15 weeks old	R18S3	A combination of R18S3 with L-glutamine & MBCD in preincubation media was able to increase fertilization (69.2%). then, the two-cell embryonic development potential of frozen stored sperm was normal (thawed 51.5%)	R18S3 modified with L-glutamine and MBCD is suitable for sperm cryopreservation of frozen strain C57BL/6J mice with a high fertilization rate.	(Takeo & Nakagata, 2010)
1997	Mice (B6D2F1 males; B6C3F1 females) (2 to 3 months old)	Raffinose and glycerol were added EY at 37°C	Development of embryos produced in vitro with spermatozoa frozen to -196°C significantly differed between frozen and fresh. Cleavage rate (68.8%) and into blastocysts (61.5%) with frozen sperm	In frozen mice sperm, the combining of raffinose and glycerol can increase the success of in vitro fertilization and the potential for embryonic development.	(Songsasen et al., 1997)

Abbreviations: ICSI = intracytoplasmic sperm injection; CrO=cryopreserved oosit; CrS=cryopreserved semen; FO=Fresh oosit; FS=Fresh semen; R18S3=18% raffinose pentahydrate and 3% skim milk; methyl-b-cyclodextrin MBCD EY=egg yolk

Table 2 shows, that currently various methods have been developed to improve the quality of embryos from frozen stored spermatozoa. In this case the authors will develop cryoprotectant modifications to suppress cell injury and improve embryo quality.

3.3. Cryoprotectant Modification

Cryopreservation occurs through a decrease in temperature to a level below the normal temperature at which all biochemical reactions take place. This proved successful because all the normal functions of the cells were preserved. The cryopreservation process exposes cells to stress caused by osmotic imbalance as well. This creates an osmotic pressure, causing the solvent to flow across the plasma membrane, from the inside to the outside of the cell. The gradient results in the extracellular need for water leading to a reduction in cell volume and further dehydration, an essential process in protecting cells from intracellular ice formation, which can cause cell deaths.

Cell injury in cryopreservation is often associated with intracellular ice formation, and slow cooling causes osmotic changes due to the effects of exposure to highly concentrated intra- and extracellular solutions or mechanical interactions between cells and extracellular ice. The procedures involved in the freezing/thawing of spermatozoa cause cell damage to temperature oscillations, oxidative injury, ice crystal formation, plasma membrane damage, DNA damage, cryoprotectant toxicity, and osmotic stress. Freezing causes changes in sperm structure, sperm function, and sperm lipids (Pini et al., 2018). Therefore, to minimize the

cellular damage that arises, in large part, from the effects of these solutes, it is important to use an added or combined cryoprotectant and antioxidant.

The modified cryoprotectant is a substance that must be present in the cryopreservation medium to minimize damage physical and chemical stress on spermatozoa cells resulting from the cooling, freezing and thawing processes. The modified cryoprotectant used is a combination of glycerol and raffinose. Permeable cryoprotectants can pass through the plasma membrane to inhibit the formation of ice crystals and reduce membrane/protein damage while reducing cytotoxic injuries. DMSO and glycerol are the most common CPAs used to freeze sperm cells but have toxic effects making them unsuitable for many clinical applications. Therefore, new non-toxic CPAs should be developed. Glycerol is a typical cryoprotectant that is frequently used to store sperm cells. Glycerol helps protect cells by penetrating cell membranes due to its small size and starting to affect water molecules (Best, 2015; Pegg, 2007). Combining glycerol with non-permeating cryoprotectants (egg yolk, raffinose, fructose, sucrose, or trehalose) appears to be the optimal method for reducing the concentration of glycerol and its negative effects in rams sperm (Rostami et al., 2020), buffalo (Iqbal et al., 2018) and bull (Hu et al., 2010).

Survival of animals under significant environmental stress often requires two primary strategies: (a) preservation of cell macromolecules via stabilizing/protective preservation mechanism and (b) inducing the hypometabolic state to reduce energy expenditure and prioritize essential vital functions (Storey & Storey, 2013). These preservation strategies are crucial in extending the lifespan of cellular components. Two protein groups involved and crucial in cellular stress response are chaperones and antioxidants (Kultz, 2005). Chaperones are constitutively present in cells and tightly regulated under stressful environmental conditions while others are induced by stress such as Heat Shock Protein (HSP). The HSP family were first defined by their molecular mass: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsps (sHsps) (sizes < 30kDa) (Bai et al., 2019; Schmitt et al., 2007; Zhang et al., 2015). Antioxidants are capable of overcoming the ROS problem. In addition, antioxidants used in semen cryopreservation are vitamins (Vitamin C and E), nanocompounds (fullerenol), amino acids (L-arginine and melatonin), minerals (selenite, selenocystine, and selenomethionine), natural (quercetin and resveratrol) and synthetic compounds (Trolox, butylated hydroxytoluene) and miscellaneous (pyruvate, butylated hydroxyanisole, n-propyl gallate (n-PG), deferoxamine mesylate dimethyl sulfoxide and glycerol), and enzyme or enzyme-based formulas (superoxide dismutase, glutathione, catalase, cytochrome c, and glutathione peroxidase) (Moradi et al., 2020). Higher concentration of ROS were detected in human, ruminant and canine spermatozoa during cooling to 5°C (Santiani et al., 2014) and following cryopreservation (Kim et al., 2010) when compared to fresh spermatozoa. Table 3 shows various attempts to overcome cryopreservation-induced cell injury due to cryopreservation. It is not yet known to determine the best course of action to treat cell injury. However, efforts such as protecting spermatozoa damage with the addition of antioxidants to minimize cell injury.

Abbreviation: ROS = reactive oxygen species, TAC = total antioxidant capacity; MMP = mitochondrial membrane potential; MDA = malondialdehyde; CPLL = carboxylated poly L-lysine; Me_2SO = dimethyl sulfoxide; GFE = good freezability ejaculates; PFE = poor freezability ejaculates; PMI = plasma membrane integrity; SVPMI = supravital plasma membrane integrity; HR = hypo-resistivity; ACR-I = acrosome integrity; LPO = lipid peroxidation; iPAM = the integrity of the plasma and acrosomal membranes; TBARS = thio-barbituric acid reactive substances; 8OHdG = e 8-oxo-7,8-dihydro-20 deoxyguanosine.

Table 3 Summary of efforts to overcome cell injury due to cryopreservation

Publication Year	Sample	Result	Conclusion	References
2022	Normozoospermic samples (n = 25)	The diluent with exosomes or microvesicles had improved sperm motility, morphology and viability compared with untreated samples. ROS levels decreased significantly with a decrease due to DNA damage. TAC activity and MMP levels also increased significantly; MDA levels and apoptotic rates remained unchanged.	Seminal plasma microvesicles and exosomes could protect spermatozoa from cryopreservation chilling injuries	(Mahdavinezhad et al., 2022)
2021	Normozoospermic semen (n=12)	The addition of 5% PRP significantly improves sperm progressive motility, viability and membrane integrity after cryopreservation	Autologous PRP has a partial protective effect on human spermatozoa cryopreservation	(Yan et al., 2021)
2021	Rabbit sperm cells	The concentration of Me2SO (5 or 8%) in the sample after thawing, significantly effected on the rate of total motility and progressive motility as well as the level of live sperm and intact acrosomes.	Supplementation of CPLL to the extender in the presence of Me2SO improved the parameters of sperm quality and DNA integrity after thawing	(Küçük et al., 2021)
2020	Normozoospermic semen (n=21)	After the sperm is stored frozen, there is rupture of the head of the plasma membrane, damage to the acrosome, decompression of the nucleus and chromatin, damaged mitochondria and disruption of the axonema, with an irregular structure.	Further research is needed to improve sperm lyophilization results. In the future, using spermatozoa lyophilization can reduce the cost of fertility preservation, because it does not need storage space and transportation is simpler.	(Bossi et al., 2021)
2019	Buffalo bull (*Bubalus bubalis*) sperm	Group (G5C0.75) containing 0.75% CPLL and 5% glycerol demonstrated an increase in total motility and progressive motility after sperm thawing, with higher plasma membrane integrity, acrosome integrity, and MMP when compared to the control group.	The addition of 0.75% CPLL in combination with 5% glycerol in extender freezing improved structure, function and fertility in vivo post-thawing (56%)	(Tariq et al., 2020)
2019	Buffalo bull (*Bubalus bubalis*) sperm (n = 32)	At post-thawing, D4 showed higher sperm progressive motility, PMI, SVPMI, HR, ACR-I, and DNA-I than the control. Sperm MMP and in-vivo fertility rate was higher in treated groups than the control	The addition of L-tryptophan improved semen quality, in vitro and in vivo fertility of buffalo spermatozoa after frozen storage.	(Ahmed et al., 2020)
2016	Ram Spermatozoa (n = 6)	There was an increase in sperm motility in the control group and the addition of canthaxanthin but did not affect the production of intracellular ROS in spermatozoa, iPAM or LPO.	The addition of 10 and 25 mM canthaxanthin which was incubated at 37°C for 2 hours after thawing, protected ovine sperm from kinetic changes.	(Souza et al., 2017)

2016	Canine sperm (n = 6)	The GSH-20 addition group resulted in lower acrosomal damage and better sperm quality with lower mitochondrial activity and higher TBARS concentrations.	The addition of 20 mM GSH increased mitochondrial activity whereas 10 mM GSH was better in the fertility aspect by showing acrosomal protection	(Lucio et al. 2016)
2015	Human sperm (n = 43)	Cryopreservation significantly reduces viability and motility, but with increased intracellular ROS and MDA of human sperm	Melatonin protects sperm by counteracting intracellular ROS and reducing the effects of MDA	(Karimfar et al., 2015)
2015	Stallion sperm (n = 24)	After frozen storage, the sperm viability of the GFE group was significantly higher than PFE group. However, there was no difference between GFE and PFE groups from the aspect of DNA fragmentation and disulfide bonding in the sperm head protein.	There was a difference in the level of reactive oxygen species between ejaculated sperm with good and bad freeze ability after cryopreservation but does not interfere to the sperm nucleus	(Yeste et al., 2015)
2014	Sea bream sperm	The ability of AFPIII to protect sea bream sperm quality is associated with a decrease in sperm protein profile during cryopreservation	The addition of AFPIII to DMSO extender improved the protection against freezing	(Zilli et al., 2014)
2013	Cat sperm (n = 18)	PMI and sperm quality decreased significantly due to the influence of cryopreservation	Ejaculated cat sperm quality is better due to the influence of 5% glycerol	(Villaverde et al., 2013)
2009	Human sperm (n = 60)	Spermatozoa DNA was not fragmented due to the supplementation of 50 and 100 mM genistein to the cryoprotectant	Genistein can reduce oxidative stress during cryopreservation	(Thomson et al., 2009)

4. Conclusions

Although no formal conclusions can be drawn regarding the cryopreservation of spermatozoa to obtain good embryo quality, our results suggest that modified cryoprotectants can be an alternative cryoprotectant compared to commercial cryoprotectants. In addition, the use of antioxidants in spermatozoa cryopreservation can also prevent cell damage due to the adverse effects of cryoprotectants. However, further researches still need to be performed to investigate the cellular mechanisms.

Acknowledgments

The authors would like to extend their appreciation to the "Center for Education Financial Services" and "Indonesian Endowment Funds for Education" for supporting the writer on this study.

References

Agarwal, A., Tvrda, E., 2017. Slow Freezing of Human Sperm. In *Cryopreservation of Mammalian Gametes and Embryos: Methods and Protocols, Methods in Molecular Biology*. New York: Springer, pp. 67–78

Ahmed, H., ahan, S., Khan, A., Khan, L., Ullah, H., Riaz, M., Ullah, K. and Ullah, F., 2020. Supplementation of L-tryptophan (An Aromatic Amino Acid) In Tris Citric Acid Extender Enhances Post-Thaw Progressive Motility, Plasmalemma, Mitochondrial Membrane Potential, Acrosome, and DNA Integrities, and In Vivo Fertility Rate of Buffalo (Bubalus Bubalis). *Cryobiology*, Volume 92, pp. 117–123

Bai, C., Kang, N., Zhao, J., Dai, J., Gao, H., Chen, Y., Dong, H., Huang, C. and Dong, Q., 2019. Cryopreservation Disrupts Lipid Rafts and Heat Shock Proteins in Yellow Catfish Sperm. *Cryobiology*, Volume 87, pp. 32–39

Best, B.P., 2015. Cryoprotectant Toxicity: Facts, Issues, and Questions. *Rejuvenation Research*, Volume 18(5), pp. 422–436

Bojic, S., Murray, A., Bentley, B.L., Spindler, R., Pawlik, P., Cordeiro, J.L., Bauer, R. and De Magalhães, J.P., 2021. Winter is Coming: The Future of Cryopreservation. *BMC Biology*, Volume 19(1), pp. 1–20

Bossi, R.D.L., Cabral, M., Oliveira, M., Lopes, S., Hurtado, R., Sampaio, M., Geber, S., 2021. Ultrastructural Analysis of Lyophilized Human Spermatozoa. *Jornal Brasileiro de Reproducao Assistida*, Volume 25(3), pp. 473–479

Critser, J.K., L.E. Mobraaten. 2000. Cryopreservation of Murine Spermatozoa. *ILAR Journal* Volume 41(4), pp. 197–206

Fahy, G.M., Wowk, B., 2015. Principles of Cryopreservation by Vitrification. In *Cryopreservation and Freeze-Drying Protocols, Methods in Molecular Biology*. London, United Kingdom: Springer, pp. 21–82

Gandini, L., Pallotti, F., Paoli, D., Lenzi, A., 2017. Cryopreservation of Spermatozoa. In *Endocrinology of the Testis and Male Reproduction*. Italy: Springer International Publishing, pp. 1–1357

Guthrie, H.D., G.R. Welch. 2005. Impact of Storage Prior to Cryopreservation on Plasma Membrane Function and Fertility of Boar Sperm. *Theriogenology*, Volume 63(2 SPEC. ISS.), pp. 396–410

Hamidi, N. 2010. Studi Inhibisi Formasi Kristal Es Dengan Krioprotektan Sukrosa dan Gliserol (Study of Ice Crystal Formation Inhibition with Sucrose and Glycerol Cryoprotectants). *Jurnal Rekayasa Mesin*, Volume 1(1), pp. 21–26

Hanifah, N.F., 2020. Pengaruh Konsentrasi Gliserol dalam Pengencer Tris Terhadap Kualitas Spermatozoa Kambing Sapera Before Freezing (Effect of Glycerol Concentration in Tris Diluent on Spermatozoa Quality of Sapera Goat Before Freezing (Effect of Glycerol Concentration in Tris Diluent on Spermatozoa Quality of Sapera Goat Before Freezing). *Jurnal Medik Veteriner*, Volume 3(2), p. 154

Höfner, L., Luther, A.M., Waberski, D., 2020. The Role of Seminal Plasma in The Liquid Storage of Spermatozoa. *Animal Reproduction Science*, Volume 220, p. 106290

Hu, J.H., Zan, L.S., Zhao, X.L., Li, Q.W., Jiang, Z.L., Li, Y.K., Li, X., 2010. Effects of Trehalose Supplementation on Semen Quality and Oxidative Stress Variables in Frozen-Thawed Bovine Semen. *Journal of animal Science,* Volume 88(5), pp. 1657–1662

Iqbal, S., Naz, S., Ahmed, H., Andrabi, S.M.H., 2018. Cryoprotectant Effect of Trehalose in Extender on Post-Thaw Quality and in Vivo Fertility of Water Buffalo (Bubalus Bubalis) Bull Spermatozoa. *Andrologia*, Volume 50, pp. 1–6

Jang, T.H., Park, S.C., Yang, J.H., Kim, J.Y., Seok, J.H., Park, U.S., Choi, C.W., Lee, S.R., Han, J., 2017. Cryopreservation and Its Clinical Applications. *Integrative Medicine Research*, Volume 6(1), pp. 12–18

Morris, G.J., Acton, E., Murray, B.J. and Fonseca, F., 2012. Freezing Injury: The Special Case of The Sperm Cell. *Cryobiology*, Volume 64(2), pp. 71–80

Karimfar, M.H., Niazvand, F., Haghani, K., Ghafourian, S., Shirazi, R., Bakhtiyari, S., 2015. The Protective Effects of Melatonin Against Cryopreservation-Induced Oxidative Stress in Human Sperm. *International Journal of Immunopathology and Pharmacology*, Volume 28(1), pp. 69–76

Kim, S.H., Yu, D.H., Kim, Y.J., 2010. Effects of Cryopreservation on Phosphatidylserine Translocation, Intracellular Hydrogen Peroxide, and DNA Integrity in Canine Sperm. *Theriogenology*, Volume 73(3), pp. 282–292

Kitporntheranunt, M., Om-Songkram, S., Srichawana, K., 2017. A Comparison of Glycerol Egg Yolk Citrate and Sperm Freeze Medium in Human Sperm Cryopreservation. *Journal of the Medical Association of Thailand*, Volume 100(10), pp. S142–S147

Küçük, N., Raza, S., Matsumura, K., Uçan, U., Serin, İ., Ceylan, A., Aksoy, M., 2021. Effect of Different Carboxylated Poly L-Lysine and Dimethyl Sulfoxide Combinations on Post Thaw Rabbit Sperm Functionality and Fertility. *Cryobiology*, Volume 102, pp. 127–132

Kultz, D., 2005. Molecular and Evolutionary Basis of The Cellular Stress Response. *Annual Review of Physiology*, Volume 67, pp. 225–257

Lestari, S.W., 2019. Journal of Global Pharma Technology Sperm DNA Fragmentation and Chromatin Maturation of Prepared Sperm with ALA Supplementation: The Effect of Embryo Development Quality.

Li, Y.X., Zhou, L., Lv, M.Q., Ge, P., Liu, Y.C., Zhou, D.X., 2019. Vitrification and Conventional Freezing Methods in Sperm Cryopreservation: A Systematic Review and Meta-Analysis. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, Volume 233, pp. 84–92

Lucio, C.D.F., Silva, L.C.G., Regazzi, F.M., Angrimani, D.D.S.R., Nichi, M., Assumpção, M.E.O., Vannucchi, C.I., 2016. Effect of Reduced Glutathione (GSH) in Canine Sperm Cryopreservation: In Vitro and in Vivo Evaluation. *Cryobiology*, Volume 72(2), pp. 135–140

Mahdavinezhad, F., Gilani, M.A.S., Gharaei, R., Ashrafnezhad, Z., Valipour, J., Nashtaei, M.S., Amidi, F., 2022. Protective Roles of Seminal Plasma Exosomes and Microvesicles During Human Sperm Cryopreservation. *Reproductive BioMedicine Online*, Volume 45(2), pp. 341–353

Miyagi-Shiohira C, Kurima K, Kobayashi N, Saitoh, I., Watanabe, M., Noguchi, Y., Matsushita, M., Noguchi, H., 2015. Cryopreservation of Adipose-Derived Mesenchymal Stem Cells. *Cell Medicine*, Volume 8(1-2), pp. 3–7

Moradi, M., Karimi, I., Ahmadi, S., Mohammed, L.J., 2020. The Necessity of Antioxidant Inclusion in Caprine and Ovine Semen Extenders: A Systematic Review Complemented with Computational Insight. *Reproduction in Domestic Animals*, Volume 55(9), pp. 1027–1043

Ni'mah, H., Rochmadi, R., Woo, E.M., Widiasih, D.A., Mayangsari, S., 2019. Preparation and Characterization of Poly (L-Lactic Acid) Films Plasticized with Glycerol and Maleic Anhydride. *International Journal of Technology*, Volume 10(3), pp. 531–540

Pegg, D.E., 2007. Principles of Cryopreservation. In *Cryopreservation and Freeze-Drying Protocols. Methods in Molecular Biology.* Totowa (NJ): Humana Press, pp. 39–75

Pegg, D.E., 2015. Principles of Cryopreservation. In *Cryopreservation and freeze-drying protocols.* London, United Kingdom: Springer, pp. 3–19

Pelufo, V., Armengol, M.L., Malcotti, V., Venturino, A. and Aisen, E.G.,2015. Effects of Glycerol and Sugar Mixing Temperature on The Morphologic and Functional Integrity of Cryopreserved Ram Sperm. *Theriogenology*, Volume 83(1), pp. 144–151

Peris-Frau, P., Soler, A.J., Iniesta-Cuerda, M., Martín-Maestro, A., Sánchez-Ajofrín, I., Medina-Chávez, D.A., Fernández-Santos, M.R., García-Álvarez, O., Maroto-Morales, A., Montoro,

V., Garde, J.J., 2020. Sperm Cryodamage in Ruminants: Understanding the Molecular Changes Induced by The Cryopreservation Process to Optimize Sperm Quality', *International Journal of Molecular Sciences*, Volume 21(8), p. 2781

Pini, T., Leahy, T., de Graaf, S.P., 2018. Sublethal sperm freezing damage: Manifestations and solutions. *Theriogenology*, Volume 118, pp. 172–181

Rostami, B., Ebrahimi, D., Sadeghipanah, H., Masoumi, R., Shahir, M., 2020. Effects of Supplementation of Tris-Egg Yolk Extender with Different Sugars and Antioxidants on Freezability of Ram Semen. *Cryobiology*, Volume 92, pp. 62–66

Santiani, A., Evangelista, S., Sepúlveda, N., Risopatrón, J., Villegas, J.S.R., 2014. Addition of Superoxide Dismutase Mimics During Cooling Process Prevents Oxidative Stress and Improves Semen Quality Parameters In Frozen/Thawed Ram Spermatozoa. *Theriogenology*, Volume 82, pp. 884–889

Schmitt, E., Gehrmann, M., Brunet, M., Multhoff, G., Garrido, C., 2007. Intracellular and Extracellular Functions of Heat Shock Proteins: Repercussions in Cancer Therapy. *Journal of Leukocyte Biology*, Volume 81(1), pp. 15–27

Setti, A.S., Braga, D.P.D.A.F., Iaconelli, A., Borges, E., 2021. Cryopreservation of Both Male and Female Gametes Leads to Reduced Embryo Development and Implantation Potential. *Zygote*, Volume 29(5), pp. 377–382

Sieme, H., Oldenhof, H., Wolkers, W.F., 2015. Sperm Membrane Behaviour during Cooling and Cryopreservation. *Reproduction in Domestic Animals*, Volume 50, pp. 20–26

Sieme, H., Oldenhof, H., Wolkers, W.F., 2016. Mode of Action of Cryoprotectants for Sperm Preservation. *Animal Reproduction Science*, Volume 169, pp. 2–5

Singer ST, Sweeters N., Vega, O., Higa A., Vichinsky, E.C.M., 2010. Fertility Potential in Thalassemia Major Women: Current Findings and Future Diagnostic Tools. *Annals of the New York Academy of Sciences*, Volume 1202(1), pp. 226–230

Songsasen, S.P., Leibo, N., 1997. Cryopreservation of Mouse Spermatozoa I. Effect of Seeding on Fertilizing Ability of Cryopreserved Spermatozoa. *Cryobiology*, Volume 35, pp. 240–254

Souza, H.M., Arruda, L.C., Monteiro, M.M., Nery, I.H., Araújo Silva, R.A., Batista, A.M., Guerra, M.M.P., 2017. The Effect of Canthaxanthin on the Quality of Frozen Ram Spermatozoa. *Biopreservation and Biobanking*, Volume 15(3), pp. 220–227

Storey, Kenneth B., and Janet M. Storey. 2013. Molecular Biology of Freezing Tolerance. *Comprehensive Physiology*, Volume 3(3), pp. 1283–1308

Storey, K.B., Storey, J.M., 2013. Molecular biology of freezing tolerance. *Comprehensive Physiology*, Volume 3(3), pp. 1283–1308

Sulistyo, H., Priadana, D.P., Fitriandini, Y.W., Ariyanto, T., Azis, M.M., 2020. Utilization of Glycerol by Ketalization Reactions with Acetone to Produce Solketal Using Indion 225 Na as Catalyst. *International Journal of Technology*, Volume 11(1), pp. 190–199

Sutijan, Wahyudi, S., Ismail, M.F., Mustika, P.C.B., Astuti, W., Prasetya, A., Petrus, H.T.B.M., 2022. Forward Osmosis to Concentrate Lithium from Brine: The Effect of Operating Conditions (pH and Temperature). *International Journal of Technology*, Volume 13(1), pp. 136–146

Sztein, J.M., 2001. Comparison of Permeating and Nonpermeating Cryoprotectants for Mouse Sperm Cryopreservation. *Cryobiology*, Volume 42(1), pp. 28–39

Sztein, J.M., Takeo, T., Nakagata, N., 2018. History of Cryobiology, with Special Emphasis in Evolution of Mouse Sperm Cryopreservation. *Cryobiology*, Volume 82, pp. 57–63

Takeo, T., Nakagata, N., 2010. Combination Medium of Cryoprotective Agents Containing L-Glutamine and Methyl-B-Cyclodextrin in A Preincubation Medium Yields A High

Fertilization Rate for Cryopreserved C57BL/6J Mouse Sperm. *Laboratory Animals*, Volume 44(2), pp. 132–137

Tariq, A., Ahmad, M., Iqbal, S., Riaz, M.I., Tahir, M.Z., Ghafoor, A., Riaz, A., 2020. Effect of Carboxylated Poly L-Lysine as A Cryoprotectant on Post-Thaw Quality and in Vivo Fertility of Nili Ravi Buffalo (Bubalus Bubalis) Bull Semen. *Theriogenology*, Volume 144, pp. 8–15

Thananurak, P., Chuaychu-Noo, N., Thélie, A., Phasuk, Y., Vongpralub, T., Blesbois, E., 2019. Sucrose Increases The Quality and Fertilizing Ability of Cryopreserved Chicken Sperms in Contrast to Raffinose. *Poultry Science*, Volume 98(9), pp. 4161–4171

Thomson, L.K., Fleming, S.D., Aitken, R.J., De Iuliis, G.N., Zieschang, J.A., Clark, A.M., 2009. Cryopreservation-Induced Human Sperm DNA Damage is Predominantly Mediated by Oxidative Stress Rather Than Apoptosis. *Human Reproduction*, Volume 24(9), pp. 2061–2070

Varisli, O., Uguz, C., Agca, C., Agca, Y., 2009. Effect of Chilling on The Motility and Acrosomal Integrity of Rat Sperm in The Presence of Various Extenders. *Journal of the American Association for Laboratory Animal Science*, Volume 48(5), pp. 499–505

Villaverde, A.I.S.B., Fioratti, E.G., Penitenti, M., Ikoma, M.R., Tsunemi, M.H., Papa, F.O., Lopes, M.D., 2013. Cryoprotective Effect of Different Glycerol Concentrations on Domestic Cat Spermatozoa. *Theriogenology*, 80(7), pp. 730–737

Wessel, M.T., Ball, B.A., 2004. Step-Wise Dilution for Removal of Glycerol from Fresh and Cryopreserved Equine Spermatozoa. *Animal Reproduction Science*, Volume 84(1–2), pp. 147–156

Whaley, D., Damyar, K., Witek, R.P., Mendoza, A., Alexander, M., Lakey, J.R., 2021. Cryopreservation: An Overview of Principles and Cell-Specific Considerations. *Cell Transplantation*, Volume 30, pp. 1–12

Yan, B., Zhang, Y., Tian, S., Hu, R., Wu, B., 2021, Effect of Autologous Platelet-Rich Plasma on Human Sperm Quality during Cryopreservation. *Cryobiology*, Volume 98, pp. 12–16

Yang, H., Ramstein, J., Smith, J., 2019. Non-Oncologic Indications for Male Fertility Preservation. *Current Urol Reports*, 20(9), pp. 1–6

Yeste, M., Estrada, E., Rocha, L.G., Marín, H., Rodríguez-Gil, J.E., Miró, J., 2015. Cryotolerance of Stallion Spermatozoa is Related to ROS Production and Mitochondrial Membrane Potential Rather Than to The Integrity of Sperm Nucleus. *Andrology*, Volume 3(2), pp. 395–407

Zhang, X.G., Hu, S., Han, C., Zhu, Q.C., Yan, G.J., Hu, J.H., 2015. Association of Heat Shock Protein 90 with Motility of Post-Thawed Sperm in Bulls. *Cryobiology*, Volume 70(2), pp. 164–169

Zilli, L., Beirão, J., Schiavone, R., Herraez, M.P., Gnoni, A., Vilella, S., 2014. Comparative Proteome Analysis of Cryopreserved Flagella and Head Plasma Membrane Proteins from Sea Bream Spermatozoa: Effect of Antifreeze Proteins. *PLoS ONE*, Volume 9(6), pp. 1–10