Senary (six) combinations of cryoprotectants for embryo, gamate, cell and tissue cryopreservation by vitrification

Oshini Basri, Nuguelis Razali, Mukhri Hamdan

[1]Department of Obstetrics and Gynaecology, Faculty of Medicine Universiti Malaya, 59100 Kuala Lumpur, Malaysia

Abstract

Introduction
Vitrification solutions (VSs) can be chemotoxic depending on its solute concentration. Commercial VSs appear to be ternary or quaternary. Using higher combinations of cryoprotectants with lower solute concentration in VS is a strategy to reduce toxicity. The present investigation aims to develop senary combinations of VSs for application in assisted reproduction technology treatment.
Materials and methods
A total 6 cryoprotectants were selected including water (solute-less). All VSs formulated contained 4 permeating cryoprotectants, namely, ethylene glycol, glycerol, DMSO, propylene glycol, and a non-permeating cryoprotectant, sucrose, in HEPES buffered Gamete Medium (Cellcura, Norway) containing 5% FCS. A total of 21 series of senary solutions were systematically formulated ranging from 2.5M to 7.0M total solute concentration consisting of all six cryoprotectants and were tested for vitrification property using a 0.5ml straw. The cryoprotectant mixture that vitrifies during cooling and remains vitrified during warming, and does not fracture either during cooling or warming shall be considered as meeting the requirements of a vitrification solution. Subsequent investigations aimed to investigate its vitrification property in smaller vehicles such as micro-vehicles and 0.25ml straws employing 1, 5, and 10ul in former and 0.25ml in latter vehicles respectively.
Results
A VS that contained 1.5M EG, 1.0M Gly, 1.0M DMSO, 1.0M propylene glycol, 1.0M sucrose and water (Senary VS7) was chosen because it met all the requirements of a vitrification solution. It vitrified on cooling at easily repeatable rate, and it remained vitreous on ultrarapid warming when using 0.5 ml plastic insemination straws. Senary VS7 also vitrified in 0.25 ml plastic insemination straws and several micro-vehicles in volumes of 1µl, 5 µl and 10 µl. Application for patent lodgment is being prepared.
Discussion and Conclusion
A senary VS with potentially useful application in ART has been formulated. Further studies are needed to determine its usefulness.

Disclaimer: The authors declare commercial interest in the findings of this investigation.
J Reprod Biotechnol Fertil 13:30-34
Correspondence: Oshini Basri; Email: oshinibasri91@gmail.com
Compliance acknowledgement: This article was edited by the Australian Editorial Services (www.nativeenglisheditor.com)
Keywords: Cryopreservation cryoprotectant, embryo, senary, vitrification solution

Introduction

Vitrification in human assisted reproduction technology is synonymous with ice-free cryopreservation. This is because in vitrification ice-crystals are not formed during cooling and/or warming. It is well documented ice formation during cryopreservation does not augur well for the survival of the cryopreserved cells, embryos, gametes or tissues. The earliest scientifically formulated attempts to cryopreserve by vitrification was by Father B.J. Luyet and co-workers beginning in the 1930's for organs as well as cells (Schmidt, 2006). Vitrification required a very high concentration of cryoprotectants often in excess of 6M or 40% solute concentration. Such high concentrations of chemicals invariably were chemotoxic.

Vitrification was ultimately abandoned by the mid-1960's due to chemotoxicity resulting in failure to cryopreserve organs and cells (see review by Ali and Shelton, 2006).

Rall and Fahy (Rall and Fahy, 1985) and Rall and coworkers (Rall et al., 1987) reported the first successful cryopreservation by vitrification of mouse embryos with moderate survival and live births due to chemotoxicity of the cryoprotectants employed. Nevertheless this report rekindled considerable interest and led to the revival of the previously abandoned technique of vitrification as a strategy for ultra-rapid cryopreservation. Subsequently Kono and co-workers (Kono et al., 1988) successfully vitrified rat embryos using the vitrification solution (VS) formulated by Rall and co-workers (Rall and Fahy, 1985; Rall et al., 1987). The following year Kasai et al (Kasai et al, 1990) reported the vitrification of mouse day 4 morulae with almost complete survival. They used an ethylene glycol-based VS.

At about this time (1989-1992) Ali and Shelton had completed a systematic and extensive work on over 3044 ternary cryoprotectant solutions in an effort to identify the least toxic and most efficacious VS. Their study identified 66 ternary cryoprotectants that could vitrify when cooled and subsequently warmed ultra-rapidly (Ali and Shelton, 1993a). Of these, one VS consisting of 5.5M ethylene glycol and 1.0M sucrose and water (called VS14) stood out as the least embryotoxic. With VS14, for the first time, all developmental stages of the mouse embryo could be vitrified with no loss of viability in vitro, day 6 sheep embryo (Ali and Shelton, 1993a-c). They also obtained live births from mouse day 4 compacted morulae and blastocysts, and day 6 sheep embryos that were vitrified using VS14 with no loss of viability in vivo. Two years later Ali vitrified the day 2 human embryo successfully for the first time, using VS14 with no loss of viability in vitro (Ali et al., 1995; Ali, 1996). All these successes in cryopreservation were achieved using ternary VSs. The composition of currently used VSs in the assisted reproduction technology appear to be ternary or quartenary vitrification solutions. Combinations of cryoprotectants need less solutes to vitrify than individual cryoprotectants. Therefore if combinations of cryoprotectants are used in vitrification solutions (VSs) the solute concentration required to vitrify is lower, and thus likely to be less toxic (Fahy, 1987). The use of combinations of cryoprotectants in present day VSs is a strategy to further reduce chemotoxicity. The VSs used in present times appear to be ternary or quaternary solutions. Using higher combinations of cryoprotectants beyond quaternary combinations in VS formulations may further reduce solute toxicity. The present investigation aims to develop VSs that contain up to six combinations of different cryoprotectants that can be safely applied for the cryopreservation of spermatozoa, oocytes and embryos with no or minimal damage. The funding agency may patent the IP products of this research to safeguard IP proprietorship.

Materials and methods

A total 6 cryoprotectants were selected including water (solute-less). All VSs formulated contained 4 permeating cryoprotectants, namely, ethylene glycol, glycerol, DMSO, propylene glycol, and a non-permeating cryoprotectant, sucrose, in HEPES buffered Gamete Medium (Cellcura ASA, Norway) containing 5% FCS (fetal calf serum). Twenty one different combinations of cryoprotectant mixtures containing six different cryoprotectants were prepared and tested for vitrification property initially in a 0.5ml straw followed by a 0.25ml straw and micro-vehicles utilizing VS volumes of 1, 5 and 10ul.

Results

Senary cryoprotectant solutions exhibit vitrification tendencies (or remained transparent) during ultra rapid cooling achieved by plunging it into liquid nitrogen beginning from the 4.0M concentration onwards however these senary cryoprotectant solutions devitrified (became milky (M) in appearance) when warmed ultra rapidly in water bath held at 25^{o}C or rapidly when warmed in air for 10 seconds. When the total solute concentration of the senary cryoprotectant was increased to 5.5M it vitrified during ultra rapid cooling and remained vitrified when warmed ultra rapidly in a water bath held at 25^{o}C but not when warmed rapidly in air. A further increase in solute concentration by 0.5M led to the fracture of the vitrified state during warming.

Table 1: The glass forming tendencies during ultra-rapid cooling and two warming protocols of twenty one combinations cryoprotectant solutions containing various molar concentrations of ethylene glycol, glycerol, dimethyl sulfoxide, propylene glycol, and sucrose.

S. No. No	Cryoprotectants (Molar Concentrations)						Cooling	Vitrification status during warming	
	EG	GLY	DMSO	PG	SUC-ROSE	Total	Vitrifi-cation Status	(25ºC)	(air-10 sec)
1	0.5	0.5	0.5	0.5	0.5	2.5	M	M	M
2	1	0.5	0.5	0.5	0.5	3	M	M	M
3	1	1	0.5	0.5	0.5	3.5	M	M	M
4	1	1	1	0.5	0.5	4	T	M	M
5	1	1	1	1	0.5	4.5	T	M	M
6	1	1	1	1	1	5	T	M	M
7	1.5	1	1	1	1	5.5	T	T	M
8	1.5	1.5	1	1	1	6	T	K	M
9	1.5	1.5	1.5	1	1	6.5	T	K	M
10	1.5	1.5	1.5	1.5	1	7	T	K	M
11	1.5	1	1	1	1	5.5	T	K	M
12	2	1	1	1	1	6	T	K	M
13	2.5	1	1	1	1	6.5	T	K	M
14	3	1	1	1	1	7	T	K	M
15	3.5	1	1	1	1	7.5	T	K	M
16	2.5	1	1	1	0.5	6	T	K	M
17	3	1	1	0.5	0.5	6	T	K	M
18	3.5	1	0.5	0.5	0.5	6	T	K	M
19	3	1	0.5	0.5	1	6	T	K	M
20	3	0.5	0.5	0.5	1	5.5	T	K	M
21	3.5	0.5	0.5	0.5	1	6	T	K	M

C = Cooling, W = Warming, M = Milky or crystallization, I = Intermediate, T = transparent or vitrified

ultra rapidly in water bath held at 25^{o}C. All further increases in the solute concentration of VSs had similar effects. Irrespective of the concentration of the senary cryoprotectant, the vitrified state devitrified (turned milky) during rapid air warming. The senary cryoprotectant solution consisting of water + 1.5M EG + 1.0M Gly + 1.0M DMSO + 1.0M PG + 1.0M Sucrose (Senary VS7) appears the most promising VS. Sucrose is used in almost all vitrification media in the present times. Sucrose being non-permeable dehydrates the embryo and keeps it dehydrated which is important to achieve intracellular vitrification. It was originally used to achieve partial dehydration initially by Mazur, Miller and Leibo (Mazur et al., 1974) in 1974 for bovine blood cells and applied in embryos by Kasai, Niwa and Iritani (Kasai et al., 1980) and in almost all subsequent work on cryopreservation of embryos and gametes.

Senary VS7 vitrified on cooling at an easily achievable and repeatable rate, and it remained vitreous on warming when using 0.5 ml plastic insemination straws. Senary VS7 also vitrified in 0.25 ml plastic insemination straws and in several microvehicles when 3 different volumes of the Senary VS7 were applied, namely in volumes of 1µl, 5 µl and 10 µl.

Discussion

The toxicity of VSs remained an insurmountable obstacle in cryopreservation until less toxic VSs were developed in the late 1980 and early 1990s, most notably the work of Kasai and co-workers in Japan, and that of Ali and Shelton in Australia. Fahy and co-workers (Fahy et al, 1987) investigated the role of combinations of cryoprotectants that could vitrify at lower solute concentrations than would single cryoprotectants. This strategy also conferred reduced toxicity to the cryoprotectant solution. He suggested that combinations of two or more cryoprotectants needed less solutes to vitrify than would a single cryoprotectant (Fahy et al., 1987). His findings led to the use of more than one cryoprotectant in VS's. In the present study the use of six cryoprotectants was an attempt to further reduce the chemo-toxicity of of the VS. Fahy's group has made considerable contributions to the study of cryoprotectant toxicity (Fahy et al., 1986, 1990, 2010, 2015). Other workers have suggested the involvement of temperature in cryoprotectant toxicity such that with higher temperature cryoprotectants act as protein denaturants (Arakawa et al., 1990). Fuller and co-workers have reviewed cryoprotectant toxicity in greater detail (Fuller et al., 2004).

The senary cryoprotectant solutions investigated in the present study that turned milky during the warming process had devitrified. In such solutions, ice nuclei may have formed either during cooling or at the onset of warming followed by their rapid growth during warming into ice-crystals. This is basically either due to lack of solutes, slower cooling or slower warming protocols and or lower warming temperatures and other physico-chemical factors that promote ice nucleation and itheir growth leading to ice crystal formation. Ice crystal formation is not compatible with life. In the present instance this occurrence is probably due to the lack of solutes in the cryoprotectant solution and to less rapid warming protocols. If the water bath was held at 37^{o}C instead of 25^{o}C may be devitrification would not have occurred in some of the VSs formulated.

Further studies will provide more insight into this phenomenon exhibited by the senary VS's. Warming temperature has a role in devitrification during warming. In the present study ultra rapid warming protocols did not exhibit devitrification as often as slower warming protocols such as those vehicles containing the VSs that were warmed in air compared to the ultra-rapid warming that occurred in the water bath. The lower solute concentrations in some of the cryoprotectant solutions investigated particularly those below 5.5M and the slower warming method that promoted devitrification cannot be applied in cryopreservation procedures because they will damage the cell or embryos irreversibly.

Furthermore, the senary cryoprotectant solutions that fractured (K) following cooling or warming are also not suitable as vitrification solutions for the cryopreservation of cell, gametes and embryos because the fracture will disrupt cellular structure of the cryopreserved material irreversibly. The fracture will break up the embryo into fragments, as a consequence of which the cell or embryo will not survive the procedure.

The present research effort led to the development of a senary VS that contained a total of six cryoprotectants. Based on the present findings it appears that the senary cryoprotectant solution consisting of water + 1.5M EG + 1.0M Gly + 1.0M DMSO + 1.0M PG + 1.0M Sucrose (Senary VS7) appears most promising senary VS but it remain to determined whether it is cytotoxic and/or embryotoxic, followed by embryo and oocyte vitrification in the mouse and other animal models before a human clinical trial can be attempted. The low solute concentration of individual cryoprotectants in the Senary VS7 solution is anticipated to be less toxic than currently used ternary and quaternary VSs. A patent application lodgement is currently in preparation.

Conclusion

A senary VS with potentially useful application in ART has been formulated. Further studies including toxicity investigations will be undertaken to determine its usefulness.

Acknowledgement

This study was funded by the University of Malaya ref. UMRG research grant RP023-14 HTM. The findings of this investigation has been presented in three different national and international scientific conferences held in Malaysia and Thailand.

References

Ali J, Shelton JN. Development of vitrification solutions. In: Vitrification in Assisted Reproduction. A User's Manual and Troubleshooting Guide. M.Tucker & J. Liebermann, Eds. Informa Healthcare Medical Books, UK,2006, p45-63

Ali J, Shelton JN. Design of vitrifcation solutions for the cryopreservation of embryos J. Reprod. Fertil. 1993a; 99:471-477

Ali J, Shelton JN. Vitrification of preimplantation stages of mouse embryos J. Reprod. Fertil. 1993b; 98:459-465

Ali J, Shelton JN. Successful vitrification of sheep day-6 embryos J. Reprod. Fertil. 1993c; 99:65-70

Ali J, Bongso A, Ratnam SS. Chromosomal analysis of day-2 human embryos vitrified with VS14. Med. Sci. Res.(UK) 1995;23:539-540

Ali J. Developmental competence of unipronuclear and triploid day-2 human embryos after vitrification with VS14. Med. Sci. Res. (UK) 1996;24:377-378

Arakawa T, Carpenter JF, Kita YA, Crowe JH. The basis for toxicity of certain cryoprotectants: a hypothesis. Cryobiology. 1990 Aug 1;27(4):401-15.

Fahy GM, Levy DI, Ali SE. Some emerging principles underlying the physical properties, biological actions, and utility of vitrification solutions. Cryobiology. 1987 Jun 1;24(3):196-213.

Fahy GM. The relevance of cryoprotectant "toxicity" to cryobiology. Cryobiology. 1986 Feb 1;23(1):1-3.

Fahy GM, Lilley TH, Linsdell H, Douglas MS, Meryman HT. Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms. Cryobiology. 1990 Jun 1;27(3):247-68.

Fahy GM. Cryoprotectant toxicity neutralization. Cryobiology. 2010;60(3):S45-53.

Fahy GM, Wowk B. Principles of cryopreservation by vitrification. Cryopreservation and freeze-drying protocols. 2015:21-82.

Kasai M, Niwa K, Iritani A. Survival of mouse embryos frozen and thawed rapidly. J Reprod Fertil. 1980;59(1):51-56.

Kasai M, Komi JH, Takakamo A, Tsudera H, Sakurai T, Machida T. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. J Reprod Fertil. 1990;89(1):91-97.

Kono T, Suzuki O, Tsunoda Y. Cryopreservation of rat blastocysts by vitrification. Cryobiology. 1988;25(2):170-173.

Mazur P, Miller RH, Leibo SP. Survival of frozen-thawed bovine red cells as a function of the permeation of glycerol and sucrose. J Membr Biol. 1974;15(2):137-158.

Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. Nature. 1985: 14-20;313(6003):573-5.

Rall WF, Wood MJ, Kirby C, Whittingham DG. Development of mouse embryos cryopreserved by vitrification. J Reprod Fertil. 1987;80(2):499-504.

Schmidt PJ. Basile J. Luyet and the beginnings of transfusion cryobiology. Transfus Med Rev. 2006;20(3):242-246..