

# The effect of glycerol and a glycerol-containing cryoprotective medium upon the motility of human sperm prior to freezing, and subsequent difficulties in assessing sperm motility following dilution

Callum Robinson<sup>1,2</sup>, Peter Roberts<sup>1</sup>, Kate Reynolds<sup>2</sup> and Phillip Matson<sup>1,2</sup>

<sup>1</sup>School of Medical and Health Sciences, Edith Cowan University, Joondalup, Western Australia 6027, Australia

<sup>2</sup>Fertility North, Joondalup, Western Australia 6027, Australia

## Abstract

Glycerol is a widely used cryoprotective agent in the cryopreservation of human semen, however it has toxic effects on sperm. The use of computer-assisted sperm analysis (CASA) software to determine sperm motility requires specific upper concentrations of sperm to allow the accurate tracking of sperm trajectories without collisions. Various diluents have been used successfully for diluting neat semen samples but the effect of dilution of semen containing glycerol for the purpose of cryopreservation is relatively unclear. The effect on sperm motility was therefore investigated for the following: the addition of a commercial, glycerol containing cryoprotective (CPM) Quinns Advantage Sperm Freezing Medium (Origio Australasia Pty Ltd, Thornleigh NSW, Australia), 5% neat glycerol (Sigma-Aldrich Pty Ltd, Sydney NSW, Australia) and 10% neat glycerol; cell-free seminal plasma, gamete handling media G-MOPS™ PLUS (Vitrolife Pty Ltd, Sydney NSW, Australia), which contains human serum albumin, and Quinn's Advantage™ Medium with HEPES (Origio Australasia Pty Ltd, Thornleigh NSW, Australia) supplemented with 5% human serum albumin (Origio Australasia Pty Ltd, Thornleigh NSW, Australia), and finally; combinations of the above cryoprotectants and handling media. The presence of glycerol was found to have a negative impact on sperm motility in all samples; this appeared to be in a dose-dependent fashion, with the CPM suffering the least, and 10% glycerol having the most severe reduction in progressive motility. All glycerol containing samples suffered a further reduction in progressive motility when either G-MOPS™ PLUS or HEPES-buffered medium were introduced. Seminal plasma suffered no further reduction in progressive motility for CPM and 10% glycerol, but a reduction in the 5% glycerol. This study has demonstrated that semen samples containing glycerol should only be diluted with seminal plasma prior to assessment by CASA, and that HEPES-buffered medium and G-MOPS™ PLUS should not be used.

**Disclaimer:** The authors declare no conflicts of interest, whether of a financial or other nature

**J Reprod Biotechnol Fertil. 7:8-13**

**Correspondence:** Callum Robinson, School of Medical and Health Sciences, Edith Cowan University, Joondalup, Western Australia, 6027, Australia. E-mail: [callum.robinson@fertilitynorth.com.au](mailto:callum.robinson@fertilitynorth.com.au)

**Funding:** The authors received no financial support for the research, authorship, or publication of this article.

**Acknowledgements:** The authors would like to thank the men who provided samples for this study, and the laboratory staff at Fertility North for their help and support.

**Keywords:** Sperm, motility, CASA, glycerol, dilution, toxicity, handling media

## Introduction

The use of the permeating cryoprotectant glycerol was a major breakthrough in enabling sperm to survive cryopreservation (Polge, Smith, & Parkes, 1949). Whilst protecting the cells from intracellular ice crystal formation, glycerol has been shown to be toxic to sperm and reduce motility (Critser, Huse-Benda, Aaker, Arneson, &

Ball, 1988; McLaughlin, Ford, & Hull, 1992). Alternative permeable cryoprotectants such as ethylene glycol have been used (Gilmore et al., 1997) but, overall, there has been greater focus on ways in which to reduce the toxic effects by either keeping the glycerol concentration at or below 10% v/v (Critser et al.,

## Glycerol and sperm motility

Robinson et al., 2018

1988; Tyler, 1973), adding the glycerol in multiple steps (Watson, 1979), or adding cold cryoprotectant (Clarke, Liu, & Baker, 2004).

In order to effectively measure thawed sperm kinetic measurements using computer assisted sperm analysis (CASA) software, there must be a defined maximum working sperm concentration to reduce collisions of sperm (Garrett, Liu, Clarke, Rushford, & Baker, 2003; World Health Organization, 2010). Dilution of samples containing sperm concentrations above the nominated upper limit is recommended to be done with seminal plasma to avoid changing the environment sperm are exposed to (World Health Organization, 2010), although various media have been found to be suitable (Farrell, Foote, McArdle, Trouern-Trend, & Tardif, 1996). Whilst the use of media of defined composition is simpler than preparing sperm-free seminal plasma, there is a paucity of work on the negative aspects of diluting the semen and potential artefacts that may be introduced, including the dilution of semen containing cryoprotectants.

The aims of this study were to examine sperm motility manually to determine (i) the toxicity in neat semen of glycerol at concentrations of 5 and 10% v/v and a commercially available glycerol-containing cryoprotective media (CPM), and (ii) the effect upon sperm motility in semen of glycerol and CPM when diluted with G-MOPS™ PLUS, Quinn's Advantage™ Medium with HEPES supplemented with 5% human serum albumin and seminal plasma. All measurements were made at room temperature and limited to the phase of the addition of cryoprotectants and diluents prior to cryopreservation.

## Materials and Methods

### Subjects and ethics

Ethics approval was gained through the Joondalup Health Campus Human Research Ethics Committee and the Edith Cowan University Human Research Ethics Committee. Fourteen men undergoing fertility investigation were recruited at Fertility North (Joondalup Private Hospital, Joondalup, WA, Australia) and each provided one semen sample with written consent for the remainder of the sample to be used in the project. Men were advised to abstain from ejaculation for two to five days before producing a sample.

### Sample preparation and assessment

Samples were allowed to liquefy ( $\geq 20$  minutes post-ejaculation) and a semen analysis was conducted for the men's fertility investigation through Fertility North. Following this, the remaining semen was then processed for the current study with all samples being completed no more than 60 minutes post-ejaculation.

Motility assessments were conducted manually according to the WHO 5th classification (World Health Organization, 2010) with sperm cells being classified as either progressive (PR), non-progressive (NP) or immotile (IM). The motility assessments were conducted with phase contrast microscopy using 10  $\mu$ l of semen applied to a glass microscope slide and covered by 22x22mm coverslip (Livingstone, Roseberry, NSW, Australia). After the initial motility assessment, aliquots of each sample were divided into the following treatment groups: (i) neat semen with nothing added, (ii) an equal volume of cryoprotective media (Quinn's Advantage™ Sperm Freezing Medium; Origio Australasia Pty Ltd, Thornleigh NSW, Australia) mixed with the semen, (iii) glycerol (Sigma-Aldrich Pty Ltd, Sydney NSW, Australia) at 5% v/v mixed with the semen, and (iv) 10% v/v glycerol mixed with the semen. The addition of both cryoprotective media and glycerol were in a drop-wise fashion at room temperature and adequate mixing of the sample and reagent were ensured. Each aliquot was then observed after one minute and the motility recorded as above.

Following this, seven men's samples containing the above cryoprotective agents were diluted (1:5) with G-MOPS™ PLUS medium (Cat-10130; Vitrolife Pty Ltd, Sydney NSW, Australia), which contains human serum albumin, and a motility measurement was taken within one minute. The other seven men's samples were diluted (1:5) with Quinn's Advantage™ Medium with HEPES (ART-1023; Origio Australasia Pty Ltd, Thornleigh NSW, Australia) supplemented with 5% human serum albumin (ART-3001-5; Origio Australasia Pty Ltd, Thornleigh NSW, Australia), or seminal plasma (1:5 dilution), and a final motility measurement recorded. Seminal plasma was obtained by centrifuging semen at 1400g for 10 minutes and observed microscopically to ensure no sperm were present in the seminal plasma supernatant.

**Statistical analysis**

Statistical analysis was conducted using SPSS Statistics Package (V.23, IBM). Data sets were first explored and considered to be either normally or abnormally distributed based on the Shapiro-Wilk test for normality score ( $\alpha=0.05$ ). If these normally distributed data sets met parametric testing requirements and passed Mauchly's tests for sphericity ( $p>0.05$ ), repeated measures ANOVA testing with Bonferroni post-hoc analysis was applied to investigate where differences occurred between groups ( $\alpha=0.05$ ), or a paired-samples t-test was employed. If data sets failed to meet the assumptions required for parametric testing, non-parametric Friedman's test was employed to identify possible significant differences between data groups. A Sign pair-wise comparison test was then used to identify where the differences occurred between measurements ( $\alpha=0.05$ ).

**Results****Effect of cryoprotectants on sperm motility**

The proportion of sperm motility for all men, with or without the cryoprotectants, is shown in Table 1. Following the addition of each cryoprotective agent, there was a significant decrease in progressive motility (PR) observed across all types of cryoprotectant added. There was no statistical difference seen between the CPM and 5% glycerol ( $p=0.79$ ), but the PR of 10% glycerol was significantly lower than both the CPM and 5% glycerol. The majority of the reduction in PR motility of samples seen was a shift directly to immotile (IM) cells, although the samples containing 10% glycerol had a significantly higher proportion of non-progressive (NP) cells than the neat semen or that containing CPM.

**Dilution with handling media and seminal plasma**

Seven men's semen samples containing an equal v/v CPM, 5% glycerol or 10% glycerol were subsequently diluted with G-MOPS™ PLUS (1:5 dilution) and loaded onto a glass slide, with a progressive motility measurement being made within 2 minutes of the dilution with G-MOPS™ PLUS. These results are shown in Table 2. The addition of G-MOPS™ PLUS to the neat semen sample did not impact on the PR motility of the sample, but the dilution of samples containing each of the cryoprotectants resulted

in a significant decrease in PR motility relative to the cryoprotectant alone.

The remaining seven men's semen samples were diluted with Quinn's Advantage™ Medium with HEPES, or seminal plasma. The neat semen sample did not show a significant reduction in PR motility when diluted with Quinn's Advantage™ Medium with HEPES or seminal plasma (SP). The addition of SP to samples with CPM or 10% glycerol did not show a statistically significant decrease in the PR motility of sperm, although addition to the 5% glycerol group did see a slight but significant decrease in PR motility. Quinn's Advantage™ Medium with HEPES had a negative impact on PR within all samples containing cryoprotectant, significantly reducing the proportion of PR sperm.

**Discussion**

The cryopreservation of sperm has always resulted in detrimental effects on post-thaw survival and fertilisation capabilities (Nijs & Ombelet, 2001; Sharma, Kattoor, Ghulmiyyah, & Agarwal, 2015). The exposure of sperm to sub-zero conditions has been shown to increase DNA fragmentation (Liu et al., 2016), increase oxidative stress (Thomson et al., 2009) and impose cytoskeletal modifications; some of which being more severe when exposed to longer periods of cryopreservation (Desrosiers, Légaré, Leclerc, & Sullivan, 2006). However even before sperm are exposed to such conditions, cytotoxicity is present due to the exposure of sperm to cryoprotective agents that are obligatory for surviving the freezing process, as shown in the current study. Glycerol is the most commonly used cryoprotective agent in the freezing of human sperm and diffuses across the cell membrane to prevent the formation of intracellular ice in sub-zero conditions (Gosden, 2011; Sharma et al., 2015), and has been noted to negatively affect sperm in various ways including alterations to mitochondria and the internal membrane of the acrosome (Di Santo, Tarozzi, Nadalini, & Borini, 2012). The data gained from this study indeed shows glycerol's direct negative influence on the motility of sperm. Even after a short exposure time, glycerol's affect can be seen with a statistically significant decrease in samples PR motility across all concentrations of glycerol used in the present study. This also appeared to

**Table 1.** Sperm motility (mean  $\pm$  sem) following the addition of no cryoprotectant (nil), cryoprotective medium (CPM), and glycerol at 5% and 10% v/v. (n=14).

Cryoprotectant	Sperm motility (%)		
	PR	NP	IM
<b>Nil</b>	64.2 $\pm$ 4.0 <sup>abc</sup>	0.6 $\pm$ 0.2 <sup>f</sup>	34.9 $\pm$ 3.9 <sup>hij</sup>
<b>CPM</b>	45.4 $\pm$ 6.1 <sup>ad</sup>	1.1 $\pm$ 0.4 <sup>g</sup>	53 $\pm$ 5.8 <sup>hk</sup>
<b>5% glycerol</b>	39.9 $\pm$ 4.9 <sup>be</sup>	3.4 $\pm$ 1.2	56.6 $\pm$ 4.7 <sup>il</sup>
<b>10% glycerol</b>	23.2 $\pm$ 3.9 <sup>cde</sup>	3.2 $\pm$ 0.9 <sup>fg</sup>	73.6 $\pm$ 3.8 <sup>kl</sup>

Motility: PR, progressively motile; NP, non-progressively motile; IM, immotile.

Groups are significantly different when they have the same superscript letter.

**Table 2.** Sperm progressive motility (mean  $\pm$  sem) following the addition of cryoprotectants and subsequent dilution using G-MOPSTM PLUS medium (n=7). The cryoprotectants were none (nil), Quinn's Advantage Sperm Freezing Medium (CPM), and glycerol at 5% and 10% v/v.

Cryoprotectant	Diluent	
	Nil	G-MOPS <sup>TM</sup> PLUS
<b>Nil</b>	62.6 $\pm$ 6.4 <sup>ab</sup>	58.3 $\pm$ 7.5 <sup>fgh</sup>
<b>CPM</b>	46.1 $\pm$ 9.3 <sup>cd1</sup>	25.9 $\pm$ 7.2 <sup>fi1</sup>
<b>5% glycerol</b>	34.0 $\pm$ 7.6 <sup>a2</sup>	16.7 $\pm$ 5.9 <sup>gj2</sup>
<b>10% glycerol</b>	17.0 $\pm$ 3.7 <sup>bcd3</sup>	3.4 $\pm$ 1.2 <sup>hij3</sup>

Groups are significantly different when they have the same superscript letter (between different cryoprotectants for same diluent) or number (between different diluents for the same cryoprotectant).

**Table 3.** Sperm progressive motility (mean  $\pm$  sem) following the addition of cryoprotectants and subsequent dilution using either Quinn's Advantage<sup>TM</sup> Medium with HEPES or seminal plasma. The cryoprotectants were none (nil), Quinn's Advantage Sperm Freezing Medium (CPM), and glycerol at 5% and 10% v/v.

Cryoprotectant	Diluents		
	Nil	HEPES	Seminal plasma
<b>Nil</b>	65.9 $\pm$ 5.1 <sup>ab</sup>	61.9 $\pm$ 7.1 <sup>de1</sup>	68.4 $\pm$ 5.6 <sup>ghi1</sup>
<b>CPM</b>	44.6 $\pm$ 8.5 <sup>2</sup>	31.3 $\pm$ 8.6 <sup>d23</sup>	46.9 $\pm$ 8.8 <sup>gjk3</sup>
<b>5% glycerol</b>	45.9 $\pm$ 6.0 <sup>ac4</sup>	20.1 $\pm$ 4.8 <sup>e45</sup>	37.4 $\pm$ 7.0 <sup>hjl5</sup>
<b>10% glycerol</b>	29.4 $\pm$ 6.3 <sup>bc6</sup>	10.9 $\pm$ 3.3 <sup>f67</sup>	25.0 $\pm$ 6.8 <sup>ikl7</sup>

Groups are significantly different when they have the same superscript letter (between different cryoprotectants for same diluent) or number (between different diluents for the same cryoprotectant).

## Glycerol and sperm motility

Robinson et al., 2018

occur in a dose-dependent fashion, in that the higher concentration of glycerol added to the semen sample resulted in the largest decrease in progressive motility. The CPM had a statistically similar effect on PR motility to the 5% glycerol even though the CPM includes several constituents beneficial for sperm motility such as glucose and sucrose (Amaral, Paiva, Baptista, Sousa, & Ramalho-Santos, 2011). Whilst no cause for the loss of motility was identified in the present study, disruption to the sperm's mitochondrial membrane and osmolarity changes leading to cell death would be consistent with this increase in shift from PR to IM, although further investigation on the mechanism would be required.

In order to effectively measure a sample's kinematic parameters using CASA software, manufacturers recommend diluting a sample to ensure a low enough concentration in order to prevent the collision of sperm which would disrupt kinematic measurements. Previous studies have illustrated the potential toxic effect of handling media on sperm motility, with factors such as the presence of transient metals negatively impacting this (Gomez & Aitken, 1996). The initial addition of both Quinn's Advantage™ Medium with HEPES and G-MOPS™ PLUS in a 1:5 dilution to the neat samples did not alter the PR motility of the samples significantly, which is to be expected and hoped for considering they are intended for the safe handling of gametes. However the addition of both of these handling media in the presence of glycerol had a statistically significant decrease on the PR motility, including the commercially engineered CPM.

Quinn's Advantage™ Medium with HEPES and G-MOPS™ PLUS had a similar detrimental effect on progressive motility when glycerol was present, suggesting that the decrease in PR results through a similar interaction. Alterations to sperm membrane that are caused by glycerol (Keel & Webster, 1990) could leave sperm more vulnerable to dilution effects brought about by further diluting with non-seminal plasma diluents. This is supported by the data in that when glycerol was not present, Quinn's Advantage™ Medium with HEPES and G-MOPS™ PLUS both had no effect on PR. More

research is needed to further uncover if this is a result of glycerol leaving sperm more susceptible to osmotic alterations when further diluted with non-seminal plasma diluents; or that there is an undiscovered interaction between glycerol and constituents of the handling media that has yet to be revealed. The use of seminal plasma as a diluent in the present study was intended for research or diagnostic purposes only, and the use of seminal plasma in samples to be used for ART therapeutic purposes is not advised unless the seminal plasma is from the same man that produced the sample.

## Conclusion

This study provided an insight into the potential effects of cryoprotective agents and sperm handling media on sperm pre-cryopreservation. Glycerol's toxic effect on sperm was highlighted and appeared to occur in a dose-dependent form. The commercially engineered CPM gave similar results to the 5% glycerol, and is probably similar to the final glycerol concentration in the CPM being around 5% after the 1:1 addition to the semen. The need to dilute semen samples containing high sperm concentrations is a necessary preparative step prior to use with CASA systems, and the present study has highlighted the danger of progressive sperm motility being reduced if culture media are used as a diluent in the presence of glycerol. The use of seminal plasma is therefore recommended.

## References

- Amaral A, Paiva C, Baptista M, Sousa AP, Ramalho-Santos J. (2011). Exogenous glucose improves long-standing human sperm motility, viability, and mitochondrial function. *Fertil Steril*, 96(4), 848-850. doi: <https://doi.org/10.1016/j.fertnstert.2011.07.1091>
- Clarke GN, Liu DY, Baker HWG. (2004). Improved sperm cryo-preservation using cold cryoprotectant. *Reprod Fertil Dev*, 15(7), 377.
- Critser JK, Huse-Benda AR, Aaker DV, Arneson BW, Ball GD. (1988).

- Cryopreservation of human sperm. III. The effect of cryoprotectants on motility. *Fertil Steril*, 50(2), 314-320.
- Desrosiers P, Légaré C, Leclerc P, Sullivan R (2006). Membranous and structural damage that occur during cryopreservation of human sperm may be time-related events. *Fertil Steril*, 85(6), 1744-1752. doi: <https://doi.org/10.1016/j.fertnstert.2005.11.046>
- Di Santo M, Tarozzi N, Nadalini M, Borini A (2012). Human Sperm Cryopreservation: Update on Techniques, Effect on DNA Integrity, and Implications for ART. *Adv Urol*, 2012, 12. doi: 10.1155/2012/854837
- Farrell PB, Foote RH, McArdle MM, Trouern-Trend VL, Tardif AL. (1996). Media and Dilution Procedures Tested to Minimize Handling Effects on Human, Rabbit, and Bull Sperm for Computer-Assisted Sperm Analysis (CASA). *J Androl*, 17(3), 293-300.
- Garrett C, Liu DY, Clarke GN, Rushford DD, Baker HW. (2003). Automated semen analysis: 'zona pellucida preferred' sperm morphometry and straight-line velocity are related to pregnancy rate in subfertile couples. *Hum Reprod (Oxford, England)*, 18(8), 1643-1649.
- Gilmore JA, Liu J, Gao DY, Critser JK. (1997). Determination of optimal cryoprotectants and procedures for their addition and removal from human sperm. *Hum Reprod (Oxford, England)*, 12(1), 112-118.
- Gomez E, Aitken J. (1996). Impact of in vitro fertilization culture media on peroxidative damage to human sperm. *Fertil Steril*, 65(4), 880-882. doi: [https://doi.org/10.1016/S0015-0282\(16\)58233-8](https://doi.org/10.1016/S0015-0282(16)58233-8)
- Gosden R. (2011). Cryopreservation: a cold look at technology for fertility preservation. *Fertil Steril*, 96(2), 264-268. doi: <http://dx.doi.org/10.1016/j.fertnstert.2011.06.029>
- Keel BA, Webster BW. (1990). CRC handbook of the laboratory diagnosis and treatment of infertility. Boca Raton, Fla. CRC Press.
- Liu T, Gao J, Zhou N, Mo M, Wang X, Zhang X, Cao J. (2016). The effect of two cryopreservation methods on human sperm DNA damage. *Cryobiol*, 72(3), 210-215. doi: <https://doi.org/10.1016/j.cryobiol.2016.04.004>
- McLaughlin, EA, Ford WC, Hull MG. (1992). The contribution of the toxicity of a glycerol-egg yolk-citrate cryopreservative to the decline in human sperm motility during cryopreservation. *Journal of Reprod Fertil*, 95(3), 749-754.
- Nijs M, Ombelet W. (2001). Cryopreservation of human sperm. *Hum Fertil*, 4(3), 158-163. doi: 10.1080/1464727012000199232
- Polge C, Smith A, Parkes S. (1949). Revival of Sperm after Vitrification and Dehydration at Low Temperatures. *Nature*. doi: 164 : 666
- Sharma R, Kattoor AJ, Ghulmiyyah J, Agarwal A. (2015). Effect of sperm storage and selection techniques on sperm parameters. *Systems Biol Reprod Med*, 61(1), 1-12. doi: 10.3109/19396368.2014.976720
- Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA, Clark AM. (2009). Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod (Oxford, England)*, 24(9), 2061-2070.
- Tyler ET. (1973). The clinical use of frozen semen banks. *Fertil Steril*, 24(5), 413-416.
- Watson PF. (1979). The preservation of semen in mammals. In C. Finn (Ed.), *Oxford Rev Reprod Biol* (Vol. 1, pp. 283-350). Oxford: Oxford University Press.
- World Health Organization. (2010). WHO laboratory manual for the examination and processing of human semen (5th ed.). Geneva, Switzerland: World Health Organization.