A Comparison of Glycerol Egg Yolk Citrate and Sperm Freeze Medium in Human Sperm Cryopreservation

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Background: *Cryoprotectant has a pivotal role in preventing cell injury during sperm cryopreservation.*
Objective: *To compare the post-thawed sperm parameters between the use of glycerol egg yolk citrate (GEYC) and Sperm Freeze medium as cryoprotectant in human sperm cryopreservation.*
Material and Method: *This was an experimental study. Thirty healthy volunteers whose sperm was collected in our clinic during August, 2009 through October, 2009 were included in the study. All of the semen samples were analysed with standard technique using the World Health Organization protocol. The semen samples were divided into 2 groups and mixed with GEYC and SpermFreeze medium respectively. Pre-freezing and post-thawed sperm parameters were analyzed with Computer-Assisted Sperm Analysis system.*
Results: *A total of 30 volunteers who could collect their semen samples participated in the study. The mean and standard deviation of sperm concentration was 53.3±27.5 million/milliliter, the percentage of sperm motility was 68.2±22.1 and the percentage of normal sperm morphology was 4.7±1.6. When compared the semen samples that freezing them with GEYC or SpermFreeze medium, there was no significant difference in mean post-thawed sperm concentration (33.7±17.4 vs. 32.7±15.6 million/milliliter, p-value = 0.827) and percentage of sperm motility (38.6±25.6 vs. 35.1±28.1, p-value = 0.616). However, it had no significant effect on normal sperm morphology before and after freezing-thawing cycle and also, there was no significant difference in percentage of post-thawed normal sperm morphology between two cryomediums (4.4±1.3 vs. 4.4±1.4, p-value = 0.849).*
Conclusion: *For normal semen samples, use of GEYC medium is an acceptable option in human sperm cryopreservation.*

Keywords: *Glycerol, Egg yolk, Cryopreservation, Sperm parameters*

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To date, fertility function is a major concern of many health organizations[1]. Several factors influence the fertility capability such as advanced age of a couple, underlying disease, and toxic exposure. In Thailand, there is also a rise of infertility problems for both men and women. In men, there is a significant role of sperm cryopreservation in clinical practice e.g. for cancer patients[2], to preserve fertility[3], sperm donor program[4], and sperm banking to assist reproductive technology treatment[5]. During freezing-thawing process, sperm may deteriorate because of damaged cell membranes, impairing sperm motility, and altering its morphology[6]. Many factors affect the sperm quality in freezing and thawing e.g. the freezing method[7], temperature control[8], sperm preparation technique[9], and type of cryopreservative agent[10].

There are two types of cryoprotectants that are commonly used. Most of the researchers have focused on permeable agents which have less toxicity[11] and can protect sperm from intracellular ice crystal formation and osmotic imbalance[12]. However, few Thai researchers have evaluated a current technique used to yield high sperm survival rates[13-15]. Most of them use a commercial sperm freezing medium in their andrology laboratory. Glycerol egg yolk citrate (GEYC) is a well known cryoprotectant and been used in human sperm freezing over the years[11,16,17]. Considering this difference, the authors aim to evaluate the ability of in-house GEYC medium as cryoprotectant in human sperm cryopreservation and to compare the post-thawed sperm quality between GEYC and Sperm Freeze medium.

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Material and Method

This study was conducted in the Assisted Reproductive Technology Unit, HRH Maha Chakri Sirindhorn Medical Center between August, 2009 and October, 2009. Healthy male volunteers were enrolled in this study. Inclusion criteria were as follows; Thai nationality, ages between 20 to 35 years, had a normal semen analysis report, and a written consent form. The exclusion criteria were men who had a history of sexually transmitted disease, vasectomy, hormonal treatment, with semen volume less than 1 mL, and had evidence of infection in the semen sample. The participants had to answer all the questions about their demographics in the data record form. The study protocol was approved by the ethic committee of the Faculty of Medicine, Srinakharinwirot University (SWUEC/EX 19-2552).

Semen analysis

The volunteers were asked to produce semen samples collected in sterile containers after 3 to 5 days of sexual abstinence. Then, the samples were placed in a 37°C incubator until complete liquefaction. Semen analysis was performed by two experienced scientists within 1 hour after the collection. Seminal fluid volume was measured with a 5 mL calibrated syringe. Each sample was assessed by Computer-Assisted Semen Analysis (CASA) with IVOS Hamilton-Thorne Analyzer (AP Tec, USA) using standard set-up parameters. The physical parameters analyzed were: semen volume, viscosity, pH, sperm count, sperm motility, and sperm morphology. Morphologic study was assessed under light microscope by using Papanicolaou staining technique. Normal morphology was counted when all of the following parameters were normal: acrosome, sperm head, mid-piece, and tail. All semen parameters were measured according to the World Health Organization guideline.

Semen cryopreservation

Each semen sample was divided into two parts. The first part of 0.5 mL volume was mixed with an equal volume of GEYC medium which was prepared according to our institute protocol (egg yolk 40 mL, glucose 2.6 gm, glycine 2.0 gm, glycerol 30 mL, sodium citrate 2.3 gm, double-distilled water 130 mL) and stored frozen at -196°C until use.

The second part of 0.5 mL volume was mixed with 0.35 mL of SpermFreeze medium (Fertipro, Belgium). It contained a HEPES buffered, glycerol, sucrose, and human serum albumin. After equilibrate for 10 minutes at room temperature, the samples were then placed into cryo-tubes and sealed with plastic plug.

All cryo-tubes were labeled and loaded into freezing canes, then placed in liquid nitrogen vapor for at least 15 minutes and then submersed in the liquid nitrogen tank (-196°C) for 24 hours (Fig. 1).

Thawing

After 24 hours of cryostorage, the cryo-tubes were removed from the liquid nitrogen tank and placed in room temperature for 5 minutes. Then, the post-thawed sperm in each cryo-tube was reassessed by CASA.

Statistical analysis

Baseline characteristics and sperm parameters were analyzed using descriptive statistics. Sample size for testing of two dependent means was calculated by N4studies program[18,19]. There was no previous report of direct comparison between two cryoprotectants, so based on an estimation from our pilot study the standard deviation of sperm concentration was 9, the difference data between two groups was 5, the α error of 0.05 and 0.80 of power. The calculated sample size

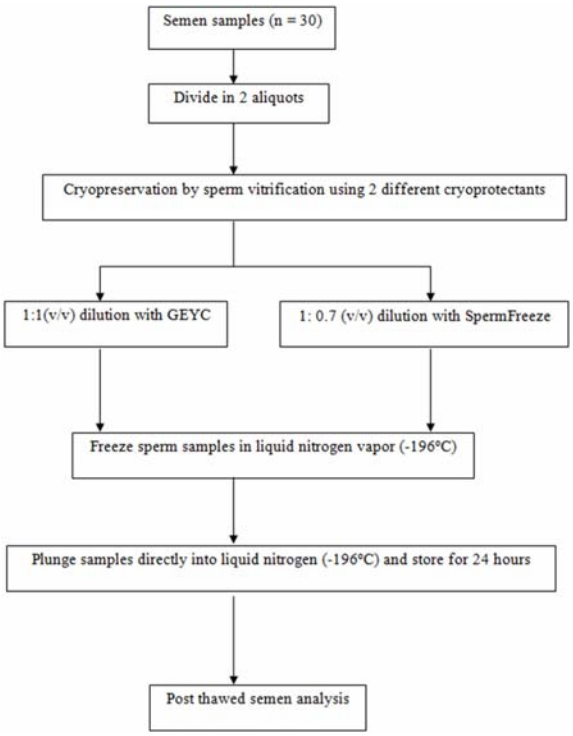

Fig. 1 Flow diagram of study.

per group was 26. The data were analyzed using the pair t-test to compare the pre and post-thawed sperm parameters in each group of cryoprotectants used; *p*-value less than 0.05 was considered statistically significant.

Results

After setting up our protocol, a total of 30 men were included in this study. Thirty semen samples were analyzed; their mean age was 31.3±6 years. When compared the semen samples between before and after sperm freezing being treated with GEYC and SpermFreeze medium, there were significant differences in mean sperm concentration and motility ($p<0.01$), but not a significant one in sperm morphology (*p*-value = 0.562). There were no significant differences in all sperm parameters measurement between GEYC-treated sample and SpermFreeze-treated sample, at the $p>0.05$ as shown in Table 1.

Discussion

This study demonstrates the protective effect of GEYC medium on human sperm cryopreservation. Sperm count and sperm motility significantly decrease after single freeze-thawed cycle while sperm morphology remains the same. In terms of each sperm parameter, it is quite interesting that the sperm count decreases significantly which influences by different ratio of semen volume and cryomedium. This dilution effect of cryomedium was previously described by Centola GM et al[20]. In theory, no loss of cells occurred during freeze-thaw process; the number of sperm counts which are not influenced by duration of freezing, should be similar after one freeze-thawed cycle. There is the possibility of different ratio on dilution technique between two cryoprotectants or abnormal cell detection by CASA analyzer [21].

In this study, post-thawed sperm motility also decreases approximately 50 percent resulting from cryoinjury during the freeze-thawed process. This finding is consistent with the result reported by Julavijitphong et al[13] and Chaiya J[22]. Talebian A et al[23] who also reported significant reduction in sperm motility treated with GEYC medium, but with a different method of freezing. In fact, the motility of sperm decreased by time[7] and the dilution effect when semen mix with the cryomedium[24]. However, there are many ways to reduce this cryo-injury by adjusting the concentration of cryoprotectant[25] or adding adjuvant e.g. vitamin E[26]. To date, researchers still look for a new agent to improve sperm recovery rate after cryopreservation[27].

When comparing between the two cryoprotectants, the use of the GEYC yields a high percentage of post-thawed normal sperm morphology which was comparable to the SpermFreeze medium. Egg yolk along with glycerol may reduce the adverse effects on cell membrane during sperm cooling phase. This suggests that GEYC medium is effective and has potential to be used in clinical practice. Further study of specific sperm tests such as DNA fragmentation and sperm vitality is needed to reassure safety of GEYC medium. A drawback of egg yolk is of animal origin and has potential of toxins or microbial contamination to the lab. Thus, other researchers have substituted egg yolk with lecithin to eliminate the health risk[28]. However, GEYC medium is easy to prepare, low cost, comparable post-thawed sperm parameters, and egg yolk component may enhance acrosome reaction[29].

The limitations of this study are a small sample

Table 1. Means and standard deviation of sperm parameters before and after sperm cryopreservation (n = 30)

Parameters	GEYC group	SpermFreeze group	*p*-value
Sperm count (x10^6/mL)			
Fresh	53.3±27.5		<0.01*
Thawed	33.7±17.4	32.7±15.6	0.827**
Overall sperm motility (%)			
Fresh	68.2±22.1		<0.01*
Thawed	38.6±25.6	35.1±28.1	0.616**
Normal morphology (%)			
Fresh	4.7±1.6		0.562*
Thawed	4.4±1.3	4.4±1.4	0.849**

* Pair t-test to compare before and after freezing in each group, ** Pair t-test to compare between GEYC-treated and SpermFreeze-treated group

size, single site, focusing only on the normal semen analysis group, exclusion of a sperm function test, and studying only initial sperm parameters which cannot predict the pregnancy rate.

Conclusion

The use of GEYC cryomedium is comparable to SpermFreeze in terms of post-thawed sperm count, sperm motility, and sperm morphology in normal semen samples.

What is already known on this topic?

Use of cryoprotectant in sperm freezing process has clear benefit. Many commercial mediums are available in the market. Those agents give an appropriate sperm recovery rate, but still have a room for improvement to get the best outcome.

What this study adds?

This study demonstrates the efficacy of in-house GEYC medium which has optimal sperm recovery and can be used for human sperm cryopreservation.

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Potential conflicts of interest

None.

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การเปรียบเทียบน้ำยากลีเซอรอลผสมไข่แดงซิเตรทและน้ำยาสเปิร์มฟรีซในการแช่แข็งอสุจิมนุษย์

เมธาพันธ์ กิจพรธีรานันท์, สุมิตรา อ้มสงคราม, เกศินี ศรีชวนะ

ภูมิหลัง: *สารป้องกันอันตรายจากการแช่แข็งมีบทบาทที่สำคัญในการปกป้องเซลล์ระหว่างการแช่แข็งอสุจิ*

วัตถุประสงค์: *เพื่อเปรียบเทียบอสุจิหลังการละลายระหว่างการใช้กลีเซอรอลผสมไข่แดงซิเตรท กับสารสเปิร์มฟรีซในการแช่แข็งอสุจิมนุษย์*

วัสดุและวิธีการ: *เป็นการศึกษาเชิงทดลองในอาสาสมัครชาย 30 คนซึ่งเก็บน้ำอสุจิในคลินิกมีบุตรยาก ระหว่างเดือน สิงหาคม พ.ศ. 2552 ถึง ตุลาคม พ.ศ. 2552 ตรวจวิเคราะห์น้ำอสุจิทุกรายด้วยวิธีมาตรฐานตามองค์การอนามัยโลก ทำการแบ่งตัวอย่างน้ำอสุจิเป็น 2 ส่วนเพื่อนำไปผสมรวมกับน้ำยากลีเซอรอลผสมไข่แดงซิเตรทและสเปิร์มฟรีซ ทำการวิเคราะห์อสุจิทั้งก่อนและหลังแช่แข็งด้วยเครื่องคอมพิวเตอร์ตรวจวิเคราะห์อสุจิ*

ผลการศึกษา: *อาสาสมัครจำนวน 30 คนที่สามารถเก็บน้ำอสุจิได้เข้าร่วมโครงการ ค่าเฉลี่ยและค่าเบี่ยงเบนมาตรฐานของความเข้มข้นอสุจิได้ 53.3±27.5 ล้านตัวต่อมิลลิลิตร มีตัวอสุจิที่เคลื่อนไหวเท่ากับร้อยละ 68.2±22.1 และตัวอสุจิที่มีรูปร่างปกติเท่ากับร้อยละ 4.7±1.6 เมื่อเปรียบเทียบค่าเฉลี่ยความเข้มข้นตัวอสุจิหลังจากแช่แข็งด้วยสารป้องกันอันตรายจากการแช่แข็งสองชนิด พบว่าไม่มีความแตกต่างกันอย่างมีนัยสำคัญ (33.7±17.4 และ 32.7±15.6 ล้านตัวต่อมิลลิลิตร p = 0.827) และร้อยละของตัวอสุจิที่เคลื่อนไหวได้นั้น (38.6±25.6 และ 35.1±28.1, p = 0.616) อย่างไรก็ตามไม่มีความแตกต่างกันในรูปร่างอสุจิที่ปกติทั้งก่อนและหลังการแช่แข็ง พบว่าเมื่อเปรียบเทียบรูปร่างอสุจิปกติหลังละลายไม่มีความแตกต่างกันอย่างมีนัยสำคัญระหว่างการใช้น้ำยากลีเซอรอลผสมไข่แดงซิเตรทและน้ำยาสเปิร์มฟรีซ (4.4±1.3 และ 4.4±1.4, p = 0.849)*

สรุป: *สำหรับตัวอย่างน้ำเชื้ออสุจิที่ปกติการใช้กลีเซอรอลผสมไข่แดงซิเตรทเป็นสารป้องกันอันตรายจากการแช่แข็งเป็นทางเลือกที่ยอมรับได้ดีในการแช่แข็งอสุจิมนุษย์*