



# Comparison of Homemade GEYC Medium and Commercial Sperm Freeze Solution™ for Cryopreservation of Human Sperm



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## Abstract

In the present study, compared the effect of two cryoprotectant medium (GEYC buffer and sperm freeze™ Solution) on sperm motility, kinematics, survival, morphology, and DNA fragmentation in a population of normozoospermia is compared. Semen samples from 20 healthy donors were divided and frozen with either GEYC or sperm freeze™ Solution. Semen characteristics were evaluated before freezing and after thawing. Percentage of motility, viability, plasma membrane integrity, and DFI decreased significantly compared to prefreeze values in both groups. Post-thaw motility following removal of the freezing media did not show difference in specimens that were frozen in GEYC buffer compared to those frozen in Sperm Freeze Solution™ ( $P < 0.05$ ). Similarly, specimens cryopreserved in GEYC buffer had no differences in sperm motility, viability, PMI, and DFI compared to aliquots that were frozen in Sperm Freeze Solution™ ( $P < 0.05$ ).

**Keywords:** Human sperm; Cryopreservation; Cryoprotectant; GEYC buffer; Sperm Freeze Solution™

## Introduction

The procedure that makes it possible to stabilize the cells at cryogenic temperature is called cryopreservation, also known as applied aspect of cryobiology or the study of life at low temperature [1]. Use of cryopreservation agent is indispensable to prevent injury to human spermatozoa during the cryopreservation process [2]. The main goal of male gamete cryopreservation is to preserve sperm viability, motility, and fertilizing ability; but it has been largely reported in the literature that the freezing thawing procedures cause severe structural and functional damage to spermatozoa, impairing cell membranes, sperm motility, and DNA integrity [3]. More complex cryoprotective buffer systems have been introduced over the years, and several appear to improve human sperm recovery upon thawing [4]. Human sperm cryopreservation has significantly improved over the last few decades, but actual protocols are neither optimal nor standardized between different laboratories [5]. However, no single comparative study has been conducted with the use of these various buffer systems for the determination of the best cryoprotective medium for human sperm freezing [6]. Glycerol was the first cryoprotective used for freezing sperm and that cells are highly permeable to glycerol to protect cells from osmotic injury [6]. Glycerol, a cryoprotectant is often extended with cryobuffers, such as citrate or egg yolk, which contain nonpermeating macromolecules [7].

A promising cryobuffer is glycerol-egg yolk-citrate (GEYC), a combination of sodium citrate tribasic dehydrate together with fresh egg yolk (20%), 72mM glucose, 1.73 M glycine, and 15% glycerol with pH 6.8-7.2 [8]. Other commercial extender without egg yolk is Sperm Freeze Solution™ (Ferti Pro, Belgium) that is a chemically defined solution for cryopreservation of semen. It contains glycerol as cryoprotective agent and HEPS, macromolecules (sucrose) reduce physical damage and help to maintain osmotic pressure of the extracellular fluid [www.fertipro.com]. In this study, we compare two cryopreservation media, GEYC standard buffer recommended by World Health Organization (WHO) Contains egg yolk and commercially medium Sperm Freeze Solution™; both contain 15% glycerol. Sperm motility, viability, morphology, cell membrane integrity, and DNA Fragmentation Index (DFI) of sperm cryopreserved with each medium were measured.

## Materials and Methods

### Subjects

This study was approved by Rayan ethics committee (No. IR.ACER.ROYAN.REC.1396.101). Sperm samples were obtained from 20 normospermia donor men Semen were collected through masturbation after 3-7 days of sexual abstinence in sterile

containers. A normal semen analysis according to the World Health Organization (WHO) classification [8].

### Semen analysis and semen cryopreservation

Sperm samples were collected by masturbation after 3-5 days of sexual abstinence. After liquefaction sperm concentration was assessed using the CASA system consists of a phase contrast microscope (Eclipse E-200, Nikon Co., Tokyo, Japan) with a heat plate equipped with Sperm Class Analyzer® software (SCA, Full Research Version 5.1, Micro tic Co., Barcelona, Spain) and motility was assessed according to World Health Organization criteria 2010. After initial semen analysis, the specimen was divided into two equal aliquots. In one aliquot, GEYC was added to aliquot at a final ratio of 1:2 (v/v), The second aliquot was cryopreserved with Sperm Freeze™ medium which was added drop wise to aliquot at a final ratio of 1: 0.7 (v/v), gently mixed, using the liquid-nitrogen vapor-freezing technique [9]. Briefly, placed into cryovials at room temperature for 10minutes to allow for proper equilibration between the cells and the medium. Then, samples were placed in nitrogen vapors (between -80 °C and -100 °C) for 15 minutes and finally immersed in liquid nitrogen at -196 °C. For thawing, the specimen was held at room temperature for 5minutes and then at 37 °C for 5minutes. Semen analysis was done on the unwashed specimens and following removal of the cryoprotective media by washing and resuspending in 1.0ml of Ham's F-10 medium (post wash) and takes place in a CO<sub>2</sub> incubator at 37 °C. After thawing, post wash specimens were analyzed for percentage motility at 30minutes.

### Assessment of sperm viability and morphology

Sperm post thaw viability was assessed using the eosin-nigrosine staining technique to establish the percentage of live spermatozoa assessing the membrane integrity of the cells [10]. Approximately equal volumes of semen and stain were mixed. One droplet of undiluted and well-mixed liquefied semen was mixed in a glass slide with one droplet of the eosin-nigrosine staining solution (50µl). After 2 minutes' incubation, slides were viewed under light microscopy at 400x. A total of 100 spermatozoa in duplicate from each sample were counted. Dead sperm appear pink; sperm without dye were counted as viable. For morphological evaluation, air-dried smears were stained with Papanicolaou stain. Sperm morphology was assessed in prefreeze whole semen specimens and in post thaw, post-wash specimens by Kruger's strict criteria [11].

### Hypo Osmotic Swelling (HOS) test

1ml of hypo-osmotic solution (150mOsm/L;0.025mM sodium citrate and 0.075mM D [12]-fructose) was added to 0.1ml suspension obtained from prefreeze whole semen specimens and in post thaw [13]. After incubation at 37 °C for 60 minimums of 200 spermatozoa per sample were examined by phase-contrast microscopy (Eclipse E-200, Nikon Co., Tokyo, Japan), and percentage of sperm with intact membranes was calculated.

### Sperm chromatin structure assay

Sperm DNA fragmentation was assessed using SCSA as previously described by Evenson [14]. Frozen semen samples were quickly liquefied in a warm water bath of 37 °C 100 l of sperm samples (2-3×10<sup>6</sup>spermatozoa/ml) were treated for 40s with 200µl of a pH 1.2 solution containing 0.1% Triton X-100, 0.15M NaCl and 0.08N HCl. Triton X-100 permeabilizes sperm cell membranes providing greater accessibility of acridine orange (AO) to DNA. The low pH solution partially denatures DNA in spermatozoa with abnormal chromatin structure. Spermatozoa with normal chromatin structure do not demonstrate DNA denaturation. After the 40 s acid treatment, 90µl of AO staining buffer (6µg AO/ml, 37m M citric acid, 126 mM Na<sub>2</sub>HPO<sub>4</sub>, 1mM disodium EDTA, 0.1mM NaCl, pH 6.0) was added to the cells before analyzing by flow cytometry. AO that intercalates into double-stranded DNA (native; normal) fluoresces green (515-530 nm) while AO that associates with single-stranded (denatured) DNA fluoresces red (≥630 nm) when excited by a 488 nm light source [15].

### Statistical Analysis

Results were expressed as mean ± SD. Sperm motility, motion characteristics, morphology abnormality, viability, and plasma membrane integrity were analyzed by "Two-way ANOVA" to determine significant differences between the groups. Spermatozoa with damaged DNA were evaluated by the chi-square test. A P value of less than 0.05 was considered significant. Statistical analyses were performed by using the SPSS version16 (SPSS, Chicago, IL, USA).

### Result

Sperm parameters distribution before and after freezing by Sperm Freeze Solution™ and GEYC were shown in Table 1. The total motile spermatozoa was decreased from %71.15 ± 11.91 in GEYC group to %42.00 ± 15.13 and from %71.15 ± 11.91 in sperm freeze group to %41.19 ± 16.00. The mean (SD) of change score in GEYC group was -29.14 ± 19.36 which was similar to the mean (SD) of change score in Sperm freeze group, -29.95 ± 15.38, (P=0.885). The progressive motile spermatozoa were decreased from %56.28 ± 10.24 to %23.52 ± 10.56 in GEYC group and to %27.83 ± 14.34 sperm freeze group. The percentage of change score regarding to progressive sperm motility was -32.75 ± 14.97 in the GEYC group and -28.44 ± 16.24 in sperm freeze group. The difference was not significant (P = 0.388). Sperm vitality decreased from %79.00 ± 10.99 in GEYC group to %51.10 ± 13.79 in sperm freeze group to %53.55 ± 12.95. There was no significant difference in the percentage change of sperm viability between GEYC -27.90 ± 16.61 and sperm freeze -24.42 ± 13.00 methods (P=0.473). The percentage of morphologically normal spermatozoa in 20 semen samples was reduced by 5.02 ± 22.35 and 5.01±22.35 after GEYC and sperm freeze methods respectively. A comparison of influence of two different freezing techniques on sperm morphology revealed no statistically significant differences between the freezing

techniques ( $P=0.990$ ). The average path velocity (VAP), curvilinear velocity (VCL) and linear velocity (VSL), compared with sperm freeze group, VCL, VAP and VSL more had fallen in group GEYC, but the differences were not statistically significant. The percentage of sperm membrane integrity (HOS test) was reduced by  $47.20 \pm (14.62)$  and  $50.15 \pm (12.42)$  after GEYC and sperm freeze methods respectively. A comparison of influence of two different freezing

techniques on sperm membrane integrity revealed no statistically significant differences between the freezing techniques ( $P=0.581$ ). The DNA fragmentation index was increased from  $14.35 \pm 7.20$  in GEYC group to  $17.40 \pm 8.48$  and  $16.50 \pm 7.89$  in sperm freeze group. The mean of change score regarding to DFI was  $3.05 \pm (2.58)$  in the GEYC group and  $2.15 \pm 1.72$  in sperm freeze group. The difference was not significant ( $P = 0.203$ ).

**Table 1:** note: GEYC= glycerol-egg-yolk-citrate; VCL= curvilinear velocity; VSL= straight-line velocity; VAP= average path velocity; DFI= DNA Fragmentation Index.  $P^* < .05$  was significant by ANOVA test.

Variables	Parameters								
	Total sperm motility (%)	Progressive sperm motility (%)	Sperm viability (%)	Sperm morphology (%)	Average path velocity ( $\mu\text{m/s}$ )	Curvilinear velocity ( $\mu\text{m/s}$ )	Straight linear velocity ( $\mu\text{m/s}$ )	Sperm membrane integrity (%) (host test)	DFI
Before freezing	$71.15 \pm (11.91)$	$56.28 \pm (10.34)$	$79.00 \pm (10.99)$	$50.49 \pm (22.34)$	$68.52 \pm (18.31)$	$100.13 \pm (18.38)$	$51.67 \pm (14.44)$	$69.95 \pm (11.19)$	$14.35 \pm (7.20)$
Post-thawing									
GEYC	$42.00 \pm (15.13)$	$23.52 \pm (10.56)$	$51.10 \pm (13.79)$	$3.05 \pm (3.51)$	$32.39 \pm (16.19)$	$57.83 \pm (28.16)$	$22.52 \pm (12.35)$	$47.20 \pm (14.62)$	$17.40 \pm (8.48)$
Sperm Freeze Solution™	$41.19 \pm (16.00)$	$27.83 \pm (14.34)$	$53.55 \pm (12.95)$	$3.35 \pm (3.78)$	$34.65 \pm (11.10)$	$62.71 \pm (31.17)$	$27.45 \pm (12.38)$	$50.15 \pm (12.42)$	$16.50 \pm (7.89)$
$P^*$	0.885	0.388	0.473	0.99	0.759	0.623	0.491	0.581	0.203

## Discussion

In the present study, we have shown that GEYC medium containing glycerol and egg yolk preserves sperm motility in post-thaw as the group of Sperm Freeze™ solution medium. In addition, post thaw-post wash viability and the percentage of spermatozoa with normal forms had no significantly difference in specimens cryopreserved in GEYC medium and in Sperm Freeze™ solution. However, we found that GEYC preserves sperm functions no difference with commercial sperm freeze medium for preserves sperm functions. Sperm Freeze™ manufactured by Ferti Pro is a ready-to-use HEPES buffered cryopreservation medium which also contains physiologic salts, glycine, dextrose monohydrate, lactate, glycerol, sucrose, and human serum albumin (3.95g/Liter) to protect sperm from damage due to the freezing procedure. Sperm Freeze™ is on the market since 1995 and is based on the formulation of the Human Sperm Preservation medium (HSPM) of Mahadevan [16]. Literature search over the years indicate that the safety and performance of Sperm Freeze™ are assured when using the product as described in the instructions for use. GEYC cryoprotectant (WHO manual, 5<sup>th</sup> edition) contains glucose, sodium citrate tribasic dihydrate, glycerol, glycine and egg yolk. The pH of this medium is 6.8-7.2. More, the pH release criteria of Sperm Freeze™ is 7.2-7.6. The pH of human semen is a matter of debate. There is considerable variation in the pH measurements reported by different researchers. Indeed, the pH of normal semen is slightly alkaline ranging from 7.2 to 8.5. During the evaluation of scientific

articles comparable devices of Sperm Freeze™ were considered. One of them is Quinn's advantage Sperm Freeze, manufactured by SAGE. Abush et al. [17] compared two different freeze media, i.e. Quinn's advantage Sperm Freeze and freezing medium-TYB which does contain egg yolk buffer (Irvine Scientific) and evaluated some basic sperm parameters [17]. The influence of the sample volume placed in the cryopreservation straws was also evaluated. When evaluating the effect of the freezing media, the investigators concluded that the motility and vitality was significantly better when freezing with Irvine Scientific medium, while no difference was found for progressive motility concentration. Again, an important note is that the sample size, especially for the group frozen with SAGE media, is very small. Consequently, the conclusions of this study should be evaluated with caution. Some clinics suggest that the medium containing egg-yolk citrate supplemented with glycerol increase the sperm survival after freezing [18], but in the literature there is no consensus of the effect of this cryoprotective medium. McLaughlin et al. [19] concluded that the toxicity of GEYC is responsible for about 50% of the loss of progressively motile spermatozoa during the complete cryopreservation process but has little effect on the quality of motility. According to the Handbook of intrauterine insemination (1997) the cryopreservation of very poor sperm should not be performed with an egg yolk-citrate containing medium [20]. The extraneous material derived from this medium could interfere. These results agree with those reported by others [17-20]. But it should be noted that the use of TEST-yolk buffer

for semen cryopreservation results in a higher recovery of motile sperm [18]. It also improves capacitation and sperm penetration into zona-free hamster oocytes [19,20]. Alteration in phospholipid: cholesterol ratio of the sperm membrane or the egg-yolk lipids' alteration may stabilize sperm membranes by reducing damage to them caused by free radicals [21]. A macromolecular interaction between egg yolk and seminal plasma proteins is another likely mechanism, cryopreservation with added egg yolk may increase stability in the membrane enzyme system, acrosin/proacrosin. The optimal concentration of glycerol for freezing human spermatozoa is from 2% to 10%. Nevertheless, extensive osmotic injury is caused by the addition and removal of glycerol, and current procedures for cryopreservation of human spermatozoa generally limit the concentration of glycerol to less than 0.8M [15]. Differences in the abilities of different cryoprotectant media to support sperm survival and preserve their fertilizing capacity are due to differences in the glycerol concentration in each medium [16]. GEYC buffer can also be used as a storage medium in the shipment of specimens to other laboratories for the sperm penetration assay and enables measurement of the functional integrity and fertilizing potential of the sperm. The extensive use of frozen spermatozoa in assisted reproductive techniques, together with the development of assisted fertilization using surgically retrieved spermatozoa, creates the need for additional studies to improve cryopreservation of human spermatozoa. In summary, semen specimens cryopreserved in GEYC buffer with glycerol had no different sperm motility, viability, percentage of normal sperm forms and DFI with specimens cryopreserved in sperm freeze but GEYC buffer is a good cryo preservative for long-term storage of human spermatozoa.

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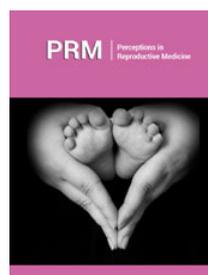
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