Comparison of Glycerol and a Zwitter Ion Buffer System as Cryoprotective Media for Human Spermatozoa

Effect on Motility, Penetration of Zona-free Hamster Oocytes, and Acrosin/Proacrosin

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This study compared the cryoprotective effect of glycerol with that of a zwitter ion buffer system (TESTCY). Spermatozoa that are cryopreserved in the presence of glycerol possess a somewhat higher progressive motility immediately after thawing than those preserved in the presence of TESTCY. However, after a 1-hour incubation in glycerol-free medium, the progressive motilities of the glycerol- and TESTCY-treated spermatozoa become essentially identical. After 2 hours in culture medium, TESTCY-treated spermatozoa possess a higher motility than glycerol-treated spermatozoa, indicating that TESTCY is a better preservative than glycerol for the long-term motility of human spermatozoa. The fertilizing potential of the cryopreserved spermatozoa was assessed by their ability to penetrate zona-free hamster oocytes *in vitro*. Spermatozoa that are cryopreserved in the presence of TESTCY produce three- to four-fold higher penetration rates than glycerol-treated, cryopreserved spermatozoa. Cryopreservation in the presence of TESTCY also results in a higher stability of the acrosin/proacrosin system than when the spermatozoa are preserved in glycerol, since about two- to three-fold higher levels of proacrosin are retained. These results indicate that TESTCY is a better cryopreservative for human spermatozoa than glycerol.

Key words: spermatozoa, human, cryopreservation, sperm motility, fertilization, acrosin.

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Bunge and Sherman (1953) reported the first pregnancy after insemination with frozen-thawed human spermatozoa. Since then, many studies have confirmed the fertility of cryopreserved human spermatozoa (eg, Behrman and Sawada, 1966; Sherman, 1973; Steinberger and Smith, 1973; Tyler, 1973; Jackson and Richardson, 1977; Leeton et al, 1980). However, it appears that the fertilizing potential of freshly ejaculated semen samples is higher than that of cryopreserved semen (Behrman and Ackerman, 1969; Ackerman and Behrman, 1975; Ansbacher, 1978; Amelar and Dubin, 1979; Quinlivan, 1979; Friedman and Broder, 1981). This may well be due to the inadequate protective effect of glycerol, the agent that is most often used for cryopreservation of human spermatozoa. For this reason, some clinicians prefer more complex cryoprotective media containing mixtures of citrate, egg yolk, glycerol and fructose (Behrman and Ackerman, 1969; Matheson et al, 1969) but good evidence that one is better than the other is lacking.

The efficacy of cryopreservative agents is almost always based on comparing the prefreeze sperm motility with the immediate post-thaw motility. However, such evaluations do not take into account other damage that may have been caused to the spermatozoa such as subtle membrane changes or an inactivation of the acrosomal en-

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zymes. Because of such damages, motile spermatozoa can lose their ability to become capacitated, undergo the acrosome reaction, and penetrate oocytes. For instance, it is well known that cryopreservation causes membrane damage in human spermatozoa (Pederson and Lebech, 1971; Schill and Wolff, 1974; Woolly and Richardson, 1978) and also results in the loss or inactivation of acrosin, an enzyme important for fertilization (Goodpasture et al, 1981). Improved test methods that have been employed include the following: 1) the cervical mucus penetration test *in vitro* (Fjallbrant and Ackerman, 1969; Zavos and Cohen, 1980); 2) determining the longevity of sperm motility (Keel and Karow, 1980); and 3) studying the effect of repeat freeze-thawing on motility (Ansari, 1976). However, these tests basically still evaluate only the motility of the spermatozoa.

The *in vitro* zona-free hamster egg test (Yanagimachi et al, 1976; Rogers et al, 1979) can be used to approximate the fertilizing capacity of human spermatozoa. Using this test, Fujita et al (1980), Binor et al (1980), Cohen et al (1981), and Jeyendran et al (1982) have shown a large decrease in the oocyte-penetrating capacity of human spermatozoa after cryopreservation even when glycerol is used as a protective agent. Cohen et al (1981) reported that with only two exceptions spermatozoa from all donors tested produced an average penetration rate of 80% before cryopreservation and 14% afterwards. Spermatozoa from the other two donors penetrated at near normal rates. It was further shown by this test that spermatozoa appear to become dependent on the glycerol and that removal of the glycerol from the medium surrounding the spermatozoa leads to a sharp decrease in their penetrating capacity (Jeyendran et al, 1982). It is most likely that glycerol is removed when freeze-thawed spermatozoa are artificially inseminated and pass through the cervical mucus (Hafez, 1976).

These results indicate that glycerol is not an optimal cryopreservative for human spermatozoa. Similar conclusions were reached in some nonhuman species, for instance the boar (Wilmut and Polge, 1977; Vazquez and Graham, 1980) and turkey (Marquez and Ogasawara, 1977). Therefore, a better preservative needs to be found. No improvement over glycerol was found when a medium consisting of glycerol, egg yolk, glucose, sodium citrate, glycine, and erythromycin (Matheson et al, 1969) was used to cryopreserve human spermatozoa as assessed by the zona-free hamster oocyte test (Cohen et al, 1981). However, a zwitter ion buffer system was reported to enhance the freeze-preservation of bull spermatozoa (Graham et al, 1972; Jeyendren et al, 1981). It was the objective of the present study to compare the relative cryoprotective efficacy of this zwitter ion buffer system (with and without glycerol) to that of glycerol in regard to the longevity of sperm motility, the ability of the spermatozoa to penetrate denuded hamster oocytes, and the sperm acrosin and proacrosin levels.

Materials and Methods

Semen was obtained by self-masturbation from healthy volunteers after three to five days of sexual abstinence. For the cryopreservation of semen, three types of preservative media were used: 1) glycerol only (10% v/v with the ejaculate); 2) TESTCY, a zwitter ion buffer system prepared by titrating a 325 mOsm aqueous solution of N-tris (hydroxymethyl) methyl 2-amino ethane sulfonic acid (TES) with a 325 mOsm aqueous solution of tris (hydroxymethyl) amino methane (TRIS) to pH 7.0, after which 12.5% 325 mOsm sodium citrate and 17.5% egg yolk were added (v/v); the mixture was subsequently centrifuged at 10,000 g for 10 minutes, the supernatant filtered and used; 3) a mixture of TESTCY and glycerol. The ejaculate was either diluted two-fold with TESTCY only (in preservative medium 2); or, after the two-fold dilution with TESTCY, glycerol was also added so that the final concentration of glycerol was 10% (v/v) (in preservative medium 3).

In order to compare the effectiveness of the cryopreservative media to maintain sperm motility and the ability of the spermatozoa to become capacitated and penetrate oocytes, ejaculates known to have good progressive sperm motility and high oocyte penetrating capacity were mixed and divided into three portions. Each portion was treated with one of the media as described above and cryopreserved. To do so, each portion was slowly cooled at a rate of 0.5 C/minute to 5 C and 0.1 ml aliquots were frozen on dry ice into pellets (Nagase and Niwa, 1964). After 10 minutes or more on dry ice, the frozen pellets were plunged into liquid nitrogen and stored for 30 minutes or more at −196 C. The frozen semen was thawed by placing the pellets in aluminum weigh boats that were floated on water at 37 C.

For the acrosin and proacrosin studies, only glycerol and TESTCY (without glycerol) were compared, with the semen divided into two portions. Aliquots of each portion were not only frozen by the pellet method but also by using straws and ampules. For the latter, 0.5 ml aliquots of the extended semen were drawn into straws or 1 ml aliquots were placed into ampules that were subsequently sealed. These were then cooled to 5 C, frozen in liquid nitrogen vapor, and then stored in liquid nitrogen.

The motility of the spermatozoa was evaluated by diluting the semen 1:20 with the medium that had been used as cryopreservative and filling a Neubauer haemocytometer. The haemocytometer was placed in a moist atmosphere and the semen sample allowed to stabilize for at least 3 minutes before observation. Since the spermatozoa with forward movements are most likely the most important ones from the standpoint of fertility, the percent spermatozoa with progressive motility was calculated. The number of spermatozoa with forward movements was divided by the total number of spermatozoa (motile and immotile) and multiplied by 100.

The ability of the frozen-thawed spermatozoa to penetrate denuded hamster oocytes was assessed as published previously (Van der Ven et al, 1982) by a minor modification of earlier methods (Rogers et al, 1979; Binor et al, 1980). Briefly, the spermatozoa were washed after addition of modified Biggers, Whitten, and Whittingham's medium containing 35 mg/ml human serum albumin (Overstreet et al, 1980) ("culture medium"), by centrifugation at 500 g for three minutes. The precipitated spermatozoa were suspended in culture medium and washed two more times. The sperm concentration was adjusted to 100×10^6 per ml in the culture medium and the mixture was incubated for 2 to 3 hours at 37 C. Aliquots containing 2×10^6 spermatozoa (0.02 ml) were then mixed with denuded hamster oocytes in 0.18 ml culture medium and incubated for another 5 hours. The oocytes were removed, washed, fixed, stained with aceto-lacmoid and examined microscopically for sperm penetration. Oocytes were considered penetrated when a swollen sperm head or a male pronucleus with a corresponding sperm tail was found within the ooplasm.

Acrosin and proacrosin levels of spermatozoa were assayed by the method of Goodpasture et al (1980) with minor modifications. Briefly, the cryopreserved semen containing $50-100 \times 10^6$ spermatozoa was layered on an 11% Ficoll gradient containing 0.1 M sodium phosphate buffer at pH 7.4, 50 mM benzamidine, and 0.02% sodium azide. The spermatozoa were then separated from the seminal plasma and cryopreservative by centrifugation at 1000 g for 30 minutes at 4 C and the supernatant was decanted. The sperm pellet was resuspended in 0.5–1.0 ml of a 1.0 M glycine, pH 2.8 buffer containing 50 mM benzamidine and incubated overnight at 4 C. The extracts were finally recentrifuged at 1000 g for 30 minutes at 4 C and the supernatants dialyzed for more than 6 hours against 1 mM HCl (pH 3) at 4 C. Nonzymogen acrosin, total acrosin and proacrosin levels were calculated by incubating the extract at pH 8.0 for 15 minutes and measuring the rates of α-N-benzoyl-L-arginine ethyl ester substrate hydrolysis of the extracts before and after spontaneous conversion of the zymogen into acrosin as described by Goodpasture et al (1980).

The data were subjected to an analysis of variance and, where appropriate, to Tukey's honestly significant difference test (Snedecor and Cochran, 1980).

Results

The immediate post-thaw progressive motility of the spermatozoa was higher when glycerol or TESTCY + glycerol were used as cryopreservatives than when TESTCY alone was employed (Table 1). In order to determine if the same was true for the longevity of sperm motility, the frozen-thawed spermatozoa were diluted four-fold with either TESTCY only or TESTCY + 10% glycerol, and observed after incubation for 1 hour at 37 C. With TESTCY + glycerol as diluent, the motility of the spermatozoa originally frozen in the presence of glycerol or TESTCY + glycerol remained higher than that of spermatozoa cryopreserved in the presence of TESTCY alone. However, when TESTCY was used as diluent, the motility of the glycerol- and TESTCY + glycerol-cryopreserved spermatozoa decreased more rapidly than that of spermatozoa cryopreserved with only TESTCY, so that after 1 hour, all samples possessed approximately equal motility (Table 1).

The ability of glycerol-treated, cryopreserved spermatozoa to penetrate denuded hamster oocytes was poor (Table 2). The penetration rate averaged 12.9 ± 0.5% which is similar to that found by Cohen et al (1981) for glycerol-treated, cryopreserved spermatozoa (14%) if the two exceptions with high penetration rates are omitted. Such exceptions did not occur in the present study. When TESTCY + glycerol was used as preservative, the penetration rate tended to be improved but was not significantly higher than that of the glycerol-treated spermatozoa. However, with TESTCY as cryopreservative, the penetration rate improved significantly and was almost four-fold higher than

TABLE 1. Progressive Motility of Human Spermatozoa After Cryopreservation*

Cryoprotective Medium	% Spermatozoa with Progressive Motility: Immediately Post-thaw	1 Hour Postincubation:† TESTCY‡	1 Hour Postincubation:† TESTCY + glycerol
Glycerol§	$45 \pm 6^{b\|}$	22 ± 5^{c}	$35 \pm 6^{a,b}$
TESTCY + glycerol¶	43 ± 5^{b}	23 ± 6^{c}	$35 \pm 5^{a,b}$
TESTCY¶	31 ± 5^{a}	24 ± 6^{c}	26 ± 6^{c}

* Number of sperm samples = 7.

† 1:4 dilutions.

‡ Zwitter Ion Buffer System: 325 mOsm solution of TES titrated with 325 mOsm Tris to pH 7.0 after which 12.5% 325 mOsm sodium citrate and 17.5% egg yolk are added. After centrifugation the supernatant is filtered and ready for use.

§ 10% v/v with ejaculate.

‖ Mean ± S.E.M. Values with common superscript are not significantly different from each other ($P > 0.05$).

¶ The ejaculate was diluted 2-fold with TESTCY. For the TESTCY + glycerol medium, glycerol was added to produce a 10% v/v glycerol concentration.

TABLE 2. Oocyte Penetrating Capacity of Cryopreserved Spermatozoa*

Cryoprotective Medium	Number of Oocytes	% Oocytes Penetrated	Number of Spermatozoa Attached to Eggs	Number of Spermatozoa and Male Pronuclei in Ooplasm
Glycerol†	160	12.9 ± 0.5[a]‡	28.0 ± 8.9	1.0 ± 0
TESTCY§ + glycerol	165	22.3 ± 2.3	37.2 ± 2.3	1.3 ± 0.1
TESTCY§	170	46.7 ± 6.4[a]	42.5 ± 8.2	1.3 ± 0.1

* Number of sperm samples = 7.

† 10% v/v with ejaculate.

‡ Values represent mean ± S.E.M. Means with common superscripts are significantly different from each other ($P < 0.05$). Untreated unfrozen spermatozoa gave a penetration rate of 75.4 ± 14.3%; with 20.8 ± 2.0 sperm attached per oocyte and 2.0 ± 0.2 sperm that had entered the ooplasm.

§ TESTCY = Zwitter Ion Buffer System: 325 mOsm aqueous solution of TES titrated to pH 7.0 with 325 mOsm Tris, after which 12.5% mOsm sodium citrate and 17.5% egg yolk are added v/v. After centrifugation the supernatant is filtered and ready for use. The ejaculate was diluted two-fold by the TESTCY. When combined with glycerol, for the TESTCY + glycerol medium, the concentration of glycerol was 10% v/v.

that of glycerol-treated, cryopreserved spermatozoa.

Even though the penetration rate of the glycerol-treated, cryopreserved spermatozoa was poor, a large number of spermatozoa had attached to the oocyte membrane (Table 2). This number tended to be even higher when TESTCY + glycerol or TESTCY were used as cryopreservative but the difference was not statistically significant. Almost no difference was seen in the number of decondensed sperm heads and/or male pronuclei that were present in the ooplasm among the three cryopreservatives (Table 2).

The progressive motility of the spermatozoa used in the *in vitro* hamster oocyte assay was also monitored (Table 3). As had been found previously (Table 1), the immediate post-thaw motility of the glycerol or TESTCY + glycerol, cryopreserved spermatozoa was somewhat better than that of the spermatozoa preserved in the presence of TESTCY. However, after being washed to remove the seminal plasma and excess cryopreservative, and after incubation for 2 hours in culture medium, the motility of the TESTCY-treated, cryopreserved spermatozoa was almost two-fold higher than that of spermtozoa cryopreserved in the presence of glycerol.

In regard to the acrosin and proacrosin content, essentially the same results were obtained whether the spermatozoa were pellet frozen (n = 3), frozen in straws (n = 10) or in ampules (n = 5). When glycerol was used as cryopreservative, the total levels of acrosin were, respectively, 23.3 ± 1.2, 27.6 ± 12, and 25.6 ± 9.9 in mIU/10^7 spermatozoa of which, respectively, 44.9 ± 7.4, 49.7 ± 16.8, and 54.1 ± 7.6% was in proacrosin form. By contrast, if TESTCY was used as cryopreservative, the total levels of acrosin were, respectively, 70.1 ± 19.4, 63.5 ± 39.1, and 74.6 ± 32.2 in mIU/10^7 spermatozoa of which, respectively, 87.9 ± 8.3, 78.6 ± 14.7, and 83.6 ± 10.4% was in proacrosin form. The results are summarized in Table 4. No difference was seen in the amount of free (nonzymogen)

TABLE 3. Progressive Motility of Cryopreserved Spermatozoa Tested in the *In Vitro* Zona-free Hamster Oocyte Assay

	% Spermatozoa with Progressive Motility*	
Cryoprotective Medium	Immediately Post-thaw	After 2 Hrs of Incubation in Culture Medium†
Glycerol‡	43 ± 3§	12 ± 3
TESTCY‖ + glycerol	45 ± 3	19 ± 4
TESTCY‖	36 ± 3	21 ± 2

* The progressive motility of spermatozoa before cryopreservation was 73 ± 3%.

† These same sperm samples were used to determine their capacity to penetrate denuded hamster oocytes (Table 2). N = 7.

‡ 10% v/v with ejaculate.

§ Mean ± S.E. All values in the second column differ significantly from their corresponding values in the first column ($P < 0.05$).

‖ TESTCY = Zwitter Ion Buffer System: 325 mOsm aqueous solution of TES titrated to pH 7.0 with 325 mOsm TRIS. 12.5% mOsm sodium citrate and 17.5% egg yolk (v/v) added and the solution centrifuged, after which the supernatant is filtered and ready for use. The ejaculate was diluted 2-fold with TESTCY; when combined with glycerol, for the TESTCY + glycerol medium, the proportion of glycerol in the medium was 10% v/v.

TABLE 4. Acrosin/Proacrosin Levels of Cryopreserved Spermatozoa*

Cryoprotective Medium	m IU Acrosin/10^7 Spermatozoa† Nonzymogen	Total	Percent of Total Acrosin as Proacrosin†
Glycerol‡	12.5 ± 0.3	25.5 ± 1.2^{a}	49.6 ± 2.7^{b}
TESTCY§	10.1 ± 1.4	69.4 ± 3.2^{a}	83.4 ± 2.7^{b}

* Number of sperm samples = 18.

† Means ± S.E. Values with common superscript in a column are significantly different from each other ($P < 0.01$).

‡ 10% v/v with ejaculate.

§ TESTCY = Zwitter Ion Buffer System: 325 mOsm TES titrated to pH 7.0 with 325 mOsm TRIS; 12.5% sodium citrate and 17.5% egg yolk (v/v) added; solution centrifuged and supernatant filtered.

acrosin on the spermatozoa but, due to the much higher levels of proacrosin, two- to three-fold higher amounts of total acrosin were found in TESTCY-treated spermatozoa as compared to those treated with glycerol.

Discussion

Our results confirm our previous data and those of others (Binor et al, 1980; Cohen et al, 1981; Jeyendran et al, 1982) that glycerol-treated, cryopreserved spermatozoa are only poorly able to penetrate denuded hamster oocytes even though the spermatozoa possess reasonably good immediate post-thaw motility. It is clear that the immediate post-thaw motility of the spermatozoa is inadequate in assessing their oocyte-penetrating capacity. A somewhat better correlation between motility and oocyte penetration appears to be present when the longevity of the spermatozoa is assessed after washing them and incubating them for 2 hours in a culture medium. Even so, a direct correlation is absent as indicated by the different ratios in % motility (Table 3) and % penetration (Table 2) of the glycerol- vs. TESTCY-treated spermatozoa. Additionally, previous experimentation has shown that even if the number of glycerol-treated, cryopreserved spermatozoa with forward progression is adjusted to equal that of untreated spermatozoa, the penetration rate of the cryopreserved spermatozoa is still greatly reduced (Jeyendran et al, 1982). As could have been expected, the hamster oocyte assay measures other parameters besides motility, such as the ability of spermatozoa to become capacitated, bind to the vitelline membrane, undergo the acrosome reaction and then pass through the vitelline membrane.

As had also been concluded earlier (Jeyendran et al, 1982), spermatozoa appear to develop a dependency on glycerol, both in regard to motility and oocyte penetration. The present data confirm that as long as this compound is present in the surrounding medium, sperm motility is retained fairly well, but when the glycerol is removed, motility decreases much more rapidly. It can be expected that the spermatozoa undergo a similar "washing" procedure when they pass through the cervix because the fluid portion of the ejaculate is excluded by the cervical mucus (Hafez, 1976). It may be possible to minimize the dependency of the glycerol-treated, cryopreserved spermatozoa on glycerol by gradual removal of this agent through repeated washing of the glycerol-treated spermatozoa in media containing decreasing amounts of glycerol. However, this procedure imposes an additional stress on the cryopreserved spermatozoa which may reduce their fertilizing capacity.

Human spermatozoa normally average 106 ± 22m IU/10^7 spermatozoa of total acrosin, of which 93 ± 2% is in proacrosin form (Goodpasture et al, 1980). Confirming our previous results (Goodpasture et al, 1981), cryopreservation with glycerol as protective agent caused a two- to three-fold decrease in the total acrosin levels. The decrease is primarily due to an inactivation of proacrosin or an inability of proacrosin to become converted to acrosin. It is known that ejaculated spermatozoa from men with infertile marriages average approximately half the amount of total acrosin and proacrosin than asymptomatic individuals (Goodpasture et al, 1982; Mohsenian et al, 1982). Further, the addition of inhibitors of acrosin or proacrosin activation to human spermatozoa ultimately prevents their penetration of denuded hamster oocytes (Van der Ven et al, 1981; Kennedy et al, 1982). Although acrosin is usually thought to be involved in the processes leading to sperm passage through the zona pellucida, the enzyme also appears to be necessary for sperm penetration into denuded, or zona-free, eggs (Wolf, 1977; Fraser, 1982), probably due to its role in capacitation and the acrosome reaction (Meizel and Lui, 1976; Perreault et al, 1982). The large decrease in the acrosin and proacrosin levels of glycerol-treated, cryopreserved spermatozoa is of serious concern and may, at least in part, be responsible for their poor *in vitro* oocyte penetrating capacity as well as their reduced *in vivo* fertility.

From these observations it appears that glycerol is not an ideal cryopreservative and a different protective agent/medium should be found if improved fertilization rates are to be obtained. Most of the published literature agrees that the *in vitro* zona-free hamster oocyte test fairly closely reflects the *in vivo* fertilizing potential of a semen sample although exceptions are certainly present (Rogers et al, 1979; Binor et al, 1980; Overstreet et al, 1980; Hall, 1981; Cohen et al, 1982; Stenchever et al, 1982). If the results obtained *in vitro* hold true *in vivo,* TESTCY appears to be a much better cryopreservative than glycerol. Use of TESTCY compared to glycerol resulted in an almost four-fold higher oocyte penetration rate for TESTCY-cryopreserved spermatozoa, an almost two-fold increase in sperm longevity when incubated for 2 hours in culture medium, and an almost three-fold increase in activatable proacrosin levels.

The addition of glycerol to TESTCY reduced the cryopreservative effect so that the oocyte penetration rate was not significantly different from that obtained with glycerol only as preservative. Apparently, the negative effects of glycerol overshadow the positive effects of TESTCY, possibly due to the dependency on glycerol that the spermatozoa develop. This may explain the results by Cohen et al (1981) who found no improvement in the hamster oocyte penetration rate when an egg yolk-citrate extender was compared to glycerol because the extender also contained glycerol. It appears that glycerol is better omitted from media used to cryopreserve spermatozoa.

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