Effect of Citrate, Tris and Earle's Salt Solutions in Cryopreservative Media on the Percentage of Postthaw Motility and Cryosurvival Rate of Human Sperm

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Abstract : *The effects of citrate, Tris, and Earle's salt solution in cryopreservative media on the postthaw motility and cryosurvival rate of human sperm were compared. Each of eighty two ejaculates from donors and husbands was divided into three aliquots. Each aliquot was mixed with one of the three different media : 1) Citrate-egg yolk-glycerol, 2) Tris-egg yolk-glycerol, 3) Earle's salt solution-egg yolk-glycerol. The mixtures were vapour frozen, and stored at - 196°C. The percentage of sperm motility and cryosurvival rate were compared 30 minutes after thawing. Average percentage of sperm motility and cryosurvival rate after thawing for citrate, Tris, and Earle's salt solution were 26.4 ± 13.1%, 44.2 ± 15.1%, 25.7 ± 11.6%, 42.7 ± 14.6% and 27.8 ± 17.2%, 54.3 ± 15.6%, respectively. No statistically significant differences were found in sperm motility and cryosurvival rate obtained following thawing according to three media.(Thai J Obstet Gynaecol 1995;7:15-23.)*

Keywords : citrate / tris / Earle's salt solution, cryopreservative media, postthaw motility, cryosurvival rate.

The freezing and thawing of human sperm is undoubtedly associated with a decrease in sperm quality. The degree of impairment, however, is dependent on several factors. A major influence on cryosurvival of human sperm are the rate of freezing[1] and the nature of cryopreservative medium.[2] Currently, several types of cryopreservative media are employed with human sperm. Glycerol was firstly introduced[3] and due to its technical simplicity is still the most widely used cryoprotectant for freezing human sperm. Different studies have revealed the necessity[4-5] and the efficiency of glycerol compared to other cryoprotectants.[6-8] The addition of extenders, such as egg yolk and buffer systems, further provides a

better cryosurvival and motility.[6,9-13]

A more complex medium containing citrate-egg yolk-glycerol later was reported to result in higher human sperm cryosurvival when compared to glycerol alone.[9,14-15] Tris (hydroxymethyl) aminomethane (Tris)-egg yolk-glycerol was a traditional media developed and noted optimal results when freezing bovine sperm.[16] Recently, Earle's salt solution has been used in several different tissue culture systems, but it has never been used as a buffer system in cryopreservative media of human sperm.

Unfortunately, no comparative study has been conducted using all of these buffer systems to determine which cryopreservative medium is the best for postthaw motility and cryosurvival rate of human sperm. Therefore, the present study was conducted to compare the cryopreservative efficiencies of these three cryoprotective buffer systems.

Materials and Methods

To determine the optimal cryopreservative media for freezing human sperm, three cryopreservative buffers were used to freeze semen samples. Samples were collected from 82 fertile donors and patients attending infertility clinic at Chulalongkorn Hospital by masturbation into sterile glass containers after 3-5 days of sexual abstinence. Each sample was allowed to liquefy in room temperature for 30 minutes. The semen was examined to determine the sperm motility according to WHO guideline.[17] Following a prefreeze semen analysis, each semen sample was divided into three portions. Each portion was mixed with one of the media and cryopreserved as described below. Samples were thawed 1 week after freezing and analyzed for the percentage of postthaw sperm motility and sperm cryosurvival rate 30 minutes after thawing by an experienced technician. The sperm cryosurvival rate was calculated from the percentage of postthaw motility / the percentage of prefreeze motility x 100. Estimates were made in triplicate.

Preparation of Cryoprotective media

The following three buffer systems were prepared as cryopreservative media for human sperm : 1) citrate-egg yolk-glycerol : sodium citrate 70% (v:v), fresh egg yolk 20% (v:v), glycerol 10% (v:v), and Kanamycin sulfate 0.05 mg/L; 2) Tris-egg yolk-glycerol : Tris (hydroxymethyl) amino-methane 70% (v:v), fresh egg yolk 20% (v:v), glycerol 10% (v:v), and Kanamycin sulfate 0.05 mg/L ; 3) Earle's salt solution-egg yolk-glycerol : Earle's salt solution 70% (v:v), fresh egg yolk 20% (v:v), glycerol 10% (v:v), and Kanamycin sulfate 0.05 mg/L. The detailed composition of three buffer Systems were shown in Table 1 Sterile glassware, vials and pipettes were used and care was taken to avoid contamination of media. Eggs were purchased farm-fresh.

Table 1 *The detailed composition of thrce buffer systems*

Sodium citrate solution

Sodium citrate	7.25 g. in 250 ml. water
Glucose	13.65 g. in 250 ml. water
Fructose	13.65 g. in 250 ml. water

Earl's salt solution

Substance	M.M	g/L
$CaCl_2 . 2H_2O$	1.75	0.2649
KCl	5.37	0.004
$MgSO_4 . 7H_2O$	0.89	0.220
NaCl	116.34	6.800
$NaH_2PO_4 . 2H_2O$	1.02	0.1583
Glucose	5.55	1.0
Phenol Pred	0.048	0.017
$NaHCO_3$	10.00	0.85

Tris solution

Tris	30.28	g.
Citric acid	17.00	g.
Fructose	12.50	g.
Demineralized water	920	ml.

Freezing Technique

Each medium was added slowly over a 10 to 15 minute period to a portion of semen sample. Samples with a sperm density > 60 x 10^6 / ml were diluted at a ratio of 1:1 with the cryopreservative medium. Those with a density < 60 x 10^6 were diluted at a ratio of 3:1. The mixtures were packaged in 0.5 - ml plastic straws and then ends were sealed with polyvinyl cement. The straws were frozen by placing the straws individually on a horizontal rack situated 3cm above the liquid nitrogen (static vapor freeze at - 120°C). After 10 minutes, the straws were plunged into liquid nitrogen for storage at - 196°C.

Thawing Technique

The straws were thawed by removing them from the liquid nitrogen container and keeping them at room temperature (30°C) for 30 minutes.

Statistical Analysis

The sperm motility and cryosurvival rate were analyzed by two-

way analysis of variance (ANOVA), using the SPSS/PC+ statistical package (SPSS Ins., Chicago, USA) with respective to the cryopreservative media and the identity of the semen. The differences of $p<0.05$ were considered to be significant.

Results

The results are summarized in Table 2. Semen preserved with Earle's salt solution-egg yolk-glycerol showed higher cryosurvival than semen preserved with the rest of cryopreservative media, although the differences among the three media were not significant after thawing.

Table 2 ***Effect of citrate, Tris, and Earle' salt solution in cryopreservative media on the percentage of postthaw motility and cryosurvival rate of human sperm.***

Cryopreservative Buffers	Prefreeze Motility (%)	Postthaw Motility (%)	Cryosurvival Rate (%)
Citrate	58.6 ± 12.5 (35 - 80)	26.4 ± 13.1 (10 - 55)	44.2 ± 15.1 (28.6 - 68.7)
Tris		25.7± 11.6 (10 - 50)	42.7 ± 14.6 (28.6 - 62.5)
Earle's Salt Solution		27.8 ± 17.2 (10 - 60)	54.3 ± 15.6 (28.6 - 75.0)

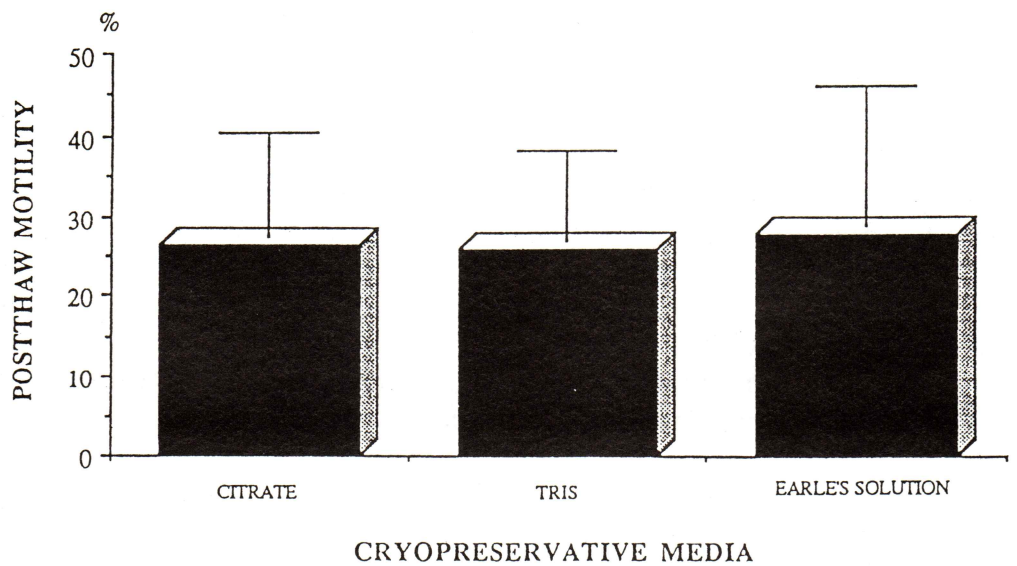

Fig. 1 Effect of Cryopreservative Media on Postthaw Motility

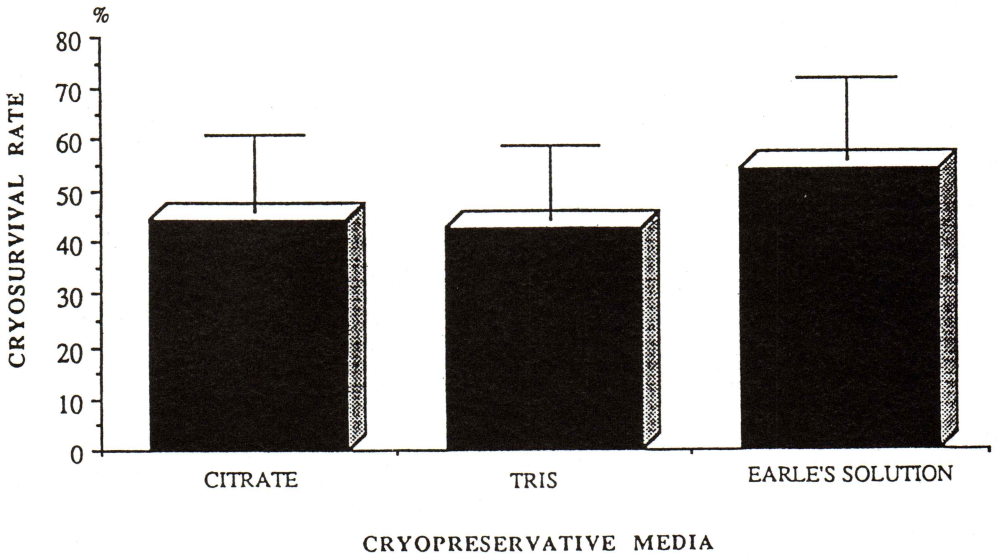

Fig. 2 Effect of Cryopreservative Media on Cryosurvival Rate

The percentage of postthaw motility and cryosurvival rate of cryopreserved sperm frozen in three different cryopreservative media are shown in Figure 1 and Figure 2, respectively. There were no statistical differences in the percentage of postthaw motility and cryosurvival rate of the cryopreserved sperm when they were frozen in three media.

Discussion

The freezing of sperm is a long-established technique and has been the subject of study for many years. Freezing of human sperm without a cryopreservative media results in severe damage to the sperm cells. Damage to the sperm cells during the freezing procedure have been demonstrated with transmission electron microscopy. When no cryopreservative medium is used the acrosome is found to be ruptured and their fine structure has been lost. Therefore, in all experiments on sperm freezing some type of cryopreservative medium has to be added to the ejaculate before freezing. Glycerol has been the main component in these media. Much efforts have been made to reduce this impairment in order to recover as many as possible progressively motile and morphologically normal sperm such as the addition of egg yolk and buffer systems to the cryopreservative media and freezing rate studies.[18]

A number of different cryopreservative media have been described for the conventional cryopreservation of human sperm based on the use of glycerol as the principal, permeable cryoprotectant in the presence or absence of extenders, which

include citrate, Tris, or other solutions, often in the presence of nonpermeable macromolecules such as egg yolk or albumin. It is possible that a medium containing egg yolk offers extra protection when freezing rates cannot be strictly controlled nor set at the optimal rates.[19]

Lipoproteins contained in the egg yolk reportedly protect sperm against cold shock[6,20], but may provide little protection during the periods of extracellular and intracellular ice formation. Cryoprotectants are required during these periods to protect sperm from the damaging effects of increasing salt concentrations and ice crystal formation. Comparative studies evaluating cryoprotectants are available[6,12], which allow several general conclusions

(1) A final concentration of glycerol between 5 and 10% with an equilibration time of 0-15 minutes at ambient temperature produces optimal results. The concentration of glycerol required for optimal survival of human sperm following cryopreservation is dependent on the type of medium used for freezing.[5]

(2) Common protocols include the direct addition of glycerol and the use of combinations of glycerol and extenders such as Tris in egg yolk buffer, usually added to semen at a 1:1 ratio. In order to minimize semen dilution inherent in the use of extenders, which will decrease the motile sperm available per insemination dose, the addition of 3 parts semen to 1 part glycerol plus extender can be employed.

Postthaw motility was found to be more highly correlated with pregnancy rates following therapeutic insemination of cryopreserved semen than prefreeze seminal quality (seminal volume, total sperm count, motility, morphology), postthaw motile sperm per inseminate or decrease in motility during cryopreservation.[21,22] Therefore, postthaw motility may be used as a clinical marker of freezing quality following observance of a significant correlation ($r = 0.98$) between postthaw motility and sperm with intact heads.[8] Although motility is not completely related to fertilizing capacity, it is generally accepted to be a sensitive parameter for evaluating freeze-thawing success.[23] Cryosurvival rate is also a sensitive parameter to assess the efficacy of cryopreservative process.

Freezing of the sperm after addition of a cryopreservative media, these solutions result in a much better preserved motility. In this study, three cryopreservative media have been compared under uniform conditions. Semen preserved with Earle's salt solution-egg yolk-glycerol showed higher cryosurvival rate and postthaw sperm motility than semen diluted with citrate-egg yolk-glycereol or Tris-egg yolk-glycerol, although the differences among these media were not significant. Previous studies using cryopreservative media for sperm cryopreservation has shown encouraging results and good postthaw motility of the spermatozoa has been ob-

tained.[24]

The choice of a cryopreservative medium for sperm should based on its ability to maintain the integrity and function of the cell during the freeze and thaw process. In the present study, we have shown that the percentage of postthaw sperm motility and cryosurvival rate were similar in all treatment groups.

The liquid nitrogen vapour technique introduced by Sherman in the early 1960s is still widely employed. This procedure involves sample exposure to liquid nitrogen vapours (-120°C) with or without the inclusion of a intermediate hold, typically at or near 4°C. the procedure is conducted without seeding, involves cooling rates of the order of 10-25°C/min depending upon the container and volume. Controlled-rate freezing (CRF) has gained popularity with increased equipment availability but has not yet proven superior for the cryopreservation of human semen. Theoretical advantages associated with the use of CRF include a more precise and reproducible cooling rate, which should lead to more effective, uniform dehydration and the prevention of intracellular ice crystal formation.[13] Although several investigators have described computer-controlled freezing methods as preserving sperm quality better than vapour freezing methods as preserving sperm quality better than vapour freezing[7,25-27], there is still controversy in the literature about its beneficial effect[10,13,28], at least for human sperm. On the other hand possible differences in cryopreservation efficiency have to be weighed against the differences in costs, time of execution and practical implication. In our hospital, liquid nitrogen vapour technique still has been used for sperm cryopreservation because it is rapid and relatively inexpensive.

The increasing risk of transmission of HIV and the concomittant risk of acquired immunodeficiency syndrome (AIDS), has now made the use of fresh donor semen unacceptable. The reported case of HIV being transmitted to recipients of fresh donor semen in a donor insemination (DI) programme in Australia underlines the risk involved.[29] Recently recommendations by the American Association of Tissue Banks and Centers for Disease Control that frozen semen be used for therapeutic insemination donor (TID) to decrease the likelihood of disease transmission[30], have resulted in renewed interest in developing better procedures and cryopreservative media for freezing human semen. Scant attentions have been given to the presence of interactions among various cryopreservation methodologies and cryopreservative media in developing optimal procedures for freezing human sperm. In contrast, the importance of these interactions in developing effective methods for freezing bovine semen is well known.

In conclusion, semen preserved with Earle's salt solution-egg yolk-glycerol showed higher cryosurvival than semen preserved with the rest of

cryopreservative media, although the differences among the these media were not significant after thawing. Further study is warranted to determine the effect of these cryopreservative media on fertilizing ability.

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