CRYOPRESERVATION AND THAWING OF HUMAN SEMEN

by

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SUMMARY

This study to find out Cryosurvival of human spermatozoa was undertaken to find out suitable method of cryopreservation and thawing of human aspermatozoa with the help of presently available equipment in India. With the advent of In-Vitro Fertilization and Embryo-transfer (test-tube baby) and newer efforts in pre-selection of sex of incoming child by segregation of x and y sperms, Cryo-preservation of semen has become an important tool, apart from its utility in Semen Bank and Artificial Insemination.

Semen samples obtained from healthy donors were transferred to the laboratory without any dilution and evaluated for count and motility and diluted with cryopreservative media. After achieving +4°C temperature, diluted semen was filled up in plastic straws. Rapid cooling of straws was done by exposing them to liquid Nitrogen Vapour for 10 minutes and then dipping them into liquid Nitrogen. These straws can be stored for almost indefinite period in Liquid Nitrogen.

After thawing i.e. warming up the semen to +37°C, motility was evaluated and compared with pre-freeze motility. The results were tabulated.

Introduction

From the begining, it has been man's wish to find out origine of life and if that is not possible, to preserve the existing life for the indefinite period. The main object of deep freezing is to arrest the process of life by decreasing metabolic needs to bear minimum, so that cell organs can be preserved for a long time. Though survival of sperms at -17°C was detected by Devenport (1897), the detailed work started only after 1940. Shettles (1940) was first to describe freezing of human semen with partial success, while Parke (1945) successfully froze human semen. First Artificial Insemination in Cattles was done by Stewart (1951). Though 6 pregnancies with frozen human semen of

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5 months were reported by Perloff *et al* as early as 1964, little progress was there because of questionable legal status of sperm bank and Artificial Insemination.

With the advent of *In-Vitro* Fertilization and Embryo-Transfer (I.V.F.+E.T.) and newer interest in pre-selection of sex of incoming child by segregation of X and Y sperms, Cryo-Preservation of human semen has become an important tool. Mahadeven *et al* (1983) has reported successful use of human semen Cryo-banking for I.V.F., as many a times it is not possible to get freshly ejeculated donor semen at very short notice. It also provides basis for cryo-preservation of human embryo and even human pregnancies following transfer of frozen-thawed embryo have been reported. (Trounson and Mohr 1983; Zeilmaker *et al* 1984).

This study to find out Cryosurvival of human spermatozoa was undertaken so as to find out ideal method of Cryo-preservation and thawing of human spermatozoa under Indian conditions using presently available facilities, without the help of expensive instrumentation. The study was conducted at Sabarmati Ashram Gaushala, Bidaj, which is about 30 kilometers from Ahmedabad, where Cryo-preservation of cattle semen is carried out regularly.

This study was carried out only to find out Cryo-survival of human sperms. The main hurdles were as follows:

1. Transfer of human semen to laboratory, involved transit time of more than one hour.

2. Non-availability of liquid Nitrogen freezer which can decrease the temperture from room temp. to -196°C. programmably, i.e.: from room temp. to +4°C at the rate of 1°C/1 min. +4°C to -30°C at 7°C/min. and then rapidly to -196°C (Planner R204 Embryo/Sperm Freezer).

Materiales and Methods

Samples are evaluated with regards to sperm count, motility, morphology and pus cells, and kept in incubator at 37°C. Dilution of media is done with Cryo-Preservative media containing cryo-protectant like glycerol etc. which protects the sperms against cold shock. Various media like Egg-yolk citrate, TRIS or HEPES buffer media, have been described. In our study we used Egg yolk citrate media. (Behrman and Sawada, 1966; Taylor *et al* 1982; Shah *et al* 1984).

Cryo-Preservative medi: (*C.P.M.*)

Contains 20% egg-yolk, 15% Glycerol, 26% glucose (5.5%) and 39% Sodium citrate of 2.9% strength.

Dilution of semen is done with above media in 1:1 ratio and allowed to equilibrate for 15 minutes. The diluted semen is cooled down to +4°C in cold handling cabinet at the rate of 2° to 3°C/minute. (cabinet similar to that used for storage of blood bottles in Blood Bank). Diluted Semen is filled in plastic straws of 0.25 to 0.5 ml capacity having one end open and other end closed but porous (sterlized in ultraviolet light and kept alongwith the media in cold handling cabinet to decrease temp. to +4°C). Semen is sucked into the straws with vacuum filler machine or by manual suction.

The straws are sealed with special moisture solidifying powder (Polyvinyl cement. Straws are now arranged on a rack and are exposed to liquid nitrogen vapour for 10 minutes, 8 cms above the surface of liquid nitrogen (2 rack high) for 5 minutes and then 4 cms above the

surface for remaining 5 minutes. This cools the straws containing diluted semen to around -100°C.

The straws are now immersed in liquid nitrogen by putting them in canister. Temperature of Liquid Nitrogen is around -196°C, and as it reduces the metabolism of sperms to bear minimum and hence theoratically they can be stored and rejuvinated even after, number of years.

Maintanence

Liquid Nitrogen, because of its extremely low temp. of -196°C, has to be handled very carefully, else it can produce burns. It evaporates slowly even when stored in high capacity vacuum flasks (Liquid Nitrogen Cylinders). The cylinders are available from 5 litre capacity onwards. Static Holding Time (i.e. time taken to evaporate given quantity of Liq. Nitrogen in stationary closed environment) of 5 litre cylinder is 20 days and that of 20 litre cylinder is 170 days. Hence refilling of cylinders has to be done prior to that period. Each cylinder can hold 6 canisters (small containers with handle which stores about 720 straws each and keep them submerged in Liq. Nitrogen). Hence one cylinder san store upto 4320 (720 x 6) straws. In 20 litre capacity cylinders, special canisters which can hold 1440 straws are also available (BHPY specifications).

Considering average semen volume of 2.5 ml, with 1:1 dilution with CPM, with one semen sample approximately 10 straws of 0.5 dl capacity are filled up. (Total 5 ml volume). Hence in one cylinder more than 400 to 800 samples of semen can be stored (depending on capacity of canisters).

Thawing

Thawing is carried out by taking out the plastic straws from the canister dipped in Liquid Nitrogen Cylinder and kept at room temperature for 10 minutes and then on 37°C hot plate for 10 mins. (Matheson *et al* 1969; Taylor *et al* 1982). Post-thaw motility is checked under the Phase Contrast (preferably) microscope.

Results

Total 12 samples were utilized for this experiment. Table I shows sperm count found in various samples of semen.

TABLE I

Count	No. of cases
20 million or less	5
21 to 40 million	3
More than 40 million	4
Total	12

Table II shows Pre-freeze, Post-thaw Motility (after 7 days) and Recovery

TABLE II

	<20%	21-49%	50-75%	>75%
Pre-freeze Motility	2	3	7**	Nil
Post-thaw Motility	3	7	2	Nil
Recovery Rate	1	2	5	4

Total cases with more than 50% Recovery Rate..... 9

** Motility after transfer to Bidaj Lab, almost 1 hour after collection of specimen.

Rate. Recovery Rate was found out by the following formula:

$$\text{Recovery Rate} = \frac{\% \text{ Post-thaw motility}}{\% \text{ Pre-freez motility}} \times 100$$

As seen in Table II, Recovery rate of 50% or more was obtained in 9 out of 12 cases (75%) after 7 days of preservation. Though theoretically, they can be preserved for indefinite period provided Liquid Nitrogen is replenished from time to time.

The factors which influenced the Recovery Rate are interval between collection of semen and time of freezing, sperm count, initial motility, presence or absence of pus cells (infection), composition of cryo-preservative media etc.

Discussion

From this study it was concluded that even without the liquid Nitrogen Freezer, successful cryo-preservation of human spermatozoa is possible by utilizing the Cryo-preservative Media as described by Behrman and Sawada (1966). The routine method used for Cryo-preservation of cattle semen as described by Polge and Loverlock (1952) which advocates for Equilibration time of 2 hours before addition of Glycerol containing media followed by 6 to 20 hours for equilibration of glycerol, is necessary in Cryo-preservation of Human Semen as can be seen from results.

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