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Effects of serial thaw-refreeze cycles on human sperm motility and viability

Cryopreservation of human spermatozoa has been used since 1954 (1). After surgical treatment, chemotherapy, or radiotherapy, permanent sterility or transient infertility of up to 5 years can occur (2). Therefore, cryopreservation is often recommended to preserve future fertility. However, sperm survival and motility are known to significantly decrease after the freeze-thaw process. Since sperm motility correlates with better pregnancy rates (3) and men presenting with systemic disease such as testicular cancer often have impaired sperm quality and quantity to begin with, these cryopreserved specimens are usually of poor quality after thawing.

Fortunately, assisted reproductive procedures require relatively few gametes. Thus, refreezing thawed specimens would provide additional opportunities for conception. The purpose of this study is to evaluate the effect that the rate of refreezing has on sperm motility and viability after repetitive thaw-refreeze cycles in specimens of varying quality.

Twenty-two semen specimens (4 paired, 14 unpaired) from men requesting cryopreservation were collected by ejaculation into sterile containers and allowed to liquefy. Repetitive freeze/thaw cycles were performed on the aliquot reserved for post-thaw analysis as per our laboratory protocol. The specimens were diluted with 12% glycerol and 20% egg yolk in 1:1 ratio at room temperature. The initial cryopreservation was performed in 1-mL plastic cryovials using a controlled-rate freezer with a standard slow freeze cycle (-1°C/min until -30°C followed by -5°C/min until -80°C, then specimens were plunged into liquid nitrogen at -196°C). All specimens were thawed using a standard thaw protocol (30 minutes at room temperature, then 10 minutes at 37°C).

Ten specimens underwent repetitive cycles of slow refreezing as described above, and 12 specimens underwent cycles of fast refreezing by being placed in a vapor freezer for 30 minutes then plunged into liquid nitrogen. The external temperature decay with the vapor freezer, was measured to be -60°C/min. The standard thaw protocol was performed for each set. The thaw-refreeze cycles were repeated until no motile and no viable sperms remained. Viability was assessed using eosin-nigrosin staining. Sperm motility and concentration were measured at 37°C under phase-contrast microscopy using a Makler Chamber (Haifa, Israel). Student's *t*-test was used for statistical analysis.

Total sperm count ranged from 1.1 to 306.0 million with a mean of 34 $\pm$ 68.6 million. Total sperm concentration ranged from 3.8 to 102 million/mL with a mean of 41.3 $\pm$ 25.5 million/mL, and the initial motility range was 3%–60% with a mean of 42.2% $\pm$13.2%. Motility and viability were present in all specimens through 2 thaw-refreeze cycles with a range of 2–7 cycles. A linear decrease of motility of 8.7% and 6.7% per cycle was noted for the slow and fast refreeze cycles, respectively. Viability decreased by 8.2% and 7.7% per cycle for the slow and fast refreeze cycles, respectively. The results were not different for paired or unpaired specimens. Fast refreezing preserved motility for an average of 2.75 cycles longer and viability for an average of 2.0 cycles longer than the slow refreezing. Mean values for motility and viability were significantly greater for specimens undergoing repetitive fast refreezing than for those undergoing slow refreezing ($P<.01$).

Cryopreservation has provided men with severely impaired semen quality or men about to undergo treatments that adversely affect spermatogenesis the means to preserve their gametes. Often, both the quality and quantity of the semen is poor and preservation of thawed specimens can offer additional chances for fertility.

Our study demonstrates that it is possible to preserve sperm motility and viability for up to 7 cycles of thaw-refreeze depending on the method of refreezing. Multiple factors such as plasma membrane damage by free radicals and superoxides, osmotic changes, and intracellular and extracellular ice formation play a role in sperm integrity during the thaw-freeze process. Some reports suggest that slow freezing allows dehydration and osmotic shrinkage to occur with minimal cell membrane damage (4).

The initial freezing method used for both cycles was by slow, controlled-rate freezer. It is not known whether results would vary if the initial freezing of specimens were by a fast vapor freezer. In addition, unlike the temperature probe in the controlled-rate freezer, which allows equilibration time of the specimen in the

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cryovial, the temperature probe used to determine fast refreezing may not represent as precisely the temperature changes occurring during vapor freezing. This study demonstrates that when the quality and quantity of an available semen specimen is limited, spermatozoa can be preserved through a refreeze cycle with preservation of motility and viability. A maximum of 7 thaw-refreeze cycles are possible with even markedly impaired semen. A fast rate of refreezing was found to be significantly better than a slow rate in preserving motility and viability of cryopreserved semen. What is still not known is whether repetitive thaw-refreeze cycles would have an adverse effect on sperm function. However, maintenance of viability as demonstrated in this study and the historical success of the use of poor-quality sperm in in vitro fertilization suggest that viable sperm remaining after repetitive thaw-refreeze cycles would be effective in assisted reproductive techniques. Therefore, efforts should be made to refreeze semen specimens when a limited quantity exists.

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