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Original Article

Analysis of the impact of cryopreservation and theophylline on motility of sperm ☆

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ABSTRACT

Objective: Sperm parameters, particularly motility, decrease during cryopreservation. Theophylline generally enhances sperm motility. We analyzed effects of theophylline and freezing on sperm motility.

Design: Experimental study.

Setting: Private IVF lab.

Setting: IVF lab of Mehrgan Hospital.

Method: 22–55 year-old men participated in this study (30 fresh ejaculation and 8 TESE samples). After sperm analysis, we added theophylline (40 mM) to half of our samples as case group to compare motility with the remaining samples as control group. Cryopreservation was performed in two groups. After thawing, motility of both groups was recorded. Furthermore, theophylline (40 mM) was applied to both groups after thawing again.

Result: After adding theophylline, sperm motility improved significantly in all samples. Sperm motility reduced in control group more than the study group after freeze-thaw procedure ($P < 0.002$, normal morphology <5%). Sperm motility was not enhanced significantly by re-adding of theophylline to the two groups. Interactions between stages and groups were statistically significant in semen and biopsy samples ($p < 0.001$).

Conclusion: Adding theophylline before freezing can preserve motility of sperms in samples with different parameters and even sperms extracted in testicular biopsy. Theophylline may have protective impact on sperms in freezing procedure.

1. Introduction

Cryopreservation is a critical procedure in ART which conserves gametes and embryos for future [1]. The main goal in freezing is the maximum survival rate and minimal harm after thawing. It has been shown that freezing-thawing procedure is associated with a variable damage to sperm viability and motility [2–7]. In this point of view, sperm freezing in oligozospermia, treatment with cytotoxic agents and radiotherapy [8,9] or those who are exposed to sperm-damaging conditions [10,11] should be considered with special care. In the case of azoospermia, cryopreservation of testicular sperm can prevent repeated testicular biopsy on the day of ICSI [8,12].

In ICSI, only a small number of viable and motile sperms are needed to inseminate the oocytes [8]. However, it has been reported that increasing severity of sperm parameters is associated with decreased rate of fertilization and cleavage, embryo quality and blastocyst formation in ICSI procedure [13]. Obviously, freezing with increasing the severity of sperm parameters and ICSI with transmitting injured sperms to the offspring may be cumulative risk factors for adverse perinatal and pregnancy outcome [14].

The most important problem in sperm cryopreservation is severe diminishing of motility especially in low motile and biopsy cases. The use of theophylline before injection or after thawing in immotile sperms for enhancing sperm motility and increasing fertilization capacity has been suggested by some authors [15–21]. Theophylline is a methylxanthine derivative that elevates cAMP

Abbreviations: ART, assisted reproductive technology; ICSI, intracytoplasmic sperm injection; cAMP, cyclic adenosine monophosphate; IVF, in vitro fertilization; GEE, generalized equation estimation; TESE, testicular sperm extraction.

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and cAMP-dependent processes of sperm, including motility, capacitation, and acrosome reaction [22–27].

In this study, we evaluated the effectiveness of theophylline to preserve sperm parameters during freezing. We believe this action can help protect human sperm from the side-effects of cryopreservation.

2. Material and methods

This experimental study was conducted on a total of 38 men (30 fresh ejaculation and 8 TESE samples) in the age range of 22–55 years who referred to IVF lab of Mehrgan Hospital, Babol, Iran. Consulting with statistician, the sample size was determined regarding to similar published papers. Fresh semen was collected with the aid of their spouse after 3–5 days of abstinence. An exclusion criterion was men with retrograde ejaculation. After liquefaction and washing, semen analysis was performed as our routine. The prepared samples were arranged into three categories; <5%, 5–15%, and >15% for normal morphology. Biopsy samples with at least one visible twitch sperm were included in the present study. A home-made (40 mM) dimethylxanthine called theophylline (anhydrous, ≥99%, powder; SIGMA) was prepared according to manufacturer's instruction and was added to half of each sample as cases and compared with the rest as controls. Sperm motility was evaluated after five minutes incubation with theophylline at 37 °C. Sperm freezing was carried out (LIFEGLOBAL® Media: Fast Freeze®) on all groups. Briefly, the sample was initially mixed in a drop-wise manner with equal volume of pre-warmed cryoprotectant (1:1). The mixture was loaded into the straws and left to incubate at 4 °C for 10 min. The straws were then placed at a distance of 10–15 cm above the level of liquid nitrogen (−80 °C) for 15 min; after that, the straws were immersed into liquid nitrogen at (−196 °C) for 15 min [15]. Sperm motility was evaluated after thawing of samples at room temperature.

In biopsy samples, we used mean of score grade instead of mean of motility:

Score grade: $\sum . n_{i.}$ Score grade$_i$/$\sum n_i$

Immotile score: grade: 0

Grade1 score: 1

Grade2 score: 2

Grade3 score: 3

Grade4 score: 4

N: percent of motility

Statistical analyses were performed using the GEE and Bonferroni tests (SPSS software, version 23, SPSS Inc, Chicago, IL, USA). The responses to theophylline were measured by conducting GEE test. Bonferroni procedure was used for paired comparisons of motility in all groups.

An alpha error rate below 0.05 was considered to be statistically significant.Fig. 1.

3. Results

The mean (±SD) of sperm concentration was 39.2 (±23) in semen samples. The mean of motility was the same in case and control groups before adding of theophylline (Fig. 2A; stage 1). The mean of motility in case group showed more increase compared with control group after adding of theophylline (Fig. 2A). The mean of motility increased significantly in <5% and >15% normal morphology categories (Fig. 2B; and Table 1). The result was the same in the biopsy specimens (1.79 ± 0.19, 0.87 ± 0.11, Table 2).

After freezing-thawing procedure (stage 3), a significant drop-off in the mean of motility was observed in fresh semen and biopsy samples which was more apparent in control group (Fig. 2A–C; and

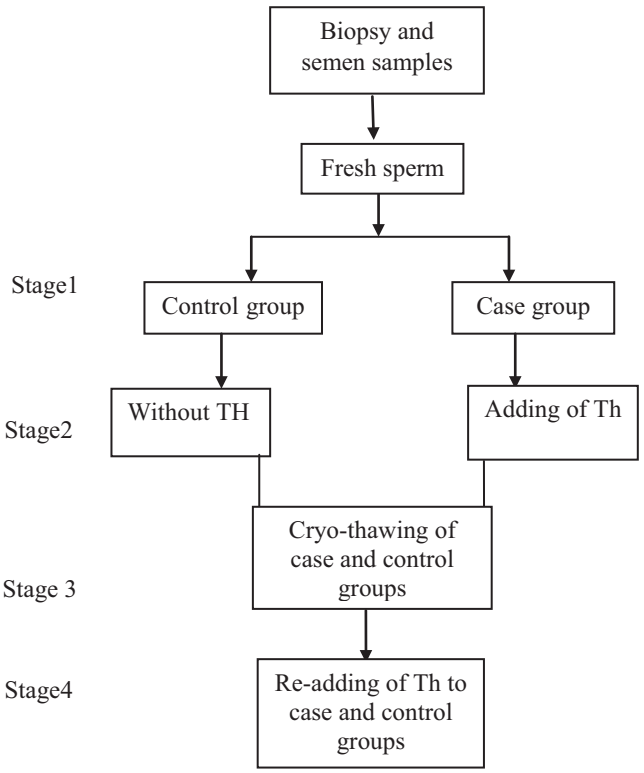

Fig. 1. Schematic diagram to illustrate our study.

Tables 1, 2). The mean of motility had the most percentage of drops-off in 5–15% of normal morphology category (Fig. 2D; and Table 1). Bonferroni test was used for multiple comparisons of the mean of score between stage two and three in case group of biopsy (1.79 ± 0.19^{e}, $1.31 \pm 0.19^{e,d}$, Table 2). Spermatozoa of frozen-thawed biopsy samples were immobilized absolutely in the control group.

Re-adding of theophylline (40 mM) at stage four to both case and control groups had no significant effects on motility. There were no differences between stage three and four in biopsy and fresh semen samples (Tables 1 and 2). The trend of mean (±SD) of score grade was illustrated in different stages (Fig. 3). Interaction between stages and groups were statistically significant at biopsy and semen samples ($p < 0.001$).

4. Discussion

Cryopreservation is a fundamental procedure in IVF clinics which preserves fertility and provides the opportunity of having a child in future. Preventing the reduction of sperm motility is one of the most attractive interests in freezing field. Generally, a methylxanthine derivative promotes sperm motility and has been routinely used to examine the vitality of immotile sperms. In the present study, we have analyzed the impacts of cryopreservation and theophylline on sperm motility.

In Ebner et al.'s report, adding of theophylline led to improvement in progressive motility [16]. Yoshioka et al. reported that fertilization rate was significant ($P < 0.05$) in the presence of theophylline [21]. Longhlin et al. showed that semen incubation with theophylline (20 mM) significantly increased the penetration rate of sperms [18]. Kenneth et al. used sperms of epididymal caput of chimpanzee and observed that thirty minutes incubation with theophylline had a small but significant effect on the improvement

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