Increased sperm motility after in-vitro culture of testicular biopsies from obstructive azoospermic patients results in better post-thaw recovery rate*

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The objective of this study was to optimize the use of testicular biopsies in 14 patients with obstructive azoospermia. Testicular specimens were retrieved from six patients (group I) and cultured at 32 and 37°C for up to 20 days; changes in percentage motile spermatozoa were compared. In four men of group I, one portion of the specimen was frozen at retrieval, and changes in post-thaw motility after 24 h of culture at 37°C were recorded. In the other eight patients (group II), testicular specimens were frozen at retrieval and after 72 h culture at 37°C. Pre- and post-freezing motility and post-thaw recovery rate were compared. No significant differences were observed until day 8 in the improvement of motility between 32 and 37°C in-vitro culture. Maximum motility was reached, under both conditions, between 48 h and 72 h. Post-thaw 24 h culture at 37°C of specimens frozen at retrieval did not improve motility; however, 72 h pre-freezing culture significantly improved initial motility (P < 0.01), post-thaw motility (P < 0.01) and post-thaw recovery rate (P < 0.001). The higher recovery rate of samples frozen 3 days after retrieval allows more economical use of the tissue that is available.

Key words: in-vitro culture/obstructive azoospermia/ testicular sperm extraction/testicular sperm cryopreservation

Introduction

Open surgery is an invasive intervention for testicular biopsies, and even fine needle aspiration is not totally atraumatic. Optimal management of testicular biopsies would therefore be to reduce surgery to the retrieval of only one sample. Until now, several strategies have been developed to improve sperm motility and to increase the motile sperm recovery rate after freezing. Several reports have described improvements in the percentage of motile testicular spermatozoa after a few days of culture at 37°C in cases of obstructive azoospermia (Craft et al., 1995; Edirisinghe et al., 1996; Liu et al., 1996; Zhu et al., 1996), although 37°C is above the physiological temperature of the testicular compartment. In the first part of this study, our aim was to compare the outcome of testicular biopsies maintained in culture for a prolonged period of 14 days. One half of each sample was cultured at 32°C (a temperature more similar to that of the testis), and the other half at 37°C. Several authors have shown similar results after performing intracytoplasmic sperm injection (ICSI) with testicular fresh and frozen spermatozoa in terms of rates for fertilization, cleavage, pregnancy and implantation (Gil-Salom et al., 1996; Friedler et al., 1997; Liu et al., 1997; Oates et al., 1997). In our attempt to develop more efficient strategies, including freezing of testicular spermatozoa, testicular samples which had been frozen on retrieval and then thawed were cultured for 24 h. It has been observed previously (Edirisinghe et al., 1996) that in the case of frozen–thawed testicular specimens, the percentage of motile spermatozoa is increased slightly by maintaining the samples in culture for one week. Furthermore, it has also been observed (Liu et al., 1997) that the number of progressively motile spermatozoa was significantly increased after 72 h of culture, even if the total number of motile spermatozoa remained constant. Therefore, in the second part of this study, we analysed the post-thaw motility and post-thaw recovery rate of testicular spermatozoa that was either frozen on retrieval (day 0), or frozen after 72 h of culture at 37°C (day 3).

Materials and methods

Patients

Fourteen patients suffering from obstructive azoospermia were included in the study. Six patients (group I) underwent treatment from July to October 1997, and eight patients (group II) underwent treatment from February to October 1998. The biopsies were obtained during open surgery under full anaesthesia. In two cases a diagnostic biopsy was taken, and in 12 cases the spermatozoa were used in fresh ICSI cycles that led to pregnancy in eight cases (two biochemical, six at term). In group I, the outcome of testicular specimen culture at 32°C and 37°C was compared, and management of the samples frozen at retrieval investigated. In group II, as a consequence of observations made in group I, the outcome of two freezing strategies was compared by freezing samples before and after culture.

Testicular biopsy preparation

Biopsy samples were placed into a Petri dish and first washed in 20 mmol/l HEPES-buffered modified Earl’s balanced salt solution

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(HEBSS), supplemented with 0.5% human albumin (Belgian Red Cross, Brussels, Belgium), 0.43 mmol/l sodium pyruvate (Sigma Chemical Co., St Louis, MO, USA) and 44 mmol/l sodium lactate (Sigma). The biopsies were mechanically dissociated with two sterile scalpels. The dissociated tissue was emptied on a 40 µm Falcon (Becton-Dickinson & Co., Meylan Cedex, France) cell strainer, placed onto a 50 ml conical Falcon tube and centrifuged at 300 g for 10 min. The pellet was then resuspended in 1 ml of modified Earl’s balanced salt solution (EBSS) buffered with 28 mmol/l sodium bicarbonate, supplemented with sodium pyruvate, lactate and 20% inactivated serum (35 min at 56°C), obtained from the same patient. The pellet was filtered through a 35 µm Falcon cell strainer and re-centrifuged. The pellet was resuspended to a final volume of 100 µl.

**Testicular biopsy culture**

After retrieval, testicular samples from group I patients were immediately frozen, with a quantity of tissue also being retained for research purposes at the same time. Two portions of this tissue were placed in culture, one at 37°C and one at 32°C. Culture was carried out in Nunc (Roskilde, Denmark) four-well dishes containing 1 ml of EBSS and 20% serum, in a humidified atmosphere containing 5% CO₂ and 5% O₂. The percentage of motile spermatozoa was assessed daily by counting 100–200 spermatozoa. At 72 h intervals, 0.5 ml of the top layer of the medium was removed and replaced with the same volume of fresh EBSS.

**Testicular biopsy freezing**

The wells containing testicular spermatozoa were each emptied into a 5 ml Falcon tube and washed with extra EBSS to remove remaining cells. The tube was centrifuged for 10 min at 300 g, and the pellet resuspended in a small volume of EBSS. The same volume of 15% glycerol (Sigma)–20% egg yolk (Difco, Grayson, GA, USA) buffer, which is used routinely for the cryopreservation of ejaculated spermatozoa was added. After 10 min of equilibration, 0.15 ml IMV straws (L’Aigle, France) were charged and cryopreserved (using a Kryo 10 Planer, Middlesex, UK), according to the following curve: from 22°C to 5°C at −1°C/min, from 5°C to −80°C at −10°C/min, and from −80°C to −130°C at −25°C/min; the straws were then plunged in liquid nitrogen. One portion of the specimens retained for research purposes was thawed by leaving the straws at room temperature for a few minutes. The cryoprotectant was removed by a centrifugation step in EBSS, after which the samples were placed in culture at 37°C in a humidified atmosphere under 5% CO₂ and 5% O₂ for 24 h. At least 100 spermatozoa were counted on the day of thawing, and 24 h later the percentage of motile spermatozoa was calculated.

In group II, one portion of each sample was frozen on the day of the biopsy (day 0), and one portion after being cultured for 72 h (day 3) at 37°C. In both cases the percentage motility was determined before freezing. Some of the samples retained for research (frozen using both strategies) were thawed, and post-thaw motility and recovery rate were determined. The percentage recovery rate was defined as the ratio of motile spermatozoa after thawing to motile spermatozoa in the fresh sample. Pre-freezing and post-thaw motility and the recovery rates in samples frozen on day 0 and day 3 were compared.

**Statistical analysis**

Statistical analysis was performed using linear regression, Student’s t-test to compare means in the case of normal distributions, and the Mann–Whitney test to compare means in the case of non-normal distributions. A SPSS software package was used. Statistical significance was defined as P < 0.05.

**Results**

**Prolonged culture at 32°C and 37°C**

Results of culturing testicular biopsies for 14 days at 37°C, expressed as percentage of motile spermatozoa on each day of culture, are shown in Figure 1. The percentage of motile spermatozoa for the same biopsies cultured at 32°C are shown in Figure 2. In all six samples a significant increase (with χ²-test used overall; P < 0.01) in the percentage of motile spermatozoa was observed at both 37°C and 32°C during the first 72 h of culture. Culture at 32°C was readily extended over a period of 10–15 days with ≥20% sperm motility, whereas during the same period in samples kept at 37°C, spermatozoa lost their motility. The slower decrease in sperm motility at 32°C followed the slope of a linear regression (y = −2.74x + 60, r = 0.58, P < 0.001) than at 37°C (y = −2.9x + 47, r = 0.43, P < 0.01). The difference in slope of the regression at the two temperatures became significant (P < 0.01) for all samples after day 8. The mean (± SD) maximum motility attained at both temperatures between 48 and 72 h was similar, namely 53.4 ± 17.3% at 32°C and 50.4 ± 16.6% at 37°C; neither were any significant differences observed in sperm motility between the two temperatures until day 8. There was a good and similar second-degree regression between sperm motility and day of culture for the first 4 days of culture, and at both temperatures.

**Culture of cryopreserved testicular spermatozoa**

The percentages of motile spermatozoa in four testicular samples (group I) frozen on the day of the biopsy and measured at thawing and after culture at 37°C for 24 h after thawing are shown in Figure 3. The samples did not show any improvement in the percentages of motile spermatozoa, and in three of the four samples the spermatozoa began to die after 24 h.
Recovery rate of testicular biopsy in obstructive azoospermia

Figure 3. Percentages of motile testicular spermatozoa immediately after thawing and after 24 h of culture are shown. Four testicular samples from group I were frozen on the day of biopsy. After thawing, the spermatozoa were cultured for a further 24 h at 37°C. No improvement in motility was observed after culture.

Figure 5. The recovery rate (% motile spermatozoa after thawing/ % motile spermatozoa before freezing) was calculated for each of eight group II samples frozen on the day of the biopsy (day 0) and after 3 days of culture (day 3). The recovery rate was significantly higher for samples frozen after 3 days of culture (Mann–Whitney test, \( P < 0.01 \)).

Outcome of testicular biopsies frozen on the day of biopsy (day 0) and after 72 h of culture (day 3)

The initial motility of the eight fresh samples (group II) on day 0 ranged from 10 to 23% (median 16%). Motility on day 3 ranged from 29 to 63% (median 48%), and was significantly higher than on day 0 (Mann–Whitney test, \( P < 0.01 \)). The post-thaw motility from samples frozen on day 0 ranged from 1 to 6% (median 4%) and this was significantly lower compared with the post-thaw motility of samples frozen on day 3 (range 7 to 25%, median 17%; \( P < 0.01 \)) (Figure 4). The recovery rate in samples frozen on day 0 ranged from 1 to 22% (median 15%) while in samples frozen on day 3 the recovery rate ranged from 16 to 45% (median 27%), and this was significantly higher than in day 0 samples (\( P < 0.01 \)) (Figure 5).

Discussion

The in-vitro culture of testicular spermatozoa in the case of obstructive azoospermia significantly increased the number of motile spermatozoa within 72 h at both 32°C and 37°C, but no significant differences were observed in changes of motility pattern within the first 8 days between the two culture temperatures. The changes in motility patterns during in-vitro culture of testicular spermatozoa has been observed previously (Craft et al., 1995; Zhu et al., 1996; Liu et al., 1997), and practical implications of using in-vitro-matured testicular spermatozoa for ICSI were also described (Urman et al., 1998; Balaban et al., 1999; Hu et al., 1999). Furthermore, it was recently observed that 48 h culture of testicular spermatozoa gives similar results to incubation with pentoxifylline and 2-deoxyadenosine (Angelopoulos et al., 1999), allowing avoidance of the use of these artificial and toxic compounds (Lacham-Kaplan and Trounson, 1993). On the other hand, in cases of non-obstructive azoospermia, no improvement in motility was observed, or else it was less pronounced—as in the case of obstructive azoospermia (Liu et al., 1996; our unpublished data). A slower decrease in sperm motility was observed at 32°C than at 37°C, but this was most likely linked to a less rapid depletion of sperm metabolic sources at 32°C.

The mechanism implicated in the observed improvement of testicular sperm motility after a short culture period remains unexplained, but it is probably mediated by a complex interaction between somatic and germinal compartments (Jutte et al., 1985; Dym, 1994; Foucault et al., 1994; Albanesi et al., 1996; Chen et al., 1997; Cudicini et al., 1997). In a recent study (Tesarik et al., 1998), it was shown that developmental changes could occur in culture of human testicular samples where germ cells lack a direct contact with Sertoli cells, indicating that the interaction is mediated by humoral factors. This necessary interaction could also explain the lack of improvement in the motility of cultured epididymal spermatozoa (Edirisinghe et al., 1996; our unpublished data). The similar motility improvement of cultured spermatozoa induced by two different temperatures suggests that it is not the temperature change that is the principal factor inducing this improvement in sperm motility, but rather the dilution of one or more inhibitory factors that occurs during the washing of samples for processing before culture.

Several authors have demonstrated that performing ICSI with fresh or frozen spermatozoa gives similar results (Gil-Salom et al., 1996; Friedler et al., 1997; Liu et al., 1997;
Oates et al. (1997), and that freezing does not affect the spermatozoa (Lin et al., 1998). Consequently, some groups have attempted to improve the protocols for testicular sperm freezing (Allan and Cotman, 1997; Crabbe et al., 1999; Gianaroli et al., 1999). When testicular spermatozoa were cultured in vitro after thawing, either a slight increase in the total number of motile spermatozoa was observed (Edirisinghe et al., 1996), or a significant improvement in the number of progressively motile spermatozoa was reported after 72 h of culture (Liu et al., 1997). In the first part of this study, thawed testicular spermatozoa frozen at retrieval were cultured for a 24 h period, and a decrease in motility was observed thereafter. The reason for this might be the destruction of somatic testicular cells. Consequently, the substitution of a glycerol-based protocol—which is designed for ejaculated spermatozoa—with a protocol adapted for somatic cell freezing may provide better results.

A previous attempt has been made to culture testicular samples for a few hours before freezing (Aslam and Fishel, 1998). In our case, the period of culture was extended to 72 h, the time at which we observed the peak in percentage of motile spermatozoa. The percentage of motile spermatozoa in samples frozen after 72 h of culture was significantly higher than that in samples frozen at retrieval and, moreover, a significantly higher recovery rate was obtained in the specimens cultured for 72 h prior to freezing. Amongst in-vitro-matured frozen spermatozoa, 27% were in fact still motile after thawing, compared with the 15% observed from non-matured frozen samples ($P < 0.01$), indicating a stronger resistance of cultured spermatozoa to freezing. A prospective randomized study, with a larger series of patients, will be necessary to compare the clinical outcome of the two freezing strategies.

In conclusion, these data indicate that a more rational management of testicular samples, in the case of obstructive azoospermia, consists of improving sperm motility of fresh samples by 3 days of culture, before ICSI or before freezing. This would allow the biopsy to be taken 2–3 days before oocyte retrieval, it would ease the selection of motile spermatozoa for ICSI, and it would reduce the volume of material necessary per cycle, offering the possibility for several further attempts with one single sample.

References


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