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Outcome of in-vitro culture of fresh and frozen–thawed human testicular spermatozoa

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The effect of in-vitro culture on the motility and morphology of fresh and frozen–thawed human testicular spermatozoa obtained from obstructive azoospermic patients and on the motility of testicular spermatozoa obtained from non-obstructive azoospermic patients was evaluated. The outcome of intracytoplasmic sperm injection (ICSI) with fresh and frozen–thawed human testicular spermatozoa was studied. The results showed that significant improvement of sperm morphology and motility was observed in culture of fresh (n = 17) and frozen–thawed (n = 15) testicular sperm samples obtained from patients with obstructive azoospermia. The motility of cultured testicular spermatozoa reached a peak at 72 h without the need for special media. In six of 20 samples obtained from patients with non-obstructive azoospermia, improvement of sperm motility was observed. When only non-motile testicular spermatozoa were cultured, they all remained non-motile (n = 9). In patients with obstructive azoospermia, fertilization rates of 80 and 81% were obtained using ICSI with fresh and frozen–thawed testicular spermatozoa respectively. Clinical pregnancies were observed in four out of nine patients with fresh testicular spermatozoa and two out of five patients after using frozen–thawed spermatozoa. When fresh testicular spermatozoa obtained from patients with non-obstructive azoospermia were used for ICSI, the fertilization rate was 68% and two out of seven patients achieved clinical pregnancies. In conclusion, the morphology and motility of fresh and frozen–thawed testicular spermatozoa in patients with obstructive azoospermia can be significantly improved after in-vitro culture. The outcome of in-vitro culture of testicular spermatozoa in patients with non-obstructive azoospermia is unpredictable. In-vitro culture of non-motile testicular spermatozoa is not successful so far. The outcome of ICSI with fresh and with frozen–thawed testicular spermatozoa was similar.

Key words: cryopreservation/intracytoplasmic sperm injection/maturation/testicular biopsy/testicular spermatozoa

Introduction

It has been shown that the combination of human testicular sperm biopsy and intracytoplasmic sperm injection (ICSI) is an efficient method for the treatment of male-factor infertility caused by azoospermia (Schoysman *et al.*, 1993; Van Steirteghem *et al.*, 1993a; Devroey *et al.*, 1994, 1995, 1996; Silber *et al.*, 1995, 1996; Kahraman *et al.*, 1996; Tournaye *et al.*, 1996a,b). The motility of fresh biopsied testicular spermatozoa is poor, often demonstrating a non-progressive, weakly shaking type of movement. The weakly shaking characteristic is an important indicator for selecting viable testicular spermatozoa for ICSI. Recently, it has been reported that the motility of testicular spermatozoa can be improved after in-vitro culture (Zhu *et al.*, 1996), especially in patients with obstructive azoospermia (Liu *et al.*, 1996a).

Currently, testicular sperm biopsy for ICSI is carried out on the day of oocyte retrieval and fresh testicular spermatozoa are often used. When no pregnancies occur, repeated procedures of testicular biopsy are required for the patients' subsequent treatment cycles. Alternatively, if sufficient numbers of viable testicular spermatozoa can be recovered after cryopreservation, repeated testicular biopsies can then be avoided. Recently, fertilization and human pregnancies have been reported after use of frozen–thawed testicular spermatozoa (Gil-Salom *et al.*, 1996; Liu *et al.*, 1996b; Romero *et al.*, 1996).

The aims of the present study were (i) to culture fresh and frozen–thawed testicular spermatozoa *in vitro* to assess whether their morphology and motility can be improved; (ii) to see the difference between the in-vitro cultures of testicular spermatozoa obtained from patients with obstructive (either fresh or frozen–thawed spermatozoa) and non-obstructive azoospermia (fresh spermatozoa); and (iii) to cryopreserve testicular spermatozoa obtained from obstructive azoospermic patients and to compare the use of fresh and frozen–thawed++++ testicular spermatozoa for ICSI.

Materials and methods

Preparation of testicular spermatozoa

Testicular spermatozoa were obtained from patients undergoing ICSI. In-vitro culture of fresh testicular spermatozoa was carried out on 37 testicular biopsies obtained from 37 patients. The mean age was 35 years (range 24–51). Of the 37 patients, 17 had obstructive azoospermia [group 1, 11 patients with congenital bilateral absence of the vas deferens (CBAVD) and six had failed vasovasotomy]; 20 had non-obstructive azoospermia (seven patients with maturation arrest of spermatogenesis, six with Sertoli-cell-only syndrome, seven unexplained), where 11 patients had weakly shaking spermatozoa (group 2) and nine patients had only non-motile spermatozoa (group 3). The

procedure of testicular sperm biopsy was similar to that described by Devroey *et al.* (1994) and the preparation of testicular spermatozoa was similar to that reported by Liu *et al.* (1995). Briefly, fresh human testicular tissue was obtained surgically from patients with obstructive or non-obstructive azoospermia. The testicular tissue was placed in a Petri dish containing ~5 ml HEPES-buffered human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA, USA) containing 3% synthetic serum substitute (SSS; Irvine Scientific), hereafter referred to as H-HTF, then shredded with microscopic glass slides under a stereomicroscope at ×40 magnification. The Petri dish was then checked for the presence of spermatozoa under an inverted microscope at ×200 or ×400 magnification. If a few spermatozoa were found, the contents of the Petri dish were transferred to a 15 ml Falcon tube. Prior to centrifugation, the larger debris of testicular tissue was removed from the tube and the tube containing sperm suspension was centrifuged for 5 min at 300 *g*. After centrifugation, the supernatant was removed and the pellet was resuspended in about 100 µl H-HTF. A 15 µl aliquot of the sperm suspension was taken and put on a glass slide for examination of the initial morphology of these spermatozoa. The morphology was assessed using strict Kruger criteria after Papanicolaou staining (WHO, 1992). The morphological abnormalities of testicular spermatozoa included (i) large heads, amorphous heads (irregular outline of the head), double heads and small heads; (ii) mid-pieces with cytoplasmic droplets and bent mid-pieces; (iii) short tails, coiled tails and double tails. For this part of the study, 100 spermatozoa were examined from each sample. However, morphological examination of spermatozoa was not done in patients with non-obstructive azoospermia because of insufficient numbers of spermatozoa in the biopsies. In clinical practice, when a patient was scheduled for ICSI, 1–10 µl aliquots of sperm suspension were used for the patient's ICSI procedure on the same day as oocyte retrieval. Otherwise, the entire sperm suspension was cryopreserved. When no pregnancy occurred in patients who had ICSI with fresh testicular spermatozoa, frozen testicular spermatozoa were then thawed and used for the patients' subsequent treatment cycles.

In-vitro culture of testicular spermatozoa

B_2 medium without additional supplements was used for culture of testicular spermatozoa. A 5 µl aliquot of testicular sperm suspension was added to each 30 µl droplet of B_2 medium covered under mineral oil (M-8410; Sigma Chemical Co., St Louis, MO, USA) and incubated at 37°C in an atmosphere of 5% CO_2.

Motility and morphology of fresh testicular spermatozoa

Motility of testicular spermatozoa was assessed at the time of testicular biopsy, at 4 h after incubation, and at five 24 h intervals under an inverted microscope (Nikon Diaphot, Tokyo, Japan) at ×400 magnification. Except in those few cases where 25–50 sperm cells were counted because there were not enough spermatozoa in the biopsies, the motility of 100 sperm cells was assessed for each sample. Different areas of the droplets of B_2 medium containing testicular spermatozoa were examined under an inverted microscope at a magnification of ×400, since progressively motile spermatozoa were often found along the edges of the droplets. The numbers of non-motile, weakly shaking spermatozoa and progressively motile spermatozoa were recorded. In-vitro culture of testicular spermatozoa was normally carried out for 120 h without change of culture medium.

For examination of the morphology of testicular spermatozoa, after 72 h in-vitro culture, four or five droplets of B_2 medium containing testicular spermatozoa were transferred into a 1.5 ml Eppendorf tube containing 500 µl H-HTF and centrifuged for 5 min at 300 *g*. The supernatant was removed, the pellet was resuspended in ~25 µl of B_2 medium, and one drop (~15 µl) of the sperm suspension (i.e. ~15 µl of the initial sperm suspension, equivalent to the amount used to assess the initial morphology) was then placed on a glass slide for examination of the morphology of the cultured testicular spermatozoa.

The original morphology of testicular spermatozoa was compared to that of the testicular spermatozoa which had been cultured for 3 days.

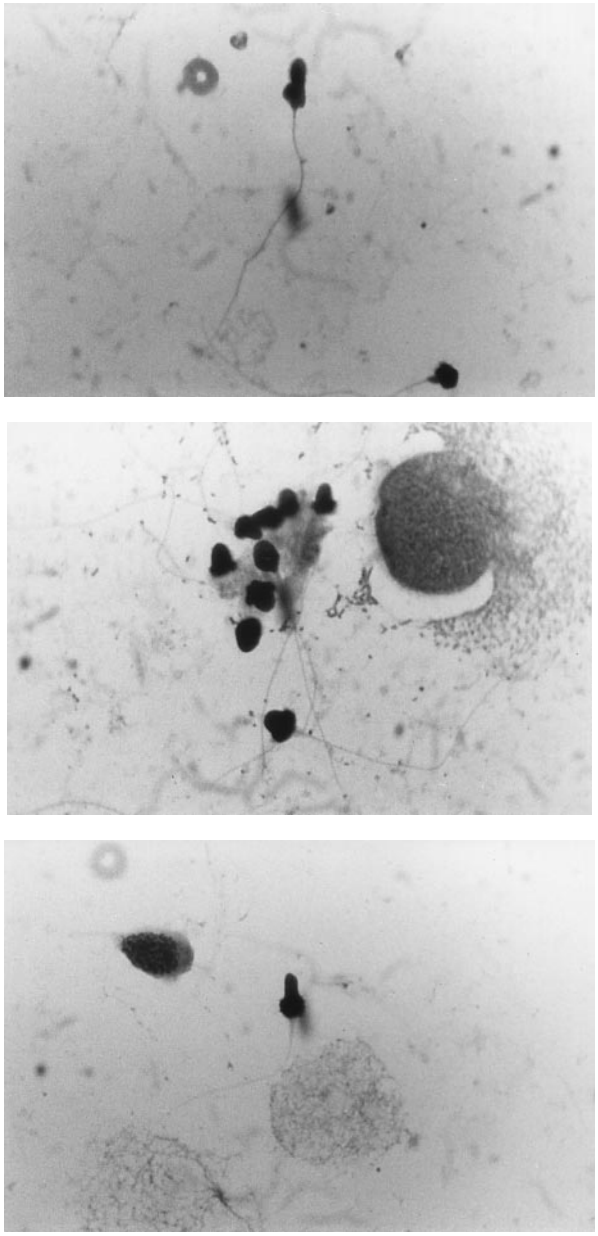

Figure 1. (**a**–**c**) Fresh (non-cultured) testicular spermatozoa obtained from patients with obstructive azoospermia (original magnification ×800).

Cryopreservation and in-vitro culture of frozen–thawed testicular spermatozoa

The number of testicular spermatozoa was much smaller in testicular biopsies obtained from patients with non-obstructive azoospermia compared to those with obstructive azoospermia. It was difficult to have sufficient numbers of testicular spermatozoa in patients with non-obstructive azoospermia to evaluate the effect of cryopreservation on them. Therefore, the sperm samples that were studied for cryo-preservation were obtained only from patients with obstructive azoospermia.

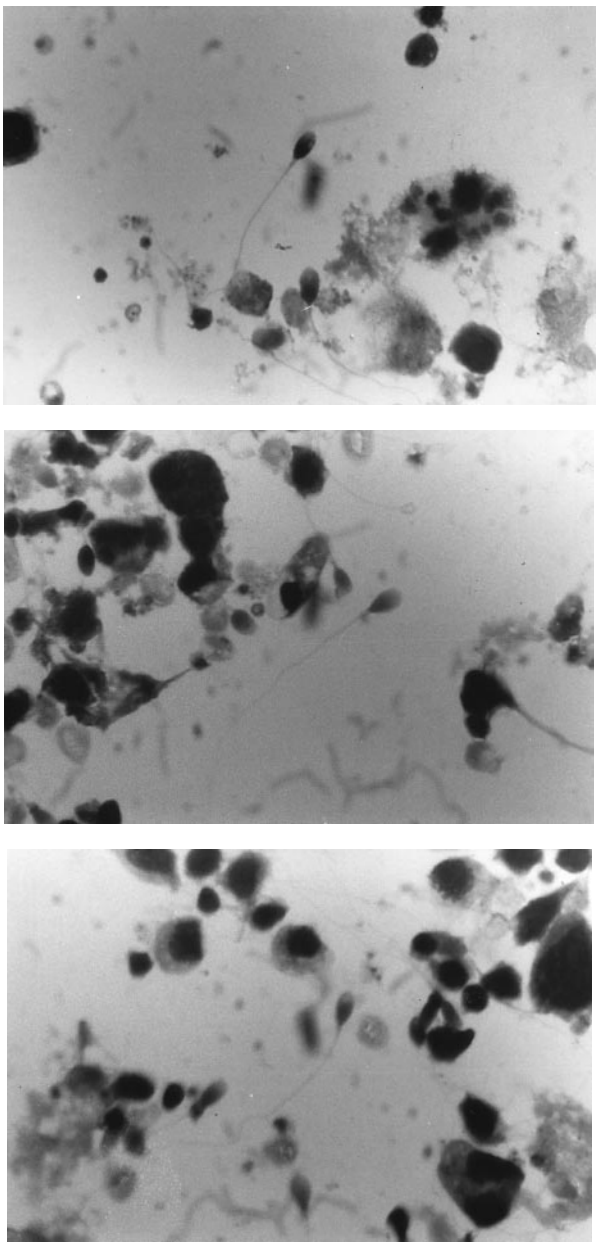

Figure 2. (**a**–**c**) Testicular spermatozoa obtained from patients with obstructive azoospermia observed after 72 h in-vitro culture (original magnification ×800).

In-vitro culture of frozen–thawed testicular spermatozoa was carried out on 15 testicular biopsy samples obtained from 15 patients with obstructive azoospermia (group 4). Of the 15 patients, nine had CBAVD and six had failed vasovasotomy. After preparation of testicular spermatozoa (as described above), the same volume of cryoprotectant solution (TEST yolk buffer with glycerol, Irvine Scientific) was added to the testicular sperm suspension (1:1, v/v), and was gently mixed. This mixture was kept at 37°C initially and at room temperature for 10 min each and then loaded into a 0.5 ml plastic straw (T.S. Scientific, Perkasie, PA, USA). The straw was kept at 4°C (in a refrigerator) for 10 min, and held horizontally ~10 cm above the surface of liquid nitrogen (N_2) in a tank for 10 min. The straws were then plunged into liquid N_2.

For thawing, the straws containing testicular spermatozoa were taken out of liquid N_2 and kept at room temperature for 10 min. The contents of a straw were transferred into a 10 ml test tube. About 5 ml H-HTF were added drop-by-drop into the tube. After gentle mixing, the tube was centrifuged at 300 *g* for 5 min. After centrifugation, the supernatant was removed and the pellet was resuspended in about 50 ml of HTF (Irvine Scientific). The suspension contained testicular spermatozoa, red blood cells, white blood cells and debris of testicular tissue. The procedures of in-vitro culture of frozen–thawed testicular spermatozoa and the examination of their morphology were the same as described above for fresh testicular spermatozoa. The examination of the morphology of cultured frozen–thawed testicular spermatozoa was carried out after 72 h in-vitro culture using the same procedure as for fresh testicular spermatozoa. The morphology of frozen–thawed testicular spermatozoa which had been cultured for 3 days was compared with that of the fresh testicular spermatozoa prior to freezing.

Motility of frozen–thawed testicular spermatozoa

The motility of testicular spermatozoa was assessed at the time of testicular biopsy, after thawing, and then after 72 h in-vitro culture under an inverted microscope (Nikon Diaphot, Tokyo, Japan) at ×400 magnification. One hundred testicular spermatozoa were counted in culture droplets for each sample. The numbers of non-motile, weakly shaking and progressively motile spermatozoa were recorded.

The concentration of testicular spermatozoa in biopsies was not taken as a parameter in the study. The reasons were (i) the number of testicular spermatozoa in biopsies was quite different from patient to patient and comparison was not easy; for instance, some patients had one small piece of testicular tissue biopsied with sufficient testicular spermatozoa for ICSI, while in others more pieces of testicular tissue were required to obtain enough spermatozoa for ICSI; (ii) since the number of testicular biopsy spermatozoa was very low, we often observed that there were no testicular spermatozoa seen in the sperm counting chamber; however, a few spermatozoa sufficient for ICSI were always found in the sperm suspension.

Viability testing

If there were only non-motile spermatozoa in the fresh testicular biopsies, the viability test was carried out by using Eosin Y staining (WHO, 1992).

Intracytoplasmic sperm injection

In clinical practice, ICSI was carried out in 14 obstructive azoospermic patients (nine with fresh testicular spermatozoa and five with frozen–thawed testicular spermatozoa) and seven non-obstructive azoospermic patients with fresh testicular spermatozoa. The procedure of ICSI was similar to that described by Van Steirteghem *et al.* (1993b). Cryopreserved testicular spermatozoa were thawed on the same day as oocyte retrieval. After thawing, the testicular spermatozoa suspension was incubated at 37°C for ~2–4 h prior to ICSI.

Oocytes were retrieved under transvaginal ultrasound guidance after ovarian stimulation (Liu *et al.*, 1997). Those oocytes that had extruded the first polar body underwent ICSI with fresh or frozen–thawed testicular spermatozoa. About 18 h after ICSI, normal fertilization was determined when an oocyte had two pronuclei (2PN). After another 24 h in-vitro culture, embryos at the early cleavage stage with fair quality (anucleated fragments were <40% of the volume of the embryo) were transferred into the uterus or cryopreserved. Pregnancy was determined by transvaginal sonography for the presence of fetal heart beat 4 weeks after embryo transfer.

Statistical analysis

A paired t-test was carried out to analyse the changes of morphology and motility before and after culture of fresh and frozen–thawed testicular spermatozoa. The χ^2 test was used to compare the outcome

Table I. Morphological change of fresh and frozen–thawed testicular spermatozoa after in-vitro culture

Time of examination	Percentages[a] of spermatozoa with normal morphology	
	Fresh	Frozen–thawed
About 1 h after testicular biopsy	3 ± 3[b]	4 ± 2[c]
72 h after in-vitro culture	21 ± 15[b]	17 ± 7

[a]Values are mean ± SD.
[b,c]The percentage of spermatozoa with normal morphology were significantly increased after in-vitro culture ($P < 0.01$).

Table II. Motility change of fresh testicular spermatozoa obtained from patients with obstructive azoospermia after culture (group 1, $n = 17$)

Time of in-vitro culture (h)	Percentage[a] of spermatozoa that were:		
	Progressively motile	Weakly shaking	Non-motile
0	2 ± 4[b,c,d,e,f]	62 ± 15	36 ± 16
4	6 ± 8[g,h,i,j,k]	59 ± 14	35 ± 16
24	19 ± 13[b,g]	50 ± 12	33 ± 15
48	33 ± 19[c,h]	37 ± 14	32 ± 18
72	53 ± 16[d,i]	19 ± 7	28 ± 15
96	48 ± 16[e,j]	21 ± 10	31 ± 15
120	31 ± 19 [f,k]	23 ± 8	46 ± 17

[a]Values are means ± SD.
[b–k]The percentages of progressively motile spermatozoa with the same superscripts were significantly different ($P < 0.05$).

of ICSI with fresh and frozen–thawed testicular spermatozoa. Probabilities <5% were considered significant.

Results

Morphology and motility changes of fresh testicular spermatozoa after culture

The percentage of morphologically normal spermatozoa was significantly higher after 72 h culture than that of fresh testicular spermatozoa in group 1 (obstructive azoospermia) as shown in Table I. The main morphological change of testicular spermatozoa after in-vitro culture was the loss of residual cytoplasm from the neck of the spermatozoa. The testicular spermatozoa with normal morphology after culture looked similar to normal ejaculated spermatozoa (Figures 1, 2). The motility of testicular spermatozoa was significantly improved after culture in all sperm samples from group 1 (Table II) and in six samples from group 2 (non-obstructive azoospermia, weakly shaking spermatozoa) (Table III). The motility of testicular spermatozoa increased with the time of incubation. The motility of cultured testicular spermatozoa reached a peak around 72 h without the need for special media. The peak motility lasted for another 24 h and then decreased. Most of the testicular spermatozoa were dead around 7–8 days after in-vitro culture. Only a few motile spermatozoa were occasionally observed after 8 days culture. No motile spermatozoa were observed in group 3 (non-obstructive azoospermia, non-motile spermatozoa) after in-vitro culture (Table IV).

Table III. Change in motility of fresh testicular spermatozoa obtained from patients with non-obstructive azoospermia and weakly shaking spermatozoa after culture (group 2, $n = 11$)

Patients	No. sperm counted	Weakly-shaking spermatozoa (%)	Progressively motile spermatozoa after different times (h) in culture (%):					
			0	24	48	72	96	120
1[a]	100	20	0	3	3	13	9	2
2	100	10	0	0	0	1	1	0
3[a]	50	10	0	0	4	8	8	2
4[a]	100	35	0	2	5	20	15	4
5	100	5	0	0	0	1	0	0
6	100	5	0	0	0	0	0	0
7[a]	50	16	0	2	2	12	10	2
8[a]	40	2	0	5	5	15	15	8
9[a]	25	16	0	4	4	16	16	4
10	100	21	1	3	0	0	0	0
11	100	2	0	0	0	0	0	0

[a]Progressively motile spermatozoa were found in these patients after culture.

Table IV. The results of fresh non-motile testicular spermatozoa obtained from patients with non-obstructive azoospermia but all non-motile spermatozoa after culture (group 3, $n = 9$)

Patient no.	No. of spermatozoa counted	Viable spermatozoa (%)	No. motile spermatozoa after culture (24–120 h)
1	30	0	0
2	100	15	0
3	50	0	0
4	50	0	0
5	40	20	0
6	50	0	0
7	40	15	0
8	25	20	0
9	40	10	0

Table V. Motility change of frozen–thawed testicular spermatozoa obtained from patients with obstructive azoospermia after culture

	Percentage of spermatozoa with:		
	Progressive motility	Weakly shaking	Non-motility
Fresh biopsies	4 ± 3[a]	52 ± 9[c,d]	44 ± 10[f,g]
After thawing	2 ± 3[b]	31 ± 7[c,e]	67 ± 9[f]
72 h of in-vitro culture after thawing	21 ± 8[a,b]	9 ± 7[d,e]	70 ± 10[g]

Values are means ± SD.
[a,b,c,d]Values with the same superscript were significantly different
[a,b,d,e]$P < 0.01$.
[c,f,g]$P < 0.05$.

Outcome of in-vitro culture of frozen–thawed testicular spermatozoa from patients with obstructive azoospermia

The number of progressively motile spermatozoa and the percentage of non-motile testicular spermatozoa significantly increased after cryopreservation and 72 h culture in samples obtained from group 4 (obstructive azoospermia) (Table V). The percentage of non-motile testicular spermatozoa did not significantly change immediately post-thaw as compared to

Table VI. The outcome of intracytoplasmic sperm injection (ICSI) with fresh and frozen–thawed testicular spermatozoa

	Testicular spermatozoa obtained from patients with:		
	Obstructive azoospermia		Non-obstructive azoospermia
	Fresh	Frozen–thawed	Fresh
No. patients	9	5	7
Mean age (years)	36	37	37
No. oocytes injected	94	54	61
No. oocytes intact	90	53	56
No. oocytes with two PN (%)	72 (80)	43 (81)	38 (68)
No. oocytes with one and three PN	6	3	6
No. embryos transferred or frozen	65	39	31
No. clinical pregnancies	4	2	2

There were no statistical differences between the groups.
PN = pronuclei.

that after 72 h in-vitro culture. The percentage of normal morphology was significantly increased after 72 h of in-vitro culture of frozen–thawed testicular spermatozoa compared to that of fresh testicular spermatozoa (Table I).

Clinical outcome of ICSI with fresh and frozen–thawed testicular spermatozoa

In patients with obstructive azoospermia, fertilization rates of 80 and 81% were obtained using ICSI with fresh and frozen–thawed testicular spermatozoa, respectively; clinical pregnancies were observed in four out of nine patients with fresh testicular spermatozoa and two out of five patients with frozen–thawed spermatozoa. When fresh testicular spermatozoa obtained from patients with non-obstructive azoospermia were used for ICSI, the fertilization rate was 68% and two out of seven clinical pregnancies resulted (Table VI). There was no statistical difference, in terms of fertilization, embryo cleavage and pregnancy rates, between patients with obstructive azoospermia (fresh and frozen–thawed testicular spermatozoa) and patients with non-obstructive azoospermia (Table VI).

Discussion

As we know, when mammalian spermatozoa (including human spermatozoa) are released from the seminiferous tubules, they are still immature and lack the capacity for effective forward motility. Spermatozoa undergo the process of maturation and gain progressive motility as they pass through the epididymis, where they undergo substantial changes in function, composition and organization (Yanagimachi, 1994). Our results provide interesting evidence that as soon as spermatozoa are released from the seminiferous tubules, if they are motile (weakly shaking) they have the potential spontaneously to gain progressive motility and improved morphology *in vitro* without the effect of the epididymis and special requirements of culture conditions.

We compared the effect of in-vitro culture on the morphology and motility of testicular spermatozoa between patients with obstructive azoospermia and those with non-obstructive azoospermia. Our results showed that after a few days' culture of fresh and frozen–thawed testicular sperm samples obtained from patients with obstructive azoospermia the morphology and motility were significantly improved. The motility of cultured testicular spermatozoa reached a peak around 72–96 h without the need for special media or treatment. Similar results, in terms of improvement of sperm morphology and motility after in-vitro culture, were also observed when testicular spermatozoa were cultured in HTF medium (Irvine Scientific) containing 3% SSS and Ham's F10 medium containing 0.5% bovine serum albumin (A9647; Sigma) (unpublished observation), which indicates that no special media are required for in-vitro culture of human testicular spermatozoa.

Our results of in-vitro culture of testicular spermatozoa obtained from obstructive azoospermic patients were in agreement with the reports by Zhu *et al.* (1996) and Edirisinghe *et al.* (1996a,b). However, as we indicated before, in-vitro culture of human testicular spermatozoa obtained from patients with non-obstructive azoospermia has been unpredictable so far (Liu *et al.*, 1996a). When weakly shaking spermatozoa were observed from fresh testicular biopsies of patients with non-obstructive azoospermia, some samples showed improved motility after in-vitro culture, whereas others did not. When only non-motile spermatozoa (without any weakly shaking spermatozoa) were present in biopsied testicular samples, no motile spermatozoa were found after culture.

The outcome of ICSI with fresh testicular spermatozoa obtained from patients with obstructive azoospermia and with non-obstructive azoospermia was similar to the reports of several groups (Devroey *et al.*, 1994, 1995, 1996; Nagy *et al.*, 1995; Kahraman *et al.*, 1996; Silber *et al.*, 1995, 1996; Tournaye *et al.*, 1996a,b). From our results, we may now suggest that in patients with obstructive azoospermia either ICSI with fresh testicular spermatozoa can be performed on the same day as oocyte retrieval, or testicular biopsy and in-vitro culture of testicular spermatozoa can be carried out 3–4 days before retrieval of oocytes so that progressively motile spermatozoa can be readily found for ICSI. However, in patients with non-obstructive azoospermia, testicular spermatozoa should be extracted and injected into oocytes on the same day as oocyte retrieval, because the results of in-vitro culture of testicular spermatozoa from these patients are unpredictable. Furthermore, if only non-motile fresh spermatozoa are found on the day of oocyte retrieval, ICSI should be carried out on the same day and fertilization can be obtained if the spermatozoa are viable.

The results reveal that cryopreservation had an adverse effect on the survival rate of testicular spermatozoa, since the percentage of non-motile spermatozoa significantly increased after cryopreservation. However, this does not affect the use of frozen–thawed testicular spermatozoa for ICSI since only a few spermatozoa are needed for ICSI. From our experience, it is not difficult to find enough frozen–thawed motile testicular spermatozoa for ICSI.

The procedures used in this study for in-vitro culture and for cryopreservation of testicular spermatozoa are simple and effective, and the chance of losing testicular spermatozoa

during the entire process is low. From our results the presence of debris from testicular tissue did not show an adverse effect on the outcome of in-vitro culture of fresh and frozen–thawed testicular spermatozoa. We believe that it may be better to incubate testicular spermatozoa with the debris containing Sertoli cells, germ cells and other kinds of cells rather than to culture them alone. This kind of culture condition may act like a co-culture system since these debris and testicular spermatozoa originate from testicular tissue. The exact influence of the debris on testicular spermatozoa under the current conditions of in-vitro culture is not clear. Normally for ICSI with cultured testicular spermatozoa, a small amount of testicular sperm suspension is added to the centre of a droplet of medium without the presence of polyvinylpyrrolidone (PVP). A motile testicular spermatozoon can be selected and transferred into a droplet of medium containing 10% PVP. Progressively motile testicular spermatozoa can also be directly transferred from the droplets of culture medium in a culture dish into a 10% PVP droplet in an injection dish by micromanipulation. In this way, a single motile testicular spermatozoon can be selected for ICSI. Therefore, we do not think that it is necessary to remove the debris for purposes of either ICSI or in-vitro culture.

A high fertilization rate was observed with ICSI using frozen–thawed testicular spermatozoa and pregnancies have resulted (Gil-Salom *et al.*, 1996; Liu *et al.*, 1996b; Romero *et al.*, 1996). Considering these results, repeated testicular biopsies are avoided in subsequent ICSI treatment cycles by cryopreservation of testicular spermatozoa in patients with obstructive azoospermia. Since frozen–thawed testicular spermatozoa do not lose their capacity for fertilizing human oocytes using ICSI, cryopreservation of testicular spermatozoa may also be useful to those patients who have testicular cancer. For patients with testicular cancer who are young or not married, prior to treatment (by surgery or chemotherapy), cryopreservation of the patients' ejaculated semen, as well as that of testicular spermatozoa is also recommended for possible use in the future if the quality of the ejaculated semen is poor. One patient had a whole testis removed because of testicular cancer, and testicular spermatozoa from his testicular tissue which was not involved with cancer were cryopreserved (unpublished results).

In conclusion, the morphology and motility of fresh and frozen–thawed testicular spermatozoa can be significantly improved after in-vitro culture in patients with obstructive azoospermia. The outcome of in-vitro culture of human testicular spermatozoa in patients with non-obstructive azoospermia is unpredictable. In-vitro culture of only non-motile testicular spermatozoa is not successful so far. Normal fertilization and pregnancy can be obtained using ICSI with frozen–thawed testicular spermatozoa.

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