Pentoxifylline Initiates Motility in Spontaneously Immotile Epididymal and Testicular Spermatozoa and Allows Normal Fertilization, Pregnancy, and Birth After Intracytoplasmic Sperm Injection

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Purpose: *Pentoxifylline (PF) has been used to enhance sperm motility in many in vitro fertilization programs. The twofold purpose of this study was to determine whether PF stimulates fresh or frozen epididymal and testicular totally immotile spermatozoa and whether it can be used to select viable spermatozoa for intracytoplasmic sperm injection (ICSI).*

Methods: *To test the effect of PF on motility, 10 samples of totally immotile spermatozoa were incubated for 10 min with 3.6 mM PF. Motility was initiated in all cases (14.8% mean motility after PF treatment of five samples of fresh or frozen epididymal spermatozoa and 13.6% mean motility of five samples of fresh or frozen testicular spermatozoa). To assess PF for selection of viable spermatozoa before ICSI, we compared the outcome of ICSI in 20 cycles using fresh or frozen epididymal or testicular PF-treated immotile spermatozoa and 139 control ICSI using fresh or frozen epididymal or testicular spontaneously motile spermatozoa.*

Results: *Fertilization rates were similar in the PF and control groups (45.2% vs. 51.0%). Embryo quality and division stages at the time of transfer were comparable. Six pregnancies occurred in PF-ICSI group (30.0% per cycle vs. 26.6% in control group) including two deliveries of healthy children and four ongoing pregnancies.*

KEY WORDS: Pentoxifylline; immotile spermatozoa; epididymal spermatozoa; testicular spermatozoa.

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INTRODUCTION

Pentoxifylline (PF) is a 3′,5′-nucleotidase phosphodiesterase inhibitor that enhances sperm motility by increasing intracellular cAMP. The stimulating effect of PF on human spermatozoa has been shown both in vivo (1) and in vitro (2). In the field of assisted reproductive technologies, PF has been used for intrauterine insemination (3) and in many in vitro fertilization (IVF) programs (4–6). Regarding the effect of PF on the percentage of motile spermatozoa in semen, conflicting results have been reported. McKinney *et al.* (7) found that PF increases the number of motile spermatozoa, but other authors including Aparicio *et al.* (1), Yovich *et al.* (4), and Tesarik *et al.* (5) observed no such increase.

The twofold purpose of this study was first, to determine whether PF increases the percentage of motility on fresh or frozen immotile epididymal or testicular spermatozoa, and second, to attempt sperm selection for intracytoplasmic sperm injection (ICSI) after PF-treatment. Indeed, random injection of immotile spermatozoa from specimens of poor vitality consistently leads to disappointing results. Liu *et al.* (8) observed that the outcome of ICSI using dead spermatozoa is poor, and this finding concerning necrozoospermia is confirmed in all our microsurgical epididymal sperm aspiration (MESA) and testicular sperm extraction (TESE) programs using immotile sperm of poor vitality. The outcome of ICSI procedures using PF-treated spermatozoa was compared to the outcome of control ICSI procedures using fresh or frozen epididymal or testicular spontaneously motile spermatozoa.

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MATERIALS AND METHODS

Protocol 1: Effect of PF-Treatment on Sperm Motility

Ten fresh or frozen epididymal or testicular spermatozoa samples displaying 0% total motility were included in this protocol. All samples displaying at least one motile sperm (even if very poor on-place motility) were excluded. Percutaneous MESA was carried out using a fine needle. After centrifugation for 25 min at 300 *g* on a monolayer 80% PureSperm solution (JCD-SA, Gauville, France), epididymal spermatozoa were washed twice in BM1 (Ellios Bio-Media, Paris, France) and suspended in 200 μl of the same medium. To obtain sufficient material, TESE was performed by surgery rather than the percutaneous route. For each patient, biopsies were performed on both testicles at several stages. Histological analysis was always performed. Biopsies were mechanically disaggregated during half an hour using sterile slides in Petri dishes containing BM1 medium. The supernatant was centrifuged for 25 min (300 *g*) on a two-layer PureSperm gradient (45%/90%) and washed twice in BM1.

Vitality was assessed by Y-eosine supravital staining on 20 μl aliquots. The remaining sperm suspension was deposited on a Petri dish and motility was analyzed as in protocol 1. If total immotility was observed, the preparation was mixed vol/vol with a 7.2 mM PF solution (Torental, Hoechst Houdé, Paris, France) diluted in BM1 and reassessed for motility after 10-min incubation at room temperature. In cases of frozen spermatozoa, freezing was performed as follows. Epididymal or testicular spermatozoa preparations were diluted vol/vol in Spermfreeze (JCD-SA, France). Spermatozoa were thawed at 37°C, centrifuged for 25 min (300 *g*) on a monolayer 80% PureSperm solution, and washed twice. Sperm motility and vitality were assessed using the same methods as for fresh sperm.

Protocol 2: Selection of Motile Spermatozoa for ICSI

The use of PF was proposed to all our patients displaying 0% total motility in fresh or frozen epididymal or testicular spermatozoa. Because of the unsatisfactory results observed after ICSI using immotile spermatozoa when sperm vitality is low (fertilization rate = 15% in cases where vitality is > 40%), the Institutional Review Board at the Institut de Médecine de la Reproduction decided for ethical reasons that PF-ICSI should be performed on all mature oocytes. Twenty couples were included in this protocol. After pituitary desensitization using a gonadotrophin-releasing hormone agonist (Decapeptyl 3 mg, Ipsen/Biotech, Paris, France), ovarian stimulation was performed using recombinant follicular stimulating hormone (Gonal-F, Serono, Boulogne, France or Puregon, Organon, St Denis, France). Follicles were transvaginally aspirated 35 h after injection of 10,000 IU human chorionic gonadotropin (hCG) (Organon). Oocyte decoronization was carried out using a 15 IU/ml hyaluronidase solution (Hyaluronidase, Choay, Gentilly, France) diluted in BM1.

Fresh or frozen epididymal or testicular spermatozoa were processed as described in protocol 1. After preparation, samples were observed on an inverted microscope to confirm total lack of motility and 7.2 mM PF solution was added vol/vol. If an adequate number of spermatozoa was available, samples were washed by centrifugation for 10 min at 1800 *g* to remove PF. For the poorest samples, the centrifugation step was omitted so as to avoid loss of spermatozoa, and the following cleaning technique was used. Three 20 μl droplets of polyvinylpyrrolidone (PVP) were deposited alongside the sperm PF preparation on the Petri dish and overlayed with prewarmed CO_2-equilibrated oil (Ovoil, JCD-SA, Gauville, France). Using an ICSI pipette, PF-treated spermatozoa were transferred into the first PVP droplet and immobilized with the tip of the pipette. Immobilized spermatozoa were then aspirated into the pipette with a minimum of PF-containing medium and transferred to another part of the PVP droplet containing no PF. Using a new pipette, immobilized spermatozoa in the first PVP droplet were aspirated with a minimum amount of PVP and transferred to the second droplet of fresh PVP. After stirring with the tip of the pipette to dilute the remaining PF-containing medium, the spermatozoa were reaspirated into the pipette. After repeating this cleaning operation in the third PVP droplet, ICSI was performed as described by Van Steirteghem *et al.* (9).

In order to assess the effectiveness of PF-ICSI, fertilization and cleavage rates, embryo quality, and pregnancy and implantation rates were prospectively compared to those observed in control ICSI performed during the same period. Control ICSI were always performed using fresh or frozen epididymal or testicular spermatozoa, but injected spermatozoa were always spontaneously motile and never PF-treated. Embryo quality was assessed using an embryo score based on the presence of anucleate fragments, blastomere irregularities in shape and/or size, and cleavage stage

(10). The embryo scores range from 0 to 4 points, 0-point embryos corresponding to uncleaved embryos and 4-point embryos corresponding to the best embryos, i.e., embryos displaying no anucleate fragments or no blastomere irregularities and which are at the 4-cell stage at the time of transfer 48 hr after oocyte retrieval.

Statistical Analysis

Statistical analysis was performed using Statistical Analysis Software (SAS 6.04). Quantitative data were compared by Wilcoxon Signed Rank test for the related samples, and Mann Withney U test for the independant samples. These tests were performed because hypothesis for parametric tests were not satisfied.

RESULTS

Protocol 1: Effect of PF Treatment on Spermatozoa Motility

Sperm motility before and after PF treatment in the ten specimens displaying 0% sperm motility are shown in Table I. Stimulation of some sperm was observed in all cases. Almost half of the live sperm were stimulated (14.8% motile out of 29.2% live epididymal sperm, and 13.6% out of 33.6% live testicular sperm).

Table I. Effect of PF on Totally Immotile Fresh or Frozen, Epididymal or Testicular Spermatozoa[a]

Specimen	Initial motility	PF Motility (%)	Vitality (%)
1 MESA	0	26	58
2 MESA	0	18	34
3 MESA	0	2	4
4 Fr. epid.	0	8	10
5 Fr. epid.	0	20	40
Mean	0	14.8	29.2
6 TESE	0	20	30
7 TESE	0	10	26
8 TESE	0	10	30
9 Fr. test.	0	18	36
10 Fr. test.	0	10	46
Mean	0	13.6	33.6

[a] MESA, Spermatozoa obtained after microsurgical epididymal sperm aspiration; TESE, spermatozoa obtained after testicular sperm extraction; Fr. epid., frozen epididymal spermatozoa; Fr. test., frozen testicular spermatozoa.

Protocol 2: Selection of Motile Spermatozoa for ICSI

One hundred fifty-nine ICSI using epididymal and testicular spermatozoa before or after freezing were performed, including 139 control ICSI using spontaneously motile spermatozoa (87.4%) and 20 ICSI using PF-treated spermatozoa (12.6%). Sperm count was low in all PF-ICSI cases (range: $50–10^4$ sperm in the whole preparation). The outcome of ICSI using PF-treated spermatozoa are summarized in Table II and compared to Tables III and IV. The mean percentage of PF-stimulated spermatozoa was of 16.4% for a mean vitality of 31.2%. Fertilization and cleavage rates were similar in PF-ICSI and control groups (respectively, 45.2% vs. 51.0% and 95.4% vs. 95.3%, NS). The number of transferred embryos was higher in the PF-ICSI group (3.2 vs. 2.6, $P < 0.05$), but pregnancy rate and implantation rate per transferred embryo were similar in both groups (respectively, 30.0% vs. 26.6, NS, and 12.3% vs. 11.2%, NS). The distribution of embryo scores was also similar (Table IV). Six pregnancies occurred in the PF group, including one miscarriage, three ongoing pregnancies, and the birth of two girls (Table II; case No.2: one fetal loss during first trimester and one healthy birth; case No. 6: one healthy birth). In two cases extra embryos were frozen and thawed, but no pregnancy occurred.

DISCUSSION

Following the report of Yovich *et al.* (2), PF was widely used to enhance spermatozoa motility in IVF programs (4–6,11). Regarding ejaculated spermatozoa, Mc Kinney *et al.* (7) found that PF increases the number of motile cells in cases of normozoospermia and asthenozoospermia. However, Aparicio *et al.* (1), Yovich *et al.* (4), and Tesarik *et al.* (5) failed to confirm this finding. This discrepancy could be due to differences in experimental design or to statistical methods. Assessment of PF-induced sperm motility is more accurate when spontaneous motility before treatment is 0% as was the case in the present report. Our data clearly demonstrate that PF stimulates immotile fresh or frozen epididymal and testicular spermatozoa, since motility was initiated in all of the 30 cases studied in the two protocols. These findings confirm those of Tasdemir *et al.* (14), who observed stimulation of motility in ten immotile testicular spermatozoa samples after longer incubation (30, 60, and 90 min) in PF, and those of Buch *et al.* (15), who observed that

Table II. Overall Results of 20 ICSI Performed After PF-Initiation of Motility in Immotile Spermatozoa[a]

Sample	PF motility	Vitality	Injected oocytes	2 PN embryos	Transferred embryos	Fetal hearts
1 TESE (MA)	19	40	15	6	4	2
2 TESE (MA)	11	26	23	11	3	2
3 TESE (MA)	50	70	13	8	5	0
4 TESE (MA)	15	34	14	2	2	0
5 TESE (GCA)	10	28	15	8	4	0
6 TESE (GCA)	16	30	7	5	4	1
7 TESE (GCA)	20	44	16	6	4	0
8 Fr. T. (GCA)	40	60	7	3	3	1
9 Fr. T. (GCA)	16	26	16	6	3	1
10 Fr. T. (GCA)	14	30	2	2	2	0
11 Fr. T. (GCA)	15	40	2	2	2	0
12 Fr. T. (NS)	5	16	14	6	4	0
13 Fr. T. (NS)	30	54	9	4	3	0
14 Fr. T. (NS)	4	10	10	7	5	0
15 MESA	16	36	9	5	2	1
16 MESA	10	26	19	7	3	0
17 MESA	1	6	19	6	3	0
18 Fr. Ep.	20	44	7	3	3	0
19 Fr. Ep.	10	20	6	3	3	0
20 Fr. Ep.	6	10	18	9	3	0

[a] TESE, Spermatozoa obtained after testicular sperm extraction; Fr. T., frozen testicular spermatozoa; GCA, germ cell aplasia; MA, maturation arrest; NS, normal spermatogenesis; MESA, spermatozoa obtained after microsurgical epididymal sperm aspiration; Fr. Ep., frozen epididymal spermatozoa.

PF enhanced motility in two cases of cryopreserved ductal spermatozoa.

Random injection of immotile spermatozoa from specimens of poor vitality consistently leads to disappointing results. Liu *et al.* (8) observed that the outcome of ICSI using dead spermatozoa is poor, with intact cells remaining present in the ooplasm 20 hr after injection. This finding concerning necrozoospermia has been confirmed in all MESA and TESE programs using spermatozoa of poor vitality. Nagy *et al.* (16), who obtained a 45% fertilization rate and a 28.6% pregnancy rate after ICSI using untreated immotile testicular spermatozoa, unfortunately did not specify sperm vitality in their study. As sperm vitality was only 32% in the present series, the fertilization rate (FR) without the use of PF probably would not have exceeded 16% (about 50% of oocytes injected with live spermatozoa after random sperm selection). Outcome of ICSI using PF-treated spermatozoa was comparable to outcome after ICSI using spontaneously motile spermatozoa (FR = 45.2% vs. 51.0%). Thus we speculate that PF treatment allowed a threefold increase in FR. Pregnancy rates were similar in the

Table III. Comparison of the Outcome of ICSI in the PF-Treatment (PF-ICSI) and Control Groups[a]

	PF-ICSI $N = 20$	Control $N = 139$
Mean female age	32.8	32.0[b]
Retrieved oocytes (mean)	323 (16.2)	1586 (11.4)[c]
Mature oocytes (mean)	241 (12.0)	1128 (8.1)[c]
Diploid zygotes (fertilization rate)	109 (45.2)	575 (51.0)[b]
Cleaved embryos (%)	104 (95.4)	548 (95.3)[b]
Transfers (% per cycle)	20 (100)	138 (99)[b]
Transferred embryos (mean)	65 (3.2)	357 (2.6)[d]
Pregnancies (% per cycle)	6 (30.0)	37 (26.6)[b]
Fetal hearts (Implantation rate)	8 (12.3)	40 (11.2)[b]

[a] In both groups injected spermatozoa were epididymal or testicular spermatozoa obtained before or after freezing. In the PF-ICSI group, all sperm used were immotile before treatment and motile after treatment. In the control group, all injected sperm were spontaneously motile.
[b] Not significant.
[c] $P < 0.01$.
[d] $P < 0.05$.

Table IV. Comparison of Embryo Quality Obtained After ICSI in the PF Treatment and Control Groups[a]

	PF-ICSI	Control
Score 0 embryos (uncleaved embryos)	4.6%	4.7%
Score 1 embryos	24.8%	19.3%
Score 2 embryos	27.5%	28.0%
Score 3 embryos	33.0%	32.5%
Score 4 embryos	10.1%	15.5%

[a] The distribution of embryo scores in both groups are not statistically different.

PF-ICSI and control ICSI groups (30.0% vs. 26.6%, NS). Nodar *et al.* (17) obtained one birth of twin males after ICSI using PF-treated immotile testicular spermatozoa. The pregnancies and births presented in this article confirm the usefullness of this method on fresh testicular spermatozoa and to our knowledge are the first published successful PF-ICSI using epididymal and frozen testicular spermatozoa.

The possibility of PF-induced embryotoxicity has been reported. Tournaye *et al.* (18,19) observed several adverse effects of PF on mouse embryos, but they (20) later reported that these adverse effects could be prevented by washing spermatozoa after PF treatment. Lacham-Kaplan and Trouson (21) observed no adverse effects on embryo development, neither when cumulus-free oocytes were inseminated with spermatozoa for up to 18–20 hr in 3 mM PF nor when subzonal insemination (SUZI) using PF-treated spermatozoa was performed. Our data demonstrate that short exposure of sperm to PF followed by sperm washing does not affect early embryo development after ICSI. Cleavage rate is normal (95.4%), the distribution of embryo scores is comparable in the PF-treatment and control groups, and the pregnancy and the implantation rates are similar (respectively, 30.6% vs. 26.6%, and 12.3% vs. 11.2%).

Use of the hypo-osmotic swelling test (HOST) has been proposed for identification of live sperm before ICSI (22). It has been questioned by Vandervorst *et al.* (23) because of possible embryo toxicity. As it allows water to enter into viable spermatozoa and induces membrane expands, HOST may cause lysis of the plasma membrane and cell death. Even short sperm incubation in water induces severe osmotic stress (24). Moreover, in cases involving very rare epididymal or testicular spermatozoa, HOST is often difficult to interpret because of absence of obvious tail swelling. PF allows more accurate and easy evaluation of sperm vitality, as it allows us to observe motile, thus obviously viable, spermatozoa. Recently, the heparin-glutathione test (HEGLUT) has been shown to allow us a selection of viable human spermatozoa, and may be another alternative to the HOST in an ICSI program (25). However, contrary to HEGLUT and HOST, PF allows us to select motile spermatozoa, and thus it may be more effective to inject gametes with apparently no axoneme defect.

In conclusion, as MESA, TESE, and cryopreservation of epididymal or testicular spermatozoa for subsequent ICSI cycle are performed in most IVF laboratories, the findings of this study suggest that PF initiation of sperm motility may be of interest in these problematic cases where only immotile sperm of poor vitality are available.

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