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Influence of pentoxifylline in severe male factor infertility

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Two in vitro fertilization sperm preparation protocols using pentoxifylline (long and short exposure before insemination) were studied in 57 couples (61 cycles) with male factor infertility. For each cycle, oocytes were divided into two groups for insemination using either pentoxifylline-treated or control semen. Fertilization rates improved over controls in the short protocol ($P < 0.001$) and fewer couples experienced fertilization failure ($P = 0.02$). Sixteen pregnancies ensued (30% per collection with the short protocol), and 4 were from cases with $<1.0 \times 10^6$ progressively motile sperm count per milliliter, 1 being as low as 0.2×10^6 progressively motile count per milliliter. Seventeen healthy infants have now delivered and pregnancy wastage is not increased. Pentoxifylline is thus a useful sperm treatment for cases of male factor infertility. Fertil Steril 53:715, 1990

It is well documented that the human fertilization rate in vitro is directly affected by the severity of oligospermia, which, if associated with asthenospermia, usually results in partial or total failure of fertilization.[1,2]

Improvement of sperm motility has been observed in oligospermic/asthenospermic males with the use of sperm stimulants such as 3-deoxy-adenosine[3] and pentoxifylline (Hoechst, Melbourne, Victoria, Australia).[4] In a previous publication,[4] we demonstrated a high incidence of pregnancy using pentoxifylline among couples in whom there was a failure of fertilization in previous in vitro fertilization (IVF) attempts, usually because of oligospermia. In addition, we observed beneficial effects of pentoxifylline on sperm motility and progressive sperm motility in this group of males. As we assimilated our experience, a revised protocol was introduced, and the present study compares the results obtained by this means with those using the original protocol. Essentially the revised protocol reduced the time between treatment of sperm and insemination, so that pentoxifylline treatment is carried out on sperm after, rather than before, a "sperm-rise" procedure to select out motile spermatozoa. Pentoxifylline is marketed by Hoechst as Trental, but was used here as the pure compound.

MATERIALS AND METHODS

Fifty-seven couples who had underlying male factor infertility were included in a PROST (pronuclear stage tubal transfer)[5] or TEST (tubal embryo stage transfer)[5] program with a total of 61 treatment cycles. The majority of the men were oligospermics/asthenospermics, however 6 had sperm counts in the normal range, but with reduced sperm activity (<1.5 on a 1 to 3 point scale) and a history of fertilization failure. Multiple follicular development was induced with an ovarian stimulation regimen combining clomiphene citrate (Clomid; Merrell-Dow Pharmaceuticals Inc., Cincinnati, OH) with human menopausal gonadotro-

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phin (hMG, Pergonal; Serono, Rome, Italy) or the gonadotropin-releasing hormone agonist leuprolide acetate (Lucrin; Abbott Pharmaceuticals, Melbourne, Victoria, Australia) used in a long-term down-regulation protocol with hMG.[6] Ovulation was induced with human chorionic gonadotropin (hCG) 10,000 IU, and oocytes were recovered transvaginally with ultrasound guidance 35 hours later. The oocytes were washed and preincubated in human tubal fluid medium[7] + 10% heat-deactivated human serum for 4 to 6 hours. Husbands were asked to produce semen samples 1 to 2 hours after oocyte recovery.

Collection of Semen Samples

Semen samples were obtained by masturbation after 3 to 4 days of sexual abstinence. Samples were allowed to liquefy for 20 minutes at room temperature, examined by routine semen analysis, and classified using a modification of the revised criteria of the World Health Organization[8] as being normospermic (>20 million total or >10 million motile spermatozoa/mL) or oligospermic (<10 million motile spermatozoa/mL). We further categorized the latter group as moderately oligospermic (>5 or ≤10 million motile spermatozoa/mL) or severely oligospermic ≤ 5 million motile spermatozoa/mL). Both overall motile sperm counts and forwardly progressive motile sperm counts were recorded.

Preparation and Treatment of Spermatozoa with Pentoxifylline

Semen samples were divided equally into two portions: one half was treated with pentoxifylline and the other used as the control. A second ejaculate was obtained if necessary from severely oligospermic patients, and this was similarly treated. Division of semen samples into treated and control groups in the present study is a modification of that described in our previous publication[4] where the first ejaculate was used as the control and the second (produced after 1 hour) for treatment with pentoxifylline. The modified method was used to eliminate any possible bias in fertilization rates between treated or control groups when comparing first and second ejaculates.

Control aliquots were treated either by the overlay[9] or modified sedimentation[10] techniques to separate highly motile sperm. With the overlay technique, the sample was washed twice with human tubal fluid medium containing 10% heat inactivated maternal serum and centrifuged after each wash at 200 × *g* for 5 minutes. The sperm pellet was placed in a 5 mL Falcon tube (Falcon No. 2001; Falcon Plastics, Oxnard, CA) and 3 mL of fresh medium was gently layered over the pellet. For sedimentation, which was used when the sperm numbers were very low, the washed pellet was placed in one well of a four-well dish (Nunc No. 134673; Kamstrup, Roskilde, Denmark) and gently overlayered with 1 mL of medium. The motile spermatozoa were harvested in the supernatant for subsequent insemination of control eggs. Experimental samples were treated either by protocol 1 or 2. The major difference between the 2 protocols related to the time of first exposure of spermatozoa to pentoxifylline before insemination. Although total incubation with pentoxifylline was the same (30 minutes) in both protocols, in protocol 1, sperm were treated with pentoxifylline *before* selection by sperm-rise, whereas in protocol 2 treatment took place *after* sperm-rise. For protocol 1, this meant that as long as 3 hours elapsed from first exposing sperm to pentoxifylline, whereas in protocol 2 it was only 40 minutes.

Protocol 1

The semen sample was washed once by centrifugation (200 × *g*, 5 minutes) in 3 mL of human tubal fluid medium + 10% serum, and the sperm pellet was suspended and incubated in 3 mL of medium containing 1 mg/mL pentoxifylline for 30 minutes. After incubation, the sample was centrifuged, the supernatant removed, and the sperm pellet was prepared by the overlay technique with human tubal fluid medium + 10% serum for normospermic and moderately oligospermic samples, and the sedimentation technique for severely oligospermic samples. Between 30 minutes and 2 hours (depending on semen quality) 1 mL of the supernatant was removed and the total, motile, and progressively motile sperm counts were recorded. The spermatozoa were washed once to remove pentoxifylline, the sperm count was adjusted to 1×10^6/mL and the suspension used for insemination.

Protocol 2

Experimental samples were washed once with medium + 10% serum and motile spermatozoa were isolated either by the overlay method or by the sedimentation technique as above. After incubation for $\frac{1}{2}$ to 1 hour, the overlay was removed, counted, and kept at 37°C. The volumes recovered

ranged from 0.8 mL (sedimentation) to a maximum of 3 mL (overlay). An equal volume of medium + 10% serum with 2 mg/mL pentoxifylline was added to the sample approximately 40 minutes before insemination was due. This made a final pentoxifylline concentration of 1 mg/mL. The sample was then incubated for 30 minutes. After this, to remove pentoxifylline, medium + 10% serum was added to take the volume up to a total of 5 mL, and the sample was then centrifuged. The sperm pellet was resuspended in medium + 10% serum to adjust the final sperm concentration to 1×10^6/mL.

Clinical Application (Insemination, Fertilization, and Embryo Transfer)

Thirty-one treatment cycles were carried out in the study group using protocol 1 and 30 treatment cycles comprised the study group for protocol 2. Six men categorized as normospermic were included (one for protocol 1, and five for protocol 2) because of reduced sperm activity and because of fertilization failure in previous IVF attempts. In most cases, washed sperm was added to approximately half the total oocytes to form the control group, whereas the remaining oocytes were inseminated with pentoxifylline-treated spermatozoa. Where feasible, oocytes were randomly selected for insemination, however, the numbers inseminated varied according to the quality of oocytes collected, the semen quality, and the numbers of motile sperm available for insemination. Furthermore, the couples' clinical history and their request for the division of oocytes into treatment and control groups were taken into consideration. Where <50% of oocytes were allocated to controls, these were selected so as to give an approximately equal balance of good quality oocytes as graded[11] on cumulus dispersal, tightness of the coronal coat, and ooplasmic appearance. The number of sperm used for insemination varied from 50,000 to 100,000 motile spermatozoa and this was carried out 4 to 6 hours after oocyte collection. In some couples with a history of consecutive failed fertilization, all oocytes were inseminated with pentoxifylline-treated sperm (five in protocol 1, and nine in protocol 2; see Fig. 1). If fertilization was demonstrated by the presence of two pronuclei in oocytes, then up to four pronuclear oocytes were transferred into the fallopian tubes in PROST or four embryos into the tube in TEST. Embryos were selected for transfer on the basis of a four-point grading system involving clarity, granularity, developmental rate, and regularity

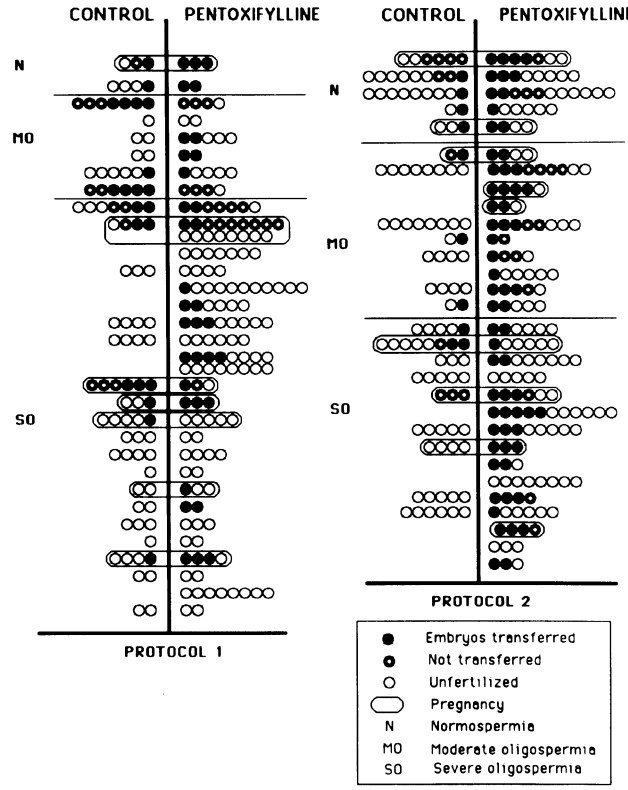

Figure 1 Embryology data and distribution of pregnancies for treatment cycles using two pentoxifylline treatment protocols; long (protocol 1) and short (protocol 2) incubation interval before oocyte insemination.

of blastomeres. Thus, in some cases only control embryos (2 couples, protocol 1), or only pentoxifylline generated embryos (2 couples, protocol 2), or a mixture of embryos (4 couples, protocol 1; 6 couples, protocol 2) were transferred, based on the policy that only the "best" embryos were to be transferred. The excess embryos were either discarded (poor quality and unsuitable for cryopreservation, 8 cases; triploid embryo, 1 case) being substandard for cryopreservation (9 cases), cryopreserved (6 cases), or donated to ethically approved research projects (2 cases). In one couple with severe oligospermia having their third attempt (protocol 2), five embryos were transferred after counseling, but one of these was of very poor quality (PIVET policy at the time was for a maximum of four good quality embryos to be replaced: this has now been reduced to three to reduce the risks of multiple pregnancy). This treatment cycle was not successful (Fig. 1). Pregnancy was diagnosed by rising levels of β-hCG in the serum 16 to 19 days after oocyte collection and confirmed 5 to 6 weeks after oocyte recovery by

Table 1 Overall Fertilization Rates and Pregnancy Outcome for Patients on Pentoxifylline Protocols 1 and 2

	Fertilization rates[a]		Embryos transferred		Pregnancy outcome[a]	
	Control	Pentoxifylline	Control	Pentoxifylline	Per transfer	Per collection
Protocol 1	33/91 (36.3)	52/154 (33.8)	21	34 [8][b]	7/19 (36.8)	7/31 (22.6)
Protocol 2	21/103 (20.4)	81/171 (47.4)[c]	10	64 [18][b]	9/27 (33.3)	9/30 (30)

[a] Values in parentheses are percents.
[b] Number of patients who had only pentoxifylline embryos transferred.
[c] $\chi^2 = 18.886$; $P < 0.001$.

ultrasound, so that only clinical pregnancies were included. Results were evaluated using the χ^2 test in appropriate contingency tables. Ethical approval for the procedures was originally given by the Human Rights Committee, University of Western Australia, and recently approval has also been granted by the Ethics Committee of Cambridge Private Hospital (formed April, 1989).

RESULTS

The number of eggs collected, fertilization rate, and distribution of pregnancies for each of the couples who were on the two pentoxifylline treatment protocols are given in Figure 1. The data (control and pentoxifylline) are arranged into three groups according to the husband's motile sperm count: normospermia, moderate oligospermia, and severe oligospermia. Overall, using protocol 1, only 19 of 31 couples achieved fertilization (61.3%), whereas with protocol 2, a significantly higher number of couples ($\chi^2 = 5.32$; $P = 0.02$) achieved fertilization (27 of 30; 90%).

Figure 1 also details the pregnancies arising in each of the three groups. The distribution of pregnancies for both protocols was 1 of 1 (100%; oligospermia), 0 of 7 (0%; moderately normospermic), and 6 of 23 (26.1%; severely oligospermic) for protocol 1; and 2 of 5 (40%; normospermia), 3 of 10 (30%; moderately oligospermic), and 4 of 15 (26.7%; severely oligospermic) using protocol 2, i.e., a greater proportion of moderately oligospermic cases were successful in the latter. In the majority of the men, pentoxifylline improved the sperm motility and progressive motility, similar to our previous observations[4] in both protocol 1 and 2.

The overall fertilization rates and pregnancy outcome for couples on pentoxifylline protocols 1 and 2 are given in Table 1. Pentoxifylline protocol 1 gave no significant improvement in fertilization rate when compared with the control group. However, in protocol 2, where pentoxifylline was added to sperm closer to the time of insemination and after a sperm-rise procedure, a significant improvement in fertilization rate was observed over that of the control group (control: 20.4% versus pentoxifylline: 47.4%; $P < 0.001$). Because of the improvement in fertilization rate with pentoxifylline protocol 2, a greater proportion of the pentoxifylline-generated embryos (86.5%) were transferred in this group compared with the group treated with protocol 1 (65.4%). Furthermore, in protocol 2, more women had pentoxifylline-generated embryos transferred (18 of 27; 66.7%) compared with pentoxifylline protocol 1 (8 of 19; 42.1%). In couples with previous fertilization failure, only pentoxifylline-treated sperm was used. These gave a fertilization rate of 7 of 48 (14.6%) for protocol 1, and 20 of 47 (42.6%) for protocol 2. When these are taken out of the data for Table 1 the pentoxifylline-generated fertilization rate for the remaining couples was 45 of 106 (42%) n = 26 for protocol 1 and 61 of 124 (49%) n = 21 for protocol 2 (no significant difference). Thus, the main benefit of pentoxifylline protocol 2 would appear to be for those couples with a history of fertilization failures.

Figure 2 shows the semen analyses for 31 men who were included in the study group for pentoxifylline protocol 1 and 30 men in protocol 2. The data are arranged by descending motile sperm count as in Figure 1. In protocol 1, two ejaculates were produced by 27 of the 31 men, and in protocol 2 the figure was 19 out of 30. Where two ejaculates were used, the means were used to calculate the sperm counts in Figure 1. Using protocol 1, pregnancies were achieved in the normospermic and severely oligospermic groups. Out of seven pregnancies, six were in the severely oligospermic group. Two of the six were achieved with motile sperm counts of 1 and 0.5×10^6/mL. These samples had 0.7 and 0.2×10^6/mL progressively motile spermatozoa, respectively. The semen analyses for the 30 men in protocol 2 are detailed in Figure 2, and it

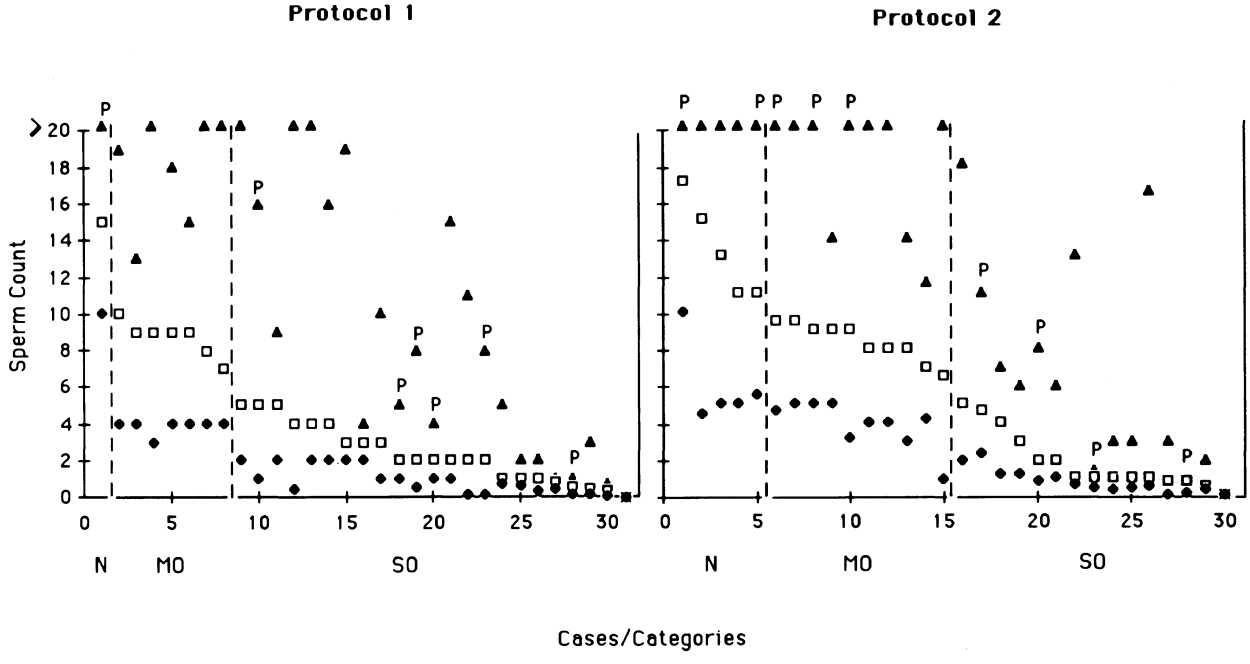

Figure 2 Semen analyses of ejaculates for patients undergoing in vitro pentoxifylline-sperm preparation arranged in decreasing order of the motile count (P, Pregnant; N, Normospermia; MO, Moderate Oligospermia; SO, Severe Oligospermia). A long (protocol 1) and short (protocol 2) pentoxifylline-sperm incubation interval was studied. ▲, total count; □, motile count; ♦, progressive motile count.

can be seen that the nine pregnancies that arose were derived from all three semen categories. The motile sperm counts for four successful attempts in the severely oligospermic group were 4.7, 2, 1, and 0.8×10^6/mL. Six pregnancies resulted from the transfer of pentoxifylline-generated embryos only.

The implantation rate, pregnancy outcome, and number of infants born using pentoxifylline are shown in Table 2. The implantation rate (calculated by number of pregnancy sacs detected on ultrasound 5 to 6 weeks after embryo transfer divided by the number of embryos transferred) was 14.5% for protocol 1 and 14.9% for protocol 2. Seven pregnancies (1 in protocol 1, and 6 in protocol 2) resulted from the transfer of pentoxifylline-generated embryos only. Overall, only 2 of 16 pregnancies were lost as blighted ovum pregnancies (early pregnancy wastage = 12.5%), there was 1 ectopic pregnancy, and all other pregnancies are ongoing or complete, with 8 women delivering 10 healthy infants to date (6 singletons and 2 sets of twins).

Table 2 Implantation Rate and Pregnancy Outcome for Pentoxifylline Protocols 1 and 2[a]

	Number of pregnancies	Implantation rate[b]	Pregnancy outcome
Protocol 1	7	8/55 (14.5)	1 blighted ovum 5 singleton 1 twin
Protocol 2	9	11/74 (14.9)	1 blighted ovum 5 singleton 2 twin 1 ectopic

[a] Number of pregnancies with only pentoxifylline embryos transferred = 7 (1 for protocol 1; 6 for protocol 2).

[b] No. sacs/no. embryos transferred; values in parentheses are percents.

DISCUSSION

Pentoxifylline, a phosphodiesterase inhibitor of the methylxanthine group, inhibits the breakdown of cyclic adenosine monophosphate (cAMP). Intracellular cAMP levels are known to play a central role in sperm motility.[12] Sperm motility is important for penetration through the zona pellucida,[13] and this function has been shown to have a high correlation with fertilization rates in vitro.[14] The addition of sperm stimulants has been shown to be beneficial for oligospermic/asthenospermic samples presumably by improving motility.[3,4] In a clinical trial, reported in our previous publication,[4] no differences in motility were noted when pentoxifyl-

line was added to normospermic samples, however, pentoxifylline significantly improved motility and progressive motility in oligospermic samples. In the present study, the observations on motility were similar. Although no improvement in fertilization rate was shown with pentoxifylline (protocol 1), a high incidence of pregnancy was noted among couples who had previous episodes of failed fertilization due to male factor infertility.[4] Similarly, in the present study protocol 1 gave no significant increase in fertilization rate, however, an increased fertilization rate for this group was obtained by modifying the pentoxifylline treatment protocol (protocol 2). The beneficial effects of pentoxifylline may not simply be limited to motility, as other sperm functions such as capacitation and the acrosome reaction may be modulated by cAMP.[15] At present, we have no clear evidence whether these functions are affected.

Using protocol 1 as described in our earlier report,[4] pentoxifylline improved the motile sperm recovery, but did not improve the fertilization rate over that of controls. In this treatment procedure, the spermatozoa were exposed to the stimulant 1 to $1\frac{1}{2}$ hours before insemination. However, when the pentoxifylline protocol was modified to expose spermatozoa to the stimulant just before insemination ($\frac{1}{2}$ hour; protocol 2), the fertilization rate was markedly improved. Work carried out by Cai and Marik[16] using the zona-free hamster penetration test, showed similar improvement in the penetration capacity of sperm when caffeine was added at coincubation with zona-free hamster eggs rather than during sperm preparation. One of the reasons for the difference in response between the two pentoxifylline treatment protocols, similar to that seen by Cai and Marik for caffeine, may be that the pentoxifylline effect on spermatozoa may not persist long enough because of a limited intracellular energy supply or because of exhaustion of the energy substrates available. Furthermore, exposure of spermatozoa to pentoxifylline may accelerate the onset of spontaneous acrosome reactions. It is generally accepted that the fertilizing sperm completes the acrosome reaction when it is close to, or on the zona pellucida,[15] and that there is a narrow temporal window after the reaction in which sperm can fertilize. In the hamster penetration test,[16] a significant improvement in sperm penetration occurred with poor motility samples when caffeine was added at coincubation as stated above. Similarly, in human IVF, the best results might well be attained by treating with pentoxifylline during coincubation of sperm and oocytes, but this approach should be treated with caution (see below).

One possible objection to these procedures is that pentoxifylline exposure might lead to detrimental effects on syngamy and early embryogenesis. Certainly we cannot remove it completely from the medium used for insemination. There are two ways by which pentoxifylline could be communicated to the conceptus: one by the fertilizing spermatozoon and the other from the culture medium. The total volume of the human sperm has been estimated at between 14 and 16 μm^3.[17] By contrast, the diameter of the human oocyte is between 130 and 140 μm[18], giving a total volume of 1.15 to 1.43×10^6 μm^3, and a mass of approximately 1.2 to 1.5 μg. The volume ratios between oocyte and sperm are thus of the order of 100,000:1, and any direct transmission of pentoxifylline by the fertilizing sperm is likely to be trivial.

Turning to the culture medium, we can only estimate the carry-over of pentoxifylline into the insemination tubes. Thus, in protocol 2, after exposure of sperm to pentoxifylline at 1 mg/mL, the suspension is diluted 5-fold to 5 mL, reducing the pentoxifylline to approximately 0.2 mg/mL. The suspension is centrifuged and the pellet of approximately 0.1 mL removed. This now contains approximately 0.02 mg "free" pentoxifylline (assuming, conservatively, that it is not preferentially concentrated by the sperm cells). For insemination, depending on the count, this is normally diluted still further. However, in the worst cases with very few motile spermatozoa, the total amount remaining would be added to the 1 mL culture tube containing the oocytes that would thus be exposed to a maximum free concentration of about 0.02 mg/mL (2 μg/mL) or 50-fold less than that used to treat the sperm. In most cases, the concentration would be much less than this level. Pentoxifylline, while not recommended for use in human pregnancy, has been tested at levels of up to 450 $mg \cdot kg^{-1} \cdot d^{-1}$ through pregnancy in laboratory animals with no significant teratogenic effects.[19] This would roughly equate to a concentration of 0.45 mg/mL in solution. Maximum therapeutic dosage for use in the treatment of vascular disease is 1,200 mg/d either orally or intravenously,[20] and this would approximate to 0.02 mg/mL for a 60 kg individual, although such an assumption is highly simplistic and does not take into account partitioning of the drug into various body compartments. Nevertheless, it would appear that the exposure of the oocyte to pentoxifylline is probably at acceptable levels,

and this would seem to be vindicated by the results described here.

It is now well-documented that naturally occurring cAMP phosphodiesterase inhibitors of follicular origin are implicated in the maintenance of meiotic arrest in mammalian oocytes.[21] One possible effect of pentoxifylline might thus be to inhibit oocyte maturation in vitro; however, oocytes are normally cultured for 4 to 6 hours before insemination, so this is unlikely to be a serious problem for the majority of cases. Certainly it would be inadvisable to expose oocytes to pentoxifylline before such a period of maturation in vitro. Further study using animal gametes is necessary before any more radical approaches (e.g., coculture of sperms and mature eggs in pentoxifylline containing medium) could be explored in human IVF.

In IVF and related techniques, the fertilization rate is significantly reduced in direct association with the severity of oligospermia and there may be total fertilization failure in some patients.[1,2] Improved pregnancy rates for oligospermics were achieved in a gamete intrafallopian transfer treatment procedure by transferring more than the standard 100,000 spermatozoa with the eggs.[22] However, in most instances, great difficulties are experienced in obtaining adequate sperm numbers for these procedures, because of the severity of the problem and the added complication of asthenospermia. Sperm stimulants such as pentoxifylline have shown improved motility, especially in oligospermic/asthenospermic samples.[3,4] In the present study, because of the enhancement of spermatozoal motility and improvement in spermatozoal recovery and fertilization rate with pentoxifylline, the pregnancy rate in the severely oligospermic group of patients is now similar to that seen in the normospermic category undergoing PROST or TEST treatments.[23]

Use of pentoxifylline did not increase the rate of early pregnancy loss (blighted ovum pregnancies 2 out of 16; 12.5%) over that found in other IVF procedures.[23] The implantation rate is comparable with that reported for normospermics having tubal transfer procedures.[23] To date, a total of 17 infants have been born after pentoxifylline procedures at PIVET (this report + 7) and 6 more are currently advanced beyond 20 weeks. All the infants (6 males and 11 females) were assessed as normal after careful examinations by specialist neonatal pediatricians and all have continued to thrive.

The use of such nontoxic sperm stimulants to assist fertilization in cases of known or suspected male factor infertility should prove an acceptable alternative to invasive methods such as micromanipulation, which to date have given rather disappointing results with respect to implantation and pregnancy.[24] Further improvement may be possible for male factor patients with very low sperm counts by reducing the culture volume to as low as 5 to 10 μL.[25] This may enable the appropriate concentration of spermatozoa to be achieved with as few as 250 to 500 spermatozoa, and this possibility is being explored.

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