

The use of chymotrypsin/galactose to treat spermatozoa bound with anti-sperm antibodies prior to intra-uterine insemination

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Anti-sperm antibodies in semen have been associated with a decrease in fertility potential. The question arises as to whether intra-uterine insemination (IUI) can improve pregnancy rates by merely allowing earlier capacitation and close timing to ovulation, or whether certain treatments of the spermatozoa add extra benefit. The study presented herein was designed to compare IUI using Percoll density separation with an albumin treatment versus chymotrypsin/galactose treatment. Sixteen patients were evaluated where IUI was randomized between both sperm treatments. Pregnancy rates/cycle were 25% (eight of 32) with chymotrypsin/galactose-treated spermatozoa compared to only 3% (one of 33) cycles with albumin-treated spermatozoa ($P < 0.01$). Since it has been reported that the proportions of spermatozoa showing immunobead binding for specific antibodies after chymotrypsin/galactose treatment remain unchanged, the exact mechanism for improvement is unknown; possibly chymotrypsin/galactose interferes with the function of the antibodies.

Key words: albumin/anti-sperm antibodies/chymotrypsin/galactose/intra-uterine insemination/pregnancy rates

Introduction

Anti-sperm antibodies have been associated with a decreased fertilizing ability, both *in vivo* and *in vitro* (Jones, 1980; Bronson *et al.*, 1982a; Ackerman *et al.*, 1984; Ayvaliotis *et al.*, 1985; Brannen-Brock and Hall, 1985; Clarke *et al.*, 1985; Kamada *et al.*, 1985; Mandelbaum *et al.*, 1987; Matson *et al.*, 1988). Approximately 15% of men undergoing infertility evaluation have significantly elevated numbers of these antibodies bound to their spermatozoa (Check and Bollendorf, 1992). One adverse effect of these antibodies on spermatozoa may be inhibition of sperm progression through cervical mucus, as demonstrated by a poor post-coital test (Moghissi *et al.*, 1980; Menge *et al.*, 1982; Wang *et al.*, 1985).

Intra-uterine insemination (IUI) may be an effective therapy for spermatozoa that have an impaired motility (Check *et al.*,

1993b). Sometimes antibodies on the sperm surface cause poor recovery of motile spermatozoa after swim-up or Percoll separation, since agglutinated and poorly progressive spermatozoa are not recovered (Kiser *et al.*, 1987). Yet, when the coagulation process of freshly ejaculated spermatozoa is altered, an increased motile sperm recovery has been obtained (Fishel and Edwards, 1982; Ver Meiden *et al.*, 1989).

Cohen *et al.* (1984) and Elder *et al.* (1990) have reported success when 50% serum was added to sperm washing medium and the patient ejaculated directly into the mixture. The coagulum was easily disrupted, resulting in reduced agglutination and increased fertilization and pregnancy rates.

Chantler *et al.* (1989) reported using chymotrypsin/galactose treatment of spermatozoa for men with sperm antibodies. Recently, Tucker *et al.* (1990) reported success after using chymotrypsin/galactose with assisted reproductive technology procedures. Seminal plasma contains the enzyme chymotrypsin which assists with liquefaction of the coagulum of freshly ejaculated semen (Cohen and Aafjes, 1982). Pattinson *et al.* (1990a) reported some enzymatic dispersal of spermatozoa which had been incubated with sperm-agglutinating, antibody-positive sera, after the addition of 500 IU/ml chymotrypsin (bovine pancreas type III). They postulated that chymotrypsin cleaves part of the immunoglobulin molecule leaving a portion recognized by the immunobead (Pattinson *et al.*, 1990b). Therefore, even though the proportions of spermatozoa with immunobead binding (especially immunoglobulin (IgG) remained unchanged, some of the deleterious effects caused by the antibodies may be removed, allowing fertilization to take place. In fact, there was some reduction in IgA binding.

The study presented herein evaluated the efficacy of treating spermatozoa naturally bound with specific antibodies using chymotrypsin/galactose, as measured by achieving pregnancy following IUI.

Materials and methods

Entry into study

Couples were prospectively entered into this study from October 1990 to September 1992. Criteria included a poor post-coital test, defined as no spermatozoa seen with progressive motion 8–12 h after intercourse. A post-coital test was performed at the time when a mature follicle was produced, as determined by sonographic demonstration, i.e. at least one follicle attaining an 18–24 mm diameter and serum oestradiol concentration of at least 200 pg/ml and a serum progesterone < 1.5 ng/ml. Only patients with normal cervical mucus were included. The mucus

was considered normal if it scored at least 10 based on the Moghissi grading system (Moghissi, 1976).

Also, the semen had to be positive for antibodies against spermatozoa. The semen samples were evaluated for antibodies using the immunobead test (Bronson *et al.*, 1984): $5-10 \times 10^6$ spermatozoa were washed three times using 0.5% bovine serum albumin (BSA) in Biggers-Whitten-Whittingham (BWW) medium. After centrifugation the final pellet was resuspended in 5% BSA/BWW. The washed spermatozoa were mixed with IgG or IgA beads and read microscopically for the percentage and attachment sites of spermatozoa binding to the beads. These initial semen results for those men whose partner conceived are shown in Table I. Four men did not have sufficient motile spermatozoa to perform a direct immunobead test, so seminal plasma was tested using an indirect assay; the IgG and IgA results of three of these men are presented in Table I. Donor antibody negative spermatozoa were mixed with the patients' seminal plasma and incubated for 30 min. The donor spermatozoa were then washed free of the seminal plasma and processed in the same manner as in the direct assay. A result of <20% was considered negative, 20-49% weak positive and >50% strong positive.

Experimental design

Sixteen males were enlisted into the randomized study. In this group, the presence of antibodies against spermatozoa was the only cause of infertility. The first cycle was randomized into groups having IUI with either albumin or chymotrypsin/galactose treated spermatozoa. From that cycle until the end of the study each patient would receive, in each succeeding cycle, the opposite treatment to the one received in the previous cycle. In addition there were 18 patients positive for anti-sperm antibodies where there were other infertility factors present. Patients in this group were not randomized but treated exclusively with chymotrypsin/galactose.

Chymotrypsin/galactose treatment

The reagents used for chymotrypsin/galactose treatment were: Earle's balanced salt solution (EBSS) (Irvine Scientific 9208), chymotrypsin type IV-s, 5 mg (Sigma CHY-5s), D(+)-galactose (Sigma G5388) and bovine albumin fraction V powder, low endotoxin BSA (Irvine Scientific 1092). The chymotrypsin/galactose pre-treatment protocol was as follows: to 5 ml of EBSS, 0.1 M galactose was added and the solution incubated at 37°C, sterilized by filtration using a 0.2 µm filter, and added to 5 mg chymotrypsin just prior to semen production. Semen was collected directly into chymotrypsin/galactose in a specimen cup and immediately disrupted with a transfer pipette until the coagulum was liquefied (30-60 s). Rapid addition of 30 mg/ml BSA stopped the enzymatic reaction, and when this was fully dissolved (~5 min), the specimen was layered onto a Percoll column.

Albumin treatment

Each patient ejaculated into 5 ml of equal parts of modified human tubal fluid buffered with HEPES (HTF-HEPES) (Irvine Scientific #9963) and 7.5% BSA (Sigma #A8412). The coagulum was immediately disrupted using a sterile transfer

pipette. The specimen was then incubated for 10 min at 37°C prior to layering onto a Percoll column.

Semen separation for IUI

Semen was prepared for IUI using a Percoll density gradient procedure. Up to 2 ml of the semen mixture was overlaid onto 1-ml columns of Percoll, made isotonic using the modified tubal fluid (modified HTF-HEPES $\times 10$, Irvine Scientific #99141) and centrifuged for 20 min at 300 g. The top semen layer was discarded and the sperm pellet was placed into a new tube. The pellet was washed and then resuspended in 0.2 ml of modified tubal fluid (Irvine Scientific #9963) for IUI.

Timing of IUI

Sonographic measurement of follicular size was started beginning 16 days from expected menses. Once a follicle with a minimum diameter of 18 mm was seen, daily measurements for serum luteinizing hormone (LH), oestradiol and progesterone were obtained. IUI was performed 36-40 h from a serum LH value rising to >35 mIU/ml, but was performed 12-24 h from the morning LH rise if the serum progesterone was >0.9 ng/ml (Check *et al.*, 1993a). Whenever the first IUI was performed an ultrasound was obtained; if the follicle was still intact, IUI was repeated 12-18 h later (Check *et al.*, 1991b).

Quality control

Each batch of media and sterile supplies was tested for its ability to support at least 75% development of 2-cell mouse embryos to hatching blastocysts in 72 h (Parinaud *et al.*, 1987).

Chymotrypsin has been used for zona drilling (Gordon *et al.*, 1988). A fine spray of chymotrypsin can bore a hole into the zona. Therefore, inactivation of the enzymatic reaction of chymotrypsin using albumin was tested using zona-intact hamster oocytes. The oocytes were incubated with chymotrypsin/galactose treated spermatozoa (as seen in Figure 1) in 100-µl droplets of 0.5% BSA/BWW under oil. After overnight incubation at 37°C in 5% CO₂ the oocytes were microscopically viewed for intact zonae.

Statistical analysis

Differences in pregnancy rates between chymotrypsin/galactose and albumin-treated specimens were analysed using Fisher's exact test. Differences in initial results between conceivers and non-conceivers were analysed using a *t*-test for independent groups (the level of significance used was $P \leq 0.05$).

Results

For the 16 patients treated and evaluated there were 65 cycles (32 with chymotrypsin/galactose and 33 with albumin treatment). Pregnancies were achieved in eight of 32 (25%) cycles following IUIs performed with chymotrypsin/galactose-treated spermatozoa compared with only one of 33 (3%) performed with albumin-treated specimens ($P < 0.01$, Fisher's exact test).

In the 18 patients who had infertility factors in addition to antibodies against spermatozoa in the male partner, four

pregnancies occurred in the 48 cycles (8.3%) following IUI with chymotrypsin/galactose treated spermatozoa.

Patients who achieved pregnancies did not have a lower percentage of either IgA or IgG antibodies, as seen in Table II. Similarly, there did not appear to be a particular location of the antibodies on spermatozoa that favoured conception (Table II) (Bronson *et al.*, 1982b).

Raw semen from three men were pre-tested by immunobead assay and then re-tested after chymotrypsin/galactose treatment. Initial results were IgG 96.7 ± 4.7 and IgA 99.3 ± 0.9 . There was no reduction measured by immunobead test (IgG 96 ± 5.0 and IgA 99.7 ± 0.5) after treatment. Therefore, pre- and post-treatment immunobead tests were not evaluated on the remaining patients, since these data corroborated those of Pattinson *et al.* (1990b).

Discussion

High doses of methylprednisolone have frequently been used to treat men with autoantibodies against spermatozoa. Successful

pregnancies have been recorded following methylprednisolone treatment (Shulman *et al.*, 1978; Shulman and Shulman, 1982; Alexander *et al.*, 1983; Hendry *et al.*, 1986; Check *et al.*, 1990), although some have claimed no benefit (Haas and Manganiello, 1987). Shulman (1976) reported that serious side-effects occurred in 3% of patients treated with methylprednisolone.

The majority of patients refuse methylprednisolone treatment. Therefore, sperm washing has been used to try to elute the antibodies on the spermatozoa; however, washing alone does not remove the antibodies (Haas *et al.*, 1988). Previously, good results were obtained using albumin pre-treatment (Cohen *et al.*, 1984; Elder *et al.*, 1990); however, chymotrypsin/galactose appears to give much higher pregnancy rates. In our study, the reason for the higher pregnancy rates with chymotrypsin/galactose treated spermatozoa in the randomized group, compared to the group exclusively treated with chymotrypsin/galactose, is probably related to the additional infertility factors in the latter group.

In a previous study, we had reported that motility, velocity and linearity of spermatozoa were significantly lower for men

Table I. Initial semen results from men whose female partner conceived^a

Patient ID no.	Specimen	IgG	IgA	Head attachment ^d	Motile density ($\times 10^6$ /ml)	No. cycles before conception achieved	Randomized	Chymotrypsin/galactose only
1	S	100	98	Y	78.4	3	X	
3	S	100	100	Y	20.8	1	X	
4	S	88	17	N	10.9	5	X	
5	S	100	94	Y	42.7	5	X	
9	S	87	60	N	9.0	5	X	
10	SP	60	26	N	4.0	2		X
11	S	75	88	N	50.4	3	X	
14	SP	9	100	Y	0.5	2		X
16	S	96	98	Y	12.6	6		X
17	SP	100	75	Y	4.0	1		X
18	S	100	100	Y	13.6	6 ^c	X	
32	S	99	93	N	4.0	2	X	
33	S	99	97	Y	1.0	4	X	

^a Semen results are from raw semen at initial testing.

^b S = spermatozoa, SP = seminal plasma.

^c Conceived during an albumin cycle.

^d Y = yes, N = no.

Table II. Comparison of initial semen parameters and characteristics of anti-sperm antibodies in males whose wives conceived following intra-uterine insemination with those failing to attain pregnancies

Results from initial semen testing	Conception group (n = 13)	Non-conception group (n = 21)
Mean count (\pm SD) ($\times 10^6$ /ml)	32.9 \pm 31.4	19.9 \pm 13.7
Mean % motility	53.5 \pm 19.6	47.3 \pm 18.2
Mean IgG % ^a	85.5 \pm 26.0	84.8 \pm 27.5
Mean IgA % ^a	81.1 \pm 29.1	64.3 \pm 33.2
Attachment sites for IgA:		
Head only	0	1
Tail only	1	2
Tail tip only	3	6
Head and tail	1	0
Head and tail tip	3	3
Head, tail and tail tip	5	9
Tail and tail tip	0	0

^aImmunoglobulin as measured in immunobead test.

with positive antibody tests than those with negative results (Check *et al.*, 1991a). The semen of some males, however, upon initial evaluation looks perfectly normal, but the spermatozoa become immobilized in the mucus several hours later. Our technique for IUI, and its timing, has resulted in good pregnancy rates for cervical factor (Check *et al.*, 1991b) and for male factor infertility other than antibodies against spermatozoa (Check *et al.*, 1993b). The fact that only one of 33 albumin-treated IUI cycles resulted in pregnancy, lends credence to the theory that specific antibodies may reduce fecundity in ways other than merely the immobilization of the spermatozoa in cervical mucus (Naz *et al.*, 1984, 1986).

Future studies need to be performed to understand how chymotrypsin/galactose treatment affects sperm physiology. Antibodies to the fertilization antigen-1 (FA-1) have been found in the serum of infertile men and women (Bronson *et al.*, 1989). This sperm specific glycoprotein may be affected by antibodies against spermatozoa, since FA-1 has been shown to be involved in sperm capacitation, acrosome reaction and sperm-zona binding (Naz *et al.*, 1984, 1992; Naz, 1987). Perhaps chymotrypsin/galactose treatment counteracts the adverse effect of the antibodies on FA-1.

Since only men whose female partner had no progressive spermatozoa in the post-coital test were included in this study, perhaps offering chymotrypsin/galactose treatment to all patients with positive antibody readings could increase the pregnancy rate/cycle in these couples.

It is difficult, even in a large infertility centre, to accumulate a large group of patients with a sperm antibody score 50% by IBT; co-operative multi-centre studies are needed to corroborate these preliminary data concerning the efficacy of chymotrypsin/galactose treatment of spermatozoa positive for antibodies which are to be used for IUI.

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