

## The effect of calcium ion channel blockers on sperm fertilization potential\* †

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**Objective:** To evaluate the effects of calcium ion ( $\text{Ca}^{2+}$ ) channel blockers on male fertility potential.

**Design:** A case comparison of the surface expression of mannose-ligand receptors on motile spermatozoa from 10 known fertile males and from 10 normospermic men taking  $\text{Ca}^{2+}$  channel blockers who were seeking infertility treatment. Examination of the effects of in vitro exposure of sperm from fertile donors ( $n = 14$ ) to antihypertensive medications.

**Setting:** Patients from a successful university hospital-based IVF-assisted reproductive technology program and from a male urology private practice.

**Interventions:** Prescription of alternate hypotensive medications for four male patients; cholesterol loading and unloading in vitro of fertile donor sperm.

**Main Outcome Measures:** Motile sperm were tested for their ability to bind fluorescein isothiocyanate-labeled, mannosylated bovine serum albumin as an index of the surface expression of mannose-ligand receptors associated with fertility potential. Acrosome status was simultaneously evaluated by fluorescence microscopy with rhodamine-labeled *Pisum sativum* lectin. Sperm were assayed before and after an 18-hour or 3-day incubation under capacitating conditions in vitro.

**Results:** Motile spermatozoa of normospermic men taking calcium antagonists for hypertension control do not express head-directed mannose-ligand receptors at high frequency, nor do they undergo spontaneous acrosome loss. Unexpectedly, mannose-ligand receptor translocation from the subplasmalemmal space over the acrosome to the sperm surface and aggregation over the equatorial-postacrosomal regions occurred in acrosome-intact sperm. This differs from fertile controls in whom receptor translocation to the equatorial-postacrosomal segment is coupled with the acrosome reaction (AR). Discontinuation of calcium antagonists results in complete recovery of parameters associated with sperm fertilizing potential: time-dependent increases in the percentages of spermatozoa exhibiting surface mannose-ligand binding and spontaneous ARs in vitro. The effects of in vivo administration of calcium antagonists is mimicked in control fertile donor sperm by inclusion of a  $\text{Ca}^{2+}$  channel blocker in the media employed during capacitating incubations.

**Conclusions:** Therapeutic administrations of calcium antagonists for hypertension control cause reversible male infertility associated with an IVF failure. A mechanism of inhibition of sperm fertilizing potential through insertion of lipophilic calcium ion antagonists into the lipid bilayer of the sperm plasma membrane is consistent with our in vitro studies.

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Within the past year, we experienced an unexpected IVF failure with normospermic semen; 15 of 15 metaphase II eggs failed to fertilize, an event that has occurred in <3% of all IVF cycles at this institution (1). Sperm-egg interaction was inhibited at the level of zona pellucida (ZP) binding. Test of the husband's sperm for their ability to bind fluorescein isothiocyanate conjugated, mannosylated albumin (Man-FITC-BSA) (2) on the day of the IVF failure after 24 hours in vitro showed that capacitation-induced expression of mannose-ligand receptors, a putative zona acceptor (1-3), had not occurred. A reduced ability to undergo acrosomal exocytosis was also observed. Subsequent review of the husband's medical history revealed that he was being treated with a calcium ion ( $Ca^{2+}$ ) channel blocker, nifedipine, for hypertension control. This raised the question of the contribution of calcium antagonist antihypertensive medications to our laboratory findings (Benoff S, Cooper GW, Hurley I, Rosenfeld DL, Hershlag A, Scholl GM, abstract).

When nonpharmacological measures fail, individualized antihypertensive therapy is possible with a wide range of drugs. First-line therapy now involves a calcium antagonist ( $Ca^{2+}$  channel blockers) or an angiotensin-converting enzyme (ACE) inhibitor as monotherapy, replacing traditional antihypertensive medications (e.g., diuretics,  $\beta$ -blockers, vasodilators) whose administration is associated with significant adverse side effects (4). Calcium antagonists inhibit vascular smooth muscle contraction by blocking the influx of calcium into vascular smooth muscle. Angiotensin-converting enzyme inhibitors block the enzymatic conversion of angiotensin I to angiotensin II. The antihypertensive effects of both types of agents are associated with a decrease in peripheral vascular resistance. They are generally well tolerated, and the choice of medication depends on the presence or absence of specific contraindications and the occurrence of adverse effects (5).

The calcium antagonists consist of at least three distinct classes of drugs as follows: 1,4-dihydropyridines (prototype: nifedipine), phenylalkylamines (prototype: verapamil), and benzothiazepines (prototype: diltiazem) (6). All bind with high affinity to distinct but allosterically interacting receptor sites on the  $\alpha_1$  core subunit of the L-subtype (long-lasting, large current) voltage-dependent calcium channel, which is present in a wide range of cells (6, 7). Therefore, observations indicating that mammalian spermatozoa possess a mechanism for calcium entry that has the same pharmacological sensitivity as the L-type voltage-dependent calcium

channel of mammalian cardiac and skeletal muscle were of specific relevance to the medical history of the signal case, given the fact that  $K^+$ -induced increases in the intracellular  $Ca^{2+}$  concentration of sperm are inhibited by prenylamine, diltiazem, nifedipine, or verapamil (8, 9). Further, activation of putative sperm voltage-dependent calcium channel apparently plays an important role in the acrosome reaction (AR) produced by its physiological agonist, the ZP, and pre-exposure of sperm to calcium antagonists inhibits ZP-induced acrosomal exocytosis in a dose-dependent manner (10).

Although calcium antagonists produce a dose-dependent decrease in human sperm motility and viability in vitro (11), the effects on sperm fertilization potential and therefore on male fecundity of the therapeutic administration of these agents for hypertension control have not been examined. The dose-dependent, reversible, partial arrest of spermatogenesis after oral administration of calcium antagonists seen in animal models (12) apparently does not occur in human subjects. A survey of 850 males in a computerized data base from an office-based urology-male infertility practice identified 23 men undergoing fertility evaluation who were being treated for hypertension by pharmacological therapy; >75% of these males displayed normal semen profiles (BR Gilbert, unpublished observations). In the current study, we used 5 men from this data base and 5 males from our practice to study the effects of calcium antagonists on two indexes for sperm fertilizing potential (1, 2): surface expression of receptor binding sites for mannose ligands and acrosome status. The characteristic behavior of specimens from the 10 men studied was mimicked in sperm from normal fertile donors by the inclusion of antihypertensive medications in the media used for sperm capacitation.

## MATERIALS AND METHODS

### Media and Chemicals

Modified Ham's F-10 medium (formula no. 90-8050PG) and Dulbecco's phosphate-buffered saline were obtained from GIBCO Laboratories (Grand Island, NY). Unless otherwise noted, all reagents were purchased from Sigma Chemical Company (St. Louis, MO).

### Human Specimens

All protocols employing human subjects were reviewed and approved by the Institutional Review

Board of North Shore University Hospital. A donor of known fertility ("Fertile Donor", serving as an intraexperimental and interexperimental control) (2, 13, 14) and patients taking antihypertensive medications while seeking infertility treatment from a university hospital-based IVF-assisted reproductive technology program and from an office-based urology-male infertility practice participated after giving written informed consent.

Semen specimens from unidentified IVF-fertile (1, 2) and responder (13) donors, collected by masturbation after 2 to 3 days of abstinence for routine semen analysis, were obtained at the point of discard, for which informed consent was not required. Only fresh specimens with the following parameters were used in these studies:  $>20 \times 10^6$  sperm/mL,  $>40\%$  motility, and at least 30% normal oval head forms with 50% or more of the head covered by the acrosome (13).

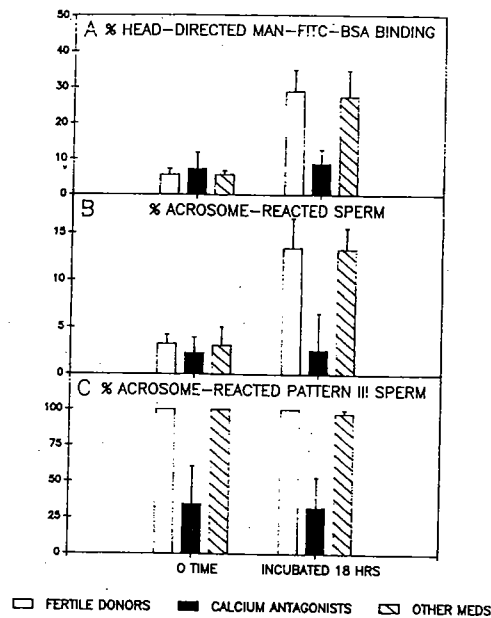
Specimens from Fertile Donor, IVF-fertile men, and responder males all "respond" to incubation in albumin-supplemented media by exhibiting time-dependent increases in the percentage of spermatozoa exhibiting head-directed mannoseylated albumin binding (Fig. 1A), accompanied by increases in the percentage of spermatozoa showing spontaneous acrosome loss (Fig. 1B) and decreases in free cholesterol content (1, 2, 13, 14).

All donors and patients were negative for the presence of immunoglobulin (Ig) A and IgG anti-sperm antibodies, as detected by direct immunobead binding (14). The presence or absence of anti-sperm antibodies is an important consideration when examining the etiology of male infertility. Prior observations from our laboratory indicated that antisperm antibodies inhibit membrane cholesterol efflux and the concomitant membrane fluidity changes needed for surface expression of sperm mannose-ligand receptors (14).

### Semen Preparation

Spermatozoa were collected from fresh semen by motile migration (swim-up) into Ham's F-10 for 1 to 2 hours at  $37^\circ\text{C}$  (2). Concentration ( $\times 10^6/\text{mL}$ ) was estimated by hemacytometer counts at  $1/20$  dilution in a 1% phenol solution. Motility in swim-up preparations ranged from 92% to 100%.

Untreated (fresh or uncapacitated) motile sperm populations were concentrated before analysis by centrifugation ( $500 \times g$  for 8 minutes). To induce capacitation and compare results with previous



**Figure 1** Comparison of sperm characteristics of fertile donors and men treated with antihypertensive medications. Group I, fertile donors,  $n = 10$ ; Group II, men treated with  $\text{Ca}^{2+}$  channel blockers,  $n = 10$ ; Group III, men from group II switched to alternate hypotensive medications and reassayed after  $>3$  months,  $n = 4$ . Analysis of variance verifies a significant difference between Group I versus Group II (% head-directed Man-FITC-BSA binding,  $P < 0.0001$ ; % acrosome-reacted sperm,  $P < 0.0001$ ; % acrosome-reacted Pattern III sperm,  $P < 0.05$ ). Sperm from group III were indistinguishable from those of group I (% head-directed Man-FITC-BSA binding,  $P = 0.7086$ , NS; % acrosome-reacted sperm,  $P = 0.8206$ , NS; % acrosome-reacted pattern III sperm,  $P = 0.1922$ , NS). Within patient groups (group II versus III), repeated measures ANOVA indicated an interaction between the type of hypotensive medication and the incubation-associated increases in the percentages of sperm exhibiting (A) head-directed surface Man-FITC-BSA binding sites, (B) spontaneous loss of acrosome content, and (C) dissociation between pattern III Man-FITC-BSA binding and loss of acrosome content (respectively,  $P < 0.0223$ ,  $P < 0.0004$ , and  $P < 0.0083$ ).

studies (1, 2, 13, 14), sperm were pelleted, resuspended in F-10 medium containing 30 mg/mL human serum albumin (HSA) at a concentration of  $12 \times 10^6$  cells/mL and incubated for 16 to 20 hours at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  in air or for 3 days at room temperature (RT).

Our prior observations indicated that after 16 hours of incubation, sufficient numbers of spermatozoa were capacitated so that tests could distinguish between fertile and infertile sperm populations (2) and that prolonged incubation (3 days at RT) in albumin-supplemented media of spermatozoa from infertile males with membrane fluidity defects resulting from cholesterol excess (13) was

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correlated with increased percentages of spermatozoa expressing surface plasma membrane mannose receptors and fertilization in IVF (2) (Benoff S, Cooper GW, unpublished observations). More than 50% of spermatozoa incubated for 3 days at RT in these studies remained motile (14).

### Visualization of D-Mannose Binding Sites

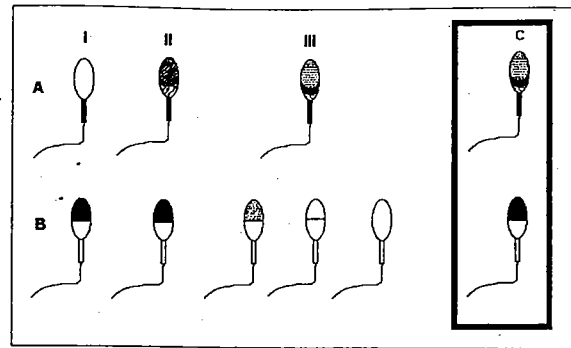
The surface of motile spermatozoa was labeled with 100  $\mu\text{g}/\text{mL}$  fluorescein isothiocyanate-conjugated  $\alpha$ -D-mannosylated BSA (Man-FITC-BSA; Sigma No. A7790) in a calcium-supplemented core buffer (2). Control reactions contained 100  $\mu\text{g}/\text{mL}$  FITC-conjugated BSA (FITC-BSA) instead of Man-FITC-BSA. Fluorescein isothiocyanate conjugate-BSA neither labels the sperm surface nor acts as an effective competitor of Man-FITC-BSA surface labeling (13).

Spermatozoa were assessed for Man-FITC-BSA binding by visual inspection of mounted slides stored at 4°C for 2 weeks before analysis. Binding was enumerated as midpiece alone (pattern I), whole head plus midpiece (pattern II), or equatorial/postequatorial regions plus midpiece (pattern III) (Fig. 2A) (1, 2, 13). All sperm in these samples exhibited one of these patterns. Coded slides from each specimen were examined at 400 $\times$  magnification by two independent observers, each scoring at least 300 sperm in 10 to 20 microscopic fields, with 5% to 7% variation in scoring between observers.

### Evaluation of Acrosome Status

Acrosome-intact and acrosome-reacted sperm surface labeled with Man-FITC-BSA were ethanol permeabilized and differentiated by reaction with 100  $\mu\text{g}/\text{mL}$  tetramethylrhodamine isothiocyanate-labeled *Pisum sativum* agglutinin (RITC-PSA; Vector Laboratories, Inc., Burlingame, CA) that binds to intra-acrosomal contents. Sperm were scored as acrosome-intact if the anterior and equatorial regions of the head were uniformly RITC-PSA labeled or as acrosome-reacted if only the equatorial segment was labeled or if sperm heads were completely RITC-PSA negative (Figs. 2B and 3). At least 300 sperm in a minimum of 20 microscopic fields were scored for Man-FITC-BSA binding and for acrosomal status by successive adjustments of the barrier and excitation filters (1, 2, 13, 14). Coded slides were examined by two independent observers.

Prior observations (reviewed in Benoff et al. (14)) indicate that the percentage of viable sperm

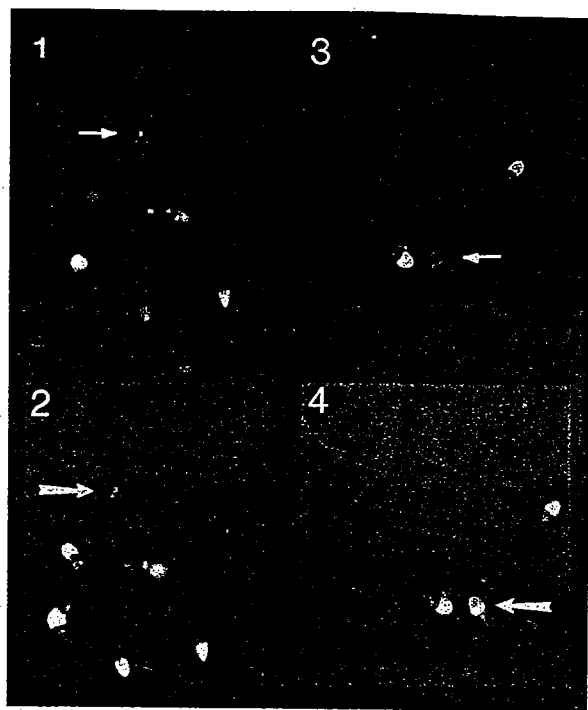


**Figure 2** Diagrammatic representation of fluorescence labeling patterns of Man-FITC-BSA/RITC-PSA double-labeled sperm. (A), Diagrammatic representation of the three distinct Man-FITC-BSA surface labeling patterns: I, II, and III. (B), Corresponding RITC-PSA labeling patterns of Man-FITC-BSA-labeled sperm from fertile donors. Sperm exhibiting FITC patterns I and II are acrosome-intact as shown by uniform RITC-PSA labeling over the acrosome and equatorial regions of the head. Pattern III displays show reduced coincident RITC-PSA labeling either over the acrosome, the equatorial segment only, or complete RITC-PSA negativity, indicating acrosomal exocytosis (2, 13). (C), Unusual occurrence of pattern III Man-FITC-BSA binding on acrosome-intact sperm from normospermic men taking  $\text{Ca}^{2+}$  channel blocker antihypertensive medications.

consistently exceeds the percentage of motile sperm in a population, and nonviable cells do not always have a reacted acrosome. In all cases, the increase in percentage of acrosome-reacted spermatozoa in 18-hour capacitated populations was greater than the percentage loss of motility associated with incubation at 37°C and was interpreted as representing an increase in spontaneously occurring, physiological ARs (14).

### Measurement of Subplasmalemmal Stores of Mannose-Ligand Binding Sites

Prior observations from our laboratory indicated that when human spermatozoa from fertile-responder donors were demembrated immediately after swim-up, cryptic mannose binding sites stored in the subplasmalemmal space were exposed (13). During capacitating incubations in media supplemented with albumin, these stores were translocated to the sperm head surface (13, 14). To determine whether the observed inhibition in capacitation-associated expression of surface mannose binding sites by spermatozoa from patients receiving calcium ion blocker antihypertensive therapy resulted from a reduction or absence of such stores, their sperm were demembrated by vortex-



**Figure 3** Comparison of the acrosomal status of sperm, labeled with Man-FITC-BSA, from fertile donors versus men receiving  $\text{Ca}^{2+}$  channel blockers for hypertension control. Paired epifluorescence photomicrographs of spermatozoa double labeled with Man-FITC-BSA and RITC-PSA. Labeled sperm were viewed at 400 $\times$  and photographed on 400 ASA film with exposure times for fluorescein and rhodamine images, respectively, of 44 and 1 seconds. In panels 1 and 3, sperm labeled in pattern III with Man-FITC-BSA are indicated by small arrows. Corresponding RITC-PSA labeling patterns for these sperm, respectively, are shown in panels 2 and 4, large arrows. 1 and 2, Man-FITC-BSA pattern III sperm (small arrow) from a fertile donor had undergone an AR (large arrow). 3 and 4, Sperm from men taking  $\text{Ca}^{2+}$  channel blocker medications show Man-FITC-BSA and acrosomal labeling patterns that differ consistently from prior observations (2, 13) in that pattern III sperm were acrosome intact.

ing, with plasma membrane integrity estimated by hypo-osmotic swelling, as previously described (13). Each specimen was aliquoted into three parts: aliquots were vortexed either before or after labeling of spermatozoa with Man-FITC-BSA with an unvortexed aliquot serving as the internal control. Spermatozoa from each aliquot were monitored for acrosomal status by ethanol permeabilization and reaction with RITC-PSA.

#### Cholesterol Loading and Unloading of the Sperm Plasma Membrane

Our prior observations have indicated that sperm membranes could be hyperloaded with nonesteri-

fied cholesterol by incubation in the presence of exogenous cholesterol with concomitant suppression of surface expression of mannose-ligand binding sites (14). When washed free of the inhibiting media and then incubated under sperm capacitating conditions, the percentage of spermatozoa exhibiting Man-FITC-BSA binding was markedly increased, comparable with that of incubated sperm never exposed to cholesterol (Benoff S, unpublished observations).

Cholesterol-saturated BSA (C-BSA) is a BSA preparation that has lost its ability to promote capacitation by presaturation with cholesterol. Liposome-free C-BSA was prepared by sonication as previously described (14). Swim-up sperm were incubated at RT for 18 to 20 hours in Ham's F-10 containing 1 mg/mL C-BSA, which increases the plasma membrane content of nonesterified cholesterol twofold to fivefold (14). At the end of incubation, specimens were washed three times with Ham's F-10 and then subjected to capacitating incubations in the presence of 30 mg/mL HSA as described above. The percentages of spermatozoa exhibiting surface labeling with Man-FITC-BSA and spontaneous acrosomal exocytosis were determined at the time of swim-up, immediately after the washes to remove the exogenous cholesterol and after incubation in capacitating media.

#### In Vitro Exposure of Spermatozoa to Antihypertensive Medications

No information is available indicating what semen levels of antihypertensive medications may be present in individuals on chronic therapy. Therefore in vitro exposure experiments were designed based on their average estimated concentration in the circulation in clinically effective regimens. Nifedipine blood plasma levels between 20 and 200 ng/mL (58 nM to 0.58  $\mu\text{M}$ ) are associated with antihypertensive efficacy (for example, see McAllister et al. [15]). Similar ranges are observed for ACE inhibitors. These levels were chosen as central values in the search for potential dose-response alterations in mannose-ligand receptor expression associated with drug exposure.

To compare the effects of calcium antagonists versus ACE inhibitors on the surface expression of mannose-ligand receptors, spermatozoa from fertile donors were exposed to antihypertensive medications by the inclusion of these drugs, at picomolar

to micromolar concentrations, in the media used for sperm capacitation or in the buffers used during the Man-FITC-BSA surface labeling reaction. To examine the relationship between membrane cholesterol content and the effect of nifedipine on expression of surface Man-FITC-BSA binding sites, nifedipine was included in the media employed for capacitation of fresh swim-up fertile donor-responder spermatozoa and in that of duplicate aliquots subjected to prior cholesterol loading. In all experiments, aliquots from each specimen of untreated spermatozoa at the time of swim-up and after incubation in Ham's F-10 supplemented with 30 mg/mL HSA served as the controls.

### Statistical Analysis

All statistical analyses were performed with the SAS/PC software package (SAS-Institute, Inc., Cary, NC). Statistical significance was set at  $P < 0.05$ . Summary statistics are presented as means and SDs.

Within the group of patients receiving antihypertensive medications and who were also seeking fertility evaluation, a repeated measures analysis of variance (ANOVA) was employed to determine the following: [1] if there was a significant difference among matched observations across time (or medications); [2] whether there was a treatment effect (medication factor); and [3] if there was an interaction between time and treatment when tested simultaneously. The interaction between time and treatment was examined before either the time or treatment effect. For infertility patients, comparisons were made between the following: [1] the percentages of spermatozoa expressing surface mannose-ligand receptors; [2] the percentages of spermatozoa exhibiting spontaneous acrosome loss; and [3] the percentages of Man-FITC-BSA pattern III sperm at 18 hours of incubation that are acrosome reacted in case-matched specimens obtained before and after discontinuation of nifedipine therapy for hypertension control. In the *in vitro* studies, comparisons were made between the percentages of fertile donor-responder spermatozoa expressing surface mannose-ligand receptors in controls versus aliquots exposed to antihypertensive medications and controls versus cholesterol-loaded specimens exposed to nifedipine.

Because the fertile donors were an independent group, repeated measures ANOVA could not be ap-

plied. Data from fertile donors and from patients treated with antihypertensive medications were compared by standard ANOVA.

## RESULTS

### Analysis of the Signal Case

The husband in the signal case, a 39-year-old male ("male 13") (1), presented to our IVF program with primary infertility of 7 years' duration. Diagnostic workup of his 35 year-old wife showed a normal in-phase endometrial biopsy, a poor postcoital test, a normal hysterosalpingogram, and minimal endometriosis at the time of laparoscopy that was removed with the CO<sub>2</sub> laser. The female partner had high titer head-directed IgG antisperm antibodies in her serum as detected by direct immunobead binding assay. The male partner's previous workup revealed normal semen parameters, with counts ranging from 63 to 123 × 10<sup>6</sup>/mL; motility: 49% to 56%; progression: 2 to 3; oval head normal morphology of 48% to 58%. Six months before the IVF cycle, the male partner was treated for hypertension with Procardia (nifedipine; Pratt Pharmaceuticals, Pfizer, New York, NY), 30 mg/d. The couple underwent three unsuccessful cycles of hMG stimulation with IUI (Pergonal; Serono Laboratories, Randolph, MA) with good follicular response. In April of 1992, the couple underwent an IVF-ET cycle with luteal phase luprolide acetate (Lupron; Tap Pharmaceuticals, Inc., Deerfield, IL) followed by three ampules of Pergonal for 10 days and 10,000 U of hCG (Profasi; Serono Laboratories). Sixteen mature oocytes were recovered at the time of vaginal retrieval 34 hours later (15 metaphase II, 1 metaphase I). A swim-up population from semen collected 1 hour after retrieval showed 58% of sperm had an acrosome cap covering ≥50% of the head surface with normal oval shape. Each egg was inseminated with ×10<sup>5</sup> sperm, cultured in the presence of 15% human fertile donor serum in a 1-mL volume in the center well of a Falcon 3037 culture dish (Falcon, Becton Dickinson, Oxnard, CA) and assessed for fertilization 16 hours after insemination. None of the 16 eggs fertilized. The oocytes were combined (5 eggs per dish) and reinseminated with ×10<sup>6</sup> sperm/mL in 1-mL volume. Sperm-egg zona binding failed to occur, and all eggs remained unfertilized.

On the day of the initial IVF failure, sperm maintained overnight at RT in media supplemented

with 10% human fertile female donor serum were assayed for Man-FITC-BSA binding. Eight percent of sperm exhibited head-directed Man-FITC-BSA binding (patterns II + III), and only 2.67% of sperm had undergone a spontaneous AR. Importantly, patterns II and III Man-FITC-BSA binding occurred on acrosome-intact sperm (Figs. 2C and 3). Less than 20% of all pattern III Man-FITC-BSA labeled sperm had undergone an AR. Thus, mannose-ligand receptor translocation from the sperm surface over the acrosomal cap to that over the equatorial-postequatorial regions occurred in the absence of an AR. This was the first time we had observed this pattern transition without an associated loss of acrosome content.

Four additional semen specimens from the signal case were analyzed at the time of swim-up and after 18 hours' incubation and/or 3 days' incubation in capacitating medium for percentage of sperm exhibiting head-directed Man-FITC-BSA surface labeling and percentage of sperm exhibiting spontaneous loss of acrosome content. In all specimens, only a low percentage of the patient's sperm expressed mannose-ligand receptors or spontaneous ARs before or after 18-hour capacitating incubations. Prolonged incubation at RT failed to elicit an increase in these parameters. Correlation between pattern III Man-FITC-BSA binding and the spontaneous AR was still not observed.

After consultation with the patient's cardiologist, he discontinued the Procardia and was placed on an ACE inhibitor, Vasotec (enalpril maleate; Merck and Co., Inc., West Point, PA) 10 mg/d (August, 1992), later increased to 20 mg/d (September, 1992). Later, a diuretic (hydrochlorothiazide and triameteren, Dyazide; SmithKline Beecham Pharmaceuticals, Pittsburg, PA), 50 mg/d, was added to the regimen (December, 1992). He was maintained on Vasotec for >3 months to allow complete renewal of the seminiferous epithelium (16). Semen specimens were obtained at 4 months and 9 months after ACE inhibitor therapy was begun. These specimens were indistinguishable from those of fertile controls (Fig. 1). His motile spermatozoa now exhibited the following: [1] capacitation-associated increases in percentage of sperm expressing surface mannose-ligand binding sites equated with fecundity (e.g., increasing from  $\leq 7\%$  at time of swim-up to  $>33\%$  by 18 hours of incubation); [2] plateau values for expression attained by 18 hours incubation in vitro; [3] time-dependent increases in the percentage of spontaneously acrosome-reacted

sperm (e.g., increasing from  $<5\%$  at time of swim-up to  $>14\%$  after an 18-hour incubation); and [4] essentially complete correlation between pattern III Man-FITC-BSA binding and acrosomal exocytosis.

#### Additional Males Studied

To further document and clarify the relationship between calcium antagonists and unexplained male infertility, semen specimens were obtained from nine other males for whom the calcium antagonists nifedipine or verapamil (verapamil HCL; Calan SR, Searle, Chicago, IL) had been prescribed for hypertension control and who were also seeking fertility evaluations. The surface membrane characteristics of their motile sperm were indistinguishable from those of the signal case (Fig. 1). No time-dependent increases in percentage of sperm exhibiting head-directed Man-FITC-BSA binding or in the percentage of sperm exhibiting spontaneous ARs were observed, and both patterns II and III Man-FITC-BSA binding occurred on acrosome-intact sperm. Nevertheless, despite these findings regarding abnormalities in the level or topography of surface-bound Man-FITC-BSA, vortexing of fresh swim-up sperm revealed the existence of subplasmalemmal stores of mannose-ligand binding sites at levels typical of sperm from fertile donors (not shown).

Alternate hypotensive medications then were prescribed for three of these additional males to date. As observed in the signal case 3 months after medication switch, their semen specimens exhibited the following: [1] capacitation-associated increases in percentage of sperm expressing surface mannose-ligand binding sites equated with fecundity (e.g., increasing from  $<7\%$  at time of swim-up to  $>19\%$  by 18 hours of incubation); [2] time-dependent increases in the percentage of spontaneously acrosome-reacted sperm (e.g., increasing from  $<5\%$  at time of swim-up to  $>10\%$  after an 18-hour incubation); and [3] essentially complete correlation between pattern III Man-FITC-BSA binding and acrosomal exocytosis (Fig. 1).

#### Statistical Analyses

To examine the significance of our findings, we performed a case comparison of the characteristics

of sperm from fertile donors versus sperm from patients taking antihypertensive medications and seeking fertility evaluation. Motile sperm populations from the 10 fertile donors employed exhibited capacitation-associated increases in percentage of sperm expressing surface mannose-ligand binding sites (e.g., increasing from  $5.49\% \pm 1.62\%$  at time of swim-up to  $28.75\% \pm 5.95\%$  by 18 hours of incubation,  $P < 0.003$ ) and percentage of sperm exhibiting spontaneous loss of acrosome content (increasing from  $3.24\% \pm 0.96\%$  at time of swim-up to  $13.44\% \pm 3.12\%$  by 18 hours of incubation,  $P < 0.0001$ ). At both swim-up and after incubation in vitro, 100% of sperm binding Man-FITC-BSA in pattern III were acrosome-reacted (Fig. 1C).

In specimens obtained from the 10 males maintained on  $\text{Ca}^{2+}$  channel blockers, no difference was detected between the percentages of sperm with surface-bound Man-FITC-BSA at swim-up versus 18 hours of incubation under capacitating conditions ( $P = 0.3177$ , not significant [NS]) or between swim-up versus 18 hours of incubation versus 3 days of incubation ( $P = 0.9434$ , NS). Likewise, no difference was detected between the percentages of sperm exhibiting spontaneous ARs at swim-up versus 18 hours of incubation ( $P = 0.2838$ , NS). Thus, the mannose-ligand binding and acrosomal antihypertensive therapy differed significantly from matched specimens from fertile donors (Fig. 1). In contrast, specimens from fertile donors or from the subgroup of the same patients assayed again after they had been maintained on other types of antihypertensive medications for more than 3 months were indistinguishable (Fig. 1). In motile sperm populations from the four males reassayed after switching medications, both the percentages of spermatozoa exhibiting head-directed Man-FITC-BSA surface labeling and the percentages of spermatozoa exhibiting spontaneous loss of acrosome content were significantly increased at 18 hours of incubation as compared with the fresh swim-up control (respectively,  $P < 0.0359$  and  $P < 0.0019$ ). The differential change in the percentages of spermatozoa expressing surface mannose receptors and those exhibiting spontaneous ARs from swim-up to 18 hours of incubation depended on the medication prescribed (respectively,  $P < 0.0023$  and  $P < 0.0004$ ). Finally, a significant medication-related dissociation between pattern III Man-FITC-BSA binding and loss of acrosome content was observed in specimens from men on  $\text{Ca}^{2+}$  channel blockers pharmacological for hypertension control (Fig. 1).

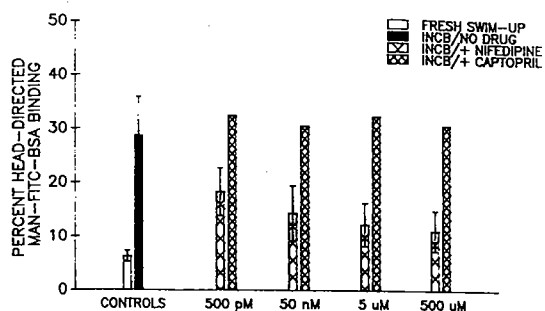
The percentage of acrosome-reacted Man-FITC-BSA pattern III sperm at 18 hours of incubation was decreased when specimens from fertile donors versus patients receiving calcium antagonist antihypertensive therapy, whereas no difference was detected between fertile donors versus patients switched to alternate hypotensive medications.

We recognize that with a maximum of 10 cases in an analysis, we cannot test the assumptions of the model. However, we present these analyses to indicate where the data appear to be statistically interesting, in preparation for further study.

#### In Vitro Effects of Calcium Antagonists

To dissect the mechanism by which calcium antagonists exert their apparent effects on parameters that correlate with the fertilizing potential of human spermatozoa, a search was made for potential dose-response alterations in surface mannose-ligand receptor expression as a result of in vitro exposure of sperm from normal fertile donors ( $n = 4$ ) to nifedipine or instead to captopril, the prototype ACE inhibitor. Comparable dose-response reductions in sperm motility were observed after 18-hour exposure to either drug (not shown). However, inclusion of captopril in the media employed during 18-hour capacitating incubations had no adverse effect on the increment increase in the percentages of sperm exhibiting surface labeling with Man-FITC-BSA (Fig. 4). These observations demonstrate that ACE inhibitors offer an alternative therapy for hypertension control that does not affect mannose-ligand receptor expression in vivo or in vitro.

In contrast, inclusion of nifedipine in the capacitating media elicited a dramatic dose-dependent decrease in the percentages of incubated spermatozoa expressing surface binding sites for D-mannose ligands (Fig. 4). Importantly, the observed inhibition of mannose-ligand receptor expression by nifedipine required long-term exposure. The simple inclusion of nifedipine in the buffers employed during the Man-FITC-BSA surface labeling reaction was without significant effect on the percentages of spermatozoa with surface-bound ligand (not shown), suggesting the requirement for the diffusion of nifedipine into the sperm plasma membrane before it exerts an inhibitory effect on Man-FITC-BSA binding.



**Figure 4** Examination of the effects on surface expression of mannose-ligand receptors of 18-hour exposure of fertile donor spermatozoa ( $n = 4$ ) to nifedipine or captopril at 37°C in Ham's F-10 containing 30 mg/mL HSA. Exposure to nifedipine significantly inhibited capacitation-associated increases in the percentage of spermatozoa exhibiting surface Man-FITC-BSA binding sites ( $P < 0.0058$ ), whereas the mannose-ligand binding characteristics of capatopril-treated spermatozoa were indistinguishable from those of control aliquots.

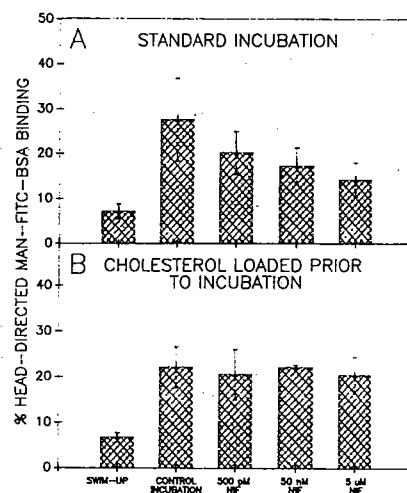
The influence of membrane cholesterol content on the inhibitory action of nifedipine was also examined (Fig. 5) to determine the importance of the partitioning of nifedipine into the sperm plasma membrane for its effects mannose-ligand receptor surface expression. There was a significant difference (group factor) in the response of control versus cholesterol-loaded aliquots from control fertile donors-responders ( $n = 10$ ) to nifedipine ( $P < 0.0270$ ). As above, nifedipine produced a dose-dependent inhibition in control aliquots in the percentages of spermatozoa expressing surface mannose-ligand receptors (Fig. 5A). In contrast, the cholesterol-loaded aliquots were resistant to the effects of nifedipine exposure during cholesterol unloading in HSA (Fig. 5B). These data support the general idea that calcium antagonist drug interactions with the plasma membrane are complex and are greatly influenced by cholesterol content (17).

## DISCUSSION

In the present study, we demonstrated a relationship between therapeutic administration of calcium antagonists for hypertension control and an unexpected IVF failure by quantitative analysis of sperm surface mannose-ligand receptor expression and acrosome status. Our IVF program has had fertilization failures in only 3% of 536 consecutive egg retrieval cycles (1). These failures were unexpected in two thirds of the failed fertilizations because the semen parameters were normal, appropriate

numbers of sperm were used, and there were no complicating immune or female factors. Without exception, after incubation under standard sperm-capacitating conditions, the spermatozoa from normospermic IVF-infertile men (noted to have fertilization failure) have failed to increase the percentages of spermatozoa with plasma membrane mannose-ligand receptor expression over the acrosome and postacrosomal regions of the sperm head and the percentages of spermatozoa exhibiting spontaneous and mannose-induced ARs (1, 2). This established a highly significant correlation between the expression of mannose-ligand receptors and acrosome status with fertilization in vitro.

Our prior studies have demonstrated that spermatozoa express surface mannose-ligand binding sites after receptor transposition from subplasma membrane storage sites during in vitro incubation. This and their ability to undergo spontaneous acrosome loss depend on the cholesterol content of the sperm plasma membrane (13, 14). Although time course studies of specimens from fertile males indicate that percentages of sperm exhibiting surface mannose-ligand binding sites plateau by 18 hours of incubation (2, 14), this percentage in specimens



**Figure 5** Effect of membrane cholesterol content on the inhibitory action of nifedipine on capacitation-induced surface expression of mannose-ligand receptors was examined in split specimens from 10 fertile-responder donors. (A), Control aliquots: exposure to nifedipine produced a dose-dependent inhibition in the percentages of spermatozoa expressing surface mannose-ligand binding sites ( $P < 0.0049$ ). (B), The percentages of spermatozoa surface labeled with Man-FITC-BSA were unaffected when specimens subjected to prior cholesterol loading were exposed to nifedipine during cholesterol unloading in Ham's F-10 supplemented with 30 mg/mL HSA ( $P = 0.1732$ , NS).

from normospermic infertile males often continues to increase for at least 3 days (2) (Benoff S, Cooper GW, unpublished observations). Thus, in the majority of unexpected IVF failures, the failure to express surface Man-FITC-BSA binding sites and to spontaneously lose acrosomal content was attributable to high levels of free or nonesterified cholesterol in the sperm plasma membrane (1, 2). These cholesterol levels decline slowly to levels similar to those of normal fertile donors only after several days' incubation under capacitating conditions (Benoff S, Hurley I, unpublished observations).

The male partner in the signal case is the first man in 10 unexpected IVF failures where a review of medical history revealed a possible cause for the failure. The male partner was taking nifedipine, a calcium antagonist used therapeutically for hypertension control. A retrospective test of the zona ligand receptor activities of his spermatozoa showed that they were clearly abnormal and differed from those of other normospermic IVF-infertile males. The percentage of sperm expressing surface mannose-ligand binding sites was not increased even upon prolonged incubation, and both patterns II and III Man-FITC-BSA binding occurred on acrosome-intact sperm, an unusual feature not seen heretofore. Our analysis of 9 additional men taking  $Ca^{2+}$  channel antagonists who were undergoing fertility evaluations has confirmed the findings of the signal case.

In the signal case and in the additional cases studied, as in animal models (12), complete recovery of the parameters associated with sperm fertilizing potential was observed after withdrawal of the calcium antagonist treatment. Therefore, it seemed likely that the mechanism of action of the calcium antagonists might simply involve blockage of  $Ca^{2+}$  channels, e.g., by preventing an elevation in intracellular ionized calcium and subsequent acrosomal exocytosis (10, 12). This, however, is not the only possibility. Calcium antagonists might also act by affecting membrane characteristics. The lipophilic nature of the calcium antagonists (17-19) makes it likely that they insert into the bilayer of the sperm plasma membrane and directly affect membrane fluidity, consequently inhibiting mannose-ligand receptor expression. Evidence to support this mechanism is based on observations obtained with dihydropyridine photoaffinity probes and with site-directed antipeptide antibodies that are consistent with intramembrane binding sites for these drugs on the  $\alpha_1$  core subunit of the voltage-dependent cal-

cium channel (reviewed in Mason [18]) and on measurements of the kinetics of binding of calcium antagonists to their receptor sites (17). Indeed, calcium antagonists most likely approach the voltage-dependent calcium channel via a "membrane pathway", e.g.,  $Ca^{2+}$  channel binding is preceded by drug partitioning into and lateral diffusion through the lipid bilayer of the plasma membrane (18, 19).

A membrane pathway is consistent with our *in vitro* studies comparing the effects of nifedipine on mannose-ligand receptor expression by control versus cholesterol-loaded sperm. Cholesterol modulates the partitioning of calcium antagonists to the sperm plasma membrane and promotes the dissociation of these drugs from the membrane. The inhibitory effect on mannose-ligand receptor expression by nifedipine inclusion in capacitating media is negated by prior cholesterol loading of the sperm plasma membrane, suggesting that the turnover of the drug within the membrane is accelerated by excess cholesterol. This hypothesis is supported by similar observations made in the dihydropyridine-sarcolemmal receptor system (17). Nevertheless, the cholesterol loading studies also demonstrate that spermatozoa clearly differ from muscle cells with regard to their expression of voltage-dependent calcium channels; in muscle, otherwise silent dihydropyridine-sensitive calcium channels are activated by cholesterol enrichment (20).

Our results are in agreement with findings from animal studies (10, 12) that  $Ca^{2+}$  channel blockers inhibit acrosomal exocytosis. Data from Florman and co-workers (10) suggested that the calcium antagonists suppress, at least in part, ZP binding-induced influx of extracellular calcium that mediates the AR in a variety of animals. We moved the point of action of these calcium antagonists to an earlier stage in the pathway in human sperm, to the suppression of externalization of putative ZP binding proteins on the sperm head during capacitation. The fact that those few sperm from the infertility patients that express mannose-ligand receptors show a distinct pattern II to pattern III Man-FITC-BSA binding shift in the absence of an AR indicates that calcium influx is not the stimulus for changes in the organization of the sperm plasma membrane. Our observations thus are in direct contrast to those of Roldan et al. (21) who reported the acceleration of the human AR by verapamil. Possible explanations for this discrepancy lie in the experimental conditions employed (10) and/or in the choice of calcium channel blocker studied. Nifedipine is a

specific antagonist that does not interact with other cationic channels (22), whereas verapamil binds with different channels and receptors (for example, see Triggle [23]) and may have activated an alternate sperm calcium transport mechanism. Our results suggest that, as observed in animal models (12), at least one of these  $Ca^{2+}$  channel blockers, nifedipine, is an effective *in vivo* contraceptive.

In conclusion, our data indicate that therapeutic administration of calcium antagonists for hypertension control cause reversible male infertility associated with an IVF failure. No information is available concerning how these drugs cross the blood-testis barrier or the levels of calcium antagonists in human semen. It is not known whether calcium antagonists concentrate in the genital tract in a manner similar to that observed for other lipophilic compounds (e.g., chlorinated hydrocarbons; reviewed in Wagner et al. [24]) or whether they reach the genital tract by transudation from the circulation as do Igs (25) and would thus be present at 1% to 2% of levels in blood plasma. Nevertheless, our *in vitro* studies demonstrate that the inhibitory effects of calcium antagonists on parameters of sperm fertilizing potential are observed at concentrations 2 to 4 orders of magnitude below the average estimated concentration of these drugs in the general circulation after long-term administration. We therefore recommend that all men taking  $Ca^{2+}$  channel blocking medications be screened for the effects of these drugs on sperm zona receptor functions and that these medications be changed to an alternate class of medications, such as ACE inhibitors, at least 3 months before IVF or natural cycle inseminations. The successful application of our laboratory findings to the management of a couple treated for infertility will be discussed in detail in a separate publication.

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