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Proteins of the Accessory Sex Glands Associated With the Oocyte-Penetrating Capacity of Cauda Epididymal Sperm From Holstein Bulls of Documented Fertility

ARLINDO A. MOURA,* DAVID A. CHAPMAN, AND GARY J. KILLIAN

Department of Dairy and Animal Science, J. O. Almquist Research Center, The Pennsylvania State University, University Park, Pennsylvania

ABSTRACT We previously reported that accessory sex gland fluid (AGF) from high fertility (HF) bulls influenced the oocyte-penetrating capacity of cauda epididymal sperm from low fertility (LF) bulls, based on in vitro fertilization (IVF) assays. The present study determined if AGF proteins were associated with these effects. Nineteen IVF assays with 12 bulls were grouped as follows. Group I ($n = 8$): assays where sperm from LF bulls exposed to AGF from HF bulls had greater oocyte penetration than exposed to homologous AGF. Group II ($n = 7$): sperm from LF bulls to AGF from HF bulls versus homologous AGF showed no significant differences. Group III ($n = 4$): sperm from LF bulls treated with homologous AGF had greater fertility than sperm treated with AGF from HF bulls. Sire fertility was based on nonreturn rates (NNR) and AGF collected by artificial vagina from bulls with cannulated vasa deferentia. Two-dimensional SDS–PAGE maps of AGF were analyzed by PDQuest and proteins identified by tandem mass spectrometry and Western blots. Differences in spot intensity between AGF of HF and LF bulls were compared across groups of IVF assays ($P < 0.05$). The expression of BSP A1/A2 and A3, BSP 30 kDa, clusterin, albumin, phospholipase A_2 (PLA_2), and osteopontin was greater in the AGF of HF bulls in Group I as compared to Groups II and III. Conversely, there was less nucleobindin in the AGF of HF bulls in Group I than in Groups II and III. This is the first report of nucleobindin (58 kDa/pI 5.6) in male reproductive fluids, using both immunoblots and mass spectrometry. Thus, the effect of AGF from HF bulls on epididymal sperm is likely the result of specific proteins expressed in the AGF. *Mol. Reprod. Dev. 74: 214–222, 2007.* © 2006 Wiley-Liss, Inc.

Key Words: albumin; BSP proteins; fertility; nucleobindin; osteopontin; phospholipase A_2

INTRODUCTION

It is well established that important attributes of the sperm are acquired during epididymal transit, such as motility, oocyte binding, and penetrating capacity (Amann and Griel, 1974) but it is also known that functions of the sperm can be modulated by accessory sex gland secretions (Yanagimachi, 1994). Analysis of accessory sex gland fluid (AGF) from mature Holstein bulls reveals a diverse set of proteins, some of which are recognized to influence sperm capacitation, acrosome reaction, motility, and sperm–oocyte interaction (Moura et al., 2006a). Moreover, removal of accessory sex glands is detrimental to cleavage of the fertilized oocyte and embryo development in mice and hamsters (Ying et al., 1998; Chen et al., 2002; Chow et al., 2003), which supports the concept that components of the accessory sex glands affect male fertility.

In the bovine, our earlier studies demonstrated that accessory sex gland secretions influenced cauda epididymal sperm function (Henault et al., 1995). More specifically, the ability of cauda epididymal sperm from low fertility (LF) bulls to penetrate zona-free oocytes was enhanced when these sperm were treated with AGF from high fertility (HF) bulls, in contrast to treatment of the same sperm with their own LF AGF. Cauda epididymal sperm used in these experiments were obtained from indwelling vasa deferentia catheters and AGF was collected by an artificial vagina (Henault et al., 1995). These findings provided clear evidence that accessory sex gland secretions influenced sperm fertility, but the specific factors present in the AGF associated with those effects remained to be identified. The present study was undertaken to determine if there were meaningful associations between the effect of AGF on cauda epididymal sperm and the expression of specific proteins in the AGF. Moreover, because we have recently shown that spermadhesin Z13, osteopontin,

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*Correspondence to: Arlindo A. Moura, Department of Dairy and Animal Science, J. O. Almquist Research Center, The Pennsylvania State University, University Park, PA 16802. E-mail: aam10@psu.edu

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phospholipase A_2 (PLA_2), and BSP 30 kDa present in the AGF was related to field fertility of Holstein bulls (Moura et al., 2006b), we also wanted to determine if these proteins were associated with the results of in vitro fertility assays using cauda epididymal sperm.

MATERIALS AND METHODS

Experimental Design

Accessory sex gland fluid collected from Holstein bulls of documented fertility and used in competitive in vitro fertilization (IVF) assays was subjected to two-dimensional SDS–PAGE. Gel images were then analyzed by a computerized system (PDQuest software) and proteins, meaningfully related to the outcome of those IVF assays, were identified by tandem mass spectrometry and Western blots.

In vitro fertilization experiments had been previously conducted to compare the oocyte-penetrating capacity of cauda epididymal sperm from LF bulls treated with homologous AGF versus treatment of the same sperm with AGF from HF bulls (Henault et al., 1995). Fertility of bulls was calculated as the percentage point deviation (PD) of its nonreturn rate (NNR) from the average NRR of all bulls in a given artificial insemination center (Killian et al., 1993; Moura et al., 2006b). We considered HF bulls those with PD = 0 and low fertility the ones with PD < 0. The number of services associated with each sire ranged from 269 to 77,321 and PD values from +7.7% to −18.1%. Cauda epididymal sperm and AGF were obtained when bulls were retired from artificial insemination centers and subjected to vasa deferentia cannulation. This procedure allowed the collection of cauda epididymal sperm from the cannulae and AGF using an artificial vagina, as explained in detail before (Henault et al., 1995; Moura et al., 2006b). The project had the approval of the Institutional Animal Care and Use Committee of the Pennsylvania State University.

There were 19 pairs of IVF comparisons, with 12 different bulls. The outcome of such assays was summarized as greatest percent of oocytes penetrated by either cauda sperm treated with AGF from LF bulls or with AGF from HF bulls (Table 1). Based on this outcome, we divided the IVF assays into three groups. Group I (n = 8): comparisons where cauda epididymal sperm from LF bulls exposed to AGF from HF bulls had greater fertilizing capacity than the same sperm exposed to homologous AGF. Group II (n = 7): comparisons where exposure of sperm from LF bulls to AGF from HF bulls versus exposure to AGF from LF bulls did not show significant differences. Group III (n = 4): comparisons where sperm from LF bulls treated with AGF from HF bulls had lower fertilizing capacity than sperm treated with homologous AGF.

TABLE 1. Competitive In Vitro Fertilization Assays Designed to Compare the Oocyte-Penetrating Capacity of Cauda Epididymal Sperm From Low Fertility (LF) Bulls Treated With Homologous Accessory Sex Gland Fluid (AGF) Versus Treatment With AGF From High Fertility (HF) Bulls

Cauda epididymal sperm from	Treated with accessory sex gland fluid from		Percent of oocytes penetrated mostly by cauda epididymal sperm		
LF bull	Its own	HF bull[a]	Comparison #	Treated with AGF from LF bull	Treated with AGF from HF bull
Group I (bull # and PD value[a])					
# 1 (−2.0)		# 6 (+6.0)	1	11	89*
# 2 (−3.3)		# 6 (+6.0)	2	7	93*
# 3 (−16.3)		# 6 (+6.0)	3	21	79*
# 1 (−2.0)		# 7 (+7.7)	4	0	100*
# 2 (−3.3)		# 7 (+7.7)	5	21	79*
# 4 (−18.1)		# 7 (+7.7)	6	0	100*
# 1 (−2.0)		# 8 (+6.9)	7	8	92*
# 4 (−18.1)		# 9 (0)	8	20	80*
Group II (bull # and PD value[a])					
# 4 (−18.1)		# 6 (+6.0)	9	27^{NS}	73
# 5 (−11.7)		# 7 (+7.7)	10	50^{NS}	50
# 3 (−16.3)		# 8 (+6.9)	11	44^{NS}	56
# 5 (−11.7)		# 10 (+1.2)	12	44^{NS}	56
# 5 (−11.7)		# 9 (0)	13	33^{NS}	67
# 5 (−11.7)		# 11 (+1.0)	14	62^{NS}	38
# 4 (−18.1)		# 10 (+1.2)	15	67^{NS}	33
Group III (bull # and PD value[a])					
# 5 (−11.7)		# 8 (+6.9)	16	100*	0
# 5 (−11.7)		# 12 (+5.3)	17	95*	5
# 5 (−11.7)		# 6 (+6.0)	18	77*	23
# 4 (−18.1)		# 8 (+6.9)	19	69*	31

The outcome of these assays was determined as the greatest percent of zona-free oocytes penetrated by either cauda sperm treated with AGF from LF bulls or with AGF from HF bulls (adapted from Henault et al., 1995).
NS: $P > 0.05$.
[a]PD: percentage point deviation of bull nonreturn rate (NRR) from the average NRR of all bulls in a given artificial insemination cooperative. High fertility bulls were those with PD = 0 and low fertility the ones with PD < 0.
*$P < 0.05$ (within each pair wise comparison).

Two-Dimensional Gel Electrophoresis of AGF Proteins and Computerized Analysis of Gel Images

Fluid of the accessory sex glands was centrifuged at 2,400*g* for 15 min right after collection and stored in liquid nitrogen. For 2-D electrophoresis, samples were thawed at room temperature, centrifuged at 10,000*g* (60 min, at 5°C) and the supernatant assayed for protein content (Lowry et al., 1951) using BSA as standards, as described before (Killian et al., 1993; Moura et al., 2005b). The procedure for SDS–PAGE consisted of isoelectric focusing carried out in tube gels (Bio Rad, Rockville Centre, NY) containing a mixture of ampholytes with pH from 3 to 7 (0.4 ml) and 3 to 10 (0.1 ml; Serva, Heidelberg, Germany). Samples containing 500 μg of protein were brought to a volume of 100 μl using a solution of β-mercaptoethanol, urea and the same ampholytes used in the gels. Gels were then subjected to 200 V for 15 min, 300 V for 30 min, 400 V for 30 min, 375 V for 16–18 hr, and 800 V during 1 hr. Following focusing, gels were removed from the tubes and placed on stacking gels that had been prepared on the top of gels containing a linear gradient of acrylamide (10–17.5%). Standards from 66 to 14 kDa were also used (Sigma Co., St. Loius, MO). Gels were stained with Coomassie brilliant blue R-250, destained in a solution of methanol, acetic acid, and deionized distilled H_2O, and scanned using a GS-670 imaging densitometer (Bio Rad). Images saved as TIFII files were analyzed using PDQuest software, 7.3.0 version (Bio Rad). For the set of AGF gels (12 images), a single master gel was generated by the software, which represented the best pattern of spots in the samples. A few additional spots, consistently present in some gels, were also added to the master so that they could be matched to all samples. Proteins in key regions of the gels were used as landmarks and final matching of spots was achieved after several rounds of extensive comparisons. Control of spot matches was done by checking each spot in each gel with the respective pattern in the master. Protein quantities were given as PPM of the total integrated optical density of the spots, according to PDQuest.

Protein Identification by Tandem Mass Spectrometry

Proteins separated by 2-D SDS–PAGE and associated with the endpoints of interest were subjected to in-gel trypsin digestion (Koc et al., 2001). Excised gel pieces were washed three times with 100 μl of ammonium bicarbonate (25 mM) and dehydrated with 100 μl of acetonitrile (50%), and dried in a speed vacuum. They were then incubated overnight at 37°C with trypsin (12.5 ng/μl in 25 mM ammonium bicarbonate). Peptides were then extracted twice with 25 μl of formic acid (5%) for 20 min. The extracts were dried in a speed vacuum again and resuspended in 10 μl of 5% acetonitrile with formic acid (0.1%). Tryptic digests were analyzed by capillary liquid chromatography–nanoelectrospray ionization–tandem mass spectrometry (CapLC-MS/MS). A Micromass Q-Tof API US mass spectrometer coupled with a Waters CapLC HPLC unit was used for the analysis. As previously described (Abbas et al., 2005; Moura et al., 2006b), the proteolytic digests (1–5 μl) were injected into solvent A (acetonitrile/water/formic acid, 5/95/0.1) supplied by the auxiliary pump of the capillary HPLC unit and trapped in a Waters Symmetry 300™ column (C-18, 5 μm film, 0.3 × 5 mm) for on-line desalting and preconcentration. After washing for 3 min with solvent A at 20 μl/min, trapped peptides were then back flushed with the gradient solvent flow on to the analytical column, a Dionex PepMap fused silica capillary column (C-18 5 μm, 0.075 × 150 mm), using a 10-port switching valve. The analytical column was run with a gradient (5–42% solvent B; acetonitrile/water/formic acid; 95/5/0.2; in 44 min). The mass spectrometry was calibrated using Glu-Fib product ion fragments as needed to maintain mass accuracy within 10 ppm. The Q-Tof mass spectrometer was operated to acquire MS/MS of Tryptic peptides in data-dependent acquisition mode for precursor ion selection using charge-state recognition and intensity threshold as selection criteria using MassLynx 4.0 SP1. In order to carry out the tandem mass spectrometric data acquisition, a survey scan (2 sec) over the m/z of 400–1,500 was performed. From each survey scan, up to four most intense precursor ions based on the selection criteria were selected for tandem mass spectrometry to obtain the production spectra resulting from collision-induced dissociation in the presence of argon. The product ion spectra (6–8 sec) collected were processed using Protein Lynx Global Server 2.1 and were converted to peak list text files for database searching. In order to identify the proteins, MS/MS ion searches were performed on the processed spectra against a locally maintained copy of the NCBI NR database using MASCOT Daemon and search engine (Matrix Science, Inc., Boston, MA). The searches were made with the assumption that there was one maximum missed trypsin cleavage and that peptides were monoisotropic and oxidized at methionine residues and carbamidomethylated at cysteine residues. Peptide mass tolerance and fragment mass tolerance were initially set to 1.2 and 0.6 Da, respectively, for MS/MS ion searching; however, peptide mass values were ensured to be within 0.1 Da (typically less than 0.05 Da) when manually reviewing MASCOT search results.

Western Blots

In addition to tandem mass spectrometry, immunoblots were used for identification of nucleobindin in the AGF. Proteins separated by 2-D SDS–PAGE were transferred to a nitrocellulose membrane (Westran™ S, Schleicher and Schuell Bioscience, Inc., Keene, NH) using a Multiphor II Nova Blot (Pharmacia, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) at 208 A for 1 hr (Moura et al., 2006a). Blots were blocked overnight at 4°C with heat-inactivated goat serum (10 ml), PBS-Tween 20 (200 ml PBS with 0.5% Tween 20) and BSA (3%, w/v), followed by incubation with the primary antibody (1:8,000) in blocking buffer for 2 hr at

room temperature. Primary antibody in this case was a rabbit anti 63-kDa nucleobindin isolated from the mineralized matrix of bovine bone (kindly provided by Dr. Dick Neinegärd, Department of Medical and Physiological Chemistry, University of Lund, Lund, Sweden). Membranes were then washed three times in PBS-Tween at room temperature (20 min each), incubated (1:7,500) with goat anti-rabbit IgG HRP (Sigma Co.) in blocking buffer for 1 hr and again washed three times with PBS-Tween (20 min each). Antibody reaction on the nitrocellulose membrane emerged with the use of chemiluminescence (ECL™ Western blotting detection reagents, Amersham Biosciences, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and exposition to a Kodak film (X-OMAT LS, Kodak Co., Rochester, NY).

Statistical Analysis

Relative expression of AGF proteins in HF bulls was compared across the three groups of IVF assays using analysis of variance and Duncan statistical test (SAS, 2003). In this case, independent variables were defined as spot intensities in the AGF 2-D maps of HF bulls minus its counterpart in the AGF of LF bulls. Within groups of pair wise comparisons, differences in spot intensity between HF and LF bulls were analyzed by *t*-test (SAS, 2003).

RESULTS

The relative expression of proteins identified as BSP A1/A2 and A3, BSP 30 kDa, clusterin, albumin, PLA_2, and osteopontin (Figs. 1 and 2A; Table 2) was greater in the AGF of HF bulls used in Group I as compared to Groups II and III. Group I had pair wise comparisons where there was an advantage of mixing cauda epididymal sperm from LF bulls with AGF from HF bulls. Both BSP 30 kDa and PLA_2 represent the average intensities of five and three spots identified as such, respectively (Fig. 1; Table 2). The most pronounced changes detected across groups were those of albumin, osteopontin, and BSP 30 kDa expression (Fig. 2A). Relative amount of albumin in the AGF of HF bulls in comparisons of Group I was 21- and 4.2-fold higher, respectively, than in those of Groups II and III. Osteopontin was 9.9- and 2.2-fold and BSP 30 kDa was 2.1- and 3.9-fold greater in IVF comparisons of Group I than in the two other groups.

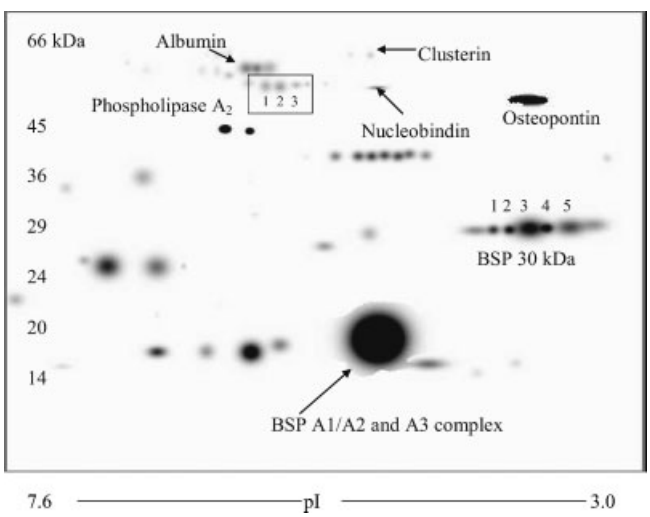

Fig. 1. Master gels constructed by PDQuest representing proteins of the accessory sex gland fluid (AGF) from Holstein bulls. Proteins were separated by two-dimensional SDS–PAGE and annotated spots identified by tandem mass spectrometry (CapLC-MS/MS).

Within Group I of pair wise comparisons, differences between AGF of HF and LF bulls were positive and significant ($P < 0.05$) for BSP 30 kDa, clusterin, albumin, PLA_2, and osteopontin. Conversely, those differences in Group II became negative, although significant only for BSP A1/A2 and A3, BSP 30 kDa, and clusterin. A similar pattern was observed in pair wise comparisons of Group III, where differences between AGF of HF and LF bulls were negative for BSP A1/A2 and A3, BSP 30 kDa, and clusterin ($P < 0.05$) or nonsignificant, in the case of albumin and PLA_2 (Fig. 2A).

Contrary to what was observed for all six proteins shown in Figure 2A (BSP A1/A2 and A3, BSP 30 kDa, clusterin, albumin, PLA_2, and osteopontin), relative intensity of nucleobindin in the AGF of HF bulls decreased in Group I ($P < 0.05$) as compared to Groups II and III of IVF assays (Fig. 2B). Moreover, differences in nucleobindin expression between AGF of HF and LF sires were negative within Group I and positive in Groups II and III, although significant only in the latter. Immunoblots confirmed a major reaction of the anti 63-kDa bovine nucleobindin antibody with a spot at 58 kDa and pI 5.6 (Fig. 3), a position matching the spot identified by tandem mass spectrometry in the 2-D gels stained with Coomassie blue (Fig. 1; Table 2).

DISCUSSION

The present study shows that AGF of HF bulls was more effective in enhancing cauda epididymal sperm from LF bulls to penetrate zona-free oocytes when it had greater amounts of BSP A1/A2 and A3, BSP 30 kDa, clusterin, albumin, PLA_2, and osteopontin. With the exception of BSP A1/A2 and A3, the relative expression of these proteins in the AGF of HF sires was significantly greater than in LF sires of Group I comparisons. This relationship was not seen in pair wise comparisons of HF and LF bulls of Groups II and III. The spot described as BSP A1/A2 and A3 complex (Fig. 1) was identified as the bovine seminal fluid protein Pdc-109 by tandem mass spectrometry and some isoforms of BSP 30 kDa generated low Mascot scores. Our previous study confirmed, however, that antibodies against BSP A1/A2 and A3 and BSP 30 kDa reacted with all spots in the respective regions of AGF 2-D maps (Moura et al., 2006a).

In agreement with the current findings, we recently reported that HF Holstein bulls expressed more osteopontin and PLA_2 in their AGF than LF bulls, as well as a quadratic association for BSP 30 kDa (Moura et al., 2006b). Less spermadhesin Z13 was related to greater field fertility (Moura et al., 2006b) and we also found it

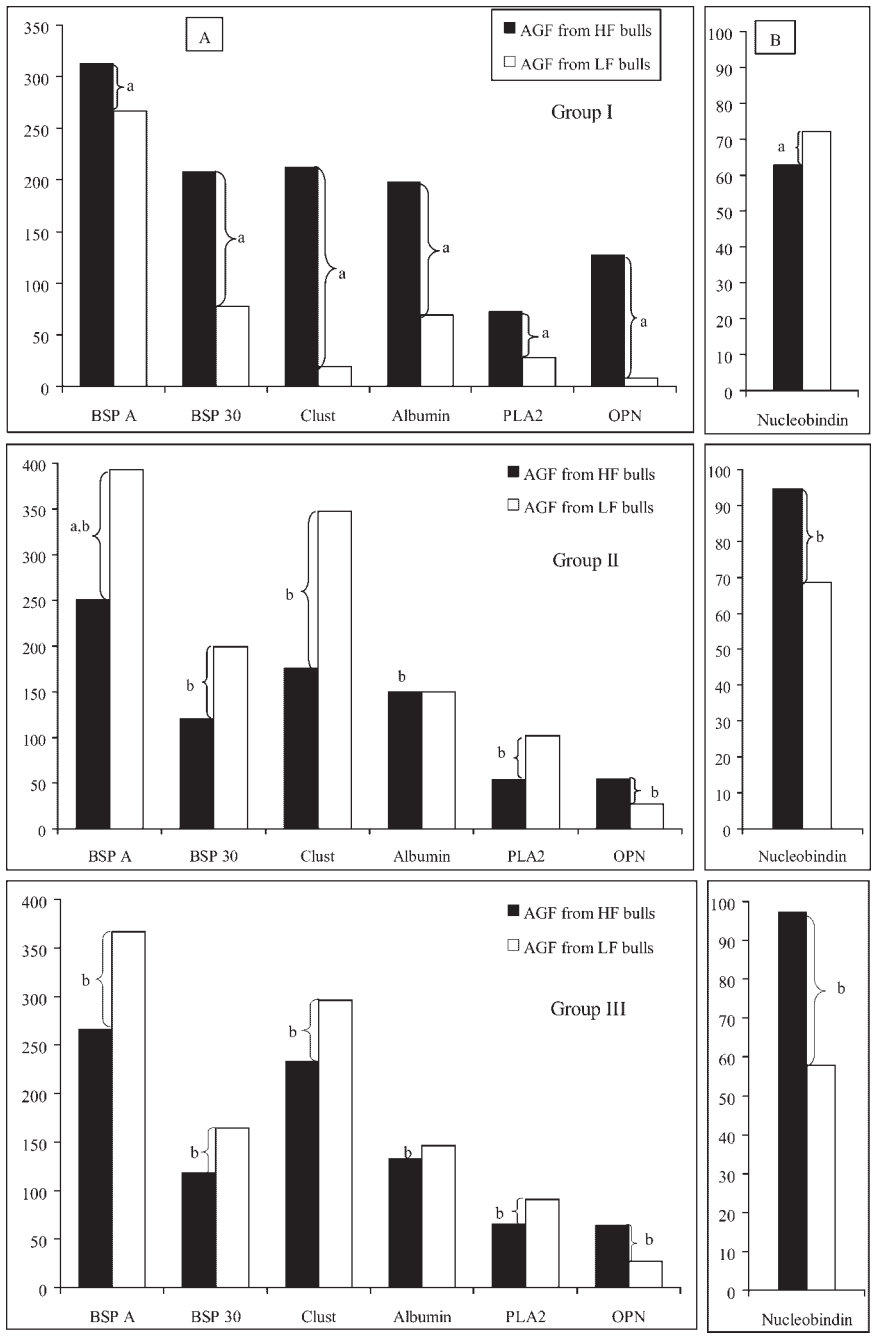

Fig. 2. Expression of AGF proteins in high (HF) and low (LF) fertility bulls used in competitive in vitro fertilization (IVF) assays. Such assays compared the oocyte-penetrating capacity of cauda epididymal sperm from LF bulls treated with homologous AGF versus treatment of the same sperm with AGF from HF bulls. Group I (n = 8): comparisons where sperm from LF bulls exposed to AGF from HF bulls had greater fertility than sperm exposed to homologous AGF. Group II (n = 7): exposure of sperm from LF bulls to AGF from HF bulls versus AGF from LF bulls did not show significant differences. Group III (n = 4): comparisons where sperm from LF bulls treated with AGF from HF bulls had lower fertility than sperm treated with homologous AGF. The independent variables evaluated across groups of IVF assays correspond to spot intensities in the AGF of HF bulls minus its counterpart in the AGF of LF bulls. Differences followed by identical letters had nonsignificant variations across groups ($P > 0.05$). BSP A, BSP A1/A2 and A3; BSP 30, BSP 30 kDa; Clust, clusterin; PLA_2, phospholipase A_2; OPN, osteopontin.

higher in the AGF of LF bulls within Groups I, II, and III of IVF comparisons (data not shown). However, because the relative expression of spermadhesin (i.e., the difference in its amount between AGF of HF and LF bulls) remained without significant variations across groups, we concluded that this peptide was not a major indicator of the effects of AGF on cauda epididymal sperm in vitro. Moreover, a fourth peptide also related to effects of AGF in vitro was identified as BSP A1/A2 and A3, which reportedly has functions similar to BSP

TABLE 2. Accessory Sex Gland Fluid (AGF) Proteins Associated With the Outcome of In Vitro Fertilization Assays Using Cauda Epididymal Sperm From Low Fertility Bulls Treated With Homologous AGF Versus Treatment With AGF From High Fertility Bulls

Protein identification[a]	GI access #	Protein kDa/pI[b]		Mascot MS/MS score[c]	Seq. cov.	Matched peptides and positions
		Theor.	Exper.			
Albumin	162648	71; 5.8	63; 6.4	178	7%	$^{(402)}$HLVDEPQNLIK$^{(412)}$; $^{(421)}$LGEYGEQNALIVR$^{(433)}$; $^{(548)}$KQTALVELLK$^{(557)}$; $^{(569)}$TVMENFVAFVDK$^{(580)}$
Bull seminal fluid protein Pdc 109	494430	5.6; 5.1	14; 5.7	107	64%	$^{(5)}$CVFPFIYGGK$^{(14)}$; $^{(22)}$IGSMWMSWCSLSPNYDK$^{(38)}$; $^{(22)}$IGSMWMSWCSLSPNYDKDR$^{(40)}$
BSP-30 kDa	28849953	22; 5.7				
Spot 1			28; 5.1	17	3%	$^{(159)}$VHSFFWR$^{(165)}$
Spot 2			28; 5.0	56	9%	$^{(159)}$VHSFFWR$^{(165)}$; $^{(167)}$WCSLTSNYDR$^{(176)}$
Spot 3			28; 4.9	19	3%	$^{(159)}$VHSFFWR$^{(159)}$
Spot 4			28; 4.8	49	15%	$^{(138)}$DEPECVPFIYR$^{(149)}$; $^{(159)}$VHSFFWR$^{(159)}$; $^{(167)}$WCSLTSNYDR$^{(176)}$;
Spot 5			28; 4.7	36	12%	$^{(138)}$DEPECVPFIYR$^{(149)}$; $^{(167)}$WCSLTSNYDR$^{(176)}$
Clusterin	116530	52; 5.7	70; 5.7	235	15%	$^{(52)}$TQIEQTNEER$^{(61)}$; $^{(62)}$KLLLSSLEEAK$^{(72)}$; $^{(63)}$LLLSSLEEAK$^{(72)}$; $^{(153)}$IDSLMENDR$^{(161)}$; $^{(177)}$ASSIMDELFQDR$^{(188)}$; $^{(259)}$TPYHFPTMEFTENNDR$^{(274)}$; $^{(332)}$LYDQLLQSYQQK$^{(343)}$
Osteopontin	19774215	31; 4.5	55; 5.0	162	35%	$^{(36)}$YPDAVATWLKPDPSQK$^{(51)}$; $^{(52)}$QTFLAPPQNSVSSEETDDNK$^{(70)}$; $^{(52)}$QTFLAPQNSVSSEETDDNKQNTLPSK$^{(77)}$; $^{(170)}$SNVQSPDATEEDFTSHIESEEMHDAPK$^{(196)}$; $^{(239)}$LSQEFHSLEDKLDLDHK$^{(255)}$; $^{(266)}$ISHELDASASEVN$^{(278)}$
PLA_2	27807045	51; 6.1				
Spot 1			58; 6.2	192	13%	$^{(42)}$IQALMAAANIGQSK$^{(55)}$; $^{(110)}$FLGTHWLVGK$^{(119)}$; $^{(144)}$YPLIIFSHGLGAFR$^{(157)}$; $^{(311)}$IPQPLFFINSER$^{(322)}$; $^{(365)}$IIGYLFTLK$^{(373)}$
Spot 2			58; 6.1	106	9%	$^{(42)}$IQALMAAANIGQSK$^{(55)}$; $^{(110)}$FLGTHWLVGK$^{(119)}$; $^{(211)}$RGEEEFPLR$^{(219)}$; $^{(323)}$FQYPSNIIR$^{(331)}$
Spot 3			58; 6.0	172	20%	$^{(42)}$IQALMAAANIGQSK$^{(55)}$; $^{(102)}$EYFLGLSK$^{(109)}$; $^{(110)}$FLGTHWLVGK$^{(119)}$; $^{(144)}$YPLIIFSHGLGAFR$^{(157)}$; $^{(268)}$IAIIGHSFGGATVIQTLSEDQR$^{(289)}$; $^{(311)}$IPQPLFFINSER$^{(322)}$; $^{(323)}$FQYPSNIIR$^{(331)}$
Nucleobindin	76641927	53.2; 5.1	58; 5.6	431	35%	$^{(51)}$YLQEVINVLETDGHFR$^{(66)}$; $^{(116)}$MDAQQEPNIQLDHLNLLK$^{(133)}$; $^{(134)}$QFEHLDPQNQHTFEAR$^{(149)}$; $^{(150)}$DLELLIQTATR$^{(160)}$; $^{(161)}$DLAQYDAAHHEEFKR$^{(175)}$; $^{(187)}$YLESLGEEQR$^{(196)}$; $^{(216)}$VNVPGSQAQLK$^{(226)}$; $^{(227)}$EVWEELDGLDPNR$^{(239)}$; $^{(308)}$LVTLEEFLASTQR$^{(320)}$; $^{(346)}$RFEEELAAR$^{(354)}$; $^{(347)}$FEEELAAR$^{(354)}$; $^{(385)}$ELQQAVLQMEQR$^{(396)}$; $^{(416)}$FHPDTDDVPVPAPAGDQK$^{(433)}$

Proteins were identified by two-dimensional SDS-PAGE and capillary liquid chromatography nano electrospray ionization tandem mass spectrometry (CapLC-MS/MS). PLA_2, phopholipase A_2.

[a]Spot # as shown in 2-D map of Figure 1.

[b]Theoretical molecular weight and pI values represent the values given for the protein identified at NCBI NR database. Experimental values were deduced from the 2-D master gel generated by PDQuest (Fig. 1).

[c]Probability-based Mowse score, according to Mascot. Individual ion score = 48 indicates identity or extensive homology ($P < 0.05$). Protein score represents the sum of the unique ion scores when more than one precursor ion was used for MS/MS analysis.

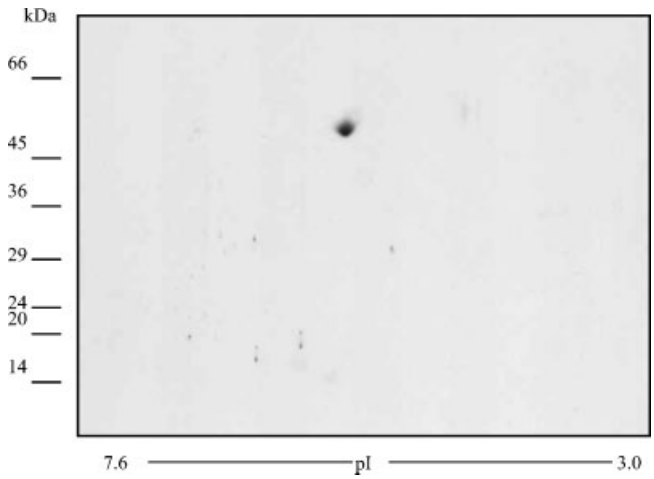

Fig. 3. Western blot of AGF from Holstein bulls. Proteins separated by 2-D SDS–PAGE were transferred to a nitrocellulose membrane and incubated with an antibody raised against a 63-kDa nucleobindin isolated from bovine bone tissue.

30 kDa in sperm capacitation, as we discuss below with more detail. Despite the obvious fact that field fertility and results of IVF assays with cauda epididymal sperm may not be affected by exactly the same variables, we present evidence that certain AGF proteins were associated with both endpoints. It is important to emphasize that fertility in our IVF study was defined by the ability of sperm to penetrate zona-free oocytes. These sperm were not challenged as sperm in vivo would be since they did not have to reach the site of fertilization or penetrate the zona before reaching the vitelline membrane. As we have suggested (Moura et al., 2006a), there could well be other AGF proteins that function in vivo to help with sperm transport in the female reproductive tract and zona penetration. Studies have reported positive correlations between the performance of bull semen in vivo and in IVF systems (Shamsuddin and Larsson, 1993; Zhang et al., 1997, 1999; Ward et al., 2003), although others questioned the existence of such links (Ohgoda et al., 1988; Schneider et al., 1999). It is likely that results from these types of experiments are influenced by the range of bull fertilities and different methods used in the IVF assays, among other factors. However, assuming that there is a certain degree of association between fertility in vivo and in vitro (Larsson and Rodriguez-Martinez, 2000), and our results support this concept, it is also possible to conclude that these associations are not necessarily linear. A complete elucidation of the potential connections between in vivo and in vitro fertility will be assessed as we improve our understanding of all factors that determine reproductive success in both environments.

Although the detailed mechanisms clarifying the associations between AGF proteins and cauda epididymal sperm function in vitro have yet to be unfolded, known attributes of AGF proteins provide explanations for the results presented here. BSP proteins, both A1/A2 and A3 and BSP 30 kDa, are implicated in cholesterol and phospholipid movement from sperm membrane (Manjunath and Thérien, 2002) and albumin influences capacitation through its ability to change membrane content of cholesterol (Go and Wolf, 1985; Visconti and Kopf, 1998). Although cauda epididymal sperm were treated with heparin in those IVF assays to promote capacitation (Henault et al., 1995), it is possible that higher expression of both BSP proteins and albumin in the AGF of certain HF bulls enhanced capacitation, facilitating sperm penetration of oocytes. The effect of albumin on sperm may have also been associated with its capacity to interact with other proteins. Albumin acts as a reservoir of other proteins and has been shown to exhibit phospholipase activity, which in turn contributes to sperm capacitation and acrosome reaction (Singleton and Killian, 1983). In support of these attributes of albumin, we detected significant correlations between the amounts of albumin and isoforms of PLA_2 in the 2-D maps of AGF ($r = 0.66–0.71$, $P < 0.01$; data not shown).

Clusterin appears in 2-D gels of accessory sex fluid of bulls as a single 70-kDa unit and as a series of isoforms at 40 kDa (Moura et al., 2006a), but we only detected associations between IVF endpoints and the 70-kDa form. Clusterin mediates prevention of damaging oxidative reactions (Reyes-Moreno et al., 2002) and protein precipitation (Ibrahim et al., 1999; Wilson and Easterbrook-Smith, 2000), agglutination of abnormal spermatozoa (O'Bryan et al., 1990, 1994), and control of complement-induced sperm lysis (Jenne and Tchopp, 1989; Ibrahim et al., 1999). The ability of clusterin to prevent oxidative damage would be of benefit to spermatozoa in the female reproductive as well as to sperm in an IVF system.

Phospholipase A_2 associated with sperm membranes appears to participate in the acrosome reaction (Breitbart and Spungin, 1997; Chen et al., 2005) and sperm-egg fusion (Riffo and Párraga, 1997), a notion supported by the report that a $PLA_2\beta$-gene knockout mouse has sperm with reduced capacity to fertilize oocytes both in vitro and in vivo (Bao et al., 2004). Exogenous PLA_2 has also been shown to enhance acrosome reaction in guinea pig sperm (Singleton and Killian, 1983). Thus, PLA_2 in the AGF may facilitate these roles when mixed with cauda epididymal sperm at ejaculation or in vitro.

Osteopontin is known to function in cell adhesion and cell-extracellular matrix communication, tissue remodeling, intra-cellular signaling and cytoskeleton dynamics, among other events (Liaw et al., 1998; Mazzali et al., 2002; Denhardt, 2004). Immunocytochemistry and Western blots of protein extracts from sperm membrane conducted in our laboratory reveal that osteopontin binds to ejaculated sperm of bulls (unpublished results). Moreover, incubation of intact bovine oocytes with oviductal fluid, which also contains osteopontin (Gabler et al., 2003), and antibodies against osteopontin inhibited both sperm-oocyte binding and embryo development (unpublished results). Interestingly, antibodies against albumin had similar negative

effects on fertility in vitro, an observation that is now substantiated by the present findings that albumin, as well as osteopontin, was among the proteins that had the most significant associations with the positive effect of AGF from HF bulls on cauda epididymal sperm.

Contrary to what was observed for the six AGF proteins mentioned above, the relative expression of nucleobindin in the AGF of HF bulls was reduced in IVF assays of Group I when compared with those of Groups II and III. Less nucleobindin in the AGF of HF bulls in Group I suggests that this protein interferes with the positive effects of AGF on cauda epididymal sperm. However, because we are the first group to describe nucleobindin in accessory sex gland secretions, its role in an IVF system or reproduction still remains to be defined. The immunoblot obtained from a 2-D gel revealed a major reaction at 58 kDa/pI 5.6, matched to the spot identified as nucleobindin by tandem mass spectrometry. As reported by Wendel et al. (1995), the same type of antibody reacted in 1-D gels with a 63-kDa nucleobindin isolated from the matrix of bovine bone. Slight differences in molecular weight between our 2-D gels (58 kDa) and 1-D gels (63 kDa) shown by Wendel et al. (1995) could have been caused by differences in the type and mobility of electrophoresis systems. In this regard, we identified a major reaction at 62 kDa when AGF proteins were separated by 1-D gel electrophoresis and blotted with the same antibody (results not shown).

Nucleobindin is found both as a Golgi protein and secreted extracellularly (Wendel et al., 1995). Its amino acid sequence is conserved across species and it binds to calcium with high affinity through its EF-hand calcium binding motif (Wendel et al., 1995; Lavoie et al., 2002; de Alba and Tjandra, 2004). Nucleobindin has been described as a component of bone and dentin extracellular matrices (Petersson et al., 2004; Somogyi et al., 2004), suggesting it interacts with extracellular matrix proteins. Based on these preliminary findings in other tissues, we conclude that nucleobindin may participate in calcium-mediated events and/or interaction with proteins on sperm, which would affect fertilization.

In summary, the present study demonstrates that the enhancing effect of AGF from HF bulls on cauda epididymal sperm from LF bulls is likely the result of AGF from HF bulls containing relatively more BSP A1/A2 and A3, BSP 30 kDa, clusterin, albumin, PLA_2, and osteopontin and less nucleobindin. Known characteristics of these proteins suggest numerous ways in which they act regarding to sperm function, from capacitation to oocyte interaction. The presence of nucleobindin in male reproductive fluids and its association with IVF endpoints are novel and further studies are needed to clarify its role. Finally, three of accessory sex gland proteins associated with the outcome of IVF assays (BSP 30 kDa, PLA_2, and osteopontin) are identical to those related to bull fertility measured in the field (Moura et al., 2006b).

REFERENCES

Abbas A, Koc H, Liu F, Tien M. 2005. Fungal degradation of wood: Initial proteomic analysis of extracellular proteins of Phanerochaete chrysosporium grown on oak substrate. Curr Genet 47:49–56.

Amann RP, Griel LC, Jr. 1974. Fertility of bovine spermatozoa from rete testis, cauda epididymis, and ejaculated sperm. J Dairy Sci 57: 212–219.

Bao S, Miller DJ, Ma Z, Wohltmann M, Eng G, Ramanadham S, Moley K, Turk J. 2004. Male mice that do not express group VIA phospholipase A2 produce spermatozoa with impaired motility and have greatly reduced fertility. J Biol Chem 279:38194–38200.

Breitbart H, Spungin B. 1997. The biochemistry of the acrosome reaction. Mol Hum Reprod 3:195–202.

Chen H, Cheung MPL, Chow PH, Cheung ALM, Liu W, O WS. 2002. Protection of sperm DNA against oxidative stress in vivo by accessory sex gland secretions in male hamsters. Reproduction 124:491–499.

Chen WY, Nia Y, Panb Y-M, Shia Q-X, Yuana Y-Y, Chena A-J, Maoa L-Z, Yua S-Q, Roldanc ERS. 2005. GABA, progesterone and zona pellucida activation of PLA2 and regulation by MEK-ERK1/2 during acrosomal exocytosis in guinea pig spermatozoa. FEBS Lett 579: 4692–4700.

Chow PH, Jiang HY, Poon HK, Lee KH, O WS. 2003. Embryos sired by males without accessory sex glands induce failure of uterine support: A study of VEGF, MMP and TGF expression in the golden hamster. Anat Embryol 206:203–213.

de Alba E, Tjandra N. 2004. Structural studies on the Ca2+-binding domain of human nucleobindin (Calnuc). Biochemistry 43:10039–10049.

Denhardt DT. 2004. The third international conference on osteopontin and related proteins, San Antonio, Texas, May 10-12, 2002. Calcif Tissue Int 74:213–219.

Gabler C, Chapman DA, Killian GJ. 2003. Expression and presence of osteopontin and integrins in the bovine oviduct during the oestrous cycle. Reproduction 126:721–729.

Go KJ, Wolf DP. 1985. Albumin-mediated changes in sperm sterol content during capacitation. Biol Reprod 32:145–153.

Henault MA, Killian GJ, Kavanaugh JF, Griel LC, Jr. 1995. Effect of accessory sex gland fluid from bulls of differing fertilities on the ability of cauda epididymal sperm to penetrate zona-free bovine oocytes. Biol Reprod 52:390–397.

Ibrahim NM, Troedsson MH, Foster DN, Loseth KJ, Farris JA, Blaschuk O, Crabo BG. 1999. Reproductive tract secretions and bull spermatozoa contain different clusterin isoforms that cluster cells and inhibit complement-induced cytolysis. J Androl 20:230–240.

Jenne DE, Tchopp J. 1989. Molecular characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: Identity to sulfated glycoprotein 2, a constituent of rat testis fluid. Proc Natl Acad Sci USA 86:7123–7127.

Killian GJ, Chapman DA, Rogowski L. 1993. Fertility-associated proteins in bull seminal plasma. Biol Reprod 49:1202–1207.

Koc EC, Burkhart W, Blackburn K, Moyer MB, Schlatzer DM, Moseley A, Spremulli LL. 2001. The large subunit of the mammalian mitochondrial ribosome—Analysis of the complement of ribosomal proteins present. J Biol Chem 276:43958–43969.

Larsson B, Rodriguez-Martinez H. 2000. Can we use in vitro fertilization tests to predict semen fertility? Anim Reprod Sci 60–61:327–336.

Lavoie C, Meerloo T, Lin P, Marilyn A, Farquhar G. 2002. Calnuc, an EF-Hand Ca^{2+}-binding protein, is stored and processed in the golgi and secreted by the constitutive-like pathway in AtT20 cells. Mol Endocrinol 16:2462–2474.

Liaw L, Birk DE, Ballas CB, Whitsitt JS, Davidson JM, Hogan BLM. 1998. Altered wound healing in mice lacking a functional osteopontin gene (spp1). J Clin Invest 101:1468–1478.

Lowry OH, Rosebrough WJ, Farr AL, Randall RL. 1951. Protein measurement with Folin phenol reagent. J Biol Chem 193:265–275.

Manjunath P, Thérien I. 2002. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. J Reprod Immunol 53:109–119.

Mazzali M, Kipari T, Ophascharoensuk V, Wesson JA, Johnson R, Hughes J. 2002. Osteopontin—A molecule for all seasons. Q J Med 95:3–13.

Moura AA, Chapman DA, Koc H, Killian GJ. 2006a. A comprehensive proteomic analysis of the accessory sex gland fluid from mature Holstein bulls. Anim Rep Sci (in press).

Moura AA, Koc H, Chapman DA, Killian GJ. 2006b. Expression of proteins of the accessory sex gland fluid as related to fertility indexes of dairy bulls: A proteomic approach. J Androl 27:201–211.

O'Bryan MK, Baker HW, Saunders JR, Kirszbaum L, Walker ID, Hudson P, Liu DY, Glew MD, d'Apice AJ, Murphy BF. 1990. Human seminal clusterin (SP-40): Isolation and characterization. J Clin Invest 85:1477–1486.

O'Bryan MK, Mallidis C, Murphy BF, Baker HW. 1994. Immunohistological localization of clusterin in the male genital tract in humans and marmosets. Biol Reprod 50:502–509.

Ohgoda O, Niwa K, Yuhara M, Takahashi S, Kanoya K. 1988. Variations in penetration rates in vitro of bovine follicular oocytes do not reflect conception rates after artificial insemination using frozen semen from different bulls. Theriogenology 29:1375–1381.

Petersson U, Somogyi E, Reinholt FP, Karlsson T, Sugars RV, Wendel M. 2004. Nucleobindin is produced by bone cells and secreted into the osteoid, with a potential role as a modulator of matrix maturation. Bone 34:949–960.

Reyes-Moreno C, Boilard M, Sullivan R, Sirard MA. 2002. Characterization of secretory proteins from cultured cauda epididymal cells that significantly sustain bovine sperm motility in vitro. Mol Reprod Dev 63:500–509.

Riffo M, Párraga M. 1997. Role of phospholipase A2 in mammalian sperm-egg fusion: Development of hamster oolemma fusibility by lysophosphatidylcholine. J Exp Zool 79:81–88.

SAS Institute, Inc. 2003. SAS/STAT® User's Guide, Version 6, 4th edition, Vol. 2. Cary, NC: SAS Institute, Inc.

Schneider CS, Ellington JE, Wright RW. 1999. Relationship between bull field fertility and in vitro embryo production using sperm preparation methods with and without somatic cell co-culture. Theriogenology 51:1085–1098.

Shamsuddin M, Larsson B. 1993. In vitro development of bovine embryos after fertilization using semen from different donors. Reprod Domest Anim 28:77–84.

Singleton CL, Killian GJ. 1983. A study of phospholipase in albumin and its role in inducing the acrosome reaction of guinea pig spermatozoa in vitro. J Androl 4:150–156.

Somogyi E, Petersson U, Sugars RV, Hultenby K, Wendel M. 2004. Nucleobindin—A Ca2+-binding protein present in the cells and mineralized tissues of the tooth. Calcif Tissue Int 74:366–376.

Visconti PE, Kopf GS. 1998. Regulation of protein phosphorylation during sperm capacitation. Biol Reprod 59:1–6.

Ward F, Rizos D, Boland MP, Lonergan P. 2003. Effect of reducing sperm concentration during IVF on the ability to distinguish between bulls of high and low field fertility: Work in progress. Theriogenology 59:1575–1584.

Wendel M, Sommarin Y, Bergman T, Heinegard D. 1995. Isolation, characterization, and primary structure of a calcium binding 63-kDa bone protein. J Biol Chem 270:6125–6133.

Wilson MR, Easterbrook-Smith SB. 2000. Clusterin is a secreted mammalian chaperone. TIBS 25:95–98.

Yanagimachi R. 1994. Mammalian fertilization. In: Knobil E, Neill JD, editors. The physiology of reproduction. New York: Raven Press. pp 189–317.

Ying Y, Chow PH, O WS. 1998. Effects of male accessory sex glands and deoxyribonucleic acid synthesis in the first cell cycle of golden hamster embryos. Biol Reprod 58:659–663.

Zhang BR, Larsson B, Lundeheim N, Rodriguez-Martinez H. 1997. Relationship between embryo development in vitro and 56-day nonreturn rates of cows inseminated with frozen–thawed semen from dairy bulls. Theriogenology 48:221–231.

Zhang BR, Larsson B, Lundeheim N, Haard MGH, Rodriguez-Martinez H. 1999. Prediction of bull fertility by combined in vitro assessments of frozen–thawed semen from young dairy bulls entering an AI-program. Int J Androl 22:253–260.