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Objective

The first objective of these studies was to characterize DAG1 and SERPINA5 immunolocalization on bovine sperm and their potential as fertility markers by evaluating variability within and amongst bulls. The second objective was to investigate the relationship of DAG1 and SERPINA5 with field fertility (sire conception rate; SCR), *in vitro* fertility (*in vitro* embryo production), and sperm parameters.

Study Description

Semen from 22 dairy bulls was used to evaluate the presence, localization, and quantification of DAG1 and SERPINA5 on sperm. Sperm motility parameters and viability was also evaluated for semen from each bull. Semen from 19 out of the 22 dairy bulls was used for *in vitro* embryo production (two Low-SCR and one High-SCR were not available for *in vitro* embryo production). Bulls were classified based on their sire conception rates (SCR) values as High-SCR (SCR > 1.0) or Low-SCR fertility (SCR < -4.0). Low fertility bulls were subdivided based on their blastocyst rate (BL) as High-BL (Low-SCR/High-BL BL ≥ 31%) or Low-BL (Low-SCR/Low-BL BL ≤ 26%), and High-SCR bulls were not subdivided. The GLM procedure in SAS was used with bull as a fixed effect to determine if variance was greater between bulls compared to within bull. Correlations were determined among DAG1 and SERPINA5 concentrations, percentage of tail labeled for SERPINA5, SCR, sperm total motility, progressive motility, and viability, and *in vitro* embryo produced cleavage rate (CL) and BL. The GLIMMIX procedure of SAS was used to evaluate the relationship of bull field fertility (High- and Low-SCR), and field and *in vitro* fertility (High-SCR, Low-SCR/High-BL, Low-SCR/Low-BL) classifications with sperm total (TMOT) and progressive (PROG) motility, viability, CL, BL, DAG1 and SERPINA5 relative concentration, and proportion of sperm tail labeled for SERPINA5. Both SERPINA5 and DAG1 were localized on the sperm head; however, SERPINA5 was also localized on the sperm tail. There was greater variance in concentration among bulls compared to within bull for both DAG1 ($P < 0.01$; 69.4 vs 49.1, respectively) and SERPINA5 ($P < 0.01$; 325.8 vs 285.4, respectively). There was a positive correlation between concentration of DAG1 and SERPINA5 ($P = 0.01$; r = 0.54). Concentrations of SERPINA5 were also correlated with CL ($P = 0.04$; r = 0.48), and percentage of sperm tail labeled for SERPINA5 was correlated with viability ($P = 0.05$; r = 0.44) and tended to be correlated with CL ($P = 0.10$; r = 0.39). There was no relationship between SCR or BL rate classifications and DAG1 ($P \geq 0.66$), SERPINA5 ($P \geq 0.54$), or percentage of sperm tail labeled for SERPINA5 ($P \geq 0.48$).

Take Home Points

Proteins DAG1 and SERPINA5 are associated with cell-to-cell interactions and were localized on the bovine sperm head, also, SERPINA5 was localized on the sperm tail. Sperm relative concentration for both proteins were correlated to each other and SERPINA5 was correlated with CL. The percentage of sperm tail labeled for SERPINA5 was correlated with CL and sperm viability; however, proteins were not associated with bull field fertility measured by SCR. Thus, SERPINA5 may be related with sperm protection and/or oocyte fertilization while DAG1 may be related to sperm transport or formation of the sperm reservoir in the oviduct.

Introduction

After differentiation, sperm lose the ability to grow, divide, repair, and synthesize proteins, and have limited metabolic function (Hammerstedt, 1993). After spermiation, sperm are stored in the tail of the epididymis in a dormant state until ejaculation (Acott and Carr, 1984; Carr and Acott, 1984; Barth and Oko, 1989). Upon ejaculation, epididymal sperm are diluted with seminal plasma from accessory sex glands and motility is initiated (Acott and Carr, 1984; Carr and Acott, 1984). Sperm with fertilizing ability reach the oviduct approximately 6-12 h after insemination, populate the isthmus portion of the oviduct and form the sperm reservoir (Hunter and Wilmut, 1984; Wilmut and Hunter, 1984; Lefebvre et al., 1995). Sperm that bind to oviductal cells have prolonged motility and fertilization ability (~30 h) compared to sperm suspended in the media (Pollard et al., 1991). Cell-to-cell interactions (i.e. sperm to oviduct and sperm to oocyte) are mediated through proteins; thus, these interactions are important for successful fertilization. The sperm's apical surface binds to oviductal isthmus and ampullary ciliated cells (Pollard et al., 1991; Lefebvre et al., 1995) and Binder of Sperm Proteins (BSP) have been reported to be involved with sperm reservoir formation (Ignotz et al., 2001; Gwathmey et al., 2003; 2006). There are only a few proteins known to be required for fertilization, and include CD9 (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000) and JUNO (Bianchi et al., 2014) on the egg, and IZUMO1 on the sperm (Inoue et al., 2005). Other proteins have been identified to be associated with mammalian fertility, but not required (see review by Sutovsky, 2009).

Previous research from our laboratory identified that DAG1 and SERPINA5 were present and loosely attached to ejaculated sperm, but they were not present on epididymal sperm, thus they coated the sperm when it was diluted with seminal plasma. The presence of DAG1 has been reported in seminal plasma but not on human sperm (Jodar et al., 2016). Beta-dystroglycan has been reported to be localized to the tail middle piece of guinea pig sperm (Hernández-González et al., 2001) and the post-acrosomal region and middle piece of mouse sperm (Hernández-González et al., 2005). The gene SERPINA5 encodes the plasma serine protease inhibitor. This protein is also known as serpin family A member 5, protein C inhibitor, and others. The presence of SERPINA5 protein has been reported in many body fluids, including plasma (blood), seminal plasma, follicular fluid, amniotic fluid, milk, and others (Laurell et al., 1992). In double knockout mice for SERPINA5, females were fertile, and males were infertile in both *in vitro* (0.5% pregnancy) and *in vivo* (0% pregnancy) experiments. Also, sperm motility (12.5% motility) and the percentage of morphologically normal sperm (5% normal morphology) were decreased in double knockout mice (Uhrin et al., 2000). Similarly, SERPINA5 concentrations were decreased in infertile men with normal sperm motility compared to fertile men (Panner Selvam et al., 2019). Nevertheless, in men, SERPINA5 has been localized to the sperm head (Zheng et al., 1994; Elisen et al., 1998). Thus, the first objective of these studies was to characterize DAG1 and SERPINA5 immunolocalization on bovine sperm and their potential as fertility markers by evaluating variability within and amongst bulls. The second objective was to investigate the relationship of DAG1 and SERPINA5 with field fertility (sire conception rate; SCR), in vitro fertility (in vitro embryo production), and sperm parameters.

Experimental Procedures

Experimental design

Dairy bulls (n = 22) with different SCR values, ranging from -7.7 to 4.45, were classified as High (High-SCR > 1.0; n = 11) or Low (Low-SCR < -4.0; n =11) field fertility. Semen from two ejaculates (average days between ejaculates 140 d; minimum difference 4 d and maximum difference 1,349 d) were used to assess sperm relative concentrations of DAG1 and SERPINA5, total (TMOT) and progressive (PROG) motility, and plasma membrane integrity (viability; n = 20; semen of two bulls had already been processed before viability could be assessed). Semen from these bulls was also used for *in vitro* production of embryos (n = 19; one High-SCR and two Low-SCR bulls' semen were not available for *in vitro* production of embryos). Cleavage (CL) and blastocyst (BL) rates were recorded. Low-SCR bulls were subdivided further based on their blastocyst rate (BL) as High (Low-SCR/High-BL ≥ 31%; n = 6) or Low (Low-SCR/Low-BL ≤ 26%; n = 3).

Sperm Analyses

Sperm motility (TMOT and PROG) analyses were performed using a computer assisted sperm analysis system (CASA). Sperm plasma membrane integrity (viability) was analyzed by evaluating a minimum of 100 sperm per sample in a Nikon Fluorescence microscope. Remaining samples not used for CASA were fixed in 2% formaldehyde solution, washed, diluted to 5 million sperm per mL and stored at 4 ºC until analyzed for DAG1 or SERPINA5.

Anti-DAG1 antibody (goat anti-human, ab136665, polyclonal, ABCAM, United Kingdom) was purified and conjugated to PE/R-Phycoerythrin (ab102918, ABCAM) according to manufacturer instructions. Anti-DAG1 and fixed sperm were incubated for 4 h at room temperature without exposure to light. Samples were evaluated with a Nikon Fluorescence microscope at 400 × magnification, and the NIS-Elements software package was used to outline 100 individual spermatozoa per sample and fluorescence intensity was determined. Also, immunolocalization of DAG1 on the sperm was determined. Anti-SERPINA5 antibody (rabbit anti-human, mouse, rat, PA579976, polyclonal, Invitrogen, Waltham, MA) was conjugated to Dylight 405 Fast (ab201798, ABCAM) according to manufacturer instructions. Anti-SERPINA5 and fixed sperm were incubated for 4 h at room temperature without exposure to light. Samples were evaluated as described for DAG1. Also, immunolocalization of SERPINA5 on the sperm was determined.

In vitro embryo production

All media for *in vitro* embryo production and *in vitro* embryo production followed previous published procedures (Ortega et al., 2016; 2018; Tríbulo et al., 2019; Stoecklein et al., 2021). Briefly, cumulus-oocyte complexes (COC) were retrieved by follicular aspiration from ovaries collected at a commercial abattoir. Tubes with COC were shipped overnight in a portable incubator (Minitube USA Inc., Verona, WI, USA) at 38.5 ºC to the University of Missouri. After approximately 24 h of maturation, groups of 100 COC were washed three times and placed in a 35-mm dish containing fertilization media. Each group of COC was fertilized with sperm from a single bull. Fertilization proceeded for approximately 18 h at 38.5 ºC in a humidified atmosphere of 5% (v/v) CO_2. Putative zygotes (oocytes exposed to sperm) were vortexed to denude from the surrounding cumulus cells at the end of fertilization. Embryos were then cultured in four-well dishes in groups of up to 50 embryos in culture medium covered with mineral oil at 38.5 ºC in a humidified atmosphere of 5% (v/v) O_2 and 5% (v/v) CO_2. Percentage of putative zygotes that cleaved (cleavage rate; CL) was determined at day 3 of development (day 0 = day of insemination) and BL at day 8 of development.

Statistical Analysis

Fluorescence intensity (concentration of SERPINA5 and DAG1) was analyzed using the GLM procedure in SAS (9.4) with bull as a fixed effect to determine the variance in mean protein concentration between bull and within bull. Protein immunolocalization was determined based on visual characterization and statistical analysis was not performed. The CORR procedure of SAS was used to evaluate correlations between SCR, TMOT, PROG, viability, CL, BL, DAG1 and SERPINA5 relative concentration, and proportion of sperm tail labeled for SERPINA5. The GLIMMIX procedure of SAS was used to evaluate the relationship of bull field fertility (High- and Low-SCR), and field and in vitro fertility (High-SCR, Low-SCR/High-BL, Low-SCR/Low-BL) classifications with sperm TMOT, PROG, viability, CL, BL, DAG1 and SERPINA5 relative concentration, and proportion of sperm tail labeled for SERPINA5. Results are presented as least square mean ± SE unless otherwise stated. Statistical differences were defined as $P \leq 0.05$, when $P > 0.05$ but $P \leq 0.10$ the results were considered as tendency.

Results and Discussion

Rate of genetic improvement in a herd is far more efficient through bull selection than female selection due to the larger number of offspring generated by one single bull versus one single female. Bull fertility, especially for use in AI, has been evaluated heavily or exclusively through semen quality which relies predominantly on sperm motility and morphology, and more recently sperm viability (Barth and Oko, 1989; Koziol and Armstrong, 2018; DeJarnette et al., 2021). However, even among bulls that pass AI quality control analysis in commercial

AI semen service centers, it is not possible to guarantee that bulls will have high fertility (DeJarnette, 2005). Thus, the study of semen characteristics that can better predict bull fertility is necessary. Animal variation is necessary for a test to be considered as a potential fertility marker. Also, any new test must not be correlated with current evaluations of semen quality or must provide a simpler method of evaluation over current analyses (DeJarnette, 2005; Harstine et al., 2018; DeJarnette et al., 2021). In the present study, a greater variation amongst bulls compared to within bull was observed for both DAG1 ($P < 0.01$; 69.4 vs 49.1, respectively) and SERPINA5 ($P < 0.01$; 325.8 vs 285.4, respectively), fulfilling the first characteristics for a potential fertility marker. Further, DAG1 and SERPINA5 were not correlated with TMOT, PROG, or viability (Table 1), fulfilling the second characteristic of a potential novel fertility marker; however, percentage of tail labeled for SERPINA5 was correlated with viability.

The objective of the bovine AI industry is to provide semen of high quality to cattle producers. Semen that passes quality control and is commercially available has met specific thresholds (Harstine et al., 2018; DeJarnette et al., 2021). With that, sperm motility, morphology and viability of commercially available semen are expected to not correlate with field fertility, especially in large samples (DeJarnette et al., 2021). In the present study; however, High-SCR bulls tended to have greater viability compared to Low-SCR bulls ($P = 0.06$; Table 2). Interestingly, it was observed that some Low-SCR bulls had good BL production with no difference from High-SCR bulls (reason for BL fertility separation); Ortega et al. (2018) reported similar findings in which one (out of three) Low-SCR bull had BL similar to High-SCR bulls. Interestingly, Low-SCR/High-BL had decreased mean SCR compared to Low-SCR/Low-BL (Table 3). It is possible that bulls with Low-SCR, but good BL (Low-SCR/High-BL), have sperm transport problems or are more susceptible to the timing of insemination (sperm longevity) or the uterine/oviduct environment compared to Low-SCR bulls with lower BL (Low-SCR/Low-BL), which the problem may be related to fertilization itself rather than sperm transport; this hypothesis is partially explained by the “compensable” and “uncompensable” characteristics of sperm previously reported (Saacke et al., 1994; Saacke, 2008; Amann et al., 2018). However, when low fertility bulls were further divided into Low-SCR/High-BL and Low-SCR/Low-BL, there was no relationship between sperm parameter or proteins with fertility classification (Table 3).

In the present study, it was identified that DAG1 was present on the sperm head; however, DAG1 was not associated with field fertility or field and *in vitro* embryo fertility in which High-SCR and Low-SCR (Table 2) or High-SCR, Low-SCR/High-BL and Low-SCR/Low-BL (Table 3) were not different, respectively. Additionally, DAG1 concentrations between fertility classification groups were almost identical (Tables 2 and 3). Furthermore, DAG1 was not correlated with SCR, CL, or BL. Thus, DAG1 may function to stabilize the acrosomal region as a decapacitating factor, preventing premature acrosomal reaction or formation of the sperm reservoir due to its localization on the sperm head.

The immunolocalization of SERPINA5 on the bovine sperm head was similar to human sperm (Zheng et al., 1994; Elisen et al., 1998); however, bovine sperm also had SERPINA5 on the sperm tail, diverging from human sperm. The protease inhibitory activity of SERPINA5 has been described in multiple body tissues and fluids (España et al., 1989; Ecke et al., 1992; Christensson and Lilja, 1994; Hermans et al., 1994; Zheng et al., 1994; Elisen et al., 1998). The activity and target enzyme of SERPINA5 can be modulated by heparin and other glycosaminoglycans (Kuhn et al., 1990; Pratt and Church, 1992; Ecke et al., 1997). Heparin and glycosaminoglycans are present in the oviduct from oviductal fluid and follicular fluid which has been reported to induce sperm capacitation (Parrish et al., 1985; 1988; Mahmoud and Parrish, 1996; Bergqvist et al., 2007). A positive correlation was observed between SERPINA5 concentration on the sperm head and CL, also, the percentage of sperm tail labeled for SERPINA5 was correlated with sperm viability and CL (Table 1). When the SERPINA5 gene was disrupted in mice, male mice were infertile both *in vitro* and *in vivo* because of morphologically abnormal sperm, lower motility, and lack of sperm-egg binding (Uhrin et al., 2000). Also, men with normal sperm motility with unknown reason for infertility had decreased concentration of SERPINA5 compared to fertile men (Panner Selvam et al., 2019). Controversially, there was no association of SERPINA5 concentration or percentage of tail labeled for SERPINA5 with field fertility or field and *in vitro* embryo fertility (Tables 2 and 3).

The ability of human sperm to bind to human zona pellucida was evaluated in the presence of different concentrations of anti-SERPINA5 or SERPINA5 in the media (Elisen et al., 1998). Interestingly, a lower concentration of anti-SERPINA5 increased the ability of sperm to bind to the zona pellucida; however, the greater the concentration of SERPINA5 in the media the lower the ability of sperm to bind to the zona pellucida (Elisen et al., 1998). Another member of the serine protease inhibitor (SERPIN) family, called glia-derived nexin or protease nexin-1 (SERPINE2), has been reported to be a decapacitating factor in mice (Lu et al., 2011). When sperm were processed for *in vitro* fertilization, the processing may have accelerated sperm capacitation and increased damage to the sperm (Baldi et al., 2020). Thus, it is possible to hypothesize that increased concentrations of SERPINA5 may have provided enough protection to the sperm; and bulls with greater concentration of SERPINA5 on the sperm head, and percentage of tail labeled, had increased CL likely due to resistance to sperm processing (protection against premature capacitation). More investigation is necessary to understand whether SERPINA5 or DAG1 could be used as a fertility marker.

Implications

DAG1 and SERPINA5 proteins that are associated with cell-to-cell interactions were localized on the bovine sperm head, also, SERPINA5 was localized on the sperm tail. Sperm relative concentration for both proteins were correlated to each other and SERPINA5 was correlated with CL. The percentage of sperm tail labeled for SERPINA5 was correlated with CL and sperm viability; however, proteins were not associated with bull field fertility measured by SCR. Thus, SERPINA5 may be related with sperm protection and/or oocyte fertilization while DAG1 may be related to sperm transport or formation of the sperm reservoir in the oviduct.

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Tables

Table 1. Pearson's correlation coefficient (above diagonal) and significance level (below diagonal) between sire conception rate (SCR), total motility (TMOT), progressive motility (PROG), sperm plasma membrane integrity (viability), SERPINA5 mean relative concentration (SERPINA5), percentage of sperm tail positive for SERPINA5 (SERPINA5 Tail), *in vitro* produced embryos cleavage (CL) and blastocyst (BL) rate, and DAG1 mean relative concentration (DAG1).

Correlation/ P-value	SCR	TMOT	PROG	Viability	SERPINA5	SERPINA5 Tail	CL	BL	DAG1
SCR		0.09	0.01	0.36	-0.13	-0.19	-0.08	0.15	-0.08
TMOT	0.69		**0.82**	0.00	0.14	0.15	0.17	0.34	-0.25
PROG	0.95	**< 0.01**		0.06	0.15	-0.07	-0.04	0.22	-0.26
Viability	0.12	0.99	0.79		0.11	**0.44**	0.24	0.15	-0.10
SERPINA5	0.56	0.53	0.50	0.65		0.28	**0.48**	0.11	**0.54**
SERPINA5 Tail	0.39	0.52	0.74	**0.05**	0.21		**0.39**	0.20	0.05
CL	0.73	0.49	0.88	0.35	**0.04**	**0.10**		**0.50**	0.33
BL	0.55	0.15	0.38	0.56	0.66	0.42	**0.03**		0.32
DAG1	0.72	0.25	0.25	0.66	**0.01**	0.81	0.17	0.18	

Table 2. Relationship of sire conception rate (SCR) fertility classification (High-SCR vs Low-SCR) on total motility (TMOT), progressive motility (PROG), sperm plasma membrane integrity (viability), *in vitro* produced embryos cleavage (CL) and blastocyst (BL) rate, SERPINA5 concentration (SERPINA5), percentage of sperm tail positive for SERPINA5 (SERPINA5 Tail), and DAG1 concentration (DAG1).

Variable	High-SCR	Low-SCR	SEM[1]	P-value
SCR, au[2]	3.4	-5.7	0.31	< 0.0001
TMOT, %	52.0	51.3	2.89	0.86
PROG, %	35.7	35.8	2.61	0.99
Viability, %	64.0	57.3	2.39	0.06
CL, %	77.4	78.3	2.39	0.81
BL, %	33.5	31.7	2.18	0.56
SERPINA5, au[2]	52.4	54.2	2.04	0.54
SERPINA5 Tail, %	32.4	35.1	3.23	0.56
DAG1, au[2]	35.6	36.5	1.41	0.66

[1] SEM = Standard error of the mean
[2] au = arbitrary unit

Table 3. Relationship of field (sire conception rate; SCR) and *in vitro* (blastocyst rate; BL) fertility classification (High-SCR, Low-SCR/High-BL, and Low-SCR/Low-BL) on total motility (TMOT), progressive motility (PROG), sperm plasma membrane integrity (viability), *in vitro* produced embryos cleavage rate (CL) and BL, SERPINA5 concentration (SERPINA5), percentage of sperm tail positive for SERPINA5 (SERPINA5 Tail), and DAG1 concentration (DAG1).

Variable	High-SCR	Low-SCR/ High-BL	Low-SCR/ Low-BL	SEM[1]	P-value
SCR, au[2]	3.4[a]	-6.2[b¶]	-4.8[b*]	0.59	< 0.0001
TMOT, %	52.0	49.3	50.0	4.86	0.81
PROG, %	35.6	34.3	32.8	4.38	0.84
Viability, %	64.0	58.8	60.3	3.79	0.32
CL, %	77.5	80.3	73.9	4.23	0.43
BL, %	33.4[a]	35.9[a]	23.9[b]	2.73	0.02
SERPINA5, au[2]	52.4	52.7	56.0	4.16	0.75
SERPINA5 Tail, %	32.6	38.5	32.7	5.60	0.48
DAG1, au[2]	35.6	36.4	36.7	2.60	0.91

[1] SEM = Standard error of the mean
[2] au = arbitrary unit
[a-b] Values within the same row not sharing a common superscript differ $P \leq 0.01$
[*,¶] Values within the same row not sharing a common superscript differ $P \leq 0.08$