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Fertility-associated proteins in male and female reproductive fluids of cattle

G. Killian[1]

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

Abstract

This paper outlines a 25 yr research strategy to better understand how proteins in male and female reproductive fluids influence fertility. Extensive fertility data available from artificial insemination cooperatives were used to identify individual dairy bulls with phenotypes of above and below average fertility. We determined whether the protein composition of reproductive fluids from these bulls differed with fertility. Surgical alteration of bulls to equip them with indwelling vas deferens cannulae enabled recovery of accessory sex gland fluid and cauda epididymal fluid and sperm for *in vitro* studies. Extensive analyses of reproductive fluids from these unique individuals enabled identification of several proteins that were associated with fertility. Methods to cannulate the oviducts of cows were also used to evaluate the composition and function of oviductal fluid. *In vitro* testing of proteins purified from male and female reproductive fluids demonstrated that they facilitated sperm capacitation, fertilization and early embryo development. Collectively these studies pursued a reductionist approach that started with the phenotype of an animal, use of surgical techniques to acquire reproductive fluids, use of analytical tools to define the composition and their relationships to fertility, and ultimately the purified proteins were tested with gametes and embryos to assess function. Using this approach, hypotheses were tested to determine fertility-related functions of proteins in reproductive fluids. The results provide an understanding of how fertility-related proteins contribute to the fertility phenotype of an individual and provide insights into potential applications to improve the fertility of cattle.

Keywords: fertility, oviduct, research strategy, seminal proteins.

Introduction

For researchers who have spent their careers interested in livestock reproduction, the importance of reproductive efficiency cannot be overstated. Production of progeny at a high rate helps to ensure the future profitability and success of a commercial livestock operation.

Reproductive efficiency of an animal population is generally defined as the number of females becoming pregnant or having offspring compared to the total number of females bred or inseminated. The "fertility" of the population is influenced by many factors, including management and environmental factors, and the inherent fertility of the males and females in the population. Because genetics influence the fertility of individuals and how they may respond to management and environmental factors, genetic selection has played a key role in improving the fertility of most species of farm animals.

Veterinary exams for reproductive soundness are an important means to eliminate animals with gross reproductive defects from the breeding population. However, more subtle reproductive deficiencies are often not detected until the sire or dam are put into service, and may manifest themselves as infertility or subfertility. These individuals contribute to a reduction of reproductive efficiency of the herd and should be eliminated to maximize future profitability.

Commercial livestock operations recognize that meticulous recordkeeping is essential to document the fertility of individuals and the reproductive efficiency of the herd. Repeated unsuccessful breeding of a female is justification for culling. Fertility of sires will be reflected in the reproductive success of the females they inseminate. Because male fertility can be based on results from a large population of females, it may be possible to distinguish degrees of fertility expressed as the ratio of the females becoming pregnant as a proportion of those bred.

Dairy cattle provide a unique opportunity for researchers interested in understanding how factors in reproductive fluids contribute to the degrees of fertility seen in reproduction. Our interest in identifying fertility factors in reproductive fluids was initially facilitated by working with "retired" dairy bulls that had been used for artificial insemination and for whom extensive fertility data were available. Bulls used for artificial insemination of dairy cows are selected for the milk production traits they impart to their daughters. Because dairy bulls of high genetic merit are often used to inseminate thousands of cows, reliable fertility data are available. The large sample size makes it possible to detect degrees of fertility that would normally be undetectable in other species.

After the gametes are formed by the testis and ovary, they are exposed to and further modified by the fluid environment in which they exist (Table 1). Sperm passing through the epididymis are known to acquire the

[1]Corresponding author: garykillian@gmail.com
Phone: +1(575)636-3151
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ability to fertilize an egg, and undergo further modifications upon exposure to accessory sex gland fluid. Inseminated sperm are also modified by the environment of the female reproductive tract. Ovulated ova are matured as they pass through the oviduct and made receptive to sperm. And the oviduct environment is also important in facilitating development of the zygote. While there is little doubt that the fluid environment to which the gametes and early embryo are exposed is beneficial, the way that reproductive fluids influence fertility is not well understood. The objective of this paper is to share experimental approaches we have used on dairy cattle to better understand how reproductive fluids contribute to fertility of bulls and cows. The purpose is to outline a 25 yr research strategy for attacking a research problem, and not necessarily to provide a detailed account of methods and results of studies that have been undertaken on the topic.

Table 1. Summary of fluids to which gametes and embryos are exposed in the male and female reproductive tract. Notably, sperm are exposed to all types of fluid environments, while oviductal fluid is a common environment for sperm, ova, zygotes and embryos. Sperm must be adapted to survive in a changing environment, while oviduct fluid must provide a suitable environment for all cell types in order for reproductive success.

	Sperm	Ova	Zygote	Embryo
Testicular fluid	X			
Epididymal Fluid	X			
Accessory sex gland fluid	X			
Follicular fluid	X	X		
Oviductal fluid	X	X	X	X
Uterine fluid	X			X

Discussion

Post testicular influence of male reproductive fluids on sperm fertility

Following formation in the testis, sperm are carried into epididymis via the efferent ducts in testicular effluent. As they traverse the epididymis they undergo further modification and are transformed both morphologically and functionally. Based on early studies with rodents, it was known that sperm recovered from the cauda epididymidis could be used for fertile *in vivo* inseminations or *in vitro* fertilization. In an early study with bulls the question asked was whether sperm entering and leaving the epididymis were similarly fertile. In other words, does the epididymal environment influence bull sperm fertility? Using surgical techniques developed for bulls (Amann *et al*., 1963, 1970; Voglmayr *et al*., 1972), sperm were recovered from the rete testis and vasa deferentia of bulls equipped with indwelling catheters, and then artificially inseminated into cows in estrus (Amann and Griel, 1974). Cows inseminated with sperm recovered from the vas deferens catheters became pregnant while those inseminated with sperm from the rete testis did not. These studies demonstrated that bull sperm were altered during epididymal passage to become fertile and capable of producing pregnancy.

Do bull accessory sex gland secretions influence sperm fertility?

While it is evident that the epididymis transforms infertile testicular sperm into sperm which are fertile, further modifications of sperm may be possible when the epididymal effluent is mixed with fluid from the accessory sex glands at ejaculation (Yanagimachi, 1994). Ejaculated bull sperm are the final natural product which is either inseminated at natural mating or are processed for artificial insemination. As such, ejaculated sperm represent the sum total of all factors contributed by the male that influence the level of fertility sperm possess. Contributions to fertility of sperm occur during formation and genetic programming in the testis, during epididymal transit and upon mixing with accessory sex gland fluid at ejaculation. Because the process is linear, each successive step is dependent on the successful completion of the prior step. While it is likely that the process as a whole has some fail-safe measures to compensate for sub-optimal performance of a prior step, these will not likely be able to correct for major defects. Considering that addition of accessory sex gland fluid to sperm at ejaculation is the last step in the process and that cauda epididymal sperm have been shown to be fertile, one would not expect that addition of accessory sex gland fluid would have a profound effect on sperm fertility.

Nevertheless, we were interested in testing in a controlled experiment whether the fertility of epididymal sperm was altered by accessory sex gland fluid. Influence of accessory sex gland secretions on sperm has been described for a variety of species as both beneficial and harmful to the fertility of sperm (Chang, 1957; Gaur and Talwar, 1973, 1975; Reddy *et al*., 1979; Corteel, 1980; Sadowski and Rogers, 1985; Shivaji and Bhargava, 1987). In general, these studies evaluated the "fertility" of sperm with and without exposure to seminal plasma. One study comparing non-return rates of cauda epididymal sperm acquired from 4 bulls at slaughter with their historical non-return rates using frozen semen showed that the rates were similar (Igboeli and Foote, 1968). Although collectively, these

reports suggested either no effect or that beneficial and detrimental fertility "factors" were present in seminal plasma, the factors involved were not identified. Difficulties in interpreting the contradictory results of previous studies were that the limited sample size made detection of small differences difficult or impossible, and the inherent fertility of the males used in the studies was unknown.

In an attempt to determine unequivocally whether accessory sex gland fluid influenced the fertility of sperm we first validated an experimental system that would enable comparing the fertility of differently treated sperm from a bull (Henault and Killian, 1995). Ideally, to assess fertility, it would have been best to inseminate the treated sperm into populations of cows, but the large numbers of cows necessary to detect small differences and the cost of such a study were prohibitive. Alternatively, we made use of sperm penetration assays using zona-free oocytes which make it possible to observe multiple sperm penetrations and pronuclear development within the same oocyte. This assay was first developed using zona-free hamster oocytes to assess the fertility of sperm from humans (Yanagimachi *et al*., 1976; Yanagimachi, 1984) and other species (Bousquet and Brackett, 1982; Parrish and Foote, 1985; Rogers, 1986; Berger and Horton, 1988). Notably, Davis *et al*. (1987) increased the sensitivity of the assay by comparing penetration rates in a single hamster oocyte by fluorescently tagging sperm from different bulls. He was able to demonstrate that the penetration rates were correlated with the relative fertility of the bull based on artificial insemination data from the field. One adaptation that we made to the assay was to validate the use of zona-free bovine oocytes (Henault and Killian, 1995) rather than oocytes from hamsters. When this assay was used in 16 competitive pairings to determine sperm penetration rates of frozen sperm from bulls of above versus below average fertility, 13 of the 16 pairings (81%) favored above average fertility sperm.

With the assay validated, we used it to evaluate the penetration rates of cauda epididymal sperm compared to that of cauda sperm exposed to accessory gland fluid from the same bull. Repeated collection of cauda epididymal sperm and fluid, and accessory sex gland fluid from the same bull was made possible by surgically equipping the bull with indwelling catheters in the vasa deferentia (Henault *et al*., 1995). When these bulls were subjected to semen collection, cauda epididymal effluent was collected in vials attached to the exterior of the scrotum, and accessory sex gland fluid was recovered from the artificial vagina (Fig. 1).

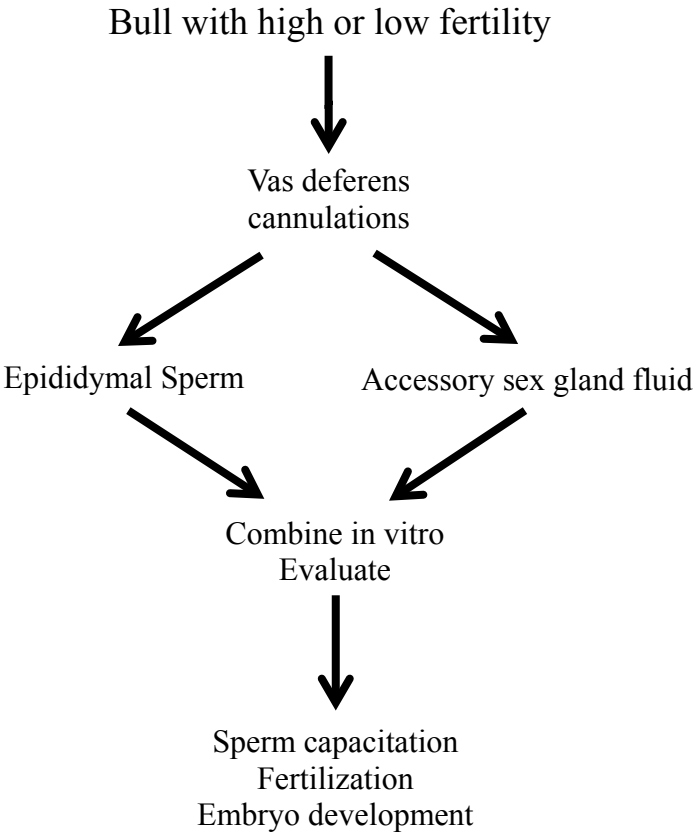

Figure 1. Schematic outline of approaches used to recover bull reproductive fluids for composition analyses and functional in vitro testing.

Dairy bulls used commercially for artificial insemination are an ideal animal model for studies of male fertility because their fertility data are often based on results of thousands of inseminations. With a large sample size, it is possible to detect smaller differences in male fertility than possible with any other species. Using surgically-altered dairy bulls of known fertility, the first question we set out to answer was whether accessory sex gland fluid was able to influence the fertility of sperm. The experiments were designed to test the effect of mixing accessory sex gland fluid from a lower fertility bull with cauda epidiymal sperm from a higher fertility bull and vice versa. For each assay, cauda sperm from a bull were mixed with either his own accessory sex gland fluid or accessory sex gland fluid from a bull of contrasting fertility (Fig. 2). Sperm in each treatment were fluorescently tagged with either rhodamine (TRITC) or fluorescein (FITC) to identify the treatment of the sperm after entering the oocyte. Following heterologous inseminations for each comparison, the number of sperm TRITC and FITC within an oocyte were counted in penetration assays involving numerous zona free-bovine oocytes. Treatments having the greatest number of sperm penetrating oocytes were considered to have a fertility advantage. These studies showed that the fertility of sperm from lower fertility bulls could be increased after exposure to accessory sex fluid from higher fertility males (Henault *et al*., 1995). Sperm from higher fertility bulls were also negatively affected by seminal plasma from lower fertility bulls (Fig. 2). Based on these experiments we concluded that accessory sex gland fluid did indeed contain factors which influenced the fertility of sperm. In a similar study comparing the effect of seminal plasma from intact bulls of contrasting fertilities on ejaculated sperm, it was observed that penetrations of sperm from low fertility bulls were generally improved when mixed with seminal plasma from a higher fertility bull (Henault and Killian, 1996).

Mixing Experiments

Bull Source	High fertility Accessory fluid	Low fertility Accessory fluid
High fertility Epididymal sperm	Homologous >	Heterologous
Low fertility Epididymal sperm	Heterologous >	Homologous

Figure 2. A matrix summarizing how cauda epididymal sperm collected from high or low fertility bulls equipped with vas deferens cannulae were mixed with either the bull's own accessory sex gland fluid (homologous) or accessory sex gland fluid from a bull of contrasting fertility (heterologous). After mixing, homologous and heterologous treated sperm from each bull were capacitated in vitro and used in competitive penetration assays using zona-free bovine ova to determine which sperm type had a penetration advantage. The results showed that sperm from high or low fertility bulls treated with accessory sex gland fluid from high fertility bulls had more penetrations.

Identification of the factors in male reproductive fluids associated with fertility

In another phase of the study we sought to identify fertility associated factors in male reproductive fluids. We analyzed the protein composition of seminal plasma from 35 dairy bulls having a wide range of documented fertilities (Killian *et al*., 1993). The bull samples were provided by three different artificial insemination centers, and fertility data for each bull were expressed as a percentage point deviation of the average fertility of all bulls at that center. To ensure that the fertility data for each bull were reliable they were based on breeding records for at least 1000 inseminations. For the 35 bulls supplying seminal plasma for analysis, the percentage point deviations ranged from -6.6 to +4.5, with average fertility designated as 0.0.

Using two-dimensional polyacrylamide gel

electrophoresis we created "maps" of the proteins present in each of the samples (Killian *et al*., 1993). These maps (2-D gels) were examined visually and analyzed by video densitometry to determine if differences existed in the amounts of individual proteins present among bulls that could be associated with fertility. We discovered that seminal fluid contained two proteins that were more prevalent in bulls of higher fertility (26 kDa, pi 6.2; 55 kDa, pI 4.5) and two proteins that were more prevalent in bulls of lower fertility (16 kDa, pi 4.1; 16 kDa, pi 6.7). We quantified by video densitometry the relative amounts of the four fertility proteins present in each sample and developed a multilinear regression equation to describe the relationship between the presence of the fertility associated proteins and bull fertility (Killian *et al*., 1993). This equation was used to estimate the fertility of bulls based on the relative amounts of the fertility-associated proteins in their seminal fluid. For the 35 bulls used in this study and another group of 35 bulls tested later, the correlation coefficient between the predicted fertility of a bull using this equation and actual bull fertility from field data was 0.89. This high correlation between predicted and actual fertilities strongly supported the conclusion that these proteins are associated with bull fertility (Killian *et al*., 1993).

In the early 1990's when we initiated studies to determine the presence of fertility-related proteins in reproductive fluids, the highly automated and sophisticated science of proteomic analyses that we know today was in its infancy. Because this technology was not available, our early efforts to identify the fertility-related proteins in seminal plasma were painfully slow and tedious. For example, to identify the 55 kDa protein, spots were cut from thirty-six 2-D gels of seminal plasma in order to provide enough protein for processing and protein sequencing, and ultimately amino acid sequence comparisons. Nevertheless, we were able to identify the 55 kDa protein that was more prevalent in high fertility bulls as osteopontin (Cancel *et al*., 1997). Using a similar approach, we also identified the 26 kDa protein, more prevalent in seminal plasma of higher fertility bulls, as lipocalin-type prostaglandin D synthase (Gerena *et al*., 1998). In both cases, the identity of the proteins determined by amino acid sequence analysis was confirmed with antibodies specific for the proteins. In addition to confirming the identity of the proteins in western blots of 2-D gels, the protein-specific antibodies proved quite valuable for determining the tissue origins of the proteins as well as tracking their association with the gametes or embryo.

Once the technology for proteomic analyses became more widely available and less costly, we were able to utilize it for extensive proteome analyses of bull accessory sex gland fluid (Moura *et al*., 2006, 2007a) and epididymal fluid (Moura *et al*., 2010). This technology greatly expanded the capacity for identifying proteins in biological fluids since the amounts of a protein and time needed for analysis were greatly reduced. Using 2-dimensional electrophoresis, computerized image analysis, and tandem mass spectrometry we compiled protein profiles of the accessory sex gland secretions of high-use dairy bulls and related them to the fertility phenotype (Moura *et al*., 2006). We determined that bovine seminal plasma protein-BSP 30 kDa, phospholipase A2 and osteopontin were more prevalent in high fertility bulls, whereas amounts of spermadhesin Z13 were inversely related to fertility.

In another study we assessed whether there were meaningful associations between the compositions of accessory sex gland fluid from high and low fertility bulls and the *in vitro* ability of treated cauda epididymal sperm to penetrate oocytes (Moura *et al*., 2007b). These studies suggested that the penetration-enhancing effect of accessory sex glad fluid from higher fertility bulls on cauda epididymal sperm from low fertility bulls was associated with the accessory sex gland fluid containing relatively more BSP A1/A2 and A3, BSP 30 kDa, clusterin, albumin, phospholipase A2 and osteopontin, and less nucleobindin than that from lower fertility bulls. Notably, three of the accessory sex gland proteins associated with the beneficial outcome of the oocyte penetration assay (BSP30 kDa, phospholipase A2, and osteopontin) were identical to those related to higher fertility bulls measured in the field (Moura *et al*., 2006). This observation justifies the use of *in vitro* assessments such as oocyte penetration, *in vitro* fertilization and embryo production for evaluating the functional effects of fertility associated proteins on the gametes, fertilization and early embryo development.

The proteome of cauda epididymal fluid collected from bull vas deferens catheters was also determined (Moura *et al*., 2010). Because more than 20% of protein detected by densitometry in the 2-D maps was albumin, a second albumin-depleted sample was also run for each sample to detect protein spots that may be masked by the albumin. Although there were more than 130 spots detected in the albumin depleted gels, many were isoforms of the same protein. Nevertheless, numerous distinct proteins were identified in both types of gels. Prostaglandin D synthase was identified in fluid depleted of albumin, confirming that the epididymis is the origin of this protein that is more prevalent in seminal plasma of higher fertility bulls (Killian *et al*., 1993; Gerena *et al*., 1998, 2000). While this study did not attempt to relate the presence and amount of the proteins detected to bull fertility, given what is known about the functions of some of the proteins it is likely that they influence sperm function. Proteins detected have the potential to play a role in sperm membrane remodeling, transport of lipophilic substances, protection of sperm against oxidative reactions and immune attack, transport of ions and prevention of premature acrosome reaction (Moura, *et al*., 2010).

Influence of bovine oviductal fluid on gamete function and fertilization

Once sperm leave the male reproductive tract and are inseminated into the female they are further modified by secretions of the female reproductive tract as they are prepared or "capacitated" for fertilization. We recently published a paper (Killian, 2011) reviewing evidence for a role of oviductal secretions of farm animals in modulating sperm function. Other review papers on oviduct function (Nancarrow and Hill, 1995; Boquest *et al*., 1999; Killian, 2004; Rodriguez-Martinez, 2007; Suarez, 2008; Aviles *et al*., 2010. Holt and Fazeli, 2010) have compiled information for a variety of species documenting the importance of the oviduct environment, not only in modulating sperm function, but providing an ideal environment for the final maturation of the ovulated oocyte, fertilization and early embryo development. Those interested in detailed information about oviduct function are encouraged to read these reviews.

A practical motivation for studying the oviduct of cattle comes from commercial interest in improving the efficiency of *in vitro* embryo production. Because the oviduct provides the environment for sperm capacitation, fertilization and early embryo development (Table 1), learning how to mimic this environment *in vitro* could improve the efficiency of embryo production.

Our research interest in the oviduct focused on how the oviduct secretions influenced sperm capacitation and fertilization, although this was later expanded to include early embryo development. The research strategy involved recovering oviduct secretions from indwelling cannulas placed in cow oviducts (Kavanaugh and Killian, 1988), so that they could be used for *in vitro* assays of gamete function and embryo development, and compositional analyses. Because the oviduct catheters provided daily samples throughout the estrous cycle, from both the ampullar and isthmic regions (Kavanaugh *et al*., 1992), stage of cycle and region of the oviduct effects of the fluid could be studied (Killian, 2011).

In vitro studies by us and others (see reviews Killian, 2004; Rodriguez-Martinez, 2007; Killian, 2011) determined that oviduct secretions had a positive effect on sperm capacitation and the acrosome reaction, oxygen uptake, sperm motility and survival, as well as rates of fertilization and early embryo development. Not surprisingly, the oviduct region (Anderson and Killian, 1994; Grippo *et al*., 1995; Way *et al*., 1997) and stage of the estrous cycle (McNutt and Killian, 1991; McNutt *et al*., 1994) when oviduct fluid was obtained affected the magnitude of the endpoint measured. Taken together, these studies confirmed in some detail that the oviduct influences the outcome of gamete function, fertilization and early embryo development.

Composition of oviduct fluid

To better understand how the oviduct influenced gamete function and fertilization, defining the composition of the secretions has received interest for several decades. Compositional analyses by our lab employed several different approaches. Early on, we used one dimensional gel electrophoresis to determine the electrophoretic profile of proteins in oviduct fluid (Gerena and Killian, 1990). By comparing protein profiles of fluid collected at different stages of the estrous cycle it was possible to determine proteins prevalent around estrus versus the luteal phase. Moreover, by comparing the oviduct fluid protein profile to that of staged serum from the same cow, and follicular fluid, it was possible to infer which proteins were synthesized by the oviduct versus those that were likely derived from serum transudate. Because a ~90 kDa oviduct fluid protein stained positive for carbohydrate and only appeared in fluid collected 3-4 days around estrus, we speculated that this oviduct-specific glycoprotein might be important for sperm capacitation, fertilization and or early embryo development. Based on numerous studies it is clear that oviductal fluid composition is complex and contains numerous amino acids, proteins, enzymes, simple and complex carbohydrates, ions, lipids, and phospholipids (see reviews, Nancarrow and Hill, 1995; Buhi *et al*., 2000; Killian, 2004, 2011; Rodriguez-Martinez, 2007; Aviles *et al*., 2010). Although for many of the oviduct fluid components it is possible to speculate on how they may influence events occurring near fertilization, few have been tested in controlled experiments. In our studies, we focused efforts to assess functional effects of proteins identified in male and female reproductive fluids on sperm capacitation, fertilization and early embryo development. The components that we found in cow oviduct of interest for further evaluation were oviduct-specific glycoprotein (Gerera and Killian, 1990), norepinephrine (Way *et al*., 2001), osteopontin and integrins (Gabler *et al*., 2003), haptoglobin (Lavery *et al*., 2004) and FAS ligand (Berqvist *et al*., 2005).

Localization of components on sperm, ova and embryos

It is not unreasonable to speculate that if components in seminal plasma or oviduct fluid affect the function of a gamete or embryo that they would associate with or interact with the gamete or embryo. It is possible to localize proteins that are present on or in cells using fluorescent antibody technology, or radiography using labeled proteins. For more common proteins, antibodies may be available commercially, but it is necessary to prepare antibodies when they are not commercially available or available from colleagues. Polyclonal antibody preparation requires a highly purified protein for immunization so that the resulting

antibodies will be monospecific. Regardless of the source of the antibody, it is prudent to establish its specificity for the protein in question. Failure to do so can result in erroneous conclusions.

Using antibodies prepared against bovine oviduct specific glycoprotein, osteopontin, prostaglandin D synthase and BSP-A3, we have determined if these proteins were present on sperm, ova or reproductive organs using western blots, immunocytochemistry and confocal microscopy. Staining for osteopontin has been detected on the post equatorial segment, acrosomal cap and to a lesser extent on the midpiece of ejaculated bull sperm (Erikson *et al*., 2007b, Souza *et al*., 2008). Although oviductal fluid contains osteopontin, and the distribution of osteopontin on sperm membranes changes after incubation in oviduct fluid (Souza *et al*., 2008), the total amount of osteopontin associated with the sperm remained unchanged (Erikson *et al*., 2007b). Prostaglandin D synthase has been localized to the apical ridge of the acrosome of ejaculated sperm (Gerena *et al*., 2000, Souza *et al*., 2011) and the acrosome of epididymal sperm (Souza *et al*., 2011). A general distribution of BSP-A3 was evident on ejaculated but not epididymal sperm, although somewhat more staining was seen on the acrosome and midpiece (Souza *et al*., 2011)

Proteins originating in the oviduct have also been shown to be present on sperm and ova. It has been shown that bull sperm membranes acquire oviduct specific glycoprotein and other proteins after incubation in oviduct fluid (McNutt *et al*., 1992; King and Killian, 1994; Rodriguez and Killian, 1998). In early studies we showed that the zona pellucida of ova recovered from follicles acquired six proteins from oviduct fluid (Staros *et al*., 1998) including the oviduct specific glycoprotein (Wegner and Killian, 1991). More recently, we showed that the zona pellucida (Souza *et al*., 2008, Gonçalves *et al*., 2008b) and oolema of ova incubated in oviduct fluid acquired osteopontin (Souza *et al*., 2008). In addition, it has been shown using western blots and immunocytochemistry that the zona pellucida of ova incubated in non-luteal oviductal fluid also acquired prostaglandin D synthase and oviduct specific glycoprotein (Gonçalves *et al*., 2008b).

Influence of proteins in seminal plasma and oviduct fluid on IVF/IVP results

A significant advantage for a researcher studying cattle reproduction is that technology exists for *in vitro* fertilization and embryo production which makes it possible to test the effects of fluid components in controlled studies. In order to gain insights into how the fertility-related proteins detected in bull and cow reproductive fluids affect fertility, *in vitro* experiments were undertaken to test the effects of purified osteopontin, prostaglandin D synthase and oviduct specific glycoprotein on events associated with gamete function, fertilization and early embryo development. We have used both purified proteins and mono-specific antibodies against the proteins in order to assess their effect on functional outcomes measurable *in vitro* such as sperm capacitation, rates of fertilization, cleavage and embryo development.

Bull sperm incubated with osteopontin purified from milk undergo capacitation (Erikson *et al*., 2007a; Monaco *et al*., 2009), although at a somewhat lesser rate than control sperm capacitated with heparin. Although incubation in osteopontin was associated with an increase in viability and rise in intracellular calcium, it was not accompanied by an in mitochondrial activity (Erikson *et al*., 2007a). *In vitro* fertilization experiments showed that sperm treated with an antibody to osteopontin fertilized fewer oocytes and had an increased incidence of polyspermy than sperm treated with control medium (Erikson *et al*., 2007b, Gonçalves *et al*., 2007). Use of bovine sperm frozen with milk osteopontin had greater rates of fertilization, cleavage and embryo development than sperm frozen without osteopontin (Gonçalves *et al*., 2008b). Pretreatment of *in vitro*-matured bovine oocytes with purified milk osteopontin, or inclusion of osteopontin in the fertilization medium improved rates of *in vitro* sperm binding to zona, fertilization, and embryo development *in vitro* (Gonçalves *et al*., 2008a, Monaco *et al*., 2009), whereas treatment of oocytes with antibody to osteopontin decreased the rates (Gonçalves *et al*., 2008b). These results suggest that osteopontin found in the oviduct and ejaculated semen functions to influence sperm capacitation and oviductal oocytes resulting in decreased rates of polyspermy and improved rates of fertilization and embryo development.

To further explore the mechanisms mediating the effects of osteopontin on sperm-egg binding and fertilization, we explored the importance of cell signaling and cell adhesion by binding to integrins through the arginine-glycine-aspartic acid (RGD) sequence (Gonçalves *et al*., 2009). Compared to untreated controls or those incubated with RGE peptides, bovine sperm and ova pretreated with the RGD sequence had reduced the rates of sperm-egg binding and fertilization. This suggested that the RGD peptide occupied binding sites on sperm and ova to which osteopontin would have bound, thereby preventing the beneficial effects of osteopontin. Treatment of ova or sperm with antibodies to integrins-alphaV and -alpha5 also reduced rates of sperm-egg binding and fertilization rates. We concluded that the mechanism by which osteopontin influenced sperm-egg binding and fertilization involved integrins binding to the RGD sequence of osteopontin.

Fewer functional studies have tested the potential role of prostaglandin D synthase *in vitro* fertilization assays. However, we have shown that pretreatment of bovine sperm and/or oocytes with antibody to lipocalin-type prostaglandin D synthase

inhibits *in vitro* fertilization, increases sperm-oocyte binding and significantly decreases rates of embryo development but not rates of cleavage (Gonçalves *et al*., 2008b).

Treatment of sperm with purified oviduct specific glycoprotein significantly increased rates of sperm capacitation, *in vitro* fertilization and embryo development of cattle and pigs (King *et al*., 1994; Martus *et al*., 1998; Kouba *et al*., 2000; Buhi, 2002; McCauley *et al*., 2003). It was also shown that exposure of either the porcine ovum or the sperm to oviduct specific glycoprotein reduced rates of polyspermy without affecting rates of penetration (McCauley *et al*., 2003).

Taken together, *in vitro* assays testing the functional effects of specific proteins present in male and female reproductive fluids have provided evidence for how they may function *in vivo* to influence fertility.

Concluding thoughts

Significant technological advances are being made every few years that greatly influence the ability of scientists to gather detailed biological data. For some scientists, it is tempting to use these powerful tools simply as a means to create large data sets, because the tools enable them to do so. However, these data sets are only useful if they are connected to answering questions or testing hypotheses.

Our research has focused on answering questions related to the success of cattle reproduction. We made use of fertility information available from commercial operations in order to identify individual bulls that may have distinguishing characteristics in reproductive fluids which could be associated with fertility. Surgical alteration of individuals enabled segregation of fluids and sperm for *in vitro* studies. Extensive analyses of reproductive fluids from these unique individuals enabled identification of several proteins that were likely related to fertility. *In vitro* testing of these purified proteins confirmed that they are capable of facilitating sperm capacitation, fertilization and early embryo development. Collectively these studies pursued a reductionist approach that started with the phenotype of an animal, made use of surgical approaches to recover reproductive fluids, used analytical tools to define the fluid composition and its relationship to fertility, and ultimately we tested purified components *in vitro* to assess if they affected the function of gametes and embryos. This approach documented specific functions of proteins found in reproductive fluids on gametes, fertilization and embryo development and provided insights into how they contribute to fertility phenotypes of animals.

Unfinished business

Based on our experience, dairy cattle have proven a valuable experimental resource for studying sub-optimal fertility. Knowledge gained from using them as an animal model is clearly of scientific value for understanding factors that influence fertility in other species. However, the practical goal of research involving livestock should be to use the information gained for improving fertility in the field. Does addition of these proteins to semen extenders or *in vitro* embryo production media improve the fertility result? Are the proteins of diagnostic value in predicting the fertility of untested sires? Is it possible to develop simple and reliable diagnostic tests for assessing fertility? Only future studies to develop related technology and assess it in the field will determine the practical value of this research.

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