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Effects of Accessory Sex Gland Fluid on Viability, Capacitation, and the Acrosome Reaction of Cauda Epididymal Bull Spermatozoa

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ABSTRACT: The effect of accessory sex gland fluid (AGF) on viability and acrosomal integrity of spermatozoa was examined with cauda epididymal spermatozoa and AGF from the same Holstein bull (n = 6). Surgical cannulation of the vasa deferentia enabled the separate collection of cauda epididymal effluent and AGF from each bull. Cauda epididymal effluent was incubated with either AGF collected from the same bull or medium alone. Following coincubation, spermatozoa (5×10^7 sperm/mL) were incubated in medium alone or under capacitating conditions (10 μg/mL heparin) for 16 hours. Every 2 hours, an aliquot of spermatozoa was exposed to lysophosphatidylcholine (100 μg/mL) to induce the acrosome reaction in capacitated spermatozoa. Sperm motility decreased over time regardless of treatment. Overall, spermatozoa incubated in AGF had fewer acrosome-intact live spermatozoa than did those not incubated in AGF. Viability was significantly ($P < .05$) compromised over time when spermatozoa were exposed to AGF, compared with those not preincubated in AGF. Significantly more ($P < .05$) acrosome-reacted live spermatozoa were seen following exposure to heparin and lysophosphatidylcholine when spermatozoa were not preincubated in AGF. We conclude that exposure of spermatozoa to AGF accelerates cell death and that rapid removal of spermatozoa from seminal plasma is critical for maximal viability.

Key words: Bovine, epididymis, vas deferens, semen.

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At ejaculation, spermatozoa previously stored in the cauda epididymidis are mixed with secretions from the accessory sex glands. These glands provide spermatozoa with nutrients and various factors that alter the sperm surface (Polakoski et al, 1982). Studies examining the influence of seminal plasma on sperm function have been contradictory, with reports suggesting both beneficial (Brackett et al, 1978; Dott et al, 1979; Corteel, 1980; Miller et al, 1990; Cross, 1993; Braun et al, 1994) and detrimental effects (Baas et al, 1983; Okamura and Sugita, 1983; Han et al, 1990; Dostalova et al, 1994a,b).

Seminal plasma components alter the sperm plasma membrane and have been implicated in preventing, as well as facilitating, capacitation and the acrosome reaction. Decapacitation factors have been found in seminal plasma (Chang, 1957; Dostalova et al, 1994a,b), as have heparin-binding proteins that adhere to the plasma membrane and are thought to be necessary for heparin-induced capacitation in bull spermatozoa (Lee et al, 1985; Miller et al, 1990; Nass et al, 1990; Thérien et al, 1995, 1997; Lane et al, 1999). Processed ejaculated spermatozoa are typically separated from seminal plasma and washed or extended in cryopreservation medium shortly after collection. This decreases the time that spermatozoa are exposed to components from the accessory sex glands, which may reduce harmful effects of AGF but may dilute potentially beneficial factors.

Although it is possible to capacitate cauda epididymal and ejaculated spermatozoa (Handrow et al, 1982; Lee at al, 1985), few comparisons have been made between the capacitation profiles of cauda epididymal and ejaculated spermatozoa from the same animal. In the following experiments, we have used a vas deferens cannulation procedure that enables collection of cauda epididymal spermatozoa (CES) and AGF from the same bull. By combining the cauda epididymal effluent from the cannulae with AGF from the same bull, we were able to compare spermatozoa from simulated ejaculation with CES that have not been exposed to AGF.

To better understand the effects of AGF on sperm function, we determined whether sperm viability was compromised by exposure to AGF and whether capacitation profiles and the ability to undergo the acrosome reaction were different for CES and spermatozoa from simulated ejaculation following in vitro capacitation with heparin.

Materials and Methods

Semen Collection

The Institutional Animal Care and Use Committee of the Pennsylvania State University approved all procedures involving the

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bulls used in this study. Holstein bulls (*Bos taurus*) of average fertility were production sires culled from breeding cooperatives in the northeastern United States. Vasa deferentia of 6 bulls were bilaterally cannulated to collect cauda epididymal effluent independent of AGF (Henault et al, 1995). Samples were collected several times during the 7 to 14 days after cannulation to ensure that the surgery did not compromise sample quality. In addition, the AGF was examined visually to determine that it was free of spermatozoa. Sample collection routinely involved a period of sexual preparation of the bull, which led to ejaculation. At ejaculation, AGF was collected from each bull with an artificial vagina, and cauda epididymal effluent was harvested via indwelling cannulae emptying into sterile vials.

Sperm Incubation and Preparation

One half of the volume of the cauda epididymal effluent collected from each bull was incubated with one half of the volume of the AGF collected from the same bull. The remaining epididymal effluent was incubated in modified Tyrode's medium (MTM; Parrish et al, 1988) equal to the incubation volume of the AGF. After incubation of each preparation for 15 minutes at 39°C, spermatozoa were washed twice in MTM (500 g, 10 minutes) and incubated in MTM (negative control) or in MTM with 10 μg/mL heparin (capacitating medium) for up to 16 hours at 39°C in 5% CO_2/air (5×10^7 spermatozoa/mL). At 0 hours and every 2 hours thereafter, an aliquot of spermatozoa in MTM or capacitating medium was exposed to 100 μg/mL lysophosphatidylcholine (LPC) to induce the acrosome reaction in capacitated sperm (McNutt and Killian, 1991). Briefly, 100 μL of sperm suspension was incubated with LPC and 50 mg/mL bovine serum albumin (BSA) for 10 minutes at 39°C. A second sample from each treatment was incubated for 10 minutes with BSA alone and served as a control for LPC.

After 10 minutes, sperm motility was evaluated subjectively by preparing a wet mount of the spermatozoa and estimating the percentage of motile spermatozoa. Additionally, a 5-μL sample was stained with 5 μL of eosin B-aniline blue, smeared onto a slide, and dried under a warm air current. Stained smears were evaluated for percentage of intact live, intact dead, acrosome-reacted live, and acrosome-reacted dead spermatozoa (Way et al, 1995). Stained slide preparations were evaluated by differential interference-contrast microscopy (Aalseth and Saacke, 1986). Live spermatozoa exclude the stain and appear white, whereas dead spermatozoa take up the stain. Spermatozoa were considered to be acrosome intact if a distinct apical ridge was observed. Because LPC is a fusogenic lipid that will induce the acrosome reaction in capacitated spermatozoa (Parrish et al, 1988), the acrosome-reacted live population of spermatozoa in each treatment were considered to represent functionally capacitated spermatozoa.

Statistical Analysis

Spermatozoa from 6 bulls were evaluated with a mixed model (SAS, 1985). Data from 6 bulls were analyzed by repeated measures analysis for each population of spermatozoa (intact live, intact dead, acrosome-reacted live, and acrosome-reacted dead). Comparisons were made with least squares and Bonferroni means comparisons, with a significance level of $P < .05$.

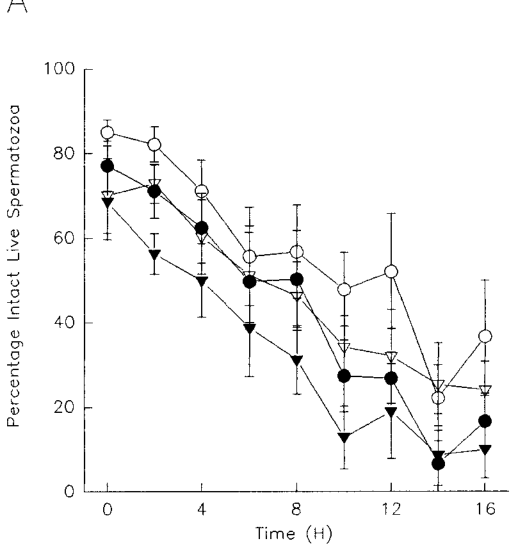
Results

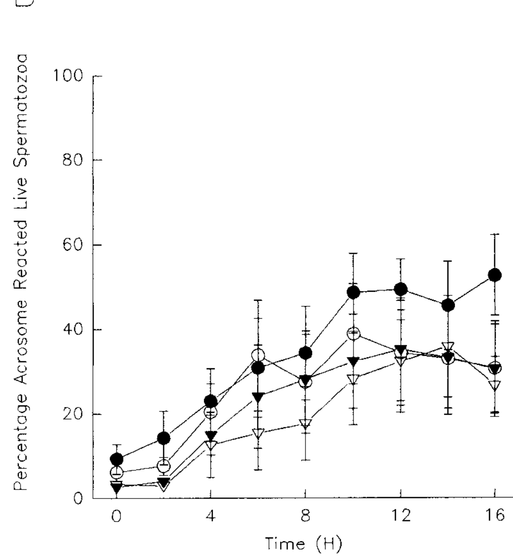
The percentage of intact live spermatozoa decreased over time for both CES and CES incubated in AGF. Regardless of treatment with heparin or LPC, spermatozoa incubated in AGF had fewer intact, live spermatozoa than did those not incubated in AGF ($P < .05$, Figure 1A). Overall, there were fewer intact, live spermatozoa in LPC-treated samples. Motility decreased over time for all treatments (67–75% at 0 hours to 30–50% at 16 hours), as did the percentage of intact, dead spermatozoa (data not shown), and there was a significant interaction between time and AGF ($P < .05$). No significant differences were observed in the intact, dead sperm population for any treatments.

By evaluating the shift in sperm populations over time, differences in viability between CES and CES exposed to AGF were observed. At 0 hours, there were significantly more dead spermatozoa in samples preincubated in AGF (Figure 2). By 16 hours, the acrosome-reacted dead population made up almost half of the population of spermatozoa incubated in AGF, whereas CES incubated in MTM had approximately equal amounts of intact-live, acrosome-reacted live, and acrosome-reacted dead spermatozoa (Figure 2). Clearly, viability was compromised when CES were incubated in AGF.

The acrosome-reacted population of spermatozoa increased significantly over time in the acrosome-reacted live and acrosome-reacted dead populations ($P < .05$, Figure 1B and C). However, the percentage of acrosome-reacted live and acrosome-reacted dead spermatozoa for samples preincubated in AGF differed greatly from the acrosome reaction profile of spermatozoa preincubated in MTM. The percentage of acrosome-reacted live spermatozoa after 16 hours of incubation was similar for CES and CES incubated in AGF. However, the percentage of acrosome-reacted live CES increased more rapidly and was maintained for a longer period than for the spermatozoa incubated in AGF (Figure 1B). Although not statistically significant ($P = .0563$), there were more acrosome-reacted live CES in preparations that were preincubated in MTM than in those that were preincubated in AGF (Figure 1B). In addition, there were significantly more acrosome-reacted dead spermatozoa in samples incubated in AGF than in those incubated in MTM ($P < .05$, Figure 1C). This was evident as early as 0 hours.

Under capacitating conditions and subsequent exposure to LPC, CES had significantly more acrosome-reacted live spermatozoa than CES incubated in AGF ($P < .05$). This was true for all time points. When compared with the controls, treatment with LPC of heparin-capacitated CES and CES with AGF increased the percentage of acrosome-reacted live spermatozoa without significantly affecting the percentage of acrosome-reacted dead sper-





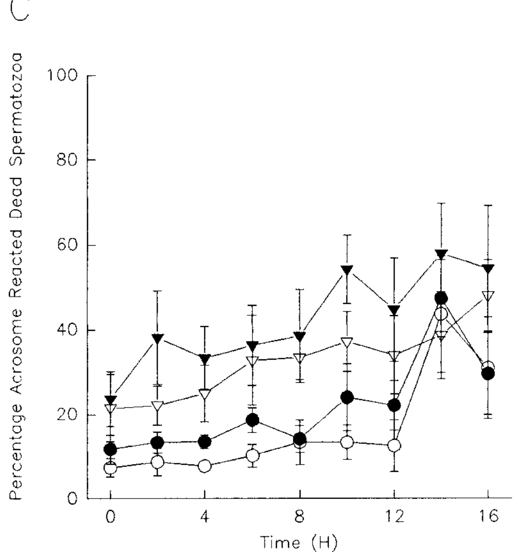


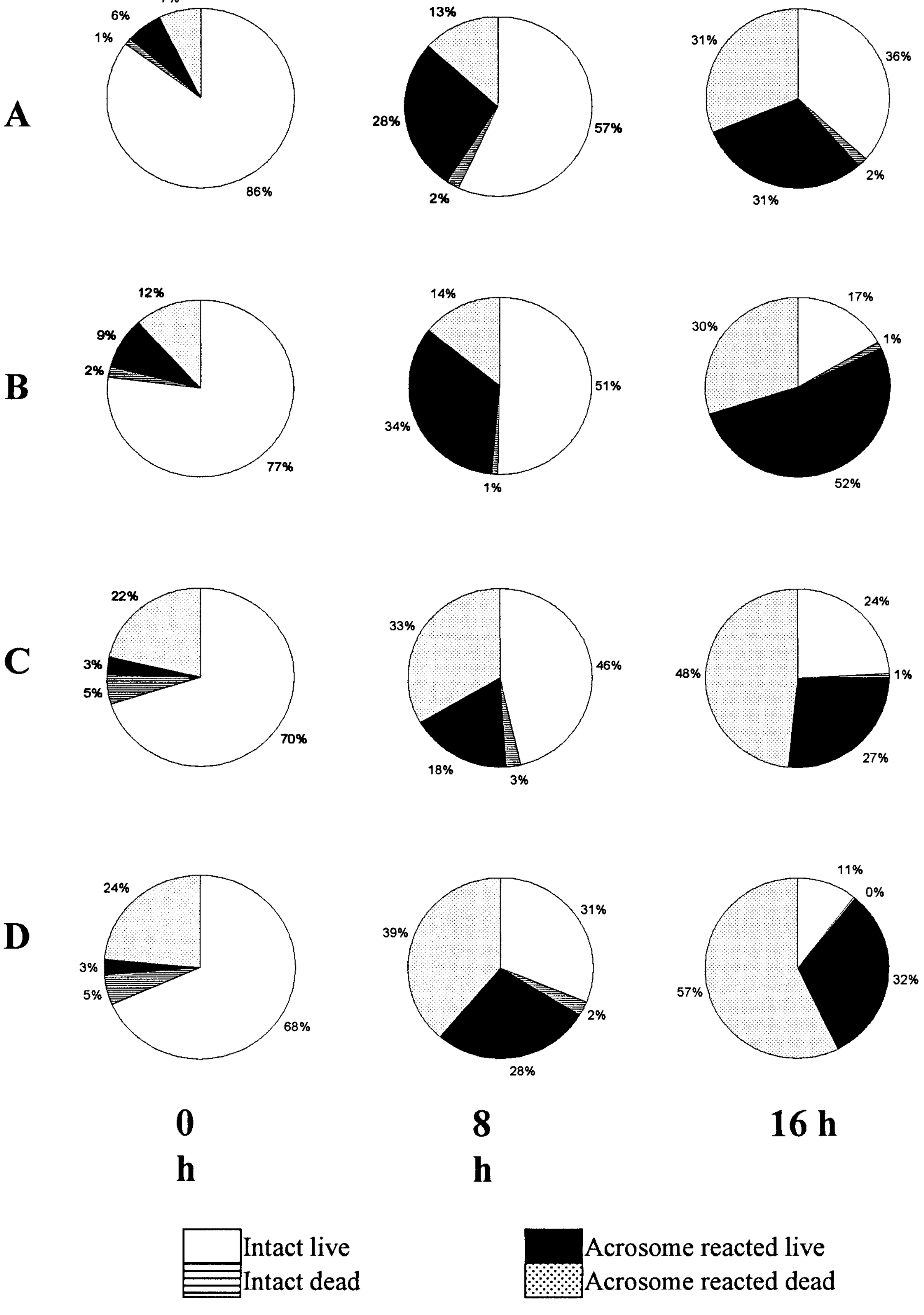
Figure 1. Percentage of intact live **(A)**, acrosome-reacted live **(B)**, and acrosome-reacted dead **(C)** spermatozoa over time (mean ± SEM).

matozoa. This indicated that the LPC was inducing a functional acrosome reaction in both sperm populations.

Discussion

Ejaculated spermatozoa differ from epididymal spermatozoa in pH (Moore and Hibbitt, 1975; Hammerstedt et al, 1979), respiration (Lardy and Ghosh, 1952), adenosine triphosphate synthesis and consumption (Cascieri et al, 1976), heparin-binding sites (Nass et al, 1990), and the profile of proteins bound to the plasma membrane (Lee et al, 1985; Shivaji, 1986; Florman and First, 1988; Veselsky et al, 1992; Dostalova et al, 1994a,b; Calvete et al, 1996). Based on the current study, ejaculated sperm and CES also differ in viability and their ability to capacitate in the presence of heparin.

AGF appears to be detrimental to sperm viability, with the percentage of acrosome-reacted dead spermatozoa being dramatically higher in populations of CES incubated in AGF. Because this effect was evident as early as 0 hours, it appears that components in AGF have an immediate toxic effect on sperm viability. This corroborates the work of others who have found that seminal plasma was detrimental to motility and viability (Chang, 1957; Dott et al, 1979; Corteel, 1980; Cross, 1993; Braun et al, 1994). If one assumes that the acrosome-reacted live population represents those spermatozoa that are functionally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32%
0 h
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Intact live
Intact dead
Acrosome reacted live
Acrosome reacted dead

capacitated, then CES appear to capacitate more quickly and are maintained in a capacitated state for a more extended period than are CES exposed to AGF. Although not statistically significant, the population of acrosome-reacted live spermatozoa in treatments of CES incubated in AGF was lower than for those spermatozoa incubated in medium alone. This suggests that incubating spermatozoa in AGF for extended periods affects their ability to capacitate in the presence of heparin. The fusogenic lipid LPC appears to induce a functional acrosome reaction in heparin-capacitated CES, a conclusion we base on the observation that inducing CES to acrosome react with LPC caused an increase in acrosome-reacted live spermatozoa without significantly affecting the acrosome-reacted dead population.

The glycosaminoglycan heparin is a potent inducer of capacitation (Handrow et al, 1982), and heparin binding to spermatozoa is likely required for it to act as a capacitating agent. Nass et al (1990) found that the major source of heparin-binding proteins in bulls is from seminal vesicle secretions; however, they are also present in secretions from the other accessory sex glands. Heparin-binding proteins are present, though in smaller amounts, on the plasma membranes of epididymal spermatozoa, and exposure of epididymal spermatozoa to seminal plasma increases the number of heparin-binding sites on spermatozoa (Lee et al, 1985). It has been reported that bovine epididymal spermatozoa are unable to undergo a zona pellucida–induced acrosome reaction after capacitation with heparin unless first exposed to seminal plasma (Florman and First, 1988). In the present study, however, lack of exposure to AGF did not compromise the ability of CES to undergo an LPC-induced acrosome reaction. This type of acrosome reaction has been shown to be functional in vitro (Parrish et al, 1988). Therefore, we conclude that the number of heparin-binding sites on epididymal spermatozoa must be sufficient to facilitate heparin-induced capacitation.

It appears that for CES incubated in AGF there is a delay in their ability to produce a population of acrosome-reacted spermatozoa equal in size to that seen in CES. This delay may be the result of the sizable acrosome-reacted dead population that is present at the beginning of the incubation for CES incubated in AGF but not for CES alone. Incubation of CES with AGF appears to have an immediate effect of decreasing viability and inducing the acrosome reaction for more than 20% of the population. One interpretation is that a proportion of this population would have remained alive and acrosome intact had the spermatozoa not been exposed to the detrimental effects of the AGF.

In contrast to the literature citing detrimental effects of AGF and seminal plasma on spermatozoa, there are reports suggesting beneficial effects. Motility-stimulating factors have been found in the seminal plasma (Baas et al, 1983; Okamura and Sugita, 1983), and decapacitation factors or acrosome-reaction inhibitory factors also are present (Chang, 1957; Han et al, 1990; Dostalova et al, 1994a,b). Some resolution of these contradictions may be found by considering the role that seminal plasma may play in the diverse reproductive strategies employed by different species. For example, in rodents, seminal vesicle secretions may be important to fertility. There was a drastic decrease in the fertility of rodent spermatozoa after seminal vesicle ablation (Curry and Atherton, 1990). This provides evidence that AGF is required for successful in vivo fertilization in rodents; this may be due in part to the semen-coagulating products of the seminal vesicles. Although semen coagulation in many species is an important reproductive strategy, bull semen does not coagulate, and it is unlikely that ejaculated bull spermatozoa remain in seminal plasma for a prolonged period in vivo.

Several studies have reported no difference between the fertilizing ability of ejaculated spermatozoa and CES in vivo (Lardy and Ghosh, 1952; Amann and Griel, 1974) and in vitro (Hosoi et al, 1981). However, others have shown that in vitro fertilization rates are higher for epididymal spermatozoa than ejaculated spermatozoa (Brackett et al, 1978). Therefore, it is possible that the main function of the accessory sex gland secretions is to provide fluids for transport of spermatozoa into the female reproductive tract and that components of AGF are not essential for fertilization in vivo. However, in vitro studies have shown that bovine CES incubated in AGF exhibit an increased ability to penetrate bovine oocytes (Henault et al, 1995). Furthermore, when CES from a bull of lower fertility were incubated with AGF from a bull of higher fertility, penetration rates were improved. Although this work was conducted in vitro, it provides an argument for the importance of AGF in bovine reproduction. Regardless of whether fertilization occurs in vivo or in vitro, fertilization by epididymal spermatozoa is not a normal circumstance. Manipulation of the events surrounding fertilization in vitro allows fertilization to occur regardless of the inherent differences between the spermatozoa in an average ejaculate and what is obtained by

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Figure 2. Viability and acrosomal status for **(A)** cauda epididymal spermatozoa (CES); **(B)** CES capacitated with heparin and incubated with lysophosphatidylcholine (LPC); **(C)** CES incubated with accessory sex gland fluid (AGF); and **(D)** CES incubated with AGF then capacitated with heparin and incubated with LPC. Each pie chart depicts the percentage of intact live, intact dead, acrosome-reacted live, and acrosome-reacted dead spermatozoa at 0, 8, and 16 hours of incubation.

electroejaculation, epididymal flushes, and other artificial methods.

Few studies have used epididymal spermatozoa from the vas deferens–cannulated bull. Rather, spermatozoa are typically flushed from the epididymis after it has been removed from the animal. CES used in this study were released from epididymal stores during ejaculation and may have provided a more physiologic representation of the sperm population that would be present in the ejaculate. An ''ejaculate'' was created by combining cauda epididymal effluent and AGF rather than by collecting an ejaculate from the bull and then using his CES, recovered at slaughter in a subsequent experiment. Although this allowed us to directly compare spermatozoa from the same collection that were exposed to AGF with those that were not, it should be recognized that it was not a true ejaculate. The normal sequential addition of secretions from the different accessory sex glands, which occurs in vivo, could not be mimicked in vitro.

Regardless of the mechanism by which AGF is detrimental to sperm viability, it is clear that spermatozoa incubated in AGF are compromised in viability. These data indicate that the more rapidly spermatozoa are removed from seminal plasma, the more viable they will be. Certainly, components of seminal plasma, such as fructose, are beneficial to spermatozoa because they provide important energy sources to fuel sperm motility. However, when spermatozoa are ejaculated directly into the cow reproductive tract, their seminal plasma fluid environment is quickly diluted because of exposure to female reproductive tract secretions. Cervical mucus acts as a barrier to seminal plasma, separating spermatozoa from the fluid portion of the ejaculate at the cervical mucus/seminal plasma interface. It is therefore unnatural for spermatozoa to spend extended periods in AGF. This suggests that after initial, brief exposure to AGF, during which sperm may obtain decapacitation factors or other beneficial factors from AGF, sperm viability is best maintained by the rapid removal of spermatozoa from the ejaculate.

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