Can Relative Spermatozoal Galactosyltransferase Activity be Predictive of Dairy Bull Fertility?

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ABSTRACT

The best and poorest bovine semen samples used commercially for artificial insemination in dairy cattle typically differ in pregnancy rates by 20 to 25% but are within a range that pregnancy rates cannot be predicted consistently by commonly used laboratory assays. Sperm motility and morphology are the characteristics most often evaluated. Laboratory assays that measure other functional traits of sperm may be useful as supplemental assays to increase the reliability of predicting fertility. One such functional trait is the ability of sperm to bind to the zona pellucida, a process mediated by complementary receptors on each gamete. On mouse sperm, $\beta_1,4$-galactosyltransferase acts as a receptor for the zona pellucida. $\beta_1,4$-Galactosyltransferase is expressed on sperm from many mammals, including bovine sperm, and is a candidate for a zona pellucida receptor. The ability of sperm to bind to the zona pellucida may be related to the amount of $\beta_1,4$-galactosyltransferase present on sperm. The aim of this work was to determine if bull sperm $\beta_1,4$-galactosyltransferase activity was related to fertility. $\beta_1,4$-Galactosyltransferase enzyme assays were performed on sperm from 24 bulls whose fertility was estimated by nonreturn rate and on sperm from a second group of seven bulls whose fertility was ranked by in vivo competitive fertilization. $\beta_1,4$-Galactosyltransferase activity varied between individual bulls but was not correlated to fertility as estimated by nonreturn rate or by competitive fertilization. These results demonstrate that $\beta_1,4$-galactosyltransferase activity on sperm varies between animals, but that $\beta_1,4$-galactosyltransferase activity alone is not an accurate indicator of fertility in dairy bulls. (Key words: $\beta_1,4$-galactosyltransferase, bovine, fertility, sperm, zona pellucida, cell adhesion, artificial insemination)

INTRODUCTION

Male infertility and subfertility are significant costs in dairy production. Because infertility is more obvious, subfertility may be a greater economic problem. Among the many factors contributing to low fertility are defects in gametogenesis, the inability of sperm to bind to and penetrate eggs, gamete asynchrony and improper egg activation during fertilization, and early embryonic death (19, 33). Fertilization rate is heavily influenced by male factors. Even among bulls used commercially for artificial insemination that have passed routine fertility tests, a difference of 20 to 25% in conception rate exists (2, 21). Nonreturn rate, which is an estimate of fertility determined by a cow’s failure to be re inseminated and assumed to be pregnant, further demonstrates that a variation of up to 25% is evident within a population of bulls that meet the normal commercially acceptable standards (26).

One long-standing objective of the cattle AI industry is to devise a simple, accurate method for predicting fertility of bovine semen before using it to inseminate cows. The most accurate assays would need to measure a substantial number of sperm attributes necessary for fertilization; a defect in one important attribute necessary for fertilization that an assay did not measure would render the assay misleading. Most often, laboratory fertility evaluations are based on subjective estimates of motility and viability of a semen sample (7, 11, 20). Another method of semen evaluation examines morphological defects in sperm associated with reduced fertility (1, 46). Although these two fertility assessment methods are simple and inexpensive, they are often incapable of accurately distinguishing between a fertile and a subfertile sample. More accurate function-based fertility assays, such as in vitro fertilization success, have been developed, but their relationship to fertility has been controversial (6, 10, 14, 41, 58, 59). The controversy and inconsistency may be because the assays are technically difficult and require uniform matured eggs.

Recent results (14, 58) suggest that the ability of sperm to bind the zona pellucida is related to fertility.
If this is so, it may be because highly fertile sperm have a greater amount of a zona pellucida receptor. Although zona receptors have not been conclusively identified in bovine sperm, in mice a zona pellucida receptor has been identified. β1,4-Galactosyltransferase (GalTase), an enzyme on the surface of mouse sperm, binds to terminal N-acetylglucosamine oligosaccharide residues on ZP3, a specific zona pellucida glycoprotein (16, 34, 35, 36, 56). GalTase binds ZP3 in a multivalent fashion, causing receptor aggregation and the initiation of the acrosome reaction (15, 25, 29, 35). The acrosome reaction is characterized by fusion of the outer acrosomal membrane with the sperm plasma membrane, resulting in the release of acrosomal enzymes, a requirement for zona pellucida penetration. GalTase-null mutant mice generated by gene targeting produce sperm whose ability to acrosome react and penetrate the zona pellucida is severely impaired (28). Therefore, GalTase is a crucial component of sperm-egg binding in the mouse.

Although present on the sperm surface, GalTase was originally described as a component of the Golgi complex where it functions during the biosynthesis of glycoproteins (49). Sperm GalTase is a product of the GalTase-I gene that has two functional ATG codons yielding two translation start sites and two different proteins (45, 48). The two proteins differ only in their amino terminal domains; the longer form has 13 additional AA on its N-terminus in the cytoplasmic domain and is more abundant on the cell surface (27, 57). Surface GalTase functions noncatalytically, due to the lack of a sugar donor (uridine 5’-diphosphogalactose) in the extracellular environment (49).

The role of GalTase and ZP3 in fertilization has been best defined in the mouse, but studies suggest that both gamete receptors function in other species as well. GalTase has been found on the surface of the head of sperm from all mammals examined to date including cattle, swine, rats, guinea pigs, horses, and humans (13, 24, 34). Likewise, ZP3 is expressed in the zona pellucida of oocytes from mammals and even fish and birds (9, 12, 22, 23, 37, 39, 40, 44, 52, 53, 54, 55). The conserved expression of GalTas and ZP3 supports the hypothesis that GalTase and ZP3 may be involved in sperm-egg binding in many species (24, 38, 43).

If GalTase on the bovine sperm surface acts as a zona receptor, the amount of GalTase on the sperm surface may be an indicator of fertility. To test this hypothesis, GalTase activity on the sperm from 31 different bulls was measured using a GalTase enzyme assay and the correlation between GalTase activity and previously determined nonreturn rates or competitive fertility indices was measured.

MATERIALS AND METHODS

Washing and Handling of Semen

Semen was obtained from 24 bulls owned by three different AI organizations, 21st Century Genetics (Shawano, WI), Noba Inc. (Tiffin, OH), and Genex Inc. (Ithaca, NY). Semen from seven bulls whose relative fertility was determined by heterospermic insemination was also obtained (47). Diluted semen was cryopreserved in 0.5-ml straws in either milk or egg-yolk citrate extender. Before assay, straws were thawed quickly in a 35°C water bath and two straws per semen sample were emptied into a 15-ml conical tube. Sperm were diluted in 10 ml of dmKRBT (120 mM NaCl, 2 mM KCl, 2 mM CaCl2, 10 mM NaHCO3, 1.2 mM MgSO4, 4.97 mM NaH2PO4, 5.6 mM glucose, 1.1 mM Na pyruvate, 25 mM TAPSO (3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid), 18.5 mM sucrose, 100 IU/ml of penicillin, 0.1 mg/ml of streptomycin, 6.0 mg/ml of fraction V BSA, pH 7.3). The samples were washed three times in 10 ml of dmKRBT by centrifugation.

GalTase Enzyme Assays

Sperm were assayed as follows for GalTase activity using a standard enzyme assay (24), as described below. After washing, the final sperm pellet was resuspended in dmKRBT containing 10 mM MnCl2 but without sucrose. Sperm concentration was determined with a hemacytometer and estimates were performed in triplicate. A 20-µl volume of 100 µM uridine 5’-diphospho-[3H]-galactose (UDP[3H]Gal; 250 Ci/m mole; DuPont-NEN Research Products, Boston, MA) and 10 µl of unlabelled 1 mM UDP-galactose were placed in 1.5-ml microfuge tubes and lyophilized. Fifty µl of dmKRBT with 10 mM MnCl2, 30 mM N-acetylglucosamine as substrate, and 1 mM 5’-AMP to inhibit nucleotidases were added to the tube of UDP[3H]Gal. Assays used 5 × 106 sperm per microfuge tube. The tubes were incubated in a 37°C water bath. At the beginning of the incubation and after 90 min, the tubes were mixed gently and 20-µl aliquots were added to 5 µl of 0.2 M EDTA-Tris-HCl, pH 7.4, to stop the reaction. Samples were spotted on 3 mm Whatman chromatography paper, allowed to air dry, and the substrate and breakdown products were separated from [3H]-galactose-N-acetylglucosamine ([3H]-lactosamine) by paper electrophoresis in the presence of sodium tetraborate as previously described (24). The assays were conducted once for each collection (two to eight assays per bull, depending upon collection availability) in triplicate. In parallel with each assay, a control sample was also assayed as an internal control to measure assay variability. The control sample consisted of a single ejaculate, extended and cryopreserved.
The electrophoresis papers were dried and the origins, containing \[^{3}H\]-lactosamine, were trimmed and placed into scintillation vials. ScintiVerse BD scintillation cocktail (20 ml; Fisher Scientific, Pittsburgh, PA) was added and the samples were counted using a liquid scintillation counter. The average counts per minute at time zero (background) were subtracted from the 90-min time point, and the data were expressed as the amount of lactosamine product formed (pmoles) per million sperm per hour.

**RESULTS**

**Relationship of Sperm GalTase to Nonreturn Rate**

Semen was obtained from 24 different bulls housed at three AI organizations whose semen is marketed jointly. Semen from these bulls was being collected routinely for commercial sale. Fertility was estimated by calculating nonreturn rate. The nonreturn rates used were based on the number of females that did not return to estrus within 60 to 90 d after insemination. Nonreturn rates are a good estimate of fertility if an adequate number of inseminations have been performed (51). Because the nonreturn rate data available for some of the individual collections of semen was from a limited number of inseminations, lifetime nonreturn rates were used for this study. These nonreturn rates were from a minimum of 1500 inseminations and as many as 167,000 inseminations. For some experiments, we examined the nonreturn rate from individual collections. For all the bulls examined, the lifetime nonreturn rates were very similar to the individual collection nonreturn rates. In only 17% of collection days where more than 200 inseminations were performed using those ejaculates did the nonreturn rate deviate from the lifetime nonreturn rate by more than 4%.

For all assays, product formation was linear with time over the course of the assay at the specified temperature and substrate concentrations (Figure 1). GalTase assays on sperm from 24 different bulls were performed to determine if the enzyme activity varied from one bull to another and to determine if GalTase activity was related to nonreturn rate. Although sperm GalTase levels from different bulls varied, no correlation between GalTase activity and lifetime nonreturn rate was observed ($r = 0.04; P = 0.89$) (Figure 2). No correlation between GalTase and lifetime nonreturn rate was observed if calculations were performed separately for each AI organization. As an estimate of assay variability and an internal control, a sample of semen from one ejaculate that was frozen-stored was assayed in parallel with each group of samples. The coefficient of variation of this sample, when assayed 21 times, was 24.6%. To

![Figure 1](image1.png)  
**Figure 1.** Sperm $\beta 1$-$4$-galactosyltransferase (GalTase) assay is linear over time (90 min). Sperm from the control bull were washed and a GalTase enzyme assay was performed in triplicate as described in Materials and Methods. The triplicates were averaged and the data were expressed as pmoles of product formed over time. Some of the standard error bars were too small to be seen.

![Figure 2](image2.png)  
**Figure 2.** $\beta 1$-$4$-Galactosyltransferase (GalTase) expression is not related to bull fertility, as estimated by lifetime nonreturn rates. Sperm from 24 bulls were washed and assayed for GalTase activity. The data represent the average of two to eight independent assays performed in triplicate. Control samples were performed in quadruplicate and in parallel to all other assays to measure assay variability. GalTase activity was plotted against nonreturn rate ranking. Bulls with nonreturn rates of <64% were grouped as below average fertility, bulls with nonreturn rates between 65 and 75% were grouped as average fertility, and bulls with nonreturn rates >76% were ranked as above average fertility for this figure. All animals assayed expressed GalTase, and there was variation between bulls; however, there was no correlation between GalTase activity and nonreturn rate ($r = 0.04; P = 0.89$). Bars indicate standard errors. Some error bars are too small to be seen.
Figure 3. \(\beta\)-1-4-Galactosyltransferase (GalTase) activity varied over time. Sperm from six or eight ejaculates from each bull, collected over 1.5 yr, were washed and assayed for GalTase activity. The data represent the average of triplicates. Control samples were run in parallel to all other assays to measure assay variability. GalTase assay results at several semen collection dates were compared. Two bulls (A and B) were used for this experiment, and the data are graphed separately. All ejaculates assayed expressed GalTase and there was slight variation over time. Bars indicate standard errors.

Changes in GalTase Activity Over Time

Even if GalTase activity was not related to the difference in fertility between bulls, GalTase may still be related to fertility changes in semen from a bull over time. To test this hypothesis, semen from two bulls, housed by the same AI organization, was collected over a period of approximately 1.5 y. Six or eight ejaculates from each bull were assayed for GalTase activity. GalTase activity varied some over time within each bull (Figure 3) but the GalTase activity was not related to the relatively small changes in nonreturn rate (\(r = -0.14\) and \(r = 0.56\), \(P < 0.10\), Figure 4).

Relationship of GalTase to Competitive Fertilization Ranking

Nonreturn rate is a relatively good indicator of fertility, but fertility can more accurately be assessed on...
fewer inseminations when equal numbers of sperm from two males are mixed together and inseminated into a female (30). After the parentage is determined the male whose sperm fertilized the oocyte is deemed more fertile. Several pairs of males can be compared, the males can be ranked, and a fertility index can be calculated. In an earlier study, nine bulls were ranked by competitive fertilization and assigned a fertility index in this manner (47). Semen from seven of the original nine bulls was available and used for this experiment. GalTase enzyme assays were performed in triplicate on all semen samples. Results showed no correlation between GalTase activity and competitive fertility index ($P > 0.10$). Therefore, GalTase activity was not related to competitive fertilization index ranking (Figure 5).

**DISCUSSION**

The results of the present study indicate that GalTase is not related to fertility, as estimated by two methods. No significant correlation between sperm GalTase levels and nonreturn rate was observed. As a second test to confirm this result, we obtained semen from seven bulls whose fertility was determined by competitive insemination. Unfortunately, few bulls have been evaluated by this method and limited semen was available (47). However, the correlation coefficient between GalTase and the competitive index was also not significant.

In this study, GalTase was present on sperm from all bulls examined, and the activity varied between bulls. When considering the potential function of GalTase, the significance of the variation is unclear, since the minimal amount of GalTase required for sperm-zona pellucida binding is unknown. Furthermore, it is not certain if GalTase functions as a zona pellucida receptor in bovine sperm. However, GalTase has been previously localized by immunofluorescence to the plasma membrane of the head of both fixed and live bull sperm, the proper location for a zona receptor (24). GalTase on bovine sperm may bind the zona pellucida, as it does during mouse and porcine fertilization (35, 43).

The results of this study revealed no correlation of GalTase activity on sperm to nonreturn rate or competitive fertility. Nonreturn rate would be expected to be less sensitive to defects in sperm-egg binding because the number of sperm in each insemination dose is in excess. In contrast, competitive fertilization emphasizes differences in sperm quality due to the nature of the measurement. But GalTase activity was not related to either fertility measure. This may be due to one or more of a variety of factors. First, GalTase may not be involved in bovine sperm-zona pellucida binding. Alternatively, it is possible that although bovine sperm surface GalTase binds the zona pellucida, there may be redundant gamete receptors that act as a fail-safe mechanism. In that scenario, changes in GalTase may not affect gamete binding. In addition, it is possible that, although a dramatic decrease in the amount of GalTase present on sperm may make males infertile, the GalTase variation observed in experiments with commercial bulls was insufficient to observe a relationship to fertility. Finally, a larger sample group may be necessary to reveal a correlation between GalTase activity and nonreturn rate or competitive fertility index.

A multitude of sperm traits are necessary for good fertility, such as normal morphology, progressive motility, hyperactivated motility, binding to and penetration of the zona pellucida, binding to the egg membrane, egg activation, and other factors that have not been studied. It is apparent that GalTase alone is not an indicator of fertility but perhaps GalTase measurements, in conjunction with several other laboratory assays of fertility developed as an index may be more correlated to fertility in vivo. It is noteworthy that, in the competitive fertilization experiments, 70 to 80% of the competitive index was already explained by differences in sperm viability (47). It would be interesting to determine if the egg binding ability of sperm is a useful predictor of fertility in semen samples with equal viability. In this regard, evidence shows that the ability of sperm to bind the zona pellucida is related to fertility...
(14, 58), so it is reasonable that the abundance of a zona receptor would be a valuable laboratory assay. The fertility of a semen sample is undoubtedly a composite of the function of a variety of sperm molecules, including proteins on the plasma membrane. There are reports (3, 4, 5, 8, 17, 18, 31, 32) that the amounts of several proteins on sperm or in seminal fluid are indicators of semen fertility.

In conclusion, we have shown that sperm from bulls with different nonreturn rates and competitive fertility index rankings express GalTase in varying amounts, although no correlation of GalTase activity to fertility was detected. Further study is required to determine whether differences in fertility can be explained by the expression of sperm proteins that have functions during fertilization.

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