Development of Real-Time, On-Farm Semen Quality Tests based on Reactive Oxygen Species
North Carolina Pork Council Matching Grants Program
Final Report

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Introduction

At the present time, insemination doses are produced in boar studs and delivered to farms at regular intervals during the week. Typically, boar studs keep a test sample from each batch of extended semen which is evaluated daily. If the quality of the test sample decreases significantly, then the boar stud instructs farms to immediately discard any remaining doses from the suspect batch. This system is good for detecting problems with boars and semen handling at the stud. In contrast, semen is typically not evaluated once it arrives on farms because of the technical expertise and cost involved. Consequently, problems that occur with semen during delivery to and storage on farms prior to insemination are very difficult to detect. When fertility decreases at the farm level, but the test samples at the boar stud are good, one of the possibilities is that something happened to the semen during transport to the farm or while it stored before insemination. In these situations, it is also possible that old semen was used instead of being discarded on the farm. In a recent survey conducted within a large corporate swine operation, an average of 35% (range = 15% to 65%) of the insemination doses in the semen storage units on breeding farms should have been previously discarded based on the production date. Development of a sensitive, accurate, and simple on-farm test for semen quality would facilitate improved semen management on the farm and help identify potential problems that arise during its transport and storage.

To make the situation even more complicated, recent work conducted at N.C.S.U. indicates that the fertilizing ability of extended semen actually begins to decrease before overt changes in its motility are observed. For both 3-day and 7-day extenders, acrosin activity and the number of spermatozoa bound to eggs (in vitro) decreased significantly before overt changes in motility were observed. This occurred after days 1 and 3 for 3-day and 7-day extenders, respectively. It is interesting to note that average motilities for semen samples in both extenders during this period were above 70%, the level often used as a decision boundary for whether to use a dose for insemination.

It is believed that this discrepancy between motility and other measures of semen quality are largely due to the production of reactive oxygen species which are also referred to as “free radicals”. Free radicals are compounds that are produced during oxidation and can cause damage to the membranes of cells. Because boar spermatozoa use both aerobic (oxygen-requiring) and anaerobic (non-oxygen requiring) mechanisms for energy after ejaculation, production of free radicals is a common occurrence in extended semen. Their concentrations in fresh semen increase during the summer months, during periods of high collection frequencies, and during bacterial contamination (Strzezek et al., 2000). Unfortunately, commercial semen extenders do not contain compounds to neutralize free radicals. Lipid

peroxide and hydrogen peroxide are the two most common free radicals found in semen from animals other than pigs (Strzezek et al., 2000). However, due to the large contribution of the seminal vesicles to the volume of semen, there are at least three other free radicals that could be elevated in boar semen compared with other species (Flowers, unpublished). Based on these observations, quantification of free radicals in boar semen might provide a more accurate and sensitive test of semen quality than motility.

Experimental Procedures

In order to accomplish this, we proposed three studies. In our first experiment, concentrations of lipid and hydrogen peroxide individually and collectively (3 tests) were measured in semen extended in 3 and 7-day extenders from boars collected at different frequencies during the summer and winter quarters. This information was important in helping us determine maximum and minimum levels of free radicals in fresh and extended boar semen. However, more importantly, it provided us with guidance in terms of whether we can target a specific free radical such as hydrogen peroxide or need to develop a test that estimates the total amount in a given sample and help us establish the relationships among free radicals and semen quality. In our second experiment, concentrations of free radicals in extended semen produced and distributed in commercial production systems were monitored. This information is important because levels of free radicals are likely to be higher in semen that is transported to and stored on farms under commercial conditions compared to those of the first study in which semen will not be transported after collection and will be maintained in a laboratory environment. Moreover, it is possible that there are environmental and genetic differences that could affect production of free radicals which can only be evaluated in a commercial production system. Finally, our third experiment will be based on the results of the first two studies. We hope to be able to develop a "dip-stick" test in which a strip of paper is dipped in semen where a change in color indicates free radical concentrations (similar to Litmus paper that measures pH). An alternative plan would be to add a marker compound to semen extender that undergoes a color change when free radicals begin to increase past a certain level.

Results and Discussion

The study originally was begun in July of 2006. Data from the summer replicate were analyzed and submitted to the NCPC as part of the interim progress report (January, 2007). Unfortunately, due to a management error, eight of the original 16 boars were inadvertently sold in December of 2006 before they were used in the winter replicate of the study for the first year. As a result, the decision was made to basically start the study over beginning with the winter replicate in February and March of 2007. This is essence basically put us 6 months behind the originally proposed time table for the study. Because of this, a no-cost extension was requested and granted by the NCPC. A key component of the study is to examine changes in reactive oxygen species in semen from the same boars over time. This is critical for two reasons. First, it will help us decide which free radical will be the best choice to serve as the basis for the development of our semen quality test. For example, if there is one free radical that changes the most or is produced in the highest quantities by all the boars in the study, then it would be logical to use it as the basis for our test. Second, we need to have some idea of the normal variation among boars that exists in the production of reactive oxygen species. The amount of variation will dictate the level of sensitivity for the test. It also will help us establish when levels of reactive oxygen species in semen are likely to cause problems with semen quality.

At the present time, we have completed the first experiment proposed during the first year of the study. Sixteen boars were collected at a frequency of either 1.5 or 4 times per week (n=8 per treatment) during a 7 week period during February and March and then again during a second 7 week period in

July and August. Each ejaculate was evaluated at collection; split into two equal volumes; and extended in BTS and Androhep. Motility, morphology, acrosin activity, lipid peroxide, hydrogen peroxide and the overall peroxide activity (MDA) were measured on days 0, 1, 2, 3, and 7 in each extender.

The initial results from the first replicate indicate that MDA, a measure of total peroxide activity and thus overall free radical concentrations, is the best indicator of the total amount of reactive oxygen species to which spermatozoa are exposed (Figure 1). The primary reason for this is that there are differences among boars in terms of hydrogen and lipid peroxide production. For example, semen from some boars has very high levels of lipid peroxide and very little hydrogen peroxide, whereas for others the opposite is true. In contrast, concentrations of MDA are high in all of these boars. Consequently, instead of developing two different tests – one for hydrogen peroxide and a second for lipid peroxide, one test can be developed that only measures MDA. The damage to spermatozoa is basically the same when they are exposed to high concentrations of free radicals, regardless of whether the free radicals are produced by oxidation of hydroxyl groups (hydrogen peroxide) or lipid molecules (lipid peroxides).

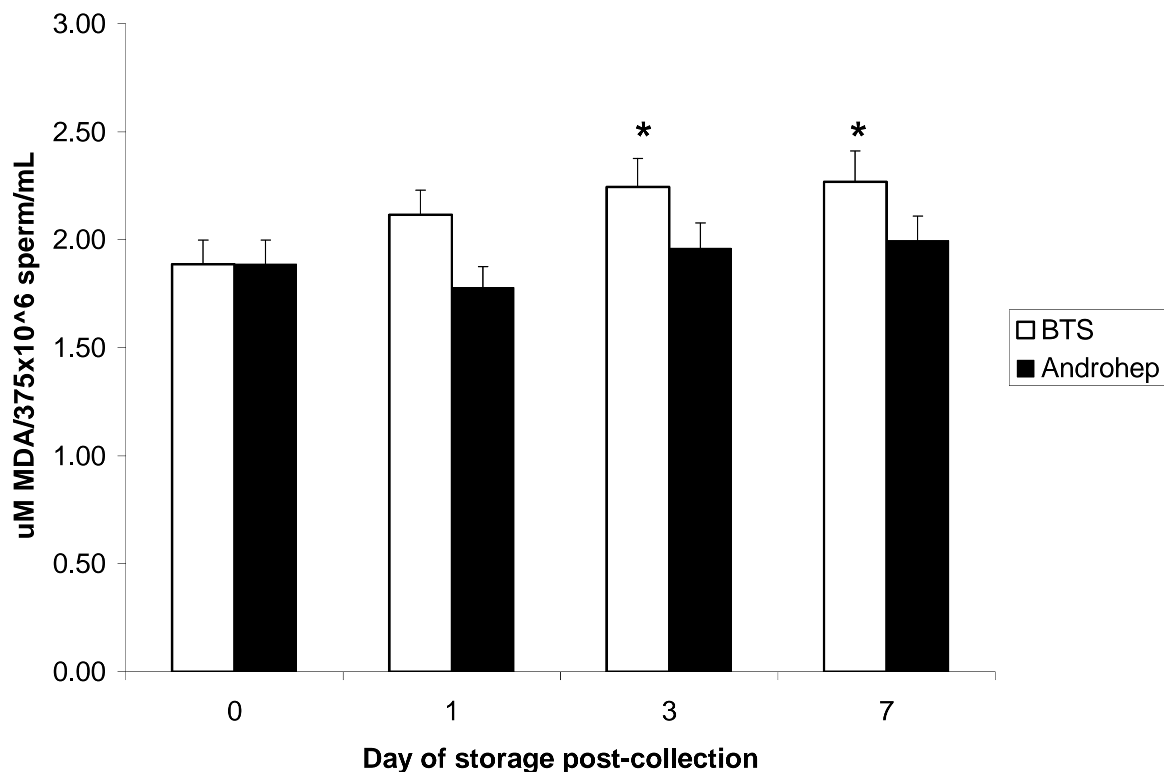

Figure 1. An extender x day interaction (P=0.03) was present for the amount of malondialdehyde (MDA) produced per 375 x 10^6 sperm/mL. There were no differences (P>0.05) between the short-term (BTS) or long-term (Androhep) extenders for any day of storage post-collection. The short-term extender had higher (P=0.0015) levels of MDA on days three and seven of storage post-collection. There was not a significant increase in the long-term extender by day seven of storage (P=0.96). *Different from day zero within extender (P<0.05)

As expected, boars with higher collection frequencies tend to have higher concentrations of MDA compared with their counterparts on a normal collection frequency. In addition, levels of reactive oxygen species in semen are elevated during the summer months compared with the winter months. For some boars, three to five fold increases were observed. Surprisingly, collection frequency does not

appear to magnify this difference. In other words, there doesn't appear to be a synergistic effect of collection frequency and exposure to elevated ambient temperatures. Since both an increased collection frequency and the ambient conditions associated with the summer months have been reported to increase production of free radicals in semen, we expected a synergistic effect – a dramatically higher increase in the summer compared with the winter when collection frequency was increased. This is an important observation from a management perspective in that reducing collection frequency and minimizing exposure of boars to high temperatures should prove to be effective treatments in terms of reducing concentrations of free radicals in spermatozoa.

Experiments proposed for the second year of funding revealed some interesting results as well. This experiment involved tracking semen samples from their production in the boar stud through transport and use on commercial swine farms. Based on results from the first year of studies, it appears, as mentioned previously that MDA concentrations in semen are the best cumulative measure of exposure of semen to reactive oxygen species. Preliminary observations indicate as expected that there is considerable variation among boars; within studs; and over time in MDA levels. However, a consistent trend is that MDA concentrations increase after extension and during transport to the farms. At the present time, there appears to be a trend for there to be slightly lower farrowing rates in sows bred with semen with increased levels of MDA. However, this seems to be farm-specific. Consequently, establishing a definitive level upon which to base the "dip stick" test may prove to be more challenging than we anticipated. Instead of a simple test in which one color change signifies a "useable" versus a "non-useable" dose, it may be necessary to develop one that exhibits a range of colors, i.e. white, pink, medium red, dark red, etc. For some farms, the decision to discard an ejaculate might be if the test turns medium red, while for others it may not be until the test turns dark red.

The initial development of a "dip stick" on-farm test linking changes in MDA levels to a color change progressed well. The first stage of this process was to combine a compound that can recognize MDA levels in extended semen with other reagents that undergo a simple color change. We tested 3 different compounds that should recognize MDA levels and 4 different compounds that are capable of undergoing a color change. All of the compounds tested can recognize very high levels of MDA with less than 10% false positives. Next, using semen samples spiked with lower levels of MDA similar to those that we have found in commercial samples, we examined the effectiveness of our "dip-stick test". It is important that any on-farm semen test be able to accurately measure changes normally seen within the normal treatment of semen from collection through insemination.

The overall goal of this grant was to develop a real-time, on-farm semen quality test in which a simple change in color would be indicative of a reduction in semen quality. Previous studies indicated that spermatozoa typically do not show overt changes in morphology or motility when MDA levels are less than 3.0 um. Consequently, this level was selected as the minimum acceptable concentration. Samples over this level should have yielded a positive test result or cause a color change. Samples under this level should produce a negative test result or not initiate a color change. The results from these studies were very promising in that they correctly diagnosed 90% of samples with high levels of MDA, a metabolite produced by sperm cells damaged by reactive oxygen species or free radicals. Data from these studies are shown in Table 1.

Table 1. Effectiveness of Real-time Semen Test for Identification of Samples with High MDA levels.

No. of Samples	MDA Levels (um/375 x 10^6 sperm)	Real-Time Test Results		
		Positive	Negative	Correct Diagnosis (%)
50	$\geq$ 5.0	48	2	96%
47	4.0	44	3	94%
38	3.0	34	4	90%
71	2.0	7	64	90%
36	$\leq$ 1.0	2	34	94%

These studies (Table 1) were performed by adding a known amount MDA to an insemination dose; allowing it to incubate; and then performing both the Real-Time test and the analytical test (MDA levels). Based on the success of these initial studies, we collected samples from commercial boar studs to test. We performed the Real-Time test and the Analytical test on each sample and, unfortunately have not been able to achieve the same results. The percentage of samples diagnosed correctly is still reasonably good at the higher levels of MDA, but is poor at the lower levels of MDA. Table 2 contains these results.

Table 2. Effectiveness of Real-time Semen Test for Identification of Samples with High MDA levels from Commercial Semen Samples.

No. of Samples	MDA Levels (um/375 x 10^6 sperm)	Real-Time Test Results		
		Positive	Negative	Correct Diagnosis (%)
24	$\geq$ 5.0	20	4	83%
37	4.0	30	7	81%
38	3.0	28	10	74%
71	2.0	29	42	59%
78	$\leq$ 1.0	30	48	62%

Collectively, these data indicate that there appears to be something in the commercial samples that is interfering with the ability of the Real-Time test to measure MDA levels accurately. The biggest problem is appears to be False Positives. This would indicate that there is something in these samples that is reacting with the test that isn't MDA. During the last 6 months, we have taken samples from our boars here in Raleigh and those obtained from commercial sources; separated them into 3 fractions – spermatozoa; seminal plasma with protein; and seminal plasma without proteins; added known amounts of MDA; and then tested MDA levels in each fraction with our analytical techniques and the real-time test. This was being done in an attempt to determine which component of semen is causing us to get False Positives. For example, if we get an increase in the seminal plasma fraction with the protein but not in any of the others, then the problem is associated with the proteins in seminal plasma and we may be able to overcome this via dilution or some other way to reduce their activity.

Based on these results it appears that for about 75% of the boars tested, there is a family of seminal plasma proteins that is causing the false positives. There are two ways that might be useful in addressing this situation. One way would be to screen boars for these seminal plasma proteins when they enter the boar stud and simply not try to use the test on them. Analysis of seminal plasma proteins currently requires a process called 2-dimensional gel electrophoresis that really can't be run in

commercial boar studs practically, so this might not be a good option. The other approach would be to try and dilute a sample of semen and then run the test on the diluted sample. This seems more practical as semen is typically diluted during extension. We have obtained additional sources of funding to continue the dilution work.

References

Rosner, B. 1989. Fundamentals of Biostatistics. PWS-Kent Publishing Company. Boston, MA, U.S.A.

Snedecor, G.W., and Cochran, W.G. 1989. Statistical Methods, Eighth Edition. Iowa State University Press. Ames, IA, U.S.A.

Strzezik, J., Fraser, L., Demianowicz, W., Kordan, W, Wysocki, P., and Holody, D. 2000. Effect of depletion tests (DT) on the composition of boar semen. Theriogenology 54, 949-963.