The Use of Testicular Fine-needle Aspiration, Histology and Immunohistochemistry for Determining Bull Fertility at an Early Age

Nicolas Negrin Pereira[1], Pawel Borowicz[1], Bryan Neville[2], Jordan Flaten[1] and Carl Dahlen[1]

[1]Department of Animal Sciences, NDSU

[2]Central Grasslands Research Extension Center, NDSU

The objective of the study was to assess the use of different techniques to allow for early, low-cost, reliable and low-invasive methodologies for the determination of Sertoli cell populations and germ cells in the bull. The techniques are: fine-needle aspiration with a fine needle (FNA-F), fine-needle aspiration with a gross needle (FNA-G), classic histology cuts and immunohistochemistry (androgen receptor expression by fluorescence). A significant correlation exists between histology and immunohistochemistry measurements, which positions the immunohistochemistry technique as a novel, very specific and reliable methodology for precocious determination of future fertility in young bulls.

Summary

Testicular parenchyma samples from 14 young peri-pubertal bulls were taken for the assessment of four different techniques: fine-needle aspiration using two different needle calibers or by open cut for histology (hematoxylin/eosin) or immunofluorescence (androgen receptor expression). For fine -needle aspiration, we used two different needle calibers attached to a syringe: 22G (FNA F) or 16G (FNA G). Once the needle was inserted in the testicular parenchyma, a vacuum was produced inside the syringe by pulling back the plunger. A smear was obtained by expelling the sample onto a glass slide. Then it was air-dried, fixed with 70 percent alcohol and stained with hematoxylin/eosin. Tissue samples were cut into 4- by 4-millimeter (mm) pieces using a microtome blade, fixed in a 10 percent formaldehyde solution, embedded in paraffin and cut into sections 5 micrometers (µm) thick using a microtome. Samples were stained with hematoxylin-eosin for histology or incubated sequentially with mouse monoclonal antibody to androgen receptor and goat-antirabbit (IgG CF633) fluorescent stain. To evaluate slides, images were taken at 200 times (FNA and histology) or 100 times magnification (immunohistochemistry) using an epifluorescence microscope equipped with a camera. Within each image, four to six seminiferous tubules within the five randomly chosen fields were selected for determination of Sertoli cell number and germ cell number. The ratio of germ to Sertoli cells was calculated and the CORR procedure of SAS was used to determine correlation of this ratio among each respective evaluation technique. Differences were considered significant at $P < 0.05$. A correlation coefficient of 0.58 ($P<0.001$) was observed among the technique of histology (5.27±0.41) and AR expression through fluorescence (4.44±0.60) for germ cells/Sertoli cell ratio. No significant correlations were found among FNA techniques and histology or immunohistochemistry cell ratios. Expression of AR by fluorescence in Sertoli cells represents a new, highly specific technique for precocious detection of more potential fertile bulls as young peri-pubertal calves.

Introduction

Fertility in the bull can be defined as the ability to produce viable calves. This characteristic of the bull is key in beef farm profitability. In the U.S., more than 95 percent of the herds rely on natural service. An early and accurate fertility prediction technique in the bull could have a great impact on profitability of beef operations (Wiltbank et al., 1986).

A bull breeding examination has proven to be a reliable and cost-effective technique to detect those animals that are satisfactory potential breeders and those not apt for their use (Barth et al., 2002). The inclusion of semen analysis has improved the effectiveness of this examination, but a significant range of different fertility levels still occurs within those bulls classified as apt for breeding (Chenoweth and McPherson, 2016).

Researchers have demonstrated for several years the importance that the final establishment of the Sertoli cell population has in determining future daily sperm production in most mammals (Berndston et al., 1987; P.J. O'Shaughnessy et al., 2011). The relationship between the size of the Sertoli cell population and number of germ cells has been used as a tool for determining fertility in different mammal species (Berndston et al., 1987). Determining the size of the Sertoli cell population in the bull at an early age can be a powerful tool in detecting those individuals with a potential higher sperm capacity.

The fine-needle aspiration technique has been used for several years in human and stallion testicles, providing a reliable, low-cost, diagnostic, low-invasive tool with minimal complications for cytology studies in the testis (Aridogan et al., 2003; Leme et al., 2012).

Expression of AR has been described in specific cells within the mammalian testis, such as in Leydig and peritubular myoid cells being expressed exclusively in Sertoli cells within the seminiferous tubules but not being expressed by germ cells (O'Hara et al., 2015).

Hardly any information is available about the application of different techniques for the determination of Sertoli and germ cell populations in the male calf and their potential use for detecting higher daily sperm producers later in adult life as bulls. The aim of this study was the comparison of four different techniques for determining Sertoli and germ cell population sizes in the peri-pubertal bulls: FNA F (22G needle), FNA G (16 G needle), classic histology cuts (hematoxylin/eosin stain) and immunofluorescence (AR expression).

Procedures

Fourteen Aberdeen Angus and Shorthorn bull calves (287±3.3 days of age, 683±29 pounds) from the North Dakota State University Beef Unit were used in the study. Previous to castration, bulls were restrained and given epidural anesthesia.

The scrotum skin was opened using a scalpel and the testicles exposed. Plexus pampiniform, testicular artery and vas deferens were crushed and severed using an emasculator to prevent bleeding.

Testicular tissue samples were obtained for evaluation via: 1) fine-needle aspiration with a fine needle (FNA F) using a sterile 22-gauge, 1¼-inch needle, 2) fine-needle aspiration with a gross needle (FNA G) using a sterile 16-gauge, 1¼-inch needle, 3) histology cut (stained with hematoxylin and eosin) and 3) immunofluorescence (androgen receptor expression).

For the FNA techniques, needles were connected to a 5- or 10-milliliter (ml) syringe and gently inserted perpendicularly into the testicular parenchyma. Once fully inserted in the parenchyma, the plunger was pulled back to produce a vacuum inside the syringe.

The needle was moved backward and forward within the testis two or three times for approximately four seconds. Once the needle was outside the testicle, a syringe filled with air was reattached to the needle and the plunger pressed to expel the sample onto a glass slide.

A smear was produced by sliding a second glass slide at an angle, extending the sample on the glass surface. The slide then was air-dried, fixed in 70 percent alcohol and stained with hematoxylin-eosin.

For histological examination, 4- by 4-mm testis parenchyma samples from the same region of each testis were taken and placed in a 10 percent formaldehyde fixative solution, embedded in paraffin and cut in 5-µm-thick sections using a microtome (Leica Biosystems Inc., Buffalo Grove, Ill.). Slides for histology were stained with hematoxylin-eosin.

Immunohistochemistry sections were submerged in sodium citrate buffer and placed in an antigen retriever (2100 Retriever, Aptum Biologics, UK) and incubated sequentially using mouse monoclonal antibody to androgen receptor (ab9474, abcam, Cambridge, Mass.) at 4 C overnight with agitation, and then stained with goat-antirabbit IgG CF633 fluorescent stain.

For each slide or tissue section, images were taken at 200 times magnification (FNA smears and histology slides) or 100 times magnification (immunohistochemistry slides) using a Zeiss Imager M2 epifluorescence microscope equipped with Zeiss piezo automated stage and AxioCam HRm camera (Carl Zeiss International, Jena, Germany). Image analysis (Image-Pro Plus, Media Cybernetics Inc., Bethesda, Md.) was performed for images of five randomly chosen fields. Within each image, four to six seminiferous tubules were selected randomly for Sertoli and germ cells individual cell counts using the Image-Pro Plus image analysis software (Media Cybernetics Inc., Rockville, Md.).

The germ cell to Sertoli cell ratio was calculated by dividing the total number of germ cells by the total number of Sertoli cells within each tubule. The mean of all ratios were determined for each testicle and analyzed using the correlation procedure of SAS (SAS Inst. Inc., Cary, N.C.). Significant differences were considered when $P < 0.05$.

Results and Discussion

The different ratios between total germ cells and Sertoli cells were determined for each technique and presented in Table 1.

A correlation of 0.58 was observed ($P = 0.001$) between germ-to-Sertoli cell ratios obtained via histology and ratios obtained via immunohistochemistry (Table 2). In addition, a correlation of 0.69 was observed in germ-to-Sertoli ratio obtained via FNA F and FNA G techniques ($P = 0.001$). No correlations were found, however, in germ-to-Sertoli cell ratios between samples obtained via FNA F and FNA G techniques (3.60±0.47 and 3.59±0.39, respectively) and histology (5.27±0.41) or AR expression by fluorescence (4.44±0.59)

Table 1. Testicular cytology determination in young peri-pubertal bulls using four different techniques.

Trait	Technique			
	FNA F[1] (Mean±SEM)[3]	FNA G[2] (Mean±SEM)	Histology (Mean±SEM)	Immuno-histochemistry (Mean±SEM)
Ratio (germ cells/Sertoli cell)	3.605[a] ±0.468 n=12	3.598[a]±0.393 n=13	5.271[b]±0.409 n=14	4.440[b]±0.599 n=14

[1]FNA F = fine-needle aspiration with a fine needle
[2]FNA G = fine-needle aspiration with a gross needle
[3]SEM = standard error of the mean

The difference found between the ratios obtained by FNA and histology contrasts with the findings of other authors (Aridogan et al., 2003), who obtained a 0.9 correlation in human patients between fine-needle aspiration and histology techniques. To our knowledge, this is the first time that a specific immunohistochemistry fluorescent method against AR has been used in peri-pubertal bulls as a specific predictive tool of potential fertility in the adult individual. AR expression through immunofluorescence has shown to be a specific and novel useful tool for determining Sertoli cell populations in the young bull.

Table 2. Correlation among four different techniques for testicular cytology determination in young peri-pubertal bulls.

Technique		FNA-F[1]	FNA-G[2]	Histology	Immunohistochemistry
FNA-F[1] (n = 22)	Pearson correlation	1.000	0.686	-0.008	-0.217
	P - value	-	0.001	0.971	0.331
FNA-G[2] (n = 22)	Pearson correlation	0.686	1.000	0.175	-0.202
	P - value	0.001	-	0.434	0.366
Histology (n = 27)	Pearson correlation	-0.008	0.175	1.000	0.581
	P - value	0.971	0.434	-	0.001
Immunohistochemistry (n = 28)	Pearson correlation	-0.217	-0.202	0.581	1.000
	P - value	0.331	0.366	0.001	-

[1]FNA-F = fine-needle aspiration with a fine needle
[2]FNA-G = fine-needle aspiration with a gross needle

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