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Research Article

The Effect of Ergot Alkaloid Consumption on Bull Sperm Chromatin Structure and Acrosome Integrity

AN DeCarlo[1], CR Burnett[1], P Rowan[1], R Johnston[1], RE Ricks[1], WC Bridges Jr[2] and Scott L Pratt[1]*

[1]*Animal and Veterinary Sciences Department, Clemson University, Clemson, South Carolina, USA*

[2]*Mathematical Sciences Department, Clemson University, Clemson, South Carolina, USA*

***Corresponding Author**: Scott L Pratt, Animal and Veterinary Sciences Department, Clemson University, Clemson, South Carolina, USA.

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Abstract

Bull sperm chromatin and acrosomal integrity when consuming ergot alkaloids (EA) was assessed. Bulls consumed rations containing or lacking EA and were subjected to semen collection every 21 to 28 days beginning at the start of feeding. Sperm samples, both fixed in formalin and post-thaw were analyzed. To determine chromatin damage, a variation of sperm chromatin structure assay (SCSA) was utilized. This technique was validated by exposing ~1 X 10^6 formalin fixed sperm to UV light at 254 nm, 302 nm or 365 nm for 0, 30, 60 and 90 minutes, in triplicate, to induce chromatin damage prior to staining. Sperm membranes of fixed and post-thaw samples were permeabilized with a 0.1% triton solution, then incubated with a staining buffer containing 6 mg/ml Acridine Orange stain solution and pelleted. Cells were resuspended in Agilent cell chip buffer and analyzed with the Agilent Cell Chip Assay. To evaluate acrosomal integrity, sperm samples from all years were incubated in the presence or absence of PNA-Alexa Fluor 594 (1 mg/ml) and counterstained with DAPI. Smears were produced and sperm were counted from five randomly chosen fields from each slide using a 40x objective on an Axio Imager 2 fluorescence microscope. Sperm were evaluated as percent stained out of total cell number. The SCSA was effective using the Agilent Bioanalyzer Cell Chip Assay; however, EA treatment exhibited no effect on chromatin structure. Sperm labeled with PNA-Alexa Fluor 594 showed 4 staining patterns, and EA altered these patterns, indicating exposure impacts acrosomal integrity.

Keywords: *Acridine Orange; Acrosome; Chromatin; Peanut Agglutinin; Semen*

Abbreviations

AO: Acridine Orange; ANOVA: Analysis of Variance; BCS: Body Condition Score; BW: Body Weight; EA: Ergot Alkaloid; KY31: Kentucky 31; mo: Month; PNA: Peanut Agglutinin; SCSA: Sperm Chromatin Structure Assay; SQA: Semen Quality Analysis

Introduction

Kentucky 31 (KY31) is a widely grazed cultivar of tall fescue in the southeastern United States. Through a mutualistic relationship with the endophyte, *Epiphloeum coenophiala* [1], KY31 is drought resistant, insect resistant, and grazing tolerant, making it very easy to establish and maintain [2]. The endophyte produces ergot alkaloids (EA) that are responsible for tall fescue's high sustainability and adaptability [2]. Cattle grazing forage containing EA exhibit detrimental reproductive effects [3-6]. Limited data are available describing the impact of EA on male reproductive physiology; however, consumption of EA may be associated with impaired semen quality [4-6] and possible reduced fertility as assessed by reduced cleavage rates when utilizing semen from E+ bulls [3]. Ergot alkaloids bind to neurotransmitter receptors [7-10] and elicit physiological responses in cattle that consume them. Unfortunately, some of the literature on semen quality is contradictory, even within labs. Studies report that cattle grazing EA show impaired semen motility, morphology [4,5] and lower semen concentrations [5], while others report no changes in sperm physiology [3,11,12]. The conflicting research on EA consumption relating to sperm physiology could be due to limited methods in which semen quality is measured. Typically, only parameters such as motility and morphology are assessed. However, other forms of quality assessment may be advantageous.

Acrosomal integrity is impaired in semen post-thaw supplemented with ergonovine in the extender [13]. Further, lectin binding sites have been identified within the acrosome in bovine spermatozoa [14]. Peanut agglutinin (PNA), is a lectin that has been shown to bind

to carbohydrates within the acrosome region of bovine sperm cells [15]. Protocols for the use of PNA to validate locations of these lectin binding sites have been performed [16], with lectin receptors identified in the acrosome of both caudal and caput epididymal sperm cells [15].

Chromatin integrity has an impact on fertility, with research showing a reduction in thermal-induced denaturation resistance in chromatin observed in sperm cells in bulls with known low fertility [17]. Further, conformation of chromatin structure plays a role in sperm physiology and therefore, bull fertility, as seen in studies utilizing flow cytometry [17]. Original assessment of sperm DNA contents utilized the sperm chromatin structure assay (SCSA). The SCSA is a flow cytometric assay that allows for assessment of DNA integrity by measuring the susceptibility of DNA contents within sperm cells after degradation by treatment with acid followed by staining with an intercalating dye, acridine orange (AO) [18]. Acridine orange is capable of a metachromatic shift, making it useful for visualization of chromatin damage by assessment of the presence of single stranded DNA. When associated with double-stranded DNA, the dye emits green (525 nm) and red fluorescence (650 nm) when bound to single stranded DNA. The AO dye is also capable of penetrating and accumulating in lysosomes [17,19,20]. Other staining protocols have been utilized, such as visualization using a lectin, PNA. Lectin binding sites are already present in the acrosome of sperm cells and may serve as a target for quality assessment of spermatozoa by staining with PNA [14]. Evaluating acrosome and chromatin integrity through these two staining methods provide an intriguing possibility to assess semen quality aside from the traditional motility and morphology testing already commonly used. Further, use of the Agilent bioanalyzer flow cytometric technology with AO staining provides an intriguing adaptation to the classic SCSA methods.

Objective of the Study

The objective of these studies was to compare two methods for staining sperm cells; a lectin, fluorescent-labeled PNA staining technique, compared to an AO staining procedure adapted to flow cytometry procedures using the Agilent 2100 Bioanalyzer Cell Chip technology. These staining profiles were evaluated in sperm obtained from bulls either consuming EA or a novel endophyte to assess whether EA play a role in male physiology pertaining to acrosome and chromatin integrity in sperm.

Materials and Methods

Animal care and use

All animal research was approved by the Clemson University Institutional Animal Care and Use Committee (Animal Care and Use Protocols ARC2010-45 and 2014-060).

Treatment

Beef bulls (12 to 24 months (mo) of age at the start of exposure to EA) were placed on a ration that included or excluded EA. The study was conducted across a 4-year period beginning in 2011. In 2011, bulls were fed a concentrate ration containing tall fescue seed as described by Stowe., *et al.* 2013. In years 2012, 13 and 14, bulls were subjected to grazing forage containing or lacking EA [21]. Prior to the initiation of treatment, all bulls passed a standard BSE, were weighed (BW) and body condition scores (BCS) recorded. Bulls were then allotted to treatment based on BW and BCS. Electro-ejaculation and semen evaluation was performed on bulls on day (d) 0 of treatment and either every 21d (2011) or every 28d (all other years).

Collection, evaluation, fixation, and freezing of semen

Standard animal handling techniques were used to restrain bulls for electroejaculation procedures. Electroejaculation was performed using the Pulsator IV (Agtech, Manhattan, KS) on a preprogrammed mode [11]. Five hundred microliters of semen from each bull collected was fixed in 9.5 mL of 10% neutral buffered formalin and stored at room temperature. Semen used for cryopreservation was extended

1:1 in a one-step Andromed one-step extender (Minitube of America, Verona, WI, USA) according to manufacturer's instructions at approximately 30 million motile sperm per milliliter. Samples were allowed to equilibrate to room temperature, followed by loading into 0.5 mL straws at approximately 10 million motile spermatozoa per straw. Straws were equilibrated over night at 4°C and frozen in liquid nitrogen vapors for 10 minutes, followed by direct contact with liquid nitrogen for long-term storage.

Acridine orange assay validation

In order to validate a method to adapt the AO staining to utilize the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to evaluate chromatin damage in semen relating to high percentage gated cells, semen with a low percent gated profile on the bioanalyzer was used for the validation assay. Sperm cells were exposed to UV light at 254 nm, 302 nm, or 365 nm for 0, 30, 60 and 90 minutes, in triplicate, to induce chromatin damage. Controls using no UV exposure were run with and without AO stain. Semen (500 ml) was fixed in 10% neutral buffered formalin and stored at room temperature. Approximately one million sperm cells were centrifuged at 10,000 x g for 30 seconds. The supernatant was removed and the sperm pellet was washed with 500 ml ice-cold 1X Tris/NaCl, EDTA (TNE) solution. Two hundred microliters of a 0.1% triton solution (0.1% triton X-100, 0.15 mol/L NaCl, .08N HCL) was added to suspended sperm to allow for permeabilization of the cell membranes and allowed to incubate for 30 minutes. Three hundred microliters of staining buffer (37 mmol/L citric acid, 126 mmol/L Na_2HPO_4, 1 mmol/L EDTA, 15 mol/L NaCl) was added directly to the samples with TNE and triton, with addition of 2 ml of a 6 mg/ml AO stain solution and allowed to incubate at room temperature for 5 minutes. Samples were centrifuged at 10,000 x g for 30 seconds; the supernatant was removed and the pellet was suspended in 100 ml of Agilent Cell Chip Buffer and suspended by gently pipetting up and down to break up the pellet. Samples were analyzed within five minutes of resuspension, using the Agilent Cell Chip Assay.

Assessment of grazing E+ forage impact on formalin fixed sperm physiology using Agilent Chip Technology

Formalin fixed semen samples were subjected to AO staining and assessment by the Agilent Cell Chip Assay as described previously, without subjection to UV light. Samples were assessed using the Agilent Cell Chip Assay according to Agilent's protocol, with samples loaded on to the chip within 5 minutes from the time of resuspension in Agilent cell buffer.

Assessment of grazing E+ forage impact on post-thaw sperm physiology using Agilent Chip Technology

Straws of semen were thawed in a thaw bath at 37°C for 60 seconds followed by fixation in 10% formalin for 30 minutes at room temperature prior to staining. Samples were subjected to AO staining and assessment by the Agilent Cell Chip Assay as described previously, without subjection to UV light. Samples were analyzed using the Agilent Cell Chip Assay according to Agilent's protocol, with samples loaded on to the chip within 5 minutes from the time of resuspension in Agilent cell buffer.

PNA labeling

Two aliquots of 500 µl of formalin-fixed semen from either NE or EA groups, pre- and post-EA exposure, from bulls on 2011 (n=14), 2012 (n = 21), 2013 (n = 12) and 2014 (n = 25) studies were centrifuged at 5000 x g for 5 minutes and washed in 1X phosphate-buffered saline (PBS). One sperm pellet was resuspended in 200 µl of PNA-Alexa Fluor 594 (1 mg/ml) (Molecular Probes, Eugene, OR) diluted 1:1000 with 1X PBS and 0.5% bovine serum albumin (BSA). The pellet was resuspended in 200 µl of 1X PBS and 0.5% BSA to serve as the negative control. Samples were incubated in the dark at RT for 1h and then washed two more times with 1X PBS and 0.5% BSA, centrifuging at 5000 x g for 5 minutes in between washes. Supernatants were discarded and the sperm pellet counterstained after the last wash with 200 µl of 1:2000 diluted DAPI (1 mg/ml) with 1X PBS and 0.5% BSA (Molecular Probes, Eugene, OR). Samples were incubated in the dark for 15 - 20 minutes and washed twice as previously stated. Final resulting sperm pellets were resuspended in 150 µl of 1X PBS.

Cell counting and fluorescence microscopy

Smears of each resuspended, stained sperm pellets were made using 15 µl of each sample per slide and smearing the sample using the edge of another glass slide. These smears were allowed to air-dry and coverslips were placed using a drop of 1X PBS. Sperm cells were examined using a 40x objective lens on an Axio Imager 2 fluorescence microscope (Zeiss, Oberkochen, Germany) using filters appropriate for Alexa Fluor 594 and DAPI staining. Five fields per slide were examined and selected at random. Photographs were taken using an Axio Cam MRm (Zeiss, Oberkochen, Germany). All photographs were saved as image files (.tif) and uploaded to Infinity Analyze software (Lumenera Corporation, Ottawa, Canada) to later count stained sperm cells.

Statistical analysis

For the PNA fluorescence data, the response variable of interest was cell counts of the stained sperm cells expressed as a percentage of total cells counted from each sample. For this response variable, the statistical model included main effects and interactions of TRT, year, month and d. For the Acridine orange-stained cell data modified for the Agilent Cell Chip Assay, the response variable of interest was chromatin damage. For this response variable, the statistical model included main effects and interactions of TRT and month. For the validation assay data, the response variable of interest was chromatin damage. For this response variable, the statistical model included main effects and interactions of UV wavelength and time of exposure.

For all response variables, an analysis of variance (ANOVA) was performed to determine significance of the main effects and interactions. T-tests among LS means were performed to further study significant main effects and interactions. All statistical calculations were performed using JMP (SAS Inst. Inc., Cary, NC). Statistical significance was defined as ($P < 0.05$).

Results

Agilent AO assay validation

Wavelength ($P = 0.0073$) and exposure time ($P = 0.0067$) were both significant in inducing DNA damage. Percentage gated cells (red dots within the gated region) increased with exposure, peaking at 302 nm wavelength compared to other wavelengths (Figure 1A). Damage increased with increasing exposure time, regardless of wavelength (Figure 1B). An example of the validation of the SCSA is shown in figure 2. These data indicate that the SCSA can be adapted to the Agilent 2100 Bioanalyzer Cell Chip technology and can detect sperm chromatin damage.

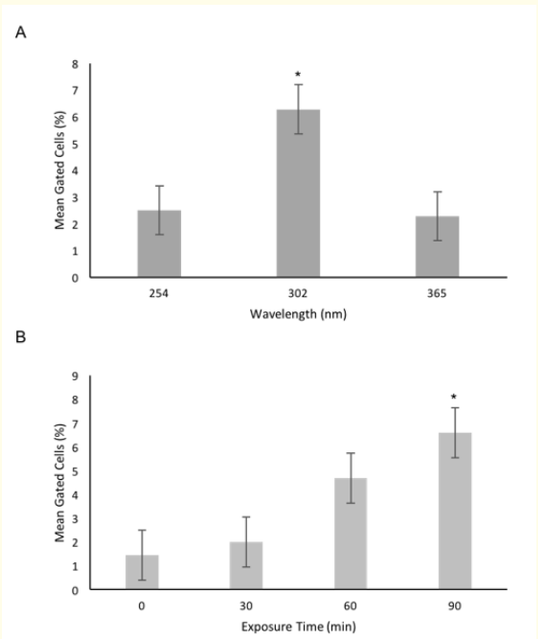

Figure1: *Validation of an Acridine Orange staining assay: (A) Effect of wavelength and (B) exposure time on the percentage of gated cells for red fluorescence indicating chromatin damage. Significant exposure time and wavelength are indicated by an asterisk (*).*

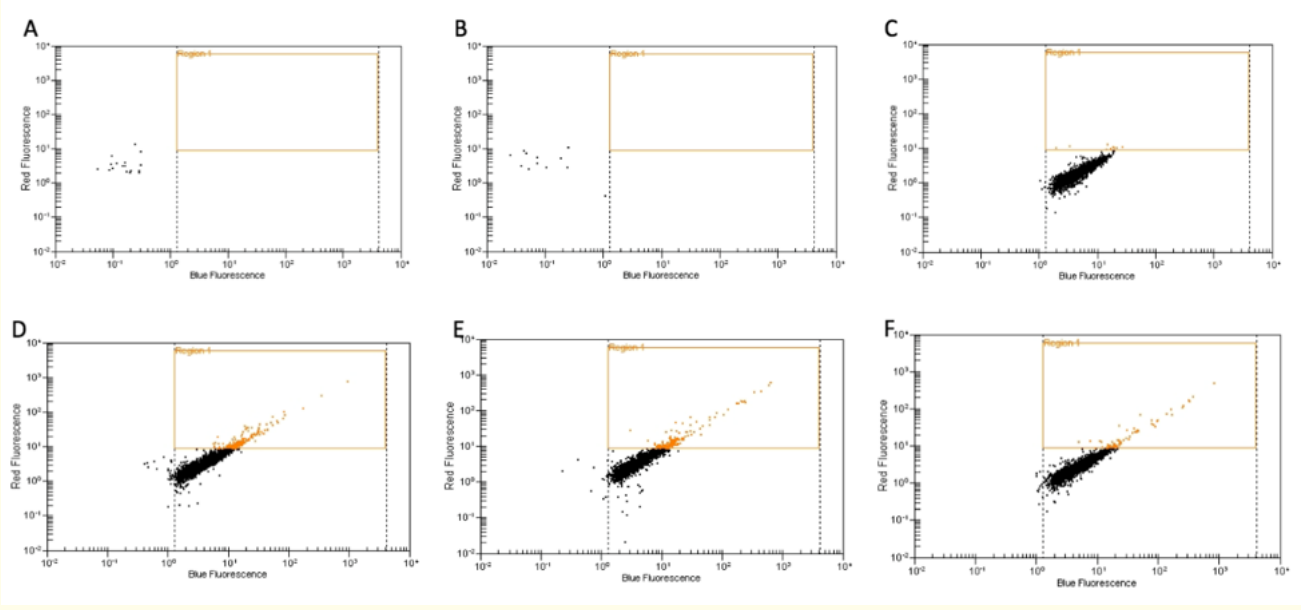

Figure2: *Sperm chromatin structure assay (SCSA) validation using semen exposed to UV light at 302 nm. Each dot represents a single sperm cell with red dots in the gated region indicating chromatin damage. Panels (A), (B), and (C) are controls for staining and UV light. Panel (A) was exposed at 302 nm for 1 hour with no Acridine Orange (AO) staining. Panels (B) and (C) were not exposed to UV light with panel (B) AO stained and panel (C) without AO stain. Panel (D), (E), and (F) were AO stained and exposed to 302nm UV light for 90, 60, and 30 minutes, respectively.*

Agilent SCSA

The Agilent SCSA was applied to sperm samples that were fixed in formalin or post-thaw. In formalin fixed samples, no difference in chromatin damage was detected for treatment, month, or treatment by month interactions ($P > 0.05$) with percent gated cells remaining fairly high across all formalin fixed semen samples. Differences in pattern formation of dot plots was present in formalin fixed semen samples, with unique population placement of percent gated cells versus normal sperm cells. Three patterns of AO gated cells were obtained for formalin fixed semen samples, depicted in figure 3. In post-thaw semen, differences were observed across month with bull samples collected in March at a higher percentage gated cells than those in April ($P = 0.0097$). However, no differences were observed due to treatment (Table 1). Post-thaw semen exhibited only a single staining pattern on dot plots that matched one out of the three patterns observed in formalin fixed samples (Figure 3C).

	E-†	E+‡
Post-Thaw Semen (%)	55.08 ± 7.43[a]	32.46 ± 5.17[a]
Formalin Fixed Semen (%)	10.17 ± 3.17[a]	12.04 ± 4.69[a]

Table 1: *Mean percent gated cells ± SEM of post-thaw and formalin fixed semen from bulls consuming a diet containing or lacking ergot alkaloids.*
Means within the same row possessing a different superscript lowercase letter differ due to treatment[1].

[1]† Diet lacking ergot alkaloids.
‡ Diet containing ergot alkaloids.

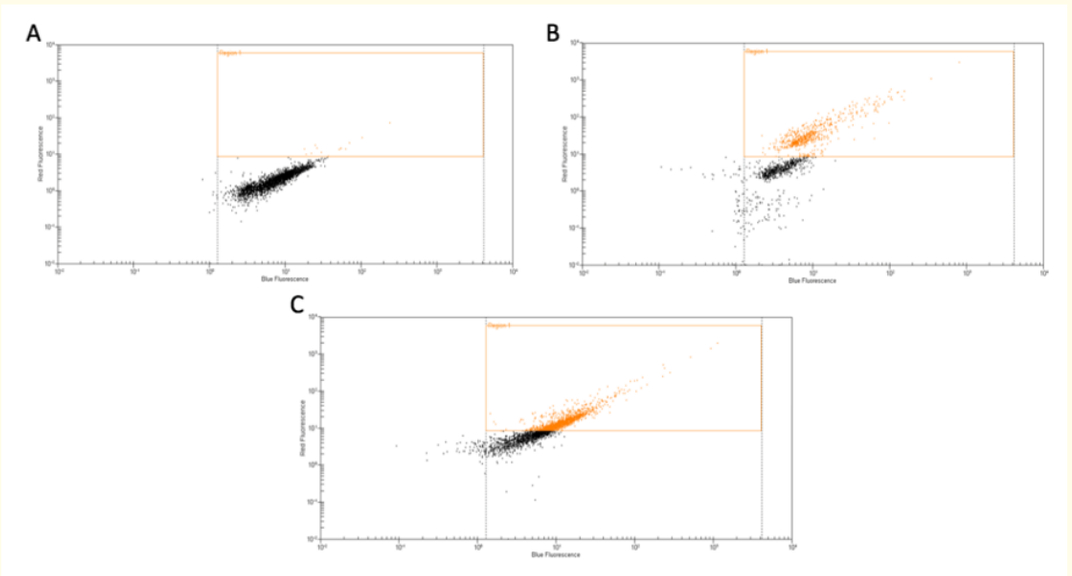

Figure3: *Examples of dot plots of sperm sample profiles from three different bulls subjected to Sperm chromatin structure assay (SCSA). Each dot represents a single sperm cell. Black indicates cells with intact chromatin and red, within the gated region, indicates impaired chromatin. Panel (A) semen (control, with known profile of low percent gated cells) category 1 depicts one grouping of stained cells below the percent gated region. Panel (B) depicts category 2 with two populations of stained cells, one within the gated region and one outside of the gated region. Panel (C) one grouping of stained cells that connects between the gated region and ungated region.*

PNA fluorescence

There were four staining profiles observed from the PNA-Alexa Fluor 594-labeled sperm cell samples (Figure 4). Patterns were defined by how much of the acrosome region was fluorescently labeled. Pattern 1 exhibited slight, irregular shaped fluorescence; pattern 2 showed a thin layer of staining directly on the anterior portion of the acrosome; pattern 3 had uniform staining of the complete acrosome; pattern 4 showed no fluorescence at all. There were significant TRT effects pertaining to patterns 2 and 3 ($P < 0.05$), with no TRT or TRT x d interaction observed for patterns 1 and 4 ($P > 0.05$) (Figure 5).

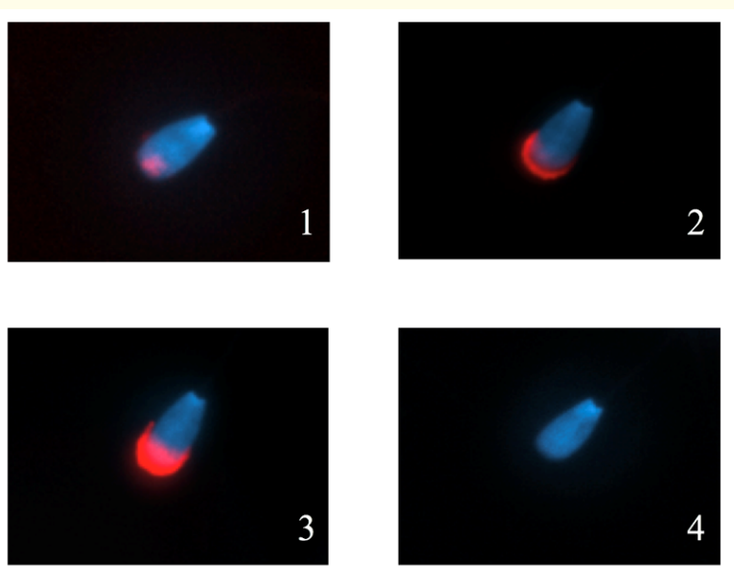

Figure4: *Acrosomal staining patterns of PNA-Alexa Fluor 594 stained bull spermatozoa, as assessed with fluorescence microscopy. The acrosome region displayed (1) patchy fluorescence, (2) uniform staining of the anterior head, (3) staining of the entire acrosomal region, (4) no staining in the acrosomal region. Sperm cells exhibited blue fluorescence due to counterstaining with the DNA dye DAPI.*

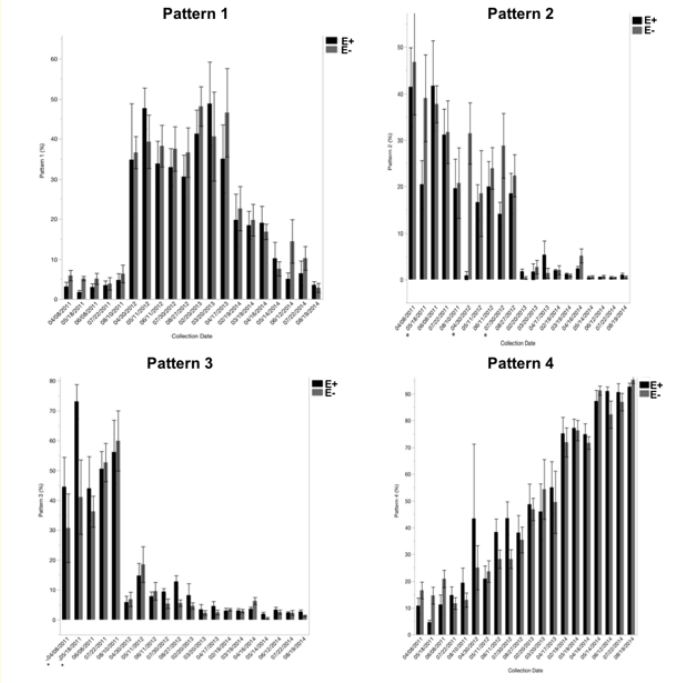

Figure5: *Mean percentages of cells labeled with fluorescent PNA as a percentage of total cells counted. Four PNA staining patterns were observed for PNA staining of sperm obtained from bulls consuming rations containing or lacking ergot alkaloids. Patterns 1, 2, 3, and 4 are shown in panel A, B, C, and D respectively. Date of collection is given on the bottom axis, the percentage of cells positively stained within each group is shown on the y-axis and consumption of ergot alkaloids is shown by E+ (black) and E- (grey).*

Discussion

Of the limited research focusing on the reproductive performance of bulls grazing toxic fescue in relation to fertility, results are contradictory. With variability of the impact of EA on male reproduction between and within labs, we tested two assays known to measure the ability of sperm to penetrate and fertilize ovum.

Chromatin integrity was evaluated using an adapted AO procedure for utilizing flow cytometry and fluorescently-labeled PNA staining for assessment of acrosomal integrity. Acridine orange has been previously validated for use in staining sperm cells [17,19,20]. There are studies identifying lectin binding sites within these germ cells [14], as well as research using PNA to stain bull spermatozoa [16]. Acridine orange staining utilizing Agilent Cell Chip technology validated a method to evaluate sperm cell chromatin damage but did not provide any evidence of an impact on EA consumption and bull spermatozoon quality. Interestingly, there seemed to be a limit as to how much UV damage can be induced before the percent gated becomes very low at 365 nm, possibly due to cross-linking of DNA within the samples at an increased wavelength. Crosslinking of samples caused an issue with consistency of results from chip to chip in which samples became difficult to resuspend fully. Although attributed to crosslinking, there is a possibility that difficulties could have been due to DNA-protein interactions as well.

Unique profiles were revealed between samples with four different population types. Population placement and scatter on dot plots depend upon light refraction angles, cell size, and cell granularity or type [22]. Larger cells depend more on angle of light with smaller

cells being hard to read. Sperm cells are approximately 50 μm making them very angular dependent in flow cytometry. The differences between population profiles could have been due to a possible morphology issue that some bulls had at the time of collection, with the sperm cells in the gated region showing up separate because the granularity and size of the damaged cells were different. However, semen quality analysis (SQA) data for semen used in the validation process was acceptable at > 70% morphology (data not shown). Differences could also be attributed to the fixation process, as formalin-fixed semen varied in profiles while post-thaw semen displayed only one profile type.

Flow cytometry is commonly used for semen analysis and research done to assess chromatin in semen samples shows differences in fluorescence populations on dot plots with increased chromatin condensation [23]. Similar research measured differences in heat treatment of sperm cells and their resistance based on chromatin and its relationship to fertility [17]. Interestingly, Evenson's lab hypothesized that misshapen sperm cell heads indicate chromatin heterogeneity and, therefore, issues with fertility. Their results show a larger scatter on the dot plot with increased heat and induction of heterogeneity, similar to findings in this study which show populations separating in some samples with damaged chromatin. Future research is needed to further explore the meaning of these population profiles.

Fluorescently-labeled PNA provided 4 unique patterns for acrosomal staining, ranging from no staining at all, patchy staining, to full acrosomal staining. This study validates PNA labeling for the use of evaluating acrosomal integrity in spermatozoa. Data were inconsistent between staining patterns and treatment impact, with only patterns 2 and 3 revealing a TRT effect. These patterns are staining of the anterior end of the acrosome (pattern 2) and full staining of the whole acrosome (pattern 3). Previous research from our laboratory shows that consuming ergot alkaloids affects survivability of semen post-thaw [5]. The observations seen here with PNA labeling indicate no effect of E+ forage on acrosomal integrity, which disagrees with what was observed in E+ bull semen post-thaw, and would suggest that acrosomal integrity may be compromised as a result of consumption of E+ forage. With prior research providing evidence of EA effects on acrosomal integrity [13], the variability between PNA staining patterns observed here, and the variability between results in this study compared to previous research from this laboratory, further investigation is warranted.

Conclusion

In conclusion, grazing E+ forage does not negatively impact sperm physiology through induction of chromatin damage, but the Agilent Bioanalyzer Cell Chip Assay can be adapted for use in detecting chromatin damage in formalin-fixed and post-thaw sperm cells using AO staining methods. Assessing acrosomal integrity utilizing fluorescently-labeled PNA is able to provide 4 unique patterns of staining to view acrosomal integrity, but there remains some variability in results concluding an effect on acrosomal integrity in regard to the identified staining patterns. These two methods may provide further insight in spermatozoa chromatin and acrosome integrity, but further research is needed to assess what role EA play in sperm cell physiology and fertility of bulls.

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Conflict of Interest

The authors state that there is no conflict of interest that could be noted as bias the results of the research reported.

Bibliography

1. Young Carolyn A., *et al*. "Characterization of *Epichloë coenophiala* within the US: are all tall fescue endophytes created equal?". *Frontiers in Chemistry* 2 (2014): 95.

2. Hoveland Carl S. "Importance and economic significance of the Acremonium endophytes to performance of animals and grass plant". *Agriculture, Ecosystems and Environment* 44.1-4 (1993): 3-12.

3. Schuenemann GM., *et al*. "Fertility aspects in yearling beef bulls grazing endophyte-infected tall fescue pastures". *Reproduction, Fertility and Development* 17.4 (2005): 479-486.

4. Looper ML., *et al*. "Influence of toxic endophyte-infected fescue on sperm characteristics and endocrine factors of yearling Brahman-influenced bulls". *Journal of Animal Science* 87.3 (2009): 1184-1191.

5. Pratt SL., *et al*. "Bulls grazing Kentucky 31 tall fescue exhibit impaired growth, semen quality, and decreased semen freezing potential". *Theriogenology* 83.3 (2015): 408-414.

6. Page Ryan., *et al*. "Ergot Alkaloid Effects on Bovine Sperm Motility *In Vitro*". *Advances in Reproductive Sciences* 7.1 (2018): 7-15.

7. Mizinga KM., *et al*. "Neural dopamine D2 receptors in rats fed endophyte-infected fescue seed". *Drug and Chemical Toxicology* 16.3 (1993): 307-319.

8. Larson BT., *et al*. "Ergovaline binding and activation of D2 dopamine receptors in GH4ZR7 cells". *Journal of Animal Science* 73.5 (1995): 1396-1400.

9. Wang Hehai., *et al*. "Involvement of signaling pathways in bovine sperm motility, and effect of ergot alkaloids". *In Vitro Cellular and Developmental Biology-Animal* 45.8 (2009): 483-489.

10. Schöning C., *et al*. "Complex interaction of ergovaline with 5-HT2A, 5-HT1B/1D, and alpha1 receptors in isolated arteries of rat and guinea pig". *Journal of Animal Science* 79.8 (2001): 2202-2209.

11. Stowe HM., *et al*. "Effects of fescue toxicosis on bull growth, semen characteristics, and breeding soundness evaluation". *Journal of Animal Science* 91.8 (2013): 3686-3692.

12. Burnett CR., *et al*. "Assessment of semen quality and fertility in young growing beef bulls exposed to ergot alkaloids". *Theriogenology* 118 (2018): 219-224.

13. Gallagher GR and PL Senger. "Effect of phenylephrine, ergonovine, oxytocin and norepinephrine as an extender ingredient on viability of bovine spermatozoa". *Journal of Animal Science* 67.6 (1989): 1573-1576.

14. Sinowatz F and AE Friess. "Localization of lectin receptors on bovine epididymal spermatozoa using a colloidal gold technique". *Histochemistry* 79.3 (1983): 335-344.

15. Cross NL and SK Watson. "Assessing acrosomal status of bovine sperm using fluoresceinated lectins". *Theriogenology* 42.1 (1994): 89-98.

16. Arya M., *et al*. "Lectin-binding pattern of bull testis and epididymis". *Journal of Andrology* 6.4 (1985): 230-242.

17. Evenson DP., *et al*. "Relation of mammalian sperm chromatin heterogeneity to fertility". *Science* 210.4474 (1980): 1131-1133.

18. Evenson Donald P. "The Sperm Chromatin Structure Assay (SCSA) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility". *Animal Reproduction Science* 169 (2016): 56-75.

19. Ballachey BE., *et al*. "Flow cytometry evaluation of testicular and sperm cells obtained from bulls implanted with zeranol". *Journal of Animal Science* 63.3 (1986): 995-1004.

20. Ballachey BE., *et al*. "Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility". *Biology of Reproduction* 36.4 (1987): 915-925.

21. Burnett CR., *et al*. "Effects of grazing tall fescue containing ergot alkaloids on bull sperm cryopreservation". *Animal Reproduction Science* 181 (2017): 24-29.

22. Davey Hazel M and Douglas B Kell "Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses". *Microbiology and Molecular Biology Reviews* 60.4 (1996): 641-696.

23. Golan R., *et al*. "Evaluation of chromatin condensation in human spermatozoa: a flow cytometric assay using acridine orange staining". *Molecular Human Reproduction* 3.1 (1997): 47-54.

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