Animal Reproduction Science 207 (2019) 107–117

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Nerve growth factor-β effects on post-thaw bull semen quality: Effects of nerve growth factor-β added to extenders for cryopreservation of electro-ejaculated and epididymal bull semen

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ARTICLE INFO

Keywords:
Bovine
Flow cytometry
Motility
Nerve growth factor-beta
Spermatozoa

ABSTRACT

Nerve growth factor-β (NGF) is a seminal plasma protein associated with improved sperm membrane integrity and motility in mammalian species. The objective of this study was to compare post-thaw semen quality from both ejaculated and pididymal-collected bull sperm incubated with purified NGF prior to cryopreservation. Semen was obtained from Angus × Simmental crossbred bulls ($n = 10$) collected by electroejaculation, followed by castration and epididymal sperm collections 3 days later. Semen samples were incubated with extender having 0 ng/mL (CONT), 0.5 ng/mL (LOW), 5 ng/mL (MED), or 50 ng/mL (HIGH) of purified NGF prior to cryopreservation. Sperm motility was assessed in each sample prior to treatment and cryopreservation and at post-thaw. Flow cytometry was used for post-thaw assessment of sperm viability (SYBR-14/PI), acrosome integrity (FITC-PNA/PI), and chromatin stability (acridine orange). Values for post-thaw sperm motility and velocity variables were decreased, while linearity was increased in samples of the HIGH compared with CONT group ($P < 0.01$), but there were no differences in epididymal samples ($P > 0.05$). Samples from the HIGH group also had a lesser amplitude of lateral head displacement at 2.5 and 3 h post-thaw ($P < 0.01$). Post-thaw sperm viability, acrosome integrity, and DNA fragmentation index were not affected by NGF treatment in either ejaculated or epididymal sperm ($P > 0.05$). In conclusion, supplementation of freezing extender with NGF had minimal effects on post-thaw sperm quality in bulls. Results indicate NGF may have a function in preventing premature sperm hyperactivation in ejaculated, but not epididymal-collected spermatozoa. Fertility studies, both *in vitro* and *in vivo*, are warranted to ascertain the relevancy of these findings.

1. Introduction

Results of several studies indicate there is an association between seminal plasma proteins and bull fertility (Killian et al., 1993; Henault et al., 1995; Moura et al., 2006; Viana et al., 2018). The role of seminal plasma proteins in regulating fertility is of special relevance to the cattle industry where artificial insemination has advanced genetic selection of desirable traits such as milk

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https://doi.org/10.1016/j.anireprosci.2019.06.010
Received 25 January 2019; Received in revised form 24 May 2019; Accepted 10 June 2019
Available online 11 June 2019
0378-4320/

production and meat quality (Hayes et al., 2009). Historically, bull semen studies have focused on improving semen freezing techniques and determining the minimum effective breeding dose for artificial insemination, while overlooking the effects that semen processing and freezing may have on seminal plasma protein functions (Schjenken and Robertson, 2014). Though early studies identified nerve growth factor-β (NGF) in the seminal plasma of bulls (Harper and Thoenen, 1980; Harper et al., 1982), little is known regarding its function in reproduction of cattle. Seminal plasma NGF has been implicated as a signaling molecule in the female reproductive tract with potential functions in increasing gene expression for gonadotropin receptors in oviduct epithelial cells of cattle (Li et al., 2014) and enhancing corpus luteum function in heifers (Tanco et al., 2012) and cows (Stewart et al., 2018b; Tribulo et al., 2015).

In addition to several other proteins, sperm *NGF* mRNA abundance in bulls was strongly associated with positive sire conception rates (Kasimanickam et al., 2012), which was attributed to beneficial effects of NGF on sperm membrane integrity (Li et al., 2010). An immunolabelling study of ejaculated bull sperm indicated that NGF was localized to the sperm head and tail and its receptor (TRKA) was present in the acrosomal cap, nucleus, and tail regions (Li et al., 2010), suggesting that NGF may have a physiological function in sperm. Consistently, in another study there was seminal plasma concentrations of NGF that were positively associated with sire conception rates (Stewart et al., 2018a). In seminal plasma, there was abundances of *NGF* mRNA positively associated with the maintenance of post-thaw functional membrane integrity in bull spermatozoa, indicating that it could be used to assess cryotolerance (Shilpa et al., 2017). In results of another study, there was greater abundances of spermatozoal *NGF* mRNA bulls with greater quality semen (< 25% discarded ejaculates) compared with those with lesser quality semen (> 40% discarded ejaculates), though there was no correlation between abundance of *NGF* mRNA and field conception rates was detected (Parthipan et al., 2017). Relative abundances of *NGF* mRNA were also positively associated with pre-freeze mitochondrial membrane potential and post-thaw sperm velocity variables in *Bos taurus* bulls (Parthipan et al., 2017). In both normozoospermic and asthenozoospermic men, supplementation of freezing extender with NGF at 0.5 ng/mL improved sperm viability and motility and decreased DNA fragmentation (Saeednia et al., 2016, 2015). Consistently, treatment of frozen-thawed bull sperm cells with 40–80 ng/mL NGF increased both leptin secretion and sperm membrane integrity (Li et al., 2010).

A recent study assessing the abundance of NGF in the bull reproductive tract determined that this protein is mainly expressed in the accessory sex glands (Bogle et al., 2018; Stewart et al., 2018a). Because these glands are the main source of the seminal plasma (Moura et al., 2006) and epididymidal-collected sperm are not exposed to these secretions of these glands, this type of semen allows for some insights on the putative functions of NGF of non-ejaculated sperm. Freezing of epididymal semen is not typically performed in bulls, but with specific cases of sudden death or injury of a genetically valuable bull, collecting epididymal sperm for cryopreservation can be useful (Martins et al., 2007). Based on all the previous findings, NGF may be an optimal candidate protein to enhance sperm cryopreservation techniques in bulls. Thus, the objective of the current study was to compare post-thaw semen quality from both ejaculated and epididymal-collected bull sperm extended with final concentrations of 0 ng/mL (CONT), 0.5 ng/mL (LOW), 5 ng/mL (MED), or 50 ng/mL (HIGH) of NGF prior to cryopreservation. It was hypothesized that supplementation of freezing extender with purified NGF would improve post-thaw motility, viability, acrosome integrity, and chromatin stability of bull sperm.

2. Materials and methods

2.1. Animal care and use

Animals in this experiment were cared for in accordance with guidelines in the Guide for the Care and Use of Agricultural Animals in Agriculture Research and Teaching (FASS, 2010). All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois in Urbana-Champaign (Protocol #17240).

2.2. Animals

Angus × Simmental crossbred bulls (n = 10; age: 15.1 ± 0.05 m) were housed at the University of Illinois Urbana-Champaign Beef Production Unit in Urbana, IL. At the time of treatment allocations, bulls weighed 585 ± 17 kg and had a body condition score of 5.8 ± 0.2 out of 9 using a previously validated scoring system (Eversole et al., 2009). A complete breeding soundness examination performed 30 days before enrollment in the experiment indicated there were no symptoms of fertility problems. Scrotal circumference was measured in cm using a scrotal tape measure placed around the widest portion on the scrotum. The average scrotal circumference was 40.5 ± 0.6 cm (range: 36–42 cm), which is in accordance with the criterion determined by the Society for Theriogenology to classify a bull as a satisfactory potential breeder (Chenoweth et al., 2010).

2.3. Semen collection by electroejaculation

All bulls were collected by electroejaculation. To obtain semen samples, a 60 mm upright weighted bull probe (Lane Manufacturing, Inc., Denver, CO, USA) was placed rectally, and the programmed cycle on the electroejaculator (Pulsator IV, Lane Manufacturing, Inc.,) was used. If an appropriate ejaculate was not obtained, the electroejaculator cycle was conducted manually by an experienced clinician. Semen was collected into 15 mL conical tubes using collecting handles coupled with a disposal plastic cone. An aliquot of each ejaculate was formalin-fixed for morphological analysis at a later time. The remainder of the sample was diluted with pre-warmed (37 °C) Optixcell extender (IMV Technologies, Maple Grove, MN) at a 1:1 ratio and transported approximately 30 min later to the investigators' laboratory in a 37 °C water bath, where further processing and analyses were performed as

subsequently described in this manuscript.

2.4. Epididymal sperm collection

Three days after semen collection, all the bulls were castrated using a Newberry knife, an emasculator and ligature of the spermatic cord. A pudendal block and local anesthesia were performed with 40 and 20 ml of 2% lidocaine, respectively. To prevent pain post-castration, the bulls were administered flunixin meglumine (3.3 mg/kg transdermal pour-on, Merck Animal Health, Madison, NJ, USA) and then repeated as needed based on bull's demeanor (*i.e.*, depressed, refusing feed, laying down). Bull demeanor, food intake, rectal temperature, and surgical incision were monitored daily by the investigators with the assistance of the farm personnel. Testicles were transported to the investigators' laboratory within 30 min after castration. One testicle from each bull was subjected to sperm collection from the cauda epididymis. Briefly, the cauda epididymis was excised and removed from the testicle and body of the epididymis using a #10 scalpel. The cauda epididymis was rinsed with saline and placed in a petri dish. A #10 scalpel was used to dissect the cauda epididymis, and ~10 mL Optixcell extender (IMV Technologies) was used to flush the semen from the tubules. The flush yielded an average concentration of 889 ± 155 million spermatozoa per mL. After the cauda epididymis was determined to be thoroughly flushed, an aliquot of the pre-diluted semen was formalin-fixed for morphological analysis, while the remaining semen underwent further processing for motility analyses and cryopreservation as described below. The epididymal sample from one bull was excluded from data analyses due to extremely poor semen quality before cryopreservation (~4% overall motility).

2.5. Semen analyses and cryopreservation

Sperm concentration from each sample was measured using a NucleoCounter (ChemoMetec Inc., Bohemia, NY, USA). Ejaculated and epididymal sperm from each bull and collection were divided into one of four treatments to achieve a final sperm concentration of 40 million spermatozoa per mL and a final NGF concentration of 0 ng/mL (CONT), 0.5 ng/mL (LOW), 5 ng/mL (MED), or 50 ng/mL (HIGH). The NGF used was purified from seminal plasma of bulls and used previously for *in vivo* studies with cows (Stewart et al., 2018b). Briefly, NGF purification from pooled bull seminal plasma was performed using a combination of anion (Q-Sepharose column) and cation (SP Sepharose column) exchange chromatography with gradient elution to fractionate proteins based on their effective charges. The purity of NGF was determined to be 59.35%, with only minor contaminants present (see Stewart et al., 2018b).

Computer-aided sperm analysis (SpermVision, MiniTube of America, Inc., Verona, WI, USA) was used to measure overall and progressive motilities in pre-freeze CONT samples using a phase-contrast microscope with a heated (37 °C) stage (20×; Olympus BX41; Olympus Corporation, Center Valley, PA, USA). Data were obtained by averaging motility measurements of a minimum of 500 cells or seven fields from various portions of the slide. Sperm output measurements used to describe sperm motion included average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), straightness of the average path (STR, %), linearity of the curvilinear path (LIN, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, μm), and beat cross frequency (BCF, number per second) (Amann and Waberski, 2014). The CASA-established setup variables were as follows per manufacturer defaults for bull sperm: frame capture speed rate, 60 Hz; cell size (min/max), 18/60 μm^2; threshold straightness, 50%; VAP cutoff, 56 μm/s; and VSL cutoff, 28 μm/s.

Immediately following the final dilution, the samples were manually loaded into 0.5-mL semen straws, sealed ultrasonically, and incubated in a cold room at 5 °C for 3 to 5 h per manufacturer's recommendation. Following incubation, straws were placed at 4 cm above the surface of liquid nitrogen for 15 min and then submerged and stored in liquid nitrogen until post-thaw analysis (~5 months). Straws were thawed in a water bath at 37 °C for 1 min. The contents of each straw were dispensed into a 1.8 mL Eppendorf tube, homogenized by reverse pipetting, and then incubated at 37 °C for 4 h, while motility parameters were assessed every 30 min intervals. The remainder of each thawed straw was formalin-fixed for post-thaw morphological analysis. One clinician evaluated 100 sperm cells from each sample under 100× magnification with a phase contrast microscope, classified each sperm cell as normal or abnormal, and further defined abnormalities as primary (proximal droplets, mal-development, midpiece defects, strongly folded tails) or secondary (distal droplets, simple bent tails, tailless) defects, as previously described (Kuster et al., 2004; Chenoweth et al., 2010).

2.6. Fluorescent probes for sperm evaluation

The flow cytometry assays used in the present study were performed according to well-established protocols published elsewhere (Martínez-Pastor et al., 2010, 2004; Robles and Martínez-Pastor, 2013). Semen straws (40 million spermatozoa/mL) from each bull, collection, and treatment were thawed individually and subjected to each staining protocol as described below. A list-mode data file for the data acquired herein is available upon request from the corresponding author. The LIVE/DEAD® Sperm Viability Kit (SYBR-14 and propidium iodide (PI) dyes; #L-7011) was purchased from Molecular Probes, Inc. (Eugene, Oregon, USA). Fluorescein isothiocyanate-conjugated pea (*Pisum sativum*) agglutinin (FITC-PNA; #L-7381) was obtained from Sigma Chemical Co. (Saint Louis, Missouri, USA). Acridine orange, C.I. (#04539-500) was purchased from Polysciences, Inc. (Warrington, Pennsylvania, USA). Dimethyl sulfoxide, anhydrous (DMSO; #D12345), triton X-100 surfact-amps detergent solution (#85111), and phosphate-buffered saline (PBS, pH 7.4) (#10010-023) were purchased from Thermo Fischer Scientific (Waltham, Massachusetts, USA). All other chemicals used were reagent grade and purchased from Sigma Aldrich. When necessary, manual compensation of the sperm flow cytometry analysis was performed with the aid of a positive control for each probe used. The instrument was set for a low flow (< 1000 cells/seconds).

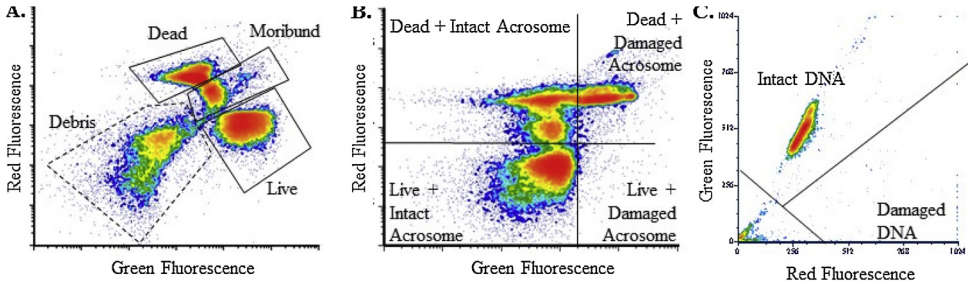

Fig. 1. Representative density plots for flow cytometric analyses performed on post-thaw bull semen. (A) Sperm viability assay using SYBR-14 (green fluorescence, X-axis) and propidium iodide (PI; red fluorescence, Y-axis). The top left gate encloses PI positive events, corresponding to dead sperm. The bottom right gate encloses SYBR-14 positive events, corresponding to live sperm. The top right gate represents events with mixed fluorescence, indicating moribund sperm. (B) Acrosomal integrity assay using FITC-PNA (peanut agglutinin conjugated with fluorescein isothiocyanate; green fluorescence, X-axis) and PI (red fluorescence, Y-axis). The lower quadrants contain live sperm (low PI) with either intact acrosomes (low FITC-PNA) on the left or damaged acrosomes (high FITC-PNA) on the right. The upper quadrants encompass the populations of dead sperm (high PI), with damaged acrosomes (high FITC-PNA) on the right and intact acrosomes (low FITC-PNA) on the left. (C) Chromatin stability assay using acridine orange. Units indicate fluorescence intensity with binding to double stranded (intact) DNA producing green fluorescence (Y-axis) and binding to single stranded (damaged) DNA or RNA producing red fluorescence (X-axis). The elongated cloud on the left is the sperm population with good chromatin integrity (Intact DNA) emitting green fluorescence. Dots to the right of the diagonal line are cells outside of the main population that bear loose chromatin (Damaged DNA) emitting red fluorescence (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.7. Assessment of sperm viability

Sperm viability was assessed using a combination of SYBR-14 and propidium iodide (PI) based on the integrity of the sperm plasma membrane (Garner et al., 1994). The SYBR-14 was used as a membrane-permeable DNA intercalating agent with maximum emission of 516 nm (green) and readily stains all nuclei. Propidium iodide was also used as an intercalating agent with maximum emission of 617 nm (red), but it only stains the nucleus if the sperm plasma membrane is damaged, which makes it a useful counterstain for dead cells. Briefly, stock solutions of SYBR-14 (0.02 mM in DMSO) and PI (2.4 mM in water) were prepared and frozen in aliquots at −20 °C for subsequent use. Staining solution was prepared on the day of flow cytometry analysis by combining 5 μL SYBR-14 (0.02 mM) and 5 μL PI (2.4 mM) per mL of HEPES buffer (10 mM Hepes, 150 mM NaCl, 0.1% BSA, pH 7.4). Working concentrations of SYBR-14 and PI were 100 nM and 12 μM, respectively. Cryopreserved semen was thawed in a 37 °C water bath for 1 min, and 25 μL of thawed semen was added to 0.5 mL staining solution to create a final concentration of 2 million spermatozoa per mL. The samples were mixed by reverse pipetting and subsequently incubated in the dark at 37 °C for 10–15 min prior to flow cytometric analysis.

Flow cytometry analysis of stained samples were done using a full-spectrum detector based (filter-less) Cytek Aurora Flow Cytometer (Cytek™ Biosciences Inc., Fremont, CA, USA). The two dyes were excited in the flow cytometer using a 488-nm argon excitation laser. Fluorescent data of all events was collected until 10,000 gated events were recorded. Two-dimensional plots of SYBR-14 (green) compared with PI (red) fluorescence events were drawn, and debris was gated out based on those events that emitted minimal fluorescence (Fig. 1A). As previously described (Robles and Martínez-Pastor, 2013), three sperm populations in the SYBR-14/PI stained sperm preparations were present. Presumptively live cells with intact cell membranes made up population 1, with only green fluorescent signal (SYBR-14 positive) detected. Population 2 exhibited a mixed green (SYBR-14 positive) and red (PI positive) fluorescence and were considered to be moribund sperm, with early or minor membrane damage. Population 3 comprised cells with only red fluorescent signal (PI positive) detected and were considered dead. The percentage of cells in each of the three populations was calculated.

2.8. Assessment of acrosome integrity

Acrosome integrity was assessed using a combination of FITC-PNA (peanut agglutinin conjugated with fluorescein isothiocyanate) and PI as previously described (Robles and Martínez-Pastor, 2013). The FITC-PNA has a maximum emission of 521 nm (green) and targets the inner leaflet of the outer acrosomal membrane, identifying damaged acrosomes. Again, PI was used as a counterstain to allow for simultaneous assessment of sperm viability and condition of the acrosome. Briefly, stock solutions of FITC-PNA (1 mg/mL in PBS) were prepared and frozen in aliquots at −20 °C for subsequent use (Robles and Martínez-Pastor, 2013). Staining solution was prepared by combining 0.625 μL PI (2.4 mM) and 1 μL FITC-PNA (1 mg/mL) per mL of PBS. Final concentrations of the stains were 1.5 μM PI and 1 μg/mL FITC-PNA. Cryopreserved semen was thawed in a 37 °C water bath for 1 min, and 25 μL of thawed semen was added to 0.5 mL staining solution to create a final concentration of 2 million spermatozoa per mL. The samples were mixed by reverse pipetting and subsequently incubated in a darkened area at 37 °C for 10–15 min prior to flow cytometric analyses.

Flow cytometric analyses were conducted as described previously in this manuscript with the exception that FITC-PNA fluorescence was detected at 515 to 545 nm fluorescence detector 1. Fluorescent data of all events was collected until 10,000 gated events were recorded. Non-sperm events were gated out of analyses as judged on scatter properties. Two-dimensional plots of FITC-PNA

(green) compared with PI (red) fluorescence events were drawn and divided into quadrants to determine the frequency of sperm in each of four populations (Fig. 1B). Population 1 included sperm that were live (PI negative) with an intact acrosome (FITC-PNA negative). Population 2 encompassed sperm that were live (PI negative) with a damaged acrosome (FITC-PNA positive). Populations 3 and 4 contained sperm that were dead (PI positive) with intact (FITC-PNA negative) or damaged (FITC-PNA positive) acrosomes, respectively. The percentage of debris was manually excluded from Population 1 based on data from SYBR-14/PI assay and validated by determining agreeance in percentages of total live spermatozoa between assays.

2.9. Assessment of sperm chromatin stability (SCSA)

Chromatin stability was assessed by meta-chromatic staining with acridine orange based on the susceptibility of the sperm DNA to acid-induced denaturation *in situ* (Martínez-Pastor et al., 2004). This is a cell-permeant nucleic acid binding dye that emits green fluorescence when bound to double stranded DNA (dsDNA) and red fluorescence when bound to single stranded DNA (ssDNA) or RNA. Cryopreserved semen was thawed in a 37 °C water bath for 1 min, and 90 µL of thawed semen was added to 110 µL PBS to create a final sperm concentration of approximately 18 million spermatozoa per mL. Acid-induced denaturation of DNA *in situ* was attained by adding 0.4 ml of an acid-detergent solution (0.17% Triton X-100, 0.15 M sodium chloride, and 0.08 N hydrogen chloride; pH 1.4) to 0.2 mL diluted semen. After 30 s, the cells were stained by adding 1.2 mL of a citric phosphate solution (0.1 M citric acid, 0.2 M sodium phosphate dibasic, 1 mM ethylenediaminetetraacetic acid, 0.15 M sodium chloride; pH 6.0) containing 6 µg/ml acridine orange, to achieve a working concentration of 2 million spermatozoa per mL, and mixed by reverse pipetting. The stained samples were subsequently incubated in the dark at 37 °C for 3 min prior to flow cytometric analyses.

Flow cytometry analysis was carried out as above using a 488-nm argon excitation laser. Fluorescent data of all events was collected until 10,000 gated events were recorded. Debris was discarded electronically and based on low fluorescence values. Two populations were discerned by drawing a diagonal line between the groups of cells with stable chromatin (intact DNA) to the left of the line (medium to high green fluorescence) and the group of cells with loose chromatin (damaged DNA) to the right of the line (medium to high red fluorescence) as depicted in Fig. 1C. The frequency of each of these populations was quantified. DNA fragmentation index was calculated as the number of cells with loose chromatin divided by the sum of both populations.

2.10. Statistical analyses

Data are presented as percentage mean ± SEM. All statistical analyses were performed using R Version 3.4.3 (https://www.r-project.org/). Homogeneity of variances among samples was established using a Bartlett test, and normality was confirmed using a Shapiro-Wilk test of the residuals. Non-normal data was transformed using Tukey's Ladder of Powers. A Kruskal-Wallis rank sum test was performed on non-parametric data. Analysis of variance was applied to parametric data using a general linear mixed model with bull ID as a random variable and fixed effect of treatment. A Tukey HSD test was performed as needed for *post-hoc* analysis. Repeated measures was used for post-thaw motility parameters to include the effects of treatment, time, and their interactions. The covariance structure that results in the smallest Bayesian information criterion will be selected from the mixed models. Significance was declared at $P \leq 0.05$.

3. Results

3.1. Ejaculated sperm

Percentage of normal sperm, primary sperm defects, and secondary sperm defects in ejaculated sperm did not differ at post-thaw between treatments with a similar distribution in specific defects ($P > 0.05$; Table 1). Post-thaw overall and progressive motilities decreased over time in all samples ($P < 0.01$) but were less in HIGH and LOW than in CONT or MED samples ($P < 0.01$; Fig. 2A and B). Values for sperm velocity variables, VAP and VCL, were least in HIGH, intermediate in LOW and CONT, and greatest in MED samples ($P < 0.05$; Fig. 2C and D). Amplitude of lateral head displacement was relatively less in HIGH samples at 2.5 and 3.0 h post-thaw ($P < 0.05$; Fig. 2E). Linearity was leas in LOW samples, intermediate in CONT and MED, and greatest in HIGH samples ($P \leq$

Table 1
Morphological analysis of sperm samples collected by electroejaculation and epididymal sperm collections prior to freezing (PRE) and at post − thaw after incubation with 0 ng/mL NGF (CONT), 0.5 ng/mL NGF (LOW), 5 ng/mL NGF (MED), or 50 ng/mL NGF (HIGH); Data are presented as mean ± SEM; Differing superscripts denote differences between treatments ($P \leq 0.05$).

	Ejaculated			Epididymal		
Treatment	% Normal	% Primary	% Secondary	% Normal	% Primary	% Secondary
PRE	63 ± 6.1	16 ± 5.4	21 ± 4.6	16 ± 2.3^{ab}	13 ± 3.5	71 ± 5.6
CONT	66 ± 7.0	15 ± 4.7	19 ± 4.1	15 ± 2.1^{b}	16 ± 4.7	69 ± 6.5
LOW	63 ± 6.4	17 ± 6.2	20 ± 4.4	18 ± 2.5^{a}	14 ± 4.5	68 ± 6.8
MED	65 ± 6.4	16 ± 5.4	19 ± 5.2	17 ± 2.5^{ab}	14 ± 5.0	69 ± 7.2
HIGH	62 ± 6.0	18 ± 5.8	21 ± 4.0	18 ± 2.9^{a}	14 ± 5.0	68 ± 7.1

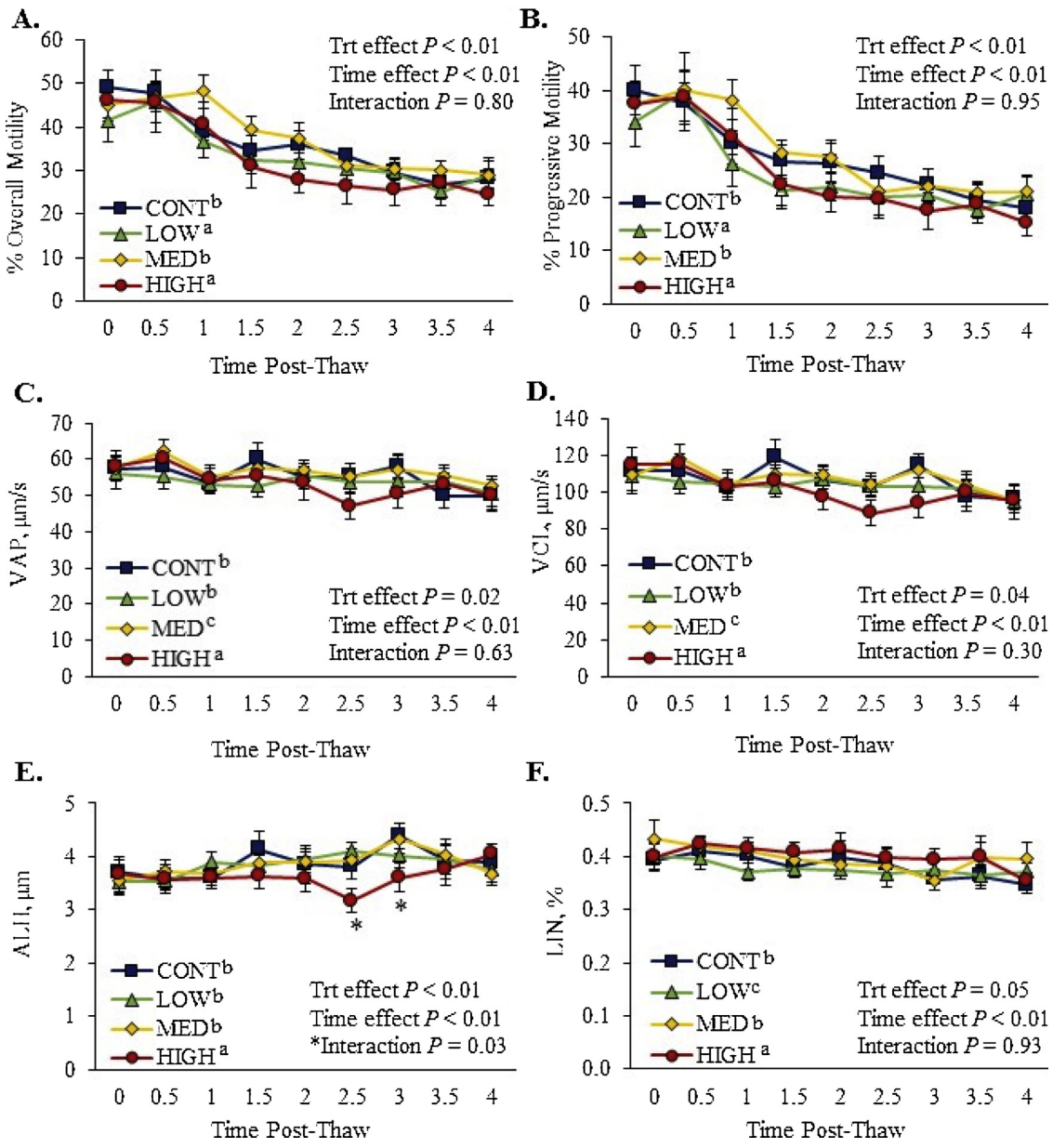

Fig. 2. Line graphs depicting changes over time in the motility and motion parameters of frozen-thawed ejaculated bull spermatozoa supplemented with 0 ng/mL NGF (CONT), 0.5 ng/mL NGF (LOW), 5 ng/mL NGF (MED), or 50 ng/mL NGF (HIGH). Data are presented as mean ± SEM. Differing letters denote treatment interactions, whereas asterisks denote treatment by time interactions ($P \leq 0.05$).
Abbreviations: VAP = average path velocity; µm/s VCL = curvilinear velocity, µm/s; LIN = linearity of the curvilinear path, %; ALH = amplitude of lateral head displacement, µm.

0.05; Fig. 2F).

Results from post-thaw flow cytometric sperm analyses indicated there were no differences in percentages of live, dead, or moribund spermatozoa amongst treatments ($P > 0.05$; Fig. 3A). Similarly, there were no differences in the percentages of live spermatozoa with intact acrosomes between treatments ($P > 0.05$; Fig. 3C). The DNA fragmentation index also did not differ between treatments ($P > 0.05$; Fig. 3E).

3.2. Epididymal sperm

There was a greater percentage of morphologically normal sperm cells in post-thaw in LOW and HIGH samples than in CONT samples ($P \leq 0.05$; Table 1). Even though this difference, there were no differences in the percentages of primary or secondary sperm

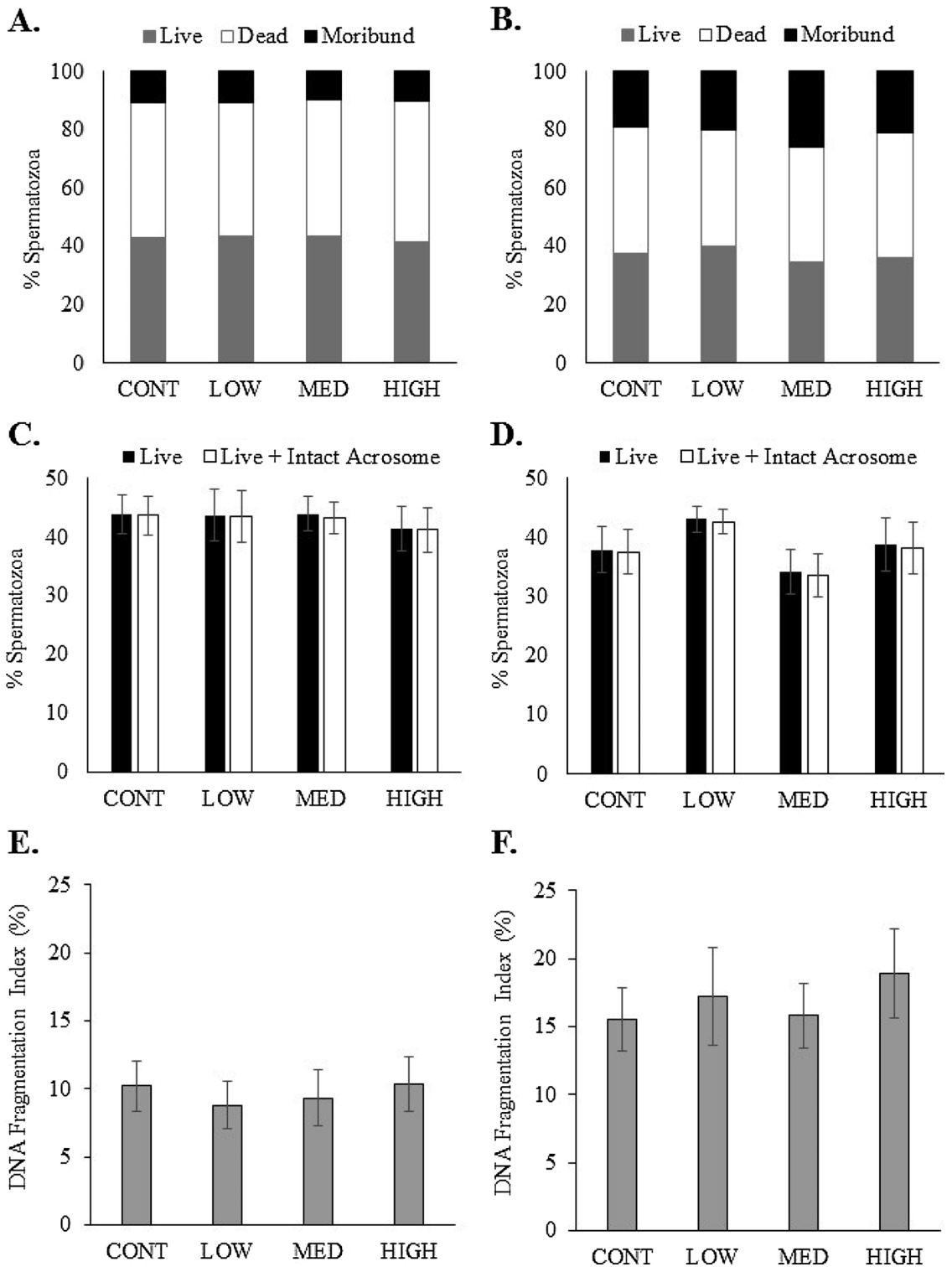

Fig. 3. Results of post-thaw flow cytometric analyses from semen samples collected by electroejaculation (A, C, E) or epididymal sperm collection (B, D, F) after incubation with 0 ng/mL NGF (CONT), 0.5 ng/mL NGF (LOW), 5 ng/mL NGF (MED), or 50 ng/mL NGF (HIGH) in bulls. Data are presented as mean ± SEM. There were no differences in sperm viability (A, B), acrosome integrity (C, D), or DNA stability (E, F) between treatments ($P > 0.05$).

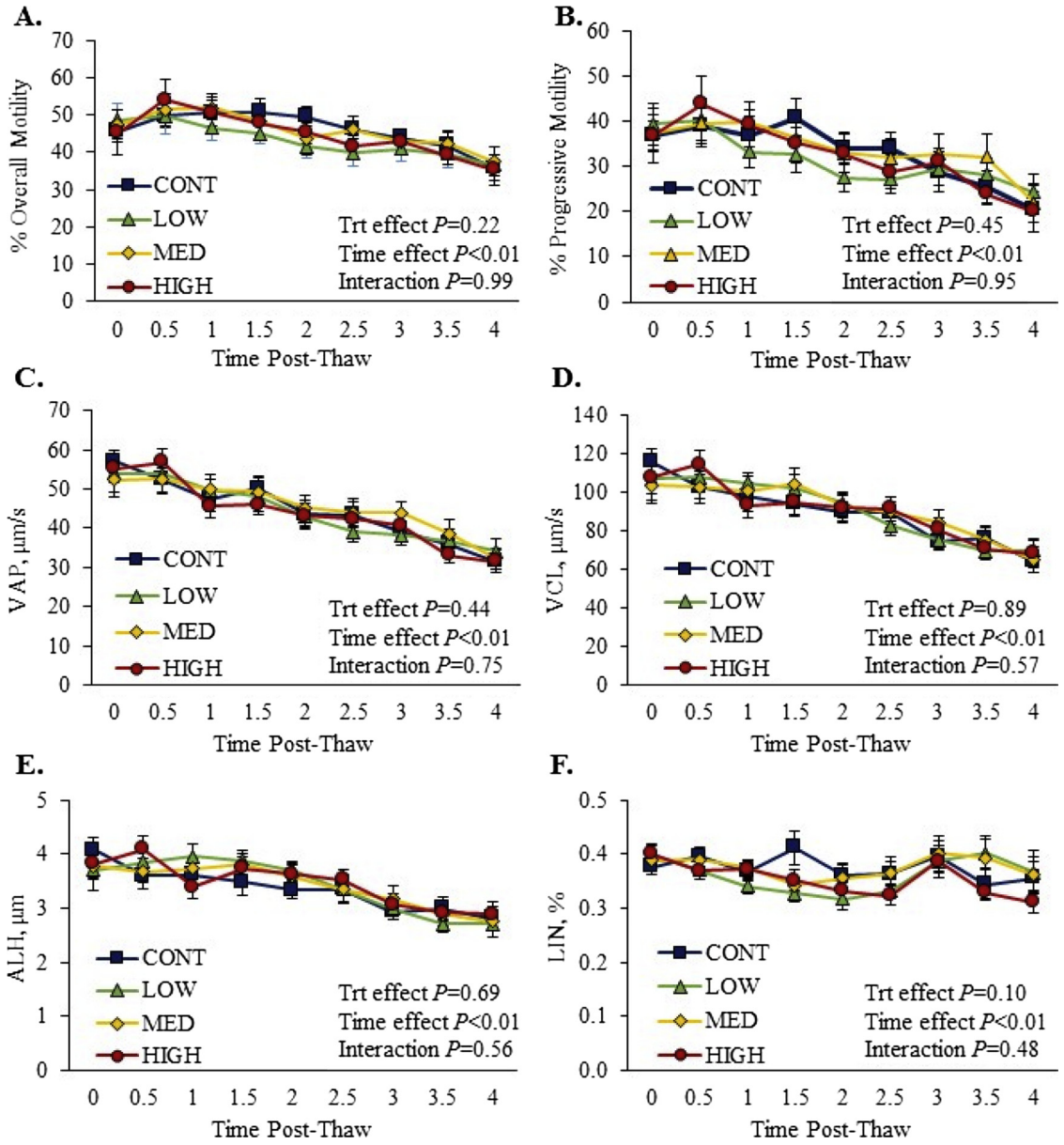

Fig. 4. Line graphs depicting changes over time in the motility and motion parameters of frozen-thawed bull epididymal spermatozoa supplemented with 0 ng/mL NGF (CONT), 0.5 ng/mL NGF (LOW), 5 ng/mL NGF (MED), or 50 ng/mL NGF (HIGH). Data are presented as mean ± SEM. There were no differences in sperm motility and motion parameters between treatments ($P > 0.05$).
Abbreviations: VAP = average path velocity; µm/s VCL = curvilinear velocity, µm/s; LIN = linearity of the curvilinear path, %; ALH = amplitude of lateral head displacement, µm

defects amongst treatments with a similar distribution of specific defects ($P > 0.05$; Table 1). Post-thaw overall and progressive motilities decreased over time in all samples ($P < 0.01$), but did not differ among treatments ($P > 0.05$; Fig. 4A and B). Similarly, treatment did not alter the values for post-thaw sperm motion variables ($P > 0.05$; Fig. 4C-F).

Results from post-thaw flow cytometric sperm analyses indicated there were no differences in percentages of live, dead, or moribund spermatozoa amongst treatments ($P > 0.05$; Fig. 3B). Similarly, there were no differences in the percentages of live spermatozoa with intact acrosomes between treatments ($P > 0.05$; Fig. 3D). The DNA fragmentation index also did not differ between treatments ($P > 0.05$; Fig. 3F).

4. Discussion

This study was designed to assess the post-thaw semen quality from both ejaculated and epididymal-collected bull sperm extended

with final concentrations of 0 ng/mL (CONT), 0.5 ng/mL (LOW), 5 ng/mL (MED), or 50 ng/mL (HIGH) of NGF prior to cryopreservation. Because ejaculated bull sperm have NGF-immunoreactivity localized to the sperm head and tail and the receptor (TRKA) in the acrosomal cap, nucleus, and tail regions (Li et al., 2010), it was hypothesized that seminal plasma NGF would function directly on the sperm improve cryotolerance. Considering the conditions in the present study, the effects of NGF on post-thaw sperm quality variables of bulls were not as beneficial as was thought might occur prior to conducting the study. It is worth noting that the ejaculated samples evaluated in the current study were collected by electroejaculation rather than by artificial vagina, which alters both seminal plasma constitution and semen quality (Austin et al., 1961; Rego et al., 2015).

Production of NGF occurs predominantly in the ampulla and vesicular glands of bulls, secreting this protein almost exclusively into the sperm-rich fraction of the ejaculate (Stewart et al., 2018a). In the current study, purified NGF was added to both ejaculated and epididymal-derived bull sperm. While NGF immunoreactivity was also detected in the myoid epithelial cells around the seminiferous tubules of the testis, the lumen and spermatids were NGF-negative, indicating there is no testicular contribution of NGF to the seminal plasma (Bogle et al., 2018). The current study was conducted, therefore, to evaluate the effects of adding purified NGF to spermatozoa exposed to accessory sex gland fluids (ejaculated) compared with spermatozoa with no exposure to accessory sex gland fluids (epididymal). As expected, the percentage of morphologically normal spermatozoa was greater in ejaculated samples (˜63%) than in epididymal-derived samples (˜16%), with the latter having a greater percentage of secondary defects (21% compared with 71%, respectively). The most common secondary defect detected in epididymal samples were distal cytoplasmic droplets, which did not differ among treatment groups. It appears, therefore, that NGF signaling does not have an important function in final sperm maturation at ejaculation and other factors need to be assessed for improving epididymal sperm quality (Harayama et al., 1996).

In one study, there was greater *NGF* gene expression in spermatozoa of bulls with greater semen quality (< 25% discarded ejaculates) compared bulls with lesser semen quality (> 40% discarded ejaculates) (Parthipan et al., 2017). In this previous study, the relatively greater *NGF* gene expression had a positive effect on values for frozen-thawed sperm velocity variables, LIN, VAP, STR, and VSL (Parthipan et al., 2017), which are strongly and positively correlated with bull fertility (Farrell et al., 1998). The results of the current study indicate there was very little change in values for motility and velocity variables of both ejaculated and epididymal-derived sperm treated with NGF before cryopreservation. Interestingly, ejaculated sperm incubated with relatively greater concentrations of NGF had an overall decrease in values for motility and velocity variables and an increase in LIN. Additionally, ALH was decreased at 2.5 and 3 h post-thaw in ejaculated semen supplemented with the relatively greater concentrations of NGF. Though the decrease in sperm motility may indicate there is a detrimental effect of NGF on sperm viability, the combination of decreased ALH and increased LIN indicates NGF could be protective against premature hyperactivation and capacitation of sperm (Marquez and Suarez, 2004; Muiño et al., 2008; Kathiravan et al., 2011). These results were not consistent in epididymal sperm, suggesting that interactions with other seminal plasma components secreted by the accessory sex glands may be necessary. Related to this, abundances of *NGF*-mRNA-transcript in sperm are positively correlated with abundances with *BMP2*, *CASP3*, and *TRADD* mRNA transcripts, which all have functions in maintaining sperm function and fertility (Parthipan et al., 2017). These data indicate there is a complex synergistic interaction of seminal plasma proteins that may affect sperm cryo-tolerance and survivability and future studies should be conducted to evaluate combinations of these factors to determine the effects on sperm motility and motion variables.

Seminal plasma *NGF* mRNA abundances in bulls was positively associated with maintenance of post-thaw functional membrane integrity in spermatozoa (Shilpa et al., 2017). Consistently, exogenous NGF added to frozen-thawed bull spermatozoa at 20, 80, or 120 ng/mL improved sperm viability after a 2 h incubation period (Li et al., 2010). Leptin secretion from bull sperm was also greater in samples incubated with 40 and 80 ng/mL NGF for 2 h (Li et al., 2010). Leptin signaling in spermatozoa has been implicated as a potential regulator of sperm capacitation and survival in pigs (Aquila et al., 2008). Supplementation of human sperm with leptin increased both motility and acrosome reaction (Lampiao and du Plessis, 2008), suggesting a role of leptin in enhancing sperm fertilization capacity. In contrast to these previous findings, supplementing freezing extender with purified NGF before cryopreservation of bull semen did not affect membrane integrity immediately after thawing, but was not assessed over time. Given the broad distribution of NGF and its receptor throughout the female reproductive tract in mammalian species (Ren et al., 2005; Weng et al., 2009; Li et al., 2012, 2014), further studies are needed to assess the relevance of these findings in regards to leptin signaling and ensuring sperm survivability throughout uterine transit.

Results of a previous study indicated there was no effect on the rate of the acrosome reaction in bull sperm treated with up to 120 ng/mL NGF (Li et al., 2010). Similarly, in the present study there were no differences in acrosome integrity of bull sperm amongst NGF treatments up to 50 ng/mL. In golden hamsters, treating semen with up to 500 ng/mL NGF improved sperm motility and induced acrosome reaction (Jin et al., 2010). Considering NGF concentrations in raw ejaculated seminal plasma of bulls is as great as 7000 ng/mL (Stewart et al., 2018a), there is a need to assess greater NGF concentrations to enhance understanding of NGF functions on sperm quality and acrosome integrity.

Sperm *NGF* mRNA abundance and seminal plasma NGF concentrations have been associated with positive sire conception rates in bulls (Kasimanickam et al., 2012; Stewart et al., 2018a). In the current study, there was no effect of NGF treatment on DNA fragmentation index, which were within the recommended 10%–20% threshold for predicting adequate bull fertility (Evenson and Wixon, 2006; Kasimanickam et al., 2006). Results of recent studies indicate there may be a pivotal role of NGF within the female reproductive tract. Systemic administration of seminal plasma derived NGF to cows improved conceptus development, potentially through enhanced luteal development (Stewart et al., 2018b). In another study, supplementing *in vitro* fertilization media with 100 ng/mL NGF induced early cleavage and improved embryo development in sheep (Crispo et al., 2016). Whether these outcomes were modulated through the effects of NGF on the sperm, oocyte, or zygote is unknown. Fertility studies, both *in vitro* and *in vivo*, are necessary to determine the relevance of these findings in cattle.

5. Conclusions

In conclusion, treatment with NGF did not significantly improve cryotolerance of sperm collected by electroejaculation or epididymal collections from bulls. Supplementation of freezing extender with relatively greater concentrations of NGF decreased post-thaw VCL and ALH and increased LIN, which may indicate there are NGF functions in preventing premature sperm hyperactivation and capacitation. Though results of the current study do not support that NGF functions in improving sperm cryotolerance, further studies should address the effects NGF may have within the female reproductive tract that could affect fertility.

Conflict of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality in conducting the experiment and publishing the manuscript.

Acknowledgments

The authors thank the staff at the University of Illinois Beef Barn and clinical year veterinary students for providing assistance during field sample collection. We also thank Som Nanjappa in the Pathobiology Department at the University of Illinois College of Veterinary Medicine for his invaluable assistance with preparing the flow cytometer for semen analyses. This research was supported with funds provided by the Department of Veterinary Clinical Medicine, College of Veterinary Medicine, the University of Illinois at Urbana-Champaign as startup funds for Dr. Fabio Lima.

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