COMPARISON OF METHODS FOR ASSESSING VIABILITY OF EQUINE SPERMATOZOA AND EFFECTS OF SEMINAL PLASMA ON VIABILITY AND MOTION CHARACTERISTICS OF EQUINE SPERMATOZOA

A Thesis

by

MARY LAUREN FOSTER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2009

Major Subject: Veterinary Medical Sciences

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Approved by:

Chair of Committee,	Charles Love
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	Katrin Hinrichs
	Dickson Varner
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ABSTRACT

Comparison of Methods for Assessing Viability of Equine Spermatozoa and Effects of Seminal Plasma on Viability and Motion Characteristics of Equine Spermatozoa. (December 2009)

Mary Lauren Foster, B.S., Texas A&M University-Commerce

Chair of Advisory Committee: Dr. Charles Love

Assessment of sperm viability is an important component for evaluating stallion sperm quality. The flow cytometer is considered the standard in the assessment of sperm plasma membrane integrity (viability); however, this instrument is costly to purchase and use, and it requires an experienced technician to operate it. The growing practice of assisted reproductive technologies (ARTs) in the equine industry has increased the need for an accurate but cost-effective means of determining sperm membrane viability. The NucleoCounter® SP-100™ is reported to be an accurate, easy-to-perform, and an efficient stallion-side test for sperm membrane viability.

To evaluate usefulness of the NucleoCounter® SP-100™ for assessing sperm membrane integrity, neat semen was subjected to four treatments with varying seminal plasma volumes and sperm concentrations. Sperm membrane viability was assessed immediately, and at 24 and 48 hours after cooled-storage using three methods: 1) flow cytometer utilizing the fluorescent vital stains

SYBR-14/propidium iodide; 2) NucleoCounter® SP-100™ utilizing the fluorescent vital stain propidium iodide; 3) eosin-nigrosin stained air-dried smears of semen. Sperm motion characteristics (total and progressive motility) were assessed using a computer assisted sperm motion analyzer (CASMA) and results were compared to sperm membrane viability to determine the relationship between sperm membrane viability and motion characteristics. Results were compared statistically by: 1) analysis of variance (ANOVA); 2) linear regression analysis; 3) coefficient of variation on untransformed and transformed data (arc sine square root); and 3) the agreement of two instruments, by means of which the difference between measurements of the two instruments were plotted on the y-axis and the average of measurements from the two instruments were plotted on the x-axis.

Results obtained with the NucleoCounter® SP-100™ agreed best with the flow cytometer, and least with eosin-nigrosin staining. Coefficients of variation were ≤ 5% for the three methods (transformed data). Sperm motion characteristics and sperm viability were similar among treatments at Time 0. At Times 24 and 48, sperm motion characteristics decreased at a more significant rate compared to viability in the treatments containing ≥ 50% seminal plasma, whereas differences among treatments were only significant at seminal plasma concentrations above 50% when only sperm membrane viability was considered.

DEDICATION

I dedicate this work to:

My parents: Paul and Mary Joyce Foster,

&

My family and friends.

Mom and Dad, thanks for providing me with the opportunity to pursue my dreams. Your guidance, support and encouragement have shaped me as a student and as an individual, and I could not have achieved my goals without you.

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I would like to thank Drs. Love, Blanchard, Varner, Hinrichs, and Brinsko for the time, support, and guidance they gave throughout the course of my graduate career. Dr. Love, thank you for your patience and direction throughout my research and time here. Dr. Blanchard, thank you for your kind and encouraging words, and the time and dedication you put forth on this project. Dr. Varner, thank you for your light-hearted humor and compassion, and for the opportunities you have provided me. Drs. Hinrichs and Brinsko, thank you for your enthusiasm and advice. Thank you to the friends I have made in the Theriogenology lab for your continued support, especially Sheila Teague and Kat LaCaze. I could not have competed this project without you two. Every moment spent as a part of the Texas A&M University, College of Veterinary Medicine and Biomedical Sciences, and Department of Large Animal Clinical Sciences will be cherished.

Many materials were provided by ChemoMetec A/S of Allerød, Denmark. Financial assistance for this study was also provided by the Link Endowment Fund, Texas A&M University; the Azoom, Corona Cartel, and Teller Cartel syndicates (through Lazy E Ranch); and the Shining Spark-Carol Rose Stallion Reproduction Fund (through the American Quarter Horse Association).

TABLE OF CONTENTS

Page

Page

Page

LIST OF FIGURES

FIGURE Page

FIGURE Page

FIGURE Page

FIGURE Page

LIST OF TABLES

TABLE Page

TABLE Page

TABLE Page

1. INTRODUCTION

The growing use of assisted reproductive technologies (ARTs) in the equine industry has increased the requirement for an accurate, repeatable, and cost-effective means of determining the quality of the sperm to be used in these procedures. These technologies include artificial insemination, low-dose, deep-horn insemination, and intracytoplasmic sperm injection (ICSI). Fluorescent probes for measurement of sperm viability have been used to label sperm compartments in several species including boars, rams, rats, rabbits, humans, turkeys [1], stallions [2, 3], and dogs [4]. Specific fluorescent probes evaluated in this study include SYBR-14, propidium iodide (PI) and JC-1, which measure sperm membrane integrity (the term viability will be used hereafter for simplicity) by demonstrating membrane permeability or mitochondrial membrane potential. The semen sample is incubated with the fluorescent labels, and results are analyzed on a flow cytometer to allow objective assessment of thousands of sperm per sample. Because of its objectivity, ability to differentiate slight variations in staining intensity, and ability to analyze high numbers of sperm, the flow cytometer is considered the “gold standard” for assessment of viability, and is commonly used to evaluate sperm membrane viability assays. The combined trends in ARTs and the continued use of stallions with moderate to poor semen quality have made proper handling, processing and evaluation of semen more

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This thesis follows the style of Theriogenology.

critical for the equine breeding industry.

Seminal plasma content and determination of sperm concentration are two important considerations when processing semen. Optimal seminal plasma concentration and sperm concentrations in extended semen are generally accepted to be between 5-20% and 25 to 50 x 10^6 sperm/mL, respectively [5, 6]. Although these ranges are considered optimal, the effects of seminal plasma levels can vary among stallions, ejaculates, and types of processing performed (i.e. cooled vs. frozen) [7-12].

The relationship between sperm membrane viability and motion characteristics in cooled-stored equine sperm is not clear, and few studies have attempted to address this issue. Brinsko et al. in 2003 found a strong correlation ($R>0.80$; $P<0.001$) between motility and membrane viability of equine sperm when assessed by computer-assisted sperm motion analysis and staining with carboxyfluoresein diacetate-propidium iodide, respectively [13]. Another study conducted in 2003 also found a strong correlation ($R=0.98$) between total motility of equine sperm as assessed by computer-assisted sperm motion analysis and membrane viability assessed by two staining techniques (SYBR-14/PI and SYBR-14/PI/JC-1) using the flow cytometer [2]. Both studies utilized treatments with varying percentages of dead sperm added, and results were assessed after a 24-hour cooling period. Results after addition of dead (frozen-thawed) sperm, which may have superphysiological changes to the sperm

membrane may not be directly applicable to findings on sperm death as it occurs after storage or in response to more physiological insults.

Many studies have also attempted to compare two or more methods for determining sperm viability in stallions [2,3,14], fowl [1,15], dogs [4], and cats [16]. Research has also been performed in an attempt to validate or evaluate a single method for determining sperm concentration and sperm viability [1].

Evaluation of sperm membrane viability using the eosin-nigrosin stain has been used as a field procedure in clinical practice for the assessment of sperm viability, as it is simple, quick, and only requires a light rather than a fluorescent microscope. However, to our knowledge, no information is available on the correlation of viability findings with eosin-nigrosin to that determined by flow cytometry of SYBR-14/PI stained sperm. Although the accuracy and repeatability of the flow cytometer are unrivaled, the cost and skills involved in using this instrument make it impractical for most laboratories and breeding farms. Another potentially objective method for determination of sperm membrane viability, assessment of propidium iodide staining using the NucleoCounter® SP-100™, is available. The NucleoCounter SP-100 determines total sperm concentration by permeabilizing the sperm using Reagent S-100 and then staining with PI; to evaluate viable sperm, this total concentration is compared to the concentration (number of PI-stained sperm) detected when a separate aliquot of the semen sample is diluted in phospate-

buffered saline and PI is allowed to permeabilize sperm membranes that are already damaged.

The objective of this study was to compare the accuracy and repeatability among three different methods for assessing equine sperm viability (eosin-nigrosin, flow cytometer, and NucleoCounter® SP-100™). Throughout this thesis, sperm that are said to be viable are defined as sperm having plasma membranes that are intact. The effects of different concentrations of seminal plasma on sperm motion characteristics using a computer assisted sperm motion analyzer (CASMA) and viability staining using each method was assessed. Two instruments, the FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA) and the NucleoCounter® SP-100™ (Chemometec A/S, Allerød, Denmark) utilize fluorescent probes as a means for determining sperm membrane viability. Eosin-nigrosin (Lane Manufacturing, Inc., Denver, CO) is an exclusion stain, a method to stain sperm if the sperm plasma membranes have been disrupted, and viability is assessed by counting sperm that exclude stain using light microscopy. Sperm that uptake eosin display a pink sperm head and are counted as non-viable, whereas sperm that remain white indicate an intact sperm membrane. Sperm motion characteristics were obtained using a computer-assisted sperm motion analyzer (CASMA; IVOS Version 12.2L, Hamilton Thorne Biosciences, Beverly, MA, USA).

1.1. Background

1.1.1. Seminal plasma

Seminal plasma is composed of proteins, enzymes, electrolytes and trace elements produced by the testes, epididymides and the accessory sex glands [17]. Seminal plasma is an alkaline buffer and is thought to play a role in suppressing the uterine inflammatory response to insemination [18-20], and motility initiation, sperm capacitation and the acrosome reaction [21, 22]. No matter the breeding technique, the presence of seminal plasma at some level may be essential for cooled storage of extended semen with most milk-based extenders [6, 23-28].

Pickett et al. in 1975 studied the effects of seminal plasma level (0% - 50%) on equine sperm motility during incubation before and after cryopreservation using a skim-milk extender, as well as the effects of centrifugation [27]. These authors found that regardless of treatment, maximal sperm motility was found in 20% seminal plasma. Complete seminal plasma removal decreased motility significantly ($P<0.05$) before and after cryopreservation [27].

In contrast other studies reported that seminal plasma removal improved sperm motion characteristics specifically when a modified Tyrode's extender was used, suggesting that extender type and seminal plasma level may interact to maintain sperm membrane viability and motility [11, 29, 30]. Padilla and Foote in 1991 compared sperm motion characteristics of stallion semen

subjected to one of four treatments after cooled-storage (4°C) for 24, 48, and 72 hours [30]. Treatments compared seminal plasma diluted in either a traditional nonfat dry skim milk extender (Kenney type) or a modified Kenney extender with Tyrode's added (KMT) [30]. When seminal plasma was removed, sperm motion characteristics were superior ($P<0.05$) in the modified Kenney extender (KMT). Rigby et al. in 2001 found that fertility was not compromised in normal mares when a skim milk-glucose extender with added Tyrode's medium was used for cooled semen storage for 48 hours prior to insemination [29]. Rigby et al. also reported that using a skim milk-glucose extender with added Tyrode's solution (KMT: 35%) was inferior to straight skim milk-glucose extender (Kenney type) for storage of uncentrifuged semen for cooled transport when the seminal plasma was 20% [29]. Consistent with other studies, Rigby et al. found that the presence of seminal plasma with modified Tyrode's extender was detrimental to the sperm motion characteristics after cooled-storage [30, 31]. Akcay et al., 2006 demonstrated that seminal plasma from different fractions of an ejaculate had different effects on viability and motion characteristics of stallion sperm compared to seminal plasma harvested from the total ejaculate. Seminal plasma from the sperm-poor fraction of the ejaculate maintained better sperm motion characteristics and higher sperm membrane integrity during cold-storage conditions when compared to seminal plasma harvested from the sperm-rich fraction of the ejaculate [11]. Similar to Padilla and Foote, and Rigby et al., Akcay et al. also found that seminal plasma was detrimental to the longevity of

cooled-stored sperm extended in KMT when sperm motion characteristics were assessed, indicating that extender type and the presence or absence of seminal plasma should be considered when processing stallion semen. Akcay et al. found that removing the seminal plasma and resuspending the sperm in KMT resulted in better motion characteristics when compared to the treatments with seminal plasma in extender with and without added Tyrode's [11]. Jasko et al. in 1992 found that removing seminal plasma completely had a detrimental effect ($P<0.05$) on total sperm motility after 24-hour cooled storage when a nonfat, dried skim milk – glucose extender (E –Z Mixin K, Animal Reproduction Systems, Chino, CA) was used to extend the semen [6]. Brinsko et al. in 2000 also found that partially removing seminal plasma (~90%) from an ejaculate by centrifugation compared to routine processing (three parts extender: one part neat semen) improved the progressive motility of cooled-stored semen after 24 and 48 hours in stallions that were considered "poor-cooling" [8]. "Poor-cooling" stallions were those that showed a ≥ 40% decrease in progressive motility as compared to fresh after 24 hours of cooled-storage [8]. Other studies demonstrated that a small percentage (5%-20%) of seminal plasma should be included when extending semen to maintain sperm motion characteristics and sperm membrane integrity [5, 6]. Seminal plasma, at most 20%, has been found to be essential for sperm motility if sperm were suspended in skim-milk glucose extender [5, 6]. Seminal plasma could be eliminated if sperm are suspended in

KMT extender, and in fact, elimination of seminal plasma in semen extended in KMT results in greater longevity of motility [11].

Previous studies have used the addition of dead sperm (intentionally frozen-thawed multiple times) to create sperm populations with different viable/non-viable ratios for testing of viability measures [2,13], however, to our knowledge, there have been no reports on research using varying seminal plasma levels to induce potentially different viable and non-viable sperm ratios. This approach uses a physiologic inducer of non-viability rather than a more severe non-physiologic method such as freezing and thawing. The research reported in this thesis utilized four treatments with varying percentages of seminal plasma and sperm concentrations (80SP – semen diluted to 80% seminal plasma; 80SP/20 – semen diluted to 80% seminal plasma thus containing 25% the sperm concentration as 80SP; 50SP – semen diluted to 50% seminal plasma thus containing 62.5% (⅝) the sperm concentration as 80SP; 20SP – semen diluted to 20% seminal plasma thus containing 25% the sperm concentration as 80SP) using un-centrifuged, neat semen diluted with extender or with extender with added seminal plasma. Measurements were obtained following 0 h, and at 24, and 48 hours of cooled storage. Increasing concentrations of seminal plasma were used in this study to induce sperm quality deterioration over time and create a range of membrane-damaged (non-viable) sperm to facilitate evaluation of the sperm quality measurement methods.

1.1.2. *Methods for detecting sperm viability*

Several studies have assessed different methods for determining sperm membrane viability [1-4, 9, 13-16, 32-37]. Most methods evaluate the condition of the sperm plasma membrane based on the ability of a non membrane-permeable dye to gain access to the sperm. Propidium iodide (PI) and eosin stain can access sperm only if the sperm plasma membrane is damaged. The dye, SYBR-14, penetrates the sperm plasma membrane regardless of its sperm membrane integrity, and so can be used to determine the total number of sperm. When used in combination with propidium iodide the SYBR-14 stain will be over-colored by PI in those sperm that are non-viable, but will not mark viable sperm, since PI will not penetrate the viable sperm membrane. The assumption that intact plasma membranes would prevent dyes such as eosin and propidium iodide from entering the cytoplasm was first addressed in using the cytosolic stain eosin and nigrosin [32-34]. Eosin is a cytosolic stain that enters disrupted plasma membranes and dyes the cytoplasm within the plasma membrane thereby marking these sperm cells with a bright pink color. Nigrosin is used as a background stain to assist in detection of the sperm cells that do not uptake eosin. Figure 1 shows the contrast between sperm that show eosin uptake (pink) or exclusion (white).

The use of eosin-nigrosin as a stain for detecting dead sperm was first studied by J. L. Hancock in 1951 in semen obtained from bulls. He found that as bull sperm were subjected to temperature shock (30°C – 0°C), the proportion of

stained (“dead”) sperm increased as the temperature decreased (N = 6 ejaculates) [32].

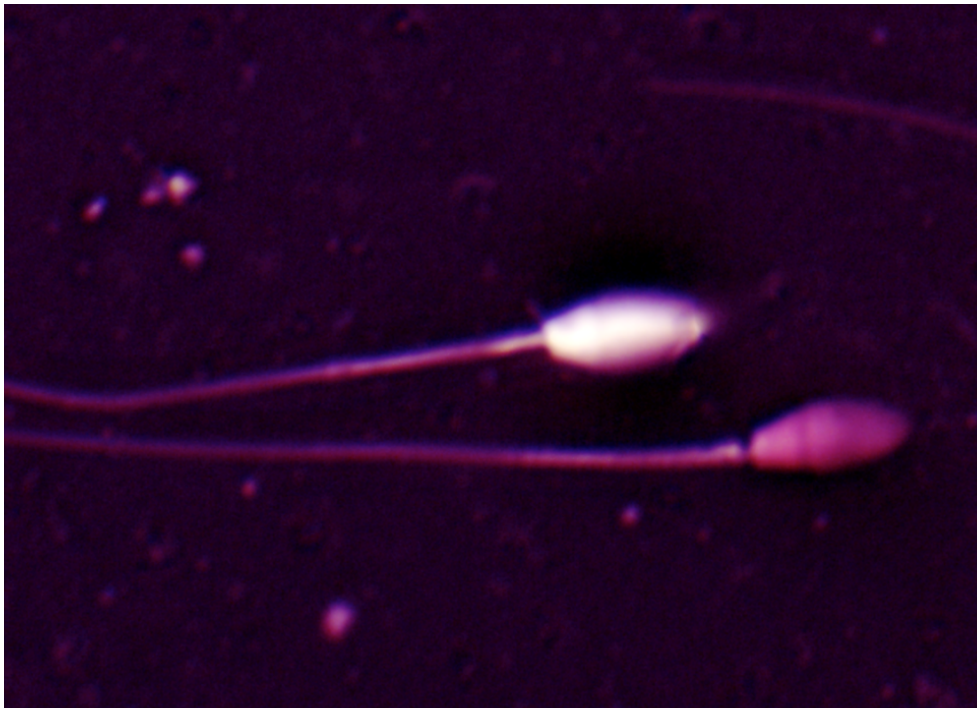

Figure 1. Sperm sample stained with eosin-nigrosin. The sperm on the top did not uptake eosin and is counted as viable (membrane intact). The sperm on the bottom did uptake eosin and is counted as non-viable (membrane not intact). The black background is due to the nigrosin stain.

Campbell et al. (1956) conducted experiments on ram, bull, and boar semen using eosin-nigrosin and light microscopy to evaluate “live “ and “dead” sperm. Measuring sample and subsample variation, and comparing different methods of preparation and counting techniques, he suggested the following guidelines: 1) sperm should be exposed to the stain for a minimum of 5 minutes; 2) microscopy fields for evaluation should be chosen at random; 3) expect larger

variation among counts when sperm clumping is present; 4) establish a well-defined interpretation of “stained” versus “unstained” sperm [33]. This interpretation was followed in this study by counting partially stained sperm separately from dead and live sperm [33]. Partially stained sperm were sperm that held a grayish color instead of white or pink, or those whose sperm heads were stained pink on the anterior portion but showed no stain on the posterior portion [33]. The clumping that was mentioned in the third guideline, and visualized more frequently when the experiments were performed on ram sperm, was thought to have accounted for the increase in “dead” sperm because a higher percentage of stained sperm were seen in clumps compared to the rest of the microscopic fields [33]. These findings are not entirely suggestive that dead sperm clump together, but that sperm that lie close together are more likely to look stained because eosin-nigrosin stain will be thicker in these areas [33]. Findings from other research have revealed that dead sperm have a tendency to clump together interfering with assessment of stained versus unstained sperm [35]. When eosin-nigrosin is used, a technician effect is likely due to variability in performing the staining procedure, including the amounts of stain and semen used, the methods for mixing the stain and semen, the pressure and methods used to make the slide smear, choice of areas to evaluate, and interpretaion of the staining results. Because viability is determined objectively by visual assessment, counting and assigning “stained” or “unstained” to sperm that are grouped in clumps could prove difficult to the human eye. When the cells lie on

top of each other, mistakes can be made in determining whether they are stained and sperm can be missed in the count. Dott and Foster (1972) conducted an experiment comparing the assessment of eosinophilic and non-eosinophilic sperm cells with eosin and 5 g of nigrosin (EN) or 10 g nigrosin (NE), and with or without clearing (immersion in aniline blue). Results indicated that both concentration of nigrosin and clearing affected the results, which was explained by the occurrence of partially stained sperm [33, 34, 36]. By the definition of the method by which eosin-nigrosin works, it can be assumed that partially stained sperm are sperm in which some form of plasma membrane damage has occurred; therefore, we chose to classify any level of pink staining of eosin as a membrane damaged cell.

Many of these earlier studies focused on examining the technique of using eosin-nigrosin; it was difficult to validate its use as a reliable method for determining sperm viability because there was no accepted standard to which to compare it [33-36]. More recent studies have compared eosin-nigrosin to other sperm viability tests [9, 14-16, 37]. Pintado et al. (2000) compared the use of eosin-nigrosin, Hoechst 33258, PI, and Hoechst 33258 with PI in bull and boar sperm. Propidium iodide (PI) is a membrane impermeant fluorescent stain that stains DNA and is excited by 488 nm wavelength of light. Because PI only enters cells with damaged membranes, it is commonly used as a test for non-viability. Hoechst 33258 is also a fluorescent dye that stains DNA and is excited by 350 nm wavelength of light. Hoechst 33258 is a membrane-permeant dye

and is more lipophilic when compared to PI therefore it is able to enter intact sperm membranes as well as non-intact sperm membranes, and relative staining is dependent upon the concentrations of Hoechst 33258 used. Visual assessment under light and fluorescent microscopy was used in the analysis of all four methods in the study conducted by Pintado et al. The eosin-nigrosin protocol employed in this study had added Giemsa despite the findings of preliminary studies done by the same group that recorded that the number of non-viable cells were not different when comparing eosin-nigrosin used alone and eosin-nigrosin used with Giemsa [37]. Three experiments were conducted to determine the correlations between the three methods of assessing sperm viability using fresh semen from boars (Experiment 1), boar semen with dead sperm added (Experiment 2), and frozen bull semen (Experiment 3). When samples from fresh boar semen were analyzed (Experiment 1), a high correlation was found among all methods; however, the highest correlation ($R=0.964$: $P<0.01$) was found between the PI and Hoechst stains [37]. When dead spermatozoa were added to fresh boar ejaculates (Experiment 2), correlations were lower ($R=0.727$, $P<0.01$) with PI showing a higher number ($66.4 \pm 2.4\%$) of stained (non-viable) cells than did either eosin-nigrosin ($56.9 \pm 1.84\%$) or Hoechst 33258 ($60.1 \pm 1.66\%$) [37]. Similarly, when frozen-thawed bull semen was evaluated, (Experiment 3), PI labeled a higher number of cells ($P<0.05$) compared to the other methods [37]. Pintada et al. concluded that PI consistently stained more non-viable sperm, and that the method type used for

determining sperm viability may influence the final value. In addition, Pintada et al. suggested that species type may also affect the results since Hoechst 33258 and eosin-nigrosin were more highly correlated in bull sperm (R=0.80; P=0.0017) than in boar sperm (R=0.84: P=0.0007 for Exp. 1; R=0.38; P=0.219 for Exp. 2) [37].

Viability results with eosin-nigrosin have also been compared to those obtained with a combination of the fluorescent dyes SYBR-14 and PI, using light (eosin-nigrosin) and fluorescent (SYBR-14) microscopy [15]. SYBR-14 is a membrane-permeant nucleic acid stain and therefore stains the DNA of all cells. It is excited by 522 nm of laser light. When used together with fluorescent microscopy or a flow cytometer, SYBR-14 and PI can allow the researcher to rapidly and definitively distinguish between non-viable and viable cells in a population. SYBR-14-stained sperm (viable) fluoresce green, and SYBR-14/PI-stained sperm (non-viable) fluoresce red (Figure 2).

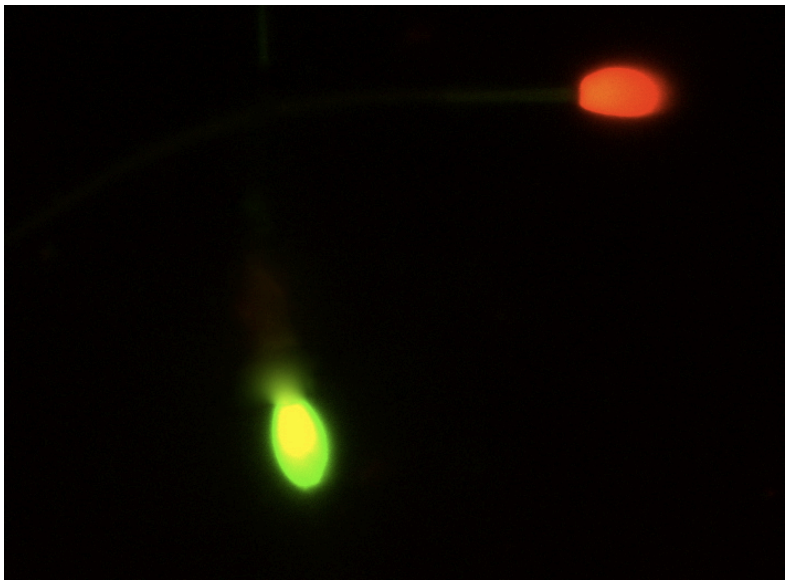

Figure 2. Equine sperm that have been treated with SYBR-14 and PI and analyzed using fluorescent microscopy. The green (SYBR-14) sperm head is considered viable and the red (SYBR-14/PI) is considered non-viable.

Chalah et al. assessed sperm viability in fowl sperm at 0, 0.5, 2, 4, and 24 hours of storage at 4^0C and after frozen semen had been thawed. Membrane viability of the samples stained with eosin-nigrosin was assessed visually using light microscopy. Membrane viability of the samples stained with the dual fluorescence (SYBR-14/PI) was assessed using a pre-scaled spectrophotometer. In this study, Chalah et al. found that the dual fluorescence of SYBR-14/PI using the spectrophotometer was a more efficient method for determining sperm membrane viability when compared to eosin-nigrosin staining ($P<0.05$) in fresh fowl ejaculates at Time 0 and Time 30 minutes indicating that SYBR-14/PI is a faster staining technique than eosin-nigrosin [15]. In cryopreserved samples, the two staining methods for sperm membrane viability were not significantly different if viability was assessed immediately after the

samples were thawed [15]. However, when sperm was assessed after 4 hours of cooled storage post-thaw, SYBR-14/PI stained samples showed a significantly higher ($P<0.05$) number of non-viable sperm when compared to the eosin-nigrosin. Chalah et al. concluded that the dual fluorescence of SYBR-14/PI is a quicker and more effective method for determining sperm viability in fowl [15].

Assessment of sperm viability using a new method, the NucleoCounter® SP-100™, is based on a computerized method of fluorescent microscopy whereby sperm is diluted in detergent to obtain total sperm concentrations or phosphate-buffered saline to obtain non-viable sperm concentration and 60µL of this sample is aspirated into a cassette containing a flow system laced with propidium iodide (PI). The PI in the cassette is dissolved by this mixture and sperm with disrupted plasma membranes uptake the PI and emits red fluorescence that is detected by the NucleoCounter® SP-100™. The only study known to us comparing the sperm membrane viability function of the Nucleocounter® SP-100™ to another method of determining sperm membrane viability was conducted by Johansson et al. in 2008 [14]. This study compared equine sperm membrane viability assessed using eosin-nigrosin staining to that obtained using the Nucleocounter® SP-100™ in eight ejaculates from nine stallions. This study also attempted to correlate membrane viability with sperm motion characteristics (progressive motility). Findings from this study included: 1) the eosin-nigrosin staining technique consistently showed a higher population

of viable cells when compared to the Nucleocounter® SP-100™ 2) the mean difference in sperm membrane viability between the two methods was 12.5%, however this difference was not statistically significant (P = 0.09; R = 0.64), and 3) sperm membrane viability assessed with both methods correlated highly with progressive motility (R=0.72 with eosin-nigrosin staining; R=0.79 with NucleoCounter SP-100) [14].

The objectives of this thesis research were to compare three methods for detecting membrane viability of equine sperm, and the effects of seminal plasma on sperm membrane viability and motion characteristics. The three methods were flow cytometry using SYBR-14/propidium iodide, eosin-nigrosin staining, and the NucleoCounter® SP-100™.

2. MATERIALS AND METHODS

Semen was collected using an artificial vagina (Missouri-model; Nasco, Ft. Atkinson, WI, USA) fitted with a nylon micromesh filter (Animal Reproduction Systems, Chino, CA, USA) to remove gel and debris from the ejaculate. The stallions were mature (age 9 to 20 years old) and sexually active. Stallions were collected daily for 2 to 3 days then allowed a 48-hour period of sexual rest prior to collection of an ejaculate for the experiments. A mare in estrus or an ovariectomized mare was used for sexual stimulation and a breeding phantom was used as a mounting source for the stallion. The penis was washed thoroughly with water and dried while erect immediately prior to collection. The artificial vagina was lubricated with a small volume (3 to 5 mL) of sterile non-spermicidal lubricant (Priority Care, First Priority, Inc., Elgin, IL, USA) before the semen was collected.

2.1. Semen processing

After collection the semen was taken to an adjacent laboratory and evaluated for: volume (using a graduated cylinder); concentration as determined by the NucleoCounter® SP-100™; viability as determined by the NucleoCounter® SP-100™ and eosin-nigrosin stain; and for sperm motion characteristics using a computer-assisted sperm motion analyzer (CASMA; IVOS Version 12.2L, Hamilton Thorne Biosciences, Beverly, MA, USA).

2.2. Seminal plasma processing

One to two ejaculates were collected from each of three stallions used in the experiments to obtain sperm-free seminal plasma. The semen was collected as previously described, and the raw semen was placed in two 15mL BLUE MAX™ polypropylene conical tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The semen was centrifuged (IEC Centra CL@; Thermo Scientific, Waltham, MA, USA) for 10 minutes at 2000 x *g*. The supernatant (seminal plasma) was aspirated off of the pellet (sperm) using a plastic 1 mL transfer pipette (Samco Scientific, Mexico) and transferred to a 15 mL syringe equipped with a 1.2 micron and 5.0 micron nylon filter (Cameo 30N Syringe Filter, Nylon, 30 mm; SIGMA-ALDRICH, St. Louis, MO, USA). These filters served to remove any sperm still remaining in the seminal plasma. The seminal plasma was filtered through the nylon filters into a separate conical tube. Aliquots (1.2 mL) of seminal plasma were placed in micro tubes (disposable/conical economy micro tubes with snap caps [1.5 mL]; VWR International, USA) and stored in a freezer at -80°C until used.

2.3. Eosin-Nigrosin slide preparation and analysis

Slides (Bev-l-edge Micro Slides; pre-cleaned; twin-frost; Propper Manufacturing Co., Inc., Hungary) were placed on a warmer for several minutes. The slides were labeled with the appropriate treatment, time, and replicate number. Slides were removed from the warmer and 15 μl of eosin-nigrosin stain was placed ¼ inch from the frosted section of the slide using a pipetter. Ten μL

of the semen sample from the treatment tube was dispensed into the eosin-nigrosin stain and the mixture was stirred five times with the pipette tip. A separate slide was held at a 45° angle and used to gently streak the sample mixture. The slide was placed back on the warmer for several minutes to allow the stain mixture to dry and then placed in a slotted slide box and evaluated within the next 48 hours. A total of 300 sperm were classified according to staining. Sperm that did not uptake the stain (remained white) were counted as viable and sperm that did uptake the stain (turned pink) were counted as non-viable sperm, using percentage to determine viability. Sperm were classified as non-viable if they exhibited any uptake of eosin therefore partially stained sperm were counted as non-viable.

2.4. Flow cytometry (SYBR-14/propidium iodide staining)

Treatment samples were prepared using the protocol from the LIVE/DEAD® Sperm Viability Kit (L-7011) (Molecular Probes, Inc., Eugene, OR) with a few modifications. The kit utilizes a prepared working solution of SYBR-14 previously diluted in sterile dimethyl sulfoxide (DMSO; SIGMA-ALDRICH, St. Louis, MO; USA) or a buffer to yield SYBR-14 working solution concentrations of 100 μM or 20 μM, respectively, and PI at a concentration of 2.4 mM as a stain for sperm non-viability. The protocol from the manufacturer recommends that the SYBR-14 dye concentrate (stock solution) be diluted 10-fold in DMSO, with 5 μl of this SYBR-14 working solution subsequently added to 5 mL of extended semen, thereby yielding a final SYBR-14 concentration of 0.1μM. The sample

was to be incubated for 10 minutes at 37°C before the addition of 25 μL of PI solution to give a final PI concentration of 12 μM. The tube was to be vortexed gently for three seconds after the addition of PI. The manufacturer recommended an additional 5 minute incubation at 37°C before analysis. For the current project, the volumes of SYBR-14, PI, and extended semen were immediately reduced to half the original volumes: this allowed use of less material while concentrations of SYBR-14 and PI were maintained at the level recommended by the manufacturer. The protocol was later modified based on the results of Experiment 2 and Experiment 3.

Prior to flow cytometric analysis a designated volume of the treatment sample, which varied dependent on sperm concentration as described below (Table 1), was combined with 500 μL of Garner's solution (130 mM NaCl, 40mM KCl, 14 mM Fructose, 10 mM Hepes, 1 mM $CaCl_2$, 0.2 mM $MgCl_2$, Bovine Serum Albumin V 1 mg/mL, pH7.38). The table (Table 1) was formulated based on a preliminary project conducted in the laboratory to determine the appropriate volume of semen to add to the 500 μL of Garner's solution to allow for an events-per-second count of 200 to 500 when processed on the flow cytometer. The volume was dependant upon the sperm concentration of the semen sample.

Table 1. Volume of the semen sample, dependent on sperm concentration, added to the flow cytometer tube containing 500 μL of Garner's solution.

Sperm concentration (million/mL)	Volume of treatment sample (μL)
< 15	10.0
16-30	5.0
31-59	2.5
60-79	2.0
80-250	1.0
>250	0.5

This sample was allowed to equilibrate for 30 seconds before being evaluated by flow cytometric analysis (FACScan Analyzer flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Flow cytometric data was stored in List-mode until analysis. A total of 5000 cellular events were evaluated per sample at a rate of between 200 to 500 cells/second and the percentages of viable and non-viable sperm were determined using WinList™ software (Verity Software House, Topsham, ME, USA).

2.5. NucleoCounter® SP-100™ (propidium-iodide staining)

Total sperm concentration was determined by adding Reagent S-100 (Chemometec A/S, Allerød, Denmark) at appropriate dilution factor volumes to Cryogenic vials ([1.2 mL and 2.0 mL]; Corning Life Sciences, Lowell, MA, USA), Reagent S-100 is a detergent that is designed to disrupt all plasma membranes and allows PI to label DNA in all sperm in the sample. The non-viable sperm concentration was determined by substituting phosphate-buffered saline (GIBCO® Dulbecco's Phosphate Buffered Saline 1X; Invitrogen; Grand Island,

NY, USA) for Reagent S-100. Dilution factors were prepared according to a manufacturer recommended dilution table (Table 2).

Table 2. Manufacturer recommended (Chemometec A/S, Allerød, Denmark) dilution table.

Sperm concentration (million/mL)	Dilution Factor	Diluent volume (μL)	Semen volume (μL)
<8	6	500	100
8-70	11	1000	100
15-125	21	1000	50
25-300	51	1000	20
50-700	101	1000	10
100-1400	201	2000	10
200-2500	401	4000	10

Each treatment sample was mixed thoroughly and a specific sample volume was added using a positive displacement pipette. The sides of the pipette tip were wiped with a Kim-Wipe® to remove any excess sperm before the sample was dispensed into the solution. The cryovial was inverted 10 times prior to analysis. An SP1-Cassette (Chemometec A/S, Allerød, Denmark) was used to determine total and non-viable concentrations (one cassette/tube). The total concentration was assessed immediately prior to determining the non-viable concentration for each replicate to allow the SemenView™ program (Version 1.21; Chemometec A/S, Allerød, Denmark) to calculate the percent viable cells. The percentage of viable cells was calculated using the SemenView™ program, or by hand calculations using the following equation supplied by Chemometec: Viability (%)

= 100% * [(TOTAL – NON-VIABLE)/TOTAL)]. In order for the SemenView™ program to calculate the viability of the sample, the total concentration and non-viable concentration must be run consecutively with the total concentration first. If an error occurred during data collection and the counts became out of sequence, the viability was calculated by hand.

2.6. Sperm motion characteristics (CASMA)

A small aliquot of the treatment sample was extended (~25 million/mL) in prewarmed (37°C) INRA 96 (GlaxoSmithKline, Research Traingle Park, NC, USA) or INRA 96 with 1 mg/mL added of Timentin (INRA-T) and aliquoted into 0.6 mL microcentrifuge tubes with a flat cap (Fisher Scientific; Pittsburgh, PA, USA). These tubes were either used for immediate anlaysis or were placed in an Equitainer® I for later analysis. For analysis, the microcentrifuge tube was placed on a slide warmer and allowed to warm for 10 minutes. Six μl of the contained sample was loaded onto warmed (37°C) analysis chambers fixed to microscope slides (Leja Standard Count 2 Chamber slides; Laja Products, B.V., Nieuw-Vennep, The Netherlands). The slide was placed onto a warmed stage and inserted into the CASMA. A minimum of 10 microscopic fields and 500 sperm were analyzed per sample. Preset values for the IVOS system consisted of the following: frames acquired – 45; frame rate – 60 Hz; minimum contrast – 70; minimum cell size – 4 pixels; minimum static contrast – 30; straightness (STR) threshold for progressive motility – 50; average path velocity (VAP) threshold for progressive motiliy – 30; VAP threshold for static cells – 15; cell

intensity – 106; static head size – 0.60 to 2.00; static head intensity – 0.20 to 2.01; static elongation – 40 to 85; LED illumination intensity – 2200. Each replicate performed was extracted from the same microcentrifuge tube prepared for the CASMA. Motility parameters assessed and of importance to this study were total and progressive sperm motility.

2.7. Statistical analyses

Percentages of non-viable and viable sperm obtained from the three methods for assessing sperm membrane viability, and the total sperm motility obtained from the CASMA were converted from percentage to decimal points. A general linear model was utilized using SAS® (Statistical Analysis Software). Percentage-data was transformed to its arc sin square root. Analysis of variance (ANOVA) was used to evaluate the main effects of treatment, time, and treatment x time interaction. Means for the coefficients of variation from the transformed data were calculated to determine method repeatability. In addition to the statistical methods discussed above, in Experiment 6, Bland and Altman plots were used to determine method agreement between the flow cytometer, NucleoCounter® SP-100™, and eosin-nigrosin staining. Bland and Altman plots were also prepared to determine any agreement between total motility (TMOT) using CASMA and sperm membrane viability using the three methods. The limits of agreement were calculated and graphed based on literature published by Bland and Altman [38, 39]. Association between methods was further described using simple regression on untransformed data and compared to the line of

equality. All equations and their associated values as well as the absolute values of the differences between methods were also reported.

3. EXPERIMENTAL DESIGN WITH RESULTS AND DISCUSSION

3.1. Experiment 1: Comparison of SYBR-14 working solution protocols

Experiment 1 was conducted to determine the best method for preparing the SYBR-14 working solution to be used for evaluation of sperm membrane viability by flow cytometry. Specifically, since the different working solutions also had varying levels of DMSO, this experiment measured the effect of concentrations of DMSO (%V/V), on sperm membrane viability.

The LIVE/DEAD Sperm Viability Kit recommends two different dilutions for preparing the SYBR-14 working solution (dilution in DMSO or a buffer). The SYBR-14 stock solution is packaged as 100 μL of a 1 mM solution in 100% DMSO. The PI solution is packaged as 5 mL of a 2.4 mM solution in water. For this study a total of 4 dilutions were prepared (Treatments 1 to 4): 1) SYBR-14 stock soloution was diluted 50-fold in Garner's solution yielding a 2% DMSO and a 20 μM SYBR-14 working solution. This solution was prepared fresh before each ejaculate; 2) SYBR-14 stock solution was diluted 10-fold in DMSO yielding a 100% DMSO and 100 μM SYBR-14 working solution. This solution was stored at -80°C and thawed before use; it was reused a maximum of one time; 3) SYBR-14 stock solution was diluted 5-fold in DMSO yielding a 100% DMSO and 200 μM SYBR-14 working solution concentration. This solution was stored at -80°C and thawed before use; it was reused a maximum of one time; 4) SYBR-14 stock solution was diluted 5-fold in PBS yielding a 20% DMSO and 200 μM SYBR-14 working solution. This solution was prepared fresh for each

ejaculate. These SYBR-14 working solutions are referred to as Treatments 1 to 4 (Table 3).

Table 3. Four different protocols for preparing the SYBR-14 working solution from the stock solution (1mM SYBR-14 in 100% DMSO) including treatment number, dilution ratio, diluent, DMSO concentration (%V/V), and concentration of SYBR-14.

Treatment	Dilution	Diluent	% DMSO	[SYBR-14] (μM)
1	50 fold	Garner's	2	20
2	10 fold	DMSO	100	100
3	5 fold	DMSO	100	200
4	5 fold	PBS	20	200

A single gel-free ejaculate was collected and processed as described above. The semen was extended to approximately 25 x 10^6 sperm/mL in INRA-96. After the addition of SYBR-14 solutions to the semen sample, the tubes were incubated for 10 minutes at 37°C and then PI was added, followed by an additional 5 minute incubation at 37°C. The tubes were prepared using the four volumes of semen and SYBR-14 working solutions as given in Table 3. The DMSO concentration (%V/V), concentration of SYBR-14 and concentration of PI within each treatment tube was calculated after being added to the semen sample and the results are given in Table 4. The samples were prepared then analyzed on the flow cytometer using the protocol described in the materials and methods at zero, 10, and 20 minutes. Two replicates were performed for each

treatment at each time interval for a single ejaculate. Percentage of sperm assessed as viable or non-viable were compared among treatments.

Table 4. Volumes of semen, concentration of DMSO (%V/V), and final volumes/concentrations of SYBR-14 working solution and PI used with the SYBR-14 working solutions (Treatments 1-4) in preparation for analysis using flow cytometer.

Tube	SYBR-14 treatment	Semen (μL)	SYBR-14 (μL)	[SYBR-14] (μM)	PI (μL)	[PI] (μM)	% DMSO
1	1	1000	5	0.1	5	12	0.01
2	2	2500	2.5	0.1	12.5	12	0.1
3	3	150	20	26.6	36	576	2.6
4	4	150	20	26.6	36	576	13.3

3.1.1. Results: Experiment 1: Comparison of SYBR-14 working solution protocols

Mean percent viable sperm was similar for Treatments 1 and 2 (75.24% and 74.73%, respectively, P>0.05) but was lower for Treatments 3 and 4 (59.3% and 38.6%, respectively, P=0.004) with increasing DMSO concentrations (%V/V) (Figure 3).

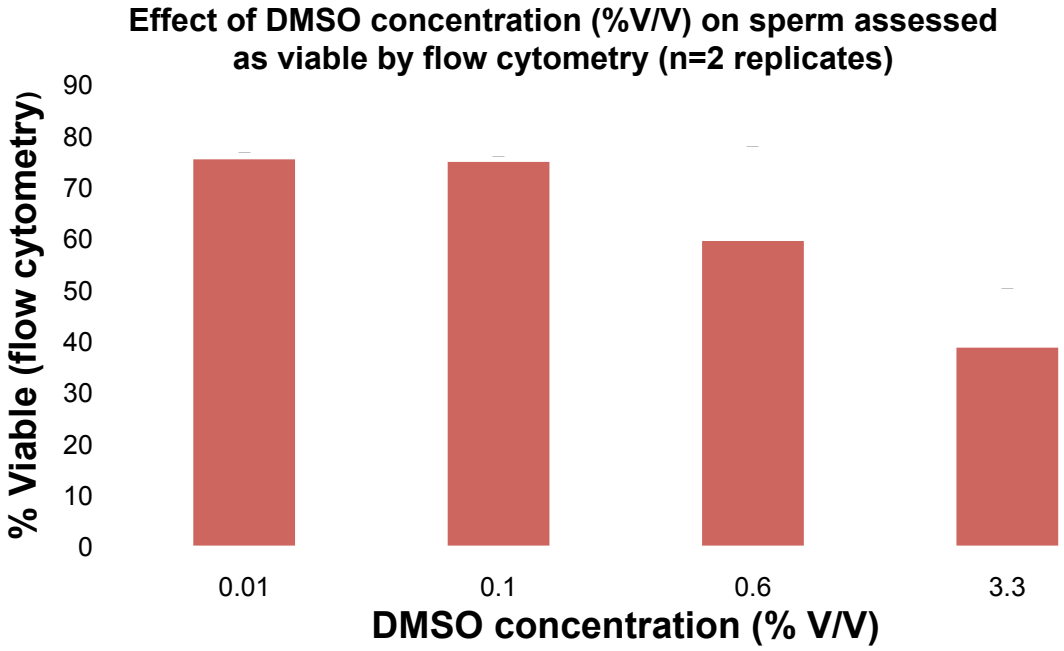

Figure 3. Effect of DMSO concentration (%V/V) on sperm assessed as viable by flow cytometry (n=2 replicates, values for three assessment times was combined) with standard deviation error bars.

3.1.2. Discussion: Experiment 1: Comparison of SYBR-14 working solution protocols

Results from this experiment were in agreement with other studies [40-42] in that DMSO at levels such as 1.0 M [40, 42], and 1.5%-3% (v/v) [41], caused increased permeability of the sperm plasma membranes. Experiment 1 was conducted to determine which SYBR-14 working solution to use in the remaining experiments, and results indicated that the protocols (Treatment 1 and Treatment 2) from the LIVE/DEAD Sperm Viability Kit were superior when compared to the experimental protocols (Treatment 3 and Treatment 4) for

determining the membrane viability of sperm, with the assumption that the higher value for viability was the correct on. Standard deviation was greater in Treatments 3 and 4. Data from Times 0, 10, and 20 minutes were combined in Figure 1 and values for sperm membrane viability decreased as incubation time increased. Treatment 2 was chosen as the protocol to be used for the remaining experiments due to the manufacturer's recommendation that if the SYBR-14 stock solution was diluted in DMSO it could be re-frozen and used at a later date. This recommendation (re-use after freezing) was not validated experimentally until a later preliminary study (Experiment 4), therefore the SYBR-14 working solution, using Treatment 2, was prepared fresh for each ejaculate in Experiments 2 and 3.

3.2. Experiment 2: Comparison of SYBR-14 working solution volumes and incubation times

To assess the difference in the staining of the DNA of the sperm at different volumes of SYBR-14 (0.5 μL, 1.0 μL, 2.5 μL) added to a 2.5-mL aliquot of semen, thus resulting in solutions with 0.02 μM, 0.04 μM and 0.1 μM SYBR-14 and 0.04%, 0.02%, and 0.1% DMSO, respectively (Table 5), and to determine if incubation time (15 minutes, 16.5 minutes, 18 minutes) affected the recorded membrane viability of the sperm, one ejaculate from each of three stallions was collected as previously described and extended to approximately 25 x 10^6 sperm/mL in INRA 96 to a total volume of 100 mL and placed in two 50 mL conical tubes. SYBR-14 was diluted 10 fold in DMSO (Treatment 2,

Experiment 1). This working solution was prepared immediately before each stallion was collected and stored away from direct light until needed. The final SYBR-14 concentrations for the three different volumes were 0.02 μM for 0.5 μL SYBR-14, 0.04 μM for 1.0 μL SYBR-14, and 0.1 μM for 2.5 μL SYBR-14 (Table 5). The final PI concentration for each volume was 12 μM.

Table 5. The SYBR-14, DMSO and PI volumes and concentrations added to 2.5 mL of extended semen prior to flow cytometric analysis.

SYBR-14 (μL)	[SYBR-14] (μM)	DMSO (%)	PI (μL)	[PI] (μM)
0.5	0.02	0.02	12.5	12
1.0	0.04	0.04	12.5	12
2.5	0.1	0.1	12.5	12

Experiment 2 consisted of four parts (A-D) performed on the same ejaculate, and three replicates were performed for each part. Part A- Three-flow cytometer tubes per replicate (9 tubes total) were labeled with treatment (0.5 μL, 1.0 μL, 2.5 μL) and prepared using the protocol previously described (2.5 mL semen, 12.5 μL PI, and one of three SYBR-14 volumes). Samples from different SYBR-14 concentration treatments were evaluated in a staggered order to ensure that the treatments were not run in the same order. Order of assessment was: Tube labels (SYBR-14 volume): 1-1a (0.5 μL), 1-1b (1.0 μL), 1-1c (2. μL); 2-2a (1.0 μL), 2-2b (2.5 μL), 2-2c (0.5 μL); 3-3a (2.5 μL), 3-3b (0.5 μL), 3-3c (1.0 μL). Three tubes were prepared simultaneously (one tube for each different SYBR-14 volume). Tubes were run on the flow cytometer continuously,

resulting in a mean incubation time of 15, 16.5, and 18 minutes for the first, second and third sets of treatments, respectively. Part B was similar to Part A with one modification. Tubes were labeled as described above and prepared seperately at a 2-3 minute interval to allow for an incubation time of 15 minutes for each treatment within replicates (all tubes incubated for no more than 15 minutes). Results obtained on the flow cytometer for sperm viability from Part B were compared to those from Part A to determine if an increase in incubation time (16.5 and 18 minutes) at 37°C would cause a change in the proportion of sperm assessed as viable. Parts A and B were performed to test the inter-assay coefficients of variation between SYBR-14 volumes (μL)/concentrations (μM), and incubation times (minutes).

Parts C and D were performed to test the repeatability, or intra-assay coefficients of variation for SYBR-14 concentrations (μM), and incubation times (minutes). Parts C and D were similar to the previous two parts (Parts A and B) however the tubes were run in a different order. Three-flow cytometer tubes/replicate (9 tubes total) were labeled with volume (0.5 μL, 1.0 μL, 2.5 μL) and prepared using the protocol previously described. Three tubes, containing the same SYBR-14 concentration, were incubated simultaneously prior to evaluation by flow cytometry. Unlike Part A and B, the three tubes prepared per replicate had the same SYBR-14 volume. Tube labels for Part C (SYBR-14 volume): 1-1a, 1-1b, 1-1c (all 0.5 μL); 2-2a, 2-2b, 2-2c (all 1.0 μL); 3-3a, 3-3b, 3-3c (all 2.5 μL). In Part C tubes were run at 15, 16.5, and 18 minutes. The

design for Part D was similar to Part C however the three tubes per replicate were prepared at 2-3 minute intervals to ensure an incubation time of 15 minutes for all tubes and SYBR-14 concentrations.

3.2.1. Results: Experiment 2: Comparison of SYBR-14 working solution volumes and incubation times

In Experiment 2 there was no incubation-time by SYBR-14 volume interaction detected for sperm membrane viability as assessed using the flow cytometer ($P>0.05$). No differences ($P>0.05$) in recorded sperm membrane viability were found between incubation times (Table 6) or between SYBR-14 working solution volumes (Table 7) when each replicate was evaluated as a single observation. There was a significant effect of stallion ($P<0.05$). Inter- and intra-assay coefficients of variation are show in Tables 8 and 9. The coefficient of variation for all observations (N=24) was 5.7%,

Table 6. Effect of incubation time on the percent of viable sperm using flow cytometer (mean ± SD). (N=72 for 15 minutes; N=18 for 16.5 and 18 minutes).

Incubation Time (minutes)	Mean % viable ± SD
15	85.1[a]±6.4
16.5	85.6[a]±6.7
18	85.4[a]±6.7

[§] Percentage data (% viable) were arc sine-root transformed prior to statistical analysis. Original means and standard deviations are presented in the table but statistical tests were conducted on transformed data.

[a] Within incubation time, means with different superscripts differ ($P<0.05$)

Table 7. Effect of SYBR-14 working solution volume on the mean percent of viable sperm using flow cytometry (mean ± SD) (N=36).

SYBR-14 working solution volume (μL)	Mean % viable ± SD
0.5	85.3[a]±6.5
1.0	85.3[a]±6.6
2.5	85.1[a]±6.4

[§] Percentage data (% viable) were arc sine-root transformed prior to statistical analysis. Original means and standard deviations are presented in the table but statistical tests were conducted on transformed data.

[a] Within working solution volume, means with different superscripts differ (P<0.05)

Table 8. Inter-assay coefficients of variation for Part A and B for incubation time and SYBR-14 volume/concentration for the measurement of sperm viability using the flow cytometer (n=3 replicates for Part A; n=9 replicates for Part B).

Part	Incubation time (minutes)	SYBR-14 volume (μL)	Coefficient of Variation (%)
A	15	0.5	10.6
		1.0	10.7
		2.5	11.3
	16.5	0.5	11.4
		1.0	9.7
		2.5	10.8
	18	0.5	10.8
		1.0	10.5
		2.5	10.1
B	15	0.5	8.8
		1.0	9.3
		2.5	8.8

Table 9. Intra-assay coefficients of variation for Part C and D for incubation time and SYBR-14 volume/concentration for the measurement of sperm membrane viability using the flow cytometer (n=3 replicates for Part C; n=9 replicates for Part D).

Part	Incubation Time (minutes)	SYBR-14 volume (μL)	Coefficient of variation (%)
C	15	0.5	7.2
		1.0	7.2
		2.5	8.5
	16.5	0.5	7.5
		1.0	6.2
		2.5	7.1
	18	0.5	6.8
		1.0	9.3
		2.5	6.6
D	15	0.5	6
		1.0	6
		2.5	5.7

3.2.2. Discussion: Experiment 2: Comparison of SYBR-14 working solution volumes and incubation times

The results from Experiment 2 indicate that similar results for sperm membrane viability can be obtained when using a smaller volume/concentration of the SYBR-14 working solution (1.0 μL/0.04 μM, or 0.5 μL/0.02 μM) compared to the original protocol volume (2.5 μL/0.1 μM). In addition, an incubation-time difference of three minutes had no effect on recorded sperm membrane viability when evaluated by flow cytometry. In Experiment 2, a change in pattern was seen with the viable population (R1 in Figure 4) on the flow cytometer scatterplots when the volume/concentration of SYBR-14 was increased to 2.5

μL/0.1 μM. It was hypothesized that this was due to a high SYBR-14 concentration causing inconsistent sperm staining.

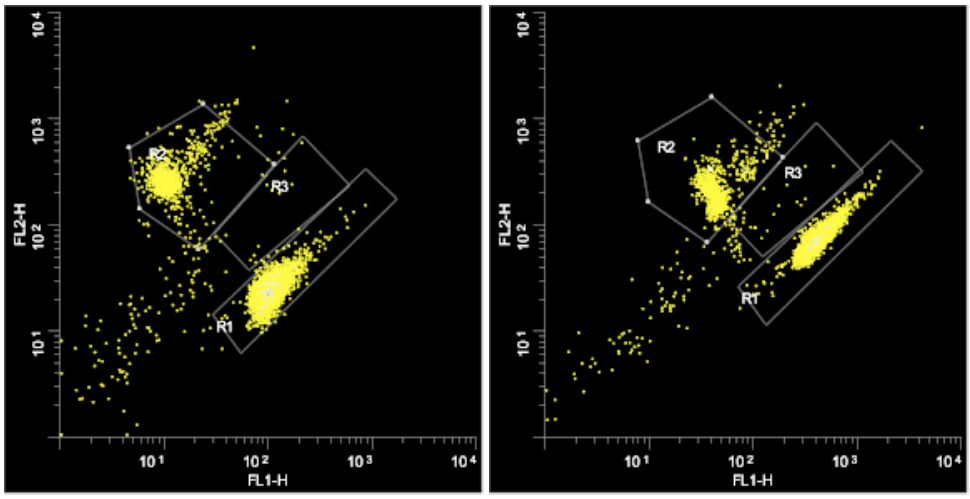

Figure 4. Flow cytometer scatterplots visually comparing the viable populations (R1) seen with 0.5 μL SYBR-14 (left) and the viable population seen with 2.5 μl SYBR-14 (right).

The scatterplot of the viable population appeared elongated rather than round as the volume of SYBR-14 was increased to 1.0 μl and then to 2.5 μl as compared to a more circular shape (indicative of consistent sperm staining) with a volume of 0.5 μl, suggesting variation in stain uptake. The percentages of viable and non-viable populations were not affected by this elongated pattern. The source of the elongated pattern was unknown. A possible explanation for this was thought to be the increased SYBR-14 concentration alone, however, since the SYBR-14 solution contains DMSO, and because concentrations of

DMSO (%V/V) also vary in the three SYBR-14 volume treatments, another experiment was needed to establish the cause of the elongated pattern.

DMSO has been known to cause changes in recorded sperm membrane viability as was evident in the findings of Experiment 1, and other studies have found DMSO to be associated with an increase in non-viable populations of sperm in rabbits [40], buffalo [41] and horses [42]. The three SYBR-14 volumes/concentrations (0.5 μL/0.02 μM, 1.0 μL/0.04 μM, and 2.5 μL/0.1 μM) used in Experiment 2 had different concentrations (%V/V) of DMSO (0.02 %, 0.04%, 0.1%). Experiment 3 was performed in an attempt to determine the cause of the elongated pattern.

3.3. Experiment 3. Effect of SYBR-14 concentration on SYBR-14 fluorescence intensity when DMSO concentration is held constant

A single ejaculate was collected from a stallion as previously described. Neat semen was extended in INRA 96 to approximately 25 x 10^6 sperm/mL to a total volume of 150 mL. The extended semen was then divided into three 50 mL conical tubes and the tubes were stored at room temperature and out of direct light until analysis. Three five-tube treatment sets were pulled from a common 50 mL conical tube. A 2.5 mL aliquot of the semen sample was placed in each of five treatment tubes. After the addition of SYBR-14, PI, and/or DMSO to the semen sample, samples were take from each of the five tubes for flow cytometric analysis in random order. When all tubes had been sampled, a second set of samples was taken from the same 50 mL conical tube. The

volumes added to 2.5 mL of semen were as follows: (A) 2 µL DMSO, 0.5 µL SYBR-14, 12.5 µL PI; (B) 1.5 µL DMSO, 1.0 µL SYBR-14, 12.5 µL PI; (C) 0 µL DMSO, 2.5 µL SYBR-14, 12.5 µL PI; (D) 2.5 µL DMSO, 0 µL SYBR-14, 12.5 µL PI; (E) 0 µL DMSO, 2.5 µL SYBR-14, 0 µL PI (Table 10). Because the percent of DMSO varied in the samples prepared for Experiment 2, a designated volume of DMSO was added to the treatment tubes to allow for a constant 0.1% DMSO in each tube. The protocol previously described was used for tube preparation, incubation, and analysis on the flow cytometer. All tubes were vortexed prior to analysis.

Table 10. Treatment tube labels with volume and % DMSO, and volume/concentration of SYBR-14 and PI for Experiment 3.

Treatment	DMSO (µL)	DMSO (%)	SYBR-14 (µL)	SYBR-14 (µM)	PI (µL)	PI (µM)
A	2	0.1	0.5	0.02	12.5	12
B	1.5	0.1	1.0	0.04	12.5	12
C	0	0.1	2.5	0.1	12.5	12
D	2.5	0.1	0	0	12.5	12
E	0	0.1	2.5	0.1	0	0

3.3.1. Results: Experiment 3: Effect of SYBR-14 concentration on SYBR-14 fluorescence intensity when DMSO concentration is held constant

No differences (P>0.05) were seen between the volumes of DMSO and SYBR-14 working solution added to the semen and PI when membrane viability was assessed (Table 11). The mean percent of viable sperm for all

observations (N=45) in Experiment 3 was 83.43% with a coefficient of variation of 1.2%. The elongated pattern noted in the previous experiment was still evident when the DMSO concentration was held constant (0.1%).

Table 11. Effect of treatment (SYBR-14 and PI volumes) on the mean percent (mean ± SD) viable sperm using the flow cytometer (N=9).

Tube	Mean % viable ± SD
A	83.7[a]±0.6
B	83.6[a]±1.2
C	83.6[a]±1.0
D	83.2[a]±1.1
E	83.2[a]±1.0

[§] Percentage data (% viable) were arc sine-root transformed prior to statistical analysis. Original means and standard deviations are presented in the table but statistical tests were conducted on transformed data.

[a] Within treatment, means with different superscripts differ (P<0.05)

3.3.2. Discussion: Experiment 3: Effect of SYBR-14 concentration on SYBR-14 fluorescence intensity when DMSO concentration is held constant

When DMSO concentration was constant (0.1%) there was no difference in sperm membrane viability due to SYBR-14 volume or concentration. The results in Treatment E were interesting since it contained only SYBR-14 and no PI. Nevertheless, there were two distinct populations (Figure 3), one behind the other. SYBR-14 is a fluorescent stain that enters sperm cells with disrupted plasma membranes as well as those cells with intact plasma membranes. A possible explanation for the two distinct populations could be that the SYBR-14 is leaking out of the disrupted plasma membranes of the non-viable cells (R2 in

Figure 5) causing these cells to fluoresce less brightly when compared to the sperm cells in the viable population (R1 in Figure 5). If PI was added to this sample it is likely that the R2 population would take the PI stain and shift to the upper left location on the scatterplot.

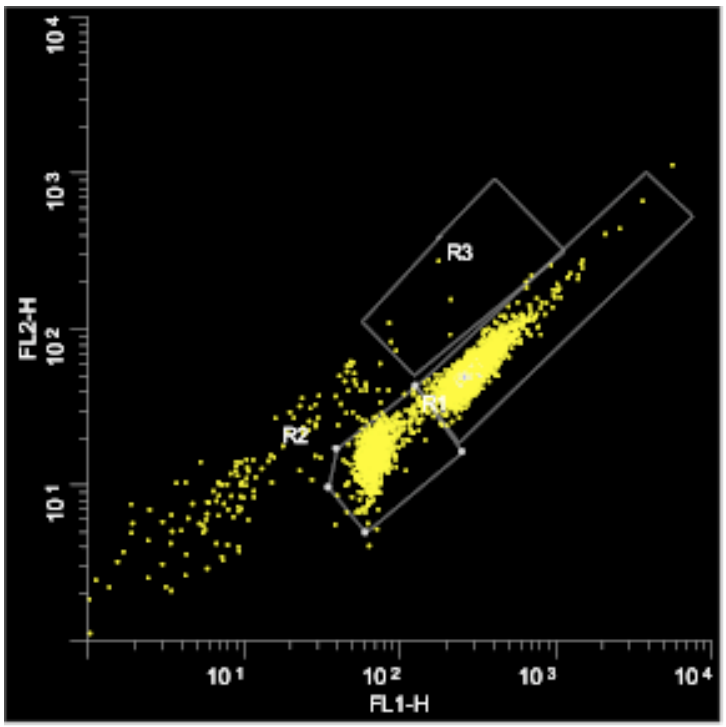

Figure 5. Flow cytometer scatterplot evaluated from Tube E of Experiment 3.

Because the elongated pattern was still evident in treatments containing 2.5 μL/0.1 μM SYBR-14 when DMSO was held constant (0.1%), it can be concluded that the probable source for the elongated pattern noted in Experiment 2 was the increase in the concentration of SYBR-14 alone. As more stain is introduced, the sperm cells uptake more stain causing an increase in green fluorescent

intensity along the x-axis. Another explaination could be that over-loading the sperm with stain could cause an uneven staining pattern seen on the scatterplot, thus a more elongated non-viable population at higher volumes as compared to a more rounded non-viable population at lesser volumes.

The results of Experiments 1, 2 and 3 supported the use of a lower volume/concentration of the SYBR-14 working solution (0.5 µL/0.02µM) in 2.5 mL of semen than previously recommended by the manufacturer (2.5 µL/0.1 µM). In addition, it was also determined that DMSO concentration alone would not affect recorded sperm membrane viability when used within the recommended volumes. Therefore the remaining experiments utilized a SYBR-14 working solution volume of 0.5 µL in 2.5 mL of semen.

3.4. Experiment 4: Effect of freezing and thawing on the SYBR-14 working solution

The LIVE/DEAD® Sperm Viability Kit gives two different methods for preparing the SYBR-14 working solution. One method utilizes Garner's solution as a buffer reagent and the second utilizes DMSO as a buffer reagent. When using the method containing Garner's solution, the SYBR-14 working solution must be prepared fresh for each experiment, whereas the manufacturer claims that the SYBR-14 working solution that contains DMSO as a buffer reagent can be frozen/thawed after preparation and re-used for later experiments. To test whether the SYBR-14 working solution could be frozen/thawed and how many times the solution could be re-used without having an effect on the results of

sperm viability obtained from the flow cytometer, an experiment was conducted using one ejaculate from each of three stallions. Prior to semen collection using the method described previously, a 10X dilution of SYBR-14 in DMSO, yielding a working concentration of 100 μM, was prepared in a single 0.5 mL Eppendorf tube to a total volume of 15 μL. Three other 0.5 mL Eppendorf tubes were labeled according to the number of times the sample would be frozen/thawed (1X, 3X, or 6X). Small aliquots (3.75 μL) were pipetted from the tube containing the SYBR-14 working solution and placed in each of the three tubes. The original tube was labeled with "no F/T" (not frozen and thawed) and served as the control for Experiment 4. A styrofoam container was filled with dry ice and the three tubes (1X, 3X, 6X) were placed in the container to allow the samples to freeze. After a period of approximately 5 minutes, all tubes were removed and allowed to thaw to room temperature. Tubes labeled with 3X and 6X were placed back in the dry ice and allowed to freeze again. The tubes were removed, thawed, then re-frozen according to their treatment then stored out of direct light at room temperature until ready for use.

After semen collection, the neat semen was extended in INRA 96 to a concentration of approximately 25 x 10^6 sperm/mL and a volume of 50 mL then placed in a 50 mL conical tube. Four flow cytometer tubes were prepared, one with each freeze/thaw treatment using the protocol previously described (0.5 μl SYBR-14/ 0.02μM), for each of three replicates performed on the same ejaculate.

3.4.1. Results: Experiment 4: Effect of freezing and thawing on the SYBR-14 working solution

There was no difference (P>0.05) in recorded sperm membrane viability among the four SYBR-14 working solution freeze/thaw treatments (Tables 12 and 13).

Table 12. Effect of treatment (number of freeze-thaw cycles) within replicates on the mean percent of viable sperm using the flow cytometer (n=12 for each replicate).

Replicate	Treatment	Mean
1	Control	87.3[a]
	1X	87.0[a]
	3X	87.6[a]
	6X	88.7[a]
2	Control	88.2[a]
	1X	88.6[a]
	3X	88.9[a]
	6X	88.6[a]
3	Control	88.0[a]
	1X	88.0[a]
	3X	88.4[a]
	6X	88.5[a]

[§] Percentage data (% viable) were arc sine-root transformed prior to statistical analysis. Original means and standard deviations are presented in the table but statistical tests were conducted on transformed data.

[a] Within replicate and treatment, means with different superscripts differ (P<0.05)

Table 13. Effect of treatment (number of freeze-thaw cycles) on the mean percent sperm viability using the flow cytometer (N=36).

Treatment	Mean % viable
Control	87.8[a]
1X	87.9[a]
3X	88.3[a]
6X	88.6[a]

[§] Percentage data (% viable) were arc sine-root transformed prior to statistical analysis. Original means and standard deviations are presented in the table but statistical tests were conducted on transformed data.

[a] Within treatment, means with different superscripts differ (P<0.05)

3.4.2. Discussion: Experiment 4: Effect of freezing and thawing on the SYBR-14 working solution

There was no effect of freezing and thawing on the stability of the SYBR-14 working solution. Despite these findings the decision was made to prepare a large volume of the working solution and aliquot out smaller, separate volumes from this common tube to ensure that the SYBR-14 working solution used for the main experiment, Experiment 6, would come from the same dilution batch and for each ejaculate and time period, the SYBR-14 working solution would have been frozen/thawed the same number of times (1X).

3.5. Experiment 5: Effect of storage temperature, time, and seminal plasma level on sperm membrane viability.

Experiment 5 was performed to compare the changes in sperm viability at two storage temperatures (23°C and 8°C), two time intervals (26 and 50 hours), and varying seminal plasma percentages (80%, 50%, 20%, 10%). Based on previous data showing a detrimental effect of increasing percentages of seminal

plasma on motility [11, 27] the percentage of seminal plasma was varied in the different treatments in an attempt to produce varying populations of viable and non-viable sperm. In addition, sperm storage for different times and at different temperatures was also expected to yield varying proportions of viable and non-viable sperm. This method was felt to be more physiological than was the method used in previous studies in which known concentrations of killed sperm were added [2, 13].

A single ejaculate from one stallion was used for this experiment, and semen was collected as previously described. Eight 5-mL cryovial tubes (four per storage temperature) were labeled with treatment group and prepared using neat semen and INRA 96 in different proportions to obtain different percentages of seminal plasma in the final preparation to a total volume of 5 mL. The treatments were: 1) 80% - 1 mL INRA 96 / 4 mL neat semen; 2) 50% - 2.5 mL INRA 96 / 2.5 mL neat semen; 3) 20% - 4 mL INRA 96 / 1 mL neat semen; 4) 10% - 4.5 mL INRA 96 / 0.5 mL neat semen (Table 14). Samples were not diluted based on sperm concentration, therefore sperm concentration varied within and between treatment groups. Room temperature samples were wrapped in a towel and stored out of direct light. Cooled-stored samples (8°C) were stored in an Equitainer® I. All samples were analyzed 26 and 50 hours after semen collection using the CASMA as described previously. In addition, eosin-nigrosin slides were made of each sample at these times.

Table 14. Treatments and volumes of semen and extender for each treatment for Experiment 5.

Tube Label	Volume (mL) semen	Volume (mL) extender
10	0.5	4.5
20	4	1
50	2.5	2.5
80	1	4

3.5.1. Results: Experiment 5: Effect of storage temperature, time, and seminal plasma level on sperm membrane viability

There were no differences (P>0.05) found among temperature (8°C versus 23°C), and no interactions (P>0.05) were found between seminal plasma content and time, seminal plasma content and temperature, and time and temperature when sperm motion characteristics (TMOT and PMOT) were analyzed using the CASMA. Significant differences (P<0.05) were found among seminal plasma content and time when sperm motion characteristics were analyzed with the CASMA and when sperm viability was assessed using eosin-nigrosin. Total and progressive motililty decreased significantly (P<0.05) with increasing proportions of seminal plasma (50% or greater). Semen stored for 26 hours maintained better motion characteristics than semen stored for 50 hours when all treatments were considered. There was a significant difference (P<0.05) when sperm viability was analyzed with eosin-nigrosin stain among temperature as well. Treatments stored at room temperature had slightly lower (85% for room temperature, 90% for cooled; P<0.05) viable populations as

compared to treatments in cooled-storage. Table 15 shows the effect of treatment (seminal plasma content) on the means ± standard deviations (SD) for sperm motion characteristics (TMOT and PMOT) using the CASMA and for sperm viability (% viable) using eosin-nigrosin stain. Tables 16 and 17 show the effects of time (26 hours versus 50 hours post-collection) and temperature (8°C versus 23°C), respectively, on the means ± standard deviations (SD) for sperm motion characteristics (TMOT and PMOT) using the CASMA and for sperm viability (% viable) using eosin-nigrosin stain.

Table 15. Effect of treatment (seminal plasma content) on sperm motility characteristics (TMOT and PMOT) and sperm membrane viability (%) using eosin-nigrosin (n=4 per treatment).

Endpoint *	Treatment ¥	Mean ± SD
TMOT	10	90.5^{a}±3.5
	20	88.8^{a}±4.0
	50	44.3^{b}±26.7
	80	16.0^{c}±12.0
PMOT	10	62.0^{a}±12.6
	20	53.3^{a}±12.6
	50	13.0^{b}±13.7
	80	1.50^{c}±1.3
EN	10	94.0^{a}±3.4
	20	93.3^{a}±2.7
	50	90.8^{a}±3.2
	80	72.4^{b}±18.0

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); EN = sperm viability (%) as assessed using eosin-nigrosin stain.

¥ 10 = 10% of total volume designated as seminal plasma; 20 = 20% of total volume designated as seminal plasma; 50 = 50% of total volume designated as seminal plasma; 80% of total volume designated as seminal plasma.

§ Percentage data (TMOT, PMOT, EN) were arc sine-root transformed prior to statistical analysis. Original means and standard deviations are presented in the table but statistical tests were conducted on transformed data.

a, b, c Within treatment and endpoint, means with different superscripts differ (P<0.05)

Treatments containing 10% and 20% seminal plasma had similar ($P>0.05$) sperm motion characteristics and sperm membrane viability. Treatments containing 10%, 20%, and 50% seminal plasma had similar ($P>0.05$) populations of viable sperm (%). The treatment containing 80% seminal plasma was significantly different ($P<0.05$) from all other treatment groups when sperm motion characteristics and sperm viability was assessed. Samples analyzed 26 hours post-collection had significantly higher ($P<0.05$) sperm motion characteristics and % viable sperm when compared to these same treatment groups analyzed 50 hours post-collection (Table 16). Treatment groups analyzed after storage in an Equitainer® I for 26 hours and 50 hours post-collection showed similar ($P>0.05$) sperm motion characteristics (TMOT and PMOT) when compared to treatment groups stored at room temperature (Table 17). As assessed using eosin-nigrosin staining, treatment samples stored at room temperature had higher populations of viable sperm ($P<0.05$) when compared to those stored in an Equitainer® I.

Table 16. Effect of time (26 hours versus 50 hours post-collection) on sperm motility characteristics (TMOT and PMOT) and sperm membrane viability (%) using eosin-nigrosin (n=8 replicates per treatment).

Endpoint *	Time ¥	Mean
TMOT	26	68.6[a]
	50	51.1[b]
PMOT	26	37.8[a]
	50	27.1[b]
EN	26	91.9[a]
	50	83.3[b]

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); EN = sperm viability (%) as assessed using eosin-nigrosin stain.

¥ 26 = sperm analyzed 26 hours post-collection; 50 = sperm analyzed 50 hours post-collection

§ Percentage data (TMOT, PMOT, EN) were arc sine-root transformed prior to statistical analysis. Original means are presented in the table but statistical tests were conducted on transformed data.

a, b, Within time and within endpoint, means with different superscripts differ (P<0.05)

Table 17. Effect of temperature (room versus cool) on sperm motility characteristics (TMOT and PMOT) and sperm membrane viability (%) using eosin-nigrosin (n=8 replicates per treatment).

Endpoint *	Temperature ¥	Mean ± SD
TMOT	room	59.0[a]±33.4
	cool	60.8[a]±39.0
PMOT	room	35.9[a]±33.2
	cool	29.0[a]±24.4
EN	room	85.2[a]±16.8
	cool	90.1[b]±5.7

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); EN = sperm viability (%) as assessed using eosin-nigrosin stain.

¥ room = sperm stored in room temperature; cool = sperm stored in an Equitainer®.

§ Percentage data (TMOT, PMOT, EN) were arc sine-root transformed prior to statistical analysis. Original means and standard deviations are presented in the table but statistical tests were conducted on transformed data.

a, b, Within temperature and within endpoint, means with different superscripts differ (P<0.05)

3.5.2. Discussion: Experiment 5: Effect of storage temperature, time, and seminal plasma level on sperm membrane viability

Increasing levels of seminal plasma have been reported to decrease sperm motility [5, 6, 8, 27, 28]. This was only true if the extender used to dilute the neat semen did not contain a modified Tyrode's medium [11, 29-31], because the presence of any seminal plasma in an extender with Tyrode's causes a decrease in sperm motility. Similar to previous studies [7-12], 20% seminal plasma supported higher motility than did higher concentrations. In addition, as storage time increased, the sperm motion characteristics and viable populations of sperm decreased. These results indicated that findings with 10% seminal plasma were similar to 20% seminal plasma. No significant differences ($P<0.05$) were found when the treatment containing 10% seminal plasma was compared to the treatment containing 20% seminal plasma when sperm membrane viability (eosin-nigrosin) and sperm motion characteristics (TMOT and PMOT) were assessed, thus is was decided to utilize 20% seminal plasma as the base percentage. Although room temperature (23°C) samples had a slightly lower viable population compared to cooled storage (8°C) storage (85.15% compared to 90.1%) when sperm membrane viability (eosin-nigrosin) was assessed, it was decided to utilize cooled-storage only for the main experiment (Experiment 6) comparing flow cytometery, the NucleoCounter® SP-100™, and eosin-nigrosin staining as methods for assessment of sperm membrane viability.

3.6. Experiment 6: Comparison of methods for detecting sperm membrane viability and seminal plasma effects on sperm motion characteristics and membrane viability

Three gel-free ejaculates were collected from each of three stallions (N=9) using the protocol previously described. The semen was divided into three 15-mL polypropylene conical tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) that had been previously prepared and placed in an incubator set at 37°C with varying volumes of a milk-based semen extender, INRA 96 (IMV Technologies, L'Aigle, France) with 1 mg/mL added timentin (GlaxoSmithKline, Research Traingle Park, NC, USA). This extender/antibiotic solution was referred to as INRA-T. Semen was placed in a fourth conical tube containing a mixture of INRA-T and frozen/thawed seminal plasma obtained from a previous ejaculate collected from the same stallion. Volumes of neat semen, frozen/thawed seminal plasma, and INRA-T were adjusted within a total tube volume of 12 mL to give the following treatments, 1) 80SP- 80% neat semen / 20% INRA-T (thus 80% seminal plasma); 2) 80SP/20 - 20% neat semen, 60% of the stallion's own frozen/thawed seminal plasma, and 20% INRA-T (thus 80% seminal plasma, but with 25% the sperm concentration of Treatment 80SP; 3) 50SP- 50% neat semen and 50% INRA-T (50% seminal plasma and 62.5% (⅝) the concentration of sperm as in 80SP); 4) 20SP -20% neat semen and 80% INRA-T (20% seminal plasma and 25% the concentration of sperm as in 80SP).

The extended semen was mixed thoroughly. In each treatment, four mL of extended semen was placed into each of three capped polypropylene tubes (Cryogenic vials [4.0 mL]; Corning Life Sciences, Lowell, MA, USA) previously labeled with treatment (80SP, 80SP/20, 50SP, 20SP) and time (T0, T24, T48). Treatment T0 was placed in a drawer for immediate analysis. Treatment T24 was placed in a commercial semen-transport container (Equitainer® I; Hamilton Research, Inc., South Hamilton, MA, USA) for cooled-storage at 8°C and analysis 24 hours post-collection. Treatment T48 was placed in a separate Equitainer® I for cooled-storage at 8°C and analysis 48 hours post-collection. Immediately prior to analysis using the methods described below, the tubes containing the treatments to be analyzed were incubated for 10 minutes at 37°C. Each treatment was incubated separately before analysis, and the remaining tubes for that time period remained in storage. For each analysis method and time interval the treatments were run in the following order: 80SP, 80SP/20, 50SP, 20SP. Three replicates (three samples from each treatment tube) were performed on each of the four treatments using the flow cytometer, NucleoCounter® SP-100™ and eosin-nigrosin for assessment of sperm membrane viability, and the CASMA for assessment of sperm motion characteristics using the protocols previously described in materials and methods. The SYBR-14 working solution used was 0.5 µL SYBR / 0.02 µM that had been previously diluted 10-fold in DMSO and frozen then thawed one time and added to 2.5 mL of semen.

3.6.1. Results: Experiment 6: Comparison of methods for detecting sperm membrane viability and seminal plasma effects on sperm motion characteristics and membrane viability

There was an effect (P<0.05) of time, stallion, ejaculate within stallion, and treatment on the mean percent of viable sperm for the flow cytometer, NucleoCounter® SP-100™, and eosin-nigrosin staining (N=324). There was an interaction of treatment and time on the mean percent of viable sperm for the flow cytometer, NucleoCounter® SP-100™, and eosin-nigrosin staining (P<0.05). Treatment 20SP (20% neat semen and 80% INRA-T; 20% seminal plasma and ¼ the concentration of sperm as in 80SP) and 50SP (50% neat semen and 50% INRA-T; 50% seminal plasma and ⅝ the concentration of sperm as in 80SP) yielding significantly higher values (P<0.05) for sperm membrane viability [all three methods of assessing sperm membrane viability (FC, NC, EN)] when compared to the other treatments within all times (0, 24, 48 hours) (Table 17). Treatment 80SP/20 (20% neat semen, 60% of the stallion's own frozen/thawed seminal plasma, and 20% INRA-T; thus 80% seminal plasma, but with ¼ the sperm concentration of Treatment 80SP) was inferior to all other treatment groups for sperm motion characteristics and membrane viability (Table 18). Total and progressive sperm motility decreased significantly as the concentration (%V/V) of seminal plasma increased (P<0.05) with 80SP/20 having the lowest total and progressive sperm motility.

At Time 0, although some treatments were statistically different ($P<0.05$), from a clinical approach all treatments were similar, with no more than a 7.5% difference for sperm motion characteristics and a 4.2% difference for sperm viability (Table 19, Figure 6). At Time 24 (Table 20, Figure 7), sperm motion characteristics (TMOT and PMOT) decreased significantly ($P<0.05$) as the concentration (%V/V) of seminal plasma increased with 80SP/20 being inferior to all other treatments ($P<0.05$). Treatments 20SP and 50SP were similar to each other ($P>0.05$) but superior to other treatments when sperm viability was assessed using the flow cytometer, the NucleoCounter® SP-100™, and eosin-nigrosin staining ($P<0.05$). Treatments 80SP and 80SP/20 were not significantly different ($P>0.05$) when viability was assessed using eosin-nigrosin, however, when analyzed using the flow cytometer and NucleoCounter® SP-100™, Treatment 80SP/20 was inferior to 80SP ($P<0.05$). At Time 48 (Table 21, Figure 8), Treatments 20SP and 50SP were not significantly different ($P>0.05$) when sperm membrane viability was assessed using the three methods (flow cytometer, NucleoCounter® SP-100™, eosin-nigrosin). Sperm motion characteristics decreased significantly ($P<0.05$) among Treatments 20SP, 50SP and 80SP, with 80SP being inferior to the treatments containing less seminal plasma (20SP and 50SP), but not significantly different ($P>0.05$) from 80SP/20.

Table 18. The effect of treatment on the mean values (mean ± SD) of sperm motility (TMOT & PMOT) and sperm membrane viability as measured by flow cytometry (FC), eosin-nigrosin staining (E-N), and the NucleoCounter® SP-100™(NC) within T0, T24, and T48 combined (n=81 for each treatment at each endpoint).

Endpoint*	20SP†	50SP†	80SP†	80SP/20†
Conc	23.8-108.2	59.2-266.7	88.9-404.0	13.1-105.8
FC	84.1[a]±11.4	83.8[a]±11.5	58.5[b]±24.8	52.6[c]±26.5
TMOT	81.1[a]±7.0	67.5[b]±22.6	47.3[c]±33.1	38.8[d]±35.5
PMOT	43.4[a]±14.9	30.9[b]±21.8	22.7[c]±26.1	18.1[d]±25.3
NC	79.3[a]±12.1	77.7[a]±12.9	51.2[b]±27.4	46.5[c]±28.4
E-N	82.6[a]±10.5	81.2[a]±12.0	64.7[b]±22.9	58.3[c]±23.7

* Conc = range of sperm concentration (x 10^6) for each treatment; FC = viable sperm assessed using the flow cytometer (%); TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); NC = viable sperm assessed using the NucleoCounter® SP-100™; EN = viable spern assessed using eosin-nigrosin staining (%).

† 20SP = contained 20% neat semen and 80% extender; 50SP = contained 50% neat semen and 50% extender; 80SP = contained 80% neat semen and 20 extender; 80SP/20 = contained 20% neat semen, 60% stallion's own seminal plasma, and 20% extender.

§ Percentage data (FC, TMOT, PMOT, NC, EN) were arc sine-root transformed prior to statistical analysis. Original means and standard deviations are presented in the table but statistical tests were conducted on transformed data.

a, b, c, d Within treatments and endpoint, means with different superscripts across rows differ ($P<0.05$)

Table 19. The effect of treatment at Time 0 on the mean values (mean ± SD) of sperm motility (TMOT & PMOT) and sperm membrane viability as measured by flow cytometry (FC), eosin-nigrosin staining (E-N), and the NucleoCounter® SP-100^{TM}(NC) (n=27).

Endpoint*	Time$^{£}$	20SP†	50SP†	80SP†	80SP/20†
Conc	0	23.8-108.2	59.2-266.7	88.9-404.0	13.1-105.8
FC	0	84.04^{a}±11	85.63^{a}±11	84.52^{a}±12	82.00^{b}±12
TMOT	0	83.04^{b}±6	85.59^{a}±6	85.74^{a}±6	84.78^{a}±8
PMOT	0	55.78^{a}±15	54.82^{a}±16	56.48^{a}±15	50.59^{b}±17
NC	0	80.26^{a}±12	80.3^{a}±13	79.89^{a}±13	77.70^{b}±15
E-N	0	82.07^{b,a}±14	81.93^{b,a}±15	82.85^{a}±14	79.59^{b}±15

* Conc = range of sperm concentration (x 10^{6}) for each treatment; FC = viable sperm assessed using the flow cytometer (%); TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); NC = viable sperm assessed using the NucleoCounter® SP-100^{TM}; EN = viable spern assessed using eosin-nigrosin staining (%).

£ Time 0 = Semen analyzed immediately post-collection.

† 20SP = contained 20% neat semen and 80% extender; 50SP = contained 50% neat semen and 50% extender; 80SP = contained 80% neat semen and 20 extender; 80SP/20 = contained 20% neat semen, 60% stallion's own seminal plasma, and 20% extender.

§ Percentage data (FC, TMOT, PMOT, NC, EN) were arc sine-root transformed prior to statistical analysis. Original means and standard deviations are presented in the table but statistical tests were conducted on transformed data.

a, b, Within treatments, endpoint at Time 0 means with different superscripts across rows differ (P<0.05)

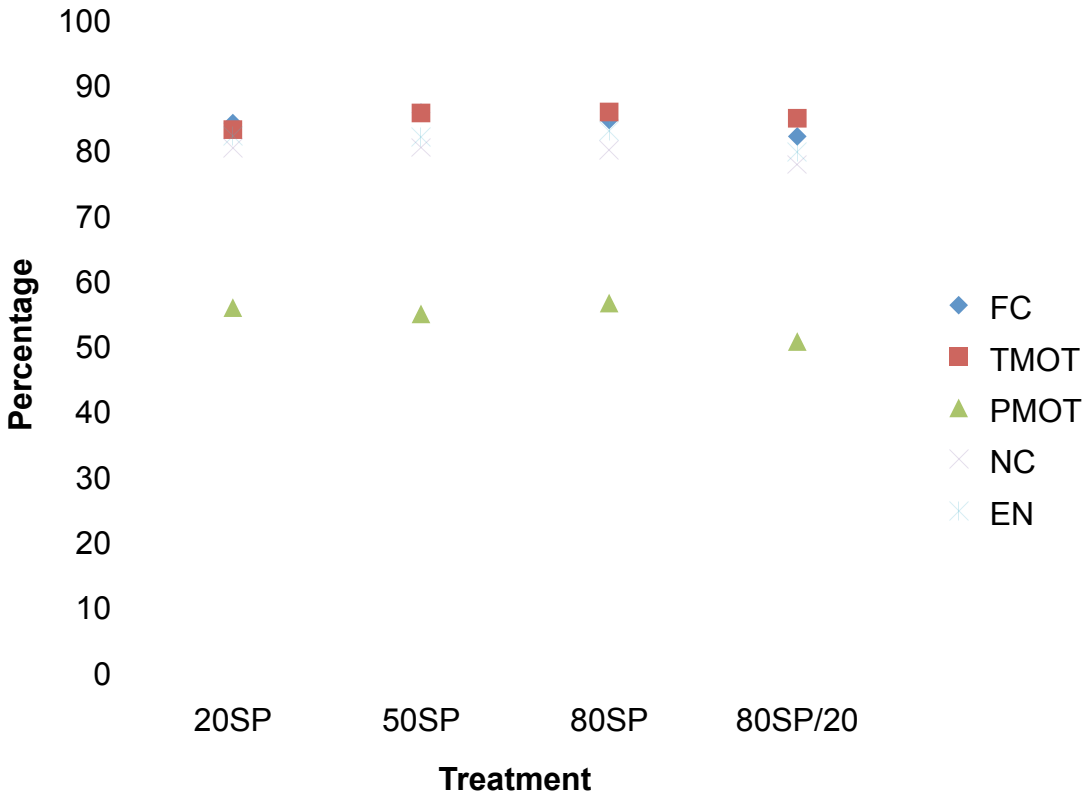

Figure 6. Comparison of findings among three different methods for assessment of sperm membrane viability (flow cytometer (FC), NucleoCounter SP-100 (NC), and eosin-nigrosin (EN)) and sperm motion characteristics (TMOT and PMOT) according to treatment (percentage of seminal plasma) at Time 0.

Table 20. The effect of treatment at Time 24 on the mean values (mean ± SD) of sperm motility (TMOT & PMOT) and sperm membrane viability as measured by flow cytometry (FC), eosin-nigrosin staining (E-N), and the NucleoCounter® SP-100^{TM}(NC) (N=27).

Endpoint*	Time$^{£}$	20SP†	50SP†	80SP†	80SP/20†
Conc	24	23.8-108.2	59.2-266.7	88.9-404.0	13.1-105.8
FC	24	83.33^{a}±12	83.26^{a}±12	58.70^{b}±14	53.96^{c}±9
TMOT	24	84.22^{a} ±6	76.93^{b}±7	48.85^{c}±10	26.11^{d}±16
PMOT	24	42.04^{a}±10	29.26^{b}±8	11.00^{c}±4	3.44^{d}±4
NC	24	79.70^{a}±13	77.37^{a}±13	51.63^{b}±15	44.56^{c}±17
E-N	24	82.74^{a}±13	80.63^{a}±16	65.37^{b}±19	60.96^{b}±16

* Conc = range of sperm concentration (x 10^{6}) for each treatment; FC = viable sperm assessed using the flow cytometer (%); TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); NC = viable sperm assessed using the NucleoCounter® SP-100^{TM}; EN = viable spern assessed using eosin-nigrosin staining (%).

£ Time 24 = Semen analyzed after 24 hours of cooled-storage in and Equitainer®.

† 20SP = contained 20% neat semen and 80% extender; 50SP = contained 50% neat semen and 50% extender; 80SP = contained 80% neat semen and 20 extender; 80SP/20 = contained 20% neat semen, 60% stallion's own seminal plasma, and 20% extender.

§ Percentage data (FC, TMOT, PMOT, NC, EN) were arc sine-root transformed prior to statistical analysis. Original means and standard deviations are presented in the table but statistical tests were conducted on transformed data.

a, b, c, d Within treatments and endpoint at Time 24 means with different superscripts across rows differ (P<0.05)

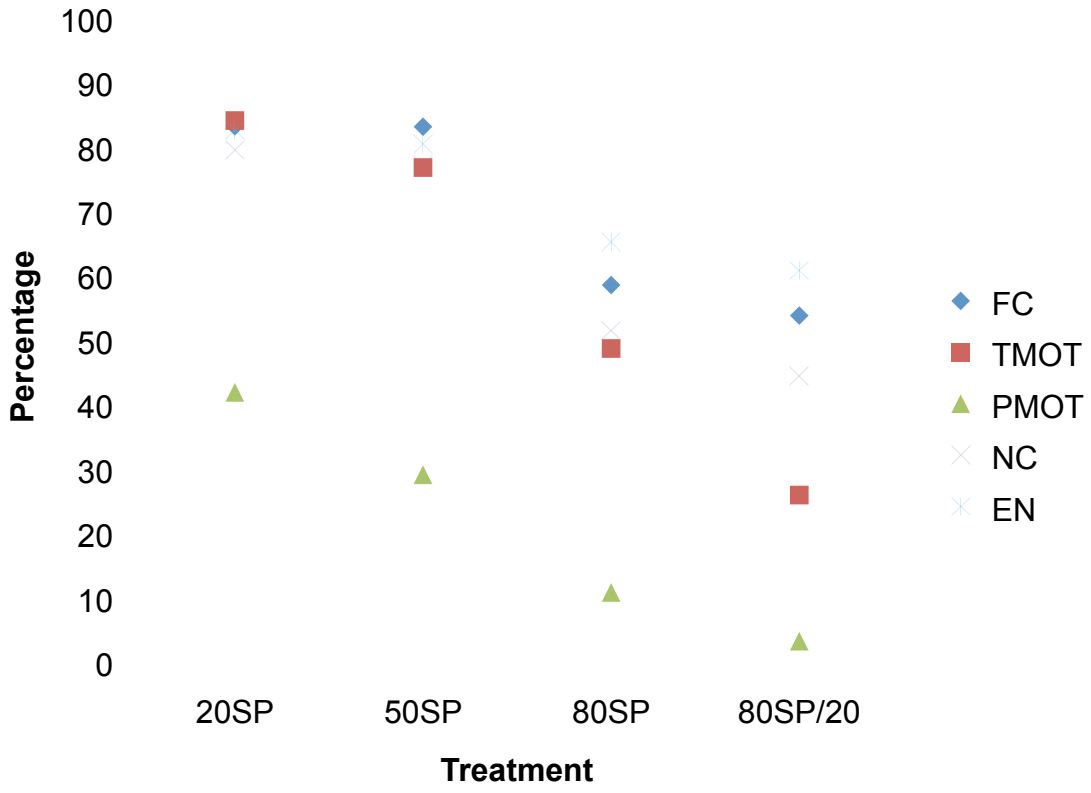

Figure 7. Comparison of findings among three different methods for assessment of sperm membrane viability (flow cytometer (FC), NucleoCounter SP-100 (NC), and eosin-nigrosin (EN)) and sperm motion characteristics (TMOT and PMOT) according to treatment (percentage of seminal plasma) after 24 hours of cooled-storage.

Table 21. The effect of treatment at Time 48 on the mean values (mean ± SD) of sperm motility (TMOT & PMOT) and sperm membrane viability as measured by flow cytometry (FC), eosin-nigrosin staining (E-N), and the NucleoCounter® SP-100^{TM} (NC) (N=27).

Endpoint*	Time$^{£}$	20SP†	50SP†	80SP†	80SP/20†
Conc	48	23.8-108.2	59.2-266.7	88.9-404.0	13.1-105.8
FC	48	84.82^{a}±11	82.41^{a}±11	32.15^{b}±11	21.70^{c}±7
TMOT	48	76.04^{a}±7	40.00^{b}±17	7.22^{c}±6	5.59^{c}±7
PMOT	48	32.26^{a}±9	8.63^{b}±5	0.63^{c}±1	0.15^{c}±0.4
NC	48	77.96^{a}±12	75.48^{a}±13	22.00^{b}±13	17.26^{b}±8
E-N	48	82.93^{a}±12	80.93^{a}±13	45.85^{b}±19	34.26^{c}±13

* Conc = range of sperm concentration (x 10^{6}) for each treatment; FC = viable sperm assessed using the flow cytometer (%); TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); NC = viable sperm assessed using the NucleoCounter® SP-100^{TM}; EN = viable spern assessed using eosin-nigrosin staining (%).

£ Time 48 = Semen analyzed after 48 hours of cooled-storage in and Equitainer®.

† 20SP = contained 20% neat semen and 80% extender; 50SP = contained 50% neat semen and 50% extender; 80SP = contained 80% neat semen and 20 extender; 80SP/20 = contained 20% neat semen, 60% stallion's own seminal plasma, and 20% extender.

§ Percentage data (FC, TMOT, PMOT, NC, EN) were arc sine-root transformed prior to statistical analysis. Original means and standard deviations are presented in the table but statistical tests were conducted on transformed data.

a, b, c Within treatments and endpoint at Time 48 means with different superscripts across rows differ (P<0.05)

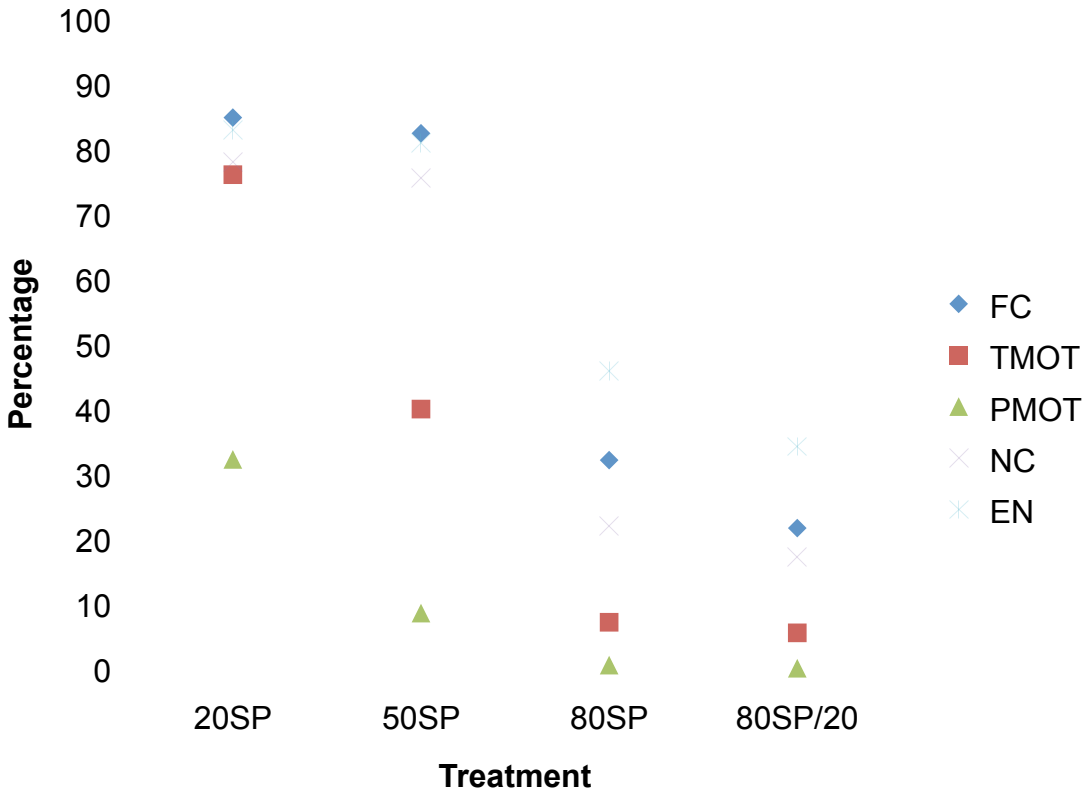

Figure 8. Comparison of findings among three different methods for assessment of sperm membrane viability (flow cytometer (FC), NucleoCounter SP-100 (NC), and eosin-nigrosin (EN)) and sperm motion characteristics (TMOT and PMOT) according to treatment (percentage of seminal plasma) after 48 hours of cooled-storage.

The coefficients of variation for the transformed data for the flow cytometer, NucleoCounter® SP-100™, eosin-nigrosin staining, and total motility were all below 7% (Table 22), with the progressive motility (PMOT) being the least repeatable (6.07%) and the flow cytometer being the most repeatable (1.42%).

Table 22. Within - sample repeatability (coefficients of variation) for the three methods for determining sperm membrane viability (FC, NC, E-N) and total and progressive sperm motility (TMOT, PMOT).

Endpoint*	Coefficient of Variation (%)
FC	1.42
TMOT	5.56
PMOT	6.07
NC	4.89
E-N	4.98

* FC = viable sperm assessed using the flow cytometer (%); TMOT = total sperm motility (%); PMOT = progressive sperm motility; NC = viable sperm assessed using the NucleoCounter® SP-100™; E-N = viable spern assessed using eosin-nigrosin staining (%).

§ Percentage data (FC, TMOT, NC, EN) were arc sine-root transformed prior to statistical analysis. 0.0125 was substituted for all values of zero. Coefficients of variation were analyzed on the transformed data.

Bland and Altman plots were prepared to determine the agreement between the methods for detecting sperm viability (flow cytometry, NucleoCounter® SP-100™, eosin-nigrosin staining) and for determining, if any, the agreement between total motility using CASMA and sperm viability using the

flow cytometer, the NucleoCounter® SP-100™, and eosin-nigrosin staining. These Bland and Altman plots as well as regression plots for each comparison were graphed at Times 0 (Figures 9-20), 24 (Figures 21-32), and 48 (Figures 33-44). Tables 23-28 show the equations and associated values used for plotting the Bland and Altman plots, as well as the absolute values of the mean differences for each plot.

At Time 0, the flow cytometer held a five percentage-point difference (absolute value) higher than the NucleoCounter® SP-100™ and eosin-nigrosin staining (Table 23 and 24). When comparisons were made with the NucleoCounter® SP-100™ and eosin-nigrosin staining, the method agreement also averaged five percentage-points, however these data points fell above and below the mean difference (Table 25). When total motility was compared to viability using the three methods at Time 0 the agreements remained between a six and eight percentage-point difference (Tables 26-28). The R-values were high for all comparisons at Time 0 (Figures 10, 12, 14, 16, 18, and 20).

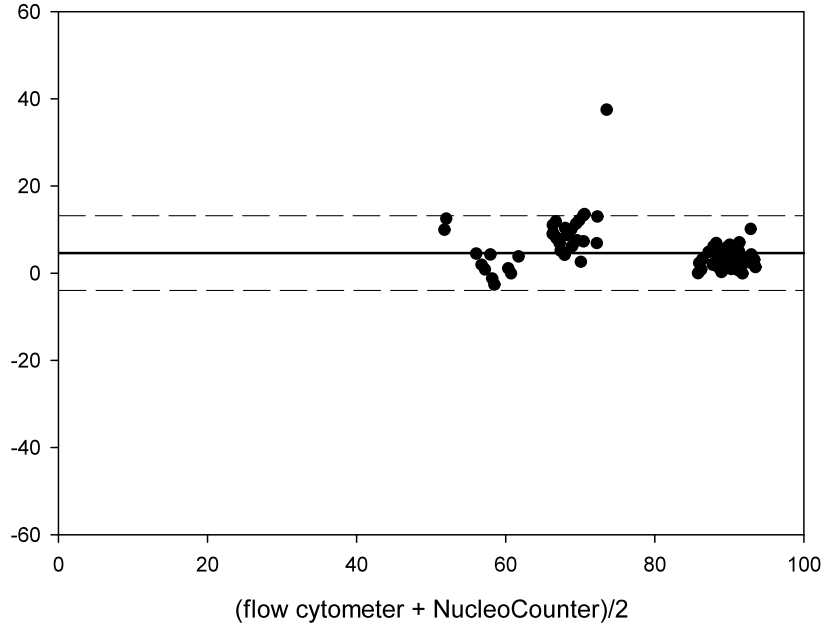

Figure 9. Bland and Altman plot comparing the difference in sperm membrane viability between the flow cytometer and the NucleoCounter® SP-100™ to the mean of the flow cytomer and the NucleoCounter® SP-100™ at Time 0 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 23. Equations and their associated values used in preparing the Bland and Altman plots for the flow cytometer (FC) and the NucleoCounter® SP-100™ (NC) at Time 0.

Value (equation)	FC-NC	absolute value (FC-NC)	(FC+NC)/2
sum	486.9	496.9	8833.5
average	4.5	4.6	81.8
standard deviation (SD)	4.7	4.6	12.1
lower limit of agreement (average-(2*SD))	-4.8	-4.5	57.5
upper limit of agreement (average+(2*SD))	13.8	13.7	106.1
Range	-3 – 37	3 - 37	52 -94
number of events	108.0	108.0	108.0

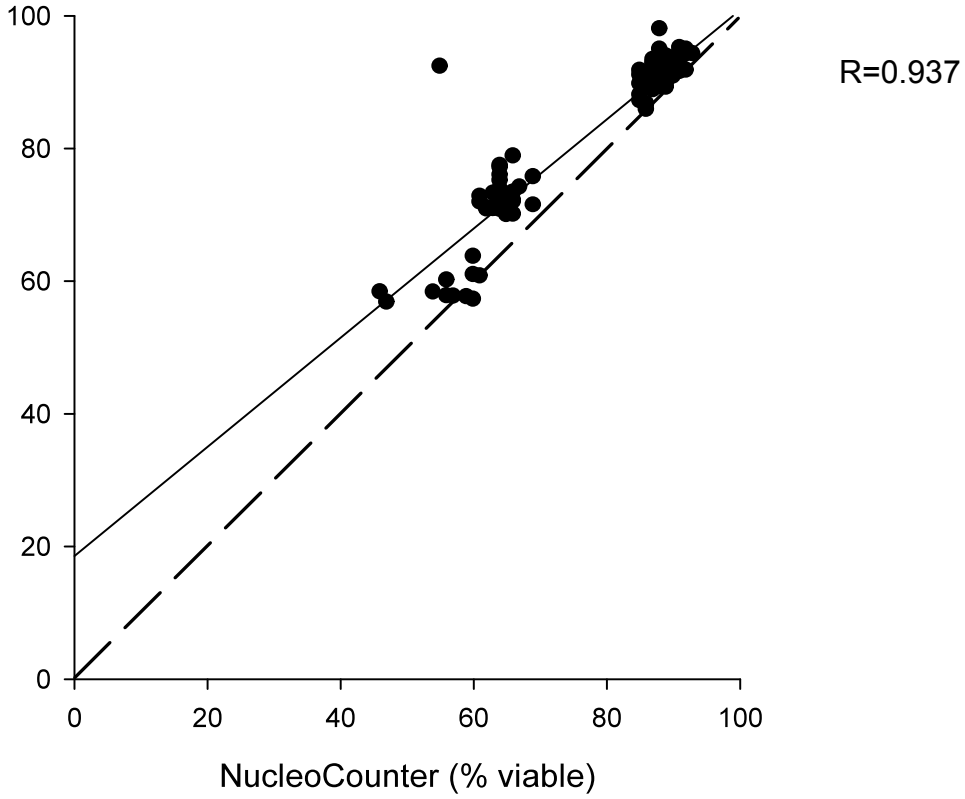

Figure 10. Regression plot comparing the mean percent viable sperm for the flow cytometer and the NucleoCounter® SP-100™ at Time 0 (N=108). Dashed line represents the line of equality.

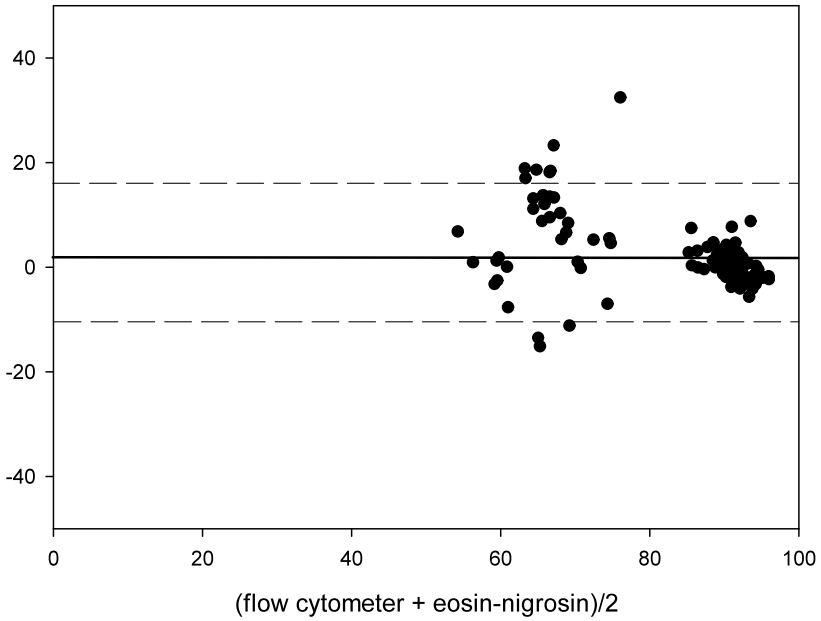

Figure 11. Bland and Altman plot comparing the difference in sperm membrane viability between the flow cytometer and eosin-nigrosin staining to the mean of the flow cytomer and eosin-nigrosin staining at Time 0 (N = 108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 24. Equations and their associated values used in preparing the Bland and Altman plots for the flow cytometer (FC) and eosin-nigrosin (EN) at Time 0.

Value (equation)	FC-EN	absolute value (FC-EN)	(FC+EN)/2
sum	259.6	524.6	8947.1
average	2.4	4.9	82.8
standard deviation (SD)	7.1	5.7	12.4
lower limit of agreement (average-(2*SD))	-11.8	-6.5	58.0
upper limit of agreement (average+(2*SD))	16.6	16.2	107.7
Range	-15 - 32	15 - 32	52 - 94
number of events	108.0	108.0	108.0

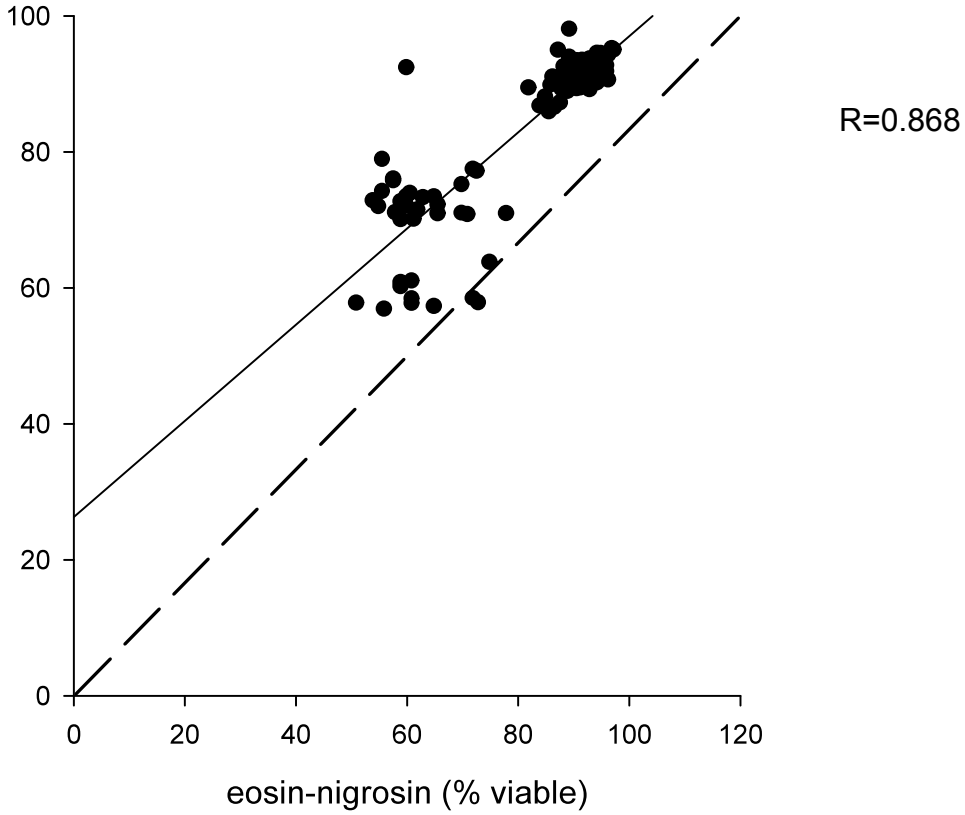

Figure 12. Regression plot comparing the mean percent viable sperm for the flow cytometer and eosin-nigrosin staining at Time 0 (N=108). Dashed line represents the line of equality.

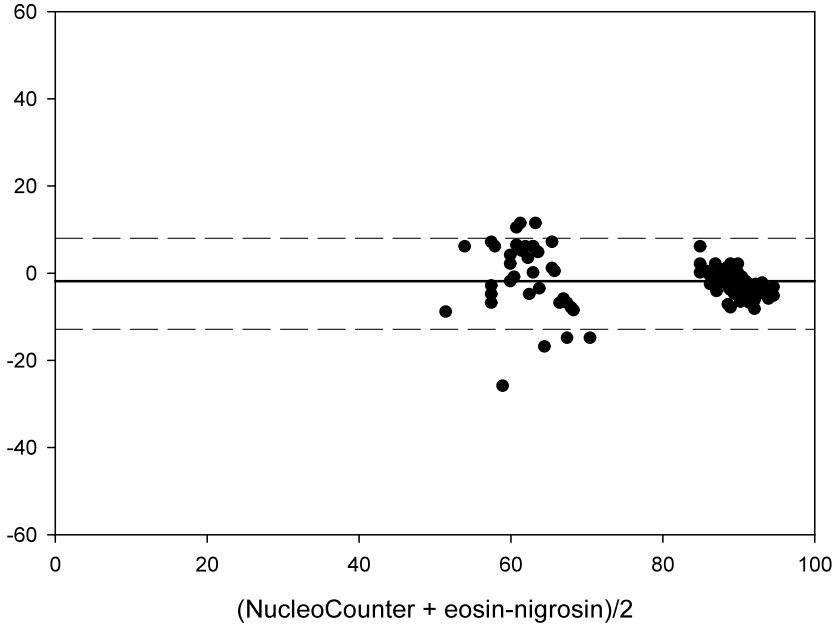

Figure 13. Bland and Altman plot comparing the difference in sperm membrane viability between the NucleoCounter® SP-100™ and eosin-nigrosin staining to the mean of the NucleoCounter® SP-100™ and eosin-nigrosin staining at Time 0 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 25. Equations and their associated values used in preparing the Bland and Altman plots for the NucleoCounter® SP-100™ (NC) and eosin-nigrosin (EN) at Time 0.

Value (equation)	NC-EN	absolute value (NC-EN)	(NC+EN)/2
sum	-227.3	470.7	8703.7
average	-2.1	4.4	80.6
standard deviation (SD)	5.5	3.9	13.4
lower limit of agreement (average-(2*SD))	-13.0	-3.4	53.8
upper limit of agreement (average+(2*SD))	8.8	12.1	107.4
Range	-26 – 11	11 – 26	52 - 95
number of events	108.0	108.0	108.0

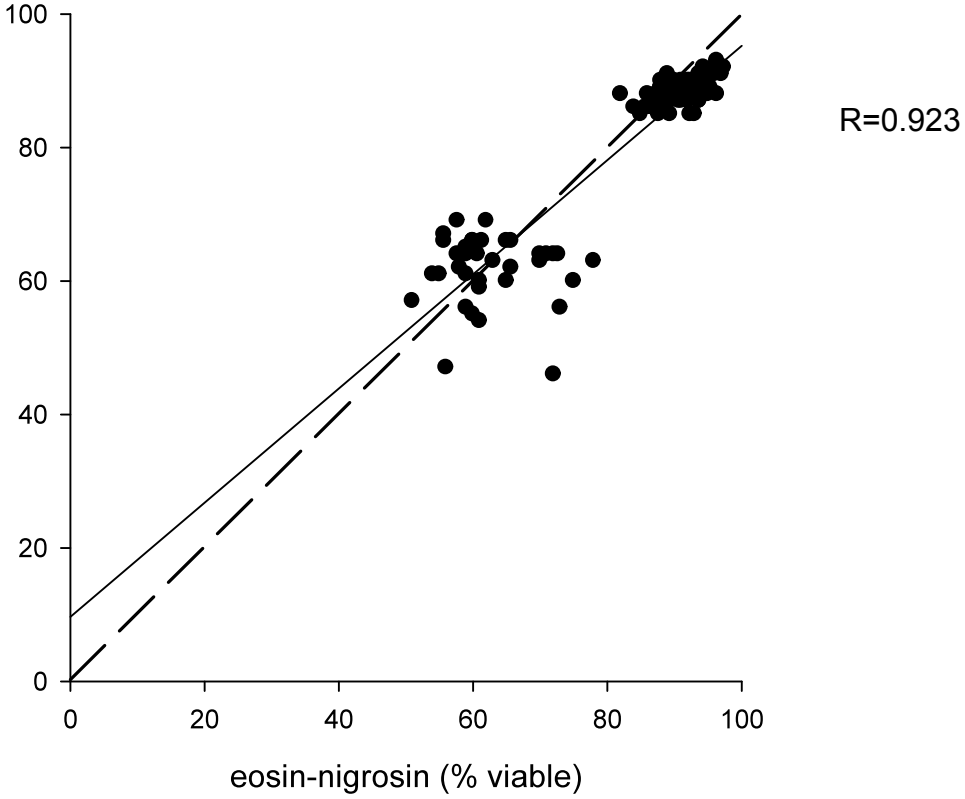

Figure 14. Regression plot comparing the mean percent viable sperm for the NucleoCounter® SP-100™ and eosin-nigrosin staining at Time 0 (N=108). Dashed line represents the line of equality.

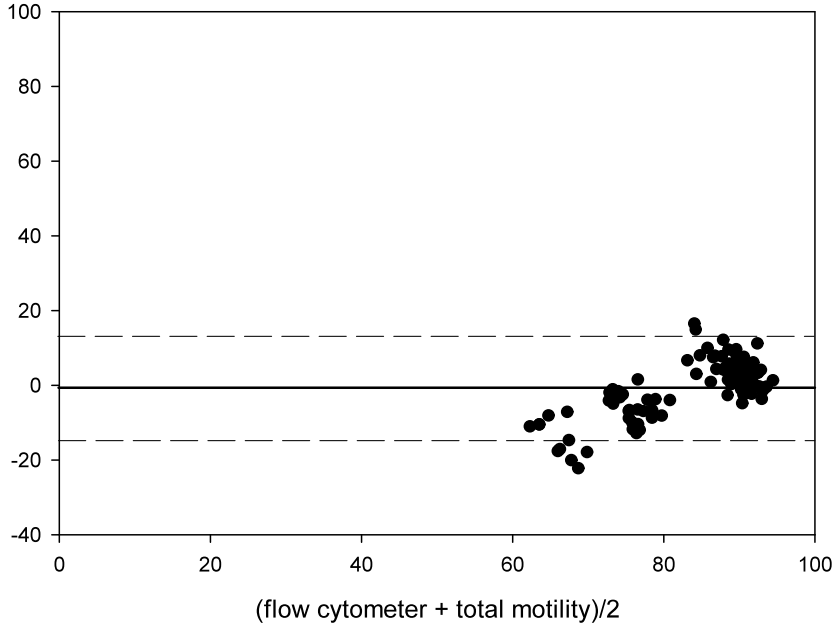

Figure 15. Bland and Altman plot comparing the difference in sperm membrane viability between the flow cytometer and total motility with the CASMA to the mean of the flow cytometer and total motility at Time 0 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 26. Equations and their associated values used in preparing the Bland and Altman plots for the flow cytometer (FC) and total sperm motility (TMOT) at Time 0.

Value (equation)	FC-TMOT	absolute value (FC-TMOT)	(FC+TMOT)/2
sum	-80.1	600.8	9117.0
average	-0.7	5.6	84.4
standard deviation (SD)	7.3	4.8	8.6
lower limit of agreement (average-(2*SD))	-15.4	-4.1	67.2
upper limit of agreement (average+(2*SD))	13.9	15.2	101.7
Range	-22 – 16	16 – 22	62 - 95
number of events	108.0	108.0	108.0

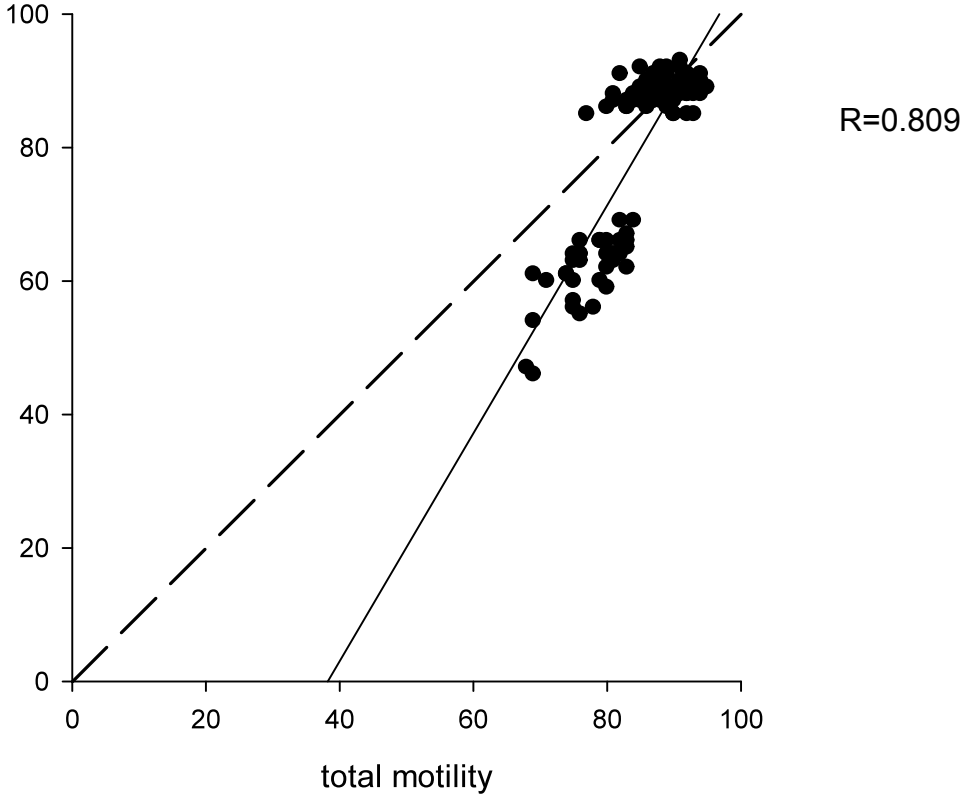

Figure 16. Regression plot comparing the mean percent viable sperm for the flow cytometer and total sperm motility with the CASMA at Time 0 (N=108). Dashed line represents the line of equality.

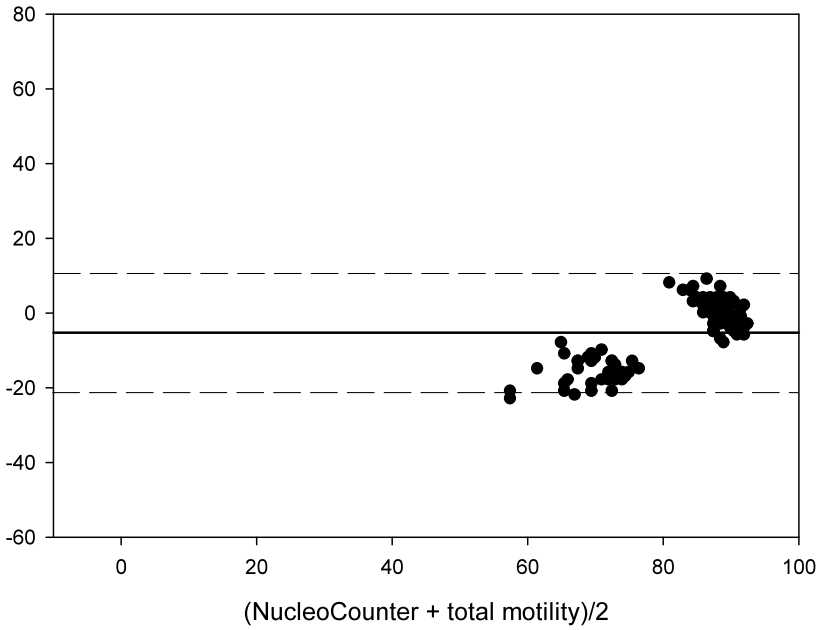

Figure 17. Bland and Altman plot comparing the difference in sperm membrane viability between the NucleoCounter® SP-100™ and total motility with the CASMA to the mean of the NucleoCounter® SP-100™ and total motility at Time 0 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 27. Equations and their associated values used in preparing the Bland and Altman plots for the NucleoCounter® SP-100™ (NC) and total sperm motility (TMOT) at Time 0.

Value (equation)	NC-TMOT	absolute value (NC-TMOT)	(NC+TMOT)/2
sum	-567.0	775.0	8873.5
average	-5.3	7.2	82.2
standard deviation (SD)	8.3	6.7	9.5
lower limit of agreement (average-(2*SD))	-21.9	-6.3	63.2
upper limit of agreement (average+(2*SD))	11.4	20.6	101.2
Range	-23 – 9	9 – 23	58 - 93
number of events	108.0	108.0	108.0

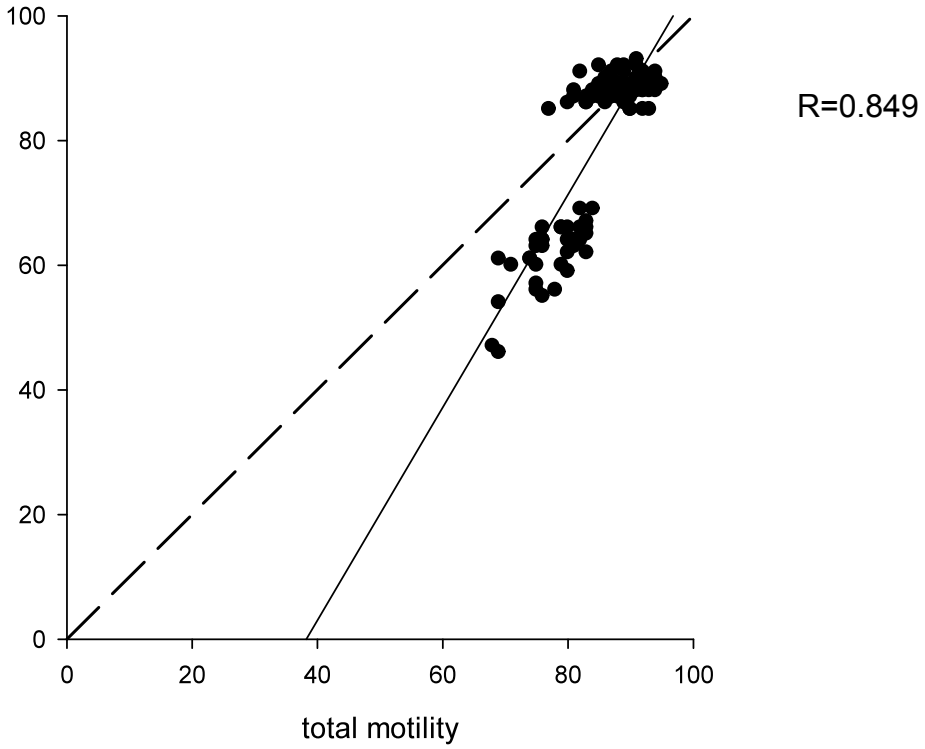

Figure 18. Regression plot comparing the mean percent viable sperm for the NucleoCounter® SP-100™ and total sperm motility with the CASMA at Time 0 (N=108). Dashed line represents the line of equality.

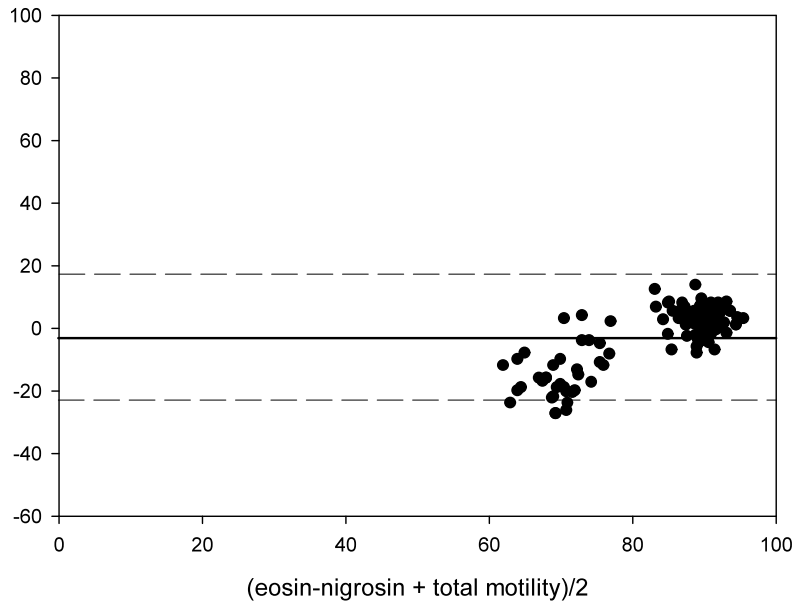

Figure 19. Bland and Altman plot comparing the difference in sperm membrane viability with eosin-nigrosin staining and total sperm motility with the CASMA to the mean of eosin-nigrosin staining and total motility at Time 0 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 28. Equations and their associated values used in preparing the Bland and Altman plots for eosin-nigrosin staining (EN) and total sperm motility (TMOT) at Time 0.

Value (equation)	EN-TMOT	absolute value (EN-TMOT)	(EN+TMOT)/2
sum	-339.7	853.7	8987.2
average	-3.1	7.9	83.2
standard deviation (SD)	10.1	7.0	9.8
lower limit of agreement (average-(2*SD))	-23.4	-6.1	63.6
upper limit of agreement (average+(2*SD))	17.1	21.9	102.8
Range	-27 – 14	14 – 27	62 - 96
number of events	108.0	108.0	108.0

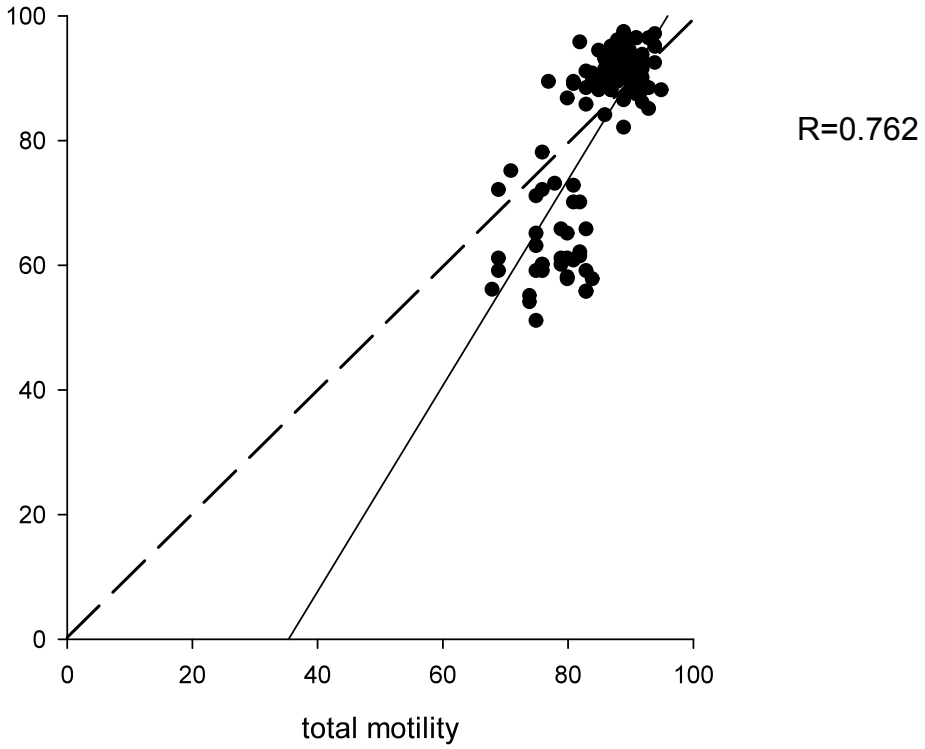

Figure 20. Regression plot comparing the mean percent viable sperm for eosin-nigrosin staining and total sperm motility with the CASMA at Time 0 (N=108). Dashed line represents the line of equality.

At Time 24, the flow cytometer held a 6.5 percentage-point difference (absolute value) when compared to the NucleoCounter® SP-100™and eosin-nigrosin staining (Table 29 and 30). Data points for the flow cytometer were above the mean difference when compared to the NucleoCounter® SP-100™, however the data points fell above and below the mean difference for the flow cytometer and eosin-nigrosin staining. When comparisons were made with the NucleoCounter® SP-100™ and eosin-nigrosin staining, the method agreement averaged an approximate 10 percentage-point, and these data points fell above and below the mean difference (Table 31). When total motility was compared to viability using the three methods at Time 24 the agreements had an approximate 10 percentage-point difference for the NucleoCounter® SP-100™ and the flow cytometer compared to total motility (Tables 32 and 33), and a 20 percentage-point difference for eosin-nigrosin and total motility (Table 34). The R-values remained high for all comparisons among methods for detecting viability (flow cytometer, NucleoCounter® SP-100™, eosin-nigrosin) at Time 24 (Figures 22, 24, 26). The R-values decreased at Time 24 for the comparisons of the three methods with total motility (Figures 28, 30, 32).

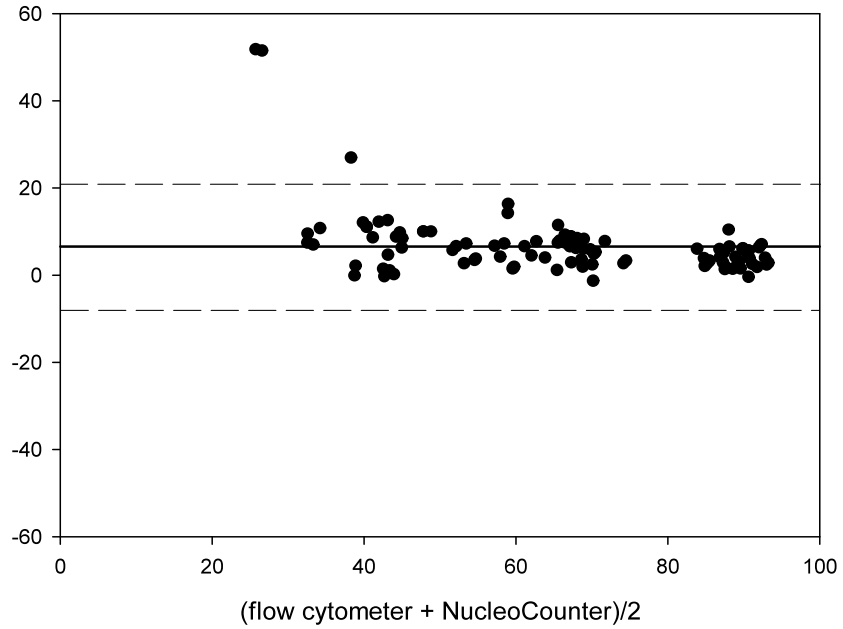

Figure 21. Bland and Altman plot comparing the difference in sperm membrane viability between the flow cytometer and the NucleoCounter® SP-100™ to the mean of the flow cytometer and the NucleoCounter® SP-100™ at Time 24 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 29. Equations and their associated values used in preparing the Bland and Altman plots for the flow cytometer (FC) and the NucleoCounter® SP-100™ (NC) at Time 24.

Value (equation)	FC-NC	absolute value (FC-NC)	(FC+NC)/2
sum	701.4	706.9	7188.7
average	6.5	6.5	66.6
standard deviation (SD)	7.4	7.3	19.3
lower limit of agreement (average-(2*SD))	-8.2	-8.1	28.0
upper limit of agreement (average+(2*SD))	21.2	21.2	105.2
Range	-1 – 52	1 – 52	26 - 93
number of events	108.0	108.0	108.0

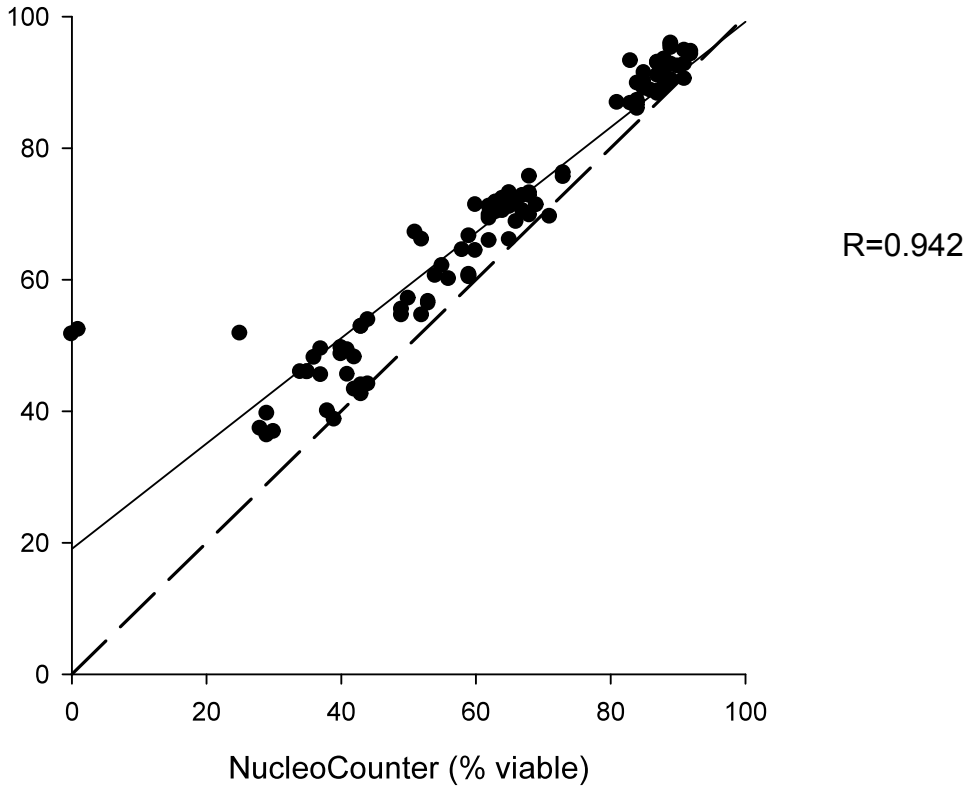

Figure 22. Regression plot comparing the mean percent viable sperm for the flow cytometer and the NucleoCounter® SP-100™ at Time 24 (N=108). Dashed line represents the line of equality.

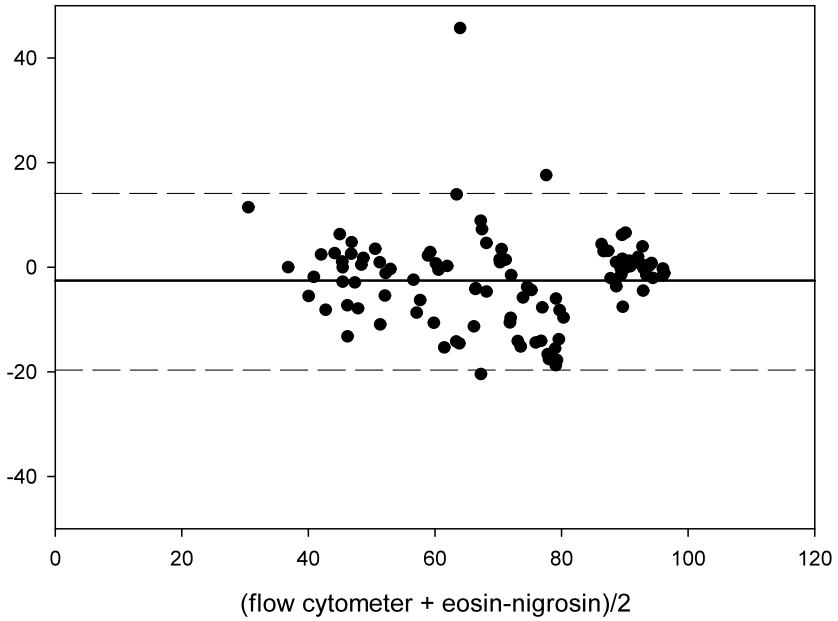

Figure 23. Bland and Altman plot comparing the difference in sperm membrane viability between the flow cytometer and eosin-nigrosin staining to the mean of the flow cytomer and eosin-nigrosin staining at Time 24 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 30. Equations and their associated values used in preparing the Bland and Altman plots for the flow cytometer (FC) and eosin-nigrosin staining (EN) at Time 24.

Value (equation)	FC-EN	absolute value (FC-EN)	(FC+EN)/2
sum	-284.6	649.4	7681.7
average	-2.6	6.0	71.1
standard deviation (SD)	8.7	6.7	17.6
lower limit of agreement (average-(2*SD))	-20.0	-7.5	36.0
upper limit of agreement (average+(2*SD))	14.7	19.5	106.3
Range	-21 – 46	21 – 46	31 - 96
number of events	108.0	108.0	108.0

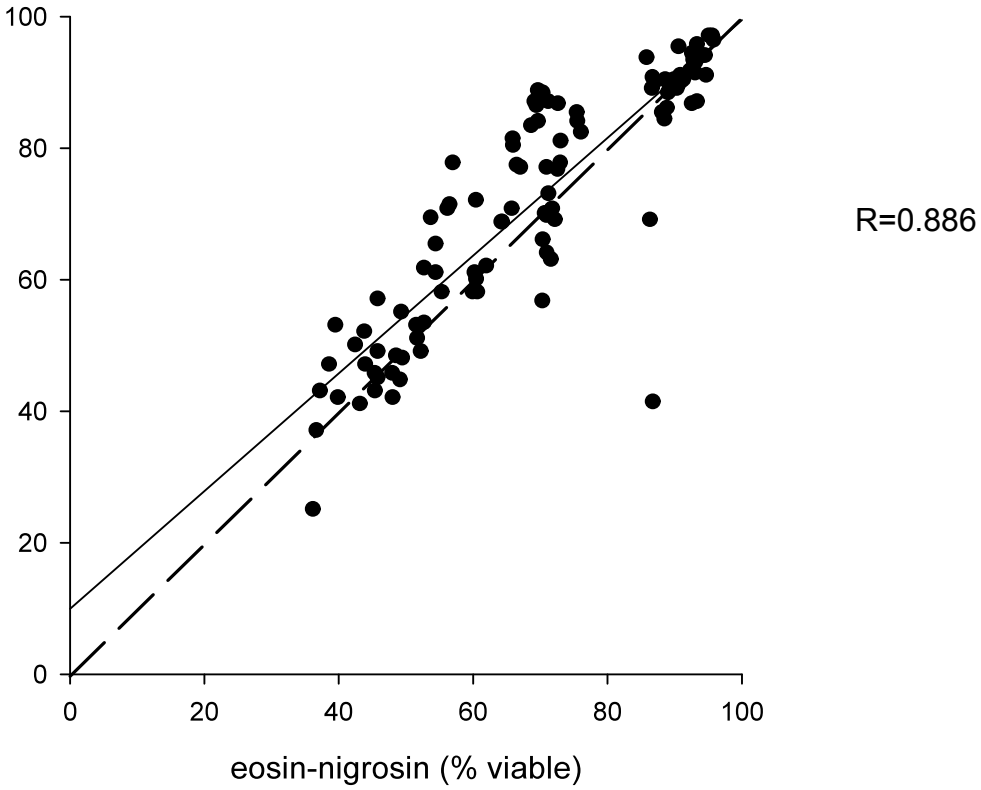

Figure 24. Regression plot comparing the mean percent viable sperm for the flow cytometer and eosin-nigrosin staining at Time 24 (N=108). Dashed line represents the line of equality.

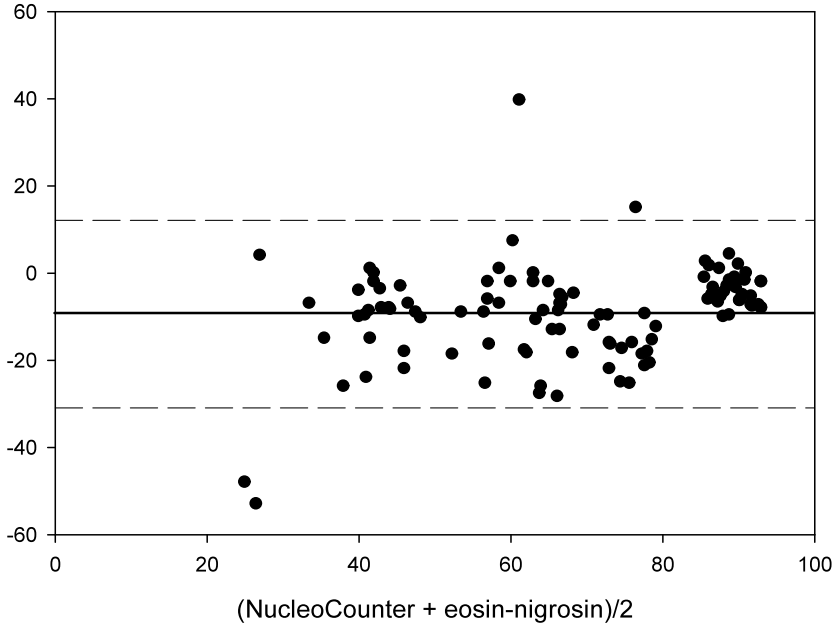

Figure 25. Bland and Altman plot comparing the difference in sperm membrane viability between the NucleoCounter® SP-100™and eosin-nigrosin staining to the mean of the NucleoCounter® SP-100™ and eosin-nigrosin staining at Time 24 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 31. Equations and their associated values used in preparing the Bland and Altman plots for the NucleoCounter® SP-100™ (NC) and eosin-nigrosin staining (EN) at Time 24.

Value (equation)	NC-EN	absolute value (NC-EN)	(NC+EN)/2
sum	-986.0	1145.3	7331.0
average	-9.1	10.6	67.9
standard deviation (SD)	11.0	9.6	18.9
lower limit of agreement (average-(2*SD))	-31.1	-8.5	30.0
upper limit of agreement (average+(2*SD))	12.9	29.7	105.8
Range	-53 – 40	40 – 53	25 - 93
number of events	108.0	108.0	108.0

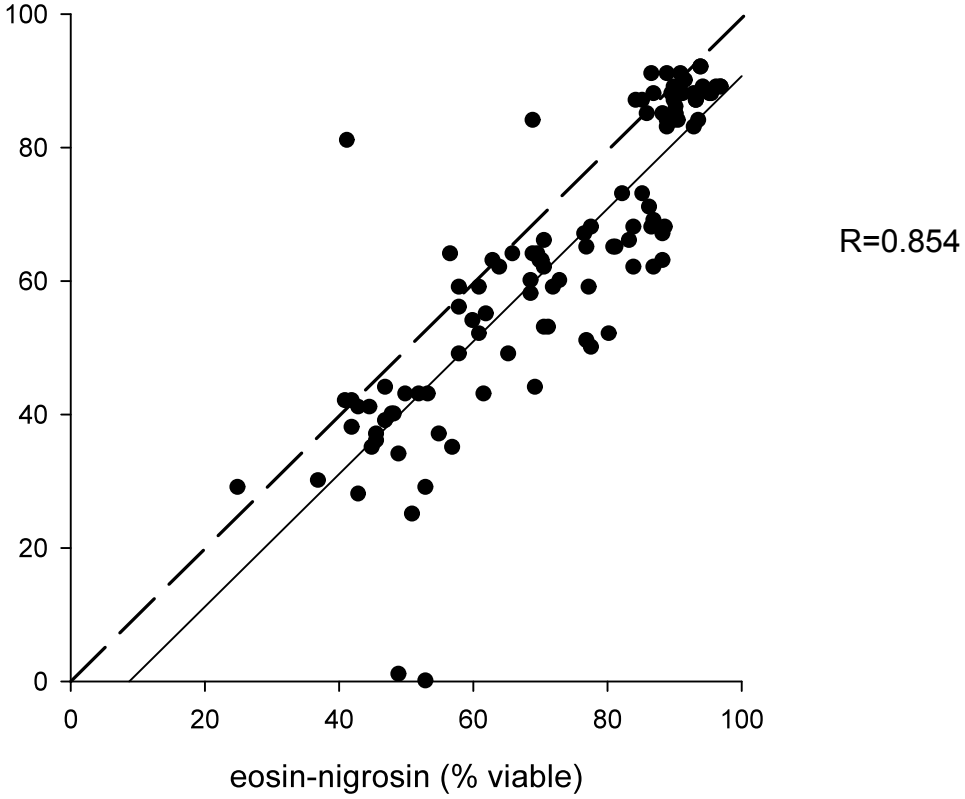

Figure 26. Regression plot comparing the mean percent viable sperm for the NucleoCounter® SP-100™ and eosin-nigrosin staining at Time 24 (N=108). Dashed line represents the line of equality.

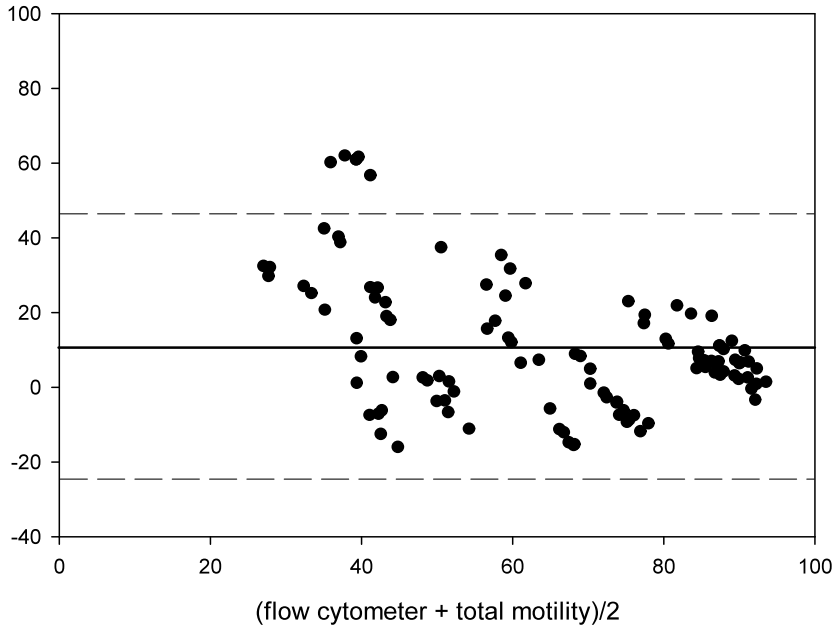

Figure 27. Bland and Altman plot comparing the difference in sperm membrane viability between the flow cytometer and total motility with the CASMA to the mean of the flow cytometer and total motility at Time 24 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 32. Equations and their associated values used in preparing the Bland and Altman plots for the flow cytometer (FC) and total sperm motility (TMOT) at Time 24.

Value (equation)	FC-TMOT	absolute value (FC-TMOT)	(FC+TMOT)/2
sum	1164.4	1628.5	6957.2
average	10.8	15.1	64.4
standard deviation (SD)	18.2	14.8	20.1
lower limit of agreement (average-(2*SD))	-25.6	-14.5	24.3
upper limit of agreement (average+(2*SD))	47.2	44.6	104.5
Range	-16 – 62	16 – 62	27 - 94
number of events	108.0	108.0	108.0

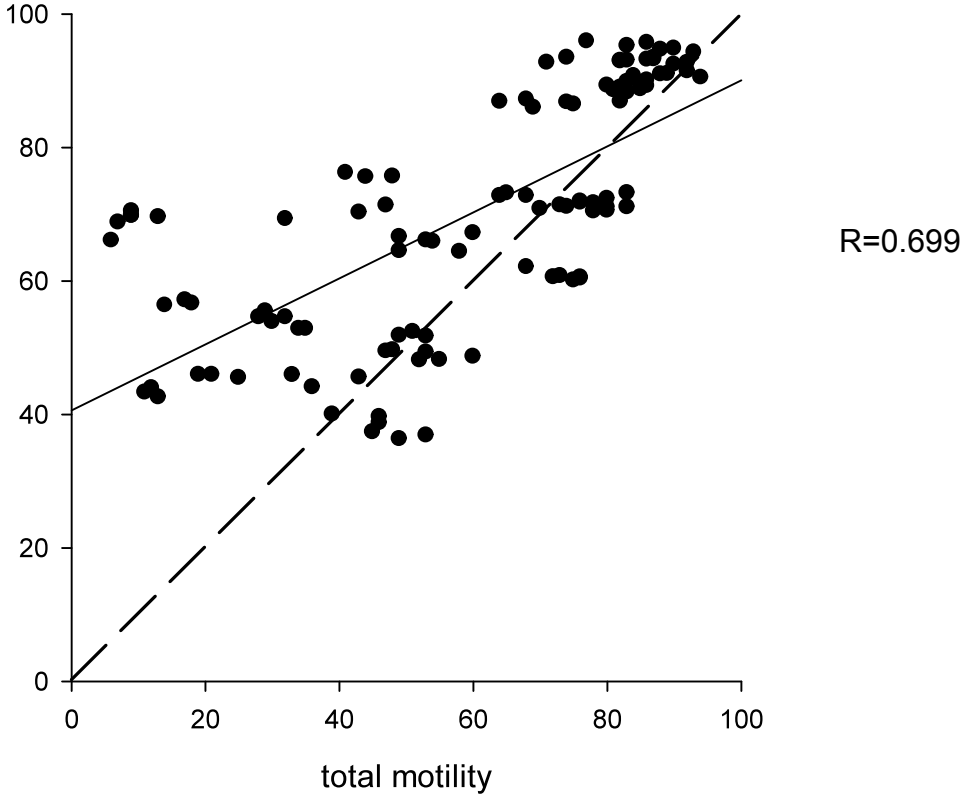

Figure 28. Regression plot comparing the mean percent viable sperm for the flow cytometer and total sperm motility with the CASMA at Time 24 (N=108). Dashed line represents the line of equality.

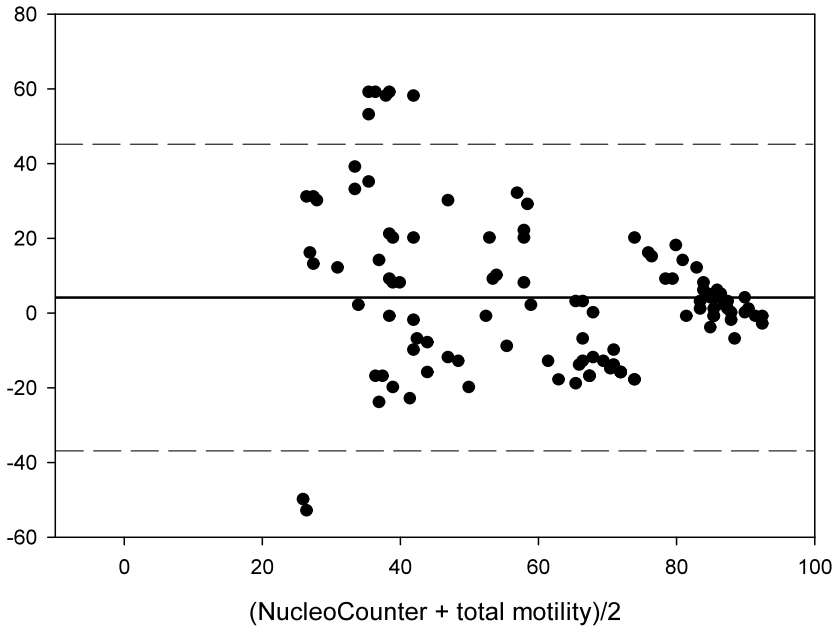

Figure 29. Bland and Altman plot comparing the difference in sperm membrane viability with the NucleoCounter® SP-100™ and total sperm motility with the CASMA to the mean of the NucleoCounter® SP-100™ and total sperm motility at Time 24 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 33. Equations and their associated values used in preparing the Bland and Altman plots for the NucleoCounter® SP-100™ (NC) and total sperm motility (TMOT) at Time 24.

Value (equation)	NC-TMOT	absolute value (NC-TMOT)	(NC+TMOT)/2
sum	463.0	1643.0	6606.5
average	4.3	15.2	61.2
standard deviation (SD)	20.7	14.7	21.0
lower limit of agreement (average-(2*SD))	-37.2	-14.1	19.2
upper limit of agreement (average+(2*SD))	45.8	44.6	103.1
Range	-53 – 59	53 – 59	26 - 93
number of events	108.0	108.0	108.0

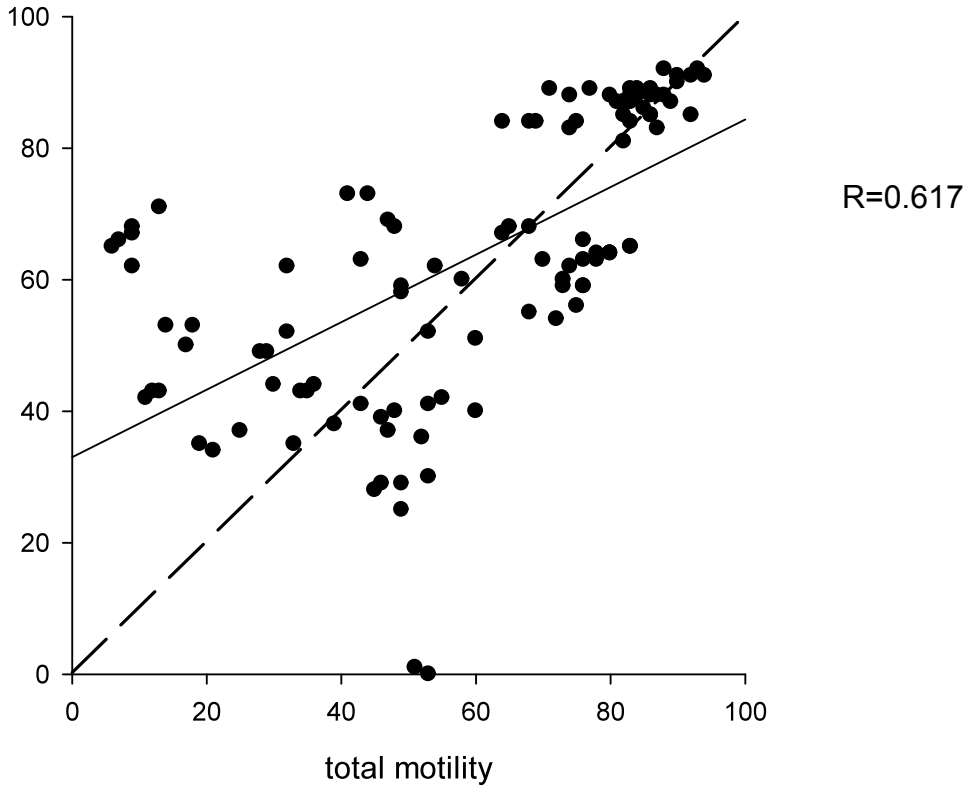

Figure 30. Regression plot comparing the mean percent viable sperm for the NucleoCounter® SP-100™ and total sperm motility with the CASMA at Time 24 (N=108). Dashed line represents the line of equality.

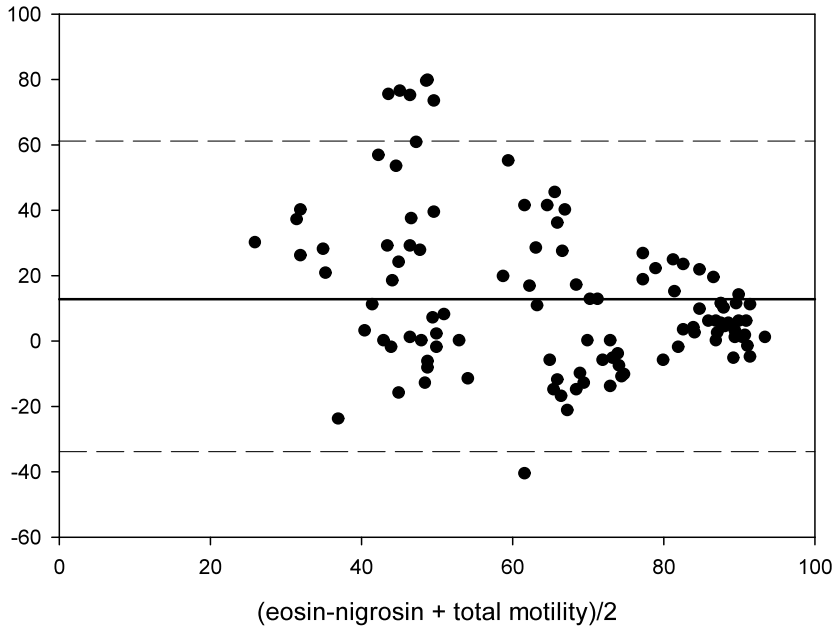

Figure 31. Bland and Altman plot comparing the difference in sperm membrane viability with eosin-nigrosin staining and total sperm motility with the CASMA to the mean of eosin-nigrosin staining and total sperm motility at Time 24 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 34. Equations and their associated values used in preparing the Bland and Altman plots for eosin-nigrosin staining (EN) and total sperm motility (TMOT) at Time 24.

Value (equation)	EN-TMOT	absolute value (EN-TMOT)	(EN+TMOT)/2
sum	1449.0	2072.3	7099.5
average	13.4	19.2	65.7
standard deviation (SD)	24.1	19.8	18.5
lower limit of agreement (average-(2*SD))	-34.8	-20.4	28.7
upper limit of agreement (average+(2*SD))	61.6	58.8	102.8
Range	-41 – 80	41 – 80	26 - 94
number of events	108.0	108.0	108.0

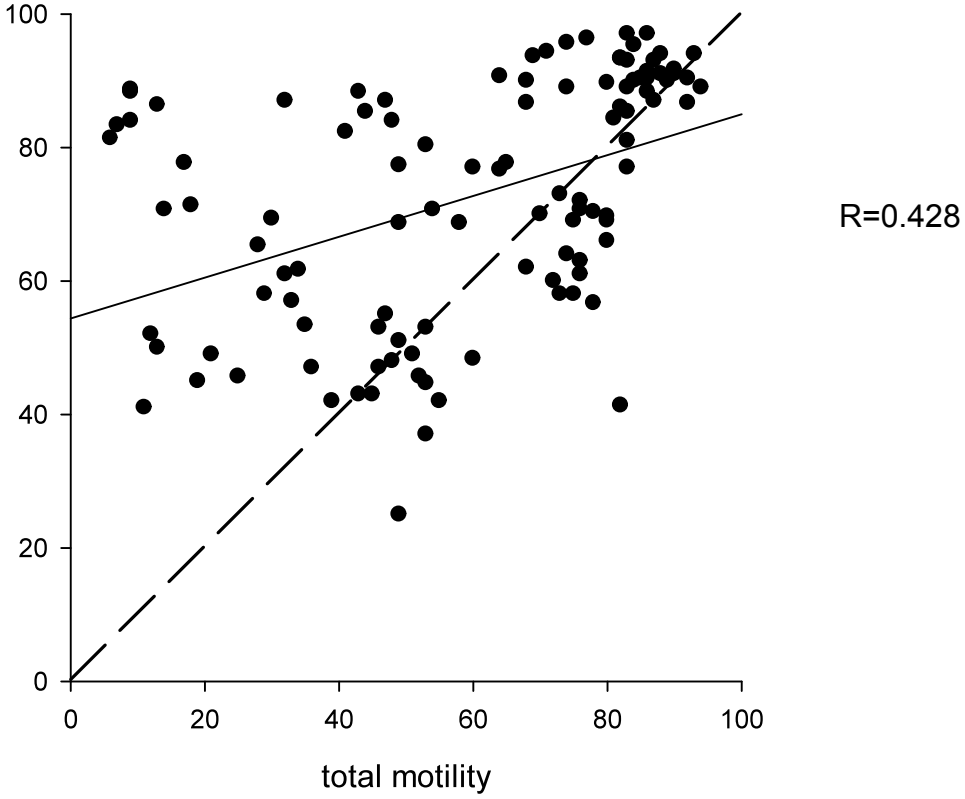

Figure 32. Regression plot comparing the mean percent viable sperm with eosin-nigrosin staining and total sperm motility with the CASMA at Time 24 (N=108). Dashed line represents the line of equality.

At Time 48, the flow cytometer held a 7.9 percentage-point difference (absolute value) when compared to the NucleoCounter® SP-100™ and an 8.5 percentage-point difference when compared to eosin-nigrosin staining (Tables 35 and 36). Values for viability using the flow cytometer were higher than those values found when the NucleoCounter® SP-100™ was used. Data points fell above and below the mean difference when comparisons were made with the flow cytometer and eosin-nigrosin staining, and with the NucleoCounter® SP-100™ and eosin-nigrosin staining. When comparisons were made with the NucleoCounter® SP-100™ and eosin-nigrosin staining, the method agreement averaged an approximate 13 percentage-point difference, and these data points fell above and below the mean difference (Table 37). When total motility was compared to viability using the three methods at Time 48 the agreements had an approximate 20 percentage-point difference for the NucleoCounter® SP-100™ and the flow cytometer compared to total motility (Tables 38 and 39), and a 30 percentage-point difference for eosin-nigrosin and total motility (Table 40). The R-values remained high for all comparisons among methods for detecting viability (flow cytometer, NucleoCounter® SP-100™, eosin-nigrosin) at Time 48 (Figures 34, 36, 38). The R-values decreased at Time 48 as compared to Time 0 however these values were higher for Time 48 when compared to Time 24 for the comparisons of the three methods with total motility (Figures 40, 42, 44).

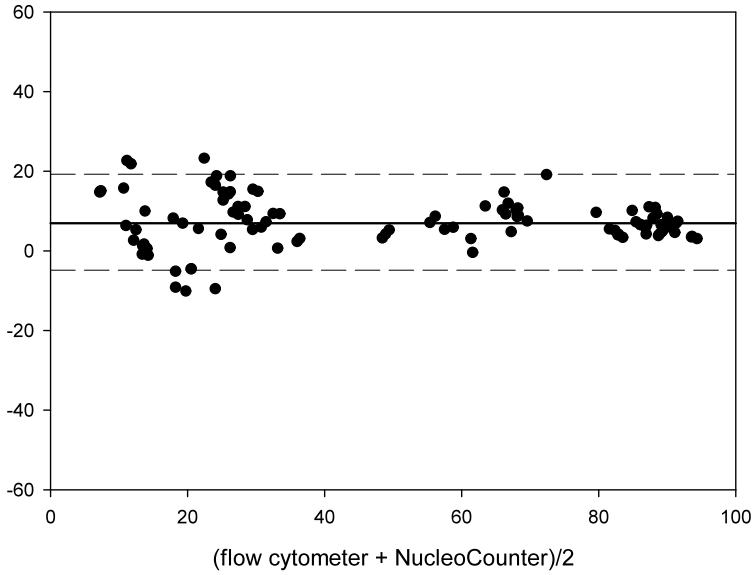

Figure 33. Bland and Altman plot comparing the difference in sperm membrane viability between the flow cytometer and the NucleoCounter® SP-100™ to the mean of the flow cytometer and the NucleoCounter® SP-100™ at Time 48 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 35. Equations and their associated values used in preparing the Bland and Altman plots for the flow cytometer (FC) and the NucleoCounter® SP-100™ (NC) at Time 48.

Value (equation)	FC-NC	absolute value (FC-NC)	(FC+NC)/2
sum	764.4	858.2	5585.2
average	7.1	7.9	51.7
standard deviation (SD)	6.2	5.0	30.6
lower limit of agreement (average-(2*SD))	-5.4	-2.1	-9.5
upper limit of agreement (average+(2*SD))	19.5	18.0	112.9
Range	-10 – 23	10 – 23	7 - 94
number of events	108.0	108.0	108.0

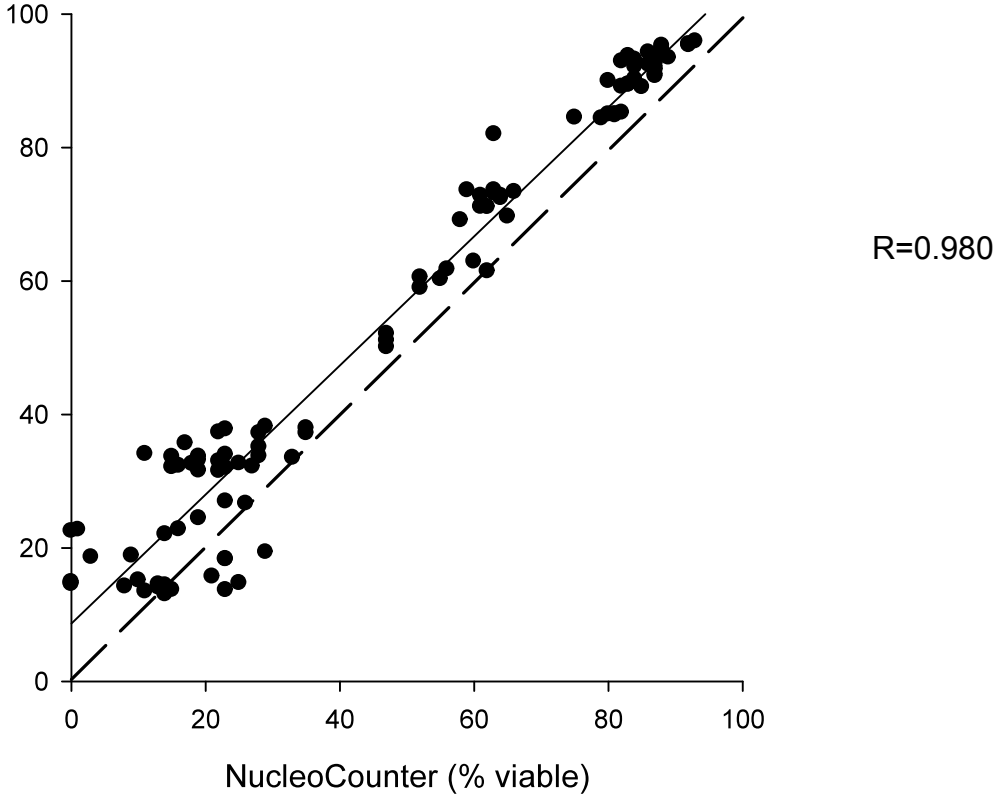

Figure 34. Regression plot comparing the mean percent viable sperm for the flow cytometer and the NucleoCounter® SP-100™ at Time 48 (N=108). Dashed line represents the line of equality.

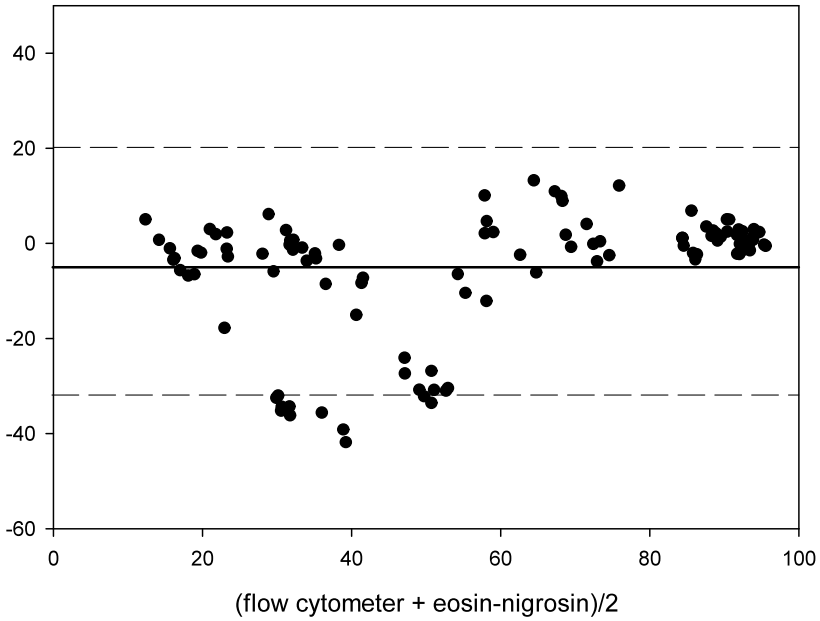

Figure 35. Bland and Altman plot comparing the difference in sperm membrane viability between the flow cytometer and eosin-nigrosin staining to the mean of the flow cytomer and eosin-nigrosin staining at Time 48 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 36. Equations and their associated values used in preparing the Bland and Altman plots for the flow cytometer (FC) and eosin-nigrosin staining (EN) at Time 48.

Value (equation)	FC-EN	absolute value (FC-EN)	(FC+EN)/2
sum	-621.6	922.3	6278.2
average	-5.8	8.5	58.1
standard deviation (SD)	13.2	11.5	27.5
lower limit of agreement (average-(2*SD))	-32.1	-14.5	3.0
upper limit of agreement (average+(2*SD))	20.6	31.6	113.2
Range	-42 – 13	13 – 42	12 - 96
number of events	108.0	108.0	108.0

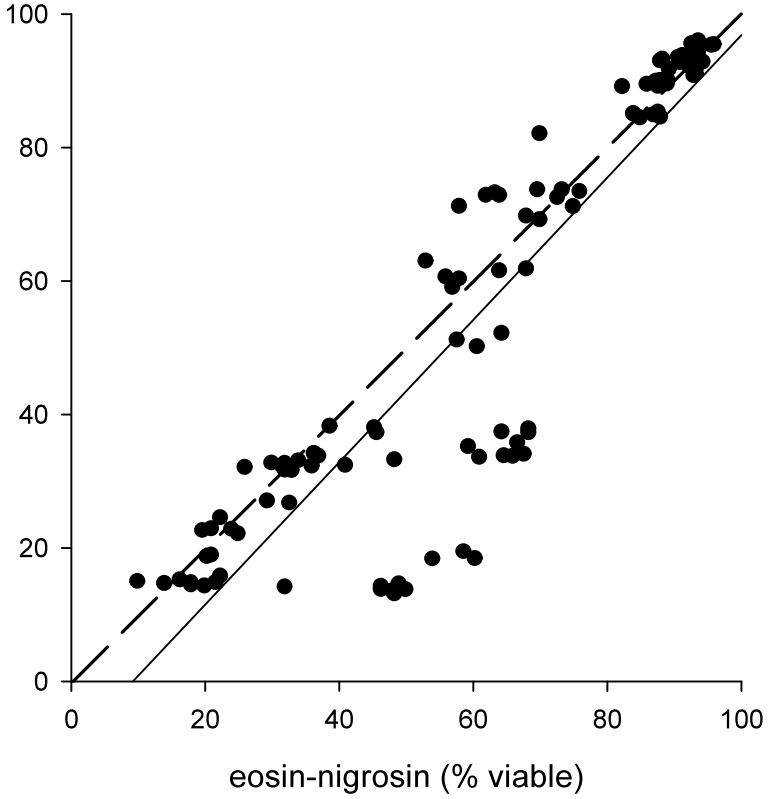

Figure 36. Regression plot comparing the mean percent viable sperm for the flow cytometer and eosin-nigrosin staining at Time 48 (N=108). Dashed line represents the line of equality.

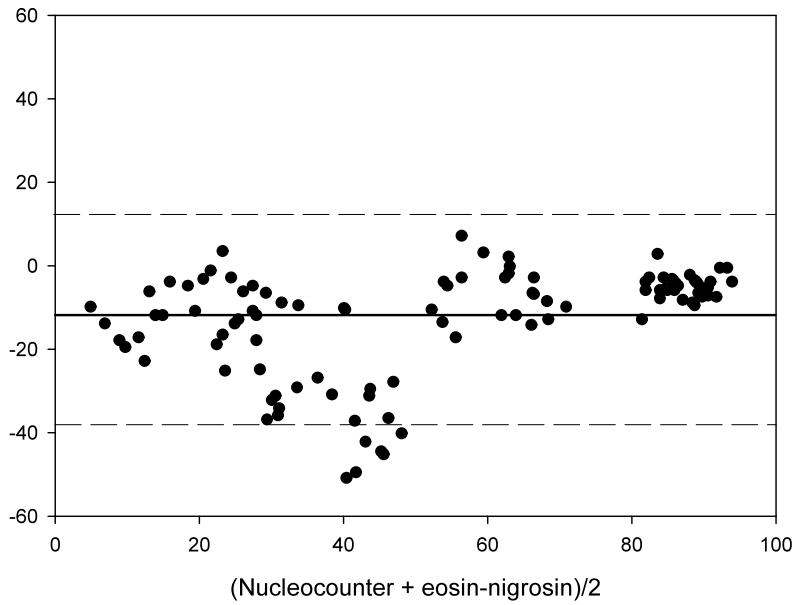

Figure 37. Bland and Altman plot comparing the difference in sperm membrane viability between the NucleoCounter® SP-100™ and eosin-nigrosin staining to the mean of the NucleoCounter® SP-100™ and eosin-nigrosin staining at Time 48 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 37. Equations and their associated values used in preparing the Bland and Altman plots for the NucleoCounter® SP-100™ (NC) and eosin-nigrosin staining (EN) at Time 48.

Value (equation)	NC-EN	absolute value (NC-EN)	(NC+EN)/2
sum	-1386.0	1422.0	5896.0
average	-12.8	13.2	54.6
standard deviation (SD)	12.7	12.3	27.8
lower limit of agreement (average-(2*SD))	-38.2	-11.5	-1.1
upper limit of agreement (average+(2*SD))	12.5	37.8	110.3
Range	-51 – 7	7 – 51	5 - 94
number of events	108.0	108.0	108.0

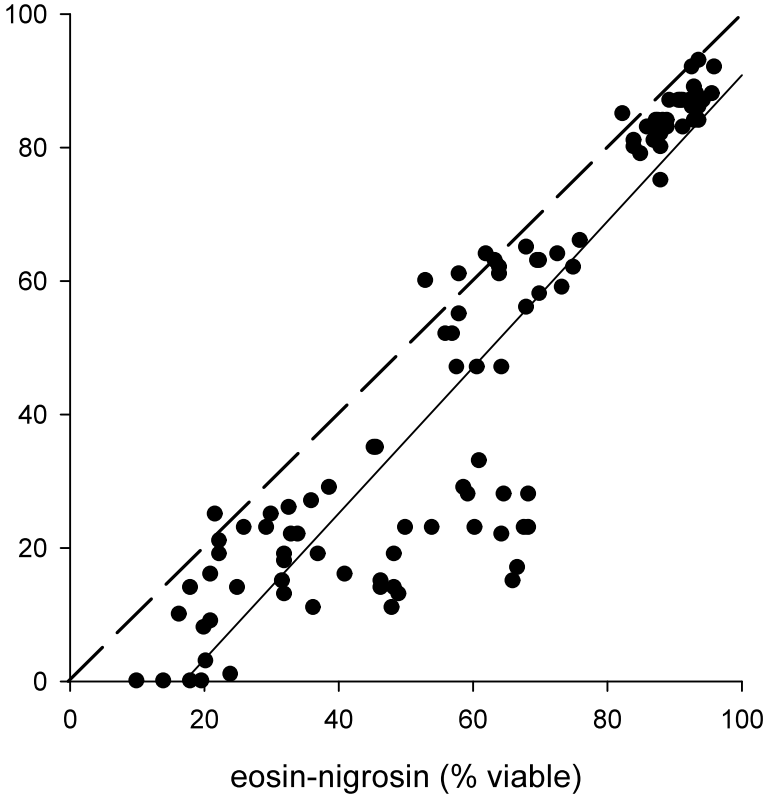

Figure 38. Regression plot comparing the mean percent viable sperm for the NucleoCounter® SP-100™ and eosin-nigrosin staining at Time 48 (N=108). Dashed line represents the line of equality.

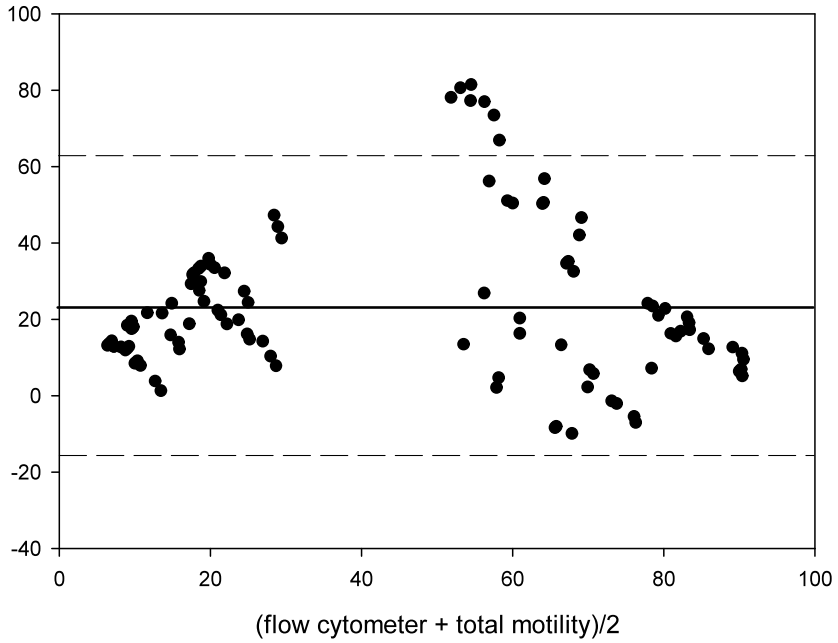

Figure 39. Bland and Altman plot comparing the difference in sperm membrane viability between the flow cytometer and total motility with the CASMA to the mean of the flow cytometer and total motility at Time 48 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 38. Equations and their associated values used in preparing the Bland and Altman plots for the flow cytometer (FC) and total sperm motility (TMOT) at Time 24.

Value (equation)	FC-TMOT	absolute value (FC-TMOT)	(FC+TMOT)/2
sum	2488.4	2575.8	4723.2
average	23.0	23.8	43.7
standard deviation (SD)	20.0	19.0	28.9
lower limit of agreement (average-(2*SD))	-16.9	-14.1	-14.0
upper limit of agreement (average+(2*SD))	63.0	61.8	101.5
Range	-10 – 81	10 – 81	6 - 91
number of events	108.0	108.0	108.0

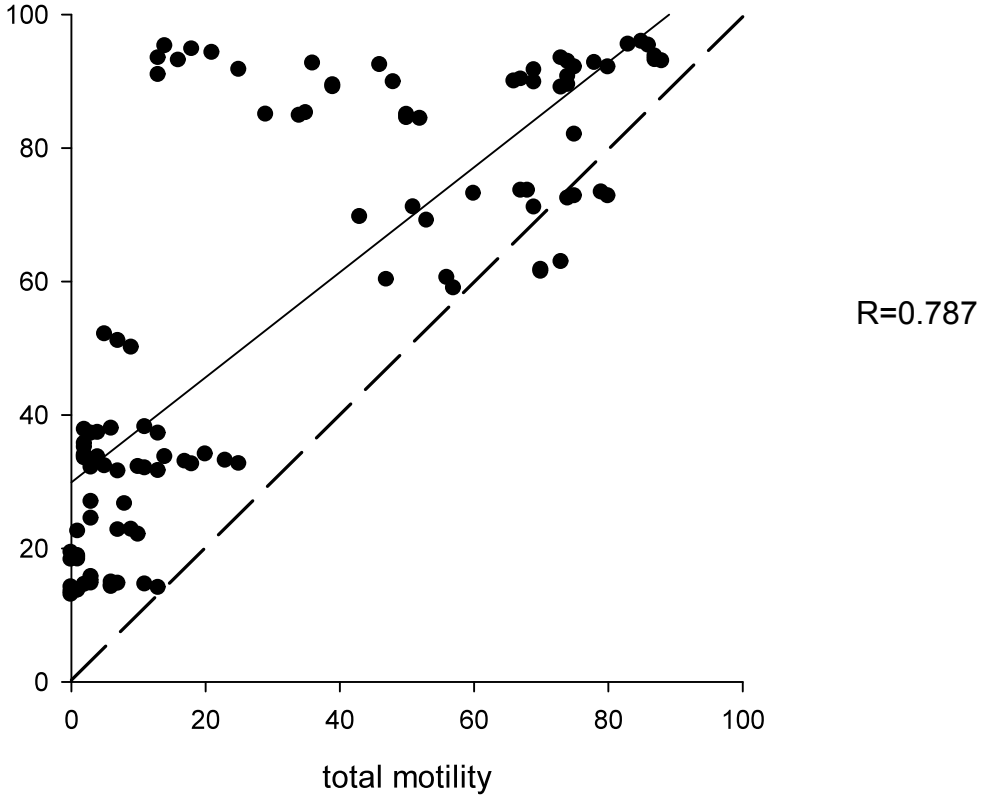

Figure 40. Regression plot comparing the mean percent viable sperm for the flow cytometer and total sperm motility with the CASMA at Time 48 (N=108). Dashed line represents the line of equality.

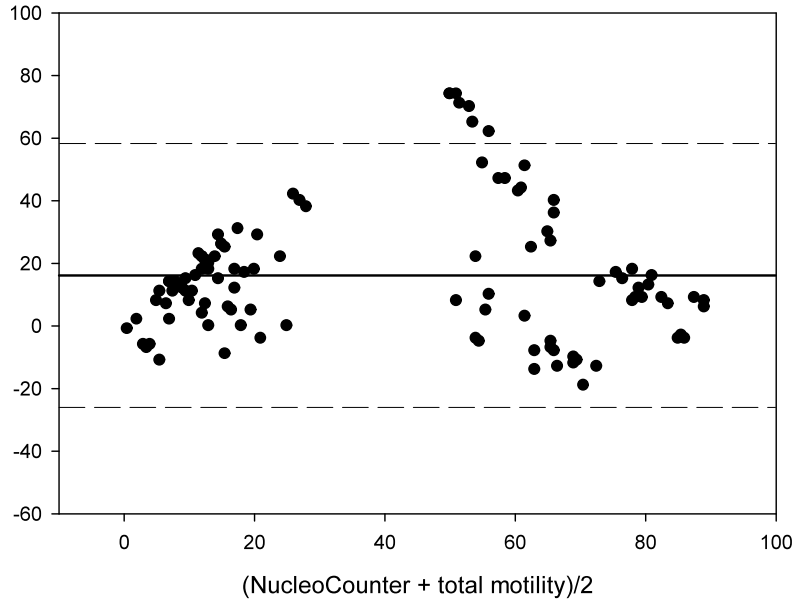

Figure 41. Bland and Altman plot comparing the difference in sperm membrane viability with the NucleoCounter® SP-100™ and total sperm motility with the CASMA to the mean of the NucleoCounter® SP-100™ and total sperm motility at Time 48 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 39. Equations and their associated values used in preparing the Bland and Altman plots for the NucleoCounter® SP-100™ (NC) and total sperm motility (TMOT) at Time 48.

Value (equation)	NC-TMOT	absolute value (NC-TMOT)	(NC+TMOT)/2
sum	1724.0	2092.0	4341.0
average	16.0	19.4	40.2
standard deviation (SD)	21.1	17.9	28.9
lower limit of agreement (average-(2*SD))	-26.2	-16.5	-17.6
upper limit of agreement (average+(2*SD))	58.1	55.3	98.0
Range	-19 – 74	19 – 74	1 - 89
number of events	108.0	108.0	108.0

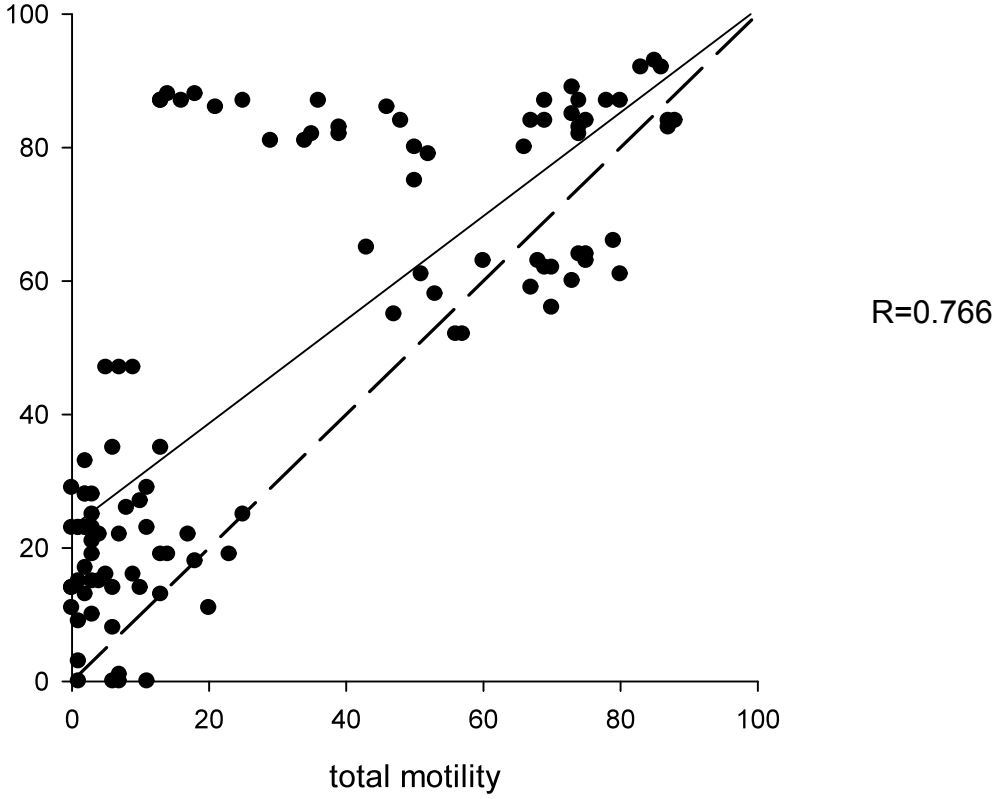

Figure 42. Regression plot comparing the mean percent viable sperm for the NucleoCounter® SP-100™ and total sperm motility with the CASMA at Time 48 (N=108). Dashed line represents the line of equality.

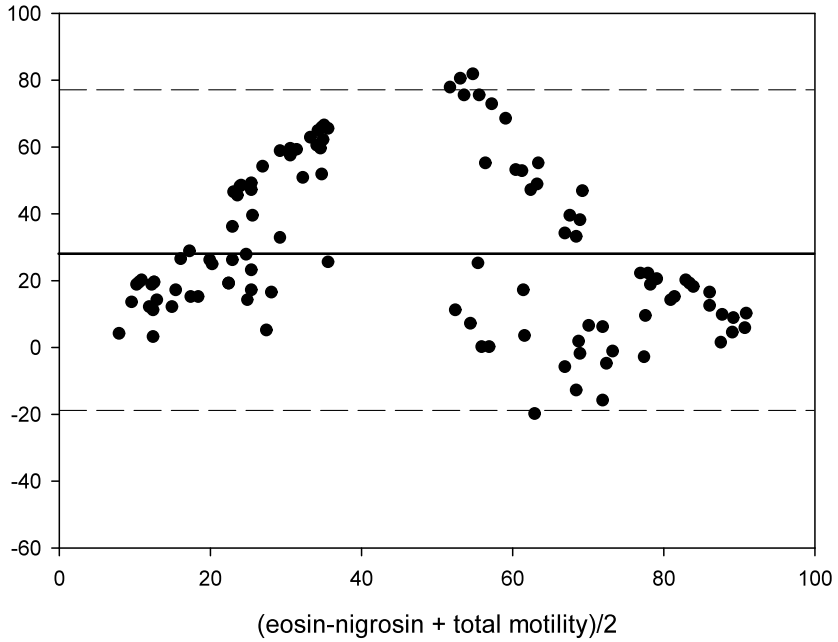

Figure 43. Bland and Altman plot comparing the difference in sperm membrane viability with eosin-nigrosin staining and total sperm motility with the CASMA to the mean of eosin-nigrosin staining and total sperm motility at Time 48 (N=108). Dashed lines represent + or – 2 standard deviations (lower and upper limits of agreement); solid line represents mean difference.

Table 40. Equations and their associated values used in preparing the Bland and Altman plots for eosin-nigrosin staining (EN) and total sperm motility (TMOT) at Time 48.

Value (equation)	EN-TMOT	absolute value (EN-TMOT)	(EN+TMOT)/2
sum	3110.0	3242.7	5034.0
average	28.8	30.0	46.6
standard deviation (SD)	24.2	22.6	25.6
lower limit of agreement (average-(2*SD))	-19.6	-15.3	-4.6
upper limit of agreement (average+(2*SD))	77.2	75.3	97.8
Range	-20 – 82	20 – 82	8 - 91
number of events	108.0	108.0	108.0

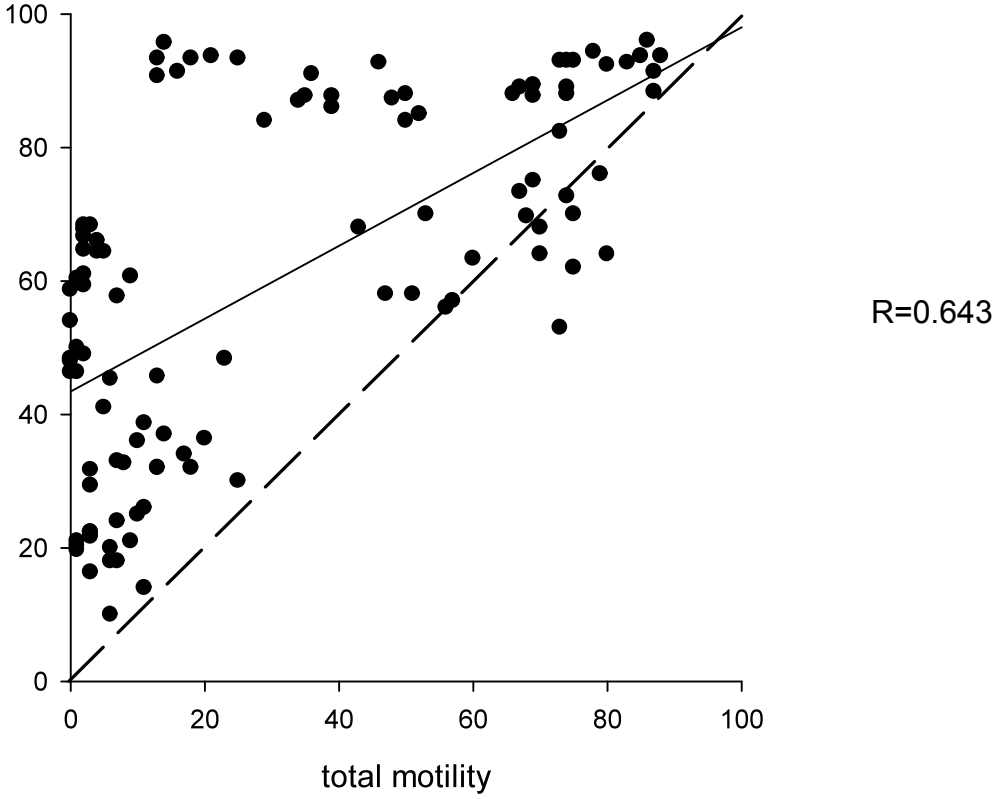

Figure 44. Regression plot comparing the mean percent viable sperm with eosin-nigrosin staining and total sperm motility with the CASMA at Time 48 (N=108). Dashed line represents the line of equality.

3.6.2. Discussion: Experiment 6: Comparison of methods for detecting sperm membrane viability and seminal plasma effects on sperm motion characteristics and membrane viability

In agreement with other studies [5, 6, 8, 27, 28], the results of Experiment 6 indicated that a seminal plasma concentration above 20% caused detrimental effects to total and progressive motility. A decrease in sperm membrane viability as measured using the three methods (flow cytometry, NucleoCounter® SP-

100™, and eosin-nigrosin staining) was also observed in Experiment 6 as seminal plasma volume increased, which to our knowledge is reported here for the first time. The decrease in proportion of viable sperm was not significant ($P>0.05$) in treatments containing 50% or less seminal plasma, however these sperm did have a significant decrease in both total and progressive motility, indicating that the sperm still had intact membranes but had been rendered immotile. This observation is an indication of sub-optimal processing of the ejaculate.

The finding of depressed motility in sperm without apparent loss of viability is in contrast to the previous reports of a strong correlation between total and progressive motility as compared to viability [2, 13]. These contrasting findings may be due to the method used to produce populations of non-viable sperm. In the studies conducted by Love et al., and Brinsko et al. in 2003, populations of non-viable sperm were produced by the addition of various proportions of killed sperm to the semen [2, 13]. The current research project is the first study, that we are aware of, using a physiological trigger, seminal plasma, to induce varying populations of viable and non-viable sperm. As evident by the findings of this study, that as seminal plasma levels were increased, the correlation between membrane viability and motion characteristics decreased, appears to reflect the sperm being rendered immotile but remaining viable. Sperm subjected to Treatment 80SP/20 (20% neat semen, 60% of the stallion's own frozen/thawed seminal plasma, and 20%

INRA-T; thus 80% seminal plasma, but with 25% the sperm concentration of Treatment 80SP) had significantly lower ($P<0.05$) populations of viable sperm and total motile sperm compared to other treatments at Time 24. This treatment (80SP/20) was used to determine if the viability and/or the total motility decreased in Treatment 80SP due to seminal plasma effects or to the increased sperm concentration in this treatement as compared to 50SP or 20SP, as storage of semen with increased sperm concentrations. The results of this study indicated that while increased seminal plasma was detrimental to motility and membrane viability; this affect was not mitigated by lowering sperm concentration and in fact the lower concentration of sperm resulted in poorer survival. The stallions used in this study were considered to have excellent semen quality and fertility and it should be noted that the effects of seminal plasma levels vary among stallions, ejaculates and processing techniques [7-12].

While many studies have compared two or more methods for determining the viability of sperm in several species including equine [2, 3, 14], fowl [1, 15], dogs [4], and cats [16], this is the first known report comparing the use of eosin-nigrosin staining to that of the flow cytometer using SYBR-14 and PI, as well as comparison of the NucleoCounter® SP-100™ to the flow cytometer and eosin-nigrosin staining. The flow cytometer has been considered the “gold standard” in terms of determining the integrity of sperm plasma membranes (viability); however, it is not of practical use for most laboratories and breeding facilities.

The Bland and Altman plots were generated to determine the amount of disagreement between the methods for comparing sperm viability in an attempt to determine if a new method (NucleoCounter® SP-100™) could replace an old method (flow cytometer or eosin-nigrosin staining) [38, 39]. Results indicate that the NucleoCounter® SP-100™ was in close agreement with the flow cytometer at all time periods (≤ 8 percentage-point difference). At higher percentages of viable sperm the agreement between the two methods was closer (≤ 6.5 percentage point difference). These results validate the NucleoCounter® SP-100™ as an accurate and repeatable test for sperm membrane viability up to a 24-hour cooling period. Although the flow cytometer is considered to be the most accurate method for determination of sperm viability, from a clinical perspective, the results of this study show that the NucleoCounter® SP-100™ can be utilized as a good substitute for measurement of sperm membrane viability, adding another important component to the evaluation of semen from breeding stallions. Eosin-nigrosin staining disagreed with the flow cytometer at a lesser degree (≤ 8.5 percentage points) than in comparison to the NucleoCounter® SP-100™ (≤ 13 percentage points). This is in agreement with another study which found a 12.5% difference between the NucleoCounter® SP-100™ and eosin-nigrosin staining [14]. A contributing factor to this finding is that the flow cytometer consistently overestimated the population of viable sperm in terms of the results obtained from the NucleoCounter® SP-100™, whereas results obtained from eosin-nigrosin staining had values that estimated above

and below the mean difference for both the NucleoCounter® SP-100™and the flow cytometer. With a decrease in viable sperm and an increase in storage (24 and 48 hours), the agreement of the three methods with total motility decreased dramatically from a 10 percentage-point difference at Time 0 to a 20-30 percentage-point difference after 24 and 48 hours of storage, respectively. These findings support the previous statement that as semen is stored at sub-optimal seminal plasma volumes (≥ 50%), the relationship between motion characteristics and viability weakens.

3.7. Experiments 7-9

A single gel-free ejaculate was collected using the procedure previously described. All initial raw semen parameters were obtained and recorded. The semen was aliquoted out into three treatments. Each treatment addressed one of the following: 1) Effect of buffer type (PBS, Garner's solution with and without SYBR-14 or INRA-T) on sperm membrane viability determination (Experiment 7); 2) Effect of incubation time after addition of SYBR-14 and propidium iodide on sperm viability measured by flow cytometry (Experiment 8); 3) Ability of the flow cytometer and NucleoCounter® SP-100™ to measure absolute zero viability (Experiment 9). For Experiments 7 and 8, semen was extended in INRA-T to a total volume of 30 mL containing 80% seminal plasma (24 mL neat semen and 6 mL extender) and was placed in two 15 mL conical tubes to be used for NucleoCounter® SP-100™ and flow cytometer analysis. In Experiment 9, a 5 mL aliquot of raw semen was dispensed into a cryovial and stored away from

direct light at room temperature (23°C) until all other experiments were complete. Three replicates were performed on each treatment in each experiment. The protocols for the NucleoCounter® SP-100™ and flow cytometer were followed according to the methods described previously unless stated otherwise in the experiments.

3.8. Experiment 7: Effect of diluent type (PBS with and without SYBR-14, Garner's solution or INRA-T)

The semen was diluted in a cryovial according to a manufacturer recommended dilution factor previously described in materials and methods in one of four diluents. The diluent treatments used included 1) PBS; 2) INRA-T; 3) Garner's solution; 4) and PBS with 0.4 μL SYBR-14 (0.04 μM). The samples were run in a staggered order dependent on treatment to rule out any incubation-time effect on viability.

3.8.1. Results: Experiment 7: Effect of diluent type (PBS with and without SYBR-14, Garner's solution or INRA-T)

The NucleoCounter® SP-100™ showed an error window and was unable to read non-viable concentrations when INRA-T was used as a diluent (Treatment 2) instead of PBS. There were no differences among the mean percent viable sperm for the other three treatments (Table 42).

Table 41. Effect of diluent type on mean percent viable (mean ± SD) sperm for treatments 1, 3, and 4 using the NucleoCounter.

Treatment	Mean (% viable)± SD
1	91.7±1.2
3	90.7±0.6
4	90.0±0

3.8.2. Discussion: Experiment 7: Effect of diluent type (PBS with and without SYBR-14, Garner's solution or INRA-T)

The opaque quality of INRA 96 is possibly the cause of the error associated with Treatment 2. Although the NucleoCounter® SP-100™ is capable of reading semen diluted in extender, the recommended use of PBS as a diluent is used to dilute out the sample allowing the NucleoCounter® SP-100™ to obtain a non-viable concentration. There were no differences among the remaining treatment groups (Treatment 1, 3, 4) indicating that PBS with and without SYBR-14, and Garner's solution did not cause detriment to sperm viability or changes in the ability of the NucleoCounter® SP-100™ to detect viability.

3.9. Experiment 8: Effect of incubation time after addition of SYBR-14 and propidium iodide on sperm membrane viability measured by flow cytometry

A 2.5 mL aliquot of extended semen was placed in each of four flow cytometer tubes and processed for analysis on the flow cytometer using one of the following treatments: 1) immediate analysis of viability after addition of the

SYBR-14 /PI to the semen sample; 2) similar to Treatment 1 except the sample was incubated for 5 minutes; 3) consisted of the addition of SYBR-14 to the semen sample, a 5 minute incubation period, then addition of PI to the semen sample followed by another 5 minute incubation period prior to analysis; 4) utilized the original protocol previously described in materials and methods (10 minute incubation time after SYBR-14 addition, then 5 minute incubation after PI addition).

3.9.1. Results: *Experiment 8: Effect of incubation time after addition of SYBR-14 and propidium iodide on sperm membrane viability measured by flow cytometry*

There was no effect of incubation time on assessment of sperm viability among treatments (Table 42).

Table 42. Effect of incubation time after addition of SYBR-14 and prodidium iodide on sperm viability measured by flow cytometry.

Treatment	Mean ± SD
1	93.9 ± 2
2	92.8 ± 0.5
3	92.7 ± 0.3
4	93.3 ± 0.2

3.9.2. Discussion: *Experiment 8: Effect of incubation time after addition of SYBR-14 and propidium iodide on sperm membrane viability measured by flow cytometry*

This experiment was conducted to determine if sperm viability, or determination of sperm viability, changed with a longer incubation time. As evident by the results for sperm viability in Table 42, there were no differences for sperm viability when immediate analysis (Treatment 1) was compared to a 15-minute incubation time (Treatment 4), therefore length of incubation does not affect determination of sperm viability via flow cytometry.

4.0. Experiment 9: Ability of the flow cytometer and NucleoCounter® SP-100™ to measure absolute zero sperm membrane viability

Data collected from Experiment 6 showed, at times, that when the NucleoCounter® SP-100™ gave a viable reading of 0%, the flow cytometer was still reading a small population of viable cells. It is therefore not clear which method was correct. To answer this question, a 5 mL aliquot of raw semen stored in a cryovial was plunged in liquid nitrogen until frozen then thawed in a water bath set at 37°C for 5 minutes. This was repeated two more times to damage sperm membranes resulting in a sperm sample with assumed zero viability. The semen was analyzed on the flow cytometer using the protocol previously used in Experiments 4-6 and with the NucleoCounter® SP-100™ (PBS as diluent) as previously described in materials and methods.

4.0.1: Results: Experiment 9: Ability of the flow cytometer and NucleoCounter® SP-100™ to measure absolute zero sperm membrane viability

When sperm viability was assessed using the flow cytometer on the raw semen sample subjected to freezing in liquid nitrogen, the average percent viable population was 0.5% with a standard deviation of 0.12. When this same sample was assessed using the NucleoCounter® SP-100™, the average percent viable population was 3% with a standard deviation of 3.6.

4.0.2: Discussion: Experiment 9: Ability of the flow cytometer and NucleoCounter® SP-100™ to measure absolute zero sperm membrane viability

From these results, it appears that the flow cytometer was able to measure absolute zero viability indicating that results from Experiment 6 were probably correct in that the flow cytometer measured the viability more accurately than did the NucleoCounter® SP-100™. In this experiment, the NucleoCounter® SP-100™ ,results indicated a population of viable cells and the how standard deviation was larger than that of the flow cytometer (3.6 versus 0.12) which is another indication of the accuracy of the flow cytometer when compared to the NucleoCounter® SP-100™. It should also be noted that one of the three replicates using the NucleoCounter® SP-100™ did give a reading of <0% non-viable.

4. SUMMARY

This was the first study to assess the relationship between sperm motion characteristics (total and progressive motility) and sperm viability utilizing varying amounts of seminal plasma to induce mimic varying populations of viable sperm, and three different methods (flow cytometer, NucleoCounter® SP-100™, and eosin-nigrosin) for detecting sperm viability. The findings of this study suggested that the proportion of motile sperm decreased the relationship between motion characteristics and sperm viability in that the motion characteristics decreased at a faster rate than did the sperm viability.

The Nucleocounter SP-100 appears to be an acceptable method for evaluating sperm viability. The advantages of using the NucleoCounter® SP-100™ as a means for detecting sperm viability are: 1) the cost and expertise associated with purchasing and using the instrument are far less than those needed for purchasing, running and maintaining the flow cytometer; 2) based on statistics and Bland and Altman plots, the NucleoCounter® SP-100™ holds better agreement with the flow cytometer and is a more repeatable method for determining sperm viability when compared to eosin-nigrosin staining; and 3) the NucleoCounter® SP-100™ is more readily available for clinical purposes and its use as an instrument for sperm concentration has also been validated. In contrast, eosin-nigrosin staining had poor accuracy in determining sperm viability at lower levels of viability, and had the highest variability among the methods examined.

5. FUTURE AIMS

This was the first known study to utilize a physiological method (varying volumes of seminal plasma and storage of semen) to induce detrimental effects to the plasma membranes of sperm. When comparisons were made between sperm viability and motion characteristics, results suggested that in higher volumes of seminal plasma the sperm are rendered immotile but remained viable (plasma membranes intact). Future studies should expand on this finding to confirm these results, and to determine if the sme findings exist when sperm motility is dereased due to factors other than storage. Previous studies have found strong correlations between motion characteristics and viability when known amounts of dead sperm were added.

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