VALIDATION OF A COMMERCIALLY AVAILABLE FLUORESCENCE-BASED INSTRUMENT TO EVALUATE STALLION SPERMATOZOAL CONCENTRATION AND COMPARISON TO PHOTOMETRIC SYSTEMS

A Thesis

by

KATHRYN LEIGH COMERFORD

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

May 2009

Major Subject: Veterinary Medical Sciences

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ABSTRACT

Validation of a Commercially Available Fluorescence-Based Instrument to Evaluate Stallion Spermatozoal Concentration and Comparison to Photometric Systems.

(May 2009)

Kathryn Leigh Comerford, B.S., West Texas A&M University

Chair of Advisory Committee: Dr. Charles C. Love

Accurate measurement of stallion spermatozoal concentration is important to equine breeding operations. The hemacytometer is considered the standard for measuring spermatozoal concentration but is time consuming and may be imprecise. The flow cytometer is considered precise and accurate, but only practical for research purposes due to sample preparation time and high cost. Photometric systems are commonly used but can be inaccurate outside a relatively narrow concentration range and can be rendered inaccurate in the presence of contaminants. A new instrument, the NucleoCounter SP-100® is reported to enumerate spermatozoa at wider concentration ranges and can identify spermatozoa in opaque semen extenders. Epididymal, neat (raw) ejaculates, and ejaculates diluted in various semen extenders were analyzed with the NucleoCounter, the Densimeter®, the Spermacue®, flow cytometric and hemacytometric methods. Results were compared statistically by: 1) regression analysis, 2) the agreement of two instruments, whereby the difference in values between two instruments was plotted on the y-axis against the mean of those values on the x-axis [26]

and 3) a modified method that measured the *percentage deviation*, whereby the percentage (of the difference in values between two instruments divided by the mean) of the same two values was plotted on the y-axis against the mean value of the two instruments on the x-axis.

The NucleoCounter showed more agreement with both the flow cytometer and hemacytometer for epididymal, neat ejaculated and extended spermatozoa over a range of concentrations than the Densimeter or the Spermacue. The NucleoCounter showed more agreement with the flow cytometer for epididymal and neat ejaculated spermatozoa and more agreement with the hemacytometer for spermatozoa diluted in semen extenders. The Spermacue showed the least agreement with both standards for all spermatozoal comparisons. All coefficients of variation for the flow cytometer, hemacytometer and NucleoCounter were >10% for all spermatozoal comparisons.

This study indicates that the NucleoCounter shows more agreement with the flow cytometer and hemacytometer than photometric systems when evaluated with epididymal, neat ejaculated and extended spermatozoa. The instrument is also more repeatable than either photometric system, but may be cost-prohibitive for some operations.

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INTRODUCTION

Accurate and precise spermatozoal concentration measurement of stallion semen is important for artificial insemination and fertility evaluations of stallions. Spermatozoal concentration is used to calculate the sperm number to be used for mares bred at the same location as the stallion as well as those to be bred with cooled or frozen shipped semen. In addition, measurement of spermatozoal concentration is used to determine total sperm numbers produced in ejaculates and to aid in calculating sperm production efficiency of the testes. Inaccurate determination of spermatozoal concentration, especially those which are too low can lead to reduced fertility resulting from the insemination of a below-threshold number of spermatozoa, as well as an incorrect interpretation of a stallion's breeding potential. The recent trend of insemination with low sperm numbers; known as low-dose insemination, is likely to continue, thus making the accuracy of spermatozoal concentration that much more important [1].

When using low-dose techniques, the insemination dose may range from 1-25 million spermatozoa [1]. Determination of spermatozoal concentration using the common artificial insemination technique requires measurement of concentration in the range of 0-800 x 10^6/mL; therefore, the determination method should be accurate over a broad range of concentrations. In addition, determination of concentration should ensure accurate identification and enumeration of spermatozoa while discriminating

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This thesis follows the style of Theriogenology.

spermatozoa from non-sperm material (i.e. debris, red blood cells, epithelial cells, etc.). The measuring instrument should also be convenient to use. Debris may artificially increase the concentration value measured, resulting in the insemination of fewer spermatozoa than intended. Contaminants can drastically affect the measurement of spermatozoal concentration if proper care is not taken to eliminate them. [2].

Methods for measuring stallion spermatozoal concentration include hemacytometry, photometric-based systems and flow cytometry. The hemacytometric method is regarded as the standard for cell counting according to World Health Organization guidelines [3]. It is the only method in which spermatozoa are directly identified in the counting process, but the set-up and counting procedures are laborious and time consuming. The flow cytometric method is not commonly used due to its high initial cost to purchase compared to other methods and extensive sample preparation time.

Two commonly used photometric systems are the Densimeter® (Animal Reproduction Systems, Chino, CA, USA) and the Spermacue® (Minitube of America, Inc., Verona, WI, USA). Both of these instruments are more affordable for commercial breeders than the flow cytometer and do not include the time consuming counting procedure of the hemacytometer. Previous studies [2, 4] indicate that the nature of photometric measurement renders these instruments more prone to error than a flow cytometer and hemacytometer and photometric systems are unable to accurately measure a spermatozoal sample in opaque semen extenders.

There is a need in the equine breeding industry for an instrument that has the affordability and ease of operation of the photometric systems without being prone to erroneous readings. Ideally, the instrument should have comparable accuracy (i.e. agreement) to a hemacytometer, but be capable of producing results more quickly and with the precision of a flow cytometer; without the large size and high cost of the latter instrument. An additional benefit would be an instrument that is able to accurately and precisely measure samples of spermatozoa that have been diluted in opaque semen extenders.

A new instrument, the NucleoCounter SP-10®, has been developed that measures spermatozoal concentration relying on the specific attachment of the fluorescent probe, propidium iodide, to spermatozoal DNA. Because of the specificity potential, it is able to analyze samples in an opaque medium, but is more compact and less expensive than a flow cytometer.

The objective of Experiment 1 was to evaluate the accuracy (correctness of results) and precision (repeatability) of the S100 dispenser mechanism. The objective of Experiment 2 was to evaluate the accuracy and precision of the NucleoCounter SP-100® for measurement of neat stallion spermatozoal concentration when samples are first inverted. The objective of Experiment 3 was to evaluate the accuracy and precision of the NucleoCounter SP-100® for measurement of neat stallion spermatozoal concentration when samples are frozen-thawed. The objective of Experiment 4 was to compare five different lengths of time for sonication of the sample prior to analysis and the objective of Experiment 5 was to evaluate the accuracy and precision of the

NucleoCounter SP-100® for measurement of neat stallion spermatozoal concentration when samples are sonicated. The objective of Experiment 6 was to evaluate the accuracy and precision of the NucleoCounter SP-100® for measurement of neat stallion spermatozoal concentration when samples are vortexed. The objective of Experiment 7 was to evaluate for use with stallion semen the company supplied dilution chart currently designed for bull semen. The objective of Experiment 8 was to evaluate the accuracy and precision of the NucleoCounter SP-100®, the Densimeter® and the Spermacue® concentration results when compared to the flow cytometer and hemacytometer for epididymal spermatozoa. The objective of Experiment 9 was to evaluate the accuracy and precision of the NucleoCounter SP-100, the Densimeter and the Spermacue when compared to the flow cytometer and the hemacytometer when measuring spermatozoa concentration of an ejaculate diluted in five different semen extenders. The objective of Experiment 10 was evaluate the accuracy and precision of the NucleoCounter SP-100, the Densimeter and the Spermacue when compared to the flow cytometer and the hemacytometer for neat stallion ejaculates over a range of concentrations as well as compare the NucleoCounter directly with the Densimeter and the Spermacue and the objective of Experiment 11was to evaluate the accuracy and precision of the NucleoCounter SP-100® when measuring spermatozoal concentration of 25 ejaculates diluted in INRA semen extender.

LITERATURE REVIEW

Stallion spermatozoal concentration is measured mainly for processing semen for use in artificial insemination. This method of breeding horses via human intervention carries with it some benefits over conventional breeding practices. In 1972, Pickett and Voss stated that collecting an ejaculate from a stallion, adding semen extenders and dividing the ejaculate among mares allows a greater number of mares to be inseminated with less stress on the stallion [5]. Artificial insemination practices have become widely used in the American horse breeding industry in the past 25-30 years. It was not previously thought to be a viable option for horse breeding operations, as stated by Anderson in 1935 [6].

Many advances have been made in this time period regarding the ideal number of spermatozoa to be used for insemination of mares and the most effective composition and volume of semen extenders. Pickett and Voss stated in 1972 that 500 million neat motile spermatozoa were most ideal for insemination but that as few as 100 million would suffice under ideal circumstances [5]. This was also the conclusion reached by Householder et al. in 1981 when using spermatozoa extended in milk-based semen extenders [7]. Demick et al. in 1976 found no difference between pregnancy rates in mares inseminated with 100 million progressively motile spermatozoa compared with 500 million progressively motile spermatozoa [8]. In 1997, Gahne et al. found no significant difference in pregnancy rates of mares inseminated with 300 million progressively motile spermatozoa compared to 500 million progressively motile

spermatozoa [9]. For spermatozoa frozen in 0.5 mL straws to be used for later insemination, Leipold et al. found in 1997 that each straw should contain 800 x 10^6 total spermatozoa and each insemination dose should contain approximately 320 x 10^6 spermatozoa [10].

In 1975, Pickett and Voss found higher pregnancy rates when using a cream-gel based semen extender versus an egg yolk based semen extender [11]. Kenney et al. in 1975 described the formulation for a milk-based semen extender containing antibiotics [12], which is now commonly used for extending stallion semen. The addition of small amounts (<5%) of seminal plasma to a spermatozoal sample also seems to aid in maintaining motility during both cooled storage and cryopreservation, according to Kareskoski and Katila in 2008 [13].

The findings of Sieme et al. in 2004 focused attention on achieving maximum fertility with the least amount of stress on the stallion throughout the breeding season. They noted that when stallions were collected more than once per day, the spermatozoal concentration and percentage of progressively motile spermatozoa were significantly higher for the first ejaculate than for subsequent ejaculates [14]. This is also the reasoning of using low-dose insemination techniques with both ejaculated and epididymal spermatozoa.

The developing research into low-dose insemination makes an accurate spermatozoal count essential. In 2000, Buchanan et al. achieved a 53% pregnancy rate with mares inseminated with 25 million spermatozoa and 35% with 5 million spermatozoa using a flexible insemination catheter [15]. In 2002, Brinsko et al.

reported a 67% pregnancy rate when mares were inseminated with 5 million progressively motile spermatozoa using a hysterscopic insemination technique and a 56% pregnancy rate when mares were inseminated with 5 million progressively motile spermatozoa using a transrectally-guided insemination technique [16]. Although studies differ on the ideal number of sperm to be deposited, there is general agreement that mares can become pregnant when deep-horn insemination techniques are used with 5-25 million spermatozoa [reviewed by 1]. Within this range, accuracy in counting spermatozoal numbers is particularly important in the lowest range.

The insemination of mares with frozen-thawed epididymal spermatozoa has also been found to achieve pregnancies by Stout et al. in 1999 [17]. Bruemmer describes a technique for harvesting and freezing epididymal spermatozoa that may be used for insemination in 2006. This method specifies surgical removal of the testes and associated epididymides using standard surgical castration procedures. The epididymides are then flushed in a retrograde fashion to collect approximately 15-20 billion spermatozoa [18]. This range of concentrations highlights the importance of accuracy in counting spermatozoa at the most concentrated ranges.

The hemacytometer has long been considered the standard for measuring spermatozoal concentration because the observer directly identifies the spermatozoa [3]. However, Christensen et al. suggested in 2005 that 300-400 cells must be counted to obtain a high level of accuracy [19]. It has also been suggested that multiple loadings and counts of the same sample are necessary to ensure accuracy and precision; as many as 10 counts have been suggested by Evenson et al. in 1993 [2], making this system

extremely time consuming and impractical for commercial use. Variations among laboratory techniques and technicians add to the inconsistencies of the hemacytometer as do differences in coverslips and hemacytometer chambers themselves [19]. Seaman et al. showed in 1996 that hemacytometers with counting chambers of different depths may show significantly different results given the same sample [20] and similar conclusions were also reached by Douglas-Hamilton et al. in 2005 [21]. Johnson et al. showed in 1996 that even the same hemacytometer may have differences between the two sides of the counting chamber [22]. The hemacytometer was noted to consistently overestimate spermatozoal concentration by Hu et al. in 2006 [23].

From a research perspective, the flow cytometer is also generally considered an accurate measurement system for spermatozoa [24, 25, 26]. Eustache [24] described the measurement technique that has been chosen for this project, whereby the spermatozoa are stained with the DNA-binding dye propidium iodide and counted via gating of light scatter to reduce the influence of other cells and debris in the sample on the concentration measurement. Prathlingham et al (2006), Hansen et al. (2002) and Christensen et al. (2004) agreed that this method may be more accurate and precise for measurement of spermatozoal concentration than the hemacytometer [25, 26, 27]. Prathlingham et al. compared the flow cytometric method with the hemacytometer and the spectrophotometer and found the flow cytometric method to be the most precise [25]. Hansen et al. validated the flow cytometric method for use with boar semen [26]. Christensen et al. validated the flow cytometric method for use boars, rams, rats, rabbits, humans and turkeys [27]. However, this instrument is not practical for use in

commercial breeding operations due to its cost when compared to other measurement systems such as a hemacytometer, which may be as low as $130 (Hausser Scientific, 2007). The requirement of using fluorospheres in the flow cytometer, which cost approximately $600 per 20mL bottle (Beckman-Coulter, 2007), also increases the cost of operation. The procedure for preparation of the sample is also laborious and time-consuming and this makes it impractical for a commercial operation.

Two commonly used instruments in the commercial equine breeding industry are the Densimeter® [28] and the Spermacue® [29], both of which measure spermatozoal concentration photometrically (measures the amount of light that is transmitted through a sample). Photometric measurement can be inaccurate due to its tendency to measure particles of contamination in addition to spermatozoa and has the disadvantage of being unable to accurately measure spermatozoal concentration in opaque semen extenders, according to Rigby et al. in 2001 [4]. Ideally, an optically clear media is required for the spermatozoa to be counted accurately. These types of instruments are used in commercial breeding operations because they are more affordable than a flow cytometer and produce results more quickly than a hemacytometer.

ChemoMetec A/S (Allerød, Denmark) has developed the NucleoCounter SP-100, a fluorescence-based instrument that measures spermatozoal concentration with a propidium iodide probe. The probe binds to the spermatozoal DNA after the cell membranes are permeabilized with a solution containing a detergent (S100 Reagent). Propidium iodide will then emit red fluorescence when excited with green light from the compact fluorescence microscope integrated in the instrument [30]. The dilution

procedure requires less initial preparation of the sample than for the flow cytometer and produces results more quickly than the hemacytometer. The NucleoCounter has been used in breeding operations for bulls, boars, poultry and several other species and has been shown to be accurate with boar semen when compared with the flow cytometer by Hansen et al. in 2006 [31].

MATERIALS AND METHODS

Experiment 1

A sample cup for each volume measurement was placed on an analytical balance (Sartorius CP64; Sartorius Mechatronics Corp., Edgewood, NY, USA) and the scale zeroed. Ten replicates of six different volumes of S100 Reagent (1, 2, 4, 5, 10 and 20 mL) were weighed using the company-recommended reagent dispenser (Dispensette® III; Brand, Germany) and air displacement pipettes (Pipetteman, Ranin Instrument, LLC, Oakland, CA, USA). A 1 mL pipetter was used for the 1 mL volume, a 5 mL pipetter was used for 2, 4 and 5 mL volumes, a 10 mL pipetter was used for the 10 mL volume and a 20 mL pipetter was used for the 20 mL volume. Volumes were chosen based on manufacturer recommendations for the dilution of bull semen with the NucleoCounter.

Experiment 2

Three ejaculates from a single stallion were collected in the following manner: each ejaculate was collected using an artificial vagina (Missouri-Model; Nasco, Ft. Atkinson, WI, USA) with an in-line micromesh nylon filter (Animal Reproduction Systems, Chino, CA, USA) to obtain gel-free semen. Immediately prior to semen collection, artificial vaginas were lubricated with approximately 3 mL of sterile non-spermicidal lubricant (Priority Care; First Priority, Inc., Elgin, IL, USA). Each ejaculate was then divided into two groups. Group 1 (n=10 separate dilutions) was prepared for analysis according to manufacturers' instructions: 10 μL of semen and subsequently 1mL of S100 Reagent (a diluents which contains Triton-X detergent to induce

membrane permeabilization in cells) was aliquoted into a sample cup after wiping the outside of the pipette tip with a KimWipe® (Kimberly-Clark, Dallas, TX, USA) to remove any additional ejaculate. Approximately 60 uL of the sample was then drawn up into a disposable SP-1 cassette laced with propidium iodide to stain the DNA of the spermatozoa. This cassette was then inserted into the instrument and analysis was initiated, using the manufacturer-supplied SemenView® software (ChemoMetec A/S, Allerød, Denmark). Group 2 (n=10 separate dilutions) was prepared by aliquoting 1mL of the S100 Reagent, then 10 µL of semen The sample vial was sealed and inverted (10x), and a sample was loaded into manufacturer-provided sample cassette and analyzed. After the sample was thoroughly mixed by inversion, approximately 60 uL of the sample was then drawn up into a disposable SP-1 cassette laced with propidium iodide to stain the DNA of the spermatozoa. This cassette was then inserted into the instrument and analysis was initiated, using the manufacturer-supplied SemenView® software (ChemoMetec A/S, Allerød, Denmark).

Experiment 3

Three gel-free ejaculates were obtained from a single stallion using the method described in Experiment 2 and divided into two groups. Group 1 (n=10) was analyzed immediately after collection (fresh) on the NucleoCounter using the method described in the previous experiment after sonicating the sample for 10 seconds. Group 2 (n=10) was frozen raw at -80°C, then thawed, sonicated for 10 seconds and then analyzed on the NucleoCounter using the same procedure as described for the group of fresh ejaculates.

Experiment 4

Three gel-free ejaculates from a single stallion were obtained according to the method described in Experiment 2 and were divided into five groups and analyzed on the NucleoCounter according to the method described in Experiment 2. All groups were inverted (10x) prior to analysis. Group 1 (n=10) was not sonicated before analysis (inverted only). Groups 2-5 (each n=10) were sonicated at 5, 10, 15 and 20 seconds, respectively, prior to analysis.

Experiment 5

Three ejaculates from a single stallion were obtained according to the method described in Experiment 2 and split into two groups. Group 1 samples (n=10) were inverted 10x and analyzed immediately on the NucleoCounter. Group 2 samples (n=10) was sonicated for 10 seconds prior to dilution to minimize spermatozoa clumping and then analyzed accordingly on the NucleoCounter. A dilution factor of 101was used for both groups of samples (10 μL of semen + 1mL of S100 Reagent).

Experiment 6

Five ejaculates were collected and frozen according to the method described in Experiment 2. Each ejaculate was divided into two groups and each sonicated for 10 seconds to reduce spermatozoa agglutination. Group 1 (n=10) was vortexed for 10 seconds after initial dilution for the NucleoCounter and an inverted (10x) before analysis; three replicates for each. Group 2 (n=10) was not vortexed but inverted (10x) only; three replicates for each. A dilution factor of 101was used for both groups of samples (10 μL of semen + 1mL of S100 Reagent).

Experiment 7

Five individual trials were conducted; each utilized 15 concentrations from 5-1000 x 10^6/mL (5, 10, 25, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000 x10^6/mL). Samples with spermatozoal concentrations of >500 x 10^6/mL were from harvested epididymal spermatozoa at an initial concentration of 4 x 10^9/mL and diluted accordingly. The flow cytometer was used to confirm that samples had been diluted to the correct concentration range. Three replicates of an ejaculate in each concentration range were analyzed on the NucleoCounter using all possible dilution factors. All samples were vortexed (10 seconds) and inverted (10x) prior to analysis. Coefficients of variation were calculated for each set of replicates. The margins of each ideal range were chosen when the coefficients of variation for NucleoCounter replicates exceeded 5%; the margins of each acceptable range were chosen when coefficients of variation exceeded 10% and the margins of each possible range where chosen when coefficients of variation exceeded 15%. Any coefficient of variation that exceeded 15% was denoted as a dilution factor range that was not recommended. Some dilution factors would not register for spermatozoal concentrations well outside their recommended range; those margins were also noted.

Experiment 8

One epididymal spermatozoal sample was obtained by retrograde flushing, using the technique first described by Carey in 2004 [32]. The sample was diluted with 0.9% NaCl (Hospira, Inc., Lake Forest, IL, USA) to a concentration of 1x10^9/mL (using the hemacytometer). Serial dilutions of this sample were performed to obtain concentrations

of $500x10^6$/mL, $250x10^6$/mL, $125x10^6$/mL, $63x10^6$/mL, $31x10^6$/mL, $16x10^6$/mL and $8x10^6$/mL. Each dilution was made with one of two diluents: NaCl or seminal plasma from one stallion and was frozen at -80ºC in a 4mL cryogenic vial (Corning cryogenic vial, Corning, Inc., Corning, NY, USA). Samples were thawed in a 37ºC water bath (Model 586A, Animal Reproduction Systems, Chino, CA, USA) and sonicated (Sonic Dismembrater F60, Fisher Scientific, Waltham, MA, USA) at 10 watts for 30 seconds to reduce spermatozoal agglutination. Spermatozoal concentrations were then determined using the NucleoCounter, Densimeter, Spermacue, flow cytometer and hemacytometer.

All samples were transferred and diluted using positive displacement pipettes (Microman, Ranin Instrument LLC, Oakland, CA, USA) and each samples was vortexed (10 seconds) and inverted (10x) prior to analysis. Each instrument (flow cytometer, hemacytometer, NucleoCounter, Densimeter and Spermacue) was operated by a different technician, and the same technician for each instrument was used for each count.

Two replicates of each sample were measured on the Spermacue (Minitube of America, Inc., Verona, WI, USA) according to manufacturer instructions which included diluting (1:1) all samples with an initial concentration of >450 x 10^6/mL with sodium citrate (Sigma-Aldrich, St. Louis, MO, USA). Aliquots (25 µL) of the raw ejaculate were placed in a micro-cuvette specific for the Spermacue and inserted into the instrument and analyzed.

Two replicates of each sample were measured on the Densimeter 591a (Animal Reproduction Systems, Chino, CA, USA). An aliquot (180 µL) of the raw ejaculate was

drawn into a positive displacement pipette (Animal Reproduction Systems, Chino, CA, USA) and dispensed into a plastic vial filled with 3.42 mL 10% buffered formalin (zeroed before addition of semen) to immobilize the spermatozoa for analysis. The vial was capped and inverted (10x) to disperse the cells evenly, then placed in the instrument for analysis.

Two replicates of each sample were measured on a hemacytometer (improved Neubauer, Hausser Scientific, Horsham, PA, USA). A 10 µL aliquot of the raw semen was diluted in 990 µL of de-ionized water, and 6 uL of the diluent was pipetted into the counting chambers and placed in humidified chamber for 15 minutes prior to being counted. Mean values of the replicates was used for statistical comparisons. Samples for which the concentration was > 200 x 10^6/mL were diluted (1:1) further to avoiding count more than 200 total cells per hemacytometer chamber.

Flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA) was used to determine the concentration of all samples (two replicates each), using a previously reported technique [24] with the modification of using distilled water instead of PBS that was used in previous studies to ensure the most optically clear sample possible, so that any particulate matter present in the PBS would not interfere with the analysis. The method used fluorospheres as a counting standard instead of actual semen to ensure the least amount of variation possible for the measurement standard. The initial dilution of the sample to 5 million spermatozoa had been previously noted to be the most appropriate concentration range for this flow cytometer procedure. Once diluted, 100 µL of the sample and 100 µL of fluorospheres (FlowCount; Beckman

Coulter, Fullerton, CA, USA) were added to a 10% Triton-X solution as well as 10 µL of propidium iodide at a fixed concentration of 0.990 µg/mL. Each sample incubated for approximately 5 minutes at room temperature after the addition of propidium iodide to ensure thorough staining of the cells before analysis. The data were analyzed using WinList Flow Cytometry Software (Verity Software House, Inc., Topsham, ME, USA). The spermatozoal population of each sample was gated on both side and forward scatter plots [2].

Two replicates of spermatozoal concentrations were measured using the NucleoCounter SP-100® (ChemoMetec A/S, Allerød, Denmark) according to manufacturer instructions [30] with modifications suggested from Experiment 2 (manually invert vial 10x before analysis.

Experiment 9

One ejaculate was collected using the method described in Experiment 2 and was diluted 1:1 in each of five different semen extenders: LE (Lactose EDTA E-Z Freezin, Animal Reproduction Systems, Chino, CA, USA), MFR5 (Modified French E-Z Freezin, Animal Reproduction Systems, Chino, CA, USA), CST (E-Z Mixin, Animal Reproduction Systems, Chino, CA, USA), INRA96 (IMV Technologies, L'Aigle, France) and NaCl. Each of these samples was serially diluted 1:1 to obtain a total of seven different concentrations for each semen extender. These samples were then frozen in 5mL cryogenic vials at -80°C. Prior to analysis, samples were thawed in a 37^{o}C water bath (Model 586A, Animal Reproduction Systems, Chino, CA, USA) for 10 minutes and sonicated (Sonic Dismembrater F60, Fisher Scientific, Waltham, MA, USA) at 10 watts

for 30 seconds to reduce spermatozoal agglutination. Each sample was analyzed on the NucleoCounter, the flow cytometer, the hemacytometer, the Densimeter and the Spermacue using the methods described previously.

Experiment 10

Ejaculates (n=120) from stallions of various breeds and ages in southeast Texas were collected as described in Experiment 2. One mL aliquots of each of the gel-free ejaculates were collected in cryogenic vials (Corning cryogenic vial, Corning, Inc., Corning, NY, USA) and frozen at -80^{o}C until analyzed. Prior to analysis, samples were thawed in a 37^{o}C water bath (Model 586A, Animal Reproduction Systems, Chino, CA, USA) for 10 minutes and sonicated (Sonic Dismembrater F60, Fisher Scientific, Waltham, MA, USA) at 10 watts for 10 seconds to reduce sperm agglutination.

Ejaculate concentrations ranged from 5 - 800 x 10^{6}/mL. Each instrument (flow cytometer, hemacytometer, NucleoCounter, Densimeter and Spermacue) was operated by a different technician, and the same technician for each instrument was used for each count.

Three replicates of each sample were measured on the Spermacue® (Minitube of America, Inc., Verona, WI, USA) and Densimeter® 591a (Animal Reproduction Systems, Chino, CA, USA). All samples with initial spermatozoal concentrations >300 x 10^{6}/mL on the Densimeter and >450x10^{6}/mL on the Spermacue were diluted (1:1) and re-analyzed. Mean values of the diluted replicates were used for statistical comparisons.

Three replicates (each replicate=mean of two chambers) were measured on the hemacytometer (improved Neubauer, Hausser Scientific, Horsham, PA, USA). Mean values of the replicates were used for statistical comparisons.

Flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA) was used to determine the concentration of all samples in triplicate, using the same method described in Experiment 8. The initial dilution of the sample to 5 million spermatozoa was estimated based on NucleoCounter measurements. Once diluted, 100 µL of the sample and 100 µL of fluorospheres (FlowCount; Beckman Coulter, Fullerton, CA, USA) were added to a 10% Triton-X solution as well as 10 µL of propidium iodide at a fixed concentration of 1.029 µg/mL. Mean values of three replicates were used for statistical comparisons.

Spermatozoal concentrations were measured using the NucleoCounter SP-100® (ChemoMetec A/S, Allerød, Denmark) according to the method described in Experiment 8. Mean values of three replicates were used for statistical comparisons.

Experiment 11

Twenty five samples from six different stallions that had been diluted in milk-based semen extenders and having post-dilution spermatozoal concentrations (using the hemacytometer) ranging from 50-100 x 10^6/mL were analyzed. Collection and storage of these samples was the same as stated previously. These samples were evaluated using the NucloCounter SP-100®, a hemocytometer and a flow cytometer in order to evaluate the ability of the NucleoCounter to analyze samples in a non-optically clear media. The methods for spermatozoa enumeration were the same as described in Experiment 8.

Data was analyzed statistically in Experiments 1-3 and 5-6 using paired samples t-tests; for Experiment 4 using one-way repeated measures ANOVA and Student-Neuman-Keuls multiple pairwise comparisons; for Experiment 7 using coefficient of variation calculations and for Experiments 8-11 using 1) regression analysis comparing intercept, slope and coefficient of determination, 2) the agreement of two instruments according to the Bland and Altman method [33], whereby the difference in values between two instruments was plotted on the y-axis against the mean of those values on the x-axis and 3) the percentage of deviation from a standard instrument according to a modified Bland and Altman method [33], whereby the percentage of difference in values between two instruments divided by the mean of the same two values was plotted on the y-axis against the mean value of two instruments on the x-axis.

RESULTS

Experiment 1

No difference among the means ($p>0.05$) was detected for volumes of 2 mL and 5 mL , but differences in means ($p<0.05$) were found at the 1 mL, 4 mL, 10 mL and 20 mL volumes (Table 1).

Table 1

Comparison of manufacturer-supplied reagent dispenser and air displacement pipettes at six volumes of S100 Reagent

Volume (mL)	Dispenser mean (g)	Coefficient of variation (%)	Pipette mean (g)	Coefficient of variation (%)	p-value
1	1.028	1.26	0.995	<0.01	<0.001
2	1.998	<0.01	2.003	<0.01	0.098
4	4.041	<0.01	4.011	<0.01	<0.001
5	5.02	<0.01	5.018	<0.01	0.55
10	10.101	<0.01	10.004	<0.01	<0.001
20	20.198	<0.01	19.94	<0.01	<0.001

Experiment 2

There was no difference ($p>0.05$) between the means of inverted and non-inverted samples (Table 2). The non-inverted samples had a higher coefficient of variation, standard error of the mean and standard deviation than the inverted samples.

Table 2

Comparison of spermatozoal concentration of neat ejaculates prepared in accordance with the NucleoCounter User's Guide versus neat ejaculates inverted 10x prior to analysis

Treatment	N	Mean (x 10^6)	Standard deviation	Standard error of the mean	Coefficient of variation: 3 replicates (%)	p-value
Inverted	30	194.7	7.63	1.4	3.92	0.248
Non-Inverted	30	188.8	29.2	5.33	15.47	

Experiment 3

Spermatozoal concentration means were different ($p<0.05$) for fresh and frozen-thawed samples (Table 3). Means of ejaculates 1 and 2 tended to be more different ($p<0.066$) than ejaculate 3. Fresh ejaculate mean values were lower than frozen-thawed ejaculate values.

Table 3

Comparison of spermatozoal concentration of fresh versus frozen-thawed neat ejaculates

Ejaculate	N	Mean (x 10^6)	Standard deviation	Coefficient of variation: 3 replicates (%)	p-value
1: Fresh	10	350.0	18.1	5.17	<0.001
1: Frozen	10	387.1	19.6	5.06	
2: Fresh	10	174.3	8.2	4.70	<0.001
2: Frozen	10	199.8	6.4	3.20	
3: Fresh	10	123.2	4.7	3.56	0.066
3: Frozen	10	126.7	4.9	3.87	

Experiment 4

Differences ($p<0.05$) were found among sonication times of samples that had been sonicated less than 5 seconds (Table 4). Comparisons of samples sonicated 10 seconds or more showed no differences ($p>0.05$).

Table 4

Multiple pairwise comparison of spermatozoal concentration of neat ejaculates sonicated for five different lengths of time

Comparison	Mean difference (x 10^6)	p-value
No sonication vs. 5s	36.78	<0.001
No sonication vs. 10s	45.29	<0.001
No sonication vs. 15s	47.43	<0.001
No sonication vs. 20s	44.57	<0.001
5s vs. 10s	8.51	0.002
5s vs. 15s	10.65	<0.001
5s vs. 20s	7.79	0.002
10s vs. 15s	2.14	0.370
10s vs. 20s	0.71	0.765
15s vs. 20s	2.85	0.456

Experiment 5

Mean sperm concentration values for sonicated samples were lower than non-sonicated samples ($p<0.05$) (Table 5). Standard deviation was lower for sonicated samples than for non-sonicated samples.

Table 5

Comparison of spermatozoal concentration of sonicated (10 seconds) neat ejaculates versus inverted only ejaculates

Ejaculate	N	Mean (x 10^6)	Coefficient of variation: 3 replicates (%)	p-value
1: Non-sonicated	10	428.9	4.66	
1: Sonicated	10	350	5.17	<0.001
2: Non-sonicated	10	220.1	4.82	
2: Sonicated	10	174.3	4.7	<0.001
3: Non-sonicated	10	140.7	6.61	
3: Sonicated	10	123.2	3.81	0.001

Experiment 6

There was no difference in means ($p>0.05$) between vortexed and non-vortexed samples (Table 6). Standard deviation and coefficient of variance were lower for vortexed samples.

Table 6

Comparison of spermatozoal concentration of inverted and vortexed neat ejaculates versus inverted only neat ejaculates

Ejaculate	N	Mean (x 10^6)	Coefficient of variation: 3 replicates (%)	p-value
1: Inverted	10	135.2	4.37	0.208
1: Vortexed	10	136.9	6.40	

Table 6

Continued

Ejaculate	N	Mean ($x10^6$)	Coefficient of variation: 3 replicates (%)	p-value
2: Inverted	10	210.1	6	0.615
2: Vortexed	10	210.6	3.9	
3: Inverted	10	166.8	2.6	0.138
3: Vortexed	10	175.2	2.2	
4: Inverted	10	230.7	1.24	0.552
4: Vortexed	10	223.1	3.98	
5: Inverted	10	181.2	1.33	0.561
5: Vortexed	10	176.2	3.95	

Experiment 7

The company-supplied dilution chart was useable for the measurement of stallion spermatozoa. The original bull chart (Figure 1) and the reformatted stallion chart (Figure 2) were similar when overlaid, but stallion chart ranges were wider at all possible dilution factors.

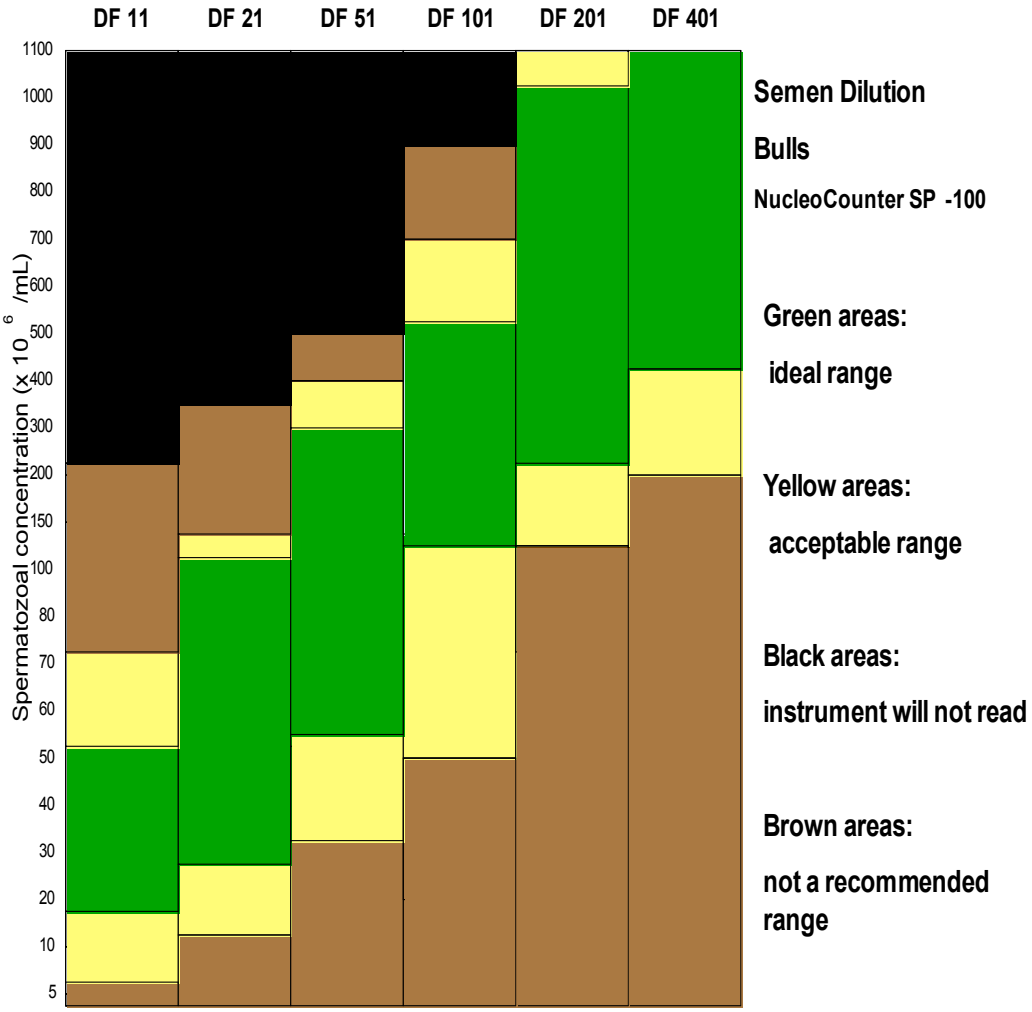

Figure 1. Company-supplied bull semen dilution chart

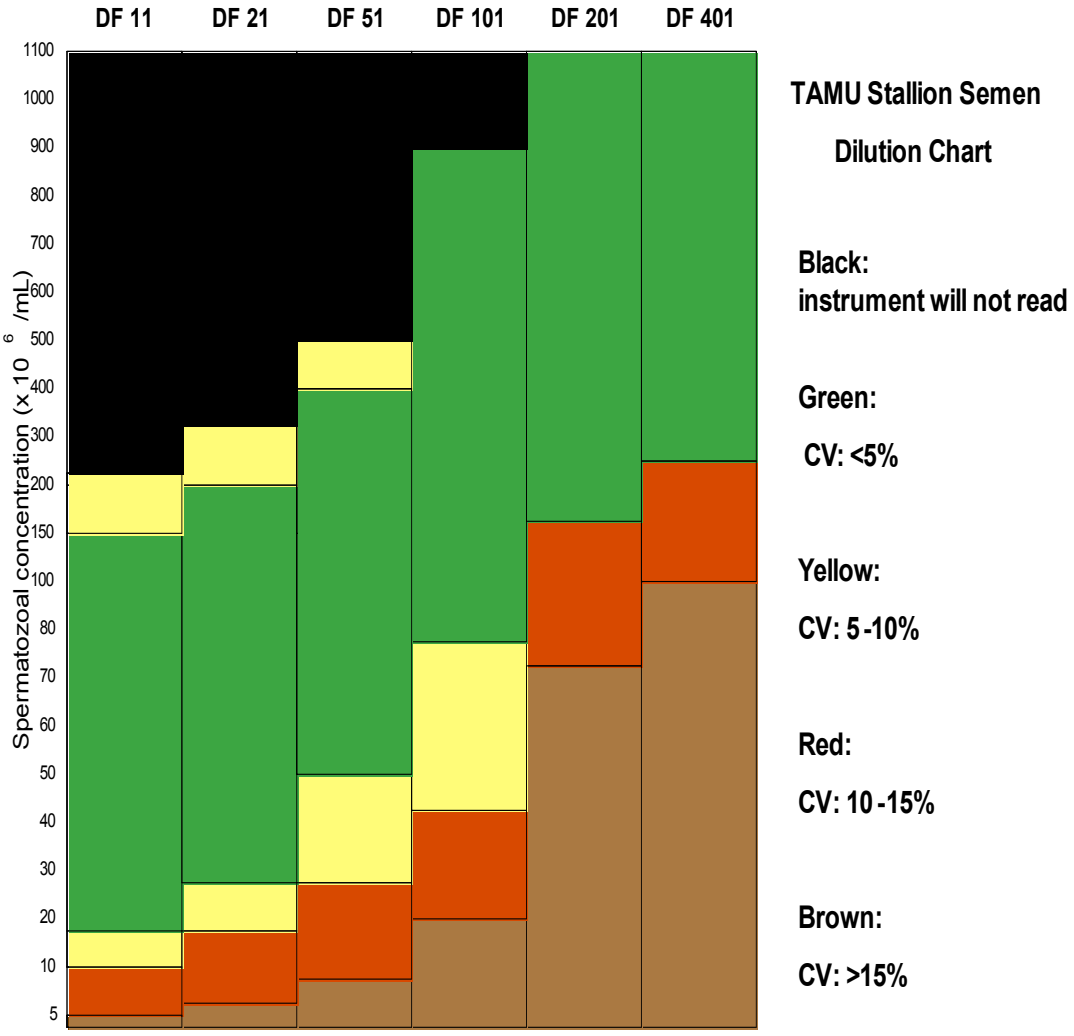

Figure 2. Modified stallion semen dilution chart

Experiment 8

Regression analysis

A combined regression plot for the NucleoCounter, flow cytometer, hemacytometer, Densimeter and Spermacue was generated for each group of samples; those diluted in NaCl (Figure 3) and those diluted in seminal plasma (Figure 4). The

data is summarized in Table 7. When epididymal spermatozoa was diluted with NaCl, the NucleoCounter, flow cytometer and hemacytometer regression plots had slopes near 1 (line of equality). The y-intercepts for all methods were close to zero. All R^2 values for both diluents were 0.99.

When epididymal spermatozoa were diluted in seminal plasma, the combined regression plot (Figure 4) showed that the NucleoCounter and flow cytometer had slopes near 1. The y-intercepts indicated that only the Spermacue showed an underestimation ($9.42x10^6$/mL). The NucleoCounter, flow cytometer, hemacytometer and Densimeter showed mean overestimations across all concentration ranges of $18.7x10^6$/mL, $7.2x10^6$/mL, $12.9x10^6$/mL and $14.6x10^6$/mL, respectively.

Table 7

Regression values for the NucleoCounter (NC), Densimeter (DENS) and the Spermacue (SC), the flow cytometer (FC) and the hemacytometer (HEM) for epididymal spermatozoa diluted in NaCl and seminal plasma (SP)

Diluent	Instrument	Slope	y-Intercept
NaCl	FC	1.06	-3.7
	HEM	1.02	-6.6
	NC	1.03	3.2
	DENS	0.92	-7.0
	SC	0.75	-0.2
SP	FC	1.00	7.2
	HEM	0.89	12.9
	NC	1.02	18.7
	DENS	0.75	14.6
	SC	1.17	-9.4

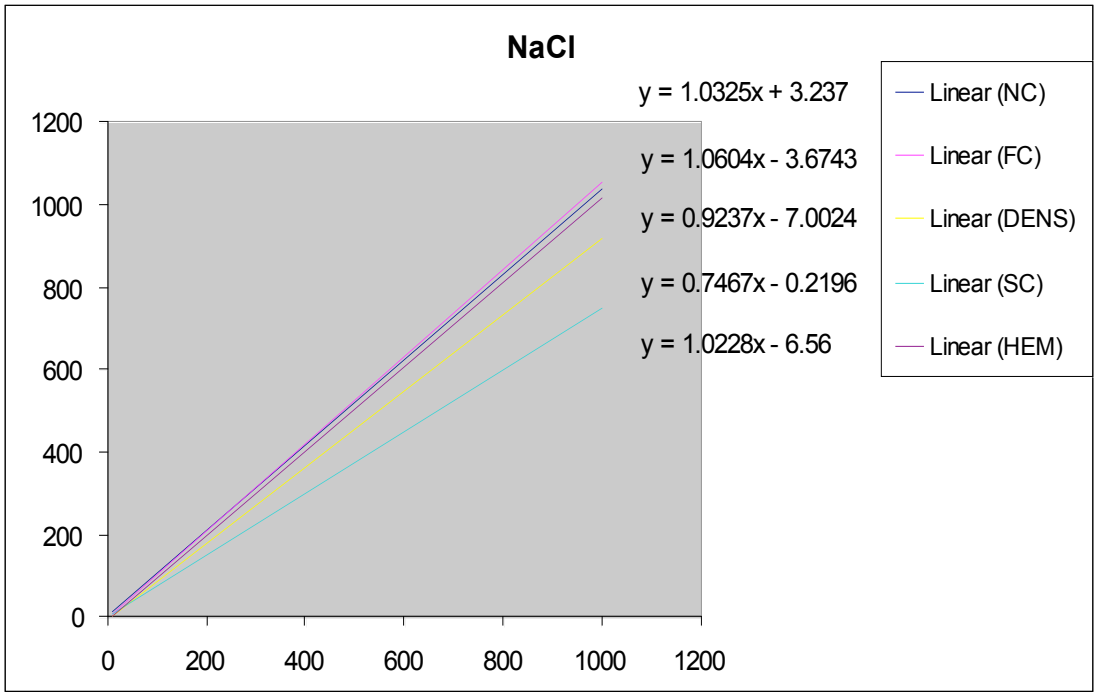

Figure 3. Regression trend lines for NucleoCounter (NC), flow cytometer (FC), hemacytometer (HEM), Densimeter (DENS) and Spermacue (SC) for epididymal spermatozoa diluted in NaCl (spermatozoal concentrations x 10^6/mL)

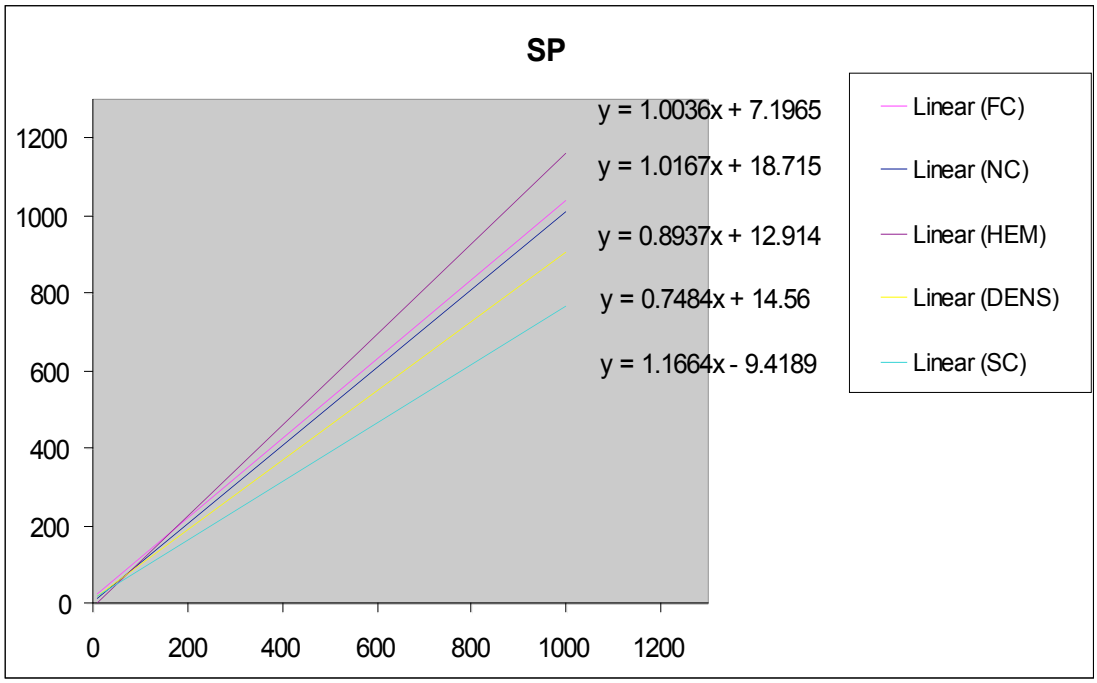

Figure 4. Regression trend lines for NucleoCounter (NC), flow cytometer (FC), hemacytometer (HEM), Densimeter (DENS) and Spermacue (SC) for epididymal spermatozoa diluted in seminal plasma (SP) (spermatozoal concentrations x 10^6/mL)

Coefficient of variation

The coefficients of variation for the flow cytometer, NucleoCounter, hemacytometer and Densimeter were all below 10%, but the hemacytometer was the least repeatable of these. The coefficient of variation for the Spermacue was greater than 10% when diluted in seminal plasma and less than 10% when diluted in NaCl (Table 8).

Table 8

Within-sample repeatability (coefficient of variation) for measurement of epididymal spermatozoal concentration using the flow cytometer (FC), hemacytometer (HEM), NucleoCounter (NC), Spermacue (SC), and Densimeter (DENS) diluted with NaCl and seminal plasma (SP)

Diluent	Instrument	Coefficient of variation: 3 replicates (%)
NaCl	FC	5.07
SP		2.94
NaCl	HEM	9.44
SP		8.73
NaCl	NC	1.46
SP		2.78
NaCl	DENS	6.19
SP		4.09
NaCl	SC	0.9
SP		41.76

Original and modified Bland and Altman plots

The hemacytometer showed the lowest percentage of difference from the flow cytometer when spermatozoa were diluted in NaCl. The NucleoCounter also showed less percentage of difference from the flow cytometer and the lowest observed actual number difference when spermatozoa were diluted in NaCl. The NucleoCounter showed the most similar percentage of deviation from the hemacytometer regardless of diluent, but showed a higher actual number difference with the NaCl diluent. The Densimeter showed more percentage and actual number agreement with the NucleoCounter than did the Spermacue.

The Densimeter showed similar agreement with the flow cytometer and hemacytometer, regardless of diluent. The percentage of deviation from both the flow cytometer and the hemacytometer was less when spermatozoa were diluted in NaCl. The Spermacue also showed similar agreement with both the flow cytometer and hemacytometer regardless of diluent. The Spermacue showed significantly greater percentage of deviation from the flow cytometer and hemacytometer when spermatozoa were diluted in NaCl. All data is summarized in Table 9.

Table 9

Mean percentage (modified Bland and Altman) and actual differences (Bland and Altman) for the NucleoCounter (NC), Densimeter (DENS), Spermacue (SC), flow cytometer (FC) and hemacytometer (HEM) for epididymal spermatozoa diluted in NaCl and seminal plasma (SP) (8-100 $x10^6$/mL concentration range)

Instrument Comparison	Diluent	Mean Difference (%)	Mean Difference (abs value x 10^6)
NC-FC	NaCl	7.17	7.20
	SP	12.31	15.23
NC-HEM	NaCl	9.91	12.23
	SP	9.51	26.50
NC-DENS	NaCl	23.56	39.50
	SP	28.1	30.74
NC-SC	NaCl	90.44	74.65
	SP	57.98	59.70
DENS-FC	NaCl	23.52	41.05
	SP	28.54	42.57
DENS-HEM	NaCl	21.97	29.24
	SP	24.96	53.03
SC-FC	NaCl	90.36	76.02
	SP	61.45	73.53
SC-HEM	NaCl	85.82	62.44
	SP	59.73	84.62
FC-HEM	NaCl	5.94	13.73
	SP	11.71	36.14

NucleoCounter Bland and Altman comparisons

When compared to the flow cytometer, the NucleoCounter showed more agreement when spermatozoa are diluted in NaCl (Figures 5 and 6). When compared to the hemacytometer, the NucleoCounter showed a similar percentage of difference,

regardless of whether the spermatozoa were diluted in NaCl (Figures 7 and 8) or seminal plasma, but showed a lower actual number difference when samples were diluted in seminal plasma. The NucleoCounter showed less agreement with the flow cytometer when spermatozoa were diluted in seminal plasma (Figures 9 and 10).

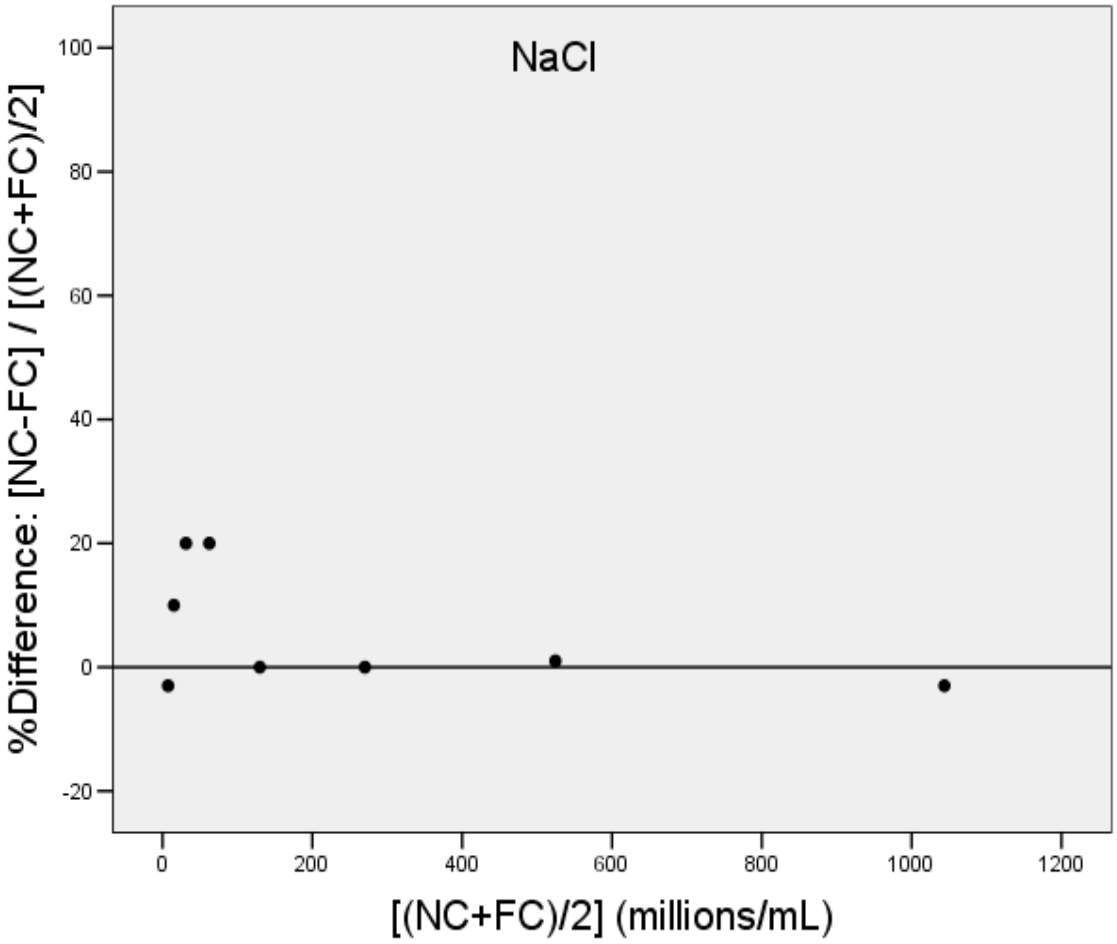

Figure 5. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the NucleoCounter (NC) and flow cytometer (FC) to the mean of the NucleoCounter (NC) and flow cytometer (FC) when spermatozoa are diluted in NaCl

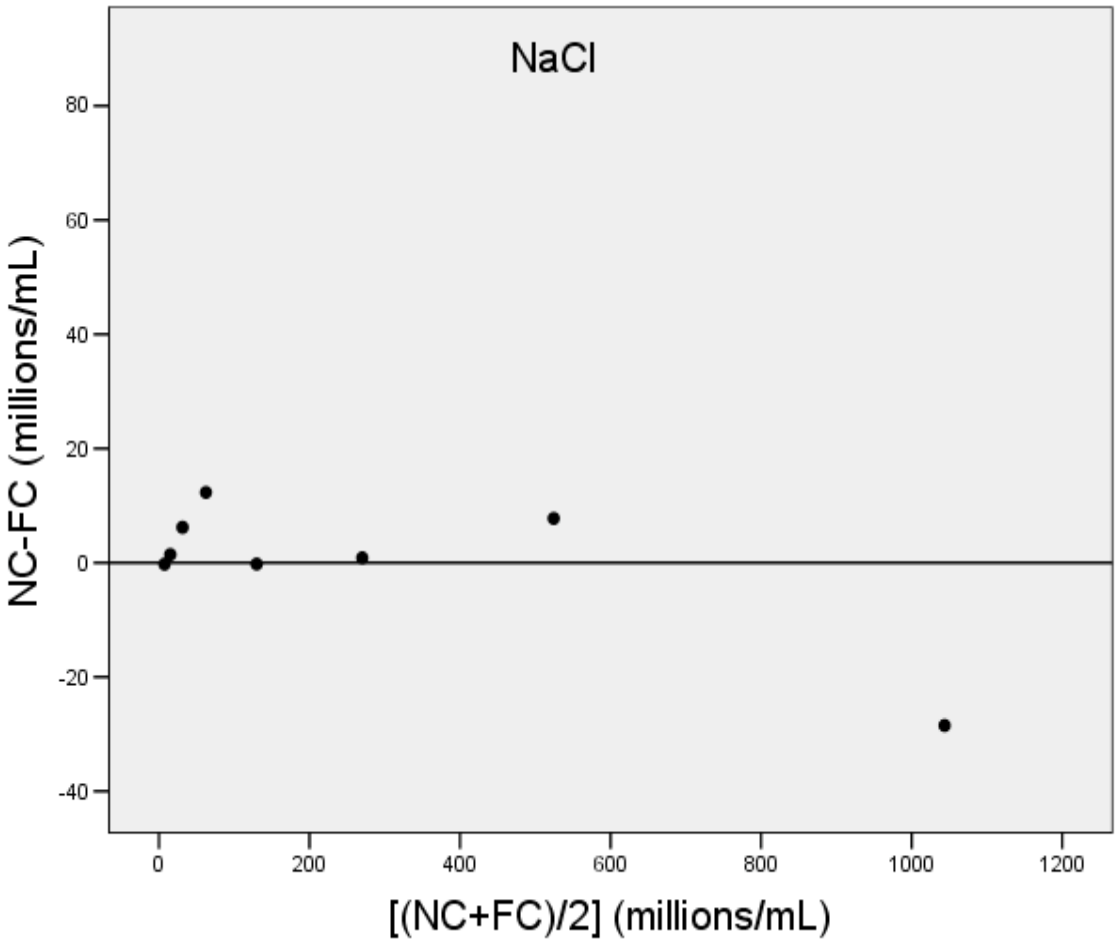

Figure 6. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the NucleoCounter (NC) and the flow cytometer (FC) to the mean of the NucleoCounter (NC) and the flow cytometer (FC)) when spermatozoa are diluted in NaCl

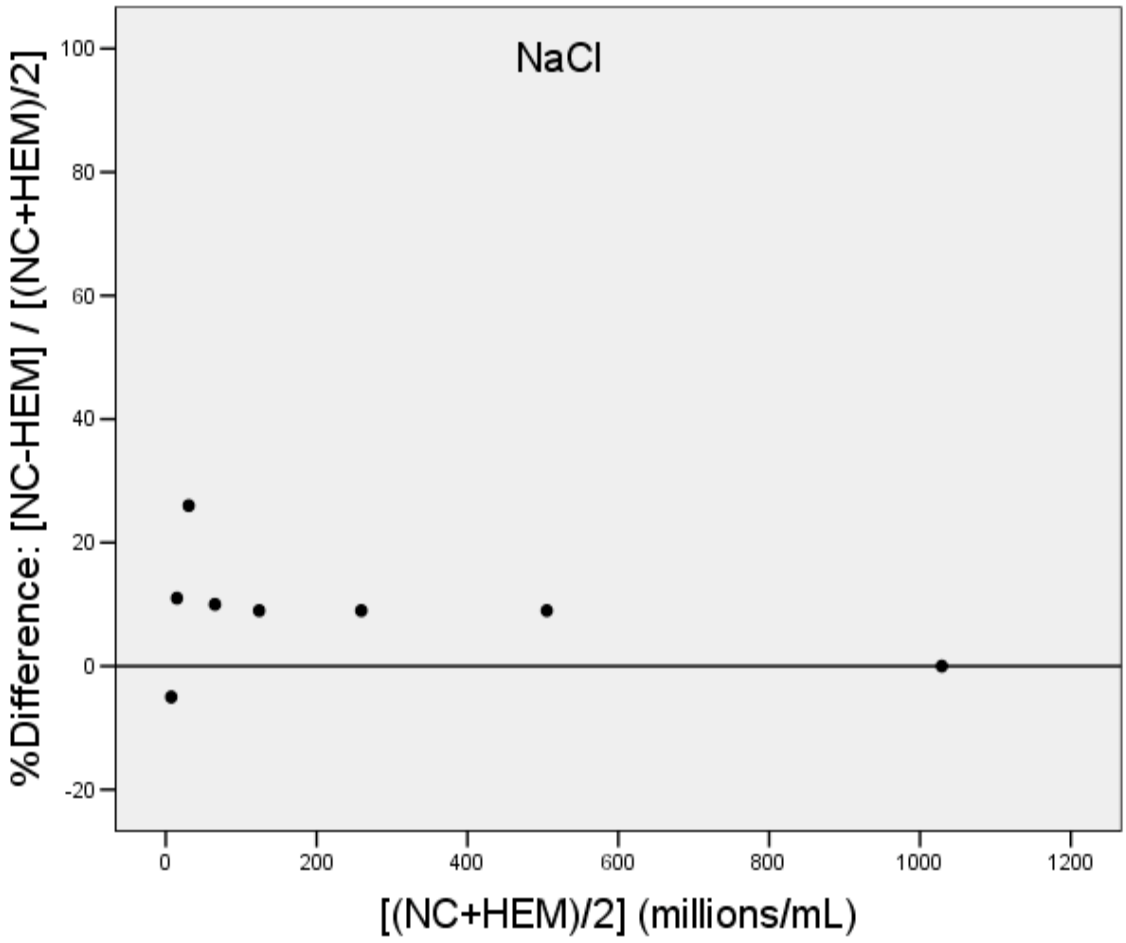

Figure 7. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the NucleoCounter (NC) and hemacytometer (HEM) to the mean of the NucleoCounter (NC) and hemacytometer (HEM) when spermatozoa are diluted in NaCl

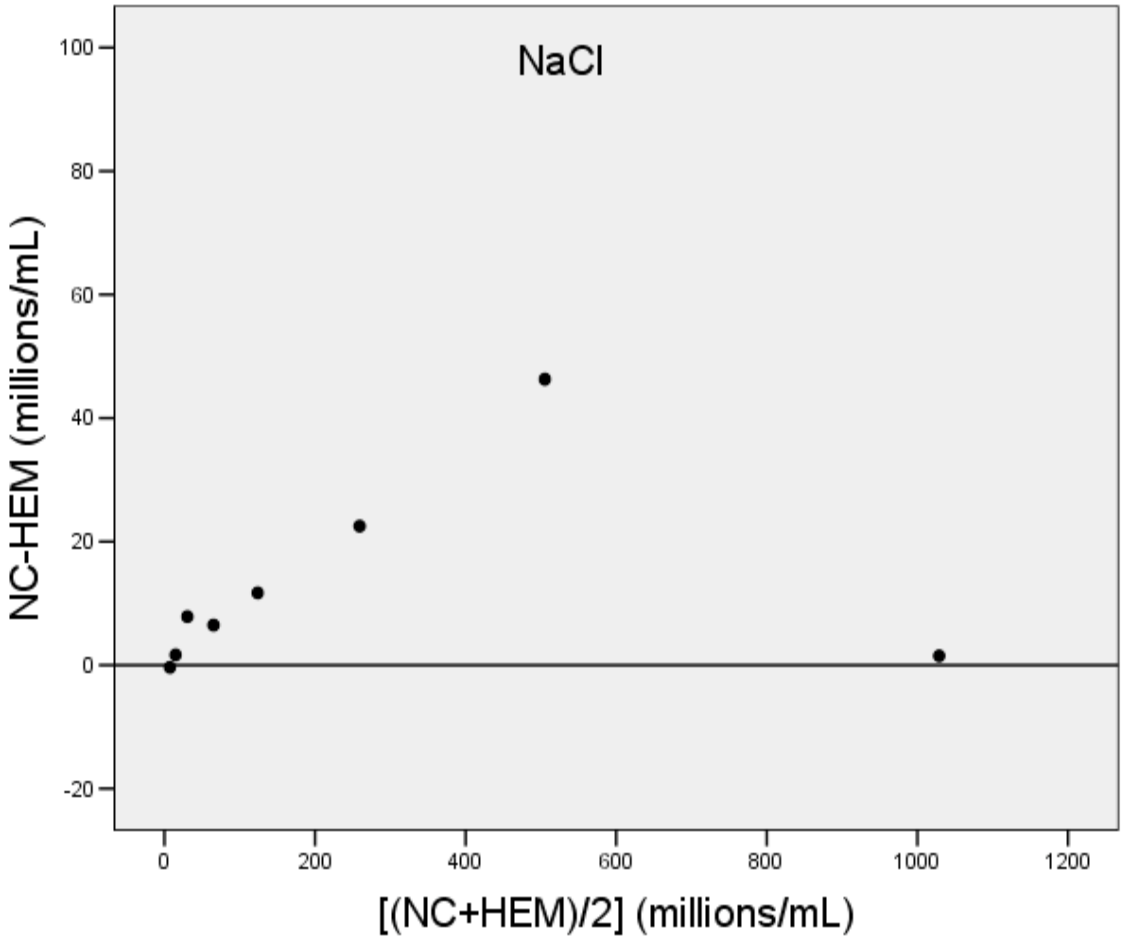

Figure 8. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the NucleoCounter (NC) and the hemacytometer (HEM) to the mean of the NucleoCounter (NC) and the hemacytometer (HEM) when spermatozoa are diluted in NaCl

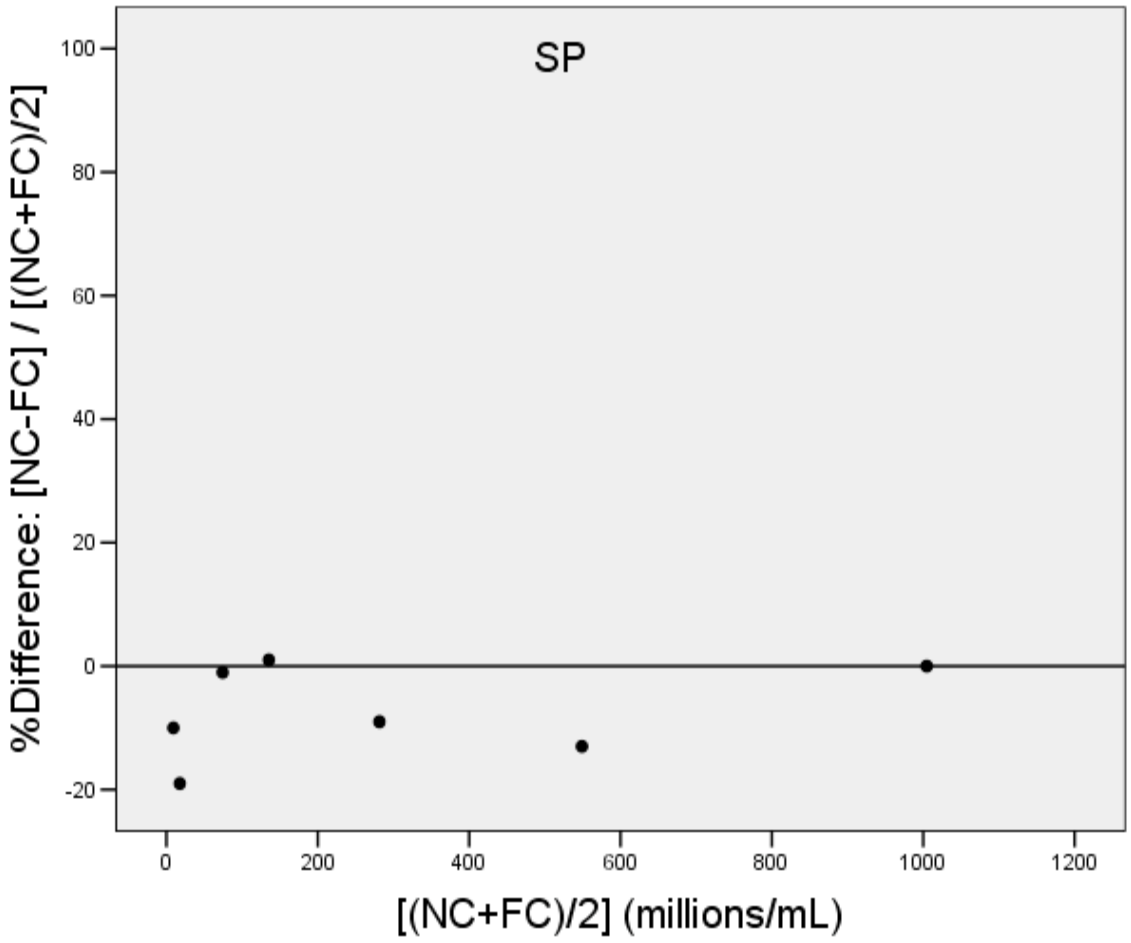

Figure 9. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the NucleoCounter (NC) and flow cytometer (FC) to the mean of the NucleoCounter (NC) and flow cytometer (FC) when spermatozoa are diluted in seminal plasma (SP)

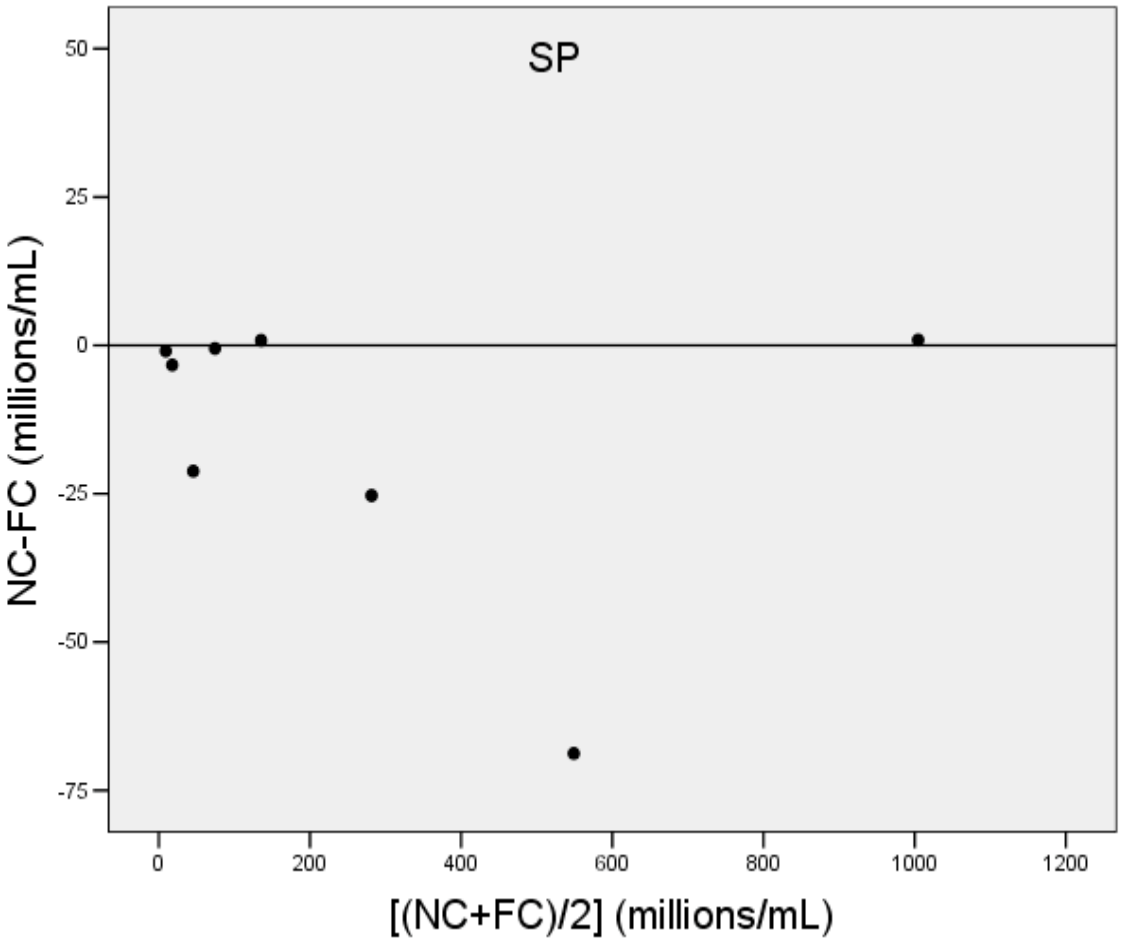

Figure 10. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the NucleoCounter (NC) and the flow cytometer (FC) to the mean of the NucleoCounter (NC) and the flow cytometer (FC)) when spermatozoa are diluted in seminal plasma (SP)

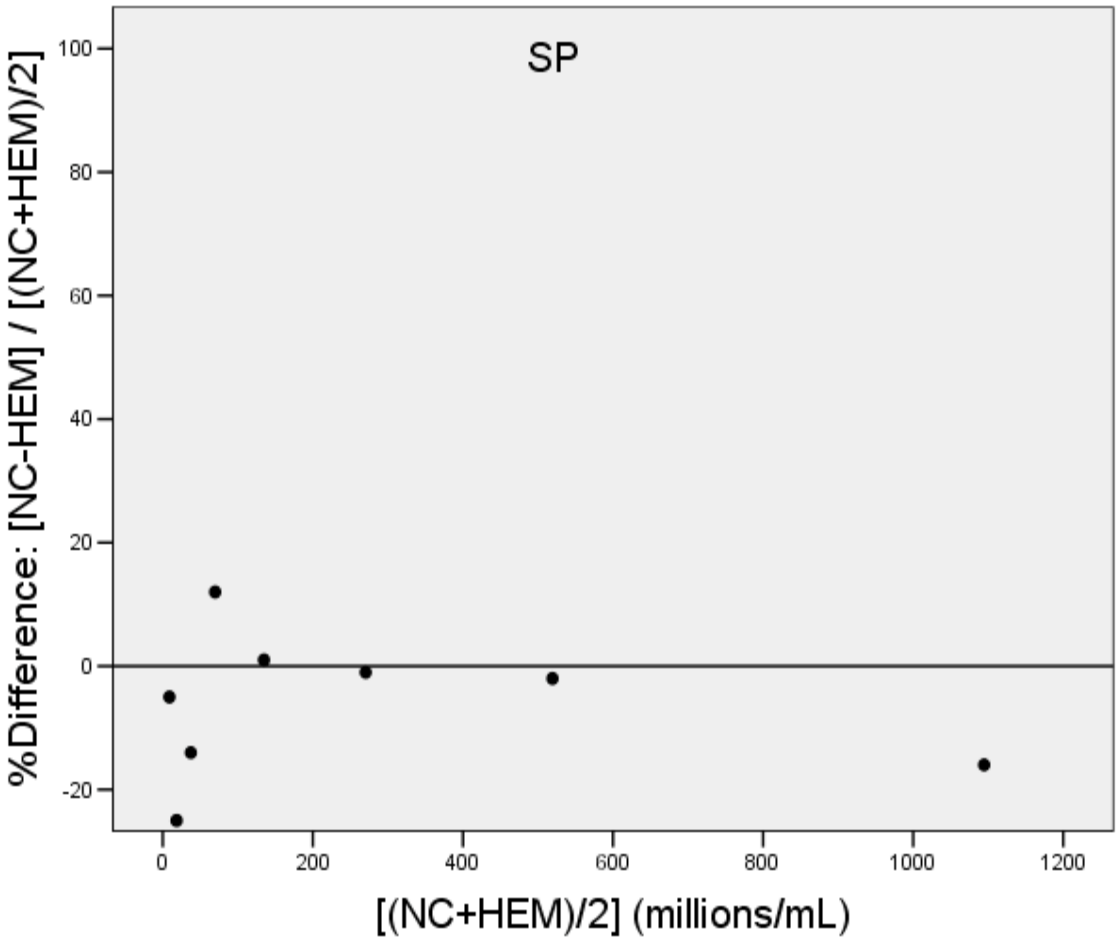

Figure 11. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the NucleoCounter (NC) and hemacytometer (HEM) to the mean of the NucleoCounter (NC) and hemacytometer (HEM) when spermatozoa are diluted in seminal plasma (SP)

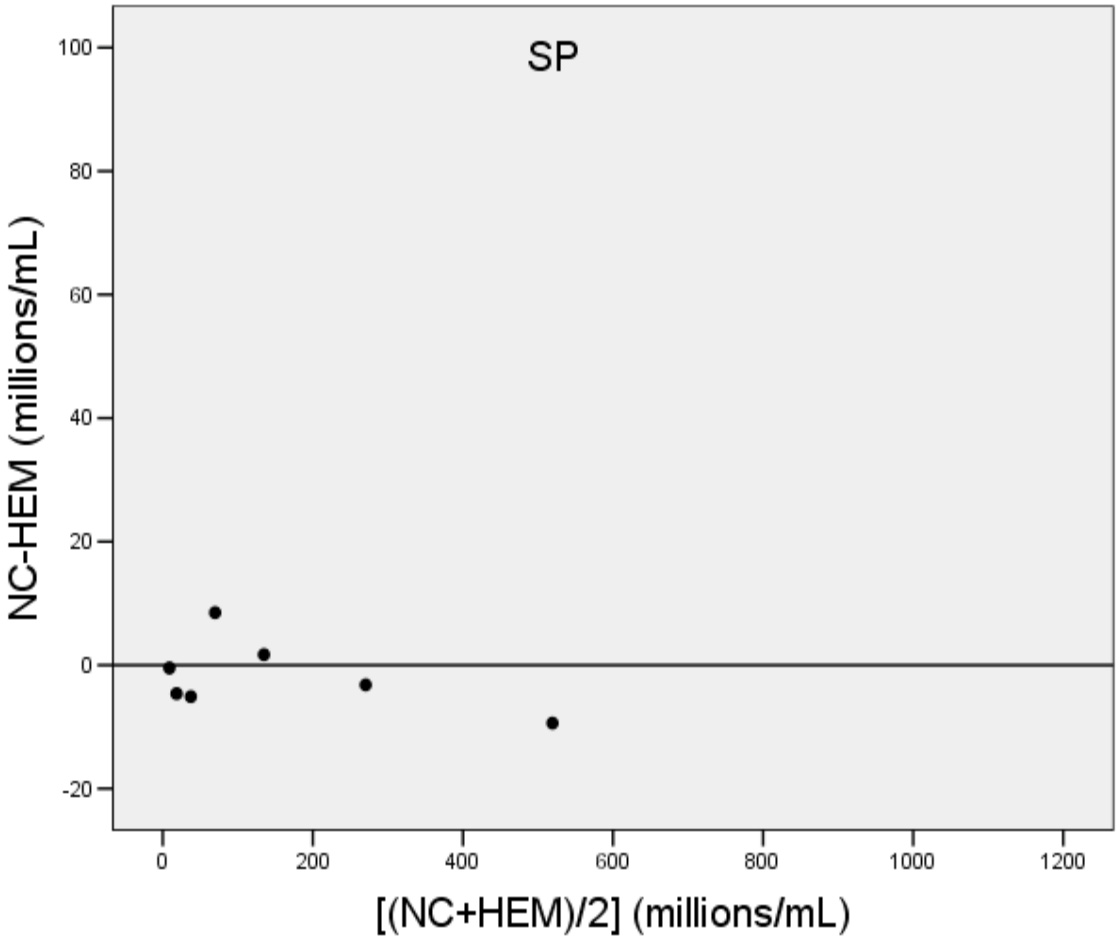

Figure 12. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the NucleoCounter (NC) and the hemacytometer (HEM) to the mean of the NucleoCounter (NC) and the hemacytometer (HEM) when spermatozoa are diluted in seminal plasma (SP)

Densimeter Bland and Altman comparisons

The Densimeter showed a similar percentage and actual number deviation from the flow cytometer, hemacytometer and NucleoCounter, regardless of diluent. Data is summarized in Figures 13-24.

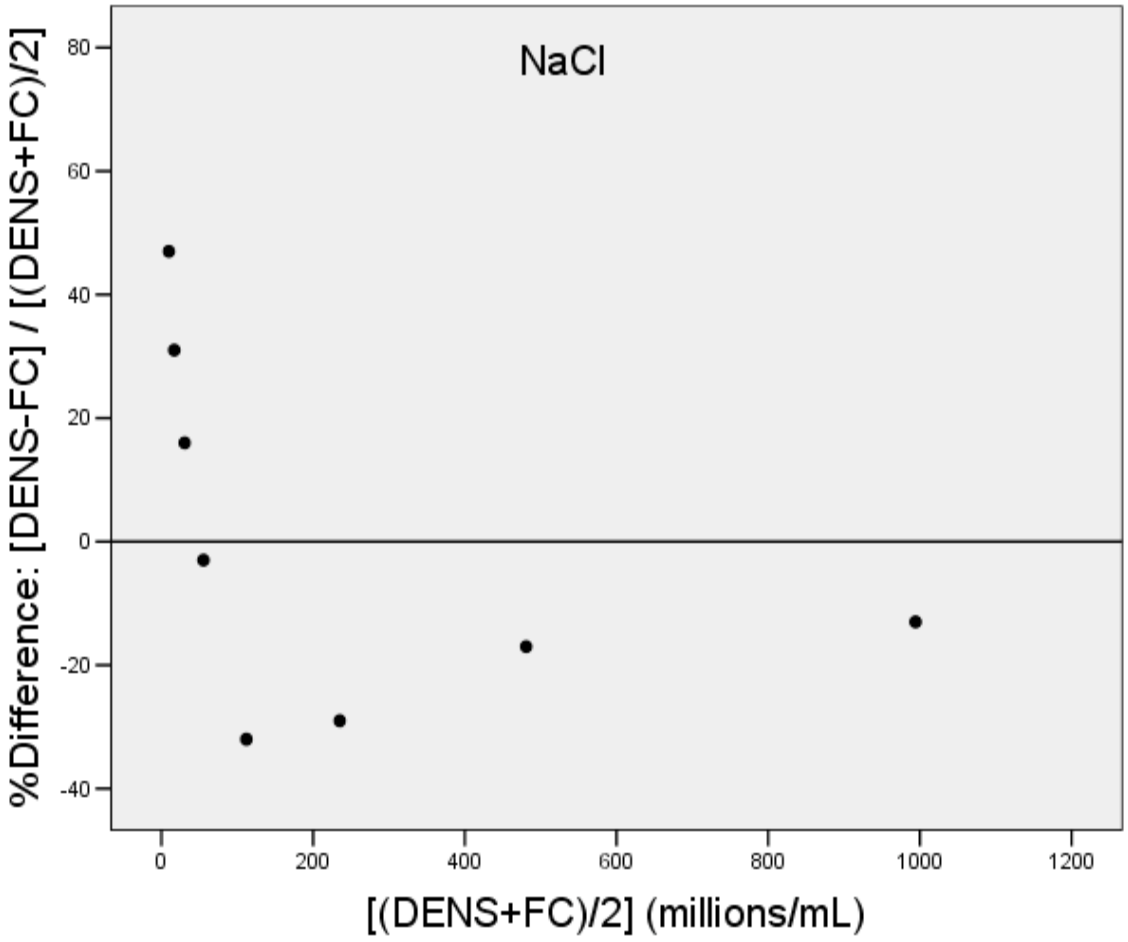

Figure 13. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the Densimeter (DENS) and flow cytometer (FC) to the mean of the Densimeter (DENS) and flow cytometer (FC) when spermatozoa are diluted in NaCl

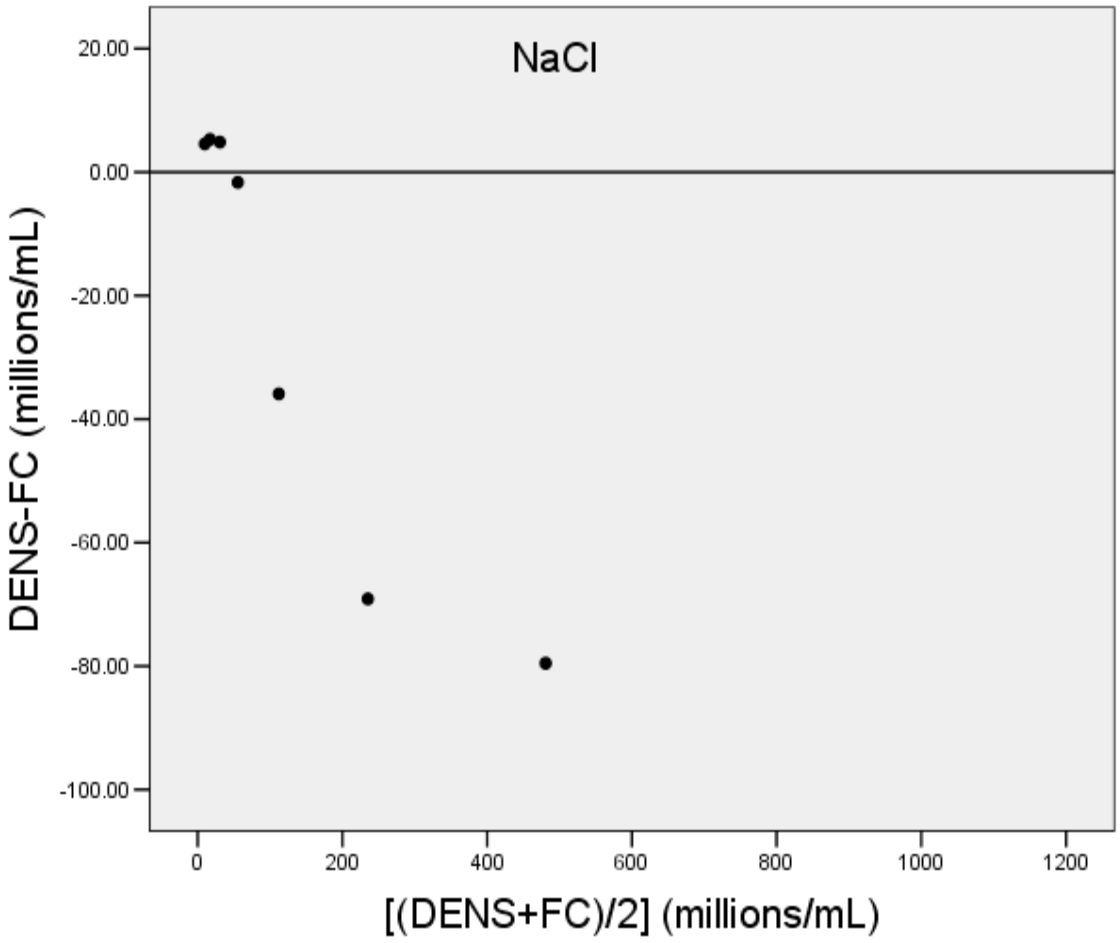

Figure 14. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the Densimeter (DENS) and the flow cytometer (FC) to the mean of the Densimeter (DENS) and the flow cytometer (FC)) when spermatozoa are diluted in NaCl

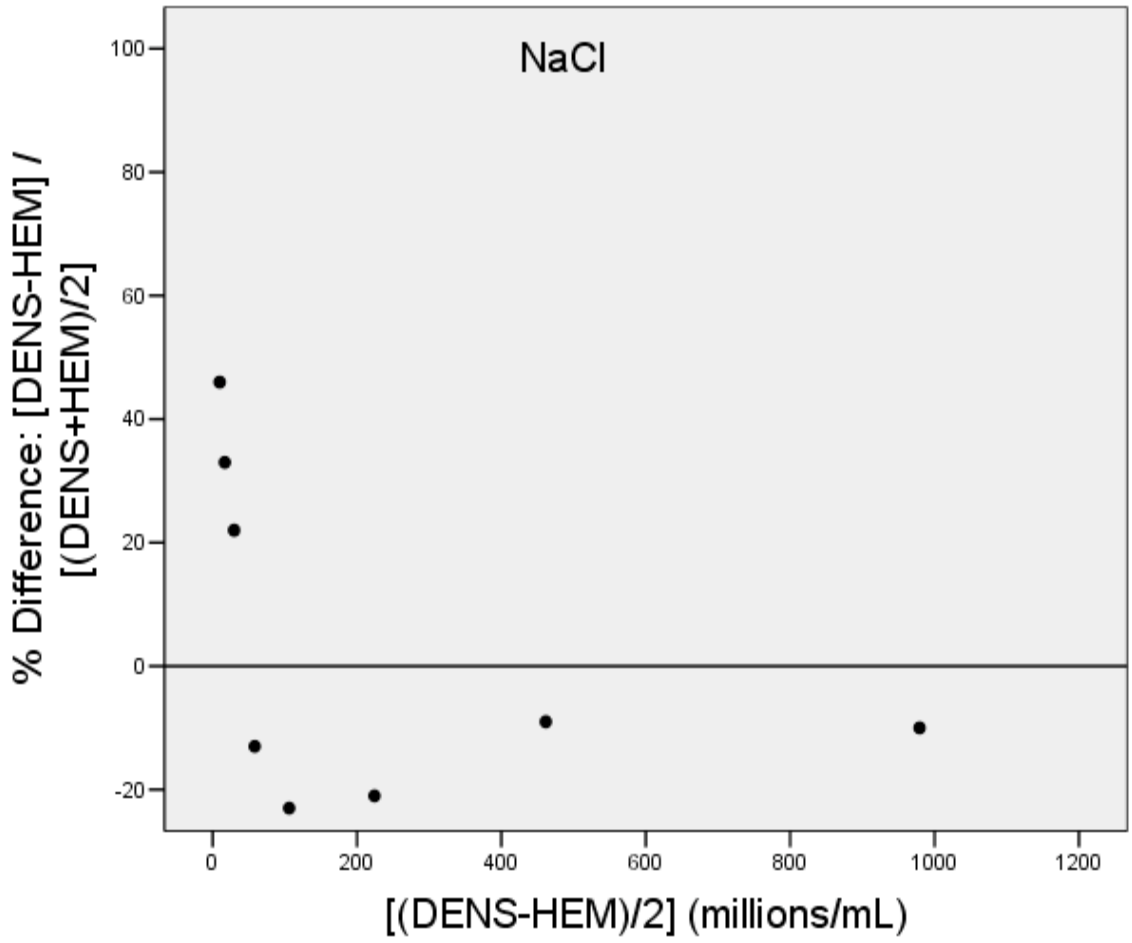

Figure 15. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the Densimeter (DENS) and hemacytometer (HEM) to the mean of the Densimeter (DENS) and hemacytometer (HEM) when spermatozoa are diluted in NaCl

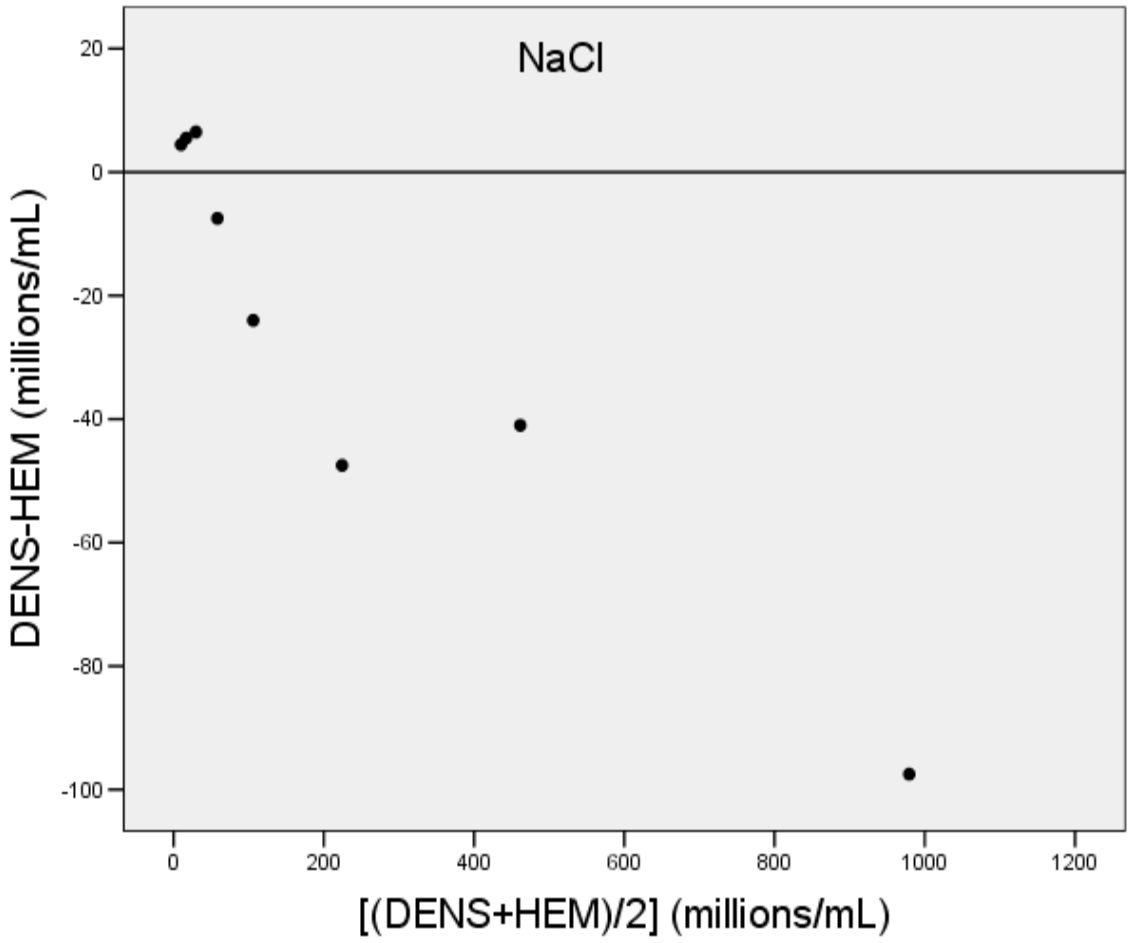

Figure 16. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the Densimeter (DENS) and the hemacytometer (HEM) to the mean of the Densimeter (DENS) and the hemacytometer (HEM) when spermatozoa are diluted in NaCl

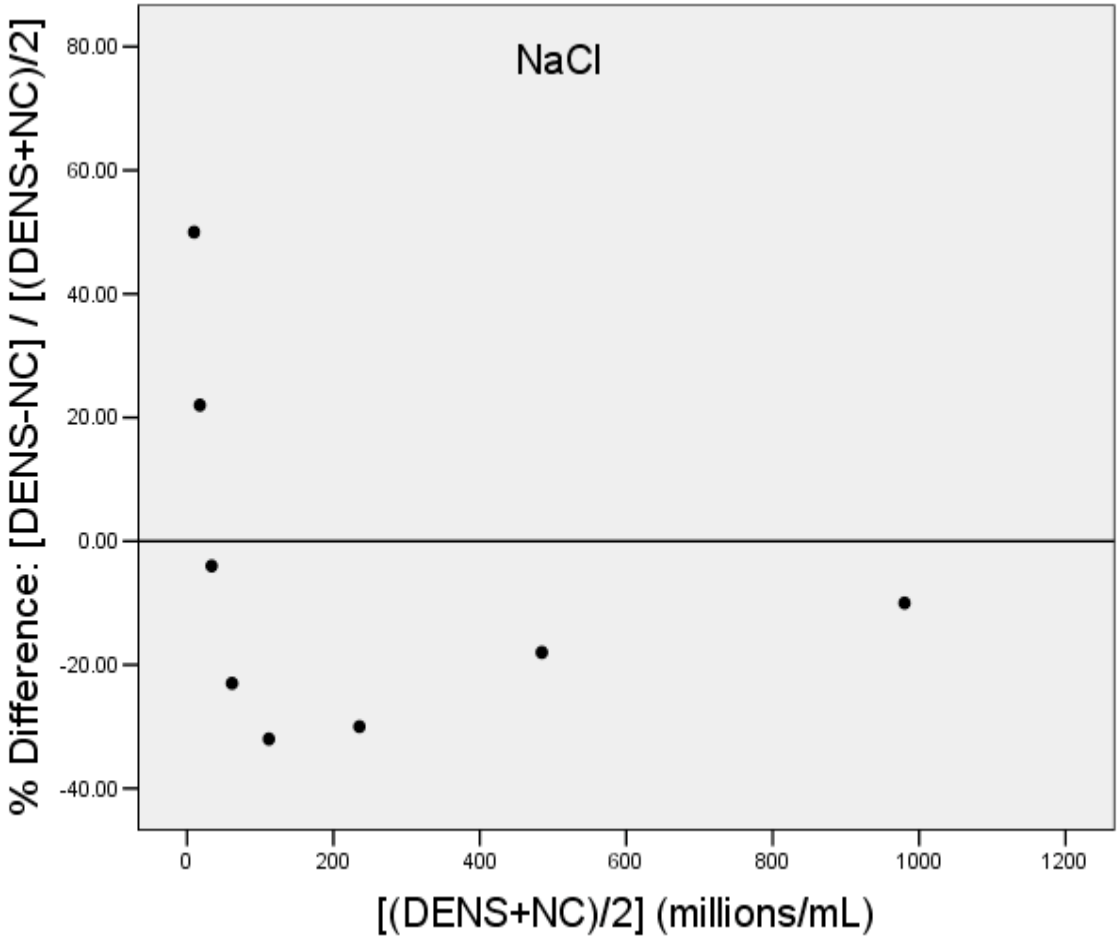

Figure 17. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the Densimeter (DENS) and NucleoCounter (NC) to the mean of the Densimeter (DENS) and NucleoCounter (NC) when spermatozoa are diluted in NaCl

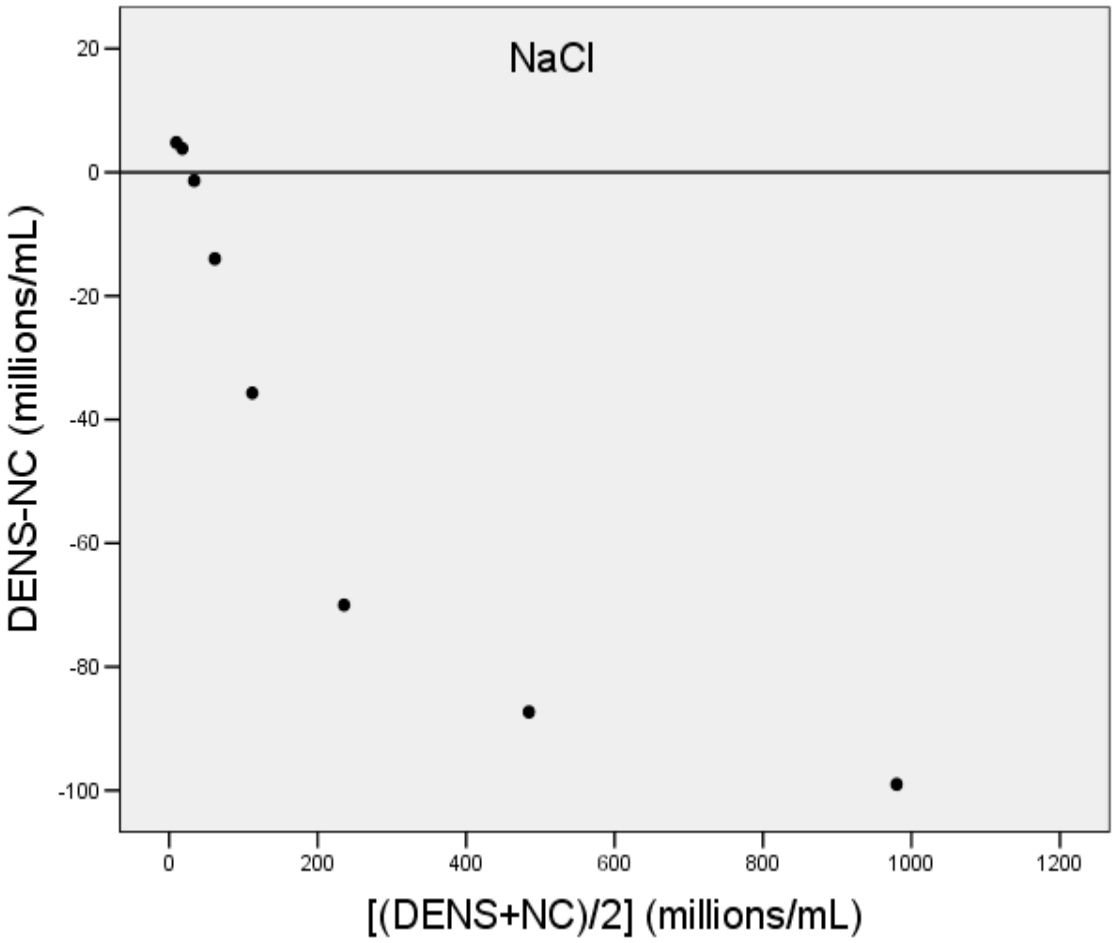

Figure 18. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the Densimeter (DENS) and the NucleoCounter (NC) to the mean of the Densimeter (DENS) and the NucleoCounter (NC) when spermatozoa are diluted in NaCl

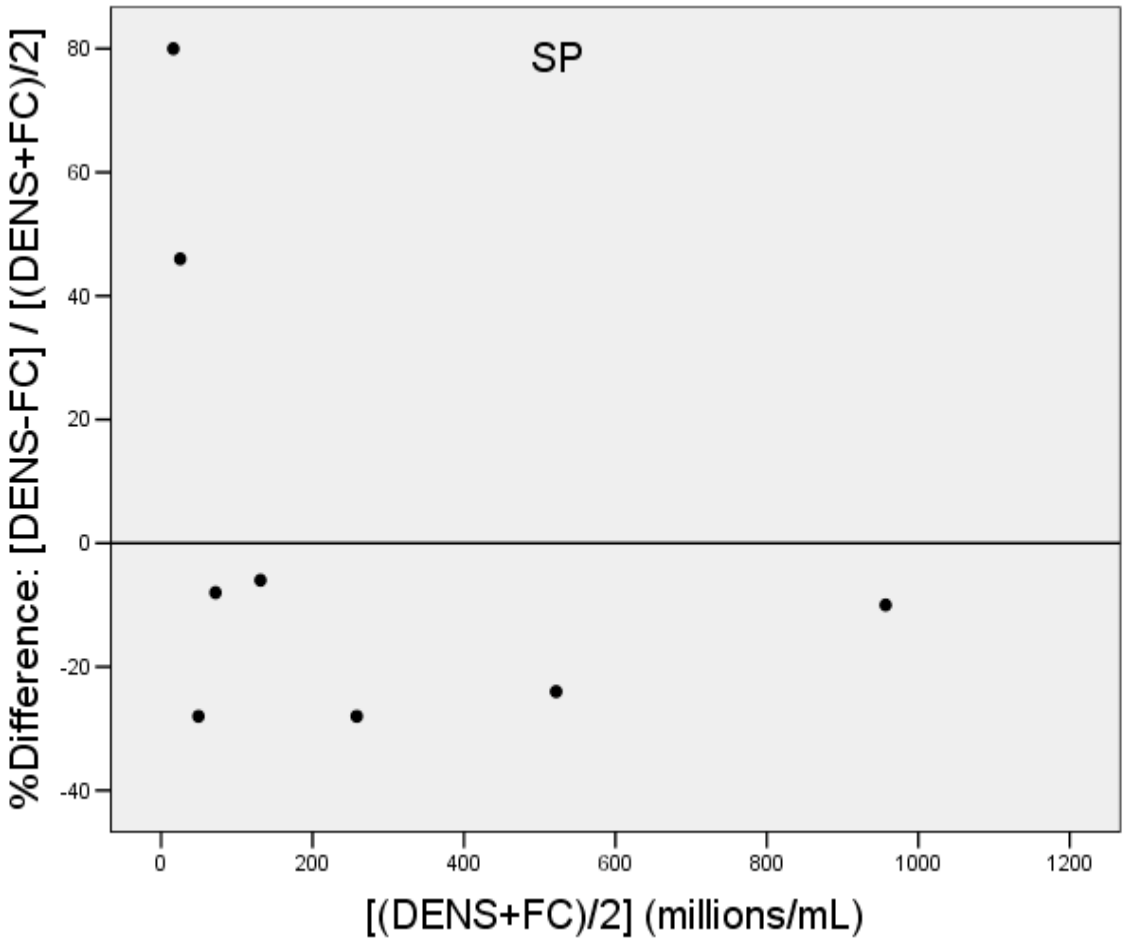

Figure 19. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the Densimeter (DENS) and flow cytometer (FC) to the mean of the Densimeter (DENS) and flow cytometer (FC) when spermatozoa are diluted in seminal plasma (SP)

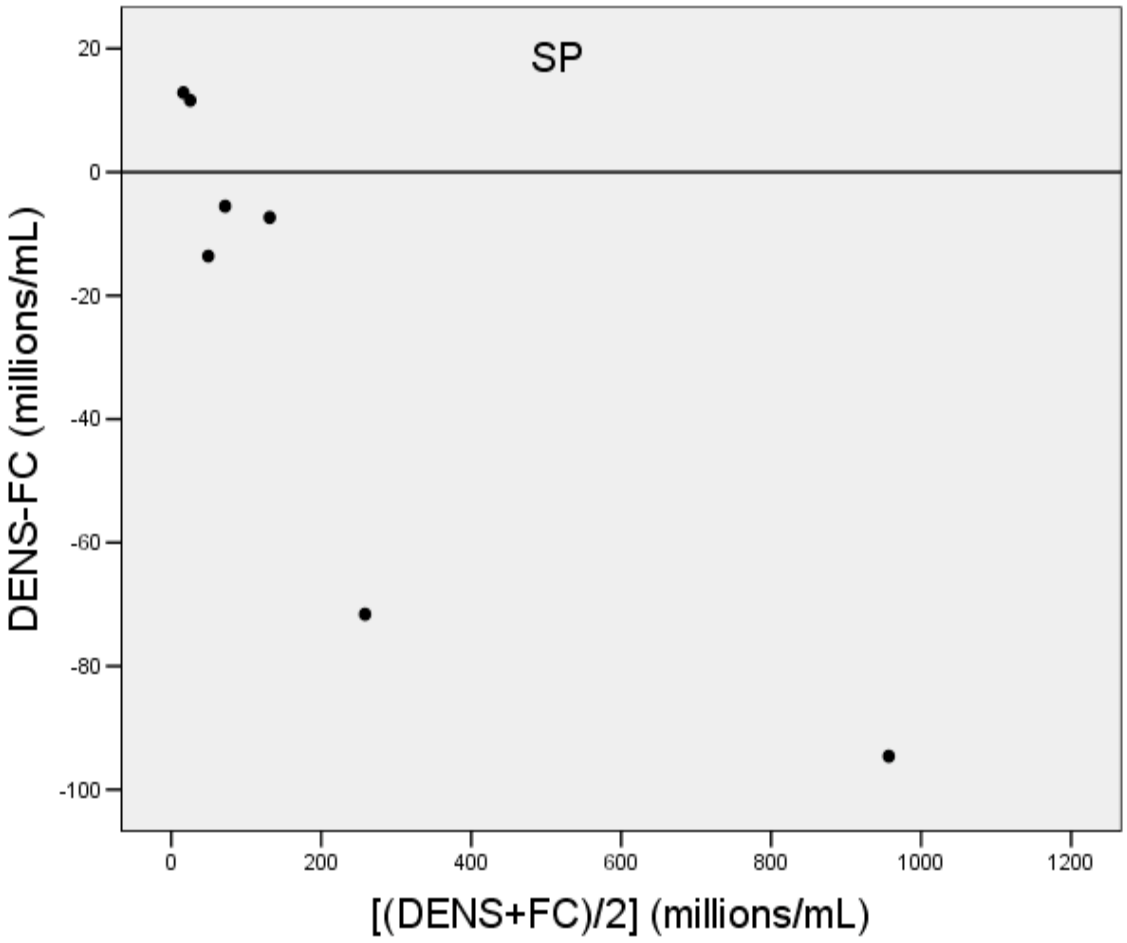

Figure 20. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the Densimeter (DENS) and the flow cytometer (FC) to the mean of the Densimeter (DENS) and the flow cytometer (FC)) when spermatozoa are diluted in seminal plasma (SP)

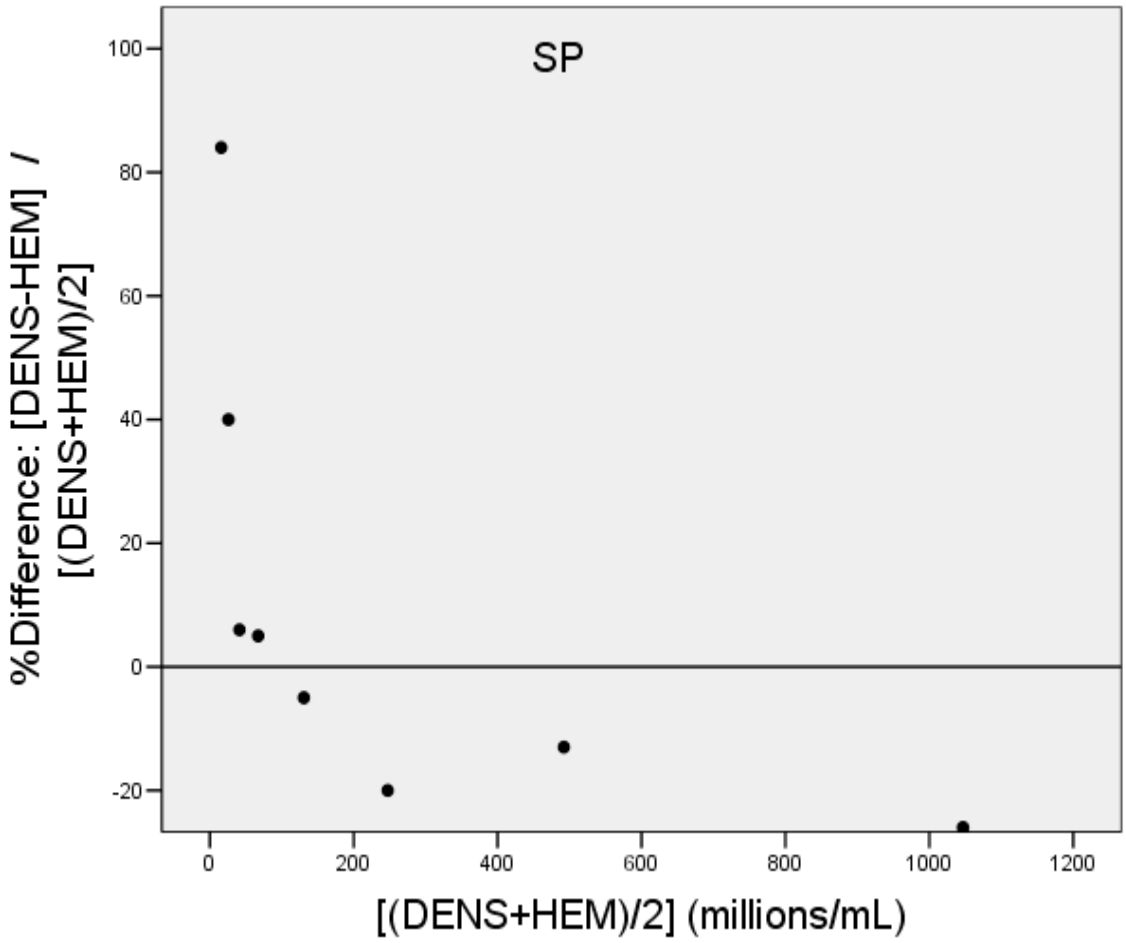

Figure 21. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the Densimeter (DENS) and flow cytometer (FC) to the mean of the Densimeter (DENS) and flow cytometer (FC) when spermatozoa are diluted in seminal plasma (SP)

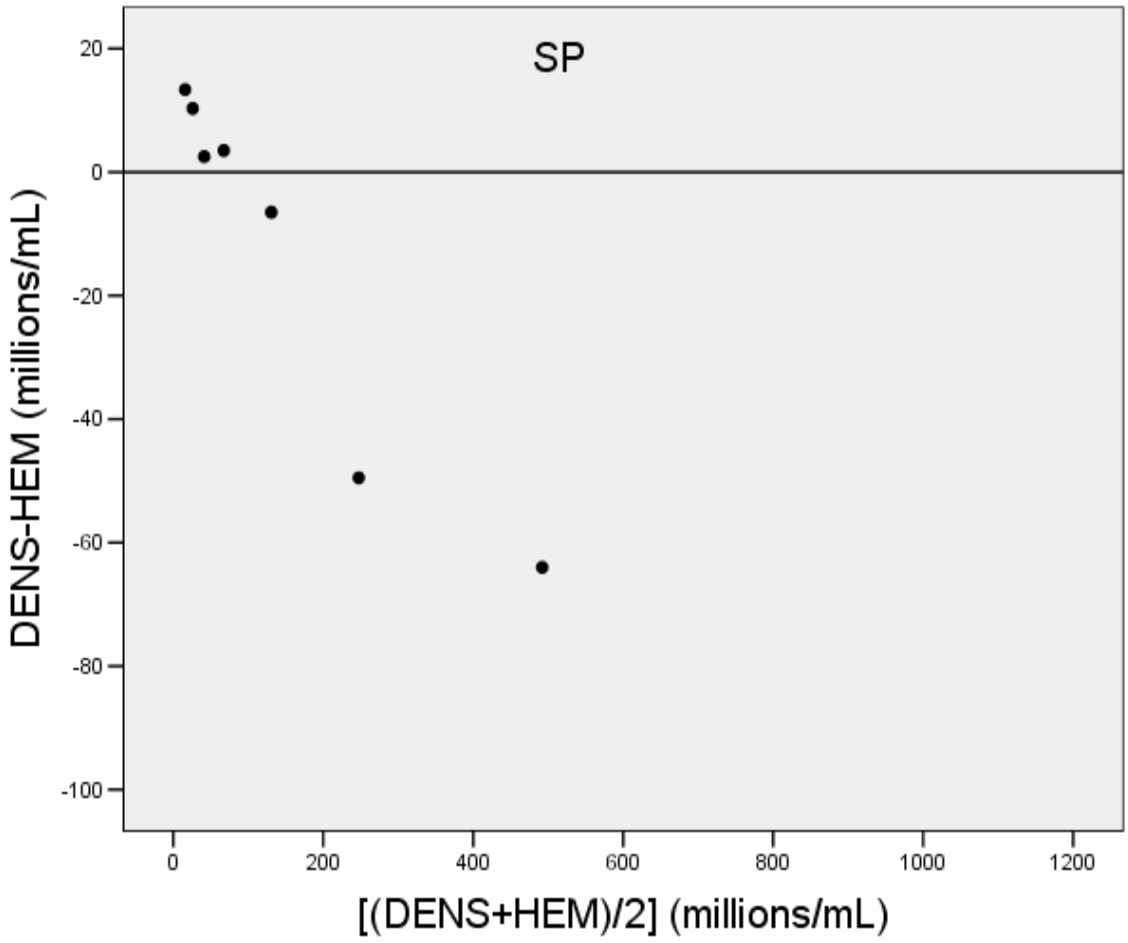

Figure 22. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the Densimeter (DENS) and the hemacytometer (HEM) to the mean of the Densimeter (DENS) and the hemacytometer (HEM) when spermatozoa are diluted in seminal plasma (SP)

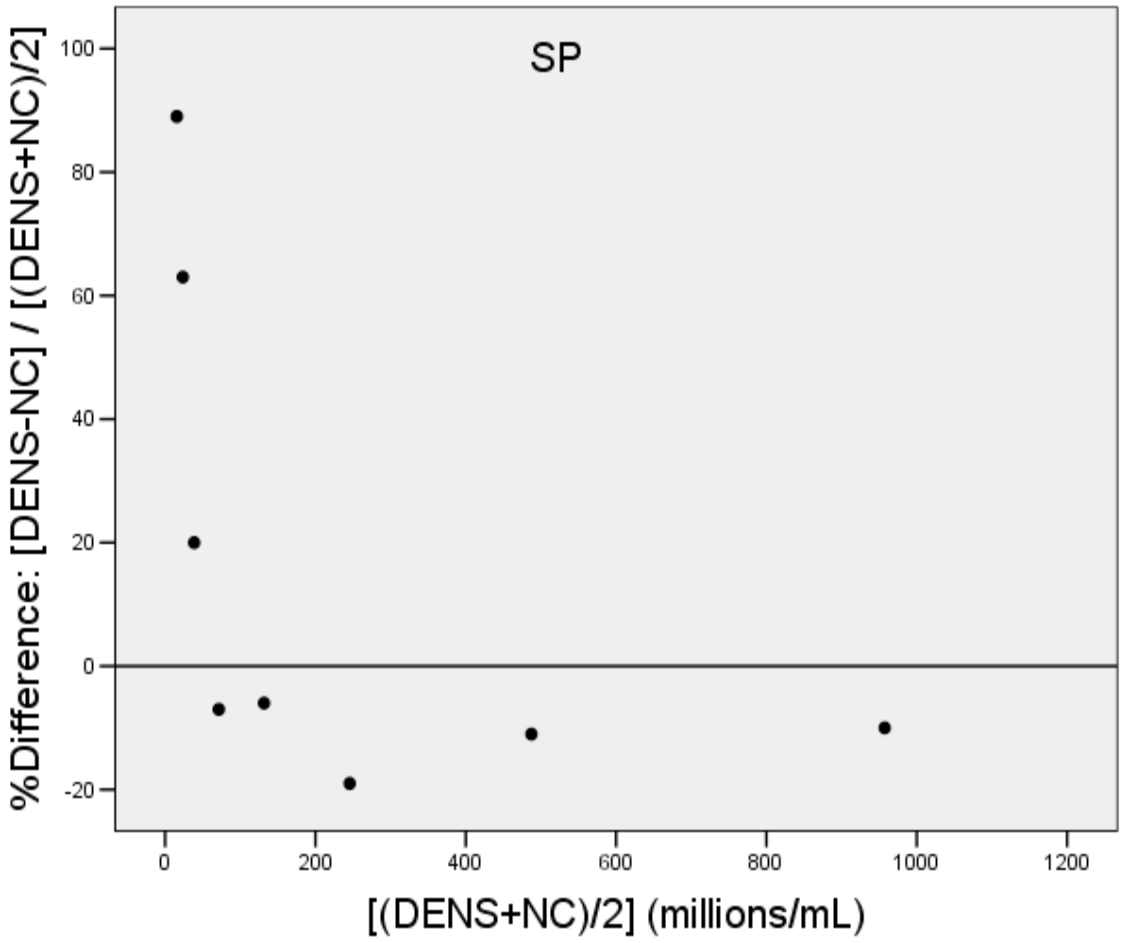

Figure 23. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the Densimeter (DENS) and NucleoCounter (NC) to the mean of the Densimeter (DENS) and NucleoCounter (NC) when spermatozoa are diluted in seminal plasma (SP)

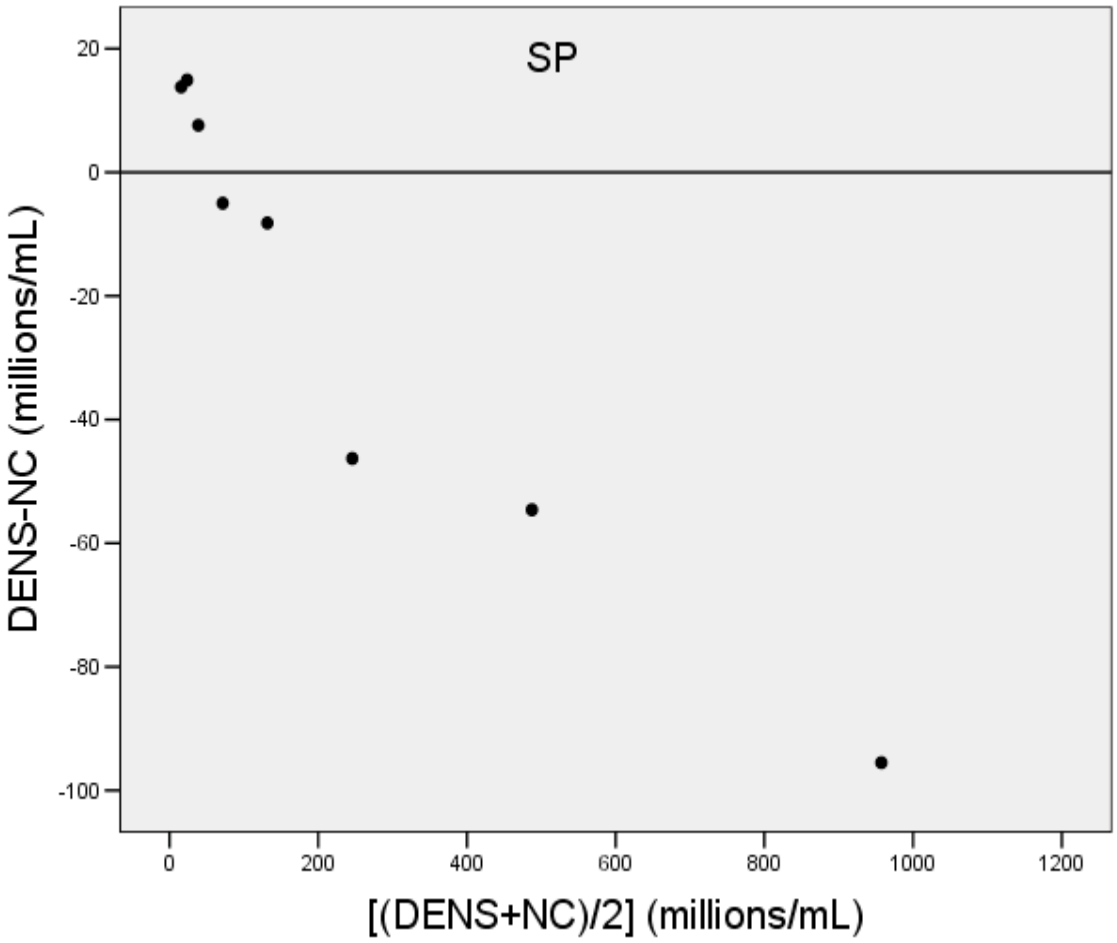

Figure 24. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the Densimeter (DENS) and the NucleoCounter (NC) to the mean of the Densimeter (DENS) and the NucleoCounter (NC) when spermatozoa are diluted in seminal plasma (SP)

Spermacue Bland and Altman comparisons

The Spermacue showed a similar percentage and actual number deviation from the flow cytometer, hemacytometer and NucleoCounter, regardless of diluent. Data is summarized in Figures 25-36.

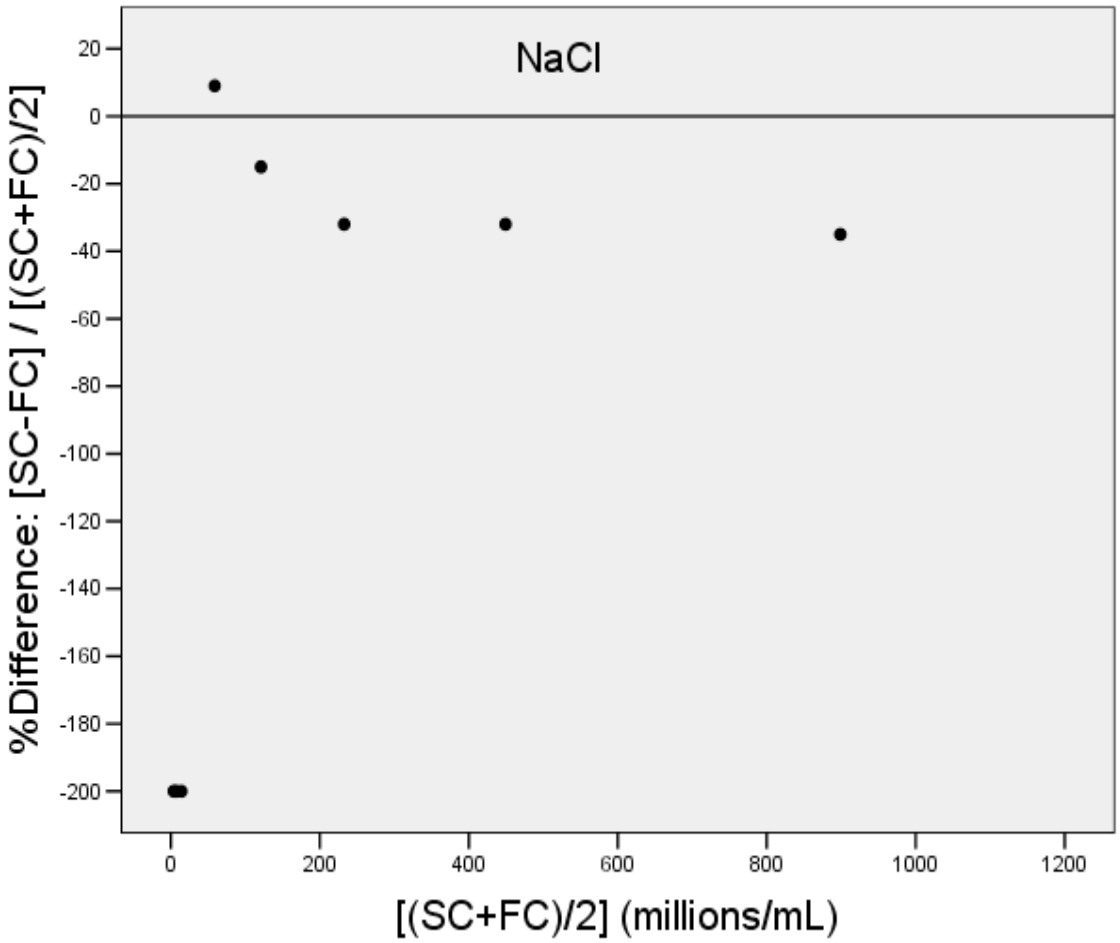

Figure 25. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the Spermacue (SC) and flow cytometer (FC) to the mean of the Spermacue (SC) and flow cytometer (FC) when spermatozoa are diluted in NaCl

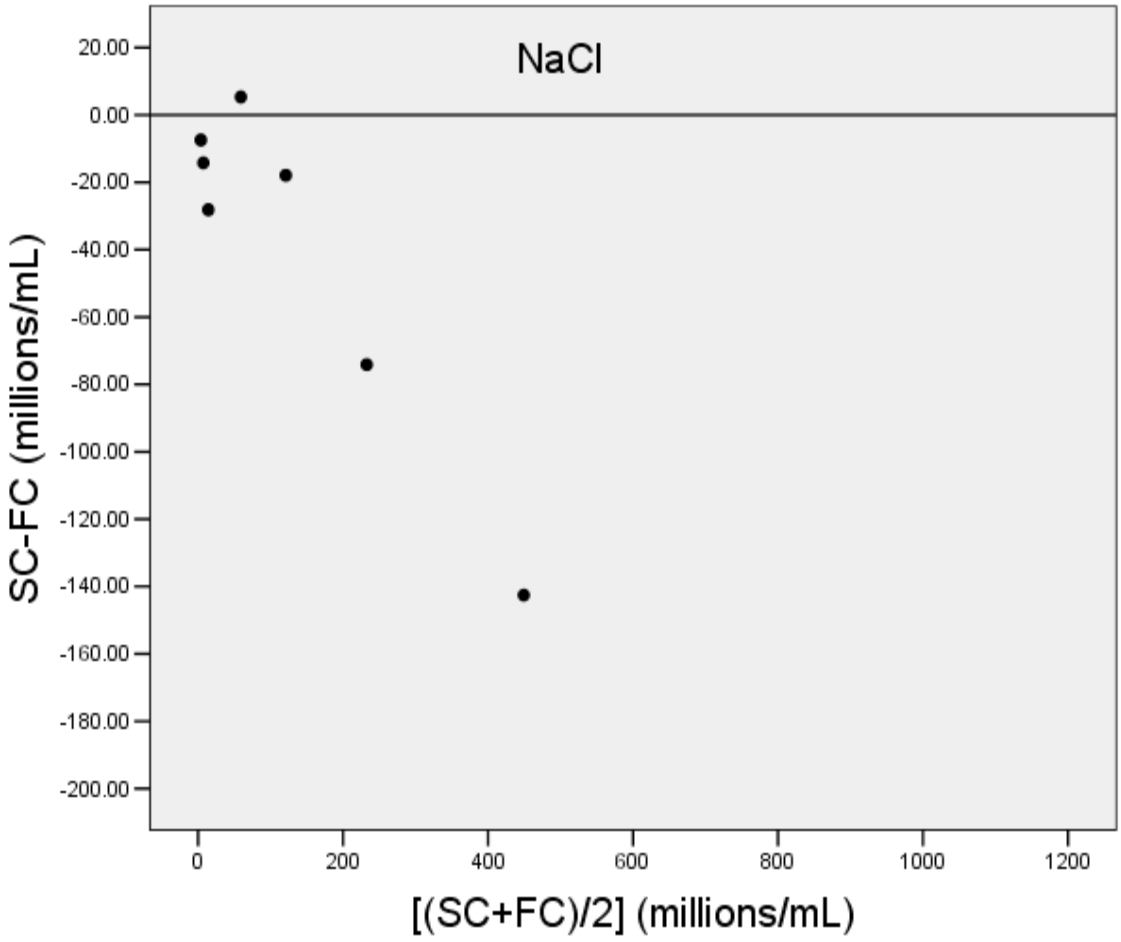

Figure 26. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the Spermacue (SC) and the flow cytometer (FC) to the mean of the Spermacue (SC) and the flow cytometer (FC) when spermatozoa are diluted in NaCl

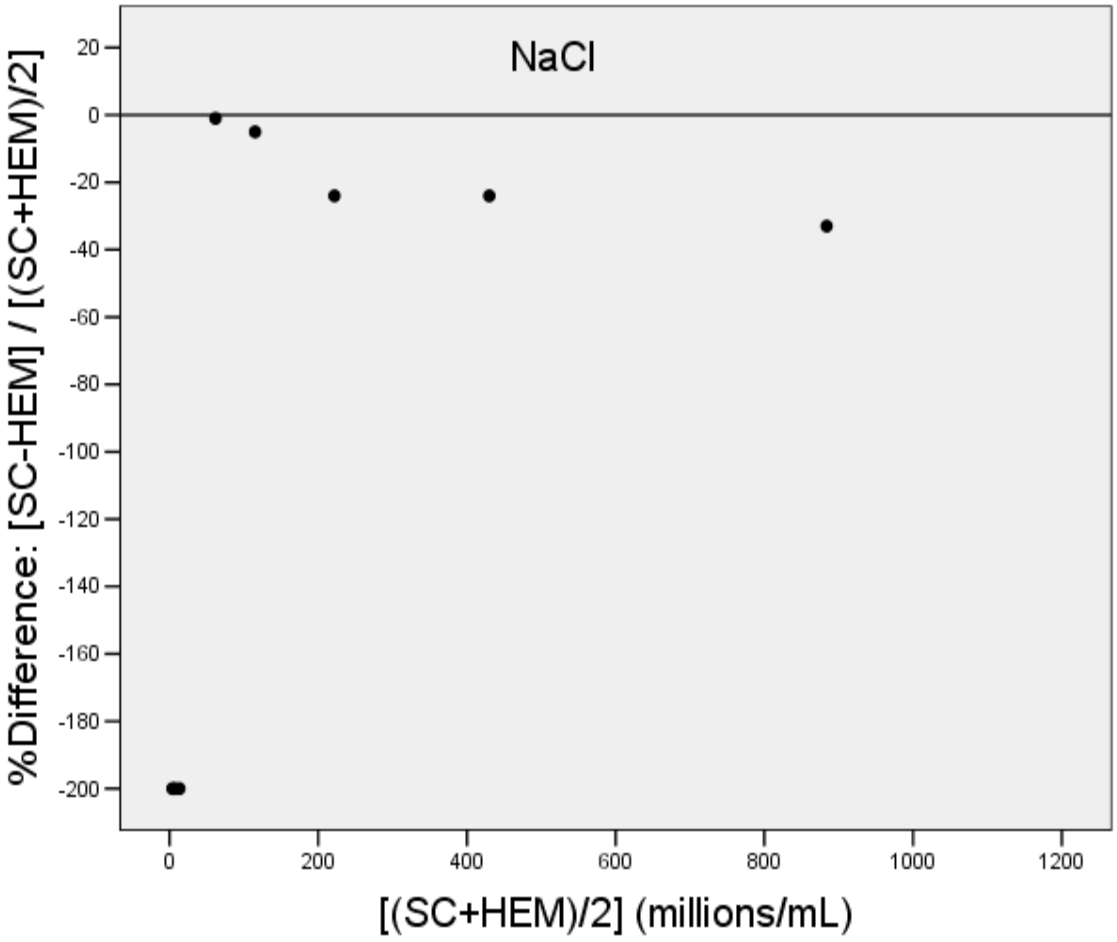

Figure 27. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the Spermacue (SC) and hemacytometer (HEM) to the mean of the Spermacue (SC) and hemacytometer (HEM) when spermatozoa are diluted in NaCl

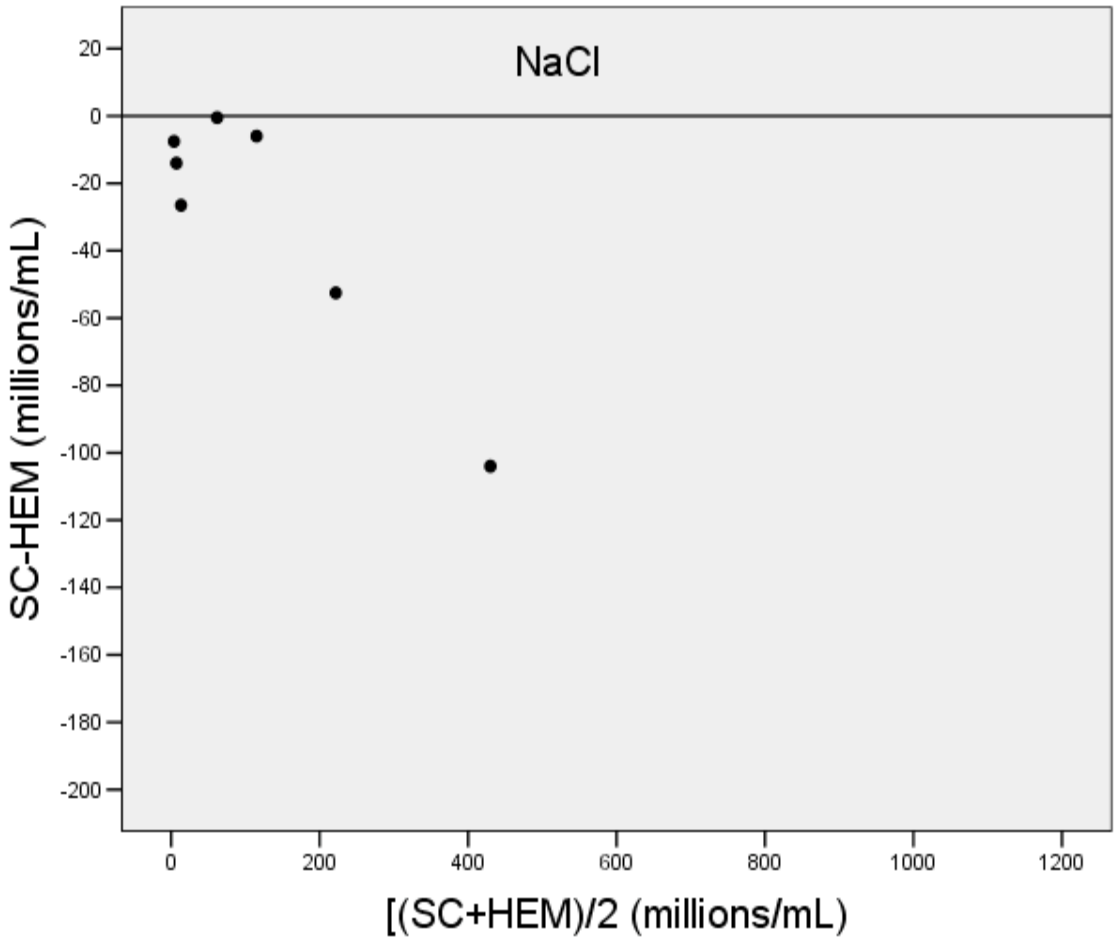

Figure 28. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the Spermacue (SC) and the hemacytometer (HEM) to the mean of the Spermacue (SC) and the hemacytometer (HEM) when spermatozoa are diluted in NaCl

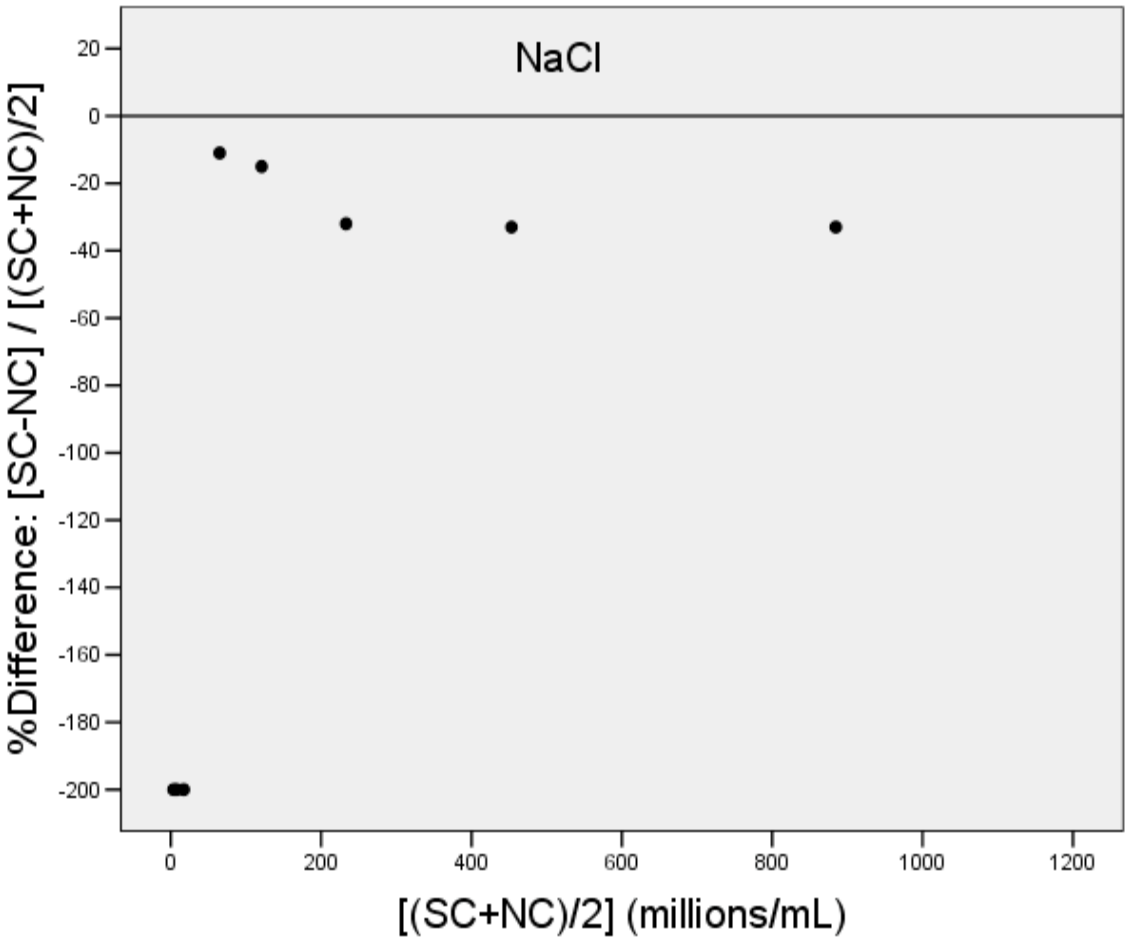

Figure 29. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the Spermacue (SC) and NucleoCounter (NC) to the mean of the Spermacue (SC) and NucleoCounter (NC) when spermatozoa are diluted in NaCl

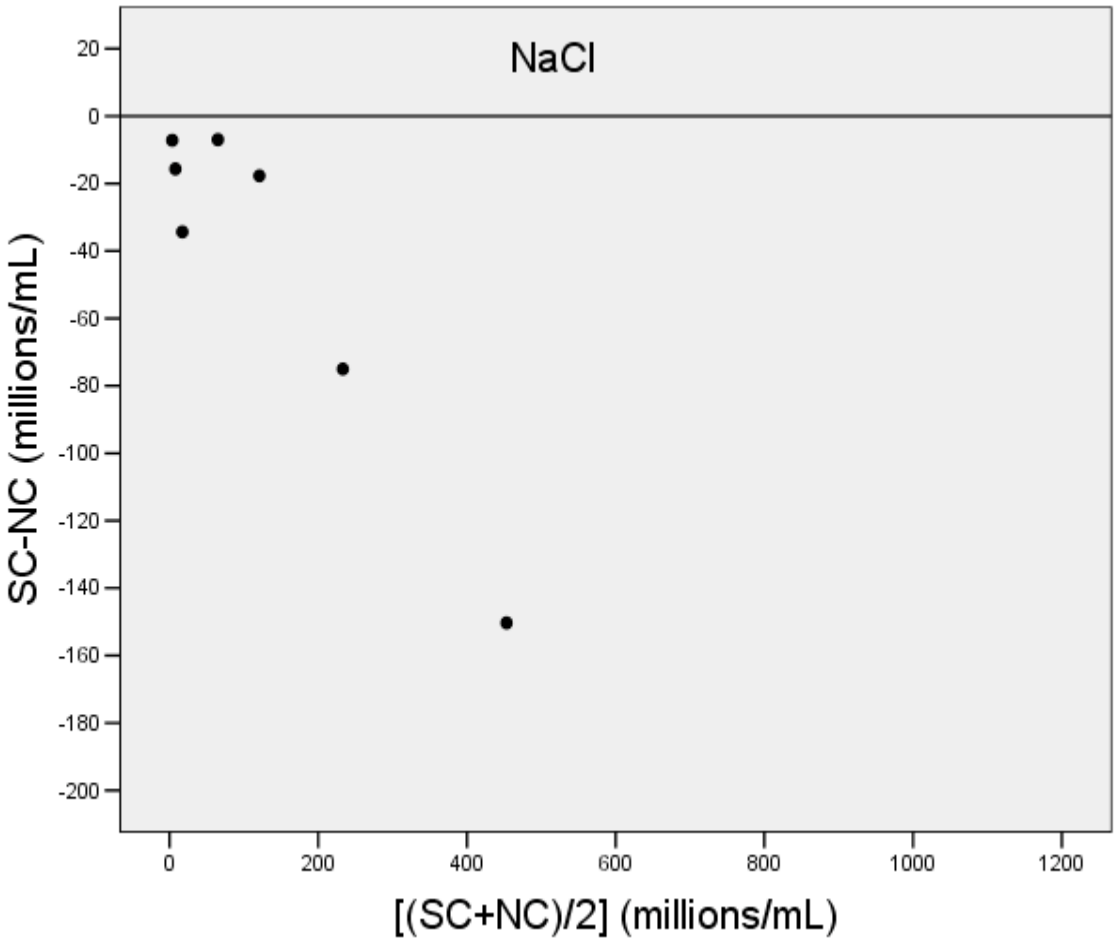

Figure 30. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the Spermacue (SC) and the NucleoCounter (NC) to the mean of the Spermacue (SC) and the NucleoCounter (NC) when spermatozoa are diluted in NaCl

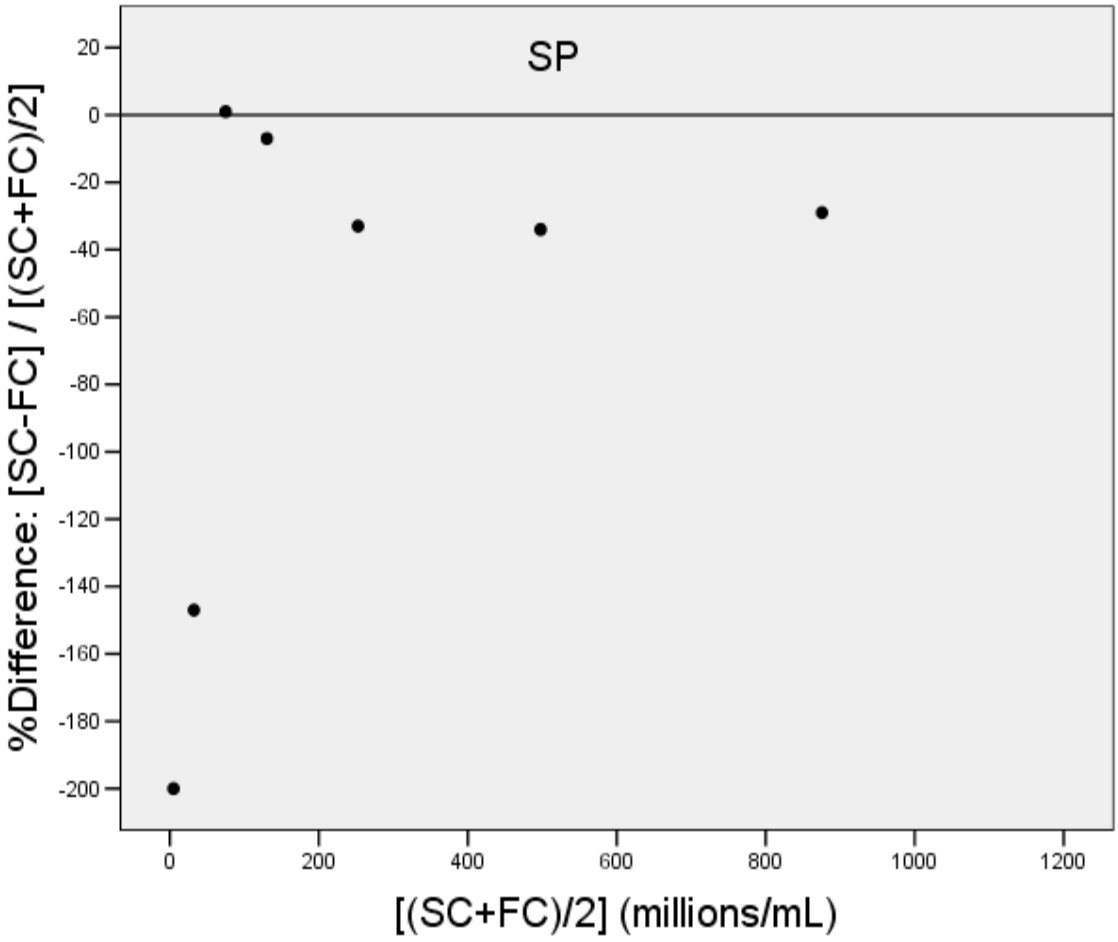

Figure 31. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the Spermacue (SC) and flow cytometer (FC) to the mean of the Spermacue (SC) and flow cytometer (FC) when spermatozoa are diluted in seminal plasma (SP)

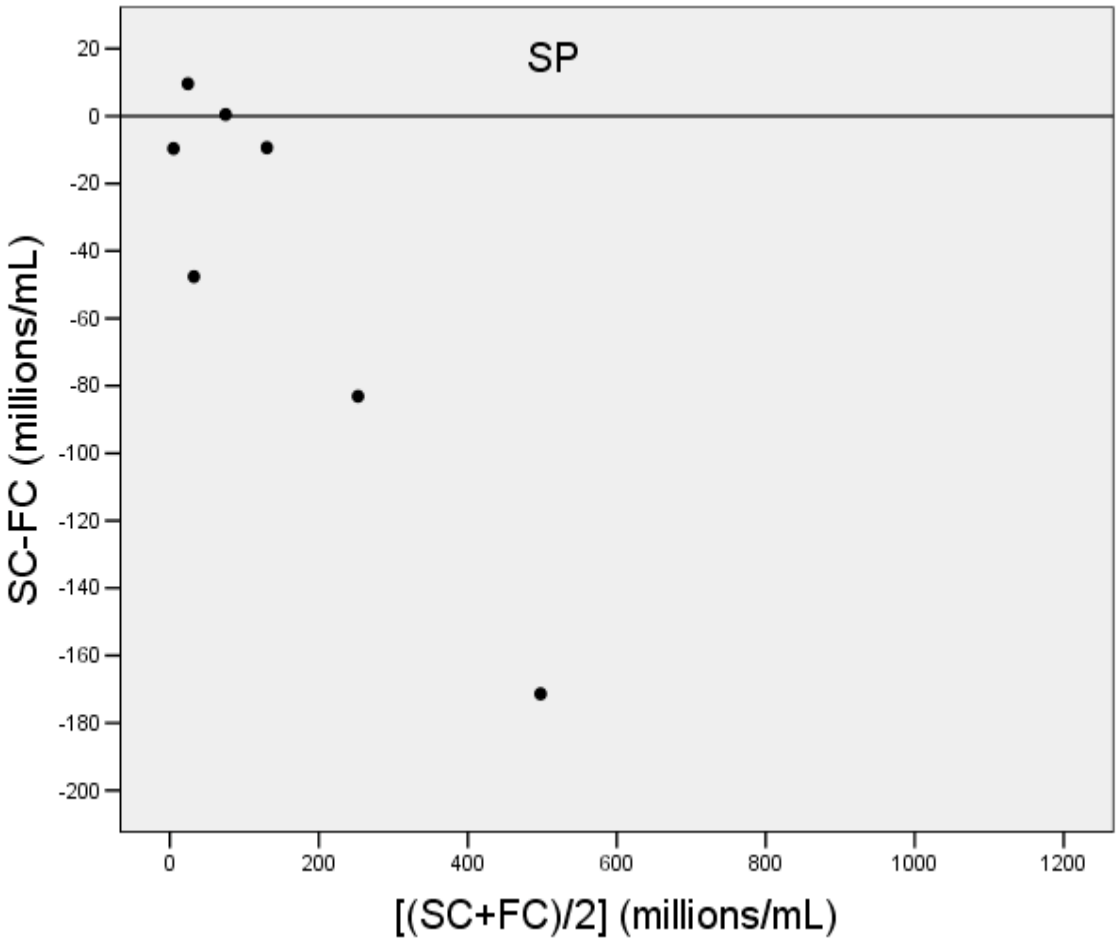

Figure 32. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the Spermacue (SC) and the flow cytometer (FC) to the mean of the Spermacue (SC) and the flow cytometer (FC) when spermatozoa are diluted in seminal plasma (SP)

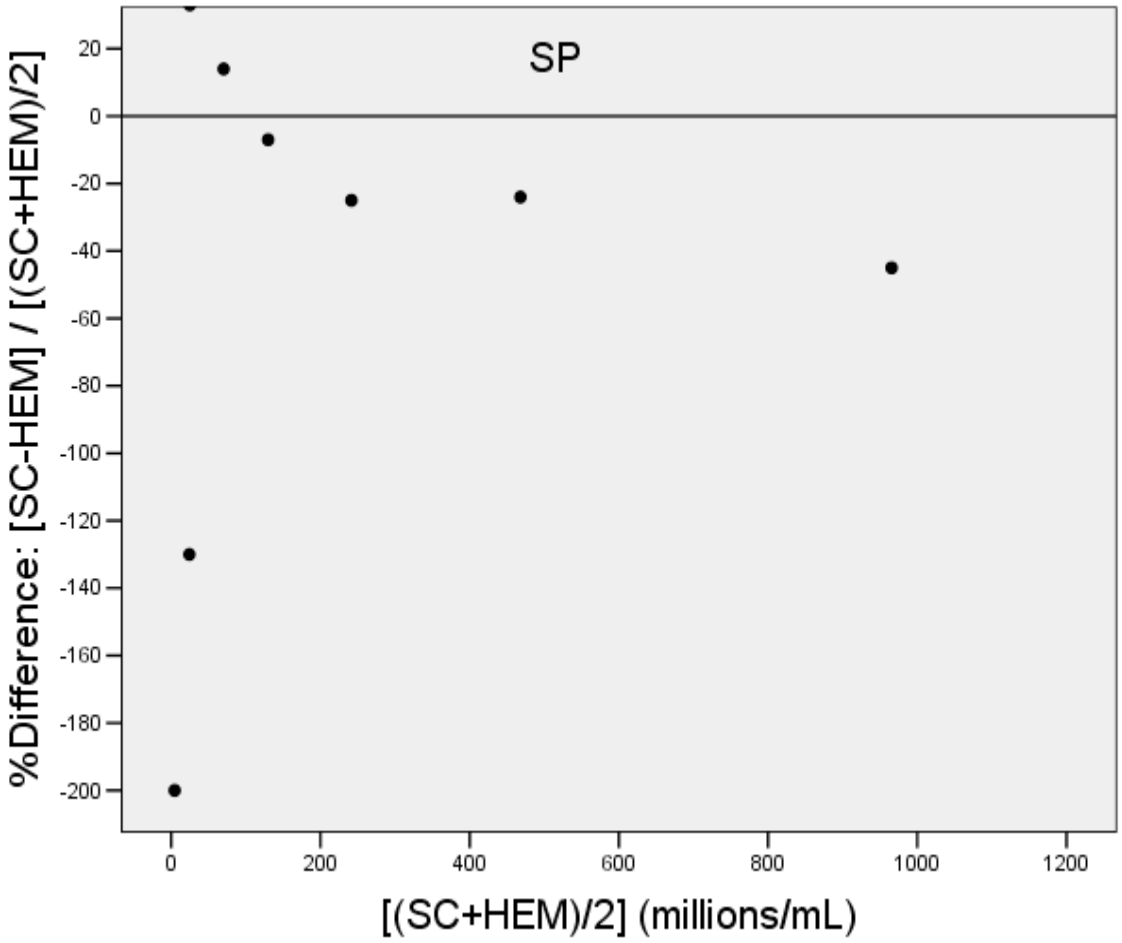

Figure 33. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the Spermacue (SC) and hemacytometer (HEM) to the mean of the Spermacue (SC) and hemacytometer (HEM) when spermatozoa are diluted in seminal plasma (SP)

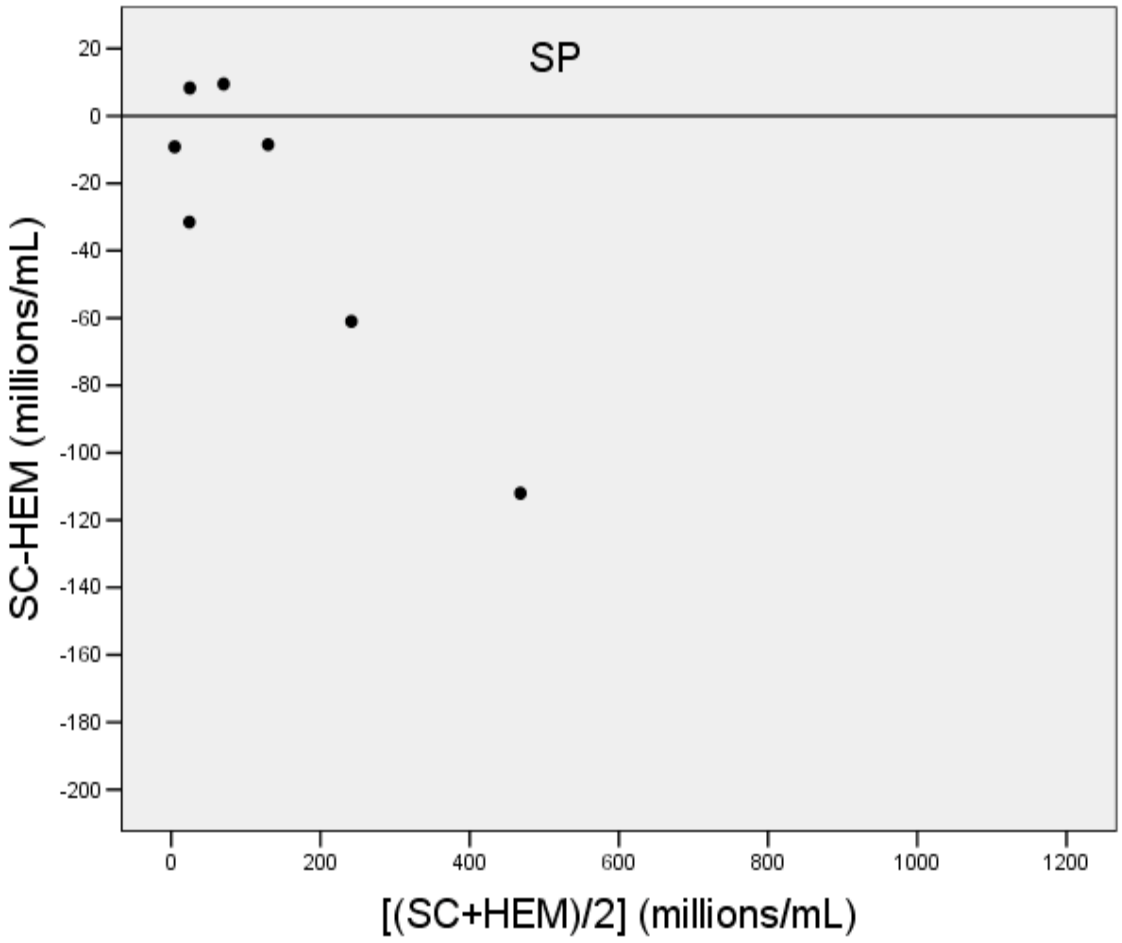

Figure 34. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the Spermacue (SC) and the hemacytometer (HEM) to the mean of the Spermacue (SC) and the hemacytometer (HEM) when spermatozoa are diluted in seminal plasma (SP)

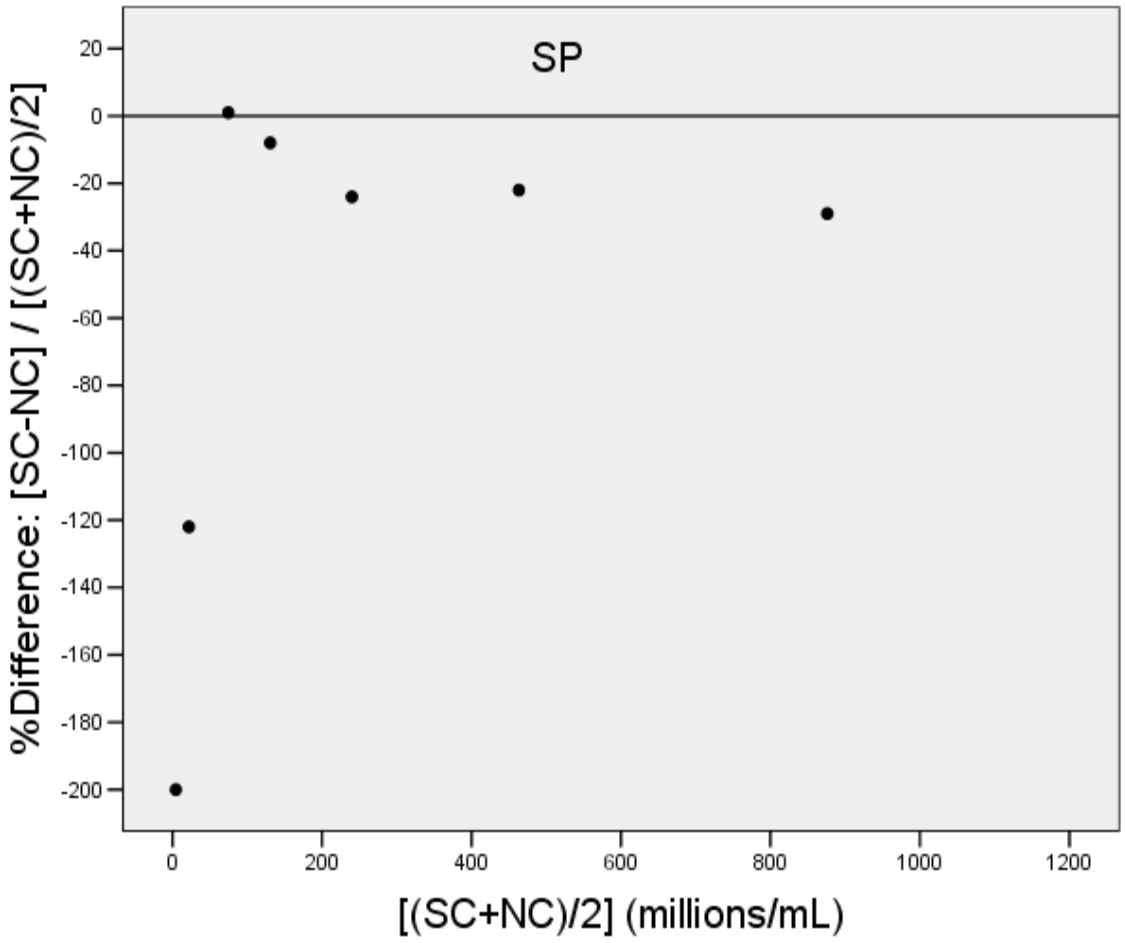

Figure 35. Modified Bland and Altman plot comparing the percent difference in epididymal spermatozoal concentration between the Spermacue (SC) and NucleoCounter (NC) to the mean of the Spermacue (SC) and NucleoCounter (NC) when spermatozoa are diluted in seminal plasma (SP)

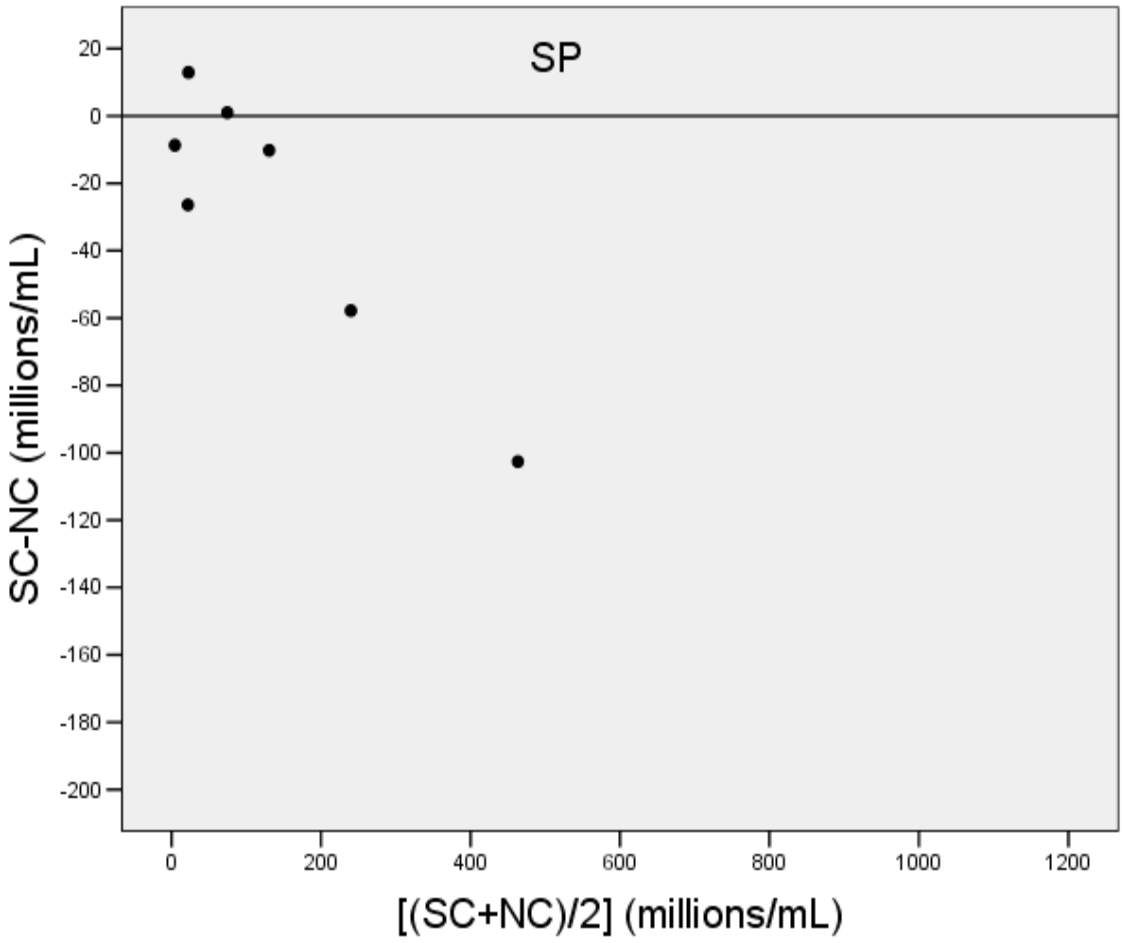

Figure 36. Bland and Altman plot comparing the difference in epididymal spermatozoal concentration between the Spermacue (SC) and the NucleoCounter (NC) to the mean of the Spermacue (SC) and the NucleoCounter (NC) when spermatozoa are diluted in seminal plasma (SP)

Experiment 9

Regression analysis

A combined regression plot for the NucleoCounter, flow cytometer, hemacytometer, Densimeter and Spermacue (Figure 37) was generated for samples diluted in NaCl only.

The data is summarized in Table 10. The NucleoCounter, Densimeter, flow cytometer and hemacytometer had slopes near 1 (line of equality). The y-intercepts indicated an underestimation of the NucleoCounter, the flow cytometer and the Spermacue of $6.1x10^5$/mL, $3.3x10^5$/mL and $7.54x10^6$/mL, respectively. The y-intercepts indicated a mean overestimation of the hemacytometer and Densimeter across all concentration ranges of $8.4x10^5$/mL and $4.51x10^6$/mL, respectively.

Table 10
Regression values for the NucleoCounter (NC), Densimeter (DENS) and the Spermacue (SC), the flow cytometer (FC) and the hemacytometer (HEM) for neat spermatozoa diluted in NaCl

Instrument	Slope	y-Intercept
FC	0.98	-0.33
HEM	0.99	0.84
NC	1.07	-0.61
DENS	0.91	4.51
SC	1.23	-7.54

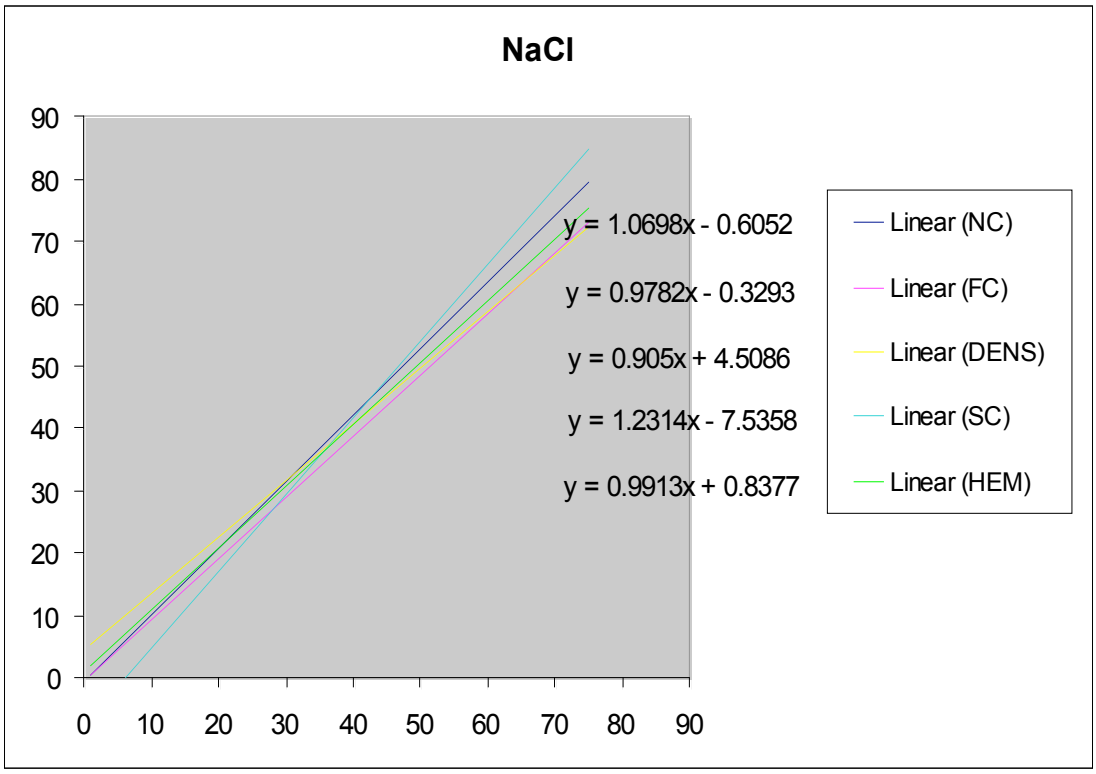

Figure 37. Regression trend lines for NucleoCounter (NC), flow cytometer (FC), hemacytometer (HEM), Densimeter (DENS) and Spermacue (SC) for ejaculated spermatozoa diluted in NaCl (spermatozoal concentrations x 10^6/mL)

Coefficient of variation

Of the instruments evaluated with samples diluted in LE extender, the Densimeter showed the lowest coefficient of variation. The hemacytometer was not evaluated. Of the instruments evaluated with samples diluted in MFR5 extender, the NucleoCounter showed the lowest coefficient of variation. The hemacytometer was not evaluated. Of the instruments evaluated with samples diluted in INRA96 extender, the Spermacue showed the lowest coefficient of variation. The hemacytometer was not evaluated. Of the instruments evaluated with samples diluted in CST extender, the

Spermacue showed the lowest coefficient of variation. The hemacytometer was not evaluated. With samples diluted in NaCl, the hemacytometer and NucleoCounter showed the lowest coefficient of variation. Data is summarized in Table 11.

Table 11

Within-sample repeatability (coefficient of variation) for measurement of spermatozoal concentration using the flow cytometer (FC), hemacytometer (HEM), NucleoCounter (NC), Spermacue (SC), and Densimeter (DENS) diluted with LE, MFR5, INRA96, CST and NaCl semen extenders

Diluent	Instrument	Coefficient of variation: 3 replicates (%)
LE	FC	5.17
MFR5		6.11
INRA96		7.73
CST		13.85
NaCl		4.76
LE	NC	4.70
MFR5		2.44
INRA96		2.71
CST		4.96
NaCl		2.63
LE	DENS	3.96
MFR5		3.18
INRA96		3.90
CST		3.73
NaCl		5.49
LE	SC	8.01
MFR5		4.62
INRA96		1.64
CST		2.45
NaCl		16.48
NaCl	HEM	2.63

Original and modified Bland and Altman plots

All data is summarized in Table 12 and scatterplots are shown for NaCl comparisons only. The NucleoCounter showed the least percentage of difference from the hemacytometer when diluted in NaCl. Hemacytometer counts were not evaluated in any other diluent. The NucleoCounter showed the least agreement with the flow cytometer when samples were diluted in CST. The NucleoCounter showed significantly less percentage and actual number difference from the hemacytometer and flow cytometer in all diluents than did the Densimeter or Spermacue. Both photometric systems showed the most agreement with both the hemacytometer and flow cytometer when the sample was diluted in NaCl. When compared to the flow cytometer, the hemacytometer showed a greater percentage of difference than did the NucleoCounter, but a lower actual number difference.

Table 12

Mean percentage (modified Bland and Altman) and actual differences (Bland and Altman) for the NucleoCounter (NC), Densimeter (DENS), Spermacue (SC), flow cytometer (FC) and hemacytometer (HEM) for neat spermatozoa diluted in LE, MFR5, INRA96, CST and NaCl semen extenders

Instrument Comparison	Diluent	Mean Difference (%)	Mean Difference (abs value x 10^6)
NC-FC	LE	9.56	2.25
	MFR5	6.83	2.87
	CST	10.97	5.13
	INRA96	7.99	3.28
	NaCl	7.86	1.66
NC-HEM	NaCl	6.09	1.15

Table 12

Continued

Instrument Comparison	Diluent	Mean Difference (%)	Mean Difference (abs value x 10^6)
DENS-FC	LE	162.34	179.51
	MFR5	122.65	37.83
	CST	119.62	34.41
	INRA96	152.30	104.69
	NaCl	66.95	4.26
DENS-HEM	NaCl	58.61	3.32
SC-FC	LE	148.94	85.59
	MFR5	150.65	87.40
	CST	156.12	115.84
	INRA96	171.37	213.12
	NaCl	147.23	7.30
SC-HEM	NaCl	146.75	8.15
FC-HEM	NaCl	11.27	1.44
DENS-NC	NaCl	64.05	4.78
SC-NC	NaCl	145.62	6.65

NucleoCounter Bland and Altman comparisons

The NucleoCounter showed a greater percentage of difference from the flow cytometer than from the hemacytometer (Figures 38 and 40), but showed similar actual number agreement with both instruments (Figures 39 and 41) when fresh ejaculated spermatozoa was diluted in NaCl.

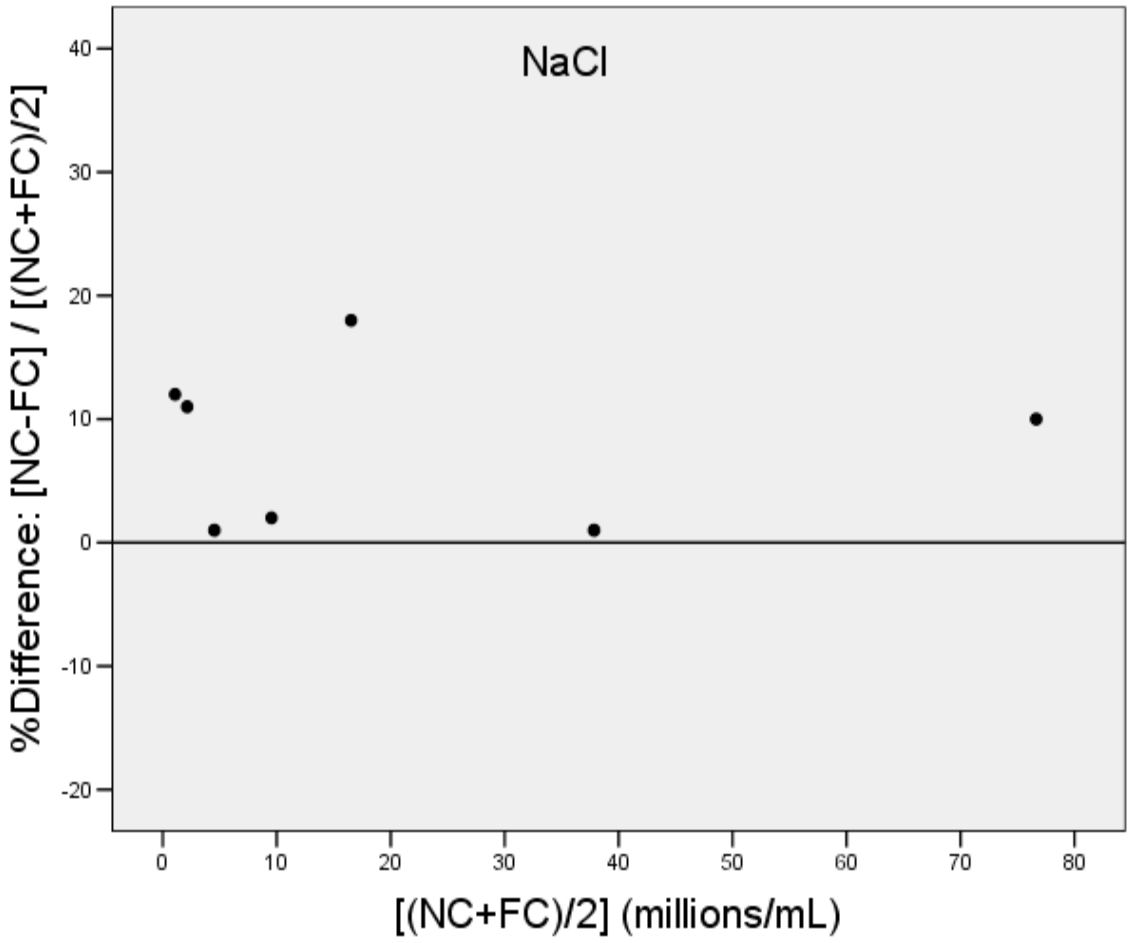

Figure 38. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the NucleoCounter (NC) and flow cytometer (FC) to the mean of the NucleoCounter (NC) and flow cytometer (FC) when spermatozoa are diluted in NaCl

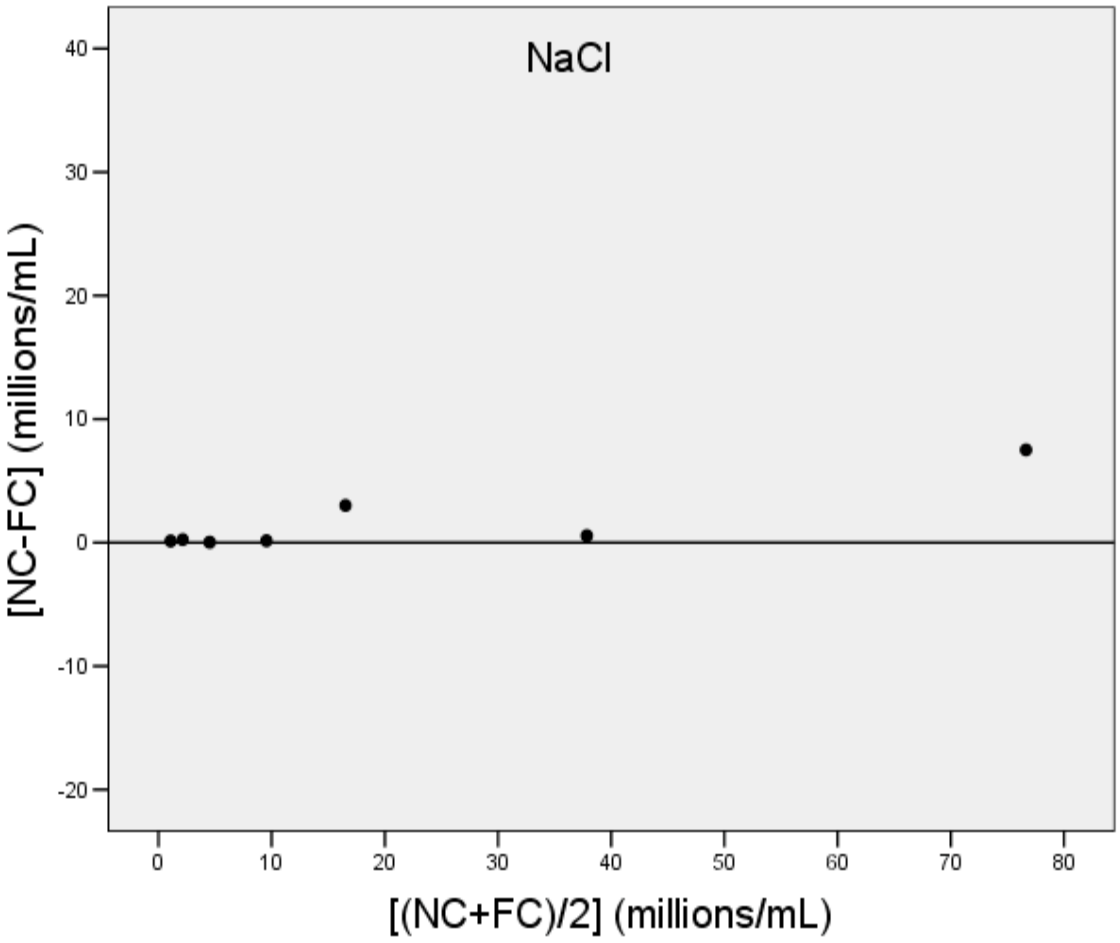

Figure 39. Bland and Altman plot comparing the difference in spermatozoal concentration between the NucleoCounter (NC) and the flow cytometer (FC) to the mean of the NucleoCounter (NC) and the flow cytometer (FC)) when spermatozoa are diluted in NaCl

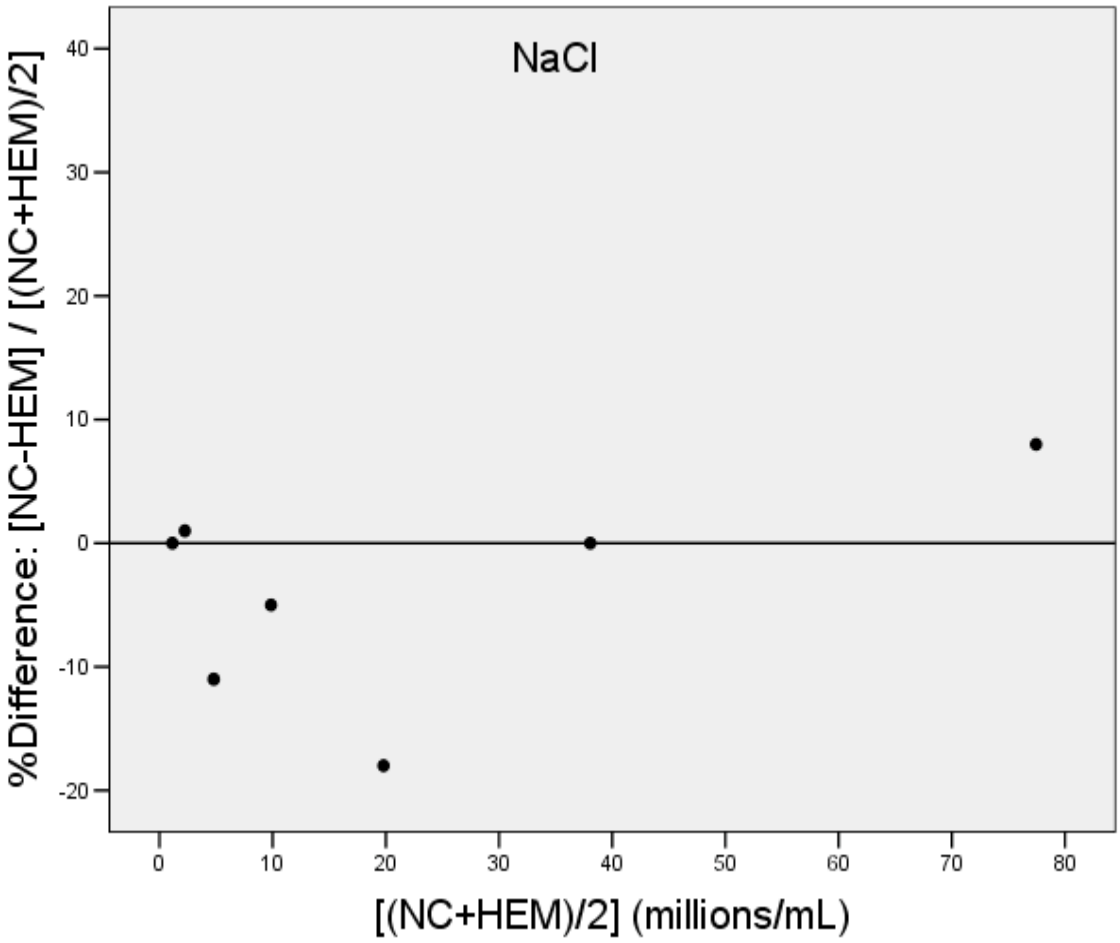

Figure 40. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the NucleoCounter (NC) and hemacytometer (HEM) to the mean of the NucleoCounter (NC) and hemacytometer (HEM) when spermatozoa are diluted in NaCl

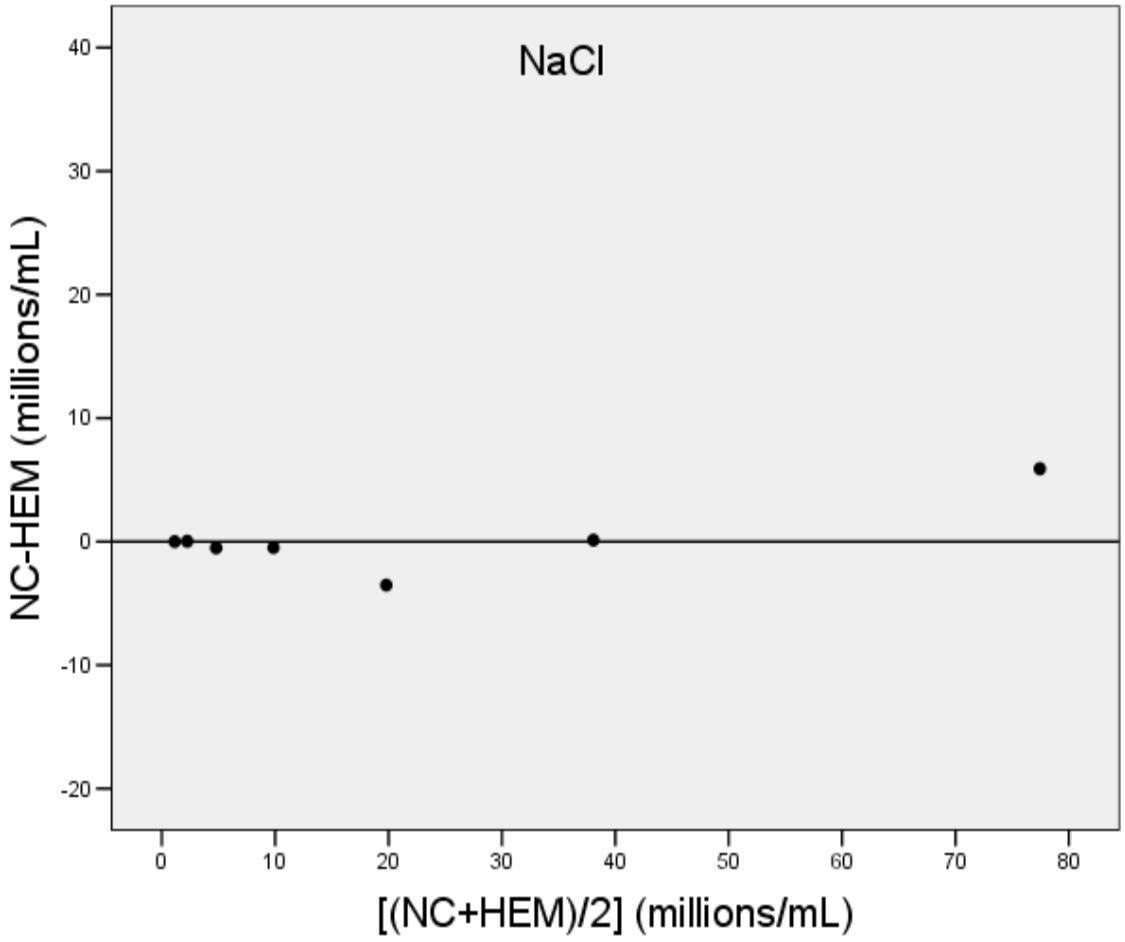

Figure 41. Bland and Altman plot comparing the difference in spermatozoal concentration between the NucleoCounter (NC) and the hemacytometer (HEM) to the mean of the NucleoCounter (NC) and the hemacytometer (HEM)) when spermatozoa are diluted in NaCl

Densimeter Bland and Altman comparisons

The Densimeter showed greater percentage and actual number agreement with the hemacytometer than with the flow cytometer when ejaculated spermatozoa were diluted in NaCl (Figures 42-45). The Densimeter showed similar percentage and actual

number differences when compared to the NucleoCounter (Figures 42 and 43).

Comparisons with the NucleoCounter are shown in Figures 46 and 47.

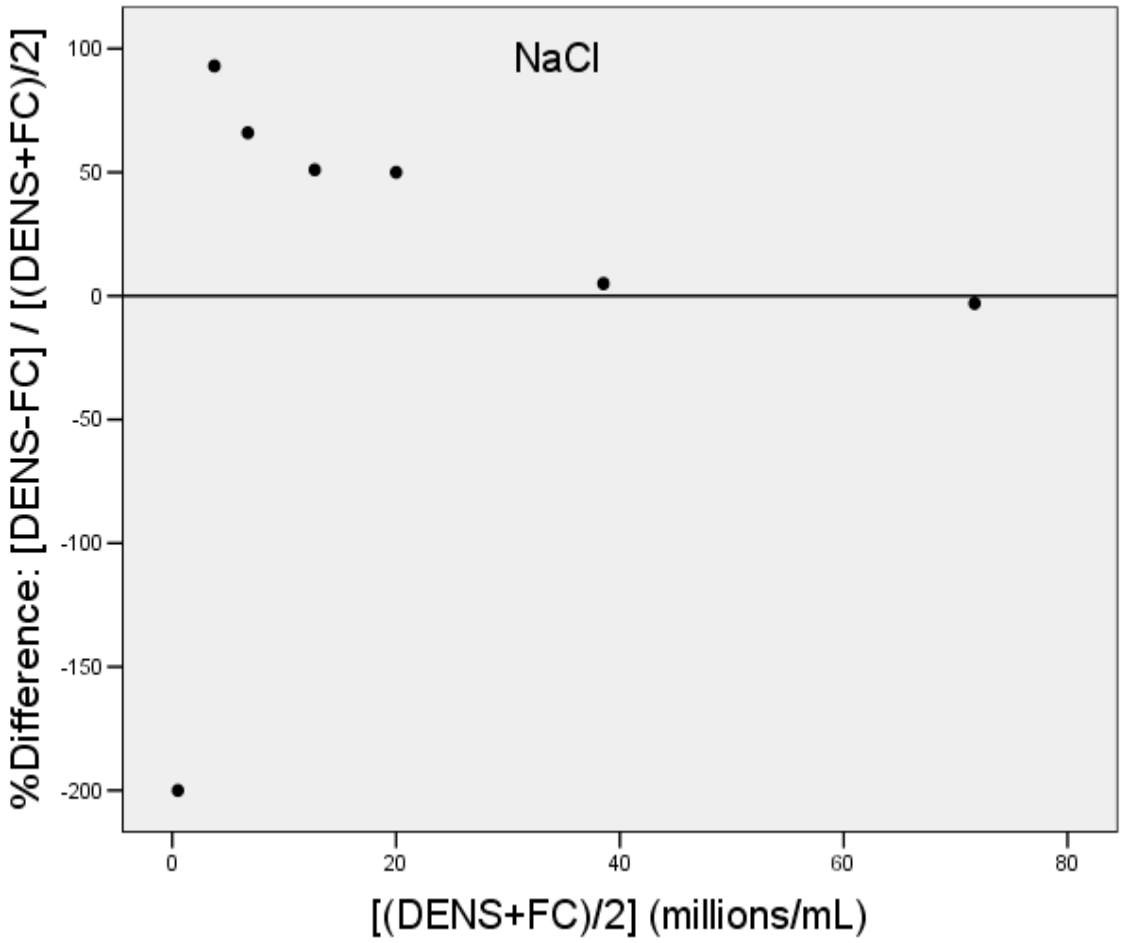

Figure 42. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the Densimeter (DENS) and flow cytometer (FC) to the mean of the Densimeter (DENS) and flow cytometer (FC) when spermatozoa are diluted in NaCl

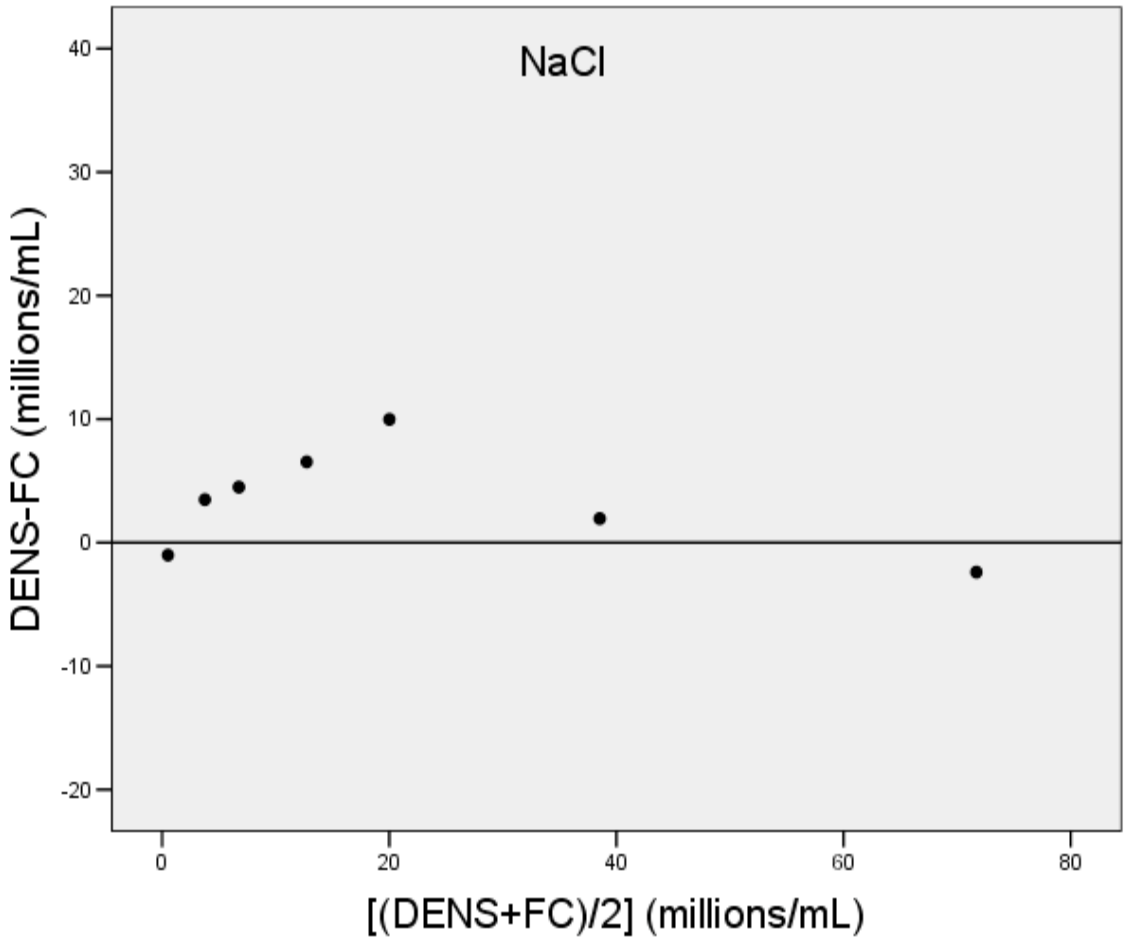

Figure 43. Bland and Altman plot comparing the difference in spermatozoal concentration between the Densimeter (DENS) and the flow cytometer (FC) to the mean of the Densimeter (DENS) and the flow cytometer (FC)) when spermatozoa are diluted in NaCl

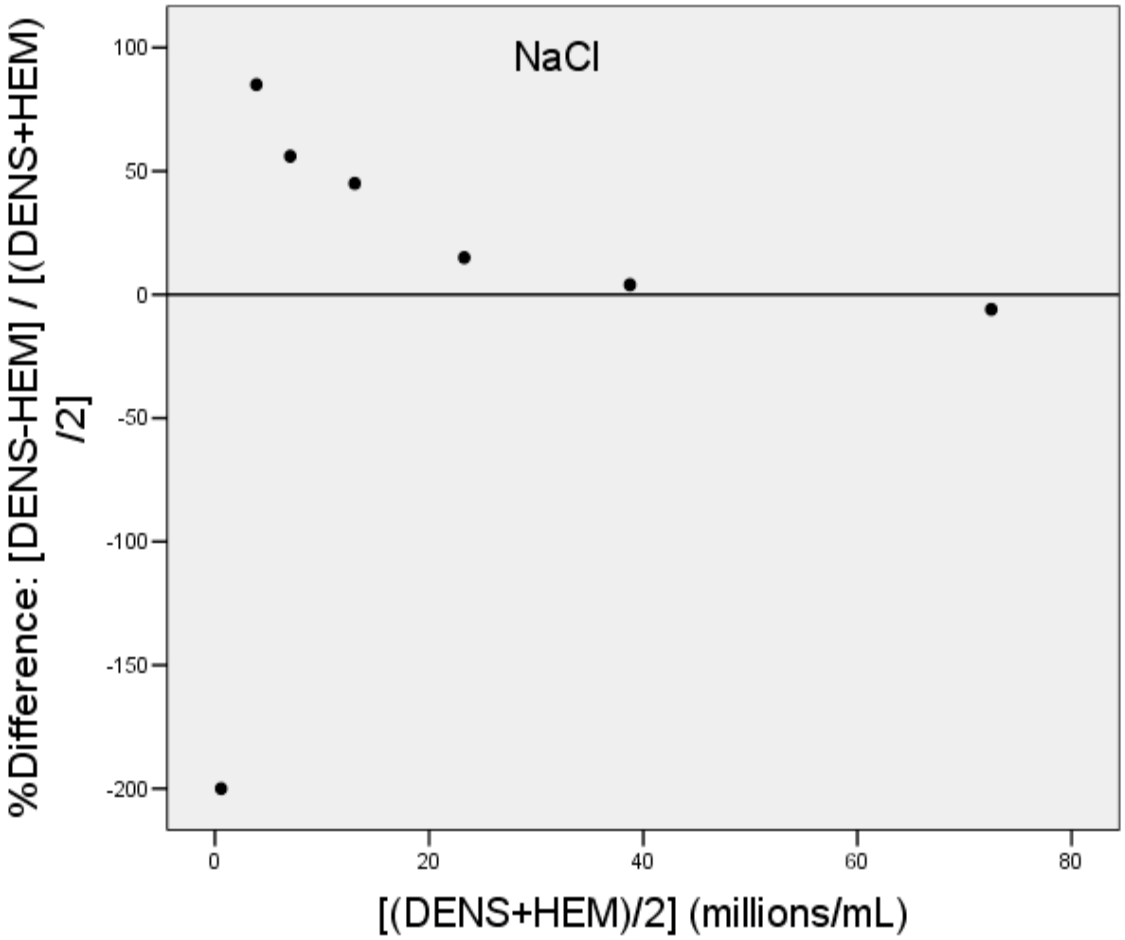

Figure 44. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the Densimeter (DENS) and hemacytometer (HEM) to the mean of the Densimeter (DENS) and hemacytometer (HEM) when spermatozoa are diluted in NaCl

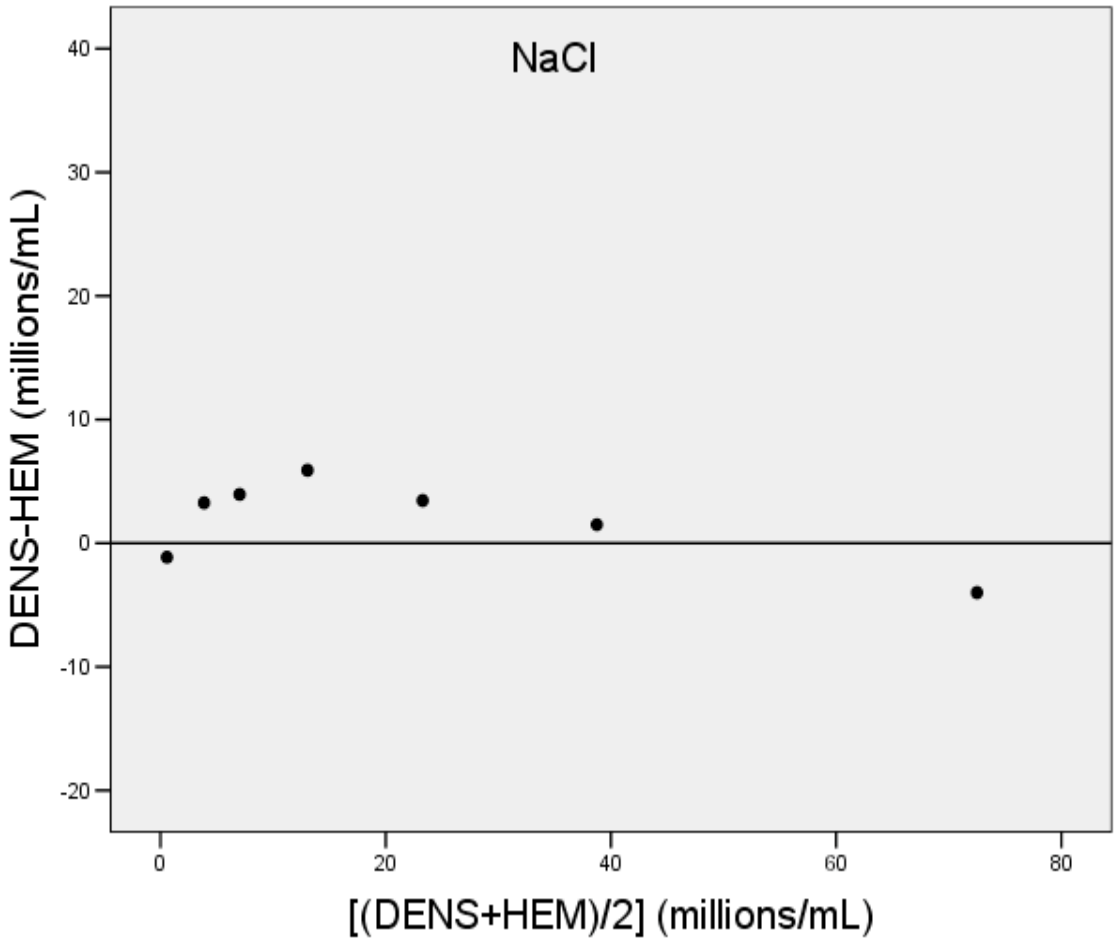

Figure 45. Bland and Altman plot comparing the difference in spermatozoal concentration between the Densimeter (DENS) and the hemacytometer (HEM) to the mean of the Densimeter (DENS) and the hemacytometer (HEM)) when spermatozoa are diluted in NaCl

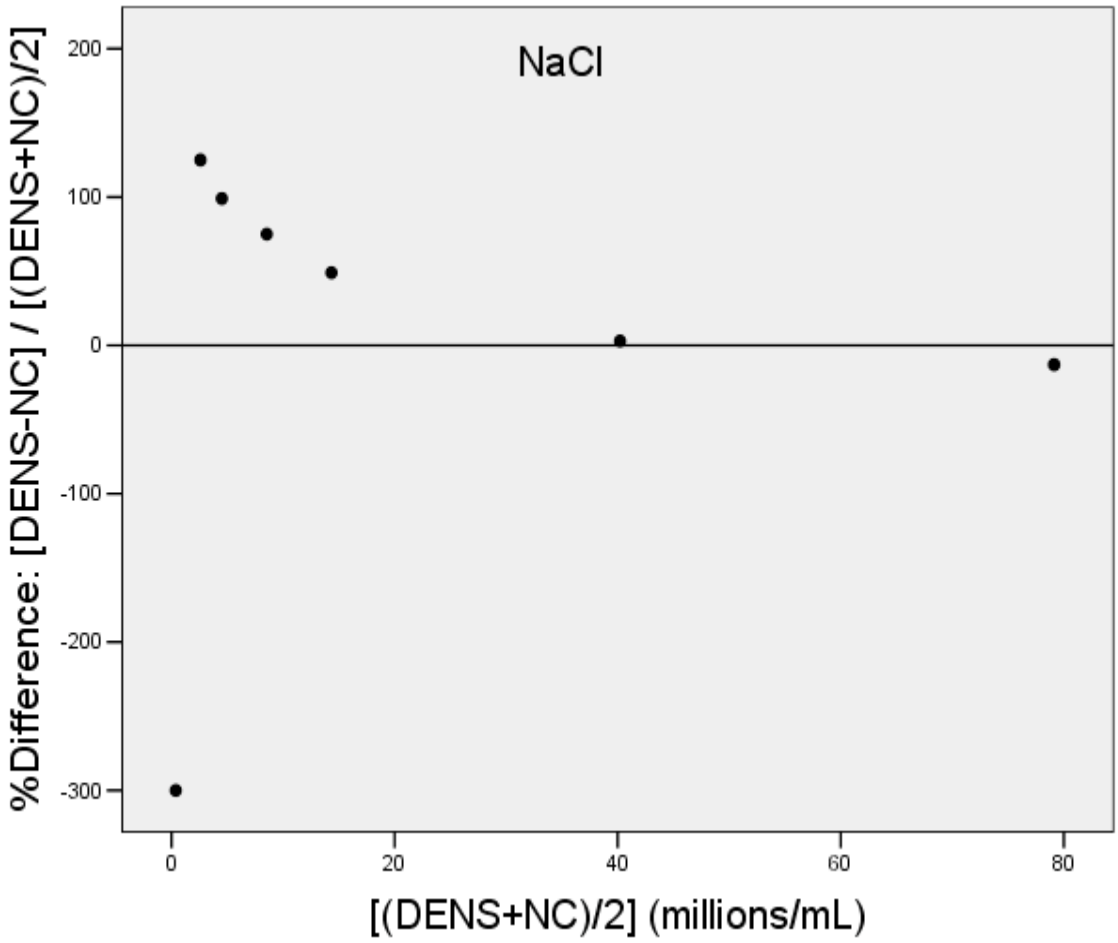

Figure 46. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the Densimeter (DENS) and the NucleoCounter (NC) to the mean of the Densimeter (DENS) and the NucleoCounter (NC) when spermatozoa are diluted in NaCl

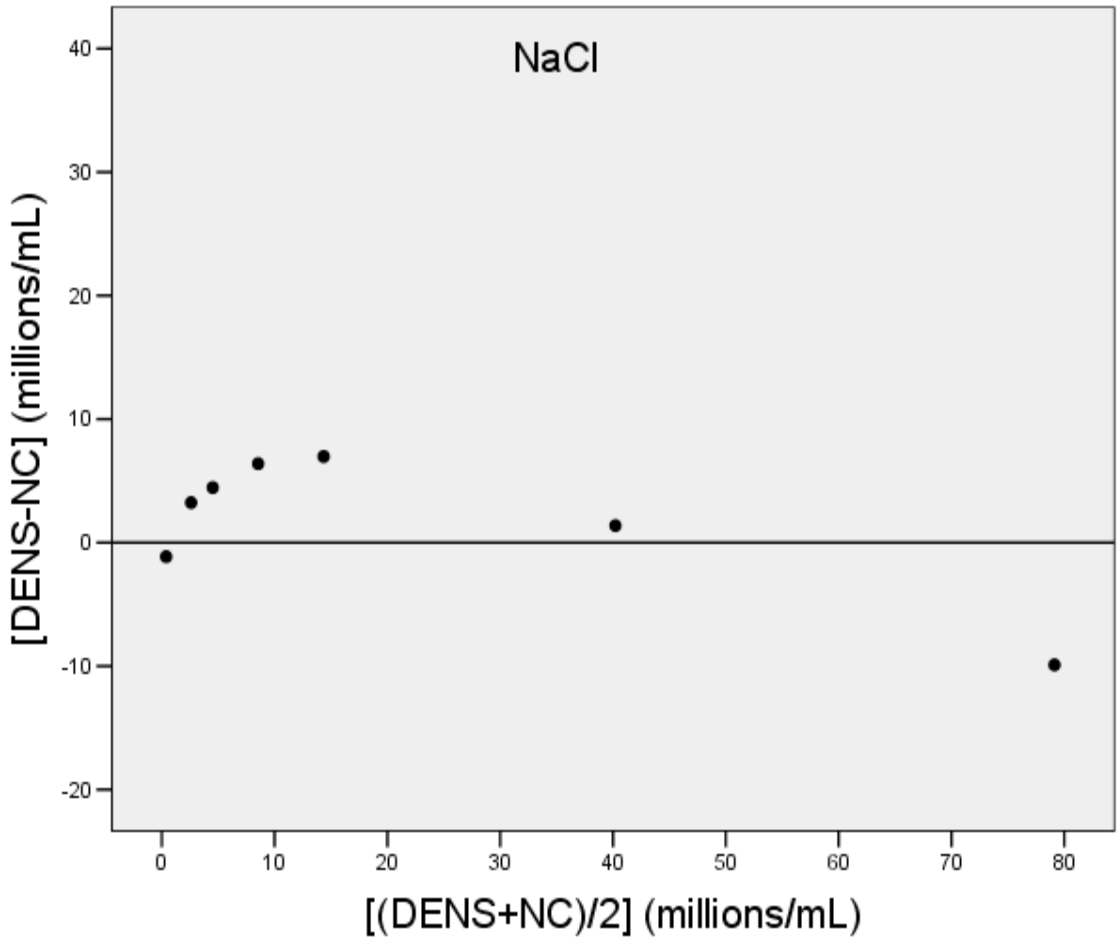

Figure 47. Bland and Altman plot comparing the difference in spermatozoal concentration between the Densimeter (DENS) and the NucleoCounter (NC) to the mean of the Densimeter (DENS) and the NucleoCounter (NC)) when spermatozoa are diluted in NaCl

Spermacue Bland and Altman comparisons

The Spermacue showed similar percentage and actual number differences when compared with the flow cytometer, hemacytometer and the NucleoCounter (Figures 48-53).

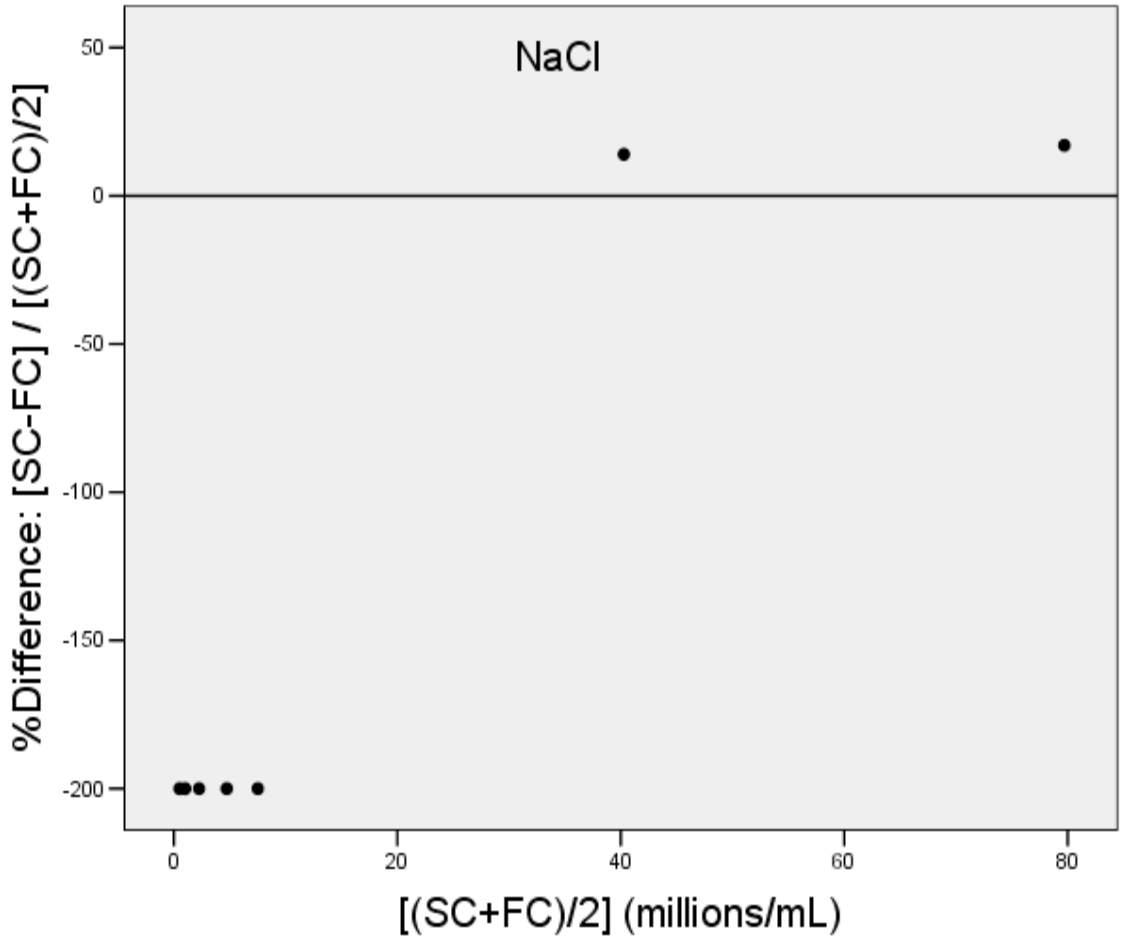

Figure 48. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the Spermacue (SC) and flow cytometer (FC) to the mean of the Spermacue (SC) and flow cytometer (FC) when spermatozoa are diluted in NaCl

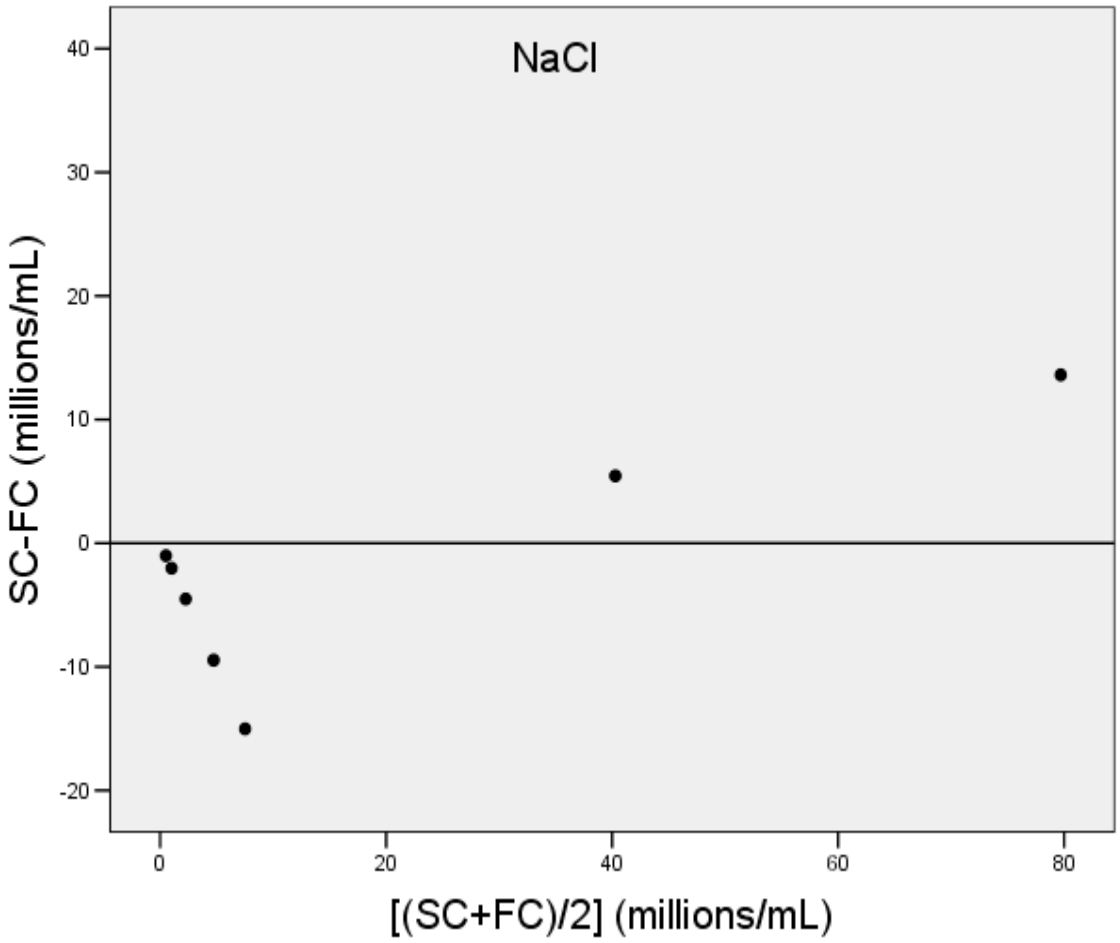

Figure 49. Bland and Altman plot comparing the difference in spermatozoal concentration between the Spermacue (SC) and the flow cytometer (FC) to the mean of the Spermacue (SC) and the flow cytometer (FC) when spermatozoa are diluted in NaCl

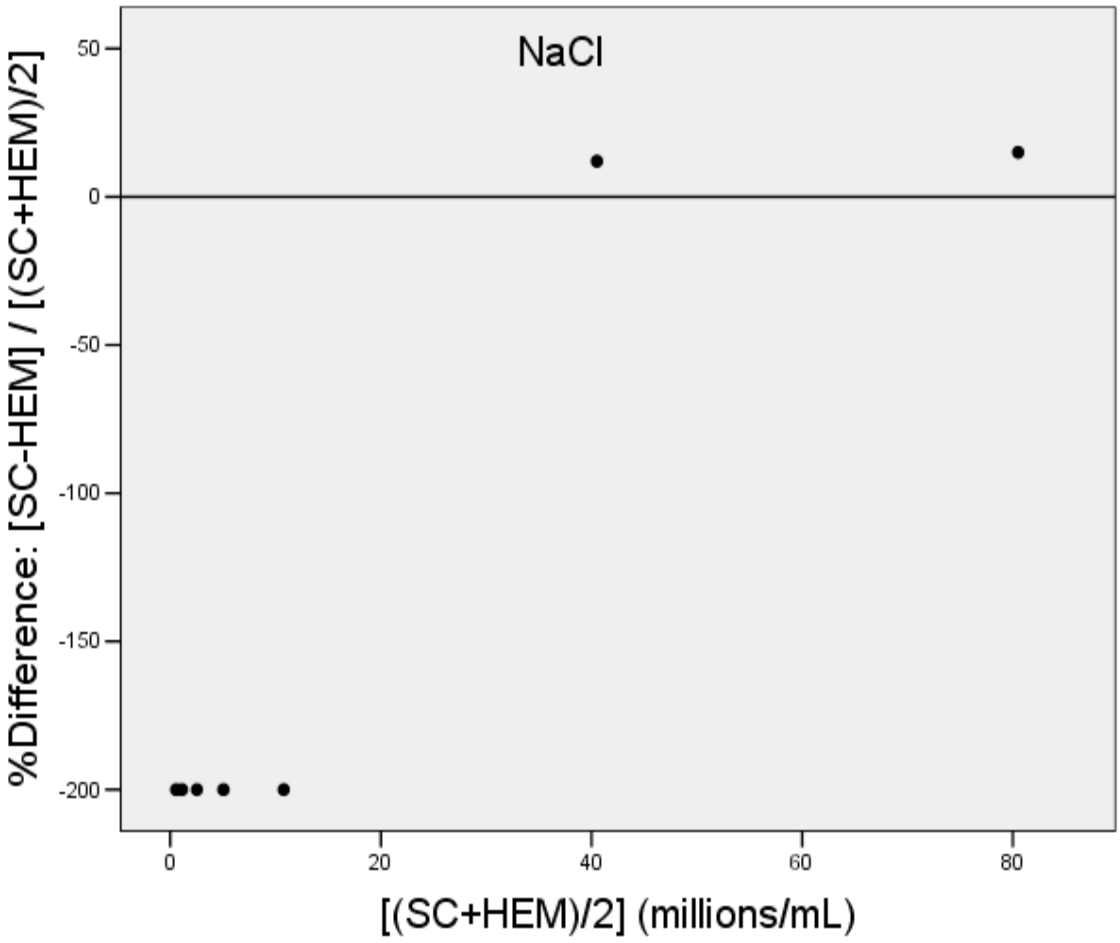

Figure 50. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the Spermacue (SC) and hemacytometer (HEM) to the mean of the Spermacue (SC) and hemacytometer (HEM) when spermatozoa are diluted in NaCl

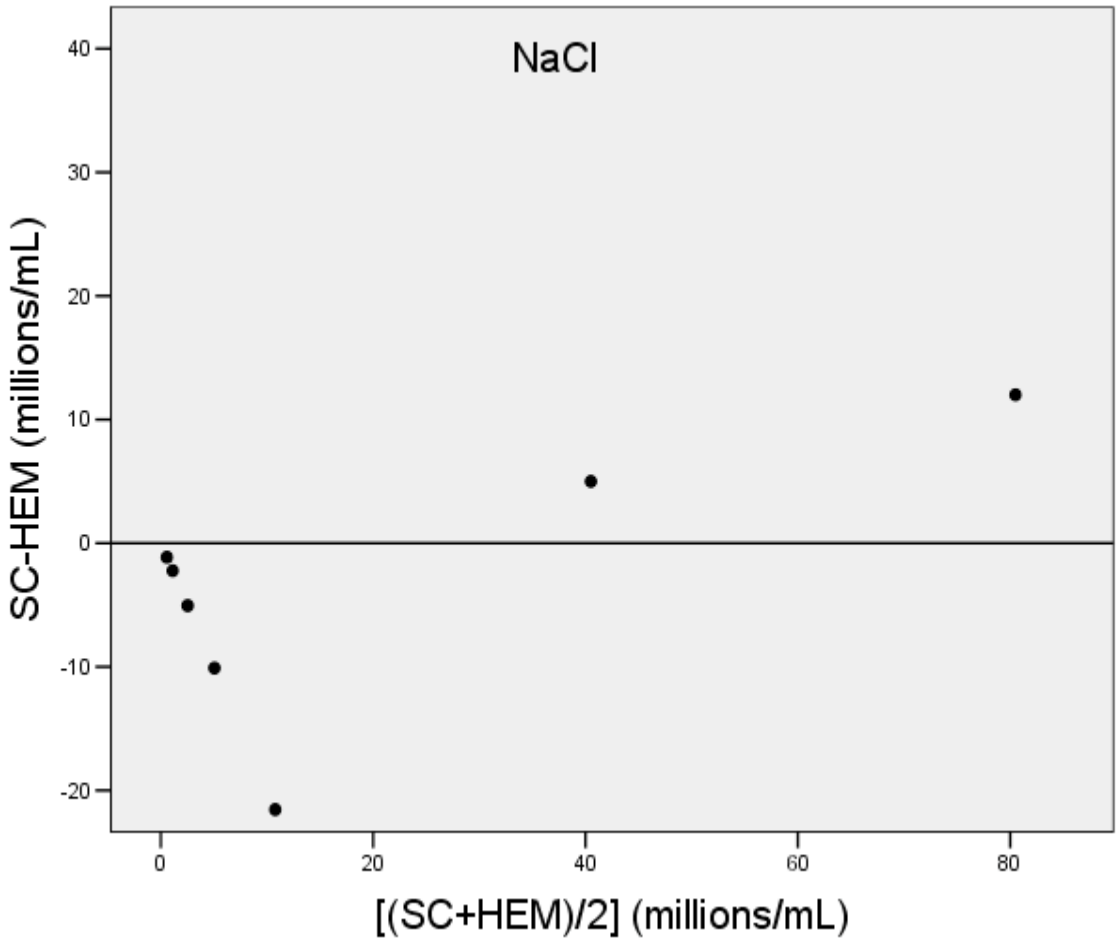

Figure 51. Bland and Altman plot comparing the difference in spermatozoal concentration between the Spermacue (SC) and the hemacytometer (HEM) to the mean of the Spermacue (SC) and the hemacytometer (HEM) when spermatozoa are diluted in NaCl

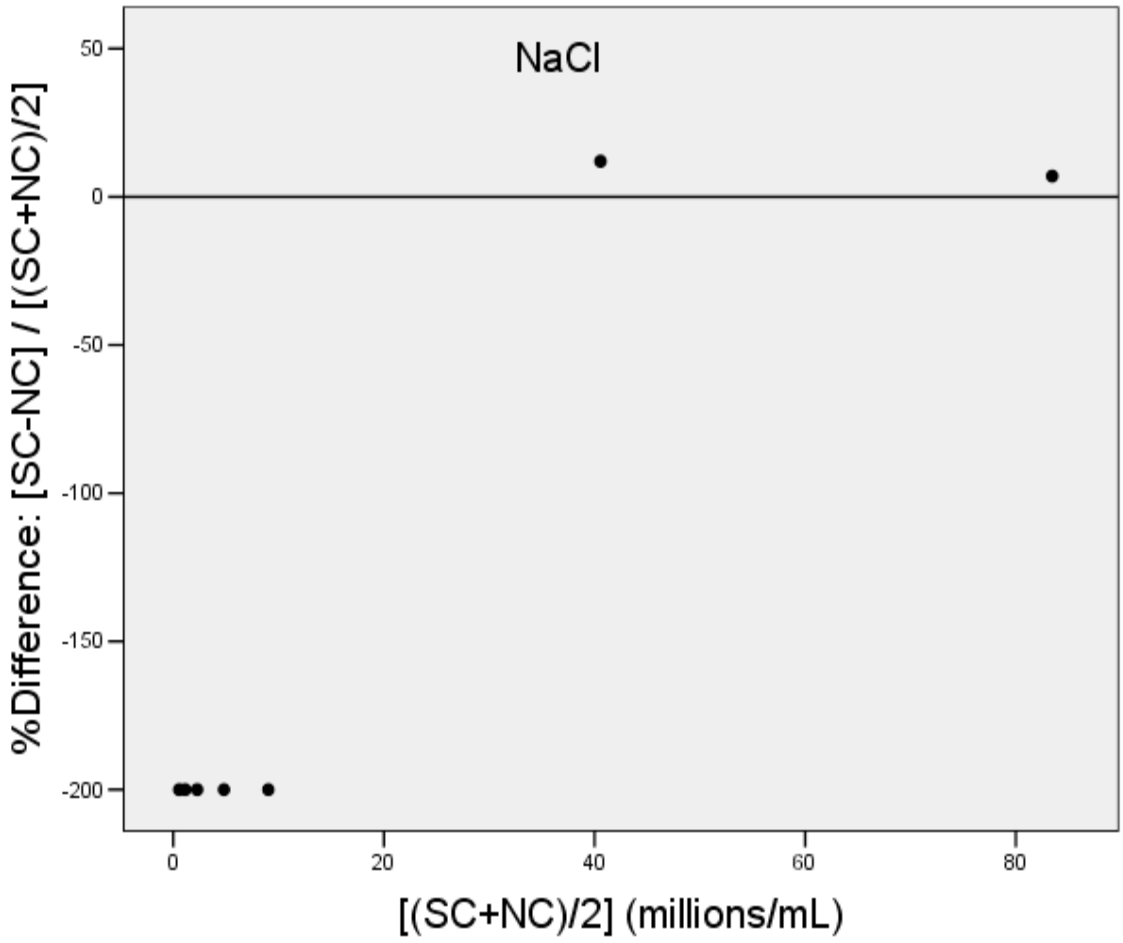

Figure 52. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the Spermacue (SC) and NucleoCounter (NC) to the mean of the Spermacue (SC) and NucleoCounter (NC) when spermatozoa are diluted in NaCl

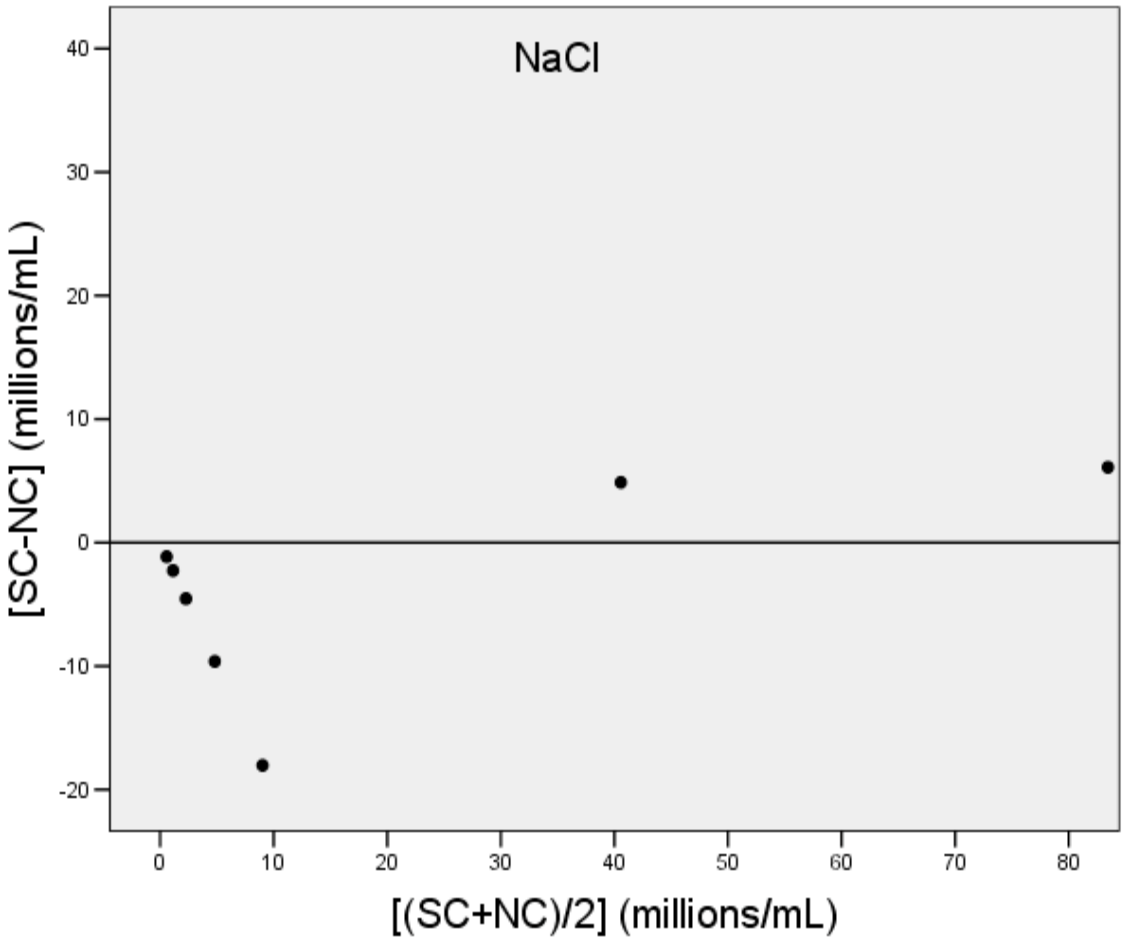

Figure 53. Bland and Altman plot comparing the difference in spermatozoal concentration between the Spermacue (SC) and the NucleoCounter (NC) to the mean of the Spermacue (SC) and the NucleoCounter (NC) when spermatozoa are diluted in NaCl

Experiment 10

Regression analysis

Regression plots were generated for the NucleoCounter compared to the flow cytometer and hemacytometer, the Densimeter and the Spermacue compared to the flow cytometer, the hemacytometer and the NucleoCounter over a 5-800 x 10^6/mL concentration range (Figures 54-56). The NucleoCounter and Densimeter had slopes near 1 (line of equality) when compared to flow cytometry and hemacytometry. The y-intercepts of the NucleoCounter indicated an underestimation of 8.61 x 10^6/mL and 6 x 10^6/mL respectively for the flow cytometer and the hemacytometer. The Densimeter y-intercepts indicated an overestimation of 4.18 x 10^6/mL and 10 x 10^6/mL respectively for the flow cytometer and hemacytometer. The Spermacue y-intercept had the largest deviation from zero with y-intercept values of 34.1 x 10^6/mL and 40.1 x 10^6/mL respectively for the flow cytometer and hemacytometer. The Densimeter had a slope of -1.01 compared to the NucleoCounter and a y-intercept of 9.0. The Spermacue had a slope of 0.81 and a y-intercept of 30.1 when compared to the NucleoCounter. Data for photometric systems reflects dilution at spermatozoal concentrations >300 x 10^6/mL. All regression data is summarized in Table 13.

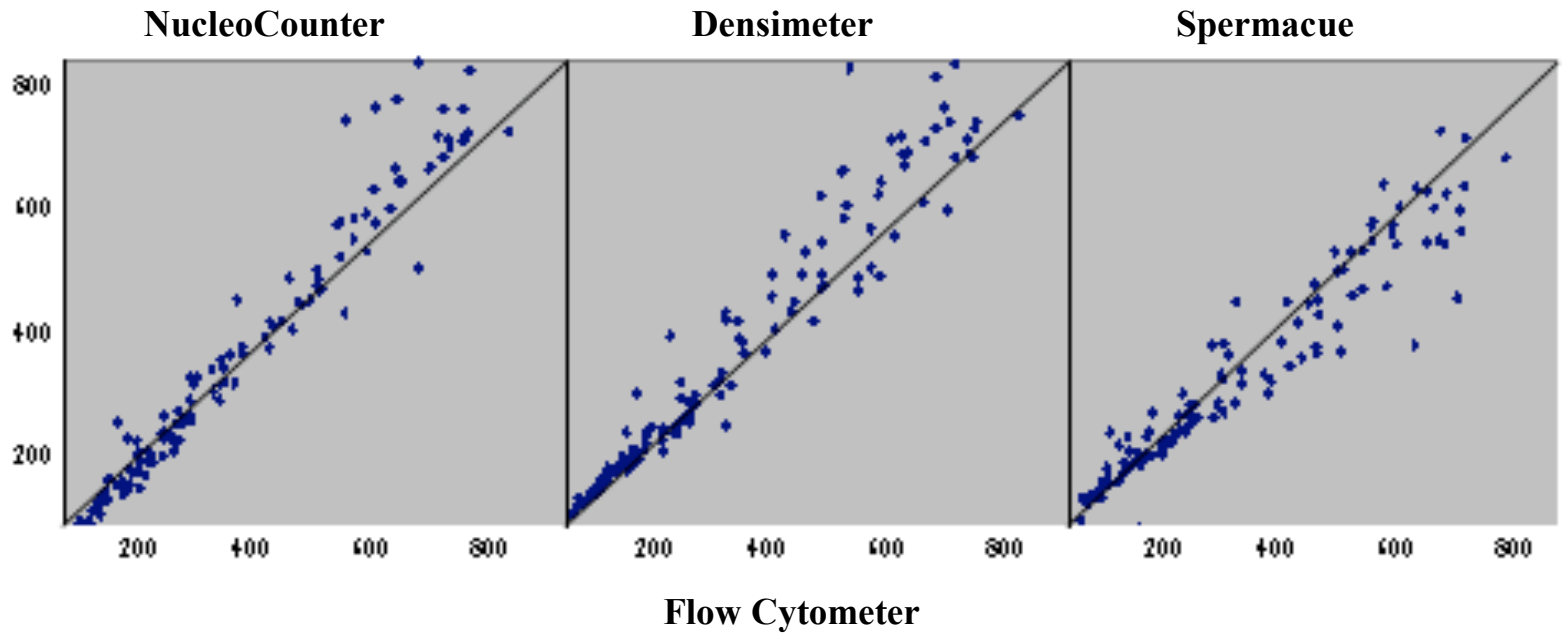

Figure 54. Regression scatterplots (5-800 x 10^6/mL concentration range) for the NucleoCounter (far left), the Densimeter (middle) and the Spermacue (far right) with the flow cytometer and plotted against a line of equality

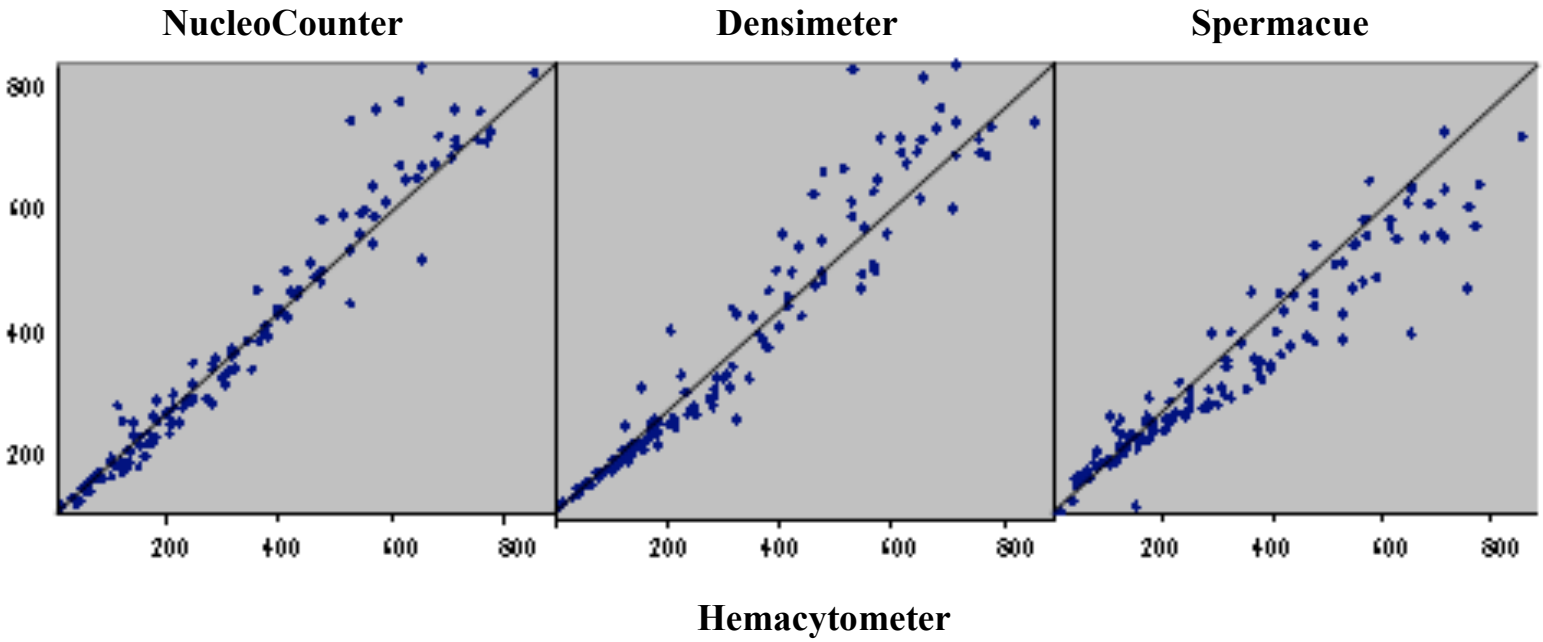

Figure 55. Regression scatterplots (5-800 x 10^6/mL concentration range) for the NucleoCounter (far left), the Densimeter (middle) and the Spermacue (far right) with the hemacytometer and plotted against a line of equality

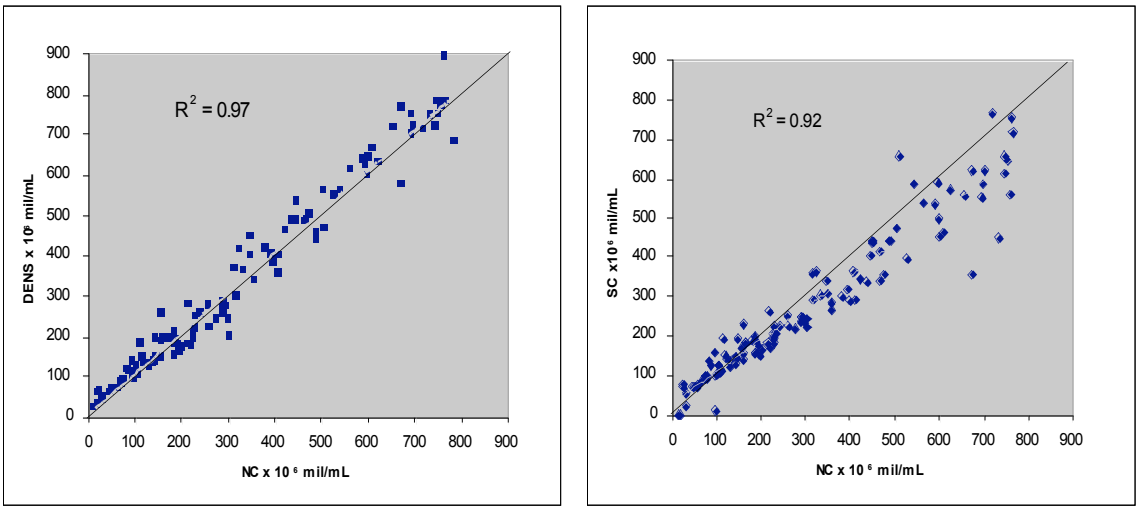

Figure 56. Regression scatterplots (5-800 x 10^6/mL concentration range) for the Densimeter (left) and the Spermacue (right) with the NucleoCounter and plotted against a line of equality

Table 13

Regression values for the NucleoCounter (NC), Densimeter (DENS), Spermacue (SC), flow cytometer (FC) and hemacytometer (HEM) (5-800 x 10^6/mL concentration range)

Instrument Comparison	R^2	Slope	y-Intercept
NC-FC	0.95	1.14	-8.6
NC-HEM	0.92	1.05	-6.0
NC-DENS	0.97	-1.01	9.0
NC-SC	0.92	0.81	30.1
DENS-FC	0.95	1.12	4.2
DENS-HEM	0.90	1.03	10.0
SC-FC	0.92	0.86	34.1
SC-HEM	0.87	0.78	40.1

Coefficient of variation

The hemacytometer was the least repeatable instrument, but all coefficients of variation are well below 10% (Table 14).

Table 14

Within-sample repeatability (coefficient of variation) for measurement of spermatozoal concentration using the flow cytometer (FC), hemacytometer (HEM), NucleoCounter (NC), Spermacue (SC), and Densimeter (DENS) (5-800 x 10^6/mL concentration range)

Instrument	Coefficient of variation: 3 replicates (%)
FC	2.95
HEM	6.69
NC	3.17
DENS	3.62
SC	2.77

Original and modified Bland and Altman plots

Over all concentration ranges (5-800 x 10^6/mL), percent difference between the flow cytometer and hemacytometer was the lowest (Table 15). In the 0-200 x 10^6/mL concentration range, the NucleoCounter showed a lower percentage difference when compared to the flow cytometer than did the Densimeter or the Spermacue and was similar to the Densimeter compared to the hemacytometer. At lower (<200 x 10^6/mL)

concentrations, the photometric methods had a greater mean percentage difference than the NucleoCounter when compared to the flow cytometer and hemacytometer. At 201-500 x 10^6/mL, the NucleoCounter showed the lowest percentage difference from the flow cytometer and hemacytometer, but photometric methods had similar but slightly greater values. At 501-800 x 10^6/mL, the NucleoCounter had similar percentage and actual number difference with the Densimeter compared to both flow cytometry and hemacytometry. When compared directly to the Densimeter and the Spermacue, the NucleoCounter had a greater percentage of difference at concentrations <200 x 10^6/mL than all other concentration ranges.

Table 15

Mean percentage (modified Bland and Altman) and actual differences (Bland and Altman) for the NucleoCounter (NC), Densimeter (DENS), Spermacue (SC), flow cytometer (FC) and hemacytometer (HEM) by spermatozoal concentration range using neat semen

Instrument (Paired Comparison)	Concentration Range (10^6 mil/mL)	Mean Difference (%)	Mean Difference (actual number x 10^6)
NC-FC	0-200	13.53	6.71
	201-500	10.66	27.99
	501-800	15.1	93.01
NC-HEM	0-200	18.03	-2.76
	201-500	11.19	7.12
	501-800	14.16	58.87
DENS-NC	0-200	19.54	-15.76
	201-500	1.14	-2.53
	501-800	3.05	-20.95

Table 15

Continued

Instrument (Paired Comparison)	Concentration Range (10^6 mil/mL)	Mean Difference (%)	Mean Difference (actual number x 10^6)
	0-200	15.18	-13.4
SC-NC	201-500	13.98	42.97
	501-800	14.42	84.17
	0-200	21.2	18.31
DENS-FC	201-500	13.13	36.99
	501-800	15.56	91.45
	0-200	18.02	6.35
DENS-HEM	201-500	14.74	-6.6
	501-800	13.82	64.87
	0-200	29.37	15.7
SC-FC	201-500	13.63	-17.01
	501-800	12.32	-48.36
	0-200	25.03	6.04
SC-HEM	201-500	19.43	-40.39
	501-800	18.59	-53.41
	0-200	7.01	-10.64
FC-HEM	201-500	9.69	-32.11
	501-800	3.60	-30.05

NucleoCounter Bland and Altman comparisons

The NucleoCounter had the greatest percentage difference at the lowest (<200 x 10^6/mL) concentration range compared to the flow cytometer and hemacytometer (Figures 57 and 59), while the original Bland and Altman plots (Figures 58 and 60)

showed a greater absolute difference at the higher (>500 x 10^6/mL) concentration range. The NucleoCounter had the greatest percentage difference at the lowest concentration range when compared to the Densimeter (Figure 61) and the greatest absolute difference at concentrations >500 x 10^6/mL (Figure 62). The NucleoCounter had similar percentage difference at all concentration ranges when compared to the Spermacue (Figure 63) and the greatest absolute difference at concentrations >200 x 10^6/mL (Figure 64).

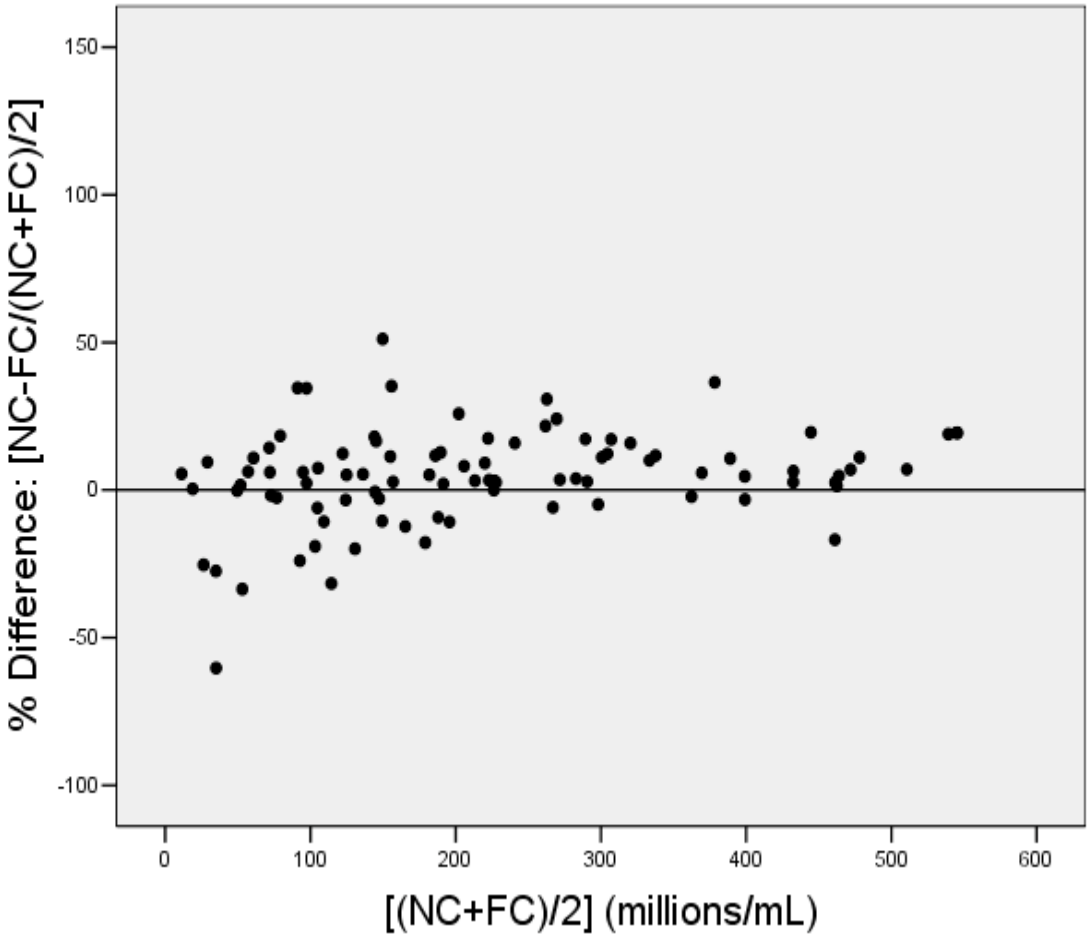

Figure 57. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the NucleoCounter (NC) and flow cytometer (FC) to the mean of the NucleoCounter (NC) and flow cytometer (FC)

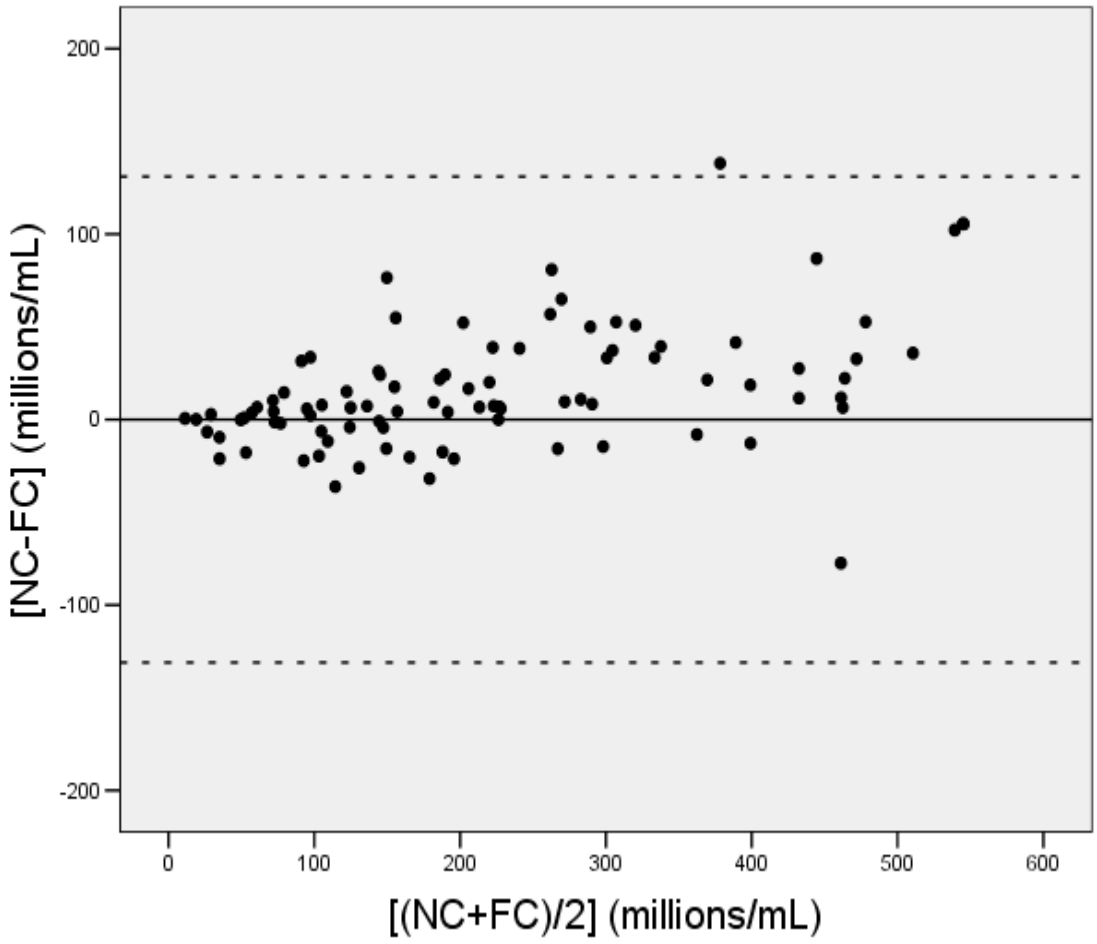

Figure 58. Bland and Altman plot comparing the difference in spermatozoal concentration between the NucleoCounter (NC) and flow cytometer (FC) to the mean of the NucleoCounter (NC) and flow cytometer (FC); dashed lines represent + or – 2 standard deviations

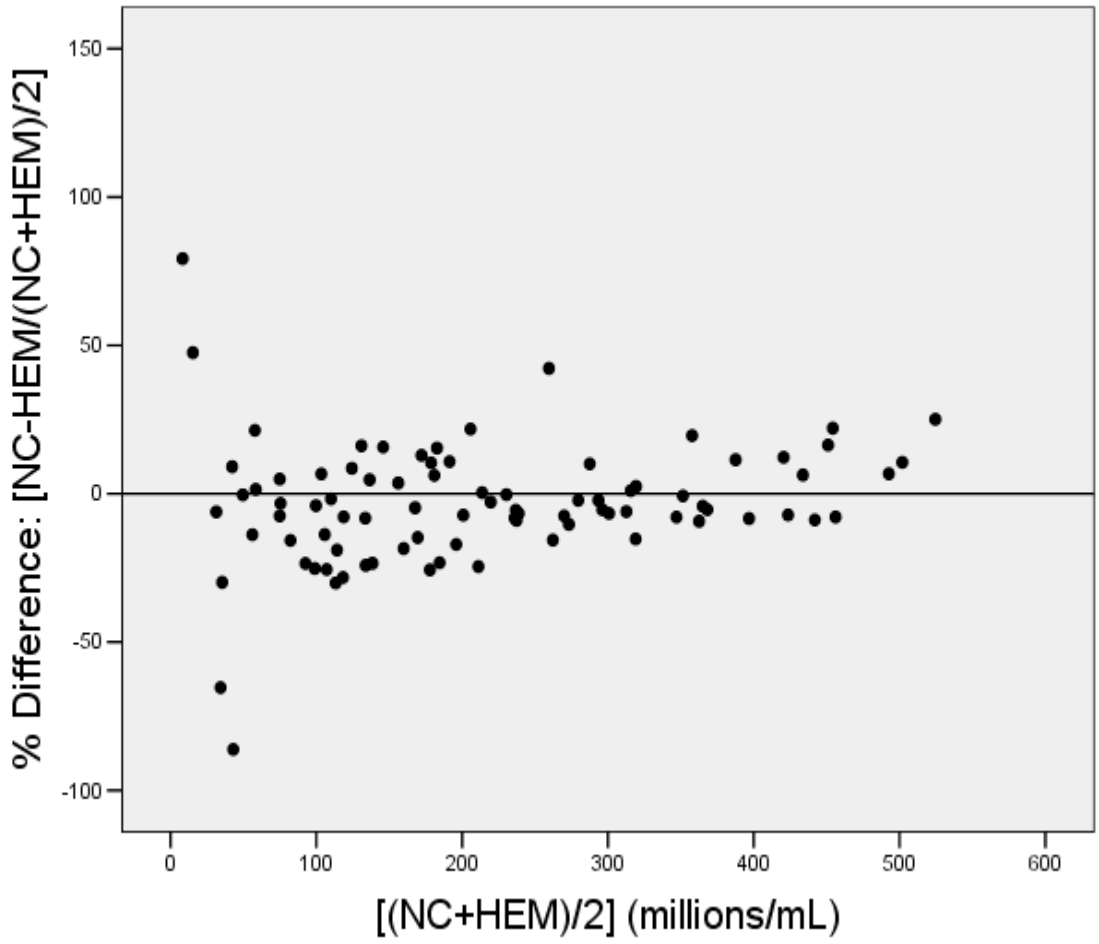

Figure 59. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the NucleoCounter (NC) and hemacytometer (HEM) to the mean of the NucleoCounter (NC) and hemacytometer (HEM)

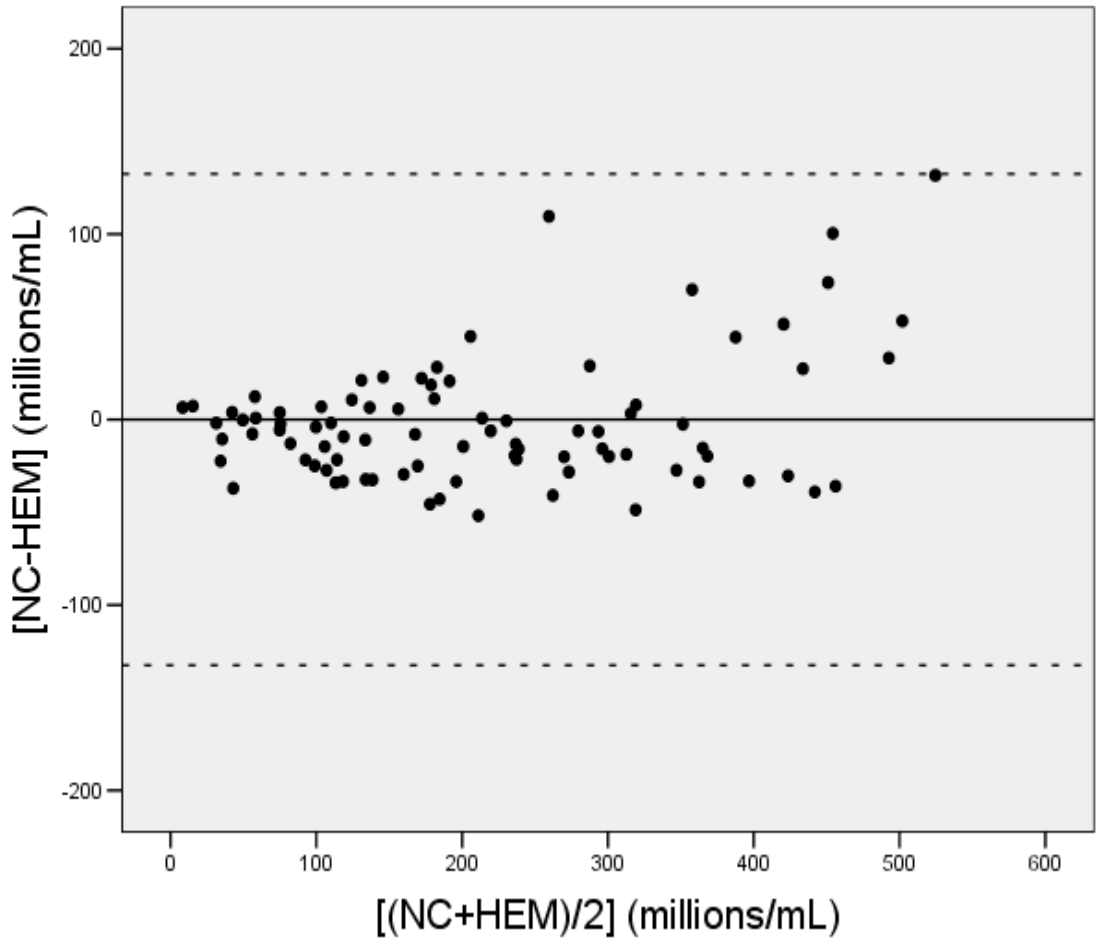

Figure 60. Bland and Altman plot comparing the difference in spermatozoal concentration between the NucleoCounter (NC) and the hemacytometer (HEM) to the mean of the NucleoCounter (NC) and the hemacytometer (HEM)); dashed lines represent + or – 2 standard deviations

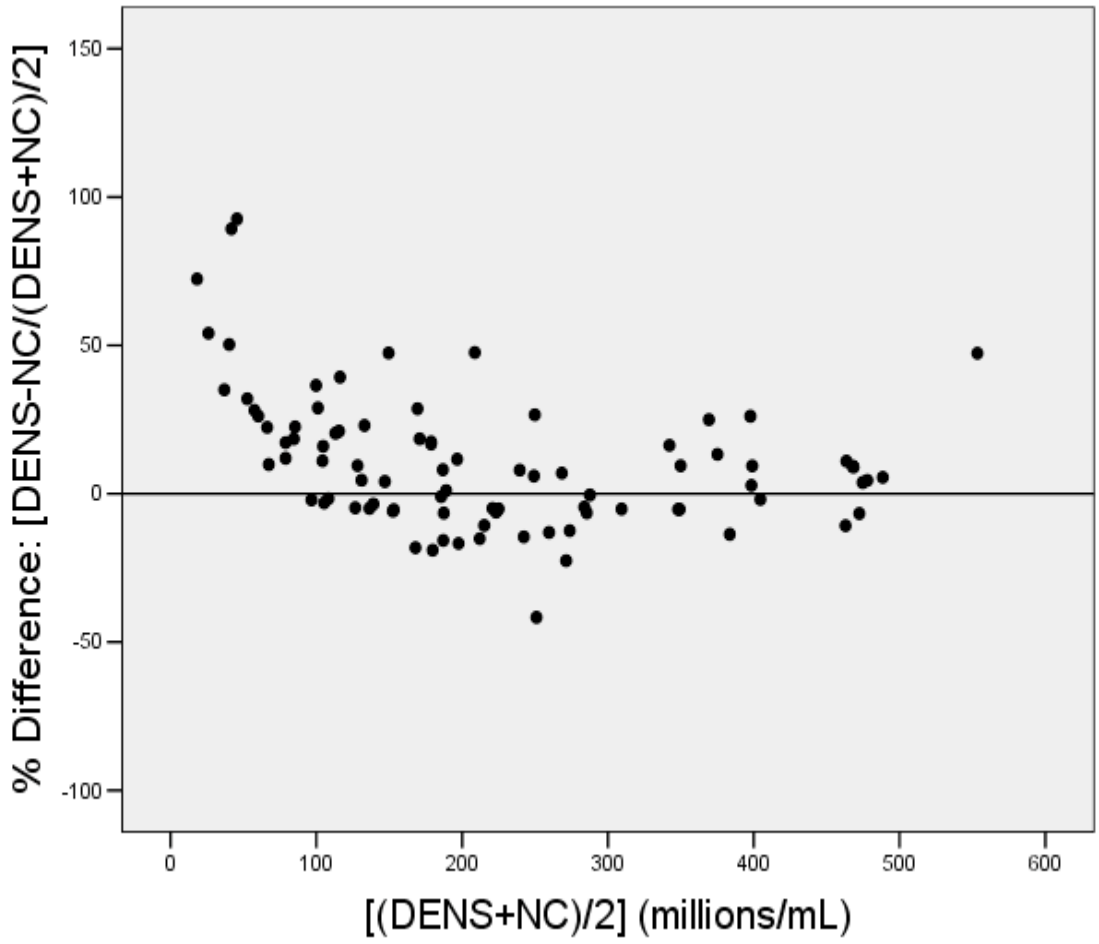

Figure 61. Modified Bland and Altman plot comparing the percentage difference in spermatozoal concentration between the Densimeter (DENS) and the NucleoCounter (NC) to the mean of the Densimeter (DENS) and the NucleoCounter (NC)

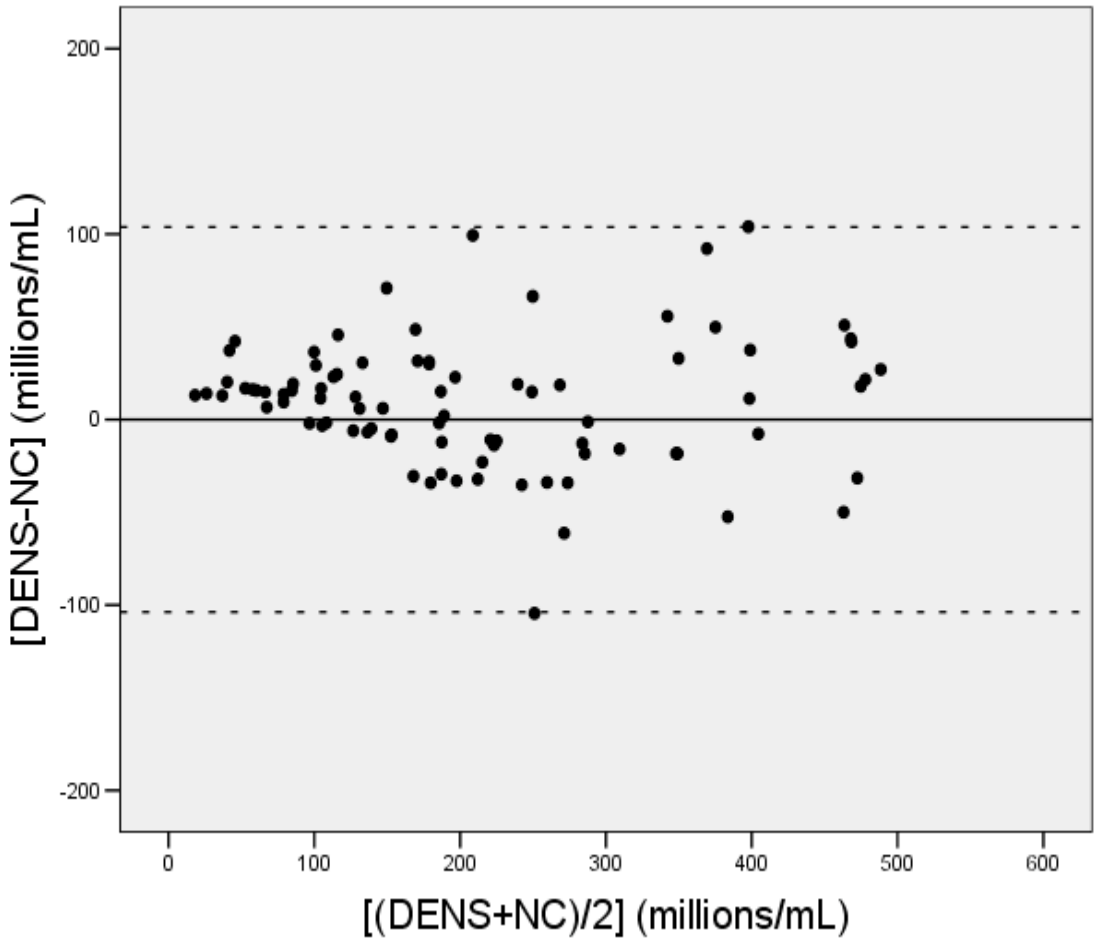

Figure 62. Bland and Altman plot comparing the difference in spermatozoal concentration between the Densimeter (DENS) and the NucleoCounter (NC) to the mean of the Densimeter (DENS) and the NucleoCounter (NC)); dashed lines represent + or – 2 standard deviations

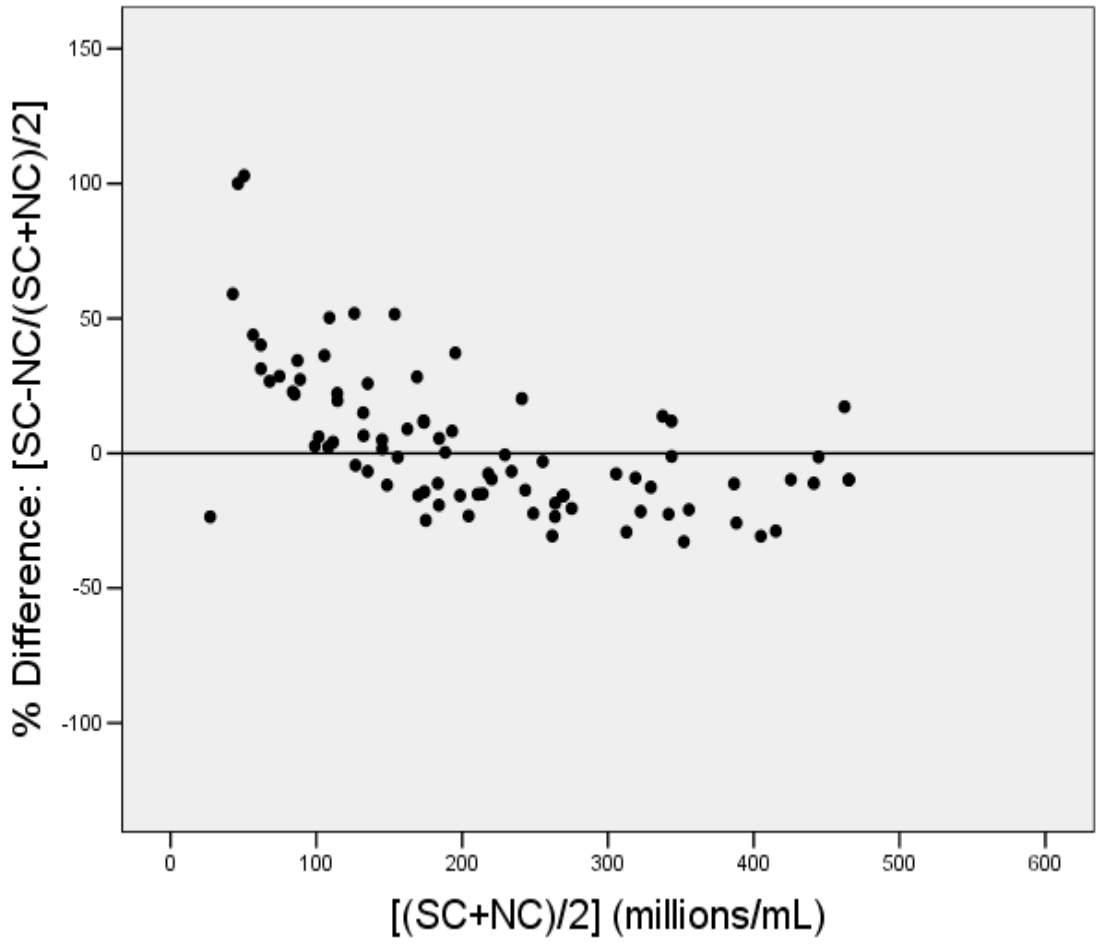

Figure 63. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the Spermacue (SC) and the NucleoCounter (NC) to the mean of the Spermacue (SC) and the NucleoCounter (NC)

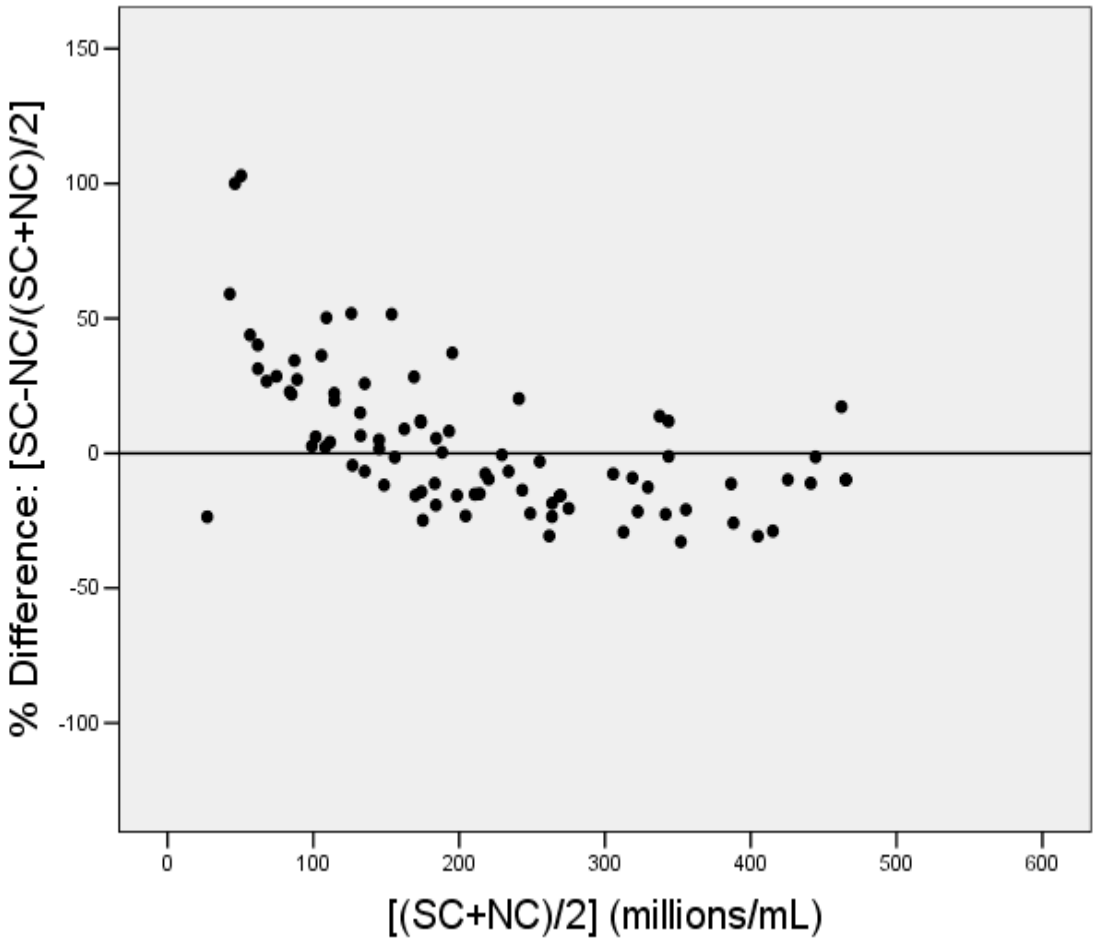

Figure 64. Bland and Altman plot comparing the difference in spermatozoal concentration between the Densimeter (DENS) and NucleoCounter (NC) to the mean of the Densimeter (DENS) and NucleoCounter (NC); dashed lines represent + or – 2 standard deviations

Densimeter Bland and Altman comparisons

The Densimeter overestimated at all concentration ranges (Figures 65 and 66). The percent difference was larger at the lower concentrations, while the absolute difference was higher at the higher concentrations. When compared to the

hemacytometer (Figures 67 and 68), the Densimeter overestimated less at the lower concentrations, but had similar overestimation when compared to the flow cytometer.

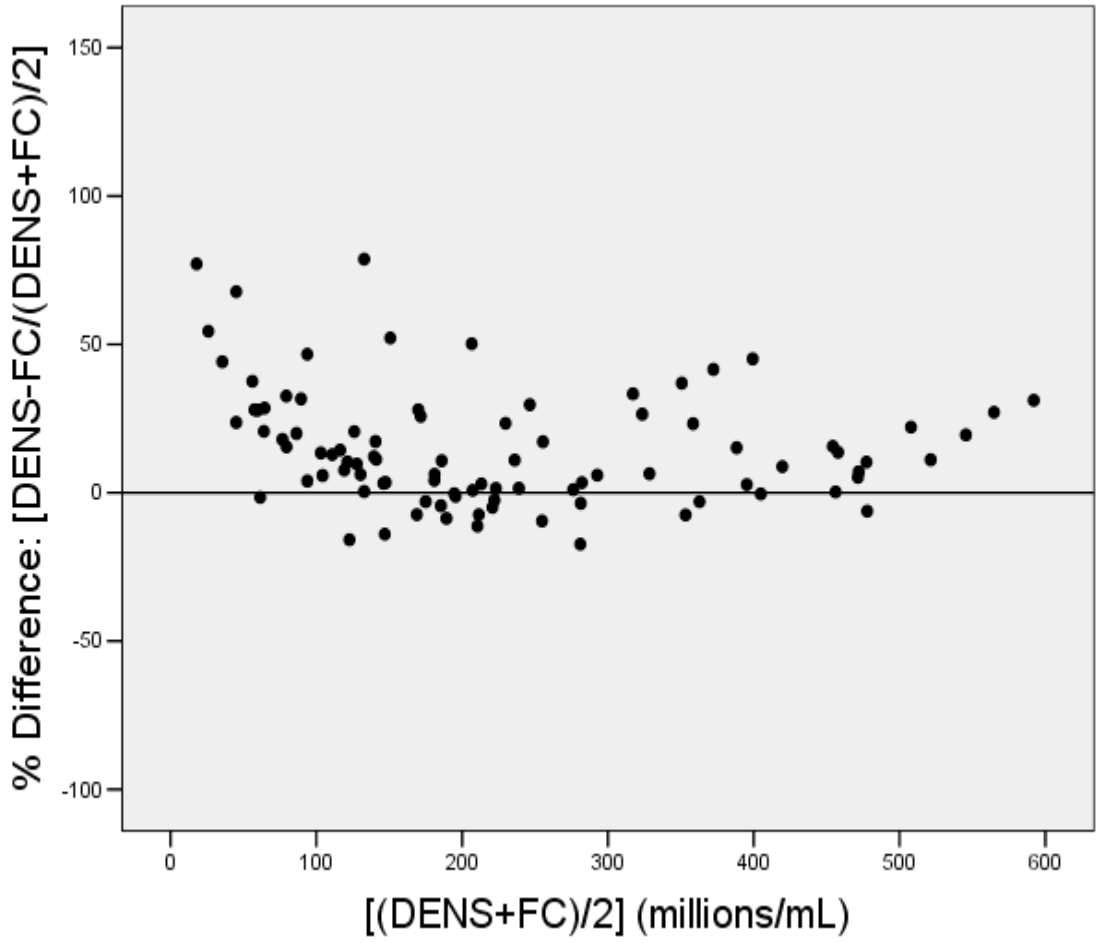

Figure 65. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the Densimeter (DENS) and flow cytometer (FC) to the mean of the Densimeter (DENS) and flow cytometer (FC)

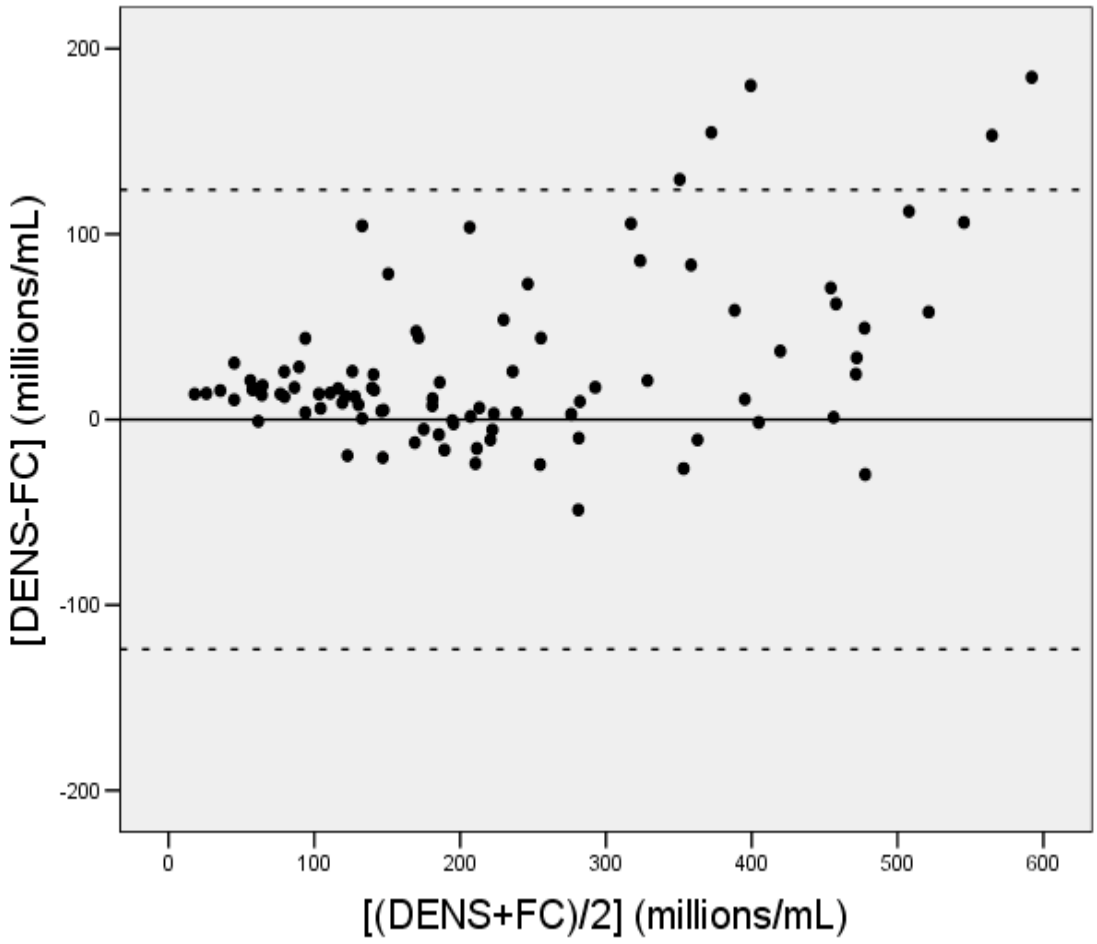

Figure 66. Bland and Altman plot comparing the difference in spermatozoal concentration between the Densimeter (DENS) and flow cytometer (FC) to the mean of the Densimeter (DENS) and flow cytometer (FC)); dashed lines represent + or – 2 standard deviations

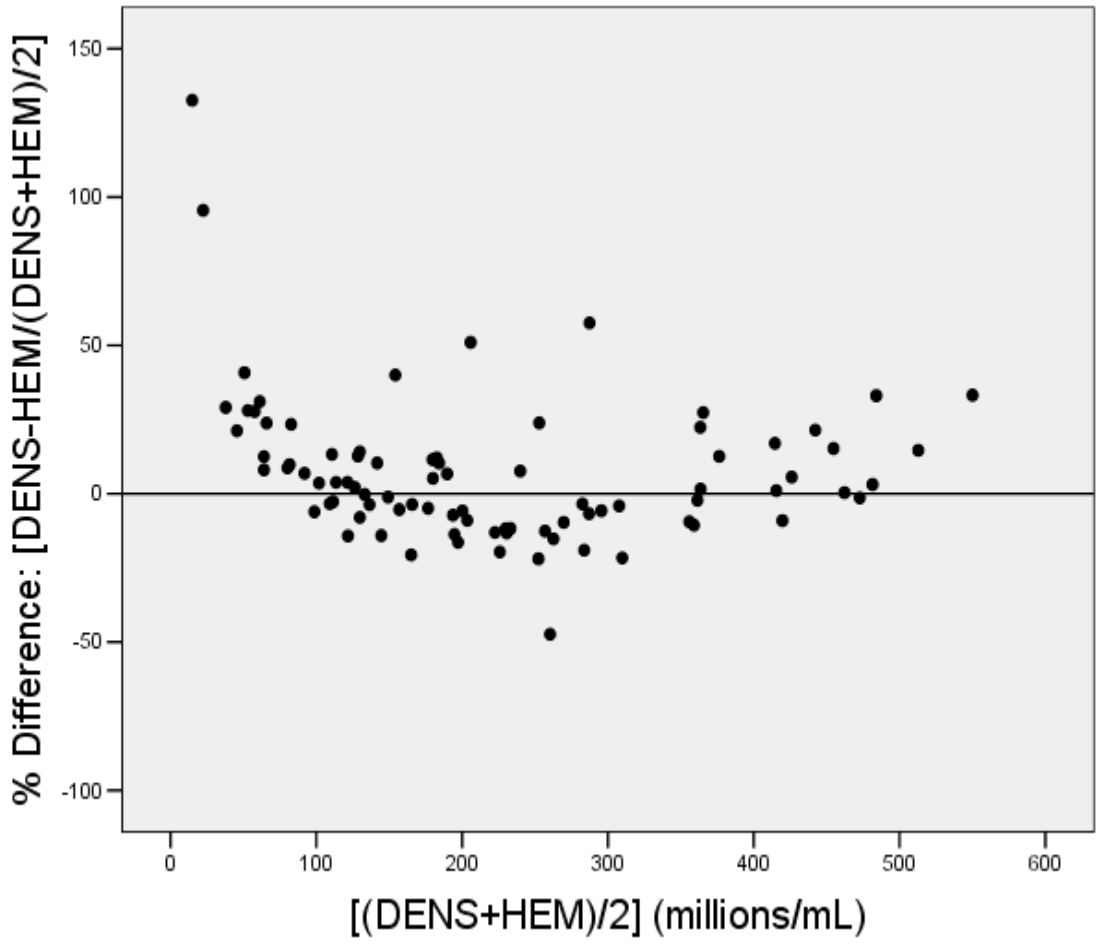

Figure 67. Modified Bland and Altman plot comparing the percentage difference in spermatozoal concentration between the Densimeter (DENS) and the hemacytometer (HEM) to the mean of the Densimeter (DENS) and the hemacytometer (HEM)

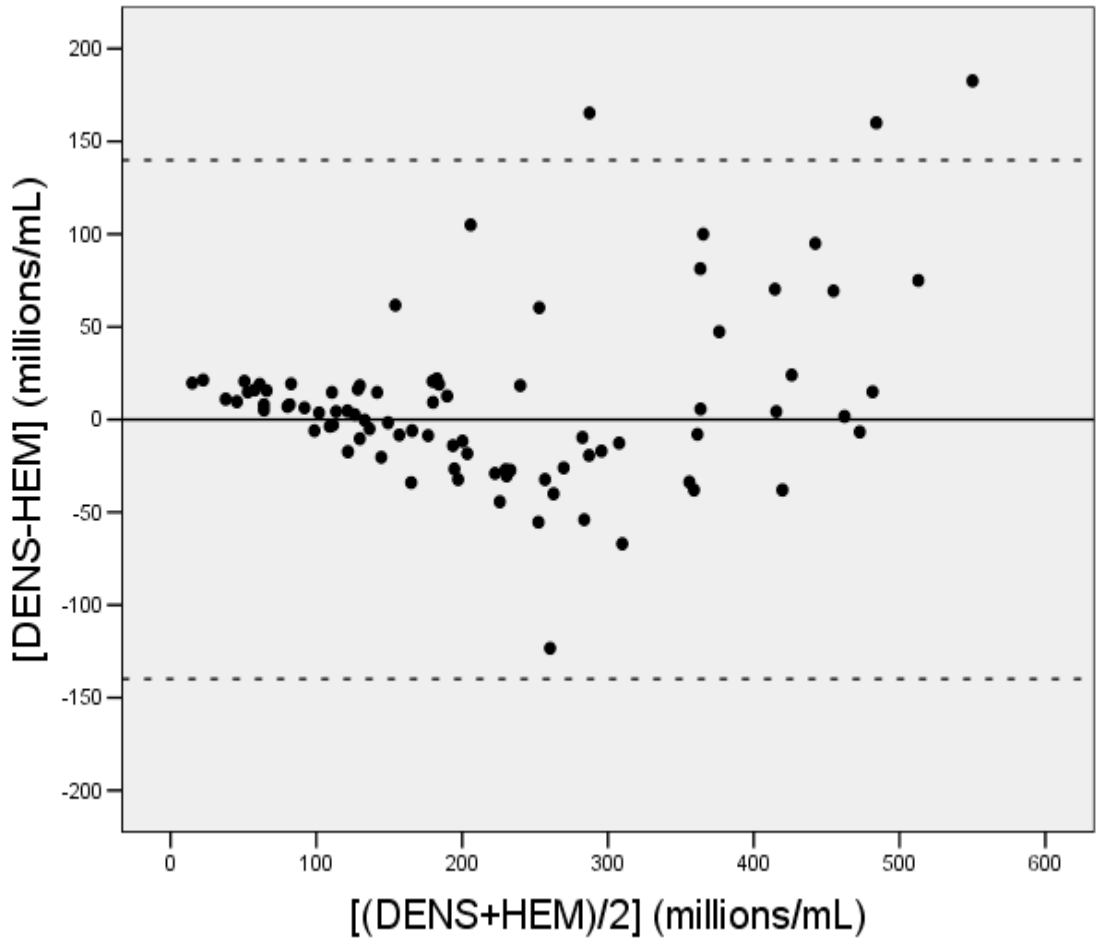

Figure 68. Bland and Altman plot comparing the difference in spermatozoal concentration between the Densimeter (DENS) and the hemacytometer (HEM) to the mean of the Densimeter (DENS) and the hemacytometer (HEM)); dashed lines represent + or – 2 standard deviations

Spermacue Bland and Altman comparisons

The Spermacue showed overestimation at low concentrations and underestimation at high concentrations compared to the flow cytometer (Figures 69 and 70) and the hemacytometer (Figures 71 and 72).

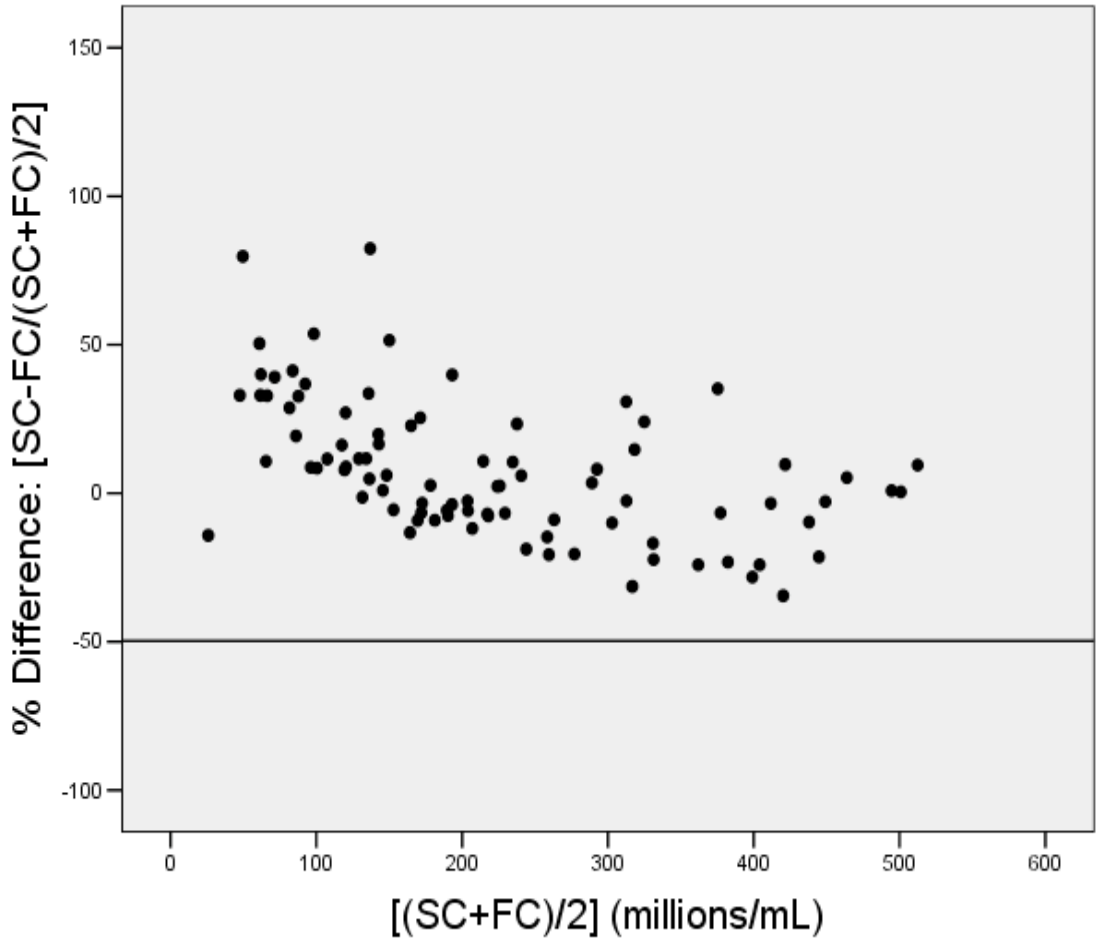

Figure 69. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the Spermacue (SC) and flow cytometer (FC) to the mean of the Spermacue (SC) and flow cytometer (FC)

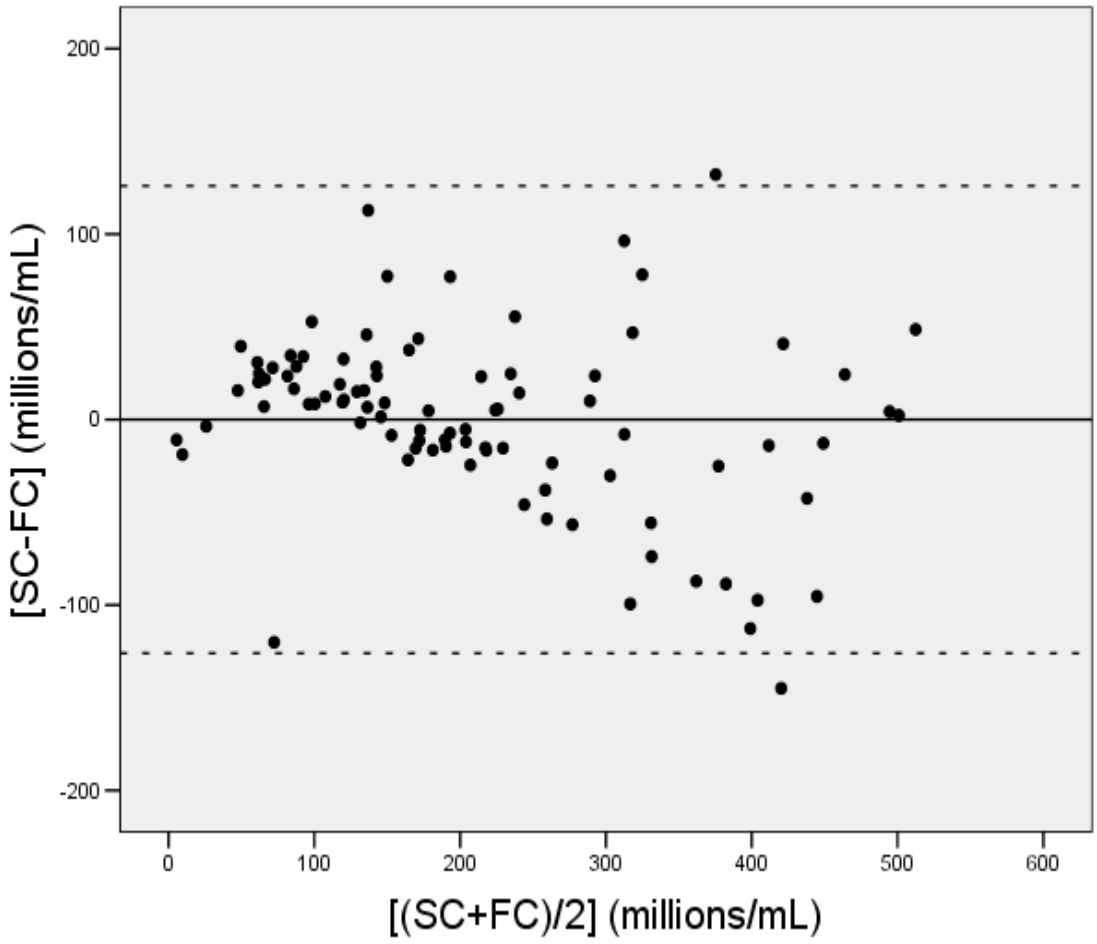

Figure 70. Bland and Altman plot comparing the difference in spermatozoal concentration between the Spermacue (SC) and flow cytometer (FC) to the mean of the Spermacue (SC) and flow cytometer (FC); dashed lines represent + or – 2 standard deviations

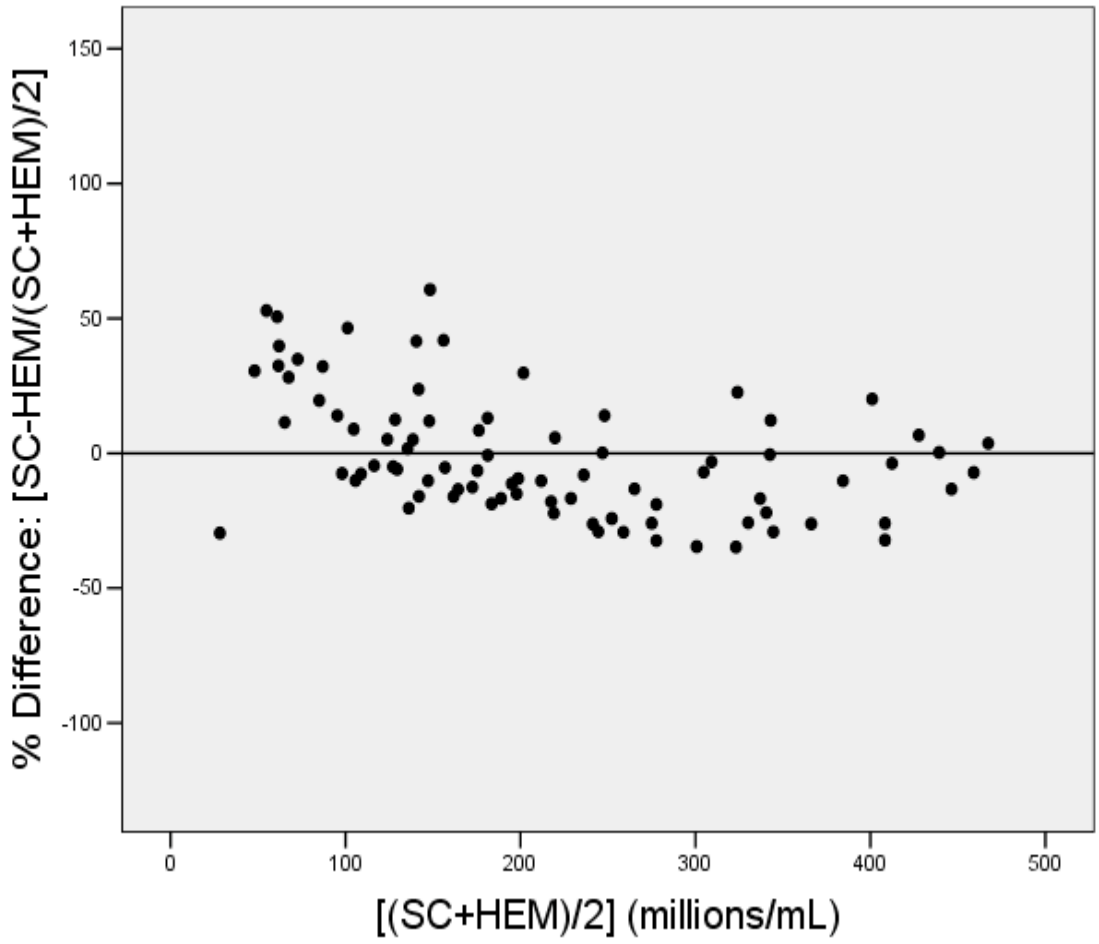

Figure 71. Modified Bland and Altman plot comparing the percentage difference in spermatozoal concentration between the Spermacue (SC) and the hemacytometer (HEM) to the mean of the Spermacue (SC) and the hemacytometer (HEM)

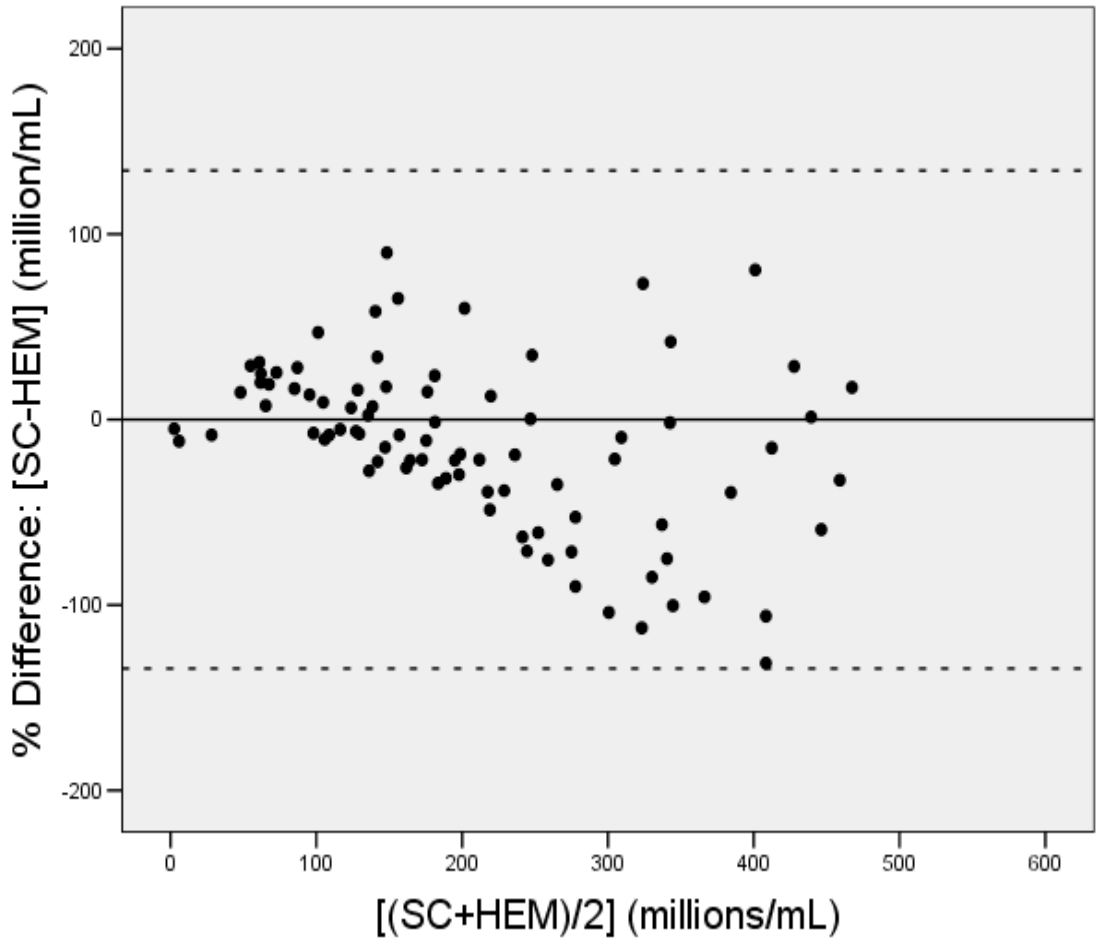

Figure 72. Bland and Altman plot comparing the difference in spermatozoal concentration between the Spermacue (SC) and the hemacytometer (HEM) to the mean of the Spermacue (SC) and the hemacytometer (HEM)); dashed lines represent + or – 2 standard deviations

Experiment 11

Regression analysis

The NucleoCounter overestimated spermatozoal concentrations when compared to both flow cytometry and hemacytometry standards. The slope and coefficients of determination were similar for both standards, but the y-intercepts were different. The

NucleoCounter slope was closer to 1 (line of equality) when compared to the hemacytometer than the flow cytometer (Table 16 and Figures 73 and 74).

Table 16

Regression values for the NucleoCounter (NC) compared to the flow cytometer (FC) and hemacytometer (HEM) for extended samples (50-100 x 10^6/mL concentration range)

Instrument Comparison	R^2	Slope	y-Intercept
NC-FC	0.79	0.81	27.2
NC-HEM	0.75	0.9	13.6

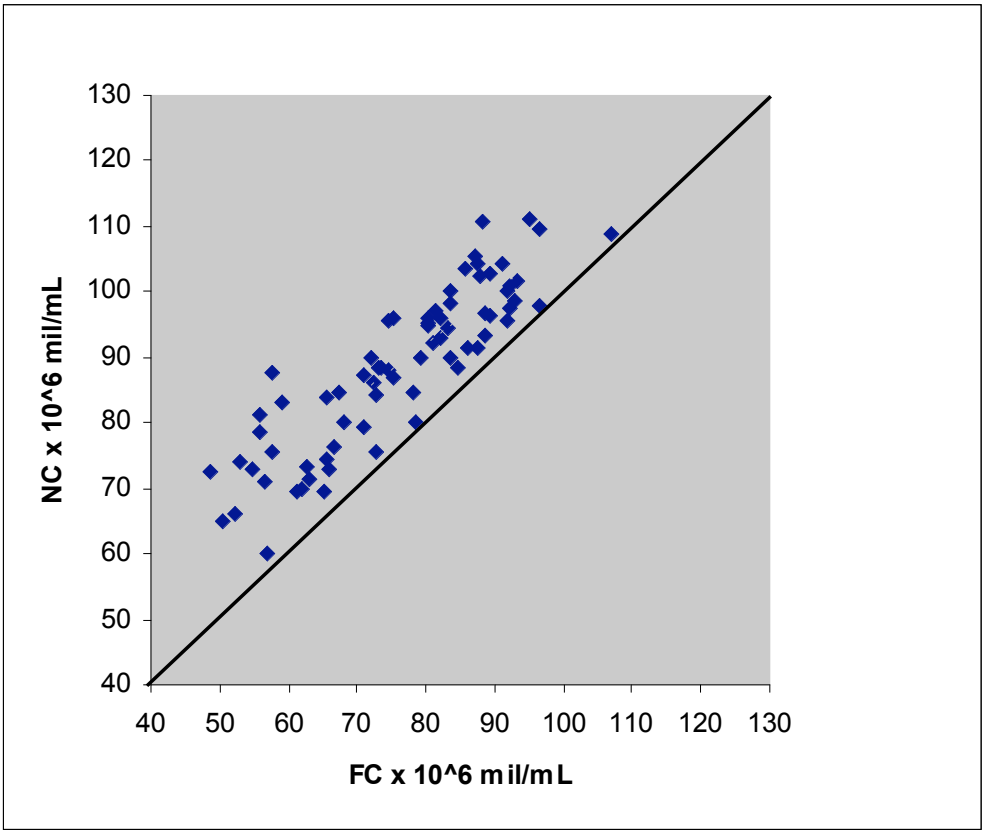

Figure 73. Extended samples regression scatterplot of NucleoCounter (NC) and flow cytometer (FC) and plotted against a line of equality

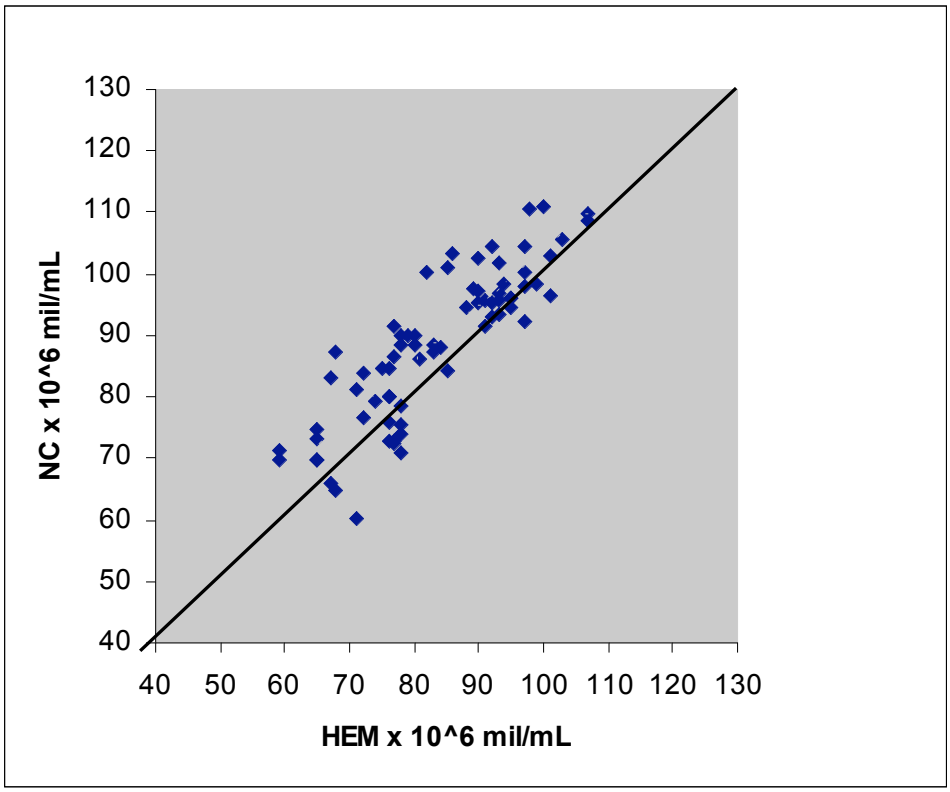

Figure 74. Extended samples regression scatterplot of NucleoCounter (NC) and hemacytometer (HEM) and plotted against a line of equality

The NucleoCounter overestimated percentage of difference and actual number difference compared to both flow cytometry and hemacytometry standards (Table 17). The flow cytometer underestimated percentage and actual number differences compared to the hemacytometer.

Table 17

Mean percent and actual differences for the NucleoCounter (NC), flow cytometer (FC) and hemacytometer (HEM) for ejaculates diluted in milk-based semen extenders

Instrument Comparison	Concentration Range ($x 10^6$/mL)	Mean Difference (%)	Mean Difference (actual number $x 10^6$)
NC-FC	50-100	15.7	12.43
NC-HEM	50-100	7.47	5.09
FC-HEM	50-100	-9.71	-7.27

Coefficient of variation

Within sample repeatability was >10% for all three instruments; the hemacytometer was the least repeatable (Table 18).

Table 18

Within-sample repeatability (coefficient of variation) for flow cytometer (FC), NucleoCounter (NC) and hemacytometer (HEM) for extended semen (50-100 $x 10^6$/mL concentration range)

Instrument	Coefficient of variation: 3 replicates (%)
FC	3.59
HEM	5.74
NC	2.42

NucleoCounter Bland and Altman analysis

The NucleoCounter showed overestimation for both original and modified Bland and Altman methods when compared to the flow cytometer (Figures 75 and 76). This was also observed (to a lesser extent) when the NucleoCounter was compared to the hemacytometer (Figures 77 and 78).

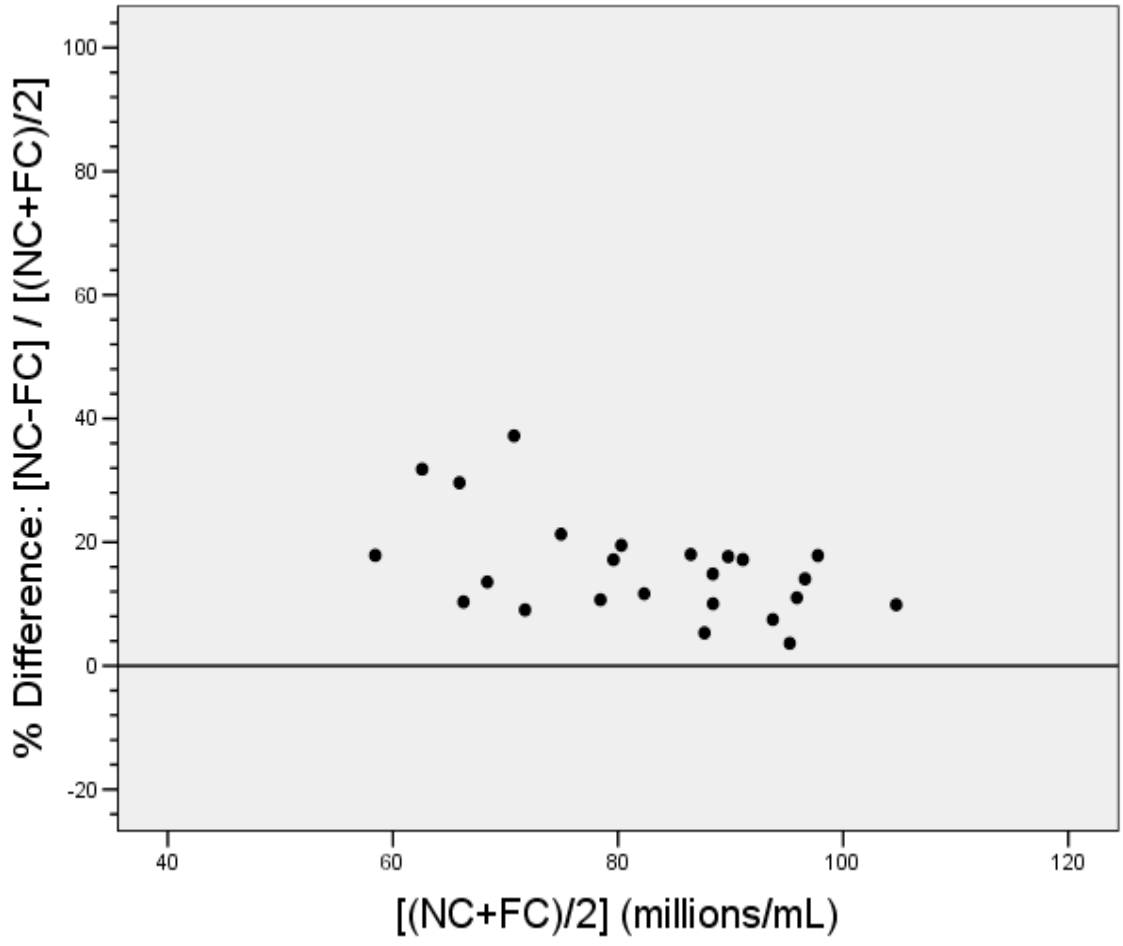

Figure 75. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the NucleoCounter (NC) and flow cytometer (FC) to the mean of the NucleoCounter (NC) and flow cytometer (FC) with extended samples

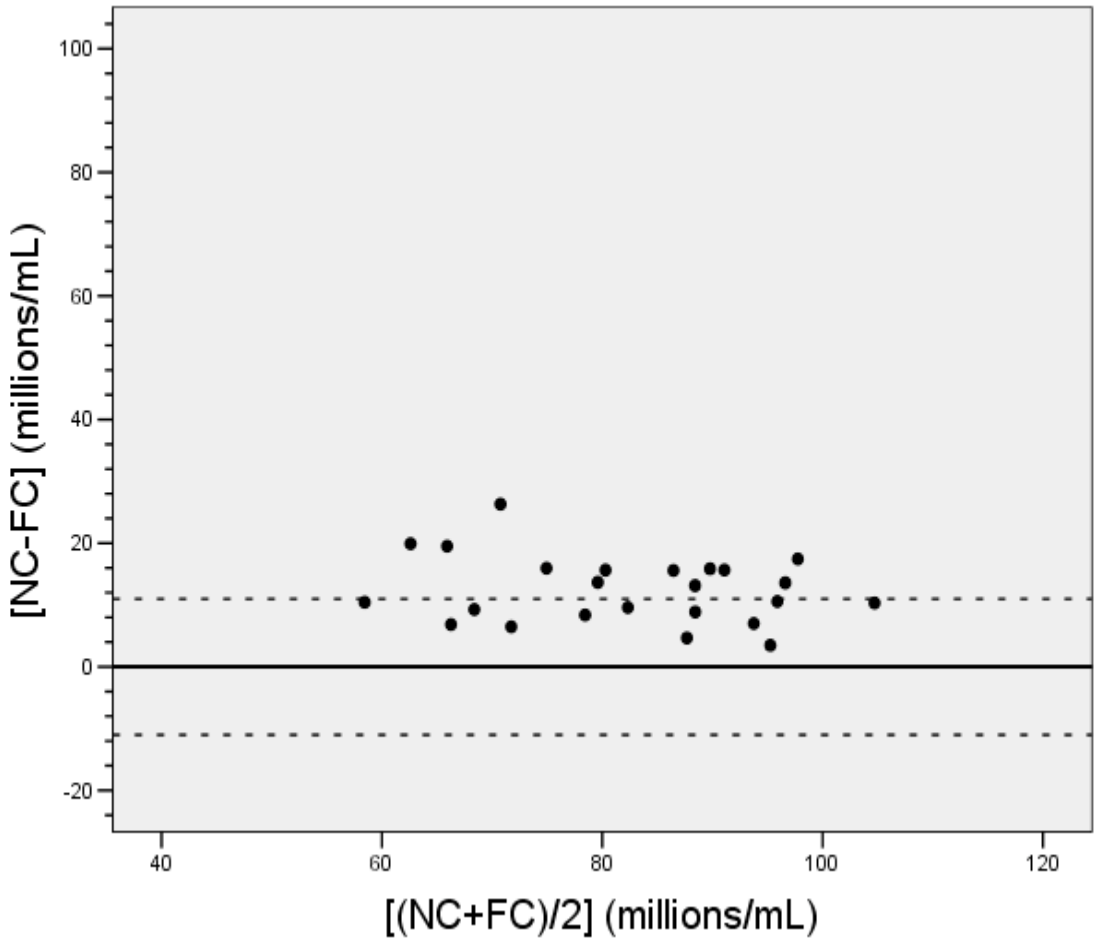

Figure 76. Bland and Altman plot comparing the difference in spermatozoal concentration between the NucleoCounter (NC) and flow cytometer (FC) to the mean of the NucleoCounter (NC) and flow cytometer (FC) with extended samples; dashed lines represent + or – 2 standard deviations

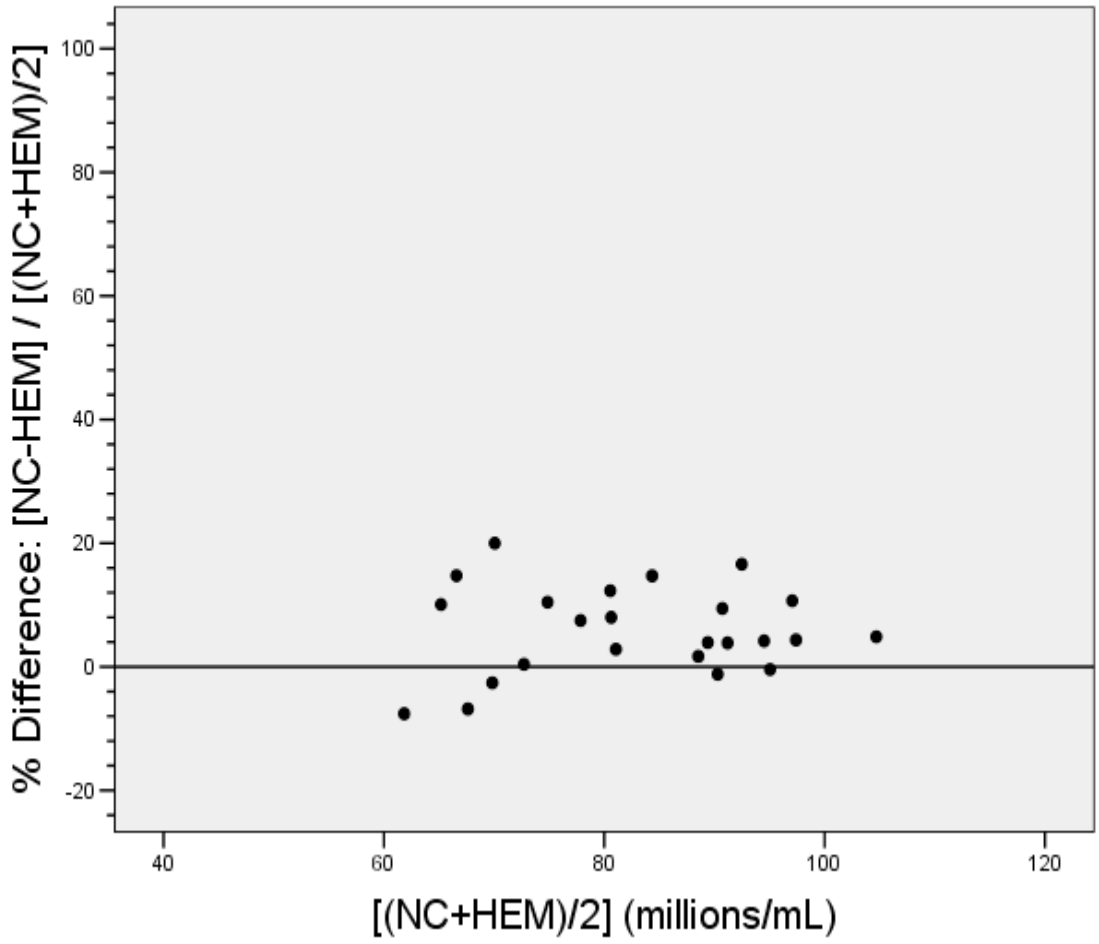

Figure 77. Modified Bland and Altman plot comparing the percent difference in spermatozoal concentration between the NucleoCounter (NC) and hemacytometer (HEM) to the mean of the NucleoCounter (NC) and hemacytometer (HEM) with extended samples

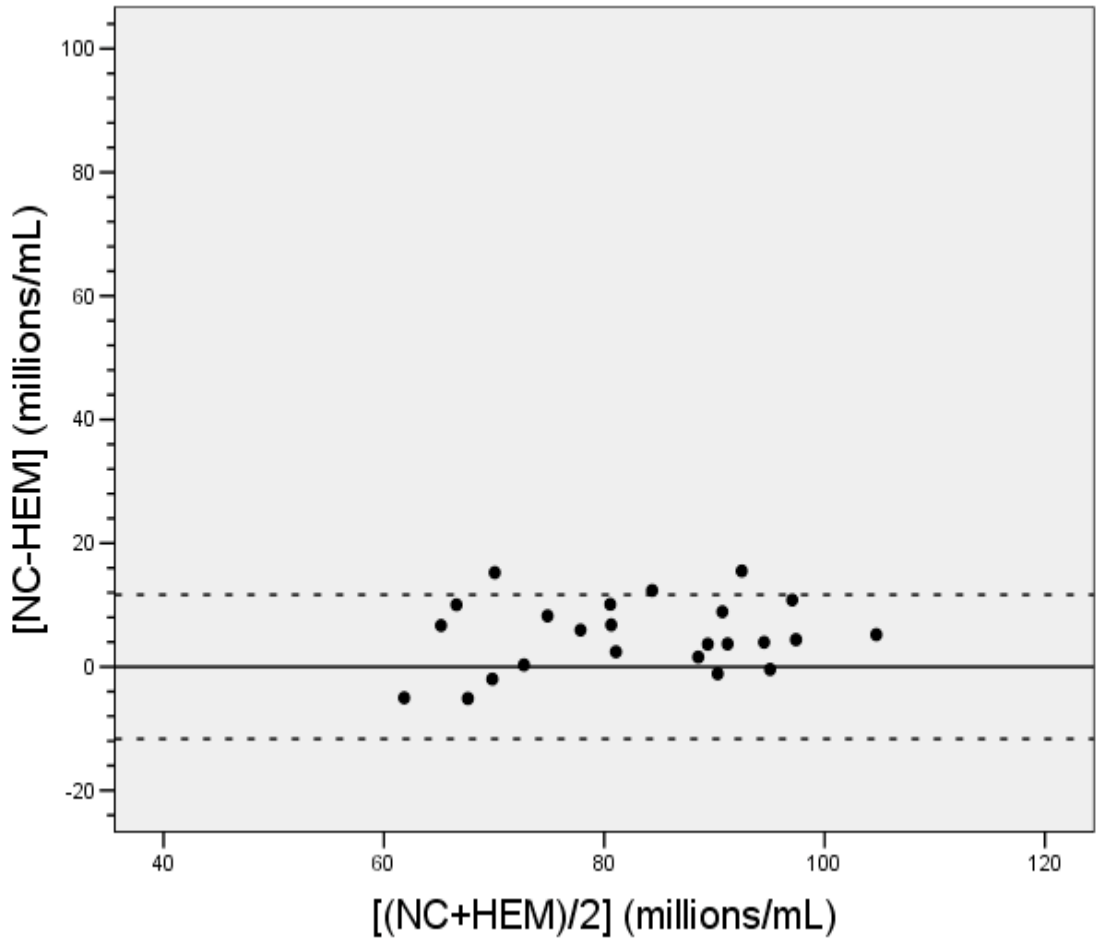

Figure 78. Bland and Altman plot comparing the difference in spermatozoal concentration between the NucleoCounter (NC) and hemacytometer (HEM) to the mean of the NucleoCounter (NC) and hemacytometer (HEM) with extended samples; dashed lines represent + or – 2 standard deviations

DISCUSSION

Previous studies have used both flow cytometry and hemacytometry as standards to which other spermatozoal concentrations are compared [4, 24-27, 31, 34-35]. In this study, both flow cytometry and hemacytometry were used as standards to determine agreement by measuring the absolute numerical difference and percent difference between the NucleoCounter and photometric methods in Experiments 8-11. The flow cytometer was selected initially as the standard for this project based on the findings of accuracy of several previous studies [24-27] and it was used as the standard for comparison for Experiment 7. However, it has been shown to consistently underestimate concentrations compared to the hemacytometer in a previous study [2]. The flow cytometer required a preliminary spermatozoal count on a different instrument (the NucleoCounter) and so was not completely independent of the NucleoCounter. The diluted spermatozoal concentration for the flow cytometer must be approximately 5 x 10^6/mL to ensure an accurate analysis.

Positive displacement pipettes (Microman, Ranin Instruments, LLC, Oakland, CA, USA) were used for most dilutions to maximize accuracy, since air-displacement pipettes have been reported to be less accurate because of the air dead space between the sample meniscus and plunger [34]. Air displacement pipettes were used for Experiment 1 because positive displacement pipettes were not available for all the necessary volumes.

The data were analyzed using several different methods, including regression analysis, Bland-Altman technique (scatterplots of the difference of the means plotted against the means) and a modified Bland-Altman method (scatterplots of the percentage of the difference of the means divided by the means plotted against the means).

Regression plots allow simple visualization of the distribution of the data points around the line of equality (slope = 1.0) by showing how close the slope of the regression line of the data is to the line of equality. The y-intercept determines a measure of one variable on the y-axis when the x-axis variable is zero and is a measure of over- or underestimation. However, the regression plots are not a technically a correct method of comparing this type of data. Data must be normally distributed for a regression to be an appropriate test [33]. This dataset was deliberately designed to have approximately the same number of ejaculates at all concentration levels and is not normally distributed (i.e. not a bell-shaped curve). The coefficient of determination (R^2) shows the amount of variation in one variable that can be explained by the variation in the other variable, which is not the same as measuring the agreement between them [33]. The regression plots were included for comparison purposes because they have been commonly used in similar previous studies [4].

The original Bland and Altman method was selected as the most appropriate method to measure agreement between two instruments, based on reports of others [24, 25, 34, 35]. The modified Bland and Altman method was used by our laboratory to remove the bias of scale of the original Bland and Altman method. For example, a

difference of 20 units is not as profound when the scale is measuring at 200 units (10% difference), as compared to a scale of 40 units (50% difference).

Log transformation of the data has been used in similar studies [26, 27, 31, 35], but is only appropriate when the differences in the means of the transformed data follow a normal distribution [33], which did not occur within this dataset.

The first experiment compared the manufacturer provided dispenser mechanism with air-displacement pipettes. There was no difference between the dispenser and pipette at 2 mL and 5 mL volumes, but the other volumes (1, 4, 10 and 20 mL) differed. However, the magnitude of the differences between the means was minimal (0.03g at 1mL and 4 mL volumes, 0.10g at 10 mL volume and 0.25g at 20 mL volume), so the dispenser mechanism may be considered adequate for dilution purposes. The differences may also be attributed to the different pipettes that were used for different volumes. A 1 mL pipette was used for the 1 mL volume, a 5 mL pipette was used for the 2, 4 and 5 mL volumes, a 10 mL pipette was used for the 10mL volume and a 20 mL pipette was used for the 20 mL volume. At larger volumes both the dispenser and pipettes tended to show a higher degree of error, therefore smaller volumes were used for dilutions in subsequent experiments.

In experiment 2, there was no effect of sample inversion on NucleoCounter values when the means were compared, but the variation around the means was dissimilar based on coefficients of variation and standard deviation. This indicates that the lack of inversion as recommended by the manufacturer caused a greater amount of variability within the sample than those that had been inverted and the cells dispersed

more evenly. These results are consistent with previous observations that inversion of a sample is necessary for adequate dispersion of spermatozoa prior to analysis.

In experiment 3, fresh samples had lower concentrations than the same frozen-thawed sample, which had not been shown to occur in previous similar studies. It was necessary to invert and sonicate these samples prior to analysis to facilitate adequate dispersion of spermatozoa, so an effect of the sonication and inversion process could have influenced these results. It was discovered in Experiment 8 that a sonicated sample must be kept still for approximately one minute before analysis to ensure that no air bubbles from the sonication process remain within the sample, thus affecting the final count.

An analysis of variance determined significant differences among groups in experiment 4, therefore pairwise comparisons were performed. The results indicated that samples must be sonicated a minimum of 10 seconds in order to have no appreciable difference among analysis results. This supports our previous observations that samples must be thoroughly mixed prior to analysis to facilitate adequate dispersion of the spermatozoa. Both experiments 4 and 5 indicated a decrease in means of fresh sonicated ejaculates. Experiment 4 was conducted first to determine the optimum sonication time. This finding was incorporated in experiment 5 and analyzed using a paired-samples t-test to confirm that sample means decrease after sonication. This was likely the result of the presence of air bubbles within the sample from the sonication process.

In experiment 6, there was no effect of vortexing and inverting frozen-thawed samples of gel-free ejaculates, therefore samples were all vortexed for ten seconds

before analysis to ensure an even spermatozoal distribution. Repeatability (coefficient of variation) and standard deviations appeared higher than expected because data presented was based on three separate ejaculates grouped together. When ejaculates were evaluated individually, all coefficients of variation were <10%.

In experiment 7, the manufacturer's dilution chart for bull ejaculates was found to be appropriate for stallion ejaculates with the exception that the suggested ranges for dilution of bull semen may actually be widened for dilution of stallion semen. After the trial was completed, new directives from ChemoMetec (manufacturer of the NucleoCounter) indicated that greater dilution factors not shown on the current chart may be used for extremely concentrated samples, such as the harvested epididymal spermatozoa that were used for the highly concentrated samples in experiments 8 and 10.

Experiment 8 was conducted using serial dilutions of epididymal spermatozoa to create a y=x regression line of equality over a broad concentration range that could be used as a comparison standard for the five instruments measured. Regression analysis indicated a high degree of correlation between the flow cytometer, hemacytometer and NucleoCounter when epididymal spermatozoa were diluted in NaCl (Figure 3). When spermatozoa were diluted in seminal plasma, all instruments showed overestimation at higher concentration ranges with the exception of the Spermacue, which showed underestimation (Figure 4). The flow cytometer and NucleoCounter had slopes nearest to 1 (Table 7). The difference in results between the two diluents was likely the result of particulate matter from the seminal plasma that affected the analysis. The use of serial

dilutions indicated that the flow cytometer, according to the regression analysis, was the most accurate instrument for measuring the concentration of epididymal spermatozoa, having a regression equation nearest to y=x.

Coefficients of variation (Table 8) were <10%, indicated that all instruments had acceptable repeatability regardless of diluent or spermatozoal concentration.

Original and modified Bland and Altman plots (Table 9 and Figures 5-36) showed that the NucleoCounter was more accurate than the Densimeter or Spermacue because it showed more agreement with both the flow cytometer and hemacytometer. The presence of seminal plasma appeared to have a greater effect on the analysis by the photometric systems than it did on the NucleoCounter analysis. Regardless of diluent, both photometric systems showed percentage and actual number underestimations from both the flow cytometer and hemacytometer, indicating that photometric measurement was less accurate than the NucleoCounter measurements (Figures 13-36).

In experiment 9, only the spermatozoa diluted in NaCl were analyzed on the hemacytometer, therefore only this comparison was evaluated with regression and Bland and Altman analysis. The flow cytometer, hemacytometer and NucleoCounter were the most accurate instruments, being nearest to the y=x line of equality.

The coefficients of variation were mostly <10%, indicating that all instruments had acceptable repeatability regardless of diluent of spermatozoal concentration. The hemacytometer and NucleoCounter were the most repeatable when spermatozoa were diluted in NaCl.

Original and modified Bland and Altman plots of ejaculated spermatozoa diluted in NaCl indicated similar agreement of the NucleoCounter compared with the flow cytometer and hemacytometer (Table 12). The Densimeter and Spermacue showed significantly greater percentage of difference from both the flow cytometer and hemacytometer, indicating that photometric measurement is not appropriate unless spermatozoa are diluted in a clear media. These results were also confirmed in Experiment 10.

Coefficients of variation were <10% for all instruments in Experiments 10 and 11, confirming that all of the measurement systems have acceptable repeatability regardless of diluent or spermatozoal concentration. The results of Experiment 11 agreed with the results of Experiment 9; showing that the NucleoCounter has acceptable agreement with the flow cytometer and hemacytometer when spermatozoa are diluted in opaque semen extenders.

The original Bland and Altman plots were included for comparison purposes with other similar studies that used this method. The modified plots were included to show the percentage of deviation of each instrument as well as the actual number difference shown in the original method. This allows for a reduction in the effect of scale at high and low concentration ranges. The Spermacue was the most inaccurate at concentration levels less than 200 x 10^6/mL and would not produce a reading when the concentration was below approximately 20 x 10^6 /mL; making this instrument impractical for enumeration of low-concentration ejaculates. All samples >300 x 10^6/mL were diluted before analysis with the Densimeter and the Spermacue based on

unpublished observations that photometric systems are inaccurate above this concentration level.

In experiment 11, spermatozoal concentration was measured in five different opaque semen extenders to test the accuracy and precision of the NucleoCounter for measuring ejaculates that are diluted in milk-based extenders. The NucleoCounter overestimated concentrations by 15.7% when compared to the flow cytometer and 7.5% when compared to the hemacytometer (Table 17). Particulate matter (debris, epithelial cells, etc.) in the ejaculate or extenders may have taken up the propidium iodide dye and erroneously counted these as spermatozoa.

A consistent pattern of overestimation of the NucleoCounter when compared to the flow cytometer was evident from both regression analysis (Figure 73) and original and modified Bland and Altman plots (Figures 75 and 76). It appeared that the propidium iodide system of the NucleoCounter was picking up particulate matter from both raw ejaculates and semen extenders and staining this as spermatozoa, which would lead to artificially high concentration results. The results were more evenly distributed about the line of equality for the regression line (Figure 74) and zero line for the Bland and Altman plots when the NucleoCounter was compared to the hemacytometer (Figures 77 and 78) for extended samples. These results agreed with previous studies [21, 34] as well as the WHO guidelines [3] and indicated that the hemacytometer, although the least repeatable of the instruments measured, is still the most ideal method for evaluation of extended semen due to the ability of the observer to directly identify the spermatozoa. However, the hemacytometer overestimated spermatozoal concentrations when

compared to the flow cytometer (Tables 15 and 17) and this is likely the reason that this method showed more agreement with the NucleoCounter.

SUMMARY

The advantages of the NucleoCounter are 1) it identified spermatozoa at all concentrations and in all different media examined (opaque and non-opaque), 2) based on regression and Bland and Altman analyses, it had the least variation at all concentrations examined compared to the flow cytometer and hemacytometer and 3) concentrations were measured more quickly because it did not require the laborious set-up and counting procedure of the hemacytometer or the extensive dilution procedure of the flow cytometer. The major disadvantage of the instrument is the initial cost, which may be somewhat prohibitive for some operations compared to that of a hemacytometer or photometric systems. However, this is currently the only automated system that is capable of measuring spermatozoa in opaque semen extenders, which has significant applications for breeding and research operations that routinely have need to confirm concentrations of cooled and frozen spermatozoa shipments as well as confirming concentrations when freezing semen. The instrument would also be very useful for any operation that uses low-dose insemination techniques. The accuracy of the concentration count is critical for these techniques and the NucleoCounter has been shown to be more accurate than conventional photometric measurement when spermatozoal concentrations are very low (i.e. $<50 \times 10^6$/mL).

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