EFFECT OF DENSITY GRADIENT CENTRIFUGATION ON QUALITY AND RECOVERY RATE OF EQUINE SPERM

A Thesis

by

ANN JULIETTE EDMOND

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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Approved by:

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Major Subject: Veterinary Medical Sciences

ABSTRACT

Effect of Density Gradient Centrifugation on Quality and

Recovery Rate of Equine Sperm. (May 2009)

Ann Juliette Edmond, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Dickson D. Varner

Density gradient centrifugation of sperm is a common assisted-reproduction procedure in humans used to improve semen quality. The technique allows sperm separation based on their isopycnic points. Sperm with morphologic abnormalities are often more buoyant, leading to their retention above centrifuged density gradients, with structurally normal sperm passing through the gradient. Three experiments were conducted to evaluate the effects of tube size, sperm number following centrifugation, and density gradient volume (height) on stallion sperm quality and recovery rate in sperm pellets following centrifugation. In all three experiments, equine semen was initially centrifuged to increase sperm concentration. In Experiment 1, one-mL aliquots were layered over EquiPure™ Bottom Layer (1-Layer) or over-tiered EquiPure™ Top and Bottom Layers (2-Layer). For Experiment 2, one-mL aliquots were layered over three different heights of EquiPure™ Bottom Layer in 15-mL or 50-mL conical-bottom tubes. For Experiment 3, four different aliquots containing a sperm load of 1-4x were layered over a constant volume of EquiPure™ Bottom Layer in 15-mL or 50-mL conical

bottom tubes. The tubes were then centrifuged. Resulting sperm pellets were evaluated for morphologic quality, DNA integrity, motility and recovery rate.

Sperm-EquiPure™ centrifugation yielded improvements in motility, morphology and DNA integrity parameters ($P<0.05$), as compared to controls. The 1-Layer method resulted in a higher recovery rate than the 2-Layer method ($P<0.05$). Sperm processed in the 15-mL tubes yielded higher velocity and higher recovery rates than sperm processed in the 50-mL tubes ($P<0.05$). Within tube type, gradient volume did not impact parameters of semen quality or recovery rate. An increase in sperm number for density gradient centrifugation resulted in a decreased recovery rate ($P<0.05$) when 15-mL tubes were used.

DEDICATION

I dedicate this work to:

My parents: Leon and Barbara Edmond,

Dr. James Marek,

&

My family and friends.

These loved ones have stood by me every step of the way with encouragement and support. Without their guidance, I would not be where I am today looking to a great future ahead. Mom and Dad, thank you for your unconditional support of my studies. I am honored to have you as my parents. Thanks for giving me the opportunity to prove and improve myself through all my walks of life. Jamie, thank you for the valuable knowledge gained through working with you. I appreciate your ability to teach and admire your expertise in all circumstances. Y'all mean the world to me. Thank you!

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TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

TABLE Page

TABLE Page

INTRODUCTION

Artificial insemination has become an integral part of the equine breeding industry. This is based on widespread acceptance by horse breed registries for insemination with fresh semen, as well as cooled and frozen semen [1]. However, the number of sperm preparation techniques utilized in horse breeding programs is far less than with corresponding human procedures [2]. Many stallions are good candidates for alternative sperm preparation techniques because stallions, like men, are not chosen for mating based on fertility. Stallions are selected as sires based on three key features: athletic performance record, pedigree, and conformation. As such, subfertility is a relatively common occurrence among breeding stallions. Some of these stallions produce semen which contains a high prevalence of sperm morphologic defects, reduced sperm motility, and reduced sperm chromatin quality. Consequently, veterinarians and stud farm managers are often faced with semen quality problems similar to that seen in human assisted reproduction laboratories. In both instances, semen-processing techniques can be applied in an attempt to improve sperm quality prior to insemination.

Density gradient centrifugation is one of the most common sperm preparation techniques performed in human assisted-reproduction laboratories, and the procedure provides for enhancement of semen sperm quality [2]. Currently, Nidacon International AB (Mölndal, Sweden) produces commonly used discontinuous density-gradient media (PureSperm®). The product contains silane-coated silica particles that are incorporated

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This thesis follows the style of Theriogenology.

into a discontinuous gradient designed to permit separation of higher-quality from lower-quality human sperm. EquiPure™ is a discontinuous density-gradient manufactured by the same company. The non-silica portion of the gradient media was modified from that used with human semen in an effort to optimize its use with stallion semen.

Using the discontinuous-gradient centrifugation method applied with these products, semen is layered over two underlying states of media containing different concentrations of silica particles. Centrifugation of this composite permits gravitational separation of sperm populations, based on their density, as well as separation of sperm from non-sperm particles such as epithelial cells, bacteria, viruses, and debris [2,3]. Sperm with various morphologic abnormalities will be trapped with greater frequency in the upper layers of the gradient whereas; morphologically normal sperm tend to pass through the gradient.

Within the last decade, extensive studies have been conducted world-wide using density gradient preparation combined with a sperm swim-up technique to remove viruses from ejaculates. In human assisted-reproductive laboratories, HIV, hepatitis C virus or hepatitis B virus in semen samples were successfully removed with this application [4]. Similarly, use of EquiPure™ in conjunction with a sperm swim-up procedure eliminated a sexually transmitted virus, equine arteritis virus, from stallion semen [5].

OBJECTIVES

The objective of this thesis project was to evaluate the performance of a discontinuous density gradient (colloidal silica–particle solution) with equine sperm. Currently, the manufacturer protocol for the product EquiPure™ (Nidacon International AB, Mölndal, Sweden) consists of layering up to 1.5 ml semen over 2 ml of the Bottom Layer (80% density gradient) and 2 ml of the Top Layer (40% density gradient) in a 15-ml centrifugation tube. Experiment 1 compared the standard two-layer protocol with a one-layer (Bottom Layer only) protocol for density-gradient centrifugation of stallion sperm in 15-mL conical centrifugation tubes. Experiment 2 evaluated the effects of gradient media height (28mm, 35mm, and 41mm) and centrifugation tube type (15-mL versus 50-mL conical centrifugation tubes) for density-gradient centrifugation. Experiment 3 studied the effects of semen volume/sperm number on quality and quantity of sperm recovered following density-gradient centrifugation. The overall goal of the study was to develop methods to utilize and simplify density-gradient centrifugation techniques in an effort to maximize reproductive performance in subfertile stallions.

LITERATURE REVIEW

Artificial Insemination

Artificial insemination plays a crucial role in the fertilization process for plants and flying insects in nature. In the equine species, artificial insemination is a development by humans that has enhanced reproduction. This process of placing stallion sperm into a mare's uterus by using artificial means has many advantages over natural breeding in the horse industry. These advantages include the ability to breed multiple mares with a single ejaculate, breeding a mare that has impediments that preclude natural mating (i.e., physical disabilities, failure to show behavioral estrus, or susceptibility to bacterial infection), increased safety for mare and stallion, and the ability to breed mares that are geographically remote from the stallion. An improvement on the technique of artificial insemination is an ongoing process that is many years in the making.

Historically, it is thought that the practice of artificial insemination dates back to 1322 and was first successful in a horse. Allegedly, an Arab chieftain stole semen from a recently mated mare of a neighboring rival, diluted it in camel milk and deposited it into one of his own mares [6-8]. Artificial insemination in horses was not recognized again for many centuries. In 1898, Walter Heape documented that a stallion with "faulty formation" was having difficulty "settling" mares. The owner was advised to artificially inseminate the mares. As a result, 26 out of 29 mares became pregnant and were able to overcome a sterility problem [9]. For the next couple of years, artificial insemination was mainly used for the treatment of subfertility. It was not until 1912 that Ivanov

compared artificial insemination to natural service in horses and yielded pregnancy rates of 79.5% and 43.2%, respectively [7].

Due to the military's need for horses, artificial insemination research began expanding in European and Asian countries in the early to mid 1900s [10]. China and Russia utilized this technique in more than 600,000 mares and expanded their national herds between 1930 and 1960 [6,8]. During this time, several types of artificial vaginas were developed [10]. This allowed for the improvement of stallion management and semen collection, evaluation and insemination.

Once equine breed registries in the United States began accepting the use of artificial insemination, research concentrated on the development of semen extenders to protect and maintain longevity of sperm. Semen extenders have the ability to protect sperm from cold shock, prevent growth of micro-organisms, and minimize detrimental effects of seminal plasma [11]. In 1957, Arhipov used a glucose-based diluent at a 1:6 (semen: diluent) ratio to extend semen prior to insemination [12]. This was one of the first reports of diluting stallion semen. Since then, milk-based semen diluents have become the most practical and effective in protecting equine sperm during storage. Batellier et al. conducted four breeding trials that tested the standard protocol with an experimental protocol by inseminating 173 mares with semen stored 24 h in INRA 82 or Kenney's diluent (standard: skim milk diluent) at 4°C in anaerobic atmosphere yielded a pregnancy rate of 40%, and 178 mares artificially inseminated with semen stored 24 h in INRA 96 (new: chemically defined, milk-free diluent) at 15°C in an aerobic environment yielded a pregnancy rate of 57%($P<0.001$) [13]. This experimental protocol showed

potential for use in the field for stallions sensitive to cold shock, by improving fertility when compared to the standard protocol [13].

Insemination Dose

Since the acceptance of artificial insemination by breed registries and its increasing popularity within the equine breeding industry, research focused on shipping semen both nationally and internationally. One main concern was the size of the insemination dose required to maintain satisfactory pregnancy rates. In 1969, Bowen states that the recommended dose varies from author to author: Berliner suggests that $1x10^9$ live sperm are adequate, while Cheng suggests $1x10^9$ motile sperm, per dose [7]. With the uncertainty and accepted death of spermatozoa during cooling when shipping, the insemination dose of $1x10^9$ progressively motile spermatozoa is one recommended standard shipping dose used today [14]. However, for mares on stud-farms the standard insemination dose is between 250 and 500 million progressively motile sperm, and is dependent on the fertility of a given stallion [14]. Colorado workers concluded from their research that an insemination dose of $500x10^6$ progressively motile sperm would achieve optimal pregnancy rates based on the management procedures of the time [15-17]. A study conducted by Gahne et al. demonstrated that reducing the insemination dose to $300x10^6$ progressively motile sperm did not yield a lower pregnancy rate [15]. An adequate insemination dose is the major limiting factor for the number of mares that can be successfully bred by an individual stallion.

Uterine body insemination is known cause a transient postbreeding endometritis in virtually all mares, which usually resolves itself with 24 hours in normal mares [18]. In cases of severe post-breeding endometritis cases (mainly older mares), mares often accumulate fluid [18]. By utilizing one of the techniques of gamete intrafallopian transfer, transrectally guided deep uterine insemination, or hysteroscopic insemination, sperm is deposited directly on the uterotubal papilla at the tip of the uterine horn ipsilateral to the ovary containing the dominant follicle [18, 19]. A goal set by inseminating a mare using one of these techniques would be to eliminate the post-breeding endometritis. Other advantages that should be considered include cryopreserved semen that is of limited supply, semen from a subfertile stallion with limited sperm numbers, stallions that have an increased mare book to service, insemination of sex-sorted semen, and insemination with epididymal sperm [18]. Even though 500 x 10^6 progressively motile sperm has become an accepted insemination dose within the industry, research has demonstrated that most of the ejaculate is expelled rapidly from the uterus due to the relaxation of the cervix during estrus and that only 0.0007% of inseminated sperm actually gain access into the oviducts [20].

Early studies involving low dose, deep uterine horn insemination were mainly with the hysteroscopic approach. In 1998, Vazquez et al. obtained 3 pregnancies out of 10 when the mares were inseminated with 4 x 10^6 sperm in a 20µl volume dose [21]. At the same time, Manning et al. also had disappointing pregnancy results when they deposited 1 x 10^6 or 10 x 10^6 sperm hysteroscopically [22]. However, Morris et al. demonstrated that when inseminated only once with 14 x 10^6 motile, frozen-thawed

sperm, the pregnancy rates were similar when mares were inseminated by hysteroscopic (9/14) or conventional (8/12) technique. [23].

In an attempt to have a more practical method and reduce time and expense required for hysteroscopic insemination, the use of a transrectally guided deep uterine technique was developed. Similar to hysteroscopic insemination, results of pregnancy rates from different studies had a wide range. Lindsey et al. deposited 5 x 10^6 sperm with this method and resulted in 0 out of 10 mares pregnant [24]. In another study, 43% (3/7) mares were impregnated when inseminated with 25 x 10^6 sperm [25]. When the transrectally guided method was compared to the hysteroscopic method in two studies, similar results in pregnancy rates were obtained. Rigby et al. showed no statistical difference in pregnancy rates of the mares inseminated hysteroscopically (13/21;62%) or after transrectally guided deep uterine insemination (10/20; 50%) [26]. Brinsko et al. reported that by depositing 3.3 – 3.6 x 10^6 progressively motile sperm 10 out of 18 mares (56%) became pregnant by the transrectally guided method and 12 out of 18 mares (67%) became pregnant when inseminated hysteroscopically [27].

When low dose, deep horn insemination techniques were used to try to help improve pregnancy rates of subfertile stallions the results have been unsatisfactory [28]. Vazquez et al. concluded from their research that it was the suboptimal number of normal motile sperm that was used in the insemination dose [21]. However, by subjecting an ejaculate to a discontinuous density gradient centrifugation method prior to hysteroscopic insemination, the percentage of normal motile sperm is increased. Morris et al. inseminated mares with 10 x 10^6, 5 x 10^6, or 1 x 10^6 Percoll® (Sigma-Aldrich)

treated motile sperm that resulted in conception rates of 60, 75 and 64%, respectively [29]. Another study with two subfertile stallions (per-cycle pregnancy rate of <20%) proved to the pregnancy rate with one stallion from 20 to 35% and resulted in 7 out of 8 mares pregnant for the other stallion [28].

Density Gradient Centrifugation

A stallion's ejaculate is known to consist of different subpopulations of sperm. Sperm need to possess many attributes (motility, normal morphological features, normal DNA quality, etc.) in order for them to fertilize an oocyte. Sperm may be infertile for a variety of reasons (i.e., one sperm may be infertile due to a lack of motility, while another due to acrosomal dysfunction) [30]. This is one reason suboptimal pregnancy rates exist in the horse breeding industry. Ley et al. claim that a stallion should be expected to achieve at least a 90% seasonal pregnancy rate when bred artificially to 120 mares in a single breeding season [31]. The success of the breeding of a stallion can be impacted when ejaculates consist of low numbers of normal, motile sperm which can contribute to a lower than expected fertility. An approach to this problem is to increase the percentages of motile and morphologically normal sperm from semen.

Discontinuous density gradient centrifugation has been a standard procedure in human assisted-reproduction laboratories for the purification of sperm. These gradient media separate sperm with differing buoyant densities into different layers. The predominant product used in previous years was a colloidal polyvinylprrolidone (PVP)-coated silica, termed Percoll® (Pharmacia Biotech AB, Uppsala, Sweden). In 1983,

Oklahoma State University workers evaluated the efficacy of Percoll® on rabbit, human and bovine semen. This study resulted that the progressively motile sperm recovered for all three species was higher when compared to the unfractionated semen [32].

Prakash et al. found that, when compared to sperm separation method by swim-up, differential gradient centrifugation with Percoll® resulted in selection of more human sperm with normal morphology [33]. Of the 74 samples, 34 showed improvement in the percentage of normal sperm after Percoll® centrifugation, while only 21 showed improvement when prepared by the swim-up method ($P = 0.009$) [33]. Chen et al., compared swim-up and Percoll® on the percentage of progressive motility, recovery of motile sperm, removal of debris, and percentage of morphologically normal sperm [34]. Although the swim-up samples had a higher percent of progressive motility, the Percoll® samples contained more motile sperm because the sperm concentration in the Percoll® samples was significantly higher ($P<0.05$) than the swim-up samples [34]. The percent of morphologically normal spermatozoa increased for both procedures, when compared to the raw sample ($P<0.05$), but showed no significant difference when compared to each other [34].

In 2000, Zini et al. concluded that even though both swim-up and Percoll® treatments improved mean sperm motility when compared to whole semen (73.0% ± 3.0% and 65.6% ± 4.0% versus 52.0% ± 3.6%, respectively, $P<0.005$), swim-up reduced sperm with denatured DNA significantly while Percoll® did not when compared to whole semen (4.8% ± 1.2% and 13.6% ± 3.6% versus 10.1% ± 2.3%, respectively, $P<0.001$) [35].

When Pharmacia Biotech removed Percoll® from commercial use in assisted reproductive technology in humans in 1996 because of high endotoxin levels, several alternative products were introduced. These included OptiPrep™(Greiner Bio-One, Axis Shield, Oslo, Norway), IxaPrep®(Medicult, Copenhagen, Denmark), Isolate® (Irvine Scientific, Santa Ana, CA), and PureSperm® (Nidacon International, Gothenburg, Sweden). OptiPrep™ and Ixaprep® consist of an iodixanol solution, whereas Isolate® and PureSperm® are colloidal silane-coated silica particles.

It was of great importance to find a substitute product for Percoll® which would give equal or better sperm separation results. Centola and co-workers compared PureSperm® with Percoll® with respect to recovery rate, sperm motility, sperm path and progressive velocities, and sperm hyperactivation. Their results showed no statistical difference for any of the motion parameters or motile count between the PureSperm® and Percoll® treatments [36]. In another study, Percoll®, PureSperm®, and swim-up method were evaluated to see how effectively each product separated out the sperm with chromatin/nuclear DNA anomalies [37]. Sperm from three fractions (wash, sediment, and swim-up) were evaluated [37]. No significant difference was observed with any fraction of the swim-up method or the 45% fraction of either PureSperm® or Percoll® [37]. However, in the 90% fraction both the PureSperm® and Percoll® possessed a significantly lower ($P<0.001$) percentage of sperm with nicked DNA and with poorly condensed chromatin [37].

About the same time, McCann and Chantler made a comparison of Percoll® and IxaPrep® density gradients. Unlike the previous experiments, IxaPrep® showed a

considerably lower recovery ($P< 0.05$) of spermatozoa in each of the samples as well as 63.7% motility and 39.4% progressive motility when compared to 76.5% and 56.5% with Percoll® ($P< 0.05$) [38]. Morphological abnormalities was 68% ± 3.2 in neat semen, and reduced to 55% ± 5.5 after Percoll® separation and to 64% ± 7.0 after IxaPrep® separation [38].

With the success in human assisted reproduction, density gradient centrifugation has increased both in research and clinical cases in other species. In 1997, Turner and Arns presented a case at the Equine Nutrition and Physiology Society Annual Symposium that demonstrated that the percentage of progressive motile sperm following Percoll® treatment was higher than the other treatments measured. Their data suggested that isolation on a Percoll® density gradient may enhance in vitro capacitation of stallion sperm [39]. Sieme et al. also tested Percoll® centrifugation on equine sperm, but had significantly lower motilities when compared to swim-up, glass wool, and glass wool sephadex filtration [40]. Percoll® was successfully used by Ock et al. to isolate round spermatids from bull testes [41]. The gradient recovered 86.7 ± 3.26% live cells compared with 70.8 ± 2.37% in the untreated cell preparation ($P<0.01$) [41].

With the success of the product PureSperm® and the promising future of density gradient centrifugation in the equine industry, Nidacon International introduced a density gradient media (labeled as EquiPure™) specifically for separating and purifying equine sperm. In 2002, Macpherson et al. examined the efficacy of EquiPure™ to separate higher quality of equine sperm by measuring morphologic and motility parameters [42]. The results revealed a higher percentage of normal sperm ($P=0.06$) and higher total

motility and progressive motility ($P<0.05$) in the processed pellet than in the raw, unprocessed semen [42]. Subsequently, Morrell and Geraghty were successful in removing equine viral arteritis from stallion semen by utilizing a double processing technique (density gradient centrifugation [EquiPure™], washing, and swim-up) [5]. Recently, Morrell et al. reported preliminary work which compared density gradient centrifugation (two layer media) to single layer centrifugation and indicated that for stallions with ejaculates within the normal range there was little difference between the two methods for motion characteristics [43]. Also, the recovery rate yielded from the single layer and the two-layer was 33.6 ± 9.8 and 31.5 ± 11.2, respectively [43].

In the experiments above, density gradient centrifugation proved to separate higher quality equine sperm into the processed pellet [5,42-43]. Since these studies have shown that density gradient centrifugation of equine semen is useful for purifying sperm from an ejaculate, the next step in improving density gradient centrifugation and processing of semen might be to assess whether the standard two-layer gradient (40%-80%) techniques could be simplified to one-layer gradient (80%). In addition, it would be beneficial to determine if the size of centrifugation tube (15mL versus 50mL), depth of the density gradient, or sperm load on the gradient has an effect on sperm recovery rate and semen quality in the post-centrifugation sperm pellets.

MATERIALS AND METHODS

Three experiments were conducted to evaluate the effects of density-gradient density (one-layer versus two-layer), tube size (15-mL versus 50-mL conical tubes), gradient height (28mm [2mL], 35mm [3mL], or 41mm [4mL]), or sperm number (250 - 2000 x 10^6) on sperm quality and recovery rate following density-gradient centrifugation. Sperm motion characteristics, sperm morphologic features, and sperm DNA quality were evaluated in neat semen, and the same experimental endpoints and sperm recovery rate were determined in sperm pellets following density-gradient centrifugation semen.

Stallions and Semen Collection

For each of the three experiments, three ejaculates from each of four stallions (n = 12) were used. All stallions were sexually mature, light-horse breed, and sexually active. Ejaculates were collected at 1- to 5-day intervals using an artificial vagina (Missouri-model; Nasco, Ft. Atkinson, WI, USA) equipped with an in-line nylon micromesh filter (Animal Reproduction Systems, Chino, CA, USA) to allow collection of gel-free semen. Immediately prior to semen collection, the artificial vaginas were lubricated with approximately 3 mL of sterile non-spermicidal lubricant (Priority Care; First Priority, Inc., Elgin, IL, USA). Each stallion was sexually stimulated by an ovariectomized mare and semen was collected using a phantom mare. Once an erection was acquired, the stallion's penis was rinsed with warm tap water and dried thoroughly.

Following semen collection, the gel-free semen sample was transported to an adjacent laboratory and placed in an incubator (37°C) prior to processing.

General Semen Processing

The total sperm number in gel-free semen was estimated by measuring semen volume with a graduated cylinder and measuring initial sperm concentration using a fluorescence-based instrument (NucleoCounter SP-100; Chemometec A/S, Allerød, Denmark). One-mL aliquots of well-mixed semen were immediately snap frozen on dry ice in 1-mL polypropylene tubes (Cryogenic vials [1.2-mL]; Corning Life Sciences, Lowell, MA, USA), then stored at -80^{o}C until analyzed for the susceptibility of sperm chromatin to denaturation (i.e., Sperm Chromatin Structure Assay; SCSA). Also, one-mL aliquots of well-mixed semen were immediately diluted in Buffered Formol Saline (BFS) in 1-mL polypropylene tubes (Cryogenic vials [1.2-mL]; Corning Life Sciences, Lowell, MA, USA), and stored at ambient temperature until analyzed for sperm morphology.

Aliquots of gel-free semen were immediately diluted with a pre-warmed (37^{o}C) extender (INRA 96; IMV, Maple Grove, MN, USA) to a final sperm concentration of approximately 20 million sperm/mL for evaluation of initial sperm motility measures, using computer-assisted sperm motion analysis. The INRA 96 extender was selected because it is free of particulate debris that could interfere with computerized sperm-motility analysis.

Cushion Centrifugation Procedures

Semen was centrifuged by cushioned method to increase sperm concentration prior to semen application to density gradients [44]. Briefly, the extended semen (40 mL) containing approximately 2-10 billion sperm was first loaded into polypropylene 50-mL conical-bottom centrifugation tubes (Corning Life Sciences, Lowell, MA, USA), then 3.5 mL of cushion media (Cushion Fluid™; Minitüb, Tiefenbach, Germany; CF) was layered beneath the extended semen, using a blunt-tipped 3.5-inch spinal needle (18 ga), attached to a sterile 5-mL syringe. The tubes were then centrifuged (IEC Centra CL2; Thermo Scientific, Waltham, MA, USA) at 1000 x *g* for 20 minutes at ambient temperature. Following centrifugation, the supernatant was aspirated to a preset volume mark in the conical tubes (7.5-mL), and then the majority of the cushion medium was removed by aspiration. The resulting sperm pellet was resuspended to a concentration of 250 – 500 x 10^6 sperm/mL with INRA 96 extender, Sperm concentration of the resuspended semen was measured using a NucleoCounter SP-100 (NucleoCounter SP-100; Chemometec A/S, Allerød, Denmark). Aliquots of resuspended semen were appropriately secured for analysis of sperm morphology, motion parameters, and chromatin quality.

Density Gradient Centrifugation Procedures

All density-gradient products were warmed to room temperature prior to use. Predetermined volumes (depending on the amount required for each experiment) of EquiPure™ Top Layer and Bottom Layers (Nidacon International AB, Mölndal,

Sweden) were transferred with a sterile 50-mL syringe from the manufacturer's storage bottle into separate polypropylene 50-mL conical tubes for ease of pipetting. EquiPure™ Bottom Layer was added to polypropylene 15-mL conical-bottom centrifugation tubes (Corning Life Sciences, Lowell, MA, USA) to volumes of two-mL (Experiments 1 and 2), three-mL (Experiment 2), or four-mL (Experiments 1, 2,and 3). The same product was added to polypropylene 50-mL conical tubes to volumes of nine-mL (Experiment 2), 12.5-mL (Experiment 2), or 16.5-mL (Experiments 2 and 3). Volumes were measured using air-displacement pipettes (Rainin Instruments, Oakland, CA, USA). For Experiment 1, two mL of EquiPure™ Top Layer were carefully layered on top of two-mL of EquiPure™ Bottom Layer for two-layer density-gradient preparation.

Previously centrifuged and resuspended semen was carefully layered onto the EquiPure™ density gradients (Fig 1; Corning Life Sciences, Lowell, MA, USA; CONICAL tubes). One-mL aliquots were layered over each of the density gradients for Experiments 1 and 2. One-, two-, three-, or four-mL aliquots were layered over density gradients for Experiment 3. The loaded tubes were then centrifuged (Marathon 10K, Fisher Scientific, Pittsburgh, PA, USA) at 200 x *g* for 30 minutes at ambient temperature, using a swinging rotor. Following density gradient centrifugation, all supernatant above the resulting sperm pellets was aspirated using a glass Pasteur pipette attached to a vacuum set at approximately 300-500 mm.Hg.

Sperm pellets were resuspended in individual 2-mL polypropylene tubes (Corning Life Sciences, Lowell, MA, USA) containing 500 µL of INRA 96 extender,

supplemented with 10% seminal plasma from a control stallion. Volumes of the post-centrifugation sperm pellets were carefully measured with an air-displacement pipette. A post-centrifugation sperm concentration was measured using a NucleoCounter SP-100. The sperm concentration and volume of the resuspended semen samples were measured, and then extended semen was prepared immediately for sperm motility analysis, was diluted in BFS solution for sperm morphology analysis, and was frozen in 1-mL aliquots at -80 ^{o}C until analyzed for sperm chromatin integrity (SCSA).

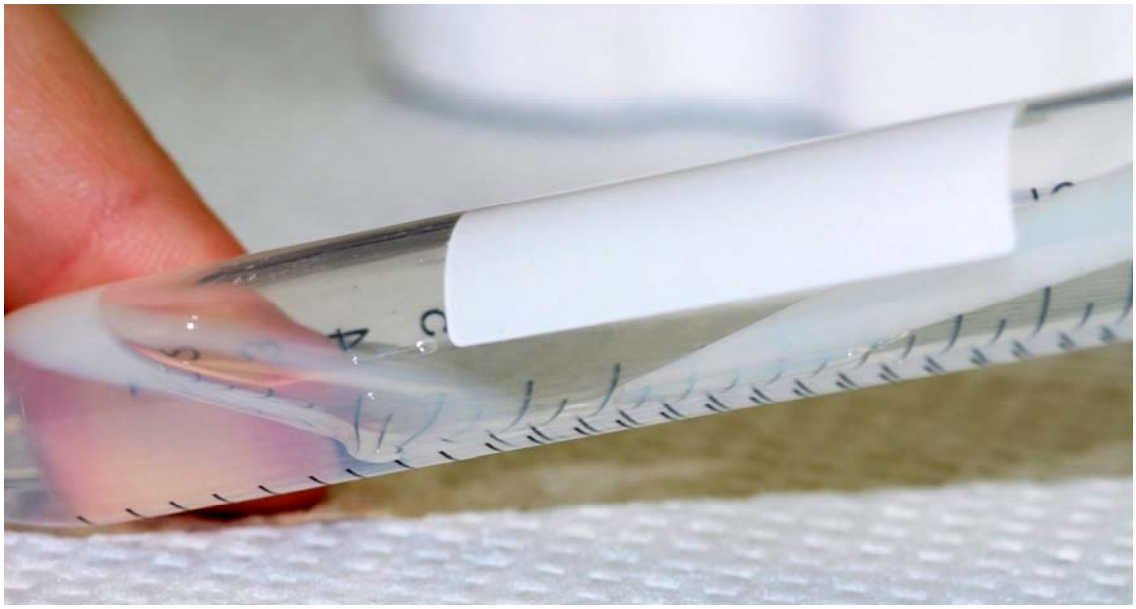

Fig. 1

Photograph of layering technique of semen over EquiPure™ gradient

Experimental Design

Experiment 1

To evaluate the effects of density gradient system on semen quality and sperm recovery rate, semen extended with INRA96 extender was subjected to cushioned centrifugation, as described above, and then sperm pellets were resuspended in INRA96 extender to a concentration of 250 – 500 x 10^6 sperm/mL. One-ml aliquots of this semen were layered over one of two density gradients ; 1) Two-mL EquiPure™ Top Layer over two-mL EquiPure™ Bottom Layer or 2) Four-mL EquiPure™ Bottom Layer. All density-gradient centrifugations were performed using polypropylene 15-mL conical centrifugation tubes. Following density gradient centrifugation, the contents of each centrifuge tube were aspirated to the level of the resulting sperm pellet. The sperm pellet was transferred into INRA 96 extender as described above.

Aliquots of resuspended semen samples were prepared immediately for CASMA, morphologic analysis or were frozen for later analysis of SCSA as described above. The effect of discontinuous density gradient composition (one-layer versus two-layer) on sperm motion characteristics, sperm morphologic features, sperm chromatin integrity, and sperm recovery rate were examined.

Experiment 2

To examine the effects of centrifuge-tube type and density gradient volume on semen quality and sperm recovery rate, semen extended with INRA96 extender was subjected to cushioned centrifugation, as described above, and then sperm pellets were

resuspended in INRA96 extender to a concentration of 250 – 500 x 10^6 sperm/mL. One-ml aliquots of this semen were layered over density gradients of specified volumes in one of two centrifuge tube types, as follows: 15-mL centrifuge tubes containing two, three, or four mL of Bottom-Layer EquiPure™ gradient medium, or 50-mL containing nine, 12.5, or 16.5 mL of gradient medium (representing similar gradient heights of 28mm, 35mm, and 41mm to the two-, three-, and four-mL gradients, respectively in the 15-mL centrifuge tubes). Following density gradient centrifugation, the contents of each centrifuge tube were aspirated to the level of the resulting sperm pellet. The sperm pellet was transferred into INRA 96 extender as described above.

Aliquots of resuspended semen samples were prepared immediately for CASMA, morphologic analysis or were frozen for later analysis of SCSA as described above. The effects of centrifugation-tube type and gradient volume on sperm motion characteristics, sperm morphologic features, sperm chromatin integrity, and sperm recovery rate were examined.

Experiment 3

To examine the effects of sperm number applied to density gradient centrifugation on semen quality and sperm recovery rate, semen extended with INRA96 extender was subjected to cushioned centrifugation, as described above, and then sperm pellets were resuspended in INRA96 extender to a concentration of 250 – 500 x 10^6 sperm/mL. One-mL to four-mL aliquots of this extended semen were layered over density gradients containing four mL of EquiPure Bottom Layer in 15-mL centrifugation

or 15 mL of EquiPure Bottom Layer in 50-mL centrifugation tubes (representing a similar gradient height to that used in 15-mL tubes). Following density gradient centrifugation, the contents of each centrifuge tube were aspirated to the level of the resulting sperm pellet. The sperm pellet was transferred into INRA 96 extender as described above.

Aliquots of resuspended semen samples were prepared immediately for CASMA, morphologic analysis or were frozen for later analysis of SCSA as described above. The effects of sperm number applied to gradients and centrifugation tube type on sperm motion characteristics, sperm morphologic features, sperm chromatin integrity, and sperm recovery rate were examined.

Computer Assisted Sperm Motion Analysis (CASMA)

Sperm were analyzed by CASMA in a manner similar to that previously described [45]. Warmed (37 ^{o}C) analysis chambers (fixed height of 20 μm) affixed to microscope slides (Leja Standard Count 2 Chamber slides; Leja Products, B.V., Nieuw-Vennep, The Netherlands) were slowly loaded with a 6-μL volume of extended semen. The slides were then placed on a stage (37 ^{o}C) and inserted into a CASMA instrument (IVOS Version 12.2L, Hamilton Thorne Biosciences, Beverly, MA, USA) for evaluation. A total of 10 microscopic fields and a minimum of 500 sperm were examined per sample. Preset values for the IVOS system consisted of the following: frames acquired – 45; frame rate – 60 Hz; minimum contrast – 70; minimum cell size – 4 pixels; minimum static contrast – 30; straightness (STR) threshold for progressive

motility – 50; average-path velocity (VAP) threshold for progressive motility - 30; VAP threshold for static cells - 15; cell intensity – 106; static head size – 0.60 to 2.00; static head intensity – 0.20 to 2.01; static elongation – 40 to 85; LED illumination intensity – 2200. Experimental endpoints included: 1) percentage of motile sperm (MOT); percentage of progressively motile sperm (PMOT); mean curvilinear velocity (VCL; μm/s); mean average-path velocity (VAP; μm/s); mean straight-line velocity (VSL; μm/s), straightness ([VSL/VAP] x 100; %; STR), and linearity ([VSL/VCL] x 100; %; LIN).

Sperm Chromatin Structure Assay (SCSA)

This assay was performed as previously described [46-48]. Individual semen samples were thawed in a water bath set at 37^{o}C. Approximately five-μL aliquots of thawed semen were mixed with 195 μL of a buffered solution which was then combined with a low pH (~ 1.2) detergent solution (400 μL) for 30 sec. A solution of the heterochromatic dye, acridine orange, was added (1.2 mL at 4.0 μg/mL) to the sample and it was processed immediately (30 sec) on a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA). The sample was allowed to pass through the tubing for two min before evaluation of cells. Semen volume was adjusted so the flow rate approximated 200 cells/sec. A total of 5000 events were evaluated per sample. The flow cytometer was adjusted so that the mean green fluorescence was set at 500 channels (FL-1 @ 500) and mean red fluorescence at 150 channels (FL-3 @ 150). Data were acquired in a list-mode and Sperm Chromatin Structure Assay values were calculated

using WinList™ software (Verity Software House, Topsham, ME, USA). Quantification of DNA denaturation in each cell was determined by the term alpha-t (αt), which is defined as the ratio of red/(red + green fluorescence). The alpha-t (αt) designation is used to describe the relationship between the amounts of green (double-stranded DNA) and red (single-stranded DNA) fluorescence. The results were recorded as both scattergrams and frequency histograms. The endpoint, Cells Outside the Main Population (COMPαt), was determined by selecting those sperm cells to the right of the main population, and represents the number of sperm cells outside the main population, as a percentage of the total number of sperm cells evaluated.

Sperm Morphology

The percentages of morphologically normal sperm and percentages of sperm with specific morphological defects were determined using differential-interference contrast microscopy (Olympus BX60, Olympus America, Inc., Melville, NY, USA; 1250 x magnification). For analysis, 2-µL aliquots of semen were applied to a microscope slide, the fitted with a 22x22-mm No. 1 ½ cover-glass. A total of 100 sperm per sample were examined to obtain the percentages of sperm with the following morphologic features: normal, abnormal heads, abnormally small heads, abnormally large heads, nuclear vacuoles (crater defects), misshaped heads, abnormal acrosomes, detached heads, proximal droplets, small proximal droplets, large proximal droplets distal droplets, small distal droplets, large distal droplets, abnormal (swollen or irregular) midpieces, bent midpieces, bent tails, coiled tails, and premature germ cells.

Statistical Analysis

For each experiment, a general linear model [49] was used to evaluate effects of treatments on experimental endpoints for semen quality and recovery rate. Semen subjected to density gradient centrifugation was also compared with semen subjected to cushioned centrifugation only and to semen that was simply diluted in extender and not subjected to any form of centrifugation. The control groups were assigned the names of Group UC for uncentrifuged semen and Group CC for cushion centrifuged semen. For Experiment 1, the EquiPure™ treatment groups were assigned the names of Group 1L for the one-layer and Group 2L for the two-layer. The EquiPure™ treated samples were assigned the names of Group 15-28mm, 15-35mm, 15-41mm, 50-28mm, 50-35mm, and 50-41mm for 2, 3, 4, 9, 12.5, and 16.5mL EquiPure™ Bottom Layer, respectively in Experiment 2. For Experiment 3, the EquiPure™ treatment groups were assigned the names of Group 15-1x, 15-2x, 15-3x, 15-4x, 50-1x, 50-2x, 50-3x, and 50-4x for the sperm load of 1-4x (250-500 x 10^6 sperm/mL). Variables measured as percentages were normalized by transformation to angles corresponding to *arc sine of the square root of percentage* for variance analyses. Tabular data are presented as non-transformed values, for ease of interpretation. The Student-Newman-Keuls multiple range test was used to separate main-effect means when treatment F ratios were significant ($P < 0.05$).

RESULTS

Experiment 1

The main effects of the density-gradient composition (One Layer [Group 1L] versus Two Layer [Group 2L]) on measures of sperm quality values are presented in Tables 1 and 2. These treatment groups were also compared to extended semen that was not subjected to centrifugation (Group UC) and to extended semen that was subjected only to cushioned centrifugation (Group CC). Group 1L yielded higher mean values for MOT, PMOT, VAP, LIN and COMPαt than did Group 2L ($P<0.05$). Mean VCL, VSL, and STR were similar between these two treatment groups ($P>0.05$). All measures of sperm morphology were also similar between the two treatment groups ($P>0.05$).

Mean values for eleven of 27 experimental endpoints (MOT, PMOT, STR, LIN, COMPαt, morphologically normal, abnormal midpieces, bent midpieces, bent tails, coiled tails, and premature germ cells) yielded improved quality in Group 1L and Group 2L, as compared to control treatments (Group UC and Group CC; $P<0.05$). Mean VCL and VAP were similar between the Group 1L and Group UC ($P>0.05$) and both were higher than Group CC ($P<0.05$). Mean VSL was lower in Group CC than Group UC, Group 2L and Group 1L ($P<0.05$). Group UC yielded slightly greater values for abnormal heads than did Groups CC, 2L, and 1L ($P<0.05$). Mean values for craters, small heads, abnormal acrosomes, proximal droplets, small proximal droplets and small distal droplets for were similar across treatment groups ($P>0.05$). Groups 1L and 2L were similar to Group CC for mean percentages of large heads, misshaped heads, detached heads, and small distal droplets ($P>0.05$). The mean percentage of large

proximal droplets and large distal droplets was slightly higher for the Group CC, as compared to the Groups UC, 1L, and 2L ($P<0.05$).

Table 1: Main effects of treatment on sperm motility and chromatin quality (COMPαt) variables for four stallions (mean ± SEM).

Laboratory Parameter*	Treatment			
	Group UC[a†]	Group CC[b†]	EquiPure™ Treated	
			Group 2L[c]	Group 1L[d]
MOT	64d (5.4)	70c (4.8)	86b (2.8)	93a (0.8)
PMOT	37d (7.3)	42c (7.1)	68b (4.1)	78a (2.0)
VCL	165a (11.9)	149c (9.0)	155bc (9.9)	160ab (10.3)
VAP	84a (7.3)	72b (5.4)	77b (6.5)	83a (6.3)
VSL	64a (6.2)	54b (4.4)	62a (4.8)	67a (4.9)
STR	74b (2.2)	73c (1.6)	81a (1.2)	81a (0.8)
LIN	39c (1.8)	36d (1.3)	41b (0.7)	43a (0.8)
COMPαt	9a (1.7)	9a (1.8)	4b (0.7)	3c (0.4)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b,c and d) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=12).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=12).

[c] Group 2L (Two Layer) = EquiPure™ gradient with Top/Bottom Layers (2 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=12).

[d] Group 1L (One Layer) = EquiPure™ gradient with Bottom Layer only (4 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=12).

† Control Samples.

Table 2: Main effects of treatment on sperm morphology variables for four stallions (mean ± SEM).

Laboratory Parameter*	Treatment			
	Group UC[a†]	Group CC[b†]	EquiPure™ Treated	
			Group 2L[c]	Group 1L[d]
Morphologically Normal	33b (5.2)	34b (5.5)	69a (3.8)	70a (3.0)
Abnormal Heads	9a (0.7)	7b (0.5)	6c (0.5)	6bc (0.6)
Craters	1a (0.2)	1a (0.4)	1a (0.2)	1a (0.2)
Small Heads	2a (0.3)	2a (0.3)	1a (0.2)	2a (0.3)
Large Heads	2a (0.5)	1b (0.4)	1b (0.1)	1b (0.1)
Misshaped Heads	4a (0.6)	3ab (0.3)	3b (0.2)	3b (0.2)
Abnormal Acrosomes	1a (0.2)	1a (0.2)	0a (0)	0a (0)
Detached Heads	6a (1.1)	4b(0.6)	3b (0.7)	3b (0.6)
Proximal Droplets	5a (0.8)	4a (0.7)	5a (0.6)	5a (0.7)
Small Proximal Droplets	3a (0.7)	2a (0.6)	4a (0.6)	3a (0.5)
Large Proximal Droplets	1b (0.2)	2a (0.4)	1b (0.2)	1b (0.1)
Distal Droplets	4b (0.7)	6a (1.3)	5ab (0.6)	5ab (0.6)
Small Distal Droplets	2b (0.5)	2ab (0.8)	3a (0.5)	3a (0.5)
Large Distal Droplets	2b (0.3)	4a (0.7)	2b (0.3)	2b (0.3)
Abnormal Midpieces	12b (1.8)	16a (1.5)	8c (0.9)	9c (0.9)
Bent Midpieces	15a (2.4)	13a (2.0)	3b (0.7)	2b (0.6)
Bent Tails	4a (1.2)	5a (1.3)	1b (0.5)	0b (0.2)
Coiled Tails	10a (0.1)	9a (1.3)	2b (0.5)	0b (0.2)
Premature Germ Cells	2a (0.5)	1b (0.3)	0b (0)	0b (0)

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b and c) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=12).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=12).

[c] Group 2L (Two Layer) = EquiPure™ gradient with Top/Bottom Layers (2 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=12).

[d] Group 1L (One Layer) = EquiPure™ gradient with Bottom Layer only (4 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=12).

† Control Samples.

Significant stallion by treatment interactions ($P<0.05$) were detected for 13 of 27 variables (MOT, PMOT, VCL, VSL, STR, LIN, COMPαt, morphologically normal, abnormal heads, distal droplets, bent midpieces, coiled tails, and premature germ cells) and are presented in Tables 3 through 10. The variable MOT was higher ($P<0.05$) in Group 1L than Group 2L for only one of four stallions. For 3 of 4 stallions, mean MOT, was higher in Group 1L and 2L than Group UC and Group CC. Mean PMOT was higher in Group 1L than Group 2L for two of the four stallions ($P<0.05$). For three of the four stallions, mean PMOT was higher in Group 1L and Group 2L than Group UC and Group CC ($P<0.05$). Mean VCL was similar across all 4 treatment groups for ejaculates from each of the four stallion studies ($P>0.05$). For three of the four stallions, no differences were detected between Group 1L and Group 2L for mean VSL ($P>0.05$). Mean VSL was higher in Group 1L than Group 2L for the remaining stallion ($P<0.05$). Mean STR was similar between Groups 1L and 2L for three of four stallions ($P>0.05$), and mean LIN was similar between these groups for all four stallions. The variable COMPαt was higher in Group 1L than Group 2L for one stallion only ($P<0.05$). The variable COMPαt was higher in the EquiPure™ treatment groups than both controls for all stallions ($P<0.05$).

No difference was detected between Group 1L and Group 2L for the mean percentage of morphologically normal sperm in semen from each stallion tested ($P>0.05$). Both EquiPure™ treatment groups (Group 1L and Group 2L) yielded a higher percentage of morphologically normal sperm than did the control groups (Group UC and Group CC) for each of the four stallions examined ($P<0.05$). The percentage of

abnormal heads was higher in Groups 1L and 2L than in Group UC for 3 of 4 stallions tested. The variable distal droplets was similar across treatment groups for three of four stallions ($P>0.05$). For all four stallions, both control groups contained a higher percentage of bent tails than did the than the EquiPure™ treatment groups ($P<0.05$). For three of four stallions, mean values for coiled tails were higher in control groups than in EquiPure™ treatment groups ($P<0.05$). The percentage of premature germ cells was higher in Group UC than all other groups for one stallion ($P<0.05$). No difference was detected among treatment groups for the remaining stallions ($P>0.05$).

Sperm recovery rate (%) was higher ($P<0.05$) in Group 1L (46.9 ± 0.08) than Group 2L (33.9 ± 0.07). A stallion by treatment interaction was detected for recovery rate ($P<0.05$). No differences were detected between Group 1L (50.7 ± 0.007) and Group 2L (45.3 ± 0.03) for mean sperm recovery rate for one stallion ($P>0.05$). For each of the remaining three stallions, Group 1L yielded a higher mean sperm recovery rate than Group 2L ($P<0.05$).

Table 3: Effect of treatment on sperm motility and chromatin quality (COMPαt) variables for Stallion A (mean ± SEM).

Laboratory Parameter*	Treatment			
	Group UC[a†]	Group CC[b†]	EquiPure™ Treated	
			Group 2L[c]	Group 1L[d]
MOT	92a (2.1)	93a (0.3)	95a (0.6)	96a (0.6)
PMOT	76b (3.8)	79ab (1.5)	83ab (1.5)	86a (0.9)
VCL	196a (14.2)	174a (5.3)	170a (1.8)	178a (1.0)
VAP	108a (10.5)	87a (3.5)	88a (0.3)	93a (0.3)
VSL	87a (8.4)	68a (3.1)	70a (0.3)	74a (1.0)
STR	81a (0.3)	78a (0.6)	80a (0.3)	78a (0.9)
LIN	45a (1.3)	40b (0.6)	42ab (0.3)	43ab (0.6)
COMPαt	5a (0.3)	4b (0.2)	2c (0.1)	2c (0.2)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b and c) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] Group 2L (Two Layer) = EquiPure™ gradient with Top/Bottom Layers (2 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

[d] Group 1L (One Layer) = EquiPure™ gradient with Bottom Layer only (4 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Table 4: Effect of treatment on sperm morphology variables for Stallion A (mean ± SEM).

Laboratory Parameter*	Treatment			
	Group UC[a†]	Group CC[b†]	EquiPure™ Treated	
			Group 2L[c]	Group 1L[d]
Morphologically Normal	60b (2.0)	62b (3.0)	89a (1.3)	85a (0.3)
Abnormal Heads	11a (1.5)	6b (0.9)	3b (0.7)	5b (0.6)
Craters	0a (0.3)	0a (0)	0a (0)	0a (0.3)
Small Heads	2a (0.9)	2a (1.2)	1a (0.3)	1a (0)
Large Heads	4a (1.0)	2a (1.5)	1a (0)	1a (0.3)
Misshaped Heads	5a (0.9)	3a (0.9)	2a (0.3)	3a (0.3)
Abnormal Acrosomes	2a (0.3)	0a (0.3)	0a (0)	0a (0)
Detached Heads	2a (0.9)	2a (0.6)	0a (0)	0a (0.3)
Proximal Droplets	3a (0.3)	3a (0)	2a (0.3)	2a (0.6)
Small Proximal Droplets	2a (0)	2a (0.3)	1a (0.3)	1a (0.3)
Large Proximal Droplets	1a (0.3)	1a (0.3)	1a (0.3)	1a (0)
Distal Droplets	4a (1.9)	5a (0.3)	2a (0.3)	2a (0.3)
Small Distal Droplets	2a (1.2)	3a (1.2)	2a (0.3)	2a (0.3)
Large Distal Droplets	3a (0.9)	3a (0.9)	1a (0.3)	1a (0)
Abnormal Midpieces	7ab (0.6)	10a (0.9)	4b (1.0)	6ab (1.8)
Bent Midpieces	6a (1.7)	5a (1.0)	1b (0.3)	0b (0.3)
Bent Tails	2a (1.2)	2a (1.0)	0a (0)	0a (0)
Coiled Tails	4a (2.0)	2a (1.2)	0a (0)	0a (0.3)
Premature Germ Cells	0a (0)	0a (0.3)	0a (0)	0a (0)

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a and b) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] Group 2L (Two Layer) = EquiPure™ gradient with Top/Bottom Layers (2 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

[d] Group 1L (One Layer) = EquiPure™ gradient with Bottom Layer only (4 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Table 5: Effect of treatment on sperm motility and chromatin quality (COMPαt) variables for Stallion B (mean ± SEM).

Laboratory Parameter*	Treatment			
	Group UC[a†]	Group CC[b†]	EquiPure™ Treated	
			Group 2L[c]	Group 1L[d]
MOT	60c (4.4)	72b (1.7)	91a (1.3)	94a (0.7)
PMOT	33b (5.8)	41b (2.6)	73a (2.0)	80a (1.5)
VCL	183a (9.9)	173a (4.6)	191a (3.5)	197a (1.9)
VAP	99a (4.4)	88b (2.0)	104a (1.5)	109a (0.3)
VSL	80b (3.2)	68c (1.5)	82b (0.9)	89a (0.6)
STR	76c (0.6)	74d (0.3)	78b (0)	81a (0.3)
LIN	42b (0.9)	39c (0.9)	44ab (0.3)	46a (0.7)
COMPαt	3a (0.2)	4a (0.5)	2b (0.003)	2b (0.1)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b,c and d) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] Group 2L (Two Layer) = EquiPure™ gradient with Top/Bottom Layers (2 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

[d] Group 1L (One Layer) = EquiPure™ gradient with Bottom Layer only (4 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Table 6: Effect of treatment on sperm morphology variables for Stallion B (mean ± SEM).

Laboratory Parameter*	Treatment			
	Group UC[a†]	Group CC[b†]	EquiPure™ Treated	
			Group 2L[c]	Group 1L[d]
Morphologically Normal	35b (1.3)	32b (3.8)	68a (0.3)	70a (0.3)
Abnormal Heads	10a (0.7)	6b (0.6)	7b (0.3)	5b (0.6)
Craters	0a (0.3)	0a (0.3)	1a (0.3)	1a (0.7)
Small Heads	2a (0.7)	1a (0.3)	1a (0.3)	2a (0.3)
Large Heads	2a (0.7)	1a (0.3)	1a (0.3)	1a (0)
Misshaped Heads	5a (1.9)	4a (0.7)	3a (0)	2a (0.3)
Abnormal Acrosomes	0a (0.3)	1a (0.3)	0a (0)	0a (0)
Detached Heads	5a (1.5)	3a (1.2)	2a (0.6)	2a (0.3)
Proximal Droplets	6a (2.4)	6a (2.5)	8a (0.3)	8a (0.3)
Small Proximal Droplets	5a (2.1)	4a (2.2)	6a (0.7)	6a (0.3)
Large Proximal Droplets	1a (0.3)	2a (0.3)	2a (0.3)	2a (0)
Distal Droplets	2b (0.6)	2b (0.7)	5a (0.3)	6a (0.6)
Small Distal Droplets	1c (0.3)	1c (0.6)	3b (0.3)	5a (0.3)
Large Distal Droplets	1a (0.9)	1a (0.3)	1a (0.3)	1a (0.3)
Abnormal Midpieces	9a (2.0)	15a (3.8)	8a (1.2)	8a (0)
Bent Midpieces	15a (2.4)	16a (0.9)	2b (0.7)	2b (0.3)
Bent Tails	3a (0.3)	4a (0.6)	0b (0.3)	0b (0)
Coiled Tails	15a (2.1)	13a (1.2)	1b (0.3)	0b (0.3)
Premature Germ Cells	1a (0)	0b (0.3)	0b (0)	0b (0)

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b and c) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] Group 2L (Two Layer) = EquiPure™ gradient with Top/Bottom Layers (2 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

[d] Group 1L (One Layer) = EquiPure™ gradient with Bottom Layer only (4 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Table 7: Effect of treatment on sperm motility and chromatin quality (COMPαt) variables for Stallion C (mean ± SEM).

Laboratory Parameter*	Treatment			
	Group UC[a†]	Group CC[b†]	EquiPure™ Treated	
			Group 2L[c]	Group 1L[d]
MOT	58c (0.9)	61c (2.9)	72b (4.4)	90a (1.7)
PMOT	24c (0.6)	28c (4.4)	48b (4.3)	70a (4.2)
VCL	102a (7.0)	101a (3.7)	104a (2.0)	106a (1.2)
VAP	50a (4.5)	46a (1.7)	47a (0.3)	53a (1.2)
VSL	41a (4.0)	36a (1.7)	41a (0.6)	45a (1.2)
STR	79b (0.3)	76b (1.0)	87a (1.0)	85a (1.2)
LIN	40a (0.9)	36b (0.6)	66a (4.7)	43a (1.0)
COMPαt	10a (0.3)	10a (1.2)	5b (0.9)	2c (0.3)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b and c) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] Group 2L (Two Layer) = EquiPure™ gradient with Top/Bottom Layers (2 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

[d] Group 1L (One Layer) = EquiPure™ gradient with Bottom Layer only (4 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Table 8: Effect of treatment on sperm morphology variables for Stallion C (mean ± SEM).

Laboratory Parameter*	Treatment			
	Group UC[a†]	Group CC[b†]	EquiPure™ Treated	
			Group 2L[c]	Group 1L[d]
Morphologically Normal	22b (0.7)	26b (1.9)	66a (4.7)	68a (0.9)
Abnormal Heads	10a (0.3)	8ab (0.6)	6b (1.2)	5b (0.3)
Craters	1a (0.3)	1a (0.3)	1a (0.3)	1a (0.3)
Small Heads	2a (0.3)	2a (0.6)	2a (0.3)	2a (0.3)
Large Heads	2a (0.3)	1a (0.3)	1a (0.3)	1a (0.3)
Misshaped Heads	4a (0.3)	3a (0.7)	2a (0.3)	2a (0.3)
Abnormal Acrosomes	1a (0.6)	1a (0.6)	0a (0)	0a (0)
Detached Heads	9a (1.2)	4a (1.2)	4a (1.7)	4a (0.7)
Proximal Droplets	7a (1.2)	4a (1.5)	5a (0.3)	4a (0.3)
Small Proximal Droplets	5a (1.5)	1a (0.9)	3a (0.3)	3a (0.6)
Large Proximal Droplets	2a (0.6)	3a (1.0)	1a (0.3)	1a (0)
Distal Droplets	6a (1.5)	11a (3.9)	6a (0.9)	7a (0.6)
Small Distal Droplets	3a (1.3)	5a (2.4)	5a (1.2)	5a (0.3)
Large Distal Droplets	3b (0.3)	6a (1.5)	2b (0.3)	2b (0.3)
Abnormal Midpieces	21a (1.8)	21a (1.0)	11b (1.5)	11b (0.9)
Bent Midpieces	11a (0.6)	10a (0.9)	2b (0.6)	1b (0.9)
Bent Tails	3a (1.5)	4a (1.0)	1a (0.6)	0a (0.3)
Coiled Tails	9a (0.7)	10a (1.0)	1b (0.3)	1b (0)
Premature Germ Cells	1a (0.7)	0a (0.3)	0a (0)	0a (0)

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a and b) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] Group 2L (Two Layer) = EquiPure™ gradient with Top/Bottom Layers (2 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

[d] Group 1L (One Layer) = EquiPure™ gradient with Bottom Layer only (4 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Table 9: Effect of treatment on sperm motility and chromatin quality (COMPαt) variables for Stallion D (mean ± SEM).

Laboratory Parameter*	Treatment			
	Group UC[a†]	Group CC[b†]	EquiPure™ Treated	
			Group 2L[c]	Group 1L[d]
MOT	46b (5.8)	52b (4.4)	87a (2.4)	92a (0.9)
PMOT	14c (3.2)	18c (2.3)	66b (2.5)	76a (1.9)
VCL	178a (6.8)	150a (0.7)	153a (10.2)	161a (6.0)
VAP	77a (3.3)	67a (6.4)	69a (5.5)	76a (3.0)
VSL	49bc (1.9)	43c (2.6)	54ab (3.5)	60a (1.2)
STR	62b (0.6)	64b (0.9)	78a (1.2)	79a (1.2)
LIN	30b (0.7)	29b (0.1)	38a (1.0)	39a (0.9)
COMPαt	17a (1.6)	17a (1.7)	6b (0.9)	5b (0.4)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b and c) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] Group 2L (Two Layer) = EquiPure™ gradient with Top/Bottom Layers (2 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

[d] Group 1L (One Layer) = EquiPure™ gradient with Bottom Layer only (4 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Table 10: Effect of treatment on sperm morphology variables for Stallion D (mean ± SEM).

Laboratory Parameter*	Treatment			
	Group UC[a†]	Group CC[b†]	EquiPure™ Treated	
			Group 2L[c]	Group 1L[d]
Morphologically Normal	16b (3.5)	13b (1.8)	56a (3.2)	58a (3.0)
Abnormal Heads	5a (0.9)	8a (1.5)	7a (0.7)	9a (0.7)
Craters	1a (0.6)	2a (1.2)	1a (0)	1a (0)
Small Heads	0b (0.3)	2ab (0.3)	2ab (0.3)	3a (0.9)
Large Heads	2a (1.0)	1a (0.6)	1a (0.3)	1a (0.3)
Misshaped Heads	3a (0.9)	4a (0.7)	3a (0)	3a (0.3)
Abnormal Acrosomes	1a (0.6)	0a (0)	0a (0)	0a (0)
Detached Heads	9a (2.2)	6a (1.0)	5a (0.3)	6a (0.3)
Proximal Droplets	2b (0.6)	2ab (0.3)	5a (0.6)	4ab (1.0)
Small Proximal Droplets	1b (0)	1b (0.3)	3a (0)	3a (0.7)
Large Proximal Droplets	1a (0.6)	3a (0.7)	2a (0.6)	1a (0.3)
Distal Droplets	4a (0.3)	6a (1.0)	6a (1.5)	5a (0.6)
Small Distal Droplets	1a (0.3)	1a (1.0)	3a (1.0)	2a (0.6)
Large Distal Droplets	3b (0)	5a (0)	3b (0.3)	3b (0)
Abnormal Midpieces	12a (2.8)	18a (2.3)	10a (0.6)	12a (2.0)
Bent Midpieces	26a (2.0)	21a (2.6)	7b (0.3)	5b (0.9)
Bent Tails	9ab (3.6)	12a (2.8)	3ab (0.9)	1b (0.7)
Coiled Tails	12a (2.0)	12a (1.2)	4b (0.9)	1b (0.6)
Premature Germ Cells	4a (1.2)	2a (0.6)	0a (0)	0a (0)

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a and b) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] Group 2L (Two Layer) = EquiPure™ gradient with Top/Bottom Layers (2 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

[d] Group 1L (One Layer) = EquiPure™ gradient with Bottom Layer only (4 mL each) in 15-mL tubes and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Experiment 2

Main effects for mean MOT, PMOT, VSL, COMPαt, morphologically normal sperm, and sperm with abnormal heads, craters, small heads, large heads, misshaped heads, abnormal acrosomes, detached heads, proximal droplets, small proximal droplets, large proximal droplets, distal droplets, small distal droplets, large distal droplets, bent midpieces, bent tails, coiled tails, or premature germ cells were not impacted by EquiPure™ treatment groups, which addressed centrifuge tube size and density gradient height ($P>0.05$; Tables 11 and 12). Mean curvilinear velocity was higher for sperm recovered in Groups 15-28mm (2mL), 15-35mm (3mL), and 15-41mm (4mL) than in Groups 50-35mm (12.5mL) and 50-41mm (16.5mL) ($P<0.05$). Mean VAP was higher for Group 15-28mm (2mL) when compared to Group 50-41mm (16.5mL) ($P<0.05$); however, sperm subjected to EquiPure™ treatment in 50-mL tubes yielded slightly higher values for STR and LIN than sperm subjected to treatment in 15-mL tubes ($P<0.05$). The percentage of abnormal midpieces was significantly lower is Group 15-28mm (2mL) treatment than in Groups 15-35mm (3mL), 50-35mm (12.5mL), and 50-41mm (16.5mL) treatment groups ($P<0.05$).

When compared to the control groups (Groups UC and CC), the EquiPure™ treatment groups yielded improved values for mean MOT, PMOT, STR, COMPαt, morphologically normal, large proximal droplets, bent midpieces, and coiled tails ($P<0.05$). Mean VCL was higher for sperm in the 15-mL conical tubes than both controls, and was higher for sperm in the 50-mL conical tubes than in Group CC ($P<0.05$). All EquiPure™ treatment groups were yielded higher values than Group CC for mean VAP, VSL, and LIN ($P<0.05$). Overall, nine of the nineteen morphological endpoints (craters, small heads, large heads, proximal droplets, distal droplets, small distal droplets, large distal droplets, bent tails, and premature germ cells) yielded similar values for all EquiPure™ treatment groups to that of both control groups ($P>0.05$). Group 15-28mm (2mL) was lower than control groups for mean abnormal heads ($P<0.05$), For the experimental endpoint, mean misshaped heads, Group 50-35mm (12.5mL) yielded lower values than did Group UC ($P<0.05$). Mean percentages of abnormal acrosomes and small proximal droplets were higher in the Group UC than all EquiPure™ treatment groups ($P<0.05$). Treatment Groups 15-41 (4mL), 50-35mm (12.5mL), and 50-41mm (16.5mL) were lower than Group UC for mean detached heads ($P<0.05$). The mean percentage of abnormal midpieces was higher in Group CC than in Groups 15-28mm (2mL) and 50-28mm (9mL) ($P<0.05$).

Table 11: Main effects of treatment on sperm motility and chromatin quality (COMPαt) variables for four stallions (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment					
	Group UC[a†]	Group CC[b†]	15-28mm (2mL)[c‡]	15-35mm (3mL)[d‡]	15-41mm (4mL)[e‡]	50-28mm (9mL)[f‡]	50-35mm (12.5mL)[g‡]	50-41mm (16.5mL)[h‡]
MOT	71b (3.4)	73b (3.5)	90a (1.8)	91a (1.9)	91a (1.8)	89a (1.9)	89a (1.8)	88a (1.8)
PMOT	42b (4.2)	46b (4.1)	73a (2.3)	73a (2.5)	74a (2.4)	72a (3.0)	72a (2.4)	70a (2.8)
VCL	202c (7.0)	188d (10.2)	228a (6.0)	224ab (4.9)	223ab (5.5)	211bc (5.1)	207c (4.9)	202c (4.9)
VAP	112ab (3.4)	96c (6.2)	122a (3.6)	121ab (3.1)	121ab (3.4)	116ab (2.9)	113ab (3.1)	111b (2.7)
VSL	87a (2.8)	72b (4.6)	96a (3.1)	94a (2.8)	96a (2.9)	93a (2.5)	92a (2.6)	91a (2.3)
STR	74d (0.6)	72e (0.6)	77c (0.8)	77c (1.0)	78c (0.9)	79ab (0.9)	79b (0.8)	80a (0.9)
LIN	43c (1.2)	38d (0.8)	43c (0.8)	43c (0.7)	44bc (0.9)	45ab (0.9)	45ab (0.9)	47a (1.0)
COMPαt	19a (3.1)	20a (3.1)	7b (1.8)	8b (1.7)	8b (1.9)	7b (1.4)	7b (1.7)	7b (1.3)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter means with different letters (a,b,c,d and e) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=12).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=12).

[c] 15-28mm (2mL) = 15-mL tube containing 2 mL of bottom-layer EquiPure™ (n=12).

[d] 15-35mm (3mL) = 15-mL tube containing 3 mL of bottom-layer EquiPure™ (n=12).

[e] 15-41mm (4mL) = 15-mL tube containing 4 mL of bottom-layer EquiPure™ (n=12).

[f] 50-28mm (9mL) = 50-mL tube containing 9 mL of bottom-layer EquiPure™ (n=12).

[g] 50-35mm (12.5mL) = 50-mL tube containing 12.5 mL of bottom-layer EquiPure™ (n=12).

[h] 50-41mm (16.5mL) = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ (n=12).

† Control Samples.

‡ Each tube had 1 mL extended semen layered on the EquiPure™ containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

Table 12: Main effects of treatment on sperm morphology variables for four stallions (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment					
	Group UC[a†]	Group CC[b†]	15-28mm (2mL)[c‡]	15-35mm (3mL)[d‡]	15-41mm (4mL)[e‡]	50-28mm (9mL)[f‡]	50-35mm (12.5mL)[g‡]	50-41mm (16.5mL)[h‡]
Morphologically Normal	38b (4.9)	38b (4.3)	63a (3.8)	61a (3.3)	61a (3.9)	62a (3.6)	61a (4.1)	59a (4.2)
Abnormal Heads	7a (0.6)	5b (0.7)	3c (0.6)	3bc (0.6)	4bc (0.5)	4bc (0.6)	4bc (0.5)	4bc (0.8)
Craters	2a (0.4)	2a (0.5)	1a (0.3)	1a (0.2)	1a (0.3)	1a (0.4)	2a (0.4)	2a (0.4)
Small Heads	2a (0.4)	2a (0.4)	1a (0.4)	1a (0.3)	2a (0.3)	2a (0.3)	1a (0.4)	1a (0.3)
Large Heads	1a (0.3)	0a (0)	0a (0.1)	1a (0.2)	0a (0.2)	0a (0)	0a (0)	0a (0)
Misshaped Heads	2a (0.4)	1ab (0.4)	1ab (0.2)	1ab (0.6)	2ab (0.3)	1ab (0.3)	0b (0.3)	1ab (0.4)
Abnormal Acrosomes	1a (0.3)	0b (0.1)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0.2)	0b (0)
Detached Heads	12a (3.1)	8ab (1.2)	7ab (2.0)	7ab (1.8)	6b (1.2)	6ab (1.3)	5b (0.7)	5b (0.6)
Proximal Droplets	14a (1.6)	17a (2.0)	14a (2.3)	14a (1.9)	15a (2.2)	14a (1.8)	14a (1.7)	15a (1.9)
Small Proximal Droplets	9b (1.5)	11a (2.1)	12a (1.8)	12a (1.5)	12a (1.9)	13a (1.8)	12a (1.7)	13a (1.7)
Large Proximal Droplets	5a (0.7)	5a (1.0)	2b (0.8)	2b (0.8)	2b (0.8)	1b (0.5)	2b (0.6)	2b (0.9)
Distal Droplets	3a (0.6)	4a (1.2)	3a (0.7)	3a (0.7)	3a (0.8)	3a (0.6)	3a (0.8)	3a (0.7)
Small Distal Droplets	1a (0.4)	3a (1.1)	3a (0.8)	3a (0.7)	3a (0.8)	3a (0.6)	2a (0.7)	3a (0.5)
Large Distal Droplets	2a (0.3)	1b (0.3)	0b (0)	0b (0.1)	0b (0.1)	0b (0.2)	0b (0.3)	4b (0.3)
Abnormal Midpieces	8bc (1.1)	11a (1.1)	5c (1.1)	8ab (0.8)	8abc (1.0)	6bc (0.8)	9ab (1.0)	9ab (1.0)
Bent Midpieces	11a (4.1)	11a (3.5)	3b (1.1)	3b (1.1)	3b (1.0)	5b (1.7)	4b (1.5)	4b (1.5)
Bent Tails	1a (0.2)	1a (0.4)	1a (0.3)	0a (0)	0a (0.3)	0a (0.3)	0a (0.3)	1a (0.4)
Coiled Tails	4a (1.0)	5a (1.7)	2b (0.6)	1b (0.3)	2b (0.5)	1b (0.3)	1b (0.4)	1b (0.5)
Premature Germ Cells	2a (0.6)	1a (0.4)	0a (0)	0a (0.1)	0a (0)	0a (0)	0a (0.2)	0a (0)

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter means with different letters (a,b and c) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=12).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=12).

[c] 15-28mm (2mL) = 15-mL tube containing 2 mL of bottom-layer EquiPure™ (n=12).

[d] 15-35mm (3mL) = 15-mL tube containing 3 mL of bottom-layer EquiPure™ (n=12).

[e] 15-41mm (4mL) = 15-mL tube containing 4 mL of bottom-layer EquiPure™ (n=12).

[f] 50-28mm (9mL) = 50-mL tube containing 9 mL of bottom-layer EquiPure™ (n=12).

[g] 50-35mm (12.5) = 50-mL tube containing 12.5 mL of bottom-layer EquiPure™ (n=12).

[h] 50-41mm (16.5mL) = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ (n=12).

† Control Samples.

‡ Each tube had 1 mL extended semen layered on the EquiPure™ containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

A significant stallion-by-treatment interaction ($P<0.05$) was detected for eleven of 27 variables (MOT, PMOT, STR, LIN, large heads, proximal droplets, small proximal droplets, large proximal droplets, large distal droplets, bent midpieces, and bent tails) and are presented in Tables 13 through 20. No differences were detected for mean MOT and PMOT among EquiPure™ treatment groups for each of the four stallions ($P>0.05$). Mean MOT was higher in all EquiPure™ treatments than control groups for two of four stallions ($P<0.05$). The EquiPure™ treatment groups for one stallion yielded similar values to Group UC ($P>0.05$) and higher values than Group CC ($P<0.05$) for MOT. Mean MOT was higher in Groups 15-28mm (2mL) and 15-41mm (4mL) than Group UC in one stallion ($P<0.05$) Mean PMOT was higher in all EquiPure™ treatment groups than both controls for three of four stallions ($P<0.05$). EquiPure™ treatment groups for one stallion had higher values than Group UC for mean PMOT ($P<0.05$). Within EquiPure™ treatment groups, no difference was detected for mean STR ($P>0.05$) for two of four stallions examined and values were higher in EquiPure™ treatment groups than control groups for two of four stallions ($P<0.05$) Mean LIN was similar among EquiPure™ treatment groups for three of four stallions.

The mean percentages of large heads, proximal droplets, and small proximal droplets were similar across treatment groups for three of four stallions ($P>0.05$). . Mean percentage of large proximal droplets was higher in Group UC than in EquiPure treatments for three of four stallions. The mean percentage of large distal droplets was similar across EquiPure™ treatment groups for each of the four stallions, and was higher in EquiPure™ treatment groups than Group UC for three of four stallions ($P>0.05$). Mean bent midpieces was higher in Group CC than all EquiPure™ treatment groups for two of four stallions ($P<0.05$), but no difference was detected among EquiPure™ treatment groups for any of the stallions examined.

A stallion by treatment interaction was detected for sperm recovery rate. Recovery rate (%) was similar ($P>0.05$) across treatment groups for two stallions, whereas recovery rate was higher in 15-mL tubes than 50-mL tubes for one stallion. For the remaining stallion, sperm centrifuged in 15-mL tubes yielded a higher recovery rate than Group 50-41mm (16.5mL) ($P<0.05$; Table 21).

Table 13: Effect of treatment on sperm motility and chromatin quality (COMPαt) variables for Stallion I (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment					
	Group UC[a†]	Group CC[b†]	15-28mm (2mL)[c‡]	15-35mm (3mL)[d‡]	15-41mm (4mL)[e‡]	50-28mm (9mL)[f‡]	50-35mm (12.5mL)[g‡]	50-41mm (16.5mL)[h‡]
MOT	87b (0.9)	89b (2.0)	96a (1.2)	97a (1.0)	97a (0.6)	97a (0)	96a (1.2)	95a (2.5)
PMOT	63b (2.0)	65b (2.7)	79a (2.3)	77a (2.9)	81a (1.9)	81a (1.5)	79a (0.6)	78a (5.5)
VCL	179a (8.7)	176a (15.0)	214a (7.3)	217a (6.4)	219a (10.6)	202a (6.2)	203a (6.4)	194a (8.9)
VAP	110ab (9.0)	96b (10.7)	122a (1.9)	122a (2.3)	125a (1.5)	119ab (1.5)	119ab (1.5)	115ab (3.7)
VSL	86a (7.8)	70b (7.8)	90a (0.9)	89a (1.2)	93a (1.5)	91a (0.3)	91a (2.3)	89a (2.6)
STR	75a (0.7)	72a (1.5)	73a (1.2)	72a (1.2)	74a (2.0)	75a (1.0)	75a (1.5)	76a (1.2)
LIN	49a (2.2)	41a (1.8)	44a (1.8)	43a (1.2)	45a (2.6)	47a (1.7)	47a (2.2)	48a (2.0)
COMPαt	8a (1.4)	7a (7.1)	2b (0.2)	4b (1.5)	4b (1.0)	3b (0.5)	2b (0.4)	4b (0.4)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter means with different letters (a and b) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-28mm (2mL) = 15-mL tube containing 2 mL of bottom-layer EquiPure™ (n=12).

[d] 15-35mm (3mL) = 15-mL tube containing 3 mL of bottom-layer EquiPure™ (n=12).

[e] 15-41mm (4mL) = 15-mL tube containing 4 mL of bottom-layer EquiPure™ (n=12).

[f] 50-28mm (9mL) = 50-mL tube containing 9 mL of bottom-layer EquiPure™ (n=12).

[g] 50-35mm (12.5mL) = 50-mL tube containing 12.5 mL of bottom-layer EquiPure™ (n=12).

[h] 50-41mm (16.5mL) = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ (n=12).

† Control Samples.

‡ Each tube had 1 mL extended semen layered on the EquiPure™ containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

Table 14: Effect of treatment on sperm morphology variables for Stallion I (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment					
	Group UC[a†]	Group CC[b†]	15-28mm (2mL)[c‡]	15-35mm (3mL)[d‡]	15-41mm (4mL)[e‡]	50-28mm (9mL)[f‡]	50-35mm (12.5mL)[g‡]	50-41mm (16.5mL)[h‡]
Morphologically Normal	61b (3.5)	60b (0.3)	80a (0.9)	78a (2.3)	79a (2.9)	78a (0.3)	81a (1.5)	80a (3.2)
Abnormal Heads	5a (1.5)	4a (0.9)	2a (0.7)	2a (1.2)	3a (1.3)	3a (1.2)	2a (1.3)	2a (1.2)
Craters	2a (0.3)	0a (0.3)	1a (0.7)	1a (0.6)	1a (0.7)	0a (0.3)	2a (1.2)	1a (0.7)
Small Heads	2a (0.3)	3a (0.9)	1a (0)	1a (0.7)	2a (0)	2a (0.9)	1a (0.3)	1a (0.6)
Large Heads	1a (1.0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)
Misshaped Heads	1a (0.7)	1a (0.3)	0a (0)	0a (0)	1a (0.7)	1a (0.7)	0a (0)	0a (0)
Abnormal Acrosomes	1a (0.7)	0b (0.3)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)
Detached Heads	5a (0.3)	4a (0.6)	3a (0.3)	4a (1.9)	2a (1.5)	4a (0)	3a (1.7)	3a (1.2)
Proximal Droplets	13a (1.2)	15a (1.2)	8b (0.7)	8b (1.2)	7b (1.5)	7b (1.2)	6b (0.6)	7b (1.5)
Small Proximal Droplets	9a (0.6)	11a (1.5)	8a (0.6)	7a (1.3)	7a (1.5)	7a (1.2)	6a (0.9)	7a (1.5)
Large Proximal Droplets	4a (0.6)	4a (1.0)	0b (0.3)	0b (0.3)	0b (0)	0b (0)	0b (0.3)	0b (0)
Distal Droplets	2a (0.9)	2a (0.6)	1a (0.7)	2a (0.3)	1a (0.9)	1a (0.3)	0a (0.3)	2a (0.6)
Small Distal Droplets	1a (0.6)	1a (0.3)	1a (0.7)	2a (0.3)	1a (0.9)	1a (0.3)	0a (0.3)	2a (0.6)
Large Distal Droplets	1a (0.3)	1a (0.3)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)
Abnormal Midpieces	5a (1.9)	8a (0.9)	4a (0.6)	5a (0.7)	6a (1.5)	5a (1.2)	6a (0.7)	6a (0.3)
Bent Midpieces	3ab (0.3)	3a (1.3)	1b (0.6)	0b (0.3)	0b (0.3)	1b (0.7)	1b (0.3)	1b (0.3)
Bent Tails	0a (0.3)	0a (0.3)	0a (0.3)	0a (0)	0a (0)	0a (0.3)	0a (0)	0a (0)
Coiled Tails	3ab (1.5)	4a (0.7)	0b (0.3)	0b (0)	1b (0.3)	0b (0.3)	0b (0.3)	0b (0.3)
Premature Germ Cells	0a (0.3)	0a (0.3)	0a (0)	0a (0)	0a (0)	0a (0)	1a (0.7)	0a (0)

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter means with different letters (a and b) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-28mm (2mL) = 15-mL tube containing 2 mL of bottom-layer EquiPure™ (n=3).

[d] 15-35mm (3mL) = 15-mL tube containing 3 mL of bottom-layer EquiPure™ (n=3).

[e] 15-41mm (4mL) = 15-mL tube containing 4 mL of bottom-layer EquiPure™ (n=3).

[f] 50-28mm (9mL) = 50-mL tube containing 9 mL of bottom-layer EquiPure™ (n=3).

[g] 50-35mm (12,5mL) = 50-mL tube containing 12.5 mL of bottom-layer EquiPure™ (n=3).

[h] 50-41mm (16.5mL) = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ (n=3).

† Control Samples.

‡ Each tube had 1 mL extended semen layered on the EquiPure™ containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

Table 15: Effect of treatment on sperm motility and chromatin quality (COMPαt) variables for Stallion II (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment					
	Group UC[a†]	Group CC[b†]	15-28mm (2mL)[c‡]	15-35mm (3mL)[d‡]	15-41mm (4mL)[e‡]	50-28mm (9mL)[f‡]	50-35mm (12.5mL)[g‡]	50-41mm (16.5mL)[h‡]
MOT	67ab (6.0)	63b (3.2)	85a (4.9)	87a (4.3)	86a (5.2)	87a (3.5)	87a (3.2)	87a (3.6)
PMOT	36b (6.7)	39b (4.9)	67a (4.6)	71a (5.9)	70a (6.4)	73a (4.8)	72a (4.1)	71a (4.0)
VCL	213a (5.3)	160b (25.4)	220a (7.0)	218a (6.9)	211a (4.5)	215a (7.5)	205a (6.4)	208a (6.6)
VAP	109a (3.2)	73b (13.0)	111a (4.6)	111a (5.5)	107a (4.4)	110a (5.2)	104a (4.9)	105a (4.3)
VSL	84a (3.5)	55b (9.9)	86a (4.1)	87a (4.9)	84a (4.3)	88a (4.9)	84a (5.2)	85a (3.8)
STR	74c (1.5)	74bc (0.9)	77abc (0.9)	78abc (0.9)	78ab (1.2)	80a (0.9)	79ab (0.3)	80a (1.2)
LIN	39a (0.9)	34b (1.2)	40a (1.2)	41a (1.5)	41a (1.5)	42a (0.9)	41a (1.2)	42a (1.0)
COMPαt	16a (2.7)	18a (2.2)	8a (2.5)	9a (3.4)	9a (4.3)	6a (1.9)	7a (2.5)	7a (2.7)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter means with different letters (a,b and c) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-28mm (2mL) = 15-mL tube containing 2 mL of bottom-layer EquiPure™ (n=12).

[d] 15-35mm (3mL) = 15-mL tube containing 3 mL of bottom-layer EquiPure™ (n=12).

[e] 15-41mm (4mL) = 15-mL tube containing 4 mL of bottom-layer EquiPure™ (n=12).

[f] 50-28mm (9mL) = 50-mL tube containing 9 mL of bottom-layer EquiPure™ (n=12).

[g] 50-35mm (12.5mL) = 50-mL tube containing 12.5 mL of bottom-layer EquiPure™ (n=12).

[h] 50-41mm (16.5mL) = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ (n=12).

† Control Samples.

‡ Each tube had 1 mL extended semen layered on the EquiPure™ containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

Table 16: Effect of treatment on sperm morphology variables for Stallion II (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment					
	Group UC[a†]	Group CC[b†]	15-28mm (2mL)[c‡]	15-35mm (3mL)[d‡]	15-41mm (4mL)[e‡]	50-28mm (9mL)[f‡]	50-35mm (12.5mL)[g‡]	50-41mm (16.5mL)[h‡]
Morphologically Normal	42b (2.2)	39b (1.2)	67a (2.3)	62a (1.5)	63a (1.7)	62a (1.7)	61a (2.4)	57a (3.3)
Abnormal Heads	6a (1.5)	6a (0.9)	3a (1.9)	5a (1.7)	4a (1.0)	5a (1.7)	4a (0.9)	6a (2.0)
Craters	1a (0.6)	2a (1.0)	1a (0.6)	1a (0)	0a (0.3)	2a (0.9)	2a (1.2)	2a (0.3)
Small Heads	1a (0.7)	2a (0.9)	1a (1.3)	1a (0.9)	2a (0.6)	2a (0.7)	1a (0.6)	2a (1.0)
Large Heads	1a (0.7)	0a (0)	0a (0.3)	0a (0)	0a (0.3)	0a (0)	0a (0)	0a (0)
Misshaped Heads	3a (1.2)	2a (1.0)	1a (0.7)	3a (2.2)	1a (0.3)	1a (0.7)	0a (0.3)	2a (1.2)
Abnormal Acrosomes	2a (0.7)	0a (0.3)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)
Detached Heads	15a (5.0)	12a (1.7)	13a (5.5)	10a (5.2)	9a (2.9)	10a (3.9)	6a (1.9)	7a (1.2)
Proximal Droplets	13a (2.3)	14a (1.9)	8a (1.2)	9a (2.0)	10a (2.5)	11a (1.2)	12a (1.7)	12a (1.5)
Small Proximal Droplets	10a (3.5)	7a (3.5)	8a (1.2)	9a (2.2)	10a (2.7)	11a (1.2)	11a (1.9)	11a (0.9)
Large Proximal Droplets	3b (1.2)	8a (2.2)	0b (0)	0b (0.3)	0b (0.3)	0b (0)	1b (0.7)	1b (0.6)
Distal Droplets	6a (0.6)	8a (4.0)	3a (1.7)	4a (1.2)	3a (0.6)	4a (1.2)	3a (0.7)	6a (1.2)
Small Distal Droplets	3a (0.3)	7a (3.3)	3a (1.7)	4a (1.2)	3a (0.3)	4a (1.2)	3a (0.7)	5a (1.0)
Large Distal Droplets	3a (0.3)	1b (0.7)	0b (0)	0b (0)	0b (0.3)	0b (0.3)	0b (0)	1b (0.3)
Abnormal Midpieces	5a (1.7)	9a (1.8)	4a (1.3)	8a (0.9)	8a (0.6)	6a (1.2)	10a (1.5)	9a (1.7)
Bent Midpieces	4ab (1.7)	7a (2.0)	1b (0)	1b (0.3)	2b (0.3)	2b (0.6)	2b (1.2)	0b (0)
Bent Tails	2a (0.3)	1a (0.3)	1a (0.7)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)
Coiled Tails	4a (0.6)	2b (0.3)	1b (0.3)	1b (0.7)	1b (0.6)	0b (0)	2b (0.6)	1b (0.3)
Premature Germ Cells	1a (0.9)	0a (0.3)	0a (0)	0a (0.3)	0a (0)	0a (0)	0a (0)	0a (0)

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter means with different letters (a and b) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-28mm (2mL) = 15-mL tube containing 2 mL of bottom-layer EquiPure™ (n=12).

[d] 15-35mm (3mL) = 15-mL tube containing 3 mL of bottom-layer EquiPure™ (n=12).

[e] 15-41mm (4mL) = 15-mL tube containing 4 mL of bottom-layer EquiPure™ (n=12).

[f] 50-28mm (9mL) = 50-mL tube containing 9 mL of bottom-layer EquiPure™ (n=12).

[g] 50-35mm (12.5mL) = 50-mL tube containing 12.5 mL of bottom-layer EquiPure™ (n=12).

[h] 50-41mm (16.5mL) = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ (n=12).

† Control Samples.

‡ Each tube had 1 mL extended semen layered on the EquiPure™ containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

Table 17: Effect of treatment on sperm motility and chromatin quality (COMPαt) variables for Stallion III(mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment					
	Group UC[a†]	Group CC[b†]	15-28mm (2mL)[c‡]	15-35mm (3mL)[d‡]	15-41mm (4mL)[e‡]	50-28mm (9mL)[f‡]	50-35mm (12.5mL)[g‡]	50-41mm (16.5mL)[h‡]
MOT	70b (2.3)	78ab (1.9)	90a (3.7)	88ab (4.6)	89a (3.5)	88ab (2.9)	87ab (3.8)	87ab (2.4)
PMOT	41b (2.6)	49ab (2.0)	73a (6.7)	71a (7.7)	72a (6.4)	71a (6.7)	70a (6.1)	69a (4.7)
VCL	231a (10.7)	227a (5.6)	255a (13.7)	243a (13.6)	245a (13.1)	229a (11.0)	222a (14.0)	216a (8.5)
VAP	127a (3.1)	118a (2.5)	137a (8.5)	131a (8.0)	132a (7.6)	125a (7.7)	121a (7.8)	118a (5.0)
VSL	99a (1.8)	89a (1.2)	108a (7.5)	105a (6.5)	106a (6.1)	102a (7.0)	100a (6.2)	98a (4.4)
STR	74d (0.9)	71e (0.7)	77c (1.0)	79bc (0.9)	79bc (0.3)	80abc (1.0)	80ab (0.3)	82a (0.6)
LIN	43a (1.2)	38b (0.3)	43a (1.0)	44a (0.9)	44a (0.9)	45a (1.3)	46a (0.3)	47a (1.0)
COMPαt	35a (3.6)	33a (5.3)	14b (4.3)	14b (4.5)	14b (4.7)	13b (2.2)	15b (2.9)	13b (1.4)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter means with different letters (a,b,c,d and e) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-28mm (2mL) = 15-mL tube containing 2 mL of bottom-layer EquiPure™ (n=12).

[d] 15-35mm (3mL) = 15-mL tube containing 3 mL of bottom-layer EquiPure™ (n=12).

[e] 15-41mm (4mL) = 15-mL tube containing 4 mL of bottom-layer EquiPure™ (n=12).

[f] 50-28mm (9mL) = 50-mL tube containing 9 mL of bottom-layer EquiPure™ (n=12).

[g] 50-35mm (12.5mL) = 50-mL tube containing 12.5 mL of bottom-layer EquiPure™ (n=12).

[h] 50-41mm (16.5mL) = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ (n=12).

† Control Samples.

‡ Each tube had 1 mL extended semen layered on the EquiPure™ containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

Table 18: Effect of treatment on sperm morphology variables for Stallion III (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment					
	Group UC[a†]	Group CC[b†]	15-28mm (2mL)[c‡]	15-35mm (3mL)[d‡]	15-41mm (4mL)[e‡]	50-28mm (9mL)[f‡]	50-35mm (12.5mL)[g‡]	50-41mm (16.5mL)[h‡]
Morphologically Normal	26b (1.2)	30b (3.2)	59a (3.7)	56a (1.9)	56a (3.1)	57a (6.7)	56a (3.9)	55a (4.0)
Abnormal Heads	8a (0.9)	7a (1.2)	4a (1.0)	3a (0.9)	5a (0)	4a (1.8)	5a (0.9)	4a (2.0)
Craters	3a (0.3)	3a (0.9)	1a (0.3)	1a (0.6)	1a (0.6)	1a (1.3)	2a (0.7)	3a (0.9)
Small Heads	1a (1.3)	1a (0.9)	2a (1.0)	1a (0.7)	1a (0.6)	2a (0.7)	2a (1.2)	1a (0.7)
Large Heads	1b (0.3)	0b (0.3)	0b (0)	1a (0.7)	0b (0)	0b (0)	0b (0)	0b (0)
Misshaped Heads	2ab (0.3)	2ab (0.9)	1b (0.3)	0b (0.3)	3a (0.6)	1b (0.7)	0b (0)	1b (0.6)
Abnormal Acrosomes	1a (0.6)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)
Detached Heads	24a (6.7)	9ab (1.3)	8ab (5.2)	9ab (4.7)	7ab (1.5)	6ab (2.6)	7ab (0.3)	5b (0.3)
Proximal Droplets	20a (2.7)	27a (1.2)	18a (2.3)	18a (2.4)	23a (2.6)	22a (2.3)	21a (0.3)	21a (1.0)
Small Proximal Droplets	13a (3.2)	20a (3.9)	16a (1.5)	18a (2.1)	21a (2.6)	21a (2.8)	20a (0.6)	21a (1.0)
Large Proximal Droplets	8a (0.9)	7ab (3.2)	2b (1.3)	0b (0.3)	2b (0.7)	1b (0.6)	1b (0.7)	0b (0)
Distal Droplets	3a (0.6)	3a (0.7)	2a (0.6)	1a (1.3)	2a (1.2)	2a (1.2)	2a (0.7)	2a (0)
Small Distal Droplets	0a (0.3)	3a (0.7)	2a (0.9)	1a (1.3)	1a (1.3)	2a (1.2)	1a (0.3)	2a (0)
Large Distal Droplets	3a (0.3)	1b (0.7)	0b (0.3)	0b (0)	0b (0.3)	0b (0)	0b (0.3)	0b (0)
Abnormal Midpieces	9a (2.7)	15a (3.0)	8a (3.8)	10a (0.3)	6a (2.0)	8a (2.4)	10a (2.0)	11a (2.6)
Bent Midpieces	4a (0.3)	3a (0.6)	0a (0.3)	2a (1.0)	1a (0.7)	3a (1.7)	1a (0.3)	2a (0.3)
Bent Tails	1a (0.6)	1a (0.3)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)
Coiled Tails	2a (0.6)	3a (2.1)	1a (0.7)	1a (0.7)	1a (0.7)	0a (0.3)	0a (0.3)	1a (0.7)
Premature Germ Cells	3a (1.8)	3a (1.3)	0a (0)	0a (0.3)	0a (0)	0a (0)	0a (0)	0a (0)

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter means with different letters (a and b) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-28mm (2mL) = 15-mL tube containing 2 mL of bottom-layer EquiPure™ (n=12).

[d] 15-35mm (3mL) = 15-mL tube containing 3 mL of bottom-layer EquiPure™ (n=12).

[e] 15-41mm (4mL) = 15-mL tube containing 4 mL of bottom-layer EquiPure™ (n=12).

[f] 50-28mm (9mL) = 50-mL tube containing 9 mL of bottom-layer EquiPure™ (n=12).

[g] 50-35mm (12.5mL) = 50-mL tube containing 12.5 mL of bottom-layer EquiPure™ (n=12).

[h] 50-41mm (16.5mL) = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ (n=12).

† Control Samples.

‡ Each tube had 1 mL extended semen layered on the EquiPure™ containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

Table 19: Effect of treatment on sperm motility and chromatin quality (COMPαt) variables for Stallion IV (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment					
	Group UC[a†]	Group CC[b†]	15-28mm (2mL)[c‡]	15-35mm (3mL)[d‡]	15-41mm (4mL)[e‡]	50-28mm (9mL)[f‡]	50-35mm (12.5mL)[g‡]	50-41mm (16.5mL)[h‡]
MOT	59b (3.5)	62b (2.3)	90a (1.2)	92a (1.8)	90a (1.2)	84a (4.0)	85a (2.5)	84a (3.5)
PMOT	29b (1.2)	30b (2.7)	73a (1.7)	74a (4.1)	73a (2.0)	63a (6.7)	65a (4.7)	63a (6.7)
VCL	185b (1.2)	188ab (11.8)	221a (3.5)	218ab (4.2)	218ab (4.2)	197ab (7.0)	196ab (8.9)	191ab (10.7)
VAP	103ab (1.2)	98b (6.3)	120a (1.9)	119a (3.5)	120a (3.0)	109ab (3.2)	109ab (5.3)	107ab (6.6)
VSL	81bc (1.2)	74c (4.0)	98a (1.2)	97a (3.0)	99a (2.5)	91ab (2.2)	92ab (3.7)	91ab (5.6)
STR	72c (0.3)	71c (1.3)	79b (0.9)	80ab (0.6)	81ab (0.3)	82ab (1.0)	82ab (0.3)	83a (0.6)
LIN	42c (0.3)	39d (0.7)	45b (0.9)	46b (0.9)	46ab (0.7)	47ab (0.9)	47ab (0.3)	49a (0.3)
COMPαt	18a (1.4)	21a (3.4)	4b (0.6)	5b (0.6)	3b (0.4)	4b (1.0)	4b (0.4)	4b (0.7)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter means with different letters (a,b,c and d) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-28mm (2mL) = 15-mL tube containing 2 mL of bottom-layer EquiPure™ (n=12).

[d] 15-35mm (3mL) = 15-mL tube containing 3 mL of bottom-layer EquiPure™ (n=12).

[e] 15-41mm (4mL) = 15-mL tube containing 4 mL of bottom-layer EquiPure™ (n=12).

[f] 50-28mm (9mL) = 50-mL tube containing 9 mL of bottom-layer EquiPure™ (n=12).

[g] 50-35mm (12.5mL) = 50-mL tube containing 12.5 mL of bottom-layer EquiPure™ (n=12).

[h] 50-41mm (16.5mL) = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ (n=12).

† Control Samples.

‡ Each tube had 1 mL extended semen layered on the EquiPure™ containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

Table 20: Effect of treatment on sperm morphology variables for Stallion IV (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment					
	Group UC[a†]	Group CC[b†]	15-28mm (2mL)[c‡]	15-35mm (3mL)[d‡]	15-41mm (4mL)[e‡]	50-28mm (9mL)[f‡]	50-35mm (12.5mL)[g‡]	50-41mm (16.5mL)[h‡]
Morphologically Normal	21b (1.8)	24b (4.1)	47a (2.8)	49a (1.5)	45a (2.3)	49a (3.2)	46a (2.6)	45a (2.8)
Abnormal Heads	7a (1.5)	3a (1.5)	3a (1.2)	2a (0.6)	4a (1.2)	4a (0.7)	4a (0.9)	4a (1.2)
Craters	2a (0.9)	1a (0.6)	1a (0.7)	0a (0.3)	1a (0.6)	0a (0.3)	1a (0.3)	1a (0.6)
Small Heads	3a (0.3)	1a (0.3)	1a (0.7)	0a (0.3)	1a (0.6)	2a (0.6)	2a (0.7)	1a (0.6)
Large Heads	1a (0.3)	0a (0)	0a (0.3)	1a (0.3)	1a (0.7)	0a (0.3)	0a (0.3)	0a (0)
Misshaped Heads	2a (0.6)	1a (0.6)	1a (0.6)	1a (0.6)	2a (0.3)	1a (1.0)	1a (0.6)	2a (0.6)
Abnormal Acrosomes	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	1a (0.7)	0a (0)
Detached Heads	4a (2.1)	6a (2.3)	4a (0.9)	4a (0.6)	4a (1.9)	5a (2.2)	4a (0)	5a (0.9)
Proximal Droplets	9a (2.5)	10a (1.7)	21a (6.0)	21a (0.6)	18a (3.3)	14a (1.5)	15a (2.3)	18a (3.5)
Small Proximal Droplets	3b (1.2)	7ab (0.7)	16a (5.8)	15a (1.5)	12ab (3.0)	10ab (2.6)	12ab (1.8)	11ab (2.5)
Large Proximal Droplets	6a (1.5)	3a (1.0)	5a (1.8)	6a (1.2)	6a (0.3)	4a (1.3)	4a (2.0)	7a (1.0)
Distal Droplets	1a (0.6)	1a (0.6)	4a (2.5)	4a (2.0)	6a (2.7)	4a (1.2)	5a (2.6)	3a (1.9)
Small Distal Droplets	0a (0.3)	0a (0.3)	4a (2.5)	3a (2.3)	5a (2.8)	3a (1.5)	4a (2.6)	2a (0.9)
Large Distal Droplets	1a (0.3)	1a (0.7)	0a (0)	1a (0.3)	0a (0.3)	1a (0.7)	1a (0)	1a (1.0)
Abnormal Midpieces	12a (0.7)	11a (1.8)	6a (2.1)	9a (2.3)	10a (3.2)	8a (1.5)	11a (3.0)	8a (1.8)
Bent Midpieces	34a (3.4)	30a (4.4)	9b (1.5)	9b (1.5)	8b (1.7)	13b (3.1)	11b (3.4)	11b (4.1)
Bent Tails	1a (0.3)	2a (1.2)	2a (0.9)	0a (0)	1a (1.3)	1a (1.0)	1a (1.0)	2a (1.5)
Coiled Tails	6ab (3.5)	12a (5.4)	5ab (1.2)	2ab (0.3)	4ab (0.9)	3ab (0.3)	2b (1.2)	4ab (0.6)
Premature Germ Cells	3a (1.7)	2a (0)	0a (0.3)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter means with different letters (a and b) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-28mm (2mL) = 15-mL tube containing 2 mL of bottom-layer EquiPure™ (n=12).

[d] 15-35mm (3mL) = 15-mL tube containing 3 mL of bottom-layer EquiPure™ (n=12).

[e] 15-41mm (4mL) = 15-mL tube containing 4 mL of bottom-layer EquiPure™ (n=12).

[f] 50-28mm (9mL) = 50-mL tube containing 9 mL of bottom-layer EquiPure™ (n=12).

[g] 50-35mm (12.5mL) = 50-mL tube containing 12.5 mL of bottom-layer EquiPure™ (n=12).

[h] 50-41mm (16.5mL) = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ (n=12).

† Control Samples.

‡ Each tube had 1 mL extended semen layered on the EquiPure™ containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

Table 21: Recovery rates for Experiment 2 (mean ± SEM).

Laboratory Parameters*	EquiPure™ Treatment					
	15-28mm (2mL)[c‡]	15-35mm (3mL)[d‡]	15-41mm (4mL)[e‡]	50-28mm (9mL)[f‡]	50-35mm (12.5mL)[g‡]	50-41mm (16.5mL)[h‡]
Total RR	43.8a (7.3)	41.2a (6.7)	41.8a (6.0)	35.1b (3.9)	32.8b (3.9)	31.3b (2.7)
RR-St I	76.3a (2.8)	72.0a (3.9)	70.0a (2.0)	45.0b (6.7)	49.3b (3.3)	39.3b (5.7)
RR-St II	21.0a (3.0)	21.0a (2.6)	24.3a (2.7)	21.0a (1.0)	19.3a (1.2)	23.3a (1.2)
RR-St III	21.0a (3.6)	20.3a (3.7)	23.0a (3.8)	26.7a (2.3)	21.7a (0.3)	24.7a (4.1)
RR-St IV	56.7a (1.2)	51.3ab (2.0)	49.7ab (3.2)	47.7abc (1.0)	40.7bc (1.2)	37.7c (1.7)

* Total RR = total sperm recovery rate for all four stallions (%); RR-St I = sperm recovery rate for Stallion I (%); RR-St II = sperm recovery rate for Stallion II (%); RR-St III = sperm recovery rate for Stallion III (%); RR-St IV = sperm recovery rate for Stallion IV (%).

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter means with different letters (a,b and c) differ ($P< 0.05$).

[a] 15-28mm (2mL) = 15-mL tube containing 2 mL of bottom-layer EquiPure™.

[b] 15-35mm (3mL) = 15-mL tube containing 3 mL of bottom-layer EquiPure™.

[c] 15-41mm (4mL) = 15-mL tube containing 4 mL of bottom-layer EquiPure™.

[d] 50-28mm (9mL) = 50-mL tube containing 9 mL of bottom-layer EquiPure™.

[e] 50-35mm (12.5mL) = 50-mL tube containing 12.5 mL of bottom-layer EquiPure™.

[f] 50-41mm (16.5mL) = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™.

† Each tube had 1 mL extended semen layered on the EquiPure™ containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

Experiment 3

The main effects of sperm number (1x, 2x, 3x, or 4x) exposed to density gradient centrifugation and centrifugation tube type (15-mL, 50-mL) on sperm quality are presented in Tables 22 and 23. Overall, twenty two of the twenty seven experimental endpoints (VCL, VAP, STR, LIN, COMPαt, abnormal heads, craters, small heads, large heads, misshaped heads, abnormal acrosomes, detached heads, proximal droplets, small proximal droplets, large proximal droplets, distal droplets, small distal droplets, large distal droplets, bent midpieces, bent tails, coiled tails, and premature germ cells) were not affected by EquiPure™ treatment group ($P>0.05$). Treatment 50-1x was yielded lower values for MOT than all other EquiPure™ treatment groups ($P<0.05$). Mean

PMOT, was slightly higher in Group 15-4x than Group 50-1x ($P<0.05$). Mean VSL was lower for Groups 15-3x and 15-4x than for Groups 15-1x, 50-1x, and 50-2x ($P<0.05$). Group 50-4x was lower than Groups 15-2x, 15-3x, and 50-2x for percentage of morphologically normal sperm ($P<0.05$). The percentage of abnormal midpieces was higher in Group 50-4x than in Groups 15-2x, 15-3x, 50-1x, and 50-2x ($P<0.05$).

All EquiPure™ treatment groups were superior to both controls for mean values for MOT, PMOT, COMPαt, morphologically normal sperm, bent midpieces and coiled tails ($P<0.05$). Mean VCL, VAP, and VSL were slightly higher in all EquiPure™ treated groups than Group CC ($P<0.05$). Mean values for STR, large proximal droplets, and premature germ cells were lower in all EquiPure™ treated groups than Group UC ($P<0.05$). The percentage of abnormal heads was significantly higher in the Group UC than in Groups 15-1x, 15-2x, 15-3x, 50-1x and 50-2x ($P<0.05$). The mean percentage of large heads was slightly higher in Group UC than in Groups 15-1x, 15-3x, 50-1x, and 50-2x ($P<0.05$). The mean percentage of detached heads was lower in Groups 15-1x and 15-3x than Group UC ($P<0.05$). The mean percentage of large distal droplets was lower in Groups 50-1x and 50-4x than in Group UC ($P<0.05$). The mean percentage of abnormal midpieces were higher in Group CC than Groups 15-2x, 15-3x, and 50-2x ($P<0.05$). Mean values for LIN, small heads, misshaped heads, small proximal droplets, distal droplets, small distal droplets, and bent tails were similar among the EquiPure™ treatment groups and the control groups ($P>0.05$).

Table 22: Main effects of treatment on sperm motility and chromatin quality (COMPαt) variables for four stallions (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment							
	Group UC[a†]	Group CC[b†]	15-1x[c]	15-2x[d]	15-3x[e]	15-4x[f]	50-1x[g]	50-2x[h]	50-3x[i]	50-4x[j]
MOT	84c (2.4)	86c (2.0)	96a (0.5)	97a (0.4)	97a (0.4)	97a (0.4)	94b (1.0)	95a (0.6)	96a (0.5)	96a (0.4)
PMOT	57d (4.0)	61c (3.5)	79ab (1.4)	81ab (1.1)	81ab (0.9)	82a (1.0)	78b (2.0)	79ab (1.4)	81ab (1.1)	81ab (1.2)
VCL	207a (11.1)	182b (6.2)	205a (7.2)	199a (7.5)	195a (7.1)	195a (7.2)	201a (6.9)	202a (6.6)	199a (6.6)	198a (6.8)
VAP	102a (5.6)	88b (2.9)	102a (3.5)	98a (3.6)	95a (3.2)	95a (3.5)	101a (3.5)	100a (3.5)	98a (3.4)	97a (3.4)
VSL	71bc (3.1)	61d (1.3)	73ab (2.4)	70bc (2.4)	68c (2.0)	69c (2.2)	76a (2.7)	74ab (2.6)	72bc (2.6)	71bc (2.6)
STR	69b (1.7)	70a (1.3)	72a (0.9)	73a (0.9)	73a (0.8)	73a (0.8)	75a (1.2)	74a (1.2)	74a (1.1)	74a (1.1)
LIN	38a (3.1)	35a (0.6)	37a (0.4)	37a (0.4)	36a (0.5)	36a (0.5)	39a (0.7)	38a (0.6)	37a (0.6)	37a (0.6)
COMPαt	5a (0.6)	5a (0.5)	2b (0.3)	2b (0.4)	2b (0.3)	2b (0.3)	2b (0.4)	2b (0.3)	2b (0.3)	2b (0.4)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b,c and d) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=12).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=12).

[c] 15-1x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[d] 15-2x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[e] 15-3x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[f] 15-4x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[g] 50-1x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[h] 50-2x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[i] 50-3x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[j] 50-4x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

† Control Samples.

Table 23: Main effects of treatment on sperm morphology variables for four stallions (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment							
	Group UC[a†]	Group CC[b†]	15-1x[c]	15-2x[d]	15-3x[e]	15-4x[f]	50-1x[g]	50-2x[h]	50-3x[i]	50-4x[j]
Morphologically Normal	46d (3.5)	49d (3.2)	69abc (2.6)	72a (2.8)	71ab (3.0)	69abc (2.4)	68abc (2.3)	71ab (2.9)	66bc (2.9)	64c (2.8)
Abnormal Heads	8a (1.0)	7ab (0.8)	4c (0.8)	5c (0.9)	5bc (0.9)	7abc (0.9)	5bc (0.7)	5bc (0.8)	7abc (0.8)	6abc (1.0)
Craters	2a (0.4)	1ab (0.1)	1ab (0.3)	1ab (0.2)	1ab (0.2)	2ab (0.3)	1ab (0.3)	1b (0.1)	2ab (0.4)	1ab (0.3)
Small Heads	1a (0.3)	2a (0.2)	1a (0.2)	1a (0.3)	1a (0.3)	1a (0.4)	1a (0.4)	1a (0.3)	2a (0.3)	1a (0.2)
Large Heads	2a (0.6)	1ab (0.5)	1b (0.2)	1ab (0.3)	1b (0.2)	1ab (0.4)	0b (0.2)	0b (0.2)	1ab (0.3)	1ab (0.4)
Misshaped Heads	4a (0.7)	3a (0.5)	2a (0.4)	2a (0.4)	3a (0.4)	3a (0.6)	3a (0.4)	3a (0.6)	3a (0.5)	3a (0.6)
Abnormal Acrosomes	2a (0.4)	2ab (0.5)	1b (0.2)	1b (0.3)	1b (0.2)	0b (0.2)	0b (0.2)	1b (0.2)	1b (0.2)	1ab (0.3)
Detached Heads	4a (0.8)	3ab (0.6)	1b (0.4)	2ab (0.5)	1b (0.5)	2ab (0.5)	2ab (0.7)	2ab (0.6)	2ab (0.7)	3ab (0.7)
Proximal Droplets	14ab (2.4)	16a (3.5)	12ab (1.9)	10b (1.7)	10b (1.3)	10b (1.3)	11b (1.6)	10b (2.0)	11b (1.6)	11b (1.6)
Small Proximal Droplets	7a (1.4)	10a (2.1)	8a (1.5)	7a (1.4)	7a (0.9)	7a (1.0)	9a (1.6)	8a (1.8)	8a (1.3)	7a (1.3)
Large Proximal Droplets	7a (1.4)	6ab (1.5)	4bc (0.9)	3c (0.7)	3c (0.7)	3c (0.6)	2c (0.4)	2c (0.5)	3c (0.6)	3c (0.5)
Distal Droplets	3a (0.8)	4a (1.2)	4a (1.5)	3a (1.2)	3a (1.2)	3a (0.8)	3a (1.0)	3a (0.8)	3a (0.7)	2a (0.6)
Small Distal Droplets	1a (0.4)	3a (1.0)	2a (0.5)	2a (0.7)	2a (0.5)	2a (0.5)	3a (0.8)	2a (0.6)	2a (0.4)	1a (0.4)
Large Distal Droplets	3a (0.5)	1ab (0.3)	2ab (1.2)	1ab (0.4)	2ab (0.8)	1ab (0.4)	1b (0.2)	1ab (0.4)	1ab (0.3)	1b (0.3)
Abnormal Midpieces	8abc (1.4)	9ab (1.4)	7abc (1.1)	6c (0.7)	6c (1.2)	8abc (1.1)	7bc (1.2)	6c (1.0)	8abc (1.2)	10a (1.2)
Bent Midpieces	5a (1.2)	4a (1.0)	1b (0.4)	2b (0.5)	1b (0.5)	1b (0.5)	2b (0.8)	1b (0.2)	2b (0.7)	2b (0.6)
Bent Tails	1a (0.4)	1a (0.3)	0a (0.1)	0a (0.1)	0a (0.2)	0a (0.1)	0a (0.2)	0a (0.1)	0a (0.1)	0a (0.2)
Coiled Tails	8a (1.3)	5b (1.1)	1c (0.4)	1c (0.4)	1c (0.4)	1c (0.3)	1c (0.5)	1c (0.4)	1c (0.5)	1c (0.4)
Premature Germ Cells	2a (0.4)	0b (0.2)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b,c and d) differ ($P<0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=12).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=12).

[c] 15-1x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[d] 15-2x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[e] 15-3x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[f] 15-4x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[g] 50-1x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[h] 50-2x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[i] 50-3x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

[j] 50-4x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=12).

† Control Samples.

Significant stallion-by-treatment interactions ($P<0.05$) were detected for 13 of 27 variables (MOT, PMOT, VCL, VAP, VSL, STR, large heads, proximal droplets, small proximal droplets, small distal droplets, large distal droplets, bent midpieces, coiled tails and premature germ cells) and these data are presented in Tables 24 through 31. Mean MOT was similar among EquiPure™ treatment groups for two of four stallions ($P>0.05$). For one stallion, MOT was higher in Groups 15-1x, 15-2x, 15-3x, and 15-4x than in Groups 50-1x and 50-2x ($P<0.05$). Mean MOT was lower in Group 50-1x than the other EquiPure™ treatment groups for the remaining stallion ($P<0.05$). Mean MOT was higher in EquiPure™ treatment groups than control groups for each of the four stallions ($P<0.05$). Mean PMOT was similar among EquiPure™ treatment groups for three of four stallions ($P>0.05$). For one stallion, Groups 15-3x and 15-4x yielded higher values than Group 50-1x for PMOT ($P<0.05$). For each of the stallions, mean PMOT in EquiPure™ treatment groups was higher than that for control groups ($P<0.05$). Values for Mean VCL, VAP, and were similar among EquiPure™ treatment groups for each of the four stallions, and mean VCL was higher in all EquiPure™ treatment groups than Group CC in one of four stallions. Mean VSL was similar among EquiPure™ treatment groups for two of the four stallions, was higher in all EquiPure™ treatment groups than Group CC in two of four stallions.

The percentages of large heads, proximal droplets, small distal droplets were similar across all treatment groups for three of four stallions ($P>0.05$). No difference was detected for percentage large distal droplets across treatment groups for any of the four stallions ($P>0.05$).Mean percentage of bent midpieces was similar among

EquiPure™ treatment groups for each of the four stallions, and was higher in EquiPure™ treatment groups than one of the control groups for two of four stallions. Mean percentage of coiled tails was similar among EquiPure™ treatment groups for each of the four stallions ($P>0.05$), and was higher in EquiPure™ treatment groups than Group UC for three of four stallions ($P<0.05$). Mean percentage of percentage of premature germ cells was similar among EquiPure™ treatment groups for each of the four stallions ($P>0.05$), and was higher in EquiPure™ treatment groups than Group UC for each of the four stallions.

Over all stallions, sperm recovery rate (%) was lower in Group 15-4x than all other treatment groups ($P<0.05$), and was higher in Group 15-1x than Groups 50-3x and 50-4x ($P<0.05$;Table 32). A stallion-by-treatment interaction was detected for recovery rate ($P<0.05$). Mean recovery rate was similar across treatment groups for two of four stallions ($P>0.05$). For one stallion, sperm recovery rate in Group 15-4x was lower than all other treatment groups. In another stallion, Groups 15-3x and 15-4x yielded lower sperm recovery rates than did Groups 15-1x, 15-2x and 50-1x.

Table 24: Effect of treatment on sperm motility and chromatin quality (COMPαt) variables for Stallion 1 (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment							
	Group UC[a†]	Group CC[b†]	15-1x[c]	15-2x[d]	15-3x[e]	15-4x[f]	50-1x[g]	50-2x[h]	50-3x[i]	50-4x[j]
MOT	90c (0.9)	90c (0.3)	96a (0.6)	97a (0)	96a (0)	96a (0.3)	94b (0.3)	94b (0.3)	95ab (0.7)	95ab (0.3)
PMOT	66b (2.0)	67b (3.8)	80a (2.3)	82a (2.7)	81a (1.9)	82a (1.3)	82a (0.7)	79a (1.5)	82a (1.2)	82a (1.7)
VCL	247a (10.2)	188b (10.4)	206b (5.2)	196b (13.9)	192b (12.5)	192b (15.0)	183b (10.3)	186b (11.0)	190b (10.4)	185b (12.0)
VAP	120a (5.8)	90ab (5.9)	99ab (3.5)	93ab (7.5)	90ab (7.2)	91ab (8.7)	88ab (6.1)	88ab (6.9)	90ab (6.7)	87b (6.4)
VSL	81a (2.6)	63a (2.0)	72a (1.7)	68a (3.5)	65a (3.5)	66a (4.9)	69a (4.4)	67a (4.6)	68a (5.0)	65a (4.0)
STR	68b (1.5)	71ab (2.3)	74ab (1.5)	74ab (2.3)	74ab (2.1)	74ab (1.7)	79a (0.9)	77a (0.9)	76a (0.7)	76a (0.7)
LIN	46a (12.5)	35a (0.6)	37a (0.7)	36a (0.6)	35a (0.3)	35a (0.3)	39a (0.6)	37a (0.6)	37a (0.9)	36a (0.3)
COMPαt	3ab (0.2)	4a (0.5)	1b (0.2)	1b (0.3)	1b (0.2)	2b (1.1)	1b (0.2)	1b (0.1)	2b (0.1)	1b (0.1)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b and c) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-1x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[d] 15-2x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[e] 15-3x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[f] 15-4x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[g] 50-1x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[h] 50-2x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[i] 50-3x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[j] 50-4x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Table 25: Effect of treatment on sperm morphology variables for Stallion 1 (mean ± SEM).

Laboratory Parameters*	Group UC[a†]	Group CC[b†]	EquiPure™ Treatment 15-1x[c]	15-2x[d]	15-3x[e]	15-4x[f]	50-1x[g]	50-2x[h]	50-3x[i]	50-4x[j]
Morphologically Normal	47b (4.7)	52ab (0.6)	68a (3.8)	68a (4.0)	67a (5.8)	66a (3.3)	64a (2.2)	66a (4.7)	65a (3.8)	61a (2.6)
Abnormal Heads	6a (1.3)	6a (0.6)	4a (1.0)	4a (1.5)	5a (0.3)	6a (0.3)	7a (1.2)	5a (0.9)	6a (0.7)	5a (0.3)
Craters	3a (1.2)	2a (0)	1a (0.3)	0a (0.3)	1a (0)	2a (1.0)	2a (0.6)	1a (0)	2a (0.7)	1a (0)
Small Heads	1a (0.3)	1a (0.3)	1a (0.7)	2a (0.9)	1a (0.3)	2a (0.3)	1a (1.0)	2a (0.7)	1a (0.7)	1a (0.6)
Large Heads	1a (0.3)	0a (0.3)	0a (0)	0a (0)	0a (0.3)	0a (0)	0a (0.3)	0a (0)	0a (0.3)	1a (0.7)
Misshaped Heads	2a (0.9)	2a (0.3)	2a (1.0)	2a (0.3)	3a (0.3)	3a (0.9)	3a (0.3)	2a (0.6)	2a (0.3)	3a (0.3)
Abnormal Acrosomes	3a (0.9)	2a (1.1)	1a (0.3)	1a (0.7)	1a (0.3)	0a (0.3)	0a (0.3)	0a (0.3)	1a (0.6)	1a (0.6)
Detached Heads	5a (1.9)	3a (0.3)	0a (0.3)	1a (0.7)	1a (0.9)	2a (1.2)	3a (1.7)	2a (0.3)	1a (1.0)	3a (1.2)
Proximal Droplets	13a (2.9)	12a (1.8)	10a (1.7)	8a (1.0)	9a (2.1)	9a (1.5)	10a (0.6)	9a (2.9)	8a (2.4)	9a (2.2)
Small Proximal Droplets	6a (1.4)	8a (2.4)	6a (1.0)	5a (1.0)	7a (0.9)	7a (2.0)	8a (0.3)	7a (2.1)	7a (1.5)	5a (2.4)
Large Proximal Droplets	7a (2.7)	3a (0.7)	4a (1.0)	3a (1.0)	2a (1.2)	2a (0.9)	2a (0.3)	2a (0.9)	1a (0.9)	3a (0.9)
Distal Droplets	5a (1.5)	10a (1.8)	11a (4.1)	8a (2.9)	9a (3.2)	5a (1.9)	8a (1.2)	7a (1.2)	5a (1.7)	4a (1.9)
Small Distal Droplets	1c (0.7)	8a (1.5)	4abc (0.9)	5abc (1.5)	4abc (0.6)	3abc (0.3)	7ab (0.3)	5abc (0.6)	3bc (1.2)	3abc (1.2)
Large Distal Droplets	4a (1.5)	1a (0.3)	7a (3.9)	3a (1.5)	5a (2.6)	2a (1.5)	1a (0.6)	2a (1.0)	2a (0.7)	2a (0.7)
Abnormal Midpieces	5a (2.1)	9a (1.5)	5a (2.3)	7a (0.3)	5a (0.6)	8a (0.6)	5a (1.2)	7a (2.0)	11a (1.5)	11a (1.5)
Bent Midpieces	6a (0.7)	3ab (1.5)	1ab (1.0)	3ab (0.9)	1ab (1.0)	1ab (1.0)	1b (0)	0b (0.3)	2ab (0.9)	4ab (0.9)
Bent Tails	2a (0.6)	1a (0.7)	0a (0)	0a (0.3)	1a (0.7)	0a (0.3)	0a (0.3)	1a (0.3)	0a (0.3)	1a (0.7)
Coiled Tails	8a (0.7)	3b (0)	0c (0.3)	0c (0.3)	1c (0.6)	1c (0.6)	1c (0.7)	2c (0.6)	1c (0.3)	1c (0.7)
Premature Germ Cells	1a (0.3)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b and c) differ ($P<0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-1x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[d] 15-2x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[e] 15-3x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[f] 15-4x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[g] 50-1x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[h] 50-2x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[i] 50-3x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[j] 50-4x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Table 26: Effect of treatment on sperm motility and chromatin quality (COMPαt) variables for Stallion 2 (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment							
	Group UC[a†]	Group CC[b†]	15-1x[c]	15-2x[d]	15-3x[e]	15-4x[f]	50-1x[g]	50-2x[h]	50-3x[i]	50-4x[j]
MOT	90b (0.9)	91b (1.2)	97a (0.6)	97a (0.3)	98a (0.6)	97a (0.7)	96a (0.3)	97a (0.6)	97a (0.3)	98a (0)
PMOT	71b (2.0)	71b (1.2)	84a (0.9)	85a (1.8)	85a (0.7)	86a (0.6)	84a (1.2)	85a (3.0)	85a (1.5)	86a (1.2)
VCL	175a (5.5)	152b (2.6)	174a (3.3)	170ab (5.2)	167ab (4.3)	167ab (4.1)	183a (1.8)	182a (5.5)	177a (7.5)	176a (6.7)
VAP	90ab (3.5)	75b (0.9)	87ab (2.7)	85ab (3.8)	83ab (3.1)	82ab (2.6)	95a (1.2)	93a (3.8)	89ab (5.2)	89ab (4.2)
VSL	68ab (2.3)	55c (0.6)	64ab (0.7)	62bc (2.0)	61bc (1.5)	61bc (1.2)	72a (2.1)	69ab (2.5)	65ab (2.2)	65ab (2.6)
STR	76a (0.6)	75a (0.3)	75a (1.7)	74a (1.2)	75a (1.2)	75a (0.7)	77a (1.3)	75a (1.7)	74a (2.5)	75a (1.2)
LIN	40ab (0)	37c (0.6)	39abc (0.3)	38bc (0.3)	38bc (0)	38bc (0)	41a (1.2)	39abc (0.9)	38bc (0.6)	39abc (0.3)
COMPαt	4a (0.6)	5a (0.7)	2b (0.5)	2b (0.1)	1b (0.2)	2b (0.2)	2b (0.2)	1b (0.1)	2b (0.1)	2b (0.1)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b and c) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-1x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[d] 15-2x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[e] 15-3x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[f] 15-4x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[g] 50-1x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[h] 50-2x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[i] 50-3x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[j] 50-4x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Table 27: Effect of treatment on sperm morphology variables for Stallion 2(mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment							
	Group UC[a†]	Group CC[b†]	15-1x[c]	15-2x[d]	15-3x[e]	15-4x[f]	50-1x[g]	50-2x[h]	50-3x[i]	50-4x[j]
Morphologically Normal	34c (2.9)	45b (2.0)	58a (2.6)	64a (5.5)	61a (1.7)	62a (0.6)	61a (3.6)	62a (0.3)	57a (1.5)	57a (1.7)
Abnormal Heads	12a (1.8)	11ab (0.3)	6b (0.3)	8ab (0.7)	9ab (0.7)	9ab (0.7)	7ab (1.5)	8ab (0.9)	10ab (0.6)	10ab (2.4)
Craters	2a (0.3)	1a (0.3)	0a (0)	1a (0.3)	1a (0.3)	2a (0.3)	1a (0.6)	1a (0.3)	2a (0.6)	1a (0.9)
Small Heads	1a (0.7)	1a (0.3)	1a (0)	2a (0.3)	2a (0.9)	0a (0.3)	2a (0.6)	1a (0)	2a (0.6)	1a (0.3)
Large Heads	4a (1.3)	4ab (0.7)	1b (0.3)	2ab (0.9)	2ab (0.3)	3ab (0.6)	1ab (0.7)	1ab (0.7)	2ab (0.3)	3ab (0.7)
Misshaped Heads	4a (1.2)	4a (0.3)	3a (0.3)	3a (0.6)	4a (0)	4a (0.9)	3a (0.3)	5a (0.9)	4a (0.3)	6a (0.9)
Abnormal Acrosomes	2a (0.6)	3a (1.2)	1a (0.6)	1a (0.3)	0a (0)	0a (0)	1a (0.7)	1a (0.7)	1a (0.3)	1a (1.0)
Detached Heads	2a (0.6)	2a (1.3)	1a (0.7)	1a (0.6)	1a (0.7)	1a (0.3)	1a (0.7)	1a (0.6)	2a (0.3)	1a (1.0)
Proximal Droplets	20a (2.6)	20a (2.3)	21a (1.5)	17a (0.9)	14a (1.8)	14a (0.3)	17a (2.9)	20a (0.3)	17a (0.6)	15a (2.3)
Small Proximal Droplets	11a (3.3)	12a (1.7)	14a (1.3)	12a (2.3)	11a (1.5)	10a (0.6)	15a (2.6)	17a (1.2)	12a (1.2)	11a (1.0)
Large Proximal Droplets	9a (1.5)	8ab (0.7)	6ab (2.7)	4ab (1.8)	3ab (0.6)	4ab (0.7)	2b (0.3)	3ab (1.2)	5ab (0.6)	4ab (1.5)
Distal Droplets	5a (1.7)	3a (1.2)	2a (0.3)	3a (2.0)	3a (0.6)	3a (1.8)	1a (0.3)	2a (0.9)	3a (1.5)	2a (0.9)
Small Distal Droplets	2a (0.9)	1a (0.9)	1a (0.6)	3a (1.5)	2a (0.7)	2a (1.2)	1a (0.6)	2a (0.3)	2a (0.7)	2a (0.7)
Large Distal Droplets	3a (1.2)	2a (0.3)	1a (0.3)	1a (0.7)	1a (0.3)	1a (0.6)	0a (0.3)	1a (0.7)	1a (0.9)	1a (0.3)
Abnormal Midpieces	12a (2.1)	12a (2.0)	10a (0.6)	6a (1.2)	12a (0.9)	11a (1.7)	12a (2.0)	7a (1.9)	10a (1.5)	13a (0.3)
Bent Midpieces	4a (1.2)	1b (0.7)	1b (0.7)	0b (0.3)	1b (1.0)	0b (0.3)	0b (0.3)	0b (0)	0b (0.3)	0b (0.3)
Bent Tails	0a (0.3)	1a (0.9)	0a (0)	0a (0.3)	0a (0)	0a (0.3)	0a (0)	0a (0)	0a (0)	0a (0)
Coiled Tails	6a (1.9)	2b (0.7)	1b (0.3)	0b (0)	0b (0.3)	0b (0)	1b (0.3)	0b (0.3)	0b (0.3)	0b (0.3)
Premature Germ Cells	2a (1.0)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)

[§] Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b and c) differ ($P<0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-1x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[d] 15-2x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[e] 15-3x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[f] 15-4x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[g] 50-1x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[h] 50-2x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[i] 50-3x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[j] 50-4x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[†] Control Samples.

Table 28: Effect of treatment on sperm motility and chromatin quality (COMPαt) variables for Stallion 3 (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment							
	Group UC[a†]	Group CC[b†]	15-1x[c]	15-2x[d]	15-3x[e]	15-4x[f]	50-1x[g]	50-2x[h]	50-3x[i]	50-4x[j]
MOT	85b (2.0)	86b (2.6)	98a (0.3)	97a (0.3)	97a (0)	97a (1.2)	96a (0.3)	98a (0.3)	97a (0.6)	97a (0.3)
PMOT	55b (0.7)	60b (1.3)	78a (1.9)	79a (1.5)	80a (0.9)	79a (2.0)	77a (2.6)	78a (1.3)	79a (2.7)	78a (2.4)
VCL	185a (29.0)	185a (3.5)	201a (2.0)	201a (5.2)	197a (8.1)	198a (6.1)	202a (6.0)	206a (1.2)	200a (3.2)	203a (4.3)
VAP	92a (17.7)	91a (2.1)	103a (2.1)	101a (2.4)	98a (3.2)	100a (2.6)	104a (2.0)	105a (0.7)	101a (2.9)	103a (2.7)
VSL	62a (9.7)	61a (2.0)	71a (2.8)	70a (2.6)	68a (2.4)	69a (2.4)	73a (1.5)	72a (2.6)	70a (2.6)	72a (3.3)
STR	69a (3.8)	68a (1.9)	69a (1.2)	70a (1.3)	70a (1.5)	70a (1.8)	71a (2.3)	69a (1.8)	71a (2.3)	70a (2.1)
LIN	35a (0.7)	34a (1.2)	36a (1.2)	36a (1.2)	37a (1.2)	37a (1.5)	38a (1.9)	37a (1.5)	37a (1.5)	37a (1.5)
COMPαt	5a (0.7)	4a (0.3)	1b (0.1)	1b (0.2)	3b (0.4)	2b (0.2)	2b (0.2)	2b (0.2)	1b (0.2)	2b (0.2)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a and b) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-1x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[d] 15-2x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[e] 15-3x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[f] 15-4x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[g] 50-1x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[h] 50-2x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[i] 50-3x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[j] 50-4x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Table 29: Effect of treatment on sperm morphology variables for Stallion 3 (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment							
	Group UC[a†]	Group CC[b†]	15-1x[c]	15-2x[d]	15-3x[e]	15-4x[f]	50-1x[g]	50-2x[h]	50-3x[i]	50-4x[j]
Morphologically Normal	60a (7.3)	52a (13.3)	79a (4.5)	83a (4.0)	83a (4.2)	80a (4.7)	78a (3.5)	84a (5.4)	79a (5.2)	77a (6.2)
Abnormal Heads	6a (2.3)	4a (1.9)	1a (1.3)	1a (1.3)	2a (1.7)	2a (1.9)	2a (0.9)	2a (1.5)	3a (1.2)	3a (1.2)
Craters	1a (0.7)	1a (0.3)	0a (0)	0a (0.3)	1a (0.7)	0a (0.3)	0a (0.3)	0a (0.3)	1a (0.3)	0a (0.3)
Small Heads	1a (1.0)	2a (0.7)	1a (0.7)	0a (0.3)	0a (0)	1a (1.3)	1a (1.0)	1a (0.7)	1a (0.7)	1a (0.6)
Large Heads	2a (1.2)	0a (0.3)	0a (0)	0a (0.3)	0a (0)	0a (0)	0a (0.3)	0a (0)	0a (0.3)	0a (0)
Misshaped Heads	2a (1.2)	1a (1.0)	1a (0.7)	0a (0.3)	1a (1.0)	1a (0.7)	1a (0.3)	1a (1.0)	0a (0.3)	1a (0.9)
Abnormal Acrosomes	0a (0)	0a (0)	0a (0)	0a (0)	0a (0.3)	0a (0)	0a (0)	0a (0.3)	0a (0)	0a (0)
Detached Heads	1a (0.9)	3a (1.5)	0a (0)	0a (0)	0a (0.3)	0a (0.3)	1a (0.6)	1a (1.0)	0a (0)	1a (0.7)
Proximal Droplets	20ab (2.3)	28a (10.3)	15ab (0.3)	12ab (1.2)	12ab (2.4)	12ab (1.8)	14ab (1.9)	9b (2.1)	13ab (3.1)	15ab (2.5)
Small Proximal Droplets	10a (0.9)	16a (5.9)	10a (1.7)	9a (0.7)	8a (0.3)	9a (0.3)	11a (1.2)	6a (1.2)	10a (2.6)	11a (1.3)
Large Proximal Droplets	11a (2.8)	12a (4.6)	5a (2.0)	3a (1.8)	4a (2.6)	4a (1.9)	3a (1.7)	3a (1.5)	3a (1.5)	4a (1.2)
Distal Droplets	2a (0.9)	3a (1.5)	1a (0.7)	1a (0.6)	0a (0.3)	2a (1.2)	2a (1.2)	1a (0.9)	2a (1.2)	1a (0.6)
Small Distal Droplets	1a (0.6)	1a (0.9)	1a (0.7)	0a (0.3)	0a (0.3)	1a (1.3)	1a (0.9)	1a (0.9)	1a (0.6)	1a (0.3)
Large Distal Droplets	2a (0.7)	1a (0.7)	0a (0)	1a (0.3)	0a (0)	0a (0.3)	0a (0.3)	0a (0)	1a (0.7)	0a (0.3)
Abnormal Midpieces	3a (0.9)	3a (3.0)	4a (2.3)	2a (1.2)	1a (0.6)	2a (0.9)	2a (0)	2a (1.2)	2a (1.9)	3a (1.5)
Bent Midpieces	1b (0.6)	4a (0.3)	0b (0)	0b (0.3)	1b (0.3)	1b (0.7)	1b (0.6)	1b (0.3)	0b (0.3)	0b (0.3)
Bent Tails	1a (0.6)	1a (0.3)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)
Coiled Tails	3a (2.4)	2a (1.9)	0a (0)	0a (0)	0a (0)	1a (0.3)	0a (0)	0a (0)	0a (0)	0a (0)
Premature Germ Cells	2a (0.3)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)	0a (0)

[§] Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a and b) differ ($P<0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-1x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[d] 15-2x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[e] 15-3x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[f] 15-4x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[g] 50-1x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[h] 50-2x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[i] 50-3x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[j] 50-4x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[†] Control Samples.

Table 30: Effect of treatment on sperm motility and chromatin quality (COMPαt) variables for Stallion 4 (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment							
	Group UC[a†]	Group CC[b†]	15-1x[c]	15-2x[d]	15-3x[e]	15-4x[f]	50-1x[g]	50-2x[h]	50-3x[i]	50-4x[j]
MOT	71c (1.7)	76c (2.0)	94a (1.2)	95a (1.0)	95a (0.9)	96a (0.9)	89b (1.5)	93a (0.6)	94a (0.9)	95a (0.3)
PMOT	37c (3.1)	43c (4.0)	75ab (3.5)	78ab (0.3)	79a (0.9)	82a (1.5)	69b (2.3)	76ab (2.0)	79ab (1.3)	79ab (2.0)
VCL	220a (8.2)	202b (5.8)	240a (4.4)	231a (6.4)	225a (4.0)	225a (4.3)	234a (4.8)	232a (4.7)	230a (2.2)	229a (2.4)
VAP	107ab (6.0)	97b (3.2)	118a (2.6)	112a (2.7)	107ab (1.8)	106ab (2.6)	117a (0.6)	114a (1.0)	112a (0.6)	110a (1.2)
VSL	71cd (3.5)	65d (0.3)	86ab (0.3)	81ab (1.5)	77bc (1.2)	77bc (1.8)	90a (2.6)	86ab (2.5)	84ab (3.2)	82ab (3.8)
STR	63b (2.3)	66ab (2.0)	72ab (2.2)	72ab (1.7)	71ab (0.9)	72ab (1.5)	75a (2.1)	74a (2.8)	74a (2.3)	74a (2.7)
LIN	32a (1.2)	32a (0.9)	36a (0.9)	36a (1.0)	35a (0.7)	35a (1.0)	39a (1.7)	38a (1.7)	37a (1.9)	37a (2.2)
COMPαt	8a (0.6)	8a (0.1)	4b (0.1)	4b (0.8)	4b (0.2)	3b (0.5)	4b (0.7)	4b (0.4)	4b (0.6)	4b (0.4)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); VAP = average-path velocity (μm/s); VSL = straight-line velocity (μm/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMPαt = percentage of sperm with αt value outside the main population (%).

§ Percentage data (MOT, PMOT, STR, LIN, COMPαt) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b,c and d) differ ($P < 0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-1x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[d] 15-2x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[e] 15-3x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[f] 15-4x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[g] 50-1x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[h] 50-2x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[i] 50-3x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[j] 50-4x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

† Control Samples.

Table 31: Effect of treatment on sperm morphology variables for Stallion 4 (mean ± SEM).

Laboratory Parameters*			EquiPure™ Treatment							
	Group UC[a†]	Group CC[b†]	15-1x[c]	15-2x[d]	15-3x[e]	15-4x[f]	50-1x[g]	50-2x[h]	50-3x[i]	50-4x[j]
Morphologically Normal	42c (2.7)	47c (4.0)	69ab (1.5)	73a (1.7)	72a (0.3)	67ab (1.2)	66ab (1.5)	71ab (1.5)	63b (2.3)	62b (1.3)
Abnormal Heads	9a (1.7)	8a (0.6)	7a (1.5)	7a (0.3)	6a (1.0)	8a (0.6)	5a (0.6)	7a (0.3)	7a (1.0)	7a (0.9)
Craters	1a (0.6)	1a (0)	2a (0.3)	1a (0.3)	2a (0.7)	2a (0.6)	1a (0.6)	1a (0.3)	1a (1.3)	2a (0.3)
Small Heads	1a (0.7)	2a (0.6)	1a (0.6)	1a (0.7)	1a (0.6)	1a (0.6)	1a (0)	2a (0.6)	1a (0.3)	1a (0.6)
Large Heads	1a (0.6)	0a (0.3)	0a (0.3)	1a (0.9)	0a (0.3)	1a (0.7)	0a (0)	0a (0)	0a (0)	1a (0.7)
Misshaped Heads	5a (1.5)	5a (0.3)	3a (0.6)	3a (0.3)	3a (0)	4a (0.7)	3a (0.6)	5a (0.9)	4a (0.3)	4a (0.6)
Abnormal Acrosomes	3a (0.7)	1a (0.3)	1a (0)	1a (0.6)	1a (0.3)	1a (0.7)	0a (0.3)	1a (0.3)	0a (0.3)	1a (0.9)
Detached Heads	7a (0.3)	5a (1.7)	2a (1.5)	4a (0.3)	3a (0.7)	4a (0.9)	5a (1.8)	5a (0.9)	6a (0.7)	6a (0.3)
Proximal Droplets	3a (0.9)	5a (0.6)	4a (0.7)	2a (0)	5a (0.7)	4a (1.2)	4a (0.6)	2a (0.7)	5a (1.5)	4a (0.9)
Small Proximal Droplets	1a (0.6)	2a (0.9)	2a (0.6)	1a (0)	3a (0.6)	2a (0.9)	2a (0.6)	1a (0.6)	2a (0.3)	2a (0.6)
Large Proximal Droplets	2a (0.7)	3a (0.3)	2a (1.2)	1a (0)	2a (0.9)	2a (0.3)	2a (0)	1a (0.3)	3a (1.3)	2a (0.3)
Distal Droplets	1a (0.7)	2a (1.7)	2a (1.2)	0a (0.3)	1a (0.9)	1a (0.7)	2a (1.5)	3a (1.7)	1a (0.9)	1a (1.0)
Small Distal Droplets	0a (0.3)	1a (1.0)	1a (0.9)	0a (0.3)	1a (0.7)	0a (0.3)	2a (1.2)	2a (1.2)	1a (0.3)	1a (0.7)
Large Distal Droplets	1a (0.6)	1a (0.7)	0a (0.3)	0a (0)	1a (0.3)	0a (0.3)	1a (0.3)	1a (0.6)	1a (0.7)	0a (0.3)
Abnormal Midpieces	11a (2.1)	12a (1.0)	10a (2.0)	7a (1.2)	6a (0.6)	11a (0.7)	7a (0.9)	8a (1.0)	9a (1.5)	12a (0.9)
Bent Midpieces	10a (2.3)	9ab (2.1)	3c (0.7)	3c (0.9)	3c (1.2)	3bc (1.2)	6abc (0.9)	2c (0.3)	5abc (1.2)	4bc (0.9)
Bent Tails	2a (1.2)	0a (0.3)	0a (0.3)	0a (0.3)	0a (0)	0a (0)	1a (0.6)	0a (0)	0a (0.3)	0a (0)
Coiled Tails	13a (1.5)	10a (0.9)	2b (1.2)	3b (0.6)	2b (1.2)	2b (0.3)	3b (1.5)	2b (1.2)	3b (1.5)	3b (0.3)
Premature Germ Cells	4a (0.3)	1b (0.7)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)	0b (0)

[§] Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter, means with different letters (a,b and c) differ ($P<0.05$).

[a] Group UC = uncenrifuged (raw) semen (n=3).

[b] Group CC = centrifuged semen in 50-mL conical tubes with 3.5 mL Cushion Fluid and 1000 x *g* for 20 minutes (n=3).

[c] 15-1x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[d] 15-2x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[e] 15-3x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[f] 15-4x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[g] 50-1x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[h] 50-2x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[i] 50-3x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[j] 50-4x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes (n=3).

[†] Control Samples.

Table 32: Recovery rates for Experiment 3 (mean ± SEM).

Laboratory Parameter*	EquiPure™ Treatment							
	15-1x[a]	15-2x[b]	15-3x[c]	15-4x[d]	50-1x[e]	50-2x[f]	50-3x[g]	50-4x[h]
Total RR	71.0a (3.8)	68.8ab (3.2)	61.7ab (3.4)	50.4c (3.2)	67.0ab (3.6)	64.9ab (2.8)	62.1b (2.9)	60.6b (3.5)
RR-St 1	71.0a (10.7)	68.0a (12.1)	69.3a (6.9)	56.7a (8.7)	57.7a (11.3)	58.7a (9.5)	61.7a (4.7)	60.3a (5.5)
RR-St 2	81.7a (2.0)	76.0ab (4.2)	56.3c (5.5)	42.3d (6.4)	75.7ab (2.4)	70.3abc (1.3)	65.3abc (2.2)	63.7bc (5.8)
RR-St 3	65.0a (3.8)	69.0a (3.6)	68.3a (6.6)	56.0a (5.1)	64.7a (0.9)	65.3a (1.8)	64.3a (5.2)	56.0a (12.1)
RR-St 4	66.3a (3.8)	62.3a (1.9)	52.7a (4.5)	46.7b (3.3)	70.0a (8.3)	65.3a (6.8)	57.0a (10.7)	62.3a (6.1)

* Total RR = total sperm recovery rate for all four stallions (%); RR-St 1 = sperm recovery rate for Stallion 1 (%); RR-St 2 = sperm recovery rate for Stallion 2 (%); RR-St 3 = sperm recovery rate for Stallion 3 (%); RR-St 4 = sperm recovery rate for Stallion 4 (%).

§ Percentage data (All Laboratory Parameters) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data. Among treatment and within laboratory parameter means with different letters (a,b,c and d) differ ($P< 0.05$).

[a] 15-1x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

[b] 15-2x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes.

[c] 15-3x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes.

[d] 15-4x = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes.

[e] 50-1x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 1 mL of extended semen containing 250 – 500 x 10^6 sperm and 200 x *g* for 30 minutes.

[f] 50-2x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 2 mL of extended semen containing 500 – 1000 x 10^6 sperm and 200 x *g* for 30 minutes.

[g] 50-3x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm and 200 x *g* for 30 minutes.

[h] 50-4x = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm and 200 x *g* for 30 minutes.

DISCUSSION AND SUMMARY

Several studies have been conducted with density gradient centrifugation in human artificial reproductive techniques [33-38]. However, there is only limited data available regarding the effects of density gradient centrifugation on the quality of equine spermatozoa [5-6,42-43]. For the equine species, three previous studies show that density gradient centrifugation yields an improvement in motion and sperm morphologic characteristics [6,42-43]. The present study was designed to evaluate sperm motion and sperm morphologic characteristics, as well as DNA integrity (SCSA) before, and following, density gradient centrifugation. Unlike previous studies that only included fertile stallions, this study encompassed both fertile and subfertile stallions. Various treatments were applied to the density gradient centrifugation process in an effort to determine ways to maximize sperm yields and semen quality, while identifying ways to simplify the technique for use in the clinical setting.

Based on the previous studies with human semen and preliminary work in our laboratory, in this study we anticipated that a number of experimental endpoints would be improved following density gradient centrifugation [33-38]. In Experiment 1, we hypothesized that single-layer density gradient centrifugation would yield similar values to the two-layer technique for sperm motility, morphology, and chromatin integrity. We selected 4 mL of EquiPure™ Bottom Layer in the one-layer treatment to compare to the two layer media (2 mL EquiPure™ Top Layer over 2 mL EquiPure™ Bottom Layer) so that the depth of the media would be comparable. In general, the one-layer treatment yielded superior values for sperm motion characteristics and sperm chromatin

characteristics, and yielded similar results to the conventional two-layer treatment for sperm morphologic characteristics, when compared to the conventional two-layer treatment. This finding is in contrast to the findings of Morrell et al. (2008), where the recovery rate was similar for both treatment groups [43]. The volume of density gradient and time of centrifugation were identical in the current study to that of Morrell et al., but the centrifugation force differed [43]. One consideration, as noted by Morrell et al., is that the interface between the density gradient layers plays an important role in selecting the sperm that pass through to the pellet [43]. However, our view is that by using the single layer method the sperm would have to pass through the same distance of the denser gradient as opposed to half the distance through the lighter gradient and half the distance through the heavy gradient. In the current study, the single layer centrifugation yielded superior sperm motion characteristics and chromatin integrity, but similar in sperm morphologic characteristics as the two-layer method. Our data support the use of a one-layer technique making it a more user-friendly procedure in a clinical setting. Instead of layering two times, by using only a single layer it is necessary to layer only once allowing the procedure to be simplified. Based on these results, we used the single layer density gradient method for Experiments 2 and 3.

The stallions used in Experiment 1 were thought to be fertile. All four stallions showed an improvement in total motility, progressive motility, morphologically normal sperm, and DNA quality. The one-layer density gradient centrifugation eliminated bent midpieces for Stallion A. For Stallion B, EquiPure™ decreased the percentage of bent midpieces and the one-layer method eliminated coiled tails. For Stallions A and B the

sperm recovery rates were similar between the two methods. Stallion C exhibited a percentage decrease in abnormal midpieces, bent midpieces, and coiled tails following EquiPure™ centrifugation. For Stallion D, there was a remarkable decrease in the percentage of bent midpieces from 26 to 5 in the one-layer method as well as a decrease in coiled tails in both methods. Stallions C and D yielded a higher recovery rate in the one-layer method when compared to the two-layer approach.

For Experiment 2, we chose to compare the 4 mL volume of EquiPure™ Bottom Layer to both a 2 mL and 3 mL volume of EquiPure™ Bottom Layer to determine if sperm quality is affected when the volume of the density gradient is altered. Reducing the volume of EquiPure™ Bottom Layer would allow the procedure to be more cost effective in a clinical setting. We found that sperm motility, morphologic characteristics and chromatin integrity were similar among the volumes of EquiPure™ Bottom Layer density gradient (2, 3, or 4 mL) when used with 15-mL centrifuge tubes. The volumes selected for use with 50-mL conical bottom tubes (9, 12.5, and 16.5 mL) were designed to correspond to density gradient heights for the three volumes used in the 15 mL-tubes. The 50-mL conical tubes were chosen to evaluate the potential of processing entire ejaculates with fewer centrifugation tubes. Measures of sperm motility, morphologic characteristics and chromatin integrity were similar among these density gradient volumes applied to the 50-mL tubes. Furthermore, experimental endpoints were similar for measures of semen quality when comparing the 50-mL and 15-mL conical tubes. However, sperm recovery rates were generally lower for semen centrifuged in 50-mL conical tubes, as compared to 15-mL conical tubes. These data suggest that 15-mL tubes

should be used to maximize sperm recovery when using the single EquiPure™ Bottom Layer centrifugation method. Based on the higher recovery rate and that there was no significant statistical difference between the volumes for semen quality the 15-28mm (2mL) would be the most cost efficient. The differences in recovery rate between the tube sizes could be due to the ability to see the pellet more clearly in the 15-mL tube.

The stallions used in Experiment 2 are different from the stallions used in Experiment 1. Stallion I is a known fertile stallion; Stallions II, III and IV are known subfertile stallions. All four stallions showed an improvement in total motility, progressive motility, morphologically normal sperm, and DNA quality. EquiPure™ processing eliminated coiled tails for Stallion I. The recovery rate differences between the 15-mL and 50-mL conical tubes for Stallion I was due to clarity of the pellet as described previously. Stallion II showed a decrease in bent midpieces. For Stallion III, density gradient centrifugation was able to decrease considerably the DNA quality percentage which means an improvement. EquiPure™ processing decreased bent midpieces from 34 to 8-13 depending on the treatment for Stallion IV. Since this experiment used known subfertile stallions, it is remarkable to see how that density gradient centrifugation can impact a stallion's semen quality.

For Experiment 3, we evaluated the effects of sperm number on sperm recovery rate and quality of sperm in recovered pellets. For this study, we selected the 41mm density gradient volume height, to allow further the distance for sperm to traverse though the gradient when increasing numbers of sperm above 1×10^9 was applied to the gradient. In this study, no major difference was detected in the resulting semen quality

as a result of sperm load added to the gradient for either the 15 mL or 50-mL tubes. Recovery rate following a 4x sperm load in the 15-mL tubes tended to be lower than that of lower sperm loads, whereas a sperm load of 1-4x (representing 500 x 10^6 to 2 x 10^9 sperm) did not affect sperm recovery rate in 50-mL tubes. The lowered sperm yield for a high sperm load in the 15-mL tubes is likely due to the reduced surface area for sperm migration into and through the density gradient, as compared to that in the 50-mL tubes. Based on the experimental endpoints' results, the 15-3x would be the most logical choice of tube combination to use. It allows you to layer between 750 x 10^6 and 1 x 10^9 sperm per centrifugation tube while keeping the usage of the density gradient medium low. Of clinical importance, density gradient centrifugation of semen with any of the techniques applied appeared to result in improved semen quality of recovered sperm, when compared to the uncentrifuged and cushion centrifuged control groups. The experimental results also suggest that the two layer protocol can be simplified to a single layer method.

The stallions used in Experiment 3 were thought to be fertile. All four stallions showed an improvement in total motility, progressive motility, morphologically normal sperm, and DNA quality. EquiPure™ processing eliminated coiled tails for Stallions 1, 2 and 3. Also, for Stallion 2 bent midpieces were eliminated. For Stallion 4, density gradient centrifugation decreased coiled tails and bent midpieces.

Of clinical importance, density gradient centrifugation of semen with any of the techniques applied appeared to result in improved semen quality of recovered sperm, when compared to the uncentrifuged and cushion centrifuged control groups. The

experimental results also suggest that the two-layer protocol can be simplified to a one-layer method. A summary comparison of best 15-mL and 50-mL treatments for Experiments 2 and 3 are presented in Table 33. For Experiment 2, by processing in 15-28mm a practitioner can use less EquiPure™ Bottom Layer and recover a higher total sperm number when the entire ejaculate is processed. Since Experiment 2 contained three subfertile stallions, the total sperm numbers were significantly lower than the stallions used in either of the other experiments. However, these would be the stallions that would utilize the EquiPure™ processing and why it is so important to show the impact the process has on subfertile stallions. For Experiment 3, the 15-3x was similar to all parameters when compared to 50-4x. From the practitioner's standpoint, even though there is a lower total number applied and one might have to use an extra tube when processing, one still would be minimizing cost by using the 15-3x treatment. This is because the volume to reach the 41mm height in the 50-mL conical tubes is 16.5mL of EquiPure™ Bottom Layer.

Table 33: Summary Comparison of Treatments for Experiments 2 and 3(mean ± SEM).

Laboratory Parameters*	Experiment 2 Treatments		Experiment 3 Treatments	
	15-28 (2mL)[a]	50-28 (9mL)[b]	15-3x (4mL)[c]	50-4x (16.5mL)[d]
MOT	90a (1.8)	89a (1.9)	97a (0.4)	96a (0.4)
PMOT	73a (2.3)	72a (2.4)	81a (0.9)	81a (1.2)
VCL	228a (6.0)	207b (4.9)	195a (7.1)	198a (6.8)
STR	77b (0.8)	79a (0.8)	73a (0.8)	74a (1.1)
Morph. Normal	63a (3.8)	62a (3.6)	71a (3.0)	64a (2.8)
Recovery Rate	44a (7.3)	35b (3.9)	62a (3.4)	61a (3.5)
TSN (x 10^9)	1.78a (0.5)	1.33b (0.3)	4.96a (0.4)	4.93a (0.5)
TSN– TMOT (x 10^9)	1.60a (0.5)	1.18b (0.3)	4.81a (0.4)	4.73a (0.5)
TSN– PMOT(x 10^9)	1.31a (0.4)	0.96b (0.2)	4.05a (0.4)	3.97a (0.4)
TSN– Morph Norm (x 10^9)	1.23a (0.4)	0.88b (0.3)	3.60a (0.5)	3.24a (0.4)

* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s); STR = straightness ([VAP/VCL]100; %); Morph. Normal = morphologically normal sperm (%); Recovery Rate = sperm recovery rate (%); TSN (x 10^9) = total number of sperm recovered following centrifugation; TSN-MOT (x 10^9) = total number of motile sperm recovered following centrifugation; TSN-PMOT(x 10^9) = total number of progressively motile sperm recovered following centrifugation; TSN-Norm (x 10^9) = total number of morphologically normal sperm recovered following centrifugation. Within treatment and within laboratory parameter, means with different letters (a and b) differ ($P < 0.05$).

[a] 15-28 (2mL) = 15-mL tube containing 2 mL of bottom-layer EquiPure™ (n=12).

[b] 50-28 (9mL) = 50-mL tube containing 9 mL of bottom-layer EquiPure™ (n=12).

[c] 15-3x (4mL) = 15-mL tube containing 4 mL of bottom-layer EquiPure™ and 3 mL of extended semen containing 750 - 1500 x 10^6 sperm (n=12).

[d] 50-4x (16.5mL) = 50-mL tube containing 16.5 mL of bottom-layer EquiPure™ and 4 mL of extended semen containing 1000 - 2000 x 10^6 sperm (n=12).

In summary, density gradient centrifugation of stallion semen appears to be a useful means of selecting sperm for superior quality. EquiPure™ improved the percentages of total motility, progressive motility, morphologically normal sperm and DNA quality for all stallions in the study. Based on the stallions used in the study, EquiPure™ processing would be suitable for a stallion that has an ejaculate with poor DNA quality, low motility, and/or morphological defects like bent midpieces, bent tails and coiled tails. The single layer can be reduced to 2 mL in the 15-mL conical tubes to be more cost efficient. No advantage was gained by centrifuging in 50-mL conical

tubes. Based on the volume used of EquiPure™ Bottom Layer used in the 50-mL conical tubes in this study it would be less cost efficient to process with these amounts when similar results can be obtained with the usage of less medium. In addition, as total sperm number layered over the density gradient increased in 15-mL tubes, the recovery rate decreased. The fertility rate following EquiPure™ Bottom Layer centrifugation in a commercial program for several subfertile stallions (unpublished data) has demonstrated that single-layer density gradient semen centrifugation in 15-mL tubes is successful and that the technique can be applied in the clinical setting.

FUTURE AIMS

While EquiPure™ appears to be satisfactory as a density gradient medium for centrifuged semen, the long term effects remain largely unstudied. To address this, future studies could be directed to evaluating density gradient processed semen after cooled storage. The relationship between laboratory measures of semen quality and fertility should also be addressed.

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