SEPARATION OF ABNORMAL SPERMATOZOA FROM SEMEN PRODUCED BY BULLS WITH IMPAIRED TESTICULAR THERMOREGULATION

BY

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(ABSTRACT)

Study 1 was conducted to determine if partial restriction of testicular thermoregulation affects spermatogenesis when compared to full restriction by mild scrotal insulation for 48 h. Study 2 and Study 3 were conducted to investigate the efficiencies of selected semen separation techniques on the removal of abnormal spermatozoa. In Study 1, semen from control and partial scrotal insulation (PI) bulls did not differ between the pre-insult and post-insult period for any variable measured. Full scrotal insulation (FI) bulls differed for several variables measured, but at different intervals and for different durations post-insulation. Spermatozoal motility and acrosomal integrity were reduced during Period 3 only of semen collection with no effect on sperm output. Full insulation caused abnormalities to be ejaculated from d 10 to 33 post-insulation. Specific morphologically abnormal cells occurred in a chronological order. The order of occurrence was: tailless heads, diadem defect, nuclear vacuole defect, pyriform shaped heads, protoplasmic droplets, severely misshapened heads, acrosomal abnormalities, and the dag defect. In Study 2, a pooled sample of frozen-thawed semen from FI bulls in Study 1 was separated using swim-up separation, glass wool filtration, Percoll® density gradients (45/90% two-layer and 50/70/90% three-layer), and Sephadex®

filtration. Glass wool filtration was basically without effect and will be excluded from further discussion. All other separation techniques improved motility from the initial sample at the expense of spermatozoal harvest. The subtle head abnormalities were unaffected by separation for all techniques and spermatozoa with nuclear vacuoles on normal shaped heads were enriched; however, head abnormalities were removed with increasing frequency as the severity of the head distortion increased. In Study 3, frozen-thawed ejaculates with a high concentration of spermatozoa with nuclear vacuoles (>20%) were separated using 45/90% Percoll® and 45/100% Percoll® density techniques. The greater the number of nuclear vacuoles per sperm head, the more efficient the removal of such sperm. The separation of spermatozoa with subtle head distortion as well as spermatozoa with nuclear vacuoles on normal shaped heads by density separation, would allow further assessment of the importance of these abnormal sperm to decreased fertility and increased embryonic mortality.

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CHAPTER I.
PREFACE AND OBJECTIVES

The focus of the studies outlined in this dissertation are several fold. The first study (Chapter III) was conducted to produce specific morphological spermatozoal abnormalities that could be used in future studies (Chapters IV and V). These abnormal spermatozoa are produced and ejaculated by bulls with full scrotal insulation as was shown by Vogler et al. (1993). Vogler et al. (1993) surmised, retrospectively, that a 48-h full scrotal insulation resulted in a chronological ejaculation of specific morphological abnormalities which was due to a disruption of the stage of spermiogenesis in which each cell was located at the time of insulation. Also, we compared the effect of partial scrotal insulation (vascular cone area only) on spermatozoal quality with that of full scrotal insulation. Skinner (1981) found that an increased fat deposition in the area of the pampiniform plexus in obese bulls increased the number of abnormal spermatozoa in the ejaculate. The increased fat deposition in the scrotal neck was related to an increase in scrotal surface temperature which reflects both subcutaneous and deep testicular temperature (Coulter et al., 1988). This study was conducted to more fully understand the role of the vascular cone on testicular thermoregulation and heat dissipation from this region.

The use of full scrotal insulation on bulls to produce specific abnormal spermatozoa has developed into a good model for testing these abnormal spermatozoa further. The second study (Chapter IV) was undertaken to determine if abnormal spermatozoa, particularly those spermatozoa with subtle distortions of the sperm head, could be removed by conventional semen separation techniques. The use of semen containing abnormal spermatozoa for insemination is associated with male-related embryonic mortality and low embryo quality (DeJarnette et al., 1992; Saacke et al., 1992). In addition, it appears that only the more subtle

abnormal forms can participate in fertilization (Howard et al., 1993; Saacke et al., 1988).

In the second study those spermatozoa with subtle distortions of the sperm head were unaffected by any separation technique used, and those spermatozoa with the nuclear vacuole defect were enriched. Therefore, we were encouraged to separate those particular spermatozoa with the nuclear vacuole defect because they have been shown to be associated with male-related embryonic death (Miller et al., 1982; Saacke et al., 1992). Thanks to the cooperation of Dr. John Chandler (Louisiana State University) and the use of image analysis systems we have found a positive relationship of spermatozoal head area to the number of nuclear vacuoles per sperm head. Thus, we speculated that spermatozoa with nuclear vacuoles were less dense than spermatozoa without nuclear vacuoles. In the third study (Chapter V) density separation techniques were used to determine if spermatozoa with the nuclear vacuole defect on otherwise normally shaped heads could be removed.

CHAPTER II.
LITERATURE REVIEW

Spermatogenesis

Spermatogenesis is defined as the sum of the transformation that result in formation of spermatozoa from spermatogonia while maintaining spermatogonial numbers (Amann, 1981) and requires a duration of 61 days in the bull (Johnson et al., 1994). There are two subdivisions of spermatogenesis, spermatocytogenesis and spermiogenesis. Spermatocytogenesis is the mitotic and meiotic divisions from spermatogonia to round spermatids and requires 44 days (Johnson et al., 1994). Spermiogenesis is the morphological transformation of the round spermatid to the elongated mature spermatozoon, requiring another 17 days (Johnson et al., 1994).

Spermatogenesis occurs within the seminiferous tubules of the testis. These tubules are divided into two compartments, the basal compartment and the adluminal compartment. The basal compartment contains the spermatogonia. The stem cell (undifferentiated) spermatogonia are diploid cells that divide mitotically giving rise to differentiating spermatogonia which undergo a series of mitotic divisions terminating in the production of primary spermatocytes. The first meiotic division results in the production of secondary spermatocytes from the primary spermatocytes. Once the primary spermatocyte reaches the leptotene stage of prophase (meiosis I) it will pass through the Sertoli-Sertoli cell junctions into the adluminal compartment of the seminiferous tubule. The pachytene stage of prophase (meiosis I) is the longest stage of prophase requiring 10.8 d in the bull (Johnson et al., 1994). During this stage the primary spermatocytes are most susceptible to damage and degeneration due to high testicular temperatures

(Setchell, 1982). The secondary spermatocytes are relatively short lived (1.9 d) and then undergo the second meiotic division forming spermatids. The spermatids contain the haploid number of chromosomes and undergo morphological transformations to produce the mature spermatozoa, a process requiring 17 d in the bull (Johnson et al., 1994). The changes that occur during spermiogenesis include condensation of nuclear chromatin thus increasing nuclear density and change in nuclear shape; spreading of the acrosome over the anterior nucleus and formation of the tail (Setchell, 1982).

Amann (1981) described the cycle of the seminiferous epithelium as the series of cellular associations within the germinal epithelium in which only certain cells or stages in their development are found in association with each other. Each cellular association is comprised of four or five major types of germ cells. Each type of cell within the association represents a generation. Several different cellular associations or stages in the cycle of the seminiferous epithelium have been identified. The number of stages are dependent upon how critically the microscopist evaluates the identifying changes (usually in the spermatid). There are two major schemes classifying the stages of the cycle of the seminiferous epithelium for the bovine (Amann, 1962; Berndston and Desjardins, 1974). The scheme set by Amann (1962) consists of eight stages of the cycle of the seminiferous epithelium. The criteria for classification is based on gross, morphological changes in the spermatids and the presence of primary and secondary spermatocytes and specific differentiated spermatogonia. Berndston and Desjardins (1974) used a more rigid criteria to classify 12 stages of the cycle of the seminiferous epithelium. These stages are based almost entirely on changes in the acrosomal cap of the developing spermatid. In the bull, one complete cycle of the seminiferous epithelium, i.e., the time required for one full generation of germ cells to be released from a given point in the seminiferous tubule and all stages to occur at that point require 13.5 days. Thus, the entire

process of spermatogenesis, from stem spermatogonia to the release of mature spermatozoa, requires approximately 61 days or approximately 4.5 cycles of the seminiferous epithelium (Johnson et al., 1994).

Testicular Thermoregulation

Regulation of testicular temperature below normal body temperature is necessary for proper spermatogenesis to occur (Coulter, 1988; Blasquez et al., 1988; Hafez, 1987, Waites, 1970 for review). Testicular temperature is maintained by special physiological mechanisms of the scrotum, the pampiniform plexus and to a lesser extent the external cremaster muscle (Blasquez et al., 1988; Waites, 1970). The scrotum is thin, lacks subcutaneous fat, and has a thin sheet of smooth muscle, the tunica dartos, which adjusts scrotal position in response to changes in ambient and body temperature. The contraction of the tunica dartos muscle can be maintained for extended periods. The scrotum also has blood vessels near the skin surface to enhance heat loss (Kastelic and Coulter, 1993). The pampiniform plexus contributes to thermoregulation by a counter-current heat exchange of the arterial and venous system in the neck of the scrotum (Waites, 1970). The extent of the counter-current heat exchange depends on the magnitude of the temperature gradient between the body and the scrotum. Cooling of the blood in the pampiniform plexus occurs if the returning venous blood is cooler than the incoming arterial blood and the incoming arterial blood can also lose heat by radiation through the skin of the scrotal neck surrounding the pampiniform plexus (Coulter and Kastelic, 1994; Kastelic and Coulter, 1993). The external cremaster muscle is similar to the tunica dartos muscle in that it can also contract, pulling the testis closer to the body; however, this muscle is striated and cannot maintain the testis in the contracted position for long periods (Kastelic and Coulter, 1993).

Bulls that have an inability to thermoregulate the testis have a propensity to produce morphological abnormalities upon exposure to increased ambient temperature (Cassady et al., 1952; Johnston et al., 1963; Meyerhoeffer et al., 1985; Skinner and Louw, 1966; Wildeus and Entwistle, 1983). Optimally, bovine testicular temperature is maintained at 34.6°C, which is approximately 4°C lower than core body temperature (38.6°C) (Waites, 1970 for review). The normal scrotal surface temperature gradient ranges from 4 to 6°C from the base to the apex of the scrotum (Coulter, 1988). The occurrence of primary (head) abnormalities increased and motility decreased as the uniformity of the temperature patterns was disrupted and the temperature gradients became less than 4°C (Coulter, 1988). The degree of spermatogenic trauma has been associated with the temperature increase and the duration of the increase caused by the testicular insult. A testicular temperature increase to 40°C results in decreased sperm production from a destruction or shedding of the germinal epithelium, especially the destruction of pachytene spermatocytes and spermatids (Setchell, 1982). In contrast, a mild thermal insult raising testis temperature to approximately 35°C can result in the production of abnormal spermatozoa without affecting sperm output (Vogler, 1993; Wildeus and Entwistle, 1983). In the ram, a mild testicular insult by scrotal insulation for 16 h per day for a total of 21 days increased the number of dead spermatozoa and decreased the percentage of motile spermatozoa without affecting sperm output (Mieusset, et al., 1992). The percentage of dead sperm from scrotal insulated rams (38.3±1.8%) increased from the same rams before insulation (17.3±3.2%) by d 15. On d 21, both the percentage of dead sperm and motility were affected by heat treatment (56.9±7.2% and 17.0±6.2, respectively) when compared to the same rams prior to heating (17.3±3.2% and 79.1±10.5%, respectively). Studies conducted in our lab using full, but mild, scrotal insulation resulting in testis surface temperatures of 34 - 36°C have shown a disruption in spermatogenesis, manifested by specific morphological abnormalities ejaculated

in a chronological order, but with no change in sperm output and little change in sperm viability (Vogler et al., 1991, Vogler et al., 1993).

Insulation of the counter-current heat exchange system of the pampiniform plexus by fat deposition in this area has been postulated to result in abnormally shaped cells, e.g., pyriform shaped heads (Barth and Oko, 1989; Skinner, 1981). Cook et al. (1994) recently found that an increase in the distance between the pampiniform plexus area and the scrotal skin is related to an increase in scrotal surface temperature. However, the extent to which spermatogenesis is hampered by insulation of the vascular cone region is unknown.

Infrared thermography has been used to assess scrotal surface temperatures. Infrared thermography is a noninvasive method of measuring scrotal surface temperature that provides a pictorial image for an object's radiated heat energy (Coulter, 1988). Subcutaneous and deep testicular temperatures and epididymal temperatures are closely associated with the scrotal surface temperatures (Coulter et al., 1988). Scrotal infrared thermograms from bulls with normal testicular thermoregulation have a uniform pattern of horizontal temperature bands across the scrotum (Coulter, 1988). The scrotal surface temperatures (SST) were warmer near the body and become cooler toward the apex of the scrotum with a temperature gradient of 2.8°C. However, subcutaneous (SQT) and intratesticular temperature (ITT) gradients from the top to the bottom of the testes were basically non-existent (.1°C and -.1°C, respectively), (Coulter and Kastelic, 1994). Intraepididymal temperature (IET) followed similar patterns as SST with a gradient of 2.3°C from top to bottom of the testis; however, temperatures were greater than SST. Intraepididymal temperatures were greater than ITT at the top of the testis but were lower than ITT at the bottom of the testis. Thus, the cauda epididymis (major extra-gonadal storage region for sperm) was the coolest organ in the scrotum, being 32.6°C. In a study to determine the role of the pampiniform plexus in thermoregulation, a partial scrotal insulation was placed around the

scrotal neck area and SST, SQT, and ITT were compared (Coulter and Kastelic, 1994). Partial scrotal insulation had little effect on scrotal surface temperature. An increase in ITT was found at all locations in the testis. The investigators relate these results found with scrotal neck insulations with that of lipid deposition over the vascular cone.

Stress and Stress Related Compounds on Spermatogenesis

Testosterone and follicle stimulating hormone (FSH) and luteinizing hormone (LH) are necessary for proper spermatogenesis to occur (Courot, 1988; de Kretser et al., 1990; Steinberger and Steinberger, 1973). Luteinizing hormone has an indirect effect on spermatogenesis through stimulation of the Leydig cells to produce testosterone (de Kretser, 1990). In adult rams, LH is necessary early in the differentiation of spermatogonia from reserve to renewing spermatogonia (Courot, 1988). Those spermatogonia more involved in an active process of differentiation and multiplication required testosterone activity and the development of B spermatogonia required FSH (Courot, 1988). The maintenance of spermiogenesis was dependent on both gonadotropins (FSH and LH) and testosterone (Courot, 1988). It would appear, from these works, that alterations in LH, FSH and testosterone could adversely affect the efficiency of normal spermatogenesis.

Stress has been shown to interfere with proper function of the reproductive system. Stress related hormones can influence sexual functions at the level of the brain by inhibition of GnRH secretion, at the pituitary by interference with GnRH-induced LH release, and at the gonad by altering the stimulatory effect of the gonadotropins on sex steroid secretion (Rivier and Rivest, 1991, for review). Many environmental factors such as increased environmental temperature, high humidity,

shipping and management problems have also been shown to increase stress hormone levels (Gwazdauskas et al., 1980, for review).

Stress imposed on bulls by inserting an electoejaculation probe into the rectum with and without electrical stimulation caused an increase in serum corticosteroid and progesterone followed by a decline in LH and testosterone (Welsh and Johnson, 1981). Liptrap and Raeside (1978) exposed boars to an estrus sow or to an aggressive boar and found a distinct rise in corticosteroid concentration with an immediate, but temporary, increase in the concentration of testosterone.

Stress-related compounds have been administered to bulls (Coulter, 1976; O'Connor et al., 1985; Thibier and Rolland, 1976), boars (Hahmeier et al., 1980; Juniewicz and Johnson, 1981; Liptrap and Raeside, 1975) and rams (Matteri et al., 1984) to determine their effects on LH (Matteri et al., 1984; Thibier and Rolland, 1976), testosterone (Hahmeier et al., 1980; O'Connor et el., 1985; Thibier and Rolland, 1976; Liptrap and Raeside, 1975), and sperm morphology (Coulter, 1976; O'Connor et al., 1985; Gwazdauskas et al., 1980). The effect of adrenalcorticotropin hormone (ACTH) administration on testosterone production and secretion is two fold. Studies have shown that corticosteroid concentration increased significantly after ACTH administration. The effect of ACTH administration on testosterone release was an initial stimulatory rise in testosterone followed by an inhibitory effect on testosterone (Hahmeier et al., 1980; Liptrap and Raeside, 1975). Johnson and associates (1982) also found a decrease in LH and testosterone and basal concentrations of testosterone were suppressed below control values after ACTH administration. Episodic secretion of both LH and testosterone returned to normal levels by 6 to 7 hr after serum concentration of ACTH had returned to basal levels. The investigators suggested from these results that ACTH-induced increases in serum corticosteroids suppress the episodic secretion of LH, resulting in a suppression of testosterone secretion by

the bull testis. Thibier and Rolland (1976) found a small peak in testosterone levels immediately after dexamethasone administration followed by a suppression of testosterone by four hours after injection along with a significant decrease in LH concentrations. The low levels of LH after injection of dexamethasone or ACTH may indirectly lower testosterone production and release. These results were similar to those found by Liptrap and Raeside (1978) for natural induction of stress.

Spermatozoal abnormalities increased following exogenous dexamethasone (Coulter, 1976) and ACTH (O'Connor et al., 1984) administration. Dexamethasone administration to bulls may have caused an increase in spermatozoa with nuclear vacuoles; however, a hormonal profile was not shown (Coulter, 1976). Similar increases in spermatozoa with nuclear vacuoles were found in control animals receiving injections of saline. Head abnormalities and protoplasmic droplets increased during the week of ACTH administration; however, the increases were relatively small in relation to a very marked depression of testosterone (O'Connor et al., 1984). The testosterone levels during the injection period decreased after 4 d of ACTH administration from 5.8±1.1 ng/ml (d 1 after injection), to .8±.1 ng/ml (d 5 to 7). The increases in head abnormalities and protoplasmic droplets were from .8±.1 and 4.2±.3% to 1.3±.2 and 5.8±.4%, respectively.

Semen Quality and Fertility

Semen quality is defined as being composed of spermatozoal viability and morphology as well as functionality resulting in the ability of spermatozoa to fertilize and sustain embryogenesis (Saacke et al., 1990). If seminal quality is low, then fertility can be compromised by failure of fertilization or embryonic development. Several investigators have concluded from breeding trials with high and low fertility bulls that both an inability of the sperm to fertilize and early embryonic death accounted for repeat breeding due to low fertility males; however,

embryonic death, almost alone, was the main cause of repeat breeding when high fertility bulls were used (Bearden et al., 1955; Kidder et al., 1953).

Infertility is not only a consequence of semen quality but also of the quantity of sperm in the inseminate. Salisbury and Van Demark (1961) proposed an asymptotic model to describe the relationship between spermatozoal quality and quantity. They stated that optimum fertility of the cow population can be increased to a threshold level by increasing the quantity of sperm with a certain (beneficial) characteristic. Once the threshold level is achieved, fertility cannot be improved further by numbers of sperm in the inseminate. If sperm numbers surpass threshold levels for that characteristic, no relationship is found between the semen characteristic and fertility; however, if sperm numbers are lower than threshold levels, usually, a direct relationship can be found between the characteristic and fertility or sperm numbers and fertility.

In support of this model, Sullivan and Elliot (1968) showed that greater numbers of progressively motile spermatozoa are needed to attain the threshold level for low fertility bulls than for high fertility bulls. Increasing numbers of spermatozoa in the inseminate cannot always ensure normal fertility of subfertile bulls. Saacke (1990) described two factors in semen quality that affect maximum fertility, i.e. compensable and uncompensable factors. The compensable factors fit the Salisbury and Van Demark (1961) model in that increasing sperm numbers improve fertility. If an inseminate has a high concentration of uncompensable semen factors, increasing sperm numbers will not improve fertility. The uncompensable semen factors have been associated with a disturbed spermatogenesis and abnormal spermatozoal production which in turn have been associated with male-related embryonic mortality (Saacke et al., 1994 for review). The uncompensable semen factors are of most concern to the artificial insemination (AI), embryo transfer (ET) and *in vitro* fertilization (IVF) industries

because many sperm in such semen are capable of fertilization but are incapable of sustaining the process of embryonic development.

Abnormal spermatozoa have been shown to reach the site of fertilization *in vivo* (Moore Smith et al., 1970; Saacke, 1990) and to penetrate ova *in vitro* (Howard et al., 1993; Kraznowska and Lorenc, 1983; Kot and Handel, 1987; Moore Smith et al., 1970). Such sperm may be the cause of male-related early embryonic death (DeJarnette et al., 1992; Miller et al., 1982; Saacke et al., 1992; Setchell et al., 1988). In the bovine, Saacke et al. (1990) evaluated the morphology of accessory sperm compared to the morphology of spermatozoa in the inseminate. In the accessory sperm population, they found an increase in morphologically normal shaped heads and a decrease in sperm abnormalities such as tapered and pyriform shaped heads. However, proportions of those spermatozoa with subtle deformities of the head or normal shaped heads with nuclear vacuoles were unchanged from the inseminate to accessory sperm. Saacke et al. (1992) compared the embryonic development of embryos from superovulated females bred with either normal semen or semen containing spermatozoa with nuclear vacuoles (38% crater defect and 19% diadem defect). They found that use of abnormal sperm resulted in a decrease in the proportions of excellent/good quality embryos and an increase in the proportions of fair/poor and degenerate embryos. This indicated that spermatozoa with the nuclear vacuole type defect may be a cause of male-related early embryonic death. Miller et al. (1982) compared the fertilization rates of a low fertility bull to seven control bulls using superovulated females. The semen for the low fertility bull had 80% spermatozoa with nuclear vacuoles. The fertilization rate was lower for this low fertility bull (18%) compared to the control bulls (72%). The pregnancy rates for the low fertility bull following a seven week natural and artificial insemination breeding trial were 5% and 8%, respectively. The use of this abnormal semen to inseminate eight superovulated females in two embryo transfer units resulted in a

41% fertilization rate. Of the 41% (30/73) fertilized ova 36.7% (11/30) were degenerate and considered nontransferable. Fifty-seven percent (11/19) of the transferred ova resulted in pregnancy. The difference between the fertilization rate and pregnancy rate may be due to embryonic mortality (Miller et al., 1982). Mieusset et al. (1992) compared pregnancy rates and embryonic loss to semen obtained from scrotally insulated rams and control rams. There was no difference in pregnancy rates between scrotally insulated and control (uninsulated) rams at 17 days after breeding; however, there were fewer ewes pregnant to treated rams at 65 days after breeding . This was interpreted as an indication of early embryonic loss.

Kot and Handel (1987) performed an *in vitro* fertilization study using abnormal mouse spermatozoa. Abnormal mouse sperm were capable of binding to the oocyte and this binding, of the most severely abnormal sperm, was tangential to the oocyte compared to perpendicular binding for normal and the subtlely abnormal sperm. Kraznowska and Lorenc (1983) used oocytes varying in vestments around the egg (i.e. zona pellucida free, cumulus free, vs intact, both vestments present) to evaluate binding and penetration of abnormal spermatozoa. Proportions of abnormal sperm reaching the oocyte vitelline membrane were lower in the intact oocyte, and the most severely abnormal cells were unable to penetrate the intact oocyte; however, they were able to bind the oocyte. The subtlely abnormal cells were able to penetrate the intact oocyte, however, to a lesser extent than normal cells. They concluded that the egg vestments were an important barrier to fertilization by abnormal sperm *in vitro*.

Howard et al. (1993) have performed some of the most recent and interesting studies on the ability of abnormal domestic cat spermatozoa to fertilize oocytes *in vitro*. The teratospermic males had fewer structurally normal spermatozoa in the ejaculate (33.3%) compared to normospermic males (70.7%) although the percent motility was not different in the two groups. The types of

abnormalities in the teratospermic samples consisted mainly of tail abnormalities and protoplasmic droplets. Both groups of semen were treated with either simple wash or swim-up separation for IVF. Swim-up separation enriched the percentage of normal sperm and percent motility. This will be discussed in more detail in a later section (Semen Separation). Spermatozoa from teratospermic males were able to penetrate the outer layer of the zona pellucida quite well compared to that of normospermic males (79.7% vs. 98.8%); however, they were less able to penetrate through the zona pellucida or reach the perivitelline space (2.7% vs. 14.9%) than spermatozoa from normospermic males. The abnormal spermatozoa were able to bind to the zona pellucida (29.2%) but were unable to reach the perivitelline space (0.0%). In spite of the fact that the spermatozoa appeared structurally normal after swim-up separation (66.5%) compared to spermatozoa of the pre-separated sample (28.6%), they were still less able to bind and penetrate the zona pellucida as compared to spermatozoa from normospermic males. Howard et al. (1993) concluded that there were unknown factors associated with the structurally normal spermatozoa from the abnormal ejaculate that could not be overcome by swim-up separation and that the zona pellucida is an effective filter to abnormal spermatozoa; however, morphological evaluation of these spermatozoa was on fixed cells using phase-contrast microscopy at 1000x magnification. The subtle abnormalities such as spermatozoa with nuclear vacuoles could not be evaluated with this microscopic technique and could explain the unknown factors of these spermatozoa resulting in decreased binding and penetration of the oocyte.

Spermatozoal morphology is an important component of semen quality and appears to play an important role in the uncompensable semen traits. An abnormality of concern to the AI, ET and IVF industries is the nuclear vacuole defect because ejaculates used for breeding and having high concentrations of these spermatozoa have lower fertilized ova and reduced embryo quality than from

normal ejaculates (Miller et al., 1982; Saacke et al., 1992). The nuclear vacuole defect has been extensively studied in the bull (Coulter, 1975; Coulter, 1976; Coulter et al., 1978; Larson and Chenoweth, 1990; Miller et al., 1982; Saacke et al., 1992), boar (Truitt-Gibert and Johnson, 1980), stallion (Johnson and Hurtgen, 1985), and human (Baccetti et al., 1989). This defect has been described as depressions or blisters at points on the sperm head (Johnson and Hurtgen, 1985). Single nuclear vacuoles are often located juxtaposition to the apical ridge or at the equatorial region of spermatozoa (Coulter et al., 1978). The presence of two or more of these invaginations at the equatorial region is specifically termed the "diadem defect" and can occur before or after head shape is established in spermiogenesis (Vogler et al., 1993). Similarly, random nuclear vacuoles can be associated with normal or abnormal head shape (pyriform defect) (Coulter et al., 1978). The nuclear vacuole and diadem defects are associated with impairment of testicular thermoregulation (Vogler et al., 1993).

Other abnormalities associated with defects in spermatogenesis are the pyriform defect, tapered heads, knobbed acrosome and decapitated heads all of which are associated with decreased fertility (Baccetti et al., 1989; Barth, 1986; Barth and Oko, 1989; Blom, 1962; Blom and Birch-Andersen, 1970; Lorton et al., 1983) and embryo quality (DeJarnette et al, 1992). The pyriform defect has been shown to occur during heat stress (Vogler et al., 1993) and in obese bulls due to fat associated with the inguinal region and the scrotum (Barth and Oko, 1989; Skinner, 1981). The pyriform shaped head can range from slightly to severely pear-shaped and is thought to be generated during the elongation process of the spermatid (stages II to IV of the eight stage cycle of the seminiferous epithelium, Amann, 1962). Tapered heads are similar to pyriform shaped heads (Barth and Oko, 1989). They range in severity and are thought to be generated during the same stages of spermiogenesis. Acrosome defects are caused from irregular spreading of the acrosomic granule during spermiogenesis (Saacke et al., 1968).

The decapitated head defect can be caused by a defect in the implantation fossa and basal groove just prior to spermiation (Baccetti et al., 1989; Blom and Birch-Andersen, 1970). An increased incidence of detached heads is due to testicular degeneration or inflammatory conditions of the seminal vesicles, ampullae and epididymides (Barth and Oko, 1989) and to impairment of testicular thermoregulation (Vogler et al., 1993). In the case of impaired thermoregulation of the testes, this is thought to occur during stage VIII of spermiogenesis (Vogler et al., 1993).

Semen Separation

In vivo, insemination with semen having a high percentage of morphologically abnormal spermatozoa resulted in lowered fertility and poor embryo quality and(or) increased male-related embryonic mortality; however, it was not known whether fertilization was by normal or abnormal spermatozoa (DeJarnette et al, 1992; Miller et al., 1982). *In vitro*, abnormal spermatozoa are capable of fertilizing oocytes (Kraznowska and Lorenc, 1983). The removal of abnormal spermatozoa from semen samples should be a high experimental priority, to provide a means to determine that morphologically abnormal spermatozoa are directly responsible for embryonic mortality and if they are, to identify the major contributing forms such that results of IVF or sperm injection techniques can be optimized. Past research has shown that cryopreserved semen is the most convenient source of spermatozoa for *in vitro* fertilization (Parrish et al., 1986). However, because the survival rate of frozen-thawed semen is relatively low, methods of viability enhancement have been developed to increase the concentration of motile sperm capable of fertilization and to remove dead or weakened sperm, cellular debris, and seminal fluid (McClure et al., 1989).

Current spermatozoal separation techniques are presented in Table 2.1. Human spermatozoa have been most frequently used to evaluate the efficiencies of these separation systems basing success on spermatozoal motility, morphology or fertility of the separated samples. The following is a description of each separation system.

Swim-Up Separation

Swim-up separation attempts to mimic *in vivo* selection of motile spermatozoa by cervical mucus. This system is also a relatively simple method of separation; however, it requires an extended incubation time (~1 h). An aliquot of semen is placed under a layer of medium. The most viable spermatozoa will swim-up into the overlying medium. After a one hour incubation period, the medium is removed to the interface (Parrish et al., 1986). This method yields a significant increase in percent spermatozoal motility and proportions of normal morphology from the initial sample with a significant decrease in sperm numbers (Berger et al., 1985; Howard et al., 1993; Katayama et al., 1989; Le Lannou and Blanchard, 1988). This method is among the least efficient at recovering all the motile sperm because many motile spermatozoa do not swim-up and remain in the lower layer.

Glass Wool Filtration

Glass wool filtration columns consist of loosely packed glass wool in the base of a column (Jeyendran et al., 1986) . If the glass wool is too tightly packed, no sperm will be able to traverse the column. On the other hand, if the glass wool is too loosely packed, dead sperm and debris will pass through the column into the filtrate (Jeyendran et al., 1986). Sherman et al., 1981, suggested that damage to the membrane and acrosome of some spermatozoa can be caused by the filtration system; however, there was still an increase in the percent motile (an increase ranging from 9 to 29%) and normal cells in the filtrate (an improvement ranging from 0 to 11%).

Table 2.1. Current spermatozoal separation techniques.

Separation Technique	Species	Literature	Mot	Morph	Fert
Swim-up	Human	Akerlof et al., 1987	+	+	N
		Berger et al., 1985	+	N	-
		Katayama et al, 1989	+	N	+
		LeLannou and Blanchard, 1988	+	+	N
		McClure et al., 1989	+	-	-
		Mortimer, 1994	+	N	N
		Serafini et al., 1990	-	N	-
		Tanphaichitr et al., 1988	+	N	+
	Cat	Howard et al., 1993	+	+	-
	Bull	Parrish et al., 1986	N	N	-
Glass Wool	Human	Katayama et al., 1989	+	N	+
		Jeyendran et al., 1986	+	N	N
		Rhemrev et al., 1989	+	N	N
		Sherman et al., 1981	+	+	N
Sephadex	Human	Byrd et al., 1994	-	--	+
	Bull	Graham et al., 1976	+	N	N
		Graham et al., 1978	+	N	N
		Graham and Graham, 1990	+	+	+
		Landa et al., 1980	+	N	N
Percoll Density Gradient	Human	Barthelemy et al., 1992	+	+	N
		Berger et al., 1985	+	N	+
		Bolton and Braude, 1984	+	+	N
		Byrd et al., 1994	+	+	+

		Forster et al., 1983	+	+	N
		Gellert-Mortimer et al., 1988	+	+	N
		Gorus and Pipelers, 1981	+	N	N
		LeLannou and Blanchard, 1988	+	+	N
		Lessley and Garner, 1983	+	+	-
		McClure et al., 1989	+	+	+
		Mortimer, 1994	+	N	N
		Pardo et al., 1988	+	N	+
		Pousette et al., 1985	+	+	+
		Rhemrev et al., 1989	+	N	N
		Serafini et al., 1990	-	N	+
		Tanphaichitr et al., 1988	+	N	+
	Boar	Grant et al., 1994	+	N	+
Nycodenz Density Gradient	Human	Gellert-Mortimer et al., 1988	+	+	N
		Mortimer, 1994	+	N	N
		Serafini et al., 1990	-	N	+

Mot = Motility
Morph = Morphology
Fert = Fertility
- = No effect
+ = Positive effect
-- = Negative effect
N = Not evaluated

Sephadex Filtration

Sephadex® filtration (G 15) has been used for the separation of bovine spermatozoa (Graham, et al., 1978; Graham and Graham, 1990; Landa et al., 1980) and human spermatozoa (Byrd et al., 1994) for assessing semen quality and recovering viable spermatozoa. The Sephadex® column retains spermatozoa by complex binding forces between the Sephadex® and spermatozoal plasma membrane. Separation is not based on motility but rather on the (-) charge of the glycocalyx of the spermatozoal plasma membrane and that of the Sephadex (Landa et al., 1980). The dead or non-viable spermatozoa have no plasma membrane with a net negative charge therefore they are trapped by the negatively charged Sephadex. The filtration time takes approximately 15 to 20 min. Graham and Graham (1990) added 0, 25, 50, 75, and 100% freeze-killed bovine spermatozoa to Sephadex® columns. The percent motile cells improved after filtration for all percentages of dead spermatozoa added both pre-freeze and post-thaw. In the sample with 75% freeze-killed sperm, the motility was lower than the 25 and 50% freeze-killed samples with less sperm recovered. The 100% freeze-killed sample had very few sperm passing through the column. The ability of abnormal spermatozoa to pass through the column was also evaluated and will be discussed in more detail in a later section.

Percoll® Discontinuous Density Gradient Centrifugation

Percoll® is a medium composed of colloidal silica particles coated with polyvinylpyrrolidone (PVP). Polyvinylpyrrolidone is used to minimize the interaction of the colloidal particles with biological material, to stabilize the colloid against freezing and thawing, and also to stabilize the colloid against the addition of salts (Rickwood, 1984). The density of 100% Percoll® is 1.13 g/ml and the osmolarity is 20 mOsM. A 90% stock solution is made isotonic by a 9:1 solution of the 100% Percoll® with 10x concentration of an isotonic medium of choice. This 90% Percoll® solution now has a density of 1.12 g/ml and an osmolarity of 290 to 300

mOsM. The lower density Percoll® layers are prepared by dilution of the 90% Percoll® with an isotonic medium of choice. The densities of the diluted Percoll® range from 1.02 to 1.13 g/ml. Some investigators consider the stock solution as 100% Percoll® (Barthelemy et al., 1992; Forster et al., 1983; Pardo et al., 1988).

There are several Percoll® gradient methods. One method is the two step method which requires centrifugation over a continuous gradient followed by a second centrifugation over a discontinuous density gradient. A one step multi-layer discontinuous density gradient and a one step two-layer discontinuous density gradient are two other methods frequently used. The two-layer method is the most popular method for separation of bovine (Mermillod et al., 1992), boar (Grant et al., 1994) and human spermatozoa (Byrd et al., 1994; McClure et al., 1989; Rhemrev et al., 1989; Tanphaichitr et al., 1988); however, the multi-layer gradient is used routinely for human spermatozoal separation (Barthelemy et al., 1992; Berger et al., 1985; Forster et al., 1983; Gellert-Mortimer, 1988; Le Lannou and Blanchard, 1988; Lessley and Garner, 1983; Mortimer, 1994; Pardo et al., 1988; Serafini et al., 1990).

The centrifugation technique is based on density gradient centrifugation or isopycnic centrifugation to separate subpopulations of spermatozoa based upon their specific gravities (Mortimer, 1994). The centrifugation is also thought to cause the spermatozoal head to align parallel with the direction of the centrifugal force. Those spermatozoa that are more progressively motile will move more readily toward the bottom of the tube than the dead or poorly motile sperm. Once the cells, normal and abnormal, and other cellular particles reach their buoyant densities they do not progress further (Lessley and Garner, 1983). Mortimer (1994) described the most dense spermatozoa as the most normal and functional and they are found in the pellet of the highest concentration of Percoll® following centrifugation. Cytoplasmic droplets and other cellular debris were found in the upper layers while dead and abnormal spermatozoa were found in the intermediate

layers. Normal spermatozoa as well as tailless heads were found in the bottom layer (Lessley and Garner, 1983; Bolton and Braude, 1984). The fact that normal spermatozoa were found in the most dense layer indicating they were the most dense cells is in disagreement with Foote et al., (1991) and Lindahl and Kihlstrom (1952) in which dead spermatozoa were found in the most dense layers and the normal cells were found in the intermediate layers of the particular medium used for separation. Because tailless heads were found in the most dense layer shows that Percoll does separate, to some extent by density; however, because dead spermatozoa were found in the intermediate layers, this does not agree with separation based solely on density. This could indicate that separation by Percoll density gradients is not only based on density characteristics of the medium and the spermatozoa but on other mechanisms which are unknown.

Barthelemy et al. (1992) used a multi-layer Percoll® gradient to evaluate spermatozoal characteristics. Motility increased in the 80% and 90% Percoll® layers from 42.5% in the initial sample to 77.2% and 83.05%, respectively. There was no improvement in normal spermatozoa following separation. Pardo et al. (1988) showed an improvement in sperm motility after separation from the initial sample for asthenospermic (deficiency in viability of sperm), 28.6% to 77.3%; oligospermic (deficiency of sperm), 29.6% to 74.2%; severe asthenospermic, 9.4% to 63.9%; and oligoasthenospermic (deficiency of viable sperm), 11.9 to 65%, semen samples. All types of samples had a decrease in the concentration of spermatozoa following separation. Forster et al. (1983) evaluated the characteristics of spermatozoa at each density layer following centrifugation. Motility increased from ~38% in the top layer (60% Percoll®) to nearly 90% in the bottom layer (100% Percoll®). The specific type abnormalities removed by Percoll® will be discussed in a later section.

Nycodenz® Discontinuous Density Gradient Centrifugation

Nycodenz® has not been used extensively for the separation of spermatozoa. Nycodenz® (5-(N-2,3-dihydroxypropylacetamido)-2,4,6-tri-iodo-N,N'bis(2,3- dihydroxypropyl)isophthalamide) is a derivative of tri-iodobenzoic acid. The carboxyl group is blocked by a covalent linkage to a dihydroxypropylamine group making it nonionic and very stable. It is readily stable in all aqueous media (Rickwood, 1984). Like Percoll®, Nycodenz® discontinuous density gradient centrifugation is an isopycnic centrifugation to fractionate subpopulations of spermatozoa based upon their specific gravities (Mortimer, 1994). Nycodenz® centrifugation improves motility (41 to 62%) with no decreases in abnormal forms(77.4 to 72%) (Gellert-Mortimer et al., 1988). On the contrary, Serafini et al., 1990 did not find improvements in motility. Foote et al. (1991), found dead spermatozoa were found in the most dense Nycodenz® layer (44%) with motile spermatozoa found in the intermediate Nycodenz® layers (23 to 25%). This also indicates that separation by Nycodenz® is based on density.

Comparisons of the Separation Systems (Viability and Fertility)

The separation techniques described above have been compared for viability and fertility to unseparated or simple washed semen and(or) to each other. When comparisons were made with swim-up separation and simple washed spermatozoa, swim-up separation yielded greater spermatozoal motility for teratospermic male cats, 90.0 vs. 64.2%, compared to simple washed samples (Howard et al., 1993). The motility for spermatozoa for normospermic male cats was not different from the initial sample. Normal cell proportions were greater after swim-up separation than simple washed, 66.5% vs. 28.6%, for the teratospermic males. Fertilization rates were not improved when swim-up separated spermatozoa were used from either normospermic or teratospermic males. In bulls, Parrish et al. (1986) did find an improvement in fertilization rates after swim-up separation compared to simple washed spermatozoa, 40% vs. 22%. In

humans, glass wool filtration yielded greater numbers of motile sperm/ml (8.7 x 10^6) than did swim-up (6.9 x 10^6) separation with motilities >85% from the initial semen sample of 36.9 x 10^6 motile sperm/ml with a motility of 54% (Katayama et al., 1989). Rhemrev et al. (1989), using human spermatozoa, showed that the recovery of motile sperm in normal ejaculates (motility >40%) increased from 57.2% to 78.7% and 72.1% for glass wool filtration and two-layer Percoll® gradient centrifugation, respectively. The recovery of motile sperm cells in below normal ejaculates (motility < 40%) increased from 31.4% to 73.6% and 57.1%, respectively. Jeyendran (1986) found similar results as Katayama et al. (1989) and Rhemrev et al. (1989) for glass wool filtration. In that study, sperm motility increased from 30% to 66% at the expense of sperm numbers (harvest). Both the two-layer (McClure et al., 1989; Tanphaichitr et al., 1988) and multi-layer (Le Lannou and Blanchard, 1992) Percoll® gradient centrifugation improved motility in oligospermic and asthenospermic ejaculates better than did swim-up separation.

Berger et al. (1985) compared the effect of simple wash, swim-up separation and Percoll® gradient centrifugation. Simple washed spermatozoa served as the control for this investigation. Both swim-up and Percoll® separation yielded higher motility than the simple wash, 62% and 88% vs. 44%, respectively. Motility following Percoll® separation was greater than both swim-up and simple washed spermatozoa. Spermatozoa following Percoll® separation penetrated more ova than either swim-up or simple washed spermatozoa, 36% vs. 14% and 18%, respectively. The low penetrability by swim-up separated spermatozoa is in agreement with Howard et al. (1993). Grant et al. (1994) evaluated boar spermatozoal motility for simple wash and Percoll® separation. They used computer-aided methods for sperm motion analysis (Hamilton Thorne Motility Analyzer, HTM). The motion traits measured by the HTM are straight linear velocity (VSL), defined as the straight-line distance between the beginning and the end of a track divided by the time elapsed; curvilinear velocity (VCL), which is the

total distance between each cell center of brightness position for a given cell during the acquisition divided by the time elapsed; path velocity (VAP), which is the total distance along the average path for each cell divided by the time elapsed; progressive motility defined as percent motility of cells having a certain speed and straightness (VAP>50 μ/s, STR>75%); and lateral head displacement (ALH), which is measured by computing twice the distance between center of brightness and the average position of this center of brightness and its four neighboring center of brightness points (Kruschwitz, 1987). The Percoll® system enhanced all velocity parameters measured. The acrosomal integrity of spermatozoa following both separation systems decreased with incubation time; however, spermatozoa from Percoll® separation exhibited higher acrosomal loss within the first hour than did spermatozoa from simple wash, 21.2 vs. 8.7%. Following Percoll® separation, spermatozoa fertilized more oocytes *in vitro* than spermatozoa from simple wash, 60.3% vs. 11.6%. The increased velocity after Percoll® separation is thought to play a significant role in zona penetration and subsequent fertilization (Grant et al., 1994).

Using another automated semen analysis system (CellTrak/S automated semen analysis system), Byrd et al. (1994) evaluated separation of fresh and cryopreserved human spermatozoa by Percoll® gradient centrifugation, Sephadex filtration, and simple washing. For the pre-freeze semen samples, there were no differences between the separation systems for percent motile cells; however, the spermatozoal velocity traits measured were lower following Percoll® separation than for simple washed spermatozoa and Sephadex filtration. These results were opposite of those found by Grant et al. (1994) for fresh extended boar spermatozoa. After cryopreservation, however, Percoll® separated spermatozoa had a higher percent motility than simple washed spermatozoa and Sephadex® filtration, 49% vs. 40% and 43%, respectively. The curvilinear velocity (VCL) of spermatozoa was higher following Percoll® separation than for simple washed

spermatozoa and Sephadex filtration, 67.0 vs. 58.0 and 63, respectively. Percoll® separated spermatozoa were also higher for straight line velocity (VSL) than the simple washed spermatozoa and Sephadex® filtration, 26.0 vs. 20.0 and 23.0, respectively. Sephadex® separated spermatozoa have a higher VSL than simple washed spermatozoa. The pregnancy rate, for the pre-freeze semen sample, was lower for Percoll® separated spermatozoa than for simple washed spermatozoa and Sephadex® filtration, 11.4 vs. 19.1 and 16.9%. Following Sephadex® filtration the percentage of normal cells decreased 47.3 to 32.7%. The specific abnormalities will be discussed in a later section.

Nycodenz® was compared to other separation systems in two investigations. Gellert-Mortimer et al. (1988) compared Nycodenz® gradient centrifugation to Percoll® gradient centrifugation. Both systems improved spermatozoal motility from the unseparated sample, ~38% to ~75%, in the most dense Percoll® layer (90% Percoll®) and ~41% to ~62%, in the 65% Nycodenz® fraction. At higher Nycodenz® densities, spermatozoal motility significantly decreased and the 100% Nycodenz® was not different from the initial sample. After 21 h of incubation, motility of spermatozoa in all Percoll® layers decreased to below 15%; however, there was no decrease in sperm motility in the 65% Nycodenz®. This decrease in spermatozoal motility may be an indication that the Percoll® system caused membrane damage to the cells. Serafini et al. (1990) also compared Nycodenz® and Percoll® gradient centrifugation, as well as swim-up separation. In contrast to Gellert-Mortimer et al. (1988) there was no difference in motility between Nycodenz® and Percoll® separated spermatozoa and spermatozoa following swim-up separation from the initial sample. In a fertilization trial using zona pellucida free hamster ova there were no differences in the number of ova penetrated between Nycodenz® and Percoll® separated spermatozoa. Swim-up separated spermatozoa penetrated fewer ova than spermatozoa from the other two systems.

Separation of Abnormal Spermatozoa

There have been few detailed analyses of morphological abnormalities, especially in bovine semen, for any of the techniques thus far described. Using human semen, McClure et al. (1989) showed an enrichment of normal spermatozoa from 41.7% to 49.7% using two-layer (47.5/95%) Percoll® gradient centrifugation; however, the types of abnormal sperm were not described. Similar results were found following a multi-layer (40 to 100%) Percoll® separation with an increase in normal spermatozoal morphology from 60% to 85% (Pousette et al., 1985®). Gellert-Mortimer et al. (1988) showed a reduction in the number of abnormal forms of spermatozoa following Nycodenz® separation. The types of abnormal sperm removed from the initial sample were mainly tertiary abnormalities (coiled tails). Le Lannou and Blanchard (1988) also showed an enrichment in normal morphology and nuclear maturity (nuclear stability) of human spermatozoa using a multi-layer (70/80/90/100%) Percoll® gradient centrifugation and swim-up separation. Nuclear maturity was measured using several techniques including: aniline blue staining to reveal lysine-rich nuclear proteins; ethidium bromide uptake for evaluation of DNA-DNP complex; nuclear swelling test using sodium dodecyl sulfate for evaluation of chromatin stability; and acridine orange staining for evaluation of DNA integrity. In this study, Percoll® proved more efficient at removing head abnormalities, while swim-up was more efficient at removing tail abnormalities. Percoll® also separated mature (stable) from immature sperm (unstable) more efficiently than swim-up separation based on the analine blue staining procedure. Percoll® density gradient separation is based on sperm densities when an equilibrium exists between the density of the spermatozoa and that of the gradient. Spermatozoa with normal morphology and having a homogeneous nucleus should pass through the lower density gradients into the higher level gradients. Whereas, spermatozoa with a morphologically abnormal head shape and having a coarsely granular nuclear appearance should remain in

the lower density layers. Forster et al. (1983) examined the effect of multi-layer (60/70/80/90/100%) Percoll® gradients on specific abnormalities and, more specifically, the density layer at which these spermatozoa were removed; however, the initial semen sample contained 78% normal spermatozoa. The number of normal heads increased from 78% to 93% in the bottom layer (100% Percoll®) with most of the abnormal cells being removed in the less dense layers. The mean motility in the 60% fraction was 38% and the mean motility in the 100% fraction was >85%. The motility in the unseparated sample was not reported. Because human semen is characterized by a morphologically diverse population of spermatozoa, many investigators do not classify specific types of abnormalities, i.e., most researchers classify human spermatozoal morphology by general gross head shape and/or tail defects. Although Forster et al. (1983) defined the type of spermatozoal abnormalities eliminated during separation, the concentration of abnormal spermatozoa in the initial semen sample was low. Byrd et al. (1994) compared Percoll® gradient centrifugation and Sephadex® filtration for removing abnormal human spermatozoa. Percoll® enriched the number of normal cells from the initial sample, 48.9 to 57.2%. This increase was predominantly due to a decrease in the percentage of tapered heads from 23.8 to 9.9%; however, there was no change in macrocephalic, amorphous head and amorphous tail defects. The percentage of normal spermatozoa decreased for Sephadex filtration from the initial sample, 47.3 to 32.7%. Sephadex® decreased the percentage of tapered heads from 22.7 to 6.7% but increased the percentage of amorphous tails from 15.3 to 44.0%. Howard et al. (1993) examined the effect of swim-up separation on teratospermic felids. Swim-up separation enriched the normal spermatozoa from 33.3 to 66.5%. The reduction in sperm abnormalities was in the percentage of sperm with coiled tails, bent midpieces, bent flagella and cytoplasmic droplets. The decrease in tertiary abnormalities was in agreement with Le Lannou and Blanchard (1988) in which tail abnormalities were better removed by swim-up

separation than Percoll® gradient centrifugation. Fertilization rates did not improve with improved morphology. The shape classification for human and cat spermatozoa did not have many equivalents with bovine spermatozoa, especially with head shape abnormalities. Graham and Graham (1990) used bovine spermatozoa, following scrotal insulation, to determine if morphology and fertility were improved following Sephadex® filtration. Spermatozoal abnormalities were more critically evaluated than those for human and cat spermatozoa. The classifications were detached heads, head shape (normal, narrow, narrow base, pear, macro, micro and other), acrosome (normal, swollen and lifted), midpiece, cytoplasmic droplet (without, proximal and distal) and tail (normal, bent, hairpin, coiled over and coiled under). Sephadex® filtration increased the percentage of normal shaped heads from 85.7 to 89.4%; detached heads from 8.3 to 15.2%; and normal acrosome from 49.7 to 58.0%. Pear shaped heads were decreased from 3.7% to 2.2%. Protoplasmic droplets and overall tail defects did not change; however, bent tails decreased from 4.1 to 1.3% at the same time as coiled over tails increased from 1.2 to 2.9%. This is in disagreement with Byrd et al. (1994) who reported normal shaped spermatozoa decreased, and tail abnormalities increased after Sephadex® filtration. The crater and diadem defects were not evaluated for any of these separation systems and because of their importance in embryonic death these cells must be evaluated.

Density of Spermatozoa

Salisbury et al. (1978) gave several specific gravities of spermatozoa found by different investigators ranging from 1.034 to 1.334. Lindahl and Kihlstrom (1952) measured the densities of bovine spermatozoa in first, second, and third ejaculates and measured the differences in densities of immature (with a cytoplasmic droplet), mature, and dead spermatozoa. The mean specific gravity

of spermatozoa of all ejaculates ranged from 1.240 to 1.334. The mean specific gravities for each ejaculate were 1.2867, first ejaculate; 1.2897, second ejaculate; and 1.2668, third ejaculate. The specific gravity of the second ejaculate was significantly greater than that of the third ejaculate but was not different from the first ejaculate. Immature spermatozoa had a lower specific gravity than the mature (intact) and dead (nonintact) spermatozoa. This was determined from sedimentation test using layers of specific gravity 1.22, 1.25 and 1.32. There were fewer immature cells in the sediment and more immature cells floating on all layers than mature and dead spermatozoa. The opposite results were found for dead cells. The mature spermatozoa were evenly distributed between floating and sedimenting for all layers. This indicates that the densities of the mature cells are evenly distributed within the range of specific gravities covered by the specific layers. Several other investigators have attempted to determine the specific gravity of bovine spermatozoa (Lessley and Garner, 1988; Oshio, 1988). These investigators used Percoll® for the separation medium. The density of Percoll® ranged from 1.02 to 1.13 g/ml. Cytoplasmic droplets and other debris were found in the upper portion of the gradient. Normospermic cells and tail fragments were found throughout the middle part of the gradient with detached heads and normal cells found in the lower portion of the gradient (Lessley and Garner, 1988). Oshio (1988) found similar results with normal spermatozoa being found in the fractions between 1.10 and 1.13 g/ml. According to Lindahl and Kihlstrom (1952), who found mature and dead sperm in media greater than 1.13, the specific gravity of bovine spermatozoa is much greater than 1.13 so the studies by Lessley and Garner (1988) and Oshio (1988) may not be accurately determining sperm density due to the limited density of the separation medium.

Although much work has been focused on the enrichment of viable morphologically normal sperm, it is clear that functional problems in fertility and embryonic mortality still exists. It is also clear that the subtle forms of

spermatozoal anomalies may be the most important to embryonic development and, therefore, deserve greater attention.

CHAPTER III.

EFFECT OF FULL AND PARTIAL SCROTAL INSULATION ON SEMEN QUANTITY AND QUALITY IN YOUNG HOLSTEIN BULLS

ABSTRACT

Thermal insulation (insult) was applied for 48 h to the entire scrotal area (full, n=9) or only to the scrotal neck (partial, n=9) of young Holstein bulls. Six bulls served as controls. Bulls ranged in age from 12 to 20 mo. Semen was collected at a frequency of 6 ejaculates per week (2 ejaculates in succession on Mon., Wed., and Fri.) for 7 wk. There was a 2 wk pre-experimental period at this collection frequency to stabilize extra gonadal sperm reserves. Sperm output, motility, acrosomal integrity, and morphology were compared for Period 1 (d -4, -2, and 0; where d 0 = first day of scrotal insulation) with Period 2 (d 3 to 7) and Period 3 (d 10 to 40). Spermatozoa ejaculated from full scrotal insulation bulls (FI) were different from control bulls and partial scrotal insulation bulls (PI) only during Period 3 of semen collection when their motility and acrosomal integrity were depressed and total sperm abnormalities were increased. Full scrotal insulation caused a reduced mean spermatozoal motility and acrosomal integrity during Period 3 compared to Period 1 from 76.5 to 62.4% (d 10 to 28) and 92.3 to 71.8% (d 10 to 31), respectively. Full scrotal insulation caused ejaculation of abnormal sperm during Period 3 from d 10 to 33 (with a peak on d 17 of 83.8%). Specific abnormalities associated with the full scrotal insulation occurred in the following chronological order during Period 3 (peak % and d of occurrence): tailless head sperm d 10 to 17, (27.6%, d 12); nuclear vacuoles/diadem d 14 to 26, (72.8%, d 17); pyriform shaped heads d 17 to 26, (8.7%, d 19); protoplasmic droplets d 19 to 33, (31.4%, d 24); severely misshapened heads d 21 - 24, (4.5%, d 24);

acrosomal defects d 24 to 31, (5%, d 28); and dag defect d 26 (5.6%). This study revealed that a relatively mild 48-h full-scrotal insulation causes specific types of abnormal sperm to be ejaculated in a chronological sequence along with a reduction in sperm viability. The same mild insult applied only to the scrotal neck was without effect.

Key Words: Holstein Bulls, Scrotal Insulation, Thermoregulation, Spermatogenesis.

INTRODUCTION

In pendulous scrotal males (e.g., bull and ram), regulation of testicular temperature, below normal body temperature, is necessary for proper spermatogenesis to occur (Coulter et al., 1988; Waites, 1970). Testicular thermoregulation is carried out by special physiological mechanisms of the scrotum, the pampiniform plexus, and, to a lesser extent, the external cremaster muscle (Blazquez et al., 1988; Waites, 1970). Bulls that have impaired testicular thermoregulation have a propensity to produce morphologically abnormal spermatozoa with or without effects on sperm output and viability (Cassady et al., 1952; Johnston et al., 1963; Meyerhoeffer et al., 1985; Skinner and Louw, 1966). Vogler et al., 1993, showed that a 48-h full, but mild, scrotal insulation caused specific morphological abnormalities which were associated with disruption of the specific stage of spermiogenesis in which the cell was located at the time of insulation. Sperm output and viability showed little change as morphological abnormalities increased. Skinner (1981) demonstrated that obese bulls with increased fat deposition between the vessels of the pampiniform plexus had higher proportions of abnormal spermatozoa in their ejaculates than bulls in normal condition. Increased fat deposition in the scrotal neck area caused an increase

in the distance between the pampiniform plexus area and the scrotal neck and was found to be related to an increase in scrotal surface temperature (Cook et al., 1994). Scrotal surface temperature is, in turn, positively related to the subcutaneous and deep testicular temperatures (Coulter et al., 1988). When a partial, but mild, scrotal insulation was placed around the scrotal neck, no increase in scrotal surface temperatures was found; however, deep testicular temperatures were increased (Coulter and Kastelic, 1994). The partial scrotal insulation was thought to mimic lipid deposition over the vascular cone which would hamper heat radiation from the spermatic artery to the scrotal surface in this region.

The aim of this study was to determine if restriction of thermoregulation using a partial (but mild) insulation of the scrotal neck affects spermatogenesis when compared to full scrotal insulation using the same insulation material.

MATERIALS AND METHODS

Animals and Semen Collection. Twenty-four Holstein bulls, ranging in age from 12 to 20 mo, were individually penned in a cold-housing, counter-slope barn. Collection of semen was by artificial vagina. Two ejaculates were collected in succession with each ejaculate preceded by a period of sexual preparation consisting of 2 false mounts separated by 2 min of active restraint.

Thermal Insult to the Testes. The purpose of the scrotal insulations was to partially or fully restrict testicular thermoregulation for 48 h. The full scrotal insulation was to mimic a mild, naturally occurring environmental interference to the entire testicular area as previously described by Vogler et al., 1991. The partial insulation was to mimic a layer of fat deposition around the pampiniform plexus interfering with the counter-current heat exchange system. Thermal insult of the testes was induced by enclosing the entire scrotum, including the neck, with a sack constructed of insulated material held in place by Velcro© Brand fasteners

and medical tape (Vogler et al., 1991). Thermal insult of the vascular cone area was induced by enclosing only the scrotal neck with a 2.5-cm strip of insulated material held in place with medical tape.

Scrotal sacks (Vogler et al., 1991) and strips were constructed from two layers of waterproof nylon taffeta filled with a 1-cm insulating layer of polyester batting. The layers were machine quilted together and then sewn into a sack or strip. The insulation was applied to the animal and then readjusted after 30 min to ensure complete coverage of the scrotum and/or neck up to the body wall. The insulation was placed loose enough not to interfere with circulation but tight enough not to be removed prematurely. Following final placement of the insulation, bulls were partially restrained by halter for the 48-h insulation period. Control bulls (without insulation) were restrained in the same manner. Bulls were monitored every 2 h for the first 24 h of insulation and every 4 h for the final 24 h to readjust insulation, if necessary, and assure dry bedding.

Experimental Design. Eight bulls were placed on the experiment at each of three different times of the year, specifically, January, May and September. The bulls were assigned to 3 treatment groups: full scrotal insulation (n = 3), partial scrotal insulation (n = 3) and control (n = 2). Semen was collected at a frequency of 2 ejaculates in succession 3 times per wk (Mon., Wed., Fri.). Collection at this frequency began 2 wk prior to initiation of the experiment in order to stabilize epididymal sperm reserves. Recording of semen data (viability, morphology and sperm output) began 4 d prior to the 48-h insulation period and continued until d 40 following application of the insulation. Semen from full insulation bulls was cryopreserved throughout the experiment and provided the abnormal semen used in the experiments outlined in Chapter IV.

Three different periods of semen collection were defined to more efficiently evaluate the effect of heat stress on epididymal spermatozoa (Period 2; d 3, 5, 7) and on testicular sperm undergoing spermatogenesis (Period 3; d 10 - 40). Period

1 (d -4, -2, 0) served as the pre-insult control. Scrotal insulation was placed on the bulls after semen collection on d 0. The duration of Period 2 (8 - 11 d) was based on estimates of epididymal transit time for spermatozoa in the bovine (Orgebin-Crist, 1962) and thus would account for ejaculation of spermatozoa in the epididymis during insulation.

Semen Evaluation. Each ejaculate was assessed for volume, concentration and percent progressively motile sperm immediately following collection. Volume was measured from the graduated 15 ml conical tube used as the collection vial (Fisher Scientific, Pittsburgh, PA). Concentration was determined using a spectrophotometer calibrated for bovine sperm using a hemocytometer. Progressive motility was estimated to the nearest 10% by averaging several microscopic fields at 250x magnification using a phase-contrast microscope equipped with a heated stage (37°C). Smears of neat semen were prepared for motility estimates by placing a drop of warm (37°C) 2.0% sodium citrate-egg yolk buffer (EYC) on a warm slide using a glass capillary tube. A wooden applicator stick was inserted into the mixed neat semen, then the tip of the stick with the semen was touched in the drop of buffer and mixed gently with the opposite end of the stick. A 22 mm x 22 mm warm cover slip was placed on top of the sample prior to evaluation. The two ejaculates were pooled, gently mixed, and a 100-µl aliquot was removed and placed in 1 ml Karnovsky's (1965) fixative in a 1.5 ml disposable microcentrifuge vial (Fisher Scientific, Pittsburgh, PA) and refrigerated until evaluated. Fixed spermatozoa were evaluated for morphological abnormalities and acrosomal integrity. For morphology, two counts of 100 cells were averaged. Cells with multiple abnormalities were only counted once; however, each particular abnormality was recorded to eliminate the preemption of one abnormality by another. Because all abnormalities are recorded in this manner the actual percentages of specific abnormalities could be greater than the total percentage of abnormal cells in the sample. Those spermatozoa that could

not be classified as having a particular abnormality were placed in the severely misshapened head category. The Percentage of spermatozoa with intact acrosomes was evaluated on the same aliquots and were declared intact based on the presence of an apical ridge (Saacke and Marshal, 1968). Two counts of 100 cells were averaged.

Statistical Analysis. Data were analyzed by the General Linear Models procedure (SAS®). Differences in means were determined using Tukey's procedure.

The model used for analysis of variance for morphology, intact acrosome, progressive motility and sperm output per treatment, collection period, day and season was as follows:

$Y_{ijklm} = \mu + S_i + T_j + ST_{ij} + B_{(ij)k} + P_l + PS_{li} + PT_{lj} + PST_{lij} + PB_{l(ij)k} + D_{(l)m} + SD_{i(l)m} + TD_{j(l)m} + STD_{ij(l)m} + e_{ijklm}$

Where:

Y_{ijklm} = percentage of initial motility, intact acrosome, abnormal sperm or sperm output per collection day (x 10^9);

μ = overall mean;

S_i= the fixed effect of the season i (i = Jan., May, or Sept.);

T_j = the fixed effect of treatment j (full, partial or control);

$B_{(ij)k}$ = the random effect of the bull k within season i and treatment j;

P_l = the fixed effect of period l (l = Period 1, d -4 to 0; Period 2, d 3 to 7; or Period 3, d 10 to 40);

$D_{(l)m}$ = the fixed effect of the day m within period l;

e_{ijklm} = random error.

Measurements for full insulation bulls were reanalyzed using the following model removing treatment from the model to eliminate variation do to treatment:

$Y_{iklm} = \mu + S_i + B_{(i)k} + P_l + PS_{li} + PB_{l(i)k} + D_{(l)m} + SD_{i(l)m} + e_{iklm}$.

The random effect of bull (k) and all interactions with bull served as error terms for the fixed effects. This study was repeated three times, once during each season to bridge a range in ambient temperatures.

RESULTS

Ambient temperature and relative humidity during the experiment ranged from -7.2°C to 28.3°C and from 47% to 100%, respectively (Data provided by WDBJ Weather Service, Roanoke, VA for Blacksburg, VA). The mean ambient temperature during the insulation period of each of the Seasons (1, 2, and 3) was 4.8, 17.9, and 20.7°C, respectively with relative humidities of 49.3, 79.0, and 97.0%, respectively. Mean testicular temperature obtained on full insult (FI) bulls at the caudal midpoint of the testis before removal of the sack was 35.5°C and ranged from 33.4°C to 36.9°C. The mean testicular surface temperatures for full insulation bulls within Season 1, 2 and 3 were 34.1, 35.8, and 36.6°C, respectively. Overall response of all bulls to the thermal insult are presented in Figures 3.1, 3.2, 3.3, and 3.4. Spermatozoa ejaculated from full scrotal insulation bulls (FI) had a lower percentage of motile cells and spermatozoa with intact acrosomes and a greater percentage of total sperm abnormalities than control bulls or partial scrotal insulation bulls (PI) during Period 3 only of semen collection; however, control bulls had a greater percentage of total sperm abnormalities than PI bulls during all Periods of semen collection and greater than FI bulls during Periods 1 and 2. All significant changes occurred during Period 3 (d 10 to 40) of collection. For FI bulls, mean percentage of abnormal sperm was above Period 1 levels from d 10 to 33 (Period 3), with a peak on d 17 of 83.8±7.0% (mean ±SE)(Figure 3.3a). Mean percentages of progressively motile sperm and acrosomally intact sperm were reduced in Period 3 ($P < .05$) when compared to Period 1 levels, from 76.5 to 62.4% (d 10 to 28) and 93.3 to 71.8% (d 10 to 31), respectively (Figure 3.1a,b).

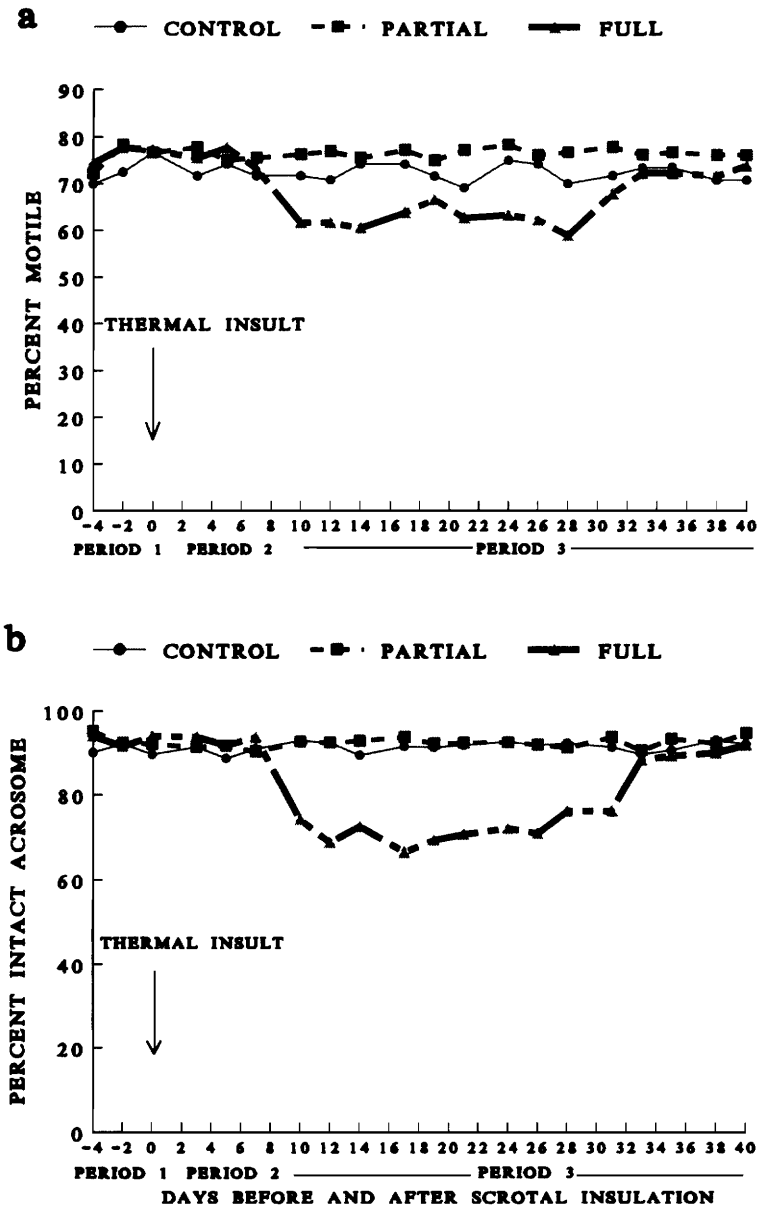

Figure 3.1 Percent sperm motility (a) and intact acrosomes (b) for control bulls and bulls treated with partial or full insulation before (Period 1) and after (Periods 2 and 3) 48-h scrotal insulation. Sperm motility and acrosomal integrity were depressed during period 3 only for FI bulls ($P < .05$); whereas, control bulls and PI bulls did not differ from throughout the experimental period.

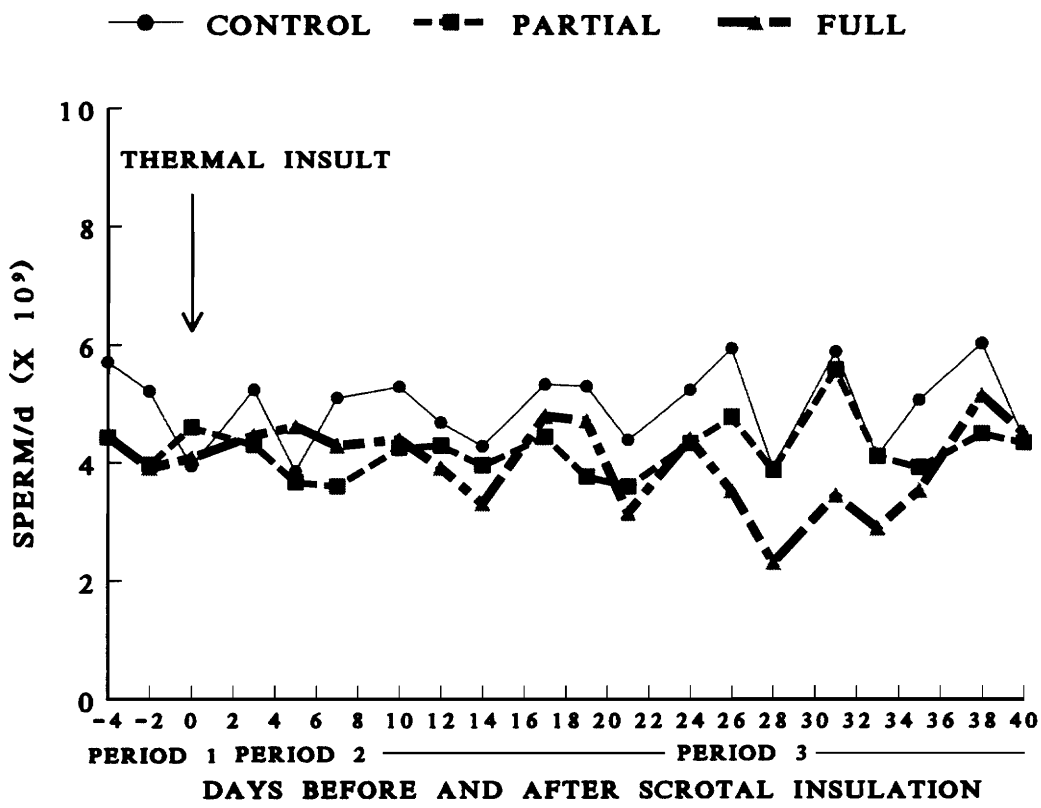

Figure 3.2. Variations in sperm output for control bulls and bulls treated with partial or full scrotal insulation before (Period 1) and after (Period 2 and 3) 48-h scrotal insulation. There were no differences in sperm output across periods for any treatment.

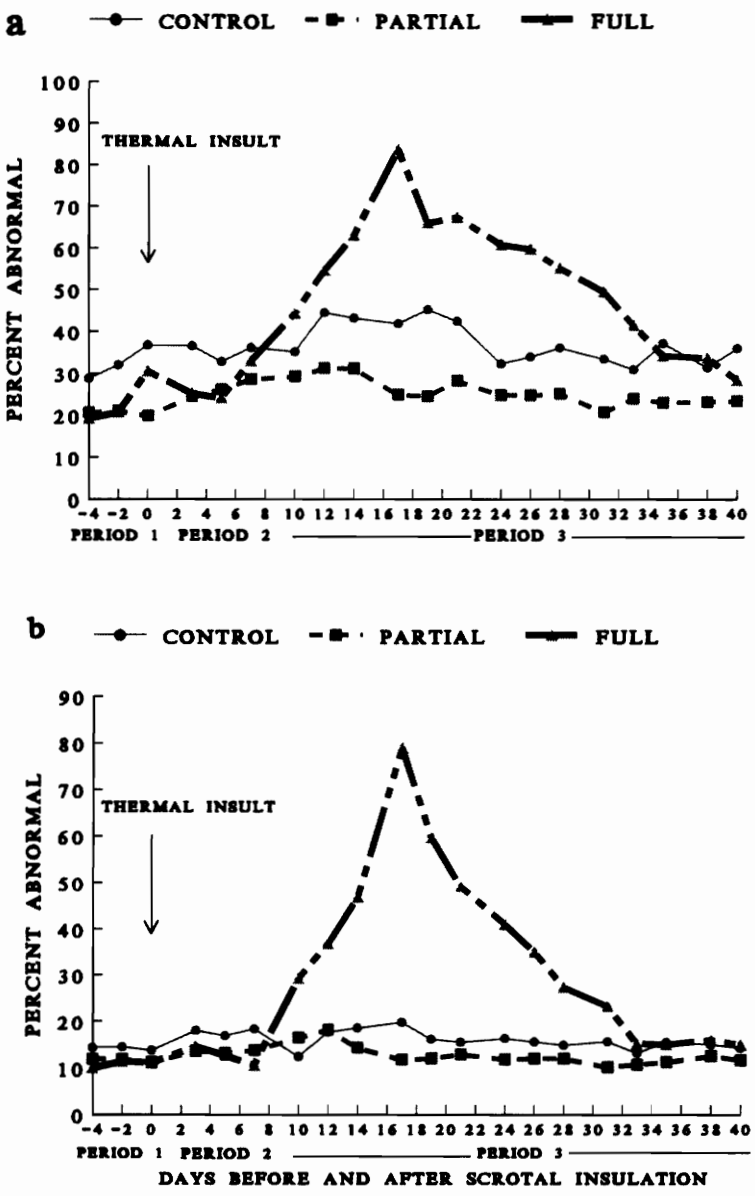

Figure 3.3. Percent total sperm abnormalities (a) and primary (head) abnormalities (b) for control bulls and bulls treated with full or partial scrotal insulation from before (Period 1) and after (Periods 1 and 2) 48-h scrotal insulation. Spermatozoal abnormalities did not change for control or PI bulls; whereas for FI bulls there was an increase ($P < .05$) in total and primary abnormalities during Period 3 only.

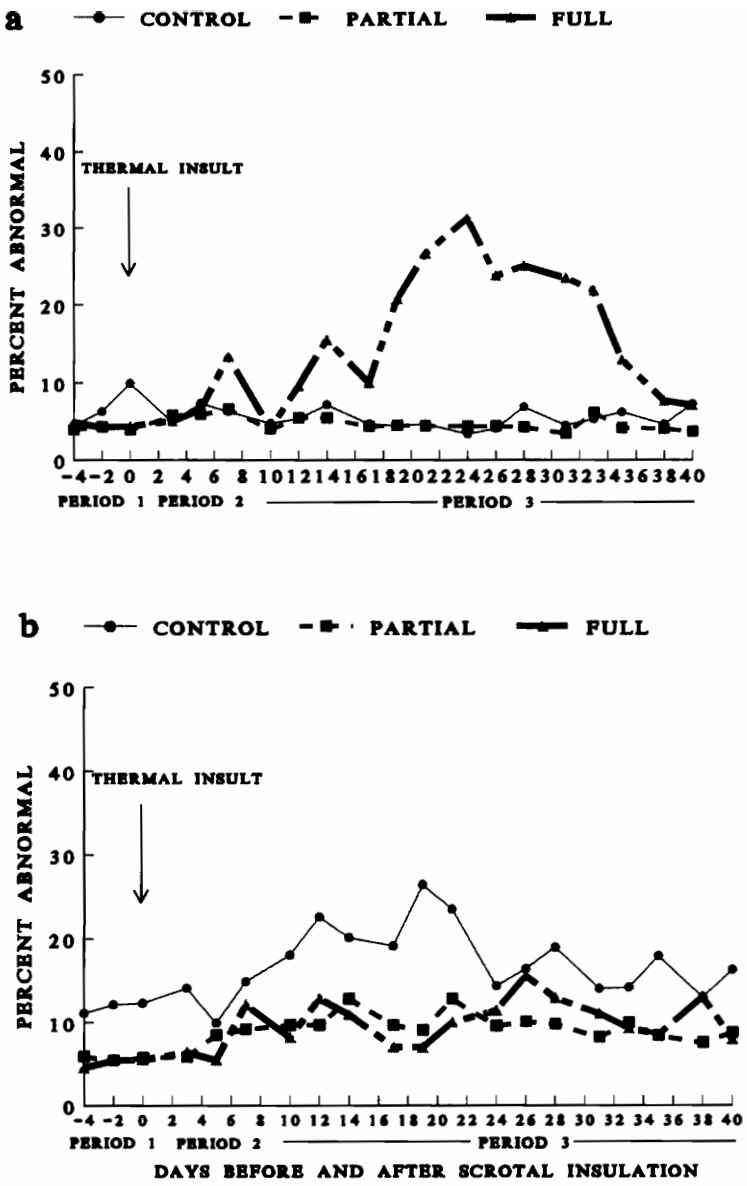

Figure 3.4. Percent secondary (protoplasmic droplets) abnormalities (a) and tertiary (tail) abnormalities (b) for control bulls and bulls treated with full or partial scrotal insulation before (Period 1) and after (Period 2 and 3) 48-h scrotal insulation. Secondary abnormalities were increased ($P < .05$) for FI bulls (Period 3) and tertiary abnormalities were above ($P < .05$) Period 1 levels on d 19 (Period 3) for control bulls only.

Sperm output (Figure 3.2) was unaffected by treatment. Motility, acrosomal integrity, and sperm output varied among replicates with the greatest adverse effect of full scrotal insulation on all variables occurring during Period 3 of Replicate 3 (Table 3.1).

Sperm abnormalities for all bulls were generally categorized into abnormal heads (primary), protoplasmic droplets (secondary), and abnormal tails (tertiary) (Figures 3.3b and 3.4). Ejaculates from FI bulls had a greater percentage of primary (head) abnormalities and secondary (protoplasmic droplets) abnormalities than PI bulls and Control bulls during Period 3 only of semen collection. There were no differences between treatments during Periods 1 or 2 of semen collection for primary and secondary abnormalities. The percentage of tertiary (tail) abnormalities was not different for PI and FI bulls across all Period; however, control bulls had a greater percentage of tertiary abnormalities than PI and FI bulls from the onset of the experiment. For FI bulls, the percentage of primary abnormalities was increased ($P < .05$) during Period 3 from d 14 to 26 with a peak on d 17 of 79.0±9.1% (Figure 3.3b). The percentage of secondary abnormalities increased ($P < .05$) during Period 3 from d 19 to 33 with a peak on d 24 of 31.4±11.1% (Figure 3.4a). The percentage of tertiary abnormalities (Figure 4.4b) did not change throughout the study. Differences between the seasons for normal morphology and primary, secondary and tertiary abnormalities are presented in Table 3.2. Again, bulls collected during Period 3 of Season 3 (September) had fewer normal spermatozoa and greater secondary abnormalities than bulls collected during Period 3 of Season 1 and 2 (January and May, respectively). Bulls collected during Period 3 of Season 3 had greater primary abnormalities than Period 3 of Season 1 but was not different from Period 3 of Season 2. There were no differences between Seasons for tertiary abnormalities.

Spermatozoal abnormalities consisting of the primary, secondary, and tertiary classification were further categorized into specific types. A chronological

Table 3.1 Means±SE for motility intact acrosome and sperm output within Periods across Season for full scrotal insulated bulls.

	Period	Season		
		1 January	2 May	3 September
Motility (%)	1	73.9±2.2	78.3±0.8	77.2±1.2
	2	76.7±1.4	77.2±1.2	72.8±2.2
	3	74.2±0.9[a]	69.8±0.9[a]	53.3±1.8[b]
Acrosome (%)	1	90.7±1.5	94.2±0.6	94.9±0.6
	2	90.6±1.2	93.4±1.0	94.7±1.1
	3	90.3±1.1[a]	82.2±2.4[a]	58.6±3.8[b]
Sperm Output ($\times 10^9$)	1	4.6±0.3	4.2±0.7	3.7±0.4
	2	5.0±0.6	4.9±0.3	3.6±2.7
	3	5.5±0.3[a]	3.6±0.2[b]	2.5±0.2[b]

[ab]Means in the same row with different superscripts differ ($P < .05$).

Table 3.2. Mean±SE for normal spermatozoa and primary, secondary and tertiary abnormalities within the Period of collection across Season for fulls scrotal insulation bulls.

Abnormality	Period	Season		
		1 January	2 May	3 September
Normal Morphology	1	82.9±1.6	78.8±1.2	77.7±2.3
	2	77.1±2.4	73.8±2.9	66.4±3.4
	3	68.8±5.0[a]	48.0±6.9[b]	23.8±8.2[c]
Primary	1	9.1±1.3	11.77±1.3	11.8±2.4
	2	13.5±2.5	10.5±3.5	14.1±2.6
	3	17.3±2.7[a]	36.2±3.7[ab]	51.4±5.1[b]
Secondary	1	3.7±1.6	3.6±0.5	6.4±0.8
	2	5.9±1.7	5.8±1.5	13.4±2.5
	3	5.2±1.3[a]	10.1±3.1[a]	36.9±7.6[b]
Tertiary	1	4.4±1.0	6.1±1.1	5.2±0.6
	2	4.0±0.6	11.4±2.7	8.8±2.6
	3	10.4±1.1	11.3±0.8	9.7±1.6

[a,b,c]Values in each row with different superscripts differ ($P < .05$).

order in the appearance of these specific abnormalities occurred in ejaculates following full scrotal insulation. The chronological order, as well as the peak and duration of appearance of each type is presented in Figures 3.5 to 3.8. The appearance of specific abnormalities began with tailless heads (Figure 3.5a) on d 10 with the peak on d 12, 28.2±8.4% (means ± SE for d 12). The diadem defect(Figure 3.5b) began on d 12 (4.6±3.7%) and peaked on d 17 (33.2±8.4%). The crater (nuclear vacuole) defect (Figure 3.6a) peaked on d 17 (39.6±7.3%) and remained above pre-insult levels until d 24. Pyriform shaped sperm (Figure 3.6b) appeared from d 17 to 24 and peaked on d 19 (8.7±3.1%). Protoplasmic droplets (Figure 3.7a) did not present a distinct peak, but were above pre-insult levels from d 19 to d 33, 31.4±11.1% (d 24). Severely misshapen heads (Figure 3.7b)increased above pre-insult levels d 21 with a peak on d 24 (4.5±2.4%). Acrosomal abnormalities (Figure 3.8a) increased during Period 3; however, they did not present a distinct peak. These abnormalities were primarily knobbed acrosomes. The dag defect (Figure 3.8b) peaked on d 26 (5.6±3.2%) and was the only specific tail abnormality to increase above pre-insult levels. Spermatozoa collected during Period 3, Season 2 and 3 were more affected by thermal insulation than those spermatozoa collected during Period 3, Season 1 (Table 3.3). All bulls varied in their response to the insulation.

Analysis of variance tables for all traits measured are shown in Appendix A, Tables 1 - 6.

DISCUSSION

Overall response to the partial and full scrotal insulation is described in Figures 3.1 to 3.4. In this experiment, insulating bulls around the vascular cone did not affect sperm motility, acrosomal integrity or morphology as did full scrotal insulation during Period 3 only of semen collection. These results are in

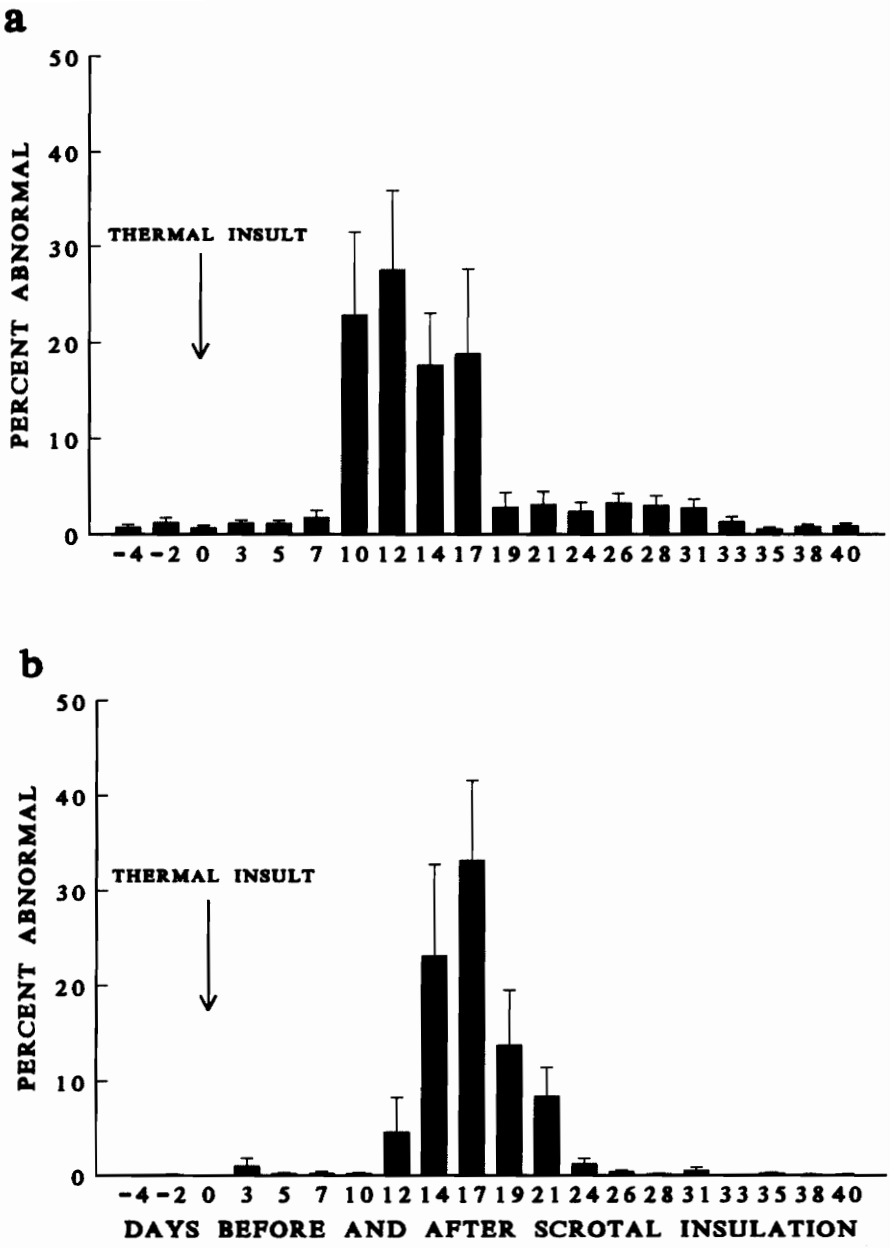

Figure 3.5. Means (±SE) for the level and duration of tailless head spermatozoa (a) and spermatozoa with the diadem defect (b) in ejaculates collected after 48-h full scrotal insulation.

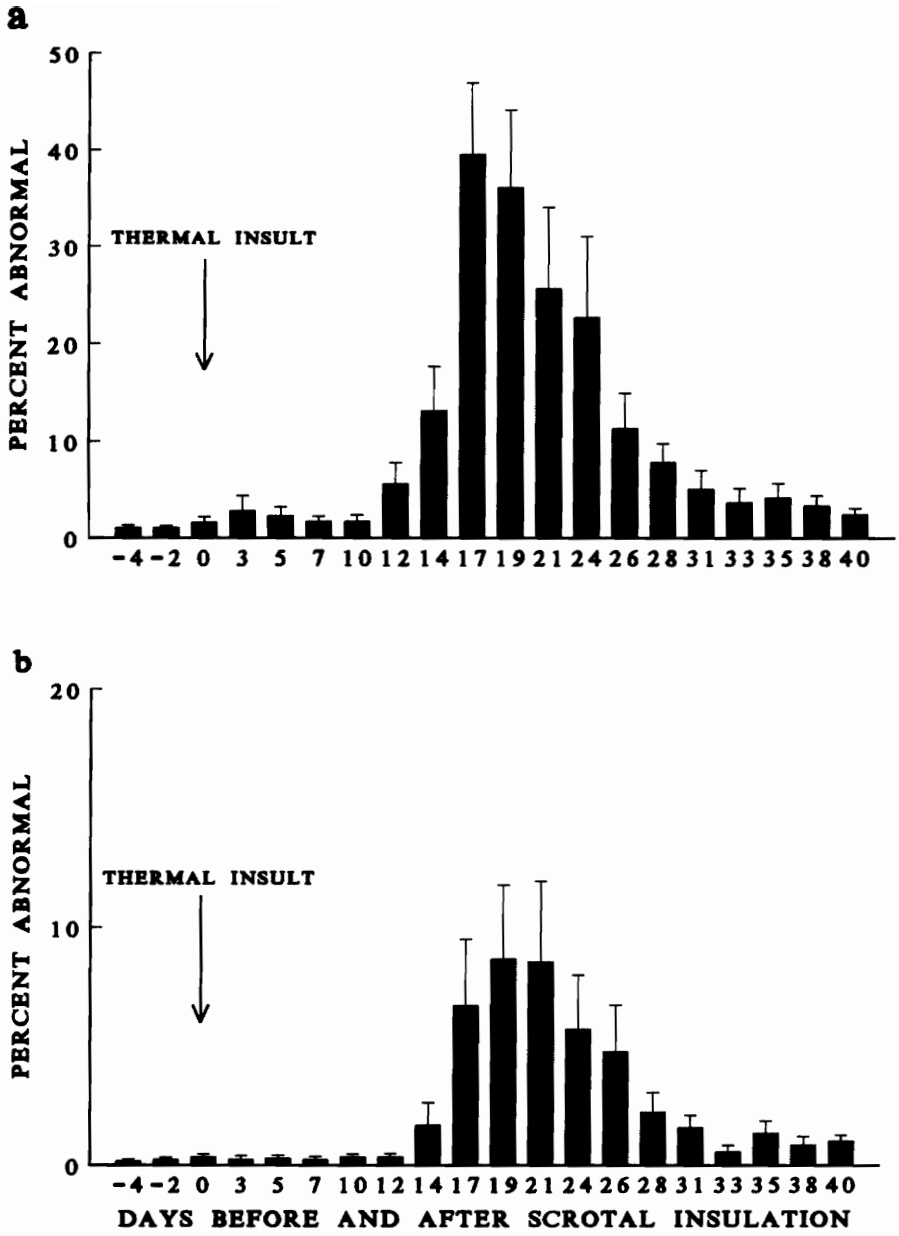

Figure 3.6. Means (±SE) for the level and duration of spermatozoa with the nuclear vacuole (crater) defect (a) and spermatozoa with pyriform shaped heads (b) in ejaculates collected after 48-h full scrotal insulation.

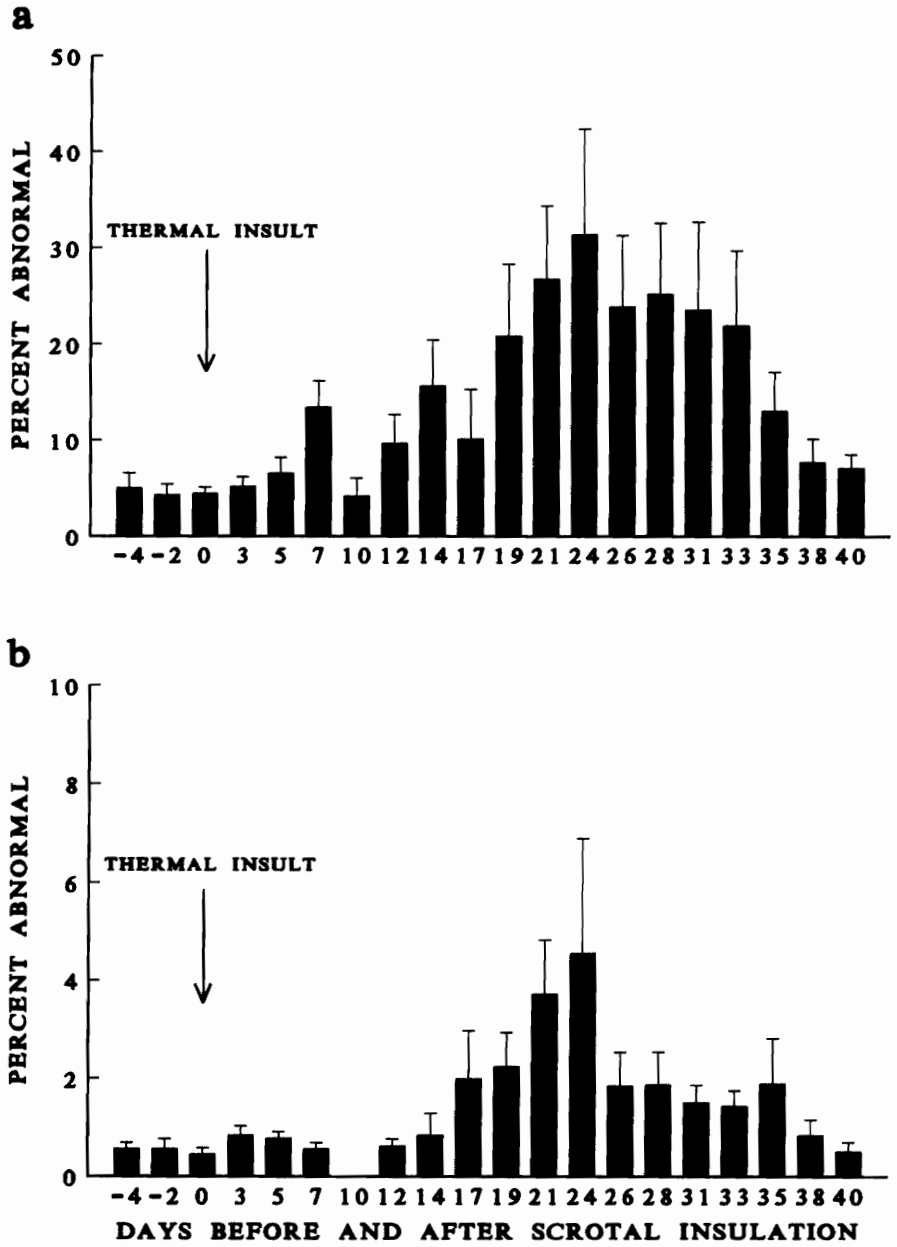

Figure 3.7. Means (±SE) for the level and duration of spermatozoa with protoplasmic droplets (a) and spermatozoa with severely misshapen heads (b) in ejaculates collected after 48-h full scrotal insulation.

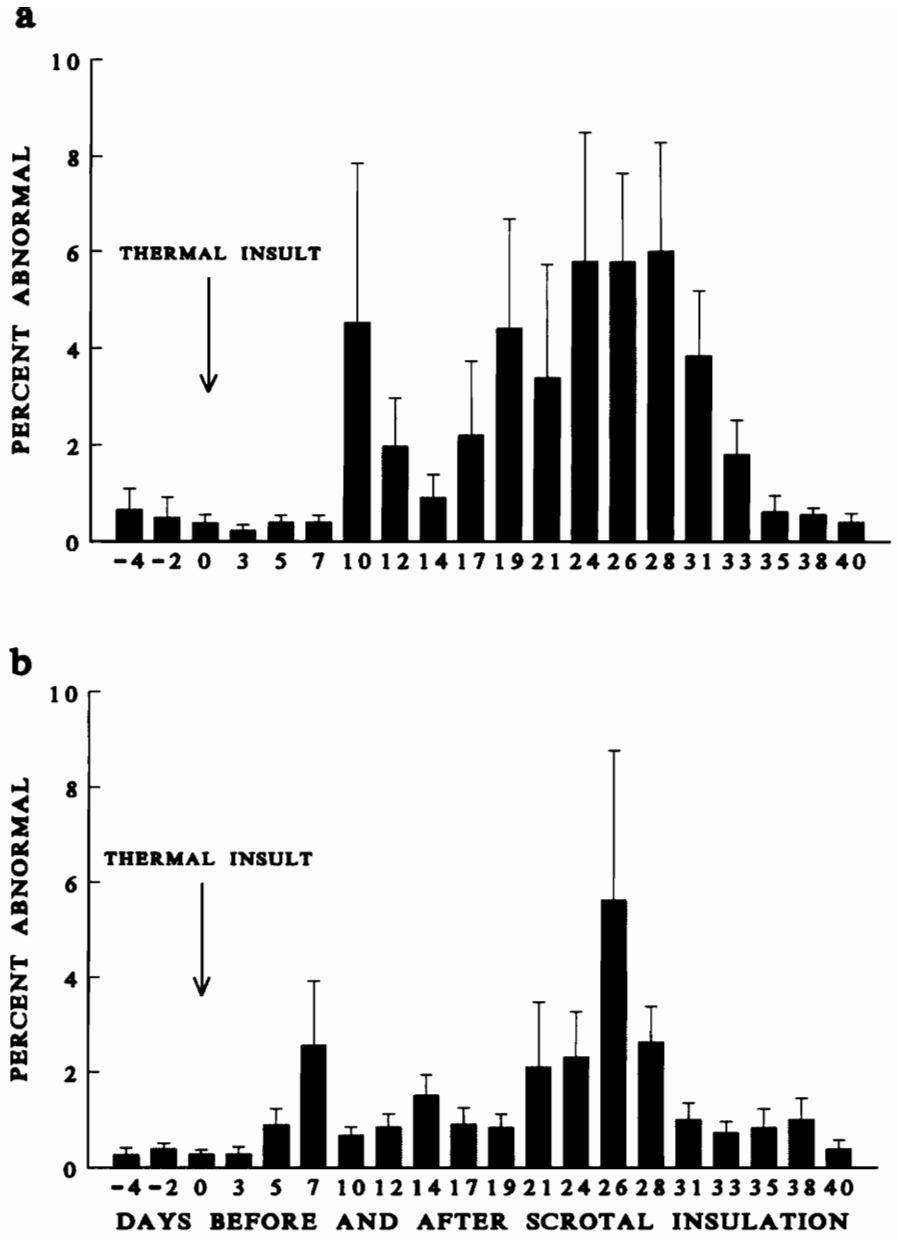

Figure 3.8. Means (±SE) for the level and duration of spermatozoa with acrosomal abnormalities (a) and spermatozoa with the dag defect (b) in ejaculates collected after 48-h full scrotal insulation.

Table 3.3. Means±SE for specific spermatozoal abnormalities ejaculated following full scrotal insulation within each Period across Season.

Abnormality	Period	Season		
		1	2	3
Tailless Heads	1	0.4±0.3	1.2±0.3	1.1±0.5
	2	1.4±0.4	1.1±0.3	1.5±0.7
	3	2.7±1.2	8.2±2.3	11.8±3.0
Diadem Defect	1	0.1±.1	0.0±0.0	0.0±0.0
	2	0.9±0.8	0.1±0.1	0.4±0.3
	3	4.0±2.0[a]	3.5±1.6[ab]	10.9±3.1[b]
Crater Defect	1	0.8±0.2	1.3±0.6	1.6±0.3
	2	2.3±1.5	2.6±1.0	1.9±0.5
	3	3.8±0.9[a]	13.6±2.8[b]	21.8±3.4[b]
Pyriform Shaped Heads	1	0.1±0.1	0.3±0.1	0.3±0.1
	2	0.2±0.1	0.2±0.1	0.3±0.2
	3	0.6±0.1[a]	2.8±0.8[b]	6.2±1.1[b]
Severely Misshapen Heads	1	0.2±0.1	0.7±0.1	0.7±0.2
	2	0.8±0.2	0.7±0.1	0.7±0.1
	3	0.8±0.2[a]	1.2±0.2[ab]	3.1±0.6[b]
Acrosomal Abnormalities	1	0.1±0.1	0.1±0.1	1.2±0.5
	2	0.2±0.1	0.2±0.1	0.6±0.2
	3	0.2±0.1[a]	2.6±0.6[a]	6.5±1.2[b]
Dag Defect	1	0.2±0.1	0.3±0.1	0.4±0.2
	2	0.4±0.3	2.1±1.4	1.2±0.4
	3	0.1±0.1	1.8±0.4	2.6±0.7

[ab]Means in the same row with different superscripts differ ($P < .05$).

agreement with Waites (1970) since the major thermoregulatory ability of the scrotum was functional and capable of dissipating heat. However, we feel that these results do not necessarily refute the response in increased pyriform-shaped sperm heads which Skinner (1981) reported for bulls with fat deposition around theinguinal region and scrotum in obese bulls. Perhaps, the mild insulation material used in this experiment was not as efficient in insulating the vascular cone as was fat (Cook et al., 1994) or that was necessary to create an effect as when used over the entire scrotum. Coulter and Kastelic (1994) found an increased intratesticular temperature following a partial scrotal insulation; however, a heavier insulation was used in their study and, unfortunately, they did not evaluate semen quality. Further research should be done in this area using a more stringent insulator which more closely mimics insulation caused by fat deposition in the scrotal neck area.

For control bulls, tail abnormalities were increased above Period 1 levels. Since all bulls were restrained during the 48-h period and tertiary abnormalities did not change for partial or full insult bulls, the change in tertiary abnormalities in control bulls was, undoubtedly, not due to the restraint. Control bulls had an unexplained greater percentage of tertiary abnormalities than either partial or full insult bulls from the onset of the experiment. No other measurement was affected in the control bulls.

Bulls treated with full thermal insult responded with a significant decrease in percent motility and acrosomal integrity, with the greatest change occurring in sperm morphology without affecting sperm output. Although bulls varied in their response to the FI, the greatest responses occurred during the third Season (Tables 3.1 to 3.3) when ambient temperature, relative humidity and, ultimately, testicular temperature were greatest. These results do not suggest any adverse effects of insulation during epididymal transport, Period 2 (d 3 to 7). However, a previous report (Vogler et al., 1991) suggests that viability of epididymal sperm are

adversely affected by a similar scrotal insulation to that used in the present study; however, the effect was only evident after semen had been cryopreserved. Sperm motility and acrosomal integrity decreased during Period 3 (d 10 to 40), as sperm abnormalities increased. These changes in morphology, motility and acrosomal integrity began 10 days after insulation during Period 3 suggesting interference with spermatogenesis. The onset of morphological abnormalities and reduced motility and acrosomal integrity on d 10 in this experiment occurred two days earlier than in a previous study (Vogler et al., 1993) and peaked on day 17, one day earlier than the same study. This was possibly due to an increased semen collection frequency in the present study with semen collection from bulls on Monday, Wednesday and Friday versus every three days in the previous study.

Full scrotal insulation increased sperm morphological abnormalities with the greatest increase in the percentage of primary (head) abnormalities during Period 3 having a peak level of 79.1% (d 17) and a duration above Period 1 levels from d 10 to 28 (Figure 3.3b). An increase in the percentage of secondary (droplet) abnormalities during Period 3 followed, reaching a peak level of 31.36% (d 24) with the duration above Period 1 levels from d 19 to 33 (Figure 3.4a). These results contradicted those found in a previous study (Vogler et al., 1993) in which no significant increase in secondary abnormalities occurred. The increase found in secondary abnormalities occurred in four bulls with testicular surface temperatures above 36°C at the termination of insulation. Three of these bulls were insulated during the third Season (September) when ambient temperatures reached 20.7°C with 97% relative humidity (Table 3.2). The fourth bull was insulated during the second Season. Tertiary (tail) abnormalities did not increase above pre-insult periods.

In this study and a previous study (Vogler et al., 1993) a specific chronological order of sperm abnormalities appeared in ejaculates during Period 3. The sequence of specific types and duration of abnormalities indicated a

possible interference with spermiogenesis. The possible cause of these abnormalities and the stages of the cycle of the seminiferous epithelium (8 stage cycle (Amann, 1962)) affected could be speculated upon. Tailless heads (appearing d 10 to 12) suggested an error in the implantation socket of the spermatozoa just prior to spermiation (Baccetti et al., 1989; Blom and Birch-Andersen, 1962). This abnormality possibly did not manifest itself until spermatozoa were activated by ejaculation because the tails, without heads, were often motile in the ejaculate. The diadem defect (appearing d 14 to 17) suggests deficiencies in chromatin condensation (Ballachey et al., 1986; Ballachey et al., 1988) during stage V to VII of the cycle of the seminiferous epithelium when the head shape was set because most diadem defects were on normal shaped heads. The crater defect (appearing d 17 to 24) and pyriform shaped heads (appearing d 17 to 24) occurred simultaneously suggesting problems with DNA condensation throughout the nucleus during the nuclear elongation process from round spermatids to elongated (stage I to IV of the cycle of the seminiferous epithelium). Protoplasmic droplets (occurring d 19 to 33) appeared in only 4 of 9 bulls and only in bulls with testicular temperatures above 36°C. Their origin is difficult to explain. The occurrence of severely misshapened heads (d 21 to 24) also suggested problems with head shape development during stages I to IV of the cycle. Acrosomal defects appeared throughout Period 3 but were greatest from d 24 to 28 suggesting alterations of the acrosomal cap on round spermatids during the cap phase of spermiogenesis (stages VII to VIII of the cycle previous to the earlier abnormal spermatozoa)(Vogler et al., 1993). The dag defect (d 26) was the only tertiary abnormality to increase and chronologically, the error could be predicted to occur on round spermatid during stage VIII of development (Barth and Oko, 1989). This was in agreement with the findings by Vogler et al. (1993) in that the dag defect was the only tertiary abnormality to increase. All bulls did not respond similarly in both the level and duration of the specific abnormalities which could be

the result of differing ambient temperature, relative humidities, and testicular temperatures within each Season. However, the type of abnormality and the order in which they occurred were similar for all bulls that responded to the insulation.

From this study we can conclude that the abnormalities which are associated with the full insulation are undoubtedly due to heat stress and not the restraint or environmental conditions under which the testes were insulated. This is apparent from the lack of response in the control bulls and PI bulls which received the same restraint and environmental conditions. This study and the previous study by Vogler et al. (1993) convince us that the use of full scrotal insulation on bulls is a good model for the production and ejaculation of specific morphological abnormalities in a chronological order that can be utilized for further experimentation.

IMPLICATIONS

A mild insulation of the testis, for a brief period (48 h), significantly alters sperm viability and morphology without affecting sperm output for 40 days post-insulation. The resulting temperature increase appears to affect young developing spermatids during different stages of spermiogenesis resulting in ejaculation of abnormal semen beginning 10 d after insulation until d 33 - 35. Although this experiment did not show an effect of partial insult around the neck of the scrotum, further research with an increased insult should be tested to mimic the effect of fat around this region in obese bulls.

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CHAPTER IV

EFFICIENCIES OF SELECTED SEMEN SEPARATION TECHNIQUES DESIGNED FOR *IN VITRO* FERTILIZATION

ABSTRACT

Three experiments were performed to critically evaluate the use of selected conventional spermatozoal separation techniques on cryopreserved bovine semen with respect to alterations in spermatozoal abnormalities and viability. Percoll® techniques evaluated in Experiment 1 were: 45/90% two-layer, 60/90% two-layer, 50/70/90% three-layer, and 45/60/75/90% four-layer Percoll® density gradients. In Experiment 2, the efficiencies of spermatozoal separation were compared for: swim-up, glass wool filtration, 45/90 two-layer Percoll® density gradient, and three-layer Percoll® density gradient. In Experiment 3, a Sephadex filtration system was studied. All Percoll® separation techniques (Exp. 1) improved sperm motility; however, acrosomal integrity was unaffected by Percoll® separation. The percentage of normal cells was improved by four-layer Percoll® separation. The percentage of secondary (protoplasmic droplets) abnormalities was reduced by all Percoll® separation techniques. The percentage of tertiary (tail) abnormalities was reduced by four-layer and 45/90% Percoll® techniques. The percentage of pyriform shaped heads was the only specific abnormality reduced and was reduced by all Percoll® techniques. The percentage of spermatozoa with the nuclear vacuole defect on otherwise normal shaped heads appeared enriched, however, not significantly so. The differences in the efficiency (sperm recovery) among the gradients were not significant. In Exp. 2, glass wool filtration was without effect. The percentage of motile cells was improved by both Percoll® techniques and Swim-up separation. No changes were found in acrosomal integrity. No technique

improved the percentage of normal cells nor reduced the percentage of primary abnormalities. Both Percoll® techniques and swim-up separation reduced the percentage of secondary abnormalities and the percentage of tertiary abnormalities was reduced by the 45/90% Percoll® technique. The percentage of pyriform shaped heads was reduced by both Percoll® techniques. The percentage of sperm with nuclear vacuoles on normal shaped heads was enriched by the three-layer Percoll® technique and Swim-up separation. The percentage of spermatozoa with tailless heads were enriched only by 45/90% Percoll® technique. Based on these results, further evaluations of techniques were based on degree of distortion in head morphology (subtle vs. distinct vs. severe). Findings were: the greater the distortion, the more effective the technique. Sephadex® filtration (Exp. 3) increased percentage motility and acrosomal integrity. Normal morphology percentage improved following Sephadex® filtration with no overall affect on the percentage of primary and secondary abnormalities; however, the percentage of tertiary abnormalities were reduced. The percentage of spermatozoa with nuclear vacuoles were enriched and the percentage of spermatozoa with pyriform shaped heads and short heads were reduced following Sephadex® filtration. Again, exclusion of abnormal spermatozoa was based primarily upon severity of the abnormality with subtle abnormalities being unaffected by any system. All differences noted were significant at $P < .05$.

Key Words: Bovine Spermatozoa, Morphology, Semen Separation.

INTRODUCTION

Abnormal spermatozoa have been shown to reach the site of fertilization *in vivo* (Moore-Smith et al., 1970; Saacke, et al., 1988; Saacke et al., 1994, for review), to bind ova *in vitro* (Kot and Handel, 1987), and to penetrate ova *in vitro*

(Kraznowska and Lorenc, 1983; Moore-Smith et al., 1970). insemination with semen containing abnormal spermatozoa has been associated with early embryonic mortality or low embryo quality (DeJarnette et al., 1992; Miller et al., 1982; Saacke et al., 1992; Setchell et al., 1988). In addition, it appears that only the more subtle abnormal forms can participate in fertilization (Howard et al., 1993; Saacke et al., 1988) The use of cryopreserved semen is the most convenient source of spermatozoa for *in vitro* fertilization (IVF) (Parrish et al, 1986). However, the survival rate of frozen-thawed semen is relatively low compared to fresh semen. Methods of spermatozoal motility enhancement have been developed to increase the concentration of motile sperm and remove cellular debris and seminal fluid (McClure et al., 1988). Conventional spermatozoal selection techniques for motility and morphology enhancement include swim-up separation (Byrd et al., 1994; Howard et al., 1993; Parrish et al., 1986), glass wool filtration (Katayama et al., 1989; Jeyendran et al., 1986; Rhemrev et al., 1989), Sephadex® filtration (Byrd et al., 1994; Graham and Graham, 1990), and Percoll® gradient centrifugation (Forster et al., 1983; Grant et al., 1994; Lessley and Garner, 1983). A detailed analysis and comparison of the most popular spermatozoal separation techniques have not been performed with bovine semen, especially on ejaculates with high concentrations of specific morphological abnormalities. Those abnormalities of particular concern are those with subtle distortions of the sperm head, including spermatozoa with nuclear vacuoles, that are implicated in male related embryonic death (Miller et al., 1983; Saacke et al., 1992).

The aim of these experiments was to evaluate the effect of different *in vitro* fertilization spermatozoal separation techniques on selected cryopreserved bovine semen containing a broad spectrum of types and severity of spermatozoal abnormalities. Semen for this study was pooled ejaculates produced by bulls collected at varying intervals after a 48-h full scrotal insulation.

MATERIALS AND METHODS

Semen Source and Preservation

Semen was collected from bulls following a 48 h full scrotal insulation (Chapter III). From each bull, ejaculates containing semen traits of interest (see Experimental Design) were pooled and prepared in the conventional manner (Robbins et al. 1976) for cryopreservation with clarified egg yolk-citrate extender containing 7% glycerol (vol/vol) at 50 x 10^6 sperm/ml. Extender was clarified by filtering first through a .45-µm Acrodisc filter (Gelman Sciences, Ann Arbor, MI) and then through a .2-µm Acrodisc filter. Semen was packaged in .5 ml French straws (Instruments de Medicine Veterinaire, L'Aigle, France), frozen 24±2 h after collection in static liquid nitrogen vapor for 10 min, and then plunged into liquid nitrogen. Straws were stored in liquid nitrogen until use.

Separation Techniques

Percoll® density gradients. A stock solution of Percoll® (90%) was prepared by mixing 45 ml Percoll® (Sigma Chemical Co., St. Louis, MO) with 5 ml of 10x (hypertonic) stock solution of sperm TALP (SPTALP), a modified Tyrode's medium (Parrish et al., 1986) (Table 4.1). The less dense Percoll® solutions were prepared by diluting the 90% stock Percoll® solution with isotonic SPTALP, without bovine serum albumin (Table 4.2) as presented in Table 4.3. The gradients were prepared by gently layering 2 ml of each solution from most dense to least dense in a 15 ml conical tube (Corning Inc., Corning, NY). Two tubes of each gradient were prepared and used for separation to accommodate the 2 ml of semen to be separated, i.e., 1 ml was placed on each of two gradients. The gradients plus semen were centrifuged at 700x g for 30 min (Mermillod et al., 1990). The

Table 4.1. 10x (hypertonic) stock solution of sperm TALP.

INGREDIENTS	STOCK CONCENTRATION	ml/100 ml
*KCl	1 M	3.090
*NaH_2PO_4	.1 M	2.920
*NaCl	80 mM	2.380
*HEPES	---	2.380
**$CaCl_2$	1 M	0.197
**$MgCl_2$	.1 M	0.394
**Lactic acid	---	0.184
**$NaCHCO_3$	---	104.5

* Add first then adjust pH to 7.3.

**For a stock solution of Percoll® (90%) mix of 90 ml Percoll® and 10 ml of 10X stock medium. Osmolarity should be between 280 - 300 mOsm.

Table 4.2. Components of sperm TALP medium (Parrish et al., 1986).

INGREDIENTS	UNITS	SPTALP
NaCl	mM	100.0
KCl	mM	3.1
$NaHCO_3$	mM	25.0
NaH_2PO_4	mM	0.3
Lactate (sodium salt)	mM	21.6
*$CaCl_2$	mM	2.0
*$MgCl_2$	mM	0.4
HEPES	mM	10.0
**Pyruvate	mM	1.0
**Bovine Serum Albumin	mg/ml	6.0
**Gentamicin	µg/ml	50.0

*Add last.
**Add on day of use.

Table 4.3. Components of Percoll® layers.

Percoll® Layer	90% stock Percoll® (ml)	Sperm TALP (ml)
45%	5.0	5.0
50%	5.56	4.44
60%	6.66	3.34
70%	8.34	1.66
75%	8.34	1.66

supernatant was removed by vacuum suction to .5 ml. The pellets from the two tubes of the same gradient were pooled and resuspended with 5 ml SPTALP and recentrifuged at 200x g for 10 min (Parrish et al., 1986). The supernatant was removed leaving 1 ml SPTALP and the sperm pellet. This wash was performed to remove any excess seminal fluid, egg-yolk, or Percoll® following the initial separation. When the interfaces were evaluated (Exp. 1) a 2 ml transfer pipet was used to remove semen at each interface separately. The equivalent interface semen and pellets from the two tubes were pooled.

Swim-up separation. Swim-up separation was accomplished by placing 1 ml of SPTALP in eight separate 15 ml conical tubes (Corning Inc., Corning, NY) followed by gently placing .25 ml of pooled semen beneath the SPTALP of each tube (Parrish et al., 1986). Eight tubes were used to accommodate the 2 ml of semen to be separated. Following a 1-h incubation at 39°C on a heated dry bath the top 0.85 ml of medium from each tube was removed and pooled. Separated semen was washed as described previously.

Glass wool filtration. Glass wool filtration was performed by packing 30 mg of glass wool microfiber (Pyrex brand wool filtering fiber, Corning Glass Works, Corning, N. Y.) into a 3-ml disposable syringe barrel, with the tip removed, to a depth of 3.0 mm (Jeyendran et al., 1986). The columns were rinsed repeatedly using SPTALP to remove loose glass wool fibers. Four glass wool columns were used to accommodate the 2 ml of semen. A .5 ml aliquot of semen was layered over the wet glass wool column and gravity filtered. Once filtration was complete the columns were rinsed with 2 ml of SPTALP to flush any remaining viable sperm that may be trapped in the column. The recovered sperm plus SPTALP were pooled and washed as described previously.

Sephadex® filtration. Sephadex® G 15 (40 - 120 µ particle size, Sigma Chemical Co., St. Louis, MO) columns were prepared as described by Graham et al. (1978) by placing a 3 - 4 mg pad of Johns-Manville Micro-Fiber, code 112,

glass wool (Compliments of Dr. Bo Crabo, Univ. of Minn.) into a 3-ml disposable syringe barrel, with the tip removed (Graham et al., 1978). The syringe was filled with a sodium citrate solution followed by the addition of .6 ml of a Sephadex® slurry and allowed to settle. The Sephadex® slurry was prepared by adding 20 g Sephadex® to 100 ml sodium citrate solution and allowed to swell at least 4 h. The column was prepared with a depth of 6 mm. Three milliliters of SPTALP were added to the column and allowed to drain. Another 3 ml of SPTALP were added to the column simultaneously with .5 ml of thawed semen. The column was rinsed twice with 2 ml SPTALP following semen filtration. Four columns were used to accommodate the 2 ml of semen to be separated, i.e., .5 ml of semen filtered on each column.

Semen Evaluation

Pooled pre-separation and post-separation aliquots of the pellet and the interface(s) were removed and evaluated for morphology, viability, and concentration/harvest. Motility was assessed using phase-contrast microscopy at 250x magnification on a heated stage at 39°C by placing a drop of extended or separated semen from a capillary tube on a warmed slide and covered with a warmed cover slip. A 100-µl aliquot was removed and placed in 1 ml Karnovsky's (1965) fixative. Morphology was evaluated on 200 fixed cells using differential interference contrast microscopy at 1250x magnification under oil. Cells with multiple abnormalities were counted once, but each abnormality on the cell was recorded in order to avoid preemption of one abnormality by another abnormality. The specific classification of severely misshapened heads was those spermatozoa that could not be classified as any other specific abnormality. Severity of head shape abnormalities were graded as normal, subtle, distinct, or severe. In this study, subtle was defined as those spermatozoa with only slight distortions in head

shape. The distinct classification included those spermatozoa with the classical distortions of the head (Barth and Oko, 1989) and the severe classification was defined as those spermatozoa with such severe distortions that the classical abnormalities were difficult to recognize. Acrosomal integrity was evaluated on the same 200 cells. A cell was declared intact based on the presence of an apical ridge (Saacke and Marshal, 1968). A hemocytometer count was taken to determine spermatozoal concentration/harvest.

Experimental Design and Rationale

Experiment 1. In preliminary studies it was found that spermatozoa from a highly abnormal ejaculate accumulated at the interface of a 45/90% Percoll® gradient, the method of choice in many laboratories for separating sperm used in IVF. Therefore, several gradients including 45/90% two-layer, 60/90% two-layer, 50/70/90% three-layer gradient, and 45/60/75/90% Percoll® gradients were tested to determine the viability and morphology of spermatozoa in the pellet of the 90% gradient and at the interface(s) of two-layer and multi-layer Percoll® gradients. Cryopreserved semen from bulls collected before and after 48-h scrotal insulation was used (Chapter III). One ejaculate from each of six bulls was selected based upon their morphological and viability characteristics. The viability of the semen selected was ≥40% after thawing. The abnormalities of interest included pyriform shaped heads, nuclear vacuole defects, tailless heads, severely misshapened heads, protoplasmic droplets, and the dag defect.

Two centrifugation tubes of each gradient were used to ensure enough sperm for evaluation following separation. One milliliter of semen was gently placed on top of the least dense layer of each gradient. After separation a 100-µl aliquot of sperm from each interface and pellet was placed in Karnovsky's (1965) fixative. The remaining sperm mix was placed in a 2.5 ml capped tube and

maintained for a minimum of 30 min at 39°C in a heated dry bath for motility analysis. The density differences between the gradient layers were visibly detected, in a separate gradient, using density marker beads (Pharmacia Biotechnology, Uppsala, Sweden) of varying colors with densities of 1.062, 1.075, 1.087, 1.098, 1.119, 1.138 g/ml. This experiment was replicated 3 times.

Experiment 2. Cryopreserved semen from bulls collected after a 48-h full scrotal insulation (Chapter III) was used to compare the efficiencies of different semen separation techniques relative to the resulting spermatozoal viability, morphology, and yield when compared to Percoll®. One ejaculate from each of five bulls was selected based upon morphological and viability characteristics. The abnormalities of interest were pyriform shaped heads, nuclear vacuole defect, tailless heads, severely misshapened heads, protoplasmic droplets, and the dag defect. The thawed semen was pooled and then separated using four separation techniques, specifically, 45/90% two-layer Percoll® density gradient (PERC45), 50/70/90% three-layer Percoll® density gradient (PERC3), swim-up separation (SU), and glass wool filtration (GW). Two Percoll® gradients were used because spermatozoal yield was not evaluated in Exp. 1 due to the manner in which the layers were removed. An unseparated sample served as control.

Two milliliters of semen were used for each separation system. A 100-µl aliquot was removed and fixed as described in Exp. 1 pre- and post-separation. The remaining sperm mix was placed in a 1.5 ml capped microcentrifuge tube (Fisher Scientific, Pittsburgh, PA) and placed in a heated dry bath at 39°C. Motility was evaluated within 30 min of separation. The experiment was replicated five times.

Experiment 3. This experiment was performed due to the poor results found following glass wool filtration. The type of glass wool used in the previous experiment was different from that recommended in the present experiment for Sephadex filtration (Personal communication, Dr. Bo Crabo, Univ. of Minn).

Sephadex filtration has been developed as an assay for spermatozoal viability (Graham et al., 1978) as well as a method of abnormal spermatozoal separation (Graham and Graham, 1990). Cryopreserved semen from the same ejaculates described in Exp. 1 was selected. Sephadex filtration was used for separation of spermatozoa in the same manner as described above. This experiment was replicated five times.

Statistical Analysis

Data were analyzed by the general linear models procedure (SAS®). The model used for analysis of variance for morphology, viability and concentration/harvest per treatment was as follows:

$Y_{ij} = \mu + T_i + R_j + e_{ij}$. Where:

Y_{ij} = percentage of motility, intact acrosome, morphology, and concentration/harvest;

μ = overall mean;

T_i = the fixed effect of treatment i (separation techniques);

R_j = the fixed effect of replication j;

e_{ij} = random error.

Differences between treatment means were determined by Tukey's analysis.

RESULTS

Experiment 1

The densities of the Percoll® solutions were 1.123 g/ml, 90%; 1.114 g/ml, 75%; 1.109 g/ml, 70%; 1.106 g/ml, 60%; 1.098 g/ml, 50%; and 1.095 g/ml, 45%. The initial, pre-separated, semen sample served as the control for all separation

techniques. It consisted of 51.4±1.4%, motile cells; 50.5±3.0%, intact acrosomes; 19.5±.7%, normal cells; 12.9±1.19%, primary (head) abnormalities; 17.9±.9%, secondary (protoplasmic droplets) abnormalities; and 8.1±1.4%. tertiary (tail) abnormalities. Each of these general classifications of abnormal spermatozoa was composed of specific abnormalities as shown in Table 4.4. Severity of head distortions was classified as subtle, distinct and severe. Alterations in the percentage of primary abnormalities are dependent upon the individual specific abnormalities and the severity of these head distortions.

Motility and acrosomal integrity were less than 25% at each interface for all Percoll® systems and the percentage of normal cells were less than 20%. Morphology and viability were not back to or above the initial semen samples until the sperm reached the 90% Percoll® layer. The spermatozoa in the pellet (bottom) of the 90% Percoll® layer were the cells evaluated in order to compare the gradients in this experiment.

The initial semen sample differed from the Percoll® separated sample for several variables measured (Figures 4.1 to 4.3). All Percoll® techniques improved ($P < .05$) the percentage of motile cells from the initial value of 51.4±1.4% to 70.0±0.0% (PERC45); 73.3±3.3% (PERC60); 76.7±3.3% (PERC3); and 70.0±0.0% (PERC4); however, acrosomal integrity was not altered from that of the initial sample by any gradient (Figure 4.1). Spermatozoal morphology was categorized into normal morphology, primary abnormalities (head), secondary abnormalities (protoplasmic droplet), and tertiary abnormalities (tail) (Figure 4.2). The percentage of normal spermatozoa was improved ($P < .05$) by PERC4 from 19.5±.7% to 34.2±4.0% with no changes in the percentage of primary abnormalities for any other Percoll® technique; however, these observations are misleading when evaluating the effects of the gradients on specific abnormalities where some are diminished and others enriched (Figure 4.3). The percentage of secondary abnormalities was deceased ($P < .05$) by all Percoll systems from the

Table 4.4. Specific abnormalities evaluated within the general sperm abnormalities.

Primary	Secondary	Tertiary
Nuclear Vacuole Defect on Normal Heads	Proximal Droplets	Dag Defects
Pyriform Shaped Heads	Translocating Droplets	Coil Tails
Tailless Heads	Distal Droplets	Bent Tails
Short Heads		Broken Tails
Asymmetric Heads		Folded Tails
Acrosomal Abnormalities		Abaxial Tails
Severely Misshapened Heads (Unclassifiable)		Auxiliary Tails
Other Head Abnormalities		Fragmented Midpiece
-Tapered Heads		Double Tails
-Pinched Neck		Broken Neck
-Micro Heads		Filiform Midpiece
-Macro Heads		Corkscrew Midpiece

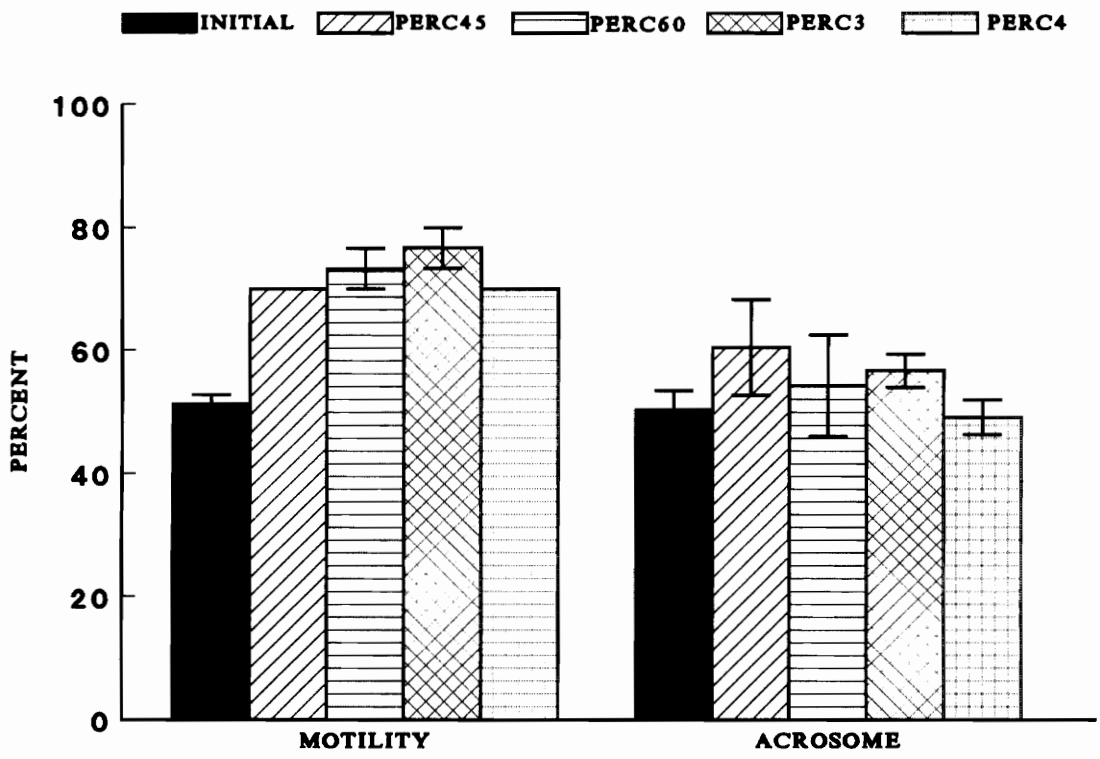

Figure 4.1. Means±SE for motility and acrosomal integrity measurements before and after Percoll® separation (n = 3). Motility improved following separation for all Percoll® techniques (P < .05) with no change in acrosomal integrity (Exp. 1).

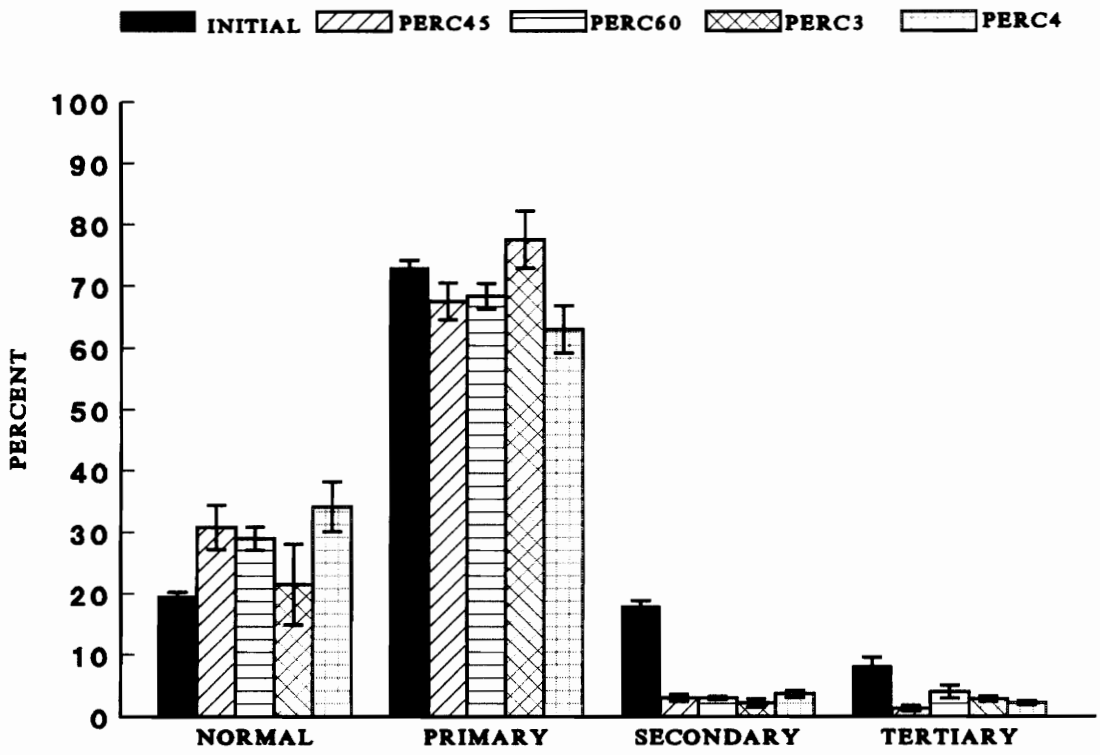

Figure 4.2. Means±SE for normal morphology, primary, secondary, and tertiary abnormalities before and after Percoll® separation (n = 3). Normal morphology was increased ($P < .05$) only by PERC4 with no changes in primary abnormalities for all techniques. The percentage of secondary abnormalities were reduced ($P < .05$) by all techniques and PERC45 was the only technique to reduce ($P < .05$) tertiary abnormalities (Exp. 1).

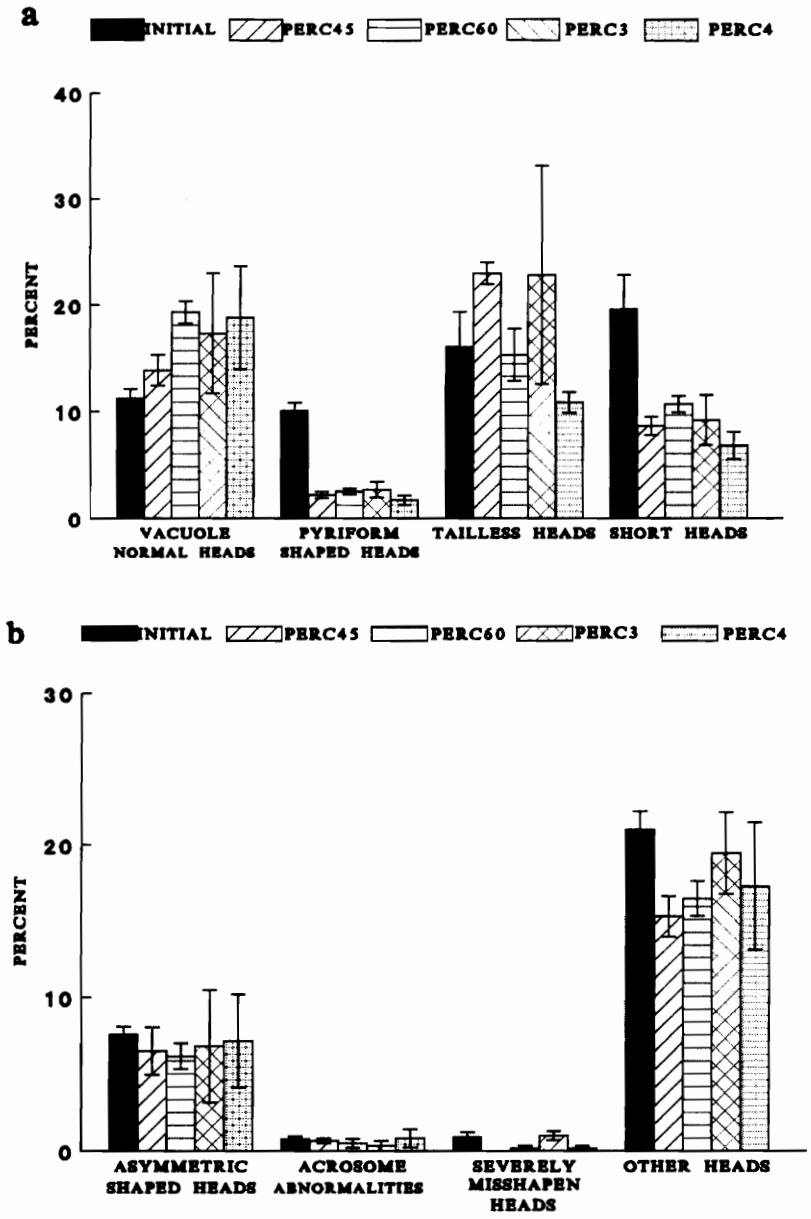

Figure 4.3. Means±SE for specific primary abnormalities before and after Percoll® separation (n = 3). The percentage of spermatozoa with pyriform shaped heads (a) was the only specific abnormality reduced ($P < .05$) by all Percoll® techniques (Exp. 1).

initial value of 17.9±.9% to 3.0±.6% (PERC45); 3.0±.1% (PERC60); 2.2±.7% (PERC3); and 3.7±.6% (PERC4); however, PERC45 (1.3±.5%) and PERC4 (2.2±.3%) were the only systems to reduce ($P < .05$) the percentage of tertiary abnormalities from the initial sample (8.1±1.4%).

The effect of the different Percoll® gradients on specific head abnormalities is shown in Figure 4.3. The percentage of spermatozoa with pyriform shaped heads (Figure 4.3a) were the only specific abnormality reduced ($P < .05$) by all Percoll® systems from the initial value of 10.1±.7% to 2.2±.2% (PERC45); 2.5±.3% (PERC60); 2.7±.7% (PERC3); and 1.7±.4% (PERC4). The percentage of spermatozoa with the nuclear vacuole defect (Figure 4.3a) on normal shaped heads was not significantly affected by the Percoll® techniques; however, there was a distinct trend towards the enrichment of these abnormal cells. This was also true for the percentage of tailless heads (Figure 4.3a) for PERC45 and PERC3. All Percoll® techniques had a distinct trend towards the reduction of spermatozoa with short heads (Figure 4.3a) although the reduction was not significantly different from initial sample.

Separation based upon severity of head abnormalities (Figure 4.4) was not influenced by the Percoll® techniques, although there was a trend toward removal of the most severe. Although Percoll® separated spermatozoa differed from the initial sample for several variables, there were no differences between Percoll® techniques.

Analysis of variance tables for all variables measured are shown in Appendix B, Tables 1 to 5.

Experiment 2

Since there were no differences in the Percoll® techniques in Exp. 1, two Percoll® gradients, 45/90% (PERC45) and 50/70/90% (PERC3), were selected as

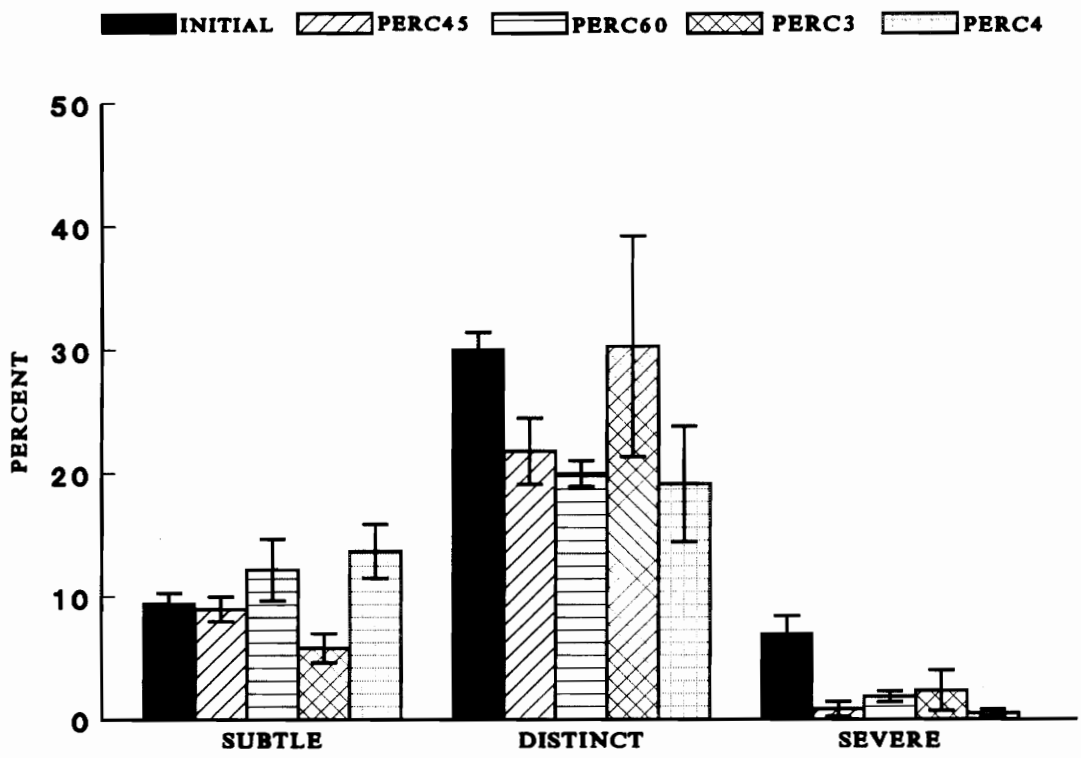

Figure 4.4. Means±SE for severity of head shape distortions before and after Percoll® separation (n = 3). There were no differences in severity from the initial sample for any Percoll® technique (Exp. 1).

a basis for comparisons with swim-up and glass wool methods. As in Exp. 1, the initial, pre-separated, semen sample served as the control for all separation techniques which was composed of 40.0±0.0%, motile cells; 36.6±1.4%, intact acrosomes; 56.0±5.0 x 10^6 sperm/ml; 7.5±.6%, normal cells; 84.4±1.3%, primary abnormalities (head); 24.5±2.5%, secondary abnormalities (protoplasmic droplets); and 7.9±.6%, tertiary abnormalities (tail) (Figures 4.5 and 4.6). Specific abnormalities within the general abnormality classification were the same as those evaluated in Exp. 1 (Table 4.4). Glass wool filtration was basically without effect.

The percentage of motile cells was increased ($P < .05$) from the initial value of 40.0±0.0% to 63.0±2.0% (PERC45); 62.0±3.7% (PERC3); and 66.0±6.8% (SU) all at the expense of sperm harvest, which decreased ($P < .05$) from the initial value of 56.0±5.0 x 10^6 sperm/ml to 18.4±1.5 x 10^6 sperm/ml, 16.6±2.6 x 10^6 sperm/ml, and 6.8±.8 x 10^6 sperm/ml, respectively (Figure 4.5). Although GW filtration did not improve motility (40.0±3.3%), sperm harvest was reduced ($P < .05$) to 29.8±11.5 x 10^6 sperm/ml. The percentage of intact acrosomes was only improved ($P < .05$) following the PERC3 technique from 39.6±1.4% in the initial sample to 57.1±6.0%.

Normal morphology and primary, secondary, and tertiary abnormalities are presented in Figure 4.6. No separation technique enriched the percentage of normal shaped heads or decreased the percentage of spermatozoa with primary abnormalities; however, as in Exp. 1, alterations in the overall response to the separation techniques for normal cells and primary abnormalities are dependent upon the sum of enrichment or reduction of specific abnormalities and the severity of those distortions (Figure 4.7 and 4.8). Again, both Percoll® techniques were capable of reducing ($P < .05$) the percentage of sperm with secondary abnormalities from 24.5±2.5% to 4.4±1.6% (PERC45) and 5.6±.6% (PERC3) and the percentage of tertiary abnormalities were reduced ($P < .05$) by PERC45 from the initial value of 7.9±.6% to 2.3±.8%. Neither SU nor GW significantly decreased

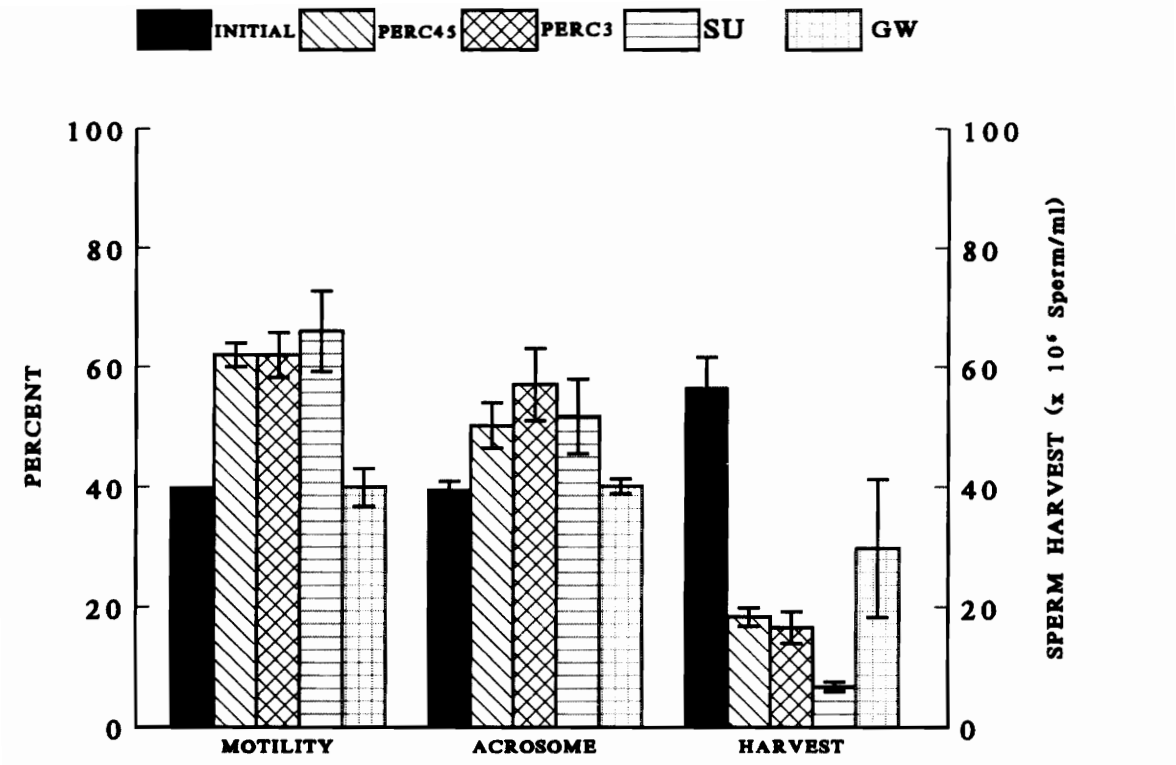

Figure 4.5. Means±SE for spermatozoal motility, acrosomal integrity and concentration/harvest before and after separation (n = 5). Motility was increased ($P < .05$) following separation with PERC45, PERC3, and SU separation. Acrosomal integrity was increased by PERC3. Spermatozoal harvest decreased for all techniques (Exp. 2).

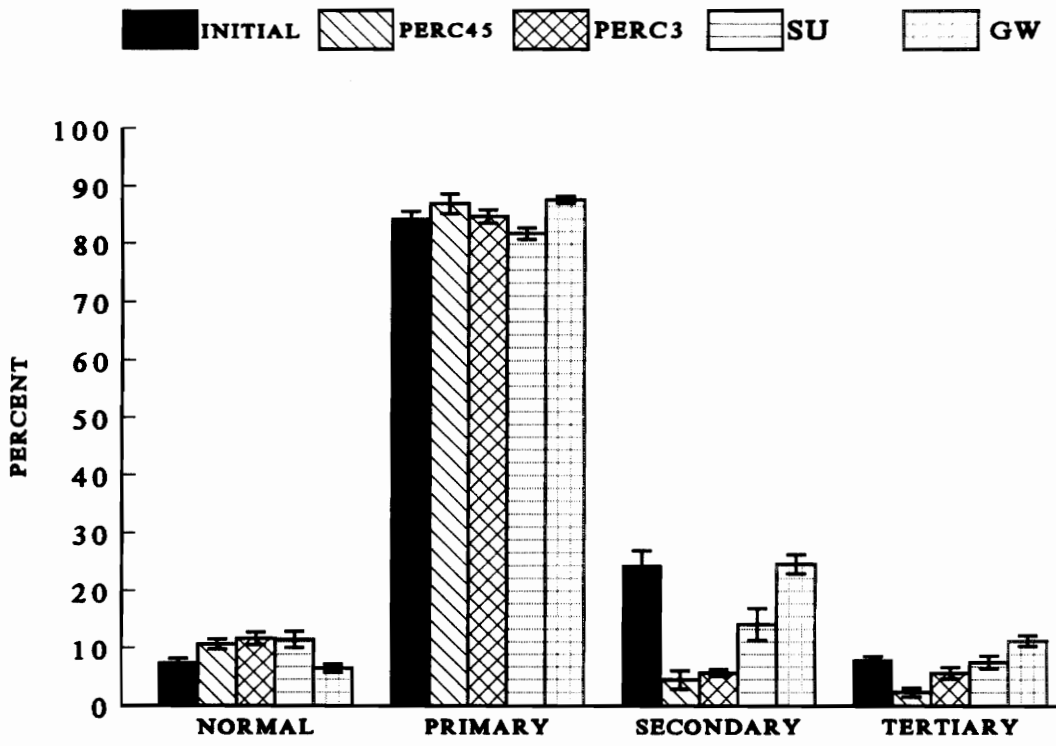

Figure 4.6. Means±SE for normal morphology, primary, secondary, and tertiary abnormalities before and after separation (n = 5). The percentage secondary abnormalities were reduced (P < .05) by both Percoll® techniques with the percentage of tertiary abnormalities only reduced by PERC45 (Exp. 2).

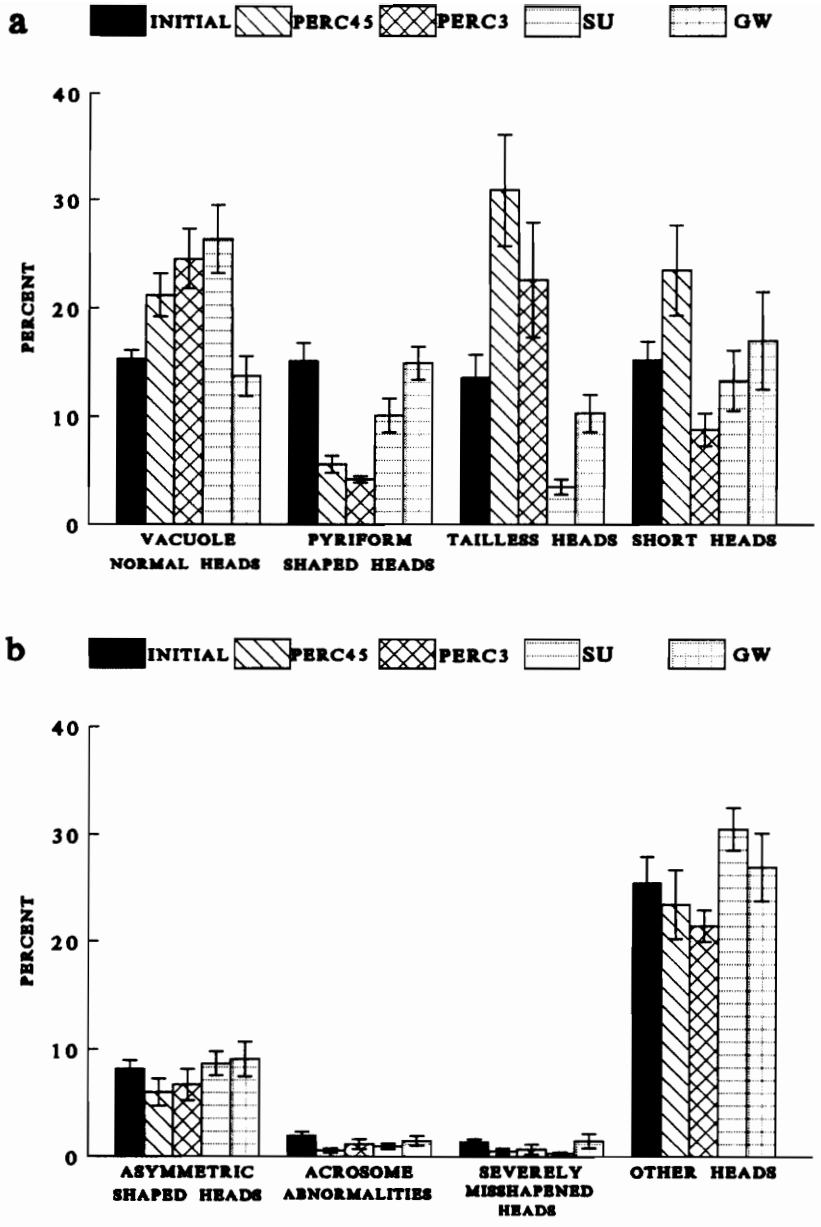

Figure 4.7. Means±SE for specific primary abnormalities before and after separation (n = 5). Spermatozoa with the nuclear vacuole defect were enriched by PERC3 and SU techniques. The percentage of sperm with pyriform shaped heads were reduced by both Percoll® techniques. The percentage of tailless heads were enriched by PERC45 ($P < .05$) (Exp. 2).

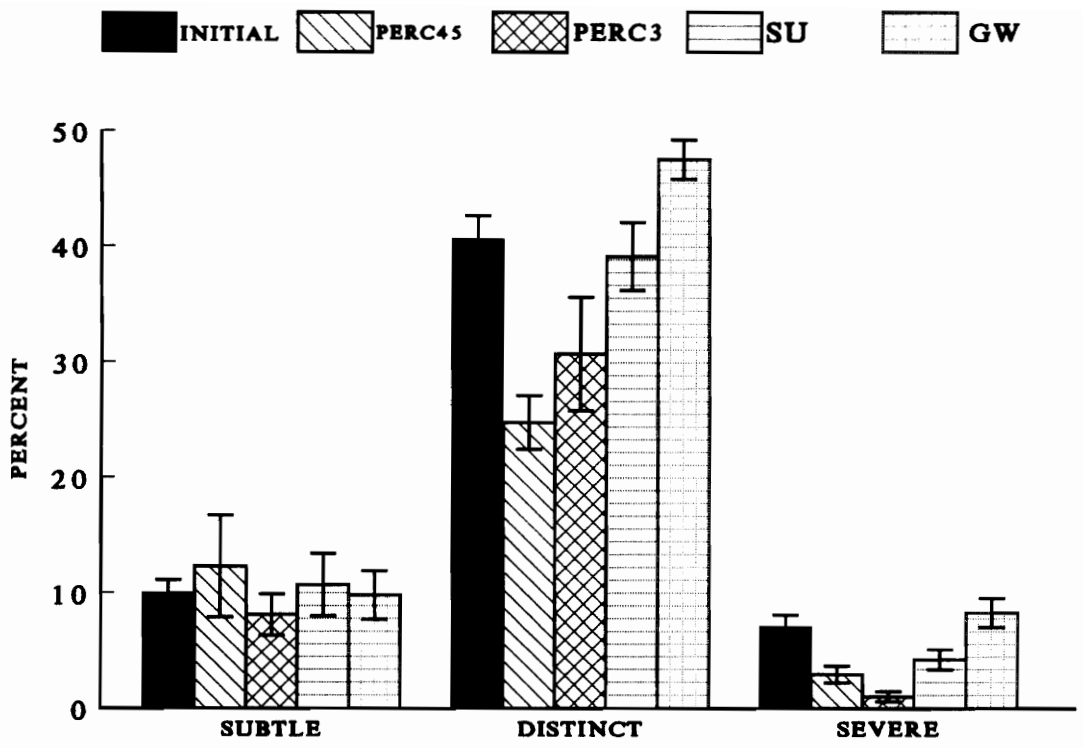

Figure 8. Means±SE for severity of head shape distortions before and after separation (n = 5). Subtle abnormalities were unchanged from the initial sample. Spermatozoa with distinct and severe abnormalities were reduced ($P < .05$) by both Percoll® techniques (Exp. 2).

the tertiary abnormalities. Primary abnormalities were further characterized into specific abnormalities (Figures 4.7). Both Percoll techniques reduced the percentage of spermatozoa with pyriform shaped heads from 15.1±1.4% to 5.6±.8%, PERC45 and 4.2±.3%, PERC3 ($P < .05$); however these abnormalities were unchanged by SU separation. The percentage of spermatozoa with nuclear vacuoles on normal shaped heads was enriched ($P < .05$) from the initial value of 15.3±.8% to 24.6±2.8 (PERC3) and 26.4±.2% (SU). The percentage of tailless heads was enriched ($P < .05$) from the initial value of 13.6±.7% to 31.0±5.2% (PERC45), but was unchanged by the other separation techniques. All other abnormalities were unchanged following separation.

Effects on the severity of head shape distortions were as shown in Figure 4.8. The percentage of subtle abnormalities was unaffected by any separation technique; however, the percentage of distinct abnormalities was reduced ($P < .05$) by PERC45 from 40.6±2.0% to 24.8±2.3% and the percentage of severe abnormalities was reduced ($P < .05$) by PERC45 and PERC3 from 7.1±1.0% to 2.9±.7% and 1.0±.4%, respectively.

Differences between the separation techniques varied depending on the variable measured. Basically, glass wool filtration was without effect and, therefore eliminated from subsequent discussion. The Percoll® separation techniques did not differ from each other for any variable measured; however, there were differences between the Percoll® techniques and SU separation (Figures 4.5 to 4.8). The separation techniques did not differ for normal shaped spermatozoa and those with primary abnormalities. The percentage of spermatozoa with secondary and tertiary abnormalities was lower following PERC45 separation than SU separation (Figure 4.6). Although both Percoll® techniques reduced the percentage of spermatozoa with pyriform shaped heads, only PERC3 was more efficient ($P < .05$) in removing pyriform shaped heads than was SU separation (Figure 4.7a).

Both Percoll® separation techniques were less efficient ($P < .05$) in removing tailless heads than SU separation.

There were no differences between separation techniques for the removal of spermatozoa with subtle or severe head distortions. Swim-up separation had a greater percentage of spermatozoa with distinct head distortions than PERC45.

Analysis of variance tables for all variables measured are presented in Appendix B, Tables 6 to 10.

Experiment 3

Due to the lack of effect from GW filtration (Exp. 2) and in recognition of its major role in semen separation and analysis system Sephadex® filtration (Graham et al., 1978) was evaluated. The initial semen sample served as the control and consisted of 36.0±2.5%, motile cells; 35.2±2.1%, intact acrosomes; 47.6±1.1 x 10^6 sperm/ml; 4.1±1.0%, normal cells; 87.6±1.2%, primary abnormalities (head); 25.2±2.7%, secondary abnormalities (protoplasmic droplets); and 10.6±.9%, tertiary abnormalities (tail) (Figures 4.9 and 4.10). All general abnormalities were further classified into specific abnormalities as shown in Table 4. 4. Severity of head distortions were classified as subtle, distinct, and severe.

Sephadex® filtration increased ($P < .05$) the percentage of motile cells from the initial value of 36.0±2.5% to 76.0±2.5% and reduced ($P < .05$) the number of sperm harvested from the initial value of 47.6±1.1 to 11.4±3.6 x 10^6 sperm/ml (Figure 4.9). Acrosomal integrity was increased ($P < .05$) following SEPH filtration from the initial value of 35.2±2.1% to 65.2±4.0%.

Sephadex® filtration increased ($P < .05$) the percentage of normal cells from 4.1±1.0%to 14.8±3.4% with no improvement in primary or secondary abnormalities. Reductions in primary abnormalities, again, were dependent upon changes in

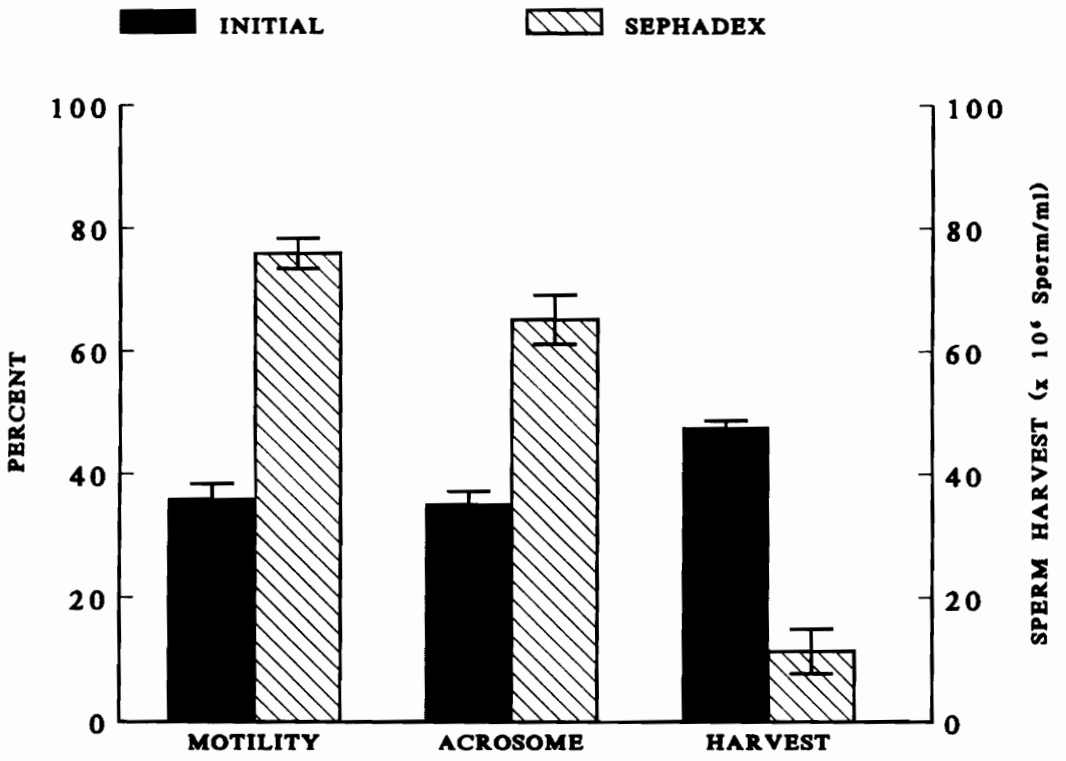

Figure 4.9. Means±SE for spermatozoal viability measurement and concentration/harvest before and after Sephadex filtration (n = 5). Motility and acrosomal integrity improved following separation with a reduction ($P < .05$) in sperm harvest (Exp. 3).

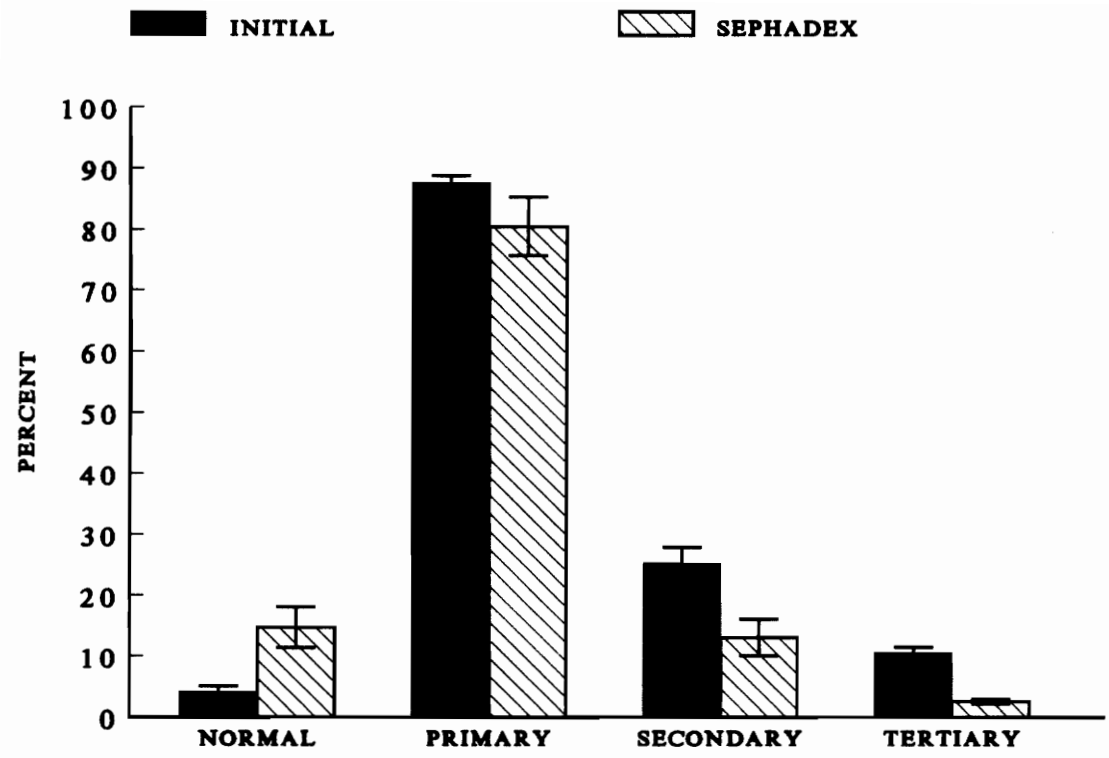

Figure 4.10. Means±SE for normal morphology, primary, secondary, and tertiary abnormalities before and after Sephadex filtration (n = 5). The percentage of normal cells were enriched ($P < .05$) while the percentage of sperm with tertiary abnormalities were reduced (Exp. 3).

specific head abnormalities and the severity of those head distortions. The percentage of tertiary abnormalities decreased ($P < .05$) from the initial value of 10.6±.9% to 2.5±.4% (Figure 4.10). There was an enrichment ($P < .05$) in the percentage of normal spermatozoa with nuclear vacuoles following filtration from the initial value of 9.3±1.7% to 21.4±3.6% and a decrease ($P < .05$) in the percentage of spermatozoa with pyriform shaped heads from the initial value of 13.4±1.1% to 5.8±.8% and short heads from the initial value of 31.2±2.7% to 8.1±1.2% (Figure 4.11a). All other specific abnormalities (Figure 4.11a and b) were unaffected by SEPH filtration. The percentage of subtle abnormalities (Figure 4.12) were unchanged from the initial sample; however, distinct (46.0±3.5 vs 32.0±3.1) and severe abnormalities (10.8±1.2% vs 2.1±.4%) were reduced ($P < .05$).

Analysis of variance tables for all traits evaluated are shown in Appendix B, Table 11 to 15.

DISCUSSION

The viability of spermatozoa at each interface of the Percoll® techniques, in Exp. 1, was extremely low (<25%), therefore limiting the practicality of evaluating sperm trapped at these interfaces in more detail. Thus, only sperm reaching the pellet in bottom gradients were evaluated fully. There were no differences between the Percoll® techniques in either Exp. 1 or 2. The results from the Sephadex® filtration study (Exp. 3) were similar to those found following Percoll® separation in Exp. 2. Motility was increased following separation using PERC45, PERC3, SU and SEPH filtration (Figures 4.5 and 4.9). The percentage of tailless heads as well as head-to-head agglutination of viable sperm in the pellet were the primary causes of the low motility and low acrosomal integrity measurements found in Exp. 2. Parrish et al. (1988) also appeared to have difficulty evaluating

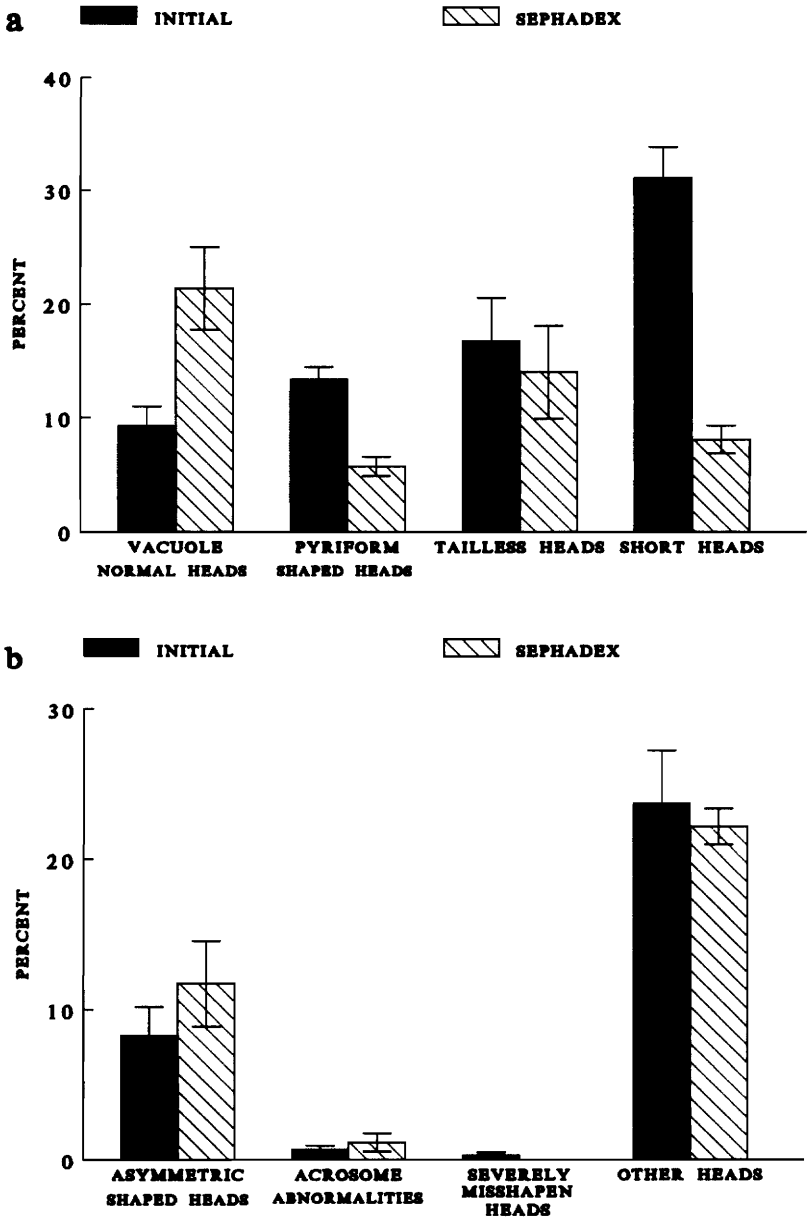

Figure 4.11. Means±SE for specific sperm abnormalities before and after Sephadex filtration (n = 5). Spermatozoa with nuclear vacuoles were enriched ($P < .05$) while the percentage of spermatozoa with pyriform shaped heads and short heads were reduced (Exp. 3).

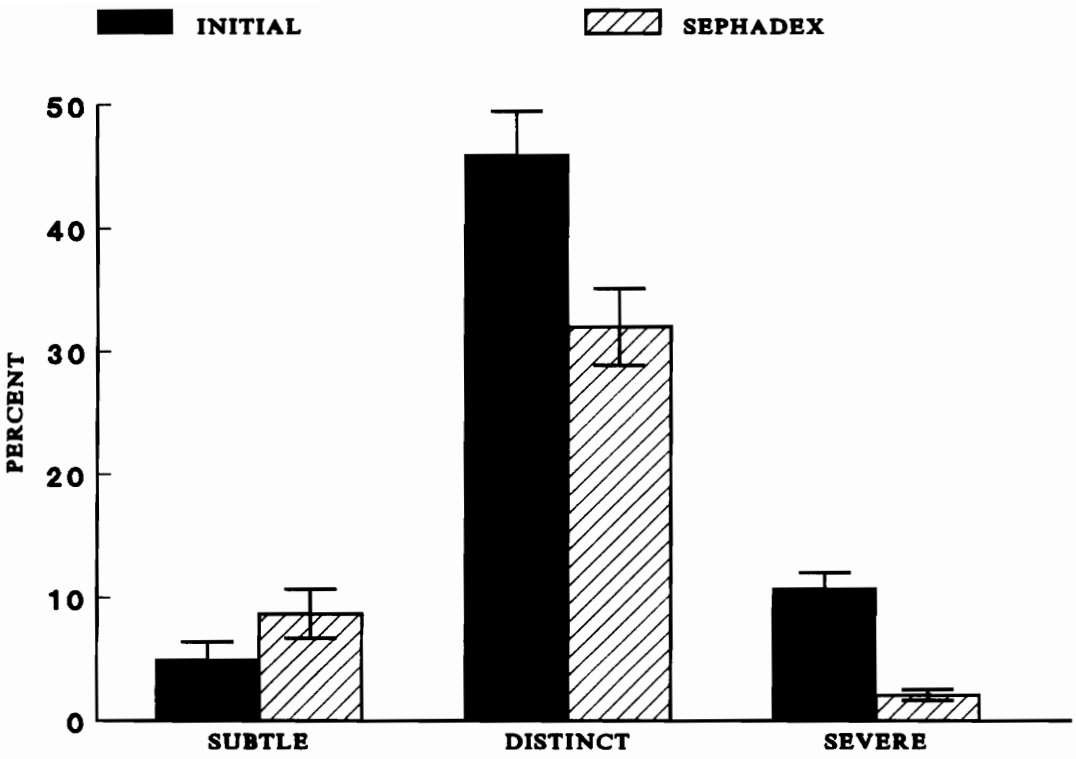

Figure 4.12. Means±SE for severity of sperm abnormalities before and after Sephadex filtration (n = 5). Sephadex filtration enriched ($P < .05$) subtle abnormalities and reduced distinct and severe abnormalities (Exp. 3).

percent motility due to head-to-head agglutination. The improvement in spermatozoal motility was in agreement with previous studies for Percoll® (Forster et al., 1983; LeLannou and Blanchard, 1988; McClure et al., 1989; Pousette et al., 1985), as well as SU (Howard et al., 1994; LeLannou and Blanchard, 1988) and SEPH (Byrd et al., 1994; Graham and Graham, 1990) separation techniques.

Evaluation of specific primary abnormalities and the severity of the distortion of these specific abnormalities have not been reported previously and are of major interest in the present study. Separation of abnormal spermatozoa appeared particularly related to severity of the head shape distortions (Figures 4.9 and 4.12) because subtle abnormalities were unaffected by separation for all techniques (Exps. 1, 2, and 3); however, distinct abnormalities were reduced by PERC45 and SEPH filtration while severe abnormalities were decreased by PERC45, PERC3, and SEPH filtration. This is further substantiated by the finding that spermatozoa with nuclear vacuoles on otherwise normal shaped heads were enriched (Figures 4.8 and 4.11). The inability of SU separation to decrease the percentage of distinct abnormalities could indicate that motile abnormal spermatozoa are capable of swimming up into the overlying selected medium.

The percentage of normal cells was increased only by SEPH filtration (Figure 4.10). Other investigators have found similar results following SEPH filtration for human (Byrd et al., 1994) and bovine (Graham and Graham, 1990) semen. The lack of improvement in the percentage of normal cells by the other separation techniques (Figure 4.7) was due primarily to the increase in spermatozoa with the nuclear vacuole defect on otherwise normal shaped heads and tailless heads even with the decrease in spermatozoa with distinct and severe head distortions and secondary abnormalities. An enrichment of normal cells was found in previous studies for two-layer Percoll® (McClure et al., 1989), multi-layer Percoll® (Forster et al., 1983; LeLannou and Blanchard, 1988; Pousette et al., 1985), and SU separation (Howard et al., 1994; LeLannou and Blanchard, 1988).

No separation technique was capable of decreasing the percentage of primary abnormalities. As with normal cells, the primary abnormalities were not reduced because of the enrichment of spermatozoa with the nuclear vacuole defect on normal shaped heads even though the percentage of spermatozoa with pyriform shaped heads was reduced with a slight reduction (however, insignificant) in short heads. Both Percoll® techniques and SU separation reduced the percentage of sperm with secondary abnormalities. The reduction in secondary abnormalities was similar to those found by Howard et al. (1993) following SU separation of cat spermatozoa. In the present study and in a previous study (Graham and Graham, 1990) the percentage of secondary abnormalities was not reduced by SEPH filtration. LeLannou and Blanchard (1988) demonstrated that SU separation was more efficient at removing tertiary abnormalities than Percoll® separation; however, in the present study PERC45 reduced the percentage of tertiary abnormalities to a greater extent than SU separation and SU separation did not differ from PERC3. Other investigators demonstrated a reduction in tertiary abnormalities by SU separation (Howard et al., 1994); no change by Percoll® separation (Byrd et al., 1994) or SEPH filtration (Graham and Graham, 1990); and an increase by SEPH filtration (Byrd et al., 1994).

In the present study, specific abnormalities of bovine spermatozoa were more critically evaluated than in previous studies separating bovine (Graham and Graham, 1990), human (Byrd, et al., 1994; Forster et al., 1983) and cat (Howard et al., 1994) spermatozoa. Comparisons of studies separating bovine spermatozoa to studies separating human or cat spermatozoa is difficult due to the diversity in classification. Human spermatozoa are classified by general gross head shape and(or) tail defects. One abnormality similarly classified by both human (Forster et al., 1983) and bovine investigators (Graham and Graham, 1990) was the pyriform shaped head. In this study and a previous study, the percentage of pyriform shaped heads was reduced by Percoll® separation (Forster et al., 1983)

and Sephadex® filtration (Graham and Graham, 1990). Sephadex filtration also reduced the percentage of spermatozoa with short heads. The enrichment of tailless heads following both Percoll® separation techniques was found in the present study and in a study using multi-layer Percoll® separation (Lessley and Garner, 1983). It is speculated that tailless heads are more dense than their normal counterparts; therefore, they are moved by the centrifugal force to the most dense Percoll® layer. Graham and Graham (1990) found an increase in tailless heads following Sephadex® filtration; however, the present study showed no such enrichment. Swim-up separated semen had the lowest percentage of tailless heads because these cells were not motile and could not swim-up into the overlying medium. The enrichment of nuclear vacuoles on normal shaped heads was found following all separation techniques. This is the only study to evaluate the separation of spermatozoa with the nuclear vacuole defect from semen samples. These spermatozoa were not separated because they were otherwise normal, viable cells.

Glass wool filtration was not an effective separation technique in this study. This directly disagrees with previous studies (Katayama et al., 1989; Jeyendran et al., 1986; Rhemrev, et al., 1989) in which the percentage of motile cells was increased. The type of glass wool used in this study was different from that used in the previous studies and could be the cause of the poor results. The type of glass wool used for semen separation is, apparently, very important (Personal Communication, Dr. Bo Crabo, Univ. of Minn.).

Several factors must be considered when selecting a separation system for preparation of spermatozoa to be used for *in vitro* fertilization. The viability and concentration of the initial sample, the percentage and type of specific abnormalities to be separated, the final concentration needed for the IVF procedure and the expense and time required to set up and perform the techniques are all important considerations for selection. Percoll® separation and

Sephadex® filtration were similar in their results in improving viability and removing abnormal spermatozoa, and both techniques required approximately the same amount of separation time, 30 and 20 min, respectively. The difference lies in the expense and time of column preparation. Percoll® media is expensive and preparation of the layers and gradients is time consuming and must be done gently so as to not mix the overlying layers. There were no differences between the two-layer and three-layer Percoll® techniques; therefore, it is recommended to use the two-layer technique which is more economical and less tedious to prepare. Sephadex column preparation is also time consuming and if not properly prepared the efficiency of separation is hampered. Swim-up separation is, by far, the simplest of the separation techniques; however, it is the most time consuming, requiring a 60 min incubation period. Swim-up separation is also the least efficient in sperm recovery. If the initial sample has a high percentage of tailless heads, the SU technique would be the only separation technique of choice. The recovery rate of the high quality semen was also low.

We can conclude from these experiments that no separation system can efficiently remove spermatozoa with subtle distortions of the head and those spermatozoa with the nuclear vacuole defect on normal shaped heads. Further research must be done in order to remove these abnormal cells from ejaculated semen.

IMPLICATIONS

Excluding glass wool filtration, all separation techniques were capable of improving motility at the expense of sperm harvest. The recovery rate of spermatozoa following SU separation was the least efficient and should not be used if there are low numbers of spermatozoa were found in the initial sample. The enrichment of normal spermatozoa with nuclear vacuoles and spermatozoa

with subtle head distortions, following separation, was an important finding. These abnormal spermatozoa have been demonstrated to play a role in male-related early embryonic death. It would be beneficial to remove these subtle abnormalities from semen samples in order to improve fertilization, both *in vivo* and *in vitro*, in bulls with a high propensity to produce these type abnormalities. Thus, more research must be performed to develop a system that will remove the spermatozoa with the nuclear vacuole defect and the more subtle abnormalities that are capable of reaching the site of fertilization if progress in fertilization and embryo development is to be obtained via the male.

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CHAPTER V

SEPARATION OF SPERMATOZOA WITH NUCLEAR VACUOLES FROM SEMEN SAMPLES USING IN VITRO FERTILIZATION DENSITY SEPARATION TECHNIQUES

ABSTRACT

Two experiments were performed to determine if normal shaped spermatozoa with nuclear vacuoles (previously implicated as a cause of embryonic mortality) could be removed from a semen sample by *in vitro* density-separation techniques. Cryopreserved semen containing >20% spermatozoa with nuclear vacuoles in normal shaped heads was used in both experiments. In Experiment 1, a 45/90% Percoll® (PERC45) density gradient and 15/30%, 15/30/40%, and 15/30/45% Nycodenz® density gradients (NYC30, NYC40, and NYC45, respectively) were used with bottom layer densities of 1.12 g/ml, 1.16 g/ml, 1.21 g/ml, and 1.24 g/ml, respectively. In Experiment 2, Percoll® density gradients of 45/90% (PERC45) or 45/100% (PERC100), with bottom layer densities of 1.12 g/ml and 1.13 g/ml, respectively were used. In Exp. 1, PERC45 did not affect spermatozoal motility or acrosomal integrity. All Nycodenz techniques reduced sperm motility and acrosomal integrity. The percentage of normal cells was not affected by any technique. In Exp. 2, the percentage of motile cells was increased by PERC100 and percent intact acrosomes were increased by PERC45 and PERC100. Both Percoll® techniques enriched the percentage of normal cells. The percentage of spermatozoa with primary abnormalities was reduced by PERC45 and the percentage of spermatozoa with tertiary abnormalities were reduced by PERC100 with no changes in secondary abnormalities. Both Percoll® techniques reduced the percentage of spermatozoa with nuclear vacuoles on normal shaped

heads. Following the initial morphological evaluation, 100 cells with nuclear vacuoles were evaluated to determine the total number of vacuoles per sperm head. Of the vacuolated spermatozoa, those spermatozoa with more than three nuclear vacuoles per sperm head were reduced while those spermatozoa with one or two nuclear vacuoles per sperm head were enriched. The vacuoles were mainly located in the equatorial region of the sperm head where aligned vacuoles are referred to as the "diadem defect". From these results, we would predict that a separation system compatible with sperm and with greater density than Percoll must be developed to more efficiently remove sperm with nuclear vacuoles. At this time, high density Nycodenz preparations are not compatible based on depressed viability.

Key words: Spermatozoa, Nuclear vacuoles, Density separation.

INTRODUCTION

Production of spermatozoa with nuclear vacuoles has been associated with increased testicular temperature (Vogler et al., 1993). Such sperm have been shown to gain access to the egg in vivo (Saacke et al., 1988) and are associated with lowered fertility and embryo quality in cattle (Miller et al., 1982; Saacke et al., 1992). It is unclear whether these abnormal spermatozoa are the cause of embryonic death or perhaps reflect incompetence in other sperm of that ejaculate. Successful efforts to remove vacuolated spermatozoa from semen would permit further assessment of the importance of this abnormality to lowered pregnancy rates as well as provide a basis to improve samples for use in the embryo transfer and in vitro fertilization (IVF) industries.

The specific gravity of a normal bovine spermatozoon has been shown to range from 1.24 to 1.33 (Lindahl and Kihlstrom, 1952). The nuclear vacuole defect

has been described as one or a series of depressions or blisters at some point on the sperm head (Coulter et al., 1978; Johnson and Hurtgen, 1985) that may be empty or fluid filled. Thus, we would speculate that spermatozoa with nuclear vacuoles have a lower specific gravity than normal sperm. If so, density gradient separation techniques should be able to remove these abnormal spermatozoa. Percoll® density gradients (Forster et al., 1983; McClure et al., 1989; Le Lannou and Blanchard, 1988) and Nycodenz® density gradients (Gellert-Mortimer, 1988; Mortimer, 1994; Serafini et al., 1990) have been used to improve viability and morphology of ejaculates. These density gradient separation techniques have not been used to critically evaluate the removal of spermatozoa with nuclear vacuoles. Thus, the present experiments were designed to determine if spermatozoa with nuclear vacuoles could be removed by Percoll® or Nycodenz® density separation techniques.

MATERIALS AND METHODS

Semen Source and Preservation

Semen was collected from a bull following a 48-h full scrotal insulation (Vogler et al., 1993). The ejaculate was prepared in the conventional manner (Robbins et al. 1976) for cryopreservation with clarified egg yolk-citrate extender containing 7% glycerol (vol/vol) at 50 x 10^6 sperm/ml. Extender was clarified by filtering through a .45-µm Acrodisc filter (Gelman Sciences, Ann Arbor, MI). Semen was packaged in .5 ml French straws (Instruments de Medicine Veterinaire, L'Aigle, France), frozen 24±2 h after collection in static liquid nitrogen vapor for 10 min, and then plunged into liquid nitrogen. Straws were stored in liquid nitrogen until use.

Separation Techniques

Percoll® density gradients. A stock solution of Percoll® (90%) was prepared by mixing 45 ml Percoll® (Sigma Chemical Co., St. Louis, MO) with 5 ml of a 10x stock solution of a modified Tyrodes medium called sperm TALP (SPTALP) (Parrish et al., 1986) (Table 5.1). The 100% Percoll® medium was prepared by mixing .7 g NaCl with 100 ml Percoll® to increase the osmotic pressure to 290- 300 mOsm. The 45% Percoll® layer was prepared by a 1:1 dilution of the 90% stock Percoll® with SPTALP without BSA (Table 5.2). The gradients (45/90% and 45/100%) were prepared by gently layering 2 ml of each solution from most dense to least dense in a 15 ml conical tube (Fisher Scientific, Pittsburgh, PA). The gradients plus .5 ml extended semen were centrifuged at 700x g for 30 min (Mermillod et al., 1990). The supernatant was removed by vacuum suction to .5 ml. The pellet was resuspended with 5 ml SPTALP and recentrifuged at 200x g for 10 min (Parrish et al., 1986). The supernatant was removed leaving .5 ml SPTALP and the sperm pellet. This wash was performed to remove any excess seminal fluid, egg-yolk, or Percoll® following the initial separation.

Nycodenz® Density Gradients. A solution consisting of .3 mM $CaNa_2EDTA$, 5 mM Tris-HCL (pH 7.5), and 3 mM KCL was used to dissolve 3.0 g, 4.0 g, or 4.5 g of Nycodenz® up to a total of 10 ml (30%, 40%, or 45% Nycodenz®, respectively) (Ford and Rickwood, 1982). The 15% Nycodenz® solution was prepared by a 1:1 dilution of the 30% Nycodenz® with SPTALP without BSA. The osmotic pressure of the 15%, 30%, 40%, and 45% Nycodenz® was 300 mOsm, 330 mOsm, 420 mOsm, and 485 mOsm, respectively. The 15/30%, 15/30/40%, and 15/30/45% Nycodenz® gradients (NYC30, NYC40, and NYC45, respectively) were prepared by gently layering 2 ml of each solution from most dense Nycodenz® to least dense in a 15 ml conical tube. The gradients plus .5 ml extended semen were centrifuged at 700x g for 30 min.

Table 5.1. 10x stock solution of sperm TALP.

INGREDIENTS	STOCK CONCENTRATION	/100 ml
*KCl	1 M	3.090
*NaH_2PO_4	.1 M	2.920
*NaCl	80 mM	2.380
*HEPES	---	2.380
**$CaCl_2$	1 M	0.197
**$MgCl_2$	.1 M	0.394
**Lactic acid	---	0.184
**$NaCHCO_3$	---	104.5

* Add first then adjust pH to 7.3.

**Add to mixture of 90 ml Percoll® and 10 ml of 10X stock media. Adjust osmolarity to 280 - 300 mOsm.

Table 5.2. Components of sperm TALP medium (Parrish et al., 1986).

INGREDIENTS	UNITS	SPTALP
NaCl	Mm	100.0
KCl	mM	3.1
$NaHCO_3$	mM	25.0
NaH_2PO_4	mM	0.3
Lactate (sodium salt)	mM	21.6
*$CaCl_2$	mM	2.0
*$MgCl_2$	mM	0.4
HEPES	mM	10.0
**Pyruvate	mM	1.0
**Bovine Serum Albumin	mg/ml	6.0
**Gentamicin	µg/ml	50.0

*Add last.
**Add on day of use.

Semen Evaluation

Pooled pre-separation and post-separation aliquots of the pellet were removed and evaluated for morphology, viability, and concentration/harvest. Motility was assessed using phase-contrast microscopy at 250x magnification on a heated stage at 39°C by placing a drop of extended or separated semen from a capillary tube on a warmed slide and, subsequently, covering with a warmed cover slip. A 100-µl aliquot was removed and placed in 1 ml Karnovsky's (1965) fixative. Morphology and acrosomal integrity were evaluated from the fixed cells. Two counts of 100 cells were assessed for morphology using differential interference contrast (DIC) microscopy at 1250x magnification under oil and averaged. Cells with multiple abnormalities were counted once, but each abnormality on the cell was recorded in order to avoid preemption of one abnormality by another abnormality. The specific classification of severely misshapened heads included those spermatozoa that could not be classified as any other specific head abnormality. Acrosomal integrity was evaluated on the same 200 cells. A cell was declared intact based on the presence of an apical ridge (Saacke and Marshal, 1968). A hemocytometer count was taken to determine spermatozoal concentration/harvest. In Exp. 2, one hundred normal shaped cells with nuclear vacuoles were counted, from the fixed sample, for the number of nuclear vacuoles per sperm head using DIC microscopy at 1250x magnification under oil.

Experimental Design and Rationale

Experiment 1. A cryopreserved ejaculate collected from one bull on d 18 after a 48-h full scrotal insulation (Vogler et al., 1993) was used to determine if spermatozoa with nuclear vacuoles could be removed from an ejaculate using *in*

vitro fertilization density separation techniques. The semen was selected based on the percentage of spermatozoa with the nuclear vacuole defect on normal shaped heads (>20%) and motility of ≥70% after thawing. The semen was composed of spermatozoa with single nuclear vacuoles, multiple nuclear vacuoles, and diadem defect. Four density separation techniques were used. The separation systems were 45/90% Percoll® density gradient (PERC45), 15/30%, 15/30/40%, and 15/30/45% Nycodenz® density gradient (NYC30, NYC40, and NYC45, respectively). The density of each bottom layer was 1.12 g/ml, 1.16 g/ml, 1.21 g/ml, and 1.24 g/ml, respectively. The unseparated semen served as control. Pre- and post-separated semen were evaluated for viability, morphology, and concentration/harvest. This experiment was replicated five times.

Experiment 2. Because a slight decrease in spermatozoa with nuclear vacuoles on normal shaped heads was found following PERC45 separation in Exp. 1 we increased the density of the bottom Percoll® layer, slight. These spermatozoa were further evaluated by counting the number of nuclear vacuoles per sperm head to determine if a separation was occurring but was not being detected by the general evaluation of nuclear vacuoles. The same cryopreserved ejaculate was used as in Exp. 1. Of particular interest was the variations in numbers of nuclear vacuoles on normal shaped heads per sperm head. The same 45/90% two-layer Percoll® gradient (PERC45) as well as a 45/100% Percoll® gradient (PERC100) were used in an attempt to remove vacuolated spermatozoa from semen. The densities of the bottom layers were 1.12 g/ml and 1.13 g/ml, respectively. This experiment was performed as described in Exp. 1. Following separation spermatozoa were evaluated for viability, morphology, and concentration/harvest. This experiment was replicated five times.

Statistical Analysis

Data were analyzed by the general linear models procedure (SAS®). The model used for analysis of variance for morphology, motility, acrosomal integrity, and concentration/harvest per treatment was as follows: $Y_{ij} = \mu + T_i + R_j + e_{ij}$. Where:

Y_{ij} = percentage of motility, intact acrosome, morphology and concentration/harvest;

μ = overall mean;

T_i = the fixed effect of treatment i (separation techniques);

R_j = the fixed effect of replication j;

e_{ij} = random error.

The same model was used for Exp. 2, except the number of nuclear vacuoles per sperm head was also evaluated. Differences between treatment means were determined by Tukey's analysis.

RESULTS

Experiment 1

The initial, pre-separated semen sample served as the control for all separation techniques. It consisted of 39.7±1.6 x 10^6 sperm/ml; 72.0±2.0% motile cells; 83.9±1.8% intact acrosomes; 62.1±2.2% normal cells; 36.0±2.8%, primary (head) abnormalities; 2.3±.6%, secondary (protoplasmic droplets) abnormalities; 2.0±.4%, tertiary (tail) abnormalities. The primary abnormalities were comprised of 22.4±2.2% nuclear vacuoles on normal shaped heads; 6.6±1.3%, tapered heads; .3±.2%, tailless heads; 1.2±.6%, short heads; 2.1±.6%, asymmetric heads; and 1.1±.2%, other head abnormalities (Figures 5.1, 5.2, and 5.3). The specific

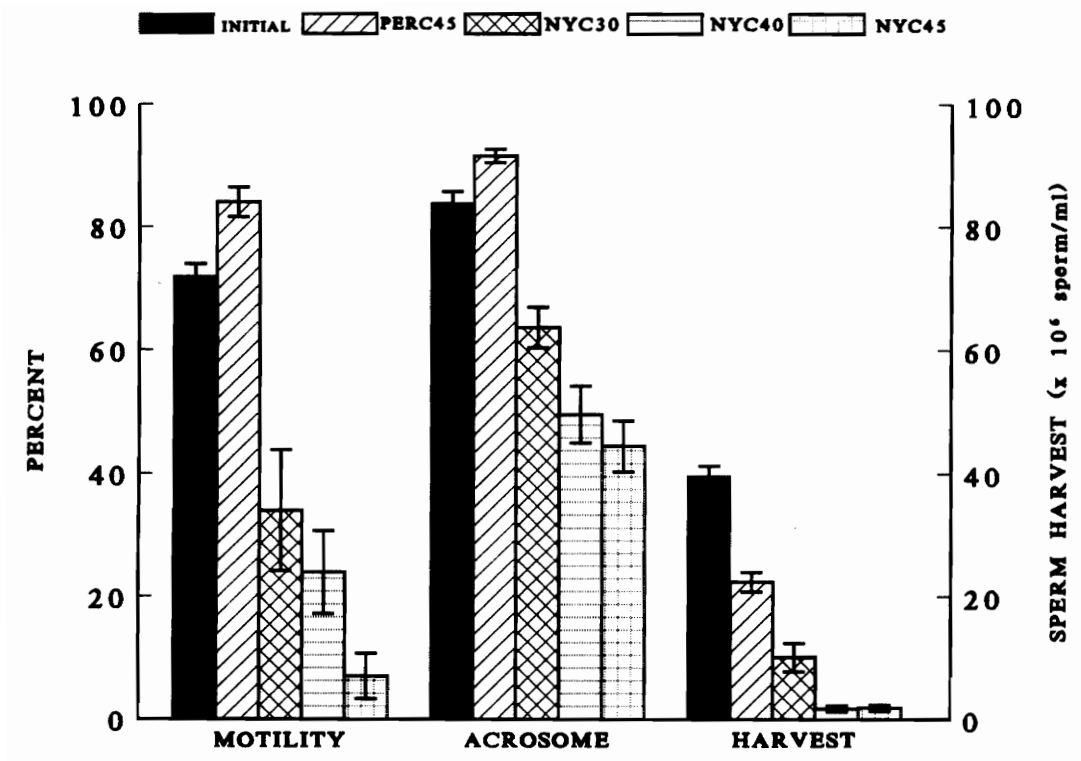

Figure 5.1. Means±SE for spermatozoal viability and concentration/harvest before and after separation (n = 5). All Nycodenz® techniques had a lower ($P < .05$) motility and acrosomal integrity than the initial sample and PERC45. All separation techniques reduced ($P < .05$) the sperm harvest from the initial sample with PERC45 recovering more sperm than any Nycodenz® technique (Exp. 1).

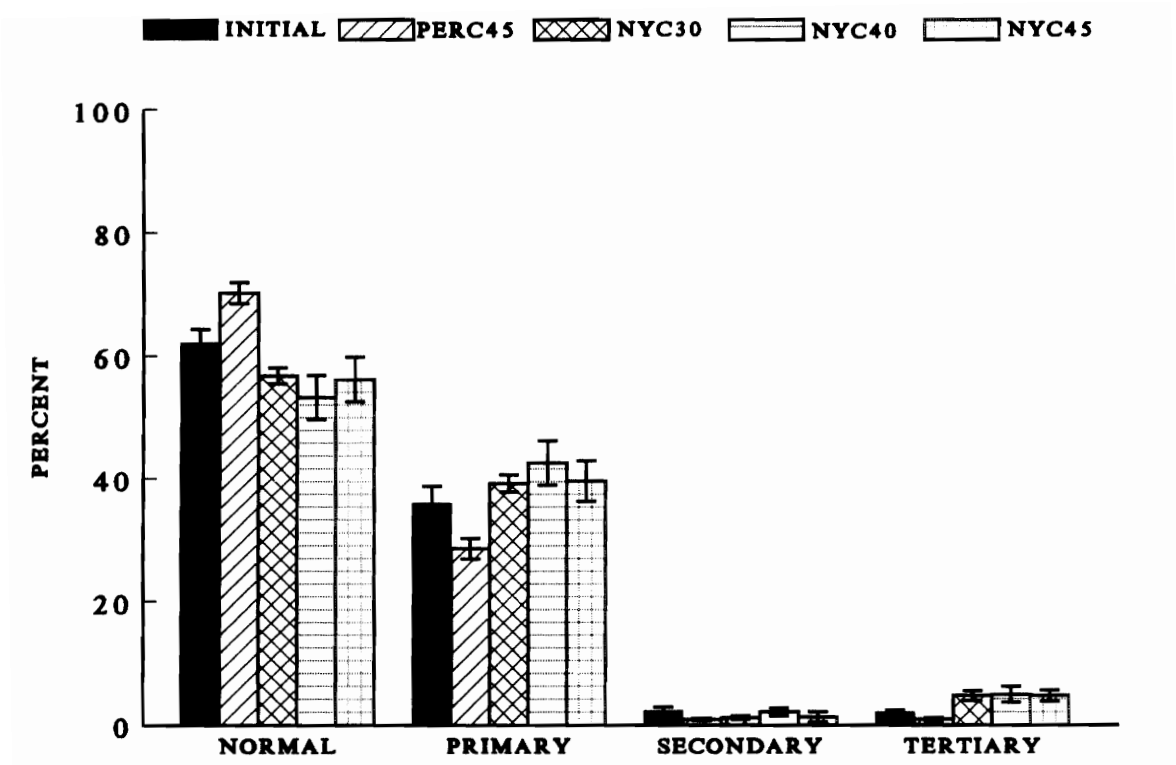

Figure 5.2. Means±SE for normal morphology, primary, secondary, and tertiary abnormalities before and after separation (n = 5). The percentage of normal cells were not different from the initial sample for PERC45 or NYC30; however, they were reduced ($P < .05$) for NYC40 and NYC45. Primary, secondary, and tertiary abnormalities did not differ from the initial sample for all techniques; however, PERC45 had fewer ($P < .05$) primary and tertiary abnormalities than the Nycodenz techniques (Exp. 1).

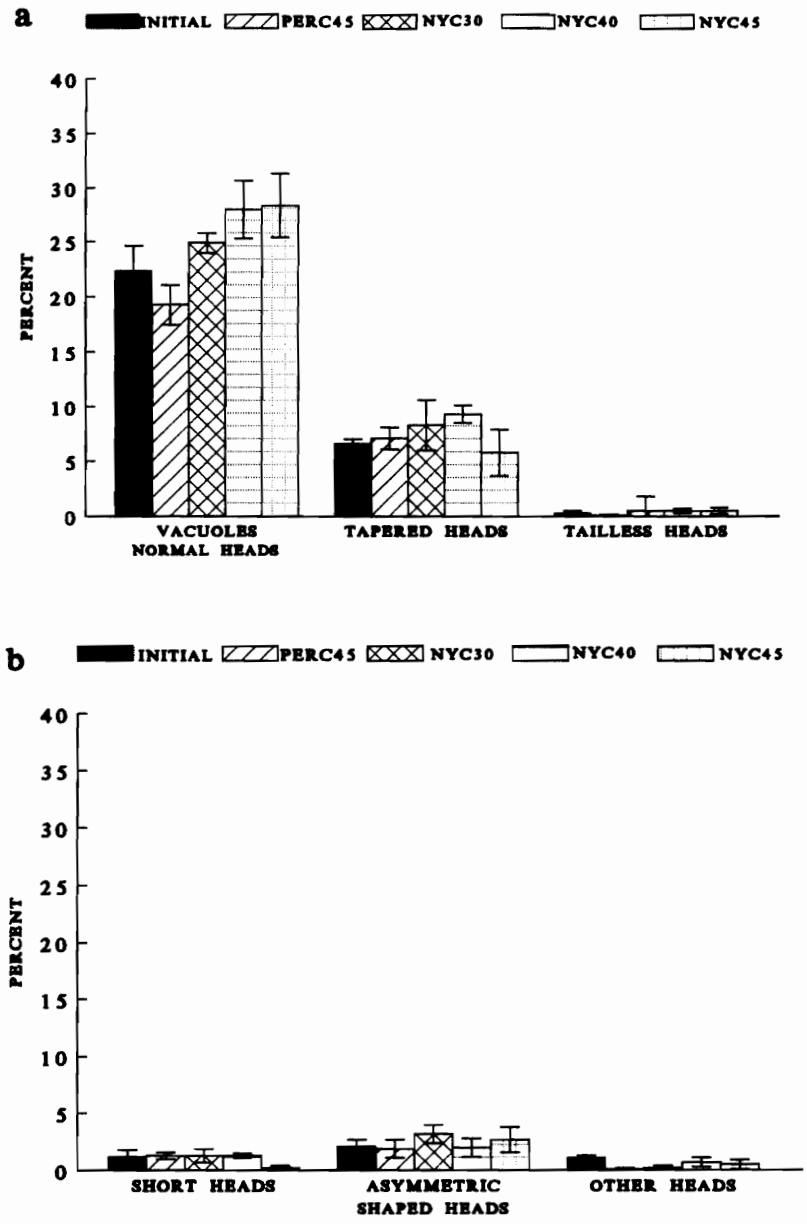

Figure 5.3. Means±SE for specific head abnormalities before and after separation (n = 5). No separation technique reduced the percentage of any specific head abnormality (Exp. 1).

primary abnormalities within the head classification, other, were pyriform shaped heads, acrosomal abnormalities, severely misshapened heads, micro and macrocephalic heads. Motility and acrosomal integrity (Figure 5.1) after separation (PERC45) were not different from those in the initial sample (84.0±2.5% and 91.6±1.1%, respectively);however, motility and acrosomal integrity were decreased ($P < .05$) after separation by NYC30 (34.0±9.8% and 63.7±3.3%, respectively), NYC40 (24.0±6.8% and 49.6±4.6%, respectively), and NYC45 (7.4±3.7% and 44.5±4.1%, respectively). The percentage of motile cells and intact acrosomes for all Nycodenz® techniques were lower than those spermatozoa from PERC45 ($P < .05$). Spermatozoa following the NYC30 technique had a higher motility than NYC45 and higher percentage of intact acrosomes than NYC40 and NYC45 ($P < .05$). Spermatozoal harvest (Figure 5.1) was reduced ($P < .05$) by all techniques 22.4±1.6 x 10^6 sperm/ml (PERC45); 10.1±2.3% x 10^6 sperm/ml (NYC30); 1.7±.5 x 10^6 sperm/ml (NYC40); and 1.9±.5 x 10^6 sperm/ml (NYC45). The Percoll® technique had a greater recovery rate than NYC30 which, in turn, had greater ($P < .05$) spermatozoal recovery than NYC40 or NYC45. The percentage of normal cells (Figure 5.2) was not different from the initial sample for PERC45 (70.2±1.7%) and NYC30 (56.8±1.3%); however, the percentage of normal cells was reduced for NYC40 (53.3±3.6%) and NYC45 (56.2±3.6%). More normal cells were recovered from the Percoll® technique than from all the Nycodenz® techniques. The Nycodenz® techniques did not differ from each other.

No separation technique reduced the percentage of spermatozoa with primary abnormalities; however, this general classification is a sum of all changes in the specific head abnormalities. The Percoll® technique (28.7±1.7%) resulted in fewer ($P < .05$) spermatozoa with primary abnormalities (Figure 5.2) than did all Nycodenz® techniques 39.3±1.4% (NYC30), 42.6±3.6% (NYC40), and 39.7±3.3% (NYC45). The percentage of spermatozoa with secondary abnormalities (Figure 5.2) was not different from the initial sample for any separation technique and

there were no differences between the techniques. The Percoll® technique (.9±.3%) had fewer spermatozoa with tertiary abnormalities (Figure 5.2) than the Nycodenz® techniques 4.7±.8% (NYC30), 4.9±1.3% (NYC40), and 4.7±.9% (NYC45); however, no technique differed from the initial sample in tertiary abnormalities. No specific abnormality differed from the initial sample (Figure 5.3).

Analysis of variance tables for all variables measured are shown in Appendix C, Tables 1 to 4.

Experiment 2

The initial sample consisted of 40.5±.5 x 10^6 sperm/ml; 72.0±2.0% motile cells; 81.6±1.5% intact acrosomes; 55.6±1.6%, normal cells; 42.4±1.5%, primary (head) abnormalities; 1.9±.7%, secondary (protoplasmic droplets) abnormalities; 2.5±.7%, tertiary (tail) abnormalities. The primary abnormalities consisted of 29.1±.7%, nuclear vacuoles on normal shaped heads; 8.1±.4%, tapered heads; .3±.2%, tailless heads; 1.2±.2%, short heads; 3.0±.6%, asymmetric heads; and .8±.1%, other head abnormalities (Figures 5.4, 5.5, and 5.6). The specific primary abnormalities within the classification, other, were pyriform shaped heads, acrosomal abnormalities, severely misshapened heads, and micro and macrocephalic heads. The percentage of Motile cells (Figure 5.4) was increase ($P < .05$) from the initial sample by PERC100 (88.0±2.0%) with no change by PERC45 (80.0±5.5%). The percentage of spermatozoa with intact acrosomes (Figure 5.4) was increased by both Percoll® techniques (90.0±2.3%, PERC45 and 90.1±1.9%, PERC100). Spermatozoal harvest (Figure 5.4) was reduced ($P < .05$) by both Percoll® techniques (19.5±1.0 x 10^6, PERC45 and 8.8±2.9 x 10^6 sperm/ml, PERC100). More spermatozoa were recovered from PERC45 than PERC100 ($P < .05$). The percentage of normal cells was enriched by PERC45 (67.1±2.3%) and PERC100 (65.5±1.7%) and the percentage of spermatozoa with primary

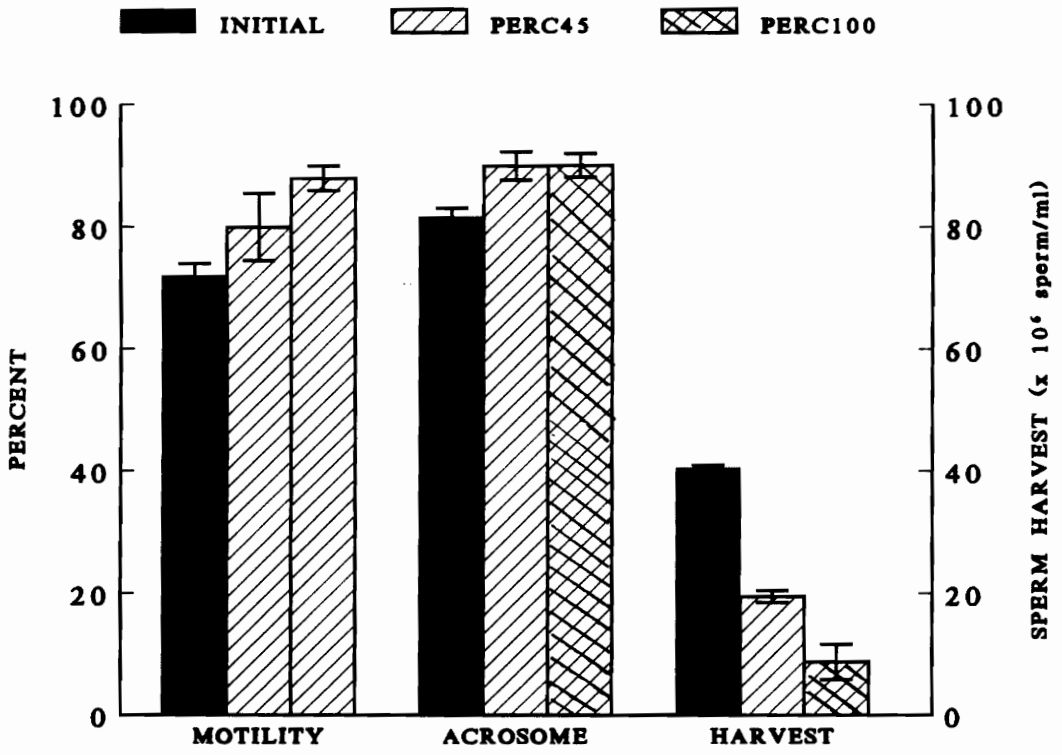

Figure 5.4. Means±SE for spermatozoal viability and concentration/harvest before and after separation (n = 5). Motility was improved by PERC100 and acrosomal integrity was increased by PERC45 and PERC100 ($P < .05$). Sperm harvest was reduced ($P < .05$) by both techniques (Exp. 1).

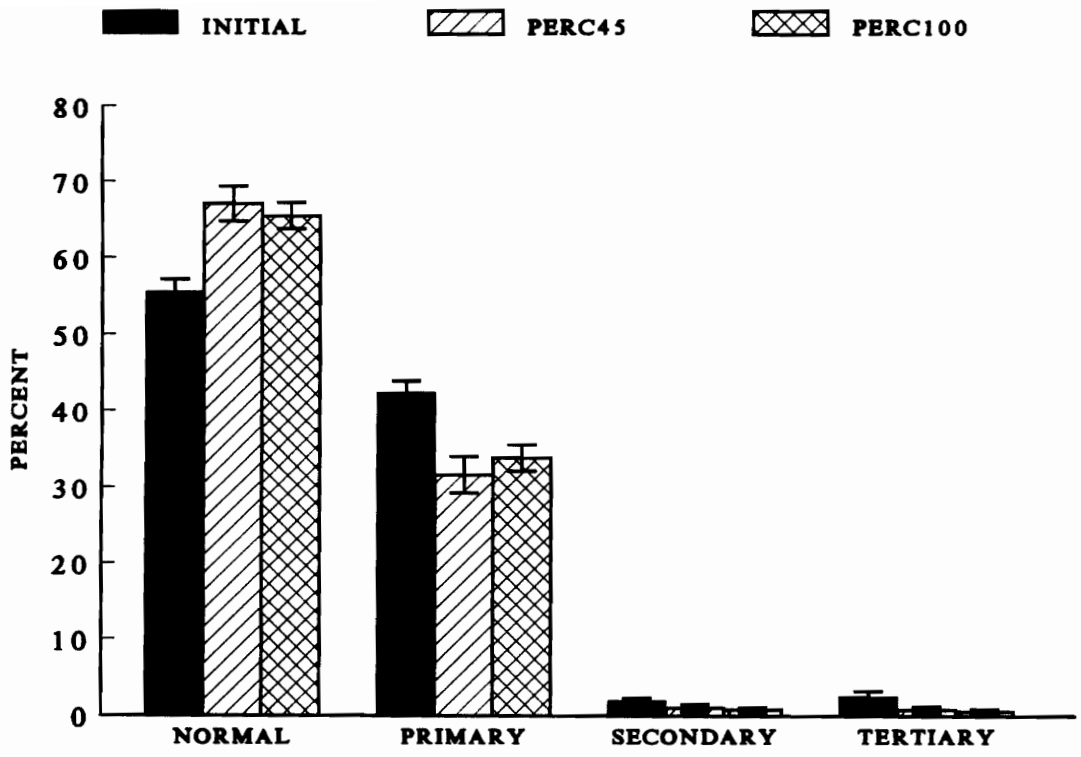

Figure 5.5. Means±SE for normal morphology, primary, secondary, and tertiary abnormalities before and after separation (n = 5). The percentage of normal cells were enriched (P < .05) by both Percoll® techniques. The percentage of spermatozoa with primary abnormalities were reduced (P < .05) by PERC45 and PERC100. The percentage of spermatozoa with tertiary abnormalities were reduced (P < .05) by PERC100 (Exp. 2).

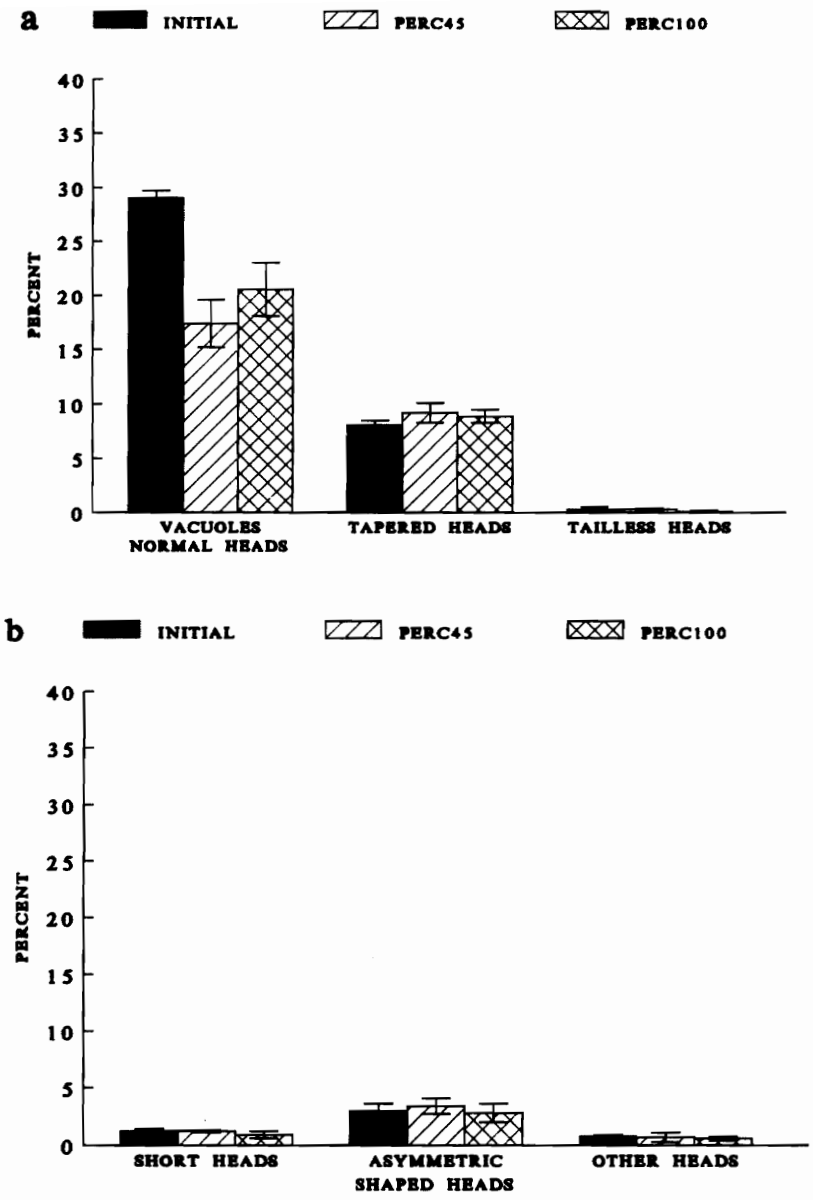

Figure 5.6. Means±SE for specific primary abnormalities before and after separation (n = 5). Only spermatozoa with nuclear vacuoles on normal shaped heads were reduced ($P < .05$) by the separation techniques (Exp. 2).

abnormalities (Figure 5.5) was reduced (P < .05) by PERC45 (31.7±2.4%) and PERC100 (33.9±1.5%). The percentage of spermatozoa with secondary abnormalities was unaffected by either Percoll® technique and the percentage of spermatozoa with tertiary abnormalities (Figure 5.5) was only reduced (P < .05) by PERC100 (.6±.3%). Both Percoll® techniques reduced the percentage of spermatozoa with nuclear vacuoles (Figure 5.6) on normal shaped heads 17.4±2.2% (PERC45) and 20.6±2.5% (PERC100). No other specific abnormality was affected by the Percoll® techniques.

The number of nuclear vacuoles per sperm head was counted on 100 cells (Figure 5.7). The number of vacuoles per head ranged from 1 to 8; however, only 1 to 5 were analyzed because of the low numbers of sperm with more than five vacuoles per head. In the initial sample proportions of spermatozoa with specific numbers of nuclear vacuoles per head were 17.0±2.3%, one; 25.83±.8%, two; 28.17±2.1%, three; 24.8±2.3%, four; and 4.5±1.1%, five. The percentage of sperm with one or two nuclear vacuoles per head was enriched (P < .05) by PERC45 (27.8±1.8% and 40.0±1.6%, respectively) and PERC100 (25.0±1.4% and 34.2±2.6%, respectively). The percentage of spermatozoa with three, four or five nuclear vacuoles per head was reduced (P < .05) by PERC45 (18.4±2.2%, 11.2±.7%, and .8±.2%, respectively). The 100% Percoll® did not reduce the percentage of spermatozoa with three nuclear vacuoles per head (27.2±1.2%) but did reduce (P < .05) the percentage of spermatozoa with four or five nuclear vacuoles per head (9.8±1.2%, and 1.0±.4%, respectively).

Analysis of variance tables are shown in Appendix C, Tables 4 - 7.

DISCUSSION

Density separation techniques have been used to improve viability and, to some extent, morphology (Forster et al., 1983; McClure et al., 1989; Gellert-

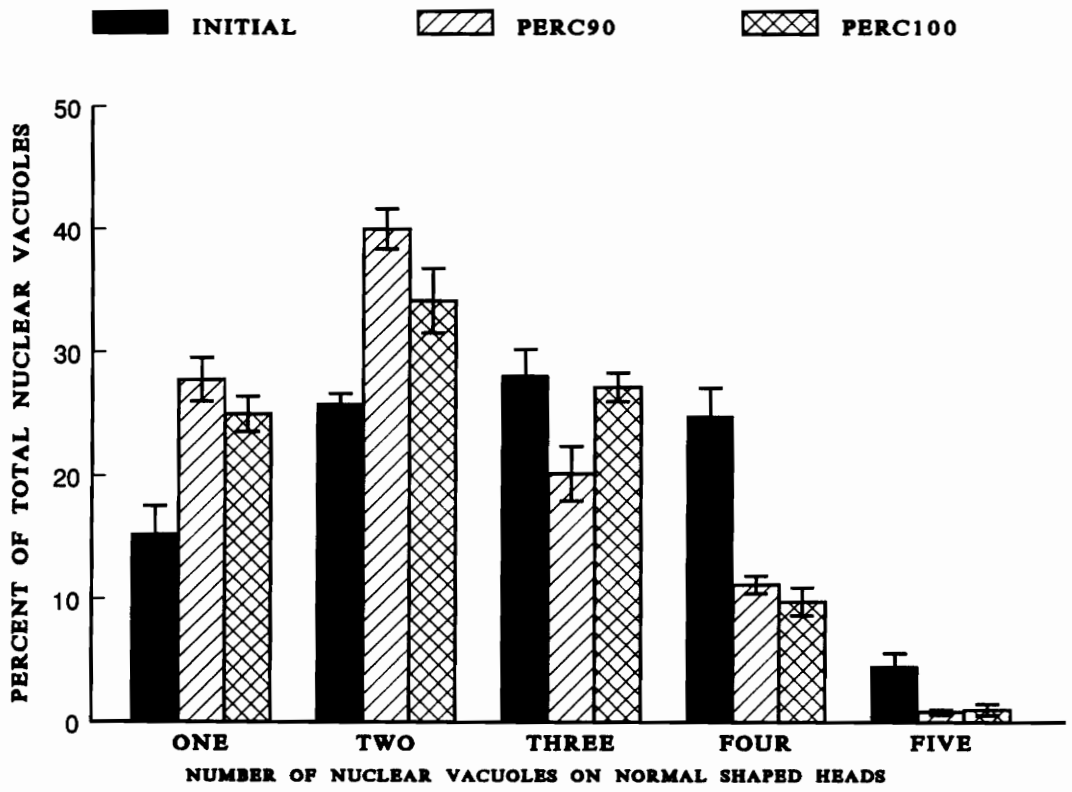

Figure 5.7. Means±SE for the number of nuclear vacuoles per sperm head before and after separation (n = 5). Spermatozoa with one and two nuclear vacuoles were enriched and those with four and five nuclear vacuoles were reduced ($P < .05$) by both Percoll® techniques ($P < .05$); however, only PERC45 reduced ($P < .05$) the percentage of spermatozoa with three nuclear vacuoles per head (Exp. 2).

Mortimer, 1988). None of these studies, however, critically evaluated the separation of spermatozoa with the nuclear vacuole defect. Because we know that these spermatozoa are implicated in male-related early embryonic death it becomes important to remove these cells from ejaculates. The bottom layer densities of the separation techniques were 1.12 g/ml (PERC45), 1.13 g/ml (PERC100), 1.16 g/ml (NYC 30), 1.21 g/ml (NYC40), and 1.24 g/ml (NYC45). The gradients in the present study were selected and prepared based strictly on the density of the medium. As the density of the Nycodenz® gradients increased above 27% the tonicity of the medium also increased above 290 - 300 mOsm. The osmolarities of the bottom Nycodenz® layers were 330 mOsm, NYC30; 420 mOsm, NYC40; and 485 mOsm, NYC45. Viability and sperm yield were greatly reduced as the density of the Nycodenz® medium increased (Figure 5.1). The reasons for the decreased viability in the lower Nycodenz® layers are not known. We speculate that the high osmotic pressure adversely affected the sperm as they passed through to the higher Nycodenz® densities. Alternately the dead sperm in the initial sample were more dense than the viable sperm; therefore, they were drawn to the higher densities. In the present study, the upper layers of the Nycodenz® gradients were not evaluated to determine the location of the viable cells.

Motility was not affected by the PERC45 separation in either Exp. 1 or Exp. 2; however, PERC100 improved the percentage of motile cells. The percentage of spermatozoa with intact acrosomes was not affected by PERC45 separation in Exp. 1; however, both Percoll® techniques in Exp. 2 increased the percentage of spermatozoa with intact acrosomes in Exp. 2 (Figures 5.1 and 5.4). These results were similar to previous studies when spermatozoal motility of the initial semen was ≥70%, as in the present study (Grant et al., 1994). When spermatozoal motilities were ≤60%, Percoll® separation techniques improved motility (Barthelemy et al., 1992; Gellert-Mortimer et al., 1988; Rhemrev et al. 1989). No separation

technique in Exp 1. improved the percentage of normal cells or reduced the percentage of primary, secondary or tertiary abnormalities (Figure 5.2). Other investigators have shown an increase in the percentage of normal spermatozoa following separation (Forster et al., 1983; LeLannou and Blanchard, 1988; McClure et al., 1989); however, these investigators did not evaluate spermatozoa with the nuclear vacuole defect on normal shaped heads which are very viable. Proportions of spermatozoa with specific head abnormalities (Exp. 1) were unaffected by any separation technique (Figure 5.3). In Exp. 2, the percentage of normal spermatozoa (Figure 5.5) was enriched by both Percoll® separation techniques. This enrichment was due primarily to the reduction in primary and tertiary abnormalities. The reduction in primary abnormalities occurred due to the reduction in the percentage of spermatozoa with the nuclear vacuoles on normal shaped heads (Figure 5.6) by both Percoll® separation techniques.

The percentage of spermatozoa with nuclear vacuoles was reduced by both 90% and 100% Percoll®; however, a complete removal of these spermatozoa did not occur. The selective removal of vacuolated spermatozoa is not completely understood. The nuclear vacuole defect has been described as one or a series of depressions or blisters at some point on the sperm head (Coulter et al., 1978; Johnson and Hurtgen, 1985). These blisters may be empty or fluid filled which would decrease the density of the cells and increase the area of the sperm head. Nuclear vacuoles can occur singly or with multiple vacuoles at different locations on the sperm head. It was found that sperm heads with increasing numbers of nuclear vacuoles increased in area measurements (Personal communication, John E. Chandler, Louisiana State University). Therefore, the numbers of nuclear vacuoles per sperm head were evaluated in Exp 2 (Figure 5.7). Spermatozoa with one and two nuclear vacuoles were enriched while the spermatozoa with three or more nuclear vacuoles were reduced. From this information we conclude that separation of spermatozoa with nuclear vacuoles on normal shaped heads did

occur; however, the Percoll® techniques were not completely effective since spermatozoa with one and two nuclear vacuoles per sperm head were enriched. These findings encourage us to further evaluate other density separation techniques with densities higher than the density of the Percoll® techniques and that are compatible to sperm survival.

IMPLICATIONS

This study shows that spermatozoa with nuclear vacuoles can be partially removed by existing density separation techniques. In view of the positive association of spermatozoa with nuclear vacuoles and male-related embryonic death, this work encourages further development of other separation procedures, with greater densities, for the removal of these spermatozoa. Successful separation of vacuolated sperm from semen would permit critical studies to determine if the vacuolated sperm are directly responsible for embryonic development failure, or if they simply reflect disturbances in other cells of the semen. If they are directly responsible for embryonic death, their removal could be of economic importance to the IVF and AI industries.

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GENERAL CONCLUSIONS

Mild insulation to the entire scrotal area of bulls for 48-h results in a decrease in the quality of spermatozoa (from day 10 to 35) with no change in sperm output; whereas, a similar mild insulation restricted to the scrotum in the area of the vascular cone is without effect. Full scrotal insulation causes an increase in ejaculation of spermatozoa with both diminished viability and normal morphology. The time to appearance and duration of appearance of specific abnormal spermatozoa in ejaculates of such bulls indicates that spermatozoa undergoing spermiogenesis, at the time of insulation, were affected. The abnormalities associated with the full insulation were due to heat stress, specifically, and were not associated with active restraint or environmental conditions under which the testes were insulated. This was apparent from the lack of response in the control bulls and bulls with partial scrotal insulation which were also restrained under the same environmental conditions. Further work is necessary to establish the importance of heat dissipation from the region of the vascular cone to sperm production.

Ejaculates from full scrotal insulation bulls were cryopreserved and used to evaluate selected *in vitro* spermatozoal separation techniques (Swim-up, Percoll®, glass wool, and Sephadex) for the removal of abnormal spermatozoa. Based on these findings, glass wool filtration was without out effect and will be eliminated from further discussion. From these studies, we can conclude that separation of abnormal spermatozoa was based on the severity of spermatozoal head distortions. The more subtle abnormalities were unaffected by any separation technique; whereas, distinct and severe abnormalities were removed by Percoll® separation and Sephadex filtration. Sephadex filtration was the only separation technique to enriched normal spermatozoa and no technique reduced spermatozoa with primary (head) abnormalities; however, these general classifications are

dependent upon the sum of enrichment and reduction of specific abnormalities. Pyriform shaped spermatozoa and protoplasmic droplets were removed by all separation techniques and short heads were removed by Sephadex filtration. Tertiary (tail) abnormalities were removed by both Percoll® techniques and Sephadex filtration. Spermatozoa with nuclear vacuoles were enriched, at least, slightly, by all separation techniques and tailless head spermatozoa were enriched by Percoll® separation. It appears from these findings, that Percoll® separation and Sephadex filtration were the most reliable systems for removal of abnormal spermatozoa, especially those with distinct and severe head distortions. These systems also had a greater yield of spermatozoa following separation than swim-up separation. Swim-up separation was very inefficient at recovering spermatozoa, although the recovered sperm were very viable.

Density separation techniques (Percoll® and Nycodenz® techniques) were evaluated for the removal of spermatozoa with nuclear vacuoles on, otherwise, normal shaped heads. Viability and morphology of Nycodenz® separated spermatozoa was reduced from the initial sample. The reason for this is not known; however, it could be due to the high osmotic pressure of the Nycodenz® medium since the medium was prepared solely on density characteristics. Percoll® separation techniques enriched viability and normal spermatozoa and reduced spermatozoa with nuclear vacuoles on normal shaped heads. When the number of nuclear vacuoles per sperm head were evaluated, we found that those spermatozoa with more than three nuclear vacuoles per sperm head were reduced; however, these techniques were unable to separate spermatozoa with one or two nuclear vacuoles per sperm head. The reason for the selective separation based on the number of nuclear vacuoles per sperm head not understood; however, we speculate that those spermatozoa with one or two nuclear vacuoles are more dense than those with more than three nuclear vacuoles per sperm head and normal spermatozoa are, in turn, more dense than

all spermatozoa with nuclear vacuoles. The density of the 90% (1.12 g/ml) and the 100% (1.13 g/ml) Percoll® media were not dense enough to separate those cells with only one or two nuclear vacuoles per head. Thus, based upon these results, there is strong indication that further research is needed on the separation of subtle abnormalities using density separation techniques with greater densities than Percoll®.

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APPENDICES

Appendix A. Table 1. Analysis of variance of initial motility, intact acrosome, and sperm output for full and partial insulated bulls and control bulls.

Source	Initial Motility		Intact Acrosome		Sperm Output	
	Mean Square	P	Mean Square	P	Mean Square	P
Season	1018.04	.0002	205.32	.1317	13.53×10^7	.0038
Trt	556.54	.0026	948.43	.0013	1.48×10^7	.4269
Season*Trt	210.06	.0354	503.87	.0053	1.12×10^7	.6158
Bull(Season*Trt)[a]	61.43	.0006	88.20	.0390	1.64×10^7	.0001
Period	584.11	.0001	1166.30	.0002	1.64×10^5	.8128
Season*Period	509.27	.0001	745.59	.0004	3.77×10^6	.0040
Trt*Period	623.16	.0001	1673.07	.0001	3.35×10^6	.0074
Season*Trt*Period	143.31	.0010	630.94	.0001	1.84×10^6	.0435
Bull(Season*Trt)*Period[a]	31.49	.0813	104.12	.0012	7.84×10^5	.9891
Day(Period)	74.12	.0001	165.84	.0001	5.99×10^6	.0001
Season*Day(Period)	39.11	.0082	77.50	.0306	2.43×10^6	.0383
Trt*Day(Period)	70.86	.0001	180.65	.0001	2.11×10^6	.1188
Season*Trt*Day(Period)	21.53	.5534	74.74	.0133	1.66×10^6	.3993
Error[a]	22.26		49.80		1.60×10^6	

[a]All interactions with bull served as the error term for the main effects and their interactions.

Appendix A. Table 2. Analysis of variance of total sperm abnormalities, primary, secondary, and tertiary abnormalities for full and partial insulated bulls and control bulls.

Source	Total Abnormal		Primary		Secondary		Tertiary	
	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Season	3639.11	.0020	243.73	.4246	1515.22	.0001	4384.16	.0014
Trt	4437.45	.0008	1348.09	.0214	1084.71	.0001	1597.36	.0445
Season*Trt	1725.34	.0130	1391.63	.0080	664.29	.0002	879.66	.1282
Bull(Season*Trt)[a]	377.45	.0001	268.59	.0001	61.10	.0010	414.14	.0001
Period	7221.36	.0001	2373.07	.0001	514.38	.0001	1252.11	.0001
Season*Period	1053.91	.0001	548.18	.0001	294.20	.0003	186.18	.0078
Trt*Period	4199.14	.0001	2345.25	.0001	852.81	.0001	84.76	.1319
Season*Trt*Period	843.09	.0001	459.30	.0001	461.83	.0001	89.08	.0776
Bull(Season*Trt)*Period[a]	89.72	.0862	56.93	.6277	40.05	.0132	44.02	.4533
Day(Period)	835.09	.0001	850.78	.0001	134.12	.0001	158.06	.0001
Season*Day(Period)	136.85	.0005	134.07	.0006	79.92	.0001	56.64	.1308
Trt*Day(Period)	453.90	.0001	824.22	.0001	166.59	.0001	119.01	.0001
Season*Trt*Day(Period)	98.76	.0088	137.38	.0001	92.89	.0001	64.89	.0144
Error[a]	63.95		63.58		23.16		43.47	

[a]All interactions with bull served as the error term for the main effects and their interactions.

Appendix A. Table 3. Analysis of variance of initial motility, intact acrosome and sperm output for full scrotal insulated bulls.

Source	Initial Motility		Intact Acrosome		Sperm Output	
	Mean Square	P	Mean Square	P	Mean Square	P
Season	838.26	.0002	1003.36	.0286	33.0×10^6	.0548
Bull(Season)[a]	16.58	.8345	145.74	.2700	6.74×10^6	.0010
Period	1934.59	.0001	4856.92	.0001	4.29×10^6	.0416
Season*Period	729.60	.0002	2115.40	.0014	6.89×10^6	.0044
Bull(Season)*Period[a]	54.44	.1298	239.16	.0223	1.02×10^6	.8145
Day(Period)	189.29	.0001	558.86	.0001	5.01×10^6	.0003
Season*Day(Period)	37.46	.4196	226.20	.0042	2.14×10^6	.1528
Error[a]	35.84		113.27		1.63×10^6	

[a]All interactions with bull served as the error term for the main effects and their interactions.

Appendix A. Table 4. Analysis of Variance of total sperm abnromalities, primary, secondary, and tertiary sperm abnormalities for full scrotal insulated bulls.

Source	Total Abnormal		Primary		Secondary		Tertiary	
	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Season	4696.60	.0035	1458.44	.1201	2661.10	.0009	216.83	.3164
Bull(Season)[a]	279.61	.0246	473.38	.0019	93.61	.0817	154.62	.0298
Period	1652.00	.0001	7634.50	.0001	2359.77	.0001	929.15	.0003
Season*Period	2196.29	.0001	1439.59	.0004	1272.12	.0001	24.56	.7554
Bull(Season)*Period[a]	147.52	.0001	121.25	.4845	87.46	.0560	52.00	.6264
Day(Period)	1662.23	.0001	2669.34	.0001	495.28	.0001	310.73	.0001
Season*Day(Period)	251.30	.0008	406.84	.0001	278.58	.0001	67.70	.3844
Error[a]	109.96		125.31		48.31		63.18	

[a]All interactions with bull served as the error term for the main effects and their interactions.

Appendix A. Table 5. Analysis of Variance of pyriform shaped heads, crater defect, diadem defect, and tailless heads for full scrotal insulated bulls.

Source	Pyriform Shaped Heads		Crater Defect		Diadem Defect		Tailless Heads	
	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Season	46.96	.0413	469.33	.0246	95.99	.0665	136.19	.1949
Bull(Season)[a]	8.75	.3475	64.20	.3281	31.79	.9545	62.64	.6830
Period	57.57	.0013	2397.09	.0001	645.85	.0001	818.50	.0014
Season*Period	47.44	.0342	503.84	.0001	113.29	.0110	125.60	.1883
Bull(Season)*Period[a]	12.84	.0856	33.87	.8230	21.49	.9942	68.69	.7265
Day(Period)	65.70	.0001	1132.07	.0001	740.17	.0001	588.53	.0001
Season*Day(Period)	27.15	.0001	243.92	.0001	141.60	.0244	111.55	.2674
Error[a]	7.70		54.85		84.09		95.09	

[a]All interactions with bull served as the error term for the main effects and their interactions.

Appendix A. Table 6 . Analysis of variance of severely misshapen heads, acrosomal abnormallities, and other misshapen heads for full scrotal insulated bulls.

Source	Severely Misshapen Heads		Acrosomal Abnormalities		Dag Defect	
	Mean Square	P	Mean Square	P	Mean Square	P
Season	8.90	.0873	82.51	.0007	18.96	.2271
Bull(Season)[a]	2.37	.6351	2.71	.9818	9.89	.0710
Period	22.58	.0020	127.53	.0001	16.35	.0085
Season*Period	8.21	.0278	48.06	.0023	8.07	.0377
Bull(Season)*Period[a]	2.06	.8156	6.09	.9589	2.24	.9357
Day(Period)	10.35	.0002	29.61	.0202	14.23	.0005
Season*Day(Period)	6.52	.0047	18.70	.2028	11.02	.0010
Error[a]	3.29		15.04		4.92	

[a]All interactions with bull served as the error term for the main effects and their interactions.

Appendix B. Table 1. Analysis of variance for motility and intact acrosome for different Percoll separation techniques.

	Motility		Intact Acrosome	
Source	Mean Square	P	Mean Square	P
Treatment	469.31	.0001	20.72	.9270
Rep	21.60	.1908	73.47	.6414
Residual	11.18		99.71	

Appendix B. Table 2. Analysis of variance for normal morphology, primary, secondary, and tertiary abnormalities for different Percoll separation techniques.

	Normal		Primary		Secondary		Tertiary	
Source	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	147.18	.0256	78.28	.0965	224.21	.0001	36.44	.0110
Rep	41.09	.3221	22.04	.5937	4.37	.1852	7.98	.2948
Error	29.41		27.41		2.23		5.37	

Appendix B. Table 3. Analysis of variance for pyriform shaped heads, nuclear vacuoles, tailless heads, and short heads for different Percoll separation methods.

Source	Nuclear Vacuoles		Pyriform Shaped Heads		Tailless Heads		Short Heads	
	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	71.11	.0311	68.59	.0001	63.16	.4403	141.77	.1091
Rep	23.95	.2718	2.74	.1894	110.51	.2095	22.27	.8452
Error	15.29		1.41		60.27		52.74	

Appendix B. Table 4. Analysis of variance for asymmetric heads, acrosomal abnormalities, pinched neck, and severely misshapen heads for separation of abnormal spermatozoa using different Percoll separation techniques.

Source	Asymmetric Heads		Acrosomal Abnormalities		Severely Misshapened Heads		Other Head Abnormalities	
	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	0.65	.9128	0.14	.6717	0.94	.1117	14.48	.6812
Rep	12.08	.4615	21.45	.2045	0.29	.5895	40.79	.2491
Error	11.53		0.23		0.35		24.65	

Appendix B. Table 5. Analysis of variance for subtle, distinct, and severe heads for separation of spermatozoa using different Percoll separation techniques.

	Subtle		Distinct		Severe	
Source	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	144.90	.0572	104.84	.1468	33.66	.0440
Rep	47.64	.3978	62.71	.3290	6.83	.5833
Residual	8.52		45.56		8.32	

Appendix B. Table 6. Analysis of variance for concentration/sperm harvest, motility, and acrosomal integrity for separation of spermatozoa using two-layer Percoll separation, three-layer Percoll separation, swim-up separation, and glass wool filtration.

	Concentration/ Harvest		Motility		Intact Acrosome	
Source	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	1833.75	.0001	830.00	.0002	292.18	.0275
Rep	300.11	.1126	70.00	.4615	136.60	.1999
Error	135.11		75.00		80.53	

Appendix B. Table 7. Analysis of variance for normal morphology, primary, secondary, and tertiary abnormalities for separation of spermatozoa using two-layer Percoll separation, three-layer Percoll separation, swim-up separation, and glass wool filtration.

	Normal		Primary		Secondary		Tertiary	
Source	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	29.84	.0041	27.29	.0295	481.49	.0001	54.46	.0001
Rep	4.99	.4422	6.17	.5413	1.24	.9945	2.46	.7100
Error	5.05		7.68		23.91		4.57	

Appendix B. Table 8. Analysis of variance for pyriform shaped heads, nuclear vacuoles on normal sperm, tailless heads, and short heads using two-layer Percoll separation, three-layer Percoll separation, swim-up separation, and glass wool filtration.

	Nuclear Vacuoles		Pyriform Shaped Heads		Tailless Heads		Short Heads	
Source	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	156.32	.0012	128.79	.0001	578.58	.0002	52.20	.4689
Rep	45.35	.1136	1.04	.9750	107.45	.1340	30.50	.7045
Error	20.49		8.94		52.15		55.86	

Appendix B. Table 9. Analysis of variance for asymmetric heads, acrosomal abnormalities, severely misshapen heads, and other head abnormalities using two-layer Percoll separation, three-layer Percoll separation, swim-up separation, and glass wool filtration.

	Asymmetric Heads		Acrosomal Abnormalities		Severely Misshapen Heads		Other Head Abnormalities	
Source	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	8.87	.1502	1.39	.1778	1.46	.1750	59.00	.0993
Rep	23.39	.0073	0.29	.8224	1.11	.2847	53.13	.1274
Error	4.53		0.77		0.80		25.22	

Appendix B. Table 10. Analysis of variance for subtle, distinct, and severe abnormalities using two-layer Percoll separation, three-layer Percoll separation, swim-up separation, and glass wool filtration.

	Subtle		Distinct		Severe	
Source	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	613.19	.0001	393.87	.0003	44.88	.0002
Rep	18.92	.7624	64.47	.2195	2.88	.6212
Error	29.49		39.98		4.28	

Appendix B. Table 11. Analysis of variance for concentration/sperm harvest, motility, and acrosomal integrity using Sephadex® filtration.

Source	Concentration/ Harvest		Motility		Intact Acrosome	
	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	3276.10	.0011	4000.00	.0002	2250.00	.0070
Rep	23.31	.7441	35.00	.3762	14.71	.9426
Error	47.16		25.00		86.31	

Appendix B. Table 12. Analysis of variance for normal morphology, primary, secondary, and tertiary abnormalities using Sephadex® filtration.

Source	Normal		Primary		Secondary		Tertiary	
	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	284.09	.0499	126.03	.2976	368.45	.0952	162.41	.0002
Rep	24.86	.6432	34.15	.8093	3.76	.9938	3.83	.0862
Error	36.78		88.03		77.86		0.85	

Appendix B. Table 13. Analysis of variance for pyriform shaped heads, nuclear vacuoles on normal heads, tailless heads, and short heads using Sephadex® filtration.

Source	Nuclear Vacuoles		Pyriform Shaped Heads		Tailless Heads		Short Heads	
	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	366.00	.0341	145.92	.0087	1334.02	.0008	19.6	.6843
Rep	43.79	.4330	2.90	.7660	28.04	.3093	53.35	.7282
Error	36.59		6.34		102.35		16.43	

Appendix B. Table 14. Analysis of variance for asymmetric heads, acrosomal abnormalities, severely misshapen heads and other head abnormalities using Sephadex® filtration.

Source	Asymmetric Heads		Acrosomal Abnormalities		Severely Misshapened Heads		Other Head Abnormalities	
	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	29.58	.4098	0.53	.4562	5.93	.7593	0.23	.2080
Rep	22.88	.6546	1.27	.3227	27.41	.7421	0.10	.5000
Error	34.98		0.78		0.10		55.07	

Appendix B. Table 15. Analysis of variance for subtle, distinct, and severe abnormalities from abnormal ejaculates using Sephadex® filtration.

Source	Subtle		Distinct		Severe	
	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	635.21	.0101	487.20	.0216	190.97	.0039
Rep	40.70	.3891	73.19	.2572	3.24	.6797
Error	21.69		36.34		5.33	

Appendix C. Table 1. Analysis of variance for the separation of spermatozoal viability and concentration/harvest from ejaculates with a high concentration of spermatozoa with nuclear vacuoles using Percoll® and Nycodenz® density gradients.

	Concentration/ Sperm Harvest		Motility		Acrosomal Integrity	
Source	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	1297.61	.0001	5278.96	.0001	2137.62	.0001
Rep	21.42	.0772	348.96	.0551	116.32	.0458
Error	8.30		37.57		119.96	

Appendix C. Table 2. Analysis of variance for the separation of normal spermatozoa, primary, secondary, and tertiary abnormalities from ejaculates with a high concentration of spermatozoa with nuclear vacuoles using Percoll® and Nycodenz® density gradients.

	Normal		Primary		Secondary		Tertiary	
Source	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	221.86	.0002	143.07	.0021	1.80	.0767	17.23	.0030
Rep	93.19	.0109	100.08	.0097	4.32	.0041	6.99	.0780
Error	19.97		20.79		0.73		2.72	

Appendix C. Table 3. Analysis of variance for the separation of specific head abnormalities from ejaculates with a high concentratin of nuclear vacuoles using Percoll® and Nycodenz® gradients.

	Nuclear Vacuoles Normal Heads		Tapered Heads		Tailless Heads	
Source	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	73.59	.0545	9.43	.2120	1.04	.1087
Rep	27.64	.3914	23.64	.0175	.60	.3108
Error	25.19		5.74		0.46	

Appendix C. Table 4. Analysis of variance for the separation of specific head abnormalities from ejaculates with a high concentration of nuclear vacuoles using Percoll and Nycodenz gradients.

	Short Heads		Asymmetric Heads		Other Heads	
Source	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	1.19	.1724	1.00	.0251	.81	.1324
Rep	1.72	.0717	13.03	.0001	.23	.6849
Error	0.65		0.43		0.39	

Appendix C. Table 5. Analysis of variance for the separation of spermatozoal viability and concentration/harvest from ejaculates with a high concentration of nuclear vacuoles using Percoll® density gradients.

	Concentration/ Sperm Harvest		Motility		Acrosomal Integrity	
Source	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	1300.32	.0001	320.00	.0098	119.02	.0026
Rep	24.90	.1674	116.67	.0767	39.03	.0337
Error	11.65		36.67		8.66	

Appendix C. Table 6. Analysis of variance for the separation of normal spermatozoa and primary, secondary, and tertiary abnormalities from ejaculates with a high concentration of nuclear vacuoles using Percoll® density gradients.

	Normal		Primary		Secondary		Tertiary	
Source	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	194.02	.0058	159.65	.0093	1.72	.1988	5.45	.0378
Rep	18.07	.4717	18.13	.4570	.78	.5077	1.23	.4038
Error	18.52		17.96		0.86		1.08	

Appendix C. Table 7. Analysis of variance for the separation of specific head abnormalities from ejaculates with a high concentration of nuclear vacuoles using Percoll® density gradients.

	Nuclear Vacuoles Normal Heads		Tapered Heads		Tailless Heads	
Source	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	82.82	.0094	1.62	.5963	.07	.6561
Rep	16.35	.5622	.69	.9102	.03	.9494
Error	20.65		2.93		0.15	

Appendix C. Table 8. Analysis of variance for the separation of normal spermatozoa and primary, secondary, and tertiary abnormalities from ejaculates with a high concentration of nuclear vacuoles using Percoll® density gradients.

	Short Heads		Asymmetric Heads		Other Heads	
Source	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	.15	.4521	.47	.8048	.05	.8393
Rep	.36	.1730	3.19	.2827	.39	.3161
Error	0.17		2.09		0.28	

Appendix C. Table 9. Analysis of variance for the separation of spermatozoa with specific numbers of nuclear vacuoles per sperm head using Percoll® density gradients.

	One		Two		Three		Four		Five	
Source	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P	Mean Square	P
Treatment	185.20	.0077	259.02	.0004	177.20	.0004	404.36	.0001	22.77	.0039
Rep	14.94	.5994	25.03	.1270	34.28	.0281	18.43	.1943	3.43	.2160
Error	19.51		10.31		7.43		9.54		1.89	

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EDUCATION

DEGREE: Doctor of Philosophy, Virginia Polytechnic Institute and State University
Advisor: Dr. Richard G. Saacke
Major: Animal Science/Dairy
Title of Proposed Dissertation: Separation of Abnormal Spermatozoa From Semen Produced By Bulls With Impaired Testicular Thermoregulation
Expected Graduation Date: May, 1995

DEGREE: Master of Science, Louisiana State University, August 1990
Advisor: Dr. John E. Chandler
Major: Dairy Science
Title of Thesis: Nanovid Microscopy and Sperm Physiology

DEGREE: Bachelor of Science, Louisiana State University, May 1987
Major: Dairy Science

EMPLOYMENT EXPERIENCE

August 1990 - May 1995

Virginia Polytechnic Institute and State University, Blacksburg VA.

Graduate Research Assistant

TEACHING:

- Assist in teaching undergraduate and graduate courses in reproductive physiology
- Subjects include: anatomy, physiology, endocrinology, histology, spermatogenesis, oogenesis, embryology, sexual differentiation, fertilization, placentation, parturition, palpation and artificial insemination

RESEARCH:

- Bovine testicular thermoregulation
- Role of the male in early embryonic mortality
- Separation of abnormal spermatozoa from bovine ejaculates used for *in vitro* fertilization

EMPLOYMENT EXPERIENCE (CONT'D)

RESPONSIBILITIES:

·Microscopic evaluation of semen using phase contrast and differential interference contrast microscopy
·Preparation of bovine semen for *in vitro* fertilization
·Preparation and collection of bulls by artificial vagina
·Preparation of semen for cryopreservation
·assisting in embryo flushing for collection of 6 d embryos
·assisting in searching and evaluating embryo quality

January 1988 - August 1990 **Louisiana State University**; Baton Rouge, LA

Graduate Research Assistant

TEACHING:

·undergraduate courses in reproductive physiology

RESEARCH:

·In vitro capacitation and acrosome reaction of Bovine spermatozoa

RESPONSIBILITIES:

·Microscopic evaluation of semen using phase contrast, differential interference contrast and nanovid microscopy and enhance video image processing
·Performing *in vitro* fertilization using human and bovine spermatozoa
·Performing sperm penetration assay test (hamster test) and assist in human semen processing

May 1985 - December 1987 **Louisiana State University Dairy Research Facility**; Baton Rouge, LA

Student employee

RESPONSIBILITIES:

·Milking, heat detection, mastitis treatment, calf management, calf delivery, and herd health
·Assist farm manager in general duties
·Training and supervising junior employees
·Assisting in preparing and showing dairy heifers and cows

PUBLICATIONS

Degelos, S.D., M.P. Wilson, and J. E. Chandler. 1994. Nanovid microscopy for assessing sperm membrane changes induced by *in vitro* capacitating and acrosome reacting procedures. J. Androl. 15(5):462.

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PRESENTATIONS

1994 Spermatozoal characteristics following separation using two-layer and three-layer Percoll gradient centrifugation and swim-up separation designed for in vitro fertilization. American Dairy Science Association, Minneapolis, MN

1993 Effect of full and partial scrotal insulation on semen quantity and quality in young Holstein bulls. American Dairy Science Association, College Park, MD

1990 Nanovid microscopy evaluation of heparin and concanavalin-A treated bull sperm. American Dairy Science Association, Raleigh, NC

1989 Effect of extender, storage time, and *in vitro* fertilization preparation on bovine semen quality. American Dairy Science Association, Louisville, KY

MEETINGS ATTENDED

1994 National American Dairy Science Association Annual Meeting, Minneapolis, MN

1994 15th Technical Conference on Artificial Insemination and Reproduction, National Association of Animal Breeders, Milwaukee, WS

1993 National American Dairy Science Association Annual Meeting, College Park, MD

1993 Annual Conference of the International Embryo Transfer Society, Baton Rouge, LA

1992 Annual Meeting of the American Society of Andrology, Bethesda, MD

1992 14th Technical Conference on Artificial Insemination and Reproduction, National Association of Animal Breeders, Milwaukee, WS

1992 Annual Conference of the Society for the Study of Reproduction, Raleigh, NC

1992 Annual Meeting of the American Society of Animal Science, Pittsburgh, PA

1990 13th Technical Conference on Artificial Insemination and Reproduction, National Association of Animal Breeders, Milwaukee, WS

1990 National American Dairy Science Association Annual Meeting, Raleigh, NC

1989 National American Dairy Science Association Annual Meeting, Louisville, KY

MEETINGS ATTENDED (CONT'D)

1989	Southern Section Meeting of the American Dairy Science Association, Little Rock, AK
1988	Southern Section Meeting of the American Dairy Science Association, New Orleans, LA

HONORS AND ACHIEVEMENTS

1994	Sigma Xi, The Scientific Research Honor Society
1993	Gamma Sigma Delta, The Honor Society of Agriculture
1993	Outstanding Teaching Assistant
1989	Judge - Louisiana State University All-American Showmanship Contest
1989	Judge - West Baton Rouge Parish Dairy Show
1985-1986	Vice-President Louisiana State University Dairy Science Club
1985-1986	Louisiana State University Dairy Cattle Judging team
1985,1986	Morgan W. Walker Scholarship
1985	Dairy Science Alumni Association Scholarship
1985	Glenn O. Dunmire Outstanding First Year Member Award and Scholarship (Dairy Science Club)
1984	Top Ten Percent in Louisiana State University College of Agriculture
1983	Alpha Lambda Delta Honor Society
1982	University of Southwestern Louisiana Agriculture Scholarship

MEMBERSHIPS

Sigma Xi
Gamma Sigma Delta
Alpha Lambda Delta
American Dairy Science Association
Society for the Study of Reproduction

COURSE WORK AND LABORATORY SKILLS

Comparative Reproductive Physiology

- comparative mechanisms of all major aspects of male and female reproductive physiology in domestic and laboratory animals

COURSE WORK AND LABORATORY SKILLS (CONT'D)

Advanced Physiology

·comparative mechanisms of all major aspects of digestion, endocrinology, and male and female reproductive physiology in domestic animals

Membrane Physiology

·general topics of membrane physiology with emphasis on mechanisms involved in the transduction of extracellular signals into physiological changes with in the cell

Physiological Experimentation

·current concepts and techniques (including surgical) used in physiological research

·surgical procedures include anesthetization, castration, vasectomy, ovariectomy, tubal ligation, total hysterectomy, and catheterization

·mechanics of grant proposal and manuscript preparation and revision

Molecular Biology of the Cell

·current concepts of the molecular organization of animal and plant cells and the biosynthesis and assembly of cellular structures

Histology

·microscopic evaluation of the structure of tissues and organs

College Teaching

·teaching/learning process and consideration of teaching methods employed to encourage, guide and evaluate college students' learning

Experimental statistics, I and II

·student's t distribution, analysis of variance, co-variance, multiple regression, curvilinear regression, experimental design including latin square design, factorial analysis, split plot design, and the use of statistical analysis system (SAS)

REFERENCES

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