Bovine sperm abnormalities: prevalence, etiology and mechanisms leading to infertility
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Introduction
Sperm morphology is an excellent predictor of the outcome of natural mating\textsuperscript{1,2} and artificial insemination (AI)\textsuperscript{3,4} and success of in vitro fertilization\textsuperscript{5} in animals and humans. Bovine sperm abnormalities are classified based on origin (primary, secondary or tertiary), effect on fertility (major vs minor), location of the sperm defect (head, midpiece, or tail), or whether its effects on fertility can be ameliorated by increasing the number of sperm in the inseminate (compensable vs uncompensable).\textsuperscript{6} Although prevalence of various types of sperm abnormalities is low in the ejaculates and ~ 83% of bulls subjected to a traditional breeding soundness examination are classified as satisfactory breeders exclusively based on sperm morphology, it is noteworthy that 17% of bulls subjected to breeding soundness evaluation were designated unsatisfactory solely on the basis of sperm morphology, highlighting its importance in Alberta.\textsuperscript{7} Although an association between sperm abnormalities and infertility has been established and functional impairments of sperm abnormalities have been documented,\textsuperscript{8} the molecular basis of impaired function of abnormal sperm remains unknown. Our recent studies demonstrated that expression of sperm proteins are altered in abnormal sperm, thereby affecting fertility. Since sperm DNA is transcriptionally inactive, content of sperm proteins in the mature sperm influences fertilizing ability. This paper summarizes current knowledge regarding the prevalence, etiology and mechanisms of infertility due to abnormal bovine sperm.

Prevalence of abnormal sperm morphology in Alberta beef bulls
We recently conducted a survey to determine the prevalence of sperm abnormalities in beef bulls in Alberta, Canada, and the percentage of satisfactory potential breeders based solely on normal sperm morphology. Eosin-nigrosin stained semen smears and evaluation reports of 1642 bull breeding soundness evaluations were procured from six veterinary clinics in Alberta. Sperm morphology was determined for at least 100 sperm per bull. The most common defects were detached head (4.86±5.71%; mean±SD), distal midpiece reflex (6.19±9.13%), and bent tail (1.01±1.54%). Overall, solely on the basis of sperm morphology, 1363 (83.0%) bulls were classified as satisfactory potential breeders, with the remainder 279 (17.0%) deemed unsatisfactory (>30% abnormal sperm, >20% defective heads, or both). That 17% of bulls subjected to breeding soundness evaluation were designated unsatisfactory solely on the basis of sperm morphology highlighted its importance.\textsuperscript{7}

Abnormal sperm morphology

Causes of abnormal sperm morphology
The most common causes of abnormal spermatogenesis in bulls include: 1) abnormal thermoregulation of the testes due to heat, frostbite or fat deposition around the scrotal neck; 2) hormonal imbalances associated with stress; and 3) effects of toxins and expression of deleterious genes.\textsuperscript{9} Consistent with this, Chenoweth et al\textsuperscript{10} listed environmental factors as the most common cause of abnormal sperm structures; however, they also noted that increasing numbers of defects are being considered as of a genetic origin.\textsuperscript{10} Increased scrotal temperature impairs spermatogenesis by increasing the metabolic rate and the oxygen demand. However, since the blood flow to the testis does not increase in response to this increased metabolic demand, this causes hypoxia of the testicular tissue, disrupts spermatogenesis, and can lead to abnormal sperm production.\textsuperscript{9} Stress can have numerous causes, including the environment, illness, or injury. Stress typically results in elevated systemic cortisol concentrations, decreased secretion of luteinizing hormone and testosterone, which cause hormonal imbalances and thus affect spermatogenesis.\textsuperscript{11} Reported effects of specific genes on sperm morphology are reviewed under specific sperm abnormalities.
Acrosomal abnormalities

Bull sperm (fresh or frozen-thawed) must have a tightly adherent, intact acrosome with a smooth surface and a distinct, uniformly shaped apical ridge. During spermiogenesis, an acrosome arises from the Golgi complex and spreads over the anterior surface of the spermatic nucleus. The abundance of microtubules in the Sertoli cell cytoplasmic processes surrounding the elongating spermatids suggests involvement of microtubules in sperm head shaping and transport of sperm organelle. Microtubules are believed to have a major role in trafficking acrosomal vesicles from the Golgi apparatus to the sperm nucleus, a critical step in acrosome development.

The acrosome contains a variety of enzymes that facilitate fertilization by digesting the oocyte zona pellucida. Microtubules are present within this region, and inhibitors interfere with acrosome biogenesis by inhibiting the ability of Golgi-derived acrosomal vesicles to dock onto the nuclear membrane and coalesce into the pro-acrosomal vesicle.

Any aberrations including incomplete or irregular apical ridge, irregular acrosomal surface, grossly distorted acrosomes (swollen, ruffled or vacuolated) are classified as acrosomal abnormalities. These researchers demonstrated that the percentage of sperm with normal acrosomal morphology improved more slowly and had not reached mature levels by 16 wk after puberty (based on a study in beef bulls conducted almost 30 years ago). Therefore, further studies are warranted as changes in genetics, environment and nutrition may have influenced spermatogenesis, influencing the time interval between puberty and age at which bulls produce sperm with normal acrosomal morphology. Although acrosomal morphology may be improved during the peri-pubertal period, various acrosomal abnormalities have also been reported in mature bulls.

Classical knobbed acrosome defect in mature bulls has been described as a beaded appearance at the apex of the affected sperm. However, the more common form of this acrosomal defect is characterized by either flattened or indented sperm apex. Although stress, abnormal thermoregulation of testes or genetic causes are potential causes for abnormal spermatogenesis, the specific causes leading to flattened or indented acrosomes remain unknown. Concurrent appearance of a variety of sperm abnormalities, along with acrosomal abnormalities, are considered to be due to adverse environmental influence (stress or injury). However, a genetic cause should be considered when a high proportion of the sperm have acrosomal abnormalities and this defect is persistent in the absence of other sperm defects.

The knobbed acrosome sperm defect was associated with an autosomal sex-linked recessive mode of genetic transmission in Friesian breed and in boars.

Based on a Canadian study on beef bulls used for natural breeding, 0.53% bulls (n =1331) had sperm with knobbed acrosomes, ranging from 25 to 100% sperm, and 1.6 % of beef and dairy bulls (n = 371) with low nonreturn rates to artificial insemination had high proportions of knobbed acrosomes (25 to 95% of affected sperm) due to unknown causes. In contrast, Chenoweth reported an estimated genetic prevalence of 6.74% in an Angus herd. A deletion in the long arm of Y chromosome has been shown to be involved in acrosomal abnormalities described as "flat heads" in mice where acrosomes appeared damaged and often contained a vesicle, but lacked acrosomal proteinase. In mice deficient for TLF (TBP-like factor), a protein that is critical for spermatogenesis, acrosomal granules did not coalesce in stage II–III spermatids, but were present in the cytoplasm, on the outer membrane of the nucleus, or invaginated in the nucleus, or elongating spermatids at stages IX-XI. In these cases, acrosomes were vacuolated or not properly associated with the head. During normal spermiogenesis, acrosomal vesicular contents should be processed (for example, removal of lysosomal proteins) for normal acrosomal biogenesis. Any impairment in these maturational changes in the acrosome may lead to the development of an abnormal acrosome.

Acrosomal abnormalities due to toxins have been reported. Subcutaneous administration of bisphenol A for six days at 20 or 200 μg/kg per injection induced a variety of acrosomal abnormalities affecting the acrosomal vesicle and acrosomal cap.

The knobbed acrosome defect has been associated with infertility in many species, including pigs, horses and sheep. Bulls with a high percentage of sperm with indented acrosomes may have normal fertility when used for AI or single-sire mating; however, their fertility may be low when breeding...
competitively with bulls with normal spermiograms.\textsuperscript{25} We used an IVF and culture system to determine the effect of bovine sperm with flattened or indented acrosome on fertilization and early embryonic development.\textsuperscript{26} Results indicated that sperm with acrosomal abnormalities had a reduced ability to bind to the zona pellucida, depending upon the severity of the defect, and that these aberrant spermatozoa did not penetrate the zona pellucida. Furthermore, apparently normal spermatozoa co-existing in the inseminate of bulls with a high percentage of sperm with acrosomal abnormalities were also functionally deficient; oocytes penetrated by these spermatozoa had a reduced potential for fertilization, and resulting zygotes had a reduced ability for cleavage and embryonic development to the blastocyst stage. We subsequently reported that bovine sperm with flattened and indented acrosomes had altered plasma membrane functional integrity, which predisposed them to premature capacitation and acrosome reaction, contributing to their inability to interact with the oocyte.\textsuperscript{27} Although proteolytic activity of the acrosomal contents in the affected sperm appeared to be normal, sperm with the flattened or indented acrosomes also appeared to have a reduced ability to fuse with oolemma (demonstrated by a zona free oocyte-sperm fusion assay, coupled with confocal microscopy), impairing the ability of sperm with flattened and indented acrosome to penetrate ooplasm and undergo sperm chromatin decondensation.\textsuperscript{28}

Abnormal sperm head shape

During spermatogenesis, haploid spermatids undergo a series of differentiation processes to achieve species-specific sperm morphology. Although chromatin compaction is achieved by replacement of nuclear histones with protamines, sperm head shaping is facilitated by the manchette, a cuff-like sleeve of microtubules emerging from a perinuclear ring located at the junction of the acrosome-postacrosomal region during the acrosome phase of spermiogenesis. Since the manchette moves caudally as sperm head elongates, it is believed that manchette sculpts sperm head shape.\textsuperscript{13} Therefore, any impairments in development and function of this structure may lead to abnormal sperm head shape. In addition, the extensive and dynamic microtubule network existing in the Sertoli cells is involved in regulating sperm head shape.\textsuperscript{13} Sertoli cell microtubules have dramatic changes in their microtubule patterns as the germ cells progress through spermiogenesis.\textsuperscript{13} The microtubules in the apical processes of Sertoli cells mediate shape changes in Sertoli cells as they respond to changes in the germ cells (migration and cell shape). Therefore, impaired function of Sertoli cells may have a major role in development of abnormal sperm head shape.

Pyriform sperm has been reported as the most common sperm defect in bulls.\textsuperscript{6} A classical pyriform sperm have a pear-shaped head, a normal acrosome,\textsuperscript{6,29-31} and a narrow post-acrosomal region.\textsuperscript{30} In Canadian beef bulls, the overall incidence of the pyriform defect was 10\%, with 1.3\% of the bulls having more than half of their sperm affected with this defect.\textsuperscript{6} Several variants of pyriform sperm have also been reported, from almost normal to varying degrees of narrowness to sperm head to severely pyriform sperm and their effects on fertility have been documented through comprehensive breeding trials.\textsuperscript{32} Based on this study it was concluded that a moderate degree of sperm head narrowness, in the absence of other seminal signs of a disturbance of spermatogenesis, is not detrimental to fertility. However, extreme narrowness of the postacrosomal region of the sperm head of most sperm, as present in two bulls without other substantial signs of a disturbance of spermatogenesis, resulted in significantly reduced fertility. We evaluated the effects of the pyriform defect on fertility in vitro and determined that they had a reduced ability to bind to and penetrate the zona pellucida. If fertilization does occur, the resulting zygotes had a reduced ability to initiate cleavage.\textsuperscript{33} It is likely that pyriform sperm have a defective centrosome, affecting defective development of sperm aster following fertilization, blocking syngamy, cleavage or defective embryos leading to early embryonic loss as reported in humans.\textsuperscript{34}

Since sperm DNA is transcriptionally inactive, sperm functions are regulated by proteins already present in sperm (without additional protein synthesis). Therefore, we conducted proteomic analyses of pyriform sperm to elucidate molecular basis of functional impairments in this defect. Expression of several proteins involved in sperm capacitation, sperm-egg interaction and sperm cytoskeletal structure were decreased in pyriform sperm, whereas proteins regulating antioxidant activity, apoptosis and
metabolic activity were increased. Furthermore, contents of reactive oxygen species and ubiquitinated proteins were higher in pyriform sperm.35

Nuclear vacuoles

Nuclear vacuoles (pouches, craters, diadems) have been described in bull sperm as well as in the sperm of other species.36-40 This is a narrow-mouthed invagination of the nuclear membrane into the nucleoplasm of the sperm head.41 The incidence of vacuoles in the spermatozoa of affected bulls varies from less than 1% to nearly 100%. However, a definitive cause for nuclear vacuolation of spermatozoa has not been detected.6 Based on electron microscopic studies, vacuole formation in bulls begins as early as step 6 of spermiogenesis.42

Nuclear vacuoles appear in bull sperm in two forms; single apical vacuoles, which can be present anywhere in the sperm head, and multiple nuclear vacuoles at the acrosome post-acrosomal junction,43-46 appearing as a string of beads.47 These vacuoles are formed during the early stages of spermatid elongation by invagination of the inner nuclear membrane into the condensing nucleus.8 Breeding trials demonstrated that sperm with nuclear vacuoles caused infertility in the bull.45,46 Superovulated cows were bred with semen from a bull with 80% nuclear vacuoles; the fertilization rate was 18% compared to 72% for control bulls.45 The diadem/crater defect was studied over several months in two related 20-month-old Angus bulls. In bull 1, diadem/crater defects were present in 2-99% of ejaculated spermatozoa at various times during the evaluation period. In bull 2, affected cells varied from 20 to 94%, with other abnormalities (head and acrosome defects, coiled tails, proximal cytoplasmic droplets) also common. Single-sire mating trials conducted over 26 days during an apparent recovery phase yielded normal fertility. Both resting and gonadotropin-releasing hormone-stimulated testosterone concentrations were within normal limits. Histopathological evaluation of testes showed no obvious hypoplastic, inflammatory, or degenerative condition. However, electron microscopy of ejaculated spermatozoa demonstrated the characteristic diadem pattern of craters in the equatorial region of the head. Many cells from bull 2 contained large craters in other regions of the nucleus. Electron microscopy of testicular tissue demonstrated nuclear invaginations lined by a single unit membrane in round spermatids. Lesions in elongated spermatids were more pronounced, with curling of the nucleus and large membrane-filled cavities in the chromatin occurring in addition to craters in the equatorial region of the nucleus.48

Saacke et al49 investigated efficiency of barriers in the female tract against spermatozoa with abnormal heads. In Experiment 1, Day 6 ova/embryos were recovered nonsurgically from superovulated and single-ovulating cows following artificial insemination with semen of bulls selected for normal spermatozoal motility (<50%) and high content (>30%) of spermatozoa with misshapen heads, random nuclear vacuoles, or the diadem defect. To assess characteristics of spermatozoa capable of traversing barriers in the female tract, accessory spermatozoa were classified morphologically (x 1250) and compared with those in the inseminate. Accessory spermatozoa from 31 ova/embryos recovered from 44 cows were more normal in head shape than those in the inseminate (76 vs 62%; P < 0.05). However, spermatozoa with normal head shape, but with nuclear vacuoles, appeared as accessory spermatozoa at the same frequency as they were found in the inseminate.

We used semen containing high percentage of sperm with multiple nuclear vacuoles in IVF and to determine subsequent embryo development. Vacuolated sperm were deficient in their ability to bind to and penetrate the zona pellucida of IVM bovine oocytes. Results of embryo culture suggested that vacuolated sperm which gained access to the ooplasm participated normally in fertilization and that the zygotes resulting from the fertilization of IVM oocytes by vacuolated sperm developed normally through morulae to blastocysts. The infertility caused by vacuolated sperm in vivo would appear to be due to a reduced ability to pass through female reproductive tract, reduced zona binding and reduced penetration of the zona pellucida.8 However, a semen sample with high percentage of spermatozoa with multiple nuclear vacuoles can be used in an IVF system for the successful production of embryos, although their developmental competence beyond blastocysts remains to be elucidated.
Detached heads

A small percentage of detached heads are routinely identified in the semen of bulls with normal fertility. A specific defect, known as the decapitated sperm defect, was inherited in the Guernsey breed, probably as a sex-limited recessive gene; affected bulls produce semen with an increased percentage of detached heads, resulting in low fertility or sterility. Evidence for the inherited nature of this defect was also noticed in a group of eight Hereford bulls diagnosed with testicular hypoplasia with no presenting genital abnormalities and maintained under ideal managemental conditions; however, they were traced back to a common ancestral origin. Blom and Birch-Anderson suggested that the head and tail separation was due to defective formation of the basal plate, resulting in severe instability of the implantation fossa; the defect became evident in the caput epididymal sperm as the cells begin to initiate motility. Conditions such as testicular degeneration or inflammation of the seminal vesicles, ampullae and epididymis have been associated with higher numbers of detached heads. Bulls that were maintained in a sexually inactive state for a prolonged interval had a higher percentage of detached heads that subsequently returned to normal percentages following successive ejaculation. In a study conducted on 1642 Alberta beef bulls (ranging from 11 to 26 months, with Angus, Simmental, Charolais, Hereford, and Limousin bulls), the incidence of detached heads was 4.86 ± 5.71 % (mean ± SEM). Similarly, in another study by Arteaga et al on 1641 yearling Canadian beef bulls, the prevalence of detached heads decreased from 4.69 to 2.89% as the age of the bulls increased from 11 to 15 months. Infertility in bulls may arise, depending upon the percentage of sperm that are affected by this defect. In a report by Cooper and Peet on a previously fertile Hereford bull, testicular degeneration secondary to toxemia and laminitis lead to 91% detached heads in the ejaculate and infertility.

Tail defects

Mitochondrial sheath defects. Sperm mitochondrial sheaths have a major role during motility. Any impairment in this structure at any given point in the tail could lead to splitting and disruption of axial fibers at that level, leading to local damage of the tail. Missing mitochondria or mitochondrial aplasia (gaps in the mitochondrial sheath) is the most common mitochondrial sheath defect in bulls. Bulls with this defect seem to have normal progressive motility, with no negative effects on pregnancy rates of inseminated cows, as reported in Charolais bulls. However, large gaps in the mitochondrial sheath result in weakness and can predispose sperm to fracture and separation of the principal piece from the midpiece, thereby reducing motility and fertility.

Proximal droplets. Cytoplasmic droplets are commonly located either in the midpiece (proximal droplets) or the principal piece of the sperm tail (distal droplets). These masses of residual spherical cytoplasm should be normally released down the tail during spermiogenesis and shed when sperm are exposed to seminal plasma during ejaculation. Presence of a high percentage of sperm with the cytoplasmic droplets is a sign of failure of maturation, abnormal spermiogenesis, or abnormal epididymal function. However, as bulls mature, the percentage of proximal droplets in their semen declines to a normal level. Based on a study in beef bulls, 67.1% of the bulls produced sperm with proximal droplets (average percentage 2.7 ± 0.25%; 8). There is general consensus that a high percentage of sperm with proximal droplets in a semen sample has a negative impact on fertility. Furthermore, when sperm with a high prevalence of proximal droplets are used for IVF, zona binding and cleavage rates were poor.

Dag defect. This is a severe deformity of the midpiece, characterized by fractures of the axonemal fibers and mitochondrial sheath disruption. It has been reported that this sperm morphological defect has a heritable basis in Jersey and Hereford (up to 100% of sperm can be affected). Defective formation of the mitochondrial sheath during spermiogenesis is considered the primary cause.

Distal midpiece reflex. Distal midpiece reflex is the most common sperm tail abnormality in bovine sperm. This defect is characterized by a bend in the distal region of the midpiece in the shape of the letter “J”. A cytoplasmic droplet may be trapped within the bend. This sperm defect is believed to be epididymal in origin. Affected sperm have impaired motility. Since this defect is often stress-induced, the prognosis for improvement is generally good. Therefore, bulls classified as unsatisfactory due to high
percentage of sperm with distal midpiece reflex in their semen should be re-evaluated on a monthly basis to establish breeding soundness.

**Distal droplets.** Distal droplets do not cause substantial impairment of fertility even at high percentages and therefore sperm with normal morphology retaining distal droplets should not be considered as abnormal. This was supported in a recent study on Swedish bulls which had higher percentages of distal droplets, although the defect was not significantly correlated with fertility status as determined by non-return rate. In another study of Zebu bulls, it was suggested that there is an age-dependent relationship of the sire to the occurrence of the distal droplets, with immature bulls displaying high percentages, whereas mature and older bulls had a low prevalence. We recently demonstrated that a high percentage of sperm recovered from cauda epididymis of bison bulls contain distal droplets. However, incubation of these sperm in the TALPH buffer resulted in shedding of these droplets. Therefore, it is very likely that sperm with distal droplets may lose their droplets in the female reproductive tract immediately after ejaculation without affecting their subsequent migration in the female reproductive tract.

**Abaxial and accessory tail.** The abaxial tail defect is observed when the tail is attached to the head at an angle and is often accompanied by accessory tails, 2-3 µm long stumps arising from a secondary implantation region lateral to the main tail. In normal spermiogenesis, suppression of the centriole replication is apparent, so that sperm develop only one axoneme. In abnormal sperm, it is probable that there is failure of suppression or partial suppression of centriole replication in the spermatid leading to development of the accessory tails. Bulls having such tail defects do not have any testicular abnormalities and do not appear affected by external factors that can adversely affect spermatogenesis. Therefore, it is assumed that the defect is due to the genetic makeup of the bull. Bulls affected with accessory tails may have impaired fertility. In contrast, abaxial tails do not have any detrimental effects on fertility and therefore they should be considered as a normal variation of bovine sperm morphology. In extreme conditions, an abaxial sperm is held at an acute angle and therefore affected sperm swim in tight circles; such cases are classified as abnormal sperm.

**Summary**

In general, bovine sperm with abnormal morphology are discriminated against during zona binding and penetration, suggesting that impairment in molecular mechanisms of sperm-oocyte interaction may be similar among various types of aberrant sperm morphologies. Therefore, abnormal morphology may be considered a marker for an accompanying functional deficiency. Sperm with abnormal morphology have impaired ability to interact with oocyte or initiate embryonic development. In addition, deleterious effects of abnormal sperm may compromise the fertilizing ability of normal sperm co-existing in the ejaculate. Therefore, abnormal sperm may be a symptom of accompanying functional deficiencies of the entire sperm population. Semen samples classified as satisfactory should contain at least 70% morphologically normal sperm, with no more than 20% of sperm having defective heads.

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**References**