A novel apical midpiece defect in the spermatozoa of a bull without an apparent decrease in motility and fertility
A case study

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Abstract

Despite some limitations as predictors of fertility, evaluation of sperm morphology and progressive motility is the commonest method to assess viability of frozen/thawed semen. In this article we describe by light and transmission electron microscopy a novel midpiece structural defect observed in 24–36% of frozen/thawed sperm cells from a Charolais bull, used in artificial insemination programs without any apparent ill effect to the fertility. After thawing, the sperm progressive motility ranged from 65 to 80% and the pregnancy rate for all artificial inseminations performed (43%) did not differ (p > 0.05) from results obtained with insemination with semen of other bulls (40%). The defect consisted in mitochondrial aplasia at the neck region, mitochondrial segmental elongation and gaps and thickening of the outer dense fibers at the apical region of the midpiece, and loss of the cementing substance and development of plasma membrane extensions in the entire midpiece. No structural abnormalities were found in the capitulum, proximal centriole, striated columns, axoneme, annulus and fibrous sheath. The thickness of the outer fibers returned to normal at the distal region of the midpiece. Based on the examination it is suggested that the alterations might be originally caused by loss of the cementing substance that links mitochondria to the plasma membrane in association with mitochondrial aplasia at the neck region of the midpiece. The abnormality appeared not related to other described sperm defect syndromes, although it shared particular characteristics with the dag defect, segmental aplasia of the mitochondrial sheath, corkscrew defect and pseudodroplet defect.

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1. Introduction

Sperm morphological abnormalities can have a detrimental impact upon fertilization and embryonic development [1]. Despite its known limitations as predictors of fertility, evaluation of sperm morphology and progressive motility is the commonest method to assess viability of frozen/thawed semen. In bulls, low percentages (≈ 5%) of midpiece defects are common [2], but higher percentages can be induced by consumption of gossypol in the ration [3–6], can be the result of some viral diseases [7], genetic abnormalities [8,9] or be of unknown origin. Many forms of midpiece defects exist, and its effect on fertility is variable. No cases of infertility have been traced to mitochondrial gaps [2] and high (>20%) incidence of other midpiece defects has a
There may be no effect, such as in the case of a bull with 25–35% “Dag-like” defects [10] and bulls with high percentages of structurally normal, abaxially implemented sperm tails [11], but subfertility [12] and even sterility can occur in some cases of “corckscrew-sperm” and Dag defects [8,9]. In the present report, midpiece defects found in a high (>24%) percentage of frozen–thawed sperm from a Charolais bull, with no apparent negative consequences for the sperm progressive motility or the conception rates of inseminated cows, were characterized by light and electron microscopy.

2. Material and methods

Semen from a Charolais bull, frozen in 0.25 French-straws was imported from a main European Artificial Insemination (AI) company. Semen of this particular bull was purchased due to its classification by staff of the company as a high fertility bull, to be used mainly in repeat breeders (cows with three or more unsuccessful inseminations) and other “problem” Holstein cows. Information on mean non-return rate for bulls of the stud was not available, and no information on the fertility of the bull’s sire or progeny was obtained. The initial assessment of the semen of this bull was done as part of routine evaluation of all semen batches newly imported by the operator of this AI sub-center.

Routine semen examination was done as follows: sperm progressive motility after thawing (10 s at 37 °C) from three straws was estimated by light microscopy (200×) by the same operator, after placing a drop of semen in a slide and covering it with a cover glass. All equipment in contact with the semen was kept at 37 °C. The remaining of the semen was fixed in 0.2% glutaraldehyde in phosphate-buffered saline (PBS) for morphology evaluation in a wet mount under oil immersion, using phase-contrast microscopy (Zeiss Axiostar SZY2; 1000×). Sperm cells with abnormal morphology were included in three broad groups in the first examination—abnormal heads, abnormal midpieces and abnormal tails.

After noticing a high percentage of midpiece abnormal forms in the first morphological analysis (33%) in the semen of the studied bull, a more detailed description of the midpiece defects was done utilizing semen from other two straws. A total of 200 sperm cells/straw were evaluated for morphology. Semen of one straw was also used to estimate the total number of spermatozoa per straw, using a hemacytometer. For transmission electron microscopy, two french mini-straws (0.25 ml) were thawed (10 s, 37 °C) and sperm washed (1500 rpm, 10 min) with 0.15 M sodium cacodylate buffer, pH 7.3 (calibrated with 0.15 M aqueous cacodylic acid) and fixed (4 °C, 2 h) with Karnowsky (2.5 ml of buffer, 0.08 M final; 2.5 ml of ultrapure water; 1 ml of 4% paraformaldehyde, 0.6% final; 0.6 ml of 25% glutaraldehyde, 2.3% final). Stock paraformaldehyde was made in buffer. After washing, sperm were post-fixed in 1–2% OsO4 in buffer containing 0.8% K3Fe3+(CN)6 dehydrated in an ethanol series (50, 70, 90, 2 × 95, 2 × 100%; 30 min each) followed by propylene oxide (2 × 15 min), impregnated (3:1, 1:1, 1:3; 1 h each) and embedded (4 h at room temperature and 3 days at 60 °C) in Epon. Semithin and ultrathin sections were cut with a diamond Diatome knife in a Leica ultramicrotome. Semithin sections were stained with aqueous azur-II and methylene blue (1:1). Ultrathin sections were collected on 300-mesh copper grids (Taab3) and stained with alcoholic concentrated uranyl acetate (20 min) and

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2 Olympus Optical Co, Hamburg, Germany.

3 Taab, Berkshire, England.
Reynolds lead citrate (10 min). Stains were centrifuged (5 min, 2000 rpm, 4 °C) and 0.2 μm filtrated before use. Sections were studied at 60 kV in a JEOL 100CXII transmission electron microscope [13]. Chemicals were from Sigma and Merck. For light microscopy analysis, sperm were fixed with Karnowsky as above, then transferred to poly-L-lisine coated glass slides and observed as wet mounts in an Eclipse E-400 microscope equipped with a CCD camera and software for automated image analysis (Cytovision Ultra).

A total of 67 inseminations were performed using the studied semen. Fifteen of these were first inseminations, 9 were second, 8 were third and 35 were the fourth or

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4 Darmstadt, Germany.

5 Nikon, Tokyo, Japan.

6 Sony, Tokyo, Japan.

7 Applied Imaging International, Sunderland, UK.
later insemination. Seventy-two inseminations were carried out with semen of 26 different Holstein bulls, for the same period and in the same farms (27 were first, 12 third and 23 were the fourth or later inseminations postpartum). The same operator performed all inseminations. Pregnancy diagnosis by palpation per rectum was performed in all cows, 45–60 days after AI. A Chi-square test was employed to compare the percentage of cows pregnant with the studied semen and with the semen of the other bulls.

3. Results

After thawing, progressive motility was estimated to be 80% for straw 1, 65% for straw 2 and 70% for straw 3, respectively. At thawing, it was noticed for all the straws that the forward movement of the sperm were very vigorous. Total abnormal sperm forms were very similar between straws (40–41%), with midpiece defects accounting for 24 and 36% of the total abnormal cells (Table 1). The total number of sperm cell per straw was $24 \times 10^6$.

Midpiece defects classified as corkscrew included sperm with various degrees of bi-lateral erosion of the mitochondrial sheath. Two types of defects were included in the group classified as unilateral segmental aplasia/flagellar gap, namely sperm showing a defect similar to the corkscrew defect but evident only in one side of the midpiece, and a second lesion, shown as a gap in one side of the midpiece.

3.1. Light microscopy analysis

Analysis of sperm defects using fixed cells and the CCD camera, confirmed the findings observed with phase-contrast microscopy (Table 1) and further detected other midpiece defects (Figs. 1–9).

The detailed analysis showed that almost all defects occurred in the midpiece. These were characterized by the presence of a pseudodroplet in the apical region of the midpiece (Figs. 4–8), which occasionally was also observed in mid (Fig. 8) and distal midpiece (Fig. 7).

In association with the local thickening of the apical midpiece, there was an increase (Fig. 6 left) or decrease (Figs. 4 and 6 right) in the thickness and extension of the distal region of the midpiece, focal (segmental aplasia of the mitochondrial sheath) or multiple (corkscrew defect) gaps which caused an irregular outline of the midpiece (Figs. 2–9), midpiece plasma membrane extensions (Figs. 2–9), and constrictions at the apical (Figs. 2 and 7) and distal (Fig. 8) regions of the midpiece.

A few defects were observed in the other regions of the flagellum, such as fracturing of the apical principal piece, reflection or bent of the principal piece over the midpiece, and coiling of the end piece.

3.2. Transmission electron microscopy

Contrary to image analysis, electron microscopy demonstrated a uniform sperm midpiece defect that was characterized by several associated structural changes mainly confined to the apical midpiece. A normal control spermatozoon is shown in Fig. 10 for better understanding of the abnormal forms seen in the transmission electron microscopy.

The absence of the most apical mitochondria that surround the proximal centriole (neck region) was a
Figs. 11–14. Ultrastructural analysis of sperm with apical mitochondrial dysplasia. Fig. 11. Nuclear envelope extensions (arrows) fill the lateral parts of the neck region. In the apical region of the midpiece, there is a slight elongation of mitochondria (black arrowheads), two mitochondrial gaps (white arrowheads) and enlargement of the outer dense fibers (ODF). Fig. 12. The left side of the neck is devoid of mitochondria (arrow), thus creating a gap in the first helix turn (white arrowhead). In the apical region of the midpiece, there is a slight elongation of mitochondria (black arrowheads) and enlargement of the outer dense fibers (ODF). The partial loss of the cementing substance (dashed arrows) creates mitochondria trapping (large black arrows) and thin plasma membrane extensions (large white arrow). Fig. 13. Nuclear envelope extensions (arrows) fill the lateral parts of the neck region. In the apical region of the midpiece, there is elongation of mitochondria (black arrowheads) and enlargement of the outer dense fibers (ODF). The partial loss of mitochondria and of the cementing substance (dashed arrow) causes the formation of plasma membrane extensions (large white arrows). Fig. 14. The neck is devoid of mitochondria (arrows). In the apical region of the midpiece, there is elongation of mitochondria (black arrowheads), a mitochondria gap (white arrowhead) and enlargement of the outer dense fibers (ODF). The partial loss of mitochondria and of the cementing substance (dashed arrow) causes the formation of plasma membrane extensions (large white arrows). Figs. 11–14. The centriole (C), the striated columns (SC), the axoneme (Ax) and the more distal mitochondria (m) have a normal appearance. Nucleus (N). 31,000×; 20,000×; 35,000×; 27,000×; respectively.
Figs. 15–18. Ultrastructural analysis of sperm. Fig. 15. Nuclear envelope extensions (arrows) fill the lateral parts of the neck region. In the apical region of the midpiece, there are two mitochondrial gaps (white arrowheads), elongation of mitochondria (black arrowheads) and enlargement of the outer dense fibers (ODF). The partial loss of mitochondria and of the cementing substance (dashed arrows) creates small and very large plasma membrane extensions (large white arrows). Figs. 16 and 17. Nuclear envelope extensions (arrows) fill the lateral parts of the neck region. The apical
constant finding. In their place there was a collar made by an extension of the nuclear envelope (Figs. 11, 13, 15–17) or a gap (Figs. 12 and 14), occasionally causing a pronounced (Fig. 16) or slight (Fig. 17) constriction at the apical region of the midpiece.

These changes appeared associated with the partial loss of the mitochondrial helix in the upper segment of the mitochondrial sheath, with mitochondria becoming elongated and swollen for a variable distal extension (Figs. 11–17). The absence of the initial mitochondria at the sperm neck region also caused the presence of mitochondrial gaps in the distal helix turns (Figs. 11, 12, 14–17).

The cementing material that links the mitochondria to the plasma membrane was also lost in variable segments of the midpiece, either in the regions with elongated mitochondria (Figs. 13–15) or in the normal mitochondrial helicoidal turns (Figs. 11 and 17). The loss of the cementing material formed plasma membrane pouches with occasional mitochondrial trapping (Figs. 12 and 18) and caused the very frequent formation of plasma membrane extensions devoid of region of the midpiece shows a pronounced (Fig. 16) or slight (Fig. 17) right side constriction (dashed arrows), a mitochondrial gap (white arrowheads), elongation of mitochondria (black arrowheads) and enlargement of the outer dense fibers (ODF). The striated columns (SC) and the axoneme (Ax) have a normal appearance. N, nucleus. 22,500×; 30,000×; 31,000×; respectively. Fig. 18. Distal region of the midpiece (MP) and proximal region of the principal piece (PP). Inset: normal spermatozoon. In the abnormal spermatozoon, the loss of the cementing substance (dashed arrows) creates membrane pouches with mitochondria trapping (large black arrows) and small and very large plasma membrane extensions (large white arrows). The mitochondria (m), the axoneme (Ax) and the outer dense fibers (ODF) of the distal MP, as well as the annulus (An) and the fibrous sheath (FS) of the PP have a normal appearance. Insert: Normal control spermatozoon 27,000×.
other structural elements (Figs. 12–14), sometimes producing very long membrane expansions (Figs. 15 and 18).

No structural abnormalities were observed in the caput, proximal centriole, striated columns and axoneme, including fracturing or shattering of the axoneme (Figs. 11–17).

Similarly, no structural changes were observed in the annulus, fibrous sheath or in the 9d + 2s axonemal arrangement and their subcomponents (Figs. 18 and 19).

However, the upper region of the midpiece was enlarged not only due to the presence of the elongated and swollen mitochondria but also by the presence of very enlarged outer dense fibers (Figs. 11–17 and 20). In the distal region of the midpiece, the outer dense fibers were of normal diameter (Fig. 18).

### 3.3. Conception rates

Conception rate at 1st AI (40% – 6/15) was higher ($p < 0.05$) for inseminations performed with the studied semen than with the semen of the other bulls (37% – 10/27), but conception rates for all inseminations did not differ ($p > 0.05$) between the studied semen (43% – 29/67) and the semen of the other 26 Holstein bulls (40% – 29/72).

### 4. Discussion and conclusions

The present results describe a bovine sperm defect syndrome of the proximal region of the midpiece of the sperm flagellum. To the authors’ best knowledge no previous documentation of this defect has been published. This structural abnormality is characterized by mitochondrial aplasia at the neck region, loss of the mitochondrial helix in the apical region of the midpiece, presence of elongated and swollen mitochondria in the apical region of the midpiece, increase in the thickness of the outer dense fibers in the apical region of the midpiece, and loss of the cementing substance that connects mitochondria to the plasma membrane along the entire midpiece, with formation of plasma membrane extensions. Of these structural changes, the mitochondrial aplasia at the neck region of the midpiece, the distal extensions of the redundant nuclear envelope, the thickening of the outer dense fibers, the loss of the cementing substance, mitochondrial trapping and the plasma membrane extensions are new descriptions. In most cases, all these changes were observed conjointly. In the few cases where only partial structural defects could be found, there were always common findings involving at least some of the newly described defects, thus clearly suggesting that there is only a single common midpiece defect albeit with a variable penetrance of all subtypes of structural defects.

The mitochondrial aplasia at the neck region is probably the obvious cause for the presence of the distal extensions of the nuclear envelope. These correspond to the redundant nuclear envelope membranes that are typical normal structures of late spermatids. These are usually lost during the final stages of sperm maturation in the testis as the neck is invaded and compressed by mitochondria [14]. The absence of mitochondria at the neck region also caused the constrictions and membrane extensions observed at the neck region, as well as the mitochondrial gaps seen in more distal regions of the midpiece. Gaps and plasma membrane extensions thus contributed to the irregular and corkscrew appearance of most midpieces.

The main cause for midpiece thickening was the presence of elongated mitochondria and enlarged outer dense fibers, as true pseudodroplets and residual bodies were not found. The loss of the mitochondria at the neck region, which initiate the helicoidal turns, could be the cause for mitochondria elongation at the apical region of the midpiece. Due to the release of helical compression forces, this would subsequently be responsible for the hypertrophy seen in the outer dense fibers. However, the loss of the cementing substance could also be the main cause of these changes. In its absence, mitochondria at the neck region could be distally displaced, thus originating loss of the apical helix, with subsequent mitochondria extension and outer dense fiber thickening at the apical region of the midpiece. Loss of the cementing substance was also associated with formation of multiple plasma membrane extensions and mitochondrial trapping along the entire length of the midpiece.

These structural abnormalities do not fit with other previously described syndromes, although there is sharing of some particular defects [2]. In common with the dag and dag-like defect, the present sperm presented a roughness of the mitochondrial sheath. However, there was only a partial disruption of the mitochondrial helix without total disorganization of the arrangement of mitochondria and loss of the mitochondrial helix, absence of folding and coiling of the midpiece, and absence of fracturing and shattering of the axoneme or of the outer dense fibers [9,10]. On the contrary, there were no common findings with the distal midpiece reflex defect, as the thickening of the midpiece was not associated with the fold back of the principal piece elements [2]. Although showing a
pseudodroplet defect at light microscopy, the present sperm did not show the characteristics described for this anomaly. At the ultrastructural level, the local thickening of the midpiece was always dependent on the presence of elongated and swollen mitochondria and thickened outer dense fibers, without any evidence of mitochondrial and dense granule clustering, or bending and fracture at the same site [2]. Sperm with an isolated mitochondrion and dense granule clustering, or bending thickened outer dense fibers, without any evidence of elongated and swollen mitochondria and disruption of the midpiece was always dependent on the anomaly. At the ultrastructural level, the local thickening of the midpiece did not show the characteristics described for this pseudodroplet defect [2]. They seem to constitute a new syndrome as the main structural defects could be found in all sperm analysed with abnormal midpieces. Even sperm that at light microscopy did not show all characteristics of the novel midpiece defect and thus seemed to correspond to other previously described defects, showed at the electron microscopical level the characteristics of the main syndrome, although at a lower level, thus representing minor variations of the syndrome here first characterized. Although seeming contradictory, this just confirms the need for a detailed ultrastructural analysis when finding such defects under the light microscope.

The present ultrastructural observations lead to the conclusion that the different abnormalities found in the sperm midpiece do not correspond to any of the defects previously described [2]. They seem to constitute a new syndrome as the main structural defects could be found in all sperm analysed with abnormal midpieces. Even sperm that at light microscopy did not show all characteristics of the novel midpiece defect and thus seemed to correspond to other previously described defects, showed at the electron microscopical level the characteristics of the main syndrome, although at a lower level, thus representing minor variations of the syndrome here first characterized. Although seeming contradictory, this just confirms the need for a detailed ultrastructural analysis when finding such defects under the light microscope.

Although the original common cause for the present structural defects is unknown, it is possible to speculate about related findings. The absence (aplasia) of the mitochondria located at the neck region of the midpiece, which are responsible for the initiation of the mitochondrial helix, would cause mitochondrion elongation and loss of the first helicoidal turns in the apical region of the midpiece. In consequence, the loss of the compression forces around the circle of the nine outer dense fibers would predispose to the deregulation observed in their thickness. However, it is also plausible that an original defect of the cementing substance could be responsible for a distal displacement of the neck mitochondria with elongation and loss of the helix in the apical region of the midpiece, followed by hypertrophy of the outer dense fibers due to the release of compression forces.

The total percentage of normal sperm cells (≤60%) found is below the minimum 70% recommended for satisfactory potential breeders, by the bull breeding soundness evaluation guidelines of the Society for Theriogenology [15] and a large field study [16] showed that using natural mating, bulls that produced the highest calf output had >70% normal spermatozoa. The low number of inseminations available, do not allow for a solid comparison between the fertility of the analyzed semen and the semen of a larger bull population. Overall, the conception rates obtained were lower than the parturition rate of 51% at first AI seen in the same area, in a long-term study [17]. However, the data suggests that the studied semen is at least as fertile as the semen of the other utilized Holstein bulls. Furthermore, this is a biased comparison, as more than half of the inseminations performed with the studied semen was used in females considered to be sub-fertile (35 out of 67 inseminations—52%), while only 32% of the semen of the other bulls (23 out of 72 straws) was used in this sub-population of cows, with 3 or more unsuccessful previous inseminations. Thus, this is an additional report that adds to previous cases [2,10,11] where high incidence of midpiece defects was not accompanied by a decrease of conception rate at AI. The fact that bulls with high percentage of “abnormal” sperm cells can reach high fertility, points out to the risk of culling bulls based on average percentages of sperm morphology, instead of careful assessments for each individual case. At least in part, the absence of a deleterious effect on fertility may be attributed to the fact that straws contained a number of sperm cells high enough to allow normal cells to compensate potential deleterious effects to fertility, of the abnormal forms. On the other hand, the fact that percentage of progressive motile sperm cells was higher than the percentage of cells with normal morphology, suggests that at least part of the spermatozoa with abnormal midpieces were motile. Data does not allow any speculation on the fertilizing ability of those abnormal sperm cells. Likewise, the origin of the defect cannot be established by the morphological analysis performed, and the data does not allow for any speculation of a possible heritability of this defect. It is very unlikely that this high incidence of midpiece abnormalities could be attributed to a lengthy and heavy consumption of gossypol or to viral diseases, as the bull was stationed in a reputed European AI station.
In conclusion, in this article a detailed morphological
description of midpiece defects of unknown origin,
without any apparent deleterious effect on the fertilizing
ability, is presented.

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