

**Review Article**

**Sperm DNA Impairment in the Bull: Causes, Influences on Reproduction and Evaluations**

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**ABSTRACT**

Conventional semen examination involving sperm motility, viability and morphology remains the backbone of assessing the fertility status of a sire. However, there remains instances where these semen parameters appear normal but cases of low conception rates or failure of pregnancy occur. This review highlights the causes of sperm DNA damage and the effectiveness of techniques designed to evaluate the contribution of sperm DNA damage to lowered fertility in bulls. Among the many causes of sperm DNA impairment are imperfect spermatogenesis, faulty apoptosis, reactive oxygen species, *in-vitro* handling, impact of environment, radiography and the stress of cryopreservation processes. Furthermore, DNA impairment impairs fertilisation, interferes with embryonic development and implantation and blocks blastocyst formation. The most frequently used tests to determine DNA damage are the acridine orange test (AOT) using acridine orange stain with examination under a fluorescence microscope and the sperm chromatin structure assay (SCSA) using the same stain but examined with flow cytometry.

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**INTRODUCTION**

The integrity of sperm DNA of bulls is critical for assisted reproductive technology in cattle. It permits agriculturalists to improve and develop their breeds in

response to rural development. Sperm are haploid cells and the chromosomes in them are mono-chromatid structures. The sperm cell nucleus is mainly occupied with DNA, which is responsible for approximately 40% of its dry matter. Bull sperm comprise 3.44 pg DNA/nucleus (Bochenek et al., 2001). The cell nucleus is the most vital component of sperm ultrastructure, as fertilisation efficiency is influenced by the presence of a normal structure. Chromatin of sperm is systematised in toroids that are steady and solid structures that are attached to nuclear matrix through toroid linker regions. These linker regions are the most vulnerable to DNA injury (Sotolongo et al., 2003) with single (ssDNA) or double strand breaks (dsDNA; Aitken et al., 2013).

In both bull and human spermatozoa, there is a high quantity of chromatin heterogeneity (Evenson et al., 1980a; Takeda et al., 2015). Even so, conventional semen examination comprising examination of sperm motility, viability, sperm count and morphology has remained the pillar of examining semen-associated male factor of infertility. However, there remain some conditions where these parameters are all in the 'normal' range but the male has low or reduced fertility (Dietrich et al., 2005). Simon and Lewis (2011) found that from all conventional parameters such as those stated above there was only one negative correlation ( $r=0.21$ ) between sperm DNA impairment and progressive motility. In addition, Venkatesh et al. (2011) found out that 15.5% of idiopathic infertile males had normal semen parameters. Hence, sperm

features are essential for considering not only the conventional parameters but also for assessment of DNA, acrosome and the fertilising ability of sperm. This review aimed to discover the causes and also the effectiveness and evaluations of sperm DNA impairment in bulls.

### **Causes of Sperm DNA Impairment**

**Imperfect spermatogenesis.** DNA deficiency in sperm could largely be due to imperfect spermatogenesis (Manicardi et al., 1995; Sailer et al., 1995). One example of imperfect spermatogenesis is the presence of vacuoles in the sperm head. This vacuole appearances could be associated with chromatin destruction (Oliveira et al., 2010; Franco et al., 2012). Furthermore, the percentage of fertility rate was correlated negatively with sperm vacuoles (Berkovitz et al., 2006). The sperm head comprises DNA almost fully and it has been reported that in case of alterations in DNA structure, morphologic abnormalities are expected (Enciso et al., 2011). Nevertheless, several studies have revealed that sperm containing DNA dicondensation do not essentially present morphologic alterations (Beletti, et al., 2005; Soares & Beletti, 2006). An earlier study by Beletti and Mello (2004) showed a great positive relationship between primary sperm defects and DNA alteration, suggesting that sperm DNA structure affects the morphology of sperm head. Furthermore, Kipper et al. (2017) found a strong relationship between compaction of DNA and the morphometry of the sperm head in Nelore bulls.

In the course of spermiogenesis, histones, the dominant protein in spermatocyte nucleus, are replaced by protamines in mature sperm (Zhao et al., 2004). The protamines of sperm are essential for providing structural rigidity and maintaining highly condensed sperm DNA packing (Miller et al., 2010). In fact, it is now known that a reduction in sperm protamine content could lead to increased sperm DNA impairment (Fortes et al., 2014). Thus, modification in chromatin rebuilding during the process of spermatogenesis might result in DNA impairment (Marcon & Boissonneault, 2004).

**Faulty apoptosis.** Programmed cell death (apoptosis) is an important factor offered to remove unnecessary or damaged sperm cells throughout spermatogenesis. In the development of the sperm germ cell through spermatogenesis, Sertoli cells take control to induce apoptosis in 50% of sperm cells that enter meiosis I (Mahfouz et al., 2009); however, due to poorly understood mechanisms, this procedure might not operate well and some imperfect germ cells that have evaded apoptosis might progress on to the process of spermiogenesis (Burrello et al., 2004). In the process of apoptosis, anti-apoptotic protein (BCI-2) and pro-apoptotic protein (BAX) deliver a signalling pathway that supports and maintains balance in a cell. The prorated levels of these proteins are important for a feedback mechanism. In fact, in the course of spermatogenesis, pro-apoptotic protein acts as a checkpoint for keeping the quality

of sperm (Oltvai & Korsmeyer, 1994; Dogan et al., 2013).

**Reactive oxygen species production before ejaculation.** It is clear that excessive reactive oxygen species (ROS) induces DNA impairment (Moustafa et al., 2004). The source of ROS before ejaculation can be from immature sperm or from epididymal epithelial cells. Studies showed that immature sperm in *cauda epididymis* produce a high amount of ROS and these could affect negatively on the DNA of mature sperm (Ollero et al., 2001). Furthermore, epithelial cells of epididymis, which could be affected by environmental factors, play an important role in increasing the amount of ROS. This would cause an increase in the antioxidant intake and thus, could reduce the harmful effect of ROS on sperm DNA (Sakkas & Alvarez, 2010). Although the internal anti-oxidant enzyme capacity of Karan Fries bulls was increased in hot dry and hot humid seasons, the ROS and malondialdehyde were significantly higher in these seasons compared to in winter or spring (Soren et al., 2016) and significantly correlated with seminal quality.

**Impact of environment.** Fluctuating environmental temperatures induce sperm DNA impairment (Karabinus et al., 1997). Bovine semen quality alterations due to season have been recorded even though bulls are not considered seasonal breeders (Menegassi et al., 2015; Malama et al., 2017). In dairy bulls there is a strong decline in semen output owing to the stress of

temperature and humidity (Al-Kanaan et al., 2015). Lucio et al. (2016) revealed that scrotal heat stress, which is induced by scrotal insulation in crossbred bulls, led to moderate to strong alterations in all sperm head morphometric measures and DNA integrity. Moreover, the roundness of the sperm head is a major reflection of heat stress. The adverse effects of rising testicular temperature might have an effect on meiotic stages of spermatogenesis and can result in remodelling of sperm DNA (Rahman et al., 2011; Lucio et al., 2016). In contrast, Malama et al. (2017) found that DNA integrity seemed to be stable over the course of the seasons. Values of DNA impairment of frozen-thawed semen of Holstein-Friesian bulls did not change in winter and summer. Whether extremes of temperature significantly affect sperm DNA is still not fully understood as Michael et al. (2013) found that 91% of bulls from tropical environments that are characterised by high environmental temperature and humidity do have stable sperm DNA. It should be stressed here that males fed based on sub-optimal feeding requirements have higher sperm DNA impairment and reduction in testicular mass, sperm motility and total sperm count in ejaculates (Aitken et al., 2012; Guan et al., 2014). The common DNA impairment in males fed sub-optimally is incomplete development of spermatid during spermiogenesis. This could be due to the positive relationship between DNA impairment and poor chromatin packaging as a result of under protamination

concentration in complete sperm (Gorczyca et al., 1993; Guan et al., 2014).

***In-vitro* handling.** Sperm DNA can undergo impairment through handling and shipment after collection (Bollwein et al., 2008; Jenkins et al., 2015). Imperfect handling of fresh, chilled or frozen-thawed semen might lead to a change in pH, decrease or increase in temperature and increase in the amount of ROS (Jenkins et al., 2015). Therefore, proper handling of semen is priceless. Collecting tubes, sperm handling, light exposure, washing, semen processing and any sub-optimal condition of ejaculated semen can increase the risk of DNA impairment (Drevet, 2016) because spermatozoa are exposed to environments that are different from the physiological.

**Radiotherapy and chemotherapy.** It has been stated that exposure to radiotherapy or chemotherapy could cause sperm DNA impairment. This is highlighted in a study by O'Flaherty et al. (2008), who found that the integrity of sperm DNA was affected in patients with testicular cancer after they were given any chemotherapy (O'Flaherty et al., 2008).

**Chilling and cryopreservation of semen.** Storage of semen in chilled temperatures results in excessive production of ROS (Crespilho et al., 2014; Daramola & Adekunle, 2015), and this directly affects sperm DNA (Morte et al., 2008). The processes of thawing frozen semen could

also lead to DNA impairment (Holt, 2000; Métayer et al., 2002; Gadea et al., 2008; Kumar et al., 2011; Papa et al., 2015; Ezz et al., 2017), which could greatly affect the fertility status of the semen.

In fact, the mechanisms accountable for freezing and chilling induced DNA impairment are not properly understood. Data suggest that the consequence of lipid peroxidation (LPO) in injurious sperm chromatin (Kasimanickam et al., 2007; Kumar et al., 2011). Simões et al. (2013) indicated that there was a negative relationship between intact DNA and the amount of ROS in bull semen. On the other hand, Gürler et al. (2016) revealed that not all kinds of ROS are harmful to sperm DNA as only H<sub>2</sub>O<sub>2</sub> is related to DNA impairment. Moreover, sperm nuclei exposure to high ionic strength in the course of cryopreservation instigates deterioration of chromatin assembly and sequentially, makes post-thawing nuclear DNA available to oxidative occurrence through extra or intracellular ROS (Gadea et al., 2008; Makker et al., 2009; Simões et al., 2013). Additionally, perhaps, unrestrained post-thawing influx of wandering calcium ions in frozen sperm (Holt, 2000) may possibly encourage additional splitting of nucleoprotein and DNA through endogenous protease and nuclease stimulation (Métayer et al., 2002).

### **Influence of Sperm DNA Impairment on Reproduction**

To date, there is no dependable data on the effects of DNA impairment in bulls during

embryo development or pregnancy product (Kipper et al., 2017). Spermatozoa lack a DNA repair mechanism, and this could be due to their unique structural differentiation. In fact, spermatozoa are metabolically silent with high condensed chromatin that is unable to self-repair damaged DNA (Smith et al., 2013). On the other hand, oocytes have the ability to repair both its own DNA and the DNA sperm, and this repair occurs after fertilisation prior to cleavage. However, sometimes, this repair is possible because of a high level of sperm DNA impairment or owing to low oocyte repair activity (Drevet, 2016). Likewise, Evenson et al. (1980a) revealed that sperm with denatured ssDNA could have decreased fertilisation efficiency both *in vivo* and *in vitro*. Around 15% of sperm population with impaired DNA is usually considered normal, whereas a value between 15% and 25% will result in decreased fertility, and much higher values such as 25% and above signify a higher chance of infertility (Larson-Cook et al., 2003; Michael et al., 2013). Despite these figures, some studies have reported decreased fertility in bulls having 10% injured sperm in their semen (Bochenek et al., 2001). Kipper et al. (2017) found that 4% to 16.15% of sperm with DNA impairment did not reduce *in vitro* embryonic development until Day 8. Similarly, Fatehi et al. (2006) confirmed that DNA impairment in bull sperm did not prevent fertilisation and early embryonic development. However, it can lead to induced apoptosis after the first cleavage.

The presence of any damage(s) to the sperm ssDNA can reduce the success of fertilisation (Simon & Lewis, 2011; Ribas-Maynou et al., 2012), while damage(s) to the sperm dsDNA could lead to a disturbance in embryonic development (Lewis & Aitken, 2005), commonly resulting in cases of miscarriage (Lewis & Simon, 2010). A contrary opinion to this has been presented by Fatehi et al. (2006), who revealed that sperm DNA impairment does not cause any problems in fertilisation of the oocyte or in first, second or third cleavage achievement, though it can stop formation of blastocysts through apoptosis induction i.e. low sperm DNA impairment can be successfully repaired by either the ovum or the embryo leading to the birth of normal offspring. Early embryonic death or abortion is only linked with higher assault to sperm DNA (Wyrobek et al., 2006). The involvement of sperm DNA damage in reducing breeding effectiveness of bulls after artificial insemination (AI) (Bollwein et al., 2008) permits consideration of factors that could lead to deviation in the chromatin structure in the course of *in-vitro* semen processing. Spermatozoa with DNA impairment have the potential to interrupt genetic and epigenetic rule of embryonic growth (Aitken & De Iuliis, 2007). Recent studies revealed that higher proportions of intact sperm chromatin correlates with improved fertilisation success and normal embryonic growth (Fatehi et al., 2006; Khalifa et al., 2008). Fatehi et al. (2006) induced impairment for sperm DNA by exposure to irradiation with X- or Gamma

rays. The results showed that embryonic growth was totally blocked at Day 7 and the blastocyst percentage reduced from 28% in non-irradiated sperm to less than 3% in irradiated sperm.

Sperm exposure to traumatic situations such as cryopreservation may compromise the mechanism, resulting in inhibited fertilisation or embryonic growth (D'Occhio et al., 2007; Kasimanickam et al., 2007; Khalifa et al., 2008). Kasimanickam et al. (2007) and Lymberopoulos and Khalifa (2010) showed that a rising proportion of sperm DNA impairment was related to declining field fertility of cryopreserved bull semen. Similarly, Khalifa et al. (2008) indicated that a substantial negative association exists between the occurrence of sperm DNA impairment in cryopreserved semen and the developing capability of bovine embryos *in vitro*. It is likely that the procedure of semen thawing could have a negative or positive effect on chromatin uncertainty. For instance, fast thawing of cryopreserved semen straws at a high temperature (45°C for 30 s), is measured by an empirically resultant procedure in minimising intracellular hyper-osmotic trauma at the course of re-warming of sperm handled at a high freezing rate (Hammerstedt et al., 1990). Furthermore, a 240-min incubation of cryopreserved semen at 25°C or 39°C doubled sperm DNA impairment frequency, with the degree of increase being intense at 39°C and advancing the biologically important limit (Bollwein et al., 2008).

It has been suggested that antioxidants safeguard frozen spermatozoa from DNA disintegration (Gadea et al., 2008; Kumar et al., 2011). The defence mechanism of sperm cell against nuclear DNA injury depends on the effectiveness of their chromatin compaction, stoppage of endogenous nucleases and degree of extra and intracellular antioxidants (Aitken & De Iuliis, 2007; D'Occhio et al., 2007). A notable decrease in sperm DNA impairment occurrence was seen after frozen-thawed semen centrifugation and extracellular milieu elimination, the main cause of post-thawing production of ROS in the existence of egg yolk and dead sperm cells (Vishwanath & Shannon, 1996).

Research shows that bull semen cooled in egg yolk extenders could not upset sperm chromatin steadiness (Waterhouse et al., 2006; Khalifa et al., 2008) and that there was no relationship between sperm movement, DNA injury incidence, agglutination and LPO (Lymberopoulos & Khalifa, 2010). DNA impairment was negatively related to the fertility of the bull sperm (García-Macías et al., 2007). Moreover, there was negative association of sperm DNA injury with capacitation status, viability and membrane reliability in buffalo (Pawar & Kaul, 2011). It should be noticed that fresh semen may contain a huge number of dead and degenerated spermatozoa with DNA impairment (Liu & Liu, 2013). Thus, the consequence of DNA injury of ejaculated sperm does not correctly replicate DNA position of motile sperm portion. Consequently, clinical evaluation of sperm

DNA impairment ought to be done in the motile sperm portion and not the entire ejaculated sperm using, for instance, the swimming up procedure (Madrid-Bury et al., 2003), self-migratory method (Makler et al., 1984) or the discontinuous Percoll gradient procedure (Berger et al., 1985) to collect solely the motile sperm before doing a DNA integrity test.

### **Evaluation of Sperm DNA Integrity**

Evaluation of sperm DNA integrity has a stronger capability to predict the fertility rate of semen than other conventional parameters such as percentage progressive motility (Simon & Lewis, 2011). A number of tests have been used for the evaluation of sperm DNA integrity including the acridine orange test, sperm chromatin structure assay, TUNEL assay, single-cell gel electrophoresis, sperm Bos-Halomax assay and sperm chromatin dispersion test.

**Acridine orange test (AOT).** The most regularly used test instrument for DNA impairment identification is the acridine orange staining test, which permits sperm chromatin steadiness detection in an acidic environment. The dye has metachromatic characteristics. Spermatozoa having double-stranded DNA discharges fluorescence in the green band, whereas those having RNA and single-strand DNA discharges red fluorescence.

DNA-intercalating dyes such as acridine orange have proved useful for examining alterations in chromatin packaging. The composition of normal double-stranded

DNA results in the spatial separation of acridine orange molecules, causing them to act like the monomeric form of the dye and emit green fluorescence. When excited by a 488-nm light source, in the presence of denatured (single-stranded) DNA, the dye molecules bind electrostatically to the strands and to each other to form aggregates, whereby dye-dye interaction causes a concentration-dependent loss of absorbed energy and a subsequent metachromatic shift to red fluorescence (Evenson et al., 1980b). The microscopic assessment of sperm chromatin integrity classifies acridine orange-stained spermatozoa as normal if fluorescing green and abnormal if red. However, this visual classification introduces some subjectivity to the assessment, since the emission spectrum from individual spermatozoa is often a mix of wavelengths, with stained spermatozoa appearing yellow to brown and not clearly identifiable with either category.

The microscopic AOT has demonstrated a significant relationship with male infertility and with fertilisation and pregnancy rates in IVF, independent of other sperm characteristics including sperm zona binding and morphology (Tejada et al., 1984; Liu & Baker, 1992).

**Sperm chromatin structure assay (SCSA).** Automation of the detection and analysis of AO fluorescence is provided by flow-cytometry (FCM) and employed in the sperm chromatin structure assay (SCSA), which was initially developed by Evenson et al. (1980b) as a potential measure of

livestock fertility. Flow-cytometry provides a powerful statistical advantage over manual microscopic methods through objective and rapid multi-parametric analysis of large numbers of cells. The SCSA assay assesses the integrity of sperm chromatin structure, using AO as an enquiry to quantify the vulnerability of sperm DNA to in-situ-induced denaturation. The original assay measured heat-induced denaturation, but this has been replaced with the acid-detergent treatment (pH 1.2, Evenson et al., 1986). Spermatozoa with a normal chromatin structure appear impervious to treatment, while spermatozoa with an abnormal chromatin structure undergo partial denaturation (Evenson et al., 1980a). The SCSA is a flow-cytometric technique, while AOT is a microscopic method, and the two quantify the metachromatic shift of acridine orange fluorescence from green (native DNA) to red (denatured DNA), and the two methods mostly determine the DNA impairment with the toroid linker region. The SCSA evaluation has been commonly used to assess sperm DNA excellence in bulls through FCM (Januskauskas et al., 2001, 2003; Waterhouse et al., 2006; Fortes et al., 2012; Michael et al., 2013; Serafini et al., 2015).

**TUNEL assay.** Another method for DNA fragmentation assessment is using the TUNEL assay. The TUNEL assay identifies double- and single-stranded DNA discontinuities by identifying a free 3-OH terminus with altered nucleotides in an enzymatic response with terminal



deoxynucleotidyl transferase (TdT) and may be examined using a microscope or through flow cytometry (Sharma et al., 2013) or fluorescence microscope (Takeda et al., 2015).

**Single-cell gel electrophoresis (COMET) assay.** In this assay, sperm DNA breaks move apart in the head region forming 'comets' after electrophoresis, while complete DNA is intact in the normal head location. The COMET assay includes embedment of sperm in agarose using a glass slide, electrophoresis and assessing DNA movement in the comet tails using a specific software programme in a computer. The COMET assays (i.e. alkaline and neutral) apparently permit stain admittance to the toroid linker and toroid regions (Shaman et al., 2007) for documentation of ssDNA and dsDNA disruption. Evenson et al. (2002) and Baumgartner et al. (2009) suggested that the neutral COMET assay recognises dsDNA disruption and closely related ssDNA disruption, while the alkaline COMET assay recognises ssDNA disruption only. In bulls, the neutral COMET assay identified higher DNA breakdown (i.e. higher tail moment) in non sex-sorted spermatozoa rather than in sex-sorted spermatozoa (Boe-Hansen et al., 2005).

**Sperm chromatin dispersion test (SCD).** SCD is a moderately new technique that is introduced to evaluate spermatozoa DNA disintegration (Fernandez et al., 2003). The SCD test is centred on the belief that spermatozoa having disjointed DNA fail to

give the distinctive halo of discrete DNA loops that are witnessed in spermatozoa with non-disjointed DNA after acid denaturation and nuclear protein elimination. The sperm tail was removed during the process of the SCD test (Fernandez et al., 2003). Fernández et al. (2005) and Pawar and Kaul (2011) modified the test's protocol, so the tail was unbroken from the sperm head. Furthermore, the scoring patterns were four different categories in the modified method vs. five in the non-modified method (Pawar & Kaul, 2011).

**Sperm Bos-Halomax assay (SBH).** The Sperm Bos-Halomax (SBH) assay was established for evaluation of spermatozoa DNA reliability in bulls, and centres on the Spermatozoa Chromatin Dispersion Test (SCDt) for humans (Fernandez et al., 2003). The SBH assay is comparable to the COMET assay with the exemption that sperm treated are not visible in an electrophoretic field. Higher DNA disintegration gives rise to more halos, while less DNA disintegration produces fewer halos (García-Macías et al., 2007).

Sailer et al. (1995), Aravindan et al. (1997) and Chohan et al. (2006) detected a solid association between SCSA and TUNEL outcomes for spermatozoa DNA disintegration, while Simões et al. (2013) indicated that a positive correlation between sperm DNA impairment and susceptibility to ROS in two different methods, the SCSA and the COMET assay, but there was no correlation between the COMET assay and the SCSA assay. Furthermore, there was a

strong relationship between SCD and AOT in the experiment that was conducted by Pawar and Kaul (2011).

## CONCLUSION

Sperm chromatin is arranged in toroids, which are steady and solid structures; these linker regions are the most susceptible to DNA damage with single- (ssDNA) or double-strand (dsDNA) breaks. DNA defects are most likely due to defective spermatogenesis (genetic disorder), faulty apoptosis, extreme reactive oxygen species production, *in-vitro* treatment, impact of the environment, exposure to radiography and chilling and cryopreservation procedures. A level of approximately 15% sperm with injured DNA is considered to be quite normal. DNA damage impairs fertilisation, interferes with embryonic development and implantation and also blocks blastocyst formation. During fertilisation the damage on the ssDNA of the sperm can be repaired by the oocyte, while the dsDNA damage leads to early embryonic death or abortion. Antioxidants present in the semen may guard frozen-thawed sperm from DNA disintegration. There is no relationship between spermatozoa motility, agglutination, LPO and DNA impairment, while DNA impairment was found to be negatively correlated with the viability, membrane integrity, capacitation and fertility of the bull sperm. Hence, clinical evaluation of sperm DNA impairment ought to be performed in motile spermatozoa fraction than in the

entire ejaculated spermatozoa. The most frequent tests that are used to determine DNA damage are the AOT and SCSA tests, as they enable sperm chromatin steadiness determination in an acidic environment. AOT and SCSA have demonstrated a significant relationship with male infertility and with fertilisation and pregnancy rates in IVF.

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