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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.

Keywords: Sperm, DNA impairment, sperm DNA evaluation

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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

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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 (BCl-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* handlin**g. 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 H2O2 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 wondering 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 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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REFERENCES

Aitken, R., Bronson, R., Smith, T., & De Iuliis, G. (2013). The source and significance of DNA damage in human spermatozoa: A commentary on diagnostic strategies and straw man fallacies. *MHR: Basic Science of Reproductive Medicine, 19*(8), 475-485.

Aitken, R. J., & De Iuliis, G. N. (2007). Origins and consequences of DNA damage in male germ cells. *Reproductive Biomedicine Online, 14*(6), 727–733.

Aitken, R., De Iuliis, G., Gibb, Z., & Baker, M. (2012). The Simmet lecture: New horizons on an old landscape – Oxidative stress, DNA damage and apoptosis in the male germ line. *Reproduction in Domestic Animals, 47*(s4), 7–14.

Al-Kanaan, A., König, S., & Brügemann, K. (2015). Effects of heat stress on semen characteristics of Holstein bulls estimated on a continuous phenotypic and genetic scale. *Livestock Science, 177*, 15–24.

Aravindan, G., Bjordahl, J., Jost, L., & Evenson, D. (1997). Susceptibility of human sperm to in situ DNA denaturation is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. *Experimental Cell Research, 236*(1), 231–237.

Baumgartner, A., Cemeli, E., & Anderson, D. (2009). The comet assay in male reproductive toxicology. *Cell Biology and Toxicology, 25*(1), 81–98.

Beletti, M. E., da Fontoura Costa, L., & Viana, M. P. (2005). A comparison of morphometric characteristics of sperm from fertile Bos taurus and Bos indicus bulls in Brazil. *Animal Reproduction Science, 85*(1), 105–116.

Beletti, M. E., & Mello, M. L. S. (2004). Comparison between the toluidine blue stain and the Feulgen reaction for evaluation of rabbit sperm chromatin condensation and their relationship with sperm morphology. *Theriogenology, 62*(3), 398–402.

Berger, T., Marrs, R. P., & Moyer, D. L. (1985). Comparison of techniques for selection of motile spermatozoa. *Fertility and Sterility, 43*(2), 268–273.

Berkovitz, A., Eltes, F., Ellenbogen, A., Peer, S., Feldberg, D., & Bartoov, B. (2006). Does the presence of nuclear vacuoles in human sperm selected for ICSI affect pregnancy outcome? *Human Reproduction, 21*(7), 1787–1790.

Bochenek, M., Smorag, Z., & Pilch, J. (2001). Sperm chromatin structure assay of bulls qualified for artificial insemination. *Theriogenology, 56*(4), 557–567.

Boe-Hansen, G. B., Morris, I. D., Ersbøll, A. K., Greve, T., & Christensen, P. (2005). DNA integrity in sexed bull sperm assessed by neutral Comet assay and sperm chromatin structure assay. *Theriogenology, 63*(6), 1789–1802.

Bollwein, H., Fuchs, I., & Koess, C. (2008). Interrelationship between plasma membrane integrity, mitochondrial membrane potential and DNA fragmentation in cryopreserved bovine spermatozoa. *Reproduction in Domestic Animals, 43*(2), 189–195.

Burrello, N., Arcidiacono, G., Vicari, E., Asero, P., Di Benedetto, D., De Palma, A., … & Calogero, A. E. (2004). Morphologically normal spermatozoa of patients with secretory oligo-astheno-teratozoospermia have an increased aneuploidy rate. *Human Reproduction, 19*(10), 2298–2302.

Chohan, K. R., Griffin, J. T., Lafromboise, M., Jonge, C. J., & Carrell, D. T. (2006). Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. *Journal of Andrology, 27*(1), 53–59.

Crespilho, A., Nichi, M., Guasti, P., Freitas-Dell'Aqua, C., Sá Filho, M., Maziero, R., … & Papa, F. O. (2014). Sperm fertility and viability following 48h of refrigeration: Evaluation of different extenders for the preservation of bull semen in liquid state. *Animal Reproduction Science, 146*(3), 126–133.

Daramola, J., & Adekunle, E. (2015). Preservative effects of pineapple and cucumber juices on viability of refrigerated spermatozoa of West African dwarf bucks. *Pertanika Journal of Tropical Agricultural Science, 38*(3), 347-359.

Dietrich, G., Szpyrka, A., Wojtczak, M., Dobosz, S., Goryczko, K., & Ciereszko, A. (2005). Effects of UV irradiation and hydrogen peroxide on DNA fragmentation, motility and fertilizing ability of rainbow trout (Oncorhynchus mykiss) spermatozoa. *Theriogenology, 64*(8), 1809–1822.

D'Occhio, M., Hengstberger, K., & Johnston, S. (2007). Biology of sperm chromatin structure and relationship to male fertility and embryonic survival. *Animal Reproduction Science, 101*(1), 1–17.

Dogan, S., Mason, M. C., Govindaraju, A., Belser, L., Abdullah, K., Stokes, J., … & Memili, E. (2013). Interrelationships between apoptosis and fertility in bull sperm. *Journal of Reproduction and Development, 59*(1), 18–26.

Drevet, J. R. (2016). Sperm DNA damage and assisted reproductive technologies: Reasons to be cautious! *Basic and Clinical Andrology, 26*(1), 11-14.

Enciso, M., Cisale, H., Johnston, S., Sarasa, J., Fernández, J., & Gosálvez, J. (2011). Major morphological sperm abnormalities in the bull are related to sperm DNA damage. *Theriogenology, 76*(1), 23–32.

Evenson, D., Darzynkiewicz, Z., Jost, L., Janca, F., & Ballachey, B. (1986). Changes in accessibility of DNA to various fluorochromes during spermatogenesis. *Cytometry, 7*(1), 45–53.

Evenson, D., Darzynkiewicz, Z., & Melamed, M. (1980a). Relation of mammalian sperm chromatin heterogeneity to fertility. *Science, 210*(4474), 1131–1133.

Evenson, D. P., Darzynkiewicz, Z., & Melamed, M. R. (1980b). Comparison of human and mouse sperm chromatin structure by flow cytometry. *Chromosoma, 78*(2), 225–238.

Evenson, D. P., Larson, K. L., & Jost, L. K. (2002). Sperm chromatin structure assay: Its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *Journal of Andrology, 23*(1), 25–43.

Ezz, M. A., Montasser, A. E., Hussein, M., Eldesouky, A., Badr, M., Hegab, A. E., … & Zaabel, S. M. (2017). The effect of cholesterol loaded cyclodextrins on post-thawing quality of buffalo semen in relation to sperm DNA damage and ultrastructure. *Reproductive Biology, 17*(1), 42–50.

Fatehi, A., Bevers, M., Schoevers, E., Roelen, B., Colenbrander, B., & Gadella, B. (2006). DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after the first cleavages. *Journal of Andrology, 27*(2), 176–188.

Fernández, J. L., Muriel, L., Goyanes, V., Segrelles, E., Gosálvez, J., Enciso, M., … & De Jonge, C. (2005). Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertility and Sterility, 84*(4), 833–842.

Fernandez, J. L., Muriel, L., Rivero, M. T., Goyanes, V., Vazquez, R., & Alvarez, J. G. (2003). The sperm chromatin dispersion test: A simple method for the determination of sperm DNA fragmentation. *Journal of Andrology, 24*(1), 59–66.

Fortes, M., Holroyd, R., Reverter, A., Venus, B., Satake, N., & Boe-Hansen, G. (2012). The integrity of sperm chromatin in young tropical composite bulls. *Theriogenology, 78*(2), 326–333. e324.

Fortes, M. R., Satake, N., Corbet, D., Corbet, N., Burns, B., Moore, S., & Boe-Hansen, G. (2014). Sperm protamine deficiency correlates with sperm DNA damage in Bos indicus bulls. *Andrology, 2*(3), 370–378.

Franco Jr, J., Mauri, A., Petersen, C., Massaro, F., Silva, L., Felipe, V., … & Oliveira, J. (2012). Large nuclear vacuoles are indicative of abnormal chromatin packaging in human spermatozoa. *International Journal of Andrology, 35*(1), 46–51.

Gadea, J., Gumbao, D., Canovas, S., García-Vázquez, F. A., Grullón, L. A., & Gardón, J. C. (2008). Supplementation of the dilution medium after thawing with reduced glutathione improves function and the in vitro fertilizing ability of frozen-thawed bull spermatozoa. *International Journal of Andrology, 31*(1), 40–49.

García-Macías, V., De Paz, P., Martinez-Pastor, F., Álvarez, M., Gomes-Alves, S., Bernardo, J., … & Anel, L. (2007). DNA fragmentation assessment by flow cytometry and Sperm-Bos-Halomax (bright-field microscopy and fluorescence microscopy) in bull sperm. *International Journal of Andrology, 30*(2), 88–98.

Gorczyca, W., Traganos, F., Jesionowska, H., & Darzynkiewicz, Z. (1993). Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: Analogy to apoptosis of somatic cells. *Experimental Cell Research, 207*(1), 202–205.

Guan, Y., Malecki, I. A., Hawken, P. A., Linden, M. D., & Martin, G. B. (2014). Under-nutrition reduces spermatogenic efficiency and sperm velocity, and increases sperm DNA damage in sexually mature male sheep. *Animal Reproduction Science, 149*(3), 163–172.

Gürler, H., Malama, E., Heppelmann, M., Calisici, O., Leiding, C., Kastelic, J., & Bollwein, H. (2016). Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm. *Theriogenology, 86*(2), 562–571.

Hammerstedt, R., Graham, J. K., & Nolan, J. P. (1990). Cryopreservation of mammalian sperm: What we ask them to survive. *Journal of Andrology, 11*(1), 73–88.

Holt, W. (2000). Basic aspects of frozen storage of semen. *Animal Reproduction Science, 62*(1), 3–22.

Januskauskas, A., Johannisson, A., & Rodriguez-Martinez, H. (2001). Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. *Theriogenology, 55*(4), 947–961.

Januskauskas, A., Johannisson, A., & Rodriguez-Martinez, H. (2003). Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. *Theriogenology, 60*(4), 743–758.

Jenkins, J. A., Draugelis-Dale, R. O., Pinkney, A. E., Iwanowicz, L. R., & Blazer, V. (2015). Flow cytometric method for measuring chromatin fragmentation in fixed sperm from yellow perch (*Perca flavescens*). *Theriogenology, 83*(5), 920–931.

Karabinus, D. S., Vogler, C. J., Saacke, R. G., & Evenson, D. P. (1997). Chromatin structural changes in sperm after scrotal insulation of Holstein bulls. *Journal of Anthology, 18*(5), 549-555.

Kasimanickam, R., Kasimanickam, V., Thatcher, C., Nebel, R., & Cassell, B. (2007). Relationships among lipid peroxidation, glutathione peroxidase, superoxide dismutase, sperm parameters, and competitive index in dairy bulls. *Theriogenology, 67*(5), 1004–1012.

Khalifa, T., Rekkas, C., Lymberopoulos, A., Sioga, A., Dimitriadis, I., & Papanikolaou, T. (2008). Factors affecting chromatin stability of bovine spermatozoa. *Animal Reproduction Science, 104*(2), 143–163.

Kipper, B., Trevizan, J., Carreira, J., Carvalho, I., Mingoti, G., Beletti, M., … & Koivisto, M. (2017). Sperm morphometry and chromatin condensation in Nelore bulls of different ages and their effects on IVF. *Theriogenology, 87*, 154–160.

Kumar, R., Mohanarao, G. J., & Atreja, S. (2011). Freeze-thaw induced genotoxicity in buffalo (*Bubalus bubalis*) spermatozoa in relation to total antioxidant status. *Molecular Biology Reports, 38*(3), 1499–1506.

Larson-Cook, K. L., Brannian, J. D., Hansen, K. A., Kasperson, K. M., Aamold, E. T., & Evenson, D. P. (2003). Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertility and Sterility, 80*(4), 895–902.

Lewis, S., & Aitken, R. (2005). DNA damage to spermatozoa has impacts on fertilization and pregnancy. *Cell and Tissue Research, 322*(1), 33–41.

Lewis, S. E., & Simon, L. (2010). Clinical implications of sperm DNA damage. *Human Fertility, 13*(4), 201–207.

Liu, D. Y., & Liu, M. L. (2013). Clinical value of sperm DNA damage should be assessed in motile sperm fraction rather than whole ejaculated sperm. *Fertility and Sterility, 99*(2), 367–371.

Liu, D. Y., & Baker, H. G. (1992). Sperm nuclear chromatin normality: Relationship with sperm morphology, sperm-zona pellucida binding, and fertilization rates in vitro. *Fertility and Sterility, 58*(6), 1178–1184.

Lucio, A. C., Alves, B. G., Alves, K. A., Martins, M. C., Braga, L. S., Miglio, L., … & Beletti, M. E. (2016). Selected sperm traits are simultaneously altered after scrotal heat stress and play specific roles in in vitro fertilization and embryonic development. *Theriogenology, 86*(4), 924–933.

Lymberopoulos, A., & Khalifa, T. (2010). Sperm chromatin stability during in vitro manipulation of beef bull semen. *Reproduction in Domestic Animals, 45*(2), 307–314.

Madrid-Bury, N., Fernández, R., Jiménez, A., Pérez-Garnelo, S., Moreira, P. N., Pintado, B., … & Gutiérrez-Adán, A. (2003). Effect of ejaculate, bull, and a double swim-up sperm processing method on sperm sex ratio. *Zygote, 11*(03), 229–235.

Mahfouz, R. Z., Sharma, R. K., Said, T. M., Erenpreiss, J., & Agarwal, A. (2009). Association of sperm apoptosis and DNA ploidy with sperm chromatin quality in human spermatozoa. *Fertility and Sterility, 91*(4), 1110–1118.

Makker, K., Agarwal, A., & Sharma, R. (2009). Oxidative stress & male infertility. *Indian Journal of Medical Research, 129*(2009), 357–367.

Makler, A., Murillo, O., Huszar, G., Tarlatzis, B., DeCherney, A., & Naftolin, F. (1984). Improved techniques for collecting motile spermatozoa from human semen. *International Journal of Andrology, 7*(1), 61–70.

Malama, E., Zeron, Y., Janett, F., Siuda, M., Roth, Z., & Bollwein, H. (2017). Use of computer-assisted sperm analysis and flow cytometry to detect seasonal variations of bovine semen quality. *Theriogenology, 87*, 79–90.

Manicardi, G., Bianchi, P., Pantano, S., Azzoni, P., Bizzaro, D., Bianchi, U., & Sakkas, D. (1995). Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. *Biology of Reproduction, 52*(4), 864–867.

Marcon, L., & Boissonneault, G. (2004). Transient DNA strand breaks during mouse and human spermiogenesis: New insights in stage specificity and link to chromatin remodeling. *Biology of Reproduction, 70*(4), 910–918.

Menegassi, S. R. O., Barcellos, J. O. J., Dias, E. A., Koetz, C., Pereira, G. R., Peripolli, V., … & Lopes, F. G. (2015). Scrotal infrared digital thermography as a predictor of seasonal effects on sperm traits in Braford bulls. *International Journal of Biometeorology, 59*(3), 357–364.

Métayer, S., Dacheux, F., Dacheux, J. L., & Gatti, J. L. (2002). Comparison, characterization, and identification of proteases and protease inhibitors in epididymal fluids of domestic mammals. Matrix metalloproteinases are major fluid gelatinases. *Biology of Reproduction, 66*(5), 1219–1229.

Michael, J., Hengstberger, K. J., Tutt, D., Holroyd, R. G., Fordyce, G., Boe-Hansen, G. B., & Johnston, S. D. (2013). Sperm chromatin in beef bulls in tropical environments. *Theriogenology, 79*(6), 946-952.

Miller, D., Brinkworth, M., & Iles, D. (2010). Paternal DNA packaging in spermatozoa: More than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction, 139*(2), 287–301.

Morte, M. I., Rodrigues, A. M., Soares, D., Rodrigues, A. S., Gamboa, S., & Ramalho-Santos, J. (2008). The quantification of lipid and protein oxidation in stallion spermatozoa and seminal plasma: Seasonal distinctions and correlations with DNA strand breaks, classical seminal parameters and stallion fertility. *Animal Reproduction Science, 106*(1), 36–47.

Moustafa, M. H., Sharma, R. K., Thornton, J., Mascha, E., Abdel-Hafez, M. A., Thomas, A. J., & Agarwal, A. (2004). Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Human Reproduction, 19*(1), 129–138.

O'flaherty, C., Vaisheva, F., Hales, B., Chan, P., & Robaire, B. (2008). Characterization of sperm chromatin quality in testicular cancer and Hodgkin's lymphoma patients prior to chemotherapy. *Human Reproduction, 23*(5), 1044–1052.

Oliveira, J. B. A., Massaro, F. C., Baruffi, R. L. R., Mauri, A. L., Petersen, C. G., Silva, L. F., … & Franco, J. G. (2010). Correlation between semen analysis by motile sperm organelle morphology examination and sperm DNA damage. *Fertility and Sterility, 94*(5), 1937–1940.

Ollero, M., Gil-Guzman, E., Lopez, M. C., Sharma, R. K., Agarwal, A., Larson, K., … & Alvarez, J. G. (2001). Characterization of subsets of human spermatozoa at different stages of maturation: Implications in the diagnosis and treatment of male infertility. *Human Reproduction, 16*(9), 1912–1921.

Oltvai, Z. N., & Korsmeyer, S. J. (1994). Checkpoints of dueling dimers foil death wishes. *Cell, 79*(2), 189–192.

Papa, P. M., Papa, F. O., Oliveira, L. A., Guasti, P. N., Castilho, C., & Giometti, I. C. (2015). Different extenders in the cryopreservation of bovine epididymal spermatozoa. *Animal Reproduction Science, 161*, 58–63.

Pawar, K., & Kaul, G. (2011). Assessment of buffalo (Bubalus bubalis) sperm DNA fragmentation using a sperm chromatin dispersion test. *Reproduction in Domestic Animals, 46*(6), 964–969.

Rahman, M. B., Vandaele, L., Rijsselaere, T., Maes, D., Hoogewijs, M., Frijters, A., … & Shamsuddin, M. (2011). Scrotal insulation and its relationship to abnormal morphology, chromatin protamination and nuclear shape of spermatozoa in Holstein-Friesian and Belgian Blue bulls. *Theriogenology, 76*(7), 1246–1257.

Ribas-Maynou, J., García-Peiró, A., Fernandez-Encinas, A., Amengual, M. J., Prada, E., Cortes, P., … & Benet, J. (2012). Double stranded sperm DNA breaks, measured by Comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor. *PloS One, 7*(9), e44679.

Sailer, B. L., Jost, L. K., & Evenson, D. P. (1995). Mammalian sperm DNA susceptibility to in situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *Journal of Andrology, 16*(1), 80–87.

Sakkas, D., & Alvarez, J. G. (2010). Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertility and Sterility, 93*(4), 1027–1036.

Serafini, R., Romano, J. E., Varner, D. D., Di Palo, R., & Love, C. C. (2015). Sperm DNA assays and their relationship to sperm motility and morphology in bulls (*Bos Taurus*). A*nimal Reproduction Science, 159*, 77–86.

Shaman, J. A., Yamauchi, Y., & Steven Ward, W. (2007). Function of the sperm nuclear matrix. *Archives of Andrology, 53*(3), 135–140.

Sharma, R., Masaki, J., & Agarwal, A. (2013). Sperm DNA fragmentation analysis using the TUNEL assay. In D. T. Carrell & K. I. Aston (Eds.), *Spermatogenesis: Methods and Protocols* (pp. 121–136). New York, NY: Humana Press.

Simões, R., Feitosa, W. B., Siqueira, A. F. P., Nichi, M., Paula-Lopes, F. F., Marques, M. G., … & Assumpção, M. E. O. (2013). Influence of bovine sperm DNA fragmentation and oxidative stress on early embryo *in vitro* development outcome. *Reproduction, 146*(5), 433–441.

Simon, L., & Lewis, S. E. (2011). Sperm DNA damage or progressive motility: Which one is the better predictor of fertilization *in vitro*? *Systems Biology in Reproductive Medicine, 57*(3), 133–138.

Smith, T. B., Dun, M. D., Smith, N. D., Curry, B. J., Connaughton, H. S., & Aitken, R. J. (2013). The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. *Journal Cell Science, 126*(6), 1488–1497.

Soares, J. M., & Beletti, M. E. (2006). Avaliação da integridade cromatínica de espermatozóides de galos (Gallus gallus, Linnaeus, 1758) de linhagem pesada em duas idades. *Brazilian Journal of Veterinary Research and Animal Science, 43*(4), 543–553.

Soren, S., Singh, S. V., & Singh, P. (2016). Influence of season on seminal antioxidant enzymes in Karan Fries bulls under tropical climatic conditions. *Turkish Journal of Veterinary and Animal Sciences, 40*(6), 797–802.

Sotolongo, B., Lino, E., & Ward, W. S. (2003). Ability of hamster spermatozoa to digest their own DNA. *Biology of Reproduction, 69*(6), 2029–2035.

Takeda, K., Uchiyama, K., Kinukawa, M., Tagami, T., Kaneda, M., & Watanabe, S. (2015). Evaluation of sperm DNA damage in bulls by TUNEL assay as a parameter of semen quality. *Journal of Reproduction and Development, 61*(3), 185–190.

Tejada, R., Mitchell, J. C., Norman, A., Marik, J., & Friedman, S. (1984). A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertility and Sterility, 42*(1), 87–91.

Venkatesh, S., Shamsi, M., Deka, D., Saxena, V., Kumar, R., & Dada, R. (2011). Clinical implications of oxidative stress and sperm DNA damage in normozoospermic infertile men. *Indian Journal of Medical Research, 134*(3), 396-398.

Vishwanath, R., & Shannon, P. (1996). Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperature. *Reproduction, Fertility, and Development, 9*(3), 321–331.

Waterhouse, K., Haugan, T., Kommisrud, E., Tverdal, A., Flatberg, G., Farstad, W., … & De Angelis, P. (2006). Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls. *Reproduction, Fertility and Development, 18*(7), 781–788.

Wyrobek, A. J., Eskenazi, B., Young, S., Arnheim, N., Tiemann-Boege, I., Jabs, E., … & Evenson, D. (2006). Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. *Proceedings of the National Academy of Sciences, 103*(25), 9601–9606.

Zhao, M., Shirley, C. R., Mounsey, S., & Meistrich, M. L. (2004). Nucleoprotein transitions during spermiogenesis in mice with transition nuclear protein Tnp1 and Tnp2 mutations. *Biology of Reproduction, 71*(3), 1016–1025.