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EVALUATION OF BUFFALO BULL SPERMATOZOAL DNA DAMAGE USING SINGLE CELL GEL ELECTROPHORESIS

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ABSTRACT

Deoxyribonucleic acid was influenced by sperm ageing and sperm storage temperature. In this study, the effect on DNA integrity at pre-freeze and post-freeze stages was analyzed by single cell gel electrophoresis during winter and summer seasons. Traditionally, the diagnosis of male infertility depends on microscopic assessment and biochemical assays to evaluate quality of semen. These tests are needed to provide the basic information for initial diagnosis. However, none of these parameters gives information about sperm function and their fertilization capability. The fertility of these sperms is questionable. The evaluation of sperm DNA damage is a promising technique to diagnose the male infertility. It has been shown to be closely associated with negative relationships with fertilization, embryo quality, implantation and positive relationships with abortion. Here we report the effect of preservation temperature and season on sperm DNA damage, using the single cell gel electrophoresis to measure DNA fragmentation. The significant difference ($P \leq 0.05$) in DNA damage among Surti buffalo bulls spermatozoa were more pronounced at post-freeze stage as compared to the pre-freeze stage. In addition, the significant difference ($P \leq 0.05$) in DNA damage was observed among Surti buffalo bulls spermatozoa during summer season as compared to the winter season. The changes on DNA integrity were dependant on temperature of preservation, period of storage and seasonal variation. The single cell gel electrophoresis can be used to detect low levels of DNA damage in Surti buffalo bulls spermatozoa. Therefore, screening of the sperm DNA damage may be used as an additional test of sperm quality that can have diagnostic importance in the male infertility.

Keywords : Spermatozoa, DNA damage, buffalo bull infertility, single cell gel electrophoresis

INTRODUCTION

DNA sequences are involved in regulating the expression of genetic information. Sperm DNA integrity is important for the success of natural or assisted fertilization, including normal development of the embryo, fetus or offspring (Morris ID et al. 2002). The semen cryopreservation is an important fundamental technique for the conservation of genetic resources in cryobanks, which used for artificial insemination. Studies aimed to optimize the procedures for semen cryopreservation therefore need a set of protocols that provide an overall assessment of the semen quality. The goal of research in laboratory evaluation of semen has been ultimately to predict fertility achievable with the use of that semen (Saacke RG, 1983). The freezing-thawing procedures cause various damages to sperm cells. In mammalian sperm, various cellular components are affected by damages induced by the nucleus. The DNA damage in spermatozoa induced by freezing-thawing is needed to be clarified for efficient

fertility success. The main factors assumed to be potentially involved in the damage at spermatozoa DNA during the freezing-thawing process are the osmotic stress and the oxidative stress that occur after thawing (Alvarez JA and Storey BT, 1992; Bilodeau JF et al., 2000). The evaluation of structural integrity of spermatozoa can predict the fertilizing ability and the capacity to withstand cryopreservation. However, none of the semen characteristic can alone be used to predict the fertility of semen with cent percent accuracy, yet some of the semen characteristics have been found to be significant1y correlated with fertility of bulls and buffalo bulls (Gopal Krishna T and Rao AR, 1979). The conventional semen quality parameters do not provide information on DNA integrity, which is important to avoid impairment at the fertilization stage, embryo development, pregnancy and reproductive outcomes. Thus, the DNA integrity of sperm cells has crucial importance.

The agarose embedded with lysed cells applied an electrophoretic field with pH conditions less than pH 10, tails were observed where the DNA migrated faster than the nuclear DNA (Ostling O and Johanson KJ, 1984). This induced damage leads to the tail of the comet consisting mainly of single-stranded DNA (Collins AR et al. 1997). The comet assay or single cell gel electrophoresis (SCGE) is a rapid, sensitive and quite simple method for detecting DNA damage at the level of mammalian cells (Ostling O and Johanson KJ, 1984). The extensive appliance of the comet assay with its various modifications led to the establishment of guidelines for its use (Tice RR et al. 2000). These have been very valuable as a basis to standardize protocols when carrying out the comet assay with a variety of cell types. However, these guidelines are not entirely applicable when investigating reproductive cells like sperm in the comet assay, unless several adjustments are made particularly to relax the highly compacted sperm chromatin structure. This review will focus primarily on the use of the sperm comet assay to evaluate sperm quality, as there is a growing interest in the evaluation of DNA structural integrity in male germ cells to access its fertilization ability. A negative relationship between the fertilization ability of spermatozoa and alterations at the DNA integrity has been observed in the number of studies conducted with different techniques including comet assay single cell gel electrophoresis.

Evaluation of DNA damage

Several techniques have been developed to detect DNA abnormalities in single male germ cells. This is generally accepted that sperm nuclear DNA tests show the most promise in of the diagnosis and treatment of male infertility (Lewis SEM et al., 2008b). The comet assay, under neutral conditions, allows the assessment of DNA integrity influenced by sperm ageing, which is manifested in DNA double-strand breaks (Fraser L and Strzez˙ek J, 2004). In the present study, a modified neutral comet assay test (single-cell gel electrophoresis) was used to assess DNA integrity of buffalo bull spermatozoa in fresh diluted semen and semen preserved at sub-zero temperature. One of its unique and important features is the ability to characterize the responses of a heterogeneous population of cells by measuring DNA damage within individual cells as opposed to just one overall measure of damaged cells versus undamaged cells as in the the Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) (Lewis SEM and Agbaje I, 2008a). Semen is one of the most heterogenous biological fluids in humans. This is important, since DNA damage may be the pivotal factor in determining the sperm's ability to fertilize (Lewis SEM and Agbaje I, 2008a). The Sperm Chromatin Structure Assay (SCSA) has been used to assess the susceptibility of DNA in ram sperm chromatin to acid-induced denaturation during cryo-preservation (Peris SI et al., 2004). It is also believed to measure both Single Strand Break (SSB) and Double Strand Break (DSB) (Evenson D et al., 2007), although primarily single-stranded DNA using a DNA fragmentation index (DFI). It can also be used to measure immature sperm (Lewis SEM and Agbaje I, 2008a). The SCSA is less specific than alkaline comet or TUNEL in determining DNA fragmentation. Furthermore, TUNEL has been used to identify DNA strand breakage in abnormal human sperm cells (Muratori M et al., 2000). In the TUNEL assay has the advantage of being relatively quick and easy to perform. However, one major limitation of this assay in sperm results from the high levels of sperm DNA compaction combined with the absence of a lysis step (Lewis SEM and Agbaje I,

2008a). There are just a few studies comparing alternative assays within the same study SCSA and alkaline comet (Aravindan GR et al., 1997), alkaline comet and TUNEL (Donnelly ET et al., 2000) and SCSA and TUNEL (Erenpreiss J et al., 2004) which give surprisingly close correlations between assays despite the differences in protocol and parameters measured by each assay.

Single Cell Gel Electrophoresis (SCGE)

To get information regarding the DNA breakage in germ cells, it is necessary to examine sperm cells for its DNA integrity. Many researchers have been used SCGE technique for DNA strand breakage due to its simple, sensitive and rapid performance (Liao W et al., 2009). The evaluation of semen with conventional methods has limitations due to lower correlation with fertility and problems with repeatability within and between evaluators (Jasko DJ, 1992). The susceptibility of sperm to alkaline DNA denaturation seems to be strongly associated with DNA strand breaks indicating an important physiological relevance of sperm quality and fertility (Aravindan GR et al. 1997). This fact suggests importance of the semen analysis. The basic method of SCGE has been adjusted for the use of human or animal sperm (Singh NP et al. 1988, 1989).

Many researchers have shown the effect of freezing on sperm DNA integrity. The cryopreservation promotes DNA fragmentation in spermatozoa. The amount of detectable DNA damage was positively correlated with the cryo-damaged cells in each treatment ($P<0.05$), when compared with fresh or processed samples (Linfor JJ and Meyers SA, 2002; Baumber J et al. 2003). The post-thaw sperm motility and plasma membrane integrity declined ($P<0.05$) with a corresponding increase ($P<0.05$) in sperm DNA damage regardless of the extender type and packaging material (Fraser L and Strzezek J, 2005). The deterioration in post-thaw sperm DNA integrity was concurrent with reduced sperm characteristics. Kotlowska M et al. (2007) reported that 48 hrs of liquid storage lead to decreases in sperm motility characteristics and increases in amidase activity (using a colorimetric assay) and DNA fragmentation (using a Comet assay) had been occurred in turkey. Comet extent and percent DNA in the comet tail were also associated with a decline in sperm concentration and motility, respectively (Trisini AT et al. 2004). A correlation was observed between increased sperm head DNA fragmentation and decreased penetration of zona-free hamster oocytes (Chan PJ et al. 2001). Low DNA damage was associated with higher hyperactivation and oocyte penetration, suggesting that failed fertilization was correlated with DNA fragmentation of the sperm (Chan PJ et al. 2001). However, Steele EK et al. (2000) have found no significant difference between the percentage of undamaged DNA of fresh testicular sperm and freeze-thawed testicular sperm. Cryopreservation of testicular sperm did not increase baseline levels of DNA damage, was observed. Freezing sperm in seminal plasma improves post-thaw motility and DNA integrity (Donnelly ET et al. 2001a).

Analysis of cryo-preserved and fresh sperm DNA integrity during winter and summer

It is more practical in larger studies to examine the DNA integrity of sperm in cryopreserved semen rather than fresh sperm (Duty SM et al. 2002); however, freezing living cells can cause unfavourable and damaging effects due to ice crystal formation and/or severe osmotic changes. Freezing seems to affect chromatin structure and sperm morphology (Hammadeh ME et al. 1999), and DNA damage from cryopreservation in semen from infertile men has been detected using the alkaline comet assay (Donnelly et al. 2001b). In the study, the effect of season was found significant ($P\leq0.05$) having higher Comet percentage of freshly diluted semen during summer (7.61±1.64%) than winter (3.00±0.55%). More percentage of Comets in summer may be due to heat stress, which leads to DNA fragmentation of spermatozoa. As shown in Table 1, the effect of preservation was significant on the Comet percentage being higher in summer at pre-freeze (11.05±1.70%) and at post-freeze (13.61±1.90%) than in winter at pre-freeze (2.88±0.60%) and at post-freeze stages (4.61±1.07%). Conventional cryopreservation and storage in liquid nitrogen caused DNA damage in thawed macaque sperm, even more without cryoprotectants (Li MW et al. 2007). However, a vital role for the integrity of the cell membrane is also based on the sperm freezing methodology in terms of speed, step-wise changes in temperature and the cryopreservative used (Morris GJ et al. 1999; Gilmore JA et al. 2000).

Table 1.*The comet percentage at different stages during winter and summer seasons*

Season	Stages of semen preservation	Comet percentage (%)
Winter	Freshly diluted semen	3.00± 0.55
	Pre-freeze stage	2.88±0.60
	Post-freeze stage	4.61 ± 1.07
Summer	Freshly diluted semen	7.61 ± 1.64*
	Pre-freeze stage	11.05 ± 1.70*
	Post-freeze stage	13.61 ± 1.90*

*The comet percentage (%) was significantly higher in summer as compared to the winter season at different stages ($P \leq 0.05$). *Student *t*- test was used for statistical analysis.

CONCLUSIONS

DNA damage is a relatively common attribute of spermatozoa that has been positively correlated with poor fertilization, impaired implantation rates and increased incidences of abortion and disease in the offspring. It is known that sperm DNA damage higher than 8% cannot be completely repaired in the oocyte and might lead to impaired embryo development and early pregnancy loss (Ahmadi A and Ng SC, 1999). It is therefore very important to have a standardized assay in reproduction technology to detect DNA damage in male germ cells. A variety of techniques has been used to detect this damage including Comet, TUNEL and SCSA. To assess DNA damage in reproductive cells, the SCGE or Comet assay has proven to be a reliable and rapid method (Fairbairn DW et al. 1995), hence being the most sensitive way to detect of DNA damage (Leroy T et al. 1996). The quality of freshly diluted and frozen semen was better during winter based on microscopic evaluation tests. DNA fragmentation of spermatozoa was lower during winter as compared to summer in freshly diluted semen and semen preserved at sub-zero temperature. Based on DNA integrity test, DNA integrity of spermatozoa was more intact in winter, and therefore quality of the semen was found better during winter as compared to summer season in Surti buffalo bulls. The factors that predispose spermatozoa to oxidative stress, such as decreased antioxidant protection or increased ROS generation by spermatozoa, are associated with oxidative DNA damage in the male germ line. In addition, genetic or environmental disruptions to spermatogenesis may result in apoptosis and the activation of endogenous endonucleases that, in turn, mediate the fragmentation of nuclear DNA in differentiating germ cells.

Although guidelines are available for the assessment of DNA damage in the Comet assay in somatic cells, unfortunately, no standardized Comet protocol for sperm is available so far, which also accounts for a wide range of related procedures like sperm storage, which may lead to different baseline damage. It is therefore crucial for future semen evaluation studies to improve existing sperm DNA damage assessments and to develop more accurate diagnostic tests. At present, the Comet assay with sperm seems to provide the necessary sensitivity, accuracy and flexibility for becoming a reliable test system for the semen evaluation.

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