Effect of age on lipid peroxidation of fresh and frozen-thawed semen of Nili-Ravi buffalo bulls

Sohail Ahmed, Muhammad Irfan-ur-Rehman Khan, Mushtaq Ahmad and Sajid Iqbal

Department of Theriogenology, University of Veterinary and Animal Sciences, Lahore, Pakistan; Department of Livestock and Dairy Development Punjab, Semen Production Unit, Qadirabad, Sahiwal, Pakistan

ABSTRACT

Buffalo bull spermatozoa are rich in polyunsaturated fatty acids and are prone to lipid peroxidation. We hypothesised that lipid peroxidation in buffalo bull semen will increase with age and will affect the semen quality. The objective was to compare malondialdehyde (MDA) concentration and the quality of fresh and frozen-thawed semen between aged (13.6 ± 1.0 years; n = 3) and young (3.4 ± 0.3 years; n = 3) Nili-Ravi bulls. The concentration of MDA did not differ (p > 0.05) between aged vs. young bulls in fresh (2.3 ± 0.2 vs. 2.9 ± 0.7 nmol mL⁻¹), frozen-thawed (53.1 ± 2.8 vs. 48.4 ± 2.6 nmol mL⁻¹) semen and seminal plasma (5.71 ± 0.97 vs. 5.19 ± 1.36 nmol mL⁻¹), respectively. In fresh semen, sperm motility and total concentration did not differ (p > 0.05) between aged and young bulls. The volume of fresh semen increased (p > 0.05) while sperm viability and DNA integrity decreased (p < 0.05) in aged vs. young bulls. In frozen-thawed semen, sperm motility, viability and DNA integrity decreased (p < 0.05) in aged vs. young bulls. In frozen-thawed vs. fresh semen, MDA level increased within young (48.4 ± 2.6 vs. 2.3 ± 0.2 nmol mL⁻¹) and aged bulls (53.1 ± 2.8 vs. 2.9 ± 0.7 nmol mL⁻¹). Conversely, sperm motility and viability decreased (p < 0.05) within the age groups between fresh and frozen-thawed semen. In conclusion, (1) lipid peroxidation (MDA) does not increase due to age; however, it is negatively associated with semen quality and (2) cryopreservation steps up lipid peroxidation irrespective of age and deteriorates semen quality of Nili-Ravi bulls.

ARTICLE HISTORY

Received 7 August 2017
Revised 29 November 2017
Accepted 1 December 2017

KEYWORDS

Malondialdehyde (MDA); Nili-Ravi buffalo; semen; age; lipid peroxidation

Introduction

The plasma membrane of buffalo bull spermatozoa is rich in polyunsaturated fatty acids and is susceptible to lipid peroxidation during freezing and thawing processes (Dhami and Kodagali 1990; Andrabi 2009). Lipid peroxidation produces malondialdehyde (MDA) (Aitken and Clarkson 1987; Beckman and Ames 1998) which damages structure and function of the plasma membrane of human (Cocuzza et al. 2008) and bull (Dawra et al. 1983) spermatozoa. The production of MDA in semen is directly related to the amount of polyunsaturated fatty acids in the cell membrane and reactive oxygen species (ROS; OH⁻, ROO⁻, H₂O₂ and O₂) in semen (Ayala et al. 2014). Therefore, MDA serves as a biomarker of oxidative stress and implicated in male infertility (Sikka et al. 1995; Beckman and Ames 1998).

As a general rule, mammalian cells experience increased production of ROS with age as the MDA (Beckman and Ames 1998). Previous studies indicate that MDA is an age-dependent agent (Cocuzza et al. 2008) and its concentration increased in blood plasma of aged buffalo (Al-Saedy and Tawfeek 2012) and ewes (Salar-Amoli and Baghbanzadeh 2010). Age-related decline in semen quality such as sperm motility, the percentage of live and morphologically normal spermatozoa is well documented in Nili-Ravi buffalo (Ahmad et al. 1987; Younis et al. 1998). Similar age-related adverse changes in semen quality were observed in Kundhi and beef bulls (Fuerst-Waltl et al. 2006; Kiani et al. 2014). Moreover, a progressive decline in semen quality and fertility of aged men (Eskenazi et al. 2003) suggests that lipid peroxidation is part of age-related oxidative stress and affects semen quality (Beckman and Ames 1998).

Therefore, we hypothesised that lipid peroxidation in semen increases with age of Nili-Ravi bulls and adversely affects the semen quality. The objective of the study was to compare MDA concentration and...
quality of fresh and frozen-thawed semen between aged and young Nili-Ravi buffalo bulls.

Materials and methods

Geographical location, experimental animals and semen collection

The current study was conducted at Semen Production Unit, Qadirabad, Sahiwal, Punjab, Pakistan (Latitude: 30.7° 42’ N/Longitude: 73° 14’ E). According to the Pakistan Metrological Department, the ambient temperature and the relative humidity during the experiment (March 2016; spring season) ranged from 23–33°C and from 16–43%, respectively. Chances of rainfall varied from 19–38% during the period. In this experiment, semen was collected from young (3.4±0.3 years; n = 3) and aged (13.6±1.0 years; n = 3) healthy Nili-Ravi bulls. A total of 12 ejaculates per age group i.e. four ejaculates per bull were collected through artificial vagina over a period of four weeks in March 2016 (Andrabi 2009). All bulls were kept in separate corrals and offered green fodder with 10% of body weight and the fixed amount of concentrates at 3 kg per day, and access to water ad libitum. All procedures used in this experiment were approved by Animal Care and Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore.

Semen evaluation and processing

Freshly collected semen samples were grossly analysed for volume, colour and consistency. All samples were subjected to microscopic analysis of sperm motility, concentration, viability and DNA integrity. Only samples having sperm motility >70% and minimum sperm concentration 500 x 10⁶ mL⁻¹ were processed further (Fayyaz et al. 2016). Based on low sperm motility (<70%), one semen sample from each age group was discarded. In the current study, TRIS-egg yolk citrate extender was used for semen dilution as it has least lipid peroxidation levels and highly recommended for buffalo semen extension (Mughal et al. 2013).

TRIS-citrate egg yolk extender (Citric acid 1.34% (wt:vol), Tris hydroxymethyl-aminomethane 2.42% (w:v), egg yolk 20% (vol:vol), glycerol 7% (vol:vol), fructose 1% (wt:vol), benzylpenicillin 1000 (U mL⁻¹) and streptomycin sulfate 1000 (µg mL⁻¹); pH 6.9) was used to extend the semen samples from each bull (Fayyaz et al. 2016). Diluted semen was cooled and equilibrated to 4°C for 4 h inside cold cabinet before packaging into straws (Andrabi et al. 2006). Semen was cryopreserved by exposing to liquid nitrogen vapours (−120°C for 10 min) and plunging into liquid nitrogen (−196°C) later. All samples were stored in a container at −196°C until further use. Thawing of frozen semen was done at 37°C for 30 s in a water bath to evaluate sperm quality parameter (sperm motility, viability, acrosome and DNA integrity) and lipid peroxidation.

Sperm motility and concentration

Sperm motility (%) in fresh and frozen-thawed semen was determined using phase contrast microscope (Olympus, Japan) at 40 X (Khan and Ijaz 2007). Sperm concentration was measured through Bovine photometer (model: n 1119, IMV, France) using 40 µL of each semen sample (Mughal et al. 2013).

Sperm viability, DNA and acrosomal integrity

Sperm viability was assessed by mixing a drop of semen sample with a relatively large drop of Eosin-Nigrosin stain onto a glass slide. After preparing a smear, a total of 200 sperm were observed under a phase contrast microscope (BX 51, Olympus, Japan) at 400 X (Khan and Ijaz 2007). Stained or partially stained (pink) head spermatozoa were characterised as dead (Khan and Ijaz 2007).

Sperm DNA integrity (%) was assessed using acridine orange (AO) staining technique. Briefly, air-dried smear of semen sample was fixed in Carnoy’s solution (methanol and glacial acetic, vol:vol, 3:1) for two hours. The glass slide was then air-dried and dipped in AO stain (0.19 mg mL⁻¹; Sigma-Aldrich Co., St. Louis, MO) for five minutes in dark. Later the slide was washed with deionised distilled water and air-dried before microscopic evaluation. A total of 200 spermatozoa were counted under an epifluorescence microscope (Olympus CX41, Japan; 480/550 nm) to differentiate spermatozoa with intact (double-stranded; green) and damaged DNA contents (single-stranded; red; Fayyaz et al. 2016).

Sperm acrosomal integrity (%) was assessed in frozen-thawed semen samples by mixing 500 µL of semen with 50 µL of 1% (vol:vol) formal citrate solution. A total of 200 spermatozoa were observed under a phase contrast microscope at 100X magnification for the integrity of apical ridge (NAR). Spermatozoa with a black crescent-shaped apical ridge were considered having intact acrosomal membrane (Khan and Ijaz 2007).
Measurement of lipid peroxidation: malondialdehyde (MDA)

Malondialdehyde concentration in fresh and frozen-thawed semen of young and aged bulls was measured by thiobarbituric acid assay (TBA; Ohkawa et al. 1979; Mughal et al. 2013). Briefly, fresh and frozen-thawed semen sample from each bull containing \(40 \times 10^6\) spermatozoa was mixed with 200 \(\mu L\) of sodium dodecyl sulfate solution (8%; wt:vol), followed by addition of 20% (vol:vol) acetic acid (pH 3.5) in a graded tube. Then, 1.5 mL of 0.8% (vol:vol) solution of thiobarbituric acid (TBA) and distilled water were added to the tube to make a final volume of 4 mL. The whole solution was heated at 95°C for 60 mins and cooled to room temperature before making up the final volume to 5 mL by adding distilled water. Later, 5 mL of butanol: pyridine solution (vol:vol; 15:1) was mixed in solution and the mixture was centrifuged at 4000 g for 10 min. Finally, 50 \(\mu L\) of a supernatant layer of the solution was aliquoted to measure the absorbance on ELISA reader (HER-480, HT Company Ltd, Essex, UK) at 492 nm wavelength (Khosrowbeygi and Zarghami 2007). The concentration of MDA (nmol mL\(^{-1}\)) in each sample was calculated from the standard curve having known concentrations of tetramethoxy propane (TMP) i.e. 0.00, 0.78, 1.5, 3.1, 6.2, 12.5, 25, 50, and 100 \(\mu M\) (Mughal et al. 2013). Malondialdehyde was measured in fresh and frozen-thawed semen (\(n=11\) per age group). In addition, seminal plasma (\(n=3\) per age group) obtained by centrifugation (3000 g for 15 min) of freshly collected semen was also processed in each replicate (Nair et al. 2006). Each replicate of fresh or frozen-thawed semen consisted of three samples per age group, an extender sample, a standard curve and a negative control (ethanol). In each replicate, each semen sample was run as a single well while standard curve (TMP), negative control and the extender were run in duplicates.

Statistical analysis

Shapiro-Wilk test was used to check the normality of data. Within age group, comparison of fresh and frozen-thawed semen quality parameters (volume, motility, concentration, viability, DNA and acrosomal integrity and MDA concentration; mean±standard error mean) was done using paired student t-test (IBM\textsuperscript{®} SPSS\textsuperscript{®}, Chicago, IL, Statistics 20). However, unpaired t-test was used to compare semen quality parameters and MDA concentration between age groups. MDA concentration of egg yolk extender was subtracted from frozen-thawed samples before comparison between age groups. Fresh and frozen-thawed semen quality parameters and MDA concentration of aged and young bulls were correlated using Pearson coefficient of correlation (IBM\textsuperscript{®} SPSS\textsuperscript{®}, Chicago, IL, Statistics 20).

Results

Age and lipid peroxidation

The concentration of lipid peroxidation (MDA; Mean±S.E.M) within fresh and frozen-thawed semen did not differ (\(p>.05\)) between aged and young bulls (Figure 1). Similarly, MDA concentration did not differ (\(p>.05\)) in seminal plasma of aged and young bulls i.e. 5.71±0.97 and 5.19±1.36 nmol mL\(^{-1}\), respectively. The mean MDA concentration of egg yolk extender was negligible i.e. 0.004 nmol mL\(^{-1}\). The week-wise variation in MDA concentration of fresh semen in each bull was also non-significant (\(p>.05\)). The inter-assay coefficient of variation between replicates (\(n=4\)) was 3%. The concentration of MDA increased within young and aged bull semen (\(p<.05\)) after cryopreservation and thawing (Figure 1). Overall, there was an adverse effect of MDA concentration on semen quality. The MDA concentration was negatively correlated with viability and DNA integrity in fresh semen. Similarly, a significant negative correlation was observed between

![Figure 1. Effect of age on concentration of MDA in fresh (\(n=18\)) and frozen-thawed semen (\(n=22\)) of Nili-Ravi Buffalo bulls.](image-url)
MDA concentration and all semen quality parameters of frozen-thawed semen (Table 1).

**Age and semen quality**

In fresh semen, the volume of the ejaculate was higher \( (p < .05) \) in aged than young bulls. Both the sperm viability and DNA integrity were lower \( (p < .05) \) in aged than young bulls (Table 1). Other parameters i.e. sperm motility and concentration did not differ due to age. In frozen-thawed semen, motility, viability and DNA integrity were lower \( (p < .05) \) in aged than young bulls. On the other hand, acrosome integrity did not differ \( (p > .05) \) due to age (Table 2). Overall, there was a negative effect of age on the quality of fresh and frozen-thawed bull semen (Table 3).

**Discussion**

In this study, we hypothesised that lipid peroxidation in semen would increase with age of bull. However, based on the results, it appeared that overall lipid peroxidation in bull semen was independent of age. According to a previous study, increased lipid peroxidation is not a universal feature of aging (Rikans and Hornbrook 1997). In mammals, lipid peroxidation has been suggested to vary according to species, strain, sex and tissue (Rikans and Hornbrook 1997). Therefore, we deduced that lipid peroxidation occurs independently of age in Nili-Ravi bull semen, and could adversely affect the spermatozoa as reported in earlier studies (Nair et al. 2006; Kadirvel et al. 2009). This fact is further substantiated by our findings that regardless of age, a strong negative association of lipid peroxidation with sperm viability and DNA integrity existed in the current study. As plasma membrane of buffalo spermatozoa are more susceptible to oxidation than cattle because of abundant polyunsaturated fatty acid (Nair et al. 2006), thereof, plasma membrane becomes permeable to ROS which damages DNA and makes sperm non-viable as observed in the current study (Table 2). Such a mechanism of loss of sperm membrane and viability due to lipid peroxidation has also been suggested in human (Wang et al. 2003).

Concurrent with the findings in Murrah buffalo (Kadirvel et al. 2009), we observed a ten-fold increase in lipid peroxidation of frozen than fresh semen irrespective of bull’s age (Figure 1). Increase in lipid peroxidation of spermatozoa during cryopreservation has been attributed to the reduction of antioxidant enzymes after semen extension (Bilodeau et al. 2000; Baumber et al. 2003), and higher activity of aromatic amino oxidase enzyme from cryo-injured or dead spermatozoa (Upreti et al. 1998). As a result, frozen-thawed spermatozoa are easily peroxidised than fresh spermatozoa (Beconi et al. 1993). In support, we found that the magnitude of the progressive decline in the semen quality of frozen-thawed spermatozoa was similar between the age groups (Table 2); correspondingly indicating that lipid peroxidation is mainly the outcome of cryopreservation rather than age.

**Table 1.** Relationship of lipid peroxidation (MDA) with fresh and frozen-thawed semen quality of Nili-Ravi buffalo bulls.

| Parameters                      | Fresh (n = 18) | Frozen (n = 22) |
|---------------------------------|----------------|-----------------|
| Ejaculatory volume, mL          | 0.20           | 0.16            |
| Sperm concentration, million mL\(^{-1}\) | 0.16           | 0.07            |
| Sperm motility, %               | 0.03           | 0.03            |
| Sperm viability, %              | 0.07           | 0.06            |
| Sperm acrosome integrity, %     | 0.03           | 0.03            |
| Sperm DNA integrity, %          | 0.03           | 0.03            |

\(^{*}\)Indicates significance level \( p < .05 \).

**Table 2.** Comparison between fresh and frozen-thawed semen quality parameters of young and aged Nili-Ravi Buffalo bulls.

| Parameters                      | Young bulls (n = 3; 3.4 ± 0.3 years) | Aged bulls (n = 3; 13.6 ± 1.0 years) |
|---------------------------------|-------------------------------------|-------------------------------------|
| Ejaculatory volume, mL          | 2.1 ± 0.34\(^{*}\)                 | 4.7 ± 0.89\(^{b}\)                 |
| Total concentration, million mL\(^{-1}\) | 2335.7 ± 1242.4\(^{a}\)            | 3550.5 ± 1342.6\(^{a}\)            |
| Sperm motility, %               | 72.2 ± 0.71\(^{a}\)                | 46.6 ± 0.39\(^{a}\)                |
| Sperm viability, %              | 82.8 ± 1.90\(^{a}\)                | 70.8 ± 1.40\(^{a}\)                |
| Sperm acrosome integrity, %     | 70.6 ± 2.16\(^{a}\)                | 65.0 ± 1.71\(^{b}\)                |
| Sperm DNA integrity, %          | 90.2 ± 1.90\(^{a}\)                | 85.9 ± 1.29\(^{b}\)                |

\(^{*}\)Within rows indicate difference \( p < .05 \) between fresh semen of young and aged bulls.

\(^{ab}\)Within rows indicate difference \( p < .05 \) between fresh semen of young and aged bulls.

\(^{ab}\)Within rows indicate difference \( p < .05 \) between frozen-thawed semen of young and aged bulls.

\(^{\cdots}\)Within rows indicate difference \( p < .05 \) between frozen-thawed semen of young and aged bulls.

\(^{a}\)Indicates significance level \( p < .05 \).
Thus far, information on the topic of an age-related decrease in semen quality of Nili-Ravi bulls is limited. Concurrent with our findings, a higher percentage of non-viable spermatozoa and increased ejaculatory volume has been reported in aged than young Nili-Ravi bulls (Younis et al. 1998; Javed et al. 2000). However, precise mechanisms regarding age-related damage to sperm DNA and viability remain elusive. It is likely that cellular or physiological alteration in the genitourinary tract and apoptosis or poor cell selection during and after spermatogenesis may be involved in age-related decline in semen quality (Singh et al. 2003). Moreover, cellular degeneration in male reproductive tract has been associated with ROS-induced lipid peroxidation due to age (Sikka et al. 1995). Therefore, the association between bull’s age and lipid peroxidation may be further explained by determining the status of ROS and antioxidants in semen.

Conclusions

Altogether, this study demonstrates that lipid peroxidation in sperm plasma membrane is independent of the age of Nili-Ravi bull. Increased lipid peroxidation in frozen-thawed semen compared to fresh semen is mainly due to the cryopreservation. In addition, both lipid peroxidation and age have a negative association with semen quality parameters of Nili-Ravi bulls; however, further investigations are required to determine the association of ROS with lipid peroxidation in aged bull semen.

Acknowledgements

The authors would like to thank Prof. Dr. Nazir Ahmad, FVS, University of Agriculture, Faisalabad for critically reviewing the manuscript. Authors also extend their gratitude to Dr. Muhammad Shahbaz Yousaif, Assistant Professor, Department of Physiology, University of Veterinary and Animal Sciences, Lahore for providing TMP for lipid peroxidation assay.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Muhammad Irfan-ur-Rehman Khan http://orcid.org/0000-0002-2432-5753

References

Ahmad M, Latif M, Ahmad M. 1987. Morphological abnormalities of spermatozoa of Nili-Ravi buffalo. Buff J. 2:153–160.

Aitken RJ, Clarkson JS. 1987. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J Reprod Fertil. 81:459–469.

Al-Saedy KA, Tawfeek FK. 2012. Role of age with some physiiological and biochemical parameters in local female buffaloes. Iraq J Vet Sci. 26:219–223.

Andrabi S. 2009. Factors affecting the quality of cryopreserved buffalo (Bubalus bubalis) bull spermatozoa. Reprod Domest Anim. 44:552–569.

Andrabi S, Siddique M, Ullah N, Khan L. 2006. Effect of reducing sperm numbers per insemination dose on fertility of cryopreserved buffalo bull semen. Pak Vet J. 26:17–19.

Ayala A, Munoz MF, Arguelles S. 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014:1–31.

Baumber J, Ball BA, Linfor JJ, Meyers SA. 2003. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J Androl. 24:621–628.

Beckman KB, Ames BN. 1998. The free radical theory of aging matures. Physiol Rev. 78:547–581.

Beconi M, Francia C, Mora N, Affranchino M. 1993. Effect of natural antioxidants on frozen bovine semen preservation. Theriogenology. 40:841–851.

Bilodeau JF, Chatterjee S, Sirad MA, Gagnon C. 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol Reprod Dev. 55:282–288.

Cocuzza M, Athayde KS, Agarwal A, Sharma R, Pagani R, Lucon AM, Srougi M, Hallak J. 2008. Age-related increase of reactive oxygen species in neat semen in healthy fertile men. Urology. 71:490–494.

Dawra RK, Sharma OP, Makkar HP. 1983. Lipid peroxidation in bovine semen. Int J Fertil. 28:231–234.

Dhami A, Kodagali S. 1990. Freezability, enzyme leakage and fertility of buffalo spermatozoa in relation to the quality of semen ejaculates and extenders. Theriogenology. 34:853–863.

Eskenazi B, Wyrobek AJ, Sloter E, Kidd S, Moore L, Young S, Moore D. 2003. The association of age and semen quality in healthy men. Hum. Reprod. 18:447–454.

Fayyaz M, Ahmad M, Ahmad N. 2016. Survival of buffalo bull spermatozoa: effect on structure and function due to alpha-lipoic acid and cholesterol-loaded cyclodextrin. Andrologia. 49:e12652.

Fuerst-Waltl B, Schwarzenbacher H, Perner C, Solkner J. 2006. Effects of age and environmental factors on semen production and semen quality of Austrian Simmental bulls. Anim Reprod Science. 95:27–37.

Javed MT, Khan A, Kausar R. 2000. Effect of age and season on some semen parameters of Nili-Ravi buffalo (Bubalus bubalis) bulls. Veterinarski Arhiv. 70:83–94.

Kadirvel G, Kumar S, Kumaresan A. 2009. Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. Anim Reprod Sci. 114:125–134.

Khan M, Ijaz A. 2007. Assessing undiluted, diluted and frozen-thawed Nili-Ravi buffalo bull sperm by using standard semen assays. Italian J Anim Sci. 6:784–787.
Khosrowbeygi A, Zarghami N. 2007. Levels of oxidative stress biomarkers in seminal plasma and their relationship with seminal parameters. BMC Clin Pathol. 7:6.

Kiani FA, Yousaf A, Zafar MA, Nawaz M. 2014. Effect of age on physical characteristics of Kundhi buffalo bull semen. Int J Curr Microbiol App Sci. 3:445–453.

Mughal D, Ijaz A, Yousaf M, Rehman H, Aleem M, Zaneb H, Wadood F. 2013. Assessment of optimal osmotic pressure of citrate egg yolk extender for cryopreservation of buffalo bull (*Bubalus bubalis*) semen. J Anim Plant Sci. 23:964–968.

Nair SJ, Brar A, Ahuja C, Sangha S, Chaudhary K. 2006. A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. Anim Reprod Sci. 96:21–29.

Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 95:351–358.

Rikans LE, Hornbrook KR. 1997. Lipid peroxidation, antioxidant protection, and aging. Biochim Biophys Acta. 1362:116–127.

Salar-Amoli J, Baghbanzadeh A. 2010. Oxidative stress in Shaal sheep of different age groups. Turkish J Vet Anim Sci. 34:379–383.

Sikka SC, Rajasekaran M, Hellstrom WJ. 1995. Role of oxidative stress and antioxidants in male infertility. J Androl. 16:464–468.

Singh NP, Muller CH, Berger RE. 2003. Effects of age on DNA double-strand breaks and apoptosis in human sperm. Fertil Steril. 80:1420–1430.

Upreti G, Jensen K, Munday R, Duganzich D, Vishwanath R, Smith J. 1998. Studies on aromatic amino acid oxidase activity in ram spermatozoa: role of pyruvate as an antioxidant. Anim Reprod Sci. 51:275–287.

Wang X, Sharma RK, Sikka SC, Thomas AJ, Falcone T, Agarwal A. 2003. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. Fertil Steril. 80:531–535.

Younis M, Samad H, Ahmad N, Ahmad I. 1998. Studies on semen quality of young, adult and old buffalo bulls during low and peak breeding seasons. Pak Vet J. 18:134–140.