

Role of antioxidant additives in the protection of DNA integrity of buck spermatozoa with RAPD assay

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Integrity of spermatozoa is an important alternative for the success of natural or assisted fertilization including normal development of the embryo, offspring, transmission of genetic information and the maintenance of good health in future generations. Cryopreservation of semen leads to the production of free radicals, which damage sperm DNA due to little cytoplasmic content and reduction of antioxidant (Bilodeau *et al.* 2000). Many assays are used to measure DNA damage in mature and immature sperm cells (Gonzalez-Marin *et al.* 2012). This study used random amplified polymorphic DNA assay (RAPD) assay to evaluate the susceptibility of buck sperm DNA to denaturation resulting from cryopreservation and whether there is any effect of antioxidants on sperm DNA. The study included 43 ejaculates of Sirohi bucks (4) aged between 2 and 3 years, subjected to routine semen collection with the aid of the artificial vagina (AV). The volume of ejaculates was measured in a graduated conical collection cups at 0.1 ml intervals and the mass activity (measured on 0–5 scale) was estimated using phase-contrast microscope (10×). The ejaculates between 1 and 2 ml in volume and with >80% progressive motility were included in this study. Each ejaculate was split into 4 equal aliquots and dilution at 37°C was done in Tris-based extender (Tris 290mM/l, citric acid 100mM/l, fructose 100 mM/l, glycerol 6%) with 10% egg yolk, reduced GSH (7 mM) named as T3, vitamin C (9 mM) named as T4, α -tocopherol (4.5mM) named as T5 and control (Saraswat *et al.* 2012). To establish comparison between freshly diluted and post thaw samples, the freshly diluted samples (5 ml each) were stored for DNA integrity in deep freezer. While remaining part (5 ml) of all the aliquots were kept for equilibration period of 4 h in cold cabinet machine. After equilibration period, prefreeze or equilibration motility was evaluated. Sufficient number of

straws were filled and sealed with polyvinyl chloride (PVC) powder. Freezing of semen straws was done in the vapour of liquid nitrogen (–120° to –130°C) for 10 min then, the straws were stored in liquid nitrogen (–196°C) for 2 days, before being thawed for evaluation of DNA integrity.

DNA from semen samples was isolated as per Rout (2004). The isolated genomic DNA was examined for purity and concentration by biophotometer. The quality of genomic DNA was checked by horizontal submarine agarose gel electrophoresis. The PCR reaction mixture was prepared using autoclaved triple distilled water (36.5 μ l), 10X PCR buffer (5 μ l), 15 mM MgCl₂ (3 μ l), dNTPs of 100mM each (1 μ l), primer of 10 picomole each (2 μ l), 0.5 μ l Taq DNA polymerase of 0.5 unit/ml and genomic DNA (2 μ l). The standardized PCR programme comprised following steps—initial denaturation at 94°C for 2 min (step1), followed by 40 cycles of denaturation at 94°C for 1 min (step 2), annealing at 40°C for 1 min (step 3), extension at 72°C for 2 min (step 4) and a final extension at 72°C for 5 min in a gradient thermal cycler. PCR products were subjected to agarose gel electrophoresis. Mean of total amplified products (bands) obtained in freshly diluted and frozen/thawed samples indicated the damage induced by cryopreservation.

Genomic DNA was isolated from 43 freshly diluted and post thaw semen samples. The DNA samples not showing smear when evaluated by agarose gel electrophoresis and OD between 1.6 to 1.8 were used in the present study while those samples showing smear were discarded (control

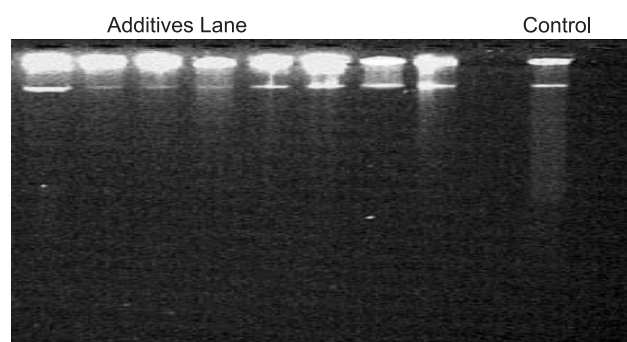


Fig. 1. Original DNA samples of semen.

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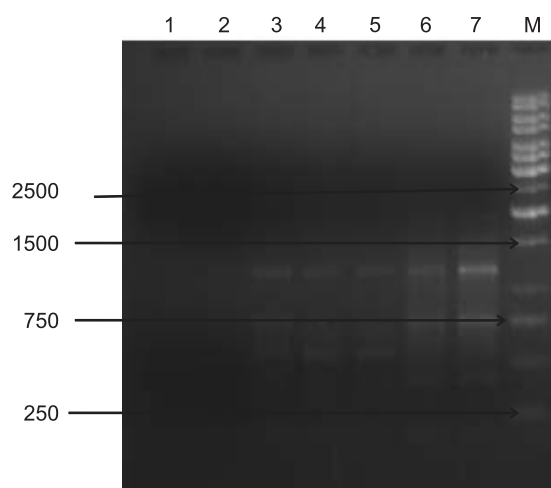


Fig. 2. Lane M-marker (1kb); lane 1–7: amplified PCR products.

samples) (Fig. 1).

Results scored as patterns of bands obtained from freshly diluted and post thaw treated samples. Both primers (OPM-5: 5'GGGAACGTGT'3 and OPM-12: 5'GGGACGTTGG'3) analyzed the presence or absence of amplification and showed differences in fragment intensity. Reduced glutathione showed more number of total bands after post thaw as compared to vitamin C and α -tocopherol (Figs 2–4; Table 1). Moreover, band analysis of individual samples was done. Total amplified products (bands) of OPM-5 were 78, the size of amplification products ranged from 190 to 1900 base pair (Fig. 2). Similarly total amplified products of OPM-12 were 42 and the size of amplification products ranged from 190–1900 base pair (Fig. 4).

RAPD assay is relatively new in its application to determine DNA damage in sperm if comet assay is not possible. With its application, we analyzed semen samples supplemented with antioxidants. Reduced glutathione (7 mM) showed more number of bands after post-thaw as compared to ascorbic acid (9 mM) and α -tocopherol (4.5 mM) (Figs 1–3). Our results indicated that ascorbic acid and α -tocopherol at respective concentration (Saraswat *et al.* 2012) have higher efficacy in protecting DNA from damage. This is due to the unique tight packaging arrangement of DNA within the sperm nucleus as sperm and spermatids no longer possess significant cytoplasm. α -tocopherol protects the sperm DNA from hydrogen

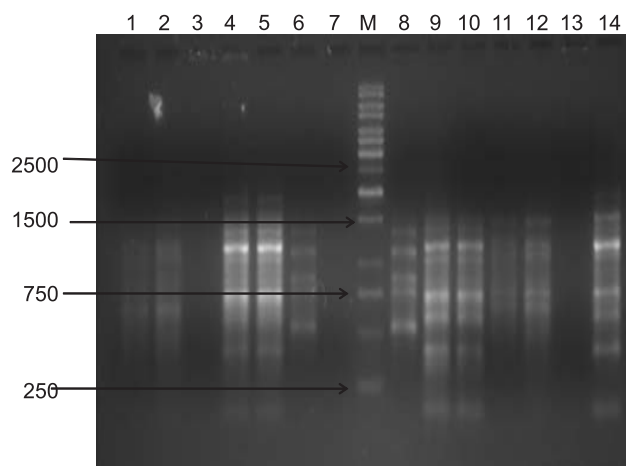


Fig. 3. Lane M-marker (1kb); lane 1–14: amplified PCR products.

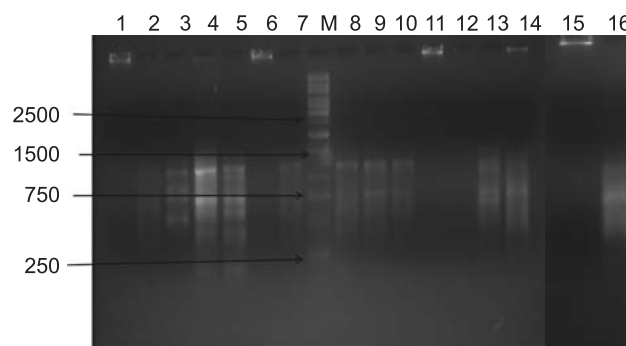


Fig. 4. Lane m-Marker (1kb); lane 1–16: amplified PCR products.

peroxide induced DNA damage *in vitro* due to its chain-breaking property so helps in protecting genomic DNA from the deleterious effects of free radicals (Kalthur *et al.* 2011, Saraswat *et al.* 2012) while, ascorbic acid reduces the production of reactive oxygen species during cryopreservation, thus protects spermatozoa from cryodamage and inhibit DNA damage (Li *et al.* 2010). Li *et al.* (2010) showed that supplementation of antioxidants (ascorbate and catalase) reduced ROS levels and sperm nuclear DNA damage (assessed by comet assay) and improved the human sperm motility in the process of freezing/thawing. In semen samples exhibiting ROS activity, ascorbic acid concentrations in the seminal plasma

Table 1. Effect of antioxidants on DNA integrity of cryo-preserved buck sperm

Treatments	Total no. of bands produced in			
	Freshly diluted with Primer OPM-5 (5'GGGAACGTGT'3)	Post thaw semen with primer OPM-5 (5'GGGAACGTGT')	Freshly diluted OPM-12 (5' GGGACGTTGG'3)	Post thaw semen OPM-12 (5'GGGACGTTGG'3)
T3 (reducedglutathione7mM)	7	10	7	10
T4 (ascorbic acid 9mM)	16	15	9	9
T5 (a-tocopherol 4.5mM)	15	15	4	3

are significantly reduced and vitamin C protects sperm DNA from damage induced by oxidative stress (Zini and Al-Hathal 2011). The presence of vitamin E in the vicinity of spermatozoa may help in effectively scavenging the highly unstable free radicals generated during the freezing and thawing process (Kalthur *et al.* 2011). Berlinguer *et al.* (2009) reported that DNA integrity represents an important parameter in the evaluation of frozen/ thawed semen functionality. Though, DNA repair occurs in developing sperm but it is terminated as transcription and translation cease post-spermatogenesis (Aitken *et al.* 2004). As a result, sperms have no mechanism to repair DNA damage incurred during their transit and storage in the epididymis, post-ejaculation or during freezing procedures (Berlinguer *et al.* 2009). The level of DNA damage with supplementation of reduced glutathione is contrary to the results obtained by Gadea *et al.* (2008), which might be due to greater oxidative damage (Van Loon *et al.* 1993).

In general, freezing affects chromatin structure (Donnelly *et al.* 2001). Chromatin stabilization occurs by intermolecular and intramolecular covalent disulphide bonds between the protamines that replace histones during spermatogenesis (Poccia 1986). Semen cryopreservation disrupts or destroys these genetic arrays rendering DNA more accessible to damage and thus impairs fertility. Furthermore, Kumari *et al.* (2008) found that PCR is one of the most reliably used techniques for detecting DNA damage as the amplification stops at the site of the damage thus amplification and analysis depends on the intensity of amplified band (Kumari *et al.* 2008). To our knowledge, this is the first in-depth study reporting, RAPD-assay to assess DNA damage in sperm from freshly diluted and cryopreserved semen samples supplemented with antioxidants (reduced glutathione, ascorbic acid and α -tocopherol) as it gives detailed information on the quantitation of DNA integrity of individual sperm.

SUMMARY

The role of antioxidants (reduced glutathione, ascorbic acid and α -tocopherol) on DNA integrity with the random amplified polymorphic DNA (RAPD) was studied. This assay relies on presence or absence of amplification product on ethidium bromide stained agarose gels. The size of amplification products ranged from 190 to 1,900 base pair. Antioxidants effectively protect sperm DNA from damage. Moreover, random amplified polymorphic DNA technique can be used for the studies of the molecular seminal characteristics of buck semen.

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