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ScienceDirect
Small Ruminant Research 75 (2008) 128–134
Small Ruminant Research
www.elsevier.com/locate/smallrumres

Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze–thawing process

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Received 5 April 2007; received in revised form 24 August 2007; accepted 7 September 2007
Available online 18 October 2007

Abstract

Oxidative damage to sperm resulting from reactive oxygen species generated by the cellular components of semen is one of the main causes for the decline in motility and fertility of sperm during the freeze–thawing process. The aim of this study was thus to determine the effects of anti-oxidants on standard semen parameters, lipid peroxidation (LPO) and anti-oxidant activities after the freeze–thawing of ram semen. Ejaculates collected from four Akkaraman rams, were pooled and evaluated at 33 °C. Semen samples were diluted in a Tris-based extender containing the anti-oxidants glutathione (GSH) (5 mM), oxidized glutathione (GSSG) (5 mM) or cysteine (5 mM) and an extender containing no anti-oxidants (control), cooled to 5 °C and frozen in 0.25 ml French straws. Frozen straws were thawed individually for 20 s in a water bath (37 °C) for microscopic evaluation. The use of an extender supplemented with cysteine led to the highest ($P < 0.01$) post-thaw motility (61.0 ± 1.9%), compared to the other treatment groups. No significant differences were observed in viability, acrosome damage and total abnormalities, and following the hypo-osmotic swelling test (HOST), following supplementation with anti-oxidants after the thawing of the semen. Following the thawing process, the levels of malondialdehyde (MDA) did not change with the addition of anti-oxidants, compared to the control. The GSH level and glutathione peroxidase (GSH-PX) activity remained significantly higher upon the addition of GSH (3.33 ± 0.14 nmol/ml and 22.02 ± 1.27 IU/g protein) and GSSG (3.24 ± 0.08 nmol/ml and 20.17 ± 3.38 IU/g protein) compared to the other treatment ($P < 0.001$) groups. Only cysteine significantly elevated the activity of catalase (CAT, 842.40 ± 90.42 *k*U/l) following the freeze–thawing process. The Vitamin E (VitE) level was significantly higher, when compared to GSSG, cysteine and the control, when GSH (4.21 ± 0.20 mg/dl) was added to the freezing extender ($P < 0.001$). It could be concluded that future efforts aimed on improving the efficiency of cryopreservation of ram sperm should concentrate on the use of anti-oxidant additives. The results obtained provide a new approach to the cryopreservation of ram semen, and could positively contribute to intensive sheep production.

Keywords: Anti-oxidants; Anti-oxidant activities; Freezing; Ram semen; Semen parameters

1. Introduction

Semen cryopreservation offers many advantages to the livestock industry, particularly in conjunction with allowing the widespread dissemination of valuable genetic material—even to small flocks by means of artificial insemination (AI). Thereby leading to increased rate

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doi:10.1016/j.smallrumres.2007.09.002

of genetic gain and the prevention or control of diseases within sheep populations (Evans and Maxwell, 1987; Holt, 1997). Transcervical insemination of frozen semen in sheep has also been shown to result in significantly lower pregnancy rates when compared to intra-uterine laparoscopic insemination—indicating a limitation in the cryopreservation process (King et al., 2004).

The mammalian sperm cell contains a high proportion of polyunsaturated fatty acids, and is therefore particularly susceptible to peroxidative damage, especially following cryopreservation—with a subsequent loss in membrane integrity, impaired cell function and decreased motility and the fertilizing capability of the sperm for AI (Aitken, 1984; Maxwell and Watson, 1996). The lipid composition of the sperm membrane is a major determinant of the viability, lipid peroxidation and cold shock experienced in spermatozoa. Cold shock of the sperm arising from freezing process plays an important role in the structure of membranes, by determining their membrane phase and the dynamic status that affect the fusion of the plasma membranes of the male and female gametes (Bakst and Sexton, 1979; Hammerstedt, 1993; Yanagimachi, 1994). During cryopreservation, semen is exposed to cold shock and atmospheric oxygen, which in turn increases their susceptibility to lipid peroxidation (LPO) due to higher production of reactive oxygen species (ROS). In addition to the damaging effects, the ROS are physiologically involved in the maintenance of the fertilizing ability and capacitation/acrosome reaction of spermatozoa. Excessive ROS impairs the motility and fertilization capacity (Alvarez and Storey, 1982; Alvarez and Storey, 1984; Baumber et al., 2000).

The improvement of semen cryopreservation technologies requires in depth knowledge of the gamete physiology and the biochemical processes occurring during semen collection, processing and freeze–thawing (Yoshida, 2000). The anti-oxidant system comprising reduced glutathione (GSH), glutathione peroxidase (GSH-PX), catalase (CAT), and superoxide dismutase (SOD) has been described as defense functioning mechanism against the LPO in semen, and important in maintaining sperm motility and viability (Bilodeau et al., 2001; Aitken and Baker, 2004; Gadea et al., 2004). This endogenous anti-oxidant capacity may however be insufficient to prevent LPO during prolonged storage (Aurich et al., 1997). In recent years, studies have also been conducted on ram semen diluents, including anti-oxidants such as cysteamine, taurine, trehalose and selenium to improve the post-thawed motility, viability and membrane integrity of spermatozoa (Semenak et al., 1999; Aisen et al., 2002; Uysal et al., 2005; Bucak et al., 2007).

Thiols such as cysteine, GSH and oxidized glutathione (GSSG) are the largest category of anti-oxidants. The glutathione or GSH is a molecule found at the mM level in a number of cells, able to react with many reactive oxygen species directly. GSH is also a co-factor for GSH-PX that catalyzes the reduction of toxic H_2O_2 and other hydroperoxides, protecting the mammalian cells from oxidative stress. However the intracellular level of thiols in sperm is reduced by cryopreservation (Meister, 1994; Bilodeau et al., 2001). The oxidized form of glutathione (GSSG) is partially regenerated from GSH by the action of the enzyme glutathione reductase, and secreted from the cell if capacity of glutathione reductase is exceeded (Meister, 1994; Rossi et al., 2002).

It is known that mammalian cells can only utilize cysteine, as it has been shown to penetrate the cell membrane easily enhancing the intracellular GSH biosynthesis both in vitro and in vivo, and protecting membrane lipids and proteins due to its direct radical-scavenging properties. It is also thought that GSH synthesis under in vitro conditions may be impaired because of the deficiency of cysteine in the media, due to its high instability and easy auto-oxidation to cysteine (Sagara et al., 1993; Mazor et al., 1996).

There are few studies on the effects of anti-oxidants containing thiol compounds in the cryopreservation of ram semen. The uses of GSH, GSSG and cysteine in ram semen cryopreservation have not previously been reported. Therefore, the present study was undertaken to investigate the effects of anti-oxidants such as GSH, GSSG and cysteine on standard semen parameters, LPO and anti-oxidant activities (GSH, GSH-PX, CAT and VitE) following the freeze–thawing process.

2. Materials and methods

2.1. Chemicals

The anti-oxidants (GSH, GSSG and cysteine) and other chemicals used in this study were obtained from Sigma–Aldrich chemicals (Interlab Ltd., Ankara, Turkey).

2.2. Animals and semen collection

The animals were housed at the Ankara University, Faculty of Veterinary Medicine, Education Research and Practice Farm and maintained under uniform feeding, housing and lighting conditions. Semen samples were obtained from four mature Akkaraman rams (3 and 4 years of age) and a total number of 48 ejaculates were collected from the rams with the aid of the artificial vagina twice a week during the breeding season (autumn to early winter).