A Study on the Cryopreservation of Stallion Semen with Alpha Lipoic Acid

Dr. Jawad Hussain1, Abdul Salam2 and Dr. Ali Gohar3

1 School of Animal, Rural and Environmental Sciences, Nottingham Trent University, England, United Kingdom
2 Department of Chemistry, Loughborough University, England, United Kingdom
3 University of Veterinary and Animal Sciences, Lahore, Pakistan

E-Mail: jawadhussain787@yahoo.com

Abstract

Horses have the lowest fertility rate in all of the domesticated species. In order to accomplish successful conception, artificial insemination, the assisted reproductive technique is the best procedure. Sperm quality during cryopreservation dramatically diminishes because of the oxidative stresses which results from the production of reactive oxygen species (ROS) during sperm metabolism. The aim of this study was to investigate if antioxidant alpha lipoic acid (ALA) supplementation increases the efficacy of fertility Parameters in cryopreserved stallion semen. For this research, a different concentrations / inclusion level of Alpha lipoic acids (0.0, 1.0, 0.5, 0.1, 0.05, 0.025 and 0.125 mmol / ml) was used in the semen of two adult Equus caballus. Post thawed semen analysis (Motility, vitality, Plasma membrane integrity, Acrosomal integrity and morphology of the semen) was conducted. The results indicate that there is no positive effect of ALA on fertility parameter of the post thawed stallion semen. In conclusion, ALA like in other bovine species, do not appear to be cryoprotectant to stallion semen.

Keywords: Artificial Insemination, Sperm Quality, Cryopreservation, Alpha Lipoic Acids (ALA)

1. Introduction

Horses have the lowest fertility rate in all the domesticated species which might be possible, as horses are mostly bread selectively for their conformation and performance characteristics rather than for their fertility accomplishment (Baczynska et al, 2007 and Blomfield, 2010). The aim of this study was to investigate if antioxidant alpha lipoic acid (ALA) supplementation increases the efficacy of fertility Parameters in cryopreserved stallion semen. Artificial insemination (AI) is considered one of the modern way of breed improvement which is use for the genetic improvement and improved production potential of horses (Stout, 2006). In the process of (AI), chilled or cryopreserved semen is used (Morel, 1999). Advantages of AI comprise the reduced risk of the spread of both venereal and non-venereal disease such as equine infectious anaemia, equine influenza and strangles (Aurich et al, 1997 and Blanchard et al, 2003).

During cryopreservation, osmotic and thermal shocks induce reactive oxygen species (ROS). A small amount of ROS is imperative for normal function of spermatozoa, but when the balance between ROS production and neutralization is disrupted, an excess of ROS leads to oxidative stress affecting the fluidity of membrane and integrity of DNA in sperm nucleus (Aitkin, 1999). These changes diminish the post-thaw sperm quality and subsequent fertility.

The seminal plasma of mammals contains an antioxidant defence system comprising of enzymatic and non-enzymatic antioxidants. However during the storage procedure semen is diluted by the addition of extender to produce multiple doses from a single ejaculate. This dilution decreases the concentration of natural antioxidants in semen, leading to the production of ROS in supra-physiologic limits.
All cellular components including lipids, proteins, nucleic acid and sugars are potential targets for ROS. Therefore, the amount of ROS should be limited to the minimum level in order to maintain the normal cell function (Agarwal et al, 2003). The excessive ROS can be neutralized by the restoration of antioxidant defence system of semen. This can be obtained through the addition of suitable antioxidants in semen extender. The addition of antioxidants such as vitamin E and cysteine to semen extender improved the longevity and quality of chilled and frozen-thawed semen in several species (Bilodeau et al, 2001).

However, there is no data regarding the effect of α-lipoic acid (ALA) on horse spermatozoa. ALA has been proved to be a potent antioxidant and capable of scavenging hydroxyl radicals, hypochlorous acid, peroxynitrite, singlet oxygen, and superoxide (Packer et al, 2001). ALA has also the potential to regenerate other antioxidants such as vitamin C (Ibrahim et al, 2008) and vitamin E (Packer et al, 2001). In spite of these promising results, the use of ALA in semen extenders used for cryopreservation is not common and recent knowledge is unable to find literature that evaluates the potential effect of ALA on frozen-thawed spermatozoa.

2. Material and Method

A total of two adult equine stallions (9 years old Welsh section C) were used in this study. These stallions had clinically normal reproductive tract and were donating semen of acceptable quality for artificial insemination. Spermatozoa from the stallions was collected with the help of artificial vagina. Immediately after collection of semen, the ejaculates were shifted to a water bath at 37°C. The semen was kept for a further of 4 hours for the spermatozoa to acquire equilibration. At the time of analysis, at least 02-04 straws of semen from each treatment were thawed at 37°C for 30 seconds to perform the semen quality parameters.

2.1. Semen Concentration

Semen concentration was measured as such freezing. For this purpose, 10 µL of semen was mixed with formal citrate in a pre-warmed Eppendorf. The dilution rate of semen and citrate solution was kept 1:10. Haemocytometer was cleaned with ethanol and a small drop of solution was put on the haemocytometer. Then a clean cover slip was placed over it and observed the slide under the Olympus BX 51 microscope to determine the concentration.

2.2. Pre and Post Thawed Semen Motility

Pre-thawed motility was determined by putting a fresh drop of semen on a pre-warmed slide and observed under phase contrast microscope (40x). For post-thawed motility, semen straws (of each treatment) were thawed in water bath at 37°C for 30 seconds. A drop (10 µL) of semen was placed on a pre-warmed glass slide and covered with a cover slip. Percentage motility was assessed under a phase-contrast microscope at 40X. A minimum of three fields were observed for percentage motility and considered as a single data point.

2.3. Vitality of Spermatozoa

To find out the live dead ratio of semen 20 µL of semen with 20 µL of stain was mixed in preheated (37°C) Eppendorf. Then a drop (10 µL) of mixture was put on a clean slide to make a thin smear and observed under a phase contrast microscope at 100X (oil emersion lens) for unstained heads of spermatozoa (live) and stained heads of spermatozoa (dead).

2.4. Acrosomal Integrity

A 500 µL portion of each semen sample was fixed in 50 µL of 1% formal citrate containing 2.9 % (w/v) tri-sodium...
citrate dihydrate. Two hundred spermatozoa were counted under a phase contrast microscope (100X) for their acrosomal integrity (NAR). Acrosome abnormalities like absent, ruffled and swollen acrosomes were observed (Rasul et al, 2001) but were counted in negative consideration.

2.5. Plasma Membrane Integrity

Plasma membrane integrity was assessed using the hypo osmotic swelling test (HOST) assay. The hypo osmotic swelling test was performed by first mixing 500 µL HOST-solution in glass tube with 50 µL of each semen sample and incubating the mixture for 45 min at 37 °C in an incubator (Ijaz et al, 2009). After incubation, one drop (~ 5 µl) of the treated mixture was examined in a thin slide-cover slip preparation under a phase contrast microscope (400x). Two hundred spermatozoa were counted per sample, and the number of spermatozoa showing clear characteristic swelling of the tail, an indication of an intact plasma membrane, was recorded. The mean of three observations was considered as a single data point.

3. Results

The fresh semen was first examined for its motility and concentration. The motility of fresh semen for horse 1 and horse 2 was 60% and 50% respectively. Fresh semen of horse 1 and 2 had shown 81.5% and 80% vitality respectively. Semen concentration was determined by haemocytometer. The concentration of semen for the horse 1 and horse 2 was 12 million/ml and 11.50 million/ml respectively. After cryopreservation of stallion semen, different parameters were analyzed. It was found that motility of the semen was reduced with increasing concentration of ALA. The detail results of research are explained in Table 1.

Different concentration of alpha lipoic acids and their effects on the sperm motility after cryopreservation are shown in the Figure 1. The result shows that, as compare to the fresh semen, the ultimate motility of the sperm after cryopreservation’s with ALA decreases.

It was also observed that sperm viability (Live/dead) with different concentration of alpha lipoic acids was also changing which shows the overall ineffectiveness of ALA on the viability of stallion semen (Figure 2).

Figures 3, 4, and 5 show the plasma membrane integrity, acrosomal integrity and morphology of the stallion sperm which were cryopreserved with ALA.

In Figure 3, for different concentration of alpha lipoic acids in equine semen, the lowest value for mean positive plasma membrane integrity starts from 01% and ends at 03% whereas Figure 4 shows 98.75% to 100% normal apical ridges of acrosomes which means that ALA did not produce worse effect on the acrosomes of equine sperm.
Similarly Figure 5, shows the abnormality of sperm treated with ALA with mean percentage range from 20.2 to 25.5%. The total no. of sperms is further investigated for thick neck, large head, and double neck and tail percentage respectively.

4. Discussion

Fertility and spermatozoa viability has been assessed by various assays in different studies (Graham & Moc’e, 2005). Some stallion may exhibit low fertility due to having defective spermatozoa parameter; these defects can be examined and eliminated via laboratory assays (Graham & Moc’e, 2005). Pregnancy itself is the best tool to assess the fertility of stallion (Samper et al, 2007). Spermatozoa their selves are highly vulnerable to oxidative stress-induced damage because their plasma membrane contain large amount of poly unsaturated fatty acids (Alvarez & Storey, 1995) and their cytoplasm contain low concentrations of scavenging enzymes (Aitken & Fisher, 2005).
equine semen. It is interesting that the seminal plasma of mammals is well capable with a set of antioxidant defence mechanism (Sikka, 1996). This antioxidant defence mechanism is composed of enzymatic antioxidants such as super-oxide dismutase (Alvarez et al, 1987), the glutathione peroxidase/glutathione reductase (GPx/GRD) system (Chaudiere et al, 1984) and catalase (Jeulin et al, 1989) and the non enzymatic antioxidants such as ascorbate (Fraga et al, 1991), urate (Thiele et al, 1994), α-tocopherol (Aitken & Clarkson, 1988), pyruvate (De-Lamirande & Gagnon, 1992), glutathione (Lenzi et al, 1994), taurine and hypotaurine (Alvarez & Storey, 1983).

This study was conducted to find out the effect of cryopreservation of semen with alpha lipoic acid in stallion. Evaluation of efficacy of ALA as an antioxidant for equine sperm motility, vitality, morphology, acrosomal integrity and plasma membrane integrity (HOS) was also a part of this project. The results of our study are as follows:

The motility of fresh semen for horse 1 and horse 2 was 60% and 50% respectively. This motility was reduced dramatically after addition of different inclusion levels of ALA in semen extender as shown in Table 1.

Similarly plasma membrane integrity, acrosomal integrity, spermatozoa vitality did not show any significant improvement as compared to control. Our findings are disappointing showing the overall ineffectiveness of ALA as an antioxidant for improving the post-thawed quality of equine semen.

5. Conclusion

In conclusion, results obtained from this study show that the motility and viability of stallion semen was decreased after cryopreservation with alpha lipoic acids which might be due to the morphological abnormalities of the sperm, particularly large headed acrosome which may be the reason of DNA damage. It should be noted that DNA damage also affects the fertility, pregnancy rate and the embryonic development of the animal (Ibrahim et al, 2008). The second reason may be the increased acidiy produced by the alpha lipoic acid which can cause the immobilization of spermatozoa. Further more, LA can be reduced to the more potent dihydrolipoic acid (DHLA) (Navari-Izzo et al, 2002), which regenerates the stock of reduced antioxidants.

Due to its multi-functional nature, it is possible that the benefits of LA could be expressed in more concrete experimental settings beyond the purpose of the present study, or in more complex media formulations, such as those used for in-vitro fertilization (IVF).

Direct addition of DHLA could be a suitable option for future experiments, since that strategy would not rely on the ability of sperm cells to convert LA to DHLA, but media formulation must be taken into an account that metal cations (e.g. iron, copper etc) must be absent. In order to achieve a synergistic effect, supplementing the medium with several antioxidants such as reduced glutathione could be a level headed approach.

References


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