Effect of trehalose and month of collection on DNA fragmentation in Holstein bull semen during dilution, cooling and thawing.

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ABSTRACT

Al-Badry KL, Aboud QM, Zalzala SJ, Ibrahim FF, Lateef WY., Effect of trehalose and month of collection on DNA fragmentation in Holstein bull semen during dilution, cooling and thawing, Onl J Vet Res., 22 (3):237-246, 2018. We report optimal levels of trehalose in diluents for freezing bull semen monitored by fragmentation of DNA during dilution, cooling and post thawing during October, November, December and January. Ejaculates with 50-55% or more individual sperm motility were collected weekly by artificial vagina. Semen was diluted with Tris containing 50, 100, 150 or 200 mM Trehalose to provide 80 million spermatozoids per ml. Semen was cooled to 5°C over 4h, packed into 0.25 ml straws and freeze stored 48hr in liquid nitrogen. DNA fragmentation was evaluated after dilution, cooling and thawing. After dilution, fragmentation in 50 (4.7%) or 100Mn (4.8%) Trehalose was less (P < 0.05) compared with 200nM (6.7%) or controls (6.7%). After cooling, fragmentation increased (P < 0.05) in all samples but in those with 50nM was 6.6% less (P < 0.05) compared with 200nM (8.1%) or controls 10.2%. After thawing fragmentation increased in all samples but in those kept in 100nM Trehalose (7.4%) was less (p<0.05). Our findings suggest that 50-100nm Trehalose in Tris-dilution may reduce DNA fragmentation in sperm and ameliorate damage due to cooling or freezing. We found that in semen collected in November and December, fragmentation was less compared with other months.

Key words: Trehalose, dilution, freezing, thawing, DNA fragmentation, bull semen.
INTRODUCTION

Artificial insemination (AI) permits a higher degree of selection and extended use of animals with a high breeding value as well as reducing risk of infection (El-Sheshtawy et al., 2015). Successful AI in cattle depends on semen quality, cryopreservation in liquid nitrogen and diluents (Haugan et al., 2002). Monosaccharide, disaccharide and polysaccharide sugars are diluents which provide energy and extracellular cryoprotection to spermatozoa (Yildizet al., 2000). Glucose has been used to freeze semen of rams (Molinaet al., 1994) and boars (De Los Reyes et al., 2000). Sucrose and Trehalose has been used for bull semen (Woelderset al., 1997, Huet et al., 2010, EL-Shehatawy et al., 2015). Trehalose replaces water at the membrane/solution interface (Bakasad Disalvo., 1991) and possibly prevents alteration to sperm membranes by maintaining the osmotic pressure in diluents (Liu et al., 1998). Trehalose is a non-reducing cryoprotectant providing energy substrate for sperm cell during equilibration, cryopreservation and post-thawing (Uysal and Bucak., 2009). When used in hypertonic solution, Trehalose exhibits an synergic effect with glycerol preventing intracellular ice crystal formation (Gutierrez et al., 2009). Improper diluents and preservatives may cause various physical changes, cell surface destruction, loss of motility, loss of DNA integrity etc. (Lyashenko and Bashchenko., 2014).

Integrity of DNA is an important criteria for success of natural or assisted fertilization including normal development of the embryo, fetus or offspring (Rahul 2007). DNA damage and enzyme inactivation can reduce sperm motility, viability and fertilization in bulls (Sariözkanet al., 2009). Luke et al., (2010) demonstrated that fertilization depended on sperm progressive motility and DNA fragmentation. However, sperm with compromised DNA integrity appears to fertilize oocytes at the same rate as normal sperm but embryos produced by fertilization of an oocyte with sperm with damaged DNA cannot develop normally (Agarwal and Said 2003). Integrity of DNA with routine sperm parameters can be used to assess quality of spermatozoa and reproductive potential of males (Agarwal and Said., 2004). Trehalose has been reported to affect the quality of diluted Holstein bull semen but to our knowledge its effect on sperm DNA fragmentation has not. We report effect of Trehalose on DNA fragmentation and fertilization in sperm frozen in liquid nitrogen collected at different months.

MATERIALS AND METHODS

This study was carried in Artificial Insemination Center, College of Veterinary Science, Baghdad, Iraq. Semen was collected during October, November, December and January. A total of 64 ejaculates with sperm individual motility equal to more than 50-55% were collected weekly by artificial vagina from five Holstein bulls aged 4-5 years and weighing 650—750kg. Bulls were fed a standard daily diet of 2 kg concentrated fodder with 12% protein at morning and evening supplemented with 10 kg green fodder. Water was provided ad libitum.
Semen samples were split into 5 equal aliquots diluted with Tris-Fructose-Egg Yolk-Glycerol (TFEG) freezing extender containing 50mM, 100mM, 150mM or 200mM Trehalose. Controls had no additive. All volumes were adjusted to 80 million sperms per ml. Semen was then cooled to 5 Cº equilibrated for 4hrs and packed into 0.25 ml polyvinyl French straw (IMV, France). After a further equilibration period of 4hrs, straws were placed horizontally on a rack and frozen in 4cm liquid nitrogen vapor for 9 min and then dipped in liquid nitrogen for 48hrs.

DNA fragmentation % was assessed by Acridine-orange (AO) staining assay as described by Hammadeh et al., (2001), Martins et al., (2007) and Varghese et al., (2011). Air-dried slides were fixed overnight in freshly prepared Carnoy's solution (3 parts methanol and 1 part glacial acetic acid) and allowed to air dry few minute and stained for 3 min with AO. Slides were viewed under fluorescence microscope at 490nm. Two hundred spermatozoa were observed with normal DNA showing green fluorescence over the head region, while DNA abnormalities appeared as varying fluorescence from yellow-green to red.

The effect of Trehalose, dilution, cooling, post thawing and month of collection on sperm DNA fragmentation were analyzed by two-way ANOVA with the general linear model method described (SAS., 2012). Comparisons between values were analyzed by Duncan’s multiple range test following an F-test in ANOVA (Duncan. 1955). Significance was set at (P<0.05).

RESULTS

Effect of Trehalose on DNA fragmentation on bull sperm diluted, cooled and then frozen in liquid nitrogen is shown in Table 1 below. After dilution, fragmentation in 50 (4.7%) or 100mM (4.8%) Trehalose was less (P<0.05) compared with 200mM (6.7%) or controls (6.7%). However, we found no dilution effect with 50, 100 or 150mM or between 150, 200mM or controls. After cooling, fragmentation in samples with 100mM Trehalose declined 6.6% (p< 0.05) compared with 200mM (8.1%) or controls 10.2%. Samples with 50mM Trehalose had less fragmentation (7.8%) (P<0.05) compared with controls (10.2%). We found no differences between 50, 100 or 150 mM and also no differences between 50, 150 or 200mM samples. After thawing we found less DNA fragmentation in semen kept in 100mM Trehalose (7.4%) (p<0.05) compared with any other treatment or controls. Data on Table 1 shows that mean DNA fragmentation was less in samples kept in 50mM trehalose (9.8%) (p<0.05) compared with 150 (8.6%), 200mM (9.7%) or controls (10.9%), but the differences were not significant between 200Mm and controls. Overall mean results show that 100Mm Trehalose 6.2% (p<0.05) caused less DNA fragmentation compared with 50, 150, 200mM or controls (8.6, 9.7, 10.9), respectively, but we found no significant differences between 50Mm Trehalose compared with 100 or 150Mm, or between 150 and 200mM, 200mM or controls.
Table 1. Effect of Trehalose on DNA fragmentation (%) of bull sperm frozen in liquid nitrogen.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Overall Mean</th>
<th>Steps of freezing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>After dilution</td>
</tr>
<tr>
<td>TFEG-50mM/L</td>
<td>7.449 ± 0.43CD</td>
<td>7.819 ± 0.65Bc</td>
</tr>
<tr>
<td>TFEG-100mM/L</td>
<td>6.234 ± 0.31D</td>
<td>7.363 ± 0.35Da</td>
</tr>
<tr>
<td>TFEG-150mM/L</td>
<td>8.570 ± 0.16BC</td>
<td>11.850 ± 0.38Ba</td>
</tr>
<tr>
<td>TFEG-200mM/L</td>
<td>7.449 ± 0.43CD</td>
<td>7.819 ± 0.65Bc</td>
</tr>
<tr>
<td>TFEG-Controls</td>
<td>4.728 ± 0.38Bc</td>
<td>5.800 ± 0.28Db</td>
</tr>
<tr>
<td></td>
<td>6.730 ± 0.25Ac</td>
<td>10.238 ± 0.20Ab</td>
</tr>
</tbody>
</table>

Table 2 shows DNA fragmentation in Semen collected at different months. We found a decline in fragmentation in semen collected during November (4.725, 10.7%) and December (4.675, 10.8%) after dilution and thawing compared with October (7.105, 12.7%) and January (6.885, 12.0%) but no differences between November and December. After cooling, we found no difference between any month. Results in Table 2 show a significant (p<0.05) decrease in mean of DNA fragmentation in semen samples collected during November and December (7.568, 7.6%) compared with October and January (9.517, 9.3%) but there was no significant difference between November and December or October and January samples.

Table (2). Fragmentation in DNA of semen collected during different months.

<table>
<thead>
<tr>
<th>Freezing</th>
<th>Months</th>
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<tbody>
<tr>
<td></td>
<td>October</td>
<td>November</td>
<td>December</td>
<td>January</td>
</tr>
<tr>
<td>After dilution</td>
<td>7.105 ± 0.26b</td>
<td>4.725 ± 0.29b</td>
<td>4.675 ± 0.41b</td>
<td>6.885 ± 0.73a</td>
</tr>
<tr>
<td>After cooling</td>
<td>8.740 ± 0.26a</td>
<td>7.325 ± 0.55a</td>
<td>7.325 ± 0.47a</td>
<td>8.520 ± 0.84a</td>
</tr>
<tr>
<td>Post-Thawing</td>
<td>12.705 ± 0.31a</td>
<td>10.695 ± 0.57b</td>
<td>10.705 ± 0.86b</td>
<td>12.640 ± 0.73A</td>
</tr>
<tr>
<td>Overall mean</td>
<td>9.517 ± 0.26a</td>
<td>7.582 ± 0.34B</td>
<td>7.568 ± 0.67b</td>
<td>9.348 ± 0.78A</td>
</tr>
</tbody>
</table>

DISCUSSION

Our findings suggest that 50-100mM Trehalose before freezing semen may reduce DNA fragmentation in sperm. These results confirm results with bovine (Bucak et al., 2010), buffalo (Badret al., 2010; Iqbal et al., 2016) or boar sperm (Menet al., 2013). El-Sheshtawy et al., (2015) found that chilling and freezing medium supplemented with Trehalose at appropriate concentrations (50-100 mM) improved sperm DNA integrity with <5% fragmentation. In boar semen, Trehalose reduced structural fluctuations of DNA and prevented its denaturation (Menet al., 2013). However in rams (Bittencourt et
al., 2014) and bucks (Tuncer et al., 2013) trehalose had no effect on sperm chromatin integrity.

We surmise that the effect of Trehalose on sperm DNA in our study may have been due to an antioxidant and not a direct effect on chromatin structure as Trehalose is a large molecule that does not penetrate sperm plasma membranes (Lechene et al., 2012). We found that 200mM Trehalose may have induced DNA fragmentation especially after thawing, confirming findings of El-Sheshtawy et al., (2015). Effects of high doses of Trehalose may be due to plasma membrane damage (Aitken and Krausz, 2001). Ramos and Wetzels (2001), maintained that motility may be a relevant physiological marker for intact DNA in sperm. Badret al., (2010) and Mahmoudet al., (2015), found a significant negative correlation between DNA damage and post-thawing motility in buffalo semen. Irvine et al., (2000), reported that sperm with low motility had greater DNA damage. Enciso et al., (2011), reported that major morphological sperm abnormalities are associated with DNA.

In our study, high concentrations of Trehalose may have increased osmotic pressures of diluents affecting DNA integrity (Dawaret al., 2017). We found dilution, cooling and thawing had a significant (P<0.05) effect on DNA fragmentation but was less in samples with 100mM Trehalose. Mahmoudet et al (2015) found that mean percent comet-detected spermatozoa with damaged DNA was significantly higher for frozen than fresh semen. Sperm DNA damage may occur during dilution, cooling, freezing and thawing due to reactive oxygen species (Duranet al., 2002).

Our results showed significant (P<0.05) differences in DNA fragmentation in sperm samples collected at different months. Semen collected during October and January months appeared to affect DNA fragmentation at cooling. Collection time, shorter photoperiods or cold stress (Barth and Waldner 2002) may affect spermatogenesis and viability of sperms. Ding et al. (2004) found Long-term exposure of semen to cold could induce sperm DNA injury. A variety of factors, including months, season have been reported to affect the quality of extended bull semen but effect of this factor on sperm DNA integrity has not been adequately assessed (Blesbois, 2007;Sheikhet al., 2008;).

Morris et al., (2002) found that the higher the semen sperm motility, the higher was DNA damage. Most of these studies have yielded results indicating an inverse correlation of DNA damage with seminal parameters including sperm count, motility and morphology (Tomlinson et al., 2001; Larson-Cook et al., 2003; Virro et al., 2004; Acharyya et al., 2005; Wyrobek et al., 2006; Evgeniet al., 2015). Aydos et al., (2015) found that spermatozoa with denatured and fragmented DNA have a significant relationship with impaired seminal parameters. Sperm viability correlates strongly with DNA fragmentation rate (Brahem et al., 2012; Samplaskiet al., 2015). Our findings confirm those of Frydman et al., (2008) and Greco et al., (2005) who found no correlation between semen parameters and DNA fragmentation. We found only a negative correlation between sperm DNA and and motility.
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AUTHORS CONTRIBUTION
All authors contributed equally in all the efforts for these articles

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