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# Effect of different sperm concentrations on the post-thaw viability and motility of turkey spermatozoa cryopreserved by the pellet method

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**ABSTRACT -** The effects of different prefreezing semen concentrations on post-thawing quality of turkey semen cryopreserved with the pellet method were investigated. Ten pooled semen samples were each divided into 6 subsamples and diluted with Tselutin extender to obtain a final concentration of 0.5, 1, 2, 3, 4 and $5x10^9$/mL respectively. Subsamples were cooled, added with 8% of dimethylacetamide as cryoprotectant and, after 5 minutes of equilibration, seminal aliquots of 80 µL were directly dropped into a liquid nitrogen bath to form frozen pellets. Thawing was performed in few seconds at 75°C. Sperm motility (Accudenz® swim-down test), viability (SyBr-Propidium Iodide staining) and sperm susceptibility to osmotic stress (Hyposmotic-water test) were assessed. Cryopreservation caused an overall loss of sperm quality, however differences in seminal parameters due to the different sperm concentration were observed in turkey spermatozoa after thawing: spermatozoa diluted to $4x10^9$/mL showed significant higher values in mobility, viable and osmotic resistant spermatozoa compared to the other concentrations. This study showed that the post-thaw quality of turkey semen cryopreserved by pellets method was affected differently in relation to prefreezing sperm concentration.

*Key words*: Turkey semen, Cryopreservation, Sperm concentration.

**Introduction -** The fertility rates of cryopreserved poultry semen are highly variable and not reproducible for use in commercial production or preservation of genetic stocks (Long, 2006). In fact, poultry spermatozoa, in particular those from turkey, appear to be much more sensitive to damage caused by cooling/freezing procedures. Researches are focused on develop a protocol for successful freezing avian spermatozoa in a practical, inexpensive manner adaptable to the field conditions. Among the freezing systems studied to cryopreserve avian spermatozoa, the pellet procedure seems to be a promising technique, however the results obtained especially in turkey aren't still very encouraging. The pellet system, previously reported by Tselutin *et al*. (1995), consists in frozen semen with dimethylacetamide (DMA) by a very rapid cooling technique that involves plunging semen droplets straight into liquid nitrogen. There are different steps during this cryopreservation process that can affect the maintenance of spermatozoa function during freezing and thawing of turkey semen, such as the choice of the diluent (Iaffaldano *et al.*, 2008a) and its dilution rate (Iaffaldano and Rosato, 2008a), the DMA concentration and its equilibration time (Iaffaldano and Rosato, 2008b), the drops volume and drops thawing temperatures (Iaffaldano *et al.*, 2008b).

Therefore, in order to optimize the pellet procedure, in this research we studied the effects of different semen prefreezing concentration on post-thawing quality of turkey semen cryopreserved with the pellet method.

**Material and methods –** Ten pooled semen samples were collected by dorsoabdominal massage from mature Hybrid Large White toms. The initial cells concentration of pools averaged from 8 to 12 billion of sperm cells in a milliliter of semen (9.8±1.6). Each pool was divided into 6 subsamples that were diluted with Tselutin extender to obtain respectively a final concentration of 0.5, 1, 2, 3, 4 and 5 billion of sperm cells per milliliter of extended semen ($1x10^9$ sperm/mL). Samples were previously cooled at 4°C for 60 minutes and then were added with an 8% (wt/vol) of dimethylacetamide (DMA) as cryoprotectant. Drops of semen (80 µL) were plunged directly into liquid nitrogen (Tselutin *et al.*, 1995). Each drop of frozen semen was catch with pliers, rapidly put in cooled cryovials and stored in liquid nitrogen before analysis. Mobility, viability, and osmotic resistance were evaluated on both fresh semen and thawed semen immediately after thawing the frozen drops by immersion of cryovials in a water bath at 75°C for 12-15 seconds.

A sperm motility assay was performed measuring spectrophotometrically the extent to which motile sperm penetrated an Accudenz® layer, according to King and Donoghue (2000), after adjusting to $0.5x10^9$ sperm/mL sperm concentration in all samples with the motility buffer. The sperm viability was measured using the Live/Dead Sperm Viability Kit (Molecular Probes, Eugene, OR, USA) as described by Blanco *et al.* (2000). The osmotic resistance of spermatozoa was assessed with a hiposmotic-water test following the same procedure of viability test, but using a 39 µL water solution instead the Tselutin extender.

All data are expressed as mean ± standard deviation (SD). Data were analyzed using ANOVA followed by Duncan's comparison test (SPSS 14 for windows, Chicago, ILL). Differences were considered to be significant at $P<0.05$.

**Results and conclusions –** Viability and stress osmotic resistance percentages of fresh turkey semen averaged to 77% and 50.5%, whereas the Optical Density (OD) measured in the Accudenz® motility procedure was 0.496. As expected, the cryopreservation worsened post-thaw quality of turkey spermatozoa, in accordance with other authors (Tselutin *et al.*, 1995; Blanco *et al.*, 2000; Blesbois, 2007). However we observed that following cryopreservation the quality characteristics examined after thawing were affected differently in relation to the prefreezing sperm concentration (Table 1). With increasing sperm concentration ranging from 0.5 to $4x10^9$/mL we observed a linear improvement in the semen quality, particularly for the viability and osmotic resistance values after thawing, whereas with the concentration of $5x10^9$/mL a decrease in the post-thawing quality characteristics of semen was observed. Therefore, the semen diluted to $4x10^9$/mL showed higher viability values ($P<0.01$) compared to the other concentrations. This concentration resulted also in significantly better motility and osmotic resistance parameters respect to the concentration of 0.5, 1, 2 and $5x10^9$ sperm/mL ($P<0.05$).

It is well known that the cryopreservation of semen lead to a series of structural and biochemical modifications in turkey spermatozoa reducing their membrane integrity, motility and consequently the fertilizing ability (Blanco *et al.*, 2000; Blesbois, 2007), but our study also indicated that the prefreezing concentration affected the post thaw semen quality. Our conclusion is not unique, since similar effects were reported in other animals (Garner *et al.*, 1997; Nascimento *et al.*, 2008).

The explanation by which the prefreezing concentration in turkey influenced the post-thawing semen quality could be attributed to a different amount of DMA per sperm cell or to the different volume of seminal plasma leading to different quantity of seminal plasma components in different diluted sperm sample.

Other researches on different animal species and with different cryopreservation systems also observed a "concentration effect" on sperm quality after freezing/thawing process. Nascimento *et al.* (2008) showed that better post-thaw equine seminal characteristics were found in lower concentrations. Instead other authors found that the increased bull spermatozoa volume may provide some protective factor during the freeze-thaw process (Garner *et al.*, 1997).

Although further studies are needed to ascertain the mechanism of prefreezing sperm densities on

the post-thawing seminal quality, we conclude that sperm concentration is one of the factors affecting the freezability of turkey semen cryopreserved with the pellet method. Only taking in the account all factors affecting semen quality during cryopreservation and their interactions will be possible to improve cryopreservation systems for avian male germoplasm.

Table 1. Effect of different sperm concentration on qualitative parameters of frozen/thawed turkey spermatozoa. Data are mean ±SD. Different letters in the same column indicate significant differences (A-C=P<0.01; a-d=P<0.05).

| Semen concentration | Motility (OD) | Viability (%) | Osmotic resistance (%) |
|---|---|---|---|
| $0.5x10^9$/mL | 0.217±0.04Bb | 9.43±1.13Cd | 5.24±0.98Bc |
| $1x10^9$/mL | 0.213±0.03Bb | 10.13±1.77Ccd | 5.71±0.96Bc |
| $2x10^9$/mL | 0.216±0.01Bb | 11.91±2.71Cc | 8.74±1.92Ab |
| $3x10^9$/mL | 0.238±0.05ABab | 18.29±2.68Bb | 9.69±1.57Aab |
| $4x10^9$/mL | 0.269±0.03Aa | 24.05±2.64Aa | 10.24±1.11Aa |
| $5x10^9$/mL | 0.223±0.04ABb | 20.06±2.92Bb | 8.54±2.04Ab |

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