

## FERTILIZING ABILITY OF PINZGAU BULL SPERM *IN VITRO* AFTER CRYOSTORAGE

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### ABSTRACT

The aim of the study was to evaluate fertilizing ability of Pinzgau bull sperm after different periods of cryostorage. The sperm samples from 16 bulls were arranged into the three groups according to cryostorage period as follows: less than 7 years (group 1), 7 to 13 years (group 2) and 14 or more years (group 3). Straws with frozen sperm doses were thawed in a water bath and the motile fraction of sperm was selected by modified swim-up method. Motile spermatozoa ( $2 \times 10^6 \cdot \text{ml}^{-1}$ ) were co-incubated with matured bovine oocytes in fertilization drops (IVF-TALP medium) under mineral oil at 39 °C in presence of 25 µg/ml heparin for 20 hours. Totally, 739 oocytes were used in the *in vitro* fertilization test. Following fertilization the presumptive zygotes were stained with DAPI nuclear stain and the status of chromatin was examined under fluorescence microscope. Penetration of the sperm head into the ooplasm of oocyte and fertilization (formation of pronuclei) were evaluated. No significant difference in fertilizing ability among the experimental groups was found. The rates of penetrated plus fertilized eggs for group 1, 2 and 3 were 79.31, 76.40 and 76.14 %, respectively. Significantly higher pronuclear formation rate was observed in the group 1, where 72.9 % of eggs had two visible pronuclei at 20 hours following incubation with sperm compared to 65.72 and 47.21 % in groups 2 and 3, respectively. Also, 4 % of fertilized eggs from the group 1 reached syngamy of both pronuclei, probably due to a faster formation of pronuclei, whilst no syngamy was observed in groups 2 and 3. In conclusion, period of cryostorage had no direct influence on penetrating and fertilizing ability of Pinzgau bull sperm doses. However, it is assumed that cryostorage length can affect the speed of pronucleus formation.

**Key words:** Pinzgau; bull; sperm; fertilizing ability; cryopreservation

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### INTRODUCTION

Cryostorage of bull semen is widely used as a useful tool to improve reproduction. In dairy cattle, the majority of inseminations are done with frozen-thawed semen. Cryopreserved semen plays a major role in genetic improvement, economics of breeding programs in the livestock industry, and preservation of endangered species and breeds. Sperm cryopreservation methods are the most efficient way for storing animal genetic resources. It is well known, that cryopreservation is stressful for spermatozoa. Although the purpose of sperm cryopreservation is to preserve sperm function and fertility, the freezing-thawing process inevitably

causes their damage and reducing fertility. During freezing and thawing spermatozoa are subjected to various stresses such as cold shock, osmotic and oxidative stress and intracellular ice crystal formation. These cause disruption of sperm integrity. Particularly, membrane structure alterations (Krogenaes *et al.*, 1994; Januskauskas *et al.*, 2003), and sperm function alterations after thawing (Garner *et al.*, 1997; Thomas *et al.*, 1998; Pons-Rejraji *et al.*, 2009) were described. In addition, excessive mitochondrial activity induces the generation of reactive oxygen species (Chatterjee and Gagnon, 2001) that affects functions of cellular compounds and organelle (Bilodeau *et al.*, 2000).

Some evidences also exist about cryodamages

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to sperm DNA integrity after cryopreservation (Gandini *et al.*, 2006; Waterhouse *et al.*, 2010). These damages ultimately result in reduced sperm motility, viability and fertilizing ability (Watson, 2000). It is assumed, that once frozen semen in the liquid nitrogen can be potentially stored unlimited time. But only scarce information was published about the influence of long-term cryostorage on quality of frozen-thawed bull semen. In humans, it was proved that sperm can be useful after even more than 28 years of cryostorage (Clarke *et al.*, 2006). But some alterations may be related to long period of storage. For example the percentage of motile sperm was lower after long-term (9–13 years) compared to short-term (1–5 years) storage (Edelstein *et al.*, 2008).

The aim of the study was to evaluate fertilizing ability of Pinzgau bull sperm after different periods of cryostorage in liquid nitrogen.

## MATERIAL AND METHODS

### Procedure of *in vitro* fertilization (IVF)

Bovine ovaries were collected at a local abattoir and transported to the laboratory within 3 hours at 25–30 °C. Ovaries were rinsed with 70 % ethanol and a sterile saline solution. Cumulus-oocyte complexes (COCs) were acquired by follicular fluid aspiration from visible large follicles (2–10 mm in diameter) on the surface of ovaries. Collected COCs with pale homogeny cytoplasm and complete cumulus were selected and washed twice in maturation medium E199 with HEPES (Biowest). For maturation, E199 medium with glutaMAX (Gibco), sodium pyruvate (0.25 mmol.l<sup>-1</sup>), gentamycin (0.05 mg.ml<sup>-1</sup>), fetal bovine serum 10 % (FBS, BioWhittaker, Verviers, Belgium) and FSH/LH (1/1 I.U., Pluset, Lab. Calier, Barcelona, Spain) was used. Oocytes were matured at 39 °C in a humidified atmosphere with 5 % CO<sub>2</sub> in air for 24 h. Matured oocytes were partially cleaned from cumulus cells by vortexing during 3 min. Frozen-thawed insemination doses from Pinzgau bulls were used for *in vitro* fertilization of oocytes. Insemination doses from 16 bulls of a proven fertility were used in experiments. Straws were thawed in a water bath at 36 °C and the motile fraction of sperm was separated in Sperm-TALP medium using modified swim-up procedure. The sperm fraction was then resuspended in IVF-TALP medium and chosen sperm concentration was adjusted. Following maturation, the presumptive matured oocytes were placed into 100 µl drops of fertilization medium IVF-TALP under sterile mineral oil and incubated with 2 × 10<sup>6</sup>.ml<sup>-1</sup> spermatozoa in the presence of 25 µg/ml heparin (Sigma-Aldrich, Germany) at 39 °C in a humidified atmosphere with 5 % CO<sub>2</sub> in air for 20 hours.

Following this time, the presumptive zygotes

were cleaned off from the excessive sperm and remaining cumulus cells by vortexing, washed twice in Sperm-TALP medium and in the PBS with 0.6 % polyvinylpyrrolidone. Zygotes were immediately fixed in 4 % formalin during 10 min and then covered with a drop of Vectashield anti-fade medium containing DAPI stain (chromatin staining; Vector Laboratories, Burlingame, CA, USA) and mounted between coverslip and microslide and stored at 4 °C until fluorescence analysis. Stained embryos were checked under a Leica fluorescence microscope using specific filter with wavelength for blue fluorescence and x20 magnification objective. Penetration of sperm head into the ooplasm of oocyte and formation of pronuclei (fertilizing ability) were evaluated.

### Statistical analysis

Differences in distribution of oocytes into either the penetrated, fertilized (presence of pronuclei) or non-penetrated among the groups of different cryostorage period were analyzed by Pearson's Chi-square test of independence. All calculations were performed using the SAS software package (SAS Institute, 2001).

## RESULTS AND DISCUSSION

Fertilizing ability estimated by *in vitro* fertilization test may be a good tool to predict potential fertility of a bull (Larsson and Rodríguez-Martínez, 2000). *In vitro* fertilization test was previously used by several authors to test fertilizing ability of sperm as a homologous system with cow oocytes and bull sperm (Fazeli *et al.*, 1997; Zhang *et al.*, 1998; Marquant-Le Guienne *et al.*, 1990) or as heterologous system with sperm of different species using cow oocytes. Such heterologous system was, for example, used to test ram (García-Alvarez *et al.*, 2009; Makarevich *et al.*, 2011), antelope (Roth *et al.*, 1998), stallion (Bromfield *et al.*, 2013) and dolphin (Sánchez-Calabuig *et al.*, 2015) sperm fertilizing ability. Major advantage of these *in vitro* tests is that the testing is not so expensive and labor intensive procedure as testing by artificial insemination. The *in vitro* fertilization technique can be very accurate to assess the sperm fertility, because the procedure evaluates the spermatozoa-oocyte interactions occurring during fertilization process.

In our previous study (Makarevich *et al.*, 2011) we used co-incubation of ram spermatozoa with bovine oocytes with intact *zona pellucida* in the *in vitro* fertilization test, where about 53–55 % of the oocytes were penetrated by ram semen and about 30–33 % of them were fertilized. Similarly García-Alvarez *et al.* (2009) documented that such heterologous *in vitro* fertilization test can be useful to predict the *in vivo* fertility of rams.

Fertilizing ability is very individual property of bull (Ax and Lent, 1987; Merckies *et al.*, 2000).

To minimize individual effect of bull and underline the effect of time we created experimental groups from minimum three bulls, according to the length of cryostorage period.

No significant difference in fertilizing ability (rate of penetrated and fertilized eggs) among the three experimental groups was found. The total rates of penetrated and fertilized eggs for group 1, 2 and 3 were 79.31; 76.40 and 76.14 %, respectively (Table 1). Similarly, period of bull sperm storage had no influence on the occurrence of polyspermy after *in vitro* fertilization. According to period of storage 6.3 %, 6.5 % and 5.1 % of fertilized eggs were polyspermic in groups 1, 2 and 3, respectively (Table 1). Similar rate of polyspermy was reported by Santos with co-workers (2008), where the polyspermy rates varied from 4.1 to 11.1 %. It was proved, that polyspermy after bovine *in vitro* fertilization is mainly influenced by individuality of a bull, heparin dose used (Marquant-Le Guenne *et al.*, 1990) and also related to oocyte quality (Santos *et al.*, 2008).

On the other hand, significant differences were found in the rate of pronuclei formation. Significantly

higher pronuclear formation was observed in the group 1 (stored for 1–6 years), where 72.9 % of eggs had two visible pronuclei at 20 hour after sperm addition compared to 65.72 and 47.21 % in groups 2 and 3, respectively (Table 1). Also, syngamy of both pronuclei was observed in 4 % of fertilized eggs in the group 1, whilst no syngamy was observed in groups 2 and 3. This difference may be probably due to faster pronuclear formation in the group 1.

Slower decondensation of sperm heads occurring in groups 2 and 3 may be related to chromatin structure alteration, described in cryostored human (Royere *et al.*, 1991) and boar (Hamamah *et al.*, 1990) spermatozoa. In particular, Royere with co-workers (1991) concluded that freezing-thawing procedure may alter the DNA/nuclear protein relationships and impair the fertilizing ability of human sperm. Similarly, Hamamah *et al.* (1990), basing on their results, hypothesize that sperm chromatin after freezing-thawing may be overcondensed; this overcondensation may be associated with the lower conception rates obtained using human and porcine semen after cryostorage.

**Table 1: Fertilizing ability of Pinzgau bull sperm after cryostorage**

Groups	Period of storage (years)	No. bulls	No. oocytes	Penetrated and fertilized (N) %	Non-penetrated (N) %	Oocytes with two pronuclei (N) %	Polyspermic oocytes from fertilized (n/N) %
1	1 to 6	3	203	(161) 79.3	(42) 20.7	(148) 72.9	(10/158) 6.3
2	7 to 13	7	339	(259) 76.4	(80) 23.6	(227) 67.0	(16/243) 6.5
3	14 to 18	6	197	(150) 76.1	(47) 23.9	(93) 47.2	(5/98) 5.1
total		16	739	(570) 77.1	(169) 22.9	(468) 63.3	(31/499) 6.2

## CONCLUSION

In conclusion, length of cryostorage had no direct influence on *in vitro* penetrating and fertilizing ability of cryopreserved Pinzgau bull sperm. However, long-term cryostorage might affect speed of pronucleus formation and syngamy in *in vitro* fertilized eggs.

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