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Cryopreservation of *in vitro* produced blastocysts from endangered Pinzgau cattle breed

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Abstract
Pinzgau cattle is a traditional livestock breed in Slovakia suitable for sub-mountain and mountain areas. Numbers of these animals have been declining. The Pinzgau Cattle was registered at the UN FAO as endangered by extinction. As a contribution to the *ex situ* strategies of conservation of Pinzgau cattle breed, our goal was to produce blastocysts of the Pinzgau cattle breed *in vitro* and to preserve them in the Gene Bank of Animal Genetic Resources. The ovaries from slaughtered Pinzgau cow were transported to the laboratory. The oocytes, isolated by aspiration of ovarian follicles, were matured *in vitro* and subsequently fertilized using a frozen sperm of a Pinzgau bull. Presumably fertilized zygotes were cultured for 6-8 days until the blastocyst stage. Totally, 20 blastocysts have been produced so far. Of them, 17 good quality blastocysts were vitrified using an ultra-rapid cooling method. Cryopreserved blastocysts were placed for storage in the Gene Bank of Animal Genetic Resources and entered into the CryoWeb database.

Keywords: Pinzgau cattle, IVF, blastocyst, cryopreservation

Introduction

Slovak Pinzgau Cattle is an undemanding, healthy and hardy breed with good ranging ability, suitable for sub-mountain and mountain areas. The beginning of breeding of the Pinzgau breed in our region dates from 1870 to 1880. In 1994, the Pinzgau Cattle was registered at the UN FAO as endangered by extinction. Pinzgau cattle in Slovakia have a long tradition, but their numbers have been declining for a long time. At present, there are less than 11000 animals. For comparison, while in 1989 more than 90,000 cows of this breed were bred in Slovakia, in 2014 there were about 2,700 purebred cows registered in the studbook of Pinzgau cattle, now it is only 2,024 cows. Therefore, this breed is among the endangered in our country. In our laboratory, we decided to contribute to the *ex situ* strategies of conservation, by creating embryos of this breed *in vitro* and their preservation in the Gene bank of animal genetic resources.

Such an approach seeks to be applied in the *ex situ* protection of several species and breeds of endangered animals. *In vitro* fertilization is used not only in cows, where this methodology is best developed. Similar methodologies have been adapted and implemented for different endangered species of livestock, domestic and wild animals. Methods of *in vitro* embryo production are described also in buffaloes (SUTEEVUN et al., 2006), horses (RODRIGUES and RODRIGUES, 2006), camel (KHATIR et al., 2004), antelope (MAHESH et al., 2011), roe deer (COMIZZOLI et al., 2021), in canine and cats (VANSOOM et al., 2014), as well as in other animal species (ANDRABI and MAXWELL, 2007).
These results show that it is possible to preserve the valuable genetic resources from a declining population and also to contribute to the improvement of genetic diversity by creating new individuals from new parent pairs *post-mortem*. Especially in combination with the use of cryopreserved male insemination doses, it is possible to produce their progeny *in vitro* for long period *post-mortem*. To enhance the genetic progress, both by selection of the most genetically valuable individuals and by increased number of offspring from these individuals is the aim of many further improvements in these reproductive technologies (SJUNNESSON, 2020).
Our laboratory participates in cooperation with several Pinzgau cattle breeders in Slovakia. Based on the agreement, the breeders provide us with information about planned slaughters of cows. After slaughter, the ovaries of such a cow are then transported to the laboratory. The oocytes obtained from them are matured *in vitro* and fertilized using an insemination dose of a Pinzgau bull registered in a Breeding book of Pinzgau Cattle. The aim of this work was to produce blastocysts *in vitro* as a gene resource of Pinzgau cattle breed and to preserve them in the Gene Bank of Animal Genetic Resources.

Material and methods

All the chemicals used in this study were purchased from Sigma-Aldrich Inc. (Saint-Louis, Missouri, USA), unless otherwise indicated.

Biological material
The ovaries were collected at slaughtering of one Pinzgau cow at the age of about 8 years and 6 lactations, kept at the Agricultural farm "PD Trstenik" (Trstena, Slovak Republic). Oocytes from an undefined population of cows slaughtered in a local slaughterhouse served as a control group.

Oocyte retrieval and in vitro maturation (IVM)
The ovaries were transported to the laboratory, washed with 70 % ethanol and then with sterile saline. The oocytes were recovered from antral follicles (2-8 mm) by the aspiration of follicular fluid using sterile 5 mL syringe with a needle (18 gauge). Cumulus-oocyte complexes (COCs) were collected into a Petri dish with a holding medium (M199-HEPES with 10% foetal bovine serum - FBS) and only COCs with several layers of cumulus cells and homogeneous ooplasm were selected for IVM. COCs were matured for 23 h in a maturation medium containing M 199 (Gibco), sodium pyruvate (0.25 mmol.L^{-1}), gentamycin (50 mg. mL^{-1}), 10% FBS and FSH/LH (1/ 1 I.U., Pluset) at 38.5°C and 5% CO2.

In vitro fertilization (IVF) of oocytes and embryo culture
Morphologically good-looking oocytes after IVM were washed in IVF-TALP medium (Tyrode-Albumin-Lactate-Pyruvate solution with 10 mg. mL^{-1} heparin, 50 mg. mL^{-1} gentamycin) and put into 100 µml droplets of IVF-TALP medium under a mineral oil, where the sperm (at $2x10^6$ per mL) and PHE solution (20 mM penicillamine, 10 mM hypotaurine,

1 mM epinephrine) were previously added, and incubated for 18 h at 38.5°C in 5% CO_2. Following IVF, presumptive zygotes were vortexed in an Eppendorf tube containing 0.5 mL of a holding medium for 30 s to remove residual cumulus cells. Denuded zygotes were transferred to the dish with the BRL-1 cell (Rat epithelial cell line, ECACC) confluent monolayer in a culture medium (prepared according to the Menezo B2 composition) with 10% FBS. Developmental ability was determined by cleavage (Day 2) and blastocyst (Days 7 to 9) rates.

Cryopreservation of blastocysts

For cryopreservation of *in vitro* produced embryos, ultra-rapid cooling technique in a minimum volume on electron microscopy grid was used (OLEXIKOVA et al. 2020). Selected embryos were washed in M199-HEPES medium with 10 % FBS. Then they were placed into equilibration solution (ES): 7.5 % ethylene glycol (EG), 7.5 % dimethyl sulfoxide (DMSO) in M199-HEPES, supplemented with 20 % FBS for 3 min. Following equilibration, the embryos were transferred to vitrification solution (VS): 16.5 % EG, 16.5 % DMSO and 0.5 M sucrose in M199-HEPES with 20% FBS at room temperature (RT) for 30 s. The embryos (2 - 4) in a small drop were transferred onto 300 mesh nickel electron microscopy grids with a glass micropipette. An excessive medium was removed by a filtration paper and then the oocytes were immediately plunged into liquid nitrogen for storage.

Results and Discussion

We present here a preliminary data, as to date only one experiment with two ovaries of a Pinzgau cow was performed (Table 1). From these ovaries, 62 cumulus-oocyte complexes (COCs) morphologically suitable for *in vitro* maturation were recovered by follicular fluid aspiration. All of these COCs following IVM were fertilized in vitro and 61.29% of them started cleavage. The cleavage rate of Pinzgau oocytes was slightly lower ($p<0.05$) than that of the oocytes derived from an undefined population of cows (control group). However, the blastocyst rate did not differ significantly between groups (Table 1).

Table 1. Preimplantation embryo development after IVF of cow oocytes

Group	IVF oocytes, n	Cleavage rate, n (%)	Blastocyst D6, n	Blastocyst D7, n	Blastocyst D8, n	Total blastocyst rate, n (%)
Ovaries	62	38 (61.29)	3	14	3	20 (32.26)
Control*	60	45 (75.00)	7	8	7	22 (36.66)

* slaughterhouse-derived ovaries from an undefined cow population

Currently, an average blastocyst rate in group culture systems is usually in the range of 30−40% from all IVF oocytes (CATTEEUV et al., 2017). Therefore, the blastocyst percentage obtained in our work (32.26%) is comparable to above reported results, what supports a validity of the protocol of in vitro embryo production in our study.

The in vitro fertilization method and embryo production are important reproduction tools in cattle breeding worldwide. According to the annual International Embryo Technology Society (IETS) statistics, in the last five years, an average of almost 470,000 bovine embryos produced *in vitro* were transferred worldwide annually (FERRÉ et al., 2020). However, most of them were directly transferred fresh without cryopreservation. It was probably caused by their lower cryotolerance compared to their *in vivo* counterparts. An important aspect of applied *in vitro* embryo production system is the use of somatic cell co-culture systems, because under this co-

culture system the embryos were more suitable for freezing, in comparison to other (cell-free) culture systems (HASLER et al., 1997). Good cryotolerance of this embryos is important for high survival rate after vitrification and high pregnancy rates after embryo transfer (FERRÉ et al., 2020).
Taking into account a reported average pregnancy rate after transfer of vitrified/thawed blastocyst in the range of 40-56% (XU et al., 2006; ZHANG et al., 2015), our yield of 17 cryopreserved blastocysts represents future potential of 6-9 new individuals of the Pinzgau breed of known origin.

Conclusion and recommendation

The described methodology of *in vitro* production of bovine embryos allowed us to extent the reproductive potential of a cow from Pinzgau breed after culling by preserving blastocysts in the Gene Bank of Animal Genetic Resources.

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