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APPLYING THE MODERN METHODS OF REPRODUCTIVE BIOTECHNOLOGY TO THE *EX SITU* CONSERVATION OF NATIONAL BREEDS OF SELECTED LIVESTOCK SPECIES

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Abstract

This article provides a comprehensive biological and zootechnical overview of novel methods of long-term conservation of biological material, as well as methods in reproductive and embryological biotechnology, used for ex situ conservation of native breeds of selected livestock species. The study focuses especially on the importance of the National Research Institute of Animal Production (henceforth 'IZ-PIB') in Balice. Projects involving biotechnology-assisted ex situ conservation allow not only for restoration, but also for the long-term preservation of the biodiversity that supports genotypic and phenotypic variation within and between subpopulations of domestic cattle.

Keywords: cryopreservation/cryopreservation, freeze-drying, reproductive biotechnology, embryological biotechnology, ex situ conservation, livestock, endangered native breeds

Biological factors in cattle genetic resource conservation, with emphasis on selected native Polish species and breeds

Divergence in genetic make-up within and between populations of cattle is an essential condition for herds to be able to adapt to intensive or widespread alterations in agricultural biotopes, caused by human activity. Given these facts, livestock subpopulations with the highest rates of genetic diversity appear to possess the best genetic backgrounds, allowing them to become accustomed to a wide range of unfavourable conditions in zootechnical habitats. This may result in the rapid evolution of livestock breeds (Comizzoli and Holt, 2019; Bolton et al., 2022).

The intensification of production, as well as the development of new breeds or lines of livestock with high genetic value or productivity, can dramatically disrupt the global variety of domesticated animals (Lauvie et al, 2011; Woelders et al, 2012; Sun et al, 2022). The progressive loss of livestock breed abundance and biodiversity is frequently the result of harsh selection aimed at genotype adjustment for high-performing phenotypic traits with genetic or utility value in individual subpopulations and herds (Mara et al, 2013; Leroy et al, 2020; Polak et al, 2021). As a result, more primitive local breeds are becoming less prevalent or replaced by high-yielding, widespread production breeds (Kikuchi et al, 2016; Dua et al, 2021; Son et al, 2021). Primary risks for reduced animal biodiversity include environmental changes connected with the growth of farmed areas, as well as the introduction of human-preferred species into the ecosystem (Chen et al., 2018; Soglia et al., 2021). For these reasons, it is critical to preserve the genetic resources of native breeds that are well suited to local, often harsh climatic and environmental conditions, and which exhibit great resilience and good health. As a result, the preservation of cattle breed biodiversity is based on the deployment of *in situ* and *ex situ* conservation measures (Polak et al., 2021; Jacques et al., 2023).

In situ conservation is the preferable approach to the protection of animal genetic resources both in Poland and worldwide. This involves preserving a given species in its natural environment, while using limited breeding methods in order to allow for the survival of a sufficient number of animals to ensure minimal genetic variability and adaptability to shifting environmental conditions (Ryder and Onuma, 2018; Elyasi Gorji et al., 2021). Programmes dedicated to the *in situ* conservation of rare native cattle breeds should be predictive and should take into account changes in climatic and ecological conditions (Bai et al., 2011; Wood et al., 2018).

Implementation of modern reproductive and embryological biotechnology methods based on cryoconservation, freeze-drying, and extracorporeal embryo extraction, for the ex situ conservation of genetic resources of selected livestock breeds in Poland

Ex situ conservation is based on the utilisation of biotechnological methods for the acquisition and long-term storage of biological material, such as sperm, somatic and stem cells, from various species and breeds of cattle. Cryoconservation and freeze-drying (sublimation drying) processes have been developed and optimised, allowing for long-term storage of biological material (Comizzoli and Wildt, 2013; Comizzoli and Holt, 2014). In contrast to cryoconservation, freeze-drying can maintain biological material at temperatures over 0˚C (León-Quinto et al., 2014; Silyukova et al., 2020).

Biological material in long-term storage, obtained from endangered livestock species and breeds can be used in the future for biotechnology-assisted restoration of lost biocenosis and its reintroduction into specific biotope niches of natural and anthropogenic agricultural ecosystems, or even for biocenotic restoration and restitution of livestock species and breeds (Comizzoli and Wildt, 2013; Keskintepe and Eroglu, 2021; Jacques et al., 2023).

Cryoconservation of biological material containing germplasm (sperm cells) is distinguished by its sophistication and effectiveness. As a result, this approach is commonly utilised in assisted reproductive technologies (ARTs) and *ex situ* conservation efforts for a wide variety of livestock species and breeds. In recent years, IZ-PIB has conducted extensive research to improve the effectiveness of cryoconservation of semen from various livestock species (Trzcińska and Bryla, 2015; Trzcińska et al., 2015). Sperm obtained from males of various livestock species undergoes a complex biological examination to assure its excellent quality. Therefore, it is critical to conduct studies aimed at the identification and accurate

assessment of cryogenically induced, ultrastructural and functional biodegradation of intracellular organelles, cytoskeleton, membrane skeleton and plasma membrane (plasmalemma). These studies also detect proapoptotic and/or pronecrotic, autophagic and mitophagic symptoms in spermatozoa (Comizzoli, 2015; Yeste, 2016; Song et al, 2021; Wu et al., 2023). Intramitochondrial processes caused by cryogenically induced biodestruction can be reduced by supplementing diluents with cryoprotective or antioxidant compounds that remove oxygen-derived free radicals (ODFRs) (Trzcińska et al, 2015; Len et al, 2019; Bolton et al, 2022).

In recent years, there has been a surge of interest in freeze-drying, also known as sublimation drying or sublimation dehydration, a novel method of preserving mammalian sperm. This approach is an alternative to sperm cryoconservation in liquid nitrogen. The biophysical mechanism underlying this technique is a low-temperature dehydration process that involves freezing semen samples and pressure reduction, resulting in the sublimation of intracellular ice crystals (formed during the freezing process) (Comizzoli and Wildt, 2013; Comizzoli et al., 2022a, b). Sublimation, on the other hand, is a direct phase transition from the solid to the gaseous state, omitting the intermediate liquid stage. Sublimation is an endothermic process that occurs at temperatures and pressures lower than the substance's triple point on its phase diagram, which corresponds to the lowest pressure at which aqueous solutions may exist as liquids (Saragusty and Loi, 2019; Keskintepe and Eroglu, 2021).

Previous research has revealed that freeze-dried (sublimation-dried) sperm cells can undergo biological destruction processes inside the plasma membrane and/or genomic DNA, one or both of which have been damaged as a result of oxidative stress and disruption during antioxidative processes. The latter may be triggered by bioaccumulation of reactive oxygen species (ROS) and reduced non-enzymatic antioxidant concentrations or impaired biocatalytic activity of endogenous isoenzymes (isozymes) with potent antioxidant activity, also known as ODFR acceptors (Olaciregui et al., 2016; Thiangthientham et al., 2023). Attempts have been made to increase the antioxidative activity of endogenous isozymes that eliminate oxidants from the ODFR group during sperm freeze-drying. This can be achieved by adding exogenous, water-soluble phenolic (polyphenolic) antioxidants with lyopreservative properties to the cell material being freeze-dried. The most noteworthy among these include 1) rosmarinic acid (RosA; a complex ester of caffeic acid, a representative of hydroxycinnamic acids, and α-hydroxyhydrocavic acid, i.e. 3,4-dihydroxyphenyl-lactic acid), which is classified as one of depsides, i.e. intermolecular esters of phenol-carboxylic acids; (2) ethylenediaminetetraacetic acid (EDTA) and (3) ethyleneglycol-bis(β-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) (Olaciregui et al., 2017; Domingo et al, 2018; Mercati et al, 2020).

It is notable that deviations in semen quality parameters, such as viability, motility and DNA stability, do not rule out the suitability of freeze-dried and then rehydrated sperm for intracytoplasmic sperm injection (ICSI) procedures performed during *in vitro* fertilisation of metaphase II (MII) oocytes in livestock species (Comizzoli and Wildt, 2013; Keskintepe and Eroglu, 2021; Thiangthientham et al, 2023). Even when male gametes exhibit a high incidence of ultrastructural and biophysical abnormalities in sperm organelles and intracellular compartments as well as hyperpermeabilisation or biochemical destabilisation of plasmalemma, maintaining conditions that facilitate a lower incidence of biodegradation or internucleosomal DNA fragmentation during sperm freeze-drying can significantly improve the utility of ICSI for *in vitro* fertilisation (IVF) (Olaciregui et al, 2016; Comizzoli et al., 2022a, b).

To date, the application of ICSI-mediated *in vitro* fertilisation (IVF) of meiotically mature oocytes using freeze-dried sperm cells, followed by re-hydration of previously freeze-dried spermatozoa, has succeeded in producing offspring in a wide range of mammalian species, including livestock. These species include hamsters (Muneto and Horiuchi, 2011), mice

(Kaneko et al., 2003), rats (Hirabayashi et al., 2005), rabbits (Li et al., 2009) and horses (Choi et al., 2011).

Unlike in the research described above, attempts to produce offspring in cattle (Hara et al., 2014), sheep (Palazzese et al., 2018) and pigs (Olaciregui et al., 2017) with *in vitro* fertilisation of oocytes by ICSI using freeze-dried/rehydrated (i.e. *de novo* hydrated) sperm cells did not actually result in progeny. Nonetheless, under *in vitro* culture conditions, these experiments resulted in the growth of embryos to the blastocyst stage.

IZ-PIB has developed effective procedures for cryoconservation and freeze-drying of cattle sperm. Cryoconserved or freeze-dried sperm, like cryo- or freeze-preserved somatic cells or stem cells, can be employed in a wide variety of ARTs based on *in vitro* embryo production (IVP).

A comprehensive IVP strategy consists of three consecutive steps: (1) *in vitro* meiotic maturation of oocytes (i.e., culture of meiotically immature oocytes at the diplotene/dictyotene stage of prophase I of meiotic division until metaphase II stage of meiotic division (MII) is achieved; (2) *in vitro* fertilisation (IVF) of oocytes or reconstitution of enucleated oocytes by somatic cloning (i.e., SCNT – somatic cell nuclear transfer); and (3) *in vitro* culture (IVC) of embryos obtained from IVF or SCNT (Glanzner et al, 2018; Samiec et al, 2019; Nguyen et al, 2020).

In order to perform successful IVF trials with reproducible efficiency, cryopreserved male gametes can be used either for conventional co-incubation of MII-stage oocytes with motile sperm or for assisted *in vitro* fertilisation using the microsurgical technique of intracytoplasmic sperm injection (ICSI), in line with standard artificial insemination procedure (AI) (Fowler et al, 2018; Magata et al, 2019; Zuo et al, 2020). Alternatively, for *in vitro* fertilisation of MII-stage oocytes by ICSI, freeze-dried (sublimation dehydrated) sperm can also be used, followed by *de novo* hydrated (rehydrated) sperm for microsurgical ooplasmic deposition (Ushigome et al, 2022; Thiangthientham et al, 2023). Similarly, when SCNT cloning is used for comprehensive IVP technology, the source of nucleus donors in the enucleated oocyte reconstitution procedure can be cryopreserved/thawed and *in vitro* cultured somatic cells, multipotent/pluripotent stem cells, as well as their previously freeze-dried (sublimation dried) and subsequently rehydrated counterparts (Ono et al, 2008; Natan et al, 2009; Zhang et al, 2017; Dang-Nguyen et al, 2020).

With regard to oocytes undergoing ICSI-assisted fertilisation, a key role is also played by the artificial stimulation of their embryonic development programme. This can be induced using physical and/or chemical activating agents, e.g. DC pulses or ionophore antibiotics, such as calcium ionomycin or calcium ionophore A23187 (calcimycin), which are responsible for the ionophoretic intraooplasmic transport of calcium cations from the extracellular environment and for inducing intracellular transduction of calcaemic signals (Ashibe et al, 2019; Ressaissi et al., 2021). Moreover, the possibility of epigenomic reprogramming of maternally (oocyte) and paternally (sperm) derived chromosomes also exerts a significant influence on the pre- and post-implantation developmental competence of embryos obtained as a result of IVF or ICSI (Kropp et al, 2017; Diao et al, 2018; Takeda et al, 2019). Additionally, two other factors of molecular nature, such as inter-transcriptomic and interproteomic communication between nuclear and mitochondrial DNA fractions (Tsai and St. John, 2018; Zuidema and Sutovsky, 2020) and the initiation of necrotic, apoptotic or autophagic processes (Jin et al, 2016; Rodríguez et al, 2019; Ramos-Ibeas et al, 2020) are undoubtedly not insignificant for the *ex vivo* and *in vivo* developmental potential and quality of IVF- or ICSI-derived embryos in a wide variety of livestock species and breeds.

In order to ensure *ex situ* conservation of a variety of biological material obtained from native breeds of selected livestock species (sheep, cattle, goats, pigs and ducks), IZ-PIB undertook measures, resulting in the accumulation of cryopreserved and/or freeze-dried

biorepositories that cover a wide spectrum of genetic resources (Table 1). The freeze-dried material also includes germplasm-containing materials, such as cryopreserved and/or freeze-dried sperm cells from rams and boars from the following breeds, respectively: Polish Heath sheep, Romanov sheep, Blackhead sheep, Olkuska sheep as well as Polish Large White pigs and Polish Landrace pigs (Table 1).

Table 1. The bioreservoirs deposited in the National Research Institute of Animal Production in Poland that encompass *ex situ* protected biological materials stemming from native breeds of selected livestock species

Species	Breed	Biological material	Method of conservation	Number of samples (semen: straws and/or pellets; cutaneous fibroblasts/stem cells: cell lines)	Number of female donors	Number of male donors
Domestic sheep	Polish Heath	Semen	Cryoconservation	587	-	8
		Cutaneous fibroblasts		15	3	3
	Romanov	Semen		968	-	5
		Cutaneous fibroblasts		32	3	3
	Blackhead	Semen		788	-	4
	Olkuska			3519	-	8
	Old Type Merino	Cutaneous fibroblasts		22	3	3
Domestic goat	Carpathian			13	3	3
Domestic cattle	Polish Red			45	5	-
Domestic pig	Polish Large White	Semen		720	-	7
		Cutaneous fibroblasts		34	7	8
	Polish Landrace	Semen	Lyophilization	645	-	6
				160	-	4
		Cutaneous fibroblasts	Cryoconservation	19	4	3
		Mesenchymal stem cells		39	20	-
	Złotnicka Spotted	Cutaneous fibroblasts		22	6	-
	Puławska			71	10	1
Domestic duck	K-2	Blastoderm-derived embryonic stem cells		10	50	-
	KhO-01			10	50	-
	P-9			8	40	-

Implementation of modern reproductive and embryological biotechnology strategies based on somatic cloning for *ex situ* conservation of genetic resources of selected Polish livestock breeds

Bioreservoirs of somatic and stem cell lines can be created through cryogenic preservation as a useful way of maintaining populations of protected wild mammalian species as well as reintroducing human-made agricultural ecosystems for moribund livestock breeds whose subpopulations are characterised by drastically shrinking numbers and high levels of inbreeding (Men et al., 2012; Men et al., 2012).

Reproductive biotechnology, which involves cloning animals using the SCNT (*somatic cell nuclear transfer*) technique, appears to be an essential booster for the long-term *ex situ* and/or *in situ* conservation of the biodiversity of native breeds of various livestock species, including endangered and even extinct livestock breeds. The latter are particularly vulnerable to:

1) severe bottleneck effects as a result of impaired genetic drift;
2) rapid genetic erosion resulting in a significant reduction in genotypic divergence across populations and between individuals;
3) a final drastic reduction in population size (Caroli and Pizzi, 2012; Leroy et al., 2020).

Given the aforementioned considerations, the production, *ex vivo* multiplication, and subsequent cryoconservation of prolifically stable somatic or stem cell lines derived from various tissue explants represent an alternative or complementary approach to gamete and embryo freezing or vitrification procedures. Such procedures are carried out not only to prevent the extinction of endangered livestock species and breeds, but also to successfully implement *ex situ* and/or *in situ* conservation projects for species- or breed-specific biodiversity (Smits et al, 2012; Enya et al, 2016; Hu et al, 2022).

On one hand, it appears that the creation of genetic resources in the form of somatic cell lines (e.g., fibroblasts) or multipotent stem cells obtained from selected livestock species is an efficient model for maintaining the biodiversity of rare native breeds. On the other hand, the SCNT technique may be the only instrument in the future that allows for the creation and extracorporeal multiplication of such genetic resources for animal cloning within and between species (Borges et al., 2020; Praxedes et al., 2021). As a result, it is essential to make efforts to precisely identify the determining factors of proliferative capacity, genetic stability, cytokinetic ageing, and genetically programmed death (apoptosis and/or autophagy) in *in vitro* subpopulation cultures of clonal somatic cell (nucleus donor) lines/strains. The discovery of the aforementioned factors may result in enhanced and rapid epigenomic reprogramming of somatic cell nuclei in clonal embryos (Jeong et al., 2020; Male et al., 2022). The goal of such procedures is to maintain the right inheritance pattern of desirable genes and enhance genotypic background within herds of endangered native livestock breeds, while also limiting inbreeding within populations. Furthermore, advances in research that result in elevated interspecies genetic diversity may cause an increase in physiological and immunological individual resistance to drastic environmental and climatic changes, as well as the spread of epidemic and pandemic infectious diseases (Leroy et al., 2016).

It is notable that IZ-PIB has developed effective methods for the *ex vivo* expansion and cryoconservation of mitotically stable fibroblastic cell lines derived from the dermal-epithalial system of individuals of both sexes representing endangered domestic livestock breeds such as the Polish Heath sheep, Romanov sheep, Old Type Merino sheep, Carpathian goat, Polish Red cow, Złotnicka spotted pig, and Puławska pig. This has enabled IZ-PIB to produce biorepositories for:

(1) cryopreserved somatic cell lines derived from biopsy specimens of dermal-epithelial tissue isolated from the auricles of the aforementioned moribund native breeds;

2) proliferation-stabilised multipotent mesenchymal stem cell (MSC) lines derived from the bone marrow and adipose tissue of young female Polish Landrace pigs;
(3) blastodermal-derived embryonic stem cells isolated from the embryonic discs of fertilised eggs of three breeds/pedigrees/lines of the domestic duck included in the national genetic resources programme for aquatic poultry.

A detailed summary of biorepositories is provided in Table 1.

The success of SCNT-mediated cloning in a wide variety of livestock species largely depends on the origin and molecular quality parameters of the differentiated somatic cell lines or undifferentiated multipotent stem cells derived and proliferated under extracorporeal conditions. It also depends on the type of methods, media and bio-organic stabilisers used for their *ex situ* preservation (cryopreservation or freeze-drying) (Das et al, 2010; Lee et al, 2019; Zhang et al, 2019; Wiater et al, 2021; Wakayama et al, 2022), as well as on the frequency of apoptotic and autophagic death symptoms in *in vitro* cultures of nucleus donor cells and/or in blastomeres of clonal embryos (Chi et al, 2017; Jeong et al, 2020). Another determining factor for SCNT effectiveness is the ability of somatic or stem cell nuclei to be epigenetically remodelled and reprogrammed in the cytoplasm of reconstructed oocytes and in the blastomeres of developing clonal embryos (Sampaio et al, 2020; Wang et al, 2020; Samiec et al, 2022). Genomic, transcriptomic and proteomic interactions between nuclear and mitochondrial DNA fractions have also been shown to significantly affect the developmental competence of embryos obtained by the somatic cloning technique (Takeda, 2019; Magalhães et al, 2020; Samiec and Skrzyszowska, 2021).

To summarise, at this point in time, the efficiency of mammalian cloning using the SCNT technique remains extremely low, ranging from a low of 0.5% to an average of 5% of the clonal offspring in comparison with the total number of oocytes reconstructed from the nuclei of different somatic and stem cell types. As a result, a thorough understanding of the genomic, epigenomic, transcriptomic, and proteomic signatures that significantly influence the ability of donor cell nuclei for correct and complete nuclear reprogramming in the blastomeres of developing clonal embryos is certainly needed to substantially improve the developmental potential of embryos, foetuses, and clonal offspring in various mammalian species, including livestock (Wiater et al., 2021; Zhang et al., 2021).

Final conclusions, future objectives and directions for further research

Human-induced environmental, ecosystemic (biocenotic and biotopic) and agricultural variables determine cattle breed improvement as a dynamic process including species-specific genetic modifications. As a consequence of the effect of excellent breeding conditions, the biodiversity of cattle breeds first grew and became more pronounced. This resulted in the perpetuation of genotypic and phenotypic divergence of individual livestock breeds, as well as the implementation of various genetically or biotechnologically assisted reproduction strategies and *ex situ* conservation programmes through the establishment of cryogenically and lyophilisogenically preserved male gametes, as well as somatic and stem cell lines. The stem cell lines were to maintain sustainable genotypic and phenotypic diversity among domesticated animal species located in anthropogenic niches of agricultural ecosystems (Mara et al, 2013; Bolton, 2013).

Following the transition from basic to applied research, strategies for generating resources such as spermatozoa, somatic cell lines, and multipotent/pluripotent stem cells, and then using them for reproductive and embryological biotechnology procedures such as IVF-based extracorporeal embryo extraction, ICSI, or SCNT may have increasing application potential in animal husbandry as well as zootechnical and interdisciplinary research combining aspects of

molecular biology, biotechnology, nanotechnology, preclinical and clinical biomedicine, biopharmaceutics, nutritional technology, human nutrition and dietetics.

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