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Innovations in cryoconservation of animal genetic resources

Practical guide

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SECTION 3

Choice of biological material to be preserved

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3.1 INTRODUCTION

The biological material preserved in cryoconservation programmes for animal genetic resources (AnGR) is expected to maintain and manage genetic diversity, and lead to its reconstitution if endangered or lost. Many routes are now available to reach this objective, and all of them have pros and cons. Determining which type of germplasm to preserve is a multifactorial consideration that must be performed so that the goals of a programme can be achieved while also identifying the maximum potential uses for any sample. Specifically, this means that a gene bank manager or curator of a collection must consider:

- costs associated with supplies and equipment required for sample collection, processing, preservation, storage, and utilization;

- sample storage capabilities such as liquid nitrogen tanks, freezers, locations that are resistant to harsh environmental conditions, and that are appropriate for the type of germplasm collected;
- expertise of the staff and its complementarity with the complexity of the collection, preservation, and utilization methods;
- success rates associated with the method of collection, the means of preservation, and the goal for utilization by the type of germplasm curated;
- utilization goal, so that the sample is appropriately preserved for its intended use (such as reconstitution or expansion of the genetic diversity within a line/breed/species in response to genetic bottlenecks, introduction of genes to enable adaptation to climate change or production demands, research, evaluation of animal health evaluation – see Section 1);
- implication of genetic background on the processes utilized, knowing that not all techniques work well, or in some instances at all, with all species or breeds (commercial, research and local populations) of livestock;
- physiological factors that determine the quality of a sample and apply to all types of germplasm, such as seasonality, health status, and age; and
- whether a collection and preservation technique meet the ethical and animal care guidelines, and if the animals/samples meet health and sanitation standards established by nation.

Therefore, this section will present perspectives on the choice of germplasm to encourage preservation activities that meet the goals of a curator and result in a valuable collection with tremendous utility. Section 6 provides details about the procedures for cryopreservation and eventual utilization of the genetic material introduced in this section. Box 3.1 provides a glossary of terms (methods and material types) and abbreviations that are discussed in both this section and Section 6.

3.2 SEMEN

Semen cryopreservation is historically the first reproductive biotechnology developed, and it is still frequently used across many livestock species, because it allows for the conservation of a large number of gametes and often at a relatively low cost. In some instances, repopulation of a line or breed based solely on semen will require initial mating with females of a different population, followed by several generations of backcrossing to reconstitute a nearly genetically pure population. The practicalities of semen cryopreservation often outweigh this shortcoming. Furthermore, semen samples are perfectly appropriate for other cryoconservation goals, such as managing the genetic diversity of *in situ* populations. Cryoconserved semen will also complement collections of other cells, such as embryos or oocytes.

Most countries have developed infrastructure to collect and freeze semen, because cryopreservation protocols are an important part of the routine breeding and reproductive programmes in almost all major agricultural species. Consequently, collection and cryopreservation of semen should be a fundamental component within a germplasm collection because of the simplicity of collection, cryopreservation and storage, the genetic variability which can be easily captured with this type of germplasm, and although the techniques are variable, the relative ease of use for artificial insemination (AI), *in vitro* fertilization (IVF),

BOX 3.1

A glossary of abbreviations for key terms in the germplasm collections

- artificial insemination (AI) – deposition of semen or sperm, which was previously collected from a male, into the vagina, cervix or uterus of a female.
- assisted reproductive technologies (ART) – the collection of methods for artificially improving the reproductive performance of animals, which may include AI, *in vitro* fertilization, embryo transfer and their related procedures.
- cryoprotectant (CPA) – a substance added to media that is used to inhibit cellular damage during freezing and thawing.
- embryo transfer (ET) – placement of an embryo into the uterus of a female for the purpose of establishing pregnancy and producing live offspring.
- *in vitro* culture (IVC) – the maintenance of cells, zygotes or embryos in an artificial environment (literally "in glass") to achieve specific stages of development.
- intracytoplasmic sperm injection (ICSI) – injection of a single sperm into an oocyte to achieve fertilization.induced pluripotent stem cells (iPSC) – somatic cells that are reprogrammed into an embryonic, pluripotent state.
- *in vitro* fertilization (IVF) – the co-culture of sperm and oocytes in culture to achieve fertilization resulting in embryos and offspring.
- *in vitro* maturation (IVM) – a stage of the IVF or vitrification process in which immature oocytes are placed in culture to achieve a specific developmental state which allows them to be fertilized.
- *in vitro* production (IVP) – the process of creating embryos via IVF.
- mesenchymal stem cells (MSC) – stem cells from non-embryonic sources that are capable of differentiating into a variety of tissues.
- primordial germ cells (PGC) – an undifferentiated stem cell that is the precursor of gametes.
- ovum pick-up (OPU) – transvaginal aspiration of follicles from ovaries with an ultrasound guided device for the collection of oocytes.
- somatic cell nuclear transfer (SCNT) – deposition of a somatic cell into an enucleated oocyte resulting in an embryo which can be transferred to produce viable offspring.

intracytoplasmic sperm injection (ICSI), or for DNA extraction. Therefore, this subsection will provide an overview of the current state of: (i) the advantages and limits of semen collection, cryopreservation and utilization for the major agricultural species; (ii) address some improvements to aspects of these processes; and (iii) discuss how these developments increase the value of this form of germplasm.

Semen collection is a simple procedure for the major livestock species. When performed correctly, for many species, it is minimally invasive (for example, by using an artificial vagina in cattle and horses) and causes no apparent physical discomfort, thus usually resulting in the collection of high-quality samples with significant quantities of sperm. Males of other species including boars, roosters and tom turkeys, can likewise be collected using

non-invasive techniques such as the gloved hand (boars) or abdominal massage (poultry). When males cannot be trained for collection using non-invasive methods, and if permitted within animal care and use guidelines, electroejaculation may be used. It is important to note that poultry and stallions should never be collected using electroejaculation, and if possible, this technique should be avoided with goats (bucks) and used only following administration of sedation in boars, as they tend to be very sensitive to the electrical stimulation. If the use of an artificial vagina or electroejaculation is not possible, then alternative methods such as transrectal ultrasonic-guided massage of the accessory sex glands (Abril-Sánchez *et al.*, 2017, 2018, 2019) or transrectal digital stimulation (Tekin *et al*., 2019) has recently been investigated with small ruminants. With these approaches for semen collection, fewer and potentially no electrical stimuli are used while a quality sample may be collected, which may mitigate animal welfare concerns. Likewise, procedures for pharmacological stimulation of stallions continue to be developed to enable semen collections from animals that may have idiopathic ejaculatory dysfunction (Cavalero *et al.*, 2020) or other issues with the collection process. Similarly, pharmacological agents are also being explored to increase the quality and quantity of semen samples when administered out of an animal's normal breeding season (Beracochea *et al.*, 2018, 2020), thus potentially acquiring semen samples more accessible.

While semen is traditionally collected via ejaculation, alternative methods may also be considered. In mammals, viable epididymal spermatozoa from excised testes can be obtained from castrated animals or from animals that experience an untimely death. For seasonal breeders, this technique is best used during the mating season, but seasonality may not be an issue for animals residing in equatorial regions. This method has been successfully applied to stallions (Cunha dos Santos *et al*., 2017), roosters (Villaverde-Morcillo *et al*., 2016), rams (Bergstein-Galan *et al*., 2017), bulls (Turri *et al*., 2012), bucks (Turri *et al*, 2014), boars and other agricultural species.

The quality of the semen sample acquired also depends greatly on collection method and on the technical knowledge of the staff who perform the collections. For example, proper and gentle animal handling techniques usually result in the acquisition of a higher quality sample. The sample quality is also greatly influenced by the genetic background, the physiological state of the males (in or out of reproductive season), the frequency of semen collection (species-specific), and many environmental factors such as photoperiod, food and water availability and temperature. All these factors should be considered prior to sample collection and cryopreservation.

Once collected, the quality of semen samples is evaluated for motility and the integrity of the plasma membrane and acrosome. In addition, the semen quality can be assigned by kinetics or cytometric, which raises the issue of establishing a threshold below which a sample will not be preserved or utilized. It is wise for each gene bank to establish this threshold for acceptability of a sample. Flexibility with these thresholds may be considered if the animal is from a particularly valuable population or if the acquisition of additional samples will be difficult. The threshold may also differ depending on the potential uses for the semen sample (for example, with AI, IVF, ICSI). When considering these factors, it may be obvious to preserve a high-quality sample. However, when some or all of these factors are substandard, preservation of a lower quality sample may still be warranted if there is limited opportunity to acquire a second collection, or if it is from a rare breed or research line.

Furthermore, the type of preservation method should also be considered. Livestock semen is frozen using a variety of freezing rates specific to each species and following the addition of internal and external cryoprotectants (CPAs, substances added to media to inhibit cellular damage during freezing and thawing). The samples are then stored in liquid nitrogen (see Section 6). The goal is for the sperm to recover their fertilizing ability after the freeze-thaw process so that they can be used to generate progeny. However, not all samples are of a high enough quality to merit freezing. In such cases, a semen sample of extremely low quality may still be useful for DNA extraction and genetic analyses, and thus may have value for gene banking. In this instance, it may be appropriate to store the sample at -20 to -80 °C in freezers rather than in liquid nitrogen. However, the latter is always a viable option regardless of the intended use. Depending on storage space, access to liquid nitrogen and the associated expenses, the species, as well as the expertise of staff in assisted reproductive technologies (ARTs), dehydration or lyophilization of samples (with ICSI being the intended use) may also be a viable option in specific circumstances in mammal species (Saragusty *et al.*, 2020a, 2020b). Recent reports have documented the financial savings of storing samples in this format while obtaining acceptable cleavage and blastocyst development rates, thus demonstrating that optimization of these techniques may come to fruition in the future (Keskintepe and Eroglu, 2021).

As previously noted, the fertility rates with frozen-thawed semen are affected by the species, cryopreservation medium, cryoprotectant, the male, the breed or genetic line (especially if there has been significant selection pressure as is the situation with some highly inbred poultry research lines), factors related to the female of the species that is inseminated (such as age, health, synchronization protocol and parity) and the type of ART utilized such as AI, IVF and ICSI. Moreover, the interaction of these influences makes optimization of ARTs for some species challenging, and this is particularly observed in non-mammalian species such as poultry and honeybees, where low post-thaw sperm quality, coupled with extended periods of storage in the female following insemination, results in highly variable and often low fertility rates. For many agricultural species, analysis of these factors should result in standardization of methodologies so that consistent fertility rates are achievable with known insemination doses (Spencer *et al.*, 2010). If fertility rates are lower than expected, the sperm dose, number of inseminations, and the model used to generate progeny (such as through backcrossing), and the number of backcrosses needed to regenerate a line or breed can potentially be increased or adjusted, to address the deficiency (Amann and DeJarnette, 2012). These factors should be considered, by species and breed/line, to ensure that enough samples are acquired based on the intended application of ARTs to meet the needs of utilizing the genetic resources encompassing recreation of populations and genetic analyses.

3.3 OOCYTES AND EMBRYOS

Oocyte cryopreservation is now commonly performed with many species, which makes this a powerful tool when conserving AnGR because it enables selection of a male (semen) and female (oocyte) from a collection to create offspring that will meet a current need (such as research goal or market demand) at the time of use. However, because they are gametes like semen, reconstitution of an extinct breed would require the use of males from another

breed followed by generations of backcrossing if semen of the extinct breed were not also cryoconserved. Cryoconservation of exclusively oocytes would also result in the loss of the breed's Y-chromosome in mammalian species. Therefore, semen should always be conserved along with oocytes, if possible.

When cryopreserved embryos are considered, their preservation captures the diploid genome of an animal and can therefore be immediately utilized to meet a particular need in the future. However, the challenge with embryos is that the re-animated animal may not be able to meet the average level of production in the future, if a significant amount of time has passed and substantial genetic gains were achieved for that breed or species. This is particularly relevant for high performing breeds where selection is actively being applied.

3.3.1 Oocyte cryopreservation techniques

Slow freezing or vitrification are both options for cryopreservation, depending on the species and developmental state as described in Section 6, i.e. cattle (Do *et al.*, 2019), buffalo (Parnpai *et al.*, 2016; Liang *et al.,* 2020), goats (Wahyuningsih and Ihsan, 2018), sheep (Menchaca *et al.*, 2016; Mogas, 2018). Success rates are often low due to the physical properties of the cell (e.g. surface to volume ratio) and non-optimized protocols that do not consider many critical factors such as the water or CPA permeabilities (Díez *et al.*, 2012; Mogas, 2018). Improvements in post-warming oocyte development and IVF rates are being achieved by considering the state of the cumulus oocyte complex at the time of vitrification with cattle (Zhou and Li, 2013; Ortiz-Escribano *et al.*, 2016) and sheep (dos Santos-Neto *et al.*, 2020), and subsequent favourable post-warming expansion rates have been attained (> 50 percent, Romão *et al.*, 2015). Still, conservation of these germplasm types may be expensive for some programmes, because the costs of the technical expertise needed for collection and preservation and the costs of the media (reagents, serum, hormones) may be considerable. Moreover, collection of these types of germplasm may also be challenging and expensive if superovulation or embryo flushing is utilized. When the success rates for producing live animals from these germplasm sources are low, then the cost of these activities may not be justified.

Equine oocytes can be harvested from live mares using ultrasound-guided ovum pick-up (OPU). Mares do not respond to superovulation like other mammals, so either one *in vivo* matured oocyte, or a dozen immature oocytes can be collected by OPU per cycle from the dioestrous mare. The latter technique is more efficient in terms of blastocyst production (Jacobson *et al.,* 2010). Equine immature oocytes can also be acquired post-mortem from excised ovaries, but it is critical to begin the process immediately following collection of the ovaries. The ovaries should be slowly cooled to room temperature and then maintained at 12 °C (Hinrichs, 2018). Following collection, immature equine oocytes can be held or shipped overnight at room temperature without affecting developmental competence.

Oocyte vitrification results in greater post-vitrification quality in the horse than slow freezing, and has resulted in live offspring (De Coster *et al.,* 2020). Currently, vitrification of *in vivo* matured oocytes provides the best results, with the first foal reported after oocyte transfer in 2002 (Maclellan *et al.,* 2002), and a blastocyst rate after *in vitro* embryo production of 33 percent resulting in live offspring after embryo transfer (ET) (Maclellan, *et al.,* 2010). However, collection of *in vivo* matured oocytes and the current efficiency of the vitrification technique is still limited (De Coster *et al.,* 2020).

Immature oocytes can either be vitrified upon collection in the immature state or after *in vitro* maturation (IVM). Vitrification of immature equine oocytes has resulted in blastocyst production (Ortiz-Escribano *et al.*, 2018; Angel *et al.,* 2020) and the birth of a foal (Ortiz-Escribano *et al.,* 2018), although efficiency was ten times lower than with fresh oocytes (Ortiz-Escribano *et al.,* 2018).

As with other mammalian species, the primary means of gene banking of pigs is by cryopreservation of male germplasm, particularly semen. Cryopreservation of oocytes and embryos is also possible, but the practical use of oocyte and embryo cryopreservation in pigs has been limited by the sensitivity to cryopreservation and the difficulty of ET technology. Various protocols for the vitrification of porcine oocytes and embryos have been reported, and the development of a standardized (optimum) protocol would be beneficial for each. To date, oocyte cryopreservation for gene banking has only been reported in two local breeds (Varga *et al.*, 2008; Somfai *et al.*, 2019).

Porcine oocytes are most frequently collected by the aspiration of follicles from ovaries removed after slaughter. However, the harvest of oocytes from live animals is possible by endoscope-assisted OPU (Brüssow *et al.*, 1997) and ultrasound guided OPU (Yoshioka *et al.*, 2020). Porcine oocytes are very sensitive to low temperatures and do not survive conventional slow freezing methods, but they respond well to vitrification (Somfai, Kikuchi and Nagai, 2012) and an optimized protocol for the efficient, rapid and inexpensive vitrification of oocytes in large groups has been developed by Somfai and Kikuchi (2021). Oocytes vitrified at the immature stage have a higher developmental competence than those vitrified at the mature stage (Egerszegi *et al*., 2013). Subsequently, use of vitrified immature oocytes for *in vitro* embryo production results has allowed the production of live piglets using surgical ET (Somfai *et al.*, 2014; Kikuchi *et al.*, 2016). Another approach was reported by Gajda *et al.* (2015) who vitrified oocytes at the mature stage using the Open Pulled Straw method and obtained piglets by the surgical transfer of warmed oocytes into recipient pigs followed by insemination.

3.3.2 Embryo cryopreservation techniques

Slow freezing methods have been successfully used for ruminant embryos in breeding and conservation schemes for decades. Acceptable pregnancy rates have been obtained with intact *in vivo* produced (IVP) embryos. However, pregnancy rates have not been satisfactory after slow freezing of OPU derived IVP embryos, especially if biopsied for genomic selection or some other purpose. Embryo vitrification has had more promising results, especially for IVP and biopsied embryos, but additional research is still needed to improve the success rates with cattle, buffalo, goats and sheep (Díez *et al.*, 2012; Menchaca *et al.*, 2016; Parnpai *et al.*, 2016; Wahyuningsih and Ihsan, 2018; Mogas, 2018; Do *et al.*, 2019). While the efficacy of this ART is improving with these species, it still requires the use of some form of *in vitro* processing (such as fertilization, maturation, co-culture, grading or cloning) which makes it more expensive (labour and resources), and more invasive (requiring ET) compared with semen preservation and AI.

Equine embryos can either be collected *in vivo* by uterine flushing or they can be produced *in vitro*. Equine *in vivo* embryos enter the uterus 144–156 h after fertilization, and are characterized by rapid expansion and the formation of an acellular glycoprotein

capsule. Both slow freezing and vitrification of small embryos (< 300 μm) generally result in pregnancy rates of 50 to 60 percent (Squires, 2020). In contrast, cryopreservation of expanded equine blastocysts has been problematic. Reduction of the blastocoel cavity fluid by aspiration via micromanipulation, followed by vitrification, has provided a breakthrough in this field, and pregnancy rates are comparable to those of fresh embryos (Choi *et al.,* 2011).

The IVP of equine embryos has substantially grown in recent years with the worldwide clinical application of OPU, followed by IVM, intracytoplasmic sperm injection (ICSI), and IVC. The advantage of IVP is that the embryos can be selected for cryopreservation upon blastocyst formation when they are still small and have no glycoprotein capsule. Consequently, cryopreservation of equine IVP blastocysts via either vitrification (Choi and Hinrichs, 2017) or slow freezing (Lazzari *et al.,* 2020) is very successful, with pregnancy rates similar to the rates obtained by fresh transfer. Cryopreservation of IVP embryos is routinely performed in the horse, which allows IVP outside the breeding season and facilitates the selection of recipient mares. Equine IVP embryos should only be cryopreserved when they have reached the blastocyst stage, which occurs between day 6 and day 10 following fertilization, adding to the complexity of the techniques. Early developing blastocysts result in higher pregnancy rates, and should be transferred on day 4 post-ovulation (Cuervo-Arango*,* Claes and Stout, 2019). Comparison of the current techniques shows that cryopreservation of IVP embryos is more efficient than cryopreservation of oocytes for conservation of equine genetic resources.

Similarly, success rates when using porcine embryo technologies have also recently increased. Porcine embryos can be obtained either *in vivo* (by flushing out from the reproductive tract after AI) or by IVP. Although *in vivo* produced porcine embryos at the blastocysts stage can be preserved by slow freezing (Fujino *et al*., 2007), much higher survival rates are obtained by vitrification (Cuello *et al.*, 2004). In the last decade, highly efficient vitrification protocols have been developed for morula and blastocyst stage porcine embryos employing chemically defined media (Maehara *et al.*, 2012; Mito *et al.*, 2015). Also, a pathogen-free closed system without the direct contact to liquid nitrogen (thus eliminating the chance for cross-contamination) has been developed (Misumi *et al.*, 2013). Although early cleavage stages are not optimal for the cryopreservation of porcine embryos (Sanchez-Osorio *et al.*, 2008), porcine zygotes (at the 1-cell stage) can be vitrified with high efficacy (Somfai and Kikuchi, 2021).

A promising component within this area of germplasm preservation is the focus on the development of new devices that either automate the process (Arav *et al.*, 2018) or aid in minimizing the volume of media during the oocyte and embryo vitrification process (Paul *et al.*, 2018; Chinen *et al.*, 2019; Olexiková *et al.*, 2020). Additional advantages with the latter devices are that they are inexpensive and enable the preservation of large numbers of oocytes or embryos at a single time, thus saving time, money and resources for a laboratory.

3.4 GONADS, TESTICULAR AND OVARIAN TISSUE

3.4.1 Poultry gonads

Historically, most of the biotechnological methods of genetic conservation with poultry have focused on semen preservation. Female gametes were neglected due to the difficulty of oocyte collection, manipulation and handling, and because of the impossibility of cryopreservation due to the large size of the oocyte (from 1 to 10 cm in diameter depending on the species) and because of the volume of the yolk which constitutes more than 95 percent of the oocyte content.

To circumvent this problem, Song and Silversides (2006, 2007a, 2007b) explored the preservation of the immature ovary of young chicks at a stage where the oocytes do not yet contain yolk. They successfully demonstrated that chick ovaries can be transplanted (by allograft) into recipient chicks of a similar age and, following puberty, the hosts will ovulate mature oocytes from the transplanted tissue. These oocytes may be fertilized and produce non-chimeric chicks within the first generation. Similarly, Song and Silversides (2007a) also demonstrated that testes of a young chick could be vitrified and transferred, and at the time of puberty, the host would produce fertile sperm. More recently, Liptói *et al.* (2020) demonstrated the successful production of chicks from fresh and frozen-thawed donor ovaries using both commercial and heritage breeds of chickens. A variable amount (8 to 100 percent) of frozen-thawed gonads of a donor were accepted by the host and produced viable offspring (9 percent). These results depended on the donor × host combination, and the type of gonad transplanted (testis or ovary). To maximize the probability of a successful outcome, the authors encouraged testing combinations of breeds prior to full experimentation or reconstitution of populations, when using limited supplies of vitrified germplasm, because of the incompatibility of some breed crosses (Liptói *et al.,* 2013, 2020). The acceptance of testicular tissue was slightly greater than that of ovarian allografts, although both types could be successfully transplanted.

Although matching donor and host genotypes is certainly a challenge, the greatest obstacle to use this type of germplasm is acquisition of and proficiency with the surgical skills. Prior experience with local and general anaesthesia, particularly with poultry, is essential, and experiences with fine surgical techniques is immensely helpful. Strict attention to the procedures listed in the Appendices for the acquisition and utilization of this type of germplasm are critical for achieving success.

3.4.2 Non-avian species

Like the cryopreservation of whole gonads, cryopreservation of excised testicular or ovarian tissue pieces has recently emerged as a viable means of preserving germplasm from prepubertal and sexually mature animals. Methods have been developed to slow freeze or vitrify whole, hemi-, pieces or follicles from ovaries as well as whole, hemi- or pieces of testes (Devi and Goel, 2016). Preservation of those tissues in those formats has been undertaken in human, wildlife, non-human primates, domesticated pets, rodents and aquatic species (Dupré *et al.*, 2016; Pšenička *et al.*, 2016), insects (Fukumori *et al.*, 2017; A. Rajamohan, personal communication, 2020), and agricultural species (Devi and Goel, 2016). However, the success is highly variable and influenced by the type of tissue (e.g. whole, hemi-, pieces or follicles), the species and the breed/line within a species (Portela *et al.*, 2019), the type of

cryopreservation process (slow freezing or vitrification), the post-thaw method of utilization (xenografting, allografting, tissue culture, organ transplantation or ICSI) and the interaction of these effects (Devi and Goel, 2016).

While births of mice have been commonly reported using frozen-thawed testicular and ovarian tissue pieces or whole gonads, this is not yet the case for other species. Still, it is important to note that success with these techniques has resulted in promising blastocyst development rates (Kaneko *et al.*, 2019) and the birth of live offspring (Kaneko *et al.,* 2013) with pigs, thus demonstrating the promise that this germplasm preservation method holds. Whole porcine ovaries can also be cryopreserved by slow freezing (Imhof *et al.*, 2004), and small segments of ovarian tissues can be cryopreserved by both slow freezing and vitrification (Gandolfi *et al.*, 2006). Furthermore, tissue segments have been xenografted into immunodeficient mice to harvest mature oocytes that have the ability for normal fertilization (Kikuchi *et al.*, 2016). However, to date, live piglets have not been produced from samples cryopreserved using that technology.

For both avian and non-avian species, cryoconservation of gonads shares the same challenge as with semen and oocytes with respect to chromosomal content. At least under today's technologies, storage of either only ovarian or testicular tissue would require generations of backcrossing to restore an extinct breed to a nearly pure state. Therefore, gene banks that conserve gonadal tissue should either store both ovarian and testicular tissues, or store the complementary gametes or embryos.

3.5 GERM, STEM AND SOMATIC CELLS

3.5.1 Preservation of diploid cells for cloning or *in vitro* formation of gametes

Cryopreservation of stem cells, embryonic and adult cells, induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs) will effectively preserve the DNA/genome of any species. Currently, not all somatic cell types and techniques are effective for regenerating live animals for all species. Somatic cells can be cryopreserved and used in somatic cell nuclear transfer (SCNT) for reproductive cloning. Cloning of mammalian livestock species has been successful for many domestic mammalian species (e.g. cattle, sheep, horses, pigs and goats) by replacing the oocyte nucleus with a somatic cell nucleus (Wilmut *et al.*, 1997; Cibelli *et al.*, 1998; Baguisi *et al.*, 1999; Polejaeva *et al.*, 2000; Galli *et al.*, 2003). However, health and development problems have been observed in many of the clones, which demonstrates the need for further refinement of this methodology. In some countries, cloned livestock may be an ethical issue that could prevent eventual utilization or require a legal exception. Nevertheless, the ease and low cost of somatic cell banking may justify their current collection and storage, while waiting to address ethical issues in the future, when and if they are ever needed.

The hope for future utilization of embryonic stem cells, iPSCs and MSCs is that techniques will be developed to directly differentiate these cells into either primordial germ cells (PGCs) or functional gametes (Pieri *et al.*, 2019) which can then be transplanted, or used for artificial insemination, IVF, and ET. Certainly, substantial progress has been made in recent years in the ability to collect and culture stem cells and to induce pluripotency, but because of species differences there is a lack of understanding of the essential elements

necessary to routinely apply this approach in livestock. Furthermore, because of suboptimal culture conditions to maintain pluripotency, reprogramming of somatic cells is often unsuccessful (Pessôa *et al*., 2019, Pieri *et al*., 2019) and consequently not practical for conserving genetic diversity. In addition, iPSCs have been used to create live, chimeric offspring in sheep (Liu *et al*., 2012) and pigs (West *et al.*, 2010, 2011; Su *et al.*, 2020), which may attest to the efficacy of the procedures used to generate the iPSCs and the quality of the cells (Sartori *et al.*, 2012), but to date these somatic cells have not been used to generate true clone (non-chimeric) animals (Pessôa *et al.*, 2019). Still, in the mouse it was demonstrated that naive pluripotent stem cells can differentiate *in vitro* into functional sperm (Hayashi *et al.*, 2011) and oocytes (Hikabe *et al.*, 2016; Hayashi *et al.*, 2017), which could be used to produce viable offspring. Potentially, functional gametes may be generated with iPSCs derived from cryopreserved somatic cells from domestic species, and be used to produce embryos that can develop into healthy animals after ET.

3.5.2 Primordial germ cells and derivatives

Perhaps some of the most promising recently developed techniques involve the use of PGCs to preserve chicken germplasm and recreate live non-chimeric chicks. With this technique the PGCs are typically collected from stage 16 HH (Hamburger-Hamilton) embryos (which amounts to approximately 2.5 days of embryo incubation), and they can then be successfully cryopreserved or vitrified (Nakamura *et al.*, 2011) or cultured prior to transplantation to increase the quantity of viable cells for more than 50 percent success rate (Whyte *et al*., 2015; Nandi *et al.*, 2016; Wang *et al.*, 2017; Tonus *et al.*, 2017). Transplantation of the PGCs can be performed to generate chimeras, but these are randomly created, result in about a 4 percent transmission rate of offspring containing the desired genotype from transplanted PGCs, and will require multiple generations of back-breeding to recreate a specific breed or line.

While use of the technology in this way is viable, it is currently inefficient and would require significant amounts of labour and chickens to attain the desired offspring. Recently, a *DDX4* knockout chicken has been created as a result of TALEN-mediated gene targeting. Taylor *et al.* (2017) demonstrated that these inefficiencies can be overcome if enough PGCs are transplanted into a host embryo and the gonad of that host is devoid (sterile) of native PGCs. Then, when a DDX4- rooster is created, he can be used as part of an AI program to produce sterile female embryos for use as sterile hosts. PGCs from a donor (fresh or frozen-thawed) can be injected into the sterile host (hen) embryo, where they migrate to the embryonic gonad and multiply, making the ovary functional (Taylor *et al.*, 2017). At sexual maturity, the recipient hen is inseminated with sperm of the same line/breed as the transfected PGCs, and the progeny will be fertile and capable of progenerating the line. This method is significantly more efficient than creating live chimeric offspring and employing multiple generations of back-breeding, and results in the production of genetically pure offspring in the first generation (Woodcock *et al.*, 2019).

A technique similar to the poultry PGCs model has recently been developed for use with mammalian species. Ciccarelli *et al.* (2020) demonstrated that spermatogonial stem cells isolated from testicular parenchyma could be cultured and transplanted into sterile hosts, and those hosts (goat, mouse and pig) would produce sperm from the donor. Bulls were also tested in similar experiments, and although donor sperm were not acquired

in that research, the prospects are high for applying these technologies to that species. While transplantation of spermatogonial stem cells and the creation of chimeras have been demonstrated in other animals (e.g. quail, trout, salmon and zebrafish), the novelty to the research reported by Ciccarelli *et al.* (2020) is that it was based on sterility induced by using CRISPR/Cas9 editing of the NANOS2 gene (knockout), rather than through chemical, environmental or radiological means. It resulted in azoospermic hosts that are capable of maintaining donor-derived spermatogenesis. Moreover, this is the first report of this combination of techniques successfully producing sperm using livestock species. This combination of results supports the current preservation of spermatogonial stem cells, even though improvements in the efficiencies of the techniques are needed. In contrast, research with oogonial stem cells and regeneration of live offspring has not progressed as rapidly or produced live offspring. Still, Hou *et al.* (2018) reported that germline stem cells from porcine ovaries can be isolated, purified, cultured and induced to differentiate into oocytes when injected into tissue grafts. However, the existence of oocyte stem cells in mammals is a controversial topic, and many reports demonstrate that they do not exist; thus validating this finding is requisite.

3.6 RECOMMENDATIONS

3.6.1 Semen

For nearly all livestock species, semen collection and cryopreservation are an inexpensive and effective option for preserving AnGR. Consequently, based on the easy access to this type of germplasm, the simplicity of preservation, and the minimal costs associated with these techniques, a gene bank should endeavour to make semen an integral part of their collection. The gene bank should also spend a significant amount of time identifying the appropriate species, breeds and males to ensure that their collection contains the suitable quantities and individuals to meet the country's AnGR conservation goals (Sections 1 and 5). When feasible, gene banks should aim to cryoconserve some complementary material (embryos or oocytes), if they wish to avoid the generations of backcrossing required for reconstitution of breeds based exclusively with semen.

3.6.2 Oocytes and embryos

The costs associated with preservation of oocytes and embryos (labour, laboratory facilities and reagents) are considerable and may be prohibitive for some gene banks. As with most types of germplasm, the success when using cryopreserved-thawed or vitrified-warmed oocytes and embryos for regenerating live animals is highly dependent on the species, the state of the germplasm (the developmental state of an oocyte or embryo) when collected or produced, and should be considered when determining the quantity of samples needed for each species and breed.

3.6.3 Gonads, testicular and ovarian tissue for avian species

Documentation of the success with this technique with vitrified-warmed gonads, rather than with freshly excised samples, was a critical step in the acceptance of this technique. Research to understand the genetic components and immune response of the donor and host will further improve the success rates. Utilization of these methods should be

considered if a gene bank has, or has access to, appropriate surgical expertise and can afford the related expenses. Even if that option is not currently available, it may still be prudent to preserve gonads from chicks, as the costs of the collection and preservation activities are minimal. The future benefit is that the offspring derived from the transplantation are non-chimeric, meaning the desired chicks are created in the first generation thus eliminating the need for backcrossing.

3.6.4 Gonads, testicular and ovarian tissue for non-avian species

Once optimized, cryopreservation and subsequent grafting of testicular or ovarian tissue could provide a potentially inexhaustible source of germplasm for the preservation of AnGR. The methods to utilize these types of germplasm in all species have not been optimized, but should be considered a viable and inexpensive means of preserving the genetics of a breed, line or species. Current preservation of this material is based on a presumed high probability that the methods for its utilization will be developed in the future (Kaneko *et al.,* 2013, 2019), and issues regarding morbidity and ethics will be resolved.

3.6.5 Germ, stem and somatic cells

Undifferentiated germ cells such as PGCs, especially from poultry, and intermediate germ cells such as spermatogonial stem cells represent legitimate opportunities that should be pursued. Nuclear reprogramming strategies that can transform germ, stem or somatic cells into germ cells or gametes are developing rapidly, but significant improvements are needed. Further research and development are especially needed in cell culture methods to allow a user to differentiate many of these cell types into functional gametes. Still, preservation of these cell types, and specifically fibroblasts or general tissue samples in the form of ear notches, should be pursued with the understanding of their potential value and utility for the future, while monitoring the technological status of these methods.

3.7 SUMMARY

Successful collection, preservation and utilization of germplasm depends on numerous factors. However, even though variable results should be expected when utilizing any assisted reproductive technology, a secure and genetically diverse collection of AnGR can be assembled for all agricultural species. Moreover, the collection, preservation and utilization techniques can be chosen to match a country's or gene bank's budget, level of expertise and the ethical perspectives of its nation. A comparison of these factors, by species, is presented in Table 3.1 to aid with decision making.

TABLE 3.1
A comparison of germplasm types according to various factors influencing their utility

Type of germplasm	Ease of acquisition	Cryopreservation expertise	Collection costs[a]	Utilization expertise	Utilization costs	Proportion of desired breed in 1st generation offspring (percent)
Semen	2	3	1	1 to 3[b]	1 to 5[b]	50–100
Oocytes	5	5	5	4	3	50–100
Embryos	5	5	5	4	2	100
Gonads	2	2	2	5	3	100
PGCs[c]	4	1	1	5	3 to 5[d]	< 10–100
Somatic cells	1	1	1	2 to 4[e]	5[e]	50–1100

Note: The types of germplasm are rated on a relative scale from 1 (lowest/easiest) to 5 (highest/hardest) within each category. The genetic profile of the resulting offspring is also reported. The information in this table generally refers to conventional collection from live animals. Collection of epidymal sperm and immature oocytes from animals after slaughter is a fairly simple and inexpensive procedure, but may not be routinely applicable in gene banking for conservation, which usually involves healthy animals of high genetic and/or financial value.

[a] Collection costs can vary greatly depending on who performs the collections and cryopreserves the samples. For example, when semen is considered, the costs of obtaining a sample for a gene bank will vary depending on whether the semen is acquired from a stud and is already cryopreserved (< USD 0.50/straw), if a sample is collected and then sent to a gene bank for cryopreservation (~ USD 1.00/straw), or if the gene bank travels and performs the collection on-site and cryopreserves the samples (~ USD 15.00/straw). These factors can be applied to any type of germplasm, and therefore this category considers the inherent costs associated with each when making the comparisons.

[b] Dependent upon if semen will be used for artificial insemination, IVF or ICSI.

[c] Only successfully demonstrated with chickens and conditionally with other avians.

[d] Variable depending on whether the laboratory is in possession of DDX4- hens.

[e] Requires cell culture and transfer of the cells into a host organism, and these methods have not currently been optimized.

Source: Authors' own elaboration.

3.8 STRATEGIC CHOICES OF STORED MATERIAL TYPE TO FIT NATIONAL NEEDS

The wide range in the types of tissues and cells available for cryoconservation offers a practically infinite number of possible strategies for countries to choose from when addressing their gene banking goals. Each type of biological samples has its advantages, disadvantages and outright limitations, and these vary depending upon the species, the cryoconservation and the technical capacity of the country. No single solution will be appropriate for all countries. In a series of case studies, gene bank managers from Brazil, Canada, China, France, Thailand and the United States of America describe their choice of stored materials in the context of their most important livestock species and national goals for management of AnGR.

3.9 EXAMPLES OF THE CHOICE OF GERMPLASM BY COUNTRY

The Brazilian Animal Gene Bank

Samuel Rezende Paiva

The Brazilian Animal Gene Bank for Animal Genetic Resources was created in 1983 and is located at Embrapa Genetic Resources and Biotechnology (Cenargen) in Brasilia. It is one of the 42 research centres of Embrapa (Brazilian Agricultural Research Corporation), which

is under the direction of the Ministry of Agriculture, Livestock and Supply. The gene bank is part of the country's conservation programme, which also has an *in situ* component that is spread nationwide in research centres, universities and private farms. The gene bank collection includes semen, embryos and fibroblasts as well as biological material such as DNA, hair and blood samples. The collection includes samples from approximately 17 000 animals, and represents 87 breeds and 12 species. Real time data can be accessed through the online information system *Alelo Animal/ Animal GRIN*[1] (see also Section 8). The repository has been mainly used for molecular biology research studies.

Current and future efforts include establishing infrastructure to fast-track legal agreements and implementing quality standards of the International Organization for Standardization to attract more interest from farmers, research institutions and industry (see Section 2). In addition, improvements to boost gene bank management and utilization are also underway. The first improvement will change the focus of the collection from breeds to species. The majority of the current gene bank samples are composed of rare local breeds, but going forward, collection activities will also include cryopreservation of specialized and commercial breeds to enhance the food security of the national livestock production system. The second improvement to the collection is that all semen samples currently in the repository have now been genotyped by medium density DNA chips (tens of thousands) and this data will be available through Animal GRIN. The third improvement is implementation of a conservation index to verify if the amounts of germplasm stored are sufficient to recover a breed, to carry out experimentation, and to enable AnGR exchange with institutions and breeders. All these changes will be monitored and evaluated over time to adapt the strategy, comply with the needs of the Brazilian population, and fulfil the main Strategic Priority Actions defined in the *Global Plan of Action for Animal Genetic Resources*.[2]

The Canadian Animal Pedigree Act and cryoconservation activities

Carl Lessard

Production of Canadian livestock used for food relies on the producers and industries. To assist them with breed improvement, Canada's government[3] adopted the Animal Pedigree Act (APA) to regulate the establishment of livestock associations and maintain breed registration records/pedigrees. One of the advantages of the APA is that the status of a breed can be monitored to identify those breeds at risk or endangered, and then actions can be taken to conserve Canadian livestock genetics. In addition, a conservation programme – Animal Genetic Resources of Canada (AnGRC) – is available for livestock associations or industries with mission to preserve germplasm or gonadal tissues from Canadian purebred animals.[4] Typically, frozen semen or embryos are sent to AnGRC's facility at no cost to the

[1] **Embrapa (Brazilian Agricultural Research Corporation).** 2021. Alelo Animal/Animal GRIN. Brasilia. Cited 8 February 2021. aleloanimal.cenargen.embrapa.br/

[2] **FAO.** 2007. *Global Plan of Action for Animal Genetic Resources and the Interlaken Declaration.* Rome. www.fao.org/3/a1404e/a1404e.pdf

[3] **Canada.** 1988. Animal Pedigree Act. Ottawa. Cited 8 February 2021. https://laws.justice.gc.ca/eng/acts/A-11.2/page-1.html

[4] **Canada.** 2021. Animal Genetic Resources of Canada. Ottawa. Cited 8 February 2021. https://agriculture.canada.ca/en/agricultural-science-and-innovation/agriculture-and-agri-food-research-centres-and-collections/animal-genetic-resources-canada

donor, and when frozen germplasm of a purebred animal is not available, AnGRC can collect and freeze semen samples on farm. Electro-ejaculation is the preferred method to harvest sperm cells. This collection process does not require the training of an animal, and the genetic material can be rapidly retrieved and analysed. Each step of the germplasm preservation process is demonstrated to producers for their education, which also serves as a tool to promote the preservation programme. Each produced dose must contain enough viable sperm cells to be used for artificial insemination to generate a new progeny. Sample acquisition requires that the owner complete consent forms which release the doses to the AnGRC group (see Section 9). Then half of the semen doses preserved by the AnGRC group can be returned to the donor to improve its herd or flock's breeding management, while the other samples become the property of the Canadian government. All information regarding the animal and the collection are entered into the Canadian Animal GRIN database, which can be consulted by the public. Only livestock associations or industries may request access to the doses stored in the Canadian repository, but they must demonstrate that the genetic material is not available on the Canadian market and the release of doses will benefit the breed. Consequently, AnGRC is a tool for Canadian producers and industries and strives to preserve their animal breeds.

The Chinese animal germplasm collection

Xueming Zhao

The Chinese animal germplasm collection[5] (China, 2021) provides a means to address the current reduction in genetic diversity of domestic livestock and poultry genetic resources, as well as to diminish the risk of resource destruction caused by the deterioration of ecosystems. The collection activities will allow for the rescue of endangered species, the recovery of populations, and maintenance of the diversity of domestic animal germplasm. These activities will lead to a better exploration and utilization of potential germplasm and genetic resources, which can then be used for the creation of new breeds or lines to meet the need of sustainable farming development.

Semen is the main form of germplasm preserved in China across all species. Because of the utility of cryopreserved semen (AI, ICSI, sperm-sexing technology), high-quality sperm can be used to breed targeted females or oocytes to manage the genetic diversity of a breed or create new breeds.

The successful application of *in vitro* embryo technologies to 16 livestock species in China makes embryo conservation very appealing. This form of germplasm is especially attractive because embryos contain the complete nuclear and mitochondrial genome of their parents. Then, when utilized, they enable recreation of a founder population for a breed in one generation. Similarly, the use of oocytes is considered practically equivalent to embryos, considered practically equivalent to embryos, but when coupled with frozen semen, *in vitro* fertilization, and semen sexing, it enables more precise and timely selection of the quality of germplasm that is used and the sex of the offspring.

[5] **China.** 2021. Chinese animal germplasm collection. Beijing. Cited 10 February 2021. db.cngb.org/

Somatic cells are considered to be most effectively preserved in conjunction with gametes and embryos. Somatic cell nuclear transfer technology has already been used to restore species. Somatic cells can also be used to produce IPSCs, which can be differentiated into gametes for preservation and used to create new breeds by gene-editing. Although both somatic cells and stem cells can be used to generate animals through nuclear transfer technology, which can regenerate populations of endangered animals and preserve genetic resources, stem cells are considered superior to somatic cells because of their greater potential for cellular differentiation.

DNA (tissue) analysis assists in improving gene bank management and population restoration, and can be used to identify potential germplasm samples, functional gene exploration to discover superior genetic variation, and as a tool for creation of new breeds or lines. The tissue is mainly used for identifying physiological and biochemical indicators, intestinal flora, and other quality traits, as well as for the acquisition of somatic cells, DNA, RNA and protein. While creation of animals exclusively using DNA is not currently possible, the gene bank preserves somatic cells, nuclear DNA and tissue for long-term preservation and scientific utilization, especially when semen and embryos are difficult to obtain from rare or endangered breeds, wild relatives and breeding groups across China.

A forward-looking type of information that is also utilized in China is via digital preservation. The structure of the physical animal body is digitized through the integration of information technology and biotechnology. This kind of new "germplasm" can be used to meet diverse requirements such as digitization of the information in multiple dimensions to provide virtual reality services and experimental animal models for future use.

The French National Cryobank of Domestic Animals

Elisabeth Blesbois

The mission of the French National Cryobank of Domestic Animals is to manage the constitution, the conservation and the distribution of domestic animal germplasm collections.[6] More specifically, the mandate states that the gene bank is responsible for restoring rare lines/breeds following catastrophic events, reintroducing genetic variability in populations in combination with *ex situ/in situ* conservation, aiding in genetic selection activities, creating new resources, and developing tools for research.

At this point in the programme's history, the main use of the bank has been via the semen collections. These collections have enabled the reintroduction of genetic variability in small populations of rare breeds and lines of pigs (Blanc de l'Ouest, Bayeux, Cu noir, Limousin, Basque pigs), sheep (Avranchin, Berrichon de l'Indre), horses (Cob normand, Trait Poitevin Mulardier), and chickens (INRAE R+ experimental lines). Reintroduction of genetic variability was also achieved using the goat embryo collection (Caprin Créole).

Increased use of the collections is foreseen in the future, and the cryobank has consequently developed a specific interface through our web portal which is a consortium of French gene banks for animal germplasm.[7] This portal offers the opportunity to view the

[6] **France.** 2007. Code rural et de la pêche maritime. France. Cited 12 February 2021. www.legifrance.gouv.fr/codes/article_lc/LEGIARTI000006596187

[7] **CRB-Anim.** 2020. Access to collections. Paris. Cited 10 February 2021. https://crb anim.fr/access-to-collection/#

contents of the collections, provides a tool to identify germplasm that should be stored, and includes a simulation tool to assess the impact on performance and predict the level of inbreeding when a particular sample of germplasm is used.

Ex situ conservation in Thailand

Rangsun Parnpai

The Department of Livestock Development (DLD), Ministry of Agriculture and Cooperative is required to follow Thailand's 20-year National Strategic Plan. Specifically, this operational plan for biodiversity management includes the policy to conserve the genetics of animals, plants and microorganisms, using *in situ* and *ex situ* conservation.

The *ex situ* conservation of animals includes the following materials:

- Frozen semen from dairy and beef cattle, native cattle and buffalo that have been developed at semen production centres serving the farmers. DLD facilities include a frozen semen storage centre that preserves samples for commercial entities and for preservation of livestock genetic diversity in general. The cryopreservation of pig semen is currently being explored.
- Frozen embryos are conserved from dairy and beef cattle, native cattle, buffalo and goats for genetic improvement and maintenance of genetic diversity.
- Tissue and DNA samples are collected for both research and biodiversity needs from all livestock species. DLD also cooperates with the Office of Natural Resources and Environmental Policy and Planning, Ministry of Natural Resources and Environment for the development of a database for banking genetics.[8] The permanent DNA storage facility is managed by the National Science and Technology Development Agency.

The National Animal Germplasm Programme of the United States of America

Harvey Blackburn

The National Animal Germplasm Programme (NAGP) is the programme of the government of the United States of America charged with the conservation of animal genetic resources. Since the programme's inception in 1999, the government has realized that an active gene bank would be required to acquire and store a variety of tissue types for current and future use, where future use may include presently unthought-of options. A key element of the programme is that germplasm samples and tissues are given to NAGP by small and large livestock producers and corporations with no compensation, and owners generally forego their rights to the germplasm. Because the many components of the livestock industry openly sell semen, either in a fresh or cryopreserved form, choosing semen as a primary germplasm type for the repository was viewed as a cost-effective approach. In addition, embryos for cattle, sheep, and to a lesser extent, swine, have been acquired, again, at no cost to the programme.

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[8] **Biotec.** 2021. Biotec culture collection laboratory. Pathum Thani, Thailand. Cited 8 February 2021. www.biotec.or.th/bcc/index.php/service

For chickens, the tissue types collected have evolved over time. Initially, semen was collected and cryopreserved, but the homogametic nature of avian males underscored the need to seek other paths for conservation. This has included collection and cryopreservation of primordial germ cells, and ovaries and testes for transplantation. Oocytes for swine have been acquired experimentally, which upon use may minimize the problem of genetics becoming obsolete over the course of decades. While the above tissue types have been collected as a means to reconstitute populations or reintroduce lost genetics, other tissues have been collected. For example, blood has been collected and stored for health tests. Genotyping animals in the collection is accomplished by using semen, blood or various tissue types (such as heart); thereby making the repository a one-stop shop for stakeholders.[9]

The National Livestock Cryobank of the Philippines

Lilian P. Villamor

The National Livestock Cryobank (NLC) was established by the Department of Agriculture in 2012 and is located at the Philippine Carabao Center in the Science City of Muñoz. The Korean International Cooperation Agency provided financial support. The NLC supports the existing genetic improvement programmes and underlies the livestock sector's response to future threats posed by climate change.

The NLC's strategy includes (i) the collection and preservation of genetic material, (ii) data banking, (iii) provision of access to stored samples, (iv) and dissemination of information. The NLC aims to preserve the diversity of native breeds, and oversee the introduction of exotic breeds that may be economically beneficial while still exhibiting resilience towards endemic diseases and the local environmental conditions. The collection currently consists of semen and oocytes and emphasizes buffaloes (91 percent), but also includes cattle (8 percent), goats and swine (<1 percent). Whole blood cells and DNA from various species are also preserved for research opportunities. The NLC envisions establishing a national repository of samples from the diverse range of livestock breeds and species, as well as threatened and wild animals in the Philippines.

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