Briefing on cryopreservation/biobanking technology for the UK National Standing Committee on Farm Animal Genetic Resources

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Purposes of cryopreserved genetic resources

Cryopreservation allows virtually indefinite storage of biological material without significant deterioration over a time scale of at least several thousands of years. This is based on the theoretical consideration that diffusion of molecules and ions at the temperature of liquid nitrogen (-196 °C) is insignificant over less than geological time spans. The oldest human semen reported to be used successfully for insemination had been stored frozen for almost 29 years. In cattle, IVF was reported with sperm that had been stored frozen for 37 years. In the Netherlands, in 2004, a calf was born after insemination of a cow with almost 26 year old Friesian Red and White semen from the Dutch CGN gene bank, This means that we can preserve the present wealth of genetic diversity in long-time storage in a biological 'safe deposit vault'. A germplasm repository may serve a number of purposes.

1) To provide the possibility of recreating lost breeds or breeding lines. Storage of germplasm for this purpose would typically be long-term storage, without frequent use of the stored material and without the need of regular updating of the collection.

2) To support *in situ* conservation. Frozen semen and embryos can be used to minimise inbreeding and genetic drift in small managed-populations, and the combination of live animals and cryopreserved germplasm can be a powerful tool in conservation of small populations

3) As a back-up in case genetic problems would occur. Decrease of effective population size and the resulting high level of inbreeding can lead to an increased relative frequency of deleterious alleles that were not apparent in a larger population. This happens not only in 'rare' breeds but can also be found in large commercial breeds, e.g. when a very small number of sires is responsible for a very high number of offspring. In such cases, the effective gene pool size is in fact very small.

4) To allow development of new lines or breeds, e.g. by introgression of specific traits from other (perhaps exotic) breeds.

Cryopreservation and storage methods

Two important cryopreservation concepts are slow-freezing and vitrification. In slow freezing methods, extracellular ice formation is induced as a means to dehydrate the cells, so that lethal intracellular ice formation is prevented. Slow cooling is needed in order to allow the cells enough time to lose much of the intracellular water. Confined between growing masses of ice, the remaining 'unfrozen fraction' containing the cells, ultimately solidifies (vitrifies) as an amorphous solid.

In so-called vitrification methods, no ice formation occurs in the entire sample, and the entire sample ultimately vitrifies. This is achieved by using extremely high concentrations of cryoprotectants like glycerol and others. In vitrification methods it is not necessary to cool slowly. In fact, it may be beneficial to cool very rapidly.

Viability or functional intactness can be impaired during freezing and thawing, but not during storage, provided that the frozen material remains stored at or very close to the boiling point temperature of liquid nitrogen (−196 °C). This applies regardless of the type of cells or the species, or the cryopreservation method. Material may be stored submersed in liquid nitrogen or in the liquid nitrogen vapour phase, but care must be taken to prevent elevation of the temperature in the vapour phase at any time.

Sperm and somatic cells (nuclei) can also be stored freeze-dried. After rehydration, the sperm and somatic cells are dead, but can be used for intracytoplasmic sperm injection (ICSI), and nuclear transfer, respectively, albeit at a lower efficiency than with not freeze-dried cells. However, freeze dried material cannot be stored indefinitely, unless stored at −196 °C.

Types of genetic resource material

Various sorts of cells or tissues are being used, or have been considered, for cryoconservation of genetic diversity, e.g. semen, embryos, oocytes, and somatic cells. There are large differences in present day feasibility, practicality and costs of these possibilities.

Semen

Semen collection and successful cryopreservation is possible in most cases in many species, including cattle, sheep, pigs, horse, goats, and poultry. Conception rates from frozen semen vary by species and between individual males within a species. An existing AI infrastructure for collecting and

use (insemination) of semen may be available, notably in cattle and pigs. Cryopreserved semen is ideal for supporting breeding schemes for conservation of rare breeds. However, only a single complement of chromosomes is preserved. For recovery of a lost breed, at least 6 generations of backcrossing are needed to restore the original genotype.

Epididymal semen can be collected post mortem or after (hemi) castration in most mammalian species. It is a cheap and easy method for gene banking, and can be used for species/breeds that cannot be trained for semen collection, or for genetically interesting animals that die or must be castrated unexpectedly. Collection of epididymal sperm in sheep is very efficient, with high semen yield (numbers of doses) per male. Freezability and fertilizing ability of epididymal semen is excellent.

Oocytes, embryos

Gene banking of embryos, or semen plus oocytes, allows restoration of a lost breed or genotype without backcrossing. Using oocytes plus semen, one remains more flexible in combining genotypes. In the most recent years, both slow-freezing and vitrification protocols for oocytes render excellent results, especially in human. In farm animals, there are not as many reports of live born young as in human. This may in part be due to species-specific problems, but it may also reflect that there perhaps has been less incentive to develop and use cryopreservation methods for oocytes in farm animal species, compared to human. Viable oocytes have been recovered after freezing and thawing in a great number of animal species. Live born young from embryos produced from cryopreserved oocytes have been reported in cattle, horse, mouse and rat. Viable offspring from frozen oocytes of avian and fish species have not yet been obtained.

Cryopreservation of embryos is a routine procedure in cattle. Both slow-freezing and vitrification protocols are effective. The success of cryopreservation is dependent on the stage of the embryo; that is, especially good results are obtained with blastocysts. The possibilities for cryopreservation of sheep embryos seem to be similar to that for cattle. Pig embryos are more difficult but recent studies resulted in live piglets from cryopreserved embryos. Other species in which live offspring were obtained from cryopreserved embryos include horse, goat, gerbil, polecat, cat, ferret, rabbit, mouse, and rat. Pregnancies have been reported in llama, and Red deer. Especially for those species for which collection and transfer techniques are available and operational, embryo banking is a very good possibility for preservation of genetic diversity, and the fastest way to restore an original genotype when needed.

In birds, semen can be effectively collected and cryopreserved. However, embryos and oocytes cannot be cryopreserved. An alternative is the use of primordial germ cells (PMG), which can be harvested from developing embryos and be injected into another embryo. The resulting chimeric animal can produce gametes of the donor animal. However, these methods are currently not very easy or practical.

Ovaries

Cryopreserved ovaries or parts of ovaries may be used as a source of oocytes. Oocytes may be harvested from heterotopically grafted ovaries for IVF and embryo transfer to a recipient. Alternatively, cryopreserved ovary tissue or whole ovaries can be grafted orthotopically in a recipient animal in order to restore fertility in that animal. This animal can then be mated and can produce offspring carrying the ovary donor genotype. Restoration of fertility after grafting cryopreserved ovaries was achieved in mice, but also in larger animals, e.g. in sheep. Gene banking of ovaries or ovarian tissue may be less efficient compared to embryos. Either laparoscopy or killing the donor animal would be needed to obtain the ovaries. To make use of the cryopreserved material to produce offspring, surgical expertise and facilities are required for the grafting of the thawed cryopreserved ovaries into recipient animals.

Somatic cells ('cloning')

Somatic cells (SC) can be used to produce embryos by nuclear transfer (NT) (SCNT 'cloning' as distinct from cloning from embryonic tissue or by embryo splitting). Cloning produces an animal that has the same genotype (except for the few genes in the mitochondria) as the somatic cell donor animal. Thus, no backcrossing would be required to regenerate a lost breed or breeding line. Cloning also offers possibilities to conserve genotypes of infertile animals, for instance that of castrated champion horses. Cloning has been demonstrated from tissue obtained post mortem, so it would be possible to regenerate the genotype of valuable animals that suddenly died, or had to be killed.

Cloning is done by transferring the nucleus of a donor somatic cell to an enucleated oocyte. The oocytes may be obtained from slaughterhouse ovaries. The breed or genotype of the oocyte donor is less relevant. In fact, in some reports oocytes from a different but related species were successfully used. Somatic cells may be used from any tissue from the body, from juvenile or adult animals (even

beyond fertile age). The oocytes 'reprogrammes' the differentiated somatic cell nucleus to obtain the totipotency needed for embryo development, and can then be cultured *in vitro* to develop into an embryo. The resulting embryos can then be transferred to recipient animals. Interspecies embryo transfer has been used when suitable females of the species are not available, e.g. in the case of wild animals.

Collection of suitable somatic cells is easy. For instance, a piece of ear can be taken using an ear tagger. Cryopreservation protocols for somatic cells are relatively simple and do not require complicated freezing equipment (or, it seems, liquid nitrogen storage). This means that establishing the collection is easy and cheap. The opposite is true for using the material when needed. This involves culturing the cells after thawing, collecting oocytes by ovum pick-up or from slaughtered animals, culture and in vitro maturation of the oocytes, enucleation of the oocytes, transfer of the somatic nucleus to (or fusion of the somatic cell with) an enucleated oocyte, culture of the resulting embryos, and finally, embryo transfer to recipients animals. The step of culturing the somatic cells (which can be re-frozen) has the added advantage of multiplication of the donor material – the cryobank is therefore extendable (though probably not inexhaustible).

Live offspring have been obtained from cloned embryos in a number of species, i.e. in sheep, goats, cattle, pigs, horse, mule, mice, rats, rabbits, ferrets, cats, and dogs. However, for cattle and sheep only a small proportion of embryos produced using somatic cells develop to become live young, typically between 0 and 5% (though the efficiency is slowly increasing). A significant proportion of pregnancies are aborted, and full-term pregnancies often result in malformed young. For pigs and horses, higher success rates are reported, with near normal rates of malformed young. Viable litters of cloned pigs are now obtained routinely by transferring large numbers of SCNT embryos into each recipient. In general though, it seems that the present cloning techniques introduce errors that affect embryonic and foetal development. On the other hand, on a long time horizon, increased understanding of nuclear reprogramming is likely to make cloning both reliable and efficient. Thus, somatic tissue cryopreserved today may be used successfully in the future. Therefore cryobanking of ear tissue can be considered as a cheap method of conserving genotypes for the more distant future, possibly even now for pigs.

The type of genetic resource material to be preserved in the gene bank may depend on the purpose of the gene bank, i.e. whether the gene bank is intended to save and preserve present day biodiversity for 'eternity' as an insurance policy, or must (also) serve to support breeding schemes of small populations. In the latter case, it seems sensible to store semen (and embryos), which can be updated regularly and can be readily used in the field. If gene banking is intended to save and preserve present day biodiversity for 'eternity' one would like to have a cheap and fast collection of as many species and breeds as possible. Wherever financial resources and existing expertise and facilities are available, embryos or gametes are probably the best choice, but collection and cryopreservation of somatic cells should be considered as a possibility, especially where the cost of collection is an issue and the material is only needed for possible disaster recovery (when presumably cost will be less of an issue, but speed will be important).

Table 1. Characteristics of several ways to cryopreserve genetic diversity.

	Semen	semen plus oocytes	embryos	somatic cells
Samples needed to restore a breed	10000	2 x 100	200	Depends on future efficiency of cloning
Backcrossing needed	Yes	No	No	No
Mitochondrial genes included?	No	Yes	Yes	No
collection possible	Mostly	Yes, various species. Routine for bovine	Yes, various species. Routine for bovine	Always
Cost of collection	££	£££	£££	£
Cryopreservation possible?	Yes	Live born young in human, cattle, horse, mouse, and rat.	Bovids, sheep, and human are routine. Live born young in many mammalian species.	Yes (easily and cheaply)
How to use	Surgical or nonsurgical Insemination. Backcrossing ≥ 6 generations needed to recover a lost breed	ICSI → In vitro culture → surgical or nonsurgical ET	Surgical or nonsurgical ET	Culture somatic cells, isolate, culture, enucleate oocytes → Transfer somatic nuclei to oocytes → in vitro culture → surgical or nonsurgical ET
Cost of use	£	£££	££	£££££
Possible?	Yes	Yes	Yes	Low efficiency and clear risks. Future development is likely!