Latest developments in cryobiology and reproductive technologies

Henri Woelders and Sipke Joost Hiemstra
Centre for Genetic Resource, The Netherlands (CGN)
Wageningen UR Livestock Research, P.O. Box 65, 8200 AB, The Netherlands

In this presentation, I will try to highlight a number of interesting and useful recent developments, but also indicate some remaining difficulties or areas of possible future research.

Semen collection

With regard to the commercial cattle and pig breeds, CGN receives regular updates (snapshots) of semen from the AI stations. Also in less mainstream breeds, bulls may be available on AI stations. But typically, in the case of rare breeds, bulls are not available on AI stations. There is now finally an official derogation for on-farm collection by CGN. For sheep, we are still happy with collection of epididymal semen of slaughtered rams, which is a very cost-effective method.

Collection of semen from geese and ducks proved to be more difficult compared with chicken, and smaller quantities are obtained per ejaculate. However, this appears to be improving as we are getting more experienced. We have investigated the possibility of using epididymal semen from bird species. There are few publications regarding collection of epididymal spermatozoa in avian species (Clulow and Jones 1988, Kwon et al. 1995, Orlu and Egbunike 2010). The morphology and function of the epididymis in birds are quite different than in mammals. In quail it was shown that most 'extragonadal' sperm are found in the vas deferens (Clulow and Jones 1988) rather than in the epididymal duct. We found that in chicken, interesting sperm numbers can be retrieved from the vasa deferentia. This appeared to be less in drakes. We concluded that collection of extragonadal semen of slaughtered drakes would not increase efficiency and cost-effectiveness for gene banking of duck, or even rooster semen.

Semen cryopreservation, birds

Cryopreservation of semen is possible in a number of bird species, but may not be possible or efficient in specific cases. For the chicken, adequate methods for freezing semen are available with good fertility being reported in a number of studies using cryopreserved semen (Tselutin et al. 1999, Woelders et al. 2006). CGN recently created a semen gene bank collection of native Dutch chicken breeds and currently has a stock of semen from 20 breeds with 5-19 males per breed (Table 1).

Table 1. Indigenous Dutch chicken breeds in the CGN gene bank.

Breed	Nr cocks	Nr doses	Breed	Nr cocks	Nr doses
Barnevelder	13	1034	Ned. Baardkuifhoen	11	862
Drents Hoen	12	578	Noordhollands Hoen	14	1489
Twents Hoen	13	902	Groninger Meeuw	10	865
Ned. Uilenbaard	12	858	Assendelfter Hoen	11	651
Welsumer	12	820	Schijndelaar	5	500
Hollandse Kriel	30	803	Hollands Hoen	12	850
Lakenvelder	9	890	Chaams Hoen	10	1004
Brabanter	13	1211	Hollands Kuifhoen	10	834
Fries Hoen	13	754	Sabelpoot Kriel	19	564
Kraaikop	10	992	Eikenburger Kriel	7	206

Both the Tselutin method (pellets) and Woelders method (straws) rely on very high freezing rates and the use of DMA (0.6 M) as cryoprotectant. We have seen that the extender that we are using also contributes to the good post-thaw sperm quality. We found that this extender, plus the freezing at high freezing rates (±200 °C/min) with 0.6 M DMA also improved freezing results in other bird species, including tragopan pheasants, cranes, duck and geese, and turkey, compared with Lake's freezing medium, lower cooling rates, and glycerol as cryoprotectant (table 2, data for turkey). We are using this method now for the freezing of drake semen for the CGN gene bank.

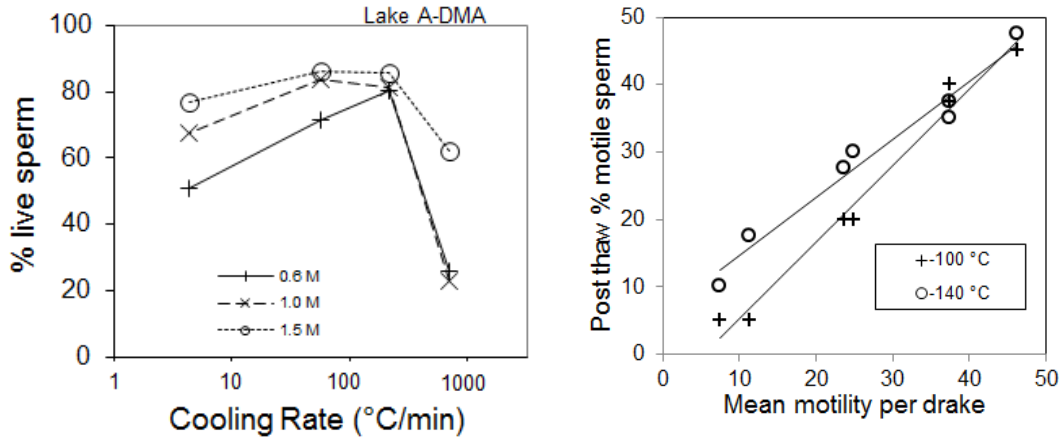

Figure 1. Role of cooling rate for poultry semen. Left panel: A relatively high cooling rate of approximately 200 °C/min (measured after dissipation of heat of fusion) was optimal for rooster semen. For routine freezing, we achieve this cooling rate in 0.25-ml straws in a constant temperature freezer at -140 °C. Right panel: We have checked that -140 °C (cooling rate ±200 °C/min) is not too much for Drake semen (7 drakes). The lowest temperature (highest cooling rate) appears to give best results, especially for drakes with poor semen/ poor freezability.

In chicken, we obtained good post-thaw fertility (86% fertile eggs) using frozen/thawed semen. However, the validation of the semen freezing method was done only using semen from a commercial chicken line, and post-thaw fertility of frozen thawed semen of the rare breeds may be lower than that of the commercial breed. In turkey, fertility results had improved using our method compared with existing methods , but there were large differences in success for different breeding lines, with no success (no hatched poults) in one of the lines studied (Long et al. in preparation).

Table 2. Fertility of frozen-thawed turkey semen with 2 extenders, each with DMA at high cooling rate (200 °C/min) or glycerol at lower cooling rate 50 °C/min). Only results of the best breeding line are shown here. Breeding line differences were significant, with one of the lines having no live hatched poults with any of the freezing methods. Long et al, paper in preparation.

Cryopreservation Treatment	Fertilized eggs(%)	Day 7 (%)	Live late embryo (%)	Hatched (%)
ASG DMA	19.0	9.5	8.9	8.9
Lake's DMA	8.6	3.6	2.9	2.9
ASG Gly	5.5	0.8	0.0	0.0
Lake's Gly	10.7	0.0	0.0	0.0

Insemination in sheep and goats

In sheep and goats, methods for freezing ram or buck semen are available and give a fair rate of success (Salamon and Maxwell 2000, Leboeuf et al. 2008). With frozen thawed ram semen good fertility results are obtained with laparoscopic insemination (surgical semen deposition in the uterine horns), but generally not with 'normal' cervical or vaginal insemination. The reason seems to be that frozen thawed spermatozoa have a shorter lifespan and probably a less efficient motility and therefore sperm migration through the cervix and uterus to reach the site of fertilization is impaired. Laparoscopic insemination is prohibited in the Netherlands, and in any case it is more complicated and time consuming than conventional AI. An alternative would be to use a non-surgical method of intrauterine semen deposition. However, this appears to be difficult because of the complicated cervix anatomy (Halbert et al. 1990a, Kershaw et al. 2005). Three approaches have been tried to reduce the difficulty of passing through the cervix. One is by using an implement to retract the cervix into the vagina to identify the cervical opening and align the funnel-shaped rings of the cervix to some degree (Halbert et al. 1990b, Halbert et al. 1990a). A second approach is the use of an agent to relax the cervix (e.g. PGE-2 or PGE analogues, oxytocin, interleukin-8, misoprostol, or hyaluronan (see several references in Wulster-Radcliffe et al. 2004, Perry et al. 2010), or the beta-adrenergic blocking agent carazolol (Gündüz et al. 2010). A third

approach is designing appropriate transcervical AI and ET equipment to overcome the physical difficulties associated with the ovine cervix (Wulster-Radcliffe et al. 2004). In what is referred to as the Guelph system, restraint of the ewe and instrumentation were adapted in order to allow transcervical deposition of the semen in at least a fraction of all tested ewes (Halbert et al. 1990b), which resulted in higher pregnancy rates compared with vaginal semen deposition in the vagina or cervical os. In later studies, both improved transcervical AI implement (Wulster-Radcliffe et al. 2004) and administration of PGE2 (Candappa et al. 2009) or PGE1 analogue (Leethongdee et al. 2007) allowed 100% penetration of the cervix, but lambing data are either missing or not dramatically improved. In the case of goats, excellent kidding rates were reported after transcervical AI with frozen thawed semen (Sohnrey and Holtz 2005).

However, in sheep there are also other reports that seem to obtain very good results with a different and quite simple approach. Paulenz et al. (2005) reported a 70% lambing rate after cervical or vaginal AI of Norwegian crossbred ewes (figure 2). Most groups reported lambing rates with frozen/thawed semen in the range of 17-23%. One of the probable causes for the good results in the Norwegian study seems to be that ewes were inseminated in natural oestrus. Furthermore, the fertility of Norwegian crossbred ewes is very high, due to environmental conditions and low milk yields, and selective culling of less fertile ewes. Lastly, the farmers monitored oestrus and performed the inseminations themselves. The flocks were small and the farmers had been well trained and were experienced inseminators. Obviously, the cervical, or especially the vaginal insemination is relatively simple, cost effective and least affects animal welfare. It is therefore interesting to see whether some of the factors that may have contributed to the high lambing rate would also apply to other breeds.

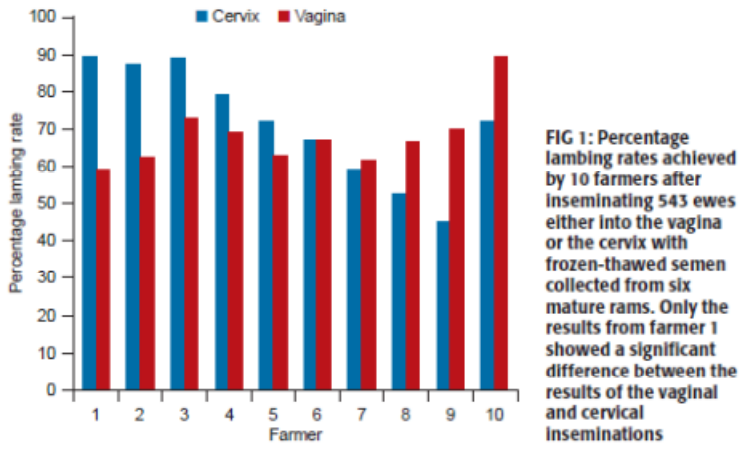

FIG 1: Percentage lambing rates achieved by 10 farmers after inseminating 543 ewes either into the vagina or the cervix with frozen-thawed semen collected from six mature rams. Only the results from farmer 1 showed a significant difference between the results of the vaginal and cervical inseminations

Figure 2. Lambing rates obtained by 10 farmers after vaginal or cervical inseminations of Norwegian crossbred ewes with frozen-thawed semen in natural oestrus. From Paulenz et al 2005.

Horse, embryos and oocytes

In the horse, recovery of a lost breed using semen is much more expensive than in other farm animal species (Gandini et al. 2007). This may be improved when using low dose (1-10 million sperm) insemination techniques e.g. by hysteroscopic insemination (Morris and Allen 2002). The possibilities of using cryopreserved embryos or oocytes appear to be poor in the horse, with relatively few reported pregnancies (see references in e.g. Choi et al. 2011) and only a few live births reported after transfer of cryopreserved embryos (Galli et al. 2007, Ulrich and Nowshari 2002). There are specific morphological and other embryo characteristics that seem to hamper cryopreservation of horse embryos. For instance Scherzer et al. (2008) and Choi et al. (2011) demonstrated that the presence of a large blastocoel in larger embryos negatively affects survival of vitrification, and success can be improved by reducing the blastocoel volume. Also the freezability of oocytes appears to be relatively low in the horse (Tharasanit et al. 2005; 2006a; 2006b) with only one report of live offspring after transfer of embryos produced from cryopreserved oocytes (Maclellan et al. 2002). Collection of horse oocytes is in itself a practical possibility, as oocytes can be collected, without knowing the stage of the oestrous cycle, by transvaginal ultrasound guided ovum pick up, allowing the collection of more than 70 oocytes per year per mare. In the Netherlands, one commercial Equine reproduction centre claims to be able to

cryopreserve horse oocytes , and CGN actually has commissioned the collection and cryopreservation of 50 oocytes from one Gelderlander mare. However, we are unable yet to know the protocol used or even the chance of success in producing embryos and obtaining offspring from these cryopreserved oocytes.

Fundamental approach to vitrification

We are trying to arrange a new research project to answer a number of fundamental research questions related to slow freeze and vitrification methods for preserving oocytes and embryos, addressing fundamental cryobiological principles and using mathematical modelling of physicochemical events involved in cryopreservation. There are some interesting advances in cryobiology that could be followed up. One is that increasingly the amount of volume that envelops the embryo or oocyte is reduced in order to increase cooling and warming rates, using open systems such as OPS, cryoloop, cryotop, or cryoleaf. However, in most clinical applications, these systems are still being used at relatively high cryoprotectant concentrations. In addition, the rationale and benefits of using either membrane permeant or impermeant cryoprotectant is little addressed in most research in which vitrification methods are applied. Recent studies by the groups of Toner (Boston), and Mazur (Oak Ridge) have shown the overriding importance of thawing rate rather than cooling rate and have indicated mechanical possibilities to substantially increase cooling and warming rates and obtain promising good results at reduced cryoprotectant concentrations. We want to address these issues using mathematical simulations combined with empirical experiments.

Ovaries, of mice and birds

Similar vitrification protocols have been applied also to pieces of ovarium cortex, or to half- or entire juvenile ovaries. The cryopreservation and use of ovaries for conserving a mutant genotype at first glance seems a less obvious option, as success may depend on fine surgical skills and requires good post-thaw survival of the ovaries that should be able to revascularize, and should not elicit graft rejection, etc. Yet, recent progress in cryopreservation and use of mouse ovaries has shown that this approach has great promise and may have several advantages over the banking of semen and embryos. Vitrification protocols very similar to those used for embryos and oocytes are simple to perform. More importantly, it was shown that grafting of vitrified/warmed ovaries into young animals is a relatively simple and successful procedure. The whole approach appears to be remarkably efficient, which was demonstrated by using cryopreserved ovaries of a large series (22) of mutant strains with various genetic backgrounds (C57BL/6, FVB, BALB/c) to recover the genotype by orthotopic grafting of the vitrified ovaries in recipient mice (Huang et al. 2010).

A similar approach also seems to work elegantly in a number of bird species. Live offspring carrying the donor genotype was obtained after transplantation of cryopreserved half-ovaries in quail (Liu et al. 2010). Both slow freeze and vitrification protocols were used in that study, but vitrification gave best result. Like in the mouse, the procedures used for collection, vitrification, and grafting of the ovaries appear to be remarkably easy to perform and successful. Graft rejection was prevented by oral administration of an immunosuppressant. This method would be ideal if it can be complemented with cryopreserved semen from the same breed, so that the original breed or breeding line can be recovered in a single generation. In that respect it is interesting that a similar approach of grafting cryopreserved testicular tissue from roosters rendered males that produced (donor testis derived) sperm sufficient for successful intramagmal insemination of hens (Song and Silversides 2007). If no semen of the breed or line is available, several generations of backcrossing would be needed and this would imply that larger stocks of ovaries need to be banked, as would be the case when only semen banking would be used to secure a breed or line.

PGCs of birds

Another possibility in birds is cryobanking of primordial germ cells (PGCs). PGCs can be simply collected from blood from day 3, Hamburger Hamilton (HH) stage 14-16 chicken embryos. (Macdonald et al. 2010, Naito et al. 1999, Van de Lavoir et al. 2006). The PGCs can be cultured and propagated as clonal cell lines, and frozen. Frozen-thawed PGCs can then be used to produce chimaeric embryos by injection of the PGCs into the cardiac tract of HH stage 14-16 embryos, which then are transferred into phase III host shells and cultured to hatching in a specialized procedure (Perry 1988). PGCs only seem to give germline transmission when transferred to a same sex embryo. Male hatchlings can be raised to sexual maturity and may then carry the donor genotype in a fraction (2-16%; Macdonald et al. 2010) of their sperm cells. Likewise, female PGCs in female embryos can generate female chimaeras with the donor genotype in a fraction (0-12%; Van de Lavoir et al. 2006) of the produced ova. Currently, more research is needed to make the procedures more easy and consistent, but in principle, the cryobanking of PGCs would have the advantage of allowing recovery of a lost breed or breeding line without the need of backcrossing, provided that also female PGCs can be cloned and propagated (c.f. Macdonald et al. 2010, Van de Lavoir et al. 2006).

Somatic cells

Lastly, somatic cells can be used in gene banking programmes to preserve genotypes or breeds and breeding lines. Eildert Groeneveld will address this topic in the WG meeting. In the Netherlands, CGN has considered cryobanking of somatic cells as a means to preserve the genotypes of unique, old mares for which other approaches to preserve their germplasm are currently not possible or impractical. The ethical acceptability of nuclear transfer 'cloning' is however debated, partly because of concerns of animal health and welfare, as cloning in many species is still associated with higher levels of embryonic loss and malformed young (Campbell et al. 2007, Oback 2008), but also because of other ethical considerations. One could still consider cryobanking of somatic cells in case no other viable route is available, on the premise that the material will not be used unless future methods regarding nuclear reprogramming have evolved to a stage that offspring can be obtained without compromising health or welfare or the genetic makeup in the first offspring and in subsequent generations. However, currently, CGN is not applying gene banking of somatic cells.

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