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REPRODUCTION TECHNOLOGY AND GENE TRANSFER IN RABBITS

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Domestication is considered to be one of the greatest experiments in evolutionary biology ever done by mankind (Nachtsheim and Stengel, 1977). In general, domestication led to the breeding of animals with new characteristics which are independent from competition for survival and less influenced by environmental factors. In addition selection was carried out for a limited set of commercial aspects. Today, the rabbits are housed for numerous purposes and fulfil multiple functions for research and industrial production. As shown by Bolet et al. (1999), Western Europe contributes around 60 per cent of the world´s commercial meat production. However, most of it is produced from hybrid animals rather than pure-bred animals, which represent the variety of existing genetic resources. Although more than one century ago the first successful mammalian embryo transfer was performed in rabbits (see Biggers 1993), artificial insemination is the most efficient applied reproduction technology used in rabbit industry. In contrast to rabbit industry, reproduction technology holds an indispensable function in research fields, where husbandry under specific conditions, generation interval and animal size leads to increasing interest in using rabbits for scientific subjects. Progress in the knowledge of reproductive physiology will have beneficial impacts on scientific and on economic development mainly based upon oocyte and embryo recovery, cryopreservation, intra cytoplasmic sperm injection, nuclear transfer, control of sex ratio and embryo transfer. Additionally, the introduction/gain of genetic information into/from mammalian species has provided the possibility of additive gene transfer and homologous recombination offering new tools by modifying the genome. In the following, the use of reproduction technology and gene transfer in rabbits will be reviewed.

EMBRYO COLLECTION AND TRANSFER

The ability to collect and transfer mammalian preimplantation embryos resulted in numerous associated applications which are inevitably linked to molecular genetics, cell biology and reproduction. “Embryo transfer” includes the generation of preimplantation embryos and the developmental continuation of embryos until term in different foster mothers. Thus in this manuscript separate attention is paid on both, donor and recipient rabbits. Moreover, it is expected, that housing, feeding, breed, age etc. are basic factors affecting reproduction (Castellini, C., 1996; Theau-Clément and Lebas, 1996; Theau-Clément et al., 1998) and have to be carefully taken into account as prerequisites for any biotechnological application.

Superovulation

Females need to be superovulated in order to achieve extra number of embryos or oocytes. Already in the forties, Pincus (1940) compared extracts from the pituitary gland and pregnant mares serum gonadotropin (PMSG) for superovulatory treatment in rabbits and found that treated rabbits delivered 3 to 4 times more oocytes than non-treated. To date, superovulation is routinely performed by the use of follicle stimulating hormone (FSH) or pregnant mares serum gonadotropin (PMSG) in varying amounts and time intervals for repeated application.

Following stimulation of follicular growth the administration of either human chorionic gonadotropin (hCG), luteinising hormone (LH) or gonadotropin releasing hormone (GnRH) sufficiently initiates induction of ovulation. Table 1 gives a summary of the effects of FSH and PMSG application prior to induced ovulation in rabbits.

Table 1: Superovulatory response of FSH and PMSG in rabbits

Induction of follicular growth	Induction of ovulation	Ovulation sites, n	Oocytes n	Embryos n	Authors
5 x 0.4 mg FSH	25 I.U. hCG	31.2	-	13.3	Beatty, 1958
3 x 50 I.U. PMSG	50 I.U. LH	38.5	-	-	Hafez, 1961
6 x 0.155 mg FSH	2.5 mg LH	56.1	40.4	-	Kennelly and Foote, 1965
6 x 12.5 I.U. PMSG	2.5 mg LH	16.6	13.7	-	Kennelly and Foote, 1965
6 x 0.5 mg FSH	2 mg LH/kg	45.1	36.8	-	Varian et al., 1967
6 x 0.3 mg FSH	2.5 mg LH	46.5	39.8	-	Maurer et al., 1968
3 x 30 I.U. PMSG	2.5 mg LH	13.6	11.0	-	Maurer et al., 1968
3 x 75 I.U. PMSG	100 I.U. hCG	14.7	12.7	11.4	Gabler, 1970
6 x 0.5 mg FSH	2.5 mg LH	45.1	30.1	27.1	Gabler, 1970
6 x 0.5 mg FSH	2.5 mg LH	36.1	29.6	25.2	Meier, 1972
6 x 0.15 mg FSH	2.0 mg LH	39.3	-	-	Michelmann and Paufler, 1974
6 x 0.3 mg FSH	2.5 mg LH	50.3	41.6	38.1	Braun, 1977
1 x 60 I.U. PMSG	60 I.U. hCG	47.8	-	-	Rottmann and Stranzinger,
6 x 0.5 mg FSH	60 I.U. hCG	26.9	-	-	1977
6 x 0.155 mg FSH	2.5 mg LH	60.8	50.3	46.0	Braun and Leidl, 1980
1 x 120 I.U. PMSG + 0.25 ml a-PMSG	60 I.U. hCG	-	-	21.3	Illera et al., 1990
1 x 80 I.U. PMSG	180 I.U. hCG	39.5	30.9	22.5	Besenfelder, 1991
5 x pFSH (Σ 2 mg)	0.2 ml GnRH	≤32	-	30.4	Joly, 1997
6 x FSH (Σ 3 units)	150 I.U. hCG	26.5	19.4	16.8	Kauffman et al., 1998
8 x FSH (Σ 4 Units)	150 I.U. hCG	23.5	23.5	21.3	Kauffman et al., 1998

Embryo collection

Highly important for collection of embryos is the exact knowledge of the timecourse of physiological order of events following induction of ovulation coupled simultaneously with artificial insemination. Ovulations are expected to occur between 9.5 – 13 h post insemination (p.i.) (Harper, 1961, Walton and Hammond, 1929). Cumulus is removed from the fertilised ova between 12-15 h (p.i.) (Gottschewski and Zimmermann, 1973). Results of Greenwald (1961) indicate, that 2 hours after ovulation, oocytes have normally migrated half the total length of the oviduct. By 18 hours post coitus (p.c.) nearly half of the embryos have entered the isthmus and are denuded, 24 hours p.c. all embryos can be found in the isthmic part (Bourdage and Halbert, 1988). The ampullary-isthmic junction is discussed to cause a delay in embryo migration through the oviduct. The first cell cycle is terminated 26 h post p.i. and

the consecutive cleavages are expected to be completed within 26 – 32 h p.i.(4 cell stage), 32 – 40 h p.i. (8 cell stage), 40 – 47 h p.i. (16 cell stage), 47 – 68 h p.i. (morula), and 68 – 76 h p.i. (blastocyst)(Gregory, 1930). 78 hours p.c. about one third of the embryos were already found beyond the uterotubal junction, whereas 84 hours p.c. more than 90 % of the embryos have reached the cornu (Bourdage and Halbert, 1988). Embryo implantation has been reported between 120 and 144 h p.i. (Blandau, 1961). Based upon this progression in embryogenesis, various techniques have been developed to collect embryos at any stage and location:

- Slaughter: final step
- Surgery: final step (removal of reproductive organs)
  repetition
- Non surgical: repetition (application of hormones)
- Endoscopy: repetition (in situ manipulation)

Embryo flushing following the slaughter of animals represents a final step. After removal and preparation of the reproductive organs, oviduct alone or combined with the uterine horns have been flushed (Kennelly and Foote, 1965, Hahn and Meier, 1973, Adams 1982). Surgical flushing aimed at the in vivo collection of embryos rather than removal of organs (Dowling, 1949; Avis and Sawin, 1951; Chang 1952; Gabler, 1970; Hafez, 1961; Meier, 1972; Adams, 1982). A more practical method was described by Takeda et al. (1977) and Gajda et al. (1988). The application of prostaglandins resulted in the extrusion of either tubal or uterine stage embryos into the vagina where vital embryos could be non-surgically flushed. Recently, we developed an endoscopic technique for the collection of oocytes and preimplantation embryos. Laparoscopy seems to be the method of choice for multiple embryo collection in rabbits since it enables free optic access to the reproduction organs and manipulation in situ and guarantees minimal invasion by using small entry into the peritoneal cavity (Besenfelder et al., 1998). In contrast to large farm animals, flushing of oviducts via uterine horns towards the vagina is not hampered by any cervical barriers. Midventral laparoscopy allows the insertion of e.g. a rigid vein catheter via infundibulum into the ampulla. This venous catheter can be easily fixed intra-ampullarly using an atraumatic grasping forceps and permit the orthograd tubal flushing with medium. First controlled study at our institute, we connected a cystendoscope to a camera monitor system, which was placed deeply in vagina for directing a silicon tube in front of the cervices or insertion about 2 cm into the cervix for medium collection. Besides flushing of the oviducts additional 40 – 60 ml medium were flushed through each uterine horn from a puncture site at the uterotubal junction in order to thoroughly flush the lumen. Due to successful collection of oocytes, zygotes and blastocysts the collecting system was further simplified by using only a laparoscope and forceps for flushing and for intravaginal medium collection a flexible embryo flushing catheter (CH 18) was used.

The collection technique will highly depend on the scientific or commercial purpose. If embryos are needed irrespective of the maternal maintenance, collection is simplified by a routine slaughter procedure. Embryo recovery from high valued animals, however, favours the use of endoscopy rather than surgery.

Embryo transfer

Walter Heap, an often cited pioneer, reported the first mammalian embryo transfer in rabbits. He succeeded in transferring embryos of Angora origin into a mated Belgian hare doe, which delivered Belgian hare and Angora coat coloured pups (see Biggers, 1991). Nowadays, embryo transfer is performed by surgery or by endoscopic methods.

- surgery — ET: lumbar/midventral → oviduct / uterine horns
- endoscopy — ET: vagina → uterine horns; laparoscopy → oviduct / uterine horns

Embryo transfer should fit to flushing concerning stage and location of embryos. Embryos collected at the tubal stage should be transferred to oviducts, whereas morulae and blastocysts are preferably transferred back to the uterine horns. Successful implantation requires optimal preparation of the uterine epithelium and the tubal stage embryo. A characteristic of this developmental stage of the embryo is an increase in diameter due to mucin layer. The mucin production during oestrus phase, the mucin discharge after ovulation, and the wrapping up of the zygotes is thoroughly triggered by the change of the ratio of steroid hormones (Greenwald, 1958). Thus, reduction or damage of the mucin layer significantly limits the success of embryo transfer (Carney and Foote, 1990; Kasai et al., 1993).
Numerous entries are available for embryo transfer via laparotomy including access to the oviducts (Chang, 1950; Hafez, 1962a; Techakumphu et al., 1987a) or uterine horns (Dowling, 1949; Chang, 1950; Hafez, 1962a) via midventral (Chang, 1950; Hafez, 1962a; Techakumphu et al., 1987a) or lumbar laparotomy (Hafez, 1962ab; Chang and Pickworth, 1969).
Techakumphu et al. (1987a) described transfer of tubal stage embryos into rabbit oviducts in detail by inserting a fine teflon catheter up to the tubular blend. This catheter was fixed by two hemostatic clamps whereas a connected Hamilton syringe guaranteed the extreme careful deposition of embryos into the oviduct. The transfer of 2-cell to morula-stage embryos resulted in 93 % (14/15) pregnant animals. Additionally, best embryo survival is obtained by transfer into a synchronised recipient (Fischer, 1989, Techakumphu et al.,1987a).
In 1969, Testart conducted embryo transfer via cervix by means of an inovulation catheter. These transvaginal experiments resulted in 60 % gravid females, however, 30 % of the recipients showed pyometra and more than half of the recipients had abnormal genital tracts (Testart, 1969).
The use of endoscopy aimed at minimising the invasion site for inspection/adspection of the reproduction organs and in situ manipulation. Endoscopy was already used in the seventies and eighties for studying reproductive physiology (Fujimoto et al., 1974; Theau-Clement and Bolet, 1987; Russel, 1988; Santacreu, 1990). In 1991 Garcia-Ximénez et al. described the first endoscopic embryo transfer into the uterine horns of rabbits. A laparoscope and forceps were used to direct a trocar cannula needle into the uterine horn and to transfer 64 to 66 hours p.i collected embryos to synchronous recipients. All of the 12 does used for transfer became pregnant. Two years later, laparoscopic embryo transfer into the oviduct was established in our lab (Besenfelder and Brem, 1993). The fixation of anaesthetised rabbits in a head down position allowed the insertion of an embryo containing glass capillary via infundibulum into the ampulla under the guidance of a midline positioned laparoscope. Both, the head down position of the recipient animal and the placement of the laparoscope 1 to 2 cm cranial of the navel region were prerequisites for adspection of the reproductive organs, the evaluation of corpora lutea, and the deposition of the embryos on both sides using ≤ 1 µl medium without further instrumentation. The transfers resulted in a pregnancy rate of 86 % and an embryo survival rate of 47 %. Meanwhile this technique has become the method of choice for transferring one-cell to morula stage embryos into the oviduct. It is expected that this technique succeeds in pregnancy rates of 80 – 100 % and is minimally invasive for rabbits simultaneously offering complete optic access to reproductive organs. The transfer itself takes

1 to 2 minutes. Moreover, the transvaginal route for successful embryo transfer via the two cervices has been already demonstrated promising embryo transfer without anaesthesia (Besenfelder et al., 1998a).

IN VITRO PRODUCTION/ IN VIVO CULTURE

Oocyte collection following in vitro production represents a more exceptional kind of embryo production in rabbits. Remarkable efforts have been made in ruminants during the last two decades in developing techniques mainly for improving offspring production utilising the enormous follicle reservoir within the female ovary at different production and reproduction stages. In rabbits, however, the generation of offspring by in vitro production of embryos has not left beneficial impacts on economic development. These oocytes/embryos are of greater scientific-associated interests such as intracytoplasmic sperm injection, the use of semen sorting, cloning and transgenesis**.**

Very rarely embryos are produced in order to maintain individual animals, lines or strains which is underlined by the fact that, to date, oocytes were recovered after slaughter. The aspiration of the follicular content assisted by a needle allows a speedy oocyte collection, whereas follicle rupture is more time consuming but leads to a higher number of oocytes (Lorenzo et al., 1996a). In contrast to bovine species, the rabbit ovaries and consequently the follicular size favours the use of mincing ovarian tissues for oocyte recovery (Fukunary et al., 1990). The maturation status is achieved in vivo rather than in vitro. After stimulation of follicular growth by PMSG (Fukunary et al., 1990) or FSH (AL Hasani et al., 1984, Zeng et al., 1999) and application of ovulation inducing hormones oocytes can be collected prior ovulation (Al Hasani et al., 1984, 1986; Zeng et al., 1999) or following ovulation from the oviduct (Al Hasani et al., 1989; Zeng et al., 1999).

During in vitro maturation numerous factors affecting synchronous maturation of cumulus cells, ooplasm and nucleus must be provided by the artificial laboratory conditions. Lorenzo et al. (1996a) recovered oocytes from unstimulated rabbit ovaries and classified them according to the characteristics of the cumulus cells and the ooplasm into one of five groups. After having matured these oocytes for 16 h in Brackett´s medium 88 % of the oocytes surrounded by layers of cumulus cells and intact cytoplasm matured successfully, whereas cumulus-oocyte complexes of poor quality showed a remarkable decrease in maturation (Lorenzo et al., 1996a). Numerous factors are discussed affecting oocyte maturation such as growth hormone (GH), Insulin like growth factor I (IGF-I), epidermal growth factor (EGF), interleukin-1-beta (IL-1-beta), angiotensin II (Ang II) and prolactin (PRL). The ovarian exposure to GH resulted in follicular growth, oocyte maturation, increase of ovarian IGF-I and estradiol production as well as enhancing the effect of gonadotropins (Yoshimura et al., 1993, 1994; Ando et al., 1994). Moreover, Ang II (Yoshimura et al., 1996), IGF-I alone or with EGF (Lorenzo et al., 1996b), IL-1-beta in absence of an ovulatory gonadotropic trigger (Takehara et al., 1994) and PRL (Yoshimura et al., 1991) induced oocyte maturation. Lorenzo et al. (1996b) showed, that the stimulatory effect is only mediated when cumulus cells are present.

Extracorporal fertilisation has been developed to an effective and successful technique (Mills et al., 1973; Al Hasani et al., 1984, 1986, 1989). Mills et al. (1973) compared IVF oocytes removed either from ovarian follicles or from the surface of the ovaries with embryos derived from natural mating. The corresponding cleavage rates were 49.5, 73.5 and 95.9 %, respectively. They also succeeded in producing offspring, however, they noted a significant difference in the sex ratio (28 females/6 males). Additional efforts on cryopreservation of oocytes demonstrated, that IVF of frozen/thawed oocytes led to a high (74 %) fertilisation rate (Al Hasani et al., 1989). Highest implantation rates (78 %) following IVF were observed

when transfer was performed to recipients synchronised 6 h later than the donor rabbits from which oocytes had been collected (Al Hasani et al., 1986). It has to be taken into account that in vitro culture (IVC) enables long term development until morula or blastocyst stage (Al Hasani et al., 1984), however, crucial for implantation is the missing mucin production during the oviductal phase (see above).
Great attention has been paid to the rabbit oviduct for in vivo culture of embryos of foreign species due to high developmental demands on culture condition. Ectors et al. (1969) compared the in vitro culture of bovine embryos versus development in rabbit oviducts. In relation to ex vivo collected blastocysts (100 %), the numbers of cells in the blastocysts were highest in tubal cultured embryos (93.3 %) than in in vitro culture conditions (67.3 – 84.6 %). This phenomenon resulted in the multiple use of rabbits for in vivo culture for species such as goat (Agrawal et al., 1983; Rao et al., 1984a), buffalo (Ahluwalia and Majumdar, 1992), mice (Brinster and TenBroeck, 1969), cattle (Trounson et al., 1977; Sirard and Lambert, 1985; Wall and Hawk, 1988; Ellington et al., 1990) and pigs (Herrmann and Holtz, 1985; Hirst et al., 1981).

SEMEN SORTING/INTRACYTOPLASMIC SPERM INJECTION (ICSI)

In addition to IVP of rabbit embryos, this technique offers basic access to the use of X/Y-sorted semen as well as intracytoplasmic sperm injection.
Gender selection is a long standing tool in agricultural industries. The separation of sperms, carrying either the X- or Y-chromosome, is preferable used for offspring production rather than by reducing the number of embryo by manipulation and selection following molecular genetic analysis. Reasons for application of sex selection may be based on sex-linked genetic diseases, differences in metabolism and growth performance, and optimal sex ratio for efficient animal production. Numerous efforts have been made during the last century, of which only some are discussed for future importance like sperm separation on the basis of head volume (van Muster et al., 1999), immunological sperm sexing due to X- and Y-sperm specific protein on the cell surface (Blecher et al., 1999), selective enchrichment of sperm by free-flow electrophoresis (Blottner et al., 1994), and sex preselection by flow-cytometry (Johnson et al., 1989). Irrespective of the invasive treatment of sperm by using flow-cytometry technique it is the only one delivering sorted semen with precision and repeatability at various locations and with different species (Johnson and Welch, 1999). The difference in relative DNA content between X- and Y-chromosome bearing sperm (3.0 % for rabbits) is made detectable for separation by using a DNA bound dye highly permeable for sperm membranes. The original method provided a total of 350,000 sperm per hour. Following intrauterine insemination, born rabbits showed sex ratio of 94 % females and 81 % males (Johnson et al., 1989). Moreover, the technology has been improved in that way, promising a 30- to 60-fold improvement over the 1989 sorting technology using rabbit sperm (Johnson and Welch, 1999). The successful use of sexed sperm for in vitro production has been shown to result in a fertilisation rate of 57 % oocytes (McNutt and Johnson, 1996).
The fertilisation by sperm microinjection into oocytes is aimed at using male gametes available in very low numbers or of minor quality from low fertile/sterile male animals. First experiments were done in hamsters (Uehara and Yanagimachi, 1976) and mouse (Markert 1983) before microfertilisation experiments were performed in rabbits (Hosoi and Iritani, 1993). The sperm injection mechanically replaces the sperm penetration into ooplasm thus acrosome reaction, sperm motility and viability are not essential (see Goto, 1993).
Generally, two different methods related to placing of sperms are described. The zona pellucida represents a glycoprotein barrier which first has to be crossed before sperm interacts to the plasma membrane. Numerous trails have been performed to successfully bypass this

barrier (Yang et al., 1988; 1990; Minhas et al., 1991). In contrast to the subzonal mircoinjection technique where sperm have first actively to traverse the ooplasm, the injection of whole spermatozoa or sperm nuclei (heads) into the ooplasm is directly followed by decondensation and pronuclei formation (Keefer, 1989; Hosoi and Iritani, 1993; Escriba and Garcia Ximénez, 1998). Rabbit spermatozoa can be used either from the cauda epididymidis (Hosoi et al., 1988) or from ejaculated semen (Escriba and Garcia Ximenez, 1998). Using the latter technique, Escriba and Garcia Ximénez (1998) collected oocytes 14 h after hormonal induction of ovulation and inserted the spermatozoa into the oocyte after having gently ruptured the plasmalemma. After 24 h 45 % of the treated oocytes cleaved of which 56 % developed to morulae and blastocysts. The developmental competence of oocytes after ICSI for production of offspring was also shown (Hosoi et al., 1988). Both, the subzonal and the intra-cytoplamic sperm injection result higher fertilisation rates, when using capacitated sperm for injection (Keefer, 1989; Minhas et al., 1991).

FREEZING OF SEMEN AND EMBRYOS

The possibility to cryopreserve gametes and embryos extends the application of animal breeding in a wide range including genome cryobanking (Joly et al., 1996), save storage to an unlimited date, save of cages, time independent access to a greater spectrum of genomes, and protection against loss through diseases.

First attempts to freeze rabbit spermatozoa were made by Polge et al. (1949) using glycerol as a cryoprotectant. However, it was also shown to be toxic for the sperm. Polge´s experiments led to tremendous progress in semen cryopreservation. Meanwhile numerous methods exist, evaluating the use of different chemicals for its cryoprotectability. Stranzinger et al., (1971) have frozen semen in a tris-yolk and dimethylsulfoxide medium and reported that after thawing there was no difference in fertilising ability compared to fresh semen. Alternative cryoprotectants such as acetamides (Hanada and Nagase, 1980; Chen and Foote, 1994) and ethylene glycol (Fox and Burdick, 1963) also resulted in post thawed fertile sperm. Due to toxicity of concentrated single cryoprotectants further investigations aimed at combination of such chemicals. Detailed experiments were done by Dalimata and Graham (1997) who combined a cell permeating cryoprotectant with nonpermeating cryoprotectants. Ethylene glycol, dimethylsulfoxide and glycerol were found to be less favourable for sperm freezing. Using acetamide together with trehalose and methyl cellulose resulted in highest rate of motile sperm (46 %) and live acrosome-intact spermatozoa (53 %). This led the authors to conclude, that a combination of permeating and nonpermeating cryoprotectants were more effective in preserving rabbit spermatozoa than acetamide alone (Dalimata and Graham, 1997). Vicente and Viudes de Castro (1996) combined sucrose plus dimethylsulfoxide (1.75 M) and obtained a sperm motility rate of 42 % after thawing. However, separate handling of cryopreserved semen is needed to achieve satisfying results e.g. reduction of the time interval between insemination with frozen sperm and ovulation led to results comparable to fresh sperm (Parish and Foote, 1986). Ultrastructural analysis of spermatozoa, frozen in tris-DMSO-glycerol (Andrieu and Courot, 1976) or acetamide-raffinose (Chen et al., 1989), resulted in cellular damages mainly due to osmotic problems. Cellular damages appeared before and during freezing for the acetamide-raffinose-technique in membrane swelling and breakage, and during thawing for the tris-DMSO-glycerol-technique in membrane and acrosome damage (Courtens and Theau-Clement, 1996). To date, protocols available for semen cryopreservation, report contradictory results. Moreover, there is no unique technique allowing the application of insemination with cryopreserved semen in the open field (Castellini, 1996) except for the availability of cryo-semen from a commercial rabbit breed, of which both cryo-technique and commercial breed are unknown.

From the genetic point of view embryos are products of successful fertilisation and early development and represent individual completeness. Thus cryoconservation of embryos has aroused increasing interest.

Remarkable progress has been made in evaluating the effects of various cryoprotectants largely to the mechanical disruption of the intracellular architecture during freezing and thawing. Balancing main factors e.g. drop in temperature, ice crystal formation, and effects of solutions resulted in the establishment of basically different techniques. Besides conventional *slow freezing* techniques routine work focused on either *rapid freezing* procedures or on *vitrification*. As for sperm freezing, low molecular weight permeating (ethylene glycol, propanendiol, DMSO glycerol etc.), low molecular weight nonpermeating (carbohydrates such as galactose, sucrose, trehalose etc.), and high molecular weight non permeating (PVP etc.) cryoprotectants have been used. The different physico-chemical properties during freezing and thawing of each group meet the basic requirements for low temperature preservation (see Palasz and Mapletoft, 1996). **Conventional freezing** protocols frequently use only one cryoprotectant (Whittingham and Adams, 1976; Prins and Fox, 1984; Techakumphu et al., 1987b; Vicente and Garcia-Ximenez, 1993) whereas **rapid freezing** (Rao et al., 1984b; Renard et al., 1984; Nowshari et al., 1994; López-Béjar et al., 1996) or **vitrification** (Rall and Fahy, 1985; Rall, 1987; Kasai et al., 1992; Gajda and Smorag, 1993; Vicente and Garcia-Ximénez, 1994; Kauffman et al., 1998) favour media for preservation with more than one cryoprotectant. Synergistic effects of cryoprotectant ingredients on the basis of water replacement, dehydration or prevention of ice crystal formation allow their sub-toxic use for shortening the equilibration time to a maximum increase of cooling rate (see Palasz and Mapletoft, 1996).

Slow freezing protocols are adapted to low cooling rates (0.1 °C/min to 2 °C/min) including distinctive steps: initial room temperature at which embryos come in contact with the media followed by temperature around ice crystal formation (-5°C to –7°C), controlled cooling to ≤ minus 30 °C and finally plunging into liquid nitrogen (Prins and Fox, 1984; Techakumphu and Heyman; 1987; Siebzehnruebl et al., 1989; Joly, 1997) . Joly (1997), for instance, used a slow freezing method for preservation of embryos from various rabbit strains after superovulation. DMSO served as a cryoprotectant in which embryos were equilibrated in three steps (0.5 M, 1.0 M, 1.5 M). The embryos were loaded into a straw (0.25 ml), kept for further 5 min. at minus 7 °C and frozen to minus 30 °C (0.5 °C/min.) before plunging into liquid nitrogen. Among several strains one strain delivered (n = 72) 25.6 ± 2.2 morulae of which 74 % were suitable for freezing (class 1 and 2 embryos). Fifty six female rabbits received 566 embryos. Eighty four per cent got pregnant and delivered 215 (38 %) live pups (Joly, 1997). Slow freezing protocols, however, have the disadvantage that they are time consuming and require accurately controlled expensive freezing units.

The term *rapid freezing* implies faster freezing protocols which achieve comparable results to conventional freezing (Nowshari et al., 1993). The mixture of cryo-components enhance the freeze-tolerance of oocytes and embryos within a shorter time, allowing rapid freezing procedures obtained by direct plunging. Frequently used chemicals for successful rapid freezing are e.g. DMSO or propanendiol combined with sucrose (López-Béjar et al., 1996; Nowshari et al., 1994). The number of steps and temperature, where the embryos are held for a time are variable. Mostly, exposure of embryos was performed at room temperature, where embryos were transferred from –6 °C to –20 °C and –196 °C (Rao et al., 1984b), or put from –27 °C direct into LN_2 (López-Béjar et al., 1996) or directly plunged into LN_2 (López-Béjar et al., 1994; Nowshari et al., 1994). When López-Béjar et al., 1994 used the two step method for rapid freezing of 2-cell stage embryos up to early blastocysts they noted a post thawed in vitro development for all stages, however, best for morulae and blastocysts.

Undoubtedly, the short time exposure to highly concentrated aqueous solutions prior to plunging into LN_2 exerts maximum demands to embryos. This is achieved by creating a completely vitreous state i.e solutions become so viscous that they solidify without the formation of ice, termed *vitrification*. Cooling rates of about 2,500 °C/min necessitate the use of multimolar concentration of permeating cryoprotectants (see Rall, 1987). As a consequence this technique eliminates any injury caused by extracellular ice, however, factors mainly such as toxicity, fracture damage, intracellular ice, and osmotic swelling threatens successful preservation (Kasai, 1996). As shown in Table 2, different vitrification solutions resulted in an embryo development and birth of offspring comparable to non treated, cultured and transferred embryos.

Table 2: In vitro and vivo development of vitrified rabbit embryos

Method	vitrified embryos	embryos used for culture	development	Authors
EG/Ficoll/ Sucrose	131	120	78 fetuses or pups	Kasai et al., 1992
EG/Ficoll/ Sucrose	611	527	149 pups	Kauffman et al., 1998
Gly+PROH	100		85 blastocysts	Dobrinsky et al., 1990
	41		16 implantations--->13 pups	
Gly+EG/	351	279/138 for ET	221 exp. Bl/59 pups	López-Béjar and López-Gatius, 2000
EG+sucr.	325	239/140 for ET	183 exp. Bl/57 pups	
PROH+	105	at 1-cell stage	27 blastocysts	Gajda and Smorag, 1993
Gly	62	at 2-cell stage	22 blastocysts	
EG+	88	79	51 blastocysts	Vicente and Garcia-Ximénez, 1994, 1996
DMSO	229		135 pups	
	184		73 pups	

Throughout the different methods of cryopreservation it was shown that either oocytes up to blastocysts (Al Hasani et al., 1989; Gajda and Smorag, 1993; Dobrinsky et al., 1990), bisected embryos (Kobayashi et al., 1990) or cloned embryos (Rao et al., 1998) can be stored at –196 successfully**.** Finally, Vicente and Viudes de Castro (1996) noted that the viability of frozen-thawed embryos recovered from females inseminated also with frozen semen did not differ significantly from those inseminated with fresh semen. Although both, semen and embryo cryopreservation are performed successfully, the handling and sperm freezing is very simple whereas embryo freezing is complicated.

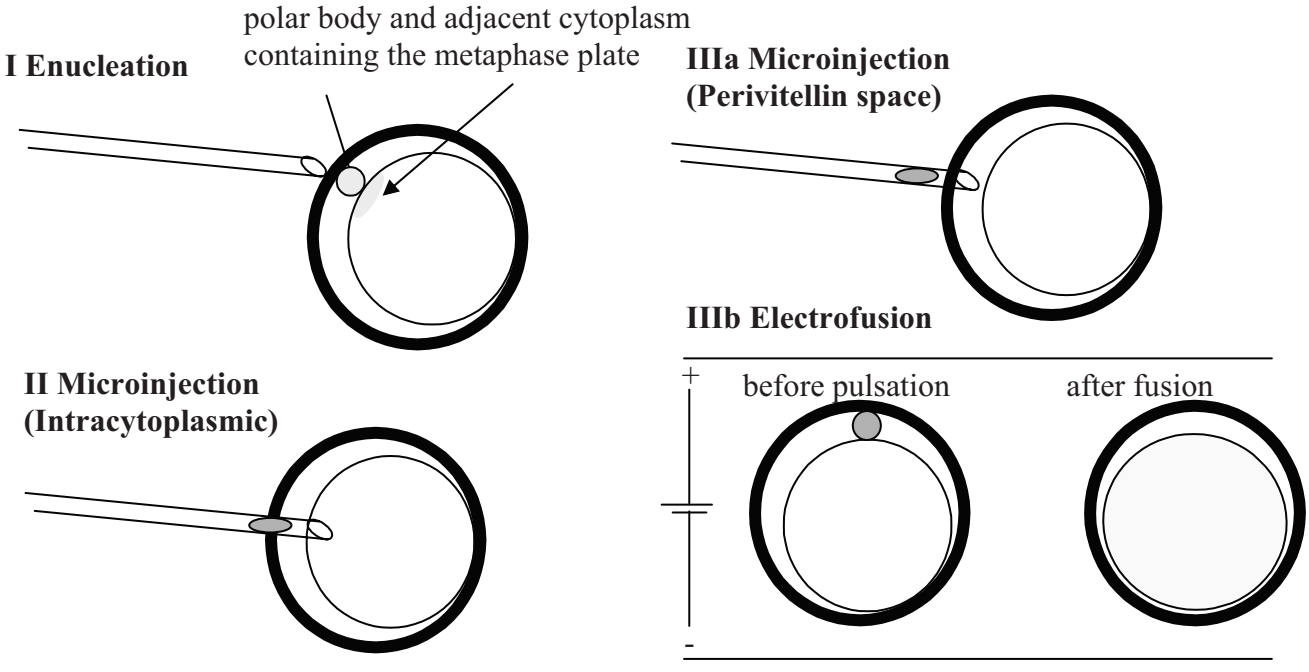
CLONING

1975 Bromhall described the successful nuclear transplantation in rabbit oocytes. Donor blastomeres obtained from morulae stage embryos were introduced into unfertilised rabbit eggs by microinjection. From nearly 600 nuclear transfer sessions more than 400 oocytes survived injection and finally 12 oocytes cleaved. These results led the author to discuss a somatic cell nucleus transplantation into an enucleated rabbit egg even 25 years ago. Presently, cloning is one of the subjects which dominates in the field of biotechnology. When using the term "clone" Campbell (1999) differentiates, that "..we must realise that the cytoplasmic contributions of the recipient oocyte will differ between animals, although the source of the donor nuclei may be identical. In addition, subtle alterations in the donor genetic material may also occur. Thus, offspring produced by nuclear transfer from somatic cells may be more aptly described as genomic copies...."

Among several nuclear transfer (NT) techniques such as virus induced and laser induced fusion, microinjection and electrofusion, the latter two are the most commonly used techniques (see figure 1). Nuclear transfer has been performed in numerous experiments in rabbits, in which a variety of different pairs of donor and recipient cells were reconstructed related to the developmental stage of the embryo, foetus or adult animal:

- embryo cloning
  - 8-cell stage embryos: (Collas and Robl, 1990)
  - 32-cell stage embryos: (Collas and Robl, 1990; Heyman et al., 1990; Adenot et al., 1997; Rao, 1998; Kang et al., 2000)
  - blastocysts (ICM, TE): Collas and Robl, 1991)
  - embryonic stem-like cells (Du et al., 1995)
- foetal cloning
  - foetal fibroblasts from D 17 fetuses: (Galat et al., 1999; Lagutina et al., 2000)
  - gonial cells (Moens et al., 1996)
- adult cloning
  - fibroblast cells adult rabbits: (Dinnyés et al., 1999; Mitalipov et al., 1999)

Figure 1: Nuclear transfer



Reconstructed oocytes must be artificially activated for their development. Activation methods include physical stimulation (e.g. electric current) as well as chemical means (e.g. ethanol, protein synthesis inhibitor, protein kinase inhibitor, strontium, calcium ionophore) to mime sperm penetration (see Hagemann et al., 1995; Kishikawa et al., 1999). However, incomplete induction of activation pathway leads to parthenogenones which arrest within the next developmental stages. Moreover, Mitalipov et al. (1999) demonstrated, that oocytes treated with tri-phosphated inositol followed by dimethylaminopurine (IP_3+ DMAP) cleaved parthenogenetically (83.9 %) and reached the blastocysts stage (50 %). Oocytes activated by ionomycin plus DMAP or multiple pulses cleaved (52.9 % vs. 61.6 %) and also developed to the blastocyst stage (5.7 % vs. 30.1 %). It has to be emphasised, that the only accurate measure of the efficiency of the NT process is the reproduction of live pups (Campbell, 1999).

This fundamental question has been proved using NT for cloning of embryos which led to the birth of pups (Collas and Robl, 1990; Heyman et al., 1990; Yang et al., 1991). It has already successfully been shown, that cloning and reprogramming of NT rabbit embryos which originated from the same donor embryos resulted in the production of multiple offspring (Heyman et al., 1990).

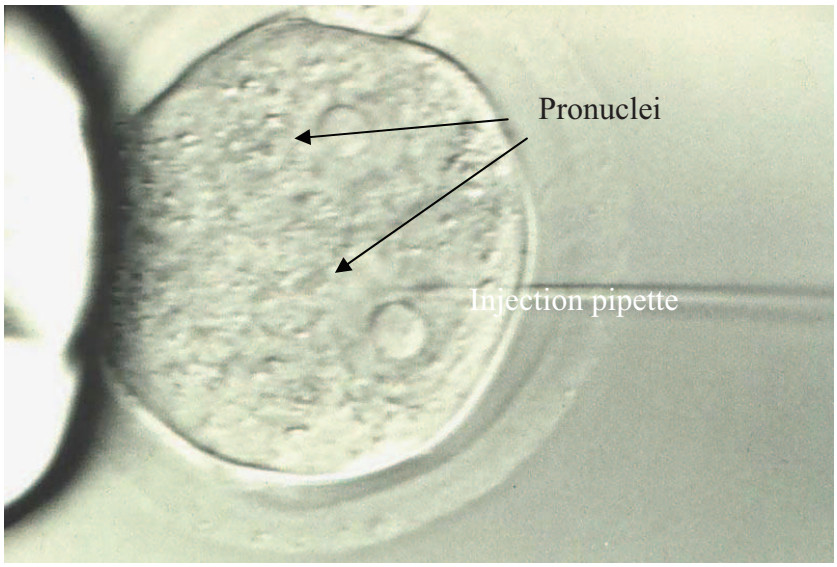
In addition, Rao et al. (1998) discussed the potential use of NT of embryos for production of large clone lines. After rabbit NT embryo production, embryos were frozen either by slow freezing or vitrification, thawed and subjected to NT again. Although the overall development was judged to being disappointing, it was concluded that recloning after cryopreservation is an excellent method for production of large numbers of genetically identical embryos. However, authors did not transfer cloned or recloned embryos to recipients.
In summary, offspring production by NT has proven, that genetic material is not irreversibly modified during differentiation and development. NT offers a powerful tool for numerous scientific as well as commercial aspects.

GENE TRANSFER

For many centuries rabbits have been used for both livestock production and animal experimental studies. Classical experimental purposes utilised them for antibody production, development of new surgical techniques, physiological studies e.g. circulation and blood pressure, and toxicity tests of new drugs. On the other hand rabbits are also important in livestock production for meat, fur, and angora wool production. Thus the ability to modify the genome of rabbits is of great interest technique for improving the performance and application in research and livestock production.
The generation of transgenic farm animals was first reported 15 years ago (Brem et al., 1985; Hammer et al., 1985). The gene transfers were carried out by microinjection of DNA constructs into pronuclei of fertilised oocytes (figure 2). In the past, this conventional technique represented the method of choice for generating farm animals (see Brem and Müller, 1994). The relatively low efficiency in creating transgenic farm animals, however, has limited its application. Recently, advances in the use of viral vectors, transformation of somatic cell lines following nuclear transfer and sperm mediated gene transfer in ruminants and pigs have entered a new era in transgene technology (see Chan, 1999; Piedrahita, 2000). Although there are many obstacles, progression in the field of production of transgenic rabbit can be expected in the near future. For microinjection, embryos have to be collected 19 to 21 h after insemination. Embryos are stored before and after manipulation is performed in PBS supplemented with 20 % calf serum. Zygotes of good quality are free of cumulus cells and

show bright cytoplasma with two easily detectable large pronuclei. About 80 to 90 % of them are suitable for microinjection. Embryo transfer into the oviduct via laparoscopy (Besenfelder and Brem, 1993) is preferred to surgical transfer (see above). Taking into account that microinjected embryos show a reduced viability (Brem et al., 1998), it is suggested to transfer additional non-injected (control) embryos of a different coat colour origin into the same recipient (Besenfelder et al., 1993). The efficiencies of gene transfer into mammals are measured by the overall efficiencies (transgenics/injected embryos transferred), the transgene integration frequency (transgenics/number of pups) and the survival rate (animals born/injected embryos transferred). For establishing transgenic lines necessarily a part of the germ line cells of the primary transgenic rabbits (founder) carry transgene copies. Transgenic founders harbouring cells with different genotypes are termed mosaics hence, the animals do not pass the transgene to their offspring at the expected rate of 50 % (Wilkie et al., 1986). Usually the hereditary mode of a transgene follows Mendelian rules.

Figure 2: Microinjection into pronuclei of fertilised oocytes



Current applications of transgenic rabbits are as follows (Besenfelder et al., 1998):

1. Gene farming – (mammary gland, blood system, bladder, seminal fluid)
    - Production of pharmaceuticals for human or veterinary medicine
    - Production of enzymes
2. Animal models for human diseases
    - Atherosclerosis
    - Tumorgenesis
    - Viral infections
3. Improvement of efficiency and quality in rabbit production
    - Meat production and growth performance

Despite the obvious benefits of transgenic rabbits for genetic engineering and disease models several problems remain to be solved. Undoubtedly, an exciting development will be the use of nuclear transfer techniques. The availability of this technique will give new impetus to gene transfer, because it will not only provide the possibility of additive gene transfer and homologous recombination but will also notably reduce problems such as low efficiency, non-expression of transgenes or insertional mutations.

CONCLUSION

The use of reproduction technologies in rabbits implies the gain of fundamental knowledge around reproduction directed towards its application and feasibility under field conditions. These techniques offer numerous possibilities especially for the maintenance of individual rabbits, lines or breeds, import of new genetic material under hygienic conditions and the delivery of gametes or embryos for various associated techniques. Based upon molecular information we are at the very early beginning of understanding gene activity and interactions, the introduction or loss of genetic information, and the totipotency/pluripotency of cells including the mechanism of differentiation and its reversibility. All together, these technologies can be of great value to agriculture and medicine and will be of immense benefit to mankind.

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