Improvement of female reproductive potential by *in vitro* embryo production technology

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One of the major factors limiting the genetic progress in cattle is relatively low female reproductivity (1 calf/cow/year). Increasing female reproductive performance (practically this concerns mainly cattle) allows to advance genetic improvement by a better use of female reproductive potential. The potential is determined during the fetal life in some species, e.g. primates, ruminants, or during the early neonatal period in others, e.g. rodents, rabbits (Erickson, 1966b; Hirshfield, 1991; Marion and Gier, 1971; Fortune, 1994) (Fig. 1). The decline in female germ cell number from the peaks typified in Fig. 1 involves oocyte atresia under differing circumstances. During the period of oogenesis, massive losses are incurred amongst oogonia in mitotic interphase and primary oocytes in meiotic prophase, especially at the stages associated with "crossing-over" (zygotene to diplotene). The steepest decline (e.g. between Days 130 and 170 of gestation in the cow; Fig. 2) is linked to both atresia and the cessation of germ cell mitosis.

Subsequently, when there are no oogonia left to degenerate, the atresia of oocytes settles down to a more gradual rhythm. Russe (1983) ascribes the loss of most germ cells to their continued maturation, after oocyte isolation, in an environment that is inadequate to support their development.

There is great variability between species, and between individuals within a species, as to the number of oocytes remaining at birth. The cow is born with an average of about 235 000 oocytes, but this figure has little meaning considering that the range in a group of 69 animals was from 0 to 724 000 (Fig. 1; Erickson, 1966b). Oocyte number continues to decline after birth and it is important to stress the variation from individual to individual; for example, Monniaux et al. (1983) found the number of follicles greater than 70 mm in diameter to range between approximately 50 and 900 per ovary in cows. Only 0.05% of these follicles reach the preovulatory stage of development. Similarly, an adult ewe may possess from 24 000 to 175 000 follicles containing oocytes, depending on the breed (Cahill et al., 1979). The understanding of the factors governing which oocytes and which follicles come to

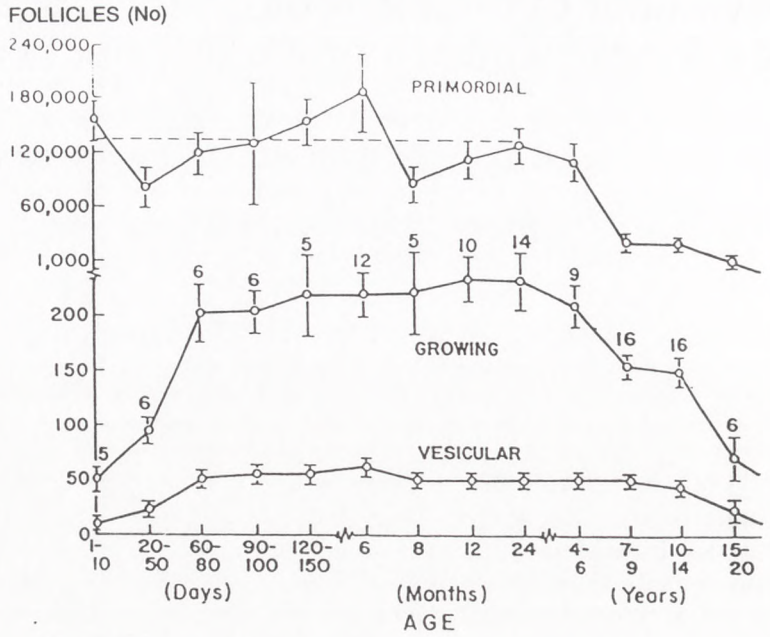

Fig. 1. Quantitative analysis of the follicles of the postnatal bovine ovary. Primordial — oocytes encompassed by a single layer of follicle cells. Broken line represents average for animals aged 0 to 24 months. Growing-follicles with two or more layers of follicle cells, but without a fully-formed vesicle. Vertical cars and numbers represent the standard error and number of ovarian pairs analyzed, respectively. Data from Erickson (1966b).

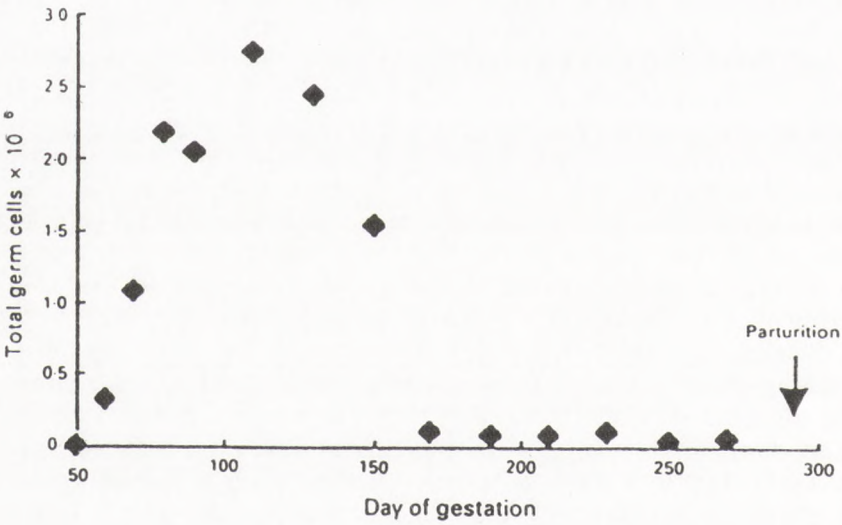

Fig. 2. Total numbers of germ cells in the prenatal bovine ovary at various stages of gestation. Data from Erickson (1966a).

fruition is at the core of all attempts to improve yields of viable eggs either during folliculogenesis or during oocyte maturation *in vitro*.

At birth, the vast majority of the thousands of primary oocytes in the ovaries are enclosed in primordial follicles, smaller than 0.06 mm in diameter, each consisting of an oocyte arrested in prophase I of meiosis and a single layer of granulosa cells. Once the cohort of primordial follicles has been established, follicles gradually and continually leave the resting pool to begin growth. The nature of the signals that initiate growth, and the mechanisms that ensure that follicles leave the resting pool gradually, are unknown (Turnbull et al., 1977; Cahill, 1982). Once a follicle begins to grow, growth seems to be continuous until the follicle meets one of two fates — ovulation or atresia. It is well known that very few follicles that begin to grow ovulate successfully; most die before reaching that stage. Follicular growth usually begins in a state of FSH and LH independence and passes through a stage of gonadotrophin sensitivity at preantral stages to one of acute hormonal dependence at the Graafian stage (Greenwald and Terranova, 1988).

It takes 6 months for a primordial follicle to reach preovulatory size in sheep, of which 4-5 months are spent in growing slowly to the size at which an antrum appears and 1-2 months in a more rapid terminal phase (Fig. 3; Scaramuzzi et al., 1993). Even in an adult female, the large growing follicles represent only a small fraction of the total population; in the ewe, for example, only about 10% of the 200-800 growing follicles exceed 1.1 mm in diameter to become visible at the ovarian surface (Cahill et al., 1979).

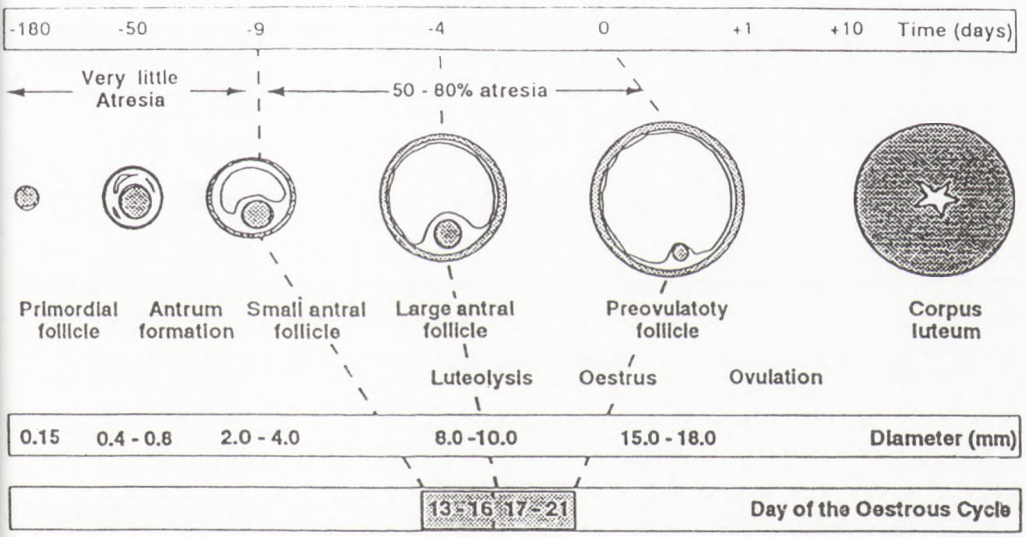

Fig. 3. The timing folliculogenesis based on information from sheep. A follicle takes an estimated 180 days to develop from a primordial follicle (0.15 mm diameter) to a preovulatory follicle (15 – 18 mm diameter). Data from Scaramuzzi et al. (1993).

The follicular antrum first appears in the cow in follicles ranging in diameter from 0.115 mm (when 10% possess an antrum) to 0.280 mm (by which stage 90% are antral) (Monniaux et al., 1983). In contrast to the oocytes of laboratory rodents, those of cows continue to grow and mature within follicles after formation of the antrum (Motlik et al., 1984; Motlik and Fulka, 1986).

There are three major factors affecting follicle abundance, namely age, species and stage of development. The consequence of a declining store of primordial follicles and a constant rate of recruitment into the growing population is that the number of follicles dwindle with age (Erickson, 1966b). Young ovaries are therefore a much richer source of follicles because they contain a higher density of follicles at most stages. Nevertheless, an evaluation of the reproductive ability of cattle of different age on the basis of the number of antral follicles (beyond 2 mm in diameter) and the quantity and quality of the recovered oocytes has shown that in 9-17-year-old cows there was only a slightly lower number of antral follicles than in heifers and 3-8-year-old cows (Tab. 1; Kątska and Smorąg, 1984). The recovery of a significant number of morphologically normal oocytes from older cows creates the prospect of utilizing these animals as oocyte, and consequently embryo donors, especially when they represent valuable breeds.

TABLE 1
RECOVERY RATE AND MORPHOLOGY OF BOVINE IMMATURE OOCYTES

Group of animals	No of animals	Follicle diameter (mm)	No of follicles examined (mean ± SD)	No of oocytes recovered (mean ± SD)	No of normal oocytes (mean ± SD)
Heifers	67	2 – 6	22.7 ± 12.6[a]	10.2 ± 6.2	4.6 ± 4.0
		7 – 20	1.2 ± 0.8	0.6 ± 0.7	0.1 ± 0.3
		>20	0.06 ± 0.24		
Cows	75	2 – 6	23.1 ± 14.0[a]	9.9 ± 6.2	5.2 ± 4.0
3-8 years		7 – 20	1.4 ± 1.0	0.8 ± 0.8	0.2 ± 0,5
		>20	0.3 ± 0.8	0.08 ± 0.38	0.03 ± 0.16
Cows	70	2 – 6	18.0 ± 10.8[b]	8.4 ± 5.9	3.9 ± 3.7
9-17 years		7 – 20	1.1 ± 1.0	0.6 ± 0.8	0.2 ± 0.4
		>20	0.4 ± 1.0	0.03 ± 0.17	

[a, b] Values within column with different superscripts differ (Duncan testy; $P < 0.05$)

Recently, two procedures for mass production of embryos for use in cattle breeding schemes are available. One consists of hormonal induction of multiple ovulation (MO) + artificial insemination (AI) + nonsurgical embryo collection + embryo transfer (ET) — this is the so-called MOET procedure. The other procedure is *in vitro* embryo production. It consists of oocyte re-

covery and *in vitro* maturation (IVM) + *in vitro* fertilization (IVF) + *in vitro* embryo culture (IVC).

Reduction of the generation interval is one of the means by which multiple ovulation and embryo transfer are already being used for genetic improvement of cattle. Superovulation reducing atresia increases the number of preovulatory follicles by the administration of gonadotrophins imitating the effect of follicle stimulating hormone (FSH). The procedure can be repeated about five times per year per animal. The yield of transferable embryos is highly variable and an average of not more than 25 embryos/year, i.e. 8 to 17 calves/donor/year can be obtained (Hasler, 1992; Kruip and Boni, 1994). In comparison to the natural reproductivity which may lead to 4 to 6 calves in a cow's life, MOET procedure distinctly improves female reproductive potential.

In spite of the fact that MOET has allowed to obtain more offspring and has found practical application in breeding programmes, the procedure has some limitations — namely the cost of production of such embryos (the cost of hormones) can be considerable and yield of embryos is highly variable.

Over the past decade, research has been focused on embryo production *in vitro* and has tremendously succeeded. The main source of oocytes used for culture studies is generally provided by the follicular oocytes (> 2 mm in diameter) collected from the ovaries of slaughtered donors — most of the time they are genetically of no interest for breeding. Recently live animals can also be used as donors of oocytes because a new technique for collecting oocytes from ovarian follicles more than 2 mm in diameter has been developed and is ready for practical application now (Kruip et al., 1994). This is the so-called Ovum Pick-Up technique by the transvaginal ultrasound guided puncture of ovarian follicles (Pieterse et al., 1988; 1991; Kruip et al., 1991). The puncturing technique is relatively easy and does not harm the animals, nor their genital tracts. It can be repeated twice a week during several months — this technique can yield more than 50 embryos in six months. This is four times more than the production *in vivo* by superovulation (Kruip, and Boni, 1994).

However, in order to reach high efficiency of the *in vitro* embryo production, a number of technical requirements must be met. Factors affecting this technology have been described as biological and environmental (Kątska, 1991). Bull variability has appeared to be one of the most important factors affecting the outcome of the *in vitro* embryo production. Results of IVF in cattle indicate that individual bulls differ in their contribution to both fertilization and embryonic development (Eyestone and First, 1989; Hillery et al., 1990; Barandi et al., 1993; Kątska and Ryńska, 1994). This means that a pre-test of the bulls on experimental oocytes has to be done in order to select the best semen donor (Tab. 2). From the practical point of view, development of methods allowing the use semen from unselected bulls for IVF seems to be a fundamental problem. Recently we have demonstrated that the removal of seminal plasma from bull ejaculate immediately after collection and before semen freezing significantly improved the *in vitro* fertilizability (Tab. 3). More-

over, it has been shown that frozen cauda-epididymal spermatozoa could be capacitated *in vitro* more easily and the yield of embryos was not as variable as for ejaculated sperm (Tab. 4).

TABLE 2
THE BULL EFFECT ON THE *IN VITRO* EMBRYO PRODUCTION EFFICIENCY

Bull	Oocytes n/Replicate	Fertilization rate	Cleavage rate	Blastocysts yield
Irak	145/4	71.3 (77/108)	49.0 (71/145)	9.6 (14/145)
Wolf	185/5	89.7 (166/185)	75.1 (139/185)	24.9 (46/185)
Irak + Wolf	768/13	82.1 (541/659)	71.2 (547/768)	20.7 (159/768)
Aldes	183/4	54.1 (99/183)	39.9 (73/183)	3.5 (5/143)
Rive	202/5	43.6 (88/202)	24.7 (50/202)	3.5 (7/202)
Stentor	131/3	77.9 (102/131)	66.4 (87/131)	22.9 (30/131)
Lizus	106/3	31.1 (33/106)	20.7 (22/106)	2.8 (3/106)
Narwik	1920/28	80.0 (1312/1639)	73.2 (1405/1920)	18.0 (346/1920)
Kurant	819/15	78.3 (409/522)	68.6 (562/819)	24.5 (201/819)
Lucek	289/8	61.6 (159/258)	53.3 (154/289)	13.1 (38/289)
Tancerz	154/4	not evaluated	13.6 (21/154)	2.6 (4/154)
Balzac	113/3	not evaluated	78.8 (89/113)	38.0 (43/113)
TOTAL	5015/95	72.9 (2909/3993)	64.2 (3220/5015)	18.0 (896/4975)

TABLE 3

THE EFFECT OF SEMINAL PLASMA ON THE *IN VITRO* FERTILIZABILITY OF BULL EJACULATED SPERMATOZOA

Bull	SP	Oocytes n/Repl.	Fertilization rate	Cleavage rate	Blastocysts/ cleaved eggs	Blastocysts yield
Luc	–	267/7	54.3 (145/267)	50.2 (134/267)	38.8* (52/134)	19.5 (52/267)
	+	254/7	59.0 (150/254)	54.7 (139/254)	24.5* (34/139)	13.4 (34/254)
Sten	–	139/3	91.4** (127/139)	79.1* (110/139)	35.5 (39/110)	28.1 (39/139)
	+	131/3	77.9** (102/131)	66.4* (87/131)	34.5 (30/87)	22.9 (30/131)
Riv	–	219/5	58.9** (129/219)	34.3** (86/219)	23.3 (20/86)	9.1* (20/219)
	+	202/5	43.6** (88/202)	24.7** (50/202)	14.0 (7/50)	3.5* (7/202)
Tan	–	158/4	not evaluated	44.3*** (70/158)	18.6 (13/70)	8.2* (13/158)
	+	154/4		13.6*** (21/154)	19.0 (4/21)	2.6* (4/154)
Total[1]	–	783/19	68.2 ± 20.2*** (401/625)	52.0 ± 19.2*** (400/783)	29.0 ± 4.8 (124/783)	16.2 ± 9.4*** (124/783)
	+	741/19	60.2 ± 17.2*** (320/587)	39.8 ± 24.8*** (297/741)	23.0 ± 8.8 (75/297)	10.6 ± 9.5*** (75/741)

[1] Data calculated for total included means ± S.D.
* $P < 0.005$; ** $P < 0.01$; *** $P < 0.0001$.

TABLE 4

THE *IN VITRO* EMBRYO PRODUCTION EFFICIENCY FOR FROZEN-THAWED CAUDA-EPIDIDYMAL SPERMATOZOA (EPI)

EPI donor	Oocytes n/Replicate	Cleavage rate	% blastocysts/ cleaved eggs	Blastocysts yield
1	2	3	4	5
A	78/3	85.9[a] (67/78)	38.8[a] (26/67)	33.3[a] (26/78)
B	294/4	80.9[ab] (238/294)	41.6[a] (99/238)	33.7[a] (99/294)
C	22/1	100[ac] (22/22)	31.8[ab] (7/22)	31.8[ab] (7/22)
D	157/3	68.1[d] (107/157)	30.8[ab] (33/107)	21.0[b] (33/157)

1	2	3	4	5
E	137/5	83.9[ab] (115/137)	43.5[a] (50/115)	36.5[a] (50/137)
F	176/4	78.4[ab] (138/176)	37.0[a] (51/138)	29.0[ab] (51/176)
G	124/3	64.5[d] (80/124)	45.0[ac] (36/80)	29.0[ab] (36/124)
Total[1]	988/23	80.2 ± 11.8 (767/988)	38.3 ± 5.5 (302/767)	30.6 ± 5.0 (302/988)

a, b, c, d Values within column with different superscripts significantly differ (x^2; $P < 0.05$).
[1] The results are presented as the mean ± S.D.

Finally, a very crucial point is the culture system after fertilization. Several systems have been developed (Kątska et al., 1995). In our protocol, the embryos are co-cultured with bovine oviduct epithelial cells in Menezo B_2 medium up to the blastocyst stage.

In spite of some limitations, large-scale *in vitro* production of cattle embryos is a reality for the cattle industry. Although the viability of the *in vitro* produced embryos is slightly lower than that of *in vivo* produced embryos, the number of calves that can result from both techniques is 40 versus 17.5 calves, i.e. more than twice as much.

The main advantage of the *in vitro* technology is the potential of producing a large number of embryos. There are numerous benefits of this technology from commercial point of view. The beef industry could fully exploit the reproductive performance of females by obtaining twins in larger numbers at a lower cost than with superovulation. The dairy industry could also have some interest in the *in vitro* produced beef embryos; these embryos could be transferred into animals of low genetic value or those destined for AI with a beef bull. In this case farmers could obtain a purebreed calf instead of crossbreed. In breeding programmes embryos of valuable breeds obtained by *in vitro* technology at a competitive cost represent a viable alternative to MOET for many farmers. *In vitro* produced embryos and *in vitro* matured oocytes have already been used in experimental embryology, i.e. in embryo cloning experiments (Heyman et al., 1994). Moreover, zygotes obtained by *in vitro* technology can be used for microinjection of DNA in programmes of transgenic animal production.

Both discussed methods of improving female reproductive potential allow to use only a tiny part of a high follicular capital of the ovary. As was indicated at the beginning of this presentation, bovine ovary contains many thousand of primordial follicles. Since the large majority of ovarian oocytes undergo atresia (> 99.9%), the possibility of resquing them before they degenerate seems to be very attractive. Recently, it has become possible to grow

preantral follicles from mouse ovary to Graafian sizes during the course of a week of culture (Eppig and Schoeder, 1989; Gosden et al., 1993). Few attempts have been made to apply these culture methods to other species, i.e. rat (Daniel et al., 1989), rabbit (Maresh et al., 1990), cat (Jewgonow and Pitra, 1993), pig (Hirao et al., 1992) and cow (Jewgonow and Pitra, 1991; Nuttinck et al., 1993; Figueiredo et al., 1994). Results of these experiments indicate that there are several problems to be solved before achieving this goal. The major problem in the culture of preantral follicles is maintenance of oocyte viability. Secondly, there is the problem of sluggish growth rates in these species. Finally, we do not expect methods that are designed for small and medium-sized follicles to be appropriate for all stages. It is important to bear in mind that it is very difficult to precisely imitate physiological conditions *in vitro*, therefore the ultimate aim of culturing preantral follicles to maturity will require much effort and is unlikely to be achieved in the near future. Nevertheless the effort will be immensely worthwhile. The ability to culture ovarian follicles from the primary stage to the preovulatory stage, followed by the techniques already available for use in *in vitro* embryo production would offer a significant means for the propagation of valuable animal stocks because it would supply a large and uniform population of oocytes from genetically superior animals. Thereby it would be possible to overcome one of the most important factors limiting genetic progress in cattle.

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Summary

Two procedures for mass production of embryos for use in cattle breeding schemes are now available, namely multiple ovulation + embryo transfer and more recently *in vitro* embryo production. Hormonal treatment for multiple ovulation, nonsurgical embryo collection and embryo transfer are widespread techniques to obtain more offspring from genetically superior cattle (MOET program). However, the costs can be considerable and the yield of embryos is highly variable. Research during the past decade has been focused on embryo production *in vitro* with oocytes from slaughterhouse ovaries and has tremendously succeed. Presently ultrasound guided technique for oocyte collection in living animals has been developed. However, both discussed procedures allow to use only a tiny part of a high follicular capital of ovary. Bovine ovary contains many thousants of primordial follicles, but the vast majority become atretic during growth and maturation. Recently is developed a technique for *in vitro* growth of preantral follicles rescuing them against atresia. It would offer a significant means for the propagation of valuable animal stocks, and would be an addition to the methods already available for use in embryo production *in vitro*, since it would supply a large and uniform population of oocytes from genetically superior animals.

Key words:
cattle, embryo production *in vivo* and *in vitro*.

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