Factors affecting the outcome of *in vitro* bovine embryo production using ovum pick-up-derived cumulus oocyte complexes

Merton, Johan Sybrand
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Factors affecting the outcome of *in vitro* bovine embryo production using ovum pick-up-derived cumulus oocyte complexes

Factoren welke het resultaat beïnvloeden van de *in vitro* productie van runderembryo's uit ovum pick-up gewonnen cumulus-eicel-complexen
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, Prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 25 maart 2014 des middags te 2.30 uur

door Johan Sybrand Merton, geboren op 23 februari 1965 te Vianen

Promotor:	Prof. dr. T.A.E. Stout
Co-promotoren:	Dr. P.L.A.M. Vos Dr. B.A.J. Roelen

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Contents

Chapter 1

General Introduction

This introduction is based on the original publication: Merton[1a] JS, de Roos[1] APW, Mullaart[1] E, de Ruigh[1] L, Kaal[1] L, Vos[2] PLAM, Dieleman[2] SJ. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry.

[1] *Holland Genetics, PO Box 5073, 6802 EB Arnhem, The Netherlands*
[2] *Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands*

Theriogenology 2003;59:651-674.

1 Introduction

Implementation of embryo technologies in cattle such as for example Multiple Ovulation and Embryo Transfer (MOET), Ovum Pick Up followed by *In Vitro* embryo Production (OPU-IVP) and embryo biopsy followed by Marker Assisted Selection (MAS), is strongly driven by the desire of the breeding industry to enhance genetic improvement in dairy and beef cattle. The production of large numbers of calves from cows of high genetic merit facilitates an increase in the selection intensity and a shortening of the generation interval, two important factors affecting genetic gain (Fig. 1). In order to increase the likelihood of producing highly valuable bulls, breeding companies are willing to invest in these types of technologies.

$$\text{GENETIC GAIN} = \frac{\text{Selection accuracy} \times \text{Selection intensity} \times \text{Variation} \times \text{Heritability}}{\text{Generation interval}}$$

Fig. 1: Formula for genetic gain.

Non-surgical embryo recovery procedures combined with non-surgical transfer techniques and embryo cryopreservation methods became available in the early 1980s and have been used world-wide ever since. An average of 6-7 transferable embryos per flush are normally recovered. Assuming 10 flushes per year, this yields to a mean of 60-70 freezable embryos per donor per year, and results in approximately 35 calves. Not surprisingly, these embryo transfer (ET) techniques were rapidly accepted by the cattle industry. The introduction of OPU-IVP techniques followed in the late 1980s and became commercially operational in the early 1990s. The advantages of OPU-IVP over MOET are obvious. More embryos can be produced per unit time. On the basis of two OPU sessions per week, production can reach about 150 embryos per year resulting in approximately 70 calves. Furthermore, each batch of collected oocytes can be fertilized with sperm from a different bull, which increases the number of genetic combinations that can be used. Moreover, embryos can also be produced during the second and third months of pregnancy. The Dutch-Flemish cattle breeding company CRV has applied this technology to their female breeding line (Fig. 2) since 1996 to produce embryos and the resultant calves. The top bull-dams are selected when the first milk production data of performance tests becomes available and their first calves are now born

shortly after to start the next breeding cycle. This procedure leads to a marked shortening of the generation interval, and therefore results in higher rates of genetic improvement.

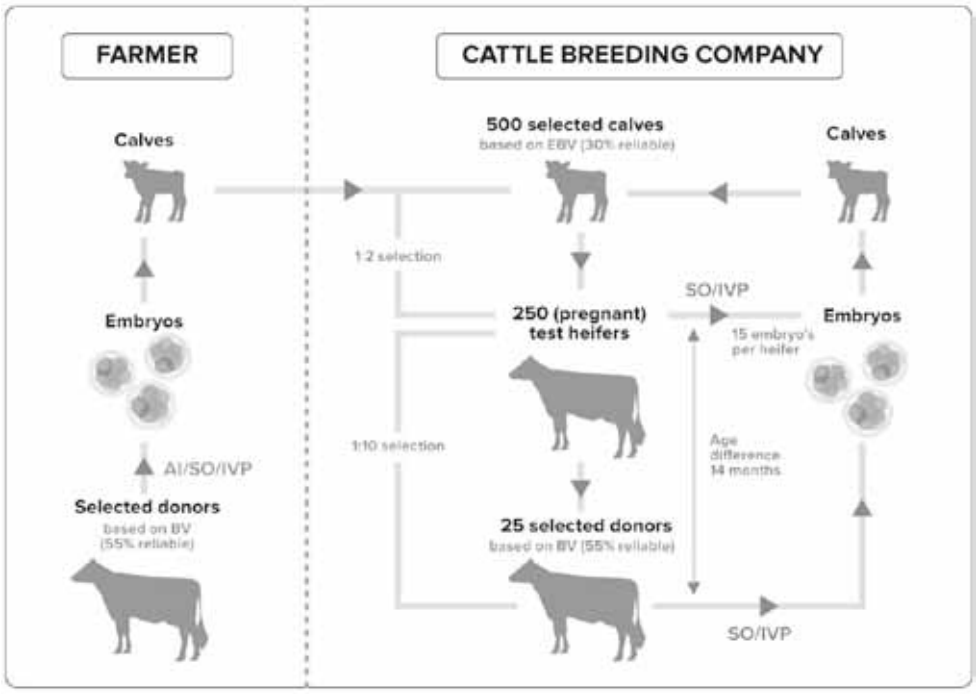

Fig. 2: Use of super ovulation (SO) and ovum pick-up followed by in vitro embryo production (OPU IVP) in an open breeding program. AI: Artificial insemination; (E)BV: (estimated) breeding value.

The application of OPU-IVP does, however, also have some disadvantages. Abnormalities of both fetal and calf development and health have been reported in cattle and sheep [1-5], and the pregnancy rate after transferring IVP embryos is lower than that for MOET. This phenotypic phenomenon, often referred to as 'Large Offspring Syndrome' (LOS), is thought to be correlated with perturbed epigenetic reprogramming during early development, which is responsible for abnormal DNA methylation and consequent abnormal expression of imprinted genes [6-10], but appears also to be related to (pre-) maturation processes of oocytes [4,11]. Although large calves are still being reported, LOS is less of a practical concern since it was established, for both cattle and sheep, that a change from a co-culture/serum system to a synthetic oviductal fluid (SOF) system, prevented many of the described problems [4,12,13].

However, epigenetic modification during early embryonic and fetal development, and the subsequent phenotypic consequences is still a subject of considerable research interest with huge potential practical implications to assisted reproduction.

In the last decade, MAS (e.g. 40 markers) quickly evolved towards genomic selection (GS) (5K–50K single nucleotide polymorphisms (SNPs)/markers). With the introduction of GS the cattle breeding industry acquired relatively accurate breeding values (50-70% reliability compared to the 30-35% reliability of the estimated breeding value based on parents' pedigree) at a relatively early age (i.e. at birth, or even at the embryo stage), creating a genetic selection opportunity that is economically more desirable than waiting for cattle to reach sexual maturity. The possibility of selecting calves for likely genetic merit at the time of birth offers the opportunity to enhance selection intensity since large numbers of young calves can be genotyped and selected based on relatively accurate genomic estimated breeding values (GEBV), with only a relatively small investment (Table 1). Subsequently, GS also creates an opportunity to decrease the costs of a breeding program by lowering the number of heifers selected for performance tests, since the data of these tests no longer significantly increase the reliability of the breeding value estimation (Table 1). The minimum size of the breeding programs can now be determined primarily by the inbreeding coefficient in order to assure sufficient genetic variation in the next generation. Finally, the introduction of GS has created a market for even more extensive utilization of OPU-IVP, in order to increase the number of embryos and offspring per selected heifer.

Table 1: Example of the impact of genomic selection on selection intensity, accuracy and size (number of animals) of the breeding program.

Breeding/selection cycle steps		Breeding program[1]	Breeding program + GS[2]	Impact on	Magnitude
1	Calves	n= 500	n= 1500	Program size	3.0x
	Reliability Breeding Value	EBV 30%	GEBV 60%	Selection accuracy	2.0x
2	Virgin heifers performance test	n= 250	n= 100	Program size	- 2.5x
	Selection factor	1:2	1:15	Selection intensity	7.5x
	Embryo production per virgin heifer	15	45	Selection intensity	3.0x
3	Donor/bull dam	n= 25	n= 10	Program size	2.5x
	Selection factor	1:10	1:10	--	--
	Reliability Breeding Value	BV 55%	GBV 60%	Selection accuracy	1.1x

[1] (E)BV (estimated) breeding value;

[2] GS genomic selection; G(E)BV: Genomic (estimated) breeding value.

2 Oocytes at different stages of follicular development as the starting material for IVF embryo technologies

Increasing the number of offspring per donor might overrule biological mechanisms in reproduction. In a normal bovine estrous cycle, one naturally-selected follicle matures and ovulates, producing an oocyte with optimal developmental potential. Embryo technologies, however, utilize oocytes that originate from follicles of different sizes and stages of the follicle developmental process (Fig. 3). It is obvious that the heterogeneity of these oocytes will lead to suboptimal embryo production compared to the natural situation.

2.1 Follicular and oocyte development during the estrous cycle

The follicle wave. The bovine estrous cycle consists of two or three follicular waves each of which is preceded by a small rise in the serum FSH concentrations resulting from the disappearance of inhibitory feedback when a dominant follicle ovulates or when a potentially ovulatory, dominant follicle undergoes atresia [14]. This rise in the FSH concentrations initiates the growth of a group of gonadotrophin-dependent follicles of ≥ 2 mm in diameter, emerging from a pool of gonadotrophin-responsive follicles; only the most advanced of these follicles will emerge concomitantly with the increase in the FSH level [15]. The first wave starts at Day 1 of the cycle, in which estrus is defined as Day 0. During the next 3 days the initial follicles, and others which reach the 2 mm stage while FSH is still elevated, continue to grow, giving rise to a population of 4-8 mm follicles at Day 3-4 [16,17]. At this stage, called deviation, one of the largest follicles starts to grow faster than the rest and becomes the dominant follicle or the future pre-ovulatory follicle (growth phase), whereas the remainder become subordinate [16]. This 'selection' of dominant follicles is based on a number of mechanisms: 1) functional hierarchy among follicles, 2) FSH concentration threshold levels of gonadotrophin-dependent follicles, 3) negative feedback at the pituitary level on FSH secretion by inhibin and estradiol secreted by the largest follicle, 4) vascularization of the largest follicle and 5) acquisition of LH responsiveness by the granulosa cells in the largest follicle, which allows these follicles to switch from FSH to LH dependence. As a result, small functional pre-existing differences among recruited follicles of a wave can be accentuated by the hormonal environment, resulting in distinct selection of a dominant follicle and atresia of the remaining follicles of the wave [18]. At Day 6 the dominant follicle reaches its maximum size of 15-20 mm and remains functional for another 2-4 days (dominant phase). When the

dominant follicle of the first wave loses its functionality (regression phase), a new follicular wave emerges. Luteal regression allows the dominant follicle of this second or third wave to stay functional and is accompanied by increased frequency of the pulsatile release pattern of LH, which ultimately results in the preovulatory LH surge. The LH surge initiates final follicular and oocyte maturation and ovulation of the dominant, preovulatory follicle. All other follicles are subordinate and do not reach a stage at which they can ovulate. Instead, they undergo atresia, which is a process of degeneration, and ultimately will regress [19]. In relation to the stage of the follicle wave in which follicles reach FSH-responsiveness, atresia occurs either in growth-arrested or in follicles that initially grew before becoming subordinate [19,20]. The period from atresia to regression takes around 1 to 2 weeks, which results in a population of ovarian follicles of which up to 85% may be atretic, at any given time in the estrous cycle [21].

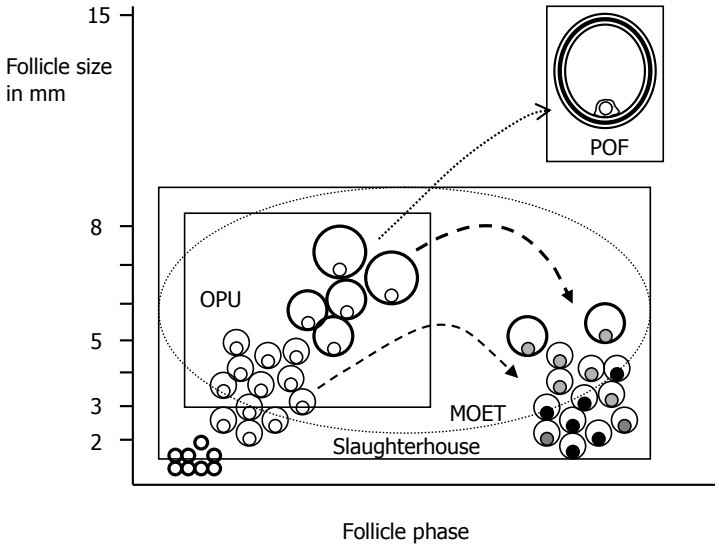

Fig. 3: The origin of the oocyte in different embryo production systems: OPU (Ovum Pick-Up=area in small rectangle); IVP with slaughterhouse oocytes (=area in large rectangle); MOET (Multiple Ovulation and Embryo Transfer=area in oval); POF (preovulatory follicle of normal estrous cycle). Follicles with blank oocytes are non-atretic; with grey are early atretic and with black are atretic.

Oocyte growth and maturation. Oocytes increase in diameter within follicles of up to 3 mm in diameter. As follicle diameter increases from 3 to 10 mm, the oocyte ceases to increase in size. During this period of growth arrest, rRNA and mRNA transcription is minimized and growth of the oocyte is essentially complete [22,23]. Additional oocyte growth accompanied by an increase in transcriptional activity, occurs in follicles of 10-15 mm in diameter [22,23]. During this second phase of oocyte growth, developmental processes occur which are referred to as pre-maturation or capacitation and are necessary for growing oocytes to achieve developmental competence, a biochemical and molecular state that allow normal fertilization, normal pre-implantation embryonic development and subsequent healthy growth of the growing embryo to term [20,24,25]. Multiple studies have described ultra-structural changes in the oocyte, including changes in Golgi complexes, cortical granules, the nuclear membrane, peri-vitelline space and positioning of the surrounding corona cells [24,26]. The pre-maturation processes also include mechanisms by which mRNAs and proteins are processed to prevent degeneration during storage in the oocyte [27]. Sub-optimal storage of these messages may affect oocyte developmental competence, primarily because transcription must cease for several cell cycles once the program of nuclear maturation begins [28]. Mourot et al. [29] showed that the relationship between oocyte mRNA storage and follicle size is not general but works through chronologically specific steps that depend on the gene involved. This observation supports the concept of an important role for final differentiation of the follicle during which specific mRNAs are produced that probably continue to accumulate after the oocyte has reached its final size [30]. Furthermore, various paracrine factors, metabolites and regulatory molecules (e.g. cyclic AMP), delivered by the cumulus cells via gap-junctions, are required for the oocyte to acquire full developmental competence within the follicle [15].

During subsequent final maturation, the oocyte nucleus resumes meiosis and progresses to the metaphase II stage; this is accompanied by several changes in cytoplasmic organization including lipid storage, alterations to Golgi complexes, migration of mitochondria and cortical granules, retraction of the transzonal processes from the corona cells and expansion of the cumulus cell mass [24,26,31-33]. Besides these ultra-structural changes, stage-dependent protein synthesis have been observed throughout maturation in oocytes cultured as cumulus oocytes complexes (COCs) [34,35], suggesting that cumulus cells are essential, especially in the first hours of oocyte maturation [36, 37].

Various research groups have investigated the expression of different growth factors and their receptors in the oocyte and cumulus/granulosa cells at different stages of oocyte and follicle

development [38-42]. In this respect, significant changes have been reported in follicles >8 mm, such as expression of LH receptors by granulosa cells, a decrease of IGF-binding proteins, an increase of IGF-I and PAP-A enzyme activity within the follicular fluid and increased expression of growth factors like TGFβ, activin, and inhibin. All these factors are involved in the complex regulation and modulation of gonadotrophin-stimulated folliculogenesis as recently reviewed by Scaramuzzi et al. [15].

Follicle-oocyte interactions. The relationship between oocyte and follicle was traditionally viewed as a passive oocyte in which growth and development are dictated by the endocrine system and the follicular somatic cells. However, this perspective has changed. Oocyte secreted factors (e.g. GDF9 and BMP15) are now known to regulate several important functions of granulosa and cumulus cells [43,44], mainly during the gonadotrophin-dependent phase of folliculogenesis [45]. The ability of the oocyte to control cumulus cell development and to maintain a regulatory loop with these cells is likely to be a critical developmental function [45].

Oocyte *in vitro* developmental competence. Oocyte quality is often defined as the competence to develop into a blastocyst within an *in vitro* production system (IVP). This definition dictates that developmental competence depends not only on the intrinsic quality of the oocyte, but is also related to laboratory specific factors such as IVP procedures, semen used and oocyte collection method (slaughterhouse vs. OPU). Some almost fully-developed oocytes from 3 mm follicles are already competent to undergo (*in vitro*) final maturation, fertilization and development to the blastocyst stage, indicating that *in vitro* developmental competence has already been acquired by oocytes at this stage. It should be mentioned however, that when oocytes are removed from the antral follicle before ovulation, the separation from the follicle wall itself triggers a pseudo-maturation event leading to completion of the first meiotic division and continuing to arrest at the metaphase II stage. This process is often called 'spontaneous maturation'. One can imagine that if the oocyte has not completed its final cytoplasmic transformation at the initiation of spontaneous maturation, it may have impaired developmental capacity. In general, oocytes progressively acquire developmental competence (the proportion of oocytes achieving competence) during follicle growth although some studies have shown equal developmental competence of oocytes collected from small or large follicles [17,38,46-49]. For example, Hagemann et al [17] found no difference in blastocyst rates between oocytes collected from 3-5 mm compared to those

from 6-8 mm follicles. However, they also showed that oocytes collected from presumptive dominant follicles (> 13mm) yielded a significantly higher blastocyst rate than oocytes obtained from 3-8 mm follicles, i.e. during the growth phase of the dominant follicle.

Developmental competence of oocytes becomes compromised when derived from atretic follicles. However, COC quality is only affected relatively late in the atretic pathway [21,49,50]. Remarkably, early atresia appears to have a positive effect on developmental competence [48-50]. This may be partly explained by the similarity in ultra-structural changes observed for oocytes undergoing pre-maturation and those entering early atresia [26]. Furthermore, some of the changes associated with early atresia mimic pre-ovulatory changes, such as a rise in follicular progesterone and a decline of 17β-estradiol as a result of the down-regulation of aromatase activity, and a progressive decrease of follicular support from the granulosa cells, which may be perceived by the oocyte as signals of impending ovulation [51,52].

2.2 The origin of oocytes in OPU-IVP

In clinical OPU-IVP, the follicles from which oocytes are harvested is determined by the resolution of the ultrasound transducer used (follicles > 2 mm in size) and the between OPU session interval. In general, with OPU performed twice a week without FSH pre-stimulation, the population of COCs generated for OPU-IVP is more homogeneous that that for MOET, as a result of the repeated sessions that eliminate both dominant and atretic follicles. Production of embryos from slaughterhouse ovaries starts with COCs collected from follicles at every possible phase of development and from all sizes visible on the surface of the ovary (Fig. 3). In contrast to MOET, COCs collected by OPU originate from follicles that lack dominance and have not undergone final preovulatory development. Furthermore, the process of final maturation has to take place *in vitro*.

In contrast to the worldwide uniform classification system used for bovine embryo evaluation (IETS system), classification systems for both slaughterhouse- and OPU-derived oocytes are much more laboratory specific. Oocytes are classified on the basis of morphology of the ooplasm and/or cumulus investment. The number of categories within a classification system may vary from 3 to 6 and, within each system, some correlation is generally found with developmental competence. For example, Wurth and Kruip [50] classified COCs into 3 classes; A: compact and bright cumulus; B: slightly expanded and darker cumulus and C: strongly expanded with degeneration of cumulus cells. With the exception of oocytes from late atretic follicles, class B oocytes resulted in higher blastocyst rates than class A oocytes.

Blondin and Sirard [48] also reported significantly better *in vitro* development using class 3 COCs (showing signs of early expansion in the outer cumulus layer and with a slightly granulated ooplasm), compared to morphologically better (class 1 and 2) or worse qualified oocytes (class 4 – 6). This further supports the suggestion that early signs of atresia have a positive effect on oocyte developmental competence.

In order to monitor the performance of the OPU-teams within CRV with respect to the evaluation of COCs, oocytes are classified solely on the appearance of the cumulus investment. Class I COCs are those completely surrounded by a compact, spherical-shaped cumulus investment, and class III COCs are defined as those in which the oocyte is completely denuded of its cumulus investment. All remaining COCs with a(n) (incomplete) cumulus investment with morphological characteristics between class I and III are defined as class II, while COCs with an expanded/degenerated cumulus are classified under class IV. The quality of the OPU-derived COCs markedly affects the *in vitro* embryo production rate (Fig. 4). Class I COCs resulted in a blastocyst rate of about 30%. Interestingly, this was independent of the number of oocytes recovered and processed as a batch during the IVP procedure. The blastocyst rate for class II COCs was significantly lower and was dependent on the number of oocytes collected per session (range: 6.2 to 18.4% for 1 to 10 COCs per batch). This group-effect has also been reported by others [49,53-55] and is based on reciprocal stimulation between co-cultured oocytes and embryos. On the basis of these results, to maximize the number of embryos produced, CRV uses all types of COCs collected from each donor and processes them as one batch, including class III (denuded) and class IV (degenerated/expanded) COCs.

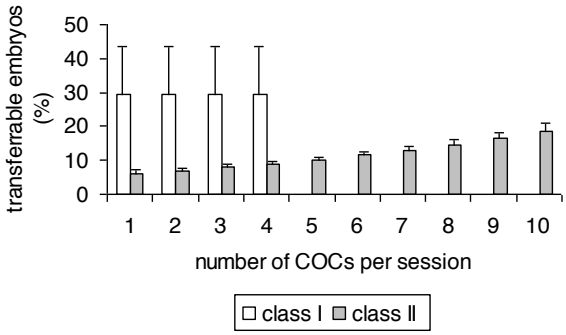

Fig. 4: Percentage of transferrable embryos per quality and number of OPU-derived COCs. Data are based on sessions in which only one type of oocyte was present; class I, *n*=61 sessions; class II, *n*=511 sessions. Data analyzed by logistic regression; mean values ± S.E.M.

Surprisingly, the distribution of classes may differ between OPU- and slaughterhouse-derived COCs, with more class I COCs recovered from the slaughterhouse group. Mullaart et al [55] showed that this difference was not related to the OPU equipment or OPU collection medium. It was suggested that, in the slaughterhouse group, the COC becomes less tightly connected to the follicle wall as a result of post-mortem changes and is therefore be collected with a more complete morphology. Other groups have also described a post-mortem effect [57,58]. For example, embryo production was doubled when ovaries were kept at 30^0C for 4 h instead of 2 h. Possibly the post-mortem effect initiates atresia which positively affects oocyte developmental competence [57]. This observation is presumably one of the reasons that better IVP results can be obtained with slaughterhouse-derived oocytes compared to those obtained by OPU. However, a lack of oocyte selection and forced use of donors selected primarily for genetic merit (i.e. of variable parity and fertility status, including the use of MOET problem donors) in most OPU-IVP programs may also contribute to the lower results obtained with OPU.

Differences between OPU- and slaughterhouse-derived COCs can also affect the outcome of IVP studies. For example, we reported [58] a significant increase in blastocyst formation rate at Day 8 when Menezo-B2 medium was used for *in vitro* culture of slaughterhouse-derived oocytes compared to TCM199 culture medium (31% and 22%, respectively). However, when this comparison was made with OPU-derived oocytes, no difference was detected (Table 2). This inconsistency is difficult to explain, but may be related to the presence of pyruvate and

lactate in M-B2 medium only. One may speculate that, as a result of the post-mortem effect (i.e. less tight attachment to the follicle wall) there is less support from the granulosa and cumulus layers; combined with subsequent *in vitro* maturation, this could result in a shortage of pyruvate and lactate within the slaughterhouse-derived zygote compared to an OPU-derived zygote. Since pyruvate and lactate are essential energy sources during the first days of embryo development [60-62], presence of pyruvate in M-B2 may compensate a deficiency of slaughterhouse-derived zygotes.

Table 2: Effect of culture media on oocytes of different origin and on in vitro embryo development.

Culture medium [1]	Slaughterhouse			OPU		
	Oocytes (n)	Cleavage (%)	Blastocysts Day 9 (%)	Oocytes (n)	Cleavage (%)	Blastocysts Day 9 (%)
TCM199	10118	62[a]	26[a]	1939	57	21
Menezo-B2	5653	73[b]	36[b]	1640	56	22

[1] Oocytes from both slaughterhouse (control IVP monitoring) and OPU (production) were processed simultaneously

[ab] Within columns values with different superscripts differ significantly (Chi-square analysis, $P<0.05$)

3 Procedural OPU factors that affect oocyte quantity and quality

Implementation of embryo technologies is primarily driven by the need to increase the number of offspring from genetically valuable animals. Therefore, the number of embryos per session is an important parameter reflecting the success of the procedure. However, as MOET and OPU-IVP are commonly used in the cattle breeding industry, improvement of current procedures with regard to both time and costs is also important. For example, reduction of the number of steps at which oocytes and embryos are manipulated, simplification of labor intensive methods and an increase in the number of oocyte batches per production step (e.g. fertilization) are measures that can lead to a reduction in the costs of embryo production. In short, not only production characteristics, but also financial efficiency, determine the value of embryo production technologies in a commercial breeding enterprise.

Session interval. The time interval between OPU sessions influences both the quality and quantity of oocytes. After OPU most follicles > 2-3 mm in diameter will have been removed, which results in removal of FSH suppression and allows induction of a new follicle wave and hence the growth of a fresh batch of follicles of 2-3 mm over the following days. A

significantly higher total number of COCs is collected when a 7 day interval compared to a 3 or 4 day interval is used between OPU sessions [30](Table 2). However, the quality of the COCs in terms of cumulus investment morphology is highest for the 3 day and lowest for a 7 day interval. Blastocyst rate and embryo developmental stage were also significantly affected (19.7 vs. 13.5% for 3 vs. 7 day intervals, respectively). This finding is in agreement with the hypothesis that a dominant follicle exerts a negative, suppressive effect on the developmental competence of the oocytes from smaller follicles, since a dominant follicle will emerge approximately 3 days after OPU. In contrast to the commonly used 3 and 4 day OPU scheme (Monday and Thursday), OPU at 2 and 5 day intervals (Monday and Wednesday) did not affect the number of oocytes collected per session. However, COC quality was higher in the 2 day interval and reflected in a higher blastocyst production rate (Table 3; personal communication, Saner and Le Gal, SVKD, Switzerland), which again may be attributed to the dominant follicle effect.

In order to increase the number of oocytes recovered over a set time-period, a scheme with OPU conducted 3 times a week (Monday, Wednesday and Friday) may be effective. Although cost efficiency will be influenced negatively, the number of embryos produced per time interval will probably be increased.

Table 3: Effect of interval between OPU sessions on oocyte quantity and quality and subsequent in-vitro embryo production rate.

Scheme	Interval in days	Sessions [1] (n)	Oocytes per session (n)	Oocyte quality index [2]	Embryos/oocytes [3] (%)
3 – 4[A]	3	516	7.2[a]	0.66	19.7[a]
	4	502	6.6[b]	0.30	17.1[b]
7[A]	7	48	9.1[a]	0	13.5[c]
2 – 5[B]	2	259	3.9	1.29 [a]	14.1 [a]
	5	259	4.0	0.97 [b]	10.5 [b]

[A] Data adapted from Hanenberg and van Wagtendonk-de Leeuw [63]; Holland Genetics

[1] Oocyte results of sessions conducted at one OPU location

[2] Index presented on a continuous scale (ordinal threshold model) with the quality of the 7 days interval as reference. The quality of a batch is calculated as the average score of oocyte quality; classes I, II, III and IV scoring 3, 2, 1 and 0, respectively. The index for the 3 days interval was higher than for the 7 days interval ($P<0.001$)

[3] Results of sessions conducted at three OPU locations (842, 778 and 90 sessions for 3, 4 and 7 days intervals, respectively); transferable embryos at Day 7 of IVP at the morula up to hatched blastocyst stages (IETS quality 1and 2)

[abc] Values within a column with different superscripts differ significantly (regression analysis, Genstat statistical program; $P< 0.05$)

[B] Unpublished data from Saner R and Le Gal, F (SVKB, Switzerland)

[2] Oocyte quality calculated as the number of COCs collected with quality grades 1 and 2

[3] Transferable embryos at Day 7 and 8 of IVP at the early blastocyst stage up to expanded blastocyst stage (IETS quality 1and 2)

[ab] Values within a column with different superscripts differ significantly (Chi-square analysis, $P<0.05$)

FSH pre-stimulation. Several groups have reported an overall beneficial effect of FSH pre-stimulation prior to OPU on the developmental competence of COCs. This has been achieved using FSH stimulating procedures which varied widely with regard to the interval between final administration of FSH and oocyte retrieval, and the time interval between OPU sessions. Blondin et al [64] described an optimum with respect to oocyte competence when the period between pre-stimulation with pFSH and oocyte retrieval was extended to 48 h. In addition to allowing growth of the cohort of stimulated follicles to beyond 5 mm, the post-FSH "coasting period" has been proposed to be essential for allowing changes to take place within the COC that corresponded to those that take place during early atresia, and resemble those occurring during pre-maturation. Bousquet et al. [65] applied this treatment in a commercial situation once every 2 weeks and reported an average number of 4.7 embryos per collection, which was similar to the average number of embryos per flush (4.3) obtained with a conventional MOET super-stimulation treatment. Goodhand et al. [66] reported an increase in embryo production

rate from 22% to 39% (embryos per oocyte used) after using a stimulation protocol for 3 days with declining FSH dosages. De Roover et al. [67] also found that FSH pre stimulation prior to OPU increased production efficiency, with significantly more follicles punctured and oocytes retrieved. However, when overall results during 2-week periods were considered (four non-stimulated sessions vs. one stimulated), oocytes were aspirated from a larger number of follicles and a significantly higher number of oocytes were retrieved using the non-stimulated protocol. No significant differences in the number of cultured embryos were detected, however, indicating that FSH/LH stimulation prior to OPU is beneficial for *in vitro* oocyte developmental competence because more embryos results from fewer oocytes. A positive effect of FSH pre-stimulation was also found in the CRV herd (Table 4). Although FSH treatment resulted in a higher number of COCs retrieved and embryos produced per session, the total production of embryos per 2-week period was higher with the standard twice-weekly sessions than with the FSH protocol (6.0 and 4.0 vs. 3.3 embryos per 2 weeks, respectively).

Table 4: Effect of FSH pre-stimulation on OPU-IVP results.

Period[1]	FSH [1] treatment	Oocytes / session (n)	Cleavage (%)	Embryos / oocytes [2] (%)	Embryos / session (n)
1	none	7.8 [a]	60.4	19.9	1.5 [a]
2	+	13.2 [b]	65.6	26.0 [a]	3.3 [b]
3	none	5.9 [c]	58.5	16.3 [b]	1.0 [c]

[abc] Values within a column with different superscripts differ significantly (regression analysis, Genstat statistical program; $P < 0.05$).

[1] Data adapted from De Ruigh et al [69]. In period 1 and 3 (control; 10 to 15 sessions in total) OPU was carried out in 5 first parity donor cows 2 times per week (Monday and Thursday). In between, in period 2 (pre-stimulation; 6 sessions) OPU was carried out once every 2 weeks following FSH administration in 4 equivalent doses 72, 60, 48 and 36 h prior to OPU (total of 10 ml Ovagen im; ICP, Auckland, New Zealand; 176 IU NIH-FSH-S1). In period 2, progesterone (PRID; Intervet International B.V., Boxmeer, The Netherlands) was applied for 7 days after each OPU session.

[2] Transferable embryos at Day 7 of IVP at the morula to the hatched blastocyst stage (IETS quality 1and 2)

Recently, the importance of the post-FSH "coasting period" was investigated by comparing the transcriptome of germinal vesicle-stage-oocytes collected from FSH-stimulated cows after coasting periods of various duration to determine which transcripts were accumulated or depleted during the rise and fall of oocyte developmental competence (optimal conditions were 44 and 68 h; under-maturation was evident at 20 h; and over-matured at 92 h). The results indicated that the 'quality' gained by using the optimal coasting interval does not last,

suggesting a possible mechanism of control by transcript degradation that could be implicated if the oocyte is not ovulated at the right time [68].

OPU team. The success of techniques involving people are usually affected by differences in the performance of those individuals. In this respect, the OPU technique is usually performed by either one or by two persons. At CRV, 8 teams have been in practice for 2.5 years using the same OPU protocol (with regard to e.g., vacuum pressure, needle diameter). The composition of the teams has varied, which allows elucidating both the effect of the technician trans-rectally manipulating the ovary and the assistant performing the actual follicle punctures. Both the technician and the assistant significantly affected the quantity of COCs collected (Figure 5). The variation among the group of technicians was larger than among the assistants, which is probably due to the leading role of the technician in determining the strategy of the team during the OPU session. Therefore, in an embryo technology program with only one or two OPU technicians, the overall results of OPU-IVP will be determined to a substantial extent by the OPU team.

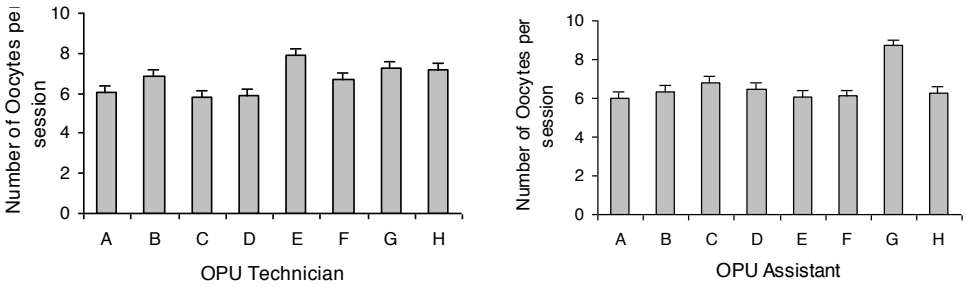

Fig. 5: Effect of OPU team (technician and assistant) on the number of oocytes per collection session. Data based on 7,800 OPU sessions ranging from 419 to 1580 for a technician and 399 to 1466 for an assistant. Data analyzed with SAS statistics program (P<0.1); means ± S.E.M.

Oocyte collection technique. The number of oocytes collected per ovary or animal is dependent on the performance of technicians and the collection technique used. At CRV the number of class I and II oocytes aspirated from the slaughterhouse derived ovaries of culled cows averaged 32 per animal (5,191 oocytes from 162 animals), while the number of oocytes recovered during OPU averaged 8 (23,130 from 2,926 sessions). This difference is primarily a

factor of the limited resolution of the OPU ultrasound transducer (range 6.5–7.5 MHz) compared to the human eye. When oocytes were aspirated from ovaries of cows that had been slaughtered immediately after the OPU session, an average of 12 extra oocytes per cow were collected in addition to the 6.7 oocytes that had already been retrieved by OPU (Table 5). The embryo production rate tended to be higher for oocytes collected by OPU from follicles of 2 to 10 mm than for oocytes from smaller follicles left over after OPU. It can be concluded that an improvement of the resolution of the OPU ultrasound transducer may facilitate the collection procedure of COCs from smaller follicles. However, the resolution of OPU transducers appears to function as a filter promoting collection of only fully-grown oocytes and allowing smaller follicles another extra 3 to 4 days to reach a size in which a higher proportion of oocytes will have reached the stadium of an improved developmental competence. Of course, even with the limited resolution, other technical factors are of significant importance, such as vacuum pressure, needle length, needle tip and type of transducer [70,71] and have to be taken into account in order to obtain optimal results.

Table 5: Effect of follicle size on in-vitro embryo production rate when using OPU followed by aspiration of the remaining oocytes after slaughter of the donor.

Oocyte collection method per animal (n=6)	Follicle size (mm)	Total number of Oocytes	Cleavage n (%)	Blastocyst [1] n (%)
In vivo by OPU	2-10	40	33 (82.5) [a]	15 (37.5)
Ex vivo by aspiration	1-2	72	31 (43.1) [b]	15 (20.8)

[1] All blastocysts at early up to hatched stage present at Day 8 of IVC

[ab] Values within a column are significantly different (Chi-square analysis, $P<0.05$).

4 Improvement of the OPU-IVP program

The outcome of MOET and OPU-IVP sessions has remained relatively constant for some years. Minimizing the interval between sessions might optimize the efficiency of MOET, but in general there has not been a major breakthrough in the number of viable embryos produced per session [72]. Similarly, in the field of IVP there have been no substantial improvements during the last decade. One has to mention however, that a comparison among laboratories is particularly difficult with regard to blastocyst formation rates. Differences in the source of ovaries (fertility status of donors, breed), oocyte collection procedures, selection of oocytes and zygotes, the reference bull used for IVF and the culture system used do influence the outcome and are the likely cause of the large differences between laboratories with regard to

the slaughterhouse-IVP results, which range from 15% to 60% blastocyst rate at Day 7 of *in vitro* culture (IVC). OPU-IVP results however, seem to be less variable, ranging from 10% to 30% transferable embryos from oocytes processed, although the definition of transferable embryos may also vary due to differences in interpretation and procedures used among laboratories (embryo stage, fresh / frozen, Day 7 / Day 8).

Since the introduction of IVP, the IVC step has been studied intensively, resulting in stable culture conditions capable of supporting early embryonic development at a rate similar to that observed in *in vivo* (Fig. 6). Many laboratories have changed the co-culture based IVC system, largely because it is thought to be at least partially responsible for the LOS [4,12,13], to a system based on SOF. The SOF system has become (more or less) the standard for IVC in commercial bovine embryo production programs. Further improvement of early embryonic development *in vitro* is likely to come from changes to the earlier steps of the embryo production process. For example, studies investigated the effects on early embryonic development of replacing the successive *in vivo* steps in the development process by *in vitro* conditions. It is evident that alongside fertilization, oocyte growth and maturation are the most critical factors influencing the outcome of *in vitro* embryo technologies (Fig. 6).

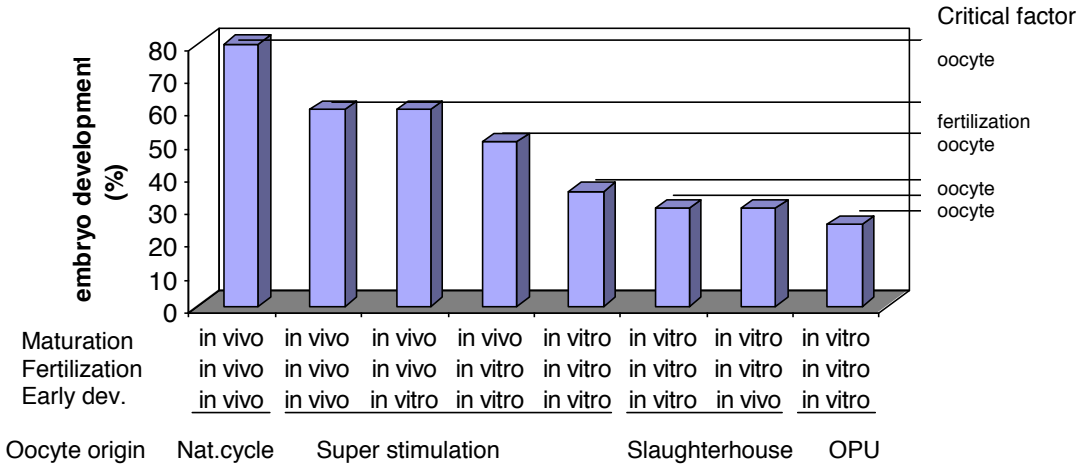

Fig. 6: Effect on embryo production of the oocyte origin and whether the successive steps of oocyte maturation, fertilization and early embryo development were performed *in vivo* or *in vitro*. The source of the respective decreases is indicated at the right of the figure.

In natural estrous cycles of healthy cattle, approximately 80% of ovulated oocytes develop *in vivo* to the transferrable embryo stage following fertilization *in vivo*. In a study by Boerjan and Merton (unpublished data), 11 out of 14 normal non stimulated cyclic cows (78%) that had been (single) flushed between Day 6 and 9 after AI produced an embryo that developed to at least the morula stage. After superovulation, approximately 60% of the ovulated oocytes develop to similar staged embryos (unpublished data CRV). Moreover, when such *in vivo* matured and fertilized oocytes were cultured *in vitro*, no decrease in embryo production efficiency was observed [73,74]. Similarly, no effect of *in vitro* culture vs. *in vivo* development has been reported for slaughterhouse-derived oocytes after IVM/IVF [75]. On the other hand, it appears that *in vitro* fertilization does compromise embryo production compared to the *in vivo* situation when the oocytes are recovered after FSH stimulation [73,74]. It should, however, be noted that the *in vivo*-matured / *in vitro*-fertilized oocytes were recovered from preovulatory follicles shortly before ovulation. Due to the spread in ovulation times after superovulatory treatment [76,77], some oocytes may not have fully completed maturation at the time of collection [78], which may contribute to the lower blastocyst yield. A relatively large decrease in embryo production rate occured when superovulation-derived oocytes were collected just after the LH surge and were matured *in vitro* in standard medium as opposed to being matured *in vivo* [73,74,79]. This finding emphasizes the importance of oocyte growth and maturation in the acquisition of developmental competence. Similarly the differences between the *in vitro* embryo production rates for immature oocytes derived either after OPU, superovulation and OPU or from slaughterhouse ovaries, indicate the importance of the pre-maturation process. It can be inferred that the techniques for oocyte of retrieval and subsequent treatment are crucial in determining the outcome of embryo production in the commercial application of embryo technologies. In summary, in order to achieve a significant improvement in both the efficiency and efficacy of an OPU-IVP program, studies should focus on the influence of oocyte (pre-) maturation on embryo production efficiency and on embryo culture with respect to the effect on embryo quality. Furthermore, research should not be limited to non-genetic factors. It is surprising that, although there are some results from MOET programs, information regarding genetic factors that do predict outcome of OPU-IVP programs is rather limited. Such an approach does, however, have the potential to yield significant improvements in the efficiency of an OPU-IVP program by selecting donors with high genetic merits for *in vitro* production suitability. Machado et al. [80] studied the variability of OPU results and *in vitro* embryo production from monozygotic twin cows. A substantially less variation was found within twin-pairs than among non-related cattle,

suggesting the presence of a genetic component and the heritability of traits associated to IVP succes. This observation is in agreement with CRV's unpublished findings, which showed less variability of OPU results and *in vitro* embryo production within full-sib heifers than among non-related animals.

5 Goals

The main goal of this thesis is to identify factors which enhance the efficiency and/or efficacy of the OPU-IVP program. Since embryo production efficiency is mainly determined by the quality of the matured oocyte, research focused on the *in vitro* maturation process, and more specifically, on the effect of 1) the composition IVM medium and 2) the duration of the maturation culture period. Since embryo quality is mainly determined during early development, studies also focused on the *in vitro* culture period, and more specifically, the culture environmental atmosphere. Finally, besides the non-genetics factors, research focused on the genetic factors that may influence the suitability of individual cows for OPU-IVP.
Importantly, the research was not limited to slaughterhouse-derived COCs but also included a commercial OPU-IVP program. Furthermore, to elucidate possible changes in the cryotolerance of in vitro produced embryos, post-transfer embryonic survival and calf characteristics, embryo transfer experiments were performed to provide more concrete evidence of ultimate success.

6 Outline of the thesis

In **Chapter 2**, the optimal maturation culture period for OPU-derived COCs was studied in terms of embryo production, embryo cryotolerance, post-transfer embryonic survival and calf characteristics. **Chapter 3** covers the effect of cysteamine supplementation during IVM and IVC on embryo production and embryo cryotolerance, including a comparison between slaughterhouse- and OPU-derived oocytes. In addition, the effect of cysteamine supplementation during IVM of OPU-derived oocytes on the post-transfer embryo survival and on calf characteristics including gestation length, birth weight, perinatal mortality and sex ratio was studied. In **Chapter 4** the effect of an intra-incubator carbon-activated air filtration system (CODA; <genX> International, Inc.) during IVC of *in vitro* matured and fertilized COCs on embryonic development and the subsequent pregnancy rate is described. **Chapter 5** describes the genetic factors influencing the outcome of OPU-IVP. Heritabilities and genetic

correlations are reported for COC quality and quantity, the number and proportion of cleaved embryos at Day 4, and total and transferable embryos at Day 7. In addition, the estimated breeding values for the number of COCs and proportion of transferable embryos are compared with the sires' breeding index for female-fertility in order to gain insight into the relationship between OPU-IVP traits and female fertility in general. Finally, in **Chapter 6** the major findings of this thesis are summarized and both their immediate implications for the cattle breeding industry and what it means in terms of future research directions are considered.

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Chapter 2

Bovine OPU-derived oocytes can be matured *in vitro* for 16-28 h with similar developmental capacity

Merton JS, de Roos A, Koenen E, Roelen B[1], Vos P[1], Mullaart E, Knijn H.

CRV BV, PO Box 454, 6800 AL Arnhem, The Netherlands

[1] Dept. of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands.

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Abstract

The aim of this study was to determine the optimal maturation culture period of ovum pick up (OPU)-derived cumulus oocytes complexes (COCs) in relation to their developmental capacity. Embryo production, embryo cryotolerance, post-transfer embryonic survival and calf characteristics such as gestation length, birthweight and sex ratio were investigated. This retrospective study covers the analyses of ovum pick up –*in vitro* production and calving results from a commercial program that took place between March 1994 and September 2004. Donors were both heifers (of which approximately 90% pregnant) and cows (of which approximately 10% pregnant). Embryo production analyses were based on 7800 OPU sessions conducted from January 1995 until January 1999. Analyses of calving rate were based on 13 468 embryo transfers performed during January 1995 until May 2002. Analyses on calf characteristics were based on 2162 calves born between March 1994 and September 2004. The *in vitro* maturation culture period ranged from 16 to 28 h. The mean production rate of transferable embryos was 16.5% (1.2 embryos per OPU session). Length of maturation culture period did not affect the production of transferable embryos. Mean calving rate was 40.9% and 38.7% for fresh and frozen/thawed embryos, respectively. Calving rate was not affected by the maturation culture period. Mean birthweight, gestation length and proportion of male calves were 46 kg, 281.9 days and 52.8%, respectively. Maturation culture period did not affect these variables. In conclusion, this study shows that the *in vitro* maturation culture period within the range of 16–28 h does not affect *in vitro* embryo production, embryo cryotolerance, post-transfer embryonic survival and calf characteristics, suggesting that all COC batches collected by OPU on the same day, can be fertilized in one IVF session without a significant loss in the production from oocyte to calf.

Keywords: Bovine; Calf characteristics; Maturation; Oocyte; OPU-IVP

1 Introduction

The success of an ovum pick up –*in vitro* production (OPU-IVP) embryo production program is determined by the number as well as the quality of the cumulus oocytes complexes (COCs) collected. It is well established that the embryo production rate relates to the intrinsic quality of the oocyte and the conditions during maturation, whereas the quality and the ability of an embryo to establish a pregnancy are more related to the conditions during early development (Merton et al. 2003; Lonergan et al. 2006). Therefore, optimal timing of COC collection and optimal conditions during IVM are key to improve the *in vitro* development of oocytes. Factors that affect *in vitro* maturation of bovine oocytes include time and temperature between slaughter and COC aspiration (Yang et al. 1990; Blondin et al. 1997) size and morphology of aspirated follicles (De Loos et al. 1991; Lequarre et al. 2005; Hendriksen et al. 2000), oocyte diameter and morphology (Fair et al. 1995; Bruynzeel et al. 1997; Hyttel et al. 1997; Hagemann et al. 1999) and composition of maturation medium, including hormones and serum (Zuelke and Brackett 1990; Bevers et al. 1997; Avery et al. 1998; Watson et al. 2000; Lonergan et al. 2006).

Duration of the maturation culture period is also an important factor with respect to *in vitro* embryo production. Embryo production rate is optimal with a maturation culture period varying between 18 and 24 h and with significantly lower blastocysts rate when this maturation culture period is exceeded (Ward et al. 2002; Merton et al. 2003; Park et al. 2005; Agung et al. 2006). These maturation culture periods seem to agree with the kinetics of bovine IVM, whereas in general, after 16–20 h most of the oocytes reached metaphase II stage of nuclear maturation (Lequarre et al. 2005; Agung et al. 2006). Exceeding this optimal maturation culture period will lead to impaired developmental competence. Although fertilization of oocytes may still be possible, development will be impaired because of ageing of the oocyte (Hunter 1989; Hunter and Greve 1997). Ageing is thought to be related to instability of nuclear and cytoplasmic organelles. Furthermore, inappropriate timing of maturation can also lead to a shifted sex ratio of embryos produced *in vitro* (Gutierrez-Adan et al. 1999; Park et al. 2005; Agung et al. 2006).

It is important to notice that in the majority of the studies described previously, results are based on slaughterhouse-derived COCs. Although several aspects of *in vitro* maturation have also been studied in relation to OPU-derived COCs, specific studies on the optimal maturation culture period are limited. It is well known that the origin and quality of the COC population are determined by the oocyte collection technique used. In case of OPU in a twice weekly

system, most of the COCs will originate from growing, healthy follicles 3–8 mm in diameter in the absence of a dominant follicle. In contrast, as animals brought to the slaughterhouse can be in any stage of the oestrous cycle, slaughterhouse-derived COCs are retrieved from a larger range of follicle sizes and at all possible stages of the follicle wave, including follicles present during the dominance phase or in their regression, atretic phase of development. Whether the optimal maturation culture period determined for slaughterhouse-derived COCs can be applied to OPU-derived COCs has to be elucidated. Moreover, in most studies described, observations were limited to the embryo production level. However, to elucidate possible changes in the cryotolerance of embryos, post-transfer embryonic survival and calf characteristics, embryo transfer experiments provide more conclusive results. It has been shown for instance that epigenetic events early in live may have long-term consequences and even be expressed only later in life (Tarin and Gomez-Piquer 2002; Thompson et al. 2007).

The aim of this retrospective study was to determine the optimal maturation culture period of OPU-derived COCs in relation to not only embryo production but also embryo cryotolerance, post-transfer embryonic survival and calf characteristics such as gestation length, birthweight and sex ratio.

2 Materials and methods

2.1 Data set

This study covers the analyses of OPU-IVP and calving results from a commercial OPU-IVP program. Oocyte donors were both heifers (of which approximately 90% pregnant) and cows (of which approximately 10% pregnant).

Embryo production analyses were based on 7800 OPU sessions conducted from January 1995 until January 1999. Analyses of calving rate were based on 13 468 embryo transfers conducted from January 1995 to May 2002. Analyses on calf characteristics (gestation length, birthweight and sex) were based on 2162 calves born during the OPU-IVP program from March 1994 to September 2004.

2.2 Oocyte collection and in vitro maturation

Immature COCs were recovered twice weekly, on Monday and Thursday, by ultrasound-guided transvaginal oocyte collection. Donors were housed at different collection centres (n = 4) and farms (n = 64) throughout the Netherlands and were not hormonally treated prior to OPU. Per donor per OPU session, all retrieved COCs were treated as one batch throughout

the whole *in vitro* production procedure. Transport and maturation of the COCs was performed in 2 ml maturation medium in transport vials (Greiner, 2 ml cryovials). Maturation was performed in TCM199, supplemented with 10% (v/v) foetal calf serum (FCS), 6 μg LH and 4 μg FSH/ml (Bovine; Sioux Biochemical Inc., Sioux Center, IA, USA) at 38.5°C under an atmosphere of 5% CO_2 in air with maximum humidity. All oocyte batches collected by OPU during the previous day were fertilized in one session resulting in maturation culture periods ranging from 16 to 28 h.

2.3 Sperm preparation and in vitro fertilization

After maturation (Day 0), the COCs were fertilized with frozen-thawed Percoll gradient-separated semen in 0.5 ml modified Tyrode-Lactate medium containing 0.6% fatty acid-free BSA fraction V and supplemented with 10 μg/ml heparin, 20 μm penicillamine, 10 μm hypotaurine and 1 μm epinephrine (all supplements: Sigma Chemical Company, St Louis, MO, USA). Sires (n = 155) used for IVF were distributed over donors dependent on the breeding programme and were used at their optimum final concentration (range 0.1–1.0×10^6spermatozoa/ml). COCs and spermatozoa were co-incubated for 24 h at 38.5°C under an atmosphere of 5% CO_2 in air with maximum humidity.

2.4 In vitro culture

After fertilization (Day 1), the cumulus cells were removed from the presumptive zygotes by manual pipetting. Dependent on the year of the programme, the culture system was either (i) TCM199, supplemented with 10% FCS with a monolayer of Buffalo Rat Liver (BRL) cells at 38.5°C under an atmosphere of 5% CO_2 in air with maximum humidity (March 1994 until January 1999) as previously described (Van Wagtendonk-de Leeuw et al. 2000) or (ii) SOFaaBSA at 38.5°C under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 (November 1997 until April 2001) (Van Wagtendonk-de Leeuw et al. 2000) or (iii) SOFaaBSA-CODA (from March 2000 onwards). This latter system was equal to the normal SOFaaBSA system but included a CODA filter unit (CODA, <genX> International, Inc., Madison, CT, USA) in the CO_2–O_2 incubator as previously described (Merton et al. 2007). As we showed that this filter significantly affected pregnancy rate for both fresh and frozen/thawed embryos, it was considered as a separate culture system. In all systems, the presumptive zygotes were cultured for 7 days in 0.5 ml culture medium which was changed on Day 4. Proportion of transferable embryos was assessed based on the number of presumptive zygotes at Day 1.

2.5 Embryo transfer

Embryos were evaluated for stage and grade on Day 7 and 8 of culture according to IETS recommendations (Robertson and Nelson 2010). Depending on the number of transferable embryos on Day 7 (IETS stage 4, grade 1 and stage 5–9, grade 1 and 2) and the number of recipients available, embryos were either transferred fresh or frozen/thawed. Embryos selected for freezing (IETS stage 5, grade 1 and stage 6–9, grade 1 and 2) were conventionally frozen in 10% glycerol as previously described (Van Wagtendonk-de Leeuw et al. 1995). Fresh embryos were transported at 25°C in either TCM199-Hepes, supplemented with 10% FCS (in combination with the TCM199/FCS/BRL culture system) or Emcare Holding Solution (ICP, Timaru, New Zealand) [in combination with the SOFaaBSA(-CODA) culture system] to participating farms throughout the Netherlands. Recipients were prepared without the use of a synchronization protocol and were evaluated and classified (good, moderate and poor) by rectal palpation based on the presence of a corpus luteum on day 6, 7 or 8 of their natural oestrous cycle. Potential recipients were only rejected for obvious reasons such as endometritis or absence of a corpus luteum. Suitable recipients received an embryo at Day 6, 7 or 8 by standard non-surgical embryo transfer procedures.

2.6 Calving rate and calf characteristics

Calf data were linked to the production/transfer database if gestation length (calving date minus date of start of oestrous prior to embryo transfer) was within 278 ± 14 days (calving rate analyses) or 280 ± 14 days (calf characteristics analyses). The following data were recorded by the farmers: calving date, birthweight (approximately 90% of calves were weighed by a balance (Bio Enterprice, Vroomshoop, the Netherlands), others were estimated and rounded to the nearest five kilograms) and sex of calf.

2.7 Statistical analysis

Data on embryo production, calving and calf characteristics were analysed using PROC MIXED with the Statistical Analyses System (SAS Institute Inc. Cary, NC, USA). Although the range in maturation culture period was equal in all three analyses, different intervals were defined to get sufficient numbers of observations per class of maturation culture period. Observations on calving rate were analysed separately for fresh and frozen/thawed embryos.

A series of variables were considered in the statistical models and were put in the model when applicable (effects were considered to be statistically significant when $p < 0.05$). The model to analyse embryo production included the effect of year of OPU session, donor, parity donor,

sire of donor, interval AI (pregnant donor) and first OPU session, lactating donor (yes/no), OPU location, OPU session interval, OPU technician, OPU assistant, maturation culture period, sire, IVF technician and IVC method. Models to analyse calving and calf characteristics included parity donor, sire, IVC method, embryo grade, embryo stage, number of embryos per OPU session, biopsied embryo (yes/no), transport time fresh embryo, quality recipient, parity recipient, day of heat recipient, ET technician, gestation length, birthweight, sex calf, month of birth and herd.

As already mentioned, in this retrospective study, OPU-IVP and calving data were analysed from a commercial OPU-IVP program covering a total period of 10 years. The three analyses performed were based, however, on different time intervals (January 1995 until January 1999 for embryo production analyses, January 1995 until May 2002 for the analyses of calving rate and March 1994 until September 2004 for the analyses of calf characteristics). To prevent confounding data, changes made in the IVC method as described in chapter 2.4 have been implemented in a time frame of at least 1 year in which both the old and new methods were used in a factorial design.

3 Results

3.1 Embryo production

During January 1995 until January 1999, 7800 OPU sessions were conducted. The mean number of COCs obtained per OPU session was 7.3 (range 1–62). The mean cleavage and transferable embryo rate was 55.7% and 16.5%, respectively. This resulted in an average of 1.2 (range 0–12) transferable embryo per OPU session. Maturation culture period did not affect mean transferable embryo rate that ranged from 11.7% to 17.5% (Fig. 1).

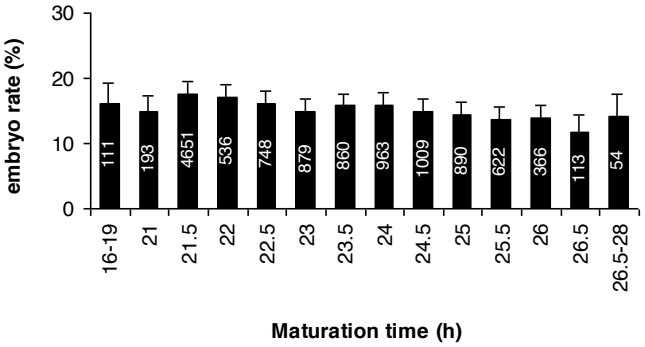

Fig. 1: Effect of *in vitro* maturation culture period on embryo formation rate (LSM ± SEM). The number of cumulus oocytes complexes batches per interval are indicated in the bars.

3.2 Calving rate

Of the 13,468 embryos transferred during the OPU-IVP programme from January 1995 until May 2002, 8889 were transferred fresh (4597 IETS grade 1 and 4292 grade 2) and 4579 transferred frozen/thawed (2750 grade 1 and 1829 grade 2). Calving rate was 40.9% for fresh embryos (44.6% and 36.9% for grade 1 and 2, respectively) and 38.7% for frozen/thawed embryos (41.1% and 35.0% for grade 1 and 2, respectively). Maturation culture period did not affect calving rate for both fresh and frozen/thawed embryos (Fig. 2).

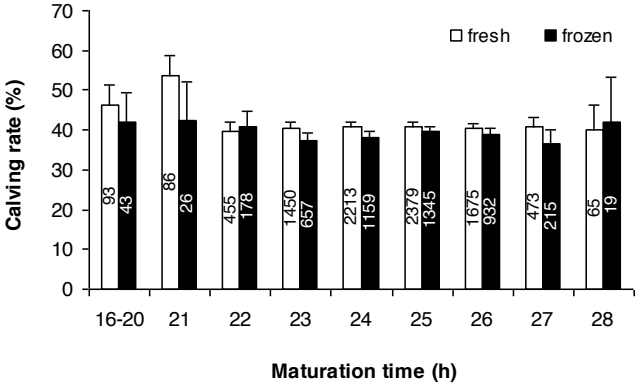

Fig. 2: Effect of *in vitro* maturation culture period on calving rate following transfers of fresh and frozen embryos (mean ± SE). The number of embryos transferred per interval are indicated in the bars.

3.3 Calf characteristics

Calf characteristics were measured on 2162 calves born between March 1994 and September 2004. Mean birthweight and gestation length were 46 ± 7 kg and 281.9 ± 5.3 days, respectively. The percentage of male calves was 52.8%. Neither birthweight, gestation length nor percentage of male calves were significantly affected by the maturation culture period (Fig. 3).

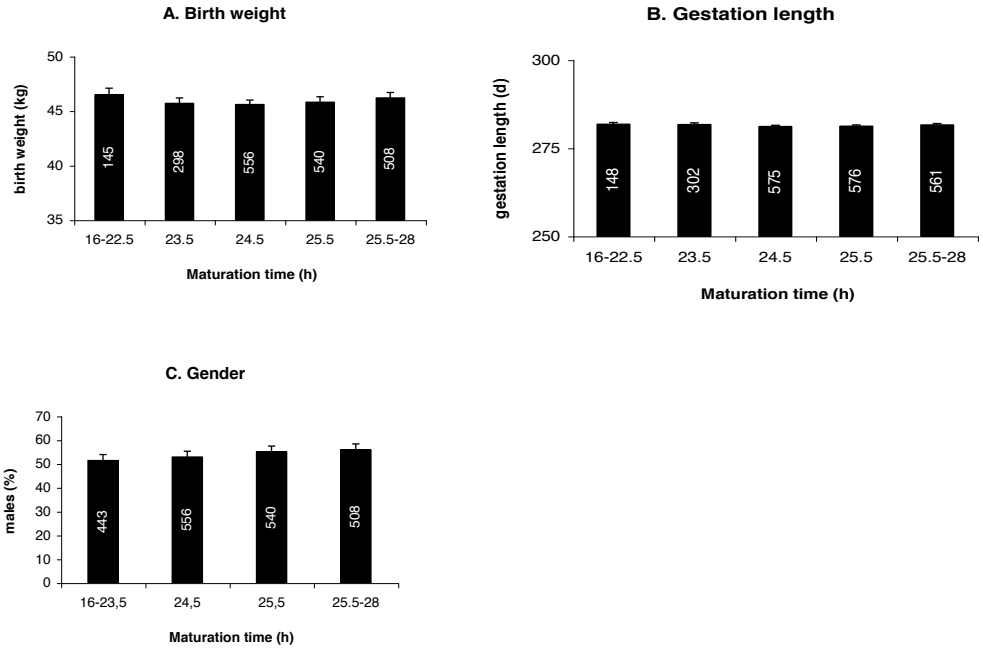

Fig. 3: Effect of *in vitro* maturation culture period on birth weight (A), gestation length (B) and percentage of male calves (C) (LSM ± SEM). The number of calves per interval are indicated in the bars.

4 Discussion

4.1 Embryo production

The results from this study show that embryo production from OPU-derived COCs is not affected when oocytes have been matured *in vitro* between 16 and 28 h. In contrast, for slaughterhouse-derived COCs, an optimal maturation culture period has been found in relation to embryo production: 18 h (Park et al. 2005), 22 h (Agung et al. 2006), 24 h (Tarin and Gomez-Piquer 2002), 18–24 h (Prokofiev et al. 1992) and 19–24 h (Merton et al. 2003).

Independent of the variation in optimal maturation culture periods as described previously, in almost all studies a relative large drop in embryo production rate was seen when the optimal maturation culture period was exceeded. We did not observe such a decline with OPU-derived COCs in our study. It is reasonable to assume that this is caused by a difference in the COC population owing to the oocyte collection technique used.

First of all, the population of OPU-derived COCs originates from a more homogeneous pool of follicles with respect to age and developmental stage during the follicle wave at the time of oocyte collection compared with slaughterhouse-derived COCs because of the twice weekly OPU recoveries sessions within a donor. This will result in a more homogeneous pool of COCs containing fewer COCs with signs of atresia. It has been suggested that the first steps in COC atresia are comparable with the first steps of maturation (Wurth and Kruip 1992; Blondin and Sirard 1995), suggesting that the optimal maturation time for those COCs will be more restricted as maturation has already started before collection. Indeed, it has been demonstrated that to optimize embryo production from class IV COCs (highly atretic COCs) (Morales-Pliego et al. 2009) maturation culture period should be shortened to 1 h. So, depending on the proportion of atretic COCs, the optimal maturation culture period may vary between studies. In this respect, the more homogeneous population of OPU-derived COCs, with probably the smallest fraction of early atretic COCs, may explain the less restricted maturation time for OPU-derived COCs.

Secondly, the time between slaughter and the start of *in vitro* maturation may be of importance. Although it is assumed that COCs are still under the influence of the follicle at the time of slaughter and that oocyte/nuclear maturation is still suppressed during the collection and transport of the ovaries, some cytoplasmic maturation processes may already have been initiated (Blondin et al. 1997). These extra interval hours and post-mortem changes in the oocyte may attribute to the more restricted culture period for slaughterhouse-derived COCs.

The results from this study suggest that OPU-derived oocytes are either matured and ready to be fertilized after 16 h of *in vitro* maturation and maintain this capacity for at least 9 h, or that oocytes complete their maturation process during the first hours of *in vitro* fertilization. Both of these processes will probably occur because oocytes already may reach the MII stage of maturity as early as 15 h and up to 24 h (Lequarre et al. 2005; Agung et al. 2006). McEvoy et al. (1998) showed that only a small proportion of both OPU-derived COCs from FSH stimulated heifers and unstimulated slaughterhouse-derived COCs reached the MII phase at 16 h of maturation (9% and 2% respectively), whereas 86% and 78% reached MII at 24 h.

Interestingly, the length of the maturation culture period did affect blastocyst development of slaughterhouse-derived COCs (4% and 35% for 16 and 24 h, respectively) but did not for OPU-derived COCs, suggesting a disruption in the balance between the nuclear and cytoplasmic maturation process caused by the post-mortem changes in slaughterhouse-derived COCs.

4.2 Calving rate and characteristics

The maturation culture period for OPU-derived COCs did not affect embryo cryotolerance, post-transfer embryonic survival and calf characteristics as measured by gestation length, birthweight and sex ratio. This lack of an optimum may seem obvious because embryo production was also not affected. However, the intrinsic quality of an embryo can be affected without being expressed morphologically and may come to expression only later in life (Tarin and Gomez-Piquer 2002; Thompson et al. 2007). For example, gene expression (Mamo et al. 2005), respiration (Lopes et al. 2007), cell number (Van Soom et al. 1997), cryotolerance (S. Merton, unpublished data) and the ability to establish a pregnancy (Merton et al. 2007) can differ between embryos within a morphologically equal population.

Park et al. (2005) reported an increased birthweight for 24 h of maturation compared with both 18 h of maturation and *in vivo* control embryos. In contrast, sex ratio was affected at 18 h of maturation (all males) compared with 24 h and *in vivo* control embryos. Different studies reported an effect of the length of the maturation culture period on sex ratio of *in vitro* produced blastocysts. A higher proportion of male blastocysts was found after 28 h of maturation compared with 16 h (68.2% vs 31.8%) (Agung et al. 2006). Also oocytes fertilized immediately after extrusion of the first polar body showed a higher proportion of female embryos compared with oocytes fertilized with an 8 h delay, resulting in a higher proportion of male embryos (Gutierrez-Adan et al. 1999; Rizos et al. 2008). It has been suggested that this result was based on differences in *in vitro* survival between male and female sperm. More female embryos can be expected with a shorter maturation culture period, assuming that, because of a delay in oocyte maturation, female sperm have an advantage compared with male sperm in overcoming this time before fertilization can take place. However, this phenomenon was not found in our study.

In conclusion, this study shows that the *in vitro* maturation culture period within the range of 16–28 h does not affect *in vitro* embryo production, embryo cryotolerance, post-transfer embryonic survival and calf characteristics, suggesting that all COC batches collected by

OPU on the same day, can be fertilized in one IVF session without a significant loss in the production from oocyte to calf.

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Chapter 3

Cysteamine supplementation during *in vitro* maturation of slaughterhouse- and opu-derived bovine oocytes improves embryonic development without affecting cryotolerance, pregnancy rate, and calf characteristics

Merton JS[1], Knijn HM[1], Flapper H[1], Dotinga F[1], Roelen BAJ [2], Vos PLAM[2], Mullaart E[1]

[1]CRV BV, PO Box 454, 6800 AL Arnhem, The Netherlands

[2] Dept. of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands.

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Abstract

Optimization of ovum pick up (OPU) followed by *in vitro* embryo production (IVP) is strongly driven by the needs of both beef and dairy cattle breeders to enhance genetic improvement. The rapidly growing use of genomic selection in cattle has increased the interest in using OPU-IVP technology to increase the number of embryos and offspring per donor, thus allowing enhanced selection intensity for the next generation. The aim of this study was to optimize embryo production through supplementation of cysteamine during *in vitro* maturation (IVM) and *in vitro* culture (IVC) of both slaughterhouse- and OPU-derived oocytes. The effects on embryo production and on embryo cryotolerance, post-transfer embryo survival, and calf characteristics, including gestation length, birth weight, perinatal mortality, and sex ratio were studied. In study 1, immature slaughterhouse-derived cumulus-oocyte complexes (COCs) were matured in IVM medium supplemented with or without 0.1 mM cysteamine, fertilized and cultured for 7 days in 0.5 ml SOFaaBSA. In study 2, cysteamine was present during both IVM (0.1 mM) and IVC (0.01, 0.05, 0.1 mM) from Days 1 to 4. In study 3, OPU-derived COCs were matured in medium supplemented with or without 0.1 mM cysteamine in a 2 × 2 factorial design (OPU week and cysteamine treatment). Embryos were evaluated for stage and grade on Day 7 and, depending on the number of transferable embryos and recipients available, the embryos were transferred either fresh or frozen-thawed at a later date. The presence of cysteamine during IVM significantly increased the embryo production rate with slaughterhouse-derived COCs (24.0% vs. 19.4%). The higher number of embryos at Day 7 was due to an increased number of blastocysts, whereas the distribution of embryos among different quality grades and cryotolerance was not affected. Embryo production rate was negatively affected when cysteamine was present during both the processes of IVM and IVC during Days 1 to 4 of culture (13.2%–19.3% vs. 26.4%). The presence of cysteamine during IVM of OPU-derived COCs also significantly increased the embryo production rate (34.4% vs. 23.4%). The higher number of embryos was again totally due to an increased number of blastocysts, whereas cryotolerance was not affected. The relative increase in embryo production rate was higher with OPU-derived oocytes compared with slaughterhouse-derived COCs (47% vs. 24%). This improvement resulted in a mean of 1.73 transferable embryos per OPU session compared with 1.06 in the absence of cysteamine. The presence of cysteamine did not affect pregnancy rate, gestation length, birth weight, perinatal mortality, and sex of calves born from either fresh or frozen-thawed embryos. This

study reported that cysteamine supplementation during IVM greatly improved the efficiency and affectivity of an OPU-IVP program.

Keywords: Bovine, Culture; Cysteamine; Gluthatione; OPU-IVP; Oxidative stress; ROS

1 Introduction

Optimization of ovum pick up (OPU) followed by *in vitro* embryo production (IVP) is strongly driven by the breeding industry to enhance genetic improvement in dairy and beef cattle. The production of large numbers of offspring from cattle of high genetic merit facilitates an increase in the selection intensity and shortening of the generation interval. In order to increase the chance of producing valuable bulls, cattle breeders are willing to invest in these technologies. OPU-IVP has become even more important with the introduction of genomic selection. Genomic selection identifies relatively accurate breeding values at birth or even from embryo biopsies, which makes the selection of donors of high genetic merit more accurate at an economically important time. Therefore, the more extensive use of successful OPU-IVP programs may lead to increases in the number of embryos and offspring per donor, leading to enhanced selection intensity for the next generation [1,2].

Since the introduction of OPU-IVP technology, widespread efforts have been undertaken to improve embryo production efficiency. Indeed many of the environmental conditions for gametes and embryos in IVP systems, e.g., media composition, pH, water and air quality, temperature, and atmospheric composition, have been relatively standardized. Because oxidative stress due to supraphysiological oxygen levels can lead to the formation of reactive oxygen species (ROS), which can compromise oocyte and embryo culture, systems have focused on a reduction of the oxygen level. However, ROS also can be formed at normal oxidative metabolism in the cell due to an imbalance of the intracellular redox potential [3]. High ROS levels may cause damage to cell components by lipid peroxidation, protein modification, and DNA damage resulting in impaired cell function and subsequently can affect oocyte maturation and further embryonic development. However, a certain level of ROS is thought to be physiologically important in processes like fertilization [4], apoptosis [5], and onset of zygotic gene transcription [6-8].

Low molecular weight thiol components such as cysteine and cystine are precursors of glutathione (GSH), which plays an important protective role in relation to ROS generated by normal oxidative metabolism in the cell. The cellular content of GSH is regulated by the gamma-glutamyl cycle as reviewed by Deleuze and Goudet [9]. Production of GSH depends on the availability and uptake of cysteine in the medium. However, cysteine is very unstable outside the cell and is auto-oxidized to cystine. Cysteamine reduces cystine to cysteine and promotes the uptake of cysteine by cells thereby enhancing the GSH synthesis. Consequently,

cysteamine plays an important role in the synthesis of GSH and is a key factor in the defense mechanism against ROS.

GSH is produced by the oocyte during maturation and reaches a maximum level at ovulation [10,11]. It has been suggested that in cattle this pool of GSH also protects the oocyte after fertilization up to the fourth cleavage until GSH is produced by the embryo [12-14].

Because oxygen levels during *in vitro* culture (IVC) affect embryo production [15-17], it seems likely that cellular antioxidant defense mechanisms that are responsible for the balance between ROS production and scavenging are insufficient to protect the oocyte or embryo from oxidative stress caused by IVC.

Therefore, in order to protect oocytes and embryos during *in vitro* conditions, antioxidants have been added to maturation media and oxygen levels, with or without the addition of antioxidants to culture media, have been reduced during culture. Presence of cysteamine in *in vitro* maturation (IVM) medium has been shown to result in enhanced embryo development and quality [18-21]. Similar results have been described for the presence of cysteamine in IVC medium [12,19,22].

It is important to note that in the majority of the studies described above, results have been on the basis of slaughterhouse-derived cumulus-oocyte complexes (COCs). It is well known, however, that the origin and quality of the COC population may be affected by the oocyte collection technique used. In case of a twice-weekly OPU system, most of the COCs will originate from growing, healthy follicles 3 to 8 mm in diameter in the absence of a dominant follicle. In contrast, animals brought to the slaughterhouse can be in any stage of the estrous cycle, and the COCs are retrieved from a large range of follicle sizes and at all possible stages of the follicle wave, including follicles present during the dominance phase or in the regressive, atretic phase of development. Furthermore, COCs are often selected on the quality of both cumulus and oocyte cytoplasm, whereas in a commercial OPU set-up, all retrieved oocytes are used for embryo production. It is therefore important to verify whether results on the basis of slaughterhouse-derived oocytes can be extrapolated to OPU-derived oocytes. Furthermore, due to practical research limitations with slaughterhouse-derived COCs, little is known about the effect of cysteamine on post-transfer embryonic survival and calf characteristics. In order to elucidate the possible changes in the cryotolerance of embryos, post-transfer embryonic survival, and calf characteristics, embryo transfer experiments provide more conclusive results. It has been shown, for example, that epigenetic events early in life may have long-term consequences and are expressed only later in life [23,24].

Therefore, the aims of this study were (1) to determine and compare the effect of cysteamine supplementation during IVM and IVC between slaughterhouse- and OPU-derived oocytes on embryo production and embryo cryotolerance and (2) to determine the effect of cysteamine supplementation during IVM of OPU-derived oocytes on the post-transfer embryo survival and the calf characteristics gestation length, birth weight, perinatal mortality, and sex ratio.

2 Materials and methods

2.1 Oocyte collection and IVM

Immature COCs were recovered twice weekly, on Monday and Thursday, by ultrasound guided transvaginal oocyte collection. The Holstein-Friesian donors, both cows and pregnant virgin heifers, were housed at a central collection center and were not hormonally treated before OPU. Per donor, all retrieved oocytes, including those (partially) denuded or with an expanded degenerated cumulus, were used for IVP and were treated as one batch throughout the production process. Transport to the central IVP laboratory and maturation of the COCs was combined and was performed in 2 mL maturation medium in transport vials (Greiner, 2 mL cryovials) for 23 to 26 hours at 38.0 °C. Slaughterhouse-derived immature COCs were recovered from ovaries 6 to 8 hours after slaughter. COCs were recovered by aspiration of all visible follicles. Denuded oocytes and those with a degenerated cumulus investment were discarded. Selected COCs were matured *in vitro* for 22 to 24 hours. Maturation was performed in TCM199, supplemented with 10% (vol/vol) fetal calf serum, 6 µg LH, and 4 µg FSH/mL (Sioux Biochemical Inc., IA, USA) at 38.5 °C under an atmosphere of 5% CO_2 in air with maximum humidity.

2.2 Sperm preparation and in vitro fertilisation

After maturation (Day 0), batches of COCs were fertilized with frozen-thawed gradient-separated semen in 0.5 mL modified Tyrode-lactate medium containing 0.6% fatty acid free BSA fraction V, 10 µg/mL heparin, 20 µM penicillamine, 10 µM hypotaurine, and 1 µM epinephrine (all supplements from Sigma Chemical Company, St Louis, MO, USA).
Sires used for IVF of OPU-derived COCs were distributed over donors dependent on the breeding program and were used at their optimum final concentration (range 0.1–1.0 × 10^6 spermatozoa/mL). For slaughterhouse-derived COCs, one reference bull was used. COCs and spermatozoa were coincubated for 22 to 24 hours at 38.5 °C under an atmosphere of 5% CO_2 in air with maximum humidity.

2.3 *In vitro culture*

After fertilization (Day 1), the cumulus was removed from the presumptive zygotes either by manual pipetting (OPU) or vortexing (slaughterhouse). The denuded zygotes were cultured for 7 days in 0.5 mL SOFaaBSA at 38.5 °C under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 as previously described [25]. Culture medium was changed on Day 4 by transferring the embryos into fresh medium.

2.4 *Embryo transfer*

Embryos were evaluated routinely in a blinded fashion in the production lab for stage and grade on Days 7 and 8 according to the International Embryo Transfer Society (IETS) recommendations [26] and were transferred either fresh or cryopreserved using conventional slow freezing in 10% glycerol as previously described [27]. Fresh OPU-derived embryos were transported at 25 °C in Emcare holding solution (ICP, New Zealand). Embryos were transferred into recipients in CRV recipient herds or at participating farms throughout The Netherlands. Recipients were prepared without the use of a synchronization protocol and were evaluated by rectal palpation on the basis of the presence of a corpus luteum on Day 6, 7, or 8 of their natural estrous cycle. Potential recipients were rejected for obvious reasons, such as endometritis or absence of a corpus luteum. Suitable recipients received an embryo at Day 6, 7, or 8 by standard nonsurgical embryo transfer procedures.

2.5 *Calf characteristics*

Calving characteristics were only recorded from embryos transferred at our own recipient herds. Clinically healthy (seronegative and pathogen-free animals for Bovine Herpes Virus-1, Bovine Viral Diarrhea Virus, Bovine Neosporosis, Salmonellosis, and Paratuberculosis) nonlactating Holstein-Friesian recipients, both heifers and first parity cows, were used for this study as described before. The following data were recorded: birth weight, calving course, sex of the calf, parity of the recipient mother, parity of the donor mother, month of calving, fresh or frozen embryo, gestation length, and calf viability 24 hour after parturition.

2.6 *Experimental design*

2.6.1 *Experiment 1: effect of cysteamine during IVM of slaughterhouse-derived oocytes on embryonic development and post-thaw survival*

Immature COCs were matured in IVM medium supplemented with or without 0.1 mM cysteamine (Sigma Chemical Company). Embryos were evaluated for stage and grade on Days 7 and 8. All grade 1 and 2 embryos (stage 6 and 7) present on Day 7 were frozen. Post-thaw survival was measured by reexpansion rate at 24 hours and hatching/hatched rate at 72 hours of culture.

2.6.2 Experiment 2: effect of cysteamine during IVM and IVC of slaughterhouse-derived oocytes on embryonic development

Denuded presumptive zygotes, matured in the presence of cysteamine (0.1 mM), were cultured for 7 days in 0.5 mL SOFaaBSA. Cysteamine was present during culture from Days 1 to 4 at either 0.1, 0.05, or 0.01 mM. Embryos were evaluated for stage and grade on Days 7 and 8.

2.6.3 Experiment 3: effect of cysteamine during IVM of OPU-derived oocytes on embryonic development, pregnancy rate and calf characteristics

The experimental design was a 2 × 2 factorial (OPU week and cysteamine treatment) randomized crossover, whereby immature COCs were matured in medium supplemented with or without 0.1 mM cysteamine. The experiment was conducted for 10 successive months from March till January. Embryos were evaluated for stage and grade on Days 7 and 8. Depending on the number of transferable embryos available on Day 7 (IETS stage 4, grade 1 and stage 5–9, grade 1 and 2), and the number of recipients available, the embryos were either transferred fresh or frozen-thawed (IETS stage 5, grade 1 and stage 6 to 9, grade 1 and 2).

2.7 Statistical analysis

The effect of cysteamine on embryo production, embryo quality, post-thaw survival, and calving rate was calculated by chi-square analysis. The effect of cysteamine on birth weight, gestation length, perinatal mortality, and sex ratio was statistically analyzed using SPSS, general linear model, univariate with fixed factors parity of the donor, parity of the recipient, sex, month of calving, and fresh or frozen embryos. Effects were considered to be statistically significant when P value was <0.05.

3 Results

3.1 Experiment 1: effect of cysteamine during IVM of slaughterhouse-derived oocytes on embryonic development and post-thaw survival

The presence of cysteamine during the IVM of slaughterhouse-derived oocytes significantly enhanced the embryo production rate (Table 1). The higher percentage of embryos at Day 7 was due to an increased percentage of blastocysts, whereas distribution of embryos among different quality grades was not affected (Fig. 1).

Table 1: Effect of cysteamine during IVM of slaughterhouse-derived oocytes on subsequent in vitro embryonic development.

Group	Oocytes [c] n	Cleavage n (%)	Embryos Day 7 n (%)			
			Morulae	Blastocysts	Total	Relative increase
Control	1056	586 (55.5)[a]	81 (7.7)[a]	124 (11.7)[a]	205 (19.4)[a]	
Cysteamine	1070	634 (59.3)[a]	79 (7.4)[a]	178 (16.6)[b]	257 (24.0)[b]	24%

[a,b] Values in columns with different superscript are significantly different, $P<0.05$.
[c] number of equally balanced replicates: 5

The intrinsic quality of the embryos was determined by measuring post-thaw survival. For parameters of post-thaw survival, percentages of embryos that had re-expanded 24 hours after thawing and percentages of embryos that had hatched from their zona pellucida 72 hours after thawing were determined. The presence of cysteamine during IVM of slaughterhouse-derived oocytes did not affect the post-thaw survival of both grade 1 and grade 2 frozen embryos (Table 2).

Table 2: Effect of cysteamine during IVM of slaughterhouse-derived oocytes on post-thaw in vitro survival of frozen in vitro-produced blastocysts.

Embryo grade	(%) re-expansion 24 hrs		(%) hatching/hatched 72 hrs	
	Control	Cysteamine	Control	Cysteamine
1	38/46 (83)[a]	56/63 (89)[a]	17/46 (37)[a]	28/63 (44)[a]
2	36/41 (88)[a]	52/56 (93)[a]	12/41 (29)[a]	12/56 (21)[a]
Total	74/87 (85)[a]	108/119 (91)[a]	29/87 (33)[a]	40/119 (34)[a]

Values in rows with different superscript (a,b) are significantly different, $P<0.05$.

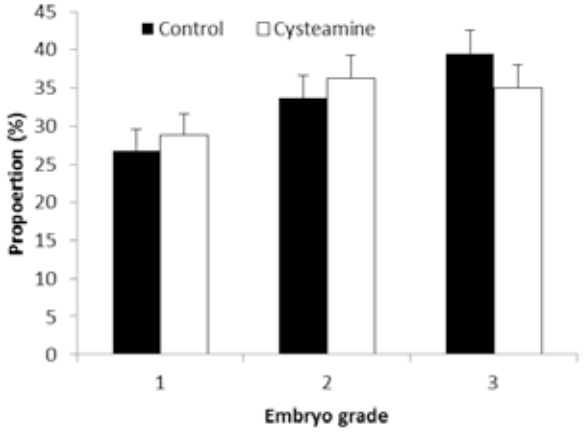

Fig. 1: Effect of cysteamine during IVM of slaughterhouse-derived oocytes on the distribution of embryo quality grades (mean percentage ± SE; number of equally balanced replicates: 5).

3.2 Experiment 2: effect of cysteamine during IVM and IVC of slaughterhouse-derived oocytes on embryonic development

In experiment 1, the presence of cysteamine during IVM enhanced the embryo production rate. In experiment 2, the effect of presence of cysteamine during IVC was investigated at various concentrations. No significant difference was found in the cleavage rate when cysteamine was present both during maturation and culture of slaughterhouse-derived oocytes (Table 3). After culture, however, the percentages of embryo produced were significantly lower for all cysteamine concentrations present during culture from Days 1 to 4 (Table 3).

Table 3: Effect of cysteamine during IVM (0.1 mM) and IVC (0, 0.1, 0.05, 0.01 mM) during Days 1-4 of slaughterhouse-derived oocytes on in vitro embryonic development.

Treatment IVM/IVC	Oocytes[d] n	Cleavage n (%)	Embryos Day 8 n (%)
0.1/0 (control)	356	197 (53.3)[a]	94 (26.4)[a]
0.1/0.01	357	179 (50.1)[a]	66 (18.4)[bc]
0.1/0.05	357	191 (52.4)[a]	69 (19.3)[b]
0.1/0.1	357	197 (53.5)[a]	47 (13.2)[c]

[a,b,c] Values in columns with different superscript are significantly different, $P<0.05$.
[d] number of equally balanced replicates: 3

3.3 Experiment 3: effect of cysteamine during IVM of OPU-derived oocytes on embryonic development, pregnancy rate and calf characteristics

In line with the results on the basis of slaughterhouse-derived oocytes, the presence of cysteamine during IVM of OPU-derived COCs significantly enhanced the cleavage and embryo production rate (Table 4). The relative increase in embryo production rate was, however, substantially higher with OPU-derived oocytes compared with slaughterhouse-derived COCs (47% vs. 24% for OPU- and slaughterhouse-derived COCs, respectively). The number of transferable embryos per OPU session increased from 1.06 to 1.73 (Table 4).

The higher embryo production rate was mainly due to an increased number of blastocysts (Fig. 2). In addition, the proportion of grade 3 embryos was significantly reduced in the cysteamine group (Fig. 3).

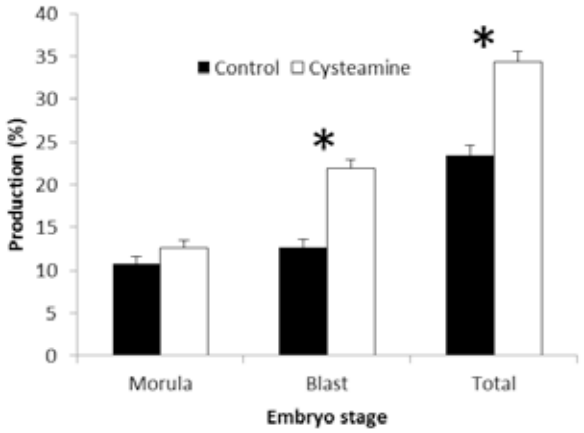

Fig. 2: Effect of cysteamine during IVM of OPU-derived bovine oocytes on the distribution of Day 7 embryo developmental stages (mean percentage ± SE). * Significantly different, $P<0.01$.

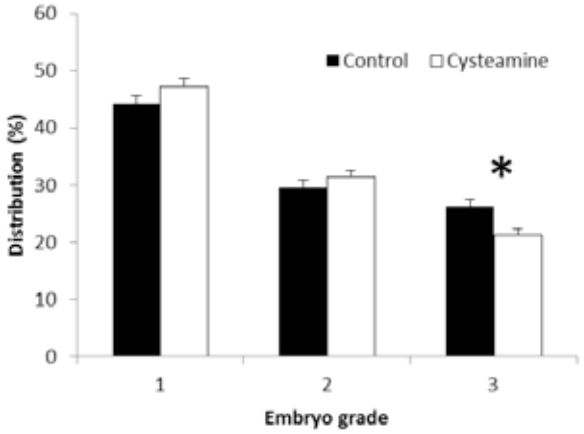

Fig. 3: Effect of cysteamine during IVM of OPU-derived bovine oocytes on Day 7 embryo quality (mean percentage ± SE). * Significantly different, $P<0.01$.

A total of 1373 OPU-derived embryos were transferred to recipients. Pregnancy rates were not significantly affected by the presence of cysteamine during IVM for either fresh or frozen-thawed embryos (fresh: 45% and 45%, frozen-thawed: 44% and 45%, for control and cysteamine, respectively; Table 5).

Table 4: Effect of the presence of cysteamine during in vitro maturation of OPU-derived bovine oocytes on subsequent in vitro embryonic development.

Group	Sessions N	Oocytes N	Cleavage n (%)	Embryo production Day 7			
				Mor + Blast n (%)	Relative increase	Transferable	
						n	Per session
Control	696	5454	3296 (60.4)[a]	1277 (23.4)[a]		740	1.06[a]
Cyst.	589	4557	2939 (64.5)[b]	1569 (34.4)[b]	47%	1017	1.73[b]

[a,b] Values in columns with different superscript are significantly different, $P<0.001$.

Table 5: Effect of cysteamine during IVM of OPU-derived oocytes on pregnancy rates of fresh and frozen-thawed embryos.

Group	Embryo		Pregnancy rate (%)
	Type	Transfers, N	
Control	Fresh	359	45 %
	Frozen	213	44 %
Cysteamine	Fresh	451	45 %
	Frozen	350	45 %

Characteristics were recorded for 95 calves born in the CRV recipient herd. A total of 45 calves resulted from embryos produced in the absence of cysteamine, whereas 53 resulted from embryos produced in the presence of cysteamine during IVM. Overall, the mean birth weights and gestation lengths were 44 ± 5 kg and 274.9 ± 6.6 days, respectively. The percentage of male calves was 58%. The presence of cysteamine did not affect gestation length, birth weight, perinatal mortality, and sex of calves born from either fresh or frozen-thawed embryos.

4 Discussion

This study found that IVP is significantly enhanced when cysteamine is supplemented during IVM of both slaughterhouse- and OPU-derived COCs. Surprisingly, the relative increase in embryo production rate at Day 7 was substantially higher for OPU-derived oocytes compared with slaughterhouse-derived oocytes (23% vs. 47%). The relative increase in transferable embryos per OPU session was even greater (63%; from 1.06 to 1.73). The greater effect on embryo development for OPU- compared with slaughterhouse-derived COCs may have been caused by a difference in the morphological quality of the COCs, on the basis of cumulus investment, at the start of IVM. In general, the amount of cumulus cells surrounding the OPU-derived COCs is lower compared with slaughterhouse-derived COCs, which is suggested to be related to a positive postmortem effect in case of slaughterhouse-derived COCs [28]. Furthermore, with slaughterhouse-derived oocytes, only those surrounded with cumulus cell layers were used, whereas with OPU, in order to maximize the embryo production, all retrieved oocytes, including those (partially) denuded or with an expanded degenerated cumulus, were used for IVP. It has been found that when present in IVM medium, cystine stimulates GSH production in oocytes surrounded by cumulus cells, but not in denuded oocytes[29,30]. Cysteamine, however, can increase the intracellular GSH levels in denuded oocytes [22,29,30]. This suggests a role for the cumulus cells in the reduction of cystine to cysteine as a substrate for GSH production. Consequently, impaired GSH synthesis may be dependent on the amount of cumulus cells surrounding the oocyte, explaining the relatively greater effect of cysteamine supplementation as found with OPU-derived COCs.

As mentioned, addition of cysteamine to the maturation medium results in an increase of GSH content in the matured oocytes [18-20,22,31]. An increase in GSH level was shown to be associated with a decrease in ROS levels within oocytes [19,32], illustrating the scavenging effect of GSH on ROS resulting in enhanced embryo production. Wang et al. [31] found that

higher concentrations of intracellular GSH led to more monospermic fertilization and less asynchronous pronucleus (PN) formation, but that nuclear maturation was not affected. Other researchers found similar results in a number of species in which increased GSH levels are correlated with improved male PN formation [33-36]. Thus, improved male PN formation can be one of the underlying factors leading to higher IVP rates, as reported. This implies that there may also be an interaction between cysteamine and sires, which may have caused the greater embryo development for OPU- compared with slaughterhouse-derived COCs. Further research is needed to elucidate the hypothesis. Improved male PN formation may also explain the small increase in cleavage rates found in this study. This finding, however, points to a discrepancy between reports [9], which make a final conclusion on the effect of cysteamine on cleavage rate hard to draw.

The higher percentage of embryos at Day 7 following cysteamine supplementation during IVM was due to an increased percentage of blastocysts, suggesting a faster rate of development. This enhanced rate of development and increased percentage of blastocyst development was observed with both slaughterhouse- and OPU-derived oocytes. The distribution of embryos among the different quality grades was slightly affected by the addition of cysteamine to the maturation medium and numerically in favor of an increased proportion of quality 1 and 2 embryos for both slaughterhouse- and OPU-derived oocytes. However, the proportion of grade 3 embryos was significantly lower only for OPU-derived oocytes.

De Matos et al. [19] found that when cysteamine was supplemented during IVM and also during Days 2 to 4 of culture, both embryo production and post-thaw embryo survival were improved at an optimal concentration of 0.05 mM, whereas with lower or higher concentrations this effect was absent or even negative. In this study, addition of cysteamine to the IVC medium did not have any beneficial effect on embryo production within the same range of concentrations tested. In fact, although cleavage rate was not affected, embryonic development to the blastocyst stage was significantly decreased for all tested concentrations. These results suggest that this culture system, with cysteamine present in the maturation medium and embryos cultured at 5% oxygen, the level of GSH is already optimal during early embryo development. Further studies on GSH levels are needed to verify this hypothesis. It can furthermore be speculated that adding extra cysteamine during the first days of culture may have resulted in a limited level of ROS. Consequently, this imbalance may have interfered with embryonic genome activation during the transition from maternal to zygotic

control of development in which ROS-induced lipid peroxidation has been shown to rise during this period *in vitro* [37] and to be involved in its mechanism [6-8].

Commercially, an important question is whether the intrinsic quality of the cysteamine-produced, morphologically similar embryos was of similar quality to those produced under control conditions. This question is particularly relevant, if due to the presence of cysteamine during IVM, COCs of poorer quality were "rescued" with a restored developmental competence. The intrinsic quality of the IVP embryos may be affected without being expressed morphologically and may come to expression only later in life [23,24]. For example, gene expression [38], respiration [39], cell number [40], cryotolerance (Merton, unpublished data), and the ability to establish a pregnancy [41] can differ between embryos within a morphologically equal population. According to the results presented here, however, the embryos from oocytes exposed to cysteamine did not appear to be different with respect to cryotolerance, post-transfer embryo survival, and calf characteristics as measured by gestation length, birth weight, perinatal mortality, and sex ratio.

In conclusion, this study shows that the apparently "simple" extra protection of the COC, by increased GSH synthesis during IVM, can have a significant effect on *in vitro* developmental competence. Moreover, the resulting enhanced production of embryos does not affect embryo cryotolerance, post-transfer embryo survival, and calf characteristics. This makes cysteamine supplementation during IVM a strong tool to improve the efficiency of an OPU-IVP program. Furthermore, enhanced oocyte competence also leads to an increase in the number of offspring. This facilitates a significant increase in the selection intensity and shortening of the generation interval, improving a major goal of the breeding industry, namely enhanced genetic improvement.

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Chapter 4

Carbon-activated gas filtration during *in vitro* culture increased pregnancy rate following transfer of in vitro produced bovine embryos

Merton JS, Vermeulen ZL, Otter T, Mullaart E, de Ruigh L, Hasler JF[1].

HG BV, Production Department, PO Box 5073, 6802 EB Arnhem, The Netherlands

[1]Bioniche Animal Health USA, Inc., 1551 Jennings Mill Rd., Bogart, GA 30622, USA

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Abstract

Many environmental conditions for *in vitro* embryo production (IVP) systems for cattle have been relatively standardised, e.g. media composition, temperature, pH, water quality, and atmospheric composition. However, little attention has been paid to the quality of ambient laboratory air and the gas environment in incubators. Although a few studies have examined the effects of chemical air contamination on IVP of human embryos, there are no published accounts for domestic animal embryos. Therefore, this study investigated the effects of an intra-incubator carbon-activated air filtration system (CODA) during *in vitro* culture (IVC) on embryonic development and subsequent pregnancy rate of bovine embryos. Immature cumulus–oocyte-complexes (COCs) were obtained twice-weekly by ultrasonic-guided transvaginal oocyte aspiration. The COCs were matured in TCM199/FCS/LH/FSH, fertilized with frozen–thawed Percoll-separated semen, and subsequently cultured for 7 day in SOFaaBSA. Day 7 embryos were transferred either fresh or frozen/thawed. The experimental design was a 2 × 2 factorial; presumptive zygotes were placed either in a conventional CO_2–O_2–N_2 incubator (Control group) or in an identical CO_2–O_2–N_2 incubator with a CODA intra-incubator air purification unit (CODA group) for IVC. The embryo production rate at Day 7 was not affected by the CODA air purification unit (23.4 and 24.7% morulae and blastocysts per oocyte for control and CODA, respectively) nor was there any significant effect on embryo stage or quality. However, the pregnancy rate was improved (P = 0.043) for both fresh (46.3% versus 41.0%) and frozen/thawed embryos (40.8% versus 35.6%). In conclusion, atmospheric purification by the CODA intra-incubator air purification unit significantly increased pregnancy rate following transfer of in vitro-produced bovine embryos.

Keywords: IVP; Cattle; Embryo; Pregnancy rate; VOC

1 Introduction

Commercial embryo transfer in cattle has grown into a large, world-wide business over the past 35 year. Statistics compiled by the International Embryo Transfer Society (IETS) indicate that more than 550,000 *in vivo*-derived embryos, obtained primarily from superovulated cattle, were transferred internationally in 2004 [1]. Improvements over the past 15 year in procedures for *in vitro* production of cattle embryos have resulted in the establishment of commercial *in vitro*-embryo transfer programs [2]. These programs include the salvage of genetics from infertile females, increased embryo production from reproductively healthy females, and the large-scale production of embryos from abattoir-derived material. Based on statistics from the IETS [1], approximately 240,000 *in vitro*-produced embryos were transferred worldwide in 2004. The largest programs are in South America, where most embryos are derived from repeated ultrasound guided transvaginal oocyte collections (ovum pickup or OPU) from live donors, and Asia, where embryos primarily are produced from oocytes obtained from abattoir-derived ovaries. In Europe, the use of OPU–IVP is limited. Only six countries reported producing IVP embryos in 2004 [3]; OPU–IVP is mainly used for breeding purposes and not for commercial production as is common in other programs.

Many of the environmental conditions for *in vitro* embryo production, e.g. media composition, temperature, pH, water quality, and atmospheric composition, have been relatively standardised in both human and animal *in vitro* production (IVP) systems. Less attention has been paid, however, to the quality of both ambient laboratory air and the gaseous atmosphere inside incubators.

Atmospheric particulate matter may affect pregnancy rates in human IVF programs. In a retrospective analysis of an ongoing program [4], pregnancy rates decreased substantially during a period of construction adjacent to the laboratory complex. Installation of ultra low penetration air (ULPA) filters resulted in a substantial improvement in pregnancy rates. In addition, a positive pressure differential system was installed in the entry and laboratory rooms. Following these changes, counts of 0.3 μm particles (particles counted for a specific length of time) ranged from <1 in the embryology laboratory rooms to >450,000 in the adjacent operating room that lacked ULPA filtration.

In addition to physical particles, it is clear that chemical air contamination and volatile organic compounds (VOCs) impact laboratory and incubator environments and may affect IVP, pregnancy rates, or both. Although many laboratories now have high-efficiency particle air filtration (HEPA or ULPA) systems, these filters do not efficiently retain gaseous organic

and inorganic molecules. The gaseous atmosphere in incubators is often a combination of room air, CO_2 and sometimes also N_2 from compressed gas tanks; all of these sources may contain contaminants. Schimmel et al. [5] showed that among the various organic compounds found in laboratory gases, benzene was derived specifically from CO_2 tanks. In some IVP systems, a high percentage (>90%) of ambient air in the incubator is obtained directly from the laboratory room through the opened door or the inlet port at the back of the incubator. Surprisingly, Cohen et al. [6] found that unfiltered, outside air may be cleaner than air both in the laboratory and in incubators; this can result from the accumulation of VOCs from the laboratory complex and from laboratory products (e.g. plastic dishes). Refrigerant gases, isopropyl alcohol fumes, various aliphatic hydrocarbons and select aromatic compounds were at higher concentrations in the laboratory air and inside the incubators compared to outside air. Cohen et al. [6] also detected a large number of VOCs in compressed CO_2 used for *in vitro* culture.

In general, air pollutants such as VOCs, small inorganic compounds (e.g. N_2O, SO_2 and CO), and heavy metals can be detected. Sources of these contaminants are VOCs produced by industrial and vehicle exhaust, but also by cleaning agents and off-gassing from plastic ware, media, laboratory furnishing, and equipment [7]. Disposable plastic petri dishes and flasks have been shown by gas chromatorgraphy to emit a number of volatile organic compounds, including ethyl-benzene and benzaldehyde [8].

To overcome possible effects of these contaminants on embryo production and pregnancy rate, some laboratories have placed filters containing activated carbon and potassium permanganate in the laboratory or inside the incubators, recycling the air while removing or diminishing toxic air pollutants. Mayer et al. [9] examined the effect of CODA intra-incubator air filter on human embryo quality and pregnancy rates. Although there was no effect on the number or quality of embryos produced, pregnancy rate was significantly higher 52% versus 30% following transfer of embryos cultured in CODA-filtered versus non-filtered incubators.

Racowsky et al. [10] also reported on human IVF-derived pregnancies resulting from embryos cultured in filtered air. Filtration of ambient, HEPA-filtered, laboratory air and incubator CO_2 gas by carbon-activated filters resulted in a slight but significant difference in embryo fragmentation and no significant difference in pregnancy rate, but a significant reduction in the spontaneous abortion rate. Most reports in humans were based on small numbers of transfers, resulting in limited statistic power to detect small differences.

The objective of the present study was to investigate the effects of an intra-incubator carbon-activated air filtration system (CODA) during IVC of bovine *in vitro* matured (IVM) and fertilized (IVF) COCs on embryonic development and subsequent pregnancy rate.

2 Materials and methods

2.1. Ovum pick up and in vitro production of preimplantation embryos

From 27 March 2000 to 05 March 2001, OPU was conducted from 193 donors at two locations in The Netherlands. Donors were 177 pregnant heifers and 16 first-parity cows (Holstein Friesian) from the delta open nucleus breeding program of Holland Genetics (Holland Genetics is now known as HG, and is a division of CRV Holding BV). An average of 7.3 OPU sessions from pregnant heifers and 30.3 OPU sessions from each first parity cow were performed. Immature COCs were obtained twice weekly, on Mondays and Thursdays, by ultrasound-guided transvaginal oocyte collection (7.5 MHz sector probe, Pie Medical, Maastricht, The Netherlands). The COCs were matured in TCM199/FCS/LH/FSH, fertilized with frozen–thawed Percoll-separated semen (Day 0), cultured for 6 day in SOFaaBSA as described previously [11], and evaluated starting on Day 7. The intra-incubator gas atmosphere for IVM and IVF was 5% CO_2 (supplied from a compressed cylinder). For IVC, the atmospheric mixture was 5% CO_2, 5% O_2, and 90% N_2, with the CO_2 and N_2 provided by compressed cylinders. Gas cylinders were provided by Air Products Nederland BV, Utrecht, The Netherlands. Gas purities were rated as 99.5% pure for CO_2 and 99.998% pure for N_2. The IVM, IVF, and IVC were all conducted at 38.5 °C.

2.2. Embryo transfer

Embryos were evaluated for stage and grade on Days 7 and 8 by IETS standards [12]. Depending on the number of transferable embryos available on Day 7 of culture (IETS Stage 4 (morula)-Grade 1 (excellent or good) and Stages 5–8 (early, mid, expanded, or hatched blastocysts)-Grades 1 and 2 (fair)) and the number of recipients available, embryos either were transferred fresh or frozen/thawed (IETS Stage 5-Grade 1 and Stages 6–8-Grades 1 and 2) using conventional slow freezing in 10% glycerol as previously described [13]. Fresh embryos were transported at 25 °C in EMCARE™ Holding Solution (ICPbio, Auckland, New Zealand) to participating farms throughout The Netherlands and to recipient herds owned and managed by Holland Genetics. On Days 7 or 8 of their estrous cycle (standing estrus = Day 0), potential recipients were evaluated by transrectal palpation for the presence of a CL; those

lacking a CL or with an overt abnormality (e.g. endometritis) were not used, whereas those deemed suitable received an embryo on Days 7 or 8 by standard non-surgical embryo transfer procedures. Because many of the embryos were transferred to farms throughout the Netherlands, it was not possible to confirm pregnancies at predetermined intervals; consequently pregnancy status was determined between Day 90 and term.

2.3. Experimental design

The experimental design was a 2 × 2 factorial randomized crossover, whereby the presumptive zygotes were placed either in a conventional CO_2–O_2–N_2 incubator (Control group) or in an identical CO2–O2 incubator with a CODA (〈genX〉 International, Inc., Madison, CT, USA) intra-incubator air purification unit (CODA group). Every 5 week, the CODA intra-incubator air purification unit was transferred to the other incubator to eliminate confounding due to incubator-specific performance.

2.4. Statistical analyses

Data were analysed by Chi-square for embryonic development and logistic regression analysis for pregnancy rates. (Genstat, Numerical Algorithms Group Ltd., Oxford, United Kingdom).

3 Results

3.1. In vitro embryo production

A mean of 8.2 COCs per collection were recovered during 1607 OPU sessions. As shown in Table 1, there was no significant difference between the control and CODA treatment groups for either cleavage or blastocyst formation at Day 8. In addition, the embryo production rate (morulae and blastocysts combined) was not affected on Day 7 by the CODA air purification unit. Also, for Day-7 embryos, the CODA air purification unit had no significant effect on the distribution of embryos among grades (Fig. 1) or stages (Fig. 2).

Table 1: Effect of CODA filter on IVP of embryos.

Group	OPU sessions	Oocytes in IVC	Cleaved Day 4		Morulae/blastocysts Day 7		Blastocysts Day 8	
	n	n	n	(%)	n	(%)	n	(%)
Control	829	6566	3825	(58.3)	1536	(23.4)	1267	(19.3)
CODA	778	6533	3901	(59.7)	1615	(24.7)	1326	(20.3)

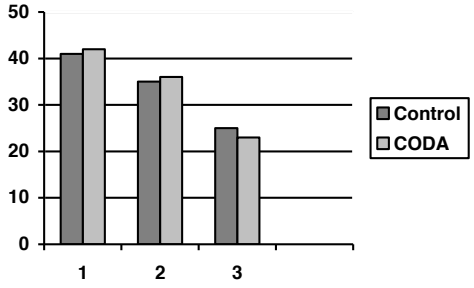

Fig. 1: Effect of CODA intra-incubator gas purification on the distribution of various grades (IETS) of Day-7 IVP bovine embryos. Key: Grade 1, excellent to good morphological quality; Grade 2, fair morphological quality; and Grade 3, poor morphological quality.

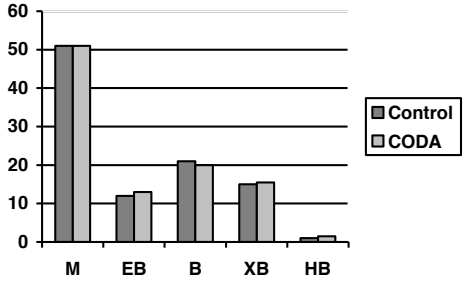

Fig. 2: Effect of CODA intra-incubator gas purification on the distribution of various stages (IETS) of Day-7 IVP bovine embryos. Key: M, morula; EB, early blastocysts; B, blastocysts; XB, expanded blastocysts; and HB, hatched blastocyst.

3.2. Embryo transfer

From the total of 3151 embryos produced on Day 7, 1666 were classified as transferable quality (820 fresh and 846 frozen; 1.04 embryos per OPU session). Although pregnancy data following transfer were not available on all embryos, data on 782 fresh and 635 frozen embryos were obtained and analysed (Table 2). There was an effect ($P = 0.043$) of the CODA air purification unit on pregnancy rate for both fresh and frozen/thawed embryos; the increase was 5.3 and 5.2 percentage points for fresh and frozen/thawed embryos, respectively (increases of 12.9 and 14.6%).

Table 2: Effect of CODA air purification during IVC on the pregnancy rate of both fresh and frozen/thawed IVP bovine embryos.

Embryo	Incubator status	No. embryos	% Pregnant
Fresh	Control	401	41.0[a]
Fresh	CODA	381	46.3[b]
Frozen/thawed	Control	298	35.6[c]
Frozen/thawed	CODA	337	40.8[a]

[abc]Means with different superscripts differ ($P<0.05$)

4 Discussion

In the present study, there was no significant effect of intra-incubator gas purification by the CODA unit on embryos, as judged by percent cleavage, embryo quality or stage or development. In previous studies, based on the transfer of large numbers of IVP [2] and *in vivo*-derived [14] bovine embryos, pregnancy rate was closely correlated with morphology. However, pregnancy rate was significantly improved following transfer of both fresh and frozen/thawed embryos produced in the CODA system; we inferred that there was an improvement in the intrinsic quality of the embryo not manifested by morphology. Similar results were reported for human embryos by Mayer et al. [9]; there was no significant effect on the number or quality of embryos produced, but pregnancy rate was significantly affected, suggesting an improved embryo quality that was manifest as a difference in morphology. In a study by Racowsky et al. [10], CODA-filtered incubation significantly decreased the apparent morphological quality of embryos, as judged by fragmentation. The authors suggested that the increase in fragmentation might provide a means by which embryos may improve their developmental competency and that the use of CODA air purification units might facilitate this process. In a recent study involving human IVF in Brazil, in-line HEPA and carbon-activated filters located between the gas cylinders and the incubators and intra-incubator filtration units resulted in higher cleavage rates, more good-quality embryos, higher pregnancy rates and lower spontaneous abortion rates than in a control lab with a HEPA filtration system and a CODA Tower [15]. In another study, the effectiveness of HEPA filtration and high-activity charcoal and potassium permanganate filters for cleaning ambient laboratory air were further improved by the addition of a CODA Tower in the laboratory [16].

The improvement included decreases in the concentrations of VOC, aldehydes and particulates.

It is noteworthy that the improvement in embryo quality in the present study was achieved with CODA filtration during only 6 day of IVC, and filtration did not include the periods of IVM and IVF. It can be hypothesised that during this early phase of development, when the embryonic genome has to become activated, the atmospheric environment affected embryo quality in a subtle manner not detectable by microscopic morphology. This was in agreement with the hypothesis that the intrinsic quality of the oocyte and conditions during IVM largely determine the proportion of oocytes that develop to the blastocyst stage, whereas conditions during IVC are more important in determining embryo quality [17,18]. The possible influence of CODA air filtration during IVM and IVF remain to be investigated.

To identify the basis for the improvement in pregnancy rate in the present study, more fundamental research is needed to identify underlying mechanisms, since at the present time, little is known about preimplantation toxicology. Based on analysis of ambient air, Cohen et al. [6] demonstrated dynamic interactive processes among air handling systems, spaces, tools, disposable materials and all items unique to their IVF laboratory. Concentrations of substances differed considerably from those of adjacent spaces and may demonstrate an interaction between water-soluble and lipid-soluble solid phases such as those in incubators. For instance, culture media and mineral oil may act as a sink for different components. Even co-culture may operate by removing water-soluble traces from the immediate environment of embryos [19].

Not all studies have demonstrated an improvement embryo quality, pregnancy rates, or both, when embryos were cultured in incubators with specialized filtration units. Battaglia et al. [20] did not observe any change in embryo quality or pregnancy rate involving human embryos cultured in CODA-equipped versus non-CODA incubators. It should be noted that the degree to which laboratory air and incubatory gas atmosphere affect embryo development may depend on many factors unique to a particular laboratory and to the specific location of the laboratory. The two programs cited in which pregnancy rates were actually improved by the use of a CODA system in the incubator were located in Norfolk, VA, USA and Campinas, SP, Brazil, whereas the program in which no difference was noted was located in Seattle, WA, USA. The present study, involving cattle embryos, was conducted in a suburban area of The Netherlands. It is not possible in the present study to separate the possible contributions of contaminants from the compressed CO_2 and N_2 from the possible effect of atmospheric air that was also part of the incubator gas mixture. Perhaps the effectiveness of laboratory air and

incubator purification systems is entirely dependent on unique and specific characteristics of each laboratory and the surrounding area.

In conclusion, although many of the environmental conditions for gametes and embryos in IVP systems have been relatively standardised, there is still room to improve the overall outcome of an IVP system. We clearly demonstrated that the use of an intra-incubator carbon-activated air filtration system (CODA) during IVC of bovine embryos improved pregnancy rates following transfer of those embryos into recipient cattle.

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Chapter 5

Genetic parameters for oocyte number and embryo production within a bovine ovum pick-up-*in vitro* embryo-production program

J.S. Merton[a], B. Ask[bc], D.C. Onkundi[c], E. Mullaart[a], B. Colenbrander[c] , M. Nielen[c]

[a]*CRV BV, PO Box 5073, 6802 EB Arnhem, The Netherlands.*

[b]*Animal Breeding and Genetics Group, Wageningen Institute of Animal Sciences, Wageningen Agricultural University, 6700 AH Wageningen, The Netherlands.*

[c]*Dept. of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands.*

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Abstract

Genetic factors influencing the outcome of bovine ovum pick-up–*in vitro* production (OPU-IVP) and its relation to female fertility were investigated. For the first time, genetic parameters were estimated for the number of cumulus-oocyte complexes (Ncoc), quality of cumulus-oocyte complexes (Qcoc), number and proportion of cleaved embryos at Day 4 ($Ncleav_{D4}$, $Pcleav_{D4}$), and number and proportion of total and transferable embryos at Day 7 of culture ($Nemb_{D7}$, $Pemb_{D7}$ and $NTemb_{D7}$, $PTemb_{D7}$, respectively). Data were recorded by CRV (formally Holland Genetics) from the OPU-IVP program from January 1995 to March 2006. Data were collected from 1508 Holstein female donors, both cows and pregnant virgin heifers, with a total of 18,702 OPU sessions. Data were analyzed with repeated-measure sire models with permanent environment effect using ASREML (Holstein Friesian). Estimates of heritability were 0.25 for Ncoc, 0.09 for Qcoc, 0.19 for $Ncleav_{D4}$, 0.21 for $Nemb_{D7}$, 0.16 for $NTemb_{D7}$, 0.07 for $Pcleav_{D4}$, 0.12 for $Pemb_{D7}$, and 0.10 for $PTemb_{D7}$. Genetic correlation between Ncoc and Qcoc was close to zero, whereas genetic correlations between Ncoc and the number of embryos were positive and moderate to high for $Nemb_{D7}$ (0.47), $NTemb_{D7}$(0.52), and $Ncleav_{D4}$ (0.85). Genetic correlations between Ncoc and percentages of embryos ($Pcleav_{D4}$, $Pemb_{D7}$, and $PTemb_{D7}$) were all close to zero. Phenotypic correlations were in line with genetic correlations. Genetic and phenotypic correlations between Qcoc and all other traits were not significant except for the phenotypic correlations between Qcoc and number of embryos, which were negative and low to moderate for $Nemb_{D7}$ (–0.20), $NTemb_{D7}$ (–0.24), and $Ncleav_{D4}$ (–0.43). Results suggest that cumulus-oocyte complex (COC) quality, based on cumulus investment, is independent from the total number of COCs collected via OPU and that in general, a higher number of COCs will lead to a higher number of embryos produced. The correlation between the estimated breeding values for Ncoc and $PTemb_{D7}$ of sires in this study and the sires breeding index for female-fertility based on the Dutch cattle population was close to zero. This study revealed OPU-IVP traits ($Nemb_{D7}$, $NTemb_{D7}$, and Ncoc) that could be of potential value for selection. Introduction of such traits in breeding programs would enhance the number of offspring from superior donors as well as improve the cost efficiency of OPU-IVP programs.

Keywords: Bovine; Genetic parameters; Oocyte; OPU-IVP

1 Introduction

Since the late 1980 s, bovine ovum pick-up followed by *in vitro* production (OPU-IVP) has become an embryo production technique well known and established in both research and commercial production programs. International Embryo Transfer Society (IETS) statistics [1] indicate that besides approximately 578,000 *in vivo*–derived embryos, obtained primarily from superovulated cows, approximately 245,000 *in vitro*–produced embryos were transferred worldwide in 2007. The success and efficiency of such a program highly depends on both the quantity and the quality of the retrieved cumulus-oocyte complexes (COCs). Oocyte quality can be expressed both morphologically and intrinsically, in which the latter often is described as developmental competence or the competence of an oocyte to develop into an embryo after fertilization. Oocyte quantity and quality are both influenced by a large number of non-genetic factors; for example, OPU technician, OPU assistant, OPU interval, hormonal treatment, and parity [2]. Furthermore, embryo production efficiency, as a parameter for oocyte quality, is considered to be determined mainly during the collection and maturation phase, whereas embryo quality is thought to be determined during the *in vitro* culture phase [2] and [3].

Since the introduction of OPU-IVP, many efforts have been undertaken to improve embryo production efficiency. These were all focused on non-genetic factors. Besides some results from multiple ovulation and embryo transfer programs (MOETs), reports focusing on genetic factors in OPU-IVP programs are very limited. This approach, however, may also lead to a significant improvement of the efficiency of an OPU-IVP program by selecting donors with high *in vitro* production results. Machado et al. [4] studied the variability of OPU results and *in vitro* embryo production from monozygotic twin cows. They found substantially less variation within twin-pairs than among nonrelated cattle, suggesting the presence of a genetic component and heritability of these traits. This is in agreement with our own unpublished findings showing less variability of OPU results and *in vitro* embryo production within full-sib heifers than that among nonrelated animals.

To explore the potential of an additional way to enhance the efficiency of an OPU-IVP program, this study aimed to investigate the genetic factors influencing the outcome of OPU-IVP. For the first time, heritabilities and genetic correlations are reported for COC quality and quantity, the number and proportion of cleaved embryos at Day 4, and total and transferable embryos at Day 7. In addition, the estimated breeding values for number of COCs and

proportion of transferable embryos were compared with the sires' breeding index for female-fertility to gain insight in the relationship between those OPU-IVP traits and female fertility.

2 Materials and methods

2.1. Oocyte donor and pedigree data

The OPU-IVP data used in this study were established by CRV (formally Holland Genetics) from the open nucleus breeding program from January 1995 to March 2006. Data were collected from 1508 Holstein female donors, both cows and pregnant virgin heifers, with a total of 18,702 OPU sessions. The pedigree data used to determine the family relations among the 1508 donor animals included multiple generations (between two and five generations) (Holstein Friesian). There were between 1 and 9 known mates per sire and between 1 and 33 offspring per dam.

2.2. OPU and classification and maturation of oocytes

Immature COCs were recovered twice weekly, on Monday and Thursday, by ultrasound-guided transvaginal OPU at two nucleus herds in The Netherlands. The OPU sessions were performed by teams of two persons, with the technician responsible for the manipulation of the ultrasound probe and ovary and the assistant responsible for the punctures of the follicles. Per donor per OPU session, all retrieved COCs were treated as one batch throughout the whole IVP system. Transport and *in vitro* maturation (IVM) of the COCs took place in 2 mL maturation medium in transport vials (2-mL cryovials; Greiner, Frickenhausen, Germany). Maturation was performed for 23 to 27 h in TCM199, supplemented with 10% (vol/vol) fetal calf serum (FCS), 6 μg luteinizing hormone (LH), and 4 μg follicle-stimulating hormone (FSH)/mL (Sioux Biochemicals Inc., Sioux Center, Iowa, USA) and 0.1 mM cysteamine (Sigma, St. Louis, Missouri, USA; since January 2004) at 38.5 °C under an atmosphere of 5% CO_2 in air with maximum humidity. Cumulus-oocyte complexes were classified into four different classes designated 1 to 4, based on the cumulus investment as described before[2].

2.3. In vitro fertilization and embryo culture

After maturation (Day 0), the batches of COCs were fertilized *in vitro* with frozen-thawed gradient-separated semen. Cumulus-oocyte complexes and spermatozoa were co-incubated for 24 h at 38.5 °C under an atmosphere of 5% CO_2 in air with maximum humidity. Bulls

used for *in vitro* fertilization (IVF) were distributed over donors based on the breeding program.

After fertilization (Day 1), cumulus was removed manually from the presumptive zygotes by pipette. Through the years different *in vitro* culture (IVC) systems were used, which have previously been described[5] and [6]: (1) TCM199, supplemented with 10% FCS with a monolayer of BRL (Buffalo rat liver) cells at 38.5 °C under an atmosphere of 5% CO_2 in air with maximum humidity (March 1994 until January 1999); (2) SOFaaBSA at 38.5 °C under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 (November 1997 until April 2001); and (3) SOFaaBSA-CODA (from March 2000 onwards). This latter system was equal to the normal SOFaaBSA system but included a CODA filter unit (CODA, <genX> International, Inc., Madison, CT, USA) in the CO_2-O_2 incubator. In all systems, the presumptive zygotes were cultured for 7 d in 0.5 mL culture medium, which was changed on Day 4. All cleaved embryos were transferred into new media on Day 4. On Days 7 and 8, embryos were evaluated for stage and grade by IETS standards [7].

2.4. OPU-IVP trait definition and (co-)variance component estimation

In Table 1, a description of the traits analyzed is given. All traits, except COC quality (Qcoc), were $\log_{10}$-transformed to improve the normality of the data. Transformation did not improve the distribution of Qcoc. The transformations of the other traits improved the normality of their distributions resulting in a Gaussian distribution and improved the standard error of estimates without significantly affecting the relative breeding values between animals.

Table 1: Description of OPU/IVP traits defined. All traits were defined per donor per session.

Ncoc	Number of COCs (class 1, 2, 3 and 4) that were successfully matured, fertilized and *in vitro* cultured.
Qcoc	Quality of COC: Class 1 COCs as a proportion of Ncoc.
$Ncleav_{D4}$	Number of cleaved embryos at Day 4.
$Pcleav_{D4}$	$Ncleav_{D4}$ as a proportion of Ncoc.
$Nemb_{D7}$	Total number of embryos at Day 7. IETS stage 4 to 9 (morula, early, mid, expanded or hatched blastocyst), Grades 1 to 3 (excellent to poor).
$Pemb_{D7}$	$Nemb_{D7}$ as a proportion of Ncoc.
$NTemb_{D7}$	Number of transferable embryos at Day 7. IETS stage 4, Grade 1 (excellent or good); and stage 5 to 9, Grades 1 and 2 (excellent - fair).
$PTemb_{D7}$	$NTemb_{D7}$ as a proportion of Ncoc.

The correlations between the estimated breeding values of the non-transformed traits and the estimated breeding values of the log-transformed traits ranged from 0.91 for number of COCs (Ncoc) to 0.99 for number of embryos at Day 7 ($Nemb_{D7}$). Variance components were estimated in univariate models, and covariances were estimated in bivariate models (see equation 1). Variance components estimated in univariate analyses did not differ substantially from variance components estimated in bivariate analyses. All models were fit as repeated-measure sire models with permanent environment effect using ASREML [8]. ASREML employs a residual maximum likelihood method (REML) with the working algorithm based on generalized linear mixed models (GLMM).

The heritability (h^2), which is defined as the proportion of phenotypic variance that is explained by the additive genetic variance, was calculated as

$$h^2 = (4\times\sigma^2_{sire}) / (4\times\sigma^2_{sire} + \sigma^2_c + \sigma^2_e)$$

where σ^2_{sire} is the variance of the sire component, σ^2_c is the variance of the permanent environment component, and σ^2_e is the variance of the residual. The heritability was considered to be low (<0.15), moderate (0.15 to 0.4), or high (>0.4). The genetic correlation (r_a), which can formally be defined as the correlation between the breeding values for trait x and trait y [9], was calculated based on the estimated (co-)variances as follows:

$$r_a = \sigma_{a,xy} / (\sigma_{a,x} \times \sigma_{a,y})$$

where $\sigma_{a,xy}$ is the covariance between the breeding values for trait x and trait y, and $\sigma_{a,x}$ and $\sigma_{a,y}$ are the additive genetic standard deviations of trait x and trait y. The phenotypic correlation (r_p), which is defined as the correlation between the phenotypes of trait x and trait y, was calculated as

$$r_p = (\sigma_{a,xy} + \sigma_{c,xy} + \sigma_{e,xy}) / [(\sigma_{a,x} + \sigma_{c,x} + \sigma_{e,x}) \times (\sigma_{a,y} + \sigma_{c,y} + \sigma_{e,y})]$$

where $\sigma_{c,xy}$ and $\sigma_{e,xy}$ are the permanent environment covariance and the residual covariance between trait x and y, $\sigma_{c,x}$ and $\sigma_{e,x}$ are the standard deviations of the permanent environment

and the residual of trait x, and $\sigma_{c,y}$ and $\sigma_{e,y}$ are the standard deviations of the permanent environment and the residual of trait y. Correlations were considered to be low (<0.2), moderate (0.2 to 0.5), moderately high (0.5 to 0.8), or high (>0.8). Two models that differed in the number and type of fixed effects were used: one for Ncoc and Qcoc (equation 1) and one for the other six traits $Ncleav_{D4}$, $Pcleav_{D4}$, $Nemb_{D7}$, $Pemb_{D7}$, $NTemb_{D7}$, and $PTemb_{D7}$ (equation 2):

$$Y_{ijklmnrst} = tech_i + assist_j + par_k + tech_i*ass_j + intOPUses_l + placeOPU_m + par_k*OPUses_n + par_k*lact_o + Sire_t + PE_u + e_{ijklmnotu} \quad (1)$$

$$Y_{ijklmnopqrst} = tech_i + assist_j + par_k + tech_i*ass_j + intOPUses_l + placeOPU_m + par_k*OPUses_n + par_k*lact_o + IVMmeth_p + IVFbull_q + IVCmeth_r + coc1_s + Sire_t + PE_u + e_{ijklmnopqrstu} \quad (2)$$

where *Yijklmnrs* (equation 1) and *Yijklmnopqrs* (equation 2) represent the trait values of the individual female offspring of sire, *t*, corrected for a random permanent environment effect (*PEu*), the fixed effects in the models (described below), and *e* is the random residual term. The sire component describes one quarter of the genetic variance and is the additive genetic component of the model. The PE component is the same for all models and accounts for covariances between repeated observations on the same individual.

Fixed effects levels are shown in Table 2. Fixed effects were technician (*techi*), assistant (*assistj*), parity (*park*), time-interval between OPU sessions (*intOPUsesl*), the place of the OPU session (*placeOPUm*), an interaction between parity and OPU session (*OPUsesn*), an interaction between parity and OPU session (*park*OPUsesn*), an interaction between parity and lactation state (*park*lacto*), an interaction between technician and assistant (*techi*assistj*), IVM method (*IVMmethp*), IVF bull (*IVFbullq*), IVC method (*IVCmethr*), and number of COC class 1 (*coc1s*). This latter was chosen to correct for phenotypic COC number and quality effects during IVP.

Table 2: Description of fixed effects used in the general model to estimate genetic parameters.

	Number of classes	Ncoc, Qcoc	$Ncleav_{D4}$, $Pcleav_{D4}$, $Nemb_{D7}$, $Pemb_{D7}$, $NTemb_{D7}$, $PTemb_{D7}$
Technician	17	*	*
Assistant	22	*	*
Technician by Assistant	118	*	*
Parity	3	*	*
Parity by OPU session	127	*	*
Parity by Lactation status	3	*	*
Interval OPU sessions	5	*	*
Place OPU sessions	2	*	*
Number of COC class 1	15		*
IVF Bull	304		*
IVM method	3		*
IVC method	3		*

2.5. Relationship and correlation of a sire's female-fertility-breeding index with his daughters' OPU-IVP traits

The estimated breeding values for Ncoc and $PTemb_{D7}$ from certain sires in this study were compared with the fertility of their daughters in the Dutch cattle population. This was done by using the breeding value for female-fertility of that sire or the single breeding values that were included in this trait.

The breeding index for female-fertility is calculated based on calving interval (CIP) and nonreturn rate after 56 d (NR56) weighted respectively for 85% and 15% [10]. The breeding value for fertility is a relative breeding value that is represented on a scale with an average of 100 and a deviation of 4 points with a reliability of 80%. Only sires with a breeding value with a reliability higher than 90% were used (n = 83).

3 Results

3.1. OPU and IVP

Overall data from OPU and IVP results are shown in Table 3. The mean number of collected COCs per session was 7.8 with a wide range. The mean for number and percentage of transferable embryos was 1.1 and 14%, again with wide ranges.

To illustrate the large variation between daughters of different sires, examples are given of mean OPU-IVP results from daughters of a few extreme sires (Table 4). Number of oocytes varied from 15.1 to 4.1 and the percentage of transferable embryos from 47% to 12%.

Table 3: Descriptive statistics of untransformed OPU/IVP traits.

Trait	Median	Mean	Range
Ncoc	7.0	7.8	0-67
Qcoc	22.2%	26.6%	0-100%
$Ncleav_{D4}$	4.0	4.4	0-62
$Nemb_{D7}$	1.0	1.8	0-29
$Ntemb_{D7}$	1.0	1.1	0-23
$Pcleav_{D4}$	55.6%	53.6%	0-100%
$Pemb_{D7}$	17.7%	22.4%	0-100%
$Ptemb_{D7}$	5.9%	14.0%	0-100%

Table 4: Examples of highest and lowest scores for OPU-IVP traits based on daughters from different sires.

Trait		Sire	Daughters N	Mean	Minimum	Maximum
Ncoc	High	A	8	15.1	9.2	26.0
	Low	B	8	4.1	1.6	5.9
$Ptemb_{D7}$	High	C	16	47 %	24	65
	Low	D	12	12 %	0	36

3.2. Heritabilities and phenotypic and genetic correlations

Estimates of heritabilities and phenotypic and genetic correlations are presented in Table 5. Low heritability (<0.15) was found for Qcoc, $Pcleav_{D4}$, $Pemb_{D7}$, and $PTemb_{D7}$. Heritability was moderate (0.15 to 0.4) for Ncoc, $Ncleav_{D4}$, $Nemb_{D7}$, and $NTemb_{D7}$.

The estimates for genetic and phenotypic correlations among OPU-IVP traits varied from positive to negative and from low (<0.2) to high (>0.8).

Genetic correlation between Ncoc and Qcoc was close to zero, whereas genetic correlations between Ncoc and the number of embryos were positive and moderate to high for $Nemb_{D7}$ (0.47), $NTemb_{D7}$ (0.52), and $Ncleav_{D4}$ (0.85). Genetic correlations between Ncoc and percentages of embryos ($Pcleav_{D4}$, $Pemb_{D7}$, and $PTemb_{D7}$) were all close to zero. Phenotypic correlations were in line with genetic correlations.

Genetic and phenotypic correlations between Qcoc and all other traits were not significant from zero except for the phenotypic correlations between Qcoc and number of embryos, which were negative and low to moderate for $Nemb_{D7}$ (–0.20), $NTemb_{D7}$ (–0.24), and $Ncleav_{D4}$ (–0.43).

Table 5: Heritabilities on diagonal, phenotypic correlations above diagonal and genetic correlations below diagonal (standard errors in parentheses).

	Ncoc	Qcoc	$Ncleav_{D4}$	$Nemb_{D7}$	$NTemb_{D7}$	$Pcleav_{D4}$	$Pemb_{D7}$	$PTemb_{D7}$
Ncoc	**0.25 (0.06)**	-0.06 (0.01)	0.71 (0.01)	0.48 (0.01)	0.41 (0.01)	0.07 (0.01)	0.01 (0.01)	0.05 (0.01)
Qcoc	-0.12 (0.16)	**0.09 (0.02)**	-0.43 (0.01)	-0.24 (0.01)	-0.20 (0.01)	0.02 (0.01)	0.00 (0.01)	0.07 (0.01)
$Ncleav_{D4}$	0.85 (0.05)	-0.32 (0.15)	**0.19 (0.04)**	0.67 (0.01)	0.56 (0.01)	0.76 (0.01)	0.70 (0.01)	0.35 (0.01)
$Nemb_{D7}$	0.47 (0.12)	0.09 (0.16)	0.85 (0.04)	**0.21 (0.04)**	0.85 (0.00)	0.51 (0.01)	0.85 (0.00)	0.70 (0.01)
$NTemb_{D7}$	0.52 (0.12)	0.04 (0.17)	0.81 (0.06)	0.99 (0.01)	**0.16 (0.04)**	0.63 (0.12)	0.86 (0.05)	0.87 (0.00)
$Pcleav_{D4}$	-0.14 (0.21)	0.30 (0.21)	0.63 (0.10)	0.83 (0.07)	0.41 (0.01)	**0.07 (0.02)**	0.55 (0.01)	0.44 (0.01)
$Pemb_{D7}$	-0.13 (0.18)	0.30 (0.18)	0.92 (0.03)	0.91 (0.03)	0.72 (0.00)	0.86 (0.07)	**0.12 (0.03)**	0.80 (0.00)
$PTemb_{D7}$	0.07 (0.19)	0.29 (0.19)	0.59 (0.12)	0.92 (0.03)	0.92 (0.03)	0.65 (0.13)	0.96 (0.02)	**0.10 (0.03)**

3.3. Relationship and correlation of OPU-IVP traits with female-fertility-breeding index

The relation between the estimated breeding values for Ncoc from sires in this study and the fertility of these sires' daughters in the Dutch cattle population (the breeding value for female-fertility of each sire) is presented in Fig. 1. For both Ncoc and $PTemb_{D7}$, correlation with the breeding index for female-fertility or the single breeding values (NR56, CIP) was close to zero.

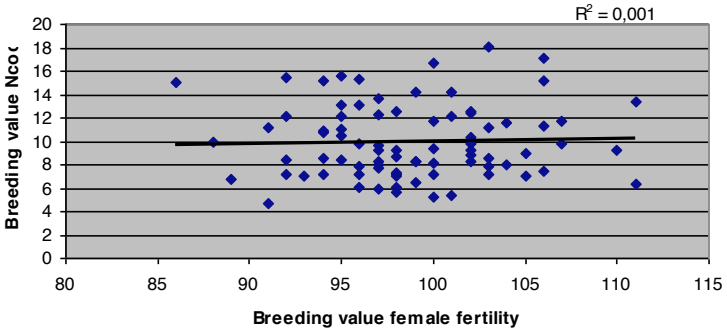

Fig. 1: Relation between the estimated breeding value for Ncoc of a sire and his breeding index for female-fertility in the Dutch cattle population.

4 Discussion

4.1. Heritability of oocyte quality and quantity and developmental competence

In the current study, heritabilities were for the first time estimated for OPU-IVP traits. In general, moderate heritabilities (0.16 to 0.40) were found for the numeric traits (Ncoc,

$Ncleav_{D4}$, $Nemb_{D7}$, and $NTemb_{D7}$), and low heritabilities (0 to 0.15) were found for the proportional traits (Qcoc, $Pcleav_{D4}$, $Pemb_{D7}$, and $PTemb_{D7}$).

The heritability for Ncoc found in this study (0.25) is in the same range as heritabilities reported for total number of flushed ova (Nova: total number of oocytes and embryos retrieved; Table 6) in MOET programs. Konig et al. [11] and Pearson et al. [12] reported a heritability for Nova in Holstein cows of 0.23 and 0.24, respectively. As both traits (Ncoc OPU and Nova MOET) share the same biological background, namely the number of follicles present in an ovary, it is reasonable to expect a similar heritability of these traits as well. It seems obvious that the number of antral follicles present during a follicular wave will subsequently affect the number of flushed ova. In fact, this has been demonstrated by Ireland et al. [16], who showed that donors can be reliably classified based on robust individual differences in numbers of antral follicles (≥3 mm) during follicular waves.

Heritabilities found for $Ncleav_{D4}$ (0.19), $Nemb_{D7}$ (0.21), and $NTemb_{D7}$ (0.16) were in line with the heritability found for Ncoc. This seems likely, as in general terms the number of embryos produced per session is limited and dependent on the number of oocytes retrieved. Again, comparable heritabilities were reported for number of transferable embryos obtained with super ovulation (Table 6; NTemb).

Table 6: Heritabilities of OPU-IVP and MOET traits (standard errors in parentheses).

Trait	Breed	OPU Ncoc	MOET Nova	OPU-MOET NTemb	OPU-MOET PTemb
This study	HF	0.25 (0.06)	-	0.16 (0.04)	0.10 (0.03)
Konig [11]	HF	-	0.23 (0.09)	0.10 (0.09)	0.10 (0.09)
Pearson [12]	HF	-	0.24	0.19	-
Piexoto [13]	Nellore	-	-	0.26 (0.13)	-
Benyei [14]	HF	-	-	0.19 (0.07)	-
Tonhati [15]	HF	-	-	0.03	-

The heritability found for Qcoc was low (0.09). Apparently, the variation in distribution of COCs among different qualities based on cumulus investment is mainly influenced by non-genetic factors other than those already used as fixed effects in the general model.

As already mentioned, all heritabilities for the proportional traits (Qcoc, $Pcleav_{D4}$, $Pemb_{D7}$, and $PTemb_{D7}$) were lower than the numeric traits (Ncoc, $Ncleav_{D4}$, $Nemb_{D7}$, and $NTemb_{D7}$). This reflects the necessity of a well-developed quality-control system as many non-genetic factors can affect the final outcome of an OPU-IVP embryo-production program.

In summary, the heritabilities found in this study do confirm previous indications for the presence of genetic variation in OPU-IVP traits as found by Machado et al. [4] and our group (unpublished) that showed less variability of OPU response and *in vitro* embryo production within monozygotic twin cows or within full-sib heifers than that among nonrelated animals.

4.2. Phenotypic and genetic correlations

The phenotypic correlation between Ncoc and Qcoc was close to zero, which indicates that the quality of the COC based on cumulus investment is independent from the total number of COCs collected via OPU.

The phenotypic correlation between Ncoc and the numeric traits was moderate for $Nemb_{D7}$ and $NTemb_{D7}$and moderately high for $Ncleav_{D4}$. This is in agreement with the numeric principle that the number of embryos produced per session cannot exceed the number of oocytes collected in that session. Similar phenomena were reported by others for superovulation [16] and [17], where in general a higher number of flushed ova also resulted in higher numbers of transferable embryos. The phenotypic correlation of Ncoc with the proportional traits was close to zero. This seems to be in contrast with observations that oocyte and embryos promote each other when cultured in groups during *in vitro* development as reviewed by Bols et al.[18]. However, in our analyses the number of Class 1 COCs was taken in the model as fixed effect to correct for this eventual (non-genetic) group culture effect, thereby removing this effect from the phenotypic correlation.

The phenotypic correlation between Qcoc and the other proportional traits was close to zero. These results are in contrast with previous findings that morphologic quality of COCs is related to oocyte developmental competence [2], [19], [20], [21] and [22]. Previously, use of Class 1 COCs resulted in a significantly higher number of embryos produced compared with that for Class 2 COCs; respectively 30% versus 19% [2] and 29% versus 8% [22]. This contradiction might be caused by the model to generate the values for the proportional embryo traits. After correction for the number of Class 1 COCs, no phenotypic correlation was left between Qcoc and the proportional traits, and a negative correlation was seen for the numeric traits.

In general, genetic correlations were in line with the phenotypic ones. First of all, this confirms the suggestion that COC quality, based on cumulus investment, is independent from the total number of COCs collected via OPU. This is an important finding as higher COC quality will result in a higher proportion of embryos produced. Although this was not shown in our phenotypic correlation (as explained above), genetic correlations were indeed found

moderate. Second, the genetic correlations confirm the suggestion that a higher number of COCs will lead to a higher number of embryos produced. Although direct selection on $NTemb_{D7}$ would be the most effective, this later finding opens the possibility for indirect selection on Ncoc due to the moderately high genetic correlation with $NTemb_{D7.}$

4.3. Correlation between OPU-IVP traits and female-fertility-breeding index

To analyze the relation of OPU-IVP traits with female fertility in the Dutch cattle population, the breeding value for female-fertility of each sire was related to their estimated breeding value for Ncoc and $PTemb_{D7}$. Correlation between female-fertility-breeding index or the single breeding values (NR56, CIP) and Ncoc was close to zero. In a normal cycle, only one growing follicle will become dominant and subsequently ovulate. Apparently, the variation in the total number of antral follicles present during a follicle wave (as indicated by Ncoc) does not influence the occurrence of an ovulation and subsequent female fertility.

We hypothesized that intrinsic oocyte quality or developmental competence, expressed in this study as $PTemb_{D7,}$ might reflect female fertility. In other words, sires with daughters having a relative high mean $PTemb_{D7}$ would also have a relatively high female-fertility-breeding index. Correlation between female-fertility-breeding index or the single breeding values (NR56, CIP) and $PTemb_{D7}$ was close to zero, however. Developmental competence of COCs is higher when maturation, fertilization, and development takes place *in vivo* compared with that *in vitro* [2] and [3]. So differences in oocyte developmental competence *in vitro*, as indicated by variation in $PTemb_{D7,}$ may not exist or be different when all developmental processes occur *in vivo*.

In conclusion, OPU-IVP traits Ncoc and $PTemb_{D7}$ do not correlate with the female-fertility-breeding index and they are therefore of no use in selection of female fertility. However, this observation also guarantees that introduction of selection on OPU-IVP traits, which could be beneficial for the efficiency of OPU-IVP programs, will not negatively affect female fertility.

4.4. Use of the heritability of OPU-IVP traits in breeding strategy

The success of breeding schemes partially depends on the number of progeny obtained from the selected superior donors by MOET or OPU-IVP. This will become even more important with the introduction of genomic selection (GS) in dairy cattle. The main advantage of genomic selection is that one can get relatively accurate breeding values already at a relatively early point in time (at birth or even at the embryo level), which makes selection possible at an economically interesting point of time. So an even more extensive use and a successful

MOET and OPU-IVP program is desired to increase the number of embryos and offspring per donor, which will subsequently allow an enhanced selection intensity for the next generation[23] and [24]. Until now, traits related to MOET or OPU-IVP are not included in breeding goals, although this may lead to a significant improvement in efficiency embryo production programs by selecting donors with high OPU-IVP efficiency results.

The current study revealed OPU-IVP traits that could be applied for selection to enhance the *in vitro* production of embryos per donor. First of all, direct selection on $Nemb_{D7}$ or $NTemb_{D7}$ will be the most effective. Second, indirect selection on Ncoc could be a good alternative due to the moderately high genetic correlation with $NTemb_{D7}$. This latter is important, because selection on Ncoc seems to be the most realistic option with respect to availability of data. Genetic evaluations require accurate estimates of parameters and, especially for OPU-IVP parameters, these data are only available from a limited number of daughters per sire and a limited number of sires. To enhance this number, one could categorize sires' daughters on the number of antral follicles present in their ovaries using a number of daily, quick ultrasound scans. This can be done irrespective of donor age as this has been shown not to influence the number of oocytes collected via OPU[25] or the number of antral follicles present during a follicular wave [26]. It will be interesting to see whether donors can be reliably classified on Ncoc before puberty in order to create more flexibility in animal logistic management and to time selection at an earlier age.

Introduction of OPU-IVP traits into a breeding goal will not only enhance the number of offspring from superior donors by OPU-IVP but also enhance cost efficiency of this specific embryo production technology. Both aspects should be taken into account by the breeding industry when weighing the pros and the cons of breeding goal adaptation.

In conclusion, in the current study we showed genetic variation in the outcome of OPU-IVP. Estimated heritabilities were found to be moderate for COC quantity and numbers of embryos produced. Heritabilities for both morphologic as well intrinsic (developmental competence) oocyte quality were lower but still present. Traits found in this study could be good candidates to be introduced in the breeding goal to enhance the efficiency and efficacy of an OPU-IVP embryo production program.

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Chapter 6

Summarizing discussion

1 Introduction

Recent statistics from the International Embryo Transfer Society (IETS) [1] indicate, that in addition to approximately 590,600 *in vivo*-derived bovine embryos, approximately 453,470 *in vitro*-produced bovine embryos were transferred world-wide in 2011. Interestingly, Ovum Pick Up followed by *In Vitro* embryo Production (OPU-IVP) has been growing rapidly in certain parts of the world, primarily in South-America during the last 5 years even though the technique has been available since the late eighties. The recent 'rediscovery' of OPU-IVP technology in Europe and North America is due, in part, to the introduction of Genomic Selection (GS) to dairy cattle improvement schemes in 2008. The success of breeding schemes depends, among other factors, on the number of progeny obtained from the selected superior donors by Multiple Ovulation and Embryo Transfer (MOET) or OPU-IVP. By using GS technology, relatively accurate breeding values can be obtained at birth or even at the embryo stage, making it possible to select desirable donors at an economically valuable point in time. As a consequence, more extensive use of successful MOET and OPU-IVP programs has become desirable to increase the number of embryos and offspring per superior donor, thereby enhancing selection intensity for the next generation [2-4]. In contrast, the growing interest in OPU-IVP in South America is due mainly to the higher efficiency of the OPU-IVP technique compared to MOET within the *Bos indicus* breed as a result of the substantially higher number of antral follicles present in the ovary compared to *Bos taurus* [5,6].

Improvements in both efficiency and efficacy of OPU-IVP programs are therefore desirable to cope with rising demand. In this respect, the success of an OPU-IVP program depends greatly on both the quantity and the quality of the retrieved cumulus oocyte complexes (COC), and both of these factors are influenced by various non-genetic factors including the expertise of OPU personnel, OPU session interval, hormonal (pre) treatment and parity of the donor cow [7]. Furthermore, embryo production efficiency is a useful indicator of oocyte quality, and is thought to be influenced primarily during the collection and maturation phase of COCs, whereas embryo quality is thought to be determined during the *in vitro* culture phase [7,8].

The work presented in this thesis focused on optimization of the efficiency and efficacy of the OPU-IVP program. Attention has been paid to both genetic and non-genetic factors, and more specifically, to the source of the COCs and other factors involved during the various steps in the OPU-IVP process. Moreover, by performing embryo transfer experiments, observations were not limited to on the embryo production stage, but also encompassed post-transfer embryo survival and calf characteristics. The results and conclusions should be of relevance

to the top end of the cattle breeding industry. In this chapter, the major findings are summarized in terms of both their immediate implications for the industry and their relevance in directing further research efforts.

2 The source of the oocyte is a key factor in the outcome of reproduction technologies

Embryo production is greatly influenced by the quality of the oocyte, which is in turn dependent on where that oocyte originated. In **Chapter 1** it was shown, by replacing the successive *in vivo* steps in the development process with *in vitro* conditions, that growth and (pre-) maturation of the oocyte is the most critical phase influencing the outcome of embryo technologies. In that respect, the quality of the population of matured oocytes ovulated during MOET is clearly superior to that generated via *in vitro* maturation of oocytes harvested by OPU.

In clinical OPU-IVP, the quality and quantity of the retrieved COCs is influenced by a large number of variables. In **Chapter 1** it was shown, for example, that OPU technician, OPU assistant, OPU interval, hormonal (pre) treatment and parity of the donor cow all affect the quality and quantity of the retrieved COCs and thereby the final outcome of an OPU-IVP session. Consequently, the choice made with respect to logistics and treatments within an OPU-IVP program will affect either the efficiency (embryos produced per OPU session) or efficacy (embryos produced per unit time). Although efficacy may seem most important with respect to shortening the generation interval and subsequent enhanced genetic gain, the efficiency of an OPU-IVP program is likewise important with regard to time and costs. In short, both production characteristics and cost effectiveness determines whether or not embryo production technologies are feasible or desirable within a commercial breeding enterprise.

In order to study oocyte growth, maturation and early embryonic development, experiments are is often performed *in vitro* using oocytes obtained from slaughtered cows. Differences between OPU- and slaughterhouse-derived oocytes can, however, affect the outcome of IVP studies. For example, in **Chapter 1**, a significant increase in blastocyst formation rate at Day 8 was observed when Menezo-B2 medium was used for *in vitro* culture of *in vitro* matured and fertilized slaughterhouse-derived oocytes rather than TCM19 [9]. However, when this

comparison was made with OPU-derived oocytes, no improvement was evident. A similar effect was found when TCM199 and SOF medium were compared (unpublished data), but the opposite effect was seen when cysteamine was introduced to the maturation medium (**Chapter 3**). The effect on OPU-derived oocytes was more pronounced than that on slaughterhouse-derived oocytes. Furthermore, in **Chapter 2** we found a more permissive limit for maturation duration for OPU-derived oocytes compared to slaughterhouse-derived oocytes.

How can these differences between OPU- and slaughterhouse-derived COCs be explained? Firstly, the origins of OPU- and slaughterhouse-derived oocytes differ. Donors undergoing regular, e.g. twice-weekly, OPU produce a more homogeneous pool of COCs, with respect to developmental stage, with fewer of them having visibly embarked on atresia. Secondly, the post-mortem period between slaughter and the start of *in vitro* maturation may be relevant. It is assumed that COCs are still under the influence of the follicle at the time of slaughter and that oocyte/nuclear maturation remains suppressed during the collection and transport of the ovaries. In fact, some cytoplasmic maturation processes may already have been initiated. Blondin et al. [10] for example, showed that embryo production was doubled when ovaries were maintained at 30^{o}C for 4 h instead of 2 h. This so called 'post-mortem effect' presumably involves a form of atresia, and is characterized by in ultra-structural changes similar to those seen in oocytes undergoing pre-maturation [11] and which are known to affect oocyte developmental competence. Thirdly, more class I COCs are obtained from slaughterhouse-derived ovaries than via OPU. Mullaart et al. [12] showed that this difference was not related to the OPU equipment or the OPU collection medium. Instead it has been suggested that, as a result of the post the post-mortem effect, the COCs become less tightly connected to the follicle wall and can therefore be collected with a higher likelihood of morphological integrity. Fourthly, a lack of oocyte selection, whereas poor quality slaughterhouse-derived oocytes are often discarded and the use of donors selected for genetic merit rather than for factors likely to promote the success of the OPU-IVP program, means that donors in many programs are of variable parity, fertility status, and may have been problem donors in a MOET program, all of which may contribute to the difference in developmental competence between OPU- and slaughterhouse-derived oocytes. In conclusion, differences between OPU- and slaughterhouse-derived oocytes may influence the outcome of embryo production experiments. This implies that results based on slaughterhouse-derived oocytes should not automatically be assumed to be valid for an OPU-IVP program.

3 Oocyte maturation determines *in vitro* embryo production efficiency

It has become evident that growth and maturation of the oocyte are the most critical factors influencing the outcome of embryo technologies. In order to improve *in vitro* embryo production, it is therefore important to focus on the period of *in vitro* maturation (IVM). Ever since the introduction of OPU-IVP technology, widespread efforts have been made to improve embryo production efficiency. Indeed, many of the environmental conditions for gametes and embryos in IVP systems, e.g. media composition, pH, water and air quality, temperature and atmospheric composition, have been studied and become relatively standardized. For example, because oxidative stress due to supraphysiological oxygen levels can lead to the formation of reactive oxygen species (ROS) and, as a consequence, may compromise oocyte and embryo culture, studies have examined the effects of reducing oxygen levels. Indeed, because excessive oxygen levels during *in vitro* culture affect embryo production [13-15], it seems likely that the cellular antioxidant defense mechanisms responsible for the balance between ROS production and scavenging are insufficient to protect the oocyte or embryo from oxidative stress imposed during *in vitro* culture (IVC) at atmospheric oxygen conditions. To protect oocytes and embryos during *in vitro* culture, oxygen levels were reduced and cysteamine was added to the culture media. In **Chapter 3** it was demonstrated that embryo production was significantly enhanced when cysteamine was supplemented during IVM of both slaughterhouse- and OPU-derived COCs. Presence of cysteamine in the maturation medium resulted in an increase in glutathione (GSH) content of the matured oocytes [16-20]. An increase in GSH level has also been shown to be associated with a decrease in ROS levels within oocytes [17,21], suggesting the scavenging effects of GSH on ROS, may enhance embryo production. Wang et al. [20] showed that increased concentrations of intracellular GSH led to higher rates of monospermic fertilization and reduce asynchrony in pronucleus (PN) formation, without affecting nuclear maturation. Other researchers reported similar findings in other species by correlating increased GSH levels with improved male PN formation [22-25]. This suggests that improved male PN formation may be one of factors contributing to improved IVP rates. Indeed, it may help to explain why there appears to be an interaction between cysteamine supplementation and sire, that results in a bigger improvement in embryo development for OPU- than for slaughterhouse-derived COCs. In a recent study Merton et al. (unpublished data) showed an interaction between the effect of cysteamine and sires used for IVF. The increase in percentages of embryo formation varied between 1% and 42% among a group of 17 bulls, where the difference may have

originated from variation in sensitivity of sperm from different bulls to suboptimal levels of ROS during fertilization. In a study by Kim et al. [26], in which GSH was supplemented during IVF, adding 1 mM glutathione during *in vitro* fertilization (IVF) affected blastocyst formation, but was bull dependent (n=4), with two bulls exhibiting negative, one neutral and one positive effects. Further research is needed to understand the impact of oxidative stress on *in vitro* embryo production and to elucidate any possible correlations with the *in vivo* fertility of bulls.

Commercially, an important question is whether the intrinsic quality of the cysteamine-supplemented medium produced embryo is similar to that of morphologically similar embryos produced under control conditions. This question is particularly relevant, if we are to determine whether the presence of cysteamine during IVM not only 'rescues' COCs of poorer quality but also restore their developmental competence. The intrinsic quality of IVP embryos may be affected without this being morphologically visible and may, therefore, only become evident later in development or life [27,28]. For example, gene expression [29], respiration [30], cell number [31], cryotolerance (Merton, unpublished data) and the ability to establish a pregnancy [32; Chapter 4] can differ among embryos within a morphologically equal population. Based on the results presented in this thesis, however, embryos originating from oocytes exposed to cysteamine during *in vitro* maturation do not differ with respect to cryotolerance, post-transfer embryo survival and calf characteristics such as gestation length, birth weight, perinatal mortality and sex ratio.

Another aspect of IVM that significantly affects *in vitro* embryo production, is the duration of the IVM incubation. In general, the optimal duration of *in vitro* maturation of bovine oocytes is thought to be 22-24 h. For slaughterhouse-derived COCs the optimal maturation duration in terms of embryo production varied among studies: 18 h [33], 22 h [34], 24 h [35], 18 to 24 h [36] and 19 to 24 h [7]. The importance of the variation in 'optimal' maturation time becomes very clear when one considers that the majority of these studies reported a marked drop in embryo production rate when the optimal maturation time was exceeded. Therefore, most IVP laboratories are now very strict on the duration of the maturation period, and this has resulted in equally strict guidelines with regard to the time of OPU collections on the previous day. As described in **Chapter 2,** embryo production from OPU-derived COCs in the CRV program was not affected when oocytes were matured *in vitro* between 16 and 28 h. Furthermore, as with cysteamine supplementation during IVM, the duration of IVM for OPU-derived COCs did not affect embryo cryotolerance, post-transfer embryo survival or calf characteristics such as gestation length, birth weight and sex ratio.

The results presented in **Chapter 2** suggest that OPU-derived oocytes are either mature and ready to be fertilized after 16 h of in vitro maturation and maintain this capacity for at least 9 h, or that oocytes can complete their maturation during the first hours of *in vitro* fertilization. Either or both of the above probably apply since oocytes have been reported to reach the MII stage of nuclear maturation as early as 15 h or as late as 24 h after the onset of culture culture [34,37,38].

In conclusion, the more permissive limits for maturation duration for OPU-derived oocytes compared to slaughterhouse-derived oocytes makes it possible to fertilize COC batches collected by OPU the previous day in one IVF session, without a significant decrease in the percentage of oocytes that give rise to a calf.

4 Culture conditions affect embryo quality

Since the introduction of IVP, the IVC step has been studied intensively, resulting in the establishment of culture conditions capable of supporting early embryonic development at a rate similar to that for *in vivo* development. However, many laboratories have changed their co-culture IVC system to SOF because the co-culture system was shown to be at least partially responsible for the LOS [39-41]. Although SOF systems have now become more or less the standard for IVC, OPU-IVP is still characterized by lower pregnancy rates compared to MOET. Moreover, it is well established that embryo quality and the ability to establish a pregnancy are determined largely by conditions during culture [7,8,41,42]. In order to improve pregnancy rates after embryo transfer, there needs therefore to be renewed focus on the IVC culture conditions.

In **Chapter 4** we studied the effect of ambient air purification on embryonic development and post-transfer survival. In general, air pollutants such as volatile organic compounds (VOCs), small inorganic compounds and heavy metals impact laboratory and incubator environments and may affect IVP outcome. The presence of the CODA intra-incubator air purification unit did not improve the embryo development rate or morphological quality of those embryos. However, pregnancy rates increased significantly for both fresh and frozen/thawed embryos. Similar results were reported for human embryos by Mayer et al. [43]. Indeed, although Mayer et al. did not find an effect on the number or quality of embryos produced, pregnancy rate for embryos cultured in the CODA system were improved significantly. These observations strongly suggest an improvement in embryo developmental potential, which may not be detectable by routine morphological evaluation. By contrast, Racowsky et al. [44]

reported a significant decrease in the morphological quality of human embryos when using a CODA system. In this respect, it has been suggested that the reported increase in the number of fragmented cells per embryo provides a means by which embryos can discard compromised cell and thereby improve their developmental competence; use of CODA air purification unit filters might facilitate this process.

It is noteworthy that the improvement in embryo quality in the present study was achieved with ambient air purification provided only during the period of *in vitro* embryo culture. It therefore seems reasonable to hypothesize that during this early phase of development, when the embryonic genome is activated, an optimal culture environment is essential to ensure subsequent establishment of a normal pregnancy. More fundamental studies are needed to identify underlying mechanisms, since at the present our understanding of pre-implantation toxic effects is limited.

In conclusion, we demonstrated that the use of an intra-incubator carbon-activated air filtration system (CODA, <genX> International, Inc.) during *in vitro* culture of embryos increased pregnancy rates in cattle resulting in an improvement of both the efficiency and effectiveness of the OPU-IVP program.

5 Heritability of OPU-IVP parameters

Most of the efforts that have been undertaken to improve embryo production efficiency have focused on non-genetic factors. However, better understanding of genetic factors may also lead to significant improvement in the efficiency and efficacy of OPU-IVP programs by enabling the selection of donors with the potential for better *in vitro* production results. Beyond some preliminary results from multiple ovulation and embryo transfer programs (MOET), however, reports focusing on genetic factors that influence the success of bovine assisted reproduction programs are limited. To explore the potential of an additional avenue to enhance the efficiency and effectiveness of an OPU-IVP program, in **Chapter 5** the genetic factors influencing the outcome of OPU-IVP were investigated. For the first time, heritability and genetic correlations were reported for COC quality and quantity, along with the number and proportion of cleaved embryos at Day 4 and the number and proportion of both the total and transferable embryos at Day 7. In addition, the estimated breeding values for the number of COCs and the proportion of transferable embryos were compared with the sires' breeding index for female-fertility in order to better understand the relationship between these OPU-IVP traits and female fertility.

In general, the numeric traits (number of COCs collected and embryos developed) were moderately heritable, whereas the proportional traits (percentage of good quality COCs collected and embryos developed) yielded low heritability estimates. It seems likely that the heritability for the number of embryos developed was related to the heritability for number of COCs (Ncoc) since, in general, the number of embryos produced per session is limited by and dependent upon the number of oocytes retrieved. This was confirmed by the phenotypic correlation between number of COCs and the other numeric traits. The weak heritability found for the proportional traits indicate that non-genetic factors other than those used as fixed effects in the general model also affect the efficiency of IVP. Therefore a well-developed quality control system should be put in place to minimize the variation in production results.

The correlation between female-fertility-breeding index or the individual breeding values [calving interval (CIP) and non-return rate after 56 days (NR56)] and Ncoc was close to zero. Apparently, the variation in the total number of antral follicles present during a follicle wave (as indicated by Ncoc) does not influence the likelihood of ovulation or subsequent female fertility. Moreover, correlation between female-fertility-breeding index or the individual breeding values (NR56, CIP) and the percentage of transferrable embryos at Day 7 (PTembD7) was also close to zero. We hypothesized that oocyte quality or developmental competence, expressed in this study as PTembD7, reflects female fertility. In other words, sires with daughters that have a relatively high mean PTembD7 would also be expected to have a relatively high female-fertility-breeding index. Surprisingly however, this assumption was not confirmed. One may conclude that differences in *in vitro* oocyte developmental competence, as indicated by variation in PTembD7, either do not exist or may be altered when all developmental processes occur *in vivo*.

6 Implications

The results presented in this thesis provide a source of useful information for future attempts to improve both the efficiency and efficacy of OPU-IVP programs in commercial breeding enterprises. Understanding the importance of the origin of the oocyte and its effect on embryo production, together with the factors that influence oocyte and embryo quality, may help to achieve an optimal balance between production characteristics *per se* and efficiency. Although efficacy may seem most important with respect to shortening the generation interval

and thereby promoting genetic improvement, the efficiency of the OPU-IVP program is arguably just as important because of its implications for both time and financial investments.

Well trained technicians both for OPU and for the laboratory processes are a major element of success. Of particular importance are situations when OPU is performed within a small enterprise with only one OPU technician/team available. The outcome of OPU-IVP will then be determined to a substantial extent (up to 50%) by the single OPU team, and will determine the quality and quantity of COCs available for IVP. With respect to the number of COCs, the effect is two-fold. Firstly, more COCs is likely to result in more embryos and, secondly, culturing larger groups of COCs leads to a beneficial group-effect that increases the number of embryos produced.

OPU interval and follicle stimulation treatment are the two major tools that determine the optimal production schedule either in a collection center or on farm. When the absolute number of embryos produced per unit time is most important (efficacy of the program), a schedule in which OPU is conducted two or even three times per week is preferable. However, if cost efficiency should take precedence, a protocol with a 2-week interval combined with FSH pre-stimulation should be considered.

The flexibility of the OPU-derived COC with respect to maturation duration time, creates a major logistical advantage. Not only is the amount of labor needed in the laboratory reduced, but fertilizing all COC batches collected by OPU on the same day in a single IVF session also creates a more efficient use of (sometimes very expensive) semen from different bulls, and complex production schedules to group donors based on the sire can be avoided.

A real breakthrough was the discovery of the apparently 'simple' extra protection of the COC provided by supplementing the medium with cysteamine during IVM. This improved the efficiency of OPU-IVP and has a major impact on the cost price of the embryos produced. In addition, the efficacy of the OPU-IVP program is enhanced since improved oocyte competence also leads to an increase in the number of offspring. This facilitates a significant increase in selection intensity and shortening of the generation interval, helping to fulfill a major goal of the breeding industry, namely enhanced genetic improvement.

Beside all the non–genetic factors, the results presented in this thesis also revealed OPU-IVP donor traits that could be amenable for selection to enhance the production of IVP embryos per donor. This can be achieved either by direct selection for the number of transferrable embryos produced (NTembD7) or indirectly for the number of COCs collected (Ncoc). This latter parameter is probably the most feasible option with respect to the availability of appropriate data. One could categorize sires' daughters by the number of antral follicles

counted (AFC) in their ovaries during two-four consecutive follicular waves using ultrasonography [45]. This can be done regardless of (1) their age, breed or country of origin; (2) season or stage of lactation; (3) span of time between AFC measurements in an individual (up to 15 months); or (4) the operator conducting the ovarian ultrasonography [45-47]. More recently, measurement of blood Anti-Mullerian hormone (AMH) concentration levels was shown to be a simpler, although somewhat expensive, approach to assess the size of the ovarian follicle pool. In cattle ovaries, ovarian AMH, a member of the TGFß superfamily of growth factors, is expressed exclusively by granulosa cells of the preantral and small antral follicles [48,49]. Moreover, circulating AMH concentrations are positively correlated with the total number of healthy follicles and oocytes in the ovaries. Since these concentrations are highly variable among animals, but highly repeatable within individuals, they can be used as a reliable biomarker for the growing follicle pool [45,49].

It may be interesting to classify potential donors by AMH concentrations before puberty, in order to select at a younger age and create more flexibility in the animal logistical management. Monniaux et al. [49] showed that, in beef heifers, plasma AMH concentrations increased between 1 and 3 months of age, remained high at 6 months, and then declined slowly until 12 months of age, the age of puberty for heifers of this breed. However, to what extend the changes in AMH concentrations reflect changes in the number of small antral follicles and/or AMH secretion capacity of individual follicles has not been fully assessed.

Because the heritability of the number of COCs collected is moderate, the number of antral follicles present in an ovary or the number of COCs collected by OPU is also influenced by other factors, known and unknown. Evans [50] for example showed that restricting the nutrition of beef heifers to 0.6 of their maintenance energy requirements, from shortly before conception to the end of the first trimester of pregnancy, resulted in calves which had 60% lower peak, minimum and mean numbers of AFC during follicular waves compared to calves born to mothers fed control diets. This low number of follicles was correlated with cow fertility later in life. Cows with a high AFC had higher pregnancy rates, shorter calving to conception intervals and received fewer services during the breeding season compared to cows with a low AFC [51]. Another study in beef heifers also showed higher pregnancy rates in heifers with high an AFC versus those with a low AFC [52]. This phenomenon is also in line with Ireland [45]'s hypothesis that low AFC was correlated with low circulating progesterone concentrations during the estrous cycle and a higher abundance of intra-follicular markers for poor oocyte quality, which provides a reasonable physiological explanation why heifers with a relatively low quantity of follicles and oocytes in their ovaries

should also exhibit suboptimal fertility. In our studies, we did not find a correlation between the Ncoc trait, which also indirectly reflects the number of follicles in the ovary, and daughter fertility. This contradictory result may have been a result of the inclusion of the bull effect and the larger scale of the experiment in our study.

In conclusion, feeding strategy, although not included in the scope of this thesis, is another very important factor affecting OPU-IVP outcome. Standardizing the housing and feeding strategy of both donors and recipients appears to be favorable in terms of enhancing the predictability of the AFC or AMH measurement.

Introduction of OPU-IVP traits into a breeding program will not only enhance the number of offspring from superior donors by, it will also improve the cost efficiency of embryo production technology. Both aspects should be taken into account by the breeding industry when weighing up the pros and the cons of breeding goal adaptation.

7 Future consideration

7.1 Use of OPU-IVP

With the introduction of GS, OPU-IVP has become of greater value to the cattle breeding industry. This has led to a new wave of interest in OPU-IVP and started a 'second embryonic revolution'. As a result, the advantages of OPU-IVP as compared to MOET have become clearer. While for many (private) breeders, the level of embryo production via MOET was satisfactory, production was spread across many donors and OPU-IVP was seen a little more than an alternative. Today, OPU-IVP is considered to guarantee production of many embryos from selected donors in a short period of time. This goal may be difficult to achieve via MOET if donors respond poorly or even at an average level to superovulation. Furthermore, even during the early stages of pregnancy in young donors, embryo production can continue, thereby provides a way of entering these elite cows into the performance testing system at a normal age. However, with increasing selection intensity, the pressure to produce embryos will become more important than the availability of performance test data, since the added value of this test over and above modern GS Breeding value is limited. Therefore, one can expect that selected bull dams will first finish their embryo production lifecycle before they are allowed to enter a milk production phase. A summary of the effects of GS and reproductive technologies on genetic gain is shown in Figure 1.

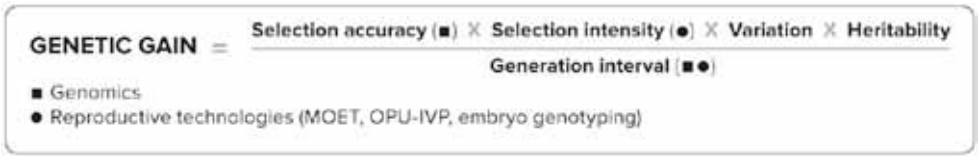

Figure 1: The relationship between genetic gain and the use of genomics and reproductive technologies.

7.2 New developments

Now that OPU-IVP programs are being used more extensively world-wide, implementation of new developments is expected to enhance genetic improvement even further:

Pre pubertal embryo production. In order to shorten the generation interval, embryo production can be started in animals that have yet to reach puberty. For example, at Trans Ova Inc. (USA) embryo production is initiated when heifers reach 6 months of age using adapted OPU equipment and hormonal pre-treatment. Although successful, efficiency is much lower than in donors at a mature age. Embryo production from even younger cows is also possible. Using a surgical approach by laparotomy, oocytes can be collected from calves as young as 2-3 months for embryo production [41, 53-55].

In vitro pre-maturation. When an immature COC is removed from its follicular environment, germinal vesicle breakdown is triggered and the oocyte enters final nuclear maturation. For a number of oocytes, however, this final maturation may be too early with regard to cytoplasmatic pre-maturation, which may not yet have been completed, but is essential for the oocyte to be developmental competent. In order to ensure the completion of pre-maturation *in vitro*, strategies have been developed to postpone meiotic resumption *in vitro*. Mermillod et al. [56] showed that the use of roscovitine (ROS), a potent inhibitor of M-phase Promoting Factor kinase activity (MPF), resulted in a meiotic inhibition, that was fully reversible and resulted in an embryo production efficiency equal to that of an unexposed control group. Ponderato et al. [57] showed that the MII kinetics of butyrolactone I and ROS-inhibited oocytes were accelerated compared to their control counterparts, with 50% of the inhibited oocytes reaching the MII stage at 13-14 h compared to 18 h for control oocytes. It was

therefore necessary to adjust maturation time to 16 h, instead of the regular 22 h, to achieve the same embryo production rate as in the control group. More recently however, Albuz et al. [58] heralded a breakthrough when they reported both a postponement of meiotic resumption and a significant improvement in embryo production rate. They developed an IVM protocol that included both a pre-IVM period and an extended IVM period. During the pre-IVM period of 2 h, the cAMP concentration in the COC was raised using the adenylate cyclase promotor forskolin (FSK) [59] and the phosphodiesterase (PDE) inhibitor IBMX, to prevent degradation of cAMP and maintain the oocyte at meiotic arrest. During the IVM phase of 30 h, COCs were cultured in the presence of cilostamide (a specific PDE inhibitor that acts only in the oocyte) and a relatively high dose of FSH to induce oocyte maturation by overriding the meiosis-inhibiting effects of the elevated cAMP levels.

Genomic selection on embryos. Although genomic analysis of embryo biopsies is still under investigation, it is already being implemented by the cattle breeding industry [60]. It will be used to enhance selection intensity at an early phase of reproduction, and will have a major impact on the costs of the total breeding program, since it will allow the size of the recipient herd to be reduced substantially. GS of embryos will also create a demand to ensure the birth of a calf with the desired genetic composition.

Embryo multiplication. Implementation of cloning technology seems to be a less desirable because it does not lead to genetic improvement in the next generation. Although embryonic cell nuclear transfer (ECNT; 'embryonic cloning') has been possible since the late eighties, it never became widely utilized commercially. Cloning by somatic cell nuclear transfer (SCNT; 'somatic cloning'), however, as first reported for the birth of 'Dolly' [61], opened up new possibilities and initiated large scale research world-wide. For strategic reasons, breeders may want to produce a genetic copy of a proven bull or bull dam to ensure the production of semen or embryos, in case the original animal dies by accident or from disease. However, the procedure of somatic cloning is associated with significant losses during pregnancy and in the perinatal period, reducing the overall efficacy to less than 5% in most cases [62]. With the implementation of GS of embryos, multiplication of a selected embryo either by splitting or embryonic cloning may become a standard procedure to ensure the birth of a calf with the desired genetic composition. Furthermore cloning techniques could have a powerful role in disseminating genes from the nucleus to the commercial herds. The advantage of ECNT compared to SNCT is that bovine morula cells are much more efficient donors than skin

fibroblasts and greatly reduce the incidence and severity of late-gestation abnormalities, such as placentome malformation, hydroallantois, and calving difficulties [63]. In conclusion, the development of GS means that embryonic cloning can now contribute to enhanced genetic improvement: this may, just as for OPU-IVP, result in a second wave of interest.

Stem cell culture. Embryonic stem cells (ES) or induced pluripotent stem cells (iPS) may facilitate embryonic multiplication. Since the efficiency of embryonic cloning is still low, the number of cloned calves born from a single embryo may not be satisfactory. Production on a larger scale using stem cells may ensure the birth of a calf with the desired genetic composition. The use of stem cells may also open up another possibility: the differentiation of stem cells into gametes. In the quest for reducing the generation interval, in 1998 Haley and Visscher [64] presented a vision they termed 'velo genetics' in which the generation interval is further reduced by producing and collecting gametes from stem cell cultures followed by IVP. Hayashi et al. [65] showed that, in the mouse, this approach is possible, although an '*in vivo* step' (embryonic stem cells or induced pluripotent stem cells aggregated with female gonadal somatic cells to make reconstituted ovaries) was necessary to initiate the process of meiosis. However, the use of this technique in cattle breeding could be 10 or even 50 years in the future. If it were implemented, this would mean a shift of the entire breeding cycle from the barn to the laboratory. As an example, Figure 2 shows the implementation of all the new developments within a breeding scheme.

Gene editing. As a result of further development of genomic technologies, complete genome sequences are now available for cattle, making it possible to comprehensively examine gene expression profiles in this species [66]. Stem cells either derived from an embryo or adult animal, may well be the basis for the next step in assisted breeding, namely gene editing. Depending on the traits of interest, genes may be introduced, or up- or down-regulated. This so called 'precision genetics' or 'precision editing' [67] will make it possible to introduce, for example, the 'polled gene' (hornless trait) into the desired next generation.

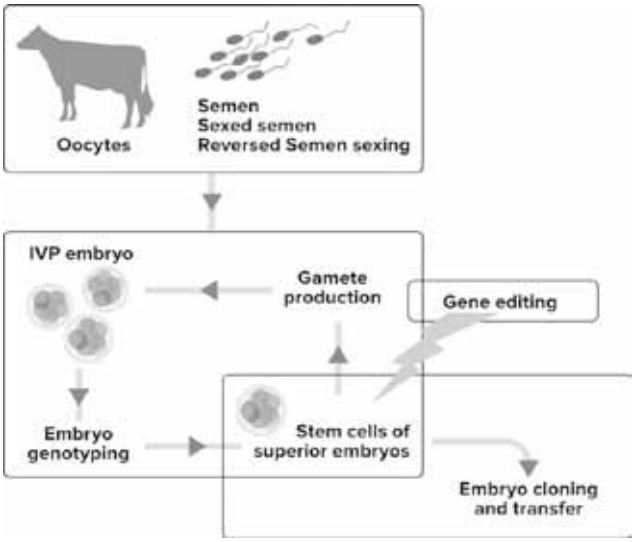

Figure 2: An example of a breeding scheme in which cutting edge developments are implemented.

7.3 ET industry

The 'second embryonic revolution' will have an enormous impact on the organization of the ET and the cattle breeding industries. Firstly, as cost is a key factor, only breeding companies or large-scale ET-IVP companies will be able to invest enough capital to provide all the services demanded. As a consequence, the role for the local ET practitioner in this chain will move towards simply transferring embryos and customer relations. Secondly, differences in regulation per continent or country with respect to the legality of new biotechnologies and their social acceptance, creates unequal playing-fields. Therefore global breeding companies may have to choose where to operate.

7.4 Ethical considerations

It is obvious that, alongside the implementation of new reproductive technologies, many ethical aspects are involved. This is reflected by the differences in regulations in various countries. In order to keep their 'license to produce', cattle breeding companies should be aware of the importance of social acceptability and act accordingly. As mentioned above, globalization of the breeding industry creates unequal playing-fields and may create huge dilemmas for specific companies. Transparency will, however, be critical in this process of entering the new era of breeding.

7.5 Research

The 'second embryonic revolution' will only be successful if further research is conducted at fundamental, applied and field-study levels. Fundamental research should address oocyte growth and (pre-) maturation, but also embryo multiplication, stem cell and gamete production. Here, it is important to take into account the existing difference among study materials. For example the enormous variation in reproductive characteristics between animals is well known. This raises the question as to which animals we should use for research. For example, do we take large numbers of animals to randomize and minimize any differential effects, or should we select specific individuals? In practice, animals with extreme embryo production results can be found. Maybe we should take both extremely good and extremely bad donors as reference material, and learn from this variation instead of ignoring it. Applied research should address the efficacy and efficiency of the OPU-IVP program, as there still seems to be room improvement of many factors, including both donor and recipient management, where both significantly affect the overall results of an embryo technology program. Within this field, research is often based on oocytes obtained from slaughterhouse ovaries. We have shown, however, that results based on slaughterhouse-derived oocytes cannot automatically be extrapolated to the OPU-IVP situation. Therefore, it is advisable, as a third step in the research approach, to perform comparative field studies based on OPU-derived oocytes prior to full implementation. Furthermore, applied research is often performed with semen from a single reference bull, selected for a high *in vitro* embryo production outcome. However, results obtained with semen from a single bull cannot automatically be extrapolated to results for other bulls. Consequently, laboratories should consider not using semen only from one proven bull, but also semen from moderate and or poorly performing bulls in order to verify any conclusions drawn.

In conclusion, the introduction of GS, the increased use of biotechnology and increasingly narrowly targeted *in vitro* embryo production schemes have become the most important and differentiating factors within the cattle breeding industry. Introduction of new technologies will enhance genetic improvement even further. An exciting future lies ahead of us.

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Samenvatting

Samenvatting

De toepassing van embryoproductietechnieken bij het rund wordt met name gedreven door de wens van fokkerijorganisaties om de genetische vooruitgang bij opeenvolgende generaties melk- en vleesvee te vergroten. Door productie van grote aantallen embryo's en daarmee geboren kalveren, afkomstig van geselecteerde stiermoeders, wordt het mogelijk om de selectie-intensiteit en het generatie-interval, twee belangrijke parameters die van invloed zijn op de genetische vooruitgang, respectievelijk te vergroten en te verkleinen. Naast de reeds beschikbare *in vivo* productiemethode genaamd MOET (*Multiple Ovulation followed by Embryo Transfer*) volgde eindjaren tachtig de introductie van de *in vitro* productiemethode van embryo's genaamd OPU-IVP (*Ovum Pick-Up followed by In Vitro embryo Production*). Deze laatste techniek heeft niet alleen het voordeel dat de embryoproductie kan worden verdubbeld in vergelijking met MOET, ook is het mogelijk embryo's te produceren tijdens de eerste maanden van de dracht van de eiceldonor. In 1996 implementeerde de Nederlands-Vlaamse fokkerijorganisatie CRV (en haar rechtsvoorgangers) deze *in vitro* embryoproductiemethode binnen haar vrouwelijke fokprogramma. Door de productie van embryo's tijdens de 2e en 3e maand van de dracht, worden kort na het bekend worden van de eerste melkproductiegegevens van deze potentiële stiermoeders, reeds de eerste kalveren geboren. Voorheen zou men pas na het verschijnen van deze eerste melkproductiegegevens met de productie van embryo's kunnen beginnen; inderdaad is er hierdoor sprake van een aanzienlijke verkorting van het generatie interval.

De toepassing van de OPU-IVP techniek brengt echter ook enkele nadelen met zich mee. Zo is het drachtigheidspercentage van getransplanteerde *in vitro* geproduceerde embryo's lager dan die van *in vivo* ontwikkelde embryo's. Bovendien werden er in de beginjaren meer kalveren geboren met afwijkingen die bekend staan als het '*Large Offspring Syndrome'*. Dit syndroom is echter voor een groot deel verholpen door de introductie van een ander embryokweekmedium eind jaren negentig. De komst van *genomic selection* (GS), het selecteren van kalveren op basis van een genomische fokwaarde berekend aan de hand van het DNA profiel van het betreffende kalf, heeft de behoefte binnen de fokkerij naar een efficiëntere en effectievere OPU-IVP methode opnieuw versterkt. Door het implementeren van GS wordt de nauwkeurigheid van selectie van de volgende generatie stiermoeders vergroot, met als gevolg dat van minder genetisch hoogwaardige donoren meer embryo's

zullen worden geproduceerd, om op deze wijze tot een nog hogere selectie-intensiteit te komen en daarmee een grotere genetische vooruitgang.
In dit proefschrift is onderzocht welke factoren van invloed zijn op de efficiëntie en effectiviteit van het OPU-IVP programma. Hierbij is met name gekeken naar de *in vitro* rijping (maturatie; IVM) van de eicel, omdat de efficiëntie van de embryoproductie voor een groot deel wordt bepaald door de kwaliteit van de gerijpte eicel. Binnen het proces van de rijping van de eicel is gekeken naar het effect van de samenstelling van het IVM medium en de duur van het *in vitro* rijpingsproces. Naast de *in vitro* eicelrijping is ook gekeken naar de omstandigheden tijdens de *in vitro* embryokweek (*culture*; IVC), daar de embryokwaliteit voor een groot deel wordt bepaald tijdens de vroeg embryonale ontwikkeling. Tenslotte zijn ook potentieel interessante erfelijke factoren bestudeerd die mogelijk de 'geschiktheid' van donoren bepalen bij de toepassing van de OPU-IVP techniek.
Een belangrijk aspect binnen het beschreven onderzoek is het feit dat resultaten niet alleen zijn gebaseerd op eicellen verkregen uit eierstokken van geslachte koeien, maar ook op eicellen van donoren uit het commerciële OPU-IVP programma. Daarnaast bevat dit onderzoek de resultaten van grootschalige embryotransplantatiestudies waarmee in het onderzoek niet alleen de effecten op de embryoproductie, maar ook op de invriesbaarheid van het embryo, de vroeg embryonale ontwikkeling en kenmerken van het geboren kalf zijn geanalyseerd.

Zoals reeds genoemd, wordt de efficiëntie van de embryoproductie voor een groot deel bepaald door de kwaliteit van de gerijpte eicel. In **Hoofdstuk 1** worden de verschillende groei fasen beschreven welke een eicel doorloopt voordat deze in staat is de finale rijping te ondergaan. Daarnaast wordt er inzicht gegeven in een aantal praktische factoren welke de kwaliteit van een eicel (ontwikkelingscompetentie) reeds bepalen voor de start van het in vitro rijpingsproces en daarmee direct van invloed zijn op het succes van een embryoproductie programma.

De efficiëntie van de *in vitro* embryoproductie wordt in sterke mate bepaald door de *in vitro* rijpingsduur van de eicel. Een optimale productie wordt bereikt wanneer slachthuiseicellen 18 tot 24 uur worden gerijpt. Een langere periode leidt tot veroudering van de eicel en daarmee een significante afname in embryoproductie. In **Hoofdstuk 2** is de optimale rijpingsduur voor OPU, *in vivo* gewonnen, eicellen onderzocht. Binnen een rijpingsduur van 16 tot 28 uur kon geen nadelig effect worden aangetoond met betrekking tot embryo productie, embryo

invriesbaarheid, draagtijd, afkalfpercentage en het gewicht en geslacht van het geboren kalf. Dit betekent dat de *in vitro* bevruchting (fertilisatie; IVF) van de verschillende OPU-eicel batches, gewonnen gedurende de gehele voorafgaande dag, beperkt kan blijven tot een sessie op de dag van de bevruchting en daarmee substantieel van invloed is op de efficiëntie van het OPU-IVP proces.

Volgens een aantal studies beïnvloedt het zuurstof niveau tijdens IVC de embryoproductie. Wanneer het intracellulaire antioxidant mechanisme te kort schiet, dan kan dit leiden tot de aanwezigheid van een te veel aan zuurstofradicalen wat ook wel bekend staat als oxidatieve stress. In **Hoofdstuk 3** is het effect beschreven van de toevoeging van cysteamine (bekend om de antioxidant werking) aan het IVM en IVC medium op de embryoproductie van slachthuis- en OPU-eicellen. Toevoeging van cysteamine aan het IVM medium leidde tot een significante toename in embryoproductie. Verrassend genoeg was dit effect bij OPU-eicellen aanzienlijk hoger dan bij slachthuis-eicellen (een relatieve toename van 47% versus 23%). De hogere embryoproductie had geen nadelig effect op het drachtigheidspercentage, draagtijd en het gewicht en geslacht van het geboren kalf bij zowel verse als ingevroren embryo's. Geconcludeerd kan worden dat een extra bescherming van de eicel tegen oxidatieve stress kan leiden tot een significante verbetering van de ontwikkelingscompetentie van de gerijpte eicel. Deze op het oog simpele aanpassing heeft uiteindelijk een groot effect op zowel de efficiëntie als de effectiviteit van het OPU-IVP programma en daarmee de gewenste grotere genetische vooruitgang.

De broedstoof in het laboratorium is als het ware de 'eileider en baarmoeder' waarin de initiële productie van embryo's plaats heeft en waarin de kweekomstandigheden voor eicel en embryo zoveel mogelijk worden gestandaardiseerd. Een van deze factoren is de samenstelling van de gasfase in de broedstoof welke in vele gevallen is samengesteld uit O_2, CO_2 en N_2 en waarbij de laatste twee afkomstig zijn uit aangesloten gastanks. Deze gasfase kan verontreinigd zijn met vaste en vluchtige deeltjes (organische en anorganische moleculen en zware metalen) afkomstig van de geïnjecteerde gassen en buitenlucht, maar ook van de gebruikte plastics, media en de broedstoof zelf. Enkele kleine studies in het veld van de humane IVF toonden een mogelijk negatief effect aan van deze verontreiniging op het zwangerschapspercentage. In **Hoofdstuk 4** wordt het effect beschreven van het gebruik van een filtratie-unit (gebaseerd op actieve kool) in de broedstoof tijdens de IVC periode. Hierbij is gekeken naar zowel de embryoproductie als het drachtigheidspercentage na transplantatie

van de geproduceerde embryo's. Het gebruik van het filter was noch van invloed op de embryoproductie noch op de morfologische kwaliteit van de embryo's. Daarentegen lieten de resultaten een significante verbetering zien in het drachtigheidspercentage van zowel verse als ingevroren embryo's.

Naast het streven tot een optimalisatie van de OPU-IVP methode en daarmee maximalisatie van de embryoproductie, kan het selecteren van stiermoeders op basis van 'geschiktheid' voor de OPU-IVP techniek in potentie een aanzienlijk effect hebben op de efficiëntie en effectiviteit van een OPU-IVP programma. De resultaten van het onderzoek naar de erfelijkheidsgraad van aan OPU-IVP gerelateerde kenmerken staan beschreven in **Hoofdstuk 5**. De erfelijkheidsgraad van de numerieke kenmerken 'eicelaantal per OPU sessie' en 'embryo aantal per OPU sessie' was matig (0.16-0.25), die van de proportionele kenmerken 'eicelkwaliteit' en 'embryopercentage' laag (<0.15). De genetische correlatie tussen 'eicel aantal' en 'eicelkwaliteit' was nagenoeg nul, die van 'eicel aantal' en diverse 'embryo aantal' kenmerken was hoog (0.47 – 0.85). Geconcludeerd kan worden dat er een genetische variatie bestaat in het te verwachten resultaat van een OPU-IVP sessie. Bovendien laten de resultaten zien dat het mogelijk moet zijn donoren te selecteren op het kenmerk 'eicelaantal' hetgeen, mede op basis van andere studies, een relatief eenvoudig meetbaar kenmerk is dat de introductie in een fokdoel mogelijk maakt.

De resultaten beschreven in dit proefschrift laten zien dat de *in vitro* embryoproductie efficiëntie voornamelijk wordt bepaald door de kwaliteit van de (gerijpte) eicel. Zowel factoren rondom de eicelwinning als ook de finale eicelrijping spelen hierbij een belangrijke rol. Embryo kwaliteit wordt met name bepaald tijdens de *in vitro* ontwikkeling van het embryo. Naast deze praktische factoren speelt ook de genetica een rol. Dit is ondermeer zichtbaar in de variatie die er bestaat van het aantal eicellen dat aanwezig is in de eierstokken van betreffende donoren. Het aantal eicellen dat per OPU-sessie gewonnen wordt, bepaald in grote mate het aantal embryo's dat *in vitro* kan worden geproduceerd. Deze variatie in aanwezige eicellen blijkt voor een deel genetisch bepaald. Hiermee is dus ook het *overall* resultaat van de *in vitro* embryoproductie deels genetisch bepaald.

De verkregen kennis door dit proefschrift is van grote technische en economische waarde in verdere pogingen de efficiëntie en effectiviteit van het OPU-IVP programma te vergroten. Dit is belangrijk omdat met de komst van *genomic selection* het gebruik van *in vitro*

embryoproductiemethoden en in bredere zin voortplantingstechnieken de onderscheidende factor zal worden in de melkvee- en vleesveefokkerij. Met de komst van nieuwe voortplantingstechnieken als prepuberale embryoproductie, in vitro prematuratie, *genomic selection* van embryo's, embryo vermeerdering, stamcelkweek en *gene-editing*, zal ook de ET industrie veranderen. Hierbij zal de rol van de lokale ET specialist waarschijnlijk beperkt worden tot het uitvoeren van embryotransplantaties en het onderhouden van de klantrelatie. Daarnaast zal ook het verschil tussen continenten of landen in regelgeving en maatschappelijke acceptatie met betrekking tot de implementatie van nieuwe voortplantingstechnieken, een ongelijkheid creëren in het wereldwijde 'speelveld' van de fokkerij. In reactie daarop zullen fokkerijorganisaties zich moeten bezinnen vanuit waar te opereren maar zeker ook op hun '*license to produce'*. Er ligt een interessante en spannende toekomst voor ons!

Dankwoord

Dankwoord

Een bijzonder moment om deze woorden te mogen schrijven. Ik sluit een fase in mijn leven af waarbij ik mij zeer bewust ben van een ieder die hierin een rol heeft gespeeld en daarmee zijn of haar bijdrage heeft geleverd aan het tot stand komen van dit proefschrift. Aan jullie allen mijn hartelijke dank. In het bijzonder wil ik een aantal mensen noemen wiens bijdrage voor mij van grote waarde is geweest. Allereerst Theo Kruip (†), die mij stimuleerde om Biologie in deeltijd te gaan studeren en mij de bevlogenheid van een onderzoeker liet zien. John Hasler, de zeer gewaardeerde adviseur van het 'CRV OPU-IVP project' en waar ik tot op heden een bijzondere vriendschappelijke band mee heb. Lisette de Ruigh en Janneke van Wagtendonk. Gedrieën bezochten we menig congres. Tijdens een van deze trips ontstond het idee om het waardevolle onderzoekswerk van CRV 'te delen met de wereld' en het uit te laten monden in een gezamenlijk proefschrift in drie delen. Het is uiteindelijk een enkelvoudig proefschrift geworden, maar ik weet hoe trots ook jullie zijn. Natuurlijk al mijn CRV collega's die betrokken zijn geweest bij het onderzoekswerk: De 'eicelwinners' op locatie en in het veld, de laboratorium medewerkers in Terwispel en Harfsen, de embryo transplanteurs, de medewerkers van de afdeling verkoop en de medewerkers van de R&D afdeling, in het bijzonder Erik Mullaart en Hiemke Knijn. Van het 'sparren' tot en met het schrijven van de artikelen, jullie bijdrage is zeer groot geweest. Maar natuurlijk ook het management van CRV, Jan Jansen en later Ate Lindeboom en Jos Koopman. Zij hebben mij de ruimte en gelegenheid gegeven hebben dit avontuur aan te gaan. De begeleiding vanuit de Universiteit Utrecht door Tom Stout, Peter Vos en Bernard Roelen. Jullie bijdrage als promotoren was niet alleen kundig en professioneel, maar vooral ook oprecht en inspirerend. Ik heb ervan genoten en geleerd.
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Sybrand

Curriculum vitea

Curriculum vitea

Sybrand Merton werd op 23 februari 1965 geboren te Vianen als zoon van Ad Merton en Jopie Merton-de Groot. In 1982 behaalde hij zijn HAVO diploma aan het Cals College te Nieuwegein, waarna hij begon aan de studie HLO Zoölogie aan het Dr.Ir. W.L. Ghijsen Instituut te Utrecht. Deze studie werd in 1986 afgerond met het behalen van het diploma. Gelijk met het in dienst treden bij Embrytec in 1989, begon Sybrand aan de deeltijdstudie Biologie aan de Universiteit Utrecht. In 1992 studeerde hij af als bioloog waarna hij vervolgens in dienst trad bij Holland Genetics (rechtsvoorganger CRV). Sindsdien is hij medeverantwoordelijk geweest voor het opstarten van het OPU-IVP programma en daaruit voortvloeiende R&D activiteiten. Vanuit die achtergrond heeft hij van 2003 tot 2010 zitting gehad in de board van de AETE (Association Europeenne de Transfert Embryonnaire), waarin hij tevens de functie van President vervulde van 2008 tot en met 2010. Binnen CRV heeft Sybrand door de jaren heen meerdere functies bekleed, sinds 2009 die van Manager Operations. Daarnaast is hij ook voorzitter geweest van de ethische commissie van CRV van 2002 tot 2013. Thans is Sybrand werkzaam als Hfd Audits & Inspecties bij Qlip te Leusden.

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