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Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry

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Abstract

With the introduction of multiple ovulation, embryo recovery and transfer techniques (MOET) plus embryo freeze–thaw methods in the early 1980s, the breeding industry has the tools in hand to increase the number of calves from donors of high genetic merit. In the early 1990s, the introduction of ovum pick-up followed by in vitro embryo production (OPU-IVP) opened up even greater possibilities. Using these technologies, we challenge biological mechanisms in reproduction. Where normally one oocyte per estrous cycle will develop to ovulation, now numerous other oocytes that otherwise would have degenerated are expected to develop into an embryo. Completion of oocyte growth and pre-maturation in vivo before final maturation both appear to be essential phases in order to obtain competence to develop into an embryo and finally a healthy offspring. In order to increase oocyte quality and quantity in embryo production technologies, current procedures focus primarily on improving the homogeneity of the population of oocytes with regard to growth and state of pre-maturation at the start of a treatment. In the case of MOET, dominant follicle removal (DFR) before superovulation treatment improves the number of viable embryos per session from 3.9 to 5.4 in cows but not in heifers and a prolonged period of follicle development obtained by preventing release of the endogenous LH surge increases the number of ova but not the number of viable embryos per session. In the case of OPU-IVP, the frequency of OPU clearly affects quantity and quality of the collected oocytes and FSH stimulation prior to OPU every 2 weeks resulted in 3.3 embryos per session. Analysis of 7800 OPU sessions demonstrated that the oocyte yield is dependent on the team, in particular, the technician manipulating the ovaries. It is concluded that an increased understanding of the processes of oocyte growth, pre- and final maturation will help to

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improve the efficiency of embryo technologies. However, somewhere we will meet the limits dictated by nature.
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1. Introduction

Implementation of smart embryo technologies in cattle e.g. 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 need of the breeding industry to enhance genetic improvement in dairy or beef cattle. The production of a large number of calves from cattle of high genetic merit, involving procedures such as oocyte collection during pregnancy and from juveniles, facilitates an increase in the selection intensity and a shortening of the generation interval. In order to increase the chance of producing the world's best bull, breeding companies are willing to invest in these types of technologies. In view of that objective, implementation of cloning technology seems to be a less useful technique because it does not lead to a genetic rise in the next generation. However, multiplication of a selected embryo may become a standard procedure to ensure the birth of a calf with the desired genetic composition and the cloning technique has a powerful role in disseminating genes from the nucleus to the commercial herds.

Non-surgical embryo recovery procedures combined with non-surgical transfer techniques and an embryo freeze–thaw method became available in the early 1980s and have been used ever since. An average of five transferable embryos per flush is to be expected. This would lead to an average of 50 freezable embryos per donor per year resulting in approximately 30 calves born following embryo transfer. Not surprisingly, this technique found its way into the industry quite easily and quickly. The introduction of OPU-IVP followed in the late 1980s to become operational in the early 1990s. The advantages compared to MOET are obvious. More embryos can be produced in the same period of time. On the basis of two OPU sessions per week, production may reach about 150 embryos per year resulting in approximately 70 calves. Also each batch of oocytes can be used for insemination with a different bull which increases the number of genetic combinations possible. Moreover, embryos can be produced during the second and third month of pregnancy. The company, Holland Genetics, applies this opportunity to produce embryos and subsequently calves from their female breeding line. At the time the first lactation results are known for selected donors, offspring of these donors are already on the ground. This procedure obviously leads to a shortened generation interval resulting therefore in higher rates of genetic improvement. However, application of the OPU-IVP technique is also accompanied by some disadvantages that still need to be solved. In general, the pregnancy rate after embryo transfer is lower than for MOET, and problems with fetuses and calves have been reported [1–3]. This latter phenomenon, often referred to as the Large Offspring Syndrome (LOS), is supposedly

correlated with imprinting errors during early development [4,5], but appears to also be related to (pre-) maturation of oocytes [6]. Presently LOS is still a subject of considerable research.

Multiple marker analysis of biopsies of embryos is still under investigation but its implementation is to be expected very soon. MAS of embryos from a certain combination of donor and bull will be used to enhance selection intensity at an early phase of reproduction. Consequently, it will have a major impact on the costs of the total breeding program.

In a seasonal breeding system, where the period of insemination and subsequent calving is limited to only 4 months per year, embryo technologies may still be valuable. Economically however, the use of these technologies in this situation will be much more difficult to justify. In such a setup, efficiency is critical and therefore a decreased pregnancy rate, for example, may prohibit introduction of such a technique.

From a retrospective view, the outcome of MOET and OPU-IVP sessions has now stabilized. Minimizing the interval between sessions could optimize the efficiency of MOET, but in general there has not been a major breakthrough in the number of viable embryos produced per session. Also in the field of OPU-IVP there has not been substantial improvement in the last decade. It must be mentioned however, that comparison of OPU-IVP systems or laboratories is particularly difficult with regard to blastocyst formation rates from IVP. There are differences in source of ovaries (fertility status of donors, breed), oocyte collection procedures, selection of oocytes and zygotes and the reference bull used. These are the likely cause of the large differences between laboratories with regard to their 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. The definition of transferable embryos also implies variation due to differences in interpretation/procedures between laboratories (embryo stage, fresh/frozen, Day 7/Day 8). The difference in success rate between slaughterhouse and OPU-derived oocytes is one of the aspects that will be discussed in this paper.

Since the introduction of IVP, the IVC step has been studied intensively, resulting in culture conditions capable of supporting early embryonic development at a rate similar to that in vivo (Fig. 1). Many laboratories have changed their co-culture based IVC system, thought to be at least partially responsible for the LOS, to a system based on synthetic oviduct fluid (SOF). The SOF system has become more or less the standard for IVC in embryo production programs. Further improvement of early embryonic development in vitro is likely to come from changes to the earlier stages of the process of embryo production. Several groups of investigators compared effects on early embryonic development of replacing the successive in vivo steps in the development process by in vitro conditions. From these studies it is evident that, besides the process of fertilization, growth and maturation of the oocyte is the most critical factor influencing the outcome of embryo technologies (Fig. 1). In the natural cycle, after fertilization, approximately 80% of the ovulated oocytes will develop in vivo to the embryo stage. In a study of Boerjan and Merton (unpublished), 11 out of 14 cyclic cows (78%) that had been flushed at Day 6–9 after AI, produced embryos that had developed to at least the morula stage. After superovulation, approximately 60% of the ovulated oocytes develop in vivo to the embryo

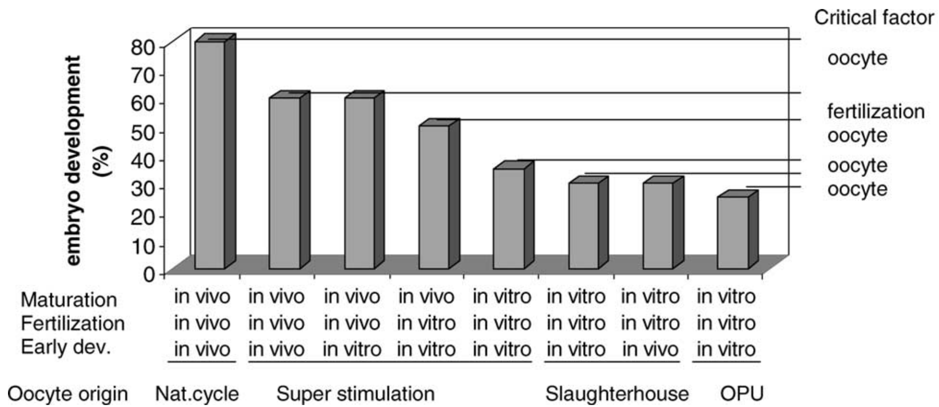

Fig. 1. Effect on embryo production of the origin of the oocyte and the successive steps of maturation, fertilization and early development performed in vivo and/or in vitro. The source of the respective decreases is noted at the right of the figure.

stage (Holland Genetics, unpublished data). When such in vivo produced and fertilized oocytes are cultured in vitro, no decrease of embryo production is observed [7,8]. Similarly, no effect of in vitro culture versus in vivo development has been reported for slaughterhouse oocytes after IVM/IVF [7,9]. It appears that in vitro fertilization decreases embryo production compared to in vivo fertilization when oocytes are used after super-stimulation [7,8]. It should be noted, however, that the in vivo fertilized oocytes concerned were ovulated oocytes in contrast to the in vivo matured/in vitro fertilized oocytes that had been obtained from preovulatory follicles shortly before ovulation. Due to the ovulation window with superovulation [10,11], some oocytes may not have completed maturation at the time of collection [12]. This may have contributed to a lower blastocyst yield. A relatively large decrease in embryo production rate occurs when superovulation-derived oocytes are matured in vitro in standard medium compared to in vivo maturation [7,8,13]. This accentuates the importance of the oocyte and its process of growth and maturation. Noteworthy also are the differences between the in vitro embryo production rate of immature oocytes derived either after superovulation, from slaughterhouse ovaries or by OPU, indicating the importance of oocyte-related processes even before maturation. In general, it can be inferred that the treatment and technique of oocyte retrieval are crucial in determining the outcome of embryo production in the commercial application of embryo technologies. In order to achieve a real breakthrough, fundamental studies are ongoing investigating the ‘golden reference’ in vivo produced oocyte at the different stages of follicular development up to ovulation. Both invasive and non-invasive techniques are used to understand the successful maturation of the oocyte leading to fertilization and subsequent embryonic development.

The objective of this paper is to present a survey of the variation in oocyte quality and quantity in relation to stage of follicular development at oocyte recovery. Possible mechanisms explaining the variation in quality and quantity will be described. Furthermore, the paper will focus on procedural factors within embryo production programs that affect the oocyte and on current research to improve their quality and quantity.

2. Oocytes at different stages of follicular development as the source of embryos for embryo technologies

The need to increase the number of offspring per donor forces us to overrule biological mechanisms in reproduction. Where normally one naturally selected follicle will come to ovulation, embryo technologies use oocytes from follicles at different sizes and stages of the follicle wave (Fig. 2). It is obvious that the heterogeneity of these oocytes will lead to suboptimal embryo production compared to the natural situation. This section will focus on the variation in oocyte quality and quantity in relation to their stage of follicular development. Furthermore, the relationship between the origin of oocytes and subsequent embryo production will be discussed with respect to the 'golden reference' oocyte. Our present knowledge in this area, however, is rather limited and this is not likely to change in the short-term. Due to the difficulty involved in obtaining 'golden reference' oocytes, this oocyte is very expensive, which makes it almost impossible to use in large experiments.

2.1. Follicular and oocyte development during the estrous cycle

2.1.1. The follicle wave

The bovine estrous cycle consists of two or three follicular waves each of which is preceded by a rise in serum FSH concentration [14]. This rise in FSH concentration initiates the growth of a group of FSH-dependent follicles of $\geq$3 mm diameter. 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 3 mm stage, continue to grow leading to a population of follicles of 4–8 mm at Day 3 [15,16]. At this stage one of the largest follicles

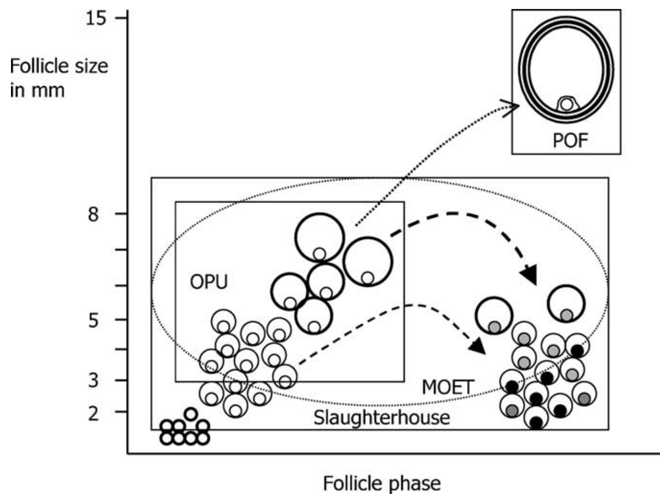

Fig. 2. The origin of the oocyte in the different embryo production systems: ovum pick-up (OPU = area in small rectangle); IVP with slaughterhouse oocytes (=area in large rectangle); multiple ovulation and embryo transfer (MOET = area in oval); preovulatory follicle (POF of normal estrous cycle). Follicles with blank oocytes are non-atretic; with gray are early atresia and with black are atretic.

starts to grow faster and becomes the future dominant follicle (growth phase) whereas the others become subordinate [15]. At Day 6, the dominant follicle reaches its maximum size and stays functional for another 2–4 days (dominant phase). When the dominant follicle of the first wave loses its functionality (regression phase) a new 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 LH surge. The LH surge initiates final follicular and oocyte maturation and ovulation of the dominant follicle. In general, all other follicles are subordinate and do not reach the stage of ovulation. They enter into atresia which is a process of degeneration and during which the follicle will be finally eliminated [17]. In relation to the phase of the follicle wave in which follicles have reached their FSH-dependency, atresia occurs either in growth-arrested follicles or in those which became subordinate [17]. The period from atresia to follicle elimination may take 1–2 weeks, which consequently leads to a distribution of follicles of which up to 85% may be atretic at any one time in the estrous cycle [18].

2.1.2. *Oocyte growth and final maturation*

Oocytes grow in diameter in follicles up to a size of 3 mm. Between 3 and 10 mm follicle size, the diameter does not change. During this growth arrest, rRNA and mRNA transcription are minimized and growth of the oocyte is almost completed [19,20]. Additional growth of the oocyte along with an increase of transcription activity again take place in follicles of 10–15 mm diameter and this is concomitant with selection for dominance of the follicle [19,20]. During this period, developmental processes occur which are referred to as pre-maturation or capacitation. Several ultra-structural studies have described changes in the oocyte including changes in Golgi complexes, cortical granules, nuclear membrane, perivitelline space and positioning of surrounding corona cells [21,22]. The pre-maturation process may also include mechanisms in which mRNAs and proteins are processed to prevent degeneration during storage in the oocyte [23]. Sub-optimal storage of these messages may affect oocyte developmental competence. During subsequent final maturation the oocyte nucleus resumes meiosis up to metaphase II, and several changes occur in cytoplasmic organization including lipid storage, alterations to Golgi complexes, mitochondria, cortical granules, retraction of the corona cells and cumulus expansion [21–26]. Besides these ultra-structural changes, time dependent protein synthesis has been observed throughout maturation in oocytes incubated as cumulus oocytes complexes (COCs) [27,28]. Cumulus cells seem to be essential, especially in the first hours of maturation [29,30] and increasingly information is becoming available on the underlying molecular processes involved in oocyte development. Several 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 [31–33]. Besides growth factors, interest is focused on regulatory candidate genes as well as the expression levels of housekeeping genes which may be correlated with oocyte quality [34]. Another approach for determining gene function is the construction of cDNA libraries from oocytes at different developmental stages in order to detect novel genes [35]. In conclusion, pre-maturation and final maturation processes occurring in the oocyte during follicular development are crucial for its quality and further ‘success in life’.

2.1.3. Oocyte developmental competence

Oocyte quality is often defined as the competence to yield a blastocyst within an in vitro production system. This definition dictates that developmental competence not only depends on the intrinsic quality of the oocyte, but is also related to laboratory specific factors such as IVP procedures, reference semen used and oocyte collection method (slaughterhouse versus OPU). Some of the almost fully-grown oocytes from follicles of 3 mm are already competent to undergo in vitro final maturation, fertilization and development to the blastocyst stage, indicating that in vitro developmental competence is acquired already at this stage by these oocytes. Whether this in vitro developmental competence (that is the proportion of oocytes achieving competence) further increases during follicular development and growth remains unclear. Several studies showed either increased or equal developmental competence of oocytes collected from small or large follicles [9,16,31,36,37]. For example, Hagemann et al. [16] found no difference in blastocyst rate between oocytes collected from follicles of 3–5 mm or 6–8 mm. However, they showed that oocytes collected from presumptive dominant follicles (>13 mm) yielded a significantly higher blastocyst rate compared to oocytes obtained from follicles of 3–8 mm during the growth phase of the dominant follicle. Developmental competence of oocytes becomes compromised when follicles become atretic. However, COC quality is only affected at the very late stage of atresia [18,38]. Remarkably, early signs of atresia apparently have a positive effect on developmental competence [9,38]. This may be partly explained by the similarity in ultra-structure observed between oocytes undergoing pre-maturation or early atresia [21].

2.2. The origin of oocytes in MOET

With MOET, super-stimulation of follicular development involves a group of responsive follicles (>3 mm; Fig. 2) of which some are in the growing phase but others in the process of atresia. As a result, a heterogeneous group of oocytes is recruited during the process of follicle growth and dominance, which for some oocytes will lead to asynchrony between development and follicular status. It is known that superovulation causes deviations in hormonal signaling and endocrine profiles. For example, Dieleman et al. [39] demonstrated that high estradiol concentrations frequently occur in the fluid of follicles shortly before ovulation which may lead to a disturbance in oocyte maturation [12,40], fertilization and subsequent early embryonic development [41]. Further, Rabahi et al. [42] showed that the expression level of bovine alpha glutathione S-transferase (GSTA) in the follicle wall after FSH stimulation decreased much less before ovulation than in unstimulated cyclic heifers.

In studies comparing in vitro and in vivo maturation, preovulatory COCs are collected either by ovariectomy (OVX) or by OPU after super-stimulation. This allows examination of the COC just before the expected time of the LH surge (40 h after prostaglandin [PG] administration) or shortly before expected time of ovulation (60 h after PG) [7,13,39]. Asynchrony between the oocyte and follicular status is clearly demonstrated at this later stage in both collection groups. The proportion of oocytes lacking an expanded cumulus may vary between 16% (61/367 COCs, 50 animals; Dieleman et al., unpublished) and 40% (141/258 COCs, 10 animals; Merton et al., unpublished), and only 3% of such COCs may reach the stage of a viable embryo compared to 42% of COCs having an expanded cumulus

(Dieleman et al., unpublished). In most of the in vivo maturation studies, experiments are conducted only with COCs showing an expanded cumulus. This may lead to some overestimation for this group of oocytes. On the other hand, selection of COCs in the pre-LH surge group, on the basis of a minimal amount of cumulus investment, may equalize this effect (13%: 47/370 COCs, 50 animals; Dieleman et al., unpublished). In general, upon OVX or OPU after super-stimulation, a recovery rate of 75% of COCs is achieved by aspiration which raises the question of the effect the uncollected oocytes would have had on embryo production. A similar question arises for the overall oocyte competence in a full MOET procedure. Fokker et al. [43] showed that 9.8 embryonic structures per animal ($n = 26$) were recovered although 17.1 corpora lutea were counted (57.3% recovery rate). One explanation for this poor recovery rate could be that the COCs lacking an expanded cumulus have a reduced chance to enter into the oviduct. On the other hand it is unknown whether COCs with an expanded cumulus always will enter the oviduct. Taking this into consideration, reported blastocyst rates of the in vivo matured, fertilized and developed group of oocytes, may in general be somewhat overestimated. Therefore, attention should be focused on the state of the oocyte at the start of superovulation treatment in order to achieve improvements in MOET results.

2.3. The origin of oocytes in OPU-IVP

In OPU-IVP practice, the source of oocytes is determined by the resolution of the equipment used (follicle size > 2 mm) and OPU session interval. The population of oocytes used in OPU-IVP is more homogeneous compared to MOET, due to repetitive sessions resulting in the elimination of dominant and atretic follicles. Production of embryos from slaughterhouse ovaries begins with oocytes collected from follicles in every possible phase of development and from all sizes visible on the surface of the ovary (Fig. 2). In contrast to MOET, COCs collected by OPU originate from follicles that lack dominance and have not undergone final preovulatory development. Final maturation has to take place in vitro.

In contrast to a worldwide uniform classification system used for embryo evaluation (IETS system), oocyte classification systems for both slaughterhouse and OPU derived oocytes seem to be much more laboratory specific. Oocytes are classified on morphology of ooplasm and/or cumulus investment. The number of categories within a classification system may vary from 3–6 and, within each system, some correlation is generally found with developmental competence. For example, Wurth and Kruip [38] classified COCs into three classes (A: compact and bright cumulus; B: slightly expanded and darker cumulus; and C: strongly expanded and degeneration of cumulus cells). Except for oocytes from heavily atretic follicles, Class B oocytes resulted in higher blastocyst rates compared to Class A oocytes. Blondin and Sirard [9] also reported significantly better in vitro development with their Class 3 COCs (showing signs of early expansion in the outer cumulus layer and with a slightly granulated ooplasm), compared with morphologically better (Class 1 and 2) or worse oocytes (Class 4–6). In order to monitor the performance of the OPU-team, oocytes are classified in our laboratory solely on the cumulus investment. Class I COCs are oocytes completely surrounded by a compacted, round-shaped cumulus investment and Class III COCs are defined as completely denuded oocytes. All remaining

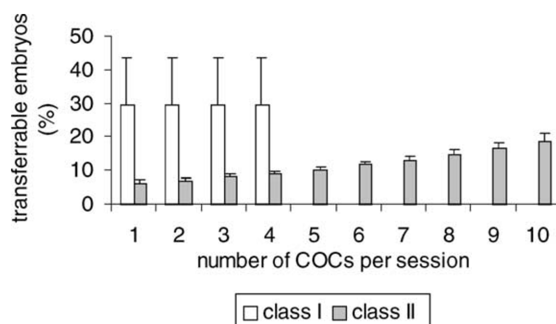

Fig. 3. Effect of the quality and number of OPU-derived COC on the in vitro embryo production rate. Data based on sessions in which only one type of oocyte was present; Class I, $n = 61$ sessions; Class II, $n = 511$ sessions. Data analyzed by logistic regression; mean values ± S.E.M.

COCs with a cumulus quality between Class I and III are defined as Class II, and COCs with an expanded/degenerated cumulus as Class IV. The COC quality as obtained by OPU clearly affects in vitro embryo production rate (Fig. 3). Class I COCs resulted in a blastocyst rate of 29.6%, independent of the number of oocytes recovered and processed as a batch throughout the IVP procedure. Interestingly, the blastocyst rate with Class II COCs was significantly lower and was dependent on the number of oocytes collected per session (range: 6.2–18.4% for 1–10 COCs per batch). In order to obtain the maximum number of embryos in our daily practice, all types of COCs collected from one donor are used as one batch, including Class III (denuded) and Class IV (degenerated/expanded) COCs.

Surprisingly, the distribution of classes may differ between OPU and slaughterhouse-derived COCs with more Class I COCs in the slaughterhouse group. Mullaart et al. [44] showed that this difference was not related to the OPU equipment or OPU collection medium. It was suggested that due to a post-mortem effect, the COC becomes less tightly connected to the follicle wall and therefore is collected with a more complete morphology. Other groups have also described a post-mortem effect [45,46]. For example, embryo production was doubled when ovaries were kept at 30 °C for 4 h instead of 2 h. Presumably, the post-mortem effect initiates atresia which positively affects oocyte developmental competence [45]. This is one of the reasons better IVP results can be obtained with slaughterhouse-derived oocytes compared to those obtained with OPU. However, a lack of oocyte selection and use of donors selected for genetic merit (i.e. having variable parity, fertility status, and 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 oocytes can also affect the outcome of IVP studies. For example, we reported [47] a significant increase in blastocyst formation rate at Day 8 when Menezo-B2 medium was used for in vitro culture of IVMF slaughterhouse-derived oocytes compared to TCM199 culture medium (31 and 22%, respectively). However, when this comparison was made with OPU-IVP oocytes, improvement was not detected (Table 1).

Interestingly, it has been reported that COC morphological classification is related to the level of gene expression. Calder et al. [48] showed a significantly higher abundance of

Table 1
Effect of COC origin and of culture media 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

Values within columns with superscript letters are significantly different (Chi-square analysis, $P < 0.05$).
[1] Oocytes from both slaughterhouse (control IVP monitoring) and OPU (production) were processed simultaneously.

Prostaglandin E2 receptor 3 (EP3) mRNA, which plays a role in induction of cumulus expansion, in Grade 1 and 2 than in Grade 3 COCs during in vitro maturation.

3. Procedural factors affecting oocyte quantity and quality in embryo production technologies

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 a production procedure. However, as MOET and OPU-IVP are already commonly used in the cattle breeding industry, improvement of current procedures with regard to time and costs is likewise important. For example, reduction of the number of steps in manipulating oocytes and embryos, simplification of labor consuming methods and an increase of the number of oocyte batches per production step (e.g. fertilization) are some issues that may lead to a reduction in costs of embryo production. Increasing the number of oocytes over a time period appears to be more important than increasing numbers per session. Thus not only production characteristics but also cost efficiency determines the feasibility of embryo production technologies in a commercial breeding enterprise. The objective of this section is to describe procedural factors of embryo production technologies which affect oocyte quantity and quality and which, if improved, may serve as a starting point for more effective technology.

3.1. Procedures affecting multiple ovulation and embryo transfer

3.1.1. Dominant follicle removal (DFR)

In order to increase the quality and homogeneity of the population of COCs at the start of a super-stimulation treatment, several groups studied the effect of DFR. The name of this method is not strictly correct as the follicle is not removed—rather it is punctured or ablated and the follicle fluid removed. Therefore, some authors prefer to use the abbreviation dominant follicle puncture (DFP). Functionality of the dominant follicle, however, ceases following DFR as reported by Van Schaik et al. [49]. When a dominant follicle is absent or removed at the start of super-stimulation a marked increase in the total

Table 2
Effect of DFR on MOET outcome

Donor	Treatment	MOET sessions (n)	Total ova/session (n)	Viable embryos[1]/ session (n)	Viable embryos/ total ova (%)
Heifers ($n = 193$)	Control	236	6.7 ± 0.5	4.0 ± 0.4	60 ± 4
	DFR	76	7.0 ± 1.0	4.3 ± 0.6	68 ± 7
Cows ($n = 60$)	Control	85	5.9 ± 0.4^a	3.9 ± 0.4^a	58 ± 3^a
	DFR	51	7.6 ± 0.6^b	5.4 ± 0.5^b	76 ± 5^b

Within group of donor values within a column with superscript letters are significantly different (SAS statistical program, $P < 0.05$; corrected means ± S.E.M.).

[1] Transferable embryos (IETS quality 1 and 2).

number of ovulations, ova and viable embryos is obtained compared with results of super-stimulation in the presence of a dominant follicle [50–52]. It has been shown that in vitro developmental competence of oocytes is impaired during the phase of follicular dominance [16,53]. The effect in cows of DFR at 38–46 h prior to superovulation treatment as performed at Holland Genetics is shown in Table 2. The total number of ova, number of viable embryos and proportion of viable embryos were significantly increased following DFR. In heifers however, an effect of DFR was not detected.

Another approach to increase the quality and homogeneity of the population of COCs at the start of a super-stimulation treatment would be the removal of all subordinate follicles that are in different stages of atresia. This can be achieved by undertaking a number of OPU sessions, at an interval of 2 or 3 days, prior to the start of a super-stimulation treatment. After a few OPU sessions only follicles that are in the growing phase of the follicle wave will be present. Such a procedure may decrease the number of non-viable/unfertilized ova and thereby increases the proportion of viable embryos. So far one report indicated a positive effect on embryo production [54]. It can be speculated, however, that the available number of responsive follicles for super-stimulation may be reduced due to the OPU sessions. Thus, although OPU before superovulation may enhance homogeneity of the oocyte/follicle population a reduction in the number of follicles may diminish this positive effect. Bols et al. [55] showed, using a 3- and 4-day interval OPU scheme in heifers, a decline from 9.6 oocytes per first session to 3.9 in the second and up to an average of 6.2 from the fourth session onwards. These findings are in agreement with results from our own breeding herd applying a similar OPU scheme with the first session performed at random in the cycle but without preceding DFR. First collection averages 14.0 oocytes per pregnant heifer declining thereafter to about 9 per session (Fig. 4). In cows, however, the yield remained constant. Therefore, provided the number of follicles present at the start of superovulation treatment is not affected, pretreatment of cows with OPU may result in a higher number of embryos per session.

3.1.2. Artificial LH surge

Due to the heterogeneity of follicles present at the start of a super-stimulation treatment (i.e. follicle ages and sizes), for some smaller follicles the period of development between PG up to ovulation may be too short to undergo full (pre-) maturation. The LH surge,

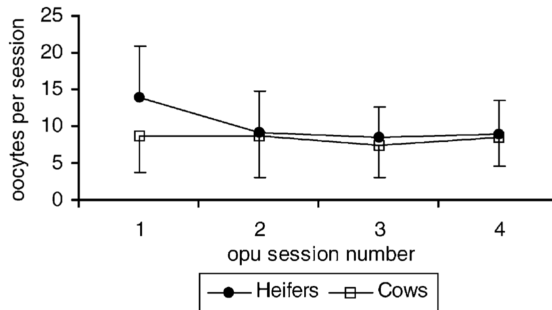

Fig. 4. Number of retrieved oocytes at the first four OPU sessions (means ± S.D.). Data shown from pregnant heifers ($n = 41$; second month of gestation) and cows ($n = 22$). OPU was performed at a 3- and 4-day interval at one OPU location.

caused by the larger follicles, may also come too early ‘in life’ for these smaller follicles, leading to a sub-optimal ovulation rate. In order to avoid this situation, several groups prevented the endogenous LH surge and then induced a delayed LH surge at a later time after PG. In general two strategies have been followed. Temporary suppression of the endogenous LH can be achieved either with a norgestomet treatment followed by GnRH-induction of the LH surge, or by a GnRH agonist treatment followed by administration of LH. Overall, an increase in total number of corpora lutea (CL) and/or of retrieved ova is observed. Embryo production, however, is not affected or is even decreased. For example, Van de Leemput et al. [56] showed an increase in the number of ova collected following a norgestomet treatment and an LH surge delayed for 6–14 h versus control (18.6 ± 3.9 versus 15.0 ± 1.7) but a reduced number of viable embryos (2.3 ± 1.0 versus 3.4 ± 1.5). In vitro experiments showed similar developmental competence of oocytes from both treatment groups suggesting a disturbed oviductal environment caused by the norgestomet treatment. D’Occhio et al. [57] used the GnRH agonist strategy and reported a higher number of ova retrieved when the LH surge was delayed for 12 h versus 0 h (15.2 ± 1.4 versus 11.0 ± 2.8) and the number of transferable embryos tended to be higher (7.3 ± 2.1 versus 5.8 ± 1.8). However, an induced LH surge with no delay or a delay of 24 h had a detrimental effect on both parameters.

3.1.3. Donor selection

As a standard procedure at the start of super-stimulation, donors are often rectally palpated to ensure the presence of a CL. This may result in the rejection of about 11% of the donors, thereby affecting the efficiency of the MOET program. De Ruigh et al. [58] compared the flushing results of 294 virgin heifers which showed a normal heat to validate the necessity of this rectal examination. No significant differences were found in both the proportion of heifers with zero-flushes (16% versus 24% for heifers with CL versus no CL and/or cystic) and the number of viable embryos per flush (4.2 versus 4.6). The combination of a check for normality of the genital tract by rectal palpation at 11 months of age with an effective heat control system enables super-stimulation treatments to be started without any rectal check. This results in an increased efficiency of the MOET program.

3.2. Procedures affecting OPU-IVP

3.2.1. Session interval

The interval between OPU sessions influences both the quality and quantity of oocytes. When OPU is performed effectively, all follicles of >2–3 mm in diameter will have been removed, which subsequently induces growth of new follicles of 2–3 mm over the following days. The length of the period between two sessions significantly affects embryo production rate. A significantly higher number of COCs is collected for a 7-day compared to a 3- or 4-day interval (30; Table 4). However, the quality of the COCs on the basis of the cumulus investment was highest with the 3-day interval and lowest for the 7-day interval. Blastocyst rate and embryo developmental stage was also significantly affected (19.7% versus 13.5% for 3 days versus 7 days intervals, respectively). This finding is in agreement with the hypothesis that a dominant follicle exerts a negative effect on developmental competence of oocytes from smaller follicles since a dominant follicles will emerge approximately 3 dyas after OPU. In contrast to the commonly used 3 and 4 days OPU scheme (Monday and Thursday), OPU at 2 and 5 days interval (Monday and Wednesday) did not affect the number of oocytes collected per session. However, COC quality was higher in the 2-day interval concomitant with 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 given time period, a scheme with OPU conducted three times a week (Monday, Wednesday and Friday) may be effective.

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

Scheme	Interval (days)	Sessions[1] (n)	Oocytes per session (n)	Oocyte quality index[2,3]	Embryos/oocytes[4,5] (%)
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}	0.0	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 [59]; Holland Genetics. Values within a column with different superscript letters are significantly different (regression analysis, Genstat statistical program; $P < 0.05$). (B) Unpublished data from Saner and Le Gal (SVKB, Switzerland). Values within a column with superscript letters are significantly different (Chi-square analysis, $P < 0.05$).

[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-day 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 of the 3-day interval was higher than of the 7-day interval ($P < 0.001$) [58].

[3] Oocyte quality calculated as the number of COCs collected with quality 1 and 2 (Saner and Le Gal, unpublished data).

[4] Results of sessions conducted at three OPU locations (842, 778 and 90 sessions for intervals 3, 4 and 7 days, respectively); transferable embryos at Day 7 of IVC at the morula up to hatched blastocyst stages (IETS quality 1and 2) [58].

[5] Transferable embryos at Day 7 and 8 of IVC at the early blastocyst stage up to expanded blastocyst stage (IETS quality 1and 2) (Saner and Le Gal, unpublished data).

Although cost efficiency may be negatively influenced due to an extra weekend shift, the number of embryos produced per time will probably be increased.

3.2.2. FSH pre-stimulation

Several groups have reported an overall positive effect of FSH pre-stimulation prior to OPU on the developmental competence of COCs. This has been achieved using procedures which varied widely with regard to the interval between last administration of FSH and oocyte retrieval, and the OPU scheme used. Blondin et al. [60] found an optimum oocyte competence when the period between pre-stimulation with pFSH and oocyte retrieval was extended to 48 h. Besides the stimulation of growth of a cohort of follicles up to a size of >5 mm, the post-FSH coasting period seems to be essential. It was suggested that this period induced changes within the COCs corresponding to those taking place during early atresia and resembling processes occurring during pre-maturation. Bousquet et al. [61] applied this treatment in a commercial situation once every 2 weeks and reported an average 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. [62] reported an increase in embryo production rate from 22 to 39% (embryos per oocyte processed) after stimulation for 3 days with declining FSH dosages. A positive effect of pre-stimulation was also found in our own 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 period of 2 weeks was higher with the standard procedure than with the FSH protocol (6.0/4.0 versus 3.3 embryos per 2 weeks).

The most extreme pre-stimulation treatment would be a complete superovulation treatment followed by OPU prior to the LH surge or even just before ovulation. Van Schaik et al. [49] investigated the restoration of ovarian cyclicity after OPU conducted before and after a controlled LH surge within a conventional super-stimulation protocol. OPU before the LH surge resulted in punctured follicles that did not luteinize. When OPU was carried out after the LH surge, shortly before ovulation, the remaining follicular tissue luteinized and, similar to a normal CL, was responsive to PG at Day 10 after LH.

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}

Values within a column with superscript letters are significantly different (regression analysis, Genstat statistical program; $P < 0.05$).

[1] Data adapted from De Ruigh et al. [63]. In period 1 and 3 (control; 10–15 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 four equivalent doses 72, 60, 48 and 36 h prior to OPU (total of 10 ml Ovagen i.m.; ICPbio, 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 IVC at the morula up to hatched blastocyst stage (IETS quality 1 and 2).

Thereafter, 60% of the cows became pregnant at first AI (unpublished data). This is of great importance in certain breeding schemes of Holland Genetics in which heifers have to become pregnant as soon as possible after the embryo production period. The advantage of this MOET-OPU approach would be the possibility to choose a number of bulls for IVF with one batch of in vivo matured oocytes. As shown by Dieleman et al. [64], the average number of embryos per session will probably be the same as normal MOET. They obtained a total of 5.1 embryos per cow/session with 2.4 embryos from oocytes collected from the left ovary 2 h before the LH surge and 2.7 embryos for oocytes collected from the right ovary 24 h after the LH surge.

3.2.3. Meiotic arrest

From the time the immature COC is removed from its follicular environment, germinal vesicle breakdown starts spontaneously and the oocyte will undergo final nuclear maturation. For a number of oocytes this final maturation may be too early 'in life'. To allow pre-maturation in vitro, strategies have been developed to control meiotic resumption. Mermillod et al. [65] showed that the use of roscovitine (ROS), a potent inhibitor of M-phase promoting factor kinase activity, resulted in meiotic inhibition which was fully reversible and led to production of embryos equal to the untreated control group. Ponderato et al. [66] showed that MII kinetics of maturation of butyrolactone I and ROS-inhibited oocytes was accelerated compared to their control counterparts with 50% of the inhibited oocytes reaching MII at 13–14 h compared to 18 h in control oocytes. Therefore, it was 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. So far however, no improvement of the embryo production rate has been reported for oocytes in which pre-maturation was mimicked by temporary meiotic arrest. An explanation for this lack of success could be that, although oocyte quality may be improved by 'pre-maturation', it may also be decreased due to concurrent in vitro oocyte aging or lack of appropriate stimuli during the in vitro pre-maturation phase. The identity of the pre-maturation stimuli is a question yet to be answered. To answer this, a complete description of the cytoplasmatic components (e.g. gene transcripts) present in the 'golden reference' oocyte from the pre-LH dominant follicle is needed. This will be a difficult task because even with the most current molecular techniques, relatively large pools of oocytes are needed to obtain enough material to identify low transcription levels. Dalbies-Tran and Mermillod (unpublished data[1]) characterized the bovine oocyte transcriptome and its evolution during in vitro maturation using heterospecific human cDNA array hybridization on pooled slaughterhouse derived oocytes ($n = 200$ per array). About 900 known genes were shown to be expressed in the bovine oocyte and these are involved in a wide range of biological functions (cell cycle regulation, signal transduction, transcription, extracellular communication, metabolism, stress response, etc.) and belong to different families (oncogenes, tumor suppressors, cell-cycle regulators, transporters, receptors, growth factors, etc.). During IVM the relative level of most identified mRNAs appeared to be stable. However, transcripts from 71 genes underwent a significant up- or down-regulation. This approach may lead to identification

[1] Meeting of the FNRS contact group on Competence of mammalian oocytes, June 2002, University of Louvain, Belgium; Research project Ex Ovo Omnia; European Commission; Grant No. QLK3-CT-1999-00104.

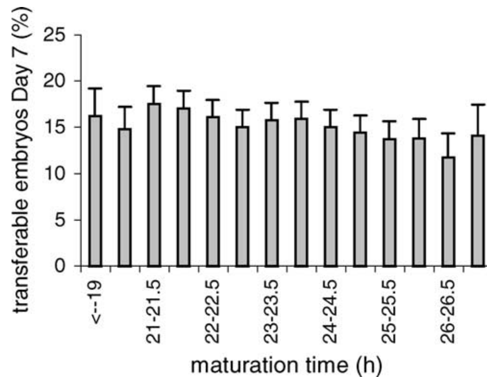

Fig. 5. Effect of duration of oocyte maturation on in vitro embryo production. Data from 7800 OPU sessions performed in 4 year, ranging from 54 sessions for the >26.5 h period to 1009 sessions for 24–24.5 h. Data analyzed with SAS statistical program (means ± S.E.M.).

of genes involved in developmental competence but using the information to derive an appropriate in vitro treatment, however, is another difficult step.

3.2.4. Duration of oocyte maturation

To improve cost efficiency of embryo production we fertilized, in one session, all oocyte batches collected by OPU during the previous day. This challenged maturation time as usually 2 or 3 IVF sessions are conducted to avoid large deviation from the 24 h maturation time. The duration of oocyte maturation varied between 16 and 28 h (Fig. 5).

Sires used for IVF were distributed over cows dependent on the breeding program and embryo culture was performed either in BRL-coculture/FCS or SOFaaBSA [7]. The proportion of transferable embryos ranged from 11.7% for 26–6.5 h to 17.5% for 21–1.5 h of maturation. Although there is no clear optimal maturation time ($P < 0.1$), it cannot be excluded that embryo production will be affected by a duration time of >24 h which is probably caused by oocyte aging. The results suggest that oocytes inseminated before 24 h of maturation continue the maturation process during the first hours of in vitro fertilization. In general, these observations were in agreement with the results obtained with slaughterhouse-derived oocytes (Table 5). Ward et al. [67] recently showed an optimum blastocyst production rate at 24 h of maturation compared to 20 and 28 h (26.4, 39.3 and 26.5% for 20, 24 and 28 h, respectively). Differences between this and our studies are difficult to explain. The relative drop in blastocyst production at 26 h reported by us here, is much higher with slaughterhouse-derived oocytes than with oocytes obtained with OPU (Fig. 5). This may be due to the procedures of oocyte collection and/or fertilization. With slaughterhouse-derived oocytes in general, one reference bull is used for insemination, and the population of oocytes may be more synchronized due to post-mortem effects.

3.2.5. In vitro maturation

Numerous experiments have been conducted to investigate the effect of different supplements during in vitro maturation on subsequent embryonic development. Effects of different types of substances, e.g. hormones, growth factors and others, have been

Table 5
Effect of duration of IVM of slaughterhouse oocytes on subsequent embryo development

Duration of IVM (h)	Oocytes (n)[1]	Cleavage (%)	Blastocyst/oocyte (%) at Day 8
19	162	61.1	25.9[a]
20	156	66.0	23.1[ab]
22	154	64.9	26.0[a]
24	161	67.7	23.6[a]
26	150	62.7	14.0[bc]
28	172	67.4	14.0[c]

Values within a column with different superscript letters are significantly different (Chi-square analysis, $P < 0.05$).
[1] Two replicates.

reported [68]. In most of the experiments, however, the effects were not cumulative or better than the Fetal Calf Serum control group. Repetition by us of previously reported [64] experiments confirms that equal embryo production rates are obtained from in vivo and in vitro matured oocytes. In the trials, COCs were produced under identical preovulatory conditions by applying OPU twice (2 h before and 24 h after the LH surge) in each cow treated for superovulation with a controlled LH surge, and in vitro maturation was carried out with 0.05 IU rec hFSH supplementation (Table 6). This supplementation may explain the high embryo production rate for the in vitro matured oocytes. The absence of a difference between in vivo versus in vitro maturation is in contrast to what has been reported in other studies [7,13,38]. Bevers et al. (unpublished data) demonstrated a beneficial effect of rec hFSH on maturation of immature slaughterhouse oocytes from follicles of 2–8 mm diameter. The proportion of oocytes at MII after IVM was significantly higher for the rec hFSH group (84% [213/253] versus 72% [163/277] for the controls), as was the proportion developing into blastocysts (32% [103/319] versus 19% [62/327], respectively).

Watson et al. [69] studied the effect of different maturation media on oocyte transcript levels. Amino acid supplementation of serum-free media was associated with an increased embryo production rate and also with elevated oocyte maternal mRNA levels for five different marker genes, indicating that the relative abundance of marker gene transcripts can be used as an oocyte quality marker in comparative studies. Transcript levels, however, were again measured on pools of oocytes.

Table 6
Effect of final in vitro vs. in vivo maturation of bovine oocytes on cleavage and embryo formation rate after IVF and IVC

Maturation procedure[1]	Number of		
	Oocytes in IVC	Cleaved on Day 4 (%)	Embryos on Day 7 (%)[2]
In vivo	344	278 (81)	155 (45)
In vitro	354	299 (85)	155 (44)

[1] Oocytes were collected in six series of OPU sessions from preovulatory follicles of treated cows ($n = 56$).
[2] Number and (proportion) of embryos/oocytes developing in IVC to between morula and expanded blastocyst stage.

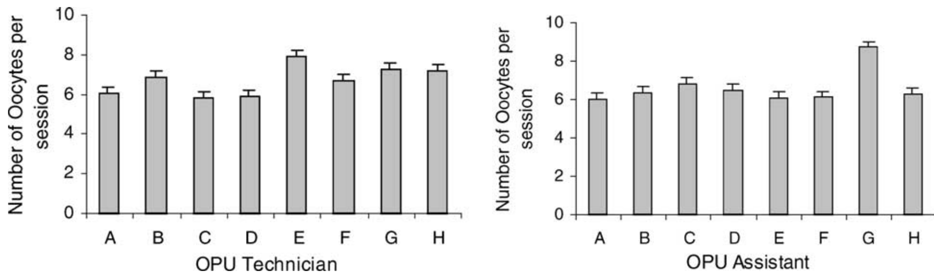

Fig. 6. Effect of OPU team (technician and assistant) on the number of oocytes per session. Data based on 7800 OPU sessions ranging from 419 to 1580 for technicians and 399 to 1466 for assistants. Data analyzed with SAS statistics program ($P < 0.1$); means ± S.E.M.

3.2.6. OPU team

A technique with people involved will always be affected by the performance of those individuals. The OPU technique is performed either by one or by two persons. At Holland Genetics, eight 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 varied which made it possible to elucidate both the effect of the technician manipulating the ovary and the assistant performing the follicle punctures. Both persons significantly affected the quantity of the COCs collected (Fig. 6). The variation in the group of technicians was larger than in 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.

3.2.7. Oocyte collection technique

The number of oocytes collected per ovary or animal is dependent on the technician and the route used. In our experience the number of oocytes of Class I and II aspirated from ovaries of culled cows averages 32 per animal (5191 oocytes from 162 animals) and from ovaries with OPU averages 8 (23,130 from 2926 sessions). This difference is mainly due to the reduced resolution of the OPU equipment compared to the human eye. When oocytes were aspirated from ovaries of cows that had been slaughtered immediately after OPU, an average of 12 oocytes per cow were collected in addition to the 6.7 oocytes that had already been retrieved by OPU (Table 7; 42). The embryo production rate tended to be higher for oocytes collected by OPU from follicles of 2–10 mm than for oocytes from smaller follicles left over after OPU. It should be noted that the blastocyst rate observed in this experiment is higher than the rate obtained in general with OPU oocytes (see Fig. 1). It can be concluded that improvement in the resolution of the OPU equipment would facilitate collection of COCs from smaller follicles. However, the current resolution of OPU equipment appears to function as a good filter promoting collection of only fully-grown oocytes and allowing smaller follicles another 3–4 days to reach a size in which a higher proportion of oocytes will have developmental competence.

Table 7
Effect of follicle size on in vitro embryo production rate 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 rate		Blastocyst rate[1]	
			%	n	%	n
In vivo by OPU	2–10	40	82.5	33[a]	37.5	15
Ex vivo by aspiration	1–2	72	43.1	31[b]	20.8	15

Values within a column with superscript letters are significantly different (Chi-square analysis, $P < 0.05$).
[1] All blastocysts at early up to hatched stage present at Day 8 of IVC.

3.2.8. *Oocyte freezing*

In countries with seasonal breeding the interest in embryo production from donor animals is focused on approximately 4 months of the year. Efficient management of an OPU-IVP enterprise is, therefore, difficult to achieve. When, however, freezing and storage of COCs is possible without decreasing developmental competence, the OPU technique may be applied more economically over a longer period per year. In the breeding season, such oocytes will then be thawed and fertilized with the bulls of interest at that time. The eventual banking of oocytes will also lead to an increased efficiency of production in the laboratory. Presently, the effect of freezing methods and oocyte developmental stage on subsequent IVP rate is being investigated by several groups. In general though, freezing/vitrification techniques result in oocytes less able to develop during IVP than fresh oocytes.

4. Concluding remarks

Successful application of embryo technologies in cattle greatly depends on both oocyte quality and quantity at the start of the procedure. This is why the oocyte and its process of growth and maturation is one of the main subjects for intensive studies. For commercial application, however, not only a high success rate is important, but so is the cost efficiency of producing the embryos.

With increasing understanding of the processes of pre-maturation and final maturation of the oocyte, it may be possible to improve in vitro embryo production procedures. One clear example is the FSH pre-stimulation of donors prior to OPU which may even be extended to the collection of in vivo matured oocytes shortly before ovulation—the OPU-IVP technique in this situation becomes very similar to super-stimulation procedures. This highlights the fact that no real breakthroughs have been made in MOET in the last decades. We may be able to enhance the homogeneity and thereby the quality of the group of oocytes at the start of the superovulation treatment, but will it be possible to increase the overall quantity of this category of oocytes? The question arises here: can we beat the limits imposed by nature? Table 8 presents a schematic overview of embryo production efficiencies of the current embryo technologies.

It is clear that most fundamental studies addressing oocyte growth, maturation and early embryonic development are performed in vitro using oocytes obtained from slaughter-

Table 8
Schematic overview of embryo production efficiencies of current embryo technologies

Treatment before oocyte collection	Number of embryos	
	Per session	Per week
Natural cycle, AI and flushing at Day 7	0.7	0.23
MOET, standard superovulation	5	1
DFR-MOET, superovulation preceded by removal of dominant follicle	5.5	1.1
OPU-MOET[1], superovulation after removal by OPU of all follicles	5–8?	1–1.6?
OPU (twice weekly) at 3 and 4 days interval	1	2
OPU (thrice weekly)[1] at 2 and 3 days interval	1?	3?
OPU after FSH pre-stimulation at 2-week interval	3	1.5
(DFR)MOET-OPU, superovulation with collection before ovulation	5.5	1.1

[1] Estimate; no data available.

house ovaries and from super-stimulated donors. Furthermore, in gene expression studies transcript levels are measured on pools of oocytes. All in all, most information obtained in fundamental studies certainly does not represent the 'golden reference' oocyte at different stages of follicle development in the normal cycling cow. Therefore, research should focus on this reference oocyte although this presently is a goal difficult to reach. Another question arises here: which animal is the 'golden standard' normal cycling cow? The enormous variation in reproductive characteristics between animals is very well known. Therefore which animal should be used for research? Do we take large numbers of animals to randomize these effects, or should we use specific individuals? In practice, animals with extreme embryo production results can be found. Maybe we should take both extremely good and bad donors as reference material and learn from variation instead of ignoring it.

In conclusion, solving the mysteries of oocyte growth and maturation will certainly raise the level of implementation of smart embryo technologies in the cattle breeding industry. However, as many other factors, from IVF up to recipient management significantly affect overall results of an embryo technology program, there is still much research to be undertaken before we reach optimum embryo production levels.

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