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Circles around the farm animal embryo – a Danish perspective

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Abstract

With focus on the farm animal embryo, a short overview is given about my research activities over the last 35 years. These activities have been described in five circles, covering different key aspects of my work. The first circle included studies on the basic biology related to oocyte maturation and follicular endocrinology in superovulated dairy cows. Methods were developed to characterize the donor cows with respect to their production of transferable embryos, and some were implemented into a Danish MOET breeding plan. The second circle dealt with in-vitro embryo production in cattle with development of a protocol to produce such embryos at high and consistent levels. Several comparisons were made to reveal consequences of the artificial in-vitro methods on oocytes and embryos, but also through studies of the newborn calves. The third circle was related to development and implementation of a number of technologies within this broad field; examples are mentioned for both oocyte recovery from donor cows, different steps in the in-vitro embryo production system, new ways to perform vitrification and nuclear transfer, and finally a new system to determine oxygen consumption in single embryos. In the fourth circle is described activities from the last years, where work was done with focus on the pregnancy rates after insemination of the dairy cow in their post-partum period, and where somatic cell nuclear transfer was developed both as a technology in itself as well as a helping technique to produce transgenic pigs as models for important human diseases. The fifth and final circle is addressing and thanking the many colleagues and collaboration partners that I have been involved with during all the years to do this work. Nothing could or would have been the same without them and their participation.

keywords: cattle, embryo technology, pig, reproductive biology, overview.

Introduction

My first AETE meeting was in 1987 in Lyon where the 3rd meeting was held. Since I have been at all but three of these annual highlights, and at every meeting I have been listening with interest to the distinguished scientists receiving the AETE award. Today, it is my privilege and honor to be that person, and since I was told this great news a year ago, I have gradually learned to use this opportunity and reflect over my scientific career in ways that I usually do not.

Having worked with farm animal embryos for more than 30 years gives many possibilities to do different things. My choice - to some extent also influenced by coincidences - has been to focus on the farm animal embryo in kind of circles around the days before, during and after fertilization, and I have always had Denmark as the center for my activities. Within those frames, most of my research interests have been divided between biology and technologies, from a basic to an applied aspect, and then using collaborations very much.

So, it has been fairly limited circles, made in a rather small country, and always with the embryo in focus. In the following, I will give a short overview of my scientific activities using some kind of a chronological approach. It will not be a real literature review, but a self-centered presentation. This is not often allowed, but on this special occasion I think it is. To prepare this has been interesting for me, but of course I hope that it will also be that for at least some of you.

The superovulated donor cow

From the beginning of the 1980ies, I started as Torben Greve's first PhD-student at the Royal Veterinary and Agricultural University in Copenhagen. He had been very much involved in establishing the basics on superovulation and embryo transfer in cattle, also in practice, but time had come to search for a better understanding of some of the reasons for the often varying and rather unpredictable results. Together with another PhD-student, Poul Hyttel, my first years in research were therefore focused on morphological and endocrine aspects of the preovulatory period in superovulated dairy cattle. What were the actions and consequences of the exogenous FSH treatment on the developing follicles and oocytes? Poul's focus was ultrastructure, while mine was the hormones and the more clinical and applied aspects.

Over several years we used more than 130 cows and heifers to study preovulatory oocyte morphology and follicular endocrinology and to describe their overall reaction patterns, and to relate these to the resulting oocyte and later embryo quality. Some of the main results were presented at my first IETS conference held in Colorado Springs in 1986 (Callesen *et al*., 1986), where characteristics of donors with good versus bad oocytes were presented. This was followed by several also more practice-related studies (e.g. Callesen *et al*., 1995) also to identify the good or bad donors. For this, donors were characterized through their patterns of progesterone or estradiol concentrations in plasma and milk (Callesen *et al*., 1988, 1990) together with detailed estrus observations (Callesen *et al*., 1993c), or by use of ultrasound examinations of follicular

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development and ovulation (Purwantara *et al*., 1993, 1994). Also in these years, problems associated with the FSH preparations used for superovulation (e.g. causing premature ovulations, Callesen *et al*., 1987b) were addressed by attempts to control the superovulatory reaction by use of anti-PMSG (Callesen *et al*., 1992) or by controlling the LH-contents (Schmidt *et al*., 1988).

On the practical side, larger projects based on MOET breeding plans were started in Europe, also in Denmark, where I got involved in such a project after having left Copenhagen and moved to Foulum in the western part of Denmark. This resulted in work on e.g. donor selection (Greve and Callesen, 1989), but we also made several studies on the embryo recipients; some of this work was presented at several AETE-meetings (Callesen *et al*., 1993a, b; 1994a, b). The contact to practice also gave some challenges when the more theoretical and experimental work met the real practical world (Liboriussen *et al*., 1995; Callesen *et al*., 1996), for example with the breeding potentials following the realized outcome of superovulation (Callesen *et al*., 1994c).

In vitro production of bovine embryos

During the 1980ies, the procedure for *in vitro* production of bovine embryos was being established based on work around the world with the first IVF calf reported from USA in 1983. In Copenhagen, Kang Pu Xu - another of Torben Greve's PhD-students - worked hard on this technology (e.g. Xu *et al*., 1987), and a prominent result was achieved when the first IVP calf in Europe was born here in 1987 (Greve *et al*., 1989).

In my Foulum-group we worked several years to optimize the bovine IVP-procedure. Peter Holm went through the tedious work of testing close-to-everything in relation to media and conditions for *in vitro* production (e.g. Holm *et al*., 1994, 1995), before this resulted in a quite useful modified SOF medium (Holm *et al*., 1999). Parallel to this work, we were still interested in morphological differences between vivo versus vitro oocytes (e.g. Hyttel *et al*., 1989b), also illustrating some of the implications for vivo and vitro fertilization (e.g. Hyttel *et al*., 1989a, 1991). At the AETE meeting in Venice, some of this work was summarized (Holm and Callesen, 1998).

With the gradual improvement of the IVP systems, we got a stronger interest in some of the consequences of such artificial *in vitro* conditions, stimulated by the disturbing reports about the so-called Large or Abnormal Offspring Syndrome. Thus, we worked on differences between vivo versus vitro embryos, both in cattle and pig (e.g. Hyttel *et al*., 2000), on chromosomal problems after *in vitro* production (Viuff *et al*., 1999), and finally also on the resulting calves (Jacobsen *et al*., 2000). As part of these studies, I spent some months in New Zealand and Australia in 2001 to study pregnancies and calves after both IVP and cloning. In Europe, these concerns were also subject for many discussions in the public with focus on animal ethics (e.g. Callesen *et al*., 1999), and also at the AETE meetings, where questionnaires were the basis for workshop discussions in e.g. Santander (den Daas and Callesen, 2000).

The tools for working with reproductive technologies

To work in such a biological area, technologies are required, and over the years I have been involved in several such technical developments in quite different fields.

Ovum pick-up

Oocyte collection through the abdominal wall was developed in the human field in the first part of the 1980ies, and together with a medical doctor we made some of the first attempts in cattle. It was a para-lumbar approach (presented at the 1987 IETS meeting; Callesen *et al*., 1987a), and several lessons were made - one was that it can result in recovery of oocytes, but also that a cow patient will kick you when you prick her with a needle; a big surprise for the medical doctor! Soon after, especially the group from Utrecht led the way into the much more convenient vaginal approach that also became routinely used in human.

In vitro *embryo culture*

We continued to work on *in vitro* culture, driven by Gabor Vajta's urge for simplicity and reliability. One area was an incubator system based on having the culture dish in a foil bag that was submerged into a water bath (Vajta *et al*., 1997b). This provided very stable temperature conditions, required minimal use of gases, and each culture dish had its own chamber. However, only few would - and will - accept a water bath in their culture lab. Another area was related to the anticipated need for an embryo to establish a local *in vitro* environment during its development. This was obtained by hand-making small holes or impressions in the bottom of standard plastic dishes (Vajta *et al*., 2000). The method was subject for a course also given at the AETE meeting in Lyon in 2004. A third interest was to see how far the embryo actually could be cultured *in vitro* (Brandão *et al*., 2004; Vajta *et al*., 2004). We learned that the trophoblastic cells grew quite well to form up to an almost 2 cm long structure … but the very early embryo proper did not.

Time-lapse systems

To study embryo morphology frequently during *in vitro* culture, Peter Holm built his own time-lapse system in the mid-1990ies (Holm *et al*., 1998). This was a fairly simple and cheap system, but it provided what was needed from the oocyte's and embryo's point of view, allowing complete, high and stable *in vitro* development for up to 9 days together with taking pictures every 20-30 min. The resulting films were rather boring during IVM, but there was much more to see when monitoring the pre-implantation period in both cattle (e.g. Holm *et al*., 2002) and pig (e.g. Callesen and Holm 2016), also with cloned and parthenogenetic embryos (Holm *et al*., 2003). Through

this work I established an interesting collaboration with the Danish company Unisense A/S that later developed the EmbryoScope®, an instrument that today is used in several human fertility clinics around the world.

Vitrification

A challenge in cryopreservation was the more fragile types of eggs, such as oocytes in general and embryos from certain species such as the pig; none of these structures really tolerated traditional slow freezing. Parallel to a visit from Masashige Kuwayama (Kuwayama *et al*., 1997), the vitrification technology was taken further by Gabor Vajta, resulting in a thin-straw system (Vajta *et al*., 1998). Now also early-stage embryos from both cattle and pig became possible to cryopreserve (Vajta *et al*., 1997a, c). An impressing illustration of the potentials of this technology was three calves born after having been vitrified/warmed two times before transfer: first as *in vitro* matured oocytes, second as blastocysts after *in vitro* fertilization and *in vitro* culture (Vajta *et al*., 1998).

Oxygen consumption

Working with embryos, it has for a long time been a wish to complement the morphological evaluations of embryo development with functional measures. Together with the company Unisense A/S, experts in microsensor technology, we established a system for measuring oxygen consumption from single embryos, using the bovine as a model. Together with a Portugese PhD-student, Ana Lopes, we used it for single-day measurements first on *in vitro* produced embryos (Lopes *et al*., 2005), but later also on flushed vivo embryos, that were afterwards transferred, illustrating the relation between embryo "respiration" and viability (Lopes *et al*., 2007b). In another approach, we installed the oxygen consumption system inside the previously mentioned time-lapse system, so repeated oxygen measurements could be made on the same embryos during seven days of *in vitro* culture, resulting in very detailed oxygen consumption curves (Gundersen *et al*., 2006, reviewed by Lopes *et al*., 2007a). The technology thus works, but its technical complexity has so far not made it useful for other than special research purposes.

Using the basic circles in a wider context

My focus on the embryo for several years, having interest in both biological and technological issues, has taken me into a number of broader applications.

One was to question our traditional approach where we attempt to make the conditions for the embryos as pleasant as possible during their stressful *in vitro* period (Callesen *et al*., 2012) and instead combine the various methods into a pro-active and challenging testing system to select the most robust embryos. This so far theoretical idea was subject for an IETS presentation in Argentina (Callesen *et al*., 2010), but it still remains theoretical.

A second area was in the post-partum cow, in which period oocyte and embryo qualities are key issues when it comes to establishment of a new pregnancy. The start of estrus cyclicity requires the endocrine systems to be in positive balance with the follicular development in the ovaries, and a successful outcome after insemination requires the whole reproductive system to be ready-for-use. In three different studies, indirect measures for these internal events were studied with particular reference to use in practice. In one, the vaginal discharge was characterized during the first period after calving and related to the cow's progesterone profiles (Gorzecka *et al*., 2011a, b, c). In another, focus was on metritis in the same period, working on the bacterial population and its effect on reproduction, as well as establishing a uterine scoring system (Elkjær *et al*., 2013a, b) as basis for deciding when to perform the first insemination (Elkjær *et al*., 2013c). In the third study, estrus cyclicity and reproductive performances were followed for a longer period after calving, namely in a system with extended lactation (Gaillard *et al*., 2016).

The third area was somatic cell nuclear transfer of pigs ("cloning"). We have been working on this complex technology over more than 20 years, first in cattle with birth of calves as a result (Smith *et al*., 1994), since in cattle and pigs with a zona-free approach (Booth *et al*., 2001a, b), and then with Gabor Vajta's handmade-cloning system (HMC; Vajta *et al*., 2003; Kragh *et al*., 2004) that resulted in the first piglets born in 2006 (review by Vajta and Callesen, 2012). The HMC system was going through a number of optimizations for example with different cytoplasmic volumes (Li *et al*., 2015) and with gilt versus sow oocytes (Li *et al*., 2014; Pedersen *et al*., 2015). Further, different pre-treatments were tested with cells and oocytes being exposed to a frog extract (Liu *et al*., 2014) or embryos to a high pressure treatment (Lin *et al*., 2014). Another very important side of our cloning work was related to the recipient animal, both in their selection and pre-treatment, but also with the transfer method used (Schmidt *et al*., 2010). Finally, the outcome was also being analyzed thoroughly (Liu *et al*., 2015), both related to the period around birth and to the piglets born with their reasons for not surviving this challenging procedure (Schmidt *et al*., 2011, 2015). Combining all these aspects, we built up a system that over four years produced very satisfying results (Callesen *et al*., 2014), and today we have a number of cloned piglets that are transgenic for different serious human diseases (e.g. Luo *et al*., 2011; Staunstrup *et al*., 2012; Al-Mashhadi *et al*., 2013; Jakobsen *et al*., 2014 - and more are coming). Over the next years, the medical doctors will reveal if these transgenic piglets can serve as useful animal models for the different diseases.

To all my collaborators

The type of research that I have described does not work well if you are sitting alone on a desert island with some paper and a pencil … no, we need each other.

In the different activities, we can have different roles, influenced by background education, experience, time etc. For all of these activities described above, I believe to have had a significant role in making them happen, but all have only been possible because we have been working as a group. We have never been a large group and Denmark is not a large country. However, Torben Greve learned me the importance of travelling around, meeting colleagues, presenting at scientific meetings. It may mean some fairly big travel expenses, but it is worth it. I first saw that as a young PhD-student at my first international meeting in 1983 in Helsinki, and since I have been at many such meetings at IETS, AETE, SBTE, ICAR and several others to meet you, discuss with you and visit you. From such an approach, even the small Danish groups have been around for some years now, and surprisingly, one of the Danes now stands here on this occasion. So, size does not always matter, if I may say so.

Final remarks

Through the years I have been working around the farm animal embryo in the days before, during and after fertilization, and this has been done in different species, in different contexts, with different technologies, in different collaborations. From such a view, I may have become a generalist in this field, but I still do consider the superovulated cow to have a special place in my scientific heart.

Speaking about my heart: My almost 35 years in research - so far - have given me so many contacts to colleagues, and today I am lucky and proud to consider quite many of you to have become friends. Thanks to all I have met during this travel around the embryo, nothing like that could or would have been done without you.

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