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Achievements and unmet promises of assisted reproduction technologies in large animals: a personal perspective

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Abstract

This paper gives an overview of assisted reproductive technologies (ART) in livestock species coming from the author's direct experience and contribution to the development of several of them. The assessment is conducted on the basis of the progress achieved since the early eighties and the impact on the clinical/practical use of such procedures. Artificial insemination (AI) is still the leading technology used on a large scale in livestock with most favourable cost benefit ratio. All the other ARTs have niche applications compared to AI. Significant progress has been achieved in embryo culture, somatic cell nuclear transfer and on the identification of the many unknown variables affecting the success rate, while in areas such as superovulation, oocyte maturation, IVF, embryonic stem cells and cryopreservation progress has been limited or absent. It is the opinion of the author that ARTs have reached a plateau whereby only minimal improvement of efficiency can be achieved. Significant advances can only come from major breakthrough in the understanding of the underlying biological mechanisms.

Keywords: large animals, oocytes, embryos, stem cells, SCNT.

Introduction

Assisted reproduction technologies (ARTs) as we know them today have been the result of a long process that started conventionally with the first artificial insemination performed by Spallanzani (1780) in a bitch and the first embryo transfer in rabbit performed by Heape (1890). The former laid down the principle and basic knowledge for the development of the artificial insemination (AI) industry on the male side while the latter marked the beginning of embryo technologies on the female side.

The theoretical and scientific basis for these developments came in fact much earlier (see (Cobb, 2012)for review) when William Harvey published his book De Generatione Animalium (Harvey, 1651) and when Leeuwenhoek discovered the spermatozoa (Leeuwenhoek, 1678).

It has been only in the second half of the last century that ARTs reached the high level of efficiency necessary to find a practical application in livestock breeding for diseases eradication and genetic selection and to serve as a model for clinical application in human clinics. Artificial insemination rapidly developed as a cost effective and reliable technique in the cattle industry (Vishwanath, 2003) where it is now the standard because of the limited amount of semen needed per individual female. However, in other species, like the pig or the horse, the requirement for higher amounts of semen per insemination dose did not allow the same widespread use of AI in breeding programmes. Moreover, significant research investments would be required still today to overcome sensitivity to cryopreservation and achieve further improvements.

Much less modest has been the impact of ARTs on the female germ line because the current state of the art allows to exploit only a very minute fraction of the oocyte pool present in the ovary to generate offspring.

In this paper I will review what were the expectations or the promises thirty years ago at the beginning of my scientific career, what has become a reality and what has remained undeveloped or has become an illusion based on a personal opinion.

The sperm

Quantity and quality of semen per se has rarely been a limiting factor for artificial insemination in cattle both as frozen or refrigerated. In other livestock species the use of frozen semen is still highly variable and in general is associated with lower fertility, due to cryo-injuries of various kind (Bailey *et al*., 2000), and often requires laparoscope or deep intrauterine insemination. Advances in insemination with lower sperm numbers has come from the developments of sperm sexing by flow cytometry (Cran, 2007). It is now possible with the refinements introduced in the sexing procedure to achieve pregnancy rates in cattle comparable to non sexed semen, with a fraction of the number of spermatozoa used for conventional artificial insemination (Gonzalez-Marin *et al*., 2016). Methods of sperm sorting other than those based on DNA content have failed to become established in practice (Seidel, 2012) so far. Male germ cell transplantation (Brinster and Zimmermann, 1994) was also proposed as a way to increase sperm production, rescue particular genotypes or to alter the germ line (Dobrinski, 2005) but it has not progressed to a level of practical use for livestock breeding.

The superovulation

The MOET (Multiple Ovulation and Embryo Transfer) is the most cost effective way to exploit the female genetics in cattle and small ruminants. The products that are used to induce superovulation have changed over the years as well as the average number of

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embryo produced (Bo and Mapletoft, 2014). What has changed over time however are the protocols that have become more user and animal friendly (Mapletoft and Bo, 2011). In the horse, the use of equine derived FSH induces a good superovulatory response that however does not correspond to the expected embryo recovery rate (Logan *et al*., 2007) probably due to the constrains typical of the anatomy of the horse ovary. Therefore horse superovulation is not being used in practice.

The oocyte

Despite being a finite number at birth, contrary to the sperm, primary oocytes are present in the order of tens of thousands (Lazzari *et al*., 1992) on livestock ovaries. In theory all of them could be harvested and recruited for maturation and fertilisation rather than being lost due to atresia. However the specific requirements, including sequential media and prolonged culture time, to grow a primordial oocyte to a stage of full competence for meiotic resumption and embryo development has been optimised only in mice (Eppig and O'Brien, 1996). In livestock species attempts have been made to grow ovarian follicles at various stages of development both in vitro and in xenografts but with very modest success (Silva *et al*., 2016). Only two calves have been produced from growing early antral follicles (Miyano and Manabe, 2007) and an improvement of blastocyst production could be obtained again by growing oocytes collected from early antral follicle cultured in vitro for 2 weeks (Makita *et al*., 2016), thus the supply of competent oocytes is still limited to the advanced antral follicles where the oocyte has completed the growth phase and acquired the full developmental competence.

The recovery of oocytes from pre-pubertal animals has been always proposed as a way to shorten the generation interval and increase the number of offspring from any given female. The advent of genomic selection, that allows the identification of superior genotypes soon after birth, has been an incentive to select oocyte donors at a very young age. For the same reason genetic screening has been optimised starting from embryo biopsies anticipating at the pre-implantation stage the identification of the required genotypes. In ruminants the competence of pre-pubertal oocytes is limited in the ability both to develop to blastocyst (Galli *et al*., 2001) and to establish pregnancy rates compared to adult donors (Ptak *et al*., 2006; Galli C. 2017; Galli and Lazzari; unpublished observations).

The protocols to mature in vitro competent oocytes capable of giving rise to offspring after fertilization, culture and embryo transfer has long been developed (Staigmiller and Moor, 1984; Gandolfi and Moor ,1987). It is well established that oocyte competence is correlated to follicular diameter (Galli and Moor, 1991), nevertheless even after the most accurate selection according to the state of the art and the best in vitro conditions, the average blastocyst production has remained fairly stable despite many scientific papers reporting every time few percentage points increase of blastocyst rates for any given treatment. After 30 years of developments of the *in vitro* technology, we still average at best 30% blastocyst rates in cattle under experimental conditions and half that in a clinical context (Galli *et al*., 2014b). The situation is even more inefficient in the buffalo and the horse where only 10% of the oocytes eventually develop into a transferable embryo (Galli *et al*., 2007, 2012). Nevertheless for these species this success rate is higher that with in vivo production of embryos by superovulation and embryo flushing.

If the oocytes are matured in vivo and harvested from pre-ovulatory follicles their developmental competence is higher (Scott *et al*., 2001; Rizos *et al*., 2002;) indicating that the majority of oocytes that we are using for in vitro maturation are either coming from regressing follicles (advanced atresia) or from growing follicles that are not yet ready for maturation and require more than the canonical in vitro maturation time necessary to reach metaphase II. The introduction of the concept of pre-maturation *in vitro* to allow time for the oocyte to complete cytoplasmic maturation before the resumption of meiosis has found no application because of the modest, if any, improvements (Dieci *et al*., 2013; Lodde *et al*., 2013;) despite the fact that such inhibited oocytes at least maintain their developmental competence (Ponderato *et al*., 2001) and allow better scheduling of the work. This approach has found application in the equine. It is remarkable that by simply holding equine oocytes at room temperature for 24 h maturation can be arrested without loss of viability (Choi *et al*., 2006; Galli *et al*., 2014a) and the same blastocyst rate can be obtained compared to freshly matured oocytes following in vitro production. This procedure allows shipping of oocytes from equine clinics where they are recovered, to a centralized in vitro production laboratory for ICSI and embryo culture, and returning to the same clinics the frozen blastocyst for transfer (Galli *et al*., 2016).

***In vitro* fertilization (IVF)**

This event critical for the successful trip of the oocyte towards becoming an embryo has been almost neglected in recent years and has not been the subject of much research. In humans IVF has become a reality after the first success obtained with the birth of Louise Brown (Steptoe and Edwards, 1978), celebrated also with the 2012 Nobel Prize to R. Edwards. This event was the turning point where human IVF began to be the model for livestock species. It was not until 1982 that the first success was reported in cattle (Brackett *et al*., 1982) and later became routine procedure with the use of heparin to capacitate bull spermatozoa (Parrish *et al*., 1986) and other ruminants but no significant progress has been made since then. Most of the bulls will fertilize in vitro under standard conditions but the variation can be high ranging from no fertilisation to polyspermy, both compromising embryo development. This problem has its origins both in the variable quality of the frozen semen batches, in the intrinsic genetic variability of the semen donors but also in the quality of the oocyte itself

and in its competence and ability to block polyspermy. In species other than ruminants IVF has remained an unreliable procedure. In pig the unresolved problem of polyspermy still dominates the field (Romar *et al*., 2016) but no solutions are on the horizon. Still porcine embryos can develop in vitro to reasonable rates (Grupen, 2014) and probably adjust to the diploid state during early embryo development. In the horse the situation has been the opposite: IVF does not work, in fact no reproducible advances have been made since the only successful IVF report (Palmer *et al*., 1991). Again the human model helped to overcome this limitation in the horse with the introduction of ICSI (Palermo *et al*., 1992) that is now largely used on a routine basis in human IVF. In the horse ICSI is currently the only option to obtain fertilization in vitro and luckily the horse is the livestock species where it gives consistent results (Lazzari *et al*., 2002a) like in humans. Given the paucity of oocytes that can be harvested from a mare, ICSI will remain the technique of choice even if IVF ever would become available in the future. With the introduction of ICSI *in vitro* embryo production in horses has become possible and it has been developed to a level of being used in the clinical practice (Galli *et al*., 2014b). ICSI would be of help also in cattle to use limited amount of sexed semen or semen of poor quality recovered from young bulls; however, so far results have been disappointing (Galli et al. 2003b), and the rates of blastocyst remains below those obtained by conventional IVF. The advent of piezo-ICSI could offer still some options that could be developed also for cattle or pigs.

The preimplantation embryo

Embryo development in vitro is the area where significant progress has been made in recent time. Forty years ago in vitro derived livestock embryos could be hardly kept alive in culture and the only successful report was from Tervit (Tervit *et al*., 1972). Then the use of embryo co-culture with oviductal cells (Gandolfi and Moor, 1987) was the beginning of a new era. To overcome the limits in early technology and lack of scientific knowledge, in vitro produced zygotes have been cultured for a long time in the surrogate sheep oviduct that ensured cryotolerance and viability after transfer to recipients comparable to that of in vivo generated embryos (Shehu *et al*., 1996; Lazzari *et al*., 2010). For many years and still now in some laboratories, co-culture with primary cells like oviductal or cumulus cells (Galli and Moor, 1989) or with established cell lines like BRL and Vero became the routine also in the human field (Menezo *et al*., 2012). The presence of a monolayer of somatic cells however required the use of complex media and the presence of serum to ensure the viability of the somatic cells. The cells function was to create a microenvironment with low oxygen, besides promoting detoxification and/or providing secretion of factors suitable for embryos to develop. It turned out however that serum (and probably some secreted factors) was detrimental for post implantation embryo development in ruminants and identified as primarily responsible for the Large Offspring Syndrome (LOS;Young *et al*., 1998). The presence of serum was also found detrimental for cryopreservation because it was responsible for the accumulation of lipids (Shehu *et al*., 1996; Lonergan and Fair, 2008). Therefore media formulations shifted towards a serum free and cell free solutions where the still undefined component remains only Bovine Serum Albumin. The medium used today by many laboratories is based on the SOF formulation of Tervit (Tervit *et al*., 1972) with the addition of amino acids and various energy substrates (Gardner *et al*., 1994) as well as the reduction of the level of oxygen to 5% by feeding nitrogen to the gas mixture to lower oxidative damage.

With the use of modified SOF medium the incidence of LOS was decreased (van Wagtendonk-de Leeuw *et al*., 2000) but still the presence of high levels of BSA is accountable for such problems (Lazzari *et al*., 2002b) and occasionally LOS is still reported. LOS is typical of ruminants. In the horse it has never been reported: the foals born out of *in vitro* embryo production are not oversize (Galli *et al*., 2007). This suggests that the problem is associated with the type of placentation and or placental abnormalities (Farin *et al*., 2006; Ptak *et al*., 2013).

The embryos that are selected for freezing or for transfer are chosen by experienced embryologists based essentially on morphological criteria. Attempt to develop more objective non-invasive measurements that could be use to select embryos for transfer have not found the way to the clinical use. Both the measurement of oxygen consumption (Lopes *et al*., 2007) or the amino acid turnover (Brison *et al*., 2004) were investigated for this purpose. Another aspect that is not taken into consideration is the embryo genotype. Usually beef breeds perform better in embryo production *in vitro* than Holstein, notoriously a breed with higher inbreeding. We have shown that crossbred embryos develop better that inbreed ones (Lazzari *et al*., 2011).

An important part of ARTs is the cryopreservation of embryos both for practical and commercial reasons. Despite the development of vitrification (Rall and Fahy, 1985), that found its application in research laboratory or in human clinics for oocyte cryopreservation (Vajta, 2013), the industry standard in livestock for both ruminants and horses embryos is still the slow cooling method (Willadsen *et al*., 1978). Embryos frozen in glycerol require very simple thawing procedures for cryoprotectant removal that any practitioner can perform on farm conditions. The development of direct transfer has further simplified the transfer of bovine embryos (Voelkel and Hu, 1992) allowing many more practitioners, without the ability to handle an embryo in a dish, to perform embryo transfer.

The stem cells and somatic cell nuclear transfer

At the beginning of my scientific career Steen Willadsen (AETE Pioneer Award recipient) had already cloned sheep and cattle using blastomeres of early stage

pre-implantation embryos (Willadsen, 1986) and companies were being set up by the breeding industry to exploit this technology. But soon it became evident that embryonic cloning had limitations (number of nuclei available, unpredictable genotype, etc.). It was from this limitation that the scientific community got interested in developing embryonic stem cells to have an unlimited source of nuclei for nuclear transfer. In collaboration with Martin Evans (later 2007, Nobel Prize for Medicine) we started a long and painful path in the attempt to establish embryonic stem cells in sheep and pig (Notarianni *et al*., 1991) and later on in cattle (Galli *et al*., 1994; Lazzari *et al*., 2006) but never succeeded to obtain naïve ESC lines and to date no one has reported success in this endeavour. At most we and others obtained ES-like cells that probably were not much different from somatic cells. Because of the difficulties in generating stable ESC lines the interest for nuclear transfer shifted towards this type of ES like cells (Galli *et al*., 1991, Campbell *et al*., 1996) and eventually to somatic cells (Wilmut *et al*., 1997, Galli *et al*., 1999). It turned out that somatic cells can be used for cloning animals and indeed surprisingly perform better that the supposedly less differentiated cells (Sung *et al*., 2006).

In the horse the development of in vitro maturation and embryo culture has also benefited the cloning of this species (Galli *et al*., 2003a) that fortunately, despite the low efficiency of development to term, as in other species, it turned out to be free of LOS and late pregnancy losses. In the pig the situation is intermediate and no dramatic phenotypes are reported (Kurome et al., 2013). The low efficiency is compensated by the transfer of many cloned embryos per recipients to ensure a high pregnancy rate and a reasonable number of newborn piglets.

The recipient

The success of any ART procedure is measured at the end of the day on the birth of a viable offspring. Therefore the recipient that is selected for the transfer plays an important part in the successful outcome. Synchronization procedures have been greatly improved and simplified with the introduction of the Ov-Synch protocols (Thatcher *et al*., 2004; Baruselli *et al*., 2010) for cattle together with a better management of the health and nutrition of the recipient animals and the collection and registration of precise information in electronic formats. Maiden recipients are generally preferred and give the highest pregnancy rates across livestock species. However in cattle there is always a struggle to maintain the pregnancy rate close to 50% especially with cryopreserved embryos or embryos produced in vitro. This relatively low efficiency seems to be associated with the gradual decrease in fertility observed in cattle herds (Diskin *et al*., 2016) that hopefully will be reversed by genomic selection for fertility traits. On the contrary in vitro produced frozen-thawed equine embryos can achieve a consistent and remarkable pregnancy rates up to 60% with a foaling rate of 50% (Galli *et al*., 2007, 2016). Also in pig the use of gilts ensures after embryo transfer a pregnancy rate in excess of 50%.

The unknowns of the laboratory

The outcome of ART procedures depends heavily, besides the gametes and the surrogate mothers mentioned above, also on two other components: the human factor and the laboratory set up. After 30 years in the profession I have meet and seen all sorts of people: the good, the bad, the ugly. Gametes and embryos are like little babies and they need a lot of care to be kept alive and thrive. Over the years this human factor has been and still is very important despite some technological advances and better equipment. Therefore it is crucial to find the right people to work in the laboratories moreover a long period of training, trial and testing is needed. Unfortunately these people are rarely found. The other critical aspects are the equipment, reagents and the disposables used in the various procedures. A great help has come from the enormous development of the human ART industry, in terms of varieties of supplies and technical solutions. However livestock gametes and embryos and in particular cattle are, in our experience, far more sensitive that mouse or human ones. This is why the most common quality control test used by the suppliers of disposable, chemicals or media, i.e. the MEA (mouse embryo assay; Punt-van der Zalm *et al*., 2009) does not detect toxicants or conditions that can affect cattle embryo development. For this reasons we have introduced our own testing for quality control using bovine embryos, both for disposables plastic, BSA and culture media once a new batch is prepared. A typical example of chemical that requires testing is mineral oil (Otsuki *et al*., 2007). After several years there is still an issue in discussion forums about its toxicity and the use of paraffin oil instead of mineral oil. We are not using oil at all for long term culture. Only during cloning or ICSI procedures we cover the micromanipulation drops with mineral oil but exposure is limited to short time. The reason is that even batches that we tested and found suitable for embryo culture, over time became toxic.

The unknowns of the biology

What has emerged with the implementation of ART in livestock, in particular in ruminants, is the incidence of some abnormalities that might result in low pregnancy rates, high pregnancy losses and abnormal offspring with a higher birth weight that can cause dystocia (Lazzari *et al*., 2002b). Several factors can contribute to this phenomenon and the causes of these perturbations are likely to be a response to stressors (Thompson *et al*., 2002) . Sub-optimal in vitro environment, inadequate culture medium and untested disposable material can contribute to alteration of cellular parameters such as pH and redox state ultimately affecting embryo development. Factors such as diet and metabolic conditions can be involved in epigenetic effects while specific procedure such as somatic cell nuclear transfer, can directly affect the

methylation status of the cloned embryos. These findings in the animal models have fuelled concerns for human ART (Thompson *et al*., 2002) where these alterations might have far greater impact on the health of the resulting babies at birth and later on during adult life on the incidence of diseases such as diabetes type 2, cardiovascular diseases and obesity (Chen and Heilbronn, 2017). This issue will be addressed more fully in the near future with the increasing amount of data collected and analysed from ART adult offspring. In livestock species the long term effects impact essentially on the efficiency of the technology while animal welfare questions emerge mainly in ruminants and in relation to pregnancies and offspring derived from somatic cell nuclear transfer procedures. On a positive note these epigenetic alterations do not appear to be transmitted to the progeny (Tamashiro *et al*., 2002; Shimozawa *et al*., 2002).

Conclusions

ARTs have come a long way in the last 30 years both in animals and humans and are well established in the clinical practice. Progress has been slow but steady especially in the area of embryo culture (Table 1). It seems that we have now reached a plateau with only small margins for improvement because of the intrinsic biological and/or technical limitation /variation of the source of gametes and of in vitro conditions. We have to live with that unless a major breakthrough occurs in our understanding of the underlying biological mechanisms.

Table 1. Current status of assisted reproductive technologies in livestock, a personal opinion.

	progress	practical use	research activity
	last 30 years	present	present
AI	+	++++	+
MOET	+	+++	-
Oocyte	++	+++	+
IVF	+	+++	+
embryo culture	++++	+++	+++
cryopreservation	+	+++	+
embryonic stem cells	-	-	+
SCNT	+++	+	++
recipient animals	++	+++	+
unknowns	+++	+++	++
	++++ intense	+++ moderate	++ low
	+ minimal	- absent	

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