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Genomic selection of *in vitro* produced and somatic cell nuclear transfer embryos for rapid genetic improvement in cattle production

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Abstract

This paper provides basic concepts of genomic selection (GS) methods in beef and dairy cattle production in combination with assisted reproductive technologies (ART) such as ovum-pick up and *in vitro* production (OPU-IVP). We first introduce genomic tools and discuss main methods of GS as practiced to-date. The general benefit from GS is that it enables selecting animals accurately early in life using genomic predictions particularly those phenotypes that are very difficult or expensive to measure. While it is known that GS increases genetic gain and profit in conventional cattle breeding, GS is much more desirable when combined with OPU-IVP in cattle production. The expected benefits of GS-OPU-IVP far exceed the benefits achieved by either GS or OPU-IVP alone mainly due to tremendous reduction in generation interval. The genetic improvement will increase even further, if genetic merit of donor cows and bulls used in OPU-IVP for key economic traits are maximal. The paper also highlights some challenges particularly with regard to embryo biopsies and quantity and quality of embryo DNA for whole genome genotyping and ways to overcome difficulties. We briefly discuss the somatic cell nuclear transfer (SCNT) technique in the context of applying GS on fibroblast cell lines from fetuses obtained from OPU-IVP techniques and provide our perspectives on how it might pave way for even more rapid cattle improvement. Main conclusion is that employing genomic selection in ARTs such as OPU-IVP of embryos coupled with embryo sexing and SCNT will lead to rapid dissemination of high genetic merit animals on a scale never been seen before. Finally, the paper outlines current research activities on combined genomic selection and advanced reproductive technologies in the GIFT project consortium (www.gift.ku.dk).

Keywords: cattle, embryo transfer, genomic selection, OPU-IVP, somatic cell nuclear transfer.

Introduction

Rapid population growth will increase the demand for food as well as other animal products, particularly in emerging economic giants like Brazil and India. Moreover, the urbanization has considerable impact on patterns of food consumption in general and on demand for livestock products in particular. Cattle (dairy and beef) production in most countries in North America and Europe has well established infrastructure and organizational structures to improve economically important animal traits for decades. This has led to substantial increase in both (the efficiency of) meat and milk production from cattle as well as the ability to attain self-sufficiency, but more importantly to a significant source of national income from export and other industries. In sharp contrast, there are several bottle necks in establishing infrastructures and organizational structures for performance data recording in farms and in central test stations/feedlots for calculation of estimated genetic merit (EBVs: estimated breeding values) and applying assisted reproductive technologies (ART) such as *in vitro* production (IVP) of embryos and Embryo Transfer (ET). In addition, both productivity and efficiency of production in developing and/or tropical countries is very low due to environmental stressors and challenges (O'Neill *et al*., 2010).

However, molecular breeding techniques such as combined genomic selection (GS) and modern ARTs such as ovum-pick up and *in vitro* production (OPU-IVP) of embryos provide a rapid and sustainable avenue for genetic improvement of both efficiency and productivity. This is to an extent that it can bypass some of the expensive data recording, progeny or performance testing and conventional genetic evaluations of large number of animals on a routine basis.

The global bovine embryo market reached 1,275,874 embryos during 2013 (International Embryo Transfer Society - IETS, 2014). Importantly, from 2000

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to 2013 the IVP of embryos went from 17.4 to 40.6% (517,587 produced embryos) of representativeness compared to the *in vivo* technology (IETS, 2014). Moreover, this global increase was related to the remarkable improvement in the Brazilian IVP market, responsible for 70.8% of the IVP of embryos (IETS, 2014). Brazil expanded over seven times the bovine IVP from 2001 (50,000 embryos) to 2013 (366,517 embryos). Considering the *in vitro* embryos produced in Brazil, in 2013, 45.7% (167,452 embryos) were obtained from dairy donors (88.6% of *Bos taurus* females) and 54.3% (199,065 embryos) from beef cattle (86.8% of *Bos indicus* females; Viana *et al*., 2015; University of São Paulo, Brazil; unpublished data). Therefore, this IVP index reflects the Brazilian potential market in *Bos indicus* and *Bos taurus* donors submitted to the OPU and IVP programs in large scale.

In addition to large scale increase in embryo production, it is now also theoretically possible to combine GS and OPU-IVP with somatic cell nuclear transfer (SCNT), taking the cattle production well beyond its current potential. The combined GS, OPU-IVP and SCNT, if applied widely, has a tremendous potential for the entire world cattle production including developed countries.

This paper provides basic concepts of using genomic selection (GS) methods applied to OPU-IVP cattle production. The paper also highlights the challenges as well as the expected benefits of genomic selection applied to bovine IVP. We briefly discuss the SCNT technique in the context of applying GS on fibroblast cell lines from fetuses obtained from OPU-IVP techniques and provide our perspectives on how it might pave way for rapid cattle improvement on a scale that has never been seen before. Finally, the Consortium on Genetic Improvement of Fertilization Traits (GIFT) in Brazilian and Danish Cattle (www.gift.ku.dk) and their main activities are mentioned.

Genomicsin cattle production and reproduction

Known genes and genetic markers influencing animal traits allow breeders to make improvements using gene assisted selection and marker-assisted selection (MAS; Kadarmideen *et al*., 2006; Kadarmideen and Reverter, 2007). However, the implementation of MAS programs has rarely been successful for several reasons (Goddard and Hayes, 2009). There are some exceptions to this case, where known genes with known functional impact on the reproduction or fertility are used. For instance, in case of bovine IVP, the oocyte quality and quantity is important. The genes that are predictive of good quality oocyte include FSHR (Izadyar *et al*., 1998), EGFR (Conti *et al*., 2006), AREG (Nautiyal *et al*., 2010; Peluffo *et al*., 2012), PR (Aparicio *et al*., 2011), COX2 (Takahashi *et al.*, 2006), GDF9 and BMP15 (Hussein *et al*., 2006; Gilchrist *et al*., 2008), H2A (Pasque *et al*., 2011), PDE3 (Richard *et al*., 2001) and OOSP1 (Tremblay *et al*., 2006). There are genes that are predictive of good quality blastocysts which include ACSL2 and HAND1 (Arnold *et al*., 2006), G6PD, GPX1, OCT4, PLAC8, SOD2 (Cebrian-Serrano *et al*., 2013), GLUT1, GLUT3, KRT8, PGK1 (Machado *et al*., 2012), GATA6, SOX2 (Ozawa *et al*., 2012), IL1-B (Paula-Lopes *et al*., 1998), LIF, LR-B (Rizos *et al*., 2003).

The Bovine Genome Sequencing and Analysis Consortium initially sequenced and assembled *Bos taurus* cattle genome with approximately 7-fold coverage - this initial assembly reported around 22,000 genes and 14,345 orthologs shared among seven mammalian species (Elsik *et al*., 2009). The benefits of genome sequencing efforts are that it has led to detection of tens of thousands of abundant markers called single nucleotide polymorphisms (SNPs). These abundant SNP markers in several thousands to a million, in the form of genotyping arrays or SNPchips, can be used in whole genomic selection rather than in MAS that uses only a few hundreds genetic markers.

Genomic selection (GS) and its benefits in conventional breeding

Genomic selection relies on 'whole genomic prediction' of breeding values and was coined in the landmark paper by Meuwissen *et al*. (2001). GS methods based on best linear unbiased prediction (BLUP) models enable us to predict the performance of animals given their genotypes at SNPs across the entire genome. These SNP effects are estimated from a large reference population with both genotypes (from SNPchip) and phenotypes of interest. Estimated SNP effects are then used to determine the merit of other genotyped animals that are not yet phenotyped. Common GS methods are Genomic best linear unbiased prediction (GBLUP; Goddard *et al*., 2011), Single-step BLUP (ssBLUP) method (Aguilar *et al*., 2010), and several Bayesian approaches (BayesA, BayesB and BayesCπ; Meuwissen *et al*., 2001; Habier *et al*., 2011).

Genomic prediction models vary based on several assumptions regarding the variance of traits of interest. GBLUP is a prediction method that assumes that all markers contribute to the additive genomic variance. This method is similar to the traditional BLUP method applied for in animal breeding for over 25 years, except that a genomic relationship matrix replaces the numerator relationship matrix computed from the pedigree information. Another method called Random regression BLUP (Meuwissen *et al*. (2001) assumes SNP effects are randomly distributed, and is considered equivalent to GBLUP (Goddard *et al*., 2011). ssBLUP jointly analyzes phenotypes and genotypes of all animals in one step (Aguilar *et al*., 2010). Inclusion of all animals (with and without genotypes) results in the better correction of genomic preselection effects; and consequently provides more accurate estimation of

GEBVs. Several Bayesian approaches have been used for genomic prediction, and these methods assume a prior knowledge about distribution of SNP effects influencing a trait. BayesA assumes that all SNPs have an effect, but each SNP has a different variance that is assumed to be equivalent to a scaled inverse-χ2 prior. The BayesB and BayesCπ assume that each SNP has either an effect of zero or non-zero with probabilities π and 1-π, respectively. Genomic prediction accuracy gets better as the trait heritability and the reference population used for calculating GEBVs increases. Overall, genomic prediction methods are improving, especially with advent of whole genome sequence data from next generation sequencing. At present, the difference between performances of different methods is marginal for most traits because they are controlled by many QTLs with small effect sizes. Genomic BLUP and its single step extension that includes non-genotyped animals (ssBLUP), remain the most commonly used methods. A new Systems Genomic BLUP or *sg*BLUP method has been introduced by Kadarmideen (2014) that accounts for biological or functional importance of SNPs in a similar framework as GBLUP.

GS has dramatically changed traditional progeny testing schemes in cattle and other species. This is because GS requires only a smaller proportion of animals to be measured for their performance (production or reproduction ability) and genotyped using SNPchip. It then predicts the performance of large proportion of animals that were not measured for performance but only genotyped. Genetic gain is increased by GS by increasing intensity of selection, accuracy of Genomic EBVs (GEBVs) and genetic variance and by reducing generation interval (Kadarmideen, 2014). Two major advantages of genomic selection compared with traditional selection based on pedigree and phenotypic data alone are (i) it can select animals accurately early in life (even at the embryonic stage) using their GEBVs, (ii) it can also predict phenotypes that are very difficult or expensive to measure, including but not limited to fertility, meat quality, disease resistance, methane emissions, and feed conversion (Hayes *et al*., 2013). In dairy cattle, for example, GS can reduce the generation interval by at least two years as we can pre-select the young bulls to be either progeny tested for production or used directly in the breeding programmes without progeny testing. It is stated that increase in genetic gain or income is 60 to 120% compared to traditional methods of progeny testing (Schaeffer, 2006; Pryce and Daetwyler, 2012), mostly achieved via dramatic reduction in costs of rearing large number of animals and selecting only a few as breeders. A recent study in Brazil (Neves *et al*., 2014) involved assessment of genomic predictive ability for 13 different weight and carcass traits, gestation length, scrotal circumference and two selection indices using 685 Nellore bulls with the Illumina Bovine HD chip SNP data (320,238 SNPs). Their results showed that accuracies of genomic predictions ranged from 0.17 (navel at weaning) to 0.74 (finishing precocity). Across traits, Bayesian regression models (Bayes C and BLASSO) were more accurate than GBLUP. The average empirical accuracies were 0.39 (GBLUP0), 0.40 (GBLUP20) and 0.44 (Bayes C and BLASSO). This study underlined and demonstrated that genomic selection can be practiced in Brazilian Nelore cattle and with this range of accuracy of selection, one can expect similar efficiency and genetic improvement as in Dairy catte and other livestock species.

Genomic selection in bovine IVP of embryos

Merging GSwith IVP production technologies can take the potential genetic improvement well beyond what can be achieved by individual methods alone (either by GS or by IVP). First of all, most genetic studies so far indicated that there is a heritable variation in donor cow's ability to produce good quantity and quality of oocytes (Merton *et al*., 2009) and recipient cows ability to maintain pregnancy and deliver IVP-calves (Spell *et al*., 2001; König *et al*., 2007). The genetic variation and heritability are very important criteria because if no genetic variation or heritability exists for a trait means, there will be no possibility for GS. Fertility in general, is a low heritable trait in both dairy and beef cattle. For instance, (Kadarmideen *et al*., 2000, 2003) reported heritability estimates ranging from 0.05 to 0.16 for traits such as non-return rates or conception rates, days to heat and first insemination, number of inseminations per conception, service period and calving interval in Holstein dairy cattle.

Age at puberty has a major effect on the productive, reproductive, and economic efficiency of female cattle (Monteiro *et al*., 2013). Eler *et al*. (2002) estimated the heritability of 0.57 ± 0.01 for the conception rate of young heifers during exposure to bulls in breeding season; this rather high heritability indicates that genetic selection could be useful to select heifers with a greater probability of precocious fertility. Additionally, studies performed in South America reported a high heritability of age at puberty in Zebu breeds (Nogueira, 2004). Therefore, heifers genetically selected for age at puberty, at first conception, and consequently at first calving should improve reproductive efficiency in cattle herds. These fertility traits would respond to GS due to existence of genetic variation between animals.

With regards to genetic basis of attributes or traits that are important for IVP, Merton *et al*. (2009) analyzed CRV (formally Holland Genetics) data from the OPU-IVP program from January 1995 to March 2006 and reported a heritability of 0.25 for number of cumulus-oocyte complexes, 0.09 for quality of cumulus-oocyte complexes, 0.19 for number and proportion of cleaved embryos at day 4, and 0.21 for number and proportion of total and transferable embryos at day 7 of

culture. These heritability estimates are on par with some of the meat and milk production traits that respond very well to GS.

As described above, the largest increase in genetic gain can be achieved by shortening the generation interval. In the simplest case of application of GS in IVP, an unborn animal's genetic merit is predicted at the embryo stage prior to implantation into recipient cows. The genetic gain is therefore improved rapidly by substantial reduction in generation interval because selection is made on an animal that was never born (Fisher *et al*., 2012; Ponsart *et al*., 2014). Furthermore, IVP embryos will be in large quantities compared to live born animals, therefore only a few animals are selected from large pool of animals (in their embryo stage) based on genetic merit (GEBVs), and rest of the embryos are discarded - this increases the selection intensity rapidly. Both reduction in generation interval and increased intensity of selection will lead to rapid genetic improvement. The large scale application of Genomic Screens of preimplantation Embryos (GSE) depends on cost-benefits of GSE to commercial producers. In fact, the use of sexed semen in IVP and combining this with GSE will transform the cattle industry.

However, GSE before embryo transfer would still be beneficial economically due to costs involved in embryo transfer (ET) of large number of embryos into recipient animals where pregnancy rates differ between uses of cows versus heifers. Further, the cost of raising ET calves of unknown genetic merit with later culling of inferior calves would also result in large logistical costs. For these reasons, GSE and selecting embryos before transfer would maximize the profit for the farmers by only transferring a "reasonable" number of embryos and raising only animals of "reasonable" genetic merit for meat or milk production. In fact, GSE will be very necessary for breeding companies to reduce costs by limiting number of ET and maintenance of unwanted calves.

The whole IVP operations can be further fine-tuned if both donor cows as well as the semen of sires used for the procedure have been genetically evaluated for a number of economically important traits (use only donor cows and sires with high GEBVs in IVP). This does not appear to be an issue for large commercial cattle breeding companies, because all animals in the breeding program go through GS and hence GEBVs should be available. However, the problem comes when IVP companies are not integrated within breeding companies, where IVP companies do not have access to GEBV information on donor cows and semen used in fertilization.

The entire workflow (depicted in Fig. 1) shows that even before GSE, one can only use the high genetic merit donor cows for OPU and use only the high genetic merit bull semen (sexed or unsexed) in fertilization; thus an IVP embryos from these parents are already high genetic merit. However, not all the full-sib embryos from the same parents will have the same genetic merit due to Mendelian sampling variance. Figure 1 illustrates that DNA can be extracted, embryos genotyped and subject to GSE prior to implantation. These assisted reproductive technologies (ART) combined with GS are expected to have dramatic impact in developing countries where traditional animal breeding, improving pregnancy rates via AI and GS is difficult to achieve or implement due to costs and infrastructural constraints. For instance, best bulls and donor cows could be identified within the large private farms or semi-private or private stud breeders or government-owned progeny testing farms. Typically IVP companies, with the help of genetic evaluation labs, can produce and deliver embryos of high genetic merit directly to places where ET takes place. The ET is usually carried out at veterinary dispensaries or hospitals and government AI centers or by technicians employed within large farms. If IVP and GS can be achieved successfully within and across several villages or townships, co-ordinated by regional centers, it will lead to overall genetic improvement rapidly and contribute to food security.

Challenges of GS in bovine IVP of embryos

There are certain technical limitations as to how widely GSE could be practical. There are critical issues in performing embryo biopsies and obtaining sufficient DNA quality and quantity for GS (Ponsart *et al*., 2014). While embryo biopsies for DNA extraction and amplification for genotyping is needed for GS, it has many technical limitations such as reduced genome coverage, allele drop-out at heterozygous loci which leads to lower SNP call rates relative to the threshold standards needed for genomic enhanced genetic analysis, missing genotypes, amplification of artifacts, or allele drop-in (Lauri *et al*., 2013; Kasinathan *et al*., 2015).

In New Zealand, Fisher *et al*. (2012) conducted a genotyping experiment using one- and three-cell biopsies from bovine morulae and using biopsy of trophectoderm from transferable quality blastocyst-stage embryos. The authors concluded that greater numbers of embryonic cells provided in the sample resulted in greater average call rate and lower replication error. The call rate for 30-40 cell embryo samples approached the 99% rates typically achieved for parental DNA obtained from blood. This provides an encouraging result.

Ramos-Ibeas *et al*. (2014) established an *in vitro* culture system to support the growth of bovine trophoblastic cells from an embryo biopsy using different cell sources of conditioned media, eliminating the risk of contamination with feeder cells. They claim that *in vitro* culture system facilitated the establishment of trophoblastic cell lines, which can be expanded (cultured) for more than 2 year and can be useful to studies in relation to placentation processes. In the context of large scale genotyping for GS, this approach

could also be employed to produce a relatively large amount of good quality genomic DNA for bovine embryo genotyping and epigenotyping.

Overall, GSE on OPU-IVP embryos is ongoing already in some private cattle breeding companies in major industrialized cattle producing countries but its widespread application is not yet optimal.

Somatic cell nuclear transfer (SCNT) in genomic selection

Recently, Kasinathan *et al*. (2015) proposed further reduction in generation interval and production of high genetic merit calves by combining advanced reproductive processes such as OPU- *in vitro* fertilzation (IVF) and GS on embryos with somatic cell nuclear transfer (SCNT). Collection of day 21-23 early stage embryos after ET from recipient cows and the establishment of cell lines from these embryos allowed rapid determination of enhanced genetic merit for a large number of candidate embryos. Kasinathan *et al*. (2015) show that fibroblast cell lines established from early stage embryos and subsequent GS on cell lines supported the production of high genetic merit calves by SCNT with efficiency comparable to IVP embryos. This method reduces the generation interval by approximately 7 months and offers the chance to produce multiple animals at the same or later time from banked, frozen fibroblast cell lines. They claimed that this approach is scalable and can lead to considerable savings for breeders by achieving substantial reduction in generation interval and selectively producing animals with the desired genetics within a timeframe of approximately one year.

Implementation

We proposed that overall there are 4 stages where GS can be applied in the entire production chain: First in donor cows, second in the bulls, third in pre-implantation embryos and fourth at the level of fibroblast cell lines. The difference between third and fourth stage is minimal because embryo or fetus genetic make-up are the same, except that a fetus could go through epigenetic and other programming events in the uterus. Hence, due to costs and practical limitations of obtaining adequate DNA for genotyping, third stage GS can be skipped if SCNT will be performed. If SCNT will not be performed, there will be only first three GS stages. At the minimal GS on pre-implantation embryos are highly recommended to improve the average genetic merit of all animals produced via OPU-IVP and reduce costs for the companies and farmers by minimizing unwanted ETs and raising calves with poor genetic potential. An overview of GS of embryos, OPU-IVP, embryos sexing and SCNT for rapid dissemination of high genetic merit animals in cattle production was given in Fig. 1.

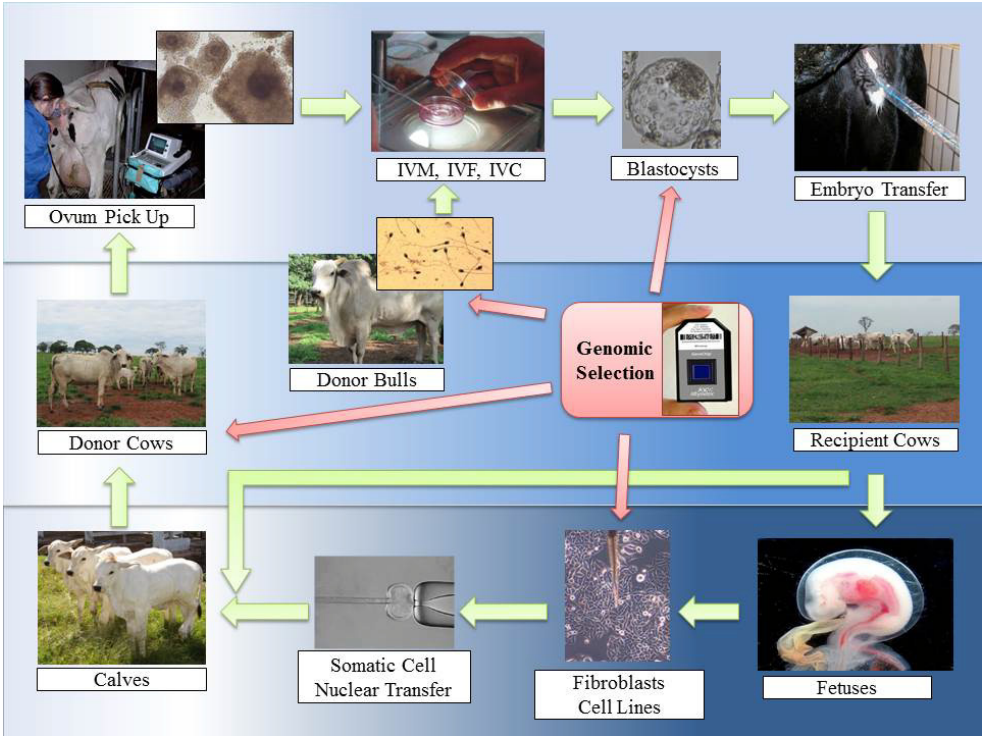

Figure 1. Work flow depicting a cycle of Genomic Selection on donor cows used in OPU-IVP, on donor bulls' semen used in IVF (either sexed or unsexed) and on pre-implantation embryos in rapid production of high genetic merit calves.Alternatively genomic selection is conducted on fibroblast cell lines from fetuses from recipient cows and used in SCNT for rapid production of calves of high genetic merit.

Danish-Brazilian bilateral GIFT project consortium

Much of the reviews provided here in this paper are actually taking place in the Danish-Brazilian bilateral GIFT project (www.gift.ku.dk). Here, we aim to deliver genomic estimating breeding values (GEBVs) and heritabilities (h^2) for key OPU-IVP traits and heifer pregnancy rates and correlations with other traits by conventional genetic evaluation tools. OPU-IVP traits include Oocyte Number (ON), Oocyte Quality (OQ), pregnancy rates in recipient cows as well as normal calving rates. We conduct Genome Wide Association Study (GWAS) to pinpoint genes and genetic variants (SNPs) influencing key IVP traits of cows and heifer pregnancy rates. We hope to deliver necessary information to develop a low density SNPchip which can improve pregnancy rates from 40 to 60% and follicles per ovary from score 1 to 5 of both ovaries. With 600 animals as a base reference population, we plan to conduct whole genomic prediction and genomic selection methods for OPU-IVP traits and with over 2000 Nellore cattle, genomic selection for heifer pregnancy is ongoing.

Legislation, ethics and costs of bovine ART

In Brazil, OPU-IVF is practiced widely and there are no legal barriers. The challenge indeed is in improving the pasture production and quality, feedlot production, Fixed Time Artificial Insemination (FTAI), IVF, ET, Fixed Time Embryo Transfer of IVF embryos, calculation and the use of genetic merit (estimated breeding values) of the cows, heifers and AI bulls in cattle breeding as well as better infrastructure and administration. This will have an important impact in the quality and amount of the Brazilian beef production. More or less, the situation is similar in most developing countries. In Denmark, a number of issues are related to the use of this range of ARTs in cattle breeding and production. Among the ARTs discussed, today's legislation in Denmark only bans the use of SCNT. However, the issue is continuously discussed both in Denmark and in the EU, both in relation to this technology itself, its influence on animal welfare and the possible effect on the resulting food products. How any future modifications will be is not known at present. The ethical discussion is related to both the general view on the techniques, their influence on the animals and the consumer's attitudes on the resulting food products. There is a generally negative view on the increasing use of ARTs in cattle breeding and production, where some of the reasons are related to concerns for the animal welfare. Examples are the repeated use of needles for anesthesia and for oocyte collection in OPU as well as the potential risk for both the recipient and the calf using IVP related to the large offspring syndrome (LOS). In particular issues related to LOS have been resolved to a high degree with the improved serum-free media for embryo culture. On the positive side are the potential achievements from a more powerful genetic selection when the breeding goals are for example focused on less mastitis, stronger legs, less digestive disease etc.

An open and public discussion about these issues is important, and in Denmark the governmental advisory Animal Ethical Council is a key player. Such discussion must have input also from those who actually working with these technologies, and such information can come from anywhere in the world. Therefore, the on-going GIFT research project can be a strong source of information, considering the huge experience found in Brazil on practical use of all these ARTs.

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