Review Report

Advances in complementary livestock artificial reproductive techniques

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Abstract

Assisted reproductive techniques are beneficial to increasing animal agricultural production and improving veterinary medicine. Foundational techniques (e.g. artificial insemination and in vitro embryo production) have paved the way for both complementary and adjunct techniques to arise as technologies and research advance. Female/offspring focused technologies include those to increase precision of estrus detection, genomic selection through embryo biopsy, and gene editing via 'clustered regularly interspaced short palindromic repeats' (CRISPR) and CRISPR associated protein 9 (Cas9), whereas male focused technologies include sex sorting after freezing and thawing ('reverse sex sorted semen'), multisire straws for artificial insemination, and spermatogonial stem cell transplantation. Although improvement in efficiency and feasibility is paramount, these recently developed and emerging techniques will likely become important to ensuring that animal products are produced efficiently and humanely. This review highlights these newer advances, with a particular focus on how they may impact mammalian agriculture and veterinary medicine.

Keywords: Assisted reproductive techniques, CRISPR-Cas9, embryo biopsy, estrus detection, reverse sex sorted sperm, spermatogonial stem cell transplant

Introduction

Assisted reproductive techniques (ARTs) include any technique and/or technology that changes the expected outcome of natural service. Purposes of ARTs in agricultural veterinary medicine and/or animal production include increasing reproductive efficiency, preserving genetics, and treating infertility.[1,2] Incorporation of ARTs to increase reproductive efficiency includes greater geographic dissemination of genetics,[2] promoting earlier conception,[3] increasing an individual's number of offspring,[4] and combating negative consequences of heat stress.[5] The goal of genetic preservation in animal production is often related to dissemination of genetics in the relatively near future. However, in certain cases, such as heritage breeds, the goal is to maintain or promote genetic diversity in the immediate and more distant future.[1] Infertility treatment is a less common purpose of ARTs in production animals compared to companion animals or humans, but may still occur for high-value individuals whose cause of infertility was not identified. Heritable conditions that impact fertility and/or performance of offspring may be grounds for classifying an animal as unsatisfactory during routine breeding soundness examinations.[6] Ultimately, utilization of ARTs for infertility treatment in production animals is only recommended when the cause of infertility is not heritable, when the fertility of offspring is not important (e.g. terminal-use sires) or in the case of extremely limited genetic diversity.

Synchronization of estrus and ovulation,[7] artificial insemination with or without the use of sex sorted semen,[8] multiple ovulation embryo transfer, and in vitro embryo production and transfer[4] are currently the most commonly used ARTs. These techniques, along with somatic-cell nuclear transfer[9] have been extensively reviewed. The purpose of this review article is to highlight and expand on ARTs that have arisen from foundational techniques, with a particular focus on how they may impact mammalian agriculture and veterinary medicine. These ARTs include: 1. those that increase precision of estrus detection; 2. genomic selection through embryo biopsy; 3. gene editing and 'clustered regularly interspaced short palindromic repeats' (CRISPR) and CRISPR associated protein 9 (Cas9); 4. reverse sex sorted semen; 5. multisire straws for artificial insemination; and 6. spermatogonial stem cell transplantation.

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Increasing precision of estrus detection

Estrus detection was vital to reproductive efficiency when artificial insemination was first implemented and still is critical in many dairy systems that are not utilizing fixed time insemination. Estrus detection is traditionally time consuming, with visual observation of the animal particularly difficult in pasture-based management systems. The simplest technique for improvement of estrus detection is to implement tail-head patches and/or paints that change colors or wear off when the female is repeatedly mounted and are commercially available in a variety of styles. Imprecise interpretation of when a patch is 'triggered' for artificial insemination can lead to variation of pregnancy outcomes, along with reduced reproductive and economic efficiency. Certain patch style designs attempt to combat this ambiguity. For example, Heat Seeker™ patches (Beacon Automation, Muswellbrook, NSW, Australia) are curved to the back to watch progression of estrus and have a larger surface area for detection. However, these still require regular visual observation of the animals and reapplication maintenance when patches fall off, either due to poor adhesion or normal wear and tear. HeatWatch® (ABS, DeForest, Wisconsin, USA) was the first patented patch technology that radiotransmitted the number of times and duration of mounting to computer software. This technology reduced the need to visually assess animals and minimized ambiguity in detecting estrus, but still required labor to analyze data on a computer. Other challenges of this technology were the short transmission radius, patches falling off, and expense, resulting in this technology being no longer commercially available. However, revival and progression of this technology have recently emerged; HEATsiecker® (HEATsiecker, Martell, Nebraska, USA) has an expected 1-2 miles radius and sends data in real-time to a cell phone app and utilizes a smaller and more cost-effective patch. Initial set up carries an expense due to purchasing the antenna utilized to send the data, but the patches utilized are not of much greater expense to those already on the market. Users are reporting that convenience and accuracy are reasons they will continue to use the system.

Precision livestock farming is the concept of using technology to constantly monitor production animals to better assess health, welfare, production, and calving.[10,11] For estrus detection, this includes pedometers/accelerometers as a tool to detect estrus-related increases in activity.[12] Rumination data have been investigated for estrus detection, based on decreases in feeding and rumination.[13,14] Activity monitoring can be a challenge due to the variability between animals and among housing systems. Regardless, other physiologically based technologies are likely to follow. One potential physiological marker that could be capitalized on includes increases in basal body temperature at and around estrus.[15] Current temperature assessment tools include rumen boluses,[16] subcutaneous RFID biosensor device,[17] and tail-head surface temp via wearable sensor[18]; these currently work well for detection of heat stress or diseases but are not sensitive nor specific enough for practical estrus detection without further calculation and analysis. Infrared thermography has been proposed as an estrus detection tool due to its ability to evaluate the whole animal and could be utilized either in a barn setting or pasture setting via infrared thermography cameras that are low maintenance, noninvasive and therefore relatively stress free on the animal, and low in labor input.[19] Other physiologic parameters investigated, but currently not practical, include vaginal conductivity, pheromones detected via trained dogs, and milk progesterone concentrations.[20] The recent development and widespread implementation of artificial intelligence presents a unique opportunity to improve estrus detection utilizing a combination of physiological data types, resulting in a practical report for the producer. Ultimately, these combinations of technologies, in conjunction with the development of artificial intelligence, will provide more accurate detection of estrus and increase reproductive efficiency.

Genomic selection through embryo biopsy

Embryo biopsies are typically performed on morula or blastocyst stage embryos after either an embryo flush or in vitro production. The goal of a biopsy is to remove enough cells needed for further analyses without damaging the embryo. Embryos are then frozen while awaiting results of the desired analyses. Viability and pregnancy rates of Grade 1 and Grade 2 (by International Embryo Technology Society standards) were not affected by embryo biopsy for either in vivo or in vitro produced embryos.[21] However, a decline in viability due to embryo biopsy may occur in Grade 2 in vivo produced embryos compared to Grade 1 embryos.[22] Method of choice for cell removal depends on the stage of embryo and/or technician experience and training[22]; acquired cells can be directly used for some single-trait analyses (e.g. sex determination) via polymerase-chain reaction. If the number of cells does not provide enough starting material of DNA to be used directly, whole genome amplification is performed prior to genomic analysis.[23] Genomic analysis is performed utilizing single-nucleotide polymorphisms (SNP)-chips of various densities; density refers to the SNPs directly tested with the specific chip. First, the genotype of each SNP is determined; proportion of SNPs that give a result (a.k.a. call rate) can range from ~ 75-95%.[22] Next, the rest of the genomic sequence is inferred from reported whole genomes. This technology depends on the fact that nucleotide sequences that exist close together on a chromosome are more likely to be transferred together during crossing-over events than sequences that exist farther apart or are on different chromosomes. Therefore, when 1 SNP is determined, the sequences that are likely to be next to it (i.e. have been correlated with that SNP) can be inferred (i.e. imputation).[24] Outcomes can then be used to generate breeding value estimations or provide direct information regarding sex, color, poll-status, milk protein variants, or genetic disease status.

Although embryo biopsy and genomic selection can be individually utilized, the combination of these 2 technologies is particularly powerful. Considerable genetic gain, the amount of increase in performance achieved per unit of time, results from increasing selection combined with a reduced generation interval. The generation interval is further reduced when biopsied embryos are generated from prepubertal heifers via oocyte-pick up and in vitro embryo production.[25] Intramuscular kisspeptin treatment has been used to manage puberty in livestock by inducing luteinizing hormone surge[26] and could be used to promote ovarian function in prepubertal females (5-10 months) resulting in mature oocytes. Combining this with round spermatids of immature males for intracytoplasmic sperm injection[27] results in multidirectional acceleration of the generational interval. The integration of genomics with such technologies can provide direct information regarding sex, color, poll-status, milk protein variants, or genetic disease status. It can also be used to generate breeding value estimations from equations for a specific trait on progeny's performance by removing environmental factors.[22] The resulting embryos could be transferred to a mature recipient

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animal, hastening access to desired genetic traits. Altogether, this technology allows for genetic progression of valuable donor animals before they are mature by transferring their embryos to mature recipients, which have had successful pregnancies, to act as surrogates.[27]

As there are several steps in genomic selection through embryo biopsy, errors can occur at many places. Technician errors are possible in terms of labeling or damage to the embryo. Errors in genotyping can occur when cells are not representative of the whole animal, such as in mixoploidy cells. The most common error that occurs during enzymatic-preamplification is when only 1 allele is amplified. This leads to a loss of heterozygosity and is commonly called allele-drop out.[22] If both sire and dam are already genotyped, the imputation step itself can be very accurate (~ 99%). Overall accuracy increases with increasing density of the SNP-chip[28] and when the reference population is larger.[29] In general, overall accuracy of the SNP call rate is very high (90-> 99%).[22,30] The accuracy of estimated breeding values also depends on the heritability of the trait and the number of progeny but may range 0.65-0.85.[31] What may be of more practical use at this stage is increased genetic testing and creating databases of dams and sires to improve future offspring.

A national or global database can be beneficial for all breeds and species as it can help producers make the best decisions. With the capabilities to ship semen anywhere in the world, the ability to find the best genetics for increased production can be of great benefit. Some examples are cattle that are more heat tolerant and genetic disposition for twins or triplets in sheep or swine with certain leptin receptor gene alleles to improve meat quality.[32] Knowing which sires have those genes can allow for their introduction into the herd or flock without extensive on-farm genetic testing.

Gene editing and clustered regularly interspaced short palindromic repeats and associated protein 9

A group of technologies that could have a large impact on livestock production and reproduction are those that enable gene editing. Although gene editing is unlikely to occur on the farm itself, it could become important in the selection of replacement animals, as some modifications can be inherited. Gene editing, genetic modification, genetic engineering and transgenics are all terms to describe changing the genomic sequence of an organism. Once the genome is modified, the organism may be called a genetically modified organism (GMO), transgenic organism, and/or a genetically engineered organism. Previously, several generations of focused breeding were required to incorporate a new trait and it was often difficult to do without impacting other traits. Gene editing can produce livestock with a desired specific trait while minimizing unintended consequences such as decreased resistance to disease.[33] When gene editing results in a modification that can then be inherited by the genetically edited animal's offspring, the procedure may be termed germline editing.[34]

Gene editing technologies act as scissors to cut DNA and then either add, delete, or change the sequence at that location. Originating gene editing technologies include zinc finger nucleases starting in 1985, followed by transcription activator-like effector nucleases. However, in 2012 the discovery of the CRISPR/Cas9 system for gene editing quickly became the most common method because of its efficiency, ease of use, and lower costs.[34–36] The CRISPR/Cas9 system was 'discovered' because it is based on the natural system that bacteria and archaea use to protect themselves against viruses; CRISPR are essentially repeated sequences of genetic code. The first CRIPSR RNA (crRNA) recognizes the target site that is ~ 20 nucleotides in size. The second RNA, called a trans-activating CRISPR RNA (tracrRNA), hybridizes with the crRNA. This essentially tells the CRISPR-Cas9 enzyme where to cut in the DNA. Once complexed with the RNAs, the Cas9 nuclease then cleaves the DNA strands. For the purpose of gene editing, a new sequence can be incorporated if desired when the DNA repairs itself. The 2 CRISPR RNAs needed are designed with online tools to minimize potential off-target sites and estimate efficiency.[33]

Gene editing has been proposed for several livestock species with several broad purposes. Gene editing could impact complex traits, such as reproductive traits, by helping to select for several genes simultaneously.[37] An example of successful gene editing is pigs that are genetically modified to lack the receptor for the porcine reproductive and respiratory syndrome (PRRS) virus were completely resistant to the disease,[33,38] increasing reproductive efficiency. Another example is poll status that has been modified in cattle with gene editing technology to improve animal welfare by removing the need to dehorn.[36] Another purpose of gene editing is to affect the composition or palatability of the final agricultural product. For example, cattle and goats have been genetically modified to lack beta-lactoglobulin in their milk; this protein is most commonly responsible for milk allergy in humans.[33] Increased production and increased production efficiency are 2 prominent goals of genetic modification in livestock. This may be through enhanced disease resistance, such as decreasing susceptibility of pigs to coronavirus, African swine fever, and senecavirus A resistance.[34]

Research is ongoing regarding reducing myostatin expression with gene editing. Myostatin negatively regulates skeletal muscle growth and is responsible for the 'double muscling' of Belgian Blue and Piedmontese cattle. Gene editing technology has been used to produce myostatin knockouts of cattle, pigs, sheep, goats, and tilapia.[33] Although the aforementioned purposes are the current focus of the majority of genetically modifying livestock, another possibility is the use of this technology to treat diseases (called 'gene therapy').

Globally, there are many regulatory agencies with oversight of GMOs to ensure safety of the products and the environmental sustainability.[35] In the US, regulation of agricultural GMOs for agricultural purposes is coordinated through FDA, USDA, and the EPA.[39] Although this review focuses on agricultural mammals, it is important to note that there are only 3 GMO animal products currently commercially available. The first is the AquAdvantage Salmon® (AquaBounty, Harvard, MA, USA) that have increased feed efficiency and grow more quickly than conventional salmon.[40] The second is GalSafe™ pigs (Revivicor Inc., Blacksburg, VA, USA), edited to have an inactive GGTA1 gene.[34,41] By inactivating the GGTA1 gene, the meat from these pigs does not contain galactose-alpha-1,3-galactose, commonly called 'alpha-gal', responsible for alpha-gal syndrome in humans and contributing to meat consumption allergies. As a result of this technology, people with alpha-gal syndrome are able to consume pork products or receive allergy-free pharmaceuticals and medical implants derived from these pigs.[41–43]

Approval by the FDA to use PRRS-resistant pigs for these purposes was granted in April 2025.[38,44]

Postthaw sex sorted sperm

Since becoming commercially available in 2003, sexed semen for cattle has grown in popularity. This is unsurprising as the difference in value between offspring of 1 sex versus the other can be quite substantial in certain industries.[45] Traditionally, the decision to offer sexed semen needed to be made prior to freezing. However, the ability to sort sperm after thawing allows for delaying the decision about offering sexed semen commercially, therefore increasing the number of sires with sexed semen available. When semen is frozen, thawed, and then sorted in the industry it is called 'reverse sex sorted (RSS) semen'. Although semen is typically not washed during cryopreservation and therefore some seminal plasma is frozen with the sperm, it is truly the sperm that are sorted, leading to the ambiguity between the industry term and the more scientifically accurate RSS sperm. The process of creating RSS semen is similar to traditional sorting that has been reviewed.[8] In very brief terms, sperm DNA is stained with Hoechst 33342 that fluoresces relative to the amount of DNA present. Utilizing flow cytometry, differences in fluorescent intensity are measured. Sperm flow single file through charged plates that direct sperm into 2 populations. The main differences in technology between traditional and RSS semen is how the semen is handled prior to and after sorting.[8] Reverse sex sorted semen must initially be removed of freezing extenders, sorted, and then is not currently refrozen in cattle. A single report after refreezing does exist, with efficiency that is not up to industry standards.[46] It has been successfully refrozen in the ram.[47] When using the same number of motile sperm (15×10^6), pregnancy rates ($p > 0.05$) were similar between controlled (i.e. frozen-thawed; 59.5%), sex-sorted frozen-thawed (51.3%), and frozen-thawed, sex-sorted and refrozen-thawed (38.9%) groups. Offspring of both sex-sorted groups were > 92% of the desired sex.[47] Future studies could explore these species-specific differences in order to make refreezing a possibility in cattle production.

In the last 20 years, advances in sperm handling and preparation for sorting have increased such that fertility of sex-sorted sperm in cattle approaches that of conventional straws. However, since RSS sperm is no longer frozen, application for artificial insemination is limited.[8] Therefore, RSS sperm is currently only used for in vitro production of embryos. New discoveries in semen handling at cooled (5°C) or at room temperature with the addition of extenders up to 6 hours could make sorting prior to freezing more accessible.[48] Conventionally, 2 frozen straws are used to make 1 dose of RSS sperm for in vitro embryo production, but this can vary with sperm number and quality. The blastocyst rate and quality are similar to conventionally sorted when RSS sperm used for in vitro embryo production. It is typically processed at ~ 90% purity. (M. Hockett, personal communication, August 15, 2022). Although RSS sperm is currently not refrozen due to extremely low efficiency, a single calf has been generated from refrozen RSS sperm (i.e. frozen-thawed-sorted-refrozen sperm).[46] Advances in sex-sorting technologies, such as sorting with Raman Spectroscopy,[27,49] will likely continue to focus on decreasing sperm damage and improving refreezing capability to ultimately improve reproductive efficiency with such techniques. If a farm is already utilizing artificial insemination, introducing sexed semen based on the needs of the herd can be readily performed and therefore cost effective.[50]

Multisire or heterospermic straws

Utilizing sperm from multiple sires (heterospermic) into 1 frozen straw for artificial insemination is another technique utilized to help increase the chances of pregnancy. This is not a new concept as the idea has been around since the 1960s, but the market has progressed such that the topic has been revived, primarily for the dairy industry's recent adaptation of beef-on-dairy.[51] Utilizing beef cattle semen on a dairy cow creates an economic advantage when the offspring is going to be utilized for beef, but the dam's value is based in her milk production. One hypothesized reason for increased fertility with this technique is that there is a greater variety of sperm survival rates with multiple ejaculates mixed together that allows for a wider ovulation window.[51] Multisire straws are currently marketed through STgenetics™ (Navasota, Texas), labeled as Super Conventional®. Several blends include: 1. beef on dairy that are male offspring focused; 2. blends focused on calving ease for young heifers to create replacement females; and 3. beef blends for mature cows to produce excellent market features in male offspring. Conception rate of first, second, and third service is described as 49%. Pooled dairy semen does not appear to have a large effect on conception rates compared to beef semen, likely due to the similar breeding ability of dairy males resulting from the practices in place for culling.[51]

Spermatogonial stem cell transplantation

Although artificial insemination is prominent in some industries, such as dairy production, a large proportion of the livestock in the world are still bred by natural service.[52,53] This practice hinders the gain in animal production since reproduction efficiency occurs rapidly when males of superior genetics are able to sire calves through artificial insemination (i.e. a wider dissemination of genetics). In some instances, an animal with the desired genetics cannot or should not be transferred to the desired environment. For example, *Bos taurus* cattle are poorly adapted to tropical environments,[54] yet *Bos taurus* x *Bos indicus* animals are economically desirable in these locations.[55] One solution that has been proposed is through 'surrogate sires' the transfer of male spermatogonial stem cells, such that 1 male can produce sperm that contains another's genetics. It has been a challenge to ensure that the recipient only produces the donor's sperm yet maintaining a high level of fertility. Recent advances in spermatogonial stem cell transplantation (SSCT) have resulted in pregnancies post natural service in the bovine.[56] The original research for this technique is decribed.[57]

There are 2 animals involved in SSCT: the donor and the recipient. Donor animals contribute testicular tissue that is injected as a single-cell suspension into the rete testis of the recipient. Recipient males have intact seminiferous cords and stroma but lack the germ cells needed to produce their own sperm. Recipients are currently derived through NANOS2 gene knockout via CRISPR-Cas9 technology. In mice, knockout females and NANOS2+/- males are fertile. If true for all species, the ability to produce recipients through breeding schemes would increase feasibility. Research is ongoing to determine how to identify the best recipients for transfer and at what age transfer will have the highest rate of success. It is currently unknown how to identify the ideal donor beyond the desire to disseminate his genetics. NANOS2 gene knockout mice, pigs, goats, and cattle have been produced. In mice, SSCT has been successful; offspring of the donor's genetics have been generated through fertile recipients. Recipient boars have generated sperm with donor genetics, but not at the number desired for

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an acceptable level of fertility. Sperm of normal morphology and motility were produced from recipient bucks. Due to the long generation interval, research with cattle was of the lowest priority. However, a NANOS2 knockout bull calf was produced with similar testicular histology to other species.[57] Recent advancement demonstrates recipient bulls ejaculate donor sperm that is capable of surviving cryopreservation.[56] In livestock, SSCT techniques could allow for superior donor genetics to be disseminated more rapidly than with artificial insemination alone. For example, a more heat stress-tolerant bull could produce sperm of a less heat stress-tolerant, but higher genetic value bull, resulting in offspring with intermediate heat stress-tolerance when bred to heat-tolerant females.

Conclusion

Growing technology in ART is a pivotal step to improve livestock management (decrease time or increase efficacy). Precision farming technology for estrus detection and reverse sex sorted semen, and multisire straws for artificial insemination are all techniques with the ability to increase reproductive efficiency. In addition to growing use of artificial intelligence, established technology can be paired together to assist with decision making on the farm. Genetic focused advances may be achieved through genomic selection through embryo biopsy, gene editing via CRISPR-Cas9, and spermatogonial stem cell transplantation. Continuing to improve on the current technologies availability will lead to a more sustainable production system, critical to increasing animal agricultural production and improving veterinary medicine.

Conflict of interest

Authors have nothing to disclose.

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