https://doi.org/10.22319/rmcp.v12s3.5918

Review

Reproductive biotechnologies in beef cattle: five decades of research in Mexico

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Abstract:

The main bovine reproductive biotechnologies are recapitulated herein in five sections, and their historical development and current status are analyzed, including the results generated in Mexico. In the 1970s, estrus synchronization and ovulation induction began; thus, the reproductive cycle started to be controlled with the resources available at that time, based on the knowledge of bovine reproductive physiology. Over the years, hormone therapy evolved as new compounds were discovered, refining methods to standardize the effect and generating new methods for the release of hormones. The most widely used biotechnology in the world, artificial insemination, owes its expansion to advances in semen processing, among which the development of diluents, cryopreservation, semen sexing, and computer-assisted sperm analysis stand out. The embryonic era began with the development of multi-ovulation and methods for collecting, evaluating, transferring, and cryopreserving embryos. The second embryonic era came with the fully *in vitro* production of embryos from immature eggs and frozen sperm, known as *in vitro* embryo production. Great research and material resources have been invested in this procedure, rendering it a pillar of genetic improvement and productivity, in combination with two other tools: sexed semen and genomic evaluations. A golden age of *in vitro* embryo production is on the horizon, with the possibility to produce accurate modifications in the embryo genome, thanks to gene editing technology.

Key words: Synchronization, Sexed semen, Embryos, Multi-ovulation, Embryo transfer, *In vitro* production, Bovines.

Received: 04/01/2021

Accepted: 30/04/2021

Introduction

The text of the FAO Convention on Biological Diversity (CBD)[(1)], in force since 1993, states that the term "biotechnology" refers to any technological application that uses biological systems, living organisms (or derivatives thereof) to make or modify products or processes for a specific use. In this sense, and for the purposes of the topics to be covered in this review, reproductive biotechnology will be understood as the technological applications that affect the physiological processes of animal reproduction, their gametes and embryos, for the purpose of achieving productive improvements.

This document will cover only the bovine species and will place particular emphasis on the research carried out by institutions and universities in Mexico, particularly the National Institute for Research in Forestry, Agriculture and Livestock (INIFAP), in its 35 years of existence, and its predecessor: the National Institute of Livestock Research (INIP).

While the authors do not claim this to be an exhaustive review of all the reproductive biotechnologies that have been applied to cattle, it is address those that have had the greatest impact on productivity and briefly mention those that have had minimal or no application in Mexico, such as transgenesis and cloning.

The topics of estrus synchronization and ovulation induction are discussed first, as they are the topics on which research has been conducted for the longest time (50 yr) and for which INIP-INIFAP has generated the largest number of technologies. Biotechnologies developed for semen collection, dilution, and cryopreservation have underpinned the massive use of bulls of high genetic merit through artificial insemination (AI). This topic will be discussed, with emphasis on recent developments such as semen sexing and computer-assisted sperm analysis. Issues related to embryo manipulation, including multi-ovulation and embryo transfer (MOET), *in vitro* embryo production (IVP), and transvaginal oocyte aspiration (TVA) have seen important developments in recent years, which will be addressed in this paper. In addition to including research results on these topics, in some cases, mention will be made of government or producer organization programs that marked a milestone in the dissemination and adoption of these reproductive biotechnologies.

Estrus synchronization and ovulation induction

In cattle, a common problem is prolonged postpartum anestrus, a condition characterized by a delay in the return to estrous cyclicity after parturition due to various factors[2-6]. Many efforts have been made to resolve this condition: hormonal treatments have been studied to induce estrus and ovulation[7,8] by controlling breastfeeding[9,10], the frequency and quality of its stimuli[11], feeding[12] and the different mating seasons[13]. This section reviews results of ovulation induction in anestrus females and synchronization of estrus and ovulation in cycling females, carried out by INIP (1971-1985) and the current INIFAP (1986-2021), as well as by other institutions.

Estrus synchronization and ovulation induction studies

The first studies date back to 1948[14], when progesterone (50 mg/d, i.m.) was used to block estrous cyclicity for a period of up to 13 d. It was observed that, at the end of its effect, a good proportion of females presented estrus at 4 and 5 d. Later, with the addition of estrogens to the treatment, estrus presentation increased, and the progesterone blocking time was reduced[15]. The use of hormonal products such as 19 alpha acetoxy-11 beta-methyl-19 norg 4-ene-3, 2-dione (a very potent progestogen known as SC21009), natural progesterone, estradiol valerate (EV), and prostaglandin F2α (PGF2α) began in the 1970s; at this time, work focused on estrus synchronization[16], the resolution of prolonged postpartum anestrus in cows, and the attempt to advance puberty in heifers[17-21]. Thus, heifers of European breeds, Creole breeds, and pre-pubertal zebu crossbreeds, treated with 5 mg of EV (i.m.) + 3 mg of SC21009 (i.m.) + 6 mg of SC21009 (auricular implant removed on d 9), had 79 % of estrus presentation in the first 48 h of implant removal, compared to control heifers, of which only 6 % exhibited estrus[21]. In another study[22] with anestrus cows and fattened zebu heifers, it was observed that, in females treated with progesterone (25 mg i.m. for 5 d) + estradiol cypionate (EC; 2 mg i.m. on the first day) or with SC21009 (3 mg for 5 d in subcutaneous implant) + EC (2 mg i.m. on the first day), the estrus was synchronized at 72 h, with an estrus rate (ER) of 61.1 and 73.7 %, and a conception rate (CR) of 44.4 and 31.3 %, respectively.

On the contrary, in females that received the control and individual treatments (progesterone, SC21009 or EC) with the same doses, estrus was not synchronized at 72 h, and they had a low TC, from 10.5 to 21.1 %, during the 30 d of the study.

In another experiment[23] with zebu cows with calf and at 60 d postpartum, the effectiveness of SC21009 + EV was evaluated. In the first 48 d, the ER in treated cows was 24 %, and 0 % in untreated cows, while the CR was 12 and 0 %, respectively. However, at the end of the AI period (d-48), 20 % of the treated cows were pregnant, and 12 % of the untreated cows were pregnant; at the end of mating (d-68; AI + natural mating), 28 % of the treated cows were pregnant, but only 12 % of the untreated cows were pregnant. However, in non-breeding zebu cows, after applying 6 mg of EV + 3 mg of SC21009 via i.m. + 6 mg of SC21009 in subcutaneous implant, it was observed that 100 % of the treated cows showed estrus in the first 48 h after the implant was removed, in contrast to the untreated cows, of which only 29.4 % exhibited estrus. The CR was 42.8 % in treated cows, and 14.7 % in untreated cows. This indicates that synchronization groups the estruses in order to facilitate AI and overall herd management[24], but provides evidence that it is more difficult to reduce the anestrus period in cows with calves, a circumstance that reveals the importance of reducing or eliminating the effect of lactation on the return to estrous cyclicity.

Estrus synchronization with melengestrol acetate

Progestogen melengestrol acetate (0.5 mg/d for 9 d) in feed, combined with EV (single dose of 6 mg i.m. on d 1) and progesterone (single dose of 50 mg on d 1), was tested in Brown Swiss x Zebu cows and heifers. It was observed that the percentage of cows inseminated at 48 days after mating was 38.9, 51.5, and 75.8 %, respectively, in control cows, in cows conventionally inseminated at detected estrus, and in cows inseminated at any sign of estrus. The percentage of cows inseminated was significantly higher when insemination was performed before any sign of estrus. CRs were statistically similar between treatments (20.9, 28.8 and 37.1 %, respectively), despite the fact that the estruses were clustered among the cows in each treatment[25]. Estrus synchronization with melengestrol acetate is not a widespread practice in Mexico, despite its proven efficacy, because it is not practical to perform it in small groups of animals, and small herds are predominant in our country.

Estrus synchronization with fixed-time artificial insemination (FTAI)

Because some females did not exhibit estrus even when treated hormonally, the decision was made to research the convenience of inseminating at pre-established schedules. Thus, in Brangus and Creole heifers, AI was performed at preset times in anestrus females because the percentage of animals in estrus in the first 5 days after removal of the implant was very low (53.1 %). Under this premise, heifers were inseminated at 48, 54 and 60 h after implant removal, resulting in CRs of 54.5, 60.6 and 47.0 %, with higher CRs at 48 and 54 h after implant removal[26]. These results set the tone for the initiation of many studies on estrus synchronization with FTAI.

On the other hand, studies were carried out with estrus synchronization in grazing beef cattle during 85-d mating seasons (matings), when AI is difficult to perform. Thus, in humid subtropical climate with non-breeding zebu cows, the ER in the first 60 h of mating was higher in cows treated with SC21009 (9 or 6 mg for 9 d) + EV (6 mg on d 1 of the 9-d treatment) than in untreated cows (84 *vs* 0 %). Similarly, CR at 26 ds after mating favored treated cows over untreated cows (59 *vs* 40 %), although at the end of the mating period there were no differences in CR between treatments[27].

Inclusion of PGF2α in estrus synchronization

The availability of PGF2α in the early 1970s made it possible to start work on estrus synchronization in cycling females, a status that was confirmed through the detection of a corpus luteum[28,29]. A CR of 34.8 % was obtained in cycling Brangus, Gyr and Charolais heifers treated with PGF2α and artificially inseminated 80 h after treatment; this percentage was similar to that of heifers synchronized and inseminated 12 h after estrus was observed (CR= 26.1 %). At the end of mating (90 d), in heifers inseminated at 80 h, a TC of 69.6 % was obtained, similar to the TC (56.5 %) of heifers artificially inseminated 12 h after estrus[30]. Studies on estrus synchronization facilitated the reproductive management of grazing cattle, since it allows females to be served in the first days of mating[13,23-26].

Use of the progestogen norgestomet in estrus synchronization

Norgestomet (CRESTAR®), (a more potent progestogen than natural progesterone), applied subcutaneously on the dorsum of the ear and removed 9 d after implantation, associated with EV and PGF2α (only in cycling cows), also proved to be useful in estrus synchronization and ovulation[31-34]. Thus, in a study[35] with zebu cows and their crosses with European bulls, without offspring, in a 63-d mating (42 d of AI and 21 d of natural mating), TES of 86.1 and 95.0 % were achieved 5 d after implant removal in anestrus and cycling cows, respectively. Subsequently, 42 ds after implant removal, the TES was 100 % in both groups of cows. When pregnancy diagnosis was performed, it was determined that 49.5 % of the anestrus cows and 54.0 % of the cyclic cows conceived during the first 5 d of mating, with the following percentages: 90.7 and 98.3 % at 42 d; and at the end of mating (63 d), with the presence of the bull from d 43 to the end of mating, 96.2 % and 98.3 %, respectively. This study demonstrated the usefulness of estrus induction and synchronization in mating cows (mainly anestrus cows) that would otherwise be delayed up to 21 d to conceive. The results with the SC21009 auricular implant combined with EV for the performance of FTAI at 56-60 h after removal of the implant (d 9) were also attractive, since a TES of 95 and a CR of 85 were achieved in 45-d matings[36].

Use of the progesterone-releasing intravaginal device (PIDR) in estrus synchronization

The introduction of the PIDR into the market revolutionized estrus synchronization[37], and, therefore, researchers from INIFAP[38,39] started using this device in cows in anestrus and cycling cows with good body condition (BC), obtaining a CR of 44.8, 77.1 and 100.0 % at 3, 30 and 60 d after PIDR withdrawal, respectively[38]. In *Bos taurus* x *Bos indicus*[39] heifers, the CR with PIDR and FTAI at 84 h after the removal of the device was higher than with conventional AI at 12 h after the detection of estrus (36.4 *vs* 18.2 %, respectively). Similarly, the CR with CRESTAR and FTAI at 84 h after device removal was higher than with conventional AI (27.3 *vs* 18.2 %, respectively). In this work, the advantage of FTAI at 84 h after the removal of the device was demonstrated, since pregnancy was achieved in some heifers that did not exhibit estrus. A frequent practice in estrus and ovulation synchronization protocols with FTAI in which PIDR and PGF2α are used in association with estradiol benzoate (EB) (which is applied when inserting the PIDR on d 0 and the day after its removal) is the substitution of GnRH for EB. This is because, like EB, GnRH synchronizes the emergence of a new wave of follicular development, which culminates in the ovulation of the mature follicle, giving good results. However, if a significant proportion of the cows are suspected to be in anestrus, it is recommended to additionally apply 400 IU of equine chorionic gonadotropin eCG on d 7 of the protocol. Furthermore, EB can be replaced with GnRH (100 µg i.m.), on the first application (d 1 of PIDR), but the second application must be carried out at the time of FTAI. An example for cycling cows is shown in Figure 1[40].

Importance of body condition in estrus induction and synchronization

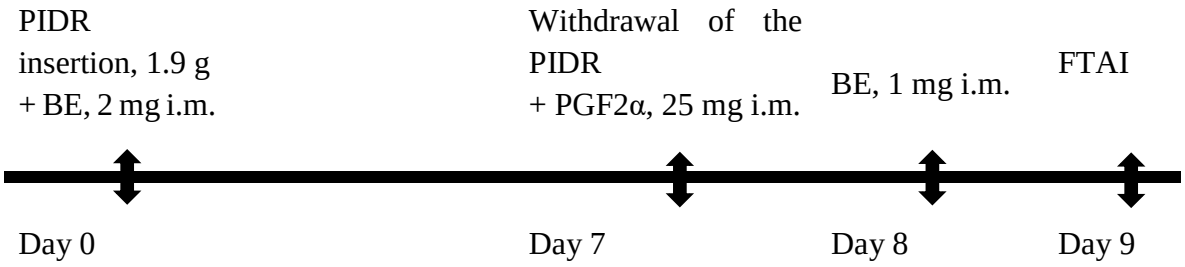
It has been shown that body condition (BC) is an indicator of the cow's nutritional status and that, if the cow has a good BC before and after calving, her fertility improves soon after giving birth[41]. In addition, this has been shown to be associated with high concentrations of insulin-like growth factor-1 (IGF-1), leptin and insulin, allowing early resumption of postpartum estrous activity[42]. Therefore, other research focused on studying changes in the blood concentration of these three hormones as metabolic indicators of the nutritional status of the animals, observing that a low BC in females (<6.0 units; scale 1 to 9) is associated with a reduction in the blood concentration of insulin and IGF-1, without changes in leptin, which diminishes the response to postpartum estrus induction in beef cows[43,44]. Therefore, females that are synchronized and enter mating should have good BC (of no less than 3.0), in order to favor the occurrence of estrus and achieve gestation[45].

Kisspeptin in estrus synchronization and ovulation induction

Kisspeptins are peptides that are named for their number of amino acids: kisspeptin-54, kisspeptin-14, kisspeptin-13 and kisspeptin-10[46]. Synthesizing neurons are considered to be integrators of signals that modulate the functionality of the somatotropic and gonadal axis[47]. Studies with intravenous kisspeptin-10 (kiss-10) (5 µg/kg bw) produced increases in LH, FSH, and growth hormone secretion in prepubertal male and female calves[48,49], LH increased with age in all calves, with mean values of 6.1, 7.2 and 11.6 ng/ml at 4, 7 and 11 mo of age, respectively[50]. The highest LH concentration was found in 11-month-old calves.

In an attempt to understand the sensitivity of the gonadotropic axis to kiss-10, other studies tested intravenous doses of 0.1, 1.0, 2.0, 3.0, 4.0 and 5.0 µg/kg bw[51] and 50.0 µg/kg[52] in prepubertal calves, showing that effective doses for inducing LH release ranged from 1.0 to 5.0 µg/kg. Therefore, the application of low doses, less than 5 µg/kg body weight, could considerably reduce the cost of treatment with this peptide[51]. In a subsequent experiment with prepubertal calves using kisspeptin-10 at a dose of 5 µg/kg-bw applied every 2 h for 84 h (3.5 d), ovulation and corpus luteum formation were induced in 28.5 % of the calves; however, the corpus luteum disappeared and the calves returned to their prepubertal state[53]. Later, it was found that, in (European x zebu) beef cows, at 78 d postpartum, with anestrus, and nursing their calf, kisspeptin-10 at a dose of 1 µg/kg-bw every 2 h for 24 h also augmented the serum LH concentration, an increase that emulated a brief LH pulse[54]. This result prompted an ovulation synchronization study, such that kisspeptin-10 was tested at a total dose of 500 µg at the time of FTAI, compared to GnRH (100 µg at the time of FTAI) and eCG (400 IU on withdrawal of PIDR on d 7), in a protocol in which each hormone was combined with PIDR, BE (2 mg at PIDR application + 1 mg the day after PIDR withdrawal), and cloprostenol (total dose of 500 µg at PIDR withdrawal), in non-calving, 180-d postpartum beef cows inseminated between 54 and 56 h after PIDR withdrawal. The ovulation rate for the eCG, GnRH and kisspeptin-10 treatments was 89.2, 96.5 and 93.8 %, while the conception rate was 43.6, 73.8 and 54.3 % respectively, with no significant statistical differences between treatments in either case[55]. This result could be of interest to the pharmaceutical industry as an alternative for ovulation synchronization and induction.

Figure 1: General outline of estrous synchronization in cows and heifers with FTAI[40]



In conclusion, estrus synchronization and ovulation induction in cattle in tropical climates revolutionized reproductive management, since it allowed the concentration of estrus between 52 and 56 h after the withdrawal of the PIDR in a mating season with FTAI in a way that facilitates the use of AI, with the possibility of re-inseminating females that did not conceive at the first service at manifest estrus, increasing the number of pregnant cows with AI, which no doubt favors the herd genetically.

Semen processing and artificial insemination

AI was the first reproductive biotechnology applied to improve production through the more efficient use of bulls of high genetic merit. The widespread use of this technique and the achievement of its full potential required the cryopreservation of semen for long periods of time.

The first report of semen cryopreservation was made in 1776[56], where it was observed that when spermatozoa from human, guinea pig and frog were cooled in snow for up to 30 min, they became inactive, but could be reactivated. In 1940[57], egg yolk began to be used to protect bull sperm cells from thermal shock upon cooling. In 1941[58], the sperm extender was improved by using egg yolk with sodium citrate, which allowed semen preservation at 5 °C for up to 3 d. In 1949, bull spermatozoa were frozen for the first time by using glycerol in the diluent. The great discovery of the cryoprotective action of glycerol opened a successful era in the cryopreservation not only of gametes of various species, but also of other cells and tissues[59]. The so-called Cornell diluent, created by Foote and Bratton in 1950[60], contained a mixture of the antibiotics penicillin, streptomycin, and polymyxin B, and was used for many years as standard. In 1952, in Cambridge, the first calf was born from frozen-thawed bovine sperm[61].

Reports of fertility with frozen spermatozoa from bulls led to an intense development of cryopreservation methods to try to improve the results obtained, so other disciplines (such as molecular biology) associated with the cryopreservation process began to be used. Such is the case of proteomics[62] and genetic engineering[63]. On these issues, a study was carried out in Mexico with INIFAP researchers, where the addition of recombinant FAA (fertility-associated antigen) and recombinant TIMP-2 (tissue inhibitor of metalloproteinases 2) to frozen bovine semen was evaluated and proved to significantly increase its fertility[64].

Sperm sexing

Advances in AI have generated interest in using AI for sex selection in dairy cattle. For this reason, over time, many researchers have tried to separate spermatozoa with "X" and "Y" chromosomes using various techniques[65]. In the 1980s, flow cytometry began to be applied, making it possible to separate spermatozoa according to their sex chromosomes and amount of DNA. It took about 20 yr for this technology to be commercialized for use in AI in cattle. This technique is based on the fact that spermatozoa with an "X" chromosome in cattle contain 3.8 % more DNA than "Y" spermatozoa[66]. This technology had an efficiency of 85 to 95 % (in terms of separation of sperm with X or Y chromosome); however, it had not been fully perfected[67].

The first commercial production of sexed semen took place at the Cogent company in the United Kingdom[65]. Although it had a relatively slow start, bovine sexed semen production increased exponentially with an estimated 4 million doses in 2008[68].

The sexed semen was marketed in 0.25 ml straws at a concentration of 2.1 million spermatozoa[69]. This concentration was due to the fact that, at the time of semen sexing, approximately 80 % of the ejaculate was lost between the sperm of the unwanted sex and the sperm that could not be differentiated, in addition to the long time that the separation process took[70]. Despite the limitations of sexed semen, it was clearly a welcomed development[68]. Acceptable gestation percentages were achieved with the reduced dose (2.1×10^6 sperm) of sexed semen in heifers, but little work was done with lactating cows[66]. Nowadays, sperm sexing technology has evolved, modifying the techniques, increasing the speed of sexing, decreasing stress on spermatozoa, increasing concentration and, therefore, improving sperm viability parameters.

AI with low dose of sexed semen

In 1997, research was conducted with two objectives: 1) to evaluate conception rates of heifers inseminated (in the uterine horn, ipsilateral to the ovary with the largest follicle) with reduced doses of semen (1 x 105; 2.5 x 105; 2.5 x 106 sperm/0.21 ml) chilled at 5 °C under ideal conditions at the field level; 2) to evaluate conception rates of heifers inseminated (in the uterine horn, ipsilateral to the ovary with the largest follicle) with reduced doses of sexed semen (1-2 x105 sperm/0.1 ml) refrigerated at 5 °C. In experiment 1, gestation percentages at 40 d were 41, 50 and 61 % for 1 x 105; 2.5 x 105; 2.5 x 106 spermatozoa/IA respectively. In experiment 2, out of 67 heifers inseminated, 22 % were pregnant, and 82 % of the offspring were of the selected sex[71]. Research indicates that AI with lower than conventional sperm doses is possible when using sexed sperm, 2.1 x 106 spermatozoa being most commonly used in the insemination dose, against at least 10x106, which is used with unsexed sperm.

Successful cryopreservation of sexed semen

Subsequently, in 1999, another research was carried out with the objective of evaluating the freezing process of the sexed semen; this was achieved because the semen was processed in a MoFlow SX™ flow cytometer, that allowed to have sufficient quantity of spermatozoa, unlike when working with the EPICS V flow cytometer. In this work it was determined that the use of the laser at a power of 100 mW had a lower impact on the progressive motility of post-thawed semen than when it was used at 150 mW. It was also observed that post-thawing progressive motility was higher when using a TRIS-based diluent than when using citrate-egg yolk or TEST. Regarding the equilibration time (adaptation to the cryoprotectant) at 5 °C prior to freezing, it was concluded that progressive motility was better after thawing for 3 to 6 h than when this took 18 h. On the other hand, it was determined that it was better to keep fresh semen for 4 to 7 h at 22 °C than to dilute it with TALP medium added with Hoechst 33342 fluorochrome. These new sperm sexing procedures yielded slightly lower results in terms of motility and acrosomal integrity than conventional semen. With these results, it was considered that the use of sexed semen for commercial AI would be available in approximately 2 yr[72].

The beginnings of sexed semen commercialization

The Monsanto Company, located in St. Louis, Mo., USA, developed a unique sperm sorting system that used 16 sorting nozzles instead of just one as in the case of the MoFlow SX™ cytometer. This equipment was intended to be commercialized, but, apparently, due to problems with low conception percentages detected in its first tests, the company decided not to commercialize it. In 2003, Genetic Resources International/Sexing Technologies in Navazota TX, USA purchased the intellectual property and the sperm sexing equipment developed by Monsanto, as well as the entire infrastructure of XY Inc. (the first company to own the intellectual property of sperm sexing and the creator of the MoFlow™ cytometers)[69]. The company has since changed its name to STgenetics®[72].

SexedULTRA™ sexed semen

The difference in fertility between conventional semen and sexed semen did not improve with increasing sperm concentration by AI. The causes of lower fertility of sexed semen have been attributed to the various biochemical changes that sperm undergo during sexing. The XY technology described in previous publications[73,74] has been modified and is now a totally new sexing system called SexedULTRA™ (Navazota, TX, USA). The SexedULTRA™ technology has been designed to be more sperm-friendly during the most critical points of the process, particularly improving pH changes (buffer system) and oxidative stress.

Modifications to the SexedULTRA™ sexing technique

Although there is currently very little data on this new technology, it has been reported that it facilitates the entry of the Hoechst 33342 fluorochrome and retains it inside the cell, allowing for greater fluorescence and thus better discrimination between "X" and "Y" populations. The protocol was modified, with a pretreatment prior to the staining process, in addition to the use of a new staining medium that maintains the pH stable for a longer period of time. The freezing medium was also modified, taking into account the dose of sexed semen per straw[72].

The success of the ultrasexing process was mainly influenced by two factors: modifications in the means and equipment used to perform sexing. The MoFlo SX™ cytometers (Cytomation Inc., Fort Collins, CO, USA) were very expensive, bulky, had low throughput and required highly trained personnel to operate them. Modern Genesis cytometers

developed by Cytonome ST™ (Boston, MA, USA) have advanced and automated electronic features with multiple heads on one machine for parallel separation. The Genesis III™ cytometer uses a solid state laser, two orthogonal detectors (0° and 90° to the laser), an orientation nozzle and a subpopulation separation of ~8000 spermatozoa/second with ~90% purity, reaching a maximum separation rate of 500 million sperm per hour[75].

Laboratory testing of SexedULTRA™ technology

The aforementioned modifications brought about sperm motility and acrosome integrity were increased with respect to the XY Legacy technology (conventional sexing) considering the same sperm concentrations[76].

On the other hand, in 2018[77], sperm quality was evaluated considering plasma membrane integrity, percentage of intact acrosomes and DNA fragmentation index of SexedULTRA™ semen compared to conventional (non-sexed) semen. In SexedULTRA™ semen at 3 h post-thawing, the percentage of intact acrosomes was significantly higher than in conventional semen. In addition, SexedULTRA™ semen had a significantly lower DNA fragmentation index at all evaluation points compared to conventional semen. The authors conclude that SexedULTRA™ technology maintains semen quality and, in many cases, has greater *in vitro* longevity compared to conventional semen.

Field testing of SexedULTRA™ technology

In the first field evaluation using SexedULTRA™ technology for AI[78,79], there was a 7.4 % increase in heifer conception rates over XY Legacy technology (conventional sexing). The second test was conducted in collaboration with the commercial company Select Sires, using eight bulls from which semen was collected and processed using both SexedULTRA™ technology and XY Legacy technology, inseminating 6,930 heifers. The results showed that SexedULTRA™ semen increased the conception rate 4.5 % over XY Legacy semen, 46.1 and 41.6 % respectively.

With these tests it was observed that the deleterious effects of the XY Legacy technology were partially ameliorated with the new SexedULTRA™ technology, so the next logical step was to increase the sperm concentration per dose, although in the past increasing sperm concentration did not improve fertility. The following test was performed in collaboration with German Genetics International: semen was collected from five bulls; each ejaculate was

divided into 4 parts and processed with XY Legacy technology of 2.1 million sperm, SexedULTRA™ of 2.1, 3 and 4 million sperm per dose. In addition, semen from these same bulls was used from contemporary conventionally frozen ejaculates, with a concentration of 15 million spermatozoa per dose. Non-return to estrus rates (NRR) at 65 d were calculated from 7,855 AI with sexed semen and 62,398 AI with conventional semen. Overall, the XY Legacy semen of 2.1 million sperm per dose resulted in lower NRRs (55.9 %) compared to all SexedULTRA™ treatments (2.1 million 59.9; 3.0 million 60.0 %; 4.0 million 66.7 %) and conventional semen (65.7 %). SexedULTRA™ treatments of 2.1 and 3 million sperm per dose were similar (59.9 and 60.0 % respectively), but lower than conventional semen (65.66 %); however, the SexedULTRA™ treatment of 4 million sperm per dose had NRRs similar to conventional semen of 15 million sperm per dose[79]. The data obtained demonstrated for the first time the effect of the response to the dose when using sexed semen.

Field tests with SexedULTRA-4M™ technology

The use of SexedULTRA-4M™ semen was evaluated in FTAI[80] using beef cows and heifers. The results show that there was no significant difference in the percentage of pregnancies between conventional semen (61.9 %) and SexedULTRA-4M™ semen (63.8 %) when the females were in heat prior to FTAI.

Another experiment[81] compared the use of conventional semen and SexedULTRA-4M™ semen in AI using three different bulls and beef cows. In this study, fertility was found to be influenced by the bull, as only one of three bulls had no difference in the percentage of gestations when comparing conventional semen and SexedULTRA-4M™, which shows that there is a difference between bulls, as well as with the sexed Legacy.

There is little research on the effectiveness of the use of sexed semen in Mexico, although it is already available from several semen processing companies and is routinely used, especially in dairy production units. Experiments have used Legacy sexed semen and found it to work for both Holstein cows and heifers, with a pregnancy rate between 80 to 90 % of that recorded for females inseminated with conventional semen and with values of 85 to 93.6 % of offspring born with the predicted sex [82-85]. It was also found that the use of sexed semen does not influence the occurrence of miscarriages or dystocic births[86].

Computer-assisted sperm evaluation

Fertility is a multiparametric event related to semen quality and quantity, the exact timing and method of AI, the intrinsic fertility of the bull and proper herd management[87]. Morphology, motility, viability, acrosome, and DNA concentration and integrity have been used as parameters to evaluate the sperm quality of bovine semen[87-90]. Some of these parameters (such as morphology and motility) can be assessed manually by conventional methods using a visible light microscope. However, these evaluations are subject to subjective criteria and technical errors that diminish their accuracy and repeatability.

In the mid-1980s, computer-assisted sperm analysis (CASA) systems were introduced commercially to maximize the accuracy and repeatability of semen evaluations[91]. The main function of these CASA systems is the objective evaluation of semen quality. The basic components of this technology consist of a microscope to visualize the sample, a digital camera to capture images and a computer with specialized software to analyze the images.

Motility is one of the most important sperm characteristics associated with the ability to fertilize[92]. With the use of the CASA system, various motility parameters describing specific sperm movements are obtained. Total and progressive motility percentages are the most important parameters in the evaluation of sperm kinetics[87]. Total motility refers to the fraction of spermatozoa that show any movement, whereas spermatozoa with progressive motility have a forward movement, essentially in a straight line.

Other specific kinetic parameters determined by the CASA system are useful to evaluate several sperm characteristics simultaneously and objectively. These kinetic parameters consist mainly of three values of the speed of movement, three speed indices, and three parameters that reflect the oscillation characteristics of the spermatozoa[93]. The three values of the velocity of motion are the curvilinear velocity (VCL), the rectilinear velocity (VSL) and the mean trajectory velocity (VAP). From these three values, three indices are calculated, linearity (LIN=VSL/VCL), straightness (STR=VSL/VAP) and trajectory oscillation (VAP/VCL), thus characterizing the quality of sperm movement. The parameters that show the oscillation characteristics of spermatozoa are the lateral displacement of the head, the frequency of tail beating, and the mean angular displacement.

CASA system movement parameters have been used for the identification of sperm subpopulations and their subsequent correlation with freezing resistance[94,95]. In addition, the effects of different media during *in vitro* processing on sperm function have evaluated[96].

On the other hand, with the use of the CASA system, several groups have reported a significant correlation between the total (r=0.26-0.61) and progressive (r=0.26-0.33) motility of bull semen and its field-associated fertility[97-101]. The CASA system collects a wide range of sperm kinetics parameters. Some research groups showed a positive correlation between VSL (r=0.17-0.67), LIN (r=0.28-0.46) and STR (r=0.33), and field fertility[98,102,103]. Correlations between motility parameters and fertility tend to be low to medium, and their use in isolation to predict the fertility of a semen sample is not recommended[101-103]. However, the combination of several motility parameters provides a better tool for predicting fertility, as the multi-parameter regression of the CASA system explained up to 0.98 (r^2 value) of the variation in fertility, compared to 0.34 explained by total motility alone[103]. On the other hand, recent analyses of sperm movement in the third dimension and the study of flagellar movement are new functional parameters that could be related to fertility[104].

Sperm morphology is one of the most important tests in semen quality control, as it reflects the physiological or pathological state of the functionality of the testicles, epididymis and accessory glands of the reproductive tract[105]; is also considered a better test to evaluate sperm DNA and genetic characteristics, compared to sperm motility[106,107]. Several studies have shown a significant correlation between sperm morphology (r=0.22-0.76) and field fertility[94,98,108]. However, most of the morphological analyses are performed through conventional methods, which remains a problem due to the subjectivity in the evaluation, as well as the inconsistency observed within and between technicians[109,110]. For this reason, morphological analysis has not been considered reliable in predicting field fertility[94, 98]. The development of specific modules for the morphological analysis of spermatozoa within the CASA system has allowed the individual evaluation of morphometric characteristics of the sperm head in terms of size (area, perimeter, length and width) and shape (ellipticity, elongation, regularity and roughness)[111]. Some systems even provide information about the mid piece (area and width) and data concerning the insertion of the mid piece into the head, such as distance and angle of insertion[112]. Within these parameters, the width of the sperm head showed a significant correlation (r=0.53) with field fertility[113]. On the other hand, sperm subpopulations have been reported based on their morphometric structure[114]. The evaluation of new parameters, as well as the identification of sperm subpopulations, could provide information on an optimal fertility-enhancing population[90].

There are other parameters in the study of sperm function, such as vitality, acrosome integrity, and DNA fragmentation. Viability classifies spermatozoa as alive or dead and shows the existence of damage to the sperm plasma membrane[115]. Acrosome integrity is one of the most important sperm function tests, since only a spermatozoon with an intact acrosome can penetrate the oocyte[116]. In the case of sperm DNA integrity, its importance in fertilization and in the early stages of embryonic development has been demonstrated, having been recognized as a parameter indicative of sperm fertilization potential[117,118]. Several studies have shown a significant correlation between vitality (r=0.19-0.40), acrosome

integrity (r=0.52) and DNA fragmentation (r=-0.49), and the field fertility of bulls[94,95,97,99]. In addition, when several parameters (sperm kinetics, vitality, DNA fragmentation and morphology) were combined in regression models, the correlation with fertility increased to more than 90%[12]. Although most of these tests are performed through the use of flow cytometry, new generations of CASA system modules have been developed for the automatic assessment of vitality, acrosome integrity, and DNA fragmentation[92]. However, few studies have been conducted in cattle using these new modules. Therefore, motility and morphology are shown to be the most important modules of the CASA system in terms of use.

These studies show the great potential of CASA systems for estimating semen quality, studying sperm function, and predicting fertility. However, the parameters provided by the CASA system have also been shown to have limitations and cannot be used in isolation as reliable predictors of the fertilization ability of the sperm. In addition, spermatozoa are complex cells that require a large number of criteria in order to be considered to achieve fertilization[119]. Therefore, the use of various parameters provided by the CASA system within a regression model is presented as the best option to attempt to predict the fertility of a semen sample. However, there is no consensus as to which parameters of sperm functionality to use in the spermatozoa[99], perhaps due to the differences found between working groups with respect to the parameters correlated with fertility.

Currently, sperm analysis through a CASA system is widely used within the quality control protocols of semen processing centers, mainly the motility module. These protocols establish thresholds for certain variables such as total and progressive motility; ejaculates below these thresholds are usually discarded before or after freezing[99].

In Mexico, several associations, institutions and companies related to the livestock sector utilize CASA systems on a regular basis mainly in the quality control of commercial doses of bovine, ovine, and porcine semen. However, studies of post-cryopreservation sperm kinetics of Pelibuey and Blackbelly rams[120], Mexican Pelon pigs[121] and Merino sheep from Socorro Island[122], and evaluations of cryopreserved semen samples from Chiapas sheep[123] and Tamaulipas Creole cattle[124] stored at the National Center for Genetic Resources of INIFAP (CNRG-INIFAP) have also been carried out. On the other hand, CASA systems have become more attractive as part of the practical evaluation of the sperm quality in semen samples of many domestic species. However, there are multiple reasons why a semen evaluation using a CASA system may vary, including system and equipment maintenance, sample handling, and technician experience[89]. For this reason, it is vitally important to corroborate the effectiveness of the analysis. To ensure that the CASA system works properly, validation of technicians, protocols and equipment is crucial. In this sense, the CNRG-INIFAP has a test validated before the Mexican accreditation entity called "Evaluation of semen from domestic animals", which is carried out with a CASA system. This allows semen evaluations to be performed with high quality standards[125].

CASA systems have evolved in recent years to become powerful tools for rapid and objective evaluation of sperm quality and function of bovine semen samples. CASA systems will continue to be developed to perform new, repeatable and increasingly accurate tests in order to contribute to fertility improvement in the field.

Multi-ovulation and embryo transfer (MOET)

This concept groups together a series of reproductive biotechnologies whose purpose is to increase the reproductive capacity on the maternal side and which are used as tools for genetic improvement. These biotechnologies include: selection of donors (both female and male) of high genetic merit, superovulation of donors, embryo collection and evaluation, embryo transfer to recipient cows, or embryo cryopreservation. This latter technique has had its greatest development in cattle; however, it is also widely applied in such species as equines, sheep, goats, and deer, among others[126]. In addition to the aforementioned purpose (genetic improvement), the MOET has also been used for the conservation of animal genetic resources, as, according to FAO[127], it is an excellent option to conserve genetic diversity and is the fastest way to restore a population at risk. For this reason, INIFAP has the National Center for Genetic Resources, where work has been done to generate germplasm banks of bovine breeds with different risk statuses[128].

The first birth from embryo transfer in mammals was achieved in 1890 with rabbit embryos[129], and the first successful surgical transfer in cattle was achieved in 1951[130]. The set of biotechnologies involving MOET developed between 1940 and 1960, laying the foundation for the embryo transfer industry, which began in 1970 as a tool for the mass introduction of continental European breeds to North America[131]. The modern activity of the MOET is the result of the efforts of two groups: a) scientists, who initially developed the procedures and techniques of embryo transfer, and b) field veterinarians, who applied this technology commercially, making it practical and available for the cattle industry, and later for other productive species as well[131].

In the early 1970s, the first embryo transfer centers were established in North America, including Alberta Livestock Transfer (Alberta, Canada), Modern Ova Trends (Ontario, Canada), Colorado State University (Colorado, United States), Carnation Genetics (California, United States), and Codding Embryological Science, Inc. (Oklahoma, United States)[132]. In our country, the first embryo transfers (ET) in cattle were performed in 1978 by North American technicians, but the results are unknown. In February 1979, the Embryo Transfer Clinic was inaugurated in Ajuchitlán, Querétaro, as part of the National Center for Animal Reproduction, under the National Institute for Artificial Insemination and Animal

Reproduction (INIARA), of the then Ministry of Agriculture and Hydraulic Resources (Secretaría de Agricultura y Recursos Hidráulicos, SARH). In that same year, MOET's first Holstein calf was born there[132]. The first TE zebu calf was born in 1981 at Carnation in Mexico[133].

The Center for Genetic Improvement and Embryo Transplantation (CEMEGEN), which is attached to the state-owned dairy company Leche Industrializada Conasupo (LICONSA), began its activities in November 1986, producing embryos of the Holstein Friesian, Brown Swiss, Jersey, Simmental, Simbrah, and F1 Holstein x Gyr dairy (Gyrholando) and Holstein x Guzerat breeds. It was estimated that, by 1993, 20,000 embryos per year would be produced from 2,200 donors. The center ceased operations in 1994; between 1987 and 1993 it produced almost 42,000 embryos. In addition, 18 training courses in the technique were given to approximately 300 professionals, and work was carried out for 23 Bachelor's and 4 Master's degree theses. Most of the embryos produced were frozen and subsequently transferred to different states of Mexico[134]. In 1990, the National Center for Animal Reproduction became part of the National Commission for Genetic Improvement and Animal Reproduction (CONAMEGRA), established through an agreement between the Ministry and the National Livestock Confederation (CNG). Several TE works were carried out for farmers in different parts of the country, and an agreement was established with LICONSA for the commercialization, between 1993 and 1994, of 1,343 embryos produced at CEMEGEN, at a price of $100.00 per embryo —an amount well below the cost of production. CONAMEGRA sold this genetic material to 33 producers from 11 Mexican states, its own technicians thawed 447 embryos, transferred them, and obtained 51 % of pregnancies[135]. In 1993, INIFAP received a donation of 1,558 embryos from CEMEGEN; these were F1 and ¾ Holstein x Zebu embryos, destined for the genetic programs of its experimental stations in the tropics. That same year, 800 embryos and 300 head of cattle were donated to the College of Posgraduates (Colegio de Posgraduados).

In 1994, LICONSA terminated the loan agreement with UNAM, auctioned the livestock, dismissed and paid off its staff, and returned its facilities to UNAM, thus putting an end to the operation of CEMEGEN[135].

The collection and transfer of embryos was initially a very complex process, since both the collection of embryos from the donor and their subsequent transfer to recipient females was done by surgical methods and using general anesthesia; this involved an enormous logistical effort, as the donors and the recipients had to be prepared for surgery at the same time[132]. In the first instance, both donor and recipient surgeries were performed under local anesthesia; subsequently, procedures for the non-surgical collection of embryos and their transfer to recipients were developed, which facilitated the more widespread use of the technique[136]. The results obtained using non-surgical methods gradually approached those obtained with surgical methods, which is why the latter fell into disuse[131].

Regarding ovarian stimulation to induce multiovulation, there has always been a high variability in the superovulatory response, despite efforts to control the sources of variation, which is one of the main problems affecting the efficiency and profitability of MOET at the commercial level[137]. A study carried out with successive superovulations in zebu cows in the "El Macho" experimental station, INIFAP, where the variables of time, FSH dose and age were considered, concluded that the initial response of a zebu cow to superovulation cannot be said to be a good predictor of subsequent responses by the same cow[138].

To date, there have been considerable advances in the study of ovarian physiology, as well as factors inherent to the donor; however, there remain some aspects to be understood in order to generate more efficient ovarian stimulation protocols[139].

In its beginnings, superovulation was induced with eCG; however, a few years later it was discovered that crude pituitary extract (FSH with 20% LH) generated a better superovulatory response than eCG[140]. Pituitary extracts are now widely used; there is a high variability in the amount of LH present in crude extracts, while purified extracts have less variation in the amount of LH. Multiple studies have been conducted to evaluate *in vivo* embryo production using crude and purified extracts[131]. In a 1995 study conducted at CEMEGEN in Mexico, the crude extract was found to produce more embryos than the purified extract in dairy cattle[141]. Conversely, in a study conducted in 2014 at INIFAP with beef cattle, the purified extract generated more embryos than the crude extract[142]; this may be due to the fact that the hepatic metabolism of dairy cattle is more accelerated compared to that of beef cattle[143].

Despite many efforts to increase embryo production per cow per year by increasing ovarian response to superovulatory treatment, little progress has been made[131]. The use of PIDR in combination with BE and prostaglandins has allowed the development of a protocol called Rapid Donor Recycling. This protocol reduces the interval between superovulations by almost half (33 to 35 d) with no decline in response, either in the number of embryos produced or in their quality, in successive superovulations for up to one and a half years[131,142]. Thus, the production of transferable embryos per donor per year is nearly doubled.

The first successful cryopreservation of embryos was reported in murines in 1972[144]. One year later, the first calf was born from a previously cryopreserved blastocyst using a 2M dimethyl sulfoxide solution with a freezing and thawing rate of 0.2 °C/min and 36 °C/min, respectively[145]. The first calves born from cryopreserved embryos in Mexico came from embryos frozen in Colorado, USA, and transferred to Nayarit, Mexico, into an experimental station of INIFAP[146].

For more than 10 yr, glycerol was the cryoprotectant of choice for bovine embryo cryopreservation. However, in 1992, a direct transfer cryopreservation system using ethylene

glycol as cryoprotectant was announced. This had a rapid and positive effect worldwide on the embryo transfer industry to the present day[131].

According to data from the American Embryo Transfer Association, in 2009, approximately 99 % of the beef bovine embryos and 94 % of the dairy bovine embryos were frozen in ethylene glycol for direct transfer[131].

In vitro production of bovine embryos (IVP)

IVP biotechnology has a variety of applications in basic and applied science; in the first instance, it has supported the production of embryos used for a wide variety of research, including the treatment of infertility in humans. It is certainly a tool to increase the productivity of farm animals by increasing the reproductive potential of animals of higher genetic merit; in addition, it plays a relevant role in the conservation of the genetic resources of animals, especially endangered ones[147].

Generically known as IVF (*in vitro* fertilization) or IVP (*in vitro* production), it is a reproductive biotechnology, which, like MOET, is composed of several biotechnologies such as: *in vitro* maturation of eggs, sperm capacitation, *in vitro* fertilization, and the culture of zygotes and embryos up to pre-eclosion stages (7 to 8 d post-fertilization)[148]. IVP consists of retrieving eggs or oocytes from ovarian follicles to be matured and fertilized under laboratory conditions; the resulting zygotes are cultured until post-compaction stages (morula or blastocyst), at which time they are transferred to a recipient cow or cryopreserved for subsequent transfer. Oocytes can be drawn from trace ovaries or live animals, by means of ultrasound-guided transvaginal aspiration (TVA)[148].

This biotechnology dates back to the 1970s, thanks to which research and achievements in the areas of culture media development, oocyte maturation, sperm capacitation and fertilization (which occurred in that decade and the following one[126]) led to the birth, in 1987, of the first calf produced entirely *in vitro*[149]. Although this biotechnology was initially oriented primarily to research and was based on tests carried out on ovaries obtained at the slaughterhouse, with the incorporation of TVA (making it possible to obtain immature oocytes from living donors), the commercial application was seen as a more promising tool than MOET for the mass production of offspring from progenitors of high genetic merit. The above is confirmed by observing that, worldwide, while the number of embryos collected *in vivo* and transferred has remained stable in recent years, the number of transferred IVF embryos has had an average annual growth rate of 12 % and, for the first time, in 2017, the number of viable embryos produced *in vitro*, exceeded the number of transferable embryos

collected *in vivo*[150]; that trend has continued as of the 2019 International Embryo Technology Society (IETS) report, with data from 2018[151]. In this same report, it is noted that the vast majority of viable IVF embryos were obtained from oocytes collected through TVA (98.9 %), in contrast to the few embryos obtained from trace ovaries[151]. The reason for this is the uncertainty of obtaining germplasm suitable for commercial production from the genetic and sanitary point of view. It is known that there are a significant number of research laboratories where viable IVP embryos are produced and discarded once they have fulfilled their function; however, this datum is not recorded by the IETS statistics committee[152].

Globally, there are two events that have been driving forces in IVP: sexed semen biotechnology and genomic evaluations. The first is that thanks to IVP it is possible to maximize the use of sexed semen by fertilizing a large number of oocytes (100+) with a single dose of semen, and the second is that it makes it possible to intensify the power of selection by shortening generation intervals (TVA in calves, enabling the use of their germplasm before the age of service) and increasing the reproductive capacity on the maternal side. Thus, these three biotechnologies (IVP, sexed semen, genomic evaluations) play a relevant role in genetic improvement and the cattle industry in general[152].

IVP had a period of great growth at the beginning of this century, especially in Brazil, where, in 2009, 85 % of the available embryos came from *in vitro* production; this amount was equivalent to 50 % of the worldwide production. The success of the Brazilian companies encouraged their expansion to other Latin American countries, including Mexico, where they settled, working independently or in partnership with Mexican companies or producer organizations. It has not been possible to replicate what has been achieved in Brazil in Mexico because this country does not have the competitive advantages that Brazil has (breeds, demand, size of production units, availability of receivers)[148]. Nevertheless, there continues to be moderate activity by these and other domestic companies; thus, in 2018, the transfer of almost 28,000 embryos was reported in Mexico, almost all from IVP, and, in contrast, only over 4,000 embryos obtained *in vivo* were transferred[151].

Although a considerable amount of research has been generated regarding the main components of this biotechnology (development of sequential culture media, control of potentially toxic agents, exclusion of serum components, inclusion of amino acids, vitamins, chelating agents and hormones, among others)[153-156], and so has research in oocyte maturation and *in vitro* fertilization processes[152-157], the fact is that it has not been possible to exceed the limit of 40 to 50 % of blastocysts obtained from fertilized oocytes, a value not very different from the 30 to 40 % that existed 20 yr ago. This has drawn attention to the lack of homogeneity in the oocyte source as the most likely cause of limited IVP success rates[155,157,158]. It is therefore imperative to continue to promote IVP-IVT (in combination with other biotechnologies such as the use of sexed semen and genomic evaluations) as a

valuable productive tool, and also to continue to conduct research for IVP in larger quantities with respect to the eggs left to mature, which achieve a better post-transfer development and have greater tolerance to cryopreservation.

Due to the complexity and high requirements of infrastructure and equipment, as well as personnel trained in this biotechnology, IVP research in Mexico has been incipient. Only a handful of universities —namely, Universidad Nacional Autónoma de México, Universidad Autónoma Metropolitana, Universidad Veracruzana, Universidad Autónoma de Nuevo León, Universidad Autónoma de Tamaulipas, Colegio de Posgraduados, and Universidad Autónoma de Chihuahua—, some producer organizations and livestock companies, and INIFAP have worked on certain IVP research topics. The following are some of the results obtained in research conducted in Mexico on topics related to bovine IVP: in relation to agents and culture conditions to reduce the production of reactive oxygen species (ROS) in the culture medium, in a study conducted at INIFAP, it was found that by reducing the O_2 tension to 2 %, the number of cells in the blastocysts increased and the production of ROS was reduced[159]. In another study also carried out at INIFAP, pterostilbene, a phytoalexin, was used as an antioxidant agent at a concentration of 0.33 μM, and it was found to reduce the production of ROS and the occurrence of intracytoplasmic lipids in 7-d-old embryos[160]. The latter is presented as an alternative for improving IVP embryo cryopreservation. In a collaborative work between INIFAP and UANL, the effect of the addition of a heat shock protein (HSP70) on the development of bovine embryos produced *in vitro* was evaluated, and the addition of HSP70 to the culture media was found to have favorable effects on the percentage of blastocysts and cell number[161]. A system for individual embryo culture called "WOW" was also evaluated at INIFAP, and, although it produces a similar number of embryos compared to group culture in microdroplets, the WOW system was found to produce a higher percentage of embryos with a better morphological quality[162]. In another study carried out at UNAM, the effectiveness of two vitrification devices for cryopreserving bovine embryos was compared. Cryotop® was found to be a more effective vitrification support than Open Pulled Straw®, resulting in higher post-vitrification viability[163]. Another study carried out between the Colegio de Posgraduados and the Universidad Veracruzana tested the alternative of utilizing the culture media used in the human IVF system to cultivate bovine embryos and found that it is possible to produce blastocysts with similar results[164]. It should be noted that the culture media used in humans are often more readily available on the domestic market. Yet another study, carried out at the Autonomous University of Chihuahua, proved that the addition of IGF-I at different times during the IVP process did not produce a beneficial effect on the percentage of blastocysts[165].

The future of IVP in cattle faces important challenges that must be resolved in order to ensure its usefulness as a productive tool, as well as support for research in multiple areas of knowledge. The evolution of culture media to provide near-physiological conditions for gametes and embryos has been one of the areas with the greatest progress[152], despite which,

difficulties are still encountered today in producing similar embryos to those obtained *in vivo*, resulting in low pregnancy rates at transfer, low tolerance to cryopreservation and alterations in fetal and placental development[157]. Research efforts have focused on the development of chemically defined culture media[166], use of oxidation level regulating compounds[167], use of delipidating compounds or lipid metabolism regulators[160,168], and mechanisms such as the use of sequential media to remove the presence of molecules that are toxic to the embryo[169], as well as on the development of cryopreservation methods that are friendlier to the embryo produced *in vitro*, such as vitrification[170]. On the other hand, with the development of biotechnologies such as embryonic nuclear transplantation (16-cell stage blastomers) and somatic transplantation (cumulus cells, fibroblasts, etc.)[171], an important future was foreseen for the genetic modification of embryos for productive, medical and research purposes (gene function studies, xenotransplantation, recombinant protein production, genetic improvement, and food production)[172] and their eventual cloning. However, despite some encouraging results, the random insertion of the transgenes generated highly variable and unpredictable results, rendering the use of this technology unviable[173]. Today, with the advent of gene editing technologies (CRISPR - Cas - 9), it is already possible to perform precise gene editing, including epigenetic reprogramming, which augurs a golden age in the genetic modification of farm animals for the aforementioned purposes, with the strong support of IVP biotechnologies[174]. There will still be ethical impediments and legal pitfalls to be resolved.

Conclusions

As a final reflection, it can be said that, although research institutions in Mexico, and especially INIP-INIFAP, have accompanied the development of reproductive biotechnologies in the world in the last five decades. With the passage of the years, these have evolved meteorically, and it has been increasingly difficult to maintain a solid research base that allows us to be aligned with the technological developments that are taking place. Although the information generated by Mexican institutions on current topics such as *in vitro* embryo production is modest, the national livestock industry is demanding immersion in these technologies. This should encourage research institutions to generate technological components that will allow the efficient use of this and other technologies under local conditions.

Literature cited:

1. Secretariat of the Convention on Biological Diversity. Handbook of the Convention on Biological Diversity Including its Cartagena Protocol on Biosafety, 3rd ed. Montreal, Canada; 2005.

2. Kiracofe GH. Uterine involution: Its role in regulating postpartum intervals. J Anim Sci 1980;51(Suppl 2):16-28.

3. Short RE, Bellow RA, Staigmiller RB, Berardinelli JG, Custer EE. Physiological mechanisms controlling anestrous and fertility in postpartum beef cattle. J Anim Sci 1990;68:799-816.

4. Wettemann RP, Lents CA, Ciccioli NH, White FJ, Rubio I. Nutritional and suckling-mediated anovulation in beef cows. J Anim Sci 2003;81(Suppl 2):E48-E59.

5. Montiel F, Ahuja C. Body condition and suckling as factors influencing the duration of postpartum anestrous in cattle: a review. Anim Reprod Sci 2005;85:1-26.

6. Lozano DRR, Asprón PMA, González PE, Vásquez PCG. Estacionalidad reproductiva de vacas *Bos indicus* en el trópico mexicano. Téc Pecu Méx 1987;25(2):192-205.

7. Lozano DF, Román PH, Castillo RH, González PE. Tratamiento del anestro posparto en vacas de ordeña en el trópico. Téc Pecu Méx 1984;46:19-24.

8. Zárate-Martínez JP, Ramírez-Godinez JA, Rodríguez-Almeida FA. Comportamiento reproductivo de vacas criollas con amamantamiento restringido y sincronización del estro. Agron Mesoam 2010;21(1):121-130.

9. Wiltbank JN. Managing beef cows to get them pregnant. Texas Agricultural Experimental Station at Beeville, USA. 1972.

10. Wiltbank JN. Getting heifers pregnant. Memorias del Seminario Internacional de Ganadería Tropical. Producción de carne. SAG-Banco de México. 1976:175.

11. de los Santos VSG, Taboada SJJ, Montaño BM, González PE, Ruíz DR. Efecto de la lactación controlada y tratamientos con hormonas esteroides en la inducción y sincronización del estro en vacas encastadas de cebú. Téc Pecu Méx 1979;36:9-14.

12. Preston TR, Willis MB. Producción intensiva de carne. Primera ed. México: Editorial Diana; 1974.

13. Rodríguez ROL, Rodríguez RA, González PE, Ruiz DR. Comportamiento reproductivo de vacas productoras de carne sometidas a diversos tipos de amamantamiento. Téc Pecu Méx 1982;43:63-69.

14. Christian R, Casida L. The effects of progesterone in altering the oestrous cycle of the cow. J Anim Sci 1948;7:540.

15. Wiltbank JN, Kasson CW. Synchronization of estrus in cattle with an oral progestational agent and an injection of an estrogen. J Anim Sci 1968;27:113.

16. Rowson LEA, Tervit HR, Brand A. The use of prostaglandins for synchronization of oestrus in cattle. J Reprod Fert 1972;29(1):145.

17. Nellor JE, Cole HH. The hormonal control of estrus and ovulation in the beef heifer. J Anim Sci 1956;15:650-661.

18. Hansel W, Malven PV, Black DL. Estrous cycle regulation in the bovine. J Anim Sci 1961;20:621-625.

19. Zimbelman RG, Lauderdale JW, Sokolowski JH, Schalk TG. Safety and pharmacologic evaluations of melengestrol acetate in cattle and other animals. A review. J Am Vet Med Assoc 1970;157:1528-1536.

20. Gonzalez PE, Wiltbank JN, Niswender GD. Puberty in beef heifers. I. The interrelationship between pituitary, hypothalamic and ovarian hormones. J Anim Sci 1975;40(6):1091-1104.

21. González PE, Ruíz DR, Wiltbank JN. Inducción y sincronización del estro en vaquillas prepúberes mediante la administración de estrógenos y un progestágeno. Téc Pecu Méx 1975;28:17-23.

22. de los Santos VSG, González PE. Combinación de cipionato de estradiol, progesterona e implantes del progestágeno SC21009 para la resolución del anestro en ganado bovino productor de carne. Téc Pecu Méx 1976;31:55-62.

23. Menéndez TM, Robles BC, González PE. Inducción del estro con esteroides en vacas cebú lactantes. Téc Pecu Méx 1977;33:15-19.

24. Menéndez TM, Robles BC, González PE. Sincronización del estro en vacas cebú con y sin suplemento de melaza + urea. Téc Pecu Méx 1977;33:9-14.

25. Rodríguez RA, Casillas TO, González PE. Empleo de acetato de melengestrol, valerato de estradiol y progesterona para el control del estro en bovinos suizo pardo x cebú. Téc Pecu Méx 1977;32:41-45.

26. Rodríguez RA, Rodríguez ROL, González PE, Ruíz DR. Inseminación a horarios predeterminados en vaquillas sincronizadas con implantes de SC21009. Téc Pecu Méx 1979;36:53-58.

27. Menéndez TM, Ruíz DR, González PE. Establecimiento de épocas cortas de inseminación artificial mediante el uso de la sincronización del estro. Téc Pecu Méx 1979;36:15-20.

28. Lauderdale JW. Effects of PGF2α on pregnancy and estrous cycle of cattle. J Anim Sci 1972;35(1):246.

29. King GJ, Robertson HA. A two injection schedule with prostaglandin F2α for the regulation of the ovulatory cycle of cattle. Theriogenology 1974;1:123-128.

30. Rodríguez ROL, Rodríguez RA, González PE. Diferentes horarios de inseminación artificial en vaquillas productoras de carne sincronizadas con prostaglandinas. Téc Pecu Méx 1982;43:55-62.

31. Heersche G, Kiracofe GH, DeBenedetti RC, Wen S, McKee RM. Synchronization of estrus in beef heifers with a norgestomet implant and prostaglandin F2α. Theriogenology 1979;11:197-208.

32. Twagiramungu H, Guilbault LA, Proulx J, Dufour JJ. Effect of Synchromate-B and prostaglandin F2α on estrus synchronization and fertility in beef cattle. Can J Anim Sci 1992;72:31-39.

33. Kastelic JP, Olson WO, Martinez M, Cook RB, Mapletoft RJ. Synchronization of estrus in beef cattle with norgestomet and estradiol valerate. Can Vet J 1999;40:173-178.

34. Stevenson JS, Thompson KE, Forbes WE, Lamb GC, Grieger DM, Corah LR. Synchronizing estrus and(or) ovulation in beef cows after combinations of GnRH, norgestomet and prostaglandin $F_2\alpha$ with or without timed insemination. J Anim Sci 2000;78:1747-1758.

35. Faustino CR, Rosete FJV, Ríos UA, Vega MVE. Inducción del estro con un implante de norgestomet y valerato de estradiol, complementado con gonadotropina coriónica equina en vacas cárnicas en una época de empadre [resumen]. Reunión Nacional de Investigación Pecuaria. Mérida, Yucatán. 2008:262.

36. Koppel RET, Rodríguez ROL. Sincronización del estro con progestágenos e inseminación a tiempo predeterminado en vaquillas cebú bajo condiciones de trópico. Téc Pecu Méx 1989;27(2):53-61.

37. Patterson DJ, Cojima FN, Smith MF. A review of methods to synchronize estrus in replacement beef heifers and postpartum cows. J Anim Sci 2003;81(Suppl 2):E166-E177.

38. De La Torre SJF, Galavíz RI, Estrada MA, Villagómez AE, Ortíz MEP. Evaluación de un esquema de inducción/sincronización del estro en vacas angus y limousin con cría al pie [resumen]. Reunión Nacional de Investigación Pecuaria. Saltillo, Coahuila. 2009:76.

39. Chaga LE, Zárate MJP, Rosas PJ, Alpírez MF, Domínguez MB. Niveles séricos de progesterona e inseminación artificial a tiempo fijo y a 12 horas posteriores al estro en vaquillas cruzadas utilizando los protocolos CO-SYNCH-CIDR y CRESTAR plus. XXII Reunión Científica-Tecnológica Forestal y Agropecuaria Veracruz 2009. Úrsulo Galván, Veracruz. 2009:478-487.

40. Vera AHR, Villa GA, Jiménez SH, Álvarez GH, De La Torre SJF, Gutiérrez ACG, *et al*. Eficiencia reproductiva de los bovinos en el trópico. En: editores Rodríguez RO, González PE, Dávalos FJL. Estado del arte sobre investigación e innovación tecnológica en ganadería bovina tropical. Primera ed. Ciudad de México, México: REDGATRO-CONACYT; 2015:153-192.

41. Rodríguez ROL, Rodríguez RA, Zambrano GR, González PE. Comportamiento reproductivo de vacas con aumentos de peso controlados antes y después del parto. Téc Pecu Méx 1979;36:40-46.

42. Ciccioli NH, Wettemann RP, Spicer LJ, Lents CA, White FJ, Keisler DH. Influence of body condition at calving and postpartum nutrition on endocrine function and reproductive performance of primiparous beef cows. J Anim Sci 2003;81(12):3107-31020.

43. Guzmán SA, González PE, Garcés YP, Rosete FJV, Calderón RC, Murcia C, *et al*. Reducción en las concentraciones séricas de insulina e IGF-1 pero no leptina, se asocia a una reducción en la respuesta a un programa de inducción de estros en vacas de carne amamantando [resumen]. Reunión Nacional de Investigación Pecuaria. Saltillo, Coahuila. 2009:50.

44. Guzmán A, González PE, Garcés YP, Rosete FJV, Calderón RC, Murcia C, *et al*. Reduced response to an estrous induction program in postpartum beef cows treated with zilpaterol and gaining body weight. Anim Reprod Sci 2012;130:1-8.

45. Rosales TAM, López CZB, Hernández CCG, Rosete FJV, Mendoza GD, Guzmán A. Short-term dietary concentrate supplementation during estrus synchronization treatment in beef cows increased IGF-1 serum concentration but did not affect the reproductive response. Trop Anim Health Prod 2017;49(1):221-226.

46. Mead E, Maguire JJ, Kuc RE, Davenport AP. Kisspeptins: a multifunctional peptide system with a role in reproduction, cancer and the cardiovascular system. British J Pharmacol 2007;151:1143-1153.

47. Navarro VM, Tena-Sempere M. Neuroendocrine control by kisspeptins: role in metabolic regulation of fertility. Nat Rev Endocrinol 2012;8:40-53.

48. Kadokawa H, Matsui M, Hayashi K, Matsunaga N, Kawashima C, Shimizu T, *et al*. Peripheral administration of kisspeptin-10 increases plasma concentrations of GH as well as LH in prepubertal Holstein heifers. J Endocrinol 2008;196:331-334.

49. Ezzat AA, Saito H, Sawada T, Yaegashi T, Yamashita T, Hirata TI, *et al*. Characteristics of the stimulatory effect of Kisspeptin-10 on the secretion of luteinizing hormone, follicle-stimulating hormone and growth hormone in prepubertal male and female cattle. J Reprod Dev 2009;55:650-654.

50. Alamilla RM, Calderón RRC, Rosete FJV, Rodríguez HK, Vera AHR, Arreguín AJA, *et al*. Kisspeptina en becerras prepúberes: I. Influencia de la edad en la respuesta de LH, FSH y GH a kisspeptina-10 y su asociación con IGF-I, leptina y estradiol. Rev Mex Cienc Pecu 2007;8(4):375-385.

51. Santos ER, Calderón RRC, Rosete FVJ, Perera MG, Murcia MC, Villagómez AME, *et al*. Evaluación de la sensibilidad del eje gonadotrópico a dosis bajas de kisspeptina (KISS-10) en becerras prepúberes [resumen]. Reunión Nacional de Investigación Pecuaria. Querétaro, Querétaro. 2012:125.

52. Villa GA, Santos ER, Rosete FJV, Calderón RRC, Perera MG, Arreguín AJA, *et al*. Kisspeptina en becerras prepúberes: 2. Respuesta de LH, FSH y GH a distintas dosis de kisspeptina-10 y su asociación con IGF-I y leptina circulantes. Rev Mex Cienc Pecu 2018;9(4):719-737.

53. Santos ER, Calderón RRC, Vera AHR, Perea MG, Arreguín AJA, Nett TM, *et al*. Hormona luteinizante y actividad ovárica en respuesta a kisspeptina-10 y su asociación con IGF-1 y leptina en becerras prepúberes. Rev Mex Cienc Pecu 2014;5(2):181-200.

54. Rosete FJV, Hernández LB, Santos ER, Gómez CBM, Perera MG, Calderón RRC, *et al*. Respuesta de hormona luteinizante a aplicaciones repetidas de kisspeptina-10 en vacas en anestro posparto [resumen]. Reunión Nacional de Investigación Pecuaria. Toluca, Estado de México. 2015:61-63.

55. Fragoso IA, Rosete FJV, Ríos UA, Santos ER. Efecto de la kispeptina-10 en la ovulación y gestación en vacas tratadas con sincronización estral [resumen]. II Congreso Internacional de Agroecosistemas Tropicales. Yucatán. 2020:70.

56. Spallanzani L. Dissertations relative to the natural history of animals and vegetables. Trans. By T. Beddoes. London: J. Murray; 1784;2:195-199.

57. Phillips EJ, Lardy HA. A yolk-buffer pabulum for the preservation of bull semen. J Dairy Sci 1940;(23):399-404.

58. Salisbury GW, Fuller HK, Willett EL. Preservation of bovine spermatozoa in yolk-citrate diluents and field results from its use. J Dairy Sci 1941;24:905-910.

59. Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 1949;164:166.

60. Ombelet W, Van Robays J. Artificial insemination history: hurdles and milestones. Facts Views Vis Obgyn 2015;7(2):137-143.

61. Polge C, Rowson LEA. Results with bull semen stored at -79°C. Vet Rec 1952;64:851-853.

62. Manjunath P, Bergeron A, Lefebvre J, Fan J. Seminal plasma proteins: functions and interaction with protective agents during semen preservation. Soc Reprod Fertil Suppl 2007;65:217-228.

63. Lenz RW, Zhang HM, Oyarzo JN, Bellin ME, Ax RL. Bovine fertility-associated antigen (FAA) and a recombinant segment of FAA improve sperm function. Biol Reprod 1999;62:137-138.

64. Alvarez GH, Kjelland ME, Moreno JF, Welsh TH Jr, Randel RD, Lammoglia MA, *et al*. Gamete therapeutics: recombinant protein adsorption by sperm for increasing fertility via artificial insemination. PLoS One 2013;8(6):e65083.

65. Seidel Jr GE, Garner DL. Current status of sexing mammalian spermatozoa. Reproduction 2002;124:733-743.

66. Garner DL. Sex-sorting mammalian sperm: Concept to application in Animals. J Adrol 2001;22(4):519-26.

67. Garner DL, Seidel Jr GE. Past, present and future perspectives on sexing sperm. Can J Anim Sci 2003;83:375-384.

68. Seidel Jr GE. Sperm sexing technology. The transition to commercial application. An introduction to the symposium "Update on sexing mammalian sperm". Theriogenology 2009;71:1-3.

69. Garner DL, Seidel Jr GE. History of commercializing sexed semen for cattle. Theriogenology 2008;69:886-895.

70. Oses MV, Teruel MT, Cabodevila JA. Utilización de semen bovino sexado en inseminación artificial, transferencia embrionaria y fertilización *in vitro*. Red Vet 2009;20:138-145.

71. Seidel Jr GE, Allen CH, Johnson LA, Holland MD, Brink Z, Welch GR. Uterine horn insemination of heifers with very low numbers of nonfrozen and sexed sperm. Theriogenology 1997;48:1255–1264.

72. Schenk JL, Suh TK, Cran DG, Seidel GE Jr. Cryopreservation of flow-sorted bovine spermatozoa. Theriogenology 1999;52(8):1375-1391.

73. STgenetics. https://www.stgen.com Accessed 15 Sep, 2020.

74. Johnson LA, Welch GR. Sex preselection: high speed flow cytometric sorting of X and Y sperm for maximum efficiency. Theriogenology 1999;52:1323-1341.

75. Vishwanath R, Moreno JF. Review: Semen sexing – current state of the art with emphasis on bovine species. Animal 2018;12(Suppl 1):1-12.

76. González MC, Lenz RW, Gilligan TB, Evans KM, Gongora CE, Moreno JF, *et al*. SexedULTRA™, a new method of processing sex sorted bovine sperm improves post-thaw sperm quality and in vitro fertility. Reprod Fert Develop 2017;29(1):204.

77. González MC, Góngora CE, Guilligan TB, Evans KM, Moreno JF, Vishwanath R. In Vitro sperm quality and DNA integrity of SexedULTRATM sex sorted sperm compared to non sorted bovine sperm. Theriogenology 2018;114:40-45.

78. Vishwanath R. 2014. SexedULTRA – raising the fertility bar of sexed sorted semen. In Proc 25th Tech Conf Artif Insem Reprod. National Association of Artificial Breeders, September 2014, Wisconsin, USA, 57-61.

79. Lenz RW, González MC, Gilligan TB, DeJarnette JM, Utt MD, Helser LA, *et al*. SexedULTRA™, a new method of processing sex sorted bovine sperm improves conception rates. Reprod Fert Develop 2017;29(1):203-204.

80. Crites BR, R Vishwanath, AM Arnett, PJ Bridges, WR Burris, KR McLeod, *et al*. Conception risk of beef cattle after fixed-time artificial insemination using either SexedUltra™ 4M sex-sorted semen or conventional semen. Theriogenology 2018;118:126-129.

81. Thomas JM, Locke JWC, Bonacker RC, Knickmeyer ER, Wilson DJ, Vishwanath R, *et al*. Evaluation of SexedULTRA 4MTM sex sorted semen in timed artificial insemination programs for mature beef cows. Theriogenology 2019;123:100-107.

82. Pérez EJI. Comparación de la frecuencia de nacimientos de hembras con el método de inseminación artificial con semen sexado y no sexado en vaquillas holstein-friesian [tesis licenciatura]. México: Universidad Nacional Autónoma de México; 2007.

83. De La Torre SJF, Hernández VR, Padilla RFJ, Reynoso CO, Maciel RMG. Uso de semen sexado en vaquillas holstein en el Estado de Jalisco [resumen]. XLIV Reunión Nacional de Investigación Pecuaria. Yucatán, México. 2008:84.

84. Callejas SJA. Evaluación del uso de semen sexado en vaquillas Holstein de reemplazo [tesis maestría]. Baja California, México: Universidad Autónoma de Baja California; 2013.

85. Villaseñor GF, de la Torre SJF, Estrada CE, Martínez VG, Coronado BH. Efecto de la presencia de mastitis en el posparto temprano sobre la fertilidad en vacas del sistema familiar inseminadas con semen sexado [resumen]. XLVIII Reunión Anual de Investigación Pecuaria. Querétaro, México. 2012:132.

86. Villaseñor GF, De La Torre SJF, Hernández VR, Martínez VG, Estrada CE. Prevalencia de abortos y distocias en vaquillas holstein inseminadas con semen sexado en el Estado de Jalisco. XLVI Reunión Nacional de Investigación Pecuaria [resumen]. Campeche, México. 2010:154.

87. Vincent P, Underwood SL, Dolbec C, Bouchard N, Kroetsch T, Blondin P. Bovine semen quality control in artificial insemination centers. Anim Reprod 2018;9(3):153-165.

88. García MV, de Paz P, Martinez PF, Alvarez M, Gomes AS, Bernardo J, *et al*. DNA fragmentation assessment by flow cytometry and Sperm–Bos–Halomax (bright-field microscopy and fluorescence microscopy) in bull sperm. Int J Androl 2007;30(2):88-98.

89. Amann RP, Waberski D. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. Theriogenology 2014;81:5-17.

90. Utt MD. Prediction of bull fertility. Anim Reprod Sci 2016;169:37-44.

91. Mortimer ST. CASA—practical aspects. J Androl 2000;21(4):515-524.

92. van der Horst G, Maree L, du Plessis SS. Current perspectives of CASA applications in diverse mammalian spermatozoa. Reprod Fert Develop 2018;30(6):875-888.

93. Lu JC, Huang YF, Lü NQ. Computer-aided sperm analysis: past, present and future. Andrologia 2014;46(4):329-338.

94. Muiño R, Tamargo C, Hidalgo CO, Peña AI. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: effects of cryopreservation and between-bull variation. Anim Reprod Sci 2008;109:27-39.

95. Muiño R, Peña AI, Rodríguez A, Tamargo C, Hidalgo CO. Effects of cryopreservation on the motile sperm subpopulations in semen from Asturiana de los Valles bulls. Theriogenology 2009;72:860-868.

96. Küçük N, Lopes JS, Soriano-Úbeda C, Hidalgo CO, Romar R, Gadea J. Effect of oviductal fluid on bull sperm functionality and fertility under non-capacitating and capacitating incubation conditions. Theriogenology 2020;158:406-415.

97. Correa JR, Pace MM, Zavos PM. Relationships among frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program. Theriogenology 1997;48(5):721-731.

98. Gliozzi TM, Turri F, Manes S, Cassinelli C, Pizzi F. The combination of kinetic and flow cytometric semen parameters as a tool to predict fertility in cryopreserved bull semen. Animal 2017;11:197-1982.

99. Januskauskas A, Johannisson A, Rodriguez-Martinez H. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. Theriogenology 2003;60:743-758.

100. Karoui S, Díaz C, González MC, Amenabar ME, Serrano M, Ugarte E, *et al*. Is sperm DNA fragmentation a good marker for field AI bull fertility? J Anim Sci 2012;90:2437-2449.

101. Gillan L, Kroetsch T, Maxwell WC, Evans G. Assessment of *in vitro* sperm characteristics in relation to fertility in dairy bulls. Anim Reprod Sci 2008;103:201-214.

102. Morrell JM, Nongbua T, Valeanu S, Verde IL, Lundstedt-Enkel K, Edman A, *et al*. Sperm quality variables as indicators of bull fertility may be breed dependent. Anim Reprod Sci 2017;185:42-52.

103. Farrell PB, Presicce GA, Brockett CC, Foote RH. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology 1998;49(4):871-879.

104. van der Horst G. Computer Aided Sperm Analysis (CASA) in domestic animals: Current status, three D tracking and flagellar analysis. Anim Reprod Sci 2020;220:106350.

105. Valverde A, Madrigal-Valverde M. Computer-assisted semen analysis systems in animal reproduction. Agron Mesoam 2018;29:469-484.

106. Menkveld R, Holleboom CA, Rhemrev JP. Measurement and significance of sperm morphology. Asian J Androl 2011;13:59-68.

107. Rodríguez-Martínez H. State of the art in farm animal sperm evaluation. Reprod Fertil Dev 2006;19:91-101.

108. Al-Makhzoomi A, Lundeheim N, Haard M, Rodríguez MH. Sperm morphology and fertility of progeny-tested AI dairy bulls in Sweden. Theriogenology 2008;70:682-691.

109. van der Horst G, du Plessis SS. Not just the marriage of Figaro: but the marriage of WHO/ESHRE semen analysis criteria with sperm functionality. Adv Androl Online 2017;4:6–21.

110. Yániz JL, Soler C, Santolaria P. Computer assisted sperm morphometry in mammals: a review. Anim Reprod Sci 2015;156:1-12.

111. van der Horst G, Skosana B, Legendre A, Oyeyipo P, Du Plessis SS. Cut-off values for normal sperm morphology and toxicology for automated analysis of rat sperm morphology and morphometry. Biotech Histochem 2018;93:49-58.

112. Gil MC, García HM, Barón FJ, Aparicio IM, Santos, AJ, García MLJ. Morphometry of porcine spermatozoa and its functional significance in relation with the motility parameters in fresh semen. Theriogenology 2009;71:254-263.

113. Gravance CG, Casey ME, Casey PJ. Pre-freeze bull sperm head morphometry related to post-thaw fertility. Anim Reprod Sci 2009;114:81-88.

114. Valverde A, Aremán H, Sancho M, Contell J, Yániz J, Fernández A, *et al*. Morphometry and subpopulation structure of Holstein bull spermatozoa: variations in ejaculates and cryopreservation straws. Asian J Androl 2016;18:851-857.

115. Garner DL, Thomas CA, Joerg HW, Mel Dejarnette J, Marshall CE. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol Reprod 1997;57:1401-1406.

116. Lange CA, Meucci A, Cremonesi F. Fluorescent multiple staining and CASA system to assess boar sperm viability and membranes integrity in short and long-term extenders. Open Vet J 2013;3:21-35.

117. Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 2003;9:331-345.

118. Gil-Villa AM, Cardona MW, Agarwal A, Sharma R, Cadavid Á. Assessment of sperm factors possibly involved in early recurrent pregnancy loss. Fertil Steril 2010;94:1465-1472.

119. Gillan L, Evans G, Maxwell WMC. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 2005;63:445-457.

120. Gómez VJC, Cuicas HR, Jáuregui PI, Gutiérrez SI, Ulloa AR, Urban DD, *et al*. Determinación de la congelabilidad de carneros de pelo (Pelibuey-Blackbelly) de acuerdo con la viabilidad y cinética espermática. Spermova 2020;10:26-31.

121. Herrera JAH. Efecto del tipo de azúcar utilizado en el diluyente sobre la criopreservación del semen de cerdo pelón mexicano [tesis doctoral]. Yucatán, México: Tecnológico Nacional de México; 2017.

122. Barragán SAL. Evaluación de cuatro criodiluyentes sobre algunas variables de respuesta seminal pre y post-descongelamiento en borregos Merino isla Socorro, con fines de preservación [tesis maestría]. Jalisco, México: Universidad de Guadalajara; 2018.

123. Urbán DD, Méndez-Gómez AC, Álvarez GH, Pérez RS, De La Torre SJF, Pedraza VJP. Conservación *ex situ*, *in vitro* y calidad seminal del borrego Chiapas. Rev Mex Agroecosistemas 2016; 3(Suppl 2):112-115.

124. Pérez RS, Urbán DD, Álvarez GH, De La Torre SJF, Palacios GMA. Conservación *ex situ*, *in vitro* y calidad seminal del bovino criollo corriente de Tamaulipas. Rev Mex Agroecosistemas 2016;3(Suppl 2):116-118.

125. Álvarez GH, Urban DD, Pérez RS, De La Torre SJF. Validación del ensayo de evaluación de semen bovino mediante un sistema "CASA" (Computer Assited Sperm Analyzer). VII Congreso Virtual Iberoamericano sobre la Gestión de Calidad en Laboratorios IBEROLAB. Madrid, España. 2014:259-260.

126. Moore SG, Hasler JF. A 100-Year Review: Reproductive technologies in dairy science. J Dairy Sci 2017;100(12):10314-10331.

127. FAO. Cryoconservation of animal genetic resources. FAO: Rome. Animal Production and Health Guidelines No. 12. 2012..

128. Villaseñor GF, De La Torre SJF, Martínez VG, Álvarez GH, Pérez RS, Palacios FJA, *et al*. Caracterización de la respuesta ovárica a la superovulación en bovino criollo coreño utilizando dosis reducidas de FSH. Rev Mex Cien Pecu 2017;8(3):225-232.

129. Heape W. Preliminary note on the transplantation and growth of mammalian ova within a uterine foster-mother. Proc Roy Soc London B 1890;48:457-9.

130. Willett EL, Black WG, Casida LE, Stone WH, Buckner PG. Successful transplantation of a fertilized bovine ovum. Science 1951;113:247.

131. Hasler JF. Forty years of embryo transfer in cattle: a review focusing on the Journal Theriogenology, the growth of the industry in North America, and personal reminiscences. Theriogenology 2014;81(1):152-69.

132. Cerda AA. Crecen saludables en México los primeros becerros de probeta. El sol del campo 22 de abril 1980:1-4.

133. Asprón PMA. Evolución histórica de la transferencia de embriones. Revista Cebú 1989;15(3):47-54.

134. Sánchez AA, Ramírez CJ. Apoyos de LICONSA a los ganaderos lecheros en diferentes regiones de México. En: Martínez BE, *et al*, coord. Dinámica del sistema lechero mexicano en el marco regional y global. México: Ed. Plaza y Valdés; 1999:270-291.

135. México Holstein. CONAMEGRA tutela el mejoramiento genético de la ganadería. 1994;25:29-31.

136. Elsden RP, Hasler JF, Seidel GE Jr. Non-surgical recovery of bovine eggs. Theriogenology 1976;6(5):523-32.

137. Córdoba SLA. Superovulación inducida en ganado bovino. Téc Pecu Méx 1988;26(1):109-119.

138. De La Torre SJF, Castro LMA, González PE, Reynoso CO. Respuesta de vacas cebú a superovulaciones sucesivas con FSH. Téc Pecu Méx 1992;30(3):223-231.

139. Mikkola M, Hasler JF, Taponen J. Factors affecting embryo production in superovulated *Bos taurus* cattle. Reprod Fertil Dev 2019;32(2):104-124.

140. Bó GA, Mapletoft RJ. Historical perspectives and recent research on superovulation in cattle. Theriogenology 2014;81(1):38-48.

141. Martínez BS, Sánchez AA, Anta JE, Berruecos VJM, Valencia MJ. Valoración de dos hormonas folículo estimulantes comerciales usadas en la superovulación de vacas en lactación y vaquillas en ganado lechero. Téc Pecu Méx 1995;33(1):34-38.

142. Polanco SR. Efecto de dos preparaciones de FSH-LH al inducir multiovulación usando reciclado rápido y tradicional en donadoras Brangus rojo [tesis maestría]. Xalisco, Nayarit, México: Universidad Autónoma de Nayarit; 2014

143. Hart CG, Voelz BE, Brockus KE, Lemley CO. Hepatic steroid inactivating enzymes, hepatic portal blood flow and corpus luteum blood perfusion in cattle. Reprod Domest Anim 2018;53(3):751-758.

144. Whittingham DG, Leibo SP, Mazur P. Survival of mouse embryos, frozen to -196°C and -289°C. Science 1972;178:411–414.

145. Wilmut I, Rowson LEA. Experiments on the low-temperature preservation of cow embryos. Vet Rec 1973;92:686–690.

146. de los Santos-Valadez S, Tervit HR, Elsden RP, Seidel GE. Transport of frozen cattle embryos from U.S.A. to Mexico. Theriogenology 1981;15(1):123.

147. Bavister BD. Early history of *in vitro* fertilization. Reproduction 2002;124:181-196.

148. Vera AHR, Santos ER, Hernández MJH, Gutiérrez ACG, de la Torre SJF, Álvarez GH, *et al*. Eficiencia reproductiva de los bovinos en el trópico. En: Estado del arte sobre investigación e innovación tecnológica en ganadería bovina tropical. 2ª ed. Ciudad de México, México: Red de investigación e innovación tecnológica para la ganadería bovina tropical. CONACYT; 2018.

149. Lu KH, Gordon I, Chen HB, McGovern H. *In vitro* culture of early bovine embryos derived from *in vitro* fertilization of follicular oocytes matured *in vitro*. Proc. Third Meet. Eur. Embryo Transf. Assoc. Lyon, France; 1987.

150. Viana JHM. 2017 Statistics of embryo production and transfer in domestic farm animals: Is it a turning point? In 2017 more *in vitro*-produced than *in vivo*-derived embryos were transferred worldwide. Embryo Transfer Newsletter 2018;36(3):8-25.

151. Viana JHM. 2018 Statistics of embryo production and transfer in domestic farm animals, embryo industry on a new level: over one million embryos produced *in vitro* IETS Data Retrieval Committee. Embryo Technology Newsletter 2019;36(4).

152. Ferré LB, Kjelland, ME, Strøbech LB, Hyttel P, Mermillod P, Ross PJ. Review: Recent advances in bovine *in vitro* embryo production: reproductive biotechnology history and methods. Animal 2020;14:991-1004.

153. De La Torre SJF, Gardner D, Preis K, Gibbons, J, Seidel, GE. Metabolic regulation of *in-vitro*-produced bovine embryos. II. Effects of phenazine ethosulfate, sodium azide and 2,4-dinitrophenol during post-compaction development on glucose metabolism and lipid accumulation. Reprod Fert Develop 2006;8:597-607.

154. Lane M, Gardner D. Embryo culture medium: which is the best?. Best Pract Res Clin Obstet Gynaecol 2007;21(1):83-100.

155. Sanches BV, Zangirolamo AF, Seneda MM. Intensive use of IVF by large-scale dairy programs. Anim Reprod 2019;16(3):394-401.

156. Ferré LB, Kjelland ME, Taiyeb AM, Campos CF, Ross PJ. Recent progress in bovine in vitro-derived embryo cryotolerance: Impact of in vitro culture systems, advances in cryopreservation and future considerations. Reprod Dom Anim 2020;55:659-676.

157. Patel O, Bettegowda A, Ireland J, Coussens P, Lonergan P, Smith G. Functional genomics studies of oocyte competence: evidence that reduced transcript abundance for follistatin is associated with poor developmental competence of bovine oocytes. Reproduction 2007;133(1):95-148.

158. Sirard MA. 40 years of bovine IVF in the new genomic selection context. Reproduction 2018;156:R1–R7.

159. Delgado TGA. Efecto de diferentes niveles de oxígeno en la atmósfera de cultivo y la adición de un antioxidante comercial en el desarrollo de embriones bovinos producidos *in vitro* [tesis maestría]. Nayarit, México: Universidad Autónoma de Nayarit; 2013.

160. Sosa F, Romo S, Kjelland M, Álvarez GH, Pérez RS, Urbán DD, *et al*. Effect of pterostilbene on development, equatorial lipid accumulation and reactive oxygen species production of *in vitro*-produced bovine embryos. Reprod Dom Anim 2020;55:1–11.

161. Garza AAJ, Pérez RS, Urbán DD, Cervantes VR, De La Torre SJF. Adición de la proteína de choque térmico HSC70 en medios de cultivo sobre la producción *in vitro* de embriones bovinos. Reunión Nacional de Investigación Pecuaria, Chiapas, México: 2019:120-122.

162. Virrueta MLM. Evaluación de dos sistemas de cultivo *in vitro* de embriones bovinos [tesis licenciatura]. Saltillo, Coahuila, México: Universidad Autónoma Agraria Antonio Narro; 2018.

163. Morales PF. Viabilidad post-criopreservación de embriones bovinos producidos *in vitro* y vitrificados mediante las técnicas de "open pulled straw" y cryotop [tesis maestría]. Ciudad de México, México; Universidad Nacional Autónoma de México; 2013.

164. Ahuja AC, Montiel PF, Pérez HP, Gallegos SJ. Medio alternativo para la producción *in vitro* de embriones bovinos. Zootecnia Trop 2009;27(3):277-284.

165. Martínez GO, Antillón RJ, Rodríguez AFA. Tasa de fertilización, desarrollo y calidad de embriones bovinos Holstein producidos *in vitro* con semen sexado y adición de IGF-I. Tecnociencia Chihuahua 2020;9(3):140-147.

166. Van der Valk J, Brunner D, De Smet K, Svenningsen ÅF, Honegger P, Knudsen LE, *et al.* Optimization of chemically defined cell culture media–replacing fetal bovine serum in mammalian *in vitro* methods. Toxicol *In vitro* 2020;24(4):1053-1063.

167. Sovernigo TC, Adona PR, Monzani PS, Guemra S, Barros FDA, Lopes FG, *et al.* Effects of supplementation of medium with different antioxidants during *in vitro* maturation of bovine oocytes on subsequent embryo production. Reprod Dom Anim 2017;52(4):561-569.

168. Torres V, Hamdi M, Millán de la Blanca MG, Urrego R, Echeverri J, López-Herrera A, *et al*. Resveratrol–cyclodextrin complex affects the expression of genes associated with lipid metabolism in bovine *in vitro* produced embryos. Reprod Dom Anim 2018;53(4):850-858.

169. Moore K, Rodriguez-Sallaberry CJ, Kramer JM., Johnson S, Wroclawska E, Goicoa S, *et al*. *In vitro* production of bovine embryos in medium supplemented with a serum replacer: effects on blastocyst development, cryotolerance and survival to term. Theriogenology 2007;68(9):1316-1325.

170. Do VH, Catt S, Kinder JE, Walton S, Taylor-Robinson AW. Vitrification of *in vitro* derived bovine embryos: targeting enhancement of quality by refining technology and standardising procedures. Reprod Fert Dev 2019;31(5):837-846.

171. Westhusin ME, Pryor JH, Bondioli KR. Nuclear transfer in the bovine embryo: a comparison of 5-day, 6-day, frozen-thawed, and nuclear transfer donor embryos. Mol Reprod Dev 1991;28(2):119-23.

172. Houdebine LM. Transgenic animal production. In: Ram LS, Sukanta M editors. Biotechnology for sustainable agriculture. UK: Woodhead Publishing; 2018:141-184.

173. Tan W, Proudfoot C, Lillico SG, Whitelaw CBA. Gene targeting, genome editing: from Dolly to editors. Transgenic Res 2016;25:273–287.

174. Yum SY, Youn KY, Choi WJ, Jang G. Development of genome engineering technologies in cattle: from random to specific. J Animal Sci Biotechnol 2018;9:1-9.