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Systematic Review

In Vitro Embryo Production and Development in Livestock and Human: A One Health Model for Human-Assisted Reproductive Technology

Jessica Looman, M.S.[1], John Gibbons, Ph.D.[2]

Abstract

Assisted reproductive technologies (ART), such as in vitro embryo production (IVP), have enabled the birth of over 8 million babies globally and address infertility, which affects 186 million individuals worldwide. In livestock, ART addresses reproductive inefficiencies while enhancing genetic progress and productivity but also aims to meet the growing global demand for food production, while tackling the environmental challenges associated with sustainable agriculture. Despite significant advancements in IVP across humans and livestock, inefficiencies remain and procedures must be optimized. By comparing the interconnections and efficiencies in IVP methodologies and developmental outcomes across species, an interdisciplinary approach can be used to better understand and refine IVP techniques for human and animal ART. This One Health perspective emphasizes the interconnected benefits of optimizing IVP techniques for human fertility treatments, animal breeding efficiency, and environmental sustainability. The objective is to compare IVP of embryos, including processes and developmental outcomes, across humans and livestock species such as sheep, pigs, cows, and horses and highlight both the efficiencies and challenges unique to each species. Oocyte collection and selection, in vitro maturation of oocytes, fertilization, and subsequent culture and development of embryos are all important steps that contribute to the success of IVP. The knowledge learned from animal research models has broad impacts that contribute to human medicine and a One Health perspective highlights how optimizing IVP benefits individual species and contributes to global health.

Keywords: In Vitro Fertilization, Embryo Production, One Health

Introduction

In the 40 years that human in vitro fertilization (IVF) has been commercially available, an estimated 8 million human babies have resulted from IVF.[1,2] The incidence of infertility continues to rise due to many factors related to lifestyle changes, environmental factors, and genetic predispositions.[3] The World Health Organization reported that in 2021, around 17.5% of the adult population (1 in 6 people worldwide) experienced infertility.[4] Projections on the growing use of Assisted Reproductive Technology (ART) suggest that by the year 2100, 3% of the world's population, 400 million people, could owe their lives to reproductive technologies.[5] As more patients build families, and their children, in turn, become parents, the number owing their existence to ART, either directly or indirectly, will expand

Corresponding Author:
Jessica Looman, M.S.
Texas Tech University School of Veterinary Medicine, Amarillo, Texas
Email: jlooman@ttu.edu

exponentially. Most advancements in ART are possible through the extensive use of animal models, allowing researchers to explore, test, and refine techniques in a controlled setting before their application to human subjects.[3] In livestock, although ART is performed for research, some techniques are commercially available and play roles in production and improve animal agriculture.[6] Unlike humans, in production animals, the focus is not to overcome infertility alone but also to enhance genetic progress, both by the selection of the most genetically valuable individuals and by increasing the number of offspring of valuable individuals.[7] Livestock production benefits from ART by increasing the dissemination of superior genetics, reducing generation intervals, and enhancing productivity, making the system more efficient.[8] By 2050, the global population is projected to rise from 7.6 to 9.8 billion, with a 20% increase in per capita meat and milk consumption.[9] Because livestock production significantly contributes to global warming, livestock producers are pushed to produce more meat and animal products from fewer animals on less land and in a shorter time, thus, ART must be optimized to increase efficiency.[9] In vitro, cattle, horses, and pigs often have few immature oocytes reaching the blastocyst stage post-fertilization, typically below 30–40%, indicating a significant rate of developmental failure.[6] Still, even though blastocyst rates are suboptimal, there is an increasing utilization of in vitro embryo production in livestock species. In 2021, worldwide, in vitro produced (IVP) embryos made up 79.7% of all transferrable cattle embryos.[10] Currently in sheep, IVP has more limited commercial application compared to cows. Still, the interest towards in vitro embryo production is constantly increasing. According to the International Embryo Technology Society (IETS) Data Retrieval Report, IVP-produced sheep embryos increased 344.0% from 2020 to 2021, compared to in vivo derived embryos which increased by 38.1%.[10] IVP can be broken into 5 crucial steps: (1) oocyte collection from the donor female; (2) in vitro maturation (IVM) of harvested oocytes; (3) in vitro fertilization with selected sperm; (4) in vitro culture (IVC) of presumptive zygotes to different stages of development; and (5) transfer or storage of embryos that develop.[8] Researchers have devoted many experiments focusing on establishing optimal conditions and protocols for each step.

This review aims to compare IVP of embryos, processes, and development rates across human and livestock species, specifically sheep, pigs, cows, and horses. By examining the similarities and differences in the methodologies and outcomes of IVP, this review seeks to highlight the efficiencies and challenges encountered in each species. Additionally, this review will explore the significance of using animal models to advance human ART. Understanding how ART techniques and success rates in livestock can inform and enhance human IVF procedures will be a key focus. Moreover, this review will emphasize the One Health perspective, illustrating how advancements in IVP not only benefit animal breeding and productivity but also enhance human fertility treatments. Additionally, IVP contributes to environmental sustainability and more efficient production of animals and animal products. By improving genetic selection and reducing the number of animals needed for efficient production, these technologies can lower greenhouse gas emissions and resource consumption, fostering a more sustainable agricultural system. This holistic approach underscores the interconnectedness of animal, human, and environmental health, demonstrating the broad-reaching impacts of optimizing IVP techniques. Through this comparative analysis, the review intends to underscore the critical role of animal research in refining and optimizing IVP of embryos, ultimately benefiting animals, humans, and the environment.

Oocyte Collection

Many different terms and abbreviations are used across species in clinical practice and research for the collection of oocytes. Some terms include transvaginal oocyte retrieval, egg retrieval, oocyte collection, transvaginal follicle aspiration, and follicle aspiration. The term

ovum pick-up (OPU) is a common broad term that can be utilized to increase consistency.[11] Oocytes are collected in one of three ways. They can be collected from the ovaries, either after a normal spay where ovaries are removed, in ultrasound-guided ovum pick-up procedures in live animals, or after the death of the animal due to slaughter, euthanasia, or after fatal trauma.[7] In many countries, animal ovaries are deemed unsuitable for human consumption, making them a byproduct of the meat industry. Consequently, they can be easily and abundantly sourced fresh from consenting slaughterhouses.[7] However, this collection method presents a limitation. It diminishes the ability to ascertain the origin of the oocyte or ovary, a critical factor considering the substantial influence of the female donor on developmental potential. Unknown factors about the donor's health or follicular status can cause significant variation in the development potential of the oocyte. Alternatively, transvaginal ultrasound-guided follicular aspiration is a safe procedure that can be performed on humans and live animals under light sedation. It was first described in humans in 1983[12], shortly before the first transvaginal OPU was successfully achieved in horses in 1987.[13] The procedure is routinely performed in human IVF and veterinary laboratories and clinics for its simplicity and effectiveness. Additionally, this oocyte retrieval method quickly became the preferred procedure due to its superior visualization, finer control, and reduced patient discomfort compared to other techniques.[14]

In sheep, oocyte collection from slaughterhouse ovaries can be common and inexpensive where access to sheep slaughterhouses are available.[15,16] This practice poses a problem regarding the heterogeneity of slaughtered females and the donor's considerable influence on in vitro blastocyst rates.[16–18] Oocytes can only be collected from live sheep by laparotomy or laparoscopy.[8] Laparoscopic ovum pick-up was first described in the ovine species in 1974.[19] Laparoscopy in the sheep allows for visualization of ovaries and oocyte collection by follicle aspiration. Most laparoscopic ovum pick-up is performed on ewes after an ovarian stimulation by multiple follicle stimulating hormone (FSH) injections.[8] Because the number and dimensions of follicles in unstimulated females is not ideal for oocyte retrieval, ovarian stimulation is practiced to enhance the follicular growth and number as well as the competence of collected oocytes.[20] The average recovery of oocytes from ewes is 10 oocytes with an oocyte recovery rate ranging from 35% to 90%.[8,20] The procedure can be safely repeated on the same ewe up to 9–10 times in a year without compromising the number of aspirated follicles, oocyte recovery rates, or quality.[21] Unlike conventional in vivo derived embryo transfer, this technique allows for the utilization of prepubertal lambs.[8]

In pigs, oocytes for in vitro embryo production are primarily sourced from the ovaries of prepubertal gilts after slaughter. These oocytes vary widely in their developmental competence and ensuring they are free from viral pathogens is challenging. There is some evidence that oocytes derived from sows compared to gilts have a higher probability to develop to the blastocyst stage following IVF, and that susceptibility to polyspermy may also be reduced.[9] Exploration of transvaginal OPU in sows has been minimal due to anatomical constraints.[22] The cramped conditions within the porcine pelvis, the short rectal mesentery, and the fact that the uterine horns are coiled with cervical folds make it difficult to reposition the ovaries transrectally in sows, especially younger sows or smaller breeds.[9] Transvaginal OPU in sows has been performed using physical restraint without sedation, but more recent approaches have included sedation and epidural anesthesia to improve recovery.[22] Either way it is crucial in the pig to ensure that the type of oocyte retrieval does not hinder or damage the cumulus oocyte complex (COC) because the cumulous cells are essential for oocyte maturation.[9]

OPU in the cow is most commonly performed via transvaginal follicle aspiration where follicles are visualized by ultrasonic examination of the ovaries. Slaughterhouse ovaries are an alternative commonly used for research. In both scenarios, oocytes are

aspirated from a heterogeneous pool of antral follicles, 2 to 8 mm in size, including follicles from both nonovulatory and ovulatory follicular waves.[23] Transvaginal follicle aspiration of an unstimulated Bos Taurus will typically result in 4 to 5 usable oocytes per session while stimulation with exogenous FSH can increase oocyte recovery rates to 20 per OPU session in some breeds (Holstein).[23] The procedure can be performed as often as two times per week and is safe for pregnant cows in the first 100 days of gestation.[23–25] In demanding OPU routines for cattle, such as biweekly sessions, it's essential to address concerns like animal welfare, repeated use of epidural anesthesia, preservation of ovarian stroma, and risk of adhesions. Such stringent protocols can enhance embryo output from OPU donors. The use of FSH in Bos Indicus cattle remains controversial, with studies showing mixed results. While FSH may boost the number of oocytes collected,[26] some evidence indicates it could impair cleavage and development to blastocyst formation and hatching.[23] However, some highlight positive effects on IVP and pregnancy rates following FSH stimulation.[27] On farms, FSH stimulation and synchronization are typically bypassed for Bos Indicus donors due to their inherent efficiency in producing more oocytes unstimulated compared to Bos Taurus.[23] Use of FSH has shown clear benefits only in Bos Taurus, emphasizing the need to balance financial considerations with biological effectiveness when deciding on these methods. Laparoscopic ovum pickup, as seen in small ruminants, has recently gained interest among commercial IVP companies as a tool to recover oocytes from prepubital calves as early as 2 to 6 months of age.[23,28] This technique could hasten the spread of superior genetics, thereby advancing genetic enhancement through the use of elite genomic young animals. Oocyte collection from slaughterhouse ovaries may be an alternative as more oocytes are recovered from slaughterhouse ovaries than harvested typically through follicular aspiration.[24] While the majority of culled females are not genetically elite, a small subset may be similar genetically to the animals still in the herd, and advancements in electronic animal identification could enable the pinpointing of specific females at slaughter facilities.[24] Current research explores how to control follicular activation and growth and to culture primordial follicles in vitro to a stage where oocytes can be efficiently harvested, potentially further increasing the yield of oocytes from abattoir-derived ovaries.[24]

In the horse, oocytes are most commonly collected from live horses via transvaginal ultrasound-guided aspiration. The transvaginal approach enables serial follicle puncture without preventing mares from subsequently conceiving and producing pregnancies themselves. Recently there has been a shift in the strategy of collecting equine oocytes from preovulatory large follicles (recovery rate of 75 to 85%) to aspirating oocytes from non-dominant smaller follicles.[29,30] Oocytes from dominant follicles have the advantage of natural selection and in vivo physiological maturation, but the oocytes are much more fragile to handle because they are actively in meiosis.[29] Furthermore, the timing for fertilization is less flexible. This poses a problem for most practitioners as there is a limited number of laboratories performing IVP in horses in the United States and thus most oocytes must be transported or shipped long distances. Time is limited and if there is not a local laboratory nearby, the time to ship the oocytes could result in oocytes aging too far past the optimal time for fertilization.[29] Thus, the strategy has now shifted to retrieving multiple immature oocytes from small follicles. Although the recovery rate is lower than mature oocytes, there is the ability to collect a larger number of oocytes from immature follicles and immature oocytes may have a higher chance of producing a blastocyst.[30] Additionally, Immature oocytes can be stored at room temperature overnight before in vitro maturation without negatively impacting maturation rates or blastocyst development. This enables the shipping of oocytes overnight to the limited number of laboratories available.[29] Horses are unique compared to other livestock species in that the COCs are tightly adhered to the follicle wall. Because of this, a double-lumen needle approach was developed in 1998, which

enables flushing fluid through the outer cannula into the follicle while aspirating the fluid out and scraping the follicular wall.[31] This technique caused the recovery rate of immature oocytes to increase significantly.[30] The fluid is kept warm (37°C) throughout the procedure, while the follicle is ideally flushed 8–10 times to maximize oocyte recovery. On average 5–12 oocytes are recovered from a mare in one session depending on operator experience, the number of follicles present, and other mare factors such as age and breed.[32] By using a 12-gauge double-lumen needle with repeated follicle flushing allows an experienced OPU team to achieve oocyte recovery rates from immature follicles between 50% and 70%.[33] Recovery rates can vary significantly across different OPU sessions and may vary between times of year as horses have seasonal cyclicity. Stout (2020) reported that in 552 commercial OPU sessions conducted over two years, there was an average recovery of 12.8 oocytes from approximately 22.2 aspirated follicles, equating to a 57.6% recovery rate.[33] Individual OPU sessions, however, saw a wide range of yields, from 0 to 49 oocytes.[33] Recovery rates can be maintained when the mare is aspirated every two weeks.

Originally, oocyte retrieval in the woman was performed laparoscopically, requiring surgery, general anaesthesia, and hospital admission. Since the early 1980s, transvaginal oocyte retrieval has become the standard.[11] Compared to transabdominal or laparoscopic methods, transvaginal retrieval offers better visualization, a shorter distance from the transducer to the ovary, a high recovery rate of quality oocytes, minimal patient discomfort, reduced risk of intestinal trauma, lower costs for patients, and quick recovery.[11] Additionally, by eliminating the need for laparoscopy, it reduced the number of personnel, time, and costs associated with the procedure and lowered the risks linked to surgery and general anesthesia, leading to higher patient acceptance. OPU procedures in humans transitioned from lengthy, hospital-based operations lasting 1-2 hours to quick 10-15 minute procedures that could be done in an office setting.[14] For outpatient procedures, conscious sedation is preferred due to shorter recovery times compared to general anesthesia and is generally suitable for both patients and operators during OPU.[11] However, patient selection is crucial, as deep sedation may be more appropriate in cases of extreme anxiety, complex pathologies, patients with psychological or social conditions, or previous issues with conscious sedation.[11] Unlike the livestock species, mature oocytes are targeted in the woman. Exogenous FSH is administered to extend the time window for follicular recruitment and encourage the growth of multiple follicles. The growth of large follicles will increase circulating estradiol (E2) concentrations and endometrial thickness.[34] Once follicles have reached mature diameter, typically at least 3 follicles 17 to 18 mm in diameter, human chorionic gonadotropin or recombinant luteinizing hormone is administered to trigger oocyte maturation.[35,36] OPU is performed approximately 36 hours later. Circulating E2 and endometrial thickness can also be used to predict pregnancy rate success but can be affected by age.[34] There is large variability among fertility clinics when it comes to the type of aspiration needle, route of retrieval, aspiration alone or aspiration with follicular flushing, type of flushing medium, and the collecting system.[35] Ovarian stimulation is very important prior to OPU in the woman. Omland et al. (2001) reports that if an unstimulated or natural cycle is used in the beginning of IVF, the likelihood of young women under 35 taking a baby home is only 3.8% and 0% if she is over 43.[37] Thus, in the last decade, different protocols for controlled ovarian hyperstimulation have been investigated to optimize the number, quality and maturity of oocytes. Research suggests that retrieving 15 oocytes optimizes pregnancy chances in a single cycle, with pregnancy rates up to 37%.[38] Optimal results still depend on the quality, specifically aiming for at least 9 mature Metaphase II (MII) oocytes. More is not necessarily better in the woman as collecting more than 16 oocytes could slightly reduce pregnancy rates and significantly increase the risk of ovarian hyperstimulation syndrome.[38] While the ideal range for mature oocytes is considered to be between 11 and 15 for IVF with single embryo transfer strategies, the overriding priority is patient safety and minimizing ovarian hyperstimulation syndrome

risks.[38]

Oocyte Quality

Research indicates that the primary determinant of the percentage of oocytes developing to the blastocyst stage is the inherent quality of the oocyte at the start of the IVP process.[39] Understanding the process that controls oocyte quality is a major challenge facing the field. The process by which mammalian oocytes reach developmental competence involves a complex series of precise, sequential events involving both nuclear and cytoplasmic maturation.[18] Selection parameters across species can include follicle diameter, endocrine concentrations in follicular fluid, and morphological characteristics of the oocyte.

Follicle diameter seems to have a large impact on oocyte quality across species. Pig oocytes that originate from small follicles (< 3 mm) are less likely to mature to MII or develop to the blastocyst stage compared to oocytes derived from medium (3–5 mm) and large (>5 mm) follicles.[40–42] Likewise in sheep, oocytes collected from follicles ≥ 3 mm had higher blastocyst development rates and a higher estradiol and progesterone ratio indicating an improvement of competence compared to those collected from smaller than 3 mm follicles.[8,15,17] On the other hand, oocytes collected from smaller subordinate follicles in the mare can produce up to 1.25 blastocysts per session, whereas oocytes from gonadotropin-stimulated dominant follicles result in only 0.58 blastocysts per session.[29] Studies in cows suggest that oocyte competence increases with follicle diameter, particularly in follicles larger than 8 mm.[43] Low developmental rates in oocytes from smaller follicles may be due to incomplete meiotic or cytoplasmic maturation or from follicles undergoing atresia. Since follicles of the same diameter can be at different stages of the estrous cycle and follicular wave be going through growth or atresia, the health of the follicles is likely more important for oocyte competence than the diameter.[44]

The morphological appearance of the ooplasm is another criterion commonly used to select competent oocytes in livestock.[6] The ooplasm appearance is influenced by lipid content, and lipid droplets act as signaling molecules influencing maturation processes and development competence.[6] Lipid droplets in livestock oocytes can form dark clusters visible as ooplasm darkness. Opacity is most pronounced in pigs, followed by cows, with sheep and goats having lighter cytoplasm.[6] Horses often exhibit lipid polarization, making it easier to visualize spermatozoa within the oocyte. Human oocytes, unlike livestock, have translucent ooplasm, making it easy to observe structures like nuclei and nucleoli, useful for pronuclear scoring. Increased lipid content in human oocytes is associated with lower cryotolerance, indicating lower quality and suitability for transfer.[45] Oocyte size is another marker for competent oocytes. Although it may be challenging to measure the precise diameter of the oocyte during conventional IVF, oocyte selection based on diameter is possible during micromanipulation protocols such as intracytoplasmic sperm injection (ICSI) in humans and horses. In the cow, oocytes can expand in diameter to over 120 µm during the growth phase. Research suggests that the critical diameter for an oocyte to attain developmental competence is 110 µm, corresponding to oocytes associated with 3 mm follicles.[44] Smaller oocytes may not develop fully after fertilization and are more prone to chromosome alterations further impairing development.[44]

Oocyte Maturation

Oocyte maturation involves both nuclear and cytoplasmic transformations within the oocyte. Nuclear maturation includes the progression of meiosis from prophase of the first meiotic division to metaphase of the second meiotic division (MII) during ovulation. Concurrently, cytoplasmic maturation occurs, marked by significant changes in the oocyte's organelles, proteins, and transcripts. Meiosis halts at MII and remains arrested until introduction to sperm cells occur, which then prompts the rapid completion of meiosis and

the expulsion of the second polar body.[46] Nuclear maturation of oocytes to MII is essential for fertilization but it must occur synchronously with cytoplasmic maturation for normal embryo development.[30] The extrusion of the first polar body is an important indicator of oocyte meiotic maturation (MII) during IVP. Identifying markers for oocyte maturation is crucial for micromanipulation procedures like ICSI and somatic cell nuclear transfer. Commonly, the selection of the oocytes is determined by morphological features that are easily assessed with light microscopy. A major advantage of conventional IVF compared to micromanipulation procedures is that fertilization can occur during oocyte and sperm coincubation. The oocyte has a window of time to reach nuclear and cytoplasmic maturity.[6] During micromanipulation procedures, the operator must accurately assess the maturity of the oocyte; however, the criteria used for grading and selecting oocytes vary among researchers and can depend on the expert's evaluation and experience.[6] Procedures and media used for IVM varies not only among species but also within the same species among laboratories. Obviously, the goal for all species is to develop a suitable single medium that combines all of the beneficial factors and components while still being chemically defined and supports both cytoplasmic and nuclear maturation. Aside from the media used in the laboratory, personnel also play a large role in the success of IVM.[47] Personnel alter two crucial practices: skilled handling and selection of oocytes and swift transition from oocyte aspiration to maturation. Experienced personnel not only choose oocytes with better developmental potential but also handle them more quickly reducing time out of the incubator. By minimizing the delay in handling time, desynchronization of the COCs and diminished oocyte quality can be avoided, as physical removal of mammalian COCs from ovarian follicles results in spontaneous resumption of meiosis.[47] Efficient training for beginners and strong teamwork are essential to maintain rapid yet effective handling and enhance IVP outcomes.

IVM conditions for sheep oocytes have not changed significantly in the last 40 years.[8,47] Incubation conditions are generally 5% CO2 and air at 38–39°C in a humidified atmosphere for 20–27 hours.[8,47] Zhu et al. (2018) reports that MII oocytes appear at 16 hours of maturation and that the maturation curve gradually increases from 16 to 24 h of in vitro maturation and reaches the maximal maturation rates of about 80% at approximately 24–26 hours of maturation.[47] Oocytes are usually cultured in groups with 2 to 10 µl of media per 1 oocyte.[48] A typical maturation environment would consist of groups of approximately 50 oocytes incubated in four well plates in 500 µl of maturation medium.[49]

Current IVM protocols for pig oocytes result in a high maturation rate, with 75–85% of oocytes reaching metaphase II (MII) and extruding the first polar body after 40–44 hours.[50] In the pig, the expansion of cumulus cells is not necessary for generating competent oocytes, but their presence is essential. Removing cumulus cells at the onset of maturation or even up to 24 hours afterward hinders the development of swine oocytes to the MII stage and affects their progression to the blastocyst stage following fertilization.[41] Cumulus cells provide a range of functions including supporting oocyte cytoplasmic maturation by allowing metabolite transfer via gap junctions, and by raising intracellular cyclic adenosine monophosphate (cAMP) levels to maintain the oocyte under meiotic arrest.[9] It is beneficial to culture oocytes in groups to share oocyte secreted factors.[51] Immature oocytes are commonly matured in vitro under about 20% O2 concentration with a 5% CO2 incubator, as the use of high oxygen tension for IVM of porcine oocytes has been shown to promote blastocyst formation.[52]

In vitro maturation rates of bovine oocytes completing nuclear maturation is greater than 80%.[24] In proper conditions 85% to 90% of immature bovine oocytes will reach MII.[23] Full developmental competence may not be reached as oocyte cytoplasmic maturation in many cases do not automatically accompany nuclear maturation leading to fertilization and development deficiencies.[23] Bovine oocytes are

commonly matured in groups for 18 to 24 hours in a 5% CO_2 incubator set to 38.5C.[53]

IVM is routinely employed in the mare. Maturation rates of immature oocytes collected from the mare average 50%,[30] but established laboratories achieve higher rates of oocyte maturation with more than 60% reaching the MII stage and being suitable for ICSI.[33] The length of time needed to reach MII differs depending on the morphology of the cumulus cells. Oocytes with compact cumuli may require 30–36 hours to reach MII, whereas oocytes with expanded cumuli require a shortened in vitro maturation period of 22–24 hours.[30] To simplify the maturation protocol and facilitate scheduling, laboratories will typically denude the oocytes after 22–24 hours of maturation and monitor the specific timing of polar body extrusion before ICSI. Studies suggest that nuclear maturation rates do not increase after 30 hours in the mare.[30] COCs are commonly incubated in groups with droplets of medium at a ratio of 10 µl medium per oocyte at 38.2°C.[54] Atmosphere conditions and research on the optimum O_2 tension for IVM has not been conclusive.[55] In practice, IVM is largely performed in atmospheric oxygen tension of about 20 to 21% in a 5% CO_2 incubator.[55]

IVM is still classified as an experimental technique for women and is not the standard practice.[56] Initially, IVM was used in reproductive medicine due to the lack of controlled ovarian stimulation protocols, which made timing ovulation challenging and the collection of mature oocytes from large preovulatory follicles difficult. However, with advancements in controlled ovarian stimulation protocols, the use of in vivo matured oocytes became the preferred method for fertilization in women.[56] IVM is typically reserved for patients who have contraindications to ovarian stimulation, such as those with severe overstimulation syndrome from conditions like polycystic ovary syndrome or those facing hormone dependent cancers who wish to preserve their fertility. IVM could be advantageous in reducing risk of ovarian hyperstimulation syndrome and polycystic ovaries, lower medication costs, reduced stress, monitoring, and labor.

Ovum Fertilization

Unlike IVM, oocytes and spermatozoa both are involved in the IVF process. One important area of research is determining the optimal timing of spermatozoa and oocyte coincubation. Human studies suggest that shorter coincubation time is better because longer times lead to increased polyspermy and poor embryo quality.[57] Still, some studies have reported contradictory results.[58,59] A meta-analysis by Zhang et al. (2013) suggests that brief coincubation (1 to 4 hours) of gametes was associated with significantly higher pregnancy rates, but good quality embryos and polyspermy were not significantly different between brief coincubation and standard insemination of 16 to 24 hours.[60] Anzalone et al. (2021) monitored spermatozoa and oocyte interactions in sheep. They reported that spermatozoa were bound to the zona pellucida in as little as 90 minutes after coincubation.[61] By 240 minutes of coincubation, a spermatozoa was within the oocyte's cytoplasm.[61] The current protocol in most laboratories producing embryos, incubate mature MII oocytes with capacitated spermatozoa overnight.[61] This is more convenient for personnel planning, rather than biological needs. Not only does prolonged exposure potentially lead to polyspermic fertilization, but the presence of millions of decaying spermatozoa in a small volume of medium exposes the oocytes to high concentrations of lytic, acrosomal enzymes, and reactive oxygen species.[61] Additionally, in IVF, success is influenced by both female and male factors. For males, variability includes individual differences, sperm morphology and functionality such as motility and membrane integrity, the source of the sperm—whether from ejaculation or epididymal collection—storage conditions such as fresh versus frozen-thawed, and seasonal variations.[8] Different species, and even individual males within the same species, have varying capacities to induce capacitation.[7] Some additives such as heparin, serum albumin, epinephrine,

penicillamine, hypotaurine, caffeine, bicarbonate, and calcium are known to aid the capacitation of sperm.[7] It is critical to expose mature oocytes to sperm that have been capacitated or are undergoing capacitation. Sperm capacitation induces biochemical modifications that allows the acrosome reaction when exposed to the zona pellucida, cumulus cells or other substances associated with matured oocytes.[62]

ICSI, a more invasive insemination technique compared with standard IVF, involves the injection of a single sperm into the cytoplasm of an egg. ICSI has become one of the most significant advancements in ART technology. Palermo and Van Steirteghem introduced this novel procedure in 1992, where a single spermatozoon could be microinjected into an oocyte after passage through the zona pellucida and the membranes of the oocyte.[63] This technique overcomes capacitation challenges specifically in the horse and has become very efficient for use in human ART. The procedure does however require micromanipulation equipment and more personnel training. The success of the technique varies across species. The success rate of ICSI in cattle (14%) is low compared to the horse (21%), goat (28%) and pig (18%).[64] Although ICSI has been used for over 2 decades, there is still much uncertainty on the implications of altering the process of natural selection of sperm. Yes, ICSI overcomes male infertility, but inheritable causes of male fertility such as constitutional chromosomal aberrations, cystic fibrosis transmembrane conductance regulator gene mutation, or missing genetic material within the Azoospermia Factor (AZF) region on the Y chromosome may be inadvertently passed on to further generations because of ICSI.[14] Additionally, potential damage to the cytoplasmic organelles of oocytes due to the procedure is not fully understood, and the long-term risk to the health of offspring is of concern as ICSI babies are only now reaching their mid-twenties. [14]

The use of frozen semen required adjustments when it came to semen preparation for IVF and ICSI. It was difficult to repeat consistent sperm numbers to coincubate with oocytes, because in frozen semen there is high sperm death and decreased motility and viability. The swim-up method of sperm preparation addressed this challenge. During swim-up procedures, sperm are placed at the bottom of a medium column. The density of the semen in the extender and cryoprotectant keeps the sperm at the bottom initially. Over time, the sperm swim up into the covering medium. Collecting this medium yields a population of nearly 100% motile or viable sperm. This population can then be counted to add a specific number of sperm to the IVF system. Although straightforward in theory, the swim-up technique was challenging for many to execute.[62] A Percoll gradient system optimized for bovine semen was introduced in the late 1980s with many labs for sperm preparation. When Percoll and swim-up methods were compared, a 9%±1 recovery rate was reported with swim-up versus 40%±4 with Percoll.[65] Despite swim-up resulting in higher IVF rates at the same sperm numbers, the higher recovery rate of Percoll made it widely adopted.[62] Increasing the sperm concentration during fertilization compensated for any deficits in Percoll separated sperm.

In sheep, the basic conditions for IVF are co-incubation of spermatozoa and oocytes in the synthetic oviductal fluid medium for 16–24 hours at 38–39°C under low oxygen tension (5% O2) or 5% CO2 in air.[8] High-quality ram semen, when regularly prepared for IVF, should result in fertilization rates of approximately 75%.[8] Specifically in small ruminants, the method of choice for frozen/thawed sperm selection is the Percoll Gradient (45%/90%) due to the greater efficiency in the sperm recovery rate.[49] The final sperm concentration most often used in the IVF drop is 1 to 4 x 10^6 spermatozoa/μl.[48] In small ruminants, in vitro sperm capacitation can be facilitated by treating sperm cells with capacitating agents for 15 to 60 minutes before coculturing with COCs. Key agents include estrus goat or sheep serum (ESS), a combination of penicillamine, hypotaurine, epinephrine (PHE), heparin, and ionomycin. Research suggests that cumulus

cells have a positive impact on IVF outcomes in small ruminants.[49] Whether directly in contact with the oocyte or simply present in the culture well, COCs improved blastocyst production. This suggests that beyond acting as a barrier to prevent polyspermy, cumulus cells may also enhance the interaction between gametes.[49] Currently, ICSI is not highly effective in sheep.[48] Nonetheless, the use of ICSI in sheep offers multiple benefits, such as preventing polyspermy, enabling the selection of superior male germ cells, utilizing sex-sorted spermatozoa, and producing viable offspring from non-motile or subfertile sperm. However, ICSI faces certain challenges, including lower fertilization rates compared to traditional coincubation methods and the need for additional oocyte activation since sperm injection alone often does not suffice.[8] To mitigate these issues, pre-treating sperm cells with detergents has been shown to enhance spontaneous activation and improve blastocyst rates.[8] Additionally in sheep, chemically activating the oocytes is a crucial component of the ICSI process.[48]

In pigs, IVF results in high rates of polyspermy that decreases development to the blastocyst stage. Currently, the occurrence of polyspermy in pig IVP remains one of the biggest and unsolved challenges in the field.[9] Recent studies have focused on reducing the coincubation time from 5 hours to 10 minutes, followed by keeping the oocytes with zona-bound spermatozoa in fresh IVF medium without additional sperm for another 5 hours. This adjustment aims to minimize oocyte exposure to excessive sperm, leading to improved penetration and higher blastocyst development rates.[66] In an attempt to ensure that only motile spermatozoa reach the oocytes and simulate more natural fertilization conditions, several alternative IVF methods have been developed that control the number of spermatozoa that can penetrate oocytes. Methods like the Climbing Over a Wall (COW) method, biomimetic microchannel IVF system, straw IVF, and a modified swim-up method reduce the occurrence of polyspermy, but they don't eliminate it entirely.[50] Generally, due to the difficulties associated with cryopreserving boar semen, most labs prefer to use fresh, extended ejaculates as the source of spermatozoa for IVF.[9] The literature suggests that IVF rates of swine oocytes are approximately 45%.[9] Because polyspermy is such a challenge in pigs, ICSI has become a favorable technique. It is also useful for creating live offspring from nonmotile sperm. However, the success rate of embryo production and the resulting embryo quality are still low. Delays in cleavage and decreased blastocyst formation of approximately 10–20% have been observed in ICSI-derived porcine embryos compared to those produced by IVF.[67] The decreases in development could be due to the selection of damaged sperm and/or incomplete oocyte activation.[41]

Successful fertilization rates of bovine oocytes are greater than 70%[24] when MII bovine oocytes are coincubated with spermatozoa for up to 18 to 24 hours.[23] Low numbers of oocytes from a donor cow can be incubated with sperm in 50 to 100 µl of fertilization media, but mass production of oocytes likely from slaughterhouse ovaries are usually fertilized in batches with approximately 400 µl of media in a well.[23] Spermatozoa undergo washing and selection through swim-up or density gradient centrifugation techniques to remove freezing media, seminal plasma, debris, and dead cells, while enriching for the most motile fraction. These sperm are then exposed to capacitating factors that enable them to penetrate the zona pellucida (ZP) of the oocyte. Although the optimal number of spermatozoa required per oocyte varies widely between bulls and breeds, a concentration of 1 to 2 million spermatozoa per ml is generally used for IVF. Fertilization rates after ICSI are extremely low in cattle.[68] This could potentially be due to a failure in pronuclear formation without supplemental activation or due to physical disruption from the ICSI procedure.[68]

Developing an efficient IVF protocol for horses is challenging due to the lack of information on sperm capacitation.[69] Equine sperm may require a longer time to achieve

capacitation compared to other species, but this is not well understood.[70] Studying equine sperm capacitation is further complicated because sperm motility decreases quickly in culture, influenced by the medium and the container used.[71] Conventional IVF is not offered commercially in the horse, rather ICSI is the approach for research and in the field. The ability to optimize the use of limited stores of frozen stallion semen has stimulated worldwide interest.[30] Hundreds of IVP blastocysts can be produced from a single straw of frozen semen, because ICSI allows semen to be used very efficiently.[72]

The utilization of ICSI in human IVF is used to overcome the low and unpredictable fertilization rates encountered with conventional IVF.[73] This technique was developed to address the low and unpredictable fertilization rates often seen with conventional IVF in cases of poor sperm quality. ICSI initially achieved fertilization rates of approximately 60%–70% when using ejaculated sperm, a rate equal to fertilization rates experienced by men with normal fertility using conventional methods.[14] The first ICSI baby was born in Belgium in 1992.[74] Over the past two decades, the use of ICSI has surged globally. In several countries, including nearly 100% of cases in the Middle East, ICSI has become the dominant method.[73] In the United States, ICSI usage grew from 36% in 1996 to 76% in 2012.[73] The advent of ICSI has become the prevalent choice for fertilization in human ART.[61] Although, IVF is a more physiological method of embryo production that guarantees a more natural spermatozoa selection for fertilization, ICSI also overcomes many male fertility challenges.[61]

Embryo Culture

Every component of the culture system is meant to optimally support the metabolic needs of the preimplantation embryo by attempting to replicate the in vivo environment.[75] The culture component is perhaps the most critical step in the process because: 1) it occurs over many days, 2) there are multiple physiological processes that take place during culture, and 3) there are multiple in vivo environments that must be mimicked correctly in vitro for culture to be successful. Embryos undergo cleavage, compaction, and cavitation, but on a molecular level, changes are more complex and not fully understood.[49] Three different methods of embryo culture have been applied to the animal species: presumptive zygotes can be transferred and cultured temporarily in recipients and subsequently recovered, cocultured in vitro with oviductal or other cells, or cultured in vitro with semi-defined or defined media.[8] Transfer of zygotes to a recipient, results in optimal embryo quality because of the optimal conditions provided by the physiological environment in the oviducts, but it involves a high number of animals and is technically challenging and labor intensive. Cultured oviduct cells may aid in advancing embryo development to the blastocyst stage by releasing embryotropic factors into the culture medium and possibly removing harmful substances.[8] The coculture, approach, however, leads to unknown exposure of the embryos and could lead to the spread of pathogens. Ideally, in vitro culture media would be fully defined and optimized to yield the highest blastocyst development across species. In vitro culture conditions affect not only short-term development, such as cleavage rates, blastocyst rates, and the average cell number per blastocyst, but also have long-term effects on pregnancy rates, fetal development, birth weight, and the overall health and size of offspring following embryo transfer. Continuous optimization of culture media for IVP embryos is essential to bring the quality of IVP embryos and offspring closer to that of their in vivo counterparts.

Many different factors and environmental conditions can impact the success of in vitro culture. Currently, the quality and quantity of embryos and the success rates are still not satisfactory and greatly lag behind the theoretically possible success rate.[76] While the female reproductive system can protect oocytes, sperm cells, and embryos from potential environmental damage, currently labs cannot completely control the environmental effect. Light is one environmental factor that impacts embryo development. Gametes,

zygotes, and embryos are exposed to a variable spectrum of light from different sources, such as ambient light, illumination of the safety cabinet, or light emitted from the microscope.[76] Light exposure occurs during oocyte retrieval, sperm preparation, fertilization checks, morphological assessment, and embryo transfer. Harmful effects of light are suggested to be associated with the generation of the embryo toxin hydrogen peroxide.[77] Additionally, cellular flavins that absorb light are likely responsible for the generation of reactive oxygen species, which can cause changes in the membrane redox state leading to membrane channel opening and together with hydrogen peroxide can cause mitochondrial dysfunction and cellular damage.[76,78] Light may also activate stress genes and damage DNA via ionization.[76] Being intentional about the wavelengths of light embryos are exposed to may be beneficial for development. Red light can be used in incubators and has been shown not to decrease the development or quality of mouse zygotes or parthenogenic-activated porcine embryos.[79] Because the biological effect of light is wavelength-dependent, filters may be used to reduce the harmful effects.[76]

Environmental gas is another environmental factor that impacts embryo development. Oxygen is an essential component of this system as it plays a central role in cellular respiration which strongly influences energy production,[75] but high oxygen concentrations cause the generation of reactive oxygen species that can cause damage to the cells and disrupt embryonic development.[80] Steptoe et al. (1971) were the first scientists to report the culture of human embryos to the blastocyst stage, and the oxygen tension in their culture system did not utilize atmospheric O2 of approximately 21%.[81] They described their gas mixture as 5% oxygen, 5% carbon dioxide, and 90% nitrogen.[81] Studies have described the in vivo oxygen tension in the female reproductive tract of mammalian species as ranging from 2 to 8%.[75] Oxygen levels in the uterus may be even lower.[80] This low oxygen range has also been confirmed in humans.[80] For IVF programs performing blastocyst transfers, a key question is whether the oxygen concentration in the female reproductive tract remains consistent as the embryo moves from the oviduct to the uterus. Overall, the literature supports that embryo culture at elevated O2 concentrations impairs blastocyst development, cell number, and embryo metabolism in a variety of species.[82] Likewise, culture at low oxygen pressure of around 5 % has been shown to aid embryo development and quality in sheep,[47] pigs,[41] cows,[44] horses,[30] and woman.[73] Developing embryos across livestock species are most commonly cultured at 38.5 °C in 5% O2, 5% CO2, and 90% N2 in a fully humidified atmosphere.

Optimizing culture media is another key to increasing development. Commercialization of culture media production has been beneficial to the industry and has increased quality control and provided more consistent outcomes, but there does not appear to be one superior formula in any species.[83] Different species provide clues and insight for culture media of other embryos; however, embryos from each species exhibit unique metabolic characteristics that must be considered when formulating media.[41] In an effort to mimic an environment similar to the oviduct or uterus, coculture with different somatic cells or the medium conditioned by these cells has been utilized for embryo culture. Somatic cells supply unknown factors that promote embryo growth or remove toxic factors from the basic medium.[84] Multiple embryokines have been identified that may aid IVP embryo development. These regulatory molecules are secreted by oviduct and endometrium cells and can influence specific developmental stages of the embryo. Embryokines are usually absent in in vitro culture mediums unless serum is added or embryos are cocultured with somatic cells that produce these specific embryokines.[24] The bovine morula has 175 receptor genes identified showing the magnitude of the different types of embryokines that may be important for optimal development.[85] Embryos may be able to share embryokines with other embryos in the same culture environment, and there has been shown to be a benefit of coculturing embryos with unfertilized oocytes and frozen-thawed

blastocysts.[86] Another approach to mimic the in vivo environment for embryo culture is synthetic oviductal fluid and human tubal fluid media based on the composition of sheep and human oviductal fluid, respectively. These media have been effective for the in vitro culture of preimplantation sheep and cattle embryos, as well as mouse and human embryos.[84] However, the composition of inorganic elements and energy substrates in the mammalian oviduct varies among species.[84] Fetal bovine serum (FBS) was once a popular additive that increased IVP embryo production but has since been linked to compromised cryotolerance and inducing large offspring syndrome. Serum replacements such as bovine serum albumin are another popular additive; however, there are many different preparations and options that may have resulted in different (or no) effects.[23] A chemically defined medium for oocyte maturation, fertilization, and embryo culture allows for precise analysis of the effects of substances like inorganic compounds, energy substrates, hormones, cytokines, and vitamins. This medium avoids the undefined factors found in biological materials like serum, improving the reliability and reproducibility of results and enabling testing of specific supplements.[84] It also ensures biosafety by eliminating protein preparations that might be contaminated by pathogens. Additionally, a chemically defined medium can help optimize the IVP system and increase the survival rate of embryos after transfer, making it beneficial for both research and commercial applications.[84] Both continuous and sequential culture systems have been utilized across species. The concept of sequential media, developed to address the evolving needs of an embryo during development, involves using different media tailored to the specific stages of growth based on the changing location the embryo undergoes from the oviduct to the uterus. In contrast, a monoculture medium system or continuous uses a single formulation designed to support the embryo to the blastocyst stage, allowing the embryo to selectively uptake the required nutrients. Both sequential and monoculture systems have been found to be effective, but monoculture systems may benefit from fewer pH and temperature fluctuations and less handling outside of the incubator.[23]

Embryos can be cultured singly with one embryo per media drop, or in groups with several embryos in the same media drop. The number of embryos cultured together or whether they are cultured individually is determined by each individual laboratory, often for historical or practical reasons. Humans, horses, and cows are primarily mono-ovulatory species. Therefore, ovulated ova undergo fertilization and development without another ovum nearby. These single embryos may secrete autocrine embryotropic factors to modify their own environment, but do not require companion embryos to develop in vivo.[83] The need for nearby companion embryos is more logical in poly-ovulatory species such as the pig. However, it has been well established that group culture increases blastocyst development for cows,[87–89] sheep,[47,90] pigs,[91] and humans.[92] Most laboratories that produce sheep, cow, and pig embryos carry out culture in groups.[48,91] Group size may be dependent on the recovery from the female donor if a single sire is selected for all. It has been suggested that the group effect may have less to do with surrounding embryos and more to do with the culture volume and density.[93] Embryos develop in vivo within the confined microenvironment of the oviduct and uterus, and highly concentrated maternal and embryo-derived factors promote preimplantation embryo development.[93] In vitro embryo development has been suggested to increase when embryos are cultured in groups due to the increased concentrations of the autocrine embryo secretions or embryotropins.[89,93] Herreros et al. (2024) reported a significant increase in blastocyst formation and usable embryos in individual culture on Day 5 compared to group culture in human embryos.[94] Currently, in humans, it is preferred to transfer a single human embryo. The application of non-invasive embryo quality markers is used to select and transfer a single euploid and competent blastocyst.[93] Because of this approach, the development of individual embryo culture systems is important to ensure an actual embryo’s individuality and allow for the application of selection methods.[93]

The length of culture varies among

species, and labs depending on the ideal time for transfer or cryopreservation. Presumptive ovine zygotes can be cultured in vitro with two different approaches. One approach cultures embryos up to 2 or 3 days and then transfers them into the oviduct of recipients.[8] Alternatively, the more common method is to culture in vitro for 6 to 8 days up to the blastocyst stage before transferring.[8] Cultures of 6 to 8 days for sheep embryos will result in fully expanded 100-cell blastocysts that developed from a single cell. Usually in sheep, the cleavage rate is evaluated 48 hours after fertilization, and blastocysts are evaluated on Days 6, 7, or 8 of culture.[49] In pigs, it is typical to transfer morula and blastocyst stage embryos into a surrogate gilt on Days 3, 4, or 5 after standing estrus.[95] The synchronization strategy of transferring into a recipient whose synchrony is at least 24 hours behind the embryonic age accounts for the developmental delay of IVP embryos.[95] More than 30 swine embryos can be deposited in the oviduct or uterus at transfer.[96] Compacting swine morulae develop about Day 5 and hatching blastocysts on Day 7. IVP bovine embryos are cultured for 7 to 8 days with blastocysts most frequently transferred to recipient females at a comparable stage after ovulation which is typically Day 7.[24] Identifying and grading equine IVP embryos can be challenging because morphological features can look different compared to in vivo embryos. It is possible to confirm a trophoblast layer through a microscope but only staining for nuclei can confirm cell number to define a blastocyst with >64 cells.[30] Expanded blastocysts with a thin trophoblast layer and a distinct inner cell mass, such as in vivo produced blastocysts, cannot be obtained in vitro.[56] Additionally, degenerate oocytes can expand with vacuoles easily mistaken for a blastocoelic cavity. Fragmentation after ICSI is also a normal occurrence and is mistaken for cleavage or parthenogenesis.[30] Equine embryos after ICSI may take 6 to 10 days to reach the blastocyst stage. Because of this wide range, it is challenging to synchronize a recipient mare for fresh transfer. Recipient mares are ideally 5 days after ovulation, though ICSI embryos ready to transfer are approximately equivalent developmentally to Day 6 in vivo embryos.[33] Thus, it is much more common to cryopreserve equine ICSI embryos to transfer at a later time. In humans, transferring blastocyst-stage embryos has resulted in higher pregnancy rates and may limit the risk of multiple gestations.[97,98] Human embryos usually reach the blastocyst stage by Day 5 or 6 after insemination.[99]

Cryopreservation of embryos enables wider propagation of valuable genes and facilitates the distribution of superior genotypes with a consequent reduction of shipping costs of live animals. This vital technology aids in the preservation of endangered species or breeds.[8] Vitrification has currently been a successful alternative to traditional slow-freezing procedures. Vitrification is a cryopreservation method that involves the treatment of embryos with high concentrations of cryoprotectants combined with very rapid cooling achieved by direct plunging in liquid nitrogen. Thus, ice crystals form around but not within the embryo.[50] In sheep and pigs, vitrification is the preferred method for IVP embryos as the viability rate of vitrified morulae and blastocysts is significantly higher compared with embryos cryopreserved by slow-freezing techniques.[8,50] Additionally, when comparing vitrification with slow freezing, vitrification does not require special equipment, reduces labor time, and is more cost-effective, making it ideal for routine use in the field.[8] While the vitrification process if more convenient, the thawing process of vitrified embryos requires cryoprotectant dilution steps in pigs, cows, horses, and humans. In comparison, slow-freezing cow embryos in ethylene glycol can be directly transferred upon thawing, with no additional media, or post-thaw processing, making the thawing and transfer process more convenient in the field. Direct transfer of vitrified embryos may be possible in sheep embryos; however, the cryopreservation and transfer vessels are not likely the same.[100] Most IVP sheep embryos are vitrified at the blastocyst stage as re-compacted and compacted morulae, are more sensitive to vitrification procedures.[8] Swine IVP embryos face challenges in cryopreservation due to their high lipid content. Success has been achieved

after centrifugation of the early embryo and removal of the lipids microsurgically prior to culture to the blastocyst stage and subsequent cryopreservation.[95] Still, cryopreservation of pig embryos has not been widely repeatable, and the industry has not adopted the transfer of frozen embryos as a method of improving or moving genetics.[41] The most popular freezing method for IVP bovine embryos is vitrification; however, slow freezing is still practiced.[23] Selection of the most viable embryos that tolerate the cryopreservation process for direct transfer, achieve the highest pregnancy rates.[23] Cryopreservation is highly utilized by the cattle industry as around half of all bovine embryos transferred in 2022 were cryopreserved.[101] In the horse, IVP embryos tolerate cryopreservation better than large in vivo, flushed embryos due to their small size, minimal blastocoele fluid content, and the absence of a confluent embryonic capsule.[33] The most common cryopreservation technique for equine embryos is vitrification.[102] Cryopreservation of IVP embryos with negligible loss of viability has allowed the development of international trade in embryos and enables much more efficient use of recipient mares.[33] Cryopreservation of embryos in human IVF was much instrumental because with the improvement of ART a frequent surplus of human IVP embryos beyond what was needed for initial IVF treatments accumulated. Initially, excess embryos were discarded, donated to other couples, or used for research. The first human pregnancy from a frozen embryo occurred in 1983, despite ending prematurely.[14] Over time, advancements in cryopreservation techniques and cryoprotective agents improved the survival of human embryos and pregnancy rates. By the late 1980s, about 50% of human embryos survived freezing and thawing, with a pregnancy rate of 13.4% per embryo transfer.[14] By 2003, frozen embryo transfers made up 17.8% of all IVF cycles in the US, with a live birth rate of 27.0% per transfer, marking significant progress in the field.[14] Both slow-freezing and vitrification are routinely used to cryopreserve human embryos and whether one technique is superior to the other, is still a matter of controversy.[103] However, Meta-analyses have found evidence that vitrification may be superior to slow freezing based on a direct comparison of embryo survival and clinical pregnancy rates.[103,104]

Success Rates

Since the beginning of ART and IVF, defining the success rate has been a challenge. The most obvious endpoint should be the delivery rate of live offspring which can be calculated in a variety of ways considering the substantial variability. While the numerator, the number of live offspring, is clear, the denominator is less obvious. The rate can be calculated based on the number of cycle starts, the number of oocytes retrieved, or the number of transfers. This variability causes inconsistency in how the success of IVF is reported.[105] Although the ideal endpoints for the success of an IVP embryo are gestation, birth, and continuing health of the resulting offspring, ethical problems arise in conducting research with these endpoints in human and animal models. Not only are they cost-prohibitive and timely, but it also takes large numbers to achieve statistical power. Some common endpoints utilized to determine success in research are nuclear maturation success, fertilization rates, the kinetics of embryo development, development to the blastocyst stage, blastocyst total cell number, inner cell mass, and trophectoderm cell numbers, oocyte and embryo metabolism, ATP content, mRNA and proteins storage, and mitochondrial activity and distribution.[18] Most commonly, blastocyst development rates are an effective measure to indicate IVF success. In the sheep, the overall efficiency of in vitro embryo production, in terms of blastocyst rates, ranges from 15% to 79%.[47] The range of success is so large because of the differences among experiments and laboratory procedures, depending on the oocyte source, age of the donor, culture conditions, reproductive status, the number of embryos transferred, and genetic background.[47] In vitro culture of porcine embryos is suboptimal, with about 30 to 40% of presumptive fertilized oocytes developing to the blastocyst stage for embryo transfer.[9,41] Additionally, cells in the blastocyst stage embryos are fewer in number

compared to blastocysts that develop in vivo.[41] Even though the first IVP porcine embryo was reported in 1986, pigs have lower success rates and little advancement over time compared to other model organisms, mainly due to insufficient synchronization of nuclear and cytoplasmic maturation and high rates of polyspermy.[106] In the cow, generally 20% to 40% of cultured presumptive zygotes will reach the blastocyst stage.[23,107] More information is vitally needed in the cow to determine real oocyte competence and subsequent factors affecting developmental competence, such as its ability to fuse with sperm, pronuclei formation, initiation of cell division, compaction and inner cell allocation, and blastulation.[23] Embryo transfer offers a way to circumvent common causes of pregnancy failure, such as incorrect estrous detection, anovulation, fertilization failure, and early embryonic death before Day 7 of pregnancy. For this reason, it is logical to expect higher pregnancy rates from transferring IVP embryos compared to artificial or natural insemination. Unfortunately, the success rate of pregnancy from IVP embryo transfers does not surpass that of artificial insemination.[107] Only in cases where fertility from artificial insemination is low, such as under heat stress or in repeat breeder cows may IVP embryos yield higher success in the cow. Blastocyst rates are even lower in the mare, with 25% to 35% of in vitro matured oocytes developing into blastocysts.[108] Laboratories across the United States and Europe routinely expect an average of about 2 blastocysts per successful ICSI session for most mares.[109] Initial pregnancy rates of IVP equine embryos range between 55% and 80% and high pregnancy rates are achievable regardless of whether the embryos are transferred fresh or after cryopreservation by vitrification or slow freezing.[33] Early pregnancy losses are generally higher for IVP embryos for a number of possible reasons causing compromised developmental competence.[33] This is also seen as slower development and a higher percentage of apoptotic cells than for in vivo-derived embryos. Human IVF reports a large range of blastocyst development. Variation in rates can be due to many factors, including lab and culture differences as well as differences in patient populations.[110] When looking for common practices between human IVF labs with high success rates in the United States, Van Voorhis et. al. (2010) found great variability and a lack of consensus regarding many aspects of IVF practice, even among high-performing centers.[111] IVF is a complex medical treatment with multiple physiological and sometimes psychological variables contributing to the outcome of a successful pregnancy and birth.[111]

The efficient production of IVP embryos is incremental and depends upon the success of all steps of the process. While some may focus primarily on the culture system, research by Rizos et al. (2002) suggests that optimal blastocyst development relies on periods of oocyte maturation and, to a lesser extent, fertilization.[112] When bovine oocyte maturation and fertilization occurred in vivo, 74% of oocytes became blastocysts. Even if oocytes were matured in vivo and IVF was performed, blastocyst rates were still 58 to 78%. However, embryos produced by in vitro maturation and fertilization did not show increased blastocyst rates when placed in the sheep oviduct (35%) compared to in vitro culture (34%).[112] Similar findings by Gad et al. (2012) and studies on mice, pigs, and rhesus monkeys underscore the crucial role of oocyte maturation and fertilization in establishing embryonic competence.[24,113]

The timing of cleavage or cell division during early embryogenesis seems to be a critical parameter for predicting subsequent developmental ability.[67] Previous studies in humans,[114,115] pigs,[67,116] and cows[117] have shown that a higher proportion of embryos with earlier cleavage reach the blastocyst stage than those with later cleavage. In sheep, the presence of more than 50% cleaved embryos within the first 24 to 30 hours of culture is a good indicator of oocyte developmental competence.[8] Slow cleavage leads to developmental arrest usually before the genomic activation, 8–16 cell stage, or at the compacted morula stage.[8] The “8 to 16 cell developmental block” is often observed in ruminant IVP embryos.[49] In pigs the same block

is observed at the 4-cell stage. Early swine embryos could be cultured before and after this critical stage while retaining viability but could not be cultured through it and maintain viability.[41] It was not until the 1990s that swine embryos were cultured from zygotes to blastocyst.[41] All mammalian species experience a developmental block during pre-implantation, often due to suboptimal in vitro culture conditions that fail to meet the embryo's dynamic metabolic and molecular needs.[118] This block can be mitigated by optimizing culture media to better mimic in vivo conditions, including adjusting nutrient compositions and supplementing factors that support mitochondrial function, energy metabolism, and gene expression critical for continued development.[118] Further, this block is likely partially responsible for the modest pregnancy rates following nonmanipulated insemination in a variety of species.

Implications for Models

Advances in animal ART have significantly contributed to human infertility treatments across four generations of biotechnologies.[3] The first generation, artificial insemination, involved collecting males and depositing semen into female reproductive tracts. The second generation, embryo transfer, enabled the transferring of embryos from donor to recipient females. The third generation, in vitro fertilization, allowed fertilization and embryo culture outside the body. The fourth generation currently includes cloning and transgenesis. To date all rely on a surrogate recipient to carry the embryos to term. Each generation was built on the previous one and all initially performed in animals before humans. The knowledge and techniques developed in animal reproduction have opened new avenues for understanding and addressing human reproductive challenges. With species comparative knowledge, the ability to understand the mechanisms behind in vitro oocyte maturation, fertilization, and early embryonic development will increase.[7] As large mammals, livestock have aspects of their embryo cell biology that are much closer in similarity to humans than the classical mouse model for fundamental biological studies. These sources of embryos are more effective as a model to improve media, culture conditions and standard operating procedures when ethical issues preclude direct experimentation on human embryos.[9] Animal models are increasingly being used to test the safety and effectiveness of new technologies, such as mitochondrial replacement therapy and gene editing. However, despite their importance, it's crucial to recognize that they don't fully replicate human biology, so results from these models must be interpreted with caution.[3]

Sheep are an excellent model for studying the underlying mechanisms of pregnancy and for developing preventative and treatment options with translational potential to humans.[119] Their basic anatomy, physiology, and manageable size allow for the insertion of physiological monitoring and sampling devices during gestation without compromising the pregnancy or causing undue stress. Unlike cattle, which are less resilient to such invasive procedures, sheep can maintain pregnancies even while being monitored or having blood and gestational fluids sampled. This resilience makes pregnant sheep particularly valuable for developing intrauterine gestational restriction models and for studying prenatal programming and parturition processes. Additionally, pregnant sheep and catheterized fetal sheep have been used extensively to investigate both normal and abnormal fetal development, as well as to explore in utero treatment options for congenital birth defects under experimental conditions that would be impossible to study in human fetuses.[120] However, one significant difference between sheep and humans is that sheep are poly-ovulatory, whereas women are mono-ovulatory, posing challenges for understanding follicle development, dominant follicle selection, and oocyte maturation.

Pigs and humans have anatomical and physiological similarities that make the pig an acceptable model for the human. Pigs are widely utilized in biomedical research as many of their organs are of similar size and function as human organs. Reproductively, the pig is a litter bearing species with a shorter generation

interval, making it possible for trans-generational questions to be answered in shorter timeframes.[119] Production and manipulation of porcine embryos in vitro is crucial for advancements in human medicine.[41] Genetically modified swine models act as powerful tools for studying genetic diseases in humans, such as cystic fibrosis, phenylketonuria, and for xenotransplantation.[41] Production of genetically modified pigs are sources of cells, tissues, or organs for xenotransplantation and may be an important donor for human transplant tissues because many pig organs and tissues are similar to humans and pig cells can be genetically engineered to overcome issues associated with the risk of transmission of zoonotic pathogens or rejection of transplant tissues.[120]

The bovine model is also valuable in understanding reproductive disorders in women. One similarity lies with the secretion of hormones during the estrous cycle in cattle and the menstrual cycle in women and both species have multiple follicular waves that result in ovulation of a single ovarian follicle.[119] The size of ovarian follicles are similar and similar reproductive pathological conditions can occur in both species. With their shared folliculogenesis characteristics, human ovarian stimulation has become reflection of the bovine model and now human ovarian stimulation is a key aspect of reproductive management.[121] The bovine model is best for investigating late folliculogenesis events that are linked to oocyte competence, and can be used as both in vivo and in vitro models.[121] The bovine is also an advantageous model to study in vitro embryo development because of the large supply of bovine oocytes obtained from either slaughterhouse material or ovum pick up procedures. The wide availability of oocytes makes it possible to produce embryos in large and small quantities.[62] Also, cows can be maintained in large groups of genetically similar females under consistent environmental conditions to reduce sources of phenotypic variation. Thus, cattle are valuable models to elucidate the endocrine and local mechanisms controlling both early and terminal stages of follicular development in mono-ovulatory species.[119] Bovine reproduction has been extensively studied for its commercial significance and the lack of ethical constraints that limit experimental studies on human embryos.[122]

Horses serve as valuable models for human reproductive technology due to their similar reproductive physiology and the parallels in certain aspects of their reproductive processes. Horses and humans have similarities in their reproduction cycles, with the length and hormonal regulation being notably similar, making them ideal for studying ovarian function and hormonal therapies.[56] Uniquely, horses share similar challenges with humans in terms of fertility issues, such as age-related declines in reproductive efficiency, providing a relevant model for investigating treatments aimed at enhancing reproductive success in aged females.[56] In humans, it is well established that factors such as advanced maternal age, obesity, excessive exercise, a sedentary lifestyle, alcohol, and tobacco use have a detrimental impact on fertility, often leading to longer times to conceive. Similarly, in horses, certain maternal conditions, including aging, obesity, and intense athletic activity, are also recognized as problematic for fertility.[56]

In reproductive medicine, animal models are essential in enhancing our understanding and leading in the advancement of next generation reproductive technologies. As the industry enters a new era defined by revolutionary tools such as CRISPR/Cas9 and cutting-edge technologies, the importance of these models is amplified. They serve as essential frameworks for studying complex physiological processes and for rigorously testing the safety and efficacy of new interventions, effectively bridging the gap between laboratory breakthroughs and clinical applications.

Conclusion

ART, especially IVP, not only provides solutions for infertility across species but also

addresses broader One Health challenges, such as promoting environmental sustainability and enabling more efficient food production. This positions IVP as a critical technology at the intersection of these fields, emphasizing the interconnectedness of animal, human, and environmental health. From an animal health perspective, IVP technology not only improves breeding efficiency and genetic dissemination in livestock, but also offers insights and innovations that are directly applicable to human fertility treatments. From a human health perspective, the refinement of IVP protocols in animals, particularly cattle, sheep, pigs, and horses, continues to serve as a critical research foundation for optimizing human ART. Animal models allow researchers to test new approaches, media, plasticware, etc. in a controlled environment before implementing them in clinical settings, reducing risks and improving the outcomes for humans. The comparative analysis of oocyte collection, maturation, fertilization, and embryo culture across species in this review underscores the importance of shared knowledge and innovations between veterinary and human medicine. Valuable insights among species guide the refinement of protocols to increase the success of these technologies. Furthermore, as global demands for animal products rise, IVP allows for increased genetic progress, producing more productive animals with fewer resources thus positively impacting the environment. By decreasing the number of animals required for efficient production and reducing resource consumption, these technologies may also help mitigate livestock's impact on climate change. This enhanced efficiency in livestock production not only alleviates the pressure on natural ecosystems but also contributes to the broader goal of reducing the environmental footprint of agriculture. Additionally, IVP of embryos provides a powerful tool for the conservation of endangered species by enabling the reproduction of animals that may face natural or logistical barriers to breeding.[123] This approach not only helps to increase population numbers but also preserves genetic diversity, which is essential for the resilience of endangered species. IVP contributes to the maintenance of complex ecosystems, safeguarding biodiversity and ecological balance for future generations.[123] Thus, IVP is a tool for creating a more sustainable food production system, balancing the need for agricultural productivity with environmental conservation.

In summary, IVP of embryos stands at the intersection of human, animal and environmental health. The knowledge learned from animal ART research have broad impacts, enabling advancements in human fertility treatments while simultaneously enhancing the efficiency of livestock production. Although the first IVF baby was born in 1978, Sir Edward Robert, credited with developing IVF, did not receive the Nobel Prize until 2010, as researchers awaited initial health data on IVF-conceived offspring.[124] Despite this milestone, the long-term health effects of IVF remain uncertain, especially regarding potential impacts on both the offspring and future generations. Although IVF technology has advanced significantly, further research is needed to optimize techniques and understand long-term health outcomes in IVF offspring. Viewed through a One Health lens, it is clear that improving IVP techniques benefits not only individual species but also contributes to global health.

Species	In Vitro Maturation Time (hours)	In Vitro Maturation Rate (%)	Semen and COC Coincubation Time (hours)	In Vitro Fertilization Rate (%)	Time of In Vitro Culture to Blastocyst Development (days)	Blastocyst Rate of IVP Embryos (%)
Sheep	20-27[8]	80[47]	16-24[8]	75[8]	6-8	15-79[47]
Pig	40-44[50]	75-85[50]	5[125]	45[9]	5	30-40[41]
Cow	18-24[53]	85-90[24]	18-24[24]	70[24]	7-8	20-40[23]
Horse	24-36[30]	50-60[30]	ICSI	>65[33]	6-10	25-35[108]
Human	In Vivo	In Vivo	ICSI	60-70[14]	5-6	High Variation[110]

Table 1. In vitro maturation time, maturation rate, coincubation time, fertilization rate, time to blastocyst development, blastocyst rate of sheep, pig, cow, horse, and human.

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Affiliations:
Texas Tech University School of Veterinary Medicine, Amarillo, Texas