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Equine Science

Applications and Implications of New Technologies

Edited by Juan Carlos Gardón Poggi and Katy Satué Ambrojo

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Meet the editors

Dr. Juan Carlos Gardón is a professor in the Department of Animal Medicine and Surgery at Catholic University of Valencia San Vicente Mártir, Spain. He develops his academic career in the areas of animal physiology, animal reproduction, and reproductive biotechnology. He is also involved in postgraduate activities at the Faculty of Veterinary Medicine, University of Murcia, Spain. His areas of research include animal physiology, reproductive biotechnology in both males and females, the study of gametes under in vitro conditions, and the use of ultrasound as a complement to physiological studies and the development of applied biotechnologies. Dr. Gardón has published two books and eighteen book chapters and has published fifty articles. He regularly supervises master's and Ph.D. students.

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Contents

Preface

Equine science has been a dynamic and constantly evolving field, deeply rooted in our shared fascination and admiration for horses. Over the centuries, humans have forged an inseparable bond with these majestic animals, relying on their strength, agility, and companionship for countless purposes. Today, during the technological revolution, we find ourselves at the crossroads of tradition and innovation, eager to explore the endless possibilities that new technologies offer to the world of equine science.

Equine Science – Applications and Implications of New Technologies is a guide to and exploration of technological advances and their impact on the equine world. It delves into the integration of science, technology, and horsemanship, offering insight into how these synergies may shape the future of our interactions with horses.

Advances in diverse fields such as reproduction, biomechanics, nutrition, veterinary medicine, and training techniques have accelerated our understanding of equine biology and behavior. In addition, advances in information technology, data analysis, and artificial intelligence have opened a new range of possibilities for improving the welfare, performance, and overall management of horses.

In this book, we bring together a diverse group of experts, researchers, and practitioners who share practical applications as well as the ethical and social implications of these advances. From the fascinating world of horse history, reproductive biotechnologies, and the interaction of horses and humans with new technologies to the study of gestational endocrinology and the application of mass spectrophotometry to study drug metabolism and kinetics, this book explores the transformative potential of technology to improve our understanding of horses and optimize their welfare.

As we embark on this journey through the applications and implications of new technologies in equine science, it is crucial to approach these advances with thoughtful reflection. We must strike a delicate balance between progress and tradition, ensuring that innovation conforms to the principles of equine welfare, ethical practices, and sustainable management. Therefore, we hope that this book will serve as a source of

inspiration, knowledge, and reflection for scientists, researchers, professionals, and horse enthusiasts alike.

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Chapter 1

History of Horses and the Biotechnologies Applied to Its Reproduction

Juan Carlos Gardón Poggi and Katy Satué Ambrojo

Abstract

The history and domestication of horses date back to thousands of years, making these animals an integral part of human life. Over time, selective crossbreeding led to the creation of different breeds with specific characteristics that made them suitable for various tasks. The advent of modern technology, such as the automobile and the railroad, reduced the dependence on horses as a means of transportation, but their importance persists in sports, recreational, and therapeutic activities. The special interest in this animal species and the need to improve its qualities have led to the development of different reproductive technologies. In recent decades, significant advances have been made, providing new opportunities to improve equine breeding and preserve valuable genetic lines. Artificial insemination, embryo transfer, in vitro fertilization, and gamete freezing, among others, have revolutionized the equine industry by facilitating selective breeding, genetic preservation, and improvement of animal quality. In this chapter, we present a historical description of the evolution of the horse and at the same time a review of the most relevant aspects of its domestication. Likewise, we make a review and description of the reproductive technologies that have marked an important advance in the knowledge of the physiology and reproductive improvement in this animal species.

Keywords: equine, history, domestication, reproductive technologies, evolution

1. Introduction

The history of horses can be traced back to over 50 million years ago when they first evolved in North America. According to a study published by Prothero in 2017 [1], horses' evolution can be traced through their teeth, and they found that the earliest known horse, called Hyracotherium or Eohippus, was about the size of a small dog and had four toes on its front feet and three toes on its back feet. Over time, horses evolved into larger and more powerful animals with a single toe on each foot.

Evolution in equids is not represented linearly, but in a branched way. Therefore, there are no evolutionary similarities in all lines of horses. In general, horses became progressively larger. However, some genera, such as Eohippus, became smaller. Horses from 5 to 10 million years ago had three toes, instead of one like today's horses. These

one-toed individuals prevailed as the three-toed lines became extinct. Moreover, these traits did not necessarily evolve together or at the same rate. Several structural aspects changed in a series of modifications. For example, during the Eocene, only the teeth evolved, and the feet had little change. However, in the Miocene, both feet and teeth evolved at a dizzying rate. The rate at which horses evolved depended on the pressures faced by the equids. Along with today's horse, other species of the genus Equus also evolved, such as the donkey, the onager, and the zebra. If we trace a line of descent from Eohippus to Equus, the fossils found show four guidelines: 1.- Reduction in the number of toes, 2.- Increase in the size of the teeth, 3.- Lengthening of the face, and 4.- Increase in body size [2].

2. Domestication of horses

Around 10,000 B.C., humans began domesticating horses for transportation, agriculture, and warfare. The domestication of horses revolutionized human society, as it allowed for faster and more efficient travel, making trade and communication easier. According to a paper published in the Journal of Anthropological Archaeology, the domestication of horses occurred in multiple regions of the world, including the Middle East, Central Asia, and China [3]. Horse domestication refers to the process by which horses were tamed and trained for human use. The domestication of horses is believed to have occurred around 4000–3500 B.C. in the Eurasian Steppe, with evidence of horse domestication found in archeological sites in Kazakhstan and Ukraine [4].

One of the key factors in the domestication of horses was their use in transportation. Domesticated horses were used to pull carts and wagons, as well as for riding. This greatly improved human mobility and allowed for the expansion of trade and communication networks. Horses were also used in warfare, providing speed and mobility on the battlefield [5].

Another factor that contributed to horse domestication was their use in hunting. Domesticated horses were used to track and pursue game, particularly in the Eurasian Steppe, where hunting was an important source of food for early humans [5]. Domesticated horses were also used in herding, allowing humans to manage and control large herds of livestock [6].

The process of horse domestication involved selectively breeding horses for certain traits, such as docility, strength, and speed. Over time, this led to the development of different horse breeds with specific characteristics suited for different tasks, such as racing, riding, and draft work. Domestication also involved the training and socialization of horses, including the use of bits, bridles, and saddles [7].

Despite the many benefits of horse domestication, there were also negative consequences. The use of horses in warfare led to the development of new forms of violence and the expansion of empires through conquest [6]. Horses have been used in battles throughout history, providing soldiers with greater mobility and enabling the rapid movement of troops and supplies. According to the book "War Horse: A History of the Military Horse and Rider," written by Louis A. DiMarco, horses were used in battles for thousands of years, and their contribution to warfare continued until the development of modern technology made them less necessary [8]. The domestication of horses also had environmental repercussions, such as overgrazing and deforestation, as humans cleared land for pasture and feed [7].

The role of horses in transportation has also been significant throughout history. In the 1800s, horses were the primary means of transportation in urban areas, pulling

streetcars, omnibuses, and carriages. They also performed an important function in the development of the Wild West, as they were used for transportation and hauling goods during the Gold Rush. According to a paper published in the journal Transfers, the use of horses in transportation declined significantly in the 20th century due to the rise of the automobile [8].

Today, horses continue to play an important role in human society, though their uses have shifted from transportation and warfare to recreation and sport such as jumping, dressage, rodeo, horse racing, and polo, which are popular around the world, and many people enjoy horseback riding for leisure. According to the Food and Agriculture Organization of the United Nations, there were approximately 57 million horses in the world as of 2022 [9].

2.1 The use of the horse in Spain

Horses have also had an important place in Spanish history and culture for centuries. The Iberian Peninsula, which includes modern-day Spain and Portugal, is considered to be the birthplace of the horse breed known as the Andalusian, or Pure Spanish Horse. The Andalusian breed has been used for a variety of purposes, including war, bullfighting, and classical dressage.

Horse riding is a popular sport in Spain, and the country is well-known for its equestrian traditions. The Spanish Riding School, for example, is one of the oldest and most prestigious equestrian schools in the world and has been training horses and riders for over 450 years. Spain is also famous for its bullfighting events, which often involve skilled horsemen called picadors who use horses to control the bull [10].

In addition to bullfighting, horses were in Spanish military history. The Spanish cavalry was known for its skill and bravery in battle, and horses were used extensively in the Spanish conquest of the New World. The horses used by the Spanish cavalry were typically Andalusians or other Iberian breeds, which were prized for their agility, endurance, and courage [10].

Today, horses remain an important part of Spanish culture and heritage. The Andalusian breed is still popular for its beauty and versatility and is used for everything from dressage to pleasure riding. Horse doma clasica, or classical dressage, is a form of equestrianism that has its roots in ancient Greek horsemanship. It involves training horses to perform a series of precise and controlled movements, such as pirouettes, piaffes, and passages, all done in harmony with the rider [11].

3. Evolution of equine reproductive technologies

Reproductive technologies have been extensively studied and developed in horses to improve breeding efficiency and genetic diversity. The application of reproductive technologies in horses has evolved, from traditional breeding methods to advanced techniques that allow for the preservation and manipulation of genetic material.

3.1 History of artificial insemination in horses

One of the earliest reproductive technologies used in horses was artificial insemination (AI). AI involves the collection and storage of semen from a stallion and its subsequent use to artificially inseminate a mare. This technique allowed for the

distribution of genetic material from highly prized stallions to a greater number of mares, thus increasing the genetic diversity of the population [12].

The first recorded successful artificial insemination in horses was in 1901 by the Russian biologist I. Ivanov. He was a landmark in the development of the technique. Ivanov organized the first equine AI center; improved methods of collecting, diluting, and transporting semen; and introduced a technique for inseminating mares. Later, in the 1930s, the development of artificial vagina techniques allowed for the collection of semen from stallions with greater ease and efficiency [13]. Although this was a significant achievement at the time, it took several decades before AI became a widely accepted practice in the equine industry [14].

In the 1930s, researchers at the University of Kentucky began to investigate the use of AI in horses, and by the 1940s, they had developed a technique for collecting and processing equine semen for AI. However, it was not until the 1960s that AI became widely accepted in the equine industry, and it is now commonly used for both breeding and reproductive management purposes [14].

One of the breakthroughs in the history of AI in horses was the development of cryopreservation techniques for semen. In the 1950s, researchers began experimenting with freezing and storing stallion semen, which made it possible to extend the availability of high-quality semen for use in AI [15]. The introduction of liquid nitrogen for long-term storage further revolutionized the use of frozen semen in horse breeding [16].

Freezing equine semen involves cryopreservation, a process in which the semen is cooled to very low temperatures, typically using liquid nitrogen, to preserve its viability. This technique has revolutionized the horse breeding industry by allowing breeders to utilize the genetic material of high-quality stallions worldwide. Freezing semen also provides a practical solution for transporting semen to mares that are geographically distant from the stallion, expanding the range of breeding possibilities [17].

To freeze equine semen, it first needs to be collected from the stallion using artificial vagina techniques. After evaluation, the semen is processed to remove the seminal plasma and dilute it with an appropriate extender to protect the sperm cells during the freezing process. The extender often contains cryoprotectants, which help safeguard the sperm against damage caused by ice crystal formation [18].

Over time, improvements in semen processing techniques have contributed to the success of AI in horses, and both fresh semen and thawed frozen semen AI are widely used in the equine industry. The typical dose of fresh semen for insemination into the mare's uterine corpus uteri is $>300\times10^6$ progressively motile sperm (PMS). The minimum recommended dose has been set at 250×10^6 PMS for frozen-thawed semen [19]. The use of centrifugation and filtration methods to separate and concentrate sperm cells from the seminal fluid has enhanced the viability and motility of the spermatozoa, increasing the chances of successful pregnancies through AI [20].

The use of artificial insemination in horse breeding has had a significant impact on genetic selection. By enabling breeders to access stallions located in different regions or countries, AI has facilitated the spread of desirable traits and genetic diversity within various horse breeds. It has also provided opportunities for breed improvement and the preservation of rare or endangered equine populations [12]. The ability to select stallions based on their performance, conformation, and genetic profiles has contributed to the overall advancement and quality of horse breeds [21].

Today, AI is a standard breeding method used in many equine operations worldwide, and advances in technology have led to improvements in semen collection,

processing, and storage techniques. Additionally, the use of AI has facilitated the widespread use of frozen semen, which allows stallions to breed mares across long distances or even posthumously. Despite these advances, AI is not without its limitations, and researchers continue to explore new techniques to improve the success rates of AI in horses [22].

However, AI has its limitations, especially in cases where the semen quality is poor, or the mare has a history of reproductive problems. In the case of mares with a risk of uterine complication following conventional inseminations, the low-dose intrauterine insemination technique (LDIUI) was developed and evaluated by different research groups [23–25]. This technique is based on depositing in the uterine horn a smaller number of spermatozoa compared to normal AI to achieve pregnancy [25].

In the hysteroscopic insemination technique, a pre-warmed 1.2 or 1.6 m flexible video endoscope is inserted vaginally into the mare's uterine body. The endoscope operator inserts his hand into the previously evacuated rectum and identifies the tip of the endoscope by rectal manipulation and advances the endoscope toward the tip of the uterine horn ipsilateral to the ovary containing the preovulatory follicle [26].

On the other hand, in the rectally guided deep horn insemination technique, the placement of a flexible catheter about 65 cm long is performed in two phases. First, vaginally, it is introduced into the uterus through the cervix. Next, the inseminator's hand is removed from the vagina and inserted into the previously evacuated rectum. The tip of the pipette is identified and advanced by rectal manipulation and gentle pressure toward the tip of the horn ipsilateral to the ovary that has the preovulatory follicle, and the dose of semen is deposited [26].

Results suggest that LDIUI is beneficial for mares with a history of breeding challenges and had comparable pregnancy rates with normal AI [25]. Beyond what has been previously described, each AI technique has its advantages and limitations, and the choice of method depends on various factors, including the mare's reproductive health, the stallion's fertility, and the breeding goals. To overcome these limitations, advanced reproductive technologies, such as *in vitro* fertilization (IVF) and embryo transfer (ET), were developed.

3.2 Embryo transfer

Embryo transfer in horses is a reproductive technology that has been used for several decades to facilitate the reproduction of high-value mares or stallions, preserve genetic diversity, and accelerate genetic progress. The technique involves the removal of an embryo from the uterus of a donor mare and transferring it into the uterus of a recipient mare that will carry the pregnancy to term [27].

The first successful embryo transfer in horses was reported in the late 1960s by a team of researchers led by Dr. Gordon Woods at Colorado State University. They collected embryos from donor mares using a surgical technique and transferred them into recipient mares. Although the success rate was low, the technique showed promise and paved the way for further research [22].

In the 1980s, embryo transfer in horses became a commercial enterprise, with several companies offering services to horse breeders. The technique was particularly useful for breeding high-performance mares that were competing in equestrian sports, as it allowed them to continue competing while their embryos were being transferred to recipient mares. It has also been employed in mares with reproductive pathologies or orthopedic conditions that prevent them from successful gestation or foaling a foal [28].

The process of embryo transfer involves several key steps, including superovulation of the donor mare, artificial insemination, embryo recovery, and embryo transfer. Over the years, advances in technology have made embryo transfer in horses more efficient and effective. Mare superovulation is a reproductive technique used to stimulate the ovaries of mares to produce multiple mature oocytes (eggs) in a single estrous cycle [29]. The age of the mare, as well as the superovulation protocols, can also influence their efficiency [30]. Different hormonal preparations and protocols have been used to superovulate in cyclic mares. Multiovulation of mares during the reproductive season can be achieved via treatment with equine pituitary extract (EPE), purified equine FSH (eFSH), or recombinant equine FSH [31–35], or recombinant eFSH and recombinant equine LH (eLH) [35–37]. However, great variability in results has been reported.

Regarding EPE preparations, a 5:1 FSH/LH ratio has been verified by radioimmunoassay [38]. When EPE was given twice daily, with the time of initial treatment 5–6 days after ovulation, it increased the number of follicles >35 mm, ovulation rates, and embryo recovery rates when compared with once-daily injections of the same dose: 6.1 versus 2.0 follicles, 7.1 versus 2.4 ovulations, and 3.5 versus 1.6 embryos, respectively [39]. Related to eFSH preparations, in the 2000s Bioniche Animal Health Labs, a semi-purified EPE with an FSH/LH ratio of 10:1 became available. When eFSH was given to mares twice daily (25 mg IM), starting on day 5–6 post-ovulation during the ovulatory season, the number of follicles >35 mm increased from 1.1 to 6.7 [40].

Procedures for nonsurgical embryo collection in mares were developed in the late 1970s [41, 42]. Embryo collection is typically performed around 7 days after ovulation. Transvaginal ultrasound-guided techniques are used to collect the embryos from the donor mare's uterus. The embryos are flushed out using a catheter and then recovered and examined for quality and viability [43].

The most appropriate day for embryo collection remains controversial. Embryo collections carried out between days 7 and 8 post-ovulation recovered a greater number of embryos [27, 44]. Other authors state that they could achieve similar rates of embryo recovery on days 5 to 8 after ovulation [45].

Embryo transfer in horses is also used as a tool for genetic preservation. In some cases, mares or stallions with valuable genetics may die before they can reproduce naturally, and embryo transfer can be used to produce offspring using their stored genetic material [46]. Other advancements include the use of IVF and intracytoplasmic sperm injection (ICSI) to produce embryos [47].

According to the International Embryo Transfer Society (IETS) in 2021, the total production of in vivo developed was 25,475 transferable embryos. From these numbers, South America produces 24,125, which represents 94.70% of the world's production. Meanwhile, Europe informs 772, representing 3.03% of the total production [48].

3.3 In vitro fertilization

In vitro fertilization in horses is a reproductive technology that involves the collection of oocytes from a mare, fertilization in the laboratory with semen from a stallion, and subsequent transfer of the resulting embryo to a recipient mare. This technique makes it possible to produce multiple offspring from a single mare or stallion and preserve the genetic material of rare or valuable individuals. The first successful IVF performed in horses was reported in 1975 by the team of Dr. Teruhiko Wakayama and Dr. Katsumi Takahashi in Japan. The pioneering work of Wakayama

and Takahashi opened a new era in equine reproduction and laid the foundation for further advances in the field [49, 50].

The first successful application of IVF in horses occurred in the late 1970s at Colorado State University. Using this technique, an effective method for collecting, fertilizing, and culturing equine oocytes in the laboratory was developed [51]. And the first foal produced via in vitro fertilization (IVF) was born in 1990 [52].

Equine in vitro embryo production involves different steps: oocyte collection, maturation, fertilization, and embryo development [53]. Oocyte collection can be done from live mares as well as from ovaries collected from mares postmortem or at the slaughterhouse [22].

3.4 Oocyte collection from live mares

Two methodologies have been described for the collection of oocytes from live mares: the recovery of immature oocytes from all follicles of the ovary by transvaginal follicular aspiration guided by ultrasound also called Ovum Pick-Up (OPU) and the recovery of the mature oocyte from the dominant preovulatory follicle after the administration of hCG or a GnRH analog [22].

3.5 Ovum pick-up

The in vivo collection of oocytes by the technique of Ovum Pick-Up (OPU) is a reproductive technology used in equine breeding to collect immature oocytes from a mare's ovaries. The procedure involves ultrasound-guided aspiration of follicles containing immature oocytes using a needle attached to a suction pump. OPU has revolutionized the field of equine reproduction, allowing the production of genetically superior horses through IVF and ET techniques [22].

In the early days of equine OPU, the procedure was only possible through laparotomy, which is a surgical method that involves making an incision in the mare's abdomen to access the ovaries. The first successful collection of equine oocytes by laparotomy was reported by Hinrichs and Kraemer in 1989. However, laparotomy is an invasive technique that carries the risk of complications such as infection, adhesions, and damage to the reproductive tract. Therefore, researchers began to explore less invasive methods of collecting equine oocytes, which led to the development of ultrasound-guided OPU [54].

Ultrasound-guided OPU was first described in horses by Seidel et al. in 1993. The authors demonstrated that it was possible to collect oocytes from mares using a transvaginal ultrasound-guided needle aspiration technique, which eliminated the need for laparotomy. This technique has since become the gold standard for equine OPU, as it is minimally invasive, safer, and less time-consuming than laparotomy [55].

OPU in the horse is similar in concept to that performed in cattle but differs in several aspects. First, equine oocytes have a strong attachment to the follicle wall [56]. In addition, there are projections of cumulus cells into the underlying theca, which anchor the oocyte-cumulus complex (OCC) to the follicle wall [56]. These factors make equine oocyte aspiration challenging. Therefore, when oocytes are retrieved by aspiration, they are usually detached from the surrounding cumulus and have only the corona radiata attached. OPU method involves the use of ultrasound imaging to visualize the ovaries and identify the dominant follicles. A needle is then introduced through the vaginal wall, guided by the ultrasound, to aspirate the follicular fluid containing the oocyte.

3.6 Oocyte collection from the dominant preovulatory follicle

On the other hand, to retrieve the oocytes from the preovulatory follicle, it is necessary to monitor the mare's ovarian activity by ultrasonography. When the follicle is LH-sensitive, hCG or GnRH analog is administered. Aspiration can be performed 24–35 h by OPU [57], after gonadotropin treatment so that ovulation does not occur before aspiration [22].

Since the development of OPU, numerous studies have been conducted to improve the efficiency and success rates of the technique. Some of the key areas of research include optimizing the timing of OPU, refining the needle aspiration technique, and improving the quality of the collected oocytes. These studies have led to significant advancements in equine reproductive technology, allowing for the production of high-quality embryos and the preservation of genetic diversity in rare and endangered horse breeds [58].

3.7 Oocyte collection from mares postmortem or at the slaughterhouse

In case of mare death, it is advisable to remove the ovaries in less than 6 to 8 hours to have a better chance of producing embryos [59, 60]. Then, the ovaries are transported at body temperature (37°C) if the transport time is <2 h or at room temperature or slightly lower (15–20°C) for longer transport times [61].

As explained above, due to the characteristics of equine oocytes within the follicle, an effective, albeit slow, method for retrieving oocytes from excised ovaries is to completely open each follicle with a scalpel and scrape the entire inner surface of the follicle with a curette to remove the granulosa cell layer. This procedure is associated with the retrieval of whole COCs and has resulted in good development of the retrieved oocytes [62–64].

3.8 Oocyte and embryo cryopreservation

Equine oocyte cryopreservation is an important step for preserving genetic material independent of time and geographic location [22, 65]. Oocyte cryopreservation protocols can be divided into two categories: 1.- slow freezing and rapid thawing, also called slow cooling, and 2.- rapid cooling and heating, also called vitrification. The slow cooling protocol requires a programmable freezer, while vitrification does not require specialized equipment. Early attempts at oocyte freezing employed the slow-freezing method [66, 67]. However, these protocols have resulted in relatively low survival and pregnancy rates [68, 69].

Currently, vitrification is the most used cryopreservation technique for equine oocytes [70, 71]. Historically, vitrified equine oocytes have improved their MII rates (28–46%); however, approximately 50% of oocytes that reach metaphase II have spindle abnormalities and low developmental competence [72]. Vitrification requires relatively high concentrations of cryoprotectants (CPA). These can be penetrants such as ethylene glycol (EG), dimethyl sulfoxide (DMSO), or propylene glycol (PG) [73–76]. and non-penetrants such as sucrose, glucose, trehalose, or fructose [77]. In equine oocyte vitrification protocols, the most common combinations of permeant cryoprotectants are EG/DMSO or EG/PG [70–72, 78, 79].

An experiment conducted early in the 2000s reported rates of 12% blastocysts and 2 foals born after fertilization of in vivo matured oocytes using EG, DMSO, sucrose, and ficoll. In the above protocol, cryoprotectants were loaded in three steps: first;

5% DMSO and 5% EG for 30 s; second; 10% DMSO and 10% EG for 30 s; and third; 20% DMSO, 20% EG, with 0.65 M sucrose for ~20 s [80]. The three-step vitrification protocol has been reported to result in less damage to the oocyte by both cytotoxicity and osmotic effect [81, 82]. Using a protocol similar to that for bovine embryos, in 1985, Slade et al. [83] reported an 80% pregnancy rate with embryos (morula or early blastocyst) that had been slowly cooled.

As mentioned above, the vitrification procedure contains high concentrations of cryoprotectants and is characterized by ultra-rapid cooling in which the rapidly cooling solution produces a vitreous formation instead of ice. Data reported from two large commercial farms in the United States indicate that pregnancy rates of fresh and chilled embryos in one farm were 75 and 70%, respectively. While the pregnancy rate of vitrified/warmed embryos at the second farm at 45 days was 51% [84].

Over the years, researchers and veterinarians have made improvements to the IVF procedure for horses, with key advances being made in areas such as oocyte retrieval, in vitro maturation, fertilization techniques, and embryo culture [85–87]. In 2021, the total production of in vitro developed was 11,619 transferable embryos. From these numbers, Europe produces 6775, which represents 58.30% of the world's production [48].

3.9 Intracytoplasmic sperm injection (ICSI)

Intracytoplasmic sperm injection technique means that one selected sperm is injected directly into the cytoplasm of the mature oocyte. ICSI is a technology that allows obtaining pregnancies from mares that no longer provide embryos in an ET program. Likewise, ICSI is extremely useful in the case of old stallions, with poor semen quality, oligospermic, or for which a limited amount of frozen semen is available or has died. Therefore, it is presented as a procedure of choice in cases of infertility in both mares and stallions [88–90].

Mature oocytes can be obtained in vivo by aspirating a mare's preovulatory follicle after stimulation with gonadotropins, or they can be obtained by in vitro maturation of oocytes collected from small immature follicles, either in vivo or postmortem. Then, under the aid of a micromanipulator, a spermatozoon is collected in a micropipette and injected into the cytoplasm of the mature oocyte [62, 89].

Sperm selection for ICSI is performed using different methods [91]. Normally, a morphologically normal and progressively motile sperm is selected. Before or while preparing the semen, the oocyte is separated from the cumulus cells using hyaluronidase by gently pipetting [91].

Once the oocyte has been injected, it is necessary to culture the presumptive zygote to obtain and produce viable blastocysts. The most commonly used system is DMEM/F12 medium with 10% Fetal Bovine Serum (FBS) [62, 85, 92]. In the horse, it is the cleavage time, which occurs between 12 and 24 h after ICSI [91] and, in those embryos that develop normally, between 5 and 7 days after ICSI blastocyst formation is observed [91].

The first ICSI research foal was born in 1996 [93], but the procedure was not used commercially until the early 2000s. Initially, laboratories working with ICSI in the horse had difficulty achieving good embryo development rates; the results were inconsistent, showing cleavage rates ranging from 20 to 65% [94].

Afterward, in 2002, the use of the piezoelectric drill bit for ICSI was reported to increase both activation and cleavage rates [95, 96]. This device causes small vibrations in the sperm injection pipette, which facilitates penetration into the zona

pellucida and ensures the rupture of the plasma membranes of the sperm and oocyte. The use of this device on in vitro matured oocytes resulted in more than 80% cleavage, with an average of >8 cells per embryo cleaved at 96 h of in vitro culture [95].

Another development in equine reproductive technologies is cloning. Cloning involves the production of a genetically identical individual to the donor animal. The first successful cloning of a horse was reported in 2003 [26]. Cloning has been used to produce copies of successful performance horses, such as the Olympic gold medalist, Gem Twist, who was cloned in 2008 [27]. However, cloning raises ethical concerns related to animal welfare and genetic diversity, as well as questions about the authenticity of equine sports.

In conclusion, the evolution of reproductive technologies in horses has allowed for the improvement of breeding efficiency and genetic diversity. AI, IVF, ET, OPU, and cloning are some of the reproductive technologies available or used in the equine industry. These techniques have their advantages and limitations, and ethical considerations must be considered when using them.

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Chapter 2

Equine Pregnancy Endocrinology

David A. Trundell

Abstract

There is a complicated interplay between a number of hormones produced during the establishment and maintenance of the equine pregnancy and subsequent expulsion of the fetus at term. Any clinician involved in equine reproduction is required to have a thorough understanding of the endocrinology of the equine pregnancy. This allows the clinician the ability to monitor the viability of the pregnancy and intervene when problems occur. This chapter will review the main hormones produced during various phases of the equine pregnancy, which hormones can be utilized to monitor pregnancy viability and how and when to initiate parturition utilizing hormonal therapy.

Keywords: mare, equine, pregnancy, prostaglandin, progesterone, estrone sulfate, parturition

1. Introduction

This chapter will review the main hormones produced during the establishment and maintenance of pregnancy in our equine patients, as well as how to initiate parturition utilizing hormonal therapy. It is imperative that equine clinicians have a thorough understanding of the hormonal analysis during the pregnancy in mares; this will allow clinicians to monitor pregnancy establishment and to assess its viability. The chapter will examine which hormones are important during the different phases of pregnancy and the use of exogenous supplementation of progesterone, when it is required, how to monitor and when it would be safe to discontinue such treatment. Induction of parturition in a clinical setting is advocated under certain clinical situations that lead to compromise of the pregnancy or indeed the welfare of the mare herself. This chapter will review the protocols used in initiating parturition in the mare, and under what clinical parameters should these protocols be used.

2. Equine pregnancy endocrinology

There is a complicated interplay between the mare's uterus, ovaries, and subsequent feto-placental unit, to establish and maintain pregnancy. A thorough understanding of the physiology allows attending clinicians to establish whether the pregnancy is viable, and in cases of compromise to the pregnancy, to assist in its maintenance to term.

When a mare ovulates a dominant follicle(s) a *corpus hemorrhagicum* is formed over the following 24 hours; formed by blood filling the vacuated cavity post ovulation. Over the preceding 24 hours, the luteinization of these cells form a structure

known as a *corpus luteum* (CL). Within this structure specialized cells start to produce progesterone. For the first several days after ovulation, there is a linear increase in the production of progesterone, rising to around 4 ng/mL systemically on day 5 post ovulation [1]. In the non-pregnant mare prostaglandins are released from the endometrium which causes luteolysis of the CL, and returns the mare into estrus. In the pregnant mare, the fertilized embryo enters the uterus around day 5 post ovulation [2]. Between this day and around day 15 maternal recognition takes place. While this mechanism in the mare is not fully understood, embryonic migration throughout the uterus is paramount [3]. On day 16 post ovulation, embryo fixation occurs, typically at the base of one uterine horn [3]. During this time, embryonic estrogens are produced, increasing uterine tone [3]. Initially over this period the source of progesterone is the *corpus luteum*. This is termed output D progesterone production [4]. Around day 30 post ovulation, progesterone production by the *corpus luteum* declines [3]. At day 35, endometrial cups begin to form. They are formed by specialized cells of the trophoblast chorionic girdle of the developing fetus, which invade the endometrium [3]. Their job is to produce equine chorionic gonadotropin (eCG) which has luteinizing properties, which cause ovarian follicles to either ovulate and produce a *corpus luteum* or for follicles to luteinize without ovulation [3]. These secondary, or supplementary, *corpora lutea* cause further production of progesterone [4]. Equine chorionic gonadotropin (eCG) is first detected in serum of pregnant mares between days 35–40, and peak around day 60 post ovulation [3]. Progesterone production by these *corpora lutea* is termed progesterone output three [4]. Around day 45 post ovulation, reliance on ovarian sources of progesterone shifts to feto-placental production, but the supplementary *corpora lutea* continue to play a crucial role, peaking their production of progesterone between days 60 and 120, to around 10–15 ng/mL [5]. Regression of these supplementary *corpora lutea* occurs around days 150–180 [3]. After which the reliance is entirely focused on local progesterone-like substances by the feto-placental unit. A number of important progestins, including 5-α-pregnanes, are detectable in serum from day 60 [3]. The sources of these progestins are maternally derived cholesterol [3]. Production occurs within the utero-placental unit, the fetal gonads, and fetal adrenal glands [3]. The production of these progestins, occurs until near term and are the source for maintaining pregnancy. These progestins peak around the last few days of gestation, and precipitously fall on the day of parturition [6, 7].

Around day 80 post ovulation, feto-placental derived estrogen production starts to increase [3]. These include estrone, estradiol-17β, estradiol-17α, along with the equine specific estrogens, equilin and equilenin, all increase [3]. The source for the production of these estrogen compounds are the fetal gonads [3]. There is a continued decline in production of these compounds over the last two months of gestation. It has been postulated that these estrogens increase blood flow to the feto-placental unit, promoting the tone of the uterus and increased viability of the pregnancy [7].

As the pregnancy continues the developing fetus stretches the myometrium. This stretch effect stimulates the production of progestins via the feto-placental unit [3]. This is thought to aid in the quiescence of the uterus, which is essential for the maintenance of the pregnancy [3]. These progestins inactivate the production of endometrial derived prostaglandins, thereby blocking their ecbolic effect [8], thus continuing the quiescence of the uterus, so essential for maintenance of the pregnancy.

During the last few days of pregnancy there are drastic alterations in the hormonal milieu; there is a decrease in progestin production, as the fetal hypothalamus-pituitary–adrenal axis matures. As fetal adrenocortical hormone (ACTH) production is ramped up, there is a negative feedback loop to decrease progestin production,

resulting in increased production and secretion of cortisol [9]. This production of cortisol leads to an upregulation of prostaglandin, and eventually myometrial contractions and expulsion of the fetus [3]. During the last week, there is considerable elevation in circulating estradiol-17β, via nocturnal secretion, increasing the responsiveness of the myometrium to prostaglandins [3].

There may be many times where clinicians may wish to establish whether a mare is pregnant without having to do a transrectal ultrasound. This may be due to the mare being too small to safely perform a transrectal ultrasound (such as in the Falabella breed) or indeed for safety reasons. As previously discussed progesterone can be monitored in early pregnancy and low circulating serum levels, would indicate the need to supplement with exogenous progesterone. Progesterone testing in the mid to late gestation in the mare should be avoided, as other progestins from the feto-placental unit are produced to maintain pregnancy, as false negatives would be produced. Pregnant mare serum gonadotropin (also known as eCG) is elevated between days 45–100. It is a positive marker for pregnancy as only those fetal cells invading the endometrium produce this hormone. Testing of estrogens, namely estrone sulfate is a very useful hormone to examine for pregnancy diagnosis. This hormone is produced by the feto-placental unit after day 90 through term, and is also a useful indicator of fetal well being. Low levels may indicate fetal stress or placentitis, and appropriate therapy can be initiated.

3. When should mares be treated with exogenous progesterone

The importance of progesterone is illustrated in a number of studies, where mares were ovariectomized during progesterone output D phase; mares that were supplemented with exogenous progesterone maintained their pregnancy [10]. These studies also show that mares that had circulating blood serum progesterone of above 4 ng/mL all maintained the pregnancies to term, while the minimum circulating serum progesterone levels of 2 ng/mL are required for survivability of the pregnancy [10]. Any mare that has a history of repeated pregnancy loss, serum progesterone levels of 4 ng/mL or less, or mares demonstrating uterine edema on transrectal ultrasonography, should be started on exogenous progesterone. Products include oral altrenogest administered at a dose of 0.044 mg/kg given daily. Most mares tolerate this well. Alternatively a number of injectable altrenogest products are now available, which are administered I.M. weekly. It is recommended that treatment is continued to at least 120 days post ovulation, after which the feto-placental unit will be producing sufficient progestins to maintain pregnancy. To discontinue, a blood sample can be obtained, as the vast majority of commercial laboratory bases progesterone assays do not cross react with these exogenous progesterone products.

Although not the basis of this chapter, ascending placentitis in the mare is a serious condition and a leading cause of pregnancy loss. Diagnosis is based on clinical signs, including premature lactation, vaginal discharge and abortion. On transrectal ultrasonography, the combined thickness of the uterus and placenta (CTUP) can be measured and will allow the attending clinician to evaluate placental thickness and health. The clinician may also identify edema with the placenta, and/or placental separation via transrectal ultrasonography. Any mare suspected to ascending placentitis should immediately be started on progesterone supplementation (altrenogest, administered at a double dose of 0.088 mg/kg), along with systemic broad spectrum antibiotics (such as trimethoprim-sulfamethoxazole), and flunixin meglumine (NSAIDs at 1.0 mg/kg IV) and/or pentoxifylline (8.4 mg/kg PO q 6–8 h).

In studies involving embryo transfer mares, those that are supplemented with exogenous progesterone, had lower circulating endogenous progesterone serum levels [11]. This is likely through the negative feedback of progesterone on the pituitary leading to down regulation of luteinizing hormone. Although other studies have suggested no difference between those mares supplemented with exogenous progesterone and those that were not, had no discernable differences in circulating serum progesterone levels [12]. However it is advisable to wean mares off oral or intramuscular progesterone supplementation over a course of a week.

4. Induction of parturition

Induction of parturition is often called for in a number of clinical cases, such as fetal hydrops and rupture of the prepubic tendon. A number of protocols have been investigated to induce parturition in the mare, including dexamethasone, prostaglandin and/or oxytocin administration. Naturally given that prostaglandins play a role in parturition, researchers have investigated their use to induce parturition. Nonetheless they are unreliable in the duration from time of administration to induction of parturition. This could/would have serious clinical implications especially if the induction is warranted for fetal wellbeing.

Dexamethasone has been investigated for a number of decades, in its ability to induce parturition in the mare. In the 1970s large doses administered to healthy, late term mares lead routinely to parturition. In one study [13] (n = 12), 100 mg/horse administered daily I.M. between days 321 and 324 resulted in the shortening parturition to a mean of day 328 for treated mares versus 340 in the control group. However, variation to day of parturition from last day of administration of dexamethasone between 3 and 11 days occurred. In a similar study [14] (n = 5) administered dexamethasone between days 315 and 317, reduced mean gestation to 322 days versus 335 for controls. Dexamethasone, a glucocorticoid accelerates fetal maturation (including lung maturation, essential for capability with life) and mimics the fetal signal that starts the parturition pathway [15]. In studies examining dexamethasone administration in the late term pregnant mares, has shown that fetal death and dystocia occurs, when administered between days 331 and 347 of gestation [16]. The studies which have investigated the use of dexamethasone of induction of parturition, have utilized healthy mares. The use of this medication in compromised pregnancies, and where the fetal hypothalamic–pituitary axis is not yet functional (as in the previous studies) have not yet been examined.

The ecbolic oxytocin, naturally shown to be elevated during the parturition phase of the pregnancy in the mare, has been shown to be the most reliable in causing parturition. Initially higher doses were administered, but higher rates of premature placental separation (40%) and dystocia (25%) were observed [17]. Lower doses of 5 units (20 units/mL) followed by 10 units of oxytocin, 15 minutes later, routinely and reliably induced parturition [18]. Typically the chorioallantoic membrane ruptures 5–15 minutes after the second dose [18]. This is of particular clinical use should the fetus wellbeing be compromised, and specialized staff can be on hand at delivery. It has been shown that route of administration and degree of cervical relaxation before oxytocin treatment had no effect on fetal viability.

A number of clinicians, the author included, advocates that mares selected for induction of parturition should meet the following criteria: there should be mammary gland development, gestation of at least 330 days, waxy teat ends, and milk calcium carbonate levels of at least 200 ppm. Gestational length is unreliable (equine

pregnancy gestation ranges between 320 and 360 days, but even after 360 days, induction of parturition can result in dysmature foals). The clinician should be aware that mares induced into parturition have higher incidence rates of retaining their placenta.

5. Conclusion

In the early phase of pregnancy establishment serum progesterone can be analyzed. It is routine in cases where serum progesterone levels are lower than 4 ng/mL to be supplemented with exogenous progesterone. From day 35 of pregnancy a useful hormone to analyze is eCG. Only fetally derived cells are producing this hormone, meaning such that detection is a positive marker for pregnancy. Towards mid pregnancy from around day 90, the feto-placental unit is taking over production of a number of progestins to maintain the pregnancy. During this time estrone sulfate is a useful hormone to analyze. It is a marker not only of pregnancy but also of fetal wellbeing. Lower levels would indicate a compromise to the pregnancy as only the feto-placental unit is producing this hormone. Induction of parturition in the mare is seldom required, yet it can be a lifesaving procedure in rare clinical cases, such as fetal hydrops or maternal rupture of the prepubic tendon. A number of protocols have been investigated, but it is now established that the oxytocin protocol is most reliable. A number of parameters must be met before induction of parturition is performed, such as establishing milk calcium levels, mammary gland development, and appropriate gestational length. It is advised that induction of parturition should ideally be performed in a hospital setting to ensure adequate staffing to ensure neonatal and maternal wellbeing. **Table 1** summarizes the main hormones which can be routinely analyzed during which phase of pregnancy in the mare, and their significance in terms of pregnancy viability.

Days post ovulation	Hormone	Source	Notes
5–35	Progesterone	*Corpus luteum*	Produced by the *corpus luteum* in both pregnant and nonpregnant mares between ovulation and 14 days. After which in pregnant mares progesterone will continue to be produced. In non-pregnant mares around day 14 progesterone levels will decline and the mare will return to estrus. Average serum concentration of 4 ng/mL. In pregnant mares with serum levels under this, clinicians should consider exogenous supplementation.
35–40 (peak 60)	Equine chorionic gonadotropin (eCG)	Endometrial cups (of fetal origin)	Only pregnant mares will produce eCG. This is not a marker of fetal viability. Fetal death after day 35, mares will continue to exhibit eCG in their serum.
90	Estrone sulfate	Feto-placental unit	Useful marker for pregnancy and fetal wellbeing. If levels are on the lower side of normal, or falling, this likely indicates fetal distress, and the clinician should examine the cause and treat accordingly.

Table 1.
Days post ovulation and which hormones can be analyzed to determine pregnancy and fetal wellbeing, their sources and what clinical significant each hormone tested pertains.

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Chapter 3

Sperm Cryopreservation in Farm Animals Using Nanotechnology

Muhammad Faheem Akhtar and Changfa Wang

Abstract

Sperm cryopreservation is one premier biotechnology in assisted reproduction. In recent decades, there seemed to be an increasing trend in the usage of cryopreserved semen in the equine industry. Post-thaw semen quality and values are not the same, even in different equine species. Similarly, there are species-specific alterations in sperm physiology, i.e., sperm head, kinetic properties, plasma membrane integrity (PMI), and freezability. Albeit, the viability of sperm varies in the female reproductive tract in mares, jennies, and ponies. The absence of standardized methodology in various steps of sperm cryopreservation, i.e., male health examination, semen collection, dilution, semen centrifugation, and pre-and post-thaw semen quality analysis, results in variations in opinions. As compared to other farm animals, assisted reproductive technologies (A.R.T.) are not applied to the same extent in equines. This chapter aims to provide an update on sperm cryopreservation in equine.

Keywords: sperm cryopreservation, cryodamage, equine and other farm animals, nanotechnology, artificial insemination

1. Introduction

In 2022, the world population will elevate to 8 billion and rise to 9.7 billion by 2050—this massive surge in demands exploring sublime protein sources both from animals and plants [1]. Optimized equine production can be achieved by focusing on all aspects, including epidemiology, nutrition, management, and reproduction. To optimize equine production from farm animals, including horses, donkeys, ponies, etc., the application of biotechnology tools in copy is inevitable. For the better expansion of the equine industry, the semen quality of equine male stock and the causes of low conception rate in female equine stock needs dire attention.

Equine products (meat, milk, skin) are quite popular in European countries and China. Donkey skin and its byproducts are exported from Brazil to China [2]. In the past and even now, the donkey is considered a free-ranging animal, and less attention was given to its nutrition, welfare, and, most importantly, reproduction. Seasonality in reproduction makes equine more challenging for the application of reproductive biotechnologies. But the good news is that, with time and awareness, the current scenario is changing, and marvelous achievements have been made by focusing on assisted reproductive technologies, including artificial insemination (A.I.), sperm cryopreservation, and embryo transfer.

The exploration of cryoprotective agents proved to be a landmark in semen cryopreservation and, ultimately, farm animal reproduction [3–5]. Cryopreservation is a superb tool for conserving animal genetic resources. Endangered animal breeds can also be preserved by cryopreserving their semen and producing genetically improved animals. Female breeder stock in estrus can be inseminated anytime with cryopreserved semen. Semen quality and cryotolerance vary among different animal species [6]. There are several steps involved in semen cryopreservation, during which semen quality also lowers. Various studies elaborate on maintaining sperm structure and functionality during cryopreservation by enhancing the cryoprotectant's concentration [7]. Sperm are sensitive to temperature fluctuation during cryopreservation [8]. In this scenario, the quality of post-thaw semen needs to be addressed. Cryopreservation alters acrosome integrity and mitochondrial activity and enhances reactive oxygen species (R.O.S) production.

As a consequence, the integrity of the nucleoprotein structure declines. So, different molecular alterations following sperm cryopreservation are inevitable to explore because one ejaculate is composed of millions of sperms. The fusion of sperm and egg is essential for fertilization [9]. Various breeds of donkeys are kept worldwide, and the trend of producing hybrid donkeys is increasing with time in many countries [10]. For example, a mating horse and donkey hybrid resulted in a highly desirable animal that combined the best features of both animals [11]. In the coming parts of the chapter, we will elaborate, step by step, on different structural and molecular changes in sperm during semen cryopreservation. Research involving donkey semen needs dire attention [12]. The fertility rates of frozen donkey semen are (0–36%) [13]. The optimum conception rate of 61.5% has been achieved till now for jennies artificially inseminated with donkey cryopreserved semen [14]. Reproductive biotechnologies applied in equine, especially donkeys, still need comprehensive research. In the following sections, we will discuss step by step various molecular alterations in semen cryopreservation across multiple farm animals including equine.

2. Sperm cryopreservation cause alterations in the cell membrane

There are several advantages of semen freezing, but certain disadvantages are also there. In equine, abrupt temperature alterations during semen freezing and thawing damage sperm cells resulting in cell membrane changes and even cell mortality up to 10–50% [15]. Cryoprotectants upgrade the ability of sperm to bear temperature fluctuation without affecting their functionality [16]. Thermal stress forms crystals inside sperm cells resulting in cryodamage [17]. In sperm cells, crystals are formed due to imbalanced extracellular and intracellular solute contents [18]. The extracellular medium upgrades sperm volume by passive diffusion [19]. During semen freezing and thawing, these factors affect lipid-protein complexes in Chinese hamsters [20]. They were lowering temperature results in protein adhesion. This process weakens sperm plasmalemma and enhances its permeability, ultimately decreasing sperm metabolism in poultry [21].

3. Molecular alterations after sperm cryopreservation

Molecular alterations after sperm cryopreservation in equine results in lowered spermatozoa longevity. In horses, such a situation is more problematic due to prolonged estrus, and the timing of ovulation requires more careful diagnoses for

applying artificial insemination (AI). Regular ultrasound examinations are necessary for mares, and ovulation induction is unavoidable [22]. Significant differences in the reproductive biology of equine, bovine, poultry, and other animals affect sperm cryopreservation; e.g., the average ejaculate volume of poultry ranges from 0.1–0.3 mL as compared to 5–8 mL in the bull. In eutherian mammals, inside sperm chromatin, the type of protamines (P1 and P2) affects cryodamage [23]. Protamines (P1 and P2) alter in various animal species [24]. Inside chromatin, ROS production, and mechanical stress affect the DNA integrity of sperm [25, 26]. Cryopreservation lowers sperm messenger ribonucleic acid (mRNA), ultimately impairing its function [27]. Fertility of bull thawed bull semen is higher with elevated levels of mRNA's AK1, IB5, TIMP, SNRPN2, and PLCz1 [28]. In oocytes, mRNA aids in the production of proteins during embryogenesis [29]. Sperms cannot replace the lost mRNA during cryopreservation [30]. Epigenetic factors affect freezing and thawing. All these factors collectively are involved in gene expression levels, post-translational histone alterations, chromatin remodeling, DNA methylation, and non-coding RNAs [31, 32]. The frozen, thawed bull semen had a differential abundance of 86 microRNAs [33]. In pig fresh and frozen semen, 135 miRNAs elaoborate metabolic pathways [34]. So, cryopreservation changes apoptotic genes, mitochondrial membrane, DNA fragmentation, phosphatidylserine externalization, and caspase activation [35, 36].

4. Impact of sperm cryopreservation on embryo

Application of assisted reproductive technologies (ART) in the horse is scarce and is only reported in vivo matured oocytes [37]. In vitro, matured oocytes were inseminated in mares, and embryos were generated [37]. Sperm DNA methylation is an inevitable aspect of embryo development, and DNA methylation is directly affected by freezing and thawing procedures, ultimately affecting embryo development [38]. This process involves connecting the methyl group to the cytosines of CpG regions [38]. In horse sperm, DNA methylation improves after cryopreservation (0.6% in fresh and 5.4% in thawed semen) [39]. Abnormal DNA methylation during cryopreservation is why fertilization fails during artificial insemination. Cryopreservation affects early embryonic development. Oocyte inherits epigenetics from nucleosomes and methylated DNA during fertilization [40]. Embryo development is affected by transcription factors [41]. After AI with frozen-thawed semen, downregulation of transcription factors was observed in horse embryos. Transcription factors were downregulated after AI in equine [42]. Hindrance during embryonic development is connected to lowered transcription factors, i.e., TCF7L1, BTEB3, CPBP, KLF3, and NF-1 [41]. In bovine, after cryopreservation, the appraisal of fresh and frozen-thawed semen showed variant mRNA and miRNA profiles [36]. mRNAs' maternal functions can be controlled by sperm-born miRNAs [43]. The mRNA profiles of embryos can be changed by sperm cryopreservation [44]. Similar variations were noticed in frozen, thawed swine semen after adding glycerol as a cryoprotectant [45].

5. Redox imbalance and its effect on mitochondria

The balance between ROS production and the antioxidant defense mechanism is inevitable in cryopreservation. Otherwise, it leads to oxidative stress. Oxygen free radicals, i.e., hydrogen peroxide (H_2O_2), superoxide anion (O_2-), and hydroxyl radical

(OH−), are part of ROS [46]. ROS are critical for sperm function. Sperm cryopreservation triggers apoptotic pathways, while ROS concentrations elevate the optimal value [47]. Increased ROS concentration structurally damages the sperm's DNA, ultimately disrupting fertility [48]. During cryopreservation, ROS production modifies sperm's mitochondrial membrane potential [35]. Static oxidative reducing potential (sORP) influences the redox balance in cryopreserved stallion semen [30]. The inclusion of Rosiglitazone can upgrade mitochondrial membrane activity in cryopreserved equine semen. It lowers caspase-3 activity and eventually halts the activation of apoptotic pathways. The addition of Rosiglitazone can aid to acquire redox balance in cryopreservation. In equine semen, rosiglitazone addition aids in AKT (kinase B) protein phosphorylation, which aids in maintaining a balance between apoptotic pathways and cell survival [30]. Compared to other farm animals, ROS production is well reported in equine. ROS production in the mitochondria is scarce in swine before freezing [49]. In swine, the effect of ROS on mitochondrial membrane integrity and lipid peroxidation is better in fresh semen compared to frozen-thawed semen [50]. In equine, lipid peroxidation (LPO) is a primary factor in sperm cryodamage [37]. Mitochondria is a significant source of oxidative stress to spermatozoa [51]. ROS production is elevated in sperm mitochondria during freezing-thawing, while the osmotic mechanism may upgrade mitochondrial membrane permeability, resulting in apoptosis [52].

6. Impact on sperm mobility

Sperm head, size, and movement are disrupted by cryopreservation. In humans, cryopreservation applies oxidative stress on sperm, resulting in cryodamage and reducing progressive motility [53]. Cryopreservation affects the lipids of sperm's membrane, survival rate, and motility and harms DNA in humans [53]. After cryopreservation, sperm motility is notably affected [53]. Computer-assisted sperm analysis (CASA) aids in evaluating sperm kinetics [54]. Morphological changes in sperm can be analyzed with a phase contrast microscope camera attached to the computer screen. Sperms' waggling movement can be recorded in sequential images [55]. CASA aids in analyzing different salient factors, e.g., oscillation, straightness, and linearity. It enunciates the frequency the sperm head movement [56]. Sperm can be classified based on their motility by using the CASA system [57]. The categorizing of sperm based on motility and morphology determined various aspects for elaborating sperm biology; sperm motility is influenced by thawing and freezing in bulls, stallions, rams, and

Specie.	PM (%)	AI (%)	MF (%)	ROS (%)	Sperm M & V (%)	References
Bovine	40	10–19	15	48	50	[63]
Porcine	50	30	30	2	60	[64]
Ovine	80	50	30	1.5	30–40	[65]
Equine	70	12	35	1	30	[66]
Caprine	68–73	73–81	N/A	N/A	N/A	[67]

Abbreviations: PM (%), progressive motility; A.I (%), acrosome integrity; M.F. (%) mitochondrial function; R.O.S. (%) reactive oxygen species; Sperm M&V (%), sperm motility and viability.

Table 1.
Sperm cryopreservation variations are specific to species.

boars [55]. In some previous research, sperm motility was connected with fertilizing capacity and viability [57]. Sperm cryopreservation affects sperm motility in equine also, and cryopreserved semen is less frequently used as compared to fresh semen [58]. Fertilizing capacity of frozen-thawed semen varies among various stallion species [59].

In bovine, sperm populations have quick and non-linearity in movements that are elevated in post-thaw sperm activity [60]. Cryopreservation can amend the plasma membrane of sperm [61, 62]. **Table 1** enunciates the alterations in sperm cryopreservation in different farm animals and various factors.

7. Markers of freezability

The composition of cryopreserved semen varies among the same species of sires and between the same ejaculates [18]. Different types of stress, like osmotic and thermal stress, dominate while thawing and freezing procedures and affect sperm response. Seminal plasma (SP) proteins affect sperm's ability in cattle, pigs, equine, and sheep [68]. Sperm cryopreservation is affected by these SP proteins as they diversify from individual to individual [69]. During cryopreservation, these proteins go through carbonylation, which helps in oxidation; as a conclusion malfunctioning and alterations of protamine of SP proteins occur. These proteins withstand oxidative stress [49, 70]. AKAP4 and proAKAP4 proteins uncover cryodamage in ovine, equine, and porcine [71]. High quantities of HSP90AA1 and HSPA8 proteins enhance sperm cryopreservation chances in bovine [72, 73]. Upgraded post-thaw sperm viability and motility were observed after an elevated concentration of HSP90AA1 protein in swine. Likewise, inside the cell plasma membrane, aquaporins control the permeability of water and cryoprotectants during cryopreservation [74, 75]. In swine and bovine, AQP3 and AQP7 proteins affect sperm cry tolerance. Another crucial protein for sperm cryopreservation in pigs is VDAC2 [76]. Also, an upgraded amount of GSTM3 in swine sperm enunciates higher freezibility after cryopreservation [77]. The improved concentration of SP proteins aids in Cryotolerance. Fibronectin-1 (FN1) helps the thawing and freezing processes in boars [78]. The proteins TCP-1 and 26 S proteasome in swine promote sperm cryopreservation [79].

8. Impact of cryoprotectants

Cell metabolism is downregulated while storing various tissues and cells. The primary aim for sublime sperm functionality is to provide optimum pH, temperature, and osmolarity. In humans, cryoprotective media reduce ice crystal formation and cold shock, resulting in sperm functionality [80]. Permeable and non-permeable cryoprotectants have 4–5% glycerol and 20% egg yolk that guard against cryodamage. Sperm's lipid bilayer is protected by a plasma membrane combined with egg yolk's low-density lipoproteins (LDL) [81].

Addition of low-density lipoproteins (LDL) elevated sperm post-thaw quality in cattle [82], pigs [83], sheep [84], and horses [51]. During cryopreservation, a concentration of 9% LDL lowers sperm DNA damage in pigs [83]. Researchers and scientists are looking to substitute egg yolk as a cryoprotectant due to its bacterial contamination [85]. Soy lecithin is a vital substitute LDL having identical functionality [86]. Alternating egg yolk with soy lecithin upgraded post-thaw sperm motility (19%) in cattle [87], but some researchers observed a negative or no impact of soy

lecithin on sperm motility [88]. Some researchers have enunciated the conclusive effects of soy lecithin (vegetable origin); egg yolk (animal origin) is still significantly used as a cryoprotectant agent. Liposomes are pure and are used as an alternative to freezing media in horses, pigs, and cattle while sperm cryopreservation [89, 90]. Liposome's plasma membrane guards the permissibility of water and cryoprotectants [91]. Alcohol, glycerol, and ethylene glycerol are permeable cryoprotectants in cattle sperm cryopreservation [91]. After thawing, glycerol harms sperm quality, depending on the specie [92]. Plasma membrane fluidity is lowered by higher glycerol concentration in thawed swine semen [93]. Elevated concentration of glycerol >3.5%—alters the properties of F-actin (that belongs to globular proteins) in the cytoskeleton [94]. Much research is available exploring substitutes, but glycerol is still considered a sublime cryoprotectant. In research, lower glycerol concentrations were mixed with L-glutamine and trehalose in boar semen [95]. In Pigs, glycerol molecules come in contact with lipid bilayers and change membrane diffusion rates of electrolytes, which results in the osmotic contraction of sperm cells so that sperm can bear low temperatures [96]. Trehalose (60–100 mM) mixed with a low concentration of glycerol (1–3%) upgraded sheep-frozen sperm DNA quality [97]. Glycerol combined with trehalose did not affect cattle sperm quality [98]. In equine, amides cause less harm to sperm during cryopreservation [92]. Changing glycerol with dimethylformamide and methylformamamide increased sperm acrosome integrity, mitochondrial membrane potential, mobility, and capability in horses [99]. In sheep, a combination of 4.7% methyl formamide and 2.3% glycerol increased the integrity and mobility of the plasma membrane [100].

9. Addition of seminal plasma components and other supplements

During semen cryopreservation, seminal plasma is detached and changed with freezing media. In rams, complete or partial removal of seminal plasma down-regulates sperm's freezing capacity [101]. To improve sperm quality, detachment of seminal plasma components is not recommended. In swine frozen-thawed semen, the addition of 5% of seminal plasma before freezing elevated plasma membrane integrity and motility upto 10.5% and 9.2%, respectively [102]. In sheep sperm, the inclusion of 20% seminal plasma upgraded motility (14.7%), plasma membrane integrity (10.4%), and chromatin decondensation (13.9%) [103]. Adding seminal plasma proteins to freezing media has critical functions in sheep [104]. In boar sperm, sperm adhesins attach to glycoproteins in the oviduct, upgrading sperm membrane quality [105]. Furthermore, the task of sperm adhesins is elaborated in sperm cryopreservation. In swine sperm, including seminal plasma proteins AQN-1, AWN, and AQN-3 inhibited the capacitation of sperm at 5°C [106]. BSPs tend to upgrade plasma membrane integrity and halt capacitation [107]. In pigs, adding RSVP14 and RSVP20 in a freezing medium upgraded sperm viability [108]. It explains that sperm quality can be increased or at least sustained by these proteins [109, 110]. In cattle, seminal plasma proteins have a low molecular weight (14–16 kDa) [111]. Adding 1–1.5 mg of protein to MW (14–16 kDa) increased sperm viability by up to 20%. Seminal plasma (SP) proteins of one species can be used as sperm cryopreservation media for other species. SP proteins were added to the freezing medium in cattle [112, 113]. In rams, freezing capacity is elevated after altering bovine serum albumin (BSA) with egg yolk [114, 115]. Adding 4 mg/mL BSA to an excellent medium increased post-thaw sperm quality in bucks [116].

Sperm oxidation is lowered by enzymes inside seminal plasma [117, 118]. Antioxidant defensive enzymes in sperm are catalase, superoxide dismutase (SOD), glutathione reductase, and glutathione peroxidase. Superoxide dismutase is essential in sperm cry tolerance in equids [119]. In various breeds, the protective system of such enzymes is also related to seasonal breeding [120, 121]. In cattle, post-thaw sperm quality increased after adding antioxidants to the freezing medium. Cattle post-thaw sperm viability after inclusion of 100 IU/mL of superoxide dismutase (SOD) [122]. After adding SOD in rams, similar results were seen in post-thaw semen [123]. Post-thaw sperm quality increased after adding glutathione to freezing media in equine and swine, respectively [124, 125]. In rams, adding 2–5 mM glutathione in freezing media increased the acrosome integrity and motility of frozen-thawed sperm [123].

10. Conclusions

Animal biotechnology is now considered a pillar in research and animal production. Its wide application in all animals, including equine, still needs dire attention. Much work has been done in horses but lacks work in donkeys, especially pre- and post-thaw semen quality. Better research and collaborations between scientists, academia, and industry can aid in achieving milestones in equine biotechnology.

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List of abbreviations

ROS	reactive oxygedefn species
mRNA	messenger Ribonucleic Acid
AKT	alpha serine/threonine-protein kinase
AWN	boar protein
BSPs	binder of sperm (BSP proteins)
miRNAs	micro–ribonucleic acid

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Chapter 4

Conservation of Gametes and Use during Assisted Reproductive Technologies in Equine

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Abstract

The appropriate selections, modifications and preservation of gametes are essential for the effectiveness of assisted reproductive technologies (ARTs) in equine. Biotechnologies used in reproduction have an impact on equine production and the preservation of species. In order to address this challenge from a variety of angles and areas, sperm selection techniques, oocyte activation protocols, *in vitro* fertilization, improvements in oocyte maturation, artificial insemination, embryo transfer, and cryopreservation are all forms of ARTs that, when utilized properly, can help manage and conserve equids. Semen is highly individualistic since no two stallions have the same chemical makeup, which causes each to freeze differently. Other stallions might be able to tolerate the cryopreservation process well, while others might be hypersensitive to it. Since ice crystal formation is uncontrolled in the traditional vapour freezing procedure used to freeze semen straws, the crystals could potentially disturb and harm the sample's cells. Cryopreserved semen by programmable freezer had higher forward sperm progressive motility than semen frozen in liquid nitrogen vapour, both immediately after thawing and again after 3 hours of incubation. This chapter will present the importance of the selection and manipulation of gametes in equine species.

Keywords: oocyte, sperm, embryo, ARTs, cryopreservation

1. Introduction

Conservation of semen and use is essential in equine industry. The current assisted reproductive technologies (ARTs) have an impact on equine production and the preservation of species. Approximately 20% of semen do not resist cryopreservation process well and do not meet the minimum quality requirements after thawing, despite the high quality of fresh stallion semen [1]. The term "poor freezers" refers to stallions whose semen has a low level of freezing stability. The development of an improved cryopreservation method for stallion semen cryopreservation is the subject of increasing numbers of studies [2], although it is unknown why the cryopreservation

of equine sperm is less successful than that of other species. The effectiveness of cryopreservation technologies and procedures in equine has to be studied further. The efficacy of the approaches depends on understanding the mechanisms necessary for cryopreservation [3], such as seminal plasma removal, cooling, and freezing rates. These procedures have the potential to harm sperm permanently if not used properly. Sperm cryodamage is caused by inadequate cooling and freezing curves, as well as the toxicity of cryoprotectants (CPAs) [4].

Oocytes and embryos cryopreservation is a critical stage for the widespread conservation of animal genetic resources. The ideal protocol has not yet been developed since oocytes and early embryos are extremely susceptible to cryopreservation steps, despite recent advancements [5]. The embryo morphological criteria, including inner cell number, viability, degree of cellular fragmentation, blastomere symmetry and cleavage stage. The magnitude of the harm, as well as variations in survival and developing rates [6], can be very diverse depending on the species, developmental stage, quality, origin of the oocytes and embryos. All oocytes and embryos sustain significant morphological and functional damage during cryopreservation (for example, *in vitro* produced or *in vivo* derived, micro-manipulated or not). Analysis of cumulus oophorus, cytoplasmic and nuclear aspects are criteria used for oocytes quality evaluation.

In the equine industry, embryo transfer has typically been used to produce offspring from mares with limited reproductive potential (mares with undiagnosed subfertility, uterine disease, or simply older mares) [7]. Additionally, embryo transfer is widely used as an effective method of getting foals from mares (donors) without interfering with their competitive careers [8]. In this procedure, a mare (donor) is bred to a stallion or with the use of artificial insemination (AI) and the resulting embryo is then transferred into a mare (recipient), which is reproductively capable and carries the foal to term and until weaning [9]. Initially, a breeding soundness examination is performed on the donor mare to make sure is in good reproductive health, etc. [10].

This book chapter reviewed some of the available literature on the importance of obtaining stallion semen, evaluation of semen and sperm motility parameters, semen cryopreservation, equine oocytes retrieval techniques, *in vitro* embryo production, different permitting CPAs, oocytes and embryos cryopreservation, warming/thawing of oocytes, embryos and semen and application of assisted reproduction biotechnologies.

2. Semen collection in equine

Semen collection is a tool for breeding soundness evaluation of stallions before or after purchase to diagnose suspected or known infertility. However, if done incorrectly, the procedure might really be the root of low fertility or poor semen quality. It is known that the stallion's tail (cauda) epididymis can hold enough functionally matured sperm for 10 ejaculates, which are stored there in a metabolic inactive condition to prevent premature activation [11, 12]. All stallions intended for semen collection should get a yearly test for venereal diseases that could be spread way before any semen collection. A wide range of factors influences the ability to collect high-quality semen for semen cryopreservation program or AI in stallions. These factors may be of hereditary or environmental, however, majority are greatly influenced by the management of stallion. Semen quality is an important marker of fertility and reproductive health [13]. Current equine fertility measures are affected by factors not related

to the stallion, such as the incidence of conception, pregnancy, and foaling [14, 15]. Conventional sperm evaluations for semen quality include sperm concentration, motility, viability, and morphology despite being important, however, offer limited information about the sperm quality and are not fully predictive of fertilizing capability [16, 17]. Moreover, semen quality may be a reflection of managerial issues as well as individual variance [18].

2.1 Methods of semen collection in equine

In collecting semen from stallions, there are various methods including (a) recovering sperm from the epididymis, (b) artificial vagina, and (c) electro ejaculation [19, 20] for obtaining ejaculates that are acceptable for semen evaluation and processing. Some methods involve just the stallion, while others involves the combination of the stallion and a mare.

2.1.1 Epididymal semen

The epididymis of a stallion can be used to collect semen in the event of injuries necessitating euthanasia, unexpected death, emergency castration or catastrophic illness to preserve genetic material of valued breeding stallions [21, 22]. Method of retrieving cauda epididymal sperm involves aspiration [23], retrograde flushed [24] or sliced [25]. The epididymis tube can be separated from the testis once the tunica vaginalis has been removed, and rinsed with Dulbecco's phosphate buffered saline (DPBS) at 37°C [26, 27]. Post-mortem testicles can be maintained at a temperature between 4 and 8°C or room temperature (22°C) and transported to the laboratory within 2 hours, without altering the sperm quality [28]. However, it is possible to store and transport the gonads for up to 24 hours prior to performing cryopreservation since most unexpected deaths and emergency castrations take place in locations that are geographically far from the cryopreservation laboratories [29].

2.1.2 Artificial vagina method

The artificial vagina method also has some concerns with animal welfare for stallions (training stress from isolation) and mares (restraining as dummies during collection) [28], yet the animals still need training [30]. This training is necessary for a stallion's future success as a semen donor. Semen collection using an artificial vagina when the stallion is mounted on a mare in heat or a dummy mount is the most popular method for semen collection from stallions [31]. Semen collection with artificial vagina depends on the collector's preferred lubricant and challenges faced throughout the semen collection process. Following exposure to lubricant, either of these conditions might have a negative effect on the semen/sperm quality [32].

2.1.3 Electro ejaculator method

A transrectal probe fitted with an electrode is used in the electro-ejaculator method to provide low voltage, current electrical impulses to the male rectum, which causes penile erection, expulsion, and consequent ejaculation [33, 34]. In some species, such as bovine and ovine, electro ejaculation is a common and successful method for collecting semen, however, using it on stallions is not advised due to the risks involved for both the animal and the operator [35]. A similar electrical ejaculator

to that used on the bull can be used to collect semen from the stallion if he is anesthetized. This method may be beneficial if an accident strikes and semen needs to be taken from an injured stallion before being euthanized or undergoing risky surgery. However, when used often, these methods trigger a stress response that has negative impacts on animal welfare [36].

2.2 Semen and sperm parameters evaluation

2.2.1 Semen volume

The amount of semen in equine is approximately 50 mL or greater [37]. However, the semen volume tends to vary with species, breed, age and environment [38]. Semen volume and sperm concentration in stallions vary according to season and photoperiod [39]. When breeding mares using AI, an adequate fertility can be obtained with as little as 1/2 mL of semen [9]. Despite the rising desire for breeding stallions to compete at the highest levels, little research has been undertaken to determine how equestrian disciplines and competition level affect semen quality and quantity [40]. Moreover, semen volume is not thought to have a direct impact on fertility [41].

2.2.2 Semen colour

The collected semen sample can be milky white, evenly turbid, free of clots and odorless [37]. Consistency changes of semen colour, from creamy or milky to watery, considered is an indicator of low sperm count/ concentration (oligozoospermia) or the complete lack of sperm (azoospermia) [42]. The physiological colour of the stallion semen is pale or whitish-grey.

2.2.3 Sperm motility and velocity parameters

To improve the sensitivity of diagnostic tests for stallion fertility, emphasis has been placed on assessing the functionality of these sperm cells *in vitro* with specific reference to their motility and their capacity to engage in the complex cascade of cellular interactions that culminate in fertilization [43]. The introduction of computer aided semen analysis (CASA) systems to objectively quantify the various elements of sperm movement has improved the diagnostic power of sperm motility assessments [44]. An analysis of sperm motility plays a significant role in determining fertilizing potential, as sperm motility is only one attribute of the sperm [45]. The objective of estimating sperm motility is to determine the percentage of motile and the proportion of progressively moving sperm [46]. The CASA technology was developed in 1980s for analyzing sperm motility and velocity parameters and has been successfully utilized in enabling this field of research focus [44]. Therefore, sperm motility should be evaluated alongside other parameters such as sperm velocity, morphology and viability, when calculating the fertility potential of the sperm [47].

2.2.4 Sperm morphology

An analysis of sperm morphology plays a significant role in any breeding soundness study [48]. Sperm morphology analysis requires specialist tools, the technician's

knowledge, experience and frequent time and patience [49]. Due to its moderate-to-high connection with stallion fertility, sperm morphology stands out as one of the most significant sperm assays [50]. The current classification approach involves keeping track of the prevalence of sperm morphologic abnormalities [47]. However, the presence of several abnormalities on a single sperm is a sign of a more serious disruption of spermatogenesis and may indicate a worsening reproductive outlook [51]. The increase in number of head defects, pyriform heads, nuclear vacuoles, midpiece defects and droplets in sperm is abnormal [52].

Threshold values for the various categories of sperm morphology analysis often fall into one of the following ranges: <30% sperm head abnormalities, <25% proximal cytoplasmic droplets, <10% premature germ cells, 30% morphologically normal sperm [53]. The Society for Theriogenology's most recent recommendations for evaluating the breeding soundness of stallions are used to pick stallions, when bred artificially or spontaneously and may produce at least 75% of 40 or more pregnant mares or 120 or more mares. Some authors have acknowledged the division of sperm abnormalities into major and minor categories, claiming that major flaws lead to early embryonic mortality or preclude conception [54].

2.3 Semen extenders and composition

The improvement in cryopreservation techniques can be obtained by optimizing temperature curves, incubation duration and also extender composition. Currently, the majority of the ingredients in the semen extenders currently in use include micellar milk proteins, egg yolk as a source of phospholipids, antibiotics, and glycerol as CPA (**Table 1**) [59]. With a maximum concentration of 3.5% glycerol is still the CPA that is typically utilized for better outcomes [1]. Glycerol turns harmful to equine sperm once it exceeds this threshold and interest in amides has grown recently [60]. Despite the lower permeability and higher molecular weight of glycerol, formamide and dimethylformamide have a lower molecular weight and higher permeability [61]. On the viability and motility of stallion sperm, fast addition and removal of CPAs have detrimental effects. In other studies, the use of protein-based treatments or modified amino acids in place of glycerol produced positive results [62, 63].

2.4 Type of cryoprotectants used for cryopreservation of equine semen

Many different CPAs and commercially accessible CPAs have been developed in response to market demands (**Table 2**). According to the latest theory, CPAs function by reducing exposure to osmotic stress, stabilizing biomolecules and their structures and limiting the impacts of reactive oxidative species (ROS) [65, 66]. The majority of CPAs exhibit some sperm toxicity. Changes in sperm motility after addition and removal of glycerol CPA may be associated with disruption of plasma membrane integrity and disruption or loss of mitochondrial membrane potential [65]. Numerous steps can be taken to reduce this toxicity; by reducing the CPA concentration as much as possible [67]. Penetrating CPAs work inside cells to replace cellular water when it is pushed out into the extracellular space, thereby preventing the production of internal ice crystals that might potentially burst the membrane [64]. The initial phase of freezing, which typically ranges from −10 to −20°C, is when non-penetrating substances or macromolecules capitalize on the elevated concentrations in the extracellular regions to osmotically drain water from the cells [68].

HF-20 [55]	**Skim milk-egg yolk** [56]	**Modified glucose-EDTA-lactose** [57]	**Tris** [58]	**EquiPlus** [58]
Glucose 5 g	Sucrose 9.3 g	Glucose 6.0 g	Tris 2.42 g	Part A: 95 mL medium
Lactose 0.3 g	Skim milk 2.4 g	Sodium citrate dehydrate 0.37 g	Citric acid 1.34 g	Part B: 5 mL egg yolk-glycerol component
Raffinose 0.3 g	Streptomycin 0.025 g	Disodium EDTA 0.37 g	Fructose 1 g	Minitube-Animal Reproduction Technologies (Germany)
Sodium citrate 0.15 g	Penicillin 25,000 IU	Sodium bicarbonate 0.12 g	Streptomycin 0.08 g	—
Sodium phosphate 0.05 g	Egg yolk 8 mL	Streptomycin 0.08 g	Glycerol 7 mL	—
Potassium sodium tartrate 0.05 g	Glycerol 3.5%	Benzyl penicillin 0.08 g	Egg yolk 20 mL	—
Egg yolk 10%	—	Double distilled water up to 100 mL	Deionized water (made up to 100 mL)	—
Penicillin 25.000 IU	—	Egg yolk 20 mL	—	—
Streptomycin 0.08 μg	—	Glycerol 5%	—	—
Glycerol 3%	—	—	—	—
Deionized water (made up to 100 mL)	—	—	—	—

EDTA = Ethylenediaminetetraacetic acid.

Table 1.
Semen extenders applied to equine semen during cryopreservation.

Penetrating cryoprotectants	**Non-penetrating cryoprotectants**
Dimethylsulphoxide	Egg yolk
Glycerol	Sugars
Methylformamide	Liposomes
Dimethylformamide	Milk proteins
—	Polymers

Table 2.
Examples of penetrating and non-penetrating CPAs [64].

3. Cryopreservation methods for equine semen

3.1 Controlled-rate method

The controlled-rate method (programmable freezer) is the alternative approach since it preserves cellular integrity by achieving the ideal freezing rate [69]. To

prevent the formation of intracellular ice crystals, an ideal rate must be slow enough to allow for sufficient cellular dehydration while being quick enough to prevent sperm from being exposed for an extended period to solutions that are overly saturated with the extracellular environment [70]. Although automated systems for maintaining temperature during cryopreservation have been developed, they are might be expensive [71]. In this regard, several procedures combining various storage quantities and freezing rates have been created and put to the test to increase the effectiveness of cryopreserved equine semen and lessen the harm caused by cryopreservation [72].

3.2 Liquid nitrogen vapour method

The Styrofoam® box might have offered a more variable freezing rate than the programmed freezer because the liquid nitrogen level inside the box is commonly assessed subjectively, subject to evaporation, and challenging to standardize for each freezing rate [73]. The quantity of straws used and the removal of the lid may both affect the temperature of the vapour inside the Styrofoam® box and consequently, the cooling rate during subsequent freezing cycles [74]. The key benefits of the Styrofoam® box freezing technology include affordability, usability, low liquid nitrogen requirements, and portability. In comparison, the programmable freezer is costly, consumes a lot of liquid nitrogen, and it might be permanently installed in the lab. For the other sperm quality factors looked at, there were no differences between the freezing techniques.

3.3 Thawing temperatures for equine semen

For stallion semen, various thawing techniques are utilized, however, it is uncertain which technique (46°C for 20 s for 0.5 mL straws, 46°C for 12 s for 0.25 mL straws, 37°C for 30 s for 0.5 mL straws, 37°C for 60 s and 38°C for 60 s, etc.) is best [75]. Different thawing temperatures must be evaluated in order to determine the optimal temperature for equine sperm and whether the thawing temperature affects sperm quality. The lower temperature (37°C) is more manageable because thawing at the higher temperature (46°C) needs specific equipment and must be done at precisely the right interval to prevent harm to the sperm [73].

4. Synchronization and artificial insemination in equine

Oestrous synchronization is important in equine AI programs. The use of hormonal protocols for synchronization of oestrus and ovulation has increased as a result of AI in the past [76].

4.1 Oestrous synchronization programs

Synchronization of mare oestrus using prostaglandin F2α [77] or progesterone releasing intravaginal devices insertion [78] or in combination with estradiol lowers financial costs and improves the effectiveness of AI by enabling AI to be applied on specified days and times. These protocols usually include the injection of human chorionic gonadotrophin (hCG) or a gonadotrophin-releasing hormone counterpart. The hCG can be utilized to induce ovulation in mares, since it stimulates luteinizing hormone (LH) receptors in the granulosa cells of the follicles [79]. The efficiency

and use of hCG as an ovulation trigger have been proven since it stimulates the final follicular maturation and ovulation and exhibits an LH-like function [80, 81].

Mares undergo seasonal cycling in the spring and summer as a result of the longest period of daylight since they are polyestrous [82, 83]. With one to two follicular waves every cycle developing. The period between ovulations in equine species is around 22 days [77, 83]. Which might be influenced by breed and reproductive stage (*e.g.*, lactation days vs. non-lactating days). From a financial point of view, the breeder's most efficient strategy is to induce and detect ovulation, inseminate at the time closer to ovulation, and use the lowest semen doses (one straw of 0.5 mL of frozen semen or one vial of 20 mL of cooled semen) for less than the cost of one pregnancy, per successful insemination [84].

4.2 Artificial insemination

One of the most effective reproductive technologies for equine breeding is which improves the genetics of the herd and the usage of stallions [76]. An effective use of AI in equine breeding programs can enhance bloodlines available for successful competition horses and significantly improve operating efficiency. The timing of insemination is critical for breeding profitability when using fresh or frozen-thawed semen. Since semen deposition in the mare reproductive tract can occur either too early or too late, the process not only waste important time but also the costs to purchase semen and mare preparation for insemination [84]. Therefore, insemination should take place right before or after ovulation to enhance pregnancy rates [35].

When fresh and cryopreserved sperm are used for AI in mares, a normal physiological inflammatory response takes place that is characterized by rapid migration of polymorphonuclear neutrophils within the uterine to clear bacteria and semen [85, 86]. Moreover, it is of great importance that AI using stallion epididymal sperm results in satisfied pregnancy rates while using low doses of sperm, to make use of all the semen that is in limited supply [35]. Cryopreserved semen has previously been reported to have lower pregnancy rates than fresh or cooled semen. According to more recent research, pregnancy rates with cooled semen are comparable to or even better than those with fresh semen, with little evidence of additional problems [87]. However, stallions are usually selected only on their performance in competitions, without consideration of fertility or semen quality. Therefore, the variable pregnancy rates related to equine AI are explained by this [88].

5. Equine oocytes collection

Oocytes may be collected from ovaries excised from mares in which ovariectomy has been performed, or who have died, in order to preserve valuable genetics that would otherwise be lost [89]. There are currently two approaches to oocyte collection. One approach is the collection of mature oocytes. This is done by aspirating a large dominant follicle right before it ovulates and typically only one follicle is aspirated per cycle. The other approach is the collection of immature oocytes. In this approach all the follicles that are present on the ovary at the time of the procedure can be aspirated regardless of size because the mare does not need to be cycling [89]. Oocyte collection from mares can involve either ovum pick-up (OPU)/ transvaginal ultrasound guided follicular, aspiration and slicing method.

5.1 Ovum pick-up method/transvaginal ultrasound guided follicular aspiration

Despite being a pricey process, ovarian pick-up is increasingly preferred for the collection of equine oocytes. The OPU often referred to as oocyte aspiration or oocyte collection, is the procedure used to transvaginally aspirate oocytes straight from a mare's ovaries. In this procedure, the ovary is visualized using a transvaginal ultrasound probe, and a long needle is passed through a guide in the ultrasound handle, through the wall of the vagina, and into the follicle. The follicle is then flushed several times and the oocyte is aspirated out of the follicle.

5.2 Slicing method

The most popular method for getting more oocytes of higher quality per ovary is by slicing the ovaries. With the aid of forceps, ovaries are typically put in a petri dish containing a collection medium. Using a scalpel blade, incisions were made along the entire ovarian surface. After that, the medium and the collected cumulus oocyte complexes (COCs) are transferred to a petri plate. When collecting the COCs by puncture and slicing methods, the ovaries are kept completely dipped in the medium.

6. *In vitro* embryo production in equine

6.1 *In vitro* maturation

In vitro oocyte maturation (IVM) may be simply performed by placing immature oocytes into the culture. The oocyte's developmental competence (ability to produce blastocysts *in vitro*) following fertilization, however, indicates that a variety of factors influence both the rate of nuclear maturation and the acquisition of cytoplasmic maturation. Oocytes retrieved after a delay of 5–9 hours require more time to culture and have lower developmental competency than those recovered immediately after slaughter [90]. It has to be noted that the base media more commonly used for equine IVM are tissue culture medium 199 or Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F-12), which are generally chosen depending on the preferences of the laboratory where IVM is performed, and core differences exist among them [91]. Furthermore, none of these media have been developed specifically for equine IVM; instead, they were developed for cell culture, albeit equine COCs are capable of maturing with similar efficiency in either medium [92]. To try to better understand the physiological conditions that equine COCs require and improve current IVM conditions, several reports have tried to address the metabolic requirements of equine COCs *in vitro*, the differences between the proteomic profiles of equine COCs maturated *in vivo* or *in vitro* [91], or the differential expression and localization of glycosidic residues in equine COCs matured *in vitro* vs. *in vivo*, among other approaches. All these reports have revealed a specific metabolic profile of equine COCs matured *in vitro* and important differences between equine COCs matured *in vitro* vs. *in vivo* [93].

6.2 *In vitro* fertilization

In terms of assisted reproductive technology, *in vitro* fertilization (IVF) is the most successful method. In IVF, mature oocytes are removed from ovaries and fertilized

in a laboratory using sperm. The most popular treatments for infertile animals are intracytoplasmic sperm injection (ICSI) and IVF. However, the transfer of many embryos and multiple births, undermine the success of IVF/ICSI treatments. Since we now understand a large number of the genes that control COCs growth, we may be able to use these genes as markers to distinguish between healthy and damaged oocytes during IVF. Oocyte IVM has promise as a substitute for IVF, but oocyte quality is still a problem, necessitating the development of new methods in the coming years. Establishment of optimal methods for equine embryo culture has been slow when compared to some domestic species [94]. In part, this delay was caused by the failure of standard IVF techniques in mares and also the scarce availability of abattoir ovaries and the lack of interest from horse breeders and breed associations have been the main reasons for this delay [94].

The fertilization rate is measured 16–18 hours following insemination or ICSI. Presumptive zygotes are cultivated in a specifically designed culture medium to support their growth. On the second and third days after retrieval, they will be assessed. If there are enough embryos that show good growth and development, they can be chosen to develop in a specially created culture medium until they reach the blastocyst stage. The benefits of blastocyst culture are numerous. Fewer embryos can be transferred on day 5 to lower the possibility of multiple pregnancies because the potential for implantation of embryos at this point is higher. Low number of embryo quality reduces the odds for good blastocyst development. Therefore, cycles with a day 3 embryo transfer are advised [95].

6.3 *In vitro* culture

The development of ICSI for the assisted fertilization of equine oocytes has resulted in a renewed interest in establishing optimal methods for embryo culture [94]. Currently, ICSI-produced equine embryos are cultured using media designed for other species or other cell cultures and typically, with the addition of serum. Although systems specifically for equine embryo culture still have not been established, ICSI-produced embryos are developmentally competent and capable of producing live offspring [96]. To promote the development of an embryo, two different types of culture media are available: sequential, or two-step, media and single step media. The key distinction between the two methods is whether the culture media is changed or cryopreserved between the fertilization check and embryo transfer. There are variations amongst laboratories in terms of preferred media, medium additions and IVF techniques [96]. A culture media system is employed in the lab to support the metabolic and physiological phases of embryo development *in vitro* prior to implantation. Equine blastocyst production can be obtained by initial culture in a modified synthetic oviductal fluid, followed by transfer to DMEM/F-12 at approximately day 5.

7. Oocytes and embryos cryopreservation in equine

7.1 Oocytes cryopreservation

There are many ways to evaluate the success of oocyte vitrification, including recovery rate (the number of oocytes discovered after thawing/warming), survival rate (the number of oocytes with an intact plasma membrane and zona after thawing/warming), maturation rate if they were vitrified at an immature stage, cleavage

and blastocyst rates after IVF [97]. When equine oocytes are vitrified at the germinal vesicle stage, it appears that a variety of vitrification techniques can support high maturation rates for example, 61% [98], 48% [99], and 46% [100]. Blastocyst rates per injected metaphase II oocyte have only been 7% [99], 10% [100], and 15%, after ICSI of vitrified-warmed equine immature oocytes that have matured *in vitro* after warming. However, very few studies have shown blastocyst production following ICSI [101].

7.2 Embryos cryopreservation

Small *in vivo*-derived equine embryos (under 300 mm) can be successfully cryopreserved by gradual freezing or by vitrification, with pregnancy rates of 45–67% following thawing/warming and transfer [102]. The usual size of equine embryos created by ICSI and embryo culture is similarly around 300 mm, and gradual freezing or vitrification of these embryos yields continuous pregnancy rates comparable to those for fresh *in vitro* production embryos [103].

7.3 Cryopreservation methods for equine oocytes and embryos

7.3.1 Slow-freezing method

During the slow-freezing method, the embryos are exposed to a cryoprotective substance gradually (i.e. stepwise) before being properly cooled in stages (**Table 3**). Glycerol (10%: 1.36 M) is the "traditional" CPA for slow-freezing equine embryos, and the embryo is equilibrated to it by incubating it in 2–4 solutions in increasing concentration [5]. There are two threats that the embryo is vulnerable to during slow-freezing which are ice crystal formation and dehydration. These approaches slowly dehydrate cells during freezing and prevent the development of intracellular ice crystals by cooling at controlled rates, typically between 0.3 and 0.5°C/min [104]. The extracellular fluid that is left behind becomes more osmolarity as cooling progresses because extracellular ice slowly develops while leaving behind solutes. Since the intracellular fluid has not yet frozen, water that flows out of the cells dehydrates them to balance the quantities of intracellular and external solutes. Slow-cooling techniques have not been routinely used in commercial programs for equine embryo transfer, except for Hinrichs [105], who successfully transferred frozen and thawed embryos of less than 220 m in diameter and recorded pregnancy.

7.3.2 Vitrification method

The main alternative to slow-freezing is vitrification, or ultra-fast freezing, which causes an immediate change in the liquid state of both intracellular and extracellular fluids to a solid, glass-like phase (or "solidification") without ice formation [5]. However, solidification is only possible when very high CPA concentrations are used (about 4–5 times greater than for slow-freezing) and when the temperature is dropped drastically, i.e. *via* submersion in liquid nitrogen [106]. Standard 0.25 mL straws generate a cooling rate of about 2500°C/min when submerged in liquid nitrogen, but the freezing rate can be increased even further to about 20,000°C/min [5], by lowering the volume of the medium surrounding the embryo (open-pulled straw technique), stretching the straw to reduce its internal diameter, or using cryotops or cryoloops [107]. Rapid passing through the severe cryoinjury zones is ensured by the

Conventional slow-freezing method	Vitrification
Standard 0.25 mL freezing straws	Numerous devices for loading embryos and oocytes (conventional straws, OPS, cryoloops, cryoleaf)
Minimal cryoprotectant concentrations	High cryoprotectant concentrations/reduced volume and time with vitrification solution
Seeding at −5 to −7°C, controlled slow cooling (0.1–0.3°C/min)	Ultrarapid cooling rates (−2500°C/min or 20,000°C/min using OPS or cryoloop)
Plunging at −30 to −70°C and storage in liquid nitrogen (−196°C)	Plunging into liquid nitrogen (−196°C)

OPS = Open pulled straw.

Table 3.
Oocyte and embryo cryopreservation methods [6].

faster cooling rate, which also lowers the necessary CPA concentration. Oocyte and embryo cryopreservation protocol are expressed on **Table 3**.

The main disadvantage of vitrification is the toxicity due to the high quantities of CPA utilized in embryos or oocytes [108]; therefore, it is crucial to strictly follow the suggested intervals for immersion in the different solutions, especially the final solution with the highest concentration of CPA [107]. This is more challenging than it appears since solutions with high CPA concentrations are highly dense and the embryo sinks unexpectedly slowly; success with vitrification consequently requires a skilled personnel in identifying and manipulating embryos in dense solutions [109].

7.4 Thawing temperature for equine oocytes and embryos

To avoid water recrystallizing during thawing, which could lead to damage to ice crystals, a rapid temperature change is desirable. Great care must be taken to prevent osmotic shock from the penetrating CPA, which is now present in the intracellular region in very high concentrations [106]. To avoid this, a second non-penetrating CPA is being utilized. The concentration of the non-penetrating CPA steadily increases as the penetrating CPA diffuses out of the oocyte and this process continues until the oocyte is placed back into the usual culture media [6].

8. Embryo transfer in equine

During embryo flushing, the entire uterine lumen is allowed to fill by gravity flow with flushing medium, such as DPBS either supplemented with a protein source (fetal calf serum or bovine serum albumin) to prevent the embryo from sticking to the silicone or plastics of the flushing tool. Currently there is commercially ready-to-use equine embryo flushing media. After filling with flushing medium, the uterus is gently massaged on the rectum to ensure that the entire lumen is flushed.

On day 7 after ovulation, the donor mare's embryos are typically removed [7]. According to Brinsko et al. [47], embryos are harvested 1 day later if the mare was bred to frozen semen as opposed to fresh or cooled semen because the mare embryo grows incredibly quickly from a morula to an expanding blastocyst (day 5–7), which results in unique characteristics defining embryo development. The use of surgical transfer to ARTs is restricted, according to studies, because nonsurgical transfer has resulted in pregnancy rates greater than 75% per embryo transferred at day 15 for

Grade	Pregnancy/transfer	Success rate (%)
1–2	2.921/3.426	85.3
3	99/154	64.3
Total	3.020/3.580	84.4

Table 4.
Success for in-clinic (on-site) transfers, 1998–2022 [111].

Grade	Pregnancy/transfer	Success rate (%)
1–2	3.266/3.866	84.0
3	212/399	53.1
Total	3.478/4.285	81.2

Table 5.
Success for transported transfers, 1998–2022 [111].

more than 8 years [9]. Additionally, on day 9 or 10 of gestation, the recipient mare is checked for pregnancy for the first time, and on day 16 of gestation (9 days after transfer), a clear positive or negative result is known [110]. Moreover, recipient mares have pregnancy rates, which are roughly 75–80% at 14 days and 6–70% at 50 days [96]. The succeeding of mare embryo transfers from 1998 to 2022 are shown in **Tables 4** and 5.

Young maiden mares or mares who have recently given birth to a foal to term are the ideal recipient mares; an older maiden mare or a mare with a history of subfertility would not be good choices as a recipient for a valuable embryo [7]. In addition, embryo transfer can be done on mares that are 2 or 3 years old or yearlings to help them start producing 1 or 2 years earlier than with traditional breeding [47]. The recipient mare should have ovulated 1 day before the donor mare to 3 days after the donor mare when choosing the right recipients for a given donor mare [10]. Prior to transfer, the recipient mare should be examined by palpation and ultrasonography per rectum; the mare should have healthy uterine tone and a tightly closed cervix, which are signs of an acceptable level of circulating progesterone [47].

9. Conclusion

Cryopreservation of embryos has the potential to significantly improve equine embryo transfer's adaptability and lower its expenses. Furthermore, given sufficient operator skill and either gradual freezing or vitrification, cryopreservation of tiny embryos can be reasonably successful. Other stallions' sperm might be able to tolerate the cryopreservation process effectively, while others might not.

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Conflict of interest

There are no conflicts of interest.

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Chapter 5

Assessment of Horse-Human Interaction with New Technologies

Cenk Aydin and Nilay Seyidoglu

Abstract

The relationship between horses and human is unique over the course of the story. The first researchers focused on the horse-human bond as a friendship and believed that it improved the welfare of horses. Today, the role of horses is different from that of history. However, the overall perception is that the horse-human connection requires significant interpretation and explanation. The most important aspect of communicating with the horse is that it is silent. To achieve that, the well-being of horses must develop a good physiological and psychological awareness. To this end, the advancement of sensors and wearable technology creates a facility for measuring and exploring the natural environment. Among the amazing advancements in this area are smartphones, flat screens, high-speed cameras, analog software, GPS tracking, and Bluetooth. In this chapter, we will attempt to explain the emphasis put on the importance of the horse-human bond and interaction for animal beings. Furthermore, we will review the roles of new technologies in the physiological and psychological situations of the horse, and studies of horse-human connection belong to recent research. In this way, we will have put a vision on the side of animals on the horse and the human connection and interaction.

Keywords: human-animal bond, horse-human interaction, welfare, equine technology, physiological and psychological horse features

1. Introduction

Horses have been domesticated about 4000 B.C. These interesting animals have been improved in a number of human areas such as horse racing, training, and assisted therapy. Humans have been attempting to understand their behaviors in a more specific way. There are several questions and suggestions for developing their benefits and potential.

The assessment of links between humans and horses is related to their differences in behavior and health status. The theory of human-horse interaction is grounded in the physiology of humans and horses. The researchers were studied to elucidate their similarity and mutual necessity. Understanding this relationship raises awareness about their behavior in all situations (**Table 1**). Therefore, it also makes it possible to interact with several professionals such as veterinarians, horse owners, horse trainers, and horse breeders.

Human facial expression	signals for social bonding, aggression, avoidance, and navigation of future encounters
Human body posture	hiding or approaching from behind of a horse / human moving helps to elicit horse behaviors
Human attentiveness	a horse walks around to face the inattentive person horse nudges the inattentive person
Human personality (for riders)	correlate with behavior patterns of the ridden horse
Human forced contact (in early life)	induce reluctance for human contact

Table 1.
Emotional cues for horses' attention with humans (prepared from kinds of literature).

The human-horse connection has been interesting to many researchers and well-documented over the years. More specifically, it is of increasing importance to monitor and analyze their physiology and psychology. Today, a variety of intelligent technologies are combined with the analytical method. Given that the technology is more useful and more recent, the results of the analysis are more telling and interesting. In this chapter, we seek to understand the role of new technologies in the connection and interaction between humans and horses.

2. Basic physiological and psychological features of the horse

Horses share the same physiological features as humans and domestic animals. However, they have some important characteristics like slow breathing and resting heart rate that ensure their long-life span. Besides that, horses have excellent senses like humans, however, some of the senses are more powerful than humans and other animals.

Horses have similar anatomical and physiological adaptations as other herbivores. They have strong and high-crowned teeth for eating plants and also have a long digestive tract for cellulose digestion. They have extremely large eyes that are set on the side of their head and peripheral vision with a 340-degree arc. It offers a panoramic view of the predators. Also, horses hear better than humans at lower frequencies. It is necessary to ensure that the horse can see you or know that you are getting closer to avoid being afraid. Horses can appreciate the senses of smell and taste with their food. In particular, their nose has a broader scent detection area than humans. All these senses may be described as making horses special [1].

Horses have important physical features such as hooves, strong leg muscles, and senses. They may sleep upright and not vomit if they wish. They continue to be alert and sensitive. One of the horses' natural abilities is movement. They have heavy muscles in the upper legs in improving their stride. Moreover, their legs are supported by powerful tendons and ligaments that allow the legs to withstand and be adaptive. Based on these physiological characteristics and the use of genetics, horses can be said to be suitable for racing. In addition, they are outstanding athletes whose performance improves as a result of regular exercise [1].

Horses are a language of their own. These are social animals. They understand humans and they can interpret human emotions. To do so, voice communication and body movements are important. Horses also have a sixth sense that helps them feel fear,

anger, joy, anxiousness, and relaxation. They are very smart and have great memories. Horses may never forget, but they forgive. There is an increase in research to elucidate the mystery of these animals in teaching, training, and behavioral characteristics.

3. The potential of the human-horse bond

The mystery of the connection between the horse and humans persists despite years of research. People have horses depending on their needs and personality. Horses have a very important place in that relationship. These are magical and gorgeous animals. They have a strong connection to the use of visual, vocal, and physical signals that make these animals unique and highly social.

A variety of people have been linked to the horse's human bond, especially professional and nonprofessional riders, veterinarians, therapists, breeders, and farriers [2]. The researchers tried to highlight the importance of knowledge and understanding the behavior of horses. The main reasons are difficulties in riding situations, different horse movements, training methods, and the need for horse-rider combinations or owner-horse relationships [2–6]. Depending on the pieces of literature, problems during riding, foot injuries, accidents followed by race or other conditions, and various occupational risks were recorded. Besides that, emotional states involving several conditions of horses, the humans' attitude toward horses' behaviors, and the relationship between horses and riders have also been reported [7, 8]. Horses are also said to have such reactions to their temperament or experience with humans. Signals based on changes in horse behavior and human approaches have a wide range of severity and still have a piece in the puzzle. But studies are still underway.

Various measures make it possible to learn the link between the horse and the human. There are a couple of target tests or reactions designed to measure physiological and behavioral situations. The most important ones are the ways of measuring the responses of the animals during approaching, such as in fitting, catching, or touching, and also veterinary practice. In this context, there are some tests performed with sensors placed on the animal's various points or ankles, as well as sensory tests [2, 9]. In addition to behavioral measurements, the researchers were interested in specific important physiological measurements, in particular, heart rate, heart rate variability, temperature, respiratory parameters, and cortisol levels [2, 9–11]. Heart rate and associated parameters of heart and breathing have been studied, especially in the context of stressful events and training. In addition, cortisol or saliva and blood glucose were measured to provide the studies for humans and horses. Therefore, all these parameters help to elucidate the behavioral differences in the human-horse interaction, as well as to improve the understanding of the human horse's connection.

Horses have been domesticated over the years and have roles, especially in work, sport, and companionship. However, horses have been critical as therapeutic animals in recent years. Horse-assisted therapy programs are conducted for a variety of therapeutic reasons [12, 13]. These programs have significant benefits such as helping people focus, calmness, mood stabilization, and improving prospects. Several important conditions in particular autism, cerebral palsy, Down syndrome, paralysis, and neuromuscular injuries are included in these treatment programs. As a result, emotional regulation, reintegrating into civilian life, and strengthening communication skills are the most beneficial outcomes for these individuals. By the way, researchers continue to be interested in many projects.

There is clear evidence that biological functioning, homeostasis, and well-being are important notions of animal feelings. Especially in the human-animal connection, all these concepts guide this relationship and give humans a vision to understand the state of animal well-being including physical, social, and emotional needs. There are several new technological processes, gadgets, and applications that can be used to clarify the potential for human-horse binding. In addition, technology can be particularly helpful for these therapeutic conditions.

4. Technology in equestrian

Equine technology is assisting horses with education, racing, and assisted therapies. This technology includes certain mechanical equipment such as sensors, wireless, trailer cameras, automatic tracking cameras, and several apps and gadgets for horses and users. Interestingly, horse technology has been of concern to humans for many years. For example, the first mechanical horse was invented in 1867, although it is unlike today's mechanical horses. Technological advances over the years have allowed equine technology to have a field that is efficient and attractive to the world of equine science [2, 14].

In earlier reviews, it was reported that studies were especially based on estrus detection, milk analysis, and improvement of livestock farming for animals. There were several cameras (digital, thermal, or high-resolution digital cameras) used for body condition scoring, early detection of diseases and lameness, and quantifying pain and stress. Also, some important gadgets such as accelerometers, positioning GPS, load cells, and electronic noses were used for these purposes. All these farm technologies allow to appear the status of animals and help animals' management and health. They were also accepted as practical and economical in this field [14–16]. Researchers suggested that lameness, inflammations, and injuries in horses can be detected by thermal imagining [14, 17]. Also, body temperature changes due to environmental differences or illness can be measured by this imagination. Besides that, to measure the changes existing by velocity and gravity, an accelerometer can be used. Leg-mounted accelerometers, commercial pedometers, and other devices can calculate the basic activity of animals. Researchers have studied the accuracy of all walk movements by this method [17–19]. However, battery power was reported as the main problem with this method. It was indicated that with the improvement of battery technology, this equipment would be more useful and technical in this area [20].

In recent years, equine technology has been increasingly more available and effective. The development of methods has changed over the years for monitoring horses for health, management, and work. Particularly, micro-technology for wearable horse gadgets and tools becomes important as well as sensors for monitoring horses in any training or at any time. The wearable belts, implantable devices, and surface sensors (electrodes) can record long-term electrocardiographic and respiration changes [19, 21–23]. Besides that, physiological parameters, especially heart rate and heart rate variability, can be used to determine the physiological or psychological stress of animals and can be drawn regarding the horse's behaviors in a comfortable manner. In addition, some studies about continuous automated monitoring systems with telemetry systems were reported [24, 25]. Using a telemetric system can support to record of electrocardiographic parameters besides recording the grazing or feeding behaviors of a horse over a long period. The telemetric system includes a wearable device or a sensor and a data collector such as a smartphone or a powerful device. The wearable

devices can collect sampling frequency from the wearable stuff onto the animals, or some of the sensors embedded into the skin [26].

Understanding how to enhance the welfare and improve the health of horses and how horses can communicate with each other and humans are important to learn them all well. In recent years, decoding the human-horse interaction has been interested dramatically. Horse-assisted therapy, horseback riding in therapeutic programs, and horse companionship on the life quality of people and horses have been studied for this interaction [2, 27–29]. Several tests have especially been designed for the human-horse bond approach as observer ratings, behavioral tests, performance tests, and, physiological measures (**Figure 1**) [9, 14].

The behavioral activities of animals such as feeding, movement, gait, or physical activities are related to their well-being. Changes in behavior may be missed by visual monitoring. Thus, certain sensors, automated acceleration sensors, collars, holters, systems, monitors, or, GPS can be used to detect behavioral models of animals. These technologies can be designed for visual monitoring, physical and behavioral measurements, and the provision of information on the physiological contents of animals.

Field studies on the grazing, feeding, or gait of horses rely mainly on observing methods. These approaches restrict the precision and amount of data. However, some researchers validate the measuring systems for 24-hour data recording, including specific behaviors (eating, drinking, etc.), grazing time, number of bites, and chewing [25]. Systems with truly wireless truck float camera systems such as EquiWatch that have a pressure sensor and a triaxial accelerometer can be integrated into a holter plate. This system allows us to understand the biological, nutritional, and environmental factors of horse grazing behavior. In addition, the monitoring of eating behavior applies to the evaluation of equine intestinal health, diet, and chewing behavior.

Some studies reported that smart devices like smartwatches wrist-worn devices, leg sensors, accelerometers, and GPS sensors can be performed for horse gait recognition. These devices have been developed for a smartwatch-based framework that can record the movement of the horse and calculate the characteristics of the gait. This means that performance and other health problems due to irregular gait can be detected and improved [28, 30]. In addition to this technology, hoof sensor systems were maintained to collect data and measure the pressure under the hooves, the gait

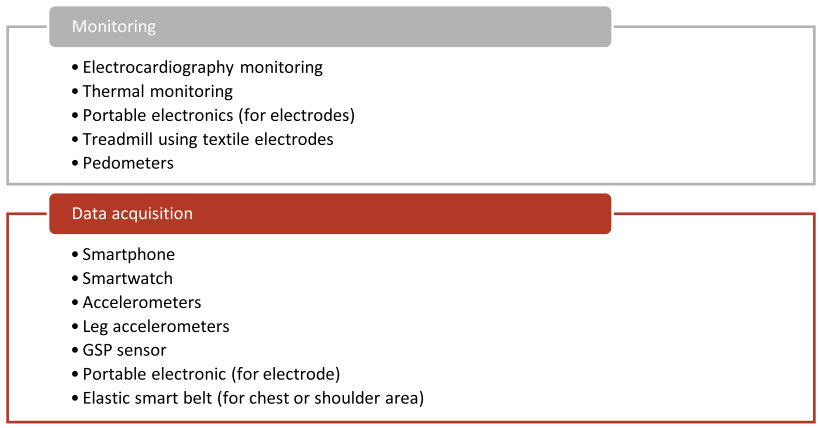

Figure 1.
The monitoring system and wearable devices for data acquisition. This can be enhanced according to literature and technological innovation.

classification, and the performance over the years [31, 32]. The researchers used the mini-wireless placed on the hooves, and evaluated the time differences between the hooves and the hooves for grading, speed, and trimming conditions [31]. Hagen et al. [32] used sensors, such as a 3D accelerometer and a gyroscope for the four hooves. They have demonstrated that this technical innovation is a practical method for observing specific motion events.

Physiological measures during human interaction with the horse can help understand the mental and psychological situation of horses as well as physiological differences. From an early age, handling by a human gets the horses a positive emotional relationship. Physical contacts, such as handling or grooming, have been reported to be important, especially in reducing heart rate (HR) and heart rate variability (HRV) [6, 33]. On the contrary, some researchers have reported an increase in HR and HRV results for that physical contact [34].

Among the studies, polar heart rate monitors and portable electrocardiograms (ECGs) were used for heart rate and HRV measurements. Hockenhull et al. [35] recorded heart rate data from all horses and handlers who wore polar monitors. The researchers positioned the data loggers on the surcingle around the girth of horses and also on the handler's wrist for recording. The beats per minute (bpm) were calculated every 5 seconds for the HR of both handlers and horses. In this study, familiar and unfamiliar handlers' differences were found in terms of HR (bpm). Another interesting study by Schmidt et al. [36] indicated that HR increases during transportation, grooming, and saddling. In addition, changes in HRV during transport resulted in an increase in friendly activity and a decrease in vagal tone only at the beginning of transport. The researchers also found that the level of change in HR and HRV was caused by transportation adaptation. In this study, the recording of heart rate was measured by a mobile system attached to the horse's chest with an elastic girth. For this purpose, the positive electrode was applied to the right shoulder and the negative electrode was located in the middle of the left thorax. Additionally, the electrodes were attached with a second girth including a pocket for the recording watch.

The researchers also applied textile sensors to portable systems such as some electrodes produced by textiles. This textile-based system for ECG monitoring of horses consisted of intelligent garments and wearable electronics. Guidi et al., [29] studied broadening our knowledge of physiological and behavioral changes in horses with this system. Two textile electrodes and a strain gauge sensor were used to scan for ECG and respiratory activity. An elastic waistband was covered around the breast behind the shoulder of the horses. ECG waveforms have been recorded from textile electrodes. In this study, it was pointed out that the emotional state of humans and horses can be measured. In addition, it was concluded that this system can be a quantitative measure of the connection and interaction between human and horses, particularly for equine-assisted activities and therapies (EAAT).

Based on the literature, it is noted that the level of stress and quality of life of the animals is important. Specifically, studies on EAAT and other therapeutic programs attempt to present stress through hormonal indicators as well as ECG changes. Malinowski et al. [37] investigated the effects of HRV in horses in EAAT programs. The ECG telemetric heart rate monitors were designed for 3 out of 5 days on horses in the study. ECG parameters were recorded for a total of 3 hours and analyzed for 10 minutes. This resulted in a decrease in HR, but no difference was observed between HRV, SDNN, and the LF/HF ratio. Apart from these findings, it has been reported that animal therapy programs improve people's

cognitive, psychological, and social situations. The assumption is that EAAT programs result in statistically significant health benefits with improved blood pressure, heart rate, and anxiety levels.

By the way, the biggest problem with wearable systems is the artifacts that are included in the noise of acquiring biomedical signals. Researchers have attempted to verify protocols and monitor environmental changes for this purpose. Stress is a significant environmental factor for horses and other animals. Evaluating stress in horses and getting control is important for studies. Indeed, the researchers aim to study these systems in animals in a reliable condition and without artifacts, even if stress. Also, the performance of the wearable systems is tried to improve.

5. Future perspectives and conclusion

Horses are so emotional animals. Humans have to learn the mindfulness of horses. Science and technology have addressed the important questions of human and horse bonds over the years. Researchers have tried to find out how and why horses react to humans for this purpose. Also, results give us features and possibilities of the human-horses bond and interaction.

There is a tendency that usage of wearable sensors and smart technology in human-horse bonds. However, the technology is based on more efficiency and more microstructure as well as convenience and cheaper. Also, it is considered more safety. Besides livestock health, the technology has room in clinical applications and the therapeutic field. Therefore, technology, wearable sensors, and monitoring devices can be called important promising strategies.

Animal monitoring systems, even for health or other issues, provide the status of animals for research. According to kinds of literature, technological devices are going to be more expected for requirements, and several health monitoring system has been developed. This gives a sight and an idea about horses to veterinarians, clinicians, and horse riders. It can be said that smart technology will improve the animal's health, behavioral features, human-horse bonding problems, and horse training.

Conflict of interest

"The authors declare no conflict of interest."

Acronyms and abbreviations

arc	A degree (in full, a degree of arc, arc degree, or arc degree)
ECG	Electrocardiogram
HR	Heart rate
HRV	Heart rate variability
EAAT	Equine-Assisted Activities and Therapies
SDNN	Standard deviation of NN interval (between two detected heartbeat detections)
LF	Low frequency
HF	High frequency

BPM	Beats per minute
HRV variables	Parameters for determining an HRV function
LF/HF ratio	The ratio reflects the sympathovagal balance.

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Chapter 6

Non-Radiolabeled Study to Evaluate the Metabolism and Residue Kinetics of Veterinary Drugs in Food-Producing Animals and Anti-Doping Analysis in Equine Sports: Application of High-Resolution Mass Spectrometry

Wei Tong, Rose Huang, Ruediger Narbe and Laura Letendre

Abstract

The chapter discusses an alternative approach to conduct the metabolism and residue kinetics of veterinary medicine for food-producing animals using high-resolution mass spectrometry in a non-radiolabeled study under the scope of VICH GL46. The application of high-resolution mass spectrometry for anti-doping analysis in horse racing is also briefly discussed. Section 1, Introduction, gives an overview of high-resolution mass spectrometry, metabolism and residue studies, and general requirements for traditional radiolabeled metabolism and residue kinetics studies. The limitations of conducting a radiolabeled residue study will also be discussed. Sections 2 and 3 review the techniques of non-radiolabeled (cold) metabolite profiling/identification with high-resolution mass spectrometry (various data acquisition functions, data mining tools, and the semi-quantitative and quantitative methods for metabolites). Section 4 presents two case studies, where high-resolution mass spectrometry was used for the marker residue and marker to total residue ratio determination in residue studies for gamithromycin in sheep and ciclesonide in horses. The application of high-resolution mass spectrometry in anti-doping analysis and the study of the metabolism of steroids in racing horses is briefly discussed in Section 5. Section 6 summarizes the advantages and disadvantages of the high-resolution mass spectrometry technique and future perspectives for the use of high-resolution mass spectrometry in other fields of veterinary drug development.

Keywords: metabolism and residue kinetics, marker to total residue ratio, tissue drug residue analysis, edible tissues, high-resolution mass spectrometry, food-producing animals, VICH GL46, anti-doping, sport horse

1. Introduction

The study of drug metabolism and drug disposition is essential in drug discovery, research, and development in both human and veterinary medicines. Drug metabolism is generally a detoxification process in which drugs are converted to molecular structures/components (metabolites) that are more readily eliminated from the body. Many of these metabolites are pharmacologically inactive and chemically stable, but in some cases, they are active, toxic and/or unstable. Metabolism impacts drug exposure, clearance, and drug-drug interactions and it is therefore essential that the formation of metabolites is investigated during veterinary drug development [1]. This should comprise not only the identification of metabolites but also the determination of the pathways involved in the metabolism of the active substance, to establish potential drug interactions. The relative contribution of the different routes of excretion of the total substance [active substance and metabolite(s)] should be quantified (e.g., expressed as a percentage of the administered dose). This is especially important to veterinary medicine because the drugs and their metabolites (collectively defined as "residues") can enter the human food chain as well as the environment. Therefore, metabolism and residue kinetics studies in veterinary drug development are needed for human food safety evaluation to ensure that food derived from treated food-producing animals is safe for human consumption [2]. The Veterinary International Conference on Harmonization (VICH) guideline GL46 [3] states that "The human food safety evaluation of veterinary drugs help ensure that food derived from treated animals is safe for human consumption. As part of the data collection process, studies should be conducted to permit an assessment of the quantity and nature of residues in food derived from animals treated with a veterinary drug. These metabolism studies provide data on (1) the depletion of residues of concern from edible tissues of treated animals at varying times after drug administration, (2) the individual components, or residues, that comprise the residue of concern in edible tissues, (3) the residue(s) that can serve as a marker for analytical methods intended for compliance purposes (i.e., monitoring of appropriate drug use), and (4) the identification of a target tissue or tissues, as applicable to national or regional programs.". One of the important objectives of these studies is to determine the marker residue to total residue ratio (M/T) in edible tissues of food-producing animals to ensure the marker used to determine the withdrawal period is representative, measurable and has a known relationship to the total residue concentration in tissue. Traditionally, *in vivo* metabolism and residue studies in veterinary drug research were conducted to obtain quantitative information using radiolabeled drugs [3]. Radiolabeled drug metabolism studies are required for product registration of all drugs used in food-producing animals to establish maximum residue limits (MRLs), drug monitoring methods, and withdrawal periods. These radiolabeled drug metabolism studies are expensive and time extensive to perform and must be conducted for every species that will be treated. In some cases, radiolabeled studies are difficult to conduct, e.g., in the case of inhaled or topically administered drugs. VICH guideline GL46 [3] also indicates that the metabolism and residue kinetics in food-producing animals may be conducted by an alternative approach [3]: "Alternative approaches (i.e., not using radiolabeled drug) to characterize the components of the residue in food derived from treated animals might be suitable." One of the promising techniques to approach this is the use of high-resolution mass spectrometry (HRMS).

HRMS has been widely used in human pharmaceutical R&D for metabolite profiling and identification during early drug discovery and first-in-human (FIH) studies during development [4]. FIH studies are a critical step in drug development to obtain an early readout of human metabolism by profiling the metabolites from plasma samples collected during single and multiple dose studies and to ensure the major metabolites are exposed in preclinical species. Recent advances in data acquisition functions and data mining tools have greatly improved metabolite profiling by HRMS [5–9]. Software packages are also available from the major mass spectrometer vendors such as Thermo Fisher Scientific, AB Sciex, and Waters. It is now a common practice to use non-radiolabeled drugs to study metabolism without the disadvantage of possibly missing major metabolites. One of the advantages of HRMS is the ability to perform a quantitative–qualitative workflow [10, 11], i.e., the simultaneous quantitation and metabolite structural identification using post-acquisition data mining.

Compared to the large number of published papers describing the use of HRMS in human pharma metabolite profiling and identification, the literature reports on the use of HRMS in veterinary drug analysis, especially with regard to metabolism and residue kinetics supporting human food safety, are scarce. Nonetheless, EU and US regulatory agencies for veterinary medicine have recognized HRMS significantly expands the scope of analytical methods used to monitor chemical residues. There are a few published guidelines containing information on the confirmation of the identity of drug residues by HRMS [12–14]. In addition, HRMS has been applied in monitoring multiple pesticides and veterinary drug residues in food including edible animal tissues [15, 16]. In the field of anti-doping in horse racing, HRMS also finds more application in studying the metabolism of anabolic/androgenic steroids (AASs) in order to find better ways of detecting the abuse of endogenous steroids, especially the new and more evasive AASs including designer steroids [17].

In order to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals in a non-radiolabeled study, the first step is to obtain the metabolite profiles for each tissue matrix without missing any major metabolite(s). Next is to quantitatively or semi-quantitatively estimate the relative amount of each residue (metabolite and the parent drug) in each tissue matrix, choose the marker residue and determine the maker to total residue ratio. In this chapter, we will review the advance of high-resolution mass spectrometry instrumentation over the past 20 years, data mining software, quantitation/semi-quantitation approaches, as well as our experience in applying HRMS in the metabolism and residue kinetics studies supporting human food safety.

2. Advance of HRMS in instrumentation and data mining strategy

The advent of liquid chromatography coupled with mass spectrometry (LC-MS) in the late 1980s and early 1990s revolutionized the way of conducting drug metabolism research in drug discovery and development, especially for metabolite profiling and identification [18]. Before the 1990s, individual metabolites were normally isolated manually by various separation and preparation methods and analyzed by mass spectrometry in an offline fashion. The emergence of commercial triple quadrupole and ion trap mass spectrometers has modernized metabolite identification methods. The application of LC-MS, coupled with the use of radiometric flow detectors enabled the generation of quantitative metabolite profiles in biological matrices

in radiolabeled absorption/distribution/metabolism/excretion (ADME) studies. A typical instrument setup and workflow are illustrated in **Figure 1**. Generally, *in vitro* (microsomal or hepatic incubations) or *in vivo* samples (plasma, urine, feces, bile, or tissue) are injected into an HPLC or UPLC after some cleanup steps (e.g., protein precipitation, solid phase extraction, etc.). For radiolabeled drugs, the metabolites in the samples are separated by LC, where the LC flow is normally split post-column to the mass spectrometer and flow scintillation analyzer. Radio-chromatograms and the corresponding total ion chromatograms (may also include data-dependent and data-independent scans) can be obtained simultaneously. For non-radiolabeled drugs, the total LC flow is normally directed to the mass spectrometer. Data mining is then carried out by surveying mass spectra near the retention times of the radio-chromatographic peaks either manually or using the software. Structural elucidation is carried out either manually or with the help of software to assign the proposed metabolite structures. Data-dependent MS/MS data or separate MS/MS or multiple-stage tandem MS (MS^n) run data are used to aid structural elucidation. If necessary, definitive structural identification is performed by isolating the metabolite of interest and performing Nuclear Magnetic Resonance (NMR) analysis.

The 2001 paper by Clarke et al., summarized the approaches starting with triple quadrupole mass spectrometry (precursor ion scan, constant neutral loss scan, and MS/MS) to further structural elucidation with ion trap mass spectrometry (MS^n) and accurate MS and MS/MS [19]. Until the late 1990s and early 2000s high-resolution mass spectrometers in drug metabolism laboratories were relatively rare, limited to big pharma, and earlier generation of quadrupole time-of-flight (QTOF) mass spectrometers. Since then, the development of the QTOF instrument allowed the production of mass spectral resolution from 10,000 to 80,000 at full width at half maximum (FWHM) with routine mass accuracy of ~2–4 ppm and wider dynamic range [20, 21]. In the mid-2000s, mass spectrometers based on the Orbitrap mass analyzer became available [22]. This mass analyzer enables very high mass spectral resolution of up to 1 million FWHM and mass accuracy <3 ppm [23].

It is the combination of a variety of powerful data acquisition functions of the modern HRMS and data mining techniques that take full advantage of the accurate mass and high-resolution capability and enable routine metabolite profiling with

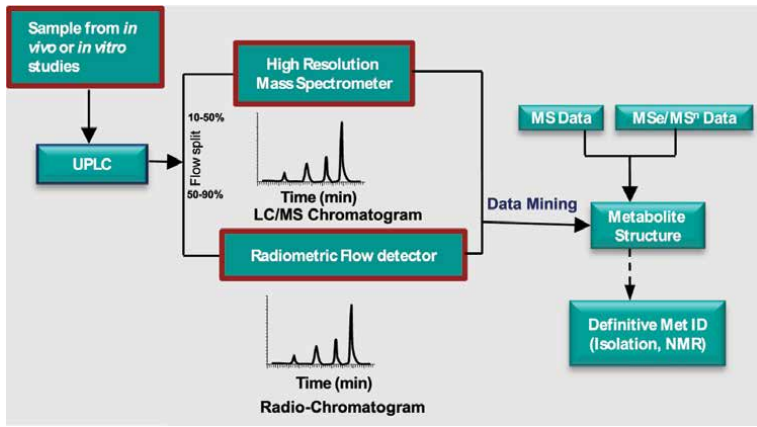

Figure 1.
Typical metabolite identification instrument setup and workflow.

non-radiolabeled samples. We will highlight current practices using HRMS as a platform for drug metabolite identification in the following sections.

2.1 Data-dependent acquisition (DDA)

DDA has been developed to reduce the data acquisition duty cycle and to increase the throughput of metabolite identification [5, 20]. Typically, a full-scan MS is acquired, and the software analyzes the full-scan mass spectra in real-time to determine which precursor ion is subjected for subsequent MS/MS scans based on the selected criteria. After the MS/MS scans were performed, the system cycles back to the full-scan. Ion intensity, accurate mass inclusion list, isotope pattern, pseudo-neutral loss, mass defect, etc. are the commonly selected criteria to trigger the switch from full-scan MS to MS/MS mode.

DDA triggered by ion intensity threshold is the most common, typically referred to as TopN DDA. This generic DDA method does not require prior knowledge of the *m/z* values of the precursors and has been relatively more effective for metabolite identification of *in vitro* samples. Direct use of intensity-dependent MS/MS acquisition for biological samples is often limited by overwhelming matrix interferences.

A more practical use of intensity-dependent MS/MS DDA is to trigger MS/MS with the accurate *m/z* values of the expected or predicted metabolites. This can be achieved by putting the accurate *m/z* values of the expected or predicted metabolites on the inclusion list. After the survey full-scan MS, the software will analyze the full-scan spectrum in real-time to determine if the *m/z* of any ions matched those in the inclusion list within a certain mass tolerance window and above a defined intensity threshold. The software will switch to MS/MS mode and acquire the MS/MS spectrum of the ion of interest. DDA with inclusion list based predicted metabolites enhances the probability of acquiring product ion spectra for low-level metabolites in complex biological matrices. While the masses of the predicted metabolites do not exist in the survey scan, the instrument will switch to intensity-dependent MS/MS acquisition. Both full-scan MS and MS/MS spectra for all the expected metabolites can be obtained from a single LC–MS/MS run [24, 25]. This approach reduces the number of steps used in a manual or traditional data acquisition process and, therefore, increases the throughput of metabolite identification.

There are numerous knowledge-based software packages (e.g., META, MetabolExpert, Meteor, etc.), that use biotransformation knowledge- and structure-dependent rules for metabolite prediction. Database-based systems (MDL, BIOVIA (formerly Accelrys), etc.) use published literature and similarity to parent structures. Structure-based system (MetaSite) considers both enzyme-substrate recognition (which is a thermodynamic factor) and the chemical transformations induced by the enzyme (a kinetic factor) and therefore tends not to over-predict the metabolism, which can be the weakness of knowledge- or data-based software. Data processing software from instrument vendors such as Metabolynx (Waters), Compound Discoverer (Thermo Fisher), MetabolitePilot™ (AB Sciex), Mass Hunter (Agilent), MetaboliteTools (Bruker), and MetID Solution (Shimadzu) all provide a metabolite detection software package to perform targeted searching of potential metabolites based on common biotransformation pathways. Mass-MetaSite (Mass Analytica) is a vendor-neutral software that is capable of handling "all-in-one" HRMS datasets from nontargeted Data-Independent Acquisition (DIA) (Section 2.2) as well as data-dependent MS/MS datasets. However, in the case of major metabolites that are formed through uncommon biotransformation or multiple-step pathways, another

DDA triggering approaches such as isotope pattern or mass defect will be needed to ensure that no metabolites are missed.

2.2 Data-independent acquisition (DIA)

DIA started with the MS^E concept using a QTOF in which two acquisitions are conducted in parallel in full-scan mode, one at low collision energy (~5 eV) and the other at high collision energy (~20–40 eV), and a collision energy ramp can be utilized to obtain wider fragmentation coverage. The mass spectra from low collision energy provide intact molecular ion information, while the mass spectra from the high collision energy contain fragmentation data useful for structural elucidation [26–28]. The MS^E approach does not require prior knowledge of the *m/z* values of potential metabolites and can perform both full-scan MS and pseudo-MS/MS spectra from a single LC–MS run. However, matrix ions and co-eluting metabolite ions make this technique challenging for assigning low-intensity product ions with their precursor ions. Ideally, baseline separation chromatography and samples with a low matrix background (such as *in vitro* incubation samples) will be extremely helpful for MS^E workflow [28]. The technique does not work well with samples from *in vivo* sources with high backgrounds from the matrix, such as samples of urine, bile, feces, and tissues.

Similar technology was subsequently implemented in Orbitrap [('all ion fragmentation (AIF)') and AB Sciex TripleTOF® systems (MS/MS^{ALL} with SWATH™ (sequential windowed acquisition of all theoretical fragment ions) acquisition)]. While AIF with Orbitrap works similarly with MS^E and therefore has similar weaknesses, SWATH™ acquisition fragments all ions across a given mass range in sequential narrow ranges as they emerge from the liquid chromatograph [29]. This approach divides a wide mass range of precursors into multiple narrower windows (~20–25 m/z). The relatively narrow mass window makes it easier to link between the product ions and the corresponding precursor ions and reduces the missing assignment of the product ions.

A hybrid MS^M approach that combines inclusion list and ion intensity-dependent acquisition and all-ion fragmentation acquisition using LTQ-Orbitrap, was developed for metabolite identification [30]. The acquired datasets were then subjected to multiple accurate-mass-based data mining tools such as precursor ion filter, multiple mass defect filter (MDF), product ion filter, and neutral loss filter to search for all metabolites of interest [31]. The MS^M approach is very powerful in achieving fast detection of common and uncommon metabolites for both *in vitro* and *in vivo* samples.

2.3 Other data-dependent acquisition

Isotope pattern-dependent acquisition is also a form of DDA. Halogen (Cl and Br)-containing molecules contain unique isotope patterns which are extremely helpful in the search for drug-related components assuming that these halogen atoms remain intact during biotransformation. With HRMS, more accurate criteria can be set to trigger the DDA. For example, criteria can be set to search for Cl-containing compounds by looking for ion pairs with *m/z* difference of 1.99705 Da and an intensity ratio of ~3:1. Similarly, searching for Br-containing compounds uses ion pairs with *m/z* difference of 1.99795 Da and an intensity ratio of ~1:1. This approach was demonstrated to be extremely effective in metabolite profiling of complex matrices

like bile with the ability to search for drug-derived components and provide MS/MS spectra for structural elucidation in a single LC-MS run [32].

Pseudo neutral loss as a trigger for MS/MS acquisition was developed on QTOF in MS^E mode as a useful way to detect metabolites that give characteristic neutral losses upon collision-induced dissociation, which is particularly useful for detecting common phase II metabolites. This DDA experiment monitors *m/z* differences of ion-pairs (neutral loss) between consecutive low and high collision energy full-scan MS, such as 176.0321of for glucuronides, 129.0426 for glutathione conjugates, and 79.9568 for sulfate conjugates, etc. within a certain mass tolerance window. The specific precursor ions are identified from the low collision energy data and the instrument automatically switches to MS/MS mode to acquire the product ion spectra of those ions [33]. All MS and pseudo-MS/MS and product ion (MS/MS) spectra are also recorded for each sample from the same LC–MS run.

Mass defect-dependent acquisition is developed as another form of DDA utilizing a precursor selection criterion to trigger MS/MS acquisition. Mass defect filter (MDF) was widely used as a post-acquisition data processing method and has been incorporated in the metabolite identification software packages from major instrument vendors. In this case, MDF is applied to analyze full-scan HRMS data and identifies precursor ions with mass defects (see Section 2.4) fall within a specific window of an MDF. Only these ions are automatically followed by MS/MS acquisition. Multiple MDFs are often required to detect different classes of metabolites. This approach performs MS/MS acquisition of both common and uncommon metabolites with little matrix interference in a lot of cases [34, 35]. Currently, this function is only available on AB Sciex TripleTOF® instrument.

2.4 Data mining strategies

HRMS-based data mining strategies can be categorized as targeted and untargeted data mining. Targeted data mining, such as extracted ion chromatogram (XIC), mass defect filter (MDF), isotope pattern filter (IPF), neutral-loss filter (NLF), and product ion filter (PIF), is based on the knowledge of drug properties and relevant metabolic pathways and more suitable for common metabolic pathways. Non-targeted approaches, such as background subtraction, do not require prior knowledge of the drug structure, m/z, or fragmentation and are therefore suitable for both common and uncommon/unknown metabolites. However, eventual structural elucidation of the metabolite will require the structure of the drug.

As indicated in Section 2.1, most of the metabolite data mining software searches for common biotransformation by extracted ion chromatograms with a narrow mass tolerance window (≤5 ppm) and compares the XICs with the control samples to minimize false positives. This is the easiest, most intuitive, and most common approach.

The mass defect of an element is the difference between the exact atomic weight and the nominal atomic weight. Carbon-12 has a mass of exactly 12.0000 Da and therefore the mass defect is "zero", but all other elements have a uniquely different mass defect. For example, the mass defect of hydrogen and oxygen are 0.007825 and − 0.005085 Da, respectively. Therefore, oxidation with an addition of oxygen will introduce a mass defect of −5.1 mDa. Mass defect of common Phase I and Phase II metabolites normally fall within the 50 mDa window of the parent drug or its cleavage product. MDF was first introduced by Zhang et al. [36] as a software filter to remove interference ions of matrices from the drug and its metabolites in LC/HRMS full scans. The drug-related ions are "enriched" in the MDF processed data for further

analysis. Based on the mass defect of the parent drug and its core templates, multiple MDFs each with a narrow MDF filter window of ~50 mDa can be applied to retain the ions derived from the parent drug and its metabolites. MDF has been widely used for both *in vitro* and *in vivo* metabolite profiling [37–45]. Most HRMS vendors provide software packages to perform mass defect filtering. The success of mass defect filtering is also dependent on how complex the matrices are and where the mass defects of the parent drug fall relative to endogenous ions [46].

As discussed in Section 2.3, an accurate mass-based isotope pattern filter is extremely powerful in filtering out the endogenous interference ions for halogen (Cl or Br) containing or stable isotope-labeled drugs [9, 47, 48]. A neutral loss filter is extremely useful for detecting Phase II metabolites. Another useful targeted data mining tool is the product ion filter (PIF). Fragment Ion Search™ (FISh) analysis function in Compound Discoverer (Thermo Fisher) is a PIF that extracts spectral peaks for structurally related features, for example, compounds related to the parent drug that share a common list of fragments.

Although intuitive, the non-targeted data mining tool, background subtraction (BS), is probably the most useful tool for detecting uncommon metabolites [7, 49]. For each ion detected in the analyte file, the algorithm searches the control file if the same ion was also present. If such a target ion is present in the control file within a predefined mass tolerance, the maximal intensity of the ion is multiplied by a predefined scaling factor and subtracted from the intensity of the ion in the analyte file. The algorithm was further improved to tolerate the retention time shift between the control and analyte acquisition runs [8]. The key factor for a better result of background subtraction is the selection of control samples, ideally from the same animals (predose) or animals dosed with the vehicles. A similar function is also built-in to some commercial software packages, for example, the Compound Discoverer (Grouping & Ratios), where the ratios of ions between sample and control are calculated and where a ratio of infinity normally represents a component in the sample but not existing in the control; it, therefore, warrants further investigation.

Although powerful instrument scan functions and data mining tools based on HRMS have been developed, it still needs an integrated approach to ensure no major metabolites are missing from the metabolite profiling and characterization in a non-radiolabeled study [46, 50]. As illustrated in **Figure 2**, full-scan mass spectra are collected followed by data-dependent MS/MS acquisition or non-selective all-ion fragmentation (or MS^E) acquisition using HRMS. Expected metabolites are filtered out by extracting the accurate *m/z* values of these predicted metabolites. The full-scan accurate mass dataset may then be further processed by multiple MDFs, background subtraction and isotope pattern filtering to detect common and uncommon metabolites selectively and effectively. In addition to this, the data-dependent MS/MS or non-selective MS/MS dataset can also be used to search for metabolites by applying an accurate mass production ion filter and neutral loss filter. In some cases, such as *in vitro* incubation samples, the UV signal, especially at higher absorption wavelengths (>250 nm), can be helpful in finding metabolites. Once the metabolite profiles are complete and tentative structures are proposed, further characterization may be warranted to understand the precise nature of the metabolic modification such as position and definitive structures.

In the case of residue studies for veterinary drugs, GL46 [3] indicates that the degree of characterization and structural identification depend on several factors which include the amount of residue present, the concern for the compound or the class of compounds to which it belongs, and the suspected significance of the residue

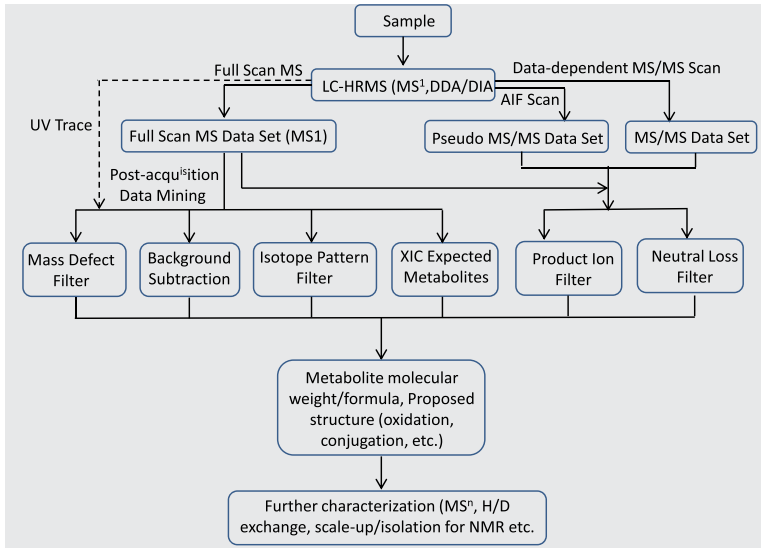

Figure 2.
Strategies of metabolite profiling and identification using high-resolution mass spectrometry.

based on prior knowledge or experience. GL46 suggests as a point of reference, major metabolites are those comprising 100 μg/kg or 10% of the total residue in a sample collected at the earliest euthanasia interval (or following attainment of steady-state or at or near the end of treatment for continuous-use drug products).

3. Approaches for quantifying metabolites

During the metabolism and residue studies, it is important to know quantitatively the relative ratios of each metabolite in each tissue to the parent at different time points. The purpose of this quantitative step is to assign the marker residue and determine the marker to total residue ratios for each edible tissue and each time point. As indicated in VICH G46 [3], an appropriate marker residue has the following properties: (1) there is a known relationship established between the marker residue and the total residue concentration in the tissue of interest; (2) the marker residue should be appropriate to test for the presence of residues at the time point of interest, i.e., adherence to the withdrawal period; and (3) there should be a practicable analytical method to measure the marker residue at the level of the MRL. Normally, this will be performed with radiolabeling. However, in a non-radiolabeled residue study, depending on the extent of metabolism and the number of metabolites formed and presented in each tissue, it is not possible to quantify every single metabolite, not to mention the analysis needs to be done with a validated method [51].

The simplest and most straightforward approach to quantify metabolite ratios is to use the peak area of each residue from the LC-HRMS full scan data with a narrow isolation window (~5–10 ppm). As it is well known, each molecule's responses in the mass spectrometers are highly structure dependent. The ion source condition and a mobile phase (solvent and pH) also play a critical role in the mass spectral response. Therefore, the quantitation with the peak area of extracted ion chromatogram is at most semi-quantitative. Practically, this approach can be combined with validated

LC-MS/MS assays for the major components in the metabolite profiles. An example is given in Section 4 for the marker to total residue ratio determination in sheep for gamithromycin using non-radiolabeled residue study data. Several methods are reported in the literature that use certain UV or radiometric signals as "calibrator."

Yang et al. [52] reported an approach to determine the extent of circulating metabolites using "metabolite standards" generated by *in vitro* incubations at relatively high concentration (50 μM) in combination with the correction for mass spectrometry response based on UV response. The *in vitro* incubation sample containing all the generated metabolites is spiked in plasma to serve as a single point calibration standard for calculating the metabolite concentrations in pharmacokinetic (PK) samples. Using buspirone as a model compound, the authors compared the concentration versus time profiles of buspirone and its metabolites in rats obtained using a UV correction approach with those obtained with synthetic metabolite standards and calibration curves. It was demonstrated that the estimated metabolite exposure of buspirone using this UV correction approach resulted in the rank ordering of metabolite exposure within three-fold of the value obtained with metabolite standards, in contrast, to eight-fold without UV correction. Although UV is not a universal response detector, its response is generally more universal than MS responses. One of the requirements is that the UV spectra of the metabolite will need to be similar among the metabolites and the parent drug also has a decent turnover in the *in vitro* incubations to generate enough metabolites for quantitation.

Another approach is to use a radiolabel response of the metabolite as the response factor calibrator for quantitation [53, 54]. In many cases, radiolabel metabolism studies were performed *in vitro* in laboratory animal-derived hepatic incubations, or *in vivo* in either laboratory animal or target animal species. The radioactive samples generated in these studies can be saved and served as a calibrator for the residue studies of minor species. In this approach, the HPLC radiometric data for metabolites obtained from profiling other species' biologic matrices (as percentages) are used in combination with the known specific activity of the parent drug to calculate the concentration of each metabolite. These data can then be used to calibrate the MS instrument response for metabolites of interest, and then metabolite concentrations in the samples of target species can be measured using this calibrated response. This is a very cost-effective method and animal welfare friendly. Radiolabeled *in vitro* or rodent metabolism studies in general require less material than a large minor species such as equine. In most cases, radiolabeled studies would be already carried out in major species such as cattle or swine and the samples from those studies can also be banked for this purpose.

NMR spectroscopy is another technology that can be used for metabolite quantification without synthetic standards. Both ^{1}H NMR [55] and ^{19}F NMR [56] have been reported in human drug development for this purpose. Fluorine is not found in endogenous compounds; therefore, this method is very selective although the sensitivity is lower compared to mass spectrometry. Quantitative NMR has been reported, validated, and accepted from a bioanalytical perspective for purity determinations. More recently, drug metabolism scientists have reported on its application to metabolites [57]. Isolated metabolites quantitated by NMR can then be used as analytical standards for quantitative LC-MS assays.

Accelerator mass spectrometry (AMS) has been used to support human drug ADME studies for many years [58, 59]. AMS, a technology that measures the intensity ratio between ^{14}C over ^{12}C, can measure extremely low levels of ^{14}C. However, AMS is a destructive technique that does not identify the metabolite, the sample preparation

(graphitization followed by combustion and detection of $^{14}C/^{12}C$ ratio) is relatively labor-intensive and the AMS system is expensive. In addition, each metabolite will need to be separated and fraction collected to be subject(ed) to AMS sample preparation and analysis. In the case of a veterinary drug residue study, this process needs to be repeated for each metabolite, in each tissue, and at each collection time point, which will greatly impede its practicality.

While chemical synthesis of metabolites is technically possible, in practice, intensive resources will be required for the synthesis, purification, and certification. Quantitative LC-MS/MS method development and validation for all metabolites is not practical either. Therefore, the synthesis of major metabolite(s) seems to be the more practical and rational approach. Some of the most effective systems for metabolite synthesis are enzyme-based, such as common *in vitro* reagents (e.g., liver microsomes), engineered P450s, and microorganisms. These systems represent a vast array of synthetic versatility and potential transformations [60]. Biocatalysts often provide better chemo-, regio-, and/or stereo-selectivity than chemical synthesis. Such bioengineered systems with better catalytic efficacy are now commercially available from CROs such as Hypha Discovery (UK) which offers this kind of metabolite biosynthesis services including chemical synthesis, mammalian biotransformations (S9s / microsomes of liver and other tissues), recombinant enzymes such as PolyCYPs, as well as the purification of metabolites from biological matrices.

4. Feasibility of conducting non-radiolabeled study to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals

4.1 Determination of marker to total residue ratio for gamithromycin in sheep with a non-radiolabeled drug residue study

Gamithromycin (GAM) is a novel azalide that belongs to the 15-membered semisynthetic macrolide antibiotics of the azalide sub-class with a uniquely positioned alkylated nitrogen atom at the 7a-position of the lactone ring. The compound was first developed for the treatment of bovine and swine respiratory diseases [61]. Thorough documentation, including food safety studies were prepared and submitted to the European Medicine Agency (EMA) to support the registration of Zactran® (Boehringer Ingelheim Vetmedica GmbH), a GAM-based product, for the treatment and metaphylaxis of bovine respiratory disease (BRD) associated with *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*; and treatment of swine respiratory disease (SRD) associated with *Actinobacillus pleuropneumoniae*, *P. multocida, Haemophilus parasuis*, and *Bordetella bronchiseptica* [62, 63]. The registered claims of Zactran® were later extended for systemic treatment of infectious pododermatitis (foot rot) in sheep associated with virulent *Dichelobacter nodosus* and *Fusobacterium necrophorum* [64].

In vivo metabolism of GAM in sheep was similar to *in vivo* metabolism in cattle, swine, and dog (which were conducted using radiolabeled GAM). The *in vitro* metabolism study demonstrated that in sheep, as with other species, GAM has limited metabolism and declad (loss of a cladinose) is the major metabolite. This data was used to establish a reliable *in vivo* and *in vitro* correlation. In addition, similar PK profiles in sheep, cattle, and swine further support the fact that GAM has similar absorption, distribution, metabolism, and elimination properties across the species

[65–67]. Considering the available data for this minor species (sheep), a non-radiolabeled marker residue study for determination of the M/T ratios in edible tissues and a combination of cold semi-quantitative LC-HRMS and quantitative triple quadrupole LC-MS/MS approach was carried out [61] and accepted by the European Medicines Agency (EMA) [64] to have adequately defined the human food safety profile of Zactran in sheep.

Metabolite profiles of GAM in edible tissues as well as excreta (urine and feces) from sheep treated with subcutaneous injections of Zactran at 6 mg/kg body weight were obtained with HRMS. Representative metabolite profiles in the sheep edible tissues (liver, kidney, injection site core, loin muscle, and peri-renal fat) collected on Day 5 expressed in extracted ion chromatograms (XIC, 10 ppm window) are shown in **Figure 3**. The relationship between the level of GAM and the total treatment-related residues was semi-quantitatively established by determining the percentages of peak areas of extracted ion chromatograms for metabolites and parent compound residues in each tissue (**Table 1**) [61].

A validated liquid chromatography/tandem mass spectrometry (LC-MS/MS) method was used to quantify major components (GAM and its major metabolite, declad). For excreta, metabolite profiles were also obtained, and the major components were quantified with an LC-MS/MS method. Taking into account previous

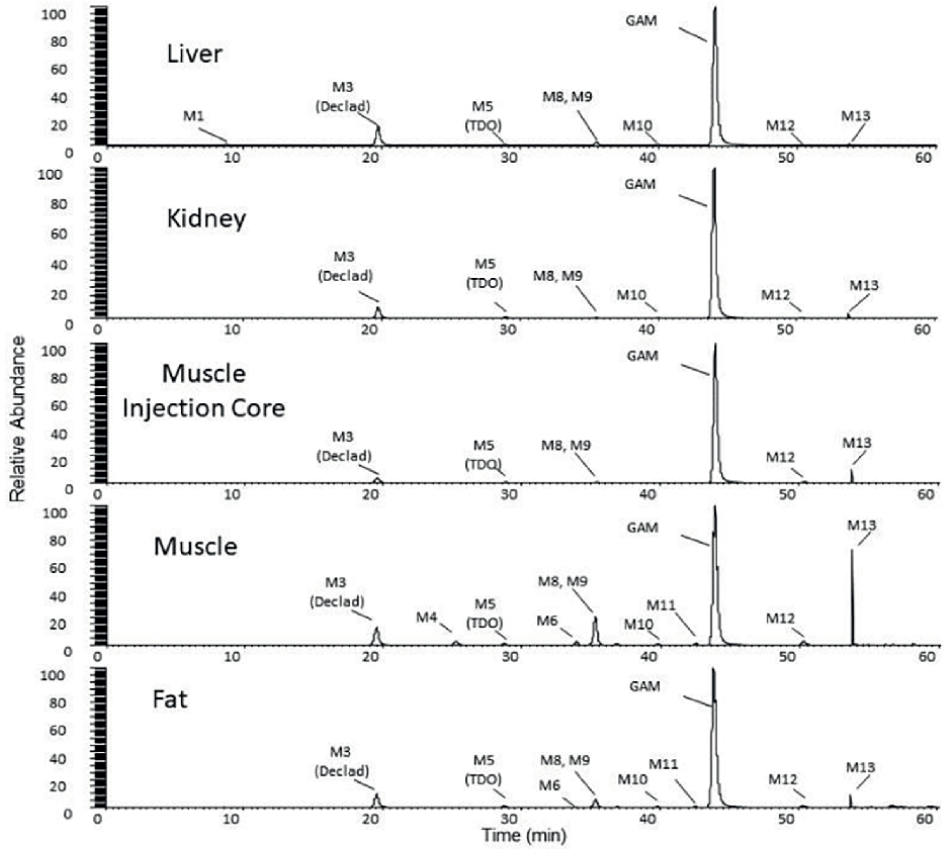

Figure 3.
Representative metabolite profiles in extracted ion chromatograms for various sheep tissues on day 5 (Ref. [61]).

Matrix	Days Post Dose	M1	M2	M3 (declad)	M4	M5 (TDO)	M6	M7	M8	M9	M10	M11	GAM	M12	M13
Liver	5	0.43	0.05	12	0.13	0.54	0.02	0.03	0.33	1.29	0.77	0.08	83.9	0.31	0.18
	9	0.48	0.05	17.4	0.08	0.61	ND	ND	0.23	1.38	0.96	ND	78.3	0.31	0.16
	14	0.86	ND	23	0.2	0.51	ND	ND	0.26	1.72	0.72	ND	72.3	0.23	0.21
	21	0.88	ND	26.2	0.2	0.52	ND	ND	0.3	2.13	1.03	ND	68.6	ND	0.11
	28	0.64	0.3	32.4	ND	0.86	ND	ND	ND	2.63	ND	ND	63.2	ND	ND
Kidney	5	0.02	0.06	7.1	0.07	0.95	0.03	0.01	0.15	0.33	0.46	0.19	89.7	0.44	0.56
	9	0.02	0.04	11.2	0.06	0.78	ND	ND	0.18	0.46	0.53	0.15	85.3	0.55	0.72
	14	0.09	ND	19.9	0.11	0.63	ND	ND	0.3	0.88	0.46	0.09	76.3	0.49	0.73
	21	ND	ND	23.2	ND	0.33	ND	ND	0.31	1.4	ND	ND	74.3	ND	0.45
	28	ND	ND	30	ND	ND	ND	ND	ND	2.22	ND	ND	67.8	ND	ND
Muscle	5	ND	ND	8.94	2.13	0.82	1.41	0.06	6.91	5.89	0.58	0.87	70.2	2.1	0.15
	9	ND	ND	11.4	3.26	0.46	2.75	ND	20.5	9.32	0.29	0.64	42.5	2.22	6.62
	14	ND	ND	13.7	1.46	0.41	13.3	ND	31.3	10.8	ND	ND	25.4	0.67	3.1
	21	ND	ND	8.57	1.73	ND	13.4	ND	48.1	18.1	ND	ND	8.19	ND	1.93
Muscle Injection Core	5	ND	ND	3.76	0.05	0.6	0.04	0.001	0.2	0.35	0.27	0.19	91.9	0.94	1.69
	9	ND	ND	6.08	0.06	0.64	0.03	ND	0.18	0.45	0.3	0.19	89.6	1.08	1.38
	14	ND	ND	7.46	0.03	0.18	ND	ND	0.36	0.74	0.13	0.14	88	0.7	2.2
	21	ND	ND	12.8	0.07	0.22	ND	ND	0.24	1.06	ND	ND	83.3	0.62	1.62
	28	ND	ND	14.2	ND	ND	ND	ND	0.19	2.49	ND	ND	81.6	0.25	1.26
	35	ND	ND	16.9	ND	ND	ND	ND	0.61	2.38	ND	ND	78.5	0.27	1.34

Matrix	Days Post Dose	M1	M2	M3 (declad)	M4	M5 (TDO)	M6	M7	M8	M9	M10	M11	GAM	M12	M13
Peri-renal Fat	5	0.1	0.06	7.3	0.11	0.99	0.2	0.02	1.61	2.79	0.67	0.48	83.1	1.21	1.35
	9	0.22	ND	14.7	0.54	0.81	0.5	ND	2.56	5.16	0.29	0.31	72.1	1.83	1.05
	14	ND	ND	27.2	ND	0.37	0.19	ND	2.63	9.8	ND	ND	59.6	ND	0.25
	21	ND	ND	37.7	ND	0.36	0.79	ND	4.47	18.7	ND	ND	37.9	ND	ND

ND: Not detected.

Table 1.
Summary of percent gamithromycin and metabolites in sheep edible tissues from extracted ion chromatogram (%XIC) (Ref. [61]).

knowledge of marker residue studies in cattle and swine, as well as metabolite data across various species from an *in vitro* comparative metabolism study, GAM is designated as the marker residue in sheep edible tissues [61].

The marker to total residue ratios were established (**Table 2**) using quantitative results for the marker residue and declad and the semi-quantitative HRMS results. The GAM residue concentrations in all tissues (liver, kidney, injection site core, injection site ring, loin muscle, and peri-renal fat) are much higher than declad. Depletion of GAM followed first-order kinetics with the liver showing the slowest depletion rate among all tissues analyzed (**Figure 4**). The half-lives of GAM depletion in liver, kidney, muscle, fat, injection site core, and injection site ring were calculated to be 5.48, 4.22, 2.55, 2.82, 4.43, and 2.39 days, respectively. The GAM tissue residue levels after Day 21 post-dose followed the order: injection site core > liver > kidney > injection site ring > loin muscle > peri-renal fat. Therefore, the liver is proposed as the target tissue because it has the slowest depletion rate and the residues are the highest after Day 21 post-dose, except the injection site.

Based on VICH GL 46 [3], GAM can be designated as the marker residue with the following rationale: (1) A known relationship was established between GAM and total residue in the edible tissues as shown in **Table 2**; (2) GAM is appropriate to test for the presence of residues at the proposed withdrawal periods estimated; and (3) An LC-MS/MS analytical method is available for the determination of GAM concentrations in all sheep edible tissues [61].

4.2 Non-radiolabeled residue study for ciclesonide in equine

The Aservo®EquiHaler® - an inhaler containing the inhalation solution, is indicated for use in horses for the alleviation of clinical signs of severe equine asthma. The dosing apparatus includes a polyurethane nostril adapter which provides 343 micrograms of ciclesonide per actuation. The Aservo®EquiHaler® is an example where safety was established and radiolabeled *in vivo* metabolism and residue kinetics studies may not be required: The active ingredient, ciclesonide, is a novel drug in veterinary medicine and is a pro-drug that is rapidly metabolized into the major active metabolite desisobutyryl-ciclesonide (des-CIC or des-ciclesonide) following inhalation. Des-ciclesonide has a large glucocorticoid-receptor affinity and demonstrates anti-inflammatory properties which are exerted through a wide range of inhibitory activities. Given that horses are a minor species with a limited

Day post dose	Liver	Kidney	Loin muscle	Fat	Injection site core
5	83.9	89.7	70.2	83.1	91.9
9	78.3	85.3	**42.5**	**72.1**	89.6
14	**72.3**	**76.3**	25.4	59.6	**88.0**
21	68.6	74.3	8.2	37.9	83.3
28	63.2	67.8	NA	NA	81.6
35	NA	NA	NA	NA	78.5

Table 2.
Estimated Average Gamithromycin (M) to Total Residue (T) Ratios (M/T) from %XIC in Sheep Tissues Using HRMS Data (Ref. [61], bold numbers stand for the selected M/T ratio for each tissue).

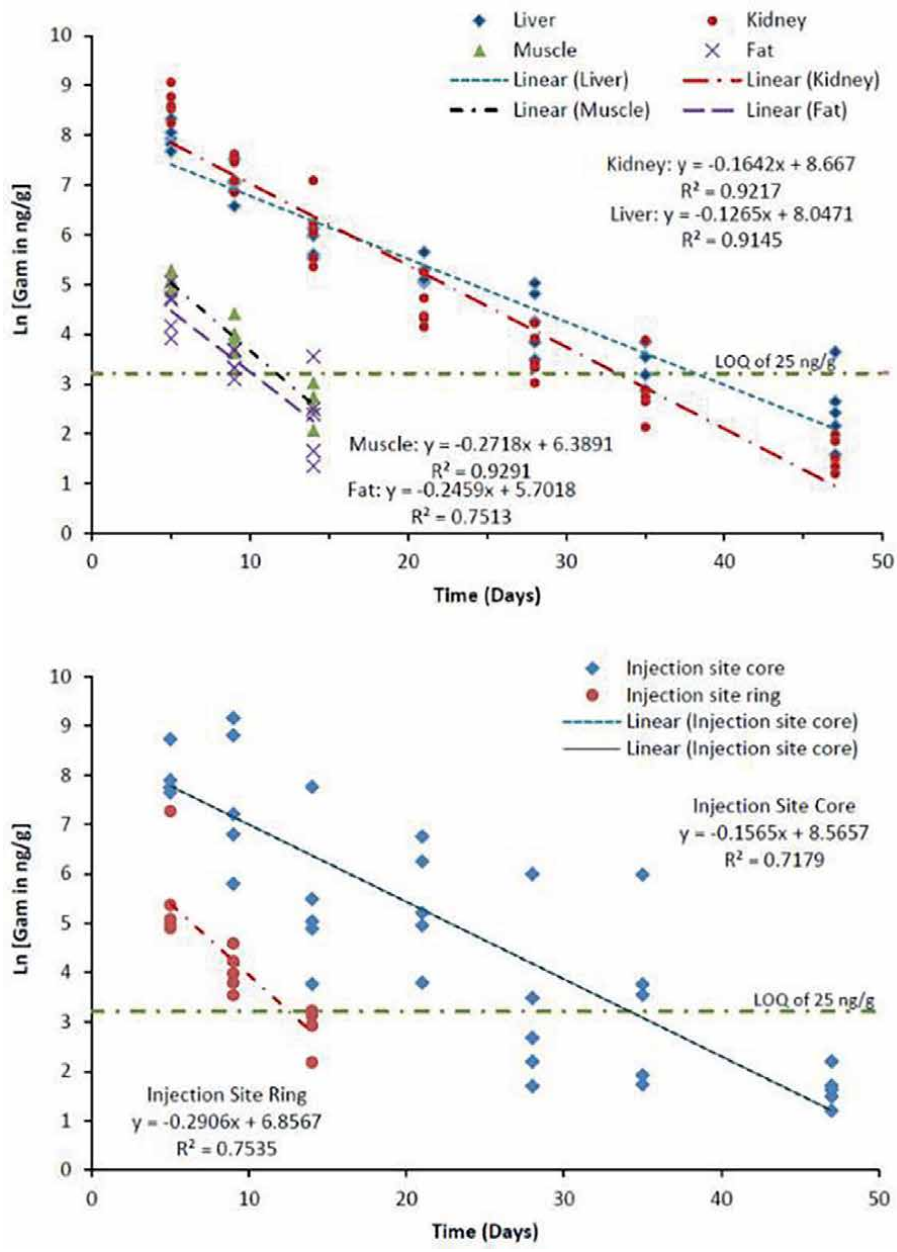

Figure 4.
Depletion of Gamithromycin residues in various sheep tissues (Ref. [61]).

market status, a minor use minor species (MUMS) market authorization could be granted. Consequently, the possibility to use HRMS could be investigated.

In vitro metabolism of ciclesonide in rat, dog, and human liver microsomes showed no significant interspecies differences. At least three metabolites were formed in all three species: major metabolite (M1, formed by de-esterification of ciclesonide), M2 (the 6-hydroxy derivative of M1), and M3 (the 23 or 24 hydroxy derivatives of M1). *In vitro* metabolism studies using horse liver slices revealed 33 different

metabolites. The three major metabolites (des-CIC and two isomers of hydroxycyclohexane des-CIC) and three minor metabolites (dihydroxycyclohexane des-CIC, isomer of hydroxycyclohexane des-CIC, and hydroxysteroid des-CIC) were identified by LC-MS/MS. These metabolites are comparable to those identified in other animal species [68].

A non-radiolabeled study was performed to establish the marker to total ratio in equine tissue samples using semi-quantitative high-resolution mass spectrometry after repeated inhalation administration of ciclesonide for 10 days in healthy male and female horses [69]. Reference standards for the major metabolite of ciclesonide (**Figure 5**) were synthesized using various approaches including the microbial system. Response factors of the M3 metabolites, desisobutyryl ciclesonide, desisobutyryl ciclesonide oleate, and desisobutyryl ciclesonide palmitate relative to the parent (ciclesonide) in a particular tissue were determined by spiking equal molar concentration of each compound in that tissue. The results showed that ciclesonide and des-CIC were the only circulating drug-derived components detected in plasma. The metabolite profiles were qualitatively similar in all selected tissues. Only very low levels of ciclesonide and/or des-CIC were detected in muscle, liver, kidney, and lung. Very low levels of desisobutyryl ciclesonide oleate were also detected in Day 10 lung samples. Relatively higher amounts of ciclesonide and des-CIC and very low levels of M3 metabolites were detected in fat. A GLP study to establish residue depletion in

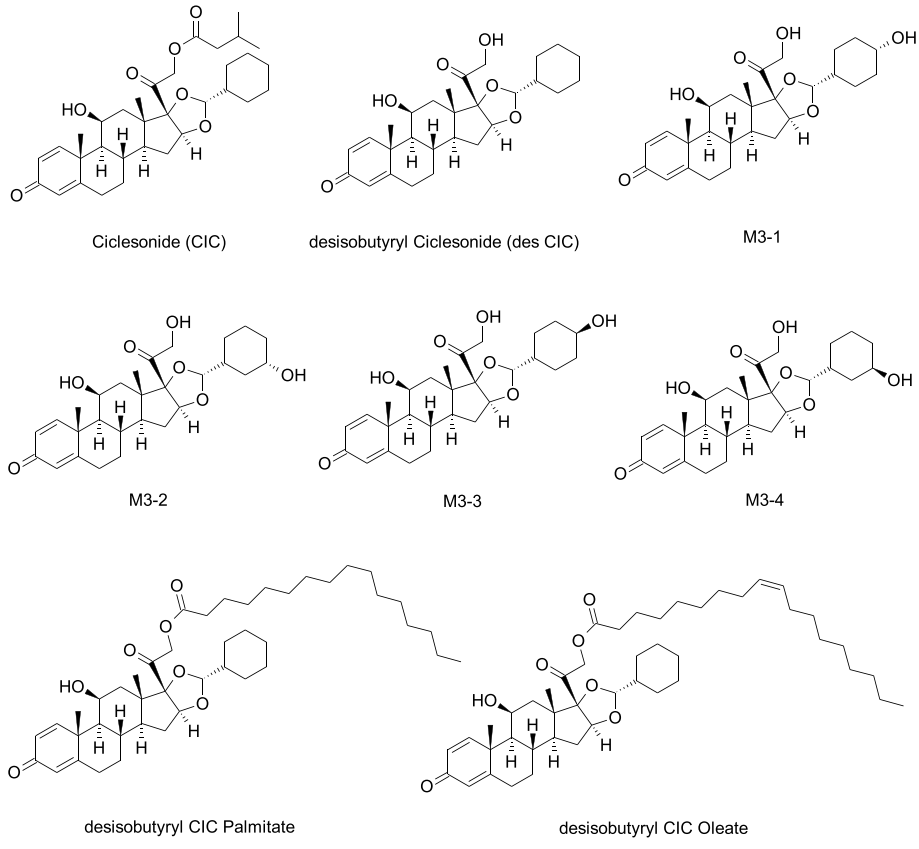

Figure 5.
Structures for reference standards of ciclesonide and its metabolites.

the horse was performed [68]. After the standard 10 days treatment period, groups of four horses were euthanized at 12 hours, 3 days, 6 days, and 9 days following the final treatment (group 1, 2, 3, and 4, respectively). Liver, kidney, fat, loin muscle, and lung were collected and analyzed for des-CIC, and the metabolites M3-2 and M3-4 using validated LC-MS/MS methods. The metabolites M3-2 and M3-4 were below or close to the limit of detection *in-vivo,* and were under the Lower Limit of Quantification (LLOQ) at the first sampling point, i.e., 12 hours after the last dose.

The results of both the semi-quantitative HRMS analysis and the quantitative analysis of the pivotal marker residue depletion study are consistent. The results of both studies show that the major metabolite of ciclesonide *in vivo* is des-CIC. Although M3-2 and M3-4 were the major observed metabolites found in *in vitro* studies, this could not be confirmed *in vivo*—neither in tissues nor in plasma most likely due to their extremely low levels. Considering the uncertainties in the HRMS study due to the limited number of metabolites detected, and the relatively low sensitivity of the full scan HRMS method, a conservative approach was used to establish a conservative marker to total residue ratio. Therefore, to support the market authorization, the ratio of marker to total residues was conservatively set to 0.5 for muscle and fat, 0.15 for liver, and 0.25 for kidney based on mean residue levels seen across the first three time points [68].

5. Application of HRMS for equine sports anti-doping

Anti-doping in equine sports poses different challenges compared to those in human sports. Compared to human sports, many more banned substances or controlled medications must be controlled in equine sports. It is not only limited to the prohibited list of banned and controlled medication substances by Fédération Equestre Internationale (FEI) [70] but also extends to other compounds with similar chemical structures or similar biological effects including designer drugs. The development of comprehensive screening methods that can detect all illegal substances is virtually impossible.

Traditionally, GC-MS has been widely used for AAS screening with sensitivity and selectivity that are ideally suited for low-level analytes and complex biological matrices [71, 72]. In addition, enormous amount of knowledge and experience were accumulated over the years for GC-MS-based AAS testing in human and sport horses. However, the sample processing is labor intensive. Although LC-MS has become increasingly more popular technique in doping screening, the detection of saturated hydroxysteroids by LC-MS is still not ideal due to their low ionization efficiency in electrospray ionization sources. In the last 10–15 years, HRMS has seen more applications in multiple-drug screening methods with the accurate mass measurement at high resolution to overcome the interferences from the matrix. The HRMS approach also enables retrospective analysis by reprocessing previously acquired data with new data mining tools when new doping agents reveal without re-processing and re-analyzing the suspect samples. The method development to incorporate new substances into the HRMS method is also much easier. Kioussi et al. [73] have developed a generic screening method for horse doping screening by LC-TOF-MS, GC-HRMS, and GC-MS. A combined implementation of LC-MS and GC-MS analysis was established using the same sample preparation method. The sample pretreatment began with two parallel procedures: enzymatic hydrolysis of sulfate and glucuronide conjugates, and methanolysis of the 17β-sulfate steroid conjugates. The extracts were subjected to LC-TOF-MS, GC-HRMS, and GC-MS analysis. Most of the prohibited substances were

identified through LC-HRMS without prior derivatization. Library searching using automated mass spectral deconvolution and identification system (AMDIS) combined with deconvolution reporting software (DRS) was used to enhance the screening. The method detects over 350 target analytes in horse urine and may easily incorporate many new substances without changes in chromatography. The full scan HRMS data allows retrospective identification of new prohibited substances by data reprocessing.

For confirmatory analysis, it is required to compare the data from the collected samples (urine or plasma) with a reference standard. Either fully characterized synthetic standards or those derived and isolated from *in vivo* or *in vitro* metabolism studies can be used for this purpose. For designer steroids, which have been synthesized for the purpose of performance enhancement but evade detection, it is difficult to detect them in a biological matrix using common GC-MS, LC-MS approaches, or by mass spectral library search since all these approaches would require establishing the analytical characteristics with suitable reference materials [74, 75]. In that regard, a good understanding of the metabolism of prohibited substances is important to target the substance itself or its metabolite(s) and more relevant matrix (urine, plasma, hair, etc.). However, due to the substantial number of potentially prohibited substances including designer steroids and over-the counter-supplements [76], it is not possible to conduct *in vivo* metabolism studies by dosing animals for each AASs not to mention carrying out these studies with radiolabeled materials. For this reason, most of the metabolism studies to understand the metabolism of the AASs were conducted *in vitro* in equine liver microsomes, S9, hepatocytes, or liver slices/homogenates and with non-radiolabeled materials and HRMS [77, 78]. After the identification of *in vitro* metabolites, mostly by searching for the accurate mass of predicted metabolites, *in vivo* metabolism studies can subsequently be performed to follow the identified metabolites in the relevant matrix to determine the longest detection time for controlling the doping abuse. A quick literature search indicated that the data mining tools described in Section 2 are underutilized. In most cases, only targeted data mining techniques are used. This could be one of the future directions by using these modern data mining strategies to ensure no major potential target metabolites are missed in metabolism studies.

Another promising method is to couple the HRMS and chemometrics for the structural characterization of AASs for early detection of unknown designer structures [79]. This method analyzes the mass spectrometric data on trimethylsilyl (TMS)-derivatives of the target AASs acquired by GC-QTOF MS with electron ionization. Five subclasses of steroids were used in the training set according to their structural similarity. The HRMS data was processed by the chemometric models (clustering, discriminant, modeling, etc.) considering over 30 variables, which allowed the identification of class-specific common fragments and structural trends. This will minimize the time needed for identifying unknown AASs by classifying them into a specific category and narrowing the scope of the investigation, which is particularly useful for completely unknown substances. It is hopeful that this approach can be adapted for the analysis of real samples such as urine if the HRMS spectrum under examination is suitably defined.

6. Conclusions and perspectives

The development of HRMS over the past two decades has made significant progress in instrument design with various scan functions, data mining tools, and

semi-quantitative and quantitative methods for metabolite quantification that make the profiling and identification of metabolites with non-radiolabeled drug routine and reliable. It has been demonstrated over the past 15 years in human drug development under the "Metabolites in Safety Testing" regulatory guidance that this method is mature and reliable. The chance of missing any major metabolite(s) in complex biological matrices is small. This development in instrumentation and data mining techniques will certainly help the understanding of the metabolism of AASs and facilitate the anti-doping analysis in equine sports although currently, the application of advanced data mining techniques in this field is still relatively rare. In fact, even with a radiolabeled drug, due to the position and nature of the label, the metabolites from the part of the molecule that does not contain the radiolabel will not show up in the radiochromatograms. There are even debates in the drug metabolism community concerning the radiolabeled mass-balance excretion and metabolism studies in laboratory animals are still necessary [80, 81]. Innovative "fit-for-purpose" studies may well be a better choice in a particular drug development program than a standard animal ADME "package." The same applies to the development of veterinary medicine. In our opinion, radiolabeled metabolism and residue studies are still the "gold standard" and should be required for at least one major species in food-producing animals. However, the regulation does allow alternative approaches (i.e., not using the radiolabeled drug) to characterize the components of the residue in food derived from treated animals. As demonstrated with the examples in Section 4 of this chapter, it is feasible that metabolism and residue kinetics in food-producing animals may be appropriately determined using HRMS without a traditional radiolabeled study. The HRMS approach has been shown here to be useful for understanding the M/T ratio and metabolism profile for regulatory purposes. It is particularly useful if prior M/T knowledge in other species is available, and the parent is not highly metabolized or when all major residues can be measured quantitatively by LC-MS/MS. It is important to demonstrate that metabolism across species is similar which can be done with the HRMS method. It is also particularly useful in certain situations when traditional radiolabeled studies are not feasible or safe. In the case of anti-doping analysis, it may be possible to conduct non-radiolabeled metabolism studies in horses directly if needed to overcome the potential disconnection of *in vitro* and *in vivo* results. It is not difficult to imagine, even for companion animals, that the "fit-for-purpose" ADME studies may also be performed with a non-radiolabeled veterinary drug to understand the major excretion pathways and safety profiles to renal or hepatic impaired animals as well as to the environment.

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