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Assisted Reproductive Technologies in Horses: A Systematic Review

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ABSTRACT

In the past decades, there have been tremendous changes in equine reproduction. Assisted reproductive techniques (ART) for horses, such as *in vitro* oocyte maturation (IVM) and *in vitro* fertilization (IVF), has been slow and with low success rates compared to other domestic species. The unique characteristics of equine oocytes such as morphology, gene expression, and developmental potential make it challenging to develop standardized IVM and IVF protocols. The aim of this review was to address all aspects of the current knowledge of IVM and IVF in horses.

Keywords: Assisted Reproductive Techniques; *In Vitro* Maturation; *In Vitro* Fertilization; Intracytoplasmic Sperm Injection; Vitrification; Oocyte; Equine

Abbreviations: ART: Assisted Reproductive Technologies; IVM: *In Vitro* Oocyte Maturation; IVF: *In Vitro* Fertilization; ICSI: Intracytoplasmic Sperm Injection; OPU: Ovum Pick Up; TVA: Transvaginal Oocyte Aspiration; FSH: Follicle Stimulation Hormone; FBS: Fetal Bovine Serum; EGF: Epidermal Growth Factor; FGF: Fibroblast growth factors; IGF: Insulin Like Growth Factor

Introduction

Assisted reproductive technologies (ART) for equine, such as *in vitro* oocyte maturation (IVM) and *in vitro* fertilization (IVF), has been slow and with low success rates compared to other domestic species [1]. This is partly because mares are long-lived animals that are gelded at an early age for management [2]. Therefore, mares are used for breeding until they became elderly making natural reproduction more difficult and, increasing the demand for ART. As a result, improving ARTs has become of utmost importance. Unfortunately, there is no standardized IVM medium due to the unique characteristics of equine oocytes such as morphology, gene expression, and developmental potential. Additionally, the zona pellucida hardening during maturation [3] and the inability to consistently capacitate sperm for penetration of *in vitro* matured oocytes [4] further hinder the development of ARTs. Until last year, standard in-vitro fertilization was ineffective in horses [5], but intracytoplasmic sperm injection (ICSI) has high success rates. ICSI requires specialized knowledge and equipment to fertilize each oocyte and achieve the MII stage of nuclear maturation [1]. Therefore, the aim of this review is to enumerate the difficulties of the equine oocyte IVM and IVF.

Oocyte Collection Techniques

The oocytes are extremely sensitive cells, and their management is crucial for achieving successful IVF outcomes. Despite improvements in oocyte collection techniques, recovery percentages, and success rates vary among authors [6,7]. For live mares, Ovum Pick Up (OPU) or Ultrasound-guided transvaginal oocyte aspiration (TVA) is used to recover maturing oocytes from dominant gonadotropin-stimulated follicles or immature oocytes from small/medium or subordinate follicles for posterior use in IVM and IVF. The average OPU recovery rates from small and medium follicles are between 8 to 13.8 oocytes per mare, recovery of preovulatory follicles is even lower [8-11]. Performing OPU involves certain risks. According to a study conducted by Texas A&M University, rectal bleeding occurred in

16% of 153 OPU sessions, but none of the mares displayed any clinical symptoms afterward. However, in another clinical trial, the incidence of rectal bleeding was only 0.4%, which may be attributed to the experience of the veterinarians performing the OPU. To reduce these risks, prophylactic antibiotics and anti-inflammatory drugs should be administered to all donor mares before the procedure [11-13].

It is also possible to obtain the oocytes from abattoir ovaries with different techniques. Oocytes can be collected from follicles by aspiration, with gentle suction created by a vacuum pump (100–150 mm Hg) through an 18-gauge needle or by ovarian scraping of the inner wall of the follicle. Both techniques offer a higher oocyte collection rate of approximately 70-80%. In a study with 1052 ovaries, 3135 oocytes were recovered from follicles with a diameter above 5 mm (2.98-3.8 oocytes per ovary) [14,15]. When oocytes are collected using aspiration, the oocytes are usually obtained with only the cells immediately surrounding the oocyte, known as the corona radiata. On the other hand, most oocytes collected by scraping contain the cumulus complex intact and are embedded in sheets of granulosa cells. Unfortunately, scraping requires more time and effort. Galli et al. reported that it takes four technicians to collect 100 horse oocytes by scraping [15,16].

Equine Oocyte Maturation

The next logical question is: Do equine oocytes have the same maturation markers as other species? The answer is no. Several maturation markers have been identified in equine oocytes. These markers include cumulus cell expansion, the formation of the first polar body and changes in the expression of specific maturation related genes. [16,17] The cumulus cell expansion is classified as compact with at least 3 to 5 layers of cumulus cells attached (having a tight, complete compact cumulus with a distinct, smooth hillock), expanded (having a granular or expanded cumulus), corona radiata (having only corona radiata present) [18]. The cumulus cell expansion is one marker that seems to work in species such as cattle. Although, in horses, there are no significant differences in blastocyst formation after IVF of oocytes with expanded or compact cumulus [15,18]. Another important marker is nuclear maturation. Nuclear maturation refers to the process by which the nucleus of an immature oocyte undergoes a series of changes, including chromosome condensation and alignment, the separation of homologous chromosomes, and the formation of the first polar body. Oocytes with an intact germinal vesicle, germinal vesicle breakdown, and metaphase I are considered immature; oocytes presenting a polar body and a well-formed second metaphase plate are considered mature [19]. Finally, cytoplasmic maturation consists of organelles replication and expression of RNAs and proteins necessary for postfertilization. Unfortunately, cytoplasmic maturation is more difficult to evaluate, but it can be performed by transmission electron microscopy [19,20].

Nuclear and cytoplasmic maturation are indicators of oocyte quality. Regardless, they aren't related to oocyte competence, including embryonic and fetal development [19]. Nonetheless, most evaluations for maturation require the oocyte to be fixed and stained, which makes them unsuitable for fertilization. Therefore, it is necessary to develop a less invasive evaluation of maturation. Carnevale and Maclellan previously reported that when maturation is complete, the ooplasm can have different shades of gray and this ca be evaluated by stereoscopic microscope. Overall, obtaining and identifying mature oocytes is utmost relevance to obtaining top-quality embryos. [16,19]. Regrettably, no research has been conducted to develop an oocyte equine-specific maturation medium. The equine industry faces multiple factors that might affect the success rates, including the type media, the hormones, and the media supplementation (Table 1). Most maturation protocols use TCM 199 with Earle's salts or DMEM/F12 with no significant differences in maturation rates between both mediums [7,14,18,21-27].

Table 1: Variations during *in vitro* oocyte maturation.

Author and year	Recovery method	Recovery rate	% of IVM	Hours in IVM	Temperature
Fernandes, et al. [16]	Aspiration		22-62%	36 h	39°C
Aguilar, et al. [25]	Scraping		12.8-43.8%	36-40 h	38°C
Foss, et al. [27]	OPU		65-88%	28-30 h	37.9°C
Galli, et al. [21]	Scraping	3.8 oocytes per ovary	51.1-60%	24-28 h	38.5°C
Lewis, et al. [7]	OPU and aspiration		67% and 32%	30 h	38.3°C
Diaw, et al. [24]	Aspiration		23.1-38.5%	24 h	38.2°C
Merlo, et al. [18]	Aspiration		59.3-61.7%	26 h	38.5°C
Campos-Chillon, et al. [28]	OPU	4.5 oocytes per cycle	70-73%	30 h	38.5°C
Cuervo-Arango, et al. [12]	OPU	11.8-12.8 oocytes per mare	56.4-60.7%	30 h	38.5°C
Agnieszka, et al. [14]	Scraping	2.98 oocytes per ovary	47%	36 h	38°C
Claes, et al. [11]	OPU	13.8 oocytes per mare	59%		

Usually, maturation media is supplemented with Follicle Stimulation Hormone (FSH) alone or supplemented with estradiol or LH. Other variables include adding sodium pyruvate, L- glutamine, different doses of fetal bovine serum (FBS) or, more recently, equine follicular fluid. Moreover, IVM protocols use growth factors like the epidermal growth factor (EGF), Fibroblast growth factors (FGF), insulin like growth factor (IGF) [18,25] or antioxidants. However, independently of the media supplementation, there are no significant differences in embryo development after IVF [7,11,12,14,15,18,19,22-28]. Finally, the time of maturation in horses has not been established and varies widely among researchers. Some studies even suggest a pre-maturation time of 0-16 hours, but most protocols use holding medium before maturation. The standard holding time ranges between 18-24 hours, allowing the shipment of immature oocytes to ICSI laboratories and facilitates injection timing. Evidence suggests that holding media can improve nuclear maturation rates. There are different options for the holding media, which include commercial holding media, synthetic oviductal fluid, and a novel shipping/maturation medium buffered with HEPES/NaHCO3 supplemented with antioxidants [7,11,19,24,26,28,29].

As for the time of maturation, some authors claim that 24 hours is enough time for oocytes with expanded or compact cumulus to mature and later develop into blastocysts. Other authors suggest that oocytes with compact cumulus require between 28-36 to mature. Dini et al. previously reported that oocytes can be monitored at different time points during maturation to identify the extrusion of the first polar body. Their study examined oocytes at 20, 22, and 28 hours, with no effects on fertilization or embryo development [12,15,23,24,29,30]. All these variations in maturation time and media prove that further research is required to establish a specific equine IVM protocol.

IVF or Vitrification?

Once the oocyte is mature, it can be either fertilized by IVF or stored by vitrification. In horses, ICSI has overcome the formidable barrier of inefficient co-culture IVF. ICSI greatly improves the use of semen (frozen, fresh or sex sorted) and is an alternative for stallions with fertility problems. However, reported rates of *in vitro* equine blastocyst production after ICSI vary from 4% to 40% among laboratories [10,11,15]. Last year, for the first time, a repeatable and effective method for equine standard IVF was reported. This new IVF method was successful (yielded >50% fertilization) and resulted in foal being born from the oocytes recovered via OPU and postmortem. To our knowledge, this is the first report of blastocyst formation *in vitro* after standard IVF in the horse and, more importantly, the birth of foals resulting from the transfer of these blastocysts [5].

On the other hand, equine oocyte vitrification is an ART of interest to the equine industry. Vitrification is a method that results in a glass-like structure without the formation of ice crystals. During vitrification, the oocytes are exposed to relatively high concentrations of permeating and non-permeating cryoprotectants, which act by lowering the freezing point of the cytoplasm and causing oocyte dehydration. Once equilibrated, the oocyte must be cooled at ultra-rapid rates by direct plunging into liquid nitrogen. Vitrification allows the preservation of the female germ line for an indefinite time but significantly compromises oocyte developmental competence [31,32]. Unfortunately, vitrification has a deleterious effect on the DNA fragmentation of equine cumulus cells, but this parameter cannot be used to determine meiotic competence [33]. For vitrification, compact equine oocytes with germinal vesicle offer the best chance of a mature oocyte with potential for fertilization but developmental competence is still reduced dramatically [34] The viability rate of oocytes after vitrification is 63-98% but embryo development rates are only 20-33.3% [14,30]. A possible cause is the increase in reactive oxygen species by the vitrification process [32]. In the horse, oocyte vitrification is still considered an experimental procedure due to its low success, with only three live foals reported [34,35].

Conclusion

In conclusion, the development of assisted reproduction techniques in horses is major relevance as they are valuable animals that are often used for breeding until they become elderly and have difficulties to reproduce. Therefore, improving the success rates of these technologies becomes essential. Moreover, advances in assisted reproduction techniques for horses may also have implications for other domestic and wild species. Unfortunately, more research is needed to standardize ART and improve success rates.

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