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EMBRYO PRODUCTION BY *IN VITRO* FERTILIZATION IN WILD UNGULATES: PROGRESS AND PERSPECTIVES – A REVIEW*

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Abstract

Wild ungulates are of fundamental importance for balancing ecosystems, as well as being the species of economic interest. Increasing concern over the accelerated population reduction of these species has resulted in the development of assisted reproduction techniques, such as *in vitro* fertilization (IVF), as a tool for conservation and multiplication. In the present scenario, IVF protocols were developed based on the methodologies used for domestic ungulates. Nevertheless, owing to the physiological and reproductive differences among the species, several factors associated with IVF and its relationship with the characteristics of the species of interest require clarification. *In vitro* conditions for the collection and selection of female and male gametes, oocyte maturation, sperm capacitation, co-incubation of gametes, and embryonic development can influence IVF results. Therefore, the present review considers the main advances in the methodologies already used for wild ungulates, emphasizing the strategies for improving the protocols to obtain better efficiency rates. Additionally, we discuss the conditions of each IVF stage, with emphasis on aspects related to *in vitro* manipulation and comparability with the protocols for domestic ungulates.

Key words: wildlife conservation, assisted reproduction, *in vitro* fertilization, gametes, embryos

The ungulates constitute a large and diverse group of mammals with hooves that are divided into artiodactyls or even-toed species and perissodactyls or odd-toed species. The first group includes the bovids, cervids, giraffes, pigs, peccaries, and camelids, whereas the second includes horses, rhinoceros, and tapirs. These animals play a key role in balancing diverse ecosystems through grazing, trampling, defecation, seed dispersal, and nutrient cycling (Mohr et al., 2005; Velamazan et al., 2020). Additionally, wild ungulates represent an important economic resource for many local communities because of their valuable meat and leather, as well as wildlife tourism (Gordon et al., 2004).

According to the International Union for Conservation of Nature (IUCN, 2021), approximately 140 species of more than 250 wild ungulates are on pathways to extinction and are classified as near threatened (32), vulnerable (52), endangered (42), critically endangered (15), and extinct in the wild (2). Moreover, the population of more than 160 species is currently decreasing. These animals are constantly affected by habitat loss, forest fragmentation, illegal hunting, and other consequences of anthropogenic activities (Malakoutikhah et al., 2020). Therefore, to conserve biodiversity, increase genetic variability, and rapidly multiply genotypes of wild ungulates, assisted reproduction techniques (ARTs) have been applied (reviewed by: Mastromonaco and Songsasen, 2020). ARTs are attractive tools for the reproduction and maintenance of wild species in zoos and reserves (reviewed by: Comizzoli et al., 2000; Herrick, 2019), as well as for efficiency of the economic activity associated with farm-raised non-domestic ungulates, such as red deer (Berg and Asher, 2003).

An interesting ART that has been developed in wild ungulates is *in vitro* fertilization (IVF). IVF can be divided into several stages, such as collection, selection, and maturation of oocytes, sperm collection, selection and capacitation, gamete co-incubation, *in vitro* embryo culture, and transfer of embryos. In general, the use of IVF associated with embryo transfer in wild ungulates has allowed the rescue of genetic material from male and female dead animals and those disabled for natural reproduction (Mahesh et al., 2011), the utilization of gametes of low quality and in low quantities (Hermes et

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al., 2009), and of gametes transported for long periods (García-Álvarez et al., 2011).

Despite many application possibilities and success in domestic ungulates, there are few reports of offspring of wild ungulates born from IVF. Overall, carrying out IVF in wild ungulates is a great challenge, primarily owing to the shortage of available male and female gametes, as well as the lack of expert knowledge to handle animals safely. These facts have hampered the development of continuous studies and the establishment of this ART (Hildebrandt et al., 2018).

There are several species of domestic or domesticated ungulates that can be used as experimental models for wild species. Nevertheless, notably, the protocols for model species are only a starting point for studies on phylogenetically close wild species (Figure 1). Therefore, as the physiology of the species of interest becomes known, it is important to adapt and test different conditions to meet intraspecific needs and achieve success in IVF. Many advances have been made in the IVF of red deer (*Cervus elaphus*) based on the usual protocols of domestic ruminants (Berg et al., 2002). However, Berg and Asher (2003) observed that the composition of the oviduct fluid of this species could be different from that of cattle and sheep. Therefore, these authors confirmed this difference and formulated culture media that were specific for red deer, achieving a significantly higher percentage of cleavage (68.0% *vs*. 49.3%) and blastocyst (12.9% *vs*. 4.5%).

The low efficiency of IVF in wild ungulates is due to technical limitations, such as insufficient knowledge about the basic reproduction and physiology of gametes and embryos, difficulty in handling animals safely and without stress, unavailability of biological material, and lack of studies establishing ideal conditions for the *in vitro* manipulation of gametes and embryos for different species (Andrabi and Maxwell, 2007; Cervantes et al., 2016; Mastromonaco and Songsasen, 2020; Thongphakdee et al., 2017). Although these factors are associated, this review focuses primarily on the aspects related to *in vitro* manipulation.

The quality and competence of gametes are fundamental to the success of fertilization and embryonic development (Berlinguer et al., 2008; Thongphakdee et al., 2017). These characteristics can be affected by several inherent factors of the technique, as well as the culture conditions at each stage of the IVF, which determine the maintenance of the quality and stimulate the development of gametes and embryos.

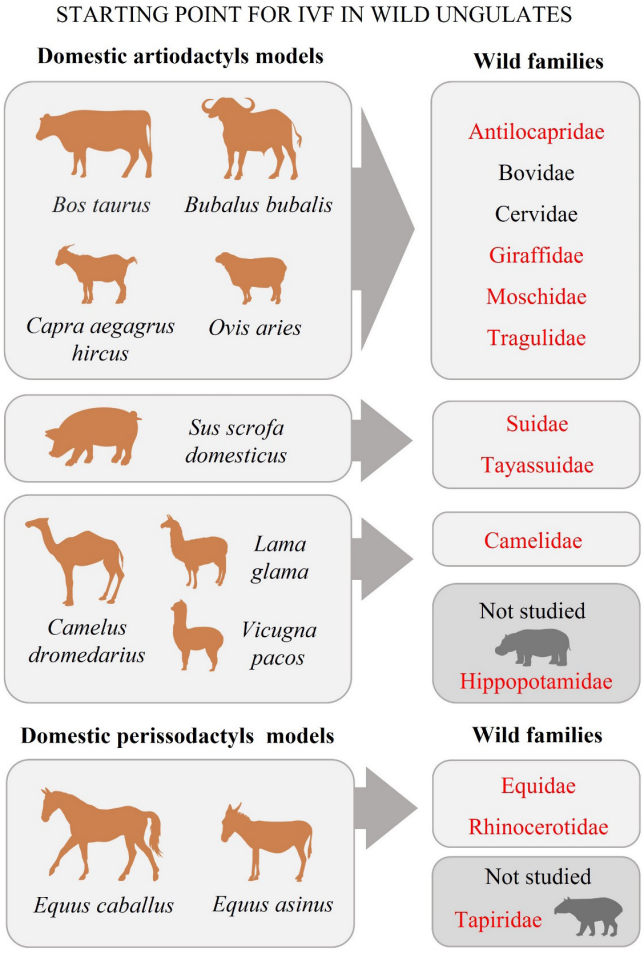


Figure 1. Domestic or domesticated ungulate species that due to phylogenetic proximity could be used as an experimental model for species of wild ungulate families, aiming *in vitro* fertilization (IVF). Family names written in red indicate that there are few or no studies

To be successful in IVF, it is particularly important to imitate the conditions found in the female reproductive tract during fertilization and pre-implantation embryonic development. If this scenario is not replicated properly, the quality and competence of gametes and embryos are negatively affected (Berg and Asher, 2003). Therefore, we will review the main advances obtained in the stages of IVF carried out in wild ungulates, emphasizing strategies for improving protocols to obtain better efficiency rates. Additionally, in parallel with the protocols of domestic ungulates we discuss the conditions for collection and preparation of gametes in wild ungulates, as well as factors related to the co-incubation of these gametes and embryo culture.

Oocyte preparation

Collection and selection of oocytes

The availability of competent oocytes, which, in other species of ungulates, has been considered as the main factor affecting blastocyst yields (Rizos et al., 2002), is a marked limitation of IVF in wild ungulates. This makes it difficult to establish ideal protocols for the manipulation of oocytes without jeopardizing their quality. Therefore, the quality of oocytes for IVF can be influenced by the conditions in which oocytes are collected *in vivo* or *post-mortem*.

In an ideal situation, the best strategy for obtaining good-quality oocytes is to collect them *in vivo*. To accomplish this, the follicular aspiration method using laparoscopy (Thongphakdee et al., 2017), laparotomy (Berlinguer et al., 2008) and transvaginal ultrasound (Cervantes et al., 2017) techniques can be used (Table 1). Nevertheless, few species have been sufficiently studied for this type of manipulation to be safe and efficient (Hildebrandt et al., 2018). This requires reliable and efficient methods for monitoring reproductive activity, which is a very challenging task in wild ungulates, as they are very temperamental and sensitive animals (Sontakke, 2018).

In domesticated camelids, such as llama (*Lama glama*, Trasorras et al., 2009) and alpaca (*Vicugna pacos*, Ratto et al., 2007), the efficiency of the laparotomy method exceeds 80%. However, in vicunas (*Vicugna vicugna*), the recovery rate was only 55.4% (46 oocytes from 83 follicles; Chaves et al., 2004). This shows that adaptation to the collection protocol is necessary to achieve greater success in the species of interest.

An interesting study was carried out on southern white rhinoceros (*Ceratotherium simum simum*) to allow safe and efficient collection of oocytes *in vivo*, to apply to the last two remaining female northern white rhinoceros (*Ceratotherium simum cottoni*) to rescue them from extinction (Hildebrandt et al., 2018). These authors developed a new protocol for ovarian stimulation, anesthesia, and transrectal oocyte recovery, as well as a specific collection instrument for rhinoceros. In this case, only the procedure to remove the oocyte from inside the follicle was similar to that performed in horses, a phylogenetically close domestic species. From these procedures, the authors achieved a promising recovery rate of 26.4% (83 oocytes from 314 follicles).

In wild ungulates, it is possible to obtain gametes from *post-mortem* females in a timely manner when individuals are euthanized, ovariectomized (in the case of hunted or slaughtered animals) or experience accidental death (Table 1). In these situations, the collection efficiency may vary, as the reproductive stage and conditions of death are typically unknown, and euthanasia or ovariectomies may be performed for health reasons (Mahesh et al., 2011). Nevertheless, this can provide a unique opportunity to obtain genetic material and study the ideal conditions for *in vitro* manipulation of oocytes from wild females. Mahesh et al. (2011) had the opportunity to obtain oocytes from a female nilgai (*Boselaphus tragocamelus*) that died during an outbreak of foot and mouth disease. Despite the presence of disease and the length of time before the ovaries were recovered after the death (7–24 h), it was possible to obtain 61.9% quality oocytes, 12% of which reached the 4–8 cell stage after *in vitro* co-incubation with sperm.

From *post-mortem* females, ovary collection and transportation conditions can also influence oocyte quality, particularly when the place of death is distant from laboratories, a common occurrence for wild ungulates. In Iberian red deer (*Cervus elaphus hispanicus*), ovaries could be stored for 12 h at different temperatures (5–8°C or 20–25°C) without affecting blastocyst rates, which ranged from 9.6 to 17.7% (García-Álvarez et al., 2011). In contrast, oocytes recovered from Hokkaido sika deer (*Cervus nippon yesoensis*) transported at 20–25°C for 12 h had a higher maturation rate (71.0%) than those transported for 24 h (31.0%, Tulake et al., 2014). Although it is possible to carry ovaries securely for a few hours, it is beneficial to reduce the transportation time to obtain better results.

Traditionally, follicular aspiration and ovarian slicing are methods used to collect oocytes *in vitro* (*post-mortem*). The choice of these methods depends on the laboratory routine, ovary anatomy, and oocyte attachment to the follicular wall, with great variation in recovery rates (number of oocytes recovered per female) (Table 1). In general, it is important that the method used permits the recovery of good-quality oocytes. For domestic ungulates, oocytes with many layers of cumulus cells and homogeneous cytoplasm are normally considered to be of good quality for maturation. In white-tailed deer (*Odocoileus virginianus*; Siriaroonrat et al., 2010) and Indian blackbuck (Rao et al., 2010), high-quality oocytes had an improved maturation rate (77.6% and 68.0%) compared to those with poor morphological characteristics (17.9% and 48.0%), such as minimal layers of cumulus cells and heterogeneous cytoplasm.

Table 1. Efficiency of oocyte collection and *in vitro* maturation in wild ungulates

Species	IUCN	Source of collection	Collection method	Oocytes/ female (%)	IVM medium*	IVM duration	IVM (%)	BL (%)	References
Collared peccary (*Pecari tajacu*)	LC	Slaughter	Follicular aspiration	16.2 (333/20)	TCM-199, 0.2 mM sodium pyruvate, 10% FBS, 20 µg/mL FSH/LH, 100 µM cysteamine, 10 ng/mL EGF	44 h	68.4	23.5	Borges et al. (2020)
Southern White Rhinoceros (*Ceratotherium simum simum*)	NT	Transrectal ultrasound guided	Follicular aspiration	4.6 (83/18)	DMEM-F12, 10% rhinoceros estrum serum	36–48 h	38.6	8.4	Hildebrandt et al. (2018)
Eld's deer (*Rucervus eldii thamin*)	EN	Ovariectomy/laparoscopy	Slicing; Follicular aspiration	44.3 (133/3)	TCM-199, 25 mM $NaHCO_3$, 0.2 mM pyruvate, 10% FBS, 100 µg/mL FSH, 100 µg/mL LH, 1 µg/mL estradiol, 0.1 mM cysteamine	24 h	60.0	7.8	Thongphakdee et al. (2017)
Iberian red deer (*Cervus elaphus hispanicus*)	LC	Hunting	Follicular aspiration	3.4 (301/88)	TCM-199, 10% FBS, 10 mU/mL FSH, 10 mU/mL LH, 50 ng/mL EGF	24 h	50.0	ND	Macías-García et al. (2018)
Wood bison (*Bison bison athabascae*)	NT	Transvaginal ultrasound guided	Follicular aspiration	7.1 (106/15)	TCM-199, 5% FBS, 5 µg/mL LH, 0.5 µg/mL FSH	24 h	71.0	ND	Cervantes et al. (2016)
Nilgai (*Boselaphus tragocamelus*)	LC	Death by disease	Slicing	73.9 (517/7)	TCM-199, 10% FBS, 0.22 mM sodium pyruvate, 10 µg/mL FSH, 6 IU/mL LH, 1.0 µg/mL 17-β estradiol	24 h	63.4	0.0	Mahesh et al. (2011)
African black rhinoceros (*Diceros bicornis*)	CR	Occasional death	Follicular aspiration and slicing	14.8 (74/5)	TCM-199, 0.1 g/L L-glutamine, 0.1% BSA, 2 mM/L pyruvate, 10% FBS, 1 µg/mL FSH, 5 µg/mL LH, 1 µg/mL estradiol	36 h	3.6	0.0	Stoops et al. (2011)
White-tailed deer (*Odocoileus virginianus*)	LC	Slaughter	Slicing	14.2 (624/44)	TCM-199, 0.33 mM pyruvate, 2 mM glutamine, 2.2 mg/L $NaHCO_3$, 10% FBS, 5 µg/mL FSH, 5 µg/mL LH, 1 µg/mL estradiol	24 h	77.6	ND	Siriaroonrat et al. (2010)
Indian blackbuck (*Antilope cervicapra*)	LC	Occasional death	Slicing	21.8 (131/6)	TCM-199, 10% FBS, 0.22 mM pyruvate, 10 µg/mL FSH, 6 IU/mL LH	24 h	68.0	13.0	Rao et al. (2010)
Dama gazelle (*Nanger dama*)	CR	Semi-laparotomy	Follicular aspiration	5.8 (35/6)	TCM-199, 10% estrus sheep serum, 10 µg/mL FSH/LH, 1 µg/mL estradiol, 0.1 mg/mL glutamine	24 h	68.9	0.0	Berlinguer et al. (2008)
Burchell's zebra (*Equus quagga burchellii*)	NT	Slaughter	Slicing and scraping	10.2 (61/6)	TCM-199, 10% FBS, 2.5 µg/mL FSH, 2.5 µg/mL LH, 1 µg/mL estradiol	27–34 h	ND	1.0	Meintjes et al. (1997)
Hartmann's zebra (*Equus zebra hartmannae*)	VU			13.8 (83/6)					

BL: blastocyst. LC: least concern. NT: near threatened. VU: vulnerable. CR: critically endangered. EN: endangered (IUCN, 2021). ND: not determined. FBS: Fetal bovine serum. *Antibiotics and antimycotics have been omitted from the composition of the medium.

In vitro maturation conditions

In domestic ungulates, such as bovines, goats, sheep, swine, and equines (including donkeys), the base medium for *in vitro* maturation (IVM) is composed of TCM-199 with 5%–10% fetal bovine serum or follicular fluid and supplements that vary among species (Table 1). Normally, the same base medium has been used in wild ungulates, such as wood bison (Cervantes et al., 2016), gazelles (Berlinguer et al., 2008), mouflon (Ptak et al., 2002), deer species (Berg et al., 2002; Locatelli et al., 2012; Thongphakdee et al., 2017), collared peccary (*Pecari tajacu*, Borges et al., 2020), and zebra (Meintjes et al., 1997). Interestingly, for black rhinoceros oocytes IVM, Stoops et al. (2011) used TCM-199 with 10% fetal bovine serum and obtained only 3.6% (3/83) oocytes in metaphase II (MII). In contrast, Hildebrandt et al. (2018) used DMEM-F12 medium with 10% rhinoceros estradiol serum, reaching a maturation rate of 38.6% in white rhinoceros.

The compounds typically used as supplements to stimulate *in vitro* oocyte maturation are FSH, LH, and estradiol, as well as epidermal growth factor (EGF). Siriaroonrat et al. (2010) evaluated the effect of 1 µg/mL estradiol during IVM in white-tailed deer oocytes and observed a higher maturation rate in the presence of this hormone (77.6% *vs.* 51.1%). An improved maturation rate was also obtained by FSH, and LH supplementation compared to hormone-free medium (68.0% *vs.* 39.6%) in blackbuck (Rao et al., 2010). These positive results can be attributed to similarities in the *in vivo* hormonal conditions in these animals.

Regarding the effect of adding EGF, in Iberian red deer, an increase in oocyte maturation rate was observed in a medium supplemented with FSH and LH and 50 ng/mL EGF (46.3%) than in the medium without EGF (19.5%, Macías-García et al., 2018). In collared peccary, we observed that the presence of 10 ng/mL of EGF during IVM did not improve meiotic development, but improved oocyte morphometry (Borges et al., 2020). Thus, different oocyte responses to this growth factor are observed in these ungulates and are likely owing to the physiological characteristics of each species.

Curiously, in sika deer (*Cervus nippon hortulorum*), there are differences in IVM media requirements owing to seasonality. In this species, it was observed that during the estrous season, the best IVM medium was supplemented with 10 µg/mL FSH, 1 µg/mL LH, 0.2 mM cysteamine, and 50 ng/mL EGF, while in the anestrous season, double these concentrations were more efficient (Yin et al., 2013). Additionally, based on the findings observed in studies with other deer species, Rola et al. (2021) observed that the IVM medium containing 5.0 µg/mL FSH, 100 µg/mL hCG and 1.0 µg/mL estradiol was efficient for maturation of brown brocket deer (*Mazama gouazoubira*) oocytes (64.5%).

The ideal time for oocyte maturation in domestic ungulates can be quite variable, ranging from 22 to 24 h for cattle, sheep, and goats, 42–44 h for pigs, and 36 h for horses. Considering this, oocyte maturation times (24 and 30 h) were compared in wood bison, and it was observed that 24 h was sufficient for 71% of the oocytes to reach MII, similar to cattle (Cervantes et al., 2016). In contrast, 27 h was necessary for 75% of red deer (*Cervus elaphus*) oocytes to reach MII using the standard medium for domestic ruminants (Berg et al., 2002). Likewise, in collared peccary, 48 h IVM was more efficient than 24 h for the nuclear stage, similar to the time for swine (76.2% *vs*. 19.0%; Borges et al., 2018). In vicunas, 41% of oocytes matured *in vitro* after 27 h, which is a shorter time than for other domesticated camelids (Chaves et al., 2004). Finally, in rhinoceros, an IVM time of 36–44 h has been reported (Hildebrandt et al., 2018), which is the approximate time for horses and donkeys (Li et al., 2021). Therefore, establishing the IVM time is an initial step in obtaining oocytes suitable for fertilization.

Sperm collection, selection, and capacitation

After collection, the main factors that influence IVF sperm quality were selection and capacitation conditions. Semen has been collected from wild ungulates primarily via electroejaculation till date (Pukazhenthi, 2016). Nevertheless, in species without established electroejaculation protocols, it is possible to obtain sperm from the epididymis in cases of occasional death, castration, and euthanasia (Table 2).

Fresh or preserved sperm can be used for IVF in wild ungulates. IVF has been consistently combined with sperm refrigeration (Hermes et al., 2009) and cryopreservation (García-Álvarez et al., 2011; Locatelli et al., 2012) for practicality and germplasm banks (Benham et al., 2021). Nevertheless, semen conservation protocols can impair sperm quality, as reviewed by Pukazhenthi (2016). Notably, sperm storage protocols have not been defined for many wild ungulate species. Thus, it is necessary to develop efficient methods to maintain the viability and fertility of these gametes.

The two most common sperm selection methods in wild ungulates are the Percoll® (colloidal silica coated with polyvinylpyrrolidone) gradient and swim-up methods, as presented in Table 2. When using the appropriate method for the species, selection increases the percentage of motility, viability, normal morphology, and fertility (Liu et al., 2013; Santiago-Moreno et al., 2014). In addition, this step is essential to remove seminal plasma (fresh semen), extender, and cryoprotectants (frozen–thawed sperm), which may negatively influence fertilization (Liu et al., 2013). For frozen–thawed spermatozoa of scimitar-horned oryx (*Oryx dammah*), better quality sperm were obtained using Percoll and swim-up compared to the washing method (centrifugation); however, the success of heterologous fertilization was similar among treatments (65–72.7% cleavage; O'Brien and Roth, 2000). For gazelles (*Gazella dama mhorr*), motility increased from 53.1% to 77.5% after the selection of frozen–thawed sperm using the swim-up (Berlinguer et al., 2008).

Table 2. Sperm collection, selection and co-incubation conditions in wild ungulates associated with the development of embryos produced by *in vitro* fertilization

Species	IUCN	Sperm collection	Sperm types	Selection method	Motile (%)	Concentration (x 10^6)	IVF medium	Efficiency (%)	References
Wood bison (*Bison bison athabascae*)	EN	ND	Frozen/thawed	Percoll® (45/90%)	60–75	5.0	Brackett-Oliphant	53.8 BL	Palomino et al. (2020)
Eld's deer (*Rucervus eldii thamin*)	EN	ND	Frozen/thawed	Percoll® (45/90%)	ND	0.05	Deer-SOF	7.8 BL	Thongphakdee et al. (2017)
Vietnamese sika deer (*Cervus nippon pseudaxis*)	LC	Electroejaculation	Frozen/thawed	Percoll® (45/90%)	ND	3.0	SOFaa, 20% ESS, 5 µg/mL heparin	29 BL	Locatelli et al. (2012)
Iberian red deer (*Cervus elaphus hispanicus*)	LC	Epididymis	Frozen/thawed	Percoll® (45/90%)	ND	1.0	Deer-SOF, 20% ESS	17.7 BL	García-Álvarez et al. (2011)
Nilgai (*Boselaphus tragocamelus*)	LC	Epididymis	Fresh	Swim-up	50–60	0.01	TALP, 100 µg⁄mL heparin	42 CL	Mahesh et al. (2011)
Black rhinoceroses (*Diceros bicornis minor*)	CR	ND	Cooling (4ºC)	Swim-up	70	0.5	SOF, 20 µg/mL heparin, 5% ERS	50 CL	Hermes et al. (2009)
Gazelle (*Nanger dama*)	CR	Electroejaculation	Frozen/thawed	Swim-up	77.5	1.0	SOF, 2% ESS, 1 µg⁄ml hypotaurin, 10 µg⁄ml heparin	30 CL	Berlinguer et al. (2008)
European mouflon (*Ovis orientalis musimon*)	VU	ND	Frozen/thawed	Swim-up	ND	1.0	SOF, 20% ESS, 2.9 mM calcium lactate, 16 mM isoproterenol	37 BL	Ptak et al. (2002)
Burchell's zebra (*Equus quagga*)	NT	Epididymis	Cooling (4 ºC)	Not selected	ND	9.0	Hsm's F10, 1 µM calcium ionophore	38 CL	Meintjes et al. (1997)

EN: endangered. LC: least concern. VU: vulnerable. CR: critically endangered (IUCN, 2021). ND: not determined. ESS: estrous sheep serum. ERS: estrous (white) rhinoceros serum. *Only centrifugation. CL: cleavage rate. BL: blastocyst rate.

When sperm selection methods in Iberian ibex (*Capra pyrenaica*) and European mouflon (*Ovis orientalis*) were compared, Capripure® (colloidal silica particles coated with silane) density-gradient centrifugation and dextran swim-up methods exhibited recovery rates of quality spermatozoa of 60.6% *vs.* 11.3% (*C. pyrenaica*) and 47.8% *vs.* 27.8% (*O. orientalis*), respectively. Therefore, density-gradient centrifugation is recommended for both species, highlighting the similarities among the different genera (Santiago-Moreno et al., 2014). Moreover, the Percoll gradient improved the linearity (64.5% *vs*. 28.5%) and viability (76.0% *vs*. 40.9%) of post-thaw sperm in Iberian red deer (García-Álvarez et al., 2016).

After sperm selection, proper oocyte-sperm interaction requires sperm capacitation, which is typically induced by different capacitating agents present in the IVF culture medium. Heparin has been widely used for sperm capacitation in domestic ruminants. However, although heparin for 1–4 h increases sperm capacitation in cattle, in the scimitar-horned oryx (*Oryx dammah*), which belongs to the same family, the rate of acrosome reaction did not increase after 6 h of incubation, suggesting that this species may require a longer incubation time (Roth et al., 1998). In contrast, when the effect of heparin (60 µg/mL) as a capacitating agent was evaluated in fallow deer (*Dama dama*), it led to an improved rate of acrosome reaction compared to the control medium (Fernández et al., 2013).

In general, equine sperm have difficulties in fertilizing oocytes *in vitro*, likely due to deficient capacitation (Leemans et al., 2019). Therefore, in domestic and wild horses, studies to improve the capacitation conditions for sperm are fundamental for the success of IVF. In Persian onager (*Equus hemionus onager*), the acrosome integrity was evaluated after sperm thawing, and good resistance of this species maintained 63% of intact acrosomes after 24 h of incubation at 22ºC (Schook et al., 2013). Although capacitation allows fertilization to occur, studies on sperm capacitation in wild ungulates are still limited.

In vitro fertilization and embryo culture

During gamete co-incubation, sperm concentration and culture medium may influence the IVF results. In the gaur (*Bos gaurus*), a higher sperm concentration (5.0 × 10^6 sperm/mL) increased polyspermy by 40% when compared to a lower concentration (1.0 × 10^6 sperm/mL, Johnston et al., 1994). In red deer, a blastocyst formation rate of 9% after IVF with 2.0 × 10^6 sperm/mL was observed, while a sperm concentration of 1.0 × 10^6 sperm/mL could not generate blastocysts (Comizzoli et al., 2001 a). However, under optimized conditions in the same species, Berg et al. (2002) evaluated sperm concentrations ranging from 0.001 to 1.0 × 10^6 sperm/mL, and the best monospermic penetration rate was obtained using 0.4 × 10^6 sperm/mL (77.0%). These controversial data show the importance of continuous studies in different conditions to achieve more satisfactory results, allowing efficient fertilization without causing polyspermy.

The composition of the IVF medium should favor the maintenance of gamete quality and stimulate fertilization. The most common IVF media in ungulates are synthetic oviduct fluid (SOF, Tervit et al., 1972), Tyrode's albumin lactate pyruvate (Parrish et al., 1988), FERT (Rath et al., 1999) and Brackett–Oliphant (Brackett and Oliphant, 1975), which are supplemented with capacitation agents. However, these conditions have seldom been studied in wild ungulates. In red deer, when the addition of sheep serum (20%), heparin (20 µg/mL), and/or bovine serum albumin (BSA; 6 mg/mL) in the IVF medium was evaluated, only the medium containing sheep serum allowed an acceptable rate of monospermic fertilization (56.2%), similar to that observed in small domestic ruminants (Berg et al., 2002).

In horses, the success of IVF is very limited because of the difficulty in penetrating the zona pellucida *in vitro* (Hinrichs et al., 2002). Compared to other domestic mammals, conventional culture conditions have not been shown to be effective in activating sperm capacitance triggers that allow efficient fertilization (Leemans et al., 2019). Therefore, alternatives have been sought to overcome this barrier. Meintjes et al. (1997) used a drilling technique with an acid solution of the zona pellucida before co-incubation with Burchell's zebra (*Equus quagga burchelli*) sperm. In this case, only 41% (31/75) of the oocytes were cleaved, and only 2% (1/75) developed to the blastocyst stage. In this respect, advances achieved with the use of intracytoplasmic sperm injection (ICSI) in the IVF stage can strongly benefit horses. Recently, a 6% rate of blastocyst formation was observed using the ICSI of Burchell's zebra sperm in horse oocytes (Gambini et al., 2020).

Additionally, the ICSI methodology can increase the success of IVF in wild ungulates with low-quality gametes. In the white and black rhinoceros (*Ceratotherium simum* and *Diceros bicornis*, respectively), the use of IVF and ICSI for embryo production has been described; although, as it was only possible to obtain a 4-cell embryo after IVF in black rhinoceros, further reproductive studies are required (Hermes et al., 2009). Subsequently, ICSI was performed on southern white rhinoceros (*Ceratotherium simum simum*) and a rate of 14.5% for cleaved embryos and 8.4% for blastocysts was obtained (Hildebrandt et al., 2018).

The time of development to the blastocyst stage *in vitro* varies among species of domestic ungulates, being 7–8 days in cattle and sheep, and 6–7 days in pigs and horses (Piliszek and Madeja, 2018). Several *in vitro* culture protocols of domestic and wild ungulate embryos use SOF medium, although in domestic swine, the medium for porcine embryos (PZM) is widely used. Nevertheless, interestingly, collared peccary embryos showed good development *in vitro* using SOF medium (Borges et al., 2020).

Few species of wild ungulates have been studied until the stage of embryo culture in IVF, and consequently,

only some species have had embryos transferred and births (Figure 2). In many cases, embryonic development does not reach the blastocyst phase, or the rate of blastocyst formation is low (Table 2). This is likely due to the conditions used in the previous steps being unsuitable for promoting efficient embryonic development. Moreover, the *in vitro* embryo culture (IVC) medium must also be carefully chosen to ensure successive cleavage of the embryos. Red sheep (*Ovis orientalis gmelini*) zygotes were cultured in Charles Rosenkrans 2 medium (CR2) or modified Brinster's ovum culture medium with bovine oviductal epithelial cells. These culture conditions did not lead to different cleavage rates, which ranged from 23.0% to 29.0%, and both media allowed the birth of offspring after embryo transfer (Flores-Foxworth et al., 1995). In red deer, the presence of ovine oviduct epithelial cells in SOF medium during IVC increased the rate of blastocyst formation compared to that in the medium alone (39.0% *vs*. 5.0%; Locatelli et al., 2005).

Techniques that assist in the establishment of IVF protocols

Parthenogenetic activation

A parthenogenetic or artificial oocyte activation strategy has been used as a model for evaluating experimental conditions that can influence embryonic development after IVF and ICSI in wild species (Yin et al., 2013). This technique can be used to assess oocyte competence and establish embryo culture media (Borges et al., 2020). Moreover, most mammals subjected to ICSI require an activation protocol to induce embryonic development. Thus, artificial oocyte activation can establish these protocols. Rao et al. (2010) evaluated the competence of mature oocytes from Indian blackbuck for embryonic development using parthenogenetic activation and obtained 58.0% cleavage and 13.0% blastocysts. In wild deer species, artificial activation was used to assess the competence of oocytes collected immediately or 12 h after animal death, with no significant effect observed on embryonic development (Brahmasani et al., 2013).

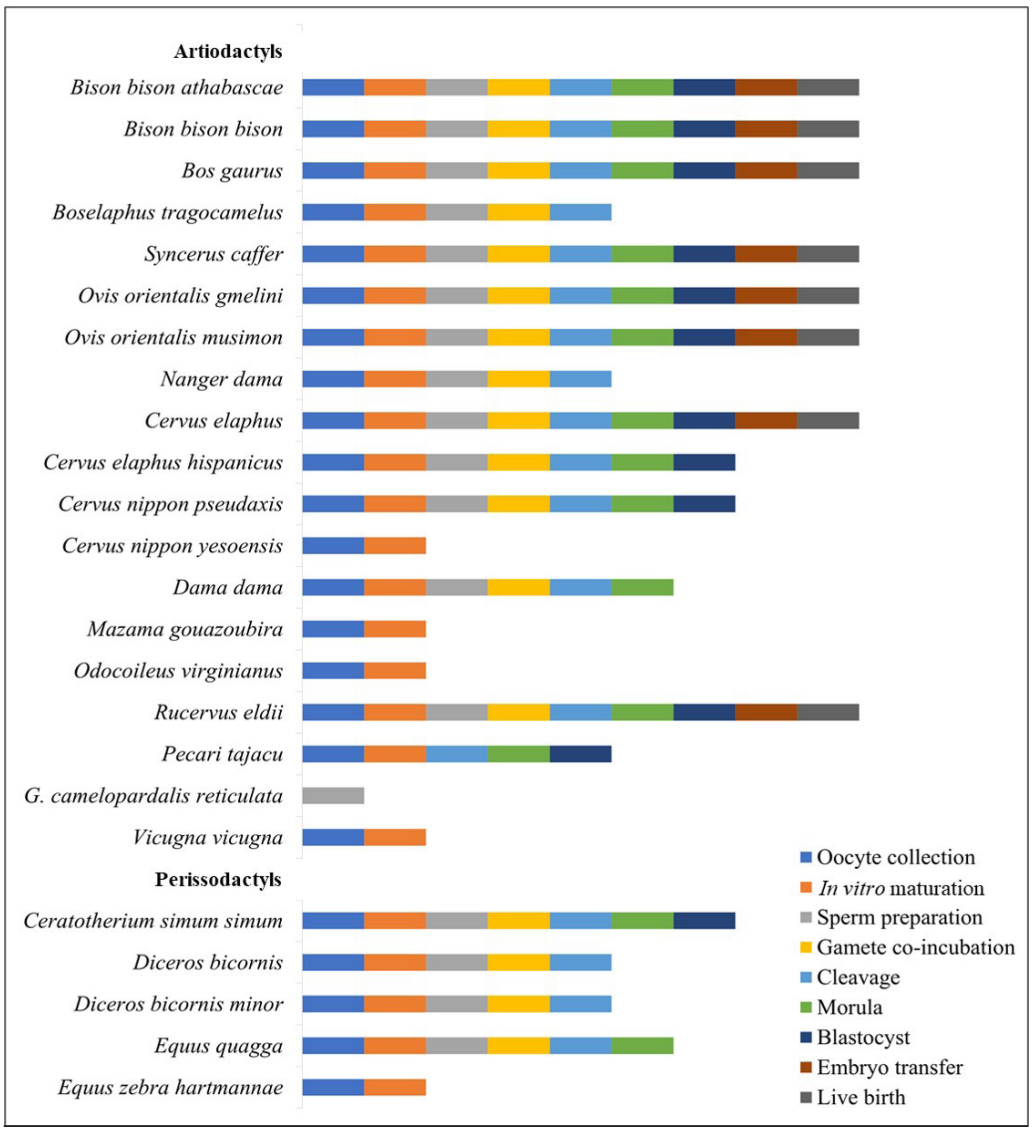


Figure 2. Development of *in vitro* fertilization (IVF) steps in several wild ungulate's species

For this type of application, it is important to establish an ideal activation protocol for different species. Yin et al. (2013) evaluated two chemical activation protocols for sika deer oocytes and obtained a higher rate of blastocysts (32.4%) using a combination of ionomycin and 6-dimethylaminopurine (6-DMAP) when compared to ethanol and 6-DMAP. They compared the quality of activated embryos with embryos produced *in vivo* and observed no difference, demonstrating the efficiency of artificial oocyte activation. Similarly, in collared peccary, we tested ionomycin and four secondary activator combinations (6-DMAP, 6-DMAP plus cytochalasin B, cycloheximide, and cycloheximide plus cytochalasin B), and ionomycin and 6-DMAP were superior in producing blastocysts (23.5%) and could be used to establish culture conditions for ICSI and cloning by somatic cell nuclear transfer (Borges et al., 2020).

Heterologous in vitro fertilization

Studies on IVF conditions in wild ungulates are scarce likely because of the challenges in obtaining sufficient mature oocytes for evaluating different experimental groups. To this end, heterologous IVF could be used, as it has already been applied as an alternative tool to evaluate sperm fertility and the response of spermatozoa to different cryopreservation and processing protocols. Hence, this technique was useful for assessing the longevity of frozen–thawed oryx sperm pre-incubated for 12 and 24 h before co-incubation with oocytes, obtaining 56.2% and 19.4% of embryos with ≥8-cell stage (O'Brien and Roth, 2000). Pradieé et al. (2018) evaluated the effect of different methods of cryopreserving Iberian ibex epididymal sperm on fertilizing capacity using bovine oocytes and observed similar cleavage rates for freezing (31.3%) and vitrification (45.1%), demonstrating that both methods can be used.

Heterologous IVF was used for sika deer sperm and zona-free bovine oocytes to assess IVF media and characterize sperm-oocyte interactions; this technique was highlighted as a tool for sperm selection that confers high development potential before homologous IVF or to understand unsuccessful homologous IVF (Comizzoli et al., 2001 b). In this context, the male reproductive seasonality of the North American bison (*Bison bison*) was also evaluated using heterologous IVF with bovine oocytes. Sperm recovered in the reproductive season led to a higher rate of blastocyst formation (30.5% *vs.* 13.7%, Krishnakumar et al., 2015).

Another interesting study with heterologous IVF aimed to evaluate the effectiveness of the cattle IVF protocol for African antelope species: springbok (*Antidorcas marsupialis*), impala (*Aepyceros melampus*), and blesbok (*Damaliscus dorcus phillipsi*). The authors observed that the protocol enabled rates above 50% penetration and cleavage, but improvements are needed to obtain embryos at the blastocyst stage in these species (Chatiza et al., 2013). This reinforces the fact that protocols of domestic species can be used as a starting point for studies on wild species.

Furthermore, many closely related species can produce viable hybrid individuals in nature, such as hybrids of sika deer (*Cervus nippon*; 2n = 68) and red deer (*Cervus elaphus*; 2n = 68), and in genetic improvement trials, such as American bison (*Bison bison*; n = 60) with domestic cattle (2n = 60) (Gabryś et al., 2021). This knowledge is also useful for studies with IVF, as observed by Meintjes et al. (1997), who produced zebroid embryos using Burchell's zebra (*Equus quagga burchellii*) sperm and Hartmann's zebra (*Equus zebra hartmannae*) oocytes for evaluating culture conditions. However, Owiny et al. (2009) evaluated the hybridization potential of African buffalo (*Syncerus caffer caffer*) sperm and cattle oocytes for genetic improvement purposes but was not successful in producing blastocysts, likely because of chromosomal disparity.

Heterologous ICSI can also provide important information for improving embryo production *in vitro*. This technique has been applied to assess the fertility of freeze-dried sperm of giraffe (*Giraffa camelopardalis reticulata*), using mouse oocytes, with 100% pronucleus formation observed (Kaneko et al., 2014). Gambini et al. (2020) provided valuable information regarding the manipulation of zebra sperm and used porcine oocytes to estimate its ability to induce activation after ICSI, which showed 30% pronucleus formation. After this evaluation, the sperm were injected into the oocytes of horses and were able to develop as far as the blastocyst stage. This result highlights that horses can be models for wild equines, as oocytes support hybrid development without compromising the expression and localization of cell differentiation markers and blastocyst cell number (Gambini et al., 2020).

Heterologous ICSI also played a key role in the development of a protocol for rhinoceros-homologous gametes. Hildebrandt et al. (2018) injected rhinoceros sperm into pig oocytes to assess fertility and the need for artificial activation. There was an increase of 30% to 90% in the fertilization rate, and the need to activate oocytes after ICSI of rhinoceros with low performance was noted.

Final considerations

IVF can be a valuable tool for the conservation of wild ungulates. In these animals, the greatest advances in IVF in terms of obtaining embryos and births occurred in species of artiodactyl from the Bovidae and Cervidae families. Among the reasons for this, we highlight the diversity of well-studied domestic models and the availability of individuals of the wild species for studies. In the other families of wild ungulates, the results are preliminary, typically with low efficiency and reproducibility.

Many factors must be considered for successful IVF to be achieved in these animals. Initially, it is necessary to promote research to understand the fundamental characteristics of reproduction and physiology of gametes and embryos from different families of wild ungulates. It is important to use advances made in domestic species

to acquire more information about the wild species. For this, techniques such as heterologous IVF, ICSI, and artificial activation can be explored in greater depth. Copying protocols from domestic models to wild species is not an effective strategy, although reproductive information for the species of interest should be combined with information from available domestic model(s). Thus, it is possible to create appropriate experimental designs to make the best use of the limited availability of gametes to establish the ideal culture and manipulation conditions for different species.

It is unlikely that IVF/embryo transfer will become a routine technique for wild ungulate management; however, it is undoubtedly an alternative that presents numerous advantages over other reproductive techniques. Recent efforts to establish IVF techniques are encouraging and will be central to the conservation of endangered ungulates and management of abundant species with economic value.

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