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Embryo transfer in cattle: status and prospects

Bachelor Thesis

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Declaration

I declare that I prepared my bachelor's thesis „Embryo transfer in cattle: status and prospects„ independently under the guidance of the supervisor of the bachelor's thesis and using professional literature and other information sources that are cited in the thesis and listed in the bibliography at the end of the thesis. As the author of the mentioned bachelor's thesis, I further declare that I have not violated the copyrights of third parties in connection with its creation.

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Martina Štěpánková

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Embryotransfer u skotu: současnost a perspektiva

Souhrn

Tato bakalářská práce poskytuje ucelený přehled o využití embryotransferu v chovu skotu. Tento postup zahrnuje přenos jednoho nebo více embryí z dárkyně do příjemkyně s cílem zlepšit genetickou kvalitu stáda. Úspěšnost postupu je ovlivněna mnoha faktory, včetně genetiky, kvality spermatu, odborných znalostí techniků a managementu recipientních krav.

Práce předkládá přehled relevantní literatury a případových studií s cílem prozkoumat současné trendy, výzvy a příležitosti v oblasti přenosu embryí skotu. Poskytuje náhled na osvědčené postupy pro zlepšení účinnosti a efektivity této důležité reprodukční technologie.

Mezi potenciální přínosy přenosu embryí skotu patří zvýšení reprodukční účinnosti a produktivity, zkrácení generačního intervalu a zvýšení míry genetického zisku. Vzhledem k rostoucímu významu přenosu embryí skotu v moderním chovu hospodářských zvířat je pro zlepšení kvality a produktivity skotu nezbytný další výzkum a vývoj v této oblasti.

V závěru se práce věnuje využití techniky embryotransferu u volně žijících zvířat, kterým by úspěch v této oblasti významně pomohl čelit jejich vyhynutí.

Keywords: Kryokonzervace, vývoj embryo, dárkyně a příjemkyně, hodnocení březosti, reprodukční biotechnologie, superovulace

Embryo transfer in cattle: status and prospects

Summary

This bachelor thesis provides a comprehensive overview of the use of embryo transfer in cattle breeding. The procedure involves the transfer of one or more embryos from a donor cow to recipient cows, with the goal of improving the genetic quality of the herd. The success of the procedure is influenced by many factors, including genetics, embryo quality, donor and management, technician expertise, and recipient cow management.

The thesis presents a review of relevant literature and case studies to explore the current trends, challenges, and opportunities in cattle embryo transfer. It provides insights into best practices for improving the efficiency and effectiveness of this important reproductive technology.

The potential benefits of cattle embryo transfer include enhancing reproductive efficiency and productivity, reducing the generation interval, and increasing the rate of genetic gain. With the increasing importance of cattle embryo transfer in modern livestock breeding, continued research and development in this field is essential for improving the quality and productivity of the cattle industry.

The thesis concludes with a look at the use of embryo transfer techniques in wild animals, for which success in this area would significantly help to counteract their extinction.

Keywords: Cryo-storage; embryo development; female donors and recipients; pregnancy rate; reproductive biotechnologies; superovulation

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1 Introduction

Embryo transfer is a key technique in the field of reproductive management of cattle, which allows significant improvement of the genetic potential of the breed. This bachelor thesis focuses on a detailed analysis of the individual steps of the embryo transfer process in cattle.

Embryo transfer began to develop in 1890 when the first transfer was performed (Seidel et al. 2003). Since then, techniques have continuously improved and progressed, which is the focus of this work.

The main objective is to understand and evaluate the importance of each step, from the selection of donors and recipients (Bó et al. 2020), through superovulation (Sakkas et al. 2017), embryo retrieval and evaluation, to preservation methods and the transfer itself (Mapletoft et al. 2003).

The thesis also discusses factors affecting the success of embryo transfer, such as genetic, environmental, and technological factors. A thorough analysis of these aspects will help to better understand the process and contribute to the optimization of reproductive programs in cattle.

In addition, the thesis provides an insight into the rules that regulate export and import of embryos, an important aspect in the global context of trade in bovine genetic material.

At last, this thesis explores the use of embryo transfer in non-domesticated species. The technique of embryo transfer does not have to be limited solely to cattle breeding for improving productivity or genetic progress; it can also serve the purpose of rescuing species on the brink of extinction (Pukazhenthi et al. 2003). This perspective extends the knowledge and possibilities of using this technique beyond livestock breeding and brings new challenges and opportunities in the conservation of endangered species.

2 Objective of work

This bachelor thesis aims to evaluate the individual steps of the embryo transfer process in cattle, including the selection of donors and recipients, superovulation, embryo retrieval, evaluation, methods of preservation, and the transfer itself.

Additionally, the thesis aims to provide insights into the international trade of embryos. The work offers a comprehensive overview of embryo transfer in the bovine species.

3 Literature overview

3.1 Historical background

In 1890 Walter Heape realized the first embryo transfer using the domestic Angora rabbits and Belgian hare rabbits as a model species (Seidel et al. 2003). Although the first calf was born from embryo transfer in 1950, the technique was not commercialized in cattle until 1970 (Betteridge 2003).

However, the new techniques were expensive and invasive as both the egg donor and the recipient had to undergo surgery. At the beginning, the success of this procedure was very low as a consequence of the high risks associated with anesthesia (either general or local) and surgical treatment, increased chances of disease incidence and transmission, and drop of milk yield as the operation process disrupted the lactation (Seidel et al. 2003).

In the late 1970s, a non-surgical method of embryo transfer was introduced, which led to great interest among dairy cattle breeders but this method still had its pitfalls, the donor and recipient had to be in the same phase of estrous cycle, which led to a significant loss of embryos when the recipient was not found after flushing, as it was not possible to freeze and properly preserve the embryos (Hasler 2014).

The focus on embryo preservation continued and there was success that led to further expansion of the method and its popularity. According to Seidel at al. (2003) today more embryos are transferred after being frozen and thawed than those that are transferred fresh.

3.2 Folliculogenesis and oogenesis

The process of follicle formation and development is referred to as folliculogenesis. The process of formation, growth and maturation of germ cells is referred to as oogenesis (Jahnke et al. 2014).

During the early stage of embryo development, primordial germ cells (PGCs) migrate from the endoderm of the yolk sac to the dorsal celomic wall. Once reached the site, PGCs stimulate the coelomic epithelium and underlying mesenchyme to proliferate forming the genital ridges, which are the precursor of the gonads (Fair 2003). After a series of mitotic division, the PGCs become oogonia, which start the oogenesis and block at the first meiotic division (MI) as primary oocytes. At this stage of their development, primary oocytes are surrounded by a single layer of follicular cells, which form the primordial follicle (Jahnke et al. 2014). Before birth, a supply of primordial follicles surrounding MI oocytes is already formed in the ovary. At the onset of puberty, a pool of primordial follicle will be cyclically recruited during each estrus cycle to resume their meiosis. Only one or two of them will complete the first meiosis and start the second one getting arrested at the metaphase (MII). At this stage, the secondary oocyte will be ovulated and, eventually, fertilized. Only the oocytes that will be fertilized will complete the second meiotic division; otherwise, they will degenerate (Fair 2003; Jahnke et al 2014).

During puberty, follicular growth and multiplication of follicular cells occurs in several primordial follicles in each estrous cycle. The interstitial cells surround the basement membrane of the granulosa cells, and the follicle envelope is formed (Sirard 2019). This envelope is divided into an outer (theca folliculi externa) and an inner (theca folliculi interna) envelope. The outer envelope becomes more vascularized and provides nourishment to the inner layer, the granulosa cells, and the oocytes (Fair 2003; Sirard 2019).

During the estrous cycle, the changes that take place on the ovaries are controlled by the gonadotropins released by the adenohypophysis. Follicle-stimulating hormone (FSH) promotes follicle growth, while luteinizing hormone (LH) is important for the ovulation process and the formation of the corpus luteum (Sakkas et al. 2017).

During the follicular phase of the estrus cycle, receptors for LH are formed on the inner lining cells of the follicle and receptors for FSH and estrogens are formed on the granulosa cells. LH induces androgen production by the cells of the theca folliculi interna. Androgens diffuse into the granulosa cells where they are converted to estrogens under the influence of

FSH (Rivera et al. 2011). The estrogens produced cause the granulosa cells to grow and divide. The primary follicle changes into a secondary, growing follicle (Andrade et al. 2011).

Oocytes stored in growing follicles begin to grow until they reach the size needed to complete the first meiotic division and start the second meiosis, getting blocked at the metaphase (MII). At this stage, the oocytes have half the number of chromosomes, hence haploid, the excess chromosomes are released at the end of the first meiosis as the first polar body (Baruseli et al. 2006).

Gradually, in the follicle, a cavity filled with a secretion of granulosa cells, also known as follicular fluid, is formed. The formation of granulosa cell fluid is stimulated by FSH and estrogens (Gosden 2002). A follicle with a cavity is referred to as an antral follicle. Increasing levels of estrogens promote LH secretion and lead to the release of LH in the wave, which induces prostaglandin production in follicular cells and oocyte maturation (Lonergan 2007). The prostaglandins cause the formation of multivesicular bodies that secrete proteolytic enzymes that cleave the proteins that bind the fibroblasts of the inner lining of the follicle, followed by the release of the egg, i.e., ovulation (Fair 2003). A follicle capable of ovulating is referred to as a tertiary (Graaff) follicle, which carries a mature and fertilizable egg blocked at, MII phase. The completion of meiosis II takes place after fertilization (Fair 2003).

After ovulation, a depression forms on the follicle and turns into a corpus luteum, and thus the luteal phase of the estrous cycle begins. The corpus luteum secretes progesterone, which inhibits the secretion of gonadotropins, stimulates the growth of the endometrial glands of the uterus, and promotes the secretory activity of the fallopian tube and endometrial glands of the uterus to provide nutrition for the developing embryo prior to nidation or implantation (Sirard 2019). If the egg has been fertilized and has nested in the uterus, the corpus luteum enlarges and remains functional throughout gestation. If the egg fails to nest, the endometrial cells begin to produce prostaglandin F2α (PGF 2α), which causes the corpus luteum to regress, causing progesterone levels to decrease and gonadotropin secretion to cease (Sakkas et al. 2017). As a result, FSH and LH levels begin to increase, inducing the starting of a new estrus cycle and the development of a new pool of follicles (Andrade et al. 2011).

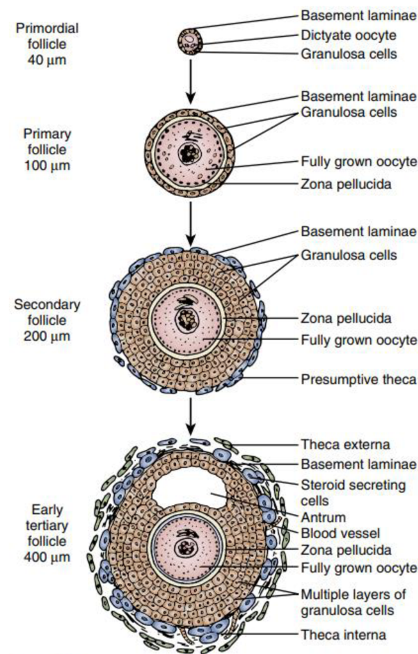

Figure 1: *Folliculogenesis (Sakkas et al. 2017)*

3.3 Estrus cycle

Cow is a polyestrous, non-seasonal breeder, with estrus cycle that lasts 21 days (Sláma et al. 2015). The estrous cycle is a recurring process throughout the reproductive period, except during pregnancy (Larson et al. 2006). The cycle can be divided into the follicular phase, comprising proestrus and estrus, and the luteal phase, comprising metestrus and diestrus. During the cycle, significant changes occur on the sexual organs of sexually mature females (Sláma et al. 2015). It causes significant changes in the reproductive organs of sexually mature females. The cycle is initiated by the onset of puberty, when gonadotropin levels rise, and the ovaries begin to produce sex hormones and release mature eggs (Bó et al. 2002). Factors that influence the age at which an animal reaches sexual maturity include environmental conditions, temperature, photoperiod (especially in small ruminants), age, nutrition, breed, body weight, and weaning weight (Paulini et al. 2011). Under normal conditions, cattle typically reach sexual maturity at 12 months (Rodgers 1999). Once the cycle stops and thus the release of eggs stops, the female has reached the end of her reproductive period. (Perry et al. 2006). Due to low economic returns to the breeder, females are often culled before they reach the post-reproductive period (Sartori et al. 2011).

The course of the estrous cycle itself is regulated by neuroendocrine mechanisms, especially gonadotropins - FSH and LH and ovarian sex hormones (Kojima & Freddie 2003).

The first phase of the cycle is called proestrus, in which the growth of follicles on the ovaries begins under the influence of gonadotropins. The concentration of estrogens increases in this phase, which promotes blood supply to the genital organs, proliferation of the uterine endometrium and vaginal epithelium. Proestrus, as a rule, lasts three days. (Larson et al. 2006).

This is followed by the estrus phase, which lasts 12-36 hours. Due to the high levels of estrogen, there is a strong blood supply to the genitals, redness of the vaginal mucosa and strong secretory activity of its mucous glands (Perera et al. 2011). When a tertiary follicle matures on the ovary, proliferation of endometrial cell peaks, the cervical canal opens, and a thick, mucoid secretion flows from the vulva. Furthermore, high estrogen levels cause the release of the preovulatory LH wave, which triggers oocyte maturation and the re-initiation of meiosis (Larson et al. 2006). The LH wave also leads to the stimulation of follicular cells to produce prostaglandins, which cause the mature follicle to rupture and release a mature oocyte through the ovulation (Kojima & Freddie 2003).

The estrus is followed by a phase called metestrus, which lasts 4 days. In this phase, the granulosa cells of the ovulated follicle begin to secrete progesterone and become luteal cells together with the theca cells (Bó et al. 2002). At this stage, the uterus enters the secretory phase, the engorgement of the genital organs begins to subside along with the discharge of mucus, and the cervical canal closes (Bó et al. 2002).

The diestrus, usually lasts 12 days. This phase is dominated by enlargement of the corpus luteum and ends with its regression (Hafez et al. 2000; Paulini et al. 2011). CL begins to produce higher concentrations of progesterone than was produced during the follicular phase and inhibits the production of FSH and LH (Paulini et al. 2011).

If fertilization does not occur, the CL regresses under the influence of PGF2α. Regression of the CL causes a decrease in progesterone levels and thus FSH and LH levels rise again. Under the influence of these gonadotropins, the proestrus period may begin again (Forde et al. 2011).

In the case of fertilization of the oocyte, there is no regression of the CL, the diestrus will continue into pregnancy (Kojima & Freddie 2003).

3.4 Fertilization

Fertilization is the process of fusion of the female and male gametes, which generally occurs in the upper third of the fallopian tube, resulting in the formation of a zygote (Barros et al. 2009). Thus, the first step of fertilization is the penetration of the sperm into the egg through the cumulus cells surrounding the oocyte (corona radiata) and through the zona pellucida by the acrosomal enzymes of the sperm (hyaluronidase and acrosin) (Florman et al. 2006). Through the resulting opening, the sperm penetrates through the plasma membrane of the oocyte and later into its cytoplasm. After penetration, the cortical granules are released into the perivitelline space and the zona pellucida hardens, preventing the passage of other sperm (Lonergan 2007; Barros et al. 2009). The oocyte thus completes the second meiotic division and releases the second polar body. In the zygote, the chromatin of the sperm and egg forms the female and male pronuclei. Subsequently, nuclear membranes are dissolved, chromosomes aggregate and the first embryonic mitosis I is initiated (Hardy 2002).

After fertilization, the zygote descends through the fallopian tube into the uterus for 3-4 days and divides. First, it divides into two cells called blastomeres, which further divide mitotically. Since the division proceeds synchronously, the embryo has 16 cells at the end of the fourth mitotic division, this formation is already referred to as a morula (Meng et al. 2023). Inside the morula, a cavity further forms and becomes a blastocyst. The cells of the blastocyst continue to divide, the cavity enlarges or expands, and this formation is referred to as the expanded blastocyst (Gosden 2002). At this stage, the blastocyst is still surrounded by the zona pellucida, but this is ruptured by contraction of the blastocyst, resulting in so-called blastocyst hatching (Niu et al. 2021). The outer layer of cells changes into a trophoblast and the inner layer into an embryoblast, which later develops into three germ layers from which the muscles, skeleton, skin, nervous system, and other organs differentiate (Florman et al. 2006).

After hatching, the embryo is nourished by endometrial secretions (uterine milk) until it attaches to the uterine lining (nidation), which is around the day 19 after fertilization (Cibulka et al. 2004). The trophoblast cells adhere to the endometrial cells and form a connection that provides nourishment for the embryo, which can continue to develop (Florman et al. 2002).

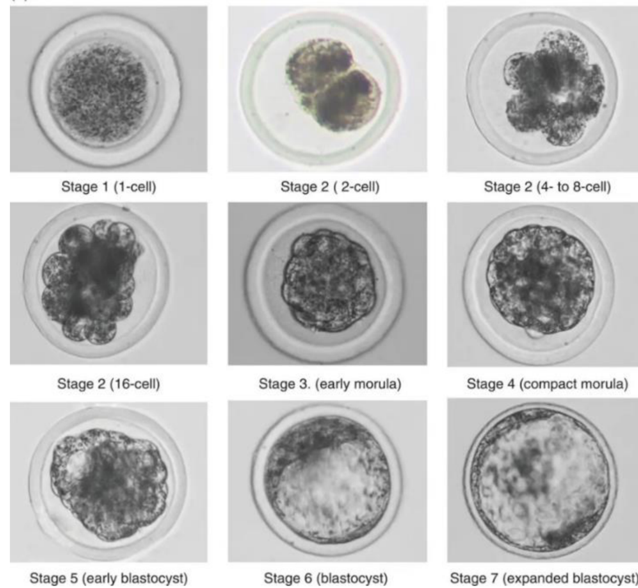

Figure2: Embryo development (Jahnke et al. 2014)

3.5 Selection and management of donors and recipients

The management of bovine embryo donors is a complex process that includes the care of the donors themselves, the collection and handling of embryos and the management of reproductive cycles (Bó et al. 2020).

3.5.1 Donor management

3.5.1.1 Selection criteria of donors

Cattle donors in embryo transfer programs are typically selected based on several criteria, including genetic merit, health status, and reproductive history (Bo et al. 2020). These criteria ensure that the selected donors i) possess desirable genetic traits, ii) are free from infectious diseases, and iii) have a history of successful reproductive performance (Bo et al. 2020).

Cattle donors are selected based on superior genetic traits related to desired production characteristics such as milk yield, meat quality, disease resistance, and other economically valuable traits (Perry et al. 2006). Rigorous health screening of potential donors is crucial to ensure they are free from infectious diseases that might affect the viability of embryos (Perry et al. 2006). Donors should have a proven reproductive history, including regular estrus cycles, successful pregnancies, and the ability to produce high-quality embryos (Bó et al. 2020).

3.5.1.2 Current protocols of superovulation

Controlled ovarian function and superovulation are commonly employed to synchronize the estrus cycles of donor cattle (Bó et al, 2020; Mapletoft et al. 2002). These hormonal treatments stimulate follicular development and potentially increase the number of available embryos for collection.

According to (Barros 2001), several hormonal procedures to induce a state of superovulation have been studied over the past 50 years. Superovulatory agents tested include pregnant mare gonadotropin in serum (PMSG) or Equine serum gonadotropin (ECG), FSH extracted from porcine, ovine, or equine pituitaries or recently, recombinant bovine FSH (Říha 1998).

The principle of hormonal treatment to induce a state of superovulation is the stimulation of the follicular system through the administration of higher doses of FSH than its endogenous levels (Sakkas et al. 2017).

Embryo transfer programs use superovulation to obtain the maximum number of transferable embryos with a high probability of pregnancy (Bó 2014).

Superovulation consists of inducing multiple ovulations, resulting in an increased number of oocytes for fertilization (Sakkas et al. 2017). Superovulation is also used to increase the efficiency of embryo transfer programs. As already mentioned, stimulating more follicles will increase the number of oocytes retrieved, which can then be used, for example, for *in vitro* fertilization (IVF), gamete intrafallopian transfer (GIFT) or intracytoplasmic sperm injection (ICSI) (Betteridge 2003).

Hormonal superovulation is a reproductive technique used in cattle breeding to stimulate the ovaries of a cow to produce multiple mature follicles, each containing an egg (Hasler 2014). This process involves the administration of exogenous hormones to manipulate the reproductive system and increase the chances of obtaining a higher number of viable ova (Hasler 2014). Commonly, FSH, LH or their analogs are used to achieve a state of superovulation (Ginther et al. 2001). FSH stimulates the development of multiple follicles within the ovaries, while LH induces ovulation (Ginther et al. 2001). The timing of hormone administration is a critical aspect to synchronize follicular development and induce ovulation (Mapletoft et al. 2002). This process involves the careful coordination of exogenous hormone administration with the natural estrous cycle of the donor cow (Sakkas et al. 2017). The goal is to stimulate the development of multiple ovarian follicles, ensuring they mature simultaneously for subsequent ovulation.

Hormones are administered at specific stages of the estrous cycle to synchronize follicular development. This ensures that a cohort of follicles matures simultaneously, increasing the likelihood of obtaining multiple eggs (Sakkas et al. 2017; Mapletoft et al. 2002).

Various gonadotropins in combination with progesterone or PGF2α have so far been used to regulate the estrous cycle. Říha (1988) mentions that in order to successfully induce superovulation, gonadotropin-based preparations that can mimic the effect of FSH must be applied. In our conditions, most often, to induce superovulation, natural preparations are used, obtained by extraction from pig or sheep pituitary glands. Specifically, these are, for example, Folicotropin (Spofa Prague, Czech Republic) or Pluset (Bioniche Animal Health, Spain) (Institute for the control of veterinary biopreparations and drugs 2024)Research has demonstrated that purified pituitary extracts containing limited amounts of LH can improve the response to superovulation in cattle (Bó 2014). Chupin et al. (1983) conducted a study in which they stimulated superovulation in three groups of cows using equal doses of 450 μg of purified FSH and varying amounts of LH. The results indicated that decreasing doses of LH led to an increase in the average frequency of ovulated oocytes, as well as the number of embryos retrieved and transferred. High levels of LH during superstimulation are believed to cause premature activation of oocytes (Kruip et al. 1984). However, doubling the dose of crude pituitary extracts containing both FSH and LH resulted in a significant decrease in the percentage of fertilized eggs and transferable embryos (Mikkola et al. 2020). Overall, these findings support the hypothesis that the negative effects of high doses of pituitary gonadotropins on egg and embryo quality are due to excess LH (Kruip et al 1984; Mikkola et al. 2020).

Superovulation is now the main source of obtaining embryos for the actual transfer. Although superovulation is said to be the main source, it is far from being the only source. Other sources of embryo's retrieval may include the retrieval of ovarian oocytes, their culture and maturation under *in vitro* conditions, *in vitro* fertilization (IVF) and their further culture to the ideal stage for transfer (Rowson et al. 1972).

To successfully induce superovulation, FSH must be administered frequently, this was decided based on the finding of the biological half-life of FSH, which is estimated to be 5 hours (Bó, 2014). Twice-daily treatment with pituitary FSH resulted in a greater super ovulatory response than once-daily treatment (Bó et al. 2020).

An integral step is the induction of luteolysis, which is induced 48-72 hours after the start of treatment by $PGF_{2\alpha}$ administration. Subsequently, estrus starts within the next 36-48 hours, and ovulation is expected after a further 24-36 hours (Bó 2014). Administration of FSH for 6 or 7 days may improve ovulation in some donors.

Superovulation protocols begin during the luteal phase of the estrous cycle (Mccue 1996). Prostaglandin F2α may be administered to induce luteolysis initiating a new follicular wave. Following luteolysis, the exogenous administration of follicle-stimulating hormone is initiated, FSH stimulates the growth and development of a cohort of ovarian follicles (Mapletoft 2002). The development is monitored through transrectal ultrasound examinations, which helps determine the optimal time for the LH administration (Mapletoft 2002; Bó 2014). The collected eggs can be fertilized either through artificial insemination (AI) or IVF (Mapletoft 2002; Binelli et al. 2001; Bó 2014).

IVF is a technique where eggs are fertilized outside the female's body. The fertilized eggs are then cultured and develop to the blastocyst stage outside the uterus. This method allows greater control over the process of fertilization and embryo development (Panzani et al. 2014). Egg collection can be done after natural ovulation or after hormonal induction of ovulation. The collected eggs are then fertilized with sperm *in vitro*. This process can take place under laboratory conditions (Lonergan et al. 2007). Sperm are added to the retrieved eggs and, if fertilization is successful, zygotes are formed. The fertilized egg (zygote) begins to divide to form a blastocyst, which is an advanced stage of the embryo containing both outer and inner layers of cells (Lonergan et al. 2007; Stringfellow et al. 2003). After reaching the blastocyst stage, the embryos are removed from the culture conditions and can be transferred to the uterus of the recipient for the subsequent gestation period (Mapletoft et al. 2002; Halser 2014).

Artificial insemination is a more traditional method where sperm are artificially introduced into the female's reproductive tract. This method is commonly used in livestock breeding to propagate genetic traits. Embryos are collected at the blastocyst stage, an advanced stage of development (Colazo et al. 2014). This stage usually occurs about 6-8 days after successful insemination (Colazo et al. 2014; Panzani et al. 2014).

Detection of estrus is important for subsequent insemination, as AI is usually performed 12-24 hours after estrus (Binelli et al. 2001). There are various protocols that have been successful in most circumstances.

Table 1*: Example of an applied schedule for preparing donor (Stádník et al. 2013)*

	preparation of donors	
Day of treatment	**Morning**	**Evening**
0	Examination of CL + Oestrophan 2 ml	
3	estrus control	
12	Examination of the size and development of CL	
13	Aplication of Pluset (3 ampules), 7 AM	Aplication of Pluset (3 ampules), 7 PM
14	Aplication of Pluset (2 ampules), 7 AM	Aplication of Pluset (2 ampules), 7 PM
15	Aplication of Pluset (1 ampule)+ Oestrophan 2 ml. 7AM	Aplication of Pluset (1 ampule) + 2 ml Oestrophanu, 7 PM
16	Aplication of FSH (1 ampule). 7 AM	
17	Insemination (12 hour after oestrus), 8 AM	Reinsemination 6-7 PM
18	Reinsemination, 8 AM	Reinsemination 3-4 PM
24	Embryo flushing	

Table 2*: Another example of a superovulation treatment schedule (Galli et al. 2003)*

	Task		**Dosage and time schedule**	
	Donor			
1.	Gynaecological examination			
2.	Applications of Oestrophane (OE)		3 ml	
3.	Estrus control			
4.	CL (corpus lutheum) examination			
			6-8 AM	**6-8 PM**
5.	Aplication of Pluset (FSH)		2 ml	2 ml
6.	Aplication of FSH		1,5 ml	1,5 ml
7.	Aplication FSH + OE (3 ml)		1 ml	1 ml + OE
8.	Aplication of FSH		0,5 ml	
9.	Insemination	4x	8-10 AM	6-8 PM
10.	Insemination		7-9 AM	2-4 PM
11.	**Embryo cellection**		**1000 ml transfermedium, 5 ml OE**	

3.5.2 Embryo recovery and evaluation

3.5.2.1 Embryo collection techniques

Non-surgical methods, such as ultrasound-guided follicular aspiration, are commonly used for the collection of oocytes from donors (Pierson & Ginther, 1984). Additionally, non-invasive transfer procedures, often guided by ultrasound, facilitate the placement of embryos into recipient animals (Hasler, 2001).

Ultrasound - guided follicular aspiration involves the use of ultrasound to visualize ovarian structures and guide the aspiration of follicles containing oocytes.

3.5.2.2 Embryo grading system

In industrial livestock farming, where technologies such as embryo transfer are used, a combination of IVF and AI may be used, depending on the specific objectives and conditions of breeding. The decision to choose between these methods may depend on genetic objectives, resource availability, and technical capabilities (Stringfellow et al. 2003).

Since the 1970s, there has been a shift in the industry towards non-surgical methods of embryo retrieval. Specialized silicone catheters are now available that are designed with precise length parameters and averages suitable for different cattle species (Fonesca et al. 2016). Unlike the original Foley catheter that was used in the 1970s, today's catheters are effectively usable and sterilizable (Nogueira et al. 2002; Fonesca et al. 2016). In addition, due to the increasing export of American genetics, the recovery systems have been adapted to remain fully closed during the embryo collection process (Nogueira et al. 2002). On average, the embryo is obtained 7 days after breeding (Szabari et al. 2008). Two methods are commonly used to recover embryos. The first is the gravity system, which involves gently flushing each uterine horn with 1 to 2 liters of a specialized medium, such as Dulbecco's phosphate-buffered saline (DPBS), in doses from 50 to 200 mL (Lonergan et al. 2007). The embryos are then gradually collected into a special filter that captures them. To prevent the catheter from falling out of the reproductive tract and to properly capture the embryos, the catheter is equipped with a balloon that inflates when the catheter is in the right place (Omidi et al. 2015). The second method of recovery is the syringe technique, in which the medium is injected into the uterine horn and then recovered into the embryo filter using a syringe (Bartolomel et. al 2003).

To successfully recover embryos and subsequently establish a pregnancy, it is essential to ensure that all surfaces and products that may come into contact with embryos or media are non-toxic and sterile. Currently, products such as media, catheters, syringes, tubing, and filters

are manufactured and evaluated to ensure their sterility and safety (Diskin et al. 2006). Media used today, such as DPBS, originally used a fraction of bovine serum albumin (BSA) as a protein additive that acted as a surfactant to help remove embryos from the endometrium and help maintain suspension during the recovery process (Fonesca et al. 2016). In the 1980s and 1990s, as world export markets expanded, there were concerns about the threat of BSA and possible contamination by the Bovine Viral Diarrhea (BVD) virus (Stringfellow et al. 2003). This problem necessitated the development of surfactants that were not of animal origin. Many have been developed, but the most used today is polyvinyl alcohol (Stringfellow et al. 2003).

One of the major improvements in the bovine embryo recovery process was the development of the embryo filter (Bartolomel et al. 2003). Originally, the obtained uterine lavage fluid was collected in a glass flask or graduated cylinder and allowed to stand for 30 to 60 minutes to allow the embryos to sink to the bottom. The supernatant is carefully aspirated, and the remaining liquid is placed in a gridded dish to look for embryos (Fonesca et al. 2016). There are many embryo filters on the market today that do essentially the same thing. They capture embryos 150 to 200 mm in size (Stringfellow et al. 2003). The filter allows fluid to escape but traps the embryos on the filter for retrieval and processing (Schenk 2006). One of the last aspects of the recovery process is the skill of the doctor (Schenk 2006).

Morphology of an embryo should be evaluated under microscope to identify any abnormalities, for example damaged zona pellucida (McGeady et al. 2017).

The assessment of embryo quality is based on visual evaluation of morphological characteristics (Van et al. 2003). An example of embryo evaluation is shown in Figure 3. This visual assessment is subjective. The most reliable indicator of the viability of an embryo is its stage of development relative to the day after fertilization (Van et al. 2003). Factors affecting embryo quality include the uniform size and color of the blastomeres, the presence or absence of intercellular spaces, the presence of extruded cells and the shape of the zona pellucida (Lundin & Park 2020).

Embryo quality codes range from 1 to 4, as illustrated by the following examples (Thomsen 2023). Embryos classified as excellent or good (1) or fair (2) have the highest probability of successful implantation. Embryos classified as excellent or good are suitable for freezing without significantly reducing the chance of a successful pregnancy (Wrenzycki 2021). Embryos with poor quality (3) should only be transferred fresh. In general, in the absence of a preference specification, priority should be given to transferring embryos with code 1. During superovulation of the cow, when embryo regeneration occurs on day 7, differences in embryo developmental stage and quality may occur (Thomsen 2003).

For an embryo to be classified as code 1, at least 85% of the cells must be intact. A code 2 embryo is scored if there is a slight abnormality in the overall condition. This category requires 50% of intact cellular material. Quality code 3 contains serious irregularities in the shape of the mass or in the size, color and density of the embryo. At least 25 % of the cellular material should be intact. If an embryo is classified as 4, this is an indication, for example, of a degenerated or single-cell embryo that is not viable (Philips et al. 2016).

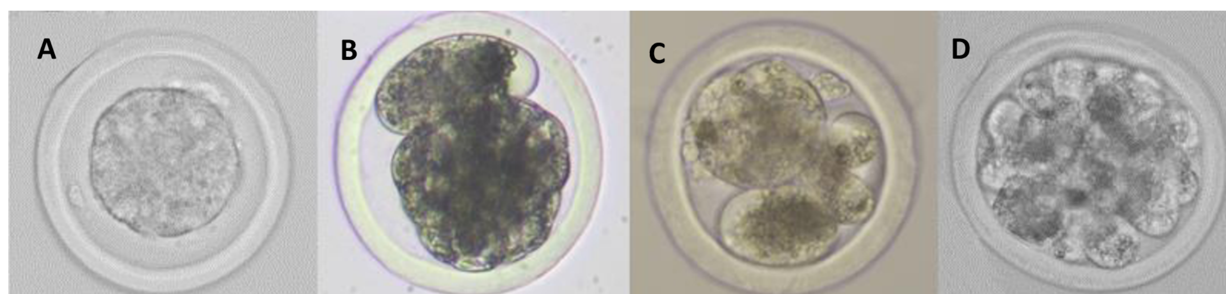

Figure 3*: Bovine embryo grading. A – Quality code 1: Excellent or good, B – quality code 2: Fair, C – Quality code 3: Poor, D – Quality code 4: Dead or degenerate. (Phillips et al. 2016)*

3.5.2.3 Embryo sex determination

To determine the sex of the embryo, detect genetic anomalies and assess the genetic material, a biopsy method may be chosen, which follows the principle of extracting a small number of cells from the embryo using a micropipette (Weikard et al. 2021).

Sex determination is used by the cattle industry to streamline trade in sex preference of offspring. However, preferences are not the same in all cattle farms, with male offspring being more prevalent in meat production farms, but female offspring being more prevalent in dairy farms (Fricke 2002). The rate of genetic progress is significantly influenced or limited by females, as their reproductive capacity is significantly lower than that of males, thus limiting the reproductive capacity of the herd (Phillips et al. 2016).

Invasive testing methods require simple biopsy techniques. Some research describes procedures to biopsy embryos at an early stage of development before morula compaction is achieved. One of these methods discusses the collection of human embryo tissue by drilling a zone followed by aspiration of a single blastomere (Phillips et al. 2016). Two micromanipulators are required for this procedure. The first one drives a pipette that retains the embryonic cells by gentle suction on the surface of the zona pellucida. The second micromanipulator controls two separate glass micropipettes (Fricke 2002; Phillips et al. 2016). One of them contains an acidic solution at pH 2.5 and is placed against the zona on the opposite

side from the holding pipette. Slow extrusion of the solution creates a hole in the zone into which the second pipette is then moved and used to aspirate a single blastomere from the embryo (Phillips et al. 2016).

Before reaching the compaction stage, a similar method is used, with the difference of pre-treating the embryos in calcium- and magnesium-free medium (Jainudeen & Hafez 2000). As with the previous method, the embryo must be fixed in a stable position using a holding pipette. A second glass pipette is used first to disrupt the zona pellucida and then to aspirate one or more cells (Phillips et al. 2016).

3.5.3 Recipient selection and management

Managing cattle recipients in embryo transfer involves various considerations to ensure successful pregnancies and optimal outcomes. One key aspect is the selection and preparation of recipient cows, which significantly impacts the efficiency of the embryo transfer process.

The selection of recipient's cows is critical for successful embryo transfer. According to research by Hansen et al. (2019), choosing healthy, cyclic, and reproductively sound animals is fundamental. Optimal recipients should be free from reproductive disorders, have a history of regular estrous cycles, and possess a suitable uterine environment for embryo implantation. According to Perry et al. (2005) young healthy cows with proven ability to conceive and carry a pregnancy to term are often preferred.

Synchronization of estrus is a common practice to ensure that recipients are in the optimal reproductive state for embryo transfer. This can be achieved using various protocols, which synchronizes estrus in a group of recipients, allowing for more efficient embryo transfer.

Proper nutrition is crucial for the well-being of recipients and successful embryo development. Research by Larson et al. (2006) highlights the importance of maintaining recipients on balanced and nutritionally adequate diet throughout the Embryo transfer (ET) process. Adequate body condition score (3,0) is associated with improved pregnancy rates. The BCS scale is shown in detail in Figure 4. Regular health monitoring is essential to identify and address any potential health issues that may affect the success of embryo transfer. According to work by Ruder (2015), routine veterinary examinations, disease prevention measures, and vaccination protocols are integral components of recipient management. Minimizing stress is vital for successful embryo implantation and pregnancy maintenance. Stress can negatively impact reproductive performance (Cooke et al. 2013). Therefore, providing recipients with a low- stress environment, proper handling, and adequate facilities is crucial.

Uterine health and preparation play a pivotal role in the success of embryo transfer. According to a study by Ferazz et al. (2016), techniques such as synchronization of recipient's estrous cycles and proper estrus induction can optimize the uterine environment for successful embryo implantation and subsequent pregnancy.

After embryo transfer, continuous monitoring of recipients is essential to detect early signs of pregnancy and address any complications promptly. Close observation of recipients during the post-transfer period allows for timely intervention, increasing the likelihood of successful pregnancies (Pierson & Ginter, 1984).

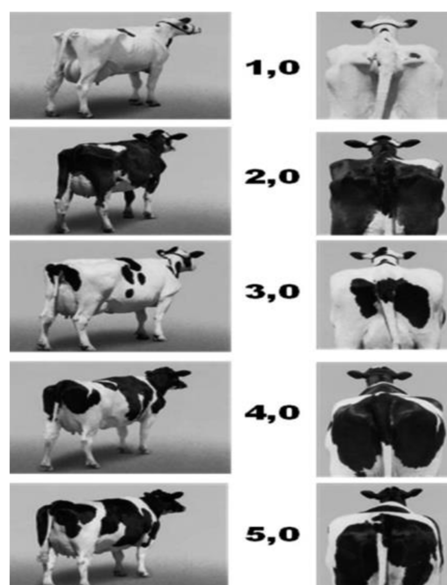

***Figure 4**: Body condition score (BCS). 1,0 – cachexy, 2,0- tendency to lose weight, 3,0 – optimal condition, 4,0- tendency to obesity, 5,0- obesity. (Edmonson et al. 1989)*

3.6 Embryo storage

Embryo storage is a crucial aspect of modern reproductive biotechnology that facilitates the preservation and transport of valuable genetic material (Hasler 2004). Embryos can be stored in cryogenic conditions, typically at -196 °C for up to several years. If immediate transfer of embryos from donor to recipient is possible, the embryos do not need to be frozen, but refrigerated for as long as necessary. Embryos that are refrigerated are usually usable within one week. Various methods, such as refrigeration, slow rate freezing and vitrification, have been developed and optimized to ensure the viability and integrity of embryos during storage (Hasler 2014). These methods play a vital role in maintaining genetic diversity, increasing breeding efficiency and accelerating genetic progress in cattle populations (Hasler 2004). The choice of storage method depends on many factors, such as the specific requirements of the breeding program, logistical aspects, and the expected storage time. Both methods have their

advantages and disadvantages, but the choice of method is one of the crucial aspects for proper preservation, maintenance of embryo viability and successful transfer (Galli et al. 2003).

3.6.1 Refrigeration

If immediate transfer of embryos from the donor to the recipients is not possible, an alternative option is to store them for a short period of time using normal refrigeration temperature, namely 0-4°C for up to week (Fasenko 2007). The use of the phrase "temporary storage" is appropriate for this method as it does not provide us with options such as cryopreservation but may serve us in the case of short-term transport between facilities or for storage before the recipient's cycle is synchronized (Porcu et al. 2002).

3.6.2 Cryopreservation

Most cryopreservation approaches rely on the use of cryoprotectants and manipulation of cooling rates and subsequent thawing. Since the beginning of successful experiments with mammalian embryo cryopreservation in the 1960s, two main groups of methodologies can be distinguished: traditional techniques based on slow freezing and the modern approach based on vitrification (Galli et al. 2003).

3.6.2.1 Slow - rate freezing

The most widely used technique for long-term embryo storage is undoubtedly cryopreservation, which involves controlled cooling of the embryo to sub-freezing temperatures using cryoprotectants to prevent cell destruction caused by ice crystal formation (Sirard & Coenen 2006). Sufficient cooling is followed by storage of embryos in liquid nitrogen at very low temperatures (-196°C) (Yungs 2011). The ultra-low temperature is important because it stops metabolic activity and thus preserves viability for a longer period (Yungs 2011). This method can preserve the embryo for several months or even years, allowing flexibility in breeding programs and facilitating international trade in embryos (Gordon 2003).

After the embryos are collected and evaluated for quality and developmental stage, they are treated with cryoprotectants. Cryoprotectants include, for example, glycerol, ethylene glycol, dimethyl sulfoxide, propylene glycol. These substances induce dehydration of the cell and at the same time limit the formation of ice crystals and thus protect the embryo from deterioration (Hansen 2020). In general, cryoprotective substances act by penetrating the cell

membrane and, by dehydrating the cell, preventing its damage during the freezing and thawing process (Gordon 2003; Hansen 2020). Not only the right choice of the appropriate substance, but also its precise concentration positively influences the whole process, minimizing cytotoxicity and maximizing vitality. Concentrations when using glycerol, ethylene glycol and propylene glycol range between 1-1.5 M, when using dimethyl sulfoxide, the concentration can be slightly higher at 1.5-2.0 M (Best 2015). As high concentrations of cryoprotectants can be toxic, they are typically used in combination to reduce the concentration of each and therefore their cytotoxicity. For this reason, cryoprotectant is added to the freezing solution in negligible amounts (Elliot et al. 2017). This procedure allows the embryos to adapt to the change in temperature and to regulate intracellular and extracellular osmotic pressure. In embryos of some species, tolerance to changes in osmotic pressure is limited to as little as 400 mOsm or less (Hasler 2014).

Of course, these active substances do not act on their own, but are included in the cryoprotective solution, that directly interacts with the cell (Fuler 2004). To stabilize the solution, a carrier is used in which the cryoprotectants are dissolved. The carrier may be saline, or a special medium designed for embryo cryopreservation, and these media may also be specially adapted for different animal species, including cattle, to best suit the needs of the particular species and to ensure optimal cryopreservation results (Fuler 2004). To make the solution complete, it is necessary to add other substances that help to stabilize the solution and reduce undesirable effects, such as antioxidants, metal chelators, and protein derivatives, among others (Yungs 2011).

Subsequently, after treatment with cryoprotectants, the embryos are cooled and frozen. First, the embryos are gradually cooled to temperatures close to zero to realize cell dehydration, the next procedure is to immerse the cooled embryos in liquid nitrogen at -196°C. In this state, embryos can be stored for up to several years (Fuler 2004).

3.6.2.2 Vitrification

In recent years, vitrification has achieved significant development (Kasai & Mukaida 2004). The advantage of this method is that it does not require expensive instrumentation and is also much less time consuming than slow freezing (Campos-Chillon et al. 2006). The vitrification technique prevents the formation of ice crystals inside the cell and in the surrounding solution during freezing. This is achieved by using a small amount of highly concentrated cryoprotectants and rapid freezing rates (Campos-Chillon et al. 2009). This process reduces the water content of the cell while reducing the cytosol becomes much more

viscous and changes to an amphoteric, glassy form (Nedambale et al. 2004). The principle is based on exposing embryos to gradually increasing concentrations of cryoprotectants as in slow freezing (Edgar & Gook 2012). The final solution is composed of highly concentrated cryoprotectants that are exposed to the cells for a maximum of 1 minute because the hyperosmotic environment could cause toxicity and osmotic damage to the cell (Edgar & Gook 2012). In the next step, the embryos are placed in very small volume (0.1-2 μl) and directly immersed into liquid nitrogen. This results in a very rapid temperature reduction (Edgar & Gook 2012). The straws are stored in cryocontainers (Nedambale et al. 2004).

3.6.2.3 Thawing

During thawing, the extracellular crystals in solution around the cell thaw, in which case the cryoprotectants from the cell are released into the surrounding environment and the water back into the cell (Hasler 2002). During the thawing process, a situation of water recrystallization may occur, this can be avoided by rapid thawing (Dochi 2019).

Embryos are placed in a controlled environment that allows thawing under stable and precisely monitored conditions. This may include the use of a water bath at a precisely set temperature or special thawing equipment. usually, frozen embryos are hold in the air for 10-20 seconds, then placed in a water bath for 20-30 seconds at 35-38 °C(Machatkova et al. 2008).

Defrosting depends on the level of cell dehydration. If the cells are gradually reduced in temperature to -30 or -40 °C, rapid rewarming is necessary to prevent the re-emergence of small, insignificant ice crystals that could coalesce into larger, more damaging structures (Machatkova et al. 2008). However, if the cells are cooled slowly to lower temperatures, rapid heating could cause excessive osmotic stress (potentially leading to cell lysis), and therefore it is necessary to heat the cells more slowly (~10 °C/min) (Hasler 2002; Dochi 2019.) Finally, the cryoprotectant is removed either by gradual dilution of the cryoprotectant or by a sucrose dilution technique that allows removal of the cryoprotectant without excessive water uptake into the cells (Hasler 2002).

After thawing, the embryos are carefully inspected and evaluated. This includes checking the morphology, vitality, and integrity of the cells. Healthy and viable embryos are characterized by good cell structure and minimal damage (Gómez et al. 2020). After successful thawing, the embryos are usually incubated in a special medium that provides suitable conditions for the recovery of their biological functions. At the same time, they are ready for immediate use for embryo transfer (Machatkova et al. 2008).

3.7 Fresh embryo or frozen embryo

The comparison between the use of fresh and frozen embryos is analyzed in detail in a study by Gustafsson et al. (1994). The research involved bovine embryos that were subjected to biopsy using a simple sectioning method and then frozen using a standard procedure using glycerol as a cryoprotectant. Viability of fresh and frozen embryos, both intact and biopsied, was assessed after *in vitro* culture, by fluorescence assay or after transfer to recipient animals. Survival after *in vitro* culture of fresh intact and biopsied embryos and frozen intact embryos and zona-free embryos was not significantly different (70%, 60%, 68% and 52%, respectively), but significantly decreased in frozen biopsied embryos (16%). Pregnancy outcomes after transfer of biopsied frozen embryos were also significantly lower (8%) compared to fresh biopsied embryos (39%).

Examination after incubation with diacetyl fluorescein showed fluorescence in both whole and biopsied embryos, with higher intensity in whole embryos. The reduced survival in frozen biopsied embryos is thought to be due to a combination of loss of zona pellucida and cell reduction caused by the simpler biopsy technique. The study concludes that if embryos need to be biopsied before freezing, advanced biopsy techniques and/or freezing should be used.

***Table 3**: Developmental rate of fresh and frozen -thawed biopsied, intact and zona free embryos (Gustafsson et al. 1994)*

	Treatment	No. of embryos cultured	No. of embryos (%) developed
Fresh	Intact	20	14 (70)[a]
	Biopsied	20	12 (60)[a]
Frozen-thawed	Intact	41	28 (68)[a]
	Biopsied	32	5 (16)[b]
	Zona-free	21	11 (52)[a]

Table 4: Pregnancy rates after transfer of biopsied embryos (Gustaffson et al. 1994)

	No. of embryos transferred	No. of animals pregnant	Pregnancy rate %
Fresh	23	9	39[a]
Frozen-thawed	12	1	8[b]

Today, the success rates are higher, and the assumption of the future is in the character of an increasing trend, but to achieve even greater success is linked to a significant effort of experimental practices.

Research has consistently shown higher success rates for both fresh and frozen embryos. Hasler (2001) states that, within the refinement and mastery of embryo freezing and thawing techniques, the increase in success rates for frozen embryos has been particularly significant. In various locations and time periods, fresh embryos have demonstrated a pregnancy rate ranging from 68.3 % to 77.1 %. The pregnancy rate for frozen and thawed embryos was slightly lower, ranging from 56.1 % to 68.7 %. However, it is worth nothing that there has been noticeable progress since a previous study conducted in 1994, particularly for frozen- thawed embryos.

Today's transfer success rates are on average around 70%, but if the embryos are of high quality and a suitable recipient is selected, success rates of up to 80% can be achieved (Hasler 2014).

3.8 Embryo transfer

3.8.1 Embryo transfer techniques

Ultrasound-guided embryo transfer (UGET) involves the non-surgical placement of embryos into the reproductive tract, specifically to the uterine horn (Selk 2002), of recipient animals using ultrasound guidance (Hasler 2001). Real-time ultrasound imaging is used to visualize the reproductive tract, allowing for precise placement of embryos into the desired location (Pierson & Ginter, 1984) ideally in the uterine horn on the side of the ovary with active CL (Selk 2002). This method allows for accurate and precise placement of embryos within the uterus, improving the chances of successful implantation (Hasler, 2001), another advantage of this method is its minimal invasiveness to the recipient animal.

Invasive methods, i.e., surgery, can be considered as another option. The surgical method utilizes laparotomy principles. The female receiving the embryo under local anesthesia. The surgeon makes an incision in the female's side via flank incision (Hasler 2014), identifies the uterus, and carefully removes the horn of the uterus under sterile conditions. Then, the embryo is confidently inserted into the uterine horn using a small plastic pipette (Strickland 2001).

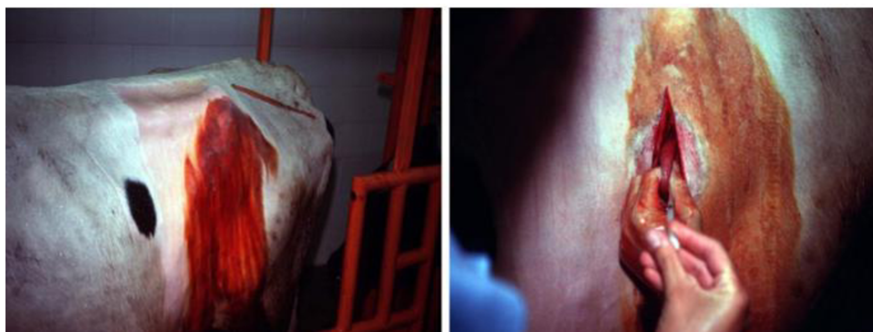

Figure 5*: This picture illustratively describes the surgical ET (Hasler 2014).*

Ultimately, the choice depends on the individual performing the action or the breeder's preference (Mebratu et al. 2020). However, it is important to note that each additional surgical operation carries a significant risk to the animal's health, whereas the non-surgical method largely eliminates this risk (Baruseli et al. 2006).

The following table shows some of the criteria that may be used to decide on the transfer itself (Mebratu et al. 2020). An illustrative comparison of surgical and non-surgical methods is shown in Table 4. A further illustrative comparison of surgical and non-surgical ET, specifically their success rates, is shown in Table 5. This example shows that heifers have a higher percentage of calving in both cases.

***Table 5**: Comparison of surgical and non-surgical methods for recovery of embryo (Mebratu et al. 2019)*

	Method of recovery	
End point	**Surgical**	**Nonsurgical**
Anesthesia	General	Epidural
Fasting	Required	Non required
Ability to recovery embryos at any stage	Excellent	Limited
Ability to accurately assess number of ovulation	Excellent	Poor
Risk of accurate complication to donor	Definite possibility	Virtually nil
Risk to future reproductive performance of donor	Yes	Probably none
Embryos recovery rate	Excellent	Good

***Table 6**: Comparison of pregnancy rates after surgical and non-surgical ET (Hasler 2014)*

Factors	Fresh embryos	
	No. transfers	% Pregnant
Surgical		
Heifers	1485	80[a]
Cows	491	70[b]
Nonsurgical		
Heifers	590	79[a]
Cows	84	61[b]

It is preferable to transfer an embryo at the blastocyst stage in the uterine horn, as the embryo has developed structures that allow it to implant successfully and develop further, but it is also possible to transfer an embryo at the morula stage into the oviduct. However, the quality of the embryo is particularly important, a study by Alvarez et al. (2008) shows that there is no significant difference in implantation of morula stage, graded as grade 1, or a cultured blastocyst. However, if we compare a grade 1 morula stage and a grade 3 blastocyst, the result is quite different. In this case, the pregnancy rate of the morula is 65 % but that of the blastocyst is only 29.4 %. In order to transfer fertilized embryos at different stages of development, it is necessary to ensure that they are transferred to the recipient at the appropriate stage of the oestrus cycle (Selk 2002). Thus, as has been said, although the blastocyst stage is more

preferred, the quality of the blastocyst must not be neglected as it has a considerable influence on success.

Pregnancy diagnosis is crucial for effective reproductive management and to accurately determine the success of ET. While several methods are available for pregnancy diagnosis, not all can be used at any stage. Although the absence of estrus is often the first observation, it may not be entirely reliable as the recipient may be in silent estrus (i.e., without any clinical sign or behavior typical of the heat) (Jainudeen & Hafez 2000).

A progesterone test can be conducted to determine the required level of progesterone for maintaining pregnancy. This test can be performed by testing blood or milk between 12 and 24 days.

Pregnancy can also be examined by rectal palpation, which detects changes characteristic of pregnancy. The changes observed are centered on the uterine positioning, size and asymmetry of the uterine horn, uterine contractility fluctuations, uterine ligament tension, uterine artery palpability and pulsation, and of course the presence of CL in the ovary (Jainudeen & Hafez 2000).

Ultrasonography is the most common and reliable method of diagnosis pregnancy, which uses high-frequency sound waves to view internal structures. The results are immediately available. The embryo can be observed for the first time on days 19 to 27 (Romano et al. 2006).

3.8.2 Factors influencing embryo transfer

The success of an embryo transfer depends on several factors, including the quality of the embryo, the synchronization of the reproductive cycle of the donor and recipient, the health of the donor and recipient, the handling of the embryo, the qualifications of the personnel and hormonal support specifically, the hormone PGF2 α, which induces luteolysis, FSH is essential for superovulation, and LH induces ovulation of oocytes.

Several protocols have been developed for synchronization of estrus, the simplest is probably the use of PGF2α, which is applied during the luteal phase, when CL has receptors for this hormone (from day 5 to 17 of the cycle), thanks to which estrus occurs in 3-4 days (Hegedüšová et al. 2010). Ovsynch, in which GnRH (gonadotropin releasing hormone) is injected at any stage of the cycle (but it is recommended in the first half of the luteal phase, which is day 5-11 of the cycle), 7 days later prostaglandin is injected and in another two days again GnRH, ovulation occurs within 24 hours (Čech 2012).

The quality of the embryo is undoubtedly a crucial factor for a successful transfer (Lonergan at el. 2001) as it is determinant for implantation and development in the recipient. Quality is influenced by embryo morphology, viability, and developmental potential. The higher the viability is, the greater the chance of success (Merton et al. 2003). It is important to note that high embryo quality does not guarantee success, but it does increase the chances. This information highlights the importance of selecting high-quality embryos for implantation. Additionally, embryo integrity, which includes structural and genetic stability, is an undeniable factor (Sartori et al. 2009). To achieve a successful outcome, it is necessary to select and evaluate embryos to identify the most suitable candidates.

Although embryo quality is a crucial factor, it is important not to overlook the health of the individuals involved. Any health complications in the donor or recipient can significantly impact the outcome of embryo transfer (Roche et al. 2009). If the donor develops a disease, such as an infection, it can lead to a decline in the quality of the oocytes and, consequently, the quality of the resulting embryos. This can reduce the success of implantation and embryo development in the recipient.

Optimal reproductive conditions are crucial for successful conception and pregnancy (Doležel 2003). Reproductive problems, such as uterine inflammation, can lead to a reduced implantation rate.

It is important to check the hormone levels of both the donor and the recipient (FSH, LH and PGF2α) to avoid any hormonal disbalances that may hinder the transfer (Coufalík 2013).

Stress is a significant factor that affects animal health. Prolonged exposure to stress can disrupt or even completely stop the reproductive cycle. Therefore, it is essential to provide optimal care and environment to achieve success (Dobson et al. 2001).

Nutrition and maintaining an ideal body condition score (BCS), as previously discussed, also have a significant impact on the health of the donor and recipient, and consequently, on reproductive success. Inadequate nutrient intake can disrupt the internal environment of dairy cows, potentially leading to reproductive failure. Excessive protein levels may adversely affect hormone production and have a toxic effect on the embryo (Stádník et al. 2013).

Late onset of estrus in cows with low or high body condition scores (BCS) can cause metabolic and endocrine changes. These changes can affect ovarian follicle dynamics, oocyte development and quality, and LH production. Infertility may result from disturbances in corpus luteum (CL) development and activity, leading to insufficient progesterone production and the inability to react the luteolytic effect of prostaglandins (Stádník et al. 2013).

3.8.3 Pros and cons of embryo transfer

Although embryo transfer has many advantages for livestock breeding, it is important to conduct a thorough analysis of its benefits and drawbacks before incorporating it into specific breeding programs.

The benefits of embryo transfer include improving genetic variability and allowing the spread of genetic material from genetically valuable individuals, which contributes to diversity in breeding (Hasler 2014). Additionally, embryo transfer can significantly reduce generation time by speeding up the reproduction process. In this case, embryo transfer allows for the transfer of several embryos from one female to other females, potentially resulting in more offspring from a genetically valuable female and reducing the time between generations, leading to accelerated genetic progress (Kasinathan et al. 2015).

Embryo transfer can enable individuals with limited reproductive capacity, such as those with musculoskeletal problems (Mebratu et al. 2020) or advanced age, to reproduce and pass on genetic material to offspring without the need to invest significant amount of energy on gestation and birth (Smith 1988).

For breeders, the economic benefits are a significant advantage in the long term, as production increases due to genetic progress and improved offspring quality (Boustan et al. 2015).

However, in economic terms, the breeder may also experience the opposite trend. This is often due to the cost of the breeding process itself, from the initial selection of individuals to the superovulation treatment, and the collection and transfer of embryos. It is important to avoid mistakes or poor planning at any stage of the project to prevent financial losses (Boustan et al. 2015).

One potential disadvantage of embryo transfer is the risk of losing embryos due to the factors such as stress conditions (Dobson et al. 2001), lack of synchronization, and poor timing of the transfer (Bó 2014), which can result in unsuccessful implantation. It is crucial to ensure that the embryo transfer is performed by a qualified professional and that every step is carefully monitored. This is because technical errors and the human factor can result in damaged embryos or even their loss. It is important to avoid overlooking these factors (Schoolcraft et al. 2001).

Neglecting the health and genetic screening of the donor and the recipient can lead to potential transmission of diseases or genetic defects (Stringfellow & Givens 2003).

The embryo transfer process raises ethical issues, as it is primarily used for more intensive production rather than the preservation of the species. This has led to debates surrounding animal welfare, particularly the five freedoms (freedom from thirst, hunger, sickness, pain, injury, discomfort, fear, and stress, and freedom to express natural behavior) (Daniel et al. 2019). The reproductive health of both the donor and recipient cows is taken into consideration to ensure that they can participate in embryo transfer without any negative impacts. Ethical concerns also arise from the fact that animals are often viewed as commodities rather than sentient beings (Chervenak et al. 2001).

As previously mentioned, embryo transfer can enhance genetic variation. However, it may also lead to a reduction on genetic diversity if there is a strong preference for the best-performing individuals, resulting in the use of limited amount of breeding material. This behavior can cause genetic diseases and reduce the population's resistance to external changes (Azkona et al. 2020).

3.9 Embryo Export and Import

In 1972, the first bovine embryo was transferred in the fallopian tubes of a rabbit, after 4 days it was removed and transferred to the recipient (Hasler 2014). Cryopreservation made longer distance transport of bovine possible. It is reported that since the first bovine embryo transfer, millions of embryos have been distributed around the world (Hasler 2003). This transportation method was made possible by the adoption of cryopreservation (Viana 2019).

The transport of embryos across national borders is significantly impacted by epidemiology. In the past, donor females had to be tested for up to 15 pathogens. Current regulations are set by the International Embryo Transfer Society (IETS) and the World Organization for Animal Health (OIE). Specifically, the IETS has developed detailed protocols and practical guidelines on how to effectively reduce the risk of disease transmission (Ponsart & Pozzi 2018). According to Ponsart & Pozzi (2018), the most effective way to prevent disease transmission is to wash embryos. It is crucial to follow the proper washing process to ensure the safety of the embryos during transport. In the European region, Council Directive 89/556/EEC, which describes the animal health conditions for the importation and exportation of bovine embryos within the community and their importation from third countries, is observed (Ponsart & Pozzi 2018). Eu legislation clearly outlines the specific requirement for the sanitary treatment of donors. Donors must have been in a herd that has been free of tuberculosis and brucellosis for the past six months. Furthermore, there should be no signs of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis observed during the previous year, and no clinical signs of enzootic leukosis in the herd for the past three years (Hasler 2003; Ponsart & Pozzi 2018). Figure 6 shows a detailed example of sanitation practices for pathogen detection.

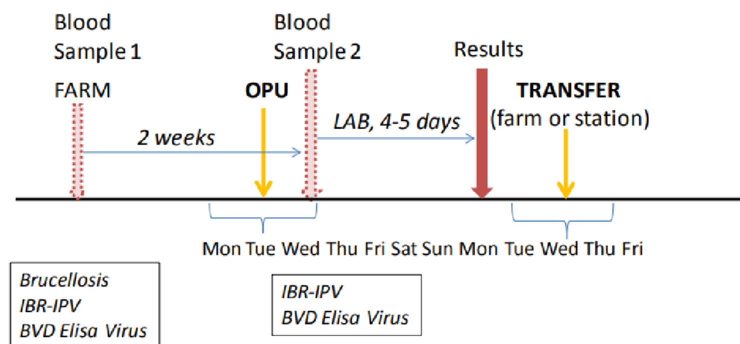

***Figure 6**: Example of sanitary control from France applied to donors (Ponsart & Pozzi 2018)*

Therefore, many pathogens have been found to remain associated with *in vitro*-derived embryos even after washing (Hasler 2003; Mapletoft & Hasler 2005).

Testing donor cows from which we obtain oocytes through OPU is not a problem. The problem arises when oocytes are obtained from the ovaries of untested cows at the slaughterhouse, the oocyte obtained in this way poses a major health risk in the form of the viruses already mentioned (Hasler 2003). To give an example, in France BVD viruses have been isolated from 1.47% of oocytes obtained from slaughter ovaries (Gard et al. 2007).

In the last decade, government regulations for importing embryos have been simplified, allowing exporters from North America to operate with regularity and, to some extent, routine. However, it is still true that the expansion of embryo exports is very much in line with health regulations (Hasler 2003; Gard et al. 2007). However, health regulations can change unpredictably, and this is mainly due to widespread disease, such as the outbreak of bovine spongiform encephalopathy (BSE) or the 2001 epidemic of foot-and-mouth disease in England and western Europe (Guerin et al. 2000). These unpleasant events have led to a complete halt in the transport of embryos and semen between countries.

A significant motivation for exporting and importing embryos has been to improve the variability of the gene pool, introduce new breeds, pass on their qualities, and enhance production (Mebratu et al. 2020).

Not to be overlooked are the economic factors that, together with government regulations, influence the embryo market (Guerin et al. 2000; Hasler 2003).

3.10 Embryo transfer in non-domestic bovids

The 1970s saw the development of IVF and ET, which sparked reflections on the potential of these and other reproductive technologies for the conservation of endangered species. While assisted reproductive methods are common in domesticated species, they have so far been difficult to adapt to wild animals due to differences in their reproductive anatomy and physiology. This limits their practical use for reproducing offspring (Pukazhenthi et al. 2003).

A significant limitation is the absence of fundamental information on numerous unexplored species, which is crucial for effective reproductive support and/or control. Reproductive technologies are now widely recognized as valuable tools for investigating the reproductive mechanism of different species, which may lead to the discovery of new and unique strategies (Loskutoff 2003).

The decline in the genetic diversity of population due to inbreeding and human activity may eventually lead to extinction (Pukazhenthi et al. 2001). Therefore, there is an ever pressing need to conserve wild population and protect biodiversity (Bhat 2021). Preserving female germplasm of wildlife, specifically oocytes and embryos, is a promising biotechnological tool for species conservation (Critser 2003). Other assisted reproductive techniques used to conserve endangered species include artificial insemination, embryo transfer technology, and sperm cryopreservation.

Cryopreservation techniques developed for genetic material from female domestic animals may also be adapted for use in wild animals (Pukazhenthi et al. 2003). Obtaining samples and access to animals for research purposes can be challenging, particularly when compared to livestock. Additionally, standardizing protocols for cryopreservation of genetic material can be difficult.

Biobanks can be used to preserve vital tissues from wild animals (Holt et al. 2004). Somatic tissue sections, ovarian tissue, sperm, oocytes, and embryos are all potential materials for vitrification preservation. Vitrification is considered the best option for preserving genetic material of female wildlife to maintaining species biodiversity due to its economic viability and ease of use (Luskutoff 2003). As a result of declining populations, efforts are being made to preserve genetic materials that can be obtained from living, but to some extent also from dead individuals (Huijsmans et al. 2023). Due to the non-invasive nature of post-mortem collection, methods of embryo retrieval, transport, preservation, fertilization can be identified and refined. Moreover, obtaining such embryos for research is economically cheaper.

On the other hand, the use of such embryos is more limited in practice because of the gradual degradation in quality (Huijsmans et al. 2023)

In the case of the American and European bison (*Bison bison* and *B. bonasus*, respectively), extinction is not far off and is the focus of conservation efforts (Acevedo et al. 2023; Huijsmans et al. 2023). They have managed to avoid total extinction but face the problem of low populations and extreme dispersal (Olech 2022). The attempt to save the European bison was a remarkable success since the population was revived from only 12 individuals. Today the populations consist of around 6,800 wild individuals in 45 herds, but only 8 herds reach the minimum viable population size (Plumb et al. 2020). Current conservation efforts focus on establishing ecologically and genetically sustainable bison herds to ensure the long-term survival of the species (Olech 2022). Assisted reproductive technologies (ART) can play a key role in this effort by allowing the transfer of genetic material in the form of gametes and embryos, while protecting the quality of life of the animals and ensuring the biosecurity of current bison herds (IUCN 2022).

The results indicate that ART can be an effective strategy for conserving the genetic material of the endangered species. If successfully applied to other endangered large mammals, it could have a significant impact on their rescue (Appeltant et al. 2023).

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4 Methodology

This bachelor thesis consists of a review of the scientific literature about bovine embryo transfer which included an overview and analysis of the individual steps of this procedure, factors influencing its success, and its potential application in non-domestic bovid species.

Data were obtained from publications available on academic databases such as Web of Science, Scopus, or Google Scholar, carefully processed and interpreted in relation to the set goals of the work, identifying main trends, challenges, and research directions in the studied area.

5 Conclusion

Embryo transfer represents a key technique in modern cattle breeding. The exploration of each stage of this procedure, including the selection of donors and recipients, superovulation, embryo retrieval, evaluation, conservation methods, and the transfer itself, provides us with valuable insights for the effective management of reproduction and genetic improvement of herds.

Analyzed factors influencing the success of embryo transfer indicate the need for further research and optimization possibilities.

Furthermore, it is important to examine the perspectives of embryo transfer utilization in international embryo trade and its application in non-domestic bovid species. This work contributes to a deeper understanding of the embryo transfer process in cattle and highlights the critical steps for future research, which may lead to further innovations and improvements in cattle breeding.

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