EFFECTIVENESS OF EMBRYO TRANSFER IN COWS - RISK FACTORS INCLUDING IN VIVO DERIVED AND IN VITRO PRODUCED EMBRYOS

Maria Wieczorkiewicz¹, Jędrzej M. Jaśkowski², Agnieszka Wichtowska¹, Monika Olszewska-Tomczyk², Bartłomiej M. Jaśkowski³

Abstract

Multiple Ovulation Embryo Transfer is a biotech method with more than 50 years of history and an established position in cattle breeding. This procedure is beneficial in many ways, but it also carries a risk of failure. The study presents the overview of the most important risk factors that may affect conception rates in the course of embryo transfer, including the factors associated with the embryo sourcing (embryo production method, embryo quality, development stage and breed, embryo storage method), embryo transfer procedure (synchrony/asynchrony, embryo transfer difficulty, the time of the transcervical insemination gun passage, depth of embryo deposition, localization and structure of the corpus luteum relative to the follicle and both individual characteristics of donors and recipients (level of concentration of progesterone, the state of health of the udder, lactation level, body condition score and age) and some environmental factors.

Running title: Risk factors for embryo transfer in cows

Keywords: embryo transfer, cattle reproduction, assisted reproduction techniques

¹Department of Basic and Preclinical Sciences, Institute of Veterinary Medicine, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University, Toruń, Poland

²Department of Clinical Sciences and Diagnostics, Institute of Veterinary Medicine, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University, Toruń, Poland

³Department of Reproduction and Clinic of Farm Animals, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, Wrocław, Poland

*Correspondence: maria.wieczorkiewicz@umk.pl

Full list of author information is available at the end of article

Introduction

Multiple Ovulation Embryo Transfer (MOET) is a biotech method with more than 50 years of history and an established position in cattle breeding. The advantages of this method include the possibility to use the genetic potential of valuable mothers, rapid valuation of bulls, creation of genetic reserves of disappearing cattle breeds and elimination of many infectious diseases [1,2]. Approximately 1 million of embryos are transmitted annually around the world. In the EU countries this number is about 140 thousand [3]. Every embryo transfer procedure carries the risk of failure. Under these conditions, any action aimed to reduce this danger to an acceptable level and increase the conception rate (CR) in recipients is fully justified. The study presents the overview of the most important individual and environmental risk factors that may affect CR in the course of embryo transfer.

Factors associated with the embryo sourcing

Conventional *in vivo* embryo deriving (IVD) is a technique used for decades. On the other hand, the following increase in *in vitro* embryo production (IVP) is a result of improvement in the technique of obtaining cumulus oocyte complexes (COC), as well as *in vitro* maturation (IVM), *in vitro* fertilization (IVM) and *in vitro* culture (IVC) [4]. Recently, numerous comparative studies on the efficiency of IVD and IVP embryos transfer have been conducted. Recorded pregnancy rates were lower in the group of recipients receiving IVP embryos than in the females after IVD embryo transfer (**Tab. 1**) [5]. These results were confirmed by Ferraz et al. [6] with conception rates higher in case of IVD embryo transfer than IVP - both fresh and frozen (49.3 vs. 39%). Significantly lower, not exceeding 30.9%, results of fertilization after IVP embryo transfer were also observed by Feres et al [4].

The best quality embryos guarantee the highest effectiveness of ET [6–9]. Lindner and Wright [10] reported that after transfer of the IVD embryos, which were evaluated as excellent, good or sufficient quality, pregnancy was obtained in respectively: 45%, 44%, 27% of recipients. This dependence was confirmed by Znaniecki, who by transferring high quality embryos reached 60% of pregnancy rate, while the transfer of good or sufficient quality embryos allowed to fertilize adequately: 43.8% and 26.1% of heifers [11]. The newer studies confirm this correlation in both dairy and meat cattle (**Tab. 1**) [12–14]. The probability of conception after the transfer of poor quality embryos was even 31% lower than in the case of 1st class embryos [9]. Spell et al. and Roper et al. do not attribute any significant effect of the embryo quality on CR in recipients [15,16].

The effectiveness of embryo transfer is also determined by the stage of their development. Em-

bryo transfer of 6- 8-day-old embryos ensured higher pregnancy rates than 5-day and 9-day-old [17]. According to Znaniecki et al. a higher CR was observed in the case a transfer of blastocyst than morula (53.8% vs. 46.2%) [18]. A similar dependence was observed by Hasler. The percentage of pregnant females after application of frozen embryos in the morula stage was 54.3% and in the early blastocyst stage 59.7% [19]. In the newest studies by Ferraz et al. [6] and Erdem et al. [14] a significantly higher probability of pregnancies after embryo transfer in the expanding blastocyst stage than in the blastocyst, early blastocyst and morula stage were recorded (**Tab. 1**). The effects of embryo transfer with a reduced number of cells, so called demi- embryos, was also studied. This method allows doubling the number of transferred germs. As a result, despite the lower CR compared to conventional embryo transfer (36.3 vs. 57.7), due to the possibility of fertilization twice as many cows, it is an interesting alternative in the case of a limited number of available embryos. However, some researchers do not attribute any significant influence of the embryo development stage on the results of conception rates in recipients [15,16].

Different CR are also recorded depending on the genetic origin of the transferred embryo [20,21]. Significantly higher results were recorded in the case of transfer the embryos obtained from meat cows, among which the best values were noted in such breeds as: Hereford, Charolaise, Angus and Brangus (**Tab.1**).

A transfer of frozen embryos results in a slightly lower CR than the transfer of fresh embryos. Ferraz et al. recorded a significant fall in conception rates (12.5 % and 6.1%) in case of frozen IVD and IVP embryos compared to the transfer of fresh ones (**Tab. 1**)[6]. Alternate results were noted by Rodrigues et al. and Roper et al., who, by transferring fresh or frozen *in vitro*-produced embryos, did not report any significant effect of this variable on CR in recipients [9,16]. The data provided by Do show that the transfer of IVP vitrified embryos did not cause any deterioration of the conception rates compared to the transfer of fresh embryos. On the 35th day after the transfer of fresh embryos, pregnancy was found in 41.3% of recipients, while after the transfer of vitrified embryos it was found in 40% [22].

The use of modern technologies has made it possible to select embryos. However, manipulations carried out on semen used for the production of IVF embryos contributed to the reduction of CR in recipients. It was shown that the procedure of sperm sexing using flow cytometry may inhibit the process of gametes capacitation, indirectly lowering the rate of blastocyst development and negatively influencing CR [23,24]. Mikkola et al., comparing the effectiveness of fresh and frozen embryos transfer

obtained after insemination by sexed semen with conventionally produced germs, proved both significant decrease in the probability of pregnancy after sorted embryo transfer and a negative effect of vitrification on the results of CR (**Tab. 1**) [24]. Similarly, Sanches et al. transferred the embryos produced from sorted semen and noted that both these processes significantly reduce the probability of pregnancy in recipients [25]. It was found, however, that the negative effects of vitrification can be minimized by using the original protocol for direct transfer of frozen embryos. The results of conception were 51% for fresh embryos and 35% and 40% for vitrified and frozen embryos - directly transferred. Gutnisky et al. also reported that using the commercial Cryotech Vitrification Kit to freeze IVP embryos, the same CR were obtained as after the transfer of fresh embryos produced in the same way (P/ET = 46.8%) [26].

Initially, when cytometric sorting of gametes was not available, the sex of embryos was determined by their biopsy [27,28]. Currently, the biopsy of commercially produced IVF embryos is mainly used to evaluate their breeding potential on the basis of biological markers present in the genome [29]. Olivei-

ra et al. determined whether the biopsied embryos are capable of implantation and whether these manipulations affect the various stages of pregnancy development. CR was evaluated three times during pregnancy, both in the group of biopsied embryos' recipients (group B, n=103) and the control group in which transferred embryos were not subjected to the abovementioned treatments (group C, n=82). In the first month of pregnancy the conception rates in the study group decreased in comparison with the control group (B=50.9% vs. C=62.5%). Pregnancy rates in the 3rd and 8th month were respectively: B=50.9% vs. C=47.6% and B=49.6% vs. C=45.2%. Birth rates recorded at 47.1% in group B and 42.0% in group C. It was found that embryos manipulation may cause their developmental incompetence and consequently disturbances in the implantation process and loss of pregnancy [29]. In turn, results presented by de Sousa et al. show that this technology can be successfully used in commercial programs aimed at associating assisted reproduction technology with genomic selection, because biopsy violation of the continuity of a pellucid zone produced both *in vivo* and *in vitro* has no significant effect on the conception rates (**Tab. 1**) [30].

TABLE 1 Effects of factors related to *in vivo* or *in vitro* embryo production on conception rates (own compilation based on: ¹Pontes et al [5], ²Ferraz et al [6], ³Donaldson et al [21], ⁴Arreseigor et al [20], ⁵Mikola et al [24], ⁶De Sousa et al [30])

FACTOR	CR FOR THE TRANSFER OF EMBRYOS PRODUCED <i>IN VIVO</i>	CR FOR THE TRANSFER OF EMBRYOS PRODUCED <i>IN VITRO</i>
Embryo production method ¹	41.5	33.5
Embryo quality ²		
Excelent	42.2	
Good	32.8	
Sufficient	23.7	
Embryo development stage ²		
Morula	36.5	
early blastocyst	38.1	
Blastocyst	45.3	
Embryo breed ^{3,4}		
Brangus ^{3,4}	47	36.8
Angus ^{3,4}	39	32.9
Nelore ⁴		36.7
HF ⁴		22.7
Hereford ³	75	
Charolaise ³	53	
Storage method ²		
Fresh	49.3	39
Frozen	36.8	32.9
Sorting process ⁵		
Sorter	40	37.3
not sorter	48.7	42.3
Biopsy ⁶		
Biopsied	54.2	26.4
not biopsied	55.9	19.8

One of the strategies to improve the survival of IVP embryos is to modify the composition of the media in order to approximate the parameters of the microenvironment of the culture to the conditions in which the embryo develops *in vivo*. Under physiological conditions, endometrial cells produce growth factors, cytokines, hormones and other regulatory molecules. Hyaluronate, Insulin Growth Factors (IGF-1, IGF-2), Colony Stimulating Factor 2 (CSF-2), Fibroblast Growth Factor (bFGF), Transforming Growth Factor (TGF- β 1), Granulocyte and Macrophage Colony Stimulating Factor (GM-CSF) and Leukemia Inhibiting Factor (LIF) are among the specific molecules whose supplementation may increase the survival of transferred embryos, both fresh and vitrified [31–34]. It has also been shown that a medium supplementation with 40 μ g/ml of heparin in the case of embryo production using sexed semen yields a pregnancy rate similar to that of conventional embryo transfer [23]. Taking into account the increased susceptibility to cryopreservation of IVP embryos caused by higher concentration of lipids in their structure, Sanches et al. investigated the effect of lipolytic factor (forslokine) on culture media. It was shown that forslokine supplementation caused an increase in the pregnancy rate in comparison to the control group (48.8% vs. 18.5%) [25].

Factors related to embryo transfer procedure

Spell et al. [15] and Frade et al. [35] report that strong expression of the heat symptoms positively correlates with progesterone levels and the conception rates. However, opinions on CR after embryo transfer after natural and synchronized heat are divided. Some authors indicate that the higher percentage of pregnancies is obtained after natural heat than that induced by prostaglandin (PGF2 α) or its analogues [36,37]. Problems associated with a poorly expressed heat observation can be eliminated by using timed embryo transfer (TET) [38,39]. It has been shown that the use of optimised synchronisation programmes results in a significant increase in the number of recipients available for transfer [40] and correlates with the amount of pregnancies per transfer session [7], as well as has a positive effect on the value of the conception rate in recipient cows, making TET suitable for transfer of both fresh (IVP-F) and vitrified (IVP-V) *in vitro* produced embryos [41].

According to some authors, the asynchronous, in other words, the difference in the time of occurrence of synchronized heat between donor and recipient, observed in field conditions, has no significant influence on the results of CR in recipients [6,15,42]. However, opinion of others, an asynchronism has a significant effect on conception rates [9,37,43]. According to Misra et al. [37], significant differences in

the percentage of pregnancies occur at $\pm$ 12 hours asynchrony. Donaldson [44] reported, that the differences in the CR for asynchronous animals of -24 and +24 hours were 6.9% and 4.8%, respectively, while in the case of -36 and +36 hours asynchronous - 4.7% and 9.8%, respectively. According to more recent studies, the conception rates for $\pm$ 30 hours asynchrony ranges from 36 to 50%[9]. Ledgard et al. [43], transferring 7-day-old blastocysts produced *in vitro* to synchronized (7 days after heat) and unsynchronized (5 or 9 days after heat) recipients, obtained the highest percentage of pregnancies in the group of recipients in whom asynchronization was +48 hours (Table 2). Additionally, the analysis of the recipients' histotroph proteom showed the highest expression of protein factors facilitating embryo implantation on the 9th day of the cycle.

The functional status of the recipient's uterus is also mentioned as an important factor that may cause the failure of ET procedure. The intensified contractility of this organ may lead to immediate or delayed expulsion of embryos. The presence of cervical mucus, which may clog the end of the catheter or surround the embryo and keep it in the cervix during the withdrawal of equipment, is also one of the factors determining the effectiveness of the procedure. Moini et al. [45] pointed out the relationship between the degree of cervical stenosis due to mucus filling and the results of embryo transfer. The removal of the clog from the cervical lumen increased the probability of implantation and favoured an improvement in pregnancy rate by 1/3.

The prolonged time of cervical catheterization, usually caused by acute uterine and cervical angulation, cervical stenosis or anatomical distortion of the cervical canal, may significantly hinder the placement of embryos in the uterine cavity [16],[46]. It is also more difficult to penetrate the cervix in the case of *Bos indicus* cows, in which the organ is longer than in *Bos taurus* cows [47,48]. The relationship between the degree of difficulty during embryo transfer procedure and its effectiveness was studied by Roper et al. [16]. The study was conducted on a three-stage scale based on the opinion of ET technicians, in which "1" meant "easy", "2" meant "moderate" and "3" meant "difficult" embryo transfer. The results of conception rates in the group of animals in which the ET procedure was found to be easy were significantly higher than in the other two (**Tab. 2**). A significant linear relationship between the time of the transcervical insemination gun passage (TTIGP) to the place of embryo deposition in the horn and the results of conception was observed. The longer the manipulation time, the fewer cows were pregnant. When TTIGP was over a minute, the percentage of pregnant females was 50% lower than in the case of manipulation lasting shorter [49]. More recent studies by Roper et al. confirm earlier reports [16]. The highest percent-

age of pregnancies was observed in cows from the group where the total time of cervical catheterization and embryo deposition was 6-9 minutes, while in cows where the procedure lasted longer (10-13 minutes and 14-25 minutes) the CR were relatively lower (**Tab. 2**) [16].

Worse results of pregnancies are obtained in the case of embryo transfer in cows than in the case of heifers [18,50]. It is suspected that one of the reasons is a longer TTIGP and larger uterus size [18,51]. Similarly, the multiple use of a female as a recipient reduces the likelihood of pregnancy after embryo transfer. The mechanism of this phenomenon is not entirely clear and it is difficult to explain it only by the relation of the time of gun passage through the cervix. Manipulations within the cervix, especially its broadening, cause an increased concentration of prostaglandin metabolites, shortened luteal phase, weakened secretory functions of the corpus luteum, and increased synthesis of oxytocin, which may lead to embryo loss [52]. Prolonged cervical manipulation causes stress and its consequences - increased concentrations of ACTH and cortisol [53,54].

The relationship between the place of embryo deposition in the uterus relative to the localization of the corpus luteum and the results of conception was also discussed [13,17,18,37,54–56]. The placement of the embryo ipsilaterally to the corpus luteum allows to obtain higher pregnancy rates in comparison with contralateral deposition (**Tab. 2**) [13]. Sanchez

et al. analyzing transcriptomes of uterine horns located ipsilaterally and contraterally to CL showed that there are differences in the expression of endometrial genes, but they are not correlated with the pregnancy survival up to 14 days after conception [55]. Trigo et al. confirmed that there are disproportions in the concentrations of progesterone, glucose and other components essential for the embryonic survival due to the asymmetry of the reproductive system, preferring the left horn of the uterus [56]. However, field studies did not show that the ipsilateral introduction of the embryo into the left horn of the uterus would guarantee a significantly higher rate of conception than when the right horn was in the ipsilateral position (53.2 vs. 51.0%) [56].

The depth of embryo insertion into the uterus may also influence the transfer effectiveness. The results of embryo placement were significantly higher in recipients in which embryos were deposited deep in the horn of the uterus than in those in which embryos were placed in the middle of the horn or in the area of the uterus body (43% vs. 31% and 24% respectively) (**Tab. 2**) [16,54].

Individual characteristics of donors and recipients

The percentage of pregnant recipients with easily palpable corpus luteum is higher than in those with less developed CL [15,57]. This is related to the level of progesterone recorded in the recipients. The

TABLE 2 Influence of factors related to embryo transfer on the conception rates ((own compilation based on: ¹Ledgard et al. [43], ²Roper et al. [16], ³Jaśkowski JM et al. [49] ³Del Campo et al. [13], ⁴Jaśkowski BM et al. [46])

FACTOR	CR
Asynchrony ¹	
- 48h (5d)	25%
none (7d)	30%
+ 48h (9d)	40%
Embryo transfer difficulty ²	
Easy	43%
Moderate	32%
Difficult	18%
TTIGP ³	
10-60s	53.4%
> 60 s	20.4%
Depth of embryo deposition ²	
Deep in the horn	43%
In the middle part of uterus horn	31%
In the uterus body	24%
Localization of the CL relative to the follicle ³	
Ipsilaterally	65%
Contrilaterally	33%
Structure of corpus luteum ⁴	
Cavitary CL	52%
Compact CL	33%

concentration of progesterone in the blood of females on the day of embryo transfer and 7 days later correlates with the conception rates [58],[59]. The higher the progesterone level on the day of embryo transfer and the lower the estradiol level, the better the results of embryo transfer [59]. There is a significant positive correlation between the degree of development of the corpus luteum measured by its volume and the level of progesterone [46,60–62]. It was shown that with a blood progesterone concentration above 2–2.5 ng/ml on the day before or on the day of embryo transfer, a higher pregnancy rate was obtained after fresh embryo transfer compared to recipients with a lower progesterone concentration than indicated [8,12,63]. Kenyon et al. [64] state the minimum level of progesterone necessary to keep pregnancy in recipients. Between 0 and 7 days after insemination it should amount to 2.71 ng/ml, and between 8–14 days after insemination 1.48 ng/ml. According to Jaśkowski et al. [65,66], the type of corpus luteum is also a significant factor correlated with the recorded progesterone levels. In recipients with cavernous structure of the corpus luteum, both P4 concentrations and CR were higher than in females with solid structure (12.1 +/- 3.58 and 8.1 +/- 3.96 for P4 and 37.3% and 30.4% for CR, respectively). Some authors [15] do not share this theory, attributing the high results of embryo transfer only to the precision of heat detection. The presence of the first wave follicle may be relevant for the conception rate. In addition to its increased size, the dominant follicle is distinguished from secondary follicles by its ability to produce more oestradiol, maintain low concentrations of intrafollicular binding proteins of insulin-like growth factor -2, -4 and -5 and follistatin and increase free intrafollicular IGF-I concentrations [67].

The results of research on the dependence of fertility rates of cows on the level of milk production indicate that in the case of donor cows with high milk production in the period preceding the superovulation protocol, the probability of fertilization the recipient after ET was lower than in the case of donor cows with significantly lower milk production before embryo flushing [49,68,69]. This relationship was explained by the lower quality of embryos collected from donors with high milk production and increased likelihood of early embryo mortality. Each subsequent day of lactation in donors increased the chances of fertilization the recipient [70].

In a study by Skrzypek et al. [71] a strong correlation between inflammation of the udder and cow's reproductive functions was found. It is the consequence of changes in the hormonal system, resulting in disorders in the sexual cycle and ovulation and early embryo mortality. Risco et al. [72] noted that the risk of losing pregnancy was 2.7 times higher in the case of udder inflammation up to 54 days after conception compared to cows who did not have any *mastitis* cases during this period.

Some authors correlate the transfer results with the age of the recipients [54]. The CR were found to be higher in recipients older than 525 days [17]. Ferraz et al. recorded significantly lower conception rates in multiparae than in primiparae and heifers (31.6% vs. 37.8% vs. 42%, respectively) [6]. A similar pattern was observed in Mikkola et al. in both conventional and sexed semen (57.8 and 54.8% for heifers, 17.7 and 18.2% for the primiparae and 24.5 and 27% for multiparae, respectively) [24].

A lot of attention was also focused on the influence of the condition of recipients (BCS) on the effectiveness of embryo transfer. It was shown that the pregnancy rates were significantly higher in recipients whose condition was evaluated at 3 and 4 (on a 5-point scale) than in those whose condition was assessed at 1, 2 or 5 points [39,73]. Fernandes et al. [74] analyzed the effect of daily weight gain of recipients on the effectiveness of pregnancy maintenance obtained from IVP embryos. It was shown that the percentage of pregnancies increases with daily weight gain to 350g/day, which suggests that this is the threshold for obtaining an optimal pregnancy rate in recipients. In addition, the assessment of daily weight gain allows the detection of even minimal fluctuations that make this method more sensitive than the evaluation of animal condition on the basis of BCS.

Environmental factors

The demand for nutrients in lactating cows rises with the increase in production, which may result in a negative energy balance. This has a significant impact on the hormonal management of the female and may cause impairment of its condition and lowering of progesterone level in blood, and consequently result in deterioration of conditions of embryo implantation in the uterus [36,75–77].

Reduced effectiveness of embryo transfer may also be associated with simultaneous occurrence of lameness or *mastitis*. Mental stress associated with changes in technological groups, transport or treatment of animals by the personnel also leads to lower pregnancy rates [76,78,79].

There are some differences between the CR as a result of embryo transfer depending on the period of time during which ET was performed [80]. It was shown that the results of embryo transfer in summer were worse than those obtained in spring and winter [17,37,79,81]. Inappropriate climatic conditions - too high temperature and environmental hyperthermia - may lead to hormonal disorders and increased embryonic mortality in the second week of pregnancy [17,78,79,82,83]. High temperature and humidity index (THI) (≥ 80) had a negative effect on the results of embryo transfer in the 8th, 7th and 6th day after heat [6]. Nabenishi et al. [84] noted differences between CR in Japanese black and HF cattle in different seasons of the year. In Holstein-Friesian cattle the conception rates in

August-October were lower than in other months. CR for Black Japanese recipients was the lowest between December and January. These results may suggest that cattle for fattening are less exposed to the negative effects of thermal stress. Fertility reduction after embryo transfer in the hot season is lower than after insemination [41]. For this reason, transfer of IVP embryos during seasonal heat may be a way to avoid fertility depression [85]. Also, the use of *in vitro* embryos obtained after insemination with sorted semen improves the conception rates in cows exposed to heat stress [86].

Lower conception rates and increased embryo mortality may also be due to infection with pathogens, among which the among which bovine viral diarrhoea virus (BVDV) is a particular nuisance [1].

Conclusions

Many years of research and population observations have allowed to identify the most important factors influencing the effectiveness of embryo transfer in cattle. This allows for the proper selection of embryos and their recipients, as well as setting the optimal date of transfer. Not without significance for the efficiency of ET also seem to be the efforts aimed at improving the technical components of the transfer - nutrient composition, freezing protocols and manipulation during embryo transfer. The conscious minimization of these factors has significant economic implications, favouring the increase of ET efficiency.

Ethical approval

The conducted research is not related to either human or animal use.

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Corresponding author

Maria Wieczorkiewicz, Department of Basic and Preclinical Sciences, Institute of Veterinary Medicine, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University, 7 Gagarina Street 87-100 Toruń, Poland, tel.: +48693-809-673, e-mail: maria.wieczorkiewicz@umk.pl.

Conflict of interest statement

The authors declare they have no conflict of interest.

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