Embryos produced with sexed semen: influence of the stage of embryos, embryo type, parity, quality of the embryo, and period of the year on pregnancy rates in dairy cattle. A longitudinal study

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SUMMARY

The objective of this retrospective study was to evaluate factors affecting pregnancy rate by embryo transfers, including both fresh and frozen, from embryos produced with sexed semen, in Holstein cattle on the same commercial dairy farm in Galicia, NW Spain. Holstein donors used in this trial were selected according to optimum body condition score (BCS) and with a normal health status and reproductive function. Each female donor was inseminated into the uterine body with two or three doses of sex sorted semen at 12 h and 24 h after the oestrus was detected. Data from 224 embryo transfers performed between 2016 to 2020 were available. To study the effect of heat on embryo transfer efficiency, all the years studied were divided into two periods, summer season and cold season. Pregnancy rate was calculated by dividing the number of cows that became pregnant after the transfer by the total number of embryo transfers, distinguishing between two dichotomous groups: pregnancy loss and not pregnancy loss. All the embryos were collected 7 days after the onset of oestrus and were identified before being evaluated following a classification system to assess the quality of embryos intended for transfer using two main criteria: the stage of development and the quality of the embryo. A logistic regression analysis was carried out to identify the different factors involved in the pregnancy rate. From all the models performed, those with the lowest value. The average gestation success reached was 51.78% for all embryos transferred in this study and produced from sexed semen. The transfer of compact morulae resulted in a lower pregnancy rate (48.57%), lower than the transfer of later stages (early blastocysts) (54.62%), and the probability of pregnancy was higher for fresh embryos (58.76%), as compared with frozen ones (46.45%). In conclusion, sexed semen was found as an effective tool to produce heifers of high genetic value, in a shorter period than with conventional semen, reducing costs and improving sustainability.

KEY WORDS

Pregnancy rate; embryo transfer; stage of development; quality of embryo; sexed semen.

INTRODUCTION

Since artificial insemination is undoubtedly considered to be the greatest technological advance in animal breeding, we could probably say that the multiple ovulation and embryo transfer technique (MOET) together with semen sexing are two tools that can contribute to major genetic improvement. Moreover, under specific circumstances such as animals subjected to heat stress or those which are classified as repeat-breeder females, the embryo transfer technique turns out to be more successful than artificial insemination (AI)[1].

In the last decades, several methods and patents have been developed to obtain-sexed semen, but most of them were discarded due to their limited efficacy [2]. Currently, the only commercially available method for the separation of X- and Y-chromosome-bearing sperm is through flow cytometry sex-sorting [3]. The primary reason for incorporating sex-sorted semen in any dairy system is to impose a desired sex bias in the resulting progeny [4], leading to a reduction of costs and an improvement in environmental sustainability [5]. However, despite all the advantages mentioned above, their use in artificial insemination of superovulated donors is limited [6], mainly due to the low sperm concentration of the sexed doses [3], combined with the higher incidence of fertilization failure on superovulated versus single ovulating cattle [7]. Also, most authors have reported a decline in the pregnancy rate and in the proportion of transferable embryos on donor cows using sex-sorted semen as compared with conventional semen [8, 9]. In

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heifers, this decline is usually smaller [8, 10,11].
However, this trend is changing in recent years, because of improved ovulation synchronisation protocols, inseminations performed at the optimal time, and more efficiently managed herds. These are the reasons for considering sexed semen in AI and embryo transfer.
Data on pregnancy rate and loss of pregnancy after the transfer of embryos produced with sexed semen are scarce [12]. In addition, there has been little information involving only the transfer of fresh and frozen embryos with sexed semen in superovulated dairy heifers and cows on the same commercial farm. This was the main starting hypothesis for the realization of this retrospective study.
The data in the present study were collected during five years and several factors were retrospectively analysed in relation to pregnancy rates following the transfer of fresh and frozen embryos. Among others, we analysed stage of development, quality of embryo, parity, embryo type, period, bulls and years as independent variables.
The objective of this experiment was to study the pregnancy rate in superovulated Holstein females following a fixed time insemination with sexed semen and how different variables (stage of development, quality of embryo, parity, embryo type, period, bulls and years) can change the pregnancy rate.

MATERIALS AND METHOD

General considerations for the study

This study is a retrospective analysis of data from n=224 embryo transfers, performed on one commercial dairy farm during a 5-year period (2016-2020). Figure 1, obtained from the Mouriscade farm s database (Provincial Council of Pontevedra, Galicia, Spain). In order to study the effect of heat on embryo transfer efficiency, all the years studied were divided into two periods, summer season (from May to September) and cold season (from October to April), obtaining the following results:
- summer season: n=70 total embryo transfers
- cold season: n=154.

Pregnancy rate was calculated by dividing the number of cows that became pregnant after the transfer by the total number of embryo transfers. Cows that had to be re-inseminated or retransferred an embryo 2 to 8 days after insemination or transfer, as well as pregnant animals that were sold or died before calving were discarded from the index calculation (n=12). Pregnancy was diagnosed at 30 days post AI using an ultrasonography equipment (Easi-scan: Go IMV imaging, L Agle, France) and confirmed at 80 and 180 days after the first positive pregnancy diagnosis.
In this study we distinguish two dichotomous groups: pregnancy loss (n=91) and not pregnancy loss (n=149). "Pregnancy loss is recognized as one of the major factors contributing to poor reproductive performance in dairy cattle" [13]. This situation includes both premature foetal loss and abortion. However, following [14], we distinguish 3 groups: early foetal loss (<45 to 60 d) (n=48), late foetal loss (60-90 d) (n=26), and abortion (>90 d) (n=17).

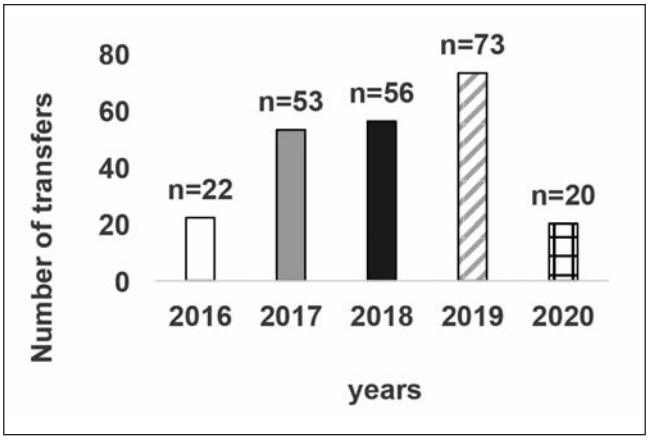

Figure 1 - Number of embryo transfers recorded during five years on a commercial dairy farm.

Superovulation synchronization protocol, embryo production and insemination

The 87 Holstein donors used in this trial were selected according to optimum body condition score (BCS) and with a normal health status and reproductive function. Most of these donors were cows (n=50), the rest were heifers (n=37).
The superovulation synchronization protocol in donors (Figure 2) began when a progesterone releasing device (Prid Delta® 1.55 g, Ceva Sante Animale, Libourne, France) was inserted in the uterus and it was kept there for 6 days. Embryos were collected from a few Holstein donors, which had previously been superovulated by intramuscular (IM) administration of FSH (Follicle-Stimulating Hormone, Pluset® Laboratorios Calier, S.A., Barcelona, Spain) at 12-hour intervals for 4 days. The doses administered were increasingly lower, totalling 800 to 900 IU FSH in the cows. In the case of the heifers, the doses were 60% to 75% of the doses in the cows. Then, the day before completing the FSH administration, 2 doses of prostaglandin F2 alpha were IM injected. The doses consisted in 15 mg of d-cloprostenol (Dalmazin® Laboratories Fatro S.P.A, Bologna, Italy) and they were administered at a 12-hour interval. PRID was removed following the second prostaglandin dose 36 h after the onset of standing oestrus was observed, GnRH (Gonadotropin hormone-releasing hormone) analogue was administered IM. The dose consisted in 1 mg of gonadorelin (Cystoreline®, Ceva Sante Animale, Libourne, France). Each female donor was inseminated into the uterine body with two or three doses of sex sorted semen at 12 h and 24 h after the oestrus was detected. Only sexed semen was used in this study. Semen was sorted out using the XY sperm sorting protocol [15]. Inseminates contained at least 30% of progressively motile sperm after thawing for 30 s in 37°C water. Sperm was inseminated into the uterine body just beyond internal cervix.
Of the total number of Holstein bulls used to produce embryos (n=23), only six were selected (ST genetics, Navasota, Texas, USA) as they were used in a representative number of inseminations (X=30). All of the inseminations and embryo transfers were performed by the same veterinarian practitioner.

Embryos

All of the embryos were collected 7 days after the onset of oestrus and were identified before being evaluated. The International Embryo Transfer Society (IETS) [16] has established a widely used classification system to assess the quality of embryos intended for transfer. The IETS embryo grading system typically uses two main criteria: the stage of development and the quality of the embryo.

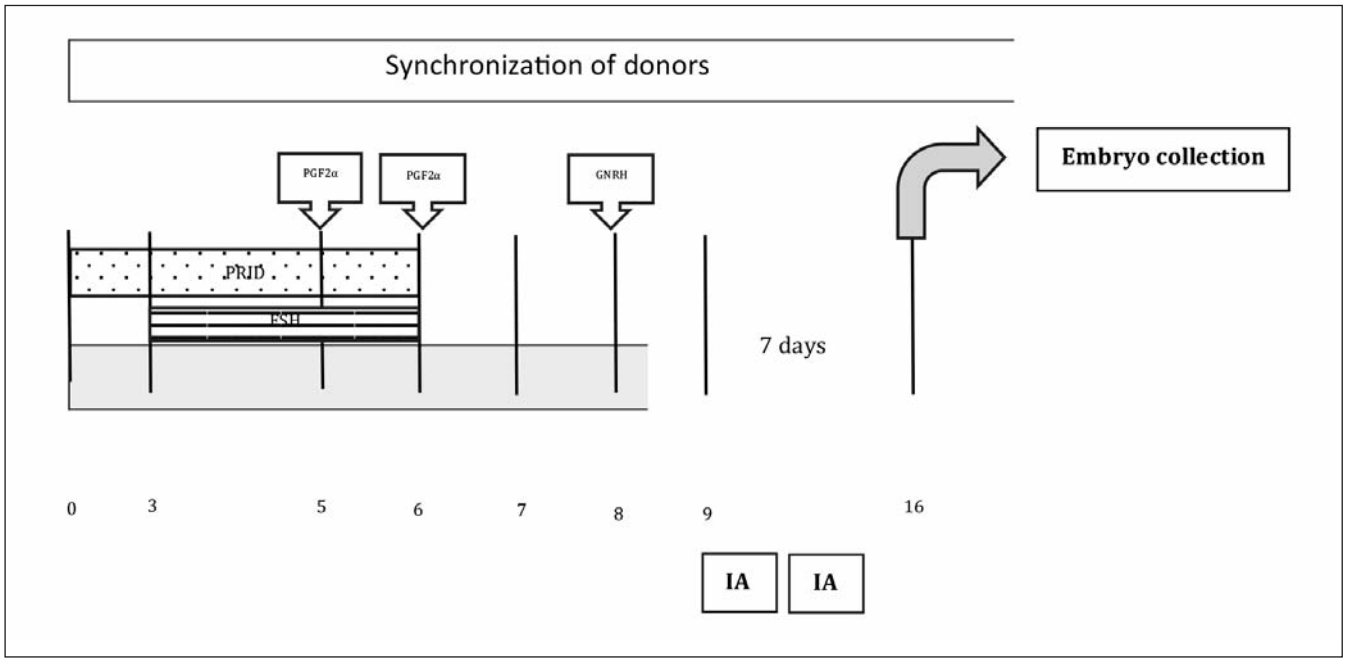

Figure 2 - Superovulation synchronization protocol in donor cows.

Bovine embryos go through several stages of development after fertilization. These stages are characterized by distinct changes in the embryo s structure and cellular composition. The developmental stages of embryos in compact morulae (n=105) and early blastocysts (n=119) were the only ones taken into consideration as expanded blastocysts, hatched blastocysts and expanded hatched blastocysts were discarded for transfer.

As regards the quality of the embryos, grade 1 and grade 2 embryos were defined as viable. However, grade 3 and grade 4 were considered degenerated and thus discarded. Therefore, in this study only embryos of quality 1 (n=176) and quality 2 (n=48) were transferred.

Most of grade 1 embryos were frozen in liquid nitrogen for preservation and subsequently transferred to the recipients (n=127). These embryos represent 56% of the collected embryos. The rest were transferred directly to the recipient females (n=49). All of grade 2 embryos (n=48) were transferred directly to the recipient females. Therefore, the number of embryos transferred fresh (n=97) represent 44% of the collected embryos.

Recipient

The Holstein recipient cows were carefully chosen basing on factors like their reproductive health, age, and genetic background. Embryos were transferred to heifers (n=16), to primiparous cows (n=113) and to multiparous cows (n=95). As we can see, the number of embryos transferred was similar in both cow groups while in heifers the number is much lower. The management and feeding of the donor and the recipient cows were the same because they were located on the same farm.

Statistical analysis

The possible influence of the different factors investigated which affect pregnancy rate results was analysed using logistic regression analyses: stage of embryos (morula or blastocyst), embryo type (fresh or frozen) parity (heifers, primiparous or multiparous cows) quality of embryo (quality 1 or quality 2), years (2016, 2017, 2018, 2019, and 2020), period (cold period or warm period) and bulls. Likewise, a logistic regression analysis was carried out to identify the different factors involved in the pregnancy rate. From all the models performed, those with the lowest value according to the Akaike's Information Criterion (AIC) were selected as the final ones. Odds ratio values were computed by raising 'e' to the power of the logistic coefficient over the reference category.

RESULTS

Influence of the different variables studied on the pregnancy rate post-ET (embryo transfer)

From the seven variables studied that could interact with pregnancy rate (stage of development, quality of embryo, parity, embryo type, period, bulls and years), we have only represented five of them (Table 1).

The transfer of compact morulae resulted in a lower pregnancy rate (48.57%) than the transfer of later stages (early blastocysts) (54.62%) ($P<0.05$) (Table 1). With an odds ratio of pregnancy of 2.44 for early blastocysts as compared with compact morulae (Table 2).

The probability of pregnancy (with a OR=2.78) (table 2) was higher for fresh embryos (58.76%) as compared with frozen ones (46.45%) ($P<0.05$).

In cows, the pregnancy rate decreased (9%) as compared with heifers (56.25%) and primiparous cows (56.33%) (Table 1). The pregnancy rate was similar in the six bulls selected as there was no effect of the bull on the pregnancy rate.

Other factors studied which did not affect the pregnancy rate were the quality of the embryo, and the period. Better pregnancy was achieved with quality 2 embryos transferred during the cold period.

Influence of the variables studied on the pregnancy loss post-ET

The pregnancy loss in this study included both premature foetal

Table 1 - Pregnancy rate (PR %) after the transfer of embryos from Holstein donors, produced with sexed semen taking into consideration the different stages of development, quality of embryo, parity, embryo type and period. a,b values in columns with common superscripts differ significantly (P<0.05).

Total TE (n=224)	Variables	Pregnancy (n=116)	Non-pregnancy (n=108)	(PR%) 51.78
Stage of development	Compact morulae (n=105)	51	54	48.57[b]
	Early blastocysts (n=119)	65	54	54.62[a]
Quality of embryo	Quality 1 (n=176)	90	86	51.13
	Quality 2 (n=48)	26	22	54.16
Parity	Heifers (n=16)	9	7	56,25
	Primiparous (n=113)	64	49	56.63
	Multiparous (n=95)	43	52	45.26
Embryo type	Fresh (n=97)	57	40	58.76[a]
	Frozen (n=127)	59	68	46.45 [b]
Period	Cold period (n=154)	83	71	53.86

loss and abortion and it was high for all the embryo transfers performed (40.6%). A total of 91 transferred embryos into recipients were not able to complete the gestation. When reviewing the data obtained, pregnancy loss was similar in all the years of the study.

The pregnancy loss in this study included both premature foetal loss and abortion and it was high for all the embryo transfers performed (40.6%). A total of 91 transferred embryos into recipients were not able to complete the gestation. When reviewing the data obtained, pregnancy loss was similar in all the years of the study.

There was no effect of the other variables studied (stage of development, quality of embryo, parity, embryo type, period and bulls) on the pregnancy loss.

DISCUSSION

To the best of our knowledge, this is the first study which uses only sexed semen to produce embryos, and which considers several factors that can affect the success of an embryo transfer programme. The first point to highlight is the fact that the pregnancy rate was high for all animals, reaching 56.25% in heifers, 56.63% in primiparous cows, and 45.26% in multiparous cows. Generally, we can confirm that our results agree with previous studies in which the gestation rates in embryo transfers in heifers were better than in adult cows [17, 18] (Hasler, 2001; Ferraz et al., 2016) and higher than those reported in adult cattle [12], who determined a pregnancy rate of 38.8% in cows inseminated with sexed embryos. What is more, our rate was even 7 points higher than that reported by the same authors (44.15%) with conventional semen transferred in the same study. In addition, the pregnancy rate obtained in our study was 56.25 %, which means that our data are much better than those reported in literature [6,8,10].

These good results seem to be due to the oestrus synchronization protocol, the timing of insemination (between 12 and 24 hours after its detection) and, above all, the application of two or three doses of sexed semen since, according to [19], an improvement in reproductive results was observed when using a higher number of doses of sexed semen. This is due to an increase in the concentration of spermatozoa, and therefore, a greater number of intact sexed spermatozoa with high fertilization capacity.

Considering the embryo preservation method, that is, fresh vs. frozen, the pregnancy results improved when applying fresh semen. In fact, according to the results obtained in similar studies previously published [17,18,20], the pregnancy rate increased by 12.29 points when transferring fresh embryos vs. frozen ones. It is well known that embryo cryopreservation techniques can greatly affect the viability of the embryo, reducing its fertility rate [21]. However, a new system has been reported by [22] for the direct transfer of frozen embryos without loss in pregnancy success as compared with fresh embryos. In this study, embryos were produced in vitro and were cultured with BSA (Bovine serum albumin) until day 6 and then without protein until day 7. Day 7 blastocysts were transferred to heifers either fresh or after being frozen in a medium using a substitute synthetic pro-

Table 2 - Representation of the probability and odds ratio for the variables studied in relation to the pregnancy rate. E (Estimator), P (Probability), OR (Odds Ratio), CI (Confidence Interval). In the case of the statistical significance, the magnitude of the difference is expressed as an OR.

	E	Z-Value	P	OR	IC-95%
Stage of development	-0.8824	-2.028	0.0205*	2.44	0.172-0.956
Quality of embryo	0.1210	0.289	0.7728	-	-
Parity	-0.3569	-0.608	0.5432	-	-
Embryo type	-1.0365	-2.518	0.0277*	2.78	0.154-0.780
Period	-0.3074	-0.870	0.3843	-	-
Interaction between embryo quality and embryo type	0.9139	1.609	0.108	0.40	0.826-7.702

tein called CRYO3. Frozen embryos were thawed and then transferred directly without removing the cryoprotectants.

According to embryo preservation and embryo quality, most of quality 1 embryos were preserved frozen based on the IETS, while quality 2 embryos were transferred in fresh according to the IETS. While most authors found better pregnancy rates when transferring quality 1 embryos [18,23] and, lower pregnancy rates when transferring quality 2 embryos, in our case, the fact that we found similar values is due to the freezing process. This process implies a damage to the embryonic viability, which will affect the pregnancy rate at the time of transfer [21]. However, if all the embryos were fresh, the pregnancy rate would be higher.

Finally, when considering the time of the year and its effect on the gestation rate, the first thing to note is that the number of transfers in this study was lower during the warm season as compared with the number of transfers performed in other seasons of the year. Furthermore, a lower pregnancy rate was recorded in embryo transfers performed in the warm season, coinciding with the results provided by [22]. Despite this decrease, it is well known that embryo transfer improves the pregnancy results, and that is why it is recommended as an alternative to Artificial Insemination in warm seasons [19]. The mechanism by which an increase in temperature causes a decline in fertility is known to be multifactorial and will depend on the magnitude of the heat stress suffered by the animals. [25] pointed out that the reason for most of the cases of infertility related to heat stress is that this causes damage to the oocyte and early embryo.

By the time an embryo is transferred, at the blastocyst stage, the embryo has acquired resistance to maternal thermal stress. [26] observed that the experimental application of heat stress to inseminated cows, reaching a rectal temperature of 41.1ºC, caused a drastic reduction in embryo development in the following 7 days after AI. As previously explained, in vivo embryos are obtained after 7 days in the donor cow, when they are at a stage of development between morula and blastocyst, and they are transferred to a recipient cow on the seventh day of the cycle. Therefore, if we transfer a day 7 embryo to a recipient cow, we will have skipped this critical period in which heat can have a serious influence [27,28]. Furthermore, this stress also affects folliculogenesis, putting at risk the superovulation procedure. Therefore, in the warm months of the year the number of good embryos obtained usually decreases. This limitation can be solved by using cryopreserved embryos that were collected at another time of the year [19,28].

CONCLUSION

In our 5-year experiment, most of the embryos implanted in dairy cows, came from sexed sorted semen and were in the early blastocyst stage. These embryos were of high quality, including all the variables in fresh and frozen semen, and the average gestation success reached 51. 78%. Therefore, sexed semen is confirmed as an effective tool to produce heifers of high genetic value, in a shorter period than with conventional semen, reducing costs and improving environmental sustainability.

Ethical Approval

Permission for the procedures of the experiment was granted by the Bioethical Committee of the University of Santiago de Compostela (USC) according to the Spanish Regulations (RD 53/2013, legal provision no. 1337), and the European Regulation of Animals for Scientific Purposes (Council of Europe, ETS no.123).

Author Contributions

Conceptualization, RM, JH, HM and CC; methodology, RM, FG, JLB and HM, formal analysis, RM, JH, CL and CC JS; investigation, FG, HM and CC; writing-original draft preparation, RM, JHB and CC writing-review and editing RM, JH and CC; All authors have read and agreed to the published version of the manuscript.

Conflict of Interest Statement

The authors declare no conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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