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Combining Embryo Transfer and Artificial Insemination to Achieve Twinning in Beef Cattle, and Effects of Different Twin Calf-Raising Methods on Neonatal Behavior and Growth

Eduarda M. Bortoluzzi [1,2,*], Kolton W. Aubuchon [2,3], Nicole D. Robben [3], Nicole Stafford [3], Mikayla J. Goering [2], Claiborn Bronkhorst [3], John A. Odde [3], Clay Breiner [4], Karol Fike [2], Lindsey E. Hulbert [2] and Kenneth G. Odde [2]

[1] Department of Anatomy and Physiology, Kansas State University, Manhattan, KS 66506, USA
[2] Department of Animal Sciences and Industry, Kansas State University, Manhattan, KS 66506, USA; mikayl3@ksu.edu (M.J.G.); lhulbert@ksu.edu (L.E.H.); kenodde@ksu.edu (K.G.O.)
[3] Odde Ranch, Pollock, SD 57648, USA; ndrobben@vet.k-state.edu (N.D.R.); nbstafford@tamu.edu (N.S.); drclins@lodivet.com (C.B.); jodde24@hotmail.com (J.A.O.)
[4] Cross Country Genetics, Westmoreland, KS 66549, USA; c.breiner@crosscountrygenetics.com
* Correspondence: bortoluzzi@vet.k-state.edu; Tel.: +1-785-532-5666

Abstract: As the beef industry moves towards efficient animal production to improve sustainability in agriculture, new production and management approaches are emerging. Among the many facets of the beef industry, cow–calf operations have the most opportunity for efficiency improvement, including improvements in fertility. This project accounts for measures and methods of (1) twinning reproductive technologies and (2) twin calf perinatal care and pre-weaning rearing. The overall objective was to produce twin calves using two reproductive technologies—embryo transfer and artificial insemination. The subobjectives were to determine accuracy of twin pregnancies embryo/fetal losses using ultrasonography, evaluate parturition and dystocia, and determine the effects of different twin-raising methods on neonatal behavior and growth. A fixed-time artificial insemination (FTAI) protocol was applied to 77 multiparous Angus-cross cows from a commercial beef herd in northcentral South Dakota during the summer of 2019. Cows were assigned to two different treatments groups: only artificially inseminated (AI) or received an embryo transfer following artificial insemination (ET + AI). They were estrous-synchronized, artificially inseminated (AI) with black Angus semen at day 0, and received and embryo transfer (ET) at day 7. Ultrasound examination detected 56% pregnancy risk for both groups, with sensitivity, specificity, and accuracy of 75%, 100%, and 90.5%, respectively, for bilateral twin detection. Calves were born during spring 2020. Twin calves (n = 34) and singleton calves (n = 11) were assigned to one of three raising methods: (1) twin born and twin raised (TT; n = 16), (2) twin born and single raised (TS; n = 18), and (3) single born and single raised (S; n = 11). Neonatal nursing behavior and birth weights were recorded, and adjusted day 200 and day 280 were calculated measures of vitality and growth. Blood samples were collected at age 24 h for colostrum intake measures (total serum protein, IgG1, and IgM). Twin calves were born 20% ($p < 0.05$) lighter in body weight than singletons; however, weights did not differ at day 280 between TT and S calves. TS calves had the shortest average latency to stand, but immunoglobulin concentrations did not differ among treatments. At weaning, cows that had birthed and raised twins produced more kilograms of live weight per pregnancy than cows birthing and raising singletons. Using ET + AI proved to increase twinning rate, and growth was maintained when raising both twins with their dam.

Keywords: artificial insemination; cattle twinning; embryo transfer

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1. Introduction

Sustainability in agriculture is a topic that is growing exponentially for both producers and consumers. The demands for more efficient animal production systems are necessary to satisfy sustainability requirements. Beef production has a higher environmental footprint compared to other animal-based proteins when considered on a per-pound basis of crude protein [1]. Cow–calf operations need the most improvement in efficiency based on the amount of greenhouse gas emissions. In 2019, Rotz et al. [2] reported that cow–calf operations contributed 77% of total cattle emissions, used significantly more fossil energy and blue water, and had more loss of reactive N (total ammonia (NH_3), nitrous oxide (N_2O), nitrate (NO_3), and nitrogen oxides (NOx)) compared to the other sectors in cattle raising (i.e., stocker background and finishing phases).

Reproductive rate was previously associated with the efficiency of beef production [3]. Discrete improvements in efficiency are related to reproductive traits that can increase economic outcomes [4]. An increase in twinning technologies could improve production and economic efficiency by 20% to 30% [5,6]. Even though twinning may result in low weaning weight per calf, an increase of 51% in weaning weight per cow was previously reported [7]. Biotechnologies applied to production of twins in cattle have been extensively studied and reported in the literature. Prior studies regarding increases in beef cattle fertility efficiency include genetic selection [4,6,8,9], hormonal superovulation [10,11], embryo transfer [7,12], and artificial insemination followed by surgical [13] or non-surgical embryo transfer [14,15]. Genetic selection was undertaken in a long-term program by selecting dams with multiple ovulations, and testing sires' progeny increased twinning frequency [6,9,16]. This program was groundbreaking, and due to the increase in ovulation, the frequency of fraternal twin and triplet births reached an annual rate of 60% [15].

Despite its positives outcomes, twinning in cattle has also raised some undesirable effects, including dystocia, greater rebreeding interval, lower birth weights, and retained placenta [6,17–19]. When genetic selection is considered, unilateral multiple ovulations (same ovary) or bilateral ovulations (both ovaries) can occur. Despite the biotechnology chosen to produce twins (e.g., genetic selection, bilateral embryo transfer), researchers reported an increased survival rate, gestation length, and birth weight when twins were gestated in bilateral uterine horns [12,20,21]. In addition, other researchers reported that unilateral twins were lighter in birth weight, which led to less dystocia [20,22]. In 2010, Tani et al. [14] produced twins in Japanese dairy cows using artificial insemination followed by embryo (produced in vitro) transfer in the uterine horn contralateral to the ovary containing the corpus luteum (CL). The reasoning for using the contralateral technique is not clear in their report; however, improved fetal and performance outcomes of embryos gestated in different uterine horns could be involved. Others [15] used a similar technique, but transferred an embryo (from donor beef cows) in the uterine horn ipsilateral to the ovary containing the CL following ET protocols.

In view of extensive previous research done on cattle twinning and the need to reopen this discussion aiming at improving efficiency of U.S. beef cattle, we developed a twinning trial in a commercial U.S. cow–calf operation. We hypothesized that transferring in vivo-produced frozen and thawed high-grade embryos from donor cows to the contralateral uterine horn to the ovary containing the CL seven days after artificial insemination would increase beef cows' conception risk, calving risk, and efficiency. Furthermore, once twins are born in commercial cow–calf operations, it is common that one of the calves becomes grafted to another cow in hopes to improve their growth rate and maintain that cow in the herd. Therefore, our secondary hypothesis is that twin calves grafted will present an improved growth rate compared to twin calves that were both kept with their dam. To the best of the authors' knowledge, few research data comparing behaviors between these two groups of calves are available.

This study aimed to use and refine technologies and methods previously described to produce twin calves in a commercial cattle operation to increase efficiency. Methods that were included and refined were artificial insemination followed by embryo transfer, ultrasound precision and accuracy of twinning diagnostics, parturition and dystocia care, evaluation of colostrum intake using behavioral observations and blood measures of passive transfer, and evaluation of the effects of weaning weight on twin calf rearing methods compared to conventional singleton rearing.

2. Materials and Methods

The commercial cow–calf operation followed animal care standards as described in the *Guide for the Care and Use of Agricultural Animals in Research and Teaching*. Additional techniques and animal care modifications for the duration of this study were approved by Kansas State University's Institutional Animal Care and Use Committee (IACUC 4282).

2.1. Animals and Treatments

Seventy-seven multiparous Angus-cross cows and their calves from a commercial beef herd in north central South Dakota were enrolled in this study. The entire study took place between June 2019 and September 2020. During the fertility treatment and gestation, cows were on pasture composed mainly of native grasslands with mineral supplementation, and water was provided ad libitum. For all procedures, including handling, sample collection, hormone injections, AI, embryo transfer, and ultrasonography, cattle were herded to a handling area with a squeeze chute (Daniels Manufacturing Co., Ainsworth, NE, USA).

2.2. Synchronization Protocol

All cows' estrous cycles were synchronized using a 7-day standardized protocol called the fixed-time artificial insemination (FTAI) method (CO-Synch + CIDR®; [23]). Synchronization was initiated with GnRH (86 mcg/2 mL, IM.; Fertagyl®, Merck Animal Health, Madison, NJ, USA). Then, an intravaginal progesterone insert was applied (CIDR®; 1.38 g of progesterone; Eazi-Breed™ CIDR Cattle Insert; Zoetis Animal Health, Kalamazoo, MI, USA). After 7 days, the CIDR was removed. Then, cows received a 25 mg intramuscular injection of prostaglandin $F_{2\alpha}$ (25 mg/5 mL, $PGF_{2\alpha}$; Lutelyse®; Zoetis Animal Health, Kalamazoo, MI, USA). A breeding indicator patch was placed halfway between the hip and the tail head (Estrotect™, Estrotect Breeding Indicators, Spring Valley, WI, USA). The breeding patch was activated when a cow was motivated to stay immobilized and allow mounting by a groupmate. Breeding patches were checked 66 h after placement, and activation of the patch was documented for each cow. Cows with less than 50% of surface ink removed were considered inactivated and therefore did not likely display standstill (standing to be breed) behavior. Cows that had inactivated patches at the 66 h check received an additional intramuscular injection of 86 mcg of GnRH (Fertagyl®, Merck Animal Health, Madison, NJ, USA). The proportion of cows presenting estrus was recorded. Nevertheless, all cows were kept enrolled in the study for the next phase: artificial insemination.

2.3. Artificial Insemination and Embryo Transfer

All cows received one dose of frozen–thawed black Angus semen at the end of the synchronization protocol. Frozen semen doses from Connealy Uptown Bull were purchased from Select Sires, Inc., Plain City, OH, USA.

Seven days after artificial insemination, the presence or absence of a corpus luteum on both ovaries was detected using transrectal ultrasonography (5–10 MHz linear array transducer; Sonosite M-Turbo, Fujifilm Sonosite, Bothell, WA, USA). A fertility specialist veterinarian used both a qualitative assessment and the corpus luteum size to place each positive-corpus luteum cow into 1 of 3 categories: excellent—palpable and firm, with >10 mm diameter; good—palpable and moderately firm, with >10 mm diameter; or poor—palpable but soft, with $\leq$10 mm diameter [24]. Cows in the poor category were not eligible to receive an embryo transfer seven days after the AI. Cows in the excellent and good categories were eligible for embryo transfer in addition to AI. Though using the CL scores to determine recipients shows conflicted results in the literature, the suitability of recipients is mainly determined by estrus and palpable CL [24–26]. Nevertheless, we recorded the CL scores as it was standard practice used by the fertility specialist veterinarian to perform the embryo transfer or not. At the time of corpus luteum assessment, cows underwent their final fertility treatments. The ineligible cows did not receive embryo transfer (AI only; n = 12), while the eligible cows received a 7-day frozen–thawed (grades 1 and 2) in vivo embryo (39 red Angus embryos and 24 black Angus embryos; Cross Country Genetics, Westmoreland, KS, USA) via non-surgical embryo transfer to the contralateral uterine horn to the ovary with a present CL (ET + AI; n = 63). The goal of the embryo transfer seven days after artificial insemination was to create two calves for one cow by producing one embryo via artificial insemination and adding a 7-day embryo through embryo transfer.

2.4. Pregnancy and Fetus Number Detection

Between 45 and 53 days after AI and embryo transfer, pregnancy risk and embryo counts were determined by transrectal ultrasonography of the uterus (4.0 MHz convex transducer; ReproScan XTC (VGA), Winterset, IA, USA). Pregnancy was considered positive when one or more embryos were visible. Then, the number of embryos detected was recorded for each cow. Pregnancy risk was defined as the proportion of cows with at least one visible embryo via ultrasonography divided by all the cows that received just a dose of semen and a dose of semen and an embryo. Pregnancy loss was determined as pregnant at time of ultrasound to open at calving time.

2.5. Periparturition Data

Six weeks before their calving date, cows were moved into a 0.4 km^2 cornstock pasture with windbreaks and easy access to a maternity barn. Cows were provided with a nutritional feed ration to meet or exceed NRC (2016) requirements for pregnant beef cows (offered once daily). Fresh water was provided ad libitum. Cows were checked for signs of parturition every 2 h in the early stages of parturition and every hour in the later stages. When cows presented signs of parturition (stage I and II of parturition), they were moved to a maternity barn and housed in individual pens. All pens were bedded with 10 cm-deep straw and re-bedded daily.

At calving time, data collection included gestation length, number of calves born per cow, birth order for twin calves (1 = first calf; 2 = second calf), sex, birth weight, calf color (black or red), and American Angus Association calving ease scores for each calf on a scale from 1 to 5 (1 = unassisted calving; 2 = some assistance; 3 = mechanical assistance; 4 = cesarean section; and 5 = abnormal presentation) [27]. Time of birth—calf on the ground, first stand—all four limbs upright, and first suckle—mouth contact with teat were recorded for each calf.

2.6. Calf Weight and Blood Collection

Before moving calves outside at 24 h of age, birth weights (BWs) were collected using a digital crane scale (Rural365, Sioux Falls, SD, USA). Then calves were gently handled and placed in lateral recumbency, and 10 mL of blood was collected via jugular venipuncture using a BD vacutainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Whole blood was centrifuged, and supernatant serum was harvested and frozen at −20 °C until analyses. At ages 6 and 9 months, calves were gathered and weighed with a squeeze chute containing a scale (Daniels Manufacturing Co., Ainsworth, NE, USA). Since calves had different birthdates, a 200-day adjusted weight was calculated using the following equation:

$$[(\text{ADG} \times 200\text{ d}) + \text{BW} = \text{A200dW}]$$

for the ~6-month weight, and a 280-day adjusted weight was calculated using the following equation:

$$[(\text{ADG} \times 280\text{ d}) + \text{BW} = \text{A200dW}]$$

for the ~9-month weight.

2.7. Nursing Behaviors

Calves born to cows enrolled (twins, n = 28; singletons n = 11) and natural twin calves (calves born as twins to cows not previously enrolled in the study, n = 6) were assigned to one of three raising groups: (1) twin calves raised by their dam (twin born–twin raised; TT; n = 10); (2) twin calves where one calf was grafted to another cow that lost her calf and one calf was left with their dam (twin born–single raised; TS; n = 18); and (3) single born calves that were raised by their dams (single born–single raised; S; n = 11). Calves grafted on twin–single groups were selected by commercial herd management to better mimic the real world and increase external validity. Calves were grafted to cows that lost their own calves at parturition (stillborn) or their calves died in the immediate postpartum period. Time of birth (calf on the ground), first stand (all four limbs upright), first suckle (mouth contact with teat), and side of first suckle were observed and recorded by a single trained observer for each calf, as previously described [28]. Calculations were made to achieve latencies relative to birth time: latency to stand (time in minutes from birth to first stand) and latency to first suckle (time in minutes from birth to first suckle). Twenty-four hours after parturition, cow–calf pairs were moved to an outside pen (twin pen), a common area for cows, and a calf nesting area with straw bedding and windbreakers. Calves could easily access the calf nesting area by using calf gates (e.g., shorter) that prevented the entrance of cows to avoid crowding and injuries.

Once cows and calves were moved outside, suckling behaviors were observed and recorded on 4 different days during a 3 h period (13:30–16:30). Two trained observers used live observation and focal methods to record the following behavioral information: cow ID, calf ID, time of suckling start, and time of suckling stop. The interobserver reliability was calculated using the intraclass correlation coefficient (ICC = 0.98). Calculations were made to achieve the total time of suckling behavior for each calf, time of non-dam nursing (suckling on a cow that was not its own dam), time of own-dam nursing, and the number of bouts (a short period of intense suckling; >1 min). For accurate measures of suckling behaviors, twin cows that lost one of their calves (calf died or was grafted to a different cow) were considered cows nursing a single calf. Only cows with two viable calves were considered cows nursing a pair of twins.

2.8. Immune Measures

Calves' sera were thawed in a refrigerator overnight and analyzed for total serum protein and immunoglobulins (IgG1 and IgM). Serum total protein was measured using a digital handheld refractometer (MISCO, Solon, OH, USA). IgG1 and IgM were measured using commercially available ELISA kits (Bethyl Laboratories Inc., Montgomery, TX, USA) using suggested dilutions. All sera samples were analyzed on one 96-well plate for each assay. Samples were randomly assigned to duplicate wells within a plate. A microplate reader was used at a wavelength of 450 nm to measure optical densities, which were converted into sample concentrations using a standard curve. The intra-assay coefficient of variation was 8.64% for IgG1 and 8.65% for IgM.

2.9. Statistical Analysis

Statistical analyses were completed in SAS® software version 9.3 and SAS® Studio (SAS Institute, Cary, NC, USA). Categorical data were arranged in tables with their frequencies, and the FREQ procedure was used to analyze proportion differences for all categorical data. Differences in pregnancy risk, fetal pregnancy loss, and calving risk were calculated for ET + AI and AI-only groups. All non-categorical data were first tested for normality using the univariate procedure and then analyzed by a Satterthwaite *t*-test assuming unequal variances. Differences were tested for gestation length variables among ET + AI and AI-only treatments. Data are expressed as means ± standard deviation, where $p \leq 0.05$ was considered significant, and if biologically appropriate, $p > 0.05 \leq 0.10$ was considered a tendency. The sensitivity, specificity, and accuracy of ultrasonography to detect twin pregnancies were tested using the FREQ and NLMIXED procedures of SAS. Results are expressed as percentages for sensitivity, specificity, positive predictive value, negative predictive value, and accuracy with their respective 95% confidence intervals.

The GLIMMIX procedure was used to analyze non-categorical data. Dependent variables of BW, A200d, A280dW, behavior measures, and measures of passive immune transfer were analyzed within a model that included the fixed effects of raising groups (twin born–twin raised, TT; twin born–single raised, TS; and single born–single raised; S), birth order (first and second born), and interaction between raising groups and birth order. Pearson correlations were calculated using the CORR procedure to determine relationships among BW, A200d, A280dW, behavior measures, and measures of passive immune transfer. Differences of $p < 0.05$ were considered significant and $p > 0.05 < 0.1$ were considered tendencies.

3. Results

The total pregnancy risk for both treatment groups was 56%. The proportions of pregnancy risk differed among the two groups (p-value = 0.026; Table 1). The ET + AI group achieved a pregnancy risk of 61.9%, where 42.9% (27/63) of the cows were pregnant with twins and 19% (12/63) were pregnant with a singleton. The AI group achieved a pregnancy risk of 25% (3/12), and all three were pregnant with singletons.

Table 1. Pregnancy risk proportions of cows submitted to two different fertility protocols.

Treatment	AI [3]	ET [4]	Pregnancy Risk		p-Value [7]
			Group [5]	Total [6]	
AI only [1]	12	-	25.0%	4%	0.026
ET + AI [2]	63	63	61.9%	52%	
Total	75 *	63	-	56%	

[1] Group of cows submitted to a 7-day CO-Synch + CIDR protocol and artificially inseminated (AI) using fixed timed AI only; [2] group of cows submitted to a 7-day CO-Synch protocol, artificially inseminated using fixed timed AI, and 7 days after AI, received an embryo transfer (ET); [3] artificial insemination; [4] embryo transfer; [5] proportion of pregnancy risk for cows within each treatment group; [6] proportion of pregnancy risk for cows in both treatment groups combined; [7] Fisher's exact test p-value; * two cows were removed from the study (7016—fail to remove CIDR; 7057—presented a possible uterine infection at ultrasound examination).

When the ultrasound was used between 40 and 53 gestational days, it detected 18 cows pregnant with twin calves of the 24 calving twins and all 11 cows pregnant with singleton calves. The sensitivity to detect twin pregnancies was 75% (18/24), while the specificity was 100% (11/11) (Table 2). Only one cow from the AI group was misdiagnosed as open once pregnant. The accuracy of ultrasonography examination in the detection of twins was 90.5% (95% CI 83.2%–97.7%).

Table 2. Sensitivity and specificity values for ultrasonography diagnostic of cows carrying twin calves.

Statistic	Estimate	SE [3]	95% Confidence Intervals	
Sensitivity [1]	75%	8.84%	58.7%	92.3%
Specificity [2]	100%	-	100%	100%

Ultrasonography effectiveness in detecting twin [1] or singleton [2] pregnancies. [3] Standard errors.

Pregnancy loss from day 53 of gestation to term was 20.5% for cows within the ET + AI group. The three cows in the AI-only group did not have any losses. When considering both treatment groups, the overall pregnancy loss was 19.05%. Gestation length tended ($p = 0.06$) to be 3 days longer for cows carrying singletons than cows carrying twins (279 $\pm$ 5 vs. day 276 $\pm$ 2 (mean $\pm$ SD), respectively). Two abortions and a stillborn parturition were removed from the data analyses to provide a better overview of gestation length. A total of 53 calves were delivered at term, and this accounts for calves born as singletons or twins in both raising groups. The calving risk for this study was 70.6% (53 calves/75 cows; Table 3).

When cows were submitted to additional embryo transfers at 7 days post-AI, the risk of calves being born as twins was 76% and the risk of calves being born as singletons was 24%. All three calves born to the AI-only cows were singletons. The twinning risk for all cows in this study was 71.7%, and the proportions significantly differed among treatments ($p = 0.01$).

Table 3. Number of calves born to AI-only and ET + AI cows.

Treatment	Number of Calves Born		*p*-Value [3]
	Singleton	Twins	
AI only [1]	3	--	0.01
ET + AI [2]	12	38	
Total	15	38	53

[1] Group of cows submitted to a 7-day CO-Synch + CIDR protocol and artificially inseminated using fixed timed AI only; [2] group of cows submitted to a 7-day CO-Synch protocol, artificially inseminated using fixed timed AI, and at 7 days after AI, received an embryo transfer; [3] Fisher's exact test *p*-value.

Calves born as second twin tended to require more assistance at delivery compared to the twin born first (85.7% and 14.3%, respectively; $p = 0.09$). Only one calf born first required minor assistance, while second-born calves required some assistance, mechanical assistance, and had an abnormal presentation (n = 4, 1, and 1, respectively).

Survival proportions during the neonatal period (first 24 days of life) for calves born as twins or singleton were not different (81.6% and 80%, respectively; $p > 0.1$). Of the total calves born during this study, 81.1% survived the neonatal period, accounting for three stillborn and four deaths of calves born as twins and three deaths of calves born as singletons.

The least-square means and their standard error of growth, neonatal behaviors, and measures of passive immune transfer are depicted in Table 4 for the three raising groups and twin birth order. Calves born as twins at birth were 20% lighter in body weight than calves born as singletons (29.3 kg and 36.6 kg, SEM = 1.79, respectively; $p < 0.001$). Heifer calves were lighter in weight than bull calves (27.4 kg and 32.8 kg, SEM = 1.34, respectively; $p < 0.001$). Adjusted 200-day weights were greater ($p < 0.05$) for singletons than calves born as twins and raised as singletons. Weight differences were not significant at 280 days for twin–twin first and second born, twin–single firstborn, and single–single calves. Additionally, second-born calves (n = 5) were more likely to be grafted to another cow than firstborn calves (n = 1) for the twin–single treatments. However, no differences were found within the twin–single group for BW, A200dW, or A280dW for grafted calves and non-grafted calves.

Table 4. Performance, behavioral observations, and measures of passive immunity for different methods of raising twin beef calves.

	n	Twin–Twin		Twin–Single		Single–Single	SEM
Birth Order	-	1	2	1	2	-	-
Weight, kg							
Birth	39	27.8 [a]	29.4 [a]	31.5 [a]	28.5 [a]	36.3 [b]	1.79
Adjusted, 200 days	39	215.7 [a]	226.6 [ab]	209.7 [a]	216.4 [a]	257.3 [b]	16.09
Adjusted, 280 days	31	310.9 [ab]	318.5 [ab]	280.3 [ab]	273.3 [a]	323.0 [b]	18.41
Behavior							
Latency to stand, min [1]	33	73.2 [a]	97.4 [ab]	34.9 [b]	80.2 [ab]	63.0 [ab]	23.52
Latency to nurse, min [2]	33	111.2	163.0	92.8	96.0	135.9	30.82
Duration of dam nursing, min/12 h [3]	28	67.2	61.0	72.0	46.2	46.0	12.35
Duration of non-dam nursing, min/12 h [4]	24	10.4	5.0	4.0	3.8	3.6	3.68

Table 4. *Cont.*

	n	Twin–Twin		Twin–Single		Single–Single	SEM
Birth Order	-	1	2	1	2	-	-
Passive immune transfer							
Total serum protein, g/dL	37	7.1 [a]	7.1 [a]	5.7 [ab]	5.3 [b]	6.1 [ab]	0.56
Serum IgG1, mg/mL	36	26.8 [ab]	28.8 [ab]	17.2 [a]	16.2 [a]	35.5 [b]	7.83
Serum IgM, mg/mL	36	1.7	2.1	0.9	1.1	1.7	0.54

[ab] LS means in a row without a common superscript differ ($p < 0.05$); [1] time between birth and first standing; [2] time between birth and first nursing; [3] time spent nursing, own dam (min) in four 3 h observation periods in the neonatal phase (first 24 days of life); [4] time spent nursing, different dam (min) in four 3 h observation periods in the neonatal phase (first 24 day of life).

4. Discussion

The pregnancy risk achieved in the ET + AI group agreed with the 56% reported by Sreenan and Diskin [13], who used AI and then non-surgically transferred embryo to either ipsilateral or contralateral uterine horns among a group of mature beef cows. Dahlen et al. [15] conducted a study with purebred Angus comparing AI, ET, and ET + AI, with the embryo placed in the ipsilateral horn to the ovary containing the CL. The ET + AI conception risk was slightly lower (48.5%) on days 30–35 than what was achieved for the current study. Others reported a much lower conception risk (30.4%) [14], but they used Japanese dairy cows, transferred the embryo into the contralateral uterine horn containing the CL, and the study took place during summer months; therefore, breed, methods, and heat stress may have influenced their results. Unlike beef cattle, dairy cows are often impregnated during lactation, which also results in lower conception risk due to their great metabolic demand for high milk yield [29,30].

Ultrasonography examinations were previously suggested as a diagnostic tool to identify twin gestations at late embryonic or early fetal stages, which resulted in better management decisions regarding cows carrying twins [31–33]. The usage of ultrasound between 40 and 53 gestational days in this study proved to be an important diagnostic tool, even though the specificity was higher than the sensitivity. In addition, since it was known that this cow population was submitted to fertility treatment, a careful scan of both uterine horns for more than one embryo was performed, resulting in high diagnostic accuracy.

Pregnancy loss has previously been attributed to reproductive inefficiency [32]. It has been described that twin pregnancy losses occur early in gestation rather than late, and pregnancy is carried to term once established [34]. The pregnancy loss percentage in this study was higher than values published previously for beef heifers and multiparous cows: 15.1% and 8–11%, respectively [5,13]. In contrast, a 60% pregnancy loss was reported when a similar twinning technique was used in dairy cows [14], and losses were attributed to twin pregnancy being prone to abortions. Based on the current study findings and previous studies that reported considerably lower losses, other factors might be associated with embryonic/fetal losses, such as breed, semen quality, and heat stress, than the twin pregnancy alone [5,13]. Twin pregnancies were previously reported as having shorter gestation lengths by 5–7 days [18,35–38] compared to singleton pregnancies. In the current study, there was only a tendency for twin pregnancies to be 3 days shorter in length than singleton pregnancies. Many factors can affect gestation length, including breed differences, sex of the calf, dam weight, parity number, and sire [39,40]. We speculate that the similar gestation length between the two groups could be attributed to bilateral pregnancies, where each calf develops on one side of the uterus, diminishing uterine crowding seen early in unilateral twin pregnancies. Uterine crowding has previously been attributed as a stimulus that causes secretion of the adrenal corticotropic hormone by the fetus's pituitary gland, which increases concentration of fetal cortisol, initiating the cascade of endocrine events that culminates in parturition [41].

Parturition assistance occurred more often for twin calves compared to singles, especially for the second twin. Nonetheless, the majority of assistances were not challenging (i.e., easy pulls). The ease during assistance could attributed due to the fact that the pregnancies were identified as twins; therefore, the importance of ultrasound technology is further amplified for high-quality management during parturition, which includes greatly reducing the time interval between births. Reduced time between the first and second calf leads to decreased morbidity and mortality risks for the second born. Only one calf was in abnormal presentation and required repositioning. Retrospective studies suggested that twin calves are more likely to present abnormal position at birth [18]. Elevated incidence of dystocia has been reported when heifers were submitted to twinning compared to cows [37]. Even though the frequency of dystocia was higher for twin calves in our study, we believe that our technique produced bilateral twins. The development of each fetus in one of the uterine horns was published as a factor decreasing abnormal presentation incidence compared with unilateral twinning [21]. Survival data of twin and singleton calves were documented by Owens et al. (1984), and the survival proportion at weaning for twin calves was lower (62%) compared to singleton calves (73.6%). Higher survival proportions were reported for twins (76.2%) and singletons (88.6%) at day 200 [21]. In our study, both twins and singleton calves had similar survival proportions (~80%); however, our data represent the neonatal period.

As expected, twin calves had lower immunoglobulin concentrations at blood collection (~24 h of age) compared to singletons, and this is consistent with results published by Adams et al. (1993) [42]. The lower concentrations have been attributed to the fact that two calves nursed colostrum from one cow, which leads to lower intake of colostrum volume by each calf [43] compared to a calf nursing alone. It is important to note that calves in our study were closely monitored during birth and first nursing to ensure passive immune transfer. This practice is not common to all cow–calf operations, especially when calving occurs at pasture. However, due to the possibility of dystocia in twin pregnancies, cows were kept in a pen with easy access and monitoring.

Neonatal behaviors are an important tool to access calf vigor in the first hours of life. Stand, seek, and nurse colostrum are essential behaviors for calf health and resilience. Latency to stand and nurse among the different raising groups are in agreement with the literature for twin and singleton calves [42]. Ewbank (1967) [44] investigated natural twin and artificially paired twin calves' behaviors once they were turned out to pasture after being kept in a calf pen and concluded that twin behavior is probably mostly associated with the system of rearing than genetics itself. In addition, others reported that the amount of enclosure space influenced mothering ability [45]. Cows nursing twins in large enclosures were less attentive to calves and did not nurse one or both twin calves compared to cows in smaller enclosures. For the current study, cows and calves were kept in the same herd, and this rearing system may have encouraged nursing behaviors that were not different between raising groups. Because this was a commercial operation, researchers were able to report that the dams with twins allowed communal nursing. Researchers reported that cows rearing twins allow communal nursing because they are acclimated to more than just one calf nursing; therefore, non-offspring are accepted at a greater rate than cows nursing singletons [45].

Lower birth weights for twin calves were expected due to limited uterine space. Nevertheless, the weights in our study were similar to the ones described in the literature that compared twins and singletons [7,46]. Lower birth weights for heifers were also previously reported in the literature, and they agree with our findings [7,43]. Weight differences among twin–single and single–single calves were still apparent at 200 days of age. These data are similar to growth rates reported by others [21,47]. However, both studies compared twin and singleton calves that were nursed by their dams until weaning without grafting one of the twin calves to another dam. There are limited reports on grafting twins. Echternkmap and Gregory [21] grafted only calves from triplet births. Growth was credited to their original dam and no comparisons were made between grafted and dam-nursed calves. In contrast to the current study, lower weight gains were observed for twin–twin calves compared with twin–single calves in a study of cattle twinning [38].

In the current study, twin–twin and twin–single calves were not different at 200 or 280 days of age.

Previous studies reported that restricted in uterus growth and restricted birth to weaning growth did not affect nutrient utilization later in life [48]. Nevertheless, the yield of twin calves could be reduced compared to singleton calves without uterine growth restriction. However, Echternkamp and Gregory (2002) [21] reported that more than three quarters of carcasses from their twin population were graded USDA-grade Choice or above, which leads us to believe that restricted growth in the uterus does not negatively affect beef quality. One of the limitations of our study is that yield-grade data were not collected or available because calves were sold at days 200–280. Thus, the present study could not confirm that hypothesis.

Weaning weights are an important measurement in beef production due to their association with cow–calf operation efficiency [49]. Cows delivering and raising twin calves can produce between 48% and 60% more total weaning weight [7,21,50] compared to cows delivering and raising singles. Our results show an increase of 71% in total when comparing calves that were twins and raised as twins and calves born singletons and raised as singletons.

5. Conclusions

Embryo transfer seven days after artificial insemination is a successful technique in producing twin calves in beef cattle. Sensitivity and specificity of ultrasound to detect twins are appropriated to assist producers in decision-making on management of cows pregnant with twins (e.g., keep cows close to barn or maternity chute, more frequent calving checks, etc.). Although twin calves are lightweight at birth compared to singletons, cows producing twins wean a greater amount of calf weight (kg/cow/pregnancy). This increases cow–calf efficiency at weaning time. The present study follows extensive cattle twinning work done in previous research and reopens the discussion of a possible efficiency improvement during the cow–calf phase in U.S. beef cattle operations. However, it is important to note that many factors need to be considered when implementing twinning in commercial herds, including time commitment, intensive management, and increase in parturition assistance for cows birthing twins. Further studies are necessary to address economic aspects of this twinning technique and effects of postpartum interval.

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Institutional Review Board Statement: The commercial cow–calf operation followed animal care standards as described in the *Guide for the Care and Use of Agricultural Animals in Research and Teaching*. Additional techniques and animal care modifications for the duration of this study were approved by Kansas State University's Institutional Animal Care and Use Committee (IACUC 4282; Approved 07/19/2019).

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Data Availability Statement: Data utilized for this research were from a commercial cow–calf operation and are not available publicly due to confidentiality.

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