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1 **Effect of progesterone supplementation in the first week post conception on**
2 **embryo survival in beef heifers**

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1 **Abstract**

2

3 Progesterone is essential for establishment and maintenance of pregnancy in
4 mammals. The objective of this study was to examine the effect of elevating
5 progesterone during the different physiological stages of early embryo development
6 on embryo survival. Estrus was synchronized in cross-bred beef heifers (n=197, ~2-
7 years old) and they were inseminated 12-18 h after estrus onset (=Day 0). Inseminated
8 heifers were randomly assigned to 1 of 3 treatments: (1) Control, n=69; (2)
9 progesterone supplementation using a Controlled Internal Drug Release Device
10 (CIDR) from Day 3 to 6.5, n=64; or (3) progesterone supplementation using a CIDR
11 from Day 4.5 to 8, n=64. Body condition (BCS) and locomotion scores (scale of 1-5)
12 were recorded for all animals. Animals with a locomotion score ≥ 4 (very lame) were
13 excluded. Embryo survival rate was determined at slaughter on Day 25. Conceptus
14 length and weight were recorded and the corpus luteum (CL) of all pregnant animals
15 was dissected and weighed. Supplementation with exogenous progesterone increased
16 ($P<0.05$) peripheral progesterone concentrations, but did not affect embryo survival
17 rate compared with controls. Mean CL weight, conceptus length and conceptus
18 weight were not different between treatments. There was a positive relationship
19 ($P<0.04$) between the increase in progesterone concentrations from Days 3 to 6.5 and
20 embryo survival rate in treated heifers and a similar trend existed between the
21 increase from Days 4.5 to 8 ($P<0.06$). There was also a positive relationship ($P <$
22 0.05) between the progesterone concentration on Day 6.5 and the embryo survival rate
23 in treated heifers. A direct correlation was seen between locomotion score and
24 embryo survival rate, with higher ($P<0.05$) early embryo survival rates in heifers with
25 a lower locomotion score. In conclusion, supplementation with progesterone at
26 different stages of early embryo development increased peripheral progesterone
27 concentration and resulted in a positive association between changes in progesterone
28 concentration during the early luteal phase and embryo survival rate. Supplementation
29 with progesterone had no effect on either CL weight or conceptus size in pregnant
30 animals. Lameness had a significant negative effect on early embryo survival.

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32 **Keywords:** Cattle, progesterone, embryo survival, locomotion score

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1 **1. Introduction**

2
3 Progesterone is an essential hormone for the establishment and maintenance of
4 pregnancy in mammals [1]. In heifers, only 55% of single inseminations lead to a
5 successful full term pregnancy despite a fertilization rate of 90-95% [2]. A major
6 portion of this conception loss occurs between Days 8 and 16 of pregnancy [3] with
7 relatively little late embryonic or early fetal mortality. Recent data from high
8 producing dairy cows suggests that the percentage of early embryonic loss in such
9 animals is even larger than in heifers [2]. The peripheral concentration of
10 progesterone in early pregnancy has a significant effect on the survival of the
11 embryo/conceptus [4]. Low concentrations of progesterone in the initial days
12 following conception (Day 3-8) are associated with smaller embryos on Day 16 of
13 pregnancy which may produce insufficient interferon- τ to suppress the luteolytic
14 secretion of prostaglandin F 2α [5]. Similarly, a delay in the post-ovulatory increase in
15 progesterone has been associated with a decreased pregnancy rate in both cows [6,7]
16 and heifers [2].

17
18 Circulating progesterone concentrations can be increased by either exogenous
19 supplementation [8-11] or ovulation of a dominant follicle, using GnRH or hCG for
20 example, to form an accessory corpus luteum (CL) [12-14]. The earliest day on which
21 a dominant follicle is large enough to consistently respond to GnRH or hCG is Day 5
22 [15]. The accessory CL created by treatment on Day 5 leads to increased plasma
23 progesterone concentrations from Day 8 which reach a maximum on Days 16-17 [16].
24 However, this increase in progesterone may be too late to influence embryo survival
25 [11,17]. A further study that involved the administration of GnRH during the second
26 wave dominant follicle did not have a consistent positive effect on pregnancy rate
27 [14].

28
29 Direct exogenous progesterone supplementation allows more control over the time of
30 administration and results in an immediate increase in progesterone concentrations.
31 This approach may therefore be a more effective method to use to increase circulating
32 progesterone concentrations. This can be achieved by injection of progesterone or the
33 insertion of a progesterone-releasing intravaginal device. Several studies have

1 described the effect of exogenous progesterone supplementation on development of
2 the embryo. One of the earliest such studies was that of Garrett et al [18] who
3 reported a dramatic advancement in conceptus development by Day 14 following
4 administration of 100 mg progesterone on Days 1 to 4 of the cycle. Mann and
5 Lamming [11] did a meta analysis of all studies that supplemented progesterone
6 during early pregnancy and examined the effect of day of initiation of progesterone
7 supplementation on the subsequent pregnancy rates. They concluded that
8 progesterone supplementation early in pregnancy leads to an overall increase in
9 pregnancy rate of 5%; however, supplementation after Day 6 had no beneficial effect.
10 The same study found that when the initial pregnancy rate was low (<50%) there was
11 a large improvement in pregnancy rate, but when the pregnancy rate was higher
12 (>50%) there was no additional benefit.

13

14 More recent studies have shown that insertion of an intravaginal device between Days
15 2 and 7 increased the survival of the conceptus by 10-15% [6,11]. However, one study
16 found that supplementation commencing within the first 2 days after ovulation and
17 continuing for 7 days suppressed fertility [19] indicating possible interference with
18 the development of the endogenous CL during this period. A recent study reported an
19 increase in pregnancy rate from 35% to 48% when progesterone was supplemented to
20 dairy cows with a used CIDR from Day 3.5 up until Day 10 [20]. This increase in
21 pregnancy rate was ascribed to the embryotrophic effects of progesterone, especially
22 in cows with low concentrations of progesterone at the time of supplementation. The
23 consensus of most studies is that the critical period for progesterone in supporting
24 increased early embryo development rates is from Day 3 to 7. In support of this, a
25 recent study from our group has demonstrated that elevating progesterone
26 concentrations from Day 3 to 7 post conception significantly increased the length of
27 the conceptus on Day 13 and Day 16, despite having no phenotypic effect on
28 development at pre-hatching (Day 5 and Day 7) stages [21].

29 The objective of this study was to examine the effect of timing of progesterone
30 supplementation relative to key stages of embryo development (morula, blastocyst
31 and elongation) within the first week of pregnancy on embryo survival rate in cattle.

32 The relationship between progesterone concentration, body condition score, degree of
33 lameness and embryo survival rate was also analysed.

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2. Materials and Methods

2.1. Animals and treatments

Cross-bred beef heifers (n=197), approximately 2 years old and weighing 487 ± 3.4 kg were used. All animals were housed in a slatted floor facility in a commercial feedlot under the same management and had ad-lib access to a total mixed ration designed to achieve an average liveweight gain of 1.26 kg/heifer/day. The body condition and locomotion score of all heifers were recorded at one time point during the trial. Locomotion was scored as described by Spreacher et al [22], where a score of 1 represented normal locomotion, 2 represented mild lameness, 3 moderate lameness, 4 lame and 5 severely lame. Heifers that showed an arched back while both standing and walking and were either non or partially weight bearing on the affected foot (locomotion score 4 and 5) were excluded from the experiment. A total of 12 animals were assigned a locomotion score 4 and 5, representing 6% of the total number of animals. Body condition was scored on a 5-point scale with increments of 0.25 as described by Edmonson et al [23].

All heifers were synchronized using two injections of a prostaglandin $F_{2\alpha}$ analogue (PG, Prosolvin, Intervet Ireland Ltd.) given 9 days apart. Heifers were checked for signs of estrus 4 times daily commencing 36 h after the second PG injection and only those recorded in standing estrus (=Day 0, n=197) within the period of 36-60 hours after the 2nd PG injection were inseminated 12-18 h later with frozen-thawed semen from a single ejaculate of a fertile bull. Over all, 59% of synchronized animals were observed in standing estrus in this time period.

Following insemination, heifers were randomly assigned to 1 of 3 treatment groups: (1) control, n=69, (2) supplemented with progesterone by means of a Controlled Internal Drug Release device (CIDR 1.9g, Pfizer UK) from Day 3 until Day 6.5 (n=64), corresponding to the transition of the embryo from the 8-cell to the morula stage of development, or (3) supplemented with progesterone from Day 4.5 until Day 8 (n=64) corresponding to the transition of the embryo from the 16-cell to the

1 blastocyst stage of development. A time line for the treatments and blood sampling is
2 shown in Figure 1.

3

4 2.2. Blood sampling

5

6 All heifers were blood sampled via jugular venepuncture for subsequent measurement
7 of progesterone at the time of CIDR insertion, 1 day post insertion and at CIDR
8 removal. Control animals were sampled at the same times as those in both treatment
9 groups (n=6 samples per animal). Blood samples were stored at ambient temperature
10 for a maximum of 6 h and at 4°C for a further 18 h. Serum was decanted after
11 centrifugation for 20 min at 1600 x g and stored at -20°C until subsequent analysis.

12

13 2.3. Early embryo survival rate

14

15 Embryo survival was determined at slaughter on Day 25. Following slaughter, the
16 uterus was opened longitudinally and, where present, the conceptus was removed.
17 Conceptus length was measured with an optical calipers and the weight was recorded.
18 The CL of all pregnant animals were dissected out of the ovaries and weighed to
19 establish whether or not there was an effect of progesterone supplementation on the
20 development of the CL of pregnancy.

21

22 2.4. Progesterone assay

23

24 Progesterone was measured using a validated time-resolved fluorescent immunoassay
25 (FIA) with an AutoDELFIA™ Progesterone kit (Perkin Elmer, Wallac Oy, Turku,
26 Finland). The inter-assay coefficients of variation (CV) were 6.5%, 6.5% and 8.6%
27 for low, medium and high quality controls serum pools, respectively. The intra-assay
28 CV were 6.4%, 6.4% and 8.5% for high, medium and low quality control sera,
29 respectively. The sensitivity of the assay was 0.01 ng/ml.

30

31 2.5. Statistical analyses

32

33 Embryo survival rate was compared using Fishers Exact Chi-square analysis as
34 described by Snedecor and Cochran [24]. Changes in serum progesterone

1 concentration between pre- and post-insertion (or control equivalent) samples were
2 calculated for individual animals in order to evaluate the incremental increase in
3 progesterone. This increment and the concentrations of progesterone on individual
4 days were compared by ANOVA with Bonferroni and one way t-test using SPSS for
5 Windows. The relationships between body condition score, locomotion score and
6 embryo survival rates were analysed using logistic regression with terms for both
7 linear and quadratic effects included in the model where appropriate.

8
9 Within treatments the relationship between concentrations of progesterone on Day
10 3.0, 6.5 and changes between Days 3 and 6.5 (Treatment 1), Days 4.5, 8.0 and
11 changes between Days 4.5 and 8 (Treatment 2) and embryo survival rate were
12 evaluated using logistic regression in SAS 9. Progesterone was initially included as a
13 linear term in the model. If significant the model was re-run with progesterone
14 included as a quadratic term. Terms used were treatment, breed, progesterone
15 concentration and pregnancy status.

17 **3. Results**

18 3.1. Progesterone

19
20 Treatment with a CIDR on either Day 3 or 4.5 increased the mean progesterone
21 concentration by 1.12 ng/ml ($P < 0.01$) on the day after CIDR insertion (Day 4 and 5.5,
22 respectively) compared with control animals (Table 1). The incremental increase in
23 progesterone from pre- to post-insertion (or equivalent) samples was higher in CIDR-
24 treated heifers versus controls ($P < 0.01$).

25
26 Within treatment 1, there was a significant relationship ($P < 0.04$) between the change
27 in serum progesterone concentration from Day 3 to 6.5 and early embryo survival
28 rate. A similar relationship ($P < 0.06$) existed from Day 4.5 to 8 within treatment 2. The
29 relationship between serum progesterone concentration on Day 6.5 and early embryo
30 survival rate in treated heifers was also significant ($P < 0.05$), while there was no
31 significant relationship between progesterone concentrations and early embryo
32 survival rate on all other days ($P > 0.4$). The relationship between change in serum
33 concentration of progesterone and embryo survival rate is shown in Figure 2.

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3.2. Embryo survival and conceptus size

The mean embryo survival rate over all animals was 41.6% and was not affected by treatment. Similarly, the weight and length of the conceptus was not affected by treatment (Table 2).

3.3. Corpus luteum weight

Treatment with exogenous progesterone did not affect the weight of luteal tissue compared with control animals, irrespective of timing of treatment (Table 2). Weight of the CL was greater ($P=0.053$) in heifers receiving progesterone between Days 3 and 6.5 compared with those supplemented between Days 4.5 and 8 (Table 2).

3.4. Body Condition Score and Locomotion Score

Of the inseminated heifers, 51% showed normal stance and locomotion and therefore had a locomotion score of 1. Forty-four percent of the heifers had a locomotion score 2; these animals showed an arched back while walking, while their gait remained normal. These animals were regarded as mildly or sub-clinically lame. The remaining 5% scored 3 on locomotion, displaying an arched back when both standing and walking. These animals had a gait that was shorter in stride and were regarded as moderately lame. There was a negative association ($P<0.02$) between locomotion score and embryo survival rate, which was independent of serum concentrations of progesterone with higher early embryo survival rates in heifers that had low locomotion scores (Figure 3).

Body condition scores of all 197 heifers are presented in Table 3. Of all heifers, 56% had a body condition score of 4 or higher. There was a significant relationship ($R^2=0.02$, $P<0.05$) between BCS and progesterone levels on Day 3, but there was no relationship between BCS and embryo survival.

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4. Discussion

With the estimated percentage of early embryonic death in dairy cows increasing from 28% in 1980 to 43% in 2006 [2], a large body of current research is aimed at understanding the factors determining embryo survival in the early stages of pregnancy. The available evidence indicates that increased progesterone concentrations during the early stages of pregnancy are associated with an advancement of embryonic development leading to an increased level of interferon- τ production and an associated increase in pregnancy rate. Many studies on progesterone supplementation, in an attempt to improve pregnancy rate have been carried out, with variable results. Part of the variation in results is probably related to the fact that only certain animals, those with naturally low circulating progesterone, may exhibit beneficial effects following exogenous progesterone supplementation. In addition, the precise physiological stage of embryo development which most benefits from high progesterone is not known. Recent studies have demonstrated that cows with low progesterone at a time when the embryo is at the morula to blastocyst stage have smaller embryos that produce less interferon- τ on Days 13-16 [5]. It has also been shown that there is an increased probability of embryo survival in heifers and cows that have elevated progesterone on Days 4-6 [2]. Thus, it appears that a critical window for progesterone supplementation is between the morula and hatching blastocyst stages. The present study was conducted to elevate progesterone during these critical stages of embryo development. Beef heifers were used to exclude the confounding factors that can occur in dairy cows, such as lactation, negative energy balance and uterine contamination. The results show that elevation of progesterone does not increase the number of animals that become pregnant, but it was seen that there was a significant relationship between the change in serum progesterone concentrations from Day 3 to 6.5 and embryo survival.

There was no beneficial effect of progesterone supplementation on conceptus survival and size on Day 25. Using a similar supplementation protocol, Carter et al. [21] reported a difference in embryo size on Day 16 when progesterone was supplemented

1 from Day 3 onwards. These two observations would support the idea that larger
2 embryos on Days 13-15 may be more effective in suppressing the luteolytic
3 mechanism around the initiation of elongation due to an advancement in embryonic
4 growth, rather than the production of larger embryos per se and that by Day 25 this
5 difference has disappeared. This suggests that progesterone may be only crucial for
6 advancing embryonic growth to ensure a strong anti-luteolytic signal (maternal
7 recognition), but once maternal recognition has occurred developmental rate is less
8 relevant. The possibility that CIDR insertion somehow negatively affected the
9 development of the endogenous CL, as has been reported in previous studies [19] is
10 unlikely as the CL weight of control and supplemented groups did not differ in the
11 present study

12 There was a relatively high incidence of subclinical lameness amongst the animals
13 used in this study, most likely due to the high energy diet that they were fed [25]. The
14 fact that the animals were housed on slats contributed to their possible discomfort
15 [26]. The amount of subclinical lameness was already evident in the low incidence of
16 estrus expression (59%) in the heifers (within 36-60 h after the second PGF2 α
17 injection) used in the experiment. Estrus expression on concrete and slats is
18 compromised by the ground surface, since cattle do not like to mount on this surface
19 [27,28]. Early embryo survival across all animals was low, irrespective of treatment
20 and the lameness could have had a negative impact on the potential positive effects of
21 treatments. A normal pregnancy rate in heifers would be expected to be up to 60-65%
22 [2,29]. It is known that lameness has a negative effect on fertility and various authors
23 have shown that dairy cows that are lame have decreased fertility, starting with lower
24 estrus expression [27,30,31] and lower conception rate with more inseminations per
25 conception [32]. To our knowledge this is the first study that has related locomotion
26 score with early embryo survival rate; there was a clear negative relationship between
27 the two parameters. This supports the hypothesis that lameness has an adverse effect
28 on fertility. Indeed, Walker et al. [31,33] demonstrated that lameness decreased the
29 intensity of estrus expression and progesterone concentrations in dairy cattle.

30 Lameness can be seen as a chronic stressor as it is a painful long term condition.
31 Chronic stress and pain has often been associated with disturbances of the
32 hypothalamic-pituitary-adrenal axis, which can lead to disrupted patterns of pulsatile
33 GnRH release as well as deprivation of the ovarian structures of gonadotrophin

1 support resulting in low progesterone concentrations [33]. When cows are lame, the
2 chance that they ovulate is reduced compared with that of normal cows. Dobson [34]
3 demonstrated that cows that did not ovulate did not have an LH surge and that their
4 LH pulse frequency was lower than that of the ovulating cows. Therefore reduced
5 embryo survival may be negatively associated with lameness in cattle.

6

7 The heifers used for this experiment were finishing heifers which was apparent from
8 their high body condition scores. These animals were fed to gain an average of 1.26
9 kg/day and to finish quickly at the appropriate slaughter weight. Animals with an
10 excess in body condition have been known to have problems breeding [35]. In the
11 abattoir it was evident that all animals that were not pregnant were cyclic. It is a
12 possibility that heifers gaining weight at a high rate on an energy dense diet may have
13 higher steroid metabolism, similar to that seen in dairy cows [36], influencing the
14 possible positive effects of the supplementation of progesterone.

15

16 In conclusion, progesterone supplementation in heifers fed a high energy diet did not
17 improve early embryo survival but resulted in a positive association between change
18 in progesterone concentration during the early luteal phase and embryo survival rate.
19 However, moderate lameness had a negative effect on embryo survival on Day 25.

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2
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9

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Table 1: Mean (\pm s.e) serum progesterone concentration (ng/ml) in all heifers treated with a CIDR versus all control heifers. Jugular blood samples were of all heifers taken on day of insertion and one day post insertion.

Treatment	N	Pre-insertion	Post-insertion	Increment*
Control (D3-6.5)	69	0.184 \pm 0.020 ^a	0.398 \pm 0.042 ^a	0.221 \pm 0.047 ^a
CIDR (D3-6.5)	64	0.189 \pm 0.016 ^a	1.235 \pm 0.071 ^b	1.073 \pm 0.060 ^b
Control (D4.5-8)	69	0.469 \pm 0.048 ^a	0.763 \pm 0.095 ^a	0.294 \pm 0.049 ^a
CIDR (D4.5-8)	64	0.420 \pm 0.032 ^a	1.563 \pm 0.086 ^b	1.166 \pm 0.059 ^b

6 *Increment from pre- to post-treatment samples was calculated for individual animals to
7 generate means and standard errors.

8 ^{a,b}Within day of assay, means without common superscripts are different (P < 0.01).

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1 Table 2: Embryo survival, conceptus length and weight and CL weight (\pm s.e) 25 days post
 2 conception in pregnant animals supplemented with progesterone from Day 3-6.5 or
 3 from Day 4.5-8 as well as the unsupplemented control animals.
 4

Treatment	No. of Animals	No. from which embryos were recovered (%)	CL weight, g	Conceptus weight, g
Control	69	29 (42.0)	5.84 \pm 0.19	0.49 \pm 0.06
P4 Day 3 to 6.5	64	22 (34.4)	6.03 \pm 0.15	0.55 \pm 0.06
P4 Day 4.5 to 8	64	31 (48.4)	5.42 \pm 0.16	0.55 \pm 0.05

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1 Table 3: Distribution of body condition scores amongst inseminated heifers (n=197).

2

Body condition score	3.25	3.5	3.75	4	4.25	4.5
Number of animals	5	20	61	78	27	6
Number of pregnant animals	3	8	20	35	14	3
Percentage pregnant	60	40	33	45	52	50

3 Body condition was scored on a 5 point scale with steps of 0.25 as described by

4 Edmonson et al [23]

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