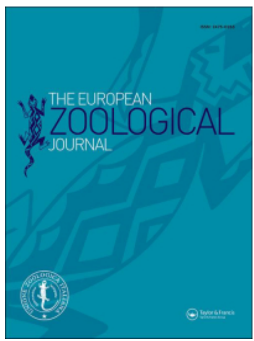

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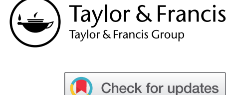

Effects of single or serial embryo splitting on the development and morphokinetics of *in vitro* produced bovine embryos

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Abstract
Embryo splitting can be used in cattle *in vitro* production (IVP) to improve embryo availability and to increase selection intensity. Despite this widespread utility, a comparative investigation of the viability of IVP embryos split at Day 2 (2-cell stage), Day 3 (8-cell stage), and blastocyst stage has not been undertaken. Similarly, the suitability of splitting Day 3 embryos with atypical numbers of blastomeres, and the feasibility of serial-splitting cleavage stage embryos, have not been investigated in cattle. Here, we demonstrate that the strategy most likely to produce the greatest output of viable embryos is the splitting of Day 3 embryos into four parts, regardless of whether embryos with exactly eight cells or an atypical number of blastomeres are used. This approach was found to produce 1.8 blastocysts per zygote on average compared to just 0.4 blastocysts per zygote for non-split controls. Single-splitting was also found to be superior to serial-splitting which, whilst feasible, impaired embryo viability as judged by cell number at day 7 post-insemination. Interestingly, zygotes (≥2 cells) split once on either Day 2 or Day 3 post-insemination, whilst resulting in smaller blastocysts than control embryos, displayed higher cell counts than expected at the blastocyst stage, suggesting a compensatory mechanism might be at play. Indeed, time-lapse imagery revealed that zygotes split at 2-cells reached the compact morula and expanded blastocyst stages earlier than either those split at Day 3 or non-split controls. Developmental events between splits originating from the same progenitor appeared well synchronized only up to the third cleavage division.

Keywords: *Reproductive cloning*, *embryo multiplication*, *twinning*, *bisectioning*, *blastomere separation*

1. Introduction

Embryo splitting has the potential to offer significant benefits to cattle *in vitro* production (IVP) by increasing the number of embryos available for transfer, thereby increasing embryo transfer success (Kippax et al. 1991). At the same time, embryo splitting can multiply the number of offspring from the most valuable gamete donors, allowing for improved rates of genetic gain by virtue of an increased selection intensity (Nicholas & Smith 1983). Moreover, the possibility of producing high numbers of monozygotic (MZ) twin embryos could have attractive applications in biomedical research, for example, by providing controls for pharmacokinetic studies (Heyman et al. 1998), or to investigate epigenetic (Haddow et al. 1999) or age-related effects (Chan et al. 2000). Additionally, the availability of MZ embryos could improve the efficiency of embryo genetic screening for either ploidy, sex selection, or for establishing the genomic breeding value of the embryo (Le Bourhis et al. 2011; Turner et al. 2019; Silvestri et al. 2021), since a single test would be simultaneously informative for each set of embryos derived from the same progenitor. Finally, embryo splitting could be employed to study the synchronization of developmental events in early embryos (Morris et al. 2012; Noli et al. 2015).

Embryo splitting was first developed in the 1980s (Willadsen 1980; Willadsen & Polge 1981) and it has been successfully applied to both cleavage

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(Johnson et al. 1995) and blastocyst (Lopes et al. 2001) stage bovine embryos. Surprisingly, however, no previous study has directly compared the developmental potentials of 2-cell, 8-cell, and blastocyst stage embryos split within the same IVP system to determine the strategy most likely to produce the greatest number of transferable embryos. A comprehensive evaluation of the developmental potential of 8-cell bovine embryos split according to different ratios (i.e. 1 × 8 vs 2 × 4 vs 4 × 2 vs 8 × 1 cells) has also not been well described in the literature, and a previous report failed to test 8-cell embryos split in more than two parts (Loskutoff & Johnson 1993). Moreover, the suitability for embryo splitting of Day 3 post-insemination embryos with cell numbers other than eight has not been reported. A further question arises as to whether or not it would be beneficial to split an embryo derived from a previous split, a procedure known as serial splitting. In cattle, the production of quarter embryos by serial blastocyst bisection has been reported (Rho et al. 1998). However, strategies for the serial splitting of embryos at the cleavage stage have not.

The aim of this study was to determine which cleavage stage embryos are most suitable for embryo splitting, and to identify the optimal embryo splitting strategy for viable blastocyst production. Moreover, the developmental potential of serially split cleavage stage bovine embryos was investigated, and a time-lapse system used to test the hypothesis that the kinetics of embryo development is affected by embryo splitting.

2. Materials and methods

All reagents were acquired from Sigma-Aldrich (Gillingham, UK) unless otherwise stated.

2.1 Oocyte recovery and IVM

Abattoir sourced bovine ovaries were transported to the University of Kent in PBS at 38°C within 3 h postmortem. Follicles ranging between 3 and 8 mm were manually aspirated using a 5 ml syringe equipped with a 19-gauge needle. Oocytes with a homogenous ooplasm and at least two compact layers of cumulus cells were selected and handled in HEPES modified Medium 199 (product code: M7528, Sigma-Aldrich) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 0.2 mM pyruvate and antibiotics. Oocytes were then washed two times in handling medium and matured for 18 to 22 h in groups of 20–30 in 90 µl drops of Medium 199 (product code: M4530, Sigma-Aldrich) supplemented with 10% FBS, 10 IU/ml PMSG, 5 IU/ml hCG (PG600, Intervet, Milton Keys, UK), 0.2 mM pyruvate, and antibiotics at 38.5°C and under 6.5% CO_2 in air.

2.2 IVF and embryo culture

Following IVM, oocytes showing a homogenous ooplasm and an expanded cumulus were washed two times in glucose-free TALP medium supplemented with antibiotics, 10 µg/ml heparin and 1:25 PHE solution prepared as described by Miller *et al.* (Miller et al. 1994), and then moved to a fertilization drop containing 90 µl of this same medium. In the meantime, frozen/thawed bull spermatozoa (Semex, Monkton, UK) were selected using the discontinuous density gradient system BoviPure™ (Nidacon, Mölndal, Sweden) according to manufacturer's instructions. An appropriate volume of the prepared sperm was added directly to the oocyte dish to achieve a final concentration of $10^{\wedge 5}$ motile sperm cells/ml. The gametes were then co-cultured at 38.5°C under 6.5% CO_2 in air for 18 to 22 h. Following this time, putative zygotes were transferred to HEPES modified, glucose-free TALP medium and mechanically denuded. Zygotes were then washed twice in pre-equilibrated SOFaaci medium (Holm et al. 1999) supplemented with 5% FBS, 5 mg/ml BSA and antibiotics and cultured in groups of 20–30 in 90 µl drops of SOFaaci at 38.5°C and under 6.5% CO_2 and 5% O_2. This medium was partially replaced after 48 h and again after 92 h of culture.

2.3 Cleavage stage embryo splitting (blastomere separation)

Cleavage stage embryos were split at either 30 h or 72 h post-insemination, when most embryos would be expected to be at around the 2-cell or 8-cell stage, respectively. All manipulations were carried out on a heated stage at 38°C with the assistance of a 125 µm wide tip (EZ-Tip, RI, Falmouth, UK). First, the embryos were briefly rinsed in PBS to remove most of the Ca^{2+} and Mg^{2+} carried over from the culture medium. The zona pellucida was then digested by exposure for up to 7 min to 0.25% w/v pronase E in PBS. At the end of the digestion, the embryos were immediately washed two more times in PBS and, if necessary, allowed to fully disaggregate by gentle agitation and pipetting. The removal of Ca^{2+} and Mg^{2+} ions by PBS washing was enough to favor complete embryo disaggregation, so that it became possible to recover individual blastomeres by simple aspiration. Following disaggregation, individual

blastomeres were washed once in 10% FBS in PBS and once in pre-equilibrated SOFaaci and then cultured in commercial well-of-the-well (WOW) culture dishes (Primo Vision 16-well culture dish, Vitrolife, Göteborg, Sweden) either alone or in pools of cells derived from the same embryo in SOFaaci at 38.5°C and under 6.5% CO_2 and 5% O_2, following the approach described by Vajta *et al.* (Vajta et al. 2000). For embryos split at 72 h post-inseminaion, the resulting cell pools and the embryos derived from them were named according to the number of blastomeres received from the parent embryo and its original cell count (not necessarily all the embryos were found to possess exactly 8 cells at 72 h post-insemination). For example, the nomenclature 3/7 indicated an embryo resulting from the grouping of three blastomeres harvested from an original embryo containing seven cells. A similar nomenclature was not required for embryos split at 30 h post-insemination, since all the embryos of that stage that were employed had exactly 2 cells.

2.4 Blastocyst stage embryo splitting (bisectioning)

Only grade I blastocysts (as defined by Nagashima *et al.* (Nagashima et al. 1989)) obtained 6 days post-insemination were used for this experiment. Embryos were rinsed once in PBS, and then positioned in a 50 µl drop of PBS under embryo grade mineral oil. A sterile, disposable P-730 ophthalmic scalpel (Feather, Osaka, Japan) was assembled on an Integra TI micromanipulator platform (RI) and used to bisect the embryos as previously described (Rho et al. 1998). Briefly, the blade was positioned so that the resulting cut would separate the blastocyst in two similarly sized halves; taking particular care that the inner cell mass would be equally divided across its middle point. The blade was then slowly lowered and the cut completed by gentle sawing (back and forward) motion. After the cut, the PBS drop containing the splits was supplemented with 10% FBS to improve handling. The splits were then washed once in 10% FBS in PBS and once in pre-equilibrated SOFaaci and cultured for a further 24 h in SOFaaci in single drops at 38.5°C and under 6.5% CO_2 and 5% O_2.

2.5 Double and triple serial embryo splitting

For double serial splitting, 2-cell embryos were split for the first time at 30 h post-insemination following the blastomere separation protocol discussed above and cultured for 24 h in single drops. After this time, embryos showing cleavage were disaggregated again, and the blastomeres were separated in two equal groups and cultured until day 7 post-insemination, resulting in the production of second serial splits. For triple serial splitting, second serial splits that showed cleavage after a further 24 h of culture were disaggregated a third time as before, resulting in the production of third serial splits.

2.6 Total cell number estimation in bovine blastocysts

Blastocysts at 171 to 175 h post-insemination were fixed overnight in 4% PFA in PBS at 4°C and stained in 0.05 mg/ml Hoechst 33342 in PBS. The embryos were then mounted under a 10-mm coverslip in 5 µl of the anti-bleaching medium Fluoroshield. Cell counts were then obtained under epifluorescence observation using an Olympus BX60 microscope equipped with a standard DAPI filter and a Hamamatsu ORCA03-G camera. The software used to collect and store the images was SmartCapture (version 3, Digital Scientific, Cambridge, UK).

2.7 Time-lapse observation

A PrimoVision EVO microscope was used together with the acquisition and analysis software provided by the supplier (Vitrolife, Gothenburg, Sweden) to annotate the timing of cleavage, compaction and blastulation events. Observation began 22 h post-insemination or immediately after splitting for control and split embryos, respectively.

2.8 Statistical analysis

Blastocyst formation frequencies were compared by classic chi-squared test, applying the Bonferroni correction when multiple tests were performed. Data for cell counts were log transformed then compared by Welch's ANOVA followed by Games-Howell's *post-hoc* test, using an approach similar to that described by Loskutoff *et al.* (Loskutoff & Johnson 1993). Finally, morphokinetic measurements were compared by one-way ANOVA followed by Tukey-Kramer's *post-hoc* test. Results were reported as means plus or minus SEM. The calculations were performed on SPSS (version 28, IBM, Armonk, NY, USA).

3. Results

In general, embryo splitting appeared to be reliable, as at least one live split embryo could be produced from 100% of the Day 2 embryos (n = 44) and 97.5% of the Day 3 embryos (n = 120). Moreover, during the splitting of blastocyst stage embryos,

95.7% of the bisections (n = 46) could be recovered. For serial splitting, 95.1% of the split embryos (n = 61) survived to produce at least one serially split embryo.

3.1 *Effects of splitting ratio on blastulation frequency and cell counts among Day 3 embryo splits*

A splitting ratio can be calculated by dividing the number of blastomeres pooled after the disaggregation of a cleavage stage embryo by the number of blastomeres originally present in the intact embryo. Therefore, Day 3 embryo splits deriving from an embryo with exactly eight cells have a splitting ratio of either 0.125, 0.25 or 0.50 for the 1/8, 2/8 or 4/8 type splits, respectively. However, the actual splitting ratio range across all tests for Day 3 embryos fell between 0.08 and 0.57 when embryos with a number of blastomeres other than eight were taken into account. These embryos were assigned to three groups based on which ideal splitting ratio (0.125, 0.25 or 0.50) they were closest to, then compared to embryos with exactly eight cells for blastocyst formation and cellularity. Embryo splits with a splitting ratio other than ideal performed similarly in terms of blastulation and cell counts to embryo splits possessing an ideal splitting ratio, as shown in Table I. In view of this, Day 3 embryos with similar splitting ratios were pooled together for the purpose of statistical analysis and will be referred to as 1/8, 2/8 or 4/8 type splits in the following sections.

3.2 *Blastulation frequency across different embryo splitting strategies*

The blastulation frequency was compared across a range of splitting methods including splitting at Day 3 (4/8, 2/8 and 1/8 type splitting), at Day 2, and at the blastocyst stage. In particular, for embryos split at the blastocyst stage, recovery events were recorded in place of blastulation events and defined as the proportion of blastocyst bisections that survived the cut and were able to re-form a blastocoel. The performance of the five treatment groups was compared against that of intact control embryos produced over the same period (n = 520); the results of this analysis are reported in Figure 1. After accounting for cleavage frequency, splitting efficiency, blastulation/recovery frequency and splitting ratio; the strategy with the greatest net blastocyst

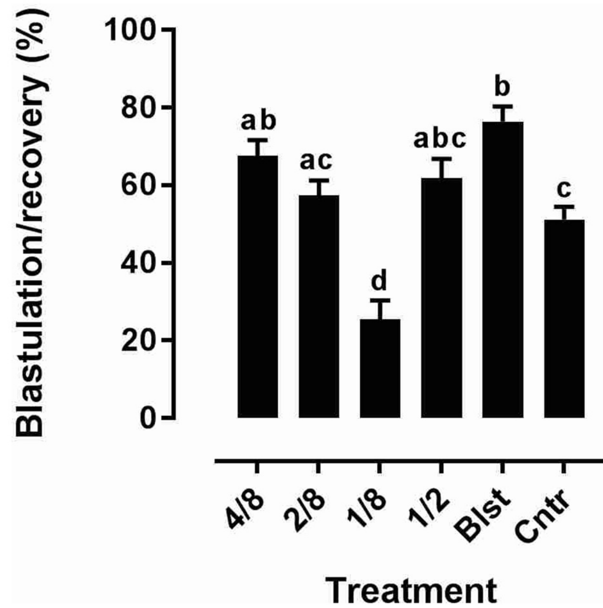

Figure 1. **Percentage of blastocysts obtained per split cleavage stage embryo or recovered per bisectioned blastocyst**. The blastulation frequency was calculated as the number of blastocysts per cleaved embryo. The recovery frequency was calculated as the number of surviving splits per bisected blastocyst. Data given as mean ± SEM. Averages with different superscripts differ significantly (chi-squared with Bonferroni correction, $P < 0.05$). Blst = blastocyst splits; Cntr = intact control embryos.

Table I. **Mean blastulation frequency and cell counts in Day 3 embryos splits based on splitting ratio**. The blastulation frequency was calculated as number of blastocysts per cleaved embryo. Embryos with exactly eight blastomeres could be split according to the ideal splitting ratios 0.125, 0.25 or 0.50. However, the splitting ratio varied for Day 3 embryos with a cell count other than eight (atypical embryo cellularity). Within the same split type, values with an asterisk (*) differ significantly (chi-squared for blastulation, Welch's ANOVA for cell counts, $P < 0.05$).

Split type	Embryo cellularity	Splitting ratio	n	Blastocyst per split embryo (%)	Cells/blastocyst
4/8	8 cells	0.50	59	66.1 ± 11.7	93.5 ± 22.3
	Atypical	0.40–0.57	52	69.2 ± 12.2	82.6 ± 15.4
2/8	8 cells	0.25	55	65.4 ± 12.2	40.7 ± 7.7*
	Atypical	0.20–0.36	95	52.6 ± 9.8	64.0 ± 11.0*
1/8	8 cells	0.125	53	28.3 ± 11.8	25.0 ± 5.8
	Atypical	0.08–0.18	45	22.2 ± 11.9	30.3 ± 8.1

output appeared to be the splitting of a Day 3 embryo in four parts (2/8 type splits), which led to the production of 1.8 blastocysts per zygote, a 3.4-fold increase as compared to intact embryos, as presented in Figure 2.

3.3 *Cell counts in blastocysts derived from different embryo splitting strategies*

For each of the splitting methods investigated (4/8 type splits, 2/8 type splits, 1/8 type splits, and blastocyst bisectioning), we recorded the number of cells per blastocyst at day 7 post-insemination and compared them against cell counts obtained from control blastocysts (n = 82), as reported in Figure 3. While no treatment group showed cell counts as high as those obtained from control blastocysts, split embryos appeared to possess, at the blastocyst stage, more cells than their splitting ratio would suggest (e.g. a 1/2 type split would possess more than 0.5 times the number of cells of a control blastocyst). To investigate this observation, a virtual experiment was carried out by comparing cell counts in embryo splits against cell counts from control blastocysts multiplied by a factor identical to the splitting ratio of the test group (for example, by 0.25 when comparing against 2/8 type splits). It was found that embryo splits had statistically greater cell counts than expected in all cases, with the exception of blastocyst stage splits. The detailed results of this analysis are presented in Table II. Furthermore, representative examples of blastocysts obtained following different splitting strategies are presented in Figure 4.

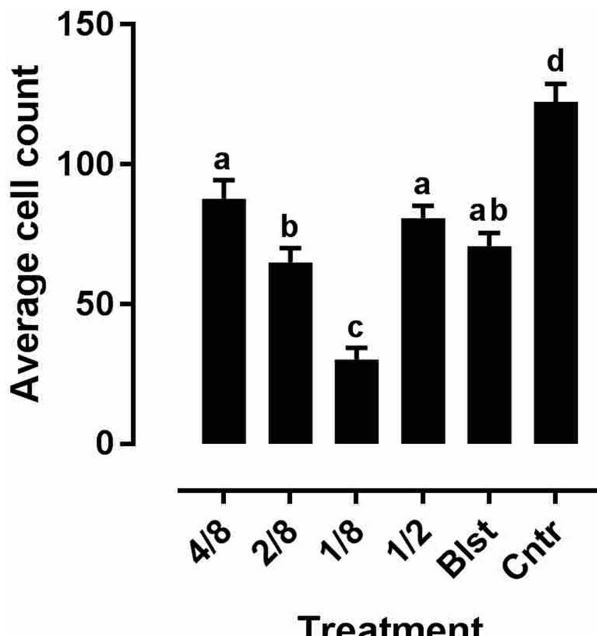

Figure 3. **Average cell counts in day 7 post-insemination blastocysts produced according to different embryo splitting strategies**. Data given as mean ± SEM. Averages with different superscripts differ significantly (Welch's ANOVA, P < 0.05).

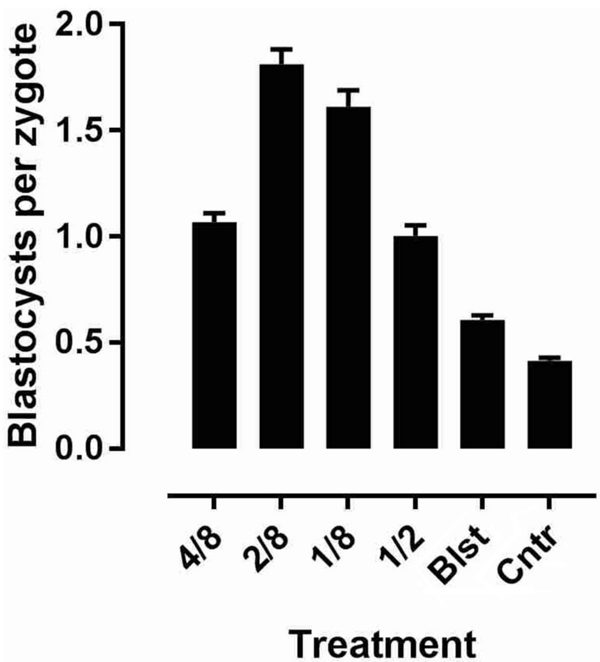

Figure 2. **Blastocysts obtained per starting zygote across different embryo splitting strategies**. Data given as mean ± SEM.

3.4 *Serial splitting of cleavage stage bovine embryos*

To determine whether the application of serial embryo splitting would be superior to single splitting in terms of blastocyst yield and viability, blastulation frequency and cell counts in blastocysts were compared between serially split embryos (second and third serial splits) and type 2/8 and 1/8 splits. Type 2/8 and 1/8 splits were chosen as a comparison due to having comparable splitting ratios to serially split embryos. The application of serial splitting led to the production of n = 67 second serial splits and n = 116 third serial splits which blastulated with a frequency of 44.7% and 24.1%, respectively. The average cell count in the blastocysts produced was 37.9 ± 3.2 cells/embryo in second serial splits and just 15.0 ± 1.0 cells/embryo in third serial splits. Second serial splits produced 1.4 blastocysts per starting zygote, while third serial splits produced 1.5 blastocysts per zygote. The frequency of blastulation for embryos serially split twice (second serial splits) did not differ significantly from the blastulation frequency achieved by 2/8 type splits (chi-squared,

Table II. **Cell counts from blastocysts derived from embryo splitting compared against adjusted cell counts from intact control blastocysts (ideal controls)**. The cell counts form intact control embryos were multiplied by 0.50 for comparison against 4/8 split, 1/2 splits, and blastocyst bisectioning splits; by 0.20 for comparison against 2/8 type splits; and by 0.125 for comparison against 1/8 type splits. Data given as mean ± SEM. Within the same row, values with an asterisk (*) differ significantly (Welch's ANOVA, P < 0.05).

Split type	n	Split embryo cells/blastocyst	Ideal control cells/blastocyst
4/8	43	87.7 ± 6.7*	61.2 ± 3.1*
2/8	58	30.6 ± 1.5*	54.3 ± 3.9*
1/8	21	27.0 ± 2.4*	15.3 ± 0.8*
1/2	47	80.7 ± 4.4*	61.2 ± 3.1*
Blastocyst	63	70.7 ± 4.6	61.2 ± 3.1

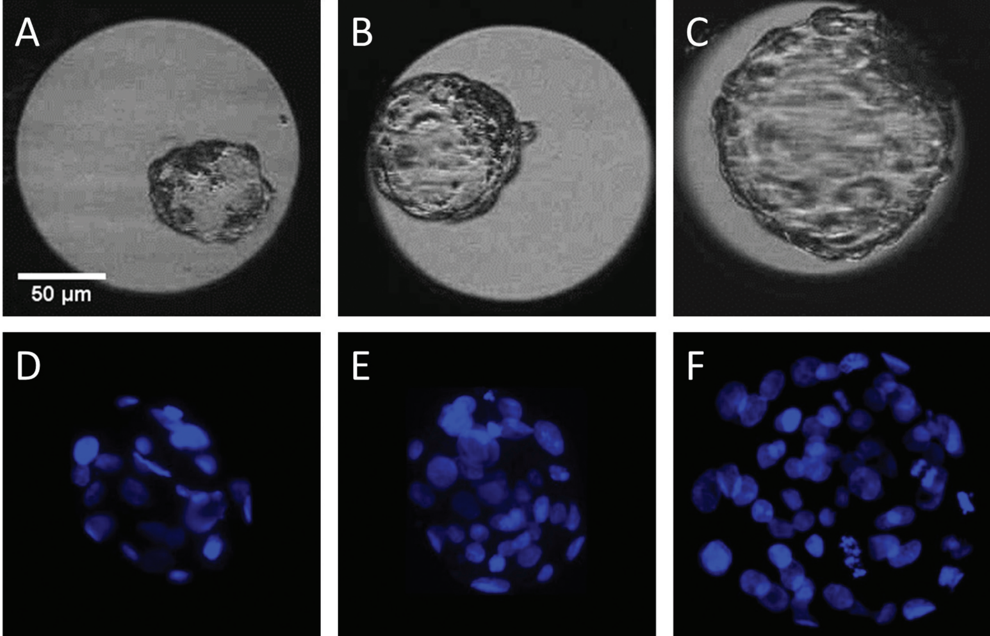

Figure 4. **Representative blastocysts derived from different splitting strategies at 168 h post-insemination**. Panels A-C: phase contrast images were captured using a PrimoVision EVO Microscope (Vitrolife). Panels D-F: images captured by epifluorescence microscopy at 200x total magnification. DNA stained with Hoechst 33342 (blue). A and D) Blastocyst derived from a 1/8 type split. B and E) Blastocyst derived from a 2/8 type split. C and F) Blastocyst derived from a 4/8 type split. The splitting ratio appeared to have a clear effect on blastocyst size.

$\chi^2_1 = 2.93$, P = 0.09). Similarly, there was no difference between the blastulation frequency of embryos serially split three times (third serial splits) and 1/8 type splits (chi-squared, $\chi^2_1 = 0.05$, P = 0.82). However, 2/8 type splits and 1/8 type splits had a greater number of cells at the blastocyst stage than second serial splits (Welch's ANOVA, $F_{1,83} = 5.84$, $P = 0.18^{-3}$) and third serial splits (Welch's ANOVA, $F_{1,43} = 28.59$, $P = 3.2\text{x}10^{-6}$), respectively.

3.5 *Developmental rate in embryo splits*

To test the hypothesis that embryo splitting affects the embryonic developmental rate and to investigate whether MZ twin embryos maintain synchronous development in cattle, a morphokinetic analysis was completed on both control and cleavage stage embryo splits for a number of developmental landmarks, which included timing of second and third cleavage, compaction, onset of cavitation, and blastocyst expansion.

It was found that the timing of developmental events in 2-cell embryo splits compared well with that of intact control embryos up to the third cleavage division. However, Day 2 splits blastulated earlier than both Day 3 splits and controls. Indeed, embryos split at 2-cells displayed a significant increase in their developmental rate after compaction and, on average, produced blastocyst stage embryos 11 hours before the control embryos did.

It is also curious to note that embryos split on Day 3 compacted later than controls but blastulated with similar timing to them. We observed that the blastomeres of Day 3 splits appeared to require additional time to come into contact with each other and to re-form the cell-to-cell junctions destroyed after disaggregation before they were able to proceed to compaction. Conversely, the blastomeres derived from mitosis of a 2-cell split tended to stay always in contact with each other (since they were all derived from the cleavage of a single blastomere); so that compaction was only slowed down in Day 3 splits. The results of this analysis are reported in Figure 5. Additionally, the development of MZ twin embryos appeared well synchronized only up to the third cleavage division, as reported in Table III.

4. Discussion

4.1 Developmental potentials of splits derived from day 3 embryos

The applicability of embryo splitting to embryos with atypical (different from eight) cell numbers at day 3 has been largely underreported in the literature. However, here we show that embryos with an atypical number of blastomeres are able to survive splitting and develop to form blastocyst stage embryos as often as Day 3 embryos with exactly eight cells. Furthermore, embryos with an atypical number of blastomeres tended to form blastocysts of

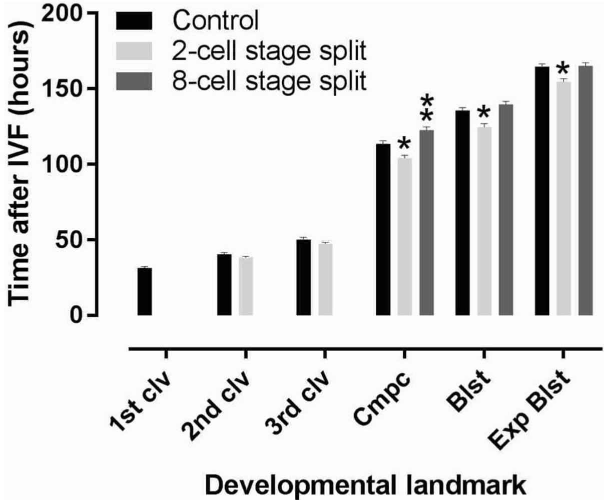

Figure 5. **Developmental rates in control and cleavage stage embryo splits**. Data given as mean ± SEM. For each landmark, columns with different superscripts differ significantly (one-way ANOVA, $P < 0.05$).

Table III. **Average difference in the time required by monozygotic twin embryos to achieve the same developmental landmark**. Results were derived from n = 9 and n = 11 pairs of twins for Day 2 and Day 3 embryo splits, respectively. Values are given as mean difference between twins in hours ± SEM.

Developmental landmark	Day 2 embryo splits (hours)	Day 3 embryo splits (hours)
2nd cleavage	0.9 ± 0.4	-
3rd cleavage	1.2 ± 0.4	-
Compaction	8.3 ± 4.2	13.0 ± 2.6
Cavitation	7.8 ± 1.8	8.9 ± 2.3
Expansion	6.0 ± 1.2	9.9 ± 2.7

similar or even better quality (in the case of 2/8 type splits) as judged by total cell counts as compared to embryos with exactly eight cells. On the other hand, the performance of embryos split at Day 3 seems to be directly correlated to their splitting ratio. The results presented, therefore, support the use of Day 3 embryos for embryo splitting, regardless of their exact cell number, but it might be advisable to avoid creating embryos with low splitting ratios (less than 0.20).

4.2 Embryo splitting production

In these experiments, the splitting of an embryo into four parts was the strategy that produced the greatest blastocyst output. This result is in agreement with previous findings suggesting that a limit of four identical embryos from a single donor embryo would be difficult to overcome (Willadsen & Polge 1981; Johnson et al. 1995; Turner et al. 2019). Split embryos outperformed controls in terms of blastocyst formed per cleaved embryo. This is an expected outcome due to embryo splitting only being applicable to embryos that achieved certain developmental landmarks. However, this finding also suggests that embryo splitting is well tolerated by early-stage embryos, a consideration in agreement with previous reports (Gray et al. 1991; Escriba et al. 2002; Tagawa et al. 2008; Velasquez et al. 2016).

4.3 Cell counts in embryo splits and control embryos

Loskutoffl *et al.* (Loskutoff & Johnson 1993) discussed how bovine blastocysts derived from quarter embryos containing about 40 cells could proceed to establish a pregnancy in 20% of cases after transfer, whilst blastocysts containing about 72 cells could establish a pregnancy in 35–40% of cases. Given the results presented, it seems plausible that embryo splits derived from most of the single splitting strategies employed here would preserve enough

developmental potential to be able to establish pregnancies with an acceptable frequency upon transfer.

Interestingly, we have here for the first time described how embryos split at cleavage stage consistently demonstrate higher cell numbers than expected at the blastocyst stage. This suggests that cleavage stage splits might experience an increased mitotic index as compared to control embryos. An alternative explanation might be that the fractions of the embryo that survive are composed of cells of better quality perhaps due to mosaicism in the embryo (Iwasaki et al. 1992). However, this hypothesis does not explain well the high cell numbers observed in multiple embryos derived from the same progenitor.

4.4 Serial embryo splitting

A form of serial embryo splitting has been previously demonstrated for bovine blastocysts, where embryos that had already formed a blastocoel were bisectioned sequentially (Rho et al. 1998). However, to the best of our knowledge, this is the first study reporting the application of serial embryo splitting to cleavage stage bovine embryos. In the present work, we found that triple serial splitting resulted in a marked decrease in the blastulation frequency as compared with double serial splitting, in agreement with the work of Illmensee and colleagues who described the serial splitting of murine cleavage stage embryos (Illmensee et al. 2006). However, in contrast to what has been described in mice, triple embryo splitting in cattle produced an increase rather than a loss in the net output of blastocysts and both double and triple serial splitting performed to similar levels in this respect. Interestingly, both double and triple serial embryo splitting appeared to have no negative effect on blastulation frequency as both strategies performed as well as type 2/8 and 1/8 splits respectively.

Nevertheless, cell counts were markedly reduced in blastocysts derived from serially split embryos as compared to single splitting strategies. The significant impact of serial embryo splitting on the number of cells per blastocyst raises questions about the viability of the embryos produced, especially in the case of triple serial splits. While the developmental potential of blastocysts with very small cell counts remains unexplored, with reference to the work of Loskutoffl *et al.* (Loskutoff & Johnson 1993), it seems likely that the transfer of serially split embryos would establish pregnancies at unacceptably low frequency. Overall, triple serial splitting appears to be a non-viable embryo multiplication strategy, which would suggest that more ambitious serial splitting strategies are likely to fail as well. On the other hand, double serial splitting could be a useful IVP strategy; however, this strategy underperformed when compared to 2/8 single splitting and had the disadvantage of requiring additional handling time.

4.5 Timing of development in split embryos

At time-lapse observation, the developmental timing of the intact control embryos closely matched that of a previously published study (Holm et al. 1998). However, we also discovered an effect of splitting on the resulting embryo's morphokinetics. Embryos split at Day 2 had faster development to blastocyst, while embryos split at Day 3 suffered from an initial reduction in their development rate but caught up to controls by the blastocyst stage. The blastomeres derived from mitosis of a 2-cell split tended to stay always in contact with each other, so that compaction was only slowed down in Day 3 splits. This observation is in agreement with previous studies performed on mouse embryos, which showed that continuous cell-to-cell interactions are important for the fate specification of blastomeres, and that blastomeres likely require re-establishment of these interactions after splitting (Johnson & Ziomek 1981). It was nonetheless interesting to note that Day 3 bovine embryo splits showed enough plasticity to be able to recover from the cell reductional event and blastulate with the same timing as intact control embryos.

The finding that it was possible to produce blastocysts with morphologically distinct cell populations (inner cell mass and trophectoderm) from subpopulations of blastomeres randomly sampled from a Day 2 or a Day 3 embryo supports both the inside-out (Tarkowski & Wroblewska 1967) and the cell polarity (Johnson & Ziomek 1981) embryo development models, which postulate that the fate of blastomeres is determined by position-dependent mechanisms. This is also in contrast with the pre-patterning model (Piotrowska et al. 2001; Piotrowska & Zernicka-Goetz 2001) which instead hypothesizes that embryonic cell lineages are pre-determined due to the uneven distribution of molecular determinants in the oocyte, or would at least suggest that cattle embryos possess enough plasticity at Day 3 to reorganize themselves correctly after a reductional event.

Finally, the developmental synchrony detected between twin embryos seems to suggest that the first three cleavage events are under a strict temporal control in bovine embryos, a finding in agreement with another study performed in human embryos (Noli et al. 2015). However, this stringent regulation seemed to relax after the third cleavage division, similar to what has been previously reported for mouse embryos (Arav et al.

2008). Therefore, the results presented here provide further evidence for the existence of a developmental clock synchronizing early developmental events in mammals (Noli et al. 2015), but also suggest that its operation might terminate at compaction, at the time of cell fate specification.

5. Conclusions

The flexible but limited ability of the bovine embryo to cope with cell reductional events suggests that perpetual embryo splitting might be impossible and likely limits the application of embryo splitting to single splitting strategies and a maximum production of quadruplets. Nevertheless, the application of embryo splitting holds promise to increase dramatically the availability of blastocysts from specific gamete donors, which would be useful to commercial breeders. Moreover, the availability of multiple MZ twin embryos might simplify the application of preimplantation genetic testing for aneuploidy (PGT-A) to cattle IVP, an advancement which in and on itself holds promise to further improve embryo transfer outcomes, as we have recently demonstrated (Silvestri et al. 2021). For example, it could be possible to biopsy or even sacrifice just one of the embryos produced by splitting and use it to obtain an indication of the ploidy status and the potential breeding value of all of its MZ twins, increasing the cost-effectiveness of the screening. Finally, the availability of multiple identical embryos would reduce the chances of a particular genetic background being lost due to an unsuccessful biopsy. In the future, it would be of interest to further corroborate the findings highlighted in the present work by performing embryo transfers. We also expect that the approach here described could be readily adopted for the IVP of other important farm animal species such as sheep and pigs.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethical review board statement

No animals were raised or sacrificed purposely for collecting organs or tissues. The procedures described are not to be considered animal experimentation and therefore do not fall under the British Animals (Scientific Procedures) Act 1986.

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References

Arav A, Aroyo A, Yavin S, Roth Z. 2008. Prediction of embryonic developmental competence by time-lapse observation and ‘shortest-half’ analysis. Reproductive BioMedicine Online 17 (5):669–675. DOI: 10.1016/S1472-6483(10)60314-8.

Chan AW, Dominko T, Luetjens CM, Neuber E, Martinovich C, Hewitson L et al. 2000. Clonal propagation of primate offspring by embryo splitting. Science 287(5451):317–319. DOI: 10.1126/science.287.5451.317.

Escriba M, Valbuena D, Remohí J, Pellicer A, Simon C. 2002. New techniques on embryo manipulation. Journal of Reproductive Immunology 55(1–2):149–161. DOI: 10.1016/S0165-0378(01)00138-3.

Gray K, Bondioli K, Betts C. 1991. The commercial application of embryo splitting in beef cattle. Theriogenology 35(1):37–44. DOI: 10.1016/0093-691X(91)90146-5.

Haddow JE, Palomaki GE, Allan WC, Williams JR, Knight GJ, Gagnon J et al. 1999. Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. New England Journal of Medicine 341(8):549–555. DOI: 10.1056/NEJM199908193410801.

Heyman Y, Vignon X, Chesné P, Le Bourhis D, Marchal J, Renard J. 1998. Cloning in cattle: From embryo splitting to somatic nuclear transfer. Reproduction Nutrition Development 38(6):595–603. DOI: 10.1051/rnd:19980602.

Holm P, Shukri N, Vajta G, Booth P, Bendixen C, Callesen H. 1998. Developmental kinetics of the first cell cycles of bovine in vitro produced embryos in relation to their in vitro viability and sex. Theriogenology 50(8):1285–1299. DOI: 10.1016/S0093-691X(98)00227-1.

Holm P, Booth PJ, Schmidt MH, Greve T, Callesen H. 1999. High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology 52(4):683–700. DOI: 10.1016/S0093-691X(99)00162-4.

Illmensee K, Kaskar K, Zavos PM. 2006. In vitro blastocyst development from serially split mouse embryos and future implications for human assisted reproductive technologies. Fertility and Sterility 86(4):1112–1120. DOI: 10.1016/j.fertnstert.2006.02.103.

Iwasaki S, Hamano S, Kuwayama M, Yamashita M, Ushijima H, Nagaoka S, Nakahara T. 1992. Developmental changes in the incidence of chromosome anomalies of bovine embryos fertilized in vitro. Journal of Experimental Zoology 261(1):79–85. DOI: 10.1002/jez.1402610109.

Johnson MH, Ziomek CA. 1981. The foundation of two distinct cell lineages within the mouse morula. Cell 24:71–80. DOI: 10.1016/0092-8674(81)90502-X.

Johnson WH, Loskutoff NM, Plante Y, Betteridge KJ. 1995. Production of four identical calves by the separation of blastomeres from an in vitro derived four-cell embryo. Veterinary Record 137(1):15–16. DOI: 10.1136/vr.137.1.15.

Kippax I, Christie W, Rowan T. 1991. Effects of method of splitting, stage of development and presence or absence of zone pellucida on foetal survival in commercial bovine embryo transfer of bisected embryos. Theriogenology 35(1):25–35. DOI: 10.1016/0093-691X(91)90145-4.

Le Bourhis A, Mullaart E, Humblot P, Coppieters W, Ponsart C. 2011. 193 bovine embryo genotyping using a 50k single nucleotide polymorphism chip. Reproduction, Fertility and Development 23(1):197. DOI: 10.1071/RDv23n1Ab193.

Lopes R, Forell F, Oliveira A, Rodrigues J. 2001. Splitting and biopsy for bovine embryo sexing under field conditions. Theriogenology 56(9):1383–1392. DOI: 10.1016/S0093-691X(01)00641-0.

Loskutoff N, Johnson W. 1993. Betteridge K.The developmental competence of bovine embryos with reduced cell numbers. Theriogenology 39(1):95–107. DOI: 10.1016/0093-691X(93)90026-2.

Miller G, Gliedt D, Rakes J, Rorie R. 1994. Addition of penicillamine, hypotaurine and epinephrine (PHE) or bovine oviductal epithelial cells (BOEC) alone or in combination to bovine in vitro fertilization medium increases the subsequent embryo cleavage rate. Theriogenology 41(3):689–696. DOI: 10.1016/0093-691X(94)90178-L.

Morris SA, Guo Y, Zernicka-Goetz M. 2012. Developmental plasticity is bound by pluripotency and the Fgf and Wnt signaling pathways. Cell Reports 2(4):756–765. DOI: 10.1016/j.celrep.2012.08.029.

Nagashima H, Kato Y, Ogawa S. 1989. Microsurgical bisection of porcine morulae and blastocysts to produce monozygotic twin pregnancy. Gamete Research 23(1):1–9. DOI: 10.1002/mrd.1120230102.

Nicholas F, Smith C. 1983. Increased rates of genetic change in dairy cattle by embryo transfer and splitting. Animal Science 36(3):341–353. DOI: 10.1017/S0003356100010382.

Noli L, Dajani Y, Capalbo A, Bvumbe J, Rienzi L, Ubaldi FM et al. 2015. Developmental clock compromises human twin model created by embryo splitting. Human Reproduction (Oxford, England) 30(12):2774–2784. DOI: 10.1093/humrep/dev252.

Piotrowska K, Wianny F, Pedersen RA, Zernicka-Goetz M. 2001. Blastomeres arising from the first cleavage division have distinguishable fates in normal mouse development. Development 128(19):3739–3748. DOI: 10.1242/dev.128.19.3739.

Piotrowska K, Zernicka-Goetz M. 2001. Role for sperm in spatial patterning of the early mouse embryo. Nature 409(6819):517–521. DOI: 10.1038/35054069.

Rho G, Johnson WH, Betteridge KJ. 1998. Cellular composition and viability of demi- and quarter-embryos made from bisected bovine morulae and blastocysts produced in vitro. Theriogenology 50(6):885–895. DOI: 10.1016/S0093-691X(98)00193-9.

Silvestri G, Canedo-Ribeiro C, Serrano-Albal M, Labrecque R, Blondin P, Larmer SG et al. 2021. Preimplantation Genetic Testing for Aneuploidy Improves Live Birth Rates with In Vitro Produced Bovine Embryos: A Blind Retrospective Study. Cells 10(9):2284. DOI: 10.3390/cells10092284.

Tagawa M, Matoba S, Narita M, Saito N, Nagai T, Imai K. 2008. Production of monozygotic twin calves using the blastomere separation technique and Well of the Well culture system. Theriogenology 69(5):574–582. DOI: 10.1016/j.theriogenology.2007.11.003.

Tarkowski AK, Wroblewska J. 1967. Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. Journal of Embryology and Experimental Morphology 18(1):155–180.

Turner KJ, Silvestri G, Black DH, Dobson G, Smith C, Handyside AH et al. 2019. Karyomapping for simultaneous genomic evaluation and aneuploidy screening of preimplantation bovine embryos: The first live-born calves. Theriogenology 125:249–258. DOI: 10.1016/j.theriogenology.2018.11.014.

Vajta G, Peura T, Holm P, Pldi A, Greve T, Trounson A, Callesen H. 2000. New method for culture of zona-included or zona-free embryos: The Well of the Well (WOW) system. Molecular Reproduction and Development 55(3):256–264. DOI: 10.1002/(SICI)1098-2795(200003)55:3<256::AID-MRD3>3.0.CO;2-7.

Velasquez AE, Castro FO, Veraguas D, Cox JF, Lara E, Briones M, Rodriguez-Alvarez L. 2016. Splitting of IVP bovine blastocyst affects morphology and gene expression of resulting demi-embryos during in vitro culture and in vivo elongation. Zygote 24(1):18–30. DOI: 10.1017/S0967199414000677.

Willadsen SM. 1980. The viability of early cleavage stages containing half the normal number of blastomeres in the sheep. Reproduction 59(2):357–362. DOI: 10.1530/jrf.0.0590357.

Willadsen SM, Polge C. 1981. Attempts to produce monozygotic quadruplets in cattle by blastomere separation. Veterinary Record 108(10):211–213. DOI: 10.1136/vr.108.10.211.