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Quality of preimplantation embryos recovered *in vivo* from dairy cows in relation to their body condition

A.V. Makarevich[1,2], *L. Stádník*[3], *E. Kubovičová*[2], *Z. Hegedüšová*[4], *R. Holásek*[4], *F. Louda*[4], *J. Beran*[3] *and M. Nejdlová*[3]

National Agricultural and Food Centre, Research Institute of Animal Production Nitra, Slovak Republic; Czech University of Life Sciences Prague, Czech Republic; and Institute for Cattle Breeding Ltd Rapotin, Czech Republic

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Summary

This study examined the impact of cow body condition on the quality of bovine preimplantation embryos. The embryos ($n = 107$) were flushed from dairy cows and classified according to a five-point scale body condition score (BCS2 $n = 17$; BCS3 $n = 31$; BCS4 $n = 11$) on the 7th day after insemination and then analyzed for development, dead cell index (DCI), cell number and actin cytoskeleton quality. The highest embryo recovery rate ($P < 0.05$) was recorded in the BCS3 group and the lowest in the BCS4 group. More transferable (morula, blastocyst) embryos were obtained from the BCS4 cows (79%), compared with the BCS2 (64%) or BCS3 (63%) animals. However, cell numbers were higher in the BCS2 and BCS3 groups ($P < 0.05$) compared with the BCS4 embryos. Conversely, the DCI was lowest in the BCS2 (3.88%; $P < 0.05$) and highest in the BCS4 (6.56%) embryos. The proportion of embryos with the best actin quality (grade I) was higher in the BCS2 and BCS3 cows compared with the BCS4 group. Almost 25% of all embryos showed fragmented morphology and a higher DCI (5.65%) than normal morulas (1.76%). More fragmented embryos were revealed in the BCS2 (28.6%) and BCS4 (31.25%) groups, and less (19.15%) in the BCS3 group. The cell numbers in such embryos were lower in the BCS4 (22.57) than in the BCS2 (46.25) or BCS3 (42.4) groups. In conclusion, the body condition of dairy cows affects the quality of preimplantation embryos. A BCS over 3.0 resulted in a higher incidence of poor (fragmented) embryos.

Keywords: Body condition score, Cow, Embryo quality

Introduction

Yield and quality of oocytes and embryos upon superovulation reflect potential fertility in dairy cows. The results of superovulation are mostly dependent on the treatment scheme used, the hormone preparation and the response of cows to hormonal treatment. The dam's response to superovulation may be affected by numerous factors including physiological state, health state, age, body condition, season and others. In particular, the nutritional condition of the donor cow can protract the length of post partum anoestrus (Řehák *et al.*, 2012) and has a substantial influence on the response of gonads to exogenous gonadotropin stimulation and production of good quality embryos (Nolan *et al.*, 1998; Rajmon *et al.*, 2012). Oocyte quality and embryo development may be negatively affected by a high-nutrition diet (Yaakub *et al.*, 1999) or by restriction of feed ration (Louda & Stádník, 2000). The nutritional status of the donor cow can be simply assessed in breeding management by evaluation of the body condition score (BCS). Primarily, the effect of the current BCS level (Stádník *et al.*, 2007) or

[1]All correspondence to: Alexander V. Makarevich. Research Institute for Animal Production Nitra, Hlohovecká 2, 951 41 Lužianky near Nitra, Slovak Republic. Tel: +421 37 6546 334. E-mail:makarevic@cvzv.sk
[2]National Agricultural and Food Centre, Research Institute of Animal Production Nitra, 951 41 Lužianky near Nitra, Slovak Republic.
[3]Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Animal Husbandry, Kamýcká 129, 165 21 Prague 6 – Suchdol, Czech Republic.
[4]Institute for Cattle Breeding Ltd. Rapotin, Vyzkumniku 267, 788 13 Vikyrovice, Czech Republic.

energy balance (Beran *et al.*, 2013; Doležalová *et al.*, 2013) on reproduction abilities of dairy cows has been considered to be the most important factor. According to Siddiqui *et al.* (2002), cows with a BCS of 2.5–3 are likely to respond better to superovulation treatment than those cows with a BCS of 4–5, because cows with higher BCS are more likely to acquire ovarian cysts and therefore have less ovulations.

Body condition can affect the yield and the quality of oocytes and embryos in different ways. In particular, the physiological status of cows (lactating cows or non-lactating heifers) has a significant effect on embryo quality (Walsh *et al.*, 2011). Embryos recovered from non-lactating heifers were of higher quality compared with those from lactating cows (Leroy *et al.*, 2005; Sartori *et al.*, 2010; Rizos *et al.*, 2010). Cows at the first lactation yielded oocytes of lesser quality than did cows at the third lactation (Snijders *et al.*, 2000).

The impact of body condition on embryo yield and quality depends on whether *in viv*o recovered or *in vitro* produced (IVP) embryos are considered. For example, for IVP embryos, cows with a higher BCS (3.3–4.0) showed higher cleavage and blastocyst rates than did cows with a lower BCS (1.5–2.5) (Snijders *et al.*, 2000). The quality of oocytes probably contributes to reduced fertility. In particular, the highest conception rates were recorded in cows with a moderate (about 3) BCS (Stádník *et al.*, 2002), whilst the cleavage rate of *in vitro* fertilized oocytes was greater in heifers with a low BCS (2.5) than in those with a moderate BCS (3.0) (Adamiak *et al.*, 2006). However, the BCS of heifers did not affect blastocyst yield. When the quality of obtained embryos was taken into account, total embryo cell number was greater in low-BCS derived oocytes (129.7) compared with moderate-BCS heifers (116.2), however the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) index (4.2 ± 0.45) did not differ between BCS groups (Adamiak *et al.*, 2006).

Kadokawa *et al.* (2008) reported no effect of heifers with a BCS of around 3 on *in vivo* embryo number yielded, whilst heifers with a BCS of 3.5–4 had poorer embryo production. More blastocysts were obtained in heifers with a BCS of 3.75 than in 3.25 and 3.5 BCS heifers, however there were no data about the quality of these embryos.

Generally, morphological and functional criteria including embryo cell number, apoptotic cell rate, level of fragmentation, cytoskeleton status, embryo diameter, developmental stage and others, are the most common parameters of preimplantation embryo quality (Van Soom & Boerjan, 2002). Data on quality of *in vivo* recovered embryos in relation to the body condition of their donors are insufficient and limited by use of subjective examinations under the stereomicroscope.

We hypothesise that the body condition of cows may have an impact on the quality of preimplantation embryos. In order to examine this hypothesis, we applied some currently used fluorescent markers (nuclear staining, cell death detection, cytoskeleton filaments) to analyze in detail the quality of *in vivo* recovered embryos. Our objective was to examine the possible relationship between BCS and the more detailed quality of *in vivo* recovered cattle embryos evaluated using fluorescent markers.

Materials and methods

Animals

In our experiments, 59 Holstein cows were classified based on BCS and palpation and visual estimation, which was performed by the same technician according to the criteria of a five-point scale of BCS (Edmonson *et al.*, 1989), and during the pre-flushing examination. In accordance with the current BCS levels, the cows were divided into groups BCS2 ($n = 17$; BCS 1.75–2.25), BCS3 ($n = 31$; BCS 2.5–3.25), and BCS4 ($n = 11$; BCS 3.5–4.5). The cows were calved from March 2009 to August 2011 and their average parity was 1.9. Daily milk yield recorded from milk parlour automatic evidence in the day of the start of hormonal treatment and was on average at the level of 29.48 kg with standard deviation (SD) of 8.04. All cows selected for observation were without reproductive disorders in the evaluated period as well as at previous lactations. Their ovarian functions were examined using an ultrasound machine ALOKA SSD-500 (Aloka Co., Ltd, Tokyo, Japan) equipped with a 5-MHz array probe before hormonal treatment. Only cows with a corpus luteum were selected for subsequent ovarian cycle synchronization and superovulation. All these facts were basic pre-selection conditions in relation to evaluation of specific reproduction traits. However, some of these animals were, during the first phase of lactation, designated for subsequent culling, mainly because of a high level of somatic cells in milk and/or the frequency of mastitis occurrence, low milk yield or an insufficient exterior of selected body parts (udder, legs, rear). The cows were loose housed in a cubicle straw-bedded barn and fed a total mixed ratio consisting of maize and alfalfa silage, straw, grass and alfalfa hay, brewery draft, bakery waste, molasses, commercial concentrates and mineral supplements. The ingredient composition of the diet corresponded to the level of the daily milk yield and feeding rations were balanced for energy, protein, fat as well as mineral and vitamin content. Feeding rations consisted of the same components throughout the year seasons during the experiments.

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Preparation of cows for flushing and embryo recovery

The oestrus of cows was synchronized by the injection of a $PGF_{2\alpha}$ analogue Oestrophan (Bioveta a.s., Ivanovice na Hane, Czech Republic) after ultrasound examination at the day 0. The cows were superovulated by application of porcine pituitary gonadotropin (Pluset® - FSHp-LHp , Lab CALIER, Barcelona, Spain) twice daily for 5 days at 8.00 and 20.00 h (given in a decreasing dosage rate; starting with the doses of 150 IU FSH +150 IU LH in the morning at the 11th day to 50 IU FSH+50 IU LH in the evening at the 15th day) of the oestrous cycle. Oestrophan was administered for luteolysis at the 13th day. One insemination and re-insemination were performed during the 17th day by the same artificial insemination (AI) technician with frozen–thawed AI doses from one sire only at 12 h intervals. The insemination was done at the standing oestrous, while re-insemination was performed 12 h later.

Estimation of BCS of cows was performed on the day of embryo flushing. It was assumed that the body condition of experimental cows did not been change during period between the date of the first insemination and embryo recovery (6 days), so that neither yield nor quality of subsequent embryos was influenced by any changes in the body condition. Embryo recovery was performed on the 7th day after the first insemination by a standard non-surgical technique to flush out the uterine horns. Uterine flushing was conducted with a complete flush solution (Bioniche, Belleville, Ontario, Canada) using a silicone two-way Foley catheter (Minitüb GmbH, Tiefenbach, Germany). Flushed ova/embryos were transferred to the holding medium, phosphate-buffered solution (PBS), with 20% fetal calf serum (FCS, Gibco BRL) and assessed using a stereomicroscope. The embryos were evaluated according to their stage of development as a transferable (i.e., morulas, blastocysts) and non-transferable (i.e. degenerated embryos, unfertilized oocytes). Thereafter, the embryos were analyzed for apoptosis occurrence (TUNEL assay), embryo cell number and quality of actin cytoskeleton.

Analysis of embryos for TUNEL, embryo cell number and actin cytoskeleton

The embryos ($n = 107$) were washed three times for 5 min in PBS–PVP washing solution (PBS with 4 mg ml^{-1} polyvinylpyrrolidone; Sigma-Aldrich Chemie, Steinheim, Germany). Then the embryos were fixed in 3.7% neutrally buffered formalin (Fluka, Buchs, Switzerland) for 10 min. Permeabilization was done by 1 h incubation of embryos in 0.5% Triton X-100 in PBS. Afterwards, the embryos were processed for cell death detection (TUNEL) using an In situ Cell Death Detection kit (Roche Slovakia Ltd, Bratislava, Slovak Republic) according to the product manual. Briefly, fixed and permeabilized embryos were incubated at 37°C in the TUNEL-detecting solution, which consisted of fluorescence-labelled nucleotides (dUTP–FITC, 37 μl) and terminal transferase (TdT, 3 μl), at 37°C for 1 h. As a positive control for TUNEL, a group of fixed and permeabilized embryos was incubated in the presence of bovine DNase I for 1 h at 37°C before the TUNEL reaction. Following this incubation, the TUNEL reaction was stopped by three-times washing of embryos in PBS–PVP solution.

For labelling of actin filaments, the embryos ($n = 107$) were put into the phalloidine–TRITC conjugate solution, which is a component of the Actin Cytoskeleton and Focal Adhesion Staining Kit (Chemicon International, Temecula, CA, USA) for 45 min. Thereafter the embryos were washed three times in PBS–PVP solution, transferred onto coverslip and covered with 5 μl of Vectashield anti-fade mounting medium, containing 4′,6-diamidino-2-phenylindole (DAPI) stain (Vector Laboratories, Burlingame, CA, USA). The coverslip was attached to microslide using nail polish. All treatments were performed at ambient temperature. The samples were stored at –20°C until fluorescence analysis.

TUNEL indexes were determined on the basis of proportion of TUNEL-positive nuclei (green fluorescence) to total embryo cell number (DAPI-stained nuclei), which have been counted under the Leica fluorescence microscope (Leica Microsystems, Germany) using specific wave-length filters. As TUNEL reaction labels both apoptotic and necrotic cells, the TUNEL-positive nuclei in our study were considered as the dead cell nuclei.

The actin cytoskeleton was classified according to Tharasanit *et al.* (2005) on the basis of appearance of actin filaments in horse embryos, with our adaptation to bovine embryos, as belonging to three grades (Makarevich *et al.*, 2012). Grade I (best quality): sharply stained actin filaments of reticular shape in cell borders; grade II (fair quality): blastomeres with less pronounced actin filaments without sharp borders on membranes; and grade III (poor quality): large areas lacking actin staining or visible actin largely aggregated into intracytoplasmic clumps.

Statistics

The experiment was performed in six replicates. A one-way analysis of variance (ANOVA) and Tukey test were used to analyze differences in TUNEL index between groups. As the variable 'TUNEL index' was normally distributed, a log-transformation of original values was not done. The hypothesis of normality was rejected for 'TUNEL cells per embryo' and for 'Embryo

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Table 1 Characteristics of dairy cows involved in the study ($n = 59$); mean ± standard error of the mean (SEM)

Parameter	BCS2 ($n = 17$)	BCS3 ($n = 31$)	BCS4 ($n = 11$)
Body condition score (BCS) range	1.75–2.25	2.5–3.25	3.5–4.5
Age (years)	3.81 ± 0.35^a	3.41 ± 0.25^a	4.8 ± 0.54^b
Days in milk (DIM)	134.7 ± 17.7^a	174.6 ± 18.6^b	328.2 ± 41.1^c
Number of lactations	2.35 ± 0.37	1.84 ± 0.23	2.38 ± 0.53

a versus *b* versus *c*: difference within a row is significant at $P < 0.5$ (*t*-test).

cell number.' Data were log-transformed for these two variables. The treatment effect on actin cytoskeleton quality was tested using the Mann–Whitney *U*-test. All calculations were performed using the SAS software package (SAS Institute, 2001).

Results

All dairy cows were grouped according to their BCS level at the time of flushing as BCS2, BCS3, or BCS4 as mentioned in Table 1, whilst BCS1 and BCS5 cows were not available in the experiment. The cows in BCS groups represented various ages: the lowest average age was recorded for the BCS3 cows (3.41 years) and the highest average age for the BCS4 cows (4.8 years). The duration of the days in milk (DIM) period was also different among BCS groups: it was the lowest in the BCS2 cows (4.5 months), gradually elevated in BCS3 cows (5.8 months) and was almost twice higher in the BCS4 cows (about 11 months). Therefore, DIM were in the range of 87–362 days of lactation with the average of 142 ± 58.9. Generally, BCS of Holstein cows at the beginning of lactation is commonly significantly lower than at the calving and reaches the previous values mainly within the second part of lactation. Therefore, only inclusion of cows with high DIM into observation enabled to evaluate effect of higher BCS scores. In average number of lactations the cows of BCS2 and BCS4 groups were not different, whilst the cows of BCS3 grade had lower number of lactations, though this difference was not statistically significant. Most of cows in all BCS grades had number of lactations ranged from 1 to 3.

The data on embryo recovery divided according to categories of BCS described during the pre-flushing examination are shown in Table 2. The embryos ($n = 245$) collected from superovulated cows were at different developmental stages, starting from pre-compaction stages, through compact morula, up to blastocyst (transferable). Degenerated embryos with arrested development and unfertilized oocytes (UFO) were considered as poor-quality (non-transferable) embryos. Average embryo recovery rate (proportion of flushed embryos to counted corpora lutea, CL) was about 58%. Most flushed embryos were represented by the morula stage (51.02%) and to a lesser amount by blastocysts (15.51%). One-third of all flushed embryos (33.47%) were degenerated embryos or unfertilized oocytes. In the parameter 'the mean CL per cow' the highest value ($P < 0.05$) was recorded in the BCS4 cows compared with the lower BCS groups. However there were no differences among BCS groups in the mean number of embryos per cow. Flushed embryos were divided according to cows as belonging various BCS categories (see Table 2). Significantly ($P < 0.05$) the highest embryo recovery rate was obtained in the categories BCS 2 and 3, compared with the BCS4 category. However, according to the developmental stage, more transferable embryos were obtained from the BCS4 cows (79%), compared with BCS2 (64%) or BCS3 (63%).

From all recovered embryos ($n = 245$), 138 embryos were either processed for other analyses or frozen and put in storage and the remainder of the fertilized embryos, either good quality as well as fragmented/degenerated embryos ($n = 107$), were subjected to viability analyses in our study.

From the total number of the embryos analyzed for quality ($n = 107$), 27 embryos (25.23%) showed signs of cytoplasmic fragmentation (Fig. 1). Fragmented embryos had substantially lower numbers of cells than viable embryos at the higher developmental stages, as is documented in Table 3.

Further, some fragmented cells can be subjected to cell death, what is evidenced by increased TUNEL index in fragmented embryos (5.65%) when compared with normally developed morulas (1.76%; Table 3). However, early blastocysts and blastocysts showed TUNEL index levels comparable with those in fragmented embryos.

At the evaluation of actin cytoskeleton in our experiments, actin filaments were localized at the cell cortex and at the cell junctions of the embryo (Fig. 2).

The proportion of embryos with best quality of actin cytoskeleton (grade I) was in the range of 75% in morulas and up to 85% in all blastocyst stages, with the highest rate of grade I embryos in early blastocysts (Table 3). Only 19% of fragmented embryos showed the highest quality of actin cytoskeleton.

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Table 2 Presence of CL, yield and quality of flushed embryos in relation to body condition score (BCS) of dairy cows

BCS	No. Cows, *n*	CL counts, *n*	Mean CL per cow, $\chi \pm$ S.E.M.	Total flushed embryos, *n*	Mean embryos per cow, $\chi \pm$ S.E.M.	Embryo recovery rate, %	Transferable embryos		Non-transferable embryos and UFO, *n* (%)
							Morulas, *n* (%)	Blastocysts (EB, B), *n* (%)	
BCS 2 (1.75–2.25)	17	105	6.17 ± 0.78	56	3.29 ± 0.8	53.33[a]	24(42.86)	12 (21.43)	20 (35.71)
BCS 3 (2.5–3.25)	31	210	6.77 ± 0.87	141	4.55 ± 0.9	69.31[b]	69 (48.94)	20 (14.18)	52 (36.88)
BCS 4 (3.5–4.5)	11	108	9.82 ± 1.88	48	4.36 ± 1.32	44.44[a]	32 (66.67)	6 (12.5)	10 (20.83)
Total	59	423	7.17 ± 0.64	245	4.15 ± 0.58	57.92	125 (51.02)	38 (15.51)	82 (33.47)

B, blastocyst; CL, corpus luteum; EB, early blastocyst; UFO, unfertilized oocyte.
Embryo recovery rate: proportion of flushed embryos to counted CL.
[a,b]Values with different superscripts within columns differ significantly at $P < 0.05$ (chi-squared test).

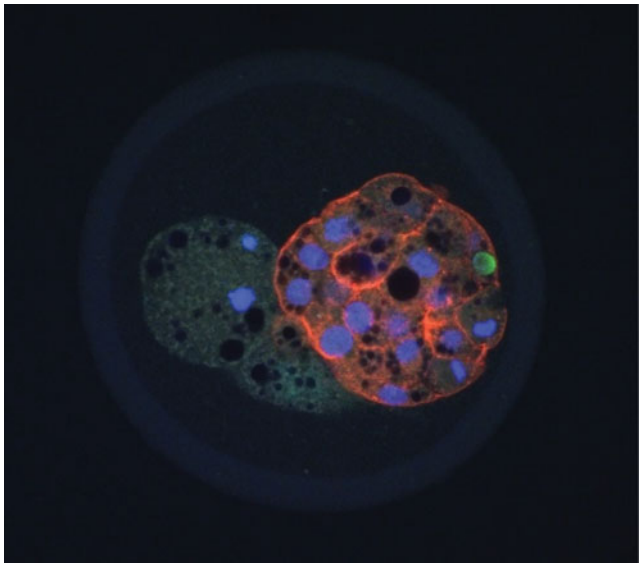

Figure 1 Bovine embryo with cytoplasmic fragments. Blue colour: cell nuclei (DAPI); red colour: actin filaments (phalloidine–TRITC); green colour: apoptotic nucleus (TUNEL–FITC); confocal microscopy, magnification ×320.

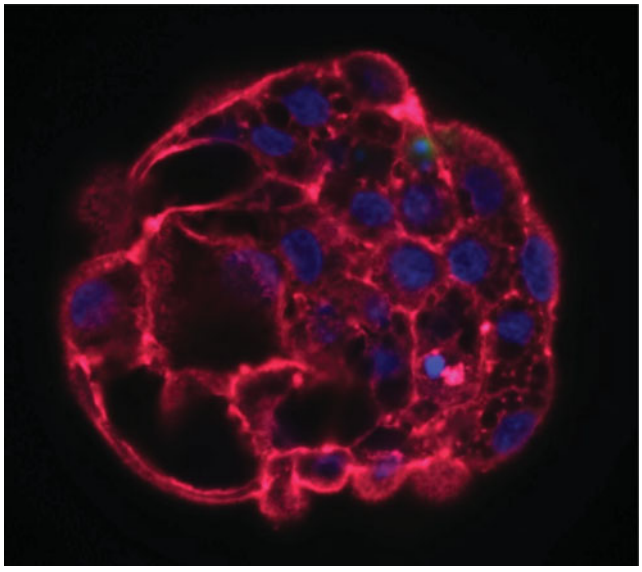

Figure 2 Actin filament localization in the bovine morula. Blue colour: cell nuclei (DAPI); red colour: actin filaments (phalloidine–TRITC); confocal microscopy, magnification ×640.

Distribution of embryos according to developmental stages within BCS groups is shown in Table 4. Morulas, blastocysts and fragmented/degenerated embryos were present in all BCS groups, but at different proportions. Generally, irrespective of BCS groups, most of embryos were represented by morulas (39.25%) followed by a lower proportion of fragmented embryos (25.23%), early blastocysts (19.63%) or blastocysts (15.9%). A higher incidence of fragmented embryos was noted in BCS2 (28.6%) and BCS4 (31.25%) groups, whilst the BCS3 group showed the lowest proportion of fragmented embryos (19.15%).

Detailed analysis of fragmented embryos showed that most of such embryos (75%) were represented

Table 3 Embryo ($n = 107$) quality according to the developmental stage (the mean ± standard error of the mean (SEM))

Quality parameter	Fragmented embryos ($n = 27$)	Morulas ($n = 42$)	Early blastocysts ($n = 21$)	Blastocysts ($n = 17$)
Total cell number	34.7 ± 4.9	51.65 ± 1.93	88.38 ± 2.73	108.08 ± 3.71
TUNEL+ cells/ embryo	2.23 ± 0.7	0.75 ± 0.23	4.46 ± 1.6	6.08 ± 1.47
TUNEL index[a], %	5.65 ± 1.9	1.76 ± 0.53	4.77 ± 1.67	5.59 ± 1.36
Actin grades I/II/III,[b] %	19/56/25	75/15/10	85/15/0	80/20/0

TUNEL+ cell: the number of TUNEL-positive cells per embryo; TUNEL index: the proportion of TUNEL-positive cells to the total cell number of the embryo.
[a]A higher TUNEL index is indicative of poorer embryo quality.
[b]Grade I actin indicates better cytoskeleton quality than grades II or III.

Table 4 Embryo ($n = 107$) distribution (n; %) according to developmental stage within body condition score (BCS) groups

Embryo stage	BCS2 ($n = 28$)	BCS3 ($n = 47$)	BCS4 ($n = 32$)
Morulas ($n = 42$)	6 (21.4)[a]	20 (42.55)[b]	16 (50.00)[b]
Early blastocysts ($n = 21$)	6 (21.4)[a]	11 (23.4)[a]	4 (12.50)[b]
Blastocysts ($n = 17$)	8 (28.6)[a]	7 (14.9)[b]	2 (6.25)[c]
Fragmented/degenerated ($n = 27$)	8 (28.6)[a]	9 (19.15)[b]	10 (31.25)[a]

a versus *b* versus *c*: differences in percentage values with different superscripts among BCS categories are statistically significant ($P < 0.05$; chi-squared test).

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Table 5 Influence of BCS on total and apoptotic cell number and proportion of apoptotic cells (the mean ± standard error of the mean (SEM))

Quality parameter	BCS2 ($n = 28$)	BCS3 ($n = 47$)	BCS4 ($n = 32$)
Total cell number	82.38 ± 7.3[a]	81.56 ± 5.43[a]	51.52 ± 5.95[b]
TUNEL+ cells/embryo	3.55 ± 0.56	2.56 ± 0.41	3.07 ± 0.44
TUNEL index,[c] %	3.88 ± 0.61[a]	4.10 ± 0.89[a,b]	6.56 ± 1.68[b]

a versus *b*: differences in values with different superscripts among BCS categories are statistically significant ($P < 0.05$; *t*-test).
[c]A higher TUNEL index is indicative of poorer embryo quality.
TUNEL+ cell: the number of TUNEL-positive cells per embryo; TUNEL index: the proportion of TUNEL-positive cells to the total cell number of the embryo.

by pre-compaction stages, having an average of 12–42 cells ($n = 20$), the rest of the embryos were represented by the 44–59 cell stage ($n = 6$) and one compact morula with a 85 cell nuclei. This fragmented compact morula occurred in the BCS2 group, and therefore the average cell number per fragmented embryo in this group (46.25) was higher (though insignificantly) than in the BCS 3 group (42.4). Average cell number in the fragmented embryos of the BCS4 group was almost twice lower (22.57) compared with either the BCS2 (46.25) or BCS3 (42.4) groups ($P < 0.05$).

Body condition of dairy cows may influence embryo quality, as is shown in Table 5. Average total cell number determined in the embryos of the same stages showed no differences between BCS2 and BCS3 cows, whilst significantly a lower cell number was recorded in the BCS4 group ($P < 0.05$). Number of dead (TUNEL-positive) cells per embryo was not statistically different among the groups, when ranged from 2.56 to 3.55 only. However, the TUNEL index was significantly ($P < 0.05$) different between the BCS2 (3.88%) and BCS4 (6.56%) embryos. In the BCS3 group, the TUNEL index has a middle value (4.10%) and was not different from both marginal BCS groups.

The proportion of embryos with the highest actin cytoskeleton quality (grade I) was twice as much in the BCS2 or BCS3 cows compared with the BCS4 group (Fig. 3). BCS4 group was also characterized by the highest occurrence of embryos with grade II actin quality compared with the lower BCS categories.

Discussion

The quality of cattle preimplantation embryos can have a significant impact on cow pregnancy rate, which directly affects the breeding economy. Data on bovine embryo quality in relation to the cow's

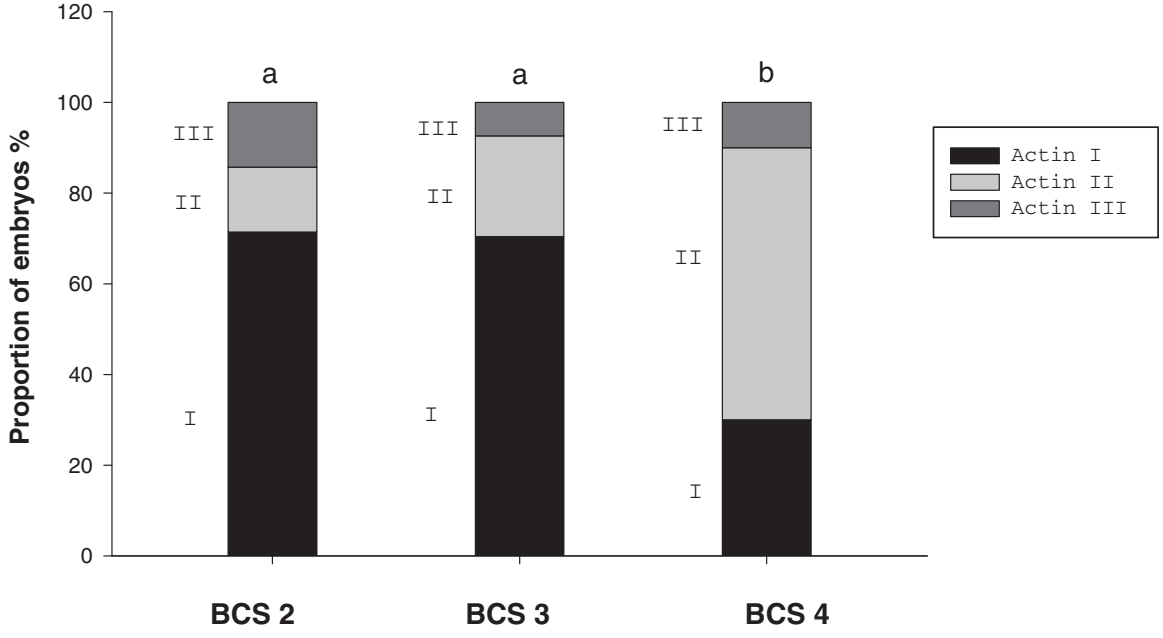

Figure 3 Distribution of embryos according to actin quality grade in relation to body condition score (BCS). a versus b: significant difference at $P < 0.05$ (chi-squared test).

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body condition have been reported in several studies (Snijders *et al.*, 2000, 2002; Starbuck *et al.*, 2004; Freret *et al.*, 2006; Roche, 2006; Kadokawa *et al.*, 2008; Bridges *et al.*, 2012). However, in these reports, the quality of embryos was estimated on the basis of visual inspection using such basic criteria as developmental stage (cleavage rate and blastocyst yield) and morphology of embryos (excellent, good, fair and poor). There are only two reports from the same research team in which functional parameters, such as embryo cell number (Adamiak *et al.*, 2005) and/or dead cell number (Adamiak *et al.*, 2006) in IVP embryos in relation to the cow's body condition, were evaluated. In our study we demonstrate the in-depth analysis of embryo quality including, in addition to the abovementioned characteristics, actin cytoskeleton status, which represents some novel aspects of this topic.

The cows involved in the embryo flushing experiment were kept at one farm under the same conditions, were fed the same components of feeding ration and were of the same breed (Holstein). However, these animals differed substantially in their BCS grouping in term of age and number of DIM with comparable parity. This fact indicated that a higher age at the first calving in cows of the BCS4 group corresponded with individual intensity of rearing. A higher age at the first calving can prolong the service period especially in the first lactation, however does not affect reproductive results during subsequent lactations (Krpálková *et al.*, 2014). Probably, differences in embryo quality parameters among BCS groups may be caused, at least in part, by differences in the age of the cows and in the length of the DIM period. Osoro & Wright (1992) determined non-significantly different conception rates in crossbreeds of Holstein cows up to 7 years of age; it is therefore possible to suppose that there are insignificant effects of age difference within the range 0.99–1.39 years. In particular, the BCS4 group was represented by the older cows and a substantially longer DIM period than the lower BCS groups. However, the average number of lactations did not differ among the BCS groups and most of the animals in all BCS categories were in the range of 1–3 lactations. Zavadilová & Štípková (2013) described differences in reproduction of Holstein primiparous cows in relation to various ages at their first calving. In our opinion, the parity effect in relation to more considerable differences in age could be more important, because it represents the interaction between age and physiological stress that appeared during milk production within individual lactations. However, as differences in parity were insignificant in our experiment, this effect may be considered as not important.

Our results indicated that cows of average BCS showed better embryo recovery rates than the cows with a higher BCS (3.5–4.5). This observation is in agreement with reports by Siddiqui *et al.* (2002) and Kadokawa *et al.* (2008). The cows with low or average BCS in our experiments differed from the higher BCS cows in their age and DIM period. Although parities did not differ, it cannot be rule out that these factors also may affect (beside BCS) the embryo recovery rate. Diskin & Morris (2008) summarized that energy balance and dry matter intake during 4 weeks after calving are critically important when cows are inseminated at 70–100 days post-calving. In our study, inseminations were performed at the time from 4.49 to 10.94 months after calving, i.e. in cows with a significantly higher DIM period. According to findings by Diskin & Morris (2008), the DIM period should not affect embryo yield and quality, because the first observed inseminations were performed at the end of the reproductively critical period, that is after the first 100 days of lactation (Stádník *et al.*, 2002; Beran *et al.*, 2013), and most inseminations were done ≥1 month after this lactation phase. The cows with a BCS from 1.75 to 4.5 were included in our study. In practice it is difficult to collect Holstein cows within the same phase of lactation belonging to such a wide range of BCS. Therefore, the mentioned differences in the length of DIM period may be necessary consequences according to the generally accepted course of BCS in dairy cows during lactation (Leroy *et al.*, 2008). However, it is important to emphasize that all observed cows were selected according to their previous non-problematic fertility as well as to the current ovarian activity documented by ultrasound examination before hormonal treatment. The high BCS group included non-pregnant cows designated for culling at the end of current lactation because of udder disorders, low milk yield, or undesirable body conformation. Therefore, Holstein cows with a BCS of 3.75 and more were available for treatment and evaluation. Based on the abovementioned fact, we can speculate that the length of the DIM period was not influenced by a combination of negative energy balance and low reproduction status, but represented the direct effect of BCS.

Several studies have documented that embryo recovery rate in superovulated cows can be influenced by level and type of feeding, or by nutritional management (Siddiqui *et al.*, 2002; Roche, 2006; Cerri *et al.*, 2009; Bridges *et al.*, 2012). However, in our study all the animals were fed at a level that corresponded to their current milk production, with the same feed ration composition and without feed restriction, therefore differences in embryo recovery rate probably were not affected by nutrition. Embryo recovery rate can be affected by many other factors, for example by a clogged catheter or an unexplained loss of the recovered fluid, thus restricting the number of

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flushed embryos. In our experiments we avoided these scenarios. The number of recovered embryos correlated ($r = 0.559$) with the number of corpus lutea revealed on the ovaries.

Using the criteria mentioned above we determined the proportion of normally developed embryos (transferable quality) or abnormally developed (fragmented, growth-arrested embryos or unfertilized oocytes (non-transferable quality) as well as the characteristics of more detailed embryo quality evaluations). The obtained embryos in our experiments were mostly at the morula or early blastocyst stages, whilst embryos at the advanced blastocyst stage occurred less frequently, because embryos were recovered on day 7 following the first insemination. When we evaluated the quality of collected embryos based on their developmental stage, a higher rate of transferable embryos was obtained from the BCS4 cows compared with BCS2 or BCS3 cows. However, such a relationship between the BCS and embryo quality was not valid when we took into account more detailed characteristics of embryo quality using viability markers. We applied invasive techniques for the evaluation of preimplantation embryos using such parameters as cell number, cell death incidence and actin cytoskeleton.

Total cell number in the embryo may characterize the developmental potential of the future fetus. Cell counts determined in our analyses for each developmental stage (Table 3) were largely in agreement with the commonly accepted ranges for cell number that were characteristic for morula, early blastocyst or advanced blastocyst stages of bovine embryo development. Fragmented embryos showed lower cell numbers compared with viable embryos. This finding means that cell fragmentation had taken place more often prior to compaction of the embryos.

Usually, the incidence of apoptotic/necrotic cells is elevated as development proceeds from the morula to the expanded (advanced) blastocyst stage (Matwee *et al.*, 2000). In agreement with this finding, the number of dead cells (TUNEL index) estimated from our observations was lowest in the morula stage embryos, and then gradually increased in early blastocysts and blastocysts. Higher proportions of best quality cytoskeleton in morulas as well as in all blastocyst stages confirmed that excellent cytoskeleton quality is a prerequisite for adequate embryo developmental dynamics.

In our experiments blastomere fragmentation was observed in each BCS group studied, but with different incidences; in the BCS4 group about one-third of embryos were fragmented, while the average body condition was less in the BCS3 group (Table 4). This observation confirms that embryo fragmentation may not only be an *in vitro* artefact, but could also occur in cattle embryos collected from the uterus of superovulated cows (Van Soom *et al.*, 2003). Abnormal cleavage starts at the first cell division. One of the most typical abnormal morphologies is embryo fragmentation (Van Soom & Boerjan, 2002). Cytoplasmic fragments can be formed due to asynchrony between the nuclear and the cytoplasmic cell cycles, as a result of a lack of karyokinesis, even though cytokinesis occurs normally (Wang *et al.*, 1999). Relatively low cell numbers in most fragmented embryos in our experiments (12–42) may suggest that these embryos were initially arrested at cleavage and then fragmented, so that they were incapable of further development. It has been found that cytoplasmic fragmentation is quite a common phenomenon in *in vitro* produced embryos, when the culture conditions or the culture media used for *in vitro* manipulations are suboptimal for normal development of human (Hardy, 1999); cattle (Van Soom *et al.*, 2003); pig (Hao *et al.*, 2003); mouse (Han *et al.*, 2005); and rabbit (Makarevich *et al.*, 2005) embryos. However, the reason for a high incidence of fragmentations in *in vivo* derived embryos in our study is unclear.

The incidence of apoptosis serves as a quality evaluation marker in preimplantation embryo development and was measured by a cell death index. The cell death (TUNEL) index is a function of total cell number and TUNEL-positive cell counts per embryo. The higher dead cell rate in the BCS4 embryos compared with the lower BCS groups, observed in our study, can be explained by fewer cell numbers in this BCS group. Generally, the incidence of dead cells increased with rise in BCS, and a maximal dead cell (TUNEL) index was noted in the BCS4 embryos. This rise in the TUNEL index can be explained by a high incidence of fragmented embryos in the BCS4 group.

There is no uniform opinion on interrelations between cytoplasmic fragmentation and apoptosis in embryos. Several researchers have claimed that both cytoplasmic fragmentation and apoptosis are interrelated mechanisms (Jurisicova *et al.*, 1996), which can be used by the embryo to eliminate undesirable or malformed cells (Hardy, 1999). Conversely, there is evidence that apoptosis and cell fragmentation are two independent processes (Xu *et al.*, 2001; Van Soom *et al.*, 2003). We did not investigate such relationships in this study, but our results showed that the incidence of fragmented embryos was associated with the occurrence of dead cells at a level comparable with the incidence of apoptosis characteristic for blastocyst stage embryos (Table 3). It is still unclear whether apoptosis and fragmentation are related processes. However, it is commonly accepted that apoptosis is a genetically programmed process, whilst cytoplasmic fragmentation is rather an embryonal response to an unfavourable environment.

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In this study we found that actin quality was significantly better in the average BCS and lower BCS groups compared with the BCS4 group. Cytoplasmic fragmentation and degeneration of embryos may be in direct association with the quality of the actin cytoskeleton. Therefore, this difference may refer to the higher incidence of fragmented or degenerated embryos in the BCS4 group, characterized by worse quality of the actin cytoskeleton; whilst in the BCS3 group the incidence of fragmented/degenerated embryos was the lowest of the groups. Actin is a major component of the cytoskeleton. Actin filaments are responsible for maintenance of the meiotic spindle, spindle rotation, polar body release, pronuclear migration, and mitotic cleavage (Maro *et al.*, 1986; Kim *et al.*, 1996; Kim *et al.*, 1997). During oocyte maturation, fertilization and embryo development, the polymerization and depolymerization of actin filaments are important processes (Wang *et al.*, 2000). Cleavage of the embryo may be affected by the distribution of actin filaments (Wang *et al.*, 1999), so that abnormal microfilament distribution results in abnormal cell function (Kim *et al.*, 1997). Therefore, we may assume that cows with a BCS higher than 3.25 may have an environment in the reproductive tract that is not favourable for proper actin filament formation. However, the exact relationship between actin cytoskeleton and cattle embryo development is still poorly understood.

In conclusion, the body condition of dairy cows affects the embryo recovery rate, occurrence of fragmented and/or degenerated embryos as well as the quality of *in vivo* recovered embryos. The elevation of BCS over an average value resulted in worse embryo quality. Our results suggest that these quality characteristics may be in direct association with the occurrence of fragmented and/or degenerated embryos, as cows in the BCS4 group had a lower proportion of blastocysts and a higher proportion of fragmented embryos relative to the BCS3 group cows.

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Statement of interest

None.

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