

Relationship of vascular perfusion of the wall of the preovulatory follicle to *in vitro* fertilisation and embryo development in heifers

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Abstract

The effect of the extent of vascular perfusion of the wall of the preovulatory follicle on *in vitro* cleavage rate of the recovered oocyte and embryo development to > 8 cells was studied in 52 heifers. Heifers received a luteolytic dose of prostaglandin F2 α (PGF2 α) when the largest follicle was ≥ 11 mm. An ovulation-inducing injection of GnRH was given 36 h later (hour 0), and collection of follicular fluid and the oocyte was done at hour 26. Vascular perfusion of the follicular wall was assessed by colour Doppler ultrasonography at hours 0 and 26. Each of the recovered oocytes (41/52; 79%) was mature (extruded polar body). Cleavage and embryo development were assessed at 48 h and 120 h respectively, after *in vitro* fertilisation (IVF). The percentage of cleaved oocytes and > 8 cell embryos was 80% (31/39) and 55% (17/31) respectively. Vascular perfusion of the follicular wall was greater (lower pulsatility index; $P < 0.001$) for follicles that produced cleaved versus non-cleaved oocytes and greater ($P < 0.04$) for follicles that produced > 8 cell versus ≤ 8 cell embryos. Percentage of follicular wall with Doppler signals of blood flow was greater ($P < 0.001$) for > 8 cell versus ≤ 8 cell embryos. Follicular-fluid concentration of free IGF1 was lower for cleaved oocytes ($P < 0.001$) and > 8 cell embryos ($P < 0.05$), and oestradiol was lower ($P < 0.05$) for > 8 cell embryos. Results supported the hypothesis that greater vascular perfusion of the wall of the preovulatory follicle was positively associated with IVF and embryo development.

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Introduction

The use of colour Doppler ultrasonography for research and clinical studies of ovarian and follicular haemodynamics is increasing in women (Fleischer 1991) and large farm animals (Ginther & Utt 2004, Miyamoto *et al.* 2006, Ginther 2007, Herzog & Bollwein 2007). In women, several studies have indicated that the blood flow in the wall of the preovulatory follicle is an indication of the potential success of assisted reproductive techniques. Preovulatory examination of colour Doppler signals of blood flow in the follicular wall (Bhal *et al.* 2001) and vascular resistance distal to an intraovarian artery by spectral Doppler indices (Du *et al.* 2006) are useful for assessing the quality of the oocyte. Bhal *et al.* (2001) reported that follicle vascularity is a factor in determining the success of pregnancy establishment after intrauterine insemination. Greater blood flow in the wall of the preovulatory follicle also has been associated with improved rates of *in vitro* fertilisation (IVF) and embryo development (Bhal *et al.* 1999, Coulam *et al.* 1999, Huey *et al.* 1999, Du *et al.* 2006, Shrestha *et al.* 2006). These studies have indicated that

colour Doppler ultrasonographic analysis of individual preovulatory follicles for collection of oocytes for IVF in women may favour selection of oocytes with better development and implantation potential of the embryos. In a few studies, a positive relationship was not found between follicular blood flow and pregnancy establishment after intrauterine insemination (Ragni *et al.* 2007) or after IVF-derived embryo transfer (Ng *et al.* 2006). However, these studies were complicated by variation in the infertility history of the women, the use of both conventional IVF and intracytoplasmic sperm injection, or the use of semen from different donors.

Follicular blood-flow assessment by colour Doppler ultrasonography has been used in mares after hCG treatment to study follicle vascularity in regard to maturity of the preovulatory follicle (Gastal *et al.* 2006) and the oocyte (Ginther *et al.* 2007), and oocyte quality in presence of circulating hCG antibodies (Siddiqui *et al.* 2009a). The vascularity tended to be greater for preovulatory follicles that contained a mature oocyte (Ginther *et al.* 2007). Mares that were negative for hCG antibodies had greater frequency of mature oocytes and greater vascularity of the follicular wall (Siddiqui *et al.*

2009a). Colour Doppler assessment of blood flow in the follicular wall in mares (Silva *et al.* 2006) and heifers (Siddiqui *et al.* 2009b) indicated that follicular blood flow was greater in the animals that became pregnant after insemination.

In summary, greater vascularity of the preovulatory follicle has been associated with greater follicular diameter (women: Bhal *et al.* 1999, mares: Silva *et al.* 2006, heifers: Siddiqui *et al.* 2009b), retrieval rate of oocytes (women: Bhal *et al.* 1999, mares: Ginther *et al.* 2007), retrieval rate of mature oocytes (mares: Ginther *et al.* 2007, Siddiqui *et al.* 2009a), fertilisation rate (women: Bhal *et al.* 1999, 2001) and pregnancy rate (women: Bhal *et al.* 1999, 2001, Coulam *et al.* 1999, Huey *et al.* 1999, Du *et al.* 2006, Shrestha *et al.* 2006, mares: Silva *et al.* 2006, heifers: Siddiqui *et al.* 2009b), and lower incidence of triploidy (women: Bhal *et al.* 2001). Studies on the relationship between blood-flow characteristics of the wall of the preovulatory follicle to oocyte retrieval and maturity have been done only in women and mares, and studies on fertilisation and embryo development of the oocyte after IVF have been done only in women.

In cattle, administration of GnRH initiates secretion of LH for 3–5 h (Thatcher *et al.* 1993), alters follicular development, and induces ovulation of the follicle (Thatcher *et al.* 1989, 1993, Macmillan & Thatcher 1991). The role of LH in the antral follicle involves stimulation of the formation of the androgen substrate by the thecal cells; the androgen is converted to oestrogen by granulosa cells (Carruthers 1986). The LH surge before ovulation is responsible for the resumption of meiosis in the oocyte, activation of enzymes for follicle rupture, and stimulation of steroidogenesis, particularly, production of progesterone by the granulosa cells (Albertini *et al.* 1993). Insulin-like growth factor (IGF)1 involves complex interactions among cumulus cells, gonadotrophins and steroids during oocyte maturation (Plancha & Albertini 1994, Webb *et al.* 2007). IGF1 stimulates oestrogen production by granulosa cells during oocyte maturation. In the preovulatory follicle of mares, concentration of free IGF1 in the follicular fluid was greater in association with

lower progesterone in follicular fluid, lower extent of blood flow in the follicular wall, less expansion of granulosa cells, and immaturity of the oocytes (Ginther *et al.* 2007). The preovulatory relationships of follicular-fluid oestradiol, progesterone, and free IGF1 and systemic LH, and oestradiol to competence of the oocyte for fertilisation and embryo development have not been reported in any species.

The present study tested the hypothesis of a positive relationship between the extent of *in vivo* vascular perfusion of the wall of the preovulatory follicle and the success of oocyte recovery and oocyte competence for IVF and embryo development in cattle. Greater vascular perfusion was based on a greater percentage of the follicular wall with colour Doppler signals of blood flow and lower spectral-Doppler pulsatility index (PI) and resistance index (RI) for a vessel of the wall (lower indices = greater perfusion). In addition, the effects of systemic LH and oestradiol and follicular-fluid factors on fertilisation and embryo development were considered.

Results

Oocyte and follicular fluid

The actual diameter of the follicle when prostaglandin F_{2α} (PGF_{2α}) was given (hour -36) was not different between groups for oocyte recovery versus non-recovery, cleavage versus non-cleavage, and embryo development to >8 cells versus ≤8 cells (combined diameter, 11.7 ± 0.1 mm; *n* = 61). Recovery of oocytes was attempted in 52 of 61 preovulatory follicles (one follicle per heifer). Nine follicles were removed from the experiment, owing to ovulation before hour 26 (hour of follicle aspiration; *n* = 6) and rupture of the follicle during the aspiration procedure (*n* = 3). An oocyte was recovered in 79% (41/52) of the follicles (Table 1). Differences that were significant (main effects and the interaction) or approached significance for the analyses of follicular diameter, percentage of the circumference of the follicular wall with blood-flow signals, and spectral Doppler indices (PI and RI) are presented (Fig. 1). For

Table 1 Relationship of granulosa cells and concentrations of follicular-fluid hormones to oocyte recovery, oocyte cleavage and embryo development 26 h after GnRH treatment when the preovulatory follicle was ≥11 mm.

Characteristics	Oocyte recovery		Oocyte cleavage		Embryo development	
	Non-recovered	Recovered	Non-cleaved	Cleaved	≤8 cells	>8 cells
Number of follicles	11 (21%)	41 (79%)	8 (21%)	31 (79%)	14 (45%)	17 (55%)
Granulosa cells						
Amount (score, 1–3)	1.7 ± 0.2 ^a	2.4 ± 0.1 ^b	2.4 ± 0.2	2.4 ± 0.4	2.4 ± 0.1	2.4 ± 0.1
Expansion (score, 1–3)	2.2 ± 0.2 ^a	2.8 ± 0.1 ^b	2.9 ± 0.1	2.8 ± 0.3	2.9 ± 0.1	2.7 ± 0.1
Follicular-fluid hormones						
Oestradiol (ng/ml)	65.6 ± 12.7	61.2 ± 3.1	55.3 ± 3.9	60.8 ± 20.2	67.1 ± 5.4 ^a	54.1 ± 4.7 ^b
Progesterone (ng/ml)	255 ± 57	203 ± 17	228 ± 69	197 ± 76	179 ± 18	217 ± 22
Free IGF1 (ng/ml)	11.3 ± 3.8 ^a	6.9 ± 0.9 ^b	13.6 ± 3.0 ^a	5.0 ± 0.6 ^b	5.8 ± 0.7 ^a	4.0 ± 0.8 ^b

^{a,b}Means with different letters within paired groups for each end point are different (*P* < 0.05 to *P* < 0.001).

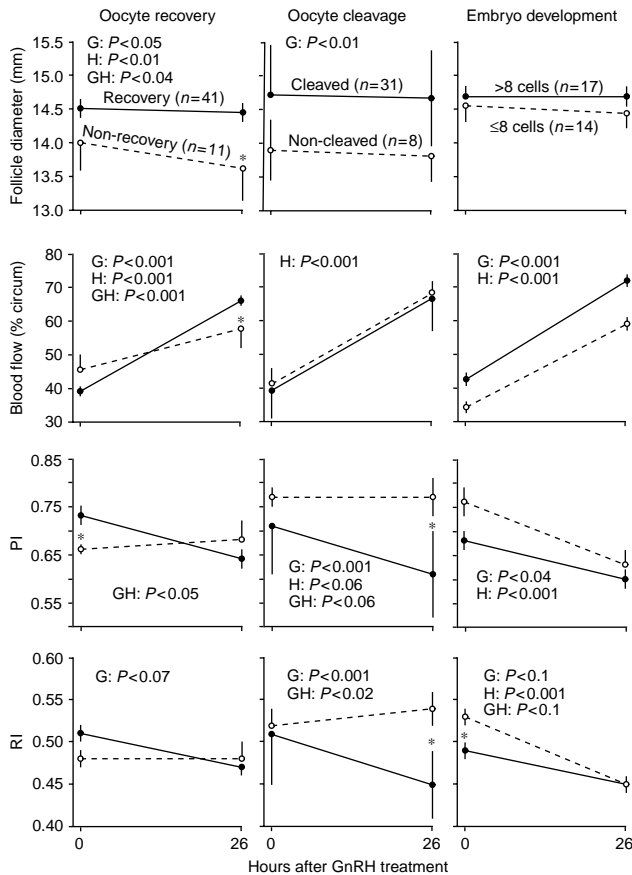


Figure 1 Mean (\pm S.E.M.) for end points of the preovulatory follicle associated with oocyte recovery, oocyte cleavage and embryo development at hour 0 (GnRH treatment) and hour 26 (oocyte recovery procedure). Blood flow = percentage of follicle circumference with colour Doppler signals of blood flow, PI, pulsatility index and RI, resistance index. The indices (PI and RI) are inversely related to the extent of tissue vascular perfusion downstream from the point of assessment at the most prominent colour spot in the follicle wall. Probabilities for significant main effects (G = group; H = hour) and the interaction (GH) are shown. An asterisk (*) indicates a statistical difference at hours 0 or 26 when a significant or approaching significant group-by-hour interaction was obtained.

oocyte recovery, both main effects (group and hour) and the interaction were significant for follicular diameter and percentage of follicular wall with blood-flow colour Doppler signals. The interactions primarily resulted from a greater ($P < 0.05$) diameter and greater ($P < 0.05$) percentage of follicular wall with blood-flow signals at hour 26 for the follicular group with oocyte recovery than with non-recovery. An interaction for PI resulted from a greater ($P < 0.04$) decrease in the index between hours 0 and 26 for follicles with oocyte recovery than with non-recovery. The difference between recovery and non-recovery approached significance for RI. No significant or approaching significant differences were found for blood velocity measurements (PSV, peak systolic velocity; EDV, end diastolic velocity; TAMV, time-averaged maximum velocity; data not shown).

At hour 26, the scores for amount and expansion of granulosa cells were greater for follicular fluid associated with oocyte recovery than with non-recovery ($P < 0.05$; Table 1). Follicular-fluid concentration of free IGF1, but not oestradiol and progesterone, was less ($P < 0.001$) for follicles with oocyte recovery than with non-recovery.

Oocyte cleavage

A polar body was detected in each of the recovered oocytes ($n = 41$). The zona of two oocytes was broken during processing, and the oocytes were not used for the fertilisation procedure. The remaining 39 oocytes were inseminated, and cleavage occurred in 31 (79%; Table 1). The results of the statistical analyses of follicular diameter, percentage of the circumference of follicular wall with blood-flow colour Doppler signals, and spectral Doppler indices (PI and RI) are shown for the non-cleaved and cleaved groups (Fig. 1). Greater follicular diameter was detected in the cleaved group, averaged over both hours (group effect), than in the non-cleaved group. An increase in the percentage of the follicular wall with blood flow signals was obtained between hours 0 and 26 (hour effect), averaged over the two groups. Significant group effects were obtained for PI and RI, owing to lower indices in the cleaved group averaged over both hours. In addition, the group-by-hour interaction for PI approached significance and for RI was significant. The interactions represented a greater ($P < 0.001$) follicular wall vascular perfusion (lower indices) at hour 26 in the cleaved group than in the non-cleaved group. No significant difference was found for PSV. The EDV and TAMV were greater (group effects, $P < 0.002$ and $P < 0.02$ respectively) for the cleaved group (4.8 ± 0.1 and 6.7 ± 0.1 cm/s) than for the non-cleaved group (4.0 ± 0.3 and 6.0 ± 0.3 cm/s). The scores for amount and expansion of granulosa cells and concentrations of oestradiol and progesterone in the follicular fluid were not different between non-cleaved and cleaved groups (Table 1). Free IGF1 concentration in follicular fluid was less ($P < 0.001$) in the cleaved group than in the non-cleaved group.

Embryo development

The percentage of embryos with ≤ 8 cells and > 8 cells was 45% and 55% respectively (Table 1). The results of the analyses of follicular diameter, percentage of the follicular wall with blood-flow colour Doppler signals and spectral Doppler indices (PI and RI) are presented (Fig. 1). No significant main effects or a group-by-hour interaction were obtained for follicular diameter between the two groups of embryos. The percentage of follicular wall with blood-flow signals was greater in the follicles that produced > 8 cell embryos averaged over hours 0 and 26 (group effect) than for ≤ 8 cell embryos.

The percentage of wall with blood-flow signals increased between hours 0 and 26 averaged over the two groups (hour effect); there was no interaction of group-by-hour. Vascular perfusion in the follicular wall increased between hours 0 and 26 (hour effect) as indicated by a decrease in PI and RI. The group effect was significant for PI and approached significance for RI, owing to greater vascular perfusion (lower indices) for the >8 cell embryos, averaged over hours 0 and 26. No significant main effects or interactions were obtained for PSV and TAMV. The main effect of hour was significant for EDV ($P < 0.01$), owing to greater velocity at hour 26 (4.9 ± 0.2 cm/s) than at hour 0 (4.4 ± 0.1 cm/s). The scores for the amount and expansion of the granulosa cells of the preovulatory follicle were not different between the two groups of embryos (Table 1). The concentration of follicular-fluid oestradiol and free IGF1 was less in the group of embryos with >8 cells than with ≤ 8 cells. The concentration of follicular-fluid progesterone was not different between the groups.

Plasma LH and oestradiol

Plasma concentrations of LH and oestradiol decreased between hours 0 and 26 (significant main effect of hour) for each set of comparisons (oocyte recovery versus non-recovery, oocyte cleavage versus non-cleavage, embryo development to ≤ 8 cells versus >8 cells). However, there was no significant or approaching significant group effect for any set of oocyte/embryo comparisons and no interaction of group-by-hour (data not shown).

Discussion

The results supported the hypothesis that greater vascular perfusion of the preovulatory follicle was associated with greater success for each of the oocyte/embryo sets for oocyte recovery, oocyte cleavage and embryo development. The hypothesis was supported statistically for each set by a group effect averaged over hour 0 (hour of GnRH treatment) and hour 26 (hour of oocyte recovery) and/or by an interaction between group and hour. The interactions were attributable to greater vascular perfusion at either hour 0 or hour 26 in association with greater success for oocyte recovery and cleavage and embryo development. The positive effect of the extent of follicle vascularity on recovery of the oocyte was indicated by a greater increase in percentage of the follicular wall with colour Doppler signals of blood flow and by a decrease in spectral indices (decreased indices = increased vascular perfusion) between hours 0 and 26 in the follicles with oocyte recovery than with non-recovery. The greater vascularity of the follicular wall in association with oocyte recovery is a novel finding in cattle and agrees with findings in

women (Bhal *et al.* 2001, Du *et al.* 2006, Shrestha *et al.* 2006) and the tendency for a similar finding in mares (Ginther *et al.* 2007).

Preovulatory follicular vascular involvement in the subsequent *in vitro* cleavage of the recovered oocytes was indicated by the lower resistance indices (greater increase in vascular perfusion) at hour 26 than hour 0 (interaction) for a vessel of the follicular wall in the cleavage group. However, the percentage of blood-flow signals in the wall increased similarly between hours 0 and 26 in the cleaved and non-cleaved groups (no interaction). The reason for this apparent contradiction is unknown. Further support for greater vascular involvement in the wall of follicles that produced fertilisable oocytes, as indicated by successful cleavage, was the greater blood velocity (EDV and TAMV) for a vessel in the follicular wall in the cleavage group for both hours of assessment. The positive effect of the extent of vascular perfusion in the preovulatory follicle on the production of cleaved oocytes that developed beyond the eight-cell stage was indicated by greater percentage of blood-flow signals and greater vascular perfusion at both hours 0 and 26 in the >8 cell group. These findings indicated that the extent of vascularity of the preovulatory follicle had a positive carry-over effect on the ability of the oocyte to become fertilised and for the cleaved oocyte to develop beyond the eight-cell stage. These are novel findings in cattle. Development of cattle embryos beyond the eight-cell stage apparently is dependent on the inherent ability of the oocyte for genome activation (Memili & First 2000). The present cleavage and embryo development results in heifers are compatible with a report in women that a greater frequency of pregnancy occurred after embryo transfer when the embryo was produced from an oocyte that was derived from a follicle with high-grade vascularity (Coulam *et al.* 1999).

Both greater percentage of blood-flow signals in the wall of the follicles and the decreased PI and RI in a vessel in the wall of the follicle indicate increased blood circulation (Ginther 2007) and presumably involved an increase in number and diameter of the arterioles that form a network encasing the follicle (Hunter 2003). The mechanisms for the effect of follicular vascularity on oocyte competence for fertilisation and embryo development are not known. It has been suggested that the competence of an oocyte for maturation, fertilisation and embryo viability after fertilisation mainly depends on the follicle (Moor *et al.* 1998, Webb *et al.* 2007). Greater blood flow in the follicle is related to the greater dissolved oxygen in the follicular fluid (Van Blerkom *et al.* 1997, Monteleone *et al.* 2008). The vascular system carries nutrients and oxygen to the follicle. Mitochondria play a vital role in the oocyte in providing ATP for activation at the time of fertilisation and for preimplantation embryo development (Dumollard *et al.* 2004). Speculatively, the association between reduced blood

flow in the follicles and lower cleavage rate of the oocyte and less development of the embryo in the present study may have reflected reduced mitochondrial oxidative phosphorylation in the oocytes for ATP production. Further study is needed.

The similarity (no significant difference) in follicular diameter at hour 36 (hour of PGF treatment), and the significantly smaller diameter in the non-recovery and non-cleaved groups at hour 0 (GnRH treatment) implies that the follicle grew at a slower rate after PGF treatment in the non-recovery and non-cleaved groups. The smaller preovulatory follicle at the time of GnRH treatment suggests that the ovulation-inducing treatment was given when the follicle was less developed. The association between greater follicular diameter and oocyte cleavage in the present study is in agreement with the previous reports of an association between greater follicular diameter and establishment of pregnancy in mares (Silva *et al.* 2006) and heifers (Perry *et al.* 2005, Siddiqui *et al.* 2009b). Although follicular diameter was not considered in the hypothesis, greater diameter was positively related to greater oocyte recovery and cleavage but not to embryo development beyond 8 cells. Greater follicular diameter would dictate greater follicular-fluid volume, which may have contributed to the greater oocyte-recovery rate. In addition, greater expansion of the granulosa cells and greater amount of granulosa cells also may have facilitated oocyte recovery. The association between less granulosa expansion and fewer granulosa cells in the non-recovery group also suggested that these follicles may have contained a high incidence of immature oocytes at the time of aspiration. The positive relationship between oocyte recovery and follicular diameter in the present study is consistent with a previous report in women (Bhal *et al.* 2001), but inconsistent with findings in mares (Ginther *et al.* 2007). In mares, non-recovery of the oocytes was associated with follicles of greater diameter and was attributed to greater immaturity of the follicle, as indicated by follicle echotexture and less expansion of granulosa cells.

In regard to the smaller follicular diameter in association with non-cleavage, the proportion of heifers that would have had a third follicular wave is not known. If the proportion was greater in the non-cleaved group than in the cleaved group, more follicles in the non-cleaved group would have been in the static phase at ≥ 11 mm and would account for the subsequent slower mean growth rate and greater incidence of non-cleaved oocytes. Although this possibility cannot be discounted, the following observations are compatible with viability of the follicles in the non-cleaved group: 1) all individual follicles increased in diameter on the day before the follicle was ≥ 11 mm and between ≥ 11 mm and GnRH treatment, 2) there was no difference in follicular-fluid concentrations of oestradiol between the cleaved and non-cleaved groups on the day of aspiration, 3) all

recovered oocytes were mature regardless of whether or not cleavage occurred and 4) the incidence of three-wave oestrous cycles before the experimental oestrous cycle was low (10%).

The greater follicular-fluid concentration of free IGF1 in the group with non-recovered oocytes and greater compactness of the granulosa cells are consistent with immaturity of the follicles. Greater concentrations of free IGF1 in the non-cleaved and the ≤ 8 cell-embryo groups indicated on a temporal basis that greater free IGF1 concentration in the follicular fluid at hour 26 was associated with less competence of oocytes for cleavage and embryo development. These results agree with findings in women, wherein lower concentration of free IGF1 in the follicular fluid was associated with better embryo development (Wang *et al.* 2006). There was a temporal negative association at hour 26 between lower follicular-fluid concentration of free IGF1 and greater vascularity of the follicular wall. However, the role of IGF1 in follicle vascularisation apparently is unknown. These *in vivo* results for free IGF1 are inconsistent with a report (Oropeza *et al.* 2004) that intraovarian application of IGF1 improves the oocyte maturation rate, fertilisation rate, and extent of embryo development in cows. The reason for this apparent difference is unknown but indicates a need for further study.

In the previous studies in cattle (Acosta *et al.* 2003), rats (Varga *et al.* 1985), rabbits (Janson 1975) and sheep (Niswender *et al.* 1976), the role of oestradiol and LH on blood flow of the follicle wall was not well established. An increase of blood-flow velocity (TAMV) in the vessel of the follicular wall occurred 0.5 h after GnRH treatment, synchronously with an increase in systemic concentrations of oestradiol and LH (Acosta *et al.* 2003); follicular blood flow remained high, while systemic concentrations of oestradiol and LH returned to basal level by 6 h and remained low until ovulation. Further study is required with frequent blood sampling and Doppler ultrasonographic scanning for a better understanding of the relationship of vascular perfusion in the preovulatory follicle and plasma oestradiol and LH concentrations between GnRH treatment and ovulation. The effect of follicular-fluid oestradiol or follicular vascular perfusion apparently is not known.

In conclusion, the study considered the effects of characteristics of the preovulatory follicle on oocyte retrieval and cleavage and on embryo development to > 8 cells. Diameter of the follicle was greater for the oocyte-recovery group than for the non-recovery group and for the cleaved group than for non-cleaved group but was not associated with embryo development beyond eight cells. Based on Doppler ultrasonographic characteristics of the follicle, greater vascular perfusion of the follicular wall was associated with a greater rate of oocyte recovery and with a higher incidence of *in vitro* oocyte cleavage and embryo development of the retrieved oocytes. Lower concentrations of free

IGF1 in follicular fluid were associated with a higher rate of oocyte recovery and cleavage and embryo development beyond eight cells.

Materials and Methods

Animals

Animals were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research. Holstein heifers ($n=52$) between 15–20 months of age and in good body condition were used. The heifers had regular oestrous cycles and no apparent abnormalities of the reproductive tract as determined by ultrasound examination (Ginther 1998). Ultrasound monitoring of the preovulatory follicle and the treatment procedures have been described (Siddiqui *et al.* 2009b). Briefly, the heifers were scanned daily by grey-scale (B-mode) ultrasonography beginning 12 days after ovulation until a ≥ 11 mm follicle of the second follicular wave was identified (mean, 15.0 ± 0.3 days postovulation). Heifers received a luteolytic dose of PGF (25 mg per heifer, i.m.; dinoprost trimethamine, Lutalyse; Pfizer Animal Health, New York City, NY, USA) when the follicular diameter was ≥ 11 mm (hour -36). The PGF was given to assure that luteolysis occurred in all heifers before the GnRH treatment. The heifers were treated 36 h later (hour 0) with GnRH (100 μ g per heifer, i.m.; gonadorelin diacetate tetrahydrate, Ovacyst; Phoenix Scientific Inc., St. Joseph, MO, USA). Follicular diameter, follicular wall blood flow and blood samples for hormone assays were collected just before GnRH treatment (hour 0) and just before the oocyte-recovery procedure (hour 26). Oocyte recovery was attempted and follicular fluid was collected at hour 26.

Ultrasonography

Heifers were sedated with a sub-dose of xylazine hydrochloride (20 mg per animal, i.m.; Xyla-Ject; Phoenix Pharmaceuticals Inc.) to minimise animal movement, as described for Doppler ultrasonic imaging (Araujo & Ginther 2009). A duplex B-mode (grey-scale) and pulsed-wave colour Doppler ultrasound instrument (Aloka SSD-3500; Aloka America, Wallingford, CT, USA) equipped with a linear array 7.5 MHz transducer (UST-5821-7.5) was used for transrectal scanning. For the grey-scale mode, the brightness and contrast controls of the monitor and the gain controls of the scanner were standardised to constant settings (Gastal *et al.* 2006). The procedure for Doppler ultrasonography of the preovulatory follicle of heifers has been described (Siddiqui *et al.* 2009b). Briefly, follicular diameter was obtained from an average of height and width of the antrum at the apparent maximal area from two frozen grey-scale images. The power-Doppler mode was used to display blood-flow signals from the vessels of the follicular wall. Percentage of follicular wall with colour Doppler signals was estimated while the entire follicle was being scanned in a slow continuous motion several times as described (Ginther 2007) at hour 0 (hour of GnRH treatment) and hour 26 (hour of follicle aspiration). The term 'circumference' refers to the periphery of a three-dimensional follicle. Colour Doppler images of relatively low and high vascularisation of preovulatory follicles are shown (Fig. 2A and D). The procedure for estimating the percentage of blood-flow signals has been used and validated by subsequent pixel analysis in the corpus luteum and follicles of heifers (Ginther *et al.* 2007, Araujo & Ginther 2009). A comparable subjective procedure has been used for the preovulatory follicle of women (Chui

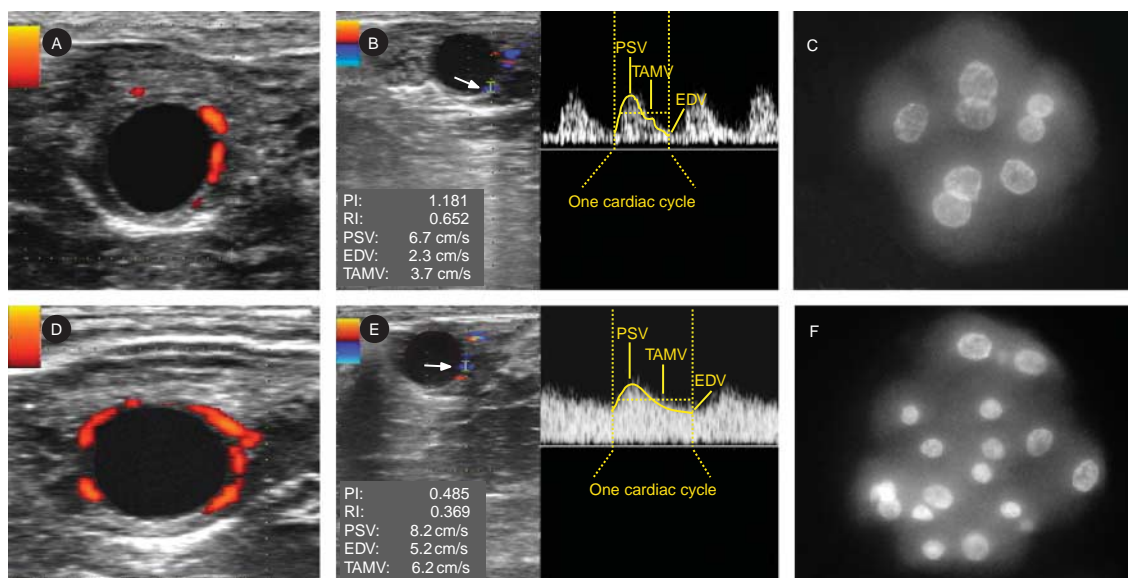


Figure 2 Representative Doppler ultrasonographic images of preovulatory follicles and embryos. The embryos were produced by IVF from the corresponding (left to right) follicle-derived oocytes. Power-Doppler ultrasonography displayed low (A) and high (D) percentage of the follicle wall with blood-flow signals at hour 26. The sample gate (white arrow) was placed on a colour spot in the wall of the preovulatory follicle to generate Doppler spectral graphs with low (B) and high (E) blood-flow velocities. The generated data in the lower-left corner of images B and E indicate the pulsatility index (PI), resistance index (RI), peak systolic velocity (PSV), end diastolic velocity (EDV) and time-averaged maximum velocity (TAMV). Nuclei of an embryo with eight cells (C) and > 8 cells (F) that were produced from the oocyte of follicles A and D respectively, are shown. The embryos were stained with DNA fluorochrome to visualise the nuclei of the blastomeres 120 h after the oocyte was exposed to spermatozoa.

et al. 1997, Miyazaki *et al.* 1998, Bhal *et al.* 2001), mares (Gastal *et al.* 2006, Silva *et al.* 2006, Ginther *et al.* 2007) and heifers (Siddiqui *et al.* 2009b).

For spectral Doppler assessment, the sample cursor or gate (width = 1 mm) was set on the most prominent colour signal in the follicular wall (Fig. 2B and E). The angle of the ultrasound beam in relation to the direction of blood flow in the vessel (insonation angle) was unknown, and therefore, relative velocities for group comparisons, rather than actual velocities, were obtained (Ginther 2007). Doppler spectrums of three cardiac cycles were generated and one of the cycles was used for spectral measurements. The mean value of two measurements for each end point was used in the statistical analyses. The velocity end points from spectral Doppler ultrasonography of a vessel in the wall of the preovulatory follicle were PSV, EDV and TAMV. The end points for blood perfusion of the follicle wall were PI and RI and were computed from the velocity measurements by the scanner (Zwiebel & Pellerito 2005). Doppler measurement techniques and other aspects of blood velocities and perfusion indices have been described previously (Zwiebel & Pellerito 2005, Ginther 2007).

Recovery of oocyte and follicular fluid

Oocyte recovery and follicular fluid collection were done at hour 26 by transvaginal ultrasound-guided aspiration of follicular contents, using an 18-gauge single-lumen needle (length 55 cm) and vacuum pump. The procedures for preparing the heifer, transrectal ovary positioning, transvaginal transducer placement and inserting the needle have been described previously (Ginther 1998). The aspirated follicular fluid and the flushing fluid (TL HEPES; Bioniche Animal Health Inc., Pullman, WA, USA) were held separately in two 10 ml silicon coated sterile tubes (BD Vacutainer, cat # 366430; Betcon Dickinson, Franklin Lakes, NJ, USA) in a 37 °C water bath until microscopic examination within 15 min after completion of the recovery procedure. The follicular fluid was searched under a stereomicroscope for an oocyte or cumulus oocyte complex (COC). If the oocyte or COC was not found in the follicular fluid, the flushing fluid was searched. After the search was completed, the follicular fluid was centrifuged (500 g, 10 min) and 200–500 µl was stored at –20 °C until assayed.

The granulosa cells were scored from 1 to 3 (minimal to maximal) for amount of cells and for the extent of expansion as described (Ginther *et al.* 2007). Extent of cell expansion was estimated as follows: score 1, tight or compact; score 2, mixture of compact and expanded; score 3, expanded with fluffy appearance. Characteristics of the preovulatory follicle and hormone concentrations were compared between oocyte non-recovery and oocyte recovery groups.

IVF and embryo development

The identified oocyte or COCs were washed three times in TL HEPES and then treated with hyaluronidase (0.05%) for 1 min to remove the cumulus. Each oocyte was fertilised and studied for embryo development in separate fertilisation drops to maintain oocyte identity as described previously (Fukui *et al.* 2000).

Briefly, drops (10 µl) of fertilisation medium were made in a 35 mm Petri dish, covered with mineral oil, and equilibrated for 4 h at 39 °C in an environment of 5% CO₂ in humidified air of a CO₂ incubator (HERAcell 150; Kendro Laboratory Products GmbH, Langenselbold, Germany). The fertilisation medium was IVF-TL solution (EmbryoMax; Chemicon International, Phillipsburg, NJ, USA) and was supplemented with fatty acid free BSA (6 mg/ml), pyruvate (0.2 mM), penicillamine (20 mM), hypotaurine (10 mM), epinephrine (1 mM), and gentamicin (50 µg/ml). Semen used for fertilisation was from a single bull with proven fertility. Spermatozoa were separated from post-thawed semen by the swim-up procedure and capacitated by heparin (2 µg/ml; Parrish *et al.* 1986). After washing, a cumulus-free oocyte was transferred into a fertilisation drop. The oocyte was fertilised by inseminating 10 × 10³ spermatozoa into each fertilisation drop. After allowing 18 h for fertilisation, the oocyte or presumptive zygote was transferred into a drop of development medium. The development medium was Synthetic Oviductal fluid (SOF, EmbryoMax; Chemicon International) and was supplemented with fatty acid free BSA (8 mg/ml), pyruvate (0.2 mM), BME (essential amino acids, 50 ×, 10 µl/ml), MEM (non-essential amino acids, 100 ×, 20 µl/ml), and gentamicin (50 µg/ml) as described previously (Parrish *et al.* 1995). The oocyte was examined for indications of cleavage under a stereomicroscope 48 h after exposure to spermatozoa. Absence of cleavage after 48 h was used to define the oocyte as non-cleaved and presumably included non-fertilisation and polyspermy. Characteristics of the preovulatory follicle and hormone concentrations were compared between the non-cleaved and cleaved groups.

The embryo was evaluated 120 h after exposure of the oocyte to spermatozoa by staining with DNA fluorochrome (Hoechst 33342, 10 µg/ml in PBS) and counting the nuclei (Fig. 2C and F) of the blastomeres under an epifluorescence microscope. The embryos were grouped as ≤ 8 cells and > 8 cells, based on *in vitro* blockage of bovine embryos at the eight-cell stage (Memili & First 2000). Blood flow and hormone concentrations of the preovulatory follicle were compared between the ≤ 8 cell and > 8 cell-embryo groups.

Follicular-fluid and plasma hormone assays

Blood samples were collected into heparinised tubes just before GnRH treatment (hour 0) and follicle aspiration (hour 26). Plasma concentration of oestradiol was determined by using a commercially available RIA kit (double antibody oestradiol; Diagnostic Products Corporation, Los Angeles, CA, USA) as described for heifers (Ginther *et al.* 2000), except for the following modifications. Standards (0.78–100 pg/ml) were prepared by serial dilution in steroid-free (charcoal extracted) bovine plasma. The standards and plasma samples (500 µl) in duplicate were extracted simultaneously with 3 ml of diethyl ether before assay. The ether was allowed to evaporate overnight, and the residue was dissolved in 100 µl of RIA assay buffer (PBS with 0.1% gelatin). The kit-supplied primary antibody (30 µl) and I¹²⁵ labelled oestradiol (75 µl) were added sequentially, vortexed, and incubated at room temperature for 2 h and 1 h respectively. The cold precipitating

solution containing second antibody (1 ml) was added to each assay tube, vortexed, and incubated for 20 min at room temperature. The tubes were centrifuged at 1700 g for 30 min and were decanted and counted in a γ -counter. Serial volumes of a pool of bovine oestrus plasma (500–200 μ l) were processed in the assay along with the experimental samples and resulted in a displacement curve that was similar to the standard curve. Plasma LH concentration was measured by RIA as described and validated in our laboratory (Adams *et al.* 1992), except that USDA-bLH-B-6 (National Hormone and Pituitary Program, Torrance, CA, USA) was used for iodination and reference standard, and USDA-309-684P was used as the primary antiserum. The intra-assay coefficient of variation (CV) and sensitivity for oestradiol and LH were 7.4% and 0.2 pg/ml and 10.37% and 0.10 ng/ml respectively. The concentrations of oestradiol, progesterone and free IGF1 in the follicular-fluid samples were assayed by radio- or enzyme-immunoassays, as described and validated in our laboratory (Beg *et al.* 2001). The intra-assay CV and sensitivity for follicular-fluid oestradiol, were 5.99% and 2.0 pg/ml respectively. For progesterone and free IGF1 in the follicular fluid, the intra- and inter-assay CV and sensitivity were 2.5%, 11.06%, and 0.05 ng/ml and 2.6%, 13.5%, and 0.01 ng/ml respectively.

Statistical analyses

Blood-flow measurements and plasma hormone concentrations during pre-ovulation were compared within each of the three sets of oocyte/embryo groups (non-recovered versus recovered groups, non-cleaved versus cleaved groups, and ≤ 8 cell versus > 8 cell groups). Quantitative data were analysed by SAS MIXED PROCEDURE to determine the main effects of group and hour and their interactions, using a repeated statement to account for the autocorrelation between measurements (version 9.1.3; SAS Institute Inc., Cary, NC, USA). Quantitative end points for hour 26 were analysed between groups and within each set by unpaired Student's *t*-tests. A probability of $P \leq 0.05$ indicated that a difference was significant and a probability between $P > 0.05$ and < 0.1 indicated that significance was approached. Data are presented as the mean \pm S.E.M., unless otherwise indicated.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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