The effect of paracrine/autocrine interactions on the *in vitro* culture of bovine preimplantation embryos

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Abstract

Bovine preimplantation embryos develop more successfully when cultured in groups, probably because of the increased production of, and exposure to, embryotrophic autocrine and paracrine factors. Using a novel embryo culture technique, this study had two aims: 1. to determine the distance over which potential paracrine interactions affect bovine embryo development in terms of blastocyst and hatching rates, cell counts and carbohydrate metabolism; 2. to investigate the effect of platelet-activating factor (PAF) supplementation on bovine embryo development and metabolism. Groups of 16 presumptive zygotes were attached to the bottom of a culture dish by the cell adhesive Cell-Tak in a 4 × 4 equidistant array. The distance between individual embryos in each group was 0–689 µm. Optimal blastocyst formation rate occurred when embryos were cultured 165 µm apart compared with control non-attached zygotes (Kruskal–Wallis followed by Mann–Whitney U test post-hoc; $P < 0.05$). Increasing the distance between embryos resulted in a further decline in blastocyst rate, which reached zero at 540 µm apart. Blastocyst cell number, pyruvate/glucose uptake and lactate production decreased as the interembryo distance increased from 240 to 465 µm ($P < 0.05$). Supplementation with PAF during conventional group culture enhanced blastocyst cell number, hatching rates and the oxidative metabolism of pyruvate and glucose. The data indicate that the distance between individual bovine embryos in culture influences preimplantation development, in particular blastocyst formation, cell number and metabolism. It is suggested that diffusible paracrine/autocrine factors, such as PAF, are in part responsible for the regulation of early embryo development.

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Introduction

The preimplantation mammalian embryo is relatively autonomous and can regulate cell division and differentiation without being in contact with the maternal reproductive tract (Schultz & Heyner 1993). *In vivo*, it is exposed to numerous factors, absent *in vitro*, which mediate maternal-embryonic dialogue (Paria & Dey 1990, Hill 2001) and could partly be responsible for the impaired *in vitro* development and viability of *in vitro* cultured preimplantation embryos (Harlow & Quinn 1982). This manifests as chromosomal abnormalities (Jamieson *et al.* 1994, Munné *et al.* 1994), inadequate oocyte nuclear and cytoplasmic maturation (Moor *et al.* 1998), altered gene expression (Wrenzycki *et al.* 1998, 2001, Niemann & Wrenzycki 2000), increased levels of apoptosis (Brison & Schultz 1997) and metabolic perturbation (Bavister 1995, Gardner & Lane 1996).

Embryo culture media do not reflect the composition of the fluids found in the maternal tract, which contain

a variety of putative mediators such that the embryo may reside in a local 'conditioned' environment (Lane & Gardner 1992). While a number of studies have attempted to establish the nutrient profile of the female reproductive tract, little is known about the nature and abundance of putative mediators (Orsi *et al.* 2005). The culture of zygotes individually in large volumes results in inferior development to the blastocyst stage and a reduced cell number compared with those cultured in groups (Paria & Dey 1990, Lane & Gardner 1992, O'Neill 1997). O'Neill (1997) suggested that the critical factor in promoting murine zygote development is the number of embryos rather than the volume of the drop. Increasing embryo density also stimulates the rate of compaction (Stoddart *et al.* 1996), cavitation (Wiley *et al.* 1986, Paria & Dey 1990), zona hatching, inner cell mass (ICM) and trophoctoderm (TE) cell number (Stoddart *et al.* 1996), and implantation rate in the mouse (Lane & Gardner 1992). A similar pattern is seen in the bovine embryo, where group culture increases blastocyst formation, cell number and the

production of interferon-tau (O'Doherty *et al.* 1997, Larson & Kubisch 1999, Khurana & Niemann 2000).

Growth factor supplementation can compensate for the adverse effects of culture in large volumes and thus enhance embryo development *in vitro* (Paria & Dey 1990, O'Neill 1997, 1998), and a number of studies have attempted to identify putative embryo-derived factors that act via paracrine or autocrine pathways. For example, addition of insulin-like growth factor (IGF)-I, IGF-II, platelet activating factor (PAF) (O'Neill 1997) or platelet-derived growth factor (PDGF) (Thibodeaux *et al.* 1993) to bovine and murine embryos cultured in decreasing embryo densities counteracted such dilution effects, ultimately leading to an increase in development. It was suggested that these embryo-derived factors acted as 'survival' factors limiting apoptosis rather than classical growth factors (O'Neill 1991, 1998).

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a low-molecular-weight ether-phospholipid which mediates a wide range of biological functions (reviewed by Hanahan 1986). It is produced by preimplantation embryos in a number of species: man (Collier *et al.* 1988), sheep (Battye *et al.* 1991), mouse (O'Neill 1985) and rabbit (Minhas *et al.* 1993). PAF is also produced by the uterine epithelium and is present in rabbit uterine fluid (Angle *et al.* 1988). It can act in an autocrine and/or paracrine manner to improve murine embryo development (Stoddart *et al.* 1996, O'Neil 1997), especially at low embryo culture densities (O'Neill 1998). Supplementation of culture medium with the biologically active C16 isoform of PAF (Stoddart *et al.* 2001) increases implantation rate (Ryan *et al.* 1990a), placental mass (Ryan *et al.* 1990a) and the viability of embryos for uterine transfer (O'Neill *et al.* 1989). Furthermore, PAF can increase mitosis (Roberts *et al.* 1993) and reduce apoptosis (O'Neill 1998), blastocyst cell number (Stoddart *et al.* 1996, O'Neill 1997) and the oxidative metabolism of glucose and lactate (Ryan *et al.* 1989, 1990b). Despite the clear effects of PAF on the embryos of a number of mammalian species, its potential as an embryotrophic factor for bovine embryos remains to be determined.

In this study, we have examined over what distance potential paracrine interactions affect bovine embryo development in terms of blastocyst and hatching rates, cell counts and metabolism. In order to undertake these experiments, a novel technique was developed to enable embryos to be fixed in a grid layout within a culture drop (Gopichandran 2004). We have also examined the effect of supplementing culture medium with PAF on bovine embryo development and metabolism and assessed its ability to enhance the development of embryos cultured large distances apart.

Materials and Methods

In vitro production of bovine embryos

All chemicals were purchased from Sigma unless otherwise stated. *In vitro* produced (IVP) embryos were derived as previously described (Gopichandran & Leese 2003). Cumulus-oocyte complexes (COCs) were aspirated from abattoir-derived ovarian follicles 3–10 mm in diameter into Hepes-buffered tissue culture medium (TCM)-199 supplemented with 50 µg/ml kanamycin monosulphate, 2 µg heparin/ml and 1.5 mg BSA/ml. COCs with an evenly shaded cytoplasm and several layers of granulosa cells were selected and transferred to TCM-199 supplemented with 10% fetal bovine serum, 0.025 IU follicle-stimulating hormone/luteinising hormone (FSH/LH) (Ferring Pharmaceuticals, Langley, UK), 0.47 µg epidermal growth factor/ml (long EGF) and 10.9 ng fibroblast growth factor/ml (bovine FGF) and incubated for 24 h at 39 °C under a humidified atmosphere of 5% CO₂ in air. After maturation, OCCs were washed in Hepes-buffered Tyrode's albumin-lactate-pyruvate (Hepes-TALP) and fertilization-TALP (Fert-TALP) (Lu *et al.* 1987) supplemented with 10 µg heparin/ml, 600 µg penicillamine/ml and 220 µg hypotaurine/ml.

Spermatozoa were prepared from a frozen-thawed semen sample with discontinuous Percoll (Pharmacia Biotech, St Albans, UK) gradient (45:90) and centrifuged at 2100 *g* for 25 min and 10 min at 1500 *g* after resuspension of the viable sperm pellet in Hepes-TALP. The sperm suspension was adjusted to give a final concentration of 1×10^6 spermatozoa/ml and added to each well of a four-well plate which contained 45–65 oocytes previously washed in Hepes-TALP and Fert-TALP. The plates were incubated at 39 °C under a humidified atmosphere of 5% CO₂ in air. After 18 h, presumptive zygotes were denuded by vortexing for 2 min in Hepes-buffered synthetic oviductal fluid (SOF) supplemented with minimum essential and non-essential amino acids (Gibco, Life Technologies, Paisley, Scotland), 1 mmol glutamine/l and 4 mg BSA/ml (Hepes-SOFaaBSA) (Tervit *et al.* 1972).

Preparation of Cell-Tak grids

A working stock of Cell-Tak (VWR International, Leicestershire, UK) was prepared by adding 1 M NaHCO₃ to an equal volume of Cell-Tak stock solution (1.45 mg in 5% acetic acid/ml). A 1 µl drop of working stock was placed in the centre of a dish, spread over a 1 mm² area, dried and washed according to the manufacturer's instructions. Once treated, the area was covered with 20 µl BSA-free SOFaa. This was used in preference to SOFaaBSA, as serum proteins can block the adhesive sites and thus reduce the attachment of the embryo to the surface. Zygotes were randomly pooled into groups of 16 and washed in BSA-free SOFaa. The first embryo was placed on the Cell-Tak grid and the second added a measured distance apart with the aid of an eyepiece graticule, calibrated with a stage micrometer. The remaining embryos

were placed on the Cell-Tak to form a 4×4 equidistant grid. The distance between individual zygotes in different dishes was 0–689 μm ($n = 12$ for each of the 10 different ranges used) (Fig. 1). The SOFaa surrounding the embryos was removed and replaced with 20 μl SOFaaBSA. Control drops containing 16 embryos cultured in the absence of Cell-Tak were placed in separate dishes at the same time. All drops were cultured continuously at 39°C in a humidified atmosphere of 5% CO_2 , 5% O_2 and 90% N_2 . Blastocyst and hatching rates were measured on days 7 and 8. Although a day-8 bovine blastocyst has only half the viability of a day-7 one (Hasler *et al.* 1995), continuing the culture to day 8 enabled hatching rates to be included.

Culture of embryos in PAF

A 1.909 mmol stock of PAF/I (C16 isoform) (Stoddart *et al.* 2001) was prepared in ethanol and diluted in SOFaaBSA to give a range of concentrations (0.5–20 μM). Controls were also prepared in which the corresponding volumes of ethanol were added to SOFaaBSA (0.026–1.04% (v/v) ethanol). Putative zygotes were cultured in groups of 16 in 20 μl culture drops of SOFaaBSA $\pm$ PAF or ethanol as described above ($n = 10$ per concentration). Blastocyst and hatching rates were assessed on days 7 and 8.

Addition of PAF to bovine embryos incubated at distances over 390 μm apart

Sixteen zygotes were cultured on Cell-Tak in a 4×4 grid array at distances greater than 390 μm apart. This point was selected, as blastocyst rates were significantly lower there than at the optimal distance apart (165 μm) or when in direct contact (Fig. 1). Embryos were cultured in SOFaaBSA $\pm$ 10 or 20 μM PAF ($n = 8$ for each concentration). These concentrations of PAF were selected because previous experiments suggested that they increased embryo development significantly, in terms of hatching rate and blastocyst cell number (Figs 4 and 5). Embryos were cultured as described above.

Figure 1 Day-8 blastocyst rate for bovine zygotes cultured at increasing distances apart. Values with different letters are significantly different ($P < 0.05$). Control values are for embryos grown in conventional group culture.

Incubation of blastocysts for carbohydrate profiling (pyruvate and glucose depletion; lactate appearance)

Single expanding to expanded blastocysts were either gently removed from their position on the Cell-Tak grid ($n = 21$ per distance) or removed from culture in the presence of PAF ($n = 12$ per concentration), transferred to 135 nl drops of SOFaaBSA and cultured for 45 min under a mineral oil overlay in a humidified atmosphere of 5% CO_2 in air at 39°C. Control droplets were incubated alongside the embryo-containing drops to account for any non-specific loss of nutrients.

Blastocyst cell counts

After incubation, the zona pellucida was removed from each blastocyst with 0.5% pronase (Van Soom *et al.* 2001). Blastocysts were transferred to bisbenzimidazole dissolved in ethanol (50 $\mu\text{g}/\text{ml}$) for 1 h, washed in 100% ethanol and mounted singly in 1 μl drops of glycerol on siliconised slides. The nuclei in each blastocyst were counted three times under a Vickers microscope.

Ultramicrofluorometric assays

The concentrations of pyruvate, lactate and glucose in incubation droplets were measured non-invasively by ultramicrofluorometric techniques (Leese & Barton 1984, Gardner & Leese 1990). The changes in fluorescence were quantified with a Leca Fluovort microscope with photomultiplier and photometer attachments.

Statistical analysis

The normality of all data was assessed by the Anderson–Darling test. Cell counts and arcsine-log transformed blastocyst and hatching rates were distributed non-parametrically and were therefore tested by the Kruskal–Wallis test followed by the Mann–Whitney U test post hoc. Significant differences in metabolic profiles (glucose, lactate and pyruvate) were tested by one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) test post hoc, as the data were normally distributed. All data were presented as means $\pm$ S.E.M. Blastocyst rates were expressed as percentage of putative zygotes cultured, while the consumption or production of glucose, pyruvate and lactate was expressed as pmol/embryo per h.

Results

Effect of culture distance between individual embryos on development and metabolism

Zygote to blastocyst rates on day 8 were significantly affected by the distance between adjacent embryos (Fig. 1). There was a significant increase in embryo development from direct contact to 165 μm apart ($P < 0.05$), at which distance blastocyst rate was maximal. Increases in

adjacent embryo distance over the range 165–540 μm resulted in a progressive decline in blastocyst rate to zero. The position of each blastocyst within Cell-Tak grids at a distance of 165 μm was examined and the number of adjacent embryos recorded (Fig. 2). Embryos with five neighbours appeared to have a higher blastocyst rate than those with three or eight neighbours, although this was significantly higher only between five and eight neighbours. Increasing the distance between individual embryos in culture from direct contact to 165 μm increased blastocyst formation but did not significantly affect blastocyst cell number (Figs 1 and 3). At the expanded blastocyst stage, there was a significant fall in cell number as the distance apart was increased from 165 to 240 μm , although this did not remain significant at higher distances.

Increasing the culture distance between individual bovine embryos significantly affected blastocyst metabolism (Table 1). At distances greater than 165 μm , there was a trend toward a decrease in glucose and pyruvate uptake, while lactate production was maximal over the range 90–240 μm and then decreased ($P < 0.05$). However, when the data were expressed on a per cell basis, any significant differences in blastocyst metabolism were lost.

The effect of PAF on development and metabolism

Day-7 blastocyst rates were significantly reduced at all concentrations of PAF (Table 2). However, this trend was not seen in day-8 blastocyst rates, where there were no significant differences between controls and PAF-supplemented drops. Day-8 hatching rates were significantly higher in the presence of 10 μM PAF, and blastocyst cell number was significantly increased by supplementation with PAF concentrations of 1 μM PAF and above (Fig. 4). At the expanded blastocyst stage, cell number was significantly increased at concentrations of PAF above 5 μM . Culture in the presence of corresponding volumes of ethanol did not affect blastocyst rates (data not shown), indicating that changes in blastocyst and hatching rates were attributable to PAF.

Figure 2 The effect of position in a 4 × 4 Cell-Tak grid on subsequent development to the blastocyst stage. Different letters indicate significant differences between distances apart ($P < 0.003$).

The uptakes of pyruvate and glucose by day-8 blastocysts were significantly increased by 10 μM PAF, while no significant differences were seen in lactate production (Table 3). When the results were expressed on a per cell basis, there was a significant increase in pyruvate uptake, but not in glucose uptake and lactate production at concentrations of $\geq 10 \mu\text{M}$ PAF. The culture of embryos at adjacent embryo distances of $\geq 390 \mu\text{m}$ apart in the presence of 10 μM PAF significantly increased blastocyst rates compared with controls (Fig. 5). However, in the presence of 20 μM PAF, there were no significant differences in blastocyst rates.

Discussion

Growth factors, either maternal or embryo-derived, have frequently been used as culture medium supplements in an attempt to improve 'suboptimal' rates of embryo development (Paria & Dey 1990, Morita *et al.* 1994, O'Neill 1998). Addition of growth factors results in enhanced blastocyst and hatching rates, cell number, metabolic activity and viability. Other strategies have relied on culturing early embryos in small volumes or in groups in order to maximise their exposure to putative embryotrophic factors (Lane & Gardner 1992, Vajta *et al.* 2000, Thouas *et al.* 2003). However, the mechanism(s) by which these agents operate remain(s) unclear. To the best of our knowledge, this is the first study to address the question of the distance over which such putative factors promote embryo development.

The effect of increasing adjacent embryo distance on development of bovine zygotes to the blastocyst stage

Our data show that both the number of adjacent zygotes and the distance between them influenced development to the blastocyst stage. In particular, there appears to be a distance, $\sim 165 \mu\text{m}$, at which blastocyst formation is highest *in vitro*. Below this distance, blastocyst rates declined, possibly due to competition for energy substrates such as pyruvate, glucose and amino acids and/or the build-up of metabolic by-products such as ammonium and lactate. Local alterations in physicochemical culture conditions (e.g. oxygen tension, pH, amino-acid profile) and paracrine mediators (e.g. nitric oxide) may also account for this reduction in development. These effects may be particularly important when embryos are in direct contact. By contrast, it seems likely that the presence of these compounds in the immediate surroundings of embryos cultured $\geq 240 \mu\text{m}$ apart would not play such a significant part in influencing embryo development, which seems, instead, to be influenced by a decrease in the availability of putative embryotrophic growth factors.

This proposition is supported by a number of observations. Limitations in pyruvate, glucose and albumin availability have all been shown to affect preimplantation development adversely (Pemble & Kaye 1986, Chi *et al.*

Figure 3 Effect of increasing the distance between individual zygotes on subsequent blastocyst cell number. Different letters indicate significant differences between distances apart ($P < 0.05$).

2002, Orsi & Leese 2004a). Similarly, ammonium resulting from amino acid breakdown, and lactate derived from glycolysis, can impair development (Lane & Gardner 1994, Orsi & Leese 2004b). Exposure of embryos to ammonium can depress oxidative phosphorylation (Lane & Gardner 2003), decrease blastocyst cell number (Gardner & Lane 1993) and affect fetal development (Lane & Gardner 1994, Sinclair *et al.* 1998). In addition, oxygen tension, pH and amino-acid profile have all been shown to influence blastocyst rate in a variety of species (Lane & Bavister 1999, Baltz 2001). Early embryos are vulnerable to oxidative stress (Orsi & Leese 2001) and have poor homeostatic regulation of acid/base balance until after compaction (Lane 2001).

The number of neighbours influenced the proportion of individual embryos developing to the blastocyst stage at the optimal distance apart, 165 μm. Intriguingly, better development was obtained when embryos had five neighbours, although this increase was significant only when compared with eight adjacent embryos. The present pattern in blastocyst rates parallels that of other studies in

which decreasing embryo density in a specific volume leads to retarded development (Lane & Gardner 1992, O'Doherty *et al.* 1997, O'Neill 1997, Khurana & Niemann 2000). These data suggest that embryo development *in vitro* is likely to be a cooperative phenomenon (Paria & Dey 1990, O'Neill 1997). As might be expected, the highest blastocyst cell numbers were recorded at an adjacent embryo distance of 165 μm; that is, the distance apart at which blastocyst rate was maximal. However, despite their marked effect on blastocyst rate, reducing the distances apart had little effect on the cell count of embryos which reached the blastocyst stage.

The effect of increasing adjacent embryo distance on metabolism

The carbohydrate metabolism of conventional group cultured bovine blastocysts is consistent with that of other studies (Gopichandran & Leese 2003). Increasing the distance between the embryos decreased metabolic activity, in terms of in glucose and pyruvate consumption and lactate production. The concentration of embryo-derived

Table 1 The effect of increasing the distance between individual zygotes on carbohydrate metabolism in subsequent day-8 blastocysts. Different superscript letters indicate significant differences between distances apart ($P < 0.05$).

Distance between embryos (μm)	Day-8 blastocyst (pmol/embryo/h)			Day-8 blastocyst (pmol/cell/h)		
	Pyruvate	Glucose	Lactate	Pyruvate	Glucose	Lactate
Control	8.30 ± 0.93 ^a	22.48 ± 1.76 ^a	39.15 ± 4.22 ^a	0.062 ± 0.007	0.170 ± 0.014	0.290 ± 0.013
0	8.08 ± 1.21 ^a	20.15 ± 2.97 ^a	27.79 ± 6.72 ^b	0.058 ± 0.008	0.144 ± 0.020	0.197 ± 0.021
15	6.27 ± 0.93 ^{ab}	17.81 ± 1.55 ^{ab}	37.38 ± 7.83 ^{ab}	0.047 ± 0.007	0.133 ± 0.011	0.258 ± 0.010
90	5.85 ± 0.94 ^b	20.90 ± 1.37 ^a	45.66 ± 7.45 ^a	0.042 ± 0.006	0.151 ± 0.012	0.319 ± 0.009
165	7.37 ± 0.52 ^{ab}	18.23 ± 1.69 ^{ab}	45.14 ± 7.54 ^a	0.054 ± 0.005	0.133 ± 0.013	0.309 ± 0.011
240	4.52 ± 0.84 ^c	15.94 ± 2.30 ^b	48.13 ± 7.99 ^a	0.036 ± 0.006	0.139 ± 0.024	0.404 ± 0.019
315	5.15 ± 0.78 ^{bc}	15.92 ± 0.74 ^b	39.55 ± 3.59 ^a	0.041 ± 0.004	0.130 ± 0.014	0.376 ± 0.007
390	4.05 ± 0.33 ^c	15.40 ± 0.50 ^b	29.37 ± 5.60 ^b	0.031 ± 0.002	0.120 ± 0.004	0.234 ± 0.003
465	3.50 ± 0.89 ^c	14.55 ± 1.57 ^b	31.23 ± 2.32 ^b	0.027 ± 0.007	0.116 ± 0.013	0.272 ± 0.013

Table 2 Effect of increasing concentrations of PAF on development of bovine zygotes to the blastocyst stage (days 7 and 8) in conventional group culture. Different superscript letters indicate significant differences between concentrations of PAF ($P < 0.001$).

PAF concentration (μM)	Blastocyst rate (%)		
	Day 7	Day 8	Day 8 (hatched)
0	17.8 $\pm$ 3.64 ^a	27.8 $\pm$ 4.65	4.4 $\pm$ 1.76 ^a
0.5	5.6 $\pm$ 1.76 ^b	23.3 $\pm$ 4.41	4.4 $\pm$ 1.76 ^a
1.0	5.6 $\pm$ 1.76 ^b	23.3 $\pm$ 4.71	7.8 $\pm$ 1.47 ^a
5.0	6.7 $\pm$ 1.67 ^b	18.9 $\pm$ 2.61	5.6 $\pm$ 1.76 ^a
10.0	7.8 $\pm$ 2.78 ^b	21.1 $\pm$ 4.55	16.7 $\pm$ 4.08 ^b
20.0	0.0 $\pm$ 0.0 ^b	21.1 $\pm$ 3.51	2.2 $\pm$ 1.47 ^a

growth factors may decrease over increasing distances, resulting in a concomitant depression in metabolic activity. For example, exogenous PAF and embryo-derived PAF – in addition to their effects in reducing cell death (O'Neill 1998) and increasing mitosis (Roberts *et al.* 1993) and blastocyst cell number (Stoddart *et al.* 1996, O'Neill 1997) – increase the oxidative metabolism of glucose and lactate in the mouse (Ryan *et al.* 1989, 1990b). Expressing metabolism on a per cell basis revealed no significant effect of adjacent embryo distance. Thus, the decrease in metabolism noted with increasing distance was most likely a consequence of decreased cell number.

These findings highlight the potential applications of this novel culture technique we developed in supporting the development of small numbers of embryos, such as those encountered during routine ovum pick-up cycles. However, we would caution against the use of this technique to culture human embryos conceived by IVF until any potential effects of the constituents of Cell-Tak have been rigorously assessed.

The effect of PAF on development and metabolism

Exposure of early cattle embryos to PAF led to a significant depression in blastocyst rate on day 7, suggesting an adverse effect on preimplantation development, in contradiction to the report that exogenous PAF increases murine embryo development in a concentration-dependent manner (Stoddart *et al.* 2001). However, the depression in cavitation rate in the bovine embryo may occur as a result of prolonged compaction (Van Soom *et al.* 1997). The duration of this morphogenetic event, which is abnormally abbreviated in bovine embryos *in vitro*, may have increased as a result of exposure to PAF. This is supported by the following observations: 1. day-8 blastocyst rates were comparable across groups; 2. hatching rate was enhanced by 10 μM PAF; 3. blastocyst cell numbers were also increased (see below). PAF-free controls containing equivalent volumes of ethanol (0.026–1.04% (v/v)) did not affect blastocyst rates on days 7 and 8, confirming that the enhanced rates were due to PAF alone, and not the presence of ethanol. It has been proposed that increasing embryo density and PAF concentration, or reducing culture volume, promotes hatching of mouse blastocysts (Stoddart *et al.* 1996, Teruel & Smith 1997, Thouas *et al.* 2003). This could be the result of an accumulation of the secretory proteolytic enzymes that mediate zona thinning (e.g. strypsin), which may act in a paracrine manner on neighbouring embryos (Perona & Wassarman 1986, Lee *et al.* 1997, Mishra & Seshagiri 1998). It is tempting to speculate that the increase in hatching associated with PAF supplementation results from such enhanced enzyme production.

An increase in blastocyst and expanded blastocyst cell number was seen at PAF concentrations above 1 and 5 μM respectively, with no further increases in cell number seen at the higher concentrations of PAF. This agrees with the finding of Roudebush *et al.* (1996) that high

Figure 4 Effect of increasing the concentrations of PAF on development of bovine zygotes to the blastocyst and expanded blastocyst stages in conventional group culture ($P < 0.001$). Different letters indicate significant differences.

Table 3 Glucose and pyruvate consumption and lactate production by day-8 blastocysts cultured in conventional groups from the zygote stage in medium supplemented with PAF (glucose $P < 0.05$; pyruvate $P < 0.001$). Different superscript letters indicate significant differences between PAF concentrations. *Conversion of glucose to lactate.

PAF (μM)	Day-8 blastocysts (pmol/embryo/h)				Day-8 blastocysts (pmol/cell/h)			
	Pyruvate	Glucose	Lactate	Glycolytic index (%)*	Pyruvate	Glucose	Lactate	Glycolytic index (%)*
0	9.86 $\pm$ 1.46 ^a	21.00 $\pm$ 2.78 ^a	42.00 $\pm$ 6.70	100.0 $\pm$ 8.2 ^a	0.126 $\pm$ 0.018 ^a	0.270 $\pm$ 0.049	0.541 $\pm$ 0.088	98.8 $\pm$ 11.1 ^a
0.5	10.32 $\pm$ 1.42 ^a	20.99 $\pm$ 1.36 ^a	41.63 $\pm$ 5.77	104.1 $\pm$ 7.0 ^a	0.124 $\pm$ 0.015 ^a	0.254 $\pm$ 0.015	0.492 $\pm$ 0.055	103.2 $\pm$ 5.4 ^a
1	12.48 $\pm$ 1.25 ^a	19.82 $\pm$ 2.11 ^a	41.63 $\pm$ 5.77	95.2 $\pm$ 7.3 ^a	0.134 $\pm$ 0.012 ^a	0.214 $\pm$ 0.024	0.449 $\pm$ 0.064	95.3 $\pm$ 7.5 ^a
5	13.25 $\pm$ 1.21 ^a	24.42 $\pm$ 1.43 ^a	40.50 $\pm$ 2.87	120.5 $\pm$ 9.9 ^b	0.135 $\pm$ 0.012 ^a	0.249 $\pm$ 0.013	0.413 $\pm$ 0.027	120.5 $\pm$ 9.6 ^b
10	18.03 $\pm$ 1.90 ^b	28.69 $\pm$ 2.02 ^b	44.72 $\pm$ 2.92	128.3 $\pm$ 13.8 ^b	0.185 $\pm$ 0.018 ^{ab}	0.294 $\pm$ 0.023	0.458 $\pm$ 0.027	128.3 $\pm$ 17.0 ^b
20	20.72 $\pm$ 1.71 ^b	29.05 $\pm$ 2.34 ^b	46.43 $\pm$ 3.54	125.1 $\pm$ 13.2 ^b	0.213 $\pm$ 0.019 ^b	0.299 $\pm$ 0.026	0.476 $\pm$ 0.037	125.6 $\pm$ 14.0 ^b

concentrations of PAF do not enhance murine embryo development. It is possible that this is a consequence of PAF receptor expression; Roudebush *et al.* (2002) found a decrease in murine receptor expression during preimplantation development, suggesting that elevated PAF concentrations are likely to saturate functional receptors. In addition, Stojanov and O'Neill (1999) reported a reduction in PAF-receptor mRNA expression after IVF, and suggested that this would lead to a deficiency of this particular autocrine signal, thereby accounting for the limited benefits of exogenous PAF supplementation.

Continuous culture of bovine embryos throughout preimplantation development in the presence of SOFaaBSA supplemented with 10–20 μM PAF led to a significant increase in the utilisation of pyruvate and glucose but did not alter lactate production. These observations suggest an

enhancement in oxidative metabolism, as reported by Ryan *et al.* (1989, 1990b), who found a similar dose-dependent enhancement of oxidative metabolism by murine blastocysts cultured in the presence of PAF, suggesting that PAF may induce the embryo to favour more energy-efficient pathways. Furthermore, the data suggest that 10 μM PAF can, to some extent, 'rescue' embryos cultured $\geq 390 \mu\text{m}$ apart. The increase in developmental potential observed in the presence of PAF may occur by increasing embryotrophic factor secretion, the cumulative concentrations of which were insufficient to enhance embryo development.

In conclusion, we have shown that varying the distance between individual bovine zygotes in culture influences their development, in terms of blastocyst formation, cell number and metabolism. Embryo development is optimal at a distance 165 μm apart, strongly suggesting a role for diffusible paracrine/autocrine factors. Moreover, it has been suggested that the 'helper' effect in embryo culture, notably in the mouse, whereby one embryo seems to help another, is a function of their 'preferred' growth environment as a polytocous species. The demonstration that similar relationships occur in the normally monotocous cow provides evidence of a more pervasive role for the autocrine stimulation of bovine embryo development.

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Figure 5 Effect of (a) 10 μM and (b) 20 μM PAF on the development of individual zygotes cultured to the blastocyst stage at varying distances apart. *Significant differences between controls ($P < 0.001$).

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