Effect of different concentrations of potassium on pig embryos developing *in vitro*

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The effects of varying the potassium concentration in Whitten's medium on the development of pig embryos *in vitro* was investigated. Osmolality was maintained by adjusting the NaCl concentration. In Expt 1, 209 one-cell to early blastocyst stage embryos were individually cultured in 50 µl microdrops of glucose-free Whitten's medium containing 0.4% (w/v) BSA and 1.5, 3.0, 6.0 or 12.0 mmol potassium l⁻¹. The percentages of embryos undergoing blastocoel formation and expansion for each cell stage evaluated did not differ ($P > 0.10$) among the various concentrations of potassium. However, more four-cell embryos commenced hatching in 3.0 mmol potassium l⁻¹ (53%) than in 1.5 mmol potassium l⁻¹ (16%) ($P < 0.05$) and 12.0 mmol potassium l⁻¹ (24%) ($P < 0.10$). The time required for embryos to develop blastocoels, expand and initiate hatching was not affected ($P > 0.10$) by the concentration of potassium in the medium. However, blastocoel formation by one- to two-cell embryos was delayed ($P < 0.05$) in medium with 1.5 mmol potassium l⁻¹. In Expt 2, 89 four-cell embryos were cultured in medium containing 6.0 or 24.0 mmol potassium l⁻¹. No differences ($P > 0.10$) were observed in the percentages of embryos undergoing blastocoel formation, expansion and hatching or in the time required to develop to these stages. The number of cells in embryos recovered at 96 h was greater ($P < 0.05$) in medium containing 6.0 compared with 24.0 mmol potassium l⁻¹ (32.9 ± 1.7 versus 26.9 ± 2.1 , respectively). These results demonstrate that pig embryos are tolerant to a wide range of potassium concentrations when the osmolality of the medium is maintained by adjusting the NaCl concentration. These results also suggest that limitations in development due to culture conditions are not due to inappropriate concentrations of this ion.

Introduction

Determination of optimal concentrations of medium constituents that will support prolonged development of early embryos such that high percentages reach the blastocyst stage is essential for successful embryo production *in vitro*. Previous studies with mouse embryos have shown that development is sensitive to the concentration of potassium in the culture medium. Wiley (1984) and Wiley *et al.* (1986) reported that reduced potassium (0.6 and 1.4 mmol l⁻¹) accelerated development of two- to four-cell stage embryos and morulae into blastocysts compared with medium with 6 mmol potassium l⁻¹ (Biggers *et al.*, 1971). However, Roblero and Rizzo (1986) reported that a high potassium concentration (25 mmol l⁻¹), similar to that found in the mouse oviduct, increased the number of cells of morulae and blastocysts developing from two-cell embryos compared with embryos cultured in medium with 4.7 mmol l⁻¹. Studies in several species have demonstrated higher potassium concentrations in reproductive tract fluids compared with the bloodstream (Iritani *et al.*, 1974;

Roblero *et al.*, 1976; Borland *et al.*, 1980). Potassium concentrations in the oviductal and uterine fluids of pigs are 12.5 and 16.0 mmol l⁻¹, respectively, and are considerably higher than the 4–6 mmol l⁻¹ traditionally found in culture media (Iritani *et al.*, 1974). This difference in potassium may contribute to the reduced development observed in embryos cultured from the early cleavage stages. Therefore, the purpose of this study was to investigate the effects of varying the potassium concentration on development of pig embryos *in vitro*.

Materials and Methods

Two experiments were conducted to evaluate the effects of potassium on numbers of embryonic cells and development to the blastocyst stage. Expt 1 was designed to evaluate the development of pig embryos collected at various cell stages to the blastocyst and hatched blastocyst stages in medium containing 1.5, 3.0, 6.0 or 12.0 mmol potassium l⁻¹. Expt 2 was designed to assess development to the blastocyst stages after 96 h and 168 h of culture and the number of embryonic cells at 96 h for pig embryos at the four-cell stage cultured in medium containing 6.0 or 24.0 mmol potassium l⁻¹. In Expts 1 and 2, cyclic Yorkshire and Hampshire cross gilts and sows were

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Table 1. Development *in vitro* of pig embryos in Whitten's medium containing 1.5, 3.0, 6.0 or 12.0 mmol potassium l⁻¹

Initial cell stage	Potassium concentration (mmol l ⁻¹)	Number of embryos cultured	Number (percentage) of embryos developing to the:			
			Blastocyst	Expanded blastocyst	Hatching blastocyst	Hatched blastocyst
One- and two-cell	1.5	10	8 (80)	2 (20)	1 (10)	—
	3.0	10	8 (80)	6 (60)	3 (30)	—
	6.0	10	7 (70)	4 (40)	3 (30)	—
	12.0	12	11 (92)	4 (33)	1 (8)	—
Four-cell	1.5	19	19 (100)	10 (53)	3 (16)	1 (5)
	3.0	17	15 (88)	11 (65)	9 (53) ^{a,b}	0 (0)
	6.0	19	16 (84)	11 (58)	6 (32)	0 (0)
	12.0	17	17 (100)	10 (59)	4 (24)	0 (0)
Morula and early blastocyst	1.5	25	25 (100)	23 (92)	14 (56)	2 (8)
	3.0	24	22 (92)	19 (79)	13 (54)	2 (8)
	6.0	22	21 (95)	21 (95)	13 (59)	2 (9)
	12.0	24	22 (92)	22 (92)	13 (54)	1 (4)

^aSignificantly different from development of four-cell embryos in medium with 1.5 mmol potassium l⁻¹ ($P < 0.05$).

^bSignificantly different from development of four-cell embryos in medium with 12.0 mmol potassium l⁻¹ ($P < 0.10$).

checked daily for oestrus and hand-mated with boars of proven fertility. In addition, in Expt 1, eight prepubertal gilts were synchronized and superovulated (Baker and Coggins, 1968) with i.m. injections of 1500 i.u. pregnant mares' serum gonadotrophin (Calbiochem-Behring Corp., San Diego, CA) followed 48 h later by 500 i.u. hCG (Sigma Chemical Co., St Louis, MO) and then artificially inseminated with commercial semen 24 h and 48 h after hCG (Baker and Coggins, 1968). Prepubertal gilts displaying oestrus after hormone treatment were also hand-mated. Reproductive tracts were recovered at slaughter and oviducts and uteri were flushed with Whitten's medium (Whitten and Biggers, 1968) without glucose (Youngs *et al.*, 1993) and BSA. Flushings were collected into embryological dishes and observed at $\times 10$ magnification with a dissecting microscope for the presence of the embryos. Embryos were transferred to microdrops of glucose-free Whitten's medium with 4 mg BSA ml⁻¹ (Sigma Chemical Co.) and their morphology was evaluated. Embryos with normal morphology ranging in development from one-cell to early blastocyst stages were randomly allocated to Whitten's medium containing 1.5, 3.0, 6.0 or 12.0 mmol potassium l⁻¹ in Expt 1. In Expt 2, four-cell embryos were randomly allocated to Whitten's medium containing 6.0 or 24.0 mmol potassium l⁻¹. Potassium concentration was varied by manipulating the amount of KCl added to the medium. Osmolality and chloride concentration were maintained by adjusting the NaCl concentration. Embryos were cultured individually in 50 μ l microdrops of Whitten's medium under paraffin oil in a humidified atmosphere of 5% CO₂ in air at 39°C. Embryos were evaluated for cell stage and morphology at $\times 100$ –200 magnification with an inverted-stage phase-contrast microscope at intervals of 24 h until the end of culture. Embryos were cultured for 168 h in Expt 1, and for 96 and 168 h in Expt 2. Embryos cultured for 96 h in Expt 2 were fixed and stained with haematoxylin and eosin for assessment of numbers of cells (McGaughey and Chang, 1969).

Statistical analyses

Differences in the percentages of embryos developing to a particular cell stage due to treatment were determined by Chi-square procedures for contingency tables (Steel and Torrie, 1980). Differences in the times required for embryos to develop to the blastocyst stages due to concentration of potassium were determined using one-way ANOVA and Fisher's least significant differences procedures for Expt 1 and the unpaired *t* test for Expt 2. Numbers of embryonic cells in Expt 2 were also analysed using the unpaired *t* test. All analyses were performed using the NCSS statistical software program (Number Cruncher Statistical System, Version 4.1, 1984, J. L. Hintze, Kaysville, UT).

Results

A total of 209 morphologically normal one-cell to early blastocyst stage embryos were recovered from 18 females for Expt 1. Sufficient numbers of embryos were collected from each female to allow allocation of embryos to all four treatments. Incidences of blastocyst formation and blastocoele expansion did not differ ($P > 0.10$) as a result of potassium concentration for any of the cell stages cultured (Table 1). Potassium concentration did not significantly ($P > 0.10$) affect the number of embryos that began hatching when embryos were cultured from the one- and two-cell or morula and early blastocyst stages. However, more four-cell embryos began hatching when cultured in Whitten's medium with 3.0 mmol potassium l⁻¹ than in media containing 1.5 ($P < 0.05$) or 12.0 mmol ($P < 0.10$) potassium l⁻¹. One- and two-cell embryos and morulae and early blastocysts were originally analysed separately but because no significant differences in development were observed, the data were combined in Table 1 for more concise presentation of the results.

Table 2. Times (h) from initiation of culture to blastocyst formation, expansion and onset of hatching for pig embryos developing in Whitten's medium containing 1.5, 3.0, 6.0 or 12.0 mmol potassium l⁻¹

Initial cell stage	Potassium concentration (mmol l ⁻¹)	Number of embryos cultured	Time to:					
			Blastocoel formation		Blastocoel expansion		Onset of hatching	
			<i>n</i>	Mean ± SE	<i>n</i>	Mean ± SE	<i>n</i>	Mean ± SE
One- and two-cell	1.5	10	8	120 ± 5 ^a	2	168 ± 15	1	168
	3.0	10	8	105 ± 5 ^b	6	140 ± 9	3	144 ± 14
	6.0	10	7	96 ± 5 ^b	4	132 ± 11	3	144 ± 14
	12.0	12	11	100 ± 4 ^b	4	138 ± 11	1	144
Four-cell	1.5	19	19	72 ± 4	10	106 ± 7	3	128 ± 13
	3.0	17	15	69 ± 4	11	96 ± 6	9	109 ± 7
	6.0	19	16	64 ± 4	11	98 ± 6	6	112 ± 9
	12.0	17	17	65 ± 4	10	89 ± 7	4	102 ± 11
Morula	1.5	13	13	28 ± 2	12	42 ± 4	8	60 ± 6
	3.0	12	10	26 ± 2	7	34 ± 5	6	56 ± 7
	6.0	10	9	24 ± 2	9	45 ± 4	6	56 ± 7
	12.0	13	11	26 ± 2	11	41 ± 4	4	60 ± 9
Early blastocyst	1.5	12	12	—	11	24	6	36 ± 6
	3.0	12	12	—	12	24	7	34 ± 6
	6.0	12	12	—	12	24	7	34 ± 6
	12.0	11	11	—	11	24	9	32 ± 5

^{a,b}Means within a column for the same initial cell stage lacking a common superscript are significantly different ($P < 0.05$).

Times required for embryos in Expt 1 to develop blastocoels, undergo blastocoelic expansion and begin hatching are reported (Table 2). One- and two-cell embryos cultured in Whitten's medium containing 1.5 mmol potassium l⁻¹ developed more slowly ($P < 0.05$) to the blastocyst stage than did embryos cultured in Whitten's medium containing 3.0, 6.0 or 12.0 mmol potassium l⁻¹. Although this delay persisted up to blastocoel expansion and onset of hatching, significant differences were not observed. Differences in developmental times among the four concentrations of potassium were not observed for four-cell embryos, morulae or early blastocysts.

Eighty-nine embryos at the four-cell stage and of normal morphology were recovered from nine females and cultured in Expt 2. No differences ($P > 0.10$) in development of four-cell embryos to a particular cell stage were observed in Whitten's medium containing 6.0 or 24.0 mmol potassium l⁻¹ at 96 h of culture (Table 3). However, embryos developing in Whitten's medium containing 24.0 mmol potassium l⁻¹ had fewer ($P < 0.05$) cells at 96 h of culture than did embryos in Whitten's medium containing 6.0 mmol potassium l⁻¹ (Table 3). When four-cell embryos were cultured for 168 h, no differences ($P > 0.10$) in development to the blastocyst, expanded blastocyst and hatching stages were observed (Table 4). Although development tended to be faster in Whitten's medium containing 24.0 mmol potassium l⁻¹, no significant differences were observed (Table 4).

Discussion

Although culture of cleavage stage pig embryos to the blastocyst stage has been accomplished in simple medium with

Table 3. Development of pig embryos from the four-cell stage after 96 h of culture in Whitten's medium containing 6.0 or 24.0 mmol potassium l⁻¹

Observation	Potassium concentration (mmol l ⁻¹)	
	6.0	24.0
Number of embryos cultured	42	47
Percentage of embryos developing to:		
Blastocyst	83 (35) ^a	77 (36)
Expanded blastocyst	40 (17)	34 (16)
Hatching blastocyst	26 (11)	15 (7)
Hatched blastocyst	0 (0)	0 (0)
Number of cells ^b	32.9 ± 1.7 ^c (19)	26.9 ± 2.1 (23)

^aValues in parentheses are the numbers of embryos.

^bTwenty-four embryos were fixed and stained for each concentration of potassium. Five embryos and one embryo cultured in medium containing 6.0 or 24.0 mmol potassium l⁻¹, respectively, either were lost or did not yield satisfactory preparations during processing.

^cSignificantly different from 24.0 mmol potassium l⁻¹ ($P < 0.05$).

BSA or serum, development of a high percentage of embryos from the early cleavage stages to hatching in such medium is limited (Reed *et al.*, 1992; Petters and Wells, 1993). The development of pig embryos has been improved with more complex systems using mouse oviduct organ culture (Krisher *et al.*, 1989; Reed *et al.*, 1992), co-culture with oviductal epithelial

Table 4. Development of pig embryos from the four-cell stage after 168 h of culture in Whitten's medium containing 6.0 or 24.0 mmol potassium l^{-1}

Observation	Potassium concentration (mmol l^{-1})	
	6.0	24.0
Number of embryos cultured	18	23
Percentage of embryos developing to:		
Blastocyst	94 (17) ^a	87 (20)
Expanded blastocyst	72 (13)	52 (12)
Hatching blastocyst	56 (10)	39 (9)
Hatched blastocyst	0 (0)	4 (1)
Time ^b (h) to:		
Blastocoel formation	75 $\pm$ 6 (17)	65 $\pm$ 4 (20)
Blastocoel expansion	103 $\pm$ 6 (13)	92 $\pm$ 5 (12)
Onset of hatching	115 $\pm$ 8 (10)	101 $\pm$ 7 (9)

^aValues in parentheses are the numbers of embryos.

^bValues are means $\pm$ SE.

cells (White *et al.*, 1989) and with medium containing oviductal fluid (Archibong *et al.*, 1989). Pollard and coworkers (1995) designed an optimal culture system that supported the development of a large proportion of pig zygotes through blastulation and hatching. In this system, pig zygotes were cultured first in simple medium (CZB; Chatot *et al.*, 1989) up to the morula stage and then transferred to modified Eagles' minimum essential medium containing glucose, non-essential amino acids, vitamins, insulin and serum (Pig-MEM; Pollard *et al.*, 1995). Difficulties encountered in designing a single culture medium that can support development of zygotes to hatching may be due to ionic imbalances or to the absence of beneficial macromolecular components such as growth factors.

The importance of the physiologically high potassium concentration in the oviductal and uterine microenvironments of the preimplantation embryo is not understood. Previous work has suggested that the high potassium concentration influences sperm motility and sperm-egg fusion (Boldt *et al.*, 1991). Roblero and Rizzo (1986) observed a dose-related increase in the rate of cleavage of mouse embryos with 4.7, 10 and 25 mmol potassium l^{-1} . Their results suggest that potassium concentrations similar to those found in the mouse genital tract improved development *in vitro*. However, conflicting results with mouse embryos have been observed (Wiley, 1984; Wiley *et al.*, 1986). Cavitation was accelerated when potassium was decreased from 60 to 0.5 mmol l^{-1} and was delayed when potassium was increased from 6 to 25 mmol l^{-1} (Wiley, 1984; Wiley *et al.*, 1986). Wiley (1984) attributed the effects of potassium on cavitation to changes in membrane potential. Decreasing the concentration of extracellular potassium would hyperpolarize the cells of the embryo, whereas increasing extracellular potassium would depolarize the cells (Cohen *et al.*, 1976; Wiley, 1984).

In the present study, only minor effects on *in vitro* development were observed by altering the potassium concentration in medium used to culture early pig embryos. When the potassium concentration was reduced to 1.5 mmol l^{-1} some devel-

opmental retardation was apparent in embryos cultured at the one- and two-cell stage. Increasing the potassium concentration to 24 mmol l^{-1} reduced the number of cells, although overall development was unaffected. The differences in onset of hatching for four-cell embryos cultured in 3.0 versus 1.5 and 12.0 mmol potassium l^{-1} may merit further investigation as 3.0 mmol potassium l^{-1} may be a more beneficial concentration for *in vitro* development. Evidence for improved development with reduced potassium was reported by Erbach *et al.* (1994) who demonstrated superior development of mouse embryos from outbred strains cultured in medium with 2.85 mmol potassium l^{-1} . It is of interest that the sodium:potassium ratio in the optimal medium described by Erbach *et al.* (1994) is 46, whereas the ratio in Whitten's medium containing 3.0 mmol potassium l^{-1} in the present study is 40. In a study investigating sodium tolerance by pig embryos, Beckman and Day (1993) concluded that the sodium concentration in modified Krebs' Ringer Bicarbonate medium (mKRB) used for embryo culture was detrimental compared with Whitten's medium (145 and 115 mmol l^{-1} , respectively). In that study the sodium:potassium ratios were not manipulated and were reasonably close for mKRB and Whitten's medium (24.4 and 19.3, respectively). Similarly, Petters and Wells (1993) have described a medium containing 118.2 mmol sodium l^{-1} and 5.4 mmol potassium l^{-1} (sodium:potassium ratio = 22.0) that supports the development of more than 90% of one- to four-cell embryos to the blastocyst stage. The effects of sodium seem related more to the absolute amount of sodium in the medium than to the sodium:potassium ratio. Biggers *et al.* (1993) increased the sodium concentration in culture medium from 85 to 125 mmol l^{-1} for mouse embryos, and observed poor development coupled with increases in intracellular sodium and decreases in intracellular potassium. However, the medium used in that study (Simplex Optimized Medium; SOM; Lawitts and Biggers, 1992), has a low potassium concentration (0.6 mmol l^{-1}). Deleterious effects on embryo survival induced by intracellular ionic imbalances would be avoided if the balance of extracellular sodium and potassium, which results in little change to intracellular values, was determined. This may explain why optimal development was observed when the potassium concentration in SOM was increased to 2.85 mmol l^{-1} (referred to as KSOM) in the presence of 130.2 mmol sodium l^{-1} in the report by Erbach *et al.* (1994). Pig embryos developing *in vitro* appear to be tolerant of a wide range of potassium concentrations when the osmolality of the medium is maintained by adjusting the NaCl concentration. However, concentrations of 1.5 and 24.0 mmol l^{-1} may represent the limits of this tolerance.

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