Differential sensitivity of one-cell and two-cell rabbit embryos to sodium chloride and total osmolarity during culture into blastocysts

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One-cell or two-cell rabbit embryos were cultured in protein-free media with various NaCl concentrations and osmolarity to determine relative sensitivity of embryos to changes in media composition. Embryos from replicates of donor rabbits were distributed randomly across treatments and cultured at 39°C. Zygotes were cultured in Expts 1, 2A and B, and 3, and two-cell embryos were cultured in Expts 4A and 4B. In Expt 1, blastocyst formation and number of cells were highest ($P < 0.05$) in the control medium with 93 mmol NaCl l⁻¹ (270 mosmols) compared with media containing 63 and 116 mmol NaCl l⁻¹ (220 and 316 mosmols). In Expt 2, embryos were cultured in media with 70 or 93 mmol NaCl l⁻¹, varying in osmolarity from 250 to 320 mosmols by adding sorbitol. In media with 70 mmol NaCl l⁻¹ and osmolarities of 250, 280 and 300 mosmols, there were 41, 56 and 50% expanded blastocysts, respectively ($P < 0.05$). With 93 mmol NaCl l⁻¹ and osmolarities of 270, 293 and 320 mosmols, embryos developed into 37, 53 and 27% expanded blastocysts, respectively, ($P < 0.05$). In Expts 3A and 3B and 4A and 4B, the osmolarity of the medium was maintained at 270 mosmols by adding sorbitol to media containing 40 or 60 mmol NaCl l⁻¹, and other components were reduced in media containing 100 and 116 mmol NaCl l⁻¹ to compensate for the higher NaCl. Zygote development into blastocysts was greatly suppressed ($P < 0.05$) in media with 40, 60, 100 and 116 mmol NaCl l⁻¹, compared with the control (93 mmol NaCl l⁻¹), whereas development of two-cell embryos into blastocysts was much less affected. These results appear to reflect a direct sodium chloride as well as osmolarity effect on embryo development; and zygotes are much more sensitive to these effects than are two-cell embryos.

Introduction

When embryos are cultured in a chemically defined protein-free medium fewer blastocysts with fewer blastomeres are formed compared with blastocysts developed *in vivo*. The causes of these differences are unknown. The ionic composition of embryo culture media has usually been derived from media developed for culture of somatic cells. These media contain a relatively high NaCl concentration, and NaCl is a major component of oviduct fluid (David *et al.*, 1969; Iritani *et al.*, 1971; Borland *et al.*, 1977; Dickens and Leese, 1994). The NaCl (Na⁺ Cl⁻) contributes to the osmolarity of cells and creates electrical potentials across cell membranes, which are important in oocyte maturation and embryo growth (Cross, 1973; Roblero *et al.*, 1976). The Na⁺ gradient across the cell membrane of mouse embryos is required for amino acid transport (Borland and Tasca, 1974, 1975; Dizio and Tasca, 1977), and an exogenous source of amino acids is required for blastocyst formation in rabbits (Kane and Foote, 1970).

Lawitts and Biggers (1991, 1992) investigated ionic composition of simple embryo culture media and found that altering

several components, such as Na⁺ and K⁺, overcame the two-cell block in culturing mouse embryos. Reduction of the Na⁺ concentration in this modified medium resulted in fully functional blastocysts from oocytes fertilized *in vitro* (Summers *et al.*, 1995). Anbari and Schultz (1993) and Ho *et al.* (1994) have shown that the pattern of RNA and protein synthesis in mouse embryos cultured in media with reduced NaCl resemble more closely synthetic patterns of embryos grown *in vivo*. Rabbit embryos grow much faster than mouse embryos and the concentration of NaCl may play an important role in rabbit embryo culture (Li and Foote, 1995).

Osmolarity of media, to which NaCl is a major contributor, has been the focus of several studies. Brinster (1965) reported that the optimal osmolarity for developing mouse embryos was 276 mOsm, considerably lower than the normal body fluid value of about 306 mOsm. Whitten and Biggers (1968) obtained most blastocysts from mouse zygotes in a defined medium with a calculated osmolarity of 256 mOsm. Maurer *et al.* (1970) found a higher rate of blastulation of rabbit embryos cultured in media with an osmolarity of 256 mOsm than in media at 310 mOsm. Other studies indicated that the optimum for rabbit embryo culture media was about 270 mosmols (Naglee *et al.*, 1969; Li and Foote, 1995).

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The relative importance of osmotic changes and NaCl concentration on embryo development is not clear, and many studies have involved culture of a mixture of one-cell and two-cell embryos. The objectives of the present study were to examine the effects of both Na^+ Cl^- and osmolarity changes on rabbit embryo development in a defined protein-free medium, and to determine whether there was a differential sensitivity of one-cell versus two-cell rabbit embryos to these changes, as reported for the mouse.

Materials and Methods

Animals and embryos

Dutch rabbits were superovulated with FSH and LH (Vetrepharm, London, Ontario) by a standard schedule (Kennelly and Foote, 1965) and inseminated at the time of LH injection. In Expts 1, 2 and 3, one-cell embryos were collected 19 h after LH injection by flushing oviducts with phosphate-buffered saline containing 0.1% polyvinyl alcohol. The embryos were cultured for 72 h in Expt 1, and 62 h in Expts 2 and 3, the latter to evaluate development rate more critically. The one-cell 'embryos' or zygotes that did not cleave during the first 24 h of culture were considered to be unfertilized oocytes. In Expts 4A and 4B, two-cell embryos were collected 24 h after LH injection by flushing the oviducts and they were cultured for 57 h, so the total elapsed time from LH injection to final evaluation was the same in Expts 2–4. All culturing was done at 39°C, the approximate body temperature of the rabbit.

Media and culture

A special macromolecule-free RD medium (RPMI 1640 with DMEM, 1:1) was prepared by Gibco BRL (Life Technologies, Grand Island, NY). The experimental medium contained no glucose, glycine or inositol and 25 mmol of NaCl l^{-1} . The medium was stored at 5°C before use and at the time of use 8 mmol glucose l^{-1} , 34 mmol $\text{NaHCO}_3 \text{l}^{-1}$, 0.5 mmol glycine l^{-1} and 0.5 mmol inositol l^{-1} were added, so that these were constant in all experiments. The other components of the control RD medium were the same as those described by Carney and Foote (1991) and Li and Foote (1995). The control culture medium, with NaCl added to a final concentration of 93 mmol NaCl l^{-1} , had an osmolarity of 270 mosmol. Osmolarity of the experimental media and NaCl concentration were varied, as indicated in the description of each experiment. Every batch of media was checked by osmometry for concurrence with the expected osmolarity.

Media for culturing embryos were equilibrated overnight in an atmosphere of 5% CO_2 :95% humidified air. Approximately 5 to 15 embryos were added to each 120 μl droplet of media in 35 mm Falcon petri dishes and cultured under 4 ml Dow Corning Medical 360 Fluid (Dow Corning, Midland, MI) at 39°C in an atmosphere of 5% CO_2 :95% humidified air.

In Expt 1, one-cell embryos were cultured in the special medium with different osmolarities (220, 250, 270, 300 and

316 mOsmols) produced by NaCl concentrations from 63 to 116 mmol l^{-1} . Embryo development was evaluated at 72 h of culture. Stage of development and cell counts were the criteria used in evaluating experimental effects. The cell counts were made on blastocysts stained with Hoechst dye 33342 by the procedure described by Pursel *et al.* (1985).

In Expts 2A and 2B effects of various osmolarities of sorbitol on embryo development, while maintaining a constant NaCl concentration of either 70 or 93 mmol l^{-1} , were studied. In Expt 2A, one-cell embryos were cultured in a 70 mmol NaCl l^{-1} medium with sorbitol added at concentrations of 0, 7.5 and 11.2 mg ml^{-1} (osmolarity: 250, 280 and 300 mOsmols, respectively). Embryos cultured in the medium with 93 mmol NaCl l^{-1} and an osmolarity of 270 mOsmol served as a control. In Expt 2B, media containing 93 mmol NaCl l^{-1} were prepared with osmolarities of 270, 293 and 320 mOsmols by adding sorbitol at concentrations of 0, 3.7 and 8.5 mg ml^{-1} , respectively. The proportion of embryos developing to different stages was recorded after 62 h of culture.

In Expts 3A and 3B, effects of NaCl concentrations ranging from 40 mmol l^{-1} to 116 mmol l^{-1} , while keeping the osmolarity of the culture medium constant, on one-cell embryo growth were investigated. The total osmolarity of the medium was maintained at 270 mosmols by adding 17 mg sorbitol ml^{-1} to the 40 mmol NaCl l^{-1} medium and 11 mg ml^{-1} to the 60 mmol NaCl l^{-1} treatment. An osmolarity of 270 mosmols was maintained with the treatments containing 100 and 116 mmol NaCl l^{-1} , by reducing the other components in the RD medium appropriately before the addition of NaCl. This experiment was divided into two parts, A and B, to limit the number of treatments so that a substantial number of embryos from each donor would be available to assign randomly to each treatment. In Expt 3A, the NaCl concentrations were 40, 60 and 93 mmol l^{-1} and in Expt 3B, the NaCl concentrations were 93, 100 and 116 mmol l^{-1} . The proportion of one-cell embryos developing to different stages was recorded at 62 h of culture to provide a more critical evaluation of the rate of development into blastocysts.

With the marked differences obtained in Expt 3, it was feasible to use the same experimental design in Expts 4A and 4B to test the effects with two-cell embryos. In addition, development of the embryos was recorded at 57 h of culture, which was the same elapsed time from LH injection as in Expt 3 in which one-cell embryos were collected 5 h sooner than the two-cell embryos.

Statistical analysis

A randomized complete block design was used in all experiments, with the embryos from each donor representing one complete block. Embryos from each donor were divided equally and randomly assigned to treatment groups. A two-way analysis of variance was performed, using the general linear models procedure of the Statistical Analysis System (SAS Institute, Cary, NC). The arcsine transformation of the proportion of blastocysts formed in each droplet was used for the percentage data analysis. When analysis of variance indicated significant mean differences, these were compared using Duncan's new multiple range test.

Table 1. Effect of increasing NaCl and osmolarity on one-cell rabbit embryos cultured in RD medium for 72 h (Expt 1)

NaCl (mmol l ⁻¹)	Osmolarity (mosmol)	Number of zygotes*	Number (%) of embryos at each stage				
			2-4- cell	Morulae	Blastocysts		
					Expanded	Hatching	Total
63	220	38	0	24 ^a	10 ^a	4 ^a	14 (37) ^a
70	250	38	0	19 ^a	13 ^a	6 ^a	19 (50) ^b
93	270	34	0	8 ^b	15 ^a	11 ^b	26 (76) ^c
106	300	34	0	11 ^{ab}	14 ^a	9 ^b	23 (68) ^c
116	316	34	0	12 ^{ab}	19 ^a	3 ^a	22 (65) ^c
		Number of blastocysts	Number of cells				
63	220	13	150 ^a				
70	250	10	190 ^b				
93	270	14	200 ^b				
106	300	12	178 ^b				
116	316	15	132 ^a				

*Zygotes were obtained from 12 donor rabbits.

^{a,b,c}Means within columns with different superscripts are significantly different ($P < 0.05$).**Table 2.** Effect of varying osmolarity with sorbitol on one-cell rabbit embryos cultured in RD medium for 62 h (Expts 2A and 2B)

NaCl (mmol l ⁻¹)	Osmolarity (mosmol)	Sorbitol (mg ml ⁻¹)	Number of zygotes*	Number (%) of embryos at each stage				
				2-4- cell	Morulae	Blastocysts		
						Early	Expanded	Total
Experiment A								
93	270	0	31	0	4 ^a	9 ^a	18 (58) ^a	27 (87) ^a
70	250	0	29	0	9 ^a	8 ^a	12 (41) ^b	20 (69) ^b
70	280	7.5	34	0	11 ^a	4 ^a	19 (56) ^a	23 (68) ^b
70	300	11.2	36	0	11 ^a	7 ^a	18 (50) ^a	25 (69) ^b
Experiment B								
93	270	0	51	0	20 ^b	12 ^a	19 (37) ^a	31 (61) ^a
93	293	3.7	54	0	13 ^a	12 ^a	29 (53) ^b	41 (76) ^b
93	320	8.5	49	0	29 ^b	7 ^a	13 (27) ^a	20 (41) ^c

*Zygotes were obtained from 8 and 12 donor rabbits in Expts A and B.

^{a,b,c}Means within columns with different superscripts are significantly different ($P < 0.05$).

Results

In Expt 1 (Table 1), optimal development of one-cell embryos was in a medium with 93 mmol NaCl l⁻¹ and 270 mOsmols, and hatching was lower in media containing lower or higher NaCl concentrations ($P < 0.05$). In addition, the blastocysts formed in the media with 70, 93 and 106 mmol NaCl l⁻¹ contained more cells ($P < 0.05$) than did blastocysts cultured in media with 63 or 116 mmol NaCl l⁻¹.

In Expt 2 (Table 2), zygotes cultured for 62 h did not have time to hatch, in contrast to Expt 1. Expanded blastocyst development in Expt 2A was lower in the 250 mOsmol

group and total blastocyst development was highest in the 270 mOsmol control group with 93 mmol NaCl l⁻¹ ($P < 0.05$). Embryos cultured in the medium with 93 mmol NaCl l⁻¹ and an osmolarity of 293 mOsmols (Expt 2B) developed into significantly more expanded and total blastocysts than in other groups ($P < 0.05$).

In Expt 3 (Table 3), one-cell embryos were cultured in media with various NaCl concentrations and constant osmolarity. In Expt 3A, the embryos cultured in the medium with 93 mmol NaCl l⁻¹ developed into significantly more blastocysts ($P < 0.05$) than did the embryos cultured in the media with 40 mmol l⁻¹ and 60 mmol l⁻¹ NaCl. Likewise, in Expt 4B, the

Table 3. Test of NaCl concentration on one-cell rabbit embryos with osmolarity maintained at 270 mosmol in RD medium for 62 h (Expts 3A and 3B)

NaCl* (mmol l ⁻¹)	Osmolarity (mosmol)	Number of zygotes [†]	Number (%) of embryos at each stage				
			2-4- cell	Morulae	Blastocysts		
					Early	Expanded	Total
Experiment A							
40	270	28	1	27 ^a	0 ^a	0 ^a	0 (0) ^a
60	270	27	0	16 ^a	6 ^b	5 ^b	11 (41) ^b
93	270	28	0	3 ^b	6 ^b	19 ^c	25 (89) ^c
Experiment B							
93	270	40	0	0 ^a	7 ^a	33 ^a	40 (100) ^a
100	270	39	0	11 ^b	11 ^a	17 ^b	28 (71) ^b
116	270	38	2	20 ^b	5 ^a	11 ^c	16 (42) ^c

*The total osmolarity of the medium was maintained at 270 mosmol by adding 17 mg sorbitol ml⁻¹ to the 40 mmol NaCl l⁻¹ treatment and 11 mg ml⁻¹ to the 60 mmol NaCl l⁻¹ treatment, and other components of the RD medium were reduced to compensate for adding more NaCl to the 100 and 116 mmolar NaCl treatments. [†]Zygotes were obtained from six and seven donor rabbits in Expts A and B.

^{a,b,c}Means within columns with different superscripts are significantly different ($P < 0.05$).

Table 4. Test of NaCl concentration on two-cell rabbit embryos with osmolarity maintained at 270 mosmol in RD medium for 57 h (Expts 4A and 4B)

NaCl* (mmol l ⁻¹)	Osmolarity (mosmol)	Number of two-cell embryos [†]	Number (%) of embryos at each stage				
			2-4- cell	Morulae	Blastocysts		
					Early	Expanded	Total
Experiment A							
40	270	29	0	14 ^a	3 ^a	12 ^a	15 (52) ^a
60	270	29	0	3 ^b	5 ^a	21 ^b	26 (90) ^b
93	270	28	0	5 ^b	6 ^a	17 ^b	23 (82) ^b
Experiment B							
93	270	30	0	7 ^a	4 ^a	19 ^a	23 (77) ^a
100	270	28	0	6 ^a	5 ^a	17 ^a	22 (79) ^a
116	270	30	0	16 ^b	5 ^a	9 ^b	14 (47) ^b

*The total osmolarity of the medium was maintained at 270 mosmol by adding 17 mg sorbitol ml⁻¹ to the 40 mmol NaCl l⁻¹ treatment and 11 mg ml⁻¹ to the 60 mmol NaCl l⁻¹ treatment and other ingredients in the RD medium were reduced to compensate for adding more NaCl to the 100 and 116 mmolar NaCl treatments.

^{a,b}Means within columns with different superscripts are significantly different ($P < 0.05$).

[†]Two-cell embryos were obtained from six and five donor rabbits in Expts A and B.

medium with 93 mmol NaCl l⁻¹ supported greater development into blastocysts than did the media with higher NaCl concentrations ($P < 0.05$).

In Expt 4A (Table 4), the medium with 93 mmol NaCl l⁻¹ promoted better growth of two-cell embryos than did the medium with 40 or 60 mmol NaCl l⁻¹. Compared with one-cell embryos (Expt 3), two-cell embryos were more able to tolerate lower NaCl concentrations in the culture medium (Fig. 1). The two-cell embryos were more similar to one-cell embryos in responding to higher NaCl concentrations in the culture medium, although the actual percentage decrease with two-cell embryos of 77 to 47% blastocysts in Expt 4B was less than the decrease from 100 to 42% blastocysts when one-cell embryos were cultured in comparable media in Expt 3B.

Discussion

Mammalian preimplantation embryos developing within the protected environment of the female reproductive tract are provided with all necessary nutrients for early embryo growth and a system for removing wastes. Embryos cultured *in vitro* must obtain all nutrients from the culture medium once the initial store in the oocyte is depleted, and also must be protected from potential toxic elements. Preimplantation rabbit embryos grow rapidly. Thus, for example, nutrient requirements may be greater for rabbit embryos than for the mouse embryos to maintain normal development, protein synthesis, intracellular organelle organization and cell division. While amino acids, vitamins, DNA precursors and glucose provide

Fig. 1. Comparison of blastocyst development from (■) one-cell and (○) two-cell embryos cultured in RD medium with NaCl concentration from 40 to 120 mmol l⁻¹, but with osmolality constant at 270 mOsmols.

materials for embryo growth, salts are also required to maintain normal cell activity, including RNA replication (Ho *et al.*, 1994).

The two-cell culture block of CFI mouse embryos can be overcome by decreasing the NaCl concentration in the medium (Lawitts and Biggers, 1991; Summers *et al.*, 1995). It is clear that transport of some amino acids and sugars is Na-dependent. The Na⁺ gradient across cell membranes furnishes the energy for this transport (Schultz and Curran, 1970).

Some rabbit embryos can develop in media containing 63 to 116 mmol NaCl l⁻¹, with an osmolality ranging from 220 mOsmol to 316 mOsmol, as shown in Expt 1. More embryos became hatching blastocysts composed of more cells when the embryos were cultured in the medium with an osmolality of 270 mOsmol and a NaCl concentration of 93 mmol l⁻¹ than in 220 and 316 mOsmol media. The results of Expts 1 and 2 indicated that osmolality and NaCl content of the culture medium may affect embryo growth, as differences observed between 250 and 270 mOsmols with NaCl were not found with NaCl constant at 70 mmol l⁻¹ and osmolality altered by sucrose (Expt 2A). However, at a constant 93 mmol NaCl l⁻¹, increasing the osmolality with sucrose to 320 mOsmols decreased the percentage of motile spermatozoa.

On the basis of these results, culture media with different concentrations of NaCl, but with osmolality held constant at 270 mOsmols were formulated for Expts 3 and 4. The optimal concentration of 93 mmol NaCl l⁻¹ in RD culture medium for culture of zygotes was verified as more blastocysts developed at these NaCl concentrations than in media with lower or higher NaCl concentrations. There was a striking difference in the sensitivity of the one-cell versus two-cell embryos to changes in NaCl concentrations. The two-cell rabbit embryos were relatively resistant to the NaCl effect. For example, in 40 mmol NaCl l⁻¹ no zygotes became blastocysts, but 52% of the two-cell embryos became blastocysts in the same medium. Although cultured rabbit zygotes do not have a two-cell block, as occurs in embryos from some strains of mice, transition from the one-cell stage to the two-cell stage was critical for future

embryo growth in these experiments. This is consistent with preliminary observations of Li and Foote (1995).

Rambhatla and Latham (1995) found differences between strains of mice in ability to cleave beyond the two-cell stage in the presence of α -amanitin. They postulated that different strains may have different supplies of needed precursors in their oocytes. It cannot be ascertained, in the present studies, whether the longer development *in vivo* provided the two-cell embryos with additional needed precursors for eventual development into blastocysts, or whether other events associated with passing the first cleavage division *in vivo* facilitated subsequent development.

The results of these studies confirm and extend previous reports on the effects of osmolality, NaCl (Na⁺ Cl⁻) or both factors on embryo development *in vitro* (Brinster, 1965; Whitten and Biggers, 1968; Naglee *et al.*, 1969; Lawitts and Biggers, 1991, 1992; Beckman and Day, 1993; Biggers *et al.*, 1993; Li and Foote, 1995). The reduction of NaCl concentration in the medium from 125 to 85 mmol l⁻¹ (Biggers *et al.*, 1993) resulted in intracellular Na⁺ and K⁺ concentrations of cultured mouse embryos resembling more closely intracellular ion concentrations of embryos produced *in vivo*. Similar reductions of NaCl in the medium resulted in increased RNA synthesis (Ho *et al.*, 1994) and protein synthesis (Anbari and Schultz, 1993).

The only compound containing Na⁺ and Cl⁻ that was varied in the present studies was NaCl. However, the RD medium also contained 34 mmol NaHCO₃ l⁻¹, 0.5 mmol NaH₂PO₄ l⁻¹ and 0.5 mmol sodium pyruvate l⁻¹. The sole source of K⁺ was 5.4 mmol KCl l⁻¹. Other compounds containing Cl⁻ were 0.9 mmol CaCl₂ l⁻¹ and trace quantities (< 1 mmol l⁻¹ total) of the hydrochlorides of vitamins. These experiments were not designed on the large scale necessary to study the concentrations and interactions of Na⁺, K⁺ and Cl⁻, all important in blastocyst formation (Borland *et al.*, 1977; Brison and Leese, 1993; Lawitts and Biggers, 1992; Summers *et al.*, 1995). However, it is clear from the current study that the concentration of NaCl in the medium is more important for culturing rabbit zygotes than is often assumed and that many commercially available culture media, such as TCM 199, may have a higher NaCl content (116 mmol l⁻¹) than is optimal for embryo culture.

It should be noted that the optimal concentration of NaCl was considerably lower than concentrations found in rabbit oviduct fluid (David *et al.*, 1969; Iritani *et al.*, 1971; Borland *et al.*, 1977; Dickens and Leese, 1994). This finding suggests that other components in oviduct fluid assist the embryo in maintaining intracellular physiological conditions.

From these studies it is concluded that osmolality of culture media has an important effect on growth and development of rabbit embryos and that a critical component of the media contributing to this effect is the NaCl concentration. Furthermore, rabbit zygotes are much more sensitive to these effects than are two-cell embryos when cultured to the blastocyst stage in defined protein-free media. These results demonstrate, in a species besides the mouse, the importance of ionic balance in a protein-free medium for culturing zygotes, and the need for caution when comparing the effects of different media supplements in different studies in which the basic ionic components of the media differ.

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References

- Anbari K and Schultz RM (1993) Effect of sodium and betaine in culture media on development and relative rates of protein synthesis in preimplantation mouse embryos *in vitro*. *Molecular Reproduction and Development* **35** 24–28
- Beckmann LS and Day BN (1993) Effects of media NaCl concentration and osmolarity on the culture of early-stage porcine embryos and the viability of embryos cultured in a selected superior medium *Theriogenology* **39** 611–622
- Biggers JD, Lawitts JA and Lechene CP (1993) The protective action of betaine on the deleterious effect of NaCl on preimplantation mouse embryos *in vitro*. *Molecular Reproduction and Development* **34** 380–390
- Borland RM and Tasca RJ (1974) Activation of a Na⁺-dependent amino acid transport system in preimplantation mouse embryos *Developmental Biology* **36** 169–174
- Borland RM and Tasca RJ (1975) Na⁺-dependent amino acid transport in preimplantation mouse embryos *Developmental Biology* **46** 192–201
- Borland RM, Hazra S, Biggers JD and Lechene CP (1977) The elemental composition of the environment of the gametes and preimplantation embryo during initiation of pregnancy *Biology of Reproduction* **16** 147–157
- Brinster RL (1965) Studies on the development of mouse embryos *in vitro*. Effect of osmolarity and hydrogen ion concentration *Journal of Experimental Zoology* **158** 49–58
- Brison DR and Leese HJ (1993) Role of chloride transport in the development of the rat blastocyst *Biology of Reproduction* **48** 692–702
- Carney EW and Foote RH (1991) Improved development of rabbit one-cell embryos to the hatching blastocyst stage by culture in a defined protein-free culture medium *Journal of Reproduction and Fertility* **91** 113–123
- Cross MH (1973) Active sodium and chloride transport across the rabbit blastocoele wall *Biology of Reproduction* **8** 566–575
- David A, Brackett BG, Garcia C-R and Mastroianni L, Jr (1969) Composition of rabbit oviduct fluid in ligated segments of the Fallopian tube *Journal of Reproduction and Fertility* **19** 285–289
- Dickens CJ and Leese HJ (1994) The regulation of rabbit oviduct fluid formation *Journal of Reproduction and Fertility* **100** 577–581
- Dizio SM and Tasca RJ (1977) Sodium-dependent amino acid transport in preimplantation mouse embryos. III. Na-K-ATPase-linked mechanism in blastocysts *Developmental Biology* **59** 198–205
- Ho Y, Doherty AS and Schultz RM (1994) Mouse preimplantation embryo development *in vitro*: effect of sodium concentration in culture media on RNA synthesis and accumulation and gene expression *Molecular Reproduction and Development* **38** 131–141
- Iritani A, Nishikawa Y, Gomes WR and VanDemark NL (1971) Secretion rates and chemical composition of oviduct and uterine fluids in rabbits *Journal of Animal Science* **33** 829–835
- Kane MT and Foote RH (1970) Culture of two- and four-cell rabbit embryos to the expanding blastocyst stage in synthetic media *Proceedings of the Society of Experimental Biology and Medicine* **133** 921–925
- Kennelly JJ and Foote RH (1965) Superovulatory response of pre- and post-pubertal rabbits to commercially available gonadotrophins *Journal of Reproduction and Fertility* **9** 177–188
- Lawitts JA and Biggers JD (1991) Optimization of mouse embryo culture media using simplex methods *Journal of Reproduction and Fertility* **91** 543–556
- Lawitts JA and Biggers JD (1992) Joint effect of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture media *Molecular Reproduction and Development* **31** 189–194
- Li J and Foote RH (1995) Effect of inositol and glycine with increasing sodium chloride and constant osmolality on development of rabbit embryos *Journal of Assisted Reproduction and Genetics* **12** 141–146
- Maurer RR, Onuma H and Foote RH (1970) Viability of cultured and transferred rabbit embryos *Journal of Reproduction and Fertility* **21** 417–422
- Naglee DL, Maurer RR and Foote RH (1969) Effect of osmolality on *in vitro* development of rabbit embryos in a chemically defined medium *Experimental Cell Research* **58** 331–333
- Pursel VG, Wall RJ, Rexroad CE and Brinster RL (1985) A rapid whole-mount staining procedure for nuclei of mammalian embryos *Theriogenology* **41** 1571–1583
- Rambhatla L and Latham KE (1995) Strain-specific progression of α -amanitin-treated mouse embryos beyond the two-cell stage *Molecular Reproduction and Development* **41** 16–19
- Roblero L, Biggers JD and Lechene CP (1976) Electron probe analysis of the elemental microenvironment of oviductal mouse embryos *Journal of Reproduction and Fertility* **46** 431–434
- Schultz SG and Curran PF (1970) Coupled transport of sodium and organic solutes *Physiological Reviews* **50** 637–718
- Summers MC, Bhatnagar, Lawitts JA and Biggers JD (1995) Fertilization *in vitro* of mouse ova from inbred and outbred strains: complete preimplantation embryo development in glucose-supplemented KSOM *Biology of Reproduction* **53** 431–437
- Whitten WK and Biggers JD (1968) Complete development *in vitro* of the preimplantation stage of the mouse in a simple chemical defined medium. *Journal of Reproduction and Fertility* **17** 399–441