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Platelet Activating Factor (PAF) Production by Mouse Embryos In Vitro and Its Effect on Embryonic Metabolism

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Factors affecting the production of platelet activating factor (PAF) by mouse embryos during culture in vitro were investigated. Detectable levels of embryo-derived PAF were produced within 1–4 hr with maximum PAF activity being observed after 6 hr of culture in vitro. The amount of PAF detected in media after 24 hr of culture of two-cell embryos was equivalent to 12.8 ng PAF/embryo. However, differences in activity were apparent with increased time in culture. Reduced synthesis of PAF during culture in vitro was supported by the observation that morulae stage embryos collected fresh from the reproductive tract displayed more PAF activity than morulae resulting from the 48 hr culture of two-cell embryos. In addition to determining production characteristics of PAF by embryos, we also show that the production of CO_2 from carbon-1 position of lactate is positively correlated with the ability of embryos to develop during subsequent culture in vitro and therefore could be used as a measure of embryo viability. Furthermore, culture of embryos in media supplemented with PAF resulted in an increase in lactate utilization demonstrating a direct effect of PAF on the embryo. As PAF is produced by preimplantation embryos, an autocoid role of PAF in regulating embryo development is implicated. Therefore, the reduced production of PAF by embryos in vitro may explain the decreased viability of embryos commonly observed following their culture in vitro.

Key words: embryo, lactate, carbon dioxide

The preimplantation mouse [1,2] and human [3] embryo produced an ether phospholipid that was homologous with 1-o-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine (platelet activating factor, PAF acether, PAF). PAF appeared to be essential for the establishment of pregnancy. In mice, the injection of inhibitors of PAF-induced platelet activation (Iloprost and SRI 63-441) throughout the preimplantation phase of development reduced the ability of embryos to undergo implantation [4,5]. It was also shown

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that a correlation existed between the production of PAF by embryos produced by in vitro fertilization and their subsequent pregnancy potential [6].

To help assess the role of PAF in embryo development, this study aims to (a) establish the effects of culture in vitro on the production characteristics of PAF by embryos and (b) to validate the use of a metabolic marker as a means of assessing embryo viability and to use this marker to determine the effects of PAF on the pregnancy potential of cultured embryos.

MATERIALS AND METHODS

Embryo Production and Culture

Six-week-old random-bred Swiss albino mice of the Quackenbush strain (QS) were superovulated by the intraperitoneal injection of 10 i.u. pregnant mare serum gonadotrophin (PMSG: Folligon, Intervet, Boxmeer, Holland) and 10 i.u. human chorionic gonadotrophin (hCG: Chorulon, Intervet) given 48 hr apart. Females were mated with males of proven fertility. Embryos were recovered by flushing the uteri and/or oviducts at various times after mating. Flushing was performed with a Hepes buffered medium [7] supplemented with 3 mg bovine serum albumin (BSA)/ml (Commonwealth Serum Laboratories, Melbourne, Vic, Australia).

Embryos were cultured in vitro in an atmosphere of 5% CO_2 in air at 37°C in either 1.0 ml aliquots of bicarbonate buffered Quinns medium [7] supplemented with 3 mg BSA/ml (Q + BSA) in 5 ml plastic tubes (Falcon, Becton, and Dickinson Co., Rutherford, NJ) or in 20 μl droplets under paraffin oil in plastic tissue culture dishes (Falcon). Some embryo culture media was supplemented with PAF (No. P-9525, Sigma Chemical Co., St. Louis, MO). PAF was not further purified prior to it being prepared as a 1 mg/ml stock solution in chloroform and stored at −20°C. Aliquots were transferred to siliconized sterile tubes under a continuous stream of nitrogen and resuspended with Q + BSA to a final concentration of 1.0 μg PAF/ml.

Detection of PAF Activity

An in vivo bioassay [8] utilizing eight-week-old QS mice was used for the detection of PAF activity in the culture media in which embryos were grown. Briefly, 200 μl of untreated culture medium was administered as an intraperitoneal injection to mice that had been splenectomized a minimum of 7 days previously. Immediately before and 30 min after the injection, 10 μl of blood was taken from the periorbital plexus and diluted with 1 ml ammonium oxalate (1% v/v). Platelet counts were then performed by the use of a Neubauer haemocytometer (Weber, England). The reduction in the peripheral platelet count was an indication of PAF activity.

Two-cell mouse embryos were collected (40–42 hr post hCG injection) and cultured in groups of 30 in Q + BSA for 1, 4, 6, or 24 hr. The activity of PAF in the culture media was measured and compared with that of culture medium alone (not used for embryo culture) and media in which degenerate two-cell embryos were cultured for 24 hr. Degenerate two-cell embryos were classified as those embryos that upon collection from the reproductive tract had cytoplasmic fragments, were of granular appearance, or had grossly misshapen blastomeres.

A second experiment was conducted, in which groups of 30 two-cell embryos were cultured in vitro for a total of 72 hr. Over three sequential 24 hr intervals, the

developmental stage of embryos was assessed and they were then transferred to fresh culture medium. PAF activity was measured in the culture medium for each of the three 24 hr culture periods representing consecutive embryonic development stages. These levels were compared with those of media from freshly collected morulae and blastocyst stage embryos recovered on days 3 and 4 of pregnancy, respectively, and cultured in vitro for 24 hr.

In an attempt to achieve approximate quantification of the amount of PAF produced by two-cell mouse embryos, known concentrations of PAF (0, 0.1, 0.15, 0.5, and 1.0 μg/ml) were injected into splenectomized mice and the percentage reduction in platelet numbers recorded. The relationship between PAF concentration and reduction in platelet numbers was used to estimate the amount of PAF present in media after groups of 10, 20, 30, and 45 two-cell embryos had been cultured for 24 hr.

Measurement of $^{14}CO_2$ From DL [1-^{14}C]Lactate

Assessment of oxidative metabolism by embryos was used to determine whether it could predict the developmental potential of embryos in culture. The decarboxylation of [1-^{14}C]lactate by two-cell embryos was measured and compared with their ability to undergo further development during 72 hr culture in vitro. The production of $^{14}CO_2$ from lactate was measured by incubating single embryos in a Hepes buffered, carbohydrate-free medium [9] supplemented with 1.12 mM DL [1-^{14}C]lactate (specific activity: 0.98 GBq/mmole, Amersham, UK). The medium also contained 3 mg BSA/ml. Incubations were carried out in 3.0 μl drops suspended from the lids of 1.5 ml microcentrifuge tubes in the presence of 1.0 ml 0.15 M NaOH. After 2.0 hr at 37°C, the NaOH was quantitatively removed, counted, and the amount of trapped $^{14}CO_2$ calculated by use of the specific activity of the radiolabelled lactate. Embryos were transferred to Q + BSA and cultured for 72 hr after which the stage of development was recorded.

To investigate the influence of culture in vitro on lactate utilization, embryos at various cell stages (two-cell, eight-cell, morula, and expanded blastocyst) were collected and the production of CO_2 from lactate assessed. These levels were compared with those of embryos collected at earlier cell stages and cultured to similar developmental stages as freshly collected embryos.

The effect of PAF supplementation of culture media and the time of exposure of embryos to PAF on lactate utilization was investigated to determine if PAF had a direct influence on embryo metabolism. Two-cell embryos were cultured for 0, 24, 48, or 72 hr in Q + BSA supplemented with 0 (control) or 1.0 μg PAF/ml. This dose was chosen because 1.0 μg PAF/ml was previously demonstrated [10] to cause maximal stimulation of lactate by two-cell mouse embryos. When PAF was present during culture in vitro it was also added to the assay incubation media at the same concentration and the lactate utilization assay was performed for 2 hr at the termination of the culture period.

RESULTS

PAF activity in embryo culture media increased with increasing time of two-cell mouse embryos in culture for up to 6 hr. However, after 24 hr culture, the percentage decrease in platelet numbers (Table I, Exp. 1: 85.5 ± 1.7 (29) and Exp. 2: 82.6 ± 1.4 (10)) did not differ substantially from that at 6 hr (81.5 ± 0.8 (4)), demonstrating no further increase in the amount of PAF present in the media. Degenerate two-cell

TABLE I. Production of PAF, As Assessed by the Reduction in Mouse Platelet Numbers, by Two-Cell, Four-to-Eight-Cell, Morula, Expanded Blastocyst, and Degenerate Mouse Embryos Cultured In Vitro for Varying Lengths of Time (Mean ± SEM)

Experiment	Time in culture (hr)	Cell stage		Percent original platelet count (n)
		Start	Finish	
1	1	Two-cell	Two-cell	94.5 ± 2.6 (8)
	4	Two-cell	Two-cell	91.8 ± 2.1(12)
	6	Two-cell	Two-cell	81.5 ± 0.8 (4)
	24	Two-cell	Four-to-eight-cell	85.5 ± 1.7(29)
	24	Degenerate	Degenerate	100.3 ± 0.5(11)
	—	Control medium		100.6 ± 1.3 (5)
2	24	Two-cell	Four-to-eight-cell	82.6 ± 1.4(10)
	24[a]	Four-to-eight-cell	Morula	85.2 ± 3.4(10)
	24[b]	Morula	Expanded blastocyst	94.0 ± 2.6(10)
	24	Morula	Expanded blastocyst	81.9 ± 1.0 (6)
	24	Blastocyst	Expanded blastocyst	84.6 ± 0.6(12)

[a]Total of 48 hr of culture.
[b]Total of 72 hr of culture.

TABLE II. Effect of Number of Embryos Cultured on the Reduction of Mouse Platelet Numbers As Compared With Known Concentrations of PAF (Mean ± SEM)

Conc. PAF (μg/ml)	Percent original platelets (n)	Number of embryos	Percent original platelets (n)
0.0	100.6 ± 1.35(5)	10	98.0 ± 1.04(3)
0.1	94.7 ± 2.33(4)	20	95.3 ± 2.73(3)
0.15	92.2 ± 1.35(6)	30	86.7 ± 1.48(6)
0.5	86.3 ± 1.16(14)	45	80.1 ± 2.58(5)
1.0	67.1 ± 2.68(7)		

embryos cultured for 24 hr and culture medium alone failed to cause a reduction in platelet numbers, indicating an absence of PAF at levels detectable in the assay system.

After 72 hr in culture, two-cell embryos developed to the expanded blastocyst stage. However, a linear ($P < 0.05$) reduction in PAF activity was observed for culture media from three consecutive 24 hr periods during this period of culture in vitro (Table I, Exp. 2). This reduction in the capacity of embryos to produce PAF appears to be a factor of culture in vitro and not associated with the advancing developmental stage since morulae and blastocysts, when freshly collected from the reproductive tract, produced the same levels of PAF as two-cell embryos (Table I, Exp. 2).

The injection of culture media supplemented with various doses of PAF (0, 0.1, 0.15, 0.5, and 1.0 μg/ml) resulted in a linear decrease in the percentage reduction of platelet numbers (Table II). The regression equation for the relationship between the percentage reduction in platelet numbers (%RPN) and concentration of PAF was as follows: PAF (μg/ml) = 2.74 − 0.0267 (%RPN).

Media from the culture of two-cell embryos in groups of 10 to 45 for 24 hr also resulted in a decrease in the percentage reduction of platelet numbers. The amount of PAF produced during the 24 hr culture period was calculated to be 12.3 ± 2.8 (3), 9.7 ±

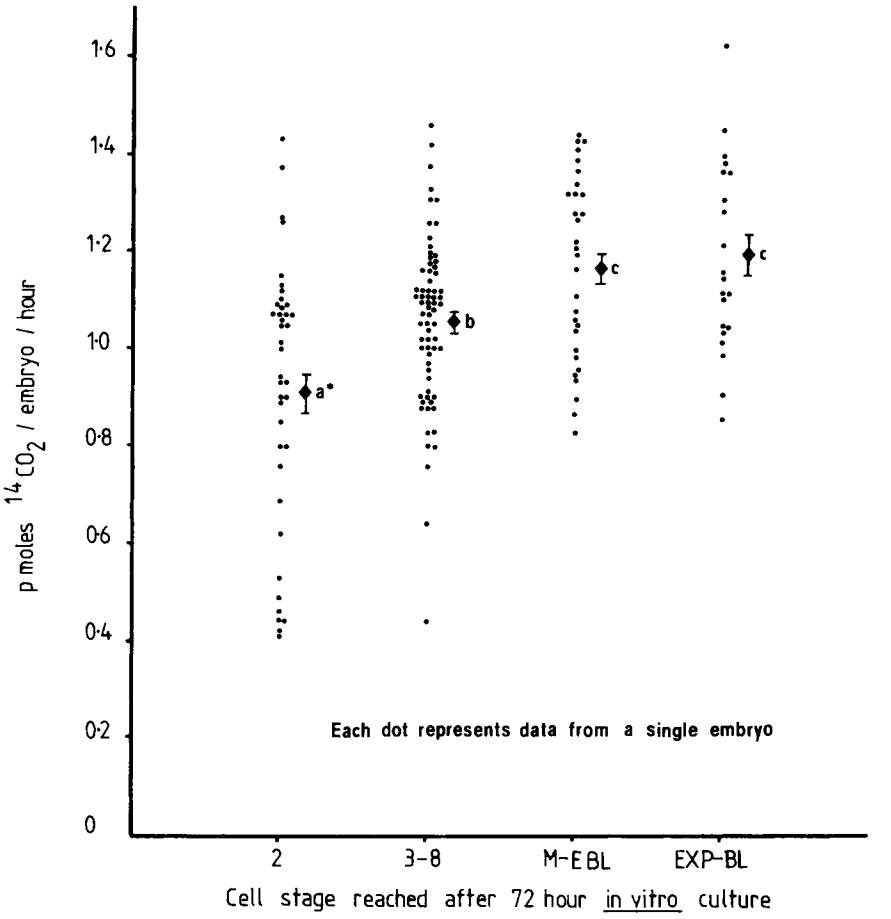

Fig. 1. Relationship between the production of $^{14}CO_2$ from [1-^{14}C]lactate by two-cell mouse embryos and their subsequent development after 72 hr of culture in vitro. *, values with different superscripts differ significantly ($P < 0.05$).

3.6 (3), 14.1 ± 1.3 (6), and 13.4 ± 1.5 ng PAF/embryo (mean ± SEM (n)) when they were cultured as groups of 10, 20, 30, and 45, respectively. No significant differences owing to number of embryos were observed. Overall, the mean amount of PAF produced was 12.8 ± 1.0 (17) ng/embryo 24 hr^{-1}.

There were marked differences between individual two-cell embryos in their capacity to utilize lactate during a two-hour assay period. To test whether this variable response was indicative of developmental potential, embryos were cultured in vitro for a further 72 hr, after which their developmental stage was recorded. In a retrospective analysis, it was observed that those two-cell embryos that produced relatively more CO_2 from lactate, grew to a more advanced developmental stage (Fig. 1). Those embryos that developed fully (expanded blastocysts) produced 1.19 ± 0.04 pmoles CO_2/embryo·hr^{-1}, whilst those failing to undergo further development produced 0.91 ± 0.04 pmoles CO_2/embryo·hr^{-1} ($P < 0.001$). However, the culture conditions during assay significantly affected subsequent embryonic development as fewer embryos developed through

TABLE III. Influence of Cell Stage and Time of Culture In Vitro on the Production of $^{14}CO_2$ From 1.12 mM [1-^{14}C]Lactate by Mouse Embryos (Mean ± SEM)

Cell stage		Time in	Production of $^{14}CO_2$ from
Collection	After culture	culture (hr)	1.12 mM [1-^{14}C]lactate
Two-cell	Two-cell	0	1.23 ± 0.04 (23)[a]
Eight-cell	Eight-cell	0	1.41 ± 0.06 (16)
Two-cell	Eight-cell	24	1.49 ± 0.04 (22)
Morula	Morula	0	2.45 ± 0.12 (20)
Eight-cell	Morula	24	1.57 ± 0.14 (13)
Two-cell	Morula	48	2.32 ± 0.12 (22)
Expanded blastocyst	Expanded blastocyst	0	2.61 ± 0.16 (18)
Morula	Expanded blastocyst	24	1.99 ± 0.12 (21)
Eight-cell	Expanded blastocyst	48	1.70 ± 0.10 (21)
Two-cell	Expanded blastocyst	72	1.72 ± 0.09 (20)

[a]pmoles $^{14}CO_2$/embryo hr^{-1} (n).

to the blastocyst stage of development than did control embryos (49 of 60—31% vs. 47 of 61—77%; $\chi^2 = 38.74$; $P < 0.001$).

The production of CO_2 from lactate by freshly collected mouse embryos increased as the stage of development at the time of collection increased (Table III). It was observed, however, that culture in vitro for varying time periods generally reduced the ability of morula and expanded blastocyst stage embryos to utilize lactate compared with embryos of similar developmental stages collected fresh from the reproductive tract, just as there was reduced net PAF production. Equivalent amounts of CO_2 were produced by freshly collected eight-cell embryos and eight-cell embryos resulting from 24 hr culture of two-cell embryos.

Supplementation of media with 1.0 μg PAF/ml resulted in a significant increase in the quantity of $^{14}CO_2$ produced by embryos from lactate compared to controls (Table IV). This response was observed for freshly collected two-cell embryos and also for embryos at the four-to-eight-cell, morula, and expanded blastocyst stage after 24, 48, and 72 hr culture in vitro, respectively. When individual embryo incubations were performed (Table IV, Exp. 1) an 11.4% increase in CO_2 production by two-cell embryos was observed whereas at the expanded blastocyst stage 50.0% more CO_2 was produced. When embryos were cultured in groups of ten (Table IV, Exp. 2) increases in CO_2 production of 15.9% and 9.7% owing to PAF were observed for embryos at the four-to-eight-cell and morula stages, respectively.

DISCUSSION

Platelet activating factor is produced during culture in vitro by mouse [1,2] and human [3] embryos. Furthermore, studies utilizing PAF inhibitors of platelet activation have suggested that PAF is essential for the establishment of pregnancy in mice [4,5]. Therefore, factors affecting PAF production by embryos during culture in vitro are of interest in understanding the role of PAF in early embryonic development.

TABLE IV. Production of $^{14}CO_2$ From [1-^{14}C]lactate by Two-Cell, Four-to-Eight-Cell, Morula, and Expanded Blastocyst Stage Embryos After Culture In Vitro From Two-Cells in Media Supplemented With 0 (Control) or 1.0 μg PAF/ml (Mean ± SEM)

Experiment	Cell stage	Time in culture (hr)	Media	CO_2 production: 1.12 mM lactate pmoles/embryo/hr
1[a]	Two-cell	—	Control	1.23 ± 0.04 (15)[c]
			1.0 PAF	1.37 ± 0.04 (19)
	Expanded blastocyst	72	Control	1.52 ± 0.08 (19)
			1.0 PAF	2.28 ± 0.13 (19)
2[b]	Four-to-eight-cell	24	Control	1.13 ± 0.06 (5)
			1.0 PAF	1.31 ± 0.08 (5)
	Morula	48	Control	2.48 ± 0.01 (5)
			1.0 PAF	2.72 ± 0.07 (5)

[a]Individual embryo incubations.
[b]Groups of ten embryos incubated.
[c]Numbers in parentheses represent number of observations.

Detectable levels of embryo-derived PAF are produced by mouse embryos in vitro within 1–4 hr with maximum PAF activity being observed after 6 hr of culture in vitro. The amount of PAF detectable in media after the culture of two-cell embryos for 24 hr was equivalent to 12.8 ng PAF/embryo representing no further increase in the PAF concentration over that present at 6 hr. This plateau in detectable PAF may have been due to reduced synthesis. This possibility was supported by the observation that freshly collected morulae stage embryos displayed more PAF activity than morulae resulting from the 48 hr culture of two-cell embryos.

PAF activity in culture media may be a reflection of both the embryos reduced ability to produce PAF with increased time in culture and its simultaneous degradation. The relative contributions of these mechanisms to the apparent loss of PAF activity are not clear from the present study. However, reduced synthesis of PAF may be due to starvation of critical substrates or growth factors in vitro or possibly to a negative feedback of PAF in the culture media on further embryonic production. Degradation is known to occur by metabolism of PAF to its key intermediate, lyso-PAF, by the enzyme acetylhydrolase [11]. This enzyme is present in serum and plasma [12] and has been detected within a number of cell types [13], though its activity within mouse embryos has not been investigated.

Adverse effects of culture in vitro on embryo development as compared with in vivo grown embryos have been previously reported [14–17]. In mice, these effects include reduced rates of cleavage from the two-cell to the expanded blastocyst stage of development, reduced rates of implantation following transfer, and decreased foetal weights on day 17 of pregnancy. These adverse effects of culture are consistent with our observations that culture in vitro reduces the capacity of embryos to produce PAF. In view of the recent observation that PAF is essential for the establishment of pregnancy in the mouse [4,5], the reduction in PAF production provides a possible explanation for the reduced viability of embryos following culture in vitro.

Various biochemical and biophysical parameters of embryos have been measured to assess whether they reflect the ability of the embryo to develop in vitro and in vivo [18–22]. In the present study, the production of CO_2 from carbon-1 position of lactate was measured to assess the rate of entry of lactate into the tricarboxylic acid (TCA) cycle [23] to determine whether metabolically more active embryos have a greater capacity to develop during 72 hr culture in vitro. Indeed, the production of CO_2 from lactate was positively correlated with the ability of embryos to develop. That is, those embryos that developed to the expanded blastocyst stage produced more CO_2 from lactate when assayed at the two-cell stage than did embryos that failed to develop or developed to the three-to-eight-cell or morula-early blastocyst stages only. Because of the suboptimal development potential of embryos after being exposed to the assay conditions, the data may more correctly reflect the ability of embryos to overcome the adverse effects of culture rather than their inherent ability for normal development. The uptake of glucose by cattle [24] and mouse [25] embryos has been measured to assess its use as an index of embryo viability and proved to be positively correlated with their ability to implant following transfer. Similarly, pyruvate utilization by healthy human embryos was greater than for embryos classed as degenerate [26].

Culture conditions in vitro known to reduce embryo viability and foetal development [14] were also responsible for a reduction in lactate utilization. A reduction in metabolic activity of blastocysts derived from cultured eight-cell embryos, as assessed by the production of CO_2 from radiolabelled glucose, compared with that of freshly collected uterine blastocysts has also been observed [27]. The adverse effect of culture in vitro was more apparent at the blastocyst stage of development than at earlier cell stages. A similar variable effect of culture owing to cell stage was observed for the viability of embryos following transfer to pseudopregnant recipients [14]. The authors suggested that a cumulative effect of suboptimal culture medium or a greater intrinsic susceptibility of blastocysts to adverse culture conditions were responsible for the phenomenon.

The current study has shown that culture in vitro reduced the ability of embryos to produce PAF and that the production of CO_2 from lactate was an indicator of developmental potential in vitro. However, although PAF appears essential for the establishment of pregnancy it has not been ascertained whether its effect is exhibited directly on the embryo or via inducing changes in maternal physiology. In this respect, the observed increase in lactate utilization by embryos in the presence of PAF demonstrates a direct effect of PAF on the embryo. Since PAF is produced by preimplantation embryos, an autocoid role of this phospholipid is implicated. No reduction in the magnitude of the response of embryos to PAF for 24–72 hr was observed. This effect may indicate that embryos are not desensitized to PAF as are other cell types such as rabbit platelets [28] and neutrophils [29], which exhibit a desensitization of their response to PAF during excessive or repeated exposure. However, in this study, it is unknown whether the PAF remained unmetabolized throughout the variable periods of culture. Furthermore, the response may also be explained by PAF switching on metabolic processes that continue to function without further PAF stimulation.

The marked affect of PAF on lactate utilization suggests that supplementation of media with PAF may influence the pregnancy potential of cultured embryos. Indeed, preliminary results suggest that PAF supplementation of culture media increases embryo cleavage rates and their ability to implant following transfer [30].

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REFERENCES

1. O'Neill C: J Reprod Fertil 73:567–577, 1985.
2. O'Neill C: J Reprod Fertil 75:375–380, 1985.
3. Collier M, O'Neill C, Ammit AJ, Saunders DM: Hum Reprod 3:993–998, 1988.
4. Spinks NR, O'Neill C: Lancet 1:106–107, 1987.
5. Spinks NR, O'Neill C: J Reprod Fertil 84:89–98, 1988.
6. O'Neill C, Saunders DM: Lancet 2:1034–1035, 1984.
7. Quinn P, Warnes GM, Kerin JF, Kirby C: Ann NY Acad Sci: 195–203, 1985.
8. O'Neill C: J Reprod Fertil 73:559–566, 1985.
9. O'Fallon JV, Wright RW: Biol Reprod 34:58–64, 1986.
10. Ryan JP, Wiegand MH, O'Neill C, Wales RG: Proc Aust Soc Reprod Biol 19:47, 1987.
11. Renooij W, Wykle RL, Blank ML, Lee T, Malone B, Fitzgerald VL, Snyder F: Fed Proc 39:2187, 1980.
12. Stafforini DM, McIntyre TM, Carter ME, Prescott SM: J Biol Chem 262:4215–4222, 1987.
13. Blank ML, Lee T, Fitzgerald V, Snyder F: J Biol Chem 256:175–178, 1981.
14. Bowman P, McLaren A: J Embryol Exp Morphol 23:693–704, 1970.
15. Bowman P, McLaren A: J Embryol Exp Morphol 24:203–207, 1970.
16. McReynolds HD, Hadek R: J Exp Zool 182:95–118, 1972.
17. Harlow GM, Quinn P: Aust J Biol Sci 35:187–193, 1982.
18. Mohr LR, Trounson AO: J Reprod Fertil 58:189–196, 1980.
19. Renard JP, Menezo Y, Heyman Y: Theriogenology 17:106, 1982.
20. Hoppe RW, Bavister BD: Anim Reprod Sci 6:323–335, 1983.
21. Trounson AO: Theriogenology 19:17–29, 1983.
22. Riegar D: Theriogenology 21:138–149, 1984.
23. Wales RG: J Reprod Fertil 76:717–725, 1986.
24. Renard JP, Philipon A, Menezo Y: J Reprod Fertil 58:161–164, 1980.
25. Gardner DK, Leese HJ: J Exp Zool 242:103–105, 1987.
26. Leese HJ, Hooper MAK, Edwards RG, Ashwood-Smith MJ: Hum Reprod 1:181–182, 1986.
27. Menke TM, McLaren A: J Reprod Fertil 23:117, 1970.
28. Henson PM: J Exp Med 143:937–951, 1976.
29. Shaw JO, Pinkard RN, Ferrigni KS, McManus LM, Hanahan DJ: J Immunol 127:1250–1255, 1981.
30. Ryan JP, Spinks NR, O'Neill C, Saunders DM, Wales RG: Proc Fertil Soc Aust 6:47, 1987.