

The role of pregnancy-specific β -1 glycoprotein (SP1) in assessing human blastocyst quality *in vitro*

R.R.Saith^{1,3}, N.A.Bersinger², D.H.Barlow¹ and I.L.Sargent¹

¹IVF Unit, Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital, Oxford OX3 9DU, UK and ²Department of Obstetrics and Gynaecology, University of Berne, Berne CH-3012, Switzerland

³To whom correspondence should be addressed

The advent of new culture techniques resulting in more than 60% of embryos developing *in vitro* to the blastocyst stage suggests that blastocyst transfer in humans with its potential to increase in-vitro fertilization success rates could be a feasible option. Blastocyst quality markers, however, are required to ensure that an increase in numbers is not achieved at the expense of lowering quality. We have previously reported a morphology based method for grading blastocysts. The current study sought to determine whether the secretion of pregnancy-specific β -1-glycoprotein (SP1) (a trophoblast product known to be associated with fetal well-being) by blastocysts of differing quality was reflected in the morphological grading. SP1 concentrations were measured in the culture medium of grade 1 ($n = 19$), grade 2 ($n = 4$) and grade 3 ($n = 4$) blastocysts as well as vacuolated morulae ($n = 6$) daily from day 1 to day 14. Cumulative SP1 concentrations secreted by blastocysts were significantly higher than those secreted by vacuolated morulae. However, SP1 levels could not distinguish between blastocysts of differing morphological grades. The inconsistent pattern of secretion suggests that at this early stage of development, SP1 secretion is probably not sufficiently defined to allow any differences in levels to be reflected by the morphological grading.

Key words: blastocyst quality/human blastocyst/IVF/pregnancy-specific β -1 glycoprotein/SP1

Introduction

After more than two decades of research, the in-vitro fertilization (IVF) live birth rate is still only 13.2% (Human Fertilisation and Embryology Authority of UK, Annual Report, 1994). One possible reason for the low success could be the transfer of four to eight cell embryos to an asynchronous uterine environment on day 2 or 3 after insemination. Blastocyst transfer is routinely practised in several domestic species e.g. cattle, giving pregnancy rates of 40–60% (Iritani, 1988). This suggests the beneficial effect of synchronizing the stage of embryo development with that of the uterine endometrium.

In humans, although initial studies by Bolton *et al.* in 1991

showed a low success rate with the transfer of blastocysts grown in conventional culture media, subsequently a number of studies have shown blastocyst transfer to yield success rates as high as 50%. Success was particularly observed in patients who had repeated failed IVF attempts (Ménézo *et al.*, 1992; Schillaci *et al.*, 1994; Ciriminna *et al.*, 1995). Besides, co-culture techniques have resulted in an increase in the percentage of embryos progressing to the blastocyst stage. Although the value of co-culture has been contested by Van Blerkom in 1993, a number of groups have reported on blastocyst formation rates >60% with improved culture techniques (Ménézo *et al.*, 1990; Bongso *et al.*, 1994; Ciriminna *et al.*, 1995). Safeguards are, however, required to ensure that an increase in blastocyst numbers does not take place at the cost of lowering blastocyst quality. This highlights the need to develop reliable methods of assessing the quality of blastocysts developed under varying culture conditions. Good quality blastocysts could then be selected for transfer.

We have previously developed a system for separating blastocysts of differing quality into three grades based on their morphological appearance (Dokras *et al.*, 1993). Morphological grading is an attractive prospect as it is non-invasive and easily performed. These rules for grading, however, have been derived from experience and are subjective. Some objective validation of the grading scheme and its ability to capture blastocyst quality has been provided by studies which show that it is related to human chorionic gonadotrophin (HCG) secretion and nuclei counts (Dokras *et al.*, 1993). Hardy *et al.* (1989) have also shown that morphologically abnormal blastocysts have significantly lower nuclei counts than their normal counterparts. However, an investigation of other indicators of blastocyst quality is required to ensure that the morphological grading captures the overall wellbeing and functional capacity of the blastocyst and not just the cell number or the capacity to secrete HCG.

Pregnancy-specific β -1 glycoprotein (SP1), like HCG, is secreted by the trophoblast, and is detectable in the serum of pregnant women as early as day 7 after the luteinizing hormone peak (Grudzinskas *et al.*, 1977). Its levels are associated with fetoplacental wellbeing and are predictive of pregnancy outcome (Ahmed and Klopper, 1984). The exact role of SP1 remains unknown, but an immunosuppressive role in protecting the fetus from rejection by the maternal immune system has been postulated (Bischof, 1984).

Secretion of SP1 by cultured human embryos as early as day 3 has been reported by Dimitriadou *et al.* (1992). In contrast, HCG secretion has not been detected before day 6 and in most embryos only after day 7 (Dokras *et al.*, 1991). This observation, as well as the likelihood that HCG and

SP1 secretion by the embryo have different biochemical and physiological regulation (Bischof, 1984), suggests that assessment of SP1 may yield information relating to aspects of embryo development that are independent from HCG secretion. Given the biological significance of SP1, this study sought to measure concentrations of SP1 present in the embryo culture fluid and determine whether the morphological grading reflects the differences (if any) in concentrations of SP1 secreted by blastocysts of differing quality.

Materials and methods

Patients

The embryos used for research were spare embryos donated by couples attending the IVF unit at the John Radcliffe Hospital, Oxford. The couples gave informed consent and the project was approved by the Central Oxford Research Ethics Committee (COREC) and the Human Fertilisation and Embryology Authority (HFEA).

IVF procedure

Details of the IVF ovulation induction regime, oocyte retrieval and embryo culture before transfer were as previously described (Dokras *et al.*, 1990). On day 2 each embryo was assessed based on its morphological appearance, cleavage rate and characteristics of the related oocyte and follicle. The three embryos assessed as being the best were selected for transfer. Embryos not so selected were assigned for research purposes on the same day.

Embryo culture

This study was restricted to the culture of bipronucleate spare embryos. Once assigned for research, these embryos were transferred to individual wells of 24-well Nunclon plates (Nunc, Delta, Denmark) each of which contained 1 ml of Tyrode's 6 (T6) medium (Quinn *et al.*, 1985) supplemented with 10% heat-inactivated maternal serum. In cases where insufficient maternal serum was available, T6 supplemented with 10% pooled serum (from women whose IVF treatment resulted in successful pregnancies) was used. Every 24 h the embryos were assessed morphologically, before being transferred to a new well containing 1 ml of fresh equilibrated medium. The culture medium from the previous well was stored at -20°C . If an embryo became attached to the culture dish, the medium was carefully removed and replaced with fresh equilibrated medium without dislodging the embryo. Medium collected from embryos when allotted for research on day 2 contained factors secreted by the embryo over the previous 24 h and was therefore referred to as day 1 embryo culture fluid. Similarly, medium collected on day 3 of culture is designated day 2 embryo culture fluid and so on.

Morphological assessment and classification

Embryos were morphologically assessed using a Wilovert inverted phase contrast microscope (Leica, Milton Keynes, UK). All embryos were assessed daily up to day 8, after which only those embryos that had formed blastocysts or were vacuolated at the morula stage were cultured up to day 14. Details on the percentage of embryos progressing to various stages of development on in-vitro culture under the conditions described here can be found in Dokras *et al.* (1991). At the blastocyst stage, embryos were graded based on their morphological appearance essentially after Dokras *et al.* (1993). Briefly, grade 1 (BG1) were blastocysts characterized by an initially eccentric cavity progressing to a well expanded cavity lined by a distinct inner cell mass (ICM) region and trophectoderm layer. Grade 2 (BG2) blastocysts were similar in appearance to BG1 but were preceded by

a transitional phase showing single or multiple vacuoles with sharp borders at the morula stage. Grade 3 (BG3) were blastocysts with or without initial vacuolation which showed several degenerative foci in the ICM on the day of formation and an incompletely expanded cavity. On observation of their developmental potential, hatching was observed in 10 out of 19 BG1 (of these, three hatched partially), one out of four BG2 and only one out of nine BG3. Also included in the study were vacuolated morulae (VM). These were embryos which showed the presence of one or more small vacuoles at the morula stage which could occasionally be mistaken for early cavitation. On further monitoring up to day 14, these six morulae did not form blastocysts and the zona remained intact. They were, however, included in the study in order to determine the SP1 secretory capacity of such 'poor quality embryos' as compared to that of blastocysts.

ELISA

A highly sensitive (10 pg/ml) double-antibody microplate enzyme-linked immunosorbent assay (ELISA) was used to measure SP1 in the embryo culture fluid. High binding capacity 96-well plates (Maxisorp, Nunc, Denmark) were coated the day before assay with excess rabbit polyclonal anti-SP1 IgG (Dako-immunoglobulins a/s, Denmark) 25–40 pg/ml, in sodium carbonate, 0.05 M, pH 9.6, 75 μl /well. The coating solution was left overnight at 4°C and aspirated next morning. Excess sites were then blocked with 200 μl of bovine serum albumin (BSA), 5 mg/ml in phosphate buffered saline (PBS), pH 7.4, for 2 h at room temperature. Plates were then washed four times with PBS containing Tween-20 (0.1% v/v, PBST). High protein carrier buffer (BSA, 20 mg/ml, and Emulsit, a non-ionic detergent, 0.05% in PBS) was added to the culture medium aliquot (1:3) and the sample was then added to microplate wells in duplicate (75 μl /well). After initial incubation at 37°C for 60 min, the plate was left overnight at 4°C . Next morning, the wells were washed three times with PBST. The enzyme conjugate, horseradish peroxidase coupled to anti-SP1 IgG (Behringwerke, Germany), was diluted 1:100 with the above high protein buffer before use. Incubation was for 60 min at 37°C after which the wells were washed four times with PBST and finally with peroxide-free Tween. The reaction was developed with 100 μl sodium perborate (0.03%) as the substrate and ortho-phenylene diamine in citrate-phosphate buffer, 0.1 M, pH 5.0 (1 mg/ml) as the chromogen, for 30 min at room temperature in the dark. The reaction was stopped with 100 μl sulphuric acid, 2 M, the plates shaken for 1 min, and the absorption measured in a Labsystems (Finland) microplate ELISA reader at 492 nm. Lyophilized aliquots made from pooled late pregnancy serum, diluted in normal male serum calibrated against the World Health Organization (WHO) reference preparation 78/610, were used as assay standards. The detection limit of the test was 10 pg/ml.

Statistical analysis

The Mann-Whitney test was used to test for differences in SP1 secretion between the blastocysts and vacuolated morulae as well as the hatched and intact blastocysts. The Kruskal-Wallis test was used to test for differences in SP1 secretion between the three grades of blastocysts. In order to determine the cumulative secretion for each embryo, the area under the curve obtained by joining points showing the concentrations of secretion for each day was calculated (Matthews *et al.*, 1990). For ease of interpretation, comparison of the cumulative concentrations of SP1 secretion by the different groups has been shown here using notched box plots. The dotted line in the plots indicates the median while the notches approximate a 95% confidence region around the median. The notched boxes contain 50% of the data, 25% above and 25% below the median. Overlap of the notches indicates the absence of a significant difference between the medians.

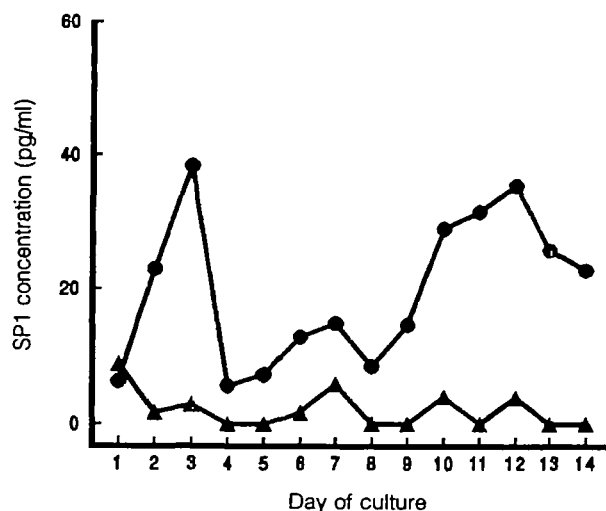


Figure 1. Mean daily pregnancy-specific β -1 glycoprotein (SP1) secretion by 32 blastocysts (\bullet) and six vacuolated morulae (\blacktriangle) from day 1 to day 14 of culture.

Results

SP1 secretion by 32 blastocysts and six vacuolated morulae was measured daily from day 1 to day 14. Concentrations of SP1 secreted by each embryo over each day were obtained by subtracting the levels detected in the respective culture medium control from the levels detected in the embryo culture fluid. Of the blastocysts, SP1 secretion was detected in 17/19 BG1, 4/4 BG2 and 6/9 BG3; 4/6 VM secreted SP1. The mean daily SP1 secretion for all blastocysts and VM is shown in Figure 1. The earliest detection of SP1 (i.e. concentrations >10 pg/ml) was on day 1 in eight out of the 38 embryos. Of these, two were BG1, two BG2, one BG3 and three VM. No individual embryo secreted SP1 over all 14 days of culture and the maximum number of days over which SP1 was detected was 10 for one BG1. Five blastocysts and one VM showed detectable secretion over 1 day only. Five out of the 38 embryos continued to secrete SP1 on day 14. Of these, three were BG1 and one each of BG2 and BG3.

The 32 blastocysts showed significantly higher ($P = 0.02$) cumulative SP1 secretion as compared to the six vacuolated morulae (Figures 2a and b). All three grades of blastocysts also showed a consistent peak in the mean concentration of secretion on day 3 which was absent in the vacuolated morulae (Table I; Figure 1). BG1 and BG2 show a trend towards higher daily cumulative SP1 concentrations than BG3 (Figure 3a). On statistical analysis, however, the three grades of blastocysts showed no significant difference in either the daily or cumulative SP1 concentrations for the three grades (Figure 3b). When the 12 blastocysts which hatched were compared with those that remained intact within the zona, there was no significant difference in the cumulative secretion (Figure 4). For individual embryos, there was no relation between the SP1 secretion and the morphology and development of the embryo.

Discussion

SP1 concentrations secreted by embryos, classified on the basis of their morphology, were measured to determine whether

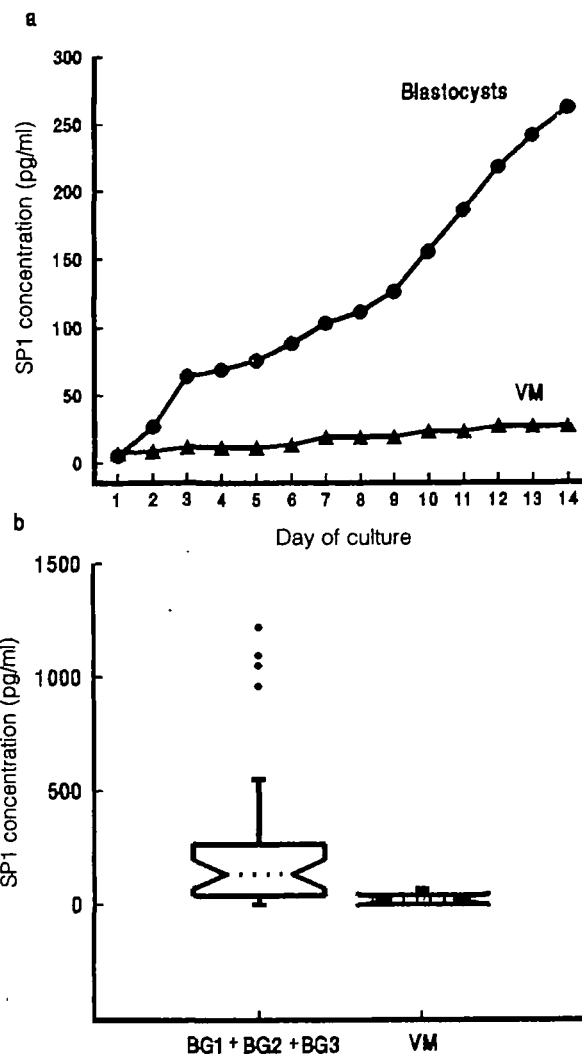


Figure 2. a Mean cumulative SP1 concentrations of 32 blastocysts (\bullet) and six vacuolated morulae (\blacktriangle) from day 1 to day 14 of culture. b Statistical comparison of cumulative SP1 secretion by 32 human blastocysts and six vacuolated morulae over 14 days of culture. For explanation of notched box plots, see statistical analysis section. BG1, BG2, BG3 represent decreasing quality grades of blastocysts; Vm = vacuolated morulae.

Table I. Daily pregnancy-specific β -1 glycoprotein (SP1) secretion (mean \pm SEM) in pg/ml of BG1, BG2, BG3 and VM from day 1 to day 14

Day	BG1	BG2	BG3	VM
1	5.93 \pm 3.25	4.26 \pm 4.25	7.56 \pm 6.40	8.80 \pm 5.43
2	21 \pm 10.61	45.67 \pm 45.67	19.22 \pm 11.66	1.67 \pm 1.67
3	38.50 \pm 21.76	71.50 \pm 60.70	23.44 \pm 16.26	2.83 \pm 2.83
4	6.59 \pm 3.28	5.25 \pm 5.25	3.89 \pm 3.89	0.00 \pm 0.00
5	7.72 \pm 3.80	0.00 \pm 0.00	9.88 \pm 6.55	0.00 \pm 0.00
6	8.68 \pm 4.87	58.00 \pm 58.00	6.44 \pm 6.44	1.67 \pm 1.67
7	22.58 \pm 7.67	7.25 \pm 7.25	2.00 \pm 2.00	5.83 \pm 4.17
8	13.44 \pm 6.58	0.00 \pm 0.00	2.22 \pm 2.22	0.00 \pm 0.00
9	22.11 \pm 9.83	7.25 \pm 7.25	2.00 \pm 2.00	0.00 \pm 0.00
10	25.16 \pm 8.38	81.50 \pm 72.78	13.89 \pm 8.35	4.00 \pm 4.00
11	40.44 \pm 16.46	58.00 \pm 58.00	2.11 \pm 2.11	0.00 \pm 0.00
12	45.22 \pm 21.87	42.00 \pm 42.00	10.88 \pm 7.17	3.83 \pm 3.83
13	36.18 \pm 21.10	7.50 \pm 7.50	13.00 \pm 13.00	0.00 \pm 0.00
14	33.35 \pm 23.40	7.75 \pm 7.75	7.75 \pm 7.75	0.00 \pm 0.00

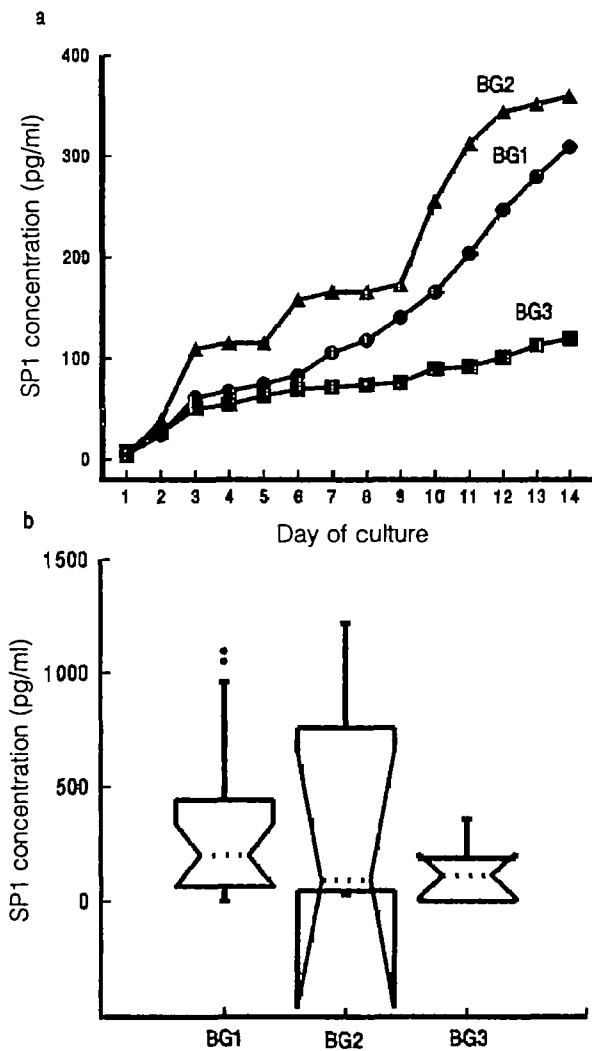


Figure 3. **a** Mean cumulative SP1 concentrations of 19 BG1 (●), four BG2 (▲) and nine BG3 (■) from day 1 to day 14 of culture. **b** Statistical comparison of cumulative SP1 secretion by 19 BG1, four BG2 and nine BG3 over 14 days of culture. For explanation of notched box plots, see statistical analysis section. See Figure 2 for definition of BG1, BG2 and BG3.

this grading system reflects variability in secretion (if any) between blastocysts of differing quality. SP1 was chosen for investigation since, later in pregnancy, serum SP1 concentrations can be used to distinguish between normal singleton pregnancies and abnormal pregnancies such as recurrent spontaneous abortions, intrauterine growth retardation, ectopic pregnancies, low birth weight and pre-term labour (Raman and Pawashe, 1986; Sinosich *et al.*, 1986; Johnson *et al.*, 1993a,b; Zhang *et al.*, 1993). Using a highly sensitive immunoassay, low concentrations of SP1 were detected in the embryo culture fluid, confirming earlier findings (Dimitriadou *et al.*, 1992). While Dimitriadou *et al.* (1992), however, studied the concentrations of SP1 in embryo culture fluid from day 3 onwards, we found that SP1 secretion in the embryo culture fluid can be detected as early as day 1. SP1 concentrations secreted by the blastocysts were significantly higher than those by the vacuolated morulae, suggesting that in general there is an association between SP1 and embryo quality. In addition

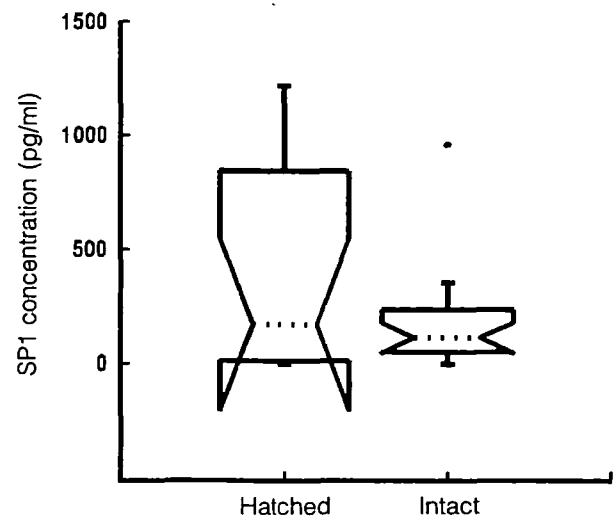


Figure 4. Comparison of cumulative pregnancy-specific β -1 glycoprotein (SP1) secretion by 12 hatched and 20 intact human blastocysts over 14 days of culture. For explanation of notched box plots, see statistical analysis section.

the blastocysts showed a pattern of secretion that differed from the VM with all three grades showing a distinct peak of secretion on day 3. This peak is of particular interest, as it could have the potential to discriminate between embryos destined to develop into blastocysts and those destined to form 12 vacuolated morulae. However, on analysis, neither the concentration of SP1 secretion on day 3 nor the cumulative day 1 to day 3 concentrations showed a statistical difference.

Blastocysts of differing developmental potential, as indicated by their ability or inability to hatch, could not be distinguished by the concentrations of SP1 secretion.

Although the better grade (as defined by morphology) blastocysts BG1 and BG2 showed a trend towards higher daily mean cumulative SP1 concentrations than BG3, there was no significant difference in the cumulative or daily SP1 secretion between the three grades of blastocysts. Despite the initial promise shown by differences in SP1 concentrations, between embryos of differing quality (i.e. blastocysts versus vacuolated morulae), the lack of a significant difference in SP1 concentrations secreted by blastocysts belonging to the three morphological grades suggests that the present grading system does not capture the ability of blastocysts to secrete SP1. Alternatively, it raises the possibility that at this early stage of development, SP1 secretion may not be sufficiently defined to be incorporated and reflected in a morphological grading scheme. This is demonstrated by the finding that SP1 was neither secreted by all blastocysts nor was it detected for individual blastocysts on all days of culture. Furthermore, the day of starting secretion was highly variable ranging from day 1 for six blastocysts to as late as day 7 for three and days 8 and 9 for one each respectively. Once secretion was detected, it did not necessarily persist, and it stopped and restarted at irregular intervals.

The concentrations of SP1 detected here were much lower than those reported by Pope *et al.* (1984) for baboon embryos and Dimitriadou *et al.* (1992) for human embryos. Possible causes for this could be the assay used and sub-optimal culture conditions. The highest concentration of SP1 detected in our

study was 391 pg/ml per day secreted by a grade 1 blastocyst on day 3 of culture, in contrast to 4800 pg/ml per 3 days reported by Dimitriadou *et al.* (1992). This large difference could have been due to the difference in culture media (they used Ham's F-10 supplemented with 7% maternal serum) which can affect embryo quality and thus secretory capacity. However, this is unlikely, as blastocysts of an obviously good quality, as indicated by their hatching, showed concentrations of SP1 secretion much lower than those reported by Dimitriadou *et al.* (1992). Another explanation could be that in our study the medium was changed daily, whereas Dimitriadou *et al.* (1992) changed the medium only once in 3 days for most embryos. It is possible that frequent changes of the culture medium could deplete embryo-derived factors which stimulate SP1 secretion. Unfortunately no direct comparison of SP1 concentrations in culture medium using the assay system employed by Dimitriadou *et al.* (1992) is possible as the Enzygnost SP1, Behringwerke kit used by them is no longer commercially available. Likewise, the media which they used to measure SP1 have also been utilized fully.

To conclude, differences in concentrations of SP1 secretion by cultured embryos are not reflected in the morphological grading. The absence of a pattern and inconsistent SP1 secretion, however, suggest that differences in SP1 concentrations secreted by blastocysts of differing quality are not defined enough to allow incorporation into the grading scheme. Other non-invasive markers of blastocyst quality therefore need to be investigated and the information obtained utilized to improve the grading scheme to allow it simultaneously to reflect differences in a number of markers. This would strengthen the case for using the grading scheme in practice for selecting blastocysts for transfer as well as for assessing the efficacy of blastocyst culture techniques. Such work is currently in progress in this laboratory.

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