Interest of co-cultures for embryos obtained by in-vitro fertilization: a French collaborative study*

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Co-cultures of human embryos, particularly with Vero cells, are used by several French groups, mainly in cases of successive failures of implantation. In most cases co-culture is continued until the blastocyst stage, expanded if possible. A total of 1603 co-cultures have been performed by 11 groups over a 2-year period. Of these, 41.8% of cleaved eggs (day 2) reached the blastocyst stage at day 5 or day 6. The mean pregnancy rate and implantation rate per transfer were 32.9 and 24.8% respectively, which represented a significant improvement compared to the transfer of 2 day old embryos. The rate of multiple pregnancies remained high (29.1%), which implies that there should be transfer of not more than two blastocysts. The rate of anomalies perceived at birth or in utero was not different from the rate observed in the general population, taking account of the maternal age.

Key words: birth abnormalities/co-cultures/human blastocysts/in-vitro fertilization/Vero cells

Introduction

The first co-cultures of mammalian embryos were performed with cellular monolayers of genital origin, originating mainly from the oviduct. Few studies concerned human embryos, because of the difficulty of collecting samples from human Fallopian tubes without bacterial or viral contamination. This problem can be overcome if the oviduct cells originate from the patient herself, but the protocol is tedious and time consuming. In 1990 a study was published showing that it was possible to use immortalized cell lines of extra-genital origin: Vero cells, which originate from kidney epithelium of African green monkey (Ménézo et al., 1990). In this study, the authors showed that >50% of embryos of even poor quality could reach the blastocyst stage when co-cultured. Further, delaying the time of transfer would permit a more efficient in-vitro selection of embryos, enabling the transfer of embryos with greater developmental potential. However, in several studies clinical results expressed in terms of clinical pregnancies were disappointing, including randomized protocols (Guerin et al., 1993; Janny et al., 1993; Sakkas et al., 1994). Therefore, some authors have proposed the use of cocultures only in cases of successive implantation failure (three or more). Some reports were positive (Ménézo et al., 1992), but control groups were generally lacking.

Clinical interest in co-cultures for human embryos has continued to be a subject of controversy. Several questions are still unresolved: (i) Is there a real benefit in the use of co-cultures, and if this is the case, must the technique be reserved for certain categories of patients, and which ones? (ii) Are cocultures strictly devoid of risks, with regard to the quality of the conceptus?

These questions prompted us to undertake a collaborative inquiry among the French teams that used co-cultures: since the majority used Vero cells, only data concerning these cells are detailed and discussed.

Materials and methods

Nature of the monolayer

Sixteen sets of answers were obtained concerning the use of co-cultures in routine practice of in vitro fertilization (IVF), among French IVF groups. The nature of the monolayer was the following: Vero cells: 11; endometrial cells from the patient: 2; granulosa cells: 2; cumulus cells: 1. Vero cells originated either from WHO Grants (four centres), or Flow laboratories (seven centres). In order to perform a homogeneous study, only data concerning Vero cells as monolayers, which represented the majority of cases and corresponded to the activity of 11 centres, were used for the study. A total of 126 co-cultures using patients’ cells (7.3% of the total) were therefore excluded from the study.

Criteria for the choice of co-cultures rather than conventional IVF procedures

All teams retained as dominant criterion the history of successive previous implantation failures, with the number 3 representing a general agreement. Other criteria were proposed by some groups: age of the female partner (two centres, the lower limits being 36 and 38 years respectively); means to reduce the risk of multiple pregnancies. These secondary criteria could represent a supplementary agreement for proposing co-cultures, but were themselves not a primary indication. Since in France the number of IVF attempts does not generally exceed the value of 4, the vast majority of patients included in this study were put through only one co-culture cycle (in other words: practically all cases corresponded to different patients).

Table I. Pooled data from 11 centres concerning co-cultures of human embryos on Vero cell monolayers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean values</th>
<th>Range of values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of co-cultures</td>
<td>1603</td>
<td>–</td>
</tr>
<tr>
<td>Blastocysts/cleaved embryos (%)</td>
<td>3828/9157 (41.8)</td>
<td>14–52</td>
</tr>
<tr>
<td>Clinical pregnancies/transfers (%)</td>
<td>480/1457 (32.9)</td>
<td>18.4–36.5</td>
</tr>
<tr>
<td>Implantation rate/blastocysts (%)</td>
<td>24.8</td>
<td>10–29.4</td>
</tr>
<tr>
<td>Multiple pregnancies (%)</td>
<td>29.1</td>
<td>21–32</td>
</tr>
<tr>
<td>Miscarriages (%)</td>
<td>15.9</td>
<td>10–21.2</td>
</tr>
<tr>
<td>Abnormalities* (%)</td>
<td>11/510 (2.3)</td>
<td>–</td>
</tr>
</tbody>
</table>

*Abnormalities detected at birth or during the pregnancy.

Table II. Pooled data for the five centres that had each performed >100 co-cultures during the study period (representing 87.4% of overall attempts)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean values</th>
<th>Range of values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of co-cultures</td>
<td>1401</td>
<td>–</td>
</tr>
<tr>
<td>Blastocysts/cleaved embryos (%)</td>
<td>42.7</td>
<td>32.9–52</td>
</tr>
<tr>
<td>Clinical pregnancies/transfers (%)</td>
<td>33.5</td>
<td>30.2–36.5</td>
</tr>
<tr>
<td>Implantation rate/blastocysts (%)</td>
<td>25.8</td>
<td>22.2–29.4</td>
</tr>
</tbody>
</table>

Table III. Details of abnormalities detected at birth or during pregnancy

<table>
<thead>
<tr>
<th>Nature</th>
<th>Outcome</th>
<th>Maternal age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymalformation</td>
<td>T.A.*</td>
<td>?</td>
</tr>
<tr>
<td>Polymalformation</td>
<td>stillbirth</td>
<td>31</td>
</tr>
<tr>
<td>Anencephaly</td>
<td>stillbirth</td>
<td>?</td>
</tr>
<tr>
<td>Hydrocephaly</td>
<td>good</td>
<td>?</td>
</tr>
<tr>
<td>Minor heart malformation</td>
<td>good</td>
<td>39</td>
</tr>
<tr>
<td>Trisomy 21</td>
<td>T.A.</td>
<td>39</td>
</tr>
<tr>
<td>Trisomy 21</td>
<td>T.A.</td>
<td>38</td>
</tr>
<tr>
<td>Trisomy 21</td>
<td>T.A.</td>
<td>37</td>
</tr>
<tr>
<td>Trisomy 21</td>
<td>T.A.</td>
<td>38</td>
</tr>
<tr>
<td>Trisomy 18</td>
<td>T.A.</td>
<td>43</td>
</tr>
<tr>
<td>Trisomy 18</td>
<td>stillbirth</td>
<td>31</td>
</tr>
</tbody>
</table>

*TA = therapeutic abortion.

**Conditions of co-culture and transfer**

The procedure for the use of Vero cell monolayers was very similar for the 11 groups: Vero cells were obtained and stored frozen; after thawing, cells were centrifuged and suspended in B2 medium (Ménézo et al., 1984), supplemented with fetal calf serum (FCS) 10% and antibiotics. Nunc multidishes (Nuncon, Denmark) were then seeded with this cellular suspension (100 000–200 000 cells per well), and placed in a thermostatically controlled chamber at 37°C, in an atmosphere composed of 5% CO₂ in air. No group overlaid the cultures with oil. Using this protocol, cell confluence was reached within 48 h. At this time, the original medium was removed and replaced with fresh B2 medium, without FCS but which contains bovine serum albumin (BSA) (10 g/l) as the source of protein. Insemination was performed by adding 50 000–100 000 motile spermatozoa to 1 ml of non-supplemented B2 medium containing oocyte–cumulus complexes and incubating at 37°C in 5% CO₂ in air. Twenty-four hours later, the cumulus layer was removed and those embryos exhibiting two pronuclear were deposited on the Vero cell monolayer (day 1).

Two centres chose to transfer embryos systematically on day 5, whereas the remaining nine waited until at least one embryo reached the blastocyst stage, expanded or not (day 5 to day 7). Nevertheless, transfers at day 7 were exceptional.

The number of embryos that were transferred varied according to the stage of evolution of the embryos (roughly two or three for blastocysts; three or four if only morulae were obtained), and to clinical situations: number of previous implantation failures, age of the female partner, etc.

**Statistics**

Comparison of pregnancy rates was made by contingency tables and χ²-tests.

**Results**

A total of 1603 co-cultures with Vero cells, corresponding to the total activity of 11 centres over a 2-year period, were included in the analysis (Table I). The mean rate of development to blastocyst stage from oocytes which had cleaved to the two, three or four cell stage at day 2 was 41.8%. The mean clinical pregnancy rate (fetal sac detected by ultrasonography) per transfer was 32.9%, whereas the implantation rate per blastocyst was 24.8%. The rates of multiple pregnancies and miscarriages were 29.1 and 15.9% respectively. For each parameter, the range of values according to the different centres was large. However, five teams out of 11 were responsible for almost 90% of the co-cultures, and the variations within these five groups were much reduced.

The mean rate of clinical pregnancies per transfer for these five groups was 33.5%. This was compared with the mean clinical pregnancy rate for these five groups over the same period, resulting from 5679 embryo transfers following conventional IVF procedures: 28.6%; the difference was statistically significant (χ² = 11.9: P < 0.001). If the results from all 11 groups were similarly compared, the difference remained statistically significant (χ² = 10.5: P < 0.002). Although complete information could not be obtained from all teams, some partial data were available. The ongoing pregnancy rates varied according to:

(i) the day on which blastocysts were transferred (three centres): 36.6 and 23.5% for blastocysts transferred at days 5 and 6 respectively (P < 0.001).

(ii) the stage of embryos that were transferred (three centres): 8.2, 22.5 and 40.1% from the transfer of morula, blastocyst or expanded blastocyst respectively (P < 10⁻⁶).

(iii) the number of blastocysts which were transferred (two centres): 22.3, 26.1 and 45.9% for one, two and three blastocysts transferred respectively (P < 0.001).

The number of abnormalities was 11/510 (2.3%). This number included malformations observed at birth and abnormalities detected during the pregnancy, and were mainly fetal trisomies (Table III). One case of anencephaly and one polynomalformation were detected by ultrasound. For five out of the six cases of trisomy, the maternal age varied from 37 to 43 years. For this age range, fetal karyotyping was routinely performed. Only one trisomy (18) was observed in a young mother (31 years old).

**Discussion**

Protocols which use co-culture with cells originating from the female genital tract (endometrial, granulosa and cumulus cells)
are difficult to perform in routine IVF procedure. In the present

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... of V ero cells, since two different sources

... are used by French groups who perform co-culture. The advantages

... are highly controlled for

... are used for the production of vaccines; (ii) they are easy to grow in a

... of media; (iii) they are immortalized, thus their physiological properties remain constant.

This study represents a retrospective analysis of co-culture practice among 11 French centres. Nevertheless, we can assume that the general biological design of both IVF and co-culture procedures was homogeneous between the teams, because of regular professional meetings where standardizations of new techniques were established. Any lack of uniformity concerns mainly the source of V ero cells, since two different sources were used in the present report and the effectiveness of a particular cell line is known to be dependent, in particular, on the number of passages. This passage number was similar between the two sources. Moreover, several teams had to change the supplier of their cell lines after they started to use co-culture systems, and these teams did not observe any variations in their data. Another source of variations may be constituted by the selection of patients to whom co-culture was proposed. In fact, the most important – if only – indication was the existence of three successive failures of implantation. The other situations mentioned by the teams (means to reduce the risk of multiple pregnancies; female age > 35 years) did not constitute sufficient criteria in themselves to induce the use of co-culture procedures.

The proportion of cleaved embryos reaching the blastocyst stage (41.8%) was not different from values reported in other studies (Guerin et al., 1993). However, it is lower than the value of 61% reported in the original study using V ero cells (Ménézo et al., 1990). This can be explained by the fact that, in this earlier work, the authors had no fixed time limit for embryos to develop to the blastocyst stage, whereas in the present study, in-vitro embryo development was restricted to 5 or 6 days. The mean rate of clinical pregnancies per transfer after co-culture was 32.9%, which was significantly higher than the mean value obtained by the same groups using conventional IVF procedures over the same 2 year period. This result is all the more interesting as the couples selected for co-cultures could be suspected of poor fertility concerning the female partners, because of successive implantation failures.

The mean implantation rate per blastocyst transferred was ~25%. This value is in agreement with data from other studies (Janny et al., 1993, 1994), although some authors have found lower values (Sakkas et al., 1994). Interestingly, this implantation rate of 25% was very consistent in the five main groups participating in the study, and represents a value twice as high as the implantation rate obtained after conventional IVF procedures, which varied between 10 and 15%.

Surprisingly, despite the publication of a large number of papers concerning co-cultures, numerous questions remain unanswered concerning the mechanisms through which somatic cells contribute to the embryo development (for review, see Bavister, 1995). Even the consequences of this interaction are still unclear: for example, it is generally observed that co-culture reduces the fragmentation rate during cleavage of embryos to blastocysts (Ménézo et al., 1990; Freeman et al., 1995). However, the clinical benefit of co-culture remains controversial; using granulosa cell co-cultures, Freeman et al. (1995) observed improved implantation rates and pregnancy rates. On the contrary, Leppens et al. (1996), using bovine kidney (MDBK) epithelial cells, showed that the inner cell mass of mouse blastocysts was larger after co-culture, but their implantation rate was not improved.

Considering the data from this multicentre study, it is tempting to propose the use of co-cultures for all patients. However, this technique is much more time-consuming than conventional IVF procedures, and offers no advantage if no more than three to four embryos are obtained. Conversely, co-cultures could be proposed following the retrieval of large numbers of embryos. However, as a higher level of heterogeneity was observed within the six groups performing co-culture only occasionally, this technique should be performed only by teams experienced in the use of cell culture, who are able to treat a sufficient number of cases to gain appropriate experience.

The rate of multiple pregnancies (29.1%) was comparable to that noted in the national French evaluation: 27.2% (FIVNAT, 1995). This result can be considered as disappointing, since one of the objectives mentioned by several groups for the use of co-cultures was precisely to reduce the risk of multiple pregnancy by transferring not more than two blastocysts. However, there was some disparity between objectives and reality, and many groups continued to transfer as many as three blastocysts. Our study indicates clearly that the maximum number of blastocysts to be transferred should be limited to two: although very high pregnancy rates were obtained after transferring three blastocysts, the risk of triplets is high (6% when data from two groups were summarized). Conversely, the chance of establishing a pregnancy after transferring two blastocysts was as high as that observed after transferring three to four four-cell embryos, especially if one or both blastocysts were expanded, while the risk of triplets was theoretically equal to zero.

To our knowledge, this collaborative study is the first one that was able to analyse the abnormalities of 500 fetuses or infants derived from embryo co-culture. The rate of abnormalities, detected in utero and at birth, is not different from that observed in the general population. Although there was an apparent high frequency of trisomies, mainly trisomy 21 (0.8%), the majority of these cases corresponded to situations in which the mother was aged > 37 years, and this rate is not different from that expected within this age bracket. Hence, all trisomies but one were detected in utero, following the establishment of a fetal karyotype. The observation of two cases of anencephaly corresponds to a frequency (0.4%) which is higher than in the general population. However, this frequency represents only two cases, and has little statistical significance.

In conclusion, the findings of this multicentre study show that almost half of the cleaved embryos were able to develop into blastocysts during co-culture with V ero cell monolayers,
and the subsequent implantation rate of these blastocysts was approximately twice as high as that of four-cell embryos. Therefore, the use of co-cultures may be useful, not only in cases of successive implantation failures, but in any cases where numerous zygotes or four-cell embryos are obtained following 1 or 2 days of traditional IVF culture respectively.

Acknowledgements


References


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