Improved cleavage rate of human embryos cultured in antibiotic-free medium

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Retarded development and blastomere fragmentation of human preimplantation embryos represent a common phenomenon in in-vitro culture systems. Even though media composition is generally formulated to meet embryo nutritional requirements, the influence of antibiotic supplementation has not been investigated thoroughly. The present study was performed to evaluate the effects of antibiotics on embryo morphology and growth in modified culture media. A total of 196 zygotes from 18 couples was cultured in three different media: (i) conventional medium (n = 99, control group); (ii) medium modified with half the standard antibiotic concentration (n = 54); and (iii) antibiotic-free medium (n = 43); 49 embryos from the control group were selected at the zygote stage and transferred to the patients on day 2. The remaining 147 zygotes were cultured to the blastocyst stage for cryopreservation; their morphology and cell number were assessed daily at 40, 64, 88, and 112 h post-insemination. Overall cleavage rate was 95% and embryo scoring revealed 91% grade 1 embryos throughout the culture period in the three media. Significantly higher cleavage rates were obtained in the antibiotic-free medium at each observation, including the blastocyst stage, when compared to the other two groups. In addition, no notable improvement was observed in the embryos cultured in a reduced concentration of antibiotics. In conclusion, antibiotic supplementation of media may interfere with the timing of cleavage events either by delaying or blocking embryo development.

Key words: antibiotics/cellular stage/culture medium/human embryos

Introduction

The in-vitro culturing of mammalian embryos was first successfully achieved in the mouse (Whitten, 1956). Since then, notable progress has been made in this field and fertilization and embryo development in vitro are now possible for many species, including humans.

A common aspect in preimplantation mammalian embryo culture is the retardation of development when compared to in-vivo conditions (Bowman and McLaren, 1970; Harlow and Quinn, 1982). This species-specific event may be expressed by a decrease in the cleavage rate, a high degree of fragmentation or, in extreme cases, a blockage to embryo development (Goddard and Pratt, 1983; Gandolfi and Moore, 1987). The cleavage arrest in vitro in the human generally occurs at the four to eight cell stage (Braude et al., 1988); only 25–30% of embryos overcome this block and cleave further to the expanded blastocyst stage (Bongso et al., 1989; Ménézo et al., 1990). Many efforts have been directed towards attenuating these, which may be effects associated with in-vitro embryo cultures. It seems logical to consider that culture media composition should reflect as much as possible the conditions (e.g. nutrients and electrolytes) present in the lumen of the female reproductive tract. Therefore, a variety of different media have been formulated with the aim of improving embryo viability on successive days of culture (Quinn et al., 1985; Gardner and Leese, 1990; Biggers, 1993; Gardner and Sakkas, 1993).

A common denominator in long-term cell culture media is the presence of antibiotics, which are routinely added in order to avoid contamination from bacteria and fungi. Sterile conditions are imperative in human in-vitro technologies, since human embryos are replaced into the maternal reproductive tract. Indeed, bacteria can easily be introduced into culture media through spermatozoa as a consequence of seminal infection or inappropriate collection of the semen sample. It has been documented that oocytes cultured in infected insemination media give reduced fertilization rates or no fertilization at all (Forman et al., 1987; Huyser et al., 1991). The addition of penicillin and streptomycin to culture media has been demonstrated to be strongly effective in reducing the incidence of microbial contamination during sperm washing and swim-up procedures (Riedel et al., 1986; Huyser et al., 1991). As a general rule, the quantities of antibiotics in culture media are kept at standard concentrations and are thought to have no detectable toxic effects on the cultured cells. However, it is well known that antibiotics are biologically active substances and the probability that they may affect cell function must always remain open to question.

We therefore decided to investigate whether the presence of penicillin and streptomycin could affect development of human embryos in culture.

Materials and methods

Ovarian stimulation and oocyte retrieval

A total of 18 normo-ovulatory patients (age ≤38 years old) entering a conventional in-vitro fertilization (IVF) treatment cycle over the
same period were included in the study. Following pituitary desensitization with gonadotrophin releasing hormone agonists (GnRHa) (Decapeptyl 3.75 mg: Ipsen Biotech, Paris, France), purified follicle stimulating hormone (FSH) (Metrodin Serono, Rome, Italy) was administered to patients (Gianaroli et al., 1994). At 36 h after human chorionic gonadotrophin (HCG) administration, oocyte retrieval was performed under ultrasound guidance. After morphological evaluation of the cumulus-corona complex, oocytes were incubated in T6 medium (Whittingham, 1971) supplemented with 10% heat-inactivated maternal serum (MS), in a 5% CO2 humidified gas atmosphere at 37°C.

**Culture medium preparation, insemination and embryo culture procedures**

A batch of conventional T6 medium was prepared as described by Whittingham (1971) with the only exception that before adding antibiotics, the same batch was divided into three aliquots: in the first, the standard amounts of penicillin (100 IU/ml) and streptomycin (50 μg/ml; Sigma, St Louis, MO, USA) were added; the second aliquot was prepared with half of the recommended antibiotic concentration (penicillin 50 IU/ml, streptomycin 25 μg/ml); whereas in the third aliquot, no antibiotics were added. The three aliquots were referred to as conventional T6 (T6-C), modified 1 T6 (T6-M1), and modified 2 T6 (T6-M2) respectively.

Semen samples were prepared by the standard swim-up procedure using T6-C with 10% MS, and insemination was carried out in microdroplets overlaid with light paraffin oil (Sigma), as previously described (Fiorentino et al., 1994). At 16 h later, oocytes were evaluated for the presence of pronuclei. Following patients' consent, each patient's pool of zygotes was randomly divided and cultured in 100 μl microdroplets according to the following scheme: 25% in T6-C, 25% in T6-M1 and 25% in T6-M2 containing 10% MS, with the remaining 25% of zygotes being cultured conventionally for embryo transfer. This protocol is illustrated in Figure 1.

The zygotes included in the three study groups were cultured and evaluated at regular intervals until the blastocyst stage, at which point they were cryopreserved.

**Embryo culture and evaluation**

All embryos were scored daily at 40, 64, 88 and 112 h post-insemination, according to the following criteria. grade 1, regular blastomeres, no fragmentation; grade 2, <50% irregular blastomeres and/or fragmentation; grade 3, >50% irregular blastomeres and/or fragmentation and grade 4, lysed embryos.

**Statistical analysis**

All data were processed by χ² analysis 2x2 contingency tables.

**Results**

As indicated in Figure 1, a total of 268 oocytes was inseminated, of which 196 fertilized normally (73%). Of these, 49 were cultured conventionally and transferred to the patients, and consequently not included in the study. Following the random allocation of 147 spare zygotes to the three different culture systems, 50 zygotes were cultured in T6-C, 54 in T6-M1 and 43 in T6-M2. Overall cleavage rate on day 2 was 95%, and embryo scoring, completed 40 h after insemination, demonstrated largely grade I embryos (95%). Further observations performed at 64, 88, and 112 h post-insemination revealed that the percentage of grade 1 embryos remained consistently high (an average equivalent to 91%) throughout the embryo culture period. Therefore, due to the small percentage of fragmented embryos, only grade 1 embryos are represented in the results.

Figure 2 indicates the first observation carried out at 40 h post-insemination which included a total of 133 grade 1 embryos. The percentage of faster cleaving embryos at the four to five cell stage was significantly higher in the T6-M2 group (80%) when compared to the T6-C group (49%; P < 0.001). An apparent difference (not significant) was also observed between the T6-M1 (56%) and the T6-M2 group, whereas similar results were obtained culturing the embryos in T6-C or in T6-M1.

The same trend in embryo growth was noted in the second observation documented at 64 h post-insemination where 91% of embryos were grade 1 (n = 121). Once more, T6-M2 embryos showed a higher rate of cell division (94% of the embryos at the six to 10 cell stage) compared to T6-C (58%).

**Figure 1** Outline of protocol.
and to T6-M1 embryos (72%; \( P < 0.01 \) and \( P < 0.05 \) respectively; Figure 3).

Figure 4 (106 grade 1 embryos) demonstrates that an increase in the blastomere number was again observed in day 4 embryos (88 h) cultured in the absence of antibiotics (100% embryos with 16–32 blastomeres) when compared to embryos which were maintained in T6-C (80%) or in T6-M1 (93%), although the differences observed were not statistically significant.

The last observation (Figure 5: 95 grade 1 embryos) indicates the percentage of blastocysts present in each culture medium at 112 h. A significant increase in the blastocyst rate was observed in embryos cultured in T6-M2 (60% blastocysts compared with 21% in T6-C; \( P < 0.05 \)). The overall rate of blastocyst formation from the 147 zygotes cultured was 18% (9/50) in T6-C, 22% (12/54) in T6-M1 and 33% (14/43) in T6-M2. Thus, at all stages of embryo evaluation, improved cleavage rates were consistently seen in the antibiotic-free medium up to the blastocyst stage.

**Discussion**

A great deal of research has been dedicated to promoting the efficiency of in-vitro culture of human embryos where delayed cleavage rates and fragmentation are commonly observed (Trounson, 1983; Hardy, 1994). In fact, identification of concentration of nutrients, electrolytes and macromolecules present in the female reproductive tract has provided useful information regarding the composition of media for in-vitro human embryo development. Indeed, several culture media ranging from simple salt solutions to enriched media (Ménézo et al., 1984; Quinn et al., 1985; Gianaroli et al., 1986) and co-culture systems (Bongso et al., 1989; Ménézo et al., 1990; Magli et al., 1995) have been developed to resemble oviduct and uterine environments in order to improve embryo viability.

Antibiotics are common to all media used in tissue culture, but are not nutritionally necessary. They are added to avoid contamination from micro-organisms. Supplementation with antibiotics is routinely carried out using concentrations determined in pioneering studies which evaluated their toxicity in cell culture systems (Jacoby, 1965). These experiments selected penicillin and streptomycin as the most useful, and generally safe drugs. The concentration recommended is 100 IU/ml and 50 \( \mu \)g/ml respectively. Subsequently, these standard concentrations have been adopted in human embryo culture media without regard to their possible effects, however small, on cell metabolism, embryo morphology or growth rate. The present study has examined the effects of antibiotics on human embryo cultures; specifically on morphology and the timing of early cleavage.

Since there was a possibility of contamination of the cultures, necessary precautions were taken to minimize any risks of microbial infection by using aseptic techniques and maintaining media isolated from the external environment through the use of oil-overlay cultures. As a result, no microbial contamination was observed even in the absence of antibiotics during the embryo culture period. This is important since bacterial and fungal contaminants are known to be common in semen samples, even when the patient has undergone antimicrobial
therapy (Huysier et al., 1991). Therefore, in this study, oocyte culture, sperm preparation, and insemination were executed in T6-C, conventional medium containing antibiotics.

The results suggest that the absence of antibiotics in culture media is associated with an increase in embryo cell division. Indeed, the elimination of penicillin and streptomycin from the media resulted in an improved cleavage rate when compared to the control group and the group whose embryos were cultured in a reduced concentration of antibiotics (Figures 2–5). Interestingly, no statistically significant difference was found between T6-C and T6-M1, implying that faster cleaving embryos can only be obtained by omitting antibiotics altogether from the culture media. Conversely, the grade of blastomere fragmentation throughout the culture period did not differ among the embryos in the three media. Hence, these observations suggest that antibiotics may interfere with the timing of cleavage events by either delaying or blocking embryo development. These effects are still present with a reduced concentration of antibiotics (T6-M1).

Faster cleaving embryos have been clearly demonstrated to be more capable of implantation in animal species (Gardner and Sakkas, 1993; McKieman and Bavister, 1994). In the human, the short culture period routinely adopted for embryo replacement may be insufficient to define whether embryos are cleaving within a time-frame compatible to vitality or undergoing retardation or blockage in development (McKieman and Bavister, 1994). However, recent studies have reported a strong correlation between cleavage delay and chromosomal abnormalities (Munne et al., 1993, 1994). Nearly 58% of these embryos are characterized by mosaicism and aneuploidy, which in turn may represent a major cause of embryo developmental arrest (Munne et al. 1994). This is consistent with results obtained by Gianaroli et al. (1996), who reported a statistically higher cleavage rate associated with an increased embryo implantation in a study where the effects of long sperm–oocyte incubation periods were investigated. In fact, conventional IVF insemination inevitably yields oocyte exposure to sperm metabolic waste products (e.g. oxygen free radicals) which as a result may subsequently interfere with embryo viability. The reduction of oocyte exposure to spermatozoa to only 1 h generated a statistically higher cleavage rate followed by a higher pregnancy and implantation rate when compared to the standard 16 h incubation period.

According to the results presented in this study, omitting antibiotics has an effect on the rate of embryo development. The inhibitory effect of antibiotics on the proliferation of eukaryotic cells cultured in vitro may occur at different stages of cell metabolism. A recent report documents the possible effect of protein synthesis inhibitors on triggering or blocking (depending on the system studied) the mechanisms of programmed cell death, which is a physiological process evolved in most, if not all, multicellular organisms (Vaux, 1993). Blastomere fragmentation and delayed division are certainly common in routine IVF, but it is not known whether the factors involved include either the culture conditions, hormonal stimulation, or if it is a specific human embryo characteristic. Studies on these aspects of cell physiology are actively in progress.

In conclusion, the analysis of the data presented in this study suggest that the culture of human preimplantation embryos without antibiotics could represent a valuable advance towards the goal of simulating in-vivo conditions. Undeniably, modifications in culture media composition in association with a reduced time exposure of oocytes to spermatozoa could prove to be advantageous. However, assessment of such beneficial effects can only be evaluated following embryo transfer.

References


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