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The influence of in-vitro culture versus stimulated and untreated oviductal environment on mouse embryo development and implantation

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A prospective randomised study was performed to evaluate stimulated versus natural oviductal environment in comparison with in-vitro culture for the developmental capacity of mouse embryos. Therefore, embryos of superovulated F1 hybrid CBAxC57Bl females were collected at 17, 22, 41 and 46 h after human chorionic gonadotrophin treatment and randomly divided into five groups. They were either transferred immediately to untreated pseudopregnant females, cultured *in vitro* for 5, 24 or 29 h before transfer, or cultured *in vitro* for 96 h to blastocysts. The transfers resulted in an impaired implantation ($P < 0.001$) and a lower numbers of living fetuses ($P < 0.001$) when embryos had been exposed longer to the stimulated oviductal environment. Similar results were obtained after a longer period of in-vitro culture ($P < 0.05$). However when embryos were flushed earlier from the superovulated mice and cultured longer in-vitro until the transfer was performed, the implantation rate was improved ($P < 0.01$). Blastocyst development, however, was better ($P < 0.001$) when embryos were flushed later. In conclusion, the stimulated oviductal environment impairs the developmental capacity of embryos in comparison with untreated pseudopregnant females. In-vitro culture is also suboptimal but better than the stimulated oviductal environment.

Key words: implantation/in-vitro culture/mouse embryo development/natural cycle/superovulation

Introduction

Studies on embryo development have shown that in-vitro culture conditions determine fertilization, cleavage and further developmental capacity. Some factors such as temperature, pH, osmotic pressure, nutrients, oxygen tension and free radical scavengers have been identified.

The oviductal fluid, which is the natural environment of the non-compacted embryo, is a specific fluid, which is totally different from the uterine fluid (Gardner and Lane, 1996, 1997). In the oviduct, several growth factors and proteins are present which interact with the oocyte and with the developing embryo (Kaye and Harvey, 1995; Gandolfi, 1995; Kane *et al.*, 1997). These growth factors (Abe, 1996) and the concentrations of the metabolites (Gardner *et al.*, 1996) change during embryo transport through the oviduct in order to support the metabolic needs of the embryo. In the pig, fertilized oocytes require a factor from the oviduct to complete cortical granule reaction and modification of the zona pellucida (Kim et al, 1996). In the mouse, in-vitro development of pronucleate ova is better when flushed later from the oviduct (Van der Auwera *et al.*, 1992) or when co-cultured with ampullar cells for at least 24 h (Minami *et al.*, 1992).

Following superovulation, the fluid from the oviduct seems to impair embryo development. Superovulation in the mouse was described as a model for intra-uterine growth retardation (Evans *et al.*, 1981). Superovulation is associated with a slower preimplantation embryo development, a later and impaired implantation and a prolonged gestation (Ertzeid and Storeng, 1992; Ertzeid *et al.*, 1993). These studies suggest that the oviductal milieu rather than the embryo quality are responsible for the adverse effects observed after superovulation. We therefore investigated the effect of the stimulated versus the natural oviductal environment in comparison with in-vitro culture on the developmental capacity of early preimplantation mouse embryos. Pronucleate ova and 2-cell embryos were collected at different times after human chorionic gonadotrophin (HCG) injection, cultured in-vitro for variable hours and transferred to foster mothers in a non-treated cycle.

Materials and methods

Animals

Four to five week-old F1 CBAxC57/Bl females were superovulated with i.p. injections of 5 IU pregnant mare's serum gonadotrophin (PMSG) and 5 IU HCG (at 17.00 hrs; 48 h apart), mated with CBAxC57Bl males and checked for copulation plugs the next morning. Mated females were killed by cervical dislocation and their oviducts flushed with HEPES buffered Earle's medium. Pronucleate mouse ova were collected at 17 or 22 h after HCG while 2-cell embryos were collected at 41 or 46 h after HCG treatment. Morphologically normal pronucleate ova and 2-cell embryos of the same age were pooled and randomly divided over the experimental groups. The embryos were either transferred immediately to the oviducts of pseudopregnant females, or cultured in-vitro for 5, 24 or 29 h before transfer, or cultured in-vitro for 96 h without performing a transfer, i.e. up to the blastocyst stage.

Pseudopregnant adult NMRI females were obtained by caging nonstimulated cycling females with vasectomized

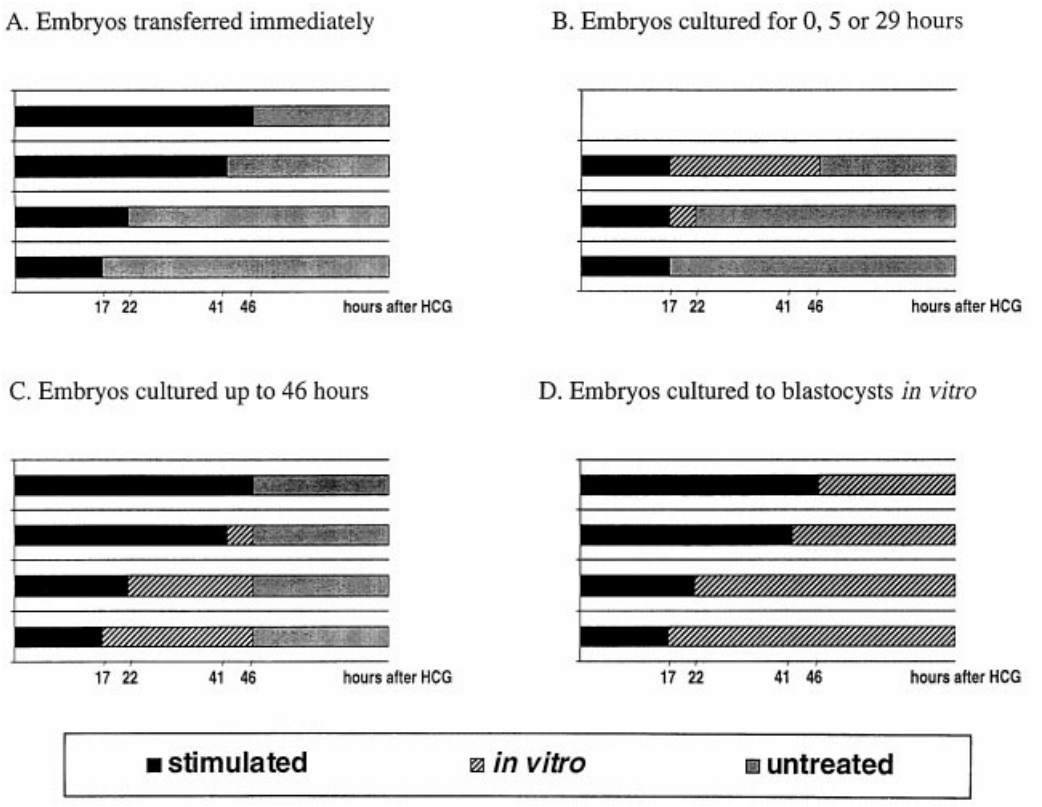

Figure 1. The influence of stimulated and untreated oviductal environment versus in-vitro culture conditions on the developmental capacity of mouse embryos in-vivo and in-vitro: experimental design.

NMRI males (Hogan *et al.*, 1986). Vasectomies were performed under sodium pentobarbital anaesthesia (100mg/kg Nembutal, Abbott, Brussels) by cutting and ligating the vas deferens. Four weeks later, their ability to copulate was controlled and uterine smears from the mated females were checked for the presence of spermatozoa. Only sterile males were used for the experiments. The NMRI strain was used for transfer experiments because of differences in eye pigmentation between NMRI×NMRI fetuses and fetuses of transferred embryos of CBA×C57Bl mice. Transfers were performed under sodium pentobarbital anaesthesia (100mg/kg Nembutal) at 17, 22, 41 or 46 h after caging with the males.

The synchronized embryos were transferred into the oviducts via the ostium tubae with a fine glass pipette under microscopic visualization. Seven to 10 embryos were transferred into each oviduct.

On day 15 of gestation, the foster mothers were killed by cervical dislocation and the number of implantation sites, the number of resorption sites and the number of living fetuses were counted.

The culture medium was Earle's Balanced Salt Solution supplemented with 15% of a pool of human cord serum (HCS), detoxified by charcoal pretreatment (Sigma, St. Louis, MO, USA; 25 mg/ml) for 1 h and resterilized over a 0.22 μm filter before use (Van der Auwera, 1994). The culture was performed at 37°C in a 5% CO_2 incubator in air, with 100% humidity.

Experimental design

In the first experiment, the effects of the stimulated oviductal environment and of the non-stimulated oviductal environment were compared. Therefore pronucleate ova and 2-cell embryos were transferred immediately from the superovulated mice to non-stimulated pseudopregnant foster mothers at exactly 17, 22, 41 and 46 h following the HCG injection. (Figure 1A).

In the second experiment, the effects of in-vitro culture and of the non-stimulated oviductal environment were compared. Therefore pronucleate ova collected 17 h after HCG injection were either transferred immediately, or after a culture period of 5 or 29 h in-vitro. (Figure 1B). The duration of exposure to the stimulated oviductal environment thus was identical for all the embryos, whereas the duration of exposure to in-vitro culture was variable.

In the third experiment, the effects of the stimulated oviductal environment and of in-vitro culture were compared. Therefore, pronucleate ova and 2-cell embryos were flushed from the stimulated oviductal environment at exactly 17, 22, 41 and 46 h after HCG and cultured in-vitro for the remaining time until the transfer was performed at exactly 46 h after HCG (Figure 1C). Therefore, the duration of exposure to the non-stimulated environment was identical for all the embryos, whereas the duration of exposure to the stimulated oviductal environment and to the in-vitro culture was variable.

In the last experiment, the effect of the stimulated oviductal environment upon the subsequent in-vitro culture was evaluated

Table I. Number of mice and embryos used per experimental group

Hours after HCG	17	22	41	46
Transferred immediately (Figure 1A)				
Mice used (*n*)	5	8	8	9
Pregnant mice (*n*)	5	5	7	6
Embryos in pregnant mice (*n*)	66	86	86	108
Embryos cultured for 0, 5 or 29 h (Figure 1B)				
Mice used (*n*)	5	7		5
Pregnant mice (*n*)	5	5		4
Embryos in pregnant mice (*n*)	66	72		60
Embryos cultured up to 46 h (Figure 1C)				
Mice used (*n*)	5	6	6	9
Pregnant mice (*n*)	4	4	5	6
Embryos in pregnant mice (*n*)	60	60	70	108
Embryos cultured to blastocysts in-vitro (Figure 1D)				
Embryos used (*n*)	100	100	100	100

HCG = human chorionic gonadotrophin.

by collecting the embryos at 17, 22, 41 and 46 h after HCG and culturing them in-vitro up to the blastocyst stage without a transfer (Figure 1D).

Statistical analysis.

Trend analysis (Mantel Haenszel's χ^2) was used for statistical evaluation.

Results

In total, 825 pronucleate ova were transferred into 54 mice. Fourteen mice did not become pregnant. Since an implantation rate of 0%, in comparison with the implantation rate of all other groups of 41%, was probably a consequence of a failure of the recipients to have become pseudopregnant or of technical problems during the transfer procedure into oviducts, these animals were excluded from further analysis. After this exclusion, there remained in each group at least four pregnant mice and 60 transferred embryos, with an overall implantation rate of 41% per transferred embryo (Table I).

The first experiment, comparing stimulated and non-stimulated oviductal environments, resulted in a higher implantation rate and a higher number of living fetuses when embryos were transferred earlier from the stimulated oviductal environment to an untreated oviductal environment (Figure 2A, $P < 0.001$). Following transfer at 17, 22, 41 and 46 h after HCG, the implantation rates were 64, 45, 36, and 28% respectively. The subsequent in-vivo development of embryos after transfer was therefore impaired by the stimulated oviductal environment of the superovulated mice.

The second experiment, comparing in-vitro culture and the non-stimulated oviductal environment, demonstrated a lower implantation rate and a lower number of living fetuses when pronucleate ova were cultured for a longer period in-vitro before transfer (Figure 2B, $P < 0.05$). Implantation rates of 64, 50 and 45% were obtained after in-vitro culture periods of 0, 5 and 29 h, respectively. The subsequent in-vivo development of embryos was thus also impaired by in-vitro culture in comparison with the non-stimulated oviductal environment.

The third experiment, comparing the stimulated oviductal environment and the in-vitro culture, showed a better implantation when embryos were flushed earlier from the stimulated oviductal environment and thus cultured longer in-vitro until transfer to an untreated pseudopregnant female at 46 h after HCG (Figure 2C, $P < 0.01$). Implantation rates of 45, 33, 31 and 28% were obtained after in-vitro culture periods of 29, 24, 5 and 0 h respectively. The number of living fetuses showed a similar trend, although not significant. The subsequent in-vivo development of embryos was therefore more impaired by the stimulated oviductal environment than in-vitro culture.

During in-vitro culture up to 96 h more blastocysts were formed when embryos were flushed later from the stimulated oviductal environment (Figure 2D, $P < 0.001$). The blastocyst formation rate was 71, 87, 89 and 95% after flushing at 17, 22, 41 and 46 h after HCG, respectively. In-vitro development up to the blastocyst stage is thus better following a longer period in-vivo, even in a stimulated oviductal environment.

Discussion

These results confirm the importance of the environment upon embryo development. They demonstrate that the untreated oviductal environment is superior to in-vitro culture, which is better than the stimulated oviductal environment.

The first experiment showed that embryos develop better after transfer to an untreated pseudopregnant female when removed earlier from the stimulated oviductal environment. This suggests that factors, e.g. high concentrations of steroids, present in the superovulated mice are detrimental for the further development of pronucleate ova. To the best of our knowledge, this is the first evidence demonstrating a toxic effect of the hormonal stimulation on the oviductal environment which reflects on the developmental capacity of pre-implantation embryos. These results could be important for human infertility treatment and IVF. We know that the quality of the endometrium and the associated probability of implantation is influenced by hormonal treatment as demonstrated by the correlation between endometrium thickness (Gonen *et al.*, 1989; Check *et al.*, 1993a,b) and pattern (Dickey *et al.*, 1992) and pregnancy outcome after hormonal stimulation for assisted

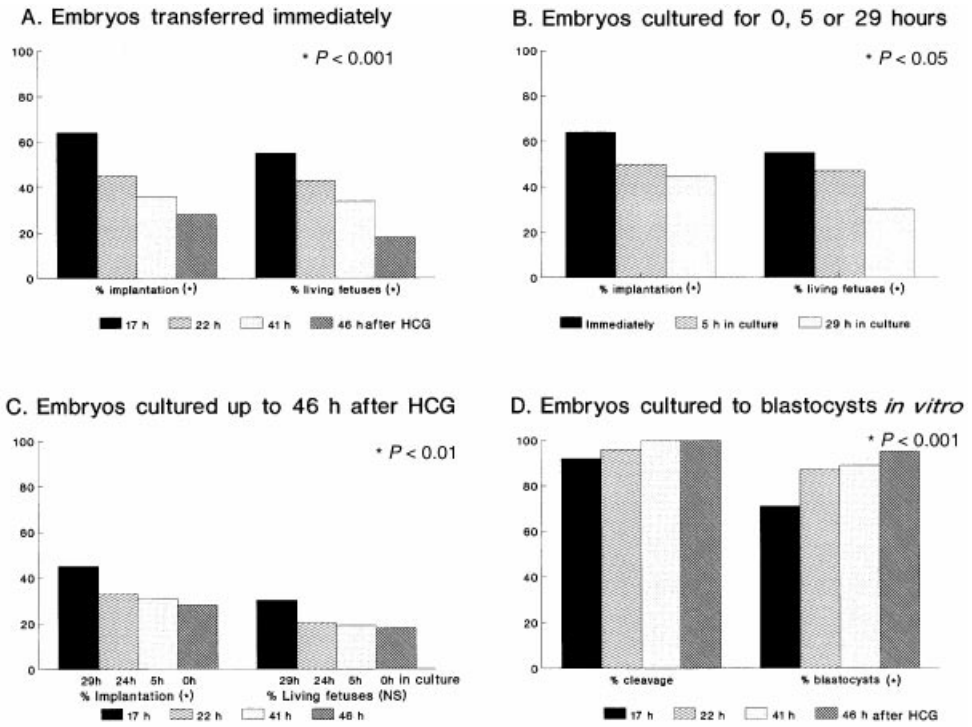

Figure 2. Implantation rates and percentages living fetuses (**A**) Pronucleate ova and 2-cell embryos collected at different ages [17, 22, 41 and 46 h after human chorionic gonadotrophin (HCG)] and transferred immediately in-vivo into pseudopregnant mice. (**B**) Pronucleate ova transferred in-vivo into pseudopregnant mice either immediately or after a culture period of 5 or 29 h. (**C**) 2-cell embryos which were previously cultured for 29, 24, 5, or 0 h in-vitro and transferred at 46 h after HCG. (**D**) Cleavage rates and blastocyst formation of pronucleate ova and 2-cell embryos collected at different ages (17, 22, 41 and 46 h after HCG) and cultured in-vitro without transfer.

reproduction. Our observations are clinically supported (Check *et al.*, 1995), where an adverse effect of the hormonal stimulation on implantation by comparing donors and recipients in a shared oocyte donation programme was demonstrated. He explained this phenomenon by a negative effect on the uterine environment. An increased uterine receptivity has been described (Simon *et al.*, 1998) by decreasing the oestradiol levels of high responders by using a step-down stimulation protocol. These studies cannot however differentiate between a decreased endometrial receptivity and an eventual detrimental uterine environment. Our studies demonstrate, in addition, a direct negative effect of oviductal factors resulting from superovulation upon embryonic development and survival.

A detrimental effect of hormonal stimulation upon the oviductal environment has not yet been demonstrated in the human. A possible potential negative effect, however, is not contradicted by observations reporting a higher pregnancy rate after ovarian stimulation and intrauterine insemination (IUI) compared to insemination alone (Guzick *et al.*, 1999). Stimulation with IUI differs theoretically from IUI alone, by enhancing the numbers of follicles, eventually improving ovulation, by improving timing of insemination at the moment of ovulation and by creating a stimulated oviductal and uterine environment. The resulting pregnancy rate obviously is the net sum of all these factors, some of which might be detrimental but counterbalanced by positive effects. Assuming that pronounced stimulation would simultaneously increase the number of follicles and decrease the quality of the environment, it could lend support to those regimens advocating low dose gonadotrophin treatment. In practice, only randomised trials can establish the optimum balance between simultaneous positive and negative effects.

In the human, there are observations that gamete intra-Fallopian transfer (GIFT) results in higher pregnancy rates in comparison with IVF (Chang *et al.*, 1996; ASRM/SART, 1996). It is difficult to interpret these data since the embryos were longer exposed to the possible deleterious stimulated oviductal environment using GIFT whereas in IVF suboptimal culture conditions were used. Since the introduction of sequential culture media, incorporating amino acids, vitamins and growth factors, the in-vitro culture of embryos has been improved, resulting in implantation rates of >50% per transferred blastocyst (Gardner and Lane, 1997). Confirmation of these data in a trial would result in higher pregnancy rates than ever reported for GIFT.

In this study, pronucleate mouse ova develop better after a shorter period of in-vitro culture before transfer to the untreated foster mothers. This negative effect of the in-vitro culture could result from a lack of necessary amino acids, growth factors, or vitamins (Gardner *et al.*, 1996, 1997; Lane and Gardner, 1998) or from oxidative stress due to high oxygen levels in the incubator (20% O_2).

The third experiment showed that embryo development is better following in-vitro culture up to 46 h, than after development in a stimulated oviductal environment. This suggests that the negative effect of factors present in the superovulated

oviductal environment, e.g. high concentration of steroids, is more important than the negative effect of in-vitro culture, e.g. lipid peroxidation processes due to the high oxygen tension and/or the absence of appropriate growth factors and/or amino acids. In contrast, the fourth experiment showed the opposite effect after in-vitro culture up to the blastocyst stage. In order to explain this apparent discrepancy, we speculate that the oviduct secretes (an) essential growth factor(s) and/or amino acids needed by pronucleate ova and 2-cell embryos for the activation of their embryonic genome to permit the subsequent development into healthy blastocysts and fetuses. These factors possibly explain the delay in development often observed in in-vitro culture systems. Recently two growth factors, insulin-like growth factor I and granulocyte-macrophage colony-stimulating factor, were shown to result in better and earlier human blastocyst formation in-vitro (Lighten *et al.*, 1998; Sjöblom *et al.*, 1998). The in-vitro culture was performed in EBSS supplemented with HCS and pretreated with charcoal which probably results in a detoxified culture medium without steroids (Van der Auwera., 1994). This medium, however, may lack these essential growth factors and/or amino acids. If embryos were cultured until the 2-cell stage and transferred 46 h after HCG, they are exposed to these growth factors in the nonstimulated oviductal environment of the foster mothers. These embryos are therefore more influenced by the toxic effects of the ovulation than by the suboptimal culture conditions in-vitro.

A possible bias in these experiments is that the cleavage rate of pronucleate ova is only 90% whereas all the 2-cell stages had a 100% capacity to develop after transfer. Taking this into account, however, the negative influence of the stimulated oviductal environment would be even more pronounced.

In conclusion, the stimulated oviductal environment has a negative influence on the implantation capacity of embryos after transfer to an untreated oviduct. In-vitro culture is better in comparison with the stimulated oviductal environment but suboptimal in comparison with the untreated oviductal environment.

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