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DIFFERENT SERUM COMPLEMENTS AFFECT THE *IN VITRO* DEVELOPMENT AND HATCHING RATES OF RABBIT ZYGOTES. A TECHNICAL NOTE.

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ABSTRACT : The effect of two different types of serum supplements to Ham's F-10 medium on the development of rabbit zygotes to the blastocyst stage was tested. The *in vitro* development was improved when ovulated-doe serum, instead young-rabbit serum was added to Ham's F-10 medium. The development to the 16-cell, compacted morula-early blastocyst, blastocyst or hatched stage at 24, 48 and 168 hours of culture were 44%, 54%, 100% and 92% *vs* 0%, 19%, 75% and 56%, respectively. Moreover, the established conditions of culture (39°C, 7% CO_2 in air and 15 mM sodium bicarbonate) associated with the ovulated-doe serum in Ham's F-10 could enhance the *in vitro* development of rabbit zygotes up to the hatching stage (90-100%).

RESUME : Le développement* in vitro *et le taux de maturation des zygotes sont influencés par les compléments de sérum. Une note technique.

Les auteurs ont comparé 2 types de sérums de lapin pour complémenter le milieu Ham F-10 lors de la culture in vitro *d'embryons fécondés de lapins. Les sérums provenaient soit de jeunes lapins (9 semaines) soit de lapines adultes ayant ovulé. Le développement des zygotes* in vitro *est possible avec les 2 supplémentations, mais est nettement favorisé par l'utilisation de sérum de lapine ayant ovulé. Avec ce dernier, les stades de développement 16-cellules (en 24h), morula (48h), blastocyste et blastocyste maturé (en 16h) ont été atteints par 44%, 54%, 100% et 92% des oeufs fécondés, respectivement, contre seulement 0%, 19%, 75% et 56% avec le sérum de jeune lapin. Ainsi, les conditions de culture adaptées (39°C, 7% de CO_2 dans l'air et 15mM de bicarbonate de sodium) et l'usage du sérum de lapine ayant ovulé permettent d'obtenir un développement* in vitro *de 90% à 100% des oeufs de lapin fécondés jusqu'au stade blastocyste mature, ce qui n'est pas réalisé avec les supplémentations classiques avec du sérum bovin.*

INTRODUCTION

For the embryo production and/or preservation techniques, efficient culture media, which sustain the *in vitro* development at every embryo stage were desired.

Some defined media support the *in vitro* rabbit embryo development from early cleavage stages (RD, Medium 199, BME, Ham's F-10). However, development up to the blastocyst stage was impaired when embryos were cultured in synthetic media devoid of chemically undefined substances (heat-unactived sera, BSA, other macromolecules, or rabbit oviduct epithelial cells-ROEC) (LI *et al.*, 1993).

Rabbit embryos have been cultured in sub-optimal media, as Ham's F-10, containing heat-treated or bovine serum. ONUMA *et al.*, (1968) cultured 2- and 4-cell rabbit embryos to the hatching blastocyst stage and almost an eighty to ninety per cent of the embryos developed a blastocel. Zygote culture rendered higher blastocysts when new-born calf serum (50% : KEEFER *et al.*, 1988), BSA (60-78% : KANE, 1974, 1975 ; 87% : KANE and HEADON, 1980 ; 86% : KANE, 1983 ; 0% : CARNEY *et al.*, 1990) or serum (MAURER, 1991) were added to the culture media because of their effect on growth (Li *et al.*, 1993). So, all data suggest higher rabbit zygote development up to the blastocyst stage when BSA was added to culture media. In our Laboratory, Ham's F-10 supplemented with 20% (v/v) ovulated-adult-doe serum is the usual embryo culture medium. Under these conditions, the developmental rates to the hatched blastocyst stage were 90-100% for 2-cell-stage rabbit embryos and latter (GARCÍA-XIMÉNEZ *et al.*, 1995 ; 1996 ; VICENTE *et al.*, 1994), being almost as efficient as other media supplemented with bovine derived compounds.

So, to test our culture condition for promoting the *in vitro* development of rabbit zygotes to the blastocyst and hatched stages at least, as later embryo stages do, was the first aim of this note.

Some seric substances were added to the embryo culture media and improved the blastocyst rates (ONUMA *et al.*, 1968 ; CARNEY and FOOTE, 1991 ; LI *et al.*, 1993). So, homologous seric undefined substances might also be beneficial to zygotic development, independently of hormonal status. Two types of serum from either young-rabbit or ovulated-adult-doe were tested to determinate their ability to support rabbit zygotes development to the blastocyst and hatched stages. If it would be, homologous sera would be more economical and available medium supplement than commercial seric compounds, especially when the infective effects of fluids from

Table 1 : Development of one-cell rabbit embryos after culture for 24 hours in Ham's F-10 supplemented with different sera

Group	No Zygotes	No searching indicated stages (%)		
		4-cell	8-cell	16-cell or more
1 : Ham's F-10 plus 20% young rabbit serum (HYR)	32	10 (31)	22 (69)	0 (0)[a]
2 : Ham's F-10 plus 20% ovulated-doe serum (HOD)	39	1 (2.5)	21 (54)	17 (44)[b]

Different superscripts indicate significant differences between groups (P<0.01)

cattle with bovine spongiform encephalopathy are called in question (WRATHALL *et al.*, 1997) and alternative newly commercial embryo tested products are required.

MATERIAL AND METHODS

New Zealand adult virgin does (4.5-5 months old) were mated with a fertile buck, after which each animal received an intramuscular GnRH injection (20 μg, Fertagyl, Ovejero, Spain). Presumptive one-cell fertilised ova were collected from does 14-16 hours after GnRH injection by flushing oviducts with phosphate buffered saline medium (DPBS ; Sigma, Cat.No. : D-5773) and 0.3% antibiotic solution (Biomicina 1:1, Ovejero Laboratory). The recovered zygotes were washed twice with DPBS. Only normal one-cell embryos judged by the absence of granular cytoplasm, the presence of a sharply defined perivitelline membrane and second polar body, were selected for culture.

Two main types of sera were used to supplement the basic medium : young-rabbit serum recovered from abattoir animals (nine weeks old) and serum from sexually-mature does at 72 hours post-hCG injection (25 IU, Coriogan, Ovejero) (ovulated-doe serum). Before use, sera were inactivated in a 56°C water-bath for 30 min and sterilised by microfiltration (0.22 μm pore size). The composition of the basic medium was Ham's F-10 (Sigma, Cat. No. : N-6635), 0.3% antibiotics solution, 15 mM $NaHCO_3$ and the pH was adjusted to 7.4.

These media were stored for up to two weeks at 4°C. Before use, media were equilibrated for almost two hours in the culture conditions : 1.5 ml of culture medium in 35 x 10mm plastic tissue culture dishes at 39°C in a humidified atmosphere of 7% CO_2 in air. All manipulations were carried out at room temperature (20°C minimal).

Two experimental groups were established : in group 1, zygotes were cultured for168 hours in Ham's F-10 supplemented with 20% young-rabbit serum (HYR). In group 2, zygotes were cultured in Ham's F-10 supplemented with 20% ovulated-doe serum (HOD) during the total culture period (168 hours). After recovery, zygotes were randomly distributed among the experimental groups . A total of 71 zygotes were used. Four replications were performed by each category of serum. Different lots of serum were used in order to consider variation between them.

At 24, 48 and 168 hours of culture the developmental stage of embryos was scored and recorded.

Statistical Analysis : Data were analysed using chi-square with Yate 's correction tests.

RESULTS

After the first 24 hours of culture, the zygotes cultured in HYR (group 1) showed lower rates of development than the group cultured with ovulated-doe serum. 24% of the zygotes in group 2 reached the 16-cell-stage and even early morula stage. In contrast, none of the one-cell ova cultured in HYR reached the 16-cell-stage by 24 hours (P<0.01, Table 1).

Table 2 : Embryo development from zygotes cultured for 48 hours in Ham's F-10 supplemented with different sera.

Group	No Zygotes	No. Reaching indicated stages (%)				
		4-cell	8-cell	16-cell	Morulae	CM and EB
1 : Ham's F-10 plus 20% young rabbit serum (HYR)	32	1 (3)	4 (12)	1 (3)	20 (62)	6 (19)[a]
2 : Ham's F-10 plus 20% ovulated-doe serum (HOD)	39	0 (0)	0 (0)	0 (0)	18 (46)	21 (54)[b]

Different superscripts indicate significant differences between groups (P<0.01)
CM and EB indicates : Compacted Morulae and Early Blastocysts

Table 3 : Endpoints after rabbit zygotes culture for 168 hours in Ham's F-10 supplemented with different sera.

Group	No of Zygotes	No. (%) Blastocysts
1 : Ham's F-10 plus 20% homologous serum (HYR)	32	24 (75) [a]
2 : Ham's F-10 plus 20% ovulated-doe serum (HOD)	39	39 (100) [b]

Different superscripts indicate significant differences between groups (P<0.01)

After 48 hours of *in vitro* culture, development to the compacted morula-early blastocyst stage observed in group 1 differed significantly from group 2 : 19% *vs* 54%, respectively ; P<0.01 , Table 2).

At the end of the culture, differences in the rate of development to the blastocyst stage were found (Table 3). Embryos of group 2 developed significantly better than group 1 (100% *vs* 75%, respectively ; P<0.01, Table 3). In group 2, 92% of embryos hatched. Embryos cultured in HYR did not reach 80% of hatching (data not shown in tables).

DISCUSSION

Development up to blastocyst stage was impaired when cells were cultured in synthetic media devoid of chemically undefined substances (sera, BSA or other macromolecules) (LI *et al.*, 1993). The Ham's F-10 medium supplemented with 20% (v/v) ovulated-doe serum supported the development of rabbit zygotes up to the blastocyst stage. The blastocyst rate was similar (100%) to that obtained in previous studies on which more advanced embryonic stages were cultured (90-100%, GARCÍA-XIMÉNEZ *et al.*, 1995, 1996 and VICENTE and GARCÍA-XIMÉNEZ, 1994).

Ham's F-10 supplemented with young-rabbit serum instead of ovulated-adult-doe would be more economical and more easily available. Unfortunately, the use of young-rabbit sera as a supplement of medium affects the development of the cultured embryos, in particular their hatching. The undefined supplements of synthetic-media could enhance the growth from supplying a desirable component or from mitigating the effect of negative components (LI *et al.*, 1993). The sustained development using different sera could depend on the variability of the hormonal status of the serum donors. Supplements as growth factors or sexual hormones exert a positive effect on biological cell cultures (KANE, 1987).

On the other hand, KEEFER *et al.* (1988) cultured zygotes in Ham's F-10 plus 10% NOS ; they achieved development up to morulae, and at 72 hours of culture were around 74%, and up to hatching blastocysts, at 120 hours of culture 62%. These results compare to our group 1 which, in our experimental conditions, gave lower rates of development throughout the culture. In this group, 81.2% of embryos reached the morula stage after 48 hours of culture. After 168 hours of *in vitro* culture, 56% of embryos were at the hatching blastocyst stage.

Our culture conditions were similar to Keefer's, except for CO_2 concentrations (7% CO_2 in air *vs.* 5% CO_2 in air, respectively). HALLDEN *et al.* (1992) established the beneficial effect of a higher concentration of CO_2 than 5% for improving the hatching stage, when rabbit zygotes were cultured in a suboptimal medium (BMS-11 plus BSA).

In conclusion, the established ovulated-doe serum added to Ham's F-10 , the established conditions of culture (39°C, 7% CO_2 in air and 15 mM sodium bicarbonate) or more possible both factors sustain *in vitro* development at every embryo stage. An impaired embryo development was obtained when young-rabbit serum, as a supplement was used.

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