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Comparison of the effect of two different handling media on rabbit zygote developmental ability

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Abstract — Despite the attention paid to culture media, the relevance of the handling medium at egg recovery/transfer is frequently overlooked. In the present work, we compare the effect of two different handling media (PBS and HEPES-buffered Ham F10, both supplemented with 20% (v/v) FCS), upon in vitro and in vivo developmental ability of in vivo fertilised rabbit zygotes. Zygotes recovered in HEPES-buffered medium (permanence 1 h as maximum) and subsequently cultured in vitro developed more efficiently to the compacted morula (100%) and blastocyst stage (92%) than those recovered in PBS (83% and 76%, respectively, $P < 0.05$). Zygotes recovered in such media were then further bilaterally transferred to recipient does following a brief in vitro culture period (for 4 hours). At caesarean section (day 28 of pregnancy), significant differences were observed in both the percentage of pregnant uterine horns (PBS: 60% vs. HEPES-buffered Ham F10: 100%) and live birth rates (PBS: 14% vs. HEPES-buffered Ham F10: 34%). Thus when early rabbit zygotes must be handled, even for short incubation periods, the medium is not innocuous.

culture media / buffer system / development / zygote / rabbit

Résumé — Comparaison entre deux milieux de récupération et de manipulation des ovocytes de lapine sur leur capacité ultérieure de développement. Malgré l'attention prêtée aux milieux de culture, il est fréquent d'oublier l'importance du milieu de manipulation lors de la récupération ou du transfert des œufs. Dans ce travail, nous comparons l'effet exercé par deux milieux de manipulation différents (PBS et HEPES-buffered Ham's F10, tous deux additionnés de 20 % SVF) sur la capacité de développement in vivo et in vitro des zygotes fécondés in vivo. Les zygotes récupérés dans le milieu tamponné par l'HEPES (1 h de permanence au maximum) et cultivés in vitro présentaient un meilleur développement jusqu'aux stades morula (100 %) et blastocyste (92 %) que les zygotes récupérés dans le PBS (83 % et 76 %, respectivement, $P < 0,05$). Dans la seconde expérience, l'effet du milieu

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de manipulation des zygotes sur le développement in vivo a été étudié jusqu'à la naissance. Les zygotes récupérés dans ces deux milieux ont été transférés bilatéralement dans des lapines receveuses après une période de culture in vitro de 4 heures. Après la césarienne à 28 jours de gestation, des différences significatives ont été observées pour les taux de gestation (PBS: 60 % vs. HEPES-buffered Ham's F10 : 100 %) et pour le nombre de jeunes nés vivants (PBS : 14 % vs. HEPES-buffered Ham's F10 : 34 %). En conclusion, quand les zygotes doivent être manipulés, même pour une courte période de temps, le milieu n'est pas inoffensif.

milieu de culture / système tampon / développement / zygote / lapin

1. INTRODUCTION

For embryo production, a number of studies have been focused on in vitro long-term culture [2, 14]. However, handling media and conditions used at egg/embryo recovery or subsequent transfer to recipient does have usually been overlooked.

Brief rabbit embryo handling (corresponding to retrieval from does and/or transfer to recipients) is carried out in balanced usually phosphate buffered simple salt solutions (Hanks, Earle, Dulbecco or Tyrode solution). However, embryo incubation in such simple media for periods longer than 1 hour penalises subsequent in vitro or in vivo developmental ability [1, 22]. For more extended handling periods, the media are usually enriched with bovine serum albumin (BSA, Fraction V), homologous or heterologous sera [8, 11, 22, 25] and even with glucose and/or sodium pyruvate [22]. Despite this, some authors prefer to use more complex, bicarbonate or HEPES-buffered media [3, 8, 9, 21, 26, 28, 32], but to our knowledge, no comparison between simple and complex media has yet been carried out.

In the present work, we compare the effect of two different handling media: Dulbecco phosphate-buffered saline (PBS) and HEPES buffered Ham F-10, both supplemented with 20% (v/v) fetal calf serum, exert upon in vitro and in vivo developmental ability of in vivo-derived rabbit zygotes.

2. MATERIALS AND METHODS

2.1. Source of zygotes

Mature rabbit does from a synthetic line, called H [7], were mated twice with fertile bucks, receiving an intramuscular GnRH injection (20 μg, Fertagyl, Intervet, Spain) at the same time. At 12–13 h after mating, eggs were recovered immediately after euthanasia of donor females by flushing oviducts with either Dulbecco phosphate buffered saline (PBS, D5773, Sigma, Spain) supplemented with 20% (v/v) FCS (hereafter: s-PBS) or with Ham F-10 (N6635, Sigma, Spain) supplemented with 20 mM HEPES (11344, GibcoBRL, LifeTech, Spain) and 20% (v/v) FCS (hereafter: h-Ham). At recovery, the maximum permanence of eggs in these media was 60 minutes.

Collected eggs were incubated in hyaluronidase solution (1 $mg \cdot mL^{-1}$; H4272. Type IV-S, Sigma, Spain) for 5 minutes to remove the corona cells. Fertilised zygotes were selected for the presence of two clearly defined polar bodies (PB1 + PB2), supernumerary spermatozoa and a healthy general appearance under light microscopy. They were then cultured in bicarbonate-buffered Ham F-10 supplemented with 20% (v/v) FCS (hereafter: s-Ham) in a 7% CO_2 in air and 95% relative humidity and at 39 °C, either for a few hours or for 5 days.

2.2. Experiment 1: in vitro evaluation

In this experiment, we studied the effect of medium used for egg-flushing upon

further in vitro development. Selected eggs showing signs of fertilisation were cultured in s-Ham for 5 days and the developmental stage reached was assessed every 24 hours, according to the flushing media used.

Six replicates were performed per group. Data were analysed by a Chi-square test. When one single degree of freedom was involved, the Yates correction for continuity was applied.

2.3. Experiment 2: in vivo evaluation

The effect of handling medium on in vivo survival to term was studied. Zygotes flushed in either of the two different tested media were cultured in s-Ham for 4–5 hours (similar to the time required for current manipulation techniques). They were then collected in the same handling medium as earlier (either s-PBS or h-Ham) and transferred to a recipient doe. The maximum handling time until transfer was 60 minutes.

Zygotes handled in different media (s-PBS or h-Ham) were bilaterally transferred into each oviduct of pseudo-pregnant virgin does (s-PBS group into one oviduct; s-Ham: into the contralateral oviduct of the same female). Recipient does (line H) were synchronised by an intramuscular injection of GnRH (20 μg, Fertagyl, Intervet, Spain) around 21–23 hours before transfer. Does were anaesthetised by injecting a 5:1 ketamine chlorhydrate (Ketolar 50 $mg \cdot mL^{-1}$, Parke-Davis, Barcelona, Spain): prometazine (Frinova 25 $mg \cdot mL^{-1}$, Rhône-Poulenc Rorer, Madrid, Spain) intramuscular solution at a dose of 1.2 $mL \cdot kg^{-1}$ body weight. This was followed 5 min later by an intravenous injection of 1.5 mL of the same solution in the marginal ear vein. Embryo transfers were carried out via a mid-line ventral laparotomy.

In recipient does, pregnancy was diagnosed by abdominal palpation on the 12th day after ovulation induction. Non-pregnant does were laparotomised on the 12th day and the uterine horn content examined. In pregnant does, the delivery of pups was carried out at 28 days post-GnRH by caesarean section, and the number of both live and dead pups or degenerated foetuses per uterine horn was assessed.

Ten replicates were performed. Data were analysed by a Chi-Square test with the Yates correction for continuity.

3. RESULTS

At 12–13 hours post-coitum, the recovered zygotes were at an early fertilisation stage, with 77 out of 322 ova showing no signs of fertilisation (fertilisation rate: 76%, data not shown in the tables).

3.1. In vitro developmental ability

The handling media used for oviductal perfusion did not affect the in vitro ability of cultured zygotes ($n = 109$) to cleave (s-PBS: 97% and h-Ham: 100%; $P > 0.05$, Tab. I). However, observed significant differences in embryo developmental ability to reach the compacted morula and blastocyst stages indicate that the HEPES-buffered Ham F-10 is a more efficient handling medium (h-Ham: 100% and 92% vs. s-PBS: 83% and 76%, respectively; Tab. I). Such differences in embryo development were also observed at the hatching stage (h-Ham: 78%; s-PBS: 69%); however, they did not reach levels of significance.

3.2. In vivo developmental ability

In this experiment, ten recipient does received a total of one hundred and thirty-six zygotes (average number of transferred embryos per doe: 10.6, ranging from 2 to 10, Tab. II). All ten recipient does became pregnant on the 12th post-ovulatory day and pregnancies progressed to term.

At day 28 of pregnancy, significant differences in the percentage of pregnant

uterine horns were observed between experimental groups (s-PBS: 60% vs. h-Ham: 100%, $P < 0.05$; Tab. II). Moreover, differences in live birth rates per transferred egg were also significant between groups (h-Ham: 34% vs. s-PBS: 14%. $P < 0.05$; Tab. II).

4. DISCUSSION

In reproductive biotechnology, amongst several factors, high quality biological material and an efficient egg transfer procedure are required to allow experimentally produced eggs to express their developmental potential, even to term [1, 29].

Zygotes of several species, including rabbits, can be readily cultured to morula or blastocyst stage in vitro [4, 5, 11, 13, 15, 21, 25, 28, this work]. Although in vitro cultured embryos of some species can be successfully transferred to recipient females, successful implantation and development to term in the rabbit depends on mucin-coat

Table I. Effect of two different flushing media on in vitro developmental ability of early rabbit zygotes.

Recoverin medium*	Initial	Nb. zygotes (%) developed to the stage of			
		Cleavage	Compacted morula	Blastocyst	Hatched blastocysts
s-PBS	60	58 (97)	48 (83)[b]	44 (76)[b]	40 (69)
h-Ham	49	49 (100)	49 (100)[a]	45 (92)[a]	38 (78)

* s-PBS: Phosphate-buffered saline medium supplemented with 20% (v/v) foetal calf serum. h-Ham: HEPES-buffered Ham F-10 supplemented with 20% (v/v) FCS.
[a,b] Different superscripts within a column differ significantly ($P < 0.05$).

Table II. Effect of two different handling media on in vivo developmental ability of early rabbit zygotes.

Recipient Does	Assessment of pregnancy on the 28th post-ovulatory day					
	s-PBS			h-Ham		
	Nb. transferred eggs	Nb. live pups (%)	Nb. degenerated foetuses	Nb. transferred eggs	Nb. live pups (%)	Nb. degenerated foetuses
1	7	2	0	7	2	0
2	7	2	0	7	2	0
3	5	1	0	8	2	0
4	8	2	1	7	5	0
5	5	0	0	7	0	1
6	2	1	1	5	4	0
7	5	0	0	8	3	0
8	8	0	0	7	2	0
9	8	1	0	7	2	0
10	10	0	0	8	2	1
Total	65	9 (14)[b]	2	71	24 (34)[a]	2

[a,b] Different superscripts between columns differ statistically ($P < 0.05$).

formation at early embryo stages during the oviductal transit [12, 24]. This particular structure of rabbit embryos can only be produced in vivo and requires the transfer of zygotes or very early embryos to definitive or temporally recipient does, which avoids the substantial reduction in the live birth rate associated with in vitro embryo culture in this species [1, 3, 22, 31, 32].

Whereas culture conditions and media have always received special attention, the medium in which eggs and embryos are handled is a factor that has usually been overlooked. However, in the present work we have demonstrated that it also affects subsequent rabbit embryo development, even when incubation is shorter than 60 min (this work). In fact, oviductal perfusion with HEPES-buffered medium leads to better in vitro and in vivo developmental rates than with a saline solution such as PBS, although both are supplemented with 20% (v/v) serum.

This effect of handling media based on balanced salt solutions cannot be due to limited amount of glucose and pyruvate, as 20% serum was added to both base media [17]. Moreover, rabbit zygotes do not require glucose or pyruvate to develop to the blastocyst stage [16].

An explanation for the phenomenon observed in our experiment would be the greater phosphate content in PBS medium (K_2HPO_4: 0.2 $g \cdot L^{-1}$ and NaH_2PO_4: 1.15 $g \cdot L^{-1}$) than in Ham F-10 (K_2HPO_4: 0.083 $g \cdot L^{-1}$ and NaH_2PO_4: 0.1537 $g \cdot L^{-1}$; Sigma), which may exert a development-inhibitory effect on early embryos. Such an effect has been described in mice [6], rat [30], cattle [10, 23], and pig [20, 27]. However, to our knowledge, such a phosphate inhibitory effect has not yet been described for rabbit embryos, in contrast with the preferred phosphate-buffered solutions recommended for this species in more classical studies [1].

Our in vivo results when rabbit zygotes were briefly handled (2 h maximum: 1 h for egg recovery plus 1 h for transfer) in s-PBS medium are comparable to those obtained by Mills et al. [19] after the immediate transfer of slightly older (24 hpc) rabbit zygotes to synchronised females (12% live pups). Better developmental rates (nearly 34% live born) were obtained in the present experiment, using HEPES-buffered Ham's F10 (h-Ham) as handling medium. Live pups per transferred egg is comparable to that obtained from more advanced rabbit embryos stages (4- to 8-cell stages), but that are immediately transferred [8, 18, 32]. The in vivo results reported in the present study support observations from in vitro assays, especially considering that embryo culture conditions and media are supported by the recipient doe.

In conclusion, when early rabbit zygotes have to be handled, the medium (in particular phosphate-buffered media) is not innocuous for further development, even for short incubation periods.

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