SHORT COMMUNICATION

Asynchrony between *in vivo* and *in vitro* rabbit embryos

Asincronía entre embriones in vivo e in vitro en conejo

Assincronia entre embriões in vivo e in vitro em coelho

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To cite this article:

García ML, Belabbas R, Muelas R, Agea I, Argente MJ. Asynchrony between *in vivo* and *in vitro* rabbit embryos. Rev Colomb Cienc Pecu 2022; 35(2): 118–123. DOI: https://doi.org/10.17533/udea.rccp.v35n2a02

Abstract

Background: Comparative features of embryos developed under *in vitro* and *in vivo* conditions are particularly important in designing embryo transfer procedures that fulfil embryo-recipient synchronization requirements. **Objective:** To determine the degree of asynchrony in rabbit embryo development between cultured and *in vivo* embryos. **Methods:** A total of 55 non-lactating multiparous female rabbits were used. Embryos were classified as 16-cells or early morulae at 48 hours post-coitum (hpc). Embryos were cultured during 30 or 32 h and embryo development was compared with *in vivo* embryos of 72 hpc. *In vitro* and *in vivo* embryos at 72 hpc were classified as early or compacted morulae. Bayesian statistics was used. Difference between *in vivo* and *in vitro* embryos and the actual probability of the difference between the *in vivo* and *in vitro* embryo higher than zero (P) was estimated. **Results:** The percentage of compacted morulae was higher in *in vivo* embryos than in *in vitro* embryos with +6 h of asynchrony (73.5 and 32.8%, P=1.00). But the percentage of compacted morulae was similar with +8 h asynchrony. **Conclusions:** *In vitro* embryos delay their development by + 8 hours compared to *in vivo* embryos.

Keywords: *assisted reproduction; embryo culture; embryonic asynchrony; embryonic development; embryo synchronization; embryo transfer; in vitro embryo production; in vivo embryo production; morulae; rabbits.*

Received: September 30, 2020; accepted: July 22, 2021

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Resumen

Antecedentes: El desarrollo comparativo de embriones producidos *in vitro* e *in vivo* es particularmente importante para el diseño de procedimientos de transferencia de embriones cuando se requiere sincronización entre el embrión y la hembra receptora. **Objetivo:** Determinar el grado de asincronía en el desarrollo embrionario entre embriones *in vivo* y cultivados. **Métodos:** Un total de 55 conejas multiparas no lactantes fueron utilizadas. Los embriones se clasificaron en 16 células o mórulas tempranas a las 48 horas después del coito (hpc). Los embriones se cultivaron durante 30 ó 32 horas y el desarrollo embrionario se comparó con embriones de 72 hpc obtenidos *in vivo*. Los embriones *in vitro* e *in vivo* a 72 hpc se clasificaron como mórulas tempranas o compactas. Se utilizó estadística bayesiana. Se estimó la diferencia entre embriones *in vivo* e *in vitro* y la probabilidad de que la diferencia sea superior a cero (P). **Resultados:** El porcentaje de mórulas compactas fue mayor en embriones *in vivo* que en embriones *in vitro* con +6 horas de asincronía (73,5 y 32,8%, P=1,00), pero el porcentaje de mórulas compactas fue similar con asincronía de +8 horas. **Conclusión:** Los embriones cultivados retrasan +8 horas su desarrollo en comparación con los embriones *in vivo*.

Palabras clave: *asincronía embrionaria; conejo; cultivo de embriones; desarrollo embrionario; mórula; producción de embriones in vitro; producción de embriones in vivo; reproducción asistida; sincronización de embriones; transferencia de embriones.*

Resumo

Antecedentes: A aquisição do desenvolvimento de embriões produzidos *in vitro* e *in vivo* é particularmente importante na concepção de procedimentos de transferência de embriões em que a sincronização entre o embrião e a fêmea receptora é necessária. **Objetivo:** Determinar o grau de assincronia no desenvolvimento embrionário entre embriões cultivados e *in vivo*. **Métodos:** Um total de 55 coelhos multíparos não lactantes foram usados. Os embriões foram classificados em 16 células ou mórulas iniciais 48 horas de gestação (hpc). Os embriões foram cultivados por 30 ou 32 horas e o desenvolvimento embrionário foi comparado com embriões de 72 hpc obtidos *in vivo*. Embriões *in vitro* e *in vivo* a 72 hpc foram classificados como mórulas precoces ou compactadas. Estatísticas bayesianas foram usadas. A diferença entre embriões *in vivo* e *in vitro* e a probabilidade de que a diferença seja maior que zero (P) foi estimada. **Resultados:** A porcentagem de mórulas compactadas foi maior em embriões *in vivo* do que em embriões *in vitro* com +6 horas de assincronia (73,5 e 32,8%, P=1,00). Mas a porcentagem de mórulas compactadas foi semelhante com assincronia de +8 horas. **Conclusão:** Embriões cultivados atrasam seu desenvolvimento em +8 horas em comparação com embriões *in vivo*.

Palavras-chave: *assincronia embrionária; coelho; cultura de embriões; desenvolvimento embrionário; mórula; produção in vitro de embriões; produção de embriões in vivo; reprodução assistida; sincronização de embriões; transferência de embrião.*

Introduction

Rabbit zygotes and early embryos have been successfully developed to blastocysts using different culture media (Saenz-de-Juano *et al.*, 2011). However, these media do not mimic the oviductal environment and *in vitro* developed embryos differ from their *in vivo* analogues. Specifically, *in vitro* cultured rabbit embryos show fewer cells, smaller diameters (Adams, 1970) and morphological signs of degeneration after one day in culture from morulae to early blastocysts (Hegele-Hartung *et al.*, 1988). Thus, embryos undergo a retarded development under *in vitro* conditions (Carney and Foote, 1990).

The female rabbit has certain physiological and anatomical characteristics that make it especially suitable for the application of embryo reproductive techniques (García, 2018). *In vitro* embryo culture is an assisted reproductive technique used frequently in embryo biology. The comparative features of embryos that develop under *in vitro* and *in vivo* conditions are particularly important for designing embryo transfer procedures that fulfil embryo-recipient synchronization requirements. Therefore, in order to maximize the results of the application of reproductive techniques, it is necessary to accurately determine the degree of delay in embryonic development of cultured embryos. Therefore, the aim of this study was to determine the degree of asynchrony in embryo development between *in vitro* and *in vivo* embryos at 72 hpc in rabbits.

Materials and Methods

Ethical considerations

All experimental procedures were approved by the Committee of Ethics and Animal Welfare of the Miguel Hernández University, Spain (Ref: 2019/VSC/PEA/0017).

In vivo embryo production and collection

Non-lactating multiparous female rabbits were used (Argente *et al.*, 2019). A total of 41 and 14 does were mated and then slaughtered at 48 and 72 hours post-coitum (hpc) by intravenous administration of 50 mg sodium thiopental/kg body weight (thiobarbital, B. Braun Medical S.A., Barcelona, Spain). The ovaries, oviducts and uterine horns were removed. To recover the embryos, the oviduct and the first third of the uterine horn were flushed with 5 mL Dulbecco phosphate buffered saline (DPBS, Sigma, Alcobendas, Madrid, Spain) supplemented with 0.2% (wt/vol) bovine serum albumin (BSA, Cod. A-3111, Sigma) and 0.2 mL of antibiotic (Penivet 1, Divasa Farmavic, Barcelona, Spain) at room temperature. *In vivo* embryos were examined. They were considered normal when homogenous cellular mass and spherical zona pellucida and mucin coat was present. A binocular stereoscopy microscope (Leica Mz 9.5-600x, Wetzlar, Germany) was used. At 48 hpc, normal embryos were classified as 16-cells or early morulae (Figure 1A). At 72 hpc, normal embryos were classified as early morulae or compacted morulae (Figure 1B).

Embryo culture

Normal embryos at 48 hpc from each doe were cultured in a one-well 4-well embryo culture dish (NUNC A/S, Thermo Fischer Scientific, Denmark) containing 1 ml culture media (TCM-199 supplemented with 10% fetal bovine serum). Culture was performed at 38.5 ºC in 5% CO2 in air saturated humidity. *In vitro* developed embryos were examined after 30 or 32 h of culture. So, the asynchrony between *in vivo* and *in vitro* embryos was +6 h or +8 h. Then, embryos were classified as early morulae or compacted morulae. A total of 20 and 21 females were used to culture their embryos with an asynchrony of + 6h and + 8h, respectively.

Statistical analyses

Early morulae and compacted morulae were expressed as a percentage of normal embryos. Differences in asynchrony between *in vivo* and *in vitro* embryos were estimated with a model including the effects of asynchrony (*in vivo* embryos, and *in vitro* embryos with +6 h and +8 h of asynchrony). Analysis was performed

using Bayesian methodology. Bounded uniform priors were used for all effects. Residuals were, a priori, normally distributed with mean **0** and variance $\mathbf{I}\sigma^{2e}$. The prior for the variance was also bounded uniform. Features of the marginal posterior distributions for all unknowns were estimated using Gibbs sampling. Inferences were derived from the marginal posterior distributions. Median, difference between *in vivo* and *in vitro* embryos (D), and the shortest interval with 95% probability of containing the true value ($HPD_{95\%}$) were provided. The $HPD_{95\%}$ showed the precision of the estimation and can be asymmetric around the estimation. The actual probability of D higher than zero (P) was estimated. The Rabbit program developed by the Institute for Animal Science and Technology (Valencia, Spain) was used.

Results

Table 1 shows differences between *in vivo* and *in vitro* embryo development. When the asynchrony was +6 h, the percentage of early morulae was lower in *in vivo* than in *in vitro* embryos (-40.7%, P = 1). So, the percentage of compacted morulae was higher. An asynchrony of +6 h was not enough for matching *in vitro* and *in vivo* embryo development. Nevertheless, the percentage of early morulae (-7.0%, P = 0.68) and compacted morulae were similar between *in vivo* and *in vitro* embryos with +8 h of asynchrony.

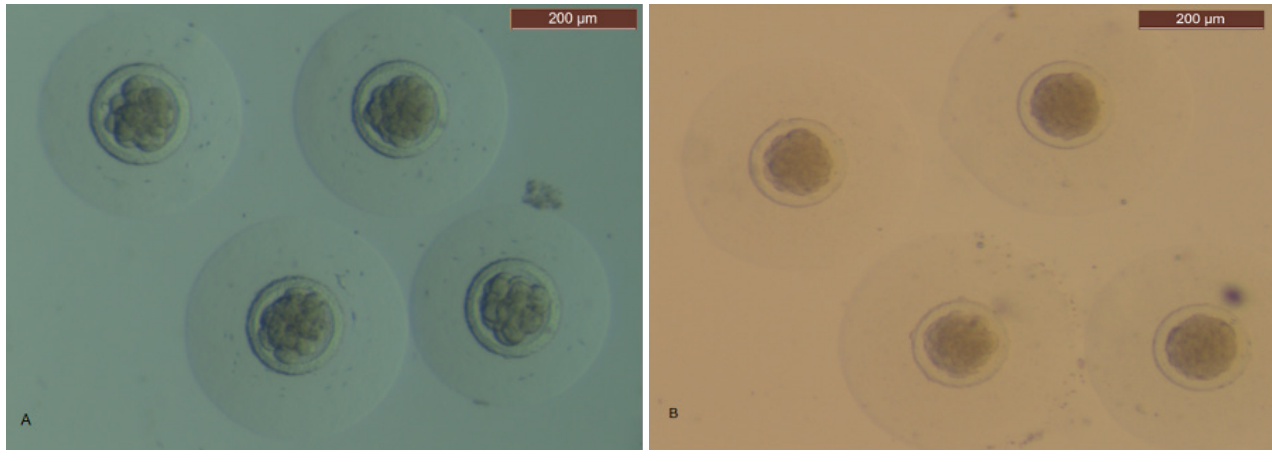

Figure 1. A) Scaled *in vivo* early morulae obtained at 48 hpc. B) Scaled compacted morulae from *in vivo* embryos at 48 hpc and cultured for 32 h.

Table 1. Differences between development between *in vivo* and *in vitro* embryos.

	In vivo	***In vitro***	**D**	**$HPD_{95\%}$**	**P**
+6 h asynchrony (N)	14	20			
Early morulae	32.8	73.5	-40.7	-65.0, -16.3	1.00
Compacted morulae	67.2	26.5	40.7	16.1, 66.2	1.00
+ 8 h asynchrony (N)	14	21			
Early morulae	32.8	39.8	-7.0	-36.0, 18.3	0.68
Compacted morulae	67.2	60.2	7.0	-20.1, 34.1	0.68

N=number of does; *In vivo*=median of *in vivo* embryos at 72 hpc; In vitro=median of *in vitro* embryos; D=median of the difference between the *in vivo* and *in vitro* embryos; HPD95%=highest posterior density region at 95%; P=probability of the difference being >0 when D>0 and probability of the difference being <0 when D<0.

Discussion

The low development of cultured embryos also occurs in cattle (Lonergan *et al.*, 2016) and pigs (Fowler *et al.*, 2018). While embryo culture systems are static, *in vivo* embryos are exposed to a constantly changing environment as it passes along the oviduct to the uterus. Concomitantly, embryos exhibit changes in physiology and energy metabolism between fertilization and blastocyst (Gardner, 1998). Thus, embryo manipulation and adaptation to *in vitro* conditions as its requirements change during development lead to a reduction in development (García, 2018). We have determined that asynchrony in rabbits is 8 hours between cultured and *in vivo* embryos. This result can be used to optimize the design of experiments in which cultured embryos are used.

In conclusion, development was lower in *in vitro* than in *in vivo* embryos. Asynchrony between *in vivo* and *in vitro* embryonic development was 8 hours.

Declarations

Funding

This study was funded by MINECO AGL2017- 86083-C2-2-P and AICO/2019/169.

Conflicts of interest

The study was funded by the Sustainability StraThe authors declare they have no conflicts of interest with regard to the work presented in this report.

Author contributions

Conceptualization: M-L García; M-J Argente. Data collection: R. Muelas; I. Agea. Funding acquisition: M-L García; M-J Argente. Writing-Original: M-L García. Writing- Editing: M-L García; M-J Argente; R. Belabbas.

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