Received: 6 March 2024 | Accepted: 13 May 2024

DOI: 10.1111/rda.14622

Reproduction in Domestic Animals WILEY

SHORT COMMUNICATION

A new hormonal protocol supports early development of in vitro-produced embryos after transfer to anoestrus mares

Carmen C. Muñoz-García[1] | Marcos Luis-Calero[1] | Pablo Fernández-Hernández[1] | Alba Pizarro-Hernández[1] | Raúl Fernández-González[2] | Alfonso Gutiérrez-Adán[2] | María Jesús Sánchez-Calabuig[3] | Juan Cuervo-Arango[4] | Lauro González-Fernández[5] | Beatriz Macías-García[1]

[1]Departamento de Medicina Animal, Grupo de Investigación Medicina Interna Veterinaria (MINVET), Instituto de Investigación INBIO G+C, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain

[2]Department of Animal Reproduction, INIA-CSIC, Madrid, Spain

[3]School of Veterinary Medicine, Universidad Complutense de Madrid, Madrid, Spain

[4]Facultad de Veterinaria, Universidad CEU Cardenal Herrera, CEU Universities, Valencia, Spain

[5]Departamento de Bioquímica y Biología Molecular y Genética, Grupo de Investigación Señalización Intracelular y Tecnología de la Reproducción (SINTREP), Instituto de Investigación INBIO G+C, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain

Correspondence
Beatriz Macías-García, Departamento de Medicina Animal, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain.
Email: bemaciasg@unex.es

Funding information
Junta de Extremadura, Grant/Award Numbers: IB20005 and PI-0152-22; Universidad de Extremadura, Grant/Award Number: RC1; Banco Santander, Grant/Award Number: RC1; Agencia Estatal de Investigación, Grant/Award Number: PID2020-112723RB-I00; European Regional Development Fund, Grant/Award Number: IB20005

Abstract

The present study aimed to evaluate whether primed anoestrus mares are suitable recipients for embryos produced by intracytoplasmic sperm injection (ICSI). Anoestrus was confirmed in four mares and daily doses of oestradiol benzoate (6 mg in total) over 5 days were administered; after 3 days of rest, oral altrenogest was administered at 0.088 mg/kg and embryos (1 to 5 embryos per mare; 15 in total) were transferred 3.5 days after progesterone onset. Uterine lavage was conducted 48 h after transfer. The results revealed an 80% embryo recovery rate, and among the retrieved embryos, 67% showed evident intrauterine development. Hence, ICSI-derived embryos can be successfully transferred to primed anoestrus mares, but more studies are required to ensure further embryo development and foaling.

KEYWORDS
anoestrus recipient, in vitro embryo, mare, transfer

1 | INTRODUCTION

Embryo transfer in horses has become more common, with a rising need for recipient mares to carry valuable embryos (Claes & Stout, 2022; Derbala et al., 2024). Interestingly, various hormonal protocols, including oestradiol benzoate, clomiphene citrate and short-acting or long-acting progesterone, enable the use of anoestrus mares as suitable recipients for in vivo-produced embryos (Derbala et al., 2024). Besides, prolonged treatment with oestradiol benzoate (5 days) followed by progesterone resulted in higher embryo survival rates compared with those using progesterone alone or short oestradiol benzoate priming in anoestrus recipient mares

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2024 The Author(s). *Reproduction in Domestic Animals* published by Wiley-VCH GmbH.

(Oquendo et al., 2022). Previous studies using anoestrus mares as recipients were conducted using in vivo-produced embryos, yielding promising results comparable to those of cycling mares (Botelho et al., 2015; Derbala et al., 2024).

For this reason, our study sought to determine whether anoestrus mares treated with oestradiol benzoate and short-acting progesterone before transfer can be used as recipients of embryos produced by intracytoplasmic sperm injection (ICSI). The study specifically aimed to observe early embryo development and morphology 48 h post-transfer.

2 | MATERIALS AND METHODS

2.1 | Recipient mares

The study involved four mares aged between 16 and 18 years old, weighing between 400 and 500 kg. The research was conducted between December of 2023 and January of 2024 at the Veterinary Clinical Hospital of the University of Extremadura (Cáceres, Spain). The mares underwent ultrasound monitoring and were included in the study when no cyclicity (anoestrus) was determined by the absence of corpora lutea for 21 days and the presence of follicles smaller than 20 mm. All experiments were conducted following institutional and European regulations.

2.2 | Hormone protocol

Oestradiol benzoate was administered by intramuscular injection once daily for five consecutive days as previously reported (Oquendo et al., 2022) with minor modifications. A total of 6 mg of oestradiol benzoate were administered to each mare. This treatment was initiated with 0.5 mg, increasing by 0.5 mg per day until reaching Day 3 (1.5 mg), and this dose was maintained on Days 4 and 5, as uterine oedema increased to 4–5/5. The presence of endometrial oedema was subjectively scored on a scale of 1 to 5 based on the intensity of the endometrial fold pattern (1 = no oedema, 2 = some oedema, 3 = obvious oedema, 4 = a lot of oedema and 5 = moderate/pathological oedema). After 5 days, the oestradiol treatment was discontinued, and 3 days later, oral altrenogest administration began at a dose of 0.088 mg/kg (considered as 'Day 0') until the day of embryo recovery.

2.3 | ICSI and embryo transfer and uterine lavage

Sixteen embryos produced in vitro by ICSI following established protocols (Sánchez-Calabuig et al., 2021) were used in this study. Embryos were vitrified on Day 9 post-ICSI using the Kitazato protocol. On the day of embryo transfer (Day 3.5 post-progesterone treatment onset), warming was performed following the Kitazato protocol. Warmed embryos (Figure 1) were washed in holding medium (Minitub Ibérica, Tarragona, Spain) and loaded into sterile 0.5 mL straws (one to five embryos per straw). Embryos were transferred following the modified Wilsher's technique, as described previously (Cuervo-Arango et al., 2018). Each mare underwent a single transfer. At the time of transfer, mares were sedated with 0.4 mg/kg of xylazine hydrochloride and received an intravenous dose of flunixin meglumine at 1.1 mg/kg. Uterine flushing was performed 48 h after transfer with warm ringer-lactate solution.

3 | RESULTS

Endometrial oedema gradually increased in anoestrus recipient mares, reaching a grade of 4–5/5 on the third day of treatment; on the day of transfer, the oedema had dropped to 1–2/5. Embryo thawing resulted in 1 Code 4 (dead or degenerating which was not transferred), and 13 classified as good Code 1 (uniform in size, colour

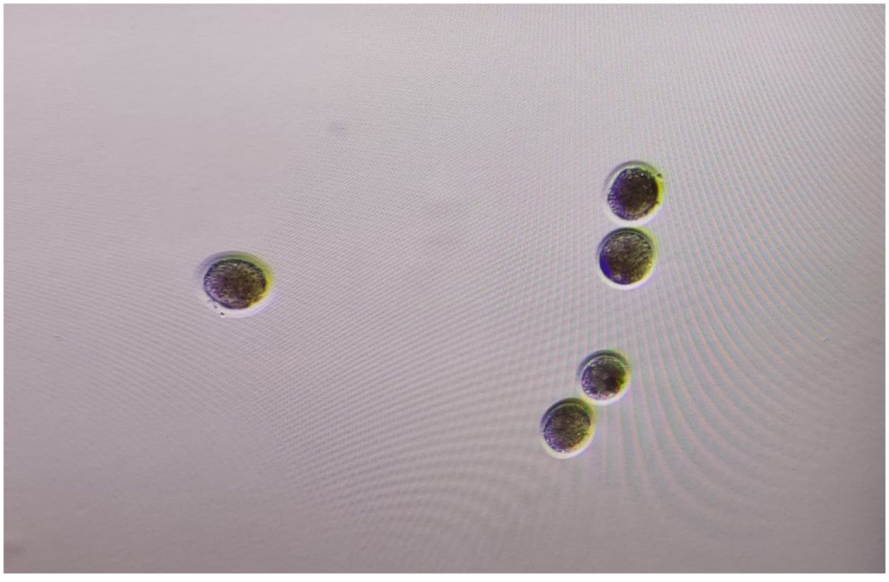

FIGURE 1 Equine embryos produced by ICSI (Day 9) after warming using the Kitazato protocol. The embryos show dark cytoplasm and similar size between them.

and density) and two embryos as Code 2 (moderate irregularities) considered to be of average quality (Lewis et al., 2023) resulting in a survival rate after warming of 93.75% (see Table 1). Out of the 15 embryos transferred, 12 were successfully recovered after 48 h (80% of embryo recovery). Among these 12 embryos, 67% (8/12) showed vivid intrauterine development, exhibiting apparent similarities with embryos produced in vivo in terms of size, transparent appearance and the presence of the inner cell mass (Umair et al., 2023; Figure 2 and Table 1). Among the remaining embryos (4/12), two exhibited small size compared with their counterparts and heterogeneous appearance (Figure 2 and Table 1), one suddenly broke during embryo handling and one seemed to be hatching.

4 | DISCUSSION

The current study is the first in the scientific literature to confirm the feasibility of transferring in vitro-produced embryos to anoestrus recipient mares treated with oestradiol and short-acting progesterone. Our protocol modifies the method described by Oquendo et al. (2022), where a final dose of oestradiol benzoate (8 mg) was administered at increasing rates for 5 days, simulating prolonged uterine oedema typical of natural oestrus. In our approach, daily ultrasound monitoring of uterine oedema revealed a peak (4–5/5) on Day 3 following the onset of oestradiol administration. To prevent excessive hormonal stimulation and possible uterine fluid accumulation, the dosage of oestradiol was not raised after reaching maximum level of uterine oedema. This result is in agreement with the data reported by Silva et al. (2023), who observed that a small dose of 2 mg of oestradiol benzoate was sufficient to induce endometrial oedema similar to that found in natural oestrus. Another modification of the protocol by Oquendo et al. (2022) was the day of transfer, while they transferred their embryos on Day 4 after progesterone administration, we transferred our embryos on Day 3.5, since embryos produced in vitro exhibit a very narrow window of recipient synchrony (Cuervo-Arango et al., 2019).

Despite all the previously mentioned modifications, the embryo recovery rate observed after 48 h of transfer (80%) was similar to that reported by Oquendo et al. (2022) even when in our report poor-quality embryos produced in vitro on Day 9 after ICSI were used. Interestingly, Oquendo et al. (2022) used Day 8 embryos ($n = 6$) produced in vivo, which are known to be of superior quality (Claes & Stout, 2022). This result suggests that the dose of oestradiol benzoate can be adjusted based on uterine oedema, resulting in good embryo recovery after 48 h, but this has to be further studied. In addition, 4 out of the 12 embryos recovered in the present study (25%), exhibited a poor-quality appearance, coinciding with the data provided by Umair et al. (2023), who reported a 33% of poor-quality embryos under similar conditions. As varying embryo quality (good to poor) were recovered from the same mare after uterine flushing, it seems that in IVP equine embryos, intrinsic embryo factors may have a significant impact on subsequent embryo development disregarding their post-warming quality (Table 1).

TABLE 1 Summarized data of equine embryo morphology post-warming and after uterine flushing.

	Transferred embryos			Recovered embryos			
		Grade			Morphology		
Mare	Total	1	2	Total	Transparent appearance	ICM	Small size/ heterogeneous
A	1	1		1	1	1	0
B	4	3	1	4	2	3	2
C	5	4	1	4	4	4	0
D	5	2	3	3	3	3	1

Note: The table presents the individual data for each mare in which embryos were transferred, indicating the number of transferred embryos, morphology post-warming, embryo recovery, gross appearance and presence or absence of inner cell mass (ICM).

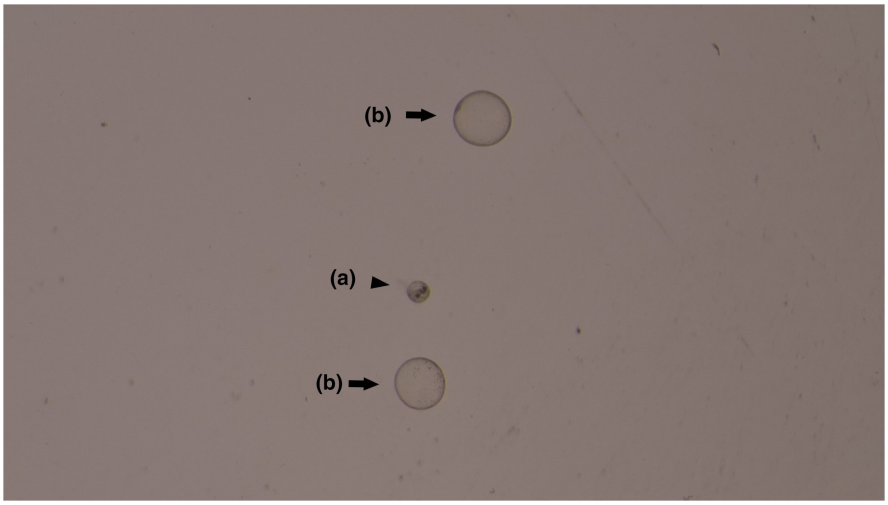

FIGURE 2 Equine expanded blastocysts recovered after 48 h of uterine transfer. Embryos were flushed from the same mare, and apparent differences are observed: (a) slow embryo development and irregular appearance (arrowhead) and (b) two embryos with significant development (arrows).

5 | CONCLUSION

Our study demonstrates the feasibility of transferring embryos produced in vitro by ICSI to anoestrus recipient mares treated with oestradiol benzoate and short-acting progesterone, offering promising prospects for embryo transfer in equine reproductive management.

AUTHOR CONTRIBUTIONS

Conceptualization, B.M.-G.; methodology C.C.M.-G., M.L.-C., P.F.-.H., A.P.-H., R.F.-G., M.J.S.-C. and B.M.-G.; formal analysis C.C.-M.-G. and B.M.-G.; investigation, C.C.-M.-G., J.C.-A. and B.M.-G.; resources, L.G.-F., A.G.-A. and B.M.-G.; writing—original draft preparation, C.C.M.-G., L.G.-F. and B.M.-G; writing—review and editing, C.C.M.-G., M.L.-C., P.F.-.H., A.P.-H., R.F.-G, A.G.-A., M.J.S.-C., J.C.-A., L.G.-F. and B.M.-G.; supervision, B.M.-G; project administration, L.G.-F. and B.M.-G.; funding acquisition, L.G.-F. and B.M.-G. All authors have read and agreed to the published version of the manuscript and to the order of authors.

FUNDING INFORMATION

This work was supported by 'Junta de Extremadura' (Spain) and 'Fondo Europeo de Desarrollo Regional (FEDER)'; Reference: IB20005, and project PID2020-112723RB-I00 funded by MICIU/AEI/10.13039/501100011033. Marcos Luis-Calero was supported by 'Plan Propio de Iniciación a la Investigación, Desarrollo Tecnológico e Innovación. Acción II' from the University of Extremadura and Banco Santander; Reference: RC1. Carmen C. Muñoz-García is funded by 'Programa Investigo' from 'Junta de Extremadura'; Reference: PI-0152-22.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available upon request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ORCID

Carmen C. Muñoz-García https://orcid.org/0009-0009-6733-4834
Pablo Fernández-Hernández https://orcid.org/0000-0001-5445-1630
María Jesús Sánchez-Calabuig https://orcid.org/0000-0002-4280-992X
Juan Cuervo-Arango https://orcid.org/0000-0001-5327-4230
Lauro González-Fernández https://orcid.org/0000-0001-5568-548X
Beatriz Macías-García https://orcid.org/0000-0001-5331-9793

REFERENCES

Botelho, J. H. V., Pessoa, G. O., Rocha, L. G. P., & Yeste, M. (2015). Hormone supplementation protocol using estradiol benzoate and long-acting progesterone is efficient in maintaining pregnancy of anovulatory recipient mares during autumn transitional phase. *Animal Reproduction Science*, *153*, 39–43. https://doi.org/10.1016/j.anireprosci.2014.12.014

Claes, A., & Stout, T. A. E. (2022). Success rate in a clinical equine in vitro embryo production program. *Theriogenology*, *187*, 215–218. https://doi.org/10.1016/j.theriogenology.2022.04.019

Cuervo-Arango, J., Claes, A. N., & Stout, T. A. (2018). Effect of embryo transfer technique on the likelihood of pregnancy in the mare: A comparison of conventional and Wilsher's forceps-assisted transfer. *Veterinary Record*, *183*(10), 323. https://doi.org/10.1136/vr.104808

Cuervo-Arango, J., Claes, A. N., & Stout, T. A. E. (2019). In vitro-produced horse embryos exhibit a very narrow window of acceptable recipient mare uterine synchrony compared with in vivo-derived embryos. *Reproduction, Fertility and Development*, *31*(12), 1904–1911. https://doi.org/10.1071/RD19294

Derbala, M. K., Mosallam, T. E., & Sindi, R. A. (2024). Embryo transfer into anestrous recipient mares prepared using clomiphene citrate and short-acting progesterone. *Journal of Equine Veterinary Science*, *133*, 105006. https://doi.org/10.1016/j.jevs.2024.105006

Lewis, N., Canesin, H., Choi, Y. H., Foss, R., Felix, M., Rader, K., & Hinrichs, K. (2023). Equine. *Reproduction, Fertility and Development*, *35*(4), 338–351. https://doi.org/10.1071/RD22224

Oquendo, P. S., Silva, E. S. M., Oquendo, F. M. G., Cuervo-Arango, J., & Beletti, M. E. (2022). The effect of priming and duration of oestradiol benzoate treatment before progesterone administration on embryo development and survival in anestrous recipient mares. *Reproduction in Domestic Animals*, *57*(11), 1474–1477. https://doi.org/10.1111/rda.14220

Sánchez-Calabuig, M. J., Fernández-González, R., Hamdi, M., Smits, K., López-Cardona, A. P., Serres, C., Macías-García, B., & Gutiérrez-Adán, A. (2021). A high glucose concentration during early stages of in vitro equine embryo development alters expression of genes involved in glucose metabolism. *Equine Veterinary Journal*, *53*(4), 787–795. https://doi.org/10.1111/evj.13342

Silva, E. S. M., Newcombe, J. R., & Cuervo-Arango, J. (2023). The effect of treatment with oestradiol benzoate on oestrus expression and endometrial oedema in anovulatory and cyclic mares. *Animals*, *13*(5), 938. https://doi.org/10.3390/ani13050938

Umair, M., Scheeren, V. F. D. C., Beitsma, M. M., Colleoni, S., Galli, C., Lazzari, G., De Ruijter-Villani, M., Stout, T. A. E., & Claes, A. (2023). In vitro-produced equine blastocysts exhibit greater dispersal and intermingling of inner cell mass cells than in vivo embryos. *International Journal of Molecular Sciences*, *24*(11), 9619. https://doi.org/10.3390/ijms24119619

How to cite this article: Muñoz-García, C. C., Luis-Calero, M., Fernández-Hernández, P., Pizarro-Hernández, A., Fernández-González, R., Gutiérrez-Adán, A., Sánchez-Calabuig, M. J., Cuervo-Arango, J., González-Fernández, L., & Macías-García, B. (2024). A new hormonal protocol supports early development of in vitro-produced embryos after transfer to anoestrus mares. *Reproduction in Domestic Animals*, *00*, e14622. https://doi.org/10.1111/rda.14622