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In vitro Developmental Competence of Adult Sheep Oocytes Treated with Roscovitine

LF Crocomo[1], F Ariu[2], L Bogliolo[2], D Bebbere[2], S Ledda[2] and SD Bicudo[3]

[1]*Institute of Agricultural Sciences, Federal University of Minas Gerais (UFMG), Montes Claros, Minas Gerais, Brazil;* [2]*Department of Veterinary Medicine, University of Sassari, Sardegna, Italy;* [3]*Department of Animal Reproduction and Veterinary Radiology, School of Veterinary Medicine and Animal Science, UNESP, Botucatu, São Paulo, Brazil*

Contents
The efficiency of *in vitro* sheep embryo production is still low compared to that observed *in vivo* and in other species. In this context, meiotic inhibition strategies emerged as a promising alternative to improve this biotechnology. So, this study aimed to evaluate, for the first time, the effects of roscovitine on *in vitro* maturation of sheep oocytes and their subsequent embryo development. For this, *cumulus*–oocyte complexes (COCs) were cultured for 6 h in the presence (Rosco) or absence (Control) of 75 μM roscovitine and, subsequently, *in vitro* matured (IVM) for 18 h with gonadotropins. At 0 (Immature), 6 and 24 h of culture, the nuclear status of oocytes was evaluated by Hoechst staining. Embryo cleavage and blastocyst formation were recorded 30 h after *in vitro* fertilization and on day 7 of culture, respectively. Blastocyst quality was evaluated by differential staining. At 6 h, the GV rate in the Rosco treatment (93.8%) was similar to that observed in the Immature oocytes (94.9%) and significantly higher compared to Control (41.3%). After IVM for 18 h, a high and similar proportion of oocytes from Rosco (93.6%) and Control (88.4%) reached the MII stage. In both treatments, approximately 70% of oocytes cleaved and 50% of them developed up to blastocyst. The mean percentage of blastocyst cells, embryoblast, trophoblast and pyknosis did also not differ between Control and Rosco. In conclusion, roscovitine, at the studied experimental conditions, was efficient to reversibly inhibit the meiosis of adult sheep oocytes without detrimental effect on development and quality of the *in vitro* produced embryos.

Introduction

Small ruminant production is an important economic activity in several developing countries. In this context, reproductive biotechnologies, as *in vitro* embryo production (IVP), represent a promising alternative to accelerate the genetic improvement and increase the productivity and profitability of livestock. However, despite this, investigations on IVP in small ruminants are still limited and the results so far obtained demonstrate that the development potential of *in vitro* produced embryos is still low compared to that observed *in vivo* or in other species (Paramio and Izquierdo 2014; Souza-Fabjan et al. 2014). According to Krisher (2004), this low developmental efficiency is directly related to the poor quality of oocytes *in vitro* matured.

In vivo, oocytes remain arrested at diplotene stage of prophase-I, morphologically recognized as germinal vesicle (GV), throughout folliculogenesis until the pre-ovulatory gonadotropin surge, when meiosis is resumed. During this period of meiotic arrest, oocytes undergo ultrastructural and molecular modifications that are essential for developmental competence acquisition and characterize the cytoplasmic maturation. Although distinct, these nuclear and cytoplasmic events are linked and occur synchronously, ensuring that only oocytes capable to drive correctly the maturation, fertilization and early embryogenesis reach ovulation. *In vitro*, on the other hand, meiosis spontaneously resumes when oocytes are removed from the follicular environment (Pincus and Enzmann 1935). However, despite the proper chromosome segregation, it alone is not enough to ensure embryo development (Krisher 2004). Furthermore, oocytes destined to *in vitro* maturation are retrieved from follicles at different stages of folliculogenesis and most of them, although meiotically competent, have not undergone all required cytoplasmic changes to support further development.

In this context, the temporary arrest of meiosis with pharmacological molecules has been proposed as a strategy to re-establish the synchronization between nuclear and cytoplasmic maturation and, consequently, improve the homogeneity and quality of oocyte population. Due to its potent and specific inhibitory action on the cell-cycle regulator M-phase-promoting factor (MPF) (Meijer et al. 1997a), roscovitine has been tested in several animal species such as bovine (Mermillod et al. 2000; Lagutina et al. 2002; Adona and Leal 2004), goat (Han et al. 2006), pig (Romar and Funahashi 2006) and cat (Sananmuang et al. 2010). The effective concentration of roscovitine, the time of exposition capable to inhibit the meiotic resumption and its effects on oocyte ability to support fertilization and pre-implantation embryo development are, however, variable among authors and studied species. While some researchers did not observe any effect on oocyte developmental competence (Mermillod et al. 2000; Lagutina et al. 2002), others reported a decrease in morula and blastocyst formation rates (Adona and Leal 2004; Romar and Funahashi 2006; Sananmuang et al. 2010) after oocyte treatment with roscovitine.

In sheep, the inhibitory potential of roscovitine as well as its effects on ultrastructure of *cumulus*–oocyte complexes has been recently investigated (Crocomo et al. 2013, 2015a,b). Irreversible alterations such as

degeneration of *cumulus* cells, swelling of mitochondria and reduction of cortical granules were reported by Crocomo et al. (2013) in sheep COCs treated for 24 h with 100 μM roscovitine. Later, maximal efficiency of meiosis inhibition was reached by Crocomo et al. (2015a,b) with 86.7% of oocytes kept at GV stage after treatment for 6 h with 75 μM roscovitine in serum-supplemented medium without oil overlay. According to these same authors, the meiotic inhibition was completely reversible and the gene expression pattern in oocytes and *cumulus* cells was not affected. Nonetheless, despite the recent scientific advances, consequences of using this inhibitor on *in vitro* embryo developmental competence still remain unknown in ovine. So, this study aimed to evaluate, for the first time, the effects of roscovitine, at concentration and culture conditions previously established, not only on nuclear maturation but, mainly, on *in vitro* production of sheep embryos.

Materials and Methods

All chemicals were purchased from Sigma Chemical. CO. (Sigma–Aldrich Corp., St. Louis, MO, USA), unless otherwise indicated.

Cumulus–oocyte complexes collection

Ovaries of adult sheep (Sarda sheep 4–6 years old) were collected at local slaughterhouses and transported to the laboratory within 1 h in sterile PBS (Dulbecco's phosphate-buffered saline) supplemented with 0.01% penicillin and streptomycin (100 μg/ml) at 37°C. COCs were collected through slicing method in sterile Petri dishes containing dissection medium composed of 20 mM HEPES-buffered TCM 199, 0.1% (w/v) polyvinyl alcohol (PVA), 50 IU/ml heparin and antibiotics. Only COCs showing intact *cumulus* cell layers and uniform ooplasm with homogenously distributed lipid droplets were selected under stereomicroscopy.

Roscovitine treatment and *in vitro* maturation (IVM)

The selected COCs were washed in HEPES-buffered TCM199 and transferred to maturation medium comprised of TCM 199, 8 mg/ml of sodium pyruvate, 100 μM cysteamine, 100 μg/ml penicillin, 100 μg/ml streptomycin and 10% foetal calf serum (Control) supplemented with 75 μM roscovitine (Rosco). The stock solution of roscovitine (1 mg/ml) was prepared in dimethylsulphoxide, aliquoted and stored at −20°C until use. To reverse the meiotic inhibition, after 6 h of culture in the presence and absence of roscovitine, COCs from each treatment were washed several times in HEPES-buffered TCM199 and *in vitro* matured for 18 h in maturation medium supplemented with 0.1 IU/ml FSH and 0.1 IU/ml LH (Pergonal, Serono Italy). Approximately 25 COCs were cultured in four-well Petri dishes (Nunclon, Roskilde, Denmark) with 400 μl of medium without mineral oil overlay, at 38.5°C and 5% CO_2 in air.

In vitro fertilization (IVF)

After *in vitro* maturation, COCs from each treatment (Control and Rosco) were denuded in TCM 199 supplemented with 300 IU/ml of hyaluronidase using a narrow bore pipette. Groups of 25 mature good quality oocytes were fertilized with frozen–thawed ram semen for 22 h in four-well Petri dishes containing 400 μl of SOF medium plus 2% heat-treated oestrous sheep serum (OSS) at 38.5°C and 5% CO_2, 5% O_2 and 90% N_2 under mineral oil. Spermatozoa were selected by swim-up technique and concentration adjusted to 1×10^6 sperm cells/ml.

In vitro embryo culture (IVC)

Approximately 20 presumptive zygotes were cultured for 7 days in four-well Petri dishes containing 600 μl of SOF medium added of essential and non-essential amino acids at oviductal concentration (Walker et al. 1996) and 0.4% BSA under mineral oil, in maximum humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C. Embryo cleavage was evaluated 30 h after the start of fertilization and blastocyst formation was recorded on day 7 of culture (D7).

Assessment of oocyte chromatin organization

Oocytes were stripped from their *cumulus* cells by repeated pipetting in HEPES-buffered TCM-199, fixed for 30 min in 4% paraformaldehyde and transferred to droplets of Hoechst 33 342 in glycerol (10 μg/ml) on a glass slide. According to the nuclear configuration identified under a fluorescence inverted microscope (Olympus IX 70), oocytes were classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII) (Shirazi et al. 2010). Those with altered nuclear structure were classified as degenerated.

Differential staining of embryos

The analysis of embryo quality was performed by differential staining of the inner cell mass (ICM) and trophectoderm (TE) of blastocysts at advanced development stages (expanded, hatching and hatched) obtained on day 7 of *in vitro* culture. Embryos were exposed, for 20 s, to 1% Triton X-100 in 20 mM HEPES-buffered TCM 199 containing 100 μg/ml propidium iodide (PI). After, they were washed for 3–5 s in ethanol 100% and transferred to a solution of ethanol and glycerol (1 : 1) containing 10 μg/ml Hoechst 33 342, for 5 min. Embryos were mounted onto a glass slide in a small drop of glycerol, gently flattened with a coverslip and examined under fluorescence microscope (Olympus IX 70). ICM nuclei appear blue due to DNA labelling with Hoechst 33 342, while TE cells appear red due to staining of nuclear DNA with membrane impermeable propidium iodide. The number of pyknotic

nuclei was determined according to the morphological appearance of blastocyst cells.

Experimental design

A completely randomized experimental design was applied with three experiments, and five replicates for each parameter were assessed (nuclear configuration, rates of cleavage and blastocyst, stages of blastocyst development and differential staining).

Experiment 1 – Efficiency of meiotic inhibition

The treatments Rosco, Control and Immature were compared in the different meiotic stages (GV, GVBD, MI, MII and Deg) according to factorial design with three treatments and five stages analysed. The nuclear configuration of oocytes from Rosco and Control was evaluated by Hoechst 33 342 staining after 6 h of *in vitro* culture with and without roscovitine, respectively. The immature oocytes were stained in the same manner but immediately after removal from follicular environment.

Experiment 2 – Reversibility of inhibitory action

Control and Rosco treatments were compared in the different meiotic stages (GV, GVBD, MI, MII and Deg) according to factorial design with two treatments and five stages analysed. The nuclear configuration of oocytes from Rosco and Control was evaluated by Hoechst 33342 staining after *in vitro* maturation for 18 h in inhibitor-free medium supplemented with gonadotropins.

Experiment 3 – *In vitro* embryo development

After *in vitro* maturation, oocytes from Control and Rosco treatments were *in vitro* fertilized. The mean percentage of cleaved embryos (30 h post-fertilization) and blastocysts (D7) was separately analysed comparing Control and Rosco treatments. The stages of blastocyst development on day 7 of culture were compared according to factorial design with two treatments (Control and Rosco) and five stages (early, blastocyst, expanded, hatching and hatched blastocyst). On the same day (D7), a sample of blastocysts from each treatment was differentially stained to evaluate embryo quality. The mean percentage of ICM, TE and pyknosis was calculated on the mean number of blastocyst cells and compared between treatments.

Statistical analysis

Data were transformed into square root of $x + 0.5$ and subjected to analysis of variance (ANOVA) according to experimental design specified in each experiment. The means were compared by Tukey test at 5% probability.

Results

Experiment 1 – Efficiency of meiotic inhibition

According to Table 1, Rosco treatment kept 93.8% of oocytes at GV stage during the first 6 h of *in vitro* culture. This result was similar to that observed in the group of oocytes stained soon after their removal from the follicles (94.9%) and significantly higher compared to those cultured for 6 h without roscovitine (Control, 41.3%). Besides, significantly higher rate of oocytes from Control was at GVBD (36.5%) and MI (22.1%) compared to the group of oocytes treated with 75 μM roscovitine, in which only 5.4% and 0.9% of oocytes resumed meiosis and reached the same stages, respectively. Similarly to Rosco treatment, only 5.1% and 0.0% of oocytes stained at 0 h (Immature) were at GVBD and MI, respectively. In all treatments, no oocyte was classified as MII or degenerated.

Experiment 2 – Reversibility of inhibitory action

No significant differences were observed between Rosco and Control treatments at the end of maturation for 18 h. A significantly higher proportion of oocytes from both treatments reached the MII stage (93.6% Rosco and 88.4% Control) compared to the other meiotic stages, and only few oocytes did not complete meiosis or degenerated (Table 2).

Table 1. Nuclear configuration of sheep oocytes at 0 h (Immature) and after 6 h of culture in the absence (Control) or presence of 75 μM roscovitine (Rosco) (5 replicates)

Treatments	COCs (No.)	Nuclear maturation stage, *No.* (%)				
		GV	GVBD	MI	MII	Deg
Immature	78	74 (94.9)^{aA}	4 (5.1)^{bB}	0 (0.0)^{bB}	0.0^{aB}	0.0^{aB}
Control	104	43 (41.3) ^{bA}	38 (36.5)^{aA}	23 (22.1)^{aB}	0.0^{aC}	0.0^{aC}
Rosco	112	105 (93.8) ^{aA}	6 (5.4)^{bB}	1 (0.9)^{bB}	0.0^{aB}	0.0^{aB}

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II, Deg, degenerated. Significant differences indicated by lowercase letters within columns and capital letters within lines ($p < 0.05$).

Table 2. Meiotic progression of sheep oocytes cultured for 6 h in the absence (Control) or presence of 75 μM roscovitine (Rosco) followed by IVM for 18 h (5 replicates)

Treatments	COCs (No.)	Stage of meiotic progression, *No.* (%)				
		GV	GVBD	MI	MII	Deg
Control	95	3 (3.2)^{aB}	0 (0.0)^{aB}	6 (6.3)^{aB}	84 (88.4)^{aA}	2 (2.1)^{aB}
Rosco	94	0 (0.0)^{aB}	2 (2.1)^{aB}	2 (2.1)^{aB}	88 (93.6)^{aA}	2 (2.1)^{aB}

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II, Deg, degenerated. Significant differences indicated by lowercase letters within columns and capital letters within lines ($p < 0.05$).

Experiment 3 – *In vitro* embryo development

As shown in Table 3, the *in vitro* culture for 6 h with 75 μM roscovitine (Rosco) did not affect the rates of cleavage and blastocyst formation, which was similar to the one observed for Control. Regarding the developmental stage of blastocysts on day 7 of culture, no significant differences were observed between Rosco and Control treatments. As expected, in both conditions, there was prevalence of expanded embryos (Table 4). The differential staining of blastocysts also revealed no differences in the mean number of embryo cells, in the mean percentage of ICM, TE, pyknosis or ICM/TE ratio between Control and Rosco treatments (Table 5).

Discussion

Despite the research efforts over the last 30 years, the efficiency of *in vitro* embryo production and offspring viability after transfer to recipient females are still low and variable in small ruminants, limiting the commercial application of this reproductive biotechnology (Paramio and Izquierdo 2014). One of the main reasons of this reduced embryonic developmental potential is the incomplete cytoplasmic maturation of *in vitro* cultured oocytes (Krisher 2004). In this context, the temporary arrest of meiosis with cyclin-dependent kinase inhibitors, as roscovitine, has been proposed as strategy to offer additional time for oocyte capacitation (Mermillod et al. 2000; Gharibi et al. 2013). The consequences of using this pharmacological molecule on the early embryogenesis, however, are still inconsistent, and no similar investigation has been performed in ovine. Based on that, the present study was designed and carried out to evaluate the effects of roscovitine on *in vitro* developmental competence of adult sheep oocytes.

Our results demonstrate that 75 μM roscovitine was efficient to arrest sheep oocytes at GV stage (93.8%) for 6 h in a proportion similar to that of Immature (94.9%) and significantly higher compared to Control (41.3%). The high GV rate and the absence of degeneration in the oocytes stained before *in vitro* culture (0 h) also indicate that meiosis had not been prematurely resumed and time interval between recovery and selection of COCs was adequate. A similar efficiency for roscovitine meiotic inhibition (GV rate ≥80%) has been reported in other species such as goat (Han et al. 2006), cat (Sananmuang et al. 2010), pig (Le Beux et al. 2003; Coy et al. 2005) and bovine (Mermillod et al. 2000; Adona and Leal 2004) but exposing the oocytes for a longer treatment (≥24 h) and to different inhibitor concentrations as 200 μM, 100 μM, 50 μM and 25 μM, respectively.

According to Han et al. (2006), the differences in the effective concentration of roscovitine observed among species are related to the probable existence of different regulatory molecules. Indeed, it has been demonstrated that high doses of roscovitine inhibit not only the MPF,

Table 3. Effect of 6-h treatment of sheep oocytes with (Rosco) or without (Control) 75 μM roscovitine, followed by 18 h of IVM, on cleavage and blastocyst rates (5 replicates)

Treatments	Oocytes (No.)	Rate of embryos (mean ± SEM)	
		Cleavage** No. (%)	Blastocyst/cleaved No. (%)
Control	207	142 (68.4 ± 3.7)	70 (48.9 ± 11.9)
Rosco	214	153 (71.6 ± 6.0)	75 (48.9 ± 9.9)

**Cleavage post-30 h was calculated on the total number of oocytes. Blastocyst rate (D7) was calculated on the number of cleaved embryos. No significant difference ($p > 0.05$).

Table 4. Developmental stages of blastocysts on day 7 of culture after 6-h treatment of sheep oocytes with (Rosco) or without (Control) 75 μM roscovitine followed by 18 h of IVM (5 replicates)

Treatments	Blastocysts (No.)	Blastocyst developmental stages (D7), *No.* (%)				
		Early	Blastocyst	Expanded	Hatching	Hatched
Control	70	2 (2.9)^{C}	8 (11.4)^{B}	42 (60.0)^{A}	18 (25.7)^{B}	0 (0.0)^{C}
Rosco	75	5 (6.7)^{C}	8 (10.7)^{B}	40 (53.3)^{A}	21 (28.0)^{B}	1 (1.3)^{C}

Differences indicated by capital letters in the lines ($p < 0.05$).

Table 5. Mean percentage of inner cell mass (ICM), trophectoderm (TE) and pyknosis of sheep blastocysts *in vitro* produced (D7) after 6-h treatment of oocytes with (Rosco) or without (Control) 75 μM roscovitine followed by IVM for 18 h (5 replicates).

Treatments	Embryos (No.)	Number of cells (mean ± SEM)				
		Total embryo cells	ICM (%)	TE (%)	ICM/TE ratio	Pyknosis (%)
Control	32	103.4 ± 8.9	23.4 ± 5.3	76.7 ± 4.3	0.31 ± 0.09	6.0 ± 0.9
Rosco	30	111.7 ± 1.9	20.5 ± 0.9	79.8 ± 4.1	0.26 ± 0.02	7.5 ± 2.2

Mean percentage of ICM, TE and pyknosis calculated on the mean number of embryo cells. No significant difference ($p > 0.05$).

but also other important kinases, such as mitogen-activated protein kinases (MAPK) (Meijer and Kim 1997b), which may be the major regulator of meiosis progression in certain species. However, despite the dose-dependent inhibition evidenced in cat (Sananmuang et al. 2010) and goat (Han et al. 2006), very high concentrations of roscovitine may be toxic and impair oocyte viability. The effects of meiosis inhibitors on COCs depend also on the conditions of *in vitro* culture, such as medium composition, incubation time and the presence or absence of oil overlay. In sheep, the maximal efficiency of meiosis inhibition was reported when COCs were treated with 75 μM roscovitine for 6 h in serum-supplemented medium without oil overlay (Crocomo et al. 2015a). This finding was reinforced in the present study once that a significantly high proportion (93.8%) of sheep oocytes treated under the same described conditions remained at GV stage.

Our results also show that the inhibitory action of roscovitine was completely reversible after *in vitro* maturation in gonadotropin-supplemented medium. The high MII rate and the low degeneration observed in both treatments (Control and Rosco) suggest that IVM for 18 h was sufficient for completion of nuclear maturation without inducing apparent deleterious effects of cellular senescence. In bovine and goat oocytes, meiosis progression is accelerated after inhibitor removal (Ponderato et al. 2001; Han et al. 2006). Accordingly, in the present study, a high rate of oocytes treated with roscovitine (93.6%) underwent GVBD and reached the MII stage after 18 h of *in vitro* maturation, which is 6 h earlier than the time considered adequate for IVM of sheep COCs (24 h) (Shi et al. 2009). The reason is probably related to the synthesis and accumulation of developmentally relevant factors during the meiosis block (Ponderato et al. 2001). The reversibility of meiotic inhibition with similar MII rate was also reported in porcine (Coy et al. 2005; Romar and Funahashi 2006), goat (Han et al. 2006) and bovine (Mermillod et al. 2000; Adona and Leal 2004) oocytes *in vitro* matured for different times after roscovitine treatment.

The similar rates of cleavage and blastocyst formation in both treatments (Control and Rosco) suggest that roscovitine did not affect the developmental competence of sheep oocytes. The reversible meiotic inhibition without any consequence on embryo development was already observed in bovine oocytes *in vitro* treated for 24 h with 25 μM roscovitine (Mermillod et al. 2000; Lagutina et al. 2002). In sheep, the development up to blastocyst stage was also not affected by treatment of oocytes with 150 μM butyrolactone for 8 h (Lu et al. 2013) or with 1 μM cilostamide for 22 h (Gharibi et al. 2013). Likewise, Rose et al. (2013) found that, despite the meiotic progression delay, cAMP modulators did not improve the ovine blastocyst rate. Similar results were reported in goat when oocytes were treated with 200 μM roscovitine for 8 h (Han et al. 2006). However, a significant reduction of embryo developmental potential was reported when the inhibition was prolonged for 16 or 24 h (Han et al. 2006), the inhibitor concentration was increased (Gharibi et al. 2013) or the duration of *in vitro* maturation was reduced to 16 h (Lu et al. 2013). Besides, in some species as cat and pig, it has been demonstrated that roscovitine may be deleterious to oocyte competence despite the reversion of meiotic inhibition (Romar and Funahashi 2006; Sananmuang et al. 2010).

Our findings further demonstrate that the kinetics of blastocyst development and embryo quality were not altered by roscovitine treatment. In fact, the values of ICM and TE as well as the ICM/TE ratio were similar between treatments (Control and Rosco) and comparable to those reported in *in vitro* fertilized sheep embryos (Bogliolo et al. 2011; Zacchini et al. 2011). Besides, the low proportion of pyknosis, characterized by the irreversible chromatin condensation of a cell undergoing apoptosis (Bogliolo et al. 2011), indicates that conditions of *in vitro* embryo culture were suitable. Likewise, no alterations in the total cells number were observed in *in vitro* produced bovine and ovine blastocysts after oocyte treatment with 25 μM roscovitine (Mermillod et al. 2000; Lagutina et al. 2002) and 150 μM butyrolactone, respectively (Lu et al. 2013). However, Rose et al. (2013) obtained a significant increase in blastocyst cells number in peripubertal ovine oocytes treated with cAMP modulators. In mouse, Sanfins et al. (2015) also reported that roscovitine treatment for 3 h followed by IVM for 15 h improved the total cells number and cell division symmetry in the blastocyst. According to these same authors, the speed of development of roscovitine treated embryos was faster compared to Control. These divergent results are probably related not only to experimental conditions but also to intrinsic oocyte quality and species peculiarities (Han et al. 2006; Crocomo et al. 2013).

In conclusion, we demonstrated for the first time that roscovitine, at the studied experimental conditions, is able to reversibly block meiosis in oocytes from adult sheep without any remarkable effect on development and quality of the *in vitro* produced embryos. Further studies are needed to investigate, at molecular level, the quality of the blastocyst *in vitro* produced after roscovitine treatment and to test their capacity to generate viable and healthy offspring after transfer to recipient females.

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Conflict of interest

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

Author contributions

All authors substantially contributed to the development of research and writing of manuscript. Professors Sergio Ledda and Sony Dimas Bicudo contributed to the conception and design of research beyond the interpretation of data obtained and revision of the article. Luisa Bogliolo and Daniela Bebbere participated in the draft and revision of the article. Federica Ariu helped with the acquisition, analysis and interpretation of data beyond the revision of the article. The corresponding author (Letícia Ferrari Crocomo) actively participated in all stages of the development of this research (design, acquisition, analysis, interpretation of data, drafting and revision of the article). The manuscript was read, revised and approved by all of us.

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Author's address (for correspondence): Letícia Ferrari Crocomo, Rua dos Girassóis, n. 278, Bairro Convívio Park Residencial, CEP: 18605-240 Botucatu, SP, Brazil.
E-mail: leticia.crocomo@gmail.com