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Overnight holding aids in selection of developmentally competent equine oocytes

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A B S T R A C T

The demand for equine in vitro produced embryos has increased over the last decade. The aim of this study was to compare the effects of an extended IVM or a prolonged period before fertilization, including holding time, on equine immature oocyte developmental competence. Oocytes, collected from abattoir-derived ovaries, were divided into 4 groups: H0/24 ($n = 165$) 0 h holding + standard 24–26 h IVM; H8/36 ($n = 160$) 8 h holding + 36 h IVM; H20/24 ($n = 187$) 20 h holding + 24 h IVM; H0/44 ($n = 164$) 0 h holding + 44 h IVM. Oocytes matured to MII were fertilized by intracytoplasmic sperm injection (ICSI) and cultured for 10 days. The oocyte degeneration rate was higher ($P < 0.05$) for H20/24 than the other groups (H0/24 38.2 %, H8/36 43.1 %, H20/24 54.5 %, H0/44 32.9 %). Cleavage was higher ($P < 0.05$) in H20/24 (70 %) compared to H0/24 (45 %) and H8/36 (54 %) but not to H0/44 (63 %). No differences among groups were observed in the number of blastocysts per oocyte. Injected oocytes that reached the blastocysts stage were higher ($P < 0.05$) for H20/24 (20 %) than H0/24 (7 %) and H0/44 (7 %) but not H8/36 (12 %). For cleaved oocytes, a higher blastocyst rate ($P < 0.05$) was observed for H20/24 (28 %) than H0/44 (11 %), while H0/24 (15 %) and H8/36 (21 %) were not different from any group ($P > 0.05$). Timing of blastocyst development was not different among groups. Overnight holding of equine immature oocytes followed by a standard IVM interval may induce a pre-selection of the most competent oocytes thereby improving cleavage and embryo development rates after ICSI.

1. Introduction

Demand for in vitro equine embryo production by intracytoplasmic sperm injection (ICSI) has increased over the last decade. In commercial programs, equine oocytes are usually obtained by ovum pick up (OPU) from immature follicles, and transported to ICSI laboratories for in vitro maturation (IVM), ICSI and embryo culture (Morris, 2018). Depending on the morphology of the cumulus oocyte complex (COCs) at the time of recovery, 24–30 h of IVM are required to reach the metaphase II (MII) stage (Hinrichs et al., 2005). Holding immature oocytes allows not only for the transportation, but also for the facilitation of timing the procedures in the laboratory.Indeed, shipment and preservation of oocytes avoid the risks and expense of animal transport, while allow the mare owner to take advantage of the most recent advances in assisted reproductive technologies (Hinrichs, 2020). Particularly, the ability to hold

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equine immature oocytes overnight has contributed greatly to the widespread use of ICSI as a clinical tool because oocytes may be recovered from mares locally and then shipped overnight to central laboratories for in vitro embryo production (Hinrichs, 2020). Overnight holding of horse oocytes at room temperature does not affect maturation and embryo development to the blastocyst stage (Choi et al., 2006a). Furthermore, it maintains meiotic arrest, viability, and mitochondrial potential of equine oocytes (Martino et al., 2014). Different holding media have been successfully used at room temperature for 18–24 h (Foss et al., 2013; Diaw et al., 2018) before an IVM of at least 22 h (Dini et al., 2016). More recently, the overnight exposure of oocytes to temperatures below room temperature (16 °C) appeared to optimize equine in vitro embryo production (IVEP) (Metcalf et al., 2020). In addition, holding of equine immature oocytes at 15 °C for 2 days slowed some morphokinetic parameters of embryo development, but did not affect blastocyst production (Martino et al., 2019).

Oocyte nuclear and cytoplasmic maturation are necessary for normal embryo development. Galli et al. in 2018 (Galli et al., 2018) observed that their current IVM protocols entailed a 24–28 h maturation time assessed by the appearance of the first polar body, despite the fact that the time from hCG administration (considered the trigger for the resumption of meiosis) to ovulation in vivo is 36–40 h. Furthermore, they observed that some oocytes matured to MII after 24–28 h of IVM did not undergo cleavage after ICSI, indicating a possible uncoupling between cytoplasmic and nuclear maturation (Galli et al., 2018). Thus, the maturation time was extended from 24 to 28–36 h, following 7–8 h holding at room temperature for logistical reasons, with a beneficial effect on the number of blastocysts produced (Galli et al., 2018). It was concluded that the additional maturation time improved the developmental competence (Galli et al., 2018). However, similar high blastocyst rates were obtained using overnight holding at room temperature from abattoir derived (Diaw et al., 2018; Brom-de-Luna et al., 2019) or OPU derived immature oocytes (Brom-de-Luna et al., 2018; Salgado et al., 2018; Metcalf et al., 2020) with IVM protocols lasting 24–30 h. There is no study comparing the effect of overnight holding and prolonged IVM in order to clarify which of the two different conditions mainly affects oocyte developmental competence. The objective of this study was to compare the effects of an extended IVM or a prolonged period before fertilization, including holding time, on equine immature oocyte developmental competence. For this purpose, different combination of holding/IVM protocols (44 h in total) were compared to control IVM (24–26 h) or direct prolonged IVM (44 h).

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.1. Collection and in vitro maturation of cumulus oocyte complexes

Ovaries from mares of unknown origin were collected at the slaughterhouse and transported to the laboratory within 2–3 h at 25 °C in an insulate container. Oocytes were collected as previously described (Merlo et al., 2018). Briefly, the ovaries were dissected free from connective tissue, washed with demineralized water, and oocytes were recovered by aspirating the contents of 10–30 mm follicles, using a 19-gauge butterfly infusion set connected to a vacuum pump (about 100 mmHg). The aspirated follicular fluid was collected into 250 ml glass flasks and filtered through a 65 µm mesh nylon filter (EmSafe, Minitube, Germany). Oocytes were then divided into four groups: 1) H0/24 (control group) ($n = 165$): no holding before standard IVM for 24–26 h; H8/36 ($n = 160$): 8 h holding at room temperature (22–25 °C) then IVM for 36 h; 3) H20/24 ($n = 187$): 20 h holding at room temperature then IVM for 24 h; 4) H0/44 (extended IVM group) ($n = 164$): no holding before IVM for 44 h. All groups except H0/24 had a total interval of 44 h from oocyte collection to injection.

For oocyte holding, sterile 0.5 ml Eppendorf tubes with screw caps (Sarstedt, Verona, Italy) were filled with HSOF (Holding Synthetic Oviductal Fluid, SOF (Tervit et al., 1972) supplemented with 20 mM HEPES, 1 mM glutamine, 10 mM glycine, minimum essential medium (MEM) essential (50x) and non-essential (100x) amino acids solutions, 6 mg/ml BSA). After oocytes deposition, tubes were closed and wrapped with aluminum foil to prevent the exposure to light. For IVM, oocytes were cultured in 500 µL maturation medium in four-well plates (Scientific Plastic Labware, EuroClone, Italy) at 38.5 °C, in a humidified atmosphere of 5 % CO_2 in air. Maturation medium consisted of Dulbecco Modified Eagle Medium Nutrient Mixture F-12 (DMEM-F12, Gibco, Life Technologies, Italy) supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS; Gibco, Life Technologies, Italy), 50 ng/ml epidermal growth factor, 100 ng/ml insulin-like growth factor 1, 0.1 IU/ml porcine FSH-LH (Pluset, Calier, Italy).

At the end of IVM, oocytes were incubated for 1.5 min in a 0.25 % (w/v) solution of trypsin in HSOF and pipetted to mechanically remove cumulus cells. Then they were transferred to HSOF supplemented with 10 % FCS for trypsin inactivation. After denuded of cumulus cells, oocytes with an extruded first polar body were considered suitable for ICSI. Oocytes with a damaged oolemma were considered degenerate, while oocytes with an intact oolemma but no polar body were considered immature.

2.2. Intracytoplasmic sperm injection and in vitro embryo culture

Frozen-thawed semen from a stallion of in vitro proven fertility was used. After cutting a piece of straw under liquid nitrogen, semen was thawed by placing the cut section of straw into 1 ml of SOF-IVF [SOF supplemented with 6 mg/ml fatty-acid-free BSA (FAF-BSA), 20 mM HEPES, 1 mM glutamine, 10 mM glycine, MEM essential (50×) and non essential (100×) amino acids solutions, 1 mg/ml heparin, 20 mM penicillamine, 1 mM epinephrine, and 10 mM hypothaurine] pre warmed at 37 °C and centrifuged at 500 g for 2 min. Supernatant was discarded leaving only 0.1 ml of medium. The sperm suspension was subsequently diluted 1:1 (v/v) with a 12 % solution of polyvinylpyrrolidone (PVP) in PBS (phosphate buffered saline) to a final concentration of 2×10^6 spermatozoa/ml. Manufactured ICSI pipettes (Biomedical Instruments, Zöllnitz, Germany) were used. ICSI was performed at 37 °C using a micromanipulator (Narishige Co. Ltd, Tokyo, Japan) equipped with a Piezo micropipette-driving unit (Prime Tech, Ibaraki, Japan) and mounted on an inverted microscope (Nikon TE 300: Nikon, Kawasaki, Japan). A motile sperm was immobilized by applying two or three piezo-pulses to the tail or midpiece regions, and it was then aspirated into the tip of the injection pipette. The oocyte was immobilized using the holding pipette and orientated with its polar body at 06:00 or 12:00 h. The injection pipette was then used to penetrate through the zona pellucida and oolemma at the 15:00 h position using the piezo-drilling motion, and the spermatozoon was released into the ooplasm.

Following ICSI, oocytes were cultured in 20 μL droplets of SOF supplemented with 20 mM HEPES, 1 mM glutamine, 10 mM glycine, MEM essential (50x) and non essential (100x) amino acids solutions and 16 mg/ml FAF-BSA (SOF-IVC) under mineral oil at 38.5 °C in an atmosphere of 5 % CO_2, 7 % O_2, and 88 % N_2 for 10 days. Culture medium was refreshed every 3 days by adding 20 μL of fresh SOF-IVC into each droplet and thereafter aspirating the same volume. On day 6 of IVC, 5 % FBS was added. Presumptive zygotes were monitored for cleavage 48 h after injection and development to the blastocyst stage was evaluated daily at day 7 through 10. At the end of the culture period, blastocysts were evaluated by staining with Hoechst 33342 (bisbenzimide 10 μg/ml in PBS) to confirm the presence of nuclear material.

2.3. *Statistical analysis*

The study was done in 10 replicates, in order to obtain an appropriate sample size of equine immature oocytes. Some oocytes were included in all groups per replicate. Data were analysed using a Chi Square test (IBM SPSS Statistics 25, IBM Corporation, Milan, Italy). Significance was assessed at $P < 0.05$.

3. Results

From the 676 recovered oocytes, 288 (42.6 %) were degenerate after IVM, 367 (54.3 %) reached the metaphase II stage and 21 (3.1 %) were determined to be immature. The highest rate of degenerate oocytes (54.5 %) and the lowest maturation rate (43.3 %) ($P < 0.05$) were observed in H20/24 (Table 1). On the other hand, H0/24 group showed an higher rate of immature oocytes (6,7 %) compared to H8/36 (1.3 %) and H20/24 (2.1 %) ($P < 0.05$) (Table 1). Consequently, analysing maturation rates without considering degenerate oocytes, the percentage of MII oocytes was lower in H0/24 group (89.2 %) than H8/36 (97.8 %) and H0/44 (96.4 %) ($P < 0.05$), while H20/24 (95.3 %) was statistically similar to all groups ($P > 0.05$) (Table 1).

Even though no differences were observed in the proportion of blastocysts per oocyte (prior to maturation) ($P > 0.05$), cleavage was significantly higher ($P < 0.05$) in H20/24 (70 %) compared to H0/24 (45 %) and H8/36 (54 %) (Table 2). Furthermore, H0/24 cleavage rate was lower ($P < 0.05$) than H0/44 (Table 2). The number of injected oocytes that developed to the blastocyst stage was higher ($P < 0.05$) for H20/24 (20 %) than H0/24 (7 %) and H0/44 (7 %) (Table 2). Considering the embryo developmental ability of cleaved oocytes, the only significant difference in blastocyst rate was observed between H20/24 (28 %) and H0/44 (11 %) ($P < 0.05$) (Table 2).

No statistically significant differences were observed in timing of blastocyst development after ICSI ($P > 0.05$) (Table 3).

4. Discussion

In vitro embryo production is becoming more popular in commercial equine breeding programmes. Shipping stored oocytes to ICSI laboratories is a requirement in ovum pick up/ICSI clinical programmes. Holding immature oocytes before the onset of IVM simplifies not only oocyte transport but also scheduling of subsequent manipulations. Although in vivo follicle maturation after induction requires approximately 36 h, the process in vitro is different. When standard IVM time (24–28 h) was extended to 36 h, including also 7–8 h holding period at room temperature for logistical reasons, the number of blastocysts produced more than doubled (Galli et al., 2018). On the other hand, similar blastocyst rates were obtained using overnight holding at room temperature followed by 24–30 h IVM (Brom-de-Luna et al., 2018, 2019; Diaw et al., 2018; Salgado et al., 2018; Metcalf et al., 2020). It remains unclear if the beneficial effect on equine immature oocyte developmental competence is related to an extended IVM or to a prolonged period before fertilization. To our knowledge, this is the first study combining different holding period and maturation times in the attempt to elucidate this aspect.

In the present study, the overall maturation rate was 54.3 %, similarly to other previous reports in the horse (Hall et al., 2013; Merlo

Table 1
Percentage of equine oocytes determined to be mature (MII), immature (IM) or degenerate (DEG) and percentage of MII oocytes per nondegenerate (MII/nonDEG) for holding and maturation groups. H0/24 = 0 h holding + 24–26 h IVM; H8/36 = 8 h holding + 36 h IVM; H20/24 = 20 h holding + 24 h IVM; H0/44 = 0 h holding + 44 h IVM.

Group	Oocytes	MII (%)	IM (%)	DEG (%)	MII/nonDEG
H0/24	165	91 (55.2)^{a}	11 (6.7)^{a}	63 (38.2)^{b}	89.2^{b}
H8/36	160	89 (55.6)^{a}	2 (1.3)^{b}	69 (43.1)^{b}	97.8^{a}
H20/24	187	81 (43.3)^{b}	4 (2.1)^{b}	102 (54.5)^{a}	95.3^{a,b}
H0/44	164	106 (64.6)^{a}	4 (2.4)^{a,b}	54 (32.9)^{b}	96.4^{a}

^{a,b}Different superscript letters within the same column differed at $P < 0.05$.

Table 2
Cleavage and blastocyst development after ICSI of equine oocytes among holding and in vitro maturation groups. H0/24 = 0 h holding + 24–26 h IVM; H8/36 = 8 h holding + 36 h IVM; H20/24 = 20 h holding + 24 h IVM; H0/44 = 0 h holding + 44 h IVM.

Group	Oocytes	Injected	Cleavage (%)	Bl/oocyte (%)	Bl/injected (%)	Bl/cleaved (%)
H0/24	165	91	41 (45)[c]	6/165 (3.6)	6/91 (7)[b]	6/41 (15)[a,b]
H8/36	160	87	47 (54)[b,c]	10/160 (6.25)	10/87 (12)[a,b]	10/47 (21)[a,b]
H20/24	187	81	57 (70)[a]	16/187 (8.6)	16/81 (20)[a]	16/57 (28)[a]
H0/44	164	104	65 (63)[a,b]	7/164 (4.2)	7/104 (7)[b]	7/65 (11)[b]

[a,b]Different superscript letters within the same column differed at $P < 0.05$.
Bl = blastocyst.

Table 3
Timing of blastocyst development after ICSI of equine oocytes among holding and in vitro maturation groups. H0/24 = 0 h holding + 24–26 h IVM; H8/36 = 8 h holding + 36 h IVM; H20/24 = 20 h holding + 24 h IVM; H0/44 = 0 h holding + 44 h IVM.

Group	Bl day 7 (%)	Bl day 8 (%)	Bl day 9 (%)	Bl day 10 (%)	Total Bl
H0/24	1 (16.7)	3 (50.0)	2 (33.3)	0 (0.0)	6
H8/36	2 (20.0)	2 (20.0)	5 (50.0)	1 (10.0)	10
H20/24	5 (31.2)	2 (12.5)	6 (37.5)	3 (18.8)	16
H0/44	2 (28.6)	3 (42.9)	1 (14.3)	1 (14.3)	7

Bl = blastocyst.

et al., 2016, 2018). Extending the IVM length (both 36 h and 44 h) increased nuclear maturation compared to direct 24–26 h IVM, but was similar to delayed (after 20 h holding) 24 h IVM. In addition, a longer IVM did not increase blastocyst production. On the other hand, comparing developmental competence of injected oocytes matured for 24 h with or without overnight holding, cleavage rate and blastocyst production were improved by 20 h holding, in spite of similar ability of cleaved oocytes to reach the blastocyst stage. Furthermore, overnight holding and 24 h IVM enhanced blastocyst rates compared to immediate placement of the oocytes in IMV for the same total interval of 44 h, even if similar cleavage ability was observed. It is likely that 24 h IVM may be shorter and 44 h IVM may be longer than optimal, depending on oocytes. Finally, short holding time (8 h) followed by extended IVM (36 h) achieved a lower cleavage rate than overnight holding followed by 24 h IVM, even if blastocyst production was not statistically lower.

Different aspects need to be considered to in order to try to explain what was observed. Firstly, meiotic competence of horse oocytes is dependent upon initial cumulus configuration, size of the follicle from which the oocyte was recovered, and the period of time the oocyte is in the ovary before recovery (Hinrichs, 2010). Oocytes recovered after being held within the ovary 5–9 h matured in larger proportion at 24 h of culture compared with oocytes recovered immediately and cultured for the same period (Hinrichs et al., 2005). Furthermore, ovary storage was associated with an increase in developmental competence of horse oocytes, as reflected in the higher blastocyst development for oocytes collected after a delay (Hinrichs et al., 2005). This suggest that pre-maturational changes may occur in horse oocytes held at room temperature within the ovary (Hinrichs et al., 2005). In the present study, oocytes were recovered after being held within the ovary 4–5 h, and this could have influenced the subsequent effects of IVM duration on oocyte developmental competence. In fact, oocytes might have matured in lower proportion at 24 h of culture.

Another important aspect is the pre-maturation period after oocyte collection. Both maturation-promoting factor (MPF) and microtubule-associated protein (MAP) kinase have been identified in horse oocytes, and MPF activity is higher in mature than in immature horse oocytes (Goudet et al., 1998a, 1998b). Several studies investigated meiotic arrest in the horse and its effect on oocyte developmental competence and blastocyst production. Cycloheximide (Alm and Hinrichs, 1996), 6-dimethylaminopurine (6-DMAP), and butyrolactone I (Hinrichs et al., 2002) have been used to maintain meiotic arrest in equine oocytes. Roscovitine was also effective in suppressing meiosis (Hinrichs et al., 2002), but decreased cleavage rates for expanded COCs (Franz et al., 2003), while compact COCs could be held in roscovitine before maturation without any harmful effect on blastocyst formation (Choi et al., 2006b). Nonetheless, when cycloheximide or roscovitine were added to the pre-IVM medium of equine expanded oocytes, although they were successful at holding oocytes at the germinal vesicle stage and maintaining the meiotic competence, a lower number of blastocysts resulted from the treated oocytes than from those held at room temperature overnight in the absence of meiotic inhibitors (Choi et al., 2006a). More recently, meiotic competence of COCs from follicles 1–2 cm in diameter was not affected by prematuration in cilostamide, whereas they yielded blastocysts with a higher number of cells than oocytes that underwent direct IVM (Lodde et al., 2019). Furthermore, the addition of forskolin and 3-isobutyl-1-methylxanthine to overnight holding medium before maturation improved blastocyst production, suggesting that management of oocyte and cumulus cell cAMP levels before IVM may be an effective means to enhance equine oocyte developmental competence and blastocyst development (Metcalf et al., 2020).

The successful method for overnight holding of equine oocytes in the absence of meiotic inhibitors was first reported in 2006 (Choi et al., 2006a). Since then, different holding periods, media, and temperatures were tested (Choi et al., 2007; Foss et al., 2013; Galli et al., 2014; Martino et al., 2014, 2019; Dini et al., 2016; Diaw et al., 2018; Campos-Chillon et al., 2019; Metcalf et al., 2020). Temperature sensitivity of equine oocytes differs depending upon the follicular stage. Oocytes recovered from subordinate follicles are less sensitive than those collected from pre-ovulatory follicles (Choi et al., 2006a; Foss et al., 2013), in which meiosis has been initiated (Mortensen et al., 2010). The progression of pre-meiotic chromatin configuration and mitochondrial status are dependent upon temperature, and resumption of meiosis is suppressed when the oocytes are held overnight at 25 °C but not at increased temperatures (30 °C and 38 °C) (Martino et al., 2014). Immature oocytes had higher developmental ability to the blastocyst stage when held overnight at room temperature (23 °C) versus body temperature (37 °C) (Foss et al., 2013). On the other hand, when holding immature oocytes below room temperatures (17 °C and 4 °C), maturation rates were not affected (Dini et al., 2016), but blastocyst production rates were lower for oocytes held overnight at 7 °C than at room temperature (Diaw et al., 2018). On the contrary, a 2 day holding at 15 °C led to similar blastocyst rates as compared to overnight holding at room temperature (Martino et al., 2019). Finally, a cooler temperature of 16 °C versus 20 °C for overnight holding of immature oocytes resulted in a similar maturation rate but in a higher cleavage rate, while dropping the temperature to 5 °C was not appropriate (Metcalf et al., 2020). The ideal temperature for the overnight holding of immature COCs is yet to be thoroughly investigated and determined. Moreover, the underlying mechanisms of maintaining meiotic arrest in the absence of inhibitors have not been clarified.

Our results employing a short holding period (8 h) and an extended maturation (36 h) compared to standard 24–26 h maturation are only partially in line with what observed by Galli et al. (2018), where the difference with the control was significant. Data from a retrospective analysis on OPU-ICSI in the same laboratory showed that increasing IVM from 26 to 28 h to 36–38 h after a 2–12 holding at 22–24 °C enhanced embryo production, and the same trend was observed for shipped oocytes, matured for 26–30 h after an overnight holding (Lazzari et al., 2020). It is likely that the present results vary from some of the clinical ICSI work because of some holding in ovaries and more variables in slaughterhouse animals. The findings that maturation kinetics and oocyte developmental competence vary with initial cumulus morphology, time of collection of oocytes from the ovary, and duration of maturation make it difficult to compare results among laboratories (Hinrichs, 2010). Different maturation media are used, and even with the same IVM medium opposite results were obtained (Hinrichs, 2018). Moreover, even when ICSI is successfully performed, the culture of equine embryos up to the blastocyst stage appears to be much more challenging, with wide differences between laboratories (Stout, 2020). An important difference that might have greatly influenced the results could be in part dependent on the use of the piezo drill. Using the Eppendorf Piezo Expert, where the settings and the functionality are reproducible each time, increasing the intensity of piezo pulses enhanced cleavage and embryo development (Galli et al., 2018). In the present study, the Prime Tech piezo was used, for which the settings depend on how the piezo pipette is mounted. Such difference could have introduced more variability in the efficiency of ICSI and reduced the significance of the differences. Indeed, in our conditions, the differences in the number of blastocysts per oocyte (prior to maturation) were not significant. Nevertheless, the developmental competence of MII oocytes matured for 24 h after overnight holding was superior to those directly matured, proving an effect of the pre-maturational holding period. As also previously observed (Galli et al., 2014), more oocytes were degenerate after 20 h holding and IVM, with a decrease of the number of matured oocytes, indicating that oocytes that are already partially compromised may be less tolerant to this treatment. Considering that a lower number of injected oocytes had a better overall development to the blastocyst stage, it is likely that the holding period may pre-select the oocytes, and the oocytes that degenerate include those that have impaired developmental competence. On the other hand, maturing the oocytes for the entire 44 h period decreases oocyte developmental competence, possibly because of oocyte aging.

5. Conclusion

Overall, an extended period of IVM is not responsible for an increased oocyte developmental competence, while an overnight holding followed by a standard IVM may induce a pre-selection of the most competent oocytes thereby improving their developmental competence after ICSI. Different protocols can be used for holding and IVM without changing embryo production per oocyte. This allows more flexibility in a clinical program.

Ethical animal research

The study did not require approval because it was performed on material obtained from an abattoir.

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Authorship

B. Merlo contributed to study design, study execution, data analysis and interpretation, and preparation of the manuscript. C. Del Prete, G. Mari and E. Iacono contributed to study design, study execution and preparation of the manuscript. All authors gave their final approval to the manuscript.

Declarations of interest

None.

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