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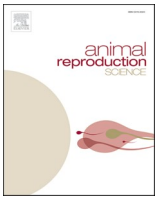

The presence of *Leptospira* spp. in the follicular fluid of naturally infected cows affects the overall efficiency of the *in vitro* embryo production technique

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ABSTRACT

The relationship between *Leptospira* infection and reproductive failures, as well as the mechanisms that lead to it, has not yet been fully established. It has been hypothesized that the presence of *Leptospira* spp. in the follicular fluid (FF) could impair the oocyte developmental competence. Thus, the impact of the presence of *Leptospira* spp. in the FF on *in vitro* embryo production (IVEP) outcomes was assessed. Dairy cows (n=244) from different farms were subjected to ovum pick-up for cumulus-oocyte complexes (COCs) collection. After PCR analysis of the FF, cows were retrospectively allocated into either: positive (POS-FF) or negative (NEG-FF) group. Statistical modeling was conducted using the farm, PCR result, and laboratory in which the IVEP was performed as effects. Noteworthy, 26.6% of the animals were positive for *Leptospira* spp., and 70% of farms had at least one POS-FF cow in the herd. POS-FF cows had a lower number of COCs recovered (22.6 ± 1.2 vs 15.0 ± 2.8, P=0.036), rate of viable COCs (85.6 ± 0.9% vs 78.1 ± 2.8%, P=0.015), number of good-quality COCs (16.0 ± 0.9 vs 9.8 ± 2.1, P=0.026), cleaved embryos (11.9 ± 0.7 vs 7.5 ± 1.5, P=0.032), and blastocysts (7.3 ± 0.4 vs 2.3 ± 0.7, P=0.044) yielded per cow. In conclusion, the presence of *Leptospira* spp. in the FF of naturally infected cows impaired the amount of COCs recovered, decreasing the overall IVEP efficiency.

1. Introduction

The productivity and consequent profitability of cattle herds are the main factors affected by reproductive performance (Hossein-Zadeh, 2013). *In vitro* embryo production (IVEP) aims to increase the production of offspring from females with greater genetic merit, including those with acquired subfertility or infertility (Sanches et al., 2019), and reduce the generation interval (Bouquet and Juga, 2013). This technology is widespread worldwide in the bovine industry and is currently the most used technique for bovine embryo production, surpassing the number of embryos produced *in vivo* since 2017 (Viana, 2023). Despite the great dissemination of

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IVEP, it is well known that the quality and quantity of the resulting embryos can be affected due to the presence of infectious agents in the female reproductive tract, which could also be transmitted to the recipients and progeny (Stringfellow and Givens, 2000). In addition, infectious diseases in the reproductive tract can impair the activation of the primordial follicle pool, follicular environment, and the development and maturation of oocytes (Gilbert, 2019).

Leptospirosis has an important role among infectious diseases that affect reproductive efficiency. This zoonotic disease is caused by spirochetes of the *Leptospira* genus, which are cosmopolitan organisms responsible for disease in animals and humans (Ellis, 2015). Due to its silent and subclinical attributes (Loureiro et al., 2016), overall reproductive outcomes can be compromised. This may contribute to major economic losses (Inchaisri et al., 2010), including fetal losses, weak offspring at birth (Lilenbaum and Martins, 2014), and irregular return to estrus (Libonati et al., 2018). In addition to these disorders, embryo death is one of the consequences that could be observed in the genital syndrome of leptospirosis (Oliveira et al., 2021). The mechanisms by which this happens, however, are still poorly understood, even though they are of vital importance for the establishment of appropriate health management measures.

In cows destined for slaughter, *Leptospira* spp. was detected by polymerase chain reaction (PCR) in 26% (11/42) of uterine fragments (Di Azevedo et al., 2020) and 11% (7/65) of follicular fluid (FF) samples (Di Azevedo et al., 2021). Interestingly, evaluating cows destined for slaughter due to reproductive problems such as chronic infertility, estrus repetition, late embryonic death, abortions, and fetal death, 37% (18/48) of the females were positive for *Leptospira* spp. in samples of cervicovaginal mucus and uterine fragments (Aymée et al., 2022). Although the number of observations was low, it is thought-provoking to note that when nine cows with a history of subfertility were examined, six (66%) were positive for *Leptospira* spp. (Aymée et al., 2021). To highlight the economic losses caused by the presence of *Leptospira* spp. in the reproductive tract, when the same cows were assessed monthly throughout one year, 53% of the animals reactive to the Sejroe serogroup had a history of embryonic death (Oliveira et al., 2021). In cattle, *Leptospira* spp. strains have been associated with the silent and chronic reproductive form of the disease, named bovine genital leptospirosis (BGL) (Loureiro and Lilenbaum, 2020), and have been reported in cervicovaginal mucus (Loureiro et al., 2017), uteri (Pires et al., 2018; Di Azevedo et al., 2020; Aymée et al., 2021, 2022), and FF (Di Azevedo et al., 2021; dos Santos Pereira et al., 2022). The economic impact of BGL has yet to be assessed, while it has been estimated that acute bovine leptospirosis can generate a loss between US$ 97 and US$ 2.611 per abortion (Ayral, 2013). Another quote not directly linked to leptospirosis points to a loss of US$ 2.000 per pregnancy loss, considering both the extended period without pregnancy and the potential of premature culling (Lee and Kim, 2007).

There are two main hypotheses that have been considered about embryonic losses and consequent return to estrus as a result of BGL. The first one, the bacterium infects the uterus, generating a local inflammation and altering the uterine environment, consequently compromising the implantation and survival of the embryo. In the second hypothesis, the bacteria penetrate the embryo and

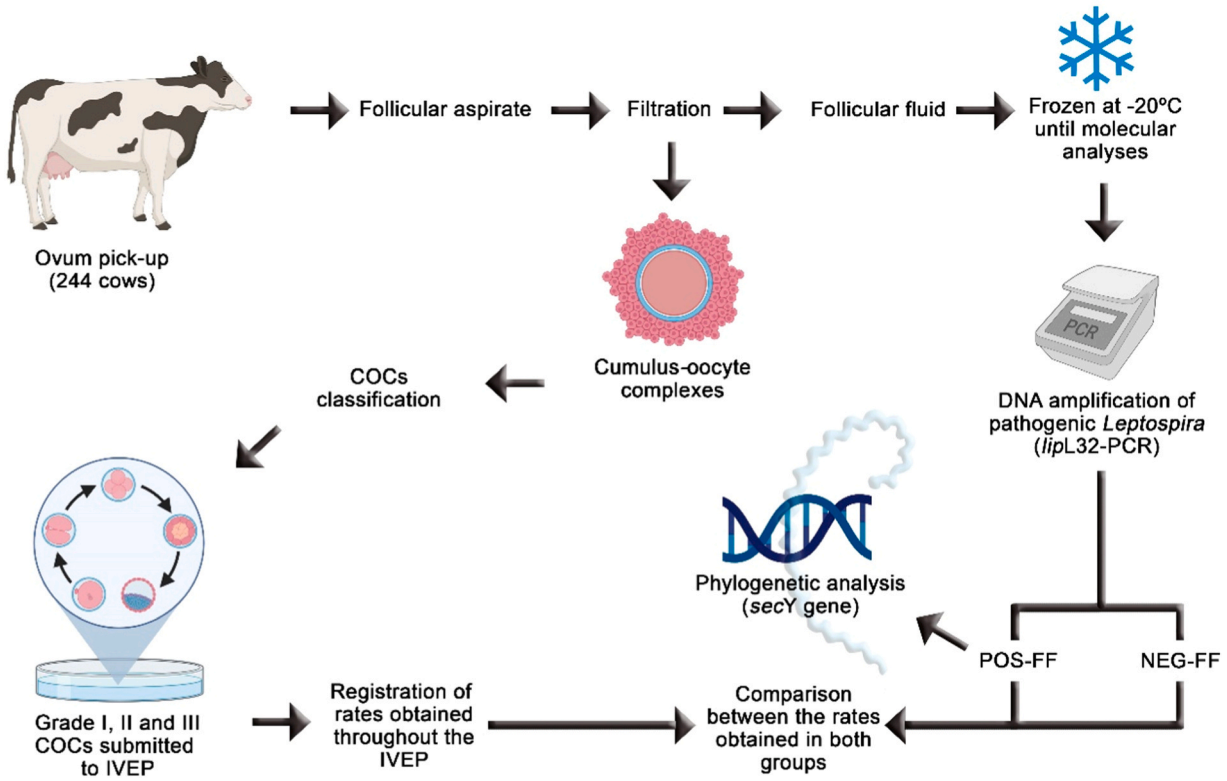

Fig. 1. Schematically representation of sample collection, molecular diagnosis, and *in vitro* embryo production. COCs: Cumulus-oocyte complexes. IVEP: *In vitro* embryo production; POS-FF: Samples with the presence of *Leptospira* spp. in the follicular fluid. DNA; NEG-FF: Samples with the absence of *Leptospira* spp. DNA in the follicular fluid.

directly damage the embryonic cells (Loureiro and Lilenbaum, 2020). Progress made by different research groups led to a third hypothesis, in which Gram-negative bacteria present in the FF cause indirect damage to the oocyte, either by experimental infection (Bromfield and Sheldon, 2011; Rincón et al., 2019) or by natural infection (Magata et al., 2014; Forrest et al., 2022). The cell and outer envelope of *Leptospira* are similar to that of Gram-negative bacteria and are composed of lipopolysaccharides (LPS) (Ellis, 2015). The presence of LPS in the FF may have negative consequences on fertility due to interference with ovarian steroidogenesis (Magata et al., 2014) and the developmental competence of oocytes (Bromfield and Sheldon, 2011; Rincón et al., 2019), causing thus reproductive failure (Forrest et al., 2022).

The relationship between *Leptospira* infection and reproductive failures, as well as the mechanisms that lead to it, have not yet been fully established. Regarding embryonic death, we hypothesize that the presence of *Leptospira* in the FF of naturally infected cows could impair the developmental competence of the derived oocytes and impair the IVEP technique outcomes. Based on this, the present study aimed to determine the effect of natural *Leptospira* spp. infection in FF on the oocyte developmental competence and overall efficiency of IVEP.

2. Materials and Methods

2.1. Ethics, Location, and Experimental Conditions

This study was carried out according to the guidelines of the Ethics Committee on the Use of Animals of the Federal Fluminense University (CEUA/UFF), being approved under the number 8688190919. Sample collections and the entire IVEP procedure were carried out in four distinct laboratories that signed partnerships. A total of 244 non-pregnant multiparous crossbreed dairy cows with no apparent clinical signs of leptospirosis, belonging to 20 different commercial farms of medium to high technification degree (Bassotto et al., 2023) in Southeast region, Brazil, were used. On a random day of the estrous cycle, cumulus-oocyte complexes (COCs) and FF collection were performed in antral follicles using the ovum pick-up technique (OPU). Within the Laboratory of Veterinary Bacteriology of the University, the FF obtained was subjected to PCR to identify the presence of *Leptospira* spp. DNA and the animals were further allocated into either: positive (POS-FF) or negative (NEG-FF) groups. The viable COCs were submitted to IVEP (COCs from each female were kept separately in each well/drop throughout the culture), and all the outcomes were compared in both groups, to demonstrate whether *Leptospira* spp. may decrease the overall effectiveness of IVEP (Fig. 1).

2.2. Collection of COCs and FF

Sacral epidural anesthesia was performed between the fifth sacral vertebra and the first coccygeal vertebra, using 4.5 mL of 2% lidocaine hydrochloride (Lidovet® - Bravet, Rio de Janeiro, RJ, Brazil). The sampling of FF was performed by the OPU technique (Arashiro et al., 2013), aided by portable ultrasound equipment (Prosound 2® - Aloka-Hitachi, Twinsburg, Ohio, USA) equipped with an intra-vaginal micro convex transducer (7.5 MHz) attached to the needle guide (Watanabe Applied Technology, WTA, Cravinhos, São Paulo, Brazil). Importantly, the transducer was protected with a sanitary sheath that was changed at every aspiration to avoid cross-contamination between animals. The aspiration was performed using disposable 20 G needles (WTA) connected to a 1.20 m Teflon circuit (WTA). This circuit was cleaned between aspirations from different cows using phosphate-buffered saline (PBS) + Gentamicin medium (IMV Technologies, Campinas, São Paulo, Brazil). The system was then connected to a vacuum pump (BV-003 WTA) that generated negative pressure (-80 mmHg and 100 mmHg). The aspirate was then deposited into a 50 mL conic tube, which was sent to the laboratory, where its contents were poured into an oocyte filter (WTA). After filtration, 2 mL of FF + aspirate medium was stored in a conical microtube and frozen at −20ºC until DNA extraction for *Leptospira* identification.

2.3. IVEP

2.3.1. Screening and Classification

The follicular aspirate was filtered into a 75 μm filter (WTA) to eliminate other cells and cellular debris, facilitating the visualization of COCs. The filter was gently washed using PBS + Gentamicin (IMV Technologies) solution heated to 37.5ºC so that the COCs retained in the filter were retrieved. The COCs were then deposited on a 60 ×15 mm plastic Petri dish, which was taken to a stereoscope microscope (NSZ 405® - Coleman, Santo André, São Paulo, Brazil) for classification. The classification of COCs was based on their number of cumulus cell layers and the appearance and texture of the ooplasm. All laboratories used the classification based on the International Embryo Technology Society (Demetrio and Barfield, 2021): Grade I (COCs with more than three compact layers of cumulus cells covering the surface of the zona pellucida (ZP) and dense ooplasm), Grade II (COCs with one or two compact cumulus cell layers and the dense ooplasm), Grade III (COCs containing less than one complete layer of cumulus cells and/or with speckled ooplasm), and Grade IV (COCs with expanded cumulus cell layers, often with an agglutinated appearance and with speckled or retracted ooplasm). Those COCs classified as Grade I and Grade II were rated as good quality; Grade I, Grade II, and Grade III were considered viable and used in the *in vitro* maturation step. Grade IV COCs were considered degenerated and discarded.

2.3.2. In Vitro Maturation (IVM) of Oocytes

All companies generally used commercial media based on the Tissue Culture Medium-199 (TCM-199) medium supplemented with hormones and growth factors. After recovery, approximately 10–30 COCs from each cow were washed and transferred to four-well dishes containing 500 μL supplemented media. The IVM step occurred under mineral oil for an average of 22–24 h at 38.5 °C and

under 5% CO_2 in humidified air.

2.3.3. In Vitro Fertilization (IVF) and In Vitro Culture (IVC) of Embryos

After IVM, the COCs from each cow were transferred to an IVF medium and co-cultured with spermatozoa for 16–18 h at 38.5 °C in a humidified atmosphere with 5% CO_2 (Day 0) (Bielanski et al., 2004). Afterward, presumptive zygotes were gently pipetted to mechanically remove the cumulus cells. Then, they were cultured in synthetic oviduct fluid (SOF) medium (Tervit et al., 1972) in 70 µL drops containing 10–30 presumptive zygotes for 8 d at 38.5 ºC, in a humidified atmosphere with 5% CO_2. The culture medium was renewed on the 4th and 6th day. Embryo cleavage was assessed at 48 h post-fertilization, and the blastocyst rate was evaluated on the 7th day. On the 8th day of IVC, the embryos that reached the blastocyst stage were transferred to 0.25 mL straws with HEPES-SOF medium (Bohlooli et al., 2015). The straws were then sealed and placed in an embryo transporter (WTA) at 38 ºC, until their transfer to recipients.

2.4. Molecular Diagnosis of Leptospira spp

The FF samples (n=244) were submitted to DNA extraction, followed by *lip*L32-PCR, according to Di Azevedo et al. (2021). DNA was extracted using a DNeasy® Blood & Tissue Kit (Promega, Madison, EUA), following the manufacturer's instructions. Polymerase chain reaction (PCR) was performed for *Leptospira* spp. DNA detection targeting the *lip*L32 gene, present only in pathogenic *Leptospira* (Stoddard et al., 2009). Ultrapure water was used as a negative control, and DNA extracted from *Leptospira interrogans* serovar Copenhageni str. Fiocruz L1–130 was used as a positive control for each set of samples. The PCR products were analyzed using 2% agarose gel electrophoresis and visualized after gel red staining under UV light.

2.5. Endpoints and Statistical Analysis

All endpoints were evaluated taking into account the possible influences that the different laboratories and farms could exert, and the cow was considered as the experimental unit. The normal distribution of all variables was determined by the Shapiro-Wilk test, and homoscedasticity was determined by the Levene test. Data were analyzed with a generalized linear mixed model (GLMM), including the molecular diagnosis (PCR) and the *in vitro* laboratory, and their interactions as main effects, while the farms were included as a random effect. The GLMM was adjusted to non-parametric data with Poisson or Gamma distribution. All analyses were performed in IBM SPSS version 25, and results are presented as LS mean ± SEM, considering $P<0.05$ as significant and $0.05<P\leq0.10$ as a tendency.

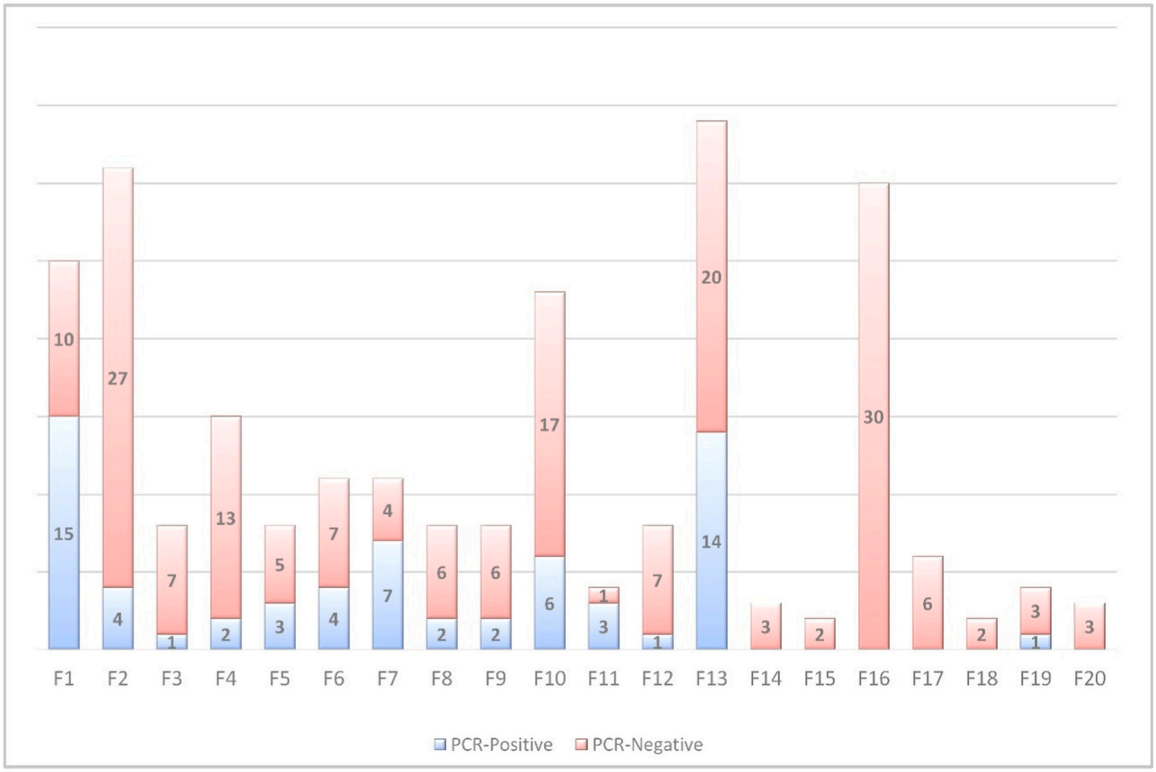

Fig. 2. Distribution of POS-FF (samples with the presence of *Leptospira* spp. in the follicular fluid) and NEG-FF (samples with the absence of *Leptospira* spp. DNA in the follicular fluid) cows, in blue and red colors, respectively, within the 20 farms (F1-F20).

Endpoints determined were: average number of COCs per cow (number of COCs recovered by OPU); average number of viable COCs per cow (number of Grade I, II, and III COCs); average number of good-quality COCs per cow (number of Grade I and II COCs); average number of cleaved embryos per cow; average number of blastocysts per cow; and rates of viable COCs (number of Grade I, II, and III COCs/ number of total oocytes recovered per cow×100); good-quality COCs (number of Grade I and II COCs/ number of total oocytes recovered per cow×100); cleavage (number of cleaved embryos/ number of viable COCs×100); blastocyst/viable COCs (number of blastocysts/ number of viable COCs×100); and blastocyst/cleaved (number of blastocysts/ number of cleaved embryos ×100).

3. Results

A total of 244 animals were evaluated, and out of those, 65 (26.6%) were positive for the presence of *Leptospira* spp. in the FF. The distribution of NEG-FF and POS-FF cows within each farm is presented in Fig. 2. Of the 20 farms evaluated, 14 (70%) had at least one POS-FF cow for *Leptospira*, with the positivity rate varying between one to 15 animals within each farm (13–60%). POS-FF cows had fewer COCs recovered (22.6 ± 1.2 vs 15.0 ± 2.8, *P*=0.036), number of good-quality COCs (16.0 ± 0.9 vs 9.8 ± 2.1, *P*=0.026), cleaved embryos (11.9 ± 0.7 vs 7.5 ± 1.5, *P*=0.032), and blastocysts produced (7.3 ± 0.4 vs 2.3 ± 0.7, *P*=0.044). The rates of COCs retrieved and IVEP compared between NEG-FF and POS-FF cows are shown in Table 1, whereas the number of structures obtained during the entire IVEP process is illustrated in Fig. 3. Viable COCs, Good-quality COCs, and Blastocysts/COCs rates tended to interact (*P*=0.054; 0.066; and 0.054, respectively).

4. Discussion

The current study applied the IVEP technique as a tool to assess the effect of *Leptospira* infection on the oocyte developmental competence of cows naturally infected by *Leptospira* spp. We demonstrated, for the first time, that the presence of *Leptospira* spp. in the FF significantly impairs the overall IVEP outcomes, confirming our hypothesis. The presence of *Leptospira* spp. affected the rate of viable COCs, as well as the total number of COCs, viable COCs, and good-quality COCs, subsequently reducing the number of cleaved embryos and blastocysts produced per cow. Even though there was a significant difference in Lab 3, however, the overall rate of blastocysts/COCs was not affected in the POS-FF group, showing that the oocyte developmental competence was probably not adversely affected.

The exact mechanism triggered by the presence of leptospires in FF on the population of oocytes available for retrieval and/or their viability is yet to be determined. This impairment can be attributed to direct damage to the oocytes, caused by the penetration of the bacterium into the oocyte (Bielanski et al., 1998), or indirect damage, which can be generated due to the presence, even in small amounts, of LPS endotoxin, which are known to be responsible for ovarian dysfunction (Sheldon et al., 2009; Shimizu et al., 2012; Magata, 2020). The former was evidenced by the presence of leptospiral DNA in oocytes from experimentally infected cows (Bielanski

Table 1
In vitro embryo production rates obtained from cumulus-oocyte complexes (COCs) recovered from follicles with either the presence (POS-FF) or absence (NEG-FF) of *Leptospira* spp. DNA, which was detected by PCR in the follicular fluid of naturally infected crossbred donor dairy cows according to the interaction between the PCR result and the laboratories (Lab). Values are presented as LS mean ± SEM.

Endpoints	PCR	Overall	Lab				*P*-value		
			1	2	3	4	PCR	Lab	PCR x Lab interaction
Viable COCs (%)	NEG-FF	85.6 ± 0.9^{x}	97.1 ± 1.5^{a}	92.3 ± 2.0^{a}	85.4 ± 2.2^{b}	70.2 ± 1.4^{c,x}	0.015	0.001	0.054
	POS-FF	78.1 ± 2.8^{y}	97.2 ± 2.3^{a}	94.3 ± 2.9^{a}	80.9 ± 2.9^{b}	50.0 ± 6.7^{c,y}			
Good-quality COCs (%)	NEG-FF	72.0 ± 1.2	89.5 ± 1.7^{a}	63.5 ± 2.4^{c}	64.3 ± 2.8^{bc}	70.9 ± 2.2^{b}	0.197	0.001	0.066
	POS-FF	66.8 ± 3.9	87.7 ± 2.6^{a}	71.0 ± 3.3^{b}	58.4 ± 3.9^{c}	50.0 ± 14.5^{bc}			
Cleaved embryos (%)	NEG-FF	66.8 ± 1.5	63.6 ± 2.3^{b}	51.5 ± 3.3^{c}	76.1 ± 3.7^{a}	75.8 ± 2.9^{a}	0.339	0.001	0.627
	POS-FF	72.0 ± 5.2	64.1 ± 3.5^{a}	49.7 ± 4.6^{b}	74.1 ± 5.2^{a}	100.0 ± 19.4^{a}			
Blastocysts/cleaved (%)	NEG-FF	40.6 ± 2.3	38.1 ± 3.4^{b}	27.8 ± 4.8^{b}	41.1 ± 5.4^{b}	55.6 ± 4.3^{a}	0.690	0.141	0.412
	POS-FF	37.5 ± 7.6	36.4 ± 5.1^{a}	30.1 ± 6.7^{a}	23.4 ± 7.8^{a}	60.0 ± 28.2^{a}			
Blastocysts/COCs (%)	NEG-FF	28.5 ± 1.7	25.3 ± 2.5^{b}	14.2 ± 3.5^{c}	32.6 ± 4.0^{ab,x}	41.9 ± 3.1^{a}	0.922	0.001	0.054
	POS-FF	27.9 ± 5.6	24.9 ± 3.7^{ab}	14.3 ± 4.9^{b}	12.5 ± 5.5^{b,y}	60.0 ± 20.7^{a}			

Within a column or row, values with different superscripts differ significantly for each endpoint (P<0.05).
^{a,b,c,d} differ among laboratories (1, 2, 3, and 4) at the same PCR results (NEG-FF or POS-FF).
^{x,y} differ between PCR results (NEG-FF and POS-FF) at the same laboratory (1, 2, 3, or 4).

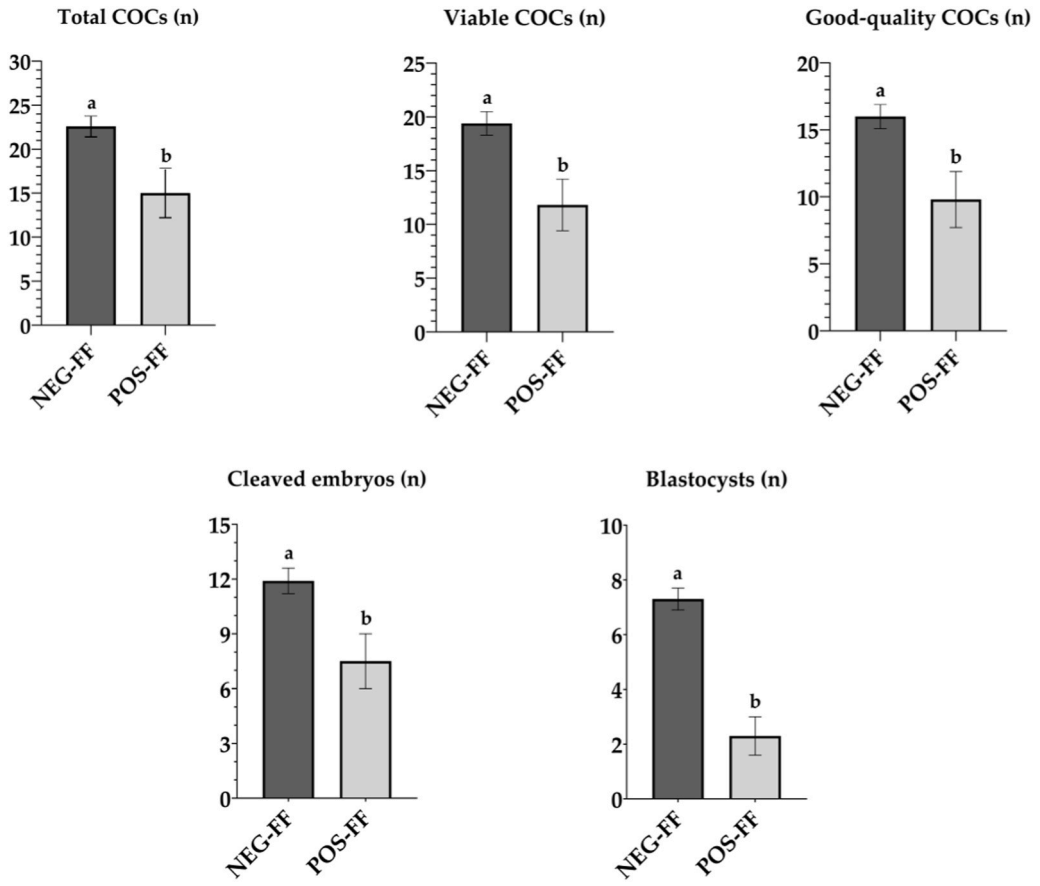

Fig. 3. *In vitro* embryo production outcomes of NEG-FF and POS-FF cows. Values are presented as LS mean ± SEM. COC: Cumulus-oocyte complex; n: number; NEG-FF: Samples with the absence of *Leptospira* spp. DNA in the follicular fluid; POS-FF: Samples with the presence of *Leptospira* spp. DNA in the follicular fluid. Total COCs: Average number of COCs per cow; Viable COCs: Average number of viable COCs per cow; Good-quality COCs: Average number of good-quality COCs per cow; Cleaved embryos: Average number of cleaved embryos per cow. Blastocysts: Average number of blastocysts per cow.

et al., 1998) or in an *in vitro* system, where penetration through the ZP and damage to embryonic membranes and cytoplasm occurred (Bielanski and Surujballi, 1996). The latter would be caused by the simple presence of this pathogen in the ovarian follicle, triggered by LPS, negatively impacting reproductive performance and fertility (Ferranti et al., 2018). The presence of this compound in FF is associated with disturbance in ovarian steroidogenesis (Magata et al., 2014) and oocyte competence (Bromfield and Sheldon, 2011; Rincón et al., 2019), causing reproductive failure (Forrest et al., 2022).

In order to evaluate the effects of LPS on bovine oocytes administered intramammarily, 6 h post-administration FF samples were collected to be supplemented into the IVM medium (Roth et al., 2020). Comparing the group with the presence of LPS (FF-LPS) with the control group, the cleavage rate did not differ. The proportion of developed blastocysts on Day 7 postfertilization in the FF-LPS group, however, was significantly lower than that in the Control group (10.6 vs 24.4%, respectively). Of note, the addition of LPS to the maturation medium of bovine oocytes or the culture medium of pre-implanted embryos had detrimental effects on their developmental competence (Soto et al., 2003). The introduction of LPS into the IVEP system could occur due to the adhesion of these bacteria in the cumulus cells or the ZP since the serial washing protocols recommended by the International Embryo Technology Society for the treatment of COCs and embryos were not capable of removing these microorganisms (Goes et al., 2012). Furthermore, LPS disrupts the synthesis of E2 within granulosa cells, causing a disturbance in the LH and FSH surge and consequently leading to abnormal follicular development, as recently reported by Guan and colleagues (Guan et al., 2021). It is well known that pre-ovulatory serum E2 concentrations have been positively associated with the establishment of pregnancy, embryonic survival (Sá Filho et al., 2011), and embryo quality (Atkins et al., 2013; Jinks et al., 2013).

It is possible that the damages induced by *Leptospira* spp. LPS in the ovary causes long-term effects, reducing the primordial follicle reserve (Bromfield and Sheldon, 2013; Fuller et al., 2017), as well as the activation and development of COCs (Bromfield and Sheldon, 2011, 2013; Rincón et al., 2019). Those impairments may lead to further reduction of antral follicles, affecting both COC number and viability at OPU. Bromfield and Sheldon (2013) demonstrated that LPS caused adverse effects on the primordial follicle reserve in the bovine ovarian in an *ex vivo* model. Indeed, administration of LPS to neonatal rats resulted in apoptosis in ovarian follicles and

reduction of primordial follicles. Importantly, LPS-induced chronic inflammation during pregnancy in rats resulted in a lower number of ovarian follicles and an increase in apoptosis in the ovaries (Shalom-Paz et al., 2017). It remains unknown whether the damage caused by *Leptospira* spp. in the ovaries is temporary or permanent. Evaluating the effect of LPS on mice ovarian tissue, it was observed that the number of primary follicles, secondary follicles, and corpora lutea were significantly decreased on the third day following LPS injection, with complete recovery on the 30th day (Shokrizadeh et al., 2019).

It is important to emphasize that the cleavage rate observed in this study (on average 69%) was similar to or slightly lower than those observed in previous studies, also in bovine: 74% (Zullo et al., 2016) and from 70% to 85% (Ferré et al., 2020). Of note, no hormonal stimulation was applied in the present study, an approach commonly used in Brazilian commercial herds since, despite affecting the number of embryos produced, the cost-benefit is not always positive (Demetrio et al., 2020). Moreover, the cleavage and blastocyst rates presented consider the use of Grade III COCs, which are usually discarded worldwide in research studies, i.e., those not performed in commercial centers. These facts may be responsible for any slight decrease in those rates compared to the literature. Of note, in the current study, the blastocyst yield in all groups was within the normal rates reported in the worldwide literature (Trounson et al., 1994; Niemann and Wrenzycki, 2000; Viana et al., 2010; Zullo et al., 2016; Ferré et al., 2020). This fact is somehow worrying since animals were asymptomatic, and their blastocyst yield was still within the normal range despite having decreased in the POS-FF group. This could mean that the true production potential of a cow infected with *Leptospira* spp. has not yet been observed.

Like most of the studies conducted in the field, this one has some limitations: In Brazil, it is common to use donor cows for OPU regardless of the stage of their estrous cycle, thus, assessment of the exact stage of the estrous cycle is not commercially performed. As a general procedure, all antral follicles are aspirated, and in the laboratory, after the classification based on oocyte morphology, the non-viable oocytes were discarded, making a homogeneous pool of COCs used for IVM. These limitations were, however, mitigated by the use of statistical modeling methodology, which included the effects of different laboratories and farms. Finally, the pregnancy rate could not be reported in the present study due to the natural bias that would be caused by this rate, as there is a strong influence of the recipient on pregnancy viability.

5. Conclusions

Under the conditions of the present study, the presence of *Leptospira* spp. in the FF of naturally infected cows decreased the number of COCs recovered by OPU, regardless of their quality, decreasing the number of blastocysts per cow and the overall efficiency of the IVEP technique. These data increase the possibility of using this biotechnology in future studies as a tool to advance the understanding of the mechanisms that lead to oocyte damage and reproductive failures. Further studies may be performed to identify if the effect of *Leptospira* spp. in the FF is either direct, indirect, or both; this definition will aid the implementation of control measurements.

CRediT authorship contribution statement

Paulo Victor dos Santos Pereira: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Maria Isabel Nogueira Di Azevedo:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Conceptualization. **Eduardo Kenji Nunes Arashiro:** Writing – review & editing, Resources, Formal analysis, Data curation. **Yeda Fumie Watanabe:** Writing – review & editing, Resources, Investigation. **Lucas Francisco Leodido Correia:** Writing – review & editing, Formal analysis, Data curation. **Walter Lilenbaum:** Writing – review & editing, Visualization, Methodology, Conceptualization. **Joanna Souza-Fabjan:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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