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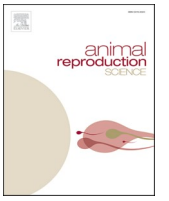

Progestogen supplementation during superovulation leads to higher embryo viability and *TGFB1* gene expression in sheep

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ABSTRACT

This study aimed to compare the effect of the administration of either medroxyprogesterone acetate (MPA) or progesterone (P4) in superovulation (SOV) treatments applied during the first follicular wave on follicular development, embryo yield, and the expression of genes related to pluripotency maintenance, differentiation of the trophectoderm, cell growth and differentiation, apoptosis and energy metabolism in sheep embryos. The estrous cycle of 36 multiparous ewes was synchronized with a short protocol, and the animals were randomly allocated to three groups. At the beginning of SOV, 12 ewes per treatment received an intravaginal sponge impregnated with 60 mg of MPA (TMPA), or an intravaginal device containing 0.33 g of P4 (TP4), or received no progestogen treatment (CON). The device was kept until the fifth dose of FSH. Ewes were mated with five fertile rams. Gene expression was performed by RT-qPCR using grade I and II blastocysts. The numbers of corpora lutea, total structures and viable embryos recovered per ewe were similar ($P > 0.05$) among groups. However, the viability rate was higher in TP4 (71.9 ± 16.3%) compared to CON (24.4 ± 16.8%; $P = 0.01$) and similar to TMPA (49.9 ± 16.3%; $P = 0.2$). Similarly, when compared with CON, treatment with P4 or MPA positively regulated the *TGFB1* transcript involved in cell proliferation and differentiation ($P = 0.01$ and $P = 0.03$, respectively). In conclusion, supplementation with P4 during the first follicular wave of the estrous cycle improves embryo viability and alters the expression of the *TGFB1* gene.

1. Introduction

The presence of large follicles at the beginning of an FSH treatment decreases the ovarian response and, thus, embryo production in small ruminants (Rubianes et al., 1995; Menchaca et al., 2002). To avoid the presence of a large follicle, Menchaca et al. (2002) proposed beginning the superovulatory treatment at the onset of the first follicular wave of the estrous cycle, the so-called "Day

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0 protocol". With this protocol, the administration of FSH begins immediately after ovulation (Day 0 of the ovulatory cycle), when there are no large follicles present. Although this treatment has been effectively used for the production of embryos (*in vivo/in vitro*) in sheep (Menchaca et al., 2009; Balaro et al., 2016; Bragança et al., 2020) and goats (Menchaca et al., 2007; Taşdemir et al., 2011; Mogase et al., 2016), the obtained embryo quality is not as good as expected. This is mainly explained by the low progesterone (P4) concentrations present during the treatment and the effects on the characteristics of the growing follicles and oocytes. According to Cuadro et al. (2018), the administration of P4 by an intravaginal device during FSH administration increases the fertilization rate, the number of viable embryos, and the proportion of high-quality embryos.

Little is known, however, about whether the administration of P4 or other progestogens during the superovulatory "Day 0 protocol" improves the response. Menchaca et al. (2018) demonstrated that greater circulating progesterone concentrations during the pre-ovulatory follicular growth period increase the developmental competence of cumulus-oocyte complexes (COC) for *in vitro* embryo production. Moreover, treatment with P4 also increases the transcript levels associated with steroidogenesis (FSH receptor, LH receptor, and α-estradiol receptor) and oocyte competence (zygote arrest 1, growth differentiation factor 9, and B-cell lymphoma 2) in sheep COC (Bragança et al., 2018). Based on this information, it could be hypothesized that the increase in the *in vitro* developmental competence observed by Menchaca et al. (2018) is associated with an increase in the steroidogenic activity during follicular growth and the accumulation of transcripts related to developmental competence. The administration of different progestogens also modifies the response since the use of P4 provides greater expression of the transcripts of the reelin protein (*RELN*; associated with oocyte competence), FSHr, and LHr than medroxyprogesterone acetate (MPA) (Bragança et al., 2018). This difference might be explained by the different actions at the cellular level, time of metabolism, and excretion (Lieberman and Curtis, 2017), although progestins are more widely used than P4 worldwide due to their availability and cost. While MPA induces similar effects as P4 by stimulating progesterone receptors, it also binds to other steroid receptors, including the glucocorticoid, mineralocorticoid, and androgen receptors (Africander et al., 2013), thus activating several pathways simultaneously. Human studies suggest that MPA, unlike P4, represses pro-inflammatory cytokine gene expression in cervical epithelial cells *via* a mechanism involving the recruitment of the glucocorticoid receptor to cytokine gene promoters (Govender et al., 2014).

Considering the aforementioned information, it might be predicted that the progestogen used during superovulatory treatment affects the competence of the *in vivo* produced embryo to establish a pregnancy and generate healthy offspring. Therefore, the hypothesis of this study was that the progestogens administered (both MPA and P4) during the superovulatory treatment of ewes alter the follicular population, quantitative and qualitative *in vivo* embryo production, and gene expression profile in blastocyst embryos. The study aimed to determine whether the administration of P4 or MPA during superovulatory treatment affects the response to the "Day 0 protocol" in ewes. The study also included the comparison of the effects of these hormones on the expression profile of genes associated with pluripotency (*OCT4* and *NANOG*), cell growth, proliferation, and differentiation (*TGFB1*), apoptosis (*BCL2* and *BAX*), mitochondrial activity (*NRF1*), and trophectoderm differentiation (*CDX2*).

2. Materials and methods

This study was approved by the Ethical Committee for Animal Use of the Universidade Federal Fluminense (#95002404/18), and it was conducted under the ethical principles of the Sociedade Brasileira de Ciência em Animais de Laboratório.

2.1. Experimental location, animals, and study design

The research comprised two repetitions, performed in September–October and in October–November 2019, respectively (spring in the southern hemisphere), at the Unidade de Pesquisa Experimental em Caprinos e Ovinos (UniPECO) in Cachoeiras de Macacu (22°2745′S), Rio de Janeiro, Brazil. In each repetition, 18 multiparous Santa Inês ewes, ranging from two to three years old, weighing 37.4 ± 4.6 kg (mean ± SD) and with a body condition score of 2.8 ± 0.3 (1–5 scale), were used. All animals were healthy, with no reproductive or clinical disorders, remained in a confined system, and were fed with access to chopped Napier grass (*Pennisetum purpureum* cv. Cameron) and 200 g/day per animal of concentrate (16% crude protein, soybean, and corn bran). Water and mineral salt (Ovinofós, Tortuga, São Paulo, Brazil) were provided *ad libitum*. Estrus synchronization was performed with intravaginal sponges containing 60 mg medroxyprogesterone acetate (Progespon, Syntex, Buenos Aires, Argentina) used for 6 days. One day before sponge removal, 300 IU eCG (Novormon 5000 MSD Animal Health, São Paulo, Brasil) and 0.12 mg cloprostenol sodium (Estron, Agner Unio, São Paulo, Brazil) were administered intramuscularly, whereas 0.025 mg lecirelin (Gestran Plus, Tecnopec, São Paulo, Brazil) was administered 36 h after progestogen withdrawal (Menchaca et al., 2009; Balaro et al., 2016).

The animals were randomly allocated into three experimental groups for the beginning of the superovulation treatment. The superovulatory protocol started 80 h after the removal of the sponge (Day 0), with 133 mg FSH (Folltropin-V, Bioniche Animal Health, Ontario, Canada) divided into six decreasing doses (33.25/33.25, 19.95/19.95, 13.3/13.3 mg) every 12 h (Santos et al., 2020). Along with the first dose of FSH, the animals received i) an intravaginal sponge containing 60 mg MPA (Progespon, Syntex, Buenos Aires, Argentina; TMPA = 12) or ii) an intravaginal device containing 0.33 g progesterone (Eazi-Breed CIDR, Zoetis, São Paulo, Brazil; TP4 = 12); both remained until the fifth dose of FSH. Twelve ewes whose only source of progesterone was the physiological P4 produced by the initial corpus luteum (CL) were used as controls (CON = 12). Regardless of the experimental group, all animals received 0.24 mg of

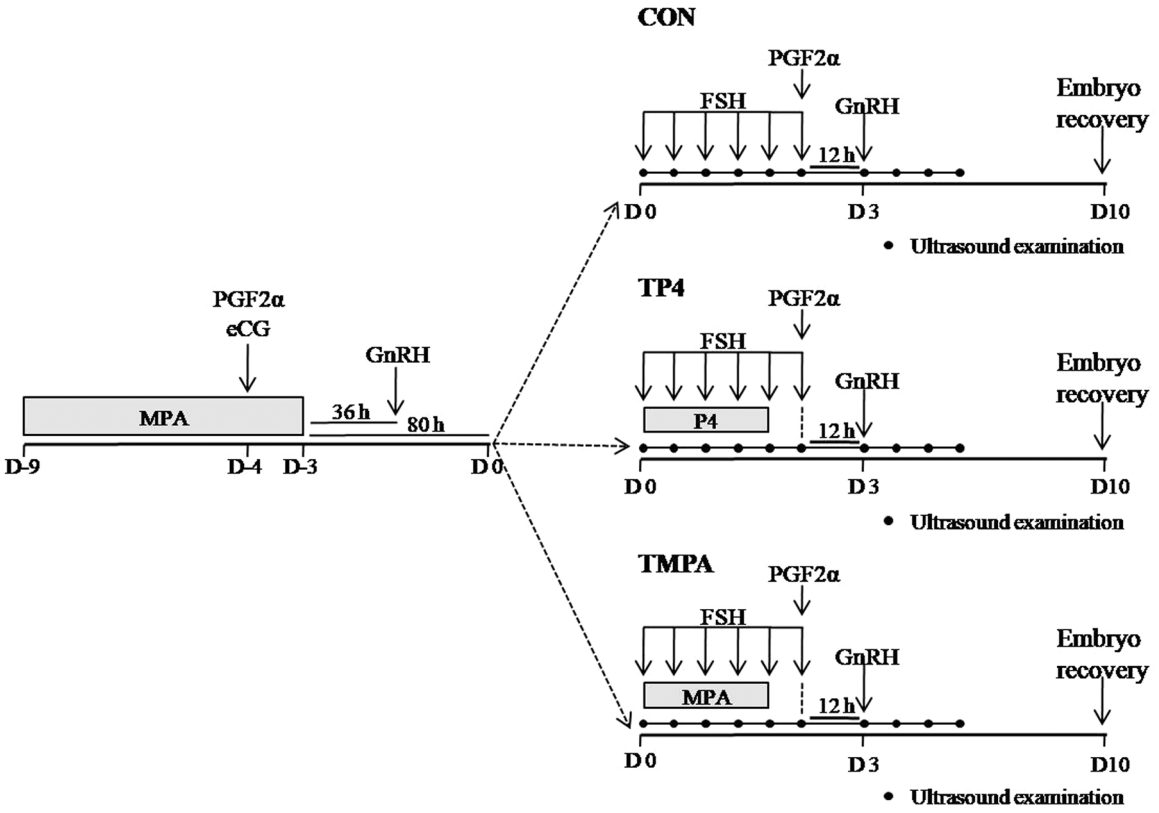

Fig. 1. Schematic representation of the experimental design. Experimental groups: CON – superovulated ewes without any exogenous progestogens; TP4 – use of an intravaginal device containing P4 during superovulation with FSH; TMPA – use of an intravaginal sponge containing progestin (medroxyprogesterone acetate; MPA) during superovulation with FSH.

cloprostenol (Estron, Agner Unio, São Paulo, Brazil) concomitantly with the application of the sixth dose of FSH, and 12 h later 0.025 mg of lecirelin (Gestran Plus, Tecnopec, São Paulo, Brazil) was administered intramuscularly (Fig. 1). The ewes were naturally mated with fertile Santa Inês rams, four times with a 12 h interval, starting immediately after the last dose of FSH.

2.2. Follicular population

All ultrasonography (US) evaluations were performed by the same operator using a portable device (Sonoscape S6, Sonoscape, Shenzhen, China) equipped with a 7.5 MHz transrectal linear transducer. The follicular population was assessed in each animal using B-mode US every 12 h, from the first FSH dose until 36 h after the GnRH administration, totaling 10 moments of evaluation (M1–M10). The follicles were classified based on their diameter as small (< 3 mm), medium (3–5 mm), or large (> 5 mm), and the number of follicles in each category was recorded, as proposed by Pinto et al. (2020).

2.3. Corpora lutea count and embryo recovery

Six days after the last mating, the ewes were deprived of food (24 h) and water (12 h), submitted to general anesthesia, and settled in the Trendelenburg position (Lima et al., 2015). The counting and evaluation of the CLs were performed by laparoscopy, as described by Bruno-Galarraga et al. (2015), and only red-colored CLs were considered functional. Ewes with ≥ 3 CLs were submitted to surgical embryo recovery through a longitudinal ventral laparotomy (Santos et al., 2020). The recovered fluid was evaluated using a stereomicroscope with 20 × to 40 × magnification. Embryos were classified according to their developmental stage and quality (Mapletoft et al., 2020) and only grade I and II blastocysts were selected and dry frozen in cryotubes (DNase and RNase free) in liquid nitrogen until molecular analysis. For gene expression analysis, a total of 10 grade I and II blastocysts were used to conform two pools comprising five blastocysts from different animals in each group.

2.4. RNA extraction, reverse transcription, and quantitative RT-qPCR amplification

Total RNA extraction was performed using an RNeasyMicro Kit (Qiagen Inc., Valencia, EUA) according to the manufacturer's instructions and treated with DNase for 15 min to avoid DNA contamination. RNAase free water (14 μL) was added, and the RNA quantification of each pool was performed using 1 μL of sample on a spectrophotometer (Nanodrop 2000, Wilmington, DE, USA); this resulted in a mean (ng) total RNA/blastocyst of 7.72 ± 2.1, 8.42 ± 1.47, and 8.16 ± 0.86 for the CON, TMPA, and TP4 groups, respectively. The SuperScript III first-strand synthesis Supermix (Invitrogen, Carlsbad, CA, USA) was used for reverse transcription, and the same RNA concentration (17.5 ng/total RNA) was used in all samples. A mixture of oligo (dT) 20 primers, dNTP mixture, Superscript III-RT, RNase OUT, $MgCl_2$, RT buffer, and RNA sample with a final volume of 20 μL was prepared to perform the reverse transcription reaction. The mixtures were first incubated at 65 °C for 5 min and then at 50 °C for 50 min. The reaction was stopped at 85 °C for 5 min, and then the samples were chilled on ice. After that, RNase H was added to the samples and they were incubated at 37 °C for 20 min

The relative quantification was done using a real-time polymerase chain reaction (ABI Prism 7300 Sequence Detection Systems, Foster City, CA, USA) in triplicate. The reactions (20 μL total volume) were achieved with a mixture of an SYBR green kit (10 μL; Power SYBR Green, Applied Biosystems), 0.1 μM primers (described in Table 1), nuclease-free water, and reverse-transcribed cDNA (1 μL). Negative controls were similarly run with each group of samples containing the RT-qPCR reaction mixture without nucleic acids. Template cDNAs were denatured at 95 ºC for 10 min, and the gene amplification was amplified by 40 cycles of thermal cycling programmed at 95 ºC for 15 s, 60 ºC for 15 s, and 60 ºC for 30 s. Fluorescence data were acquired during the extension steps. After each RT-qPCR run, a melting curve analysis was performed to confirm that a single specific product had been generated. The primer efficiency was calculated using LinRegPCR software (Ramakers et al., 2003) for each reaction. The primer efficiency standard was 1.96, 1.92, 1.94, 1.93, 1.91, 1.91, 1.95, 1.93, and 1.98 for *OCT4*, *NANOG*, *TGFB1*, *BCL2*, *BAX*, *NRF1*, *CDX2*, *GAPDH* and *H2AFZ*, respectively. Relative quantification was performed *via* the comparative Ct method ($2^{-\Delta\Delta Ct}$) using the REST 2008 software (Livak and Schmittgen, 2001) and the efficiencies were obtained with the LinRegPCR software. The expression of each target gene was normalized using the standards *GAPDH* and *H2AFZ*. The stability of the reference genes was calculated according to the methodology described by Pfaffl et al. (2004) using the BestKeeper - Excel tool. The values of the Pearson correlation coefficient observed for the *GAPDH* (r2 = 0.778) and *H2AFZ* (r2 = 0.765) genes demonstrate the stability ($P < 0.01$) of these reference genes.

2.5. Calculations and statistical analysis

The numbers of small, medium, and large follicles were compared with a mixed model, including treatment, time and their interaction as the main effects, considering time as a repeated measure. The recovered structures were classified as embryos (all stages and quality; Mapletoft et al., 2020), non-fertilized oocytes or free zona pellucida. Only embryos grade I and II were considered as

Table 1
The sequence of the specific primers used in the RT-qPCR for the gene expression evaluation of *in vivo*-derived embryos in sheep.

Gene	Sequence of primers (5'-3')	Annealing temp. (°C)	Size (bp)	Refs.
TGFB1	GGAATTCATGCCGCCCTCGGGGCTGCGG GGTCTAGATCAGCTGCACTGCAGGAGCG	63	390	Juengel et al. (2004)
OCT4	GAGGAGTCCCAGGACATCAA CCGCAGCTTACACATGTTCT	56	204	Bebbere et al. (2010)
NANOG	TTCCCTCCTCCATGGATCTG AGGAGTGGTTGCTCCAAGAC	53	501	Sanna et al. (2010)
NRF1	GCAGGTCCTGTGGGAATG CTGGGATAAATGCCCGAAG	61	412	Nau et al. (2002)
CDX2	GCCACCATGTACGTGAGCTAC ACATGGTATCCGCCGTAGTC	60	140	Sakurai et al. (2010)
BAX	CCTGGGATCTTGAAACTCTCCTT CTGAGCCAGGCTGAAATCAAAA	60	566	Chakravarthi et al. (2015)
BCL2	GCCGAGTGAGCAGGAAGAC GTTAGCCAGTGCTTGCTGAGA	60	214	Chakravarthi et al. (2015)
GAPDH	ATGTTTGTGATGGGCGTGAA ACAGTCTTCTGGGTGGCAGT	60	176	O'Connor et al. (2013)
H2AFZ	GTCGTGGCAAGCAAGGAG GATCTCGGCCGTTAGGTACTC	57	182	O'Connor et al. (2013)

TGFB1: transforming growth factor beta 1; *OCT4*: octamer-binding transcription factor-4; *NANOG*: nanog homeobox; *NRF1*: nuclear respiratory factor 1; *CDX2*: caudal type homeobox 2; *BAX*: *BCL2* associated X, apoptosis regulator; *BCL2*: B-cell lymphoma protein 2; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; *H2AFZ*: H2A histone family, member Z.

viable. The recovery rate was calculated as recovered structures * 100/number of CLs and the viability rate as viable embryos * 100/recovered structures. Data were compared with a mixed model (SAS University Edition). The model included the treatments (CON, TMPA, and TP4) as the main effects and the repetition as a random factor. The results are expressed as LSmeans ± SEM. For all tests, $P < 0.05$ was considered significant, and $P < 0.1$ was considered to indicate a tendency.

3. Results

3.1. Effect of progestogens on the follicular population during SOV

There was no effect of treatment or an interaction of the treatments with time in the number of follicles from each size. However, the number varied with time ($P < 0.0001$ in the three categories of follicles) (Fig. 2). Regardless of the experimental group, there was a progressive increase in the population of medium and large follicles, accompanied by a decrease in the population of small follicles

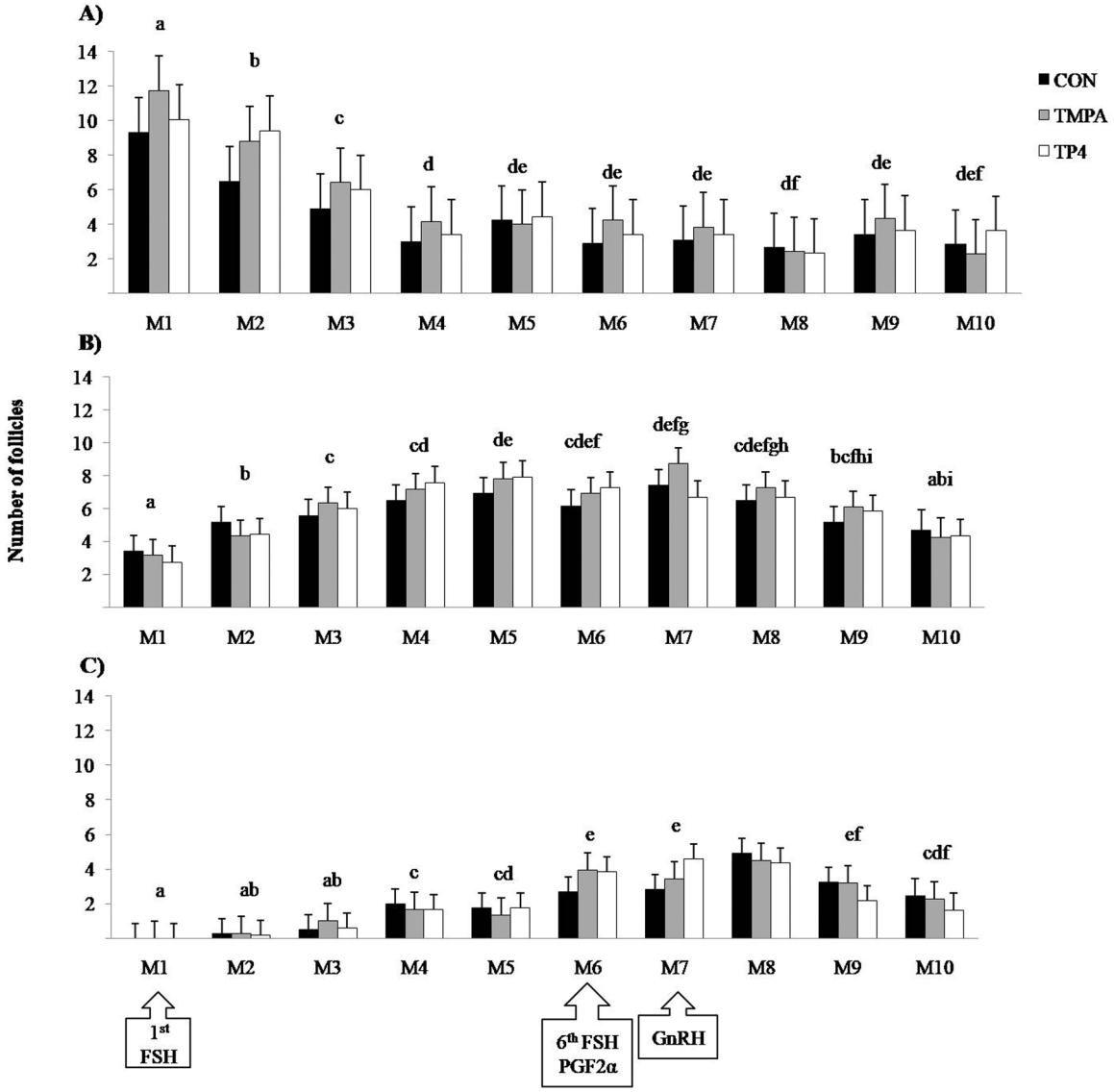

Fig. 2. Follicular population of the different experimental groups throughout a superovulatory treatment. Number of A) small (< 3 mm), B) medium (3–5 mm), and C) large (> 5 mm) follicles throughout a superovulatory treatment, from the Day 0 protocol in sheep. The moments of FSH, GnRH, and PGF2α administration are indicated with arrows. Ewes were treated with an intravaginal sponge containing medroxyprogesterone acetate (grey bars) or an intravaginal implant containing P4 (white bars), or were given no exogenous progestogens (black bars). The follicular population was assessed by transrectal B-mode ultrasonography (US) every 12 h, from the first FSH dose until 36 h after the GnRH administration (M1 to M10). Different letters: P < 0.05 regardless of the treatment.

Table 2
Superovulatory response and embryo production from Santa Inês ewes.

	CON	TMPA	TP4	P
CLs (n)	6.0 ± 1.2 (0–11)	7.7 ± 1.1 (0–14)	8.2 ± 1.1 (0–18)	ns
Recovered structures (n)	2.4 ± 0.9 (0–5)	4.8 ± 0.8 (1–10)	4.9 ± 0.8 (1–13)	0.08
Viable embryos (n)	1.0 ± 0.7 (0–4)	1.9 ± 0.7 (0–7)	3.3 ± 0.7 (0–9)	0.08
Recovery rate (%)	44.5 ± 9.5 (0–100)	62.4 ± 9.1 (25–100)	62.5 ± 9.1 (12–100)	ns
Viability rate (%)	24.4 ± 16.8^{b} (0–100)	49.9 ± 16.3^{ab} (0–100)	71.9 ± 16.3^{a} (0–100)	0.04

Different letters within the same row differ statistically (P < 0.05); within the brackets are the absolute numbers; CON – ewes superovulated without exogenous progestogen; TMPA – ewes with a medroxyprogesterone acetate sponge during superovulation; TP4 – ewes with a progestogen (P4) implant during superovulation. Recovery rate: structures recovered * 100/number of CLs; viability rate: viable embryos recovered * 100/recovered structures. Data are presented as mean ± SEM.

throughout the studied period.

3.2. Effect of progestogen treatments in the superovulatory response

The treatments did not affect the number of CLs or the recovery rate (Table 2). However, there were tendencies for differences in the number of structures recovered and the number of viable embryos ($P = 0.08$ for both) (Table 2). Only grade I and II embryos were considered as viable. From those, 41.25% were morula, 56.25% were blastocysts, and 2.5% were hatched blastocysts in the three groups. The treatments affected the viability rate ($P = 0.046$), which was greater in TP4 than in CON ($P = 0.014$) and intermediate in TMPA, without any differences with the other groups (Table 2).

3.3. Effect of progestogens on the gene expression profile

The administered progestogens did not affect the number of transcripts associated with pluripotency (*OCT4*), maintenance of pluripotency (*NANOG*), apoptosis (*BCL2*), mitochondrial activity (*NRF1*), or trophectoderm differentiation (*CDX2*). However, the *TGFB1* transcript was less expressed in the CON group embryos than in the TP4 and TMAP embryos ($P = 0.01$ and $P = 0.03$, respectively) without differences between both progestogens (Fig. 3).

The high concentrations of progestogen during follicular growth affect oocyte competence, probably increasing the capacity of the oocyte to undergo cleavage and embryonic development *in vitro* (Menchaca et al., 2018), most likely through the positive regulation of *TGFB1* – a gene involved in cell growth, proliferation, and differentiation, contributing to the increase in oocyte competence.

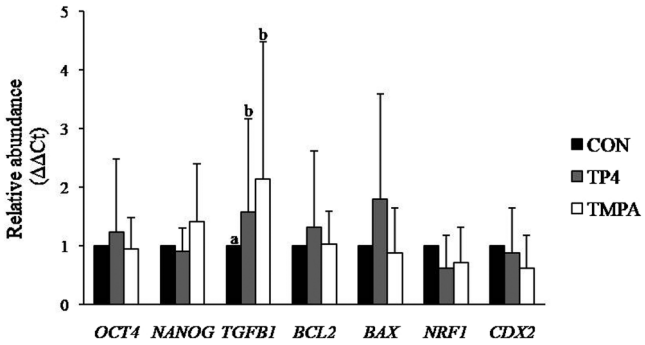

Fig. 3. Expression of embryonic competence markers in *in vivo*-produced blastocysts. Genes associated with pluripotency (*octamer-binding transcription factor 4 – OCT4* and *Homeobox protein – NANOG*), cell growth, proliferation, and differentiation (*transforming growth factor beta 1 – TGFB1*), apoptosis (*B-cell lymphoma protein 2 – BCL2 and BCL2 associated X protein – BAX*), mitochondrial activity (*Nuclear respiratory factor 1* – NRF1), and trophectoderm differentiation (*Caudal Type Homeobox 2 – CDX2*) were evaluated in grade I and II blastocysts. CON – ewes superovulated without exogenous progestogen (black bars); TMPA – ewes with a medroxyprogesterone acetate sponge during superovulation (white bars); TP4 – ewes with a progestogen (P4) implant during superovulation (grey bars). Different letters differ statistically (P < 0.05).

4. Discussion

The results of the study demonstrate that the administration of progesterone at the beginning of SOV increases the rate of embryonic viability. Previously, *in vitro* studies have demonstrated that plasma P4 concentrations in animals from which oocytes are collected also affect *in vitro* embryo production (McEvoy et al., 1995; Menchaca et al., 2018; Saad et al., 2019), which is consistent with the findings of the present study. Additionally, previous *in vivo* studies have reported a markedly higher pregnancy rate in dairy cows with a higher concentration of P4 compared to a lower concentration before artificial insemination (Inskeep, 2004; Wiltbank et al., 2011). This beneficial effect of P4 may be explained by in follicular fluid composition (Cerri et al., 2011), cumulus expansion, and oocyte competence (Fair and Lonergan, 2012). Relatively lower concentrations of P4 lead to the disruption of oocyte nuclear maturation (Rajamahendran and Manikkam, 1994) and the impairment of fertilization (Rivera et al., 2011) and early embryonic development (Mihm et al., 1994; Ahmad et al., 1995).

The gene expression data, specifically the *TGFB1* gene, whose expression was higher in embryos from the TMPA and TP4 groups, also suggest a greater capacity for cell growth, proliferation, and differentiation of these embryos compared to embryos from the CON group. Furthermore, these data suggest a greater capacity for the implantation and post-implantation development of these embryos, since *TGFB1* also mediates the invasion of the endometrium by the trophectoderm during implantation (Jones et al., 2006; Li, 2014; Monsivais et al., 2017). *TGFB1* acts as an autocrine/paracrine factor during embryonic development, modulating the microenvironment in which the embryo develops and implants (Chow et al., 2001). It is likely that higher progestogen concentrations during follicular growth can modify the composition of both the follicular and uterine fluids (Lonergan and Sánchez, 2020), impacting the oocyte and, consequently, embryo quality (Cuadro et al., 2018). Moreover, Kim et al. (2006) reported that the addition of progesterone to a co-culture of human endometrial epithelial and stromal cells also increases the concentration of *TGFB1* in media culture. Therefore, the addition of progestogens might have also increased the concentration of *TGFB1* in the uterus and due to the previously mentioned embryonic maternal interaction characteristics (Chow et al., 2001), potentially leading to the progressive increase of this gene in TP4 and TMPA embryos. Although this study does not allow us to confirm whether these pathways are involved, it should be considered that the treatments ended before ovulation, and so should have acted in the characteristics of the ovulated oocytes and/or the milieu in which that oocyte/embryo developed.

In line with previous reports (Bragança et al., 2019), the tested treatments did not affect the follicular population. Although Bartlewski et al. (2015) have reported that the administration of P4, in comparison with MPA, increased the number of follicles $\geq$ 3 mm, this difference was only transient and did not alter the ovulatory response. However, while P4 increased the viable embryo rate compared with the controls, MPA did not. Therefore, these results suggest that the difference in progestogen metabolism and clearance (Husein and Kridli, 2002) does not modify the superovulatory response of ewes, although it might modify the final embryo quality.

5. Conclusions

In conclusion, the administration of P4 during the Day 0 superovulatory treatment protocol enhances the embryo viability rate. The effect of progestogens ended before ovulation, however, the use of MPA/P4 modified the milieu of development of the oocytes, increasing the final quality of the embryos by increasing the expression of the *TGFB1* gene.

CRediT authorship contribution statement

A.R. Taira: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **R.I.T.P. Batista**: Conceptualization, Methodology, Formal analysis, Supervision, Visualization, Writing – review & editing. **J.D.R. Santos**: Investigation, Data curation. **P.H.N. Pinto**: Investigation, Data curation, Writing – review & editing. **M.F.A. Balaro:** Investigation, Data curation, Writing – review & editing. **C.G. Espírito Santo:** Investigation, Data curation. **V.L. Brair**: Investigation, Data curation, Writing – review & editing. **J.M.G.S. Fabjan**: Investigation, Data curation. **R. Ungerfeld**: Methodology, Writing – review & editing. **J.F. Fonseca**: Methodology, Writing – review & editing. **F.Z. Brandão**: Project administration, Supervision, Resources, Visualization, Conceptualization, Methodology, Writing – review & editing.

Declaration of Competing Interest

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

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