



FEMALE BOVINE DONOR AGE INFLUENCE ON QUALITY MARKERS' EXPRESSION AND *PPARS* ABUNDANCE IN DAY 7 BLASTOCYSTS

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Abstract

In bovine females, the production of embryos derived from oocytes obtained by ovum pick-up (OPU) is becoming a frequent procedure. Collection of oocytes from prepubertal animals enables shortening the genetic distance, especially in high-value animals. Nevertheless, the oocyte and later embryo developmental potential differ between oocytes derived from prepubertal and adult heifers. The aim of this study was to determine the possible underlying causes for differences in quality between embryos obtained from prepubertal and pubertal heifers. Cumulus oocyte complexes were collected via OPU. The expression of *PLAC8*, *IFN τ* , *IGFR1*, *SOX2*, and *OCT4* in blastocysts was evaluated. The transcriptome of peroxisome proliferator-activated receptors (*PPARs*), the major genes involved in the metabolism and development of the embryo, and the embryo quality marker gene expression (*PLAC8*, *IFN τ* , *IGFR1*, *SOX2*, and *OCT4*) in the day 7 blastocysts derived from oocytes collected from prepubertal or pubertal heifers differed significantly. The expression of genes involved in basic biological processes of early embryonic development differed between embryos derived from oocytes collected from prepubertal and pubertal heifers. The obtained data may be used to adjust culture conditions to the biological requirements of the embryos derived from oocytes collected from prepubertal heifers, and to predict the possibility of successful development of the embryo.

Key words: cow, embryo, ovum pick-up, *PLAC8*, *PPARs*

The production of embryos *in vitro* (IVEP) in combination with oocyte collection by transvaginal ovum pick up (OPU) is becoming a more popular method of livestock production, especially in the bovine industry (Galli et al., 2014). The latest molecular innovations, such as genomic predicted transmitting ability (GPTA) or genomic selection of embryos (GS), alongside the continuous search for production (Jiang et al., 2019; Kawamoto et al., 2022) or wellness (McNeel et al., 2017) genetic traits in cattle, allow those involved to evaluate animals at the very early stage of life (Jiang et al., 2019; Ferré et al., 2021). Hence, the use of OPU-IVEP followed by molecular evaluation of the produced embryo may help to breed only the most valuable animals.

OPU allows us to recover oocytes from calves as young as two months of age, upon which they are able to resume meiosis and, if fertilized, develop to blastocysts. Together, OPU-IVEP in combination with GPTA or GS may allow the generation of large numbers of valuable animals and significantly shorten the time needed for ge-

netic improvement in cows (Galli et al., 2014; de Silva et al., 2022).

Nevertheless, the age of the donor is suggested to be an important factor influencing oocyte quality and, most importantly, the subsequent development of the embryo and successful pregnancy (Rizos et al., 2002; Baruselli et al., 2016; Currin et al., 2021). Oocytes from immature heifers are less adequate in comparison to those from adult ones (Zaraza et al., 2010; Baruselli et al., 2016) and they may differ in metabolism, cytoplasmic and nuclear maturation or electric potential of the membrane, which may further have implications on the rate of subsequently obtained blastocysts resulting in the establishment of successful pregnancies (Khatir et al., 1996; Ax et al., 2005).

Both economic and scientific interest has led to the continuous search for better understanding of embryonic development, as well as embryo quality markers (Orozco-Lucero and Sirard, 2014; Ferré et al., 2021). The analysis of differentially expressed genes has revealed that peroxisome proliferator-activated receptors

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(PPARs), which are major receptors involved in the development of the embryo (Jawerbaum and Capobianco, 2011), mammalian target of rapamycin (mTOR), and nuclear factor (erythroid derived) like 2 (NRF2)-mediated oxidative stress response signaling pathways may be altered in embryos produced from oocytes collected from prepubertal heifers (Morin-Doré et al., 2017). In consequence, downstream effectors of PPAR signaling pathways may also be inhibited in these embryos (Morin-Doré et al., 2017). PPARs regulate the expression of genes associated with metabolic (Bensinger and Tontonoz, 2008), developmental (Abbott et al., 2009), and inflammatory processes (Bensinger and Tontonoz, 2008) and have been detected in the early stages of rodent, human and, recently, bovine embryo development (Abbott et al., 2009). PPAR δ is the first to be expressed in the developing embryo, followed by expression of PPAR γ . Both are activated by prostaglandin I₂ (PGI₂, prostacyclin) and long-chain polyunsaturated fatty acids (LC-PUFAs) (Kowalczyk-Zięba et al., 2020). An additional ligand specific for PPAR γ activation is the bioactive metabolite of prostaglandin D₂, 15deoxyD_{12,14} and prostaglandin J₂ (15dPGJ₂). Among other candidate genes, whose expression may relate to embryo development and quality, are those involved in cell proliferation and apoptosis, such as insulin-like growth factor 1 and 2 (IGF1, IGF2) and their receptors (IGF1R, IGF2R) (Wang et al., 2009); genes encoding octamer binding transcription factor-4 (OCT-4) and SRY-related HMG-box containing factor (SOX2), which are involved in maintaining cell pluripotency, a signal of maternal recognition of pregnancy in cattle; and interferon tau (IFN τ) or glutathione peroxidase 4 (GPX4), whose expression is considered protective against oxidation and apoptosis of the embryo. Another gene which may help to predict the outcome of pregnancy is placenta associated 8 (PLAC8), which is involved in processes such as placenta formation (Chang et al., 2018) and growth of cells (Mao et al., 2021).

Despite ongoing research, the number of pregnancies obtained from embryos delivered from oocytes collected from prepubertal heifers is still lower in comparison to embryos delivered from oocytes collected from mature animals.

Here we sought to determine the expression of PPAR γ , PPAR δ , and chosen embryo quality marker genes related to important biological processes present in embryos derived from oocytes collected *in vivo* from prepubertal and pubertal heifers by OPU.

Material and methods

Animals

Experimental procedures were approved by the Ethical Committee in Olsztyn with the approval number Agreement No. 76/2014/DTN.

Clinically healthy Holstein Friesian heifers from a dairy cattle farm located in north-east Poland were used in this study. All the females were kept together and had

unlimited access to water and haylage. Heifers were assigned either to the prepubertal or pubertal group based on the age (Morin-Dore et al., 2017) and ultrasound examination performed by a veterinarian. Prepuberty was confirmed if there was no corpus luteum or dominant follicle present in the ovary. There were six females assigned to the prepubertal group (PP) and six to the pubertal group (P). The mean age of the females in the PP group was 6.2 $\pm$ 1.3 and weight was 187.5 $\pm$ 21 kg (37% of the adult cow body weight), while in the P group these values were 11.7 $\pm$ 0.5 months and 397.2 $\pm$ 5 kg (79% of the adult cow body weight), respectively.

Ovum pick-up

All the ovum pick-up sessions were performed between October and December 2021. Every heifer in the prepubertal and pubertal groups were used four times for the cumulus-oocyte complex (COC) collection. The OPU procedure was performed as described by Cavalieri et al. (2018). Briefly, every animal was epidurally anesthetized and the perineal area was cleaned. The ovary was positioned between the transvaginally inserted ultrasound probe with the aspiration needle and the rectally inserted operating hand. Aspiration of all visible follicles less than 5 mm diameter was performed with a real-time B-mode ultrasound scanner (Draminski 4Vet, Poland) equipped with an 8 MHz micro-convex transducer connected to a follicular aspiration guide and a stainless-steel guide. Follicles were punctured with a 18 G disposable hypodermic needle connected to a 50 mL conical tube (Corning, Acton, MA, USA) via a silicon tubing system (Minitube, Germany). Negative pressure was maintained at the level of 90–100 mmHg. Once all the visible COCs were collected from both ovaries, the aspiration system was washed with tissue culture medium (Merck, Germany), 0.05% sodium heparin (Merck, Germany), and 1% fetal bovine serum (Gibco®, Thermo Fisher Scientific, USA).

Oocytes were inspected and washed twice with Wash Medium (Merck, Germany) supplemented with 20 mM HEPES (Merck, Germany), 25 mM sodium bicarbonate (Merck, Germany), 0.4% bovine serum albumin (Merck, Germany), and 40 μ g/mL gentamicin (Merck, Germany). All COCs, regardless of their quality, were used for *in vitro* maturation.

In vitro maturation

There were 192 and 168 immature COCs collected from the prepubertal and pubertal heifers, respectively. These were placed into four-well plates (Thermo Fisher Scientific, MA, USA) containing 500 μ L of maturation medium, with approximately 25 gametes in one well of each plate. The medium was supplemented with 0.02 IU/mL of a pregnant mare's serum gonadotropin (PMSG, Merck, Germany), 0.01 IU/mL human chorionic gonadotropin (hCG, Merck, Germany), and 5% fetal bovine serum (FBS, Merck, Germany). COCs were then incubated for 22 h at 38.5°C in a 5% CO₂ humidified air atmosphere.

In vitro fertilization

Frozen-thawed semen from the same bull was used for all the oocytes. Semen was thawed in a 38°C water bath for 60 s, and then was carefully placed under the capacitation medium (TL sperm capacitation medium, Minitube, Germany) supplemented with 1 mM sodium pyruvate, 0.6% BSA and 0.1 mg/ml gentamicin. Incubation was performed at 38.5°C in 5% CO₂ for 1 h. A swim-up procedure was used for the recovery of motile sperm. Once the incubation was finished, the upper two-thirds of the capacitation medium were collected and centrifuged at 200 × g for 10 min. The supernatant was discarded, and the pellet of sperm was diluted with fertilization medium to a final concentration of 10⁶ motile sperm/ml.

COCs after *in vitro* maturation were transferred to 400 µL fertilization medium (TL fertilization medium; Minitube, Germany), supplemented with 10 µg/mL heparin (Merck, Germany), 20 mM sodium pyruvate (Merck, Germany), and 0.5% BSA and co-incubated with spermatozoa at 38.5°C in a 5% CO₂ humidified air atmosphere for 24 h. The day of *in vitro* fertilization was considered Day 0.

In vitro embryo culture

After 24 h of insemination, embryos were denuded of cumulus cells and washed twice in a wash medium. Then, they were cultured until they reached the blastocyst stage (Day 7) in four-well dishes in 500 µL of culture medium (SOF, synthetic oviduct fluid medium; Minitube, Tiefenbach, Germany) supplemented with amino acids, 10 µL/ml BME (Sigma Aldrich, Munich, Germany) and 20 µL/ml MEM (Sigma Aldrich, Munich, Germany), 3.3 mM sodium pyruvate and 5% FBS under 500 µL of mineral oil (NidOil, Nidacon, Sweden) at 38.5°C in an atmosphere of 5% CO₂, 5% O₂, 95% N₂ and high humidity.

The morphological quality and developmental stage of the embryos were assessed by visual examination using an inverted and a stereo microscope at 20, 50, or 100× magnification, according to the International Embryo Technology Society (IETS; International Embryo Technology Society 1998). Only expanded blastocysts were selected for the study. Results are shown in Table 1.

RNA isolation, reverse transcription (RT) and real-time quantitative PCR (qPCR) analysis

Total RNA was isolated from all expanded blastocysts from both groups (P and PP; Table 1) with the PicoPure RNA Isolation Kit protocol (Applied Biosystems, USA) according to the manufacturer's instructions. For mRNA expression analysis, total RNA was extracted from 6 pools of 5 blastocysts from the prepubertal animals (n= 6 × 5 blastocysts) and 9 pools of 5 blastocysts from the pubertal animals (n= 9 × 5 blastocysts). The RNA samples were stored at -80°C until RT. Before use, RNA content and quality were evaluated via spectrophotometric measurement. The average RNA level from each sample (pool of 5 blastocysts) was 20 ng, and the purity was confirmed through measurement of the 260:280 ratio in the range of 1.8–2.0. Reverse transcription was performed using SuperScript™ III First-Strand Synthesis SuperMix for qPCR (Thermo Fisher, USA). RT products were diluted and stored at -20°C until qPCR amplification.

qPCR was performed in duplicate in 384-well plates using the ABI Prism 7900 sequence detection system (Applied Biosystems, Life Technologies, USA), and 500 ng/ml of cDNA was amplified for every sample. Primers used for cDNA amplification are listed in Table 2. The results of qPCR were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, an internal control) and mRNA expression is presented in arbitrary units.

Table 1. Developmental rates of embryos derived from oocytes collected from prepubertal and pubertal heifers. Cleavage rate and blastocysts rate are presented in percentages (%). Total number of selected quality blastocysts is presented as a number/percentage of all blastocysts (%)

Experimental group	Total number of oocytes	Cleavage rate (%)	Average number of oocytes per one animal	Total number of blastocysts	Blastocyst rate (%)	Total number of selected quality blastocysts (%)
Prepubertal (n = 6)	192	81.77	8	30	15.62	Grade 1/76 (39.6) Grade 2/86 (44.8) Grade 3/16 (8.3) Grade 4/14 (7.3)
Pubertal (n = 6)	168	84.52	7	45	26.78	Grade 1/64 (38.1) Grade 2/80 (47.6) Grade 3/16 (9.5) Grade 4/8 (4.7)

Table 2. List of primers, product length, and GenBank accession numbers of the amplified genes

Gene symbol	Gene name	Primer sequence 5'-3'	Fragment size, bp	GenBank accession no.
1	2	3	4	5
IGF1R	Insulin like growth factor 1 receptor	GAGTGGAGAAATCTGCGG AAATGAGCAGGATGTGGAGG	110	NM_001244612.
IGF2R	Insulin like growth factor 2 receptor	ACCTCCGATCCTCAATCCC TGTAAGTTGAAGTGCCGGTC	82	NM_174352.2

Table 2 – contd.

1	2	3	4	5
<i>OCT4</i>	Octamer binding transcription factor 4	GAGAAAGACGTGGTCCGAGTG GACCCAGCAGCCTCAAAATC	101	NM_174580.2
<i>PLAC8</i>	Placenta associated 8	TTTACCGCTCTGTGCCCTTT CCATGTGAACCTGACCAAGCAT	95	NM_001025325.2
<i>SOX2</i>	SRY-Box transcription factor 2	TGGATCGGCCAGAAGAGGAG CAGGCGAAGAATAATTGGGGG	89	NM_001105463.2
<i>PPARγ</i>	Peroxisome proliferator activated receptor γ	AGTGGAGACCGCCAGGTTTG GGGAGGACTCGGGGTGGTTC	104	NC_037349.1
<i>PPARδ</i>	Peroxisome proliferator activated receptor δ	TGCGGGGCTTCTCCCATGAC AGCCGTGAGTCTGCCAAGT	81	NM_001083636.1
<i>CAT</i>	Catalase	CTGGGACCCAACTATCTCCA GATGCTCGGGAGCACTAAAG	112	NM_001035386.2
<i>GPX4</i>	Glutathione peroxidase 4	GTGCTCGTCCATGCACGA CCTGGCTCCTGCCTCCCA	120	NM_174770.4
<i>IFNτ</i>	Interferon τ	TTGACAAACCCAAATTTATTGG GCAAGGCTTTTAAATAGGGAAGA	172	NM_001015511.3
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase	CACCTCAAGATTGTCAGCA GGTCATAAGTCCCTCCACGA	103	NM_001034034.2

Statistical analysis

The analysis was performed using the R programming language (version 4.1.0, R Development Core Team, Vienna, Austria, 2008; <https://www.r-project.org/>) and GraphPad Prism software (version 9.0, San Diego, USA), with a type I error rate set at 5%. Data are presented as mean $\pm$ standard deviation (SD). The normality of the data was assessed using the D'Agostino–Pearson and Shapiro–Wilk tests, while the homogeneity

of variance was checked using Levene's test. The effect of pubertal status on the expression of selected genes was estimated using a linear mixed-effects model, with pubertal status as a fixed effect (prepubertal or pubertal) and heifer ID and ovum pick-up session as random effects. Repeatability of the ovum pick-up results performed on different sessions was analyzed using PDIF: Pairwise Difference function of the R package “sasLM”.

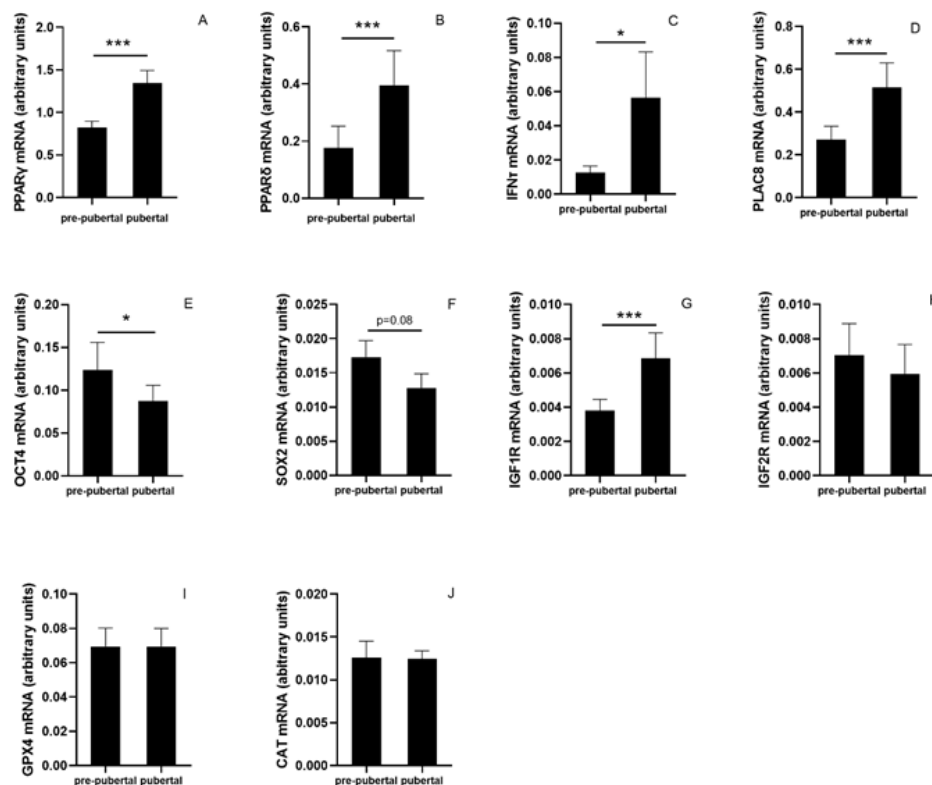


Figure 1. mRNA expression of *PPAR γ* (A), *PPAR δ* (B), *IFN τ* (C), *PLAC8* (D), *OCT4* (E), *SOX2* (F), *IGF1R* (G), *IGF2R* (H), *GPX4* (I), *CAT* (J) in day 7 bovine blastocysts derived from prepubertal and pubertal heifer oocytes. Values are presented as arbitrary units and displayed as mean $\pm$ SD. Significant differences between gene expression were determined by two-tailed Student's t-test with α error set at 5% (* P <0.05; *** P <0.001).

Results

Both *PPAR* γ and *PPAR* δ were expressed in blastocysts from both groups (prepubertal and pubertal heifers), and the expression of both isoforms was significantly higher in the blastocysts derived from pubertal heifers (Figure 1 A, B).

The mRNA expression of the chosen embryo quality genetic markers was present in blastocysts from both the prepubertal and pubertal heifers. mRNA abundance was significantly higher for the *IFN* τ , *PLAC8*, and *IGFR1* genes in the P-blastocysts (Figure 1 C, D, G), while for the *OCT4* and *SOX2* ($p=0.08$) genes expression was significantly higher in the group of PP-embryos (Figure 1 E, F). There were no differences in mRNA expression of *IGF2R*, *GPX4*, and *CAT* between the analyzed groups of blastocysts (Figure 1 G, I, J).

Discussion

This study demonstrated that *PPAR* δ and *PPAR* γ are significantly less expressed in bovine embryos derived from oocytes collected from prepubertal heifers in comparison to those from pubertal heifers. This result corresponds to research by Morin-Doré et al. (2017) who showed that *PPARs* pathways are inhibited in embryos derived from oocytes collected from prepubertal heifers in comparison to adult heifers. The crucial role of *PPAR* γ/δ in the development of early embryos and the placenta has been demonstrated in studies where *PPAR* $\gamma/PPAR$ δ knockout mice had embryo lethality (Barak et al., 1999, 2022). Signaling pathways of both *PPAR* isoforms are involved in essential processes during the maturation of an oocyte and embryo development, such as lipid and glucose metabolism (Bensinger and Tontonoz, 2008), adipogenesis (Barak et al., 1999), and development of trophoblast and placenta (Barak et al., 1999, 2002; Ax et al., 2005). Embryos developed from oocytes collected from prepubertal heifers are less viable and of lower quality in comparison to those from pubertal ones (Morin-Doré et al., 2017; Feire et al., 2020). We propose that the lower abundance of *PPAR* expression may in part explain this phenomenon. Interestingly, activation of *PPAR* δ by a synthetic ligand GW501516 has been proposed to improve the quality of day 8 bovine embryos via the lipid metabolism maintenance (Indrees et al., 2019). A similar supporting role of the activated *PPAR* δ was demonstrated in porcine blastocysts (Guo et al., 2017). Whether the supplementation of the culture medium with factors supporting *PPARs* activation/expression would improve the quality of embryos derived from prepubertal heifers is beyond the scope of the current study, but is an interesting research possibility.

Interferon tau (*IFN* τ) secreted by bovine embryos supports its elongation and is a signal of maternal recognition of pregnancy (Brooks et al., 2014). Abundance of *IFN* τ may be influenced by both genetic and environ-

mental factors (Brooks et al., 2014; Kasimanickam and Kasimanickam, 2020). The results of previous research are inconclusive and some report differences in *IFN* τ secretion between embryos produced *in vitro* with the use of various assisted reproduction techniques, and *in vivo* (Arnold et al., 2006), while others do not (Yao et al., 2009). It is suggested that these possible variations in *IFN* τ secretion may be attributed to differences in pregnancy rates, for example, after embryo transfers *in vitro* vs. *in vivo* (Forde and Lonergan, 2017). Here we showed that expression of *IFN* τ was significantly higher in the pubertal embryos than in the prepubertal embryos. In general, pregnancy rates after transferring embryos derived from prepubertal heifers are lower in comparison to those in pregnancies using embryos derived from pubertal ones (Khatir et al., 1998; Majerus et al., 1999; Palma et al., 2001; Ax et al., 2005; Morin-Doré et al., 2017). Lower expression of *IFN* τ by PP embryos may be one of the factors influencing successful pregnancy establishment. Conceptus-derived *IFN* τ is suggested to affect expression of various molecules in the endometrium (Hansen et al., 2017). As the other type of interferon, *IFN* τ supports the synthesis of phospholipase A2 (*PLA2*) and has been shown to upregulate expression of prostaglandin-endoperoxide synthase 2 (*PTGS2*) and, in consequence, PG synthesis in the endometrium, which in turn supports embryo elongation (Brooks et al., 2014). It is likely that *IFN* τ , besides having a paracrine influence on the endometrium, also has similar autocrine functions within the developing trophoblast (Rocha et al., 2021). It has been shown that in ewes it supports proliferation of the trophoblast in an autocrine manner (Wang et al., 2013). Moreover, it has been suggested to be involved in the functioning of both innate and adaptive immune systems, and influences, for example, the chemotaxis of the immune cells in the uterus (Rocha et al., 2021). Notwithstanding, it remains unknown whether these functions of *IFN* τ may depend on its abundance; however, this is a feasible possibility.

Placenta associated 8 (*PLAC8*) is a factor whose sequence is conserved across vertebrates. It is associated with apoptosis, carcinogenesis, and embryonic and placental development (Chang et al., 2018), and is one of the key regulators of cell growth across species (Mao et al., 2021). It is considered as one of the markers which helps to predict the outcome of pregnancy in cows (El-Sayed et al., 2006). *PLAC8* is present in both bovine embryos and the placenta. Its higher expression in day 7 embryos corresponds to successful pregnancies, resulting in giving birth to live calves (El-Sayed et al., 2006). We determined that *PLAC8* had significantly higher abundance in the group of embryos derived from pubertal heifers in comparison to the ones from prepubertal heifers, which may indicate that the transfer of these embryos might result in higher numbers of successful pregnancies. Nevertheless, the mechanism by which *PLAC8* promotes embryonic and placental development remains unknown. Collected evidence implies that the functions of *PLAC8*

may differ depending on the type of cell (Jia et al., 2018). In some types of cancer-transformed cells or in fibroblasts, *PLAC8* (Jia et al., 2018) inhibits apoptosis and/or increases cell proliferation (Shi et al., 2017; Jia et al., 2018), while in others it has the opposite action (Wu et al., 2010; Jia et al., 2018). In human placentas, *PLAC8* is expressed in invasive trophoblasts and it is suggested that its expression might be upregulated by lower oxygen tension (Chang et al., 2018). In our experiment, the *in vitro* culture conditions, including oxygen tension, did not differ between the groups. Moreover, the implantation of bovine trophoblasts, unlike in humans, does not invade the endometrium. Studies involving other cell types than trophoblasts indicate that *PLAC8* is one of the regulators of cell survival and apoptosis, possibly via the c-Myc-p53 pathway (Mourtada-Maarabouni et al., 2013). It is possible that *PLAC8* exerts a similar protective role in day 7 bovine blastocysts. Another explanation of the supporting role of *PLAC8* may be based on cancer research (Mao et al., 2021). In some types of cancer, such as colorectal, prostate or lung, *PLAC8* works as an oncogene, promoting the growth and survival of cancer cells (Yu and Cui, 2016; Huang et al., 2020). Moreover, it was recently reported that *PLAC8* may disrupt the autophagy of cancer cells via the serine/threonine protein kinase B/mechanistic target of rapamycin (AKT/mTOR) pathway in nasopharyngeal carcinoma (Huang et al., 2020). It is noteworthy that the Akt/mTOR pathway is essential in early embryonic development and genetic knockout of some of its components leads to embryonic lethality (revised in Chang et al., 2018). In pluripotent stem cells, AKT/mTOR signaling contributes to the self-renewal and differentiation of these cells (Yu and Cui, 2016). Detailed mechanisms of *PLAC8* involvement in very early development, in the pre-implantation stage of embryos, remains unknown; however, a positive effect of the AKT/mTOR pathway may be hypothesized. It has also been proposed that, in some types of cancer, *PLAC8* may interact with stem cell markers such as Krüppel-like factor 4 (KLF4) and, as a consequence, mediate the stemness of cancer cells (Jia et al., 2018). While in tumorigenesis the above functions of *PLAC8* are inevitably negative, they may have a positive effect in growing blastocysts.

The gene encoding octamer binding transcription factor-4 (OCT4) and SRY-related HMG-box containing factor (SOX2) are two of the essential factors regulating early embryogenesis in mammals. It is generally agreed that both cooperate in order to maintain the pluripotency of embryonic stem cells during embryogenesis (Rizzino and Wuebben, 2016). OCT4 is expressed from fertilization, through the blastocyst stage, and, as the pregnancy progresses, it is suggested that it is only expressed in the germ cell lines (Patra et al., 2020). SOX2 expression starts at the morula stage and at the blastocyst stage is limited to inner cell mass cells (Keramari et al., 2010). OCT4, together with SOX2 and another pluripotency-related gene Nanog, maintains self-renewal and pluripotency of inner cell mass and embryonic stem cells in mammals (Kera-

mari et al., 2010). Both OCT4 and SOX2 are crucial for embryonic development, and OCT4/SOX2 null embryos do not survive (Rizzino and Wuebben, 2016). Surprisingly, our results showed that the expression of *SOX2* and *OCT4* was lower in embryos derived from pubertal animals in comparison to those derived from prepubertal ones. Because both genes are markers of pluripotency and are required for differentiation of inner cell mass and trophectoderm lineages in order to lead to physiological formation of the blastocyst (Beyhan et al., 2007), it could be suggested that their higher expression would be associated with better development of the embryo. However, embryos derived from prepubertal heifers are still characterized by lower developmental potential and survival (Morin-Doré et al., 2017). On the other hand, growing evidence also suggests that the expression of SOX2 and OCT4 must be tightly controlled in order to maintain pluripotency as well as further physiological development of the embryo; too high expression has a negative impact (Rizzino and Wuebben, 2016). Indeed, increased levels of OCT4 result in the lack of self-renewal and pluripotency of embryonic stem cells. Elevated levels of SOX2 inhibit activity of OCT4 and Nanog as well as the SOX2 gene itself (Rizzino and Wuebben, 2016). A similar result was obtained by Velásquez et al. (2019), who showed that the expression of OCT4, SOX2 and Nanog was higher in less adequate bovine embryos. Lower expression of *OCT4* and *SOX2* in the group of blastocysts obtained from oocytes from prepubertal donors in our study might be explained by their lower developmental competence.

Insulin-like growth factor 1 and 2 receptors (IGF1R and IGF2R), together with insulin-like growth factors 1 and 2 (IGF1 and IGF2), are a part of a specialized system which is involved in cell proliferation and apoptosis as well as mutagenesis (Wang et al., 2009). In the analyzed embryos, expression of *IGF1R* was significantly higher in the group derived from pubertal heifers, while expression of *IGF2R* did not differ between both groups. Pre-implantation embryos express IGF1R; however, they do not express its ligand, IGF1, which is maternally delivered (Wang et al., 2009). A study by Ludwig et al. (1996) suggested that IGF1 acts mainly via the IGF1R. It is feasible to speculate that the expression of IGF1R may influence the effect of IGF1 on the developing embryo. The supplementation of media with IGF1 improves the development of bovine embryos (Wang et al., 2009). Moreover, IGF1 is found to work as a protective factor against apoptosis in developing human embryos (Spanos et al., 2000). Experiments by Oropeza et al. (2004) demonstrated that intraovarian injection of IGF1 increased the numbers of blastocysts from oocytes derived from prepubertal heifers to levels matching those generated from oocytes collected from mature heifers. Higher expression of *IGF1R* by embryos derived from pubertal heifers in comparison to those derived from prepubertal ones may result in their better development, even though the culture conditions of both groups of embryos are the same.

Both *PPAR* isoforms and blastocyst quality marker genes were co-expressed in embryos from both groups. Studying the possible associations between the expression of these genes and *PPARs* is beyond the scope of this experiment. However, evidence from research in sheep shows that *IFN τ* supports the secretion of *PTGS2*, which is needed for the production of *PGI2* and *PGJ2*, both of which activate *PPAR γ* (Dorniak et al., 2011). Expression of *PPARs*, including *PPAR γ* isoform, which may be activated by *PGI2*, was confirmed in pre-implantation bovine and ovine embryos (Huang, 2008). Additionally, the involvement of *PPAR γ* in regulation of development and differentiation of the growing trophoblast has been acknowledged (Brooks et al., 2014). Nevertheless, whether *IFN τ* and *PPAR γ* indeed cooperate in order to support embryonic development requires further investigation. A similar possibility comes from studies on the involvement of *PLAC8* in the development of cancer (Mao et al., 2021). Recent results show that upregulated expression of the inhibitor of DNA binding 1 (*Id1*) promotes expression of *PLAC8* in colorectal cancer and might maintain stemness of the cancer cells through the *Id1*-c-Myc-*PLAC8* axis via the Wnt/ β -catenin pathway (Sun et al., 2019). The Wnt/ β -catenin pathway operates in early-stage embryos (Sidrat et al., 2021). Furthermore, its activation increases the expression of *PPAR δ* and in turn the expression of genes related to embryonic development (Sidrat et al., 2020, 2021). If *PLAC8* may be involved in *PPAR δ* mechanisms of action through the Wnt/ β -catenin pathway remains unknown, but is a feasible possibility.

Clearly, our results demonstrate that transcripts of chosen marker genes as well as *PPAR γ* and δ , which regulate a variety of crucial cellular functions, differ between embryos derived from pubertal versus prepubertal heifers. Knowledge of mechanisms which differ in the blastocysts derived from prepubertal and adult animals is already being utilized to work on methods which might compensate for these differences, such as intraovarian injection of *IGF1* in prepubertal heifers (Oropeza et al., 2004) or supplementation with melatonin of the cultured COCs (Gutiérrez-Añez et al., 2021). We propose that, next to genomic selection of the desired production and/or wellness traits of the embryo quality markers, other useful predictors might be used to determine successful pregnancies.

Conclusions

The need for shortening the bovine generation interval, as well as the production of higher numbers of genetically valuable animals, requires the collection of oocytes from immature heifers. However, blastocyst rate as well as subsequent pregnancy success ratio varies between transfers of embryos derived from oocytes collected from prepubertal versus pubertal heifers. Here, we showed that the expression of *PPAR γ* and *PPAR δ* , which belong to the group of nuclear receptors involved in basic cellular functions such as metabolism, as well as embryo quality marker genes i.e., *PLAC8*, *IFN τ* , *OCT4*, *SOX2*, *IGFIR*, differ between day 7 blastocysts derived from

oocytes retrieved from prepubertal and pubertal heifers. The obtained data might be utilized in further research on optimization of the culture conditions of embryos and oocytes collected from immature bovine donors.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

JJ wrote the paper. AN performed OPU. IK-Z was involved in OPU, and *in vitro* embryo production, DB was involved in *in vitro* embryo production, AS performed the RT-qPCR. MT took part in the *in vitro* embryo production, KŁ contributed to the writing of the paper; IW-P the main designer of the study, performer of OPU, took part in the *in vitro* embryo production, contributed to writing of the paper.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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