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Differential expression of insulin-like growth factor family members in immature cumulus–oocyte complexes from dairy cows with different genotypes

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Contents

It has been evident the improvement of in vitro embryo production (IVEP) in dairy cows. Nevertheless, it is known that differences in the number and quality of oocytes between taurine and zebu females impact the efficiency and economic viability of IVEP. As the insulin-like growth factor (IGF) system is related to follicular and oocyte development, we aimed to quantify mRNA abundance of IGF system members and pregnancy-associated plasma protein-A (*PAPPA*) in the cumulus–oocyte complexes (COCs) of Gir, 1/2 Holstein × 1/2 Gir and Holstein cows. Four pools of 30 immature COCs from Gir, 1/2 Holstein × 1/2 Gir and Holstein cows were obtained by ovum pickup (OPU), and the oocytes and cumulus cells (CC) were mechanically separated and stored at −80°C. Total RNA was extracted from pools of 30 oocytes and their respective CC. Expression of target genes was assessed by real-time RT-PCR. In oocytes, the abundance of *IGFR1* mRNA was higher ($p < .05$) in Gir cows compared with the other breeds. In contrast, in CC, mRNA encoding *IGF2* ($p < .05$), *IGFR2* ($p < .05$) and *IGFBP4* ($p < .01$) was higher in Holstein donors compared with Gir and 1/2 Holstein × 1/2 Gir cows. Additionally, the abundance of *PAPPA* mRNA was higher in oocytes ($p < .001$) and CC ($p < .01$) in Gir and 1/2 Holstein × 1/2 Gir cows compared with the Holstein donors. In conclusion, the higher abundance of *PAPPA* mRNA in the oocytes and CC from Gir and cross-breed donors combined with the low expression of *IGFBP4* in the CC suggests an enhancement of the bioavailability of IGF-free when compared with Holstein COCs.

1 | INTRODUCTION

Although a proportion of the Brazilian dairy herd is composed of animals of taurine breeds (*Bos taurus*), such as Holstein and Jersey cows, dairy production is mainly contributed by cross-breed cows. In this context, breeds of cattle originating from crosses between zebu (*Bos indicus*), mainly Gir (a zebu breed), and Holsteins are predominant. The favourable effects of heterosis and complementarity of the breeds guarantee milk production and greater adaptability (Madalena, Peixoto, & Gibson, 2012).

There has been a progressive increase in the use of in vitro embryo production (IVEP) in dairy breeds, including Holstein, Gir and 1/2 Holstein × 1/2 Gir cross-breed cows (Viana, Siqueira, Palhão, & Camargo, 2012). In the last 5 years, the most rapid progress in production of embryos using the IVEP technique has been observed in the Girolando breed. Girolando is a synthetic breed developed by crossing of the Gir and Holstein breeds and is the only cross-bred with significant participation in IVEP activity (Sartori et al., 2016; Viana et al., 2012).

One of the major challenges for most dairy farmers with cross-bred cattle is the difficulty of maintaining a herd at the desired breeding

purity level. Therefore, the use of biotechniques such as IVEP is an alternative for the establishment and maintenance of herds with the aim of using cross-breed females for milk production. Indeed, there is an ongoing push for the use of IVEP on a large scale to produce 1/2 Holstein × 1/2 Gir female calves (Pontes et al., 2010). The resulting first-generation crosses (F_1) can also be used as oocyte donors to produce other crosses, such as 3/4 and 1/4 (Viana et al., 2012).

It is known that differences in the number of follicles obtained by ovum pickup (OPU) and the oocyte competence of taurine and zebu females have a significant effect on the efficiency and economic viability of IVEP. Indeed, in Gir donors, a higher number of oocytes are recovered by aspiration and there is a higher cleavage rate and blastocyst production, based on the number of oocytes recovered, when compared with Holstein donors (Camargo et al., 2007; Pontes et al., 2010; Sales et al., 2015). Interestingly, the number of oocytes recovered and the embryos produced by 1/2 Holstein × 1/2 Gir females is similar to, or better than, those for Gir donors (Pontes et al., 2010).

Among the factors identified as regulators of follicular and oocyte development are the insulin-like growth factors (IGFs). In cattle, the mRNAs encoding *IGF1* and *IGF2* and their cognate receptors (*IGFR1* and *IGFR2*) have been described in cumulus–oocyte complexes (COCs) (Nuttinck et al., 2004; Yoshida, Miyamura, Hamano, & Yoshida, 1998) and embryos (Lonergan et al., 2000, 2003). Studies have also shown that addition of IGF1 during in vitro maturation (IVM) in bovines results in an increase in cumulus cell expansion, an improvement in nuclear maturation and a reduction in oocyte apoptosis rates (Sakaguchi et al., 2002; Wasielak & Bogacki, 2007; reviewed by Velazquez, Zaraza, Oropeza, Webb, & Niemann, 2009). In addition, supplementation of the in vitro culture medium with IGF1 or IGF2 increases the number of morulae and blastocysts and reduces the incidence of embryonic apoptosis in bovines (Byrne, Southgate, Brison, & Leese, 2002; Bonilla et al., 2011; Sirisathien, Hernandez-Fonseca, & Brackett, 2003; Sirisathien & Brackett, 2003; reviewed by Paula-Lopes, Lima, Satrapa, & Barros, 2013).

The transport and function of IGFs are modulated by interaction with at least six IGF-binding proteins (IGFBPs 1–6), which are present in bovine oocytes (Satrapa et al., 2013) and embryos (Winger et al., 1997; Sawai et al., 2005). The increase in IGFBP expression is related to a lower bioavailability of IGF-free, whereas the degradation of IGFBP-2, IGFBP-4 and IGFBP-5 through the proteolytic action of pregnancy-associated plasma protein-A (PAPPA) increases the IGF bioavailability (Fortune, Rivera, & Yang, 2004; Monget et al., 2003).

Previous studies have demonstrated the importance of the IGF family in folliculogenesis, including growth and follicular deviation (Fortune et al., 2004; Itoh, Kacchi, Abe, Sendai, & Hoshi, 2002; Zhao, Taverne, van der Weijden, Bevers, & van den Hurk, 2001). At the time of deviation, the dominant follicle produces high concentrations of oestradiol and shows increased synthesis of PAPPA and, subsequently, lower concentrations of IGFBP4 and 5 (Fortune et al., 2004; Rivera & Fortune, 2001). The low concentrations of IGFBP4 and 5 lead to a higher amount of free IGF1, which aids in follicular growth and enhances the effects of follicle-stimulating hormone (FSH) and the synthesis of oestradiol (Fortune et al., 2004).

Given the remarkable importance of the IGF family members in follicular and oocyte development and the differences in reproductive performance among breeds, the aim of this study was to quantify the mRNA abundance of IGF family members (*IGF1*, *IGF2*, *IGFR1*, *IGFR2*, *IGFBP2* and *IGFBP4*) and *PAPPA* in oocytes and cumulus cells obtained by OPU from Gir (*B. indicus*), 1/2 Holstein × 1/2 Gir (*indicus taurus*) and Holstein (*B. taurus*) donor cows.

2 | MATERIAL AND METHODS

2.1 | Experimental design

To assess the differential mRNA abundance of IGF family members and PAPPA in oocytes and cumulus cells, three different dairy donor breeds were used to obtain immature COCs, which were recovered by ultrasound-guided follicular aspiration. For this, cumulus cells and oocytes from the immature COCs were separated and four pools of 30 oocytes and their corresponding cumulus cells were stored for each experimental group: Gir (GIR), 1/2 Holstein × 1/2 Gir (HOL × GIR) and Holstein (HOL) groups.

Follicular aspiration was performed at a farm in the southern region of the state of Minas Gerais, Brazil, which has a humid temperate climate, dry winter and temperate summer (Alvares et al., 2013). None of the donors were submitted to any type of hormonal stimulation prior to follicular aspiration. Non-lactating pluriparous cows of the Gir (n = 12), 1/2 Holstein × 1/2 Gir (n = 11) and Holstein (n = 16) breeds with a body condition score ranging from 3.0 to 4.0 (1 to 5 scale; Edmonson, Lean, Weaver, Farver, & Webster, 1989) were used.

All cows were cycling regularly and were free of clinical and reproductive abnormalities. During the experimental period, the cows were maintained on pasture (*Brachiaria* spp.) and supplemented with corn silage and 20% protein concentrate, with ad libitum access to water and mineral salts.

The study was approved by the Animal Ethics Commission of the University of José Rosário Vellano, Brazil (Protocol 41A/2015).

2.2 | Follicular aspiration and recovery of cumulus–oocyte complexes

The follicular aspiration procedure (OPU) was conducted as previously described by Bols, Ysebaert, van Soom, and de Kruif (1997). Briefly, after caudal epidural anaesthesia (5 ml of 1% lidocaine; Hertape, Juatuba, MG, Brazil), follicular aspiration was performed using a portable ultrasound device and a biopsy guide with a 7.5-Mhz intravaginal sectorial transducer (Mindray Medical International Ltd, Shenzhen, China).

Follicles with a diameter greater than 3 mm were identified and punctured using 20-G needles and a vacuum pressure of 80 mmHg, which corresponds to a flow of water of 11 ml/min. The follicular fluid was recovered in 50-ml plastic tubes containing 15 ml of modified Dulbecco's PBS medium (DPBS; Nutricell, Campinas, SP, Brazil), supplemented with 125 UI/ml of heparin (Liquemine; Roche Lab., São Paulo, SP, Brazil) and heated to 37°C.

TABLE 1 Information of specific primers used for amplification in real-time polymerase chain reaction

Target	Sequence	Fragment size (bp)	Primer concentration (nM)	Annealing/extension temperature (°C)	Reference
IGF1	F 5′- ACCCTGGAGTTGGTAGATTGCTGT-3′ R 5′- CACCCATGCATTTGTGGCTCTTGA-3′	108	300	60	AH009378.2
IGF2	F 5′- GACCGCGGCTTCTACTTCAG-3′ R 5′-AAGAACTTGCCCACGGGGTAT-3′	162	300	60	NM_174087.3
IGFR1	F 5′-TTGCAAGAACCATGCCTGCAGAAG-3′ R 5′-TGGCATTCTCAGGTTCTGGCCATT-3′	110	300	60	XM_005907389.2
IGFR2	F 5′-TGCGGTGGTGGCCAGAAGATAATA-3′ R 5′-TCAAACTCGTAGAAGCAGCCGTCA-3′	115	200	62	NM_174352.2
IGFBP2	F 5′-CAGCGGCAGATGGGCAA-3′ R 5′-GAAGGCGCATGGTGGAGAT-3′	122	300	60	NM_174555.1
IGFBP4	F 5′-TGTGTGCGTGTGTGTTAATGAGCC-3′ R 5′-TGGGAAACATACCAGGGCTCTCCT-3′	105	200	62	NM_174557.4
PAPP A	F 5′- TCCAGATGTTGAGCAGCCCTGTAA-3′ R 5′-ACCCAAACGGTCAAAGACTCAGGA-3′	161	300	60	AF421141.1
CYCA	F 5′- GCCATGGAGCGCTTTGG-3′ R 5′-CCACAGTCAGCAATGGTGATCT-3′	65	300	60	NM_178320

F, forward primer; R, reverse primer.

After puncturing, the COCs were separated using an 80-μm-mesh collection filter for embryos (Millipore, São Paulo, SP, Brazil) and morphologically classified according to Viana, Palhão, Siqueira, Fonseca, and Camargo (2010). Only COCs classified as Grade 1 (COC compact, with more than three layers of cumulus cells and an oocyte with homogeneous cytoplasm) and Viable (COC with at least one layer of cumulus cells and an oocyte with homogeneous or slightly heterogeneous cytoplasm, suitable for IVEP) were used in this study.

Cumulus cells and oocytes from immature COCs were mechanically separated by repeated pipetting in PBS. Denuded oocytes were recovered and washed three times in PBS. Cumulus cells were transferred to a 1.5-ml tube and centrifuged twice for 5 min at 700 × *g*. The supernatants were discarded, and 350 ml of RNA extraction lysis buffer was added to the cell pellets. The cell suspension and oocytes were stored at −80°C until RNA extraction.

2.3 | Transcript level evaluation

Total RNA was extracted from pools of 30 oocytes and their corresponding cumulus cells using the RNeasy® Micro kit (Qiagen, Mississauga, ON, Canada), according to the manufacturer's instructions, and eluted in 30 μl of RNAse-free water. As cumulus cell number may vary between samples, the total RNA concentration of the cumulus cells was measured using a NanoDrop® ND 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Total RNA (100 ng for cumulus cells and all the RNA for the 30 oocyte pools) was incubated with DNase (1 U/μg; Invitrogen, São Paulo, SP, Brazil) to eliminate possible contamination with genomic DNA and then subjected to reverse transcription (RT) using random primers and the High Capacity cDNA Reverse Transcription Kit® (Applied Biosystems, São Paulo, SP, Brazil), according to the manufacturer's instructions. The reagents were incubated at 25°C for 10 min, 37°C for 120 min and finally 85°C for 5 min to inactivate the enzyme.

Expression of the target genes (*IGF1*, *IGF1*, *IGFR2*, *IGFBP2*, *IGFBP4* and *PAPPA*) in oocytes and cumulus cells was investigated by real-time polymerase chain reaction (PCR) with an ABI 7500 thermocycler using Power Sybr Green PCR Master Mix (Applied Biosystems). Reactions were performed in a final volume of 25 μl, and the genes were amplified using the following conditions: 95°C for 10 min (1 cycle), denaturation at 95°C for 10 s and annealing and extension for 1 min (40 cycles). The primer sequences, fragment sizes, and annealing and extension temperatures for each target gene are shown in Table 1.

The reactions were optimized to provide maximum amplification efficiency for each gene. Each sample was analysed in duplicate, and the efficiency and specificity of the oligonucleotide primers of the genes were evaluated by the amplification and dissociation curves, respectively.

The genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), peptidylprolyl isomerase A (*PPIA*) and histone H2AFZ (*H2AFZ*) were examined using the geNorm program (Microsoft®; Vandesompele et al., 2002) to select the most stable endogenous control for each cell type analysed. The results indicated that *PPIA* was the best reference gene for oocytes and cumulus cells.

The relative abundance of each target gene was calculated using the ΔΔCt method with efficiency correction and a control sample for calibration (Pfaffl, 2001). The average efficiency values for each gene were calculated through the amplification profile of each sample using the LinRegPCR program (Ramakers, Ruijter, Deprez, & Moorman, 2003).

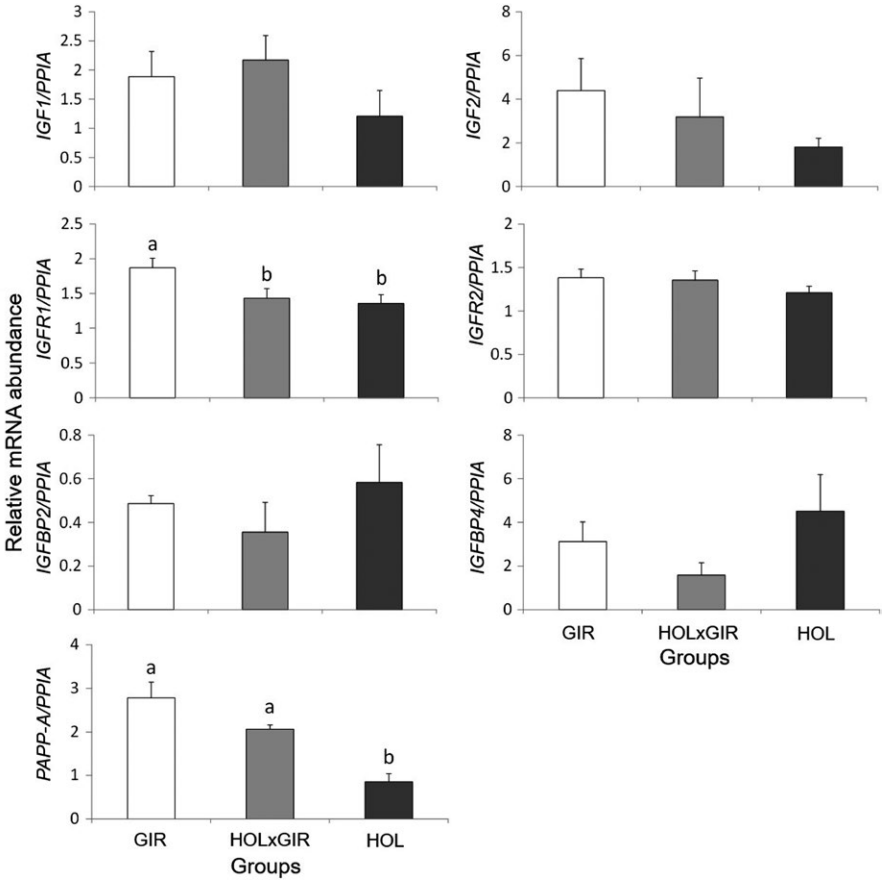

FIGURE 1 Abundance of *IGF1*, *IGF2*, *IGFR1*, *IGFR2*, *IGFBP2*, *IGFBP4* and *PAPPA* mRNAs in the immature oocytes of Gir (GIR; white bars), 1/2 Holstein × 1/2 Gir (HOL × GIR; grey bars) and Holstein (HOL; black bars) cows. The abundance of mRNA was measured by real-time polymerase chain reaction. Data are presented as mean values (±S.E.M.) relative to a calibrator sample and were calculated using the ΔΔCt method and relative to a housekeeping gene (peptidylprolyl isomerase A [*PPIA*]). Bars with different letters are significantly different ($p < .05$). Data were derived from four replicates for each experimental group

2.4 | Statistical analysis

The effects of different donor breeds on the mRNA abundance of target genes in oocytes and cumulus cells were tested by ANOVA, and the means of relative mRNA abundance were compared using the Tukey–Kramer HSD test.

The mRNA abundance data showing a non-normal distribution were transformed to a logarithmic scale. When the values did not have a normal distribution even after log-transformation, a nonparametric test was used (Kruskal–Wallis).

All statistical analyses were performed using the JMP program (SAS Institute, version 7.0). The results are presented as the mean ± standard error of the mean (S.E.M.). Differences with $p < .05$ were considered significant.

3 | RESULTS

There were no differences among breeds in the abundances of oocyte *IGF1*, *IGF2*, *IGFR2*, *IGFBP2* and *IGFBP4* mRNAs. However, the relative abundance of *IGFR1* mRNA was higher in oocytes recovered from Gir donors. Furthermore, *PAPPA* mRNA abundance was higher in oocytes from Gir and 1/2 Holstein × 1/2 Gir donors compared with Holstein donors (Figure 1).

A higher abundance of *IGF2*, *IGFR2* and *IGFBP4* mRNAs was observed in the cumulus cells of Holstein cows compared with Gir and 1/2 Holstein × 1/2 Gir cows. In addition, the abundance of *PAPPA* mRNA was higher in the cumulus cells from Gir and 1/2 Holstein × 1/2 Gir donors compared with Holstein donors. There was, however, no difference in the mRNA abundance of *IGF1*, *IGFR1* and *IGFBP2* genes in the cumulus cells of the different breeds (Figure 2).

4 | DISCUSSION

The present study compared the transcriptional profiles of IGF family members in COCs obtained by OPU in Gir (*B. indicus*), Holstein (*B. taurus*) and cross-bred (1/2 Holstein × 1/2 Gir, *B. indicus/taurus*) dairy cows. Several authors have demonstrated that the IGF family plays an important role in oocyte maturation (Lorenzo, Illera, Illera, & Illera, 1994; Sirotkin, Dukesová, Makarevich, Kubek, & Bulla, 2000; Walters, Binnie, Campbell, Armstrong, & Telfer, 2006). The members of the IGF family expressed in oocytes accelerate nuclear maturation of the oocyte and promote increased oocyte competence for embryonic development (Lorenzo et al., 1994; Sakaguchi et al., 2002; Sirotkin et al., 2000; Walters et al., 2006).

In immature oocytes, there was a higher abundance of *IGFR1* mRNA in Gir donors compared to 1/2 Holstein × 1/2 Gir and Holstein donors, whereas *PAPPA* mRNA abundance was higher in oocytes from Gir and 1/2 Holstein × 1/2 Gir donors compared to Holstein donors. Similarly, Sales et al., 2015; evaluating genes related to cellular metabolism in immature oocytes, showed a higher abundance of *IGFR1*

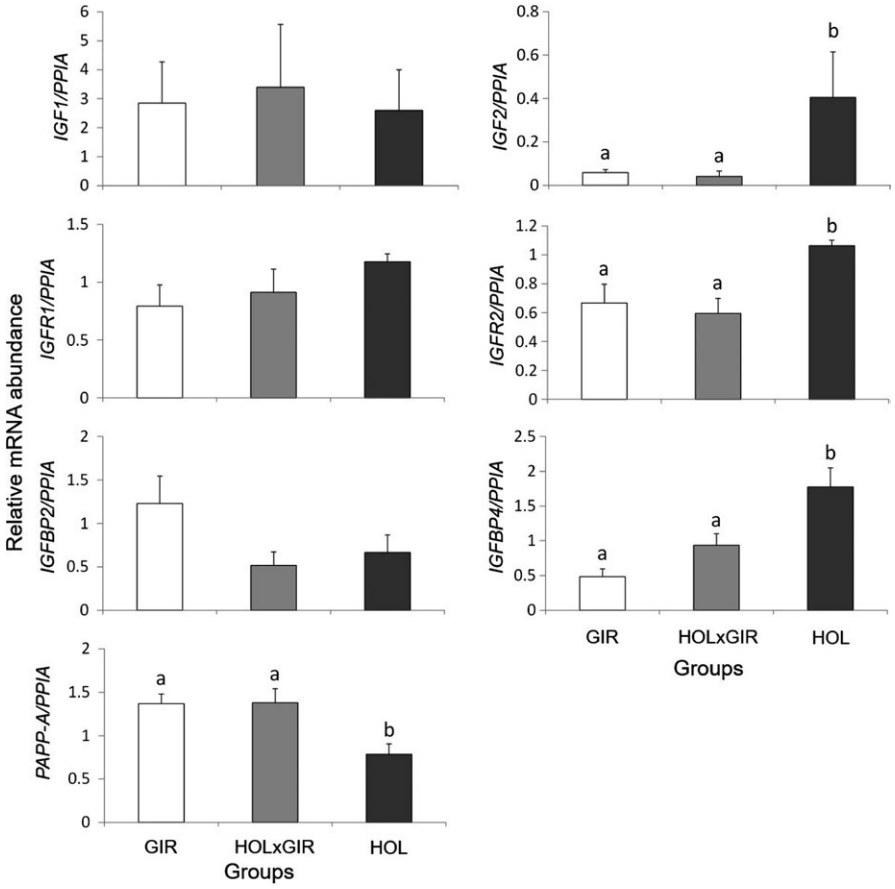

FIGURE 2 Abundance of *IGF1*, *IGF2*, *IGFR1*, *IGFR2*, *IGFBP2*, *IGFBP4* and *PAPPA* mRNAs in the immature cumulus cells of Gir (GIR; white bars), 1/2 Holstein × 1/2 Gir (HOL × GIR; grey bars) and Holstein (HOL; black bars) cows. The abundance of mRNA was measured by real-time polymerase chain reaction. Data are presented as mean values (±S.E.M.) relative to a calibrator sample and were calculated using the ΔΔCt method and relative to a housekeeping gene (peptidylprolyl isomerase A [*PPIA*]). Bars with different letters are significantly different ($p < .05$). Data were derived from four replicates for each experimental group

mRNA in Gir cows compared to Holstein. Furthermore, Satrapa et al. (2013), who compared the IGF family transcript profiles in immature oocytes from *B. indicus* (Nelore) and *B. taurus* (Holstein) cows, observed a higher abundance of *PAPPA* mRNA in the oocytes of Nelore cows compared to Holstein animals. As PAPPA is responsible for the degradation of IGFBPs (Monget et al., 2003), Satrapa et al. (2013) suggest that these results indicate a greater bioavailability of free IGF in Nelore oocytes, which may be a factor contributing to the superior competence of the oocytes of this breed compared with the Holstein breed. Interestingly, Nyegaard et al. (2010) demonstrated that the loss of a functional *Pappa* gene reduces fertility potential, based on observations in PAPPA knockout mice, which have lower litter size and lower numbers of COCs ovulated upon gonadotropin stimulation. These data indicate a possible role of PAPPA in oocyte competence and female fertility.

Satrapa et al. (2013) also demonstrated that mRNA abundance of *IGF1*, *IGF2*, their *IGFR1* and *IGFR2* receptors, and *IGFBP2* and *IGFBP4* binding proteins was higher in the immature oocytes of Holstein cows compared with the Nelore breed. However, in the present study, using immature oocytes from *B. indicus* cows of the Gir breed and *B. indicus/taurus* cross-breed cows, no differences were observed in the expression of *IGF1*, *IGF2*, *IGFR2*, *IGFBP2* and *IGFBP4* mRNA compared with the Holstein donors. This discrepancy may be due to the fact that different *B. indicus* breeds were used.

It is important to note that a key role in the process of development and quality of oocytes is played by adjacent somatic cells, the cumulus cells. Such cells are specialized in providing nutritional support to the oocyte, thereby controlling its growth and metabolism (Haghighat & Van Winkle, 1990). This is mediated via gap junctions, which provide a favourable follicular microenvironment, and premature loss of this communication impairs the progression of oocyte maturation and subsequent embryonic development (Lolicato et al., 2015; Wigglesworth et al., 2013). Therefore, in the present study, we investigated the mRNA expression pattern of members of the IGF family, not only in oocytes but also in cumulus cells, which are able to produce IGFs and express their receptors. It is worth mentioning that no previous studies, to our knowledge, have compared the expression patterns of IGF family members in cumulus cells of *B. indicus* and *B. taurus*.

The relative abundance of *IGF2*, *IGFR2* and *IGFBP4* mRNAs was higher in the cumulus cells of Holstein cows compared to Gir and 1/2 Holstein × 1/2 Gir cows. However, *PAPPA* mRNA abundance was higher in cumulus cells from Gir and 1/2 Holstein × 1/2 Gir donors compared to Holstein donors. These results are similar to those obtained for immature oocytes by Satrapa et al. (2013) when evaluating IGF gene expression in Holstein and Nelore cows. Although the abundance of *IGF2* and *IGFR2* mRNAs was higher in the cumulus cells of Holstein cows, these animals had lower *PAPPA* expression and higher *IGFBP4* abundance. It is possible that a lower bioavailability of IGFs in COCs, due to a lower degradation of IGFBPs by PAPPA, may have been one of the factors leading to the lower oocyte quality and embryonic development normally observed in Holstein animals compared with Gir and *B. taurus/indicus* cross-breed animals (Pontes et al., 2010; Sales et al.,

2015). Indeed, in women, high concentrations of IGFBP1 and 4, which are associated with low IGF1 levels in the follicular fluid, are correlated with reduced embryonic development in vitro (Wang et al., 2006).

It is well established that the phosphatidylinositol-3-kinase/serine-threonine kinase (PI3K/Akt) signalling pathway is activated to mediate the action of IGF1 and IGF2 in different cell types (Laviola, Natalicchio, & Giorgino, 2007; Mu et al., 2015). A recent study by Khan et al. (2017) demonstrated that induction of the PI3K pathway plays a positive role in in vitro blastocyst development and increases their resistance under heat stress conditions. Akt is a serine/threonine-specific kinase that regulates a wide variety of substrates to alter gene transcription, modulate the usage of cellular energy and inhibit the activity of pro-apoptotic proteins (Manning & Cantley, 2007). Indeed, the addition of IGF1 during IVM in bovines has been shown to reduce the rate of apoptosis in oocytes (Wasielak & Bogacki, 2007). Furthermore, when IGF1 or IGF2 supplementation was performed in the culture medium in vitro, there was a reduction in the incidence of embryonic apoptosis (Bonilla et al., 2011; Sirisathien & Brackett, 2003).

In conclusion, the higher abundance of *PAPPA* mRNA in cumulus oocyte complexes from Gir and cross-breed donors suggests a more efficient degradation of IGFBPs, which together with a lower abundance of *IGFBP4* mRNA in cumulus cells, results in greater bioavailability of IGF-free in COCs, and may contribute to the improvement of oocyte competence when compared with Holstein dairy herds.

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CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

Ovum pickup sessions were performed by CAC Fernandes and MP Palhão and assisted by ES Caixeta and AC Lopes. Gene expression assays were performed by ES Caixeta and supported by ACS Castilho. Data were analysed by a group effort, with notable contributions by ES Caixeta and MJ Sudano. Preparation of the manuscript was also a collective undertaking by ES Caixeta, ACS Castilho and MJ Sudano, with subsequent revisions by the entire group.

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