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Interleukin-4 Supplementation During In Vitro Culture Modulates the Expression of Stress- and Immune-Related Genes During Porcine Blastocyst Development

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ABSTRACT

The aim of this study was to evaluate the effect of supplementing in vitro culture medium of porcine zygotes with interleukin-4 (IL-4) on their developmental competence and quality. Cumulus-oocyte complexes (COCs) were matured and fertilized in vitro. The presumptive zygotes were then cultured in vitro for 7 days with different concentrations of IL-4 (25 pg/mL, 100 pg/mL, 25 ng/mL and 100 ng/mL) or without IL-4 (control). Cleavage and blastocyst formation rates were evaluated on days 2 and 7 of culture, respectively. On day 7 of IVC, some control and 25 pg/mL blastocysts were used to assess the expression of a panel of genes related to embryo quality by real-time quantitative PCR (RT-qPCR). The results did not show any positive effect of IL-4 on embryo developmental competence, regardless of its concentration. RT-qPCR revealed a significant downregulation of genes involved in cell cycle regulation (*PCNA*), DNA damage response and immune pathways (*STAT6* and *GATA3*) in blastocysts treated with IL-4. In conclusion, supplementation of IVC medium with IL-4 did not improve the developmental competence of IVP embryos; however, IL-4 modulated the expression of stress- and immune-related genes in blastocysts.

1 | Introduction

The in vitro production (IVP) of pig embryos remains inefficient, yielding blastocysts of lower quality than those derived in vivo (Martinez et al. 2022). Refinement of the in vitro culture (IVC) media continues to be essential to obtain embryos suitable for application in gene editing or embryo transfer (Chen et al. 2022). In this context, there is a growing trend of enhancing culture media by cytokine supplementation (Garcia-Canovas et al. 2024), molecules playing crucial roles for reproduction functions in mammals. Interleukin-4 (IL-4) is an anti-inflammatory cytokine secreted by different cell types and is detectable during pregnancy in multiple species (Lin et al. 1993; Vélez et al. 2019). Further, Okuyama et al. (2024) observed that inseminated sows showing delayed peaks of IL-4 expression failed to conceive. These findings suggest a key role of IL-4 in reproductive processes in pigs, including maternal recognition, embryo implantation and the maintenance of pregnancy. However, studies evaluating whether IL-4 supplementation in IVP media improves embryonic development potential and embryo quality are lacking. The present study aimed to evaluate the developmental competence of

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in vitro-fertilized porcine oocytes in the presence of IL-4, as well as its impact on the expression of a panel of genes related to embryo quality.

2 | Materials and Methods

2.1 | In Vitro Production of Embryos and IVF Parameters Assessment

The in vitro maturation (IVM) of cumulus-oocyte complexes, in vitro fertilization (IVF), and IVC were performed as previously described by Garcia-Canovas et al. (2025). Putative zygotes were cultured sequentially in vitro in a chemically defined IVC medium for 7 days. The fertilization parameters evaluated 18 h after IVF included rates of maturation (proportion of matured and penetrated oocytes among the total oocytes), penetration (ratio of penetrated oocytes to matured) and monospermy (ratio of monospermic zygotes to the number of penetrated oocytes). The IVF overall efficiency was calculated as the ratio of monospermic to the total number of inseminated oocytes.

2.2 | Embryonic Development Assessment

Cleavage rate (2–4 cell embryos relative to inseminated oocytes) was evaluated on day 2, whereas blastocyst formation rate (ratio of total blastocysts to cleaved embryos), blastocyst efficiency (ratio of blastocysts to total inseminated oocytes) and hatching rate (ratio of total hatched and hatching to the total of blastocysts) were assessed on day 7.

2.3 | Quantitative Real-Time PCR Analysis

The sample preparation, primers design and real-time quantitative polymerase chain reaction (RT-qPCR) analysis were made as previously described in Cambra et al. (2020). The RT-qPCR reactions were performed on a QuantStudioTM 5 system using iTaqTM Universal SYBR Green Supermix. Additional samples were used to assess each primer set amplification efficiency. The relative expression of each gene was calculated using the $\Delta\Delta C_t$ method with efficiency correction (Pfaffl 2001). The sequences of the primers are shown in Table 1, being *RPL19* and *PPIA* as the housekeeping genes.

2.4 | Experimental Design

The IVC medium was supplemented with different IL-4 concentrations: 25 pg/mL ($N=354$), 100 pg/mL ($N=358$), 25 ng/mL ($N=366$) and 100 ng/mL ($N=369$). The control group was unsupplemented ($N=454$). The experiment was performed in 4 replicates. As no differences in embryo developmental potential were observed among IL-4 concentrations and the control group, gene expression analysis was performed using blastocysts from

TABLE 1 | Primers sequences used for real-time quantitative PCR analysis (RT-qPCR).

Gene symbol	Accession number	Primer sequences (5′-3′)		Size (pb)
PPIA[a]	XM_021078519.1	Forward	CTGAAGCATACGGGTCCTGG	100
		Reverse	CCAACCACTCAGTCTTGGCA	
RPL19[a]	XM_003131509	Forward	AGCCTGTGACTGTCCATTCC	95
		Reverse	AGTACCCTTCCGCTTACCGA	
GATA3	NM_001044567.1	Forward	TACGTCCCCGAATACAGCTC	227
		Reverse	GGTCGATTCTGTCCGTTCAT	
LIF	AY585336.1	Forward	GCCAACGCCCTCTTTATTCT	222
		Reverse	GTTCACAGCACCAGGATTGA	
PCNA	NM_001291925.1	Forward	CTCGTCTCATGTCTCTTTGGTG	179
		Reverse	GTGCCAAGGTGTCTGCATTA	
SOD1	NM_001190422.1	Forward	GGATCAAGAGAGGCACGTTG	159
		Reverse	CTGCCCAAGTCATCTGGTTT	
STAT6	FJ972620.1	Forward	GGGGTTTACTCCCAGAGCAC	153
		Reverse	ACCACTGCCAAAAGGTGAAG	
TGFβ1	NM_214015.2	Forward	GGACTACTACGCCAAGGAGGT	159
		Reverse	TCTGCCCGAGAGAGCAATAC	
TP-53	NM_213824.3	Forward	CTTCGAGATGTTCCGAGAGC	106
		Reverse	CTTAGACTTCAGGTGGCTGGA	

[a]Housekeeping genes.

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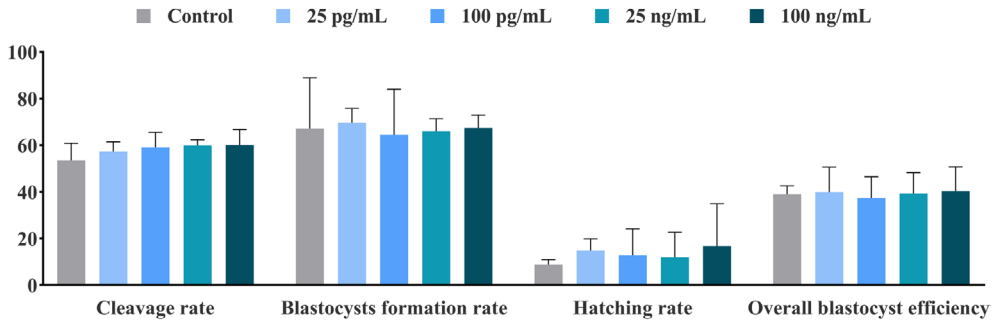


FIGURE 1 | Effects of supplementing in vitro culture medium with different concentrations of IL-4 on embryonic development after 2 and 7 days. Data are presented as means ± SD.

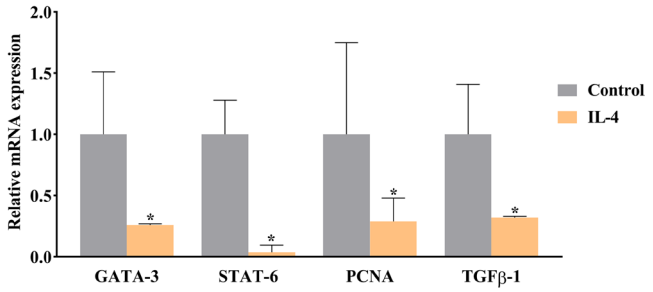


FIGURE 2 | Effects of supplementation with 25 pg/mL of IL-4 during IVC on the transcriptome profile of porcine blastocysts. Data are presented as means ± SD. *$p < 0.05$.

the control and 25 pg/mL groups. Three biological replicates, each consisting of a pool of seven blastocysts, were analysed per group.

2.5 | Statistical Analysis

Data were analysed using program IBM SPSS 28.0 (IBM, Chicago, IL, EEUU). Variables were calculated with percentage in each well of each experimental group in each replicate (4 replicates). Normality was evaluated by the Kolmogorov–Smirnov test. Groups were compared using non-parametric statistics by the Kruskal–Wallis test with the Bonferroni post hoc test. All data are presented as means ± standard deviation (SD). Significant differences were considered when p-value < 0.05.

3 | Results

The IVF parameters were similar to those previously obtained in our laboratory (87.6% ± 6.2%, 75.3% ± 14.0%, 53.9% ± 15.2% and 34.7% ± 9.5%, respectively). Regardless of the concentration used, IL-4 did not affect IVC outcomes (Figure 1).

RT-qPCR data showed a significant ($p < 0.05$) downregulation of *PCNA*, *TGFβ1*, *GATA3*, *STAT6* genes (Figure 2) in blastocysts from IL-4 (25 pg/mL) compared to control blastocysts. Although no significant differences were observed for *SOD1*, *TP-53* and *LIF* expression levels, there was a trend ($p = 0.13$, 0.14 and 0.13; respectively) toward downregulation of these in the IL-4 blastocysts.

4 | Discussion

To the best of our knowledge, this is the first study to evaluate the effects of IL-4 supplementation during IVC on embryonic development of porcine oocytes fertilized in vitro and its effects on the blastocyst transcriptome. The concentrations used in this experiment were selected based on the concentrations reported previously by Parrilla et al. (2020), which described IL-4 levels in ranges of pg/mL. Additionally, higher concentrations (ng/mL) were tested to ensure the potential effects beyond the reported range were not overlooked.

In the present study, none of the concentrations of IL-4 affected embryonic development. To date, no studies have evaluated the effects of supplementing with IL-4 the IVP media of mammals to compare. Only one previous work has evaluated the effects of IL-4 supplementation on human endometrial stromal cells in vitro, and reported that its addition increased the production of IL-6 (Nasu et al. 2001), associated with the maintenance of pregnancy and the improvement of embryonic developmental competence (Yoo et al. 2017). In this context, we previously reported that IL-6 supplementation during the IVC of in vitro-fertilized porcine oocytes had no effects on embryonic development or quality (Garcia-Canovas et al. 2025), whereas a significant increase in embryonic development was observed when added during the IVM of porcine oocytes. Consistently, in our laboratory, supplementation with low concentrations of IL-4 during IVM has also been associated with improved embryonic development of porcine oocytes fertilized in vitro (unpublished data). Together, these findings support the hypothesis that the potential effects of cytokines on the embryonic development may occur primarily when interleukins are added during IVM, likely through interactions with membrane receptors on cumulus-cells, which play a key role in oocyte maturation (Yuan et al. 2017).

Our findings indicate that IL-4 supplementation during IVC modulates the transcriptome of porcine blastocysts, downregulating the expression of genes involved in cell cycle regulation and DNA damage response (*TP-53*, *PCNA*), and key immune pathways (*TGFβ1*, *GATA3* and *STAT6*). Decreased expression of *STAT6* and *GATA3* suggests reduced activation of immune signalling. This response indicates that IL-4 may promote intracellular modulation, as alterations in these genes have been associated with oxidative damage, apoptosis and impaired embryonic development (Luo et al. 2021). The implications for this

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apparent increased intracellular modulation following embryo transfer of these blastocysts is not known, but intriguing; could they develop best than routine counterparts in the recipients? Future studies in this respect are planned.

Although the downregulation of *SOD1*, *TP-53* and *LIF* did not reach statistical significance, the overall trend supports an effect of IL-4 on the modulation of stress response. In addition, *PCNA* plays a key role in DNA replication and cell proliferation; therefore, its repression may suggest decreased DNA damage in embryos cultured with IL-4 (Strzalka and Ziemienowicz 2011).

In conclusion, although supplementation with IL-4 during IVC did not improve the developmental competence of porcine embryos, it altered the expression of stress- and immune-related genes, suggesting that IL-4 may modulate specific molecular responses to the in vitro culture environment.

Author Contributions

M.G.-C., C.C., E.A.M. and M.A.G. conceived and designed the study. M.G.-C., M.T.-F., A.L.-J., I.P., C.C. and M.A.G. performed the experiments. M.G.-C., E.A.M., C.C. and M.A.G. analysed and interpreted the data. M.G.-C. wrote the primary manuscript. H.R.-M., E.A.M., C.C. and M.A.G. revised, discussed and edited the manuscript. All authors revised and approved the manuscript for publication. M.A.G., C.C., E.A.M. and H.R.-M. obtained the funding to carry out the study.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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