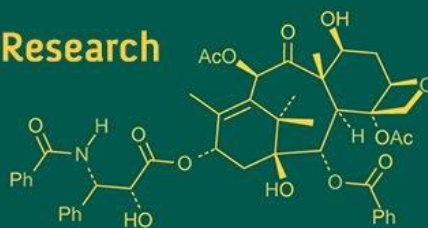


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Interleukin-6 modulates *in vitro* development of the cattle oocytes and embryo: A review

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Abstract

In vitro embryo production (IVP) has become a cornerstone of modern dairy cattle breeding, with over 1.6 million IVP bovine embryos transferred worldwide in 2022. Despite its widespread adoption, IVP faces significant hurdles, including suboptimal embryo development efficiency and reduced post-transfer survival rates. This review emphasizes current research on interleukin-6 (IL-6), a multifunctional cytokine within the IL-6 family, and its role as an embryokine in enhancing bovine oocyte maturation, folliculogenesis, and early embryo development. IL-6 exerts its effects via the JAK/STAT3 signaling pathway, with studies demonstrating that its supplementation during *in vitro* maturation (IVM) and embryo culture increases inner cell mass (ICM) cell numbers and improves developmental competence. However, its influence on blastocyst formation and trophectoderm (TE) cell populations remains inconsistent. While elevated IL-6 levels in follicular fluid correlate with improved oocyte quality and higher implantation rates, excessive supplementation may impair maturation, potentially due to adequate endogenous IL-6 production. These findings underscore the context-specific nature of IL-6's effects, highlighting its promise for optimizing IVP outcomes while revealing challenges, such as variable embryo responsiveness and unresolved questions about long-term developmental impacts. This review emphasizes the need for further investigation to refine IL-6 supplementation strategies, offering insights into its potential to advance bovine reproductive technologies and improve livestock productivity.

Keywords: Cytokine, interleukin-6 (IL6), inner cell mass (ICM), embryo quality, *in vitro* embryo production (IVP)

Introduction

The use of *in vitro* embryo development to produce calves with the correct genetic composition is being quickly adopted by the dairy industry. This can be achieved by using assisted reproductive technologies such as embryo transfer (ET), ovum pickup (OPU), and *in vitro* production (IVP). Over the past years, there has been a significant increase in the use of *in vitro*-produced (IVP) bovine embryos worldwide; according to the most recent estimate, over 1.6 million IVP embryos were transferred in 2022 (Viana, 2022) [53]. Since 1981, it has been possible to produce calves from *in vitro* produced (IVP) bovine embryos (Brackett *et al.*, 1982) [11], resulting in a breakthrough in cattle reproductive biotechnology. However, there are two main drawbacks to *in vitro* embryo technologies for production. Firstly, the generation of embryos is currently not very efficient. Numerous factors, including the culture media's composition, the environment (such as oxygen content), seasonal fluctuations, and the degree of technical expertise, can cause the success rate to fluctuate significantly. Only between 20 to 45 percent of oocytes employed in *in vitro* embryo development are known to effectively grow into transferable blastocyst-stage embryos (Lorengan *et al.*, 2003 and Lorengan *et al.*, 2008) [25, 26]. Secondly, after embryo transfer, a significant number of transferable IVP embryos do not survive. Wooldridge *et al.* (2019) [54] found that only 27 percent of IVP embryo transfers produced live calves in dairy industry. Some researchers hypothesize that adding biologically active compounds made by the reproductive system in the early stages of pregnancy to media formulations could improve post-transfer IVP embryo competency and *in vitro* embryo production efficiency. Hansen and colleagues introduced the term "embryokine" to refer to substances that control embryo growth that are produced by the oviduct, uterus, or embryo (Hansen *et al.*, 2014) [21].

There are various growth factors, signalling molecules and cytokines identified as an embryokine. Growth factors such as IGF1, EGF, and FGF greatly impact the *in vitro* embryo production when it is used in without combination (Xie *et al.*, 2017) [57]. In cattle, insulin-like growth factor 1 (IGF1) supplementation increases the amount of blastocyst cells, IVP blastocyst development rates, and post-transfer pregnancy rates throughout the summer (Block *et al.*, 2003, Jousan *et al.*, 2007 and Xie *et al.*, 2017) [10, 23, 57]. A pleiotropic cytokine, interleukin-6 (IL-6) is essential for inflammation, immunological responses, and cellular communication. Cytokines that have been identified and explored as an embryokines basically belong to two cytokine families, first the beta common cytokine family which includes CSF2 or GM-CSF, IL3 and IL5. Members of the second cytokine family belong to the interleukin 6 (IL6) family which are differ from the β -common cytokines in both structure and function. IL6, IL11, IL27, IL31, leukemia inhibitory factor (LIF), oncostatin-M (OSM), and ciliary neurotrophic factor (CNTF) are all members of the IL6 family (Eulenfeld *et al.*, 2012 and Rose-John, 2018) [18, 37]. However, in the IL6 family, IL6 and LIF are the molecules that attract the most interest as embryokines. This review provides insights into *in vitro* embryo development and livestock productivity by supplementing interleukin 6.

1. Evidence of IL-6 involvement in folliculogenesis, oocyte maturation, and embryo development *in vivo*

In reproductive biology, IL-6 has been connected to follicular development, oocyte maturation, and early embryogenesis. For decades, preimplantation embryos of various species, including humans, mice, rabbits, and cattle, have been cultured *in vitro* to the blastocyst stage (Tervit *et al.*, 1972) [51]. However, *in vitro* culture systems lack the complexity of the oviductal environment, which can hinder ICM development. It is therefore not surprising that defects of the ICM can result in lethality (Feldman *et al.*, 1995 and Takeda *et al.*, 1997; Arman *et al.*, 1998) [19, 50, 7]. Interleukin 6 (IL-6) is a pleiotropic cytokine; It has multiple cellular and tissue effects and leads to pro-inflammatory response. IL-6 may also play a crucial role in follicular development (Yang *et al.*, 2020) [58]. Some studies have shown that a higher level of IL-6 correlated with worse quality of embryos, and patients were less likely to get pregnant (Semeniuk *et al.*, 2018) [41]. In contrast, high levels of IL-6 in the follicular fluid were good for oocyte maturation and were associated with increased rates of clinical pregnancy and embryo implantation (Wu *et al.*, 2017) [55]. Indeed, during the peri-implantation period, immunological balances must be regulated, including regulation of pro-inflammatory/anti-inflammatory chemokines and IL17/regulatory T cells (Ali *et al.*, 2021) [5]. Some studies have shown that high levels of IL-6 in follicular fluid have a positive effect on oocyte maturation (Kawasaki *et al.*, 2003 and DeMatos *et al.*, 2008) [15, 16]. High levels of IL-6 in embryo culture are associated with an increased rate of clinical pregnancies and implantation of embryos (Wu *et al.*, 2017 and Qin *et al.*, 2016 and Wu *et al.*, 2007) [55, 35, 56]. Higher levels of IL-6 correlate with poor embryo quality, and patients are less likely to become pregnant (Altun *et al.*, 2011 and Semeniuk *et al.*, 2018) [6, 41]. It has been concluded that IL-6 inhibits oocyte development (Banerjee *et al.*, 2012) [9]. In addition, some studies have suggested that IL-6 does not affect the clinical rate of pregnancy, as well as the quality of oocytes

(Alhilali *et al.*, 2019 and Asimakopoulos *et al.*, 2008) [4, 8]. On the basis of studies, that IL-6 might be improve oocyte developmental competence and embryo quality in *in vitro* conditions by mimicking *in vivo* conditions.

2. Expression of IL-6 receptors (IL-6R/gp130) and Signaling in bovine ovarian cells and embryos

Cytokines may be significant autocrine and paracrine regulators of ovarian activities, as evidenced by their production in the ovary, accumulation in ovarian follicular fluid, and impact on some ovarian processes (Qiao *et al.*, 2011, Rechard *et al.*, 2010, Sirotkin 2011, Smolikova *et al.*, 2012 and Sheldon *et al.*, 2014) [34, 36, 47, 43]. Among all Interleukins (ILs) are cytokines that seem to play a key role in regulating reproductive function and a variety of physiological and pathological processes in the ovary (Smolikova *et al.*, 2012) [47]. Leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), IL-11, and cardiotrophin-like cytokine factor 1 (CLCF1) are among the members of the IL-6 family (Senaldi *et al.*, 1999) [42]. They have similar biological roles and share the receptor IL-6 signal transducer (IL-6ST, also called gp130) (Taga and Kishimoto, 1997) [49]. LIF has been linked to the early development of follicles (Skinner, 2005) [46] and oocyte quality (De Matos *et al.*, 2008) [15, 16] and implantation (Hu *et al.*, 2007) [22]. Since oocytes can be direct targets of cytokine action, mice with a conditional deletion of the gp130 receptor in their oocytes show reduced zygotic cleavage to the two-cell stage (Molyneaux *et al.*, 2003) [31]. In granulosa cells, by attaches itself to the IL-6 receptor, which is made up of IL-6R α and another protein on the cell membrane. The precise ligands that control JAK/STAT3 activity in bovine embryos are unknown. The JAK/STAT3 pathway is crucial for the ICM lineage in mice and calves (Takeda *et al.*, 1997; Meng *et al.*, 2015) [50, 29]. Members of the IL6 cytokine family appear to fulfill this function in a number of ways. Certain members of the family act as pluripotency factors for mouse embryonic stem cells (Shmitt *et al.*, 1991; Conover *et al.*, 1993; Nichols *et al.*, 1994) [39, 14, 32]. Additionally, in pig blastocysts, IL6 supplementation promotes STAT3 phosphorylation and raises the quantity of ICM cells (Shen *et al.*, 2012) [44]. Because embryonic genome activation had already taken place by this stage of development (the 8-16 cell stage), allowing embryos to respond to external signals, and because these embryos were getting ready for compaction and embryonic lineage specification, the majority of the studies started on day 5 of embryonic development (Graf *et al.*, 2014) [20]. This is an ICM-specific activity. In trophectoderm (TE) cells, no similar IL6-induced STAT3 activation is seen. Additionally, ICM development in bovine embryos is inhibited by pharmacological suppression of JAK2, which cannot be reversed by IL6 supplementation. (Wooldridge *et al.*, 2019 and Meng *et al.*, 2015) [54, 29].

3. Interleukin 6 supplementation effect on *in vitro* maturation of bovine oocytes

Bovine embryos created *in vitro* (IVP) have lower post-transfer survival and developmental potential. The absence of growth, differentiation, and cytokine factors that are present in the follicle but not in oocyte maturation culture systems could be one explanation for this. The cumulus-oocyte complex (COC) gene expression, fertilisation success, embryo development potential, and maturation

success are all impacted when certain members of the interleukin-6 (IL6) cytokine family are supplemented during *in vitro* bovine oocyte maturation. According to Vendrell-Flotats *et al.* 2020^[52] supplementation of IL6 or IL11 during IVM enhanced the production of many microRNAs that promote the maturation of bovine oocytes. According to a different study, IL6 might play a role in regulating when meiosis completion occurs in cattle under heat stress (Rowinski *et al.*, 2021)^[38]. McKinley *et al.* (2023)^[28] reported that Oocyte maturation and COC gene expression were all negatively impacted by IL6 administration results could be explained by the fact that IVM produces enough endogenous IL6 to eliminate the requirement for further supplementation.

4. Interleukin 6 supplementation effect on *in vitro* fertilisation of bovine oocytes

Influences of Supplementing Selective Members of the Interleukin-6 Cytokine Family on Bovine Oocyte Competency investigated the effects of leukemia inhibitory factor (LIF), interleukin-11 (IL-11), and IL-6 supplementation during the *in vitro* maturation of bovine oocytes. McKinley *et al.* (2023)^[28] discovered that although supplementing with LIF and IL-11 boosted blastocyst growth rates and the expression of many transcripts linked to oocyte competency, it had no appreciable impact on the embryo's development or the success of fertilization. According to a different study on IL-6 supplementation during *in vitro* embryo development, it increased the number of inner cell mass cells and appeared to have a tendency to increase the number of trophectoderm cells in blastocysts, but it had no effect on blastocyst formation rates.

5. Interleukin 6 supplementation effect on *in vitro* culture of bovine embryo inner cell mass and trophectoderm

Supplementation with various growth factors and cytokines during *in vitro* embryo production can have positive effects on pregnancy retention, calf birth weight and dystocia (Loureiro *et al.*, 2009; Denicol *et al.*, 2014)^[27, 17]. It was intriguing to see that on day nine, IL6 treatment increased the amount of undifferentiated cells. This might be a result of IL6's capacity to increase ICM cell counts before epiblast/ primitive endoderm specification, as demonstrated most recently in research done on blastocysts analyzed on day 7 after fertilization (Wooldridge *et al.*, 2019)^[54]. However, after they have committed to one of the two cell fates, they lose this flexibility. The TE was slightly impacted by IL6 treatment as well. Given that the majority of studies did not identify effects of IL6 on TE cell counts, this finding is noteworthy even though just a statistical tendency for increased TE cell numbers was found (Wooldridge *et al.*, 2019)^[54]. However, when comparing the EPI and PE figures at days 9 and 10 to those at day 8, no improvements were found. Therefore, it's possible that undifferentiated cell differentiation only partially restored the missing PE and/or EPI cells. Alternatively, apoptosis of undifferentiated cells might have taken place. When undifferentiated cells continue to exist in the ICM, this is a typical occurrence (Plusa B, *et al.*, 2008)^[33] When 100 ng/ml of recombinant bovine IL6 was added to *in vitro*-produced bovine embryos on days 1, 3, or 5, the number of inner cell mass (ICM) cells and the ICM: trophectoderm

(TE) ratio increased ($p < 0.05$), but not the number of TE cells. After taking 1 or 10 ng/ml of IL6 starting on day 1 or day 5, there was no increase in the number of ICM or TE cells. The number of ICM cells was raised ($p < 0.05$) by sequential supplementation with 100 ng/ml IL6 on days 1 and 5 (for a total of 200 ng/ml IL6) more than using IL6 supplements for a single time in one research but not in another. Furthermore, compared to supplementing with 100 ng/ml IL6, giving 200 ng/ml IL6 starting on day 1 or day 5 did not result in any additional increase in ICM cell counts. In group culture, cleavage and blastocyst formation were unaffected by IL6 therapy. However, when bovine embryos were cultivated separately, IL6 treatment enhanced cleavage and the development of day 8 blastocysts (Wooldridge *et al.*, 2019)^[54]. Once EPI/PE cell fates are determined, IL6 controls ICM development. IL6 targets the PE cells and increases PE populations by means of a JAK-dependent signal. Once the PE lineage has been identified in the bovine blastocyst, PE development requires this JAK2-dependent signal. Although it is unknown if IL6 is required for primitive endoderm development during the bovine embryo's growth inside the uterus, a subgroup of IVP bovine embryos have impaired yolk sac development upon transfer (Alberto *et al.*, 2013 and Mess *et al.*, 2017)^[3, 30].

6. Effect of the IL 6 on the expression of gene

Additionally, STAT3 activation in mouse blastocysts is decreased by antibody-based immunoneutralization of embryo-derived IL6 (Do *et al.*, 2013)^[61] Additionally, there are some signs that IL6 may promote the pluripotency of induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESC) (Do *et al.*, 2013; Tang *et al.*, 2020)^[61, 59]. When human fibroblasts are reprogrammed, IL6 mRNA increases 50-fold (Brady *et al.*, 2013)^[2]. The same study found that IL6 supplementation activated a number of STAT3-induced targets, including c-Myc, one of the four elements necessary to cause human and mouse somatic cells to become pluripotent (Liu *et al.*, 2008). When the other three components (Oct4, Klf4, and Sox2) were overexpressed, cells were able to undergo pluripotency by substituting IL6 for c-Myc expression. RNA sequencing results did not show the SOCS3 rise (Wooldridge *et al.*, 2019)^[54]. RNA sequencing investigation involved embryos exposed to IL6 for 72 hours and that SOCS3 expression is known to increase initially but then decline following prolonged IL6 treatment, this is not surprising (Starr *et al.*, 1997)^[48]. Additionally, the RNA sequencing dataset demonstrated that IL6 supplementation had negligible effects on the development of embryo lineages, early pluripotency, and differentiation potential. Little information on the processes behind IL6's effects on the ICM was revealed by the RNA profiling study. To interpret changes in gene expression linked to IL6 treatment, time-response studies and studies that specifically look at the ICM are probably required. The most widely expressed ligand-specific receptor was IL6R. Furthermore, IL6R expression improved in response to IL6 supplementation. While changes in PE lineage markers were observed (Wooldridge *et al.*, 2019)^[54]. IL6 supplementation had no effect on the expression of lineage markers linked to ICM and TE development. The yolk sac will be formed by the PE, and compared to embryos created *in vivo*, the yolk sac of IVP bovine embryos is not as well vascularized (Alberto *et al.*, 2013, Mess *et al.*, 2017)^[3, 30]. IL 6 treatment control the expression of the transcription regulator gene like STAT1, STAT3 and OTX2 and it is expected to detect

these responses to IL6 treatment in bovine embryos (Brocke-Heidrich *et al.*, 2004 and Zang *et al.*, 2005) [12, 60].

7. Limitations and future challenges using IL6 as supplement in bovine embryo production

IL6 acts as an embryokine in bovine embryos, promoting STAT3 phosphorylation in the ICM. Its effects on ICM growth are blocked by JAK/STAT3 inhibition, but its reliance on this pathway remains unclear, as STAT3 is essential for ICM survival (Wooldridge *et al.*, 2019) [54]. IL6 transcripts are highly expressed in the oviduct on days 3 and 5 and in the endometrium on day 5 during early pregnancy (Starr *et al.*, 1997; Shen *et al.*, 2012; Wooldridge & Alan, 2019) [48, 44, 54]. While IL6 enhances ICM development *in vitro*, its necessity for overall embryo development is uncertain. IL6-treated embryos show no adverse effects on fetal or placental development during the first 10 weeks of gestation. Instead, it may support embryo and fetal development, though studies with limited pregnancies prevent definitive conclusions (Wooldridge *et al.*, 2019) [54]. Further research is needed to assess its impact on neonatal health and post-transfer pregnancy survival. Individual embryos showed varied responsiveness to IL6 supplementation, indicating that not all embryos may benefit equally from this treatment, which could limit its effectiveness in practical applications. IL6 supplementation did not significantly affect cleavage rates or blastocyst formation in group cultures, suggesting that its benefits may be context-dependent and not universally applicable. Future studies are necessary to explore the optimal conditions for IL6 supplementation, including timing and concentration, to maximize its benefits for embryo development in various culture environments (Wooldridge *et al.*, 2019) [54]. Recently in a study examined cytokines IL6, IL11, and LIF in embryos post-fertilization. Results showed IL6 and LIF increased ICM cell quantity, indicating better embryo quality. However, no treatments affected IVP embryo survival post-freezing and thawing (Wooldridge *et al.*, 2019) [54].

Conclusion

Interleukin-6 (IL-6) plays a complex role in embryo development, exerting both beneficial and detrimental effects on oocyte maturation and embryo quality. It, along with related cytokines like LIF and IL-11, regulates ovarian function, folliculogenesis, and embryogenesis via pathways such as JAK-STAT3. In bovine models, IL-6 supplementation can enhance developmental competence and inner cell mass (ICM) formation; however, excessive levels may impair maturation due to adequate endogenous IL-6. While LIF and IL-11 improve oocyte competence and blastocyst development, IL-6's influence on embryo quality and post-transfer outcomes remains promising yet inconclusive. IL-6 also affects ICM and trophoderm cell numbers, pluripotency markers, and early gestation, but its role in ensuring overall embryo viability appears context-dependent. Despite its potential to improve *in vitro* embryo production and fertility outcomes, the precise mechanisms and optimal conditions for IL-6 use remain unclear. Thus, balanced cytokine supplementation and further research are crucial to harness IL-6's full reproductive potential.

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