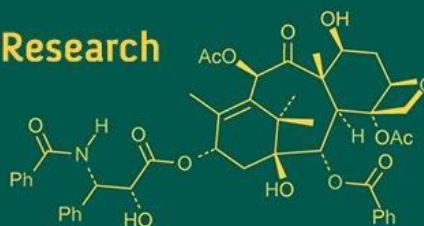


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From lab to livestock: Harnessing hormones to revolutionize *in vitro* embryo development

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Abstract

The development competence of embryos is crucial for livestock reproduction and agriculture productivity. Hormone supplementation has an additive effect on improving embryo quality and enhancing developmental rates. This review focuses on the impact of hormones such as progesterone, estradiol, follicle-stimulating hormone, and triiodothyronine and thyroxine. Optimized concentrations of these compounds impact *in vitro* oocyte maturation and enhance the competence of embryos across various livestock species and their potential to improve reproductive outcomes.

Keywords: Embryo, maturation, cumulus cells, hormones, competence

Introduction

In recent years, optimization and improvement of *in vitro* oocyte maturation and embryo production in livestock species has been focused mainly due to increased infertility problems. The use of various assisted reproductive technologies like *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* embryo culture (IVC) has been progressively increasing for enhanced *in vitro* embryo production (IVEP). Yet, the transfer of yielded embryos is very low. Many factors are responsible for the success of IVP embryos, including embryonic factors, maternal/paternal factors, and in-vitro culture factors like light exposure, media composition, and temperature fluctuations (Ealy *et al.*, 2019; Hansen, 2020) [30, 36]. Most *in vitro* culture media do not contain formulations like hormones, growth factors, and cytokines that mimic the oviduct during the first 7 to 8 days post-estrus (Martins *et al.*, 2018) [53]. Supplementation of hormones in livestock embryo culture media plays a significant role in mimicking *in vivo* conditions, enhancing the maturation of oocytes, and promoting embryonic development, including the quality and development of blastocysts (Choi *et al.*, 2024; Bahrami and Cottee, 2022; Shabankareh *et al.*, 2011) [23, 7, 38]. Progesterone (P4) is crucial for maintaining pregnancy and plays a role in uterine receptivity, fertilization, and embryonic development (Barrera, Avila, and Díaz, 2007; Loutradis *et al.*, 2008) [10, 49]. Estrogen, particularly estradiol (E2), is essential for oocyte maturation and has a complex role in IVM (Bevers *et al.*, 1997) [14]. Guo *et al.*, 2009 [34] reported that E2 increases the proportion of oocytes that undergo nuclear maturation. Thyroid hormones are important for post implantation-development and may improve embryo development, and hatching rates, and reduce apoptosis in cryopreserved embryos (Ashkar *et al.*, 2010; Medina, Buttler, and Gilbert, 2014) [6, 1]. The addition of gonadotropin like follicle-stimulating hormone (FSH) can improve nuclear maturation in oocytes, meiotic resumption, and early embryonic developmental competency (Pereira *et al.*, 2013) [60]. Most studies reported that supplementation of FSH in IVM media enhances the maturation rates in bovine (Sirard *et al.*, 1988; Calder *et al.*, 2003; Takada *et al.*, 2012) [71, 18, 74]. This review summarizes the importance of supplementing different hormones for *in vitro* embryo production in various livestock species.

Progesterone

Progesterone is a 21-carbon cholesterol-derived steroid hormone synthesized within several cell types like corpus luteum, adrenal gland, and placenta (Golub *et al.*, 2006) [33] and too much lower levels in Leydig cells (Rossato, 1999) [62] and adipose tissues (Zhang *et al.*,

2009) [34]. P4 secretion from the ovary regulates gonadotropin secretion (Mahesh, 1987) [51], influences mating behavior (Dudley, 1980) [28], and stimulates uterine and placental development (Spencer, 2004) [73]. P4 is essential for high rates of embryogenesis (Dumesic *et al.*, 2003) [29], embryo implantation, and sustaining pregnancy (Bulletti *et al.*, 2022) [15]. Some studies suggest that co-cultured cumulus cells (Shimada and Terada, 2002) [67] produce P4 that enhances the *in vitro* maturation rates and blastocyst development in cattle (Ryan *et al.*, 1992) [64], sheep (Kyasari *et al.*, 2012) [43], goat (Wang *et al.*, 2011) [80] and porcine (Yoon *et al.*, 2015) [83]. Yuan *et al.*, 2016 [85] reported that P4 inhibits the apoptosis of blastocyst by enhancing the quality of porcine oocytes and minimizing the polyadenylation. The *in vitro* supplementation of P4 had no positive effect on the bovine secondary follicular growth but it enhanced the gene expression levels of GDF9 and cyclin B1 in bovine oocytes (Paulino *et al.*, 2020) [58]. P4 and EGF did not show any synergic effect in bovine preantral follicles. Progesterone mediates its effect by interacting with genomic P4 receptors A (nPR-A) and B (nPR-B) (Bain *et al.*, 2007) [9] in female reproductive organs. However, classical activation of genomic receptors is a slow and complex process as nPR expression in the follicle is species-specific (Lodde *et al.*, 2023) [48]. In sheep, nPR is expressed in the granulosa cells of growing antral follicles (Ding *et al.*, 2022) [27] and in granulosa cells of primordial, primary, secondary, and tertiary follicles in cattle (Haeseleer, Simoens, and Broeck, 2007) [25]. P4 interaction with non-genomic membrane progesterin receptors (NPR α , β , and γ) (Zhu *et al.*, 2003) [87] and non-genomic receptor membrane components 1 (PGRMC 1) and 2 (PGRMC 2) had shown rapid signaling events void of the transcriptome (Cahill, 2007) [16]. Aparicio *et al.*, 2011 [3] reported the localization of both genomic and non-genomic P4 receptors in bovine cumulus-oocyte complexes (COCs) and supplementation of P4 enhanced the maturation rates in bovine. PGRMC 1, the most abundant P4 non-genomic receptor membrane transcripts were found in bovine morula (Sang *et al.*, 2021) [65] and porcine embryos (Cho *et al.*, 2020) [22] depicted their role in the cleavage by regulating cell division (Lodde *et al.*, 2022) [47]. Larson *et al.*, 2011 [44] reported that *in vitro* supplementation of P4 did not improve the embryo characteristics in the *in vitro*-produced bovine embryos. Ferguson, Kesler, and Godke, 2012 reported the positive effect of P4 supplementation for the *in vitro* production of bovine embryos. There are a lot of gaps in knowledge to determine the optimum concentration of *in vitro* supplementation of P4 due to different genomic and non-genomic progesterone receptors in livestock species.

17 β -Estradiol

17 β -Estradiol (E2) is an isomer of estradiol produced mainly in the ovaries, promotes the growth of the uterine lining, and enhances the LH surge for ovulation. It also regulates the release of pituitary hormones through negative feedback. The proportion of E2 produced in testes is much lower. Maksura *et al.*, 2021 [52] reported the effect of 17 β -Estradiol on nuclear maturation of buffalo and goat oocytes. In Buffalo, the MetaphaseII (MII) stage oocytes number progressed with the increased supplementation of 17 β -Estradiol in a concentration-dependent manner. Still, in the goats, treatment with 1 μ g/ml of 17 β -Estradiol increased the proportions of MII stage oocytes than the control and other

treatment groups. Supplementation of 1 μ g/ml of 17 β -Estradiol is considered to mimic the presence of estradiol in the preovulatory follicle *in vivo*. 1 μ g/ml of E2 is commonly supplemented in an *in-vitro* maturation medium for the maturation of oocytes in bovine (Beker *et al.*, 2002) [12], ovine (Leoni *et al.*, 2007, Catala *et al.*, 2012, Wang *et al.*, 2013) [45, 20, 81], and caprine (Hammami *et al.*, 2013) [35]. 17 β -Estradiol also enhances the cumulus cell expansion as well as the nuclear and cytoplasmic maturation of oocytes in bovine (Sirotkin, 1992) [72], caprine (Pawshe *et al.*, 1996) [59], equine (Carneiro *et al.*, 2001) [19], and porcine (Kubo *et al.*, 2015) [42]. Vlu and Kuz'mina, 2005 studied the interaction of 17 β -Estradiol with prolactin and theophylline release of calcium ions from intracellular stores of porcine oocytes. E2 supports the growth and viability of oocyte granulosa complexes in bovine (Taketsuru *et al.*, 2012) [75]. Showed a significant inhibitory effect of 10-100 μ g/ml concentration of 17 β -Estradiol on the progression of oocyte maturation to MetaphaseII (MII) in prepubertal goats. No effect was observed with 1 μ g/ml E2 supplementation on the progression of *in-vitro* matured oocytes to MII stage compared to control groups. Transient supplementation of E2 improved the developmental competence of oocytes and enhanced the blastocyst formation rates in porcine (Kim *et al.*, 2012) [41]. In conclusion, supplementation with 1 μ g/ml of E2 is the best concentration recommended in the *in-vitro* development of oocytes and embryos in several livestock species.

Follicle-stimulating hormone (FSH)

Follicle-stimulating hormone (FSH) is a gonadotropin and a glycopeptide protein hormone (Cahoreau, Klett, and Combarous, 2015) [17] synthesized and secreted by the anterior pituitary gland. In several vertebrates, FSH is essential in reproductive processes (Ulloa-Aguirre *et al.*, 1999; Loof *et al.*, 2001) [77, 26]. FSH consists of two polypeptide chains (α and β subunit) associated non-covalently and exhibit no biological activity when present alone; however, they regain this activity upon recombination. FSH is involved in the development process, like folliculogenesis and steroidogenesis in the ovary (Santi *et al.*, 2018) [66], by acting on the ovarian granulosa cells and the sertoli cells in the testis. Supplementation of FSH in the *in vitro* maturation studies showed improved fertilization rates, enhanced embryonic developmental competency (Izadyar, Zeinstra, and Bevers, 1998) [37], and cumulus cell expansion (Calder *et al.*, 2003) [18]. The action of FSH on oocyte maturation is poorly understood, but many reports have suggested that its action is mediated by cumulus cells. Observations have shown that FSH receptor mRNA is present in granulosa and cumulus cells but not detected in oocytes isolated from bovine antral follicles (vanTol *et al.*, 1996) [78]. Ferreira *et al.*, 2016 [32] reported that *in vitro* supplementation of FSH (100 μ g/ml) with 10 μ g/ml insulin improved follicular and oocyte growth in caprine. Bahrami, Morris, and Day, 2019 [8] reported that supplementation of 1 μ g/ml FSH to the culture media enhanced the number of metaphase II oocytes in bovine. *in vitro* supplementation of FSH (10 U/ml), LH (10 U/ml), and glutathione (GSH) enhanced the maturation rates of Grade B and Grade C oocytes in ovine (Ren *et al.*, 2021) [61]. No significant increment was observed in Grade A oocyte maturation rates. Takada *et al.*, 2012 [74] reported 0.5-1 μ g/ml FSH as the best-suited concentration for *in vitro* developmental

competency of bovine embryos. FSH (20 µg/ml) supplementation in oocyte maturation media resulted in higher MII rates (Roth and Hansen, 2005) comparable to 0.05 IU/ml FSH (Beker, Colenbrander, and Bevers, 2002) [12]. Singha *et al.*, 2015 reported that 5 µg/ml FSH-supplemented media showed significantly higher maturation rates than the gonadotrophin-supplemented counterpart in indigenous zebu cattle. FSH is essential for *in vitro* maturation of oocytes and cumulus cell expansion in buffaloes (Pandey *et al.*, 2010) [56]. Some studies reported no increment in maturation rates after supplementing with FSH (Younis *et al.*, 1989) [84]. FSH supplementation enhances the *in vitro* maturation rates. It helps in the activation and survival of preantral follicles in ovarian tissues of equine (FSH-50 ng/ml) (Aguair *et al.*, 2016) [2], caprine (FSH-50 ng/ml) (Celestino *et al.*, 2011; Barros *et al.*, 2013) [21, 11], ovine (FSH-100 ng/ml) (Patel *et al.*, 2013) [57], and bovine (FSH-50 ng/ml) (McLaughlin *et al.*, 2010) [54]. Widayati and Pangestu, 2020 [82] studied the effect of supplementation of FSH (50 IU/L) on *in vitro* maturation and embryonic development of caprine. In conclusion, FSH enhances the preantral follicular development, *in vitro* maturation of oocytes, and developmental competency of embryos in livestock species. No fixed concentration is found for the individual supplementation of FSH due to species variation and different research contexts.

Triiodothyronine and Tetraiodothyronine

Triiodothyronine (T₃) and Tetraiodothyronine (T₄) are the two tyrosine-containing thyroid hormones produced by the thyroid glands and are primarily responsible for metabolism regulation. T₃ and T₄ are produced by thyroid-stimulating hormone (TSH) released from the anterior pituitary gland (Li *et al.*, 2021) [46]. Both T₃ and T₄ with their active metabolites are essential for promoting the development of the brain, supporting the functions of heart, muscle, and bone health, and regulating metabolic rates in the body (Kim and Mohan, 2013; Mullur *et al.*, 2014) [40, 55]. Thyroid hormone supplementation in the culture media plays a vital role in the enhancement of *in vitro* maturation, fertilization, and blastocyst development in various livestock species. Asadpour *et al.*, 2021 [5] reported that the addition of thyroid hormone to the IVM medium had controversial effects on the development of ovine oocytes. The lower dose of T₃ (10 ng/ml) reduced the apoptosis rate in blastocysts, and the higher dose (100 ng/ml) had detrimental effects significantly ($p < 0.001$) decreasing cumulus expansion and blastocyst rates compared to control and other treatment groups. Some studies reported that *in vitro* supplementation of culture media with thyroxine hormone at 100 ng/ml showed a significant increase in cleavage rates and early embryo development (Singh *et al.*, 2020) [69]. The cleavage and morula rates were significantly higher ($p < 0.05$) in the T₃ (50 ng/ml and 100 ng/ml) supplemented groups, suggesting that triiodothyronine may have increased the morula rate after fertilization (Singh *et al.*, 2017) [68]. Conducted a study to evaluate the effect of 1,000 ng/ml recombinant bovine somatotropin (rbST) and 20 ng/ml thyroxine (T₄), which did not exert beneficial effects on the survival and growth of bovine preantral ovarian follicles (PAOF) cultured *in vitro*. Combinatorial effect of T₃ (50 ng/ml) and T₄ (50 ng/ml) in the IVC improved embryos' developmental competency in bovine (Ashkar *et al.*, 2010) [6]. Costa *et al.*, 2013 [24] reported that adding 50 nM and 100 nM T₃ to the IVM

medium significantly increased ($p < 0.05$) the rate of hatched blastocysts compared to the control and 25 nM groups. The thyroid hormone-treated embryos exhibited a significant ($p < 0.05$) increase in blastocyst formation and hatching rates, with embryo quality demonstrated by higher total cell counts and reduced proportions of apoptotic cells (Ashkar *et al.*, 2010) [6]. Supplementation of T₄ (1 µg/ml) combined with follicle-stimulating hormone (FSH), insulin growth factor 1 (IGF-I), and growth hormone (GH) enhanced the maturation of oocytes to the Metaphase-II stage in preantral follicles of ovine (Arunakumari, Shanmugasundaram, and Rao, 2010) [4]. Bera *et al.*, 2024 [13] reported the combination of T₃ (100 ng/ml) and T₄ (100 ng/ml) enhanced the developmental competence of immature cattle oocytes and increased the blastocyst rate. The optimum concentration of T₃ and T₄ for the development of embryos in bovine ranges between 50-100 ng/ml. Very few studies reported the *in vitro* supplementation of T₃ and T₄ in porcine, caprine, and ovine embryo development.

Conclusion

In conclusion of this overview, the supplementation of different hormones in the *in vitro* culture media has shown promising results in the *in vitro* maturation of oocytes, enhancing the embryonic developmental competency and promoting a higher success rate of *in vitro* embryo production. Including these hormones in the maturation medium can improve the nuclear and cytoplasmic maturation of oocytes, enhancing the quality of oocytes and influencing the success of embryogenesis. However, it is important to note that the supplementation of these hormones in the culture medium needs to be optimized in combinations and concentrations, as they may vary based on individual circumstances such as age, breed, species, and reproductive history. Further studies need to be done to understand the mechanism of their effects on *in vitro* embryo development.

Author contributions

Writing-Original draft preparation, S.B., R.S., S.D; reviewing and editing-S.B., R.S. All authors have read and agreed to the published version of the manuscript.

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