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Use of Colony Stimulating Factors 1 and 3 to Improve oocyte *In vitro* Maturation

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Abstract

We have recently shown that CSF-2 can improve *in vitro* oocyte maturation (IVM) and subsequent development in a mouse model. The present study was undertaken to investigate whether CSF-1 and CSF-3 can also improve IVM in this model. Cumulus oocyte complexes (COCs) from CBAF1 mice were matured with 0, 2 or 10 ng/ml of CSF-1 or CSF-3, fertilised and then cultured in vitro for 96 h. Cumulus expansion, cleavage rate, blastocyst development and inner cell mass (ICM) and trophectoderm (TE) cell numbers were examined. The addition of CSF-1 during IVM tended to increase ICM numbers in both treatment groups and significantly increased the ratio of ICM:TE cell number in the 2 ng/ml group compared with control. The addition of CSF-3 during IVM had no effect on embryo development. In conclusion, our results suggest that the addition of CSF-1 can improve IVM.

Keywords: *In vitro* maturation; Oocytes; Embryo; Colony stimulating factors

Introduction

Oocyte in vitro maturation (IVM) is increasingly used as an assisted reproductive technology in humans where hormonal stimulation is unsuitable including for PCOS patients and for those wishing to preserve their fertility, for example prior to undergoing chemotherapy [1]. IVM is also an integral component of in vitro embryo production (IVP) which is used widely in the cattle industries as a breeding tool [2]. Current IVM systems do not replicate the follicular environment and it is this stage in vitro embryo production (IVP) that holds the most potential for improvement [1, 3]. The Colony Stimulating Factors (CSF 1, 2 and 3) are pleiotropic cytokines belonging to the hematopoietic growth factor family and are secreted by the uterine epithelium in a variety of mammalian species including humans [4-6]. Furthermore, all three have been shown to improve embryo development when added during in vitro embryo culture [4-6]. Of these, CSF-2 (also known as GM-CSF) has been the most widely studied and is considered to be an embryokine [5]. The Colony Stimulating Factors are also present in the ovarian follicle [7-9], suggesting they may have a role in oocyte maturation. We have recently shown that the addition of recombinant mouse CSF-2 during IVM in a mouse model can improve embryo development which results in increases in implantation and birth rate following embryo transfer [10]. We have also demonstrated that CSF-2 can improve embryo development as well as mitochondrial activity in a mouse model of advanced maternal age [11]. Whether CSF-1 and CSF-3 can also improve IVM has not been fully determined. However, follicular

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concentrations for both have been correlated with IVF outcomes in humans and as such been suggested as predictive markers of IVF success rates [12, 13]. The present study was undertaken to determine whether the addition of CSF 1 or 3 during IVM can improve oocyte maturation. To examine this hypothesis, we undertook dose response experiments for both cytokines using concentrations shown previously to improve embryo development when added during in vitro culture [4, 6]. The effect of both cytokines on IVM was determined by examining cumulus expansion, cleavage and blastocyst development.

Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Media

The base medium for ovary collection (handling), and IVM was alpha Minimal Essential Medium (αMEM, Gibco by Life Technologies, Carlsbad, CA) supplemented 3 mg/ml bovine serum albumin (BSA, MP Biomedicals, AlbumiNZ, Auckland, NZ), and 1 mg/ml fetuin. Handling medium was HEPES-buffered. IVM medium was bicarb-buffered, with 50 mIU/ml recombinant human follicle stimulating hormone (rhFSH; Parlow, Los Angeles Biomedical Research Institute, Los Angeles, CA), and 2 ng/ml and 10 ng/ml of either CSF-1 or CSF-3 (R&D Systems, Minneapolis, MN: Cat# 416ML010; Cat#: 414-CS). CSF-1 and 3 were reconstituted as per supplier instructions in PBS plus 0.1% BSA (10 µg/ml) and frozen down in 10 µl aliquots and stored at –80°C. The concentrations of 2 and 10 ng/ml were chosen as these have been previously shown to improve embryo development when added during culture [11-14]. Fertilization, wash, and culture media (Vitrofert, Vitrowash, and Vitrocleave) were purchased from ART Lab Solutions (Adelaide, SA, Australia) and supplemented with 4 mg/ml BSA.

Animals

Female CBA × C57BL/6 first filial generation (F1) mice (CBAF1) mice (3-4 weeks old) were obtained from the University of Adelaide Laboratory Animal Services and maintained under 12L: 12D conditions with wet and dry food and water provided ad libitum. All experiments were performed in accordance with Australian Code of Practice for the Care and the Use of Animals for Scientific Purposes and the study was approved by the University of Adelaide Animal Ethics Committee (M-2020-032).

In vitro maturation

At 48 h post intraperitoneal (i.p.) injection (5 IU/100ul) of equine chorionic gonadotropin (eCG; Folligon Intervet, Bosmeer, The Netherlands), mice were culled by cervical dislocation and dissected ovaries collected in warmed handling medium for COC isolation. The COCs were isolated from ovaries by puncturing antral follicles in handling medium. Oocytes were matured in maturation media containing 0 (control), 2 or 10 ng/ml of CSF-1 or CSF-3. Culture media drops were prepared in 60 mm culture dishes with 10 COCs per 500 µl of IVM culture medium under paraffin oil (Merck Group, Darmstadt, Germany). IVM dishes were pre-equilibrated for a minimum of 4 h in 20% O_2, 6% CO_2, N_2 balance, in a humidified incubator at 37°C. The COCs were matured for 16 h. Following maturation, cumulus expansion was assessed using a scale as previously described [15]. COCs were graded as 0 = no expansion, 1+ = outer layer of cumulus cells expanded, 2 = outer half of cumulus expanded, 3 = all layers expanded apart from corona radiata and 4+ = maximum expansion of all layers of cumulus cells.

In vitro fertilisation and embryo culture

Prepared dishes were equilibrated for 4 h pre-IVF in 5% O_2, 6% CO_2, N_2 balance, in a humidified incubator at 37°C. The sperm capacitation dish contained 1 ml of fertilization media under paraffin oil. Fertilization dishes had 2 x fertilization wash drops (180 µl), and 5 x fertilization drops (90 µl). One CBAF1 male was euthanized as described above, and the corpus epididymis, cauda epididymis, and vas deferens removed, trimmed of fat, and transferred into the sperm capacitation drop where sperm were released by squeezing with tweezers. Sperm were left to capacitate for 45 min at 2-5 million sperm/ml density at 37°C in 5% O_2, 6% CO_2, N_2 balance. COCs were transferred from IVM, washed, and then placed into the fertilization drops (10/drop), then fertilized with between 200,000-500,000 sperm and incubated for 4 h in 5% O_2, 6% CO_2, N_2 balance, in a humidified incubator at 37°C. Following fertilization (4 h later), presumptive zygotes were moved into wash drops and denuded by gentle pipetting with a hand pipette, then placed into culture media drops (Vitrocleave, previously equilibrated) at 10 COCs/20 µl drop and cultured in 5% O_2, 6% CO_2, N_2 balance, in a humidified incubator at 37°C. On day 2, embryos were moved into a new equilibrated culture dish at 10 COCs/20 µl drop in 5% O_2, 6% CO_2, N_2 balance, in a humidified incubator at 37°C and cleavage rate was assessed. Cumulus expansion, cleavage rate (estimate of fertilisation rate) and blastocyst rate at 72 and 96 h were determined as well as the number of expanded plus hatched (good quality) blastocysts and blastocyst cell numbers. The experiments were replicated at least five times with a minimum of 35 oocytes per group for each replicate.

Differential staining of blastocysts

Inner cell mass (ICM) and trophectoderm (TE) cell numbers were determined as previously described [16]. Briefly, blastocysts (96 h) were placed into 20 µl of 0.5% pronase for 2-3 min until the zona pellucida was dissolved, then placed in protein free 3-(N-morpholino) propanesulfonic acid (MOPS) media. The embryos were then transferred into 10 µl of 2,4,6-trinitrobenzenesulfonic acid (TNBS) and 90 µl

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of polyvinylpyrrolidone (PVP) and cultured at 4°C for 10 min. Blastocysts were then incubated in 20 µl of anti-dinitrophenyl (Anti DNP) for 10 min at 37°C followed by complement (50 µl propidium iodide (PI) and 50 µl guinea pig serum) at 37°C for 10 min. Blastocysts were then transferred to 500 µl of bisbenzimide and incubated overnight at 4°C. The following day embryos were transferred into 500 µl of 100 % ethanol and placed into a 3 µl drop of glycerol on a slide. A coverslip was gently placed on top of the drop and the number of ICM cells (blue), and TE (pink) were imaged using an Olympus BX 51 (Olympus, Victoria, Australia). Bisbenzimide was excited and emitted at 338 nm and 505 nm to visualise ICM cells and PI at 537 nm and 619 nm to visualise TE cells. TE and ICM cells were counted using Microsoft Paint.

Statistical analysis

All statistics were performed using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). Developmental and cell data was analysed using one way ANOVA with Dunnett's multiple comparisons test and proportions were arcsine transformed (Arcsine (SQRT)).

Results

We showed that addition of 2 or 10 ng/ml of CSF-1 during IVM did not alter cumulus expansion (Fig. 1 A), cleavage rate (Fig. 1 B) and blastocyst rates (Fig. 1 C) or the number of good quality blastocysts (Fig. 1 D) compared with the control group. There was no difference between groups for TE (Fig. 1 E), or TCN (Fig. 1 F). In both CSF-1 treatment groups there was a trend for increased ICM cell numbers compared with control (Control: 20 vs 2 ng/ml: 23, P = 0.08; Control: 20 vs 10 ng/ ml: 20, P = 0.06; Fig. 1 G). For the 2 ng/ml group the ICM/TE ratio was significantly higher compared with the control (Control: 0.40 vs 2 ng/ml: 0.43; P=0.04; Fig. 1 H).

The addition of 2 and 10 ng/ml of CSF-3 during IVM did not increase cumulus expansion (Fig. 2 A), fertilisation (Fig. 2 B), blastocyst rate (Fig. 2 C), the number of good quality blastocysts (Fig. 2 D) or blastocyst cell numbers (Fig. 2 E-H) compared with control.

Discussion

The present study was undertaken to determine whether the addition of CSF-1 or 3 during IVM can improve oocyte maturation. To examine this hypothesis, we undertook dose response experiments for both cytokines using doses shown previously to improve embryo development when added during in vitro culture [4, 6]. The effect of both cytokines on IVM was determined by examining cumulus expansion, cleavage and blastocyst development.

The major finding from this study was that both CSF-1 concentrations tended to increase ICM number. Although preliminary, this is an important finding as ICM number has

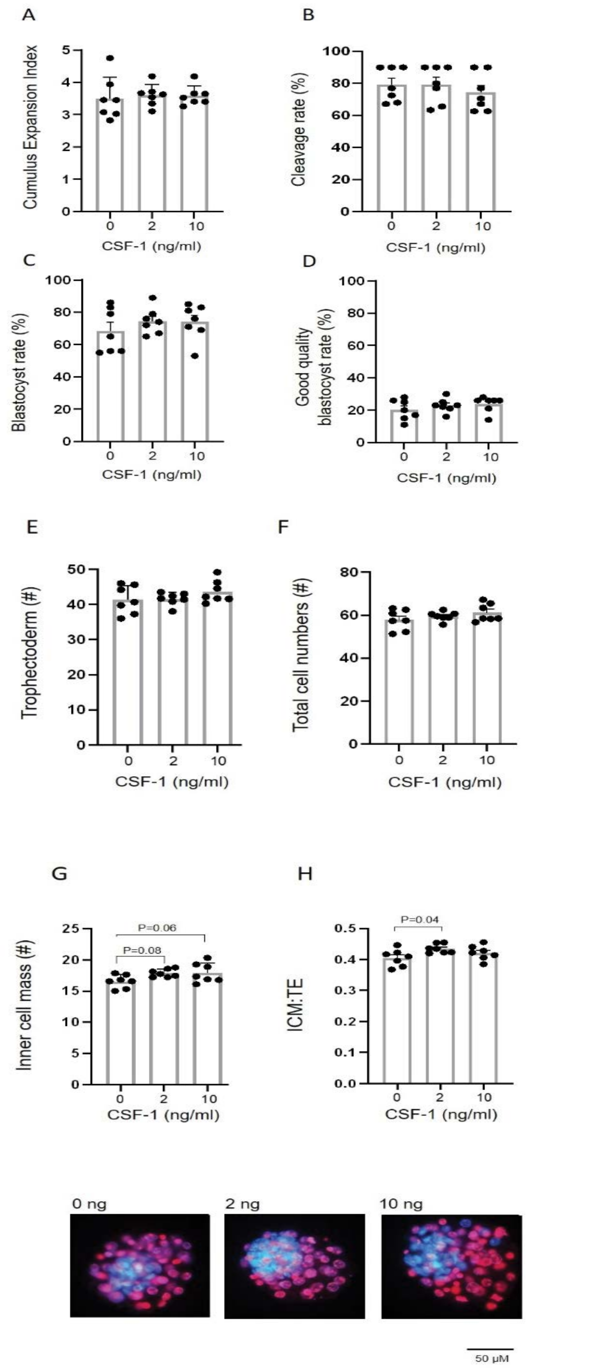

Figure 1: Oocyte maturation and embryo development following CSF-1 treatment during IVM. The addition of 2 and 10 ng/ ml of CSF-1 did not affect cumulus expansion (A), cleavage or blastocyst rates (B and C), or the number of good quality blastocysts at 96 h post fertilization (D). There was no difference in TE cell numbers (E), or total cell numbers (F) between treatment groups. Blastocyst ICM numbers tended to be increased in both CSF-1 treatment groups compared with the control group (G). The ratio of ICM:TE cell numbers was increased in the 2 ng/ml CSF-1 group (H) compared with the control. Values are expressed as percentage of total oocytes and are the mean ± SEM of seven replicates, with a minimum of 35 oocytes per group for each replicate. Representative images are differentially stained blastocysts for control and treatment groups.

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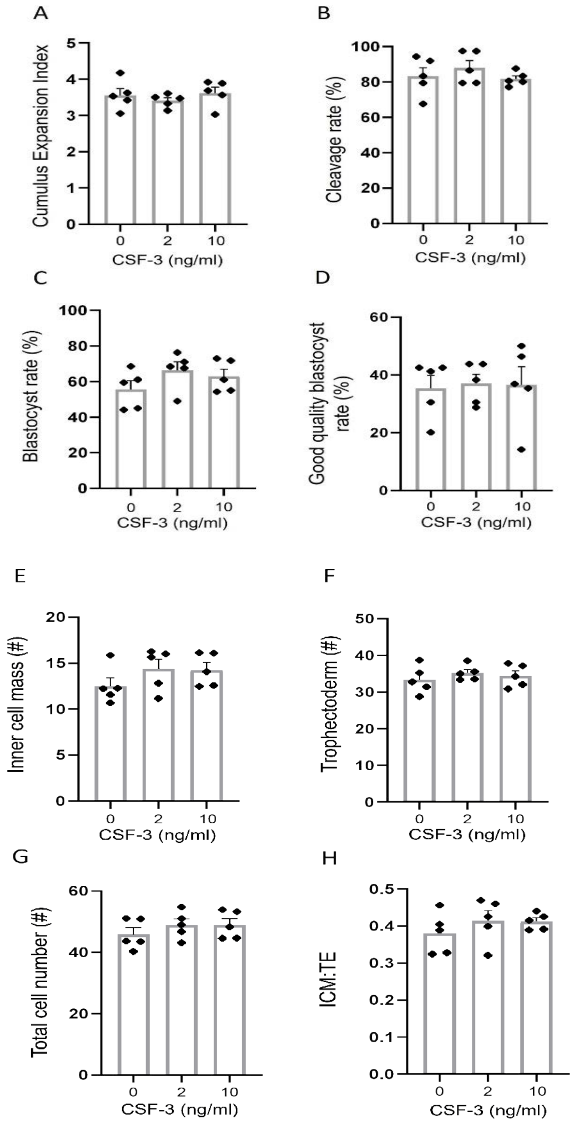

Figure 2: Oocyte maturation and embryo development following CSF-3 treatment during IVM. There was no effect of CSF-3 addition to IVM on cumulus expansion (A), cleavage or blastocyst rates (B and C), or the number of good quality blastocysts at 96 h post fertilization (D) compared with the control. CSF-3 did not have any effect on ICM cell numbers (E), TE cell numbers (F), total cell numbers (G), or the ratio of ICM to TE cell numbers (H). Values are expressed as percentage of total oocytes and are the mean ± SEM of five replicates, with a minimum of 35 oocytes per group for each replicate.

been previously correlated with implantation and birth rates in a range of species [14, 15]. We also showed that 2 ng/ml significantly increased ICM to TE ratio suggesting that cell allocation is influenced by CSF-1 during oocyte maturation. Further studies are required to confirm our initial findings and to determine whether these improvements increase implantation and birth rates, as follicular concentrations of CSF-1 have been correlated with human IVF outcomes [12].

Interestingly, the addition of CSF-1 during embryo culture has been previously shown to increase trophectoderm and total embryo cell numbers, with no increase in ICM cell numbers in mice [4], This suggests that CSF-1 during IVM influences embryo development differently compared with that for embryo culture, suggesting CSF-1 may have additive effects on blastocyst development, in particular cell numbers and also warrants further investigation .

CSF-3 had no effect on any of the parameters measured in the present study suggesting that it does not influence IVM in our mouse model. In contrast, Cai et al. [17] showed that the addition of 10 ng/ml of human CSF-3 during IVM increased fertilisation and blastocyst rate in oocytes derived from medium follicles in the pig. In contrast, Jannaman et al. [18] demonstrated that the addition of 1 and 10 ng/ml of CSF-3 during IVM, had no effect on fertilisation or blastocyst rate in cattle. The reason for these differences between these species is unclear. However, this may reflect differences in CSF-3 receptor expression between species. The CSF-3 receptor has been found to be present on porcine oocytes and cumulus cells [6], while its expression has been found to be non-existent in bovine oocytes [17], and at very low levels in bovine cumulus cells [18].

Conclusion

In conclusion our results suggest that CSF-1 may improve IVM outcomes. However, evidence to support a role for CSF-3 in improving IVM remains equivocal. Further studies examining the expression of CSF-1 and 3 and their receptors in oocytes and cumulus cells may provide further insights into their role in oocyte maturation as well as a rationale for using these to improve IVM outcomes.

Conflict of interest

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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