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Effect of cyanocobalamin on oocyte maturation, *in vitro* fertilization, and embryo development in mice

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Research Article

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Summary

The aim of this study was to investigate the effect of cyanocobalamin supplementation on *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and subsequent embryonic development competence to the blastocyst stage, and *in vitro* development of mouse 2-cell embryos. Cumulus cells were prepared from mouse cumulus–oocyte complexes (COCs) and incubated for 24 h in an *in vitro* culture (IVC) medium that contained different concentrations of cyanocobalamin (100, 200, 300 or 500 pM). We collected 2-cell embryos from superovulated NMRI mice and cultured them in the same concentrations of cyanocobalamin (100, 200, 300 or 500 pM). After 42 h of IVM, we observed significantly increased oocyte maturation in the 200 pM cyanocobalamin-treated group compared with the control group ($P < 0.0001$). Mature oocytes cultured in 200 pM cyanocobalamin were fertilized and cultured in IVC medium with cyanocobalamin (100, 200, 300 or 500 pM) during early embryogenesis. The matured oocytes that were cultured in 200 pM cyanocobalamin had significantly higher 2-cell development rates compared with the control oocytes ($P < 0.01$). Embryos obtained from *in vitro* mature oocytes and *in vivo* fertilized oocytes that were cultured in 200 pM cyanocobalamin had significantly greater frequencies of development to the blastocyst stage and a significant reduction in 2-cell blocked and degenerated embryos compared with the control embryos ($P < 0.0001$). Embryos derived from oocytes fertilized *in vivo* with 200 pM cyanocobalamin had a higher percentage of blastocyst embryos compared with those derived from matured oocytes cultured *in vitro* ($P < 0.0001$). These finding demonstrated that the effects of cyanocobalamin on oocyte maturation, fertilization, and embryo development in mice depend on the concentration used in IVC medium.

Introduction

Embryo culture is an important aspect of assisted reproductive technology (ART) because it allows the zygote to mature, divide, and reach a stage where it can be transferred to the uterus (Cagnone and Sirard, 2016). It is important to optimize the composition of embryo culture media and settings for incubation during *in vitro* development to improve the quality of these embryos (Lan *et al*., 2019). Suboptimal conditions for cultivation often decrease embryonic output during early development due to inadequate control of homeostasis (Summers and Biggers, 2003). Despite the widespread use of embryo culture medium, *in vitro* culture (IVC) continues to develop embryos of lower viability relative to natural conception (Cagnone and Sirard, 2016), therefore it is essential to improve IVCs to reduce the effect of ART on embryo viability.

Oocyte maturation appears to be of key importance for oocyte competence. Follicular fluid (FF) prepares an extremely important microenvironment for oocyte development. Follicular fluid reflects metabolic and hormonal processes that occur in the maturing oocyte microenvironment prior to ovulation and is a predictor of outcome parameters such as fertilization, embryo cleavage, and pregnancy rates of *in vitro* fertilization (IVF) (Wiener-Megnazi *et al*., 2004). Consequently, certain biochemical characteristics of the FF that surrounds the oocyte play a critical role in determining oocyte quality and the subsequent potential for fertilization and development of the embryos.

Reactive oxygen species (ROS) develop inside the follicle, particularly during the ovulation phase (Sugino, 2005), and it is presumed that oxidative stress is responsible for poor oocyte quality (Sugino, 2006). Early mammalian embryos cultured *in vitro* increase the level of oxygen free radicals and it is believed to play a crucial role in IVF success (Sellens *et al*., 1981; Agarwal *et al*., 2006b). ROS sources in the culture medium originate from embryo metabolism and the

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culture environment (Agarwal *et al.*, 2006a). However, certain levels of ROS are required for sperm function, normal ovarian follicle activity, oocyte maturation, normal sperm–oocyte interactions, and sperm capacitation, in addition to compaction, blastocyst development, and implantation (Riley and Behrman, 1991; Agarwal and Gupta 2005; Agarwal *et al.*, 2006a; Gupta *et al.*, 2006). It has been reported that minimal oxidative stress levels are beneficial for adequate growth and development of the embryo (Agarwal and Gupta, 2005). However, excessive amounts of oxidative stress may have adverse effects on sperm DNA, fertilization, and embryo quality and they may block or retard early embryonic development, damage the embryo cell membrane, alter the mitochondria, and result in apoptosis (Noda *et al.*, 1991; Gupta *et al.*, 2006; Askoxylaki *et al.*, 2013; Lan *et al.*, 2019). Embryos in commercial culture media produce ROS at different levels, depending on the media composition (Martín-Romero *et al.*, 2008; Shih *et al.*, 2014). Therefore, IVC could be optimized by using antioxidants to reduce the effects of ART on embryo survival and quality.

Most vitamins act as antioxidants and prevent oxidative stress from mammalian cells (Sinbad *et al.*, 2019). Antioxidant vitamins help reduce oxidant damage by acting as waste electron sinks (Thiyagarajan and Valivittan, 2009). Vitamin B_{12} (B_{12}), or cobalamin, is a water-soluble vitamin that functions as a coenzyme in various biochemical reactions such as methionine synthesis and the metabolism of branched amino acids (Van De Lagemaat *et al.*, 2019). Cyanocobalamin is the form of B_{12} that is typically used in vitamin supplements (Hamedani *et al.*, 2013) and it plays a role in cell replication and DNA synthesis (Mello *et al.*, 2018). Cyanocobalamin is currently used as a treatment for human infertility (Gaskins *et al.*, 2015). Additionally, research has shown that B_{12} is a crucial nutrient for fetal development (Reese Pepper and Black, 2011). Bovine and sheep studies have shown that administration of cobalamin during the time of oocyte growth and post-fertilization development to the blastocyst stage plays an important role in the offspring's health (Sinclair *et al.*, 2007; Kwong *et al.*, 2010). Zacchini and colleagues reported that cobalamin supplementation during *in vitro* maturation (IVM) improved the developmental competence of sheep oocytes (Zacchini *et al.*, 2017). Roy and colleagues demonstrated that cobalamin treatment prior to IVM improved the developmental competence of porcine oocytes (Roy *et al.*, 2017). It was also shown that cyanocobalamin supplementation had positive effects during the thawing of frozen boar semen on spermatozoa, IVF, and embryonic development (Mello *et al.*, 2018).

Of note, only the optimal concentration of cobalamin supplementation can prevent the active forms of oxygen generation and membrane lipid peroxidation and scavenge against ROS (Hamedani *et al.*, 2013). The effect of cyanocobalamin has not been evaluated in the development of mouse embryos through its supplementation in maturation and embryo culture medium; therefore, the aim of this study was to evaluate effects of cyanocobalamin supplementation in various concentrations on *in vitro* mouse maturation, fertilization and embryo development, and to determine the optimum concentration of this vitamin.

Materials and methods

Adult female (6–10-week-old) and male (8–12-week-old) NMRI mice were maintained according to the Guidelines for the Care and Use of Laboratory Animals. All mice were kept in cages with standard conditions of 12 h : 12 h, light : dark and a temperature 22 ± 2°C.

Effect of cyanocobalamin on *in vitro* maturation (IVM)

Ovulation was induced in the female mice with intraperitoneal (i.p.) injections of 5 IU pregnant mare serum gonadotropin (PMSG; Sigma-Aldrich, Saint Louis, MO, USA). At 48 h after the injection, cumulus–oocyte complexes (COCs) at the germinal vesicle (GV) stage (Fig. 1*Ca*) were aspirated from the ovaries by puncturing the follicles with a sterile 28-gauge needle. The COCs were washed three times in maturation medium droplets that included αMEM (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 7.5 IU/ml recombinant human follicular stimulating hormone (rhFSH, Sigma-Aldrich, Saint Louis, MO, USA) and 100 IU/ml human chorionic gonadotropin (hCG, Sigma-Aldrich, Saint Louis, MO, USA). The immature oocytes were divided into five groups according to the dose of cyanocobalamin (Sigma-Aldrich, Saint Louis, MO, USA) administered [0 (control) and 100, 200, 300 and 500 pM]. The oocytes in each group were exposed to cyanocobalamin for 24 h, after which we assessed viability in metaphase M II (Fig. 1*Cb*) with an inverted microscope.

Effect of cyanocobalamin on IVF and development of embryos

The matured oocytes (Fig. 1*Cb*) were assessed by IVF. Sperm were collected from the cauda epididymides of the fertile NMRI males and allowed to capacitate for 1 to 1.5 h at 37°C. The sperm were subsequently diluted in human tubal fluid (HTF; Millipore) to a final concentration of 0.7–1.3 × 10^6 sperm/ml. The collected matured oocytes in the control and 200 pM cyanocobalamin groups were incubated with spermatozoa for 4 h and then washed to remove the excess spermatozoa. The oocytes were cultured overnight in separate dishes that contained a drop of modified potassium simplex optimized medium (KSOM Sigma-Aldrich, St. Louis, MO, USA) supplemented with the 100, 200, 300, and 500 pM concentrations of cyanocobalamin. At 24 h after fertilization, we determined the percentage of 2-cell embryos to evaluate the fertilization rate. The embryos were subsequently cultured at 37°C and 5.5% CO_2 until they reached the blastocyst stage.

Superovulation

Each female mouse was superovulated by an i.p. injection of 8 IU PMSG, followed by an injection of 7.5 IU hCG 48 h later.

Two-cell embryo collection for the *in vitro* study

In vivo embryos at the 2-cell stage were obtained from superovulated female mice that were individually mated with fertile males of the same strain. The following morning, successful mating was confirmed by the presence of a vaginal plug in the female mice. This was considered to be gestation day 1. The 2-cell embryos were flushed and collected with M2 (Sigma-Aldrich, St. Louis, MO, USA) medium supplemented with bovine serum albumin (BSA) on day 2 post-coitum (p.c.), 48 h after hCG administration. The 2-cell embryos that had normal morphology, which consisted of equal-sized blastomeres with no apparent fragmentation as observed under an inverted light microscope, were divided into five groups – control and 100, 200, 300 and 500 pM of cyanocobalamin.

Statistical analysis

The data were analyzed by Tukey's post-hoc test using one-way analysis of variance (ANOVA), and are presented as mean ± standard deviation (SD). The differences in the values of maturation,

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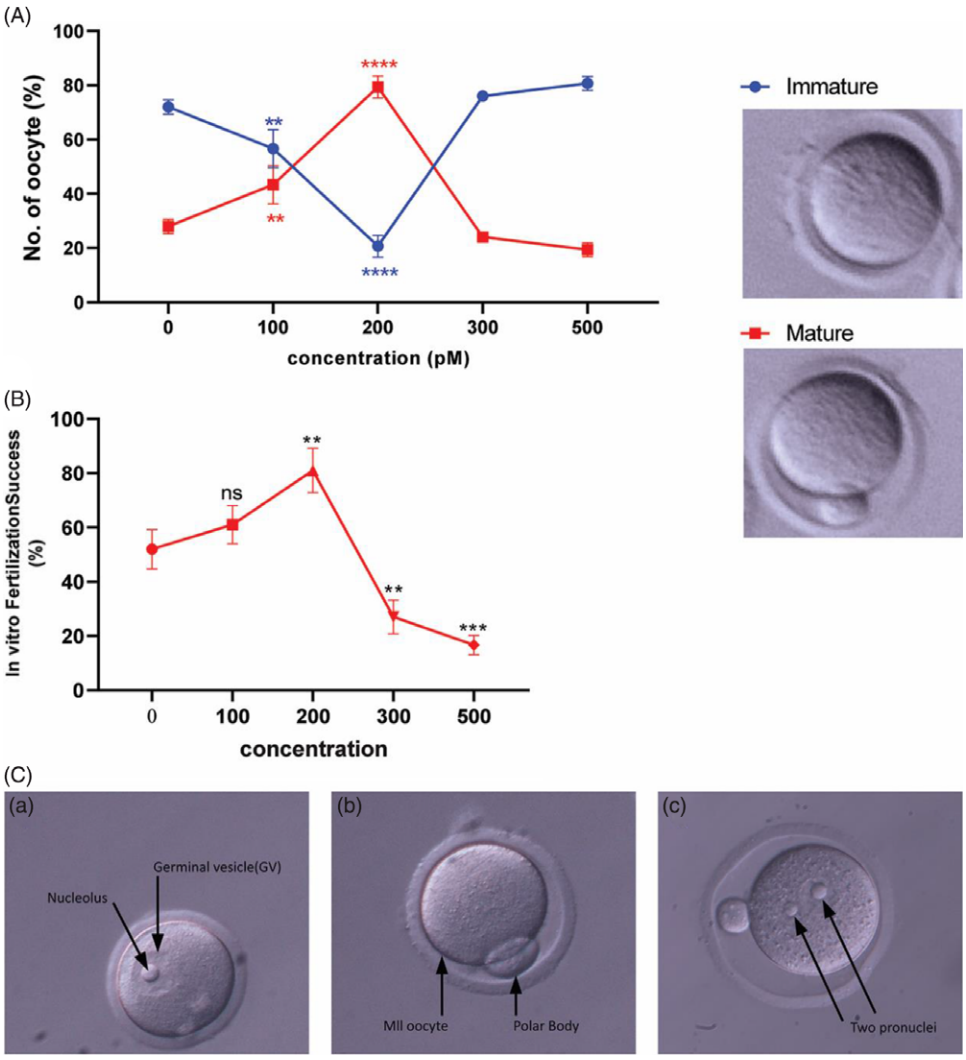

Figure 1. (*A*) Effect of cyanocobalamin on nuclear maturation during in *vitro* maturation in mouse oocytes $^{**}P < 0.001$, $^{***}P < 0.0001$. (*B*) Effect of cyanocobalamin on *in vitro* fertilization success $^{**}P < 0.01$, $^{***}P < 0.001$. Data are expressed as mean ± SEM. (*C*) Different stages of mouse oocyte after *in vitro* maturation and fertilization. (*Ca*) Germinal vesicle (GV, mature oocyte). (*Cb*) Extruding the first polar body of mouse oocyte. (*Cc*) Fertilized mouse oocyte.

fertilization, and developmental rates were considered to be significant at $P < 0.05$. For Windows, all computations were performed using Statistical Package for the Social Sciences (SPSS) 25. All data are presented as mean ± SD in the graphs from a minimum of three independent experiments.

Results

Effect of cyanocobalamin on nuclear maturation of mouse oocytes

Figure 1(*A*) shows the effect of cyanocobalamin during IVM on nuclear maturation of mouse oocytes. The mature oocytes were evaluated at the MII stage, which was morphologically characterized by germinal vesicle breakdown (GVBD) and the release of a distinct first polar body (Fig. 1*Cb*).

The 100 pM (43.33%, $P < 0.001$) and 200 pM (79.33%, $P < 0.0001$) cyanocobalamin groups had significantly more MII oocytes compared with the control group. Treatment with 100 pM cyanocobalamin significantly reduced oocyte maturation compared with the 200 pM cyanocobalamin group ($P < 0.0001$). Although the 300 pM (24%) and 500 pM (19.33%) cyanocobalamin groups had fewer immature oocytes than the control group (28%), these findings were not significant (Fig. 1*A*).

IVF and development of mouse embryos

The success rate for IVF (Fig. 1*Cc*) was measured by the number of embryos that entered the 2-cell stage. As shown in Fig. 1(*B*), high IVF success rate was observed in the 200 pM cyanocobalamin treatment group compared with other groups ($P < 0.01$).

Table 2 and Fig. 2(*A*) show that the morula (Fig. 2*Bb*) rates were significantly different between the 100 pM cyanocobalamin (29.33%) group and the control group ($P < 0.005$). The rate of embryo development to the blastocyst stage (Fig. 2*Bc*) was greater in the 100 pM cyanocobalamin group (37.33%) compared with the control group (32.67%), however this finding was not significant.

The results of our study demonstrated that mouse embryos cultured in the medium that contained 200 pM cyanocobalamin (49%) developed faster and had increased development to the blastocyst stage compared with mouse embryos cultured in the control group (32.67%; $P < 0.0001$). The 200 pM cyanocobalamin group compared with the 100 pM cyanocobalamin group had the lowest percentage of 2-cell (6%; $P < 0.01$) and degenerated embryos (8.67%; $P < 0.01$). In the 200 pM cyanocobalamin group, we observed the development of the 4-cell (2.3%), 8-cell (6%, $P < 0.05$), and morula (28%, $P < 0.01$) stages. This finding suggested that the 100 pM cyanocobalamin group delayed embryonic cleavage when compared with the 200 pM group (Table 1 and Fig. 2*A*).

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Table 1. Assessment of the effect of different concentrations of cyanocobalamin on the percentage of early mouse embryos *in vitro*

	 2-cell stage	 4-cell stage	 8-cell stage	 Morula	 Blastocyst	 Degenerated
0 (control)	14.00 ± 1.00	4.33 ± 2.08	12.33 ± 1.15	20.00 ± 2.00	32.67 ± 4.16	16.67 ± 1.53
100 pM	8.33 ± 1.53	2.33 ± 0.58	10.33 ± 1.53	29.33 ± 3.51***	37.33 ± 3.79	12.33 ± 1.53
200 pM	6.00 ± 0.00**	2.33 ± 2.52	6.00 ± 1.00*	28.00 ± 1.73**	49.00 ± 1.00****	8.67 ± 0.58**
300 pM	29.67 ± 2.52****	5.67 ± 1.15	1.00 ± 1.00****	7.00 ± 2.00****	16.33 ± 4.04****	40.33 ± 7.77****
500 pM	31.33 ± 3.06****	0.00 ± 0.00	0.00 ± 0.00****	0.00 ± 0.00****	0.00 ± 0.00****	68.67 ± 3.06****

*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.005$ and ****: $P < 0.0001$: significant differences within the same column with control. Data are presented as mean ± SEM.

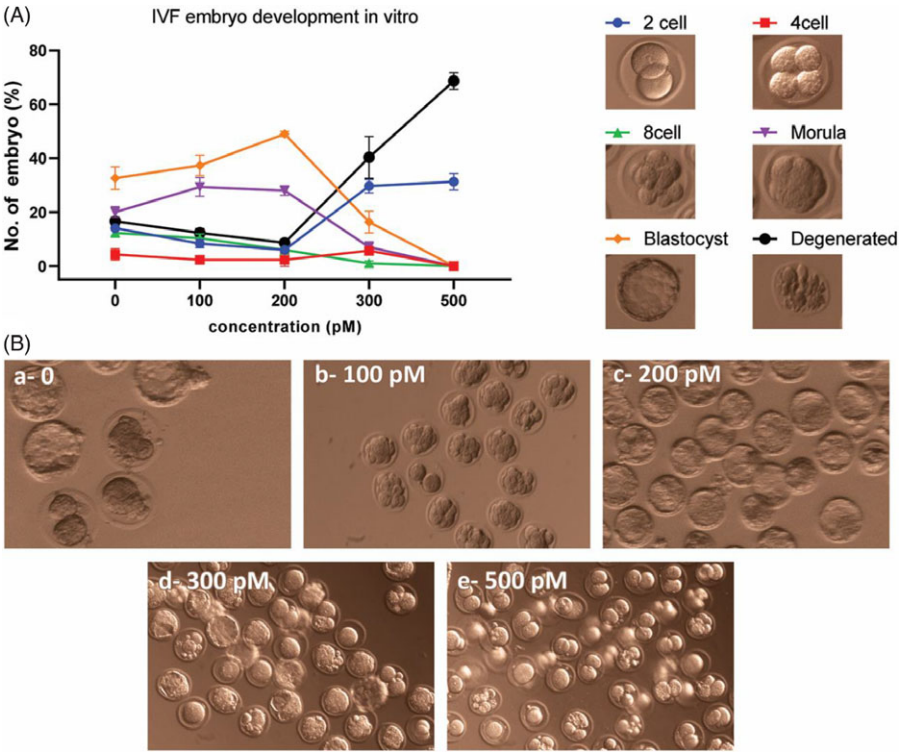

Figure 2. (*A*) Effect of cyanocobalamin during early embryogenesis after *in vitro* fertilization. Each point represents the mean ± SEM of four repeats. *P*-values are shown in Table 1. (*B*) Bright field photographs of embryos cultured under different concentrations of cyanocobalamin.

There were significant differences between the 300 pM cyanocobalamin and control groups in terms of the 2-cell (29.67 % vs 14%), 8-cell (1% vs 12.33%), morula (7% vs 20%), blastocyst (16.33% vs 32.67%), and degenerated (40.33% vs 16.67%) embryos ($P < 0.0001$) (Table 1 and Fig. 2*A*, *Bd*). The 500 pM cyanocobalamin group had similar 8-cell, morula, and blastocyst rates (0%; $P < 0.0001$) (Table 1 and Fig. 2*A*, *Be*). However, the 300 and 500 pM cyanocobalamin groups yielded similar results (29.67% and 28.9%, respectively) in terms of blocking to the 2-cell stage (Table 1 and Fig. 2A). However, the 500 pM cyanocobalamin group had a significantly higher 2-cell block and degenerated embryos compared with the other groups ($P < 0.0001$) (Table 1 and Fig. 2*A*).

There were no significant differences in the rate of the 4-cell stage between the four different treatment groups compared with the control group at the end of the culture period (Table 1 and Fig. 2*A*).

The effect of cyanocobalamin on 2-cell mouse embryo development *in vitro*

The developmental rates of 2-cell embryos in medium that contained different concentrations of cyanocobalamin are shown in Table 2 and Fig. 3. The embryos cultured in 100 pM cyanocobalamin had a significantly greater frequency of development to the morula stage (29%) compared with the control group (23%, $P < 0.05$) and blastocyst stage (52%) compared with the control group (39.5%, $P < 0.0001$) (Table 2 and Fig. 3*A*, *Ba*, *Bb*). We also observed a significant decrease in 2-cells blocked in the 100 pM group (3%) compared with the control group (14.5%, $P < 0.0001$), in the 8-cell embryos (9%) compared with the control group (14%), and degenerated embryos (7%) compared with the control group (8%) (Table 2 and Fig. 3*A*).

Treatment with 200 pM cyanocobalamin during early embryogenesis increased the blastocyst rate (70%) of embryos compared

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Table 2. Assessment of the effect of different concentrations of cyanocobalamin on the development of mouse 2-cell embryos *in vitro*

	 2-cell stage	 4-cell stage	 8-cell stage	 Morula	 Blastocyst	 Degenerated
0 (control)	14.50 ± 3.54	1.00 ± 1.41	14.00 ± 1.41	23.00 ± 1.41	39.50 ± 0.71	8.00 ± 4.24
100 pM	3.00 ± 2.83****	0.00 ± 0.00	9.00 ± 2.83	29.00 ± 2.83*	52.00 ± 1.41****	7.00 ± 1.41
200 pM	1.00 ± 1.41****	0.00 ± 0.00	2.50 ± 2.12****	24.00 ± 1.41	70.00 ± 2.83****	2.50 ± 0.71
300 pM	46.50 ± 2.12****	4.00 ± 1.41	4.00 ± 2.83***	13.50 ± 2.12***	7.00 ± 1.41****	25.00 ± 2.83****
500 pM	58.50 ± 0.71****	4.50 ± 2.12	0.00 ± 0.00****	0.00 ± 0.00****	0.00 ± 0.00****	37.00 ± 1.41****

*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.005$ and ****: $P < 0.0001$: significant differences within the same column with control. Data are presented as mean ± SEM.

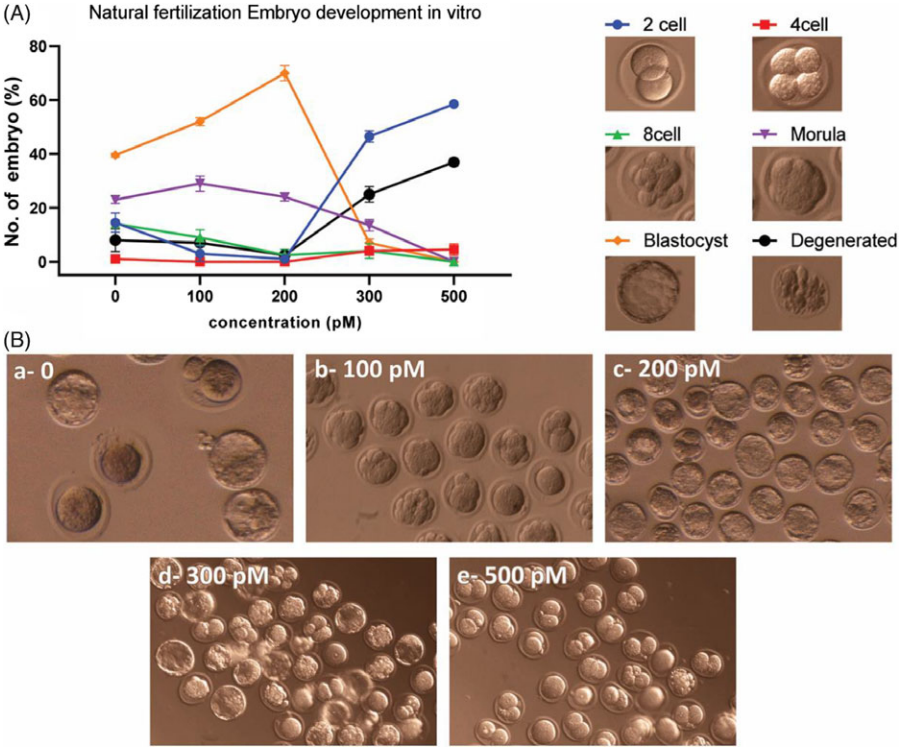

Figure 3. (*A*) Effect of different concentrations of cyanocobalamin on the development of mouse two-cell embryos *in vitro*. Each point represents the mean ± SEM of three repeats. *P*-values are shown in Table 2. (*B*) Morphology of embryos resulted from different concentrations of cyanocobalamin.

with the control group (39.5%; $P < 0.0001$). There were no significant differences in the percentages of morula (24%) and degenerated (2.5%) embryos compared with the control group (Table 2 and Fig. 3*A*, *Bc*). The blastocyst rate of embryos increased in the 200 pM cyanocobalamin group (70%) during late embryogenesis when compared with the 100 pM cyanocobalamin group (52%; $P < 0.0001$). The lowest percentage of blocked 2-cell (1% vs 14.5%) and 8-cell (2.5% vs 14%) embryos was observed in the 200 pM cyanocobalamin group ($P < 0.0001$) (Table 2 and Fig. 3*A*).

The percentage of the 2-cell blocked (46.5% vs 14.5%) and degenerated (25% vs 8%) embryos were significantly higher in the 300 pM cyanocobalamin group compared with the culture medium alone ($P < 0.0001$). In the 300 pM cyanocobalamin group, there were significant differences in the embryos that reached the 8-cell (4%), morula (13.5%), and blastocyst (7%) stages compared with the control group ($P < 0.0001$) (Table 2 and Fig. 3*A*, *Bd*).

There were significantly more embryos in the 2-cell block and degenerated in the 500 pM cyanocobalamin group compared with the control and the other treatment groups ($P < 0.0001$). There were no embryos observed in the 8-cell, morula, and blastocyst stages in the 500 pM cyanocobalamin group (Table 2 and Fig. 3*A*, *Be*). No significant differences existed in the rate of embryos that reached the 4-cell stage between the four different treatment groups compared with the control group at the end of the culture period (Table 2 and Fig. 3*A*).

Discussion

Frequently, in routine IVF, a few morphologically mature MII oocytes obtained from mature follicles remain unfertilized or, if fertilized, they result in low quality embryos under the same culture conditions (Rizzo *et al.*, 2010). Follicular fluid constitutes

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the actual pre-fertilization environment of the mature oocyte and may affect IVF outcome parameters of fertilization, embryo cleavage, and pregnancy rates (Agarwal *et al.*, 2003). Follicular fluid, as well as granulosa cells, growth factors and steroids hormones include leukocytes, cytokines and macrophages, all of which can produce ROS (Attaran *et al.*, 2000). ROS may be produced by the environment, impaired oocyte metabolism, or both (Rizzo *et al.*, 2010). Pre-treatment with cobalamin has a positive effect on the microenvironment of the maturing oocyte by reducing total homocysteine (Hcy) concentrations in the pooled FF and increasing follicular diameter (Rizzo *et al.*, 2010). Studies have shown that Hcy mediates ROS accumulation (Van De Lagemaat *et al.*, 2019). The potential antioxidant properties of cobalamin can be attributed to direct scavenging of ROS and reduction of Hcy-induced oxidative stress (Van De Lagemaat *et al.*, 2019). Glutathione plays a significant role in oocyte maturation (Luberda, 2005) and is essential for fertilization, preimplantation, and embryonic development (Nakamura *et al.*, 2011). The results of studies indicate that elevated levels of GSH in matured mouse and hamster oocytes are needed to form the male pronucleus after fertilization and for early embryo development (Gardiner and Reed, 1994; Zuelke *et al.*, 2003). There is significantly less glutathione in matured *in vitro* oocytes compared with those matured *in vivo* (Luberda, 2005). Cobalamin can indirectly stimulate ROS scavenging by maintaining glutathione levels (Van De Lagemaat *et al.*, 2019). Oxidative stress causes a negative impact on fertilization and the *in vitro* preimplantation developing embryo (Lopes *et al.*, 2010). During IVF, gametes and embryos are in culture media (Martín-Romero *et al.*, 2008) that contain serum or serum synthetic replacements (SSR), albumin, vitamins, and other elements such as buffers or heavy metal chelators. Consequently, the medium itself can be a source of ROS during handling and culture (Burton *et al.*, 2003). Several researchers have reported that high intracellular ROS concentrations have adverse effects during maturation and early cleavage, and result in changes in chromosome segregation during meiosis, disrupted fertilization, 2-cell embryo blockage, and low pregnancy levels (Martín-Romero *et al.*, 2008). During the IVC of preimplantation embryos, the absence of maternal antioxidants also leads to a breakdown in the balance between ROS production and clearance, and results in developmental arrest. Therefore, antioxidants are commonly monitored during the cultivation of preimplantation embryos to remove excessive ROS (Ye *et al.*, 2017).

The present study showed that exposure of mouse oocytes to 200 pM cyanocobalamin improved progression of maturation in mouse oocytes. We also observed an increased fertilization rate following IVF in the 200 pM cyanocobalamin group, which was associated with quantitative improvement in blastocysts and the rate of blastocysts from matured oocyte *in vivo*. Minimizing oxidative stress is especially important when IVF is performed for assisted reproduction (Tsunoda *et al.*, 2014).

The 2-cell block identified functionally the failure of fertilized eggs to develop *in vitro* after the 2-cell stage in the medium (Biggers, 2004). Arrest of embryos developed *in vitro* is commonly seen in several strains of mice and other species (Betts and Madan, 2008). The increased levels of ROS in mice were related to the 2-cell embryo block (Nasr-Esfahani *et al.*, 1990). The 2- to 4-cell embryos had immature (undifferentiated) mitochondria and elevated intracellular ROS levels (Betts and Madan, 2008). An immature mitochondrial electron transport system can also result in increased ROS production, which leads to the formation of superoxide (Kimura *et al.*, 2010). As mentioned earlier, one of the most important potential antioxidant properties of cobalamin is direct scavenging of ROS, specifically superoxide (Van De Lagemaat *et al.*, 2019). We found that the rates of 2-cell block and degenerated embryos were substantially reduced in the presence of 200 pM cyanocobalamin. Subsequently, the current study found that doses higher that 200 pM cyanocobalamin (300 and 500 pM) significantly decreased early embryo developmental potential and increased the amount of degenerated embryos. As well, treatment with 100 pM cyanocobalamin showed a slight increase in the embryo development rate compared with the control. When the concentration of cyanocobalamin was increased to 200 pM, we observed a protective effect of cyanocobalamin on embryo development. These data suggested that cyanocobalamin has a dose-dependent antioxidant feature.

Hormesis refers to a biphasic dose response to an environmental agent with a low-dose stimulus or beneficial effect and a high-dose inhibitory or harmful effect (Kendig *et al.*, 2010; Assadollahi *et al.*, 2019). It has been shown that vitamins, including B_{12}, are hermetic nutrients (Hayes, 2007). The results of the present study agree with findings of previous studies. In our study, we observed that oocytes and embryos were healthier in the 200 pM cyanocobalamin dose. According to previous studies, the optimal amount of cobalamin is 200 pM (Hannibal *et al.*, 2016; Green *et al.*, 2017). Zacchini and colleagues reported that 200 pM of cobalamin supplementation during IVM improved the developmental competence of sheep oocytes (Zacchini *et al.*, 2017). Excessive antioxidants are ineffective and often have harmful effects (Tsunoda *et al.*, 2014). It appears that high doses of antioxidants serve as enzyme inhibitors and mutagens by inhibiting topoisomerases, proteasome synthesis, or the synthesis of fatty acids (Crespo *et al.*, 2008, Sameni *et al.*, 2018).

Improving the formulation of the embryo culture medium leads to an increase in the ability of mammalian embryos to survive *in vitro* and preimplantation (Farin *et al.*, 2001). The culture system and the compounds used in it could affect the quality of the embryo, while the quality of the oocyte is considered to be the main determinant of blastocyst function (Rizos *et al.*, 2002). However, the environment to which the embryos are exposed in the preimplantation stage plays an important role in determining blastocyst quality (Pereira *et al.*, 2005).

In conclusion, the present study demonstrated that supplementation of 200 pM cyanocobalamin in IVC medium enhanced nuclear maturation of oocytes, IVF, and embryo development in mice. However, higher concentrations of cyanocobalamin (300 and 500 pM) in IVC negatively affected oocyte maturation, IVF, and embryo development. Therefore, our findings suggest that 200 pM cyanocobalamin might improve *in vitro* production systems of mouse embryos.

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Conflicts of interest. The authors declare they have no competing financial interests.

Ethical standards. The experiments were conducted with the approval of the Ethics Committee of the Kurdistan University of Medical Sciences.

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