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The effects of cyanocobalamin supplementation during the thawing of frozen boar semen on spermatozoa, *in vitro* fertilization, and embryonic development

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Abstract

The objective of this study was to assess the *in vitro* fertilization (IVF) of pig oocytes using frozen-thawed boar sperm in media supplemented with cyanocobalamin. Frozen semen pellets were thawed and incubated for 1 h in fertilization media containing cyanocobalamin (0, 0.5, 1.0 or 2.0 µm) and evaluated for forward progressive motility, viability, and embryo cleavage. Forward progressive motility of the 0.5 and 1.0 µm cyanocobalamin supplements was higher ($P < 0.05$) than the 0 and 2.0 µm cyanocobalamin supplements. Membrane viability of sperm supplemented with 0.5 µm cyanocobalamin was higher ($P < 0.05$) than all other groups. Oocytes were matured and fertilized with frozen-thawed boar semen that was previously incubated for 1 h in fertilization media containing cyanocobalamin (0 or 0.5 µm; 100 oocytes/treatment). Fertilization characteristics were evaluated 12 h after IVF of oocytes and embryo development was analyzed at 48 h and 144 h post-IVF. There were no significant differences between treatment groups when evaluating fertilization, polyspermic penetration or male pronucleus development. Embryos derived from oocytes fertilized with 0.5 µm cyanocobalamin supplemented sperm had a higher percentage ($P < 0.05$) of cleaved embryos compared to those without cyanocobalamin supplementation at 48 h after IVF. There were no significant differences in the percentages of embryos reaching the blastocyst stage by 144 h after IVF between treatment groups. The results of this study suggest that there are positive effects of 0.5 µm cyanocobalamin supplementation during the incubation of frozen-thawed boar semen on early development of IVF derived pig embryos.

Keywords: antioxidants, cyanocobalamin, embryo, polyspermic, sperm.

Introduction

Reactive oxygen species (ROS) formed from the freezing and thawing of boar semen (Fraser and Strzeżek, 2005) reduce spermatozoa motility (Guthrie and Welch, 2012) and membrane function (Chatterjee and Gagnon, 2001), and increase nuclear DNA fragmentation (Fraser and Strzeżek, 2007; Whitaker *et al*., 2008). Creating a favorable environment for spermatozoa during *in vitro* fertilization (IVF) could improve the success rate of embryo production (Abeydeera, 2002; Casey *et al*., 2011).

Most vitamins can act as antioxidants and protect mammalian cells against oxidative stress (Dalvit *et al*., 2005), such as α-tocopherol (vitamin E), which has been shown to increase embryonic development in pigs when supplemented during oocyte maturation (Tao *et al*., 2010). Supplementation of α-tocopherol to boar semen during the freezing process (Jeong *et al*., 2009) increases subsequent capacitation and energy production in frozen-thawed spermatozoa (Satorre *et al*., 2009) and decreases DNA fragmentation during the thawing process (Whitaker *et al*., 2012).

Cyanocobalamin (vitamin B12) is active during cellular replication and DNA synthesis but may also protect α-tocopherol and is already being used as a treatment for male infertility in humans (Eskenazi *et al*., 2005). Additionally, research has shown that there is a positive correlation between the total cyanocobalamin concentration in seminal plasma and spermatozoa concentration in semen (Boxmeer *et al*., 2007). Supplementation of cyanocobalamin decreases the amount of ROS produced from oxidative stress in human semen (Chen *et al*., 2001a, b) in addition to improving post-thaw motility and membrane integrity during cryopreservation in rams and bulls (Ha and Zhao, 2003a, b; Hu *et al*., 2011).

Research has not been published focusing on the supplementation of cyanocobalamin to frozen-thawed boar spermatozoa prior to IVF. Therefore, the objective of this study was to determine if supplementation of 0, 0.5, 1.0 or 2.0 µm cyanocobalamin to frozen-thawed spermatozoa would affect IVF and embryonic development. Forward progressive motility, viability, penetration, polyspermic penetration, and male pronuclear (MPN) development were observed in addition to embryonic cleavage and blastocyst formation.

Materials and Methods

Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The oocyte maturation medium was tissue culture medium 199 (TCM199) with Earle's salts (Invitrogen, Carlsbad, CA, USA) supplemented with 5 µg/ml FSH, 1 µg/ml insulin, 50 ng/ml Gentamicin

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sulfate, 10 ng/ml EGF, and 10% fetal calf serum (v/v; FCS).

The IVF medium used was a modified Tris-buffered medium (mTBM; Abeydeera and Day, 1997) containing 113.1 mM NaCl, 3.0 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris, 11 mM D(+)-glucose, 5.0 mM sodium pyruvate, 1 mg/ml BSA (fraction V; 129K1977, initial fraction by heat shock) and 0.38 mg/ml caffeine.

The embryo culture medium used was NCSU 23 medium (Petters and Wells, 1993) containing 0.4% BSA (fraction V; 129K1977, initial fraction by heat shock). All media were filtered through a 0.2 mm pore HT tuffryn membrane Acrodisc (Fisher Scientific, Pittsburgh, PA, USA) and allowed to equilibrate at 39°C in an atmosphere of 5% CO_2.

Cyanocobalamin was purchased from Sigma-Aldrich Co. (V6629; 117K15209).

Spermatozoa preparation

Frozen semen pellets (International Boar Semen, Eldora, IA, USA) were thawed in Dulbecco PBS containing 0.1% BSA (w/v), 75 µg/ml potassium penicillin and 50 µg/ml streptomycin sulfate at 39°C and centrifuged at 36.3 x *g* for 5 min. The semen was then washed twice at 553 x *g* for 5 min. After washing, the spermatozoa pellet was re-suspended with mTBM to a concentration of 1.0 x 10^7 cells/ml and incubated for 2 h at 39°C in 5% CO_2. Sperm were analyzed for forward progressive motility using a phase-contrast microscope at 400X magnification. Viability/membrane integrity was assessed by staining spermatozoa with 0.6% Eosin red (w/v) and 5.0% Aniline blue (w/v) dye and then smeared on a microscope slide to determine viability. Spermatozoa that had intact membranes did not incorporate the dye and stained pink, whereas spermatozoa that had degraded membranes incorporated the dye and stained purple (Colenbrander *et al*., 1992).

Maturation of oocytes

Oocytes aspirated from mature follicles (3-6 mm diameter) were obtained commercially (Applied Reproductive Technologies, Madison, WI, USA) from adult sows (at least 18 months of age). Ovaries were transported to the laboratory in PBS containing 100 U/ml Penicillin G sodium and 100 mg/ml Streptomycin sulfate at 23-25°C. The average elapsed time between ovary collection and follicular aspiration was 5 h. At the laboratory, ovaries were washed three times in PBS and medium sized follicles (3-6 mm in diameter) were aspirated using an 18-gauge needle fixed to a 10-ml disposable syringe. Oocytes surrounded by a compact cumulus cell mass and uniform ooplasm were washed three times and shipped overnight to our laboratory in TCM199 at 39°C.

After 20-24 h from initial placement in media, oocytes (n = 300) were washed three times in TCM199 and randomly placed (45-50 oocytes/well) into 500 µl of TCM199 equilibrated at 39°C and 5% CO_2 for an additional 18-26 h under mineral oil without FSH or FCS. After incubation, cumulus cells were removed from the oocytes by repeat pipetting in TCM199 containing 0.1% hyaluronidase (w/v).

IVF and embryo culture

Prior to the end of oocyte maturation, sperm were diluted to a concentration of 4 x 10^5 cells/ml and incubated for 1.0 h at 39ºC in 5% CO_2 before 50 µl was added to each group of oocytes. The final concentration was approximately 2,000 sperm/oocyte. After 4-6 h of IVF, the zygotes were washed three times in embryo culture medium, placed (20 zygotes/well) into 500 µl of culture medium under mineral oil and incubated at 39°C in an atmosphere of 5% CO_2. Cleavage and blastocyst formation were evaluated 48 h and 144 h post-IVF, respectively, under a stereomicroscope.

Examination of fertilization

Approximately 12 h after IVF, oocytes (n = 100) were mounted and fixed with 25% acetic acid in ethanol (v:v) at room temperature. After 48 h of fixation, oocytes were stained with 1% orcein (w/v) in 45% acetic acid (v:v) and examined using a phase-contrast microscope at 400X magnification. Oocytes were characterized by visualization of polyspermic penetration and MPN formation.

Experimental design

The effects of supplementing 0.5, 1.0 or 2.0 µm cyanocobalamin to frozen-thawed spermatozoa were studied. Supplementations were added to the spermatozoa thawing (Dulbecco PBS) and mTBM prior to addition of the spermatozoa. The percent of forward progressive motile and membrane intact spermatozoa were determined 2 h after thawing. A total of 9 semen pellets from three 5.0 ml straws from the same boar and ejaculate were thawed for this study. Three samples from each pellet were used to evaluate forward progressive motility and morphology. A total of 100 sperm were counted from each sample.

The cyanocobalamin supplementation that elicited spermatozoa with the highest forward progressive motility and viability was used for the remainder of the study. Fertilization endpoints (n = 100 oocytes) measured were the number of oocytes penetrated, number of oocytes penetrated with a MPN, number of polyspermic oocytes, and the number of embryos (n = 200) cleaved at 48 h post-IVF and blastocysts at 144 h post-IVF.

Statistical analyses

Data in all experiments were analyzed by one-way ANOVA using the PROC ANOVA procedures of SAS (SAS Institute, Cary, NC, USA) because the data were balanced in all cases. When there was a significant effect of treatment, significant differences were determined using LSMEANS statement and Tukey adjustment for multiple comparisons. The effects included in the initial model were treatment, straw, pellet, and sample. Straw, pellet, and sample effects were not significant ($P > 0.05$) and deleted from the final model. In all analyses, a $P < 0.05$ was considered significant. Results are expressed as the least-squares mean ± SEM.

Results

Spermatozoa supplemented with 0.5 µm (57.0 ± 1.2%) and 1.0 µm (68.9 ± 5.0%) cyanocobalamin displayed higher ($P < 0.05$) forward progressive motility compared to no supplementation (control; 49.5 ± 6.2%) and 2.0 µm (60.0 ± 3.8%) cyanocobalamin supplemented spermatozoa. Viability of spermatozoa supplemented with 0.5 µm cyanocobalamin (77.2 ± 3.5%) was higher ($P < 0.05$) than all other treatment groups (Table 1).

Table 1. Effects of cyanocobalamin supplementation on boar spermatozoa characteristics.

Concentrations of cyanocobalamin[†]	Forward progressive motile cells (%)	Live spermatozoa (%)
0 µm	49.5 ± 6.2[a]	52.2 ± 12.3[a]
0.5 µm	57.0 ± 1.2[b]	77.2 ± 3.5[b]
1.0 µm	61.7 ± 5.0[b]	68.9 ± 4.7[a]
2.0 µm	45.3 ± 3.8[a]	60.0 ± 5.0[a]

[†]Treatment groups were the final concentration of cyanocobalamin supplemented to the spermatozoa thawing and incubation (IVF) media. [a,b]Means with different superscripts differ ($P < 0.05$). Data are expressed as mean ± SEM.

There was no significant difference in penetration rates between oocytes fertilized by sperm without cyanocobalamin supplementation (76.0 ± 17.3%) and sperm with 0.5 µm cyanocobalamin (62.0 ± 5.8%). No significant differences were observed in the percent of polyspermic oocytes obtained when fertilized by sperm without cyanocobalamin supplementation (36.8 ± 17.0%) and sperm with 0.5 µm cyanocobalamin (32.3 ± 9.4%), or MPN (36.8 ± 17.4% and 35.5 ± 9.7%, respectively). There was a higher ($P < 0.05$) percentage of embryos cleaved by 48 h after IVF when fertilized with the 0.5 µm cyanocobalamin supplemented spermatozoa (42.0 ± 3.1%) compared to the control group (34.0 ± 4.8%; Fig. 1). There were no significant differences in blastocyst formation by 144 h after IVF between treatment groups.

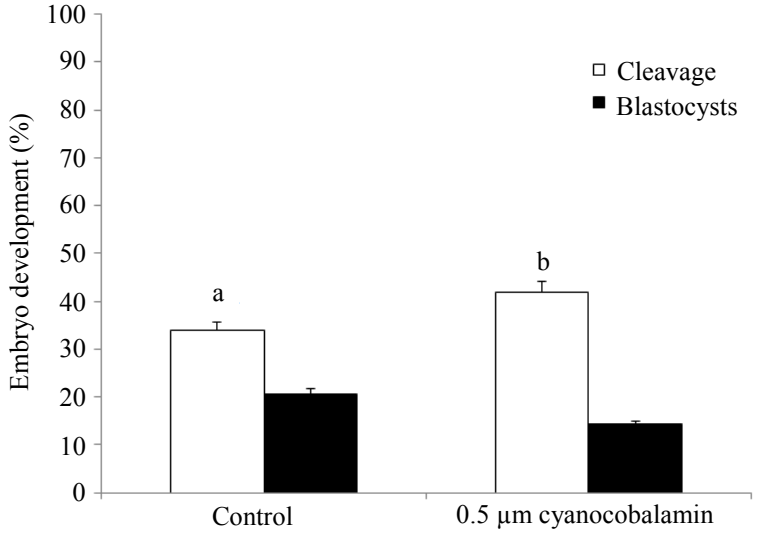

Figure 1. Effects of 0.5 µm cyanocobalamin supplementation during boar spermatozoa incubation on embryo development. Control (n = 100), no supplementation during spermatozoa incubation; 0.5 µm cyanocobalamin (n = 100), 0.5 µm cyanocobalamin supplemented to the spermatozoa during incubation; Cleavage, observed at 48 h after IVF; Blastocysts, observed at 144 h after IVF. [a,b]Means with different superscripts differ ($P < 0.05$). There were no significant differences in blastocyst production between groups. Data are expressed as mean ± SEM.

Discussion

The successful IVF of porcine oocytes is an arduous and intricate technique that continues to have inefficiencies in oocyte cytoplasmic maturation, monospermic penetration rates and culture conditions throughout the entire *in vitro* process (Abeydeera, 2002; Gil *et al*., 2010; Dang-Nguyen *et al*., 2011). Fertilization of the oocyte is subjected to spermatozoa quality and environmental conditions, both of which affect subsequent embryonic development (Wang *et al*., 2003; Gil *et al*., 2008). Research has shown that the processes associated with freezing and thawing boar semen generate above normal levels of ROS (Fraser and Strzeżek, 2005) which reduces spermatozoa quality (Bilodeau *et al*., 2000; Chatterjee and Gagnon, 2001; Whitaker *et al*., 2008; Guthrie and Welch, 2012).

Previous studies have shown that supplementation of antioxidants like cyanocobalamin to the freezing extender improves thawed spermatozoa quality and subsequent fertilization ability in cattle and pigs (Dalvit *et al*., 2005; Gadea *et al*., 2005; Malo *et al*., 2010). Although to our knowledge, this is the first report of supplementing cyanocobalamin to the thawing and IVF media of pigs. In agreement with those studies, our results showed that supplementing low doses (0.5 and 1.0 µm) of cyanocobalamin improved spermatozoa quality; specifically, it increased frozen-thawed spermatozoa motility and viability. Supplementing the thawed spermatozoa with 0.5 µm cyanocobalamin improved embryonic cleavage rates by 48 h after IVF compared to unsupplemented spermatozoa. However, cyanocobalamin supplementation did not improve MPN or decrease polyspermic penetration rates. The significant increase in embryonic development observed at the 2-cell stage with the cyanocobalamin supplemented spermatozoa was not observed at the blastocyst stage of development. This lack of improvement to embryonic development success is not surprising because the antioxidant was supplemented to the spermatozoa, independent of the oocyte and zygote. The supplementation of antioxidants to the media aims to establish a stress-free environment (Coyan *et al*., 2010; du Plessis *et al*., 2010) and cyanocobalamin was supplemented only to the spermatozoa, which were improved.

A deficiency in cyanocobalamin decreases the motility of spermatozoa in rats (Watanabe *et al*., 2003) and human males (Chen *et al*., 2001a). Supplementation of 2.5 mg/ml cyanocobalamin to bull semen extender increased motility after thawing (Hu *et al*., 2011), similar to our reported 0.5 µm supplementation to the thawing media in boars. The process of thawing induces ROS production in semen (Fraser and Strzeżek, 2005), and in the present study the optimal supplementation of cyanocobalamin could scavenge ROS, thus decreasing the deleterious effects of thawing cryopreserved boar semen.

Excessive levels of ROS also impair normal spermatozoa membrane function (Bilodeau *et al*., 2000; Chatterjee and Gagnon, 2001), which can be alleviated by the addition of antioxidants to the environment. The use of antioxidants to reduce oxidative stress is not a new treatment (for review, see Agarwal *et al*., 2005) and many vitamins act as antioxidants to protect cell membranes against ROS (Dalvit *et al*., 2005; Jeong *et al*., 2009; Tao *et al*., 2010; Casey *et al*., 2011). It is not surprising that our results indicated that supplementation of another antioxidant, cyanocobalamin, increased the thawed spermatozoa membrane integrity (viability) versus no supplementation of antioxidants. Our findings are similar to those found previously in humans (Boxmeer *et al*., 2007). These results, together with the improvement of post-thawing motility described above, suggest an opportunity where antioxidants can play a crucial role in reducing the oxidative stress associated with semen thawing.

Swine is an attractive animal model for research, and requires a large and steady supply of viable, high-quality, and efficiently-produced pig embryos. However, despite extensive research, polyspermic penetration continues to plague the *in vitro* system, creating a critical need to develop methods for blocking polyspermic penetration during IVF. In our results, supplementation of cyanocobalamin improved frozen-thawed spermatozoa characteristics but did not appear to influence embryonic development. Further investigation to provide more evidence as to how cyanocobalamin is acting in the thawing media is necessary to fully understand the mechanisms of oxidative stress.

To our knowledge, this is the first study to provide information on the effects of cyanocobalamin supplementation to the thawing media of frozen boar semen. Our results indicated that supplementation with 0.5 µm cyanocobalamin during thawing had beneficial effects on forward progressive motility, membrane viability, and early embryonic development. It is possible that the beneficial effects observed were due to the reduction of oxidative stress in the environment during thawing. These findings will improve our knowledge of alleviating oxidative stress associated with semen thawing in boars and could be used to develop new methods to modify the IVF conditions to improve the *in vitro* production of pig embryos.

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