

Coenzyme Q10 Supplementation Effects on In Vitro Maturation, Fertilization, and Early Embryonic Development in Pigs

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ABSTRACT. The objective of this study was to determine the reduction of polyspermic penetration, and increase of mitochondrial activity, in early pig embryonic development by supplementing different concentrations of coenzyme Q10 during oocyte maturation. Oocytes (n = 1,100) were supplemented during the last 24 h of maturation with 0 (control), 10, 50, or 100 μ M of coenzyme Q10. After in vitro fertilization (IVF), embryos were evaluated for fertilization kinetics (penetration, polyspermic penetration, male pronuclear formation), and subsequent embryonic development and mitochondrial activity. Supplementation of 100 μ M coenzyme Q10 was detrimental to the oocytes, as they had significantly lower ($p < 0.05$) fertilization kinetic and early embryonic development rates to the other treatment groups. There were no differences in fertilization kinetic and early embryonic development rates between the 0, 10 and 50 μ M coenzyme Q10 treatment groups. Oocytes, matured in medium supplemented with 50 μ M coenzyme Q10, ultimately developed into embryos with a significantly greater ($p < 0.05$) presence of intact mitochondrial membranes (observed at both 48 and 144 h post-IVF) compared to oocytes not supplemented with coenzyme Q10. In summary, supplementation of 100 μ M coenzyme Q10 during oocyte maturation is detrimental, yet supplementation of 50 μ M coenzyme Q10 leads to a higher occurrence of intact mitochondrial membranes in the in vitro produced pig embryos.

Publication Date: April 2019

<https://doi.org/10.18061/ojs.v119i2.6366>

OHIO J SCI 119(2):2-8

INTRODUCTION

Pig embryos produced from in vitro fertilization (IVF) are an ideal biomedical research model due to the similar genetics, organ development, and disease progression as seen in humans (Whyte and Prather 2011). In vitro fertilization in pigs (*Sus scrofa*) has been as little as one-half as efficient compared to other species such as cows or sheep (Mtango et al. 2002). There are several contributing factors to inefficient IVF including lack of oocyte cytoplasmic maturation, high rate of polyspermic penetration (polyspermy), and insufficient blastocyst formation (Niemann and Rath 2001). Therefore, in vitro techniques in pigs need to be improved in order to increase the efficiency and success of in vitro derived embryos.

Changing the in vitro maturation conditions can improve IVF success and increase efficiency in producing pig embryos (Gruppen 2014). Oxidative stress is a major contributing factor to both the lack of cytoplasmic maturation and to an increase in reactive oxygen species (ROS) development. Oxidative stress has also been implicated in causing protein and DNA breakdown, resulting in apoptosis (Whitaker and Knight 2008). Reducing detrimental levels of ROS and decreasing oxidative stress can promote oocyte development and embryo survivability.

Antioxidants reduce oxidative stress and are attractive additives to a maturation media to promote cytoplasmic maturation and blastocyst development. As an example, Tatemoto et al. (2001) observed this effect by supplementing their maturation media with the antioxidant ascorbic acid 2-O- α -glucoside, which reduced the ROS and improved embryonic development.

Coenzyme Q10 (also known as ubiquinone) is an antioxidant that has been used as a supplement (30 and 100 μ M) to the IVF media in cows, resulting in increased blastocyst formation and ATP production (Stojkovic et al. 1999). Coenzyme Q10 is also an integral component for the production of ATP in the electron transport chain (ETC) and is imbedded in the lipid core of the mitochondrial membrane (Turunen et al. 2004). In the ETC, coenzyme Q10 acts as an electron acceptor for electrons from NADH in complex I and from succinate in complex II. In complex III of the ETC, cytochrome c accepts electrons from coenzyme Q. Electrons continue to flow through complex IV where they are used to generate ATP from ADP (McKee and McKee 2012). Coenzyme Q10 acts as a cofactor for uncoupling proteins to translocate hydrogen

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ions into the mitochondria to establish a proton gradient for oxidative phosphorylation to produce ATP (Turunen et al. 2004). Oxidative stress can also result when unpaired electrons are released from the ETC.

The synthesis of ATP is disrupted when a mitochondrial permeability transition pore (PTP) expands which results in mitochondrial membrane depolarization, ATP depletion, and ultimately cellular death. Coenzyme Q10 prevents the opening of the PTP and thus facilitates an adequate supply of ATP (Papucci et al. 2003).

No reports could be located concerning the supplementation of coenzyme Q10 to pig oocyte maturation media. ***The objective of this study was to reduce polyspermic penetration, and improve both male pronuclear (MPN) formation and early embryonic mitochondrial function, by supplementing 10, 50, or 100 µM coenzyme Q10 into pig oocyte maturation media.*** Following oocyte maturation and IVF, zygotes were evaluated for penetration, polyspermic penetration, and MPN formation to determine IVF effectiveness. Embryos were evaluated for cleavage, blastocyst formation, and mitochondrial membrane potential.

METHODS AND MATERIALS

Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich® Inc. (St. Louis, Missouri, USA). The oocyte maturation medium was Medium 199 (M199) with Earle's salts (Fisher Scientific® Co. LLC, Pittsburgh, Pennsylvania, USA) supplemented with 5 µg/ml follicle stimulating hormone (FSH), 1 µl/ml insulin, 50 ng/ml gentamicin sulfate, 10 ng/ml epidermal growth factor, and 10% fetal calf serum (v/v; FCS). The IVF medium was a modified Tris-buffered media as originally developed by Abeydeera and Day (1997). The embryo culture medium was North Carolina State University (NCSU)-23 medium (Petters and Wells 1993) containing 0.4% (w/v) bovine serum albumin (BSA).

All media were filtered through a 0.22 µm pore mixed cellulose esters membrane syringe filter (Fisher Scientific® Co. LLC, Pittsburgh, Pennsylvania, USA). All incubations were carried out in media droplets under mineral oil at 38.5 °C in a humid atmosphere of 5% CO₂ unless otherwise indicated.

Maturation of Oocytes

Oocytes (n = 1,350; Applied Reproductive Technology LLC, Monona, Wisconsin, USA) were aspirated from mature follicles (3 to 6 mm diameter) obtained and pooled from adult crossbred sows (at least 18 months of age) at an abattoir. The average elapsed time between ovary collection and follicular aspiration was 5 h. The first phase of maturation (from 0 to 22 h) began when the oocytes were shipped overnight, in oocyte maturation media at 38.5 °C, to our laboratory. Only oocytes observed with uniform ooplasm and compact cumulus cells 22 h after initial placement in oocyte maturation medium by the supplier were selected using a micropipette.

The second phase of maturation (from 22 to 46 h) began when selected oocytes (n = 1,100) were washed 3 times in oocyte maturation medium and placed (50-oocytes per well) in 500 µL of oocyte maturation medium without FSH, insulin, and FCS. At this point—the start of this second phase of maturation—the oocytes were divided into 4 treatment groups; a different concentration (0, 10, 50, or 100 µM) of coenzyme Q10 was supplemented to the medium of each group. After 24 h of additional incubation, cumulus cells were removed from the oocytes by repeat pipetting in M199 containing 0.1% (w/v) hyaluronidase. Oocytes with cumulus cells removed, but observed to have uniform ooplasm, were immediately washed in IVF media and used for further analysis as described below.

In Vitro Fertilization and Embryo Culture

Approximately 1 h before the completion of oocyte maturation, a frozen semen pellet (International Boar Semen, Eldora, Iowa, USA) was thawed in IVF medium. The semen was then centrifuged at 36.3 g for 5 min, washed in IVF medium, and again centrifuged twice at 553 g for 5 min. After washing, the sperm pellet was re-suspended in IVF medium to a concentration of 4×10^5 spermatozoa per mL. A volume of 50 µL of this IVF medium (with the spermatozoa) was added to groups of 30 oocytes. The final concentration was approximately 65 spermatozoa per oocyte. After 4 to 6 h of IVF, the zygotes were washed 3 times with NCSU-23 medium and placed (in groups of 50) into 100 µL of NCSU-23 medium for culture.

Experimental Design

Experiment 1: effects of different doses of coenzyme Q10 on embryo production.

This experiment studied the effects of supplementing 0, 10, 50, or 100 μM coenzyme Q10 to maturation media during the second phase of oocyte maturation (the period from 22 to 46 h after follicular aspiration, but before IVF).

The first part of Experiment 1 examined and recorded initial fertilization kinetics. Approximately 12 h after the completion of IVF, the zygotes ($n = 400$; 100 per treatment group, times the 4 treatment groups) were removed from the NCSU-23 medium and mounted and fixed with 25% acetic acid in ethanol (v/v) at room temperature. After 48 h of fixation, the zygotes were stained with 1% orcein (w/v) in 45% acetic acid (v/v) and examined using a phase-contrast microscope at $400\times$ magnification. Endpoints measured were the percent of oocytes penetrated, the percent of polyspermic oocytes, and the percent of oocytes penetrated with MPN formation. Oocytes were considered penetrated when they had one or more swollen sperm head(s) or a MPN formed and their corresponding sperm tails.

The second part of Experiment 1 examined subsequent embryonic development. Embryos ($n = 400$; 100 per treatment group, times the 4 treatment groups) in NCSU-23 medium were observed—under a stereomicroscope—for the percent of embryos cleaved 48 h after the completion of IVF and blastocysts developed at 144 h after the completion of IVF.

Experiment 2: test of the effects of coenzyme Q10 on embryonic mitochondrial activity.

Experiment 2 applied the results of Experiment 1. Experiment 1 determined the most advantageous

concentration of coenzyme Q10 which (when supplemented to the oocyte maturation media during the second phase of oocyte maturation) elicited the lowest rate of polyspermic penetration and highest rates of penetration, MPN formation, cleavage, and blastocyst formation. This optimal concentration of coenzyme Q10, ultimately determined to be 50 μM , was used exclusively for Experiment 2.

Mitochondrial membranes must remain intact to ensure proper functionality throughout embryonic development. In Experiment 2, mitochondrial activity was measured—as described below—by determining the ratios of embryos that displayed red fluorescence in the mitochondrial matrix (indicating intact, proper functioning, membranes) versus embryos that had green fluorescence (indicating damaged/disrupted membranes) dispersed throughout the cell. This measurement, quantifying mitochondrial functionality, was taken at 12, 48, and 144 h after the completion of IVF.

Oocytes used in Experiment 2 ($n = 300$ total as follows: a coenzyme Q10 treatment group of $n = 150$; 50 per time interval, times 3 intervals. Plus an untreated control group of $n = 150$; 50 per time interval, times 3 intervals) were incubated in maturation media supplemented with 50 μM coenzyme Q10 during the second phase of oocyte maturation (the period from 22 to 46 h after follicular aspiration), this was followed by IVF and culture in NCSU-23 medium.

At 12, 48, and 144 h after the completion of IVF, embryos were removed from the NCSU-23 medium, stained with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarboxyanine iodide (JC-1; Sigma-Aldrich® Inc.), and mounted onto slides. Embryos were examined using fluorescent microscopy (excitation maximum wavelength = 525 nm and emission maximum wavelength = 590 nm). Images

Table
Effects of coenzyme Q10 on oocyte fertilization kinetics 12 h after completion of IVF

| Treatment group [†] | Oocytes penetrated (%) | Polyspermic oocytes [§] (%) | Oocytes with MPN [§] (%) |
|--------------------------------|-------------------------------|--------------------------------------|-----------------------------------|
| No coenzyme Q10 | 86.00 \pm 4.97 ^a | 26.67 \pm 6.35 ^a | 46.51 \pm 9.54 ^a |
| 10 μM coenzyme Q10 | 80.00 \pm 9.43 ^a | 26.25 \pm 3.65 ^a | 53.75 \pm 4.33 ^a |
| 50 μM coenzyme Q10 | 95.00 \pm 7.14 ^a | 33.68 \pm 8.32 ^a | 57.89 \pm 4.54 ^a |
| 100 μM coenzyme Q10 | 35.00 \pm 4.94 ^b | 5.71 \pm 10.35 ^b | 5.71 \pm 11.85 ^b |

[†] Treatment groups were the concentration of coenzyme Q10 supplemented to the oocyte maturation media in the second phase of maturation from 22 to 46 h ($n = 400$; 100 per treatment group) in Experiment 1.

[§] Percentage of the number of oocytes penetrated.

^{a,b} Data are expressed as least-squares mean \pm SEM. Means with different superscripts, and within the same column, differ significantly ($p < 0.05$). Significant differences between columns are not comparable.

were recorded digitally and the fluorescence brightness at the equatorial section of each embryo (stained by JC-1) was calculated using computer software (Nikon® NIS-Elements; Nikon Instruments Inc., Melville, New York, USA). The data were presented as the ratio (red to green) of relative fluorescence intensity.

Statistical Analysis

Data were analyzed by one-way ANOVA using the PROC GLM features of the GLM procedure in SAS/STAT® software (SAS Institute Inc., Cary, North Carolina, USA). When there was a significant effect, significant differences were determined using the LSMEANS statement and TUKEY adjustment for multiple comparisons. The effects included in the initial model were treatment, well, and replicate. Well and replicate effects were not significant ($p > 0.05$) and were deleted from the final models. A chi-square test was used to determine percentages of embryos reaching the different developmental stages for each treatment. In all analyses, $p < 0.05$ was considered to be significant. Results are expressed as the least-squares mean \pm the standard error of the mean (SEM).

RESULTS

Experiment 1

Measured approximately 12 h after completion of IVF, oocytes supplemented with 100 μM coenzyme Q10 had significantly lower ($p < 0.05$) incidences of penetration ($35.00 \pm 4.94\%$), polyspermy ($5.71 \pm 10.35\%$), and MPN formation ($5.71 \pm 11.85\%$) compared to all other treatment

groups (Table). There were no statistical differences between the other treatment groups for all IVF endpoints considered.

Embryo development results are shown in Fig. 1. The 0, 10, and 50 μM coenzyme Q10 treatment groups showed no statistical differences in the percentage of embryos cleaved by 48 h after IVF or percentage of blastocysts formed by 144 h after IVF. However, the oocytes supplemented with 100 μM coenzyme Q10 had a significantly lower ($p < 0.05$) percentage of embryos cleaving by 48 h after IVF ($3.00 \pm 19.06\%$) and no oocytes reached the blastocysts stage of development by 144 h after IVF.

Experiment 2

Supplementation of 50 μM of coenzyme Q10 was the highest level that did not have detrimental effects on sperm penetration, MPN formation, and cleavage and blastocyst formation—and thus was the supplementation level used to determine mitochondrial activity. Supplementation of 50 μM coenzyme Q10 resulted in a significantly higher ratio ($p < 0.05$) of intact mitochondrial matrixes (fluorescing red) to dispersed mitochondrial matrixes (causing the whole cell to fluoresce green) as compared to the control (0 μM coenzyme Q10 supplementation). This was observed at both 48 h post-IVF (0.51 vs. 0.45 relative fluorescence intensity, 50 μM vs. 0 μM respectively) and 144 h post-IVF (0.49 vs. 0.42 relative fluorescence intensity, 50 μM vs. 0 μM respectively) (Fig. 2). There was no significant difference in mitochondrial activity between treatment groups at 12 h post-IVF.

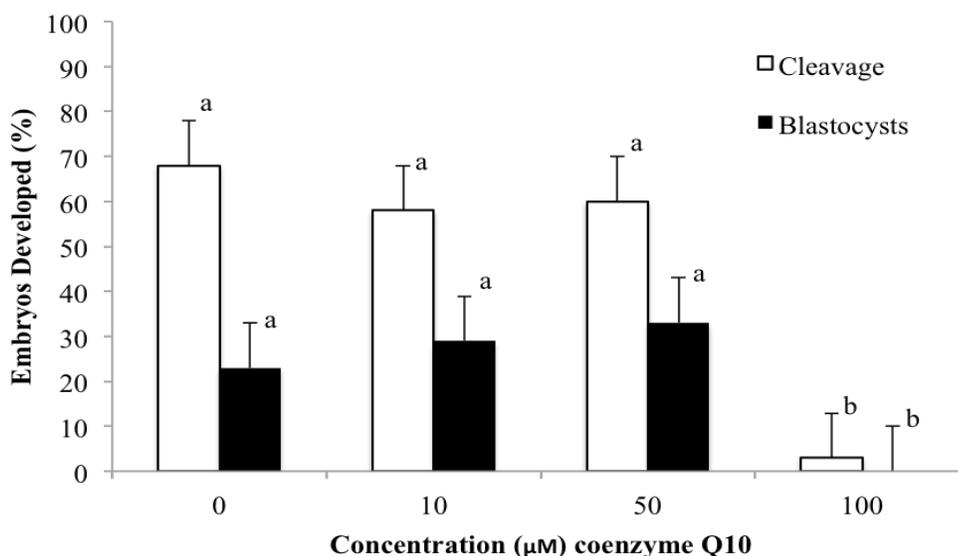


FIGURE 1. Effects of different coenzyme Q10 concentrations (supplemented in the oocyte maturation media in the second phase of maturation from 22 to 46 h) on embryo development ($n = 400$) in Experiment 1. Data expressed as least-squares mean \pm SEM. Cleavage observed 48 h after completion of IVF; blastocysts developed 144 h after completion of IVF. Significant differences between cleavage and blastocyst data are not comparable. ^{a,b}Means with different superscripts differ significantly ($p < 0.05$).

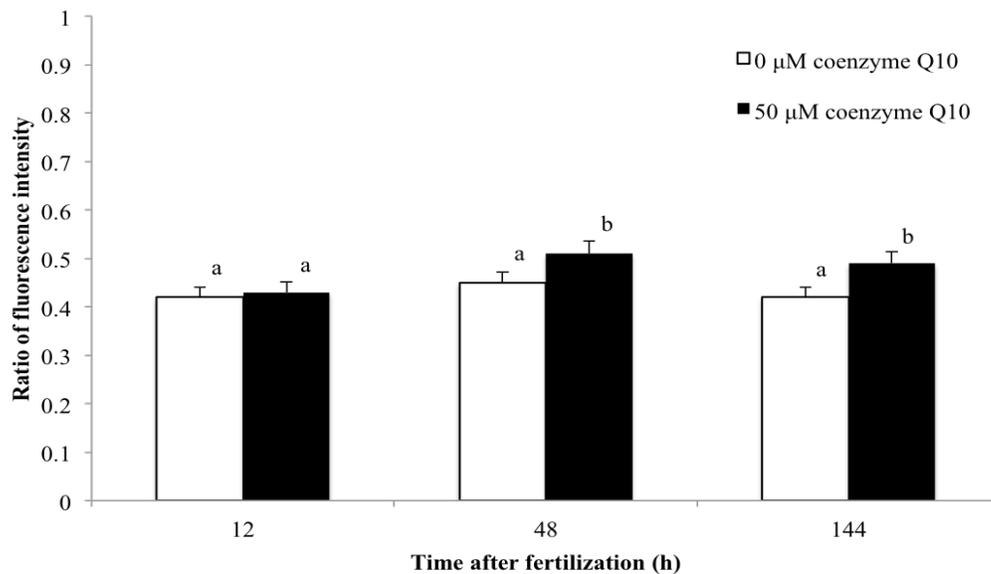


FIGURE 2. Effect of 50 μM coenzyme Q10 (supplemented in the oocyte maturation media in the second phase of maturation from 22 to 46 h) on embryo mitochondrial activity ($n=300$) at 12, 48, and 144 h after IVF in Experiment 2. Data, expressed as least-squares mean \pm SEM, show the ratio of red fluorescent intensity (intact mitochondria) to green fluorescent intensity (dispersed mitochondria) in the stain color of JC-1. There were no significant differences in the ratio of fluorescence intensity at 12 h post-IVF.

^{a,b} Means with different superscripts, and within the same time interval, differ significantly ($p < 0.05$).

DISCUSSION

Coenzyme Q10 is located in the cytoplasm and mitochondrial membranes that carry hydrogen ions and electrons. It plays a role in cell growth, energy metabolism, and preventing oxidative damage (Crane et al. 1993). During the production of in vitro derived pig embryos, the embryo culture step is the longest step and fails to mimic the in vivo environments of the oviduct and uterus. Pig embryos produced in vitro continually have lesser developmental competence—including lower cleavage and blastocyst development rates—than embryos cultured in vivo (Gil et al. 2010). Previous studies have shown that culture media containing lower levels of ATP and glucose during the early stages of embryonic development improve success rates (Abeydeera 2002; Kikuchi et al. 2002).

Reducing oxygen tension during culture could reduce the oxidative stress placed on the embryos; however, there are conflicting reports on the optimal levels of oxygen in the system (Machaty et al. 1998; Karja et al. 2004; Yoshioka et al. 2008). Stojkovic et al. (1999) reported that 30 μM or 100 μM coenzyme Q10 supplementation during in vitro embryo culture in cows improves cleavage, blastocyst, and hatching blastocyst rates. Stojkovic et al. (1999) also reported that supplementation with coenzyme Q10 also increased the number of embryonic cells and ATP content per cell. In partial agreement with these studies, the results of this study indicate

that supplementation of the maturation media with 50 μM coenzyme Q10 increases the ratio of intact mitochondrial matrixes during embryonic development in pigs.

Results of the current study indicated that supplementation of oocyte maturation media with 100 μM coenzyme Q10 was detrimental to the oocyte and resulted in death. Maturation media supplemented with 50 μM coenzyme Q10 did not have significant effects on oocyte fertilization kinetics (sperm penetration, polyspermic penetration, or MPN formation) compared to the control. Therefore, 50 μM coenzyme Q10 was chosen as the appropriate level of supplementation for the mitochondrial study. These findings were expected: coenzyme Q10 is located in plasma and mitochondrial membranes and would not have an immediate impact or function during oocyte maturation or fertilization. Coenzyme Q10 potentially could be acting as an antioxidant in the media during oocyte maturation (Frei et al. 1990), however these effects were not considered in the current study.

Embryos, developed from oocytes incubated in maturation media supplemented with 50 μM coenzyme Q10 (during the second phase of maturation), did show significant improvement in mitochondrial activity. At both 48 and 144 h after IVF the embryos sourced from the 50 μM coenzyme Q10-matured oocytes displayed a significantly higher ratio ($p < 0.05$) of intact mitochondrial matrixes compared

to dispersed mitochondrial matrixes (as compared to the control). This is similar to two previous findings: First, coenzyme Q10 supplementation improves embryonic development beyond the cleavage stage—most likely due to mitochondrial regulation (Hwang et al. 2016). Second, antioxidants could reduce ROS that would induce PTP openings in the mitochondrial membrane (Vianello et al. 2012).

Although there have been advances in the in vitro production of pig embryos, the success rate is still below the corresponding rates in vivo. Suboptimal defined culture conditions create a stressful environment for developing embryos in vitro and inhibit their ability to adequately progress to a hatched blastocyst (Gil et al. 2010).

During early embryonic development, mitochondria are essential in providing enough energy for the cell to function properly. High concentrations of ATP are required for perinuclear mitochondria clustering for cleavage and then homogenous distribution in a blastocyst (Sun et al. 2001). Additionally, the plasma membrane ion transport system found in the mitochondria depends on coenzyme Q10 to carry electrons and, in the form of ubiquinol, scavenge free radicals to alleviate peroxidative damage. Increasing the availability of coenzyme Q10 during oocyte maturation could increase its incorporation into the mitochondria during subsequent embryonic development and act as an antioxidant in the environment. At this point, however, it is uncertain which mechanism of action the coenzyme Q10 supplementation is affecting; a topic which warrants further studies.

CONCLUSION

This is the first study that considers the effects of separately supplementing 4 different concentrations (0, 10, 50, and 100 μM) of coenzyme Q10 into the maturation media of pig oocytes intended to subsequently undergo IVF and embryo culture. ***Test oocytes, initially incubated in maturation media supplemented with 50 μM coenzyme Q10, later experienced similar IVF kinetic, cleavage, and blastocyst success rates—but ultimately yielded embryos with significantly higher proportions of intact functioning mitochondrial membranes (at both 48 and 144 h post-IVF)—than the control oocytes.*** These findings may improve success rates for producing in vitro derived pig embryos.

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