Improvement of bovine *in vitro* embryo production by vitamin K₂ supplementation

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Abstract

Mitochondria play an important role during early development in mammalian embryos. It has been shown that properly controlled follicular preparation increases the likelihood of *in-vitro*-produced bovine embryos reaching the blastocyst stage and that competent embryos exhibit heightened expression of genes associated with mitochondrial function. We hypothesized that apparently incompetent embryos could be rescued by restoring mitochondrial function. It has been shown that vitamin K₂ (a membrane-bound electron carrier similar to ubiquinone) can restore mitochondrial dysfunction in eukaryotic cells. The aim of this study was therefore to investigate the effects of vitamin K₂ on bovine embryonic development *in vitro*. The vitamin was found most effective when added 72 h after fertilization. It produced a significant ($P < 0.05$) increase in the percentage of blastocysts (+8.6%), more expanded blastocysts (+7.8%), and embryos of better morphological quality. It improved the mitochondrial activity significantly and had a measurable impact on gene expression. This is the first demonstration that current standard conditions of *in vitro* production of bovine embryos may be inadequate due to the lack of support for mitochondrial function and may be improved significantly by supplementing the culture medium with vitamin K₂.

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Introduction

Mitochondria are intracellular organelles that ensure a multitude of key cellular functions such as energy production, control of apoptosis, lipid metabolism, steroid synthesis, and production of metabolites having important regulatory roles in various signaling pathways (Dumollard *et al.* 2009, Van Blerkom 2009). Although mitochondria are involved in several vital functions, it is their role in balancing oxidative stress and producing ATP that have been most investigated (Thompson *et al.* 2000, Trimarchi *et al.* 2000, Dumollard *et al.* 2009, Van Blerkom 2009). An atypical mitochondrial contingent composed of immature organelles having limited cristae and hence a limited potential for ATP production via oxidative phosphorylation and glycolysis has been observed in oocytes (Trimarchi *et al.* 2000, Fair *et al.* 2001, Crocco *et al.* 2011). This lower level of energy production lasts apparently until these organelles undergo a transition to mitochondria of mature form, which coincides with the activation of the embryonic genome (Thompson *et al.* 1996, Tarazona *et al.* 2006, Dumollard *et al.* 2009). Culture media produce adverse effects on the structure and function of mitochondria,

with consequences for the quality of embryos produced *in vitro* (Crosier *et al.* 2001, Abe *et al.* 2002a). The expression of genes associated with embryo viability may be modified (Rizos *et al.* 2003, Plourde *et al.* 2012). However, variations in mitochondrial function in association with immature structure have also been observed in embryos produced *in vivo* (Crosier *et al.* 2001, Fair *et al.* 2001, Abe *et al.* 2002a,b). Meanwhile, oxidative stress has been shown to perturb mitochondrial function leading to a marked reduction in the embryonic development (Bavister 1995, Trimarchi *et al.* 2000, Van Blerkom 2004). This problem has been studied extensively, and several modifications to culture media (primarily the addition of antioxidants) have been proposed (Olson & Seidel 2000, Sudano *et al.* 2010). However, this approach has brought only marginal improvements in embryo competence thus far.

Vitamin K₂ has been used recently to counteract the deleterious effects on mitochondrial function in a modified strain of *Drosophila* mutated for a gene that is known to be involved in Parkinson's disease (Vos *et al.* 2012). It was thus found that vitamin K₂ played an electron carrier role in the mitochondrial electron

transport chain complex, resulting in more efficient oxygen use and production of ATP.

Based on our previous study (Plourde *et al.* 2012), we hypothesized that vitamin K₂ could rescue dysfunctional mitochondria, which would translate into a higher percentage of embryos reaching the blastocyst stage. The objectives of this study were to characterize, for first time, the effects of vitamin K₂ on bovine embryo development and mitochondrial function.

Materials and methods

All chemicals were purchased from Sigma–Aldrich unless specified otherwise.

Recovery of oocytes for embryo production *in vitro*

Ovaries removed immediately from cows slaughtered in a commercial abattoir were transported in saline (0.9% NaCl) containing 1% antifungal agent. Oocytes were collected and matured, followed by fertilization and culture according to standard *in vitro* techniques in our laboratory (Plourde *et al.* 2012). Cumulus–oocyte complex (COC) was retrieved from follicles 2–6 mm in diameter using a 38 mm 18 G needle attached to a 10-ml syringe. Homogeneous COCs with at least five layers of cumulus surrounding the oocyte completely were selected, while COCs exhibiting fragmented cytoplasm, pyknotic cumulus, pale nuclei, or abnormal morphology were automatically rejected.

Maturation of oocytes

COCs were placed in HEPES-buffered Tyrode's lactate medium (TLH) supplemented with 10% bovine serum, 200 µM pyruvate, and 50 µg/ml gentamycin and washed twice thoroughly to ensure total removal of follicular liquid. Groups of ten healthy COCs were placed in 50-µl droplets of the medium under 9 ml of filtered mineral oil (#8410, Sigma). The maturation medium was composed of TCM199 (Gibco product 11150-059, Invitrogen) plus 10% FCS (Sterile Foetal Bovine Serum for Cell Culture, Medicorp, Montreal, QC, Canada), 200 µM pyruvate, 50 µg/ml gentamycin, and 0.1 µg/ml follicle-stimulating hormone (Gonal-f, Serono Canada, Inc.). Droplets containing COCs were incubated for 24 h at 38.5 °C in a humidified (100%) atmosphere containing 5% CO₂ and 20% O₂.

Fertilization of oocytes

Mature COCs were washed twice in TLH. Groups of five mature COCs were added to 50-µl droplets of TLH supplemented with 0.6% BSA (Sigma fraction V) and 200 µM gentamycin (IVF medium) and covered with filtered mineral oil. Two microliter of solution containing 1 mM hypotaurine, 2 mM penicillamine, and 250 mM epinephrine were then added to the COC-containing droplets. Oocytes were fertilized with frozen semen after 15–18 h of incubation at 38.5 °C in a humidified atmosphere containing 5% CO₂ and 20% O₂. Cryopreserved pooled bull ejaculate was thawed at 37 °C in a water bath, laid

on a discontinuous Percoll gradient (2 ml of 45% Percoll over 2 ml of 90% Percoll) and centrifuged at 700 *g* for 30 min at room temperature. The supernatant was discarded and the pellet was re-suspended in the *in vitro* fertilization medium to obtain a concentration of 10 000 spermatozoa per COC (five COCs per droplet) based on counts using a hemocytometer.

Culture of embryos

Presumptive zygotes were denuded of cumulus cells and spermatozoa by gentle pipetting, washed three times in TLH supplemented with fatty-acid-free BSA, and then cultured in three steps in a synthetic oviduct fluid (SOF) containing essential and non-essential amino acids, 0.5 mM of glycyl-glutamine and 0.4% fatty-acid-free BSA (ICP-Bio, Auckland, New Zealand) under filtered mineral oil at 38.5 °C in a humidified atmosphere containing 6.5% CO₂, 5% O₂, and 88.5% N₂. Embryos were placed in groups of ten in droplets of SOF (10 µl). Embryonic culture was following three media change. For the first change, the SOF contained only non-essential amino acids at the standard concentration plus 3 µM EDTA. For the second change (72 h post-fertilization), the SOF also contained essential amino acids at half this concentration (SOF2). For the third change (120 h post-fertilization), both the non-essential and essential amino acids were present at the standard concentration (SOF3).

Vitamin K₂ was added in accordance with Vos *et al.* (2012) with some modifications. Briefly, vitamin K₂ dissolved in 95% ethanol solution (HPLC grade, Fisher Scientific, Fair Lawn, NJ, USA) and stored in the dark at –20 °C as a 400× stock solution was diluted in SOF to obtain final concentrations of 0.5 mM vitamin K₂ and 0.25% ethanol.

At 72 h post-fertilization, embryos were transferred randomly to droplets (10 µl) of SOF2 containing added vitamin K₂ or not. The vitamin-supplemented and control groups were thus both supplemented with 0.25% ethanol (previous tests indicated that 0.25% ethanol had no effect on bovine embryo development *in vitro*). Embryos were transferred at 120 h post-fertilization to 20 µl droplets of SOF3 again containing added vitamin K₂ or not (with 0.25% ethanol in either case). This medium was replaced three times to prevent toxicity due to ammonium accumulation and nutrient (amino acid) depletion due to assimilation. Embryos remained under these conditions until day 7, when blastocyst percentages were recorded and early, expanded, and hatched blastocysts were categorized according to the IETS system and used in the various tests in this study. Embryos were either processed fresh or collected, snap-frozen in liquid nitrogen, and stored at –80 °C until use.

Characterization of embryo mitochondrial activity

Under an atmosphere containing 5% CO₂ at 38 °C, live embryos in SOF were stained for 40 min with the active mitochondrial dye CMX–rosamine (Mitotracker Red, Molecular Probes, Eugene, OR, USA) at a concentration of 300 nM (same atmosphere and temperature). This dye has been found to be more sensitive to the mitochondrial membrane potential and mitochondrial protein (thiol group abundance) than other dyes and to exhibit better retention in the organelle and more homogenous dispersion throughout all embryonic cells

(Poot *et al.* 1996). The fluorescence excitation wavelength was 594 nm and emission was read at 608 nm. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which uncouples mitochondrial membrane potential, was used at a concentration of 100 nM in order to provide a negative control. The CCCP treatment provided background values against which the different measurement runs were calibrated. Embryos were selected randomly for treatment with CMX–rosamine or with CCCP+CMX–rosamine.

Confocal microscopy

Confocal microscopy was performed using a Nikon TE2000 confocal microscope (Nikon, Mississauga, ON, Canada) with a water-immersion objective at an optical magnification of 60. Embryo morphological phenotype was recorded in photos in gray scale taken with the same settings to estimate color (dark or pale) based on the IETS system. Mitochondrial activity was recorded as an epifluorescent image of CMX–rosamine dye in gray scale.

Digital images of embryos stained with CMX–rosamine were obtained using a LSM 700 confocal system with a Zeiss Axiovert 200 M inverted microscope (Carl Zeiss GmbH, Jena, Germany). The embryos were viewed with the Plan-Apochromat 40× lens (NA=1.2) at 10% of the utilizable laser intensity (maximum power: 1.2 W, output: 25% of the maximum tube current) and an HFT dichroic beam splitter (458/514 nm). Images of orthogonal projections consisting of 21 slices (1 µm each) were acquired as 'lambda stacks' using the Lambda Mode scanning procedure at a resolution of 1024×1024 pixels. The microscope settings and the lambda mode scanning procedure were the same for all collected lambda stacks. The intensity of CMX–rosamine fluorescence was measured using the mean gray scale in the freely available Image J Software (Abramoff *et al.* 2004). Results are expressed in mean fluorescence intensity arbitrary units (AUs) based on all samples within a group.

Extraction of total DNA and RNA

Additional blastocysts from each group were used to obtain values for total genomic DNA and RNA. The nucleic acids were extracted simultaneously using the AllPrep DNA/RNA Micro Kit (Qiagen) according to the manufacturer's instructions. Genomic DNA was used in the mitochondrial DNA

(mtDNA) quantification procedure described below and total RNA was reverse transcribed for quantification by RT-PCR.

Quantification of mtDNA

mtDNA in individual embryos ($n=10$ biological replicates per treatment) was quantified using a PCR (qPCR) method. The 12S rRNA gene (GenBank accession number: J01394) was selected as a mitochondrial target and the *MX1* gene (GenBank accession number: AY340484) as a nuclear target (Table 1). The mtDNA and nuclear DNA (nDNA) were used to calculate the relative concentration of mtDNA in each embryo, which was expressed as the mtDNA/nDNA ratio. The LightCycler 2.0 (Roche Diagnostics) was used to monitor qPCRs. The reaction mixture (20 µl) contained 0.5 µl of each primer solution (0.25 µM), 1.2 µl of 1.5 µM MgCl₂, 2 µl of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics), and 5 µl of DNA sample. The average DNA concentration of each sample was 0.00335 ng/µl. The following cycling conditions were applied for amplification: initial denaturation at 95 °C for 10 min followed by 50 cycles of 95 °C for 5 s, 5 s (12S rRNA) at 58 or 60 °C (*MX1*), followed by 72 °C for 20 s and 76 °C (12S rRNA) or 85 °C (*MX1*) for 5 s. The presence of amplicons was verified using melting curve analysis: Following the last amplification cycle, the internal temperature of the LightCycler was increased rapidly to 94 °C then decreased to 72 °C for 30 s, followed by an increase to 94 °C at a rate of 0.1 °C/s, with continuous fluorescence reading. Copy numbers of mtDNA and nDNA were obtained from a standard curve, which was based on the linear relationship between the crossing point cycle values and the logarithm of the starting copy number.

Determination of differential gene expression in blastocysts

Total RNA extracted from embryos ($n=4$ biological replicates for each group) was purified using the PicoPure RNA Kit (Molecular Devices, Downingtown, PA, USA) according to the manufacturer's instructions. DNA was digested with DNase I (Qiagen). The RNA concentration and quality were measured using the 2100-Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with the RNA PicoLab Chip (Agilent Technologies). Only RNA of very good quality (RIN over 8) was used for amplification.

Table 1 Characteristics of the primers used for gene candidate transcript quantification.

Symbols	Accession numbers	Primer sequences		Annealing temperature (°C)	Acquisition temperature (°C)	Product size (bp)
		Fw (5'–3')	Rv (5'–3')			
ACAA2	NM_001035342	TGGTTCCAGTCCATTGTGA	TTCCACCTCTGCTGTGATTG	57	81	291
ATP2B4	NM_001172594	ACGGCTGTGGGTATCAACTC	TCAGACCAGCTTCCCAATC	57	85	256
NDUFS7	NM_001038022	GTCCGACGTGATGATTGTGG	CCCGTTTGATCTTCTCTGC	57	89	263
CERS2	NM_001034667	CACCCAGTTGCCCTTTAAACC	CCACAGGTGGGACAGTAAGG	57	82	254
MRPS7	NM_001034343	TGAGTGCAGGGAGAAGAAGC	CTGGCCTTAAGAGGGAACG	57	89	256
MX1	AY_340484	ATGCGTGCTATTGGCTCT- TCCTCA	CAAACAGACGAAGG- GAGTTTGGCA	60	85	181
12S rRNA	J0_1394	TCGATAAACCCCGATAAACC	TTCTGTGCTTGATCTCTTGG	58	76	186

The concentration of purified RNA amplified for two rounds (with T7 RNA polymerase) using the RiboAmp HSPlus RNA Amplification Kit (Life Science, Foster City, CA, USA) was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Antisense RNA labeled with Cy3 or Cy5 using the Universal Linkage System Kit (Kreatech Diagnostic, Amsterdam, The Netherlands) was hybridized (825 ng) on Agilent-manufactured EmbryoGENE slides (Robert *et al.* 2011) in a two-color dye-swap design in a hybridization oven for 17 h at 65 °C. A simple direct comparison of the treatments was made using four independent biological replicates (each consisting of one blastocyst) per treatment. Microarray slides were then washed and scanned with the PowerScanner (Tecan, Männedorf, Switzerland) and analyzed using the Array-Pro Analyzer Software (MediaCybernetics, Bethesda, MD, USA).

Microarray data were pre-processed as described in previous studies (Plourde *et al.* 2012). Briefly, data intensity files were analyzed using FlexArray 1.6.1 (<http://genomequebec.mcgill.ca/FlexArray>), in which raw data corrected by background subtraction were pre-processed by performing a Lowess intra-array and Quantile inter-array normalizations. Statistically significant variations were detected using Limma (Bioconductor, hosting provided by Fred Hutchinson Cancer Research Center, Seattle, WA, USA). Gene expression differentials were considered significant when they were at least 1.2 and the cut-off adjusted *P* value was <0.01. Pathway analyses and downstream exploitation of gene lists were carried out using Ingenuity Pathway Analysis Software Version 8.6 (Ingenuity Systems, Inc., Redwood City, CA, USA).

Validation of mRNA levels by quantitative RT-PCR

Microarray results were validated using single embryos (*n*=5 biological replications per group). Total RNA (extracted as described above) was reverse transcribed using the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) with oligo-dT to prime the reaction as per the manufacturer's recommendations. Primers of candidates (acetyl-CoA acyl-transferase 2 or *ACAA2*, Ca^{2+} transporting plasma membrane 4 ATPase or *ATP2B4*, NADH dehydrogenase (ubiquinone) Fe-S protein 7 or *NDUFS7*, 20 kDa NADH-coenzyme Q reductase, ceramide synthase 2 or *CERS2*, and mitochondrial ribosomal protein S7 or *MRPS7*) were designed using the Primer3 Web interface (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized at IDT (Coralville, IA, USA). The reaction mixture was composed of the LightCycler FastStart DNA Master SYBR Green I Kit components (Roche Diagnostics) and real-time measurements were performed in a LightCycler 2.0 apparatus (Roche Diagnostics). GenBank accession numbers, primer sequences, annealing temperatures, and product sizes are given in Table 1.

For quantification, real-time PCR was performed as described previously (Bermejo-Alvarez *et al.* 2010). The comparative cycle threshold (ΔCT) method was then used to quantify expression levels, as described by Schmittgen & Livak (2008). Quantification was normalized to the endogenous control (β -actin to account for cell numbers). A change in the relative level of gene expression of the target was calculated as $2 - \Delta\Delta\text{CT}$.

Electron microscopy

Early blastocysts (*n*=5 biological replicates for both treatments) classified as good quality were washed three times in PBS containing 10% FCS at 4 °C. Embryos were fixed in freshly prepared 3% glutaraldehyde (Electron Microscope Solutions, Ft. Washington, PA, USA) in 0.1 M PBS for 1 h at 4 °C, stored in 0.1 M PBS and later embedded individually at 37 °C in 4% (w/v) agarose (low melting temperature, prepared at ~55 °C), and isolated as blocks using a razor blade and a stereomicroscope. The blocks were washed twice in PBS for 5 min at room temperature, post-fixed for 1 h at room temperature in 4% osmium tetroxide (OsO_4) in PBS, dehydrated gradually in a series of acetone solutions, and then embedded in Epon 812 epoxy resin. Sections of 1 μm thickness were sliced through the entire embryo using a glass knife on a Reichert Jung Ultracut E ultramicrotome and stained with toluidine blue. Sections of 80–100 nm thickness were sliced with a diamond knife and collected on Formvar-carbon-coated 200 mesh nickel grids. Sections were then stained with uranyl acetate and lead citrate and examined using a FEI Tecnai 12 (Eindhoven, The Netherlands) transmission electron microscope operated at 80 kV. Observations were made on four different sections per embryo.

Statistical analysis

Samples were distributed randomly among the vitamin-treated and control groups. The blastocyst rate (percentage) was calculated from the total number of oocytes selected for maturation and data were analyzed using a paired *t*-test. The mitochondrial activity based on Mitotracker Red intensity was analyzed using Student's *t*-test to identify significant differences between groups. All tests were performed using the Prism Version 5.0 statistical software package (GraphPad Software, La Jolla, CA, USA). Differences between groups were declared significant when *P*<0.05.

Results

Effects of vitamin K₂ on bovine embryo development in vitro

Timing of vitamin K₂ supplementation of the culture medium was based on preliminary experiments that showed detrimental effects when the vitamin was added during oocyte maturation or early post-fertilization stages (data not shown). It was noted that vitamin K₂ supplementation was effective from the eight-cell stage onward. All measurements were carried out on embryos treated at 72 h post-fertilization.

Supplementation of the culture medium with vitamin K₂ increased the percentage of blastocysts by 8.6%. The overall appearance of the blastocysts is shown in Fig. 1. Visible differences were few and trivial (e.g. extruded blastomeres) and were observed more frequently in the control group. On day 7, the percentage of expanded blastocysts (Fig. 2) was significantly higher in the vitamin-treated group (17.19%) than in the control group (10.0%). The quality of the embryos in the

Figure 1 Bovine embryos produced *in vitro* and matured for 7 days in a synthetic oviduct fluid, (A) control group and (B) treated with vitamin K₂. Images were taken at 40× magnification.

vitamin-treated group may be classified as 'excellent' compared with the control group, based on IETS criteria.

Blastocyst rate and mitochondrial activity are both increased in embryos treated with vitamin K₂

Figure 3A and B shows the distribution of active mitochondria (red) in bovine embryos obtained *in vitro*, as observed using confocal microscopy. As expected, mitochondrial activity (based on Mitotracker fluorescence intensity, Fig. 3C) was greater in embryos cultured in the vitamin-K₂-supplemented medium ($12\,603 \pm 88$ AU) than in control embryos (8960 ± 92 AU). The mtDNA contents normalized on a per-cell basis (Rao *et al.* 2009) did not differ, suggesting that the mitochondria were more active and not simply greater in number (Fig. 3D).

Vitamin K₂ supplementation of the early embryo in vitro culture medium influences gene expression at the blastocyst stage

Of the 37 238 gene transcripts surveyed in the bovine *in vitro* embryos using the microarray, only 37 showed a significant ($P < 0.01$) change at the expression level (by a factor of at least 1.2) in association with the addition of vitamin K₂ to the culture medium.

Enrichment analysis showed that the cellular functions with the most significant values were calcium transport and β -oxidation of fatty acids. These suggest that vitamin K₂ had an impact on mitochondrial functions. The microarray results were validated by qRT-PCR analysis using five candidate genes (*ACAA2*, *ATP2B4*, *NDUFS7*, *CERS2*, and *MRPS7*) associated with mitochondrial functions (Table 1). The increased expression of *ACAA2* in the vitamin K₂ group based on hybridization results was thus confirmed (Fig. 4). In the case of the other genes, qRT-PCR analysis tended to confirm the increased expression, albeit with a lesser degree of certainty.

Effect of vitamin K₂ on mitochondrial morphology in bovine embryos in vitro

The potential impact of vitamin K₂ on mitochondrial maturation was investigated using electron microscopy. Mitochondria of different morphological types in

bovine blastomeres have been reported in several studies (Crosier *et al.* 2001, Fair *et al.* 2001, Abe *et al.* 2002a,b, Crocco *et al.* 2011, 2013). The two most frequently noted types are known as 'mature' (i.e. round or elongated with transverse cristae distributed either in only part of or in the whole organelle) and 'immature' (i.e. rounded and swollen forms in which the matrix is less dense, cristae area is small and retracted toward the periphery or absent, and small vacuoles may be present).

Both of these mitochondrial types were present in blastocysts obtained by culture in both the medium supplemented with vitamin K₂ and the control medium. We observed a larger proportion of mitochondria of swollen shape in embryos in the control group (Fig. 5A and C), while the blastomeres of embryos cultured in medium with added vitamin K₂ contained more mitochondria of the mature shape (Fig. 5B and D).

Discussion

Mitochondria are vital organelles that play crucial roles in eukaryotic cells not only by generating metabolic energy and managing lipid reserves but also by controlling apoptotic equilibrium and thereby determining cell fate (Dumollard *et al.* 2009, Van Blerkom 2009). Mitochondrial disorders can lead to the arrest of early development (Bavister 1995, Trimarchi *et al.* 2000, Van Blerkom 2009). In cattle embryos, effects of the suboptimal *in vitro* microenvironment on mitochondria have been shown to decrease developmental competence (Crosier *et al.* 2001, Fair *et al.* 2001, Abe *et al.* 2002a,b, Crocco *et al.* 2011, 2013). We have observed that developmental competence during *in vitro* production of embryos is improved to 55% for immature oocytes collected by transvaginal pick-up (OPU) following a dedicated ovarian stimulation regimen, compared with 35% for those collected from ovaries post-mortem (Plourde *et al.* 2012). Analysis of the transcriptome showed that the resulting early blastocysts

Figure 2 Effect of vitamin K₂ on bovine embryo development *in vitro*. Embryos were cultured in a synthetic oviduct fluid without (white) or with vitamin K₂ (gray). Values are expressed as mean $\pm$ S.E.M.

*Significantly different ($P < 0.05$).

Figure 3 Confocal microscopic images of bovine blastocyst-stage embryos obtained *in vitro* and stained with Mitotracker Red (CMX-rosamine) to show active mitochondria: (A) embryo cultured in a synthetic oviduct fluid (SOF), (B) embryo cultured in a SOF enriched with vitamin K₂ 72 h after fertilization, (C) Mitotracker Red fluorescence intensity (carbonyl cyanide m-chlorophenylhydrazine (CCCP), used as a negative control), and (D) ratio of mitochondrial DNA to nuclear DNA in single blastocysts. Values are expressed as mean $\pm$ S.E.M. *Significantly different ($P < 0.05$). AU, arbitrary unit.

were very similar regardless of the oocyte collection method, except for the expression levels of genes associated with mitochondrial functions, which were not affected when the oocytes were collected by OPU from stimulated ovaries. It is well accepted that developmental competence is influenced greatly by the quality of the oocyte (Rizos *et al.* 2002). We hypothesized that this maternal preparation is associated in part with the state of readiness of the mitochondrial contingent found in the oocyte and that providing support for mitochondrial functions might compensate for suboptimal preparation and thus result in higher developmental competence.

The possibility that the impaired mitochondrial functions observed in embryos developed under *in vitro* conditions could be due to increased free radical stress has been investigated in several studies. Reducing oxygen tension and adding antioxidants such as vitamins C and E to the culture medium have been investigated as means of reducing the free radical load (Olson & Seidel 2000, Sudano *et al.* 2010) and reducing free radical generation associated with lipid catabolism (Phongnimitr *et al.* 2013, Takahashi *et al.* 2013). The antioxidant approach thus far has brought only marginal improvements in developmental competence. This indicates either that free radicals are not the source of the problem or that the ability of mitochondria to fulfill

their multifaceted role is compromised even in the presence of lower levels of free radicals.

In this study, we tested the effect of vitamin K₂ on bovine embryonic development *in vitro*. Vitamin K₂ has been shown recently to restore the mitochondrial function by acting as an electron carrier (Vos *et al.* 2012). We have shown herein that adding vitamin K₂ to the culture medium 72 h after fertilization significantly increased the percentage of embryos that reach the blastocyst stage. Preliminary tests suggested that adding the vitamin earlier does not have this effect and may even be detrimental. The timing of the supplementation thus coincides with the slight increase in mitochondrial activity observed at the morula stage (72–168 h after fertilization), which happens when activation of the embryonic genome occurs in cattle (Thompson *et al.* 1996, Tarazona *et al.* 2006, Crocco *et al.* 2011). It is known that this transition is critical for bovine early development, as most developmental failures occur before this activation (Niemann & Wrenzycki 2000, Meirelles *et al.* 2004, Gad *et al.* 2012). Our results suggest adding vitamin K₂ at the time of genome activation, a phase marked by an extended cell cycle and substantially increased demand for metabolic energy (Thompson *et al.* 1996, Tarazona *et al.* 2006), helps embryos complete this developmental step by increasing the mitochondrial activity.

As the treatment did not increase the number of hatched blastocysts, it appears that vitamin K₂ did not stimulate developmental kinetics but rather rescued embryos that would otherwise have failed to develop. It has been proposed that development is affected by lower numbers of mitochondria or at least of copies of mtDNA (Ge *et al.* 2012). However, vitamin K₂ was shown not to have any impact on the mtDNA/nDNA ratio (Rao *et al.* 2009).

Embryo quality is evaluated routinely on the basis of morphological criteria in order to select embryos most

Figure 4 Validation of the differential expression of bovine embryo genes indicated by microarray analysis by quantitative RT-PCR: embryos were produced *in vitro* and cultured in a synthetic oviduct fluid without (white) or with vitamin K₂ (gray). Values are expressed as mean $\pm$ S.E.M. Quantification was normalized to endogenous β -actin transcript levels. *Significantly different ($P < 0.05$).

Figure 5 Electron micrographs showing morphological types of mitochondria in bovine embryos (blastomere stage) produced *in vitro*, (A and C) embryo cultured in a synthetic oviduct fluid (SOF) without added vitamin K₂, (B and D) embryo cultured in a SOF with added vitamin K₂. The mitochondria of the control group have fewer cristae than those of the vitamin-K₂-treated group. N, nucleus; M, mature mitochondria; Im, immature mitochondria.

likely to survive cryopreservation (Hasler 2001, Abe *et al.* 2002a, Rizos *et al.* 2003) and produce gestation (Lindner & Wright 1983, Hasler 2001, Abe *et al.* 2002b, Van Soom *et al.* 2003). Culture conditions are known to have a major impact on the quality of embryos (Abe *et al.* 2002a,b, Rizos *et al.* 2003, Van Soom *et al.* 2003). We observed that supplementation of the culture medium with vitamin K₂ resulted in a high proportion of high-quality embryos.

Abe *et al.* (2002b) observed that the blastomeres of bovine embryos obtained *in vivo* and classified as 'excellent' quality contained a larger proportion of mature mitochondria. Similarly, we observed a higher yield of high-quality embryos and increased proportion of mature mitochondria in blastomeres, in association with vitamin K₂ supplementation of the culture medium. Furthermore, the improved mitochondrial activity in the supplemented culture was most likely due to this increased proportion of mature mitochondria. The conditions that provide the signal for the maturation of the mitochondrial contingent are unknown. Mitochondria adopt their swollen immature shape with limited cristae during oocyte growth and keep a limited capacity to produce ATP until genome activation and thereafter acquire the mature shape (Mohr & Trounson 1981, Plante & King 1994). It has been reported in several studies that the developmental competence of

mammalian oocytes and embryos is related to the state of maturity of the mitochondrial cohort (Shepard *et al.* 1998, Van Blerkom 2004, 2009, Crocco *et al.* 2011).

One reported manifestation of improved mitochondrial activity is increased β -oxidation of fatty acids, which generates ATP necessary for embryo development (Ferguson & Leese 2006). Accordingly, the greater mitochondrial activity in the vitamin-K₂-treated embryos coincided with increased expression (based on microarray analysis) of *Acaa2*, a gene known to play a role in β -oxidation of fatty acids in rat hepatocytes by regulating the generation of acetate (Yamashita *et al.* 2006). Embryos in this group also tended to show greater abundance of transcripts of *CERS2*. This gene is also involved in regulating mitochondrial function, as its ablation disrupts respiratory chain activity and leads to chronic oxidative stress and activation of stress-signaling pathways (Pewzner-Jung *et al.* 2010, Zigdon *et al.* 2013). Consistent with these findings, embryos cultured in the presence of vitamin K₂ also tended to exhibit heightened expression of *NDUFS7* and *MRPS7*. Dysregulation of the former has been associated with impairment of the mitochondrial complex I of the electron transport chain (Triepels *et al.* 2001), while the latter gene has been associated with resistance to oxidative stress (Harding *et al.* 2003).

Based on transcriptome analysis, vitamin K₂ supplementation was also found to have an impact on the transport of calcium, which plays a central role in the regulation of mitochondrial oxidative metabolism and is a co-factor of a major rate-limiting dehydrogenase of the TCA cycle (Duchen 2000, Dumollard *et al.* 2004). Impaired mitochondrial uptake of Ca²⁺ alters the spatiotemporal characteristics of cellular [Ca²⁺] signaling and mitochondrial metabolism and might trigger pathological states that lead to cell death (Duchen 2000). Our findings might be related to differential expression of the *ATP2B4* gene, the transcripts of which tended to be more abundant in the vitamin-K₂-treated embryos. Several studies confirm that intracellular Ca²⁺ levels are related to the expression of *ATP2B4* (Usachev *et al.* 2002, Schuh *et al.* 2004).

In conclusion, bovine embryos produced *in vitro* benefit significantly from vitamin K₂ supplementation of the culture medium. This vitamin appears to improve embryonic phenotype when added at the onset of embryonic genome activation, a stage at which the initiation of mitochondrial maturation is also known to occur. The succeeding step in this research is to determine the potential of the resulting embryos to lead to healthy gestations that go full term.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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