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Resveratrol–cyclodextrin complex affects the expression of genes associated with lipid metabolism in bovine in vitro produced embryos

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Contents

Antioxidants have been widely used during in vitro production to decrease the negative effect of reactive oxygen species. It was reported that the complex resveratrol–methyl β-cyclodextrin (RV-CD) improves resveratrol's stability and bioavailability and increases its antioxidant activity. This study evaluates the effect of RV-CD during in vitro oocyte maturation (IVM) or in vitro embryo culture (IVC) on developmental competence and quantitative changes in gene expression of developmental important genes. In experiment 1, RV-CD was added to IVM media and maturation level, embryo development and oocytes, cumulus cells, and blastocysts gene expression by RT-qPCR were examined. In experiment 2, presumptive zygotes were cultured in SOF supplemented with RV-CD and embryo development and blastocysts gene expression by RT-qPCR were studied. A group without RV-CD (control$^-$) and a group with cyclodextrin (control$^+$) were included. No differences were found in cleavage rate or blastocyst yield between groups. However, the expression of *LIPE* was higher in blastocysts derived from oocytes treated with resveratrol compared with control groups ($p < .05$). Blastocysts produced by IVC with resveratrol showed that RV-CD could modify the expression of genes related to lipid metabolism (*CYP51A1*, *PNPLA2* and *MTORC1*) compared with control groups ($p < .05$). RV-CD in the IVM and IVC media could reduce accumulated fat by increasing lipolysis and suppressing lipogenesis of blastocysts.

1 | INTRODUCTION

In vitro fertilization (IVF), manipulation of gametes and embryos and in vitro culture conditions expose cells to environmental factors that may affect antioxidant defence mechanism and therefore, negatively impacting the oocyte and the subsequent embryo, compared with those produced in vivo (Agarwal, Durairajanayagam, & du Plessis, 2014). To improve the quality of the oocyte and the developmental capacities of embryos cultured in vitro, antioxidant compounds have been used to supplement the culture medium. One of the most important polyphenols found in red wine is resveratrol (3,4,5-trihydroxy-trans-stilbene). Besides its cardioprotective, anticancer, antiapoptotic and anti-inflammatory effects, it has also an antioxidant and antilipogenic effect (Gambini et al., 2015). Its use has been reported during the IVM at concentrations of 0.1–10 μM in pig oocytes (Kwak et al., 2012), cattle (Wang et al., 2014) and goats (Mukherjee et al., 2014), increasing the concentration of glutathione within the oocyte, leading to a decreased level of ROS and increasing the blastocyst rate. In addition, the use of resveratrol during IVC at lower concentrations of 0.25–1 μM reduced the ROS level, whereas high concentrations of resveratrol during the embryo culture have toxic effects and are detrimental to the developing embryos (Lee, Wang, Chaille, & Machaty, 2010).

Routinely, antioxidants are dissolved in Dimethyl sulfoxide (DMSO) or ethanol. However, these diluents are well known to have embryotoxic effects in both in vivo and in vitro conditions. Normal DMSO concentrations for dissolving compounds range from 0.01% to 1%. Adler, Pellizzer, Paparella, Hartung, and Bremer (2006) proved that DMSO and ethanol have cytotoxic effect at a concentration as low as 0.125% and 1.07%, respectively. Cyclodextrins, a group of cyclic oligosaccharides, could be an excellent alternative to standard diluents when using resveratrol, solving both bioavailability and embryotoxic problems. The main property of Methil-β-Cyclodextrin is that the central cavity of these molecules is hydrophobic, while the rims of the surrounding walls are hydrophilic. This hydrophobic cavity forms inclusion complexes with a wide range of organic and inorganic guest molecules and has been used to improve the solubility of drugs, vitamins and food colourants (Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, & Simal-Gandara, 2009). Accordingly, given resveratrol's many health-promoting activities, this complexation has been previously used for increasing its bioavailability and stability in pharmaceutical (Li et al., 2011), cosmeceutical (Moyano-Mendez et al., 2013) and food industries (Davidov-Pardo & McClements, 2014) containing resveratrol. Thus, it would be very interesting its use in in vitro embryo production keeping in mind it would avoid the deleterious effect of standard diluents such as DMSO. Therefore, the purpose of this work was to evaluate the effect of RV-CD complex supplementation during IVM or IVC on developmental competence and embryonic quality. Indeed, we investigated the effects Resveratrol-cyclodextrin complex on the expression of genes associated with apoptosis, oxidative stress, transcription factors, cell junction, mucification, epigenetics and metabolism in a system of bovine embryo production in vitro.

2 | MATERIAL AND METHODS

Unless otherwise stated, all chemicals and media were purchased from Sigma Chemical Co. Ethical approval was not required as the experiments were in vitro performed.

2.1 | Recovery and IVM of cumulus–oocyte complexes

Immature cumulus–oocyte complexes (COCs) were obtained by aspirating follicles (2–8 mm) from the ovaries of matured heifers and cows collected from a local abattoir. A total of 2,487 COCs with homogenous cytoplasm and intact cumulus cells matured in four-well dishes (Nunc, Roskilde, Denmark) in 500 μl of TCM-199 maturation medium, supplemented with 10% (v/v) foetal calf serum (FCS) and 10 ng/ml epidermal growth factor (EGF) in groups of 50 COCs per well for 24 hr at 38.5°C under an atmosphere of 5% CO_2 in air with maximum humidity (Rizos, Ward, Duffy, Boland, & Lonergan, 2002).

2.2 | Nuclear oocyte staining

After 24 hr of maturation, COCs were gently vortexed for 2 min to remove cumulus cells, washed twice in PBS, fixed and stained with Hoechst 33342 as previously described (Coy et al., 2008). Nuclear maturation was observed in an epifluorescence microscope (Nikon 141731) equipped with a fluorescent lamp (Nikon HB-10104AF) and UV-1 filter (excitation and emission wavelengths of 352 and 455 nm, respectively) and oocytes classified as follows: germinal vesicle stage (GV), metaphase I (MI) or metaphase II (MII).

2.3 | In vitro fertilization (IVF)

The matured COCs were transferred in groups of 50 to four-well plates each containing 250 μl of fertilization medium (FERT-TALP medium [Merck, Darmstadt, Germany]) supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/ml fatty acid–free BSA and 10 μg/ml heparin. Frozen straws from an Asturian Valley bull previously tested for IVF were thawed, separated on a density gradient Bovipure™ (Nidacon, Sweden) and resuspended in fertilization medium. Then, 250 μl of this suspension was added to each fertilization well to obtain a final concentration of 1×10^6 spermatozoa/ml. Gametes were co-incubated for 18–22 hr at 38.5°C under an atmosphere of 5% CO_2 in air at maximum humidity.

2.4 | In vitro embryo culture

Presumptive zygotes were denuded and cultured in synthetic oviductal fluid (SOF) (Holm, Booth, Schmidt, Greve, & Callesen, 1999), supplemented with 3 mg/ml bovine serum albumin, in groups of 40–50 in 500 μl SOF under mineral oil and incubated under an atmosphere of 5% CO_2 and 5% O_2 in air at maximum humidity.

2.5 | RNA extraction, reverse transcription (RT) and real-time PCR (qPCR)

Gene expression was examined in oocytes, cumulus cell and blastocysts matured and cultured with resveratrol as later described. Pools of 10 oocytes and their cumulus cells and pools of 10 Day 7–8 expanding blastocysts per experimental group (three replicates per group) were used for mRNA extraction.

Poly (A) RNA was extracted using the Dynabeads® mRNA DIRECT™ Micro Kit (Ambion®, Thermo Fisher Scientific Inc., Norway) following the manufacturer's instructions with minor modifications (Bermejo-Alvarez, Rizos, Rath, Lonergan, & Gutierrez-Adan, 2010). After 10 min of incubation in lysis buffer with Dynabeads, poly (A) RNA attached to the Dynabeads was extracted with a magnet and washed twice in washing buffer A and washing buffer B. RNA was eluted with Tris–HCl. After extraction, RT reaction was carried out following the manufacturer's instructions (Epicentre Technologies Corp., USA) using poly (T) primers, random primers and MMLV High Performance Reverse Transcriptase enzyme in a total volume of 40 μl, to prime the RT reaction and produce cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 50 units of reverse transcriptase. Next, the tubes were incubated at 25°C for 10 min to promote the annealing of random primers, 60 min at 37°C to allow the RT of RNA and finally at 85°C for 5 min to denature the enzyme.

All mRNA transcripts were quantified using quantitative polymerase chain reaction (qPCR) and were run in the following conditions: 95°C for 3 min, 35 cycles of 94°C for 15 s, 56°C for 30 s and 72°C for 15 s, followed by a final extension step for 10 s and carried out with two repetitions for all genes of interest in the Rotorgene 6000 Real Time Cycler TM (Corbett Research, Sydney, Australia) by adding a 2-ml aliquot of each sample to the PCR mix (GoTaq qPCR Master Mix, Promega Corporation, Madison, WI, USA) containing the specific primers selected to amplify the selected genes. The comparative cycle threshold (CT) method was used to quantify expression levels. Values were normalized to the housekeeping gene (histone *H2AFZ*). According to the comparative CT method, the ΔCT value was determined by subtracting the housekeeping mean CT value for each sample from each gene CT value of the sample. The calculation of ΔΔCT involved using the highest treatment ΔCT value, that is the treatment with the lowest target expression, as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula 2–ΔΔCT (Livak & Schmittgen, 2001). The primers used for qPCR are provided in Table 1.

2.6 | Experimental design

Based on the current literature, where concentrations between 1 and 10 μM during IVM and of 0.25–1 μM during ICV increased the blastocysts rates and reduced the ROS levels, different concentrations of resveratrol, 1 and 10 μM, (RV-CD1 and RV-CD10, respectively) were used to supplement IVM medium (experiment 1) and 0.5 and 1 μM (RV-CD0.5 and RV-CD1, respectively) were used to supplement IVC medium (experiment 2). Resveratrol stock solutions were dissolved with 7% Methyl β-cyclodextrin in acetone/water (1:1, v/v) (Ou, Chang, Huang, & Prior, 2013). A group without RV-CD complex (control$^-$) and a group with cyclodextrin (control$^+$) were also included in both experiments. A final concentration of Methyl β-cyclodextrin in IVM and IVC was used in the same proportion of resveratrol in 7% Methyl β-cyclodextrin solution (0.001% and 0.0001%, respectively).

In experiment 1, a total of 1,743 COCs in six replicates were used. Four groups were established during IVM: (I) no treatment (Control−) (II) Methyl β-cyclodextrin 0.001% (Control+) (III) resveratrol 1 μM (RV-CD1) and (IV) resveratrol 10 μM (RV-CD10).

After 24 hr of IVM, 10 oocytes from each group were stained to evaluate nuclear maturation (*n* = 330), 10 COCs were gently pipetted to separate their cumulus cells (CC), snap frozen separately in LN_2 for gene expression analysis by RT-qPCR (*n* = 120) and the remaining were destined to IVF and posterior embryo development (*n* = 1,293). Cleavage rate was recorded at 33 and 48 hr post-insemination (pi) and cumulative blastocyst yield was recorded on Days 7, 8 and 9 pi.

To determine effects of RV-CD supplementation during IVM on embryo quality, pools of 10 expanding blastocysts per experimental group obtained on Day 7 and Day 8 pi (*n* = 120) were snap frozen in LN_2 for gene expression analysis by RT-qPCR.

In experiment 2, a total of 744 COCs in four replicates were obtained, matured (as control$^-$ group) and fertilized in vitro as previously performed in Experiment 1. Presumptive zygotes were cultured in groups of 40–50 as: (I) no treatment (Control$^-$) (II) Methyl β-cyclodextrin (Control$^+$) (III) resveratrol 0.5 μM (RV-CD0.5) and (IV) resveratrol 1 μM (RV-CD1).

Cleavage rate was recorded at 48 hr pi and cumulative blastocyst yield was recorded on Days 7, 8 and 9 pi. Pools of 10 expanding blastocysts per experimental group obtained on Days 7 and 8 pi (*n* = 120) were snap frozen in liquid nitrogen and kept at −80°C until mRNA extraction.

2.7 | Statistical analysis

The differences in the data relating to cleavage rate, blastocyst yield, nuclear maturation and relative mRNA abundance in fold increase in the genes followed a normal distribution and had homogeneous variances, thus, were compared using one-way analysis of variance ANOVA; when the normality test failed, a Ducan's test was conducted using the Sigma Stat software package (Jandel Scientific, San Rafael, CA, USA). Differences in $p < .05$ were considered significant.

3 | RESULTS

3.1 | Nuclear maturation

Nuclear maturation was unaffected by the presence of RV-CD, and similar MI and MII rates were recorded for RV-CD-treated groups (for the MI rates RV-CD 1 and RV-CD 10 groups were 28.6 ± 3.6% and 29.8 ± 6.0%, respectively; the MII rates were 57.1 ± 5.9% and 53.6 ± 4.4%, respectively) and control groups (for the MI rates were 24.3 ± 4.8% and 32.6 ± 3.8% in control$^-$ and control$^+$, respectively; the MII rates were 57.1 ± 4.3% and 71.4 ± 4.2%, respectively). In contrast, RV-CD10 led to an increase in number of oocytes with intact GV (16.6 ± 2.6%) ($p < .05$) compared with RV-CD1 (9.03 ± 0.6) and control groups (4.22 ± 0.6 and 10.3 ± 0.7 for control$^-$ and control$^+$, respectively ($p < .05$) indicating that a higher percentage of oocytes remained arrested or had not initiated meiosis at this time.

3.2 | Effect of RV-CD on gene expression levels in oocytes and cumulus cells

Oocytes from RV-CD1 and RV-CD10 groups had lower expression of *BAX* compared with the control$^+$ ($p < .05$). Moreover, *BMP15* expression in oocytes was significantly lower in RV-CD10 compared with control$^+$ ($p < .05$, Figure 1).

In cumulus cells from oocytes matured with RV-CD, we found that the *SOD1* expression in RV-CD1 group was significantly

TABLE 1 Summary of gene symbol, accession number, primer sequences and amplicon product length for the target genes examined

	Gene name	Entrez gene	Primer sequence (5′-3′)	Product length (pb)	Accession number (NCBI)
Oxidative Stress	*Glutathione Peroxidase 1*	*GPX1*	F-TCGCCAAGGCAGATGTGAGAG R-TTCATGCCAGTGGGTCAGGGT	115	NM_174076.3
	Superoxide Dismutase 2, Mitochondrial (former MnSOD)	*SOD2*	F-GCTTACAGATTGCTGCTTGT R- AAGGTAATAAGCATGCTCCC	101	NM_201527.2
	Glutathione S-transferase, alpha 4	*GSTA4*	F-CGCAGACAAGCACCATCTCTTCG R- TGCTGCCAGGTTCAAGGAATTTC	383	BC109801.1
	Superoxide dismutase 1, soluble	*SOD1*	F- CCCATGAAGCCTTTCTAATCC R-TTCAGAGGGGCTACTATTTCC	309	NM_174615.2
Apoptosis	*BCL2-associated X protein*	*BAX*	F-CTACTTTGCCAGCAAACTGG R- TCCCAAAGTAGGAGAGGA	158	NM_173894.1
	B-cell CLL/lymphoma 2	*BCL2*	F- ACTATAAGCTGTCGCAGCGG R- TACAGCTCCACAAAGGCGTC	517	NM_001166486.1
Growth Factor	*Bos taurus bone morphogenetic protein 15*	*BMP15*	F- ATCATGCCATCATCCAGAACC R- TAAGGGACACAGGAAGGCTGA	72	NM_001031752.1
Cell Junction	*gap junction protein alpha 1 (former CX43)*	*GJA1*	F-TGGAATGCAAGAGAGGTTGAAAGAGG R- AACACTCTCCAGAACACATGATCG	293	NM_174068.2
Mucification	*Prostaglandin-endoperoxide synthase 2*	*PTGS2*	F- ATCTACCCGCCTCATGTTCCT R- GGATTAGCCTGCTTGTCTGGA	187	NM_174445.2
Lipid Metabolism	*ytochrome P450, family 51, subfamily A, polypeptide 1*	*CYP51A1*	F-GGCCCAAGGTGATTTCCATTTC R-CTCCCAAGAAACCCTGCACTGG	168	BC149346.1
	Adipose triglyceride lipase	*PNPLA2*	F-TGCCAGTACCTGATGATACGC R-CCTCCCATTTGGCCAGTACAT	193	FJ897536
	Lipase, hormone-sensitive	*LIPE*	F-GGGATATCTGAAGAGGCCTGG R- TGTGTCCAGGTCAAAGAGGTG	241	NM_001080220
	Acetyl-CoA carboxylase alpha	*ACACA*	F-AAGCAATGGATGAACCTTCTTC R-GATGCCCAAGTCAGAGAGC	196	FN185963.1
	Fatty acid desaturase 1	*FADS1*	F-GCTGCCCAATCTGAGCAAAGC R-TCCTGTCATGGTGTGGGTCCTG	208	XM_005197347.3
	Solute carrier family 2 (facilitated glucose transporter) member 1	*SLC2A1*	F- CTGATCCTGGGTCGCTTCAT R-ACGTACATGGGCACAAAACCA	68	NM_174602.2
Transcription Factor	*Transcription factor A, mitochondrial*	*TFAM*	F- CAAATGATGGAAGTTGGACG R- AGCTTCCGGTATTGAGACC	150	NM_001034016.2
	Sirtuin 1(silent mating type information regulation)	*SIRT1*	F-TTACAGGGCCTATCCAGGGAG R-GCATGCGAGGCTCTATCATCT	185	NM_001192980.1
	Mechanistic target of rapamycin (serine/threonine kinase)	*MTORC1*	F-CTTTGAGGCTGTGCTCCACTA R- TCCTCAGTGACCTTCTTCTGC	228	XM_015466778.1
Epigenetic	*DNA (cytosine-5-) methyltransferase 3 alpha*	*DNMT3A*	F- CTGGTGCTGAAGGACTTGGGC R- CAGAAGAAGGGGCGGTCATC	318	AY271299.1
	Insulin-like growth factor 2 (somatomedin A)	*IGF2*	F-GACCGCGGCTTCTACTTCAG R: AAGAACTTGCCCACGGGGTAT	176	XM_010838191.1
	Insulin-like growth factor 2 receptor	*IGF2R*	F-GCTGCGGTGTGCCAAGTGAAAAAG R-AGCCCCTCTGCCGTTGTTACCT	201	NM_174352.2
Housekeeping	*H2A histone family, member Z*	*H2AFZ*	F-AGGACGACTAGCCATGGACGTGTG R- CCACCACCAGCAATTGTAGCCTTG	212	NM_016750

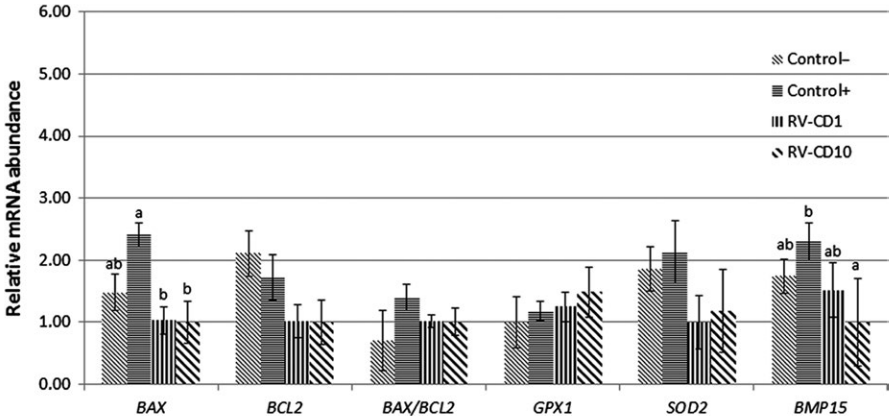

FIGURE 1 Relative mRNA transcription in bovine oocytes in vitro matured with RV-CD resveratrol. Results are expressed as mean ± *SEM*. ^{a,b}Different superscripts letters indicate significant differences ($p < .05$) between groups

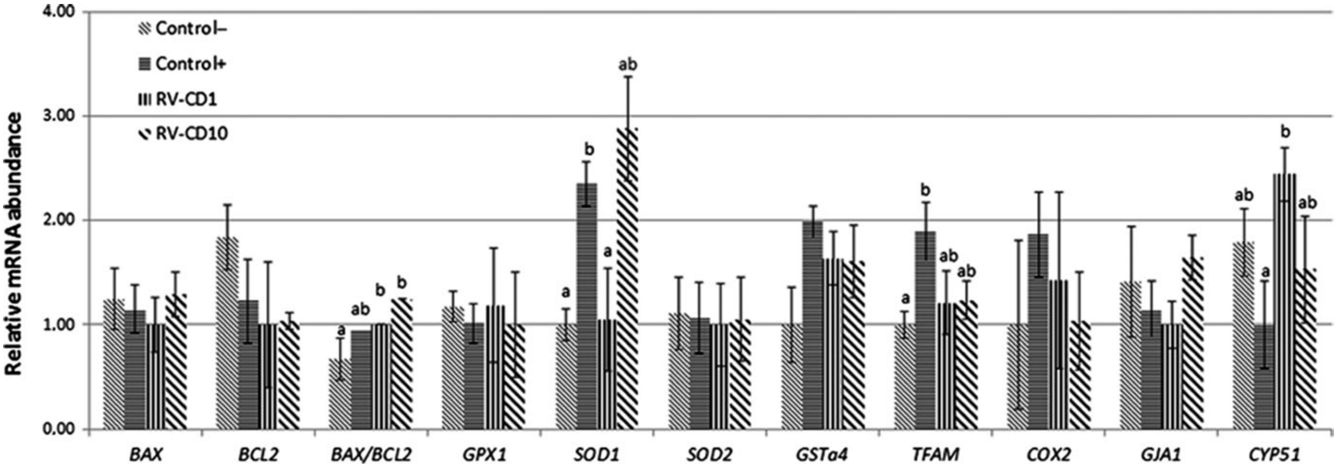

FIGURE 2 Relative mRNA transcription in bovine cumulus cells in vitro matured with RV-CD. Results are expressed as mean ± *SEM*. ^{a,b}Different superscripts letters indicate significant differences ($p < .05$) between groups

lower compared with control$^+$ ($p < .05$). Moreover, *CYP51A1* expression was increased compared with control$^+$ ($p < .05$, Figure 2).

3.3 | Effect of RV-CD on developmental competence and gene expression levels in blastocysts derived from oocytes matured with RV-CD

No significant effect on cleavage rate nor blastocyst rates were observed among RV-CD-treated groups and control groups ($p > .05$, Table 2).

The level of *SCL2A1* expression was significantly lower in RV-CD1 than in the control$^+$. Also, *LIPE* was increased in the RV-CD-treated groups compared with the control groups ($p < .05$). *PNPLA* expression was higher in control$^+$ compared with control$^-$, but not differences were found between RV-CD groups and control$^-$. Finally, the levels of expression of *BAX*, *BAX/BCL2* ration and *DNMT3A* were significantly higher in RV-CD10 compared with other groups ($p < .05$; Figure 3).

3.4 | Effect of RV-CD on developmental competence and gene expression levels in bovine blastocysts cultured with RV-CD

No differences were found in terms of cleavage rates nor blastocyst yield between RV-CD0.5, RV-CD1 and control groups, as shown in Table 3 ($p > .05$).

The level of *CYP51A1* and *PNPLA2* expression was significantly higher in RV-CD0.5 and in RV-CD0.5 and RV-CD1 groups respectively compared with control groups ($p < .05$). Moreover, SIRT1 expression was significantly lower in RV-CD1 than in the control$^-$ while *MTORC1* expression was higher in control groups compared with RV-CD-treated samples ($p < .05$, Figure 4).

4 | DISCUSSION

The present study investigated for the first time the influence of resveratrol–cyclodextrin complex supplementation in the medium

TABLE 2 Effect of RV-CD supplementation during in vitro maturation on cleavage rates and blastocyst yield (n = 1,293). Cleavage rate at 33 and 48 hr′ post-insemination (hpi) and blastocyst rate at Days 7–9 pi (in vitro fertilization = Day 0) derived from experimental groups (I) no treatment (Control$^-$) (II) Methyl β-cyclodextrin (Control$^+$) (III) Resveratrol 1 μM (RV-CD1) and (IV) Resveratrol 10 μM (RV-CD10). *N*, Total number of presumptive zygotes placed in culture. Results are expressed as mean ± *SEM*

		Cleavage		Blastocyst yield		
	Oocytes cultured for maturation	33 hpi Mean ± *SEM* (%)	48 hpi ± *SEM* (%)	Day 7 Mean ± *SEM* (%)	Day 8 Mean ± *SEM* (%)	Day 9 Mean ± *SEM* (%)
Control$^-$	364	229 (73 ± 6.0)	298 (81.5 ± 3.0)	33 (11.4 ± 3.2)	63 (19.7 ± 3.8)	76 (21 ± 5.1)
Control$^+$	275	148 (75.4 ± 4.3)	223 (80.8 ± 3.1)	40 (15.3 ± 2.8)	57 (22.7 ± 2.8)	67 (24.3 ± 2.7)
RV-CD1	342	180 (75.9 ± 2.4)	291 (85.1 ± 1.1)	67 (18.2 ± 2.8)	84 (25.3 ± 1.5)	96 (28.2 ± 2.3)
RV-CD 10	312	152 (72 ± 3.3)	261 (84.2 ± 2.8)	62 (18.7 ± 1.9)	82 (26.2 ± 2.4)	83 (26.3 ± 4.5)

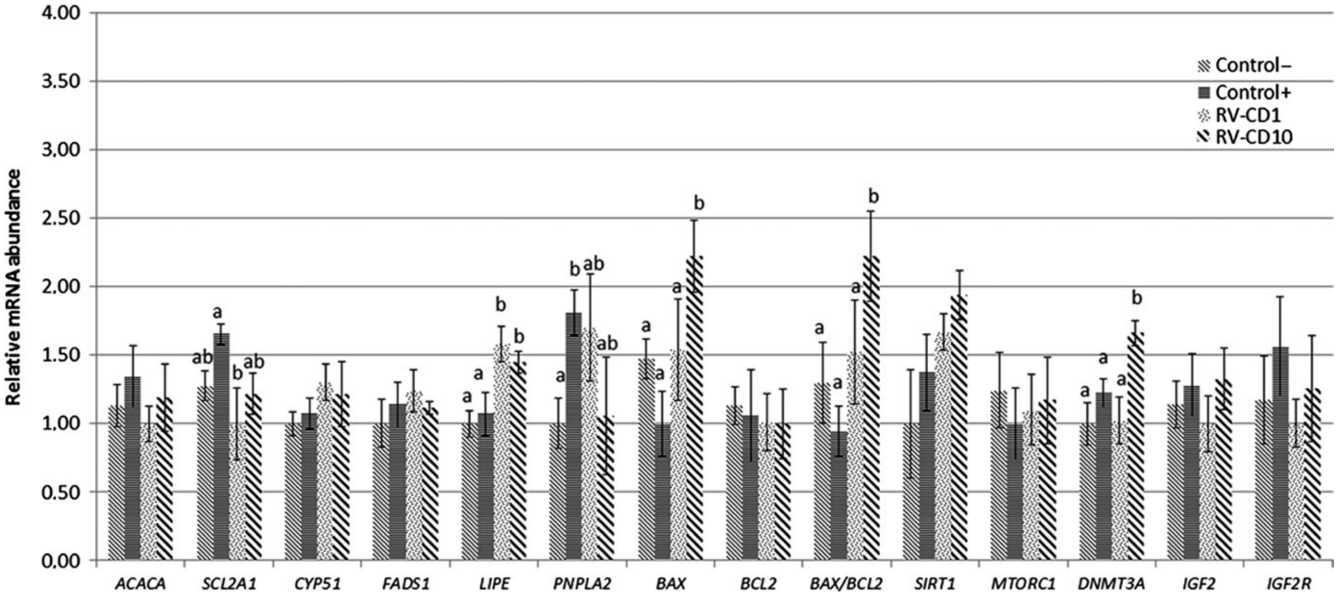

FIGURE 3 Relative mRNA transcription in bovine in vitro blastocysts (D7–D8 pi) matured with RV-CD. Results are expressed as mean ± *SEM*. ^{a,b}Different superscripts letters indicate significant differences (p < .05) between groups

TABLE 3 Effect of RV-CD supplementation during in vitro culture on cleavage rate and blastocyst yield. Cleavage rate at 48-hr post-insemination (pi) and blastocyst rate at Days 7–9 pi (in vitro fertilization = Day 0) derived from experimental groups (I) no treatment (Control$^-$) (II) Methyl β-cyclodextrin (Control$^+$) (III) Resveratrol 0.5 μM (RV-CD0.5) and IV) Resveratrol 1 μM (RV-CD1). *N*, Total number of presumptive zygotes placed in culture. Results are expressed as mean ± *SEM*

		Cleavage	Blastocyst yield		
Treatment	*N*	48 hpi Mean ± *SEM* (%)	Day 7 Mean ± *SEM* (%)	Day 8 Mean ± *SEM* (%)	Day 9 Mean ± *SEM* (%)
Control$^-$	183	155 (85.3 ± 2.9)	28 (15.2 ± 1.0)	44 (24 ± 3.9)	45 (24.7 ± 3.6)
Control$^+$	212	176 (82.9 ± 2.7)	38 (18 ± 2.5)	56 (26.4 ± 2.3)	57 (26.9 ± 2.2)
RV-CD0.5	169	137 (81 ± 4.6)	31 (18.3 ± 3.0)	44 (26.1 ± 2.7)	45 (26.7 ± 2.6)
RV-CD1	180	152 (85.1 ± 3.0)	33 (18 ± 2.7)	42 (22.7 ± 3.2)	43 (23.3 ± 3.0)

of maturation and culture in vitro on developmental competence and embryonic quality. Results showed that 10 μM of RV-CD significantly increased the percentage of oocytes that remained arrested in germinal vesicle but did not reduce metaphase II rate. Similar effect has been reported using the same concentration of resveratrol during porcine IVM (Kwak et al., 2012). Similarly, Pocar, Augustin, and Fischer (2004) reported that 10 μM resveratrol did not induce significant differences at any stage compared with the

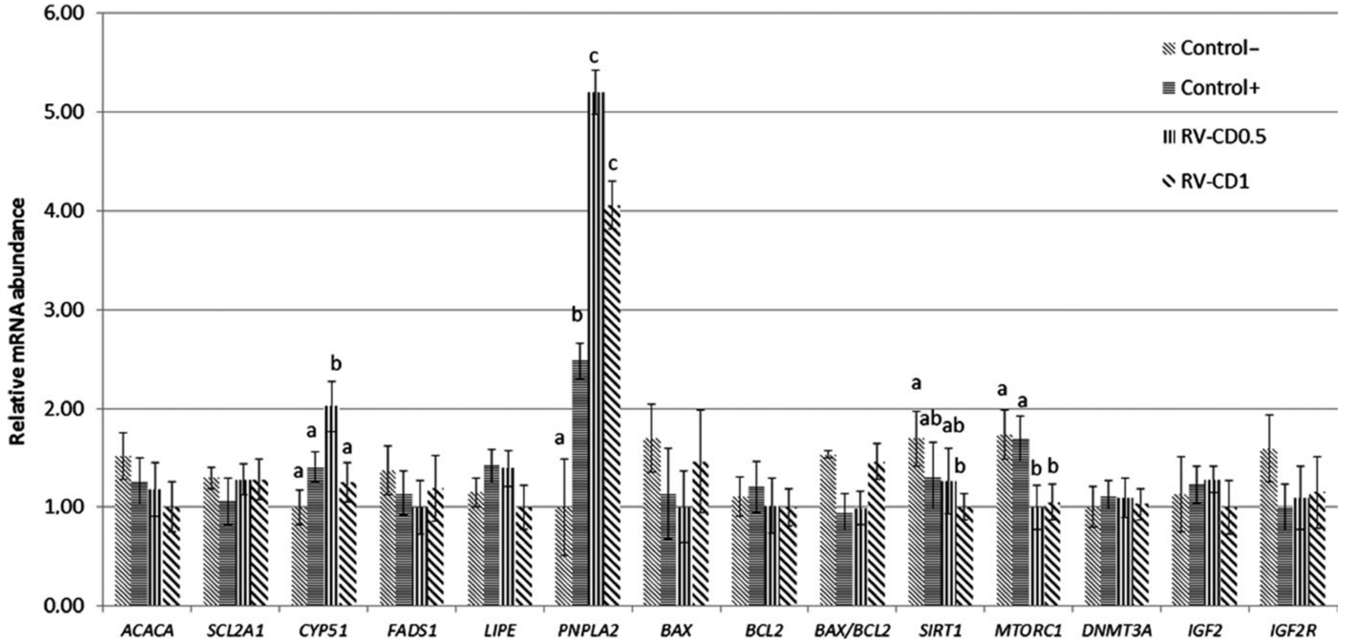

FIGURE 4 Relative mRNA transcription in bovine in vitro blastocysts (D7–D8 pi) cultured with RV-CD. Results are expressed as mean ± *SEM*. ^{a,b,c}Different superscripts letters indicate significant differences ($p < .05$) between groups

control group whereas resveratrol at higher concentrations (20 and 40 μM) reduced the percentage of oocytes that reached the metaphase II stage after 24 hr of culture.

Furthermore, we observed that supplementation of RV-CD during IVM and IVC at different concentrations did not negatively affect neither cleavage rate nor embryo development. Similar results were found when different concentrations of resveratrol were evaluated during culture (0.25–1 μM) (Salzano et al., 2014). Conversely, other studies reported an improvement of developmental competence of resveratrol at different concentrations (0.1–10 μM) on bovine (Wang et al., 2014), porcine (Kwak et al., 2012), goat (Mukherjee et al., 2014) oocyte maturation and subsequent embryo development. Concerning our study, we postulate that due to the properties of Methil-β-Cyclodextrin to encapsulate resveratrol, this oligosaccharide could improve its water-dispersibility and bioavailability in the culture media, leading to an increased effect compared with previous works using standard diluents. Thus, a concentration lower than 1 μM of resveratrol when used with Methil-β-Cyclodextrin during IVM of bovine embryos could be more appropriate to obtain better results. Moreover, it has been recently investigated that RV-CD inclusion increases the antioxidant activity of the resveratrol and provides further stability, which could improve its advantages (Kumar, Kaur, Uppal, & Mehta, 2017). Despite no effect was observed in blastocyst rate in both experiments, a positive effect of the RV-CD was observed concerning gene expression. In fact, we observed in oocytes treated with RV-CD during IVM a lower expression of *BAX* compared with control^{+}. This result suggests that RV-CD in the IVM medium may exert an antiapoptotic effect on oocytes. These results are in accordance with the previous studies in porcine (Kwak et al., 2012) and goat oocytes (Mukherjee et al., 2014).

Besides concerning gene expression in cumulus cells, we observed that *SOD1* was down-regulated when RV-CD1 was added to the maturation media compared with control^{+}. It was recently suggested that *SOD1* neutralizes superoxide anions in the cytoplasm of cells, and the oocyte is probably an abundant producer of ROS as it is metabolically active (Combelles et al., 2010). As mentioned above, oocytes exposed to RV-CD1 during maturation decreased the expression of proapoptotic gene *BAX*; thus, we suggest that cumulus cells matured in this media are less exposed to ROS (Papler et al., 2014). Finally, the expression of *CYP51A1*, involved in cholesterol synthesis pathway, was increased in RV-CD1 compared with control^{+}. This result is in accordance with lower expression level of *BMP15* found in oocytes exposed to high concentration of RV-CD during maturation as far as oocytes are probably dependent upon cumulus cells to provide them with cholesterol, and oocytes stimulate this activity in cumulus cells via *BMP15* and *GDF9* (Su et al., 2008).

Furthermore, we examined the expression of mRNAs to determine effects of RV-CD supplementation during IVM on embryo quality. The gene related to lipid metabolism *LIPE* (also known as *HSL*) is a key enzyme in the mobilization of fatty acids from acylglycerols in adipocytes as well as nonadipocytes. It has been recently described the beneficial effects of resveratrol against metabolic diseases reducing body weight and body fat accumulation (Ahn, Cho, Kim, Kwon, & Ha, 2008). Lasa et al. (2012) found that *HSL* expression was not altered in murine 3T3-L1 adipocytes after incubation with resveratrol. Interestingly, the present study revealed that RV-CD increased the expression of *LIPE*, suggesting it could play a crucial role in the metabolic effect of RV-CD. Moreover, RV-CD10 during IVM increased the expression of *BAX* and the ratio *BAX*/*BCL2* in blastocysts. It has been reported that the expression of *BAX*, an apoptotic regulatory gene, was higher in blastocysts produced in SOF than those developed in coculture or in vivo, indicating that these embryos were of lower quality (Rizos, Lonergan et al., 2002). However, its deficiency seems to be compensated by increased production of *DNMT3A*, responsible for the establishment of de novo genomic DNA methylation patterns (Kaneda et al., 2004). This suggests that RV-CD10 could improve the quality of embryo, through the regulate de novo methylation that is an essential process for mammalian development.

Surprisingly, we demonstrated that RV-CD could decrease triglycerides (TG) accumulation in blastocysts cultured with RV-CD. In fact, we observed that *CYP51A1* (required for sterol biosynthesis) and *PNPLA2* were significantly higher in the 0.5 μM RV-CD-treated samples during in vitro culture. Moreover, *PNPLA2* also known as *ATGL* selectively performs the first step in TG hydrolysis resulting in the formation of diglycerides and free fatty acids. In the same line, Lasa et al. (2012) found that decreased TG accumulation in 3T3-L1 adipocytes after the three incubation periods with resveratrol and enhanced *PNPLA2* expression level in 3T3-L1 and SGBS adipocytes. Therefore, our findings are also in accordance with those reported by Picard et al. (2004) and Szkudelska, Nogowski, and Szkudelski (2009) in 3T3-L1 adipocytes, observed that resveratrol increased the glycerol release in rat adipocytes.

Moreover, *MTORC1* (mechanistic Target of Rapamycin) plays a significant role in protein synthesis, lipid biosynthesis and inhibition of triacylglycerol lipolysis (Soliman, 2013). The present study revealed that RV-CD decreased the expression of *MTORC1* in blastocysts. Chakrabarti, English, Shi, Smas, and Kandror (2010) reported that inhibition of *MTORC1* signalling via rapamycin decreases triacylglycerol storage by increasing lipolysis in 3T3-L1 adipocytes and that activation of *MTORC1* signalling in 3T3-L1 adipocytes inhibited expression of *ATGL* and *HSL* at the level of transcription, suppressing lipolysis, increasing de novo lipogenesis and promoting intracellular accumulation of triglycerides. These results suggest that RV-CD exerts a positive effect on lipid metabolism and can reduce accumulated fat of blastocysts produced in vitro.

In conclusion, we demonstrate that the complex RV-CD during both the IVM and IVC not negatively affects the embryonic development. In addition, RV-CD during the IVC of bovine embryos induced a positive effect on the expression of genes related to lipid metabolism, increasing lipolysis (*PNPLA2*, *CYP51A1*) and decreasing lipogenesis (*MTORC1*). Interestingly, the bovine oocyte maturation medium supplemented with resveratrol generates an increase in the expression of *LIPE* gene in in vitro produced embryos. This result suggests that resveratrol could stimulate lipolysis in bovine embryos obtained from oocytes matured with resveratrol. However, to obtain a complete gene expression profile and to better understand the mechanisms for regulating transcription of RV-CD as a supplement to culture media, more studies are needed.

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AUTHOR CONTRIBUTIONS

A Gutiérrez-Adán, D Rizos and MJ Sánchez-Calabuig designed the experiment. V Torres, M Hamdi, M Millán de la Blanca and MJ Sánchez-Calabuig performed the experiment and analysed data. V Torres and MJ Sánchez-Calabuig drafted the manuscript. R Urrego, J Echeverri and A López-Herrera contributed with the experiment design and the manuscript corrections.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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