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EMBELIN SUPPLEMENTATION OF *IN VITRO* MATURATION MEDIUM DOES NOT INFLUENCE NUCLEAR AND CYTOPLASMIC MATURATION OF PIG OOCYTES

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Oxidative stress caused from *in vitro* culture contributes to inadequate oocyte maturation which leads to a poor embryo development. Therefore, it is important to protect oocytes and embryos against oxidative stress. This study was aimed at evaluating the effect of Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone), an antioxidant with various pharmacologic activities, on nuclear and cytoplasmic maturation of pig oocytes as well as on steroidogenesis of cumulus cells (CCs). Another objective was to determine the influence of Embelin on developmental competence of pig oocytes as well as the expression levels of three key genes (Nanog, Sox2 and Oct4) involved in the control of pluripotency in parthenogenetically activated embryos. Embelin (0, 10, 20 and 40 μM) was added during *in vitro* maturation of cumulus oocyte complexes; media of both the first and the second day of culture were collected and assayed for progesterone and estradiol-17β. At the end of the maturation period, the oocytes were fixed (to determine nuclear maturation) or partenogenically activated to evaluate cytoplasmic maturation and genes expression. Embelin did not exert any effect on the proportion of MII oocytes, steroidogenesis of CCs, percentage of embryos that developed to blastocyst stage and the number of blastomeres/blastocyst. Moreover, no significant differences of Oct4, Nanog and Sox2 transcripts were detected in blastocyst stage embryos. In conclusion, Embelin did not influence the reproductive parameters assessed, confirming that it is not possible to predict whether the beneficial effect exerted by an antioxidant in a particular tissue could be present also in another one.

Key words: *Embelin, blastocyst, oocyte in vitro maturation, pluripotency genes, steroidogenesis*

INTRODUCTION

The production of reactive oxygen species (ROS), oxygen-derived molecules formed as intermediary products of cellular metabolism, usually increases during *in vitro* oocytes and embryos culture due to light exposure, medium composition, temperature and pH variations and high oxygen concentration. The consequent development of oxidative stress is one of the most important factors causing defective gametes or poorly developing embryos *in vitro* (1). In fact ROS have been reported to induce mitochondrial dysfunction, cell membrane damage by lipid peroxidation, DNA fragmentation, RNA and protein damage thus reducing the developmental competence of embryos (2, 3).

To alleviate this situation, special attention has been accorded to the addition of antioxidants to the culture system. Various compounds with antioxidative properties such as β-mercaptoethanol, cysteine (4, 5), vitamin C (6) and vitamin E (7) were added during oocyte/embryo culture to improve the maturation of oocytes and the developmental competence of embryos.

Another approach is to supplement the culture medium with a natural antioxidant. Many plant molecules have been tested during oocyte/embryo *in vitro* culture on the basis of their well known antioxidant properties, clearly manifested in the prevention of cancer, cardiovascular disorders and other pathological conditions. Positive results have been obtained by adding to the culture media of oocytes and embryos plant antioxidants such as resveratrol (8, 9) and green tea polyphenols (10, 11). However, it is not possible to predict whether the beneficial effect exerted by an antioxidant in a particular type of cell could be manifested also in another cell, making it necessary to test each antioxidant during *in vitro* culture of oocytes/embryos.

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone), an alkyl-substituted hydroxyl quinone, is the main active constituent of *Embelia ribes* seeds (common name: Vidanga; family: Myrsinaceae); it is indigenous to India and found in the hilly parts of the Himalayas.

In addition to antioxidant property (12), various pharmacological activities of embelin, commonly used in Ayurvedic medicine, have been demonstrated in mammals and cell lines, such as anti-inflammatory and analgesic (13), hepatoprotective (14), anticancer (15-18), chemopreventive (19), anti-pyretic and anti-inflammatory activities (20).

To our knowledge, no studies have used Embelin as antioxidant agent during *in vitro* maturation of pig oocytes. Therefore, the present study was aimed at evaluating the effect of Embelin on nuclear and cytoplasmic maturation of pig oocytes as well as on steroidogenesis of cumulus cells (CCs).

Another objective was to determine the influences of Embelin on developmental competence of pig oocytes as well as the expression levels of three key genes (Nanog, Sox2 and Oct4) involved in the control of pluripotency in parthenogenetically activated embryos (21-23). Pluripotency is crucial for the survival and development of preimplantation embryos.

MATERIALS AND METHODS

In vitro maturation (IVM) of cumulus-oocyte-complexes (COCs)

Ovaries were collected at a local abattoir and transported to the lab within 2 hours in a thermos filled with physiological saline at 30 – 35°C. Cumulus oocyte complexes (COCs) from follicles 3 – 6 mm in diameter were aspirated using a 18 gauge needle attached to a 10 ml disposable syringe. Under a stereomicroscope, intact COCs were selected and transferred into a petri dish (35 mm, Nunclon, Roskilde, Denmark) prefilled with 2 ml of modified PBS supplemented with 0.4% BSA. Only COCs with complete and dense cumulus oophorus were used. After three washes in NCSU 37 supplemented with 5.0 mg/ml insulin, 0.57 mM cysteine, 10 ng/ml epidermal growth factor, 50 µM β-mercaptoethanol and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500 µl of the same medium per well and cultured at 39°C in humidified atmosphere of 5% CO_2/7% O_2 in air. For the first 22 hours of *in vitro* maturation, the IVM medium was supplemented with 1.0 mM dibutyryl cyclic adenosine monophosphate (db-cAMP), 10 IU/ml eCG (Folligon, Intervet, Boxmeer, The Netherlands) and 10 IU/ml hCG (Corulon, Intervet). For the last 20 hours COCs were transferred to fresh maturation medium (24).

Embelin (Sigma-Aldrich; 0, 10, 20 and 40 µM) solubilized in dimethyl sulfoxide was added during IVM of cumulus oocyte complexes; media of both the first and the second day of culture were collected, centrifuged and the supernatants were assayed for progesterone and estradiol-17β by validated RIAs. At the end of the maturation period oocytes were fixed to determine nuclear maturation or partenogenically activated.

Unless otherwise specified, all the reagents were purchased from Sigma-Aldrich (Milan, Italy).

Parthenogenetic activation

After IVM, oocytes were denuded as described above, washed three times IVF in medium and then parthenogenetically activated according to the method described by Boquest *et al.* (25) slightly modified. Briefly, the oocytes were transferred to IVF medium containing 5 mM ionomycin for 5 min, then washed twice and incubated in NCSU-23 containing 2 mM 6-dimethylaminopurine (6-DMAP) for 3 h at 39°C. Presumptive parthenotes were washed twice in NCSU-23 (26) and cultured in groups of 50 in 500 µl of the same medium. On day 5 post activation, 250 µl of medium were replaced with fresh pre-equilibrated NCSU-23 containing 20% (v/v) FCS to reach a final FCS concentration of 10% (v/v).

At day 7 post activation, percent of blastocysts and number of blastocyst nuclei were determined after staining with 5 µg/ml Hoechst 33342 and mounting on microscope slides by using a

Table 1. Forward and reverse primer sequences, PCR product length (bp), accession number (Acc.No.) and reference.

Gene	Primer Sequence (5'-3')	PCR Product (bp)	Acc. No.	Reference
β-ACT	For.: GTCCTCCTCCTCCCTGG Rev.: GTGGTCTCGTGGATGCC	141	AJ312193	(27)
NANOG	For.: ACTGTCTCTCCTCTTCCTTCCTC Rev.: CTCCTTGTCTGTGCTCTTCTCTG	117	ENSSSCT00000005489	Present study
OCT4	For.: TGTTCAGCCAAACGACCATC Rev.: ATACTTGTCCGCTTTCTCTTCC	178	ENSSSCT00000001516	Present study
SOX2	For.: ACAGCCCAGACCGAGTTAAG Rev.: ATCTCCGAGTTGTGCATCTTG	106	ENSSSCT00000012883	Present study

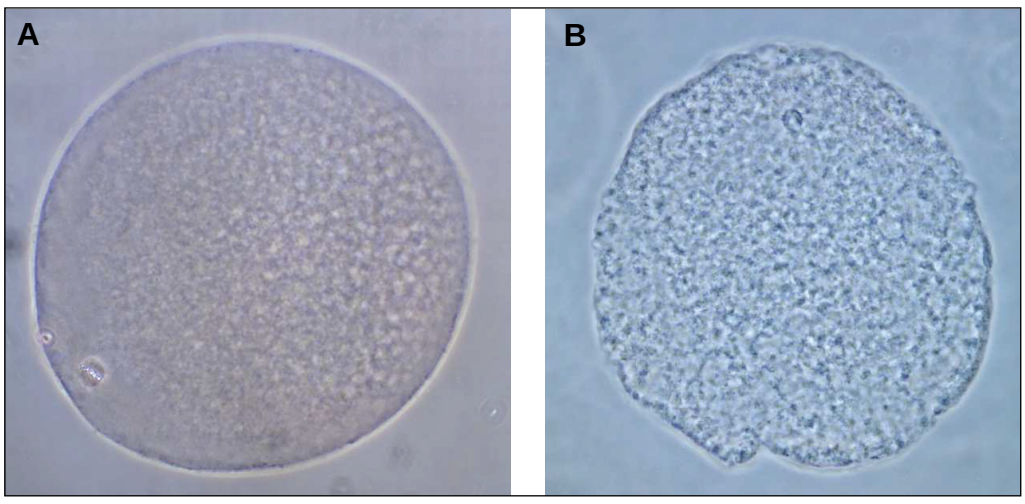

Fig. 1. Example of mature oocyte (A) and degenerated oocyte (B).

Nikon Eclipse E600 epifluorescence microscope equipped with a UV set filter. Embryos with at least 20 blastomeres and a clearly visible blastocoel were considered as blastocysts.

RNA isolation and gene expression of Nanog, Oct4 and Sox2

Blastocysts were treated with pronase 1 mg/ml PBS supplemented with 0.4% BSA, in order to remove zona pellucida, rinsed twice in nuclease free water, and groups of 10 blastocysts for each treatment (Embelin 10, 20 and 40 µM) and control (Embelin 0 µM) were frozen in 5 µl water, and stored at –80°C until analysis. We analyzed 5 groups for each treatment. Blastocysts were lysed in 200 µl of SideStep lysis and Stabilization buffer (Agilent Technologies, Santa Clara, CA, USA), then 3.5 µl were retrotranscribed with iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., California, USA), following the manufacturer's instructions, in a 20 µl final volume to obtain cDNA. Quantitative PCR was carried out using a CFX96 (Bio-Rad) thermal cycler. Primers sequence for β-actin (reference gene), Nanog, Oct4, and Sox2 were reported in *Table 1*. A master mix of the following reaction components was prepared in nuclease free water to the final concentrations indicated: 0.2 µM forward primer, 0.2 µM reverse primer, 1X iTaq Universal SYBR Green Supermix (Bio-Rad). One µl of cDNA was added to 19 µl of the master mix. All samples were analysed in duplicate. The qPCR protocol used for the transcriptional characterization was: 10 min at 95°C, 40 cycles at 95°C for 15 s and at 60°C for 30 s, followed by a melting step from 55°C to 95°C (80 cycles of 0.5°C increase/cycle).

Relative expression was calculated as fold of change ($2^{-\Delta\Delta Ct}$ method) in respect to blastocyst obtained under standard condition in absence of Embelin. Error bar represents the range of relative expression.

Experiment 1: effect of Embelin on nuclear maturation

In order to assess the effect of Embelin on nuclear maturation, pig COCs were exposed to 0, 10, 20 and 40 µM Embelin during *in vitro* maturation period (44 – 46 h).

At the end of the maturation period the oocytes were mounted on microscope slides, fixed in acetic acid/ethanol (1:3) for 24 h and stained with lacmoid. The oocytes were observed under a phase contrast microscope in order to evaluate the meiotic stage achieved. Oocytes with a nuclear morphology corresponding to MII were considered mature, those with abnormal cytoplasm or chromatin morphology or no chromatin were considered as degenerated.

Experiment 2: effect of Embelin on cumulus cell steroidogenesis

IVM media of both the first and the second day of culture were collected, centrifuged at 900 × g for 5 min and the supernatants were stored at –20°C until assayed for progesterone (P4) and estradiol-17β (E2) by validated radioimmunoassays (28). At the end of the maturation period, cumulus cells were counted using a Thoma's hemocytometer, after being freed from matured oocytes by gentle repeated pipetting.

For P4, intra and interassay coefficients of variation were 7.8% and 10.1%, respectively; assay sensitivity was 1.65 pg/tube. Intra and interassay coefficients of variation for E2 were 4% and 12%, respectively; assay sensitivity was 1.1 pg/tube. Steroid concentrations are expressed as ng/10^6 cells.

Experiment 3: effect of Embelin on embryo development and gene expression

In order to assess the effect of Embelin during IVM on embryo development, blastocysts formation and quality were evaluated 7

Table 2. Effect of Embelin on pig oocytes IVM.

Embelin (µM)	**0**	**10**	**20**	**40**
No. of oocytes examined	232	230	228	230
% of mature oocytes	94.6 ± 2.7	92.5 ± 3.3	93.8 ± 2.9	93.4 ± 3.6
% of degenerated oocytes	7.5 ± 2.4	8.0 ± 1.9	8.4 ± 2.1	7.9 ± 2.0

Values are mean ± S.E.M. of at least five replicates.

Table 3. P4 and E2 (ng/10^6 cells) production by cumulus cells after 24 and 48 h of *in vitro* maturation of oocytes in absence or in presence of 10, 20 and 40 µM Embelin. Data represent the mean ± S.D. of at least six replicates.

Embelin (µM)	**0**	**10**	**20**	**40**
P4 24 h	174.7 ± 79.3	155.0 ± 21,5	127.8 ± 38.5	83.1 ± 77.8
P4 48 h	931.8 ± 275	969.0 ± 268	870.3 ± 174	701.4 ± 469
E2 24 h	10.2 ± 4.5	8.6 ± 2.4	10.5 ± 6.6	10.6 ± 3.0
E2 48 h	8.9 ± 5.1	7.6 ± 2.8	7.02 ± 1,2	10.3 ± 3.2

Table 4. Effect of Embelin during *in vitro* maturation on developmental competence of embryos.

Embelin (µM)	**0**	**10**	**20**	**40**
No. of oocytes	235	270	257	267
% of blastocysts	19.4 ± 9.4	22.2 ± 8.1	15.0 ± 5.9	22.7 ± 9.9
cells per blastocyst	37.9 ± 12.2	38.2 ± 11.7	37.1 ± 9.6	41.4 ± 14.5

Values are mean ± S.D. of at least six replicates.

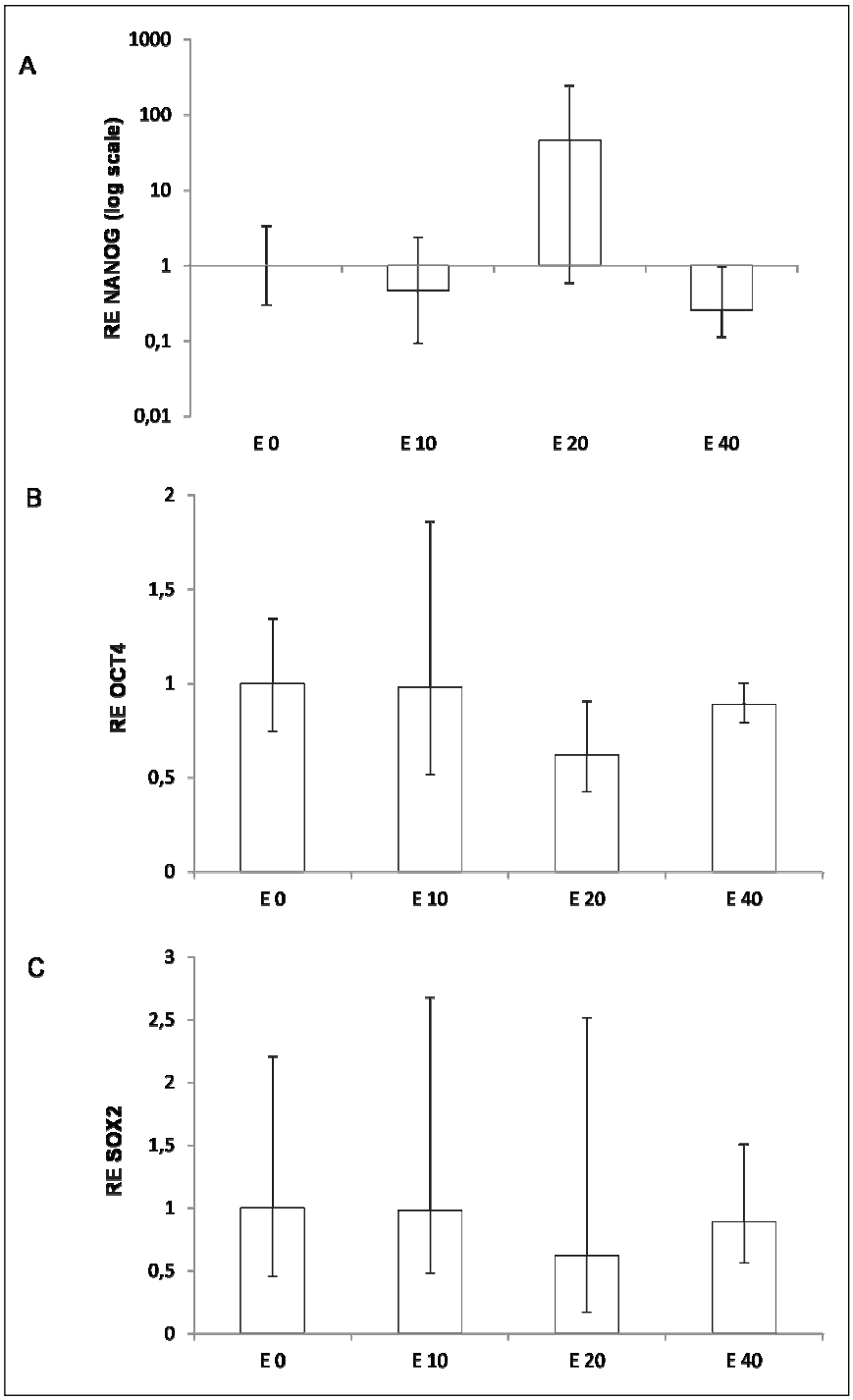

Fig. 2. Relative gene expression (RE) of Nanog (A), Oct4 (B) and Sox2 (C) in swine blastocysts obtained in absence (E0) or in presence of increasing amounts of Embelin (10, 20 and 40 µM). Results are expressed as fold of change ($2^{-\Delta\Delta Ct}$ method) in relation to control group (E 0). Error bar represents the range of relative expression.

days after activation as above described. Moreover, expression of Nanog, Oct4 and Sox2 was evaluated in blastocysts derived from oocytes matured in the presence or absence of Embelin.

Statistical analysis

Data were analyzed using R version 3.0.3. (29) and significance was set at $P < 0.01$ unless otherwise specified. Data were assayed for normal distribution using Shapiro-Wilk test; subsequently an ANOVA test was used to assess differences between the percentage of blastocysts in control and treated groups. The difference in blastomeres number in blastocysts from control and treated groups was compared by a general mixed effects model with Poisson distribution. A repeated measures ANOVA was used to assess differences in progesterone and estrogen production after 24 and 48 hours of *in vitro* culture. mRNA data were analyzed using one-way ANOVA; the level of significance was set at $P < 0.05$.

RESULTS

Experiment 1: effect of Embelin on nuclear maturation

None of the concentrations of Embelin induced significant variations in either the proportion of oocytes completing

nuclear maturation or the percentage of degenerated oocytes (*Fig. 1*, *Table 2*).

Experiment 2: effect of Embelin on cumulus cell steroidogenesis

Embelin did not induce any significant effect on steroid production by cumulus cells after 24 and 48 hours of culture, as shown in *Table 3*.

Experiment 3: effect of Embelin on embryo development and gene expression

Embelin did not exert any effect on the proportion of embryos that developed to blastocyst stage or the number of blastomeres per blastocyst (*Table 4*).

Quantitative PCR data demonstrated that Nanog, Oct4 and Sox2 mRNA were detectable in all analysed samples. As shown in *Fig. 2*, no significant differences of Oct4, Nanog and Sox2 transcripts were detected in blastocyst stage embryos produced by parthenogenetic activation from oocytes matured in absence or presence of increasing amounts of Embelin.

DISCUSSION

In the last years the interest of the researches towards antioxidants used to improve oocyte IVM has been gradually increased together with their use in the prevention of certain diseases, such as cancer, cardiovascular disorders and other pathological conditions in which oxidative stress plays a major role (30).

The first aim of this study was to investigate the possible influences of Embelin on nuclear and cytoplasmic maturation of pig oocyte and on steroidogenesis of cumulus cells. In fact, during the *in vitro* culture both oocytes and embryos are inevitably more exposed than *in vivo* to light and high oxygen concentrations that may lead to an increase in the production of ROS that cross the cellular barriers and can damage lipids, proteins, nucleic acids thus blocking embryonic development (31). Therefore, as reported by Abeydeera *et al.* (4) and Wang *et al.* (10), antioxidant supplementation during oocyte IVM-IVF can improve the developmental competence of embryos that are deprived of maternal antioxidant protection and therefore are more exposed to ROS damage than *in vivo*.

In our study, Embelin neither influenced the percentage of oocytes in metaphase II at the end of IVM, nor modified the percentage of blastocysts and the number of blastomeres per blastocyst as compared to control.

Embelin seems therefore to be ineffective in favoring IVM of pig oocytes. However, the lack of a beneficial effect can be attributed, at least in part, to β-mercaptoethanol and cysteine present in the maturation medium; these substances act as antioxidants and have been demonstrated to enhance the developmental competence of embryos (4, 10). Moreover, in our study IVM and embryo culture were performed at a low oxygen tension (7%), that resembles physiological oxygen concentration *in vivo*, minimizing the oxidative stress that can be induced *in vitro* by the use of atmospheric oxygen concentrations (32).

In our model Embelin did not induce any effect on estradiol-17β and P4 secretion by cumulus cells after 24 and 48 hours of culture; irrespective of Embelin treatment, progesterone production dramatically increased during the second half of culture, likely due to cumulus cell differentiation/luteinization.

Our results indicating that Embelin is not effective in modulating steroidogenesis are not in agreement with other studies. In fact, in *in vivo* model, Embelin is a potent oral contraceptive which possesses anti-implantation activity in rats when administered at 50 mg/kg for 7 days and also inhibits pregnancy at single dose regimen (33). These Authors suggest that administration of Embelin can prevent implantation by either altering steroidogenesis and therefore estrogen/progesterone balance required for egg implantation or modifying the uterine environment thus inhibiting or interfering with the implantation process (33). Similarly, in the male Embelin inhibits testosterone production. Sonawane *et al.* (34) have shown that in rat treatment with Embelin increases mRNA expression for the aromatase, without affecting the enzymes involved in steroidogenesis and androgen receptors. These authors speculated that the decrease in testosterone is due to an increased clearance of the steroid-induced hydrolase produced by the liver.

Another set of experiments has been performed aiming at evaluating whether Embelin can influence the expression of some genes related to pluripotence (Nanog, Sox2 and Oct4) in parthenogenetically activated embryos produced from oocytes matured in the presence of different concentrations of Embelin. Transcript levels of these genes may be linked to embryo quality and embryonic development. In fact, Oct4 plays an important role on nucleus reprogramming during the first cell divisions and during embryonic genome activation and its normal expression is crucial for maintaining undifferentiated state (35, 36). Deletion of Oct4 induces failure of pluripotent ICM formation in the blastocyst (37).

Nanog behaves as a core factor of the pluripotent transcriptional network, and its expression is essential for somatic pluripotency (38). Hu *et al.* (6) found that the addition of vitamin C increases the expression of the Nanog gene in porcine embryos, with a fivefold increase in 20 μg/ml vitamin C treatment compared with the control.

In pig, appropriate Sox2 expression levels are needed to maintain pluripotency and prevent embryonic stem cells from differentiating toward the trophectoderm lineage (39).

In our experiments the relative abundance of Nanog, Oct4 and Sox2 in blastocysts of control group (E0) was in agreement with previous study (40); moreover none of the Embelin doses tested modified significantly the levels of gene expression.

In contrast to that observed for Embelin, other substances like plant-derived phytoestrogens including genistein and daidzein adversely affect folliculogenesis, follicle/oocyte health, steroidogenesis and glucocorticoid synthesis by disrupting adrenal steroidogenesis (41-43). All these studies confirm that reproductive function is dramatically influenced by nutrition. In addition to these natural substances, numerous hormones and metabolites are implicated as potential mediators affecting reproductive function and leptin has been considered as the appropriate signal to inform the reproductive system about the metabolic status (44, 45).

In conclusion, despite the positive antioxidant properties described (12, 46), Embelin does not influence the reproductive parameters assessed, confirming the difficulty to predict whether the beneficial effect exerted by an antioxidant in a particular tissue could be repeated also in another one. Therefore, to evidence possible positive actions on *in vitro* culture of oocytes/embryos, each promising molecule has to be tested.

Abbreviations: CCs, cumulus cells; E2, estradiol-17β; FCS, fetal calf serum; ICM, inner cell mass; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; MII, metaphase; P4, progesterone; ROS, reactive oxygen species

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