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Nobiletin enhances the development and quality of bovine embryos in vitro during two key periods of embryonic genome activation

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In vitro culture can alter the development and quality of bovine embryos. Therefore, we aimed to evaluate whether nobiletin supplementation during EGA improves embryonic development and blastocyst quality and if it affects PI3K/AKT signaling pathway. *In vitro* zygotes were cultured in SOF + 5% FCS (Control) or supplemented with 5, 10 or 25 μ M nobiletin (Nob5, Nob10, Nob25) or with 0.03% dimethyl-sulfoxide (C_{DMSO}) during minor (2 to 8-cell stage; MN_{EGA}) or major (8 to 16-cell stage; MJ_{EGA}) EGA phase. Blastocyst yield on Day 8 was higher in Nob5 ($42.7 \pm 1.0\%$) and Nob10 ($44.4 \pm 1.3\%$) for MN_{EGA} phase and in Nob10 ($61.0 \pm 0.8\%$) for MJ_{EGA} phase compared to other groups. Mitochondrial activity was higher and lipid content was reduced in blastocysts produced with nobiletin, irrespective of EGA phase. The mRNA abundance of *CDK2*, *H3-3B*, *H3-3A*, *GPX1*, *NFE2L2* and *PPAR α* transcripts was increased in 8-cells, 16-cells and blastocysts from nobiletin groups. Immunofluorescence analysis revealed immunoreactive proteins for p-AKT forms (Thr308 and Ser473) in bovine blastocysts produced with nobiletin. In conclusion, nobiletin supplementation during EGA has a positive effect on preimplantation bovine embryonic development *in vitro* and corroborates on the quality improvement of the produced blastocysts which could be modulated by the activation of AKT signaling pathway.

In vitro culture (IVC) of bovine embryos is one of the most important processes in the development of assisted reproductive techniques due to the fact that postfertilization culture conditions can dramatically alter the quality of the resulting blastocysts^{1,2}. *In vitro*, gametes and embryos are exposed to spatial and temporal unnatural conditions, whose scope is not completely known³. Although many improvements have been made, *in vitro* culture systems are still not as efficient as *in vivo* embryo production⁴. In cattle, the proportion of embryos reaching the blastocyst stage is around 30–40%⁵ and are often compromised in quality and competence manifested by a darker morphology¹ or altered gene expression patterns⁶ when compared to their *in vivo* counterparts. The factors that most influence the quality of the embryos are the conditions after fertilization; which include physicochemical (temperature, osmolality, and pH), oxidative (antioxidant balance), and energetic (production, utilization, and storage) stresses⁶.

Under *in vitro* conditions, the dynamics of embryo development and the kinetics of cleavage are related to the subsequent developmental stages: the faster-cleaved embryos have a higher chance to develop to the blastocysts stage⁷. Therefore, the morphological and metabolic changes that occur during the first 4 days of preimplantation development of the bovine embryo are the most important; besides, during this same period the embryonic genome activation (EGA) occurs^{2,8}. At the start of early embryogenesis, all mRNAs and proteins controlling development are of maternal origin, and as development progresses, these reserves gradually degrade while

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embryonic transcripts are synthesized; this process is called maternal-to-embryonic transition and involves EGA⁹. The EGA occurs in distinct waves, which are species-specific. Bovine preimplantation embryo development is characterized by two distinct phases: (i) minor EGA (MN_{EGA}) (2-cell to 8-cell stages) where zygotes and early embryos are transcriptionally and translationally active; (ii) major EGA (MJ_{EGA}) (8-cell to 16-cells stages) which includes a gradual degradation of mRNA molecules of maternal origin, together with a change in the protein synthesis, and these events are key factors for successful embryonic development and differentiation^{2,8}. EGA is a prerequisite for correct compaction, that leads to an increase in intercellular adhesion mediated by adherent junctions and embryonic polarization¹⁰, as well as the formation of the blastocyst, with its trophoblast (TE), and the inner cell mass (ICM)¹¹.

In recent years, the role of different signaling pathways in preimplantation development has been analyzed, suggesting the existence of a complex network of signals that control and are responsible for cell division, differentiation, cytoskeleton rearrangements, cell proliferation and apoptosis^{11,12}. One of the most important signal transduction pathways that regulate cell survival is PI3K/AKT. PI3K/AKT pathway consists of several molecules, including kinases, phosphatases, and transcription factors that are fundamental in processes such as migration, metabolism, and cell cycle progression^{13,14}. During embryonic development, PI3K/AKT regulates cell survival and its inhibition can cause a significant delay in blastocyst hatching¹². In this context, the quality of the embryos produced *in vitro* depends not only on the proper functioning of the signaling pathways but also on the post-fertilization culture environment.

To improve the blastocysts rates and quality, several studies have probed the addition of different types of natural antioxidants to the IVC medium, such as vitamin C¹⁵ or crocetin¹⁶. These compounds improved embryonic quality in terms of increase in blastocysts rates and embryo cell number, as well as reduction in reactive oxygen species (ROS) levels and apoptotic cells in embryos. In recent years, nobiletin a class of polymethoxylated flavone identified from the citrus peel (chemically known as 5,6,7,8,3',4' hexamethoxyflavone), has drawn increasing attention since it is easily absorbed across the cytoplasmic membranes due to its structure and lipophilic nature¹⁷. In addition, it has been reported that nobiletin has a broad spectrum of biological activities, that include antioxidative functions and cell cycle regulation¹⁷. We observed that supplementation of *in vitro* maturation (IVM) medium with nobiletin counteracts the effects of the increase in ROS production during IVM, improves oocyte nuclear and cytoplasmic maturation, and subsequent embryo development and quality in bovine¹⁸. Other studies using cultured cell lines have demonstrated that nobiletin can modulate signaling cascades, including PI3K/AKT signaling pathway^{17,19}. Nevertheless, the mechanism of specific action by which nobiletin modulates this signaling pathway is not fully understood, and, to our knowledge, there is no evidence of any developmental effect of nobiletin supplementation during post-fertilization embryo culture *in vitro*. Thus, in this study, we aimed to evaluate whether supplementation of nobiletin to the *in vitro* culture medium during the two EGA phases improves embryonic development and blastocyst quality and if its action is related to the PI3K/AKT signaling pathway. The parameters evaluated in blastocysts were, (i) lipid accumulation, (ii) mitochondrial activity, (iii) quantitative changes of key genes related to quality and development, (iv) immunolocalization of phosphorylated-AKT (p-AKT) and (v) level by western blot analysis for AKT and p-AKT (Thr308/Ser473).

Results

Nobiletin during MN_{EGA} or MJ_{EGA} enhances early embryo development *in vitro*. For all experimental groups, only embryos that reached the 8-cell stage at 54 h post-insemination (hpi) were selected for the study. As shown in Fig. 1a for MN_{EGA} phase, no differences were observed in cleavage rate at 54 hpi, which ranged from 82.3 ± 1.0 to 85.5 ± 0.5%. At 54 hpi, no differences were observed either in the proportion of embryos that reached the 8-cell stage, which ranged from 57.1 ± 1.4 to 60.4 ± 0.7%. Consequently, a similar proportion of embryos with a delayed development (< 8 cells), which ranged from 22.6 ± 0.9 to 26.6 ± 1.2%, was observed (Fig. 1b). Blastocyst yield at Day 7 and 8 (Fig. 1c) was significantly higher ($P < 0.001$) for Nob5 (39.7 ± 0.8 and 42.7 ± 1.0%, respectively) and Nob10 (41.0 ± 1.0 and 44.4 ± 1.3%), compared to Control (32.7 ± 0.7 and 34.6 ± 0.7%); C_{DMSO} (32.8 ± 0.5 and 34.9 ± 0.4%) and Nob25 (31.8 ± 1.7 and 34.6 ± 1.2%) (Supplementary Table S1).

During MJ_{EGA}, cleavage rate at 54 hpi was 86.6 ± 0.2% and the proportion of embryos that reached the 8-cell stage was 71.1 ± 0.4% while the proportion of embryos with a delayed development (< 8 cells) was 15.5 ± 0.3% (Fig. 1d). At 96 hpi a significantly ($P < 0.001$) higher proportion of embryos reached the 16-cell stage in Nob5 and Nob10 groups (70.1 ± 0.5% and 69.9 ± 0.4%, respectively) compared to Control (60.0 ± 0.4%), C_{DMSO} (60.7 ± 0.4%) and Nob25 (60.8 ± 0.8%) groups (Fig. 1e). As a consequence, a significantly lower proportion of embryos with a delayed development (<16 cells) was observed in Nob5 and Nob10 compared to the other groups (Nob5: 29.9 ± 0.5% and Nob10: 30.1 ± 0.4% vs Control: 40.0 ± 0.4%, C_{DMSO}: 39.3 ± 0.4% and Nob25: 39.2 ± 0.8%, $P < 0.001$). On Day 7 and 8, blastocyst yield was significantly higher ($P < 0.001$) for Nob10 (54.5 ± 1.1% and 61.0 ± 0.8%, respectively) compared to Control (38.4 ± 1.1% and 47.3 ± 1.4%), C_{DMSO} (35.8 ± 1.0% and 44.0 ± 1.1%), Nob5 (46.6 ± 0.8% and 52.5 ± 1.5%) and Nob25 (35.9 ± 1.5%–42.5 ± 1.3%) groups, while Nob5 was higher ($P < 0.001$) compared to Nob25 and both control groups (Fig. 1f) (Supplementary Table S2).

Nobiletin during MN_{EGA} or MJ_{EGA} increases the quality of *in vitro* produced blastocysts. Only the experimental groups that showed better blastocyst yield in the previous experiment (Nob5 and Nob10 during MN_{EGA} or MJ_{EGA}) were used for embryo quality evaluation in comparison with both control groups (Control and C_{DMSO}).

The mitochondrial activity was higher ($P < 0.001$) in blastocysts from Nob5 and Nob10 groups, from either MN_{EGA} or MJ_{EGA} phase, compared with both control groups (Fig. 2).

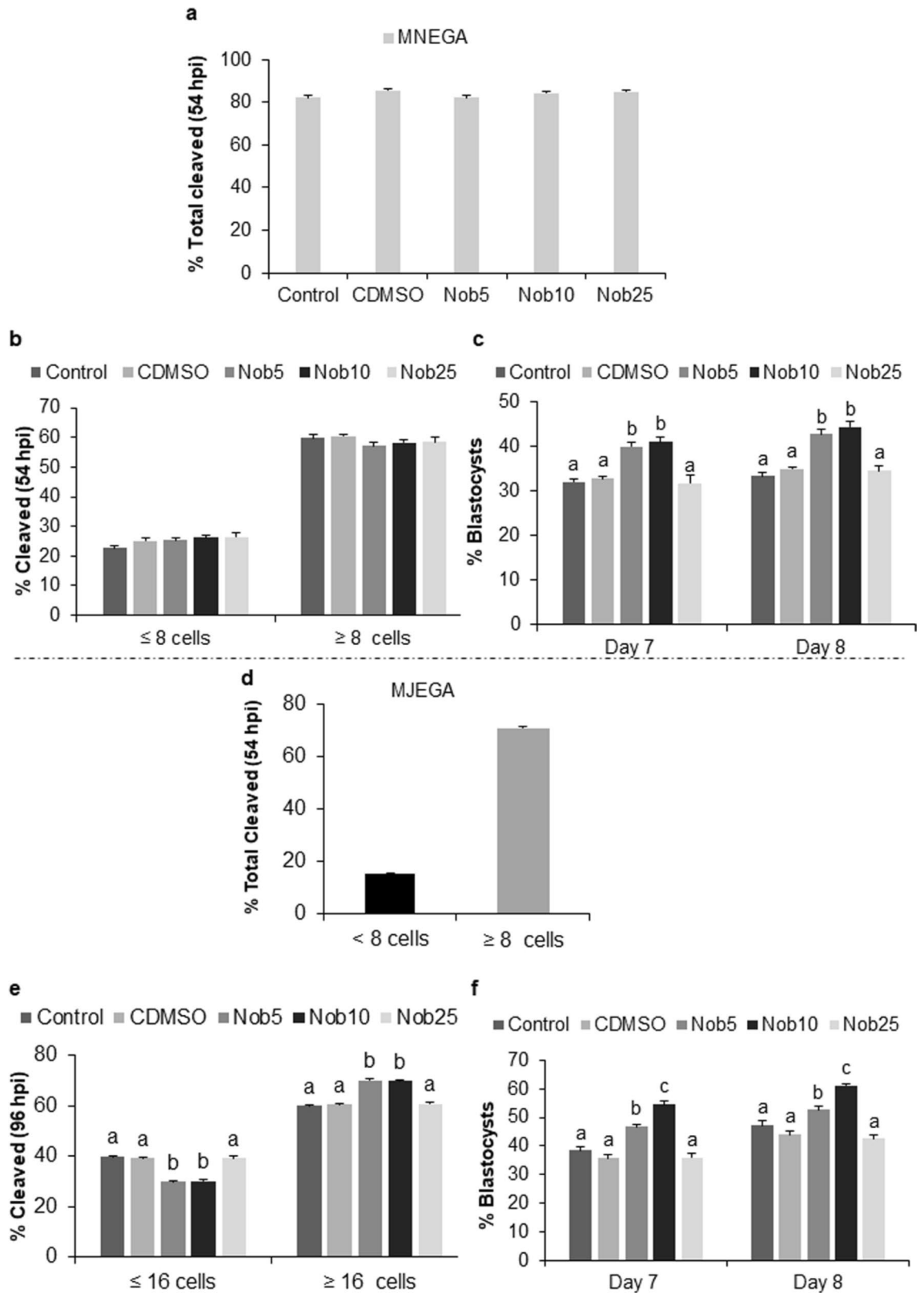


Figure 1. Nobiletin effect in embryonic development. Developmental rates of in vitro produced bovine embryos cultured during 21–54 h post-insemination (hpi) (MN_{EGA}: **a–c**) or during 54–96 hpi (MJ_{EGA}: **d–f**) with or without nobiletin. (**a,d**) Total cleavage rate at 54 hpi; (**b,e**) embryos ≥ or ≤ 8-cell stage at 54 hpi and ≥ or ≤ 16-cell stage at 96 hpi; (**c,f**) blastocyst rate on Days 7–8 pi (in vitro fertilization = Day 0), from embryos cultured in SOF + 5% FCS (Control), supplemented or not with 5 (Nob5), 10 (Nob10) or 25 μM (Nob25) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}) during MN_{EGA} or MJ_{EGA} respectively. Results are expressed as mean ± s.e.m. Significant differences (*P* < 0.001) are indicated with different letters.

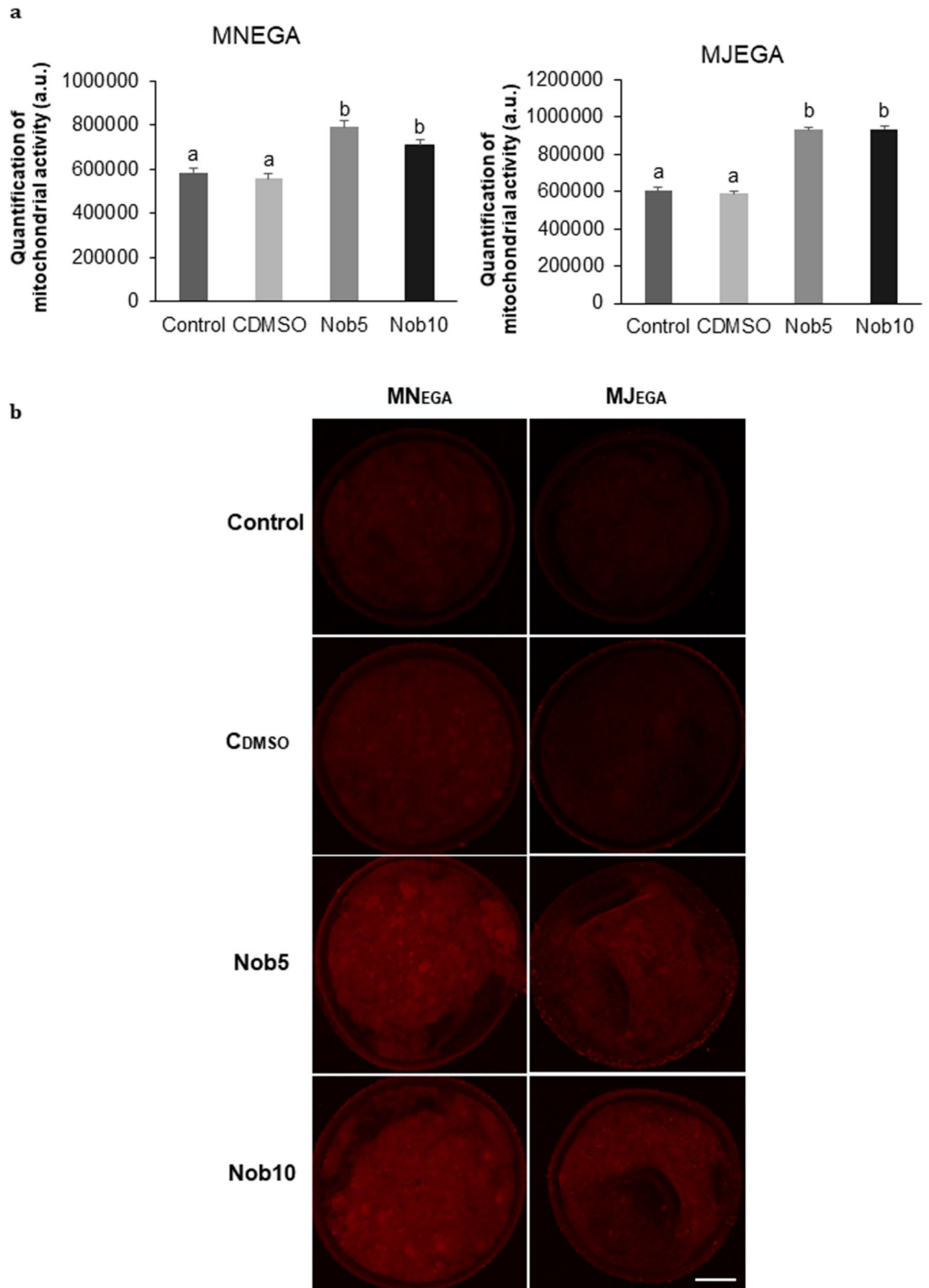


Figure 2. Nobiletin effect in blastocysts mitochondrial activity. **(a)** Quantification of mitochondrial fluorescence intensity in arbitrary units (a.u.) in Day 7 blastocysts cultured in SOF + 5% FCS (Control), supplemented or not with 5 (Nob5) or 10 μ M (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}) during 21–54 hpi (MN_{EGA} : presumptive zygote to 8-cell stage) or during 54–96 hpi (MJ_{EGA} : 8- to 16-cell stage). Data are the mean \pm s.e.m. Significant differences ($P < 0.001$) are indicated with different letters. **(b)** Representative fluorescence images of mitochondrial activity in Day 7 blastocysts from all experimental groups (Control, Nob5, Nob10, C_{DMSO}) in both phases (MN_{EGA} or MJ_{EGA}). Images were captured on 63 \times objective. Scale bar 50 μ m.

When analyzing the lipid content, we observed that the total area of lipid droplets in blastocysts resulting from treatments during MN_{EGA} or MJ_{EGA} was significantly reduced ($P < 0.001$) in Nob5 and Nob10 groups compared with the control groups (Fig. 3).

The total number of cells was greater ($P < 0.001$) in blastocysts from MN_{EGA} phase produces with 5 μM of nobiletin (137.3 ± 0.6) compared to all other groups (Control: 105.7 ± 0.7 ; C_{DMSO}: 106.4 ± 0.8 ; Nob10: 126.7 ± 0.8), while blastocysts from Nob10 group had more cells ($P < 0.001$) compared to control groups, but less ($P < 0.001$) when compared to Nob5. However, during MJ_{EGA} phase the total number of cells was higher in blastocysts from Nob5 and Nob10 groups (133.2 ± 0.9 and 134.2 ± 0.7 , respectively) compared to control groups (Control: 104.9 ± 0.7 and C_{DMSO}: 104.6 ± 0.6) ($P < 0.001$) (Supplementary Table S3).

Gene expression in ≥ 8 -cell embryos and blastocysts produced with nobiletin during MN_{EGA}. The mRNA abundance of *CDK2*, *H3-3B*, *H3-3A*, and *GPX1* was significantly increased in 8-cell stage embryos from Nob5 and Nob10 groups compared to both controls ($P < 0.05$) (Fig. 4a). The expression of *PPAR α* and *GPX1* was significantly higher in blastocysts from Nob5 and Nob10 groups when compared with both controls ($P < 0.05$) (Fig. 4b). No differences were observed for the *PPARGC1A*, *PPAR α* , *RPS6KB1*, and *NFE2L2* transcripts in 8-cell stage embryos and *PPARGC1A*, *RPS6KB1*, *CDK2*, *H3-3B*, *H3F3A*, and *NFE2L2* in blastocysts.

Gene expression in ≥ 16 -cell embryos and blastocysts produced with nobiletin during MJ_{EGA}. The expression level of *CDK2*, *H3-3B* and *NFE2L2* transcripts was significantly increased in 16-cell stage embryos from Nob10 group compared to Nob5 and both control groups. While the expression of *GPX1* gene was higher in Nob5 and Nob10 compared to control groups ($P < 0.05$) (Fig. 5a). In blastocysts the expression of *PPAR α* was significantly higher in Nob10 group compared to all other groups ($P < 0.05$), while *CDK2* and *GPX1* were upregulated in both nobiletin groups compared with controls ($P < 0.05$) (Fig. 5b). No significant differences were observed for *PPARGC1A*, *PPAR α* , *RPS6KB1* and *H3-3A* in 16-cell stage embryos, and for *PPARGC1A*, *RPS6KB1*, *H3-3B*, *H3-3A* and *NFE2L2* in blastocysts.

Nobiletin during MN_{EGA} or MJ_{EGA} increases AKT phosphorylation in blastocysts produced in vitro. Immunofluorescence analysis revealed immunoreactive proteins for p-AKT in bovine blastocysts. In Day 7 blastocysts, AKT increased its phosphorylation levels when nobiletin was present in the culture medium (Nob5 and Nob10 groups) during MN_{EGA} or MJ_{EGA}. While p-AKT levels were weaker in blastocysts produced from control groups during MJ_{EGA} phase (Fig. 6).

Similarly, the western blot analysis showed that both p-AKT-Thr308 and p-AKT-Ser473 phosphorylation levels were significantly higher in blastocysts produced with nobiletin supplementation (Nob5 and Nob10) during MN_{EGA} phase when compared with control groups ($P < 0.05$) (Fig. 7a–c). A similar pattern was observed in response to nobiletin treatment during MJ_{EGA}, as p-AKT-Thr308 and p-AKT-Ser473 phosphorylation levels were significantly higher in blastocysts produced with Nob5 and Nob10 compared with control groups ($P < 0.05$) (Fig. 7d–f).

Discussion

Under in vivo conditions, cells have antioxidants levels in equilibrium and possess physiological mechanisms to hinder excessive free radical formation²⁰. During in vitro culture this mechanism suffers disturbances, in which the redox balance is altered with an increase in the production of free radicals and, as a consequence, a decrease in embryo development⁶. Several studies, aiming to identify the most effective antioxidants to reduce the alteration of the redox balance and ROS levels during the in vitro production of embryos, have shown that the addition of quercetin, resveratrol, vitamin C or carnitine to the culture media have beneficial effects on early embryonic development^{15,21}. To our knowledge, the present study is the first that investigates the antioxidant effects of nobiletin supplementation in the culture medium during the two main phases of EGA (MN_{EGA}: minor activation from 2- to 8-cell stage and MJ_{EGA}: major activation from 8- to 16-cell stage)^{2,8} in bovine embryo developmental competence in vitro and quality of the produced blastocysts, as well as its possible interaction with the AKT signaling pathway.

Irrespective of concentration, addition of nobiletin to culture media during MN_{EGA} phase (21–54 hpi) did not affect cleavage rates at 54 hpi as well as the percentage of embryos reach the 8-cell stage but increased blastocyst production, whereas nobiletin supplementation in culture media during MJ_{EGA} phase (54–96 hpi) significantly increased the percentage of embryos that reach the 16-cell stage and blastocyst production. Several studies have shown that during EGA the bovine embryo actively synthesizes transcription factors and this process directly links to chromatin changes, protein allocation, nuclear reorganization and cell proliferation^{8,22}. Since in our results developmental kinetics were stimulated with more 16-cell embryos by nobiletin during MJ_{EGA}, we could hypothesize that nobiletin activates early embryonic genes important for the proper genomic function of the embryo during major EGA. Although with our experimental design we cannot link this effect specifically with either of the two activation phases of the embryonic genome.

The evidence that nobiletin supplementation improves blastocyst production is in line with other studies showing increased embryo development in vitro when culture medium was supplemented with biological antioxidants similar to nobiletin^{16,23,24}. Another effect of nobiletin was to induce a significant increase in mitochondrial activity and a lower content of lipid droplets in blastocysts from both EGA phases analyzed. Mitochondria play a central role in the generation of adenosine triphosphate (ATP), so, they are considered as energy control units necessary for cell division, pluripotency and differentiation²⁵. Cagnone and Sirard⁶, reported that in vivo, during the early cleavages, mitochondria and intracellular metabolism are quiescent. Nevertheless, in vitro, this metabolic quiescence is altered due to the presence of nutrients in excessive amounts that overstimulate mitochondria

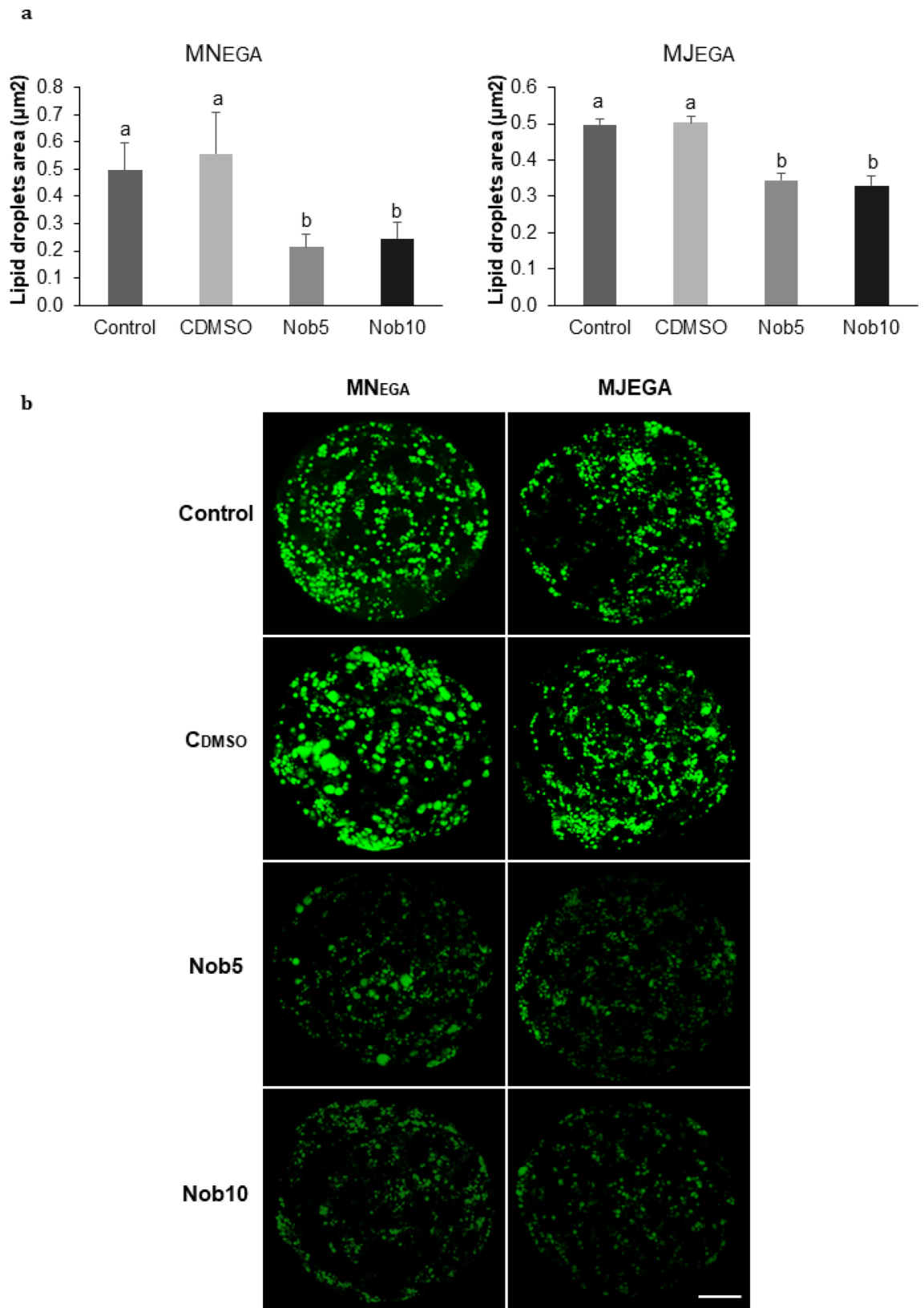


Figure 3. Nobiletin effect in blastocysts lipid content. **(a)** Quantification of the total area of lipid droplets (μm^2) in Day 7 blastocysts cultured in SOF + 5% FCS (Control), supplemented or not with 5 (Nob5) or 10 μM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}) during 21–54 hpi (MN_{EGA} : presumptive zygote to 8-cell stage) or during 54–96 hpi (MJ_{EGA} : 8- to 16-cell stage). Data are the mean \pm s.e.m. Significant differences ($P < 0.001$) are indicated with different letters. **(b)** Representative fluorescence images of lipid droplets in Day 7 blastocysts from all experimental groups (Control, Nob5, Nob10, C_{DMSO}) in both phases (MN_{EGA} or MJ_{EGA}). Images were captured on 63 \times objective. Scale bar 50 μm .

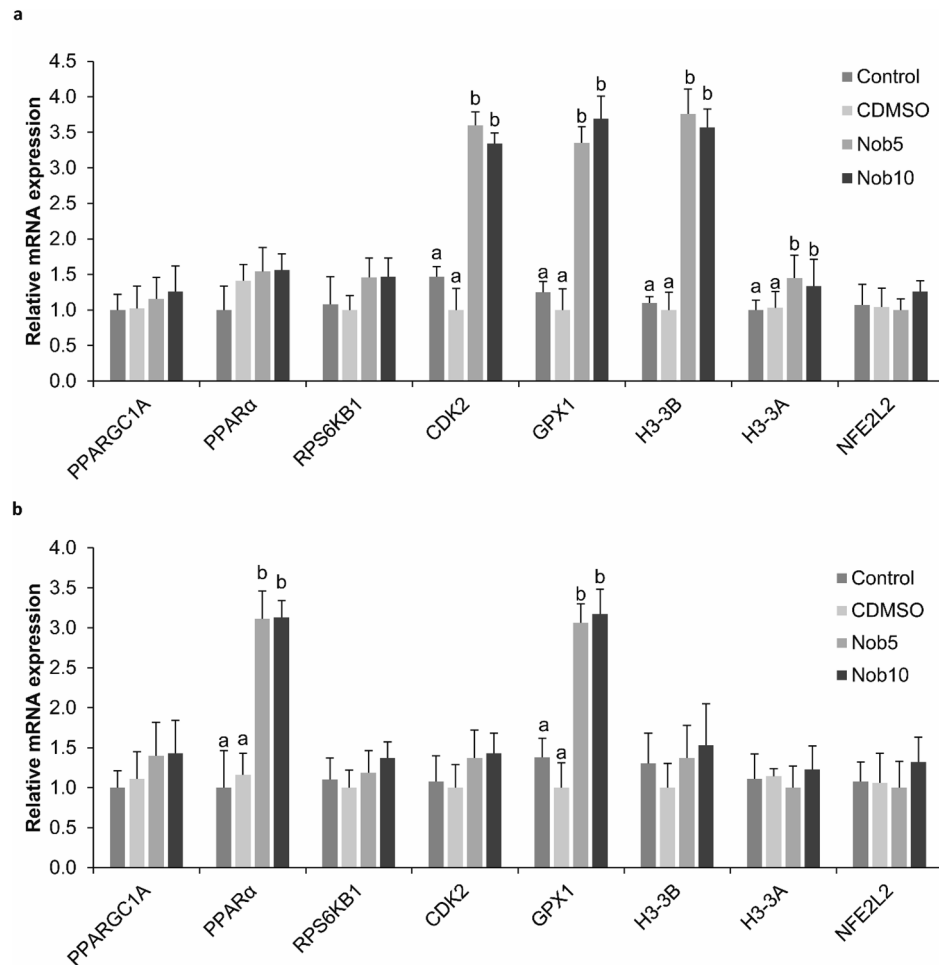


Figure 4. Relative mRNA transcript abundance of embryo development-related genes in in vitro produced embryos cultured during 21–54 h post insemination (MN_{EGA} : presumptive zygote to 8-cell stage) with or without nobiletin. **(a)** Relative abundance in 8-cell stage embryos cultured in SOF + 5% FCS (Control), supplemented or not with 5 (Nob5) or 10 μ M (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}) during MN_{EGA} phase. **(b)** Relative abundance in blastocysts from Control, Nob5, Nob10, and C_{DMSO} experimental groups from MN_{EGA} phase. The relative abundance of the transcripts was normalized to *H2AFZ* and *ACTB* as housekeeping genes. Data are the mean \pm s.e.m. Different letters indicate significant difference ($P < 0.05$) between treatments.

and alter their efficiency to respond to oxidative phosphorylation⁶. Mitochondria also sense changes in redox potential and force embryos to adapt versus the decreased production of ATP by oxidative phosphorylation during the transition from morula to blastocyst^{6,25}. Besides, some studies reported that changes in mitochondrial activity may affect the development of energetic metabolism in the embryo, in terms of availability of glucose, lipids, amino acids and DNA methylation^{6,26}. Although the nobiletin action mechanism in mitochondria has not been fully elucidated, in a previous study we observed that increased oocyte mitochondrial activity was related to the cytoprotective effects of nobiletin and its intrinsic ROS-scavenging property¹⁸. Nevertheless, the effect in the blastocyst could be explained based on the fact that nobiletin is a hydrophobic compound, which easily penetrates through cell membranes directly affecting mitochondrial bioenergetics. Nobiletin can modify intramitochondrial proteins (e.g. acetylated proteins localized within the mitochondria in the brain of rats)²⁷ or alter the mitochondrial membrane potential by changing the activities of mitochondrial enzymes, like succinate dehydrogenase and cytochrome c oxidase as it has been demonstrated in human blood lymphocytes²⁸. However, to verify if this mechanism occurs in bovine blastocysts, further investigation is necessary.

Lipid content is a crucial factor for early embryo development in vitro in bovine since energy metabolism is abnormal under such conditions, resulting in an excessive accumulation of lipids associated with reduced embryonic quality²⁹. Lipids are stored in intracellular droplets and are metabolized via β -oxidation in the mitochondrial matrix. A large amount of lipid droplets increases the production of ATP necessary for the formation of blastocysts but this can affect its quality; thus, a lower number of lipid droplets in blastocysts is considered as a criterion of good quality embryos¹⁶. Our results showed for both EGA phases, nobiletin supplementation in culture medium reduced the amount of lipids in blastocysts. Furthermore, we analyze the expression of peroxisome proliferator-activated receptor alpha transcript (*PPAR α*), belonging to one of the 3 key nuclear receptors

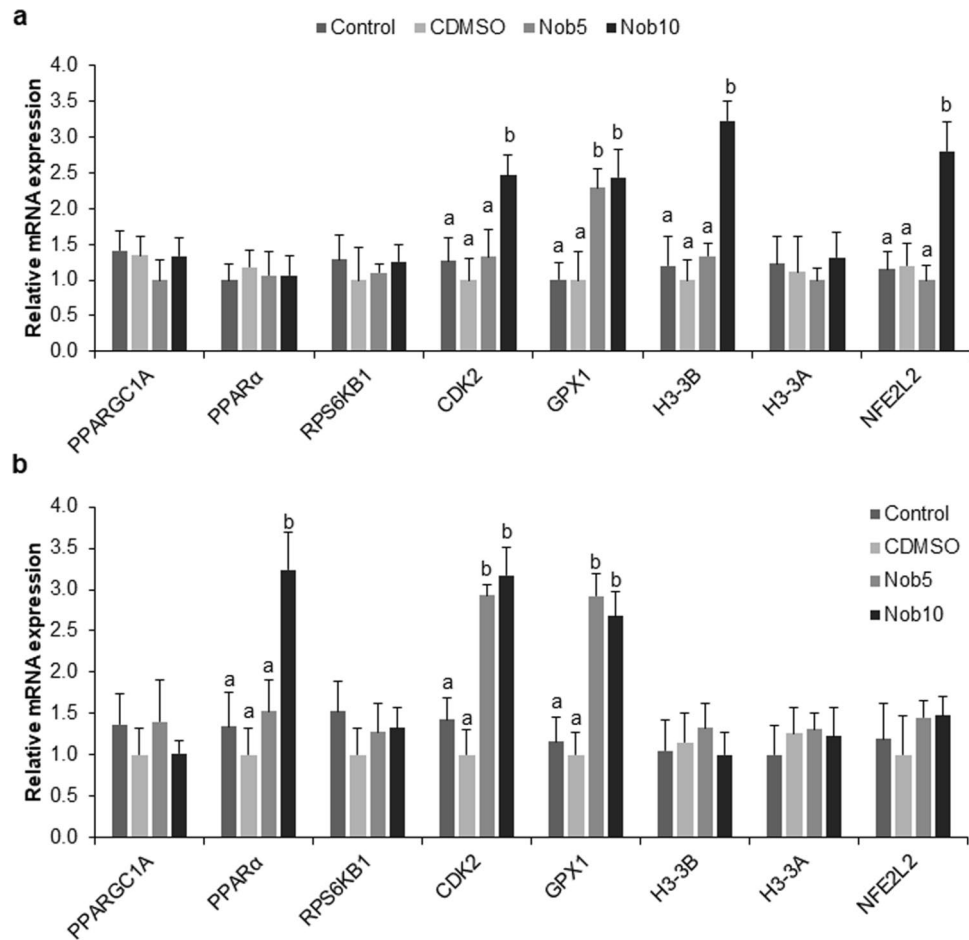


Figure 5. Relative mRNA transcript abundance of embryo development-related genes in in vitro produced embryos cultured during 54–96 hpi (MJ_{EGA}: 8-to 16-cell stage) with or without nobiletin. **(a)** Relative abundance in 16-cell stage embryos cultured in SOF + 5% FCS (Control), supplemented or not with 5 (Nob5) or 10 μ M (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}) during MN_{EGA} phase. **(b)** Relative abundance in blastocysts from Control, Nob5, Nob10, and C_{DMSO} experimental groups from MJ_{EGA} phase. The relative abundance of the transcripts was normalized to *H2AFZ* and *ACTB* as housekeeping genes. Data are the mean \pm s.e.m. Different letters indicate significant difference ($P < 0.05$) between treatments.

in the modulation of transcription for lipid metabolism-related genes³⁰. *PPARα* was previously detected in cattle embryos and has been associated with embryo quality³¹. In our study, *PPARα* was significantly upregulated in blastocysts produced with both concentrations of nobiletin supplementation during MN_{EGA} phase or 10 μ M of nobiletin supplementation during MJ_{EGA} compared to controls. These results together are in line with other studies which demonstrated that antioxidant supplementation in IVC medium, like crocetin²³ and L-carnitine²¹, improved embryo quality by decreasing their lipid content. Regarding nobiletin, studies in mice showed its ability to reduce hepatic lipid accumulation, prevent lipoprotein overproduction and normalize insulin sensitivity when supplied in the diet³². Moreover, it has been demonstrated that nobiletin reduces lipid accumulation and regulates lipidic metabolism in hepatic cell lines^{17,33}. There is evidence that nobiletin upregulates the expression of *PPARα* in white adipose tissue of mice¹⁷. An explanation for the reduction of lipids by nobiletin has been proposed indicating that full methoxylation of the A-ring of nobiletin seems to be the most optimal structure to express potent effects on modulating hepatic lipid metabolism via primarily suppressing lipoprotein secretion in HepG2 cells³³. Therefore, it appears that the ability of nobiletin to reduce lipid content and improve mitochondrial activity in blastocysts may be related to the properties of its chemical structure that allows modulation of lipid metabolism and mitochondrial activity. Moreover, activation of *PPARα* by nobiletin could result in increased embryo lipid turnover through the β -oxidative pathway, preventing accumulation of lipoperoxides despite peroxisomal induction. Recent studies have shown that response of embryos to IVC involves a variety of metabolic factors that act as signals of extracellular and intracellular conditions to which the early embryos can adapt cell programming, signaling pathways, mitochondrial metabolism (mitochondrial production of Acetyl-Coenzyme A (Acetyl-Co A) and methyl groups, which are dependent on the availability of glucose, lipids and amino acids) or peroxisome proliferator-activated receptors (PPARs) in response to lipid content. These factors in the embryo are translated into effects on developmental speed or epigenetic modifications^{6,34,35}. Consequently, these results

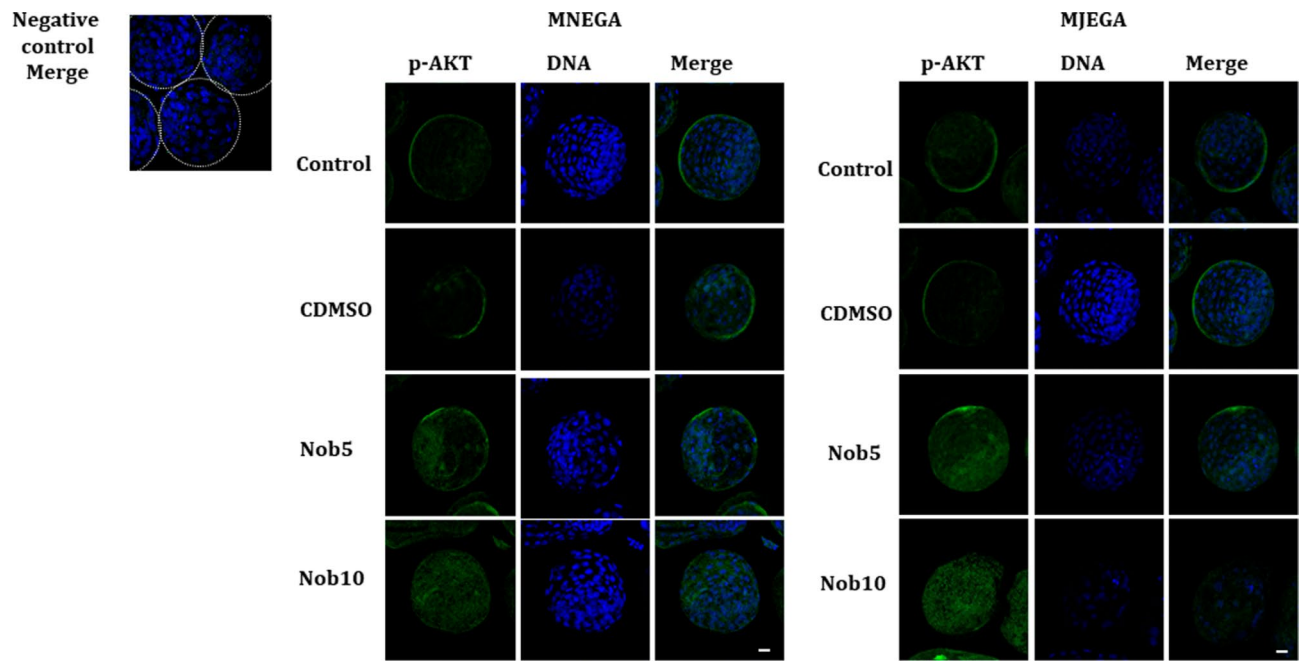


Figure 6. Nobiletin effect in the phosphorylated-AKT (p-AKT) in blastocysts. Representative images of immunofluorescence detection of p-AKT in in vitro produced bovine blastocysts cultured during 21–54 hpi (MN_{EGA} : presumptive zygote to 8-cell stage) or during 54–96 hpi (MJ_{EGA} : 8- to 16-cell stage) in SOF + 5% FCS (Control), supplemented or not with 5 (Nob5) or 10 μ M (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}). Positive staining for p-AKT proteins shown in green. Cell nuclei were counterstained with Hoechst stain (blue). Images were captured on 63 \times objective. Scale bar 20 μ m.

can reinforce the antioxidant-defense role of nobiletin during early embryo development in vitro in bovine and could indicate an improvement of the quality of the produced blastocysts.

Embryo cell number is a parameter correlated with embryonic development and quality. Also, it has been reported for different cellular lines (MOLT-4, HUVEC, PC12D, K-N-SH cells) that nobiletin exert its activity by modulation of cell cycle progression¹⁷. We observed that regardless of EGA phase (MN_{EGA} and MJ_{EGA}), nobiletin supplementation in culture media increased the total cell number of produced blastocysts. This increase rate was similar to that observed with other antioxidants such as vitamin C¹⁵ or crocetin^{16,23}, suggesting that nobiletin could directly stimulate the cell cycle during EGA and improve embryo quality.

To verify if the effects of nobiletin during MN_{EGA} or MJ_{EGA} were related to gene expression changes, we analyzed the expression of candidate genes for oxidative stress, embryo development and quality. Glutathione peroxidase (*GPX1*) and Nuclear Factor Erythroid 2-Like 2 (*NFE2L2*), are oxidative-stress-response-related genes. *GPX1*, considered the major antioxidant enzyme within the Glutathione peroxidase family, is ubiquitously expressed in the cytosol and also has been found in mitochondria²⁰. Furthermore, *GPX1* acts as a scavenger of hydrophilic peroxide species, can be transformed into an enzymatically inactive cellular structural component, and protects cells against oxidative damage²⁰. During in vitro production ROS generation increases and one of the defenses to counter excess ROS in the embryo is *GPX1*; therefore, *GPX1* overexpression has been positively linked with embryo quality^{36,37}. In our study, gene expression analysis revealed the upregulation of *GPX1* in 8- and 16-cell embryos as well as in blastocysts produced with nobiletin supplementation during MN_{EGA} or MJ_{EGA} phases. A similar response has been reported in sheep and bovine embryos treated with other types of antioxidants like L-carnitine²¹ or crocetin¹⁶. *NFE2L2* transcript (also known as *Nrf2*) is important for embryo tolerance to oxidative stress during EGA as well as for its competence for development². PI3K/AKT pathway plays a role in regulating *NFE2L2* activation and is involved in the regulation of protein kinases, which may induce nuclear translocation³⁸. Harris and Hansen³⁹, and Ghanem et al.²⁹ reported in mice that up-regulation of *NFE2L2* transcript may protect embryos from oxidative stress through preservation of intracellular redox states to ensure normal embryonic development. In the same line, our results showed the relative abundance of *NFE2L2* transcript increased in 16-cell stage embryos cultured with 10 μ M nobiletin during MJ_{EGA} , while remained unaltered in 8-cell stage embryos as well as in blastocysts from both treatments. Moreover, data obtained in cancer cells of mice showed that *NFE2L2* mRNA levels were upregulated when nobiletin was supplemented in culture medium⁴⁰. Taken together, these data suggest that nobiletin plays an antioxidant-defense role via distinct pathways during the different phases of early embryo development in vitro. However, it is necessary to confirm this antioxidant action by measuring ROS levels in the embryos.

Cyclin Dependent Kinase 2 (*CDK2*) is necessary for cell cycle progression, and is a major kinase that governs AKT phosphorylation, while it also participates on EGA⁴¹. In our study, *CDK2* mRNA expression was upregulated in 8-cell (MN_{EGA}), and blastocysts (MJ_{EGA}) cultured with 5 μ M or 10 μ M of nobiletin as well as in 16-cell (MJ_{EGA}) cultured with 10 μ M of nobiletin. This is in line with previous data obtained in bovine embryos

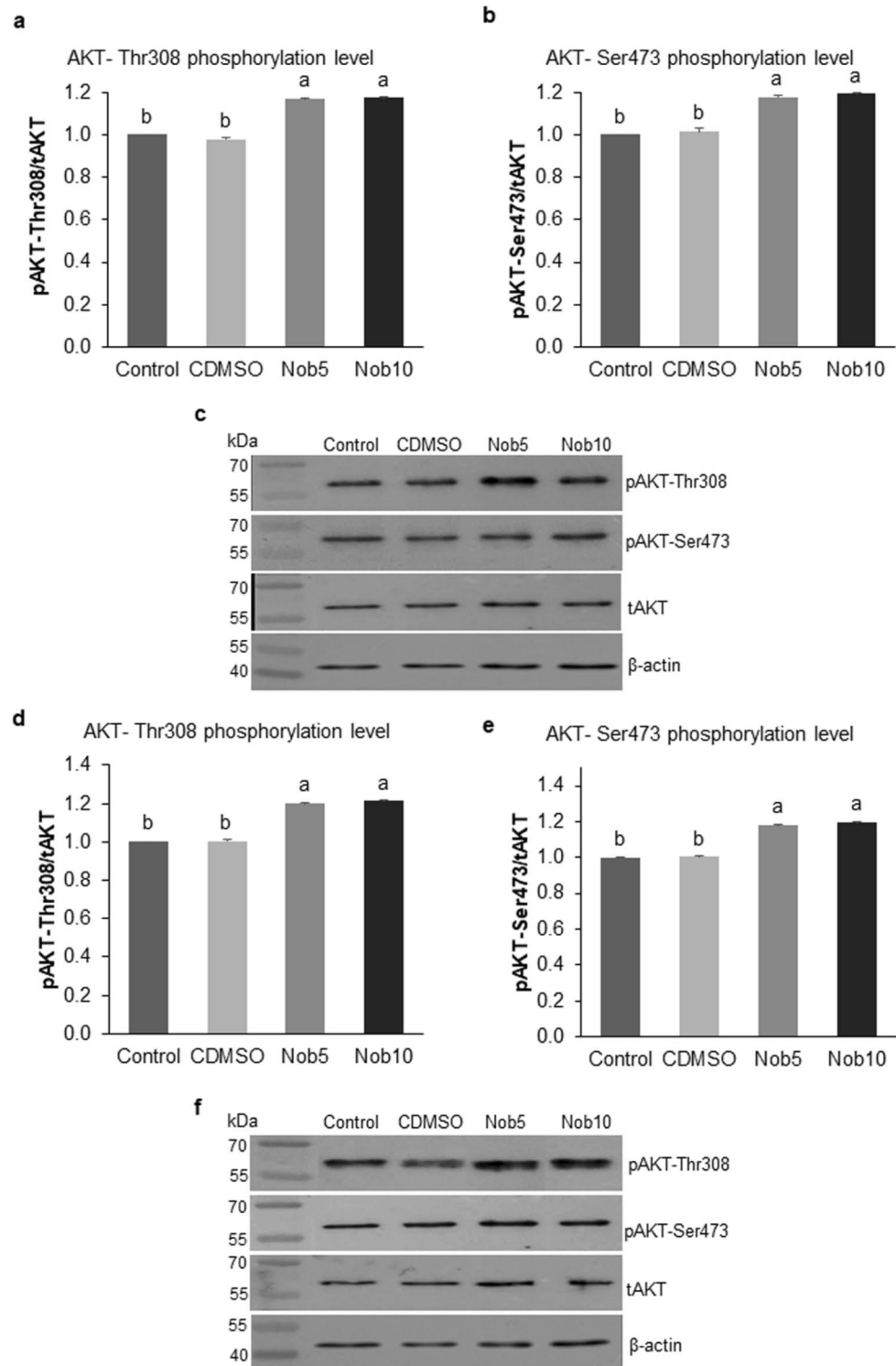


Figure 7. Nobiletin during MN_{EGA} or MJ_{EGA} increases AKT phosphorylation in blastocysts. **(a,b)** Quantification of phosphorylation levels of pAKT-Thr308/tAKT and pAKT-Ser473/tAKT in in vitro produced bovine Day 7 blastocysts cultured during 21–54 hpi (MN_{EGA}: presumptive zygote to 8-cell stage) in SOF + 5% FCS (Control), supplemented or not with 5 μ M (Nob5) or 10 μ M (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}). **(c)** Western blot images showing the expression of pAKT-Thr308, pAKT-Ser473, (t)AKT and β -actin in Day 7 blastocysts from MN_{EGA} phase. **(d,e)** Quantification of phosphorylation levels of pAKT-Thr308/tAKT and pAKT-Ser473/tAKT in in vitro produced bovine Day 7 blastocysts cultured during 54–96 hpi (MJ_{EGA}: 8- to 16-cell stage) in Control, Nob5, Nob10, and C_{DMSO}. **(f)** Western blot images showing the expression of pAKT-Thr308, pAKT-Ser473, (t)AKT and β -actin in Day 7 blastocysts from MJ_{EGA} phase. Data were normalized relative to the abundance of β -actin and p-AKT phosphorylation levels. Samples derive from the same experiment and gels were processed in parallel. Cropped western blot membrane images are shown here, while full-length blots are presented in Supplementary Figure S1. Data are the mean \pm s.e.m. Different letters indicate significant difference ($P < 0.05$) between treatments.

that showed changes in the levels of transcription in genes associated with cell cycle and observed an increase in *CDK2* expression during early embryo development (8 and 16-cell embryos, and blastocysts)⁴². Conversely, studies using nobiletin in cancer cells (U87, Hs683) showed a decrease in *CDK2* expression^{17,43}. Hence, nobiletin seems to respond differently depending on the cell type.

Histone H3.3 is encoded by H3.3 histone A (*H3-3A*) and H3.3 histone B (*H3-3B*) genes and is related to DNA synthesis and integrated into embryonic nucleosomes to mark genes for subsequent expression in development⁴⁴. We observed that supplementation of 5 or 10 μM nobiletin during MN_{EGA} increased the expression of *H3-3B* and *H3-3A* genes in 8-cell embryos while supplementation of 10 μM nobiletin during MJ_{EGA} increased the expression of *H3-3B* gene in 16-cell embryos. This is in corroboration with results from a recent study where characterization of the expression of both genes that encode *H3.3* (*H3-3A* and *H3-3B*) was performed in early bovine embryos, demonstrating that *H3-3B* mRNA is very abundant throughout early embryogenesis, being two to three times higher than *H3-3A* mRNA during the major wave of EGA⁴⁵. Additionally, a higher abundance of *H3-3B* compared to *H3-3A* was found in mouse embryos⁴⁶, suggesting that the protein encoded by *H3-3B* gene may be critical for initiating the transcription of embryonic genes during EGA. As mentioned above, EGA is crucial for further embryo development and regulated by several important factors⁴⁷. One crucial factor is histone modification, including methylation and acetylation⁴⁸. Likewise, Acetyl-Co A is a central metabolite linking glucose oxidation and long-chain fatty acid or cholesterol synthesis, providing energy and materials for cell growth and proliferation. Furthermore, Acetyl-Co A, as a donor of an acetyl group, can be utilized by histone acetyltransferases for histone acetylation⁴⁹. A recent study showed that Acetyl-CoA synthases are essential for maintaining histone acetylation under metabolic stress during EGA in pigs and they corroborated that β -oxidation is crucial for porcine embryo development by contributing to energy metabolism and histone acetylation⁵⁰. This suggests one more time that nobiletin could prefer the β -oxidation pathway as an energy production mechanism.

During in vitro development, embryos have a series of metabolic factors that are required in proliferation, differentiation, and survival of cells^{13,51}. In this context, the quality of the embryos produced in vitro depends on many factors, among them the expression of different genes, as we have shown in this study. Gene expression depends of different signaling pathways that play important roles in the formation of the blastocyst, for example, PI3K/AKT^{12,13,51}. AKT regulates cellular processes such as glucose metabolism, transcription, cellular growth and proliferation⁵¹. In blastocysts, AKT inhibition alters their development and AKT activation triggers the differentiation and migration of trophoblast cells^{14,52}. Other studies showed that the AKT appear to have an important role in early embryonic development, in double-knockout mice deletion of any of the AKT isoforms leads to embryonic death or exhibiting more severe phenotype and earlier lethality⁵³. In addition, PI3K/AKT regulates the development of preimplantation embryo by mediating the effects of autocrine factors⁵⁴. Previous studies in cell lines have shown that nobiletin can act through various signaling pathways, including AKT¹⁷. However, as far as we know, nobiletin action on AKT pathway in bovine blastocysts produced in vitro is unknown. In this study, we established the presence of the AKT pathway in bovine blastocysts. Based on immunofluorescence images, p-AKT protein appears to be predominantly localized in the cytoplasm of embryos cultured with 5 and 10 μM nobiletin during MN_{EGA} and MJ_{EGA} , suggesting constant stimulation of this pathway during the preimplantation period. Expression of the AKT protein and its phosphorylation status were confirmed by western blot analysis of bovine blastocysts produced with or without nobiletin supplementation during MN_{EGA} or MJ_{EGA} phases. Similar results were found by Ashry et al.¹⁴, who investigated the relationship between AKT signaling and the embryotrophic actions of follistatin, and indicated that it plays an important role in the regulation of AKT signaling in early bovine embryos. Together, these results suggest that nobiletin is associated with increased AKT phosphorylation and, as it has been shown in cell lines studies, nobiletin has the ability to interact with this pathway, and regulate specific genes. In our study increased AKT phosphorylation might be related to the increase in the production and the expression of genes that favor the progression of the cell cycle (*CDK2*) and to the improvement in embryo quality by increasing mitochondrial activity and genes related to oxidative stress (*NFE2L2*), and reducing lipid content (*PPAR α*). However, further studies are needed to fully elucidate its mechanism of action in early embryos.

In conclusion, nobiletin supplementation during MN_{EGA} or MJ_{EGA} has a positive effect on preimplantation bovine embryonic development in vitro by increasing blastocyst production and also corroborates on the increase in transcription level of genes related to cell division. Besides, this effect is reflected on the blastocysts quality improvement by (i) stimulating mitochondrial activity and expression of genes related to the protection of oxidative stress, and (ii) reducing the cytoplasmatic accumulation of lipids and promoting the expression of genes that regulate lipid metabolism. In addition, these positive responses of nobiletin on embryonic development and quality of the produced blastocysts in vitro could be modulated by the activation of AKT signaling pathway (Fig. 8). Therefore, nobiletin could constitute a suitable supplement to overcome oxidative stress in bovine IVP and improve ARTs in mammals.

Methods

Unless stated otherwise, all reagents were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA).

Oocyte collection and maturation. Immature cumulus-oocyte complexes (COCs) were obtained by aspirating follicles (2–8 mm) from the ovaries of mature heifers and cows collected from a local abattoir. COCs (homogeneous cytoplasm and intact CCs) were selected and matured in four-well dishes (Nunc, Roskilde, Denmark) in 500 μL maturation medium (TCM-199), supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/mL epidermal growth factor (EGF), in groups of 50 COCs per well for 24 h at 38.5 °C and an atmosphere of 5% CO_2 in the air with maximum humidity⁵⁵.

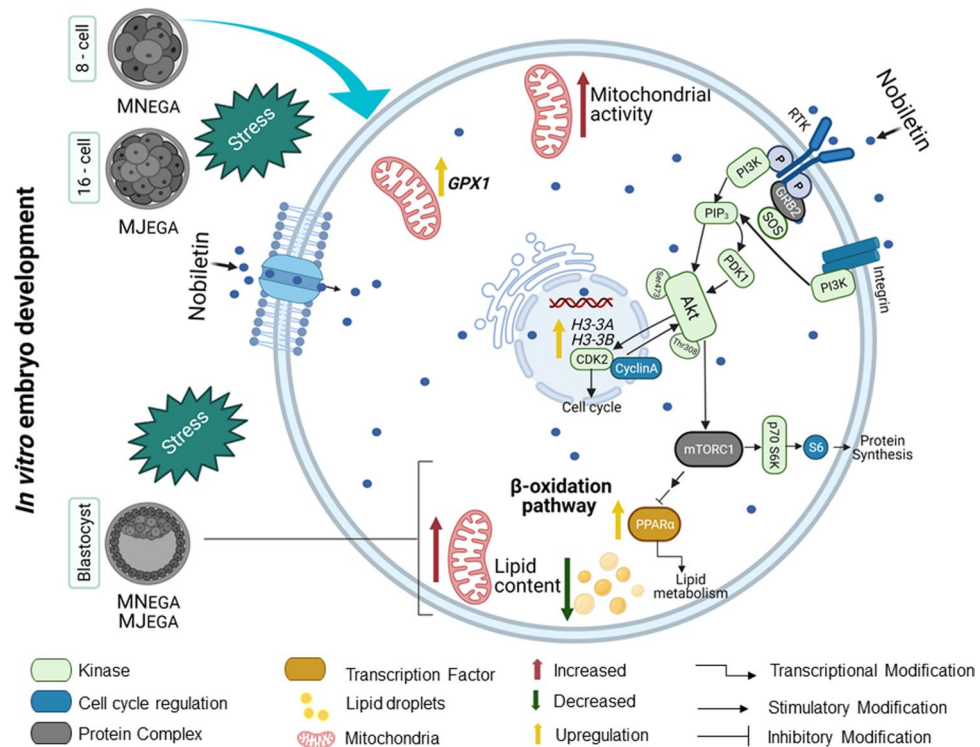


Figure 8. Schematic diagram illustrating the effect of nobiletin on bovine embryo development during the two EGA phases (MN_{EGA} and MJ_{EGA}). Nobiletin a class of polymethoxylated flavone, is easily absorbed across the cytoplasmic membranes due to its structure and lipophilic nature¹⁷, but the specific mechanism of action is not clear yet. Nobiletin supplementation during EGA has a positive effect on preimplantation bovine embryonic development, also increase the abundance of *CDK2* (cell cycle progression), *H3-3A*, *H3-3B* (development) and *GPX1* (oxidative stress) transcripts in 8-cells and 16-cells embryos. In addition, the nobiletin effect in the produced blastocysts was reflected by stimulating mitochondrial activity, decreasing the cytoplasmatic accumulation of lipids and promoting the expression of genes that regulate lipid metabolism and protect against oxidative stress. Besides, these positive responses of nobiletin on embryonic development and quality of the produced blastocysts in vitro could be modulated by activation of the AKT signaling pathway. In our study increased AKT phosphorylation might be related to the increase in the production and the expression of genes that favor the progression of the cell cycle (*CDK2*) and reducing lipid content (*PPARα*). However, further studies are needed to fully elucidate its mechanism of action in early embryos. Figure created with BioRender.com.

Sperm preparation and in vitro fertilization (IVF). IVF was performed as described previously⁵⁶. Briefly, frozen semen straws (0.25 mL) from an Asturian Valley bull previously tested for IVF were thawed at 37 °C in a water bath for 1 min and centrifuged for 10 min at 280×g through a gradient of 1 mL of 40% and 1 mL of 80% Bovipure (Nidacon Laboratories AB, Göttenborg, Sweden) according to the manufacturer's instructions. The sperm pellet was isolated and washed in 3 mL of Boviwash (Nidacon Laboratories AB) by centrifugation at 280×g for 5 min. The pellet was re-suspended in the remaining 300 μL of Boviwash. The final concentration of spermatozoa was adjusted to 1×10⁶ spermatozoa/mL. Gametes were co-incubated for 18–22 h in 500 μL fertilization medium (Tyrode's medium) with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate and 6 mg/mL fatty acid-free bovine serum albumin (BSA) supplemented with 10 mg/mL heparin sodium salt (Calbiochem) in four-well dishes in groups of 50 COCs per well in an atmosphere of 5% CO₂ in the air with maximum humidity at 38.5 °C.

In vitro culture of presumptive zygotes. At approximately 21 hpi, a total of 7237 (3398 for MN_{EGA} phase and 3839 for MJ_{EGA} phase) presumptive zygotes were denuded of cumulus cells (CCs) by vortexing for 3 min and then cultured in groups of 50 in a four-well dish containing 500 μL per well of culture medium (synthetic oviductal fluid (SOF);⁵⁷ supplemented with 5% (v/v) FCS, 4.2 mM sodium lactate, 0.73 mM sodium pyruvate, 30 μL/mL basal medium eagles (BME) amino acids, 10 μL/mL minimum essential medium (MEM) amino acids and 1 μg/mL phenol red, in the presence (MN_{EGA}) or absence (MJ_{EGA}) of nobiletin (MedChemExpress, MCE, Sweden) or with 0.03% dimethyl sulfoxide (DMSO vehicle for nobiletin dilution). At 54 hpi those embryos that reached the 8-cell stage were selected and randomly cultured in groups of 50 in SOF + 5% FCS until Day 8 (MN_{EGA}) or in presence of nobiletin or DMSO until 96 hpi (MJ_{EGA}). At 96 hpi those embryos that reached the 16-cell stage were selected and randomly cultured in groups of 50 in SOF + 5% FCS until Day 8 (MJ_{EGA}) (see

'Experimental design' described below for more details). Culture took place at 38.5 °C in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂.

Assessment of embryo development and quality

Embryo development. Developmental rate was recorded at 54 hpi from MN_{EGA} and MJ_{EGA} (≥ 8-cell) and 96 hpi from the MJ_{EGA} phase (≥ 16-cell). For both phases, cumulative blastocyst yields were recorded at Day 7, and 8 pi under a stereomicroscope.

Embryo quality: mitochondrial activity measurement, lipid content quantification and total cell number of blastocysts.

Day 7 blastocysts (~ 30 per group) were simultaneously evaluated regarding mitochondrial activity, the number of lipid droplets, and total cell number. Blastocysts from each treatment were first suspended in 100 µL phosphate-buffered saline (PBS) without calcium or magnesium supplemented with 0.1% polyvinylpyrrolidone (PVP). Next, blastocysts were equilibrated for 15 minutes in culture media supplemented with 5% FCS and then incubated for 30 min at 38.5 °C in 400 nM MitoTracker DeepRed (Molecular Probes, Eugene, USA) for mitochondrial activity; blastocysts were then fixed in 4% paraformaldehyde (PF) for 30 min at room temperature. For lipid content analysis, fixed blastocysts were permeabilized with 0.1% saponin for 30 min and stained for 1 h with 20 µg/mL Bodipy 493/503. For analysis of total cell number, blastocysts were stained with Hoechst 33342 (10 µg/mL) for 30 min, washed in PBS + 0.1% PVP three times for 5 minutes each, and then mounted in 3.8 µL mounting medium between a coverslip and a glass slide which was sealed with nail polish. Slides were examined using a laser-scanning confocal microscope (Leica TCS SP2) equipped with an argon laser excited at 488 nm and with an emission spectrum of 500–537 nm for visualization of lipid droplets. For mitochondria, we used excitation and emission set at 644 nm and 625–665 nm, respectively. All images were captured using the same parameters, performing sequential acquisition.

For the assessment of mitochondrial activity, the fluorescence signal intensity (pixels) was quantified. Serial sections of 5 µm were made for each blastocyst and a maximum projection was accomplished for each one. Images obtained were evaluated using the ImageJ program (NIH; ImageJ version 1.52k software (<http://rsbweb.nih.gov/ij/>)). After selection using the freehand selection tool, each blastocyst was measured to determine its area and its integrated density (IntDen), which corresponds to pixel intensity. Also, the background fluorescence of an area outside the blastocyst was measured. Fluorescence intensity in each blastocyst was determined using the following formula: Relative fluorescence = IntDen – (area of selected blastocyst x mean fluorescence of background readings). Fluorescence intensities are expressed in arbitrary units (a.u.).

The lipid quantification in blastocysts was obtained by analysis of the total area of lipids in each blastocyst. We captured three images of each blastocyst: one in the middle of the blastocyst (the image with the largest diameter) and the other two in the middle of the resulting halves. We used a 63× objective at a resolution of 1024 × 1024 and images were analyzed using the 'nucleus counter' tool, set to detect, distinguish, and quantify droplet areas with the ImageJ program. For blastocysts, lipid quantity was corrected by total embryo area, to account for varying blastocyst sizes. After verification of a significant correlation ($r^2 = 0.84$ and $P < 0.0001$ by Pearson's correlation test) between lipid quantity of three sections in 30 blastocysts (10 per group) we chose the section with the largest area per blastocyst to be analyzed⁵⁸. Simultaneously, the total number of cells per blastocyst was determined by counting the Hoechst stained cells under an epifluorescence microscope (Nikon 141731) equipped with a fluorescent lamp (Nikon HB-10104AF) and UV-1 filter.

Embryos at 8- and 16-cell stage and blastocysts for gene expression analysis.

Gene expression analysis was performed using 3 pools of 10 embryos at 8-cell (MN_{EGA}); 16-cell (MJ_{EGA}); and Day 7 blastocysts of both phases (MN_{EGA} and MJ_{EGA}) per treatment group: Control, C_{DMSO}, Nob5, and Nob10. Poly(A) RNA was extracted using the Dynabeads mRNA Direct Extraction Kit (Ambion; Thermo Fisher Scientific) with minor modifications⁵⁹. Immediately after poly(A) RNA extraction, reverse transcription (RT) was performed using a Moloney murine leukemia virus (MMLV) Reverse Transcriptase 1st-Strand cDNA Synthesis Kit according to the manufacturer's instructions (Epicentre Technologies Corp, Madison, WI, USA). Tubes were heated to 70 °C for 5 min to denature the secondary RNA structure, allowing Poly(T) random primers and Oligo dT annealing, and the RT mix was then completed by adding 0.375 mM dNTPs (Biotools, Madrid, Spain), 6.25 U RNasin RNase inhibitor (Promega, Madison, WI, USA), MMLV HP RT 10x reaction buffer, 5 mM DTT and 5 U MMLV high-performance reverse transcriptase (Epicentre Technologies Corp, Madison, WI, USA). Samples were incubated at 25 °C for 10 min, and then at 37 °C for 60 min, to allow the RT of RNA, and finally at 85 °C for 5 min to denature the enzyme. All mRNA transcripts were quantified in duplicate using a Rotorgene 6000 Real-Time Cycler (Corbett Research, Sydney, Australia). RT-quantitative polymerase chain reaction (qPCR) was performed by adding a 2 µL aliquot of each cDNA sample (~ 60 ng µL⁻¹) to the PCR mix (GoTaq qPCR Master Mix, Promega) containing specific primers to amplify the genes of interest. Primer sequences are provided in Supplementary Table S4. The selection of genes to be evaluated was carried out considering the expression of key genes in preimplantation embryonic development. All primers were designed using Primer-BLAST software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to span exon-exon boundaries when possible. For quantification, RT-qPCR was performed as described previously⁶⁰. The PCR conditions were tested to achieve efficiencies close to 1. Relative expression levels were quantified by the comparative cycle threshold (CT) method⁶¹. Values were normalized using two housekeeping genes (*H2AFZ* and *ACTB*) selected according to previous studies^{56,62}, while their stabilities were evaluated using the geNorm software for microsoft^{63,64}, ranking the genes based on the internal control gene stability parameter M. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to a dou-

bling of the amplified PCR product. According to the comparative CT method, the Δ CT value was determined by subtracting the mean CT value of the two housekeeping genes from the CT value of the gene of interest in the same sample. The calculation of $\Delta\Delta$ CT involved using the highest treatment Δ CT value (i.e. the treatment with the lowest target expression) as an arbitrary constant to subtract from all other Δ CT sample values. Fold-changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta$ CT}.

Immunofluorescence of phospho-AKT (p-AKT) in blastocysts. Immunolocalization of p-AKT was performed according to López-Cardona et al.⁶⁵ with minor modifications. Day 7 blastocysts (n = 10 per group) were washed twice with PBS + 0.1% PVP and fixed in 4% PF for 10 min at room temperature. Next, they were permeabilized by incubation in PBS with 10% FCS and 1% Triton X-100 for 45 min at room temperature. After permeabilization, blastocysts were incubated overnight at 4 °C in PBS + 0.1% PVP and 5% FCS and 1:100 rabbit polyclonal antibody against p-AKT (Thr308/Ser473) (D9E) XP® Rabbit mAb (Cell Signaling Technology, #4060). Following incubation, blastocysts were washed twice in PBS + 0.1% PVP and incubated in PBS supplemented with 5% FCS and 1:250 goat anti-rabbit polyclonal antibody Alexa Fluor 488-conjugate (Molecular Probes, Eugene, OR, USA), for 2 h at room temperature followed by washing again three times in PBS + 0.1% PVP. In all cases, nuclei were stained with Hoechst 33342 (10 µg/mL). Finally, blastocysts were mounted in microdrops with Fluoromount G (EMS, Hatfield, UK) and examined by confocal microscopy (Leica TCS-SPE). Negative control was prepared to omit the primary antibody before adding the secondary antibody.

Western blot of AKT in blastocysts. The western blot analysis was performed as described previously by Ashry et al.⁶⁶ with minor modifications. Day 7- 8 blastocysts (n = 20 blastocysts/group, n = 3 replicates/ EGA phase) were lysed in 1 × RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris [pH 7.6]), supplemented with 1 × protease, phosphatase Inhibitor Cocktail (Roche, Basel, Switzerland), for 1 h at 4 °C. The samples were mixed with 1 × sample buffer and then denatured at 95 °C for 10 min. Proteins were resolved by SDS-PAGE (12% acrylamide gel loading 45 µL of total protein per well) and transferred onto a nitrocellulose membrane. After the transfer, membranes were blocked for 30 min in 3% BSA in PBS + 0.1% Tween-20 (PBS-T) at room temperature, and was incubated overnight at 4 °C with a total (t)AKT rabbit polyclonal antibody [1:1000 (Vol:Vol), Cell Signaling Technology, #9272S]; or a p-AKT (Thr308) polyclonal antibody [1:1000 (Vol:Vol), Cell Signaling Technology, #9275S]; or a p-AKT (Ser473) polyclonal antibody [1:1000 (Vol:Vol), Cell Signaling Technology, #9271S]. Then, incubation with the secondary antibody goat anti-rabbit IgG-HRP [1:2500 (Vol:Vol), Cell Signaling Technology, #7074S] was conducted for 2 h at room temperature revealed by Enhanced Chemiluminescence kit (RPN2109, ECL™, Amersham GE Healthcare) and detected by an ImageQuant LAS 500 chemiluminescence CCD camera (GE Healthcare Life Sciences, USA, 29005063). The monoclonal anti-β-actin–peroxidase antibody produced in mouse was used as the loading control.

Membranes were probed sequentially with primary p-AKT (Thr308), p-AKT (Ser473) and (t)AKT antibodies. For this purpose, after detection of an antibody membranes were stripped by washing extensively in TBS-T, three times for 10 minutes each, and repeating the blocking step, and then the membranes are re-probed with the next antibody. After detection of (t)AKT, membranes were stripped and re-probed with anti-β-actin–peroxidase antibody. In all cases, intensities of protein bands (optical density (OD)) were quantified by ImageJ software and the relative abundance of each protein was normalized to the total-actin expression in the corresponding lane and phosphorylation level was expressed as phosphorylated (p) AKT/(t) AKT. The ratio of the OD of the protein concerned (AKT/p-AKT) in relation to actin is presented in the form of bar charts.

Experimental design. *Experiment 1: effect of nobiletin on early embryo development in vitro.* In this experiment the effect of nobiletin supplementation on embryo development during two developmental periods: (a) MN_{EGA}: from 2-cell to 8-cell stage, minor EGA phase; and (b) MJ_{EGA}: from 8-cell to 16-cell stage, major EGA phase was determined by evaluating the cleavage rate at 54 hpi and blastocysts yield at Days 7 and 8 (Fig. 9).

For this purpose, presumptive zygotes/embryos from 2- to 8- cell stage (MN_{EGA}: 21-54 hpi) or embryos from 8- to 16-cell stage (MJ_{EGA}: 54-96 hpi) were cultured in SOF + 5% FCS alone (Control: n = 730 and 621 for MN_{EGA} and MJ_{EGA} respectively) or supplemented with 5, 10 or 25 µM nobiletin (Nob5: n = 757 and 518; Nob10: n = 695 and 553; and Nob25: n = 521 and 424 for MN_{EGA} and MJ_{EGA} respectively), or 0.03% DMSO (C_{DMSO}: n = 695 and 622 for MN_{EGA} and MJ_{EGA} respectively). For MJ_{EGA} phase groups, embryo culture until 8-cell stage (21-54 hpi) was performed in SOF + 5% FCS. At 54 hpi (MN_{EGA} - Control: n = 388; Nob5: n = 386; Nob10: n = 352; Nob25: n = 254; C_{DMSO}: n = 368) or 96 hpi (MJ_{EGA} - Control: n = 331; Nob5: n = 315; Nob10: n = 347; Nob25: n = 210; C_{DMSO}: n = 331), embryos that reached the 8- or 16- cell stage, respectively, were transferred to SOF + 5% FCS and cultured until Day 8, maintaining the different experimental groups separately (Fig. 9). Embryos were cultured in groups of 50 under an atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 38.5 °C.

Considering that during the experiment it was necessary to preselect the embryos at different stages of development (≥ 8 cells and ≥ 16 cells), the developmental parameters were calculated as follows: (I) developmental rate at 54 hpi: percentage of presumptive zygotes that developed to the 8-cell stage; (II) developmental rate at 96 hpi: percentage of selected 8-cell embryos at 54 hpi that developed to the 16-cell stage; and (III) blastocyst yield: percentage of selected 8-cell embryos (54 hpi) or 16-cell embryos (96 hpi) that continued in culture and developed to the blastocyst stage at Day 7 and 8. A representative number of 8-cell (MN_{EGA}), 16-cell (MJ_{EGA}), and Day 7 blastocysts from both phases for each experimental group were frozen in liquid nitrogen (LN₂) in three groups of 10 and stored at - 80 °C for gene expression (See Experiment 2). Additionally, Day 7 blastocysts from both phases were selected to (i) evaluate quality (See Experiment 2); (ii) immunolocalization of p-AKT or (iii) for western blot analysis (See Experiment 3).

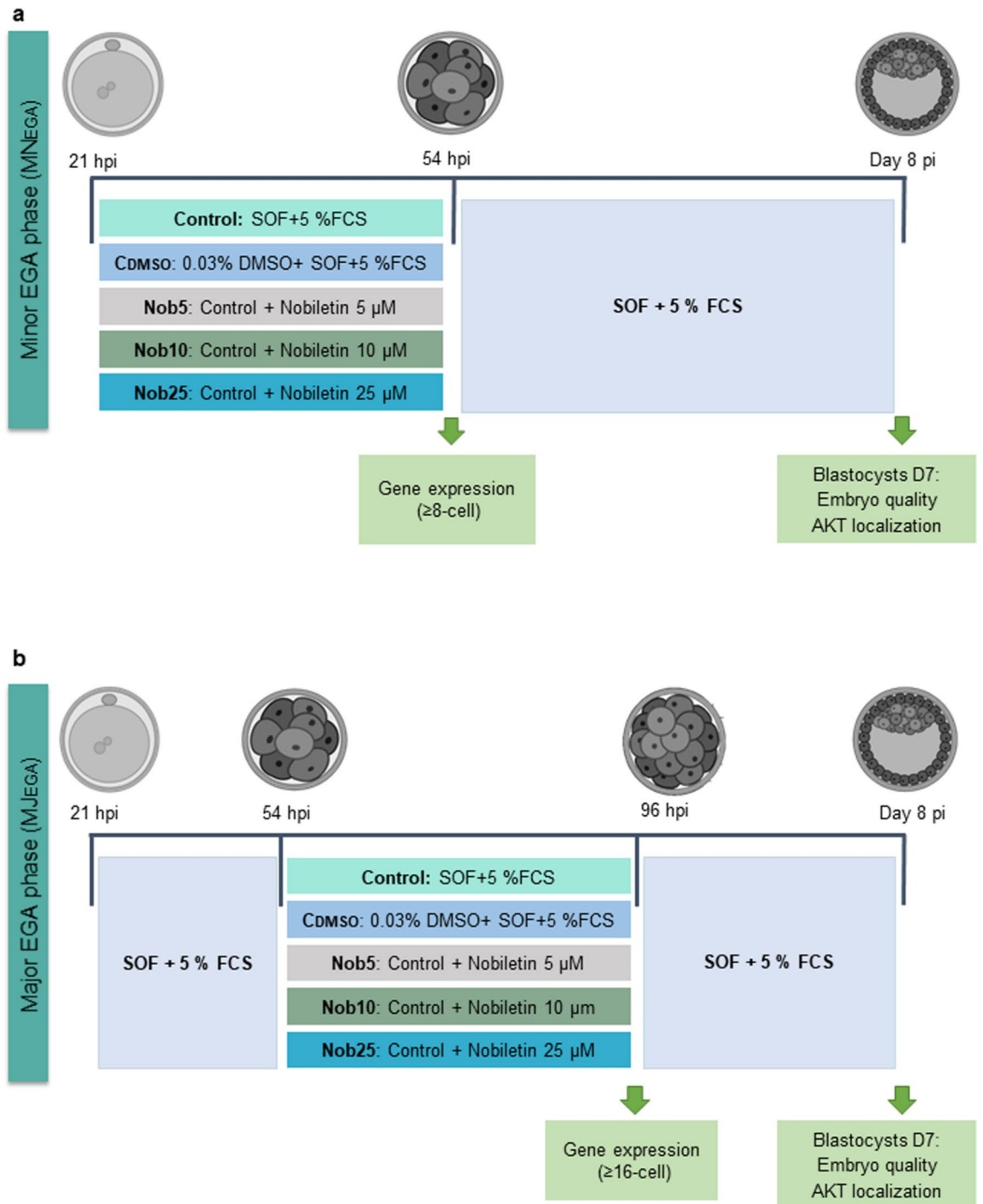


Figure 9. Experimental design. **(a)** MNEGA: Presumptive zygotes were cultured during the minor EGA phase (2- to 8-cell stage: 21–54 hpi) in synthetic oviductal fluid (SOF) with 5% fetal calf serum (FCS) (Control); supplemented or not with 5, 10 and 25 μ M nobiletin (Nob5, Nob10 and Nob25, respectively), or with 0.03% dimethyl sulfoxide [control for DMSO vehicle for nobiletin dilution (C_{DMSO})]. At 54 hpi, embryos that reached the 8-cell stage were transferred to SOF + 5% FCS and cultured until Day 8 maintaining each experimental group separately. **(b)** MJEGA: Presumptive zygotes were cultured in SOF + 5% FCS (Control) until 54 hpi. At 54 hpi, embryos that reached the 8-cell stage were cultured during the major EGA phase (8-cell to 16-cell stage: 54–96 hpi) in SOF + 5% FCS supplemented or not with Nob5, Nob10 and Nob25, or with 0.03% dimethyl sulfoxide (C_{DMSO}). At 96 hpi, embryos that reached the 16-cell stage were transferred to SOF + 5% FCS and cultured until Day 8 maintaining each experimental group separately.

Twelve and ten replicates for MN_{EGA} and MJ_{EGA} phases, respectively, were performed under the same assay conditions.

Only the experimental groups that showed higher blastocyst yield in this experiment (Nob5 and Nob10) in comparison with both control groups (Control and C_{DMSO}) were used for experiments 2 and 3.

Experiment 2: effect of nobiletin on the quality of in vitro produced blastocysts. To evaluate blastocyst quality of embryos produced in vitro with or without nobiletin supplementation during MN_{EGA} or MJ_{EGA}, a representative number of Day 7 blastocysts (n ≈ 30 per group/Experiment 1) were stained with MitoTracker DeepRed, Bodipy and Hoescht to evaluate mitochondrial activity (intensity recorded in arbitrary units (a.u)), lipid content (lipid droplet area in μm²) and total cell numbers, respectively. Blastocysts were examined using a laser-scanning confocal microscope or an epifluorescence microscope and images obtained were evaluated using the ImageJ program.

To evaluate if nobiletin induces changes in the expression levels of genes related to embryo development and quality, three independent pools of 10 embryos per stage (8-cell, 16-cell, and blastocyst) obtained from each experimental group cultured with or without nobiletin during MN_{EGA} or MJ_{EGA} (Experiment 1), were used for gene expression analysis by qRT-PCR according to the procedures described above.

The selected genes have been linked to embryonic development and are essential in cell proliferation, differentiation, and embryo quality, such as PPARG coactivator 1 alpha (PPARGC1A); Peroxisome Proliferator-Activated Receptor Alpha (PPARα); Ribosomal Protein S6 Kinase Beta-1 (RPS6KB1); Cyclin Dependent Kinase 2 (CDK2); H3 Histone Family Member 3B (H3-3B) and H3 Histone Family Member 3A (H3-3A), including Nuclear Factor Erythroid 2-Like 2 (NFE2L2) and Glutathione Peroxidase 1 (GPX1) related with oxidative stress.

Experiment 3: nobiletin effect on the AKT pathway in blastocysts produced in vitro. To assess if nobiletin can interact with AKT pathway during in vitro embryo development, Day 7 blastocysts (n = 10 - Experiment 1) from each group were stained with p-AKT (Thr308/Ser473) for immunolocalization. To evaluate the phosphorylation level of AKT (Thr308 and Ser473), Day 7 blastocyst (n = 60 - Experiment 1) from each group were frozen in LN₂ for western blot analysis.

Statistical analysis. All statistical tests were performed using the software package SigmaStat (Systat Software Inc., San Jose, CA, USA). Cleavage rate, blastocyst yield, mitochondrial activity, lipid content, number of cells per blastocyst, relative mRNA abundance levels, and AKT phosphorylation level, were normally distributed with homogeneous variance, so one-way analysis of variance (ANOVA) with arcsine data transformation, followed by Tukey's test, was performed to evaluate the significance of differences between groups. The correlation analysis for lipid quantification in blastocysts was determined by Pearson's correlation coefficient test. Values were considered significantly different at $P < 0.05$. Unless otherwise indicated, data are presented as the mean ± s.e.m.

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Author contributions

K.C.-B., Y.N.C., D.R. and E.M.G. conceived and designed the study. K.C.-B., Y.N.C., S.P.-C., C.L.V.L. and E.M.G. performed the experiments. K.C.-B., Y.N.C., E.A. and D.R. analysed and interpreted the data. K.C.-B. and Y.N.C. wrote the first draft of the manuscript. C.L.V.L., E.A., A.G.-A., E.M.G. and D.R. revised and discussed the manuscript. A.G.-A. and D.R. Funding acquisition. All authors read and approved the manuscript for publication.

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Competing interests

The authors declare no competing interests.

Additional information

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