Effect of polymethoxylated flavone Nobiletin in bovine oocyte maturation and subsequent development in vitro

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1. Summary

The ability of an oocyte to undergo successful cytoplasmic and nuclear maturation is fundamental for fertilization and embryo development.

The aim of this study was to evaluate the effect of Nobiletin supplementation during *in vitro* maturation (IVM) of bovine oocytes on nuclear and cytoplasmic maturation and their developmental competence. Nobiletin is a polymethoxylated flavonoid isolated from citrus fruits exhibiting a wide biological effect in cell adhesion, cell migration, cell cycle regulation and inhibition of reactive oxygen species (ROS) production; important factors for oocyte IVM.

Immature cumulus oocytes complexes (COCs) were aspirated from ovaries of slaughtered heifers. Selected COCs were *in vitro* matured in TCM-199+10% foetal calf serum (FCS) and 10 ng/ml epidermal growth factor (EGF) (Control) supplemented either with 10, 25, 50 and 100 µM of Nobiletin (N10, N25, N50 and N100 respectively) or 0.01% dimethyl sulfoxide (CDMSO), vehicle for nobiletin dilution. After 24 h of IVM at 5% CO2 in air at 38.5 °C, a representative number of oocytes from each group were fixed and stained to evaluate nuclear and cytoplasmic maturation. In addition, oocytes were stained to measure oocyte metabolism in terms of ROS and glutathione (GSH) content. The remaining oocytes were fertilized and cultured *in vitro* to evaluate their developmental competence by cleavage rate and blastocyst yield.

Significantly higher percentage of matured oocytes were observed in metaphase II when N25 (87±0.6%) or N50 (89.3±0.3%) were added to the IVM medium compared to N10 (72.9±0.3%), N100 (71.5±0.8%), Control (71.7±0.7%) and CDMSO (70.5±0.5%) groups. Furthermore, N25 and N50 showed higher rate of oocytes with peripheral migration of cortical granules (85.7±0.3% and 89.9±2.2% respectively) and mitochondria (86.7±0.6% and 88.9±1.2% respectively) compared to the remaining groups (P<0.05). In addition, the supplementation of N25 and N50 showed a significant reduction (P<0.05) in the ROS (2.53±0.8; 2.62±1.2 a.u. respectively), and GSH (2.84±0.4; 3.09±0.1 a.u. respectively) content in comparison with all other groups. Cleavage rate was significantly higher (P<0.05) for N25 (89.9±0.3%) and N50 (91.3±0.3%) compared to all other groups (N10: 75.6±0.3%; N100: 74.0±0.6%; Control: 74.2±0.4%; and CDMSO: 73.6±0.4%). Similarly, cumulative blastocyst yield at D8 was significantly higher (P<0.05) for N25 (32.1±0.8%) and N50 (35.5±0.8%) compared to N10 (23.1±0.7%), N100 (24.5±0.9%), Control (25.9±0.4%) and CDMSO (26.1±0.6%) groups. In conclusion, supplementation of 25 µM or 50 µM of Nobiletin to the IVM medium improves oocyte nuclear and cytoplasmic maturation, reduces oxidative stress and improve embryo development.
2. Resumen

La habilidad del ovocito para lograr una maduración nuclear y citoplasmática exitosa es fundamental para la fertilización y el desarrollo embrionario.

El objetivo de este estudio fue evaluar el efecto de la suplementación con Nobiletina durante la maduración in vitro de ovocitos bovinos. La Nobiletina es un flavonoides aislado de las frutas cítricas, con vastos efectos en adhesión celular, migración celular, regulación del ciclo celular e inhibición de la producción de radicales libres de oxígeno (ROS); todos ellos son factores importantes en la maduración in vitro (MIV) de ovocitos.

Complejos cumulo-ovocitarios (COCs) fueron aspirados de ovarios provenientes de terneras sacrificadas para consumo. Los COCs seleccionados fueron madurados in vitro en TCM-199+10% suero fetal bovino (FCS) and 10 ng/ml factor de crecimiento epidermal (EGF) (Control) suplementado con 10, 25, 50 y 100 µM de Nobiletina (N10, N25, N50 and N100 respectivamente) y 0.01% dimetilsulfóxido (CDMSO), como diluyente de la Nobiletina. Después de 24 h de MIV a 5% CO₂ y 38.5 °C, un número representativo de ovocitos de cada grupo fueron fijados y teñidos para evaluar maduración nuclear y citoplasmática. Asimismo, un número representativo de ovocitos fueron teñidos para medir metabolismo ovocitario en términos de contenido de ROS y glutatión (GSH). Los ovocitos restantes fueron fertilizados y cultivados in vitro para evaluar su capacidad de desarrollo a través de la tasa de división y el rendimiento de blastocistos.

Un porcentaje significativamente alto de ovocitos fueron observados en metafase II en los grupos N25 (87±0.6%) y N50 (89.3±0.3%) comparado con los grupos N10 (72.9±0.3%), N100 (71.5±0.8%), Control (71.7±0.7%) y CDMSO (70.5±0.5%). Asimismo, los grupos N25 y N50 mostraron una tasa superior de migración de gránulos cíticos (85.7±0.3% y 89.9±2.2% respectivamente) y mitochondrias (86.7±0.6% y 88.9±1.2% respectivamente) comparado con los restantes grupos (P<0.05). Los grupos N25 y N50 también mostraron una reducción significativa (P<0.05) en los niveles de ROS (2.53±0.8; 2.62±1.2 a.u. respectivamente), y GSH (2.84±0.4; 3.09±0.1 a.u. respectivamente) en comparación con los otros grupos. La tasa de división fue significativamente superior (P<0.05) en los grupos N25 (89.9±0.3%) y N50 (91.3±0.3%) comparado con los otros grupos (N10: 75.6±0.3%; N100: 74.0±0.6%; Control: 74.2±0.4%; y CDMSO: 73.6±0.4%). De forma similar, el rendimiento acumulativo de blastocistos fue significativamente más alto (P<0.05) para los grupos N25 (32.1±0.8%) y N50 (35.5±0.8%) comparado con N10 (23.1±0.7%), N100 (24.5±0.9%), Control (25.9±0.4%) y CDMSO (26.1±0.6%).

En conclusión, la suplementación con 25 µM y 50 µM de Nobiletina en el medio de MIV mejora la maduración nuclear y citoplasmática de los ovocitos, y reduce el estrés oxidativo mejorando el desarrollo embrionario.
3. **Resum**

L’habilitat de l’òcit per aconseguir una maduració nuclear i citoplasmàtica reeixida és fonamental per a la fertilització i el desenvolupament embrionari.

L’objectiu d’aquest estudi va ser avaluar l’efecte de la suplementació amb nobiletina durant la maduració in vitro d’òcits bovins. La nobiletina és un flavonoide aïllat de les fruites cítriques, amb vastos efectes en adhesió cel·lular, migració cel·lular, regulació del cicle cel·lular i inhibició de la producció de radicals lliures d’oxigen (ROS); tots ells són factors importants en la maduració in vitro (MIV) d’òcits.

Complexos cúmul-ovocitarios (COCs) van ser aspirats d’ovaris provinents de vedelles sacrificades per a consum. Els COCs seleccionats van ser madurats in vitro en TCM-199 + 10% sèrum fetal boví (FCS) and 10 ng/ml factor de creixement epidermal (EGF) (Control) suplementat amb 10, 25, 50 i 100 micres de nobiletina (N10, N25, N50 and N100 respectivament) i 0,01% dimetilsulfòxid (CDMSO), com diluent de la nobiletina. Després de 24 h de MIV a 5% CO2 i 38.5 ° C, un nombre representatiu d’òcits de cada grup van ser fixats i tanyits per avaluar maduració nuclear i citoplasmàtica. Així mateix, un nombre representatiu d’òcits van ser tanyits per mesurar metabolisme ovocitario en termes de contingut de ROS i glutatió (GSH). Els òcits restants van ser fertilitzats i cultivats in vitro per avaluar la seva capacitat de desenvolupament a través de la taxa de divisió i el rendiment de blastocists.

Un percentatge significativament alt d’òcits van ser observats en metafase II en els grups N25 (87 ± 0.6%) i N50 (89.3 ± 0.3%) comparat amb els grups N10 (72.9 ± 0.3%), N100 (71.5 ± 0.8%), Control (71.7 ± 0,7%) i CDMSO (70.5 ± 0.5%). Així mateix, els grups N25 i N50 van mostrar una taxa superior de migració de grànuls corticals (85.7 ± 0.3% i 89.9 ± 2.2% respectivament) i mitocòndries (86.7 ± 0.6% i 88.9 ± 1.2% respectivament) comparat amb la resta de grups (P < 0.05). Els grups N25 i N50 també van mostrar una reducció significativa (P <0.05) en els nivells de ROS (2.53 ± 0.8; 2.62 ± 1.2 au respectivament), i GSH (2.84 ± 0.4; 3.09 ± 0.1 au respectivament) en comparació amb els altres grups. La taxa de divisió va ser significativament superior (P <0.05) en els grups N25 (89.9 ± 0.3%) i N50 (91.3 ± 0.3%) comparat amb els altres grups (N10: 75.6 ± 0.3%; N100: 74.0 ± 0.6%; control: 74.2 ± 0,4%, i CDMSO: 73.6 ± 0.4%). De manera similar, el rendiment acumulatiu de blastocists va ser significativament més alt (P <0.05) per als grups N25 (32.1 ± 0.8%) i N50 (35.5 ± 0.8%) comparat amb N10 (23.1 ± 0,7%), N100 (24.5 ± 0,9%), Control (25.9 ± 0.4%) i CDMSO (26.1 ± 0.6%). En conclusió, la suplementació amb 25 micres i 50 micres de nobiletina en el medi de MIV millora la maduració nuclear i citoplasmàtica dels òcits, i redueix l’estrès oxidatiu millorant el desenvolupament embrionari.
4. Introduction

4.1 Oocyte developmental competence and early embryo development in vivo

In cattle, the process of oogenesis begins in the fetal ovary (day 70-80 of pregnancy) and female calves are born with thousands oocytes as primordial and primary follicles until puberty (Van Eetvelde et al., 2017). During oogenesis, oogonia experiment mitotic divisions and early stages of meiosis becoming oocytes arrested at the diplotene stage of prophase I (the germinal vesicle stage), until they are committed to ovulation or atresia (Lonergan & Fair, 2016).

As the oocyte progresses through oogenesis, it maintains a relationship within the ovarian follicle ensuring that the two processes, oogenesis and folliculogenesis, are integrated. The follicular growth begins when the shape of the granulosa cells change from flattened to cuboidal constituting the primary follicle (Braw-Tal, 2002). Then, the increase of granulosa cells in layers in the follicles, originates the secondary follicles. Granulosa cell proliferation finish in the tertiary follicles, also called antral follicles (Lussier et al., 1987). The bovine oocyte reaches its full size when the follicle enclosing it reaches a diameter of approximately 3 mm (Fair et al., 1995).

Resumption of meiosis and progression through maturation result in arrest at the metaphase II stage, with the extrusion of the first polar body and a DNA complement of 1n2C. Penetration of the sperm leads to extrusion of the second polar body and establishment of a 1n1C state in the oocyte, leading to a diploid embryo (2n2C) after the first mitotic division following fertilization as shown in Figure 1 (Lonergan & Fair, 2016).

During oogenesis, oocyte passes from different phases:

a. Growth phase

The major factors that regulate development during the growth phase (pre-antral), involve paracrine and gap–junction mediated signalling mechanisms.

During the growth phase, the mitotic proliferation of oogonia occurs in the prenatal gonad and is accompanied by entry into meiotic prophase I (PI). In the dictate arrest, the oocyte transit from a quiescent non-growing state within the primordial follicle to an active growth one, start with an expansion in size, because of new proteins and RNAs synthesized, and hyperplasia and organelles.

This period of gene expression becomes repressed and the oocytes remain in meiotic arrest with a germinal vesicle (GV), and the pre-granulosa cells form primordial follicles.

Figure 1. Prophase I arrest (the germinal vesicle stage). Preovulatory surge of LH, and further arrest at the metaphase II (MII). Lonergan & Fair, 2016.
The follicles wait in this state until the correct endocrine cues to resume division, or activation. Meiosis does not resume in mammals until after puberty (Reviewed by D. Albertini, 2015).

b. Pre-maturation phase and maturation stage

During pre-maturation and maturation phases (antral follicles), follicle-stimulating hormone (FSH) promotes growth and differentiation of somatic cells, ensuring that they eventually acquire sensitivity to LH, the primary trigger for ovulation and oocyte maturation.

The estrous cycle in cattle is composed of two or three waves of follicular growth involving emergence of a new wave of follicles in association with a transient rise in follicle-stimulating hormone (FSH), growth of a follicular cohort, and selection of a dominant follicle. Each follicular wave culminates in development of a single nonovulatory or ovulatory dominant follicle (Ireland et al., 2000). If the dominant follicle develops at a time when progesterone concentration is low, it is exposed to an appropriate LH pulsatility pattern and will go on to ovulate. The trigger for resumption of meiosis in the oocyte within the dominant follicle is the preovulatory surge of LH, which activates the breakdown of the germinal vesicle (GVBBD) and progression to metaphase II. Each of these phases in maturation has been identified as error-prone in terms of nuclear (genetic, epigenetic) or cytoplasmic quality and have been implicated as determinants of embryonic developmental competence (Krisher, 2004).

Approximately 27 h after the onset of the LH surge, ovulation takes place, and the now-matured oocyte (arrested in MII) is released along with the cells that surround it (cumulus oophorous) and the fimbria of the infundibulum that surrounds the ovary allows the passage of the cumulus-oocyte complex (COC) into the oviduct. Is only upon fertilization, via the influx of calcium ions that is triggered by sperm penetration, that MII stage progress once the sperm enters through the zona pellucida (ZP), the glycoprotein coat surrounding the oocyte.

The oviduct plays a key role in the finale maturation and the transport of the gametes. The mucosa epithelium is formed by secretory and ciliary columnar cells, responsible for the secretions and the movement of the gametes and embryos through the oviduct respectively (Leese et al., 2008). In the bovine species, the proportion of ciliated and secretory cells as well as their morphology changes during the oestrous cycle under hormonal control (Abe, 1996; Mukherjee et al., 2014b). Apart from the own oviduct secretions, the oviductal fluid (OF) has also components from the plasma such as albumin or amino acids. On one hand, ciliated cells and both muscular layers, guide the oocyte to the ampullary isthmic junction, where fertilization takes places (Reviewed by Croxatto, 2015). On the other hand, and at the same time, sperm progress in a counter-current from the distal portion of the oviduct. While a portion migrates to the ampulla, the majority remains in the isthmus establishing a sperm reservoir, where the sperm cells adhere to the ciliated cells delaying capacitation (Coy et al., 2012).
Finally, after fertilization, the ciliary activity of the ampulla moves the zygote to the isthmus (Kölle et al., 2009). Approximately 24 hours post fertilization, the first cleavage occurs, this process lead the embryo to a 2-cells, 4-cells (36 hours), 8-cells (72 hours), and 16-cell stage (84-96 hours). This is the most critical period of mammalian development because it includes embryonic genome activation, which occurs at the 8- to 16-cell stage in the bovine (Memili & First, 2000a) and is critical for future cell differentiation, embryo implantation, and fetal development (Niemann & Wrenzycki, 2000). Then, the embryo enters the uterus and by day 7 forms a blastocyst consisting of an inner cell mass, which gives rise to the foetus, and the trophoectoderm (TE), which forms the placenta. On days 9–10, the blastocyst hatches from the ZP and soon begins the process of elongation, which involves transitions from a spherical blastocyst on day 7 of gestation, through ovoid (days 12–13), tubular (days 14–15), and finally filamentous forms around days 16–17 before implantation which begins at Day 19 (see Figure 2) (Degrelle et al., 2005; Senger, 2005).

4.2 In vitro embryo production (IVP)

In 1988 Lu et al. (1988) developed a method to produce embryos entirely in vitro with the advances in the knowledge of oocyte maturation and sperm capacitation. The fact of being able to mature oocytes in vitro also opened the possibility of recovery oocytes from ovaries collected at the slaughterhouse, therefore significantly decreasing the cost of the IVP. Therefore, the IVP became quickly a commercial reality and a tool for breeding-improvement purposes. Moreover, the IPV in mammals allow us to enhance our understanding of oocyte maturation and early embryo development during the preimplantation stages.

Production of embryos in vitro is a three-step process involving oocyte maturation (IVM), oocyte fertilization (IVF), and subsequent culture of the zygote to the blastocyst stage (in vitro culture, IVC).

Even when reproductive research has led great progress during the past 30 years, the production process of in vitro embryos is far from optimal nowadays. From the oocytes recovered from
slaughterhouse ovaries, 90% undergo matured (nuclear and cytoplasmic maturation). Of those, 80% are successfully fertilized and divide into 2 cells stage (Lonergan et al., 2003). However, only 30% of the matured oocytes manage to reach the blastocyst stage (Rizos et al., 2008). In addition, in vitro embryos are of inferior quality compared to in vivo counterparts, in terms of morphology, cryotolerance, gene expression patterns (Rizos et al., 2008), and inner cell mass (ICM)/trophoectoderm (TE) cells ratios (Plourde et al., 2012). Moreover, after transfer of these embryos into recipient cows, pregnancy rate is between 40-60% compared to about 70% when in vivo embryos are transferred (Hasler et al., 1995). There is convincing evidence that the quality of the oocyte is the principal factor determining the blastocyst yield, while postfertilization culture environment, within limits, does not have a major influence on the ability of the immature oocyte to ultimately form a blastocyst, but have a major impact in the quality of the blastocysts (Rizos et al., 2002).

4.2.1 In vitro maturation (IVM)

Although the rate of oocytes maturation is high (90%) (Lonergan, et al. 2003), there are many factors to consider achieving it, and adverse consequences from in vitro maturation have been noted in several mammalian species (Plant et al., 2015). Oocyte origin is what determines the final embryo production; those which have matured in vivo and then have been fertilized and cultured in vitro are more likely to reach the blastocyst stage than those which have undergone the whole process in vitro (Rizos et al., 2002).

As part of the procedures for the in vitro production of bovine embryos, IVM is initiated immediately following the removal of the immature oocyte from small to medium-sized antral follicles. The quality and therefore the blastocyst rate, is influenced by the size of the follicle aspirated. Oocytes recovered from follicles of more than 6 mm yield a higher proportion of blastocyst compared with follicles between 2-6 mm (65.3% and 34.3% respectively) (Lonergan et al., 1994). Cumulus-oocyte complexes (COCs) for research purpose are from a very heterogeneous pool of 2-6 mm follicles and are still, several days away from possible ovulation. In contrast, the follicle that ovulates a mature oocyte at metaphase II grows to a size of 15–20 mm (Reviewed by Lonergan, 2016).

Once obtained COCs are selected for in vitro maturation by morphological criteria based on the appearance of the cytoplasm, and the presence or absence of cumulus cells (CCs).

According to these features the COCs are classified into 4 groups (de Loos et al., 1989):

- Grade 1: oocytes with more than 4-5 layers of cumulus cells, complete and compact, and a homogeneous cytoplasm.
- Grade 2: oocytes with fewer layers of cumulus cells, between 1 and 3, with homogeneous cytoplasm or a darker area on the perimeter of the oocyte.
- Grade 3: oocytes without cumulus cells or not surrounded by them, with darker and/or irregular cytoplasm.
- Grade 4: expanded oocytes, and pyknotic or very dark and/or irregular oocyte cytoplasm.

The cumulus cells play a critical role in the development of the oocyte, the cellular communication between oocytes and cumulus cells is complex, and both sides have active
regulatory roles (Fernando H. Biase & Kimble, 2018). During folliculogenesis, CCs provide metabolites and nutrients and support meiotic arrest and cytoplasmic maturation of the oocyte, for instance, by exporting cyclic AMP (Conti et al., 2012), calcium (Amireault & Dubé, 2005), and other metabolites (Gilchrist et al., 2004; Wigglesworth et al., 2013).

Cumulus cell expansion is also an important marker for oocyte maturation and is induced by gonadotrophin stimulation in vivo and in vitro leading to massive production of mucoid extracellular matrix protein (Chen et al., 1990). This process, in vivo, facilitates COC removal from the follicle wall, its extrusion during ovulation and its capture by the oviductal fimbriae. In cattle, in vitro cumulus cell expansion was shown to be essential for fertilization and subsequent cleavage and blastocyst development (Gutnisky et al., 2007; Nagyova, 2012).

Oocyte maturation involves (i) nuclear maturation, i.e., progression from prophase I to metaphase II with extrusion of the first polar body, (ii) cytoplasmic maturation which includes organelle redistribution, and (iii) molecular maturation that involves the accumulation of specific mRNAs (Sirard, 2001).

(i) Nuclear maturation

Nuclear maturation implicate the transition from a germinal vesicle nucleus to a second metaphase arrangement of the chromosomes and formation of a first polar body by the time of ovulation in most species so far studied (Andreu-Vázquez et al., 2010).

In vivo the trigger for resumption of meiosis in the oocyte within the dominant follicle is the preovulatory surge of LH, which triggers GVBD and progression to metaphase II (Lonergan & Fair, 2016).

The follicular environment is responsible both for maintaining meiotic arrest of the oocyte at prophase I (germinal vesicle stage) and for resumption of meiosis. High levels of intraoocyte cAMP keep the oocyte in meiotic arrest by suppressing maturation promoting factor activity via stimulation of cAMP-dependent protein kinase A. The preovulatory gonadotrophin surge causes a drop in follicular and oocyte cGMP levels, leading to upregulated oocyte phosphodiesterase activity, which causes a fall in intraoocyte cAMP and meiotic resumption (Norris et al., 2009; Vaccari et al., 2009).

However, when the oocyte is removed from the follicle before the LH surge, as in IVM, spontaneous resumption of meiosis occurs (Pincus & Enzmann, 1935) before the completion of cytoplasmic maturation, and this compromises developmental competence (Sánchez & Smitz, 2012). Therefore, strategies to maintain meiotic arrest before initiating IVM have used to improve embryo development (Soares et al., 2017).

(ii) Cytoplasmic maturation

Typical oocytes submitted to IVM, although capable of high rates of nuclear maturation, have had insufficient time to undergo normal cytoplasmic maturation (Lonergan & Fair, 2016).

It has been hypothesized that by delaying spontaneous resumption of meiosis in vitro, continued mRNA and/or protein accumulation in the oocyte may enhance cytoplasmic maturation (Bilodeau-Goeseels, 2012). However, although it is possible to reversibly inhibit meiotic resumption, evidence for a positive effect on oocyte competence is relatively sparse.
Also, the addition of pharmacological compounds that allow prolong the oocyte maturation period to promote a longer interaction between the immature oocyte with adequately conditioned cumulus cells have reported promising results (Gilchrist, 2011).

Despite the attempts to recapitulate in vitro some of the events that occur naturally during oocyte maturation in vivo, during IVM oocytes may have neither the time nor the correct environment to complete the necessary changes required for subsequent successful development (Lonergan & Fair, 2016).

Those include relocation and modification of organelles, acquiring functional Ca2+ release mechanisms, capacity to decondense the chromatin of the fertilizing sperm (Eppig et al., 2008).

**Organelles modification during maturation:**

- **Oocyte mitochondria**

Maternal mitochondria play a key role in the ability of oocytes to be competent because the whole preimplantation period is sustained by mitochondria produced during oogenesis, and only when the embryo begins implantation, their production is resumed (Jansen, 2000). Organization and continued metabolic activity of mitochondria are necessary features of cytoplasmic maturation and resumption of meiosis (Cummins, 1998; Hyttel et al., 1986), affecting subsequent development after fertilization (Bavister, 2000).

- **Mitochondrial distribution**

In bovine oocytes, the major relocation of mitochondria occurs during in vitro maturation (IVM) and is influenced by hormones and energy substrates in the maturation medium (Bavister, 2000).

As shown in Figure 3, grade 1 and 2 oocytes (selected for IVM) show a peripheral uniform distribution of mitochondrial foci (A1), and a peripheral but weaker mitochondrial signal (A2) respectively, after recovery from the follicles. After maturation, grade 1 and 2 oocytes (B1, B2) show large clusters of mitochondria in the periphery (large arrows) but also foci of mitochondria in the more central cytoplasm (arrowheads).

In addition to oocyte quality, maturation conditions may affect the distribution of mitochondria.

Confocal studies revealed a higher incidence of mitochondrial clustering in the cytoplasmic periphery of oocytes matured in vitro in standard maturation medium (TCM199 with serum) and chemically defined medium containing glucose and lactate, whereas mitochondria of oocytes matured in chemically defined poor medium containing glucose and lactate, often appear homogeneously distributed (Krisher & Bavister, 1998).
- Metabolic activity

The energy status, i.e., ATP content, of oocytes is critical for their maturation and has been suggested as an indicator for the developmental potential in human (Steeves & Gardner, 1999) and mouse oocytes (Leese et al., 1984). In bovine, the mitochondria existing within the oocyte must provide adequate ATP for successful maturation of the cytoplasm and nucleus in preparation for fertilization (May-Panloup et al., 2007; John et al., 2010), and to fuel the first few days of embryonic development (Silva et al., 2011; Tamassia et al., 2004). Embryos with less ATP in the cytoplasm had slower development and resulted in a smaller number of cells (Liu et al., 2000; Stojkovic et al., 2001).

Increasing evidence shows the role of mitochondria as determinants of developmental competence for mammalian oocytes (Eichenlaub-Ritter et al., 2011; Ramalho-Santos et al., 2009; Van Blerkom et al., 2002), and are involved in ATP synthesis, reactive oxygen species (ROS) production, calcium signalling, and apoptosis (Ramalho-Santos et al., 2009).

- Maternal mitochondrial transcripts

The oocyte has the largest number of mitochondria and mtDNA copies of any cell (May-Panloup et al., 2007). Evidence from heteroplasmic murine and human oocytes, showed that the segregation of mtDNA variants occurs as early as during the first mitotic divisions of germinal cell precursors and that these variants are then rapidly transmitted to future generations (Ferreira et al., 2009). Thus, maternal transcripts deposited in the oocyte during oogenesis affects mitochondria events. For example, mouse oocytes lacking maternal-effect genes such as NLR family pyrin domain containing 5 (NLRP5), peptidylarginine deiminase 6 (PADI6), or heat shock transcription factor 1 (HSF1) showed altered redox homeostasis that can lead to altered mitochondrial functions due to elevated ROS levels (Bierkamp et al., 2010; Fernandes et al., 2012; Kan et al., 2011).

Mitochondrial dysfunctions or abnormalities may compromise developmental processes by inducing chromosomal segregation disorders, maturation and fertilization failures, or oocyte/embryo fragmentation resulting in mitochondria-driven apoptosis.

- Oocyte cortical granules

Ultrastructural investigations in bovine oocytes have indicated that cortical granules (CGs), change significantly during meiotic maturation (Hyttel et al., 1986; Assay et al, 1994). Such granules are, in fact, small vesicles that contain enzymes. During the resumption of meiosis, the CG migrate from the Golgi apparatus close to the vitelline surface, assuming a position 0.4–0.6 μm below the plasma membrane (Ducibella & Buetow, 1994). Only when situated just beneath the plasma membrane can they undergo exocytosis by fusing with the oocyte membrane. This fusion enables release of the CG contents into the perivitelline space, an important step in membranous maturation and in instigating a block to polyspermy (Hosoe & Shioya, 1997; Szollosi, 1967; Wang et al., 1997). The exocytosis of cortical granules (cortical reaction) is one of the most common mechanisms used by the oocyte to prevent polyspermy. If fertilization with more than one spermatozoon occurs, the resulting zygote will undergo abnormal cleavage and will become non-viable, eventually degenerating at the beginning of mitotic divisions (Ferreira et al., 2009).
Cortical granules distribution

CGs were found to be densely distributed as irregular aggregates of particles throughout the peripheral cytoplasm immediately after collection. And as oocyte maturation proceeded, the aggregated CGs collapsed and dispersed in the cortex of the cytoplasm (see Figure 4) (Hosoe & Shioya, 1997).

Grade 1 and 2 oocytes (selected for IVM) show similar ultrastructural appearance of in vivo oocytes after maturation (Kruip et al., 1983; de Loos et al., 1989; Hyttel et al., 1986) exhibiting a wide distribution of CGs (Hosoe & Shioya, 1997).

Dispersion of CGs (type III) is regarded as being essential for the proper course of oocyte maturation (Sathananthan & Trounson, 1982).

Type II oocytes showed to be protected against polyspermy but may have been immature in some other cytoplasmic factor. Thus, oocytes type I, metaphase II may not have developed to the blastocyst stage (Hosoe & Shioya, 1997a).

Improvements of maturation culture conditions may enable dispersion of CGs in all oocytes and would allow developing more to blastocyst stage.

(iii) Molecular maturation

Molecular maturation consists of transcription, storage and processing of maternal mRNA, which is stored in a stable, inactive form until translational recruitment (Ferreira et al., 2009). The maternal mRNA population is highly diverse and supports a range of different functions during oocyte maturation and after fertilization, such as pronuclear formation and fusion (Philipps et al., 2008), the first cell division (Tang et al., 2007), embryonic gene transcription (Bultman et al., 2006) and cleavage-stage embryogenesis (Ma et al., 2006).

It has been hypothesized that the quality of an oocyte is based on the presence of the appropriate set of mRNA and proteins stored during folliculogenesis (Wrenzycki et al., 2007). A defined oocyte-specific gene expression pattern arising during folliculogenesis is crucial for the acquisition of oocyte developmental competence; conversely, deficiencies in gene expression or dynamics that occur during follicle development may be linked to impaired oocyte competence (Eichenlaub-Ritter et al., 2006).
While maternal mRNA population supports a range of different functions in early stages, the expression of embryonic messages gradually increases during the progression of embryonic development from the zygotic stage to the blastocyst stage and the major onset of embryonic genome activation (EGA) occurs at a species-specific stage of 8-cell (Memili & First, 2000).

Lonergan (2003) had found differences in the relative abundance of polyadenylated mRNA between in-vivo and in-vitro matured oocytes. Generally, oocytes destined to IVM may not have received sufficient exposure to hormones and growth factors in vivo to have the resulting accumulation of maternal mRNAs to develop well in vitro (Dieleman et al., 2002). In addition, maturing oocytes are essentially transcriptional quiescent and RNA pools are regulated by posttranscriptional modifications, such as poly-A tail elongation (Assou et al., 2006; Eichenlaub-Ritter et al., 2002). It has been shown that a shorter poly-A tail is correlated with low developmental competence indicating the importance of adenylation and deadenylation processes during in vitro maturation of bovine oocytes (Brevini et al., 2007).

Gene expression profiling of in vivo- and in vitro-matured bovine oocytes can identify transcripts related to their developmental potential. Adona et al., (2016) suggested that dysfunctional gene expression is not random and mostly affect the metabolism of oocytes. Some of the most important genes related with metabolism are the glucose transporter (GLUT1) (Augustin et al., 2001); two enzymes involved in anaerobic glycolysis [Glyceraldehyde-3-Phosphate Dehydrogenase: GAPDH (You et al., 2012) and Lactate Dehydrogenase A: LDHA (Valckx et al., 2015)]; and the enzyme that catalyses the first and irreversible step of the pentose phosphate pathway [Glucose-6-Phosphate Dehydrogenase: G6PD (Guerin et al., 2001)]. In addition, those associated with oxidative response are manganese superoxide dismutase (MNSOD) related to mitochondrial activity and glutathione peroxidase (GPX1) (Farin et al., 2001) which protects the organism from oxidative damage. Insulin-like growth factors have showed to play a major role in the autocrine and paracrine regulation of folliculogenesis. Insulin Like Growth Factor 2 Receptor (IGF2R) is a negative regulator of excess levels of IGF2 (Pantaleon et al., 2003), and has been showed that higher expression in mature oocytes displayed higher embryo developmental rates in bovine (Warzych et al., 2007).

Biase et al., (2014) by comparing the transcriptome of oocytes that sustained embryo development to blastocyst stage in vitro with those that arrested development before blastula formation, associated specific gene products in the oocyte to its ability to undergo normal development. They reported over expression of Serine/threonine-protein phosphatase 2A regulatory subunit A beta (PPP2R1B) implicated in the negative control of cell growth and division; Discoidin domain receptor family member 1 (DDR1) involve in the regulation of cell growth, differentiation and metabolism; and IGF2R in competent oocytes. Furthermore, Bermejo-Álvarez et al., (2010) reported that Cyclin B1 (CNB1), the protein encoded by this gene is a regulatory protein involved in mitosis related with developmental competence in goats (Anguita et al., 2008); Prostaglandin-endoperoxide synthase 2 (PTGS2), also known as cyclooxygenase, key enzyme in prostaglandin biosynthesis; and Gremlin 1 (GREM1), which encodes a member of the BMPs (bone morphogenic proteins) antagonist family, were related with oocyte competence.
**Environment and maturation medium**

In vivo the oocytes are contained in the follicular antrum, formed early in folliculogenesis. This antrum is filled with follicular fluid derived from the bloodstream and from the components secreted by somatic cells inside the follicle (Hennet & Combelles, 2012). Follicular fluid (FF) contains a variety of proteins, cytokine/growth factors and other peptide hormones, steroids, energy metabolites and other undefined factors (Leroy et al., 2012; Van Hoeck et al., 2013). The composition of follicular fluid varies between follicles and depends on their size and structure (Orsi et al., 2005). Despite several experimental studies, it is still not clear what constitutes a follicular environment that allows oocytes to develop successfully (Van Blerkom, 1998). Furthermore, oocyte-secreted factors regulate cumulus cell function and developmental competence of the oocyte and subsequently the composition of the follicular fluid (Gilchrist et al., 2008).

During IVM experimental evidence in bovine demonstrated that the medium, its supplements, and other factors (e.g. the oxygen atmosphere) used for oocyte maturation can influence mRNA expression and development of the resultant embryos (Russell et al., 2006; Wrenzycki et al., 2007; Hashimoto, 2009). Oocytes are normally matured between 22 and 24 hours, shorter or longer periods were associated with reduced development (Ward et al., 2002) in a suitable environment with 38.5°C, 5% CO₂ in air (~20% oxygen) in a humidified atmosphere and saturated humidity (Gordon, 2003).

Despite the lower oxygen tension in the follicular fluid (McNatty, 1978), IVM is generally conducted under atmospheric oxygen tension in both humans and bovine. Although low oxygen concentration (5%) is more similar to in vivo conditions, considerably increases the cost of IVM and was shown to have detrimental effects on maturation rates (Pinyopummintr & Bavister, 1995). It is well known that in the female genital tract the oxygen concentration is 40% or even less than that in the atmospheric air (<8%), (Mastroianni & Jones, 1965; Fischer & Bavister, 1993). High oxygen tension triggers oxidation, which is manifested by the production of ROS.

ROS are usually free radicals containing one or more unpaired electrons, the most common include anion superoxide radical (O₂⁻) hydrogen peroxide (H₂O₂), hydroxyl radical (·OH) and peroxinitrate (Forman & Fisher, 1981; Fridovich & Porter, 1981). High oxygen tension during IVP favours the formation and accumulation of ROS, which could inversely affect both gametes and the early embryo (Agarwal et al., 2005; Yanagisawa et al., 1990). Damages caused by increased ROS production include cell membrane damage, mitochondrial dysfunction, RNA damage and cytoskeleton alterations (Fruehauf & Meyskens, 2007; Yanagisawa et al., 1990).

On the other hand, it has been reported that ROS at well balanced levels might be beneficial for oocyte maturation by modulating the mitogen activated protein kinase (MAPK), and maturation promoting factor (MPF) (Fissore et al., 1996). It has been also shown that certain low amount of ROS induces sperm hyperactivation, capacitation, acrosome reaction and sperm–oocyte fusion, which finally enhances fertilization rate in vitro (de Lamirande et al., 1997). Within the cell, redox, i.e. the balance between oxidizing and reducing species is achieved by various enzyme systems that neutralize or scavenge toxic oxidants, such as ROS (Fruehauf & Meyskens, 2007).

To eliminate the side effects of ROS during the IVP, two major approaches have been employed; firstly, oxygen concentration, especially in embryo culture, has been reduced up to 5%, and
secondly, various antioxidant compounds have been used (Takahashi, 2012; Sovernigo et al., 2017).

Medium composition

The widest media use for IVM is tissue culture medium 199 (TCM199) supplemented with:

(I) Protein source such as bovine serum albumin and serum. This kind of media are semi-defined, by the addition of biologicals components as foetal calf serum (FCS) and BSA which composition varies between batches and is not fully known (Gordon, 2003).

(II) Hormones such as gonadotropins (FSH, LH), steroids and growth factors (e.g., epidermal growth factors) (Lonergan & Fair, 2016). The gonadotropins FSH and LH are supplemented to induce cumulus cell expansion, nuclear and cytoplasmic maturation (Machado et al., 2015; Zuelke & Brackett, 1990). Steroid hormones also play an important role in vivo and in vitro. In mammalian preovulatory follicles, the LH surge initially stimulates the secretion of both androgens and oestrogens (Dieleman et al., 1983). Oestrogen concentrations decrease 6 h after the LH surge, followed by a decrease in androgens and an increase in progesterone concentration. By 18 h after the LH surge, progesterone constitutes 90% of intrafollicular steroid content (Osborn & Moor, 1983). It has been shown that cumulus cells of bovine COCs are able to secrete estradiol and progesterone in culture for in vitro maturation, and this steroidogenesis is modulated by the steroids progesterone, testosterone and estradiol.

(III) Antioxidants, most of these substances are endogenous, such as various vitamins (retinols and retinoids, a-tocopherol and derivatives, ascorbic acid), glutathione and other thiols (Agarwal et al., 2005; Dalvit et al., 2005; Wang et al., 2002), which apart from their antioxidant properties they could differentially influence the maturation process of the oocyte and/or the developmental competence of the embryo. Blondin el al. (1997) showed that during IVM, controlled levels of some less harmful ROS such as superoxide radicals induced by hypoxanthine–xanthine system, may have some beneficial effects on oocytes as they might remain developmentally more competent. Sovernigo et al. (2017) assessed different antioxidants to reduce the harmful effects of ROS on the in IVP and found that the supplementation of the maturation medium with quercetin, vitamin C or resveratrol decreased ROS levels in oocytes.

The evidence suggests that the use of antioxidants during IVM may reduce oxidative stress either by decreasing ROS levels directly or by increasing glutathione (GSH) levels in oocytes (Jeong & Joo, 2016; Wang et al., 2014) which in bovine oocytes acts as the main non-enzymatic defence system to reduce ROS levels (Deleuze & Goudet, 2010; Rocha-Frigoni et al., 2016).

Polymethoxylated flavones (PMFs)

Flavonoids are a family of phytochemicals that exhibit a broad spectrum of pharmacological properties (Middleton et al., 2000; Manthey & Grohmann, 2001). The highest concentration of PMFs is found in the citrus peel with much lower amounts found in the juice (Kanes et al., 1993; Rouseff & Ting, 1979). Tangerine and nobiletin are two polymethoxylated flavones (PMFs) that are relatively common in citrus and both are present in sweet orange peel.

Citrus fruit-derived flavonoids and their metabolites have been shown to impart significant protective biological activities including anticancer, antiviral, anti-inflammatory and antiatherogenic activities (Manthey & Grohmann, 2001; Middleton et al., 2000; Whitman et al., 2005).
Naringenin, has been shown that can support the trophectoderm migration in porcine embryos, crucial for orchestrating conceptus-uterine interactions during the peri-implantation stage of pregnancy (Lim & Song, 2016).

**Nobiletin**

Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), was first identified from the peel of citrus fruits, belongs to polymethoxyflavones (PMFs) and along with other members of the family has been reported to have a broad range of biological effects. Those include anti-inflammatory, anticarcinogenic, and antiatherogenic properties (Wang et al., 2008). For instance, it has been shown that PMFs can downregulate gene expression of some proinflammatory cytokines such as interleukins (IL-1α, IL-1β, and IL-6) and tumour necrosis factor-a in mouse macrophages (Whitman & Daugherty, 2005). Furthermore, nobiletin acts as an anti-carcinogenic compound through anti-proliferative activity, induction of apoptosis and cell cycle deregulation (Yoshimizu et al., 2004), and was shown to have anti-proliferative activity in human lung cancer (Luo et al., 2008) and colon cancer in mice (Miyamoto et al., 2008). In neurobiology, nobiletin has demonstrated a positive effect in rescuing memory deterioration in the Alzheimer’s disease rat model (Matsuzaki et al., 2006) and olfactory-bullectomized mice, as well as improved prevention of memory impairment in the Alzheimer’s disease rat model (Onozuka et al., 2008).

Lam et al., (2011) identified for the first time that nobiletin has potent anti-angiogenic activity in vivo in zebrafish embryos and in vitro in human endothelial cells through regulating cell cycle arrest and the vascular endothelial growth factor (VEGF) pathway.

It has been demonstrated that Nobiletin has an evident effect in airway inflammation (Wu et al., 2006), and as an inhibitor of ROS production (Choi et al., 2007).

When oocytes or embryos are cultured in vitro, they are exposed to manipulation, light temperature, medium constituents, sperm and O2 concentration, which favour increase in ROS and may lead to downregulated of their defence mechanism (Guerin et al., 2001; Yu et al., 2014). This increase of ROS and decreased intracellular GSH pools (Hashimoto et al., 2000) in the oocytes have adverse effects on subsequent embryo development.

Thus, the gaseous environment to which the oocyte is exposed varies considerably between in vitro and in vivo conditions (Wrenzycki & Stinshoff, 2013). In addition, the female reproductive system is naturally rich in antioxidants such as catalase, glutathione, among others (Carbone et al., 2003), and it is considerably different from the synthetic medium used for in vitro culture of oocytes and embryos. Therefore, the use of substances with antioxidant properties during the in vitro production of embryos may avoid the excessive increase in ROS and improve the embryo production efficiency (de Matos et al., 2002; Kere et al., 2013).

Oxidative stress is a major cause of low efficiency in oocyte maturation and embryo development in several species (Luberda, 2005). Balanced ROS act beneficially as signalling molecules in physiological processes (Agarwal et al., 2008). However, excessive amounts of ROS have damaging effects on cells and lead to cell change and death (Agarwal et al., 2005).

Based on the above, we hypothesize that the addition of nobiletin as an antioxidant during IVM may improve the conditions in which oocytes mature and providing a better oxidative stress.
response and allowing them to consume critical points during cytoplasmic maturation such as organelle relocation.

4.2.2 In vitro fertilization (IVF)

The IVF is designed to facilitate the union of the gametes. In this step, the most important factor is to make a good selection of sperm to remove nonmotile sperm cells and unwanted components of semen. There are several products and protocols to do so: the swim-up (Parrish et al. 1986), and gradients such as Percoll (Saeki et al., 1991) 12 and BoviPure™. The Percoll and BoviPure™ have similar results regarding the final rate of blastocysts, but the advantage of BoviPure™ is that it is less toxic (Samardzija et al., 2006). After selection, the sperm concentration is calculated, and the proper quantity of sperm is added to the fertilization medium to have a final concentration of 1x10⁶ sperm/ml. The IVF medium have a specific ionic balance for oocyte and sperm requirements, the most commonly used is the TALP (Bavister & Yanagimachi, 1977), a modification of the Tyrode that contains sodium bicarbonate, albumin as a source of protein and lactate and pyruvate as an energy source (Gordon, 2003). Heparin is also added to the medium to induce sperm capacitation (Parrish et al., 1986) and prepare it for the acrosome reaction and fertilization (Parrish et al., 1985). The selected spermatozoa and matured oocytes co-cultured for 18-22 hours under the same conditions as maturation: 38.5 °C, 5% CO₂ and saturated humidity (Gordon, 2003). During this period spermatozoa cross all physiological barriers, fuse with the ooplasm, the oocyte is activated, and the pronuclear formation begins (Galli et al., 2003).

4.2.3 In vitro culture (IVC)

While oocyte origin is the main factor affecting the rate of blastocysts, the factors that most influence the quality of the embryos are the conditions after fertilization (Rizos et al., 2002). Some of the main factors affecting the development and the embryo quality are: the culture medium, the number of embryos in culture (ratio: embryo/µl of culture medium), the temperature and the gas balance in the incubator (Loneran et al., 2006). The most used media for the culture of presumptive zygotes/embryos is synthetic oviductal fluid (SOF) which is usually supplemented with 5% of FCS (foetal calf serum) and/or BSA (bovine albumin serum) (Holm et al., 1999; Tervit et al., 1972). Both have pros and cons; their use increases the final percentage of blastocysts compared to serum free media. However, clear evidence has shown that serum, affects negatively the embryo quality compared to the once produced with albumin in a concentration of 3mg/ml (Rizos et al., 2002). The addition of FCS to the culture media has also been linked to the large offspring syndrome, which is characterized by large calves that can cause dystocia and have a high postnatal mortality due to defects in many organs (Young et al., 1998; Lazzari et al., 2002); which represents an important economic loss to the farmer. Beside the defined or semi-defined media, the embryos can also be cultured with oviductal, granulosa or Vero cells; or with conditioned media (Rizos et al., 2017; Mermillod et al., 2010). The conditioned media is the result of the co-culture of the media with somatic cells. This co-culture systems are supposed to help overcoming the embryonic blockage that happens in the stage of 8-16 cells (Rizos et al., 2017; Vansteenbrugge et al., 1994). In Lopera-Vasquez et al., (2016) provided evidence that extracellular vesicles (EVs) isolated from the conditioned medium of an
extended culture BOEC monolayer improve embryo quality and induce cryoprotection in *in vitro* cultures. Also, has been reported that embryo development was not affected by the presence of oviductal fluid-extra cellular vesicles (OF-EV), but the quality of the produced embryos in terms of cryotolerance and the expression of genes related to metabolism and epigenetics were improved (Lopera-Vasquez et al., 2017). In the same line, to optimise the bovine embryo IVC, supplementation of the post-fertilisation culture medium with oviductal fluid (OF) and uterine fluid (UF) has been used, reporting that a low concentration of OF and/or UF supports embryo development and improves embryo quality (Hamdi et al., 2018).
5. **Motivation and objective of the project**

Some of the differences between in vivo and in vitro matured oocytes have led to the identification of specific competency deficits. Therefore, this study proposed to assay the antioxidant role of Nobiletin to improve in vitro maturation (IVM) of bovine oocytes and determine the consequences in further embryo development.

6. **Experimental design**

Cumulus-oocyte complex in IVM medium were treated with different concentration of Nobiletin leading the following experimental groups:

- **Control group (C):** TCM199 + 10% FCS + 10 ng/ml EGF.
- **Grupo DMSO (CDMSO):** Medio TCM199 + 10% FCS + 10 ng/ml EGF. Supplemented with 0.001% DMSO (as a Nobiletin vehicle).
- **Group 10 (N10):** TCM199 + 10% FCS + 10 ng/ml EGF. Supplemented with Nobiletin to a final concentration of 10 μM.
- **Group 25 (N25):** TCM199 + 10% FCS + 10 ng/ml EGF Supplemented with Nobiletin to a final concentration of 25 μM.
- **Group 50 (N50):** TCM199 + 10% FCS + 10 ng/ml EGF. Supplemented with Nobiletin to a final concentration of 50 μM.
- **Group 100 (N100):** TCM199 + 10% FCS + 10 ng/ml EGF. Supplemented with Nobiletin to a final concentration of 100 μM.

The concentration of Nobiletin was based on the findings of other studies in which these polymethoxylated flavonoid from citrus fruits, in vivo in zebrafish embryos and in vitro in human umbilical vein endothelial, showed an anti-angiogenic activity at concentrations between 30 and 100μM (Qu et al., 2018).

After 24 h of IVM part of the oocytes were denudes from CCs and fixed for measuring nuclear and cytoplasmic maturation, ROS and GSH levels and gene expression, while the reminding was in vitro fertilized and culture to the blastocyst stage. The factors examined in all groups were:

(1) **Oocyte nuclear maturation**: percentage of oocytes that reach meiotic metaphase II after 24 h of IVM.

(2) **Oocyte cytoplasmic maturation**: mitochondria and cortical granules were stain after 24 h of IVM. The organelle distribution was used as a cytoplasmic maturation parameter.

(3) **Oocyte ROS and GSH levels**: the levels of intracellular GSH and ROS were assessed by fluorescence intensity after 24 h of IVM as oxidative stress parameters.

(4) **Embryo development**: cleavage rate (48 h post IVF) and blastocyst yield at day 7 and 8 post IVF.

Oocytes and their cumulus cells (24 h IVM) along with blastocyst from day 7 and 8, were frozen in liquid N2 and stored at −80°C for:

(5) **Oocyte and cumulus cell (CC) gene expression (RT-qPCR).**

(6) **Blastocyst gene expression (RT-qPCR).**
7. Materials and methods

7.1 In vitro embryo production

a) Oocyte collection and IVM

Immature COCs were obtained by aspirating follicles (2–8 mm diameter) from the ovaries of mature crossbred heifers (i.e. at least one corpus luteum or remained scars from previous ovulations in one or both ovaries) collected at slaughter from local abattoirs.

Class 1 and class 2 COCs (homogeneous cytoplasm and intact CCs) were matured for 24 h in 500 µL of maturation medium, TCM 199 supplemented with 10% (v/v) foetal calf serum (FCS) and 10 ng/ml epidermal growth factor in four-well dishes, in groups of 50 COCs per well at 38.5°C under an atmosphere of 5% CO$_2$ in air, with maximum humidity (Lopera-Vasquez et al., 2016).

Each experiment consisted of six groups of around 100 COCs, in accordance to each treatment: (I) no treatment (control); (II) dimethyl sulfoxide (control DMSO, 0.01% DMSO as Nobiletin vehicle); (III) 10 µM Nobiletin, (Medical Chemical Express, MCE); solubilized in DMSO to obtain a stock solution 70mM; (IV) 25 µM Nobiletin; (V) 50 µM Nobiletin and (VI) 100 µM Nobiletin.

COCs matured under different conditions were employed to evaluate: nuclear maturation, cortical granules (CGs) distribution patterns, mitochondria (Mt) distribution patterns, gene expression in oocytes and CCs, as well as levels of ROS and GSH in oocytes after in vitro maturation (IVM), developmental competence after in vitro fertilization and culture.

From each experimental group, after maturation, 10 oocytes and their respective CC´s were used to study gene expression analysis. Other 10 oocytes were employed for nuclear maturation and CG distribution, 10 oocytes for mitochondria distribution, 10 for ROS and 10 for glutathione. The remains were destined to in vitro fertilization and posterior embryo development. At least 6-8 replicates of each experiment were performed.

b) Sperm preparation and IVF

For in vitro fertilization, COCs were washed in fertilization medium before being transferred in groups of 50 into four well dishes containing 250 µl of Tyrode’s fertilization medium composed by 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate and 6 mg/mL fatty acid-free bovine serum albumin (BSA) supplemented with 10 mg/mL heparin sodium salt (Calbiochem) (Lopera-Vasquez et al., 2016).

Motile spermatozoa were obtained from frozen semen straws (0.25 mL) thawed at 37°C in a water bath for 1 min and centrifuged for 10 min at 280 xg through a gradient of 1 mL of 40% and 1 mL of 80% Bovipure (Nidacon Laboratories AB, Göthenborg, Sweden Bovipure) 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 xg for 5 min. The pellet was re-suspended in the remaining 300 µL of Boviwash. Spermatozoa were counted and diluted in the appropriate volume of fertilization medium to give a concentration of 2x10$^6$ spermatozoa/ml. A 250 ml aliquot of this suspension was added to each fertilization well to obtain a final concentration of 1x10$^6$ spermatozoa/ml. Gametes were co-incubated for 18–22 h at 38.5°C under an atmosphere of 5% CO$_2$ in air and maximum humidity.
c) **In vitro culture of presumptive zygotes**

At approximately 20 h post-insemination (hpi), presumptive zygotes were denuded of CCs by vortex for 3 min and then cultured in groups of 25 in 25 µL droplets SOF containing 4.2 mM sodium lactate (L4263), 0.73 mM sodium pyruvate (P4562) 30 µL/mL BME amino acids (B6766), 10 µL/ml minimum essential medium (MEM) amino acids (M7145) and 1 µg/ml phenol red (P0290). under mineral oil at 38.5°C under an atmosphere of 5% CO₂, 5% O₂ and 90% N₂, as the embryo culture is routinely (Lopera-Vasquez et al., 2016). SOF was supplemented with 5% FCS.

7.2 Embryo development

Cleavage rates were recorded on Day 2 (48 hpi) and cumulative blastocyst yields on Days 7 and 8 post-insemination under a stereomicroscope. At least 6-8 replicates of each experiment were performed.

7.3 Oocyte quality assays

i. Nuclear maturation and cortical granules distribution patterns

Nuclear maturation and CGs distribution, as one parameter of cytoplasmic oocyte maturation, were assessed by confocal microscopy (Coy et al., 2005). Briefly, first in vitro matured COCs from each treatment (≅ 50) were suspended in 2000 µL of phosphate-buffered saline (PBS) without calcium or magnesium supplemented with 0.3% BSA and hyaluronidase, and their CCs were removed by vortex. Next, oocytes were treated with 0.5% w/v pronase in PBS to digest the zona pellucida. Zona-free oocytes were washed in PBS three times and fixed in 3.7% w/v buffered neutral paraformaldehyde solution (pH 7.2–7.4) for 30 min at room temperature and treated with permeabilization solution (0.2% v/v Triton X-100 in PBS) for 10 min. The oocytes were washed in PBS without calcium or magnesium supplemented with 0.3% BSA and incubated in 100 µg/mL FITC-LCA (Vector Laboratories, Burlingame, USA) for 30 min in a dark chamber. After staining, oocytes were washed, mounted in 3.8 µl of mounting medium (50% v/v PBS, 50% v/v glycerol (Sigma G-S150), 0.0025 µg/mL Hoëchst) between a coverslip and a glass slide and 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 whose detection spectrum is 515–530 nm. Nuclear maturation was observed in an inverted epifluorescence microscope (Nikon Eclipse TE 300) equipped with a DA-U3 Digital Camera, and UV-1 filter. For the nuclear maturation, all the nucleus and polar bodies were evaluated, oocytes were classified as follows: 0: GV germinal vesicle stage (nucleus well defined); 1: GVBD; 2: Metaphase I (first metaphasic plate visible); 3: Metaphase II (nucleus mature, represented by the presence of first polar body, or second metaphasic plate). Those having a metaphase plate and the first polar body were classified as metaphase II stage and were considered mature. As a parameter of cytoplasmic maturation, CGs were analysed and the distribution of cortical granules was classified as three types (type I, distributed in clusters or no migrated; type II, dispersed and partly clustered or partially migrated; and type III, small CG arranged at the periphery or migrated)(Hosoe & Shioya, 1997b). At least 5 replicates were performed.

ii. Mitochondrial distribution patterns

Mitochondrial distribution patterns as parameter of cytoplasmic oocyte maturation, were assessed by confocal microscopy. Briefly, first matured COCs from each treatment (≅ 50) were suspended in 2000 µL of phosphate-buffered saline (PBS) without calcium or magnesium supplemented with 0.3% BSA and hyaluronidase, and their CCs were removed by vortex. Next, oocytes were equilibrated for 15 minutes in MIV supplemented with 5% FCS and then incubated for 30 minutes in MitoTracker DeepRed (Molecular Probes, Eugene, USA). Oocytes were fixed in
3.7% w/v buffered neutral paraformaldehyde solution (pH 7.2–7.4) for 60 min at room temperature and darkness. After staining, oocytes were washed, mounted in 3.8 μL of mounting medium (50% v/v PBS, 50% v/v glycerol (Sigma G-S150), 0.0025 μg/mL Hoëchst) between a coverslip and a glass slide and sealed with nail polish. Slides were examined using a laser-scanning confocal microscope (Leica TCS SP2) equipped with an argon laser excited at 644 nm and whose detection spectrum is 665 nm. Mitochondrial patterns were analysed, and the distribution was classified as three types (type 0, no migrated; type I, partially migrated; and type II, migrated). At least 5 replicates were performed.

iii. Levels of reactive oxygen species (ROS) and Glutathione (GSH)

Matured oocytes (≅ 50) from each treatment were denuded, for evaluation of ROS and GSH. The oocytes were fixed with 3.7% formaldehyde for 15 minutes and incubated in CellROX Deep Red Reagent (Invitrogen, Eugene, USA) at a final concentration of 5 μM, and CellTracker™ Blue (Invitrogen, Eugene, USA) at final concentration of 10 μM, for 30 min at 37°C. After staining oocytes were washed 3 times with PBS and mounted in 3.8 μl of mounting medium (50% v/v PBS, 50% v/v glycerol (Sigma G-S150), 0.0025 μg/mL Hoëchst) between a coverslip and a glass slide and sealed with nail polish. Fluorescence emitted from the oocytes was captured using B-2E/C (ROS) and UV-2A (GSH) filters ten seconds after exposure to UV light. Samples were examined with an inverted epifluorescence microscope (Nikon Eclipse TE 300) equipped with a DA-U3 Digital Camera. The fluorescence intensities expressed were analysed in arbitrary fluorescence units (pixel) (Rocha-Frigoni et al., 2016) using the IMAGE J software. At least 5 replicates were performed.

7.4 Oocytes and cumulus cells for gene expression analysis

After 24 h of IVM, pools of 10 COCs (≅ 50) were collected from each treatment group and CCs physically separated from oocytes by gentle pipetting with hyaluronidase and vortex 2 min in PBS. Oocytes, in pools of 10 per treatment group, were washed in PBS, snap frozen in liquid N2 and stored at −80°C until mRNA extraction. Their corresponding CCs were also washed in PBS, centrifuged at 10,000 x g and then snap frozen in liquid N2 and stored at −80°C until mRNA extraction. At least 5 replicates were performed.

7.5 Statistical Analysis

Statistical analysis was performed using Sigma Stat (Jandel Scientific, San Rafael, CA, USA). Data for IVC (cleavage rates and blastocyst yield), as well as nuclear maturation, CG distribution patterns and mitochondrial distribution patterns were compared by one-way analysis of variance (ANOVA). When a statistically significant difference (P = <0.001) in the mean values among the treatment groups was detected, Tukey’s test was performed to determine whether the groups showed significant differences. Values were considered significantly different at P<0.05. As results from ROS and GSH fluorescens failed normality test, pairwise multiple comparison procedures were performed by Dunn’s method. Values were considered significantly different at P<0.05. Unless otherwise indicated, data are presented as the mean±s.e.m.
8. Results

8.1 Nuclear maturation

Significantly higher percentage of matured oocytes (P<0.05) were observed in metaphase II when N25 (87±0.6%) or N50 (89.3±0.3%) were added to the IVM medium compared to N10 (72.9±0.3%), N100 (71.5±0.8%), Control (71.7±0.7%) and CDMSO (70.5±0.5%) groups (Figure 5).

![Figure 5. Percentages of bovine oocytes that reach Metaphase II after 24 h of IVM with or without Nobiletin. Data are the mean±s.e.m. performed in 5 replicates. Different letters above columns indicate significant difference (P<0.05) between treatments.](image-url)
8.2 Organelle Relocation

N25 and N50 showed higher rate of oocytes with peripheral migration of cortical granules (85.7±0.3% and 89.9±2.2% respectively) and mitochondria (86.7±0.6% and 88.9±1.2% respectively) compared to the remaining groups (P<0.05) (Figure 6). Mitochondrial and cortical granules distribution patterns in bovine oocytes after 24 h of IVM for all experimental groups is showed on Figures 7 and 8 respectively.

![Figure 6. Percentages of mitochondrial (red) and cortical granules (green) migration in bovine oocytes after 24 h of IVM with or without Nobiletin. Data are the mean±s.e.m. performed in 5 replicates. Different letters above columns indicate significant difference (P<0.05) between treatments for mitochondrial and cortical granules migration.]
Figure 7. Representative fluorescent images of mitochondrial distribution patterns in bovine oocytes after 24 h of IVM with or without Nobiletin- (A) Migrated oocytes: show large clusters of mitochondria in the periphery; (B) Partially migrated oocytes: show clusters of mitochondria in the periphery, but also foci of mitochondria in the more central cytoplasm; (C) Non-migrated oocytes: show a peripheral uniform distribution of mitochondrial. In each category can be observed the differences in the fluorescens emissions between treatments (1. Control; 2. CDMSO; 3. N10; 4. N25; 5. N50; and N100). Scale bar 50 µm.
Figure 8. Representative fluorescent images of cortical granules distribution patterns in bovine oocytes after 24 h of IVM with or without Nobiletin - (A) Migrated oocytes: show high periphery migration of the cortical granules; (B) Partially migrated oocytes: cortical granules individually dispersed; (C) Non-migrated oocytes: uniform distribution cortical granules. In each category can be observed the differences in the fluorescens emissions between treatments (1. Control; 2. CDMSO; 3. N10; 4. N25; 5. N50; and N100). Scale bar 50 µm.
8.3 Oxidative stress

The results expressed in arbitrary fluorescence units showed that the supplementation of N25 and N50 presented a significant reduction (P<0.05) in the ROS (2.53±0.8 a.u. ; 2.62±1.2 a.u. respectively), and GSH (2.84±0.4 a.u.; 3.09±0.1 a.u. respectively) content in comparison with all other groups (Figure 9). Fluorescens emissions of ROS (red) and GSH (blue) in bovine oocytes after 24 h of IVF for all experimental groups is showed in Figure 10.

**Figure 9.** Levels of ROS (red) and GSH (blue) fluorescence emissions in bovine oocytes after 24 h of IVM. Data are the mean±s.e.m. performed in 5 replicates. Different letters above columns indicate significant difference (P<0.05) between treatments for ROS and GSH.

**Figure 10.** Representative fluorescent images of ROS (red) and GSH (blue) in bovine oocytes after 24 h of IVM with or without Nobiletin (A, G: Control; B, H: CDMSO; C, I: N10; D, J: N25; E, K: N50; F, L: N100). Scale bar 50 µm.
8.4 Embryo development

As showed in Table 1, cleavage rate was significantly higher (P<0.05) for N25 (89.9±0.3%) and N50 (91.3±0.3%) compared to all other groups (N10: 75.6±0.3%; N100: 74.0±0.6%; Control: 74.2±0.4%; and CDMSO: 73.6±0.4%). Similarly, cumulative blastocyst yield at D8 was significantly higher (P<0.05) for N25 (32.1±0.8%) and N50 (35.5±0.8%) compared to N10 (23.1±0.7%), N100 (24.5±0.9%), Control (25.9±0.4%) and CDMSO (26.1±0.6%) groups (Table 1).

Table 1. Embryo development evaluation at 48 h post IVF (Cleavage) and blastocyst yield at day 7 and 8 of IVC. Effect of Nobiletin supplementation in bovine oocyte in vitro maturation on cleavage rate and blastocyst yield.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total no. presumptive zygotes in culture</th>
<th>Cleavage rate</th>
<th>Blastocyst yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>C</td>
<td>359</td>
<td>267</td>
<td>74.2±0.4bc</td>
</tr>
<tr>
<td>CDMSO</td>
<td>378</td>
<td>278</td>
<td>73.5±0.5b</td>
</tr>
<tr>
<td>N10</td>
<td>397</td>
<td>300</td>
<td>75.6±0.3c</td>
</tr>
<tr>
<td>N25</td>
<td>372</td>
<td>335</td>
<td>90.0±0.4a</td>
</tr>
<tr>
<td>N50</td>
<td>336</td>
<td>307</td>
<td>91.3±0.3a</td>
</tr>
<tr>
<td>N100</td>
<td>414</td>
<td>306</td>
<td>74.0±0.6bc</td>
</tr>
</tbody>
</table>

Data are the mean±s.e.m. performed in 6-8 replicates. Within columns, values with different superscript letters differ significantly (P<0.05).
9. Discussion

One of the greatest challenges for scientists across the world is to mimic in vivo conditions for in vitro assisted reproductive technologies. One of the main differences between in vitro and in vivo conditions is the level of oxidative stress, which is higher in the former than in the latter (Goto et al., 1993). Antioxidants and ROS are found in equilibrium in cells under normal physiological conditions, cells possess mechanisms to hinder excessive free radical formation including specific enzymes which control their intracellular levels (Liu et al., 2016). However, in certain situations in vivo (such as diseases, pathogens, etc.) or in vitro (such as excessive exposure to light, high oxygen tension, etc.), physiological mechanisms suffer disturbances in redox equilibrium with the increased production of free radicals (David et al., 2016; Guérin et al., 2001). Such increases may cause depletion of intracellular antioxidant concentrations (Kurutas, 2015; Liu et al., 2016; Wang et al., 2014).

During IVP of embryos, in which the entire process is done in vitro (IVM, IVF, IVC), the ROS production increases and results in decreased in vitro embryo development (Guérin et al., 2001; Yu et al., 2014). Thus, the physiology of oocytes can be protected through supplementation of antioxidants in the culture medium. Supplementation with quercetin, resveratrol or vitamin C during in vitro maturation act as a defence mechanism against ROS in porcine oocytes (Sovernigo et al., 2017). Other results reported a reduction in ROS levels associated with an increase in GSH levels in porcine and bovine oocytes (Kere et al., 2013; Kwak et al., 2012; Wang et al., 2014).

Antioxidants provide protection on premature aging of oocyte by the increased production of ROS (Agarwal & Majzoub, 2017). Premature aging of oocytes before MII stages are detrimental to oocyte nuclear and cytoplasmic maturation process, causes poor fertilization and retarded embryo development. Oxidative stress has showed to have contradictory results in the effect over meiotic progression, promoting or inhibiting germinal vesicle breakdown (Takami et al., 1999). On the other hand, negative effects have been reported in the assembly and function of the meiotic spindle, mitochondrial defects, apoptosis, and DNA integrity (Combelles et al., 2009).

Therefore, the addition of antioxidants to effectively control the losses in the in vitro embryo production due to oxidative stress is interesting for both research and commercial production purposes. Aiming to reduce the harmful effects of ROS on the in vitro maturation of bovine oocytes, the current study assessed the use of Nobiletin, a member of polymethoxylated flavones family, as an antioxidant agent. Flavonoids are most commonly known for their antioxidant activity in vitro (Bagchi et al., 1999). It has been reported that members of the family, Quercetin and Taxifolin, were effective in reducing ROS levels in mature porcine oocytes and the rate of blastocyst formation from treated oocytes (Kang et al., 2016).

Ours results show that intracellular ROS levels in in vitro matured oocytes were reduced by the supplementation of the IVM medium with Nobiletin. The levels of intracellular ROS were assessed by fluorescence intensity as oxidative stress parameters, finding a significant reduction (P<0.05) in the groups supplemented with 25 µM and 50 µM of Nobiletin (N25 2.53±0.8 a.u; N50 2.62±1.2 a.u. respectively). These results corroborate the findings of other studies in swine, in which oocytes matured in vitro with quercetin, vitamin C or resveratrol showed lower intracellular ROS levels (Kang et al., 2013; Kwak et al., 2012).
The synthesis of intracellular GSH is a critical part of oocyte cytoplasmic maturation (Eppig, 1996), and the presence of high levels of GSH in oocytes at the end of maturation is considered a biochemical marker for improved oocyte quality (de Matos et al., 1995) as this reservoir will protect the zygote and early embryo against oxidative damage before genomic activation and de novo GSH synthesis (Deleuze & Goudet, 2010). However, studies in porcine (Choi et al., 2013; Kere et al., 2013; Kwak et al., 2012), caprine (Mukherjee et al., 2014) and mice (Yu et al., 2014) oocytes matured in vitro showed no increase in GSH levels. On the contrary, Rocha-Frigoni et al., (2016) reported a depletion of intracellular GSH in mature bovine oocytes among all treated groups with antioxidants compared to immature oocytes. This is in agreement with our results observing a significant (P<0.05) decrease of GSH levels in 25 μM and 50 μM Nobiletin supplemented groups. Based on the above, the expression of GSH is not a clear marker to evaluate oocyte quality as the results are contradictory and depended to the antioxidant use.

Nuclear maturation rate (metaphase II) was not affected in oocytes treated with any antioxidant tested so far (Sovernigo et al., 2017; Kang et al., 2013; Kere et al., 2013; Mukherjee et al., 2014; Wu et al., 2011) suggesting that antioxidants do not affect nuclear maturation, even with reduced ROS levels (as with quercetin, vitamin C or resveratrol) or even with increased GSH levels (as observed with cysteamine or carnitine) (Sovernigo et al., 2017). However, we observed significantly higher percentage of matured oocytes (P<0.05) in metaphase II when 25 μM and 50 μM Nobiletin was supplemented (N25: 87±0.6%; N50: 89.3±0.3%) to the IVM medium.

Antioxidants supplementation during in vitro maturation is essential for improved cytoplasmic maturation, which is associated with the embryonic development competence of oocytes (Furnus et al., 2008; Dimitrios Rizos et al., 2002). In the present study, cytoplasmic maturation was evaluated in terms of organelle relocation. In the same line as nuclear maturation, oocytes matured with 25 μM and 50 μM Nobiletin supplemented medium showed to have significant higher incidence of mitochondrial clustering in the cytoplasmic periphery (N25 86.7±0.6% and N50 88.9±1.2%) and dispersion of cortical granules (N25 85.7±0.3% and N50 89.9±2.2) comparing to control and other Nobiletin supplemented groups. Both characteristics happen to be essential for the proper course of oocyte maturation (Krisher & Bavister, 1998; Sathanathan & Trounson, 1982).

Our study clearly showed also that supplementation of IVM medium with Nobiletin improves the embryo development. In agreement, Rocha-Frigioni et al., (2016) postulated that supplementation of IVM medium with cysteine, cysteamine and catalase antioxidants improves the mitochondrial membrane potential, the intracellular levels of ROS and GSH in the bovine oocytes at the end of maturation, and thereby affects the subsequent embryonic development. Here we reported a significantly higher cleavage rate for 25 μM and 50 μM Nobiletin supplemented in maturation medium (N25: 89.9±0.3%; N50: 91.3±0.3%) compared to all other groups. Similarly, cumulative blastocyst yield at D8 was significantly higher for N25 (32.1±0.8%) and N50 (35.5±0.8%) groups. In the same line, Kang et al., (2016) reported that the inclusion of Quercetin in the IVM medium increased blastocyst formation, presumably because Quercetin reduced ROS and increased intracellular GSH more effectively. However, no beneficial effect has been found on blastocyst formation when applied only during IVC, which may be confirming the impact of oocyte IVM improvement on in vitro embryo culture.
Based on our results, supplementation with Nobiletin during IVM improves bovine oocyte quality and the subsequent embryo development. Studies on gene expression of treated oocytes and their produced embryos are in progress to confirm the quality improvement.
10. Conclusions

Supplementation of the maturation medium with 25 µM and 50 µM Nobiletin used as an antioxidant improves:

- Nuclear maturation, the percentage of oocytes that reach metaphase II.
- Cytoplasmic maturation, oocyte organelle distribution (mitochondrial and cortical granules migration).
- Embryo development in terms of cleavage rate and blastocyst yield.
11. Bibliography


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