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RESVERATROL SUPPLEMENTATION DURING *IN VITRO* MATURATION: EFFECT ON THE QUALITY OF OOCYTES IN SPECIES OF VETERINARY INTEREST

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Abstract

The quality of oocytes plays a pivotal role in determining *in vitro* embryo production (IVEP) outcomes. Different intrinsic and extrinsic factors can impair the quality of mammalian oocytes, affecting their developmental competence.

The aim of the study was to evaluate the effect of supplementation of resveratrol, a natural antioxidant, during *in vitro* maturation (IVM) on the quality of oocytes in species of veterinary interest (domestic cat, prepubertal goats and sheep).

Three studies were performed to test the potential beneficial influence of resveratrol to improve the *in vitro* developmental competence of poor quality oocytes such as under sub-optimal condition or with low developmental competence.

Specifically, the effect of resveratrol addition to the IVM medium was tested on:

-STUDY I: IVEP from domestic cat oocytes retrieved from ovaries stored at 4°C for 24 and 48h.

Oocytes retrieved from stored ovaries for 24 and 48h were IVM with or without 5µM resveratrol. Meiotic competence, intracellular levels of reactive oxygen species (ROS) and glutathione (GSH), blastocyst yield and the blastocyst cell number were evaluated. The results showed that resveratrol treatment had not effect on the meiotic competence of the oocytes. Resveratrol groups had lower ($P<0.05$) intracellular ROS levels and higher ($P<0.05$) GSH content compared to untreated oocytes, both at 24 and 48h. Moreover, resveratrol supplementation significantly increased blastocyst yield in 48h group and improves blastocyst cells number in both groups.

-STUDY II: IVEP from prepubertal goat oocytes selected by brilliant cresyl blue (BCB) staining.

Oocytes were classified by BCB staining as BCB+ (fully grown oocytes) or BCB- (growing oocytes) and IVM with or without 1μM resveratrol. ROS and GSH levels, mitochondrial activity and distribution and ATP content were analyzed in metaphase II oocytes (MII). After *in vitro* fertilization (IVF), the blastocyst rate and the blastocyst quality were assessed. No differences were found in ROS levels, ATP content and mitochondrial activity among groups. GSH levels were significantly higher in both BCB groups treated with resveratrol than their respective controls. Oocytes treated with resveratrol showed a higher proportion of clustered active mitochondria than control groups. The development to blastocyst stage was significantly higher in BCB+ oocytes matured with resveratrol compared with the other groups. No differences were observed in blastocyst quality among groups.

STUDY III: *In vitro* fertilization outcome of prepubertal sheep oocytes under cadmium exposure.

Oocytes were expose to 2μM cadmium (Cd) and IVM in the presence of different concentration of resveratrol: 0μM (Cd), 1 μM (Cd-Resv 1μM) and 2 μM (Cd-Resv 2μM). Oocytes matured in absence of Cd were used as control. Fertilization outcomes, cortical granules (CGs) and mitochondria distribution, mitochondria activity and ROS level were evaluated. Oocytes of control, Cd-Resv 1μM and Cd-Resv 2μM groups had higher normal fertilization compared to Cd group ($P<0.05$). The percentage of MII oocytes with CGs distributed in the cortex of the oocytes was higher ($P<0.05$) in control and Cd-Resv 1μM groups than Cd group. The percentage of MII oocytes that exhibited a homogeneous mitochondria distribution throughout the cytoplasm was higher in control, Cd-Resv 1μM and Cd-Resv 2μM groups than Cd group ($P<0.05$). Lower activity ($P<0.05$) of mitochondria was recorded in control and Cd-Resv 1μM oocytes compared to Cd oocytes. The intracellular ROS levels were lower in control, Cd-Resv 1μM and Cd-Resv 2μM groups than Cd group.

Taking all these results into account, we conclude that resveratrol supplementation during IVM constitutes a useful strategy to improve oocyte quality and IVEP outcome in species of veterinary interest. The mechanism underlying resveratrol effects included the regulation of bioenergetic/redox status of the oocytes by the modulation of ROS and GSH levels and mitochondria function and the distribution of cytoplasmic organelles.

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Resveratrol supplementation during in vitro maturation: Effect on the quality of oocytes in species of veterinary interest.

Chapter1:

General background

1- General background

The application of Assisted Reproductive Technologies (ARTs) have experienced growing interest in human to treat infertility and in animal to increase the rate of selective breeding and in safeguard of endangered species. In the livestock industry, the use of methods such as artificial insemination (AI), *in vitro* production of embryos (IVP), embryo transfer (ET) and cryopreservation allow to increase the productivity of the animals and to obtain offspring from sub-fertile subjects of high genetic value (Wu and Zan, 2012). *In vitro* embryo production using oocytes derived from prepubertal donors in conjunction with *in vitro* embryo transfer, termed as juvenile *in vitro* embryo transfer (JIVET), is also applied with the aim of increasing the rate of genetic gain through a reduction of the generation gap (Morton, 2008; Paramio and Izquierdo, 2014). In the general interest of biodiversity conservation, ARTs represent an important tool for management and preservation of endangered species (Pukazhenthi *et al.*, 2006; Andrabi and Maxwell, 2007; Cocchia *et al.*, 2015). The possibility of producing embryos *in vitro* from germplasm of endangered species recovered even after death allows the propagation of genotypes that would otherwise be lost. However, despite the great potential application, the efficiency of ARTs and in particular the *in vitro* production of embryos is still low. The quality of oocytes plays a pivotal role in determining ARTs outcomes. The oocyte quality is unanimously defined as the oocyte ability to mature, to be fertilized and to develop to the blastocyst stage and give rise to healthy offspring (Duranthonn and Renard, 2001). The availability of good quality oocytes is a prerequisite to ensure satisfactory blastocyst yields in *in vitro* embryo production programs. Selection of oocytes with the best developmental potential has been the objective of intense research in the last years (Goovaerts *et al.*, 2010; Labrecque and Sirard, 2014; Paramio and Izquierdo, 2016; Melo *et al.*, 2017). The most widely used selection criterion is the morphological evaluation of the oocytes (size, homogeneity and regularity of the cytoplasm, number of cumulus cells). However, the still low efficiency of the IVP and the variability of the results suggest the need to identify other non-invasive and more reliable selection methods.

In this regard, the selection with the brilliant cresyl blue (BCB), a vital dye that exploits the action of the enzyme G6PDH to separate growing oocytes from fully grown oocytes, has allowed to improve the *in vitro* production of embryos in different species (Opiela and Kątska-Książkiewicz, 2013a). The potential developmental competence of oocytes can be greatly affected by a large number of intrinsic and external factors including follicle size (Lonergan *et al.*, 1994; Crozet *et al.*, 1995; Bagg *et al.*, 2007; Töpfer *et al.*, 2016), donor's age (Armstrong, 2001; Grupen *et al.*, 2003; Tatone *et al.*, 2008; Iwata *et al.*, 2011; Mohammadzadeh *et al.*, 2018), nutrition (Moussa *et al.*, 2015), season (Al-Katanani *et al.*, 2002; Comizzoli *et al.*, 2003; Di Francesco *et al.*, 2011; Mara *et al.*, 2014), exposition to environmental contaminants (Gandolfi *et al.*, 2002).

Moreover, the *in vitro* conditions themselves for their inadequacy to recreate the physiological environment where the oocyte matures and develops can contribute to affect the oocyte quality, thus decreasing the success of IVP protocols (Agarwal *et al.*, 2014).
All these factors act at multiple levels and may modify the bioenergetic/redox status of oocytes leading to oxidative stress. This condition can determine structural and molecular damages of the physiological arrangement of nuclear and cytoplasmic compartments of the female gamete impairing its quality (Combelles *et al.*, 2009).

Major efforts have been made to ameliorate the quality of oocytes and to protect against oxidative damage (Zavareh *et al.*, 2015). Among these approaches, the addition of antioxidants to the maturation medium represents a therapeutic strategy of research interest.

Chapter2:

Bibliographical revision

2.1 ACQUISITION OF OOCYTE DEVELOPMENT COMPETENCE

The acquisition of developmental competence of the oocyte is orchestrated by a complex series of events, which include molecular and morphological changes to both the oocyte and the surrounding follicle. Mammalian oocyte development begins during foetal life through to a diploid primordial germ cell (PGC) which differentiates into a specialized haploid cell, called oocyte (Picton, 2001).

The reservoir of female gametes is limited and defined in the foetal life in some species (e.g. primates, ruminants) or in early neonatal period in others ones (e.g. rodents, rabbits) (Fortune, 1994). The oocytes enclosed in primordial follicles are arrested at the prophase of the first meiotic division until puberty, when cyclically, few follicles are recruited to resume growth (Fortune, 1994). A series of autocrine and paracrine stimuli promote the exit of primordial follicles from the pool of follicles not growing towards the transition to primary follicles (Kawashima and Kawamura, 2018). In this phase the oocyte undergoes a series of morphological and structural changes: it increases in size, the cytoplasmic organelles begin to take on more mature forms, the granulosa cells proliferate and the zona pellucida appears (Paulini *et al.*, 2014). Non-gonadotropic signals promote oocyte and follicle growth to the secondary follicle stage (Kawashima and Kawamura, 2018). The cytoplasm of the oocyte is enriched with organelles such as cortical granules, polyribosomes and a larger amount of lipid droplets (Paulini *et al.*, 2014). Mitochondria elongated active form becomes more frequent and a well-developed endoplasmic reticulum and Golgi cisternae become aggregated forming a complex (Paulini *et al.*, 2014). At this stage the oocyte is metabolically active and the mRNA synthesis begins (Fair *et al.*, 1997). Also the somatic compartment undergoes changes, the granulosa cells become sensitive to gonadotropins thanks to the expression of the receptors for follicle-stimulating hormone (FSH) (Yamoto *et al.*, 1992), the layer of the theca cell is formed accompanied by blood vessel network establishment (Orisaka *et al.*, 2009). Follicular cells begin to secrete follicular fluid that fills the spaces between granulosa cells, the accumulation of follicular fluid leads to the formation of the antrum (Paulini *et al.*, 2014).

When antrum is formed, the oocytes become able to resume meiosis. Although, the competence to complete meiosis and support embryo development after fertilization is acquired in dominant follicle. In the antral follicle, the oocyte is immersed in the follicular fluid which provides important regulatory substances produced by follicular cells or deriving from the blood, such as hormones, growth factors, lipoproteins and proteoglycans necessary for oocyte growth (Van Den Hurk and Zhao, 2005).The pre-antral and early-antral phases of folliculogenesis are mainly regulated by autocrine/paracrine factors while progression through antral and pre-ovulatory phase is gonadotropins dependent (Hutt and Albertini, 2007). Only follicles expressing luteinizing hormone (LH) receptors and that are sensitive to low FSH levels will become dominant follicles. These follicles produce large amount of estradiol and inhibin and are potentially able to ovulate, while the remaining follicles undergo atresia.
In the dominant follicle (pre-ovulatory) the oocyte is arrested at the prophase I stage by the action of the cyclic adenosine monophosphate (cAMP). The pre-ovulatory LH peak induces the reduction of cAMP in the oocyte triggering a series of reactions that lead to the recovery of meiosis by the oocyte.

2.1.1 The final phase of oocyte maturation

The final phase of oocyte maturation is a complex process involving both the progression of the meiotic cycle and the reprogramming of cytoplasmic events of the fully-grown oocytes. Although nuclear and cytoplasmic maturation are distinct processes, they are interlinked and both events are required for appropriate acquisition of oocyte developmental competence.

- **Nuclear maturation**

In mammals, oocyte is arrested in prophase of the first meiotic division until the per-ovulatory period, when LH-surge induce meiosis resumption of fully-grown oocyte. The ability to resume meiosis is acquired stepwise during folliculogenesis. Studies in animal model have showed that oocytes become able to resume meiosis at the time of antrum formation.

However, only oocytes derived from large antral follicles are competent to advance beyond metaphase I and reach metaphase II (Wickramasinghe *et al.*, 1991; De Smedt *et al.*, 1994; Handel and Eppig, 1998; Marchal *et al.*, 2002).

Instead, cow oocytes seem to acquire both competence at the same time, but the two events need different activators (Sirard *et al.*, 1997). Once the oocyte becomes meiotically competent its arrest in prophase I is imposed by the action of granulosa cells and additional time is required to acquire complete developmental competence (Conti and Franciosi, 2018). Pincus and Enzmann (1935) first proved the involvement of granulosa cells in suppression of meiosis in oocytes derived from large antral follicles. In fact, when cumulus-oocyte complex (COC) is isolated from an antral follicle and cultured, it spontaneously resumes meiosis without needing hormonal stimulation (Pincus and Enzmann, 1935). This observation led to the hypothesis, later confirmed, that some factors produced by granular wall cells are necessary to block the meiotic progression of fully-grown mammalian oocytes.

Cyclic guanosine monophosphate (cGMP) produced by granulosa cells has been identified as key factor in meiosis arrest. Indeed, cGMP diffuses into the oocyte thought gap junctions and inhibits phosphodiesterase 3 (PDE3A) action. This enzyme is responsible for cyclic adenosine 3', 5' monophosphate (cAMP) degradation. Cyclic AMP is the main molecule responsible for oocyte arrest in prophase I. High concentration of cAMP due to inhibition of its degradation maintains a high protein kinase A (PKA) activity. In turn, PKA phosphorylates activating key cell cycle factors prevent maturation promoting factor (MPF) activation causing meiotic arrest (Conti and Franciosi, 2018).

In vivo, this arrest is maintained until the oocyte into pre-ovulatory follicle acquires complete developmental competence. LH surge induces rapid modification in mural granulosa cells leading to the shut off cGMP production. The drop-in cGMP levels actives PDE3A that degrades cAMP. The loose of the cAMP- induced arrest permits the oocyte to proceed through meiotic cycle until reaching metaphase II stage (Conti and Franciosi, 2018).

- **Cytoplasmic maturation**

Cytoplasmic maturation gives the oocyte the ability to be activated, fertilized and support embryonic development (Eppig, 1996).

The mains events associated with the acquisition of oocyte cytoplasm competence are the intracellular reorganization of organelles, storage of mRNAs, proteins and transcription factors (Gosden *et al.*, 1997; Zuccotti *et al.*, 2011; Reader *et al.*, 2017). During the growth, the oocyte transcripts, and accumulates mRNAs necessary not only for its maturation but also for the early embryo development (Zuccotti *et al.*, 2011). It is well established that oocyte undergo ultrastructural changes during its growth and maturation. Cytoplasmic organelles redistribution take a place under the action of cytoskeletal microfilaments and microtubules in order to locate the organelles in the areas where their action is required. In particular, mitochondria distribution and activity is necessary for cytoplasmic maturation and subsequent embryo development (Stojkovic *et al*. 2001).

Mitochondria are the mainly producer of ATP in the cell and they are also involved in calcium control and redox homeostasis (Dumollard *et al.*, 2007). Mitochondria number increases during oocyte growth from 10 units in the primordial germ cells to more 100000 units in the mature oocyte (Poulton and Marchington, 2002).Mitochondria replication ends during embryo cleavage. Therefore, an adequate number of mitochondria in the mature oocyte is crucial for embryo development. These organelles are distributed into each blastomere of the embryo in order to supply the energy necessary for the early embryo development (Dumollard *et al.*, 2007). Moreover, cytoplasm maturation is associated with mitochondria re-distribution. Mitochondria move from peripheral localization in the immature oocyte to diffuse distribution throughout the cytoplasm in the mature oocyte (Reader *et al.*, 2017). This reorganization is necessary not only to support oocyte function during maturation but also to ensure that each blastomere contains an adequate number of mitochondria (Dumollard *et al.*, 2007). The endoplasmic reticulum (ER) is another component of the cytoplasm that undergoes redistribution during oocyte maturation. This organelle is involved in several functions likely protein folding and degradation, lipid metabolism, membrane synthesis, nucleus compartmentalization and regulation of calcium signaling pathway.

In the oocyte at the GV stage, ER is located in cortical region. As the oocyte progress through meiosis up to MII stage, ER forms small clusters and diffuse throughout the cytoplasm (Mehlmann *et al.*, 1995).

Fertilization triggers a marked release of Ca^{2+} from ER leading to cortical granules exocytosis, hardening of the zona pellucida and the beginning of embryonic development. Cortical granules migration and localization close to the plasma membrane is crucial for blocking polyspermy and for normal embryo development (Wessel *et al.*, 2001).

2.2 METHODS OF OOCYTE QUALITY ASSESSMENT

Assessment of oocyte quality is an important matter of investigation in assisted reproduction technology (Goovaerts *et al.*, 2010; Labrecque and Sirard, 2014; Melo *et al.*, 2017). Current methods for assessing oocyte quality as morphological classification or molecular techniques have significant limitations. Morphological classification of oocytes is highly subjective and not always reflected the oocyte ability to develop (Wang and Sun, 2007). Molecular biology methods enable to identify markers of oocyte developmental potential, but they lead to destruction of the cell (Coticchio *et al.*, 2004).

2.2.1 Morphological assessment of oocytes

Several studies have shown a relationship between the morphology of the cumulus-oocyte complex and oocyte developmental competence. Morphological characteristics as structure and number of cumulus cell layers, oocyte diameter and cytoplasm homogeneity allow to select oocytes with different grade of quality (Lasienė *et al.*, 2009). Oocytes with several layers of compact cumulus cells and homogeneous cytoplasm are classified as good quality, grade A, oocytes and developed to blastocyst stage at higher rate than other classes of oocytes (Blondin and Sirard, 1995; Wood and Wildt, 1997; Warriach and Chohan, 2004; Nagano *et al.*, 2006).

The morphology of the first polar body (PBI) has been also proposed as a marker for the evaluation of the quality of the human oocyte in the ICSI protocols (Coticchio *et al.*, 2004; Borini *et al.*, 2005). Ebner *et al.* (2000) showed that oocytes with the intact polar body had higher fertilization rates after ICSI, and gave rise to higher quality embryos (Ebner *et al.*, 2000).

The correlation between PBI fragmentation and post-ovulatory aging of human oocytes has been suggested by Eichenlaub-Ritter *et al.* (1995). These Authors reported an increase of the implantation and the pregnancy rates, following the transfer of embryos derived from oocytes selected on the basis of the PBI morphology (Eichenlaub-ritter *et al.*, 1995).

However, results by other authors demonstrated no relationship between the morphological characteristics of the PBI and the rate of fertilization, the development of blastocysts and the quality of the embryo (Verlinsky *et al.*, 2003; Ciotti *et al.*, 2004).

Another marker of the oocyte quality is the morphology of the meiotic spindle of the oocyte arrested in the second metaphase stage. The meiotic spindle plays a pivotal role in the correct segregation of chromosomes in metaphase I and II, as well as in the fertilization process. Polarized light microscopy (Polscope) allows to analyze the macromolecular structures of the cell, such as spindle microtubules, on the basis of their birefringence (Liu *et al.*, 2000). Several authors observed a positive correlation between the visualization of the birefringence of the meiotic spindle and the quality of the human oocyte. It has been reported that oocytes with a birefringent spindle have greater development competence after *in vitro* fertilization or ICSI compared to those without spindle birefringence (Wang *et al.*, 2001 a, b; Shen *et al.*, 2006; Fang *et al.*, 2007). The morphological selection of oocytes is an easy and inexpensive practice applies routinely in the IVP laboratories. However, the still low efficiency of IVP programs suggests the need to find alternative methods.

2.2.2 Brilliant cresyl blue selection

The brilliant creasyl blue (BCB), a vital dye, has been proposed as good predictor of oocyte quality. BCB is a blue compound which is reduced by the action of glucosio-6-phosphate-dehidrogenase (G6PDH) in a colorless substance. Studies in mouse (De Schepper *et al.*, 1985), rat (Tsutsumi *et al.*, 1992) and cattle (Ferrandi *et al.*, 1993) have been demonstrated that G6PDH activity decrease during oocyte growth. Indeed, growing oocytes having high G6PDH activity reduce BCB and present a colorless cytoplasm (BCB-), while grown oocytes with low G6PDH activity are unable to metabolize the stain and exhibit a blue cytoplasm (BCB+) (Ericsson *et al.*, 1993). It has been demonstrated that BCB+ oocytes have larger diameter than BCB- oocyte (Rodríguez-González *et al.*, 2002; Pujol *et al.*, 2004; Catalá *et al.*, 2011).

Furthermore, after IVM more BCB+ oocyte reach metaphase II than BCB- oocyte in goat (Rodríguez-González *et al.*, 2002), sheep (Mohammadi-Sangcheshmeh *et al.*, 2012), horse (Mohammadi-Sangcheshmeh *et al.*, 2011), cattle (Alm *et al.*, 2005), mouse (Wu *et al.*, 2007) and human (Duarte Alcoba *et al.*, 2018).

Several authors evaluated the percentage of oocytes selected by morphological assessment that were positive to BCB test. This percentage varies depending on the species and the laboratory from 50% to 79% (Roca *et al.*, 1998; Rodríguez-González *et al.*, 2002; Pujol *et al.*, 2004; Alm *et al.*, 2005; Ishizaki *et al.*, 2009). These data suggest that morphological selection is not satisfactory to identify the most competent oocytes.

An increasing number of studies indicated that BCB test is a useful tool to select high quality oocytes for *in vitro* embryo production. Indeed, it has been widely demonstrated that fertilization and blastocyst production were significantly improved in BCB+ oocyte compared to BCB-.oocytes in cattle (Pujol *et al.*, 2004; Silva *et al.*, 2013) buffalo (Manjunatha *et al.*, 2007) sheep (Catal *et al.*, 2012; Mohammadi-Sangcheshmeh *et al.*, 2012) mouse (Wu *et al.*, 2007) and goat (Rodríguez-González *et al.*, 2002).

However, the efficacy of BCB test as non-invasive method to assess oocytes quality is still a subject of debate. Wongsrikeao *et al.* (2006) showed that double exposure to BCB impaired fertilization and embryonic development in pig oocytes (Wongsrikeao *et al.*, 2006). Moreover, an increased in chromosome abnormalities has been observe in the porcine oocytes exposed to BCB test (Pawlak *et al.*, 2011). Furthermore, blastostocysts derived from bovine oocytes selected with BCB had higher caspase-3 activity than blastocysts of the control group, suggesting a possible negative effect of BCB staining (Opiela *et al.*, 2010). Therefore, although the usefulness of BCB test as a method for selecting high-quality oocytes has been widely described, further studies are needed to evaluate a possible adverse effect.

2.2.3 Cumulus cells transcriptome analysis

It has well recognized the importance of thighs communication between oocyte and cumulus cells on oocyte quality (Albertini *et al.*, 2003; Gilchrist *et al.*, 2008). Transcriptome analysis of cumulus cells has been proposed as non-invasive approach to assess oocyte competence. Several studies, overall in human, have been aimed to find differences in gene expression of cumulus cells between high or low quality oocytes (Patrizio *et al.*, 2007; Li *et al.*, 2008). Although numerous genes have been candidates as biomarkers, no agreement has yet been reached on which are really predictors of oocyte competence (Dumesic *et al.*, 2015). Currently, despite the great potential, the analysis of cumulus cells transcriptome is not yet routinely used in the human field (Goovaerts *et al.*, 2010). Moreover, the need to separately analyze cumulus cells of each oocyte makes this technique laborious, time-consuming and expensive for the application in animal field (Goovaerts et al., 2010).

2.2.4 Follicular fluid analysis

The analysis of follicular fluid (FF) is another non-invasive technique to evaluate oocyte quality (Revelli *et al.*, 2009). The follicular fluid is easily available at the time of the oocyte retrieval from the ovary and may provide important information on the state of the oocyte.

In recent years, the application of molecular, proteomic and metabolomic analyzes enabled to better characterize the complex composition of follicular fluid (Dumesic *et al.*, 2015). Hormones, growth factors, proteins, peptides, amino acids, reactive oxygen species, antioxidants sugars and prostanoids are the main constituents of FF (Revelli *et al.*, 2009). Several studies have been performed to identify any components differently expressed in the FF that can be associated with high or low oocyte development competence. Mendoza *et al.* (2002) reported high levels of LH and growth hormone (GH) in the FF of follicles from which the oocytes resulting in transferable embryos were derived (Mendoza *et al.*, 2002). In cattle, FF analysis of individual follicles showed that lower content of palmitic acid and total fatty acids and higher levels of linoleic acid were present in FF of competent oocytes than that of incompetent oocytes (Matoba *et al.*, 2014).

Furthermore, L-alanine, glycine and L-glutamate were positively correlated and urea was negatively correlated to blastocyst formation (Matoba *et al.*, 2014). The concentration of D-Asp in human follicular fluid has been indicated as a marker for oocyte quality in patients undergoing IVF programs (D'Aniello *et al.*, 2007). D'Aniello *et al.* (2007) reported a relationship between the age of patients and D-Asp levels in follicle fluid. Nicholas *et al.* (2005) described different levels of Insulin-like growth factor-blinding protein (IGFBP) in FF of bovine follicles in various development stages and demonstrated that correlation between the expression profiles of IGFBP in FF and oocyte developmental competence (Nicholas *et al.*, 2005). The concentration of Insulin-like growth factor-1 (IGF-1) and IGFBP-1 in human FF has been associated with oocyte quality and pregnancy rate (Oosterhuis *et al.*, 1998; Fried *et al.*, 2003). The concentration of anti and pro-oxidized agents in FF has also been proposed as a possible predictive parameter of oocyte quality. Hight total antioxidant capacity (TAC) and low reactive oxygen species (ROS) levels in the FF has been positively related to the success rate of IVF and ICSI procedures and the quality of the embryos (Oyawoye *et al.*, 2003; Nuñez-Calonge *et al.*, 2016).

2.3 FACTORS AFFECTING OOCYTE QUALITY

Mounting evidence highlights the negative impact of multiple factors on the quality of the oocyte. *In vivo* influences as lifestyle factors, nutrition, age, exposition to environmental contaminants may compromise the developmental competence of oocytes. Similarly, the *in vitro* external environment that is associated with ART technique (e.g. *in vitro* culture condition, handling of gamete/embryo, exposition to sub optimal temperatures during cryopreservation) may determine a deterioration of oocyte quality.

2.3.1 Donor Age

Maternal age is one of the most important factors influencing the success of assisted reproductive technology both in human and in animal. It is well documented that advanced maternal age is linked to a decline of physiological fertility, IVF outcome and achievement of a normal pregnancy (Tatone *et al.*, 2008; Cimadomo *et al.*, 2018).

In animal field, the possibility of using juvenile subjects as a source of oocytes for the *in vitro* embryo production (JIVEP) is of great interest in breeding programmes. This technology offers the advantage of reducing the generational interval and increasing the rate of genetic gain. However, the low competence of oocytes derived from prepubertal animals makes the efficiency of this technique very low (O'Brien *et al.*, 1997; S. Ledda, *et al*., 1997; Marchal *et al.*, 2001; Palma *et al.*, 2001; Leoni GG, *et al.*, 2009). Several studies have been aimed to investigate the causes of sub-optimal developmental competence, after IVF, of oocytes derived from juvenile animals in comparison with adult ones.

Compared to the oocytes of adult donors, oocytes from prepubertal animals are smaller in diameter and, although have similar *in vitro* meiotic maturation ability, show a higher percentage of abnormal fertilization and a lower embryo developmental competence (Morton, 2008). The impaired development competence is mainly attributed to incomplete or perturbed cytoplasm maturity (Morton, 2008).

It has been demonstrated that oocytes from prepubertal animals exhibit a precocious decline of protein synthesis and a reduced store of mRNA and proteins required to support normal fertilization and embryonic development (Amstrong, 2001; Morton, 2008). Jiao *et al.* (2013) demonstrated that oocytes from prepubertal animals have a decreased ability to synthesize glutathione that is reflect in their impaired ability to decondense sperm head, form male pronucleus and develop until blastocyst stage (Jiao *et al.*, 2013). Moreover, difference in the quantity and distribution of cytoplasmic organelles between adult and prepubertal oocytes have been identified. O'Brien *et al.* (1996) reported that oocytes from juvenile ewes showed a reduction in the volume fraction and size of cortical granules and mitochondria in comparison with those of adult animals after *in vitro* maturation (O' Brien *et al.* 1996).

Cortical granules exocytosis is necessary for zona hardening reaction and polyspermy block. Higher frequency of polyspermy has been observed in oocytes from prepubertal than adult animals in goat (Mogas *et al*., 1997), sheep (O' Brien *et al.* 1996), pig (Marchal *et al.*, 2001) and mouse (Jiao *et al.*, 2013). Damiani *et al.* (1996) explained that high frequency of polyspermy in calf oocytes is due to an altered Ca^{2+} oscillations which in turn altered exocytosis of cortical granules (Damiani *et al.*, 1996). Mitochondria represent important organelles linked to oocyte quality and embryo development (Reader *et al.*, 2017). Mitochondria provide the oocytes with energy in form of ATP, control of redox potential in the cytoplasm as well as intracellular Ca^{2+} level (Dumollard *et al.*, 2007). In ovine (O' Brien *et al.,* 1996) and bovine (Paz *et al.*, 2001) models a lower volume density of mitochondria has been described in oocytes from prepubertal animals than adult ones. It has been observed that during maturation mitochondria are redistributed in the cytoplasm in bovine (Stojkovic *et al.*,2001), dog (Valentini *et al.*, 2010), goat (Velilla *et al.*, 2006), horse (Torner *et al.*, 2007), human (Dell'Aquila *et al.*, 2009) mice (Calarco, 1995) and pig (Torner *et al.*) oocytes. Leoni *et al.* (2015) for the first times observed differences of active mitochondria organization between sheep and lamb oocytes. At the GV stage both types of oocytes showed a fine or granular homogeneous distribution. After maturation most of the sheep oocytes presented a cluster mitochondrial organization while in the lamb oocytes persisted a fine configuration (Leoni *et al.*, 2015).

2.3.2 Environmental contaminant

Exposure to environmental contaminants are also implicated in the reduction of reproductive performances. Currently, a large number of chemical pollutants as polychlorinated biphenyls (PCBs), organochlorine pesticides as 1,1,1-trichloro-2,2-bis (p-chlorophenyl)-ethane (DDT), polychlorinated dibenzodioxins (PCDDs), bisphenol A, phthalates, metals including cadmium, mercury and lead, have been identified as endocrine disruptors (EDs) (Schantz and Widholm, 2001; Brevini *et al.*, 2005).

EDs are defined as exogenous agents that interfere with the synthesis, secretion, and activity of hormones involved in homeostasis, reproduction and developmental processes (Colborn *et al*., 1993). The exposure to EDs occurs as result of ingestion of contaminated water and food or breathing contaminated air (Brevini *et al.*, 2005). The environmental concentration of these compounds is constantly increasing due to their long half-live. In addition, the stability and lipid solubility of EDs lead to their bioaccumulation in fat tissues, which could compromise human and animal health and reproduction processes. Environmental pollutants have been found in ovary and in the follicular fluid of human and animals (Kamarianos *et al.*, 2003; Henson MC, 2004; Brevini *et al.*, 2005 a; Petro *et al.*, 2012).

Environmental contaminants may exert a negatively effect on primordial and primary follicles in the ovary compromising their healthy development to antral follicles (Hooser *et al.*, 1994). During follicular growth, the finely regulated endocrine/paracrine interaction among oocytes, cumulus and follicular cells may be disrupted by the exposition to EDs hindering oocyte developmental ability (Brevini *et al.*, 2005 a; Petro *et al.*, 2012). In humans, high EDs levels in the follicular fluid have been associated with a decreased oocytes retrieval , *in vitro* fertilization and embryo development rate (Petro *et al.*, 2012; AL-Hussaini *et al.*, 2018)

The effects of metal and chemical contaminants have been studied in animal both *in vitro* and *in vivo*.

Alterations of the oocytes nuclear maturation associated to spindle abnormalities have been documented after *in vitro* exposition to these chemicals (Alm *et al.*, 1998; Leoni *et al.*, 2002; Picard *et al.*, 2003; Rossi *et al.*, 2006; Nandi *et al.*, 2010; Liu *et al.*, 2014; Machtinger and Orvieto, 2014; Aker *et al.*, 2018). Further recent findings also described the deleterious impact of these substances on oocyte cytoplasmic maturation. EDs exposure during *in vitro* maturation impaired transcript abundance as well as, mitochondria and cortical granules re-organization in bovine and swine oocyte (Pocar *et al.*, 2006; Kalo and Roth, 2015, 2017). Furthermore, alteration of oxidative status and mitochondria activity of oocytes has been reported as consequence of pollutants exposition in bovine and ovine (Kalo and Roth, 2015; Martino *et al.*, 2017).

2.3.3 ARTs procedures

The procedures and conditions to which the oocyte is exposed during ARTs may also contribute to reduce its developmental competence. *In vivo*, the oocyte is enclosed within the follicle, where an intense bi-directional communication between the oocyte and the surrounding environment modulates its growth and maturation. The follicular fluid derived from the bloodstream and from the secretion of cumulus and granulosa cells provides the oocytes with nutrients, growth factors, hormones, antioxidants and other undefined factors necessary for its development (Krisher, 2013). The *in vitro* culture condition cannot exhaustively simulate the follicular environment both for the formulation of maturation media and for the multitude of physicochemical factors that are present in the ex-*vivo* environment. During ART setting, oocytes are exposed to variation of pH and temperature, high oxygen tension, visible light and handling which can compromise the oocyte quality and the outcome of fertilization (Combelles *et al.*, 2009; Agarwal *et al.*, 2014). Exposition to different environmental conditions reflected in the lower fertilization and blastocyst rate of *in vitro* matured oocytes compared to *in vivo* matured oocytes, observed in different mammalian species (Wang *et al.*, 1998; van de Leemput *et al.,* 1999; Zeng *et al.*, 2009; Sanfins *et al.*, 2014; Arias-Álvarez *et al.*, 2017).

Despite the oocyte resumes, the meiotic progression once removed from the follicle and cultured *in vitro,* the synchronous achievement of cytoplasmic maturation is not achieved. Comparative studies aimed to identify possible changes induced by *in vitro* maturation, revealed molecular and structural differences between *in vitro* and *in vivo* matured oocyte. Mouse oocytes matured *in vitro* showed a delayed and lower progression of development up to the blastocyst stage. This reduction in development competence has been associated with an alteration of cytoskeletal patterns of the oocyte and embryo, probably due to an asynchrony between nuclear and cytoplasmic maturation (Sanfins *et al.*, 2014). The analysis of the global transcriptome of *in vivo* and *in vitro* matured bovine oocytes, identified alterations in the expression of the genes involved in cell cycle regulation, glucose metabolism, in the production of ATP, osmoregulation and stress-induced apoptosis (Katz-Jaffe *et al.*, 2009). In addition, differences in the expression of redox-related gene in pig (Yuan *et al.*, 2012) and rabbit (Arias-Álvarez *et al.*, 2017) oocytes have been identified following *in vitro* maturation. Consistent with the results of the genetic analysis, other studies reported that *in vitro* matured oocytes had a lower activity of the two major proteins involved in the regulation of meiotic process as maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) (Bogliolo *et al.*, 2004), as well as a reduced content of ATP (Combelles and Albertini, 2003; Nishi *et al.*, 2003) and GSH (Brad *et al.*, 2003) compared to oocytes matured *in vivo*. The organization, number and activity of mitochondria are others important markers of cytoplasmic quality. Zeng *et al.* (2009) reported that rat oocyte matured *in vitro* exhibited a reduction in the mitochondrial DNA copy number and the intracytoplasmic ATP content (Zeng *et al.*, 2009). Furthermore, the alteration in the mitochondria distribution has been described in rat (Zeng *et al.*, 2009), rabbit (Arias-Álvarez *et al.*, 2017) and swine (Sun *et al.*, 2001) oocyte after *in vitro* maturation. All together, these results suggest a modification of oocyte quality in response to the surrounding *in vitro* environment, which could affect the *in vitro* embryo production efficiency.

Moreover, techniques such as cryopreservation of the oocyte can may lead to an even more serious deterioration in the quality of the animal and human oocyte.

The oocyte exposure to low temperature, the osmotic stress and the high concentration of cryoprotectancts may cause structural and molecular damage impairing oocyte developmental competence. A reduction in the ability to form blastocysts after *in vitro* fertilization of vitrified/thawed oocytes with respect to non-vitrified oocytes has been observed in various species such as porcine (Shi *et al.*, 2007), bovine (Zhao *et al.*, 2011), ovine (Shirazi *et al.*, 2016), feline (Merlo *et al.*, 2008). The cytoskeleton and in particular the meiotic spindle are sensitive to the low temperatures that can cause de-polymerization of microtubules and microfilament, with consequent scattering of the chromosomes and aneuploidies (Saunders and Parks, 1999; Bogliolo *et al.*, 2007; Shi *et al.*, 2007; Gomes *et al.*, 2008; Luciano *et al.*, 2009; Mikołajewska *et al.*, 2012). Another factor that may impair the competence of vitrified/thawed oocytes is the oxidative stress generated during the cryopreservation process (Tatone *et al.*, 2010). In fact, an increase in reactive oxygen species (Somfai *et al.*, 2007; Tatone *et al.*, 2011; Zhao *et al.*, 2011; Dai *et al.*, 2015; Succu *et al.*, 2018) and a reduction of antioxidant system (Somfai *et al.*, 2007; Dai *et al.*, 2015; Cao *et al.*, 2017; Succu *et al.*, 2018) has been observed in vitrified oocytes, suggesting an alteration of the mechanisms involved in the regulation of the redox status of the oocyte. Other evidences indicated damage to the bioenergetic system of the oocyte.

Mitochondria are the main producers of oocyte energy, through oxidative phosphorylation. The negative effect of vitrification of mitochondria function, organization and their membrane potential has been described in human (Jones *et al.*, 2004), fox (Cao *et al.*, 2017), swine (Dai *et al.*, 2015), ovine (Succu *et al.*, 2018) and bovine (Rho *et al.*, 2002) oocytes. Mitochondria damage has been associated with drop of ATP content, alteration in Ca^{2+} release, ROS production and decreased of oocytes developmental competence (Jones *et al.*, 2004; Zhao *et al.*, 2011; Dai *et al.*, 2015; Succu *et al.*, 2018).

2.4 OXIDATIVE STRESS AND OOCYTE QUALITY

Intrinsic and extrinsic factors can affect the quality of the oocyte by influencing its oxidative status. In physiological condition, the equilibrium between reactive oxygen species (ROS) production and degradation is maintained by the antioxidant defence system of the *in vivo* environment.

The reactive oxygen species such as superoxide anion (O_2^-), hydroxyl radical (**.**OH), and hydrogen peroxide (H_2O_2) are normally produced as by-products of cellular metabolism (Kohen and Nyska, 2002; Zavareh *et al*., 2015). The process of oxidative phosphorylation by which the cell synthetizes adenosine triphosphate (ATP) is one of the main sources of ROS (Murphy, 2009; Zavareh *et al*., 2015). Approximately, 1 to 3% of electrons crossing the mitochondrial electron transport chain leak from the system at the level of the complex I and III and creates superoxide anion (O_2^-) (Kowaltowski and Vercesi, 1988; Rhoads *et al.* 2006; Murphy, 2009; Birben *et al.*, 2012). Several other organelles such as peroxisomes and endoplasmic reticulum, and enzymes, as xanthine oxidase, lipoxygenase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and cytochrome P450 enzyme, contribute to ROS generation (Lewis, 2002; Schrader and Fahimi, 2004; Santos *et al.*, 2009; Manea, 2010).

At low concentration ROS regulate cellular functions and act as signaling molecules (Hancock ' *et al.*, 2001), whereas their over-production is detrimental for the oocyte. The maintenance of redox homeostasis within the oocyte is given by the collaboration of numerous enzymatic and non-enzymatic antioxidants. The main enzymatic antioxidants are superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px). The SOD and its cytoplasmic (Cu-SOD and Zn-SOD) and mitochondrial (Mn-SOD) variants by the dismutation reaction of O_2^- to H_2O_2, represent the main defence system against superoxide anion (Khazaei and Aghaz, 2017; Gajalakshmi *et al.*, 2016). The hydrogen peroxide produced by SOD or generated by the action of oxidases such as xanthine oxidase is reduced to H_2O by catalase and GSH-Px activity (Brigeliu-Flohè, 1999; Chelikani *et al.*, 2004).

The GSH-Px performs its detoxifying activity thanks to the action of glutathione (GSH) that acts as a cofactor of different enzymes. In fact, the GSH gives an electron oxidizing to GSSG to convert H_2O_2 to H_2O and scavenge other free radical.

GSH together with other non-enzymatic antioxidants such as vitamin E and carotenoids protect the cell membrane from lipid peroxidation (Birben *et al.*, 2012) . The presence of a micro-environment in which the pro and anti-oxidant agents are in equilibrium is important to support the correct growth of the oocyte and the subsequent embryonic development.

Tonic levels of ROS are required for modulate key cellular functions as cell cycle or apoptosis (Hancock *et al.*, 2001). In the oocyte, a certain threshold level of ROS is required for resume meiosis from the diplotene-arrested stage (Pandey and Chaube, 2014). Pandey *and* Chaube (2014) reported that the reduction of intra-oocyte cAMP is associated with a moderate increase in H_2O_2 which, in turn, through phosphorylation and dephosphorization events, could induce the inactivation of the Maturation Promoting Factor (MPF) and restarts the meiosis (Pandey and Chaube, 2014). Conversely, the excessive increased of H_2O_2 levels inhibits oocyte maturation (Tamura *et al.*, 2008), first polar body extrusion and promotes apoptosis in rat oocytes (Chaube *et al.*, 2005). Tarín *et al*. (1996) hypothesized that the increase in the frequency of anomalies such as aneuploidy, the inhibition of first polar body extrusion and fragmentation in the aged mammalian oocytes was attributable to the negative effect of oxidative stress on cytoskeleton (Tarín, 1996). A recent study (Mihalas *et al.*, 2017) underlined the contribution of the electrophilic aldehyde 4-hudroxynonenal (4HNE) produced by lipid peroxidation in the reduction of tubulin polymerization and in the destabilization of the meiotic spindle in mouse oocytes. In addition, oxidative stress induced mitochondrial damage, reduction of ATP levels, increase in cytosolic Ca^{2+} and fall in the GSH/GSSG ratio (Tarín, 1996; Zhang *et al.*, 2006). All these effects acting separately or synergistically can interfere with the assembly/disassembly processes of the microtubules and/or microfilaments inducing cytoskeletal alterations (Tarín, 1996; Zhang *et al.*, 2006). The integrity of the meiotic spindle is necessary for the correct alignment and segregation of chromosomes during meiosis.

Oxidative stress may also lead to alterations of fertilization and embryo development process by affecting adequate cytoplasmic maturation. Mitochondrial dysfunction, low ATP levels (Zhang *et al.*, 2006) and the alteration of Ca^{2+} oscillations (Takahashi *et al.*, 2003) induced by oxidative stress can interfere with key processes of fertilization such as migration of cortical granules and their exocitosis.

Appropriate glutathione content is crucial during fertilization and initial embryonic development (Luberda, 2005; Adeoye *et al.*, 2017). The depletion of GSH levels prevented the decondensation of the sperm head and the formation of the male pronucleus in bovine oocyte (Sutovsky and Schatten, 1997), as well as, gave rise to abnormalities in female pronucleus in hamster oocytes (Zuelke *et al.*, 1997a). Embryo development could be compromised by oxidative stress proportionally to the stress intensity (Gardiner and Reed, 1994; Fujitani *et al.*, 1997; Sakatani *et al.*, 2008; Bain *et al.*, 2011).

The exposure of mouse oocytes 6 hours after fertilization to a moderate increasing of ROS levels induced a reduction in mitochondrial membrane potential and an increase in DNA damage with a consequent negative effect on blastocyst formation (Qian *et al.*, 2016). Oxidative stress could also cause arrest, fragmentation and apoptosis in human (Yang *et al.*, 1998), bovine (Velez-Pardo *et al.*, 2007) and mouse (Noda *et al.*, 1991) embryos (Bain *et al.*, 2011).

Favetta *et al.* (2007) showed that bovine embryos cultured under 20% oxygen exhibited a 2-4 cell arrest 2-fold higher than those cultured under 5% oxygen. The increase of embryo arrest is associated with a 20-fold rise of ROS levels and to an increase in mRNA and protein levels of $p66^{shc}$ but not p53 (Favetta *et al.*, 2007).

In contrast, the results of Velez-Pardo *et al.* (2007) demonstrated that oxidative stress damaged the mitochondria membrane potential by inducing the activation of the prosthesis/caspase, thereby leading to the fragmentation and arrest of the bovine embryo development (Velez-Pardo *et al.*, 2007).

In order to prevent oocyte oxidative damage during ARTs procedures therapeutic approaches should be established. In this sense, the oral antioxidant therapy and the supplementation of medium for culture during ARTs is an ongoing area of research interest (Agarwal *et al.*, 2014).

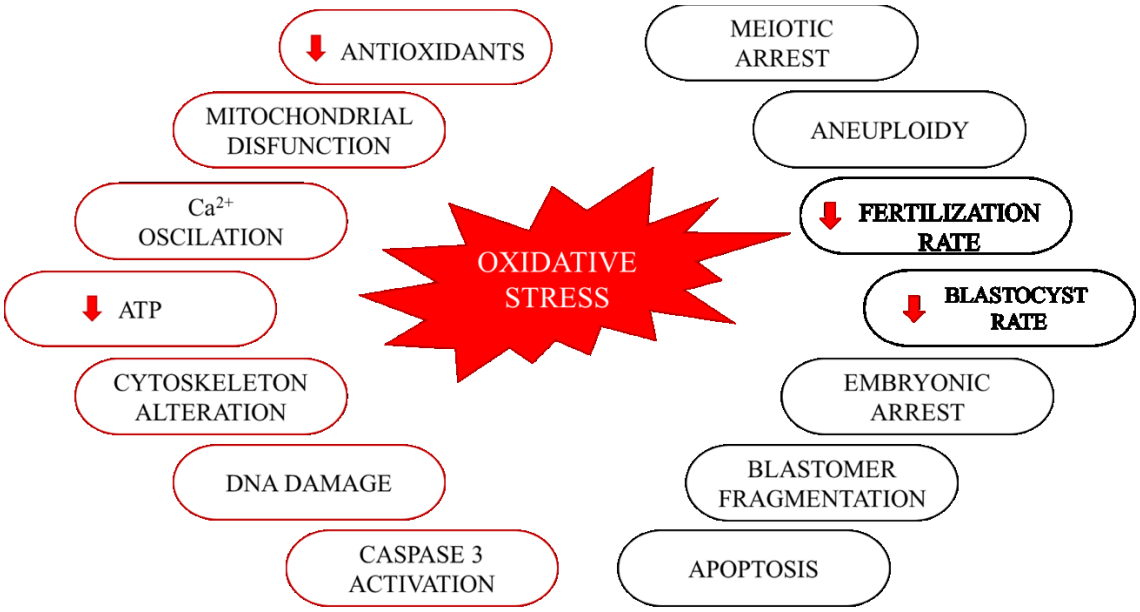


Fig.1. Schematic representation of the detrimental effects on oocyte quality induced by oxidative stress exposure.

2.5 UTILITY OF ANTIOXIDANTS DURING ARTs

The oxidative stress due to intrinsic and external factor may affect oocyte quality and ARTs outcome. *In vivo*, the oocyte is protected by endogen and exogenous antioxidant system. Indeed, the enzymatic (such as SOD, glutathione peroxidase, and catalase) and non-enzymatic (including taurine, hypotaurine, vitamin C, and glutathione) antioxidants present in the oocyte, in the ovary, follicles, follicular, tubal and peritoneal fluid and endometrial epithelium (Rakhit *et al.,* 2013), preserve the oocyte to ROS injuries during all steps of its developmental process. These defence systems are loose during *in vitro* culture condition. The supplementation of environmental medium with antioxidant compounds represent a useful therapeutic approach to protect oocytes against oxidative damage. In the last years, the effect of several antioxidants has been tested in order to improve the efficiency of IVP system. The beneficial effect of adding cysteamine to the maturation medium has been demonstrated in several species (Deleuze and Goudet, 2010). Cysteamine promotes GSH synthesis by increasing the oocyte cysteine content. An increase of GSH levels in cysteamine-treated oocytes has been detected in buffalo (Gasparrini *et al.*, 2003), dog (Hossein *et al.*, 2007), goat (Zhou *et al.*, 2008), mouse (De Matos *et al.*, 2003), sheep (De Matos *et al.*, 2002) and pig (Kobayashi *et al.*, 2007). Furthermore, cysteamine promoted male pronuclear formation and embryo development in goat (Urdaneta *et al.*, 2003; Zhou *et al.*, 2008), buffalo (Gasparrini *et al.*, 2003; Anand *et al.*, 2008), pig (Bing *et al.*, 2001; Kobayashi *et al.*, 2007) and sheep (De Matos *et al.*, 2002). Hu *et al.* (2012) reported the beneficial effect of Vitamin C supplementation to the maturation medium on the competence of pig oocytes (Hu *et al.*, 2012). The rate of blastocyst formation after parthenogenetic activation and the total cell number/blastocyst were higher in oocytes treated with vitamin C compared to control. Similar findings have been described by Sovernigo *et al.* (2017) in bovine after *in vitro* fertilization (Sovernigo *et al.*, 2017). The improved developmental competence of the oocyte induced by vitamin C has been associated with its antioxidant activity, as indicated by the reduction of ROS levels observed in pig embryos (Hu *et al.*, 2012) and the increase of GSH content in bovine oocytes (Sovernigo *et al.*, 2017). Melatonin is another antioxidant widely studied in various animal species (Cruz *et al.*, 2014).

In mice, treatment with melatonin at various stages of the *in vitro* embryo production process helped to improve oocyte maturation (Bahadori *et al.*, 2013) and embryonic development (Ishizuka *et al.*, 2000; Bahadori *et al.*, 2013; He *et al.*, 2016). Similarly, the addition of melatonin to the maturation medium increased embryonic development and improved the quality of embryos produced also from oocytes of prepubertal goats (Soto-Heras *et al.*, 2018), bovine (Tian *et al.*, 2014) and pig (Li *et al.*, 2016). Melatonin protected the oocyte from oxidative stress by reducing the intracytoplasmic levels of ROS and increasing GSH levels (He *et al.*, 2016; Li *et al.*, 2016; Soto-Heras *et al.*, 2018). He *et al.* (2016) reported that the use of melatonin during *in vitro* maturation regulated mitochondria activity, increased ATP production, promoted the assembly of the meiotic spindle and protected DNA from oxidative damage.

Interesting results have also been reported for L-carnitine. Recent studies in cattle (Knitlova *et al.*, 2017) and sheep (Reader *et al.*, 2015) described the positive effect of L-carnitine on the embryonic development of low-quality oocytes. In cattle, the use of L-carnitine during the *in vitro* maturation of oocytes deriving from small follicles (2-5 mm) improved fertilization and embryo development compared to control (Knitlova *et al.*, 2017). Treatment with L-carnitine positively affected embryonic development compared to control and induced an increase in cytoplasmic volume of the lamb oocyte (Reader *et al.*, 2015). Furthermore, Wu *et al.* (2011) observed that L-carnitine improved pig oocytes developmental competence following parthenogenetic activation, promoting redox homeostasis of the oocyte and embryo (Wu *et al.*, 2011). Several other antioxidants such as quercetin (Kang *et al.*, 2013; Banihosseini *et al.*, 2017; Sovernigo *et al.*, 2017), superoxide dismutase (Ochota *et al.*, 2016), and Coenzyme Q10 (Abdulhasan *et al.*, 2017; Liang *et al.*, 2017) protected the oocyte against oxidative stress and improved embryo development.

2.5.1 Resveratrol

In 1940, resveratrol (3,5,4'-trihydroxystilbene) was isolated for the first time from the root of white hellbore (Takaoka, 1940). Two decades later, the growing interest in the biomedical properties of chinese herbs led to the identification of resveratrol in the medicinal plant Polygonum Cuspidatum (Nonomura *et al.*, 1963; Timmers *et al.,* 2012). However, the real interest in resveratrol began in the 90s when its presence was discovered in large quantities in red wine (Siemann and Creasy, 1992; Timmers *et al.,* 2012). In fact, the involvement of resveratrol in the cardio-protective action of red wine known as French paradox was soon suggested (Meishiang Jang *et al.*, 1997; Timmers *et al.,* 2012). Further studies led to the discovery of the multiple biological effects of resveratrol such as anti-inflammatory, anti-cancer, blood glucose-lowering and antioxidant (Kuršvietienė *et al.*, 2016; Timmers *et al.,* 2012). The identification in 2003 of resveratrol as a powerful activator of SIRT1 (Howitz *et al.*, 2003; Timmers *et al.,* 2012), a NAD+ - dependent deacetylases belonging Sirtuin family, led to an increased interest on this stilbene. In fact, Sirt1 acts, inside the cell as the master controller of metabolism, gene silencing, energy homeostasis, genomic stability, and cell survival, thanks to the multiplicity of targets on which it acts (Canto and Auwerx, 2012). To date, numerous research groups are involved in the study of the effects and mechanisms of action of resveratrol.

- **Chemistry**

Resveratrol is a phytoalexin produced by plants in response to biotic stresses such as fungal and bacterial infections or UV radiation (Murakami et *al.*, 2013) . Resveratrol is mainly produced by the shikimic pathway in which the p-coumaroyl CoA, its precursor, is produced starting from phenylalanine (Donnez *et al.*, 2009). The p-coumaroyl CoA and 3 molecules of malonyl CoA are condensed by the enzyme stilbene synthase to form resveratrol (Fig.2) (Donnez *et al.*, 2009). Resveratrol is present both in *cis* and *trans*- configuration; however, the *trans* isoform is more stable and bioactive as well as the predominant one (Fig.3) (Camont *et al.*, 2009). Indeed, *trans* isoform is stable for a month when it is protected from light and high pH (Soleas *et al.*, 1997). The structure of resveratrol is stilbenic, and consists of two phenolic rings linked by a methylene bridge on which 3 OH groups are inserted in positions 3, 5 and 4 '.

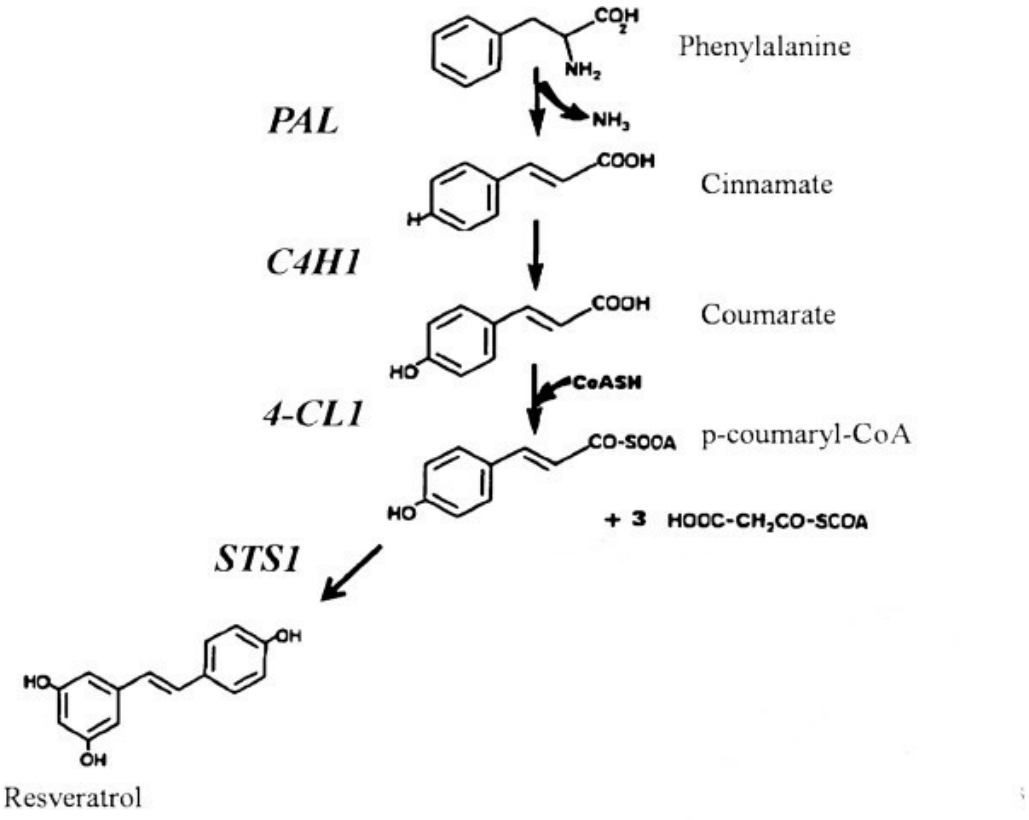


Fig.2. Representative image of biosynthetic pathway from phenylalanine to resveratrol. Adapted from (Soleas *et al.*, 1997).

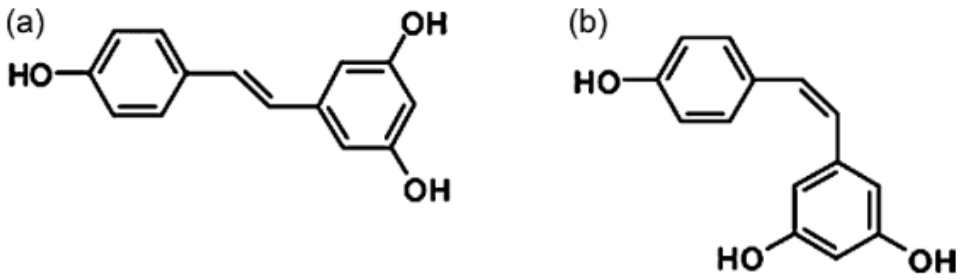


Fig.3. Chemical structures of (a) *trans*-resveratrol and (b) *cis*- resveratrol, from (Camont *et al.*, 2009).

- **Antioxidant property of resveratrol**

One of the multiplicity biological activities attributed to resveratrol is represented by its antioxidant activity. *In vitro* studies have shown that resveratrol exerts its antioxidant action mainly through three mechanisms:

1) free radical scavenger

2) regulation of enzymatic and non-enzymatic antioxidants

3) reduction of mitochondrial ROS production (Xia *et al.*, 2017; Truong *et al.*, 2018).

Thanks to its polyphenolic nature, resveratrol is able to exert a free radical scavenger function against a variety of oxidant such as hydroxyl radical (●OH), superoxide anion ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$) hydrogen peroxide (H_2O_2), nitrogen oxide ($NO^{\bullet}$), and peroxynitrite (Truong *et al.*, 2018).

Antioxidant properties is conferred to the presence of phenolic rings with three hydroxyl groups in positions 3, 4, and 5, and with conjugated double bonds, as well as potential for electron delocalization in the structural molecule (Truong *et al.*, 2018; Papuc *et al.*, 2017). Indeed, it has been demonstrated that hydrogen donation (or atom hydrogen abstraction) is one of the mechanisms that explain the antioxidant activity of trans-resveratrol.

Two ways of hydrogen abstraction are possible: 1) from *para*-OH group located in position 4' and 2) from a *meta*-OH located in position 3 or 5 (Fig 4) (Papuc *et al.*, 2017). In the first case, the delocalization of the unpaired electron occurs on the whole molecule generating a more stable semiquinone than the one produced when the unpaired electron is delocalized in a single ring. Thus, it is believed that the *para*- OH path is the most favorable mechanism to resveratrol scavenger action. Another important mechanism by which resveratrol exerts its antioxidant action is the regulation of endogenous antioxidant defense system (Papuc *et al.*, 2017). Resveratrol works by activating molecular targets involved in the regulation of the expression and activity of cellular antioxidants (Xia *et al.*, 2017). Currently, the best-known pathway of resveratrol is mediated by SIRT1. It has been demonstrated that resveratrol, by the activation of SIRT1 induces the up-regulation of SOD enzymes (SOD 1 and SOD2), GSH peroxidase 1(GPX1) and catalase (CAT) (Xia *et al.*, 2017).

Recent findings have shown that SIRT1 up-regulated SOD2 through the activation of the transcription factor FOXO1 (Xia *et al.*, 2017). Instead, the activation of FOXO3a causes the up-regulation of CAT. Resveratrol treatment was also associated with the up-regulation of NAD(P)H: quinone oxidoreductase 1 (NQO1), and ɣ-glutamylcysteine synthetase (Xia *et al.*, 2017). Although the direct target of resveratrol in this process has not yet been identified, the involvement of Nrf2 has been demonstrated. Furthermore, resveratrol increased intracellular content of glutathione, the major non-enzymatic antioxidant of the cell (Xia *et al.*, 2017). As well known, within the cell one of the main ROS-producing system is constituted to the electron transport chain of mitochondria (mtETC) (Murphy, 2009; Zavareh *et al*., 2015). There is a growing evidence that most of the $O_2^{\bullet -}$ generated by mitochondria occurs at the levels of NADH dehydrogenase and coenzyme Q, respectively, in the complexes I and III of electron transport chain (Kowaltowski and Vercesi, 1988; Rhoads *et al.* 2006; Murphy, 2009; Birben *et al.*, 2012, Xia *et al.*, 2017). To counteract the continuous production of $O_2^{\bullet -}$ that occurs physiologically at the mitochondrial level, this organelle is equipped with an efficient antioxidant system (Xia *et al.*, 2017). However, when the rate of electrons leaving the mtETC through terminal oxidases is slowed down or the rate of electron passing through the chain exceeds its capacity, an overproduction of ROS occurs. The beneficial effect of resveratrol on mitochondria function has been described in different cell types. Resveratrol through SIRT1 activates PGC-1α by stimulating mitochondrial biosynthesis (Ungvari *et al.*, 2011). The increase in mitochondria content reduces the flow of electrons per unit of mitochondria by decreasing the production of ROS.

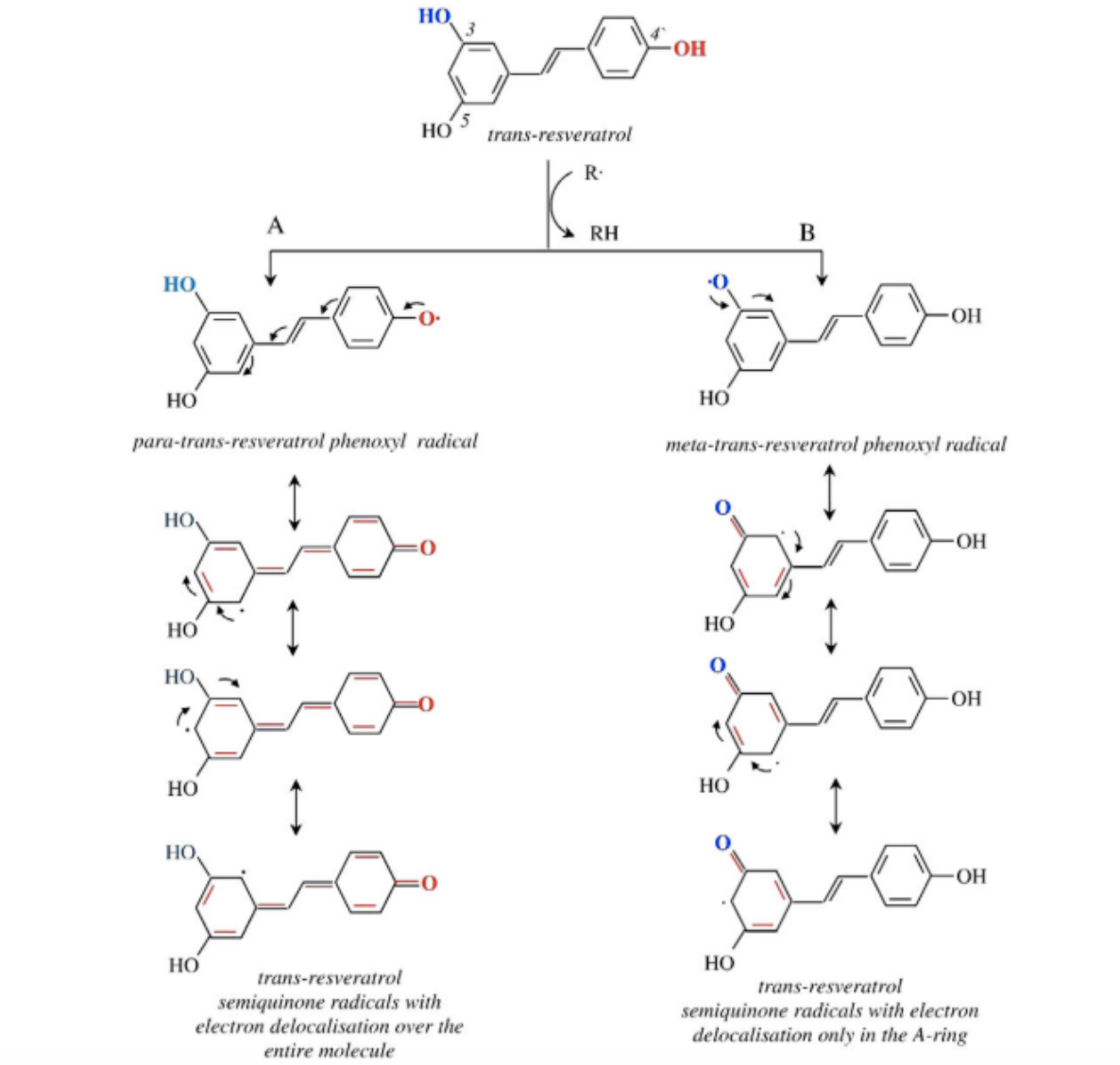


Fig.4. Representative image of two possible mechanism for scavenging action of *trans*-resveratrol from (Papuc *et al.*, 2017). A) hydrogen abstraction from the *para*-OH group and unpaired electron delocalization over the entire molecule. B) hydrogen abstraction from the *meta*-OH group (position 3 or 5) and unpaired electron delocalization in a single ring.

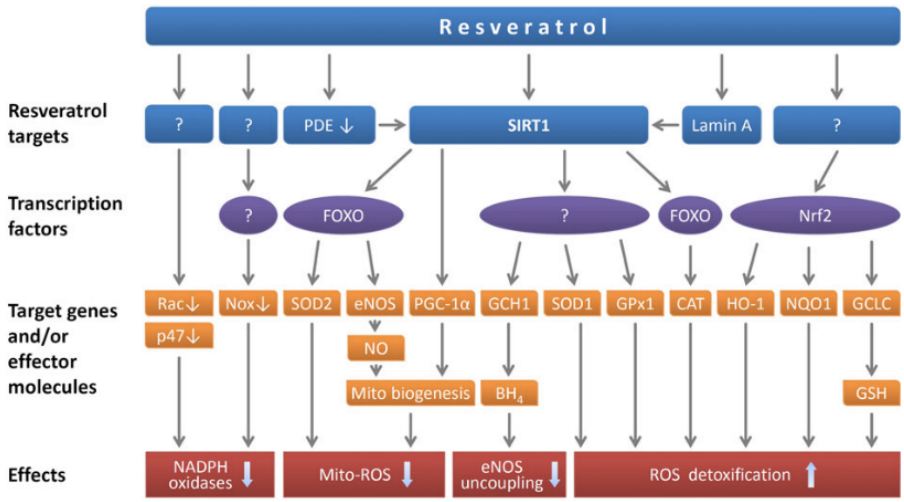


Fig.5. Schematic representation of the mechanisms through which resveratrol up-regulates endogenous antioxidant systems (Xia *et al.*, 2017).

2.5.1.1 Resveratrol effect on oocyte quality and *in vitro* embryo production

The antioxidant properties of resveratrol and its ability to regulate numerous cellular processes have led numerous researchers to investigate its effect on *in vitro* maturation of oocyte and embryonic development. *In vitro* culture conditions may affect oocyte quality leading to poor embryo development compared with *in vivo* produced embryos.

An increasing number of evidence indicate that resveratrol is a useful agent for oocyte maturation and subsequent embryonic development. Supplementation of culture medium with resveratrol had beneficial effect on *in vitro* embryo production in different domestic species (Galeati and Spinaci, 2015,) as demonstrated by improved blastocysts rate, hatching blastocyst rate, and number of blastocyst cells after *in vitro* maturation and fertilization (Galeati and Spinaci, 2015).

Resveratrol promoted nuclear maturation of bovine and goat oocytes (Mukherjee *et al.*, 2014; Wang *et al.*, 2014) by inducing progesterone secretion from cumulus cells that, in turn, enhanced the expression of the Mos/ MEK/p42 MAP kinase cascade genes (Wang *et al.*, 2014).

Beneficial effect of resveratrol on cytoplasm maturation of oocytes and subsequent embryo development has been also described. Downregulation of apoptosis-related genes such as Bax/Bcl-2, Bak, and Caspase-3 expressions in matured oocytes resulted in the significant improvement of the blastocyst formation rate and blastocyst quality after parthenogenetic activation or *in vitro* fertilization (Lee *et al.*, 2010; Kwak *et al.*, 2012; Sugiyama *et al.*, 2015).

Recently, Takeo *et al*. (2014) observed that resveratrol improved the distribution of cortical granules *in vitro* matured bovine oocytes enhancing fertilization outcome via reinforcement of the mechanisms responsible for the blockage of polyspermic fertilization.

Furthermore, the increase of the mitochondria number, mitochondria activity and ATP content of oocytes under resveratrol treatment underlined its positive effect on oocyte quality (Takeo *et al.*, 2014).

The molecular mechanisms of resveratrol's effects on oocyte quality might be mediated by SIRT1 activation. Sato *et al*. (2014) demonstrated that activation of SIRT1 by resveratrol enhanced the biosynthesis and degradation of mitochondria in porcine oocytes (Sato *et al.*, 2014). Resveratrol also increased ATP content and mitochondrial membrane potential, thereby improving mitochondrial function and the developmental ability of oocytes (Sato *et al.*, 2014).

In addition to its positive influence on bioenergetic status of the oocyte, resveratrol is also an antioxidant. Resveratrol addition to the IVM medium has been associated with a reduction of intracellular ROS levels and an increase of GSH content in cattle (Wang *et al.*, 2014), goat (Mukherjee *et al.*, 2014) and porcine (Kwak *et al.*, 2012) oocytes. Moreover, the up-regulation of the expression of other antioxidant enzymes as CAT, GPx4 and SOD1 has been demonstrated in porcine oocytes (Kwak *et al.*, 2012).

Taken together, all these data clearly indicated that the use of resveratrol during *in vitro* maturation may be an effective treatment to improve the oocyte developmental potential and the *in vitro* embryo production outcome in different mammalian species.

2.5.1.2 Protective effect of Resveratrol on oocyte quality under sub-optimal conditions

Recent studies provide new evidences that resveratrol may be considered a useful candidate to protect the oocytes from the deterioration of their quality induced by sub-optimal conditions.

Using a murine model Jia *et al*. (2018) proved that the negative impact of diet-induced obesity on the quality of oocytes can be alleviated by the addition of resveratrol.

Specifically, resveratrol attenuated the obesity-induced oxidative stress, mitochondrial misdistribution and abnormal spindle and chromosome arrangement of mouse oocytes (Jia *et al.*, 2018).

Administration of resveratrol can also be a useful strategy to protect oocytes against postovulatory aging *in vivo* and *in vitro* as reported in the mouse (Liang *et al.*, 2018) and the porcine model (Ma *et al.*, 2015). Such effect was mediated by the prevention of ROS production (Liang *et al.*, 2018) and the activation of SIRT1 expression which counteract the decline of the oocyte quality.

Moreover, both *in vivo* and *in vitro* experiments, showed that resveratrol can protect rat and mouse oocytes against toxic substances such as methylglyoxal, 2-bromopropane, and ethanol through preventing oxidative damage and apoptosis (Huang *et al.*, 2007, 2011; Chan, 2011; Liu *et al.*, 2013). Promising results have also been reported with regard to resveratrol ability to alleviate the negative impact of cryopreservation on the female gamete. Giaretta *et al.* (2013) showed that resveratrol supplementation to the IVM medium improved the resistance of MII porcine oocytes to damage induced by vitrification by modulating the apoptotic process (Giaretta *et al.*, 2013).

Beneficial effect of resveratrol has been also reported in vitrified/warmed prepubertal bovine oocytes, (Sprícigo *et al.*, 2017) and immature vitrified/warmed porcine oocytes (Santos *et al.*, 2018).

Resveratrol can also protect the oocytes against the harmful effects induced by hyperthermia. The oxidative stress generated by heat stress compromised the quality of the oocyte and determined a reduction of embryo development (Nabenishi *et al.*, 2012). Li *et al.* (2016), demonstrated that resveratrol treatment during *in vitro* maturation improved nuclear maturation and embryo development of pig oocytes under heat stress condition through its antioxidant activity (Li *et al.*, 2016).

Chapter 3:

Objectives

3- Objectives

The objective of the study was to evaluate the effect of resveratrol supplementation during *in vitro* maturation on the quality of oocytes in species of veterinary interest (domestic cat, prepubertal goats and sheep).

The research includes three different experimental studies which were performed to test the potential beneficial influence of resveratrol to improve the *in vitro* developmental competence of poor quality oocytes such as oocytes with low developmental competence (oocytes from prepubertal animals) or oocytes under sub-optimal condition (oocytes retrieved from cold stored ovaries and oocytes exposed to environmental contaminants).

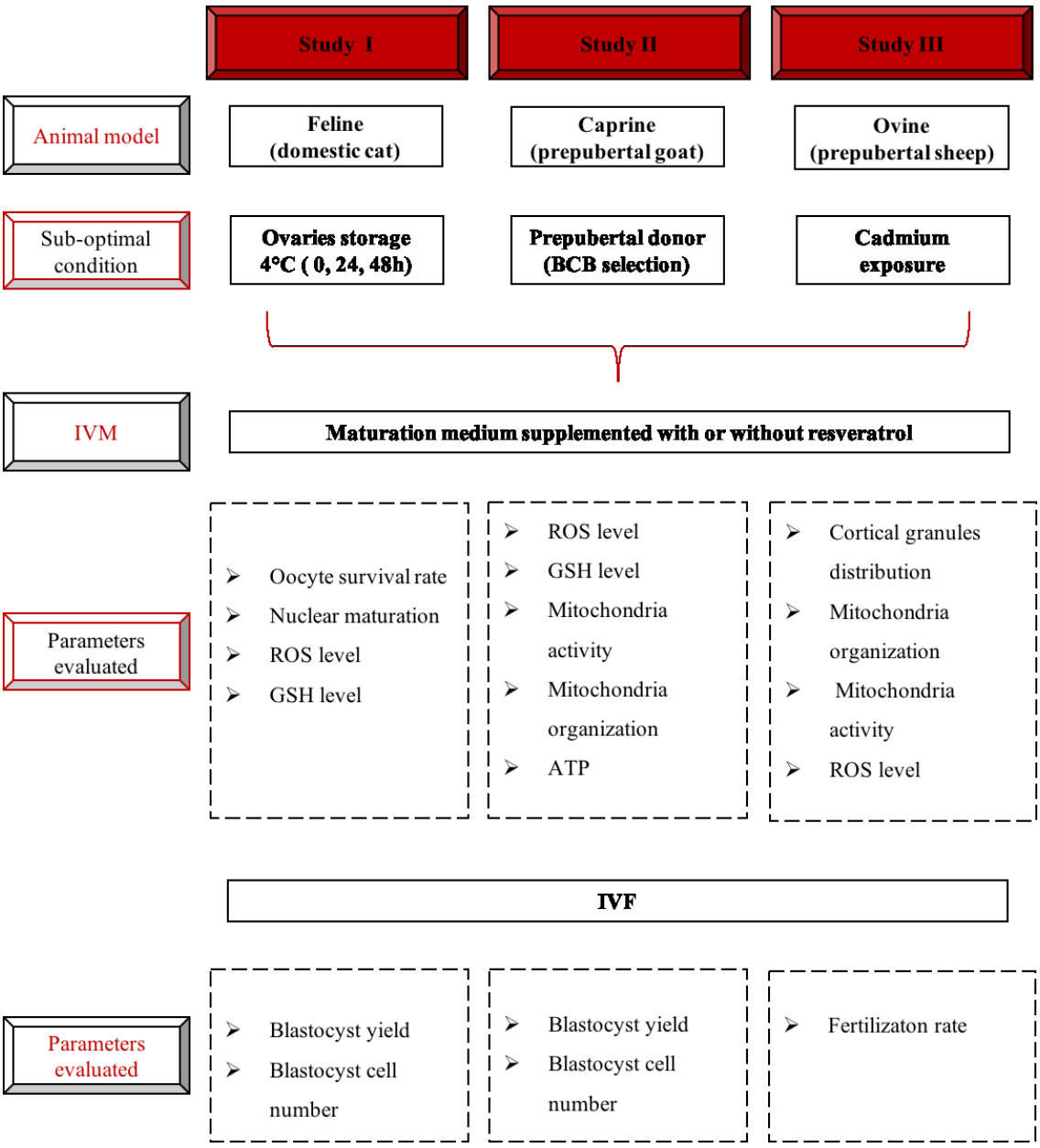
SPECIFIC OBJECTIVES

To evaluate the effect of resveratrol addition to the *in vitro* maturation medium on:

STUDY I: *in vitro* embryo production from domestic cat oocytes retrieved from ovaries stored at 4°C for 24 and 48h

STUDY II: *in vitro* embryo production from prepubertal goat oocytes selected by brilliant cresyl blue (BCB) staining

STUDY III: *In vitro* fertilization outcome of prepubertal sheep oocytes under cadmium exposure


Study I
Study II
Study III
Animal model
Feline (domestic cat)
Caprine (prepubertal goat)
Ovine (prepubertal sheep)
Sub-optimal condition
Ovaries storage 4°C (0, 24, 48h)
Prepubertal donor (BCB selection)
Cadmium exposure
IVM
Maturation medium supplemented with or without resveratrol
Parameters evaluated
Oocyte survival rate
Nuclear maturation
ROS level
GSH level
ROS level
GSH level
Mitochondria activity
Mitochondria organization
ATP
Cortical granules distribution
Mitochondria organization
Mitochondria activity
ROS level
IVF
Parameters evaluated
Blastocyst yield
Blastocyst cell number
Blastocyst yield
Blastocyst cell number
Fertilizaton rate

Chapter 4:

STUDY I

Resveratrol supplementation during in vitro maturation: Effect on developmental competence of oocytes retrieved from domestic cat ovaries stored at 4°C for 24 and 48h

ABSTRACT

Background: Feline oocytes have a unique tolerance to cold storage at 4°C. Domestic cat oocytes collected from ovaries stored at 4°C up to 72h maintain the ability to mature *in vitro*. However, the extension of the storage period beyond 24h decreases the oocyte ability to produce blastocysts after *in vitro* fertilization. Oxidative stress of *in vitro* matured oocytes is one of the factors affecting their potential developmental competence following ovary storage for more than 24h.

Aim: To evaluate whether supplementation of maturation medium with resveratrol would improve the *in vitro* embryo production after cold storage of cat ovaries up to 48h.

Methods: Ovaries were recovered from domestic queens during ovariectomy and stored at 4°C for 0 (control), 24 and 48h. After recovery cumulus-oocyte complexes (COCs) were cultured in maturation medium supplemented with 0 (24h-; 48h- groups) and 5 µM resveratrol (24h+; 48h+ groups) for 24h. COCs collected from fresh ovaries were matured *in vitro* (IVM) in standard condition as control. The meiotic competence and intracellular levels of reactive oxygen species (ROS) and glutathione (GSH) of oocytes were evaluated. After IVM, oocytes were fertilized *in vitro* and presumptive zygote culture for 7 days. The percentages of embryos cleaved and developing to the blastocyst stage and the blastocyst cell number were determined.

Results

There were no significant differences in the maturation rates of oocytes among the groups, irrespective of resveratrol supplementation.

The levels of GSH were similar between 24h+ and 24h- groups and higher ($P<0.05$) in 48h+ group respect to its counterpart 48h-. Significantly increased ($P<0.05$) GSH levels were recorded both in 24h+ and 48h+ oocytes compared to those of fresh control ones. Resveratrol supplementation decreased significantly ($P<0.05$) intracellular ROS levels in both 24h+ and 48h+ stored groups compared with their respective counterparts (24h-; 48h-).

The rate of blastocyst formation from oocytes of ovaries stored for 48h and matured with resveratrol was higher ($P<0.05$) than that of oocytes matured without resveratrol and similar to that of fresh control oocytes. Resveratrol did not affect blastocyst rate of 24h+ stored group compared to the respective counterpart 24-. Resveratrol treatment significantly ($P<0.05$) increased blastocyst cell number in both 24h and 48h groups compared to their respective counterparts

Conclusion: The antioxidant effect of resveratrol ameliorated the *in vitro* embryo production of cat oocytes recovered from ovaries stored at 4°C for 48h. These results provide a basis for improving culture conditions and extent the storage of cat ovarian tissue up to 48h ensuring the *in vitro* embryo production outcome.

INTRODUCTION

The accelerating decline in wild animal species through the word is an alarming problem. Destruction or fragmentation of habitat (Brooks *et al.*, 2002), climate change (Parmesan, 2006; Root *et al.*, 2003), invasive species (McGeoch *et al.*, 2010), diseases (Smith *et al.*, 2009) and poaching (Oldfield, 1988) are some of the causes which have led to an increase of the number of species threatened, vulnerable or endangered of extinction.

As regard to feline species, most of the 36-living species of wild cat are included in the Red List of Endangered Species of the IUNC (IUCN, 2018).

Assisted reproductive techniques (ARTs), such as *in vitro* embryo production and embryo transfer, cryopreservation of gametes and embryos, represent a valuable tools for conservation breading of endangered species (Pope, 2000;2006; Cocchia *et al*, 2015).

Recent progress has been made in the successful application of these technologies in domestic cat which represent a model for development of ARTs in non-domestic Felidae species (Prochowska *et al.*, 2017; Veraguas *et al.*, 2017). However, the efficiency of reproductive technologies applied to non-domestic and especially to endangered species remains low. Temporal storage of explanted gonads is a critical step in the setting of fertility preservation programs in wild felids. Indeed, the possibility of rescuing germplasm from feline females after death in the field may require the transport of ovaries, even for long distance, from the retrieval site to a specialized laboratory.

In this context, studies were addressed to establish the appropriate transport conditions (temperature, duration, medium) (Otoi *et al.*, 2001; Naoi *et al.*, 2007; Evecen *et al.*, 2009; Luu *et al.*, 2013, 2014; Cocchia *et al.*, 2015b). Naoi *et* colleagues (2007) investigated the effect of different temperatures (4°C, 23-25°C, 38°C) during ovaries storage on the meiotic and developmental competence of cat oocytes and concluded that only oocytes derived from ovaries maintained at 4°C maintained the ability to develop *in vitro* up to blastocyst stage.

Other authors have further confirmed the temperature of 4 °C as optimal for the temporary storage of the ovaries of both the domestic cat and the wild felines (Johnston *et al.*, 1991; Wolfe and Wildt, 1996; Otoi *et al.*, 2001; Evecen *et al.*, 2009; Luu *et al.*, 2014).

The storage of the ovaries at 4°C for 24 and 48h did not affect the ability of the oocyte to reach the metaphase II stage *in vitro* (Wolfe and Wildt, 1996; Otoi *et al.*, 2001; Luu *et al.*, 2014; Piras *et al.*, 2018), but decreased significantly when ovarian tissue was stored for longer periods (Wolfe and Wildt, 1996; Piras *et al.*, 2018) and is closely related to oocytes diameter (Otoi *et al.*, 2001). In fact, it has been observed that small oocytes are more sensitive to low temperatures. However, only oocytes retrieved from ovaries stored at 4°C for 24h could produce blastocysts following *in vitro* fertilization (Wolfe and Wildt, 1996; Piras *et al.*, 2018) and developed into live offspring after transfer into recipients (Pope *et al*., 2003).

The addition of relaxin, a member of the insulin-like family of hormones, to the maturation medium improved the blastocyst formation after *in vitro* fertilization of cat oocytes stored within the ovaries at 4°C for 24h (Luu *et al.*, 2013). Recently, Cocchia *et al*. (2015) reported that superoxide dismutase (SOD) supplementation in the transport media of domestic cat ovaries stored at 4°C for 72h reduced cellular apoptosis, enhanced oocyte survival and *in vitro* embryo production (Cocchia *et al.*, 2015).

In a previous study, we explored the effect of ovaries storage a 4°C up to 96h on redox state of *in vitro* matured oocytes (Piras *et al.*, 2018). We observed that preservation of the ovarian tissue for more than 24h induced a progressive increase of reactive oxygen species (ROS) associated with elevated lipid peroxidation of *in vitro* matured oocytes and reduced their ability to develop to the blastocyst stage. Increased levels of ROS beyond the physiological range may lead to oxidative stress and can cause a wide range of damages at the structural and molecular components of the oocyte, resulting in deterioration of its quality and reduction of developmental competence (Combelles *et al.*, 2009).

In order to counteract the negative effect of oxidative stress, the present study was addressed to explore whether treatment with resveratrol during *in vitro* maturation of domestic cat

oocytes could ameliorate the *in vitro* production after storage of the ovaries at 4°C for 24 and 48h.

MATERIALS AND METHOD

All chemicals in this study were purchased from Sigma Chemical Company (St. Louis. MO, USA) unless stated otherwise.

Ovary collection and storage

Ovaries were harvested from domestic queens (*Felis catus*, 8 months - 2 years of age) at random stage of oestrus cycle during routine ovariectomy at the Veterinary Teaching Hospital of the University of Sassari (Italy). Ovaries were placed in sterile 15-ml tubes containing PBS (Dulbecco's Phosphate Buffered Saline) with penicillin (100 IU/mL) and streptomycin (100mg/mL) and immediately transported to the laboratory. Ovaries were randomly divided in two groups:

-fresh ovaries (0 h, control group);

-ovaries to be stored at 4°C (stored groups).

For storage, each ovary was transferred under sterile conditions to a tube containing 4 ml of fresh pre-cooled PBS and held at 4°C in a refrigerator for 24 and 48h.

Oocyte *in vitro* maturation.

Ovaries from fresh and stored groups were sliced with a scalpel blade to release the cumulus-oocyte complexes (COCs). COCs were collected in sterile Petri dishes in dissection medium (DM; 25 mM Hepes-buffered TCM 199) supplemented with 0.1% (wt/vol) polyvinyl alcohol (PVA) and antibiotics (100 μg/ml penicillin and streptomycin). Only COCs with darkly pigmented ooplasm and completely surrounded by at least one layer of cumulus cells were selected for *in vitro* maturation (IVM).

COCs were matured in groups of 25-35 in 650 µL of IVM medium (TCM 199 supplemented 0.36 mM pyruvate, 2 mM glutamine, 2.2 mM calcium lactate, 1.2 mM cysteine, 4 mg/mL BSA and FSH 1 IU/mL and LH 1 IU/mL), in four-well Petri dishes in a humidified atmosphere of 5% CO_2, at 38.5°C for 24h.

Assessment of oocyte nuclear maturation

At the end of IVM, groups of oocytes were completely denuded of granulosa cells via gentle pipetting with a fine bore glass pipette in DM, stained with Hoechst 33342 (1µg/mL) in 1:1 (v/v) glycerol/PBS solution, placed on a slide and overlaid with a coverslip supported by four droplets of Vaseline. The nuclear configuration was classified under an epifluorescent microscopy (Olympus IX 70, Italy) as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII).

Measurement of intracellular ROS and GSH levels

Groups of matured oocytes (MII) of all experimental groups were selected on the basis of the presence of the first polar body and sampled for intracellular reactive oxygen species (ROS) and glutathione (GSH) level measurement. Briefly, 2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes Inc., Eugene, OR, USA) and 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CellTracker Blue CMF2HC, Molecular Probes) (Sovernigo *et al.*, 2017) were used to detect intracellular ROS as green fluorescence and GSH levels as blue fluorescence. A total of 30-35 oocytes from each treatment group were incubated in the dark for 30 minutes in DPBS-PVA containing 10µM H_2DCFDA and 10µM CellTracker Blue. After incubation, the oocytes were washed with DPBS-PVA; each oocyte was placed in a single 50 µL droplet of DPBS and observed using an epifluorescence microscope (Olympus IX 70) with UV filters (460 nm for ROS and 370 nm for GSH). Oocytes were positioned in the plane of focus, and the area of measurement was adapted to the size of the oocyte. Microscope adjustments and photomultiplier settings were kept constant for all experiments. The data of emission intensity/oocyte were reduced by compensation for the background fluorescence.

The fluorescent images were saved as graphic files in TIFF format. Intensities of fluorescence were analyzed using Image J software (version 1.40; National Institute of health, Bethesda, MD) and normalized to that of control oocytes.

In vitro **fertilization and embryo culture**

In vitro matured oocytes were fertilized in synthetic oviductal fluid (SOF) containing 4mg/mL BSA, 100 IU/mL penicillin, 50µg/mL gentamicin, at 38.5 °C and under a 5% CO_2, 5% O_2, 90% N_2 atmosphere (Sananmuanga *et al.*, 2011) in four-well Petri dishes with frozen-thawed spermatozoa (1×10^6 motile spermatozoa/mL) selected by the swim-up technique. Semen was collected from cauda epididymis of adult cats following routine orchiectomy (Bogliolo *et al.*, 2001; Pope *et al*, 2003) and frozen according to the procedure described by Tsutsui et al. (2003).

The sperm were co-incubated with the COCs for approximately 22h at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Presumptive zygotes were washed and cultured in 650 µl of SOF containing 4 mg/mL BSA, and 100 IU/mL penicillin. On day 3 after IVF (day=0) the embryos were transferred to SOF supplemented with 10% fetal calf serum (FCS) and 2% MEM essential amino acids, and cultured at 38.5°C in a humidified atmosphere of 5% CO_2 in air for additional 4 days (Sananmuanga *et al.*, 2011). The embryos that cleaved and reached the blastocyst stage were evaluated morphologically on day 2 and on day7 after IVF, respectively.

Assessment of blastocyst cell number

Analysis of blastocyst cell number was performed by differential staining of the inner cell mass (ICM) and trophectoderm (TE) cell compartments (Bogliolo *et al.*, 2011). To differentially stain ICM and TE nuclei, blastocysts derived from control and stored groups were exposed to 1% Triton X-100 in 20 mM HEPES-buffered TCM 199 containing 30 mg/mL propidium iodide (PI) for 35 to 40 second.

The blastocysts were then transferred into ice-cold ethanol for 2 to 5 second. Finally, blastocysts were incubated in medium with 50% (v/v) glycerol and ethanol containing 0.1 mg/mL bis-benzimide (Hoechst 33342) for 5 min. The blastocysts were directly mounted into a small droplet of glycerol on a glass slide and examined under epifluorescent microscope (Olym-pus IX70, Italy). A digital image of each embryo was taken, and the numbers of TE (red) and ICM (blue) nuclei were counted.

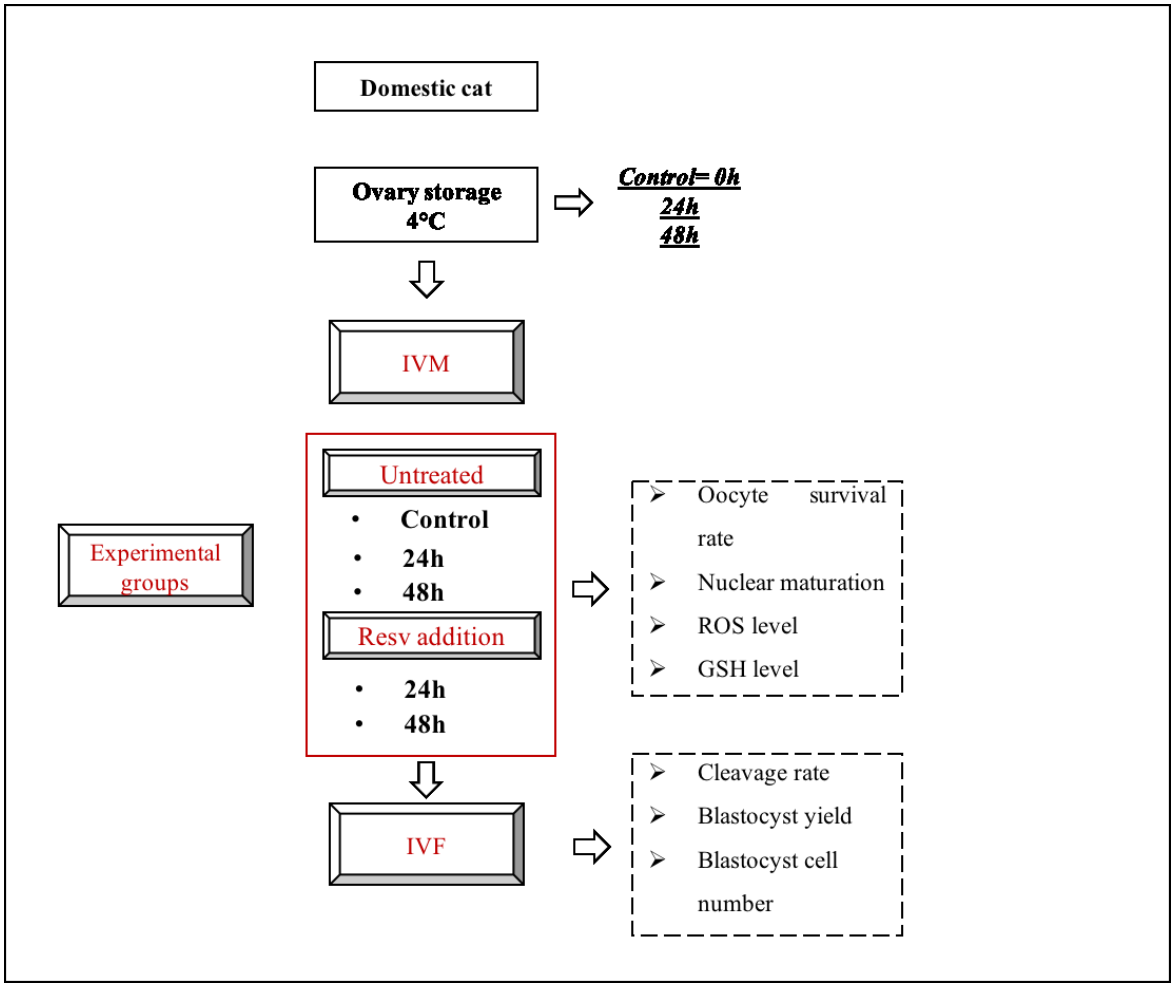
Experimental design

Cumulus-oocyte complexes recovered from ovaries stored at 4°C for 24 and 48h were matured *in vitro* in presence (+) or absence (-) of 5µM Resveratrol (Resv). Oocytes from fresh ovaries (0h stored) were matured *in vitro* in standard condition.

Experiment 1 was performed to evaluate the effect of Resv on oocyte survival, nuclear maturation, and intracellular levels of glutathione and reactive oxygen species.

In **Experiment 2**, we analyzed the effect of Resv on oocyte developmental competence. After *in vitro* fertilization, cleavage rate, blastocyst formation and cell number were assessed.

EXPERIMENTAL DESIGN



Statistical analysis

All statistical analyses were performed using Stata/IC 11.2 (StataCorp LP, USA). Categorical data of survival, maturation, cleavage and development to blastocyst stage were analyzed by chi-square test. The data of blastocyst cell number, intracellular ROS and GSH levels are expressed as mean ± standard error (SEM) and analyzed using a one-way univariate analysis of variance (ANOVA) followed by Bonferroni's as post hoc test. $P < 0.05$ was considered statistically significant. All the experiments were replicated at least for three times.

RESULTS

Effect of resveratrol on oocyte survival and nuclear maturation

After *in vitro* maturation, the survival rate (Table 1) was significantly lower (P<0.05) in oocytes retrieved from ovaries stored at 4°C for 24h- and 48h- compared to control group. The percentage of viable oocytes of 24h+ group was similar to that of control oocytes. Treatment with resveratrol did not significantly affect the survival rate of the oocytes of 24h+ and 48h+ stored groups compared to their respective counterpart (24h-, 48h-). The number of oocytes reaching MII stage did not differ among groups (Table 1).

Storage time (h)	Resv	N° oocytes	Survival (%)	MII (%)
0 (control)		85	80 (94.12) [a]	52 (61.18)
24	-	63	47 (74.60) [bc]	38 (60.32)
	+	66	54 (81.81) [ab]	41 (62.12)
48	-	69	41 (59.42) [c]	41 (59.42)
	+	77	58 (75.32) [bc]	48 (62.34)

Tab.1 Effect of resveratrol supplementation during IVM on survival and nuclear maturation of cat oocytes retrieved from ovaries stored at 4°C for 24h and 48h. Different superscript letters in the same column (a, b, c) indicate a significant difference P<0.05. MII: metaphase II stage.

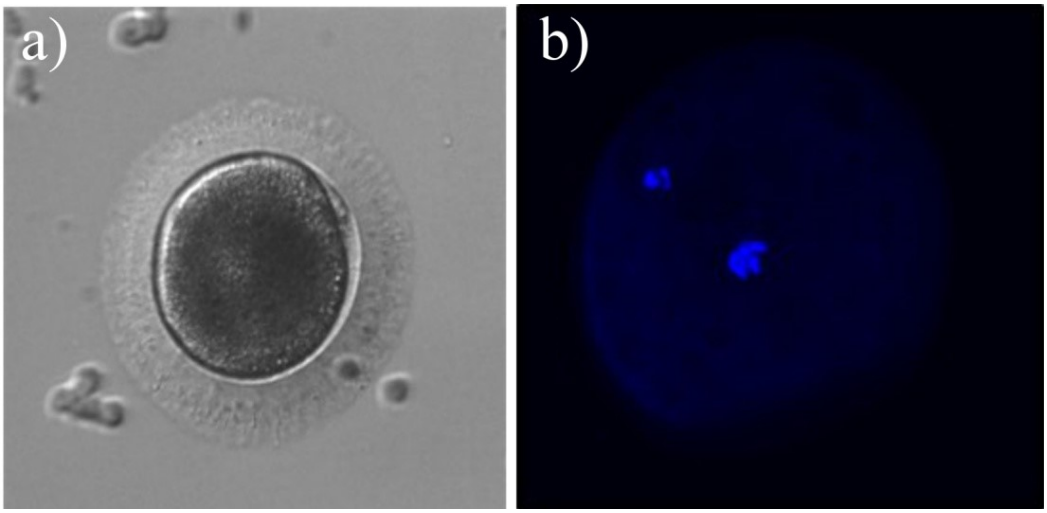


Fig.1. Representative images of *in vitro* matured cat oocytes under phase contrast microscopy (a) and under epifluorescent microscopy after staining with Hoechst 33342 (b).

Effect of resveratrol on intracellular levels of GSH and ROS of *in vitro* matured oocytes

The levels of GSH of *in vitro* matured oocytes (Figure 2 a, a') were similar among control, 24h-, 48h- stored groups. Treatment with resveratrol significantly ($P< 0.05$) increased GSH level of oocytes from ovaries stored for 24h+ and 48h+ compared to control group. Significantly higher levels of GSH were recorded in 48h+ stored group respect to its counterpart 48h-.

ROS levels were higher ($P< 0.05$) in the oocytes of 48h- stored group, compared to those found in 24h- stored and control groups (Fig.2 b, b'). Resveratrol supplementation decreased significantly ($P<0.05$) intracellular ROS levels in both 24h+ and 48h+ stored groups compared with their respective counterparts (24h-; 48h-).

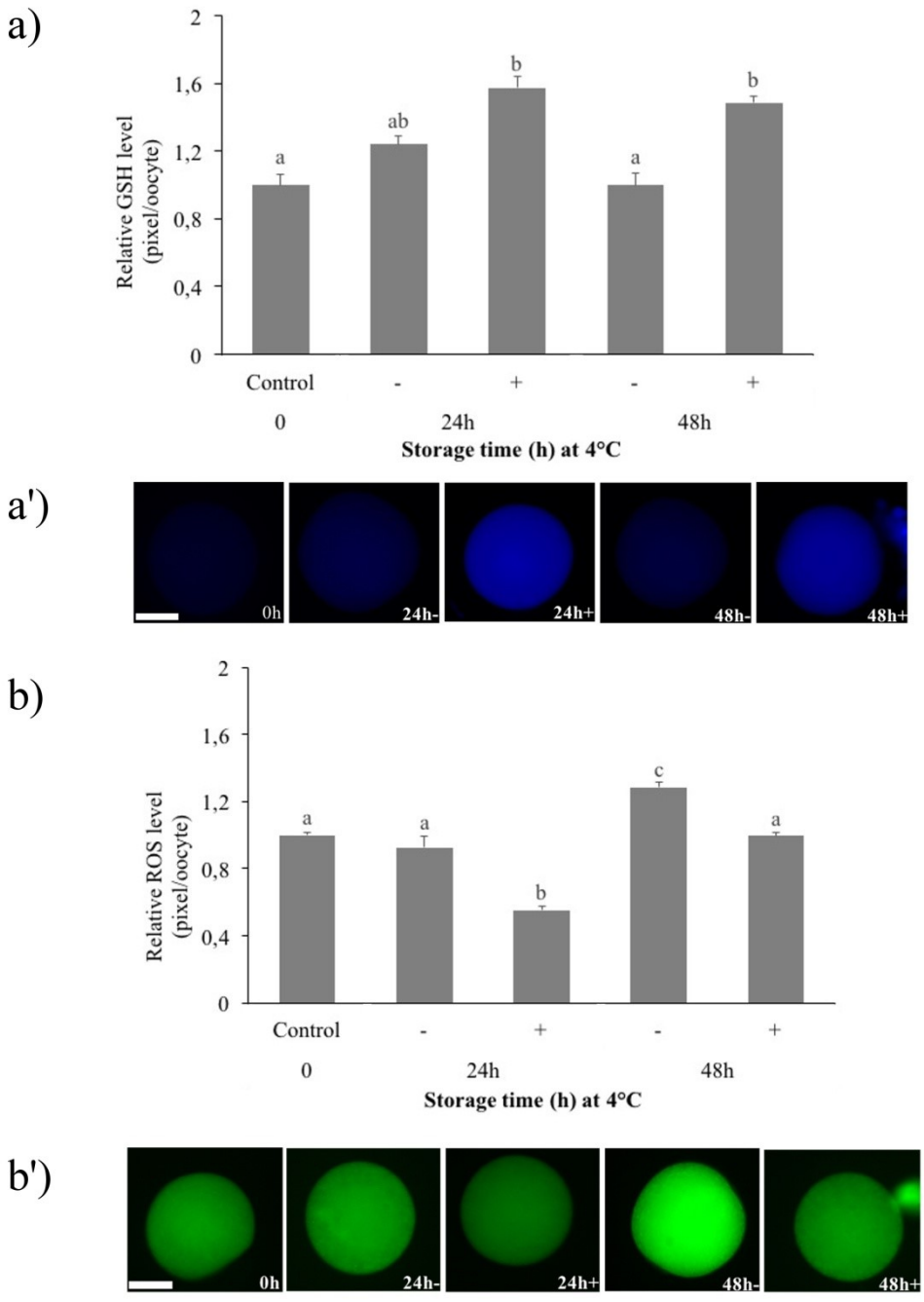


Fig.2. Effect of resveratrol on intracellular GSH **(a)** and ROS **(b)** levels of *in vitro* matured oocytes retrieved from ovaries stored at 4°C for 24h and 48h. Epifluorescence photomicrographs of MII oocytes that were stained with CellTracker Blue to determine the level of GSH **(a')** and with 2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) to detect ROS **(b').** Different superscripts among storage times indicate $P<0.05$.

Effect of resveratrol on oocyte developmental competence and blastocyst cell number

The developmental stages of embryos across treatments are shown in Table 2. The proportion of oocytes that progressed to the first cleavage stage was similar among groups. The percentages of blastocysts relative to the number of cleaved embryos (developmental competence) and to the total number of oocytes (blastocyst yield) was lower in 48h- group compared to those of control, 24h- and 24h+ groups. Resveratrol treatment significantly ($P<0.05$) increased blastocyst development of the 48h+ group at rates comparable to those of control, 24h- and 24h+ groups and higher than those of the 48h- respective counterpart. The mean number of total cell/blastocyst, ICM and TE (84.8±16.7; 16.3±1.5; 68.5±3.5, respectively) were significantly ($P<0.05$) lower after ovary storage for 48h compared to control (186.4±7.8; 43.1±2.5; 143.3±6.8, respectively) and 24h (170.54±10.8; 39.4±4; 131.09±8.3, respectively) stored groups. Resveratrol treatment significantly ($P<0.05$) increased blastocyst cell number in both 24h (234.15±15.2; 57.7±3.2; 173.4±11.2, respectively) and 48h (147.6±10.1; 36.3±3.7; 110.5±8.2, respectively) groups compared to their respective counterparts. These values are similar to those of fresh control group (Figure 4).

Storage time (h)	Resv	N° oocytes fertilized	N° cleaved embryos (%)	N° blastocysts/ cleaved embryos (%)	N° blastocysts/ total oocytes (%)
0 (Control)		82	36 (43.90)	22 (61.11) [a]	22 (26.83) [a]
24	-	79	32 (40.50)	18 (56.25) [a]	18 (22.78) [a]
	+	77	32 (41.56)	19 (59.37) [a]	19 (24.67) [a]
48	-	84	31 (36.90)	4 (12.90) [b]	4 (4.76) [b]
	+	74	28 (37.84)	14 (50.00) [a]	14 (18.92) [a]

Tab.2 Effect of resveratrol supplementation to IVM medium on embryo development of cat oocytes retrieved from ovaries stored at 4°C for 24h and 48h. Different superscript letters in the same column (a, b) indicate a significant difference $P<0.05$.

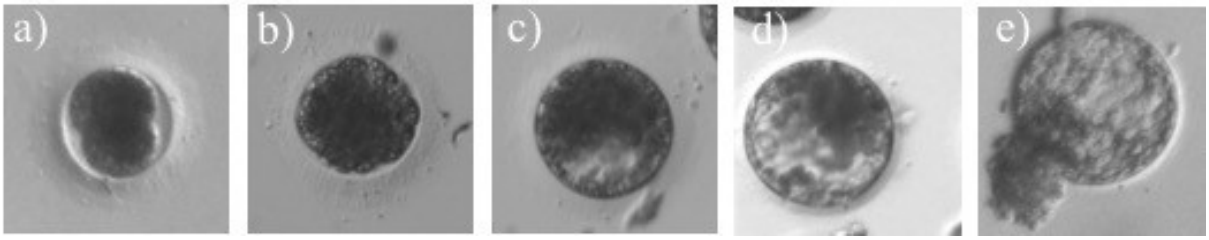


Fig.3. Representative images of cat *in vitro* produced embryos at different development stages, a) 2-cell stage; b) morulae; c) early blastocyst; d) blastocyst; e) hatched blastocyst.

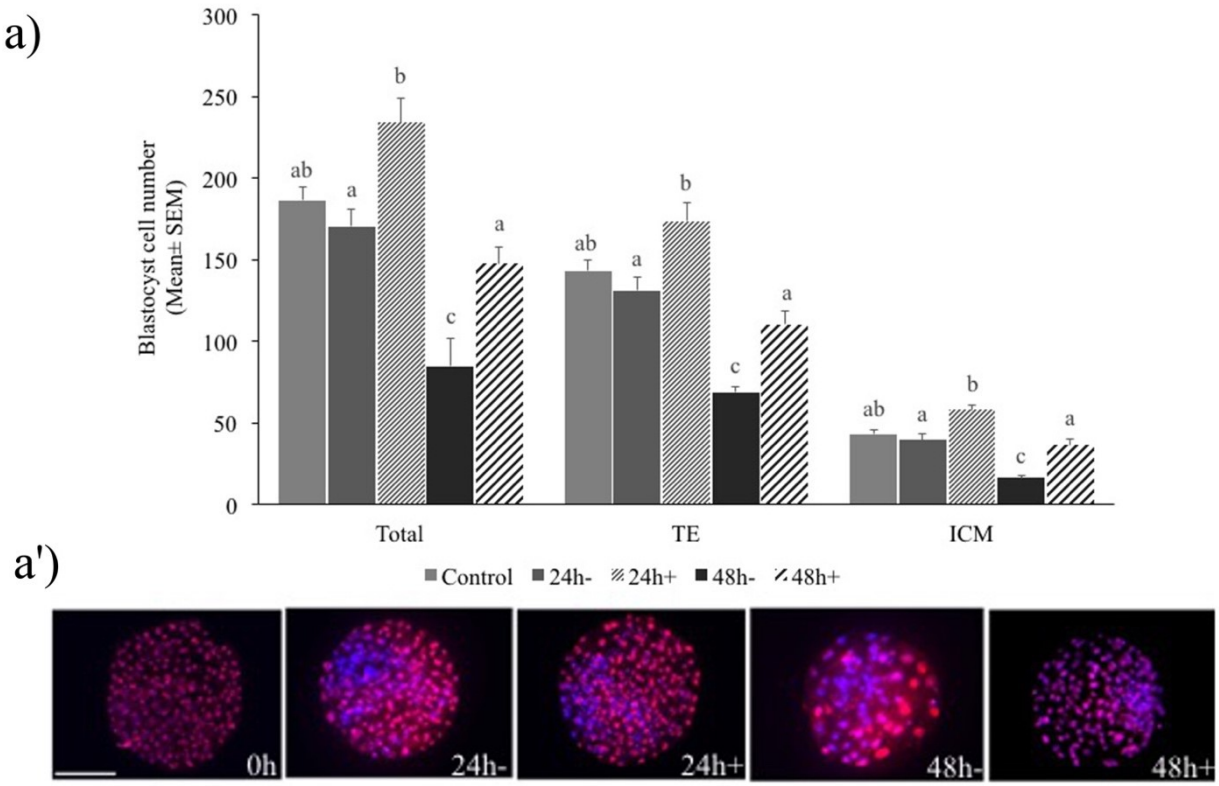


Fig.4. Effect of resveratrol supplementation in IVM medium on number of cell of blastocysts developed from cat oocytes retrieved from ovaries stored at 4°C for 24h and 48h. a) Total, inner cell mass (ICM) and trophoectoderm (TE) cell number a'). Representative epifluorescent images of blastocysts after differential staining with Hoechst 3342 and propidium iodide. Different superscripts among storage times indicate $P<0.05$. Scal bar =100μm.

DISCUSSION

In the present study, we explored the potential beneficial effect of resveratrol addition to the *in vitro* maturation medium on embryonic development, after IVF, of cat oocytes recovered from ovaries stored at 4°C for 24 and 48 h.

The main findings of the addition of resveratrol to maturation medium of cat oocytes were: *a)* increased the GSH content and reduced the ROS levels of *in vitro* matured oocytes after 24h and 48h of ovary storage *b)* improved blastocyst rate after IVF of oocytes retrieved from ovaries stored for 48h and *c)* increased the blastocyst cell numbers in both 24 and 48 h groups.

Low temperature (4°C) is considered the most suitable for temporary storage of feline ovarian tissue (Naoi *et al.*, 2007; Luu *et al.*, 2014). However, when the ovaries refrigeration exceeded 24h embryonic development markedly declined (Wolfe and Wildt, 1996; Cocchia *et al.*, 2015; Piras *et al.*, 2018). In the current study, the presence of resveratrol during maturation not only had a positive effect on the *in vitro* embryo production after 48h of ovaries storage, but also supported oocyte developmental competence with percentages of blastocyst formation comparable to that obtained from fresh control oocytes.

However, we found that the supplementation of IVM with Resv did not affect oocyte meiotic competence; this finding is consistent with the results of other authors in different species reporting the absence of positive effect of resveratrol on *in vitro* nuclear maturation (Kwak *et al.*, 2012; Mukherjee *et al.*, 2014; Sprícigo *et al.*, 2017).

To the best of our knowledge, only one study explored the effect of resveratrol in the domestic cat. In particular, the Authors reported that percentages of cleaved embryos and ability to form more advanced embryo stages (48.7±6.4 %; 6.9 %, respectively) were improved ($p < 0.05$) when immature cat oocytes were exposed to Resv before vitrification compared to not exposed oocytes (17.7± 2.5%; 0%, respectively) (Comizzoli *et al.*, 2009).

The rationale for the resveratrol treatment derived from the results of our previous research which highlighted the involvement of the oxidative stress in the loss of oocyte developmental competence after ovaries storage for more than 24h (Piras *et al.*, 2018). Indeed, a low *in vitro* embryo production after 48h ovary storage was associated with a high level of ROS and lipid peroxidation. of IVM oocytes (Piras *et al.*, 2018).

Resveratrol is a strong antioxidant which maintains the levels of antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) (Robb *et al.*, 2008; Wang *et al.*, 2014) and improves the distribution and function of mitochondria (Gerhart-Hines *et al.*, 2007; Price *et al.*, 2013).

Different studies in other species underlined the beneficial impact of resveratrol addition during IVM on the developmental potential of oocytes after fertilization or artificial activation, by increasing their intracellular GSH and decreasing ROS content (Galeati and Spinaci, 2015).
GSH is the main non-protein sulphydryl compound in oocytes and embryos protecting them from oxidative damage induced by ROS attack. The presence of high GSH content in matured oocytes is a marker of cytoplasm maturity and it is associated with high *in vitro* blastocyst production in several species (De Matos and Fumus, 2000; De Matos *et al.*, 2002, 2003; Li *et al.*, 2014).

Recent studies have been addressed to improve transport and culture medium conditions with the aim to enhance *in vitro* embryo production after cold storage of cat ovaries. The study of Luu *et al.* (2013) found that the addition of relaxin to the *in vitro* maturation medium ameliorated the blastocyst yield after cat ovaries storage at 4°C for 24h (Luu *et al.*, 2013). In fact, the rate of blastocyst formation from oocytes matured with relaxin (16.0%) was higher than that from oocytes matured without relaxin (5.9%).
Moreover, superoxide dismutase (SOD) supplementation to the transport medium of cat ovaries positively affected oocyte quality and reduced cellular apoptosis (Cocchia *et al.*, 2015).

The presence of SOD during 48h and 72h storage supported the development to blastocyst stage (7% and 4% blastocysts/total oocytes respectively) whereas in the SOD-free groups embryos did not developed to blastocyst.

In the current study, the percentage of blastocyst yield obtained after addition of resveratrol after 24h (24%) and 48h (18%) was higher than those of the above-mentioned studies (Luu *et al.*, 2014; Cocchia *et al.*, 2015).

In conclusion, resveratrol treatment during *in vitro* maturation of cat oocytes recovered from ovaries stored at 4°C for 48h enhanced *in vitro* embryo production. These data provide a basis for extending the possibility of ovary collection and storage, especially for feline wild species, which die in geographic areas far from specialized laboratories.

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Chapter 5:

STUDY II

Resveratrol supplementation during in vitro maturation: Effect on developmental competence of prepubertal goat oocytes selected by brilliant cresyl blue staining

ABSTRACT

Background*:* *In vitro* embryo production using oocytes derived from prepubertal animals in conjunction with *in vitro* embryo transfer (JIVET) may allow accelerating genetic gain in livestock breeding programs decreasing the generation intervals. A widely used animal model for the JIVET research field is the caprine species, but the efficiency of this technique remains low mainly due to reduce *in vitro* embryo development competence of prepubertal oocytes. Brilliant cresyl blue (BCB) staining has been proposed as a non-invasive method for the selection of high quality immature oocyte in animal studies.

Aim: to investigate the effect of resveratrol supplementation to maturation medium on developmental ability of prepubertal goat oocytes selected by brilliant cresyl blue (BCB) staining.

Methods: Oocytes collected from slaughterhouse-derived ovaries of prepubertal goat were selected with 13 µM BCB and classified as grown BCB+ (blue cytoplasm) and growing BCB- (colourless cytoplasm) oocytes. After BCB selection, oocytes were *in vitro* matured (IVM) in conventional maturation medium (TCM-199 with hormones and serum) and supplemented with 1 µM (BCB+R and BCB-R) and without (BCB+C and BCB-C) resveratrol. The meiotic competence and intracellular levels of reactive oxygen species (ROS) and glutathione (GSH), mitochondrial activity and distribution and ATP content of oocytes were evaluated. After IVM, oocytes were fertilized with fresh semen and presumptive zygotes cultured for 8 days. The number of embryos cleaved, blastocyst rate and blastocyst cells numbers were determined.

Results: The development to blastocyst stage was significantly ($P<0.05$) higher in BCB+R oocytes matured with resveratrol compared with its control BCB+C and with those of BCB- groups. Resveratrol supplementation significantly ($P<0.05$) increased blastocyst development of BCB- oocytes which was similar to that of BCB+ oocytes. No difference was found in the blastocyst cells numbers among groups.

GSH levels were significantly ($P<0.05$) higher in both BCB groups treated with resveratrol than their respective controls. No differences were found in mitochondrial activity, ROS level and ATP content among groups. Resveratrol modified the distribution of active mitochondria in BCB+ and BCB- groups.

Conclusion: Supplementation of resveratrol during *in vitro* maturation improved embryo development to blastocyst stage with more significance in better quality oocytes (BCB+). The increase of GSH level and the modification of active mitochondria organization induced by resveratrol could be some of the mechanisms underlying the effect on oocyte quality.

INTRODUCTION

Goat production in Mediterranean countries is economically and socially important. In this species, artificial insemination (AI) is the most used reproductive technology. However, *in vitro* embryo production using oocytes derived from prepubertal animals in conjunction with *in vitro* embryo transfer, termed as juvenile *in vitro* embryo transfer (JIVET), can be used by breeders to accelerate genetic gain in livestock breeding programs. These technologies allow an increase of selection intensity placed on females and they can also reduce the optimal age at which animals are selected, and thus decrease the generation intervals. The addition of JIVET to a AI yielded an extra 25 to 60 % genetic gain in sheep programs (Granleese *et al.*, 2017). However, although in the late 70' the first births using JIVET have been obtained (Trounson *et al.*, 1977), the efficiency of this technology remains low mainly due to reduce *in vitro* embryo development competence of oocytes coming from prepubertal females compared to those from adult counterparts (O'Brien *et al*., 1996; 1997; Ledda *et al.* 1997; Marchal *et al.*, 2001; Palma *et al.*, 2001; Leoni *et al.*, 2009). The reduction of developmental competence of oocytes from prepubertal animals after *in vitro* maturation and fertilization has been related to oocyte structural and molecular abnormalities (O' Brien *et al.,*1996;Gandolfi *et al.*, 1998; Ledda *et al.*, 2001; Kochhar *et al.*, 2002; Velilla *et al.*, 2004, 2005, 2006; Morton, 2008;) which are signs of their poor quality.

In order to improve *in vitro* embryo production, the selection of high quality oocytes is crucial. Brilliant cresyl blue (BCB) staining is a non-invasive method used for the selection of immature oocyte in animal studies (Opiela and Kątska-Książkiewicz, 2013b). BCB is a glucose-6-phosphate dehydrogenase (G6PD) substrate, from which it is reduced from blue to a colorless compound. G6PD activity gradually decreases as oocytes reach their growth phase (Mangia and Epstein, 1975). Thus, grown oocytes present a low G6PD activity and cannot reduce BCB, so exhibit a blue cytoplasm, while growing oocytes that have high G6PD activity reduce BCB and present an unstained cytoplasm (Ericsson *et al.*, 1993).

Several studies in cattle (Pujol *et al.*, 2004; Alm *et al.*, 2005), sheep (Catalá *et al.*, 2011; Wang *et al.*, 2012), horse (Mohammadi-Sangcheshmeh *et al.*, 2011), goat (Rodríguez-González *et al.*, 2003), buffalo (Manjunatha *et al.*, 2007), and mice (Wu *et al.*, 2007) showed that BCB+ oocytes have higher embryo development competence compared to BCB- oocytes. However, in adult goat was observed a 3.6% of morphological good oocytes showed signs of degeneration following the staining with BCB (Opiela and Kątska-Książkiewicz, 2013 b).

An important factor which contributes to the poor quality of *in vitro* matured oocytes may be oxidative stress (Khazaei *et al.*, 2017).

Oocytes from prepubertal females oocytes are less able to maintain an appropriate redox homeostasis in response to oxidative stress generated by the *in vitro* condition compared to those from adult females (Yuan *et al.*, 2012). This is due to an altered synthesis of endogenous antioxidants (Yuan *et al.*, 2012; Jiao *et al.*, 2013). The addition of antioxidants to the maturation medium has been proposed as a good strategy to overcome the effect of oxidative stress increasing embryo development (Khazaei *et al.*, 2017; Sovernigo *et al.*, 2017).

Resveratrol (3,4,5-trihydroxy-trans-stilbene) is a small polyphenol synthesized by several plants as nuts, mulberry and grapes (Jeandet *et al.*, 2012). This phytoalexin is a potent antioxidant that, by activation of SIRT1, a NAD+ - dependent deacetylases belonging Sirtuin family, induces the upregulation of endogen antioxidant system (Tatone *et al.*, 2014). Recent studies highlighted that, SIRT1 acts as sensor of the redox state in oocytes and granulosa cells (Tatone *et al.*, 2014). Furthermore, resveratrol is involved in the regulation of energy homeostasis (Price *et al.*, 2013) metabolism (Liang and Ward, 2006; Gerhart-Hines *et al.*, 2007) level of estrogen (Bhat *et al.*, 2001) and genomic stability (Ortega and Duleba, 2015). It has also been observed that resveratrol supplementation during *in vitro* maturation positively affected oocyte quality, fertilization and embryo development outcomes in goat, cattle and pig (Galeati and Spinaci, 2015).

This study was addressed to investigate the effect of resveratrol supplementation to the *in vitro* maturation medium on developmental ability of prepubertal goat oocytes selected by BCB. To this end the bioenergetic/oxidative status of *in vitro* matured oocytes, oocyte cleavage, blastocyst formation and quality following *in vitro* fertilization were analyzed.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich Chemical Co.

Oocytes collection and brilliant cresyl blue staining

Oocytes were collected from ovaries of slaughtered prepubertal (30 to 45 – days old) goats (Capra hircus). Oocytes with two or more complete layers of compact cumulus cells and with a uniform cytoplasm were selected for IVM. In the experiment 2 and 3 morphological selected oocytes were incubated with BCB 13 µM for 45 minute under 5% CO_2 in air at 38.5°C. After BCB exposure, oocytes were classified as cytoplasm coloration, BCB+ (blue) or BCB- (colorless), and then disposed to IVM (Catalá *et al*., 2011).

In vitro maturation (IVM), in vitro fertilization (IVF) and embryo culture (IVEC)

Groups of 25-30 oocytes were matured in TCM-199 supplemented with 5µg/ml FSH, 5µg/ml LH, 1µg/ml 17 ß estradiol, 10ng/ml EGF, 10% fetal bovine serum, 5µg/ml gentamycin, 1mM L-glutamine, 0,2mM sodium pyruvate for 24h under 5% CO_2 in air at 38.5°C. After IVM, the oocytes were inseminated with fresh semen, obtained from three Murciano-Granadino bucks of proven fertility. Highly motile spermatozoa were selected by Bovipure density gradient (Nidacon EVB S.L., Barcelona, Spain). Oocytes were transferred in BO-IVF medium (IVF Bioscience; UK) for fertilization with $1X10^6$ spermatozoa/mL for 20h under 5% CO_2, 5% O_2 and 90% N_2 atmosphere at 38.5°C. At the end of the *in vitro* fertilization, presumptive zygotes were cultured in BO-IVC (IVF Bioscience; UK) for 8 days under 5% CO_2, 5% O_2 and 90% N_2 atmosphere at 38.5°C. The cleavage rate and blastocyst rate/blastocyst cell number were recorded at 48h and on day 8.

Assessment of nuclear status

After IVM, denuded oocytes were fixed in ethanol and stained with 1µM Hoechst 33342 solution (Invitrogen) for 1h. The nuclear configuration was classified under an epifluorescent microscopy (Olympus BX50) as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII).

Blastocyst differential staining

Analysis of blastocyst cell number was performed by differential staining of the inner cell mass (ICM) and trophectoderm (TE) cell compartments (Thouas *et al.*, 2001). Briefly, blastocysts were first incubated for 15 second in TCM199 with 1% Triton X-100 and 100µg/ml propidium iodide and then transferred into an ethanol solution with Hoechst 33342 for 3h. A digital image of each blastocyst was taken by epifluorescence microscope and the numbers of TE (red) and ICM (blue) nuclei were counted by Image j software (ImageJ 1.5Oi).

Measurement of GSH and ROS levels

Denuded oocytes at the MII stage (presence of the first polar body) were incubated in the dark for 30 minute with 10 µM 2'7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes, Eugene, OR, USA) or 10 µM 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (Cell Tracker Blue; CMF_2HC; Molecular Probes, Eugene, OR, USA) for ROS and GSH detection respectively. Epifluorescence microscope with UV filter (460nm for ROS and 370 nm for GSH) was used to take a digital images and fluorescence intensities of the oocytes were analyzed using Image j software (Soto-Heras *et al.*, 2018).

Quantification of ATP intracellular content

Groups of 6 MII oocytes were completely denuded by gently pipetting and placed into Eppendorf with 200 µl ultrapure water and stored at -80°C until their analysis. The ATP content of oocytes was measured using the adenosine 5-triphosphate bioluminescent somatic cell assay kit (FLASC) as described previously (Catalá *et al.*, 2013).
The sample light generated by ATP-dependent luciferin-luciferase reaction was immediately measured with a luminometer.

Evaluation of mitochondrial distribution and activity

After IVM, MII oocytes were denuded and incubated in the dark for 30 minute with 200nM MitoTracker Orange CMTMRos (Molecular Probes, Eugene, OR, USA) under 5% CO_2 in air at 38.5°C. After incubation, oocytes were fixed in 3% paraformaldehyde for 60 minute at 38°C and stained with 1µM Hoechst 33342 solution. Oocytes were stored at 4°C in the dark until their analysis (Catalá *et al.*, 2011). Mitochondria analysis was performed using Leica TCS SP5 CLSM with LAS lite 170 Image software equipped with a 405-nm diode laser and a multiphoton laser. In each individual oocyte MitoTracker Orange CMTMRos fluorescence intensities were measured at the equatorial plane as described previously (Martino *et al.*, 2016). Leica LAS AF Lite image analysis software package (Leica Microsystems GmbH, Wetzlar, Germany) was used for the quantitative analysis of fluorescence intensity. Mitochondrial distribution patterns were classified in two groups, as previously reported, with some modifications (Leoni *et al.*, 2015): 1) Pattern A: homogeneous fine, with small granulations spread throughout the cytoplasm; 2) Pattern B: heterogeneous clustered, with large granulations spread throughout the cytoplasm or located in specific cytoplasmic domains.

Experimental design

Experiment 1: *Effect of resveratrol supplementation at different concentrations on oocyte developmental competence*

The dose-responsive effects of resveratrol on oocyte developmental competence was evaluated. Resveratrol was added in the IVM medium at the concentration of 0,5 μM and 1 μM. A group of oocytes were cultured in absence of resveratrol, as control (C). After *in vitro* fertilization (IVF) cleavage rate, blastocyst formation and cell number were evaluated.

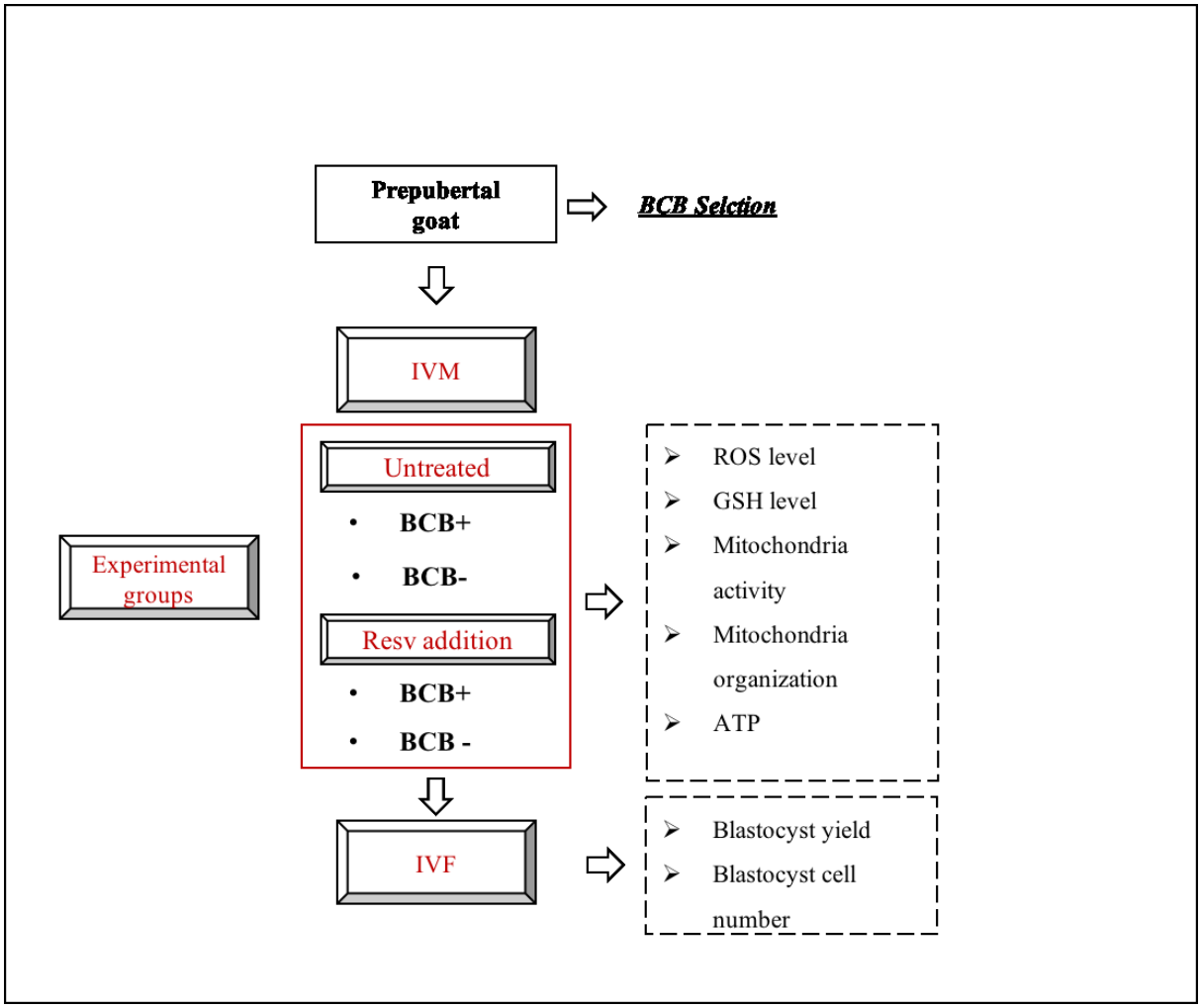
Experiment 2: *Effect of* 1 μM Resv *supplementation on developmental competence of oocytes selected by* brilliant cresyl blue staining

Based on the results of experiment 1, we evaluated the effect of 1 μM resveratrol on developmental competence of oocytes selected by brilliant cresyl blue staining. COCs were matured *in vitro* with (BCB+R; BCB-R) or without (BCB+C; BCB-C) 1 μM resveratrol. After IVF and IVEC, cleavage rate, blastocyst formation and cell number were assessed.

Experiment 3: *Effect of* 1 μM Resv *supplementation on bioenergetic/oxidative status of oocytes selected by* brilliant cresyl blue staining

Oocytes at the Metaphase II (MII) stage from the different groups were analyzed for: intracellular ROS and GSH levels (Experiment 3a), intracellular ATP content, mitochondrial activity and organization (Experiment 3b).

EXPERIMENTAL DESIGN



Statistical analysis

For each experiment, at least three replicates have been carried out. The oocytes used in each replicate derived from the same group of abattoir-derived ovaries collected on the same day. After BCB selection, BCB+ and BCB- oocytes were randomly distributed across resveratrol groups.

Statistical analysis was performed using STATA\IC 11.0 software package. Data were first checked for normally distribution and were analyzed using Shapiro-Wilk test. Data about maturation, cleavage, blastocyst rates and blastocyst cell number expressed as mean values ± standard errors of mean (SEM) were normally distributed and were analyzed using a one-way ANOVA followed by Bonferroni's as post hoc test. Data about intracellular ROS and GSH levels, ATP content and mitochondrial activity expressed as mean values ± standard errors of mean (SEM) were not normally distributed and were analyzed with a nonparametric Kruskal-Walli's test. The active mitochondria distribution was analyzed by Chi-square test and Fischer's exact test where appropriate. The overall chi-square was calculated and was found to be significant before performing the Fischer's exact text to detect differences among experiment groups. Differences with probability value of 0.05 or less were considered significant.

RESULTS

Eperiment 1: Effect of resveratrol supplementation at different concentrations on oocyte developmental competence and blastocyst cell number

The results on the effect of resveratrol supplementation at different concentration on oocytes developmental competence after IVF are reported in Table 1. Cleavage and blastocyst rates were higher ($P<0.05$) when resveratrol was added in the IVM medium at the concentration of 1 μM compared to 0 μM and 0.5 μM. Total cell numbers of blastocysts did not differ among groups (Table 1).

Table 1

Effect of Resveratrol concentrations added to IVM medium on embryo development and blastocyst cell number of prepubertal goat oocytes

Resveratrol concentration (μM)	N° total oocytes	N° cleaved (mean ±SE)	N° blastocysts/ cleaved (mean ±SE)	N° blastocysts/ total oocytes (mean ±SE)	total cell number (mean ±SE)
0	164	100 (61.2±2.0)[a]	11 (10.9±2.5)[a]	11 (6.8±1.6) [a]	127±10.8
0,5	152	89 (56.7±5.3)[a]	11 (14.5±3.4)[a]	11 (7.9±1.7) [a]	167.5±23.8
1	163	137 (83.5±2.8)[b]	32 (24.5±2.0)[b]	32 (20.1±1.3) [b]	156.5±13.9

Values with different superscript letters [(a, b)] within a column differ significantly ($P<0.05$).
[a] 3 replicate trials were performed.

Experiment 2*:* <u>Effect of 1 µM Resv supplementation on developmental competence of oocytes selected by brilliant cresyl blue staining</u>

Supplementation with 1 µM resveratrol during IVM did not affect meiotic progression and the ratio of nuclear maturation within BCB+ or BCB- groups (Table 2). No differences were found in the cleavage rate among groups (Table 3).

The blastocyst rate (blastocyst/total oocytes) was higher (P<0.05) in BCB+C group compared to that found in BCB-C group. Resveratrol supplementation increased blastocyst rate of BCB- oocytes at the same rate of BCB+C. BCB+R presented higher (P<0.05) blastocyst rate than that of BCB+C, BCB-C and BCB-R groups (Table 3). Total, ICM and TE blastocyst cell numbers did not differ among groups (Table 3).

Table 2 Effect of 1 µM Resveratrol added to the IVM medium on meiotic progression of prepubertal goat oocytes selected by brilliant cresyl blue staining

Treatment	N° of oocytes	GV (mean ±SE)	GVBD (mean ±SE)	MI (mean ±SE)	MII (mean ±SE)
BCB+C	53	0	1(2.2±1.3)	4(7.3±2.3)	48(90.4±1.1)
BCB+R	46	0	0	3(6.5±0.1)	43(93.5±0.1)
BCB-C	47	0	1(1.9±1.1)	6(12.9±0.5)	40 (85.2±0.6)
BCB-R	50	1(1.9±1.1)	1(1.9±1.1)	8(16.4±3.1)	40(79.9±1.0)

GV, germinal vescicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphaseII.
[a] 3 replicate trials were performed.

Table 3 Effect of 1 μM Resveratrol added to the IVM medium on embryo development and blastocyst cell number of prepubertal goat oocytes selected by brilliant cresyl blue staining.

Treatment	N° inseminated oocytes	N° cleaved (mean ±SE)	N° blastocysts/ cleaved (mean ±SE)	N° blastocysts/ total oocytes (mean ±SE)	Total cell number (mean ±SE)	ICM (mean ±SE)	TE (mean ±SE)
BCB+C	110	87 (78.4±3.6)	14 (16.0±0.5)[a]	14 (13.0±0.7)[a]	134.6±7.4	30.2±3.5	106.8±21.8
BCB+R	116	103 (88.3±2.6)	32 (32.1±1.3)[b]	32 (28.3±0.9)[b]	167±12.6	43.4±4	133.4±12
BCB-C	95	67 (71.1±6.9)	4 (8.3±1.5)[a]	4 (4.7±0.4)[c]	136±4.9	32.5±0.5	122.5±28.5
BCB-R	88	67 (78.0±8.9)	8 (11.1±1.4)[a]	8 (8.3±0.8)[ac]	120.5±12.4	31.3±5.5	89.2±8.2

Values with different superscript letters [(a, b, c)] within a column differ significantly (P<0.05).
[a] 4 replicate trials were performed.

Experiment 3: Effect of 1µM Resv supplementation on bioenergetic/oxidative status of oocytes selected by BCB staining

Experiment 3a: intracellular level of GSH was higher ($P<0.05$) in both BCB+ (36554.6±3049.2) and BCB- (34946.8±1877.8) groups treated with resveratrol during IVM compared to their respective counterpart BCB+C (27624.0±1513.7) and BCB-C (27655.42±1489.8) groups (Fig.1). We did not find any difference ($P>0.05$) in ROS levels among experimental groups (BCB+C: 32740.3± 3165.0; BCB+R: 26314.1±2857.0; BCB-C: 59071.3±14079.0; BCB- R: 30587.3±3337.0) (Fig.1)

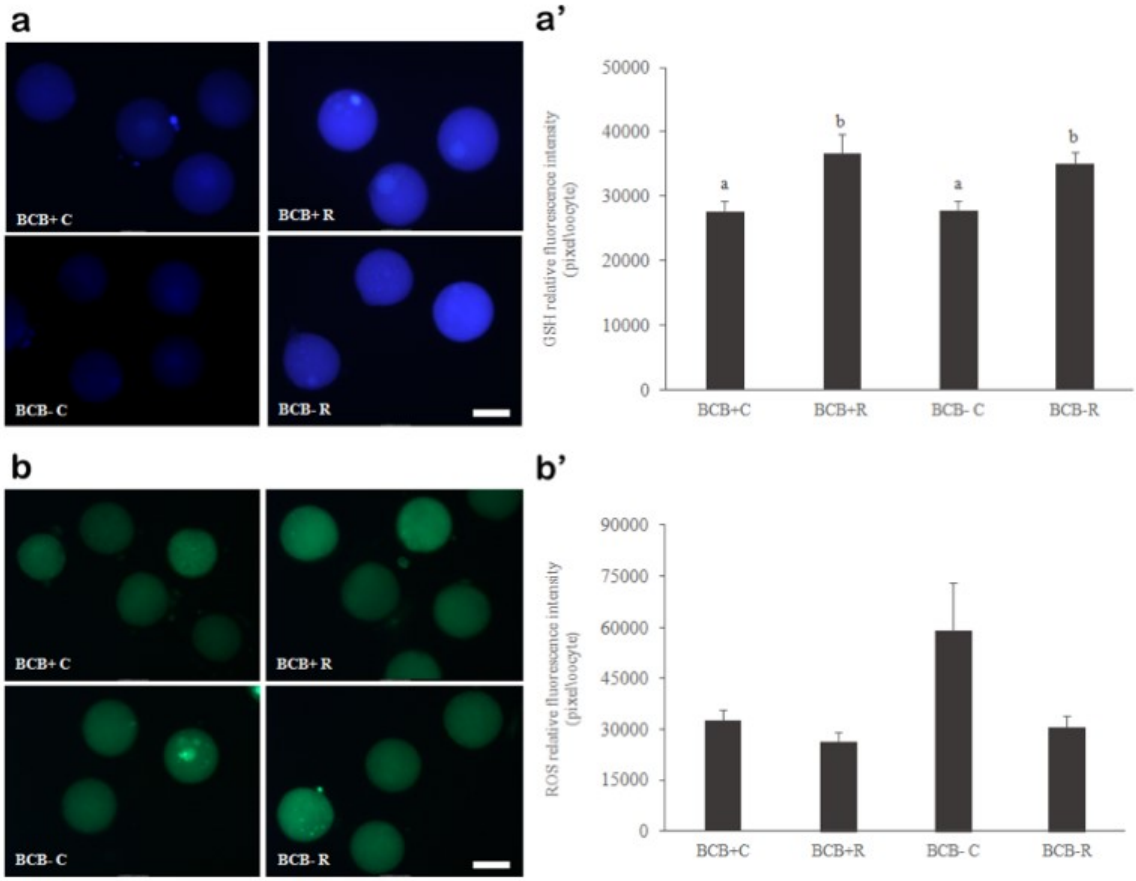


Fig. 1. Effect of 1 µM Resveratrol added to the IVM medium on GSH and ROS intracellular levels of prepubertal goat oocytes selected by brilliant cresyl blue staining (Experiment 3a): intracellular GSH **(a)** and ROS levels **(b)** of *in vitro* matured prepubertal goat oocytes. Epifluorescence photomicrographs of MII oocytes that were stained with CellTracker Blue to determine the level of GSH **(a')** and with 2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) to detect ROS **(b').** Values with different superscript letters $^{(a\ vs\ b)}$ are significantly different ($P<0.05$). Scale bar=100 µm.

Experiment 3b: The intracellular ATP content of the oocytes (Fig.2) was not different among groups (BCB+C: 3586.4±203.6; BCB+R: 3219.0±171.9; BCB-C: 3769.2±267.6; BCB-R: 4083.1±291.6).

We did not find any difference in fluorescent image intensity of active mitochondria (BCB+C: 17.2± 2.6; BCB+R: 13.5± 1.4; BCB-C: 16.5± 2.5; BCB-R: 16.0± 2.4) (Fig. 3). Mitochondrial distribution pattern was different (P<0.05) between control and resveratrol in both BCB groups.

Resveratrol treated oocytes had a higher (P<0.05) rate of pattern B mitochondria distribution (BCB+R: 73.07%; BCB-R: 79.16%) compared to controls (BCB+C: 19.35% and BCB-C: 40%, Fig.4).

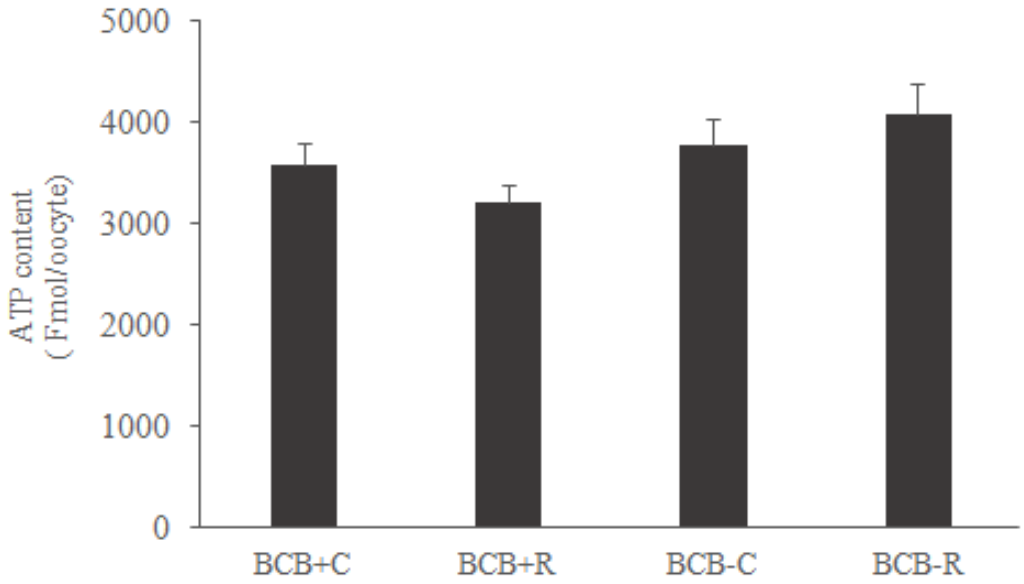


Fig. 2. ATP content (mean± SEM) of brilliant cresyl blue (BCB) selected prepubertal goat oocytes *in vitro* matured with or without 1 µM Resveratrol (Experiment 3b).

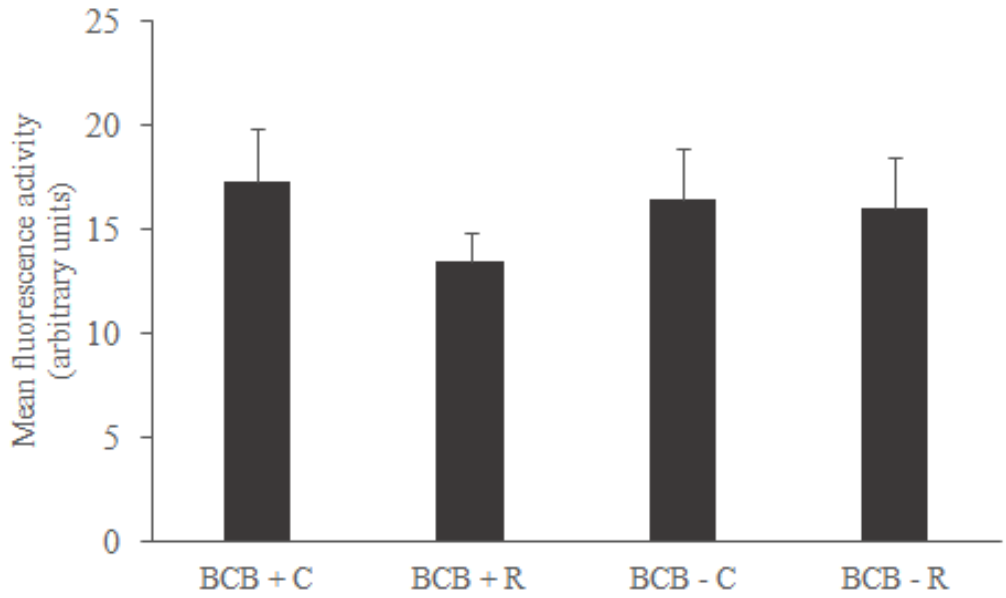


Fig. 3 Mitochondrial activity of brilliant cresyl blue (BCB) selected prepubertal goat oocytes *in vitro* matured with or without 1 µM Resveratrol (Experiment 3b). Fluorescence intensity was measured at the equatorial plane. Values are expressed as arbitrary units (Means± SEM).

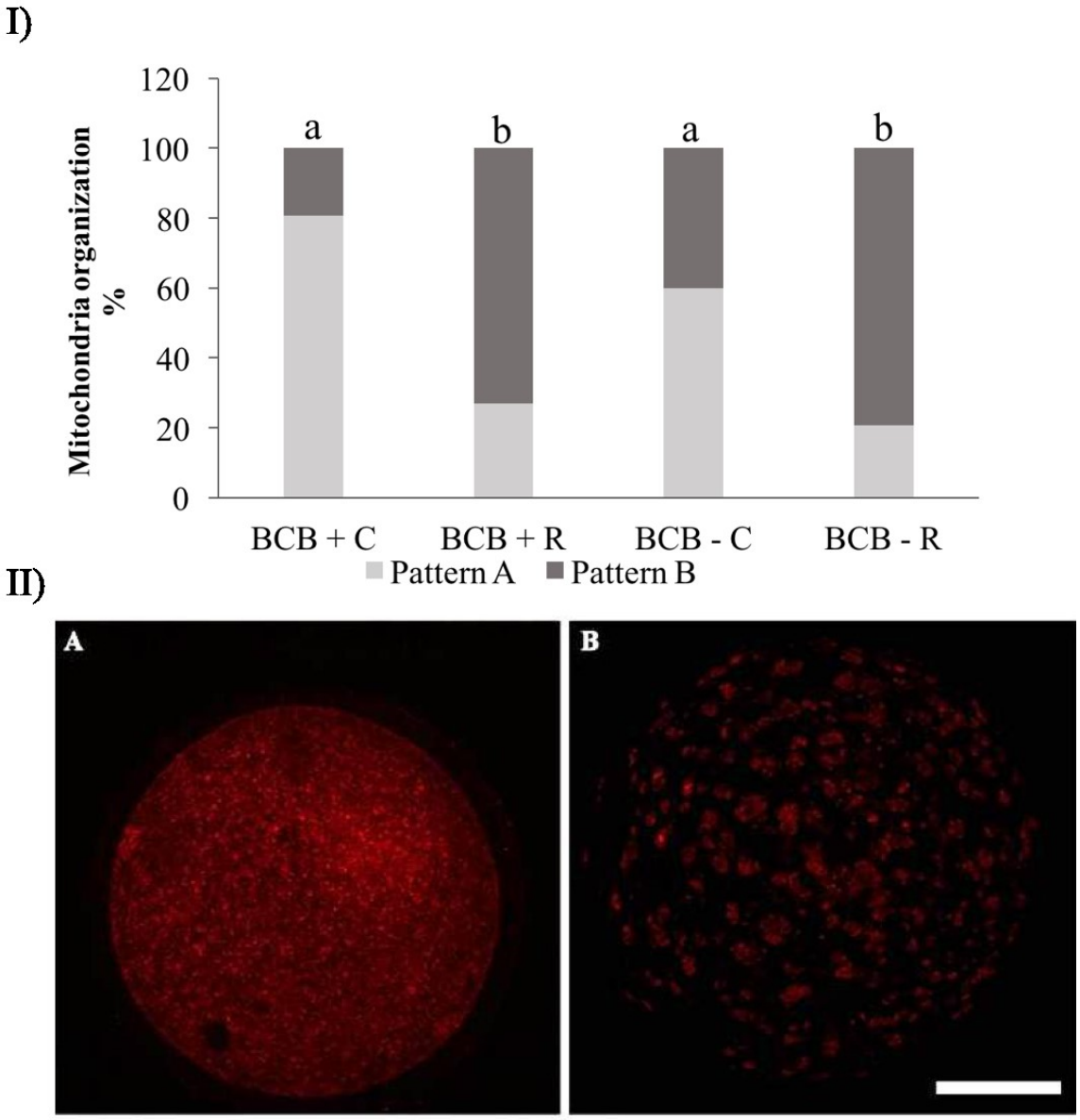


Fig. 4. Mitochondrial organization of brilliant cresyl blue (BCB) selected prepubertal goat oocytes *in vitro* matured with or without 1 µM Resveratrol (Experiment 3b). I) distribution of mitochondria aggregation patterns in metaphase II prepubertal goat oocytes, ($P<0.05$). II) representative CLSM images of mitochondrial aggregation patterns in prepubertal goat oocytes after staining with MitoTracker orange CMTM Ros: A) homogeneous small granulations spread throughout the cytoplasm (pattern A); B) heterogeneous large granulations spread throughout the cytoplasm or located in specific cytoplasmic domains (Pattern B). Different superscript letters $^{(a\ vs\ b)}$ are significantly different Scale bar=50 µm.

DISCUSSION

In the present study, we investigated the potential beneficial effect of resveratrol supplementation to the maturation medium on embryo development competence of prepubertal goat oocytes selected by BCB test.

An increasing number of evidence proved that the addition of resveratrol during *in vitro* maturation has positive effect on *in vitro* embryo production in different species (Galeati G and Spinaci, 2015). In adult goat, resveratrol improved the developmental potential of parthenogenetic derived blastocyst and hand-made cloned blastocysts (Mukherjee *et al.*, 2014). Resveratrol acts in a dose-dependent manner and the optimal concentration is species-specific (Galeati and Spinaci, 2015). We have demonstrated that, resveratrol at the concentration of 1 μM, significant increased both cleavage and embryo development of prepubertal goat oocytes. After oocyte selection with BCB, BCB+ oocytes matured in presence of resveratrol (BCB+R) developed to the blastocyst stage at percentages higher than those of the respective counterpart (BCB+C). Moreover, resveratrol treatment positively affects BCB- oocytes improving their competence to blastocyst development up to BCB+ oocytes.

In our study the nuclear maturation of oocytes was not affected by resveratrol treatment, which is in lines with results of other authors in bovine (Sprícigo *et al.*, 2017), goat (Mukherjee *et al.*, 2014) and pig (Kwak *et al.*, 2012). On the contrary, Wang *et al.* (2014) found that resveratrol promoted the oocyte nuclear maturation due to its antioxidant properties and the induction of progesterone secretion (Wang *et al.*, 2014). We observed that oocytes with low G6PDH activity (BCB+) had higher developmental competence than those with high enzyme activity (BCB-). These findings have been previously shown in our laboratory in goat (Rodríguez-González *et al.*, 2003; Urdaneta *et al.*, 2003), cattle (Pujol *et al.*, 2004) and sheep (Catalá *et al.*, 2011) and also by other authors in different species (Alm *et al.*, 2005; Manjunatha *et al.*, 2007; Wu *et al.*, 2007; Mohammadi-Sangcheshmeh *et al.*, 2011; Wang *et al.*, 2012).

A recent study observed that in spite mitochondria distribution was similar between both BCB groups, mtDNA content experienced a 1.9-fold increase in BCB+ cattle oocytes which could confirm the high competence of them compared to BCB- oocytes (Lamas-Toranzo *et al.*, 2018).

On the other hands, some findings contradicted the utility of this test on selecting competent oocytes in bovine and pig (Opiela and Kątska-Książkiewicz, 2013). Moreover, the existence of a high caspase-3 activity in the bovine blastocysts that developed from the BCB+ oocytes and a higher BAX protein level in the BCB+ oocytes could imply a detrimental effect of this staining on oocytes (Opiela and Kątska-Książkiewicz, 2013).

In order to understand the reasons of the positive effect of resveratrol on embryo development of prepubertal goat oocytes, we evaluated the oxidative and bioenergetics status of oocytes.

Our findings demonstrated that resveratrol significant increased intracellular GSH levels of *in vitro* matured oocytes in both BCB groups. In pig (Kwak *et al.*, 2012), cattle (Wang *et al.*, 2014) and goat (Mukherjee *et al.*, 2014), the beneficial effect of resveratrol on oocyte developmental competence has been associated to its antioxidant activity by increasing the intracellular GSH levels and decreasing the ROS levels. In our study, beside the increase of GSH content, resveratrol treatment did not affect the level of ROS in both BCB+ and BCB- groups. Antioxidants do not always act in a univocal manner; indeed, an increase in GSH levels is not always associated with a reduction in ROS levels how it was observed by other authors (Sovernigo *et al*., 2017; Soto-Heras *et al*., 2018;).

Many reports suggest that supplementation of IVM media with other antioxidants alleviated oxidative stress during *in vitro* maturation of poor quality oocytes and improved the early embryo development through a mechanism with included an increase of GSH content (Nabenishi *et al.,* 2012; Li *et al.,* 2015; Park *et al.,* 2018).

GSH is the major non-protein sulphydryl compound in mammalian cells and protects cells from oxidative damage (Luberda, 2005). The level of GSH in oocytes increased as the oocyte resumes meiosis, and higher concentrations are found in mature oocytes than in immature (Zuelke *et al.*, 2003). The intra-oocytes level of GSH can be considered a marker of cytoplasmic maturity due to the close correlation with embryonic development (De Matos *et al.*, 1996; 2000; Furnus *et al.*, 2008). In adult goat, the more competent oocytes (BCB+) presented higher intracellular GSH level and capacity to develop to the blastocyst stage after parthenogenetic activation (Hossein Abazari-Kia *et al.*, 2014). Moreover, a reduction of GSH levels has been correlated with low developmental competence of oocytes derived from prepubertal mice and pigs (Yuan *et al.,* 2012; Jiao *et al.,* 2013).

Several studies proved that GSH promotes the decondensation of the sperm head and the male pronucleus formation during fertilization (Sutovsky *et al*., 1997; Rodriguez-Gonzalez *et al*., 2003) but also play an important role in the development of parthenogenetic embryos (Kwak *et al.,* 2012; Hossein *et al.,* 2014; Mukherjee *et al.,* 2014).

Furthermore, GSH is involved in many biological processes as DNA and protein synthesis, cell proliferation and protection of mitotic spindle from oxidizing agents (Lafleur *et al.*, 1994; Zuelke *et al.*, 1997 b).

Another finding of our study was the effect of resveratrol on mitochondria organization. In fact, the supplementation of resveratrol to the maturation medium induced a modification of active mitochondria distribution in the cytoplasm of BCB+ and BCB- oocytes from a fine homogeneous pattern to a clustered phenotype. It has been shown that activation of SIRT1 by resveratrol enhanced the biosynthesis and degradation of mitochondria, thus improving mitochondrial function and the developmental ability of oocytes (Takeo *et al.*, 2014; Sato *et al.*, 2014). In addition, resveratrol treatment could efficiently correct the defective phenotypes of mitochondria organization in *in vitro* aged or methylglyoxal-treated mouse oocytes (Liu *et al.*, 2013; Ma *et al.*, 2015). Mitochondrial distribution and activity are considered good markers of oocyte quality.

During *in vitro* maturation, changes in mitochondrial distribution and activity occurs supporting oocytes maturation in cattle (Stojkovic *et al.,* 2001; Tarazona *et al.*, 2006), dogs (Valentini *et al.*, 2010), goat (Velilla *et al.*, 2006), sheep (Catalá *et al.*, 2011), horse (Torner *et al.*, 2007), pig (Brevini *et al.*, 2005b) and human (Dell'Aquila *et al.*, 2009).

In a comparative study, Leoni *et al*. (2015) documented different active mitochondria organization in ovine MII oocyte with high (adult) and low (prepubertal) developmental competence (Leoni *et al.*, 2015). A fine homogeneous dispersion of active mitochondria was observed at GV stage in both types of oocytes. This organization persisted in prepubertal MII oocytes while adult MII oocytes acquired a clustered distribution. A clustered active mitochondria organization was associated with oocytes maturation and high developmental competence in horse (Torner *et al.*, 2007), dog (Reyes *et al.*, 2011), pig (Torner *et al.*, 2004) and human (Dell'Aquila *et al.*, 2009).

The presence of large clustered granules in MII oocytes treated with resveratrol which showed the highest GSH levels and developmental competence suggest that also in our study, the mitochondria clustered phenotype may reflect a correct cytoplasmic maturity. The quantitative analysis revealed that resveratrol did affect neither the ATP content nor the mitochondrial activity of prepubertal goat oocytes, indeed no significant difference was found among groups. By contrast, resveratrol treatment increased ATP content and the mitochondrial membrane potential in bovine *in vitro* matured oocytes (Takeo *et al.*, 2014).
In summary, the results of the present study proved that supplementation of resveratrol during *in vitro* maturation improved embryo development to blastocyst stage with more significance in better quality oocytes (BCB+). The increase of GSH level and the clustered of mitochondria organization induced by resveratrol could be some of the mechanisms underlying the effect on oocyte quality.

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Chapter 6:

STUDY III

Resveratrol supplementation during in vitro maturation of oocytes under cadmium exposure: Effect on fertilization outcome in the ovine model.

ABSTRACT

Background: Cadmium (Cd) is a highly toxic heavy metal with adverse effect on female reproduction. *In vitro* studies demonstrated that Cd exposure during maturation impaired oocyte fertilization by inducing mitochondria hyper-activation and oxidative stress.

Aim: to investigate the effect of resveratrol (Resv) supplementation during *in vitro* maturation on the fertilization outcome of prepubertal sheep oocytes under Cd exposure.

Methods: Ovaries collected from slaughterhouse-derived ovaries of prepubertal sheep were exposed to 2 μM Cd and *in vitro* matured in the presence of 0 μM (Cd), 1 μM (Cd-Resv 1 μM) and 2 μM (Cd-Resv 2 μM) resveratrol. Oocytes matured in absence of Cd were used as control (CTR). Fertilization outcomes, cortical granules (CG_S) and mitochondria (mt) distribution, mt activity and reactive oxygen species (ROS) level were evaluated.

Results: Oocytes of CTR, Cd-Resv 1 μM and Cd-Resv 2 μM groups had higher normal fertilization and lower polyspermic fertilization rates compared to Cd group ($P<0.05$). The percentage of metaphase II (MII) oocytes with CGs distributed in the cortex of the oocytes was higher ($P<0.05$) in control and Cd-Resv 1μM groups than Cd group. The percentage of MII oocytes that exhibit a homogeneous mitochondria distribution (normal distribution) throughout the cytoplasm was higher in CTR, Cd-Resv 1 μM and Cd-Resv 2 μM groups than Cd group ($P<0.05$). Lower activity ($P<0.05$) of mitochondria was recorded in CTR and Cd-Resv 1 μM oocytes compared to oocytes matured under Cd exposure in absence of Resv. The intracellular ROS levels were lower in CTR, Cd-Resv 1 μM and Cd-Resv 2 μM groups than Cd group.

Conclusion: Resveratrol addition to the IVM medium at both 1 μM and 2 μM concentrations protected oocytes against the toxic effect of Cd on *in vitro* fertilization of oocytes by preventing inadequate distribution of cortical granules and mitochondria, dysfunction of mitochondria and oxidative stress.

INTRODUCTION

Anthropogenic activities have increased environmental toxicants pollutants levels in terrestrial and aquatic ecosystems. Among inorganic pollutants, heavy metals (HMs), plays a major role as they can affects reproductive performances and are associated with infertility in human and animals (Ma *et al.*, 2018).

Cadmium (Cd) is a highly toxic heavy metal of particular concern for human and animal health due to its ubiquitous occurrence in air, water, soil, vegetables, food and feed. Anthropogenic sources of Cd include combustion of fossil fuels, waste incineration, leachate from landfill sites mining, smelting, rubber processing, galvanization process, nickel-Cd batteries, plastic stabilizer and pigment (Flora and Agrawal, 2017). Cd is also a constituent of pesticides and fertilizer (Järup, 2003) and is one of the major components of cigarette smoke (Flora and Agrawal, 2017). Its long biological half-life (15-30 years) and its low rate of excretion from the body cause its accumulation over time in organs and tissue.

Several studies underlined the negative effect of this metal on female reproduction (Thompson and Bannigan, 2008). Long-term exposure to Cd damages reproductive system and affects fertility (Thompson and Bannigan, 2008; Flora and Agrawal, 2017). Bioaccumulation of Cd has been found in the ovaries and follicular fluid of both humans and animals (Zenzes *et al.*, 1995; Piasek *et al.*, 2001; Martino *et al.*, 2017). It has been shown that Cd had a direct effect on the ovaries of hamster, resulting in the reduction of the ovarian weight, and necrosis (Thompson and Bannigan, 2008). Furthermore, ovarian steroidogenesis and ovulation was greatly altered by Cd exposure in rat (Zhang and Jia, 2007; Thompson and Bannigan, 2008; Zhang *et al.*, 2008). Cadmium influenced follicular development by promoting follicular atresia in mouse (Wang *et al.*, 2015) and inducing apoptosis of granulosa cells of chicken ovarian follicles (Jia *et al.*, 2011). The suppression of hyaluronic acid synthesis and the reduction of FSH-induced cumulus cells expansion has been also reported in porcine oocytes (Vršanská *et al.*, 2003; Mlynarcikova *et al.*, 2005) following Cd exposure.

By using *in vitro* models, previous studies investigated the influence of Cd on the female gamete. Cd exposure have been proved to modify the morphology of buffalo oocytes (Nandi *et al.*, 2010) and to affect oocyte and cumulus cell viability in both buffalo and ovine oocytes (Leoni *et al.*, 2002; Nandi *et al.*, 2010). Other evidences indicated a deleterious effect of Cd exposure on the *in vitro* nuclear maturation of oocytes in sheep (Leoni *et al.*, 2002), buffalo (Nandi *et al.*, 2010) and bovine (Ahmad and Khal, 2018 b). The disruption of periodic changes of the Maturation Promoting Factor (MPF) activity by increasing mRNA and protein expressions of MPF-related genes has been suggested as one of the mechanism involved in the inhibition of meiotic progression of metaphase I mouse oocyte exposed to Cd (Liu *et al.*, 2018).

The exposure of ovine and bovine oocytes to Cd during *in vitro* maturation impaired *in vitro* fertilization and embryo development (Leoni *et al.*, 2002; Nandi *et al.*, 2010; Martino *et al.*, 2017; Ahmad and Khal, 2018 b). In a recent study in juvenile and adult sheep, Martino *et al.* (2017) reported age-related bioaccumulation of Cd, with detectable harmful levels even in ovarian tissues of juvenile sheep. These Authors found that exposure to nanomolar concentration of Cd during *in vitro* maturation reduced fertilization of oocyte retrieved from both juvenile and adult sheep with a slight increase in the number of abnormally fertilized oocytes (Martino *et al.*, 2017). The deleterious effect of Cd on fertilization has been related to mitochondria hyper-activation associated with two specific indicators of oxidative stress, such as reactive oxygen species (ROS) levels and liporeroxidation (Martino *et al.*, 2017).
We hypothesized that resveratrol could counteract oxidative stress and investigated the effect of resveratrol supplementation during *in vitro* maturation of prepubertal sheep oocytes under Cd exposure on the fertilization outcome, cortical granules distribution and bioenergetic/redox status of oocytes.

MATERIAL AND METHODS

Chemicals

All chemicals, in this study, were purchased from Sigma Chemical CO. (St. Louis, MO, USA) unless stated otherwise.

Oocyte collection

Ovaries from prepubertal sheep (*Ovis Aries,* 30-40 days of age) were recovered from local abattoirs and transported within 3h to the laboratory in Phosphate Buffered Saline (PBS) with penicillin (100mg/mL) and streptomycin (100mg/mL) at 34-37°C. Ovaries were washed three times in PBS and processed by the slicing procedure for cumulus oocytes complexes (COCs) retrieval in dissection medium (DM; 25μM Hepes-buffered TCM199) supplemented with 0.1% (w/v) polyvinyl alcohol (PVA) and antibiotics (100 μg/mL of penicillin and streptomycin). Only COCs with several intact cumulus cells layers and homogenous cytoplasm were selected for *in vitro* maturation (IVM).

In vitro maturation (IVM)

Cumulus oocytes complexes were matured *in vitro* in conventional maturation medium (CMM) consisting of TCM 199 supplemented with 10% heat-treated estrous sheep serum (ESS), 0.36 mM pyruvate, FSH 1 IU / ml and LH 1 IU / ml (Pluset; Bio98, Milan, Italy) under mineral oil, in 4-well dishes (Nunc Cell Culture, Thermo Fisher Scientific, Waltham, Massachusetts, USA) in a humidified atmosphere of 5% CO_2, at 38.5°C.

In vitro fertilization (IVF)

After maturation, COCs were partially stripped of the granulosa cells and co-incubated with fresh ram semen (1 x 10^6 sperm cells/ml) in SOF medium (synthetic oviductal fluid, Tervit *et al.*, 1972) + 2% ESS + 1 μ g/mL heparin + 1 μ g/mL hypotaurine, under mineral oil in a humidified atmosphere of 5% CO_2, at 38.5 °C for 16h.
All experimental procedures were performed with the semen of one single ram of proven fertility.

Fertilization assessment

After IVF, the presumptive zygotes were denuded from cumulus cells by aspiration in and out of finely-drawn glass pipettes, fixed with ethanol and stained with 1 μM Hoechst 33342 (Comizzoli *et al.*, 2001). They were thereafter, placed on a slide and classified (Martino *et al.*, 2017) under an inverted epifluorescent microscope (Olympus IX70, Italy).

The presence of 2 pronuclei (2PN) and two polar bodies (2PB) indicated a normally fertilized oocyte, the presence of more than two pronuclei (>2PN) and two polar bodies (2PB) was designated polyspermic fertilization, one PN and a sperm head (SH) that failed chromatin decondensation and 2PB or one PN and MII plate and 2PB were considered as abnormal fertilization. Oocyte with the Metaphase II (MII) plate with the first PB indicated unfertilized matured oocytes (representative image in Fig.1).

Assessment of cortical granules distribution

Analysis of cortical granules (CGs) was performed according the methodology of Hosseini *et al*. (Hosseini *et al.*, 2012). After IVM, MII oocytes from each experimental group (n=20-25) were incubated with 0.5% pronase on a warm plate (38.5°C) to digest zona pellucida and fixed with 3.7% paraformaldehyde in PBS at RT for 30 minute. After fixation, oocytes were washed three times in PBS containing 0.3%BSA and 100-mM glycine.

Oocytes were permeabilized in 0.1% Triton X-100 in PBS for 5 minute, and incubated in 100 µg/mL Alexa Fluor 488-conjugated Lectin PNA (Molecular probes, Invitrogen) in PBS for 30 minute in the dark. The unbounded dye was removed by 3 washes in PBS containing 0.3% BSA, 100-mM glycine and 0.01% Triton X-100. Finally, oocytes were mounted on glass slides with 2.5 mg/mL Hoechst 33342 in PBS and glycerol solution (3:1 v/v), overlaid with a coverslip supported by four droplets of vaseline and sealed with nail varnish.

The localization of CGs (excitation 490 nm) and chromatin configuration (excitation 361nm) were evaluated with Leica TCS SP5 CLSM with LAS lite 170. Image software equipped with a 405-nm diode laser and a multiphoton laser. The CGs configuration was classified as previously reported (Takeo *et al.*, 2014), with some modifications in: Pattern A: cortical granules distributed in the oocyte cortical region; Pattern B: cortical granules diffused throughout the oocyte cytoplasm (representative images Fig.2).

Evaluation of bioenergetic/oxidative status of oocytes

In order to elucidate the effect of resveratrol on bioenergetic/oxidative status of oocytes matured under Cd exposure, oocytes underwent laser scanning confocal microscopy (LSCM) analysis of mitochondria (mt) distribution and activity and intracellular ROS levels. After IVM, MII oocytes from each experimental group (n=25-30) were subjected to a triple staining with MitoTracker Orange CMTMRos (Molecular Probes, Inc., Eugene, OR, USA), 2′7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Cat n° D 6883) and Hoechst 33342 for detection of mt distribution and activity, intracellular reactive oxygen species levels and chromatin configuration respectively (Succu *et al.*, 2018). MitoTracker Orange CMTMRos passively diffuses across the plasma membrane and is readily sequestered only by active mt depending on their membrane potential. H_2DCF-DA is a cell-permeable non-fluorescent probe. Inside to the oocyte this probe is de-esterified and turns to highly fluorescent 2′,7′-dichlorofluorescein due to oxidation by H_2O_2 (Succu *et al.*, 2018).

Briefly, the oocytes were washed three times in PBS with 0.1% bovine serum albumin (BSA) and were incubated for 30 minutes in PBS with 3% BSA containing 200 nM MitoTracker Orange CMTMRos, at 38.5°C under 5% CO_2. After the first incubation, oocytes were wash again in PBS with 0.1% BSA and incubated for 30 minutes in PBS with 0.1% BSA and 10 μM H_2DCFDA. After washing in in PBS with 0.1% BSA oocytes were fixed for 60 minutes at 38°C in 3% paraformaldehyde and stained for 5minutes with 1 μM Hoechst 33342 solution. Particular attention was paid to avoid sample exposure to the light during staining to reduce photo-bleaching. Oocytes were stored at 4°C in the dark until their analysis.

The oocytes were placed on a slide and covered with a drop of a solution of PBS and glycerol (3:1v/v), overlaid with a coverslip supported by four droplets of vaseline and sealed with nail varnish.

The mitochondria distribution patterns classified as: normal: homogeneous mt distribution, with small granulation spread throughout the cytoplasm; abnormal: mt distribution, with large granulations located in specific cytoplasmic area (representative images in Fig.3). Mitochondria and intracellular ROS level quantification were performed by a confocal laser-scanning fluorescence microscope (Leica TCS SP5 CLSM), equipped with 543 nm He/Ne, 488 nm Argon and 405 nm diode lasers using an oil immersion 40x objective and recorded on a host computer.

A helium/neon laser ray at 543 nm and an argon-ion laser ray at 488 nm were used to detect the MitoTracker Orange CMTM and DCF fluorescence signals respectively.
In each individual oocyte MitoTracker Orange CMTMRos (excitation 554nm) and H_2DCFDA (excitation 460 nm) fluorescence intensities were measured at the equatorial plane as described previously (Dell'Aquila *et al.*, 2014) and microscope objective, pinhole, filters, offset, gain, and laser potency were kept constant throughout the experiment. Acquisition, storage and image analysis were made with the LAS lite 170 Image software. The fluorescence intensity of the cytoplasm area of the oocyte was measured and reduced by compensation for the background fluorescence.

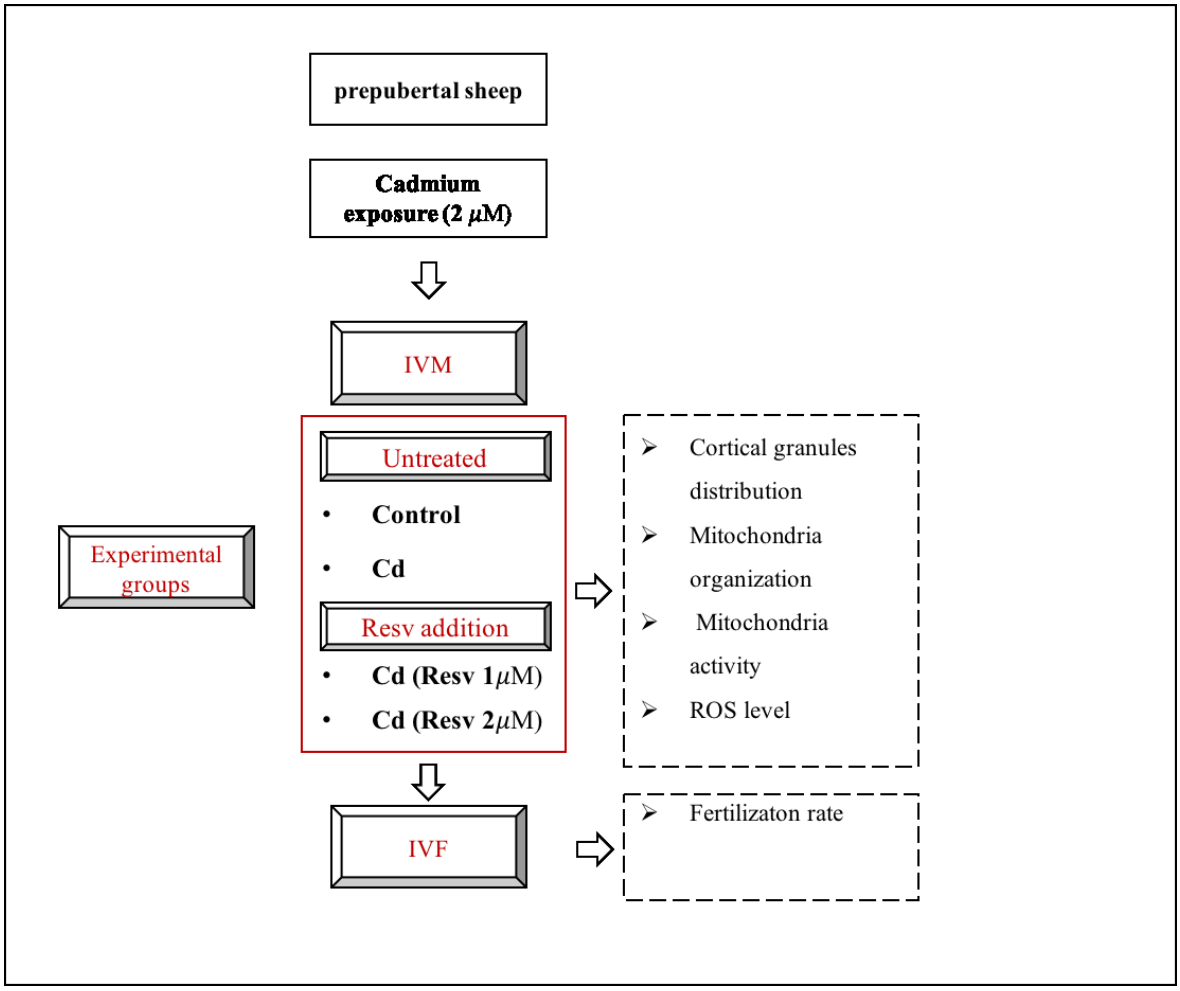
Experimental design

Cumulus oocytes complexes were randomly divided in four groups for IVM:
-control (CTR) group: oocytes were matured in CMM
-Cd- group: oocytes were matured in CMM medium supplemented with 2 μM $CdCl_2$
-Cd-Resv 1 μM and Cd-Resv 2 μM groups: oocytes were matured in CMM medium supplemented with 2 μM $CdCl_2$ and 1 μM or 2 μM resveratrol.
The concentration of cadmium used in the present study was selected by referring to earlier *in vitro* study in ovine specie (Leoni *et al.*, 2002).

The Experiment 1 studied the effect of Resv supplementation (1 μM and 2 μM) to the IVM medium on fertilization rates of oocytes exposed to Cd during maturation.

The Experiment 2, assessed: the CGs distribution, bioenergetic/oxidative status of oocytes (mitochondria distribution and activity, and ROS levels) of *in vitro* matured oocytes exposed to Cd

EXPERIMENTAL DESIGN



Statistical analysis

All statistical analyses were performed using Stata/IC 11.2 (StataCorp LP, USA). Categorical data of fertilization, mitochondria and cortical granules distribution were analyzed by chi-square test. Data of intracellular ROS levels and mitochondria activity were not normally distributed and were analyzed by non-parametric Kruskal-Walli's test.

$P < 0.05$ was considered statistically significant. All the experiments were replicated at least for two times.

RESULTS

Experiment 1

Effect of resveratrol on fertilization of oocytes under Cd exposure

Results about *in vitro* fertilization of oocytes in the different experimental groups are reported in Table 1. Representative images of normal, polyspermic and abnormal fertilization are shown in Fig.1. Cadmium exposure during *in vitro* maturation of the oocytes significantly (P<0.05) reduced the percentage of normally fertilized oocytes and increased the polyspermic fertilization compared to control group. Resveratrol addition to the maturation medium, at both 1 μM and 2 μM concentrations, enhanced normal fertilization and reduced polyspermy (P<0.05) compared to Cd group. Normal and polyspermic fertilization rates of oocytes treated with 1 μM and 2 μM Resv were similar to those of control oocytes. No difference was found in the percentages of abnormal fertilized and unfertilized oocytes among groups.

Table 1. Effect of resveratrol supplementation to IVM medium on fertilization of prepubertal sheep oocytes under cadmiun-exposure

Groups	N° of oocytes	Fertilization N° (%)			
		Normal (%)	Polyspermy (%)	Abnormal (%)	Unfertilized (%)
Control	149	92 (61.7) [a]	36 (24.2) [a]	10 (6.7)	11 (7.4)
Cd	147	53 (36.0) [b]	66 (44.9) [b]	10 (6.8)	18 (12.3)
Cd-Resv1 μM	141	82 (58.2) [a]	37 (26.2) [a]	4 (2.8)	18 (12.8)
Cd-Resv2 μM	99	58 (58.6) [a]	23 (23.2) [a]	8(8.1)	10 (10.1)

Different superscripts in the same column (a, b) indicate a statistical significant difference P<0.05
[a] 3 replicate trials were performed

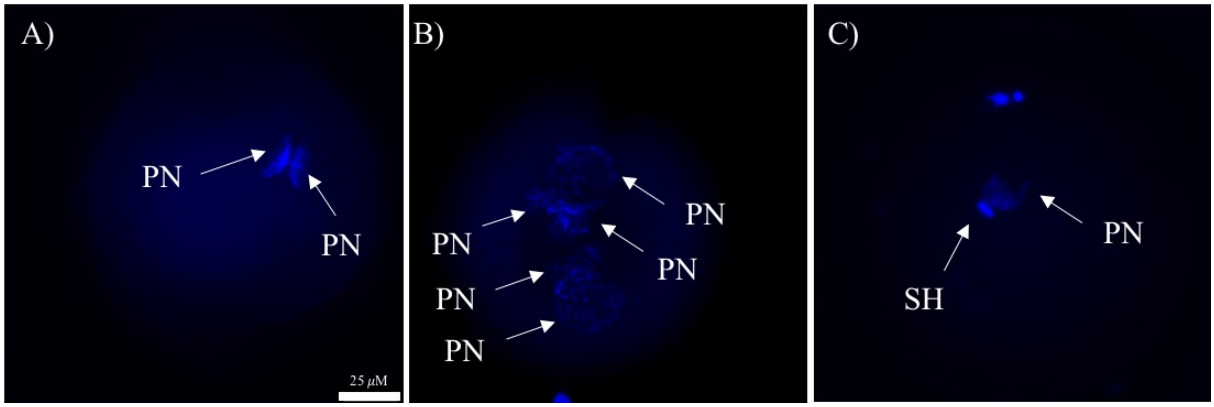


Fig.1. Representative image of nuclear configuration of prepubertal sheep oocytes 16h post-*in vitro* fertilization showing A) 2PN (normal fertilization), B) > 2PN (polispermy) C), 1PN and a sperm head (SH) with no signs of nuclear decondensation (abnormal fertilization). Scale bar= 25 μm.

Experiment 2

To understand cadmium and resveratrol mechanism of action on oocyte fertilization, we analyzed Cortical Granules (CG)s distribution, mitochondria distribution and activity, and intracellular ROS levels in *in vitro* matured oocytes of prepubertal sheep.

Effect of resveratrol on cortical granules distribution of oocytes under Cd-exposure

Figure 2 shows representative images of the two-different patterns of CGs distribution. Most of control oocytes (n=23/24, 95.8%) showed a distribution of the CGs under the oocyte cortex (pattern A). Cd-exposure induced a significant reduction ($P<0.05$) of this pattern of distribution (n=15/23, 65.2 %). The ratio of oocytes exposed to Cd with CGs distributed in the peripheral region was higher ($P<0.05$) for oocytes matured with Resv 1 μM (n=21/23; 91.3%) and similar for oocytes matured with Resv 2 μM oocytes (n=17/21, 80.95%) compared to those matured without Resv (Cd-group).

No differences were observed among resveratrol (Cd-Resv1 μM: n=21/23, 91.3%; Cd-Resv2 μM: n=17/21, 80.95%) and control groups (n=23/24, 95.8%).

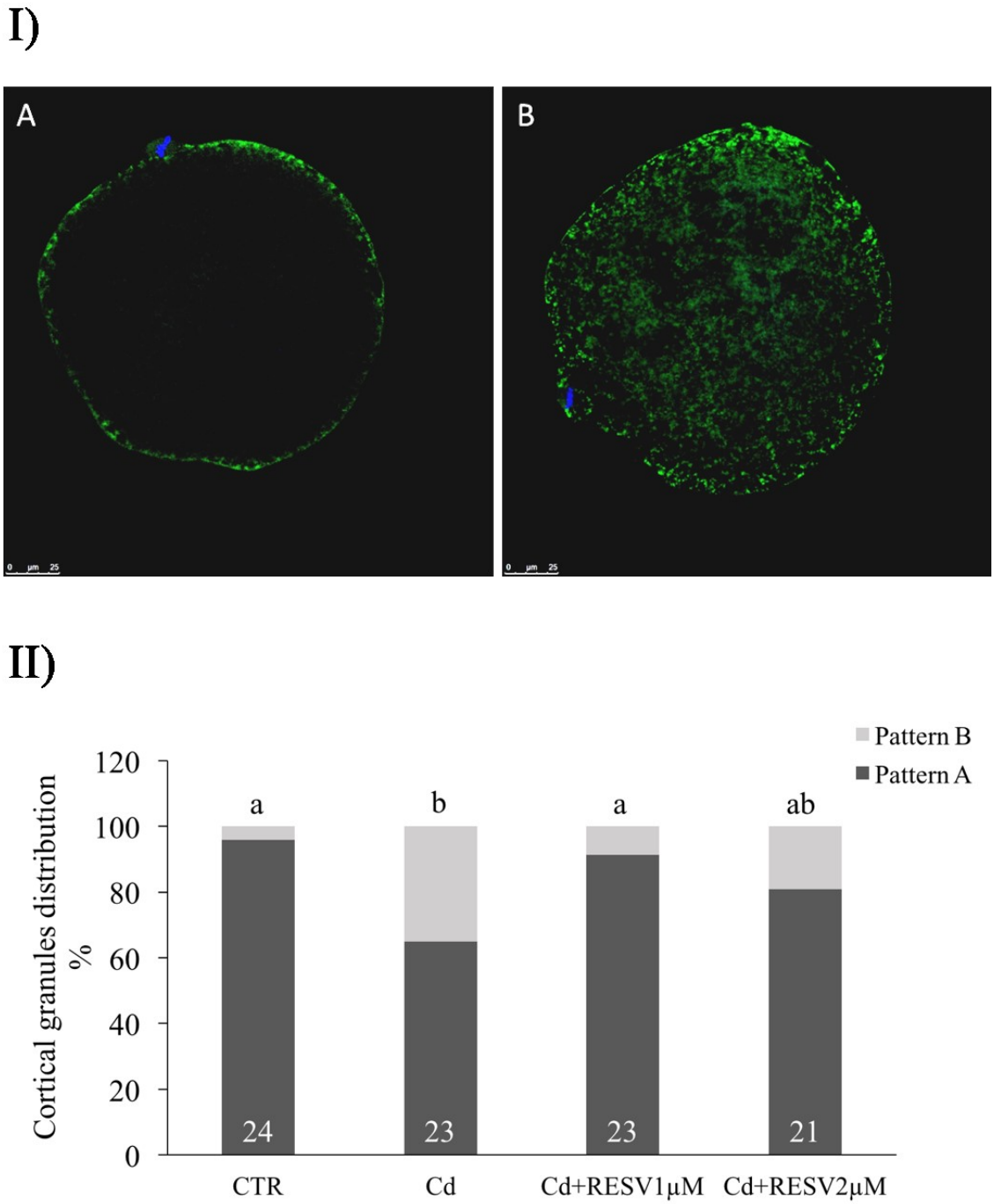


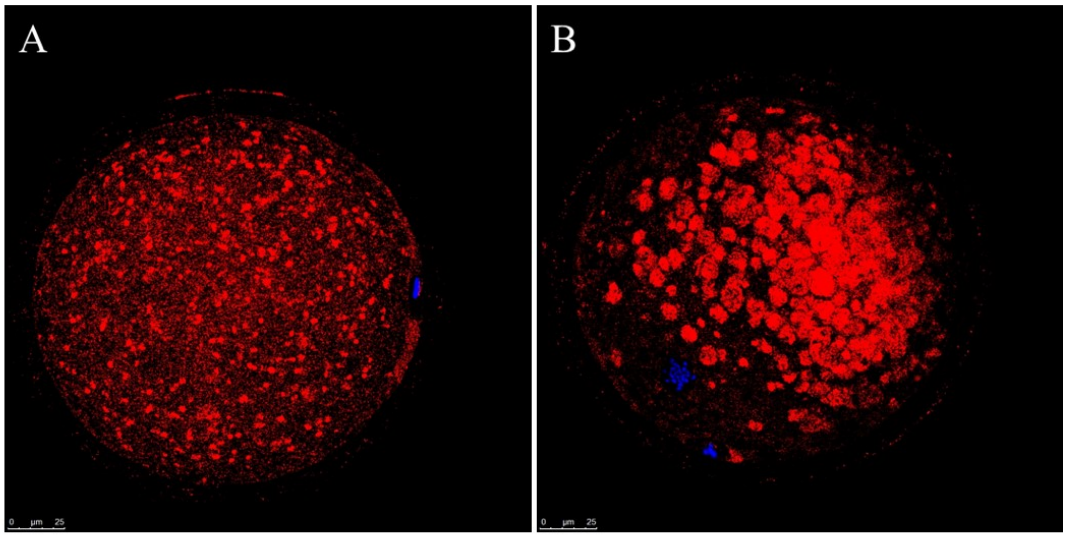
Fig.2. Effect of resveratrol supplementation to IVM medium on CGs distribution of prepubertal sheep oocytes under Cd-exposure. I) Representative images of CGs distribution patterns showing A) CGs distributed in the cortical region (pattern A); B) CGs diffused throughout the cytoplasm (pattern B). II) Percentage of MII oocytes with different pattern of CGs distribution. Numbers of examined oocytes /group are indicated at the bottom of each histogram column.
Different superscript letters $^{(a\ vs\ b)}$ indicate a significant difference ($P<0.05$).
Scale bar= 25 µm.

Effect of resveratrol on bioenergetic/oxidative status of oocytes under Cd exposure

Figure 3 shows representative images of the mitochondria distribution in the oocyte cytoplasm. After *in vitro* maturation 73.1% of CTR oocytes (n=19/26) showed a homogeneous distribution of the mitochondria with small granulation spread throughout the cytoplasm (normal distribution). Cd-exposure during maturation induced a decrease ($P<0.05$) of normal mt distribution (n=14/32, 43.7%) compared to control. Cd-Resv1 μM (n=27/32, 84.4%) and Cd- Resv2 μM (n=21/27, 77.8%) groups displayed a percentage of normal mt distribution higher ($P<0.05$) than Cd-group and similar to CTR oocytes.

Mitochondria activity (103.1 ± 1.4 ADU) and ROS levels (122.4 ± 1.5 pixel/oocyte) were significantly higher ($P <0.05$) in Cd-exposed oocytes compared to the CTR (67±0.6 ADU; 71.1±2.3 pixel/oocyte, respectively). Mt activity (79.3±1.1 ADU) and ROS level (74.4±1.3 pixel/oocyte) of oocytes exposed to Cd and treated with Resv 1 μM were lower ($P<0.05$) than those without Resv and similar to CTR. Similar rate of mt activity was recorded between Cd-Resv1 μM and Cd-Resv2 μM groups. In the oocytes treated with resveratrol 2 μM mt activity (83±0.9 ADU) was higher than CTR and similar to that of oocytes of Cd and resveratrol groups. Cadmium exposure during maturation increased ROS level (122.4 ± 1.5 pixel/oocyte) compared to CTR oocytes. The ROS levels of the oocytes treated with resveratrol 2 μM (96.6± 2.4 pixel/oocyte) were lower than Cd group and similar to CTR (Fig.4).

I)



II)

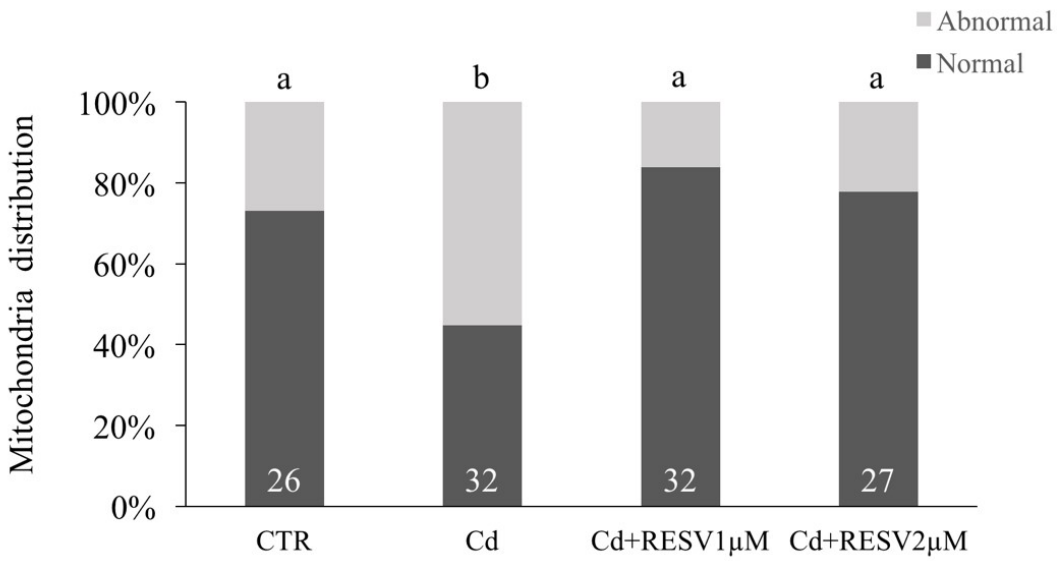


Fig.3. Effect of resveratrol supplementation to IVM medium on mt distribution of prepubertal sheep oocytes under Cd exposure. I) Representative images of mt distribution patterns after staining of oocytes showing homogeneous mitochondria distribution, with small granulation spread throughout the cytoplasm (normal distribution) and b) large mt granulations located in specific cytoplasmic area abnormal distribution). II) Percentage of oocytes with normal and abnormal mitochondria distribution. Numbers of examined oocytes/group are indicated at the bottom of each histogram column Different superscript letters (a vs b) indicate a significant difference ($P<0.05$). Scale bar=50 µm.

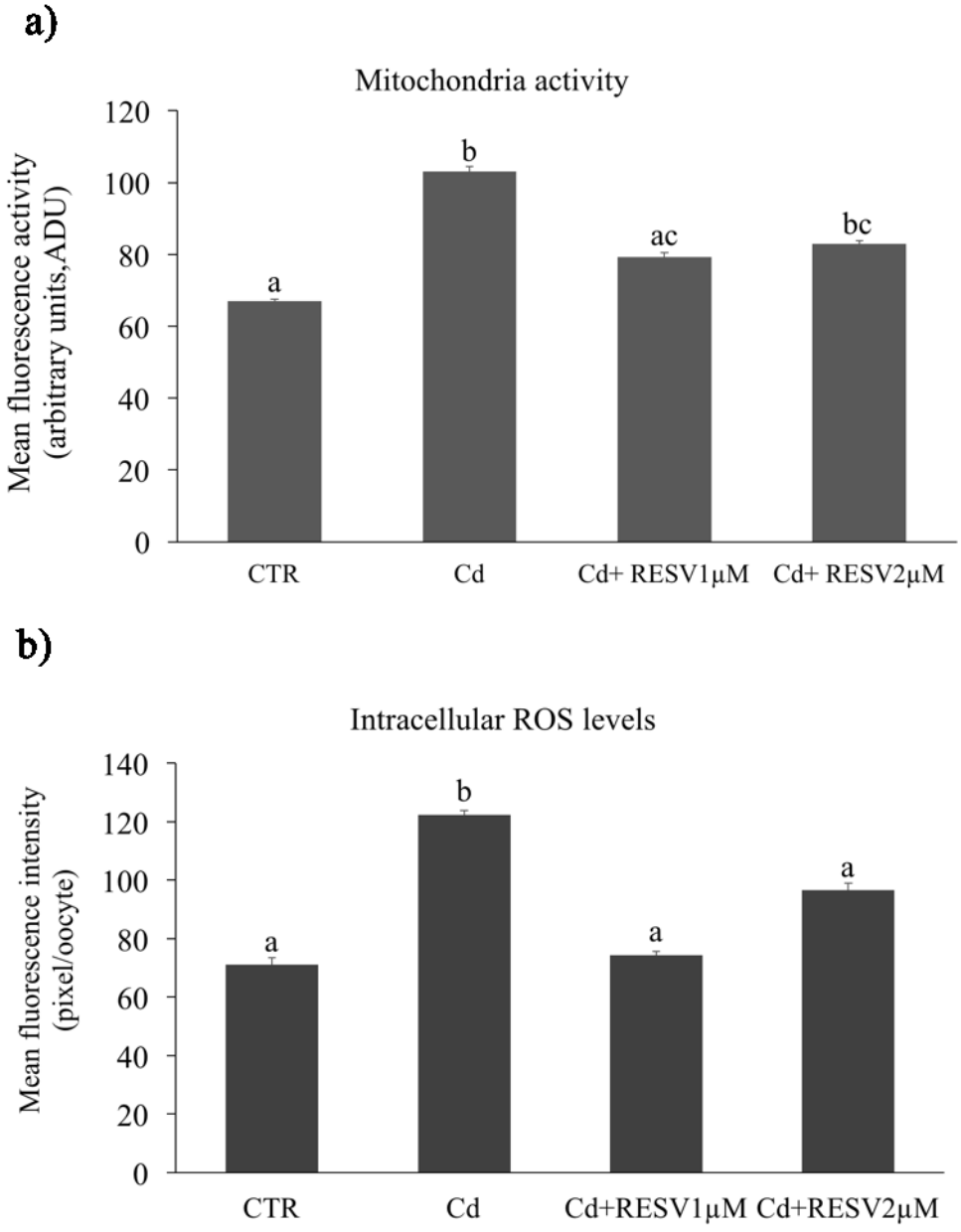


Fig.4. Effect of resveratrol supplementation to IVM medium on mitochondria activity (a) and ROS intracellular levels (b) of prepubertal sheep oocytes under Cd exposure. Different superscript letters (a, b and c) indicate a statistical difference $P<0.05$. Data are expressed as mean ± SEM.

DISCUSSION

In this study, the potential protective effect of resveratrol (1 μM and 2 μM) against Cd (2 μM) toxic effect on fertilization and bioenergetic/oxidative status of prepubertal sheep oocytes was investigated.

Results demonstrated that resveratrol at both concentrations has the following effects: *a)* increased normal fertilization outcome preventing polyspermy and abnormal fertilization via mechanisms which include cortical granules redistribution; *b)* prevented inadequate mitochondrial distribution and mitochondria dysfunction; *c)* reduced the intracellular ROS levels of oocytes.

Cadmium is a widely diffuse pollutant which concentration in the environment and in the body, depends to the level of industrialization and urbanization (Patra *et al.*, 2006).
A recent study, (Martino *et al.*, 2017) investigated the levels of 19 trace elements in ovarian tissues of prepubertal and adult sheep living in low contaminated areas and found that Cd is the trace metal with the highest age-dependent ovarian bioaccumulation. In detail, 1.34 ± 1.36 and 16.14 ± 8.25 ng/g were found in the ovaries of prepubertal and adult sheeps, respectively.

Previously studies reported the deleterious effect of Cd added during IVM at nanomolar and micromolar concentrations on *in vitro* fertilization of ovine oocytes (Leoni *et al.*, 2002; Martino *et al.*, 2017). Leoni *et al.* (2002) examined the effect of sheep oocytes exposure during *in vitro* maturation to two concentration of $CdCl_2$ (2 and 20μM) and found that both concentrations decreased *in vitro* fertilization rate while polyspermy was increased in the 2μM $CdCl_2$ group (Leoni *et al.*, 2002).

The negative impact of Cd on *in vitro* fertilization outcome was further confirmed by a recent paper of Martino *et al.* (2017) reporting a reduced rate of normal fertilization after exposure of ovine oocytes even to Cd nanomolar concentrations (1 and 100 nM) (Martino *et al.*, 2017).

In contrast to the findings of Leoni *et al.* (2002) these authors did not observe an increase in polyspermic fertilization, possibly due to the lower concentrations of $CdCl_2$ used. In buffalo oocytes, the effect of different Cd concentrations (ranging from 27.3 nM to 54.60 μM) was also assessed and a decline of fertilization and cleavage rates were observed starting from the concentration of 1.0 μg/ml (5.5 μM) (Nandi *et al.*, 2010).

Successful fertilization requires the correct function and distribution of cytoplasmic organelles. Specifically, calcium release from the endoplasmic reticulum, CGs distribution under oocyte oolema and proper exocytosis are crucial for inhibit polyspermic fertilization and support normal embryonic development (Blerkom and Davis, 2007; Dumollard *et al.*, 2008; Calì *et al.*, 2013). It has been demonstrated that Cd competes with calcium for protein-binding sites disturbing intracellular calcium activity (Biagioli *et al.*, 2008). Moreover, Cd has adverse effects on microfilaments of oocyte cytoskeleton which drive CGs migration (Prozialeck and Niewenhuis, 1991; Abbott and Ducibella, 2001).

Our results showed that the exposure to Cd during IVM disturbed CGs redistribution in the cortex of the oocyte and that resveratrol was able to reverse this effect therefore avoiding polyspermic fertilization. Confirming our findings, resveratrol improved the correct CG distribution before *in vitro* fertilization of bovine oocytes (Takeo *et al.*, 2014) and during *in vitro* aging of porcine oocytes (Ma *et al.*, 2015).

The protective effect of resveratrol on CGs migration and fertilization may be mediated by the maintenance of the proper distribution of mitochondria during oocyte maturation (Ma *et al.*, 2015). It is well known that mitochondria distribution, function and interaction with endoplasmic reticulum are involved in the process of CGs migration and exocytosis (Blerkom and Davis, 2007; Dumollard *et al.*, 2008; Calì *et al.*, 2013).

In this respect, our results demonstrated that resveratrol addition in the culture medium counteracted the adverse effect of Cd on mitochondria distribution and organization in the oocyte cytoplasm.

A prevention effect of resveratrol to the inadequate translocation of mitochondria has been described in mouse oocyte exposed to methylglyoxal (MG), during *in vitro* maturation (Liu *et al.*, 2013). Oxidative stress may be another factor underlying the alteration of fertilization induced by the Cd exposure (Martino *et al.*, 2017). In this regard, Martino *et al.* (2017) demonstrated that cumulus-oocyte mitochondria over-activity associated with an increase of ROS levels and membrane lipid peroxidation were related to impaired *in vitro* fertilization of ovine oocytes exposed to nanomolar Cd concentrations during maturation.

The findings of our study corroborated the negative impact of Cd on bionergetic/oxidative status of oocytes as showed by the increased mitochondria activity and cytosolic ROS levels, which occurred in oocytes under Cd exposure. We demonstrated the beneficial action of resveratrol to alleviate oxidative stress induced by Cd and, even more important, this effect was proved using micromolar concentration of Cd using a model of low competence consisting of oocytes derived from prepubertal donors. This highlights the powerful antioxidant properties of resveratrol and its potential use to improve the quality of oocytes. The beneficial effect of resveratrol on oocytes redox status has been described in several species (Galeati and Spinaci, 2015) in standard *in vitro* embryo production protocols or after oocyte exposition to toxic substances (Huang *et al.*, 2007; Chan, 2011; Liu *et al.*, 2013). The mechanism of resveratrol action included the reduction of ROS levels by up-regulation of Sirtuin 1, which in turn modulated the activity of antioxidant enzymes, the increasing of GSH (Wang *et al.*, 2014; Xia *et al.*, 2017), the regulation of biosynthesis, degradation and activity of mitochondria (Sato *et al.*, 2014).

In conclusion, resveratrol is a useful therapeutic agent to counteract the toxic effect of Cd on *in vitro* fertilization of oocytes by preventing inadequate distribution of cortical granules, dysfunction of mitochondria and oxidative stress.

Further experiments are requiring to better understand the exact mechanisms underlying the resveratrol effect on fertilization outcome.

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Chapter 7:

General discussion

7- General discussion

In recent years, the interest of the scientific community is focused on the developmental of new strategies to improve the outcome of ARTs techniques. The techniques of *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) are routinely used for *in vitro* production of embryos in several animal species, but the success in terms of births is still sub-optimal.

The oocyte quality certainly represents the most important determinant of *in vitro* embryo production. Many attempts have been made to optimize the *in vitro* environment by formulation of appropriate culture conditions (Bermejo-Alvarez *et al.*, 2010) including the formulation of culture media which can minimize stress imposed upon gametes and enhances oocyte developmental competence (Sovernigo *et al.*, 2017).

In the present work, we have aimed to improve the oocyte competence by using a potent antioxidant as is resveratrol, in different veterinary animal species. Thus, we have designed the following studies:

Study I: Resveratrol supplementation during *in vitro* maturation: effect on embryo developmental competence of oocytes retrieved from domestic cat ovaries stored at 4°C for 24 and 48h.

Study II: Resveratrol supplementation during *in vitro* maturation: effect on embryo developmental competence of prepubertal goat oocytes selected by brilliant cresyl blue staining.

Study III: Resveratrol supplementation during *in vitro* maturation of oocytes under cadmium exposure: effect on fertilization outcome in the ovine model.

As detailed in the introduction, an increasing number of studies have recently indicated resveratrol as a useful agent to ameliorate oocyte quality during *in vitro* maturation and to protect the gamete against stressful events. Resveratrol improved *in vitro* maturation of oocytes and *in vitro* embryo production (Galeati and Spinaci, 2015) and enhanced the oocyte resistance to chemical reagents (Huang *et al.*, 2007; Liu *et al.*, 2013), heat stress (Li *et al.*, 2016) and cryopreservation-induced damage (Comizzoli *et al.*, 2009; Santos *et al.*, 2018) in various species.

The results of the present study further confirmed the utility of resveratrol supplementation to the *in vitro* maturation media, expanding the knowledge on its beneficial effect even on oocytes of animal species not yet been investigated and in specific sub-optimal conditions which may affect the oocyte quality. In particular, the effect of resveratrol has been studied in association with ovary cold storage in the domestic cat, oocytes from prepubertal goats and exposure to cadmium in prepubertal sheep.

Resveratrol has been widely studied for its many beneficial properties, including antioxidant properties. Like any other antioxidant, resveratrol is considered a redox agent, which can produce a pro- or anti-oxidant effect depending on the administered dose. With regard to the oocyte the overall results demonstrate that supplementation of *in vitro* maturation medium with low concentration of resveratrol improves oocyte maturation and blastocysts formation (Galeati and Spinaci, 2015). However, species-specific differences have been reported with different resveratrol concentrations.

Kwak *et al.* (2012) investigated the effect of supplementation of five different concentrations (0, 0.1, 0.5, 2.0 and 10 μM) of resveratrol during *in vitro* maturation of pig oocytes and reported the positive effect of 2 μM resveratrol on blastocyst formation and total cell number both after IVF and after parthenogenetic activation. On the contrary when the concentration was increased to 10 μM, despite not having a toxic effect, a reduction of nuclear maturation and no effect on blastocyst production has been recorded (Kwak *et al.*, 2012).

In bovine was observed that among all the concentrations tested (0.1, 1.0, 10 μM), that 1 μM was the best to improve nuclear maturation, embryo development and blastocyst quality (Wang *et al.*, 2014). As demonstrated for pig (Kwak *et al.*, 2012), the beneficial effect of resveratrol is lost at a concentration of 10 μM.

In the goat, even lower resveratrol concentrations (0.25 and 0.5 μM) positively affected embryo production following parthenogenetic activation and somatic cell nuclear transfer, while negative effects have been found at a concentration of 5 μM (Mukherjee *et al.*, 2014). In contrast with most results, some other studies reported that high resveratrol concentration (20 μM) may exert a positive influence on fertilization and embryonic development in cattle and pig oocytes (Sato *et al.*, 2014; Takeo *et al.*, 2014). These differences within the same species could depend on several factors including different culture conditions existing among laboratories.

In the present research, appropriate resveratrol concentrations have been identified according to each experimental study.

In the domestic cat, (Study I), the supplementation of 5 μM resveratrol to the IVM medium of oocytes retrieved from ovaries stored at 4°C for 24 and 48 h improved blastocyst yield. To the best of our knowledge, this is the first study evaluating the effect of resveratrol on *in vitro* embryo production in the domestic cat. A previous study (Comizzoli *et al.,* 2009) was designed to examine the influence of *in vitro* compaction of GV chromatin using various resveratrol exposures (0, 0.5, 1.0 or 1.5 mM) for 1.5 h before *in vitro* maturation, on preservation of oocyte meiotic and developmental competence during vitrification (Comizzoli *et al.*, 2009). Results of this study showed that, in fresh oocytes, treatment with 0, 0.5, 1.0 mM resveratrol had no effect on maturation and embryonic development, and the concentration of 1.5 mM was found to be detrimental. In vitrified oocytes, exposure to resveratrol 1 mM before vitrification allowed to improve the rates of survival, maturation and development after thawing (Comizzoli *et al.*, 2009). In pig, Santos *et al.* (2018) described a positive effect of 2 μM resveratrol on the blastocyst rate of vitrified/thawed oocytes, while no effect was observed on fresh oocytes (Santos *et al.*, 2018).

In our study, the exposition of cat oocytes to 5μM resveratrol during in *vitro* maturation allowed to improve the embryonic development only after storage of the ovaries for 48h but had no effect on the 24h group.

These results suggest that, in addition to the species-to-species variation to the dose effect of resveratrol, the sensitivity of oocytes to resveratrol concentration should be evaluated specifically according to each specific stress conditions, which may impair oocyte quality differently. Moreover, the optimal concentration of resveratrol may vary according to the intrinsic competence of the oocytes.

In the caprine species, 0.5 μM resveratrol addition to the IVM medium increased the embryonic development of oocytes retrieved from adult animals (more competent) (Mukherjee *et al.*, 2014). Consistently, in our second study (Study II), resveratrol treatment at the concentration of 0.5 μM had no effect in prepubertal goat (less competent). Our findings revealed that prepubertal goat oocytes required a higher dose of resveratrol than adult ones. Indeed, resveratrol at the concentration of 1μM significant increased both cleavage and embryo development.

Based on the results obtained in the prepubertal goat the concentration of 1 μM was also selected for the prepubertal sheep. In fact, for the best of our knowledge, there is not study on the effects of resveratrol in the ovine specie. Taking into account to both the low competence of oocytes from prepubertal donors and also the sub-optimal condition induced by cadmium exposure during maturation, it was decided to test also a higher concentration of resveratrol (2μM). The two concentrations tested positively affected the competence of prepubertal sheep oocytes under Cd exposure, favouring the normal fertilization and preventing polyspermy (Study III).

The antioxidant action is one of the main mechanisms underlying resveratrol effect. In our studies, resveratrol supplementation during maturation reduced the intracytoplasmic levels of ROS both in cat oocytes collected from cold stored ovaries and in prepubertal sheep oocytes exposed to cadmium.

It has been shown that resveratrol can directly scavenge free radicals or can stimulate targets molecular involved in the regulation of the endogenous antioxidant system (Xia et *al.,* 2017). Sirtuin 1 (SIRT1), a key regulator of the redox state in the oocytes and granulosa cells(Tatone *et al.,*2015), represent the main target of resveratrol. SIRT1 activation by resveratrol induces the up-regulation of the antioxidant enzymes such as superoxide dismutase, GSH peroxidase and catalase (Xia et *al.,* 2017). Wang *et al*. (2014) reported that in the cumulus cells of pig oocytes the SIRT 1 levels and the expression of antioxidant genes CAT, GPx4 and SOD1 were significantly increased after maturation in presence of resveratrol. Furthermore, an increase in GSH content has been reported in cattle (Wang *et al.*, 2014) in pig (Kwak *et al.*, 2012) and goat oocytes (Mukherjee *et al.*, 2014).

In agreement with these findings, we found higher GSH content in resveratrol treated groups both in cat and goat oocyte. GSH is the major non-protein sulphydryl compound in mammalian cells and protects cells from oxidative damage. The intra-oocytes level of GSH can be considered a marker of cytoplasmic maturity due to the close correlation with embryonic development (Luberda, 2005). In our studies, the increased of GSH content in cat and goat oocytes treated with resveratrol was associated to the enhancement of *in vitro* blastocyst formation.

Numerous studies demonstrated the involvement of resveratrol in mitochondrial regulation (Ungvari *et al.*, 2011). Resveratrol up-regulate SIRT1 which modulatating mitochondrial biogenesis and degradation promotes mitochondrial turnover and enhanced mitochondrial activity in pig oocytes (Sato *et al.* 2014). Parameters such as mitochondrial activity, number and distribution are considered good markers of the quality of the oocyte.

The data of this study underlined the influence of resveratrol in the regulation of active mitochondria organization in the oocyte. In prepubertal goat, resveratrol addition to the maturation medium induced a modification of active mitochondria organization in the cytoplasm of BCB+ and BCB- oocytes from a fine homogeneous pattern to a clustered phenotype.

A clustered active mitochondria organization was associated with oocytes maturation and high developmental competence in several species (Reyes *et al.*, 2011; Leoni *et al.*, 2015). This phenotype is related to a higher development competence also in the prepubertal goat, as demonstrated in Study II by the increase in the *in vitro* embryo production.

In the third study (Study III) resveratrol protected prepubertal sheep oocytes against the Cd induced toxicity at the mitochondria level. Indeed, resveratrol prevented inadequate translocation of mitochondria and modulate their activity by restoring a condition similar to that of control. A prevention effect of resveratrol to the inadequate translocation of mitochondria has been described in mouse oocyte exposed to methylglyoxal (MG), during *in vitro* maturation (Liu *et al.*, 2013).

Taking all these results into account, we conclude that resveratrol supplementation during *in vitro* maturation constitutes a useful strategy to improve oocyte quality and *in vitro* embryo production outcome in species of veterinary interest. Furthermore, resveratrol may represent an effective therapeutic agent to protect oocytes against external injuries, which can deteriorate oocyte quality. The mechanism underlying resveratrol effects included the regulation of bioenergetic/redox status of the oocytes by the modulation of ROS and GSH levels and mitochondria function and the distribution of cytoplasmic organelles.

Chapter 8:

General conclusion

8- General conclusion

Study I. Resveratrol supplementation during *in vitro* maturation of domestic cat oocytes recovered from ovaries stored at 4°C for 24 and 48h:

- Increased the GSH content and reduced the ROS levels of *in vitro* matured oocytes after 24h and 48h of ovary storage;
- Improved blastocyst rate after IVF of oocytes retrieved from ovaries stored for 48;
- Increased the mean number of cells/blastocyst both after 24h and 48h of ovary storage.

Study II. Resveratrol supplementation during *in vitro* maturation of prepubertal goat oocytes selected by BCB test:

- Increased GSH content of both BCB+ and BCB- selected oocytes;
- Modified active mitochondria organization;
- Improved embryo development to blastocyst stage with more significance in BCB+ than in BCB- oocytes.

Study III. Resveratrol supplementation during *in vitro* maturation of prepubertal sheep oocytes under cadmium exposure:

- Increased normal fertilization rates preventing polyspermy fertilization via mechanisms which include cortical granules redistribution;
- Prevented inadequate mitochondrial distribution and mitochondria dysfunction;
- Reduced the intracellular ROS levels of oocytes.

Chapter9:

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9- Bibliography

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