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Dynamics of nuclear and cytoplasm maturation of bovine oocytes cultivated *in vitro* in medium supplemented with fulerol

[Dinâmica da maturação nuclear e citoplasmática de oócitos bovinos cultivados in vitro *em meio suplementado com fulerol]*

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ABSTRACT

The aim of this work was to evaluate, *in vitro*, the dynamics of nuclear and cytoplasmic maturation of bovine oocytes in traditional IVM medium (CT) and supplemented with fullerol (MF50), for 36 hours. The nuclear maturation of CT (n=300) and MF50 (n=270) every 6 hours, stained with Hoechst33342 and cytoplasmic, the mitochondrial distribution of CT (n=197) and MF50 (n=159) at every 12 hours, stained with Mitotracker Orange. At 6 hours, CT oocytes (19%) were in MI (metaphase I), while in MF50 they were in GV (germ vesicle) or GVB (GV breakeage), repeating at 12 hours. At 18 hours, 46.3% were matured in CT, and 20% in MF50. At 24 hours, 43.9% of maturation was observed in the MF50 group, and 63.8% in the CT. At 30 and 36 hours, the maturation pattern was stable, but with the onset of oocyte degeneration. There was a delay in cytoplasmic maturation with 36 hours ($P<0.05$) in MF50 (53.9% of mature gametes), compared to CT (69.8%). With immature cytoplasm, they were 10.4% and 31.7% for CT and MF50 ($P<0.05$), respectively. It was concluded that fullerol possibly interfered in the expansion of cumulus oophorus cells, as well as delayed the meiotic progression and cytoplasmic maturation.

Keywords: bovine oocytes, fullerol, meiosis inhibition, *in vitro* oocyte maturation

RESUMO

O objetivo deste estudo foi avaliar, in vitro, a dinâmica da maturação nuclear e citoplasmática de oócitos bovinos em meio MIV tradicional (TC) e suplementado com fulerol (MF50), durante 36 horas. Na maturação nuclear do TC (n=300) e do MF50 (n=270) a cada seis horas, corados com Hoechst 33342, e na citoplasmática, avaliou-se a distribuição mitocondrial do TC (n=197) e do MF50 (n=159) a cada 12 horas, corados com Mitotracker Orange (Life® Technologies). Às seis horas, oócitos do TC (19%) se encontravam em MI (metáfase I), enquanto no MF50 estavam em VG (vesícula germinativa) ou QVG (quebra VG), repetindo com 12 horas. Às 18 horas, 46,3% estavam maturados no TC, e 20% no MF50. Com 24 horas, verificaram-se 43,9% de maturação no grupo MF50, e 63,8% no TC. Às 30 e 36 horas, o padrão de maturação foi estável, mas com início de degeneração oocitária. Houve retardo na maturação citoplasmática com 36 horas ($P<0,05$) no MF50 (53,9% de gametas maduros), comparado ao TC (69,8%). Com citoplasma imaturo, foram 10,4% e 31,7% para TC e MF50 ($P<0,05$), respectivamente. Conclui-se que o fulerol possivelmente interferiu na expansão das células do cumulus oophorus, *bem como retardou a progressão meiótica e a maturação citoplasmática dos oócitos.*

Palavras-chave: bovinos, bloqueador de meiose, fulerol, maturação oocitária in vitro, oócitos

INTRODUCTION

The *in vitro* production of embryos (IVP) depends on the insemination of viable, mature, and competent oocytes to develop embryos so that they are then transferred to the recipient females. The reduction in oocyte competence impairs the production of blastocysts which, despite the variability of results between commercial *in vitro* production systems, range from 40 to 50% (Watson, 2007). In these *in vitro*

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systems, oocyte maturation (IVM) is an important stage of reproductive biotechnology that aims to obtain mature oocytes from cumulus oophorus complexes (COCs) mechanically removed from antral follicles after follicular aspiration guided by ultrasound or ultrasound. slaughterhouse ovaries. At maturation, oocytes are cultured for 24 hours until they reach metaphase II, when they become ready to be fertilized and can develop into embryos. The oocytes matured *in vitro* are mostly meiotically competent, however, variations in culture media can influence embryonic development, altering the number of blastocyst cells and the rate of apoptosis (Watson, 2007). Therefore, it is necessary to understand the *in vitro* maturation process of oocytes (Gilchrist *et al.*, 2008).

One of the great challenges for increasing the results of *in vitro* embryo production is to understand the mechanism that confers competence for the oocyte to develop, including the role played by the follicular environment in vivo. The understanding of the dynamics of the maturation process aims to obtain mechanisms to modify and increase the quality of *in vitro* maturation media, resulting in a higher rate of blastocyst production. Given the context, IVM means have been supplemented and tested to improve the oocyte potential for IVP. One of the last technologies that has been tested is nanotechnology, which has been gaining notoriety in several scientific researches, mainly in the biological and medical areas.

Ladeira (2013) verified the antioxidant property of fullerol for the medical field, in cardiovascular dysfunctions induced by oxidative stress. As the *in vitro* environment for embryo production has a high concentration of reactive oxygen species, Prata (2019) recently developed a study to evaluate the effect of adding the fullerol nanoparticle to the *in vitro* maturation medium of bovine embryos, at different concentrations, on production rates and quality of embryos produced. Prata (2019) concluded that the addition of fullerol did not change the rates of cleavage and blastocyst production, however, at the highest concentration of 50nM, it reduced the rate of apoptotic cells of blastocysts produced *in vitro*. In view of this concentration, the author was also able to observe a greater amount of unmatured oocytes in relation to the other treatments, after 24 hours of *in vitro* maturation, which leads to the assumption that fullerol could have a probable blocking effect on *in vitro* nuclear maturation, when used at higher concentrations.

The aim of the present study was to describe the dynamics of nuclear and cytoplasmic maturation of bovine oocytes cultured *in vitro* in a medium supplemented with 50nM fullerol, to test the hypothesis that this nanoparticle can be used as a reversible blocker of meiosis, delaying the resumption of meiosis of oocytes mechanically removed from the antral follicles.

MATERIAL AND METHODS

The study was carried out at the *In vitro* Embryo Production Laboratory of the Department of Veterinary Clinic and Surgery of the Veterinary School of UFMG (Federal University of Minas Gerais), in Belo Horizonte/MG. The procedures were approved by the CEUA (Protocol No. 308/2018). (St. Louis, MO, USA), except when specified in the methodology.

The synthesis of fullerol (C60OH22-24) occurred from the chemical functionalization of fullerene (C60), by the polyhydroxylation process, which was carried out at the Nanomaterials Laboratory of the Physics Department of UFMG. To obtain the solution at the proposed concentration (50nM), the fullerol was subjected to serial dilutions in Phosphate buffered saline (PBS) solution (D1408 Sigma).

The ovaries were collected soon after the evisceration of the animals in slaughterhouses in the region with authorization from the Federal Inspection Service. They were transported to the laboratory in warm saline solution (0.9% NaCl). In the laboratory, follicles with diameters of 3 to 8 mm were aspirated and grades I and II oocytes were screened and subjected to maturation for 36 hours.

In the control treatment (CT) the IVM was performed in TCM-199 bicarbonate base medium (Gibco® Life Technologies, Grand Island, USA) plus some substrates. To the maturation treatment with 50nM fullerol (MF50) 50nM fullerol (TCM 199 Bicarbonate + 50nM fullerol) was added to the IVM medium. This dose was proposed based on a previous study by the same team (Prata, 2019), when various

concentrations of fullerol added to the maturation medium were tested, and the dose of 50nM provided a better result in IVP, similar to those obtained in the control treatment of that one. study.

During the 36 hours, nuclear maturation was evaluated every 6 hours of incubation by Hoechst 3334 staining. Nine laboratory routines were performed to evaluate nuclear (n=6) and cytoplasmic (n=3) maturation. The oocytes, based on the methodology of Hewitt and England (1997), were classified according to the stages of maturation into germinal vesicle (GV), germinal vesicle breakage (GVB), metaphase I (MI), metaphase II and degenerated oocytes (DEG). as shown in figure 1.

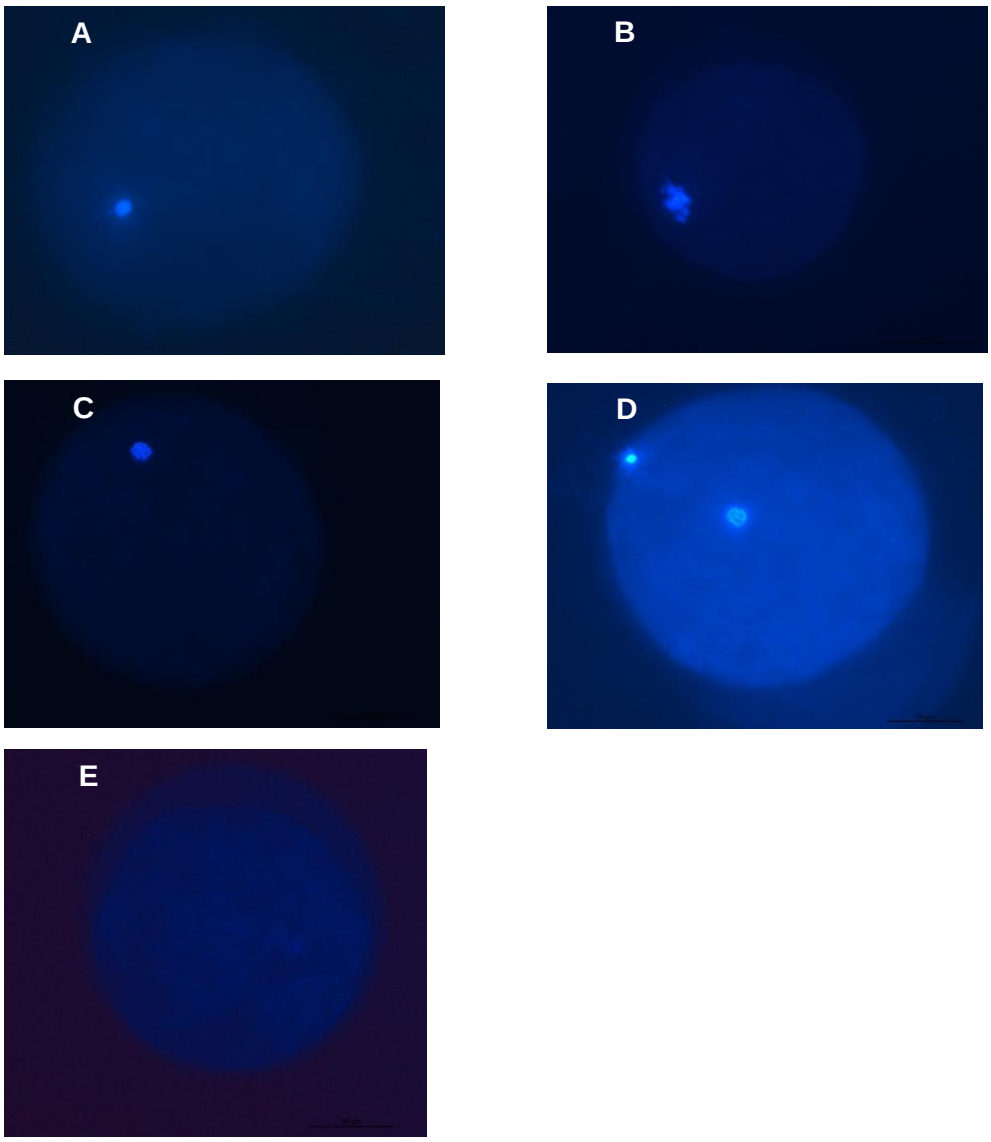

Figure 1. Photomicrographs obtained under a fluorescence microscope of the different phases of nuclear maturation of bovine oocytes stained with Hoechst 33342. (A) Oocyte in germinal vesicle (*GV - Prophase I of meiosis): decondensed chromosomes; (B) Germinal vesicle breakage (GVB*); (C) Oocyte in metaphase I: highly condensed chromosomes; (D) Oocyte in metaphase II: chromosomes condensed in the metaphase plate and extrusion of the first polar body (E) Degenerated oocyte: oocyte retraction and absence of nucleus (400x magnification).

The evaluation of cytoplasmic maturation was performed with Mitotracker Orange staining (Life® Technologies, Carlsbad, CA, USA), according to the methodology proposed by Jeseta *et al*. (2014). From 0 h, and every 12 h (up to 36 h) of maturation, a group of oocytes from each treatment (CT and MF50) was stained. Maturation was evaluated according to the distribution of mitochondria in the cell's cytoplasm, being homogeneous and peripheral in immature oocytes and heterogeneous in matured ones, according to the pattern adopted by Katska-Ksiazkiewicz *et al*. (2011). The images in figure 2 represent the different stages of cytoplasmic maturation evaluated.

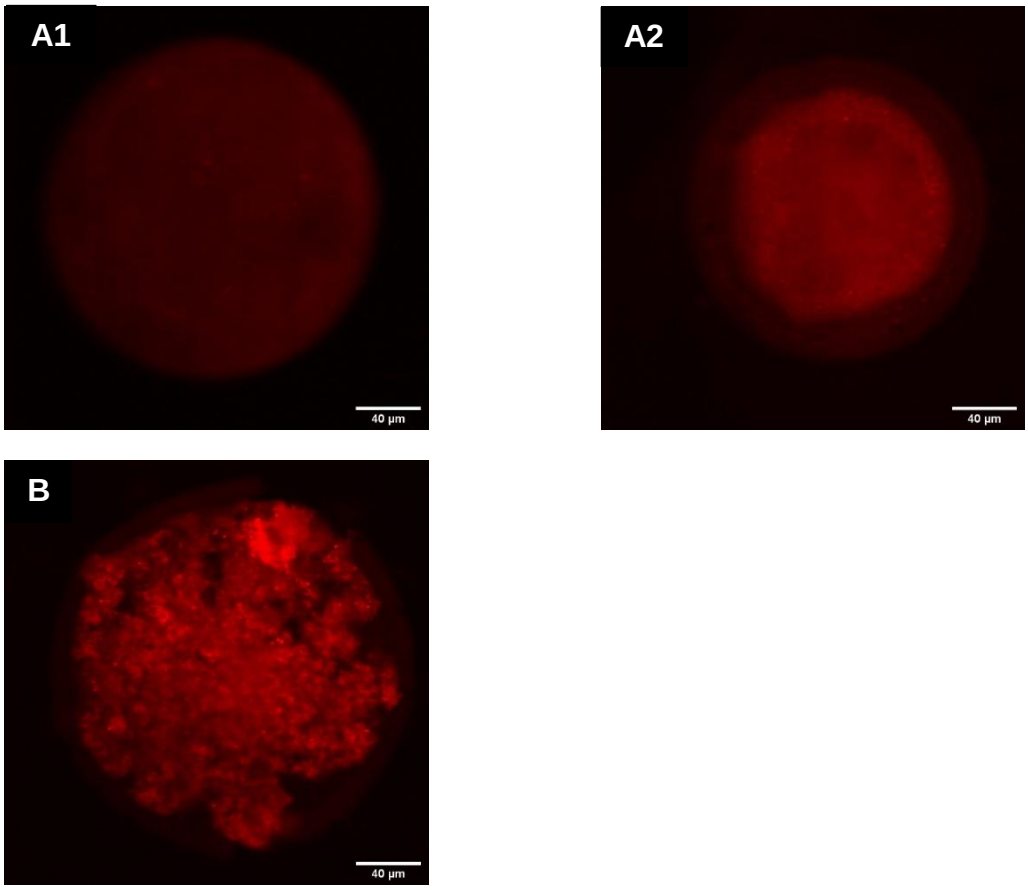

Figure 2. Equatorial plane microscopy images of bovine oocytes showing the cytoplasmic distribution pattern of mitochondria stained with MitoTracker Orange. (A) Immature oocytes with a homogeneous pattern (A1) and peripheral distribution of mitochondria (A2); (B) Heterogeneous pattern in matured oocytes. (400x magnification).

The experiment followed a completely randomized design. The percentage of *in vitro* nuclear and cytoplasmic maturation of the oocytes, over the hours of incubation, was reported in a descriptive way. The comparison between treatments, Control and Fullerol 50nM, of the rates of nuclear and cytoplasmic maturation, within each hour, was evaluated by Fisher's Exact test, using the statistical program GraphPad Instat version 3.06.

RESULTS AND DISCUSSION

Figure 3 shows the temporal comparisons of the different maturation stages of bovine oocytes cultured *in vitro* in control media and supplemented with 50nM fullerol.

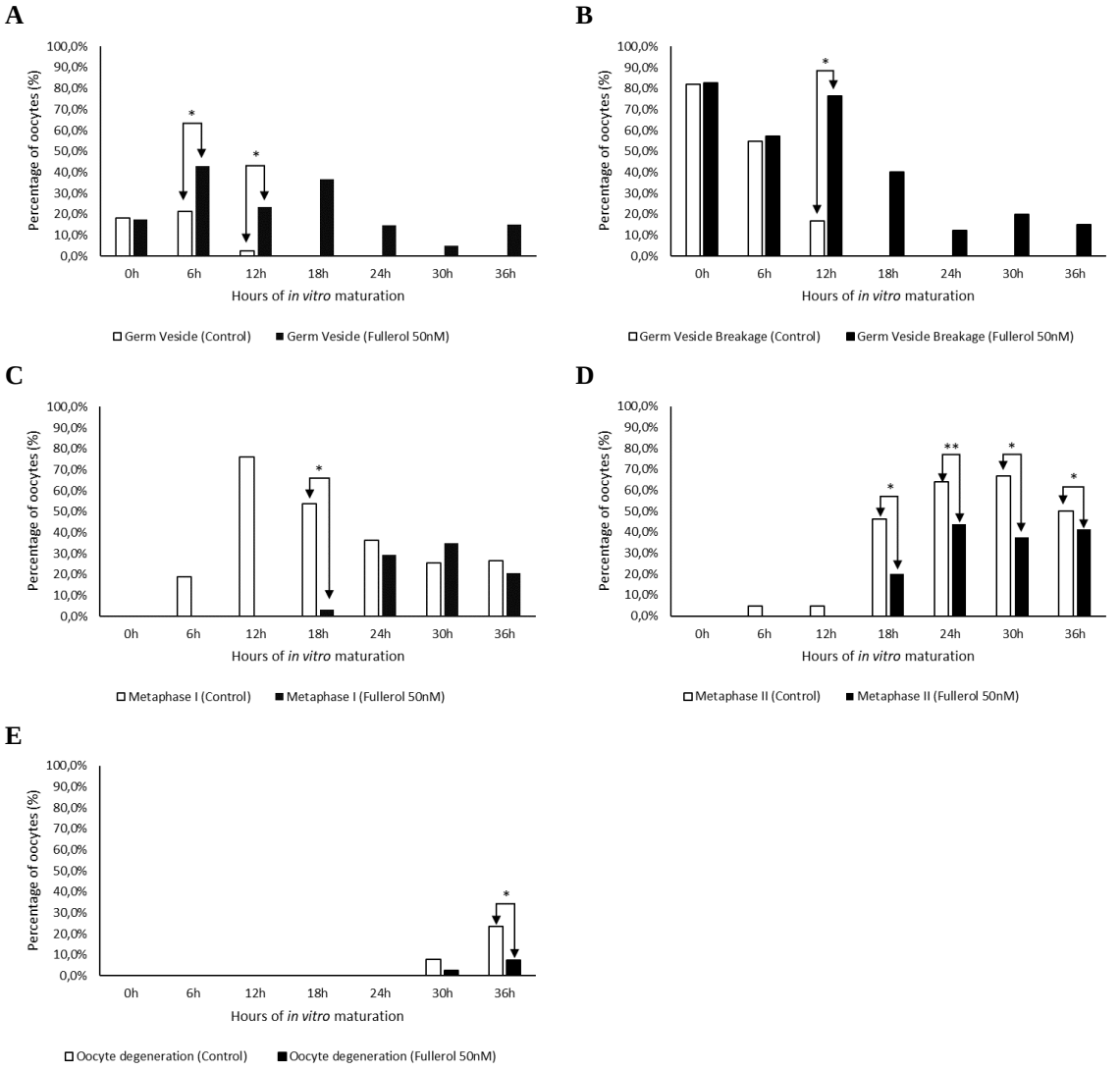

Figure 3. Percentage distribution of the different stages (A=germ vesicle; B=germ vesicle breakage; C=metaphase I; D=metaphase II) of nuclear maturation and oocyte degeneration (E) of bovine oocytes cultured *in vitro* in conventional medium or containing 50nM of Fullerol, depending on the hours of incubation. Data analyzed by Fisher's Exact Test (GraphPad Instat 3.06) at 5% significance. *P<0.05; ** P=0.08.

The retention of oocytes in the GV stage (P<0.05) is evidenced at 6 and 12 hours (Figure 3A), and for GVB (P<0.05) at 12 hours (Figure 3B) for the 50nM fullerol treatment, when compared to the control treatment, and did not differ (P>0.05) for the other evaluation times. Considering the 12 hours of culture, the treatment with 50nM fullerol maintained higher rates of oocytes in the germinal vesicle (23.5% vs 2.4%) and germinal vesicle breakage (76.5% vs 16.5%). The progression to metaphase I (Figure 3C) is seen as early as 6 hours of cultivation of the control treatment, while for the fullerol 50nM treatment it started at 18 hours, that is, a time difference of 12 hours after the start of the *in vitro* incubation. Likewise, fullerol is suggested to act as a meiosis blocking agent. At 12 hours of incubation, when 76.2% of oocytes from the control treatment were in metaphase I, no oocytes from the 50nM fullerol treatment had resumed meiotic division. At 18 hours there was also a statistical difference (P<0.05), when 53.7% and 3.3% of the oocytes

were in metaphase I, for the control and 50nM fullerol treatment, respectively.

The nuclear maturation (Figure 3D) started at 18 hours of incubation for the two treatments and remained statistically different until the end of the study. The percentage differences were higher for the control treatment at 26.3% ($P<0.05$), 19.9% ($P<0.08$), 29.3% ($P<0.05$) and 11.5% ($P<0.05$) for 18, 24, 30 and 36 hours of incubation, respectively. These results again suggest fullerol as a meiosis blocking agent.

The degeneration of oocytes, initiated at 30 hours of *in vitro* incubation for the two treatments, differed ($P<0.05$) at 36 hours, when the control treatment had 16% (23.5% vs 7.5%) more of degenerated oocytes than 50nM fullerol treatment (Figure 3E).

In IVM, when follicles are aspirated from the ovaries, meiotic division resumes in those competent oocytes that have reached the minimum diameter for the species. It is believed that this acquisition of competence of the oocyte occurs before the resumption of meiosis, and the removal of the oocyte from the follicular environment, which has inhibitory factors that keep them stationary in the meiotic division, provides the progression of maturation (Sirard, 2001). After oocyte removal, meiosis spontaneously resumes, regardless of gonadotropins, due to the absence of inhibitory factors. In *in vitro* maturation, the resolution of meiosis occurs, on average, in 24 hours. The high intraoocyte concentrations of cAMP, which are accumulated during follicular growth, are associated with oocytes with high competence for fertilization (Luciano *et al*., 1999). In contrast, under *in vivo* conditions, after the preovulatory LH surge or mechanical removal of the oocyte from the follicle, cAMP concentrations gradually decrease, unblocking meiosis (Richard and Sirard, 1996). However, only the resumption of the nuclear maturation process is not enough to guarantee the later embryonic development, being necessary that the cytoplasmic maturation occurs, which is a step that needs longer duration. Therefore, studies with meiosis blockers have been carried out to allow cytoplasmic capacitation, before the resumption of meiosis, as a way of providing additional time for oocytes to undergo prematurity changes (Hyttel *et al*., 1997; Lonergan *et al*., 2000; Albarracín *et al*., 2005). Butyrolactone I is one of the substances used for this purpose, used in pre-maturation, reversibly blocking the meiosis of oocytes in GV. This allows the improvement of oocyte competence, through more time for the oocyte to undergo the necessary changes to maintain its further development after fertilization (Adona and Leal, 2004).

Figure 4 and 5 show the distribution of cytoplasmically considered immature and mature oocytes, respectively, after evaluation at 12-hour intervals.

It can be observed that there was a significant difference ($P<0.05$) between the percentage of cytoplasmic maturation for the control media and those supplemented with 50nM fullerol, at 36 hours of incubation. In the control treatment, 69.8% of cytoplasmic maturation was observed, which was 16.0% higher than that found in the 50nM fullerol treatment, which was 53.9%. This result suggests that, possibly, fullerol may also be acting in the control of cytoplasmic maturation, but later than that observed for nuclear maturation, since the difference only appears at 36 hours.

Adona (2006) evaluated the effect of meiotic blockade by butyrolactone I (10 and 100 µM) on the distribution of mitochondria in the cytoplasm of oocytes. The oocytes were blocked for 24 hours and then divided into two groups: the first for immediate evaluation and, in the other, the oocytes were submitted to 24 hours of IVM, before evaluation. In the control group, the evaluation was performed at 0 hours and after 24 hours of IVM. After 24 hours of blockade, oocytes with peripheral distribution of mitochondria amounted to 81.5% for the 10µM butyrolactone I group, and 86.9% for the 100µM butyrolactone I group, and were lower than the control group (100%). After 24 hours of IVM, mitochondria migrated throughout the cytoplasm of the oocytes. In the control group, migration was lower (81.5%) than the 10µM butyrolactone I (95.2%) and 100µM butyrolactone I (98.2%) groups, which did not differ from each other. The authors concluded that although butyrolacone I blocks meiosis, mitochondrial migration is not completely blocked.

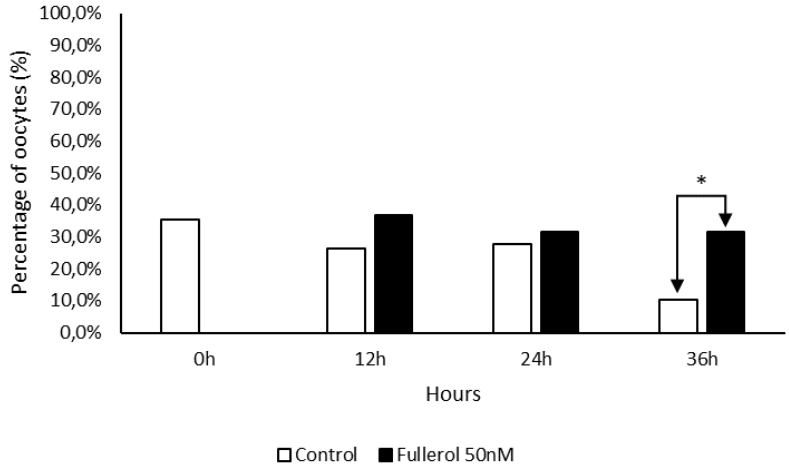

Figure 4. Temporal distribution of the percentage of immature oocytes (homogeneous + peripheral mitochondrial distribution) cultured under conventional conditions (control medium) or in medium supplemented with 50nM fullerol (*P<0.05).

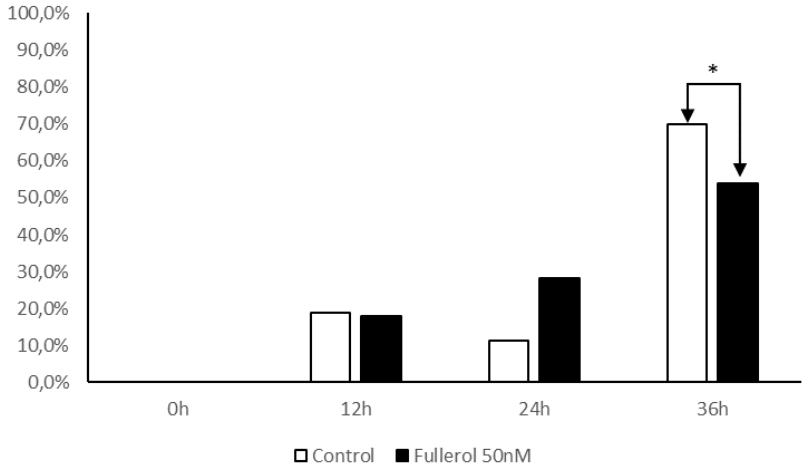

Figure 5. Temporal distribution of the percentage of mature oocytes (heterogeneous mitochondrial distribution) cultured under conventional conditions (control medium) or in medium supplemented with 50nM fullerol. *P<0.05.

Based on the aforementioned study and on the data of the present work, it is suggested that although fullerol interferes by blocking nuclear maturation, its effect on cytoplasmic maturation is not so intense. Compared to butyrolactone I, which is used as a meiotic blocker, but which has little interference in cytoplasmic maturation, fullerol at a concentration of 50nM also showed a similar action.

CONCLUSION

The addition of 50nM fullerol to the *in vitro* maturation medium delayed meiotic progression and cytoplasmic maturation of bovine oocytes. Fullerol seems to interfere with the mechanism of expansion of cumulus oophorus cells, since it was observed that it was difficult to denude the oocytes during the laboratory routine. If

experimentally confirmed that fullerol has a reversible function in blocking meiosis in bovine oocytes, it can be used in laboratory routines of IVP to increase the competence of later development of oocytes. However, further studies should be carried out to adjust the ideal dose and time of action, as well as to elucidate the pathway that this nanoparticle uses in the female gamete to delay the resumption of meiosis *in vitro*, and later, to present the contribution of its use in the results of *in vitro* production of embryos.

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