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ANTIOXIDANT EFFECT OF LYCOPENE ON *IN VITRO* MATURATION AND FERTILIZATION OF BOVINE OOCYTES

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ABSTRACT

In vitro embryo production (IVEP) is a vital assisted-reproductive technology with significant applications in producing genetically superior animals through *in vitro* maturation (IVM), fertilisation (IVF) and culture of zygote. This study evaluated the antioxidant potential of lycopene during in vitro maturation (IVM) and fertilisation (IVF) of bovine oocytes. Fifty ovaries from Belgian Blue cows were collected from a slaughterhouse in Moeskroen, Belgium, and transported to the laboratory. Cumulus-oocyte complexes were aspirated from follicles (4–8 mm diameter) and incubated in modified bicarbonate-buffered TCM-199 with supplements (50 µg/mL gentamycin and 20 ng/mL epidermal growth factor) at 38.5°C in 5% CO_2 for 22 hours. Four experimental groups were established: control (C), lycopene (supplemented with 0.2 µM lycopene), menadione (supplemented with 5 µM menadione), and lycopene plus menadione (L+M), each with 40 oocytes. Following maturation, oocytes were fertilized in media containing BSA and heparin with diluted sperm, co-incubated at 38.5°C in 5% CO_2 for 21 hours. Maturation and fertilisation were assessed under a fluorescent microscope after staining with Hoechst solution. The results showed that oocytes in the lycopene group (L) achieved significantly higher maturation (86.66 ± 5.09%) and fertilisation rates (84.99 ± 5.65%) compared to the other groups ($p < 0.05$). These findings suggest that lycopene supplementation enhances oocyte maturation and fertilisation outcomes. The study recommends incorporating lycopene into maturation media to improve IVEP efficiency in bovine reproductive technologies.

Key Words: Antioxidant, Lycopene, maturation and oocytes

INTRODUCTION

In vitro embryo production (IVEP) is a crucial assisted reproductive technology with various applications, particularly in producing animals of superior genetic quality. It involves processes such as *in vitro* maturation (IVM), *in vitro* fertilisation (IVF), and the *in vitro* culture of presumptive zygotes (Falchi *et al*., 2022).

Oocytes, the female germ cells essential for reproduction, are among the largest cells in mammals (approximately 0.12–0.50 mm in diameter) and can often be seen without magnification (Roy *et al*., 2019). These oocytes are surrounded by cumulus oophorus cells, which protect the oocytes and facilitate their follicular development and maturation (Robert, 2021) . A dense layer of cumulus cells supports the oocyte by supplying nutrients and other essential factors during development (Robert, 2021).

For large-scale embryo production via IVM and IVF techniques, the ovaries of slaughtered animals provide a cost-effective and abundant source of primary oocytes (Moulavi *et al*., 2020). In contrast, in vivo-matured oocytes can be collected through surgical or laparoscopic methods, but these are expensive and yield a low number of oocytes per ovary (Piekarski *et al*., 2023). The practical and economic advantages of embryos produced *in vitro* make them a preferable alternative to those recovered in vivo (Gardner *et al*., 2015).

Notably, earlier research highlighted the potential harmful effects of exogenous estrogen. For instance, culturing bovine COCs with 1 µg/ml estradiol for 22 hours significantly reduced the proportion of oocytes reaching metaphase II and increased nuclear aberrations (Asimaki *et al*., 2022). This suggests that endogenous hormone production by CCs is preferable for optimal oocyte development.

The *in vitro* maturation stage is particularly critical in the IVEP process. For ruminants, oocyte maturation typically occurs at 39°C in a humidified atmosphere with 5% CO_2 (Baldassarre, 2021). Menadione promotes reactive oxygen species (ROS) production through enzymatic processes that generate semi-quinone free radicals, converting oxygen into superoxide anions and further producing hydrogen peroxide (Krylova *et al*., 2016).

On the other hand, lycopene, a potent natural antioxidant, has demonstrated the strongest free radical-scavenging properties among carotenoids and is highly effective in chemically quenching singlet oxygen—10 times more effective than α-tocopherol

(Black *et al*., 2020). Lycopene is a natural carotenoid found mainly in ripe tomato, watermelon and pink grapefruit, it was previously used as food colourant, but its antioxidant potentials has been discovered (Ilahy *et al.*, 2019). Lycopene is a highly unsaturated, straight-chain hydrocarbon containing 13 double bonds, 11 of which are conjugated. This unique structure makes it a powerful and highly effective antioxidant, with potency twice that of β-carotene (Leh & Lee, 2022).

Supplementing antioxidants like lycopene in culture media can help achieve optimal ROS neutralization, maintaining balance during oocyte maturation and embryo development *in vitro*. This study focuses on evaluating the antioxidant effects of lycopene supplementation during the *in vitro* maturation and fertilisation of bovine oocytes.

METHODOLOGY

Tissue culture media (TCM)-199-medium, gentamycin, and kanamycin were purchased from Life Technologies Europe (Ghent, Belgium). Phosphate-buffered saline (PBS) was obtained from Gibco™ 20012019, Thermo Fisher Scientific, (Waltham, MA, USA). All other chemicals were obtained from Sigma-Aldrich (Diegem, Belgium). All media were filtered before use (0.22 M Pall Corporation, Ann Arbor, MI, USA).

In vitro maturation

Bovine ovaries were obtained from slaughterhouse (Moeskroen, Belgium). The ovaries were transported to the laboratory in a thermos container. They were then processed within two hours after collection. In the laboratory, the mesovaria were removed with scissors, and ovaries were disinfected with 70% alcohol and washed three times in warm (37ºC) physiological saline supplemented with Kanamycin (25 mg/mL). Cumulus-oocyte complexes (COCs) were aspirated from antral follicles with diameter ranges between 4 and 8 mm using an 18-gauge needle attached to a 10 mL syringe. The COCs aspirated together with follicular fluid were transferred in to 15 mL tube containing 2.5 mL of wash medium (HEPES-TALP). The oocytes were transferred in a Petri-dish containing 7 mL of HEPES-TALP. Thereafter, only oocytes with homogeneous cytoplasm and surrounded by more than three compact layers of cumulus cells were selected under a stereo-microscope (Olympus ACH 1X, Japan) at X 40 magnification (Sidi *et al*., 2022). A total of 160 oocytes were randomly assigned and incubated in 4 well plate in groups of 60 COCs in 500 µL modified bicarbonate-buffered TCM-199 (supplemented with 50 µg/mL gentamycin and 20 ng/mL epidermal growth factor) in 5% CO_2 in air for 22 h at 38.5 ºC. Four groups were made: group C: control, group L: lycopene, group M: menadione and group L+M: lycopene plus menadione. Group L was supplemented with 0.2 µM lycopene as earlier described by Chowdhury *et al*. (2018), group M was supplemented with 5 µM menadione and group L+M combination of lycopene and menadione.

Determination of maturation and fertilisation rate

Oocytes/Zygote fixation

Oocytes were fixed 22 h post-maturation/fertilisation, and were transferred in a sterile 15mL tube, containing 2.5 mL of HEPES-TALP. They were then denuded with vortex machine for 5 minutes, after which they were washed in a small Petri-dish, thereafter the oocytes were transferred into 4 well plate containing 4% paraformaldehyde and kept overnight at 4 ºC.

Staining (Hoechst)

A 10% Polyvinyl pyroledone (PVP) was prepared in phosphate buffered saline (PBS), 1 µL of hoechst (a cell-permeable DNA stain that is excited by ultraviolet light and emits blue fluorescence at 460 to 490 nm) was added into 10 mL of PVP/PBS solution. Exactly 500 µL of hoechst/PVP/PBS was transferred into each well of a 4 well plate. The oocytes were transferred in to the hoechst solution and allowed to stain for 10 minutes. A drop of Dabco (1 µL) was placed on a glass slide and oocytes were transferred into the droplet, which was then covered with cover slip. Evidence of maturation (presence of second polar body) was determined using fluorescent microscope (Leica DM 5500B Wetzlar, Germany).

In vitro fertilisation Process

Percoll 90 %, SP-TL (-) BSA and SP-TL (+) BSA were placed on the lamina flow at 28^0C.

Preparation of percoll gradient

Two conical tubes (15 mL) were labeled as percoll 45% (tube 1) and percoll gradient (tube 2), 2 mL serological pipette was used to pipette 1.5 mL percoll 90% (GE Healthcare Biosciences, Uppsala, Sweden) into tube 1 and the same pipette was used to pipette 2 mL of percoll 90% into tube 2, fresh 2 mL serological pipette was used to dispense 1.5 mL of SP-TL (-) BSA into tube 1. The same pipette was used to mix the two solutions to achieve 45% percoll. Exactly 2 mL of 45% percoll was aspirated and transferred into tube 2 (Pavani *et al*., 2019).

Semen preparations

For each replicate a straw of semen was taken from liquid nitrogen tank, the straw was swung in air for 5 seconds and then transferred in water bath (37 ^{o}C -38 ^{o}C).

The semen straw was emptied into the Eppindorf tube. Thereafter 1 mL syringe connected to 22 gauge needle was used to dispense the semen from eppindorf tube into the percoll gradient, the tip of the needle was placed on top of the percoll 45% layer and semen was released very slowly. Thereafter, the semen in the percoll gradient was centrifuged twice.

First centrifugation: the semen on the percoll gradient was centrifuged for 447 (g) for 30 minutes at 22 ^{o}C.

Second centrifugation: The tube was removed out of the centrifuge, the supernatant was aspirated and 5 mL of SP-TL (+) BSA was added to the sediment using 5 mL serological pipette. The tube was returned into the centrifuge and the machine was adjusted for 63 (g) for 10 minutes at 22 ^{o}C and the supernatant was removed and the pellet was adjusted to ±100 µL. The sperm concentration was determined with heamocytometer. The volume of the fertilisation medium and sperm concentration needed for *in vitro* fertilisation was determined using the formula: $C_1V_1 = C_2V_2$. The calculated volume of IVF medium and sperm were mixed in a dilution tube.

Fertilization

A total of 160 oocytes were used in this experiment. Fertilisation medium was prepared by adding BSA (Sigma A8806; 6 mg/mL) and heparin (25 mg/mL) in TCM 199. Four well Plates were prepared by dispensing 250 µL of IVF–TALP into all wells of the 4 well plates. Diluted sperm (250 µL) at concentration of 500,000 spermatozoa was added into well 2 and 4. The gametes were co-incubated for 21 h in 5% CO_2 in air at 38.5^{o}C.

RESULTS

Effect of Lycopene and Menadione on Maturation Rate

The maturation rate in all tratment groups (n = 160) is shown in Table 4.1. After 22 h of incubation in maturation medium, the mean ± SE maturation rate of oocytes in L (86.66 ± 5.09) is significantly ($P < 0.05$) different from other treatment groups

Table 4.1: Effect of lycopene and menadione on maturation rate (Mean ± SE, n = 160)

Group	Number of oocytes	Mean ± SE
C	40	63.33 ± 5.09^{a}
L	40	86.66 ± 5.09^{b}
M	40	53.33 ± 5.09^{a}
L+M	40	56.66 ± 5.09^{a}

Values along the same column with different superscript letters $^{(a,b)}$ are significantly different

Key: C: Control; L: Lycopene 0.2 µM; M: Menadione 5 µM; L+M: Lycopene 0.2 µM + Menadione 5 µM

Effect of Lycopene and Menadione on Fertilisation Rate

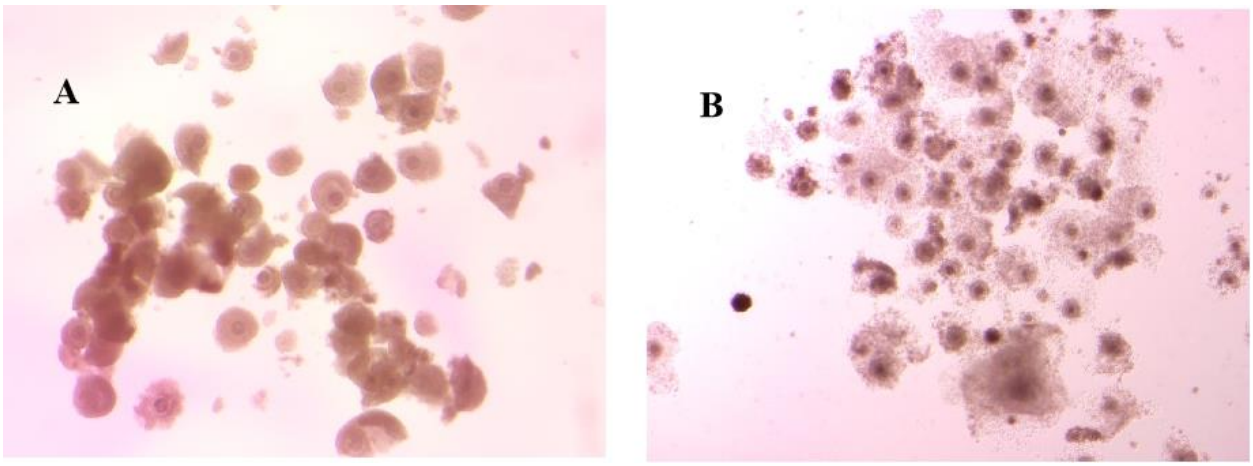
The fertilisation rate in all tratment groups (n = 160) is shown in Table 4.2. After 21 h of incubation in fertilisation medium, the mean ± SE fertilisation rate of oocytes in L (84.99 ± 5.65) is significantly ($P < 0.05$) different from other treatment groups

Table 4.2: Effect of lycopene and menadione on fertlisation rate (Mean ± SE, n = 160)

Group	Number of oocytes	Mean ± SE
C	40	58.33 ± 5.65^{a}
L	40	84.99 ± 5.65^{b}
M	40	56.66 ± 5.65^{a}
L+M	40	64.99 ± 5.65^{a}

Values along the same column with different superscript letters $^{(a,b)}$ are significantly different

Key: C: Control; L: Lycopene 0.2 µM; M: Menadione 5 µM; L+M: Lycopene 0.2 µM + Menadione 5 µM



Degree of cumulus cells expansion in bovine COCs evaluated under a stereo-microscope. (A) Unexpanded aspirated cumulus cells. (B) Expansion of cumulus cells after maturation. (×40 magnification).

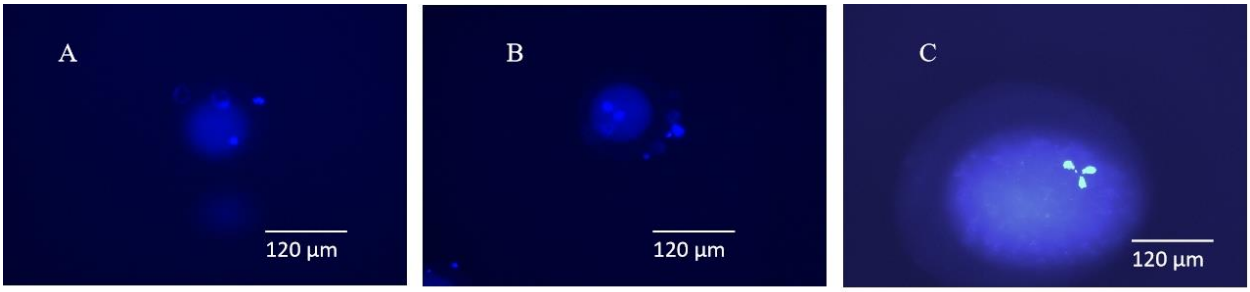


Fig. 2. Nuclear configuration in bovine oocytes stained with Hoechst 33342 and evaluated under a fluorescence inverted microscope: (A) MII: metaphase II; (B) Zygote; (C) Degenerate (×100 magnification)

DISCUSSION

Lycopene, a type of β-carotenoid, exhibits diverse biological effects, including cell cycle regulation, anti-apoptotic properties, and antioxidative activity. This study is the first to examine the impact of lycopene supplementation during serum-free *in vitro* maturation (IVM) on bovine oocyte maturation and developmental potential. Lycopene was found to enhance steroidogenesis in cumulus-oocyte complexes (COCs), improve nuclear and cytoplasmic maturation of oocytes, and reduce intracellular reactive oxygen species (ROS) levels (Chowdhury *et al*., 2018). Additionally, it improved developmental competence and the quality of resulting zygotes.

Cumulus cells (CCs) play a vital role in oocyte growth and maturation, supplying nutrients and mediating hormonal effects. Previous studies demonstrated that CCs in bovine COCs secrete estradiol (E2) and progesterone (P4) during maturation (da Silva Rosa *et al*., 2024). These hormones, produced endogenously or added exogenously, directly support oocyte development. The current findings suggest that lycopene may interact directly or synergistically with endogenous hormones to modulate CCs steroidogenesis. Enhanced production of P4 and E2, even in the absence of exogenous hormones, likely contributes positively to nuclear and cytoplasmic maturation.

The improvement in nuclear maturation observed with lycopene supplementation aligns with studies on other

antioxidants, such as resveratrol, astaxanthin, and melatonin (Vr *et al*., 2022). This variability may stem from differences in the ability of antioxidants to activate the mitogen-activated protein kinase (MAPK) pathway (Supruniuk *et al*., 2023). In mammals, MAPK plays a crucial role in meiotic progression, microtubule organization, spindle assembly, and chromosomal distribution (Bury *et al*., 2016). Lycopene has been shown to activate MAPK in various cell types, potentially explaining its positive effects on meiosis resumption. Further studies are needed to confirm if lycopene enhances androgen secretion by CCs and activates MAPK to improve oocyte maturation.

Additionally, lycopene reduced ROS levels in oocytes exposed to oxidative stress induced by menadione and partially preserved their developmental competence (Sidi *et al*., 2022). These findings underscore the detrimental effects of oxidative stress on oocyte quality and highlight the protective role of antioxidants. Excessive ROS production from pro-oxidants and heat shock has been shown to impair oocyte cleavage after fertilisation and compromise subsequent embryo development (Lin & Wang, 2021).

Meiotic events in oocytes, which are sensitive to stressors such as pH changes, oxidative damage, and toxins, are critical for genetic competence. Fully grown oocytes must reach a certain size and maturity, including sufficient cyclin B levels, to progress through meiosis (Bouftas & Wassmann, 2019). For bovine oocytes, this competence is associated with a follicle size of 2-3 mm and an oocyte diameter of at least 110 µm.

This study concludes that lycopene significantly enhances the maturation and fertilisation of bovine oocytes, while menadione has a negative impact on both nuclear and cytoplasmic maturation. We recommend supplementing 0.2 µM lycopene in the *in vitro* maturation medium to support bovine embryo development. Additionally, incorporating lycopene-rich substances into animal feed may improve reproductive performance. Further research is encouraged to explore the effects of lycopene in *in vitro* fertilization and *in vitro* culture media.

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