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Abstract. The objectives of this study were to evaluate the effect of low-level laser irradiation (LLLI) on bovine oocyte and granulosa cells metabolism during *in vitro* maturation (IVM) and further embryo development. Cumulus-oocytes complexes (COCs) were subjected (experimental group) or not (control group) to irradiation with LLLI in a 633-nm wavelength and 1 J/cm² fluency. The COCs were evaluated after 30 min, 8, 16, and 24 h of IVM. Cumulus cells were evaluated for cell cycle status, mitochondrial activity, and viability (flow cytometry). Oocytes were assessed for meiotic progression status (nuclear staining), cell cycle genes content [real-time polymerase chain reaction (PCR)], and signal transduction status (western blot). The COCs were also *in vitro* fertilized, and the cleavage and blastocyst rates were assessed. Comparisons among groups were statistically performed with 5% significance level. For cumulus cells, a significant increase in mitochondrial membrane potential and the number of cells progressing through the cycle could be observed. Significant increases on cyclin B and cyclin-dependent kinase (CDK4) levels were also observed. Concerning the oocytes, a significantly higher amount of total mitogen-activated protein kinase was found after 8 h of irradiation, followed by a decrease in all cell cycle genes transcripts, exception made for the CDK4. However, no differences were observed in meiotic progression or embryo production. In conclusion, LLLI is an efficient tool to modulate the granulosa cells and oocyte metabolism. © 2014 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: [10.1117/1.JBO.19.3.035006](https://doi.org/10.1117/1.JBO.19.3.035006)]

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1 Introduction

The development of *in vitro* produced bovine embryos depends on the quality and competence of gametes to successfully accomplish the events involved in oocyte maturation, fertilization, and early embryonic development.¹ For the increment of production systems, the low-level laser irradiation (LLLI), also known as photobiomodulation, arises as a new alternative. Based on a special mechanism of electromagnetic emission, LLLI may increase cellular metabolism and improve structural characteristics, which has been proven effective in different cell types.²

The laser irradiation introduces the photonic energy into a biological system, which is converted to ATP and may be then available for cellular metabolism. In this context, the dose of irradiation is an essential parameter for the success of laser therapy, since if insufficient energy is applied no effect will be observed. On the other hand, if more energy is applied, the biostimulation may disappear and be replaced by bioinhibition.²

The optimal dosimetry for the laser therapy is still controversial. The influence of electromagnetic radiation (e.g., laser) in biological systems has been demonstrated in inflammatory processes, tissue repair, and stimulation of cellular respiratory chain.³ The mechanism involved in the influence of laser

therapy in cellular cycles is still a matter of debate. However, studies indicate that the cellular photoreceptors, sensitive to certain wavelengths, are capable of absorbing photons, triggering chemical reactions such as glycolysis and oxidative phosphorylation. This could result in acceleration of RNA transcription and DNA replication. Furthermore, the laser light is known to accelerate the mitosis and induce changes in mitochondrial structure, which are reflected in different metabolic responses.⁴ The effect of laser radiation has been shown to increase the proliferation rate and viability of fibroblasts for both *in vivo* and *in vitro* conditions.⁵ The LLLI was also able to increase the amount of ATP produced by cell lines maintained *in vitro*, demonstrating the intense action in cellular metabolism.⁶

Studies regarding the effect of LLLI on reproductive parameters were conducted mainly in sperm cells. In dog sperm, increase in motility and changes in anaerobic metabolism for energy production were observed 15 to 45 min after irradiation.⁷ Increase in sperm cell viability and motility was also observed in turkeys and horses after irradiation.⁸ Low-power He-Ne laser was also used to treat immature oocytes. The use of laser at doses of 0.4 and 2 J/cm² had a negative effect in the maturation process and induced nuclear damage.⁹ Besides these data, the pathways involved in gamete stimulation and the consequences for embryo production induced by the laser remain unclear.

The *in vitro* embryo production includes three major processes: *in vitro* maturation (IVM), *in vitro* fertilization (IVF),

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and *in vitro* culture (IVC). The process still results in reduced embryo production (around 40%), suggesting that not all the oocytes have the capacity to mature and fertilize properly. The oocyte quality seems to be a key factor that determines embryo production, development, pregnancy establishment, and the production of healthy offspring.¹⁰

Thus, this work aimed to characterize biochemical and molecular changes induced by LLLI in bovine oocytes and its effect on *in vitro* produced bovine embryos.

2 Material and Methods

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical (St. Louis, Missouri). Tissue culture media (TCM) (Hepes and bicarbonate) and fetal calf serum (FCS) were obtained from Gibco (Grand Island, New York).

2.1 Oocytes Collection

Ovaries were obtained from slaughtered cows and washed several times with sterile saline [0.9% (w/v) NaCl containing 100 U/ml penicillin-G and 100 µg/ml streptomycin] at 25 to 30°C to remove blood and debris. Cumulus-oocyte complexes (COCs) were collected by slicing the surface of each ovary and agitating in a beaker containing oocyte collection medium [phosphate-buffered saline (PBS) supplemented with 10% FCS]. After follicle slicing, COCs were allowed to settle for 5 min in oocyte collection medium. Selection of COCs was based on morphological status according to the cytoplasm aspect and the number of granulosa cell layer.¹¹

2.2 Effects of LLLT on Oocytes and Granulosa Cells during IVM

COCs were mechanically dissociated (granulosa and oocytes separation) and submitted to LLLT (experimental group). Evaluations were performed after 30 min, 8, 16, and 24 h of IVM. Granulosa cells were evaluated for cell cycle status (flow cytometry and real-time PCR), mitochondrial activity (flow cytometry), and viability (flow cytometry). Oocytes were assessed for meiotic progression (optical microscopy), cell cycle genes content (real-time PCR), and signal transduction status (western blot). To assess the efficiency of LLLT during IVM in increasing blastocyst rates and qualities, COCs were submitted to irradiation and, after 24 h of IVM, *in vitro* fertilized. Cleavage and blastocyst rates were assessed at days 4 and 7, respectively. All experiments were performed in at least three replicates.

2.3 In Vitro Embryo Production

2.3.1 IVM

Groups of 30 COCs were matured in 90 µl droplets of TCM-199 bicarbonate supplemented with 10% FCS and 0.5 µg/ml follicle-stimulating hormone (FSH) (Folltropin-V, Bioniche, Belleville, Canada), 100 IU/ml human chorionic gonadotropin (hCG) (Chorulon, Merck Animal Health, Boxmeer, The Netherlands), and 1.0 µg/ml estradiol under mineral oil for 18 h at 38.5°C and 5% CO₂ in air and high humidity. Cumulus cells were enzymatically removed using 0.2% hyaluronidase in calcium-free PBS for 10 min and repeated pipetting for 2 min and divided into experimental groups as described in experimental design.

2.3.2 LLLI

After placing the COCs in IVM medium droplets, irradiation was performed as previously described in Ref. 6. Briefly, irradiations were performed with a He-Ne laser (TEM00, Unilaser, Brazil) at a wavelength of 633 nm with a single dose. In order to homogenize the wavefront intensity distribution, the light was passed through a home-made spatial filter projected for this purpose. In the present experiment, a power of 7.5 mW was used. The delivered light power was verified with a power meter (Newport 841-PE, Irvine, California). A 450-mirror deflects the light beam to the individual dish. The corrected fluency applied was 1 J/cm².

After irradiation, samples from experimental and control groups (nonirradiated) were cultured at 38.5°C under an atmosphere of 5% CO₂ in air and high humidity. Samples derived from control groups were named GC followed by the period of incubation. Samples derived from treated groups were named GT followed by the period of incubation. After 30 min (GC30 or GT30), 8 (GC8 or GT8), 16 (GC16 or GT16), or 24 (GC24 or GT24) h of incubation, control and experimental groups were mechanically dissociated with an automatic pipette and immediately evaluated. For embryo production, after 24 h, COCs were removed from IVM and submitted to IVF.

2.3.3 IVF

The IVF procedures, reagents, and media formulations were as described elsewhere.¹² All incubations were performed in an atmosphere of CO₂ in humidified air. Groups of 30 oocytes were inseminated with 1 × 10⁶ Percoll-purified spermatozoa from a pool of three bulls.

2.3.4 IVC

Approximately 18-h postinsemination, putative zygotes (groups of ~30) were cultured in 90 µl droplets of KSOM containing 10% FCS under mineral oil at 38.5°C in a humidified atmosphere with 5% CO₂ for 7 days. Cleavage rate (cleaved/oocyte) was determined 36 h after the beginning of IVC and blastocyst rates (blastocyst/oocyte) at D7 of IVC.

2.4 Assessment of Cell Cycle Status, Viability, and Mitochondrial Membrane Potential by Flow Cytometry

2.4.1 Cell cycle status

For cell cycle analysis, after dissociation, granulosa cells were centrifuged at 500 × *g* for 10 min and resuspended with cold 70% ethanol for fixation. Cells were maintained for at least 24 h at 4°C. Fixed granulosa cells were washed in PBS for ethanol removal, and each sample was resuspended in 500 µl of PBS. Cells were stained with 50 µg/ml of propidium iodide, and 0.5 mg/ml of RNase were added and samples were incubated at 42°C for 1 h.¹³ Samples were analyzed using Guava Easycyte Mini Flow Cytometry System (Guava Technologies, Hayward, California). Data were obtained from 10,000 events from each sample. Histograms of red fluorescence versus counts were generated to evaluate percentages of cells in each phase of the cell cycle.

2.4.2 Mitochondrial membrane potential

For mitochondrial membrane potential analysis, granulosa cells were centrifuged at $500 \times g$ for 10 min and resuspended in $500 \mu\text{l}$ of PBS. Mitochondrial function was assessed with the JC-1 probe, which marks mitochondria that have high-membrane potentials as fluorescent red and mitochondria with low-membrane potentials as fluorescent green. A volume ($2 \mu\text{l}$) of JC-1 ($76.5 \mu\text{M}$ in DMSO) was added to cells samples and incubated for 10 min at 37°C . Samples were washed in PBS at $6000 \times g$ for 2 min and resuspended in PBS. Flow cytometry analyses were performed using the Guava EasyCyte Mini Flow Cytometry System (Guava Technologies).

2.4.3 Cell viability

For viability assessment, granulosa cells were centrifuged at $500 \times g$ for 10 min and resuspended in $500 \mu\text{l}$ of PBS. Cells were stained with $50 \mu\text{g}/\text{ml}$ of propidium iodide, and samples were incubated at 42°C for 1 h. Samples were analyzed using Guava EasyCyte Mini Flow Cytometry System (Guava Technologies). Data were obtained from 10,000 events from each sample. Histograms of red fluorescence versus counts were generated to evaluate percentages of live and dead cells.

2.5 Oocytes Meiotic Progression

After maturation, oocytes were denuded of granulosa cells by mechanical removal. The oocytes were then mounted on glass slides and coverslips using one drop of $10 \mu\text{g}$ Hoechst 33342 ml^{-1} and examined under microscopy (IX-75, Olympus, EUA, Center Valley, Pennsylvania) with epifluorescence (filter with maximum excitation of 365 nm and maximum emission of 480 nm) to evaluate nuclear maturation rates.

2.6 Gene Expression Analysis: Real-Time PCR

2.6.1 RNA isolation and cDNA synthesis

Cells were pooled in four replicates containing 15 oocytes or their respective granulosa cells from the following groups: control 30 min, irradiated 30 min, control 8 h, irradiated 8 h, control 16 h, irradiated 16 h, control 24 h, and irradiated 24 h. RNA from each pool was isolated using Illustra RNAspin MiniRNA Isolation Kit® (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. From purified RNA, reverse-transcription PCR was performed to synthesize the cDNA using Superscript First-Strand Synthesis System for RT-PCR® (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. Briefly, total RNA ($8 \mu\text{l}$), oligo-dT primer ($50 \mu\text{M}$), and dNTP (10 mM) were denatured for 5 min at 65°C . After that, the cDNA synthesis was performed by adding SuperScript III RT (200U), followed by incubation at 50°C for 50 min and terminated by heating for 5 min at 85°C . For the RNA removal, samples were incubated at 37°C for 30 min with RNase H (2U).

2.6.2 Real-time PCR

The expression of target and control genes was determined by real-time PCR. Previously, the PCR efficiency for each pair of primers was calculated. For that, random oocyte and blastocyst cDNA were amplified in serial dilutions (2, 1, 0.5, 0.25, and

$0.125 \mu\text{l}$) and a standard curve was generated. The PCR protocols were considered standardized if (1) only one slope was generated after dissociation; (2) the slope value of the standard curve was between -3.1 and -3.4 ; and (3) the R^2 value was higher than 0.95. Amplification products were sequenced in order to be sure that the correct segment had been amplified.

Amplification cycles were carried out on Real Time Thermocycler (Mastercycler EP, Eppendorf, Germany) using Platinum SYBR Greener qPCR SuperMix (Invitrogen). The reaction (total volume of $25 \mu\text{l}$) contained Master Mix ($12.5 \mu\text{l}$), reverse and forward primers (100 to $200 \mu\text{M}$), and cDNA template ($1 \mu\text{l}$ —equivalent to ≈ 0.15 oocyte or embryo). Reaction conditions included initial activation at 95°C for 10 min; cycling: denaturation at 94°C for 15 s; annealing at 60°C for each set of primers¹⁴ for 15 s; and extension at 68°C for 20 s, followed by a melting curve step. For each sample, three technical replicates were performed. Amplification products were verified by melting analysis and gel electrophoresis on 2% agarose gel.

2.6.3 Protein expression analysis: western blot

Total protein from oocytes was obtained from 10 oocytes using a digestion buffer (NP40 1%, NaCl 135 mM, Tris 20 mM pH 8, glycerol 10%). Protein integrity was analyzed by the Bradford method.¹⁵ Total protein from these oocytes was resolved by electrophoresis on 5% stacking/15% polyacrylamide-SDS gels, and the resolved proteins were transferred to nitrocellulose membrane (Bio-Rad, Hercules, California). The membrane was stained with Ponceau solution to demonstrate that the protein concentration was similar in the different lanes. The membrane was then washed with TBST (Tris 50 mM, NaCl 150 mM, pH 7.5, and Tween-20 2%) for 10 min at room temperature. After this, the membrane was incubated at 4°C overnight with polyclonal antibody against mitogen-activated protein kinase (MAPK) (1:700) (SC-7199, Santa Cruz Biotechnology, Santa Cruz, California) and GAPDH (1:1000) (SC-322233, Santa Cruz Biotechnology) in TBST. After washing the membranes, the secondary anti-rabbit and anti-mouse, respectively, conjugated with peroxidase (Jackson, Pennsylvania) at a 1:10,000 dilution in TBST was added for 1 h at room temperature. The membranes were washed again with TBST and incubated with ECL detection reagents (Amersham Biosciences, Piscataway, New Jersey), which produced a chemiluminescence's signal that was detected by exposure to x-ray film. The protein bands were quantified by densitometry, and the band densities were calculated and expressed as arbitrary units.

2.7 Statistical Analysis

Data from flow cytometry were analyzed by analysis of variance (ANOVA) using PROC GLM of SAS computer software. Dependent variables were percentage of cells in G0/G1, S, G2/M, and dead cells for cell cycle status and high- and low-mitochondrial membrane potentials for mitochondrial function. Comparison of mean values was performed using Tukey's test with 5% significance level. Data from embryo development were evaluated by ANOVA and submitted to Tukey's test for cleavage and blastocyst rates. Quantitative PCR data were analyzed using REST MCS beta software by pairwise-fixed reallocation randomization test. Reactions were normalized by the expression of housekeeping gene beta actin.

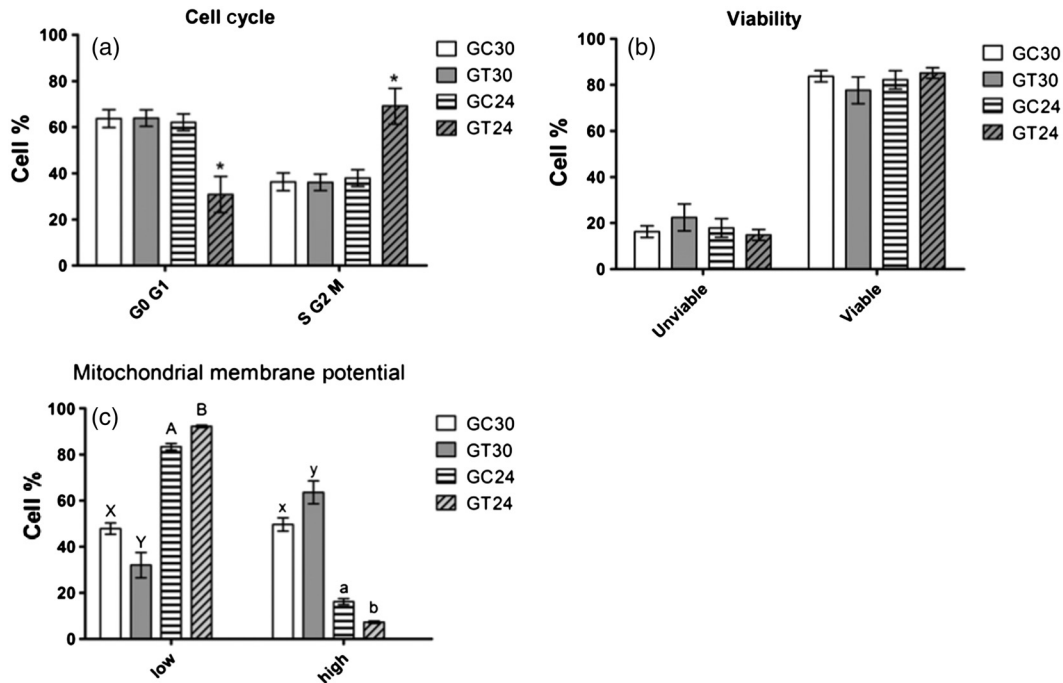


Fig. 1 Granulosa cells evaluation after 30 min and 24 h of IVM submitted to laser irradiation. GC30—nonirradiated (control) group after 30 min of IVM; GT30—irradiated (treated) group after 30 min of IVM; GC24—nonirradiated (control) group after 24 h of IVM; and GT24—irradiated (treated) group after 24 h of IVM. (a) Effect of laser irradiation on granulosa cell cycle. * $p < 0.001$. (b) Effect of laser irradiation on granulosa cell viability. (c) Effect of laser irradiation on mitochondrial membrane potential. Different letters represent $p < 0.005$. X and Y represent comparison of cells presenting low mitochondrial membrane potential between control and treated groups 30 min after the irradiation. x and y represent comparison of cells presenting high-mitochondrial membrane potential between control and treated groups 30 min after the irradiation. A and B represent comparison of cells presenting low mitochondrial membrane potential between control and treated groups 24 h after the irradiation. a and b represent comparison of cells presenting high mitochondrial membrane potential between control and treated groups 24 h after the irradiation.

3 Results

3.1 Granulosa Cells

3.1.1 Evaluation of viability, mitochondrial membrane potential, and cell cycle

There was no effect of irradiation on cell cycle progression after 30 min of incubation ($p > 0.05$). However, after 24 h, an

increase in the percentage of cells committed to the cycle was observed in the irradiated group when compared with the control group ($p < 0.001$ —Fig. 1). No difference in the percentage of viable and unviable cells was found between any of the studied groups (GC30 x GT30 and GT24 x GC24) x ($p > 0.05$ —Fig. 1). For mitochondrial membrane potential, GT30 presented more cells with high-mitochondrial membrane potential than GC30, and GT24 presented more cells with

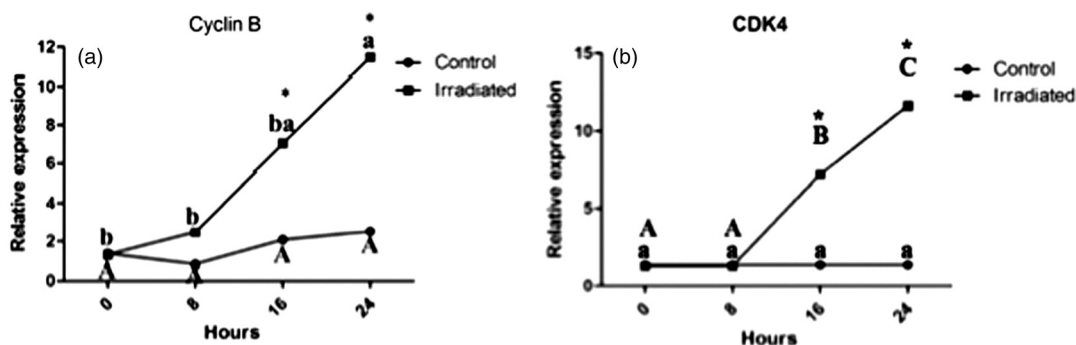


Fig. 2 Granulosa cell cycle genes evaluation after 30 min (group 0), 8 h, 16 h, and 24 h of IVM submitted to laser irradiation. Only groups presenting a significant interaction time x treatment are represented. (a) Effect of laser irradiation on granulosa cyclin B relative expression. (b) Effect of laser irradiation on granulosa CDK4 relative expression. Different letters represent differences within the group. * represents difference between groups at the same time.

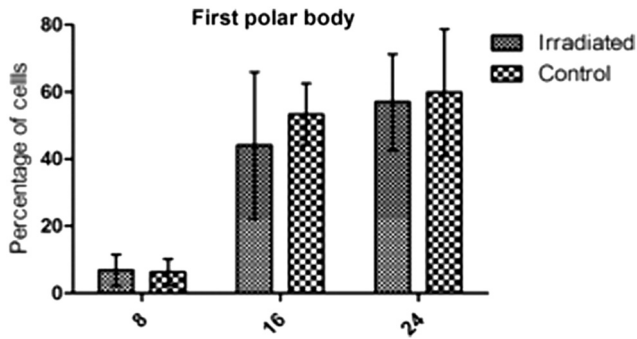


Fig. 3 First polar body extrusion after 8 h, 16 h, and 24 h of IVM submitted to laser irradiation. No differences were found among groups related to this characteristic.

low-mitochondrial membrane potential than GC24 ($p < 0.05$ —Fig. 1).

3.1.2 Differential gene expression

Due to the tendency found for cyclin B, data were evaluated considering the effect of a significant interaction ($p = 0.06$). Also, a time \times treatment interaction was found for cyclin-dependent kinase (CDK4) ($p < 0.05$). Granulosa cells from irradiated group increased the number of both transcripts after 8 h of irradiation. The results are shown in Fig. 2.

3.2 Oocyte

3.2.1 Meiotic progression

There was no difference in the meiotic progression of oocytes among the control and treated groups over time (Fig. 3).

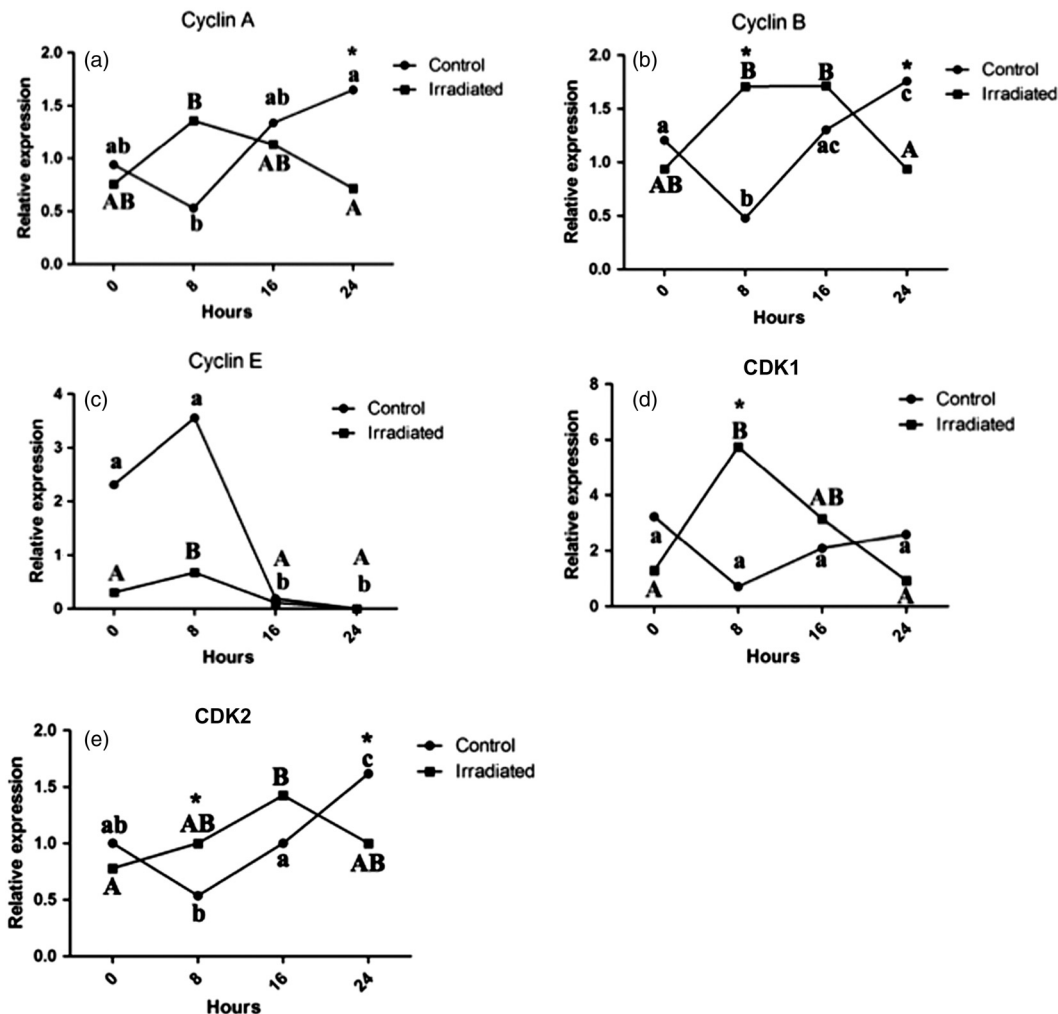


Fig. 4 Oocyte cell cycle genes evaluation after 30 min (group 0), 8 h, 16 h, and 24 h of IVM submitted to laser irradiation. Only groups presenting a significant interaction time \times treatment are represented. (a) Effect of laser irradiation on oocyte cyclin A relative expression. (b) Effect of laser irradiation on oocyte cyclin B relative expression. (c) Effect of laser irradiation on oocyte cyclin E relative expression. (d) Effect of laser irradiation on oocyte CDK1 relative expression. (e) Effect of laser irradiation on oocyte CDK2 relative expression. Different letters represent differences within the group. * represents difference between groups at the same time.

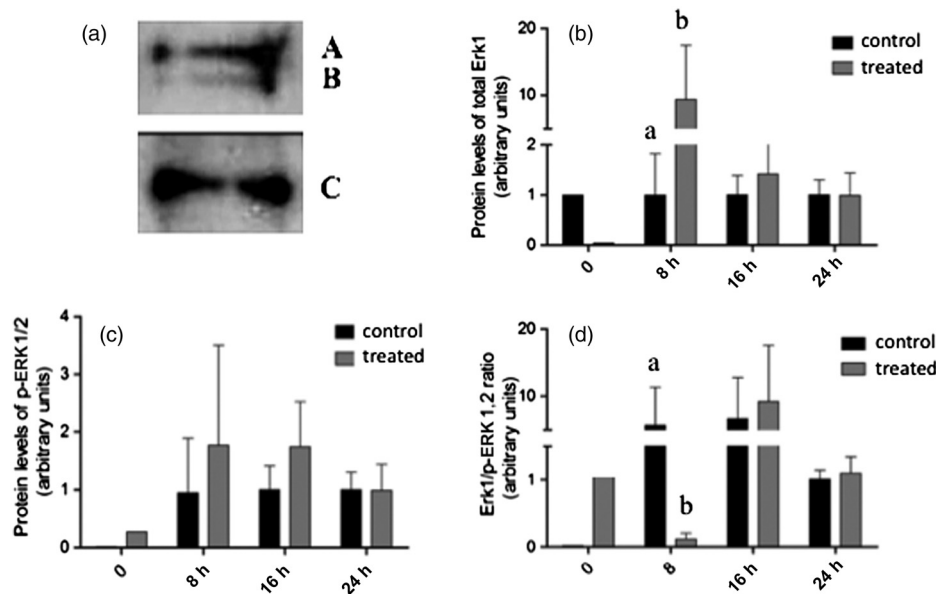


Fig. 5 Oocyte total MAPK and phosphorylated MAPK quantification after 30 min (group 0), 8 h, 16 h, and 24 h of IVM submitted to laser irradiation. (a and b) Total MAPK quantification after normalization by beta actin in oocytes throughout *in vitro* maturation (IVM). (c) Phosphorylated MAPK quantification after normalization by beta actin in oocytes throughout IVM. (d) Total MAPK/phosphorylated MAPK ratio. Different letters represent difference between control and treated samples within the same time.

3.2.2 Differential gene expression

An interaction between time and treatment was observed for all transcripts ($p < 0.05$), exception made for the CDK4 ($p > 0.05$). Oocytes from irradiated group presented a decrease in transcripts for cyclin A and cyclin B along the maturation, whereas the control group had an increase in these transcripts. For cyclin E, irradiated oocytes presented an increase at 8 h, followed by a decrease very similar to the control group. CDK1 remained unchanged during all IVMs for the control group, while the irradiated oocytes had a peak of expression at 8 h. CDK2 also presented an increase in transcript level at 8 and 16 h, followed by a decrease in irradiated oocytes; an opposite behavior when compared with its control (Fig. 4).

3.2.3 Western blot

To assess the status of MAPK phosphorylation, oocytes were collected from all experimental groups after irradiation in

three independent experiments. In Fig. 5, the reaction of WB for total, phosphorylated MAPK and the normalizer, beta actin was represented. An increase in total MAPK levels can be observed after 8 h of irradiation. This is directly reflected in the lower proportion of phosphorylated MAPK/total MAPK.

3.3 Embryo

To assess the effect of laser irradiation on embryonic development, three independent experiments were performed, evaluating cleavage and blastocyst rates. No difference was found between treated and control groups for cleavage and blastocyst rates (Fig. 6).

4 Discussion

The maturation and fertilization processes of bovine oocytes include nuclear and cytoplasmic changes that are dependent, besides other factors, on an appropriate culture system. Until

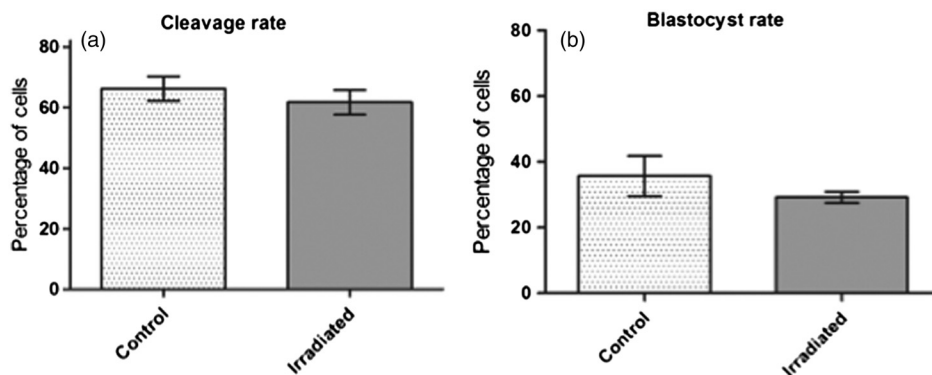


Fig. 6 Embryo production after irradiation of oocytes before IVM. (a) Cleavage rates of control and irradiated groups evaluated 36 hpi. (b) Blastocyst rates of control and irradiated groups evaluated after 168 hpi.

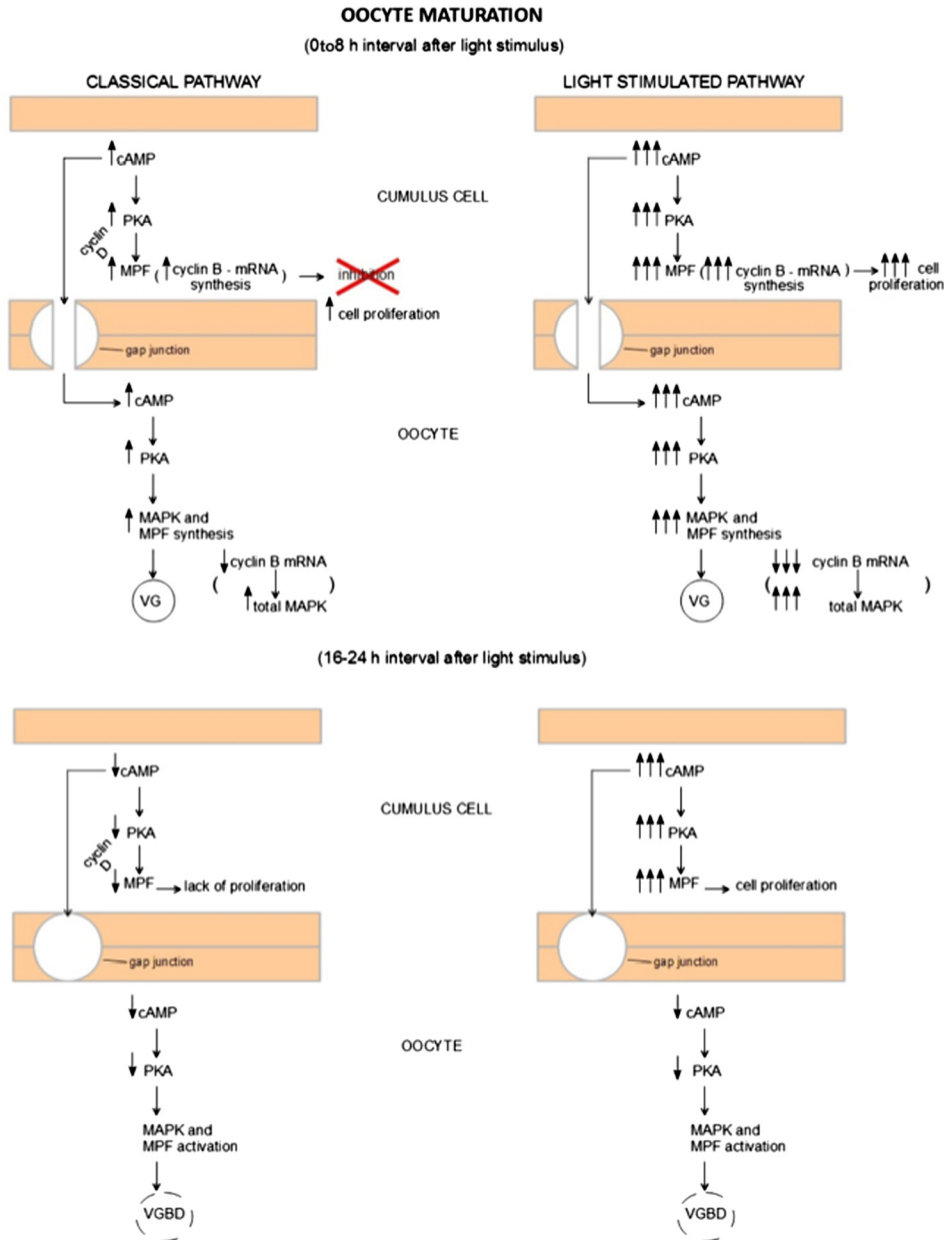


Fig. 7 Diagram of the proposed light effect on oocyte and granulosa cells during maturation. During the first hours of IVM, mainly due to specific hormones and growth factors, granulosa cells increase cAMP levels, triggering to PKA and MPK synthesis. Since there is no inhibition of cell proliferation, granulosa cells are subjected to a moderate replication. This cAMP also reaches oocyte cytoplasm due to the communication established by gap junctions. cAMP induces PKA, MAPK, and MPF syntheses in a concatenated way. Besides the increase of MPF levels, other factors maintain the oocyte blocked on prophase I. During the last hours of IVM, the granulosa cells change their response to hormone and growth factors, diminishing cAMP levels, PKA, and MPF syntheses, and, consequently, leaving the cell cycle. At this time, gap junctions are closed and cAMP and PKA decrease. It promotes MAPK and MPF activations, leading to the breakdown of the germinative vesicle and meiosis progressions. The light stimulate pathway induces an increase in cAMP synthesis (due to the changes in mitochondrial membrane potential) and consequently the PKA and MPF syntheses and activations, resulting in a higher number of cells committed to the cycle. This higher level of cAMP also crosses the GAP junction, penetrating the oocyte. It leads to higher level of cAMP inside the oocyte, inducing the fall of cyclin B mRNA levels due to the higher recruitment of it to be converted into protein. The same increasing happens to MAPK. At the final portion of the IVM, the light effect remains, maintaining higher levels of cAMP, PKA, and MPK in granulosa cells, avoiding the exit of the cell cycle. Besides this higher level of cAMP, the gap junctions are closed, so the decay in cAMP and PKA levels, added to MAPK and MPF activations, promotes the meiosis progression. It is important to point out that despite the increase in MAPK levels, the amount of activated MAPK (phosphorylated) does not change, suggesting other mechanisms regulating the meiosis progression.

now, the application of lasers to improve the *in vitro* produced embryo system has been poorly studied. Thus, studies are necessary to understand the metabolic pathways associated with laser stimulation in order to establish a possible routine application of this biotechnology.

The energy density is one of the key points to the success of biomodulation by LLLT.² In this study, by using a single energy chosen by its effect in other cell types, we observed changes in granulosa cells' physiology as well as in the synthesis, by the oocyte, of essential molecules for fertility. The use of this single energy was essential for oocyte viability due to the time required for the irradiation. Once we get the ovaries at the laboratory, the IVM procedure must occur as soon as possible to avoid the loss of cellular viability. Moreover, freezing such structures, while maintaining its characteristics, is not an efficient process. The inclusion of other groups in the same experiment would result in irradiation at different times after the beginning of IVM and, consequently, results that could not be compared among them, especially those related to activation of signaling pathways. It is important to highlight that this is the first report, to our knowledge, of the analysis of gene expression and signaling pathways in bovine oocytes subjected to LLLT, with very promising results.

Several modifications occur during oocyte maturation, necessary for the acquisition of competence to undergo fertilization and embryo development. Most of these changes are driven by the granulosa cells, which present a direct communication to the oocyte via gap junctions.¹⁶ In our experiment, LLLI was able to increase the mitochondrial membrane potential of granulosa cells and the number of granulosa cells committed to cell cycle. These data corroborate with those of Gavish et al.,¹⁷ which reported that the photons from the laser irradiation are absorbed by proteins of the mitochondrial respiratory chain that converts this energy into chemical energy. They also described in isolated mitochondria that the LLLI increased mitochondrial membrane potential, the proton gradient, and the rate of ADP/ATP replacement. Gao and Xing¹⁸ reported that the LLLI can induce increased intracellular Ca^{2+} , as well as an increase on reactive oxygen species and cyclic-adenosine-monophosphate (cAMP) resulted from an increase on mitochondrial activity. This increase on mitochondrial activity, in addition to the stimulatory effect of FSH (supplemented during IVM) in granulosa cells, seems to increase the cAMP levels, leading to the higher number of cells in S-phase, G2, and M (committed to the cycle). The increased levels of cyclin B during maturation in this group could confirm this result, since cyclin is responsible for entry and progression to the M phase.¹⁹

In the oocyte, the more evident effect of irradiation could be observed after 8 h with the increase on total MAPK, resulting in the decrease in the extracellular signal-regulated protein kinase (ERK) 1/2-phosphorylated/total ERK1 ratio. Gerits et al.²⁰ reported that the levels of MAPK are dependent of protein kinase A (PKA), which in turn is dependent on the cAMP levels. In this case, we can propose two pathways for this increase on ERK. The first resulted from the action of radiation in the mitochondria of the oocyte itself, leading to increased mitochondrial membrane potential and consequent increase on cAMP. The second would be derived from the action of irradiation in the mitochondria of granulosa cells, also increasing cAMP production, which would be transported to the oocyte via gap junctions.¹⁶ In both cases, the elevation of cAMP would lead to increased PKA that, in turn, would increase the levels of ERK. Despite this

increase in total ERK, no increase on ERK phosphorylation was observed, reinforcing the idea that, in oocyte, the activation of ERK will take place only after the luteinizing hormone (LH) peak.

It is important to point out that the LH-induced MAPK signaling is essential for the resumption of meiosis, ovulation, and luteinization. Failures in this process imply infertility.²¹ In our article, based on the proposed model, we demonstrate the modulation of MAPK in the oocyte and several physiological changes in granulosa cells. Despite these changes, meiotic progression and blastocyst development remain the same. Nevertheless, we must take into account that we have used in this work only COCs with excellent quality. The ability to increase the levels of MAPK by laser irradiation opens the possibility to stimulate the increase in the amount of this molecule in poor-quality oocytes and, consequently, the potential of these cells to be converted into blastocysts.

In traditional oocyte maturation, LH has the effect of closing the gap junctions, contributing to the reduction of cAMP and, in consequence, PKA. This culminates in activation of maturation promoting factor (MPF) and germinal vesicle breakdown. In our experiment, concomitantly to the overall increase in ERK in the oocyte, we found an increase followed by a decrease in cyclin B mRNA, suggesting that this transcript was recruited at that point in a disordered manner. Nonetheless, no physiological effect could be observed, since all these transcripts were recruited from the maternal stock. However, it seems that no new cyclin B transcription was stimulated. Moreover, the activation of MPF would happen only after ERK phosphorylation by the inhibition of myt1 and, consequently, the inhibition of cdc2 phosphorylation.²²⁻²⁴ In fact, the correct balance between kinases and phosphatases is a crucial step in the control of oocyte meiotic resumption.²³

Regarding the other genes of the cell cycle in the oocyte, similarly to the cyclin B, the laser induced a rapid increase followed by a decrease on mRNA expression. There was a decrease in the levels of cyclin A mRNA, suggesting a disordered recruitment. This could be due to the high levels of PKA, which diminished the accumulation of cyclin E mRNA and, in consequence, cyclin A, since the latter is driven by the first. Anyway, despite these data, there was no difference in the meiotic progression or in embryo production rates (cleavage and blastocyst) for the irradiated group. This may have occurred due to the high plasticity of the oocyte in reversing harmful events (including the absence of cell cycle-related genes), allowing the embryo progression besides this disordered recruitment.²⁵⁻²⁷ Taken these results together, we proposed a model to the effect of LLLI in oocyte and granulosa cells, as described in Fig. 7.

We conclude that the LLLI is capable of modulating events in granulosa cells that may result in changes in the oocyte. Despite the fact that we found no changes in meiotic progression or embryo production, our data indicate the laser as a promising tool for improving the system of *in vitro* embryo production.

Acknowledgments

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