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The impact of near-infrared photobiomodulation therapy on human oocyte rescue *in vitro* maturation

Sara Stigliani [a], Matilde Balbi [b], Silvia Ravera [b,c], Ida Casciano [a], Francesca Bovis [d], Claudia Massarotti [a,e], Paola Anserini [a], Andrea Amaroli [f,g,*,1], Paola Scaruffi [a,1]

[a] SSD Physiopathology of Human Reproduction, IRCCS Azienda Ospedaliera Metropolitana, 16132 Genova, Italy
[b] Department of Experimental Medicine, University of Genova, 16132 Genova, Italy
[c] IRCCS Azienda Ospedaliera Metropolitana, 16132 Genova, Italy
[d] Department of Health Sciences (DISSAL), University of Genova, 16132 Genova, Italy
[e] Department of Neuroscience, Rehabilitation, Ophthalmology, Genetics and Maternal-Child Health (DiNOGMI), University of Genova, 16132 Genova, Italy
[f] Department of Earth, Environmental and Life Sciences (DISTAV), University of Genova, 16132 Genova, Italy
[g] Interuniversity Center for the Promotion of the 3Rs Principles in Teaching and Research (Centro 3R), 56122 Pisa, Italy

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ABSTRACT

This prospective study investigated the effects of photobiomodulation therapy (PBMT) at 810 nm on rescue *in vitro* maturation (rescue-IVM) of human immature oocytes following controlled ovarian stimulation (COS). A total of 260 immature oocytes (germinal vesicles (GV): 143; metaphase I (MI): 117) were collected from 114 women undergoing COS between December 2023 and July 2025, denuded, and randomized into control or PBMT groups; ten *in vivo*-matured MII oocytes served as references. PBMT was applied at 810 nm, 1.0 W, 60 J/cm^2 for 1 min, and nuclear maturation and morphology were assessed up to 6 h of IVM. ATP content, mitochondrial oxidative phosphorylation (OxPhos) activity, and lipid peroxidation (MDA) were measured using luminometric, oximetric, and spectrophotometric assays. PBMT accelerated maturation, with GV and MI oocytes progressed to the next maturation stage 112.5% and 92.3% faster, respectively, within 1 h. ATP content increased markedly in PBMT-treated oocytes ($P < 0.0001$), with early-resuming MI oocytes exceeding levels of *in vivo* MII oocytes. OxPhos activity increased by 240% (GV) and 194% (MI) immediately after irradiation without mitochondrial uncoupling, while MDA levels remained stable. Collectively, these results demonstrated that PBMT accelerated cell cycle progression to GV and MI oocytes by enhancing mitochondrial OxPhos and ATP production without inducing oxidative stress. Limitations include the need for larger cohorts, evaluation of safety through assessment of potential DNA damage, and validation in poor-prognosis or advanced maternal age patients. This pioneering proof-of-concept study highlights the potential of PBMT to temporally accelerate rescue-IVM of immature oocytes - and possibly cumulus-oocyte complexes - in poor-responder patients undergoing IVF or fertility preservation.

1. Introduction

Retrieval of immature oocytes after controlled ovarian stimulation (COS) remains a persistent, unsolved challenge in *in vitro* fertilization (IVF) treatments, as immature oocytes are usually considered unsuitable for reproductive purposes and are therefore discarded in IVF programs. Approximately 20% of retrieved oocytes are immature, remaining arrested either at the germinal vesicle (GV) stage, corresponding to prophase I of the first meiotic division and characterized by a large nucleus, or at the metaphase I (MI) stage, identified by the absence of both a visible GV and the first polar body (PB) [1]. These oocytes have not yet completed meiosis resumption and remain at the earliest stage of maturation. Progression to the metaphase II (MII) stage during oocyte development is a highly regulated process involving both nuclear and cytoplasmic maturation [2]. Nuclear maturation entails progression through meiotic stages from prophase I arrest to MII, and is characterized by chromosomal condensation, spindle assembly, and extrusion of the first PB. Cytoplasmic maturation involves the accumulation and

* Corresponding author at: Department of Earth, Environmental and Life Sciences (DISTAV), University of Genova, Viale Benedetto XV, 5, 16132 Genova, Italy.
E-mail address: andrea.amaroli@unige.it (A. Amaroli).
[1] A.A. and P.S. contributed equally to this work.

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relocation of RNAs, proteins, and organelles such as mitochondria, ribosomes, endoplasmic reticulum, as well as cytoskeletal reorganization and microtubule assembly required for calcium release [3,4]. These biochemical and structural changes are necessary for successful fertilization and subsequent embryo development. Metaphase I oocytes may spontaneously complete the first meiotic division, extrude the first PB, and reach the MII stage within several hours of *in vitro* culture. Classical *in vitro* maturation (IVM) typically involves immature oocytes retrieved from unstimulated or minimally stimulated cycles and mainly applies to patients with polycystic ovary syndrome [5]. More recently, a type of IVM known as rescue-IVM, referring to the IVM of immature oocytes retrieved following conventional COS, has been developed to improve fertilization outcomes in patients with a poor prognosis [6].

The reasons why immature oocytes after COS fail to respond to exogenous triggering *in vivo* and to undergo nuclear maturation to the MII stage remain unclear. This may stem from asynchrony among developing follicles, premature retrieval timing, aspiration of smaller antral or degenerating follicles, and insufficient ovarian response to stimulation [7].

Mitochondria represent the primary source of ATP in oocytes and early embryos up to the blastocyst stage, as anaerobic glycolysis is limited during these phases [8]. During oocyte maturation, ATP levels dynamically change approximately one hour after germinal vesicle breakdown and during extrusion of the first polar body. ATP consumption rates are higher in MI and MII oocytes compared with those arrested at GV stage [9]. Mitochondrial dysfunction and reduction of mitochondria-derived ATP production have been associated with suboptimal developmental competence [10].

Photobiomodulation therapy (PBMT), the use of low-level near-infrared (NIR) light to energize mitochondria, has been studied for over four decades [11,12]. This therapy targets mitochondrial photoacceptors, primarily cytochrome *c* oxidase (CCO, Complex IV), the terminal enzyme of the electron transport chain. When CCO absorbs photons, it enhances oxidative phosphorylation, boosting ATP production and generating low, non-toxic levels of reactive oxygen species (ROS), which act as signaling molecules to activate prosurvival pathways such as Nrf2 [13,14]. In particular, diode laser light at 810 nm or 980 nm primarily modulates Complex IV (with minor effects on Complex III), while 1064 nm light stimulates Complexes I, III, and IV [15–19]. PBMT has been investigated in diverse *in vitro* and *in vivo* models, improving mitochondrial function, calcium regulation, and membrane order, and accelerating tissue repair *via* anti-inflammatory and redox-regulating mechanisms [13,20]. In medical and veterinary fields, PBMT supports recovery from diseases associated with mitochondrial dysfunction by restoring energy metabolism and redox homeostasis [21–25]. Studies investigating the effects of PBMT on reproductive parameters have been conducted primarily in sperm cells. Considerable evidence indicates a photobiomodulatory effect of low-level laser therapy on human sperm function *via* an "energy modulation effect" [24,26–28]. Our group recently demonstrated that PBMT improves progressive motility in cases of asthenozoospermia by increasing mitochondrial metabolism and energy production in human spermatozoa, without inducing detrimental oxidative stress [29,30]. In contrast, few studies have explored the effects of PBMT on the female reproductive system. The earliest study, published in 1983 in a porcine model, reported that application of a 630 nm laser to ovarian granulosa cells increased estrogen levels and modulated progesterone levels [31]. The same laser wavelength was also shown to increase colony formation in Chinese hamster ovary cell cultures [32]. Furthermore, exposure to an 830 nm laser increased vascular endothelial growth factor (VEGF) levels and mitogen-activated protein kinase (MAPK) activity in a human granulosa cell line [33]. When applied to a more complex system of IVM of bovine cumulus-oocytes complexes, PBMT increased the mitochondrial membrane potential in cumulus cells and enhanced ovarian cellular metabolism [34]. More recently, PBMT has been proposed as a potential novel therapeutic strategy for female reproductive disorders by enhancing endometrial receptivity and regeneration [35], as well as by modulating follicular dynamics through the regulation of apoptosis and vascular stability in the mouse ovary [36]. However, to date, no studies have specifically investigated the effects of PBMT on human immature oocytes during rescue-IVM, nor the underlying mechanisms involved.

The aim of this study was to evaluate the effects of PBMT at a wavelength of 810 nm on the rescue-IVM of human immature oocytes retrieved from stimulated cycles. The efficacy of the treatment was assessed by monitoring the temporal maturation and the oxidative phosphorylation (OxPhos) activity. The safety was evaluated through the morphological assessment of oocytes and the detection of oxidative stress damage measured by quantification of malondialdehyde (MDA) as a marker of lipidic peroxidation.

2. Material and methods

2.1. Study population and study design

We collected 260 immature oocytes from 114 women undergoing oocyte retrieval for assisted reproduction technology (ART) cycles at the SSD Physiopathology of Human Reproduction, IRCCS Azienda Ospedaliera Metropolitana, Genova, Italy, from January 2024 to July 2025. The average age was 34.7 ± 3.7 years (range: 23–39 years). Inclusion criteria: patient's age < 40 years; infertility factor: tubal, male, or idiopathic factor. Exclusion criteria: women with ovulatory endocrine disorders, endometriosis, and diminished ovarian reserve.

2.2. Experimental protocol

As outlined in Fig. 1, immature oocytes were randomized into two groups: the control group (74 GV from 53 patients, 62 MI from 42 patients), which received laser treatment with 0.0 W, and the test group (69 GV from 48 patients, 55 MI from 48 patients), which received laser treatment with 1.0 W for 1 min. A second operator trained in laser therapy treated the samples. Oocyte nuclear maturation and oocyte morphology were assessed in a blinded manner after 1 (T1), 2 (T2), 4 (T4), and 6 (T6) hours of IVM, with oocyte denudation considered as time zero. In parallel, the evaluation of OxPhos and oxidative stress damage in oocytes was performed as detailed in Fig. 1. We also included in the study 10 MII collected after oocytes retrieval as a reference for *in vivo* matured oocytes.

2.3. Ethical approval

The study complied with the Declaration of Helsinki and was approved by the Ethics Committee of Regione Liguria (ID 219/2024). Written informed consent was obtained from all donors, and participation was voluntary.

2.4. Patient treatment

Standard COS protocols were used [37]. Briefly, pituitary suppression was achieved with either GnRH agonists or antagonists. Stimulation with gonadotrophins was monitored by measuring serum estradiol levels and follicle growth. HCG or GnRH agonist was administered when patients reached the individual clinic's trigger point for follicular growth. Cumulus-oocyte complexes (COC) were collected 36 h following trigger injection by ultrasound-guided transvaginal follicular aspiration.

2.5. Oocyte preparation and rescue-IVM

Immediately after retrieval, COC were washed in G-MOPS™ buffer (Vitrolife, Sidney, Australia) and incubated in G-IVF™ medium (Vitrolife) at 37 °C in a humidified atmosphere of 6% CO_2, 5% O_2, 89% N_2 using MINC Benchtop incubators (Cook Medical, Sydney, Australia).

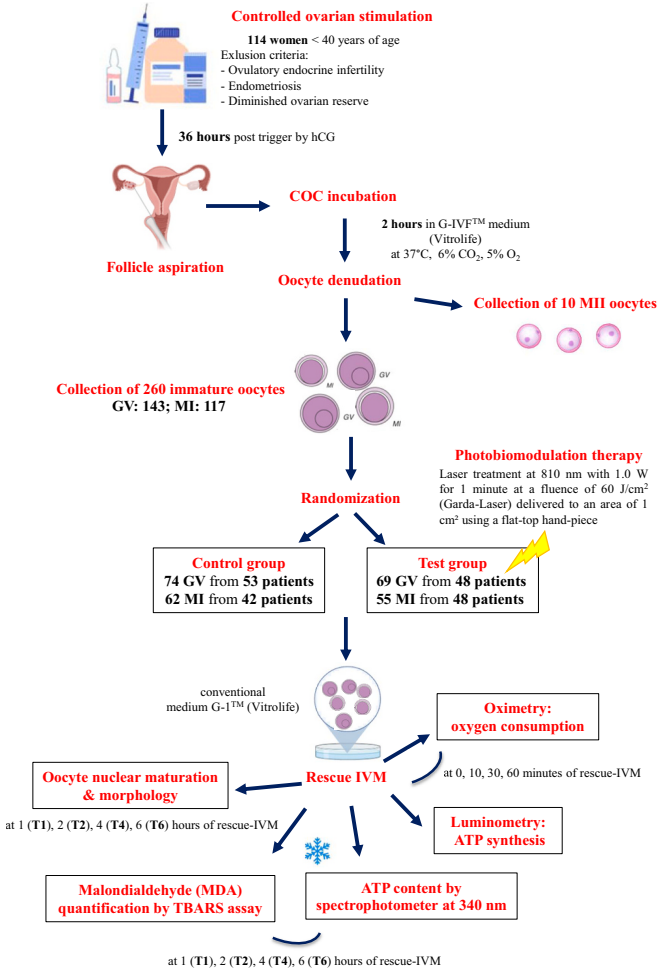

Fig. 1. Summary of the experimental setup.

Two hours after oocyte retrieval, cumulus cells were removed by mechanical pipetting with 150 μm diameter pipettes (Origio, Målov, Denmark) in 40 IU/ml hyaluronidase (Origio) in G-MOPS™ buffer (Vitrolife). The nuclear maturation status of oocytes was assessed under an inverted microscope (Eclipse TE2000-S, Nikon Instruments Europe BV, Amsterdam, Netherlands) at 400× magnification using morphological criteria. The maturity status was classified into germinal vesicle (GV) (presence of an intracytoplasmic nucleus characteristic of prophase of the first meiotic division), metaphase I (MI) (absence of a visible germinal vesicle or a polar body) or MII (presence of a polar body) [38]. Oocyte denudation was considered as time zero.

Immature oocytes (GV and MI) were randomized into test or control group and individually placed into 4-wells Nunc™ IVF plate (ThermoFisher Scientific Inc., Waltham, MA, USA) containing 500 μl of pre-equilibrated conventional culture medium G-1™ (Vitrolife) without any hormone supplementation under mineral oil (OVOIL™, Vitrolife). Oocytes were incubated in a humidified atmosphere of 6% CO_2, 5% O_2, 89% N_2 using MINC Benchtop incubator (Cook Medical).

2.6. Technical characteristics of the equipment used and the irradiation setup

The irradiations were performed using the ENEA GaAl-As diode laser system (Garda Laser S.A.S., Verona, Italy). The device allowed irradiation at a wavelength of 810 ± 2 nm for 60 s in continuous wave mode. The irradiation power was set at 1.0 W to generate an energy of 60.0 J (power density of 1.0 W/cm^2; fluence of 60.0 J/cm^2). Treatments were performed at 810 nm, 1 W, and 60 J/cm^2 as we had previously demonstrated these parameters to be effective and safe in a sea urchin *in vitro* model [39]) and in human sperm from patients with asthenozoospermia [29,30]. Control samples were subjected to a power setting of 0.0 W to ensure baseline comparisons. Irradiations were delivered using a flat-profiled handpiece (FT-HP). Prior characterization of the FT-HP demonstrated its ability to provide a uniform and stable energy distribution across a spot area of 1 cm^2, regardless of the distance from the target surface, thereby limiting localized energy peaks and reducing thermal increases under equivalent irradiation conditions [17]. A 635 nm red light pointer (negligible power, < 0.5 mW) was used in both treatments to visualize the exposed area and maintain experimental blinding. The accuracy of the laser parameters was verified using a Pronto-250 power meter (Gentec Electro-Optics, Inc., G2E Quebec City, Canada). The irradiations were carried out with the handpiece fixed to a stand and in contact mode with the surface of the multi-wells plate (diameter of a single well approximately 1.1 cm). All irradiations were performed with the multi-wells plate placed on a Metal Velvet light-absorbing plate (Acktar Ltd., 8643 Kiryat-Gat, Israel). The extremely low reflectance of the plates, which is guaranteed to be equal to or less than 1% in the near-infrared band, significantly reduces reflections from the top of the bench. Thermal effects were monitored using a thermal monitoring system with the following specifications: operating range from −200 °C to 390 °C, resolution (internal and displayed) of 0.5 °C, and accuracy of ±0.5 °C (OM-EL-USB-TC-LCD, OMEGA Engineering Limited, M44 5BD Manchester, UK).

2.7. ATP content quantification in frozen oocytes

At the end of observations (change from GV to MI and from MI to MII) the oocytes were immediately frozen at −20 °C in 10 μl of culture medium. They were cryopreserved until the time of analysis. The ATP concentration was measured in 59 GV (control group: $n = 29$; test group: $n = 30$) and 80 MI (control group: $n = 40$; test group: n = 40) oocytes after addition of 0.01% digitonin by the luciferin/luciferase ATP bioluminescence assay kit CLSII (Roche Diagnostics Corp., Indianapolis, IN, USA) on a Promega GloMax® 20/20 Luminometer (Promega Italia, Milano, Italy). ATP standard solutions were used in the range 10^{-10}–10^{-7} M for calibration. Protein quantification was performed using the Bradford method. We also evaluated the ATP content in 6 frozen MII collected after oocytes retrieval as a reference marker of the energetic status of *in vivo* matured oocytes.

2.8. OxPhos evaluation in fresh oocytes

To evaluate the OxPhos activity, ATP synthesis and oxygen consumption rate (OCR) were assessed in a total of fresh GV ($n = 25$) and MI ($n = 15$) oocytes immediately and after 10, 30, and 60 min from the laser administration after permeabilization with 0.01% digitonin and resuspension in phosphate-buffered saline (PBS). To establish a reference for the energetic status of *in vivo* matured oocytes, we also evaluated the OxPhos in 4 fresh *in vivo* matured oocytes. ATP synthesis was measured by a GloMax® 20/20 Luminometer (Promega, Milan, Italy) at 30-s intervals over 2 min using the luciferin/luciferase methods. OCR was measured using an amperometric electrode (Unisense Microrespiration, Unisense A/S, Aarhus, Denmark) in a sealed chamber. For both assays, 10 mM pyruvate and 5 mM malate were used as respiring substrates and 0.1 mM ADP was added just before the start of the evaluations [40]).

To assess the OxPhos efficiency, the P/O value was calculated as the ratio between the produced ATP and the consumed oxygen. Values around 2.5 indicate a whole coupling between energy synthesis and respiration; lower values indicate an uncoupling status [41].

2.9. Lipid peroxidation quantification in frozen oocytes

The intracellular concentration of malondialdehyde (MDA), a marker of lipid peroxidation, was measured in 59 GV (control group: *n*

= 29; test group: $n = 30$), 80 MI (control group: $n = 40$; test group: n = 40), and 6 MII collected after oocyte retrieval as a reference for *in vivo* matured oocytes by the thiobarbituric acid reactive substances (TBARS) assay [42], with minor modifications. The TBARS solution was prepared with 15% trichloroacetic acid (TCA) in 0.25 N HCl and 26 mM thiobarbituric acid. To assess the baseline MDA concentration, 600 µl of the TBARS solution was added to 50 µg of total proteins dissolved in 300 µl of Milli-Q water. The mixture was incubated for 45 min at 99 °C, followed by centrifugation at 14.000 rpm for 2 min. The supernatant was then analyzed spectrophotometrically at a wavelength of 532 nm.

2.10. Statistical analyses

To analyze differences in time to maturation (measured in hours), we used cumulative incidence curves and Cox proportional hazards models. Robust standard errors were applied by adjusting for patient's age and by clustering on patient ID to account for correlation between oocytes derived from the same woman. Metabolic data analysis was conducted using one-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test. Statistical processing was performed using R software (version 4.3.2) and Prism 10 software (GraphPad Software, Boston, MA, USA). Statistical significance was determined for $P < 0.05$.

3. Results

3.1. Temporal acceleration of rescue in vitro maturation of immature oocytes induced by photobiomodulation

The immature oocytes underwent rescue-IVM with or without PBMT. No degeneration, cytoplasmic granularity, perivitelline space alterations, or zona pellucida changes were observed in the PBMT-treated oocytes compared to controls (Fig. 2).

Within a 6-h timeframe compatible with clinical application, no GV oocytes reached full MII maturation to the MII stage. Only after overnight incubation of 131 GV oocytes we observed complete maturation in approximately 50% of cases, with a non-significant 21.7% increase in full maturation to MII in the PBMT group (36/64, 56%) compared with the control group (31/67, 46%) ($P = 0.332$). Regarding MI oocytes, full maturation to the MII stage within 6 h of IVM was observed in 52% (32/62) and 64% (35/55) of oocytes in the control and PBMT groups, respectively.

Cox proportional hazards model (Table 1) and cumulative incidence curves (Fig. 3) showed that PBMT enhances cell cycle progression in both GV (Fig. 3A) and MI (Fig. 3B) oocytes. Differences in time to maturation of GV reached a statistical significance value ($P = 0.043$) even after application of robust standard errors to account for the patient's age and correlation between oocytes derived from the same woman. MI oocytes showed a similar trend, likely due the smaller

Table 1
Cox proportional hazards model to analyze differences in time to maturation for GV and MI oocytes.

	Univariable		Adjusted*	
	HR (95%CI)	*p*-value	HR (95%CI)	*p*-value
PBMT *vs* control (GV)	1.53 (0.99–2.36)	0.054	1.52 (1.01–2.28)	0.043
PBMT *vs* control (MI)	1.43 (0.88–2.31)	0.146	1.44 (0.91–2.30)	0.122

* Adjusted for patient's age and clustered on patient ID to account for correlation between oocytes derived from the same woman.

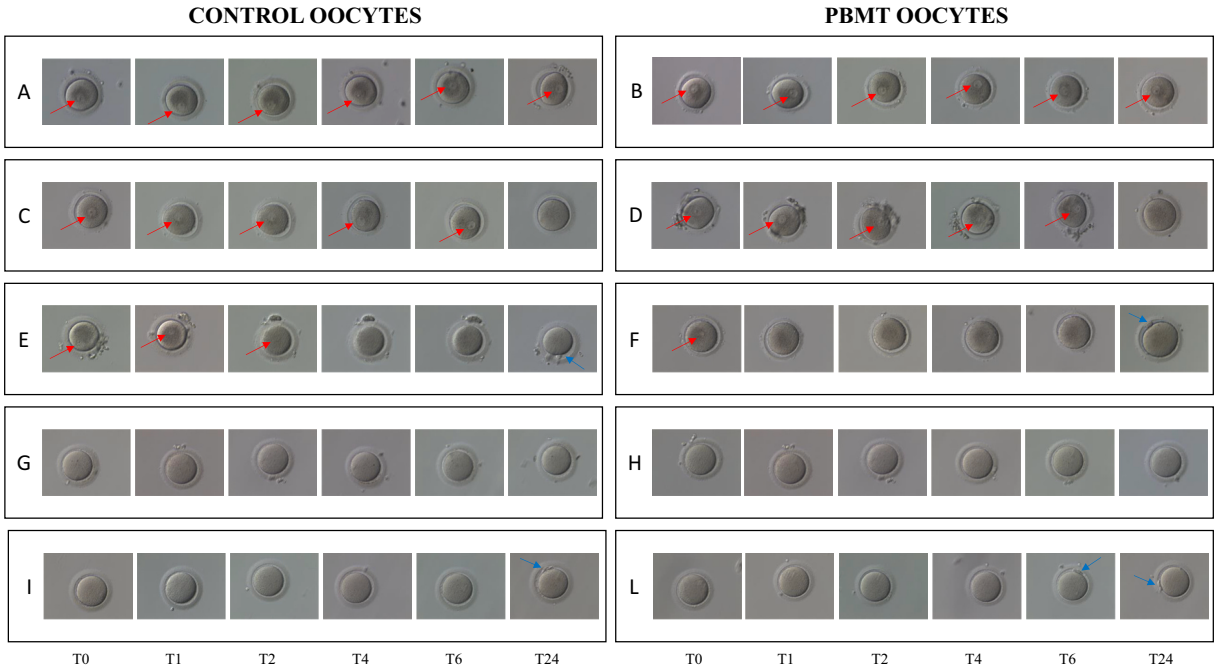

Fig. 2. Representative examples of immature oocytes of both the control (panels A, C, E, G, I) and PBMT (panels B, D, F, H, L) groups evaluated after decumulation (T0), and after 1 (T1), 2 (T2), 4 (T4), 6 (T6), 24 (T24) hours of IVM (400× magnification). (A, B) Typical denuded GV oocytes with a nucleus and a single nucleolus (red arrow). They did not mature after IVM. (C, D) Typical denuded GV oocytes with a nucleus and a single nucleolus (red arrow). Progression to MI stage was observed at T24 since oocytes have no visible nucleus and have not as yet extruded the PB. (E, F) Denuded GV oocytes with a nucleus and a single nucleolus (red arrow). At T4 (E) and T1 (F) progression to MI stage was observed since oocytes have no visible nucleus. Progression to MII stage was observed at T24 since the PB (blue arrow) is clearly visible in the perivitelline space. (G, H) Typical denuded MI oocytes without a visible nucleus and PB. The perivitelline space is narrow. They did not mature after IVM. (I, L) Denuded MI oocytes. Progression to MII stage was observed at T24 (I) and T6 (L) since the PB (blue arrow) is clearly visible in the perivitelline space.
There are no signs of degeneration, cytoplasmic granularity, perivitelline space alterations, or zona pellucida changes in the PBMT-treated oocytes compared to controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

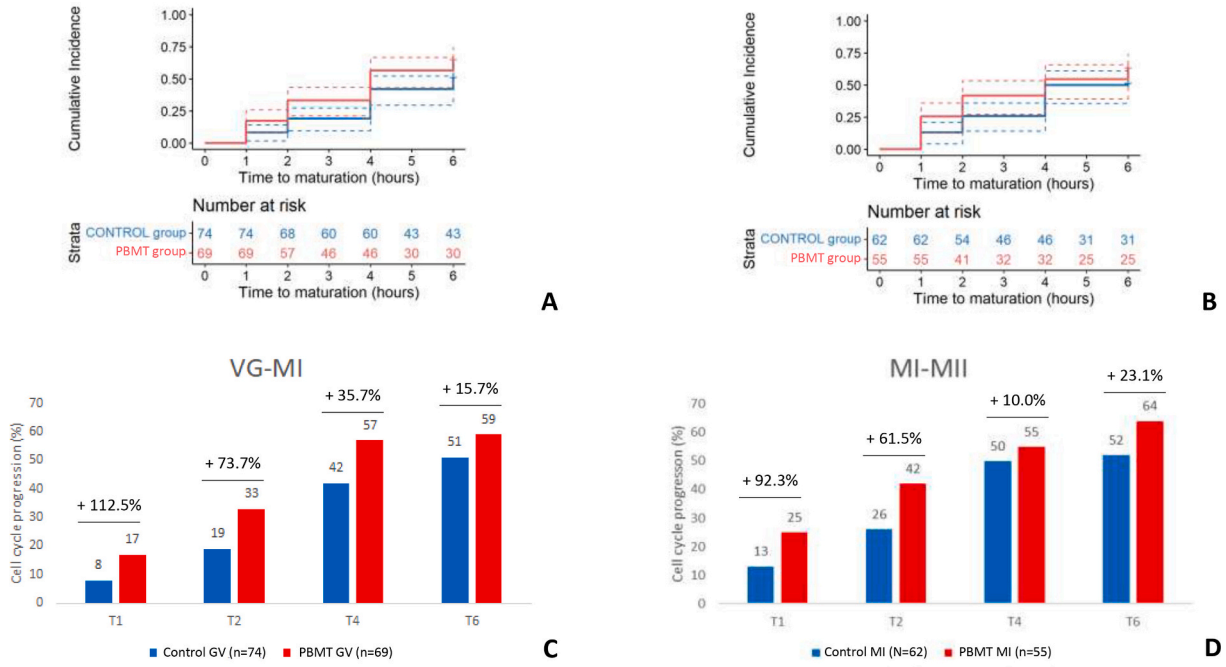

Fig. 3. Cumulative incidence curves of oocyte maturation from GV to MI stage (A) and from MI to MII stage (B) in PBMT and control groups during IVM, with oocyte denudation as time zero. Bar graphs show the cell cycle progression rates for GV (C) and MI (D) oocytes in both groups evaluated at 1 (T1), 2 (T2), 4 (T4) and 6 (T6) hours of IVM. For each time of observation, the percentage increase is also shown.

sample size of MI oocytes ($n = 117$ compared with 143 GV oocytes). The effect on cell cycle progression from GV to MI and from MI to MII was obtained 1 h after irradiation (percentage increase: GV = + 112.5%, MI = + 92.3%) and kept higher in the PBMT group within 4 h for GV (percentage increase: + 35.7%) and 6 h for MI oocytes (percentage increase: + 23.1%) with respect to controls (Fig. 3C, D).

The same pattern of temporal maturation was observed in 79 sibling GV (43 after PBMT and 36 controls) from 29 patients and 54 siblings MI (26 after PBMT and 28 controls) from 20 patients (Supplementary Table S1, Supplementary Fig. S1).

3.2. *Increased ATP content in oocytes in response to photobiomodulation*

We investigated potential variations in ATP concentration in oocytes following laser irradiation at 1, 2, 4, and 6 h after PBMT, always compared with the controls. The ATP content was also evaluated in 6 *in vivo* matured MII oocytes as a reference for *in vivo* maturation (Fig. 4).

ATP content was significantly higher in photoactivated GV and MI oocytes compared to their respective controls ($P < 0.0001$). This significant increase was observed in untreated oocytes at various stages of cell cycle progression and at different time points following irradiation. Interestingly, an earlier progression of GV and MI stages within the cell cycle was associated with a significantly higher ATP content, a trend

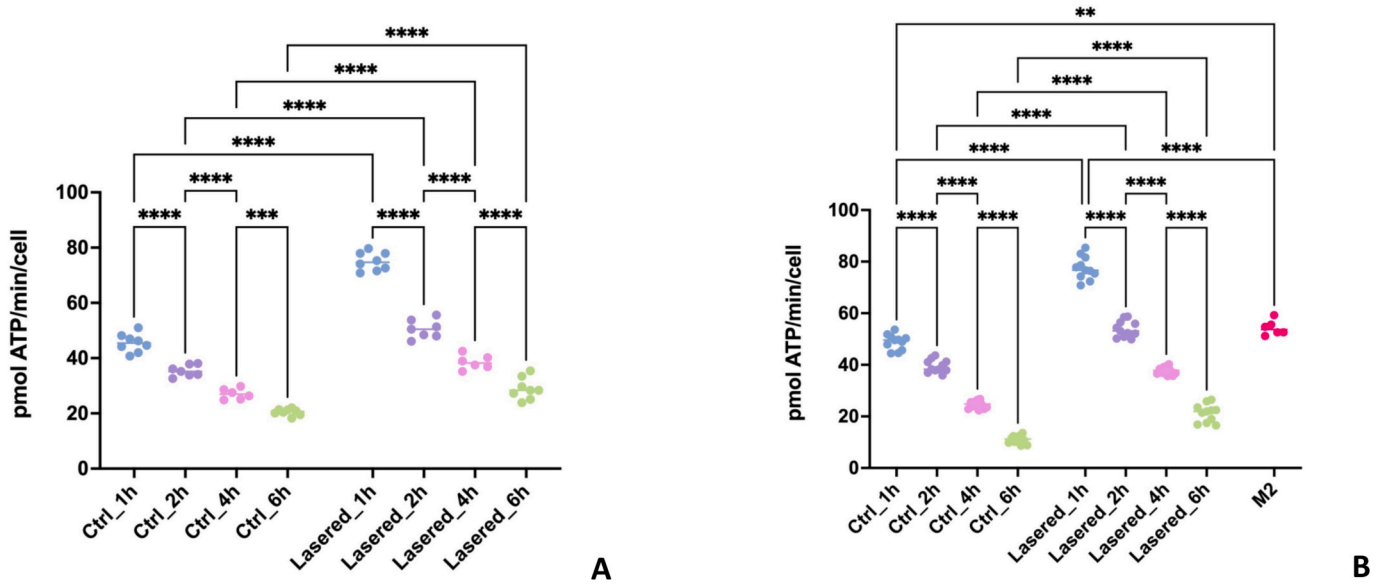

Fig. 4. ATP content in GV (A) and MI (B) oocytes that progress cell cycle in PBMT and control groups at 1–2 - 4 - 6 h of IVM. In panel B the energetic status of 6 *in vivo* matured MII oocytes is also shown. Ctrl: control group; Lasered: PBMT group. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. h = hour(s).

consistently observed in both the control and PBMT groups. The *in vivo* matured MII oocytes showed a significantly higher ATP content than untreated MI oocytes, even those that matured after 1 h of rescue-IVM ($P < 0.01$). Following irradiation, MI oocytes that completed maturation within 1 h exhibited a significantly higher intracellular ATP content compared to retrieved MII oocytes ($P < 0.0001$). MI oocytes maturing within 2 h of laser treatment showed ATP levels comparable to those of *in vivo* matured MII oocytes. Conversely, ATP content decreased in MI oocytes that completed maturation 4 or 6 h after irradiation.

3.3. Photobiomodulation induces increased OxPhos activity in oocytes

Biochemical data showed that the ATP synthesis and OCR increased by approximately 180% in GV and 162% in MI immediately after irradiation, reaching a maximum of 240% in GV and 194% in MI after 10 min, and remained unchanged even 1 h after treatment (Fig. 5A, B, D, E). The observed increase in OxPhos activity did not lead to uncoupling between energy production and respiration, as P/O values remained stable at approximately 2.5, the standard value observed when OxPhos is coupled under pyruvate and malate stimulation (Fig. 5C, F).

The *in vivo* matured MII oocytes exhibited significantly higher OxPhos activity than untreated MI oocytes ($P < 0.0001$). However, irradiation induced a significant increase in OxPhos in MI oocytes, surpassing that of MII oocytes immediately post-treatment and persisting for up to 1 h.

3.4. Absence of lipid peroxidation in oocytes following photobiomodulation

MDA as a marker of lipid peroxidation was evaluated at 1, 2, 4, and 6 h after PBMT irradiation. Data analysis showed no statistically significant differences between the treated group and the control group both for GV and MI ($P > 0.05$), indicating that the PBMT under our experimental conditions did not significantly influence MDA levels (Fig. 6). In both control and treated MI oocytes, MDA concentrations remained at a physiologically basal level comparable to that of *in vivo* matured MII oocytes, with no significant change observed over time (Fig. 6B).

4. Discussion

This study is the first to investigate the potential of PBMT at 810 nm as a tool for rescue-IVM of human immature denuded oocytes following controlled ovarian stimulation. Our findings demonstrate that PBMT enhances cell cycle progression of GV and MI oocytes by activating mitochondrial metabolism. The improved bioenergetic efficiency results in increased ATP production through upregulated oxidative phosphorylation (OxPhos). This occurs without a concomitant rise in oxidative stress. Importantly, under our experimental conditions, temperature variations were negligible (remaining below 1 °C), indicating that the observed effects are attributable to photobiomodulation rather than to thermal mechanisms associated with near-infrared irradiation. ATP is a key indicator of metabolic activity and is essential for oocyte maturation, supporting processes such as organelle redistribution, cytoskeletal

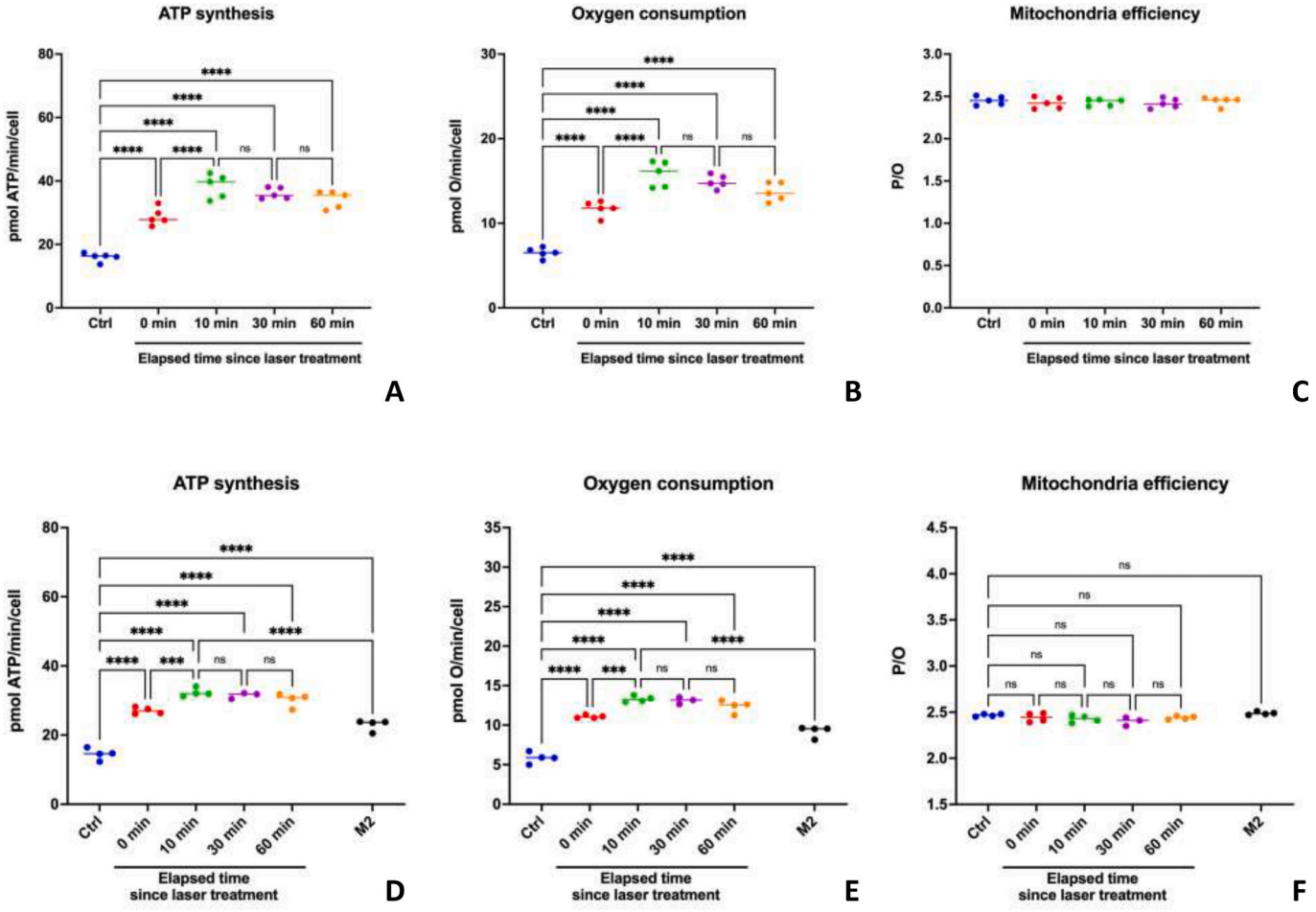

Fig. 5. ATP synthesis in GV (A) and in MI (D) and oxygen consumption rate (OCR) in GV (B) and in MI (E) evaluated in the presence of pyruvate plus malate, as respiring substrates. P/O ratio (a marker of OxPhos efficiency) in GV (C) and in MI (F). Panels D-F also show data of 4 *in vivo* matured MII oocytes. *** $P < 0.001$; **** $P < 0.0001$. n.s. indicates no statistically significant difference. Min = minute(s).

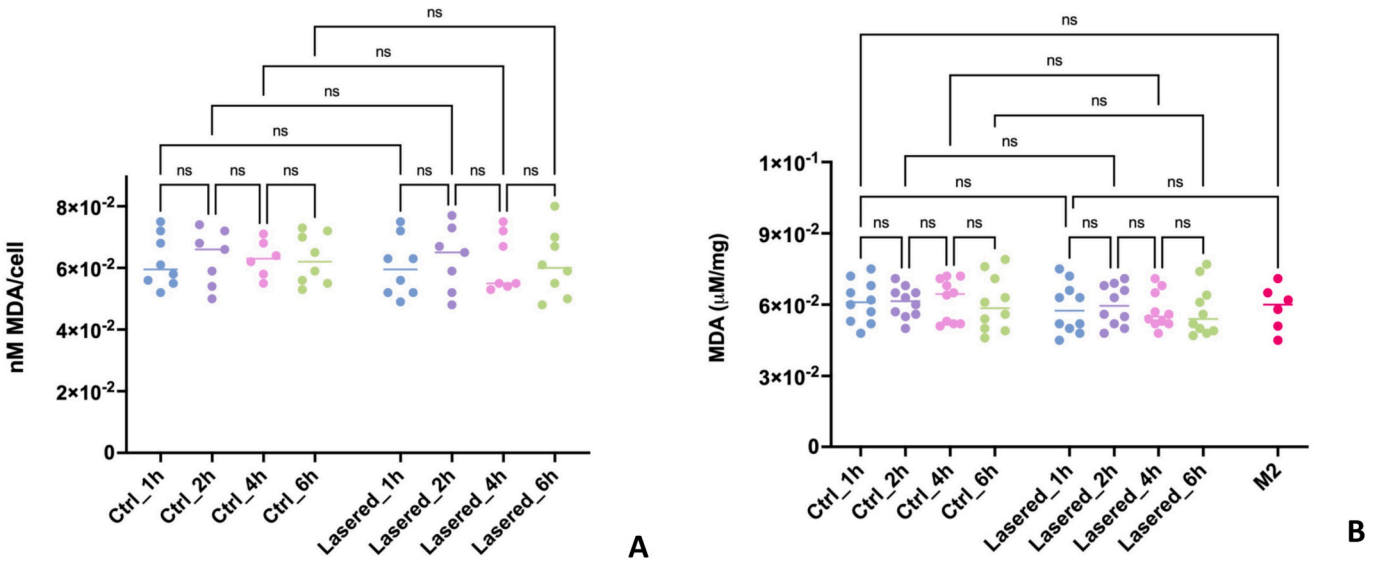

Fig. 6. Effect of photobiomodulation on malondialdehyde accumulation in controls and treated GV (A) and MI (B) oocytes at 1, 2, 4, and 6 h after the irradiation. In panel B the malondialdehyde content of 6 *in vivo* matured MII oocytes is also shown. Ctrl: control group; Lasered: PBMT group. No significant differences were observed (ns: $P > 0.05$). h = hour(s).

reorganization, spindle formation, chromosomal segregation, and fertilization competence [43–45]. Unlike surrounding cumulus and granulosa cells, which primarily utilize glycolysis to supply the oocyte with pyruvate, the oocyte predominantly relies on OxPhos for ATP generation during maturation [46,47]. Our data show a clear relationship between the timing of meiotic progression and ATP availability: GV and MI oocytes resuming meiosis earlier exhibit significantly higher ATP levels in both control and PBMT-treated groups. This suggests that metabolically competent oocytes respond more efficiently to maturation stimuli, highlighting the central role of mitochondrial function in early meiotic progression [45,48]. In GV oocytes, PBMT induced a sustained increase in mitochondrial oxidative metabolism, with higher ATP levels compared to controls, regardless of meiotic resumption or time elapsed post-irradiation. Importantly, the increase in OxPhos was not associated with uncoupling between ATP synthesis and oxygen consumption, suggesting an enhancement of mitochondrial function rather than uncoupling. However, PBMT alone was insufficient to achieve full maturation of GV oocytes to the MII stage within a clinically relevant rescue-IVM timeframe, with complete maturation occurring in only approximately 50% of GV oocytes after overnight incubation. This suggests that additional stimuli (*i.e.,* growth factors), beyond PBMT are required to complete the maturation process. These findings are consistent with the concept that nuclear maturation and acquisition of cytoplasmic competence must be tightly coordinated [49]. Key processes such as mitochondrial biogenesis, calcium homeostasis, and cytoplasmic remodeling are temporally regulated and may not be accelerated solely by increasing mitochondrial activity [10]. While the timing of first polar body extrusion is commonly used as the primary indicator of oocyte nuclear competence [50], other parameters, including the timing of GV breakdown and duration of the MI stage, may also be critical predictors of overall oocyte competence. Research has shown that the progression of GV oocytes to developmentally competent MII oocytes is influenced by patient-specific biology [51] and culture medium composition [49]. Skepticism regarding the reproductive potential of GV oocytes primarily stems from concerns about their capacity to progress to the MII stage within a timeframe compatible with clinical application. Nonetheless, some authors have reported successful rescue-IVM of GV oocytes for clinical use, resulting in healthy live births [52–54]. However, research on the developmental potential and biological characteristics of human cumulus-free GV oocytes retrieved from stimulated cycles remains limited, and prospective studies evaluating culture conditions for GV rescue-IVM are scarce. For all the above reasons, the following discussion focuses on MI oocytes, which are of a greater clinical relevance in ART and fertility preservation programs.As expected, *in vivo* matured MII oocytes showed significantly higher ATP content than untreated MI oocytes, reflecting optimal mitochondrial function. Notably, PBMT established a critical temporal window for oocyte's metabolic activation: MI oocytes maturing within 1 h post-irradiation exhibited ATP levels exceeding those of *in vivo* matured MII oocytes, while those maturing within 2 h had comparable ATP levels. In contrast, MI oocytes completing maturation at 4 or 6 h post-irradiation exhibited a marked ATP depletion. These findings identify an optimal ~2 h temporal window in which PBMT enhances oocyte bioenergetics. Delayed maturation beyond this period may be associated with compromised energy metabolism, leading to impaired spindle formation, chromosome misalignment, and, ultimately, reduced fertilization potential and developmental competence [44,55]. Consistent with previous studies [55,56], MII oocytes exhibited significantly higher OxPhos activity than MI oocytes. Importantly, PBMT induced a marked increase in ATP synthesis and OCR in MI oocytes immediately after treatment, indicating that immature oocytes retain a latent capacity for mitochondrial activation. The laser treatment triggers a rapid metabolic activation in MI oocytes beyond that of MII oocytes up to at least 1 h post-treatment, potentially enhancing their bioenergetic state to meet increased energetic demands for maturation. Overall, these data highlight a strong interplay between the timing of meiotic progression and the ATP availability, with metabolic status acting both as driver and a consequence of oocyte maturation. Biochemical analyses demonstrated that the increase in OxPhos activity was not associated with an uncoupling between oxygen consumption and ATP synthesis, indicating that the increased mitochondrial respiratory activity effectively translated into a higher ATP production, without compromising the OxPhos efficiency. This is consistent with the known mechanism of PBMT, where near-infrared light beneficially modulates cellular metabolism *via* mitochondrial photoacceptors, primarily cytochrome C oxidase, enhancing electron transport and ATP production [57,58]. Clinically, prolonged *in vitro* maturation (overnight culture) of MI oocytes often leads to aging-associated decline in quality, reflected by low fertilization rates and poor embryonic development, likely due to increased chromosomal abnormalities [59]. In contrast, MI oocytes maturing within 2–6 h post-denudation may contribute positively to embryo yield [1,60,61]. Leveraging the ~2-h metabolic "priming" window induced

by PBMT may therefore improve rescue-IVM outcomes by mitigating mitochondrial dysfunction and oxidative stress [62–64].

Importantly, PBMT did not induce oxidative damage, as shown by unchanged lipid peroxidation levels comparable to *in vivo* matured MII oocytes. The preservation of redox homeostasis supports the safety of our PBMT system to enhance rescue-IVM outcomes without inducing harmful mitochondrial overstimulation in irradiated immature oocytes. This important safety issue is consistent with previous findings in human sperm using the same parameters [29,30].

Overall, our results support the potential clinical relevance of PBMT as a strategy to improve the quality and developmental competence of rescue-IVM oocytes. Although not yet routinely used in ART, rescue-IVM is currently gaining renewed interest by the scientific community [65]. Despite its limitations - including lower fertilization and blastocyst formation rates [1,59–61,66] and altered embryonic kinetics [53,66,67] - recent expert consensus [65] has cautiously suggested it may increase embryo yield [1,61] and has resulted in live births [52,66,67]. Accordingly, the recent update of the ESHRE-Alpha Istanbul consensus [65] recommends its use mainly in poor-prognosis patients or when no alternatives are available. In this context, PBMT may complement emerging strategies to optimize rescue-IVM, including improved culture media, supplementation with growth factors and cytokines supplementation, co-culture systems with ovarian support cells or follicular fluid, antioxidant use, and pre-IVM protocols [68–70]. Beyond identifying mitochondrial activation as a key mechanism underlying the beneficial effects of PBMT on the rescue-IVM of immature oocytes, a further strength of this study is the development of a standardized and reproducible PBMT protocol. The short exposure to the laser minimizes suboptimal culture conditions while ensuring rapid effects and procedural consistency. Moreover, the innovative FT-HP delivery system enables uniform, distance-independent irradiation across the entire 1 cm^2 surface. Together, these properties improve reproducibility and reduce operator dependency compared to conventional PBMT systems [17].

5. Challenges and future perspectives

Despite the promising results obtained in this proof-of-principle study, several critical challenges must be addressed before the proposed approach can be reliably translated into clinical practice. First, a comprehensive assessment of the safety profile associated with laser irradiation was not performed. This represents an important gap, as the potential biological effects of PBMT on oocyte genomic stability remain uncharacterized. To overcome this limitation, future studies should specifically investigate whether PBMT influences the incidence of aneuploidy. This could be achieved through the analysis of the first polar body of MII oocytes obtained following rescue-IVM, comparing treated and untreated conditions. In addition, the absence of molecular-level investigations in the treated oocytes constitutes a major limitation of the present work. Several key aspects remain unresolved, including the impact of PBMT on nuclear maturation processes, epigenetic remodeling, and the preservation of the molecular cargo within the treated oocytes. In particular, the integrity and functionality of RNAs and proteins – both of which are essential for the regulation of early embryonic development - should be evaluated in future research.

A second limitation of this study is the absence of a formal *a priori* sample size calculation and the relatively small number of *in vivo*-matured MII oocytes included as a physiological reference group. This reflects the ethical and clinical constraints inherent to research involving donated human gametes in an ART setting. Future studies with larger cohorts will be necessary to confirm and extend the present findings. Importantly, such studies should incorporate dynamic embryo assessment tools, such as morphokinetic analysis obtained through time-lapse imaging systems, rather than relying exclusively on conventional static morphological evaluation. This would allow a more precise and objective characterization of developmental competence.

A third limitation of this study is the lack of direct evaluation of the developmental competence of rescued MII oocytes. This constraint is primarily due to current Italian legislation, which prohibits the insemination of human oocytes outside standard, non-experimental clinical procedures. Consequently, functional outcomes such as fertilization, embryo development, and implantation potential could not be assessed. Evidence from previous studies conducted in the sea urchin - a well-established experimental model for investigating the molecular mechanisms underlying fertilization and early embryogenesis [71] – has demonstrated that PBMT can significantly enhance fertilization rates without inducing detectable embryonic or larval abnormalities [39]. Nevertheless, extrapolation to human systems remains limited, and further validation in additional animal models is warranted. In particular, studies using immature mouse oocytes could provide valuable mechanistic and translational insights.

Fourth, this study was performed exclusively in young oocyte donors with a favorable reproductive prognosis. Therefore, the applicability of these findings to more clinically relevant populations remains uncertain. Future investigations should aim to include women with poor reproductive prognosis and/or advanced maternal age, in order to determine whether the observed effects of PBMT are maintained across different patient populations and clinical conditions. Lastly, we have identified two open questions that warrant further investigation. The first concerns whether MII oocytes matured *in vitro* and exposed to PBMT acquire the capacity for full activation during MII arrest more rapidly than those subjected to conventional rescue-IVM. In fact, the final maturation of human oocytes arrested at the MII stage is critical for acquiring the competence to undergo normal activation and cleavage divisions. This period is significant for the proper assembly and function of the meiotic spindle [60]. The second question involves the potential of PBMT in classical IVM protocols using COC collected *i.e.,* in conjunction with ovarian tissue cryopreservation. To this end, studies investigating the application of PBMT directly on the physiological COC model will be required, in order to assess whether and how laser treatment may exert direct effects or effects mediated by cumulus cells on the oocyte maturation process. Investigating this application could broaden the application of PBMT in fertility preservation strategies [72–74].

6. Conclusions

This work provides pioneering insights into the mechanisms and role of PBMT in promoting cell cycle progression of human immature oocytes retrieved from stimulated cycles, likely through increased ATP production mediated by the upregulation of mitochondrial OxPhos activity. This field of study is undeniably still in its early and challenging stages. The safety validation – especially at DNA level - is a prerequisite for any clinical translation, before the innovative application of PBMT may be considered clinically meaningful in IVF protocols and fertility preservation programs.

CRediT authorship contribution statement

Sara Stigliani: Investigation, Formal analysis. **Matilde Balbi:** Investigation, Formal analysis. **Silvia Ravera:** Writing – review & editing, Resources, Data curation. **Ida Casciano:** Investigation. **Francesca Bovis:** Formal analysis. **Claudia Massarotti:** Conceptualization. **Paola Anserini:** Supervision. **Andrea Amaroli:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Data curation, Conceptualization. **Paola Scaruffi:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Garda Laser S.A.S. had no role in the study's design, data collection, analysis, interpretation, manuscript writing, or decision to publish the results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotobiol.2026.113452.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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