Stimulation of the oxygen consumption by photobiomodulation in the chicken embryo chorioallantoic membrane during hypoxia

Emmanuel Gerelli\textsuperscript{a}, Georges Wagnières\textsuperscript{a*} and Jaroslava Joniová\textsuperscript{a}

\textsuperscript{a}Laboratory for functional and metabolic imaging, Swiss Federal Institute of Technology (EPFL), Station 6, 1015 Lausanne, Switzerland

*Correspondence:
MER Dr Georges Wagnières
Laboratory for functional and metabolic imaging
Swiss Federal Institute of Technology (EPFL)
CH H5 595 (Bâtiment CH)
EPFL SB IPHYS LIFMET/BMO
Station 6
CH-1015 Lausanne / Switzerland
E-mail:
georges.wagnieres@epfl.ch

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ABSTRACT

The main objective of this project is to study the effects of photobiomodulation (PBM) on the oxygen consumption in the \textit{in vivo} chick’s embryo chorioallantoic membrane (CAM) under hypoxia to improve understanding of the mechanisms involved in PBM therapy of various conditions, including strokes and ischemic wounds. Protoporphyrin IX (PPIX), produced endogenously after administration of aminolevulinic acid, was used to probe the oxygen partial pressure ($pO_2$) in the CAM by time-resolved spectroscopy of its delayed fluorescence. This approach enables exploring and optimizing conveniently different radiometric and spectral parameters at play in PBM. Interestingly, since PPIX is produced in the mitochondria, an organelle playing a key role in cell respiration, and then diffuses in different compartments, the $pO_2$ can be determined in different (sub-) cellular/tissue locations. Our results showed that PBM applied between 6 and 16 minutes (5 mW/cm$^2$) after the beginning of hypoxia with light at 730 or 820 nm sustains the oxygen consumption in the CAM for more than one hour, an effect that is not observed in control eggs. This may explain the positive effects induced by PBM in hypoxic tissues, including those that are subject to ischemia-reperfusion injuries.
1. INTRODUCTION

Photobiomodulation (PBM), also called low-level laser therapy (LLLT), is based on the administration of “low” light doses and/or irradiances, in most cases in the near-infrared (NIR) range (600-900 nm), to treat a large number of different conditions [1]. This spectral range is usually selected because it corresponds to the maximal penetration depth of light in most soft tissues [2]. PBM effects are observed at the molecular, cellular and tissue levels [3,4]. Among many applications, it has been shown that PBM can be used to stimulate wound healing and tissue repair, as well as for the treatment of strokes [1]. In addition, PBM seems to generate positive effects to relieve inflammation and edema due to injuries or chronic diseases [5], for sedation, for tissues subject to ischemia-reperfusion injuries and for the treatment of neurological conditions [6,7]. Recently, beneficial effects of PBM have been demonstrated for the treatment of thrombocytopenia [8].

An important common feature of these diseases is in relation with mitochondrial dysfunctions, as it is also the case for cells affected in heart failure, diabetes, and cancer [9,10]. It is worth noting that PBM seems to generate effects directly in this organelle [1,11,12]. Among the reported biochemical effects of PBM one can mention: an increased production of adenosine triphosphate (ATP), as observed in various cell lines [13]; a reduced generation of reactive oxygen species (ROS) in hypoxic cells; effects preventing apoptotic cell death [8]; a stimulation of cells proliferation and, finally, a better cells oxygenation [14,15].

Although PBM has been explored and used for several decades, its clinical use remains relatively limited. This situation is surprising considering its high potential, its simplicity, as well as the width of its applications. Different factors could explain this situation, among which one can mention: a limited understanding of the (photo)biochemical mechanisms involved in PBM; a
large variety of light administration protocols (chronograms) used to treat a broad spectrum of lesions affecting different organs; the fact that, very often, the light dosimetry and radiometry are very poorly optimized and mastered, thus leading to limited effectiveness of the treatment, if not even negative treatment outcomes, which contribute to controversial and/or non-reproducible results [16]. Therefore, reproducible model systems enabling to perform rapidly such optimization in controlled radiometric and spectral conditions while assessing relevant parameters/outcome are highly needed.

The oxygen consumption rate (OCR) along with the ATP level, the mitochondrial membrane potential, the concentration and flux of ions and metabolites reflect the respiratory activity of living organisms and give relevant information about their bioenergetic status [1]. Changes of the standard OCR level can be due to mitochondrial dysfunction or disease [10,17]. Similarly, changes of cell or tissue oxygenation are associated with many common pathological conditions, including stroke, cancer, as well as neurological and metabolic disorders. Mitochondria play a key role in the OCR and ATP production. This organelle contains an important chromophore which is likely to be the precursor in the cellular response to PBM – cytochrome c oxidase (CcO) [14]. CcO is a proton pump, a unit of the organized supercomplexes forming the respiratory chain (OXPHOS) [18]. CcO reduces oxygen to water without ROS production, thus permitting an efficient production of ATP thanks to a sustained mitochondrial membrane potential [19,20]. Moreover, it can modulate the level of ROS and reactive nitrogen species (RNS) [21,22], which are considered to have the second major retrograde signaling function [23] after the mitochondrial unfolded protein response [9,24].

An interesting hypothesis to explain PBM effects is based on the assumption that small perturbations of the electron tunneling effect in CcO [25] induced by IR light could modulate
various retrograde signaling and, therefore, generate biological responses by activation of biological cascade pathways, and generation of transcription factors. This could explain the long-term beneficial macroscopic effects. It should be noted that the wide variety of biological and photochemical concepts which must be considered to validate this hypothesis is very challenging and complex. Thus, this situation explains why the mechanisms at play during PBM are not yet completely understood, in particular the various potencies of different wavelengths.

Vos and al. observed an increase of OCR induced by various NIR wavelengths in the *pink 1* fruit fly model, with maximal potencies around 660 and 860 nm, whereas a minimum was observed at 750 nm [6]. Inhibiting sequentially each complex of the OXPHOS chain indicated that CcO is likely to be the key complex responsible for the PBM effects [6,14], an observation that is consistent with the hypothesis mentioned above. Interestingly, the action spectrum reported by Vos et al. is in agreement with the CcO absorption spectroscopy measured in the NIR by Mason et al. [26].

The study reported here is based on the chick’s chorioallantoic membrane model (CAM) that is well characterized, relevant, and convenient for numerous *in vivo* studies [27]. Its membrane can conveniently be probed with optical imaging or “point” spectroscopic systems to study its response to different treatments. Among the wide uses of this model, we have demonstrated that its membrane produces protoporphyrin IX (PPIX), a fluorescing photosensitizer, following the administration of its precursor, aminolevulinic acid (ALA). Since the lifetime of the PPIX triplet state is very sensitive to the partial pressure of oxygen (pO$_2$), this parameter can be measured by time-resolved spectroscopy of the PPIX delayed fluorescence (DF). Only the PPIX DF lifetime can be measured in physiological conditions because its phosphorescence is too weak to be detected *in vivo* [28]. PPIX is of high interest for such measurements since: 1) ALA, or certain
derivatives thereof, are approved PPIX precursors for diagnostic and therapeutic applications; 2) the production of PPIX takes place in the mitochondria, an organelle playing a key role in the cell respiration as mentioned above, and 3) since PPIX diffuses in different cellular and tissue compartments after its endogenous production, the pO$_2$ can be determined at different (sub-)cellular/tissue locations by changing the time between ALA administration and the pO$_2$ measurements.

Following our historical results obtained while assessing the modification of the mitochondrial membrane potential induced by PBM on the pink 1 fruit fly model [6], we wanted to improve our understanding of the mechanisms involved in PBM treatments of various conditions associated to hypoxia, such as strokes and ischemic wounds. Winter et al. reported that the persistent reduction in the cerebral metabolic rate of oxygen after hypoxia-ischemia is likely to be due to mitochondrial dysfunctions [29]. Interestingly, this group showed that the persistent reduction in the cerebral metabolic rate of oxygen after hypoxia-ischemia is probably due to a combination of impaired mitochondrial function and reduced energy demands during acute phase.

Therefore, in this study, we examined the effects of light at 730 and 820 nm on the CAM OCR during a prolonged hypoxia. This study was performed on the CAMs placed in a thermoregulated and air-tight closed chamber for 1 hour during which the pO$_2$ in the chamber gently decreased as the CO$_2$ concentration increased due to the breathing of the embryo, a phenomenon called hypercapnia. PBM illumination was performed between 6 and 16 minutes after closing of the chamber. Changes of the pO$_2$ (OCR) were assessed by time-resolved spectroscopy of the PPIX DF.
2. MATERIALS AND METHODS

2.1 Setup to measure the PPIX delayed fluorescence lifetime

Time-resolved measurements of the PPIX DF luminescence have been performed with the spectrometer developed by our group and described in detail by Piffaretti et al. [30] (Figure 1). Briefly, the light source consists of a nitrogen laser-pump dye laser emitting at 405 nm (LTB HP 110) (pulse duration: < 10 ns; repetition rate: 3 Hz). Excitation (0.4 µJ/pulse) and luminescence signals were carried out to and from the sample by a single quartz optical fiber (core diameter: 550 µm) that probed the sample perpendicularly to its surface and in contact. The detection of the PPIX luminescence was performed by a gated photomultiplier (PMT) placed after two emission filters (LP 610 nm + BP 628±16 nm) to reject the excitation light, to minimize perturbations induced by the setup and the sample “autoluminescence”, as well as the PPIX photoproducts [31]. This photomultiplier was kept switched off during 600 ns after the laser pulse to prevent its saturation and the resulting loss of dynamic range due to the strong PPIX prompt fluorescence. The detected DF was converted by the PMT (model R928, Hamamatsu) and sampled by a LeCroy (model LT342; 500 MHz – 24 bits - 50 Ohms) oscilloscope. Ten decays (10’000 points per decay; time resolution: 20 ns; recording windows: 200 us) were acquired and averaged for each measurement. These data were automatically recorded in the form of an Origin file (OriginLab Software) via a home-made LabView interface (National instrument). The lifetimes were determined by fitting the DF PPIX decays tails with a mono exponential function using a Levenberg-Marquardt algorithm implemented by OriginLab software [30]. Then the pO₂ was derived using the Stern-Volmer expression. The Stern-Volmer coefficients were determined according to a calibration procedure similar to the approach described by Piffaretti et al. [32].

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2.2 Chicken embryo chorioallantoic membrane (CAM) preparation

The preparation of the CAMs has been described in detail elsewhere [31]. Briefly, fertilized chicken eggs (Animalco AG, Switzerland) were transferred into an automatic turn incubator (FIEM snc, Italy) and incubated blunt end up during 3 days at 37 °C with a relative humidity of 65 % and at atmospheric oxygen pressure (155.4 mmHg). On the 3rd embryo development day (EDD) a small part of the pointed end of the shell was removed, thus creating a hole (approximately 3 mm in diameter), which was then covered by a tape (Scotch® MagicTM, St. Paul, Minnesota, USA). Eggs were then returned into the incubator blunt end down in a static position until further use. At EDD 10, the hole in the shell was enlarged to approximately 2.5–3 cm in diameter, enabling an easy topical application of an ALA solution (20 mg/mL of isotonic solution (pH 7.4); 20 μl). The CAMs were then covered with a Parafilm M barrier film (Pechiney Plastic Packaging Company, Chicago, Illinois, USA) for 3 hours to enable the endogenous production of PPIX in the CAM. This time is such that the level of the PPIX DF emission is strong enough for an easy detection with an acceptable signal-to-noise ratio.

2.3 Measurement of the oxygen consumption rate and PBM protocol

pO₂ measurements of the CAM were performed in a Poly(methyl methacrylate) (PMMA + stainless steel) thermo-stabilized air-tight gas chamber (preventing gases exchanges) presenting an inner geometry fitting the egg shell (Figure 2). Such a geometry minimizes the quantity of gas (typically 2 mL) in the chamber that is not dissolved in the egg and enables to have a fast decrease/depletion of oxygen in the chamber due to the breathing of the embryo. Importantly, the minimal volume in the air-tight chamber prevents the desiccation of the surface of the CAM during PBM. A glass window located 5 mm above the CAM surface was placed in the upper wall
of the chamber to enable the homogenous illumination of the CAM with a 25-mm diameter spot (Figure 2A). This homogenous spot was produced by an optical fiber-based frontal light distributor (Model FD1; Medlight SA, Ecublens, Switzerland) positioned 5 cm above the window. The light distributor was connected to a Ti:Sapphire (Mai Tai, Spectra-Physic, Santa Clara, CA, USA) emitting light at 730 or 820 nm. The irradiance at the CAM surface was 5 mW/cm² for both wavelengths, and the irradiation lasted 600 s (3 J/cm²). In addition to the probe dedicated to pO₂ measurements performed in the CAM with PPIX, mentioned thereafter as mitochondrial pO₂ (mtpO₂), a commercially available calibrated pO₂ and temperature probe (Oxylab pO₂ ETM probes BF/OT/E; Oxford Optronix; Abingdon, UK) was positioned in the air space just above the CAM, referred thereafter as the environmental pO₂ (epO₂). This pO₂ probe served as a control probe not only to monitor the environmental pO₂ around the egg in the gas chamber, but also to check that the chamber was air-tight closed. This probe enabled measuring the pO₂ and temperature in real time during the whole duration of the experiment. Finally, this epO₂ probe was used to determine the Stern-Volmer constant (Kq_DF PpIX = 3700 ± 50 mmHg⁻¹.s⁻¹), used to derive the mtpO₂ from the PPIX DF lifetime, according to a procedure described by Piffaretti et al. [30].

A stainless-steel tube (inner/outer diameters: 1 / 1.5 mm) introduced through a glass window enabled flushing the gas (air) in the chamber for 1 minute before the induction of the hypoxia. This tube was surrounded by a removable seal enabling the gas outflow.

The PMMA + stainless steel thermoregulated gas chamber containing the egg was positioned in a much larger (height x width x depth: 36 x 47 x 62 cm³) glove box for a precise control and stabilization at 36 °C of the outer temperature, and to avoid condensation on the optical window mentioned above.
The optical fiber used to measure the mtpO$_2$ was placed in a gentle contact against the CAM, and recordings of this parameter, as well as of the epO$_2$, were performed synchronously every 10 s. The total light dose at 405 nm necessary to measure one DF PPIX lifetime was determined with a joule meter (Model FieldmaxII-TOP, Coherent, Santa Clara, CA, USA). The measured value of 1 mJ/cm$^2$ per lifetime measurement, leading to a total dose of 360 mJ.cm$^2$ after 60 min, did not induce any photobleaching or phototoxic effects [31].

The experimental protocol used in this study was as follows (Figure 2B):

1) ALA was applied topically on the CAMs at EDD 10. Eggs were then returned to the incubators.
2) Eggs were transferred to the gas chamber 3 h after ALA administration.
3) Air was flushed into the air space in the chamber one minute before its closing to establish the initial epO$_2$ of the experiments.
4) After stopping air flush, the chamber was closed, including the seal surrounding the stainless-steel tube for gas flushing. This defined the time $t = 0$ min.
5) Eggs were maintained in this condition for a 6 minutes resting time.
6) Then, PBM irradiations were performed on the CAM for 10 minutes.
7) N$_2$ was flushed at $t = 60$ min, followed by a flush with air at $t = 66$ min to perform a second calibration and a control of the experiment, respectively.
8) Eggs were returned into the incubator. All the explored eggs (102 in total) were alive one day after the experiment.

< Figure 2 >
3. RESULTS AND DISCUSSION

3.1 Value of the initial mitochondrial and environmental \( \text{pO}_2 \) just after closure of the chamber.

The \( \text{mtpO}_2 \) as well as the \( \text{epO}_2 \) were measured in 102 CAMs before the start of the experiment (4\(^{th}\) point in the protocol presented above; \( t = 0 \) min). As mentioned in chapter 2.3, in a first step, the chamber was flushed with air during one minute to establish an initial \( \text{epO}_2 \) before air-tight closing the chamber. The mean value of the \( \text{epO}_2 \), thereafter mentioned as the “initial \( \text{epO}_2 \)”, was typically 116 ± 5 mmHg (Figure 3). It is not surprising that the value of the initial \( \text{epO}_2 \) in our conditions was lower than the atmospheric \( \text{pO}_2 \) (155 mmHg) due to the breathing of the embryo and shell oxygen conductance. In the avian embryos, the exchanges of gases take place primarily by diffusion [33]. The diffusion rate depends on the partial pressure of the gases between the two sides of the eggshell barrier, and on the permeability of this barrier to oxygen (conductance). Shell conductance is dependent on the size and number of pores present in the shell [34]. It has been observed that \( \text{pO}_2 \) varies in different parts of the shell due to the diverse number and sizes of these pores in the eggshell [35]. Hence, even though the air was flushed in the chamber for 1 minute before its closing, the depletion of oxygen (due to the chicken embryo respiration and the limited shell conductance) was much faster than the oxygen delivery, causing the decrease of the initial \( \text{epO}_2 \) down to 116 ± 5 mmHg.

As shown in Figure 3, the initial \( \text{mtpO}_2 \) values measured on the surface of the 102 CAMs ranged typically between 20 and 90 mmHg, with a mean value of 50 ± 15 mmHg.

As described above, it should be noted that all measurements were performed 3 h after ALA administration. As previously reported in the literature, the vast majority of the PPIX luminescence is of mitochondrial origin at this time interval after ALA administration [28,36].
These reports support the name of mtpO\(_2\) we give in the text for pO\(_2\) values derived from PPIX DF lifetime measurements performed in our conditions.

Such a broad distribution of the initial mtpO\(_2\) can be explained by a pO\(_2\) gradient that is present in the epithelial layer of the chorion membrane. As the embryo develops, a tree-like venules and arterioles evolve from an initially homogeneous capillary plexus [37]. Also, with the embryo development, blood vessels network involves more bifurcations, resulting in a heterogeneous distribution of the pO\(_2\) within the CAM. It has been reported that at early EDDs, the CAM pO\(_2\) varies between 0 and 45 mmHg [38]. At EDD 10, the arterial blood (embryonic venous) pO\(_2\) is typically 30.9 mmHg [39]. Moreover, during the embryo development, extraembryonic organs as well as embryo grow at different speed. More precisely, in the early days of incubation, the extraembryonic organs develop at faster rate than the embryo itself. Then, in the middle of the incubation time (EDD 12), the embryo starts to develop faster. This “phase shift” also contributes to the changes of the pO\(_2\) (evolution of gradient) in the CAM [40]. It should be noted that Piffareti et al. [30,32] measured in the CAM the dependence of the PpIX’s DF reciprocal lifetime on the environmental pO\(_2\). Their results showed significant variations which increased at large pO\(_2\) values, i.e. with increasing concentration of oxygen, the standard deviation increased as well. This phenomenon is linked to a heterogenous PPIX distribution along with physiological inter- and/or intra-sample variations, like vascular and microvascular density in the CAM probed area. Considering all these factors, the wide range of the initial mtpO\(_2\) in the CAM (Figure 3) is expected.

In summary, the reasons mentioned above suggest that the probe was placed in low vascularized area of the membrane (extravascular space) when low initial mtpO\(_2\) values were measured in the CAM. On the other hand, high initial mtpO\(_2\) values were probably measured when the
placements of the probe were next to blood vessels (artery or vein). This statement is also supported by pO₂ measurements we have performed in different tissue compartments of the CAM with a Clark electrode (Model OX100; Unisense, Denmark). These measurements demonstrated the presence of a positive pO₂ gradient going from extravascular space to arteries and veins (data not shown).

Due to the constrains of our experimental setup, the fiber used to probe the mtpO₂ (probed surface area: about 1 mm²) had to be placed in the air-tight chamber in the center hole of the optical window (Figure 2). This constrain prevented us to choose the tissue structures probed by the fiber.

< Figure 3 >

3.2 PBM effects on the evolution of the mitochondrial and environmental pO₂ after closure of the chamber.

As Figure 4A (blue line) shows, the epO₂ (typical initial value of 116 ± 5 mmHg) decreased slowly and gently during one hour following the closure of the chamber (with a final value equal to 70 ± 5 mmHg). Consequently, the difference of the epO₂ caused by the respiration of the embryo was typically ΔepO₂ = 46 ± 10 mmHg.

Standardly, in our conditions, the volume occupied by the egg was about 58 mL, whereas the total volume of the chamber is 60 mL. Hence, the remaining volume of 2 mL contains the equivalent of 0.4 mL of oxygen. Assuming that the oxygen dissolved in the egg is equal to the concentration of this gas in water at saturation, in the order of 0.4 mL of oxygen is dissolved in the whole egg. Therefore, the corresponding total amount of oxygen contained in the chamber right after its closure is less than 1 mL. According to Motorla and Labbe [41], the oxygen
consumption of chicken embryo at EDD 11 is 1 mL/min. Therefore, all oxygen contained in the chamber should be consumed in about one minute after its closure if the embryo would consume oxygen at a rate corresponding to normal conditions. The much smaller OCR observed in figure 4A through our measurement of the epO$_2$ can be explained by the observations of Andrewartha at al. [42]. This group reported that a hypoxic and hypercapnic environment quickly and strongly reduces the oxygen consumption of the embryo.

Although the inter-egg fluctuations affecting the ΔepO$_2$ were relatively small (in the order of 22 %), the evolution of the mtpO$_2$ was, however, very different and more complex. As mentioned in chapter 3.1, the initial mtpO$_2$ values measured in the 102 CAMs varied significantly (between 20 and 90 mmHg). We observed that, for the 75 eggs presenting an initial mtpO$_2$ larger than 40 mmHg, the mtpO$_2$ never decreased under the value of 5 mmHg during the whole course of the experiment. As discussed above, this is probably due to the placement of the fiber probe next to densely vascularized locations of the CAM (vein or artery), where the mtpO$_2$ never reached values close to zero due to the blood flow which sustained a constant delivery of oxygen in the CAM, even if the egg was in hypoxia. However, 27 eggs presenting “low” initial mtpO$_2$ values (between 20 and 40 mmHg) exhibited a fast decrease of the mtpO$_2$ below 5 mmHg (point 1 in figure 4A) about 25 minutes (time called t’ thereafter) after the closure of the gas chamber. This evolution is probably due to the fact that the fiber probe was positioned “far” from blood vessels, as discussed above. In the absence of PBM light, these CAM then demonstrated a marked increase of the mtpO$_2$, up to 10 mmHg, which reached its maximum about 10 minutes later, called t’’ thereafter (see point 2 showing the “bump” in Figure 4A). Then, the mtpO$_2$ went down back to 0 mmHg after 20 additional minutes (point 3 in Figure 4A). The mtpO$_2$ maximum observed at the time t’’ for CAMs kept in the dark can be explained by a reduction, if not a stop,
of the CAM’s respiration as a consequence of its hypoxic environment. This effect, reported by Chance and later by Sarti as “cushioning effect” [43,44], occurs in certain tissues when oxygen is depleted, which leads to a slowing down, and eventually stopping, of the respiration through a partial inhibition of cytochrome c oxidase by nitric oxide [38,45–47]. This slowing down of the OCR suggests a decrease of the metabolic activity, an effect that is more important in embryos than in hatchlings subjected to hypoxia [38]. It is probable that this reduced metabolic activity observed at time t’’ is due to mitochondria dysfunctions induced by the hypercapnia resulting from the hypoxia. Indeed, due to the air-tight closing of the gas chamber and breathing of the embryo, the partial pressure of carbon dioxide (pCO₂), although not measured, certainly increased with time. Ultimately, the eggs were probably suffering from increased levels of pCO₂, which is known to induce a disruption of the acid-base balance. These concepts are of relevance since it has been reported that hypercapnia, or hypercapnic acidosis, promotes mitochondrial dysfunction and, among others, impairs cell proliferation and precludes mesenchymal stem cells ability repair functions [48,49]. Interestingly, impaired mitochondrial function in combination with reduced energy demands during the acute phase (hypoxia) was shown to be responsible for the persistent reduction in the cerebral metabolic rate of oxygen after hypoxia-ischemia [29].

The most interesting aspect of this study is in relation with the disappearance of the “bump” observed at time t’’ when the CAM was treated by PBM (see figure 4A). The parameter we established to quantify the PBM potency to induce an increase of the OCR is the difference of mtpO₂ between the points 1 and 2 (ΔmtpO₂) in Figure 4A divided by their time differences (t’’ – t’ = Δt; Δt was equal to 10 min., as mentioned above for the CAMs that were kept in the dark). This parameter is presented in Figure 4B for 9 eggs dedicated to a control group, 9 irradiated at 820 nm, and 9 at 730 nm. It is interesting to note that the dispersion of the parameter
(ΔmtpO₂/Δt) varies significantly between the illuminated and non-illuminated groups. Indeed, the interquartile ranges (25th to 75th percentile) corresponding to the black chart box is much larger for the control group (56.3 mmHg·h⁻¹) than the groups illuminated with 730 nm (13.1 mmHg·h⁻¹) and 820 nm (14.8 mmHg·h⁻¹). Interestingly, the mean value for the control group is 26.9 mmHg·h⁻¹, whereas these values are much lower for the groups illuminated at 820 and 730 nm, i.e. -11.7 mmHg·h⁻¹ and -1.2 mmHg·h⁻¹, respectively. Since these values are negative for both illuminated groups, it can be assumed that light at 730 and 820 nm induces a marked modification of the mechanisms involved in the consumption of O₂ after a long (about 30 minutes) hypoxia. In other words, we have shown that the arrest of the OCR caused by hypoxia and/or hypercapnia, evidenced by the mtpO₂ “bump” observed at the time t”, is prevented by PBM. This effect is possibly in relation with the “rescuing” by PBM of cells/tissues suffering from a prolonged hypoxia [50–52]. Although not statistically significant, comparing the PBM effects induced at 730 or 820 nm suggest that the later wavelength is more potent to maintain the OCR after hypoxia in our conditions. This observation is in agreement with some of our previous results which indicated that light at 820 nm is more potent than 730 nm to induce other PBM effects [6,7].

< Figure 4 >

4. CONCLUSION

We have developed a method to study the PBM effects on the CAM OCR during a prolonged hypoxia. The cellular pO₂ (the mtpO₂) was monitored by time-resolved spectroscopy of the PPIX DF, whereas the CAM environmental pO₂ (the epO₂) was measured with a commercially available probe in the volume surrounding the CAM that was confined in an air-tight chamber.
We have shown that, when the CAM is placed in this chamber for one hour, the gradual decrease of the epO$_2$ almost stopped as a result of the significant reduction of the CAM OCR. Importantly, our measurements of the mtpO$_2$ showed that the decrease of the OCR induced by hypoxia can be avoided with PBM performed at 730 or 820 nm. These results have important implications to better understand the mechanisms leading to the positive effects induced by PBM when treating strokes or ischemic wounds, as well as other tissues subject to hypoxia.

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Disclosures
Authors have no conflicts of interest to declare.

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**Figure legends**

**Figure 1:** Optical fiber-based time-resolved spectrophotometer developed to measure the pO$_2$ in solutions and/or *in vivo*.
Inset lower right corner: Fluorescein angiography showing a typical microvasculature network observed on the CAM at EDD 10.

**Figure 2:** Experimental setup and chronogram of the experiment

A: Picture of the gas chamber with the CAM that is visible through the glass window
B: Chronogram of the egg treatments and measurements in the gas chamber (mtpO$_2$: measurement of the PPIX DF lifetime; epO$_2$: measurement of the pO$_2$ in the cavity located above the CAM with the Oxylab probe).

**Figure 3.** Distribution of the initial mtpO$_2$ measured in 102 eggs

**Figure 4.** Effect of PBM on the CAM mtpO$_2$ and epO$_2$.

A: Illustrative evolution of the mtpO$_2$ and epO$_2$ recorded after closing the chamber (t = 0). Blue line shows the epO$_2$, red line the mtpO$_2$ when the CAM was irradiated with 820 nm (irradiance 5 mW/cm$^2$ during 600 s). Black line represents the control CAM (no light applied).
B: Box chart presenting the values of $\Delta$mtpO$_2$/Δt in control and PBM treated CAMs.
A

Oxylab $pO_2$ probe - $epO_2$

PBM frontal light distributor

Optical fiber for PPIX luminescence excitation and detection - $mtpO_2$

B

Thermoregulated gas chamber

PBM: 730 or 820 nm

flush of the air - start of the experiment

PBM irradiation

flush of $N_2$ followed by a flush of air for the calibration of the $pO_2$ probe - end of the experiment
