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Title: MODULATION OF THE ROD OUTER SEGMENT AEROBIC METABOLISM DIMINISHES THE PRODUCTION OF RADICALS DUE TO LIGHT ABSORPTION.

Article Type: Original article

Keywords: ATP synthase; diabetic retinopathy; dim light; light; metformin; reactive oxygen intermediates; rod outer segment; resveratrol; oxidative stress.

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Abstract: Oxidative stress is a primary risk factor for both inflammatory and degenerative retinopathies. Our previous data on blue light-irradiated retinas demonstrated an oxidative stress higher in the rod outer segment (OS) than in the inner limb, leading to impairment of the rod OS extra-mitochondrial aerobic metabolism.
Here the oxidative metabolism and Reactive Oxygen Intermediates (ROI) production was evaluated in purified bovine rod OS in function of exposure to different illumination conditions. A dose response was observed to varying light intensities and duration in terms of both ROI production and ATP synthesis. Pretreatment with resveratrol, inhibitor of F1Fo-ATP synthase, or metformin, inhibitor of the respiratory complex I, significantly diminished the ROI production. Metformin also diminished the rod OS Complex I activity and reduced the maximal OS response to light in ATP production.
Data show for the first time the relationship existing in the rod OS between its -aerobic- metabolism, light absorption, and ROI production. A beneficial effect was exerted by metformin and resveratrol, in modulating the ROI production in the illuminated rod OS, suggestive of their beneficial action also in vivo. Data shed new light on preventative interventions for cone loss secondary to rod damage due to oxidative stress.

Professor Rafael Radi, MD
Associate Editor
Free Radical Biology and Medicine

Genoa, January , 17th 2018

Dear Editor,

thank you for Your interest in our manuscript Free Radical Biology and Medicine

Manuscript ID: FRBM-D-17-00543R2, Entitled: MODULATION OF THE ROD OUTER SEGMENT AEROBIC METABOLISM DIMINISHES THE PRODUCTION OF RADICALS DUE TO LIGHT ABSORPTION.

We are now ready to submit the revised manuscript. In the answer to reviewers the changes we have made and why are indicated in detail.

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***Detailed Response to Reviewers**

Reviewers' comments:

Reviewer #1: I am glad the authors have followed my suggestion and happier still that this has proved useful!...

We thank the reviewer for the good evaluation of our revised version of the manuscript.

It would be useful to determine how the effects of metformin and resveratrol vary with their concentrations, as this might give some clue as to their utility in combatting oxidative stress in vivo. However this is not a major concern.

We are grateful to the Reviewer for his suggestion. Indeed, due to the very fast reaction kinetics of the DHR dye, in our opinion non-significant variations of ROI production related to different concentrations of resveratrol or metformin would be expected, by citofluorimetric analysis in the presence of concentrations of inhibitor that lay in the same order of magnitude. Furthermore, a dose-response study of the oxidative stress in a subcellular fraction, as rod outer segments are, may not be indicative for the determination of the best concentration of resveratrol or metformin to recommend in the clinical practice to hinder the oxidative stress *in vivo*.

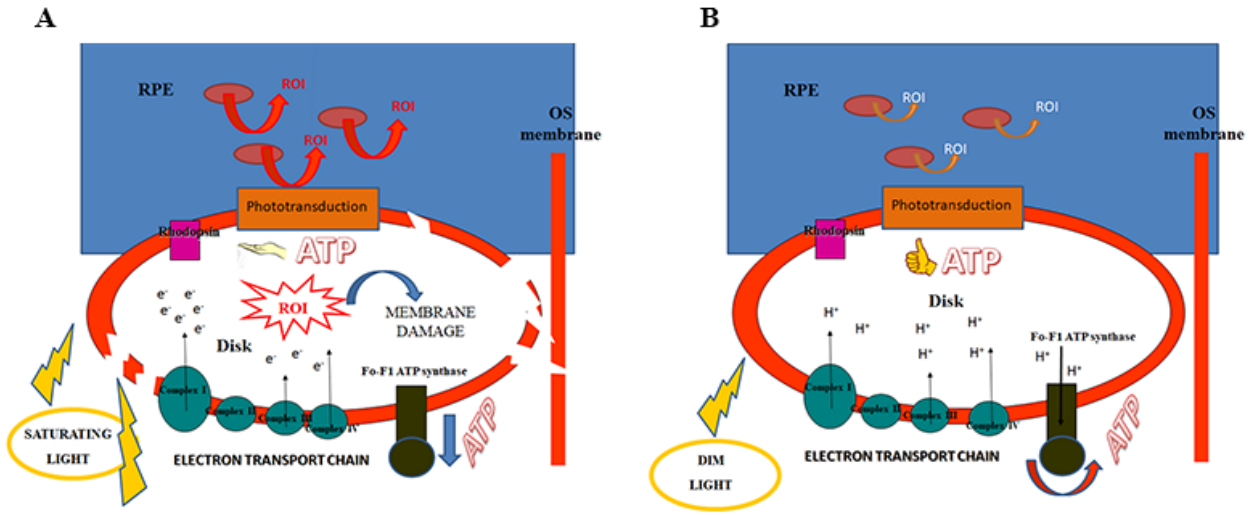
Indeed, here we aimed at investigating for the first time the relationship among the rod outer segment (OS) aerobic metabolism and illumination, together with the related oxidative stress in an isolated system housing both photransduction and OXPHOS proteins, as the rod OS is. In turn, Resveratrol and Metformin were used as known inhibitors of FoF1-ATP synthase and ETC Complex I respectively, allowing a negative control. Moreover, concerning Metformin, its therapeutic dose is only clinically determined, related to its hypoglycemic action. Notably, the novelty of the present study is to give a possible experimental and molecular explanation of the effect of Metformin (MTF) in effectively reducing the cumulative oxidative risk damage (a common pathogenic factors of diabetic retinopathy, DR) in type 2 diabetes mellitus patients MTF-treated.

For these reasons, especially considering that the issue was not considered a major concern, we have preferred not to attempt those experiments.

It would be useful to redraw the graphic to emphasise the effects of light and on mitochondrial ROS production in RPE and rods as those unfamiliar with retinal anatomy would benefit from a fuller understanding of the crucial details of this separate but close association of these cells within the retina

We are grateful to the Reviewer for his suggestion. We are agree with the Reviewer about the contribution of RPE mitochondria in the ROI production in the outer retina *in vivo*, in fact we mentioned this in the Discussion section.

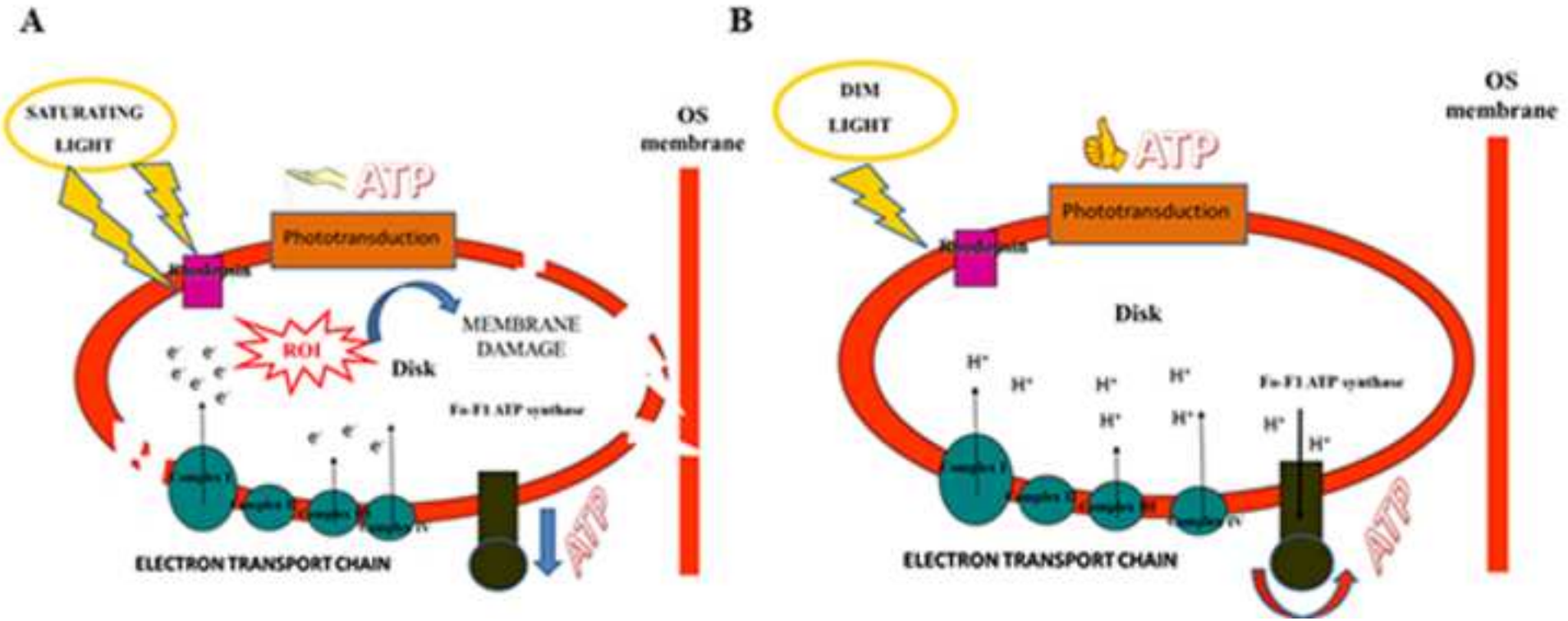
Therefore, considering that the Graphical Abstract should be representative of the paper data, as reported by FRBM "Guide for Authors " (*A graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article*.), addition of the RPE cells in the graphic may be in our opinion misleading about the content of the paper. In fact our experiments were not performed on whole retinas, rather on purified Rod OS, where RPE cells are absent and their contribution in the oxidative stress generation reported in the paper is negligible. So for a better comprehension of our paper we would prefer to maintain the original version of Graphical Abstract. Nonetheless, a Graphical Abstract modified according to the Reviewer's suggestion is attached below to give the opportunity to the Reviewer to decide whether using the revised graphic or not. Notably, in Panel A (saturating light conditions) of the revised version of Graphical Abstract, the contribution of RPE cells in ROI production is represented as tentatively higher (big arrows) than those in Panel B (dim light condition, small arrows), because in our opinion the faster photransduction rates stimulated by saturating light illumination may increase RPE mitochondrial metabolism (and so ROI production) due to the higher energy demand necessary to accomplish the faster photo-pigment (11-cis retinal) renewal. However, we have no data about this fact, as we did not work on RPE, and therefore it is just our hypothesis.



HIGHLIGHTS (3-5 BULLET POINT)

- Saturating ambient light induces oxidative stress in the rod outer segments (OS)
- Resveratrol and Metformin reduce radical production in OS
- Positive correlation between light exposure and ROI production in OS in vitro
- Modulating OXPHOS in OS may prevent cone loss secondary to rod damage

*Graphical Abstract



MODULATION OF THE ROD OUTER SEGMENT AEROBIC METABOLISM DIMINISHES THE PRODUCTION OF RADICALS DUE TO LIGHT ABSORPTION.

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Abbreviations

AMD, age-related macular degeneration; Ap5A, di(adenosine)-5-penta-phosphate; ATP synthase, FoF1-ATP synthase; ; DR, diabetic retinopathy; ETC, electron transport chain; MTF, Metformin:

OS, outer segment; PUFA, polyunsaturated fatty acids; ROI, reactive oxygen intermediates; RP, retinitis pigmentosa; RV, resveratrol; TEM, transmission electron microscopy.

ABSTRACT

Oxidative stress is a primary risk factor for both inflammatory and degenerative retinopathies. Our previous data on blue light-irradiated retinas demonstrated an oxidative stress higher in the rod outer segment (OS) than in the inner limb, leading to impairment of the rod OS extra-mitochondrial aerobic metabolism.

Here the oxidative metabolism and Reactive Oxygen Intermediates (ROI) production was evaluated in purified bovine rod OS in function of exposure to different illumination conditions. A dose response was observed to varying light intensities and duration in terms of both ROI production and ATP synthesis. Pretreatment with resveratrol, inhibitor of F_1F_o-ATP synthase, or metformin, inhibitor of the respiratory complex I, significantly diminished the ROI production. Metformin also diminished the rod OS Complex I activity and reduced the maximal OS response to light in ATP production.

Data show for the first time the relationship existing in the rod OS between its -aerobic- metabolism, light absorption, and ROI production. A beneficial effect was exerted by metformin and resveratrol, in modulating the ROI production in the illuminated rod OS, suggestive of their beneficial action also *in vivo*. Data shed new light on preventative interventions for cone loss secondary to rod damage due to oxidative stress.

Key Words: ATP synthase; diabetic dim light; light; metformin; reactive oxygen intermediates; rod outer segment; resveratrol; oxidative stress.

INTRODUCTION

The Electron Transfer Chain (ETC) is a major producer of Reactive Oxygen Intermediates (ROI) [1,2], in turn responsible for the oxidative stress production, a set of alterations occurring in tissues, cells and macromolecules upon alteration of the physiological equilibrium between production and elimination of ROI. These, typically, trigger a vicious circle, since oxidative stress aggravates ROI production, causing hypo-metabolism, in turn lowering the ability of the cell to scavenge the damage. Markers of oxidative damage include malondialdehyde (MDA), and 4-hydroxynonenal (4-HNE) [3]. The body's antioxidant defense capacity relies on different Vitamins and antioxidants of both exogenous and endogenous origin among which polyphenols [4,5].

The retina is the central nervous system (CNS) tissue displaying the highest oxygen (O_2) consumption per gram of tissue [6], especially the outer retina adapted to darkness, particularly sensitive to damage by ROI. In fact, the rod OS membranes display an elevated content of polyunsaturated fatty acids (PUFA) [2,7]. In particular, photoreceptors consume about 4 times more O_2 than the other neurons of the CNS, thereby comprising the retina [8]. Consistently, rod OS may be responsible for the high O_2 consumption of the outer retina [6], as suggested by experimental evidence from biochemical proteomic and imaging analyses [9–12]. The ectopic expression of active of F_1F_o-ATP synthase (ATP synthase), as well as electron transfer chain (ETC) in the rod disks points to the occurrence of an aerobic metabolism, devoted to providing ATP for photo-transduction. Notably, irradiation of organotypic eye cultures (explants) with short wavelength blue light (BL) elicited a larger ROI production in the rod OS, than in the IS [7]. Exposure to BL for 6 hours impaired the ETC and uncoupled ATP synthase, likely due to OXPHOS overwork caused by prolonged irradiation, with membrane damage. In fact, a dysfunctional oxidative phosphorylation increases the level of ROI [1].

ROI are believed to play a role in the aging process, both in acute and chronic conditions [13–15][16][4-6] . Furthermore, oxidative stress plays a central role in the retinal degenerations [17]

[18] Retinal degenerative diseases include Age-related macular degeneration (AMD) [17] and diabetic retinopathy (DR) [19]. DR is a progressive complication during the course of type 1 or 2 diabetes and the leading cause of blindness in developed countries [6,20]. About 60% of individuals with type 2 diabetes have some degree of retinopathy and develop initial DR within 20 years of diagnosis. DR is a microvascular disease, characterized by progressive changes in the retinal microvasculature with accompanying damage of neurons and Muller glial cells, that are eventually lost by apoptosis [6]. The ischemic retina releases growth factors that lead to pathological angiogenesis, neovascularization resulting in edema and intraretinal hemorrhages. A number of interrelated pathways that are the target of hyperglycemia are involved in the pathobiology of diabetic complications; among the most actively studied you include oxidative stress, the polyol pathway and the advanced glycation products (AGE) [21,22].

The American diabetes Association (ADA) and the European Association for the Study of diabetes (EASD) recommend metformin (MTF) now used for more than 60 years, as a first-line therapy of type 2 diabetes mellitus in non-pregnant adults [23]. Its benefits include low-cost, minimum hypoglycemic risk, and a decrease in low-density lipoproteins (LDL) and glycated hemoglobin (HbA1c) levels. MTF improves insulin sensitivity in muscle and adipose tissue and suppresses hepatic gluconeogenesis mainly through activation of adenosine monophosphate-activated protein kinase (AMPK) [24]. AMPK regulates metabolism and lipid biosynthesis, inactivates acetyl-CoA carboxylase and induces the expression of muscle hexokinase [25]. However, the metformin mechanism of action involved in insulin-sensitizing effects and bioactive lipids is not completely understood. By AMPK, MTF regulates metabolism and lipid biosynthesis, inactivating acetyl-CoA carboxylase and inducing the expression of muscle hexokinase [24]. MTF treatment improves insulin sensitivity and dyslipidemia, regulating the expression of the genes associated with lipid metabolism [26] (also promoting hepatocyte and pancreatic β cell regeneration [27]. However, MTF suppresses liver glucose-6-phosphatase expression by AMPK-independent mechanism involving complex I inhibition [28]. In fact, another MTF mechanism of

action is ETC Complex I activity inhibition [29], recently suggested to be its main action also *in vivo* [30] [31][32]. Resveratrol, a stilbene natural compound, was shown to act as an inhibitor of ATP synthase, and was shown to be able to inhibit the ectopic ATP synthase of the rod OS[33].

Here we present *in vitro* data on bovine rod OS homogenates showing, for the first time, a dependence of the rod OS aerobic metabolism and ROI production on absorbed light at varying intensities. We also report an increase in surface protein oxidation in the rod OS under saturating ambient light and show that both metformin and resveratrol can diminish the ROI production and the oxidative phosphorylation rates.

MATERIAL AND METHODS

Sample preparations

Extraction of retinas

Retinas were extracted as previously described [34]. Briefly, the eye semicup, including the retina, from freshly enucleated bovine eyes (from a local certified slaughterhouse) after vitreous and lens removal, were incubated for 10 min with Mammalian Ringer (MR, 0.157 M NaCl, 5 mM KCl, 7 mM Na_2HPO_4, 8 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$ pH 6.9 plus protease inhibitor cocktail (Sigma-Aldrich, S. Louis, MO, USA) and 50 μg/ml Ampicillin). Each retina was then cut free of the optic nerve with scissors and collected.

Purified bovine rod OS preparations

Purified bovine rod OS were prepared under dim red light at 4 °C from 14 retinas, by sucrose/Ficoll continuous gradient centrifugation [12] [35]in the presence of protease inhibitor cocktail (Sigma–Aldrich, S. Louis, MO) and ampicillin (100 μg/ml). Rod OS preparation were characterized for integrity of plasma membrane as reported[35]. Rod OS homogenates were

obtained by Potter-Elveheim homogenization on ice in 1:1 (w/v) hypotonic medium (5 mM Tris-HCl, pH 7.4 plus protease inhibitor cocktail and 100 µg/ml ampicillin).

Osmotically intact rod OS disk preparations

Osmotically intact disks were prepared by Ficoll flotation [36] from purified rod OS. After letting the rod OS burst for 3 h in 5% Ficoll solution with 70µg/ml leupeptin, and 100µg/ml ampicillin, at 4°C 2 ml of distilled water were layered onto Ficoll and sample centrifuged for 2 h at 25,000 rpm in a Beckman FW-27 rotor (100.000 x g). Disks were collected in the distilled water phase, under sterile conditions. Both purification procedures were carried out in the absence of Cyclosporin A and 2-Aminoethoxydiphenyl borate. These are inhibitors of the mitochondrial permeability transition pore (MTP)[37,38]. Such conditions therefore would promote the MTP formation in contaminant mitochondria, if any, so that these would not be functional.

Transmission Electron Microscopy

The front half of bovine eyes were excised and the vitreous humor and lens removed. The eye cups were then filled with fixative consisting of 4% paraformaldehyde and 0.1% glutaraldehyde in PBS buffer solution. After fixation (1,5 hr), retinas were removed from the eye capsule, cut into small pieces, washed overnight with 50 mM NH4Cl, dehydrated and embedded in LR White Resin [39] and polymerized at 58°C. Ultrathin sections were placed on Formvar-coated nickel grids and used the next day for immunogold labeling.

For immunostaining of sections, the postembedding immunogold method was applied. Sections were treated with blocking solution (1%BSA, 0.1% Tween 20, PBS 1X), then incubated with mouse monoclonal anti-rhodopsin (1:100) (Sigma Aldrich, St.Louis, MO, USA) and rabbit polyclonal anti-ND1 subunit of ETC I Antibody (Ab) (diluted 1:50) (Abcam, Cambridge, UK) overnight at 4°C. Ab binding was detected using secondary anti-rabbit IgG (British BioCell International) (diluted 1:100) coupled to gold particles (25) nm, and goat anti-mouse IgG (British BioCell International) (diluted 1:100) coupled to gold particles (5 nm). Sections were analyzed at a

FEI Tecnai G12 transmission electron microscope operating at 100KV. In negative controls the preimmune serum was applied to the sections instead of the specific primary Ab. Images were acquired with OSIS Veleta cameras, collected and typeset in Corel Draw X4. Controls were performed by omitting primary Ab, which resulted in absence of crossreactivity (data not shown).

Light exposure conditions for cytofluorimetric and ATP synthesis assay.

For cytofluorimetric measurements and ATP production assays, rod OS were subjected to the following illumination conditions: i) dim light; ii) saturating ambient light exposure for 5 minutes; iii) saturating ambient light exposure for 1 hour under a lens filter (lens filters, belonging to category 3 defined by NBR15111 Maximum value of spectral transmittance is 1 tV Maximum value of solar UVA transmittance (315-380 nm) is 0,5 tV Visible Light Transmission (VLT) is from 8% to 18%.) (according to: ABNT. Protec¸a˜o pessoal dos olhos – Oculos de sol e filtros de protec¸a˜o contra raios solares para uso geral. NBR15111/2004. Associaca˜o Brasileira de Normas Te´cnicas ,2004), to mimic human sunglass wearing conditions; iv) saturating ambient light exposure for 1 hour. For oxygen consumption assay rod OS were treated with ambient-sunlight exposure for 1 hour or to dim light.

ROI production assay by cytofluorimetric measurements

Cytofluorimetric measurements of ROI production in the rod OS was performed using dihydrorhodamine 123 (DHR) probe (Molecular Probes, Life Technologies). Changes in ROI production can be evaluated. Rod OS (40 µg) treated as above specified were washed in phosphate-buffered saline (PBS) and resuspended in 50 ml HEPES buffer (10 mM HEPES, 135 mM NaCl, 5mM $CaCl_2$) and stained for 20 minutes with DHR (2.5 mg/mL) and kept at room temperature, washed with PBS and re-suspended in a total volume of 400 µl of PBS. Flow cytometry was performed in a CyAn ADP cytometer (Beckman Coulter) equipped with three laser lamps. The plot of all physical parameters (forward scatter (FSC) versus side scatter (SSC) is used to set the gate

that limits debris and aggregates. When necessary, isolated rod OS were preincubated with 30 µM Resveratrol (RV) for 15 minutes or 5 mM MTF for 2 hours

Spectrophotometric assay of rod OS Complex I activity

Complex I (NADH-ubiquinone oxidoreductase) activity was measured on 50µg of protein following the reduction of ferricyanide at 420nm [40]. A molar absorption coefficient of 1 (mM−1 cm−1) at 420 nm was used. Measurements were conducted on a double beam spectrophotometer (UNICAM UV2, Analytical S.n.c.).

Surface protein oxidation assay

Rod outer segment disks (100 µg) were washed in 20 mM Phosphate, 150 mM Nacl, 5 mM EDTA at 6,8 pH was exposed or not at the light for 10 min. Then samples were labeled with EZ-link Maleimide PEG_2-biotin (Thermo Scientific) overnight at 4°C in a dark box. Oxidative positive control was done treating the sample with 5 mM H_2O_2 and then labelling with biotin probe. Excess of probe was removed from the samples by micro-dialysis using 100-500 Da membrane (SpectrumLab). Finally the samples were transferred to nitrocellulose membrane using a Dot-Blot apparatus (Bio-Rad, Hercules, CA). Membranes were saturated in 5% BSA, rise and incubated with 1:10000 neutravidin HRP conjgate (Thermo Scientific) in 1% w/v BSA, PBS and Tween 0.05% v/v. Chemiluminescence was used for detection and image was acquired with the ChemiDoc Touch Imaging System (Bio-Rad). Quantitative densitometry was performed using Image Lab software (Bio-Rad).

ATP synthesis assay in rod OS

The formation of ATP from ADP and inorganic phosphate (Pi) was assayed in purified rod OS (0.04 mg protein/ml) treated as above specified according to Mangiullo et al. [41] and as reported previously (Calzia et al., 2013). Rod OS were incubated for 5 min at 37 °C in 50 mM Tris/HCl (pH 7.4), 5 mM KCl, 1 mM EGTA, 5 mM $MgCl_2$, 0.6 mM ouabain, 0.25 mM

di(adenosine)-5-penta-phosphate (Ap5A, adenylate kinase inhibitor), and 25µg/ml ampicillin. ATP synthesis was induced by addition of: 5 mM KH_2PO_4, 10 mM glucose and 0.1 mM ADP at the same pH of the mixture. After stopping the reaction with 7% perchloric acid final concentration, and neutralizing samples with K_2CO_3 the ATP concentration was measured in a luminometer (Lumi-Scint, Bioscan) by the luciferin/luciferase chemiluminescent method. Calibration was done with ATP standard solutions (Roche Diagnostics Corp., Indianapolis, IN) between 10^{-9} and 10^{-7}M. When necessary, isolated rod OS were preincubated with 30 µM RV for 15 minutes or 5 mM MTF for 2 hours.

Oxygraphic measurements

O_2 consumption by rod OS (0.04 mg protein/1.7 ml) was measured at 23 °C in a closed chamber, using a thermostatically controlled oxygraph equipped with an amperometric electrode (Unisense–Microrespiration, Unisense A/S, Denmark) under continuous electromagnetic stirring (Calzia et al., 2013). A specific software (MicOx, Unisense) was used to convert data in Excel files. Rod OS were previously diluted 3:1 in ultrapure water, then added to the mixture by means of a Hamilton syringe, which granted a partial disruption of rod OS, allowing substrates to permeate. Incubation medium was: 50 mM HEPES pH 7.3, 100 mM KCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 25 µg/ml ampicillin, and 0.3 mM di(adenosine-5′) penta-phosphate to inhibit adenylate kinase (Aicardi and Solaini, 1982). Respiring substrate was 5 mM glucose. When necessary, rod OS were preincubated with 5 mM MTF for 2 hours

Data analysis

Results are expressed as mean ± SD and n refers to the number of assays in any particular condition. In ATP synthesis, statistical analysis was performed using Student's t-test to compare data obtained using different light conditions and ATP synthase inhibitors (resveratrol and Metformin). Results were considered significant if $P < 0.05$.

Materials

Ampicillin, Ap5A, metformin, and resveratrol were all supplied by Sigma-Aldrich (St. Louis, MO, USA

RESULTS

The complex of our previous biochemical and imaging data suggests that an ATP synthesis and O_2 consumption sensitive to classical mitochondrial inhibitors, and to polyphenols as inhibitors of F_oF_1-ATP synthase, are detectable in rod OS, which are devoid of mitochondria. The rod OS may be regarded as a “specialized kind of mitochondrion", displaying active Electron transport Chain (ETC) complexes and a transmembrane electrochemical proton gradient [9].

A TEM characterization of a bovine retina confirms that ETC complex I is an integral part of the disk membranes. Figure 1 showing immunogold colocalization experiment on bovine retina with antibodies (Ab) against Rh (5 nm diameter gold particles) and ND1 subunit of complex I (25 nm diameter gold particles) reports that ND1 is expressed not only in mitochondria, as expected, but also in rod OS. Panel A shows an over view of a labelled bovine retina, while Panel B and C are magnifications of a mitochondria and a portion of a rod OS respectively, included in Panel A. Colocalization of Rh and ND1 is more visible in Panel D and its inset, that are a magnification of a portion of a rod OS included in the black squares in Panel C and Panel D, respectively.

The ROI production in a suspension of rod OS homogenized homogenates (subjected to shearing to avoid large aggregates) in the presence of various substrates was studied by flow cytometry, in the presence of a dihydrorhodamine 123 (DHR). This probe can detect the presence of ROI, in particular H_2O_2 and superoxide [42]. In saturating ambient light a significant production of ROI was detected in rod OS, which increased in parallel with the time of sample incubation with

respiring substrates (Glucose or NADH + succinate) and even in their absence (Figure 2). On the other hand, in dim light, the best condition to activate rod signaling, ROI production was negligible in the presence of the same substrates. Moreover, pre-treating rod OS with Resveratrol (RV) for 15 min in the same experimental conditions, a significant decrease in intracellular ROI production was observed especially under light conditions. Notably, in dim light DHR signal was similar in untreated and treated samples.

Considering that a main action of Metformin (MTF) has been reported to be the inhibition of Complex I of the ETC [32], we tested the effect of MTF also on extramitochondrial ETC Complex I activity in rod OS. Complex I activity in rod OS accounted for about 1,51 ± 0.2 IU/mg of total protein (µmol of reduced ferricyanide/min/mg). Upon preincubation with MTF for 2 hours Complex I activity decreased to 0.77±0.08 IU/mg of total protein with an inhibition of about 50%, consistently with its inhibitory actin on Complex I [32]. Moreover we studied the effect of an incubation with MTF (5 mM) on of ROI production and ATP synthesis in the rod OS, both in dim and ambient light varying intensity using a lens filter (see Material and methods section) and time of exposure (Figure 3). In the presence of glucose, at intermediate light intensity after 1 hour of saturating ambient light exposure under lens filters (Figure 3) (see Materials and Method section), ROI production was slightly lower (although not as low as in dim light) than after exposure to saturating ambient light for the same period of time. On the other hand, interestingly, after 5 minutes of saturating ambient light exposure, ROI production was similar to that in dim light and therefore consistently lower than after 1 hour of saturating ambient light. Moreover, rod OS preincubated with MTF produced significantly less ROI in all conditions tested, with respect to untreated samples (Figure3). In saturating ambient light, preincubation with N-acetyl-L-cysteine (NAC), an antioxidant acting as a precursor to glutathione restoring GSH levels, inhibited ROI production by 37% .

Considering that the ETC is embedded into the rod OS disk membranes, to further test the actual site of oxidative stress, purified disks were assayed for the presence of oxidized proteins by the EZ-

linked Maleimide PEG_2-biotin assay, that labeled reduced thiols (sulfhydryl groups,—SH) of the disk surface protein. Densitometric analysis showed that the signal of samples exposed to ambient light in the same experimental conditions was significantly lower than that of disks in dim light. Disks treated with H_2O_2, used as positive control, showed a negligible signal corresponding to a maximal oxidation state of the sample (Figure 4).

In the presence of glucose as a respiring substrate, the ATP synthesis in the rod OS was assayed varying light intensity and time of exposure (see Materials and Method section) in the presence or absence of MTF. Results are shown in Figure 5. It can be noted that the difference in rod OS ATP synthesis after 1 hour of saturating ambient light exposure with respect to dim light was higher than after 1 hour of saturating ambient light exposure under lens filter. On the other hand, interestingly after 5 minutes of light exposure, ATP production was already similar to that after 1 hour of saturating light exposure. The presence of MTF depressed ATP synthesis by about 70% in saturating ambient light, 46% in dim light and about 50% after 5 minutes of saturating ambient light exposure or under lens filters, suggesting that this probably depends considerably on the activity of Complex I.

O_2 consumption assay can follow the whole process resulting from the transfer of electrons to O_2 by the ETC with the consumption of the proton gradient with aerobic ATP synthesis from added ADP. In this case the difference between the conditions of light and dark was more pronounced. Figure. 6 Panel A, shows a representative amperometric graph of the O_2 consumption in coupled conditions by the rod OS homogenates subjected to saturating ambient light for 1 hour, O_2 consumption increases in the presence of substrates (glucose 5 mM) and then by addition of ADP (0.3 mM). Panel B shows a representative experiment conducted under the same conditions of Panel A, but in dim light. O_2 consumption was reduced, as a result of the lower energy requirement.

Panel C and d are oximetric traces of O_2 consumption in the rod OS pre-treated for 2 hours with of MTF under saturating ambient light and dim light respectively. The effect of MTF, which limits the ROI production modulating the ATP synthase, shows a little decrease in O_2 consumption more

evident under light condition than in dim light, as showed in Table 1 reporting the mean O_2 consumption in rod OS treated or not with MTF under saturating light or dim light.

By subjecting the discs to saturating light (Panel C) an uncoupling was observed, probably due to an oxidative damage of the disc membrane by prolonged exposure light which dissipates the proton gradient

DISCUSSION

The main result of the present work is the finding of a correlation among light absorption, aerobic metabolism and oxidative stress in the rod OS, with a significant impact on the current understanding of the photoreceptor rod OS physiology. This is, to the best of our knowledge, the first study able to show that the photo-transduction functioning in the rod OS, consequent to light absorption at varying intensities, requires an increase in ATP production and O_2 consumption and can elicit an increase in ROI production, with respect to dim light conditions. The rod OS metabolism would in fact be oxidative, as suggested by data on ATP synthesis and O_2 consumption, and the ectopic functional expression of ND1 (Figure 1), consistently to previous proteomic and biochemical data [43,44]. On the other hand, the ectopic expression of the ETC and F_oF_1-ATP synthase would not be exclusive of the rod OS (reviewed in [45][46]). Notably, it is undeniable that the ETC, especially Complex I, is a main source of ROI production [47].

It was reported that the expected increase in glycolytic flux in the rod OS (currently believed to contribute exclusively to ATP supply therein) upon light absorption, could not be observed [48]. Maximum glycolitytc production in the light was 84 uM of ATP/s, lower than the energy cost of the visual process, estimated to range around 127 uM ATP/s [48,49]. On the other hand, ATP diffusion through the cylium seems unlikely [50], when considering that only the distal disks are active in phototransduction, being devoid of cholesterol [43].

The unexpected consistent ROI production observed in the rod OS *in vitro* in function of the time and intensity of light exposure and also proportional to the length of exposure to the respiring substrates, i.e. the physiological rod OS functioning conditions. Lowering light intensity (i.e. for 1 hour under a lens filter) ROI production and ATP synthesis decrease respect to those after saturating ambient light exposure for 1 hour, while interestingly after 5 minutes of saturating ambient light exposure ATP production was already similar to that after 1 hour of light exposure, ROI production is similar to that in dim light and so consistently lower than after 1 hour of saturating ambient light. This may mean that to support the energy demand of phototrasduction

saturating ambient light induces ATP synthesis increasing respect to dim light by 5 minutes of exposure but this time is not enough to elicit oxidative stress production. This suggests that the ROI production inside rod OS is not an instantaneous, but cumulative phenomenon, due to the increasing commitment of physiologically active oxidative phosphorylation in the rod disk membranes, especially detectable in *in vitro* conditions that limit the ability of the cell to scavenge ROI. Results are very interesting, in that they show that the ROI production more than the ATP synthesis is the most sensitive index of involvement of OXPHOS in the light-induced photo-transduction.

In vitro, in the absence of the dioptric means of the eye, light may overstimulate the OS causing phototransduction and ETC overwork, with increased ROI production. Moreover, homogenization dilutes the rod OS cytosol, rendering antioxidant systems less efficient.

Vision, OXPHOS and ROI appear to be inextricably intertwined: light increases cGMP turnover, and hence ATP demand, conceivably sustained by the ectopic OXPHOS, main producer of ROI. It was shown that irradiation of mouse eye explants with blue light (405 nm) (a model of AMD) for 6 hours, causes a considerable retinal oxidative stress more intense in the OS than in the IS [7,51]. In those extreme illumination conditions there was a dramatic late impairment of ATP production and O_2 consumption, likely as the result of disk membrane peroxidation [52][7] , causing cytochrome *c* to escape the disk, activating the apoptosome [53]. It is known that sustained illumination causes irreversible damage, and photoreceptor apoptosis [54].

Data imply that the Retinal pigmented epithelium (RPE) mitochondria, currently believed to be the main source of ROI for the rod OS, may not play this role, being instead the rod OS the main ROI producer. The rod OS would house an important potential source of ROI, being therefore prone to oxidative stress as its membranes are enriched with polyunsaturated fatty acids. Thus, the rod OS *in vitro* appear a good model of light damage, useful to understand the molecular basis of the generation of oxidative stress inside the rod, common denominator of retinopathies [6,12] [55]. Retinal diseases have oxidative stress as an etiopathogenetic moment [56,57]. It has been demonstrated that the oxidative stress is among the risk factors contributing to the pathogenesis of

retinopathy of prematurity (ROP), and also the degenerative retinal dystrophies. Incidence of ROP, a major cause of blindness especially in premature infants, exposed to conditions of systemic oxidative stress, having poorly developed antioxidant systems [58]. The main contributing factors in the pathogenesis of ROP would be hypoxia, hyperoxia and reperfusion damage [59].

Degenerative retinopathies are expected to constitute a serious medical problem in the next future, with significant economic implications [17,60]. The human retinal dystrophies are currently irreversible. Therapies are focused on delaying the onset or progression [61]. One approach is aimed at preventing cell death, for example by administering neurotrophic compounds, among which antioxidants, to prolong the viability of retinal cells [62]. In this context, the effectiveness of resveratrol, a polyphenol present in many natural compounds, in scavenging ROI production in the rod OS homogenates is promising and consistent with its protective action in many animal models of retinal damage [10] . In fact, slowing down the degenerative process by modulating the ROI production inside the rod OS may be crucial in all stages of the disease.

Increased oxidative stress is one of the common pathogenic factors of diabetic retinopathy (DR) one of the main diabetic disease complications. In particular hyperglycemia is a cause of overproduction of superoxide anion, although mechanisms by which hyperglycemia increases oxidative stress are not fully understood [63]. In addition, recent studies have identified abnormalities in diabetic patients in the retinal neurons before the marks of microvascular changes. DR Patients usually show reduced responses in multifocal electroretinography, decreased sensitivity to blue-green color, and contrast, before microvascular injury, suggesting that the damage to photoreceptors is the “primum movens”. Here we have tested the action of metformin (MTF) the first-line therapy for type 2 diabetes. The best known mechanism of MTF action, is activation of AMPK [24,26,27]. However, besides its hypoglycemic and metabolic effects recently MTF inhibitory action on mitochondrial complex I has received attention and has also been indicated as its main action [31] [32].

MTF intracellular transport is mediated by isoforms of the organic cation transporters (OCT), but within the cytosol, the ETC would be the primary objective of MTF [64]. Notably, data show that MTF is able to inhibit ectopic Complex I in rod OS and significantly diminish the production of ROI levels in the rod OS, as a probable consequence of its inhibitory action on Complex I [32]. MTF would produce a decrease in NADH oxidation, proton pumping, and consumption of O_2, with reduction of ATP synthesis and hypometabolism. This is consistent with the reports that MTF produces a hepatic depletion of ATP, also reducing the lipogenesis. It appears that the use of MTF in patients suffering from type 2 DM, and thus likely also from DR, may reduce the of cumulative oxidative risk damage.

Conclusions

The increase in energy demand for phototransduction in varying ambient light conditions and the consequent overfunctioning of ETC would causes increased oxidative stress rates, representing a major risk factor for retinopathies, both inflammatory and degenerative such as ROPDR and AMD, respectively.

Moreover although the human retina also contains cones, not studied here, the rods are vital, as the dystrophies always start with the loss of the rods, which irreversibly causes the death of cones. In fact, rods produce a cone survival factor, called rod-derived cone viability factor (RdCVF), which was identified as a truncated thioredoxin-like protein encoded by the nucleoredoxin-like-1 gene (*NXNL1*) [65]. Rod-driven cone death, triggered by the loss of RdCVF expression, appears to be the first phase of the disease [66] . In fact, rod survival is a crucial point in rod-cone dystrophies, such as AMD and retinitis pigmentosa (RP) where first loss of ability to see in dim light conditions due to degeneration of rod photoreceptors was observed and then central vision was compromised caused by a loss of cone function with a potentially complete blindness. RdCVF injection in animal models of RP prevented the shortening of cone outer segments which precedes cone loss [67].

A prevention of cone secondary loss by intravitreal administration of RdCVF is one of the latest therapies bearing a nice clinical rational, even though many patients have already lost most of the rods when consulting an ophthalmologist. Unfortunately, visual acuity is subjectively reduced when more than 50 % of cones become non-functional, a late phase [68].. Therefore again it appears of vital importance to be able on one hand to "take care of the rods"[69] with accurate screening of occurrence of hemeralopia and, on the other hand, to implement preventative strategies allowing to modulate the ROI production in the rod. It is tempting to presume that also antioxidants may be administered intravitreally.

However, any approach of this kind can only be accomplished when the awareness of the oxidative risk that exists in the retinal rod OS due to the presence of an oxidative metabolism supported by the expression of ETC and ATP synthase, unknown to date, will have been achieved.

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FIGURE LEGENDS

Figure 1. Transmission Electron Microscopy (TEM) on bovine retina.

Panel A. Bovine retina double-labelled with antibodies anti-Rh (small, 5 nm width gold particles) and anti-ND1 (large, 25 nm width gold particles). IS: inner segment; m: mitochondrion; OS: rod outer segment. Panel B: enlarged area of Panel A to show a detail of a mitochondrion in rod inner segment (IS) labelled with anti-ND1 (arrow). Panel C: enlarged area of Panel A showing the rod OS, characterized by co-localization of Rh signal and ND1 signal (arrows). Panel D: enlarged area of Panel C to show a magnification of the rod OS, expressing both rhodamine (arrowheads) and ND1 (arrows). Inset in Panel D: detail of a large gold particle (indicating the presence of ND1) close to two smaller ones (indicating the presence of Rh; arrowheads).

Figure 2. ROI production in rod OS in light or dim light.

Kinetic of the increase in ROI production in rod OS incubated in presence of respiring substrates: NADH (0.29mM) +succinate (20mM) (Panel A) or glucose (20mM) (Panel B) under ambient light (1 h) or dim light. ROI production was measured following oxidation of the dye rhodamine 123. The reaction kinetic was also followed for both reaction mixtures in presence of ATP synthase inhibitor Resveratrol (RV) .

Figure 3. Effect of Metformin (MTF) on ROI production in rod OS in different light conditions Kinetic of the increase in ROI production in rod OS under ambient light (1 hour or 5 minutes), dim light, ambient light under lens filter (1 h) in presence of glucose (20mM). ROI production was measured following oxidation of the dye rhodamine 123. The reaction kinetic was also followed for both reaction mixtures in presence of MTF .

Figure 4. Oxidation of disks surface proteins by EZ-link Maleimide PEG$_2$-biotin labeling.

Rod OS disks (100 □g) exposed to dim light or saturating ambient light or treated with 5mM H_2O_2 (as positive control) were labeled with EZ-link Maleimide PEG_2-biotin o.n. at 4°C in dark box and transferred on nitrocellulose. Protein oxidation was revealed with neutravidin HRP conjugate.

Figure 5.. ATP synthesis in rod OS under dim light or different conditions of ambient light exposure

ATP synthesis was tested in the presence or absence of MTF under ambient light (1 hour or 5 minutes), dim light, ambient light under lens filter (1 h) .Histogram shows ATP formation in rod OS (40□g) over 1 min at 37°C in the presence of glucose (20 mM) as substrate. Addition of MTF inhibited ATP production in all conditions. Data shown are means ± SD n = 4. *P < 0.01, paired Student's t-test.

Figure 6. Oxygen consumption in rod OS under saturating ambient light or dim light. Representative amperometric tracing of O_2 consumption in coupled conditions in purifed rod OS homogenates (0.04 mg total protein) irradiated with ambient light for 1 h (Panel **A**); or in dim light (Panel **B**); ambient light plus MTF (Panel C); dim light plus MTF (Panel D) Additions: Glucose (5 mM); ADP (0.3 mM), in 1.7 ml volume.

Table 1

Table 2 Oxygen consumption by rod OS under saturating ambient light or dim light

Table reports the coupled respiration rates in rod OS (0.05 mg total protein) under saturating ambient light (light) or dim light in the presence or absence of Metformin (MTF). To stimulate O2 consumption Glucose (5mM) was added. Data are the mean ± SD of four experiments.

	Light		Dim light	
	Glucose	ADP	Glucose	ADP
OS	172.72±25.91	195.42±29.54	117.64±15.29	153.68±22.85
OS+MTF	91.8±11.93	111.52±15.61	63.24±8.85	106.76±13.78

Figure 1

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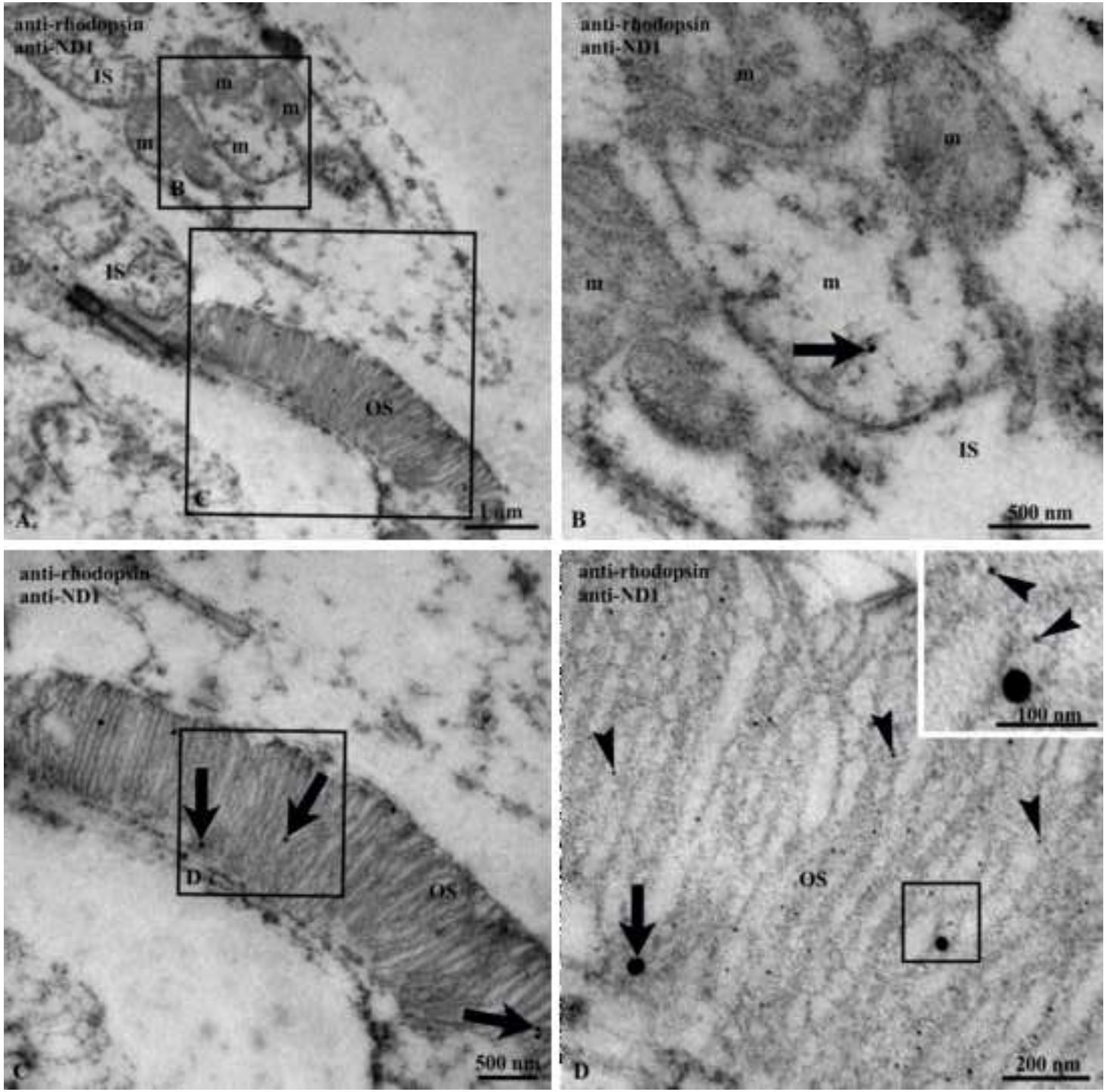

Figure 2

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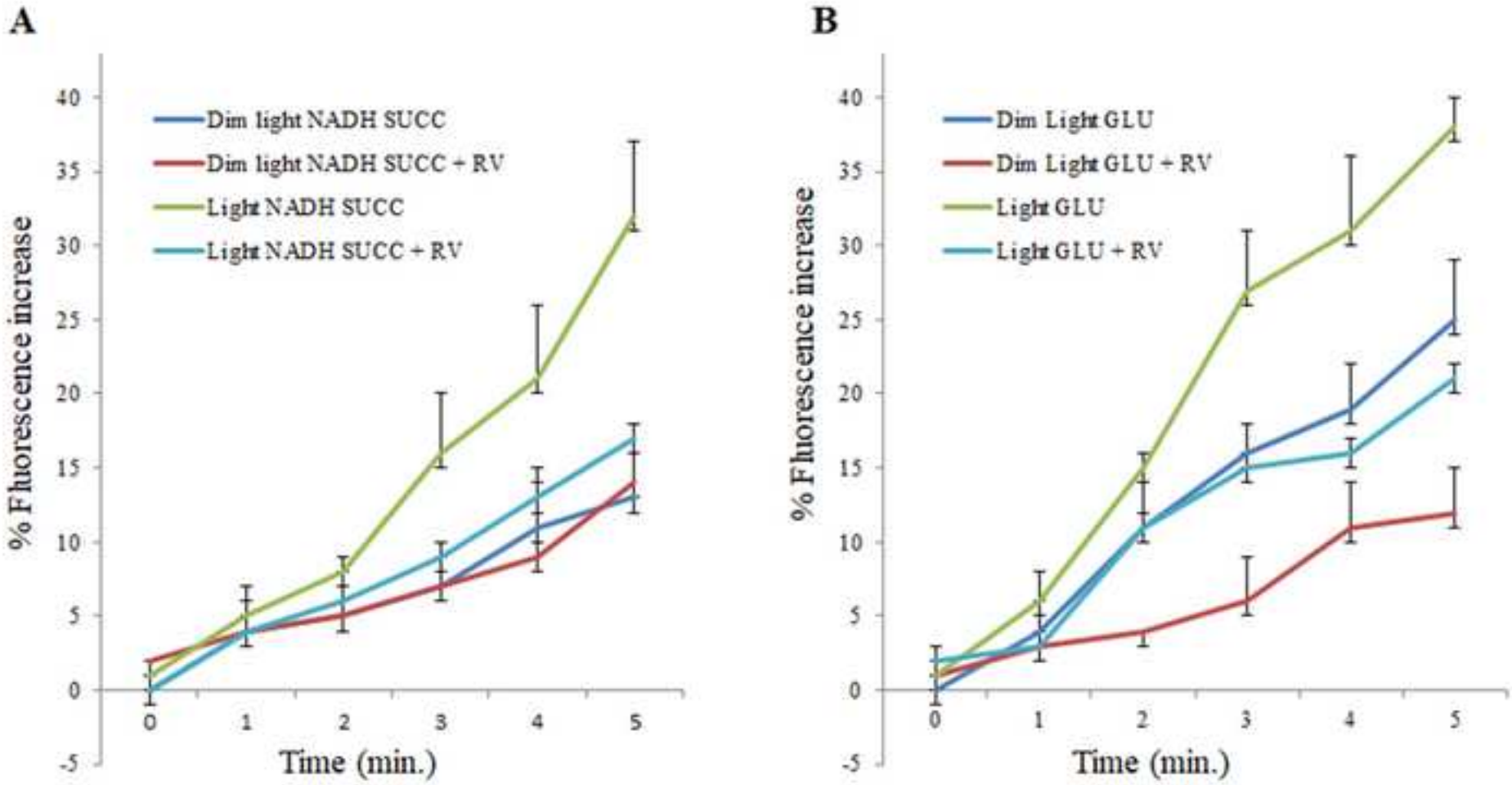

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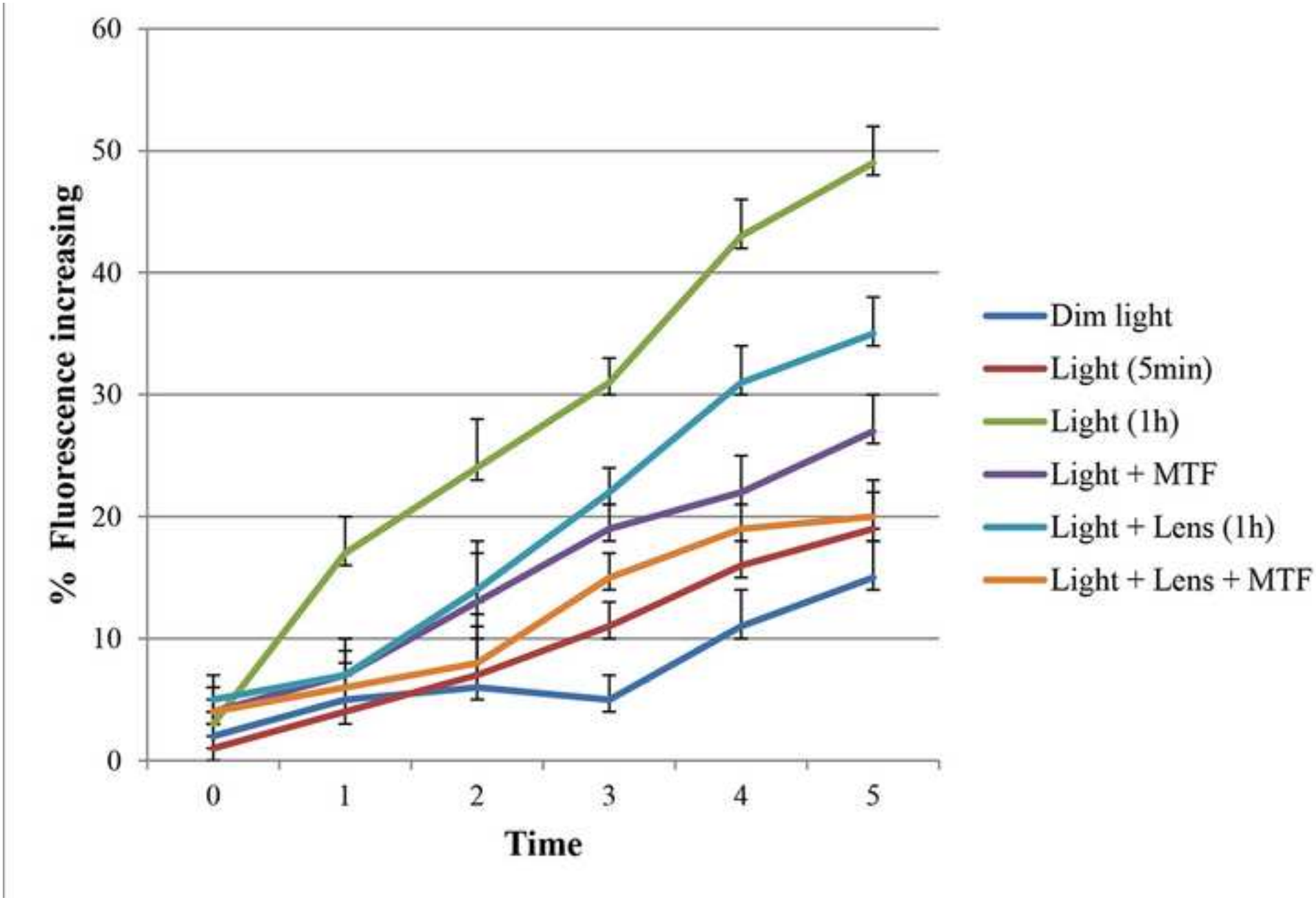

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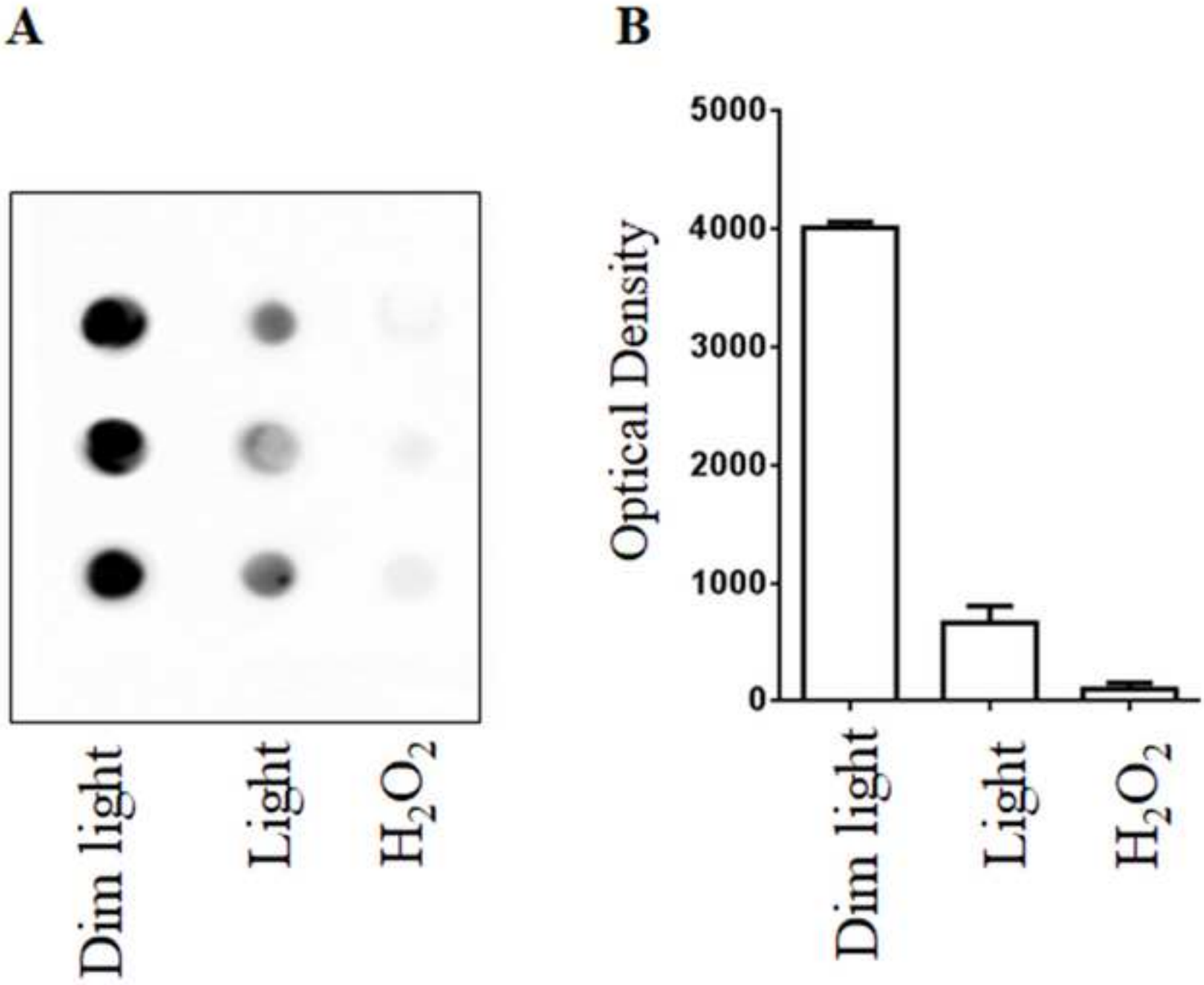

Figure 5

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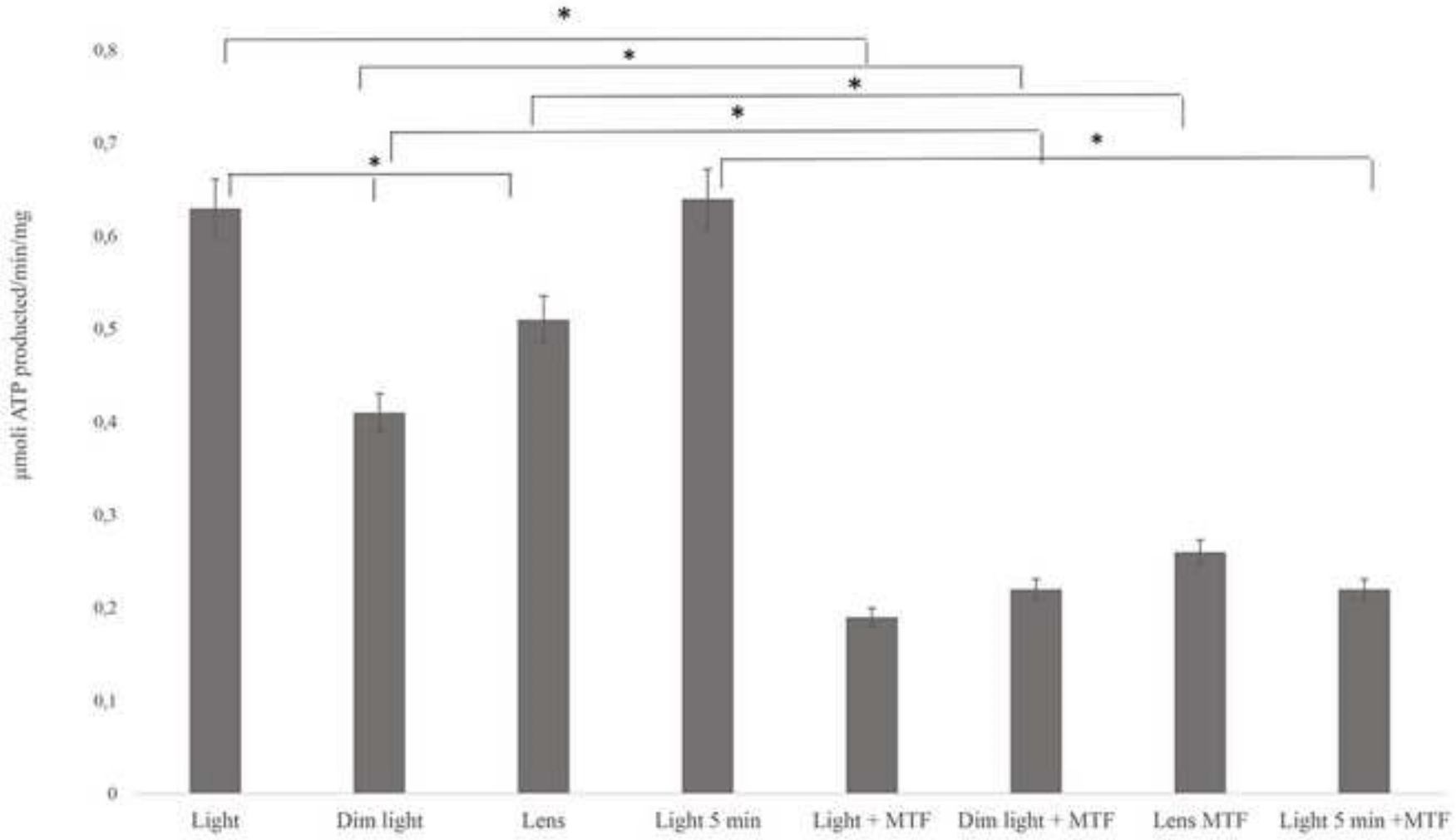

Figure 6

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