Biphasic dose responses in low level light therapy

Michael R Hamblin PhD

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Disclosures

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Outline

- Introduction to mechanisms of LLLT
- Survey of biphasic dose responses in LLLT
- Mechanistic studies in mouse embryonic fibroblasts
- NF-κB activation in HEK293 cells
- HeLa cells and neurons
- Conclusions
What’s in a name?

Low level laser therapy
Low reactive-level laser therapy
Low intensity laser therapy
Low level light therapy
Low energy laser irradiation
Photobiomodulation
Photobiostimulation
Biomodulation
Biostimulation
Cold laser
Soft laser
Laser therapy
Phototherapy

It is called “LOW” because a little light is better than a lot of light

Biphasic dose response?
Mechanisms of LLLT

- Red near infrared light
- Growth factor production
- Extracellular matrix deposition
- Cell proliferation
- Cell motility

\[ \text{NO} \rightarrow \text{cAMP} \rightarrow \text{mitochondrion} \rightarrow \text{Jun/Fos} \rightarrow \text{Gene transcription} \]

\[ \text{ATP} \rightarrow \text{ROS} \rightarrow \text{NF-kB} \rightarrow \text{nucleus} \]
POWER GAMES

There's a fight going on inside all our cells for each breath of air. **Nick Lane** sheds therapeutic light on the implications for cancer and degenerative diseases.

"The finding that the body could poison one of its own enzymes was initially shrugged off as an imperfection."

Yet over the past decade, researchers have come to appreciate that cells often use CO, and to an even greater extent NO (nitric oxide), to block respiration. Not only that, but light has striking counter-effects on cytochrome oxidase. And all these suitors to the enzyme turn out to be critical to our understanding not just

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**Nitric Oxide and the Control of Firefly Flashing**

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The results reported here document an important role for NO in firefly flash control. It is well established that O₂ availability is the immediate biochemical trigger for light production, and we propose that the role of NO is to transiently inhibit mitochondrial respiration in photocyes and thereby increase O₂ levels in the peroxisomes. This is consistent with the distinctive spatial arrangement of NOS-containing cells, the known NO-mediated inhibition of cytochrome c oxidase (21–23), and the fact that firefly luminescence can be induced by cytochrome c oxidase inhibitors, such as cyanide and carbon monoxide.
Mitochondrial respiratory chain

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Cytochrome c oxidase can act as a photoreceptor allowing the photolytic dissociation of any bound nitric oxide.


The nitric oxide present in the heme-CuIIa3 center of CytC ox can be photolysed by visible light.


Near infrared light protects cardiomyocytes from hypoxia and reoxygenation injury by a nitric oxide dependent mechanism.
Zhang et al. Journal of Molecular and Cellular Cardiology 46 (2009) 4–14
Basic mechanisms

Mitochondria are primary photoreceptors
Cytochrome c oxidase activity is increased
NO is dissociated from COX + heme proteins
ATP and cAMP increased
Reactive oxygen species are produced
Transcription factor induction
Growth, repair, survival, less inflammation
Biphasic dose response?
How is dose measured?

Power (W) x Time (sec) = Energy (Joules)

\[
\frac{\text{Power (mW)}}{\text{Beam Area (cm}^2\text{)}} = \text{(irradiance)} \times \text{time} = \text{fluence (J/cm}^2\text{)}
\]

Arguments have been made for total energy, fluence, irradiance and illumination time to be most important parameters in measuring dose.
- Dose response curve


*Arndt-Schulz curve*
Stimulation/Inhibition of Wound Healing in Mice

635-nm Laser for wound healing in mice


Low-Level Light Stimulates Excisional Wound Healing in Mice

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Wavelength response (action spectrum?)

Biphasic dose response may be different at each wavelength
Wound healing in vitro  980-nm laser - scratch in fibroblast monolayer

Mark D. Skopin & Scott C. Molitor
Effects of near-infrared laser exposure in a cellular model of wound healing
Photodermatol Photoimmunol Photomed, 25, 75-80.(2009).

Constant time - irradiance and fluence vary
810-nm laser for arthritis in rats

<table>
<thead>
<tr>
<th>Irradiance (mW/cm²)</th>
<th>3 J/cm²</th>
<th>30 J/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mW/cm²</td>
<td>10 minutes</td>
<td>100 minutes</td>
</tr>
<tr>
<td>50 mW/cm²</td>
<td>1 minute</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

Hypothesis: length of illumination is more important than total fluence or irradiance in LLLT effect


Low-Level Laser Therapy for Zymosan-Induced Arthritis in Rats: Importance of Illumination Time

Ana P. Castano, MD, Tianhong Dai, PhD, Ilya Yaroslavsky, PhD, Richard Cohen, MD

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HeLa DNA synthesis
633nm
0.1J/cm²
10 - 1000mW/cm²

- Dose response curve

Motility changes over time with different irradiation times using 104 x LED Cluster mixed 660nm & 850nm 45mW/cm²
Reduction of infarct size (heart attack) by low-energy laser

Oron U, et al

*Michael R Hamblin, PhD, Wellman Center for Photomedicine. Dose Response Conference 2009*
• Dose response curve

![Dose response curve diagram]

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- Dose response curve

![Dose response curve diagram](image)

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A 3D model for Low Level Laser / LED therapy
Biostimulation and Bioinhibition
(a dose sweet spot)

Stimulation

Inhibition

Anders Island

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Mitochondrial ROS induces transcription factor NF-κB
NF-kB target genes

**Anti-apoptotic**
c-IAP1, c-IAP2, survivin, Bcl-2, Bcl-xL, Bcl-xS, Bfl-1/A1, XIAP, c-FLIP, E2F3A, NR13, IEX-1, GADD45β, TRAF1, TRAF2

**Anti-oxidant**
Mn-SOD, heme oxygenase 1, Glutathione peroxidase, Ferritin heavy chain, NQO1, γ-glutamylcysteine synthetase

**Pro-inflammatory**
iNOS, TNF-α, COX2, LTA, LTB, phospholipase A,

**Pro-proliferation**
MCSF, GCSF, GMCSF, c-myc, VEGF-C, PDGFβ, BMP2, c-myb, cyclin (D1, E)

**Adhesion molecules**
E-selectin, ICAM1, VCAM1, ELAM, MADCAM1

**Adaptive immunity**
MHC-I, MHC-II, IgG κ light chain, IGHG3, CD3γ, CD105, TAP1, CD69,

**Cytokines & chemokines**
IL1, IL2, IL6, IL2-R, IL8, IL9, IL11, GRO, IP10, MIP1, MCP, RANTES, eotaxin

**Acute phase response**
C reactive protein, serum amyloid A, angiotensin, tissue factor, MMPs, complement (B, C4)

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Use of luciferase and bioluminescence

Luciferase is a generic name for enzymes commonly used in nature for bioluminescence.

Firefly luciferase (EC 1.13.12.7) from the firefly Photinus pyralis.

Light is produced by the oxidation of luciferin substrate consuming adenosine triphosphate (ATP) and catalyzed by luciferase

\[
\text{luciferin + ATP} \rightarrow \text{luciferyl adenylate + Ppi}
\]

\[
\text{luciferyl adenylate + O}_2 \rightarrow \text{oxyluciferin + AMP + light}
\]
Review

Molecular imaging of the transcription factor NF-κB, a primary regulator of stress response

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Received 3 October 2003; received in revised form 23 February 2004; accepted 23 February 2004

NF-κB response element on Ig-k light chain promoter drives luciferase

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Does LLLT activate NF-kB?

1. Establish a fibroblast cell line (3T3 protocol) from NF-kB luciferase reporter mice (HLL)
2. Deliver different fluences of 810-nm light from a laser (or other light source)
3. Keep illumination time constant at 5 min (vary irradiance)
4. After various times assay for luciferase expression (NF-kB activation) and cellular ATP
810-nm laser
1. Effects on ATP production
0.3 J/cm² 810-nm delivered over 5 min

Increase in ATP after 0.3 J/cm²

ATP Time C

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Fluence effect (dose-response) of ATP increase (measured at 5 min post laser)
Azide/deoxyglucose mimics hypoxia
0.003, 0.03, 0.3, 3 and 30 J/cm²

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2. Time course of NF-κB activation
Fluence response of NF-kB activation
What is mechanism of NF-κB activation?

Hypothesis is reactive oxygen species
DCFDA
Sensitive to lipid hydroperoxides
Quantification of ROS induced by laser
MEF ROS Time Course
MEF ROS Time course

• 1. Irradiate MEF with 810nm Laser
• 2. At each time point, 2x washing with PBS and add the DCFDA probe.
• 3. Incubate the MEF with the probe for 1 hour.
• 4. Microscope Imaging
Results

1. 810 nm laser promoted ROS production.

2. Loading the Probe 30 minutes after the irradiation showed the highest fluorescence.
Ascorbic Acid abrogating induced ROS Production?

- 1. Apply 100 µM Ascorbic Acid into the medium and pre-incubated for 30 minutes.
- 2. Irradiate the cells. Since the time course showed the best fluorescent results when loading the probe 30 min after irradiation, cells were incubated for 30 minutes before loading the probe.
- 3. 2x washing with PBS and incubate cells in the loading buffer for 1 hour.
- 4. microscopy imaging
Results

• 1. Ascorbic Acid decreased the DCFDA fluorescence in both control and the laser irradiated cells.
MitoSOX Red Assay
Laser induced superoxide production in the mitochondria.

• 1. Irradiate cells
• 2. 2x washing with PBS and incubated cells in the loading buffer for 1 hour before imaging
• 3. Apply Antimycin A as a positive control
Mitochondrial Superoxide Production
By
MitoSOX Red
Real Time Fluorescence of Mitochondrial Superoxide by MitoSOX Red
Results

• Fluorescence in the first minute did not show significant difference from the control.

• Fluorescent difference started to appear from the third minutes

• This test serves an additional evidence supporting mitochondrial superoxide was actually produced by 0.3 J/cm$^2$ laser during the irradiation.
Do mitochondrial inhibitors increase NF-kB activation?
Do antioxidants prevent NF-kB activation?
What is effect of mitochondrial inhibitors and antioxidants on ATP?
Do antioxidants abrogate laser induced ATP?
**NFκB activation by Lasers**

<table>
<thead>
<tr>
<th>Control</th>
<th>Laser 0.3j/cm²</th>
<th>NAC + Laser 0.3j/cm²</th>
<th>Positive Control</th>
<th>Antimycin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>5min</td>
<td>15min</td>
<td>30min</td>
<td>5min</td>
<td>15min</td>
</tr>
</tbody>
</table>

Positive Control: Cells (Primary MEFs) treated with TNFα for 15min

**Phospho IKK α/β**

**Phospho NFκB p65**

**Total NFκB p65**

**Conclusions:** Laser (810nm 0.3j/cm²) can activate NFκB.

**Note:** NAC did not neutralize this expression completely suggesting either;
1. incomplete inhibition of ROS
2. alternative (GF) mediated activation of NFκB.
Human Fibroblasts

ATP Time Course

ATP Synthesis (RLU/μg)

0 5 min 60 min 240 min

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810 LED induced Nitrate Production in HeLa

![Graph showing nitrate production over time for different light intensities.](attachment:image.png)
Experiments with HEK293 cells

• Why HEK293?
  – Lack of any Toll-like receptors by nature
  – Easy to grow and transfect (transiently and stably)
• Stably transfected TLR9 and NF-kB Luciferase reporter genes into HEK293. (by Marc Lamphier at Eisai.)
• Transiently transfected NF-kB Luciferase reporter
• Irradiated by 810nm LED, 10mW/cm² 5 min.
980-nm CW laser  810-nm and 970-nm LED array
810 Laser induced NF-κB inhibition in HEK293

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Light effect on TLR9 signaling

CpG and light added at same time

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Laser pre-illumination abrogates effects of TNF-alpha 24 h later

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Light effect on TLR9 Signaling

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ATP change by 810nm 3J/cm² Light

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ROS in Murine Embryonic Neurons
DCDHF-DA

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MitoSox Red

before 0' 30' 60' 120' H 2O2

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Conclusions I

1. 810-nm laser increases cellular ATP
2. 810-nm laser activates NF-kB
3. 810-nm produced mitochondrial ROS
4. Mitochondrial inhibitors and (H$_2$O$_2$) activate NF-kB without increasing ATP
5. Mitochondrial inhibitors produce ROS
6. Antioxidants abrogate laser and mitochondrial inhibitor-induced NF-kB activation but have no effect on ATP
7. Preliminary evidence of NO production 15 and 30 min post-light
8. Different cell types (HEK293 cells and neurons) may behave differently
Mechanisms of LLLT

Red near infrared light

NO

NF-kB

Gene transcription

ATP

cAMP

mitochondrion

Jun/Fos

growth factor production
extracellular matrix deposition
cell proliferation

ROS

IkB

molecule

cAMP

nucleus

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What is the mechanism for biphasic dose response?

ROS have been shown many times to stimulate at low doses but to be harmful at high doses.

NO (and peroxynitrite) may also have biphasic response: stimulate at low dose and inhibit at high dose.

Protective transcription factors may be induced at low dose (NF-kB) and additional different harmful pathways activated at high dose.
Future work

Test other cell types: neurons, leukocytes, epithelial cells

Test other wavelengths and non-coherent light sources

Investigate other redox sensitive transcription factors (AP1)

Repeat experiments (bioluminescence imaging, effect of antioxidants) in vivo

Study mouse model of traumatic brain injury
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