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Review

Laser Mediated Production of Reactive Oxygen and Nitrogen Species; Implications for Therapy

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Laser therapy has gained wide acceptance applications to many medical disciplines. The side effect-effects from laser therapy involve the potential for interaction with cellular and extracellular matrix molecules to generate reactive oxygen species and reactive nitrogen species which in turn can initiate lipid peroxidation, protein damage or DNA modification. These issues are addressed in this short overview in the context of experimental models of laser-induced thrombosis.

Keywords: Free radicals; Laser therapy; Thrombosis; Low-intensity laser; Lipid peroxidation; DNA damage; Mutation

Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; NO-Hb, nitrosyl complex of hemoglobin (NO-Hb); DDT, dithiotreitol; GSH, glutathione; SSB, DNA single-strand breaks; DSB, DNA double-stranded breaks; MDA, malondialdehyde; LEI, laser-induced electron-impact ionization; IR-MALDI, infrared matrix-assisted laser desorption/ionization; PAHs, polyaromatic hydrocarbons; CO, cytochrome oxidase; LILR, low intensity laser radiation

INTRODUCTION

Laser therapy is increasingly used for biomedical treatments which involve skin,[1,2] diabetes,[3] cornea[4] and surgery.[5,6] However, the possible damaging effects of laser irradiation are still highly contested and the possible mechanisms of action remain unclear. Previous reports support the hypothesis that one mechanism for damaging effects involves the reaction of light with hemoglobin, resulting in oxygen radical production. Moreover, a free radical-based mechanistic hypothesis focused on the stimulation of low-intensive laser irradiation, such as that used for therapy of a variety of inflammatory diseases has been proposed.[7] The implications for laser-dependent oxidant production are discussed in this review.

Laser-induced Free Radicals are Potentially Damaging

The fundamental role played by free radicals in a wide range of pathologies has been widely reported and is generally accepted.[8–10] Laser-induced free radicals have been demonstrated in association with low level laser irradiation involving the He–Ne laser,[11,12] diode laser[13] infrared laser[14] and excimer laser.[15,16] In order to investigate the potential for cellular damage, *in vivo* experimental models of laser-induced thrombosis or platelet aggregation, tumoral disease, and vascular injury models were developed. In these cases lasers caused damage including formation of cutaneous pigmented cells,[17] induction of thrombosis,[18,19] platelet deposition,[20,21] cancer[12] and lipid peroxidation.[16,22,23] It has been suggested that such changes could be from reactive oxygen species/reactive nitrogen species (ROS/RNS) generated by the infiltrating polymorphonuclear cells at the site of tissue damage. However, it is important to recognize that light absorption *per se* induces the production of

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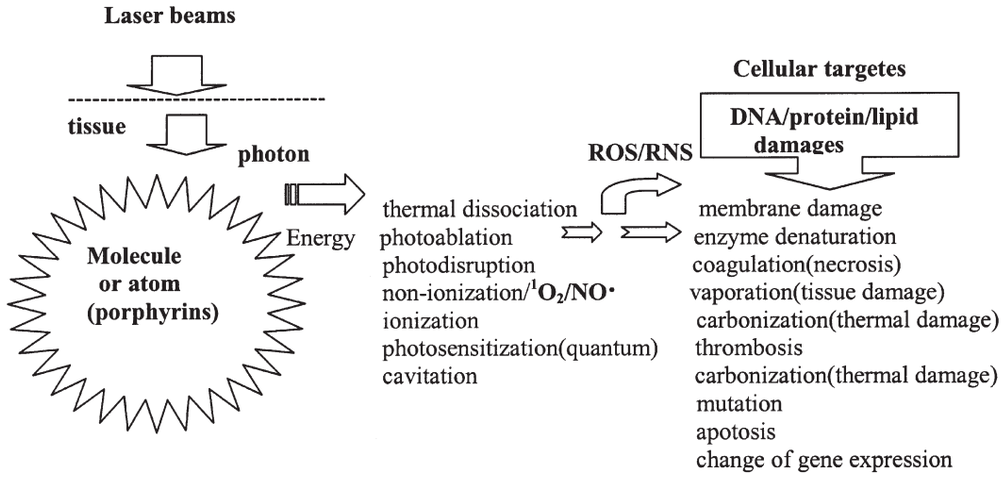

FIGURE 1 Interaction mechanism of laser light to biological tissue by potential ROS/RNS. Laser beams will generate the photons at different dose rates and energy transfer occurs depending on both frequency and energy charge. Within the cells, interaction with endogenous chromophores including porphyrins will be affected by cellular thermal dissociation, non-ionization/ROS/RNS systems etc. these events lead to modification of biomolecules and change in cell function.

initiating radicals that are involved in subsequent free radical reactions, such as lipid peroxidation.[7] Such effects were evaluated and assessed by measuring indices of lipid peroxidation,[24] by electron paramagnetic resonance spectroscopy[25,26] and by electron microscopy.[17,22] Therefore, despite the success of laser therapy in treatment against many diseases[27–29] there are still confounding issues concerning the choice of wavelength, fluence, pulse width, and dose rate.[30,31] Here, we discuss the possibility of generating ROS/RNS in response to laser treatment (Fig. 1).

Role and Toxicity of ROS and RNS

The overall biochemical properties of ROS/RNS have been discussed extensively in a number of excellent reviews.[32–34] Of growing biomedical interest are near ultraviolet radiation (UVA, 320–400 nm) and visible radiation (400–700 nm) as sources of ROS/RNS since this radiation can generate oxidative injury through photodynamic action with endo- or exogenous sensitizers and other molecular oxygen species.[35–37] Although irradiation of cells with selected visible light wavelengths at low doses promotes photobiostimulation[38] and thus is useful for clinical applications,[39] such therapy is highly controversial.[40] Therefore, Kohli *et al.*[41] suggested that the oxidative stress due to He–Ne laser irradiation-induced generation of singlet oxygen leads to sublethal damage of DNA in cells, which may trigger DNA repair processes. Recent report have suggested that laser damage can result from free radicals by He–Ne laser in treatment of cancer by He–Ne laser,[12] by excimer laser therapy in the cornea[16] and by Nd:YAG laser photodisruption.[42] Therefore, mechanisms for generation of free radicals from low-intensive lasers have been scrutinized,[7] and the available does rates studied.[11,12,22] However, it is not clear how laser irradiation causes cellular damage. We can assume that whenever the energy of a photon exceeds the energy needed to remove an electron from a molecule, a collision with that molecule might then lead to generation of free radicals; this would be feasible in the case of visible and even UV light or laser irradiation. Therefore, the initial cellular damage may be due to the local formation of hydroxyl or other reactive radicals that may then generate longer lived organic radicals including peroxyl or alkoxyl radicals. If this is the case, then DNA and cellular membranes may be particularly important targets.[10] Laser intensity and frequency are additional factors that can initiate bond breakage between molecules if low laser irradiation is exceeded. Generalized light emission, molar absorption coefficient values, and quantum yields of photodecomposition and peptide bond scission were determined for a number of aromatic and aliphatic depeptides under 93 nm laser irradiation in neutral agron-saturated aqueous solution.[43]

Nitric oxide complexes with metalloproteins and *S*-nitrosothiols are sensitive to light and may be targets for laser-dependent generation of ROS/RNS. For example, the nitrosyl hemoglobin complex is formed *in vivo* and may be degraded on exposure to laser irradiation to form free nitric oxide.[44] It was shown recently that the nitrosyl complex of hemoglobin (NO-Hb) is photochemically sensitive and hence may serve as a source of free NO

TABLE I Damaged effects of lasers without photosensitizers

Laser type	Parameter	Samples	Effects	References
Nd:YAG	1064 nm/10 mJ	Plasma	Hydroxyl radical formation	[50]
He–Ne	630 nm	*In vitro*	Membrane	[51]
	630.1 nm	Tumor/human	LP	[12]
	632.8 nm/3–5 J	Skin/mouse	LP/antioxidants	[22]
	632.8 nm	Cell membrane	LP/ion permeability	[7]
Diode	810 nm/0.15–0.3 J	Skin/Guinea pig	Melanosome damage	[17]
Argon-ion	488 nm	Thrombosis/mouse	Platelet formation	[38]
	150 mJ/5 Hz	Cornea/rabbit	LP	[16]
Argon	514.5 nm	Rat	Thrombosis formation	[52]
CO_2	10,600 nm	Pig/urethra, bladder	Tissue injury	[53]

under He–Cd laser irradiation (441 nm).[45] This is further supported by observations with photosensitized red-cell ghost using detection of luminescence of singlet oxygen inside of the cell membrane.[46] At low irradiation doses, it is possible that energy transfer from porphyrin which is known to be present in the cell produces singlet oxygen.[47,48]

It was further determined that imidapril, an inhibitor of nitric oxide synthase, delayed He–Ne laser-induced cerebral thrombosis and increased significantly the plasma concentration of nitric oxide metabolites.[19] With respect to oxidative stress induced by laser irradiation singlet oxygen is clearly an important ROS mediator since it is involved in many reactions with exogenous sensitizers and visible light.[35] Singlet oxygen is generated by ~22.5 kcal of energy transfer from a relatively long-lived sensitizer triplet to ground state oxygen. Photodynamic reactions may also give rise to reduced oxygen species such as lipid peroxyl radicals, $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}HO$ (Table I).[49]

Laser Therapy and Lipid Peroxidation

Several studies have suggested a link between laser irradiation, oxygen radicals and antioxidant enzymes in the animal models (Table I). Oxygen free radical damage in the rabbit cornea after argon fluoride laser ablation was associated with lipid peroxidation including a 198% increase in ketodiene.[16] In pathological and photochemical studies of laser injury to rabbit retina, high levels of malondialdehyde (MDA) were reported following exposure to a 0.49 W argon laser,[54] Although laser photocoagulation is beneficial in laser treatment, the mechanism remains unclear. It is possible that the treatment induces a sudden, temporary increase in free radical activity with increasing MDA levels, either by direct thermal damage or by oxygen reperfusion. These responses could explain the reported complications of photocoagulation which lead to direct or indirect damage to vascular endothelium, resulting in increased vascular permeability[55] Shimmura *et al.*[56] suggested that hydroxyl radicals may be partially responsible for stromal fibroblast cell apoptosis *in vitro* after excimer photoablation. Thus, evidence is mounting that the efficacy of laser therapy is at least partly related to the ability to initiate lipid peroxidation.[16,57–59] Belougne-Malfatti *et al.* have attempted to identify the protective effects of inhibitors against free radicals via the laser-induced free radical model or the laser-induced thrombosis model.[18] They found that intravenously injection of adrenaline or serotonin (1 ng/kg) stimulated arterial thrombosis formation, whereas injection of high dose of acetylcholine (5 mg/kg) slackened the thrombosis formation. Schulte-Frohlinde *et al.* concluded that photoionization of the uracil moiety in poly U leads eventually to the same chemical pathway for single-stranded breaks (SSB) formation as that induced by hydroxyl radicals. Furthermore, they proposed that protection by DTT and GSH occurs via H donation to the C-4′ radicals of the sugar moiety of DNA and to the C-4′ and the C-2′ radicals of poly U.[60]

Laser-induced DNA Damage and Mutagenic Effects

It is well known that all forms of ionizing radiation deposit energy stochastically along their paths.[61] The resulting distribution of energy deposited in a small target molecule, such as the DNA helix, leads to a corresponding spectrum in the severity of damage produced. Critical energies for SSB and double-stranded breaks (DSB) in DNA by low-energy photons can be determined. These energies can also provide estimates for the energy dependence of strand-break formation, which is important for track-structure simulations of DNA damage.[62] Modeling and calculations are presented as a first step towards mechanistic interpretation and prediction of radiation effects. Such modeling is based on the spectrum of initial DNA damage produced by low energy electrons (100 eV–4.5 keV); this can be compared with experimental information. Relative yields of single and clustered strand breaks are related to the complexity and source of damage

(e.g. direct energy deposition or reaction of $^{\bullet}$OH radicals), dependence on the activation probability of $^{\bullet}$OH radicals, and the amount of energy required to give a SSB.[63] Potential formation of breaks in DNA chains under conditions of visible laser radiation (532 nm) on DNA-dye-intercalator complexes has been predicted theoretically and verified experimentally.[64] This was assessed on Poly[A,G] at the guanine moiety by using acetone as a photosensitizer.[65] Without a photosensitizer, release of bases from (a) calf thymus DNA and (b) three polynucleotides induced by 20 ns excitation in aqueous solution (193 nm; pH 7) was also detected by HPLC.[66] Further analysis revealed that the quantum yields of free base (phi B) formation from double-stranded DNA (0.4 mM) was independent of intensity, indicating a one-quantum mechanism of *N*-glycosidic bond cleavage.[67] When human lymphocytes in culture were irradiated with laser pulses of an Xe–Cl excimer laser (308 nm) or a dye laser at wavelengths from 312 to 640 nm, the number of photons necessary for DNA damage increased about 10,000 times from 2.14×10^{8} photons at 308 nm to 2.85×10^{12} photons at 450 nm. Cao *et al.*[68] have measured the photon statistics of random lasers with resonant feedback. With an increase of the pump intensity, the photon numbers distribution in a single mode change. Conversely, it was reported that the yields of SSB per incident photon increased from 1.4×10^{-15} SSB per plasmid per photon/cm^2 at 7 eV to 7.5×10^{-14} SSB per plasmid per photon/cm^2 at 150 eV. Direct induction of DSB also increased from 3.4×10^{-17} DSB per plasmid per photon/cm^2 at 7 eV to 4.1×10^{-15} DSB per plasmid per photon/cm^2 at 150 eV.[62] These observations suggested that laser-induced damage was dependent on wavelength[69] and photon.[62] The damage to DNA is thought to result from the attack on DNA bases by $^{\bullet}$OH radicals generated by photoelectrons. Previously it was postulated that a key step in the mechanism of change could be an interstrand radical transfer, with single stand break formation caused by the direct effect of high-energy irradiation associated with laser-induced photoionization producing a radical cation.[70] Release of bases from both DNA and polynucleotides was detected by HPLC with the effect being markedly larger for pyrimidines than for purines.[71] Furthermore, DNA-dye-intercalated complexes enhanced the formation of DNA breaks because of induction of high-energy yield. However, it should be noted that the threshold electron energy for induction of single-strand breaks is <25 eV; for double-strand breaks the threshold is between 25 and 50 eV, similar to monoenergetic electrons with low energies.[62]

There has been a recent report of a laser-induced electron-impact ionization (LEI) signal in the mass spectra that yields information relative to the inorganic bulk constituents of a sample (e.g. compounds such as water, oxygen, nitrogen, and carbon dioxide).[72] Infrared matrix-assisted laser desorption/ionization (IR-MALDI) of the polyaromatic hydrocarbons (PAHs) anthracene, benzo[a]pyrene, and dibenz[a,h]anthracene was also performed using a CO_2 laser and a liquid matrix.[73]

Recently Plappert *et al.*[74] and Stocker *et al.*[75] suggested that laser pyrolysis products from porcine tissues induced very potent genotoxins and were therefore potentially hazardous. Pyrolysates liberated during vaporization of tissue induced DNA damage. In addition, such pyrolysis products also have mutagenic properties as determined by the Salmonella mutagenicity assay. A previous study also demonstrated that laser smoke condensates are mutagenic.[76] Pyrolysis may cause the generation of ROS that are formed by decomposition of water molecules due to high temperature cavitation. The formation of double-strand breaks in DNA may result from the mechanical effects of cavitation, while single-strand breaks may result primarily from the free radicals. It will be further supported that laser-induced cavitation was generated from Nd:YAG laser-driven flyer-plate technique to generate shock waves.[77] Although low laser irradiation is used in treating lesions, its high frequency will also produce ionization; thus this treatment may be a potential cause of biological damage.

Laser-induced Protein Damage

Subtoxic ROS and RNS production can lead to alteration in cellular and extracellular redox state, and it is such alterations that have been shown to alter redox cell signaling pathways. Particular attention has been paid to the importance of thiols and thiol-containing molecules in these processes.[78]

Cytotoxic singlet oxygen can be produced during photoactivation by deeply penetrating 670 nm light.[79] Its detection was performed by means of a spin trap, nitronyl nitroxyl radical.[45] He–Ne laser irradiation at fluence ~ 100 J/m^2 has been reported to lead to generation of singlet oxygen in Friend erythroleukemic cells. Therefore, it was suggested that endogenous porphyrins are involved in generation of singlet oxygen.[80] Retinal laser lesions on cytochrome oxidase (CO) activity are good example.[81] Previous report demonstrated that the phototoxicity of the porphyrin peptide fraction derived from haematoporphyrin derivative could be potent photosensitizer.[82] Protein modification may be caused from the quenching of singlet oxygen, which leads to oxidative destruction of the aromatic ring, correlates with amino acids such as tryptophan, tyrosine, histidine, methonine, cysteine and their derivatives.[83]

Laser-induced Thrombosis Model

Recently, we reported the possible induction of damage by low intensity laser radiation (LILR).[84] Schindl[85] emphasized the non-destructive ability of LILR's and questioned whether low intensity laser irradiation really caused cell damage.[86,87] Although LILR has many beneficial applications for disease treatment, the possible initiation of oxidative stress and free radical formation by LILR should be noted. Recently the laser-induced thrombosis model has been correlated with laser thermal angioplasty,[88] platelet and coagulation[89,90] and vascular injury.[21] This is thought to be due to inadequate safety precautions[91] and rapid removal of clots by using inhibitors.[19,92] In contrast, intracellular low-intensity laser therapy in rheumatoid arthritis had a negative effect on articular syndrome and inflammation.[93] Schindl *et al.* described positive effects in cancer[87] and diabetic-neuropathic foot ulcers.[86,94] It is possible that thrombosis growth may be a reflection of platelet recruitment in response to oxygen radicals generated on laser light exposure. This idea is further supported by investigation of free radical damage caused by laser irradiation.[12,16,22,95,96] The stress-limiting action of low-intensity laser irradiation was reported in-patients with ischemic heart disease and with breast cancer.[87,97,98]

PROSPECTS AND CONCLUSIONS

Despite the success of laser therapies against a wide range of diseases, these treatment options remains controversial. Although many regard laser treatments as beneficial and benign, laser-induced thrombosis models are well accepted. The summaries discussed here suggest that local platelet deposition should be restricted to the level of optimal laser energy, with the most appropriate method of laser thermal ablation.[20,99] As with free radical mechanisms of low-intensity laser radiation, controversy still exists about specific side effects associated with the chosen contrast intensity. An evaluation of these adverse side effects has been reviewed here, based on laser-induced ROS/RNS and laser-induced thrombosis models. This review indicates that laser therapy should be applied to diseased patients by carefully selecting both the general treatment and the specific laser protocols. Antioxidants should also be investigated for their potential to eliminate laser-induced free radical damages.[100,101] Additional investigation of these and related phenomena are required for further laser therapy refinement and application.

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