Effects of Low-Level Light Therapy on Hepatic Antioxidant Defense in Acute and Chronic Diabetic Rats

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ABSTRACT: Diabetes causes oxidative stress in the liver and other tissues prone to complications. Photobiomodulation by near infrared light (670 nm) has been shown to accelerate diabetic wound healing, improve recovery from oxidative injury in the kidney, and attenuate degeneration in retina and optic nerve. The present study tested the hypothesis that 670 nm photobiomodulation, a low-level light therapy, would attenuate oxidative stress and enhance the antioxidant protection system in the liver of a model of type I diabetes. Male Wistar rats were made diabetic with streptozotocin (50 mg/kg, ip) then exposed to 670 nm light (9 J/cm^2) once per day for 18 days (acute) or 14 weeks (chronic). Livers were harvested, flash frozen, and then assayed for markers of oxidative stress. Light treatment was ineffective as an antioxidant therapy in chronic diabetes, but light treatment for 18 days in acutely diabetic rats resulted in the normalization of hepatic glutathione reductase and superoxide dismutase activities and a significant increase in glutathione peroxidase and glutathione-S transferase activities. The results of this study suggest that 670 nm photobiomodulation may reduce, at least in part, acute hepatic oxidative stress by enhancing the antioxidant defense system in the diabetic rat model. © 2009 Wiley Periodicals, Inc. J Biochem Mol Toxicol 23:1–8, 2009; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10:1002/jbt.20257

KEYWORDS: **Oxidative stress; Hyperglycemia; Photobiomodulation; Liver; Antioxidant Enzymes**

INTRODUCTION

Recent years have seen an increased interest in using low-power near-infrared light or light-emitting diodes (LED) as a vehicle for inducing beneficial effects in biological systems. Photobiomodulation, a low-level light therapy (LLLT), has been shown to improve cell proliferation in vitro [1]. Previous biochemical and molecular studies have indicated that 670 nm light treatment affects the redox balance in cells, reduces oxidative stress and radical damage, may alter cell cycling and transcription factor activity or production [2–4] and promotes various cellular processes including collagen synthesis and mitochondrial metabolism [5]. In clinical settings, LLLT is employed in the reduction of pain and in the treatment of edema, eczema, dermatitis, and persistent ulcers in patients [6].

LLLT has particularly important ramifications for the treatment of diabetes mellitus, a growing epidemic in Western societies [7,8]. Insulin-dependent diabetes mellitus is a degenerative disease characterized by the persistent state of hyperglycemia, which promotes glucose metabolism and generates reactive oxygen species (ROS) in the tissues by activating multiple signaling pathways, such as mitochondrial oxidative respiration, glucose auto-oxidation, advanced glycation end-product (AGE) formation, polyol pathway, and protein kinase C (PKC) activation [9–11]. Oxidative stress has been implicated in the damage of organelle membranes within the cell, leading to a decline in cellular activity and overall loss of energy metabolism [12]. Oxidative stress affects various organs in diabetics. The principal complications of diabetes include coronary heart disease, cardiomyopathy, retinopathy, nephropathy, cerebro-vascular disorders, and neuropathy of the peripheral nervous system [13,14].

Antioxidant defense system in the cell includes vitamins and glutathione (GSH), as well as antioxidant

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enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S transferase (GST), and glutathione reductase (GRx). Because of their ability to scavenge for and attenuate the effects of ROS, these enzymes serve as biomarkers of oxidative stress.

In the present study, we investigated the effects of LLLT on oxidative stress in the streptozotocin-induced diabetic rat model as a possible alternative antioxidant therapy. Since light treatment may have beneficial clinical applications, we hypothesized that irradiation with 670 nm light would improve some of the effects of oxidative and energy stress in the liver. We analyzed various biochemical markers of oxidative and energy stress to determine the efficacy of LLLT in both acute and chronic diabetes.

METHODS

Animals

Adult male Wistar rats weighing 250–275 g (Harlan Sprague Dawley, Madison, WI), maintained under controlled temperature (21–23°C) and light (12:12 h light:dark, lights on at 07:00) conditions in social housing (two per shoebox cage), habituated to the laboratory environment and apparatus for a week after arrival. Throughout the experiment rats received ad libitum access to food and water, and the University of Wisconsin–Milwaukee Institutional Animal Care and Use Committee reviewed and approved all procedures. On day 3 of habituation blood glucose measurements from tail blood samples assessed euglycemia. Body weights on day 8 informed counterbalanced pseudorandom group assignment to control, streptozotocin (STZ)-diabetic, or light-treated STZ-diabetic (STZ + LED). Rats then received a single intraperitoneal injection of either 20 nM sodium citrate (control) buffer or 50 mg/kg STZ in 20 nM sodium citrate buffer at pH 4.5 (STZ and STZ + LED). Glucose levels three days after injection indexed hyperglycemia. A blood glucose level of ≥300 mg/dL was defined as hyperglycemia.

670 nm Photobiomodulation

Diabetic rats received photoirradiation treatment once per day over a period of 18 days (14 total treatments) for the acute study and, beginning three days after injection, Monday through Friday over a period of 98 days (70 total treatments) for the chronic study with 300 s of 670 nm light (Quantum Devices, Inc., Barneveld, WI) at a 60% power intensity of 50 mW/cm^2 (an energy density of 9 J/cm^2). Clear Plexiglas restraint tubes restricted movement, while STZ + LED rats received photoirradiation treatment; control and STZ rats received equal exposure to restraint tubes. Body weight and blood glucose levels were assessed twice weekly. Since previous research [2,15] indicated that normal controls were not affected by photoirradiation treatment, a separate control and light treatment group was not set up.

Tissue Harvest and Preparation

All rats received a euthanizing dose (50 mg/kg) of sodium pentobarbital at a rate of 8 rats per day, counterbalanced across groups. Livers were extracted, flash frozen, and stored at −80°C until assayed. Although tissues were not perfused in the acute experiment, experimental design for extrahepatic tissues required perfusion with phosphate buffered saline in the chronic experiment.

For antioxidant enzyme analyses, 300 mg of each liver was homogenized in 4.7 mL of 0.1 M sodium phosophate/5 mM EDTA buffer (pH 7.6) using a Polytron homogenizer (Glen Mills Inc., Clifton, NJ). The homogenates were centrifuged (1500×g, 10 min), and the supernatant was recentrifuged (100,000×g, 1 h) in a Beckman L7–55 centrifuge (Beckman Coulter Inc., Fullerton, CA). The second supernatant fraction (5% cytosol) was used to determine protein concentrations and enzyme activities. For GSH and GSSG contents, 100 mg of liver tissue was homogenized in a mixture of 0.1 M sodium phosphate/5 mM EDTA buffer (pH 8.0) and 25% HPO_3, and centrifuged at 100,000×g for 30 minutes. For ATP measurement, 50 mg of tissue was homogenized in 2.5% trichloroacetic acid, vortexed, and centrifuged at 10,000–12,000 rpm for 10 min at 4°C. The remaining pellet was resuspended with 1 N NaOH and assayed for protein using the Bradford protein assay kit (Bio-Rad, Hercules, CA). For cytochrome c oxidase (CCO) measurement, 300 mg of tissue was homogenized in 1.2 mL of ice cold 1.15% KCl and centrifuged at 1500×g for 10 min at 4°C. The supernatant was recentrifuged at 100,000×g for 1 h. The remaining pellet was resuspended with enzyme dilution buffer (10 mM Tris–HCl/250 mM sucrose, pH 7.0) and stored at −80°C until used. For lipid peroxidation (LPO) measurements, 100 mg of liver tissue was homogenized in 1.0 mL of cold 1.15% KCl, and fresh homogenates (10%) were used for the assay. Total protein was determined spectrophotometrically [16] using a bovine serum albumin standard.

Biochemical Assays

Antioxidant enzyme activity was measured spectrophotometrically and expressed as units/mg protein. GPx activity was determined by the method of Tappel [17]. One GPx activity unit was defined as the

amount of enzyme that transforms 1 μmol of NADPH to NADP in 1 min at 37°C. GRx activity was determined by the NADPH-dependent reduction of oxidized glutathione (GSSG) to GSH [18]. The oxidation of 1 nmol NADPH/min is defined as a unit of GRx activity. Cytosolic SOD activity was measured using xanthine and xanthine oxidase as a superoxide generator and cytochrome *c* as an indicator in the absence of SOD [19]. The 50% inhibition (IC_{50}) of cytochrome *c* in the presence of superoxide anion was defined as a unit of SOD activity. CAT activity was determined by measuring the absorption of hydrogen peroxide in the presence of catalase at 240 nm [20]. One unit of CAT activity was defined as the amount of enzyme that liberated half the peroxide oxygen from hydrogen peroxide solution (~0.03%) in 100 s at 25°C. GST activity was determined by the GST-catalyzed reaction between GSH and 1-chloro-2,4-dinitrobenzene (CDNB), which produced a dinitrophenyl thioether [21]. One unit of GST activity was defined as the amount of enzyme producing 1 mmol of CDNB–GSH conjugate/min. GSH content was determined by a fluorometric method in the presence of *o*-phthalaldehyde [22]. CCO activity was analyzed using the protocol of the Sigma cytochrome *c* oxidase assay kit (product no.: CYTOCOX1). One unit of CCO activity was defined to oxidize 1.0 μmol of ferrocytochrome *c* per minute at pH 7.0 at 25°C. ATP content was analyzed using a modification of the luminescence method of Strehler [23] and components of the Sigma bioluminescence somatic cell assay kit (Sigma Chemical, St. Louis, MO). The supernatant was neutralized with 1 M Tris base and mixed (1:1) with luciferase ATP assay mix (in dilution buffer) and measured with a luminometer. LPO level was determined spectrophotometrically by measuring the production of malondialdehyde, a thiobarbituric acid reactive substance [24].

Statistical Analysis

Statistical analyses were performed using the SAS system 9.13 (SAS Institute, Cary, NC). Data are expressed as mean ± SEM. Mean differences were obtained using one-way ANOVA (PROC GLM with Duncan's post hoc multiple comparisons) to compare STZ and STZ + LED treatment groups to controls. A p-value of <0.05 was considered statistically significant.

RESULTS

Acute Study

There was a significant increase in blood glucose levels in all diabetic rats compared to control rats (Table 1), typical of the study design, but no changes in body weights of diabetic rats over this short period. Livers of diabetic rats showed evidence of decreased GRx, SOD, and CAT activity compared to control rats (Figures 1 and 2). Light treatment restored GRx and SOD activity. CAT activity increased by 53% after light therapy, but the change was not significant due to high variability. There was a significant increase in the activity of GPx and GST in diabetic rats after light treatment (Figure 1). Neither the diabetic condition nor light treatment appeared to have an effect on GSH or GSSG levels (Figure 2). The levels of LPO in diabetic and light-treated animals showed no significant changes compared to controls (Figure 3). Light treatment increased the content of ATP by 12% and 22% as compared to control and diabetic groups, respectively, but the changes were not significant (Figure 3).

TABLE 1. Blood glucose and body weights of diabetic rats and light-treated diabetic rats compared to controls, analyzed with one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test (PROC GLM; n = 6-9 for acute and 8 for chronic). Data are expressed as mean ± S.E.M.

	Blood Glucose (mg/ml)		*Body Weight (g)*	
Treatment	*Acute*	*Chronic*	*Acute*	*Chronic*
Control	71.7 ± 1.7	64.5 ± 2.0	331.97 ± 5.02	508.4 ± 11.3
Diabetic	348.2 ± 7.5[a]	409.8 ± 29.9[a]	296.96 ± 3.58	304.6 ± 21.4[a]
Diabetic + LED	350.9 ± 14.4[a]	368.1 ± 14.2[a]	302.14 ± 8.01	307.8 ± 18.6[a]

[a] Significantly different from control group.

Chronic Study

A significant increase in serum glucose and a decrease in body weights were found in diabetic rats as compared to control rats (Table 1). Light treatment did not drastically alter any of the characteristic features of diabetes. Livers of diabetic rats showed evidence of decreased hepatic GST and CAT activities compared to control rats (Figure 1), which were not improved by light treatment. Neither light treatment nor the diabetic condition appeared to have an effect on other antioxidant enzymes (Figures 1 and 2). No marked changes of GSH, GSSG, and LPO levels were found in either light-treated or untreated diabetic groups (Figures 2 and 3). The increased activity of CCO when diabetic rats were treated with 670 nm LED (Figure 3) was not significant.

DISCUSSION

In the present study, we found that the activity of hepatic antioxidant enzymes was altered by acute

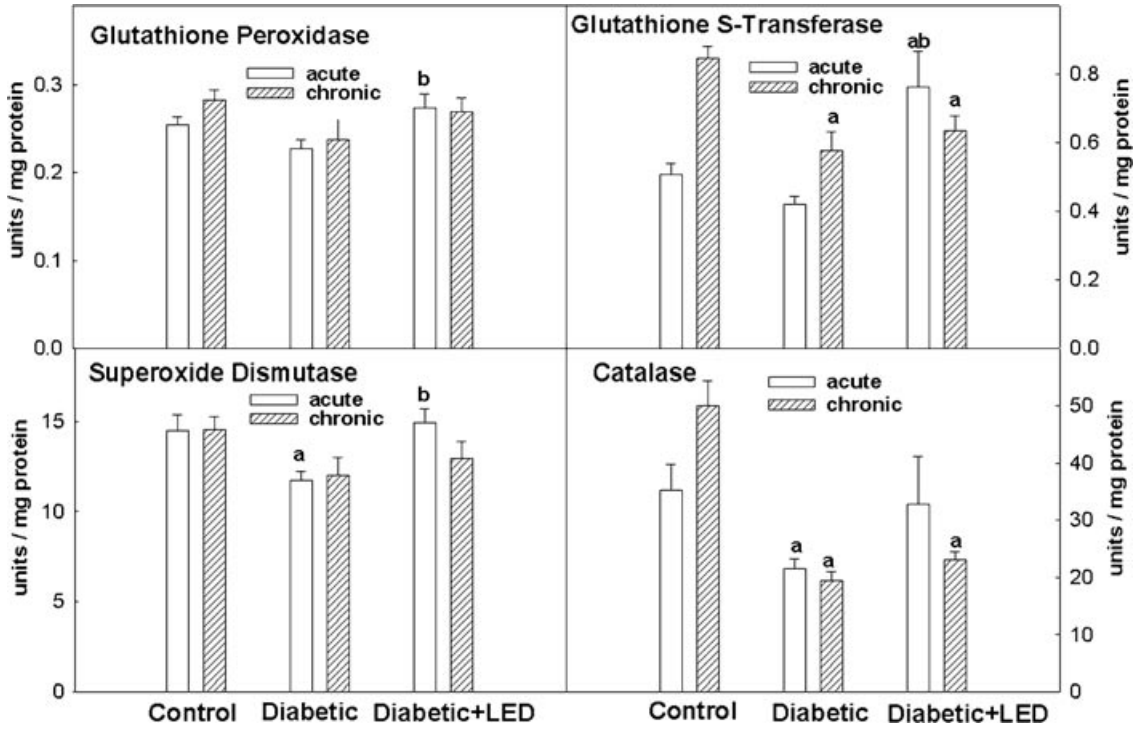

FIGURE 1. Activities of glutathione peroxidase, glutathione S-transferase, superoxide dismutase, and catalase in livers of control, diabetic and diabetic rats treated with low-level light during acute (18 days) or chronic (14 weeks) disease. [a]Significantly different from normal control at $p \leq 0.05$. [b]Significantly different from diabetic control at $p \leq 0.05$.

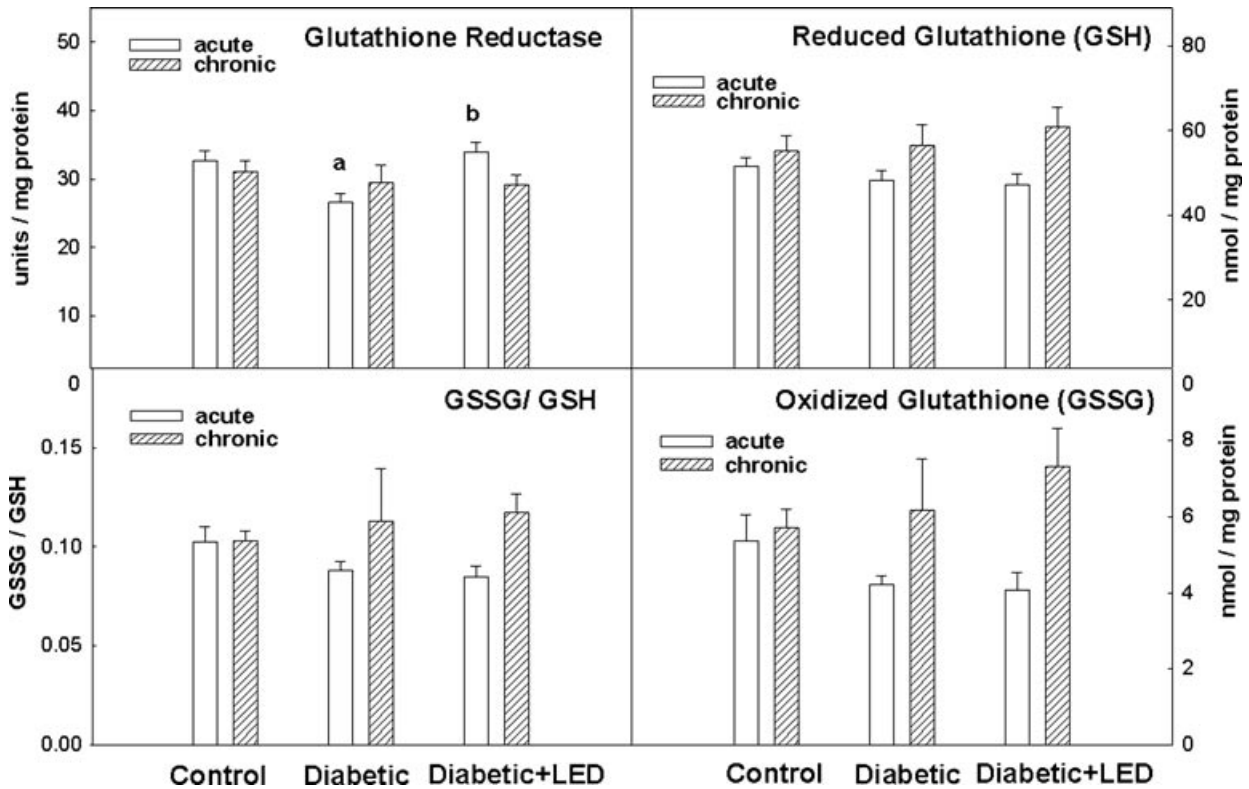

FIGURE 2. Activity of glutathione reductase and levels of reduced (GSH) and oxidized (GSSG) glutathione, as well as ratio of oxidized to reduced glutathione (GSSG/GSH) in livers of control, diabetic and diabetic rats treated with low-level light during acute (18 days) or chronic (14 weeks) disease. [a]Significantly different from normal control at $p \leq 0.05$. [b]Significantly different from diabetic control at $p \leq 0.05$.

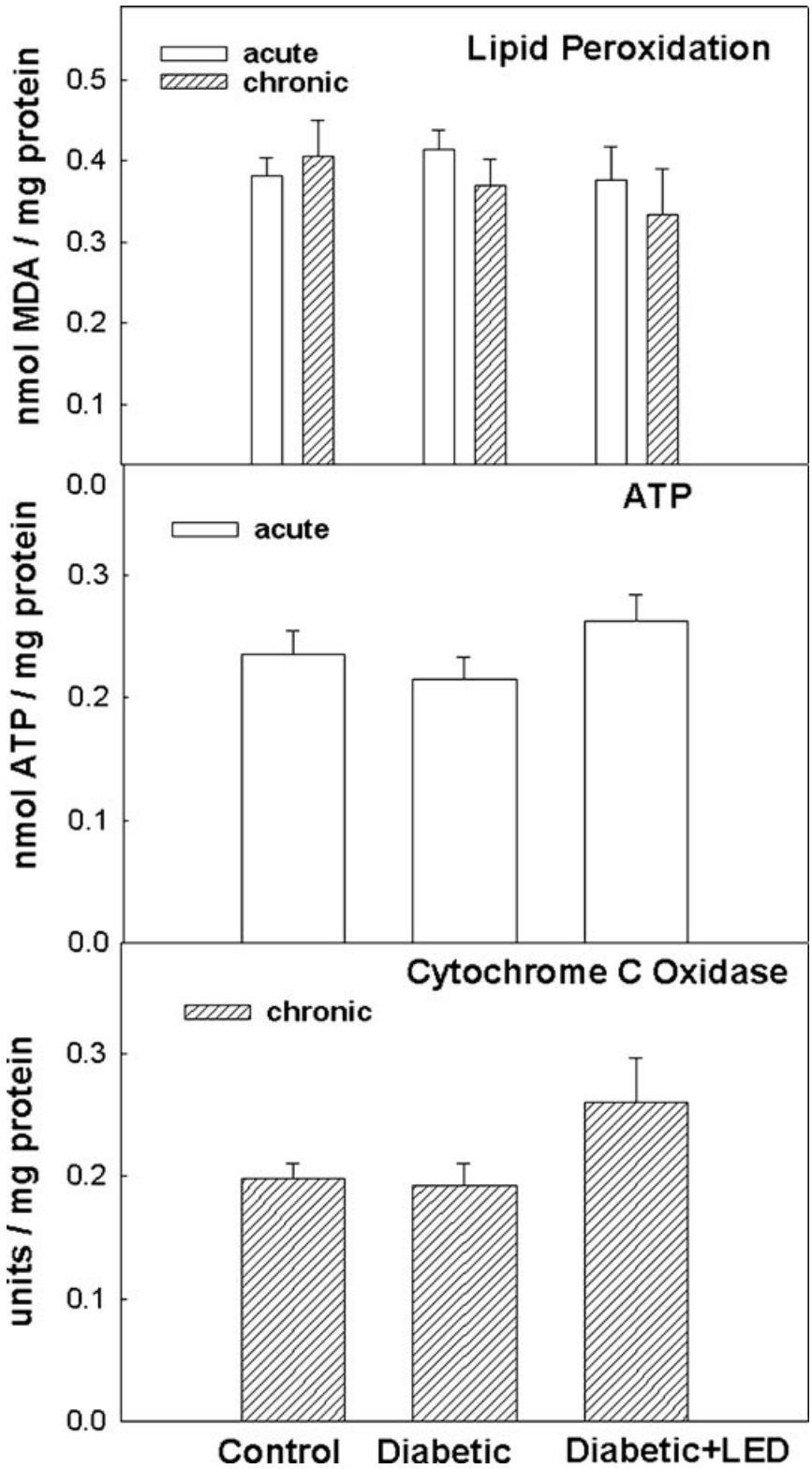

FIGURE 3. Level of malondialdehyde as a measure of lipid peroxidation and activities of ATP and cytochrome *c* oxidase in livers of control, diabetic and diabetic rats treated with low-level light during acute (18 days) or chronic (14 weeks) disease. *Significantly different from control at $p \leq 0.05$. [a]Significantly different from normal control at $p \leq 0.05$. [b]Significantly different from diabetic control at $p \leq 0.05$.

diabetes or LED light therapy while the hepatic levels of antioxidants and antioxidant enzyme activities in chronic diabetes were not changed by LLLT. We hypothesized that the levels of oxidative and energy stress would increase in untreated diabetics compared to normal controls and become normalized by the protective antioxidant pathways enhanced by low-level light treatment. The results from our study demonstrated that although 670 nm LED treatment increased cellular energy production (ATP), it did not significantly affect the state of oxidative and energy stress in livers of acute and chronic diabetic rats.

670 nm LED irradiation has been found to be an optimal absorption wavelength since it promotes mitochondrial energy metabolism, thereby increasing available cellular ATP and enhancing wound healing in endothelial and epithelial tissues [2,25,26]. LED treatment activates cytochrome *c* oxidase, the mitochondrial photoacceptor molecule that mediates photobiomodulation and elevates cellular ATP production [27,28], which may be one mechanism underlying the healing powers of photobiomodulation. Evidence from previous biochemical and molecular studies indicates that 670 nm LED treatment affects the redox balance in cells, reduces oxidative stress and radical damage, may alter cell cycling and transcription factor activity or production, and affects the production of proteins that control cell–cell and cell–extracellular matrix interactions [2–4].

Excessive cellular ROS are implicated in the development of diabetic complications. Concurrent activation of the multiple pathways such as mitochondrial oxidative respiration under a hyperglycemic condition, glucose auto-oxidation, AGE formation, polyol pathway, and PKC activation contribute to the elevated production of ROS in diabetes. In addition, ROS may also further activate the pathogenetic pathways involved in the production of ROS, resulting in exacerbation of diabetic complications by the enhanced ROS production cycles. Thus, inhibition of ROS production at the cellular level may be a key mechanism of beneficial light treatment in attenuation of diabetic complications. The results from cultured bovine endothelial cells showed that blockade of ROS formation by inhibitors of mitochondrial oxidative respiration normalizes mitochondrial ROS levels, preventing hyperglycemia-induced activation of PKC, AGE formation, sorbitol accumulation, and nuclear factor-kappa B (NF-κB) activation [29].

The effects of diabetes on liver function have been well documented. Diabetic rats secrete higher levels of bile acids, cholesterol, phospholipids, and organic anions in bile [13,30]. Activities of most antioxidant enzymes in the liver significantly decrease with the progression of untreated diabetes [31]. This suggests that the severity of oxidative damage in tissue may be related to the duration of the disease. Our previous studies showed that oxidative and energy stress decreased the activity of antioxidant enzymes, and 670 nm LED treatment normalized it by enhancing protective antioxidant defense system [4,15]. In this study, GPx, CCO, and SOD activities followed this expected trend in chronic diabetes, but the changes were not significant. The significantly decreased activities of GST and CAT compared to controls (32% and 61%, respectively) were typical of oxidative stress in diabetes [32]. Light treatment was ineffective at reversing hepatic CAT or GST activity. Our results from the study of chronic diabetes are in good agreement with other antioxidant studies that treated rats longer than 3 weeks with isoeugenol [33], quercetin [34], piperine [35], coenzyme Q_{10} [36], vanadate [37], acetylsalicylic acid (ASA) [38], and various other antioxidants [32]. These studies also did not report improvement in hepatic CAT activity. The reduced LPO levels in diabetic rats in the present study were not indicative of oxidative stress, but they do suggest that lipid peroxides may be reduced as a result of increased hepatic lipolysis in diabetes [39].

Although chronic light treatment may have been ineffective as an antioxidant in the liver, light treatment for 18 days in acutely diabetic rats resulted in the normalization of oxidative stress-induced suppression of hepatic GPx, GRx, GST, and SOD enzyme activities and to a lesser extent, CAT activity. The disparity between our acute and chronic studies suggests that LLLT may stimulate hepatic antioxidant enzymes to initially intensify cellular defense against oxidative damage to the diabetic tissue. However, progression of the disease may eventually increase resistance to oxidative stress, reduce the efficacy of LED irradiation, and significantly weaken the antioxidant capabilities of the cell as it alters hepatic function and intensifies oxidative stress.

Although LED irradiation did not appear to effectively ameliorate the effects of oxidative stress in diabetic rats, its potential benefits have been widely reported. Cytochrome *c* oxidase has been implicated as a photoacceptor that brings about the beneficial changes of photobiomodulation, including increased oxygen consumption and energy charge of rat liver mitochondria [5,40]. Light irradiation at 670 nm may therefore improve hepatic function in healthy and acutely diabetic rats with no adverse effects.

Our previous studies suggest that the attenuation of oxidative stress plays an important role in protecting the cell at a microscopic level and protecting the tissue at a macroscopic level from the deleterious effects of diabetes mellitus. Several intracellular mechanisms correlate oxidative stress with the activation of stress-activated signaling pathways involving NF-κB and motigen-activated protein kinase. These pathways in turn activate transcription factors that ultimately

contribute to cellular damage as a consequence of increased oxidative stress [41]. Recent research also implicates diabetes and oxidative stress in the formation of AGEs, which may contribute to endothelial and vascular dysfunction as a result of the cross-linking of structural proteins such as collagen [42–45]. Studies that use antioxidant treatments in reducing the effects of oxidative stress [46], like our chronic light therapy study, fail to show consistent prophylactic or therapeutic benefits.

In conclusion, acute light therapy improved the hepatic antioxidant defense system, indicating that photobiomodulation at 670 nm may be effective at inducing short-term changes in the hepatic antioxidant defense system. Therefore, 670 nm photobiomodulation may be broadly applicable to reverse hepatic complications induced by acute hyperglycemia that disrupt the antioxidant defense mechanisms. Additional studies are necessary to elucidate the mechanisms by which photobiomodulation attenuates deleterious effects of oxidative and energy stress in diabetes mellitus.

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