Photobiomodulation on Bax and Bcl-2 Proteins and SIRT1/PGC-1α Axis mRNA Expression Levels of Aging Rat Skeletal Muscle

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Objective. This study aimed to analyze the effects of low level laser irradiation (LLLI) on Bax and IGF-1 and Bcl-2 protein contents and SIRT1/PGC-1α axis mRNA expression levels to prevent sarcopenia in aged rats. Material and Methods. Twenty female Sprague Dawley rats (18 months old) were randomly divided into two groups (n = 10 per group): control (CON) and LLLI groups. The gallium-aluminum-arsenium (GaAlAs) laser irradiation at 810 nm was used in the single point contact mode (3.75 J/cm²; 0.4 cm²; 125 mW/cm²; 30 s). Bax, Bcl-2, and IGF-1 proteins and SIRT1/PGC-1α axis mRNA expression were assessed 24 h after LLLI on gastrocnemius in aged rat. Results. Gastrocnemius muscle weights, gastrocnemius mass/body mass, Bcl-2/BAX ratio, Bcl-2 protein, IGF-1 protein, and the mRNA contents in SIRT1, PGC-1α, NRF1, TMF, and SOD2 were significantly (P < 0.05) increased by LLLI compared to CON group. However, levels of BAX protein and caspase 3 mRNA were significantly attenuated by LLLI compared to CON group (P < 0.05). Conclusion. LLLI at 810 nm inhibits sarcopenia associated with upregulation of Bcl-2/BAX ratio and IGF-1 and SIRT1/PGC-1α axis mRNA expression in aged rats. This indicates that LLLI has potential to decrease progression of myocyte apoptosis in sarcopenic muscles.

1. Introduction

Sarcopenia, the age-related decline in muscle mass and function, represents a significant health issue due to its associated high prevalence of frailty and disability [1]. An altered regulation of myocyte apoptosis has recently emerged as a possible contributor to the pathogenesis of sarcopenia [2]. Studies in animal models [2] and human [3] have shown that the severity of skeletal muscle cell apoptosis increases over the course of aging and correlates with the degree of muscle mass and strength decline. Several apoptotic pathways are operative in aged muscles, with the mitochondria-mediated pathways being the most likely relevant to sarcopenia [4].

Sirtuin 1 (SIRT1), a nicotinamide adenine dinucleotide (NAD⁺)-dependent histone deacetylase [5], is implicated in the prevention of many age-related diseases such as sarcopenia, cancer, Alzheimer’s disease, and type 2 diabetes, by maintaining mitochondrial homeostasis [6]. SIRT1/PGC-1α (peroxisome proliferator-activated receptor-γ coactivator 1α) pathway plays a vital role in regulating mitochondria oxidative stress, biogenesis, and apoptosis, thereby contributing to maintain skeletal muscle homeostasis and human longevity [5]. Therefore, disruption of SIRT1/PGC-1α pathway may result in age-related loss of muscle function and sarcopenia. Indeed, muscle wasting could be prevented by reducing myocyte apoptosis and proteasome degradation if the transcriptional activity and protein content of PGC-1α were enhanced in skeletal muscle [7].

To regulate the deficiencies of myocyte activity in elderly subjects, many methods are applied such as physical training,
amino acid treatment, myostatin inhibition, testosterone treatment, calorie restriction, and noninvasive physical procedures like ultrasound therapy, electrical current modalities, and phototherapy [8]. The phototherapy of photobiomodulation (PBM) can effectively stimulate or inhibit biological functions, without irreducible damage [9]. It is well known that low level laser irradiation (LLLI) or monochromatic light has excellent therapeutic response in sarcopenia of ovariectomized rats [8] and cryoinjured rats [10]. However, the underlying molecular mechanisms are still unknown. We hypothesize that the LLLI may be effective in preventing sarcopenia in aged rats by reducing myocyte apoptosis, especially in type II fiber containing skeletal muscles, which are more susceptible to muscle mass losing via intrinsic apoptotic pathway. We further hypothesize that the age-related loss of muscle mass and myocyte apoptosis could be delayed by LLLI, an effect involving upregulation of insulin-like growth factor 1 (IGF-1) protein and SIRT1/PGC-1α axis messenger ribonucleic acid (mRNA) expression in skeletal muscle of aged rats. Therefore, this study was intended to investigate the alterations in BAX, Bcl-2, and IGF-1 protein expression under the irradiation of GaAlAs (810 nm; 125 mW/cm²) in aged rats. Therefore, this study was intended to investigate the alterations in BAX, Bcl-2, and IGF-1 protein expression under the irradiation of GaAlAs (810 nm; 125 mW/cm²; 3.75 J/cm²; 0.4 cm²; 30 s). The effect of LLLI on SIRT1/PGC-1α axis mRNA expression in skeletal muscle of aged rats was also investigated.

2. Materials and Methods

2.1. Animals. Twenty female Sprague Dawley rats (18 months old, 378 ± 11 g) obtained from the experimental animal center in Guangzhou University of Traditional Chinese Medicine (GUTCM) (Guangzhou, China) were housed in plastic cages (five rats per cage) in a temperature controlled room (22 ± 2°C), with lights switched on from 6 am to 6 pm. All experimental procedures and animal care were approved by the Experimental Animal Care and Use Committee of GUTCM.

2.2. Low Level Laser Irradiation. The gallium-aluminum-arsenide (GaAlAs) laser (λ = 810 nm) was operated at 5 W, using a continuous wavelength of 810 nm with a 50 mW irradiation over each illuminated area of 0.4 cm². Phototherapy was started on day 1. The center of the greater trochanter was located by palpation. The intensity of 125 mW/cm² was performed punctually, through a single point contact mode in the center of the greater trochanter of the right femur [8] for 30 s every 24 h over an 8-week period.

2.3. Tissue Sampling and Preparation. On the last day of the experimental period, all rats were anaesthetized with pentobarbital sodium (40 mg/kg) 24 h after LLLI irradiation. Bilateral gastrocnemius medialis muscles were then extracted and weighed. The proximal halves of the muscles were embedded in tragacanth gum, after which the samples were frozen in isopentane cooled by liquid nitrogen and were stored in a deep freeze (−80°C). And the distal halves of the muscles were immediately separated into the two blocks of superficial and deep regions, with each trimmed into 50 mg tissue samples. The superficial region of gastrocnemius muscle was organized with fast twitch fibres almost exclusively; however, the deep region included slow- and fast-twitch fibres [II]. The superficial region of gastrocnemius was homogenized in 0.01 M phosphate buffer (PBS; pH 7.4). And then the homogenates were centrifuged at 4°C at 5600 g for 10 min; the supernatants were harvested and stored in a deep freeze (−80°C). The amount of protein in each muscle supernatant was determined by a BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

2.4. Bax and Bcl-2 and IGF-1 Proteins Content. Proteins content for Bax, Bcl-2, and IGF-1 were determined by western immunoblot analysis (Figure 1). Separating gel (375 mM Tris-HCl; pH 8.8; 0.4% SDS; 10% acrylamide) and stacking gel (125 mM Tris-HCl; pH 6.8; 0.4% SDS; 10% acrylamide monomer) solutions were made, and polymerization was initiated by TEMED and ammonium persulfate. Then separating and stacking gels were quickly poured into a Bio-Rad Protein III gel-box (Bio-Rad; Hercules, CA, USA). Eighty micrograms of protein from skeletal muscle homogenates in sample buffer (Tris pH 6.8 with 2% SDS, 30 mM DTT, 25% glycerol) were then loaded into the wells of 10% polyacrylamide gels and electrophoresed at 150 V. The gels were then transferred at 30 V onto a nitrocellulose membrane (Bio-Rad) overnight. The membranes were blocked in 5% nonfat milk in PBS with 0.1% Tween-20 at room temperature for 6 h. After blocking, the membranes were incubated at room temperature in PBS, with appropriate primary antibodies for 12 h: rabbit polyclonal Bax (1:200, Santa Cruz Biotechnology, CA, USA), mouse monoclonal Bcl-2 (1:250, BD Transduction Laboratories, KY, USA), and rabbit polyclonal IGF-1 (1:200, Santa Cruz Biotechnology). After three times of washing in PBS with 0.4% Tween-20, the membranes were incubated with horseradish peroxidase- (HRP-) conjugated secondary antibodies (Santa Cruz Biotechnology) in PBS at room temperature for 90 min. After three times of washing in PBS with 0.4% Tween-20, an enhanced chemiluminescence (ECL) detection system (Amersham: Piscataway, NJ, USA) was used for visualization. Densitometry (as area time's grayscale relative to background) was performed by using a Kodak film cartridge and film, a scanner interfaced with a microcomputer, and the NIH Image J Analysis program. The consistent loading of wells was confirmed with Ponceau-S-staining, and protein expression was quantified as area time's grayscale relative to background per mg protein.

2.5. SIRT1/PGC-1α Axis mRNA Content. Total RNA was prepared from 100 mg of frozen muscle tissues using TRIzol (Invitrogen, Singapore) and was purified according to the instructions included. The RNA purity was verified by the OD260/OD280 on the ultraviolet spectrophotometric module of the Tecan Microplate Reader (Infinite 200, Switzerland). Double-stranded cDNA was synthesized from −1 μg of total RNA using ReverTra Ace qPCR RT Kit (Osaka, Japan). Real-time PCR reactions were set up by using the SYBR-Green PCR kit (Osaka, Japan), and were cycled in StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The mRNA abundance of the targeted genes was
Figure 1: Western blot analysis and densitometry ratios of BAX, Bcl-2, and IGF-1 in gastrocnemius. Western blot analysis is used to determine the level of proteins extracted from gastrocnemius, relative to the endogenous control β-actin. Western blots and values are means ± SE representative from one rat from each group. n = 10 for each group. * P < 0.05 versus control group.

3. Results

3.1. Gastrocnemius Wet Weight and Gastrocnemius Index.
Table 2 summarizes the effects of 8-week LLLI on body mass and gastrocnemius muscle weights. The gastrocnemius muscle weights of the LLLI group are significantly increased compared to the CON group (P < 0.05), although the animal body mass was not changed (P > 0.05). There is a significant
Table 1: Primers used for PCR amplification of cDNA.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRT1</td>
<td>CTGTTTCTGTGGGATACCTGACT</td>
<td>ATCGAACATGGCTTGAGGATCT</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>CTACAATGAATGCAGCGGTCTT</td>
<td>TGCTCATGATTCTCGGTCTT</td>
</tr>
<tr>
<td>NRF1</td>
<td>GCATTGAGCTACTGACAGAC</td>
<td>CTGTGTCCTGGATCTTCCTT</td>
</tr>
<tr>
<td>TFAM</td>
<td>TGGAAACACGCCACATGCTT</td>
<td>ACCATGTTGCGAAAATGCTT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CTATCGGCAATGAGCGGTT</td>
<td>TGTGTTGGCATAGAGGTCTTTA</td>
</tr>
<tr>
<td>SOD2</td>
<td>CGTCATTCACTTCGAGCAGA</td>
<td>AAAATGAGGTTCTGACAGTG</td>
</tr>
<tr>
<td>Caspase3</td>
<td>TGGTTCATCCAGTCGCTTTGT</td>
<td>CAAATTCTGTTGCCACCTT</td>
</tr>
</tbody>
</table>

Table 2: Weight, gastrocnemius weight, and gastrocnemius mass/body mass.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body mass (g)</th>
<th>Gastrocnemius weight (g)</th>
<th>Gastrocnemius mass/body mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>378.8 ± 65.7</td>
<td>0.61 ± 0.16</td>
<td>1.60 ± 0.20</td>
</tr>
<tr>
<td>LLLI</td>
<td>378.0 ± 27.6</td>
<td>0.84 ± 0.03*</td>
<td>2.20 ± 0.11*</td>
</tr>
</tbody>
</table>

*P < 0.05 versus the CON groups.

increase in the gastrocnemius mass/body mass in the LLLI group compared to the CON group (P < 0.05).

3.2. Protein Level of Bax, Bcl-2, IGF-1, and Bcl-2/BAX Ratio. Bcl-2 and IGF-1 protein expression in the gastrocnemius of the LLLI group are significantly elevated, while BAX expression is significantly inhibited, compared to the CON group (P < 0.05). Therefore, LLLI significantly increases Bcl-2/BAX ratio compared to CON group (P < 0.05).

3.3. Expression of SIRT1/PGC-1α Axis Genes mRNA. The mRNA contents of SIRT1, PGC-1α, TFAM, NRF1, and SOD2 in the gastrocnemius of LLLI group are significantly upregulated compared to the CON group (P < 0.05). However, caspase 3 mRNA content in the gastrocnemius of the LLLI group significantly decreased compared to CON group (P < 0.05).

4. Discussions

4.1. LLLI Effects on Age-Related Loss of Muscle Mass in Aged Rats. It has been confirmed in many studies that 30% or more of skeletal muscle myocytes, particularly in fast-twitch fibers, may be lost with aging due to apoptosis. Andersen et al. [12] found gastrocnemius mass/body mass ratios significantly decreased in aged rats (22 months) by 22.8% compared to young rats (6 months). Gastrocnemius muscle weight and gastrocnemius muscle wt/body were 24% and 20% lower in aged rats (22 months) compared to young rats (3 months), respectively [13]. Song et al. [14] found that gastrocnemius mass decreased by 11.2% in aged rats (22 months) compared to rats of 18 months.

It has been suggested that the age-related loss of muscle mass may be inhibited by LLLI [9]. Corazza et al. [8] demonstrated that LLLI improved the muscle volume of middle aged ovariectomized rats. In present study, the gastrocnemius muscle weight and gastrocnemius mass/body mass ratios of the LLLI group are 30% and 37.5% higher than the CON group, respectively. In other words, PBMT from LLLI retarded the loss of skeletal muscle mass from aged rats (18 months) and has larger muscle mass in old rats (22 months) compared to younger ones (6 months).

4.2. LLLI Effects on Apoptotic Signal in Aging Skeletal Muscle. A large number of studies using both rodent model and human subjects have confirmed that the mechanism for age-associated skeletal muscle fiber atrophy and myocyte loss is due to apoptosis [2, 3]. A critical event in mitochondrial-driven apoptosis is the formation of permeable membrane pores, regulated by the balance between competing antiapoptotic Bcl-2 family proteins such as Bcl-2 and proapoptotic proteins including Bax. Increased proapoptotic signaling through the mitochondrial Bcl-2 family has been implicated as an important mechanism leading towards muscle cell loss and atrophy with aging [2]. Previously, elevation of Bax and caspase 3 protein expression and Bax/Bcl-2 ratio in aging skeletal muscle was reported by Alway et al. [15]. They showed that the dysregulation of Bcl-2 pathway signaling leads to caspase 3 activation and intrinsic apoptotic pathways in age-associated skeletal muscle [15]. And Song et al. found that the mitochondrial Bcl-2 protein levels were reduced by 20% in aged rats compared to young rats, but the mitochondrial Bax protein level was 11% higher in aged rats compared to young rats. Moreover, the Bcl-2/Bax ratio in white gastrocnemius was increased by 98% with age [14]. In addition, caspase 3 mRNA content in aged rats (22 months) was 72% higher than that in young rats (4 months) [16].

Theoretically, the apoptotic signal in aging skeletal muscle may be partly wiped out by the LLLI [9]. Data from aged rats indicate that LLLI can provide protection against sarcopenia [8, 10]. Studies on experimental animal and various cell types revealed that LLLI acted as a preventive tool against cell apoptosis [17, 18]. And other studies indicated that LLLI may inhibit muscle cell and myoblasts apoptosis through upstream Bcl-2 family signaling [19, 20]. LLLI inhibited apoptosis of muscle cell after irradiation with fluency of 133.3 J/cm² in the rats gastrocnemius before high intensity exercise [19]. In the present study, LLLI resulted in a reduction of Bax protein and elevation of Bcl-2 protein expression and
SIRT1 has been found to increase the transcription of PGC-1α and activate PGC-1α by deacetylation [37, 38].

The mechanism of LLLI promotion of SIRT1 expression and NAD⁺ level has not been well elucidated. LLLI was found to induce a significant increase in cAMP level via upregulating ATP content and mitochondrial membrane potential [31]. As a second messenger, cAMP may initiate beneficial cell signaling pathways leading to the activation of redox sensitive transcription factors such as nicotinamide phosphoribosyltransferase (NAMPT) and protein kinase A (PKA) signaling pathways, which may increase NAD⁺ level and induce expression of IGF-1, respectively [32]. IGF-I may enhance muscle cell survival signaling in terms of protection from oxidative stress-induced mitochondrial-driven apoptosis through upregulating the SIRT1 expression [33]. We then proposed that the downregulation of the cAMP-NAMPT-NAD⁺ signaling pathway may contribute to mitochondrial deterioration in aged muscle, whereas LLLI may ameliorate the deficits. In addition, the overexpression of anticancer genes cyclin-dependent kinase inhibitor p16INK4a, in satellite cells irreversibly may affect their intrinsic regenerative and self-renewal capacities in sarcopenic muscles. Recent studies showed that cAMP-PKA signaling was shown to inhibit the expression of p16INK4a [34], which appeared to be positively associated with reduced myogenic potential and increased cellular senescence in satellite cells from physiologically aged individuals with sarcopenia through downregulation of the SIRT1 expression [35, 36]. We further proposed that LLLI may promote the muscle-regeneration process by regulating PKA-p16INK4a-SIRT1 pathway in sarcopenic muscles (Figure 3).

SIRT1 has been found to increase the transcription of PGC-1α and activate PGC-1α by deacetylation [37, 38].

**Figure 2:** mRNA quantification of SIRT1/PGC-1α axis genes in skeletal muscle subjected to 8-week LLLI. Real-time PCR is used to determine the expression of genes in gastrocnemius from the LLLI group or the CON group, relative to the endogenous control GAPDH. Values are means ± SE. *n = 10 for each group. *P < 0.05, **P < 0.01 versus CON group.

![Figure 2: mRNA quantification of SIRT1/PGC-1α axis genes in skeletal muscle subjected to 8-week LLLI. Real-time PCR is used to determine the expression of genes in gastrocnemius from the LLLI group or the CON group, relative to the endogenous control GAPDH. Values are means ± SE. *n = 10 for each group. *P < 0.05, **P < 0.01 versus CON group.](image-url)
The mRNA expression of PGC-1α is significantly decreased by 35% in skeletal muscles of old rats (22 months) compared to young rats (4 months) as previously described [16]. As expected, the mRNA expression of PGC-1α and SIRT1 was found to be significantly correlated [28]. Interestingly, the expression of mitochondrial biogenesis genes was known to be under the control of the PGC-1α and their activator SIRT1. And increased PGC-1α activity may enhance its DNA binding and thus induce the expression of genes involved in mitochondrial biogenesis. And nuclear respiratory factors 1 (NRF1) may promote the expression of most nuclear-encoding mitochondrial proteins, as well as mitochondrial transcription factor A (TFAM) that may directly stimulate mitochondrial DNA replication and transcription. Thus, our study suggested that the LLLI may increase the transcription of NRF1 and TFAM, probably through upregulating the transcription of SIRT1 and PGC-1α. Nguyen et al. [30] confirmed that SIRT1 and PGC-1α expression itself was elevated in C2C12 cells following 4 days of LLLI. Their results showed that modulation of mitochondrial regulation via LLLI represented one of the molecular and cellular mechanisms contributing to the clinical therapeutic benefits of LLLI in patients with musculoskeletal injuries [30]. Furthermore, our data indicated that 8 weeks of LLLI were useful for the treatment of other skeletal muscle disorders with dysfunctional mitochondria contributing towards their etiology in sarcopenic muscles probably through modulation of SIRT1/PGC-1α axis and its key regulators. However, further studies are needed to verify the mechanism of action.

The skeletal muscle of aged rats may be characterized by enhanced reactive oxygen species production compared with young rats. Aging may be associated with increased oxidative damage, leading to muscle dysfunction [39]. Aged animals displayed a significant decrease by 59.3% in the transcript levels of mitochondrial antioxidative enzyme SOD2, compared to the younger ones [28]. In the present study, we found that SOD2 mRNA level increased 2.8-fold by the LLLI (Figure 2). Thus, age-related loss of SOD2 mRNA may contribute to the oxidative damage with aging, which could be inhibited by LLLI. Since SIRT1 has been found to increase the transcription of SOD2 by activating PGC-1α [40], we proposed that LLLI may promote SIRT1-mediated gene transcription of SOD2.

We recognize that our study evaluated skeletal muscle mass, marker of apoptosis, and gene expression of the expression of a group of genes involved in mitochondrial function and biogenesis in an animal model, so we understand that this represents a limitation and we express caution at extrapolating our findings into humans at this stage. Nevertheless, LLLI has a strong safety profile and reports of side effects in an evidence base of over 200 randomized controlled clinical trials are few and minor. Therefore, we believe serious consideration should be given to the potential of LLLI as a treatment option of long-term conditions like sarcopenia. Future studies would include investigation of the effects of LLLI on protein expression of mitochondrial biogenesis and oxidative capacity and functional aspects of sarcopenia and the determination of optimal parameters to inform the design of robust clinical trials. We hope that our findings may initiate interest in the use of LLLI as a potentially useful adjunct for sarcopenia.
5. Conclusion

LLLI at 810 nm inhibits sarcopenia associated with upregulation of Bcl-2/BAX ratio and IGF-1 and SIRT1/PGC-1α axis mRNA expression in aged rats. This suggests that LLLI may inhibit sarcopenia in the aged rat by promoting the expression of IGF-1 and SIRT1/PGC-1α axis genes. Further studies are needed to verify the mechanism of action and effects on functional outcomes and to establish optimal parameters of application to inform clinical use.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Fang-Hui Li and Yan-Ying Liu contributed equally to this study and share first authorship.

Acknowledgments

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