MEASUREMENT OF GaAlAs DIODE LASER ACTION ON PHAGOCYTIC ACTIVITY OF HUMAN NEUTROPHILS AS A POSSIBLE THERAPEUTIC DOSIMETRY DETERMINANT

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Recently, there has been an increasing amount of interest in the application of low reactive level laser therapy (LLLT) for pain attenuation or pain removal. However, the mechanisms by which LLLT works are not yet fully understood.

We measured the phagocytic rate using the luminol-dependent chemiluminescence method and a Neutroprobe 48-cell chemotaxis chamber to examine the chemical dependent motion of neutrophils with and without LLLT irradiation.

This study showed that at a 15-s exposure, 15, 30, 45, and 60 mW output powers (0.25, 0.45, 0.67 and 0.9 J) shortened the peak time compared with control; peak height increased at all four output powers; and neutrophil random motion count also increased across the board. At a 30-s exposure, doses of 0.45, 0.9, 1.356 and 1.8 J caused no significant shortening of peak time compared with control, but the peak height increased significantly for output powers of 15, 30 and 45 mW. Neutrophil random motion only increased for the dose of 0.45 J (15 mW). For a 60-s exposure at the same output powers as above, doses of 0.9, 1.8, 2.7 and 3.6 J respectively, neither peak time nor peak height nor neutrophil random motion count showed any significant difference compared with the controls.

KEY WORDS Laser therapy Chemiluminescence Chemotaxis Neutrophils Reactive oxygen species

Introduction

Recently, there has been an increasing amount of interest in the application of low reactive level laser therapy, or LLLT, for pain attenuation or pain removal. We have been obtaining consistently good pain relief results in the Shiroto clinic using the GaAlAs diode laser. 1-2 Although the mechanisms by which LLLT works are not yet fully understood, some studies have been undertaken which have shown some of the possible pathways. This group previously reported the enhanced metabolic rate and phagocytic activity of LLLT-irradiated normal human neutrophils in vitro. In the present study, we once more measured the phagocytic rate using the luminol-dependent chemiluminescence method (a simultaneous multiple measurement system of neutrophil chemiluminescence), in which the degree of luminescence in the solution is indicative of the strength of the phagocytic action, due to the release of superoxide and the other reactive oxygen species.

In addition, we used a Neutroprobe 48-cell chemotaxis chamber to examine the chemical dependent motion of neutrophils with and without LLLT irradiation. The upper and lower cells of the chamber were separated by a polycarbonate micropore filter, and the number of neutrophils trapped in the filter as they migrated between the cells was an indication of the degree of chemotaxis.

Material and Method

We used the MLD-2001 GaAlAs diode laser, manufactured by the Mochida Pharmaceutical Company Ltd. (Figure 1). This machine emits a continuous wave at 830 nm, and at an output power variable from 15 mW to 60 mW in 15 mW steps.

Luminol Dependent Chemiluminescence Method

Whole blood samples were taken from 20 healthy male volunteers in their 20's (mean of 23.5 ± 2.3 years). The blood sample was mixed with the same amount of HBSS buffer (Hanks balanced salt solution) and the neutrophils were isolated using Histopacks 1077 and 1119 in the density-gradient method (Figure 2). After isolation
presence of the luminol causes a luminescent reaction, and degree of luminescence could therefore be measured as a direct indicator of the degree of phagocytosis.

The solution (150 µl) was pipetted into each well of a 96-well flat-bottomed microtitre plate from Coster company. The experimental wells were irradiated directly from above (Figure 3). The laser powers were 15, 30, 45 and 60 mW, with exposure times of 15, 30 and 60 s. The unirradiated control solution was put in exactly the same plate, but was not irradiated.

Figure 4 shows on the vertical axis the degree of chemiluminescence, converted by the measuring system into millivolts on a meter and recorder. Time is shown along the horizontal axis. The maximum or peak values for luminescence (hereafter referred to as PH) and the time to reach PH (hereafter called PT) were measured, and analysed statistically using the Student’s t-test.

Chemotactic Activity

A Neuroprobe 48-cell chemotaxis chamber from Ieda Trading Company (Figure 5), was used to assess the degree of neutrophil chemotaxis. Chemotactic peptide (FMLP) was placed in the lower part of each chamber, and the 3 µm micropore filter inserted. The neutrophil solution with constant cell density of 2 × 10⁹/ml was added to each upper chamber, and irradiation was carried out at the same parameters as before, with one set left

<table>
<thead>
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<th>Table 1. Peak height (mV)</th>
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<tr>
<td><strong>Irradiation time (s)</strong></td>
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<tr>
<td><strong>Power (mW)</strong></td>
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<td>45</td>
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<td>60</td>
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NS: not significant (paired t-test).
* p < 0.05
† p < 0.01.

<table>
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<th>Table 2. Peak time (min)</th>
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<tr>
<td><strong>Irradiation time (s)</strong></td>
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<td><strong>Power (mW)</strong></td>
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NS: not significant (paired t-test).
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* p < 0.01.

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Figure 2. Luminol-dependent chemiluminescence method

Table 3. Chemotactic activity (neutrophil count)

<table>
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<tr>
<th>Power (mW)</th>
<th>Irradiation time (s)</th>
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<th>15</th>
<th>t-test</th>
<th>30</th>
<th>t-test</th>
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<td>15</td>
<td>21.1 ± 11.1</td>
<td>31.7 ± 6.1</td>
<td>*</td>
<td>27.9 ± 8.9</td>
<td>†</td>
<td>23.7 ± 12.4</td>
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<tr>
<td>30</td>
<td>21.1 ± 11.1</td>
<td>28.3 ± 8.6</td>
<td>†</td>
<td>21.3 ± 7.4</td>
<td>NS</td>
<td>19.4 ± 13.6</td>
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<tr>
<td>45</td>
<td>21.1 ± 11.1</td>
<td>36.5 ± 11.6</td>
<td>*</td>
<td>30.7 ± 19.3</td>
<td>NS</td>
<td>23.0 ± 12.9</td>
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<tr>
<td>60</td>
<td>21.1 ± 11.1</td>
<td>33.0 ± 11.3</td>
<td>†</td>
<td>27.8 ± 15.3</td>
<td>NS</td>
<td>21.9 ± 6.2</td>
<td>NS</td>
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</tbody>
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NS: not significant (paired t-test).  
* p < 0.05  
† p < 0.01.
unirradiated as control. The solutions were then incubated at 37°C for 20 min in a CO₂ oven.
Following incubation, the micropore filter was removed, treated with ethanol and processed with Giemsa stain. The numbers of the neutrophils in the filters at five random points were counted and tabulated, and the data statistically analysed using the Student's t-test.

Results
The mean values for PH, PT and chemotactic activity in the chemiluminescence study at the various settings can be seen in Tables 1-3.

The P1 for the 15- and 30-s exposures at 15 mW output power were statistically significantly higher compared with the control at a risk factor of 5% (Figure 6). The PT mean values for the control four exposure times at 15 mW output power can be seen in Figure 7. The PT for the 15-s exposure, at 8.88 min, was statistically significantly shorter than the control at 10.17 min, with a risk factor of less than 1%. The neutrophil count was higher for all three exposure doses compared with the control, but was statistically significantly higher for the 15-s exposure, with a risk factor of less than 1%, and for the 30-s exposure, with a risk factor of under 5% (Figure 8).

The same values for the 30 mW output power are shown in Figures 9-11. PH was significantly higher for the 15- and 30-s exposures, with risk factors of less than 1% and 5% respectively. PT mean value
Figure 6. The peak height for the 15- and 30-s exposures at 13 mW output power were statistically significantly higher compared with the control at a risk factor of 5%.

Figure 7. The peak time mean values for the control four exposure times at 13 mW output power is shown. The PT for the 15-s exposure of 8.88 min was statistically significantly shorter than the control at 10.17 min, with a risk factor of less than 1%.
Figure 8. The neutrophil count at 15 mW output power was higher for all three exposure doses compared with the control, but was statistically significantly higher for the 15-s exposure, with a risk factor of less than 1%, and for the 30-s exposure, with a risk factor of under 5%.

Figure 9. Peak height mean value at 30 mW output power was significantly higher for the 15- and 30-s exposures, with risk factors of less than 1% and 5%, respectively.
Figure 10. Peak time. at 30 mW output power, mean value for the 15-s irradiation was significantly shorter than the control, with the risk factor of less than 5%.

Figure 11. Neutrophil count at 30 mW output power was significantly higher for the 15-s exposure with a risk factor of less than 5%; for the 30-s exposure it was practically the same as the control, and for the 60-s dose, it was less.
Figure 12. Peak height at 45 mW output power was significantly higher at 15- and 30-s. PT significantly shorter at 15-s.

Figure 13. Peak time at 45 mW output power.
Figure 14. Neutrophil count at 45 mW output power

Figure 15. Peak height at 60 mW output power
Figure 16. Peak time at 60 mW output power

Figure 17. Neutrophil count at 60 mW output power
for the 15-s irradiation was significantly shorter than the control, with the risk factor of less than 5%. Neutrophil count was significantly higher for the 15-s exposure with a risk factor of less than 5%; for the 30-s exposure it was practically the same as the control, and for the 60-s dose, it was less.

The same significant differences were found in the 45 mW irradiated group. PH was significantly higher at 15- and 30-s, PT significantly shorter at 15-s, and cell count greater for 15-s (Figures 12-14).

At 60 mW output power, less of a difference was shown in Figures 15-17. PH was significantly higher at 15-s, but there was no statistically significant difference in the PT values. Cell count was significantly greater at 15-s.

**Discussion**

We used the luminol-dependent chemiluminescence method to give an indication of neutrophil phagocytic activity. As already said, when phagocytosis occurs, reactivated oxygen is released in volumes varying directly with the strength of the reaction. The reactivated oxygen reacts with the luminol, and luminescence results, the degree of which also varies directly with the phagocytic reaction. Thus by measuring the degree of luminescence and converting it into an electric current, measurable in millivolts, an accurate record of strength of phagocytic reaction can be arrived at. The peak level of luminescence, peak height (PH) in this study, is thought to occur when the neutrophils are at their most active stage of phagocytosis.

The time taken to reach the peak of phagocytic activity is referred to in this study as peak time (PT), and is considered to be the mean time between the recognition by the neutrophil of a foreign body and the release of reactivated oxygen. The authors have previously reported that LLLT has less effects on the PH value than doses on PT; in other words, the laser used at these milliwatt levels shortens the time to reach PH, rather than significantly increasing the actual level of phagocytic activity.

Chemotaxis is recognized especially as the unidirectional movement of white blood cells towards an attractant, and is the factor behind the motion of lymphocytes towards an infected area. Recently researches have clarified this phenomenon as the basis of the biodefence mechanism, and further investigations into this phenomenon may well be one of the key points towards solving many disease problems, including immunodeficiency and other diseases of the immune system.

In summary, this study showed that at 15-s exposure, 15, 30, 45 and 60 mW output powers, which represent doses of 0.225, 0.45, 0.678, 0.9 J respectively, shortened the PT compared with control; PH increased at all four output powers, and neutrophil random motion count also increased across the board.

At 30-s exposure, doses of 0.45, 0.9, 1.356, and 1.8 J caused no significant shortening of PT compared with control, but the PH increased significantly for output powers of 15, 30 and 45 mW. Neutrophils random motion only increased for the dose of 0.45 J (15 mW).

For the 60-s exposure at the same output powers as above, doses of 0.9, 1.8, 2.7 and 3.6 J respectively, neither PT nor PH nor neutrophil random motion count showed any significant difference compared with the controls.

It seems from this *in vitro* study that, for shorter irradiation times and weaker output powers, the phagocytic action of neutrophils is higher, the time to reach maximum activity shorter, and neutrophil chemotactic action stronger. When neutrophils attack an attractant and phagocytose it, reactivated oxygen is released by xanthine oxidase, aldehyde and NAD(P)H-oxidase. It could be presumed that the laser beam acts on these enzymes, increasing their activity level and thus activating the increased superoxide production. Naturally, further studies with reduced irradiation times and doses are necessary to fully explore this precept.

It does seem from this study that, especially with longer irradiation times, neutrophil random motion was retarded. This could be explained by the theory that, while the neutrophil membrane surface may be damaged, and viability is retarded, in fact the cell's level of activation is still being raised.

Naturally, we have set ourselves the task of studying the effects of different combinations of exposure times and output powers on cell viability and activation levels, before we can really analyse these data in a meaningful way to provide further insight into the pain attenuating powers of LLLT.

**References**