NITROGEN LASER IMPROVES THE PHAGOCYTOTIC FUNCTION OF "LAZY" POLYMORPHONEUCLEAR LEUKOCYTES IN DIABETIC PATIENTS.

R Sachdeva¹, NS Bhagwanani², and DS Chitnis³

1: Department of Microbiology & Immunology; 2: Medical Director; and 3: Choithram Hospital and Research Centre, Indore, India

Polymorphonuclear leukocytes (PMNL) from ten diabetic cases were tested for phagocytic activity by the NBT reduction test and the activity was found to be grossly reduced ($p < 0.001$) in comparison to PMNL from matched healthy volunteers. *In vitro* experiments were then carried out on PMNL from 21 diabetic subjects to find out if the phagocytic activity in terms of intracellular killing of phagocytosed challenged bacteria was improved following irradiation with low incident doses of nitrogen (N_2) laser energy. It was demonstrated that N_2 laser exposure for 15 minutes at an output power of 6 mW, $\lambda = 337$ nm, could accentuate the killing of internalized *Staphylococcus aureus* when compared to the non-irradiated control PMNL ($p < 0.001$). Furthermore, it was also seen that this enhanced phagocytic activity occurred without affecting the viability of the PMNL cells. Thus to conclude, N_2 laser therapy accentuated the *in vitro* phagocytic activity in neutrophils from diabetic patients. This study may have therapeutic potential in various groups of immunocompromised patients.

Key words: N_2 laser, diabetes, neutrophils, phagocytosis, intracellular killing, NBT reduction test

Introduction

Low level laser therapy has recently been used extensively for the treatment of various ailments and conditions.^(1,2) The nitrogen (N_2) laser, a pulsed beam with a wavelength of 337 nm in the UVA waveband, has been reported previously by the authors as being effective for the treatment of pulmonary tuberculosis⁽³⁾ as well as non healing wounds⁽⁴⁾ and N_2 LLLT is now being used on a regular basis at our institute.

The enhanced bactericidal activity and improved phagocytic function of N_2 laser irradiated human polymorphonuclear leukocytes (PMNL) have been documented.⁽⁵⁾ PMNL form one of the first and very important defence mechanisms against invading microorganisms and reduced PMNL phagocytic activity in diseases like diabetes is largely responsible for susceptibility to recurrent pyogenic infections and decreased healing capability. In this direction,

preliminary experiments were carried out to verify the reduced phagocytic activity of PMNL from diabetic subjects by performing the NBT reduction test, and further experimental work was designed to find out if low levels of N_2 laser energy *in vitro* could accelerate the intracellular killing of bacteria by the 'lazy' PMNL from diabetic subjects without affecting the cells' viability.

Materials and Methods

The medium used consisted of Hanks balanced salt solution (HBSS), nutrient agar and Mueller Hinton agar (HiMedia, India), gentamicin (American Schering), 6% dextran (Sigma with molecular weight 150,000 in normal saline, 0.2% nitroblue tetrazolium (NBT) salt (Sigma), 0.2% trypan blue (Loba Chemicals, India), and 0.1% acridine orange-ethidium bromide (Sigma) were used. The lipopolysaccharide (LPS) used during NBT reduction was extracted from *Staphylococcus typhi*

Addressee for Correspondence:

Dr. D.S. Chitnis,
Head of Pathology, Choithram Hospital & Research Centre, Manik
Bagh Road, Indore, India.

Manuscript received: June, 1998

Accepted for publication: August, 1998

grown in a nutrient broth (HiMedia) in the laboratory by Westphal's hot phenol water method.⁽⁶⁾

The N₂ laser system used for the study was fabricated by the Centre for Advanced Technology (CAT), Indore, India. The laser had a power output of 6 mW at 337 nm and the incident energy density at an exposure time of 15 minutes was 5.4 J/cm² in all the experiments.

Separation of PMNL

5-7 ml of heparinized blood from diabetic patients was centrifuged at 1000 rpm for 10 min to separate the platelet rich plasma. The platelet rich plasma was further centrifuged at 3000 rpm to remove the platelets from the autologous plasma which was then used as the opsonizing agent during the experiment. The packed cells were diluted with HBSS to the original volume of the heparinized blood and one-tenth of the volume of 6% dextran was added to the cell suspension. The cells were incubated at 37°C for 45 min to separate the neutrophil rich plasma. The neutrophil rich plasma was centrifuged to separate out the PMNL which were then washed twice with HBSS and density adjusted to 5×10^6 cells/ml in presence of 50% autologous serum in HBSS.

NBT reduction test

The PMNL from ten diabetic patients (average age 52.8 years) diagnosed as being diabetic for periods from 4 months to 10 years and on insulin therapy were randomly selected for the NBT reduction test.⁽⁷⁾ Ten normal subjects of matching age, sex and economic status were used as controls for the NBT reduction test to confirm the diminished activity of PMNL from diabetic patients. Briefly, 100 µl of the neutrophil suspension from both the diabetic patients and healthy volunteers was suspended in a test tube containing 200 µl of 0.2% NBT and 5 µl of LPS (1 mg/ml for activation of the neutrophils). The unactivated control consisted of neutrophil suspension with NBT but without the LPS. The solutions were incubated at 37°C for 45 min and then centrifuged at 2000 rpm for 10 min. The supernatant was discarded and a smear from the cell pellet was made on a slide glass, fixed with methanol and counterstained with 0.5% safranin for 2 minutes. The NBT positive neutrophils were counted under an oil immersion lens and expressed as the percentage of the total neutrophils seen.

Bacterial suspension for the phagocytic function test

An overnight culture of *Staphylococcus aureus* NCTC 6538 grown on Nutrient agar slant was harvested in

normal saline to a density of 2500 million/ml using Browns opacity standards.

Experimental design for phagocytic function study

100 µl of the neutrophil suspension was irradiated with the N₂ laser for 15 min in duplicate microwells. The irradiated suspension was then transferred to a test tube containing *S. aureus* 50 ml. The suspension was incubated at 37°C for 30 min to allow the neutrophils to phagocytose and kill the engulfed *S. aureus*. The extracellular bacteria in the medium as well as those adherent to the outer surface of the neutrophils were washed twice with HBSS containing 30 µg/ml gentamicin. Gentamicin was selected as the antibiotic component of the wash as this antibiotic does not penetrate into intact neutrophils.⁽⁸⁾ The neutrophils were finally washed with plain HBSS to eliminate the extracellular gentamicin from the medium. The neutrophils were next lysed by three cycles of freeze thawing. The viable bacteria were plated on Mueller Hinton agar plates after serially diluting the suspension. The control unirradiated neutrophil suspension in the duplicate microwells was handled in the same manner as the irradiated test group.

Viability test

The viability of the irradiated neutrophils from the healthy as well as the diabetic patients was checked using the 0.2 % trypan blue dye exclusion test⁽⁹⁾ and 0.1% acridine orange-ethidium bromide fluorescent staining.⁽¹⁰⁾

Results

The data on the NBT reduction test in PMNL from ten diabetic subjects and ten healthy volunteers are shown in Table 1 and Figure 1. NBT reduction in the diabetic group was significantly less compared with PMNL from the normal subjects ($p < 0.001$ by unpaired t test). Further, the NBT reduction in the LPS activated PMNL from the diabetic subjects was not significantly different from their unactivated control (without LPS) counterparts ($p < 0.1$ by paired t test). Thus, the diabetic patients had reduced phagocytic activity.

The experimental results on the phagocytic levels of PMNL from twenty-one diabetic patients are given in Table 2 and Figure 2, comparing laser irradiated and unirradiated cells. The N₂ laser irradiated PMNL demonstrated a statistically significantly greater intracellular killing of *S. aureus* ($p < 0.001$ by paired students t test). The results suggest clearly increased killing of bacteria by N₂ laser irradiated PMNL from diabetic patients. The viability of the neutrophils was checked after irradiation with N₂ laser and 98% - 99%

Table 1: Comparison in the NBT reduction in PMNL from diabetic patients and healthy volunteers, unactivated and activated with polyliposaccharide.

	Healthy Volunteers (n=10)		Diabetic Patients (n=10)	
	Control (no LPS)	Test (with LPS)	Control (no LPS)	Test (with LPS)
Mean NBT Reduction (%)	26.023	49.730	15.880	17.113
±SD	8.639	9.288	5.017	6.567

Fig 1: Results of the NBT reduction test on polymorphonuclear leukocytes from diabetic and healthy subjects (n=10 each), comparing both unactivated and lipopolysaccharide activated PMNL. The diabetic PMNL demonstrated significantly reduced phagocytic activity compared with the normal PMNL ($p < 0.001$), and there was no significant difference between the LPS activated and unactivated diabetic PMNL ($p > 0.1$).

Fig 2: Intracellular phagocytosis and killing of *S. aureus* compared for unirradiated and N₂ laser irradiated N₂ diabetic PMNL from 21 patients with diabetes. N₂ laser irradiated PMNL demonstrated significantly higher killing rate ($p < 0.001$).

viability was confirmed in the test group from both the diabetic patients as well as the healthy volunteers.

Table 2: Intracellular killing of *S. aureus* compared for unirradiated and N₂ laser irradiated diabetic PMNL (n = 21 patients).

	Unirradiated PMNL	N ₂ Irradiated PMNL
Mean Bacterial Counts	504.185	289.445 [†]
±SD	317.487	198.307

[†] $p < 0.001$, paired t-test

Discussion

Phagocytosis by the polymorphonuclear leukocytes forms one of the most important non-specific defence mechanisms in the human body to counteract invading microorganisms. There is a PMNL dysfunction in diabetics with reduction in adherence to and diapedesis through the capillary walls.⁽¹¹⁾ Phagocytosis and the intracellular killing of bacteria is also depressed during uncontrolled metabolic states such as ketoacidosis in diabetic patients.⁽¹¹⁾ The diminished phagocytic activity of PMNL from diabetic subjects was confirmed *in vitro* by decreased NBT reduction in the present study. Further, PMNL from the diabetic subjects, following exposure to N₂ laser, showed enhanced and accelerated

microorganisms. There is a PMNL dysfunction in diabetics with reduction in adherence to and diapedesis through the capillary walls.⁽¹¹⁾ Phagocytosis and the intracellular killing of bacteria is also depressed during uncontrolled metabolic states such as ketoacidosis in diabetic patients.⁽¹¹⁾ The diminished phagocytic activity of PMNL from diabetic subjects was confirmed *in vitro* by decreased NBT reduction in the present study. Further, PMNL from the diabetic subjects, following exposure to N₂ laser, showed enhanced and accelerated intracellular killing compared with unirradiated control diabetic PMNL *in vitro*, and this finding may have therapeutic value for the improvement of lethargic PMNL *in vivo* in patients with diabetes. The increase in the intracellular killing of *S. aureus* following irradiation with the N₂ laser could possibly be due to the increase in the oxygen dependent or independent mechanisms⁽¹²⁾ which lead to intracellular bacterial killing. The exact mechanism of the acceleration of the intracellular killing of bacteria in the PMNL from diabetic subjects exposed to N₂ laser, however, needs to be explored further.

Diabetic foot lesions are very common and mostly the infections are polymicrobial: the longer the duration of diabetes, the higher is the risk of infection.⁽¹³⁾ We have previously documented the bactericidal effect of N₂ laser therapy on a broad spectrum of microorganisms.⁽¹⁴⁾ We have additionally demonstrated the increased microvascular circulation with accelerated wound healing following N₂ laser irradiation.⁽⁴⁾

N₂ laser therapy has given encouraging results in the management of diabetic ulcers in our institute (personal communication) and this appears to be due to the direct bactericidal effect of N₂ laser, faster wound healing with increased microvascular circulation and the enhanced intracellular bacterial killing in N₂ laser irradiated PMNL.

Acknowledgements

The authors are thankful to Dr. A.G. Bhujle and Mr. S. Shyam Sunder from the Centre for Advanced Technology, Indore, India, for designing and fabricating the ni-

trogen laser instrument and to Dr. D.D. Bhawalkar for providing the same for the study.

References

- 1: Gitmore SJ (1991): Laser produced plasma in medicine. *IEEE Transactions on Plasma Science*, 19 : 1209-1219.
- 2: Fisher AMR, Murphree AL, and Gomer CJ (1995): Clinical and preclinical photodynamic therapy. *Lasers in Surgery and Medicine*, 17: 2-31.
- 3: Bhagwanani NS, Bhatia GC, and Sharma N (1996): Low level laser therapy in pulmonary tuberculosis. *Journal of Clinical Laser Medicine & Surgery*, 14: 23-25.
- 4: Chamania S, and Sachdeva R (1996): Prospective controlled trial of nitrogen laser on infected wounds. *Laser & Technology*, 6: 35-40.
- 5: Sachdeva R, Bhagwanani NS, and Chitnis DS (1995): Low incident energy levels of the nitrogen laser enhances the biocidal activity of human neutrophils on internalized bacteria: An *in vitro* study. *Laser Therapy*, 7 : 107-112.
- 6: Luderitz O, Staub AM, and Westphal O (1966). Immunochemistry of O and R antigens of Salmonella and related Enterobacteriaceae. *Bacteriological Review*, 30: 192-255.
- 7: Bhuyan UN (1992) : Evaluation of neutrophil function. In (eds Talwar GP and Gupta SK): *A Handbook of Practical and Clinical Immunology, volume 1, Second edition*. CBS Publishers & Distributors, Delhi, India. pp 301-307.
- 8: Lorian V, and Gemmell GC (1991): Effect of low concentrations of antibiotics on bacteria. In (ed. Lorian V): *Antibiotics in Laboratory Medicine, third edition*. Williams and Wilkins, Baltimore, USA. pp 493-555.
- 9: Hunt SV (1987): Preparation of lymphocytes and accessory cells. In (ed. Klaus GGB): *Lymphocytes: A Practical Approach*. IRL Press Limited, Oxford. pp 1-34.
- 10: Taylor PM, Brain Thomas D, and Mills KGH (1987): In vitro culture of T cell lines and clones. In (ed. Klaus GGB): *Lymphocytes A Practical Approach*. IRL Press Limited, Oxford. PP 133-147.
- 11: Leslie CA (1986): Infections. In (eds. Beigehnan PM and Kumar D): *Diabetes Mellitus for the House Officer*. Williams and Wilkins, USA. pp 129-136.
- 12: Roitt IM (1988): The basis of Immunology. 1 - Innate Immunity. In (ed. Roitt IM): *Essential Immunology, Sixth edition*. Blackwell Scientific Publications, Oxford. pp 1-14.
- 13: Leichter SB (1995): Diabetic foot infections. In (eds. Leslie RDG and Robbins DC): *Diabetes: Clinical Science in Practice*. Cambridge University Press, Great Britain. pp 250-255.
- 14: Sachdeva R, Bhagwanani NS, and Chitnis DS (1995): The nitrogen laser inhibits the growth of a wide range of microbes *in vitro*. *Laser Therapy*, 7: 23-26.