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# Anti Inflammatory Effect of Low Level Laser Irradiation on the LPS-stimulated Murine Immunocytes

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## Abstract

**Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin-12 (IL-12) and interleukin (IL-1)-β, play a key role in causing inflammatory diseases, which are rheumatoid arthritis, Crohn's disease and sepsis. Accumulating evidences suggest that low level laser irradiation (LLLI) may have an anti-inflammatory action. However, there are few data regarding down regulation of Th1 immune response by using the diod typed laser emitting device for human patients. As a fundamental step in order to address this issue, we investigated immunological impact of the low level laser irradiation (10 mw laser diode with a wavelength of 630 nm) on expression of pro-inflammatory cytokines in murine immunocytes (splenocytes and peritoneal macrophages) *in vitro*. The LLLI on lipopolysaccharide (LPS 100 ng/ml)-stimulated murine splenocytes and macrophages, clearly down regulated mRNA expression of TNF-α and IL-12 in dose-dependent manner. In addition, LLLI significantly inhibits the NO production in the LPS-stimulated murine macrophages. This data suggests that LLLI (wavelength of 630 nm) may exert an anti-inflammatory action via modulation of pro-inflammatory cytokine and NO production pathway.**

**Keywords:** low level laser irradiation(LLLI), pro-inflammatory cytokine, nitric oxide (NO), RT-PCR

Low Level Laser Therapy (LLLT) has been widely used in medicine, mostly focused on recovery from pathological condition, such as injury of biologic tissues (skin, connective tissue, cartilage, bone, neuron)[1]. The innate immune cells comprising of such biologic tissues is essential to rapid recovery of damaged tissues. In that context, nanophoton from diverse sources should have interaction with molecular components of innate immune cells. This biostimulative application using nanophoton such as lower power laser has been limited to the relief of particular symptom/disease entity, such as pain and trauma. In relation with such limitations, one of the major reasons is the magnitude of immune responses in the nanophoton activated-innate immune cells has been generally difficult to explore. Another reason is that an ascription to vast diversities and complexities of nanophoton system device (sources, wave length, energy, way of irradiation)[2-5]. For instance, He-Ne laser irradiation with various wavelengths (600-1,000 nm) affects biologic behaviors (proliferation, differentiation) of innate immunocytes, such as macrophages, lymphocytes, fibroblasts and keratinocytes[6-9], consequently regulating the secretion of hormone, cytokine and growth factor from innate immunocytes *in vitro* as well as *in vivo*[10,11]. Of the diverse nanophoton systems, we hypothesized that the diode typed lower power laser with 630 nm wave length may have anti-inflammatory function. In clinical setting, pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin-6 (IL-6), interleukin-12 (IL-12) and interleukin-1 beta (IL-1β) play a pivotal role in causing inflammatory, which are such as rheumatoid arthritis, diabetes, Crohn's disease, multiple sclerosis and sepsis. However, there are few data regarding the down-regulation of pro-inflammatory or possible Th1 immune response triggered by diode (10 mw, 630 nm) laser irradiation.

Beside pro-inflammatory cytokines, nitric oxide (NO) has been implicated in a variety of pathophysiological conditions including atherosclerosis, inflammation, and carcinogenesis[13-15]. Since iNOs is responsible for the overproduction of NO in inflammation, it has become a new target to develop new substances for the treatments of chronic inflammatory diseases[18]. Here, we report that the diode typed-lower power laser irradiation may affect on mRNA profiles of pro-inflammatory cytokines, NO production in LPS-sensitized mouse splenocytes and peri-

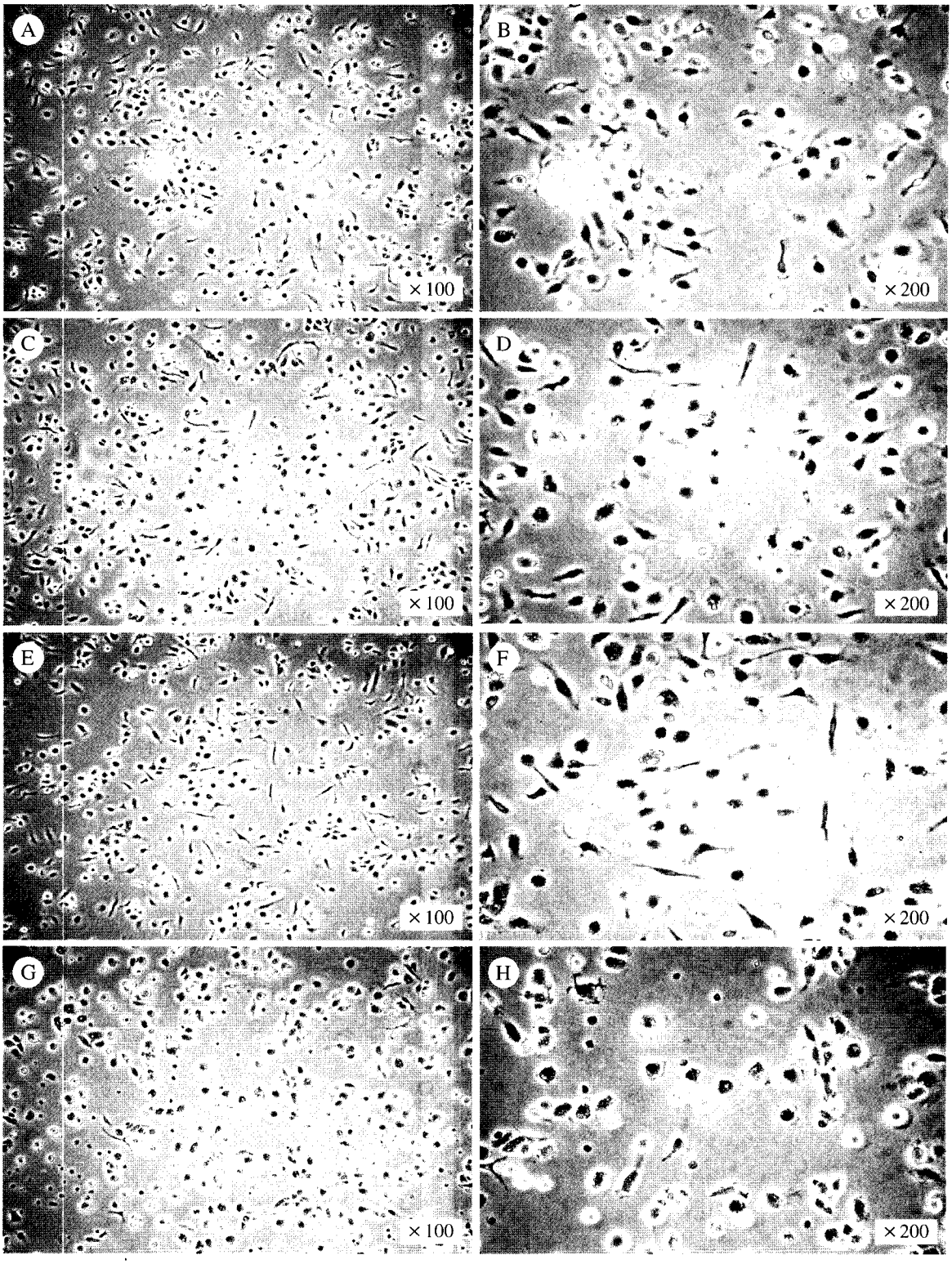


**Fig. 1.** Microphotographs of the mouse peritoneal macrophages after laser exposure. Mouse macrophages ($3.5 \times 10^6$ cells/ml) were isolated from 3% thioglycollate medium-induced C3H/He N mouse peritoneal cavity, and were stimulated with 100 ng/ml of LPS for 24 h then treated with low level laser irradiation for the last 10 min, 30 min of culture. A and B: control; C and D: 100 ng/ml of LPS; E and F: LPS +irradiation 10 min; G and H: LPS + irradiation 30 min.

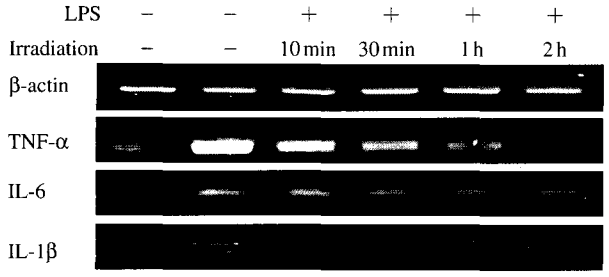


**Fig. 2.** Low level laser irradiation down-regulates pro-inflammation cytokines-TNF-α, IL-1β and IL-6 mRNA expression in LPS-stimulated mouse splenocytes. Mouse splenocytes ($5 \times 10^6$ cells/ml) were isolated from C3H/He N mouse, then stimulated with 100 ng/ml of LPS for 24 h, and treated with low level laser irradiation for the last 10 min, 30 min, 1 hours, 2 hours of culture. The gene expression was analyzed by semi-quantitative RT-PCR as described in Methods. The PCR products were electrophorased in 1.2% agarose gel.

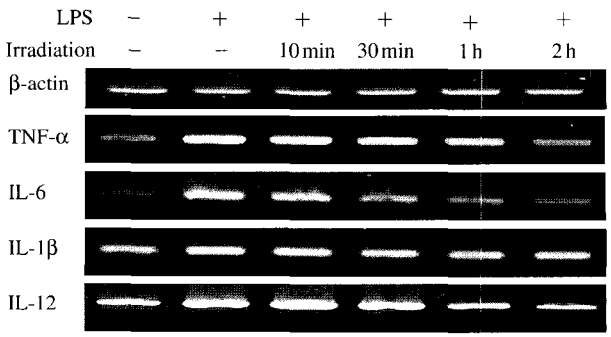


**Fig. 3.** Low level laser irradiation down-regulates pro-inflammation cytokines-TNF-α, IL-1 β, IL-6 and IL-12 m RNA expression in LPS-stimulated mouse macrophages. Mouse macrophages ($5 \times 10^6$ cells/ml) were isolated from 3% thioglycollate medium-induced C3H/He N mouse peritoneal cavity, then were stimulated with 100 ng/ml of LPS for 24 h and treated with low level laser irradiation for the last 10 min, 30 min, 1 hours, 2 hours of culture. The gene expression was analyzed by semi-quantitative RT-PCR as described in Methods. The PCR products were electrophorased in 1.2% agarose gel.

toneal macrophages implying that this type of laser irradiation *in vivo* may hold potential to attenuate pro-inflammatory dominant condition.

## Downregulation of Pro-inflammatory Cytokine Gene Expression by Lowe Level Laser Lrradiation

The pro-inflammatory cytokines-TNF-α, IL-6 and IL-1β mRNA expression was clearly upregulated in both the 100 ng/ml LPS-stimulated murine splenocytes (Fig. 2) and peritoneal macrophages (Fig. 3). However, with the laser irrardiation on the same type of cells, the mRNA expression of pro-inflammatory cytokine were profoundly reduced. Moreover, their mRNA expression were proportionally in decrement depending on the exposure interval (10 min, 30 min, 1 hours and 2 hours). In concert, the mRNA expression of pro-inflammatory IL-12 was decreased in LPS-stimulated macrophages. To elucidate the cytotoxicity effect evoked by laser irradiation, we examined the viability of murine splenocytes and peritoneal macrophages. There are no significant cellular reduction in numbers as well as morphological alteration (Fig. 1).

It has been well known that laser irradiation with various wavelengths (600-1,000 nm) affects biologic behaviors (proliferation, differentiation) of innate immunocytes, such as macrophages, lymphocytes, fibroblasts and keratinocytes[6-9], which consequently regulates the secretion of hormone, cytokine and growth factor from innate immunocytes *in vitro* as well as *in vivo*. Despite growing body of evidences in benificial effect of laser irradiation, the impact on innate immune systems by LLLI was not formulated. For instance, it was reported the Ga-Al-As diode low-energy laser (830 nm, 3.95-7.90 J/cm$^2$) inhibited IL-1 (production and gene expression in human gingival fibroblasts[12]. Conversely, Funk *et al.* reported Helium -neon (632.8 nm) laser irradiation (energy : 18.9 J/cm$^2$) increased the m RNA level of IL-1alpha, IL-2, TNF-alpha and INF-gamma in human periphearal blood mononuclear cells (PBMC)[5]. These results suggest that low laser irradiation might enable to modulate the expression of cytokine gene in the innate immunocytes. However, there is clear limitation to generalize the immuno-biologic effects elicited by the specified laser irradiation. To clarifiy this issue, we first examined the sensitive innate immune parameter [mRNA expression of proinflammatory cytokine (TNF-α, IL-6, and IL-1β)] in murine splenocytes and macrophages by usage of the diode typed low level laser (630 nm of wavelength), which would be easily applicable to human patients with chronic inflammatory disease. Because those cytokines have been play an important role in causing inflammatory disease, which are rheumatoid arthritis, diabetes, Crohn's disease, multiple sclerosis and sepsis. Our RT-PCR results showed that TNF-α, IL-6 and IL-1β mRNA in the primary murine splenocytes were constitutively expressed and further upregulated following treatment with 100 ng/ml of LPS. When the cells were irradiated with the diode typed laser, the mRNA expressions of cytokines (TNF-α, IL-6 and IL-1β) were definitely decreased when compared to those of LPS-unstimulated cells. This inhibitory effect is well proportional to the duration in laser irradiation. This

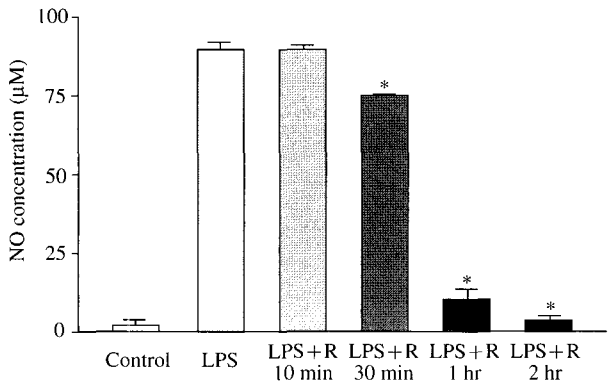


**Fig. 4.** Suppression of the NO production in 24 hr LPS sensitized murine macrophages after laser exposure. Mouse macrophages ($5 \times 10^6$ cells/ml) were isolated from 3% thioglycollate medium-induced C3H/He N mouse peritoneal cavity, then were stimulated with 100 ng/ml of LPS for 24 h and treated with low level laser irradiation for the last 10 min, 30 min, 1 hours, 2 hours of culture. Data are mean ± S.D. of a representative experiment repeated three times. *$p < 0.01$, vs. LPS.

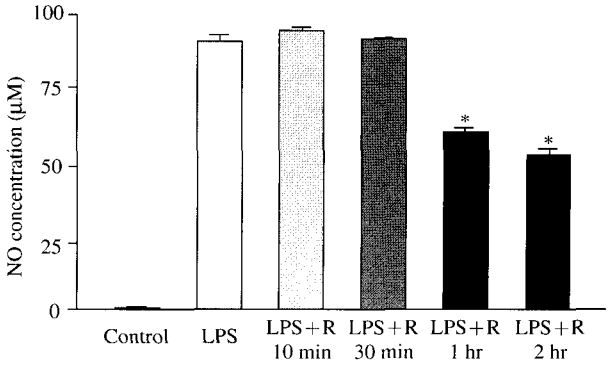


**Fig. 5.** Suppression of the NO production 48 hr LPS sensitized murine macrophages after laser exposure. Mouse macrophages ($5 \times 10^6$ cells/ml) were isolated from 3% thioglycollate medium-induced C3H/He N mouse peritoneal cavity, then were stimulated with 100 ng/ml of LPS for 48 h and treated with low level laser irradiation for the last 10 min, 30 min, 1 hours, 2 hours of culture. Data are mean ± S.D. of a representative experiment repeated three times. *$p < 0.01$, vs. LPS.

effect was more clear in primary murine peritoneal macrophages. In murine macrophages exposed to laser light, there was a clear attenuation of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) mRNA expression. Interestingly, the IL-12 mRNA expression was down-regulated in LPS-stimulated murine macrophages by LLLI. IL-12, is a key immunoregulatory molecule in Th1 responses, which is produced by phagocytic cells and antigen-presenting cells within a few hours of infection (particularly, intracellular parasites). Furthermore, IL-12 favors the differentiation and function of Th1 cells while inhibiting the differentiation of Th2 cells. Thus this data holds an important clinical implication that LLLI would be a effective, noninvasive strategy to counteract Th1 dominant disease setting. Unfortunetly, we did not peform *in vivo* experiment using Th1 dominant animal model.

The anti-inflammatory effects of low-level laser irradiation in *in vivo* experimental models have been previously reported. For instance, Honmura *et al.*[6] demonstrated that Ga-Al-As diode laser irradiation inhibited carrageenin-induced inflammation in rats. Furthermore, low-level laser therapy is widely used clinically for rheumatoid arthritis with satisfactory results[6,7]. These findings suggest that the inhibitory effect of LLLI on TNF-α, IL-6 IL-12 and IL-1β gene expression can be regarded as an anti-inflammatory action reflecting an *in vivo* event, thereby conferring scientific relevance to our LLLI *in vitro* study.

## Suppression of NO Production from Murine Macrophages by Lowe Level Laser Lrradiation

Nitric oxide (NO) has been implicated in a variety of pathophysiological conditions including atherosclerosis, inflammation, and carcinogenesis[13-15]. Since iNOS is responsible for the overproduction of NO in inflammation, also LPS and/or cytokines induces the expression of the iNOS isoform in many cell types[16], including macrophages[17]. Therefore, NO has become a new target to develop new substances for the treatments of chronic inflammatory diseases[18]. In our experiments, we demonstrate that LLLI inhibits the production of NO in cultures LPS-stimulated murine primary macrophage cells in a dose-dependent manner (Figs. 4, 5). As NO is generally known as pro-inflammatory mediators and oxidative stressors in cells, their excessive extracellular production result in the damages of a variety of normal defense cells. In this point, the NO suppressive result by simple laser exposure suggests that LLLI might own additional anti-inflammatory mechanism.

As we observed, this combined data indicate that LLLI (wavelength of 630 nm) may exert anti-inflammatory action via down-regulation of pro-inflammation cytokines gene expression in LPS-stimulated primary murine splenocytes and peritoneal macrophage cells, as well as through the suppression of NO production in LPS-stimulated primary murine peritoneal macrophages *in vitro*.

**Table 1.** primer sequences used for the amplification of target gene and β-actin

| Target gene | Sequence (5′ → 3′) | Size |
|---|---|---|
| β-actin sense | GGAATCCTGTGGCATCCATGAAAG | 348 bp |
| Anti-sense | TAAAACGCAGCTCAGTAACAGTCCG | |
| TNF-α sense | GGCAGGTCTACTTTGGAGTCATTG | 299 bp |
| Anti-sense | ACATTCGAGGCTCCAGTCAATTCGG | |
| IL-1β sense | CTAAAGTATGGGCTGGACTG | 177 bp |
| Anti-sense | GGCTCTCTTTGAAC GAATG | |
| IL-6 sense | AATGATGGATGCTACCAAAC | 281 bp |
| Anti-sense | TAGCCACTCCTTCTGTGACT | |
| IL-12 sense | GAGGTGGACTGGACTCCCGA | 618 bp |
| Anti-sense | CAAGTTCTTGGGCGGGTCTG | |

## Methods

### Preparation of Peritoneal Mouse Macrophages and Splenocytes

Three percent thioglycollate medium (3ml) was injected intraperitoneally into C3H/He N mice as a stimulant to recrunt peritoneal macrophages. Three days after injection, the inflamed peritoneal cells were harvested by peritoneal lavage with PBS. The exudates were centrifuged at 1,000 rpm, 25°C for 20 min. The erythrocytes in the cell pellets were lyzed by sodium ammonium (0.83% $NH_4Cl$). Isotonicity was restored with PBS. The cell suspension was centrifuged and the cells were washed twice and re-suspended in RPMI-1640 (Invitrogen corporation, USA) supplemented with 10% heat-inactivated FBS. The cell number was adjusted to $1 \times 10^6$ cell/ml. The trypan-blue dye exclusion was used to determine the viability of macrophages.

The mice were sacrificed and their spleens were removed aseptically. The cell suspension was prepared by menans of lose potter and flushing. After centrifugation at 1,000 rpm for 10 min at 25°C, erythrocytes were lysed by sodium ammonium (0.83% $NH_4Cl$) and the cell pellets were washed twice with RPMI-1640. The cells were re-suspended in complete RPMI-1640 medium, and the cell number was adjusted to $1 \times 10^6$ cell/ml. The viability of splenocytes was determined by the trypan-blue dye exclusion.

### Laser Irradiation

A diode laser, which has a continuous wavelength of 630 nm and a maximum power output of 3 mW, was constructed and used in this study. The duration of exposure were 10 min, 30 min, 1 hr and 2 hr, respectively. The total energy in this experiment with these exposures were corresponded to 5.1 J/cm$^2$ (1.8 J/plate), 15.3 J/cm$^2$ (5.4 J/plate), 30.6 J/cm$^2$ (10.8 J/plate) and 61.2 J/cm$^2$ (21.6 J/plate). After irradiation, the cells were washed twice with PBS and treated with TRIzol® for the RNA extraction.

### RNA Preparation and Semi-quantitative. RT-PCR

RNA was extracted from the LPS-stimulated-primary mouse macrophages and splenocytes, with or without treated LLLI, using the TRIzol® extraction method (Invitrogen, Burlington, ON), and cDNA reverse was transcribed from 2 μg of total RNAs. Reverse transcriptase system with oligo d (T) 12-18 primer at 42°C for an 1 hr. resulting cDNA was amplified by the manufacturer under the following conditions: One hold at 94°C for 5 min; 30-35 cycles at 94°C 30 s, 55-60°C 30-60 s, and 68-72°C for 30-90 s; and one hold of 72°C for 7 min. The primers for PCR amplification show in Table 1. PCR-amplified DNA fragments were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

### Nitric Oxide Assay

Nitric oxide (NO) production was evaluated by measuring the accumulation of nitrite, the stable metabolite of NO. The amount of NO produced by mouse peritoneal macrophage under the different conditions, was determined by examining the culture supernatants for the stable end product, nitrite, using an automated procedure based on the Griess reaction. Briefly, 100 μl of the culture medium was mixed with an equal volume of Griess reagent (0.1% N-1-aphthyl -ethylenediamine dihydrocholate and 1% sulfanilamide in 5% phosphoric acid) (Sigma, USA). After a 10 min incubation period at room temperature, the absorption was measured at 540 nm. The nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

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