



## Differential sensitivity of polytene chromosomes to Nd:YAG laser

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### ABSTRACT

A fundamental understanding of the interaction of Nd:YAG lasers with biological tissues have remained a challenging field demanding further investigation, especially exploring the threshold level of chromatin damage. The present study aims at addressing these questions using an insect derived model tissues, namely salivary glands (SG) and malpighian tubules (MT) of a midge *Chironomus ramosus*. Both SG & MT have polytenized chromosomes in their nuclei with a variable number of chromatids. Upon Nd:YAG ( $\lambda = 1064$  nm) laser irradiation, using a fixed dose with variable time of exposure, SG & MT showed different levels of sensitivity. The onset and rate of induction of pyknotic nuclei and the threshold level of laser-induced pyknosis of chromatin material were dependent on exposure time. Parameters used for laser irradiation were non-ablative in order to avoid thermal injury. The temperature of the samples was kept controlled within  $\pm 1$  °C and did not vary during laser exposure. Our findings suggest that non-ablative laser-induced cell death is dependent on chromatin organization.

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**Keywords:** Nd:YAG laser, Polytene chromosomes, Chironomus, chromatin integrity, ploidy level.

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### INTRODUCTION

Almost four decades ago, laser emission from neodymium-doped glass was first produced. Later on, crystals of yttrium, aluminum and garnet (YAG) were found to be a better lasing host for neodymium (Nd). Since then Nd:YAG laser has been used for various clinical applications (Mainman, 1960; Mester et al., 1985; Beach et al., 1993; Knappe et al., 2004). The application of Nd:YAG laser in hair removal (Wanner, 2005), skin resurfacing (Papadavid and Katsambas, 2003; Trelles et al., 2005; Bellew et al., 2005), cartilage reshaping (Wright et al., 2005), ophthalmology (Schwartz and Budenz, 2004; Aslam et al., 2004) and cancer therapy (Ishikawa et al., 2003; Foroulis and Thorpe, 2006) have rapidly developed over the last couple of years. Since 1996, besides Nd:YAG, other sources of laser had been explored as effective devices for hair

removal and cosmetic applications. But long-pulsed Nd:YAG ( $\lambda = 1064$  nm) laser has been preferred for hair removal (Wanner, 2005). It is noteworthy that many conflicting reports have also generated doubts regarding its efficacy and safety (Liew, 2002). Nevertheless, optimal safety parameters continue to demand further investigation. A large number of studies, reported during the past one and half decade, extensively dealt with different factors that determine laser-tissue interaction (Niemz, 2003; Welch and van Germert, 1995; Beach et al., 1993; Dederich, 1991). However, less attention was paid to the tissue death at the cellular level, especially with reference to chromatin damage.

Safety parameters for different types of lasers and its interaction with various types of tissues have remained a debatable issue and there is a considerable need for a review on

the doses for safe application modality. We also need to ensure a safety regime for the workers in order to avoid occupational hazards resulting from accidental exposures (Mainman, 1960; Niemz, 2003; Lucas et al., 2002). Despite the increasing and successful application of laser in medicine, deleterious side effects of Nd:YAG irradiation on glandular tissues remain mostly unknown. Moreover, there are no therapeutic clinical procedures that use laser irradiation to treat glandular tissues. The present study aims at investigating the effect of Nd:YAG ( $\lambda = 1064$  nm) laser on simple tissues like salivary gland and malpighian tubules of an insect *Chironomus*, that has been reported to be an excellent model for cytotoxicity studies (Nath et al., 2005). Interestingly, the chosen tissues have variable quantity of chromatin materials (Macgregar and Varley, 1988) rendering them suitable for a comparative study to investigate the effect of laser irradiation on chromatin integrity. The present study also addresses the question whether a variable amount of chromatin material has any relevance to non-ablative laser-induced tissue death.

## MATERIALS AND METHODS

### Model organism and tissue sample

The *in vitro* study was carried out using a tropical insect *Chironomus ramosus*, commonly called 'midge'. An inbred line of this insect was reared under laboratory conditions following the methodologies described by Nath and Godbole (1998). Aquatic larvae of fourth developmental instar were selected from a synchronously grown culture. Tissue samples from two different sources, namely salivary glands and malpighian tubules were chosen for laser irradiation. Both salivary glands and malpighian tubules remain suspended in the hemolymph, and the dissection was carried out in 0.67% NaCl, isotonic to hemolymph.

### Experimental set up for Nd:YAG laser irradiation

Both salivary gland and malpighian tubules were irradiated using a CW mode Nd:YAG laser ( $\lambda = 1064$  nm) with a fixed dose of 1.8 W for a variable time of exposure (0 minute to 30 minutes) to laser. A non-ablative moderate dose was chosen based on the literature survey of different clinical trials

reported till date, which did not lead to thermal injury to tissues. The sample was placed 50 cm away from the laser source and irradiated as per the focal spot sizes encompassing salivary gland and malpighian tubules. The size of two experimental samples viz. salivary gland and malpighian tubule were different which necessitated the variation of exposure areas in order to fit these samples under the focal spot of the laser beam. The resultant fluences were 0.77 kJ/cm<sup>2</sup> and 0.101 kJ/cm<sup>2</sup> for the salivary glands and malpighian tubules respectively.

The experiments were carried out in a temperature-controlled room. During the laser irradiation treatment, ambient temperature remained constant around  $25 \pm 1$  °C. In order to validate the extent of temperature variation of the surrounding fluid in which the samples were incubated, a thermocouple microelectrode (OMEGE Engineering India, Hydrophobic probes) was used. It was ensured that the temperature varied very little within a narrow range of  $\pm 1$  °C, during the entire exposure time.

### Cytological assay to estimate percent pyknosis

Control (without laser treatment) and treated (laser irradiated for varying interval of time) salivary glands and malpighian tubules were transferred from 0.67% NaCl ringer solution to 1:3 acetic acid-methanol fixative for 1 minute. The fixative treated samples were stained in 2% aceto-orcein for 30 minutes, and then these stained samples were dissociated in 45% acetic acid. A clean cover glass was placed onto the preparation for thumb squashing. The cytological preparations were examined under a Zeiss 135 Axiovert microscope. Pyknosis is a cytological parameter of chromosomal damage, which ultimately results in cell-death. Both salivary glands & malpighian tubules possess polytene chromosomes in their cell nuclei. The loss of typical band-interband demarcation and condensation of chromatin material were taken as the endpoint while identifying and scoring the pyknotic nuclei. Percent pyknotic nuclei (mean  $\pm$  S.E) were plotted against the exposure time and the LT<sub>50</sub> ('Lethal Time' taken to obtain a score of 50% pyknotic nuclei) values were determined. Ten

replicates of each set of experiments were performed.

## RESULTS AND DISCUSSION

For the *in vitro* Nd:YAG laser irradiation experiment, salivary glands and malpighian tubules of *Chironomus* were chosen as target tissues due to differences in their DNA content. Unlike diploid cells of other tissues, cells of salivary glands and malpighian tubules contain nuclei with polytene chromosomes. These are giant chromosomes produced by multiple cycles of replication without division, and consist of identical sets of chromatids aligned side by side in a banded cable-like pattern (Macgregar and Varley, 1988). Interestingly, in *Chironomus*, the degree of polytenization varies between malpighian tubule and salivary gland cells. There are thirteen rounds of continuous replication and as a result, polytenization occurs in the nuclei of salivary gland ( $2^{13} = 8192$  chromatids aligned side by side). On the contrary, in malpighian tubules, there occur only nine rounds of continuous replication cycles ( $2^9 = 512$  chromatids form polytene chromosomes). Therefore, polytene nuclei in malpighian tubules contain 16 fold less chromatin material in comparison to those of salivary glands of *Chironomus* (Macgregar and Varley, 1988; Deneholt and Edström, 1967).

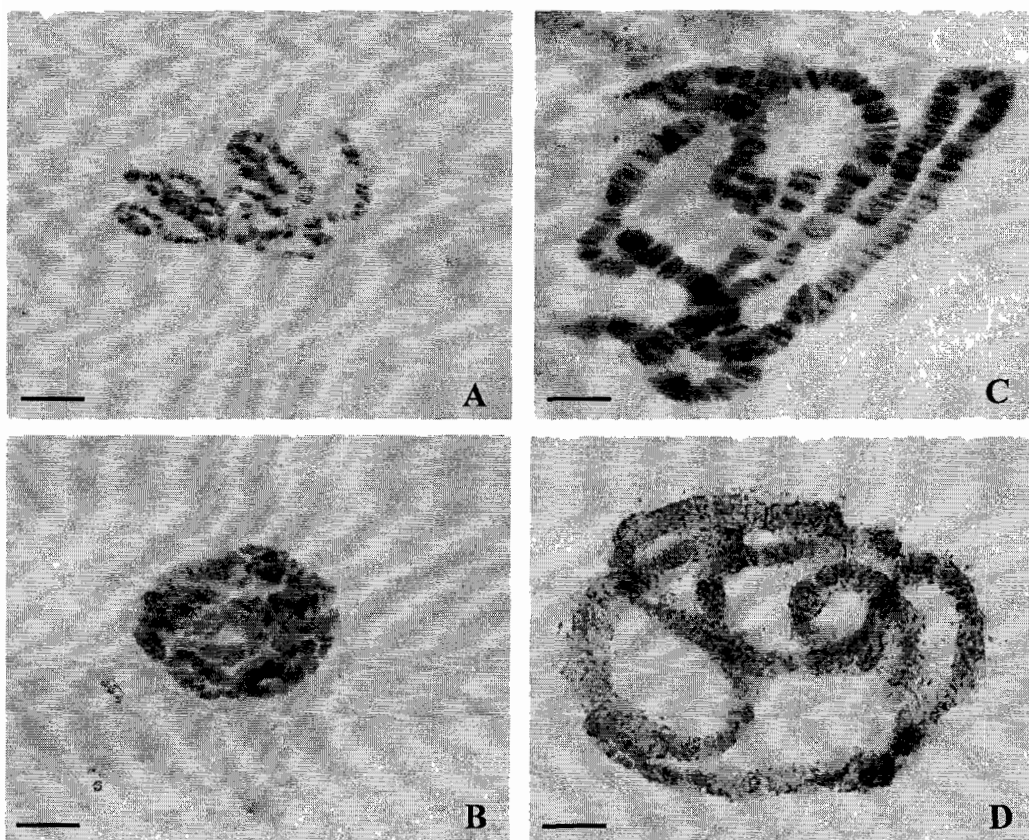
Nd:YAG laser irradiation of malpighian tubule leads to rapid pyknosis during the initial few minutes of exposure, which gradually reaches a plateau after 10 minutes. The time required for 50% pyknosis ( $LT_{50}$ ) was calculated as 3 minutes 22 seconds as extrapolated from the graph (Figures 1 A,B and 2). On the other hand, when the salivary gland was subjected to Nd:YAG laser irradiation using the same dose as applied to malpighian tubule, the nuclei did not show any sign of pyknosis till 10 minutes of initial exposure. Subsequently, the nuclei showed a sign of pyknosis at a slow rate and only after 22 minutes, a sharp increase in the number of pyknotic nuclei was observed. Thereafter, the pyknotic nuclei population reached a saturation level. The time required for a 50% pyknosis ( $LT_{50}$ ) for salivary gland nuclei was calculated as 23 minutes 15 seconds (Figures 1 C,D and 3 ). The difference in  $LT_{50}$  values between salivary gland and malpighian

tubules was approximately 7 fold, whereas the degree of polytenization differed by approximately 16 times (Macgregar and Varley, 1988; Deneholt and Edström, 1967). The data clearly indicated that the more the amount of chromatin material (ploidy level), the longer the time required for pyknosis.

Three decades ago, there had been attempts to target chromosomes using laser irradiation (Berns, 1974). These manipulative techniques aimed at isolation, fragmentation and gene-mapping without addressing the relative role of chromatin damage in cell/tissue viability. Later on, condensed chromosomes were manipulated during cell division using laser-mediated optical trapping (Vorobjev et al., 1993). Most of these studies associated chromatin damage with thermal injury resulting from laser-irradiation.

Although in the past, there had been studies on cytotoxic and mutagenic effects of Nd:YAG laser on mammalian cells (Kaido et al., 2002), the wavelength used was shorter ( $\lambda=213$  nm) than what was applied in the present study. DNA damage in diploid cells by Nd:YAG laser microbeam was reported in NC31 lymphoblast cells by laser irradiation, although no DNA damage was observed by Mohanty et al., (2002) within the wavelength range of 800 – 1064 nm range. Mozdarani and Monfrated (2001) reported that clastogenic effects could be enhanced by Nd:YAG laser irradiation coupled with hyperthermia in HeLa cells. However, the paper did not focus directly on chromosome damage. Elevation of temperature in laser-irradiated tissues depends on a number of parameters like power, spot size and irradiation time. The aim of our study was to optimize laser-parameters in such a way so as to avoid thermal injury due to hyperthermia. This was achieved using a fixed dose and fixed distance of irradiation from the source without any rise in ambient temperature. The only variable parameter was the duration of exposure to laser. Therefore, this is a significant finding where pyknosis, i.e. chromatin damage, could be induced without thermal intervention using non-ablative mode of laser irradiation.

The effect of Nd:YAG laser irradiation was seen on monolayer fibroblast cell cultures using vital staining and cytomorphological analysis (Gulknecht et al., 1998). However,

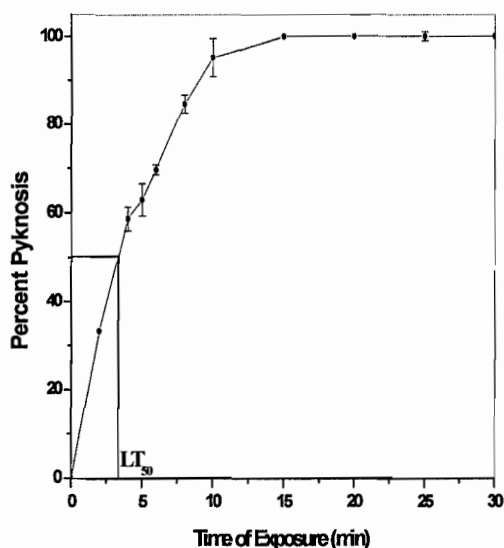


**Figure 1:** Photomicrographs showing polytene chromosomes from (A) control and (B) irradiated pyknotic nuclei of malpighian tubule and (C) control and (D) irradiated pyknotic nuclei of salivary gland cells. Bar represents 10 $\mu$ m.

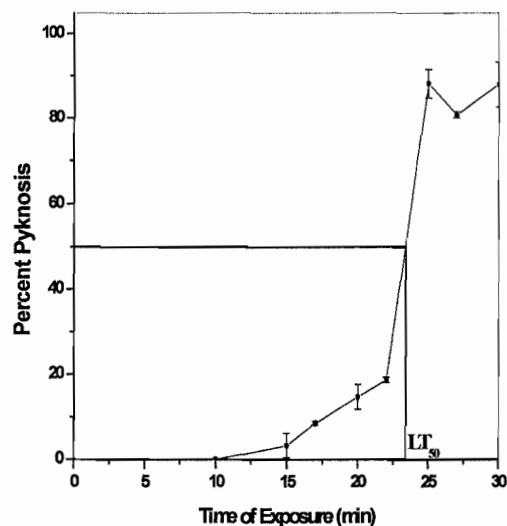
parameters used for these cultured cells cannot be equated with the intact tissues, as attempted by us. Moreover, the pyknotic cell death reported by Gulknecht et al. (1998) did not clearly demarcate the nuclear zone with that of cytoplasmic area while referring to chromatin damage. Their study also did not address the question of threshold level of Nd:YAG laser for effective chromatin damage in a diploid cell. Keeping these earlier reports in the background, our study is the first of its kind to report that the effect of Nd:YAG laser ( $\lambda = 1064$  nm) on the chromatin material depends on its mass. The nuclei with a variable amount of compact chromatin materials have differential threshold level of laser-induced damage, which is a function of duration of exposure.

Now-a-days, Nd:YAG laser has been introduced in cancer therapy (Foroulis and

Thorpe, 2006; Ishikawa et al., 2004) especially for organs like liver (Nikfarjam et al., 2005) which is known to possess polyploid cells with a variable number of chromosomes. In order to block tumor progression augmented by laser irradiation, one can optimize an effective dose for aiming at chromatin damage based on the ploidy level of the cell leading to tumour regression, rather than relying on the conventional thermal laser ablation which can lead to side-effects. The polytene model system with variable chromatin content, chosen in the present study provided the opportunity to test this hypothesis using non-ablative laser-induced cell death. The preliminary results presented here clearly indicated that resistance of chromatin material to Nd:YAG laser radiation is a function of its compactness and density. Our study opens up a future avenue of



**Figure 2:** Effect of increase in exposure time on the extent of pyknosis of malpighian tubules' polytene chromosomes irradiated by Nd:YAG laser.



**Figure 3:** Effect of increase in exposure time on the extent of pyknosis of salivary glands' polytene chromosomes irradiated by Nd:YAG laser.

investigation to find out the molecular nature of laser-induced chromatin damages, and the extent of DNA and protein denaturation.

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