Laser-induced hyperthermia for treatment of granulation tissue growth in rats

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OBJECTIVE: We aimed to develop a new technique for treatment of granulation tissue (GT) growth using local hyperthermia.

METHODS: A temperature-controlled diode laser system was developed for induction of mild hyperthermia in real time. GT was generated by harvesting the skin over the gluteal fascia in rats. Histopathological analysis was used to estimate the effect of hyperthermia on the tissue.

RESULTS: In untreated rats, GT was detected within 3 days and reached maximal thickness after 12 days. Hyperthermia at 43°C and above significantly decreased GT thickness (n = 8 per group). Hyperthermia at 48°C for 3 minutes was the most efficient parameter for treatment of GT (51% reduction), with minimal (5%) muscle necrosis.

CONCLUSIONS: Hyperthermia can significantly inhibit GT growth, with minimal damage to surrounding structures. Our findings suggest a possible role for hyperthermia as a therapeutic model against GT. Further research and long-term studies are needed to explore the utility of laser-induced hyperthermia for inhibition of GT growth.

Granulation tissue (GT) can be macroscopically described as small, red, granular foci that bleed easily and are commonly seen within freshly healing tissue. Granulations typically develop after removal of the skin or mucosal epithelium. Microscopically, GT involves clusters of evolving capillary blood vessels, along with fibroblasts and inflammatory cells, which migrate and proliferate into the wound during the early stage of healing.1 In most cases, GT proliferation is associated with bacterial invasion. In the trachea, GT may develop after insult to the mucosa, with removal of the respiratory epithelium deep to the basal membrane. The tracheal epithelium can be damaged by prolonged intubation, by crush or penetrating injury, by endoscopic surgery, or after open surgical procedures (laryngotracheal reconstruction, cricoid split, cartilage grafts, or end-to-end anastomosis). Iatrogenic injury may result from either blunt or sharp dissection, or from laser surgery. Development of GT in the larynx, subglottis, trachea, or bronchi can cause hoarseness, stridor, or hemoptysis. In severe cases, GT may cause significant airway obstruction.

The treatment of GT is difficult. Antibiotics and systemic steroids are the mainstay of treatment in mild to moderate airway obstruction, and surgical procedures are often necessary for severe cases. Mitomycin C has been used as an antiproliferative agent and applied topically for laryngotracheal GT.2 However, none of these methods offers definitive eradication of the process, and frequent surgical procedures are common owing to high recurrence rates.3

In recent years, controlled hyperthermia has been used for tumor ablation. Hyperthermia may be induced by different methods, including radio waves, microwaves, ultrasound, and lasers.4 With the use of this modality, several studies have demonstrated regression and even complete eradication of tumors with minimal damage to neighboring organs.5 A possible explanation for the increased susceptibility of cancer cells to hyperthermia is based on their impaired ability to repair thermal damage compared with normal tissue.6 In theory, the effect of hyperthermia on GT may be comparable to that on neoplastic tissue owing to similar proliferative qualities of both tissues.

A temperature-controlled laser system is a relatively new technique for heating biological tissues by means of a laser beam. It has been used in the repair of bladder and urethral defects, skin, and dura.7,9 Our objective was to develop a temperature-controlled laser heating system for the treatment of GT growth by inducing local hyperthermia. A novel rat model was developed to identify the optimal parameters (temperature and duration) for GT ablation without damaging adjacent tissue.
MATERIALS AND METHODS

We developed a fiberoptic laser system for temperature-controlled heating of biological tissue. The diode laser (800 nm, infrared) was chosen for this project, because its radiation can penetrate up to 2 to 4 mm into tissue, and because it can be easily used by the surgeon without injuring deeper layers. The basic idea was to use a noncontact laser that can be delivered endoscopically through an optical fiber for heating GT for different durations.

Temperature-Control Laser Apparatus

The temperature-controlled laser system has been fully described in our previous publications for the carbon dioxide laser. In brief, the system includes a diode laser (Lasers Industries Ltd, Tel Aviv, Israel) emitting at \( \lambda = 0.8 \mu \text{m} \), an infrared (IR) detector, an IR fiber, and a personal computer. The laser power usually reached 10 to 12 W. For laser delivery, conventional fibers with a diameter of 0.7 mm and lengths of 2 m were used. IR radiation was measured with a parallel optical fiber that was prepared in our laboratory from poly-crystalline silver halides (AgCl, Br, \( 0 < x < 1 \)). These fibers were highly transparent in the IR range, were flexible, and were nontoxic and biocompatible. The two fibers were placed in a holder (10 cm long by 1 cm wide) so that the distal ends of the fibers were placed 2 mm from the surface of the GT. The proximal end of one fiber (“power fiber”) was attached to a diode laser, and the laser radiation was focused into the proximal end of this fiber. The laser beam emitted from the distal end heated a spot on the surface of the tissue. The heated spot emitted IR radiation whose intensity \( I \) was determined by the temperature \( T \). The emitted radiation was collected by the distal end of the second IR fiber (“sensor fiber”). The radiation was transmitted to the proximal end of this fiber and then focused onto an IR detector. The computer measured the voltage signal from the detector and used it to determine the temperature of the heated spot. A computer program was used for feedback control so that if the temperature was too low, the laser power increased and vice versa. This noncontact measurement and control of temperature allowed an accurate investigation of induced hyperthermia and simulated the possibility of endoscopic application.

Laser-induced hyperthermia of the tissue was achieved by holding the laser apparatus 2 mm above the wound, reaching the predetermined temperature within 30 to 35 seconds (42°C-51°C). The method of holding the laser above the tissue requires training and was practiced multiple times before the experiments were initiated. This working distance can be easily maintained, resulting in a relatively stable heating temperature over time.

Animal Model

All protocols were approved by the institutional committee for experimentation on animals. Sprague-Dawley rats (200-250 g) were used in all experiments. Surgery and laser irradiation were performed under general anesthesia. Animals were anesthetized with an intramuscular injection of 20 mg/kg Ketalar and 2 mg/kg Rompun. A portion of skin (15 mm) superficial to the gluteal fascia was excised bilaterally under general anesthesia. Following skin harvesting, GT was left to proliferate in the area of the wound.

In the experimental designs presented for comparing two groups of animals with or without hyperthermia, power analyses have been performed to predict the minimal number of animals or samples required to obtain statistical significance. This value was based on preliminary data of difference between groups. The following equation for continuous variables was used: \( N = \left( \frac{1/\alpha + 1/q_2}{\sigma_{\alpha}^2 + \sigma_{\beta}^2} \right)^2 \frac{E^2}{\sigma^2} \), where \( N \) is the required sample size, \( q_1 \) is the proportion in group 1 (0.5), \( q_2 \) is the proportion in group 2 (0.5), \( S \) is the estimated standard error of the mean for the two groups, \( z_{\alpha} \) is the standard normal deviate for alpha (1.96 when \( \alpha = 0.05 \) [95% confidence interval]), \( z_{\beta} \) is the standard normal deviate for beta (power of the test; 0.84 when \( \beta = 0.8 \) or 80%), and \( E \) is the estimated difference between the means. Utilizing data from our preliminary experiments (\( S = 0.1-0.2 \) and \( E = 0.5-0.8 \) in the formula), we determined that each group would require a sample size of at least 5. We therefore used a sample size of 8 in each treatment group. In the control group, we used a sample size of 12.

In the first phase of the study, we evaluated the rate of GT growth during a period of 20 days. We evaluated 20 animals (two wounds per day). Next, we evaluated the effect of hyperthermia on GT growth. The control group (no treatment) included 12 wounds. There were 30 treatment groups (8 wounds per group). Each group was treated with a single set of parameters including specific temperature (°C) and duration (minutes) of hyperthermia. Various temperatures were investigated, ranging from 42°C to 51°C. At each temperature, hyperthermia was induced for different time periods, until immediate tissue burn was noticed. In the 42°C hyperthermia group, the maximal duration was 20 minutes because there was no clear effect of temperature on GT growth at a shorter duration. The various parameters (temperature/duration) are provided in the Results section. Increasing tissue temperature to 56°C induced immediate tissue burn.

Histological Analysis

Histological analysis was performed on representative sections stained with hematoxylin and eosin. Five-micron sections were cut from the blocks and placed on glass slides. Two investigators including an attending pathologist (A.S.) and the first author (R.L.) reviewed and analyzed the slides independently by estimating the morphological localization of the GT and muscle. The staining characteristics of the tissue were determined by consensus among the reviewing investigators. The GT was characterized by capillary proliferation in a background of acute and chronic inflammatory cells. In some specimens, the surface was ulcerated with fibrinous exudates on the surface. Measurements were performed by each investigator independently with image analysis software (Metamorph, Molecular Devices, Down-
ingtown, PA). Five slides were analyzed in each experiment.

**Statistical Analysis**

Statistical analysis was performed with SPSS software (SPSS Inc, Chicago IL). The nonparametric, Mann-Whitney U test (two-tail) was used for independent comparison between the control and treatment groups at each variable (temperature/duration). Kruskal-Wallis test (two-tail) was used for comparison of multiple \((k)\) samples. The \((+/−)\) values and the error bars in the figure are standard deviation.

**RESULTS**

We aimed to investigate the effect of hyperthermia on GT growth. First, we established an animal model for development of GT in rats. We chose the area of the gluteus muscle fascia for two reasons: First, it is readily available bilaterally and easy to manipulate. Second, the relatively large bulk of the fascia and muscle can absorb the laser radiation, protecting internal organs from thermal damage. The thickness of the GT layer was evaluated daily for 20 days. **Figure 1** shows the wound 8 days after surgery. GT was detected within 3 days after surgery \((0.25 ± 0.1 \text{ mm})\) and reached maximal thickness 12 after surgery \((2 ± 0.3 \text{ mm})\). The time for developing 50 percent of the maximal GT thickness \((1 ± 0.2 \text{ mm})\) was 8 days. Because we aimed to evaluate the effect of temperature on GT growth, hyperthermia was induced at day 5, and its effect was evaluated 3 days later. The average thickness of GT in the control group was \(1.1 ± 0.2 \text{ mm} (n = 12)\). Tissue temperature of \(42°C\) for up to 20 minutes did not cause significant change in GT thickness \((1 ± 0.08 \text{ mm}, P = 0.7; \text{Mann-Whitney } U \text{ test})\). However, maintaining tissue temperature at \(43°C\) for 6 minutes caused significant decrease in GT thickness \((0.66 ± 0.3 \text{ mm}, P = 0.001, \text{compared with the control, Mann-Whitney } U \text{ test})\), with only minor necrosis \((5%)\) of the underlying muscle \((0.25 ± 0.1 \text{ mm thickness of muscle necrosis, Fig 2})\). Increasing the duration of hyperthermia \((at 43°C)\) to 9 minutes further reduced the GT thickness \((0.4 ± 0.08 \text{ mm}, P = 0.0001, \text{Kruskal-Wallis test})\). Further histological examination of the specimens revealed significant damage \((45%)\) to the gluteus muscle with coagulation necrosis thickness of \(2.2 ± 0.6 \text{ mm} (P = 0.0001; \text{Kruskal-Wallis test})\). The effect of hyperthermia on GT growth was further evaluated at \(44°C\) to \(51°C\) for various durations \((n = 8 \text{ per experiment})\). Laser-induced tissue hyperthermia above \(50°C\) resulted in immediate tissue burn, inducing full-thickness coagulation necrosis of the muscle (Fig 3). The results of the

**Figure 1** Granulation tissue 8 days after removal of the skin over the gluteal muscle fascia. Granulation tissue (GT) layer and muscular layer are shown (scale bar 0.5 mm).

**Figure 2** Granulation tissue at day 8, following 43°C hyperthermia applied for 6 minutes. Hyperthermia induced superficial necrosis of the granulation tissue (GT) layer. The underlying GT is congested with inflammatory infiltrates. The striated muscle is intact (scale bar 0.25 mm).

**Figure 3** Granulation tissue at day 8, following 50°C hyperthermia applied for 2 minutes. Hyperthermia-induced necrosis of the granulation tissue (GT) layer and the underlying muscular layer. The striated muscle is covered by inflammatory exudate (scale bar 1 mm).
experiments and their statistical analysis are summarized in Figures 4 and 5. The effect of hyperthermia on GT growth and on the underlying muscular layer was both temperature- and time-dependent.

Figure 6 summarizes the parameters required to reduce GT thickness by 50 percent. Our data show that the most efficient parameter for treatment of GT was 3 minutes of hyperthermia at 48°C. This induced 51 percent reduction in GT thickness and 5 percent muscle necrosis. In addition, a lower temperature (46°C) applied for 5 minutes resulted in 45 percent GT reduction with 4 percent muscle necrosis.

**DISCUSSION**

We investigated the utility of laser-induced hyperthermia for treatment of GT growth. Our working hypothesis was that controlled superficial hyperthermia delivered endoscopically by a diode laser can provide physiological and technical benefits by 1) reducing GT growth shortly after the procedure and 2) causing minimal damage to neighboring organs.

Previous studies have examined the use of hyperthermia mainly for tumor ablation. These techniques have been
used for ablations of both benign and malignant tumors and as sensitizers before chemotherapy or radiotherapy.\cite{4,11} Our study is the first to evaluate the use of laser-induced hyperthermia for ablation of GT. For this purpose, we have used a novel laser system with an optic sensor for controlling superficial tissue temperature. In our setup, an optically delivered diode laser was used to induce hyperthermia at 42°C to 51°C with accuracy of $\pm1$°C. The diode laser was chosen because its beam could penetrate through the surface and cause hyperthermia to cells lying in deeper layers. The diode laser has properties similar to those of the neodymium: yttrium-aluminum-garnet (Nd:YAG) laser; however, it is smaller, lighter, and more compact than the Nd:YAG laser, making it ideal for treating tracheobronchial granulations.

Our results indicate that prolonged hyperthermia at 42°C has no effect on GT growth. However, increasing tissue temperature to $\geq 43°C$ can promote cell destruction, de-

**Figure 5** The effect of hyperthermia on the underlying muscle at day 8. Increasing tissue temperature from 43°C to 51°C induced muscle necrosis ($n = 8$ wounds per point). $P < 0.0001$ for 43°C to 48°C, $P = 0.02$ for 49°C, $P = 0.001$ for 50°C to 51°C (Kruskal-Wallis test).
creasing GT thickness. We also showed that prolonged hyperthermia can cause significant damage to the underlying tissue. Taken together, our experiments show that hyperthermia at 48°C applied for 3 minutes was the most efficient parameter against GT growth, with minimal damage to the underlying muscle.

Increasing tissue temperature to 43°C to 51°C is known to induce a delayed cellular death, whereas exposure of the tissue to 52°C can cause irreversible cell damage from protein denaturation, inhibition of protein synthesis, breaking of DNA and RNA molecules, and loss of cell membrane integrity. The effect of hyperthermia on cellular death was demonstrated previously in culture after Nd:YAG laser irradiation. One explanation for the high susceptibility of GT to hyperthermia may arise from the dense capillary framework found in this tissue. The effect of laser-induced hyperthermia on blood vessels was recently explored with the use of a scanning electron microscope. Hyperthermia induced a complete destruction of the tumor microvascular architecture in mice. Similar results were observed by histopathological assessment of the skin and subcutaneous tissue after hyperthermia at 44°C. These results indicate that inhibition of GT growth and profound cellular death can be induced as a direct result of capillary destruction.

Another explanation for the effect of hyperthermia can arise from changes in cell metabolism and activation of a family of heat shock proteins (HSPs), causing cell death and apoptosis. Recent experiments have demonstrated that HSP-70, a member of that family, is overexpressed after laser irradiation. HSP-70 can also promote GT destruction, improving the tissue-healing process. The effect of hyperthermia on tissue is a cell-type specific feature that may be related to the proliferation rate of cells. In accordance with this idea, we have found that GT is more sensitive to hyperthermia than nonreplicating striated muscle cells.

A proposed application of our system is for treatment of subglottic, tracheal, or suprastomal granulations that induce airway obstruction. This system may be especially beneficial in these cases because cartilage cells are known to be resistant to thermal damage.

Our paper highlights a new technique for ablation of GT using an animal model of wound healing. The future use of hyperthermia in the larynx or trachea warrants significant further evaluation because the effect of our system on the underlying superficial lamina propria, vasculature, and cartilage are unknown. Furthermore, the long-term effects of hyperthermia on wound healing were not assessed in this study, and its impact on deposition of collagen, mucosal scarring, and wound contracture should be addressed in future studies. Such changes may have a long-term impact on the physiology of the larynx and trachea, including voice changes, accumulation of secretions, and airway obstruction. Our experiments on rats may pave the way for future in vivo experiments on larger animals. The next step will be to directly explore the value of laser-induced hyperthermia for treatment of tracheal and laryngeal granulations. Other issues that will need clarification are 1) What is the effect of mild hyperthermia on the tracheal cartilage? 2) Can hyperthermia induce similar effect on a thicker tissue? and 3) Does the effect of hyperthermia prevent regrowth of GT weeks or months after treatment? Repeated treatments may be required for complete GT ablation.

CONCLUSIONS

We demonstrated the utility of hyperthermia for ablation of GT growth in rats. Controlled hyperthermia is a nontraumatic and tissue-selective procedure, which can be applied by a flexible endoscope. The current study may help to set the ground for future use of lasers for treating GT in the trachea, larynx, and subglottis.

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Ziv Gil, study design, data analysis, manuscript author; Roee Landsberg, data collection; Ari DelRowe, planning of experiments; Abraham Katzir, planning of experiments, development of system; Alexander Shtabsky, pathological analyses; Dan M. Fliss, critical review of the manuscript.

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