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Original Article

Temperature processing and distribution in larynx thermal inhalation injury with analogy to human airway cells: a mechanism of protection

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Abstract: Objective: Inhalation injuries, especially laryngeal injuries, threaten the lives of burn patients. Unlike studies on temperature distribution in the upper airway, studies on temperature development in different laryngeal layers, including the mucosa, lamina propria, cartilage, muscle, and subcutaneous layer, are lacking. Method: For the *in-vivo* study, 16 healthy adult male beagles were divided into four groups: control, low-, medium-, and high-heat groups, inhaling dry air at 26, 80, 160, and 320°C for 20 min, with temperature probes punctured through skin into layers as mentioned, and heat energy was calculated. For the *in-vitro* study, we heated human lung fibroblasts and bronchial epithelial cells using a similar heating profile with heat energy of 15-90 J/g to investigate cell survival and viability for clinical comparison. Results: No statistical difference emerged between the temperatures of different laryngeal layers at each timepoint. The temperatures decreased significantly and shortly before increasing unevenly in the low- and medium-heat groups. The survival rates and viability of the two cell lines correlated negatively with heat energy. The heat energy absorbed in the low-, medium-, and high-heat groups of beagles were 12, 29, and 44 J/g, with calculated *in-vitro* human cell survival rates of 114%, 90%, and 69%, respectively, for the corresponding energy levels. Conclusions: The abnormal temperature processing and lack of a difference between layers indicate an effective self-protective mechanism of heat conduction in larynx. The *in-vitro* results demonstrate a high survival rate of lung cells at comparable heat energy levels to those measured in the larynx.

Keywords: Inhalation injury, larynx, heat tolerance

Introduction

Inhalation injury threatens the survival of burn patients, along with infection and shock [1]. Inhalation injuries include respiratory tract injury caused by heat or inhaled chemicals. Among these, the inhalation of hot air causes upper airway injury [2]; however, the most intense thermal injuries usually localize in the upper airway and can be managed with conservative treatment [3].

There have been studies on temperature distribution and development, as well as heat dissipation of blood circulation and mucus in the mucosa of the epiglottis, glottis atrium, and vocal cords, usually with the *in-vivo* model of beagle dogs [4-8]. Various models have been set for thermal inhalation injury where temperature distribution is key to the model setting, but most of them assume a consistency of airway walls in different parts and lack detailed data, some leading to the result that the nasal cavity is damaged the most during thermal inhalation injury, which contradicts clinical observations [9-11]. Currently, damage is not well characterized in the different layers of the larynx, including mucosa, lamina propria, cartilage, muscle, and subcutaneous layer, which may reveal the mechanism of laryngeal heat resistance and dissipation.

Additionally, as clinical practitioners, we wanted to evaluate cell survival of the human larynx. For clinical analogy, we chose an *in-vitro* experiment with human airway cell lines. The survival

of laryngeal cells after temperature-changing process has not been investigated. This is different from the cumulative equivalent minutes at 43°C (CEM43°C) model, an accepted thermal dose parameter for hyperthermia treatment monitoring usually in a temperature-consistent heating process of long duration with low heat [12, 13]. In contrast, the rapid temperature-changing heating process in common laryngeal thermal inhalation injuries occurs within a short period, and thus may not be appropriately modeled in the CEM43°C. The calculation of heat energy involves only the product of tissue mass multiplied by specific heat, and the difference between the initial and final temperatures, without concern of a temperature-time process [14]. Therefore, we chose to verify this using heat energy in our study instead of CEM43°C to connect an *in-vivo* and an *in-vitro* study.

Beagle dogs are commonly used to model laryngeal injury. Therefore, in this study, we aimed to evaluate the change in temperatures in different laryngeal layers during inhalation of heated air in beagle dogs to understand the temperature distribution, heat dissipation, heat energy absorbed and heat resistance. For clinical analogy of laryngeal immediate injuries of temperature-changing thermal insults, we used heat energy as the unit for *in-vitro* experiments of human airway cells measuring survival and viability after different heat energy absorption, and compared them to similar temperature-changing heating processes according to the heat energy amount calculated from the *in-vivo* study.

Materials and methods

In-vivo experiments and equipment

Sixteen healthy male adult beagle dogs (weight: 11.43±0.56 kg, Beijing Vital River Laboratory Animal Technology Co. Ltd.,) were randomly allocated into four groups (four dogs in each) using the random number table method: control group (group 0), low-heat group (group I), medium-heat group (group II), and high-heat group (group III), to inhale air through the mouth for 20 min at room temperature (26±2°C), 80±5°C, 160±5°C, and 320±5°C, respectively. The study was approved by the Medical Bioethics Committee of Beijing Jishuitan Hospital (No. 201911-02). The *in-vivo* study was conducted according to local and international guidelines for animal care and welfare, with the use of humane methods of euthanasia as follows.

The heating and ventilation instruments are the same as those used by Wan et al. [4]. Briefly, room air was inhaled by the beagles through a high aluminum tube, in which the air was heated by an electric heater within. The temperature of the inhaled air was monitored and stabilized by the device. Before each experiment, we started and operated the device at accordingly set temperatures without contacting the experimental animals, until it had stabilized for at least five minutes.

In-vivo experiments

All beagles were anesthetized with 3.5% pentobarbital sodium 1 mL/kg and Su-Mian-Xin 0.15 mL/kg (1 mL Su-Mian-Xin contains Dihydroetorphine Hydrochloride 4 ug and haloperidol 2.5 mg). After general anesthesia and application of vital sign monitoring equipment, the temperature probes (tp100; length, 3 cm; diameter, 0.3 cm; Beijing Kunlun Hengye Electronic Technology Co. Ltd.) were placed in four locations through ultrasound-guided skin punctures: the laryngeal mucosal surface, inner surface of the thyroid cartilage, outer surface of the thyroid cartilage, and subcutaneously marked as T1, T2, T3, and T4. One extra probe was placed in the rectum for the core temperature. All probes were connected to a digital temperature indicator (XMT-7; Beijing Kunlun Hengye Electronic Technology Co. Ltd., China).

We waited until the temperature readings of all probes were stable for five minutes before the following procedures. After the preparation, the upper and lower jaws were held open with an opener, the nostrils were plugged with rubber plugs, and a stabilized preheated heating device was inserted through the mouth, 5 cm away from the epiglottis, with the end of the temperature probe facing the epiglottis. Each dog was ventilated for 20 min in control or heated air, during which the temperature of each probe was recorded every minute, and the respiratory rate and oxygen saturation data of the beagles were maintained. The beagle dogs were then executed with an intravenous potassium chloride injection. Temperature-time data were recorded and plotted. To unify the heat energy calculation, we calculated heat energy

per tissue mass, as the product of the difference between the initial and final temperatures, multiplied by the specific heat [14, 15].

In-vitro preparation and experiment

The cells were provided by the China Infrastructure of Cell Line Resource (CIC), Chinese Academy of Medical Sciences, and included human embryonic fibroblasts (MRC) and human bronchial epithelial cells (HBE). Following CIC instructions, MRC cells were cultured in MEM with 10% (v/v) fetal bovine serum. The HBE cells were cultured in DMEM-H containing 20% (v/v) fetal bovine serum. MEM and DMEM-H were provided by Thermo Fisher Scientific Inc., USA, and fetal bovine serum (FBS) was provided by CIC. Both culture mediums contained 100 U/mL penicillin and 100 μg/mL streptomycin (Solarbio Science & Technology Co., Ltd). The cells were cultured at 37°C in a humidified 5% CO_2 incubator. Before each heating experiment, exponentially growing cells after three passages were washed with phosphate-buffered saline (Solarbio Science & Technology Co., Ltd.), digested with 0.25% trypsin with EDTA (Solarbio Science & Technology Co., Ltd.), added to their original culture medium, and made into cell suspension samples. The cell suspensions were slightly diluted to 80,000 cells/mL for the MRC samples and to 100,000 cells/mL for the HBE samples.

All samples were heated in 1.8 mL cell cryogenic vials with closed caps, completely immersed in a water bath, and were uniformly preheated to an initial temperature of 34°C before heating.

Thermal samples were used to modulate the temperature of the water bath to reach 40, 50, 55, 60, 65, and 70°C at the end of five minutes of heating in the corresponding water baths, without overheating or a constant-temperature stage. According to the literature based on specific heat as 2.5 J/(g*°C), the corresponding experimental groups absorbed 15, 40, 53, 65, 78, 90 J/g heat energy (n=3 for each group) with one control sample containing an unheated cell suspension and one blank sample containing non-cell culture medium.

After the water bath, all experimental samples were returned to room temperature (20°C), when cell counts and viability measurements started immediately. Cell viability was measured by WST-8 assay with a CCK-8 kit (Dojindo Laboratorise) which targets mitochondrial dehydrogenase of viable cells [16]. Each sample was added into five wells of a cell culture plate as repeats. Each well contained 100 μL sample and 10 μL CCK-8, cultured in the CO_2 incubator for one hour per instructions of Dojindo, before the absorbance was read at 450 nm using an ELISA reader. Viability was calculated according to the ratio of two differences: the difference between experimental samples and blank samples, and the difference between control samples and blank samples. The cell morphology and count were observed using an optical microscope and trypan blue staining before doing two repeat counts using a cell counting chamber (1.0×1.0×0.1 mm, Shanghai Qiujing Ltd,.), and cell survival rate was calculated.

Statistical analysis

We used Microsoft Excel (version 2010, Microsoft Corporation) software to process and summarize the data, and SPSS Statistics (version 19, SPSS Corporation) software for analysis. Variables that conformed to a normal distribution were described using the mean and standard deviation. ANOVA test was used to distinct the difference between different laryngeal layers of beagle dogs. Spearman's correlation analysis and one-way linear regression were performed. Statistical significance was set at $P<0.05$.

Results

In-vivo experiment

Before the inhalation of heated air, the core body temperatures of all groups were close to 37.57±0.21°C. After 20 min of ventilation, the core temperatures of the control, low-, medium-, and high-heat groups reached 37.83±0.25°C, 38.40±0.20°C, 38.97±0.15°C, and 39.13±0.42°C, respectively. All dogs remained alive.

After the inhalation of heated air in group I, the low heat group, there were no significant differences between the temperatures of each layer of the larynx at the same time points (ANOVA $F=0.559$, $P=0.993$), although the temperature showed a decreasing trend from the laryngeal mucosa to the subcutaneous tissue. The tem-

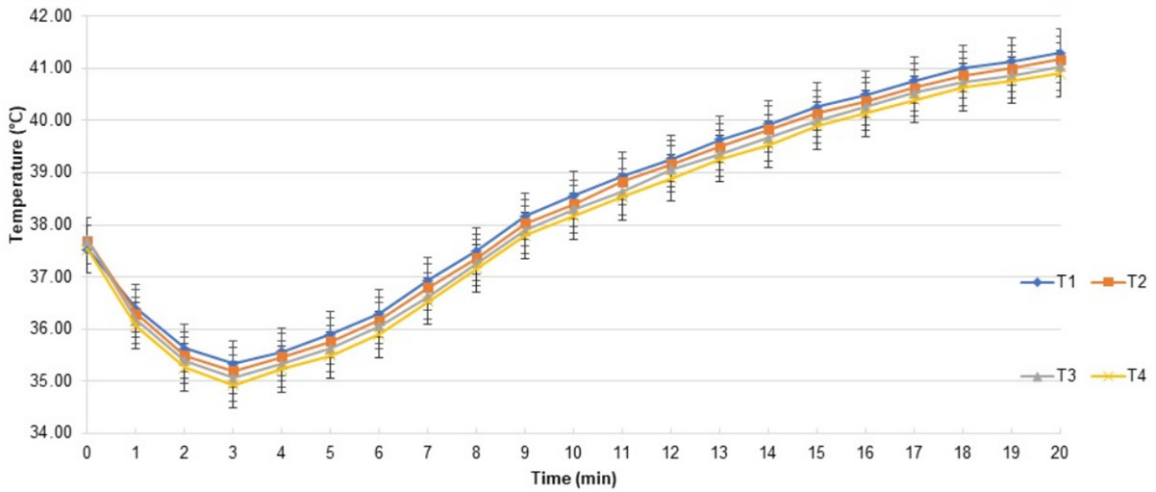

Figure 1. Increase in temperature with time in different laryngeal layers of the low-heat group (group I) during the inhalation of heated air.

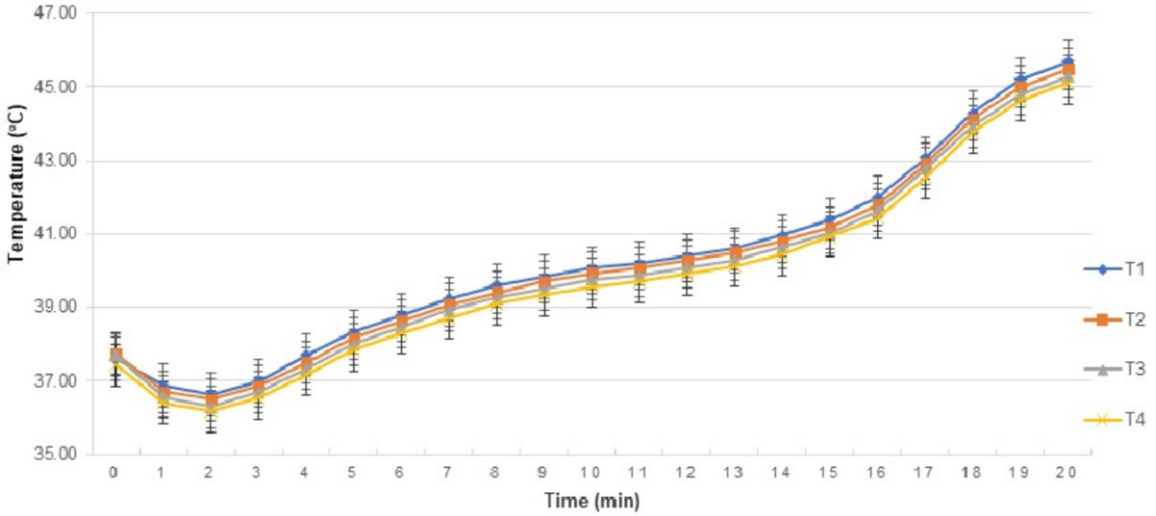

Figure 2. Increase in temperature with time in different laryngeal layers of the medium-heat group (group II) during the inhalation of heated air.

perature of each layer varied with time; a significant temperature decrease was observed from mins 0 to 4 before a slow increase, and the laryngeal temperature was lower than the core body temperature during the initial 8.67± 0.58 minutes. It kept increasing thereafter, as shown in **Figure 1**. At the end of the heated air inhalation experiments, the average temperature of the laryngeal layers was increased by 3.23±0.25°C, corresponding to an average heat absorption of 12.27 J/g.

In group II, the medium heat group, there were no significant differences between the temperature of each anatomical level of the larynx at the same time points (ANOVA F=14.270, P=0.163), and the temperature layer-decreasing trend remained. The temperature of each layer varied over time. There was a significant temperature decrease from mins 0 to 2 before a slow increase, and the larynx temperature was lower than the core body temperature during mins 0 to 4.33±0.61. The temperature gradually increased, as shown in **Figure 2**. At the end of the inhalation experiments, the average temperature of the laryngeal layers was increased by 7.83±0.15°C, corresponding to an average heat absorption of 29.75 J/g.

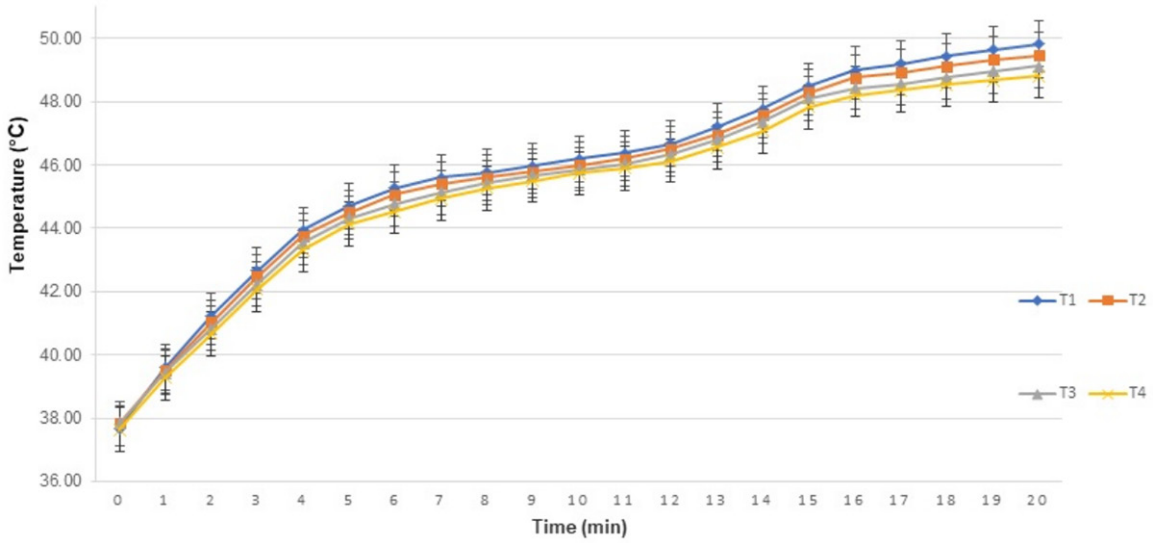

Figure 3. Increase in temperature with time in different laryngeal layers of the high-heat group (group III) during the inhalation of heated air.

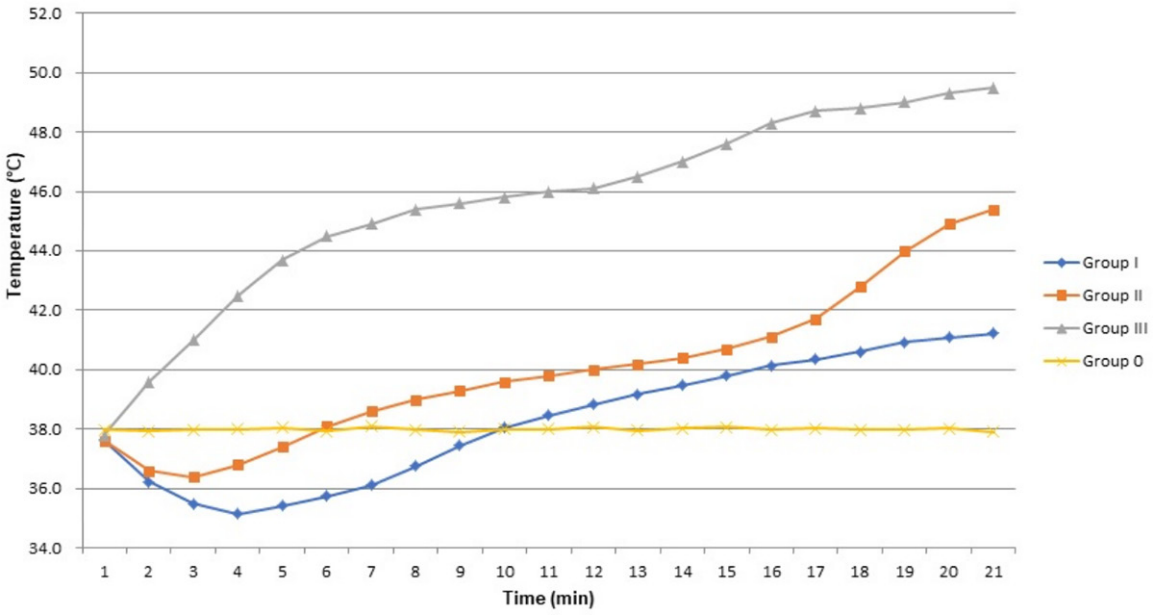

Figure 4. The average change in temperature with time in the laryngeal layers of the experimental groups.

In group III, the high heat group, there still were no significant differences between the temperatures of each anatomical level of the larynx at the same time point (ANOVA F= 4.943, P=0.379), while the temperature layer-decreasing trend remained. The temperature of each layer varied with time, but there was no temperature decrease. A rapid increase in each tissue layer was seen from mins 0 to 5, with a subsequent slowdown in the increase rate, as shown in **Figure 3**. At the end of the inhalation experiment, the average temperature of the laryngeal layers was increased by 11.67± 0.21°C, which is equivalent to 44.35 J/g of heat absorption.

The average change in temperature with time in the laryngeal layers of the experimental groups is shown in **Figure 4**. The severity of larynx injury increased with the temperature inhaled, as shown in **Figure 5**, where the mucosa of plicae vestibuli of beagles in the low

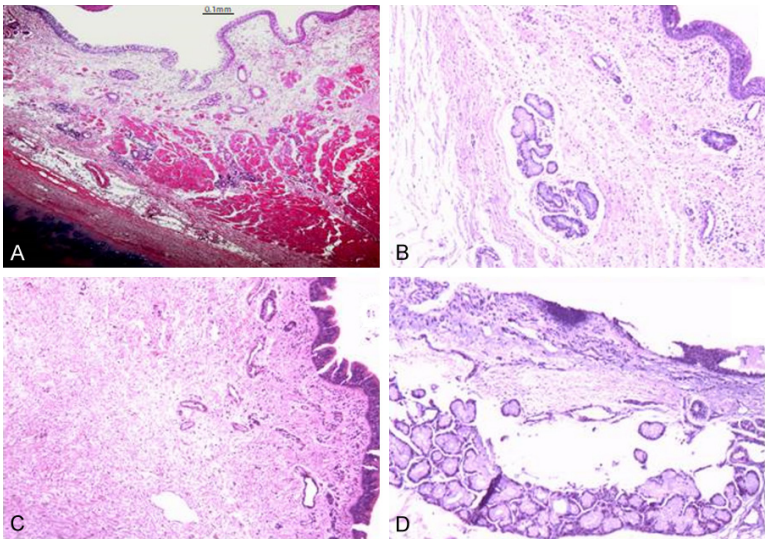

Figure 5. Representative images of mucosa of plicae vestibuli of beagles after thermal inhalation, hematoxylin-eosin staining. A. Control group, with normal mucosa. B. Mucosa in low heat group showed decreased cilia and mild congestion. C. Mucosa in the medium heat group showed thinner mucosal epithelium, infiltration of white blood cells, obvious congestion, and thrombosis. D. Mucosa in the high heat group showed degenerated epithelium and submucosal edema.

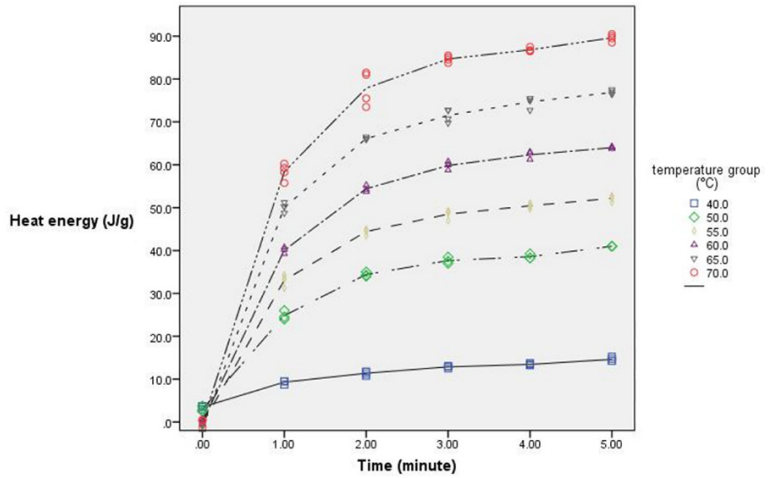

Figure 6. Cell heat energy absorption during the heating procedure. No constant-temperature stage was seen.

heat group showed decreasing cilium and mild congestion. The mucosa in the medium heat group showed thinner epithelium, infiltration of white blood cells, obvious congestion and thrombosis. The mucosa in the high heat group showed degenerated epithelium and submucosal edema.

In-vitro experiments

In the respective temperature water baths, the thermal samples were warmed up rapidly within 1-2 minutes and approached their target temperatures at the end of heating, without a constant-temperature stage recorded, similar to the in-vivo experiments, as shown in **Figure 6**. The results of cell viability measured by CCK-8 are shown in **Table 1**. The survival and viability of both MRC and HBE cells decreased with the amount of heat energy absorption, as shown in **Figure 7**. The viability of cells with average heat absorption of 15 J/g, corresponding to 40°C, was slightly higher than that of unheated cells, and the viability of cells with heat absorption up to 90 J/g was the lowest. The Spearman correlation coefficient between MRC cell viability and heat was -0.945, $P<0.001$. The R-square of the regression equation was 0.896. The linear regression equation was:

MRC cell viability = -0.011 × absorbed heat + 0.295

The Spearman correlation coefficient between HBE cell viability and heat was -0.784, $P<0.001$. The R-square of the regression equation was 0.614. The linear regression equation was:

HBE cell viability = -0.020 × absorbed heat + 0.390

The cell count results were similar to the cell viability results, as shown in **Table 2**. We observed a negative linear correlation between the cell count and heat absorption in both cell lines. The Spearman correlation coefficient for MRC cell survival rate and heat was -0.798, $P<0.001$. The R-square of the regression equation was 0. 558. The linear regression equation was:

MRC cell survival rate = -0.014 × heat absorption + 1.411

Table 1. Viability of human embryonic fibroblasts (MRC) and human bronchial epithelial cells (HBE)

Average heat absorption (J/g)	15	40	52.5	65	77.5	90
MRC viability	0.113±0.128	-0.152±0.096	-0.282±0.156	-0.427±0.087	-0.649±0.091	-0.737±0.218
HBE viability	0.365±0.301	-0.583±0.155	-0.844±0.091	-0.920±0.175	-1.095±0.050	-1.369±0.479

The data are mean ± standard deviation. For each cell line, n=3.

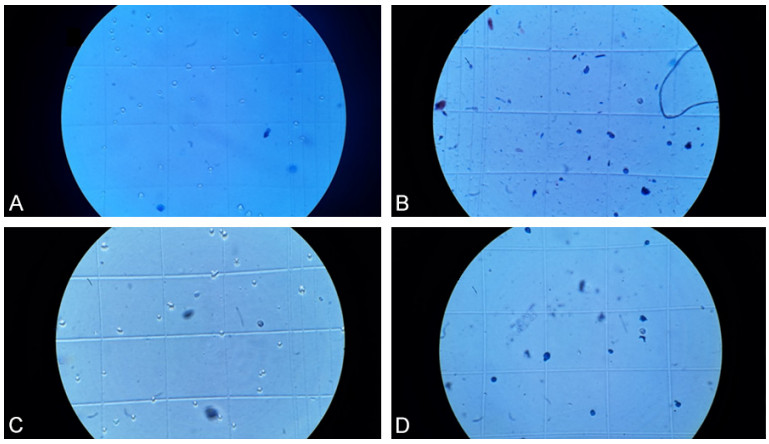

Figure 7. MRC cells and HBE cells, before and after heating. A, MRC after 15 J/g heat energy. B, MRC after 90 J/g heat energy. C, HBE after 15 J/g heat energy. D, HBE after 90 J/g heat energy. Cell death increased with the energy absorbed.

The Spearman correlation coefficient of the cell survival rate and heat for HBE cells was -0.783, P<0.001. The R-square of the regression equation was 0. 561. The linear regression equation was:

HBE cell survival rate = -0.014 × absorbed heat + 1.320

According to the regression equations above, we calculated the cell survival and viability rates of human cells in the conditions of beagle dogs as a clinical analogy. According to the heat energy amounts of low-, medium-, and high-heat groups, the average cell survival rates of MRC and HBE are shown in **Table 3**. The average cell survival rates were 114%, 90%, and 69%, respectively, while the average cell viabilities were 0.152, -0.119, and -0.345, respectively.

Discussion

In the model of laryngeal burns in beagle dogs, the temperature-time change curve was not linear, especially in the low- and medium-heat groups at 80°C and 160°C, where the tissue temperature first decreased with time and then the temperature increase varied with different speeds. Such a temperature decrease during consistent heating is abnormal, and we believe it is a self-protective mechanism in the larynx against thermal damage, resulting in a significantly lower tissue temperature in all laryngeal layers than that of the hot air during the inhalation process. There were no significant differences in the temperatures of the different laryngeal layers, suggesting that the laryngeal layers have good heat conduction, meaning that the heat energy could transfer quickly through laryngeal tissue layers in the early stage of injury to confer protection. The detailed temperature recording of efficient heat conduction may contribute to better models of thermal inhalation with location-specific details [9, 11, 17].

As stated, the temperature-changing heating processes in the *in-vivo* experiments were different from the conventional CEM43 temperature-consistent processes. Therefore, we conducted the *in-vitro* experiments to investigate the results of cellular thermal damage at fixed time intervals and heat energy, using human embryonic lung fibroblasts and human bronchial epithelial cells for clinical human analogy. The heat energy per unit mass can be calculated by specific heat multiplying the difference between the initial and final temperatures; thus, it does not deal with temperature fluctuations or time intervals during heating. Our results showed a slight increase in human cell counts and viability after absorbing the same amount of heat energy as the low-heat group. Although this is similar to the results of previous studies of heating below 43°C [12], it could also result from shrinking spontaneous death or a counting error. We also found a significant

Table 2. Survival rates of human embryonic fibroblasts (MRC) and human bronchial epithelial cells (HBE)

Average heat absorption (J/g)	15	40	52.5	65	77.5	90
MRC survival rate	107±13%	91±16%	81±21%	47±13%	31±11%	12±5%
HBE survival rate	102±11%	92±15%	76±18%	14±3%	13±4%	8±3%

The data are mean ± standard deviation. For each cell line, n=3.

Table 3. Calculations of MRC and HBE cells of heat energy level of *in-vivo* experiments

Energy level	Average heat absorption (J/g)	MRC viability	HBE viability	MRC survival (%)	HBE survival (%)
Low heat	17.1±0.4	0.157±0.004	0.148±0.008	117.2±0.5	108.1±0.5
Medium heat	30.3±1.2	-0.183±0.013	-0.215±0.023	92.7±1.6	89.6±1.6
High heat	47.5±2.1	-0.228±0.023	-0.560±0.042	74.6±3.0	65.5±3.0

decrease in the cell viability and counts in the higher-heated groups; however, at least 69% of the cells remained alive in the *in-vitro* set corresponding to the heat energy of high-heat beagles, which may explain the self-healing phenomenon of laryngeal tissue observed clinically after thermal injury [1].

In this study, we observed that the curve of temperature against time was not linear; the temperature first decreased to a value lower than the core temperature, and then slowly increased in the low- and medium-heat beagle groups. Heat dissipation includes heat solid conduction, liquid convection (heat energy flows from high temperature object to the low temperature in both conditions), and radiation (could be ignored under 1000°C) [14]. Since the core temperature, namely the blood flow temperature, was higher than that of the larynx, the heat of the larynx could not be dissipated by the blood flow. In the three groups, the temperatures of different laryngeal layers increased at different rates, which could be due to the self-protective mechanism described above and the blood flow heat dissipation. Without circulation dissipation, the statistically similar temperatures between laryngeal layers proves an effective convection between layers. Such protection appeared rapidly and earlier than the blood circulation dissipation. Combining the humid natural airway mucus, and the study of Wan et al. who proved that mucus evaporation has a protective effect during inhalation of low-heat dry air [8], these findings suggest that the larynx is protected from heat by a combination of mucus evaporation, circulation dissipation, and rapid convection through different anatomical layers from the laryngeal cavity to the environment. For clinical human analogy, the human cells we experimented on could retain at least 69% survival after absorbing the same amount of heat energy as in the high-heat group. There have been studies describing fine mathematical models in contact burns of the skin with detailed physical parameters [18, 19], but the models of inhalational thermal injury have long been of low accuracy due to the complexity of temperature, humidity, the complex structure of the larynx, and the air flow of respiration [20-23]. Our result should be helpful for the improvement of these models.

In addition, the cell count survival rate and cell viability of both cell lines responded differently to heat. For example, in MRC cells, the correlation coefficient and the regression equation R-square for the cell viability were larger than those for cell survival, indicating that the same amount of heat energy absorbance influences cell count survival and CCK-8 result differently. Similar results were observed in HBE cells. The CCK-8 assay is based on the activity of mitochondrial dehydrogenase enzymes, but such a difference in cell count survival and cell viability could result from numerous possibilities, including vulnerable mitochondria, delayed cell death, and incomplete cell function as a suspension. Further studies are required to reveal the mitochondrial and cell death roles in such injury. There were studies concerning the combination of radiation with hyperthermia in cancer cells [24], and the difference between rapid and slow heating at different temperature ranges [25]. Therefore, the heat energy model

might explain the thermo-tolerance of cells, and such results might be useful in models of upper airway heat injury [26, 27].

Our study has several limitations. In the *in-vitro* experiments, a thermocouple with a diameter of approximately 1.6 mm was used as the temperature probe, which may lead to an underestimation of the true values compared to the subcutaneous tissue with a thickness of less than 10 mm. However, non-invasive temperature measurements, such as magnetic resonance imaging or infrared imaging, are time-consuming procedures or have insufficient detection depth [28]. We used human airway cells in suspension for clinical analogy of the injury process, but the analogy could be optimized with attached cells in a physiological state in 3D-printed models. Heating time was constant in both *in-vitro* and *in-vivo* experiments, which may confine our understanding of longer injuries and exponential or fractional decreases during cell heating [13, 29]. The airway could be compromised by the late stage of severe inflammation [30] and a detailed temperature recording is of value to injury model building. Therefore we ignored the delayed injury and focused on the temperature distribution during the insult, so as to better understand the heat transfer. Further studies involving more combinations of heating time and temperatures, more stable and accurate heating equipment, and cells cultured at higher densities are needed.

Conclusion

In the heating process of a laryngeal burn model in beagle dogs, there was a temperature decrease followed by an increase in different laryngeal layers with no statistical difference between layers, a short period of temperature lower than core temperature, as well as an uneven change in temperature with time. These abnormal temperature developments a self-protective mechanism in the larynx against thermal injury including heat conduction, mucus evaporation, and blood circulation of layers in our experiments. Cellular studies as a clinical analogy showed that the human airway cells after absorbing a large amount of heat energy as in animal models, could maintain a relatively high cell survival rate, which may indicate a strong self-healing ability of the larynx after thermal injury.

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Disclosure of conflict of interest

None.

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