Original Contribution

Effects of Experimental Hyperthermia on Biodynamics and Immunity in Dogs and Cats

TOYOHIKO URAKAWA1, CHIYOKO NUKUZUMA2, KOHEI OHTSUKE2, MANABU KAWATA3, AKIRA SHIBAZAKI3, TAKASHI HASEGAWA3, HIROMU KATAMOTO3

1Laboratory for Thermotherapy, Nippori Kako Co. Ltd., 2-38-1 Kyuhouen, Yao, Osaka 581-0817, Japan
2Division of Molecular Oncology and Virology, Medical Research Institute, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan
3Laboratory of Advanced Therapeutics, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan

Abstract: A high-accuracy hot water immersion-type device for hyperthermia has been developed. In order to provide strong evidences that raising body temperature causes heat injuries at higher temperature and enhances immunity at proper combinations of temperature and duration, a series of accurate experimental hyperthermia trials in dogs and cats was carried out using the device. The biological responses to heating were similar in both dogs and cats. A typical sign of heat related injuries, rapid granulocyte collapse (granulocytolysis), was observed while the body was heated at the rectal temperature 42.5°C and more than 42.5°C. In addition, the increase of liver enzymes and the tendency of hemorrhage were notable. Heating at 43.0°C was rather fatal even one hour heating. The degradation of heat shock proteins, HSP90 and Hsc73, was detected on 1 and 3 days post treatment. On the other hand, in the cases of the mild (fever-range) heating less than 42.0°C, the animals were rather stable during the treatment, recovered quickly from anesthesia and showed the good condition and appetite after the treatment. Increase in number and percentage of lymphocytes was remarkable at the proper heating temperature 41.5°C. The enhancement of cellular immunity and the induction of Hsp72 were also confirmed by delayed type hypersensitivity skin test and Hsp72-specific ELISA, respectively. Contrary to our expectations, even the slightly mild heating at the normal body temperature range of these small animals, 38-39°C for 3.5 hours, also provided clear enhancement of immuno-competence. In conclusion, there were no significant differences in biodynamics and immunity in both dogs and cats to heating and we regard fever-range hyperthermia as a mighty immunotherapy.

Key Words: granulocytolysis, high-accuracy water immersion-type device, HSPs, immuno-competence, mild hyperthermia

Received 19 December 2005, Accepted 2 February 2006. *Corresponding author, Tel.: +81-729-23-8699; Fax.: +81-729-99-0106; E-mail: urakawa@nipppi.co.jp
Introduction

It is widely accepted that fever, which is inherent in the homeothermal animals, is one of the strong self-defence mechanisms\(^1\). Hyperthermia is a medical application treatment to artificially induce higher temperature to a whole body or a part of body to cure cancer or other various diseases. Despite years of grinding, hyperthermia hasn’t become one of the standardized cancer therapies yet. The basic idea of the present hyperthermia is based on a hypothesis that cancer cells might be “heat-labile”. However, heating or warming up a body is a double-edged sword depends on its combinations of temperature and duration. The noble water-immersing type whole body hyperthermia device mentioned in this paper has made it possible to control rectal temperature indirectly but precisely. Using this device, a serial hyperthermia experiment of dogs and cats was designed and carried out in order to quantify and determine the heat injuries to animal body and evaluate from the immunological point of view. Heat shock proteins (HSPs), including HSP90, HSP70 (Hsc73 and Hsp72) and HSP60, which might interact closely with immunity were analyzed in parallel. As a result from these experimental hyperthermia trials, we would like to point out misunderstanding of the present mainstream concept to hyperthermia and provide a clear-cut conclusion that mild (fever-range) hyperthermia apparently elicits immuno-competence in dogs and cats.

Materials and methods

Water immersion-type hyperthermia device

A high-accuracy water immersion-type device for hyperthermia has been developed. The device consists of heat-generation unit (Fig. 1A), bathtub (Fig. 1B), electric elevating lift (Fig. 1C) and computer.

Fig. 1. Photographs of the high-accuracy water immersion-type device for hyperthermia and a scene of experimental hyperthermia in dog. The device consists of heat-generation unit (A), bathtub (B), elevating lift (C) and computer. The dog was immersed in water which temperature was controlled precisely and was laid on the back under general anesthesia.
system. A newly developed software running on a Linux OS operates the device and processes data from accurate temperature sensors and a patient monitor including blood pressure, heart rate, electrocardiogram (ECG), hemoglobin saturation (SpO₂), end-tidal CO₂ (EtCO₂) and anesthetic gasses. The bathtub has the water capacity of 500 liters and ten scattered nozzles to jet out around 75 liters per minute for homogenizing water temperature. The water temperature is set with one decimal place and is controlled with a high degree of accuracy (±0.05°C).

**Experimental animals and anesthesia**

Healthy Beagle dogs, aged 10.7±1.7 (mean ± SD) months, and mixed cats, aged 24.3±6.5 months, were utilized. The experiments were conducted according to the guidelines for the care and use of laboratory animals, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University. All procedures for hyperthermia were performed under general anesthesia to diminish animal pain and stress. Food, but not water, was withheld for at least 12 hours before the experiment. Ten minutes after atropine (0.05 mg/kg, subcutaneously) injection, diazepam (0.5 mg/kg, intravenously) and acepromazine (1.0 mg/kg, intramuscularly) were administered to dogs and cats, respectively. Anesthesia was induced by propofol (5.0 mg/kg, intravenously) and was maintained by isoflurane. Vecuronium bromide (initial dose; 0.1 mg/kg, intravenously) was injected and artificial ventilation with isoflurane and 100% O₂ was done when the animals showed panting with the increase of body temperature. ECG, rectal temperature, heart rate, respiratory rate, blood pressure, SpO₂ and EtCO₂ were monitored and all animals were administered lactated Ringer’s solution intravenously at 5.0 ml/kg/hr during treatment. The animals were observed carefully after recovery from anesthesia and if they showed depression or other clinical problems, adequate treatments and care were done until their clinical conditions improved.

**Control of rectal temperature**

Rectal temperature was used as one of the core temperature and indirectly controlled by changing the bathtub temperature precisely. After the initiation of anesthesia, animals were immersed into the bathtub. The maximal water temperature which was 0.5 or 1.0°C higher than the target rectal temperature was employed to introduce the target temperature. In many cases, it took around one hour to elevate rectal temperature to the target temperature (raising stage). After maintaining the target temperature by changing water temperature little by little basically for two hours (maintaining stage), water temperature was set to 38.0°C (cooling stage). When rectal temperature dropped down to 40.0°C, usually within 30 minutes, animals were moved out of the bathtub, recovered from anesthesia and dried. A total of 3.5 hours were needed to accomplish a hyperthermia treatment.

**Differential counting of leukocytes and leukokinetics**

Blood was collected at the different points including just before the treatment (pre), one hour after reaching the target temperature (1hr), after the treatment (post), one day post treatment (1dpt), three days (3dpt), seven days (7dpt) and fourteen days (14dpt). Blood cell counts were determined by an automated hematology analyzer. For the estimation of leukocyte differential count, the blood smears were made
and microscopically analyzed. The increased numbers of lymphocytes were statistically analyzed by using Student’s t-test.

**CD4 and CD8 flow cytometry**

Separation of peripheral blood mononuclear cells (PBMC), which consisted mainly of lymphocytes, was performed by a density gradient centrifugation method (NycosPrep 1.077 Animal, AXIS-SHIELD). One million of isolated lymphocytes were reacted with the combination mixture of anti-dog CD4-FITC (MCA1038F, Serotec) and CD8-PE (MCA1039PE, Serotec) or that of anti-feline CD4-FITC (MCA1346F, Serotec) and CD8-PE (MCA1347PE, Serotec) for double staining. Then flow cytometric analyses were performed. The numbers of cells were statistically analyzed by using Student’s t-test.

**Delayed type hypersensitivity (DTH) skin test**

To determine immunological response to exotic substances, DTH responses to phytohemaggglutinin (PHA) were tested. The hair on the lateral side of the chest was clipped and the area was wiped with 50% isopropyl alcohol. All animals were injected intradermally with 0.1 ml of PHA (1.0 mg/ml) or saline as the negative control. Skin-fold thickness was measured at 24 and 48 hours after injection with a digital micrometer. The response was expressed as a increase ratio of skin thickness compared to the same injection site at 0 hour. The DTH skin tests were performed on 3 and 7 days post treatment.

**Western blot analyses of HSPs**

Protein samples of lymphocyte lysate were resolved by 10% SDS-PAGE. The proteins separated were transferred onto an Immobilon-P transfer membrane (Millipore) by semidry electric procedure. The membrane was blocked with 5% skimmed milk PBS. Then the membrane was soaked in the mixture of three monoclonal antibodies, anti-HSP90 (Cat. 610719, BD Biosciences, at the final concentration 1 : 10,000), anti-HSP70 (Cat. 610608, BD Biosciences, 1 : 100,000) and anti-HSP60 (Cat. 611563, BD Biosciences, 1 : 50,000). After multiple washing with Tween20-PBS, the membrane was soaked in the peroxidase conjugated second antibody (peroxidase conjugated anti-mouse immunoglobulin-goat IgG at 1 : 50,000, Cat. A-9452, Sigma). The gel images of antigen-antibody reactions were obtained by a chemiluminescence system using Western Blotting Detection Kit (ECL Advance, Amersham Biosciences). For further analysis of HSP70, anti-Hsp72 specific monoclonal antibody (SPA-810, StressGene Biotechnologies) was used. The anti-HSP monoclonal antibodies used in this study were confirmed to cross-react to mouse, rat, cat, dog and human HSPs.

**Hsp72-specific ELISA**

Anti-Hsp70 Immunoassay Kit (EKS-700M, Stressgen Biotechnologies) was used for the specific quantification of Hsp72 in lymphocytes and blood plasmas. The preparation of lymphocyte lysate samples and the actual procedures were followed by the manufacturer’s instruction manual.

**Results**

Whole body hyperthermia by the way immersed in hot water was successfully achieved. The rectal
temperature was indirectly controlled by changing the bathtub temperature frequently and precisely (Fig. 2). These experimental hyperthermia trials by manually controlling were progressively improved by gaining our experiences and in almost all cases the differences between the target temperature and the averages of rectal temperature during the maintaining stage were within a range of 0.1°C.

Table I indicates the temperature-specific summary of the animal experiments. Significant clinical signs related to the heat-injuries were detected and observed when the target temperature was 42.5°C and exceeded 42.5°C. One of the typical clinical signs was mucous and bloody stool releasing a strong and unpleasant odor in several hours after the treatment. One dog and one cat out of two treated at 43.0°C

![Fig. 2. A result of the experimental hyperthermia of dogs. The water temperature and rectal temperature are indicated. The average temperature during 125 minutes at the temperature-maintaining stage in this experiment was 41.52 ± 0.025°C. A total treatment time was approximately 3.5 hours.]

<table>
<thead>
<tr>
<th>Rectal temperature</th>
<th>Canine cases</th>
<th>Feline cases</th>
<th>Clinical signals</th>
<th>Invasiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal 38.3-39.3°C</td>
<td>5</td>
<td>5</td>
<td>Stable during the treatment, good condition and appetite after awakening</td>
<td>Slight</td>
</tr>
<tr>
<td>41.0°C</td>
<td>1</td>
<td>1</td>
<td>Stable during the treatment, good condition and appetite after awakening</td>
<td>Slight</td>
</tr>
<tr>
<td>41.5°C</td>
<td>6</td>
<td>12</td>
<td>Stable during the treatment, good condition and appetite after awakening</td>
<td>Slight</td>
</tr>
<tr>
<td>42.0°C</td>
<td>2</td>
<td>7</td>
<td>Rapid heart rate, dispirited on the treatment day</td>
<td>Minor</td>
</tr>
<tr>
<td>42.5°C</td>
<td>1</td>
<td>1</td>
<td>Dispirited for several days after the treatment, mucous and bloody stool, mild increase in liver enzymes</td>
<td>Severe</td>
</tr>
<tr>
<td>43.0°C</td>
<td>1 (dead)</td>
<td>2 (1 dead)</td>
<td>Dispirited for a week after the treatment, mucous and bloody stool, remarkable increase in liver enzymes, severe hemorrhage of many organs</td>
<td>Fatal</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
for one and two hours, respectively, died within 12 hours after the treatment despite intensive care. As a result of autopsy, the pathogenesis of death due to hyperthermia was investigated. Severe hemorrhagic lesions and coagulation disorders including disseminated intravascular coagulation (DIC) were observed in many organs such as small intestine, kidney, liver, lung and others. Therefore, the direct cause of their death was hemorrhagic shock and DIC in their cases. Another cat treated at 43.0°C showed severe clinical signs but survived a life-or-death crisis by intensive care.

Granulocytolysis

The rapid denaturation and necrosis of granulocytes while animal was heated at 43.0°C, even at 42.5°C, was detected by microscopic analysis (Fig. 3). The degranulation, the loss of cytoplasm, and the condensing of nucleus were microscopic common features. The percentage of the denatured granulocytes maximally reached 72% after the treatment. These abnormal cells almost disappeared from peripheral blood and were replaced by normal granulocytes on 3dpt. This span corresponds to the lifetime of apoptic granulocytes in peripheral blood.

Transient hepatitis

A transient surge of three hepatic enzymes (ALP, AST and ALT) in the same cat as shown in Figure 3 was detected (Fig. 4). This cat was recovered from fulminant hepatitis within a couple of weeks.

Degradation of HSPs

While the kinetics of HSPs expression in lymphocytes was analyzed, the degradation of HSPs was

![Graph showing percentage of granulocyte and lymphocyte over time](image)

**Fig. 3.** One typical sign of the harmful heat injuries, granulocyte collapse (granulocytolysis) was seen in the heating at 42.5°C and/or more. This is the case of a cat treated at 43.0°C for 2 hours. The appearance of the degranulated and necrotized granulocytes was observed during the heating. The clearance of the denatured cells was within 3 days post treatment.
confirmed in the 1dpt, or both 1 and 3dpt in some cases, samples of dogs and cats treated at 42.5°C or 43.0°C. When a sheet of film was exposed for longer time, extra smaller protein bands were appeared in the lane of the 1dpt sample in this case (Fig. 5A). For further analyses to identify the degradation of HSPs, the 1dpt sample was reacted with the individual monoclonal antibodies, including anti-HSP90, anti-HSP70 (both Hsc73 and Hsp72), anti-Hsp72-specific and anti-HSP60 (Fig. 5B) at the different concentrations of the individual antibodies. HSP90 and Hsc73 were confirmed to be disintegrated into the smaller degradation products. However, the degradation of newly synthesized inducible Hsp72 was not detected (Fig. 5B). The degradation of HSP60 was not confirmed in this experiment because of the

![Figure 4](image_url)

**Fig. 4.** The hepatic injury of the same cat shown in the figure 3. Increased activities of three hepatic enzymes showed a clear sign of transient fulminant hepatitis after the treatment.

![Figure 5](image_url)

**Fig. 5.** Western blot analyses showed the degradation of heat-shock proteins, HSP90 and Hsc73. A sheet of film was over-exposed in western blot analysis of the serial cat lymphocyte samples treated at 42.5°C for 2 hours (A). A cocktail of three different anti-HSP monoclonal antibodies (anti-HSP90, 70 and 60) were used in this experiment. The sample one day after the treatment was analyzed furthermore using individual monoclonal antibodies (B).
shortage of reaction.

**Leukokinetcs**

In leukokinetcs effected by the treatments in dogs, a transient leukopenia by leukopedesis or other mechanisms during the treatments was always seen despite heating or none-heating. All animals in none-heating anesthesia group (n=3), whose temperatures dropped down by 2-3°C during the treatment for 3.5 hours, showed a transient leukopenia during the treatment and the increase of granulocytes on 1dpt and 3dpt. On the other hand, the numbers and percentages of lymphocytes were dropped for two weeks (Fig. 6A). The normal temperature heating group (consisted of 4 dogs), whose temperatures were maintained at 38.3-39.3°C during the treatment for 3.5 hours, showed a transient and slight leukopenia mainly due to the decline in granulocytes during the treatment. The decrease of granulocytes, 20% down from the initial level in number on 14dpt, and the sharp increase of lymphocytes was observed and the high level of lymphocytes were maintained for more than two weeks, 134% up from the initial level in number on 14dpt (Fig. 6B) (p<0.05). The proper hyperthermia group (consisted of 4 dogs), whose temperatures were maintained at 41.5°C for 2 hours, showed a transient leukopenia and the subsequent increase of lymphocytes (p<0.05 on 3dpt and 7dpt), 94% up from the initial level in number on 14dpt (Fig. 6C). In addition, the trend of the increase of lymphocytes and the decrease of granulocytes after heating was also observed in the cats heated at normal body-temperature for 3.5 hours or at 41.5°C for 2 hours (data not shown).

**Kinetics of the numbers of CD4+ and CD8+ T cells**

The dog CD4+ and CD8+ T cells were increased along with the increase of the total lymphocytes in the normal temperature-heated group (n=4) and the 41.5°C-heated group (n=4) (Fig. 7). The increase ratios of CD4+ and CD8+ T cells increased by 158% and 118% in the normal temperature-heated group.

![Fig. 6. Kinetics of leukocyte numbers in dogs. The non-immersion control group (n=3) was treated with general anesthesia (A). Another control group (n=4) was treated at the normal body temperature (38.3-39.3°C) for 3.5 hours (B). The hyperthermia group (n=4) was treated at 42.5°C for 2 hours. Actual numbers and percentages of cells in blood were indicated. Asterisks indicate significance (* : p<0.05).](image)
and also increased by 122% and 138% in the hyperthermia group on 14dpt, respectively. The significant change of CD4⁺ and CD8⁺ T cells in the non-heating anesthesia group (n = 3) was not detected (data not shown).

Fig. 7. Kinetics of the numbers of CD4⁺ and CD8⁺ lymphocytes post treatment at 41.5°C for 2 hours in dogs (n = 4). Actual numbers of cells in blood were indicated. Asterisks indicate statistical significance (*: p < 0.05, **: p < 0.01).

Fig. 8. The results of the delayed type hypersensitivity (DTH) skin test in dogs. a: non-immersion anesthesia (rectal temperature dropped down by 2-3°C) group (n = 5), b: normal body temperature (38.3-39.3°C) heating group (n = 4) and c: hyperthermia (at 41.5°C for 2 hours) group (n = 4). The change in skin thickness was calculated by subtracting the original thickness from that at 24 or 48 hours post injection of PHA and dividing by the original thickness. Asterisk indicates statistical significance (*: p < 0.05).
**DTH skin tests**

DTH skin tests were carried out as an in vivo cellular immunity assay in dogs (Fig. 8). PHA and the same volume of saline were intradermally injected on 3dpt (Fig. 8A) and 7dpt (Fig. 8B). The change in skin thickness was calculated by subtracting the original thickness from that at 24 or 48 hours after injection and dividing by the original thickness. The responses were always greater in order of the none-heating anesthesia only group (Fig. 8 Column a, n=3), the normal temperature-heated group (Fig. 8 Column b, n=4) then the 41.5°C-heated hyperthermia group (Fig. 8 Column c, n=4).

**Kinetics of Hsp72 in lymphocytes and blood plasma**

Hsp72-specific ELISA of the lymphocyte lysates and blood plasmas was done. Figure 9 showed a typical pattern of the kinetics of the Hsp72 expression in a cat case treated at 41.5°C for 2 hours. In this case the similar diphasic patterns, one of them was during the heating and another was on 3dpt both in lymphocytes and blood plasma, were observed. However, some animals had different patterns, e.g. single peak alone. None of remarkable change in the Hsp72 kinetics of the normal temperature-heated group was observed so far as we tested.

![Graph showing the kinetics of Hsp72 in lymphocytes and blood plasma](image)

*Fig. 9.* Typical kinetics of the quantification of Hsp72 in lymphocytes (per one million cells) and blood plasmas (per ml) in a cat case treated at 41.5°C for 2 hours.

**Discussion**

The original idea of hyperthermia was initiated from the careful observations of natural healing from cancer with a high self-fever caused by the infections of bacteria or viruses. Hyperthermia practitioners of early date had artificially used live/killed bacteria as a vaccine in order to induce high fever and inflammation. Thereafter, the present basic concept of hyperthermia is to “burnout” (eliminate) malignant cancer cells entirely with a combination of higher temperature and longer duration because cancer cells should be regarded as being heat-labile and a hypothetical point of temperature, which kills malignant cells and doesn’t kill normal cells, might exist. The initial idea has been transformed from the way inducing high fever to the “burnout” way of killing entire cancer cells by heating. On the other
hand, many ways for heating have been applied and examined so far such as microwave, radio-wave, far infrared rays, extracorporeal circulation and so on. The whole body (= systemic) hyperthermia, however, had so many difficulties. Thereafter, major trend has been shifting from whole body hyperthermia to partial hyperthermia because the results were disappointing despite the big-scaled facilities.

A water immersion-type device for whole body hyperthermia that we employed in this study was not a new one. Since 1983 Oita Medical University (the present Faculty of Medical, Oita University) has examined the pioneering heat therapy in three cases of the patients with malignant tumors with a hot water vessel\(^3\). Due to the historical restrictions of those days, especially in the points of the accuracy of temperature control and monitoring devices, the whole body hyperthermia by an immersion method did not accept proper assessment. However, the computed device has been developed and available to precisely control the liquid temperature in a bathtub. Using the newly developed device, we have been able to maintain the elevated core temperature of animal or human bodies, represented by the rectal temperature, with accuracy within 0.1°C.

While repeating the animal experiments using both dogs and cats, our research members have noticed that the conditions of higher body temperature, more than 42.0°C, and longer duration, 1 or 2 hours, which may be employed for the “burnout” way of the present main stream for hyperthermia are highly harmful to both immune system and some normal cells of organs. One of the initial noticeable symptoms caused by heat injuries might be a rapid degradation of granulocytes, granulocytolysis, which we firstly report in this paper. The previous reports\(^4\) using water-immersing way suggested that the lethal mechanism was endotoxic shock and DIC as we experienced in this study. The neutrophil has been implicated as an important mediator of tissue injuries including vascular injury and mucosal injury by releasing elastase and active oxygen species\(^5,6\). It might be possible to explain that the release of a mass of active oxygen species and enzymes such as elastase from the cytoplasm of granulocytes in a short time could cause the destruction of the intestinal barrier function and subsequent endotoxic shock. Additionally, the degradation of even HSP90 and Hsc73 was detected in the samples of lymphocyte lysate on 1dp and 3dp, when animals were treated at 42.5°C or 43.0°C. It suggests the molecular-level damages that a great deal of cellular proteins might denature and break down under these heating conditions.

The fact that mild (fever-range) hyperthermia elicited and enhanced immunity by raising the number of lymphocytes, reducing the number of granulocytes and stimulating cellular immunity was reconfirmed by our experiments. Moreover, fever-range hyperthermia enhances L-selectin-dependent adhesion of lymphocytes\(^7\) and stimulates the secretion of cytokines and the expression of adhesion molecules in endothelial cells\(^8\). The interactions among fever, heat shock response and immune response are complicated and are not always dissolved\(^9\). Especially, it was surprising that even a slightly mild heating condition, around 38-39°C for 3.5 hours, also elicited immunity for several weeks in our experiments. On the contrary, the artificially induced transient hypothermia by anesthesia caused the decrease of lymphocytes and the increase of granulocytes in number for a couple of weeks. These phenomena could explain the desirable relationship between health and proper body-heating habit such as daily bathing.

HSPs had been considered as an “enemy” that produces heat-resistance or tolerance to reduce the
effect of sequential treatments by conventional hyperthermia. In recent years, however, there are many
evidences that HSPs are closely related to immunity. For instance, HSP-peptide complexes derived from
cancer tissues work as self-cancer vaccine\(^{11-19}\). HSPs promote the expression of MHC class I-peptide
complexes on cell surface\(^{10}\), the maturation and cross-presentation of dendritic cells and also activate NK
cells and the innate and specific immunity as a danger signal\(^{17-19}\). HSPs stimulate macrophages to
secrete inflammatory cytokines\(^{20}\). In addition, there is a fact that the locus of a HSP70 gene is embedded
in the MHC class III region of genome which contains a lot of principal genes modulating immunity, e.g.
TFN and complements\(^{21}\). As a matter of fact, the aspect of conventional hyperthermia completely
opposes to the “new” concept of hyperthermia in regard to the point that HSPs are regarded as desirable
molecules.

It has been suggested that the mechanism of hyperthermia does not always consist in the direct killing
activity by heating against cancer cells or virus-infected cells based on their “heat-labile” property.
Proper whole body heating less than 42.0°C, even if it is normal body temperature, elicits
immuno-competence in both dogs and cat. We have considered that the enhancement of
immuno-competence by proper heating is much more important in hyperthermia treatments than the
“burnout” way. The new concept of hyperthermia that the proper heating enhances the
immuno-competence all over the body by way of the effective antigen presentations due to heat-induced
HSPs, the increased numbers and activities of lymphocytes and good peripheral blood circulation might
be more essential for anti-tumor effects.

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実験的全身温熱療法による生体と免疫への影響

浦川 豊彦1・奴久魁美智子2・大塚浩平3・川田 学3
柴崎 哲3・長谷川貴史2・片本 宏3

1）日立製作所（株） 寒冷療法研究所
2）金沢医科大学総合医学研究所 分子チーム研究部
3）大阪府立大学 農学生命科学研究科特殊診断治療学研究室

要旨：我々は温水浸漬療法によるハイパーサーマーの新たに高精度温浴装置（0.1℃単位で設定、温度誤差±0.05℃）を開発した。より正確な温度の温水を使い直腸温を間接的にコントロールすることにより、全身麻醉下のインネコの実験的な一回の全身加温を行った。まず高温による傷害を調べ、41.0℃から0.5℃刻みで43.0℃まで2時間の加温実験を行った。傷害はインネコとも24.5℃からはじめ、43.0℃では極めて重篤な症候を呈し懸念する治療にいかなかった死亡例が見られた。特に高齢者の粘液便、血便、肝酵素値の異常上昇、各種臓器の出血である。特に今までに報告の無い顆粒球の変性・壊死率が加温後中から顕著に増加し、加温後3日目に正常に戻った。この急激な顆粒球の崩壊による全身の活性化、好酸球や好塩基球の放出と腸管バリアの破壊が傷害発生の機序の一つになっている可能性が示唆される。加えて高温によるHSPsの分解も42.5℃から検出され、今回HSP90とHSP70（Hsc73）の分解が加温後1〜3日目に確認された。そこで生体への浸透性と安全性に考慮し、至適加温温度を直腸温41.5℃として加温し免疫に与える影響を調べた。加温群（41.5℃で2時間間隔、合計5.5時間）、平熱領域加温群（38.3〜39.3℃で3.5時間間隔）、それに対照群として全身麻醉だけを施した非加温群（3.5時間）の3グループとした実験を行った。平熱加温群では2週間には顆粒球数が20%程度減少し、リンパ球数は加温直後から変化を示す2週目では2倍以上に上昇し、4週間程度で元のレベルに戻った。直腸温41.5℃2時間加温群では、加温直後に一過性のリンパ球減少と3日目に顆粒球数の一過性の上昇が見られたものの、2週間目でリンパ球数が2倍強上昇した。加温後2週目のCD4、CD8陽性リンパ球数も著明に増加した。HSP70（Hsp72）の発現量は41.5℃加温群で加温中と加温後3日目の二峰性の上昇を示す個体が存在したが、対照群と平熱加温群では変化しなかった。PHAを使った遲延性過敏症皮内試験では3日と7日目に接種、その後24時間目と48時間目の判定で、いずれも41.5℃加温群が最も高い値を示し、次は平熱加温群であった。伝があればインネコとほぼ同様の結果を得た。これらの正確な加温による動物実験の結果から、直腸温41.5℃の加温はもろん平熱レベルで数時間の加温でも十分免疫を高めること分かった。反対に、わずか数時間の実験的な2〜3℃の低温では顆粒球増加とその後2週間にわたる免疫低下を来たすことが明らかになった。