A Possible Reno-protective Effect of Systemic Thermal Stimulation in a Mouse Remnant Kidney Model

Yoshihiro IWASHITA1, 3, Yoshiyasu YOZA1, Hiroki KAMEYAMA2, Masashi MUKOYAMA3, Junich IIYAMA1, Kenichiro KITAMURA3

Abstract

Objective: Sauna bathing is a popular recreational activity and has long since been used to relieve stiff necks and low back pain. Recently, low-temperature sauna has been used to treat congestive heart failure (CHF), coronary artery diseases, chronic fatigue syndrome, and chronic pain. During 1960–1970, thermal stimulation was applied to the patients with renal failure. We could not find the subsequent reports, and the long-term effects are unclear. The purpose of this experiment was to verify the safety of systemic low-temperature sauna treatment (ST) for the 5/6 remnant kidney mouse and to examine the effect of ST on urinary protein excretion.

Materials and Methods: The C57BL/6 mice were divided into the following 4 groups; group 1: sham-operated and non-sauna treatment mice (sham+non-ST group: n = 5), group 2: sham-operated and ST mice (sham+ST group: n = 5), group 3: Nx and non-ST mice (Nx+non-ST group: n = 5), and group 4: Nx and ST mice (Nx+ST group: n = 5). Mice received ST at 41°C for 15 min and at 32°C for 20 min for 12 weeks using a natural convection dry sauna system.

Results: After 12 weeks of ST, no differences were observed in creatinine clearance, body weight, fluid intake, urine volume, serum sodium and potassium levels between ST and non-ST groups. Our results showed a significant increase in eNOS mRNA expression in the Nx+ST group compared to that in the Nx+non-ST group. These results suggest the possibility that mild sauna treatment induces thermal vasodilation effects on glomerulus. Systolic blood pressure and urine protein levels in the Nx groups did not change throughout the intervention.

Conclusion: There are no clear adverse events associated with low-temperature sauna. Therefore, this study setting is safe in the CKD model mouse. Renal eNOS mRNA expression was increased by the low-temperature sauna. The present results suggest the possibility that ST might provide a renal protective effect by suppressing glomerular hypertension via stimulation of renal NO production in the CKD model mouse.

Keywords: low-temperature sauna, 5/6 nephrectomized model mouse, eNOS mRNA

I Introduction

Sauna bathing is a popular recreational activity and has long since been used to relieve stiff
necks and low back pain. However, sauna bathing has been contraindicated in patients with hypertension and heart diseases. Heart failure with sympathetic hypertonia has been thought to deteriorate with systemic sauna. In addition, there have been no recommendations for exposure time, heat intensity, and frequency in traditional sauna.

Recently, low-temperature sauna has been used to treat congestive heart failure (CHF), coronary artery diseases, chronic fatigue syndrome, and chronic pain\(^1\),\(^2\). Tei et al. reported the acute effects of low-temperature sauna treatment on hemodynamics in patients with severe heart failure\(^1\). In recent years, this low-temperature treatment has been established as "waon therapy," which means soothing and warmth in Japanese. With repetitive waon therapy, vascular endothelial function was improved not only in animal models with heart failure but also in patients with CHF. Although the safety and efficacy of the low-temperature sauna for the treatment of the cardiovascular diseases have been reported, there are no reports investigating its effects on the progression of the kidney diseases.

During 1960‒1970, thermal stimulation was applied to the patients with renal failure. Researchers attempted to excrete urea nitrogen and potassium through sweating\(^3\)–\(^5\). However, this approach was intolerable to the patients because it was a high-intensity therapy with prolonged exposure time (load setting ≥ 70℃ and treatment time 1–2 h). We could not find subsequent reports, and the long-term effects are unclear.

It is known that the vascular endothelial function is already impaired at the early stage of the chronic kidney diseases (CKD)\(^6\),\(^7\) and the endothelial damage leads to the vascular rarefaction in the capillary system of the kidney\(^8\). Reduced availability of nitric oxide (NO) as a result of decreased synthesis by endothelial cells is thought to be a key event in vascular damage\(^9\),\(^10\). We hypothesized that the low-temperature sauna could prevent the progression of CKD. The purpose of the current study is to verify the safety of systemic low-temperature sauna treatment (ST) for the 5/6 remnant kidney mouse and to evaluate the effect of ST on the urinary protein excretion.

II MATERIALS AND METHODS

1. Animals

All animal procedures were conducted in accordance with the guidelines for care and use of laboratory animals approved by Kumamoto Health Science University. Male C57BL/6 mice (Kyudo, Kumamoto, Japan) with initial body weights of 23‒26 g were used in this study. All animals were housed under controlled humidity and temperature with a 12:12-h light/dark cycle and were given free access to standard mouse chow and tap water.

2. Remnant kidney model and general parameters

The 5/6 nephrectomized (Nx) model mouse was created by surgical renal reduction following standard procedures. In brief, 2/3 of the left kidney was excised with scissors, and the right kidney was removed after a 2-week recovery period. Two weeks after right kidney removal, 20 mice were randomly divided into the following 4 groups; group 1: sham-operated and non-ST
mice (sham+non-ST group: n = 5), group 2: sham-operated and ST mice (sham+ST group: n = 5), group 3: Nx and non-ST mice (Nx+non-ST group: n = 5), and group 4: Nx and ST mice (Nx+ST group: n = 5). At the end of the 12-week study period, all mice were weighed and systolic blood pressure was assessed by the tail-cuff method using the MK-2000ST manometer (Muromachi Kikai, Osaka, Japan).

Twenty-four-hour urine samples were collected in metabolic cages and fluid intake was determined. Blood samples were collected from the tail vein or the inferior vena cava, and the levels of serum creatinine, sodium, potassium, and chloride were measured with a Hitachi 7180 Biochemistry Automatic Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). Urinary albumin levels were measured by the Lbis Albumin Mouse ELISA Kit (Shibayagi, Gunma, Japan).

3. Sauna treatment

The mice received ST at 41°C for 15 min and then at 32°C for 20 min to increase the rectal temperature by approximately 1°C using the EKK-450 (AS ONE, Osaka, Japan) as a natural convection dry sauna system. Unprecedented report that was applied to the low-temperature sauna CKD model mice. We have a reference to the protocol of the sauna treatment for cardiomyopathy hamster to set the sauna\(^{11}\). In same studies on Waon therapy, deep body temperature by about 1°C increase, improvement of vascular function of systemic\(^{11,12}\), correction of hormonal activity and autonomic nervous system was confirmed\(^{13}\). In addition, such as activation of biological defense mechanism and autoimmunity has been confirmed in studies on hyperthermia\(^{14}\). It is believed to be to hold the rise in core body temperature, these effects to be maintained and enhanced. We have confirmed that the rectal temperature increase of 0.8 ± 0.2°C is maintained after 20 minutes kept at the pre-experiment on sauna setting. The sham+ST and Nx+ST groups underwent daily ST for 12 weeks starting 2 weeks after right kidney removal.

4. Quantitative real-time reverse transcriptase-polymerase chain reaction

Total RNA was extracted with SV Total RNA Isolation Kit (Promega, Madison, MI), and 1 µg of total RNA was transcribed with PrimeScript RT Master Mix Kit (Takara Bio, Otsu, Japan). TaqMan probes for mouse TGF-β1, NPHS1, and NOS3 were purchased from Applied Biosystems. Statistical analyses of results were performed with the ΔCt (threshold cycle) value (Ct\(_{\text{gene of interest}}\) - Ct\(_{\text{GAPDH}}\)). Relative gene expression was obtained by the ΔΔCt method (Ct\(_{\text{sample}}\) - Ct\(_{\text{calibrator}}\)).

5. Statistical analysis

For statistical analysis, data between the Nx+non-ST and Nx+ST groups were compared by using the Mann-Whitney U test. Groups were compared by using the 1-way analysis of variance (ANOVA) followed by the Tukey test to identify differences. A value of P < 0.05 was regarded statistically significant. Data were expressed as mean ± standard deviation (SD). Statistical analysis software was used Stat Mate IV for windows (ATMS, Tokyo, Japan).
III RESULTS

1. General parameters

The Nx mice showed significantly lower body weight compared with the sham mice during the whole experimental period. The sauna treatment had no effect on the body weight both in the sham and Nx mice (Fig 1A). Fluid intake was significantly increased in the Nx mice compared with that in the sham mice (P < 0.001, sham+non-ST vs. Nx+non-ST, sham+non-ST vs. Nx+ST, sham+ST vs. sham+non-ST, and sham+ST vs. Nx+ST at weeks 0, 4, 8, and 12) (Fig 1B), but ST had no effect on the fluid intake. Accordingly, urine volume was markedly increased in the Nx mice compared with that in the sham mice (P < 0.001, sham+non-ST vs. Nx+non-ST, sham+non-ST vs. Nx+ST, sham+ST vs. sham+non-ST, and sham+ST vs. Nx+ST at weeks 0, 4, 8, and 12) (Fig 1C), but ST had no effect on the urine volume. The Nx+ST group appeared to have reduced systolic blood pressure compared to the other groups although statistically significant difference was not observed among the 4 groups at the end of the study period (Fig 1D).

![Fig. 1](image)

Fig. 1 Changes in the body weight (A), fluid intake (B), 24-h urine volume (C), and systolic blood pressure (D) over time throughout the treatment period. The 24-h urine samples were collected in metabolic cages. No significant changes were observed in body weight, fluid intake, urine volume, and systolic blood pressure by sauna treatment. At the end of the study period, systolic blood pressure was measured by the tail-cuff method. Symbols are: (■) before sauna treatment; (□) after 12 weeks sauna treatment. Sh, non-sauna-treated sham-operated mice; Sh+S, sauna-treated sham-operated mice; Nx, non-sauna-treated 5/6 nephrectomized mice; Nx+S, sauna-treated 5/6 nephrectomized mice.

No. of mice: Sh=5, Sh+S=5, Nx =5, Nx+S=5.
2. Blood and urine analysis

Serum creatinine levels in the Nx groups were significantly increased compared to those in the sham groups (P < 0.001, sham+non-ST vs. Nx+non-ST, sham+non-ST vs. Nx+ST, and sham+ST vs. Nx+ST; P < 0.05, sham+ST vs. Nx+non-ST). No significant difference in the serum creatinine levels was observed between the Nx+ST and Nx+non-ST groups. No differences in serum sodium, potassium, and chloride levels were detected in any groups. Although there was no statistically significant difference in the creatinine clearance (CCr) in both Nx and sham groups (NS), Sham+ST and Nx+ST groups tended to have decreased CCr compared with the non-ST groups. There was no difference in urinary albumin levels between the Nx+ST and Nx+non-ST groups (Table1).

3. Real-time PCR

Chronic progressive kidney diseases typically are characterized by active renal fibrosis and inflammation. TGF-β1 is a key mediator in the development of renal fibrosis and inflammation\(^{15, 16}\). Podocyte is functioning as the glomerular filtration barrier, and nephrin is a major constituent protein of the podocyte and a marker for the glomerular barrier function in the kidney disease\(^{17-19}\). Endothelial nitric-oxide synthase (eNOS) is implicated in numerous aspects of renal vascular control and function\(^{20}\). It is thought to exert a vasculoprotective effect by modulation of kidney blood flow through counterbalancing the effects of the renin-angiotensin system\(^{21}\). The ST had no effect on the mRNA expressions of TGF-β1 and nephrin in the kidney. mRNA expression of eNOS in the kidney was significantly increased (approximately 1.3-fold) in the Nx+ST group compared with the Nx+non-ST group (Fig 2).

IV. DISCUSSION

We attempted to demonstrate the safety of systemic thermal stimulation intensity in the sauna setting and the effect of systemic thermal stimulation against the progression of the CKD. There were no adverse events, dehydration primarily, in the sauna setting in this time. The ST significantly induced the mRNA expression of eNOS in the Nx mice kidney.

1. Possibility of dehydration with sauna treatment

In this study, no differences were observed in CCr, body weight, fluid intake, urine volume, serum sodium and potassium levels between the ST and non-ST groups. Although not statistically significant, CCr levels tended to decrease in Nx+ST group at 12 weeks when compared with 0 week in sauna settings used in this study. This trend might indicate the potential dehydration with sauna treatment. It is well known that angiotensin II induces drinking behavior. On the other hand, systemic administration of angiotensin II in C57BL/6 or BALB/c mice does not stimulate drinking of water\(^{22, 23}\). It is suggested that C57BL/6 mice were relatively insensitive to angiotensin II for the drinking behavior. Therefore, we speculate that mice might not be able to fully compensate the dehydration status by stimulating the drinking behavior. Further evaluation and consideration will be needed to yield any findings about the dehydration by the sauna treatment.
CCr (mL/min) is calculated by the formula urine creatinine (mg/dL) × urine volume (mL/min) / serum creatinine (mg/dL). In general, it is considered the equivalent of the creatinine production amount if the body weight is almost the same. Decrease in blood creatinine points to an increase in the excretion of creatinine by the kidneys. The CCr can be significantly

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<tr>
<td>Na</td>
<td>Sh</td>
<td>146 ± 2.8</td>
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<td>(mEq/L)</td>
<td>Sh−S</td>
<td>148 ± 2.2</td>
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<td>Nx</td>
<td>151 ± 4.2*</td>
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<td>Nx−S</td>
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<td>K</td>
<td>Sh</td>
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<td>(mEq/L)</td>
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<td>Cl</td>
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<td>Nx</td>
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<td>Urine microalbumin</td>
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<td>Nx−S</td>
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Values are means ± SDs. No. of mice: Sham+non-ST = 5, sham+ST = 5, Nx+non-ST = 5, and Nx+ST = 5. Na: serum sodium, K: serum potassium, Cl: serum chloride, creatinine: serum creatinine, CCr: creatinine clearance. Urine albumin is adjusted for urine creatinine concentration. Sham+non-ST: non-sauna-treated sham-operated mice, sham+ST: sauna-treated sham-operated mice, Nx+non-ST: non-sauna-treated 5/6 nephrectomized mice, and Nx+ST: sauna-treated 5/6 nephrectomized mice. *P < 0.05: vs. sham+non-ST, **P < 0.01: vs. sham+non-ST, † P < 0.05: vs. sham+ST, and ‡ P < 0.01: vs. sham+ST.
affected by a slight change in blood creatinine levels. This quantity is therefore affected by the accuracy of the measurement of serum and urine creatinine levels. As we can see in Table 1, despite the serum creatinine being reduced at 12 weeks, a contradiction in terms of reduced

![Fig. 2](image-url)

**Fig. 2** Expression of TGF-β1, nephrin, and eNOS mRNAs in the kidney. mRNA expression of transforming growth factor (TGF)-β1, nephrin, eNOS, and GAPDH was determined by real-time PCR. The abundance of each mRNA was normalized for GAPDH, and data are expressed as fold increase over sh. Sh, non-sauna-treated sham-operated mice; Sh+S, sauna-treated sham-operated mice; Nx, non-sauna-treated 5/6 nephrectomized mice; Nx+S, sauna-treated 5/6 nephrectomized mice.

No. of mice: Sh = 5, Sh+S = 5, Nx = 5, Nx+S = 5. *P < 0.05.
CCr was observed. One of the factors considered to be contributing to this contradiction was that the slope of a calibration curve (linear equation when assuming that the horizontal axis is the concentration) prepared at the time of measuring creatinine had decreased. Maybe there was a problem in the standard solution. Compared to the volume of blood collected by the mouse tail cut method, only trace amounts of creatinine were seen (serum volume approximately 40 µL). The sample volume could not be re-examined as none was left over. However, we believe the CCr values at 12 weeks value of true are almost the same as those 8 weeks (Sh: 0.36 mL/min, Sh-ST: 0.39 mL/min, Nx: 0.26 mL/min, Nx-ST: 0.28 mL/min).

2. Effect of ST on eNOS upregulation

The renal eNOS mRNA expression was significantly increased in the Nx+ST group compared with the Nx+non-ST group. NO plays an important role in the regulation of renal blood flow (RBF) and glomerular filtration rate (GFR) in the kidney[24, 25]. Endothelial cell dysfunction occurred in CKD[7] leads to the reduction in NO production and NOS expression, and it accelerates the progression of CKD. Therefore, endothelial dysfunction is a treatment target for CKD[7, 26-29]. Sufficient amount of renal blood flow is an important factor for the eNOS expression and the activation of NO production[25, 30]. NO production is increased by an increase in cardiac output (CO) and shear stress of large vasculature due to sauna treatment[11, 31]. The systemic thermal stimulation greatly increases cutaneous circulation. On the other hand, it decrease circulation in kidney, other splanchnic and muscle vasculature[32]. Interestingly enough, Iiyama et al.[33] demonstrated that warm water bathing (the subjects bathed in 41°C water for 10 min and then kept warm with a blanket for 30 min) increased RPF and did not change GFR. Considering the hemodynamic changes shown after bathing, our results might support their conclusion. The low temperature sauna may not increase stress in glomerulus and shear stress in renal vasculature.

3. 5/6 nephrectomized C57BL/6 mice

Renal failure, whatever primary disease, is progress for excessive stress on remaining glomerular through decrease nephron by renal failure. 5/6 nephrectomy as CKD model was reproduced it condition by nephron reduction due to operation. Remnant kidney of mouse and rat in this model occur hyperfiltration and glomerular hypertrophy, decrease in GFR, glomerulosclerosis are observed failure if progress[34, 35]. The features of this experimental procedure are common to CKD observed in humans[36]. Increase in serum creatinine, decreased CCr were observed our 5/6 nephrectomy C57BL/6 mouse, we considered that it has reproduces the basic pathology of CKD.

In this study, systolic blood pressure and urine protein levels in the Nx groups did not change throughout the low temperature sauna period. Ma and Fogo[37] reported that systolic blood pressure was not increased after the 5/6 nephrectomy in C57BL/6 mice, and urine protein excretion returned to normal without treatment. Mouse strains such as 129 strain have two renin genes, while C57BL/6 mouse strain has one renin gene. The mice with two renin genes have 10-fold higher plasma renin activity, angiotensin-dependent hypertension, increased
blood pressure, and cardiac and renal hypertrophic responses to salt compared with the mice with one renin gene\textsuperscript{38, 39}. Hemodynamics and urinary protein excretion are influenced by the renin gene polymorphisms. The C57BL/6 mouse would not be a suitable CKD model. Thus, the mouse strain should be carefully considered for the future studies.

\textbf{V CONCLUSION}

There are no clear adverse events associated with low-temperature sauna treatment. Therefore, it is safe for the CKD model mouse in this study setting. Kidney eNOS mRNA expression was increased by the low-temperature sauna. The present results suggest a renal protective effect caused by suppressed glomerular hypertension in the CKD model mouse.

\textbf{Conflict of Interest}

We declare that there is no conflict of interests regarding the publication of this paper.

\textbf{References}

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抄録

一般にサウナは健康に良いとされ、腰痛や肩こりのみならず、慢性心不全など従来禁忌とされてきた疾患にも適応されるようになってきた。一方、腎不全に対するサウナの影響を検討した論文は1970年代以降みられない。本研究では、5/6腎摘除（Nx）マウスに対する低温サウナの安全性、および腎組織での内皮型一酸化窒素合成酵素（eNOS）の発現を確認し、サウナによる腎保護作用について検討する。

我々は、マウス（C57BL/6）を用いて5/6腎摘除モデルを作成し、サウナ治療群、非治療群に分け、同様に偽手術を施したマウスをサウナ治療群と非治療群に分け、4群（各n=5）で実験を行った。低温サウナ治療には電熱式自然対流温熱装置を用い、41℃、15分間の加温、その後32℃、20分間の保温を12週間連日実施した。

 Nxモデル完成時の血清クレアチニンは偽手術群に比して有意な上昇を示した。12週間のサウナ治療後、サウナ治療群と非治療群でクレアチニンクリアランスに低下傾向がみられたが統計学的有意差は認められなかった。体重、水分摂取量、24時間尿量、血清ナトリウムなどの電解質にも有意差はなく、今回のサウナ設定において明らかな有害事象はなかった。収縮期血圧、尿タンパクいずれも有意差は認められず、期待した尿タンパク減少効果は確認できなかった。しかし、eNOS mRNAの発現量は、サウナ治療群で有意な増加が認められた（p<0.05、Nx治療群 vs. Nx非治療群）。腎でのeNOS mRNAの発現増は輸出入細動脈の温熱性拡張による糸球体にかかる圧ストレス軽減が期待でき、糸球体硬化を生じやすい種では尿タンパク減少が期待できるかもしれない。

キーワード：低温サウナ治療、5/6腎摘除モデルマウス、eNOS mRNA