Original Contribution

Evaluation of Heat Shock Protein 70 Induced in Spa Therapy with a Simple Thermal: Preliminary Experience

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Abstract: To clarify the effects of spa therapy on biodefense, we evaluated the induction of Hsp70 and Hsp70mRNA and hematological and immunological changes in 10 healthy males and females after bathing in an alkaline simple spring (40°C). Compared with the control value before bathing, the amount of Hsp70 expression in the group that bathed once (10 minutes) increased 1.2-fold and 1.6-fold after 48 and 96 hours, respectively. The amount of Hsp70mRNA expression in the group who bathed once increased 1.4-fold after 96 hours, and that in the group that bathed twice increased 1.7-fold after 48 hours and 1.82-fold after 96 hours. Blood examinations in the group that bathed once showed an increase in lymphocytes after 48 hours (p < 0.05) and decreases in peroxylipid, total ketone body, acetoacetic acid, and 3-hydroxylactic acid after 96 hours (p < 0.05). In this group, NK activity decreased after 48 hours but recovered to the pre-bathing level after 96 hours. The group that bathed twice showed an increase in white blood cells after 48 hours (p < 0.05) and an increase in catalase and decreases in peroxylipid and acetoacetic acid after 96 hours (p < 0.05). This group also showed a decrease in NK activity after 48 hours and a further decrease after 96 hours (p < 0.05).

These results suggest the involvement of Hsp70 in the disorder-preventive effects of spa therapy as well as in the anti-oxidative defense mechanism.

Introduction

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In modern society, there are many people with decreased biodefense ability due to environmental contamination, food pollution, and lack of exercise. Attention has been directed to alternative therapies that have a non-invasive effect on the balance of the body, strengthen immune capacity, and enhance spontaneous healing ability in humans.\textsuperscript{1)} There are various types of alternative therapy with purposes from healing to relaxation. Spa therapy is one of these methods. Spa therapy has been long called "tōji" in Japanese and has been reported to have effects on some disorders.\textsuperscript{2, 3)} Pain relief, sedation, rehabilitation, improvement in constitution, and maintenance of health have been shown. Kato\textsuperscript{4)} reports that spa therapy for the maintenance/promotion of patient health should be the main therapy, and western medicine rather than spa therapy should be regarded as an alternative therapy.

In this study, to scientifically evaluate the effects of spas, we investigated heat-shock protein 70 (Hsp70) and evaluated its induction in the body after spa bathing.

HSP is produced when cells undergo stress and is involved in the repair of stress-induced damage. Therefore, Hsp70 is one of the proteins that has recently attracted special attention regarding biodefense.\textsuperscript{5, 6)}

Materials and methods

I. Subjects

The subjects were 5 males and 5 females aged 21-28 years (mean, 25.5 ± 1.6 years) from whom informed consent for this study was obtained.

II. Place of test

The test was performed in an alkaline simple spring (about 40°C) in a spa center. Informed consent was obtained from the appropriate authorities for the purpose of this study.

III. Experimental methods

The subjects were allocated into two groups who bathed once (10 minutes) or twice (10 minutes × 2; 1-hour rest between bathing). Hsp70 and Hsp70mRNA were measured, and a blood examination was performed.

1. Measurement of Hsp70 and Hsp70mRNA

Heparinized blood (7 ml) was diluted 1/2 with phosphate-buffered saline (PBS), mixed with the same volume of lymphocyte separate solution (Separate L: Muto Pure Chemicals Co.), and centrifuged at 2,500 rpm for 30 minutes. The lymphocyte layer was obtained, washed with PBS twice, homogenized with 10-volume 0.5% SDS, and centrifuged again at 10,000 rpm for 10 minutes. The obtained lymphocytes were solubilized by adding 10-volume 0.5% SDS. Hsp70 expression was measured by ELISA, and Hsp70mRNA was measured by the light cycler method.

2. Blood examination

In both groups, blood was collected 3 times (before bathing as a control value, and 48 and 96 hours after bathing). To determine the effects of spa bathing, the white blood cell count (WBC), red blood cell
count (RBC), hematocrit (Ht), hemoglobin (Hb), platelets, and differential leukocyte count (6 items) were measured. The serum biochemical examination items were albumin (ALB), uric acid, IP, catalase, SOD, peroxylipid, total ketone body, acetocetic acid, and 3-hydroxylactic acid. As immunological parameters, CD4, CD8, the CD4/CD8 ratio, and NK activity were measured.

IV. Analysis method
Statistical analysis was performed by the t-test using an SPSS Base System 10.07 J, and p< 0.05 was regarded as significant.

Results
I. Hsp70 expression
1. Hsp70 expression in the group who bathed once
In the 5 subjects who bathed once, the amount of Hsp70 expression after 48 hours was 1.2 ± 0.65 times (mean; no significance, p = 0.27) the pre-bathing control value (Fig. 1-a). The values in the 5 subjects were 2.3, 1.1, 0.85, 0.95, and 0.75 times the control value, showing an increase in 2 subjects (Fig. 1-c).

The value after 96 hours was 1.6 ± 0.79 times (mean; N. S., p = 0.11) the control value (Fig. 1-a). The values in the 5 subjects were 2.4, 1.4, 2.5, 0.94, and 0.87 times the control value, showing an increase in 3 subjects (Fig. 1-c).

2. Hsp70 expression in the group who bathed twice
In the group who bathed twice, the amount of Hsp70 expression after 48 hours was 0.94 ± 0.45 times (mean; N. S.) the pre-bathing control value (Fig. 1-c). The values in the 5 subjects were 0.86, 0.39, 1.1, 1.6, and 0.75 times the control value, showing an increase in 2 subjects (Fig. 1-c).

The value after 96 hours was 0.94 ± 0.28 times (mean; N. S.) the control value (Fig. 1-b). The values in the 5 subjects were 0.64, 1.3, 1.1, 0.7, and 0.98 times the control value, showing an increase in 2 subjects (Fig. 1-c).

Fig. 1-a : Mean Hsp70 expression in the group who bathed once (40°C, 10 minutes)

Fig. 1-b : Mean Hsp70 expression in the group who bathed twice (40°C, 10 minutes, twice)
Fig. 1-c: Changes in Hsp70 induction by ELISA in the groups who bathed once or twice (each subject)
II. Expression of Hsp70mRNA

1. Hsp70mRNA in the group who bathed once

In this group, the amount of Hsp70mRNA expression after 48 hours was 1.0 ± 0.8 times (mean; N. S.) the control value (Fig. 2-a). The values in the 5 subjects were 0.22, 1.0, 2.1, 1.5, and 0.17 times the control value, showing an increase in 2 subjects (Fig. 2-c).

The value after 96 hours was 1.4 ± 0.82 times (mean; N. S., p = 0.13) the control value (Fig. 2-a). The values in the 5 subjects were 1.1, 1.9, 1.3, 2.5, and 0.32 times the control value, showing an increase in 4 subjects (Fig. 2-c).

2. Hsp70mRNA expression in the group who bathed twice

In this group, the amount of Hsp70mRNA expression after 48 hours was 1.7 ± 1.9 times (mean; N. S., p = 0.24) the control value (Fig. 2-b). The values in the 5 subjects were 0.18, 0.92, 0.82, 1.5, and 4.9 times the control value, showing an increase in 2 subjects (Fig. 2-c).

The value after 96 hours was 1.8 ± 1.5 times (mean; N. S., p = 0.39) the control value (Fig. 2-b). The values in the 5 subjects were 2.6, 0.47, 0.42, 1.8, and 3.9 times the control value, showing an increase in 3 subjects (Fig. 2-c).

III. Results of blood examination before and after spa bathing (Tables Ia, Ib, Ic)

1. Results of blood examination in the group that bathed once

No changes were observed in WBC, RBC, Hb, or Ht. The platelet count was 254,000/µl before bathing and increased to 374,000/µl (about a 50% increase) after 48 hours but returned to 256,000/µl after 96 hours. The mean lymphocyte percentage was 28.8 ± 4.2% before bathing and significantly increased to 31.0% after 48 hours (p < 0.05), but decreased to 27.2% after 96 hours.

A serum biochemical examination revealed no change in ALB but a slight decrease in uric acid after 96 hours. IP showed a 70% increase (mean) after 48 hours but a 50% increase after 96 hours. The mean SOD level was 8.4% after 48 hours, showing no change, but increased slightly to 10.6% after 96 hours. The mean peroxylipid level was 0.8 nmol/ml before bathing, slightly decreased to 0.66 nmol/ml after 48 hours.
Fig. 2-c: Changes in Hsp70mRNA induction by light cycler in the groups who bathed once or twice (each subject)
hours, and further decreased to 0.42 nmol/ml (about 50% of the pre-bathing value) after 96 hours. The mean total ketone body level was 35.4 μmol/l before bathing, decreased to 33.8 μmol/l after 48 hours, and significantly decreased to 21.6 μmol/l after 96 hours (p < 0.05). The mean acetoacetic acid level was 11.0 μmol/l before bathing and decreased to 6.2 μmol/l after 48 hours, rising to 7.4 μmol/l after 96 hours. The mean 3-hydroxylactic acid level was 24.4 μmol/l before bathing, and increased to 27.6 μmol/l after 48 hours; however, it significantly decreased to 16.8 μmol/l after 96 hours (p < 0.05).

An immunological examination showed a decrease in the mean NK activity from 16.6% before bathing to 10.7% after 48 hours but recovered to 15.5% after 96 hours. The mean CD4 percentage increased once but returned to the pre-bathing level after 96 hours. The mean CD8 percentage was 35.9% before bathing but decreased to 32.2% after 48 hours and 30.6% after 96 hours. The mean CD4/CD8 ratio was 1.1 before bathing but slightly increased to 1.3 after 48 hours and remained 1.3 after 96 hours.

2. Results of a blood examination in the group that bathed twice

A routine blood examination showed a significant increase in the mean WBC from 6,442/μl before bathing to 7,268/μl after 48 hours (p < 0.05) but recovered to 6,442/μl after 96 hours. No change was observed in RBC, Hb, or Ht. The mean platelet count decreased from 330,000/μl before bathing to 225,000/μl after 48 hours and to 209,000/μl after 96 hours. The mean lymphocyte percentage was 31.8% before bathing and increased to 35.9% after 48 hours and 40.2% (about a 30% increase) after 96 hours.

A serum biochemical examination showed no change in ALB or uric acid. The mean IP level was 3.7 mg/dl before bathing, 4.1 mg/dl after 48 hours, and 5.1 mg/dl after 96 hours. The catalase level was 1.4 U before bathing and 1.2 U after 48 hours; however increased to 2.3 U (about a 90% increase) after 96 hours. The mean SOD percentage was 9.6% before bathing and 9.5% after 48 hours but 13.2% (about a 37% increase) after 96 hours. The mean peroxylipid level was 0.88 nmol/ml before bathing, 0.66 nmol/ml after 48 hours, and 0.72 nmol/ml after 96 hours. The mean acetoacetic acid level was 10.8 μmol/l before bathing, 5.2 μmol/l after 48 hours (p < 0.05), and 5.6 μmol/l (about a 50% decrease) after 96 hours.

An immunological examination showed a decrease in the mean NK activity from 20.4% before bathing to 15.8% after 48 hours and a significant decrease to 10.4% after 96 hours (p < 0.05). CD4 and CD8 changed only negligibly, and the mean CD4/CD8 ratio was 1.1 before bathing, 1.0 after 48 hours, and 1.0 after 96 hours.

Discussion

I. Hsp70 expression

HSP is a group of proteins initially found to be induced by heat shock but also induced by other environmental changes due to physical or chemical factors other than heat. In this study, to evaluate the effects of spa bathing on health, Hsp70 that is expressed after heat stress was measured. In addition, for molecular level analysis, Hsp70mRNA was added as a measurement item.

As a result, the Hsp70 level increased 1.20-fold after 48 hours and 1.6-fold after 96 hours in the group who bathed once (Fig. 1-a). In the group who bathed twice, such an increase was not observed, but 3 of the 5 subjects showed an increase (Fig. 1-c).
Hsp70mRNA in the group that bathed once showed negligible changes after 48 hours but increased 1.41-fold after 96 hours, confirming a slight expression. In the group that bathed twice, a 1.7-fold increase over the pre-bathing level was observed after 48 hours, and a 1.82-fold increase was seen after 96 hours, showing marked expression. Therefore, Hsp70mRNA expression was more marked after 96 hours than after 48 hours, suggesting that the effects of spa bathing continued for a long time. However, in the
group that bathed twice, Hsp70 after 48 hours was 0.99 times, and that after 96 hours was 0.94 times the pre-bathing value, showing decreases. This suggested that bathing once had a more marked effect than bathing twice. Thus, Hsp70 induction may be an effect of spa bathing, and the induced Hsp70 may reduce the degree of injury due to subsequent increased stress. As a disorder-preventive effect of spa therapy, the involvement of Hsp70 should be considered.

Ito \(^5\) heated the entire bodies of rats using a heating apparatus by electromagnetic radiation (40-45°C) for 30 minutes and observed a 2-fold increase in Hsp70 after 48 hours. However, it is almost impossible for humans to endure spa bathing at that temperature for 30 minutes. Further studies are thus necessary to evaluate various types of bathing, from 10-minute bathing to repeated sessions of 3-5 minutes for subjects who experience discomfort with 10-minute bathing, or the effects of long-term spa therapy (10 minutes daily) in terms of Hsp70 expression.

Concerning the correlation between Hsp70 and Hsp70mRNA, it can be assumed that their increases occur nearly simultaneously because Hsp70 expression occurs in the G1/S border area of the cell cycle and Hsp70mRNA increases in the S phase in cells without stress \(^7\). The group that bathed twice showed no change in Hsp70 but a slight increase in Hsp70mRNA expression after 96 hours. Based on the above assumption, there is a possibility that Hsp70 increases after more than 96 hours.

II. Results of blood examination before and after spa bathing
1. Routine blood examination

A routine blood examination showed significant differences in WBC and the lymphocyte percentage among the values before bathing and 48 and 96 hours after bathing. In addition, RBC, Ht, and Hb data, such as that from anemia patients showed no changes in the group that bathed once, while RBC and Hb slightly increased after 48 hours and 96 hours compared with the pre-bathing value in the group that bathed twice. The mean WBC in the group that bathed twice was 6,422/μl before bathing and significantly increased to 7,258/μl after 48 hours. The mean lymphocyte percentage in the group that bathed once was 28.8% before bathing but significantly increased to 31.0% after 48 hours. These results are the opposite of those reported by Oh et al.\(^8\) who studied short-term spa bathing (41°C, 20 minutes, once or twice) in healthy subjects. However, a direct comparison between the two studies is difficult because of differences in the age of the subjects (≤ 35 years or less in this study), the frequency of bathing, spa quality, and blood collection times (after 48 and 96 hours in this study).

2. Serum biochemical examination

A serum biochemical examination showed decreases in total ketone body, acetoacetic acid, and 3-hydroxyxylactic acid, which increase in the blood mainly during fatigue. The decrease was particularly significant in the total ketone body after 96 hours in the group that bathed once. These results suggest that bathing once reduces substances produced as a result of fatigue. In this study, a correlation between Hsp70 expression and decreases in fatigue substances was observed only in the group that bathed once. In the group that bathed twice, Hsp70 expression was slight, and fatigue substances increased. However, since Hsp70mRNA increased, slightly further studies are necessary to evaluate methods of bathing and conditions, such as bathing and resting times.
The catalase level did not markedly change in the group who bathed once but showed about a 60% increase from 1.4 U (mean) before bathing to 2.3 U (about a 60% increase) after 96 hours in the group who bathed twice. The SOD percentage also increased from 8.5% (mean) before bathing to 10.6% (about a 20% increase) after 96 hours in the group that bathed once and from 9.6% before bathing to 13.2% (about a 40% increase) after 96 hours in the group that bathed twice. These results suggest that bathing at 40°C for 10 minutes once or twice increases anti-oxidation effects.

The peroxylipid level decreased after bathing in the group who bathed once, reaching 50% of the pre-bathing level after 96 hours, and also decreased slightly in the group that bathed twice, suggesting the in vivo inhibition of active oxygen.

Since the association between oxidation stress and aging or various disorders such as diabetes mellitus and carcinogenesis, has been suggested,9) biodefense due to the anti-oxidation effects of spa therapy can be expected.

3. Immunological examination

The favorable effects of spa therapy, spa bathing, and spa water itself on immune function have been reported.8) 10) 11) Matsuno et al.11) reported an increase in the CD4/CD8 ratio 24 hours after short-term spa bathing (once or twice) at 41°C for 20 minutes in healthy subjects aged ≥ 36 years but a decrease in this ratio in those aged ≤ 35 years. In our study, all subjects were younger than 35 years old, and negligible changes were observed in CD4+ cells or CD8+ cells with no characteristic changes in the CD4/CD8 ratio after 48 or 96 hours.

The NK activity significantly decreased after 96 hours in the group who bathed twice. This finding may be similar to the results of the study by Shirakura,12 who reported that the CD4/CD8 ratio decreased after subjects bathed at 47°C 3 times daily for 21 consecutive days and showed a general decreasing trend although the increase/decrease was repeated during a 6-week course. In this study, spa bathing was performed once or twice in order to evaluate the involvement of Hsp70 on the effects of bathing. Therefore, measurements were performed only until 4 days after bathing. However, since the decrease in the CD4/CD8 ratio is contradictory to Hsp70 expression or biodefense/protection, long-term studies on changes in immune capacity are also necessary.

Conclusion

To clarify the effects of spa therapy on biodefense, we evaluated the induction of Hsp70 and Hsp70mRNA and hematological and immunological changes after spa bathing. In the group that bathed once (10 minutes), the amount of Hsp70 expression increased 1.2-fold after 48 hours and 1.6-fold after 96 hours, and the amount of Hsp70mRNA expression increased 1.4-fold after 96 hours. In the group that bathed twice (10 minutes × 2), the amount of Hsp70mRNA expression increased 1.7-fold after 48 hours and 1.82-fold after 96 hours. Blood examinations showed an increase in lymphocytes and decreases in peroxylipid, total ketone body, acetoacetic acid, and 3-hydroxyacetic acid.

These results suggested the involvement of Hsp70 in the disorder-preventive effects of spa therapy as well as in the anti-oxidative defense mechanism.

For this study, the results of bathing once were compared with those of bathing twice on a single day.
The results of this short-term spa bathing may differ from those of long-term spa therapy. Therefore, further studies are necessary to evaluate changes in Hsp70 expression after long-term spa therapy or those depending on bathing methods with attention directed toward comprehensive bioregulatory effects as a characteristic of spa therapy.

References

単純泉による温泉療法により誘導される
Heat Shock Protein 70 の評価

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要　旨：温泉療法の生体防御に及ぼす効果を評価する目的で、健康的な男女 10 名を対象に 40°C、アルカリ性単純温泉における温泉入浴後の Hsp70 および Hsp70mRNA の誘導と血液学的、免疫学的動態について検討した。温泉入浴 1 回目の (10 分間) における Hsp70 の発現量は入浴前のコントロールに比べて 48 時間後に 1.2 倍、96 時間後に 1.6 倍の増加を認めた。Hsp70mRNA の発現量では、温泉入浴 1 回目において 96 時間後に 1.4 倍、2 回目において 48 時間後に 1.7 倍、96 時間後に 1.82 倍の増加を認めた。血液検査においては温泉入浴 1 回目の 48 時間後にリンパ球の増加 (p < 0.05)、96 時間後には過酸化脂質、総ケトント体、アセト酢酸、3-ヒドロキシ酢酸 (p < 0.05) の低下を認めた。NK 活性は 48 時間後に低下したが、96 時間後に入浴前値までの回復を認めた。2 回目では 48 時間後に、白血球の増加 (p < 0.05)、96 時間後にはカタラーゼの増加、過酸化脂質およびアセト酢酸 (p < 0.05) の低下を認めた。NK 活性は 48 時間後に低下し、96 時間後には更に低下 (p < 0.05) していた。以上の結果から、温泉療法の疾病予防効果における Hsp70 の関与が示唆され、温泉療法が抗酸化的防御機構にも関与している可能性が明らかになった。