Abstract

Widely distributed electrolytic-generators for hydrogen-water are not fully considered for the dependencies of post-electrolytic values of the dissolved hydrogen concentration (DH) and the oxidation-reduction potential (ORP) on the properties of the pre-electrolytic water. We investigated the dependencies of DH and ORP on mineral-based hardness, temperatures and the container materials, and effects on the oral cavity by oral washing or drinking. Along with an increase in mineral-based water-hardness, DH decreased from 960 to 870 μg/L and the ORP unexpectedly increased from −400 to −320 mV. Purified water of almost zero hardness, however, caused a post-electrolytic DH as low as 80 μg/L and an ORP as high as +20 mV. Post-electrolytic DHs were not significantly changed (780–900 μg/L) upon electrolysis at 1.5–30°C and decreased at 40–50°C. The diffusion of hydrogen from the inside to the outside of the container was extremely small even after 12 hours for an aluminum- or stainless steel-made container, but not for containers made of diverse plastics. The ORP of the intact saliva was +136 mV, and decreased to +90 mV at 20 minutes after 1-minute oral-cramming of hydrogen-water, but returned to +135 mV after 60-minute leaving, showing a transient ORP-decrease in the saliva. Drinking-pause for 4 weeks after drinking hydrogen-water, however, saliva ORP, gradually but not instantaneously, increased to +60 to +80 mV, but upon drinking-resumption and 2 weeks thereafter, decreased again to −100 to −110 mV, suggesting that several-week hydrogen-water drinking caused a certain decrease in the saliva ORP. Thus, the present study provided the appropriate conditions such as hardness and temperatures for hydrogen-water production by the electrolytic generator, and the container materials suitable for hydrogen-water preservation. Furthermore, we clarified ORP changes of human saliva, being an indicator for human oxidative stress. The study was approved by the Medical Ethics Committee of the NPO (Non-Profitable Organization)-Corporate Japanese Center for Anti-Aging Medical Sciences (approval No. 09S02) on May 2, 2012.

Key words: dissolved hydrogen concentration; hydrogen-water; hydrogen-water generator of the portable type; oxidation-reduction potential; oxidative stress; saliva; storage of hydrogen-water; water quality; water temperature; water-hardness

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Introduction

Hydrogen can be used for medical applications without surplus worries by several ingestion methods because inhalation of 1–4% hydrogen gas exhibits a great efficacy from a viewpoint of absorption into human body, especially through the respiratory tissue.1,2 There are several methods to ingest or consume hydrogen: inhaling hydrogen gas, drinking hydrogen-water, injecting hydrogen-dissolved saline (hydrogen-saline), taking a hydrogen bath, or dropping hydrogen-saline into the eyes. The method of drinking is a relatively easy way.

Hydrogen-water may be beneficial since it is a portable, easily be administered and proposes a safe way to ingest hydrogen.3,4 A hydrogen-water generator of the portable type has the convenience of utilizing the functional water arbitrarily regardless of location or time. On the other hand, the user does not usually have the correct information on the dissolved hydrogen concentration (DH) or oxidation-reduction potential (ORP), related to environmental factors on hydrogen generation, such as the mineral-based hardness of water, thus the hydrogen-water provider is considered to be obliged to provide accurate information to users.

We conducted basic experiments on DH and ORP of hydrogen-water under a variety of conditions of pre-electrolytic water and post-electrolytic hydrogen-water and investigated the influence of washing/drinking hydrogen-water on the living body.

We first examined the correlation between the dependencies of post-electrolytic DH and post-electrolytic ORP of hydrogen-water on differences of water quality during electrolytic manufacture by means with a hydrogen water generator to obtain the best conditions of retain hydrogen in the process of production and storage of hydrogen-water.

Drinkable hydrogen-water first enters the mouth and reaches the stomach through the esophagus, and then be absorbed into the intestine. In this process, it is thought that hydrogen passes through various tissues and migrates to body fluids and blood.5,6

It has been reported that ORP of saliva is a barometer of general health conditions.7,8 Owing to the limited amount of drinking water being supplied, we next examined the change
in ORP of saliva when hydrogen-water was contained in the mouths of aged persons, and then examined the ORP change upon swallowing or drinking the hydrogen-water.

Hydrogen-containing water is easy or feasible to be incorporated into the blood via the saliva glands, which are well-known to be pharmacologically utilized as the absorption route for “buccal” or “troche,” in spite of aging/senescence-dependent decline for the saliva-gland functions. Thus, dependencies of the saliva in aged persons on hydrogen-based ORP are worthy for research concerning gerontological saliva redox-potentials.

**Materials and Methods**

**Relationship between types of water and DH**

To investigate the correlation between the dependencies of post-electrolytic DH, and the post-electrolytic ORP of hydrogen-water on differences of water quality during electrolytic manufacture, we utilized a hydrogen water generator of the portable type: an H-AQUA hydrogen-water server (Good Well Engineering Ltd., Hong Kong, China; **Figure 1**). Commercial mineral water of five types with diverse qualities such as hardness (18, 60, 83, 150, and 304 mg/L), pure water and tap water were calculated for mineral-based hardness by an equation “(Ca^{2+}) \times 2.5 + (Mg^{2+}) \times 4.0.” They were electrolyzed at a water temperature of 20°C, and immediately each 300-ml aliquot was estimated for DH and ORP, by a DH meter ENH-1000 (Trustlex Co., Ltd., Osaka, Japan) and an ORP meter, TL-60 (Trustlex Co., Ltd.), respectively.

![Figure 1: Hydrogen-water generator of the portable type.](image)

**Note:** The generation of hydrogen-water by electrolysis was performed using the hydrogen-water generator of the portable type, H-AQUA hydrogen-water server.

**Influences of water temperatures during the generation of hydrogen-water on post-electrolytic DH and ORP**

Water temperatures on hydrogen-water generation from mineral water with a pre-electrolytic hardness of 83 mg/L were examined for the influences on the DH and ORP, before and after generation when the water temperature is changed from 1.5°C to 50°C. Measurements for each condition were performed more than three times.

**The influence of material differences of hydrogen-water storage containers on migration of hydrogen gas into the atmosphere and migration of hydrogen gas into the outside water from a container for hydrogen-water storage**

We examined the influence of material differences of hydrogen-water storage containers on fading-out migration of hydrogen gas into the atmosphere, and relationship between hydrogen storage times and hydrogen storage amounts in various hydrogen-water containers (polyethylene (PE) container, polyethylene terephthalate (PET) container, polypropylene (PP) container, glass container, aluminum container, and stainless-steel container). Under the condition of hydrogen-water of 150 mL, at 20°C, hydrogen-water having DH of 830 μg/L was placed in each container and left to stand for 12 hours.

Changes in DH and ORP were observed in order to examine the leakage migration of hydrogen gas into the water of outside, from hydrogen-water inside a PE-made hydrogen-water storage container. Hydrogen-water in a PE storage container was 50 mL, and the water outside a PE storage container in a stainless-steel-made tank was 150 mL. Measurements for each condition were performed more than three times.

**ORP changes of human saliva in time of oral washing with hydrogen-water**

The total number of person-examinations was five. However, by the limitation of burdens on elder persons, who were selected for the purpose of the research on saliva ageing, one or two subjects could continue each experiment schedule.

Changes in ORP of saliva by washing with hydrogen-water were examined for a 66-year-old woman (normal health) and a 100-year-old woman (normal health) with their informed consents being examined further for 60 minutes. The subjects crammed 50 mL of hydrogen-water (DH, 825 μg/L; ORP, –395 mV for the former, DH, 825 μg/L; ORP, –392 mV for the latter) in the mouth for 1 minute and then rinsed with the mineral water (ORP, +242 mV).

For the 66-year-old woman, the saliva ORP was examined under the following conditions in order to further observe the detailed change in the ORP of the first 10 minutes; the subject included hydrogen-water of 50 mL (DH, 960 μg/L; ORP, –440 mV) for 1 minute, in the mouth, and then washed with the mineral water (ORP, +190 mV). The research was officially approved, under conditions of noninvasive research such as no hemorrhage, painless treatment, and no trace/sign, using a commercially available apparatus, as an Approval by the Medical Ethics Committee of the NPO (Non-Profitable Organization)-Corporate Japanese Center for Anti-Aging Medical Sciences (approval No. 09S02) May 2, 2012 which was an officially authenticated by the Hiroshima Prefecture Government of Japan. All subjects provided the informed consent.

**ORP changes of human saliva in times of start, temporary pause and resumption for hydrogen-water drinking**

We examined the ORP changes in human (a 66-year-old woman, normal health, with an informed consent) saliva upon controlled, placebo and drinking of hydrogen-water for an 8-week study. The subject ingested 200 mL of soft water (hardness: 83; DH: below the detection sensitivity limit of 10 μg/L) for the first 2 weeks as a control, further drank the same amount of placebo soft water as pseudo hydrogen-water for the next 2 weeks, and then drank hydrogen-water (200 mL; DH, 902.2 ± 32.2 μg/L; ORP, –382.2 ± 19.7 mV) before breakfast.
We further examined the ORP changes in human (a 68-year-old man, normal health, with an informed consent) saliva upon initiation, pausing and resumption of drinking of hydrogen-water for an 8-week study. The subject ingested hydrogen-water (200 mL; DH, 890.8 ± 29.8 μg/L; ORP, –388.9 ± 9.8 mV) at random amounts and time for the first week, and then drank 200 mL of hydrogen-water before breakfast, lunch and dinner for the second week. The intakes of hydrogen-water refrained for four weeks, and then the subject drank hydrogen-water (200 mL; DH, 913.4 ± 28.3 μg/L; ORP, –391.9 ± 10.1 mV) before breakfast, lunch and dinner, for the last 2 weeks. The research was officially approved as above-mentioned. All subjects provided the informed consent.

Statistical analysis

For every clinical study or in vitro research, the Student’s t-test was used to compare the difference in mean ± standard deviation (SD) between the pre-treatment and post-treatment groups using a Microsoft Office Excel 2010 software (Microsoft, Albuquerque, NM, USA) or a software package SPSS 11.0 (SPSS Inc., Chicago, IL, USA) for Windows. A P-value that is below 0.05 was regarded to be statistically significant.

For every clinical study or in vitro research, multiple comparison tests (Tukey-Kramer method) were used for a relationship between water-temperatures during hydrogen-water production and concentrations of resultantly generated hydrogen, and for DH and ORP changes of human saliva in times of the start, temporary pause and resumption of hydrogen-water drinking.

RESULTS

Influences of difference in hardness-based quality on the post-electrolytic DH and ORP

Hydrogen was scarcely generated in purified water (20 μg/L), but, in tap water and mineral water, DH was above 800 μg/L, which was much closer to the saturation of approximately 1600 μg/L under the normal temperature and normal atmospheric pressure (Figure 2A). As the hardness of mineral water increased, DH tended to decrease, and a negative correlation (r = –0.499) was estimated (Figure 2B). In the case of tap water, notably, the concentration of chlorine immediately after electrolysis dropped to about half, and it decreased to almost zero after 1 hour (data not shown).

In purified water, the ORP was in the oxidation state of +15 mV, but in the case of tap water and mineral water, ORP was –320 mV or less being indicative of the reduced state (Figure 3A). As the hardness of mineral water increased, the ORP increased and a strong positive correlation was observed (r = 0.888) (Figure 3B).

Influence of water temperatures during the generation of hydrogen-water on post-electrolytic DH and ORP

The average value of DH was about 800 μg/L at water temperatures of 1.5°C to 30°C, and showed a tendency to decrease when the water temperature was 40°C or more, but the standard deviation was large (Figure 4A).

There was no significant difference in ORP from 1.5°C to 30°C, but in ORP between 1.5°C and 40°C, between 10°C and 50°C, between 20°C and 50°C, between 30°C and 50°C,
there were significant differences of 1% in the dangerous rate. Significant differences of 5% were found between 10°C and 40°C, 10°C and 50°C (Figure 4B).

**Figure 4: Relationship between water temperature and post-electrolytic DH or ORP.**

Note: (A) DH exerted a tendency to decrease along with water temperatures above 40°C. The pre-electrolytic DH showed to be below the detection sensitivity limit. At the time of producing hydrogen-water, there was the amount of water of 300 mL. DH was thereafter measured always at 20°C not so as to be deferred and affected by hydrogen-generating temperatures. (B) ORP tended to increase as the water temperature increased. Data were expressed as the mean ± SD. The number of data at each water temperature was three (n = 3) for a typical converged one chosen out of independent three-time-repeated experiments. *P < 0.05, **P < 0.01. DH: Dissolved hydrogen concentration; ORP: oxidation-reduction potential.

**Influence of difference of materials of hydrogen-water storage containers on fading-out migration of hydrogen gas into the atmosphere and migration of hydrogen gas into the water of outside from a PE hydrogen-water storage container.** Various hydrogen-water containers made of PE, PET, PP, glass, aluminum and stainless steel, were used to measure migration of hydrogen (Figure 5A). Hydrogen diffusion from hydrogen-water in a plastic container and a glass container is fast, about half the hydrogen diffuses after 4 hours and after 12 hours almost zero in the glass container and PE container. On the other hand, in the case of metal (aluminum and stainless steel) containers, the diffusion from the vessel was extremely small even after 12 hours, and the value just after production was maintained (Figure 5B). In plastic containers and a glass container, ORP increased rapidly, and after 12 hours indicated the oxidized state except for PET container. On the other hand, in the case of metal (aluminum and stainless steel) containers, the values of ORP immediately after generation were maintained even after 12 hours (Figure 5C). The migration rates of hydrogen from the inside to outside of various containers are shown in Table 1.

The migration rate of hydrogen fluctuated depending on the material of the container, relatively slow for PET containers and relatively fast for PE containers for up to one hour. At four hours or more, PP and PET containers have relatively slow moving speeds, and PE and glass containers have relatively fast speeds.

**Table 1: The migration rate of hydrogen (μg/L per hour) from the inside to outside of various containers.**

| Material of container | 0–2 h | 4–12 h |
|-----------------------|-------|--------|
| Polyethylene          | 225   | 27.5   |
| Polyethylene terephthalate | 135 | 23.1   |
| Polypropylene         | 180   | 20     |
| Glass                 | 190   | 30     |
| Aluminum              | 0     | 1.3    |
| Stainless steel       | 0     | 0.6    |

Note: The aluminum- or stainless steel-made container repressed the movement of hydrogen from inside to outside. Measurements for each material of container were performed more than three times and typically the mean values were described.
which caused finally to diminish to almost zero for the inside DH. It was therefore interesting, in a case for the PE-container, to examine whether the outside-hydrogen resultant-amount might accord with the outside- and inside-hydrogen amount, from a viewpoint of stoichiometry.

Therefore, we investigated the leakage-migration of hydrogen-water in a PE container to outside water in a stainless steel tank (Figure 6A), resulting that DH and ORP were almost the same between both the sides of the PE container for hydrogen-water storage after 12 hours. However, the DH remained at almost 200 μg/L and the ORP was still kept near –100 mV inside the PE container (Figure 6B and C), meaning that the hydrogen of outside water has an effect of retaining hydrogen in the hydrogen-water inside the container, which is different from the case of air atmosphere without the diffusion limit (Figure 5B and C).

In the case of a 66-years-old female, the ORP of the saliva prior to containing hydrogen-water in the mouth was 136 mV, but dropped to 90 mV after 20 minutes by including hydrogen-water, but returned to 135 mV after 60 minutes. It was found that ORP of saliva diminishes even without drinking hydrogen-water, but it gradually returned to the initial value after about 60 minutes (Figure 7A). Similarly, in the case of 100-years old, female, the ORP formed a bottom in 10 to 20 minutes and returned to its original state in 60 minutes (Figure 7B).

ORP decreased only for saliva containing hydrogen-water in the mouth. Until 3 minutes after 1-minute washing with hydrogen-water, ORP was decreased, but, until 3 to 10 minutes, ORP increased and returned to the value immediately after washing (Figure 8).
ORP changes of human saliva in times of start, temporary pause and resumption for hydrogen-water drinking

For a 66-years-old female, the average of saliva ORPs upon the control drinking and the placebo drinking were 128.8 ± 11.9 mV and 130.8 ± 9.7 mV, respectively. Thereafter, by continuation of drinking hydrogen-water, saliva ORPs decreased to about 80 mV or less (Figure 9A). For a 68-years-old male, the time and amount of drinking hydrogen-water for one week from the start were irregular (random). Hydrogen-water (DH, 902.2 ± 32.2 μg/L; ORP, –382.2 ± 19.7 mV) was further drunk for one week (periodical to drink). After a temporary pause for the drinking of hydrogen-water for 4 weeks (drinking stop), and drinking hydrogen-water (DH, 913.4 ± 28.3 μg/L; ORP, –391.2 ± 10.1 mV) for 2 weeks (drinking resumption), for each situation, ORP was measured (Figure 9B). There was no significant difference between “random drinking” and “periodic drinking,” and saliva ORP gradually increased when stopping drinking, and increased to about 80 mV after 4 weeks. It is unexpected that the ORP-increase was gradual but not steep even though 4-week hydrogen stoppage. Then after resuming drinking, saliva ORP gradually decreased, and decreased to about –115 mV after 2-week periodic drinking. Thus, saliva ORP showed almost stably low values when drinking for a long period of time, even though the time and amount of drinking hydrogen-water are irregular. On the contrary, it was found that saliva ORP gradually increased when stopping drinking, but decreased again when drinking was resumed.

DISCUSSION

Hardness in drinking-water is defined by the World Health Organization (WHO) as Guidelines for Drinking-water Quality. It is known that Ca²⁺ and Mg²⁺ along with their forms of carbonates, sulfates and chlorides confer hardness to the water. Hardness is most commonly expressed as milligrams of calcium carbonate equivalent per liter. Water containing calcium carbonate at concentrations below 60 mg/L is generally considered as soft; for 60–120 mg/L, moderately hard; 120–180 mg/L, hard; and more than 180 mg/L, very hard. We conducted experiments using five types of mineral water (hardness: 18, 60, 83, 150 and 304 mg/L).

In the present study, we aimed to explore the most suitable conditions necessary for hydrogen-water generation and storage of hydrogen-water upon the manipulations for a hydrogen-water generator of the portable type, and also to investigate the time-dependent effects of hydrogen-water oral-washing and intake on the saliva ORP.

The usefulness of hydrogen on organisms includes the protection against post-ischemic reperfusion injury, protection against neurodegeneration, preventive effects on metabolic syndrome, improvement of lipid metabolism, anti-inflammatory effects, and reductive relieving effects on side-effects in oxidative cancer-treatment and so on.

We turned this concept in a publication in 2007 that hydrogen acts as a therapeutic and preventive antioxidant by selectively reducing highly strong oxidants such as hydroxyl radical (•OH) and peroxynitrite (ONOO⁻) in cells, and that hydrogen exhibits cytoprotective effects against oxidative stress. These published papers cover many biological effects against oxidative stress in most of organs. Moreover, it has been revealed that hydrogen has more functions, including anti-inflammatory, anti-apoptotic and anti-allergic effects, and that hydrogen stimulates energy metabolism, in most tissues of model animals.

Compared to commercially available hydrogen-bubbled water, the electrolytically produced hydrogen-water and nano-bubble hydrogen-water (nano-H water) showed the superiority over other types of hydrogen-water in terms of •OH scavenging activity. That is, significant reduction activities of hydrogen-water and nano-H water were shown. A hydrogen-water-producing portable-type apparatus has the convenience that functional water can be arbitrarily provided irrespective of locations and times. However, the correct information has scarcely been provided concerning the DH and ORP, in relation to environmental factors such as mineral-based water hardness and temperatures on hydrogen electrolytic generation in commercially available hydrogen-water products.

In the present study, first, basic experiments in the generation of hydrogen-water were performed using a portable type hydrogen-water generator. When the pre-electrolytic water had the quality of softness or hardness or tap water, post-electrolytic DH exceeded 800 μg/L. However, as the hardness of mineral water increased, the post-electrolytic DH tended to decrease. It turned out that the usage of mineral water with lower hardness is more suitable for hydrogen-water production. In addition, since hydrogen was hardly generated in purified water (20 μg/L), it was found to be unsuitable to
hydrogen-water production. When examining the relationship between hardness and post-electrolytic ORPs, a strong correlation was observed, in which the ORP gradually increased as the hardness of the mineral water increased. It was surmised that as the mineral concentration in the electrolytic water increased, the conductivity increased and hence the electrolysis efficiency was promoted. On the contrary, the DH decreased and the ORP simultaneously increased, both of which were unexpected. The results may be attributed to the alternative possibility that the existence of more abundant minerals obstructs the stability of hydrogen-bubbles. No existence of minerals in the purified water however, generated extremely DH-lowering together with extremely ORP-heightening, suggesting that optimal hydrogen-water generation may need the smaller mineral-amounts, but is unsuitable for no mineral or excess mineral in the electrolytic water.

Lower-hardness mineral-water (soft water) and tap water in Osaka-city waterworks were at the same ORP level. However, tap water was mixed with various impurities, and it was considered not to be highly suitable for preservation of hydrogen-water from the viewpoint of hygiene. Therefore, results for ORP also indicated that it was better to use commercially available mineral water with lower hardness for hydrogen-water production.

For the relationship between water temperature and post-electrolytic DH or ORP, DH decreased and ORP increased, either of which being proportional as the water temperature during electrolytic generation was artificially heightened. These revealed that lower water-temperatures of the examined range (1.5–30°C) were suitable for the production of hydrogen-water. The results are reasonable because of Henry’s law that the amount of gas dissolved in water correlates inversely with water-temperatures.

In addition, in our investigation for permeability-based hydrogen diffusion (migration), if plastic or glass containers are used, hydrogen in hydrogen-water is likely to diffuse out of the containers, but it scarcely diffused outside the metal container even after 12 hours. From these experiments, in the electrolytic generation of hydrogen-water, hydrogen-water with a higher DH is generated as the temperature of the water is lower, and the hydrogen-water after generation is advantageously stored in containers made with metals such as aluminum or stainless steel over organic polymers such as PE. Hydrogen is the smallest molecular size in the earth, and therefore permeates easily through the sparse and light-weight PE, but can be blocked by high-density metals.

We next examined the effect of oral irrigation/drinking of hydrogen-water on the ORP of the saliva. It was reported that ORP of saliva is a barometer of general health conditions in terms of amylase activities, stress-based cortisol and dry mouth. In addition, saliva analysis as an indicator of oxidative stress has been suggested as an effective diagnostic tool.24,25

The major salivary glands, including the parotid, submandibular and sublingual glands, and minor salivary glands that distribute throughout the oral cavity are open in the oral cavity. The daily secretion rate is about 1000 mL/day, and the normal secretion rate is 10 mL/10 minutes or more. Saliva that can be easily collected as fluid in the present study was used to ORP measurement. At about an hour after 1-minute washing the mouth with hydrogen-water and the subsequent mineral-water-rinse, the mouth returned to the same ORP as that before containing the hydrogen-water in the mouth. However, it was found that just containing hydrogen-water in the mouth reduced the ORP of the saliva, even after spitting-out of hydrogen-water and thereafter rinsing. The results suggest that hydrogen-water heightened and retained the ORP-based reducing-ability of the saliva within the definite period as short as 2 minutes even after transient oral washing and thereafter rinsing, suggesting the transient durability of hydrogen with an effect-exerting range wider than the inside of an oral cavity. In addition, even if the time and amount of drinking hydrogen-water were irregular, saliva ORP showed a nearly stably low value by drinking it for a long time. The salivary ORP which increased by stopping the supply of hydrogen-water also recovered relatively quickly upon resumption.

Ordinarily, the ORP of human saliva is around +40 mV.26 It was found that even single-frequency intake of hydrogen-water can significantly reduce the ORP of saliva, which is an indicator of the oxidative stress. Additionally, it was found that the duration of the hydrogen-water drinking effect was relatively short to sustainably reduce oxidative stress. Owing to the benefit-utilization of hydrogen-water against oxidative stress, it may be necessitated to continuously supply hydrogen-water and maintain the effect of diminishing the oxidative stress. The gradual ORP-increases without instant and steep restoring suggest that the effects of hydrogen on the reducing and anti-oxidant hallmark of the saliva are spreading with a range wider over the inside of an oral cavity.

In conclusion, the most suitable conditions for electrolytic hydrogen-water production by the hydrogen-water generator were found as temperatures from 1.5°C to 30°C and water-hardness of 83 mg/L, and those for the preservation of hydrogen in hydrogen-water after production as the aluminum- or stainless-steel-made container. Furthermore, we clarified the ORP change of saliva, which is well-known to be an index of the oxidative stress in living organisms, from two viewpoints such as oral-containing and swallowing/drinking of hydrogen-water.

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Author contributions
Literature search: YT; data collection: FT, MN; funding acquisition: FT; manuscript writing: YT, NM. All authors read and approved the final version of manuscript for publication.

Conflicts of interest
None declared.

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Institutional review board statement
The study was approved by the Medical Ethics Committee of the NPO (Non-Profitable Organization)-Corporate Japanese Center for Anti-Aging Medical Sciences (approval No. 09S02) on May 2, 2012.

Declaration of patient consent
The authors certify that they have obtained participants’ consent
forms. In the form, participants have given their consent for their images and other clinical information to be reported in the journal. The participants understand that their names and initials not be published and due efforts will be made to conceal their identity.

**Reporting statement**

The writing and editing of the study report was performed in accordance with the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) statement.

**Biostatistics statement**

The statistical methods of this study were conducted and reviewed by the biostatistician of Osaka Prefecture University, Japan.

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**Data sharing statement**

Datasets analyzed during the current study are available from the corresponding author on reasonable request.

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