Kidney-Qì deficiency diagnosed by Deng's diagnosis standard was not correlated with aging based on clinical observation of 90 participants

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Abstract

OBJECTIVE: To verify the Traditional Chinese Medicine (TCM) theory that kidney-Qì deficiency (KQD) is considered to be the main cause of aging using cross-sectional study.

METHODS: Demographic and lifestyle characteristics of 90 healthy participants were collected with a self-administered questionnaire. KQD syndrome was diagnosed according to Deng’s diagnosis standard. Creatinine-adjusted urinary 8-hydroxy-2’-deoxyguanosine (8-OH-dG) and 8-isomeric-prostaglandin2α (8-isopGF2α), salivary advanced oxidation protein products (AOPPs), malondialdehyde (MDA) and dehydroepiandrosterone-sulfate (DHEA-S) were selected as aging markers and measured using enzyme-linked immunosorbent assay.

RESULTS: No significant differences were observed in participant characteristics between the KQD group and non-KQD (NKQD) group (P > 0.05). Levels of 8-OH-dG, 8-isopGF2α, AOPPs, and MDA increased with age, except for a slight decrease in 8-OH-dG in the older group. The increase in 8-isopGF2α was significant (P < 0.05). DHEA-S significantly decreased with increasing age (P < 0.01). 8-OH-dG levels were higher in the KQD group compared with the NKQD group. Levels of urinary 8-isopGF2α, salivary AOPPs, and MDA in the KQD group were lower than in the NKQD group. Salivary DHEA-S was higher in the KQD group compared with the NKQD group. However, differences between KQD group and NKQD group were not significant.

CONCLUSION: The current results suggested that KQD syndrome, as diagnosed by Deng’s standard, does not underlie the aging phenotype.

Keywords: Aging; Kidney-Qì deficiency; Advanced
oxidation protein products; Malondialdehyde; Dehydroepiandrosterone sulfate; Cross-sectional studies

INTRODUCTION

The clinical application of Traditional Chinese Medicine (TCM) is guided by a unique theoretical framework. In TCM, health is considered to be a function of the smooth flow of Qi, which reflects the vital activity of life energy, through a series of pathways or meridians. According to the visceral manifestation theory, the body is divided into five organ systems (heart, spleen, liver, lungs and kidneys) which include the organs and their physiological and pathological functions. Different systems contain different kinds of Qi. Among these, kidney-Qi is the basic and vital energy needed for the body to survive. Kidney-Qi controls the growth, reproduction and aging of the body and the metabolism of body fluid. When Qi in the kidney system is not sufficient to support physical activity, a number of clinical symptoms and signs are observed, such as weakness in the waist and knees, tinnitus or deafness, frequent and profuse urine, enuresis, aconuresis or dribbling urine, frequent nocturia, spermatorrhea, premature ejaculation, metrorrhagia, pale tongue with a white coating, and weak pulse. This specific cluster of symptoms and signs has been summarized as kidney-Qi deficiency (KQD) syndrome. According to TCM theory, kidney-Qi and aging are closely related, and the aging process and life span are determined by the amount of kidney-Qi. It has been long believed that people with enough kidney-Qi age at a slower rate, whereas people with KQD syndrome exhibit accelerated aging. Moreover, invigorating kidney-Qi is considered to be an effective anti-aging method. Nevertheless, in the era of evidence-based medicine, TCM has encountered strong challenges from biomedical science due to a shortage of evidence-based theoretical interpretations and solid proof of TCM-based efficacy. Importantly, our team has studied kidney-Yang deficiency (KYD) by using advanced modern techniques for nearly 50 years. KYD is a TCM syndrome caused by an insufficiency of Yang Qi in the kidney, and is considered to reflect the progressive stage of KQD, characterized by cold and stagnant symptoms. Previous studies in our laboratory confirmed that the mechanisms underlying KYD include a decrease in urinary 17-hydroxy-corticosteroid and dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, and that KYD is closely related to the aging process. Moreover, we reported that the classic therapeutic treatment for KYD, Epimedium flavonoids (EF), which contain icariin as a major component, can improve cognitive deficits and activate quiescent neural stem cells in aging rats, as well as delaying aging and extending the health span in D. melanogaster, C. elegans, and mice. However, the relationship between KQD and aging is poorly substantiated. Here, we used the diagnosis standard of KQD syndrome from Deng’s Standard for Traditional Chinese Medicine Syndromes. We quantitatively tested KQD aging theory by evaluating the relationships between several aging markers [8-hydroxy-2-deoxyguanosine (8-OH-DG), 8-isoprostaglandin 2α (8-iso-PGF2α), advanced oxidative protein products (AOPPs), malondialdehyde (MDA) and dehydroepiandrosterone-sulfate (DHEA-S)] which have been commonly applied in scientific research and KQD syndrome. In the current study, we used the diagnosis standard of KQD syndrome described in Deng’s Standard for Traditional Chinese Medicine Syndromes, which is widely accepted in China. However, the current results indicated that KQD exhibited no correlation with these aging markers. Thus, the current findings suggested that KQD syndrome, as diagnosed by Deng’s standard, does not reflect the aging process.

MATERIALS AND METHODS

Design and subjects

After approval by the ethics committee of Huashan Hospital, Fudan University, participants were recruited from the physical examination center of Huashan Hospital through announcements seeking volunteers to take part in the study. The inclusion criteria were as follows: (a) people who live in Shanghai and nearby regions; (b) aged over 20 years old; and (c) having no disease history. The exclusion criteria were as follows: (a) participants who had oral or urinary tract infections; (b) people who were currently taking antioxidants or other drugs; (c) people with a cognitive disorder; (d) pregnant women; (e) people with abnormal results in routine blood examination, liver function, renal function, electrocardiogram or chest fluoroscopy. Information was obtained by self-administered questionnaire. Educational attainment was divided into three levels: high (master’s degree or higher), moderate (college degree), and low (high school degree and lower). Alcohol/tobacco consumption was recorded as yes or no. According to the age division criteria of the World Health Organization (WHO), we divided participants into three groups [young (< 45), middle age (45-59) and old (> 60)]. The diagnosis of KQD was based on the “Standard for TCM Syndromes”. We created a scale according to this standard, as follows (Table 1). People with typical manifestations, or who had four items of primary symptoms, main tongue manifestation, main pulse manifestation, or who had three items of primary symptoms, two items of secondary symptoms, any kind of tongue demonstration and pulse condition above, or who had two items of primary symptoms, three items of secondary symptoms, main tongue demonstration, main pulse condition, or who
had two items of primary symptoms, four items of secondary symptoms, any kind of tongue demonstration and pulse condition above were diagnosed with KQD. This diagnosis was conducted by more than two TCM doctors. Thus, participants were divided into a KQD group and a non-KQD (NKQD) group.

Sample collection
Saliva samples were collected after fasting from 22:00 of the day before by using Salivette cotton swabs (Sarstedt, Germany). Specifically, participants were asked to gargle three times using water and take a 5-min rest before collecting. After that, sterile cotton swabs were given to participants and they were instructed to chew the swab for 5 min and spit it into a tube. The swab was then centrifuged for 10 min (4 °C, 3000 rpm) and saliva was pipetted into another tube. Samples were stored at −80 °C until testing. First morning urine samples were collected in 50 mL polypropylene tubes and stored at −80 °C until analysis.

Analysis of urinary 8-OH-dG
The level of urinary 8-OH-dG was determined using an enzyme immunoassay assay (EIA) kit (Stressmarq, Canada). In brief, the bulk standard (30 ng/mL) was gradient diluted into eight tubes. 100 μL EIA buffer was added to non-specific binding wells and 50 μL EIA buffer to maximum binding wells. 50 μL standard (10.3 pg/mL-30 ng/mL) from each tube was added to the standard wells in order, until all the eight standards were aliquoted. 50 μL of the sample and 8-OH-dG AChE tracer (except for the total activity and blank wells) and 8-OH-dG monoclonal antibody (except for the total activity and the non-specific binding wells) were added to each well. Each sample was assayed at a minimum of two dilutions, in triplicate. The plate was incubated for 18 h at 4 °C. The wells were emptied and rinsed five times with wash buffer. 200 μL of Ellman’s Reagent was then added to each well. 5 μL of tracer was added to the total activity wells. Optimum development was obtained using an orbital shaker equipped with a large, flat cover to allow the plate to develop in the dark. This assay typically developed in 90-120 min. Absorbance of each well was read at 420 nm by a microplate reader.

Analysis of urinary 8-iso-PGF2α
The urine samples were analyzed for 8-iso-PGF2α lev-
els using a competitive enzyme-linked immunoassay (ELISA) kit (Enzo Biochem, New York, NY, USA). The kit used a polyclonal antibody to bind, in a competitive manner, 8-iso-PGF2α in a sample or standard (6.1-100 000 pg/mL). After simultaneous incubation at room temperature, the excess reagents were washed away and substrate was added. After a short incubation time, the enzyme reaction was stopped and the yellow color generated was read on a microplate reader at 405 nm.

**Analysis of salivary AOPPs**

Saliva AOPPs were assayed using an OxiSelect™ AOPP Assay Kit. Samples were prepared according to the manufacturer’s protocol. 200 μL of samples or standards (0-250 μM) were added to separate wells of the microtiter plate and 10 μL of Chloramine Reaction Initiator to each well. Samples and reagents were then mixed thoroughly and incubated on a table top rotator or shaker for 5 min. 20 μL of stop solution was added to each well. Absorbance was read immediately on a spectrophotometric plate reader using 340 nm as the primary wavelength. 0 μM chloramine standard was used as an absorbance blank.

**Analysis of salivary MDA**

MDA was assayed using a commercially available ELISA kit (Oxford Biomedical Research, USA). Briefly, samples were prepared following the manufacturer’s protocol, 200 μL of standard (0-20 μM) or sample and 200 μL of indicator solution were added into each well, then incubated at room temperature for 45 min. 150 μL of each solution was transferred to the microplate and read at 540 nm.

**Analysis of salivary DHEA-S**

Saliva DHEA-S was assayed using an ELISA kit (LDN, Germany). In brief, 50 μL of standard (0-12 ng/mL) or sample and 150 μL of diluted conjugate was added to the wells. The plate was incubated at 37 °C for 15 min. The content of each well was removed. The wells were washed three times with 0.3 mL of diluted wash solution. During each washing step, the plate was gently shaken for 5 s and excess solution was removed by tapping the inverted plate on an absorbent paper towel. 100 μL 3,3’5,5’-tetramethylbenzidine (TMB) substrate was added. After incubating at room temperature (22-28 °C) for 15 min in the dark, 100 μL of stop solution was added into the wells. Absorbance was read at 450 nm.

**Analysis of creatinine**

Creatinine was assayed using an enzymic creatinine assay method. Briefly, 6 μL of sample or standard and 180 μL of enzyme solution was added to each well. The plate was then incubated at 37 °C for 5 min and the absorbance was read at 546 nm.

**Statistical analysis**

All data were analyzed using the SPSS 20.0 statistical package (IBM, Armonk, NY, USA) for Windows. Mean ± standard error of mean were used for descriptive statistics. For the analysis of demographic and lifestyle characteristics of participants, Pearson’s χ² test was used to determine the significance of the categorical variables. The relationship between aging markers and age was analyzed using one-way analysis of variance (ANOVA) or non-parametric Kruskal-Wallis tests according to the distribution of the data. Each marker’s concentration was compared between the KQD and NKQD groups using a t-test. A criterion of P < 0.05 was considered to indicate statistical significance.

**RESULTS**

Although a total of 142 people were examined, 52 were excluded as a result of the exclusion criteria or missing data. Thus, 90 healthy participants were included in the final analysis. The mean age of participants was (45 ± 13) years old, including 47 young people, 28 middle-aged people and 15 older people. Based on Deng’s diagnosis standard for KQD, 22 people were diagnosed as KQD and 68 people were diagnosed as NKQD (Figure 1). The percentage of KQD individuals in the three different aged groups was 33% in the older group, 14% in the middle-aged group, and 27% in the young group, revealing that the number of KQD individuals did not increase steadily with age (Figure 2). Demographic and lifestyle characteristics of the two groups are shown in Table 2. No differences in participant characteristics were observed between the KQD and NKQD groups (P > 0.05).

8-OH-dG, 8-iso-PGF2α, AOPPs, MDA and DHEA-S are widely used as markers of aging. To confirm the efficacy of these five markers for evaluating aging in humans, we evaluated the variation in each marker along with increasing age using ELISA. The results revealed that the contents of the four oxidative markers (8-OH-dG, 8-iso-PGF2α, AOPPs, MDA) increased with age, except for a slight decrease in 8-OH-dG in the older group. The increase in 8-iso-PGF2α was significant (8-iso-PGF2α, P < 0.05). DHEA, an endocrine aging marker, was found to significantly decrease with increasing age (DHEA-S, P < 0.01) (Figure 3). The mean levels of 8-OH-dG, 8-iso-PGF2α, AOPPs, MDA and DHEA-S were 15 ± 0.5 (ng/mmol, creatinine), 3976 ± 183 (pg/mmol, creatinine), 2283 ± 191 (μM), 2.15 ± 0.14 (μM), and 2.89 ± 0.15 (ng/mL), respectively.

To investigate whether KQD can be used as an aging indicator, we analyzed the levels of each marker in the KQD and NKQD groups. The results revealed that the average level of 8-OH-dG in the KQD group was 16.5 ± 1.0 (ng/mmol), while that in the NKQD group was 14.5 ± 0.6 (ng/mmol). The level of 8-OH-dG was higher in the KQD group compared with the NKQD group, but this difference was not significant (P >
Participants \((n = 142)\)

Excluded \((n = 52)\)
42 were excluded according to exclusion criteria; 10 were excluded because data missing

Included in final analysis \((n = 90)\)

According to TCM
Kidney-Qi Deficiency group
Non-kidney-Qi Deficiency group

According to age
Young \((< 45)\)
Middle age \((45-59)\)
Old \((> 60)\)

Figure 1 Flow chart of this study
TCM: Traditional Chinese Medicine.

Figure 2 Percentages of kidney-Qi deficient people in the three age groups.
The diagnosis of KQD was based on the KQD scale based on "standard for TCM syndromes". Percentages of kidney-Qi deficient people in young group, middle age group and old group were calculated. KQD: kidney-Qi deficiency; TCM: Traditional Chinese Medicine.

Discussions:
We evaluated aging at several levels, measuring DNA, protein, lipid and endocrine aging markers. Oxidative stress plays a central role in human aging. Oxidative damage to DNA was reflected in the formation of 8-OH-dG. Numerous studies have revealed that 8-OH-dG in plasma or in other organs increases with

<table>
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Notes: information of participants included in this study was obtained by self-administered questionnaire. Educational attainment was divided into three levels: high (master’s degree or higher), moderate (college degree), and low (high school degree and lower). Alcohol/tobacco consumption was recorded as yes or no. All participants were divided into three groups (young (< 45), middle age (45-59) and old (> 60)) according to their age. n: sample size; %: percentage; KQD: kidney-Qi deficiency; NKQD: non-kidney-Qi deficiency.

0.05). The urinary level of 8-iso-PGF2α and the salivary levels of AOPPs and MDA in the KQD group were lower than those in the NKQD group. The salivary level of DHEA-S in the KQD group was higher than that in the NKQD group. However, these differences were not significant (Figure 4).

**DISCUSSION**

We evaluated aging at several levels, measuring DNA, protein, lipid and endocrine aging markers. Oxidative stress plays a central role in human aging. Oxidative damage to DNA was reflected in the formation of 8-OH-dG. Numerous studies have revealed that 8-OH-dG in plasma or in other organs increases with
In the current study, we examined urinary creatinine-adjusted 8-OH-dG levels. The results revealed differences in urinary creatinine-adjusted 8-OH-dG levels between the younger group and the other two age groups, in accord with previous findings. Most previous studies reported a tendency for increased 8-OH-dG with age, because of an increase in the rate of oxidative DNA damage.24 However, the levels of oxidized bases may be affected not only by changes in the rate of oxidative DNA damage, but also by alterations in the rate of repair.25 Furthermore, urinary 8-OH-dG levels are a direct reflection of repair ability, which declines in older people, potentially explaining why we observed that urinary creatinine-adjusted 8-OH-dG in the older group was slightly decreased compared with the middle-aged group. Regarding lipid peroxidation, in the current study, we measured urinary-creatinine adjusted 8-iso-PGF2α and salivary MDA. In addition, we found that 8-iso-PGF2α, which is generated by cyclooxygenase-independent free radical attack of arachidonic acid, significantly increased with age, indicating that 8-iso-PGF2α also can be a good marker of aging, in addition to oxidative markers. Yavuzer et al26 reported that, in an older control group, 8-iso-PGF2α levels were significantly higher than those in a young control group, consistent with the current findings. Salivary MDA exhibited a tendency to increase with age in the current study, although this difference was not significant. However, MDA has
long been suggested as an aging marker.\textsuperscript{30–34} Regarding protein oxidation, the level of AOPPs was chosen as an aging marker because it was found to increase in a dose-dependent manner following \textit{in vitro} exposure of hypochlorous acid.\textsuperscript{35} The current findings were in accordance with these previous studies. DHEA-S is an important precursor of sex steroids, and functions as an important regulator of the immune system. Serum DHEA-S is a protective factor that is negatively correlated with age in primates and humans,\textsuperscript{36,38} and was found to significantly decrease with age in the current study. We measured the five aging markers using ELISA. The ELISA kits used in the current study are considered to have high cross-reactivity, poor sensitivity and high variation, and the best way to assess these biomarkers was by using high performance liquid chromatography (HPLC) or chromatography coupled to mass spectrometry. The current results were consistent with the results of studies using HPLC or chromatography coupled to mass spectrometry, indicating the reliability of our results. Overall, the current findings confirmed the role of 8-OH-dG, 8-iso-PGF2\alpha, AOPPs, MDA, and DHEA-S in aging evaluation.

According to TCM theory, kidney-Qi deteriorates gradually with the process of aging, and people with KQD exhibit a more severe aging phenotype than those without KQD.\textsuperscript{7} Our results revealed that concentrations of 8-OH-dG in KQD group were higher than those in the NKQD group, in line with TCM theory, although this difference did not reach significance. Moreover, we found that concentrations of 8-iso-PGF2\alpha, AOPPs, and MDA in the KQD group were lower than those in the NKQD group, contradictory to TCM theory. As an endocrine aging marker, DHEA-S is considered to exert positive anti-aging effects, and would be expected to be lower in KQD individuals. However, we obtained the opposite finding. In addition, the middle-aged group (45-59) in the current study contained the lowest percentage of KQD individuals. Huang Di Nei Jing, the first classic description of TCM, states that 28-year-old females and 32-year-old males exhibit the most kidney-Qi, suggesting that the young group (20-44) in the current study would be expected to contain the lowest number of KQD individuals.\textsuperscript{39} The current results did not support the well-known TCM theory that KQD reflects an acceleration of the aging process. Rather, the current results suggest two main possibilities: first, Deng’s standard may not be appropriate, or second, KQD does not underlie the aging phenotype.

To our knowledge, the current study is the first attempt to evaluate the relationship between KQD and established aging markers. Although there are several limitations in the current study relating to its small sample size and potential reminiscence bias, the experiment is the first quantitative study to test the relationship between KQD and aging.

In conclusion, in this cross-sectional clinical study, we examined KQD, a TCM syndrome, in 90 healthy participants in different age groups. The results confirmed that 8-OH-dG, 8-iso-PGF2\alpha, AOPPs, MDA and DHEA-S were appropriate aging markers. Analysis of the relationship between KQD and the five aging markers revealed no correlation between KQD and aging, based on Deng’s diagnosis standard. More quantitative studies should be conducted to test the theories underlying TCM, and provide a scientific way to examine TCM.

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