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Development of personalized health monitoring using ultra-weak photon emission based on systems medicine concepts

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Chapter 1

Introduction

Background

In the past century, medical science has improved considerably and has provided tools for managing nearly all major diseases, including autoimmune diseases, cardio- and cerebrovascular disease, and cancer. At the same time, adverse drug reactions and side effects have become increasingly important issues. For example, in the United States, adverse drug reactions range from the sixth to the fourth leading course of death among hospitalized patients.[1] In addition, adverse drug reactions are closely related to drug safety and efficacy in the human body. Strikingly, most drugs work as intended in fewer than half of patients, meaning that a particular drug can be effective in some individuals (the so-called responders), but fail to be effective in others (i.e., non-responders), thereby leading to the possibility of adverse drug reactions. This situation has prompted us to rethink the conventional drug development model of "one drug fits all", a model that is based on double-blind clinical trials designed to predict a drug's average therapeutic outcome in the broadest, most homogenous groups of patients. However, a growing body of evidence supports the notion that a patient's response to a given drug can depend on a variety of integrated factors, including the patient's age, nutritional state, lifestyle, emotional state, and the surrounding environment.[2] Given that each individual has a unique profile with respect to these complex factors, we must revise our thinking to achieve the concept of "the right therapy, with the right drug, in the right patient".[2] Therefore, individualized predictive medicine—i.e., personalized medicine—is becoming the focus of shifting away from the "one drug fits all" approach.[3]

Personalized medicine

The goal of personalized medicine is to develop a therapy using the right drug, at the right dose, at the right time, in the right patient.[1] Personalized medicine is based on the notion that each patient's disease can be unique, and therefore each patient requires individual treatment.[4] This notion suggests that disease profiles vary among individuals with respect to the cause, progression, and response to therapy.[4]

Therefore, the key to achieving truly personalized medicine lies in the diagnosis of disease. Developing a novel, effective strategy for diagnosing disease in individual patients can lead to a more effective personalized approach to disease management and prevention.[5] To understand the importance of effective diagnostics, we must first understand the notions of dynamic health and disease. Fig. 1 schematically illustrates a dynamic system that can respond automatically and effectively in the resilient state (allostasis), thereby maintaining healthy status (homeostasis).[5,6] When this dynamic system experiences a reversible pathological event, the appropriate intervention returns the system to homeostasis.[5] However, of this dynamic system loses its ability to regulate itself, irreversible pathology and disease develop.[5,6]

The presence of a dynamic system that regulates health and disease indicates that many chronic diseases involve processes that are long-term, nonlinear, multifactorial and highly complex.[5,7] For example, type 2 diabetes is multifactorial in origin and is associated with genetic factors, metabolic disorders, and lifestyle-related risk factors.[8,9] Type 2 diabetes can be present in an early, undetected form for more than ten years, during which dysglycemia increases the risk of severe complications.[10]

Therefore, conventional diagnostics and treatment may not be completely effective in this dynamic state, and personalized diagnostics may enable the clinician to monitor the dynamic state in both health and disease; moreover, it may also help identify biomarkers in the early stages of chronic disease, as well as helping develop a suitable intervention for individual patients.[5,11]

Importantly, personalized diagnostics can be used to capture phenotype information for specific patients, thereby leading to the development of personalized healthcare strategies (Fig. 2).[5,11] Recently, a metabolomics-based approach has provided researchers with the opportunity to understand the changes that occur in the comprehensive metabolite profile in body fluids during various conditions, including changes in nutrition, environment, psychological state, and disease stage.[11] Thus, it is now possible to stratify patient populations into distinct phenotypes based on

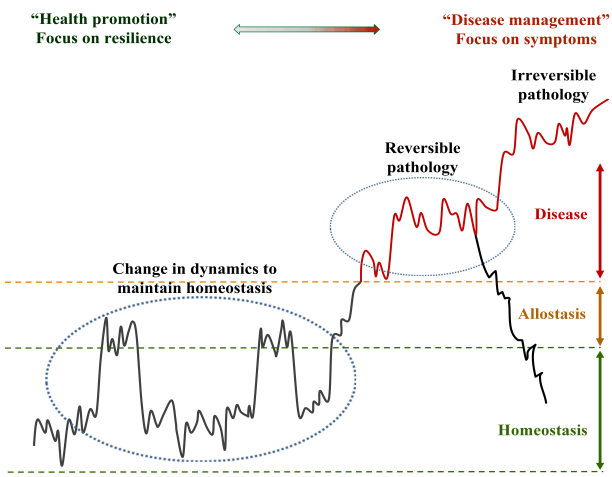

Fig. 1 Schematic diagram illustrating a dynamic system of regulation in both health and disease.

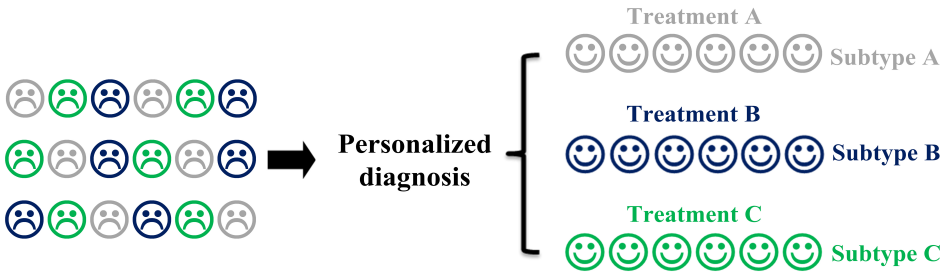

Fig. 2 Schematic diagram illustrating the use of personalized diagnostics to stratify patient populations. The treatment used corresponds to each patient's subtype, ultimately leading to personalized medicine for individual patients in each subtype.

molecular biomarker profiles, an approach that has been proposed for solving the aforementioned responder/non-responder issue, thereby achieving personalized medicine.[11] Interestingly, many studies regarding patient phenotypes are guided by the concepts of traditional Chinese medicine (TCM)–based diagnostics.[12–18] This indicates that TCM-based diagnostics can contribute extremely valuable information regarding personalized medicine.

Traditional Chinese medicine–based concepts

Traditional Chinese medicine (TCM)-based concepts are based on a holistic view of health and disease and therefore represent systems-based medicine.[5] Because TCM

and Western medicine differ with respect to their views regarding diagnostic goals, they also use different diagnostic methodologies.[19] In Western medicine, pathological factors are believed to cause disease; therefore, research and the development of therapeutic strategies focus primarily on these pathological mechanisms.[19] In contrast, TCM-based diagnostics focuses on the body's *response* to these pathological factors; therefore, TCM-based diagnostic strategies aim to examine the patient's overall functional state.[19] According to the TCM theory, one's overall functional state defines whether one is experiencing health or disease.[20] This state depends on the dynamic balance among complex interactions between one's physiological state and other factors such as age, gender, emotional state, seasonal fluctuations, and pathological factors.[20] The patient's dynamic response to these interactions provides a functional profile of the patient's disease-related symptoms and/or signs.[21] In addition, these symptoms and/or signs can be diagnosed by physicians trained in TCM using several approaches, including palpation, listening and smelling, inspection, and inquiry, thereby identifying specific "syndrome subtypes" within a specific disease.[22] For example, rheumatoid arthritis can be differentiated generally into "Heat syndrome" and "Cold syndrome" in order to describe different patterns within patient populations based on TCM-based diagnostics.[5]

Importantly, the identification of "syndrome subtypes" is the most important concept guiding personalized interventions using Chinese herbal medicine (CHM),[21] an approach that has been used in China for thousands of years to maintain health and treat disease.[23] In this long history, the Chinese have observed a wide variety of responses to herbs in many clinical settings and have established many important concepts with respect to the pharmacological actions and therapeutic effects of CHM.[23] For example, so-called "indigenous medicinal materials"—which refers to herbal medicines produced in a specific geographic region—may actually represent the optimal responses and therapeutic effects in the human body.[24] Another crucial

concept is based on the therapeutic categories of herbs, which are closely related to "syndrome subtypes".

CHMs are usually marked by specific descriptors to classify their therapeutic properties according to their effects in patients.[23] Herbs with specific descriptors are targeted to specific syndromes. For example, herbs with a "Heat-cleansing effect" can be used to treat "Heat syndrome", and these herbs are often marked by a "cold" descriptor.[25] In addition, herbs with a "sweet" descriptor are generally used to treat "Deficiency syndrome", as these herbs can nourish the body, thereby promoting health.[26] Herbs with different therapeutic descriptors can be combined to prepare a Chinese herbal formula, which can be used to treat complex syndromes such as "Deficiency of both Qi and Yin syndrome", providing personalized interventions for treating unique "syndrome subtypes" within a specific disease.[21,27] In summary, the basic concept in TCM is to combine individual diagnostics with individual interventions. Therefore, exploring TCM-based concepts may provide fresh insight into personalized healthcare and management strategies. However, TCM-based diagnostics and CHM-based treatments are descriptive, phenomenological concepts; therefore, objective, evidence-based data is needed in order to support these concepts. Applying a systems biology–based approach has been proposed to achieve this goal.[5]

Systems biology–based approaches

The aim of systems biology is to understand living organisms at the systems level,[28,29] thereby promoting personalized medicine.[3,5] The field of systems biology is extremely broad, and multi-technology approaches should be used for specific research purposes.[28] To develop a systems biology–based approach, we must first understand the hierarchical structure of a living organism, ranging from the biochemical and molecular levels to the cellular and organ levels, all the way to the integrated systems level, as well as the system's dynamics over time.[7,29] To date, so-called "omics"-based technologies (e.g., genomics, proteomics, metabolomics, etc.) have been used to study different hierarchical structures in a biological system.[5,29,30]

In addition, statistical tools such as clustering and network tools have been used successfully to analyze large amounts of data obtained using omics-based technologies.[31] Thus, a research approach for measuring and analyzing biological systems has been established.

Because TCM-based diagnostics (i.e., "syndrome subtypes") reflects the body's physiological and pathological status at a large-scale organizational level, systems biology–based approaches may provide a suitable method for studying this status. Recently, metabolomics-based approaches were used to investigate "syndrome subtypes" of specific diseases guided by TCM-based diagnostics.[12–18] Metabolomics provides a comprehensive overview of the molecular metabolites in a biological system and can be used to establish the physiological and/or pathological status of a living organism.[32] In principle, this approach is suitable for studying TCM-based diagnostics. However, because metabolomics can only be performed using currently available analytical platforms, it may not necessarily cover the entire metabolome. In addition, metabolomics provides a picture of the system's physiological and/or pathological status at a single point in time, but does not provide information regarding the system's dynamics. Therefore, metabolomics may not completely reflect TCM-based diagnostics, and complementary tools are still needed in order to further characterize TCM-based diagnostics at an integrated, dynamic systems level. In this thesis, we propose that measuring ultra-weak photon emission may provide a suitable technique for studying TCM-based diagnostics.

Ultra-weak photon emission

Measuring ultra-weak photon emission (UPE) is a non-invasive method for recording the physiological state in living organisms.[33–37] Recent studies suggest that UPE may reflect the biological rhythms of the human body with time-dependent dynamics.[38] In addition, UPE may provide insight into nonlinear regulatory processes (e.g., coherence) at a high systems level.[7] Moreover, UPE can be used to measure a variety of physiological states, thereby providing potential diagnostic

applications.[36] Therefore, measuring UPE may approximate the organizational level of TCM-based diagnostics, providing objective data that can be used to characterize "syndrome subtypes". On the other hand, TCM-based diagnostics—which focuses on obtaining a holistic pattern of systems—may improve our understanding of UPE patterns measured in the human body, thereby contributing to the development of novel strategies for achieving personalized medicine.[7] To study CHM-based characteristics at the same systems level as TCM-based diagnostics, a comprehensive measuring tool is also needed. Photon-induced delayed luminescence (DL), which is the long-term emission of photons from various materials following excitation with light, has been proposed for use in studying the therapeutic properties of Chinese medicinal herbs.[39] Therefore, combining a systems biology–based approach with photon emission and robust statistical tools may provide fresh insight into TCM-based concepts, leading to personalized diagnostics and interventions and ultimately achieving personalized medicine.

Scope and outline of this thesis

The studies described in this thesis were performed in order to develop personalized approaches to health monitoring using UPE and DL methods in combination with TCM-based concepts. Fig. 3 provides a schematic overview of the following two research aspects in this thesis: *i*) studying TCM-based diagnostics using UPE measurements (Fig. 3, *left*) and *ii*) studying CHM-based characteristics using DL analyses (Fig. 3, *right*). To investigate systems-based diagnostics by combining UPE with TCM-based concepts, we first studied the Chinese literature published from 1979 through 1998, as a vast amount of research in this period was based on UPE and/or TCM-based diagnostics; these results are presented in **Chapter 2**. Our analysis revealed that UPE has clear potential in terms of improving our understanding of the systems-level view regarding health and disease, guided by TCM-based diagnostics. Next, we investigated whether UPE can be used as a tool to identify the three distinct "syndrome subtypes" in subjects with early-stage type 2

diabetes; the subtypes were diagnosed by physicians who were trained in TCM-based diagnostics. In this preliminary study, which is presented in **Chapter 3**, statistical analyses such as logistic regression and correlation networks provide the first evidence that UPE parameters can be used to differentiate between "syndrome subtypes" identified using TCM-based diagnostics.

In **Chapter 4**, we discuss systems interventions regarding CHM-based concepts (e.g., herbal therapeutic properties) using DL based on a stable, reproducible experimental protocol to optimize DL excitation time, herbal powder size, and the remaining water content in the dried herbal materials. DL is a promising method for examining both the quality and therapeutic properties of dried Chinese herbal materials. In **Chapter 5**, we used an established protocol to study the feasibility of using DL to measure differences in the quality of herbal medicines. In this chapter, both DL measurements and chemical analysis were used to reflect differences in the therapeutic quality of rhubarb due to differences in environment factors. We then linked DL parameters to specific bioactive compounds in rhubarb. In **Chapter 6**, we used DL to characterize the therapeutic categories of herbal materials. To provide biological relevance to the DL results, we also performed an in vitro dendritic cell−based immunomodulatory assay. Finally, in **Chapter 7** we present and discuss conclusions and future perspectives regarding the studies presented in this thesis.

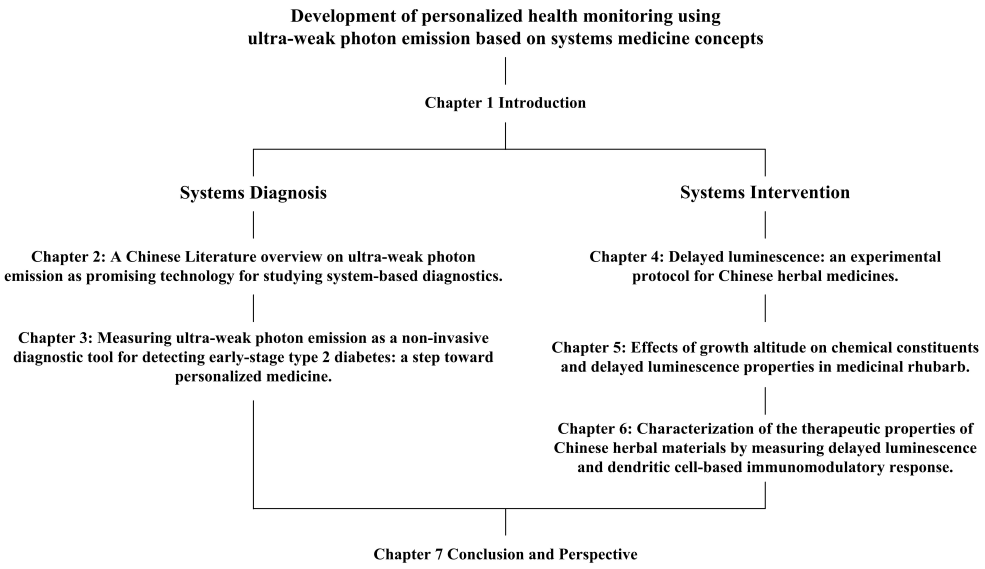

Fig. 3 Schematic overview of the framework in this thesis.

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Chapter 2

A Chinese literature overview on ultra-weak photon emission as promising technology for studying system-based diagnostics

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*, These authors contributed equally to this work.

Abstract

To present the possibilities pertaining to linking ultra-weak photon emission (UPE) with traditional Chinese medicine–based diagnostics principles, we conducted a review of Chinese literature regarding UPE with respect to a system's view of diagnostics. Data were summarized from human clinical studies and animal models published from 1979 through 1998. The research fields can be categorized as follows: 1) human physiological states measured using UPE; 2) characteristics of human UPE in relation to various pathological states; and 3) the relationship between diagnosis (e.g., syndromes) and the dynamics of UPE in animal models. We conclude that UPE has clear potential in terms of understanding the systems view on health and disease as described using traditional Chinese medicine–based diagnostics, particularly from a biochemistry-based regulatory perspective. Linking UPE with metabolomics can further bridge biochemistry-based Western diagnostics with the phenomenology-based Chinese diagnostics, thus opening new avenues for studying systems diagnostics in the early stage of disease, for prevention-based strategies, as well as for systems-based intervention in chronic disease.

Keywords: Ultra-weak photon emission (UPE), traditional Chinese medicine research, diagnosis, reactive oxygen species (ROS).

1. Introduction

The use of ultra-weak photon emission (UPE) in living organisms was first described by Gurwitsch in 1923.[1] At that time, the technical capabilities for measuring radiation using physical devices was rather limited. This technology became more feasible when sensitive photomultipliers were developed in the 1960s in the former Soviet Union. The early data were published primarily in Russian journals,[2,3] with only a fraction of the reports translated into English.[4] Since the 1970s, UPE has been used by research teams in Germany,[5] Australia,[6] Poland,[7] Japan,[8] the United States,[9] and China.[10] UPE has been used successfully in a wide variety of organisms, including bacteria, yeast, plants, animals, and humans, as well as in cells and cellular homogenates derived from living organisms.[5–11]

UPE occurs spontaneously in living organisms, without the need for external intervention.[12] The emission range of UPE is approximately 10–10^3 photons/sec/cm^2. The spectral range of the photons emitted from living systems is 300–750 nm;[13] the photons emitted from human tissue ranges from 420–570 nm.[14] The source of UPE is closely related to the electronic transport and the generation of reactive oxygen species (ROS) during oxidative metabolic processes, with UPE originating from the transition from either the singlet excited state (such as singlet oxygen $^1O_2$) or the triplet excited level of carbonyl species ($^3R{=}O^*$) to the singlet ground state.[15,16] Biological ROS—including the reactions of superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO\cdot$)—are produced dynamically during chemical metabolic redox reactions, including lipid peroxidation and protein/nucleic acid generation; moreover, during these metabolic processes, electrons can become excited, and energy is emitted in the form of photons.[17] Similar to the ROS theory described above, photons can also be released during the metabolism of radical nitrogen species (RNS). ROS causes the oxidation of biomolecules such as nucleic acids, proteins, and lipids, which play essential roles in many cellular processes, including cell signaling, apoptosis, and pro/anti-inflammatory regulation.[18,19]

Therefore, UPE can be measured in order to detect the physiological state of the human body and to measure dynamic changes in health.[12,13,20]

In humans, UPE is usually measured using a photomultiplier tube (PMT) or a charge-coupled device (CCD). Emitted photons can be measured directly through the skin in a light-tight, dark environment.[21,22] The use of UPE as a diagnostic tool for health-related issues in humans has been reviewed recently.[23] The intensity of UPE emitted from the human body can be influenced by several physiological states, including age,[24] gender,[25] biological rhythms,[22,26–29] and conscious activities,[30–32] thus leading to the discovery of putative diagnostic properties of photon emission. For example, hypothyroidism can be diagnosed by measuring the emission of photons from the index finger of human subjects.[33] Furthermore, differences in the intensity of photon emissions have been measured between patients with multiple sclerosis and healthy subjects.[34,35] Moreover, patients with hemiparesis have asymmetrical UPE intensity between the left and right hands, suggesting that measuring photon emission symmetry could be used as a novel diagnostic parameter in addition to measuring UPE intensity.[36,37] Based on the aforementioned experimental observations, UPE has been proposed as a non-invasive indicator of the integrated states and dynamic changes in human health.[12,20,38]

In the newly emerging systems-based view of health, biology can be considered a hierarchy of various levels of organization, ranging from low levels (e.g., biochemistry and molecules) to the cellular and organ levels, all the way up to the integrated systems level.[38] In Western medicine, “omics” technologies are often utilized to study genes, proteins, and metabolites at relatively low organizational levels.[39] Recent work suggests that the dynamic distribution of UPE emissions from the human body can reflect both the health status at a large-scale organization level and the dynamics of the system.[13,20] Similar to UPE, traditional Chinese medicine (TCM) integrates physiological and pathological information at a higher level of organization—i.e., the phenotype level—in order to obtain a holistic description of

the body's state. Two important types of descriptions are frequently used: constitution differentiation and syndrome differentiation.[39–41] However, TCM–based diagnostics is a descriptive, phenomenological approach based on many clinical observations, and the insights regarding molecular and mechanistic biology have been explored only recently.[42] Given that UPE may provide important insight into health at a high level of organization, measuring UPE parameters may provide novel scientific insights into TCM–based diagnostics and may help guide Western medicine towards a systems-based view of life, both from a diagnostic perspective and from an intervention perspective. Therefore, it is important to explore the history of this relationship between UPE and TCM–based diagnostics.

Applications in which UPE has been used to understand and measure systemic organization can be found in Chinese literature; these publications have generally focused on the relationship between UPE and TCM–based concepts in both human and animal studies. In this review, we summarize these studies published in Chinese scientific journals from 1979 through 1998. In studies published between 1979-1998, TCM–based concepts were used to establish UPE experimental designs. After the turn of the century, there was a shift in UPE research interests in China from healthcare to the agricultural area.[43,44] As a result, no more Chinese literature was found regarding UPE and TCM–based concepts after 1998. Because much of the clinical data was published in Chinese, UPE research is relatively unknown among scientists in non-Chinese-speaking countries. By reviewing this literature, we hope to educate scientists in terms of the possibilities regarding linking UPE with TCM–based diagnostics principles. Furthermore, because Western UPE researchers rarely study TCM–based diagnostics from a systemic regulatory perspective, this review will also provide a basis for further research in this specific area.

2. Temporal variations in UPE intensity among healthy human subjects

According to the TCM theory, one's health depends on a dynamic balance between one's physiological state and the surrounding environment. The human body can

adapt in response to many environmental factors (e.g., changes in the seasons) and internal environmental changes (e.g., emotional variations). These patterns of change that result from changes in the internal and external environments are essential for obtaining a diagnosis in TCM. Therefore, Chinese physicians are taught to make a comprehensive diagnosis that includes an evaluation of how the body responds to the surrounding environment at various ages, as well as the effect of seasonal fluctuations.[45–47]

In China, UPE measurements have been used to study temporal changes in human physiological states since the 1980s. Zheng[48] investigated the effect of gender and age on UPE measured from the fingertips of seven groups of healthy subjects; these results are summarized in Fig. 1. In general, the intensity of UPE was higher among males than among females, and UPE intensity tended to increase with age. This association between age and UPE was later confirmed by Sauermann et al..[24] In a separate study, Yan[49] examined the relationship between age and UPE by measuring the specific acupuncture point LI1 (also known as the Shangyang acupuncture point); Yan found higher UPE intensity among young subjects (17-49 years of age) compared with both older subjects (50-72 years age) and children (11-16 years of age).

Yang measured UPE intensity at various acupuncture points located at the extremities and on the torsos of male and female children and adults.[50,51] Consistent with the studies described above, Yang found that UPE intensity was higher in men than in women and higher in adults than in children. The association between UPE intensity and season (i.e., higher photon emission in the summer compared to the winter) that was originally reported by Zheng[52] for the fingers of healthy subjects has been later confirmed with UPE measurements of other body locations by Popp and Cohen,[34] Van Wijk,[53] Bieske et al.,[54] and Jung et al.;[55] importantly, these authors did not refer and probably had no prior knowledge of Chinese literature regarding UPE measurements. These findings indicate that measuring UPE can provide insight

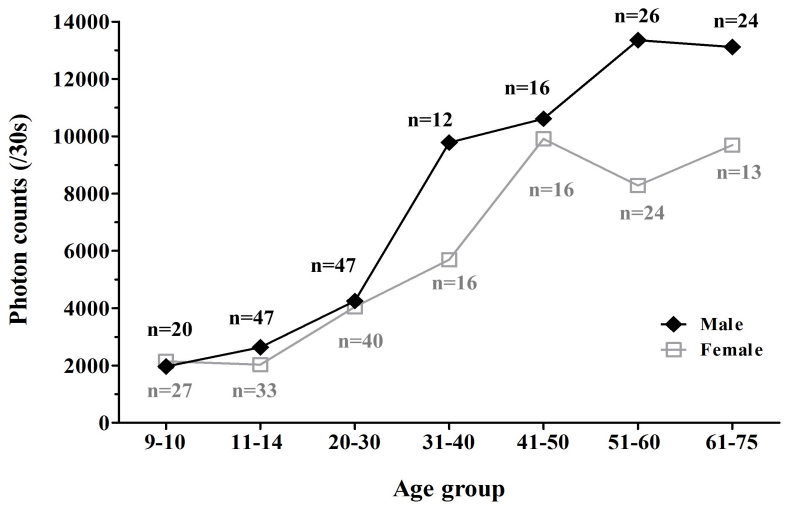

Fig. 1 UPE intensity measured in male and female human subjects at the indicated ages (in years). UPE intensity was measured as the average photon counts (per 30 seconds) of the total photon emission from ten fingertips; the data are the average of five separate measurements per subject.[48]

into the state of harmony between the human body and the environment. Thus, deviations from these temporal rhythms in UPE intensity might be utilized further in order to study the pathological state and TCM–based diagnostic patterns.

3. The association between UPE and pathological state based on TCM–based diagnostic principles

In TCM, illness is viewed as a disruption of the body's dynamic balance. The body's dynamic balance is an abstract way to describe the flow of energy through the entire body, as well as the exchange between the body and the external environment. Measuring this flow of energy—particularly interruptions in this flow—provides important diagnostic information regarding the occurrence of specific illnesses. The aims of acupuncture are to regulate this flow of energy, remove blockages that interrupt energy flow, and help the ailing body re-establish its dynamic homeostasis.[56–60] In Western medicine–based terms, this might indicate a dysregulation of processes, which can be experienced as chronic disease.

The dynamic balance concept was recently correlated with symmetry—and asymmetry—in UPE intensity between the left and right sides of the human body.[13,37,61,62] As far back as the early 1980s, this UPE left-right symmetry was identified by Chinese researchers as an important parameter for distinguishing between health and disease.[52] Thus, healthy subjects can be characterized by a symmetry in UPE intensity between acupuncture points on the two sides of the body.[63–65] Significant differences in UPE intensity at acupuncture points between the left and right sides of the body have been observed in typical "Western" diseases, including hypertension, facial nerve paralysis, and constipation.[63–68] Fig. 2 shows an example of UPE asymmetry measured using acupuncture points on the hand. The left side of the figure shows disease states diagnosed using Western medicine. These specific diseases correspond to acupuncture point locations at which significant UPE asymmetry was measured. The right side of the figure shows the acupuncture point numbers and related meridian channels. These meridian channels always correspond with a diagnosis of the specific corresponding diseases in TCM.[46,63–65,67] Here, UPE may serve to bridge the Western medicine and Chinese medicine concepts. In other words, because UPE can be used to demonstrate potential deviations from homeostasis in a meridian, and because these deviations can also be related to specific Western diseases, UPE provides the opportunity to connect TCM–based diagnoses with specific Western diseases;[61,69] in this way, the long history of knowledge regarding TCM can be used to enrich Western medicine.

Other studies have shown an uneven distribution of UPE intensity at acupuncture points at various body locations.[50,51,70] Higher intensity UPE has been measured at acupuncture points compared with non-acupuncture points; this difference was based on measurements of more than 150 acupuncture points together with their surrounding non-acupuncture points. Thus, the authors suggested that acupuncture points with higher UPE intensity generally coincide with the theoretical meridians.[71–73] Interestingly, Guo et al. used chemical indicators to obtain fluorescence-based

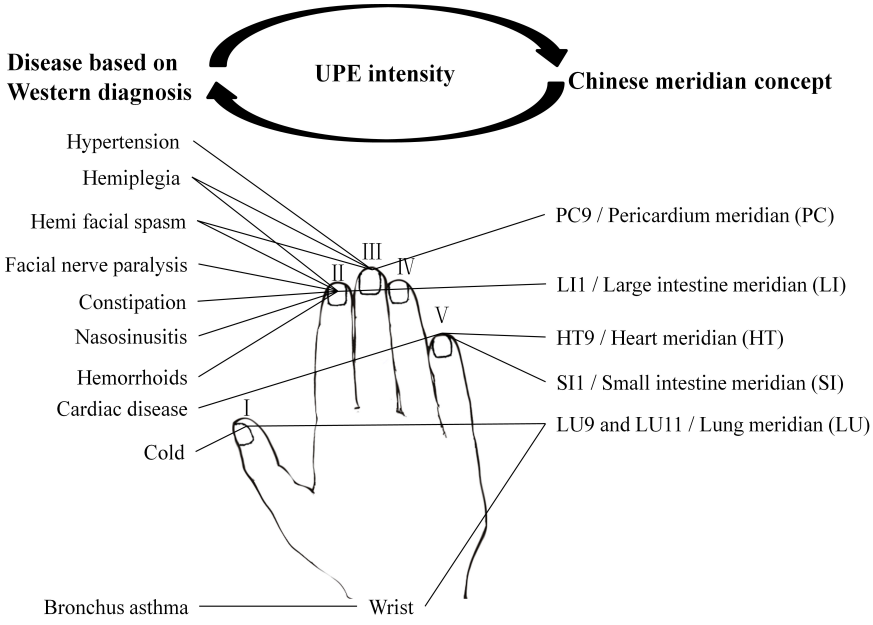

Fig. 2 UPE patterns are related with both the Western medical concept of disease and TCM concepts.[63-65,67] The Western medicine description of diseases corresponding to Chinese acupuncture points and specific UPE intensity asymmetries. I: Thumb; II: Index finger; III: Middle finger; IV: Ring finger; V: Pinkie. PC9: Zhongchong acupuncture point on the middle fingertip; LI1: Shangyang acupuncture point on the index fingertip; HT9: Shaochong acupuncture point on the pinkie fingertip; SI1: Shaoze acupuncture point on the pinkie fingertip; LU9: Taiyuan acupuncture point on the wrist; LU11: Shaoshang acupuncture point on the index fingertip.

images of visible ROS distributions in an animal model and found that the areas with the strongest fluorescence were superimposable on human meridian lines.[74] Given that ROS content defines UPE intensity in living systems,[9,18,19,75,76] the meridian-like lines of ROS activity measured in animals support—albeit indirectly—the correspondence between meridians and UPE intensity in humans.

In traditional Chinese medicine, needles are used to stimulate acupuncture points and to trigger a dynamic interaction between the acupuncture points and the connective tissue along the meridian.[77,78] This dynamic interaction was measured in several Chinese studies by measuring changes in UPE intensity.[79,80] After placing needles in

the acupuncture points of the forearm or calf, UPE intensity will change significantly at the acupuncture points of a finger or toe, respectively. In addition, UPE asymmetry can also be used to measure the therapeutic effect of acupuncture in patients. For example, left-right UPE asymmetry was measured at various acupuncture points on both sides of the body and was found to change following acupuncture.[81] Some studies also examined the therapeutic effect of acupuncture treatment by comparing the concentration changes in ROS-related enzymes and endogenous metabolites before and after treatment; these studies have been performed in both human subjects and animal models.[82–86] In addition, adiposity decreased when ROS-related anti-oxidant products (e.g., a recombinant superoxide dismutase protein) were applied to specific acupuncture points in obese subjects, and this therapeutic effect is similar to the effect of Chinese acupuncture.[86] The aforementioned studies of the therapeutic effect of acupuncture based on UPE and ROS measurements suggest that linking UPE parameters to changes in ROS may provide more opportunities to study the effect of acupuncture at the biochemical level.

4. UPE in relation to Chinese syndromes based in studies using animal models

Chinese studies have provided examples for how to study basic TCM diagnostics concepts using UPE measurements, and this has been supported by similar UPE studies conducted in both Japan[87] and Korea.[37,88] The pattern of UPE in the human body—and the changes in UPE intensity at specific body locations following acupuncture—appear to coincide with the meridian theory of TCM. Thus, the question arises whether UPE can also reflect the Chinese diagnostic syndrome theory.

The term “Chinese syndrome” refers to a combined pattern of physiology, psychology, and pathology in relation to a specific condition. The goal of syndrome differentiation is to understand illness as a pattern of relationships. Typically, several diagnostic procedures are used in order to identify the syndrome; these procedures include inspection, listening and smelling, inquiry, and palpation. Correctly

identifying a Chinese syndrome is the basis of personalized therapies that use Chinese herbs, nutritional advice, acupuncture, physical exercise, and medication.[89,90] To obtain a better understanding of Chinese syndromes from a modern biological perspective, several Western analytical tools—for example, omics-based approaches—have been used to study basic Chinese syndromes in patients with chronic diseases such as rheumatoid arthritis and diabetes. Using this approach, chemical biomarkers have been identified successfully for subtypes of patients with diabetes or rheumatoid arthritis.[91,92]

Given its potential for measuring overarching regulatory processes, UPE may be a useful diagnostic tool for identifying Chinese syndromes. In the Chinese literature, UPE has been used in three animal models to study deficiency syndromes.[10,93–95] Marked reductions in UPE intensity at the acupuncture points located at the governor vessel (gV) and the conception vessel (cV) meridian channels were observed in Yang deficiency rats and Blood deficiency rats, respectively; an increase in UPE intensity was measured after stimulating these acupuncture points.[96] In another study, a rabbit model of Qi deficiency was established by excessive intake of rhubarb. In this model, a rapid decline in UPE intensity, followed by a slow rise in intensity, was measured in the rabbit's ears, reflecting the rabbit's altered dynamics as it progressed from illness to a healthy state.[97] In addition, the UPE level of the rabbit's organs (e.g., the spleen and stomach) decreased considerably, suggesting that UPE can also reveal changes in organs induced by treatment with herbs.[98] The Chinese research showed an intriguing change in UPE intensity related to the specific dynamics of deficiency syndromes. As more UPE parameters are identified in the future, they will likely provide more information regarding Chinese syndromes.

5. Perspective: UPE-guided metabolomics based on TCM –based diagnostics

In this review, we discussed the UPE research that has been performed in China within the past century with respect to physiological and pathological conditions.

Importantly, our review revealed that UPE experimental observations are closely correlated with TCM–based diagnostic concepts. Some researchers have hypothesized that this correlation may be due to the concordance between the coherence theory of photon emissions in humans and the energetic properties of living organisms as developed in TCM.[99,100]

Here, we propose that a UPE-guided metabolomics approach based on TCM diagnostic theory may improve the dialogue between Western medicine and TCM. UPE parameters and TCM diagnostics reflect dynamic responses that arise as a result of internal and/or external disturbances in the human body at a relatively high organizational level. In addition, because its origin lies in oxidative metabolic processes, UPE has been proposed to link to metabolic networks.[20] Various ROS-regulating metabolites have been detected in several diseases, including cardiovascular disease, hypertension, rheumatoid arthritis, and type 2 diabetes.[91,92] Several metabolomics platforms—such as platforms based on amino acids and oxylipins—have been established, and these platforms reflect ROS/oxidative stress products, as well as their biosynthetic pathways.[101–103] Given that ROS play an important role in mechanisms associated with UPE and metabolic processes, they might serve as a direct biochemical bridge between UPE and metabolomics. If UPE parameters can be linked to ROS-related metabolic pathways, the TCM diagnostic principle, which is characterized by UPE, may be related to biochemical mechanisms. Thus, UPE might be used to detect early perturbations, even before they can be detected using metabolomics. In this way, UPE measurements could be used to indicate when metabolomics measurements would be warranted. Alternatively, depending on the UPE parameter that is changed, a specific metabolomics platform can be used for further analysis. In other words, by characterizing TCM diagnostics using UPE parameters, and by studying the relationship between UPE and metabolomics, UPE-guided metabolomics based on TCM diagnostics can be used to improve healthcare.

6. Conclusions

In this review, we discussed the UPE research linked to TCM that was published in the Chinese literature in the last century. Several experimental observations using UPE were found to be highly correlated with TCM–based diagnostic concepts. A UPE-based metabolomics approach guided by the TCM–based diagnostic concept may provide a biochemical bridge between Western medicine and TCM. From this perspective, three areas of UPE-based research should be explored further: *i*) the UPE-based methodologies should be developed and optimized; *ii*) experimental work should bridge UPE with TCM–based diagnostics and metabolomics; and *iii*) dynamic UPE-based data should be integrated with other system-based diagnostic measurements.

Linking UPE, a dynamic diagnostics tool, with omics measurements in systems biology studies will increase our understanding of the diagnosis, prediction, and treatment of many diseases. Moreover, combining UPE with metabolomics based on ROS production might provide an effective approach for studying the relationship between health and disease and will help improve our understanding of the healthy state.

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Chapter 3

Measuring ultra-weak photon emission as a non-invasive diagnostic tool for detecting early-stage type 2 diabetes: a step toward personalized medicine

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Abstract

The global prevalence of type 2 diabetes is estimated to reach 4.4% by 2030, placing a significant burden on our healthcare system. Therefore, the ability to identify patients in early stages of the disease is essential for both prevention and effective management, and diagnostic methods based on traditional Chinese medicine (TCM) may be suitable for identifying patients with early-stage type 2 diabetes. Here, a panel of three physicians trained in TCM classified 44 pre-diabetic subjects into three syndrome subtypes using TCM-based diagnostics. In addition, ultra-weak photon emission (UPE) was measured at four anatomical sites in each subject. Ten properties encompassing 40 parameters were then extracted from the UPE time series. Statistical analyses, including multinomial logistic regression, were performed using the results of each parameter measured at the four sites. Sixteen UPE parameters were then selected and used to discriminate between the three subtypes of pre-diabetic subjects. Next, Spearman's correlation coefficient was used to quantify the correlation between the 16 UPE parameters and the TCM-based diagnoses. The resulting correlation networks accurately reflected the differences between the three syndrome subtypes. These results suggest that UPE is a suitable tool for detecting subtypes in early-stage type 2 diabetes. In addition, our results provide evidence that TCM may represent an important step toward personalized medicine.

Key words: traditional Chinese medicine–based diagnostics, personalized medicine, type 2 diabetes, ultra-weak photon emission (UPE), correlation network

1. Introduction

Type 2 diabetes (T2D) is multifactorial in origin and is associated with genetic factors, metabolic disorders, and lifestyle-related risk factors such as obesity, inactivity, and poor diet.[1–4] Currently, the oral glucose tolerance test and established glucose criteria are the golden standard for diagnosing T2D.[5] However, T2D can be present in an early, undetected form for more than ten years, during which dysglycemia increases the risk of severe complications,[6] including hypertension, blindness, renal failure, and cardiovascular disease.[3] Glycemic control can prevent some—but usually not all—of the aforementioned complications.[7,8] It has therefore been argued that a more personalized diagnostic approach may provide the opportunity to effectively manage T2D in its early stages, before the onset of complications.[9,10]

The diagnostic approach used in traditional Chinese medicine (TCM) is highly personalized and takes into account the interactions between the patient and his/her environment and pathogenic factors.[10] The patient's response with respect to these interactions provides a functional profile of the disease-related signs and/or symptoms and can be used to identify specific "syndrome subtypes" within a specific disease.[11,12] Thus, using the principles of TCM, several syndrome subtypes have been identified within T2D.[13] Recent studies combined TCM-based syndrome subtyping with modern metabolomics technologies, which are commonly used in Western medicine,[14] thereby combining TCM-based subtyping of pre-diabetes with metabolomics-based medicine.[15,16] The ability to identify and characterize these syndrome subtypes is an important step toward encouraging changes in unhealthy lifestyle on a personalized basis.

Measuring ultra-weak photon emission (UPE) is a non-invasive method for recording changes in metabolic processes within the human body and can reflect the dynamics of metabolic organization.[17] Photobiology (the metabolic effects of absorbed photons) and low-level biological luminescence (the production and

emission of photons) are complementary manifestations of the photons' role in metabolism.[17,18] Thus, recording photon emissions provides a measure of the net activity of these types of metabolic reactions, which reflects the body's current physiological state. We hypothesized that this technology may provide an alternate method for subtyping early-stage T2D in combination with TCM-based concepts. The equipment needed to continuously measure UPE in human subjects is relatively simple and includes a sensitive photomultiplier tube (PMT) in a sealed dark environment.[19] Importantly, the current technology for measuring UPE is rapid, highly sensitive, relatively inexpensive, and non-invasive.[20] In addition, several studies have calculated parameters (e.g., mean signals and signal variance) in UPE signals measured in both healthy subjects and in the pathological state,[21–30] providing a baseline for comparison.

Here, we asked whether UPE parameters can be used as a tool for identifying syndrome subtypes in subjects with early-stage T2D diagnosed using TCM-based diagnostics. An explorative, non-intervention urine metabolomics study at TNO (https://clinicaltrials.gov/ct2/show/NCT00469287) was designed in which 44 pre-diabetic male subjects were diagnosed with three distinct syndrome subtypes by a panel of three TCM-trained physicians.[15] We then measured UPE parameters in this same cohort of 44 pre-diabetic subjects in order to investigate the relationship between UPE signal parameters and the syndrome subtypes identified using TCM-based diagnostics. Our results suggest that measuring UPE represents a non-invasive method for distinguishing between the three syndrome subtypes of pre-diabetes. Thus, UPE may provide key insight into personalized diagnostics and personalized medicine, ultimately improving the management of diabetes and other diseases.

2. Materials and Methods

2.1 Subjects and pre-diabetes subtypes

A total of 44 male Dutch subjects were recruited and screened as described previously.[15] Each subject provided written informed consent, and the study was approved by the Medical Ethics Committee of Tilburg, the Netherlands. Pre-diabetes was defined as a fasting glucose level of 6.1–6.9 $mmol \cdot L^{-1}$ in the absence of other clinical evidence of diabetic complications measured during several examinations.[15] Three physicians who were trained in TCM independently diagnosed the subjects in a blinded fashion.[15] The physicians asked the subjects questions about their various symptoms, and then grouped these symptoms into 26 TCM-based diagnostic items (Table 1). Next, the three physicians assigned specific scores to these diagnostic items based on their severity and frequency.[15] The consistency of TCM based diagnosis between the three physicians was 85% based on the generalized procrustes analysis (GPA),[15] resulting in the following three syndrome subtypes of pre-T2D: subtype A ("Qi-Yin deficiency", n=15 subjects), subtype B ("Qi-Yin deficiency with dampness", n=20 subjects), and subtype C ("Qi-Yin deficiency with stagnation", n=9 subjects).15 In addition, UPE measurements and urine metabolomics analysis were carried out by the other two independent teams in a blinded fashion.

2.2 Photomultiplier system and UPE measurements

For this study, a tabletop photomultiplier system specifically designed for measuring UPE signals in the hands (Type PMS06.1) was provided by Meluna Research (Geldermalsen, the Netherlands). The detection head is located at the top of a dark chamber and includes a custom-designed shutter system. To measure UPE, we used a model 9235QA photomultiplier tube (ET Enterprises, Uxbridge, UK) fitted with a 48-mm diameter window, with spectral sensitivity in the range of 200–650 nm. The background noise measured by the PMT was 4-5 counts/s. A ring was constructed at the end of the PMT. Subjects were asked to position their hand against the ring in order to fix their hands at a specific placement to secure that the same areas on subjects' hands were measured. This avoids errors related to the positioning of the hands below the PMT. The distance between the ring and skin was 27 mm. The PMT

Table 1. The 26 diagnostic items (symptoms) associated with T2D based on traditional Chinese medicine.

ID	Diagnostic item (symptom)
C1	Blood stagnation
C2	Damp heat in the liver
C3	Damp Heat in the Middle Jiao
C4	Damp heat in the Spleen
C5	Damp heat in the stomach
C6	Damp Heat
C7	Dry Heat consumes Yin
C8	Heart Qi deficiency
C9	Heart Yin deficiency
C10	Heat in the Heart
C11	Kidney Yin deficiency
C12	Liver fire
C13	Liver Qi stagnation
C14	Liver Yang ascending
C15	Liver Yin deficiency
C16	Lung Yin deficiency
C17	Lung Qi deficiency
C18	Qi and Yin deficiency in the Middle Jiao
C19	Spleen Qi deficiency
C20	Stomach Qi deficiency
C21	Stomach Yin deficiency
C22	Yin deficiency
C23	Yin deficiency in the Middle Jiao
C24	False heat consumes Yin
C25	Qi deficiency
C26	Lung fire run

was operated in the single-photon counting mode, and the signals were recorded using a model 6602 PCI card (National Instruments, Austin, TX). The temperature within the measuring chamber was maintained at 20±1.0°C.

All UPE signals were recorded between 11 a.m. and 3 p.m. in order to minimize any possible effects of diurnal rhythms.[31,32] Each subject wore light-tight gloves on both hands for 30 min prior to recording the signal in order to minimize the effect of

ambient light exposure. During the measurements, the subject placed the hand below the PMT, and the following four sites were recorded, resulting in a total of four measurements per subject: right dorsal (RD), right palm (RP), left dorsal (LD), and left palm (LP). Each signal was recorded for 5 min by counting the number of photons emitted in 6000 consecutive 50-ms bins. Background noise was recorded in the same manner for each subject.

2.3 Gas chromatography-mass spectrometry analysis of urine metabolomics

Gas chromatography-mass spectrometry (GC-MS) analysis was used to study the urine metabolomics in the 44 pre-diabetic subjects. GC-MS was performed using an Agilent 6890 GC system with an Agilent 5973 MS detector (Agilent Technologies, Palo Alto, CA). The urine sample preparation and GC-MS analysis methods have been reported previously.[15] The concentration of urine metabolites was processed using an Agilent ChemStation software.[15]

2.4 Data processing and statistical analysis

2.4.1 Statistical analysis of UPE parameters

Ten properties of the UPE signal—strength, FF0, FF1, FF2, alpha, rho, theta, phi, SSI, and SSR—were calculated for each signal recorded. The specific calculations used to obtain these UPE properties have been reported previously.[25–27] The number of photons detected in a time series is to indicate the strength of the UPE signal. It is the photon emission intensity subtracted by the background noise. The photon distribution characteristics can reflect using the Fano Factor.[33] The three parameters FF0, FF1 and FF2 can be extracted from a Fano factor curve fitted by a second order polynomial function.[27] FF0 is the intercept of the Fano Factor curve, and determines theoretically the feature of photon distribution at zero bin size (time series).[27] FF1 and FF2 are parameters to express the slope of the Fano factor curve which is an indication for the structural patterns in the signal dynamics. FF1 is the slope of the Fano Factor curve, and determines the photon distribution changes in the different

time series compared to the photon distribution in the zero-time series.[27] FF2 is a coefficient in the second order polynomial function, and influences the shape of Fano Factor curve in specific samples.[27] The photon number distribution was also analyzed for non-classicality by procedures that have been developed to link quantum features to photon statistics, hence constructing portraits of quantum optical states. The approach followed in [25,27] and resulting in the parameters alpha, rho, theta, phi, SSI and SSR have been used in the present study. The use of these parameters is not meant to argue about optical characteristics of UPE because reliable evidence for coherence or non-classicality is missing.[34] Instead, they are only used as ways to analyze the photo count distribution

All ten properties were calculated in the signals obtained from each of the four anatomical sites for each subject, yielding a total of 40 UPE parameters for each subject. Thus, signal strength (for example) was defined as follows: Str_RD, Str_RP, Str_LD and Str_LP, corresponding to the signal strength measured in each of the four anatomical sites. The other nine parameters were defined using this same nomenclature.

The 40 UPE parameters were used to identify possible predictors in the multinomial logistic regression.[35] To build the model, we used forward neural networks to identify the maximum of a joint posterior distribution over the candidate predictors' coefficients, using the R package nnet (version 3.2.2).[36–38] To reduce the number of possible predictors, backward elimination was performed using the Akaike information criterion for variable selection.[39] The multinomial logistic regression was validated using cross-validation in order to avoid overfitting of the model.[40]

2.4.2 Statistical analysis of urine metabolites

In the previous urine metabolomics study,[15] both principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were used to identify a total

of 24 urine metabolites which contributed to the discrimination of the three TCM-based syndrome subtypes in the 44 pre-diabetic subjects.[15]

2.4.3 Statistics of the correlation network

To determine the correlation between the UPE parameters and TCM-based diagnoses, the median score was calculated for each of the 26 diagnostic items based on the scores assigned by the three physicians. Spearman's rank correlation coefficient (ρ) was used to quantify the correlation between the value of the predicted UPE parameters and the median score of each TCM-based diagnostic item using SPSS version 23.0 (IBM Corp., Armonk, NY). A linear relationship was defined as Spearman's $|\rho| > 0.30$.[41] Thereafter, Cytoscape version 3.2.1 (www.cytoscape.org) was used to draw a network view, which was used to visualize these correlations.[42] The Spearman's rank correlation coefficient was also used to study the correlation between the UPE parameters and the urine metabolites, the identified correlations were visualized using Cytoscape.

3. Results and Discussion

The three physicians trained in traditional Chinese medicine classified the 44 pre-diabetic subjects into three distinct syndrome subtypes. To predict these subtypes using UPE parameters, we used these subtypes as the response in multinomial logistic regression. From the original 40 UPE parameters (corresponding to ten parameters measured at four anatomical sites each; see Materials and Methods), 16 parameters were identified in the logistic regression model as effective predictors (Table 2). “Qi-Yin deficiency with dampness” was used as the reference category for the dummy coding in multinomial logistic regression.[43] Compared to this reference category, the estimated coefficient values indicate the features of each UPE parameter in the other two categories (“Qi-Yin deficiency” and “Qi-Yin deficiency with stagnation”). The coefficient values of these 16 UPE parameters accurately

identified the three syndrome subtypes identified by the TCM-trained physicians, and cross-validation of the model revealed predictive accuracy of 97.81%.

Table 2. Estimated coefficients for the multinomial logistic regression model for the pre-diabetes subtypes based on traditional Chinese medicine–based diagnostics.

UPE Parameter[1]	Estimated coefficients[2]		
	Qi-Yin deficiency (subtype A)	Qi-Yin deficiency with stagnation (subtype C)	Total effect[3]
Alpha_LD	-11704.69	7397.46	19102.15
Str_RD	4027.06	-12698.77	16725.83
Str_LD	8629.85	2795.39	11425.24
Alpha_LP	-3331.47	4651.03	7982.5
Alpha_RP	2718.85	-4134.38	6853.23
SSI_LP	2205.62	-2990.89	5196.51
SSI_RP	-3523.47	1590.54	5114.01
FF0_RP	2633.09	1759.27	4392.36
Phi_RP	-2284.12	1631.2	3915.32
Theta_RD	1692.18	971.66	2663.84
SSI_LD	1583.92	817.23	2401.15
FF1_RP	-1514.28	323.49	1837.77
Rho_RD	-343.77	-1066.63	1410.4
FF0_RD	1121	-89.01	1210.01
FF0_LP	-444.23	668.98	1113.21
FF2_LD	-373.06	53.1	426.16

1 LD, left dorsal; LP, left palm; RD, right dorsal; RP, right palm.

2 Qi-Yin deficiency with dampness (subtype B) was used as the reference category.

3 Total effect refers to the sum of the absolute values of the estimated coefficients in subtype A and subtype C.

In traditional Chinese medicine, syndrome subtype is the outcome reached by the physician after analyzing the patients' symptoms and signs comprehensively by inspection, listening, smelling, inquiry, and palpation.[12,13] Syndrome type is used to interpret the body's holistic state at both the large-scale and systematic organization levels in order to guide the choice of treatment in each patient using acupuncture and/or Chinese herbal medicines.[12,21] Similar to syndrome subtype, UPE is a highly sensitive holistic measurement that accurately reflects the dynamics of the human

body and the disease status at the systems level.[17,21] Importantly, some TCM concepts (for example, the body's response to acupuncture or herbal medicines) have been characterized using UPE intensity, particularly with respect to basic syndrome subtypes in animal models.[21] Therefore, UPE can provide a close approximation of the system level structures identified using TCM-based diagnostics. This may explain—at least in part—why the three syndrome subtypes of pre-diabetic subjects could be classified using UPE parameters measured in this study. These UPE parameters, which were derived from ten properties of the UPE signal, have been found that most of these UPE properties can predict pre-T2D subtype. This finding may indicate that obtaining a comprehensive UPE profile may be useful for predicting syndrome subtypes identified using TCM concepts.

In traditional Chinese medicine, many diseases can have two or more syndrome subtypes, and dynamic changes are likely to occur among these subtypes under specific conditions.[12,13] This suggests that TCM-based syndrome subtypes are not independent but rather are interrelated.[12] This notion supports our finding that 16 of the 40 UPE parameters (40%) were needed in order to identify the three syndrome subtypes with high predictive accuracy. This also underscores the importance of studying the relationships between syndrome subtypes in further detail. Therefore, we used correlation network-based analyses in order to identify the relationship between UPE parameters and TCM-based diagnostic items (symptoms) in the three pre-diabetic syndrome subtypes.

Network-based analysis is an emerging approach in biomedical research, and correlation networks have been used to discriminate between disease phenotypes and to understand the interactions that reflect parts of a complex biological system.[44,45] Fig. 1 illustrates the differences in the overall profile of correlations in the three pre-diabetes syndrome subtypes. Different distributions of correlations between UPE parameters and diagnostic items are observed in the three subtypes, with relatively fewer correlations in subtype B ("Qi-Yin deficiency with dampness"). This indicates

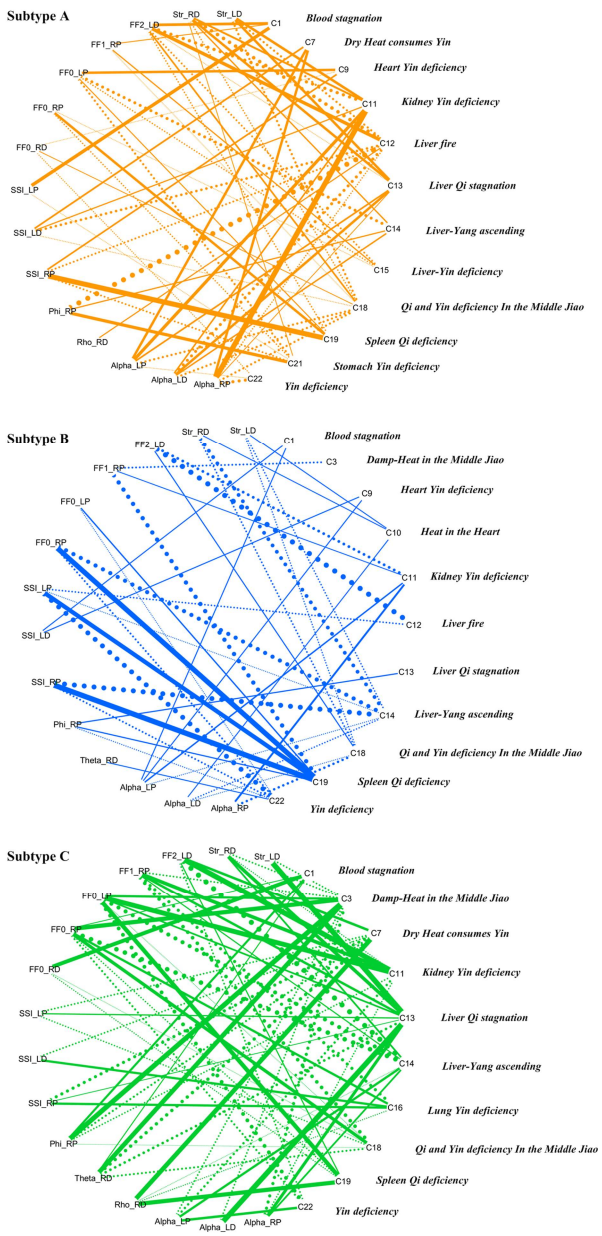

Fig. 1 Correlation network of the UPE parameters and traditional Chinese medicine–based diagnostic variables for three pre-diabetes syndrome types. Visualization of the data is concentrated on the correlations between UPE parameters and TCM-based diagnostic items (symptoms) using Cytoscape. Linear relationships are drawn for correlations in which Spearman's $|\rho|$ was >0.30. Negative correlations are indicated with dotted lines, and positive correlations are indicated with solid lines; thicker lines indicate a higher correlation (i.e., a bigger Spearman's $|\rho|$). The length of each line has no meaning. Specific diagnostic items are indicated with the letter "C" followed by the ID corresponding to the diagnostic item based on TCM (for example, C1 corresponds to blood stagnation); see also Table 1. Fifteen UPE parameters are correlated with 12 diagnostic items resulting in 61 correlations in subtype A. Fourteen UPE parameters are correlated with 11 diagnostic items, resulting in 39 correlations in subtype B. Sixteen UPE parameters are correlated with 10 diagnostic items resulting in 66 correlations in subtype C.

that TCM-based syndrome subtypes can also be characterized using UPE properties in a systematic network. According to Chinese medicine, "Qi deficiency" and "Yin deficiency" are key factors that can underlie the onset of T2D.[46] The combined deficiency of both Qi and Yin—which generally exists in patients with T2D—can be used to reflect a reduced functional level resulting in specific symptoms due to pathological factors such as heat, dampness, and stagnation.[13,15,46] Therefore, the three pre-diabetic subtypes identified by TCM-based diagnostics share a common basic, cross-biological background as well as individual features with respect to overall symptoms identified.

To interpret this pattern further, we focused on the identical and unique correlations between the three syndrome subtypes with respect to their correlation network structures. Table 3 summarizes the correlations that were common among the three syndrome subtypes. In these identical pairwise correlations, specific UPE parameters were correlated with nine diagnostic symptoms, most of which (e.g., C7, C11, C18, C19, and C22) reflect the functional effect of Qi deficiency and/or Yin deficiency measured at specific body locations. From the perspective of traditional Chinese medicine, pre-diabetic patients often show aggravated emotional tension, which is closely associated with liver-related T2D symptoms (C12, C13, and C14).[13,47] In particular, liver Qi stagnation (symptom C13) has been proposed as the first stage in the onset of diabetes.[13] These liver-related T2D symptoms were also observed in the identical pairwise correlations with UPE parameters among the three syndrome subtypes.

To visualize the unique pairwise correlations in each syndrome subtype, we removed the identical correlations and integrated the remaining pairwise correlations using Cytoscape. Fig. 2 shows that different syndrome subtypes can also share diagnostic symptoms; however, the pairwise correlations have non-overlapping, unique correlated UPE parameters with different correlation strength and/or direction (e.g., positive or negative correlations). Each subtype appears to correspond to a unique

set of diagnostic symptoms as reflected by the pairwise correlations. For example, the correlation between UPE parameters and the diagnostic item C21 was present in subtype A, but not in subtype B or subtype C. Thus, these unique pairwise correlations could potentially be used to identify the individual features of each pre-diabetes syndrome subtype, and UPE could be used to standardize the TCM-based symptoms, reflecting distinct syndrome subtypes.

Table 3. Overview of identical correlations between UPE parameters and diagnostic items among the three syndrome types.

Pairwise correlation		Subtype A	Subtype B	Subtype C	Pairwise correlation		Subtype A	Subtype B	Subtype C
UPE parameter	Diagnostic item				UPE parameter	Diagnostic item			
Alpha_RP	C11	+	+	+	SSI_LD	C7	+		+
Alpha_LP	C11	+	+	+	Str_RD	C11	+		+
FF2_LD	C11	+	+	+	Alpha_LD	C13	+		+
Str_RD	C14	+	+	+	Alpha_RP	C13	+		+
FF0_RP	C19	+	+	+	Str_LD	C13	+		+
FF0_LP	C19	+	+	+	Str_RD	C13	+		+
FF2_LD	C12	+	+		FF0_LP	C14	+		+
Alpha_LD	C14	+	+		FF0_RD	C18	+		+
Alpha_LD	C18	+	+		FF2_LD	C19	+		+
Alpha_RP	C18	+	+		Alpha_LP	C22	+		+
Str_LD	C18	+	+		FF1_RP	C3		+	+
Str_RD	C18	+	+		FF1_RP	C11		+	+
FF2_LD	C18	+	+		Alpha_LP	C14		+	+
SSI_RP	C19	+	+		FF0_RP	C22		+	+
					FF0_LP	C22		+	+

"+" indicates specific syndrome types that have the same correlations between UPE properties and TCM-based diagnostic items.

Although the physical meaning of UPE parameters has been well interpreted previously,[25–27] understanding the biochemical meaning of UPE parameters is of great importance to identify potential applications for UPE in disease characterization. A strategy of combination between UPE and TCM-based diagnostics may improve our understanding of UPE patterns in the biochemical relevance. In traditional Chinese medicine, the syndrome of "Qi-Yin deficiency" has similarities with "chronic fatigue syndrome" and/or "mild inflammatory status", both of which are induced by hyper-metabolism.[15] Qi-Yin deficiencies with a

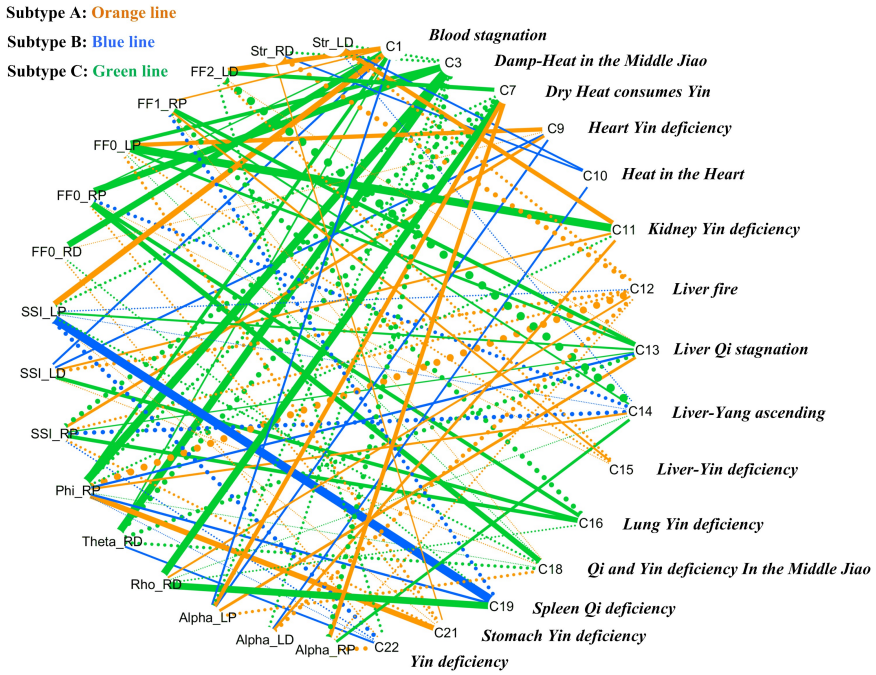

Fig. 2 Unique correlations between UPE parameters and traditional Chinese medicine–based diagnostic variables for the three pre-diabetes syndrome types. Negative correlations are indicated with dotted lines, and positive correlations are indicated with solid lines; thicker lines indicate a higher correlation (i.e., a bigger Spearman's $|\rho|$). The length of each line has no meaning. Specific diagnostic items are indicated with the letter "C" followed by the ID corresponding to the diagnostic item based on TCM (for example, C1 corresponds to blood stagnation); see also Table 1.

pathological factor is closely associated with an unhealthy lifestyle, and these subjects usually prefer raw, cold, greasy, and/or high-energy food.[13,15] Dampness is a sub-health status and can lead to development of metabolic syndrome and hypertension.[15] Another pathological factor is stagnation which may lead to emotional problems, impaired blood circulation, and diabetic peripheral neuropathy.[15,48] TCM syndrome-based biological relevance can be linked with TCM-based diagnostic symptoms (Table 1), the UPE parameters correlated with these diagnostic symptoms (Fig. 1) may therefore link to the biological interpretation of T2D from the disease cause, progression, to potential complications. Thereby, UPE parameters may be used to predict disease in a personalized basis.

Moreover, the biological and molecular interpretation of UPE parameters requires further study, and one promising strategy is to link UPE to metabolomics.[17,21,49] Metabolomics provides a comprehensive analysis of various low molecular weight compounds in biological systems based on specific metabolomics platform.[50] In addition, UPE results from the metabolic processes occurring in living organisms.[51,52] Therefore, UPE parameters may theoretically correlate to specific metabolites identified in metabolomics analysis. To test this hypothesis, we carried out a Spearman's correlation analysis between the identified 16 UPE parameters and the concentration of 24 urine metabolites in the 44 pre-diabetic subjects. The selected 24 urine metabolites contributed to the discrimination of TCM-based T2D syndrome subtypes in the previous urine metabolomics study.[15] Fig. 3 illustrates the correlation network which has a total of 26 pairwise correlations between 10 UPE parameters and 13 urine metabolites including amino acids (e.g., L-cysteine and L-serine), organic acids (e.g., Citric acid and Pyruvic acid) and sugars (e.g., D-Glucose and D-Ribose). These results indicate that the biological interpretation of the UPE parameters may be correlated to the biological pathways reflected by these specific urine metabolites in the T2D subjects, and thereby UPE parameters may provide more biological information to the TCM-based diagnostics. However, further validation of this strategy is required. Given that additional metabolomics platforms (e.g., amino acid and oxylipin platforms in plasma sample) are used to identify more metabolites such as oxidative stress/ reactive oxygen species (ROS) products etc., as well as their biological pathways in the disease state,[21,53–55] it may be possible to establish a comprehensive correlation between UPE parameters and multi-metabolic biomarkers. Therefore, UPE may serve to complement metabolomics, providing an integrated strategy for interpreting both UPE properties and TCM-based diagnostics, ultimately contribute to personalized medicine.[17,21]

4. Conclusions

Phenotyping is believed to play an essential role in realizing the goal of personalized

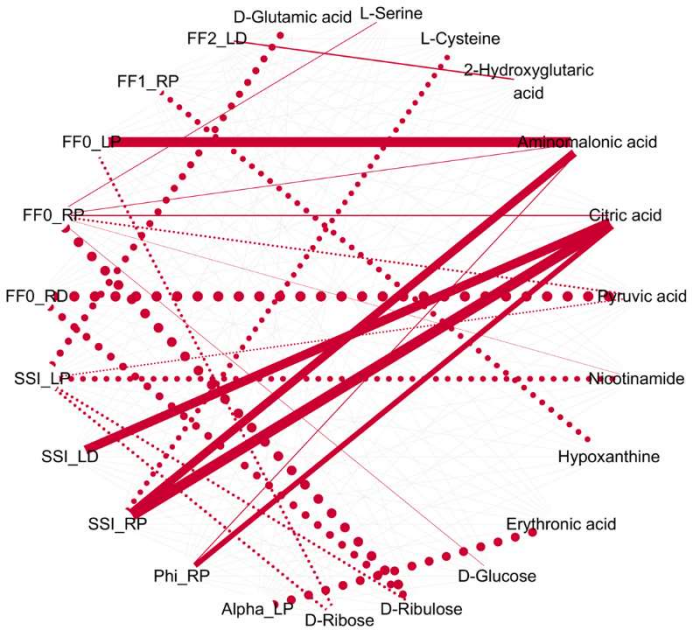

Fig. 3 Correlation network of the UPE parameters and the urine metabolites for pre-diabetic subjects. Visualization of the data is concentrated on the correlations between UPE parameters and urine metabolites using Cytoscape. Linear relationships are drawn for correlations in which Spearman's $|\rho|$ was >0.30. Negative correlations are indicated with dotted lines, and positive correlations are indicated with solid lines; thicker lines indicate a higher correlation (i.e., a bigger Spearman's $|\rho|$). The length of each line has no meaning.

medicine,[10] and traditional Chinese medicine–based diagnostics provides important information (e.g., syndrome subtype) regarding personalized phenotypes.[10] Given that TCM-based diagnostics is a phenomenological and descriptive approach used to reflect regulation at a systems level—including dynamics—it can be used to monitor changes in lifestyle and system-based treatments.[12,21] The key challenge is to both distinguish between and standardize TCM-based syndrome subtypes using modern technology.[12] Here, we present the first study combining UPE parameters with TCM-based diagnostics in order to investigate syndrome subtypes among pre-diabetic subjects. Several main conclusions can be drawn from our study.

First, based on the characteristic nature of the UPE signal, ten properties were

extracted from four anatomical sites, resulting in 40 separate UPE parameters measured in 44 subjects with pre-T2D; 16 of these 40 parameters were able to discriminate between the three pre-diabetes subtypes. Second, our correlation network between UPE parameters and TCM-based diagnostics provides key insight into the identification of three pre-T2D syndrome subtypes. These results indicate that UPE profiles may reflect personalized TCM-based diagnostics. A future strategy to reveal powerful UPE parameters is combing TCM-based diagnostics, UPE measurements and metabolomics analysis. The correlation between UPE parameters and metabolomics data for a given subtype as determined by TCM-based diagnostics, could lead to a better understanding of the underlying biochemistry and regulatory processes on which TCM diagnosis is based. The previously published urine metabolomics study reported only limited differentiation between three TCM-based syndrome subtypes in the pre-diabetic subjects.[15] In the present study it seems that a better segregation is obtained in the personalized diagnosis using 44 subjects, when a combination of UPE parameters and urine metabolites data is used. Further study should focus on the biological interpretation of UPE parameters.

Finally, the results of our study create new avenues for investigation. Future studies should include larger patient cohorts. In addition, similar analyses can be performed with other disease cohorts. In conclusion, UPE is a highly sensitive, non-invasive, robust technique that represents a new technological platform for diagnosing disease and disease subtypes, and for studying the efficacy of various therapies and healthcare approaches.

Acknowledgments

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Chapter 4

Delayed luminescence: an experimental protocol for Chinese herbal medicines

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Luminescence, 2016, 31:1220-1226.

Abstract

In traditional Chinese medicine, raw herbal materials are used in processed and unprocessed form aiming to meet the different requirements of clinical practice. To assure the chemical quality and therapeutic properties of the herbs, fast and integrated systematic assays are required. So far, such assays have not been established. Delayed luminescence (DL) refers to a decaying long-term ultra-weak photon emission after exposure to light. Its decay kinetics under certain conditions may be a sensitive indicator reflecting the internal structural and chemical/physiological state of a biological system. DL measurements have been used in many applications for quality control. However, relatively little research has been reported on dried plant material such as Chinese herbs. The objective of the present study is to establish a protocol for direct and rapid DL measurements of dried Chinese herbal materials, including the determination of the dependence on: a.) the optimal excitation time utilizing a white light source; b.) the optimal size of the grinded herbal particle; and c.) the humidity conditions before and during measurement. Results indicate that stable and reproducible curves of DL photon emission depend mainly on the water content of herbal materials. To investigate the application of the established DL measurement protocol, non-processed and processed *Aconitum* (*Aconitum carmichaelii* Debx.), wild and cultivated rhubarb (*Rheum palmatum* L.) and ginseng (*Panax ginseng* C.A. Mey.) of different ages were measured using DL. The results suggest that DL technology is a potential tool for assessment of dried Chinese herb qualities. The results warrant a further exploration of this technique in relation to therapeutic properties of the herbs.

Keywords: delayed luminescence (DL), Chinese herbal medicine, quality control, excitation time, particle size, water content.

1. Introduction

Delayed luminescence (DL) refers to a long-term, decaying, weak photon emission shown by various materials after exposure to light. It is a characteristic property of some inorganic and organic materials and has also been described for living organisms.[1–6] The difference between DL and fluorescence lies in the time of decay of the excited state. Fluorescence usually refers to photon emission that fades in nanoseconds or picoseconds. Light that fades in milliseconds or more is called DL.[7] The kinetics of the DL decay depend on the excitation conditions including excitation energy and excitation time.[8,9]

DL has been utilized as a tool for rapid examination of a biological system and found to be a sensitive indicator for its chemical/physiological state.[10–12] A typical application is in the field of food quality control.[13] Estimations of DL from various water-rich vegetables and fruits showed relationship of DL to development and maturity (ripeness).[14–16] Another DL application is in the field of germination capacity of seeds.[17–20] DL measurement may be able to correlate to the functional state of biological system in living materials.[18] Therefore, we ask whether DL could also represent specific properties of dried plant materials such as Chinese herbal medicines.

A few recent studies have focused on the DL signatures of dry powders of Chinese herbal medicines.[21,22] They focused on therapeutic properties ("heat" and "cold") of herbal medicines and demonstrated that DL dynamics differ between "heat" and "cold" herbal medicines.[22] Chemical analysis (LC/MS, GC/MS and NMR) has also been used to study the different properties of herbal medicines. However, it is still not evident why herbs with different therapeutic properties can have similar chemical constituents, while herbs with the same therapeutic effect have completely different chemical constituents.[23–25] Therefore, DL may be a candidate technology to indicate additional, unknown properties of Chinese herbal medicines.

At present, there is no detailed experimental protocol described the use of DL technology in dried herbal materials. The aim of this paper is to present such protocol. A description of the DL signal from dry powders of *Aconitum* root is presented, focusing on how DL depends on a.) time of excitation; b.) the size of grain in powdered herbal materials; c.) moisture; as well as d.) reproducibility of the signal when samples are stored under specific dry conditions. Based on an established protocol, materials from dried roots were compared: a.) in case of *Aconitum* the effect of processing; b.) in rhubarb wild and cultivated materials from different areas; c.) in ginseng, different ages were compared. The results show that the different DL dynamics appear between processed and unprocessed herbs, as well as the herbs with different environmental factors and ages.

2. Materials and methods

2.1 Herbal materials

Raw roots of *Aconitum* (*Aconitum carmichaelii* Debx.) were collected by Sichuan new Lotus Pharmaceutical Co. Ltd. in the Jiangyou district, Sichuan province, P. R. China. Other processed root samples of *Aconitum* with white (salt processed method) and black (black bean processed method) colors were obtained from Xiansheng Company, Nanjing city, the P. R. China.

Raw root samples of rhubarb (*Rheum palmatum* L.) were supplied by the China Academy of Chinese Medical Sciences, which harvested in three locations of Gansu province and Sichuan province, the P. R. China. Samples of raw ginseng roots (*Panax ginseng* C.A. Mey.) were provided by Changchun University of Chinese Medicine, which were obtained in Fusong city, Jilin province, the P. R. China.

2.2 Sample preparation

Samples were prepared prior to the measurements by crushing the herbal materials using a grinder (Yili Company, Zhejiang province, the P. R. China, type QE-100).

Different diameter sizes of herbal particles were selected by different-sized sieves (Tongrentang Company, Beijing, the P. R. China, Standard sieve with the size of 74μm, 150μm, 355μm and 600μm). Thereafter, herbal samples were stored in a dark box together with silica gel (Boom BV, Netherlands, 3-5mm) at room temperature for at least 16h before the DL measurements started. Self-made processed *Aconitum* samples were prepared from powders of raw *Aconitum* roots by sterilization at 127℃ for 60 minutes under high pressure,[26] using an autoclave device (Prestige Medical, UK, Series 2100 Classic). After processing the powder was stored in a silica environment.

2.3 Delayed luminescence technology, measurement procedure

Fig.1 illustrates schematically the instrumental set-up for DL measurements. The device includes a dark sample chamber (9.5cm×15cm×16cm) with a vertically positioned photo-multiplier tube (PMT) (Electron Tubes Enterprises Ltd, Ruislip, UK, type 9558QB). The sample chamber was kept at 22°C. The cathode opening of the PMT has a diameter of 44 mm. The sensitivity of the PMT is in the range between 160 and 870nm. The PMT was cooled to -25°C for reducing the dark count rate to 10 counts per second. A fast preamplifier (ORTEC, U.S.A., type 9301) was used to enlarge the signal of photon emission. A PC with a counting card (National instruments, U.S.A., type 6602) was used for data acquisition.

Samples were measured utilizing a petri dish (diameter: 35mm) placed in the dark chamber at 12cm from the shutter of PMT. One-gram herbal material was used to cover the bottom of the Petri dish. For excitation of the powder, a white halogen excitation source (PHILIPS, German, NO. 284-2812) was used at 10 cm distance from the Petri dish.

The protocol for sample preparation immediately prior to DL measurement included that each herbal batch was used to prepare five samples of 1 g. The DLs of such samples were consecutively measured three times. The total number of 15

measurements of each herb batch was used for analyzing the DL of the particular herb. DL kinetics were obtained by recording the number of counts over consecutive periods of 0.05 sec for a total period of 5min resulting in 6000 data points.

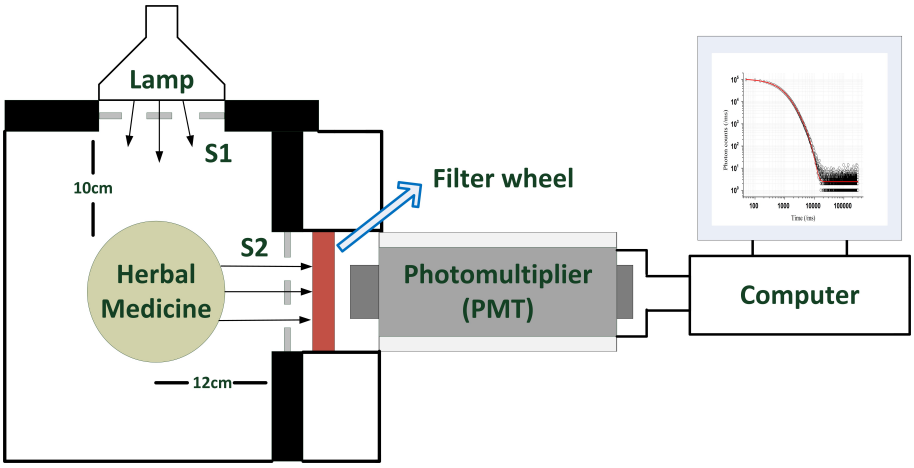


Fig. 1 Schematic diagram of delayed luminescence set-up. Excitation stage: When the excited light irradiated the herbal sample, shutter 1 (S1, shutter between excitation source and Petri dish) was opened and shutter 2 (S2, shutter between Petri dish and photomultiplier) was closed. Emission measurement stage: It begins immediately after the end of the light exposure period. When photon emission from sample radiated into the photomultiplier, S2 was opened and S1 was closed. The response time of S2 was 0.1s.

2.4 Data analysis and statistics

The photon counts during the 5min of each decay curve were used to calculate the parameters of the two-exponential decay function:[27]

$$y = y0 + A1e^{-\frac{x}{t1}} + A2e^{-\frac{x}{t2}}$$

where A1 and A2 are the amplitudes of photon emission of exponential decay components and t1 and t2 are time constants in those exponential decays, while y0 represents the final value of photon emission in DL decay curve. Curves fitting was performed by using ExpDecay 2 (http://www.originlab.com/doc/Origin-Help/ExpDec2-FitFunc) of Origin software (OriginLab Corporation, Northampton, MA, United States, Version 9.0).[28] Statistical analysis was carried out using SPSS

Statistics software (IBM, Armonk, New York, United States, Version 23.0).[29] Unpaired two-tailed tests were performed to estimate differences between conditions (e.g., *Aconitum* samples with different water contents etc.). Paired two-tailed tests were conducted to estimate differences in repeated samples (e.g., *Aconitum* samples at different times). The significant difference was estimated when p-value <0.05.

3. Results

For calculating parameters of herbal DL kinetics, a two-exponential decay function was used to fit the observed decay curve. Fig. 2 illustrates a fitting result taking the *Aconitum* root sample as an example. The raw data measured in consecutive 0.05 seconds periods were too scattered for the visualization of the DL decay kinetics. Therefore, 20 consecutive data points were accumulated, resulting in the decay curves represented in Fig. 3 for the three Chinese herbal medicines after the excitation. Data show that both the background noise and the empty Petri dish noise were far lower than the herbal DL signal.

3.1 Procedure for obtaining reproducible DL measurements of dried herbs.

Two types of explorative experiments were performed to establish the measurement protocol. These experiments were carried out with the powders of *Aconitum* root samples. These powders were divided into five independent samples. These samples were stored in separate Petri dishes under dry conditions in the presence of silica gel (500 g). The measurements were made with the same amount (1.0 g) of herb powder.

The first explorative study focused on the effect of excitation time on the kinetics of the delayed emission of the sample. Different excitation times were applied ranging from 1sec to 60 sec. The DL curves of the five independent samples were highly similar under each excitation time. Fig. 4 shows the DL behavior after illumination. Additionally, Table 1 presents the DL parameters for different excitation times. Based on the data in Fig. 4 and Table 1, the excitation time resulting in the highest total photon counts (i.e. 10 sec) was selected for further studies.

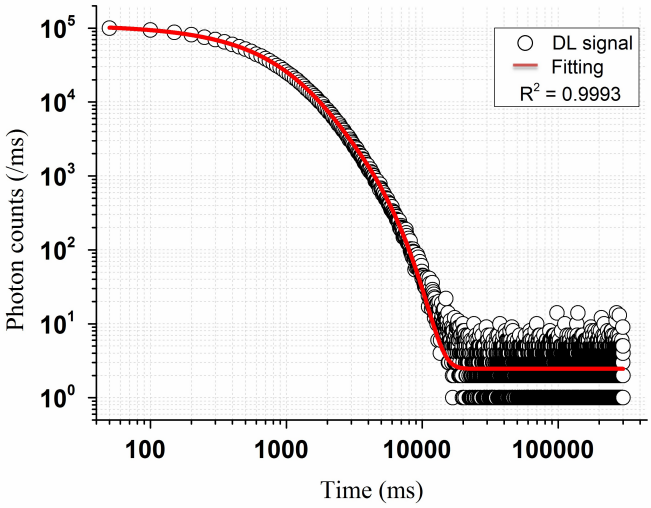


Fig. 2 Fitting effect of the DL decay curve of an *Aconitum* root sample.

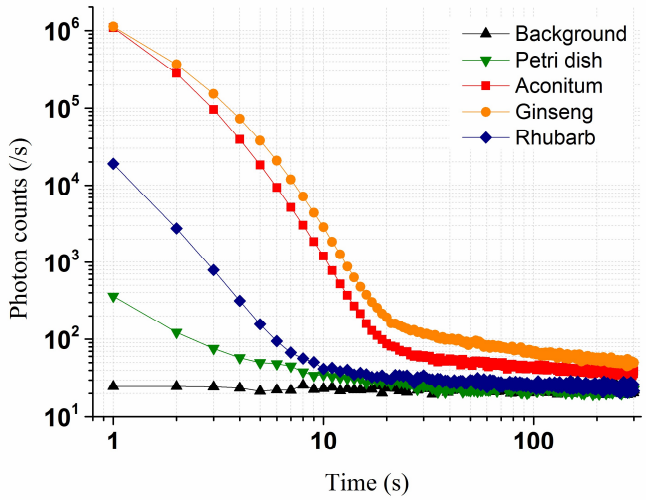


Fig. 3 Comparison of herbal DL signal and the noise from an empty Petri dish and background. Each herbal sample was grinded to approximate 150 μm, and saved with silica gel (500 g) for 16h before measurement. The excitation time was 10 s.

Table 1 DL parameters of *Aconitum* root samples under different excitation time

Excitation time (s)	Photon counts (Mean ± SD)	A1 (Mean ± SD)	A2 (Mean ± SD)	t1 (Mean ± SD)	t2 (Mean ± SD)	y0 (Mean ± SD)
1	1256463.33 ± 91231.79	96614.67 ± 8763.66	11648.81 ± 3820.62	508.67 ± 29.30	1398.16 ± 186.34	1.49 ± 0.24
3	1545725.47 ± 106928.89	103114.50 ± 5024.72	10388.50 ± 2407.08	604.72 ± 33.82	1674.23 ± 127.87	1.51 ± 0.23
5	1621452.20 ± 70209.91	100366.40 ± 6125.15	13067.23 ± 3856.46	616.72 ± 36.31	1668.37 ± 156.14	1.74 ± 0.27
10	**1642700.67 ± 62888.33**	**99917.40 ± 4313.14**	**13155.96 ± 1919.77**	**623.01 ± 24.13**	**1688.84 ± 99.30**	**1.85 ± 0.27**
30	1587917.93 ± 89464.48	95751.15 ± 3785.44	14634.53 ± 1345.91	604.34 ± 30.84	1611.06 ± 95.25	2.31 ± 0.30
60	1542705.53 ± 120026.23	94100.47 ± 4122.80	15185.18 ± 1490.20	587.85 ± 41.86	1564.39 ± 126.88	2.56 ± 0.44

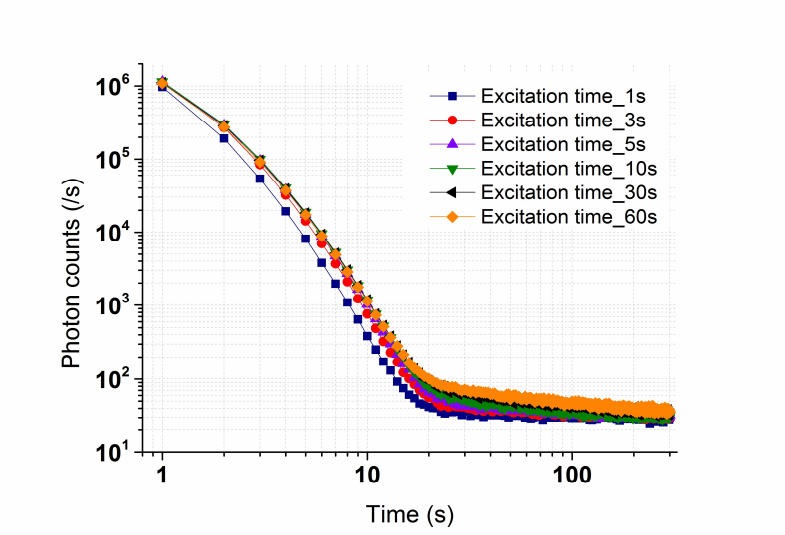


Fig. 4 Relationship between *Aconitum* DL-dynamics and excitation time. In total, 15 measurements of five independent *Aconitum* samples were averaged to obtain the DL dynamics under each excitation time.

The second type of explorative experiments was related to test reproducibility as well as stability of samples over a period of 3 weeks. For this purpose, the aforementioned five independent *Aconitum* root samples were used. Each sample was repeatedly excited and analyzed for its DL within 2 h utilizing a protocol including four periods, with each period including again three consecutive tests within 17min, and an interval of 17min between each two periods. In order to maintain the same excitation conditions in consecutive exposures, samples were kept

in the dark chamber before the next measurement period took place. The 12 curves of each sample were highly similar within 2 h suggesting that excitation has no influence on the DL properties of the sample. Therefore, the 12 DL curves of the same sample were averaged in order to represent the DL of that sample. To study the stability of the samples, this procedure was repeated with the same samples over a period of 3 weeks. Fig. 5A shows the results from the five independent samples measured with this protocol in the first week, and the DL decay curves are similar. Similar DL curves were also obtained for these five samples in the second and third weeks (data not shown). Therefore, the estimated differences of DL parameters between the 3 weeks were estimated using paired two-tailed tests (Fig. 5B). The results showed that most DL parameters could significantly differ (p-value < 0.05) between weeks, except y0 between week 1 and week 3 (p-value = 0.067) and y0 between week 2 and week 3 (p-value = 0.140). The differences were not unidirectional and must be attributed to yet unknown storage conditions.

3.2 Effects of powder grain size on DL

In the previous section, the DL decay kinetics was estimated using a 150μm grain size of the powdered *Aconitum* root. In studies utilizing organic materials, it has been reported that grain size has influence on DL features.[30,31] In the present study the effect of grain size of *Aconitum* powder on DL was estimated using different particle sizes, ranging from 74μm to 600μm. Herbal particle size larger than 600μm has also been explored. However, with a powder size larger than 600μm, the surface of the Petri dish could not be completely covered with the standard 1.0 g of herbal material. Each measurement of a specific particle size involved five independent *Aconitum* samples. Fig. 6 illustrates the DL curves of the *Aconitum* powder samples with particles of different size after 10 sec excitation time. The data demonstrate that initial values of emission differ for the various grain sizes. Table 2 demonstrates the relationship between DL parameters and powder size. The maximum of total DL was observed with grain size of 150μm. The parameters reflecting DL dynamics are

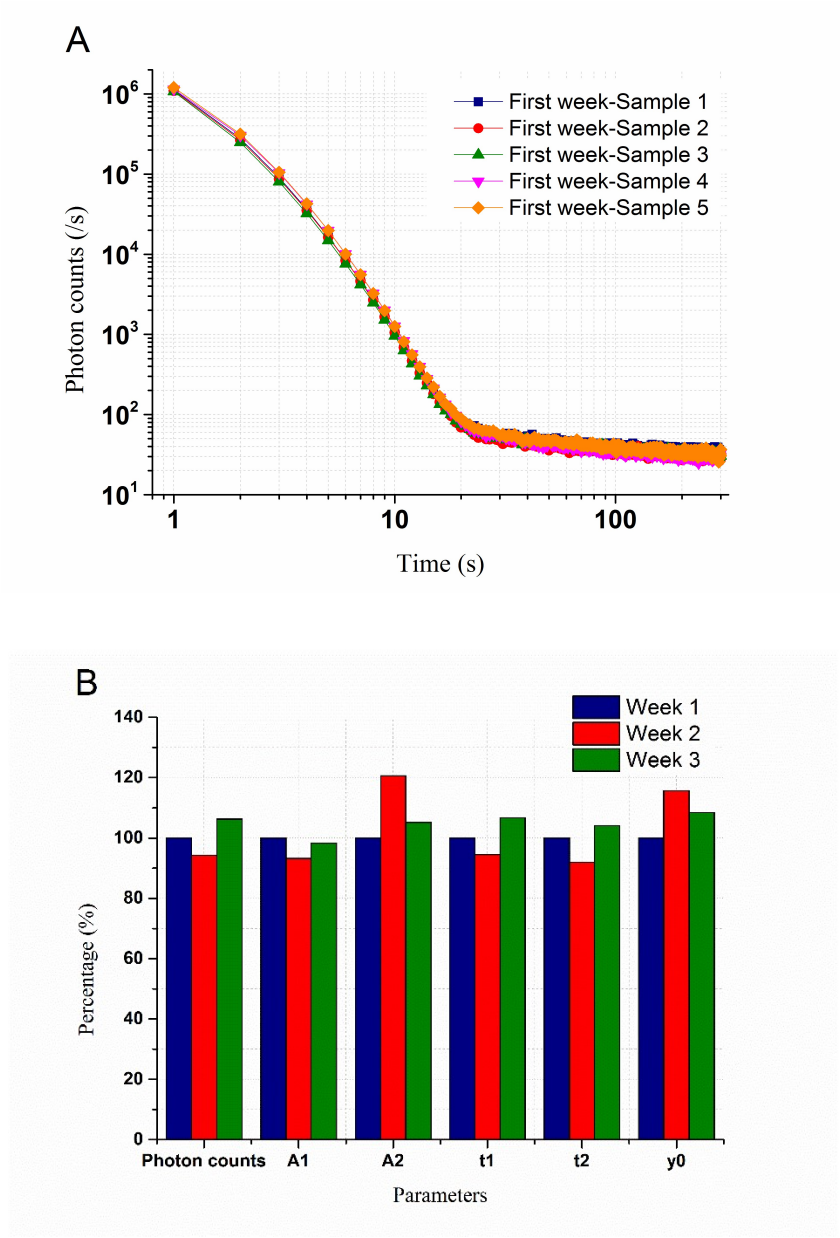


Fig. 5 Stability and repeatability tests of *Aconitum* root samples. (A) DL dynamics of five independent *Aconitum* samples in the first week. (B) Variations of DL parameters of *Aconitum* root samples in 3 weeks. The value of each parameter in the first week was fixed as 100%. The relative percentage of each parameter in both second and third week was compared with that in the first week.

shown in Table 2. They also demonstrate that DL dynamics varies with grain size. Interestingly, A1 and A2 show opposite behavior. The size of 150μm was selected for standardizing the grain size in further studies.

Table 2 DL parameters of *Aconitum* root samples under different powder size

Size (μm)	Photon counts (Mean ± SD)	A1 (Mean ± SD)	A2 (Mean ± SD)	t1 (Mean ± SD)	t2 (Mean ± SD)	y0 (Mean ± SD)
74	788134.00 ± 45833.48	62255.12 ± 6167.34	14930.44 ± 5572.62	375.69 ± 15.86	1280.78 ± 174.00	2.16 ± 0.52
150	1642700.67 ± 62888.33	99917.40 ± 4313.14	13155.96 ± 1919.77	623.01 ± 24.13	1688.84 ± 99.30	1.85 ± 0.27
355	984808.60 ± 57288.06	67765.75 ± 3675.07	20335.40 ± 2555.35	397.93 ± 14.43	1160.66 ± 41.07	2.94 ± 0.25
600	777038.60 ± 95133.52	52951.56 ± 3610.42	20726.16 ± 1597.95	344.95 ± 22.35	1030.68 ± 57.45	3.02 ± 0.16

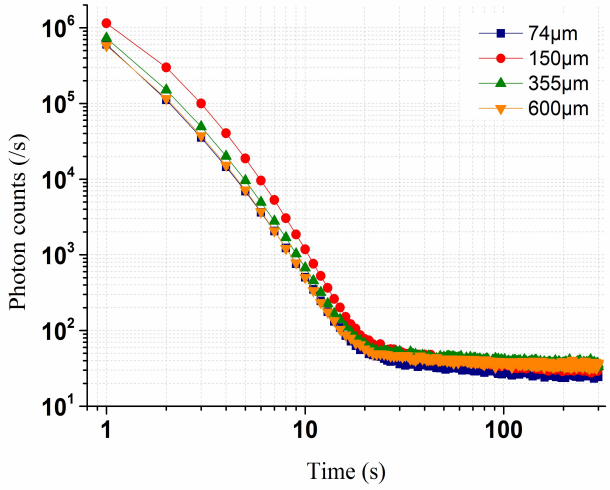


Fig. 6 Effects of powder size on DL of *Aconitum* root samples. In total, 15 measurements of five independent *Aconitum* samples were averaged to obtain the DL dynamics under each powder size.

3.3 Effects of water content on DL

As described in literature, there is a strong influence of the water content of the samples on the DL characteristics.[32–34] For that reason the herbal samples for research were usually stored in a silica-dried environment. However, to study the effect of silica to decrease humidity, the DL of *Aconitum* was estimated from silica-dried samples and samples that were measured without silica for storage. The

condition without silica-dried storage resulted in a sample water content of 9.4% as compared to 6.6% with silica-dried conditions. The measurement protocol was the same as that used in previous tests of reproducibility and stability. Fig. 7 illustrates the changes of photon emission of those two *Aconitum* samples during the four measurement periods. For each measurement period the difference in response between the two water content conditions were significant (p-value <0.05). The variations of four periods were evaluated using relative standard deviation (RSD). The variations of silica-dried samples (RSD:1.55%) were far lower than that of non-silica-dried samples (RSD: 9.26%) in all 12 measurements. Table 3 represents the data of other parameters over the four periods. The differences of parameters were significant (p-value < 0.05) between the two humidity conditions. In addition, the RSD values of all parameters were smaller than 5% with the silica-dried condition, which indicated relatively small variations over the four measurement periods. In contrast, four parameters (A1, t1, t2 and photon counts) showed RSD values higher than 5% without silica-dried condition, which reflected relatively large variations. In conclusion, whereas DL of silica-dried *Aconitum* was stable over time, the *Aconitum* samples without silica, show relatively strong changes which can be related to an increasing water content. It is obvious that non-controlled humidity affects DL, hence the strictly control of humidity is important and necessary.

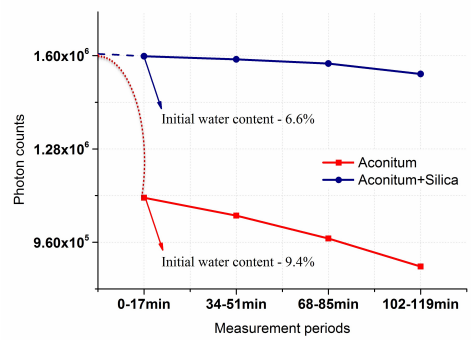


Fig. 7 Effects of water content on photon counts of *Aconitum* root samples. Solid lines present the real variations of photon counts in the measurements of the four periods. Dotted lines show possible variations of photon counts with changes in water content.

Table 3 DL parameters of *Aconitum* root samples under different stored conditions

Initial water content	Periods (Mean ± SD)	Photon counts (Mean ± SD)	A1 (Mean ± SD)	A2 (Mean ± SD)	t1 (Mean ± SD)	t2 (Mean ± SD)	y0 (Mean ± SD)
6.60%	0-17min	1597930.67 ± 1422.59	101174.11 ± 397.45	602.15 ± 3.31	12835.77 ± 499.19	1648.63 ± 24.73	2.11 ± 0.02
	34-51min	1587248.33 ± 5192.25	100722.81 ± 179.98	599.33 ± 0.53	12917.13 ± 319.59	1640.59 ± 11.27	2.04 ± 0.02
	68-85min	1572848.33 ± 3105.36	100221.05 ± 302.59	598.67 ± 1.74	12634.88 ± 329.98	1648.26 ± 16.99	2.02 ± 0.03
	102-119min	1537294.00 ± 8357.14	97800.83 ± 238.82	582.25 ± 2.71	14048.24 ± 89.75	1568.78 ± 5.29	2.14 ± 0.02
9.40%	0-17min	1112804.33 ± 8656.56	71425.34 ± 184.69	439.41 ± 0.71	21483.04 ± 397.43	1198.79 ± 5.30	2.12 ± 0.07
	34-51min	1051625.33 ± 18365.84	68117.43 ± 1293.34	424.23 ± 9.73	21550.34 ± 1122.90	1166.43 ± 27.71	2.54 ± 0.10
	68-85min	972914.67 ± 16900.15	63106.75 ± 417.84	397.71 ± 2.93	22524.24 ± 746.20	1104.48 ± 7.46	2.59 ± 0.08
	102-119min	877027.33 ± 4906.83	57554.02 ± 987.62	373.95 ± 4.10	22186.04 ± 880.45	1060.28 ± 17.69	2.56 ± 0.09

3.4 Effects of *Aconitum* processing on DL

In Chinese herbal medicine, "processing" refers to a pharmaceutical technique that alters the properties of herbal materials to meet the requirements of therapeutic application. It includes methods such as steaming, boiling and stir frying etc. of herbal materials to reduce toxicity, decrease side-effects and enhance the pharmacological efficacy and stability etc..[35,36] For instance, a raw root of *Aconitum* requires specific processing in order to reduce the content of toxic components. In this section, we studied the effect of processing of herbal materials in relation to DL dynamics. For this purpose, *Aconitum* roots powder (150μm) was processed by autoclaving (127℃, 60 minutes). A further comparison was made with two other commercially processed *Aconitum* samples resulting in white and black colored *Aconitum* material.[36] Fig. 8 shows that DL drastically decreased as a result of these processing methods. The differences in DL parameters between the raw *Aconitum* samples and processed *Aconitum* samples are demonstrated in Fig. 9. Only parameter A2 does not show significant difference (p-value = 0.102) between raw *Aconitum* and processed *Aconitum* by autoclaving (Fig. 9C). The other parameters of processed *Aconitum* samples show significant differences compared with the raw *Aconitum* sample (p-value <0.05) (Fig. 9).

3.5 DL of other herbs (rhubarb and ginseng)

In the present research, we also included rhubarb root and ginseng root samples,

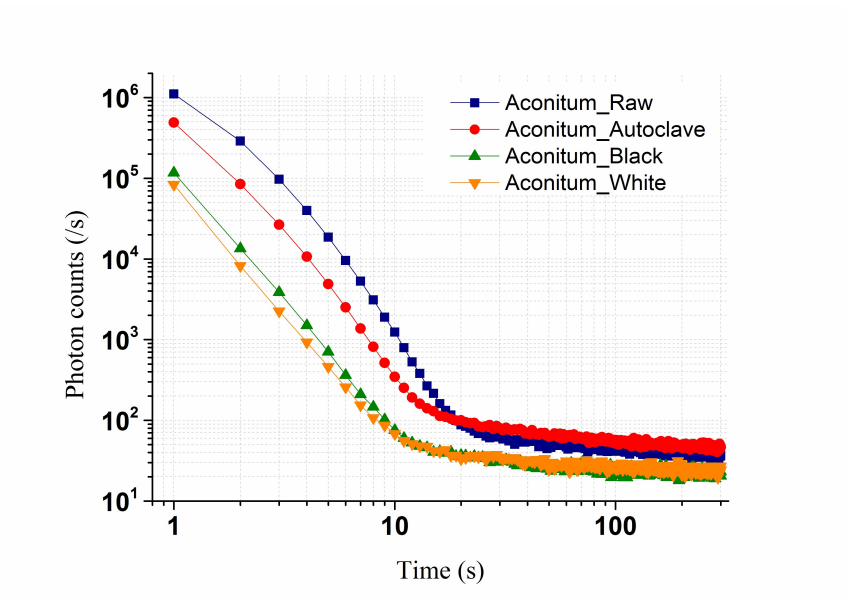


Fig. 8 Effects of *Aconitum* processing on DL. In total, 15 measurements of each sample were averaged to obtain the DL dynamics for different *Aconitum* samples.

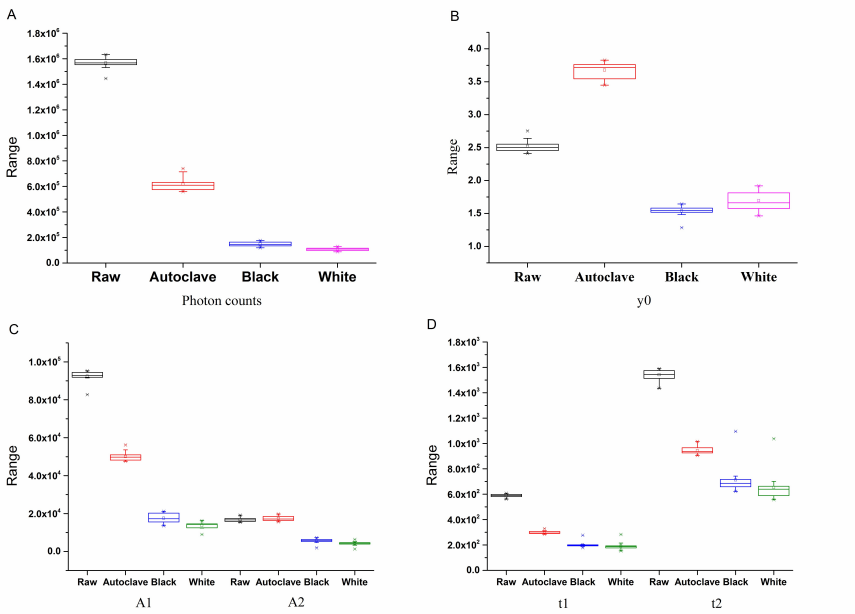


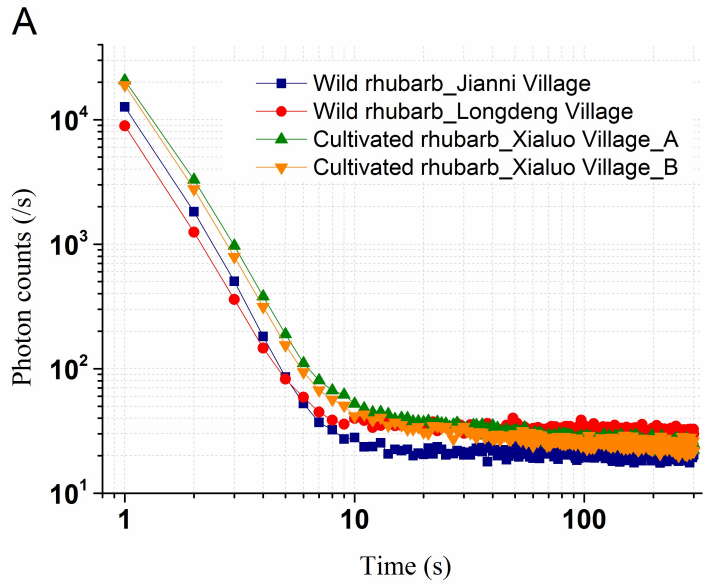
Fig. 9 DL parameters of *Aconitum* root samples and processed *Aconitum* root samples. (A-D) Show the value of DL parameters of raw and processed *Aconitum* samples.

because these two herbal materials are widely used and easily collected. In the case of rhubarb roots we compared roots from plants, grown under different conditions. Fig. 10A illustrates the DL curves of two wild and two cultivated rhubarb samples respectively. It is obvious that one wild rhubarb samples (grown in Longdeng village) demonstrate different decay behaviors compared with other rhubarb samples. The DL parameters show significant differences (p-value <0.05) between two wild rhubarb samples (grown in different location) except the parameter of photon counts (p-value =0.19). Two cultivated rhubarb samples grown in the same location and with the same age demonstrated only two parameters (t1 and t2) with significant differences (p-value <0.05). These results may indicate that different growth condition may be reflected in DL differences.

In the case of ginseng, two samples were studied. The two samples were grown in the same location but had different growth ages (10 years and 16 years). Fig. 10B illustrates the different shapes of two ginseng sample curves. In addition, significant differences in all DL parameters (p-value <0.05) were found. Therefore, herbal age may be also reflected in the DL dynamics.

4. Discussion

A DL measurement protocol of dried herbal materials (*Aconitum*, ginseng and rhubarb) has been developed in which the particle size of dry powder is defined as well as the excitation and measurement conditions. Utilizing this protocol, the effects of different processing conditions as well as differences in growth conditions of the herbs (locations, growth age) were evident. DL measurements show typical long-term kinetic patterns depending on the duration of excitation. A long measurement time (5 min) led to a long DL tail (Fig. 3). As these tails do not completely overlap with the DL signal of the empty Petri dish, these tails may represent specific features of different herbal medicines. DL curves of the same sample measured at weekly intervals demonstrated small differences. Further investigation on the reproducibility



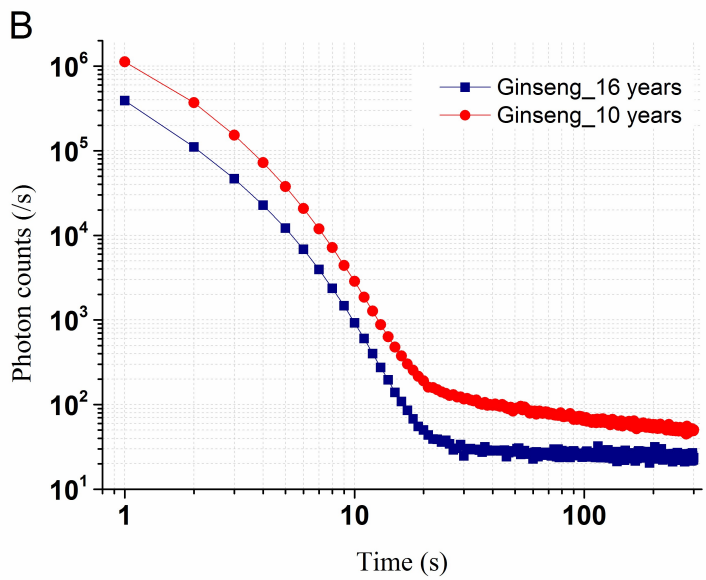


Fig. 10 Effects of growth location and different age on DL. (A) Shows two wild rhubarb samples grown in different location compared with two cultivated rhubarb samples grown in the same location and with the same age. (B) Shows two ginseng samples grown in the same location but different age.

of measurements is needed to elucidate effects of time of the day, season and weather.[37] However, compared with the differences that were measured between experimental conditions presented in the processing, location and age sections, the weekly variation was relatively small and thus seems not to be very relevant.

Luminescence properties are influenced by molecular interactions. The main reason for changes in emission may be due to the conformational changes of cell proteins. Such interactions may refer to radiation-less (resonance) transfer of energy from an excited molecule to another molecule. Therefore, DL (similar to fluorescence) depends on a whole series of factors that affects the luminescence properties of the aromatic residues.[38] Here hydrogen bonding as a specific interaction needs to be considered. Hydrogen bonding is represented widely between water and macromolecules such as proteins, RNA and DNA. In the literature it is reported that an increasing water content is accompanied with a decreasing DL intensity in collagen and lanthanide samples.[32–34,39] These data correspond to the experimental results described for *Aconitum* samples. Moreover, the changes of carbohydrates (cellulose) may also affect the molecular structure, thereby influence the DL signatures in herb.

It is well known that the excited energy pattern formed by light exposure depends on the molecular absorption of the excitation energy.[38] The changes of chemical composition may change the internal structure of herbal medicines, thus may change the molecular interaction and absorption and storage capacities of the excitation energy leading to different DL dynamics. Chinese herbal medicines of different ages and from different growth locations as well as subjected to different processing procedures may differ in chemical composition. For instance, the contents of major toxic compounds (i.e. aconitine, mesaconitine and hypaconitine) are decreased significantly after processing.[40] Ginsenosides contents are shifted by the different ages of the ginseng.[41] With the changes in environment factors, the contents of anthraquinone derivatives are also changed on rhubarb.[42] Importantly, such chemical

compounds may directly influence the pharmacological effects of those herbal medicines, and hence the therapeutic properties.[43–45] For instance, ginseng roots demonstrate growth age-dependent therapeutic effects on diabetic rats, which may be the result of the variation in both the concentrations and ratios of ginsenosides in ginseng roots of different growth ages.[43] The dynamics of DL may not only be an indicator of the changes of herbal age etc. but also may relate to the therapeutic effects of those bioactive compounds. Therefore, if a correlation between DL parameters and active compound contents could be established, DL might become a candidate technology for herbal quality control.

At present, the chemical changes of herbal medicines are typically measured by tools such as liquid chromatography and gas chromatography.[40,43,46–49] Chemical analysis is the method of choice to estimate the content of specific substances (either as medical or toxic substance) of herbal materials. However, chemical analysis requires isolation and extraction procedures, which may lead to losses of substances and incomplete identifications. In addition, existing chemical analysis platforms are not able to evaluate the entire integral profile of chemical compounds. Moreover, complex structural changes of chemical molecules are not reflected by chemical analysis. While DL measurement does not require isolation and extraction procedures, and DL can reflect the overall properties of a biological system.[50] Therefore, combing DL and chemical analysis may provide new insight for herbal quality control in the future.

5. Conclusion

The data represented in this article show that DL characteristics from Chinese herbal medicines can be influenced by the excitation time, herbal particle size and herbal water content. The established DL measurement protocol is both stable and reproducible and demonstrates the sensitive response of several herbs under the different conditions. We suggest that the combination of DL and chemical analysis may point to a possible new herbal quality control method in the future.

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Chapter 5

Effects of growth altitude on chemical constituents and delayed luminescence properties in medicinal rhubarb

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Abstract

To improve the quality control of herbal drugs, there has been a major shift from evaluating individual chemicals to evaluating multiple-constituent chemicals, given the multi-pharmacology nature of herbal drugs. Therefore, rapid, systematic assays are needed in order to assess the quality of medicinal herbs using a comprehensive, integrated approach. Light-induced delayed luminescence (DL) is used to measure decaying long-term ultra-weak photon emissions following excitation with light. DL is considered to be a sensitive indicator for characterizing the properties of biological systems and herbal medicines with various therapeutic properties. The aim of this study was to examine the feasibility of using DL as a novel quality-assessment tool using rhubarb material as a model system, and to establish the correlation between DL parameters and the chemical constituents of rhubarb. Raw roots and rhizomes were collected from rhubarb (*Rheum palmatum* L.) at various elevations in western China. HPLC analysis was used to identify fourteen bioactive constituents. Five DL parameters were calculated from the DL decay curves of the rhubarb samples. Statistical tools, including principal component analysis, were used to classify the rhubarb samples using data obtained using two different assays. Finally, Spearman's correlation coefficient was calculated to quantify the correlation between the bioactive compounds and corresponding DL parameters. We found that both the chemical analysis and DL measurements reflect variations in the quality of rhubarb due to environment factor. The DL parameters were correlated significantly with the bioactive chemical constituents. Our results indicate that DL is a promising tool for evaluating multiple constituents and for assessing the therapeutic properties of herbal medicines. Thus, DL may be used as part of a comprehensive system for assessing the quality and/or therapeutic properties of herbal medicines.

Key words: rhubarb, delayed luminescence, quality control, active constituents, environmental factor.

1. Introduction

Environmental factors related to growth conditions are closely associated with the quality and therefore therapeutic properties of medicinal plants.[1,2] Since ancient times in China, medicinal plants have been produced and/or collected in specific geographic regions with unique ecological conditions; these so-called "indigenous medicinal materials" are used to represent the optimal quality and therapeutic properties of Chinese herbal drugs based on clinical practices and experiences.[1] It is generally accepted that secondary metabolites are important pharmacologically active constituents in medicinal plants.[3] Plants produce secondary metabolites as a means to adapt to their growth conditions, for example to provide protection from environmental stressors.[2] In addition, changes in environmental factors can be reflected in the accumulation of secondary metabolites.[4] Therefore, focus should be shifted towards controlling environmental conditions in order to manage quality and standardize herbal drugs.[5]

Rhubarb (scientific name: Rhei Rhizoma) is an herbal drug that has been widely used for thousands of years throughout China.[6,7] The dried roots and rhizomes of *Rheum palmatum* L., *Rheum tanguticum* Maxim. ex Balf., and *Rheum officinale* Bail. are officially included in the Chinese Pharmacopoeia[8] and European community monograph.[9] According to the principles of traditional Chinese medicine, rhubarb is a typical herbal drug with many therapeutic properties, including catharsis, heat-clearing effects, detoxification, and removal of blood stasis.[8] In recent decades, chemical and pharmacological studies have shown that the pharmacological activities of secondary metabolites in rhubarb—including anthraquinone derivatives and polyphenol constituents—correspond with the traditional therapeutic functions associated with rhubarb (Fig 1).[10–14] For example, rhubarb's detoxification property is reflected largely by its antibacterial activity, which is related primarily to free anthraquinones such as rhein, emodin, and aloe-emodin.[10] Rhubarb's cathartic property is due to the presence of both anthraquinone glycosides and

sennosides.[10,11,13] Rhubarb's blood stasis–relieving properties have been attributed to (+)-catechin.[14] In addition, gallic acid has been proposed to have antiplatelet activity,[15] which may also explain rhubarb's blood stasis–relieving properties. Moreover, additional pharmacological effects have been attributed to compounds isolated from rhubarb, including anti-cancer activity,[16] anti-inflammatory activity,[17] antioxidant activity,[18] liver-protective properties,[19] and improved renal dysfunction.[20]

Given these important therapeutic properties, rhubarb has become one of most commonly used herbal medicines, and the demand for rhubarb has grown both in China and in global markets.[11] However, rhubarb is produced primarily in a limited region in western China at an altitude of 1000-5000 m,[21] and overharvesting has caused a significant decline in rhubarb crops; in addition, overharvesting also causes considerable damage to the growing environment, causing variation in the quality of rhubarb grown.[11] Therefore, developing an effective tool for measuring quality control in rhubarb is very important. Given the high number of bioactive constituents in rhubarb and their high number of therapeutic properties, comprehensive, systematic methods for analyzing rhubarb quality are needed.[11]

Delayed luminescence (DL) is long-term decaying, weak photon emission from various materials following exposure to light with a wavelength of 400–800 nm.[22–25] DL has been used as a tool for directly and rapidly assessing biological systems and has been found to provide a sensitive indicator of food quality.[24–26] Recent studies examined the DL signatures of dry powders prepared from Chinese herbal medicines.[27,28] These studies demonstrate that distinct DL properties can be measured between the same herbal medicines prepared under different conditions, including the age of the herb, environmental factors, and the processing method.[27] These differences in DL properties reflect variations in both the bioactive constituents contained in the herb and the therapeutic properties of herbal drugs.[29–32]

Importantly, DL can be used to predict the herb's energetically therapeutic properties

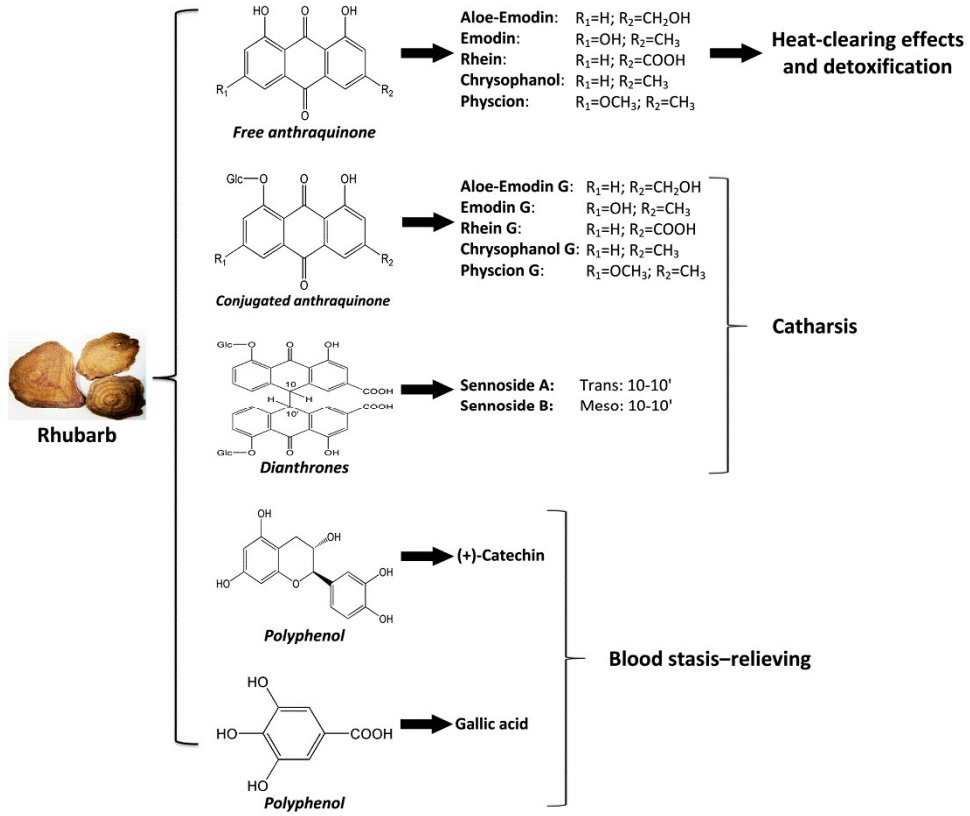

Fig. 1 Schematic diagram of the chemical constituents in rhubarb and their role in traditional Chinese medicine-based concepts. Fourteen chemical constituents in rhubarb correspond to various therapeutic properties in the principle of traditional Chinese medicine. The terms in italics under the chemical structure indicate the structural characteristics of the chemical constituents. "Glc" in the chemical structure indicates a glycoside. "G" in the names of chemical constituents indicate glycosides. Conjugated anthraquinone represents anthraquinone glycosides with O-glycosides, where the aglycone moiety is an 8-dihydroxyanthraquinone derivative. Additional structures of conjugated anthraquinones not shown here are published elsewhere.[11]

(i.e., "Cold" or "Heat") based on the principles of traditional Chinese medicine.[28] Thus, DL may contribute to our understanding of why herbs with different chemical constituents can have the same therapeutic properties and—conversely—why herbs with different therapeutic properties can contain similar chemical constituents.[28] Importantly, DL may provide an integrated, comprehensive picture of the bioactive constituents, thereby reflecting the herbal medicine's therapeutic properties.

Here, we performed both chemical analyses and DL measurements in order to evaluate the effect of the growth environment on the constituents contained in rhubarb (*Rheum palmatum* L.) The aim of this study was to determine whether DL parameters can be used to create a signature of rhubarb quality, and to determine whether these parameters correspond to key chemical constituents. Our results show a clear correlation between DL and the chemical constituents, reflecting variations in the quality of rhubarb, thus providing new insights into measuring quality control in herbal medicines.

2. Materials and Methods

2.1 Rhubarb materials and chemicals

Rhubarb (*Rheum palmatum* L.) samples were collected from twelve locations in western China (Table 1) during the summer months (from July through August) to minimize the effect of seasonal changes on the concentrations of chemical constituents.[33] All rhubarb samples were verified by Prof. Chunsheng Liu (Beijing University of Chinese Medicine) and deposited at the Beijing Institute of Chinese Medicine.

Seven reference compounds (aloe-emodin, rhein, emodin, chrysophanol, physcion, gallic acid, and (+)-catechin) were supplied by the National Institutes for Food and Drug Control (Beijing, China). Two additional reference compounds (sennoside A and sennoside B) were purchased from Nature Standard Biotechnology Co., Ltd. (Shanghai, China). The purity of all reference compounds was >98%.

2.2 High-performance liquid chromatography (HPLC)

2.2.1 Specimen preparation for determination of bioactive constituents

Extraction of total anthraquinones: Total anthraquinones contain five constituents (aloe-emodin, rhein, emodin, chrysophanol, and physcion). Powdered rhubarb samples (250 mg each) were extracted with 25 ml of 15% sulfuric acid (v/v) and

Table 1. Summary of the 12 sampling sites in China, including the number of batches collected at each site

Sample ID	Location	Batch	Longitude	Latitude	Altitude (m)
SXCJW	Caojiawan, Shanxi	3	E106°38′45.02″	N35°51′14.43″	1441
GSLX	Lixian, Gansu	3	E104°52′52.12″	N33°57′16.91″	2136
GSTC	Tanchang, Gansu	5	E104°43′50.33″	N33°57′17.50″	2331
NXLD	Longde, Ningxia	3	E106°07′01.69″	N35°33′28.71″	2358
GSMX	Minxian, Gansu	5	E104°06′54.69″	N34°23′01.86″	2530
SCLH	Luhuo, Sichuan	5	E100°39′56.91″	N31°23′54.51″	3166
GSDB	Deibu, Gansu	3	E103°39′40.62″	N34°05′41.26″	3227
SCLD	Luding, Sichuan	5	E102°02′14.81″	N29°51′50.07″	3590
SCDB	Danba, Sichuan	4	E101°32′55.86″	N30°51′35.83″	3636
SCKD	Kangding, Sichuan	3	E101°45′40.80″	N30°13′36.41″	4077
SCDF	Daofu, Sichuan	4	E101°22′01.14″	N30°41′16.71″	4094
SCLT	Litang, Sichuan	4	E100°15′09.17″	N30°13′35.91″	4265

methanol at a ratio of 2:3 (v:v) using a model KQ5200D ultrasonication device (200 W, 40 HZ; Kunshan Ultrasonic Instruments Co., Ltd., Kunshan City, China) for one hour. The resulting solution was extracted four times with 10 ml chloroform, and the combined extract was then dried by evaporation. The residue was dissolved in methanol and transferred to a 25-ml volumetric flask. The solution was then filtered through a 0.22-μm membrane and analyzed by HPLC.

Extraction of free anthraquinones:[34] The five aforementioned constituents were also extracted as free anthraquinones. Powdered rhubarb samples (250 mg each) were extracted in 60% methanol (v/v) using ultrasonication for one hour. The extracted solution was prepared by the method of weight relief, in which we compensated for any weight lost during the extraction procedure. The weight lost during the extraction procedure was replaced with 60% methanol (v/v). The solution was then filtered through a 0.22-μm membrane and analyzed by HPLC. This extraction method was also used to isolate gallic acid, (+)-catechin, sennoside A, and sennoside B using the same powdered rhubarb.[34]

2.2.2 HPLC analysis

HPLC analysis was performed using an Agilent 1100 system (Agilent Technologies, Palo Alto, CA) equipped with a micro-vacuum degasser, quaternary pump, auto-sampler, thermostatted column compartment, and diode array detector, which was connected to an Agilent ChemStation. Chromatographic analysis was conducted using an Agilent Zorbax SB-C18 column (4.6 mm × 250 mm, particle size: 5 μm; Agilent Technologies) maintained at 40°C. The detection wavelength was 280 nm. The mobile phase consisted of 0.05% (v/v) aqueous phosphoric acid (A) and acetonitrile (B) with a gradient program of 3%–11% (B) at 0–10 min, 11%–15% (B) at 10–30 min, 15%–17% (B) at 30–45 min, 17%–22% (B) at 45–60 min, 22%–36% (B) at 60–75 min, 36%–60% (B) at 75–90 min, 60% (B) at 90–105 min, and 60%–3% (B) at 105–110 min. The injected volume was 10 μl, and the standard solution containing nine bioactive reference compounds were prepared in methanol. The method used to analyze the aforementioned chemical constituents is well-established and validated,[34] showing reasonable reproducibility and repeatability for each chemical constituent.

2.3 Delayed luminescence measurements

2.3.1 Sample preparation

Rhubarb samples were prepared by crushing using a model QE-100 grinder (Yili Company, Zhejiang Province, China), and 150-μm particles were selected using a standard sieve (Tongrentang Company, Beijing, China). Thereafter, the samples were stored in a dark box containing 3-5-mm silica gel (Boom BV, Meppel, the Netherlands) at room temperature for ≥16 hours before DL measurements were performed.[27]

2.3.2 DL measurement

DL was measured using a previously established protocol for herbal drugs.[27] The device for measuring DL (Meluna Research, the Netherlands) included a dark sample chamber with a vertically positioned photomultiplier tube (PMT) (model

9558QB; Electron Tubes Enterprises Ltd., Ruislip, UK). The sample chamber was kept at 22°C. The cathode end of the PMT has a diameter of 51 mm and is sensitive at 160-870 nm. The PMT was cooled to -25°C in order to reduce the dark count rate to 10 counts per second. The photon emission signal was amplified using a model 9301 fast preamplifier (ORTEC, Oak Ridge, TN). A personal computer containing a model 6602 counting card (National Instruments, Austin, TX) was used for data acquisition. Each batch of rhubarb was used to prepare five 1-g samples. Each 1-g sample was placed in a Petri dish and illuminated for 10 seconds using a model 284-2812 white halogen excitation source (Philips, Germany). The DL of each sample was measured three consecutive times. The total number obtained from all fifteen measurements in each batch was used to analyze the DL parameters of that particular rhubarb batch. DL kinetics were obtained by recording the number of counts in consecutive 0.05-second periods for a total of 30 seconds, resulting in a total of 600 data points.

2.4 Data processing and statistical analysis

2.4.1 Statistics of fourteen bioactive compounds

The semi-quantitative contents of the five anthraquinone glycosides—aloe-emodin glycosides, rhein glycosides, emodin glycosides, chrysophanol glycosides, and physcion glycosides—were calculated using the difference between the total anthraquinone and free anthraquinone contents measured using HPLC.[10] The calculated five anthraquinone glycosides and nine constituents identified using HPLC were investigated using statistical tools. Hence, the contents of fourteen compounds in each rhubarb sample were analyzed. Principal component analysis (PCA) was used to discriminate among the fourteen compounds in rhubarb using the tools provided in the MetaboAnalyst software package (http://www.metaboanalyst.ca).[35] Thereafter, the rhubarb samples were stratified into two groups based on their growing altitude (i.e., above or below 3000 m). Partial least square discriminant analysis (PLS-DA) was used to further investigate the

differences in the chemical compositions of rhubarb (http://www.metaboanalyst.ca). The PLS-DA model was validated using cross-validation in order to avoid overfitting of the model.[36] Variable Importance in the Projection (VIP) scores based on the PLS-DA analysis were used to indicate the compounds that significantly contributed to group separation.[37] Subsequently, a two-tailed, unpaired Student's *t*-test was performed (SPSS version 23.0; IBM, Armonk, NY) to compare the two altitude groups using the contents of each constituent; differences were considered significant at $p<0.05$.

2.4.2 Statistics of five DL parameters

The photon counts measured during the first 30 seconds of each decay curve were used to calculate the parameters of the following double-exponential decay function:[27]

$$y = y0 + A1e^{-\frac{x}{t1}} + A2e^{-\frac{x}{t2}}$$

where t1 and t2 are time constants for the exponential decays, A1 and A2 are the amplitudes of the exponential decay components, and y0 is the final value of photon emissions in the DL decay curve. Curve-fitting was performed using ExpDecay 2 in Origin version 9.0 (OriginLab Corporation, Northampton, MA). The parameters of the fifteen batch measurements were averaged and used to represent the DL properties of each rhubarb batch. PCA was used to indicate the level of discrimination between DL parameters using tools provided in the MetaboAnalyst software package (http://www.metaboanalyst.ca). A two-tailed, unpaired Student's *t*-test was used (SPSS version 23.0) to compare the DL parameters between the two altitude groups; differences were considered significant at $p<0.05$.

2.4.3 Correlation between compounds and DL parameters

Spearman's rank correlation (ρ) was used to quantify the correlation between the bioactive constituents and DL parameters (SPSS version 23.0). Moderately strong,

significant correlations were defined as Spearman's $|\rho|$ >0.35 and p<0.01, respectively.[38,39] Thereafter, Cytoscape version 3.2.1 (www.cytoscape.org) was used to draw a network view, which was used visualize these correlations.[40]

3. Results

To evaluate the effects of growth environment on the quality of rhubarb, chemical analyses and DL measurements were conducted using 47 batches of rhubarb collected at various altitudes. The concentrations of 14 chemical constituents were quantified in each batch of rhubarb using HPLC. Unsupervised PCA was applied to the chemical data in order to visualize the variations between the different batches of rhubarb. Fig 2A displays the PCA results in the form of a score plot. The results show that the variance in chemical components of rhubarb due to the first two principal components (PC1 and PC2) accounted for 34% and 18.2%, respectively, of the total variance. The results also show two clusters based on the growth altitude, with a threshold of 3000 meters. Given the significant clusters obtained using PCA, a supervised clustering approach (PLS-DA; Fig 2B) was performed to optimally separate and identify the constituents that contributed significantly to the separation. PLS-DA evaluation with cross-validation revealed predictive accuracy of 0.90 and goodness-of-fit (R^2) of 0.80. Six chemical constituents with a VIP score >1 were found to contribute to the classification; three of these constituents (gallic acid, emodin, and rhein) had a VIP score >1.5 (S1 Table). The results obtained from the PLS-DA analysis revealed a similar classification effect as the results obtained from the PCA analysis. This is likely because the most variation in response to altitude were well represented by the first two principal components of the PCA. In addition, the PCA and PLS-DA analyses contained the same misclassified batches of rhubarb. Specifically, three batches (GSLX1, NXLD2, and NXLD3) in the <3000-m group were misclassified into the >3000-m group, and one batch (SCLD5) in the >3000-m group was misclassified into the <3000-m group. Next, the estimated differences in chemical data between the two altitude groups were analyzed using a two-tailed,

unpaired Student's *t*-test. The analysis revealed that the contents of nine constituents in the >3000-m group were significantly higher than in the <3000-m group ($p<0.05$); these results are shown in Fig 3.

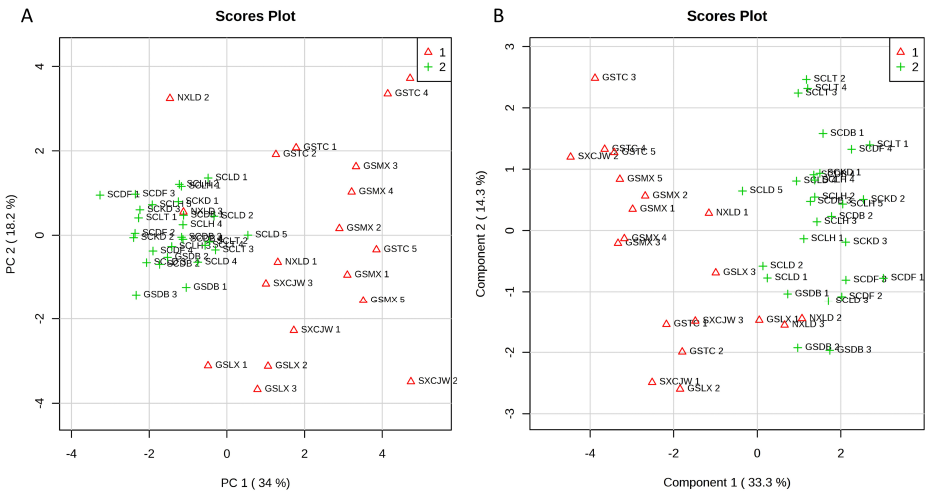

Fig 2. PCA and PLS-DA scores obtained from the chemical data. (A) PCA and (B) PLS-DA score plots of the chemical data obtained from all rhubarb batches, showing two general clusters separated by growth altitude (3000m). The individual samples were marked with a "Δ" (<3000 m) or a "+" (>3000 m) symbol, together with the sample ID number.

Next, to calculate the parameters of the rhubarb DL curves, a double-exponential decay function was used to fit the observed decay curves; an example of such a fit is illustrated in Fig 4. To display the differences in DL parameters using an unsupervised approach, PCA was used to obtain a focused view of the variance in the five DL parameters. Fig 5 shows a score plot of PCA using DL parameters with PC1 and PC2 values of 60.1% 32.9%, respectively. The reasonable group clusters in the PCA plot also indicates the differences in DL parameters between the two groups, with a threshold altitude of 3000 m. However, the misclassified rhubarb batches based on the DL parameters differed from the misclassified batches based on the PCA analysis using chemical data. Specifically, DL parameters of three rhubarb batches from location SXCJW were misclassified into the >3000-m group, whereas

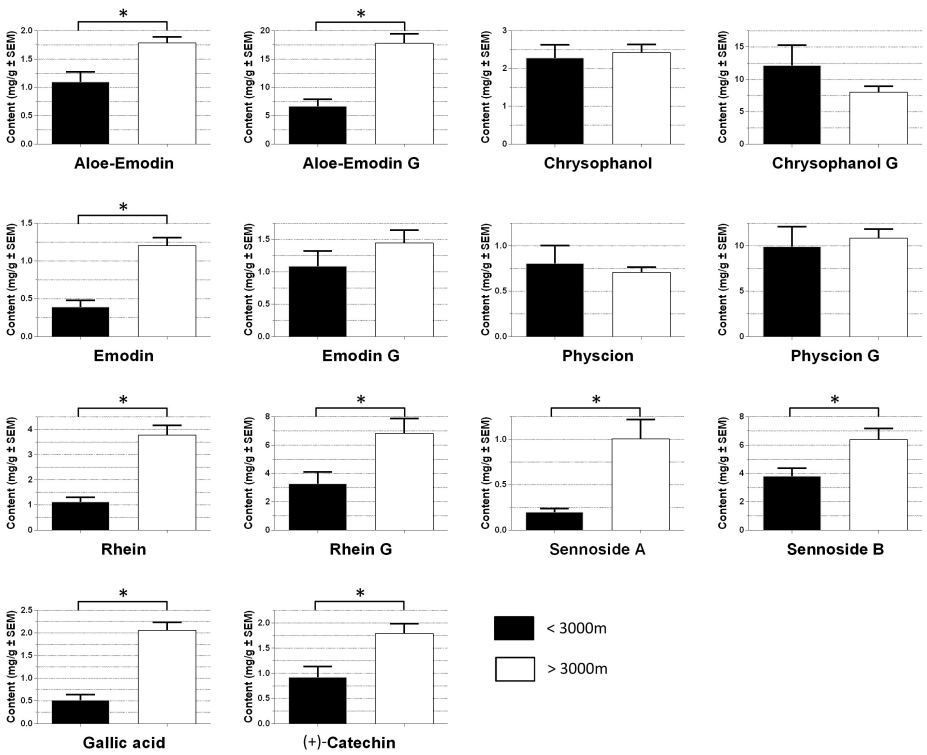

Fig. 3 Histograms comparing the contents of chemical constituents between rhubarb samples in two altitude groups. *, $p<0.05$ (two-tailed, unpaired Student's t-test). "G" in the names of chemical constituents indicate glycosides.

the other three batches (SCLH1, SCKD1, and GSDB3) were misclassified into the <3000-m group. To visualize the differences in DL kinetics caused by altitude, 20 consecutive data points were accumulated and are expressed as photon emissions per second.[27] Fig 6 illustrates the different decay curves between the two altitude groups following excitation. To analyze further the difference between DL parameters, a two-tailed, unpaired Student's *t*-test was used to compare the five DL parameters between the two altitude groups. The analysis revealed that four parameters differed significantly between the two groups; only one parameter (t2) did not differ significantly (p=0.42). On average, the DL parameters in the <3000-m group were larger than in the >3000-m group (Fig 7, S2 Table).

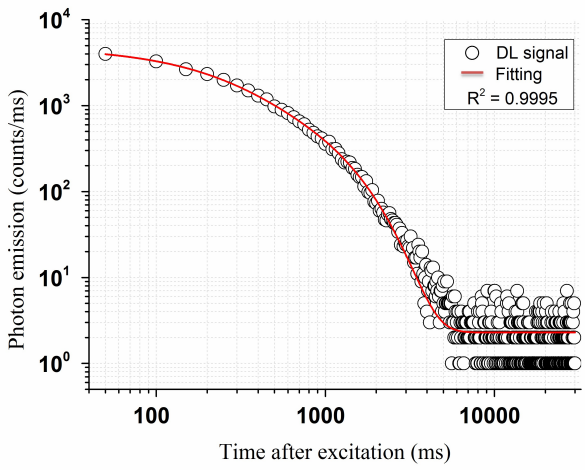

Fig. 4 Fitting effect of the DL decay curve of a rhubarb sample (ID: GSTC5). The sample was excited with light, after which photon emissions were counted. The red line shows a double-exponential fit of the data. Note that the data are plotted on a log-log scale.

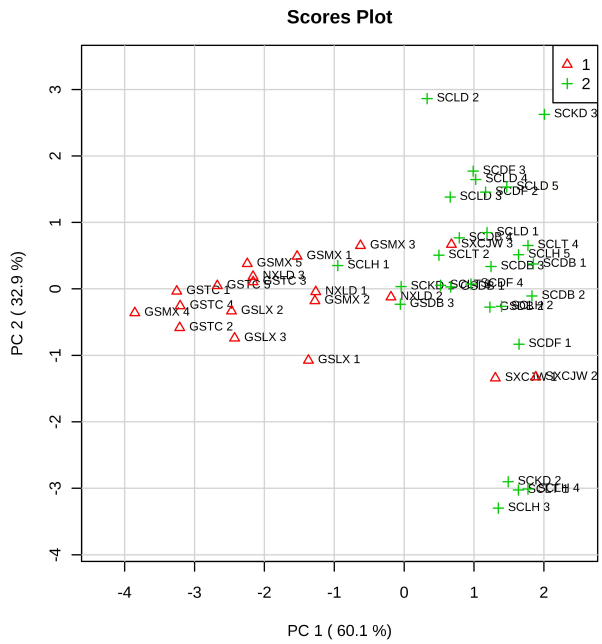

Fig. 5 PCA scores of the DL parameters measured for the individual rhubarb batches. The PCA score plot of DL parameters shows two general clusters. The individual samples are marked with a "Δ" (<3000 m) or a "+" (>3000 m) symbol, together with the sample ID number.

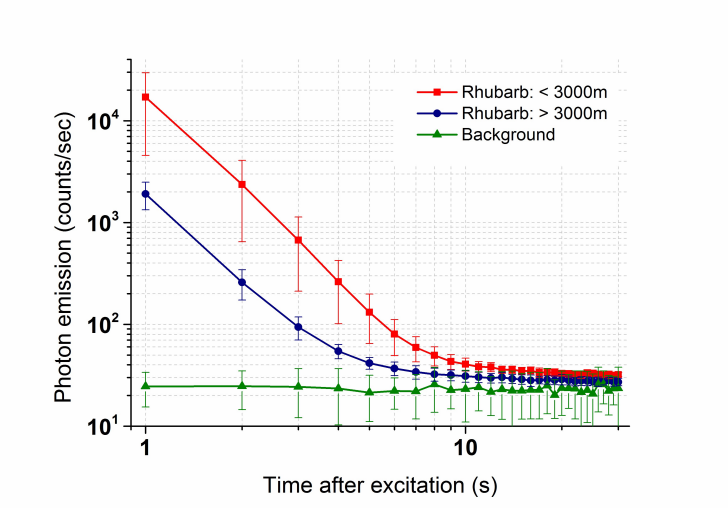

Fig. 6 DL decay curves obtained from rhubarb samples collected at an altitude <3000 m and >3000 m. The sample was excited with light, after which photon emissions were counted. Background emission were stable throughout the experiments. Note that these data are plotted on a log-log scale.

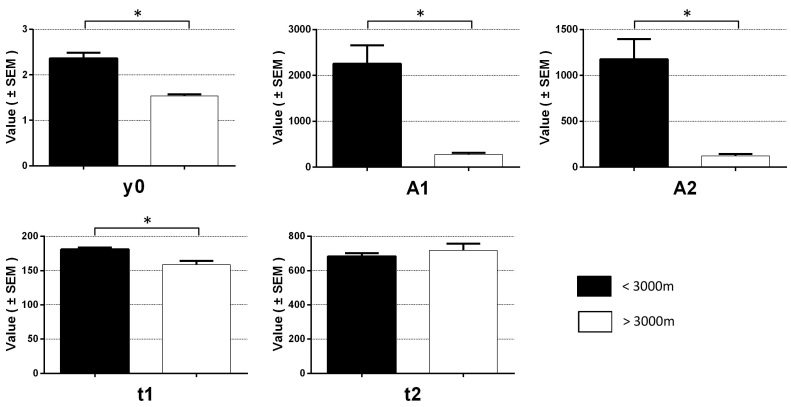

Fig. 7 Histograms comparing the DL parameters obtained between the two different altitude groups. *, $p<0.05$ (two-tailed, unpaired Student's t-test).

Both the chemical analysis and the DL measurements successfully identified differences in rhubarb based on altitude. Next, we determined the correlations between the chemical constituents and DL parameters for all rhubarb batches using the Spearman's correlation method. We found moderately strong and significant correlations between DL parameters and constituents (Table 2). These correlations

are depicted visually in Fig 8. Interestingly, the compounds that differed significantly due to altitude generally had a negative correlation with the significantly different DL parameters; in particular, gallic acid, emodin, and rhein—the three compounds with the highest VIP scores—had stronger correlations with the DL parameters related related to photon counts (i.e., y0, A1, and A2). On the other hand, the DL parameter that was not significantly different (t2, which represents the decay time of the DL curve) had a relatively stronger positive correlation with sennoside A.

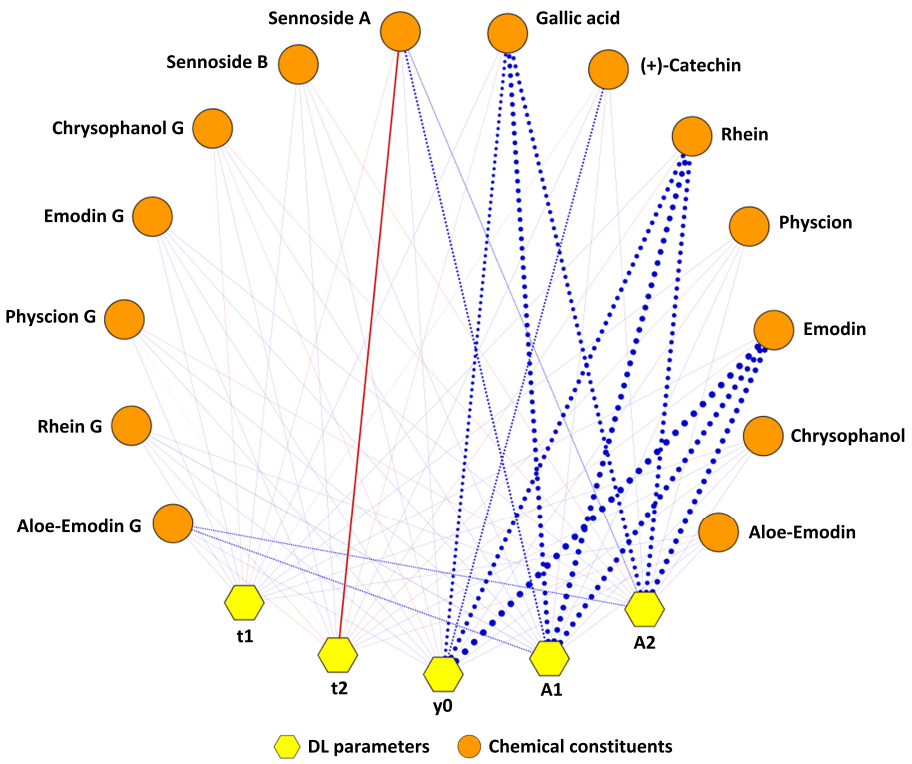

Fig. 8 Correlation network between the 14 chemical constituents and 5 DL parameters measured in the rhubarb samples. Visualization of data concentrated on the correlations between chemical constituents in relation to DL parameters. The negative correlations are indicated with blue lines, and positive correlations are indicated with red lines; thicker lines indicate a higher correlation. The length of each line has no meaning. “G” in the names of chemical constituents indicate glycosides.

Table 2. Moderately strong and significant correlations between DL parameters and chemical constituents

Chemical constituent	DL Parameter			
	y0	A1	A2	t2
Rhein	ρ=-0.57 p<0.01	ρ=-0.63 p<0.01	ρ=-0.66 p<0.01	
Emodin	ρ=-0.67 p<0.01	ρ=-0.61 p<0.01	ρ=-0.62 p<0.01	
Gallic acid	ρ=-0.51 p<0.01	ρ=-0.58 p<0.01	ρ=-0.55 p<0.01	
(+)-Catechin	ρ=-0.43 p<0.01			
Aloe-Emodin G		ρ=-0.41 p<0.01	ρ=-0.40 p<0.01	
Sennoside A		ρ=-0.44 p<0.01	ρ=-0.39 p<0.01	ρ=0.41 p<0.01

4. Discussion

Our preliminary analysis indicates that altitude is an important environmental factor, significantly affecting both the chemical composition of rhubarb and its DL parameters. Specifically, the concentrations of nine chemical constituents in rhubarb were significantly higher in rhubarb samples collected at an altitude higher than 3000 m. The increased contents of two anthraquinone derivatives (aloe-emodin glycoside and rhein glycoside) in rhubarb grown at high altitude are consistent with results reported by Wang et al..[30] It has been reported that altitude is able to affect the contents of anthraquinone glycosides in all three medicinal rhubarb species (*Rheum palmatum* L., *Rheum tanguticum* Maxim. ex Balf., and *Rheum officinale* Bail.) included in the Chinese Pharmacopoeia[30], therefore we may hypothesis that altitude is able to influence the other chemical constituents in these three rhubarb species. Since different species of rhubarb may have variations in their chemical constituents.[41] Therefore, in our present study only *Rheum palmatum* L. is used. Our analysis of rhubarb DL curves revealed that four of the five DL parameters decreased in value at the higher altitude. Moreover, the misclassified batches of rhubarb based

on the PCA analysis differed between the chemical data and DL data. This indicates that DL may be correlated with unknown mechanisms that may not be characterized completely by chemical analysis. In addition, our data suggest that chemical constituents in rhubarb are correlated with DL parameters. The most significant negative correlations were found between DL intensity parameters and gallic acid, rhein, and emodin, the three constituents with the highest contributions in the PLS-DA analysis (S1 Table, Fig 8). One significant positive correlation was also identified; this correlation was between the DL parameter t2 and the compound sennoside A. Significant correlations between chemical constituents and DL properties indicate that specific DL parameters are correlated with the contents of bioactive constituents and that these parameters may be correlated with specific therapeutic properties of rhubarb.

In our study, we found that the contents of anthraquinone derivatives and polyphenols in rhubarb are affected by altitude. Variations among such chemical constituents have also been reported in other plants that are affected by altitude and altitude-related environmental factors. Altitude is believed to be an important environmental factor, as altitude is related both comprehensively and closely to several environmental features, including the intensity of solar radiation and fluctuations in ambient temperature.[42,43] In turn, these environmental factors cause the accumulation of phenolic compounds in plants that grow at the timberline.[44,45] As altitude increases, temperature gradually decreases, whereas light intensity increases.[4] This increasing light intensity increases the contents of free anthraquinones and sennosides in *Rhamnus purshiana* and *Cassia angustifolia* Vahl., respectively.[46,47] Additionally, an increase in the concentration of anthraquinone glycosides with increasing altitude (and the resulting decrease in temperature) has also been demonstrated in *Rumex dentatus* L. and *Lavandula officinalis* L..[43] Moreover, many other pharmaceutically active secondary metabolites in medicinal plants—including quercitrin, total flavonoid, total hypericin, terpenes, and

antioxidant compositions—are positively correlated with altitude.[4,43,48,49] Importantly, variations in the concentration and/or ratio of specific chemical constituents can directly affect the therapeutic effects of herbal drugs;[29,50] thus, growing conditions and environmental factors are likely also closely correlated with the pharmacological activity of herbal drugs. For example, ethanol extracts of rhubarb (*Rheum tanguticum* Maxim. ex Balf.) grown at 4500 m are more potent at inhibiting the proliferation of adenocarcinoma cells than extracts of rhubarb grown at 3200 m.[51] Therefore, the idea of using indigenous medicinal materials is deeply rooted in traditional Chinese medicine. In ancient China, based on clinical observations, doctors believed that using only indigenous medicinal materials could achieve optimal medical results.[1] Although the notion of producing regions of indigenous medicinal materials (for example, growing specific medicinal in mountain areas, valleys, or in a specific province) was relatively simple in ancient times,[1] even today this notion indicates that growing conditions and environmental factors play an important role in the quality and therapeutic properties of medicinal herbs. Therefore, because environment factors can be closely correlated with the overall quality of the herbal drug, comprehensive quality control requires measuring both the chemical constitutes and the chemical bioactivities under specific growth conditions.[52]

DL is believed to reflect the overall properties of a biological system,[22,53] and differences in the physiological status of plants in response to environmental stressors have been detected by measuring DL in fresh leaves and twigs.[54,55] Interestingly, altitude-related variations in dried herbal materials can also be measured using DL. The underlying environmental factor could be the different growth rates of rhubarb depending on the growth altitude. Variation in plant growth rates are closely related to ambient temperature.[56,57] Since average temperatures differ at different altitudes[4], the growth rates of rhubarb will vary in the 1000 – 5000 meter range. As a consequence, this results in varying concentration of plant

metabolites but possibly also in polysaccharide / protein content i.e. carbon-to-nitrogen ratio.[58,59] Hence, the concentration of rhubarb chemical composition is related to altitude and at the same time it partially correlated to DL parameters suggesting DL might be a suitable tool for quality control. However, whether variations in DL parameters could be correlated to specific chemical constituents requires further research and validation.

The molecular absorption of the illuminating energy defines the dynamics of delayed luminescence, whereas changes in emission may be due to conformational changes in macromolecules in the cell, including proteins and nucleic acids.[60] These conformational changes can influence the interactions between molecules, thereby affecting the radiant (resonance) transfer of energy from one excited molecule to another.[60] On the other hand, secondary metabolites in herbal plants often interfere with protein conformation via highly reactive functional groups such as aldehydes and phenolic hydroxyls.[61,62] For example, polyphenols contain several hydroxyl groups and phenolic OH groups, which can form hydrogen bonds with amino acid residues in proteins.[61,62] Similarly, most anthraquinones contain phenolic OH groups and can also affect the conformation of proteins.[62] Accordingly, we conclude that amino acid residues in rhubarb contain more hydrogen bonds with increasing contents of anthraquinone derivatives and polyphenols, and the resulting changes in conformation and molecular interactions cause a change in DL dynamics. It may partially explain the correlation between DL parameters and the chemical constituents.

In our study, the relative high correlations have been found between DL properties and chemical constituents such as gallic acid, rhein, and emodin. This results suggest that DL parameters may be suitable for estimating both the quality and therapeutic properties of rhubarb, however, further validation is required. Rhein and emodin are two free anthraquinones that are commonly used to evaluate the quality of rhubarb in the Chinese Pharmacopoeia.[8] The significant correlations between DL parameters

and both rhein and emodin indicate that DL may serve as a tool for assessing the quality control of rhubarb. In addition, both rhein and emodin have heat-clearing and detoxifying properties, which are rooted in traditional Chinese medicine–based therapies,[10] as well as more general pharmacological properties, including anti-cancer and antioxidant activities.[63–73] In addition, gallic acid may have similar properties, inhibiting both human prostate carcinoma cells and human glioma cells, as well as scavenging free radicals.[74–76] Therefore, measuring DL parameters may reveal one or more specific therapeutic properties that are related to specific chemical constituents in rhubarb. If DL parameters can be comprehensively linked to both the chemical constituents and bioactivities of other Chinese herbs, these parameters may serve to explain why different Chinese medicinal herbs can have different bioactive constituents but can synergistically affect similar therapeutic targets and diseases.[77] Depending on the identified correlations between DL and both the chemical constituents and therapeutic properties of Chinese herbal medicines, DL may provide new opportunities to understand Chinese medicinal herb–based concepts based on biochemistry, thereby improving our understanding of healthcare.

5. Conclusions

Environmental stressors—particularly altitude—can have significant effects on the quality of many plants, including rhubarb. Here, we found that both chemical analyses and DL measurements revealed that the quality and composition of rhubarb are affected by altitude. Thus, DL parameters may represent a novel tool for assessing the quality of rhubarb. The identified potential correlations between DL parameters and chemical constituents suggest that DL may also be correlated to the bioactive properties of rhubarb. To date, approximately 200 constituents have been isolated from rhubarb species, including stilbenes, tannins, and anthocyanins.[11,78] Therefore, this proof-of-concept study should be repeated using a wider range of rhubarb samples and species, and the comprehensive network between secondary metabolites and DL parameters should be established. Additional studies for

validation of this methods are also required. Because of the multi-pharmacology nature of herbal drugs, there is currently a shift toward analyzing multiple constituents in herbal drugs.[79] Therefore, DL provides a rapid, direct, systematic tool for analyzing overall property, which may provide a novel means to measure quality control in medicinal herbs.

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Supplementary Table

S1 Table. The concentrations of each bioactive constituent measured in the rhubarb samples collected below 3000 m and above 3000 m

Chemical constituent	Altitude (m)			
	<3000	>3000	*P*–value [a]	VIP score [b]
Aloe-Emodin	1.10 ± 0.18	1.78 ± 0.11	**<0.05**	**1.09**
Rhein	1.11 ± 0.19	3.78 ± 0.39	**<0.05**	**1.55**
Emodin	0.39 ± 0.09	1.21 ± 0.10	**<0.05**	**1.58**
Chrysophanol	2.28 ± 0.34	2.43 ± 0.21	>0.05	0.36
Physcion	0.80 ± 0.20	0.71 ± 0.06	>0.05	0.49
Aloe-Emodin G	6.67 ± 1.25	17.80 ± 1.66	**<0.05**	**1.24**
Rhein G	3.28 ± 0.83	6.83 ± 1.05	**<0.05**	0.57
Emodin G	1.09 ± 0.24	1.45 ± 0.20	>0.05	0.33
Chrysophanol G	12.16 ± 3.10	8.00 ± 0.91	>0.05	0.17
Physcion G	9.90 ± 2.21	10.86 ± 1.00	>0.05	0.49
Sennoside A	0.20 ± 0.04	1.01 ± 0.21	**<0.05**	0.99
Sennoside B	3.80 ± 0.58	6.38 ± 0.80	**<0.05**	0.43
Gallic acid	0.52 ± 0.12	2.06 ± 0.18	**<0.05**	**1.7**
(+)-Catechin	0.92 ± 0.21	1.79 ± 0.19	**<0.05**	**1.12**

Values are presented in mg/g (mean ± SEM). [a] Two-tailed, unpaired Student's t-test. Significant *p*-values are shown in bold. [b] VIP score obtained by PLS-DA. A VIP score >1 (shown in bold) indicates a significant contribution to the classification. "G" in the names of chemical constituents indicate glycosides.

S2 Table. Values obtained for the five DL parameters measured in the rhubarb samples collected below 3000 m and above 3000 m

DL parameter	Altitude (m)		
	<3000	>3000	*P*-value [a]
y0	2.36 ± 0.13	1.54 ± 0.03	**<0.05**
A1	2254.64 ± 405.30	279.69 ± 34.81	**<0.05**
t1	180.95 ± 2.86	158.88 ± 5.20	**<0.05**
A2	1176.64 ± 218.37	124.95 ± 17.92	**<0.05**
t2	683.70 ± 18.39	718.49 ± 38.84	>0.05

Values are presented as the mean ± SEM. [a] Two-tailed, unpaired Student's t-test. Significant *p*-values are shown in bold

Chapter 6

Characterization of the therapeutic properties of Chinese herbal materials by measuring delayed luminescence and dendritic cell-based immunomodulatory response

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Abstract

Based on the traditional Chinese medicine theory, the Chinese pharmacopeia assigns a therapeutic description of "taste" to all herbs; thus, a herb's "taste" is valued in traditional Chinese medicine as a major ethnopharmacological category and reflects the herb's therapeutic properties. These properties guide the practitioner with respect to preparing a specific herbal formula in order to provide each patient with a personalized intervention. The key challenge in evidence-based medicine is to characterize herbal therapeutic properties from a multi-target, multi-dimensional systems pharmacology perspective. Here, we used delayed luminescence (DL, the slowly decaying emission of photons following excitation with light) as a rapid, direct, highly sensitive indicator to characterize the properties of herbal medicines. The DL parameters were able to reliably identify a specific category of herbal materials with the so-called "sweet" taste. To support the DL results and provide biological relevance to the DL results, we used a murine bone marrow-derived dendritic cell–based assay to examine the immunomodulatory effects of herbal extracts from various "taste" categories. Our results indicate that DL may serve as a robust and sensitive tool for evaluating the therapeutic properties of herbs based on the traditional Chinese medicine classification of "taste". Thus, DL provides a promising technological platform for investigating the properties of Chinese herbal medicines both qualitatively and quantitatively.

Key words: Chinese herbal medicine, therapeutic classification, delayed luminescence, immunity, dendritic cell

1. Introduction

Chinese herbal medicine has been used in China for thousands of years to maintain health and treat disease.[1] In this long history, practitioners of traditional Chinese medicine have accumulated a wealth of knowledge regarding herbal therapeutic effects based on clinical observations, resulting in the classification of herbal medicines into specific therapeutic categories.[1,2] Chinese herbal medicines are traditionally classified according to the sensations they evoke and the patient's response, resulting in descriptive characterizations such as taste, warm/cold, and toxic/non-toxic.[1–3] The taste category includes descriptors based on the perception in the mouth and include sweet, bitter, pungent, salty, and sour.[4] Our current understanding of the herbal descriptor "taste" developed from a long history of clinical experience and is linked to specific therapeutic properties in humans.[3,4] Hence, in traditional Chinese medicine, herbs are not necessarily classified according to their perception in the mouth, but rather according to their therapeutic properties in the human body.[4] Thus, the five taste descriptors have been used to classify the specific therapeutic properties and pharmacological actions of Chinese herbal medicines in clinical practice.[5] As a result, the taste descriptors of some herbal medicines currently described in the herbal materia medica can differ from how they actually taste in the mouth.[6]

Based on the classification of taste in traditional Chinese medicine, herbs with the same taste descriptor generally possess similar therapeutic properties, and herbs with different taste descriptors generally have different therapeutic properties.[6] For example, the so-called "sweet" class of herbs is associated with a tonic effect that can nourish the body, hence promoting a healthy status, boosting the immune system, and helping fight the aging process.[4,7,8] On the other hand, herbs in the so-called "pungent" class eliminate pathological agents and therefore treat the corresponding symptoms (e.g., stagnation) by promoting the circulation of energy and blood.[4]

Herbs in the so-called "bitter" class have heat-cleansing and detoxification effects and are used to treat constipation, inflammation, infection, and other conditions.[5,9]

Herbs that have a single taste descriptor generally have basic therapeutic properties. However, many herbs belong to two or more taste classes and therefore have a wider therapeutic range than herbs with a single taste descriptor.[5] These taste descriptor–based therapeutic classifications are often used to help the practitioner prepare specific herbal formulas in order to achieve personalized intervention.[1] Therefore, the taste descriptor is an important concept for understanding an herb's therapeutic effect and clinical application, and it is listed both in Chinese medicine textbooks and in the Chinese Pharmacopoeia.[10,11] Interestingly, traditional Indian medicine (i.e., Ayurveda) also uses taste descriptors to indicate the pharmacological activity of herbal medicine.[12,13] Thus, both Ayurveda and traditional Chinese medicine use a common system of taste descriptors. In addition, statistical analyses support the use of herbal taste descriptors for predicting the pharmacological activity of herbs.[12] However, evidence-based scientific data is still needed in order to understand the therapeutic properties of herbal medicines based on taste descriptors.

Delayed luminescence (DL) is the long-term decay of weak photon emissions from materials following exposure to light with a wavelength of 400–800 nm.[14] DL provides a holistic, integrated, comprehensive method for measuring materials and biological systems, and provides a direct, rapid, and sensitive indicator of a wide range of processes, including food quality, seed germination, and cancerous cells.[15–17] Recently, we used DL to study the features of dry powders prepared from Chinese herbal materials.[14,18] The results suggest that specific DL properties can be used to indicate differences in herbal materials prepared under different conditions, including the processing method and the age of the herb.[18] Importantly, DL can be used to detect differences in the overall signatures of a given herb grown under various environmental conditions, and distinct DL properties have been correlated to the specific bioactive constituents extracted from these herbal samples.[14] These

differences in DL properties indicate the presence of different bioactive constituents as a result of environmental factors.[14] Because these studies confirm that DL can reflect herbal characteristics at the systems level,[14] we hypothesized that DL may be used to increase our understanding of herbal therapeutic properties based on the taste descriptor.

Here, we measured DL in Chinese herbal materials with different taste descriptors. In addition, to support our finding of distinct clusters of sweet descriptors and other herbal taste descriptors based on our DL data, we used murine bone marrow-derived dendritic cells (DCs) in an assay to examine the immunomodulatory effect of herbal extracts from various taste descriptor categories. DCs are specialized leukocytes that play a key role in initiating the adaptive immune response,[19] and the production of cytokines such as TNFα and IL-6 by DCs has been used as an indicator of the immunomodulatory capacity of herbal medicines.[20] The results obtained with our DC-based immunomodulatory assay generally support the results of our DL experiments; therefore, DL may provide both qualitative and quantitative insights into the therapeutic properties of herbal medicines.

2. Materials and Methods

2.1 Herbal materials

A total of 90 herbal materials (roots and/or rhizomes), ginseng leaves, and ginseng flowers were purchased in five batches from TongRenTang Co., Ltd. (Beijing, China). These 90 herbal medicines are listed in Table 1 and are classified according to six taste descriptor groups—sweet, bitter, pungent, sweet & bitter, sweet & pungent, and bitter & pungent—in accordance with the 2015 Chinese Pharmacopoeia.[11] The identities of all herbal samples were verified by Dr. Wen-Te Chang (China Medical University) and were deposited at China Medical University (Taichung, Taiwan).

Table 1. List of the 90 herbal medicines used in this study, including the Latin names and taste classifications.

ID	Name (English)	Name (Latin)	Taste descriptor
S1*	ASTRAGALI RADIX	*Astragulus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao	Sweet
S2	HEDYSARI RADIX	*Hedysarum polybotrys* Hand. - Mazz	Sweet
S3	GLYCYRRHIZAE RADIX ET RHIZOMA	*Glycyrrhiza uralensis* Fisch.	Sweet
S4*	CODONOPSIS RADIX	*Codonopsis pilosula* (Franch.) Nannf.	Sweet
S5	POLYGONATI RHIZOMA	*Polygonatum kingianum* Coll. et Hemsl	Sweet
S6	DIOSCOREAE RHIZOMA	*Dioscorea opposita* Thunb.	Sweet
S7	GASTRODIAE RHIZOMA	*Gastrodia elata* Bl.	Sweet
S8	ADENOPHORAE RADIX	*Adenophora stricta* Miq.	Sweet
S9	STELLARIAE RADIX	*Stellaria dichotoma* L. var. *lanceolata* Bge.	Sweet
S10*	POLYGONATI ODORATI RHIZOMA	*Polygonatum odoratum* (Mill.) Druce	Sweet
S11	IMPERATAE RHIZOMA	*Imperata cylindrica* Beauv. var. *major* (Nees) C. E. Hubb.	Sweet
S12	REHMANNIAE RADIX	*Rehmannia glutinosa* Libosch.	Sweet
S13	PHRAGMITIS RHIZOMA	*Phragmites communis* Trin.	Sweet
S14	NOTOGINSENG RADIX ET RHIZOMA	*Panax notoginseng* (Burk.) F.H. Chen	Sweet
S15*	GINSENG RADIX ET RHIZOMA RUBRA	*Panax ginseng* C. A. Mey.	Sweet
S16	GINSENG RADIX ET RHIZOMA	*Panax ginseng* C. A. Mey.	Sweet
S17*	PSEUDOSTELLARIAE RADIX	*Pseudostellaria heterophylla* (Miq.) Pax ex Pax et Hoffm.	Sweet
S18	CYATHULAE RADIX	*Cyathula officinalis* Kuan	Sweet
S19	PANACIS QUINQUEFOLII RADIX	*Panax quinquefolium* L.	Sweet
S20	CHANGII RADIX	*Changium smyrnioides* Wolff	Sweet
S21*	OPHIOPOGONIS RADIX	*Ophiopogon japonicas* (L.f) Ker-Gawl.	Sweet
S22	TRICHOSANTHIS RADIX	*Trichosanthes kirilowii* Maxim.	Sweet
S23	GLEHNIAE RADIX	*Glehnia littoralis* Fr. Schmidtex Miq.	Sweet
P1	CURCULIGINIS RHIZOMA	*Curculigo orchioides* Gaertn.	Pungent
P2*	ANEMONES RADDEANAE RHIZOMA	*Anemone raddeana* Regel	Pungent
P3*	ALPINIAE OFFICINARUM RHIZOMA	*Alpinia officinarum* Hance	Pungent
P4	ZINGIBERIS RHIZOMA	*Zingiber officinale* Rosc.	Pungent
P5	ANGELICAE DAHURICAE RADIX	*Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook. f.	Pungent
P6*	CHUANXIONG RHIZOMA	*Ligusticum chuanxiong* Hort.	Pungent
P7	ASARI RADIX ET RHIZOMA	*Asarum heterotropoides* Fr. Schmidt var. *mandshuricum* (Maxim.) Kitag.	Pungent
P8	CYNANCHI PANICULATI RADIX ET RHIZOMA	*Cynanchum paniculatum* (Bge.) Kitag.	Pungent
P9*	LIGUSTICI RHIZOMA ET RADIX	*Ligusticum sinense* Oliv.	Pungent
P10	KAEMPFERIAE RHIZOMA	*Kaempferia galanga* L.	Pungent
P11*	LINDERAE RADIX	*Lindera aggregata* (Sims) Kosterm.	Pungent
P12	CYPERI RHIZOMA	*Cyperus rotundus* L.	Pungent
P13	CIMICIFUGAE RHIZOMA	*Cimicifuga foetida* L.	Pungent
B1	DRYNARIAE RHIZOMA	*Drynaria fortunei* (Kunze) J. Sm.	Bitter
B2	DIOSCOREAE SPONGIOSAE RHIZOMA	*Dioscorea spongiosa* J.Q.Xi,M.Mizuno et W.L.Zhao	Bitter
B3*	SALVIAE MILTIORRHIZAE RADIX ET RHIZOMA	*Salvia Miltiorrhiza* Bge.	Bitter
B4	PAEONIAE RADIX RUBRA	*Paeonia lactiflora* pall.	Bitter
B5	PARIDIS RHIZOMA	*Paris polyphylla* Smith var.*yunnanensis* (Franch.) Hand. -Mazz	Bitter
B6	AMPELOPSIS RADIX	*Ampelopsis japonica* (Thunb.) Makino	Bitter
B7	POLYGONI CUSPIDATI RHIZOMA ET RADIX	*Polygonum cuspidatum* Sieb. et Zucc.	Bitter
B8*	RHEI RADIX ET RHIZOMA	*Rheum palmatum* L.	Bitter
B9	PULSATILLAE RADIX	*Pulsatilla chinensis (Bge.) Regel*	Bitter

(continued on next page)

Table 1. *(continued)*

ID	Name (English)	Name (Latin)	Taste descriptor
B10	KNOXIAE RADIX	*Knoxia valerianoides* Thorel et Pitard	Bitter
B11	SOPHORAE FLAVESCENTIS RADIX	*Sophora flavescens* Ait.	Bitter
B12	ISATIDIS RADIX	*Isatis indigotica* Fort.	Bitter
B13*	PICRORHIZAE RHIZOMA	*Picrorhiza scrophulariiflora* Pennell	Bitter
B14	RUBIAE RADIX ET RHIZOMA	*Rubia cordifolia* L.	Bitter
B15	BELAMCANDAE RHIZOMA	*Belamcanda chinensis* (L.) DC.	Bitter
B16*	SCUTELLARIAE RADIX	*Scutellaria baicalensis* Georgi	Bitter
B17*	COPTIDIS RHIZOMA	*Coptis chinensis* Franch.	Bitter
B18	PHYTOLACCAE RADIX	*Phytolacca acinosa* Roxb.	Bitter
B19	STEPHANIAE TETRANDRAE RADIX	*Stephania Tetrandra* S. Moore	Bitter
B20	PHAPONTICI RADIX	*Rhaponticum uniflorum (L.) DC.*	Bitter
BP1	ACONITI RADIX	*Aconitum carmichaelii* Debx.	Bitter & Pungent
BP2	ACONITI KUSNEZOFFII RADIX COCTA	*Aconitum kusnezoffii* Reichb.	Bitter & Pungent
BP3	AUCKLANDIAE RADIX	*Aucklandia lappa* Decne.	Bitter & Pungent
BP4	NOTOPTERYGII RHIZOMA ET RADIX	*Notopterygium incisum* Ting ex H.T. Chang	Bitter & Pungent
BP5	CURCUMAE LONGAE RHIZOMA	*Curcuma Longa* L.	Bitter & Pungent
BP6	ASTERIS RADIX ET RHIZOMA	*Aster tataricus* L.f.	Bitter & Pungent
BP7	ATRACTYLODIS RHIZOMA	*Atractylodes lancea* (Thunb.) DC.	Bitter & Pungent
BP8	ACORI TATARINOWII RHIZOMA	*Acorus tatarinowii* Schott	Bitter & Pungent
BP9	CYNANCHI STAUNTONII RHIZOMA ET RADIX	*Cynanchum stauntonii* (Decne.) Schltr.ex Lévl.	Bitter & Pungent
BP10	ANGELICAE PUBESCENTIS RADIX	*Angelica Pubescens* Maxim.f.*biserrata* Shan et Yuan	Bitter & Pungent
BP11	GENTIANAE MACROPHYLLAE RADIX	*Gentiana macrophylla* Pall.	Bitter & Pungent
BP12	BUPLEURI RADIX	*Bupleurum chinense* DC.	Bitter & Pungent
BP13	CURCUMAE RADIX	*Curcuma wenyujin* Y. H. Chen et C. Ling	Bitter & Pungent
BP14	HOMALOMENAE RHIZOMA	*Homalomena occulta* (Lour.) Schott	Bitter & Pungent
BP15	POLYGALAE RADIX	*Polygala tenuifolia* Willd.	Bitter & Pungent
BP16	DIPSACI RADIX	*Dipsacus asper* Wall. ex Henry	Bitter & Pungent
BP17	PLATYCODONIS RADIX	*Platycodon grandiflorum* (Jacq.) A.DC.	Bitter & Pungent
BP18	ZANTHOXYLI RADIX	*Zanthoxylum nitidum* (Roxb.) DC.	Bitter & Pungent
BP19	PEUCEDANI RADIX	*Peucedanum praeruptorum* Dunn	Bitter & Pungent
BP20	DICHROAE RADIX	*Dichroa febrifuga* Lour.	Bitter & Pungent
SP1	ACONITI LATERALIS RADIX PRAEPARAIA	*Aconitum carmichaelii* Debx.	Sweet & Pungent
SP2	NARDOSTACHYOS RADIX ET RHIZOMA	*Nardostachys jatamansi* DC.	Sweet & Pungent
SP3	SAPOSHNIKOVIAE RADIX	*Saposhnikovia divaricata* (Turcz.) Schischk.	Sweet & Pungent
SP4	ANGELICAE SINENSIS RADIX	*Angelica sinensis* (Oliv.) Diels	Sweet & Pungent
SP5	RANUNCULI TERNATI RADIX	*Ranunculus ternatus* Thunb.	Sweet & Pungent
SP6	MORINDAE OFFICINALIS RADIX	*Morinda officinalis* How	Sweet & Pungent
SP7	PUERARIAE THOMSONII RADIX	*Pueraria thomsonii* Benth.	Sweet & Pungent
SP8	PUERARIAE LOBATAE RADIX	*Pueraria lobata* (Willd.) Ohwi	Sweet & Pungent
SB1	STEMONAE RADIX	*Stemona sessilifolia* (Miq.) Miq.	Sweet & Bitter
SB2	ASPARAGI RADIX	*Asparagus cochinchinensis* (Lour.) Merr.	Sweet & Bitter
SB3	SEMIAQUILEGIAE RADIX	*Semiaquilegia adoxoides* (DC.) Makino	Sweet & Bitter
SB4	CIBOTII RHIZOMA	*Cibotium barometz* (L.) J. Sm.	Sweet & Bitter
SB5	ATRACTYLODIS MACROCEPHALAE RHIZOMA	*Atractylodes macrocephala* Koidz.	Sweet & Bitter
SB6	ANEMARRHENAE RHIZOMA	*Anemarrhena asphodeloides* Bge.	Sweet & Bitter

*, Herbs that were examined in the in vitro cell assay. Taste descriptors are based on the 2015 Chinese Pharmacopoeia.[11]

2.2. Delayed luminescence (DL)

2.2.1. Sample preparation

Each herbal sample was crushed using a model QE-100 grinder (Yili Company, Zhejiang Province, China) and passed through a standard sieve to obtain150-μm particles.[18] These herbal samples were kept in a dark, light-tight box containing 35-mm silica gel (Boom BV, Meppel, the Netherlands) at room temperature for 16 h before DL measurements were conducted.[18]

2.2.2. DL measurement

DL was measured in the herbal samples as described previously.[18] The instrument used to measure DL was obtained from Meluna Research (Geldermalsen, the Netherlands) and included a photomultiplier tube (PMT) (type 9558QB; Electron Tubes Enterprises Ltd., Ruislip, UK) vertically positioned on a dark sample chamber kept at 22°C. The PMT contains a cathode end (51 mm diameter) with sensitivity at 160–870 nm. The PMT was cooled to −25°C in order to reduce the dark count rate to 10 counts per second. The DL signal was amplified using a type 9301 fast preamplifier (ORTEC, Oak Ridge, TN). Data were acquired using a personal computer containing a model 6602 counting card (National Instruments, Austin, TX). Each herbal sample (1 g) was placed in a plastic Petri dish (35-mm diameter) and excited for 10 s using a model 284-2812 white halogen light source (Philips, Germany). For each sample batch, the DL signal was measured three consecutive times, and a total of fifteen measurements in five batches were used to examine the DL properties of that particular herbal medicine. The DL decay signature was obtained by recording the number of photon counts in consecutive 0.05-s periods for a total of 60 s, yielding a total of 1200 data points.

2.3 Dendritic cell assay

2.3.1 Preparation of extracts

Sixteen herbal samples were tested using the DC assay (Table 1). Each herbal sample was divided into three batches, and each batch of a specific herb was used to prepare four 4-g samples; hence, each herbal sample was divided into 12 independent samples, which were crushed into powder form using a type QE-100 grinder (Yili Company, Zhejiang Province, China). Each powdered herbal sample was extracted with 80 ml distilled water at 100°C for 1 hour. The resulting extraction was then centrifuged, filtered through No. 1 filter paper (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and concentrated using a model R-210 rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland).[20] Thereafter, the water extract was mixed with 95% ethanol at a ratio of 1:5.7 (v:v), chilled at 4°C overnight, and then centrifuged at 5000 rpm for 30 mins to precipitate polysaccharides and proteins. The resulting supernatant was then filtered through No. 1 filter paper (Toyo Roshi Kaisha, Ltd.). Finally, 12 independent water extracts were obtained from each original herbal sample, dried in a vacuum evaporator overnight, and stored at 4°C until further use in the DC assay.

2.3.2 Mice

ICR mice (8-12 weeks of age) were purchased from BioLASCO Taiwan Co., Ltd (Taipei, Taiwan). All animals were housed in a specific pathogen-free facility at the Animal Center of China Medical University (Taichung, Taiwan) and handled in accordance with the Institutional Animal Care and Use Committee of China Medical University (Taichung, Taiwan).[20]

2.3.3 Preparation of mouse dendritic cells (DCs)

The methods for preparing and culturing bone marrow-derived dendritic cells have been described previously.[20] In brief, bone marrow cells were isolated from the tibias and femurs. After the red blood cells were removed, the remaining cells were seeded on 6-well culture plates (Costar) with RPMI 1640 medium (Hyclone) supplemented with HEPES, penicillin/streptomycin (Gibco), 10% fetal bovine serum (Hyclone),

recombinant mouse GM-CSF (10 ng/ml, PeproTech), and IL-4 (10 ng/ml, PeproTech). After 7 days in culture, the DCs were collected and used for the experiments described below.

2.3.4 Effects of herbal extracts on DCs

Each herbal extract was dissolved in DMSO to prepare a 500 mg/ml solution. DCs were treated with each herbal extract at 500 μg/ml in the absence or presence of 100 ng/ml lipopolysaccharide (LPS) for 6 h. The concentrations of TNFα and IL-6 in the medium were measured using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA) in accordance with the manufacturer's instructions.[21] Control standards (10 μg/ml in PBS) were used to treat DCs in the absence or presence of LPS.[20] Each herbal extract sample was tested in two independent experiments; thus, a total of 24 datasets for TNFα and IL-6 were obtained for each specific herb.

2.4 Data processing and statistical analysis

2.4.1 DL Properties

The DL decay curve for each sample, measured over a 60-second period, was fit to the following double-exponential function:[18]

$$y = y0 + A1e^{-\frac{x}{t1}} + A2e^{-\frac{x}{t2}}$$

where y0 is the final value of DL emission in the decay curve, A1 and A2 are the amplitudes of the exponential decay components, and t1 and t2 are time constants for the exponential decays.[18] The R package nnet (version 3.2.2) was used to perform the DL curve-fitting.[22] The median of each DL property from the fifteen measurements was calculated and used to represent the DL signature of each herbal medicine. One-way analysis of variance (ANOVA) with least significant difference (LSD) post hoc analysis[23] was used to compare the DL properties between the six

taste groups; differences were considered significant at $p<0.05$. Principal component analysis (PCA) was used to indicate the level of discrimination between DL properties, using the tools provided in the MetaboAnalyst software package (http://www.metaboanalyst.ca).[14,23]

2.4.2 Cytokines measured in the DC assay

To quantify the immunomodulatory effect of the herbal samples on DCs, the concentrations of the cytokines TNFα and IL-6 were normalized to the control standard (to measure activation) or the control standard plus LPS (to measure inhibition); these control samples were set to 100%. The percentage of each herbal sample relative to the control value was used to reflect the relative secretion of cytokines. Thereafter, a one-sample Student's *t*-test was used to analyze the difference between each specific herbal sample (or taste group) and control standard (or control standard plus LPS) using SPSS (version 23.0; IBM, Armonk, NY). Differences were considered significant at $p<0.05$.

3. Results

In traditional Chinese medicine, various components in plants can be used as herbal materials, including the roots and/or rhizomes, leaves, flowers, fruits, and/or seeds.[11] From a biological perspective, different parts of a plant play distinct roles in the plant's physiology.[24] Because DL can be used to detect the holistic signatures of plant materials,[25,26] we first investigated whether different parts of a plant have different DL signatures. Therefore, we measured the dry roots and rhizomes (in one sample), as well as the leaves and flowers of ginseng; the DL decay curves for the various dry materials are shown in Fig. 1. The roots and rhizomes sample produced a significantly different DL signature compared to the leaves and flowers; in particular, the DL features in the tail of the curve differ considerably. Therefore, in order to minimize differences in the DL signatures obtained from different parts of plant, we focused our analysis on the roots and/or rhizomes, as these two structures

grow beneath the soil, and the corresponding herbs are commonly used in traditional Chinese medicine. Table 1 lists the 90 Chinese herbal materials that were analyzed, their taste groups, and their main descriptors (sweet, bitter, or pungent), as well as the combinations of these three tastes in accordance with the 2015 Chinese Pharmacopoeia.[11]

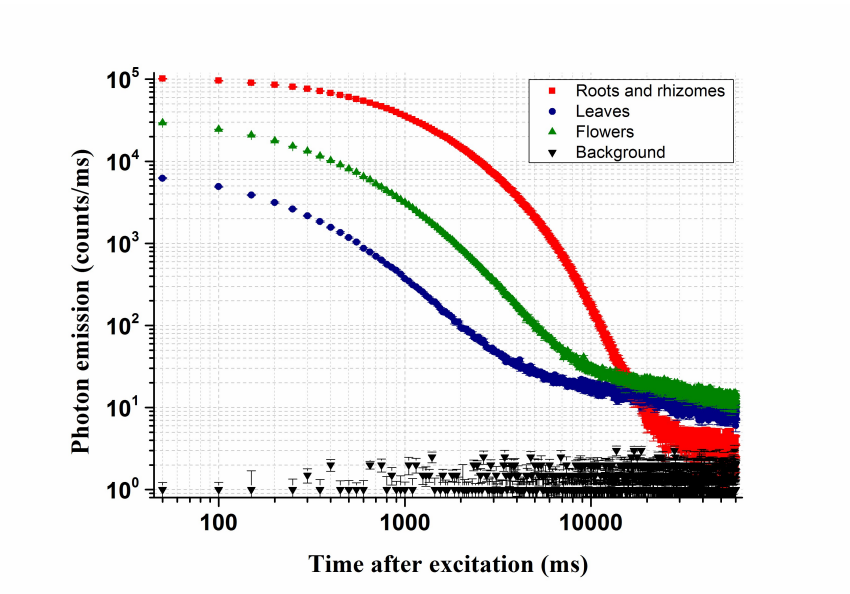

Fig. 1 DL decay curves for ginseng roots and rhizomes, ginseng leaves, and ginseng flowers. Data are plotted as the mean ± SEM. Note that the data are plotted on a log-log scale.

Next, we measured the DL profiles of these 90 herbal samples. The results show that herbal samples in the same taste descriptor category produce generally similar DL curves, with strikingly different curves between “sweet” herbs and “bitter” herbs (Fig. 2). To compare the overall DL curves obtained from the three individual taste descriptor groups, we pooled the data from the herbal samples within a specific taste descriptor group; these results are shown in Fig. 3. These results show that the DL dynamics in the “sweet” descriptor group differ from both the “pungent” and “bitter” taste groups; moreover, the “pungent” and “bitter” groups have similar DL decay profiles (Fig. 3).

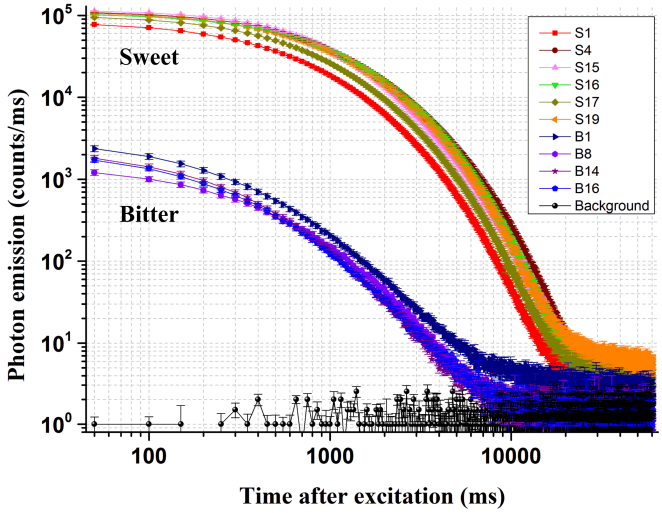

Fig. 2 DL decay curves of several herbal samples with "sweet" (S) descriptor and "bitter" (B) descriptor. Data are plotted as the mean ± SEM. Note that the data are plotted on a log-log scale. Each specific herbal sample is indicated by ID number (see Table 1).

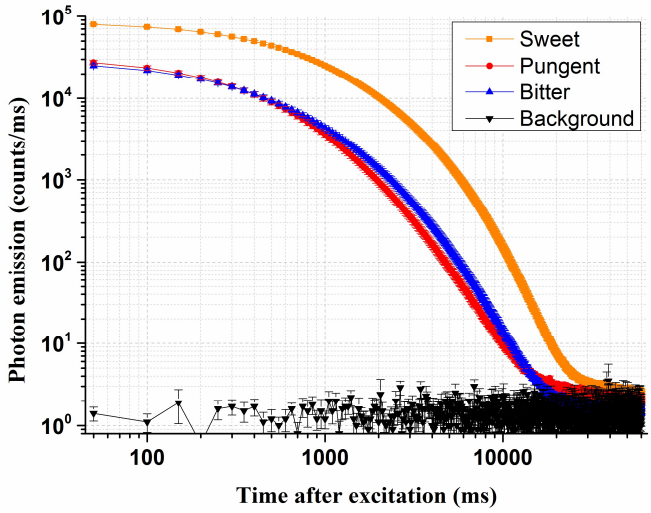

Fig. 3 DL decay curves for pooled samples from the sweet, bitter, and pungent groups. Data are plotted as the mean ± SEM. Note that the data are plotted on a log-log scale.

Five parameters were then derived from the DL curves by fitting the decay curves with a double-exponential function. We then compared these five parameters between all six taste groups using a one-way ANOVA; the results are summarized in Fig. 4. Fig. 4 reveals that the five parameters (i.e., A1, A2, t1, t2 and y0) can be used to differentiate between the "sweet" descriptor group and the other five groups.

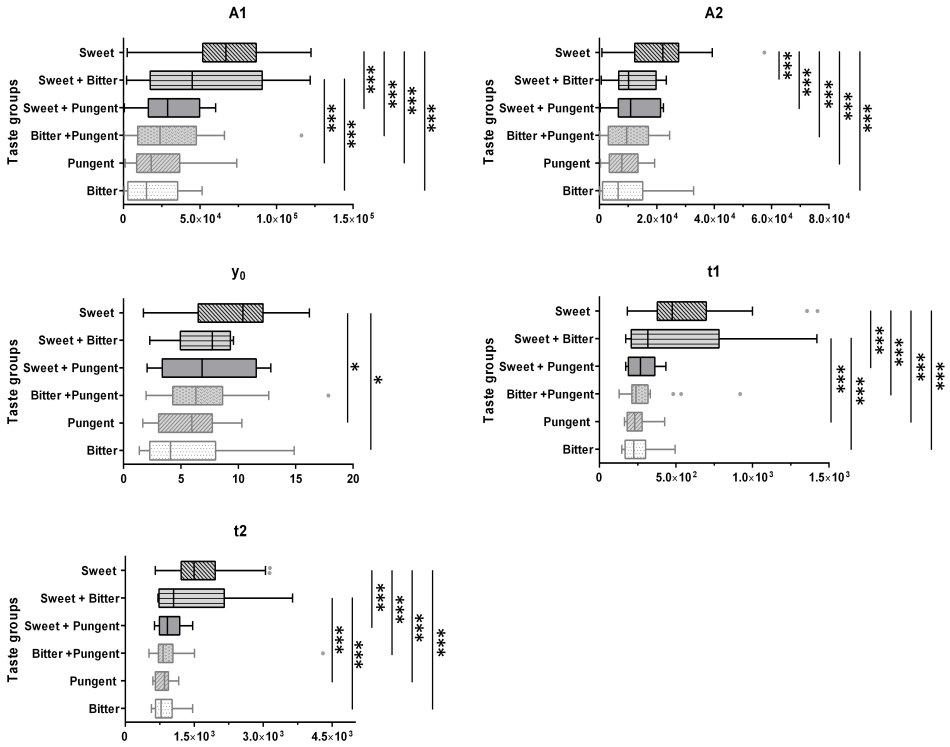

Fig. 4 Box plot summarizing the five DL properties measured in the six "taste" groups. *, $p<0.05$; ***, $p<0.001$ (one-way ANOVA with LSD).

To visualize the overall differences in DL properties between the various groups using an unsupervised method, we applied principal component analysis (PCA) for each herbal sample in the taste groups "sweet" and "bitter" (Fig. 5A) and in the taste groups "sweet" and "pungent" (Fig. 5B). Fig. 5A shows the PCA score plot using DL parameters in which PC1 and PC2 account for 70.9% and 20.4% of the total variance, respectively. This plot shows that the DL properties of the sweet descriptor

group and the bitter descriptor group generally form distinct clusters, with minor overlap. The DL parameters of five “sweet” herbal samples (S5, S6, S12, S14, and S18) were misclassified as belonging to the “bitter” descriptor group, whereas two “bitter” herbal samples (B4 and B12) were misclassified as belonging to the “sweet” descriptor group. Fig. 5B shows the PCA score plot in which PC1 and PC2 account for 67% and 23.6% of the total variance, respectively. These results show that the DL properties of the sweet descriptor group and the pungent descriptor group generally form two distinct clusters, with some overlap. None of the “pungent” herbal samples were misclassified as belonging to the “sweet” descriptor group, whereas five “sweet” herbal samples (S5, S6, S12, S14, and S18—the same five samples that were misclassified as belonging to the “bitter” group) were misclassified as belonging to the “pungent” descriptor group. No indication of separate PCA clusters was found between the “bitter” and “pungent” descriptor groups. Moreover, a PCA analysis between the “sweet” group and the “bitter & pungent” group revealed significant clusters between these two groups (Supplemental Fig. S1). We did not perform PCA analyses using the “sweet & pungent” descriptor group or the “sweet & bitter” descriptor group due to the relatively small numbers of herbal samples in these two groups. These results show that the “sweet” descriptor is unique among the descriptor groups with respect to both the DL curves and the DL parameters. Therefore, we conclude that DL can be used to differentiate between various taste descriptor classifications and can be used identify therapeutic properties in specific herbal materials at the systems level.

Next, to provide biological relevance to these findings, we performed an *in vitro* assay using mouse dendritic cells (DCs) and examined the immunomodulatory effects of select herbal samples. To reduce the complexity of herbal properties, we excluded the herbal materials with double taste descriptors (e.g., “sweet & bitter”, sweet & pungent”, etc.), and we examined 16 herbal materials with single taste descriptors.

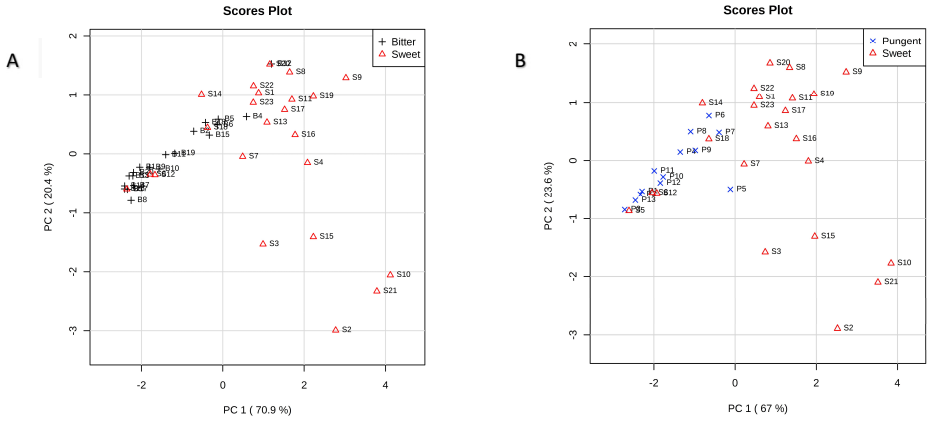

Fig. 5 PCA scores of the DL properties measured for the indicated "taste" herbs. A) "Sweet" herbs and "bitter" herbs are plotted. B) "Sweet" herbs and "pungent" herbs are plotted. Each symbol represents an individual herbal material with the corresponding ID number (see Table 1).

Water extraction is the traditional method used to prepare medicinal herbs and is well-suited to evaluating pharmacological effects based on clinic experience. In this study, we treated DCs using herbal water extract either in the absence or presence of LPS. These measurements were performed using two independent cell batches, and 12 measurements were obtained for each herbal sample in a specific cell batch.

To study the immunomodulatory response to a specific "taste" group as a whole, we pooled data from the individual herbal materials in each group. Fig. 6 summarizes the immunomodulatory responses of the three "taste" groups measured using secreted TNFα and IL-6. To study the ability of each group to inhibit the immune response, we first treated the cells with LPS, a potent activator of DCs.[19] Fig. 6A shows that both the "bitter" and "pungent" taste groups significantly inhibited the LPS-induced increase in TNFα and IL-6 secretion, with the pungent group having the strongest effect. In contrast, the "sweet" taste group caused higher IL-6 secretion compared to LPS alone (Fig. 6A). With respect to activation, Fig. 6B shows that both the "sweet" and "bitter" groups significantly increased TNFα secretion, whereas only the "sweet" group significantly increased IL-6 secretion. The "pungent" group

had no significant effect on either TNFα or IL-6 (Fig. 6B). In summary, these results show that herbs in the "sweet" group are primarily immunostimulatory, whereas herbs in the "bitter" and "pungent" groups are primarily immunosuppressive. These results generally correspond to the functional indications of herbal "taste" classifications based on traditional Chinese herbal medicine.[4]

Lastly, we examined the immunostimulatory and immunosuppressive effects of individual sweet, bitter, and pungent herbal medicines. With respect to the sweet herbal materials (Fig. 7A1), five samples—Codonopsis radix (S4), Polygonati odorati rhizoma (S10), Ginseng radix et rhizoma rubra (S15), Pseudostellariae radix (S17), and Ophiopogonis radix (S21) —significantly increased TNFα production, and three samples—Codonopsis radix (S4), Polygonati odorati rhizome (S10), and Ginseng radix et rhizoma rubra (S15)—significantly increased IL-6 production.

Moreover, two sweet herbs—Codonopsis radix (S4) and Ginseng radix et rhizoma rubra (S15)—significantly inhibited the effect of LPS on TNFα (Fig. 7A2). This result suggests that Codonopsis radix (S4) and Ginseng radix et rhizoma rubra (S15) can act as both an inhibitor and activator of the immune response. Interestingly, Astragali radix (S1) increased the secretion of TNFα in the presence of LPS but significantly decreased the secretion of IL-6 (Fig 7A2). In addition, Codonopsis radix (S4), Pseudostellariae radix (S17), and Ophiopogonis radix (S21) significantly inhibited TNFα in the presence of LPS, but significantly increased IL-6 secretion.

With respect to the "bitter" herbal samples, the majority did not significantly increase TNFα or IL-6 secretion (Fig. 7B1); indeed, two "bitter" herbs—Picrorhizae rhizoma (B13) and Coptidis rhizoma (B17)—led to significant decreases in TNFα and IL-6, respectively. Only one herb in the bitter group—Rhei radix et rhizoma (B8)—significantly increased TNFα secretion and significantly decreased IL-6 secretion (Fig. 7B1). A similar pattern was observed for this bitter herb with respect to inhibition in the presence of LPS—Rhei radix et rhizoma (B8) significantly increased TNFα secretion and significantly decreased IL-6 secretion (Fig. 7B2).

Moreover, the majority of bitter herbal samples significantly inhibited both TNFα and IL-6 (Fig. 7B2); Scutellariae (B16) and Coptidis (B17) had the strongest immunosuppressive effect among the five bitter herbal materials tested. Finally, the "pungent" herbal samples generally inhibited both TNFα and IL-6 secretion under both control (Fig. 7C1) and LPS (Fig. 7C2) conditions.

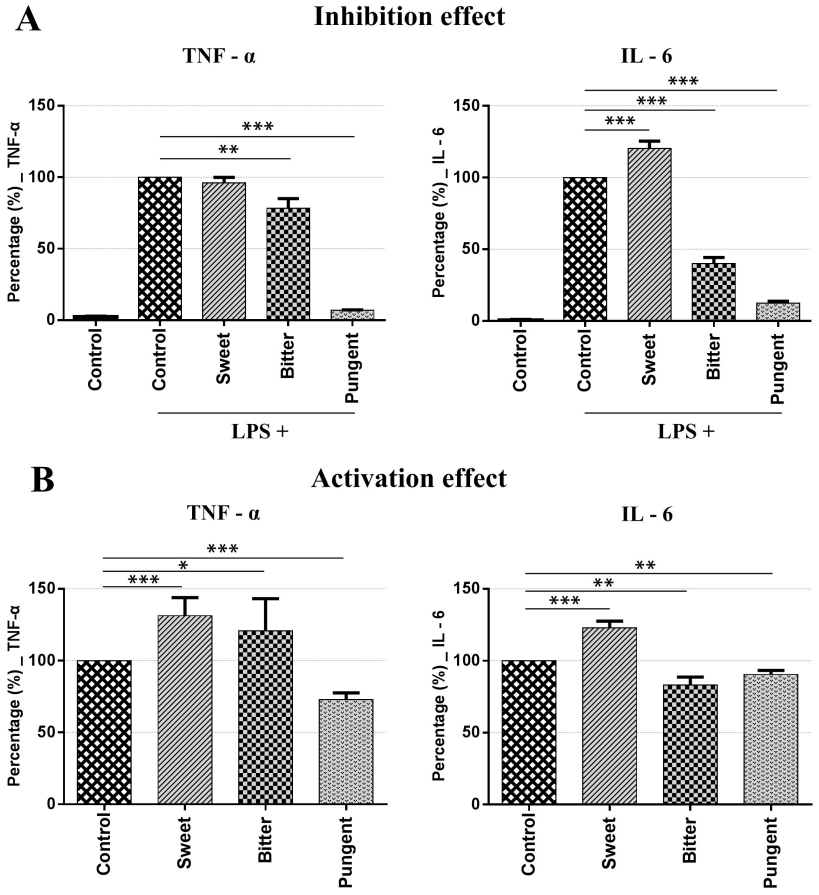

Fig. 6. Immunomodulatory effects of the various taste groups. A) Summary of the inhibitory effect of each taste group compared to cells stimulated with LPS. B) Summary of the stimulatory effect of each taste group compared to control (unstimulated) cells. The secretion of TNFα and IL-6 was measure using ELISA and is plotted relative to the respective control. Data are plotted as the mean ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (one-sample Student's t-test).

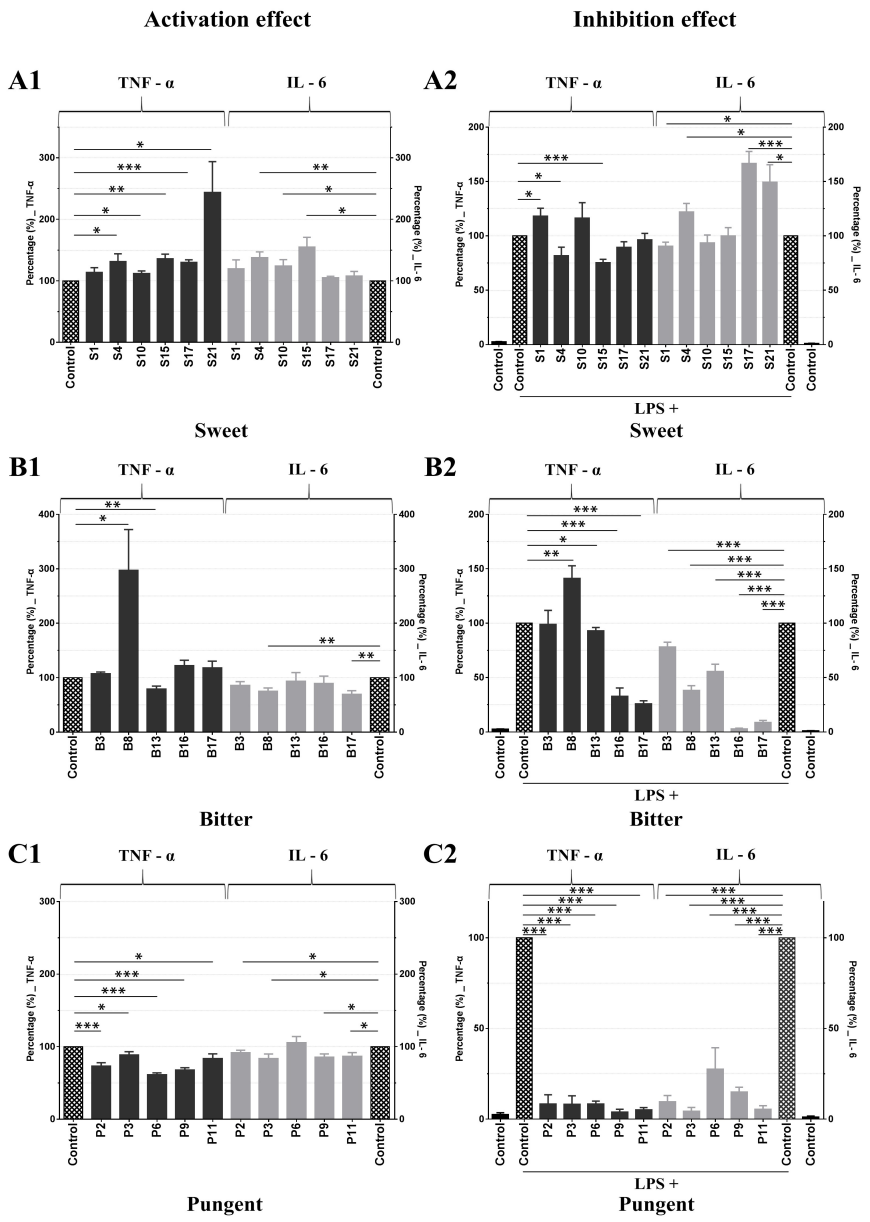

Fig. 7. Immunomodulatory effects of individual herbs in various taste groups. A) Summary of the activating (A1) and inhibitory (A2) effects of "sweet" herbs. B) Summary of the activating (B1) and inhibitory (B2) effects of "bitter" herbs. C) Summary of the activating (C1) and inhibitory (C2) effects of "pungent" herbs. Activation and inhibition were measured as described in Fig. 6. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (one-sample Student's t-test).

4. Discussion

In traditional Chinese medicine, the therapeutic effects of herbal medicines have been classified into distinct categories using different "taste" descriptors.[2] According to the traditional use of Chinese medicines, the taste descriptor provides a specific therapeutic description based on a long history of experience. This type of description usually applies to multi-dimensional pharmacological effects in the human body as a whole. DL is a systematic measurement that may be suitable for determining an herb's ethnopharmacological effect based on the taste descriptor. Here, we found that herbal materials in the same "taste" group generally produce similar DL curves. Interestingly, some herbs with the same taste descriptor (e.g., Codonopsis and Ginseng) can be interchangeable in some prescriptions for clinical applications.[10,27,28] In addition, the therapeutic effects of herbal medicines have been attributed to bioactive compounds,[29] and herbs within the same descriptor category can have very different profiles of bioactive compounds, as reflected in the Traditional Chinese Medicine Database (http://tcm.cmu.edu.tw/zh-tw/index.php). Moreover, the therapeutic effects of herbal medicines are not simply related to the identified bioactive compounds, as other chemical components within herbal medicines can combine synergistically with bioactive compounds to increase overall therapeutic efficacy.[30] Therefore, measuring an herb's DL signature may provide information regarding the biological characteristics and thus the specific therapeutic property indicated by the taste descriptor.

In principle, the molecular absorption of excitation energy defines the dynamics of the subsequent DL emission.[14,18] Changes in a compound's DL signature can be due to conformational changes in the cellular macromolecules, including proteins and nucleic acids.[31] Because both the primary and secondary metabolites in plants can interact with proteins and the cell surface by forming hydrogen bonds,[29] the molecular conformation of herbal cells can be changed, resulting in complex interactions between molecules. These interactions can affect the radiant (i.e.,

resonance) transfer of energy from one excited molecule to another, causing a change in the plant's DL dynamics.[31] Our analysis indicates that DL parameters can be used to identify herbs with a "sweet" descriptor. Since sweet taste can be derived from different types of compounds such as aldehydes, ketones, and sugar alcohols. The relationship between DL parameters and the intrinsic structure of "sweet" herbs needs to be explored further. This is also true with respect to the difference between herbs in the "sweet" category and herbs in the other "taste" categories. In addition, the finding that some herbs were misclassified in the PCA analysis may indicate the presence of other factors, other unknown mechanisms in DL, and/or the number of plant materials that we tested.

Another method for characterizing "taste" is to measure the biological response *in vivo*. However, this approach is currently limited. We therefore used an *in vitro* cell system to support our DL results. Herbal medicines with different taste descriptors have been reported to yield different effects with respect to their antioxidant and anti-inflammatory properties.[32,33] Here, we studied the immunomodulatory effects of herbs using a DC-based assay with water extracts. It is important to note that polysaccharides were removed before the samples were applied to the cells. Polysaccharides are a primary metabolite generally present in plants,[34,35] and polysaccharides in herbs can cause a significant immunostimulatory effect by boosting the immune system.[36–42] In addition, because secondary metabolites are the principle bioactive constituents in herbal medicines,[29] we focused on immunomodulatory responses induced by herbal water extracts without the influence of polysaccharides.

DCs are commonly used as a model cell system for antigen-presenting cells, which are activated to initiate an adaptive immune response.[43] The expression of the cytokines TNFα and IL-6 plays an essential role in the activation of DCs,[20,44,45] and the secretion of TNFα and IL-6 is a hallmark feature of DC activation used to measure immunostimulatory effects.[20,44] TNFα and IL-6 are tightly coupled, as

TNFα induces IL-6 production; therefore, TNFα is often used as an early marker of an immune response.[46] In addition, secretion of high levels of TNFα can harm the immune system, and when combined with other cytokines such as IL-6, pathological damage can occur.[47] However, in our study we measured the secretion of TNFα and IL-6 in response to LPS stimulation primarily to observe the ability of herbal medicines to suppress the immune response.

The "sweet" herbal materials tested here had an immunostimulatory effect corresponding to their traditional tonification action. The immunostimulatory effect in the "sweet" group differed significantly from the other groups; specifically, Ginseng radix et rhizoma rubra (S15) had both activating and suppressing effects on the immune response. Interestingly, this ambiguity regarding the effects of ginseng extract in vascular pathophysiology has been reported previously[48] and is consistent with our results. The immune response elicited by Codonopsis radix (S4) was similar to the immune response elicited by Ginseng radix et rhizoma rubra (S15). In traditional Chinese medicine, Ginseng and Codonopsis have a similar clinical action ("Qi tonifying") and have been categorized into the same group.[27] In addition, Codonopsis is usually substituted for Ginseng, although it has a weaker pharmacological effect in some applications in clinical practice.[27,28] This difference in pharmacological strength is supported by our results, which revealed differences in TNFα secretion between Codonopsis and Ginseng. In the presence of LPS, Astragali radix (S1) both increased TNFα secretion and inhibited IL-6 secretion; the latter effect is consistent with a report that a water extract of Astragali radix inhibits LPS-induced IL-6 release in human amniotic cells.[49] Several other studies indicate that polysaccharides derived from Astragali radix reduce LPS-induced TNFα release in various cell cultures.[50–52] Here, we found that a water extract of Astragali radix (without polysaccharides) increased the secretion of LPS-induced TNFα in DC cells, possibly indicating an opposing active principle in Astragali radix, similar to reports regarding ginseng.[48]

According to traditional Chinese medicine, the typical ethnopharmacological effects of "bitter" herbs are heat-cleansing.[9] Herbs with heat-cleansing effects are often used to treat infection, as well as to decrease tissue damage following inflammation.[53] Inflammation is a key component of autoimmune diseases such as rheumatoid arthritis and diabetes,[54,55] and increased secretion of TNFα and IL-6 are important inflammatory markers.[56] In our study, the majority of "bitter" herbs tested inhibited the secretion of both TNFα and IL-6. This result indicates that these "bitter" herbal samples have an immunosuppressive function consistent with their traditional pharmacological effects. In addition, these "bitter" herbs are reported to reduce the levels of TNFα and/or IL-6 in various pathological states.[57–60] In this respect, it is worth noting that Rhei radix et rhizoma (B8, rhubarb) significantly increased TNFα secretion and significant inhibited IL-6 secretion, thereby presenting opposing immunomodulatory effects. This finding may indicate that the immunosuppressive effects of rhubarb occur in a later stage in the immune response. On the other hand, Kounsar et al. reported that an ethyl acetate extract of rhubarb (*Rheum emodi* Wall. ex Meissn) increases the secretion of TNFα, thereby enhancing the immune response,[61] which is consistent with our results.

According to the principles of traditional Chinese medicine, "pungent" herbs can promote health by removing and expelling pathogenic factors such as heat, dampness, wind, and cold,[4] pathogenic factors that are closely related to diseases associated with inflammation.[62] Although both "pungent" and "bitter" herbs can reduce harmful factors, their pharmacological actions are not interchangeable.[4] The "pungent" herbal materials tested here inhibited both TNFα and IL-6 secretion, which is similar to the "bitter" herbal materials tested (although the effect was stronger for the "pungent" herbs). Similar results regarding these two herb groups were found in a study of NO production-based anti-inflammatory effects using herbal ethanol extracts.[33] In their study, the authors found that "pungent" herbs were slightly stronger anti-inflammatory agents than "bitter" herbs.[33] Thus, their results

are consistent with our findings and suggest that the Chinese herbal property of "taste" can be used to predict an herb's therapeutic effects.

The results regarding the immunomodulatory effects based on our DC assay are partially in agreement with our DL measurements, however, cell-based assay cannot fully represent the systemic response of herbs in the human body. Therefore, in this study, the use of DCs-based immunomodulatory assays is used to support the principle of herbal "taste" properties in TCM only, but supports—albeit indirectly—the results obtained from the holistic DL measurements. Thus, it is not meant to correlate DL results with specific immune responses (e.g., opposing effects in S4, S15 and B8) in DCs using herbal extracts. For further studies on correlations between herbal "taste" properties, therapeutic effects and DL characteristics, a total system-based model (e.g., zebrafish or other animal models) may provide a suitable option for future studies.

5. Conclusions

An interesting study discussed common pharmacological activity between different compounds such as ibuprofen and oleocanthal and noted their similarity regarding taste.[63] This approach is similar to the concept of "taste" in traditional Chinese medicine. The concept of herbal taste originates from traditional ethnopharmacological medical practices. The key challenge is to characterize and standardize herbal taste as a qualitative and quantitative predictor of pharmacological activity. Here, we present data supporting the use of DL to perform a systems level measure of an herb's pharmacological activity. In addition, we report the first study combining DL measurements with a cell-based assay in order to investigate herbal taste properties with respect to biological effects. Several main conclusions can be drawn from our study.

First, the parameters of the DL profiles were able to accurately discriminate the "sweet" category from other taste categories. Second, the results of our DC-based

immunomodulatory assay support the results obtained from our DL measurements. It is worth noting that DC-based assay is sensitive to LPS, therefore, possible contamination of herbal samples should be avoided. Finally, the results of this study may provide a suitable foundation for follow-up studies. For example, future studies can include a larger number of samples, more herbal taste categories, and/or more herbal plant components. In addition, additional platforms for screening the activity of herbal materials and the *in vivo* biological response should be performed in order to support the DL data using a large number of herbal materials. In this respect, a total system-based model (e.g., zebrafish) may provide valuable information to support the systematic observation of taste based on DL data. Moreover, improvements and developments of additional DL parameters and DL spectral analyses may provide a clearer distinction between different "taste" herbs such as "bitter" and "pungent" herbs. In conclusion, evidence-based interpretation of traditional herbal pharmacological effects is important for exploring the multi-dimensional pharmacological effects of herbs and for providing quality control.[64] DL is a robust, new technique for studying the ethnopharmacological effects of Chinese herbal medicines, thereby facilitating the move toward personalized healthcare.

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Supplementary Figure

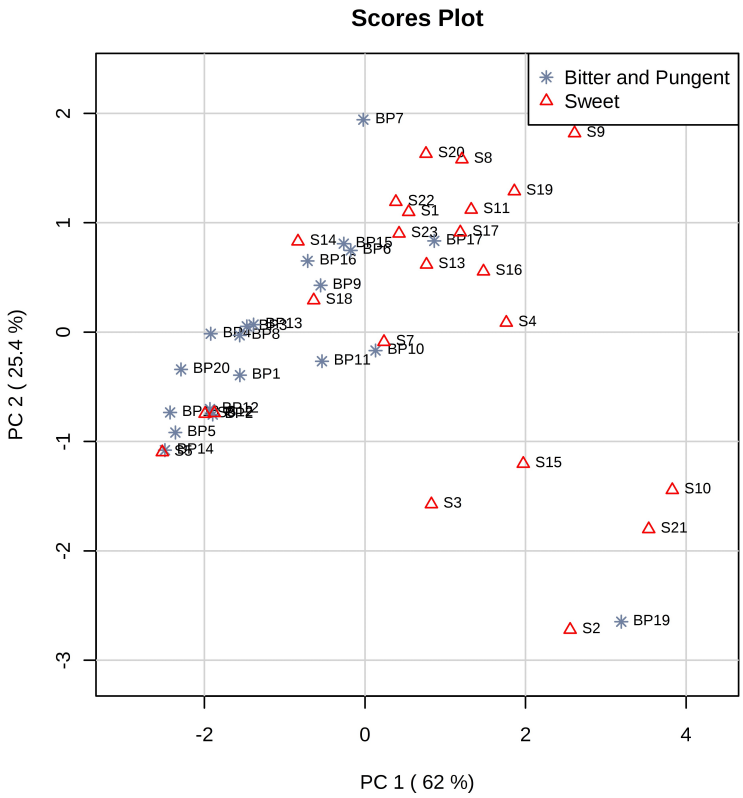

Fig. S1 PCA scores of the DL properties measured for “sweet” herbs and “bitter & pungent” herbs. Each symbol represents an individual herbal material with the corresponding ID number (see Table 1).

Chapter 7

Summary, conclusions, and perspectives

Personalized medicine is the key to moving away from the conventional medical concepts of "one disease, one target" and "one-size-fits-all".[1] Personalized medicine also includes monitoring the individual patient's comprehensive profile of nutritional, psychological, lifestyle, and other factors, and it can facilitate the development of multiple therapeutic strategies aimed at providing the best possible course of treatment.[1] Phenotyping patients is believed to play an essential role in achieving personalized medicine.[2] Traditional Chinese medicine (TCM)–based diagnostics uses important information regarding personalized phenotypes (so-called "syndrome subtypes") at a holistic level, and TCM can theoretically be explored using systems biology–based approach.[2] To investigate TCM-based diagnostics, a systematic, dynamic measurement is needed. Ultra-weak photon emission (UPE) provides a non-invasive, comprehensive measure of dynamics with respect to the physiological state of a living organism.[3] In addition, because UPE can be used to measure various physiological states, it can be used to identify putative diagnostic properties.[3–6] Measuring UPE may therefore approximate the organizational level of TCM-based diagnostics. Thus, UPE may serve as a potential tool for characterizing "syndrome subtypes", providing objective data to support TCM-based diagnostics.

To test this hypothesis, we performed an explorative study in 44 pre-diabetic subjects. In this study, which is presented in **Chapter 3**, we combined UPE measurements with TCM-based diagnostics in order to identify personalized phenotypes ("syndrome subtypes") in these subjects. Three physicians who were trained in TCM achieved 85% diagnostic consistency by standardizing 26 symptoms in pre-diabetic subjects, thereby identifying the following three "syndrome subtypes": Qi-Yin deficiency, Qi-Yin deficiency with dampness, and Qi-Yin deficiency with stagnation.[7] Based on TCM-based diagnostic concepts, these three subtypes share a common fundamental cross-biological background— Qi-Yin deficiency—which can be used to reflect hypermetabolism-induced mild inflammatory status and

chronic fatigue syndrome.[7] The additional pathological factor is closely related to an unhealthy lifestyle and poor diet. Dampness can lead to complications such as metabolic syndrome, hypertension, and angionosis.[7–9] Another pathological factor—stagnation—may be associated with pressure and tension, thereby leading to emotional problems, impaired blood circulation, and diabetic peripheral neuropathy.[7,8,10] Thus, TCM-based diagnostics (i.e., the identification of specific "syndrome subtypes") may provide important information with respect to developing a personalized profile for predicting pre-diabetic patients, thereby helping prevent the onset of diabetes-related complications.

A total of 16 UPE parameters were measured at four anatomical sites in our cohort of 44 pre-diabetic subjects; this analysis reliably identified the three TCM-based "syndrome subtypes". In addition, a correlation network between these 16 UPE parameters and 26 symptoms (i.e., items) was also performed to indicate the associations and differences between these three phenotypes. These results indicate that UPE is a promising methodology for studying TCM-based diagnostics. This is the first evidence demonstrating the feasibility of combining UPE parameters with TCM-based diagnostics in order to investigate syndrome subtypes among pre-diabetic subjects. Future studies should include larger cohorts, more TCM-trained physicians and syndrome subtypes, and other disease cohorts in order to strengthen the value of this approach. Moreover, measuring UPE signals at additional anatomical sites in the human body may provide additional information in support of TCM-based diagnostics. Given the relationship between specific diseases and acupuncture points (as discussed in **Chapter 2**), combining UPE with TCM-based acupuncture theory may help with the selection of suitable anatomical sites for measuring UPE; this approach may also help characterize and standardize TCM-based acupuncture treatment both qualitatively and quantitatively. Moreover, similar analyses can be performed in combination with metabolomics based on various platforms in order to establish a comprehensive molecular and/or biochemical basis

of UPE and TCM-based diagnostics. If this molecular and individual phenotype information can be correlated with UPE parameters, then UPE might represent a highly sensitive, non-invasive technological platform for diagnosing disease and for studying the efficacy of various therapeutic agents, ultimately achieving the goal of personalized medicine.

In Chinese medicine, "personalized intervention" refers to the use of various Chinese herbal prescriptions for treating various disease "syndromes".[11] The Chinese herbal prescription is prepared depending on the herb's ethnopharmacological effects observed in clinical practice, thereby providing Chinese herbal medicine (CHM)–based concepts such as "indigenous medicinal materials" and herbal "taste" properties.[12,13] These CHM-based concepts are closely related to TCM-based diagnostics and reflect the individual's holistic response to herbs from a multi-target, multi-dimensional systems pharmacology perspective. Although these CHM-based concepts are often interpreted using specific bioactive compounds present in the herbs, the herb's ethnopharmacological effects may be due to more than just the bioactive compounds in the herb; indeed, other chemical components in the herbs may act synergistically with bioactive compounds to increase the herb's therapeutic efficacy.[14] Therefore, a comprehensive, systematic measurement is needed in order to investigate CHM-based concepts. In this thesis, we used photon-induced delayed luminescence (DL)—a rapid, direct, systematic measurement—to study the holistic properties of a medicinal herb.

In **Chapter 4**, we discuss the development of a protocol for measuring DL in dried herbal materials. Our results indicate that the water content of herbal materials is an important factor for achieving stable, reproducible DL measurements. Therefore, the conditions used to store the herbal materials should be considered when analyzing DL data. To demonstrate the feasibility of our DL measurement protocol, we used DL to measure several herbal materials prepared under various conditions. The DL parameters reliably distinguished between herbs of different ages, herbs grown under

different environmental conditions, and herbs processed using different methods. Given that these conditions are closely related to the quality and therapeutic properties of Chinese herbal medicines,[15–20] we hypothesized that measuring DL may provide a novel technological platform for studying CHM-based concepts. To test this hypothesis, we conducted the experiments described in **Chapter 5** and **Chapter 6**. It worth noting that in Chapter 4 we measured DL for 5 min in order to investigate the long-term properties of herbal DL measurements. To identify differences in DL properties between herbs, it is not necessary to measure herbal samples for such a long time; indeed, measuring DL for as little as 1 min can usually provide sufficient information regarding DL parameters to allow for meaningful analyses.

In traditional Chinese medicine, the term "indigenous medicinal materials" is used to indicate medicinal plants that are produced under unique environmental conditions, therefore providing optimal quality and therapeutic properties.[13] This ethnopharmacological concept is based on the premise that environmental factors can directly influence the quality of the herb. In Chapter 5 both chemical analyses and DL measurements reflected that the quality and composition of medicinal rhubarb are influenced by altitude variations. The identified correlations between DL parameters and chemical constituents suggest that DL may also be correlated with the bioactive properties of rhubarb. To test this hypothesis, future studies should use a wider range of rhubarb samples and should include a comprehensive profile of secondary metabolites contained in rhubarb. Hence, identifying the correlation network between a comprehensive panel of secondary metabolites and DL parameters may reveal the true potential of using DL to predict the quality of rhubarb. The approach presented in Chapter 4 can also be used to investigate the effect of growing and/or processing conditions on the quality of other medicinal herbs. These studies may provide further evidence that DL is a powerful tool for assessing the quality of herbal medications.

In accordance with the theory of traditional Chinese medicine, all herbs are assigned a therapeutic description of "taste", which is used to reflect the herb's major ethnopharmacological category, thereby revealing the herb's therapeutic properties.[21] This so-called "taste" property can help guide the preparation of various herbal formulas in order to realize personalized interventions.[21] As discussed in Chapter 6, DL parameters were able to distinguish between herbs in the "sweet" category and herbs in the other "taste" categories, and measuring dendritic cell–based immunomodulatory responses revealed that the immunostimulatory effect of "sweet" herbs differed significantly from "pungent" and "bitter" herbs, which generally support the results obtained using DL measurements. However, DL cannot be used to distinguish between "pungent" and "bitter" herbs. This indicates a possible limitation of using DL, and it may reflect the limited number of herbal materials that we studied, which justifies further investigation. First, another fitting method can potentially be used to extract different DL parameters in the herb's DL profile.[22] These additional DL parameters may help differentiate between different "taste" herbs. In addition, DL spectral analysis may provide a potential method for studying the therapeutic properties of herbs based on their ethnopharmacological effects.[23]

Second, studying larger numbers of herbs, more categories of herbal "tastes", and more components in herbal plants will help support the feasibility of this DL-based approach. Moreover, cell-based assays are not necessarily sufficient for providing a complete system-based approach for observing the pharmacological activity of herbal medicines. A complete system-based model (for example, zebrafish) may provide an additional platform for observing the comprehensive bioactivities of herbs that have been classified using DL. The preliminary experiments discussed in Chapters 5 and 6 were designed using DL measurements combined with CHM-based concepts. Our results indicate that DL may be widely applicable to the study of herbal medicines; in addition to reflecting the quality of specific herbs. Therefore,

DL is a promising technique that represents a robust technological platform for investigating Chinese herbal medicines both qualitatively and quantitatively.

In conclusion, both UPE and DL have high potential for studying the concepts of medicine at the systems levels. The results reported in this thesis can be used to develop future research strategies guided by traditional Chinese medicine–based concepts. As analytical and statistical methods improve, and as UPE and DL approaches are integrated further into medical research, UPE and DL will likely provide valuable new insights into personalized medicine.

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Samenvatting

Diagnose op maat is de sleutel om het huidige concept van medicijnontwikkeling gebaseerd op “one disease – one target” en “one-size-fits-all” te verbeteren. Ontwikkeling van geïndividualiseerde gezondheidsbewaking op basis van verfijnde diagnostische strategieën geeft een basis voor nauwkeurig afgestemde behandelingen. Concepten gebaseerd op traditionele Chinese geneeskunde (TCM) voor zowel de diagnose als de interventie kunnen belangrijke bijdragen leveren aan “personalized medicine”. Om TCM concepten te bestuderen zijn diagnostische methoden nodig, die een uitlezing van de fysiologische toestand van een dynamisch systeem kunnen registreren op een niet-invasieve wijze. Ultra-lage foton emissie (Ultra-weak photon emission, UPE) is ’een technologie met een uitstekend potentieel om systeem-gebaseerde (TCM) diagnose te meten.

Om deze hypothese rond UPE te testen is er allereerst een litteratuurstudie uitgevoerd over de literatuur zoals verschenen in de periode van 1979 tot 1998, omdat er in die periode omvangrijke wetenschappelijke studies verricht zijn rond UPE en TCM gebaseerde diagnostiek. Deze resultaten zijn terug te vinden in Hoofdstuk 2. De conclusie daarvan was een bevestiging van de mogelijkheid om UPE in te zetten voor het beter begrijpen van gezondheid en ziekte vanuit een systemisch perspectief, gebaseerd op TCM diagnose. In aanvulling hierop zijn de resultaten van UPE-metingen geanalyseerd uit een exploratieve studie met 44 personen met een vroeg-diabetes type 2 stadium ziektebeeld, zoals gepresenteerd in Hoofdstuk 3. In deze studie werden UPE-metingen gecombineerd met TCM-diagnostiek om de individuele fenotypen te onderscheiden. Drie artsen, met expertise in TCM, diagnosticeerden de personen op basis van 26 symptomen en bereikten een consistentie van ~85% in de diagnostische klassering voor de vroeg-diabetes patiënten. Op basis hiervan konden drie “syndroom-subtypen” geïdentificeerd worden, die een gemeenschappelijke biologische basis delen maar verschillen in de

oorzaak en progressie van het vroeg-diabetes ziektebeeld. De UPE metingen werden verricht op 4 verschillende anatomische plekken van de hand voor elk van de 44 personen in het cohort en in totaal konden er 16 relevante UPE-parameters geïdentificeerd worden met statistische methodieken, welke de drie TCM-gebaseerde subtypen konden beschrijven. Op basis hiervan werd de indicatie verkregen dat UPE een veelbelovende methode kan zijn voor bestudering van TCM-diagnostiek. Verdere studies zijn nodig waarbij grotere cohorten worden betrokken met een groter aantal TCM-experts en voor verschillende syndroomtypen. In aanvulling daarop kunnen deze analyses worden gecombineerd met metabolomics metingen, zodat een meer omvattend beeld verkregen kan worden met een biochemische onderbouwing van zowel UPE als TCM-diagnose.

In Chinese geneeskunde heeft "personalized medicine" betrekking op een diagnose op maat en het gebruik van een voor elk individu geoptimaliseerde kruidenmengselextract. Het Chinese kruidenextract is gemaakt op basis van de etnofarmacologische observaties in de klinische praktijk. Op basis hiervan zijn er concepten ontwikkelt zoals "inheemse medische preparaten" en op kruiden-"smaak" gebaseerde interventie eigenschappen. Deze concepten zijn nauw gerelateerd aan TCM-diagnose en reflecteren de persoonlijke holistische respons op kruiden. Uitgebreide en systemisch-gebaseerde metingen zijn nodig om deze TCM-concepten te kunnen onderzoeken. In dit proefschrift is foton-geïnduceerde vertraagde luminescentie (Photon-induced Delayed luminescence, DL) gebruikt als snelle, directe en systemische meetmethodiek om deze eigenschappen van kruiden te karakteriseren.

In Hoofdstuk 4 wordt de ontwikkeling van een op DL gebaseerd protocol beschreven. De resultaten geven aan dat het watergehalte van kruidenmaterialen een belangrijke factor is om stabiele en reproduceerbare DL gegevens te verkrijgen. Derhalve is er veel aandacht besteed aan de bewaarcondities van de materialen, die door DL gemeten worden. Om de toepasbaarheid van de DL-methode te exploreren zijn er

experimenten uitgevoerd zoals beschreven in Hoofdstuk 5 en 6. In Chinese geneeskunde wordt de term “inheemse medische preparaten” gebruikt om aan te duiden dat de medicinale planten gegroeid zijn onder unieke omgevingsfactoren, waardoor de optimale kwaliteit en daaraan verbonden optimale therapeutische werking gegarandeerd kan worden. Het etnofarmacologische concept is gebaseerd op de premisse dat omgevingsfactoren een directe invloed hebben op de constitutie en kwaliteit van medicinale planten. In Hoofdstuk 5 reflecteren zowel de chemische analyses als de DL-metingen dat de compositie en kwaliteit van rabarber beïnvloed worden door de hoogte waarop deze gekweekt is. De geïdentificeerde correlaties tussen de chemische componenten en DL-metingen wijzen op een mogelijke correlatie van DL-metingen met de bioactive eigenschappen van rabarber. Toekomstige studies met een bredere selectie van rabarbermonsters in combinatie met een meer omvattende analyse van secondaire metabolieten kunnen hier meer inzicht in geven. Met name het verkrijgen van gedetailleerde correlatienetwerken tussen secondaire metabolieten en DL-parameters kunnen het potentieel van de DL-methodiek verder onderbouwen om kruidenkwaliteit te karakteriseren. Dergelijke studies kunnen ook verdere onderbouwing geven om DL als methodiek in te zetten voor evaluatie van kruideninterventies.

In TCM worden aan alle kruiden een therapeutische toepassing toegeschreven op basis van “smaak”, welke aangeeft tot welke hoofdgroep van de etnofarmacologische indeling deze behoort. Dit geeft dan tevens aan welke therapeutische werking aan dit kruid zijn toegeschreven. Deze zogenaamde “smaak”-eigenschap geeft een mogelijke handvat voor de ontwikkeling van “personalized medicine” concepten. In Hoofdstuk 6 is beschreven hoe DL-parameters gebruikt kunnen worden om kruiden te onderscheiden binnen de “zoete”-categorie en tussen andere categorieën. In aanvulling werden immuun stimulerende effecten gemeten met behulp van dendritische cellen en het bleek dat “zoete” kruiden significant verschillen van “scherp prikkelend” en “bitter” smakende kruiden. Deze

observatie ondersteunt de resultaten van de DL-metingen. Echter, DL kan geen significant verschil aantonen tussen de “scherp prikkelende” en bittere” kruiden. Dit geeft een mogelijke limitering aan van de toepassing van DL, maar kan ook een resultaat zijn van de beperkte kruidencollectie die voorhanden was in deze studie. Bovendien zijn cel-gebaseerde studies mogelijk niet voldoende om systeem farmacologische effecten te kunnen waarnemen van kruidenextracten. Meer systeem gebaseerde studies (bijvoorbeeld met het zebravismodel) zouden een additioneel platform kunnen bieden om de omvattende werking van bioactieve kruiden te weerspiegelen zoals waargenomen met DL. De resultaten in Hoofdstuk 5 en 6 laten zien dat DL breed toepasbaar zou kunnen zijn in de studie van kruidengeneeskunde. Derhalve is DL veelbelovend als robuuste technologische methodiek om kruiden zowel kwalitatief als kwantitatief te onderzoeken.

In conclusie, zowel UPE als DL zijn veelbelovend om systeemgeneeskunde te onderbouwen en te onderzoeken. De resultaten uit dit proefschrift kunnen gebruikt worden om toekomstige research programma’s te ontwikkelen met als rode draad de concepten vanuit TCM. Verdere ontwikkeling van de analytische technieken en statistische methoden gaan het mogelijk maken om UPE en DL te integreren in biomedisch onderzoek en beide methoden kunnen zo een waardevolle bijdrage leveren aan en nieuwe inzichten bieden in “personalized medicine”.

Curriculum Vitae

Mengmeng Sun was born on March 23rd, 1985 in Changchun, Jilin province, P.R. China. In 2003, he received his senior high school degree from No.11 High School of Changchun and started to study in Shandong University of Technology, majoring in biology. He received his Bachelor's degree in 2007. In 2008, he was admitted by Changchun University of Chinese Medicine as a master student in the major of pharmaceutical chemistry and drug delivery. He received his Master's degree in 2011.

In 2012, he got a scholarship from the China Scholarship Council and started his research as a PhD candidate at Analytical BioSciences, Leiden Academic Centre for Drug Research, Leiden University. His research is mainly focusing on development of personalized health monitoring using ultra-weak photon emission based on traditional Chinese medicine-based concepts.

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