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Dual-Index Evaluation of Character Changes in *Panax ginseng* C. A. Mey Stored in Different Conditions

Chen-yang Li,[†,‡,§,#] David Tai-wai Lau,[⊥,#] Tina Ting-xia Dong,[⊥] Jian Zhang,[†,§] Roy Chi-yan Choi,[⊥] Hai-qiang Wu,[∥] Li-yan Wang,[∥] Rui-sha Hong,[†] Shi-he Li,[†] Xun Song,[†,§] Tian Yu,[†,§] Wei-wei Su,[‡] Karl Wah-keung Tsim,[*,⊥] and Zhen-dan He[*,†,§]

[†]Department of Pharmacy, School of Medicine, Shenzhen University, Shenzhen 518060, China

[‡]School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China

[§]Institute of Biotherapy, Shenzhen University, Shenzhen 518060, China

[⊥]Division of Life Science, The Hong Kong University of Science and Technology, Hong Kong, China

[∥]College of Life Science, Shenzhen University, Shenzhen 518060, China

ABSTRACT: *Panax ginseng* C. A. Mey has been used as a traditional medicine and functional food in Asia for thousands of years for its improvement of human immunity and metabolism and its antitumor and antifatigue activities. This study reports the impact of storage conditions and storage period on the quality of *P. ginseng*. The contents of four major ginsenosides in *P. ginseng* and phosphorylation activities of Akt of ginseng extracts were affected by both storage conditions and storage period. In contrast, the ATP generation capacity of ginseng extracts was affected by storage conditions, but not by storage period. The results showed that the quality of *P. ginseng* could be well maintained at a relative humidity between 70% and 90%, and dry conditions might decrease the quality of *P. ginseng*. Through dual-index evaluation, the present study extended our knowledge on the changes of ginsenosides and bioactivities in *P. ginseng* with respect to different storage conditions and storage periods.

KEYWORDS: *Panax ginseng, ginsenosides, bioactivity, storage condition, storage period*

INTRODUCTION

Panax ginseng C. A. Mey, which belongs to the family Araliaceae, has been used as an ancient and famous traditional Chinese medicine and functional food in Asia for more than 4000 years. *P. ginseng* has been proved to have the effects of boosting health and prolonging life by many clinical trials. Researchers have found the ginsenosides are the main active constituents in *P. ginseng*. Until now, more than 60 kinds of ginsenosides have been isolated and identified from *P. ginseng*, which are divided into three categories: protopanaxadiol-type ginsenosides, i.e., ginsenoside Rb1, ginsenoside Rd, etc.; protopanaxatriol-type ginsenosides, i.e., ginsenoside Re, ginsenoside Rg1, etc.; and oleanolic-type ginsenosides, i.e., ginsenoside Ro. A great number of studies indicated that ginsenosides can promote learning and memory, enhance nerve growth,[1,2] modulate nerve transmission by decreasing the availability of neurotransmitters,[3−5] have anticancer[6−9] and immune effects,[10] etc. Ginsenosides Rg1, Re, Rb1, and Rd are four major active compounds in *P. ginseng*, whose bioactivities coincide with the effects of *P. ginseng*. The research showed that ginsenoside Rg1 can selectively enhance immune function in aged animals, ameliorate age-related alterations in both behavior and motor response, promote hippocampus neuronal function of aged rats, and provide partial protection against the excitotoxic effect of glutamate;[11] ginsenoside Re improved learning and memory abilities of natural aging and was able to relieve fatigue by decreasing SOD content and increasing SOD activity;[12,13] ginsenoside Rb1 can significantly enhance the levels of NO, IL-6, and TNF-α to improve the human immunity;[14] ginsenoside Rd also has many bioactivities, such as radical scavenging activity and antiaging.[15] So the content of these four ginsenosides directly influences the bioactivities and quality of *P. ginseng*.

The research has demonstrated that the effect of storage period on total ginsenosides content was significant in all four ginseng types (fresh ginseng, white ginseng, taeguek ginseng, and red ginseng), and total ginsenosides was 20−80% of the original concentration after one-year storage.[16] Individual ginsenoside content and chemical profiles were different with respect to storage periods. The storage period was observed to affect the content of each ginsenoside, except for Rc, Rb1, Rg2, and F2. The effect of storage period on major ginsenosides of fresh ginseng was significant.

On the other hand, the storage conditions always influence the quality of medicine and food, referring to the content of chemical profiles and their bioactivities. So more and more attention was attracted to the study of storage conditions on the quality of medicine and food, such as storage humidity, storage temperature, isolation air, sunlight, and storage time.

The present study was conducted to discuss the influence of storage humidity, storage temperature, and storage period on the content of ginsenosides and bioactivities of *P. ginseng*.

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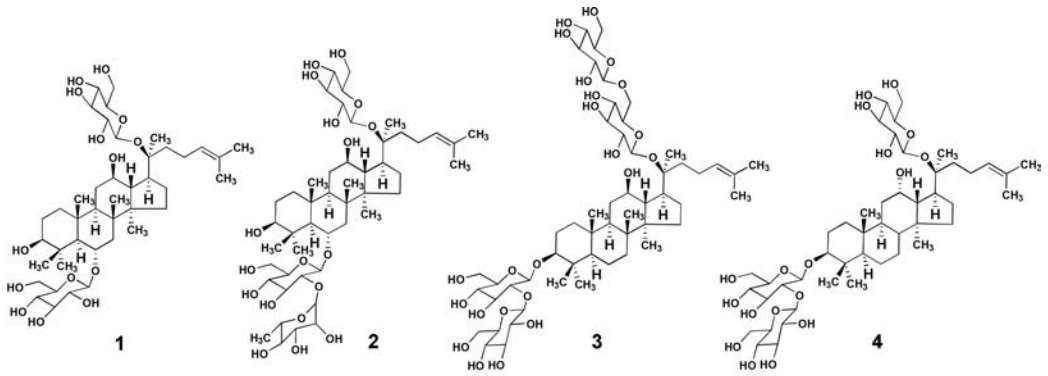
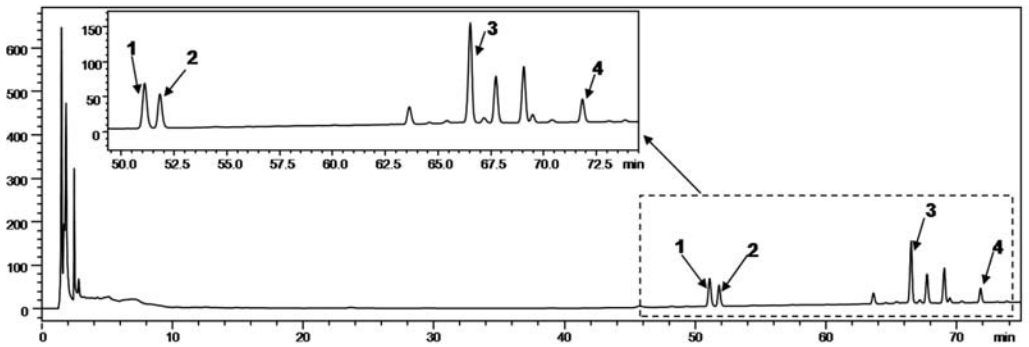
Table 1. Average Temperature and Rainfall in Guangdong Province during the Past Three Years

year/ month	average temperature (°C)	rainfall (mm)	year/ month	average temperature (°C)	rainfall (mm)	year/ month	average temperature (°C)	rainfall (mm)
2009.1			2010.1	14.2	85	2011.1	9.6	17
2009.2			2010.2	16.0	79	2011.2	14.4	42
2009.3	17.7	142	2010.3	12.5	41	2011.3	15.3	52
2009.4	21.4	152	2010.4	19.7	27	2011.4	22.2	19
2009.5	25.1	203	2010.5	25.8	299	2011.5	24.5	259
2009.6	27.4	299	2010.6	26.2	391	2011.6	28.3	208
2009.7	28.7	183	2010.7	28.7	142	2011.7	28.7	211
2009.8	29.3	218	2010.8	28.7	147	2011.8	29.0	90
2009.9	28.8	120	2010.9	27.5	332	2011.9	27.1	89
2009.10	25.2	24	2010.10	24.2	49	2011.10	23.3	118
2009.11	17.2	64	2010.11	18.7	9	2011.11		
2009.12	14.9	39	2010.12	14.0	38	2011.12		



A Structures of four main active ginsenosides



B HPLC chemical profile of *P. ginseng*

Figure 1. (A) Structures of four ginsenosides. (B) HPLC chemical profile of *P. ginseng*. The methanol extract of *P. ginseng* was subjected to HPLC analysis. HPLC was obtained with an Ultimate XB-C18 (4.6 mm × 250 mm, 5 μm). Mobile phase: acetonitrile (A)−water (B) [0−35 min, 17% A; 35−75 min, 17% A −42% A], employing gradient elution at a flow rate of 1.0 mL/min. Chromatograms were record at 203 nm. The four ginsenosides are separately denoted as 1 (Rg1), 2 (Re), 3 (Rb1), and 4 (Rd). The contents of the four ginsenosides were separately determined with SD values less than 5% of the mean (n = 3).

MATERIALS AND METHODS

***P. ginseng* and Chemicals.** The cultivated crude herbs of *P. ginseng* were collected from Paektu Mountains in Jilin City, China, and authenticated by Prof. Hong Xu, Hong Kong University of Science and Technology. A voucher specimen has been deposited with the School of Medicine, Shenzhen University, Shenzhen, China. The standards of ginsenosides Rg1, Re, Rb1, and Rd were isolated from *P. ginseng* in our laboratory, with purities of >98%. Chemicals used in this study were of reagent grade from XingKe Biochemistry Co. (Shanghai, China) and Fisher Scientific (Bridgewater, NJ, USA).

Storage Conditions of *P. ginseng*. *P. ginseng* was stored in specific constant temperature and humidity cases (version: LHS-250SC, Qixin, Shanghai), which were used as four sets of different storage conditions according to the natural climatic conditions in southern China (Table 1): mild condition (70% relative humidity, 30 °C; similar to summer), moist condition (90% relative humidity, 25 °C; similar to rainy season), dry condition (50% relative humidity, 25 °C; similar to spring or autumn), and dry and cold condition (50%

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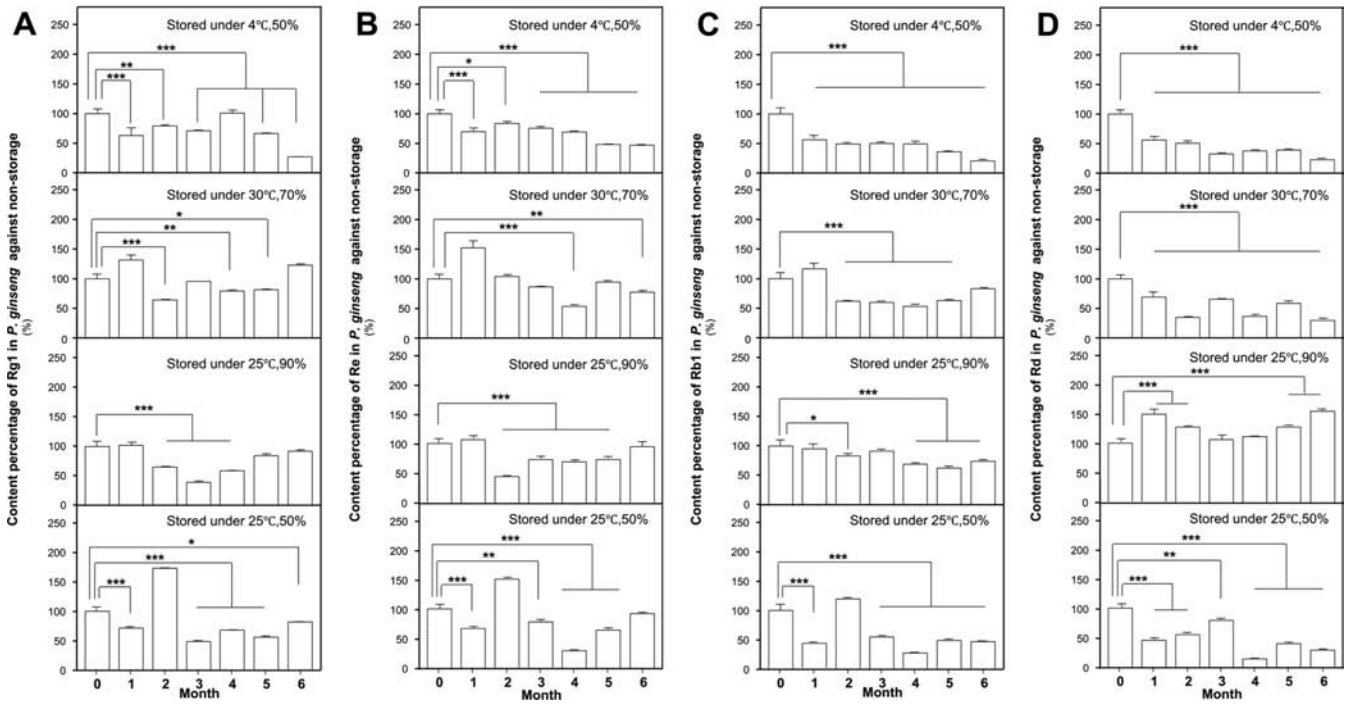

Figure 2. Comparison of content percentages (% compared with nonstored ginseng) of four major ginsenosides in *P. ginseng* stored under four different conditions depending on storage periods. The content percentages of four major ginsenosides (Rg1, Re, Rb1, and Rd) in nonstored ginseng (sampling in month 0, T0) were regarded as 100%, respectively. Other data are expressed as the content percentage against nonstored ginseng, mean ± SEM, $n = 3$. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ as compared to the nonstored ginseng.

relative humidity, 4 °C; similar to winter) [in a garage at the School of Medicine, Shenzhen University, Shenzhen, Guangdong Province, China]. The range of relative humidity was 50−90% (average: 65%), and the range of temperature was 4−30 °C (average: 21 °C) during the storage time. These conditions were selected to represent a mild, a moist, a dry, and a dry and cold environment, because harvested *P. ginseng* might be stored and/or transported under various environmental conditions before processing.

Sample Preparation. The ginseng samples were collected for six months after placing them in the constant temperature and humidity equipment, while nonstorage ginseng samples were also analyzed, which were defined as T0 samples, and others were defined as T1−T6.

Sample Preparation for Ginsenoside Analyses. The ginseng samples were crushed to a coarse powder and weighed out accurately at 0.5 g of pulverized ginseng samples. The powder was mixed with 10 mL of methanol and extracted three times by ultrasonic methods for 30 min each. The mixture was centrifuged at 540 rpm for 5 min. The supernatant was diverted to a flask. Then the operation was repeated twice. The residue was washed twice with 5 mL of methanol. The extracted liquid and scrubbing liquid were merged and evaporated to dryness. The residues were transferred into 100 mL volumetric flasks and brought to volume with methanol.

Sample Preparation for Bioactive Analysis. One gram of *P. ginseng* was weighed out, and then the ginseng samples were soaked in 50 mL of ultrapure water for 1 h. The ginseng samples were boiled for 2 h at 250 °C with frequent agitation. Meanwhile hot ultrapure water (volume of water is TCM dependent) was added and then filtered. The extract was lyophilized into a powder, and then ultrapure water was added to the extract powder to give a 20 mg/mL concentration. The samples were disrupted by ultrasound for 1 h and centrifuged, and the supernatant was collected into a 15 mL flask and filtered using a 0.22 μm filter.

HPLC Analysis of Ginsenosides. The analysis of ginsenosides was conducted with an Agilent 1260 Infinity HPLC system coupled with a DAD detector. The separation of ginsenoisdes was performed with an Ultimate XB-C18 analytical column (5 μm, 250 × 4.6 mm i.d.), and the absorbance was measured at 203 nm. The mobile phase was water (solvent A) and acetonitrile (CH_3CN, solvent B). The injection volume was 10 μL, and the gradient (A:B = V_A:V_B) of the mobile phases was as follows: initially, A B = 83 17; 0−35 min, A B = 83:17; 35−75 min, A:B = 58:42. Total HPLC run time was 75 min, and the flow rate was 1 mL/min.

A typical HPLC chromatogram of *P. ginseng* is shown in Figure 1.

Cell Culture. H9c2 (purchased from American Type Culture Collection, ATCC) is a subclone cell line derived from embryonic BD1X rat heart tissue and exhibits many of the properties of skeletal muscle. Dulbecco's modified Eagle's medium (DMEM) [Gibco: 12100-046], supplemented with 10% heat-inactivated fetal bovine serum (HIFBS), 100 IU/mL of penicillin [Sigma P3032], and 100 μg/mL of streptomycin [Sigma S9137], and 0.6 mM $NaHCO_3$, pH 7.4, were used. Incubation temperature was 37 °C, and the CO_2 concentration was 5%.

Human umbilical vein endothelial cells (HUVEC) were obtained from Hong Kong Baptist University and cultured in medium 199 supplemented with 20% fetal bovine serum, 20 μg/mL endothelial cell growth supplement, 90 U/mL heparin, and 1% penicillin−streptomycin in a humidified incubator at 37 °C with 5% CO_2. HUVEC between passages 3 to 8 were used in these studies to ensure the genetic stability of the culture. A stock solution of VEGF (50 mg/mL) was prepared in phosphate buffer saline, as a positive control.

ATP Generation Capacity Assay. Cell viability after drug treatment (24 h) was measured using the Trypan Blue exclusion assay. The H9c2 cell line was cultivated in DMEM, supplemented with 10% HIFBS under 37 °C, 5% CO_2. After the indicated periods of drug incubation time, the ATP-GC assay was performed. The medium was aspirated, and the cells were treated with digitonin (50 μg/mL) in incubation buffer (120 mM KCl, 5 mM KH_2PO_4, 2 mM EGTA, 10 mM HEPES, 0.1 mM $MgCl_2$, 0.5% BSA, pH 7.4) for 3 min at 37 °C. After aspirating the digitonin, glutamate (5 mM), malate (5 mM), and ADP (60 μM) were added to the cells for mitochondrial ATP generation, which was terminated by the addition of 60 μL of perchloric acid (30%, w/v) at specific time points (0, 7.5, 15 min), and the reaction mixtures were centrifuged at 600*g* for 10 min at 4 °C. An aliquot (120 μL) of the supernatant was mixed with 90 μL of 1.4 M

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$KHCO_3$ for neutralization. The mixtures were centrifuged again at 600*g* at 4 °C, and the supernatants were measured for ATP content using a bioluminescence assay.[17,18]

Activation of the PI3K-Akt Signaling Pathway. Cells were dissolved in 2× direct lysis buffer (100 mM Tris-Cl (pH 6.8), 8% sodium dodecyl sulfate (SDS), 20% glycerol, β-mercaptoethanol, and bromophenol blue). Lysates were boiled at 95 °C for 10 min. An equal amount of cell lysates was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. The blot was then probed with phospho-Akt (Ser473) and total Akt, followed by reaction with horseradish peroxidase-conjugated secondary antibody. These antibodies were purchased from Cell Signaling, Inc. The signal was detected using enhanced chemiluminescence and was determined by densitometry.

Statistical Analysis. *P. ginseng* samples from all storage conditions were extracted at least in triplicate. All data were analyzed by using analysis of variance (ANOVA) in the general linear models procedure of the Statistical Analysis Systems software package (SAS, version 8.0). Differences between group means were analyzed by Duncan's multiple-range test. Statistical significance was set at the 0.05 probability level.

RESULTS AND DISCUSSION

Change in Each Ginsenoside Content. The contents of ginsenosides Rg1 and Re under all conditions fluctuated during storage; however, the content reductions of ginsenosides Rg1 and Re in the conditions of 30 °C, 70% humidity were the least; in this condition ginsenoside Rg1 was reduced by up to 60%, and Re was reduced by up to 55% (Figure 2A and B). The content of ginsenoside Rb1 decreased over the storage period under all the conditions, which suggested that the storage period was a major influencing factor on the content of ginsenoside Rb1 in *P. ginseng*. However, the change in the conditions of 25 °C, 90% humidity was the slightest, with the rate of change being 3%−18% every month, and the change in the conditions of 30 °C, 70% humidity was less than the other two conditions, which suggested that hotter and wetter storage conditions could stabilize ginsenoside Rb1 in *P. ginseng* (Figure 2C). The content of ginsenoside Rd under the conditions of 25 °C, 90% humidity was approximately stable during storage, even increasing slightly, compared with the conditions of 25 °C, 90% humidity. The other three conditions all showed quick decreases (Figure 2D). The experimental data showed that the four constituents changed most severely under conditions of 4 °C, 50% humidity.

Change in Total Ginsenoside Content and Its Rate of Change. The total content of four major ginsenosides stored under the conditions of 4 °C, 50% humidity decreased quickly (Figure 3). After one month, the total content was reduced to 60% of the original concentration, after maintaining stability for three months, and then continued to fall, to nearly 30% of the original concentration. Under the conditions of 25 °C, 50% humidity, the total content of the four ginsenosides also decreased significantly; the content remained fluctuating at 40−60% after three months. Furthermore, the change rates (CR) of total ginsenoside content of each month were calculated by the formula CR = $(C_n - C_{n-1})/C_{n-1} \times 100\%$. The results showed that change rates under the conditions of 25 °C, 50% humidity during month 1 to month 5 were higher than other storage conditions, which meant the content of total ginsenosides was unstable if stored under the conditions of 25 °C, 50% humidity (Figure 4). Meanwhile, the change rate of total ginsenoside content under the conditions of 25 °C, 90% humidity was the lowest among the four storage conditions, and the total ginsenoside content was stable when stored under this condition.

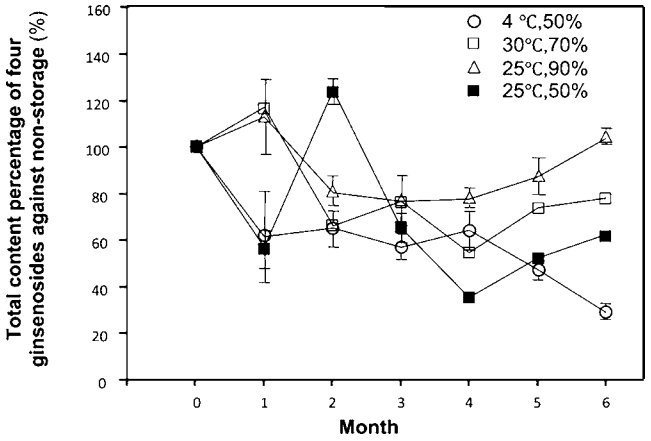

Figure 3. Comparison of total content percentages (% compared with nonstored ginseng) of four major ginsenosides in *P. ginseng* stored under four different conditions depending on storage periods. The total content percentages of four major ginsenosides (Rg1, Re, Rb1, and Rd) in nonstored ginseng (sampling in month 0, T0) were regarded as 100%, respectively. Other data are expressed as the content percentage against nonstored ginseng, mean ± SEM, $n = 3$.

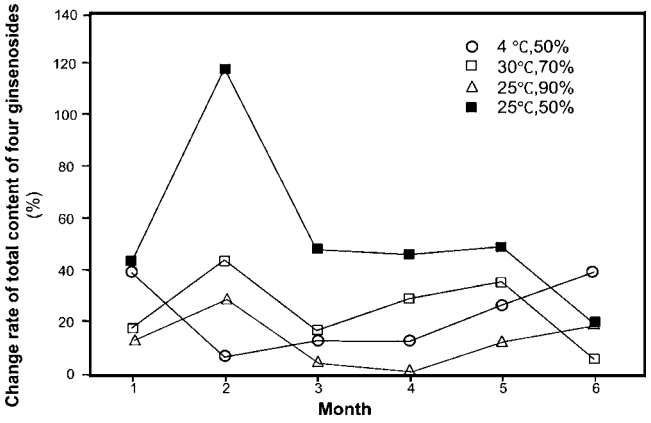

Figure 4. Comparison of change rate of total content of four major ginsenosides in *P. ginseng* stored under four different conditions depending on storage periods. The change rate (CR) was calculated by the formula CR = $(C_n - C_{n-1})/C_{n-1} \times 100\%$.

Change in Activity of ATP Generation Capacity. The results of ATP generation capacity demonstrated that the activities under the conditions of 25 °C, 50% humidity and 4 °C, 50% humidity dropped faster than the other two conditions during just four months. After two months the results under all the conditions rose again, but the conditions of 25 °C, 50% humidity and 4 °C, 50% humidity were also worse than the two other conditions (Figure 5).

Change in Activation of PI3K-Akt Signaling Pathway. We tested the phosphorylation activities of Akt of storage samples under four conditions. The activities of Akt phosphorylation under all conditions declined dramatically during four months, but after two months the activities rose back slightly (Figure 6). The results of Akt phoshorylation showed that the decline of phoshorylation capacity under 25 °C, 50% humidity was the sharpest, and the change in the conditions of 30 °C, 70% humidity was greater than the other two conditions.

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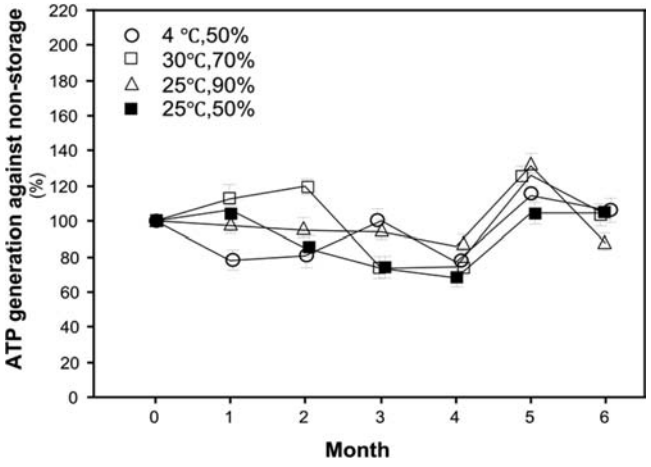

Figure 5. Comparison of ATP generation capacity of *P. ginseng* stored under four different conditions depending on storage periods. The ATP generation capacity of *P. ginseng* in nonstored ginseng (sampling in month 0, T0) was regarded as 100%. Other data are expressed as the active capacity percentage against nonstored ginseng, mean ± SEM, $n = 3$.

In summary, Guangdong Province is the main port of TCM's export in southern China; the exports of TCM in 2010 were estimated as approximately 114 000 tons, which were valued at $130 million. *P. ginseng* is Guangdong's main TCM export; the amount of annual exports in 2009 reached $15.8 million. So it is very necessary and important to investigate the influence of storage conditions on the quality of *P. ginseng*.

According to the content data of chemical components, we found that ginsenosides Rg1, Re, and Rb1 remained stable under the conditions of 30 °C, 70% humidity, and ginsenosides Rb1 and Rd and the total content of the four ginsenosides remained stable under the conditions of 25 °C, 90% humidity, even increasing slightly. On the other hand, the experimental data showed the four constituents changed most severely under the conditions of 4 °C, 50% humidity. This implied that the dry air and cold temperature could damage the ginsenosides in *P. ginseng*, so the crude ginseng herb should not be kept in dry and cold conditions. As the change of each ginsenoside was different, the separate evaluation of each ginsenoside was not comprehensive, so the total content variance of these four ginsenosides was also examined. The total content of four major ginsenosides stored under the conditions of 4 °C, 50% humidity and 25 °C, 50% humidity decreased quickly. Furthermore, the change rates under the conditions of 25 °C, 50% humidity during month 1 to month 5 were higher than the other storage conditions. The change rate under the conditions of 25 °C, 90% humidity were the lowest among the four storage conditions, which meant the total ginsenoside content was unstable if stored under the conditions of 25 °C, 50% humidity and was stable when stored under the conditions of 25 °C, 90% humidity. Compared with the bioactivities of crude extracts of *P. ginseng*, we also found that the ATP generation capacity under the conditions of 25 °C, 50% humidity and 4 °C, 50% humidity declined faster than the other conditions, and the change in phosphorylation activities of Akt was the highest under the conditions of 25 °C, 50% humidity. The change of both chemical content and activities was coincident. So the four types of ginsenosides (Rg1, Re, Rb1, and Rd) might be the main active compounds for ATP generation capacity and phosphorylation activity of Akt. Further research on the bioactivity of each compound will be carried out. The results showed that the storage conditions of 4 °C, 50% humidity and 25 °C, 50% humidity might decrease the quality of *P. ginseng*, so dry conditions are not recommended for storage of *P. ginseng*. By the way, the quality of *P. ginseng* could be well maintained at a relative humidity of between 70% and 90%.

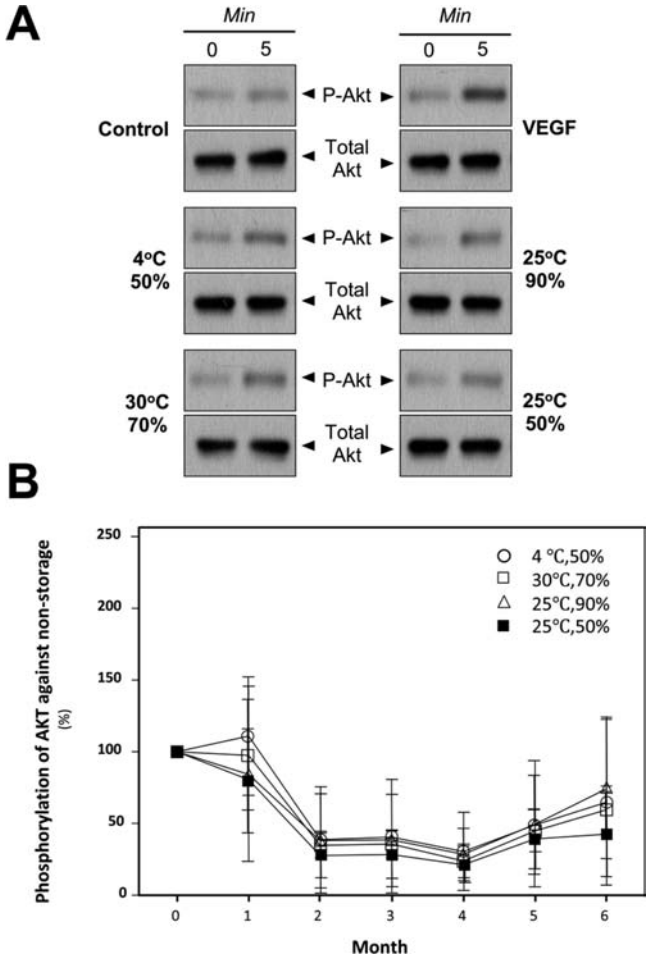

Figure 6. Comparison of phosphorylation activity of Akt of *P. ginseng* stored under four different conditions depending on storage periods. (A) HUVEC cells were serum starved and then treated with the extracts for 5 min. Cultures were collected to measure the phosphorylation of Akt (serine 473) and total Akt by Western blotting. Representative images from the samples at the month 0 (T0) are shown. VEGF (10 ng/mL) served as a positive control. (B) Phosphorylation activity of Akt of *P. ginseng* in nonstored ginseng (sampling in month 0, T0) was regarded as 100%. Other data are expressed as the phosphorylation activity percentage against nonstored ginseng, mean ± SEM, $n = 3$.

AUTHOR INFORMATION

Corresponding Author

*(Z.-D.H.) Tel: +86-755-86671916. Fax: +86-755-86671906. E-mail: hezhendan@gmail.com. (K.W.-K.T.) Tel: +852-23587332. Fax: +852-23581559. E-mail: botsim@ust.hk.

Author Contributions

#C.-Y. Li and D. T.-W. Lau contributed equally to this work.

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Notes

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■ ABBREVIATIONS USED

SOD, superoxide dismutase; NO, nitric oxide; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; TCM, traditional Chinese medicine; HPLC, high-performance liquid chromatography; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; HIFBS, heat-inactivated fetal bovine serum; HUVEC, human umbilical vein endothelial cells; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ANOVA, analysis of variance; SAS, Statistical Analysis Systems software package

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