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Effects of deuterium-depleted water on skin fibroblasts and melanoma cells

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Abstract: The effects of different concentrations of deuterium-depleted water on the proliferation and lactate metabolism of normal skin fibroblasts CCD-1095sk, and on the tyrosinase activity and melanin production of B16 melanoma cells were studied. At the same time, the effect of deuterium-depleted water on the repair of UV-damaged cells was observed. The MTT method was used to detect the effects of three different concentrations of deuterium-depleted water (25, 50 and 105 ppm) on the proliferation of CCD-1095sk cells, and the lactate content in the culture medium was determined; B16 melanoma cells of mice in the logarithmic growth phase were added with different concentrations of deuterium-depleted water for 72 h, and the melanin content and tyrosinase activity were determined; the tyrosinase activity was determined by the in vitro oxidative dopa reaction method; the cell proliferation rate was determined by treating skin fibroblasts irradiated with UV light with different concentrations of deuterium-depleted water. In the early stage of cell culture, 50 ppm and 105 ppm deuterium-depleted water promoted the growth of CCD-1095sk cells and inhibited lactate metabolism. In vitro biochemical experiments showed that three different concentrations of deuterium-depleted water could inhibit tyrosinase activity to varying degrees. Cell experiments showed that after 72 hours of action, 50 ppm and 105 ppm deuterium-depleted water could significantly inhibit tyrosinase activity and reduce the production of melanin in B16 melanoma cells. The difference was statistically significant ($P < 0.05$). UV damage experiments showed that 50 ppm deuterium-depleted water could reduce the inhibitory effect of UV radiation on cell proliferation. The difference was statistically significant ($P < 0.05$). Deuterium-depleted water has potential value in cosmetic applications. Keywords: deuterium-depleted water, skin fibroblasts, melanoma cells B16, tyrosinase activity, UV damage. Chinese Library Classification Number: R969.4 Document Identification Code: A

Effect of Deuterium-depleted Water on Skin Fibroblast and Melanoma Cells

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Abstract: To study the effect of different concentrations deuterium-depleted water (DDW) 1 on normal skin fibroblast proliferation and LD metabolism and the UV damage repair, as well as on tyrosinase activity and melanin of melanoma cell. Using MTT method, measure the effect of 25ppm, 50ppm , 105 ppm DDW on the CCD-1095sk cell proliferation and lactate metabolism, on the tyrosinase activity in vivo and in vitro and the formation of melanin. The effects of DDW on normal skin fibroblast cells which was injured by UV were also detected. At early step of cell culture, 50 ppm and 105 ppm DDW increased CCD-1095sk cell proliferation, and inhibited the cell content of lactic acid metabolism. In vitro, three different concentrations DDW inhibited tyrosinase activity; In 72 h , 50 ppm and 105 ppm DDW can inhibit tyrosi-nase activity in B16 cells. And the inhibition radio of DDW on the formation of melanin was significant, $P < 0.05$. For normal skin fibroblast injured

by UV, 50 ppm DDW played an important role in the restoration, which relieves growth in-hibitor caused by UV injured, P ＜ 0. 05. The DDW deserved going further study in the direction of cosmetic value. Key words: Deuterium-depleted water, Skin fibroblasts, melanoma cells B 16, tyrosinase activity, UV injured

There are many reports on the biological effects of deuterium-depleted water. The earliest report by Somlyai et al. [1] was that deuterium-depleted water can inhibit the growth rate of mouse fibroblast L929 cells and cause tumor tissue regression in transplanted tumor mice. Russian researchers recently [2-4] found that if the volume fraction of deuterium in ordinary water is reduced by 65%, it will show certain anti-tumor properties. The results of tumor inhibition mouse experiments also showed that deuterium-depleted water can inhibit tumor growth and prolong the survival of mice. Cong Fengsong [5] et al. reported that deuterium-depleted water can inhibit tumor growth and prolong the survival of mice. Deuterium water can inhibit the growth of lung cancer transplanted tumors in mice. This paper intends to study the effects of deuterium-depleted water on normal skin fibroblast proliferation, lactate metabolism, tyrosinase activity and melanin production in melanoma cells through experiments, and study the repair effect of deuterium-depleted water on cells damaged by ultraviolet radiation, and preliminarily explore the application value of deuterium-depleted water in cosmetics.

1 Materials and methods

1.1 Materials

CCD-1095sk cells and B16 melanoma cells were purchased from Cell bank of the Chinese Academy of Sciences. Deuterium-depleted water (DDW) was provided by Shanghai Chitian Ultralight Water Bioengineering Co., Ltd., with deuterium contents of 25, 50 and 105 ppm, respectively; Hyclone serum was purchased from Thermo Fisher Scientific Biological Products Co., Ltd.; MTT kit was purchased from Nanjing Keygen Biotechnology Co., Ltd.; LD detection kit was purchased from Nanjing Jiancheng; MEM and RPM1640 medium powders were purchased from GIBCO. Tyrosinase (25KU) was purchased from Worthington, and L-Dopa was purchased from

SIGMA. 1.2

Methods 1.2.1 Cell culture

Human normal skin cell line CCD-1095sk was cultured in MEM medium containing 10% imported fetal bovine serum at 37°C and 5% CO2 . B16 melanoma cells were cultured in RPMI1640 medium containing 10% fetal bovine serum at 37°C and 5% CO2 .

1.2.2 MTT assay for CCD-1095sk cell proliferation

CCD-1095sk cells (1 × 104 cells/well) were inoculated in 96-well culture plates

containing 100 ÿL culture medium. The control group was cultured with normal MEM medium, and the experimental groups were cultured with MEM medium prepared with 25, 50 and 105 ppm deuterium-depleted water, respectively. After treatment for 8, 10, 12, 24 and 48 h, 50 ÿL MTT solution was added and cultured for 4 h. After the culture medium was aspirated, 150 ÿL DMSO was added to each well, and the plates were shaken for 10 min. The absorbance OD value of each well was measured at a wavelength of 490 nm. Five replicate wells were set for each group.

1.2.3 Determination of lactic acid metabolism of CCD-1095sk cells by LD kit Collect

logarithmic phase CCD-1095sk cells, suspend them, and take equal amounts of cell suspensions into MEM medium prepared

with 25, 50 and 105 ppm deuterium-depleted water. Detect the lactic acid content of cell metabolism in the culture medium at 8, 10, 24 and 48 h. Set up three parallel controls for each group. Lactic acid (LD) content = (absorbance value of the assay tube - absorbance value of the blank tube) ÷ (absorbance value of the standard tube - absorbance value of the blank tube) × standard concentration (3 mmol/l) × sample dilution factor

1.2.4 In vitro tyrosinase activity assay [6, 7] According to the

references for tyrosine activity assay, in a 3 ml reaction system, 0.05 mol/L phosphate buffer (pH 6.8) was contained, 0.5 mmol/L L-DOPA was used as the active substrate, and the final concentration of tyrosinase was 15.29 ÿg/mL. The optical density at a wavelength of 475 nm was detected by a spectrophotometer to measure the change in reaction time. The enzyme activity was calculated from the slope of the linear portion. The extinction coefficient ÿ was 3700 (mol/L cm) 1.2. 5 Cellular tyrosinase activity assay [8-10] B16 cells were cultured in 1640 ÿ medium prepared with deuterium-depleted water at 25, 50, and 105 ppm, respectively. After 72 h, the supernatant was discarded. The cells were washed twice with PBS at pH 7.4. 10 ml/L Tri-90 ÿL of onX-100 solution was added and shaken for 5 min to dissolve the cells. After incubation at 37 ÿ, 10 ÿL of 1% L-Dopa was added to each well and incubated for 30 min. The absorbance was measured at 490 nm. Five replicate wells were set for each group. Tyrosinase activity inhibition rate = (1-average absorbance value of each concentration ÷ average absorbance value of control group) × 100% 1.2.6 Determination of melanin content [10, 11] The modified Hideya Ando method was used to culture B16 cells with different concentrations of deuterium-depleted water for 72 h, and the cell concentration was adjusted to 105 cells /mL. The cell suspension was aspirated and placed in centrifuge tubes, centrifuged and the supernatant was discarded. After resuspending the cell pellet with 200 ÿL PBS buffer, 1 mL of 1:1 ethanol ether solution was added and placed at room temperature for 30 min. Centrifuged at 3000 rpm for 5 min and the supernatant was discarded. 1 mL of 1 mol/L NaOH solution containing 10% DM-SO was added, and the absorbance value was measured at a wavelength of 470 nm after 45 min of water bath at 80 ÿ. Three parallel controls were set up for each group.

Melanin synthesis inhibition rate = [1-(absorbance value of drug well ÷ cell density of drug well) ÷ (absorbance value of control well ÷ cell density of control well)] × 100% 1.2.7 UV damage experiment CCD-1095sk cells in the logarithmic phase were collected and the cell concentration was adjusted to 105 cells /mL.

1(104 cells/well were inoculated in a 96-well culture plate containing 100 ÿL culture medium, and cultured with MEM medium prepared with 25, 50 and 105 ppm deuterium-depleted water and normal MEM medium for 4 h, respectively, and then irradiated with UV light. Irradiation conditions: UVC lamp power 15 W, wavelength 254 nm, vertical irradiation distance 20 cm, irradiation time 2 h. Then, culture was continued for 8 h, and the growth and proliferation of cells were detected by MTT. Non-irradiated cells were used as the positive control group. Each group was set up with 6 replicate wells. 1.2.8 Statistical methods All data are expressed as means ± SD, and SPSS 11.0 statistical software was used for analysis. Data were analyzed using a computer software package, and comparisons among groups were performed using analysis of variance.

2 Results

2.1 Effect of deuterium-depleted water on normal skin fibroblasts CCD-1095sk CCD-

1095sk cells were cultured with deuterium-depleted water at different concentrations. In the early stage of culture, deuterium-depleted water promoted cell growth. At 8 h, the cell proliferation rates of 25 ppm and 50 ppm deuterium-depleted water were 102.27% and 104.09%, respectively, which were statistically significant compared with the control group, P < 0.05, as shown in Figures 1A and 1B. At 10 h, the cell proliferation rate of 25 ppm deuterium-depleted water was 104.75%, which was statistically significant compared with the control group, P < 0.05. After 12 h of culture, the cells cultured with deuterium-depleted water graduallyWhen the culture was continued for 48 h, the deuterium-depleted water began to promote cell growth. Among them, the effect of 105 ppm deuterium-depleted water was the most obvious, with a proliferation rate of 103.66%, which was significantly higher than that of the control group. The difference was statistically significant, P < 0.05, as shown in Figure 1C.

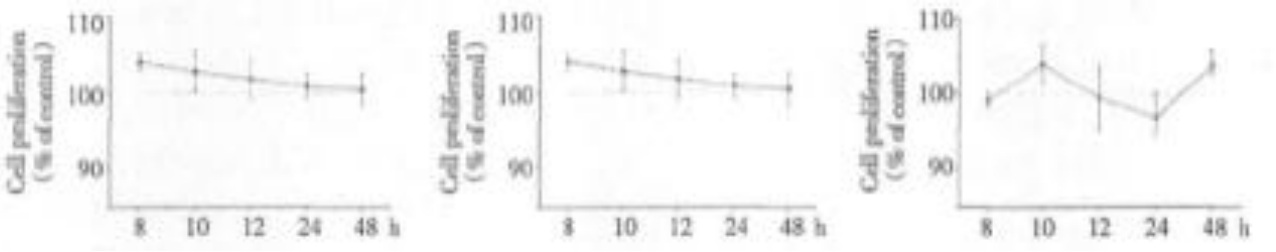

Fig. 1 Effects of DDW on the proliferation of CCD-1095sk cells.

A: 25 ppm DDW; B: 50 ppm DDW ; C: 105 ppm DDW. * : P ＜ 0. 05，vs con

2.2 Effect of deuterium-depleted water on lactic acid production in CCD-1095sk cells

CCD-1095sk cells were cultured with deuterium-depleted water of different concentrations, and the supernatant culture fluid was taken to detect the content of lactic acid (LD) metabolized by the cells. The results showed that at 10 h of culture, the lactic acid production in the cell fluid cultured with 50 ppm and 105 ppm deuterium-depleted water was significantly lower than that in the normal control group, and the lactic acid production rates were 68.47% and 85.87% of the normal group, respectively, with statistical significance, P < 0.05. At 48 h of culture, the lactic acid production rates in the CCD-1095sk cell fluid treated with 25 ppm and 50 ppm deuterium-depleted water were 81.22% and 82.64% of the normal group, respectively, with statistical significance, P < 0.05, as shown in Figure 2. The actual lactic acid content is shown in Table 1.

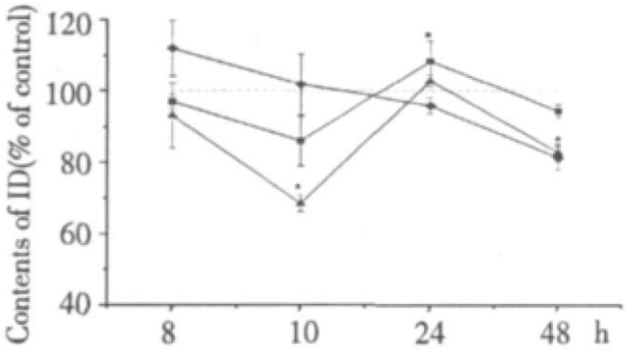

Fig. 2 DDW affects contents of LD of CCD-1095sk cells.

: 25 ppm DDW group; ▲: 50 ppm DDW group; ■: 105 ppm DDW group, * : P ＜ 0. 05，vs control

Table 1 LD content of CCD-1095sk cell (mmol /L)

	25 ppm group	50 ppm group	105 ppm group	150 ppm group
8 h	0.801 ± 0.0061	0.666 ± 0.0043	0.693 ± 0.0001	0.715 ± 0.0018
10 h	0.962 ± 0.0066	0.647 ± 0.0004*	0.812 ± 0.0252	0.945 ± 0.0229
24 h	1.319 ± 0.0008	1.415 ± 0.0005	1.493 ± 0.0058*	1.376 ± 0.0013
48 h	2.051 ± 0.0067*	2.087 ± 0.0041*	2.382 ± 0.0022	2.525 ± 0.0347

* p < 0.05. vs control (150 ppm group)

2.3 Effect of DDW on tyrosinase activity In the in vitro biochemical

reaction system, the inhibition rate of 25 ppm and 105 ppm deuterated water on tyrosinase activity was 3.67%, and the inhibition rate of 50 ppm deuterated water on tyrosinase activity was 11.89%, as shown in Fig. 3.

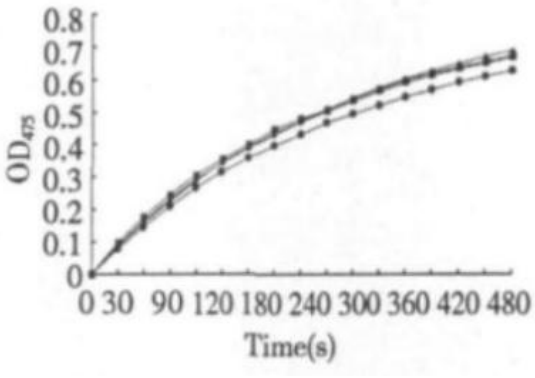

Fig. 3 Effects of DDW on tyrosinase activity.

: 25 ppm DDW group; ●: 50 ppm DDW group; ▲: 105 ppm DDW group; ■: control group

2.4 Effect of DDW on tyrosinase activity in B16 cells

After B16 melanoma cells were treated with deuterated water of different concentrations for 72 h, L-DOPA was used as the active substrate to detect the activity of tyrosinase in the cells. The inhibition rates of 50 ppm and 105 ppm deuterated water on tyrosinase were 44.32% and 24.51%, respectively, with statistically significant differences (P < 0.05), Fig. 4.

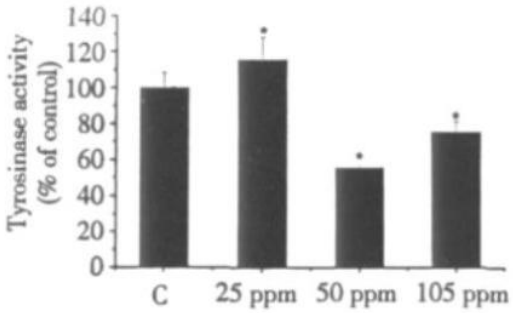

Fig. 4 Effects of DDW on tyrosinase activity in B16 cells. * : P ＜ 0. 05, vs control

2.5 Effect of deuterium-depleted water on melanin production in B16 cells

After B16 melanoma cells were treated with deuterium-depleted water of different concentrations for 72 h, the inhibition rate of melanin production in B16 cells by 50 ppm deuterium-depleted water was 39.59%, and the inhibition rates of melanin production in B16 cells by 25 ppm and 105 ppm deuterium-depleted water were 26.45% and 24.61%, respectively. Compared with the control group, the differences were statistically significant (P < 0.05), as shown in Fig. 5.

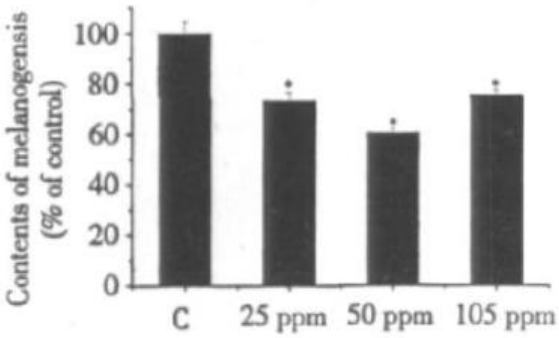

Fig. 5 Effects of DDW on melanogenesis.

2.6 Repair effect of deuterium-depleted water on cells damaged by ultraviolet light

Compared with the positive control group, the cells irradiated by ultraviolet were damaged, and the cell proliferation decreased by 30.43%, and the difference was statistically significant (P < 0.01), indicating that the model of ultraviolet

damage was successful (the cells in the positive control group were not irradiated by ultraviolet). The CCD-1095sk cells irradiated by ultraviolet were treated with different concentrations of deuterium-depleted water. The results showed that the proliferation rate of cells cultured with 50 ppm of deuterium-depleted water was 105.37%, which was statistically significant compared with the control group, $P < 0.05$, as shown

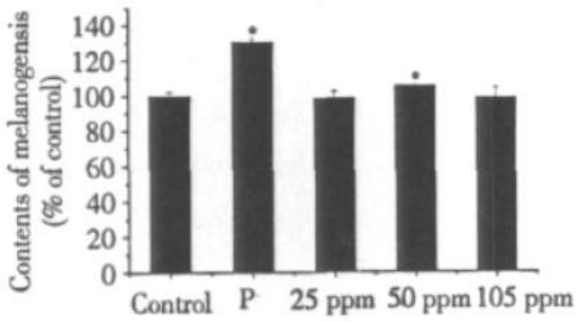

Fig. 6 Effects of DDW on cell proliferation after irradiation of UVC

C: control group; P: positive group (no UV injured) . * : P ＜ 0. 05, vs control group .

3 Conclusion

The rapid development and widespread application of modern science and technology have brought new development opportunities to the cosmetics industry. Cosmetics have evolved from basic skin care products for the purpose of cleansing and moisturizing to functional cosmetics for the purpose of delaying aging and beautifying skin tone [12]. People's attention to cosmetics It is no longer simply about beauty, but about safety. In ordinary water, the ratio of deuterium to protium (D/H) is about 1:6600, that is, the volume fraction of deuterium in water is 0.015 % [13]. We call water with a deuterium volume fraction below 0.015% deuterium-depleted water (DDW). The different masses of deuterium and protium lead to different physical and chemical properties between these two stable isotopes of hydrogen [14, 15].

The elasticity and smoothness of the skin are determined to a certain extent by the proliferation and division of the cells that make up the different components of the skin [16]. The results of this experiment showed that in the early stage of cell culture, different concentrations of deuterium-depleted water promoted cell proliferation and reduced the production of lactic acid in the cells.

The main factor affecting skin tone is melanin in the skin. The depth of skin color is mainly determined by the ability of melanocytes to synthesize melanin [16]. Tyrosinase, commonly known as polyphenol oxidase, is widely present in humans, animals and plants and is an essential enzyme in the biosynthesis of melanin. It can oxidize L-dopa into dopaquinone. Dopaquinone is unstable and can undergo a series of non-enzymatic reactions to form a heteropolymer, melanin, composed of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid units [17]. If the enzyme activity is too high, melanoma may form, which has attracted people's attention to tyrosinase. In vitro biochemical experiments have shown that three different concentrations of deuterated water can inhibit tyrosinase activity to varying degrees. Cell experiments have confirmed that 50 ppm and 105 ppm of deuterated water can significantly inhibit the tyrosinase activity of B16 cells and reduce the amount of melanin produced by cells. The difference is statistically significant ($P < 0.05$).

As the ozone layer is depleted, the amount of ultraviolet light reaching the earth's surface is increasing. Ultraviolet radiation (UVR) can increase the reactive oxygen species (ROS) in skin cells, causing DNA and corneal damage, promoting skin photoaging and cancer, as well as the occurrence of diseases such as cataracts and

immunosuppression [18-20]. Bild et al. [21] fed mice with 30 ppm deuterium-depleted water for 15 days and then irradiated them with 8.5 Gray half-lethal dose of radiation. The mice in the control group were fed with ordinary water and irradiated with the same dose of radiation. The results showed that the survival rate of mice in the experimental group was 61%, while the survival rate of mice in the control group was only 25%. Our experiment also confirmed that 50 ppm deuterium-depleted water can promote the proliferation and growth of CCD-1095sk fibroblasts damaged by ultraviolet light. Compared with the normal control group, the difference was statistically significant, $P < 0.05$. The above results suggest that deuterium-depleted water has a certain repair effect on cell DNA damage, but its specific molecular mechanism needs further study.

References

1 Somlyai G, Jancsó G, Jákli G, et al. Naturally occurring deu-terium is essential for the normal growth rate of cells, FEBS, 1993, 317: 1-4.

2 Siniak IuE, Turusov VS, Grigorev AI, et al. Consideration ofthe deuterium-free water supply to an expedition to Mars. Aviakosam Ekolog Med, 2003, 37(6) : 60-63.

3 Turusov VS, siniak IuE, Grigor ' ev AI, et al. Low-deuterium water effect on transplantable tumors. Vopr Onkol, 2005, 51: 99-102.

4 Tyrysov VS, Siniak IuE, Antoshina EE, et al. The effect of preliminary administration of water with reduced deuterium content on the growth of transplantable tumors in mice. Vopr Onkol, 2006, 52: 59-62.

5 Cong FS, Zhang YR, Wang JY, et al. Inhibitory effect of deu-terium-depleted water on proliferation of lung carcinoma cells and the possible mechanism. Chin J Cancer Biotherapy, 2009, 16, 484-489.

6 Qiu L, Chen Qx, Wang Q, et al. Irreversibly inhibitory kinetics of 3, 5-dihydroxyphenyl decanoate on mushroom (A gari-cus bisporus) tyrosinase. Bioorganic & Medicinal Chemistry, 2005, 13: 6206-6211.

7 Zhuang JX, Qiu L, Zhong X, et al. Inhibitory Effect of Ginkgolic Acids GA1 on Mushroom Tyrosinase and B-16 Cell. J Xiamen Univ, Nat Sci, 2009, 48: 103-106.

8 Emest V, Cecil K, Heino H, et al. Inhibitors of mammalian Melanocyte tyrosinase: in vitro comparisons of alkyl esters of gentisic acid with other putative inhibitors. Biochem Pharm, 1999, 57: 633.

9 Nakajima M, Shinoda I, Fukuwatari Y, et al. Arbutin increases the pigmentation of cultured melanocytes through mecha-nisma other than the induction of tyrosinase activity . Pigment Cell Res, 1998, 11: 12.

10 Ma JB, Feng SF, Li F, et al. Effects of Glabridin on B16 Cell Metablism. Fudan Univ J Med Sci, 2003, , 30: 353-355.

11 Hidey A, Akira I, Yutaka M, et al. Correlation between the number of malanosomes, tyrosinase mRNA levels, and tyrosi-nase activity in cultured murine melanoma cell in response to various melanogenesis regulatory agents. J Cellular Physiol, 1995, 163: 608.

12 Yan SX Beijing: Science Academic Press. 1995

13 Yurtsever Y and Gat JR. Stable Isotope Hydrology (JR Gatand R Gonfiantini, Eds.) , International Atomic Energy A-gency, Vienna, 1981, 103-142.

14 Collins CJA and Bowman NS (Eds) : Isotope Effects in Chemical Reactions. Van Nostrand Reinhold, New York, 1971.

15 Winberg KB. The deuterium isotope effect . Chem Rev, 1955, 55: 713-43.

16 Wang ZM, Li L, Guo SY. Application of bioactive polysaccharides in cosmetics. China Surfactant Detergent & Cosmetics, 2004, 245-248.

17 Sánchez-Ferrer A, Rodríguez-López JN, García-Cánovas F, etal. Tyrosinase: a comprehensive review of it's mechanism. BBA-protein and Molecular Enzynology, 1995 . 1247: 1-11.

18 Kim TY. Kripke ML, Ullrich SE. Immunosuppression by factors released from UV-irradiated epidermal cells: selective effects on the generation of contact and delayed hypersensi-tivity after exposure to UVA or UVB radiation. J Invest Der-matol, 1990, 94: 26-32.

19 Moan J, Peak M J. Effects of UV radiation of cells. J Photochem Photobiol B, 1989, 4: 21-34.

20 Shimmura S, Suematsu M, Shimoyama M , et al. Subthreshold UV radiation-induced peroxide formation in cultured corneal epithelial cells: the protective effects of lactoferrin. Exp Eye Res, 1996, 63: 519-526 .

21 Bild W, Stefanescu I, Haulica I, et al. Research concerning the radioprotective and immunostimulating effects of deuteri-um-depleted water. Romanian J Physiol, 1999 , 36: 205-218.

9 Pan J, Kai G, Yuan C, et al. Separation and determination of the structural isomers of m adecassoside by HPLC using β-cyclodextrin as mobile phase additive. Chromatographia , 2007, 66: 121-123.

10 Shao Y(Shao Yan), Chen YY(Chen Yunyan), Huang WW(Huang Wenwu), et al. Quantity on Extract of Centella Triterpenes and Its Preparation. I. Determination of Asiaticoside and Madecasso-side in Centella Triterpenes Cream by HPLC

Study on the content of its preparations. I. Centella asiatica and hydroxy Determination of Centella asiatica by HPLC) ÿ Chin J Pharm(China Pharmaceutical Industry Magazine), 2010, 41: 205.