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Ganoderma lucidum polysaccharide prevents oxidation and skin aging*

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Abstract

BACKGROUND: Studies have shown that Ganoderma lucidum polysaccharides have biological activities of anti-viral, anti-tumor, enhance immunity, anti-oxidative and anti-aging.

OBJECTIVE: To observe the biological effect of Ganoderma lucidum polysaccharides on the antioxidant capacity of D-galactose induced aging mice skin tissues, and to observe the effect in postponing skin aging.

METHODS: Forty-four 2-month-old Kunming mice were randomly divided into four groups: normal control group, aging model group, vitamin E group, and Ganoderma lucidum polysaccharide group. Rats in the last three groups received subcutaneous injection (nape area) of Ganoderma lucidum polysaccharide to establish rat aging models, and intragastric administration of corresponding drugs or saline was performed. After 42 days, pathological sections of back skin were obtained to assess the morphological changes of skin tissues, measure the epidermal and dermal thickness, and detect the superoxide dismutase levels and the expression of CuZn-superoxide dismutase mRNA.

RESULTS AND CONCLUSION: The epidermal and dermal thickness in the vitamin E group and Ganoderma lucidum polysaccharide group were increased when compared with the aging model group. The superoxide dismutase levels in the Ganoderma lucidum polysaccharide group were significantly higher than those in the other two groups. The decreased degree of cycle threshold value in the Ganoderma lucidum polysaccharide group was lower than that in the other two groups. The results indicate that Ganoderma lucidum polysaccharide can increase the epidermal and dermal thickness, improve the skin tissue structure, and enhance the superoxide dismutase levels and the expression of CuZn-superoxide dismutase mRNA.

Subject headings: skin aging; superoxide dismutase; galactose; RNA, messenger

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INTRODUCTION

Aging is the accumulation of gradual changes in morphology, structure, and function of an organism. Skin is exposed on the body surface and exhibits the earliest signs of aging. At the same time, skin is the largest organ of human body and is the “window” to internal organ diseases. Skin aging can not only influences beauty, but also etiologically correlates with many skin diseases. Therefore, preventing and postponing skin aging is a rapidly growing area of research[1-2].

D-galactose-induced aging animal models have been successfully developed by Chinese researchers based on the metabolic theory of aging. Intracellular D-galactose is converted to melampyrin by aldose reductase and becomes stacked in cells, changing osmotic pressure and causing cellular swelling, dysfunction, metabolic disorders, and eventually organism aging[3]. This model requires short development time, has good comparability, and provides stable and reliable results[3].

The fungi ganoderma has been used as a drug for more than 2000 years in China. Ganoderma lucidum polysaccharide, the primary biological component, exists in the natural ganoderma fruiting body as well as in the mycelium and broth following fermentation. Ganoderma lucidum polysaccharide has anti-virus, anti-tumor, anti-oxidant, and anti-aging properties and can improve immune function[4], indicating it may have potential for treating skin aging.

MATERIALS AND METHODS

Design
A randomized controlled animal experiment.

Time and setting
The experiment was completed in Guangdong Medical College between December 2008 and July 2009.

Animals
Forty-four female Kunming mice, aged 2 months old, weighing (25.8±2) g, were provided by the Laboratory Animal Center, Guangdong Medical College. Prior to experimentation, all mice were fed for 3 days to acclimate.

Main instruments and reagents
LeicaRm-2135 LEICA RM 2135 rotary microtome, computer image digital analysis system, FluorChem SP chemiluminescent gel image analysis system, ABI PRISM® 7300 fluorescence quantitative PCR Instrument, and B×51 fluorescent microscope were used in this study. Ganoderma lucidum polysaccharide was provided by the Hubei Provincial Research Institute of Microbiology and Sino-German Baoding Shidake Bioengineering Co., Ltd., China. Vitamin E was provided by the Medical Science and Technology Development Center, Guangdong Medical College, China. D-galactose was purchased from Shanghai Second Reagent Factory, China. Superoxide dismutase was purchased from Nanjing Jiancheng Bioengineering Research Institute, China. Power SYBR Green PCR two-step kits were sourced from ABI, USA.

Preparation of medicine and reagents
A total of 31.5 g D-galactose was dissolved in 250 mL physiological saline to prepare 12.5% D-galactose solution; 1 g sodium carboxymethyl cellulose and 5 mL 100% pure Vitamin E oil were triturated, and distilled water was added to 100 mL to prepare 5% vitamin E oil.

Methods
Group management and medication
Forty-four mice were randomly and evenly divided into four groups: normal control group (one mouse died), aging model group, vitamin E group, and Ganoderma lucidum polysaccharide group. All mice had freely available food and water. All mice received daily subcutaneous injection (in the nape area) or intragastric administration as follows: normal control group: 1 000 mg/kg physiological saline+250 mg/kg physiological saline; aging model group: 1 000 mg/kg 12.5% D-galactose solution+250 mg/kg physiological saline; vitamin E group: 1 000 mg/kg 12.5% D-galactose solution+250 mg/kg 5% vitamin E oil; Ganoderma lucidum polysaccharide: 1 000 mg/kg 12.5% D-galactose solution+250 mg/kg Ganoderma lucidum polysaccharide. All administrations were performed under sterile conditions. After 42 days, all mice were sacrificed by decapitation. An area of 0.5 cm×0.5 cm back skin was harvested, fixed in 10% neutral formalin, and sliced into sections for morphologucal observation and quantitative analysis. The remaining back skin was washed in double distilled water, dried, frozen in liquid nitrogen, and stored at −70 ℃. Tissue homogenate was used to determine the superoxide dismutase activity and CuZn-superoxide dismutase mRNA expression.

Preparation of 10% tissue homogenate
Following back hair shaving, a 0.5 g skin block was taken and washed with pre-cooled physiological saline. Following removal of connective tissues (subcutaneous fat included), skin was dried and weighted. The skin block and pre-cooled physiological saline (nine times volume of tissue block) were made into 10% tissue homogenate using a homogenizer. Freeze-thawing in iced water was run into triplicate to thoroughly disrupt cells.

Hematoxylin-eosin staining [5]
Paraffin sections were stained by hematoxylin-eosin to observe skin structure under a fluorescent optical microscope. Digital analysis was performed on five randomly selected visual fields from each section to determine the average epidermal and dermal thicknesses.

Determination of skin biochemical indicators
Superoxide dismutase activity was determined following the manufacturer's instructions. The calculation formula is as follows:
Superoxide dismutase activity in tissue homogenate (U/mg prot)=(absorbancecontrol tube−absorbancetest tube)/absorbancecontrol tube÷50%×total volume of reaction solution/sample solution (mL) ÷ protein level in the tissue homogenate (mg prot/mL)

CuZn-superoxide dismutase mRNA expression
Isolation and identification of total RNA: following the manufacturer's instructions for Trizol reagent kits, absorbance at 260 nm and 280 nm was determined using an ultraviolet spectrophotometer to determine RNA purity.

Reverse transcription reaction: The first strand reaction was performed according to the manufacturer's instructions of PrimeScript™ reverse transcription-PCR kits provided by Takara Biotechnology (Dalian) Co., Ltd., China. Total reaction volume was 20 μL. Three parallel tubes were designated for each sample.

Primer design and synthesis: Primer sequences are as follows:

CuZn-superoxide dismutase (amplified size 252 bp)

Upstream primer: 5'-CCAGTGCAGGACCTCATTTT-3'

Downstream primer: 5'-TCCCAGCATTTCCAGTCTTT-3'

Internal control: β-actin (amplified size 211 bp)

Upstream primer: 5' AGG CCA ACC GCG AGA AGA TG 3'

Downstream primer: 5' CGG CCA GCC AGG TCC AGA 3'

Quantitative reaction: Power SYBR® Green PCR Master Mix and reverse transcription-PCR two-step kits were used for quantitative PCR, using 5 μl cDNA as template and β-actin as internal control. The PCR reaction system (50 μL) contained 20 μmol/L upstream, downstream primers (each 3 μL), mixture (25 μL), and tri-distilled water (added till 50 μL).

Reagent	Volume (μL)
PCR Master MIX	25
5 μmol/L forward primer	0.5
5 μmol/L reverse primer	0.5
Template	5.0
Deionized water	19.0
Total volume	50

PCR reaction conditions are as follows:

Step	AmpliTaq Gold® Polymerase Activation	Cycle (40 cycles)	
		Denature	Anneal/Extend
Temperature	95 ℃	95 ℃	60 ℃
Time	10 min	15 s	1 min
Volume		50 μL	

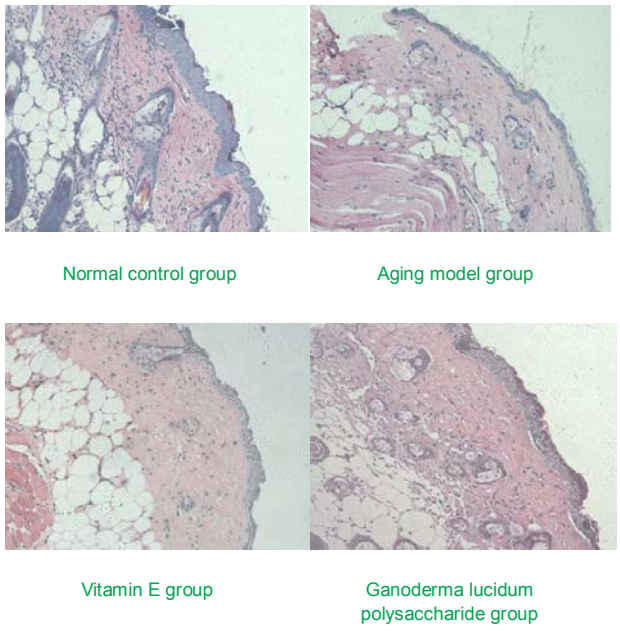
Following PCR, the data were statistically processed using an ABI 7300 system (v1.4) using the cycle threshold value method. The cycle threshold value represented the cycle number in quantitative PCR at which the fluorescence generated within a reaction well exceeded the defined threshold. There was a linear relationship between the cycle threshold value and the log of template gene copies, according to the formula: cycle threshold=[cycle threshold CuZn-superoxide dismutase mRNA−cycle threshold β-actin]$_{\text{tested sample}}$−[cycle threshold CuZn-superoxide dismutase mRNA−cycle threshold β-actin]$_{\text{corrected sample}}$, CuZu-superoxide dismutase mRNA expression $2^{-\triangle\triangle}$cycle threshold

Statistical processing

All data were statistically processed using SPSS 13.0 software (SPSS, Chicago, IL, USA) and were expressed as Mean±SEM. One-way analysis of variance was used for comparisons. Homogeneity of variance was adopted for pair wise comparison among the means. The Bonferroni method was adopted for equal population variances and Tamhane's T2 test for unequal population variances. The significance critical level was set at α=0.05, with $P < 0.05$ considered significant.

RESULTS

Observation of skin structure through pathological sections (Figure 1)



Ganoderma lucidum polysaccharide induced thickening of the skin structure, each layer of epidermal cells exhibited good structure, the dermal layer was thick, there was a clear boundary between epidermal and dermal layers, with an obvious basement membrane borderline, and the dermal collagen fibers were uniformly distributed. Compared with the normal control group, chromatin was more uniform, compact, cell layers were increased, subcutaneous fat was richer, and the structure of hair follicle, sebaceous gland, and sweat gland was more complete in the Ganoderma lucidum polysaccharide group.

Figure 1 Observation of pathological sections of skin tissues in the normal control group, aging model group, vitamin E group and Ganoderma lucidum polysaccharide group (Hematoxylin-eosin staining, ×200)

Quantinative analysis on the computer image of the thickness of mouse epidermis and dermis (Table 1)

Table 1 Computer image analysis results of mouse epidermal and dermal thicknesses ($\bar{x}\pm s$, μm)

Group	*n*	Epidermal thickness	Dermal thickness
Normal control	10	19.90±4.54	151.70±21.58
Aging model	11	12.02±1.21[a]	110.58±10.49[a]
Vitamin E	11	15.3±1.41[c]	139.24±13.81[bc]
Ganoderma lucidum polysaccharide	11	17.61±1.82[cd]	148.66±11.63[c]

[a]$P < 0.01$, [b]$P < 0.05$, *vs.* normal control group; [c]$P < 0.01$, *vs.* aging model group; [d]$P < 0.05$, *vs.* vitamin E group.

The epidermal and dermal thickness were decreased in the aging model group compared to normal control group ($P < 0.01$), indicating the model was successfully established. The epidermal and dermal thicknesses were increased in the vitamin E group and Ganoderma lucidum polysaccharide group ($P < 0.01$); compared with vitamin E group, the epidermal thickness was increased in the Ganoderma lucidum polysaccharide group, and the difference was significant ($P < 0.05$), the dermal thickness was increased in the Ganoderma lucidum polysaccharide group, but the difference was not significant ($P > 0.05$).

Detection of oxidation- and anti-oxidation-related protein levels in skin tissue

The superoxide dismutase activity in skin tissues is shown in Table 2.

Table 2 Superoxide dismutase activity in mouse skin tissues

Group	*n*	Superoxide dismutase activity (U/ mg prot)
Normal control	10	58.43±3.83
Aging model	11	41.68±5.44[a]
Vitamin E	11	48.07±3.71[ab]
Ganoderma lucidum polysaccharide	11	54.17±5.61[cd]

[a]$P < 0.01$, *vs.* normal control group; [b]$P < 0.05$, [c]$P < 0.01$, *vs.* aging model group; [d]$P < 0.05$, *vs.* vitamin E group.

The superoxide dismutase activity in the mouse skin tissues of aging model group was decreased compared with normal control group ($P < 0.01$), indicating the model was successfully established. The superoxide dismutase activity in the Ganoderma lucidum polysaccharide group was more than that in the aging model group ($P < 0.01$) and vitamin E group ($P < 0.05$).

CuZu-superoxide dismutase mRNA expression was detected by real-time reverse transcription-PCR (Firgue 2; Tables 3, 4).

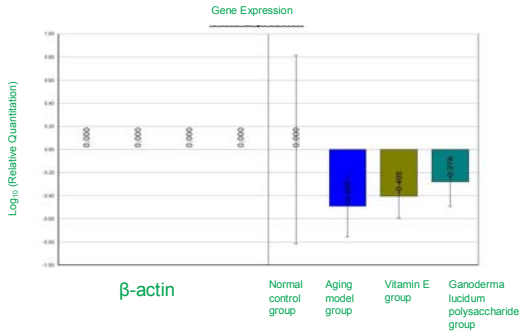


Figure 2 CuZu-superoxide dismutase mRNA expression detected by real-time reverse transcription-PCR

Table 3 Relative expression of CuZu-superoxide dismutase mRNA in mouse skin tissues from each group

Group	△△Ct	$2^{-\triangle\triangle Ct}$	$Log_{10}2^{-\triangle\triangle Ct}$	Avg Ct
Normal control	0.00	1.000	0.000	22.22
Aging model	1.62	0.33	−0.49	25.31
Vitamin E	1.34	0.40	−0.40	24.12
Ganoderma lucidum polysaccharide	0.92	0.53	−0.29	23.53

Table 4 Relative expression of CuZu-superoxide dismutase mRNA in mouse skin

Group	*n*	Ct
Normal control	10	21.78±1.12
Aging model	11	25.63±0.78[a]
Vitamin E	11	24.39±0.99[ab]
Ganoderma lucidum polysaccharide	11	22.79±1.21[cd]

[a]$P < 0.01$, *vs.* normal control group; [b]$P < 0.05$, [c]$P < 0.01$, *vs.* aging model group; [d]$P < 0.05$, *vs.* vitamin E group.

The CuZu-superoxide dismutase mRNA cycle threshold values in mouse skin were decreased in the aging model group compared with the normal control group ($P < 0.01$). The cycle threshold value in the Ganoderma lucidum polysaccharide was more than that in the aging model group ($P < 0.01$) and the vitamin E group ($P < 0.01$).

DISCUSSION

Ganoderma lucidum polysaccharide, an effective component of ganoderma, can enhance immune capacity, block free radicals, inhibit tumorigenesis, resist radiation, and boost the capacities of liver, bone marrow, and blood to synthesize DNA, RNA, and protein.

Ganoderma lucidum polysaccharide maintains the immune balance by directly or indirectly influencing the immune system to enhance disease resistance (Liu, 2000). Vitamin E is a natural, effective antioxidant and free radical scavenger that exists in biological tissues, blocks free radicals, and stabilizes cell membranes[6]. Therefore, we used vitamin E as a control for postponing skin aging.

Aging skin shows poor elasticity and softness, keratinization, dryness, and excessive pigment sedimentation. Decreased dermal collagen and elastic fibers contribute to poor elasticity and fragile fibers[7], leading to wrinkled, flabby skin. The free radical theory proposed by Harman in 1956 indicates that aging is caused by injury from active oxygen components generated by metabolism[8-9]. A key way to prevent or postpone skin aging is to reduce free radicals generation and remove residual free radicals. Free radical theory accounts for wrinkle formation[10]: (1) Free radicals can promote the cross linking and polymerization of collagen protein, particularly to stabilize cross linking. (2) Free radicals can increase the elastase activities and degrade the elastic fibers by reducing elastin gene expression. (3) Free radicals can directly degrade the sodium hyaluronate. These functions can change dermal water preservation, tension, elasticity, and softness and are the structural basis of wrinkle formation.

Superoxide dismutase, an important antioxidase, can defend cells and plays a critical role in organism balance between oxidation and anti-oxidation. Superoxide dismutase activity is gradually decreased with aging[11-12], and CuZn-superoxide dismutase mRNA expression in human skin is gradually decreased with aging[13]. Superoxide dismutase exists in human erythrocytes only as Cu, Zn- superoxide dismutase. As the expression of inhibitor-synthesizing genes in skin cells is increased, many genes related to cell activity are suppressed, and oxidative stress injury to DNA influences DNA replication, transcription, and expression, so gene regulation provides a basis for preventing skin aging[1]. Ganoderma lucidum polysaccharide can up-regulate the activity of superoxide dismutase, is an important part of the anti-oxidant system, and block to D-galactose-induced aging. Ganoderma lucidum polysaccharide can reduced aging levels to near normal controls. Ganoderma lucidum polysaccharide can up-regulate CuZn superoxide dismutase mRNA to overcome the decrease that occurs during aging, superoxide dismutase mRNA expression is up-regulated, which can accelerate the intracellular superoxide dismutase protein synthesis, and finally increase superoxide dismutase contributes to enhance enzymatic activity. The mechanisms of Ganoderma lucidum polysaccharide changes in transcription deserve further study.

Ganoderma lucidum polysaccharide can improve the outcomes in an aging model in mice by increasing the epidermal and dermal thicknesses, superoxide dismutase activity, and CuZn-superoxide dismutase mRNA expression. Ganoderma lucidum polysaccharide can repair the severe oxidative damage which cannot be repaired by vitamin E, indicating the clinical potential for Ganoderma lucidum polysaccharide in aging.

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灵芝多糖抗氧化、抗皮肤衰老*

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文章亮点：

1 实验拟观察灵芝多糖延缓皮肤衰老的作用。

2 维生素 E 具有清除自由基和稳定细胞膜的作用，故实验选择维生素 E 作为对照组。

3 由于衰老小鼠机体中超氧化物歧化酶基因表达水平逐渐降低，通过给预灵芝多糖后逆转了基因表达水平降低的趋势，说明灵芝多糖对 Cu-Zn SOD mRNA 基因在转录水平的表达量上有正调节作用，能提高该基因的表达量。

关键词：

组织构建；组织构建细胞学实验；皮肤衰老；灵芝多糖；超氧化物歧化酶；CuZn-SODmRNA；维生素 E；D-半乳糖；表皮；真皮

主题词：

皮肤衰老；超氧化物歧化酶；半乳糖；RNA，信使

摘要

背景：研究结果表明，灵芝多糖具有抗病毒、抗肿瘤、提高免疫力、抗氧自由基、抗衰老等生物活性。

目的：观察灵芝多糖对 D-半乳糖致衰老小鼠皮肤组织抗氧化能力的生物学效应和延缓皮肤衰老作用。

方法：将 44 只 2 月龄昆明种小鼠随机分为 4 组，即正常组、衰老模型组、维生素 E 组、灵芝多糖组，后 3 组颈背皮下注射 D-半乳糖建立小鼠衰老模型，同时灌胃给予相应药物或生理盐水，42 d 后取小鼠背部皮肤作病理切片，观察皮肤组织形态变化，检测表皮、真皮厚度，测定超氧化物歧化酶含量及 CuZn-SODmRNA 在皮肤中的表达水平。

结果与结论：维生素 E 组和灵芝多糖组小鼠表皮、真皮厚度均较衰老模型增加。灵芝多糖组小鼠皮肤超氧化物歧化酶活力明显高于其他组。灵芝多糖组小鼠皮肤 Ct 值降低显著低于其他组。说明灵芝多糖能增加表皮和真皮厚度，改善皮肤组织结构；提高皮肤组织超氧化物歧化酶水平及 CuZn-SOD mRNA 的表达。

*作者贡献：*设计、实施、评估为本文作者，均受过专业培训。

*利益冲突：*课题未涉及任何厂家及相关雇主或其他经济组织直接或间接的经济或利益的赞助。

*伦理要求：*实验过程中对动物的处置符合 2009 年《Ethical issues in animal experimentation》相关动物伦理学标准的条例。

*学术术语：*灵芝多糖-目前已分离到的有 200 多种，其中大部分为 β-葡聚糖，少数为 α-葡聚糖，多糖链由 3 股单糖链构成，是一种螺旋状立体构形物，其立体构形和 DNA、RNA 相似，螺旋层之间主要以氢键固定，相对分子质量从数百到数十万，除一小部分小分子多糖外，大多不溶于高浓度乙醇，在热水中溶解，大多存在于灵芝细胞内壁。

*作者声明：*文章为原创作品，数据准确，内容不涉及泄密，无一稿两投，无抄袭，无内容剽窃，无作者署名争议，无与他人课题以及专利技术的争执，内容真实，文责自负。

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