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ORIGINAL ARTICLE

Evaluation of Hair Growth Properties of Glycyrrhizic Acid

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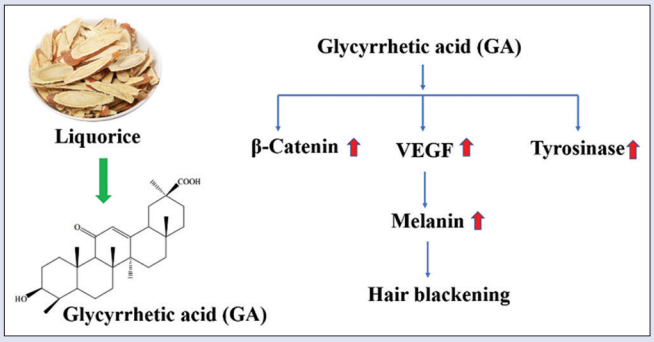
ABSTRACT

Background: Many studies have indicated that hair growth and color regulation are cyclical processes involved in the strict regulation of multiple signaling pathways, such as the transforming growth factor and Wnt/β-catenin protein pathways. It has also been reported that *Glycyrrhiza uralensis* extract can promote hair growth, but its active components are still unclear. **Objectives:** The aim of this study was to investigate the potential hair blackening effect and mechanisms of glycyrrhetic acid (GA) extracted from *Glycyrrhiza uralensis*. **Materials and Methods:** Different concentrations of GA solution (1.0 wt%, 10.0 wt%, and 20.0 wt%) were coated onto the skin surface of fur model mice induced by H_2O_2 for two weeks. After that, the number and depth of follicles and cortex thickness were observed in the skin tissue stained by hematoxylin and eosin (H&E). The relative mRNA levels of β-catenin, vascular endothelial growth factor (VEGF), and tyrosinase in skin tissue were analyzed using quantitative real-time polymerase chain reaction. The protein levels of β-catenin, VEGF, and tyrosinase were also evaluated by western blotting and immunohistochemistry. **Results:** There was a marked increase in the number of hair follicles and the blackening effect upon exposure to 10.0 wt% and 20.0 wt% GA. Moreover, the β-catenin, VEGF, and tyrosinase expression levels in the skin tissue varied significantly compared to those in the model group. **Conclusion:** GA showed positive effects on hair growth factor expression levels in mice, which meant that GA could promote hair blackening.

Key words: Glycyrrhetic acid, hair blackening, liquorice, tyrosinase, melanin

SUMMARY

- The efficacy of glycyrrhetic acid (GA) as a hair growth promoter is estimated.
- The effect of GA on hair growth is estimated by histological and morphological analysis.
- GA can promote β-catenin, VEGF and tyrosinase expression, causing hair blackening.



Abbreviations used: GA: Glycyrrhetic acid; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; 4-ABP: 4-aminoaminobiphenyl; VEGF: Vascular endothelial growth factor; TGF: transforming growth factor.

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INTRODUCTION

Melanocytes are mainly found in the anagen bulb and dermal papillae of the hair follicle. The melanin and hair stromal cells play key roles in the process of premature hair growth.[1] If melanin is not produced, the hair turns pale, leading to hair whitening.[2] Many researchers have explored the underlying mechanisms of premature hair greying. The apparent causes of white hair are generally identified as genetic factors, physical stress, low levels of immunity, and aging, but the underlying mechanisms remain largely elusive.[3-7]

In practice, to beautify the white hair, many chemically synthesized hair colorants are applied.[8] However, they can cause serious damage to the human body due to their high toxicity and difficult degradation of the human body. For example, naphthalenediamine and 4-aminoaminobiphenyl (4-ABP) are recognized as human carcinogens and are currently used in most hair colorants.[9] However, blackened hair is nondurable because of the physical effects of these chemical dyes.[10] Therefore, the products with low toxicity, easy degradation, and high durability to treat grey hair and promote hair growth are crying needs.

Chinese herbal extracts have the advantages of low toxicity and easy biodegradability.[11] Herbal cosmetics have been rapidly developed in recent years, and the active ingredients of various plant extracts have been identified, characterized, and utilized. Natural hair dyes are increasingly popular, but their underlying mechanisms and potential adverse reactions are still unclear.[12,13] The hair follicles of C57BL/6 mice are often used to assess the mechanisms of hair growth.[14] Hair growth and color regulation are cyclical processes which involve the strict regulation of multiple signaling pathways, including the transforming growth

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factor (TGF) and Wnt/β-catenin protein pathways. Dermal nipples (DP) are the primary mesenchyme components and are importantly located at the deepest end of the hair follicle.[15]

Glycyrrhiza glabra (liquorice) is a renascent herb found in the Mediterranean region, including Asia Minor, Iran, and central to southern Russia. This herb is now widely cultivated in Asia and the Middle East.[16] Liquorice is also a commonly used plant in traditional Chinese medicine and is well known as the "father of Chinese herbal medicine."[17] The main bioactive compounds in liquorice are Glycyrrhetic acid (GA) and glycyrrhizic acid glycosides, which reveal a variety of pharmacological effects such as anti-inflammatory, anticancer, anti-immunogenic, and anti-allergic activities.[18] Furthermore, these compounds have recently been found to inhibit the growth of skin tumors,[19] and they have good antioxidant properties, as evidenced by decreased lipid peroxidation.[20] However, there are a few reports on the effects of these compounds on the promotion of hair growth.

Therefore, the purpose of this study is to estimate the effect of GA on hair growth by virtue of the macroscopic and histopathological assessments. Specifically, the influence of GA on H_2O_2-induced hair greying is investigated by measuring inflammatory parameters and oxidative stress responses. The efficacy of GA as a hair growth promoter is also compared with an FDA-approved medication (2.0 wt% minoxidil). This study will provide a basis for research on prematurely greying hair.

MATERIALS AND METHODS

Plant materials and chemicals

Glycyrrhizic acid (GA, purity >98.0%) was provided by Guangdong Heji Biotechnology Company (Guangzhou, China). Three different concentrations of GA solution (1.0 wt%, 10.0 wt%, and 20.0 wt%) were prepared using 30 wt% ethanol as the solvent. Minoxidil liniment containing 2.0 wt% minoxidil was obtained from Sichuan Meidakang Medical Company (Deyang, China).

Experimental animals and modeling

Five-week-old-male (17 g–21 g) C57BL/6 mice were obtained from the Experimental Animal Center of Guangdong Province (SCXK/20130002, Guangzhou). All mice housed in a room with a temperature of 23°C ± 2°C and with a 12/12 hour light/dark cycle were fed adaptively and provided standard laboratory water and food.

Hair in an area of approximately 3 cm × 2 cm on the back of each mouse was shaved and wiped with warm water without injuring the skin and residual hair. The skin of the mice being in the growth phase was estimated according to the reported literature, where the skin of the mice turned pink during the initial period, black during the growth phase, and grey during the period of decline.[21] Male mice were randomly divided into six groups with ten mice, namely, GA-1%, GA-10%, GA-20%, control, H_2O_2-5%, and minoxidil. A pathological white-hair model was established. The model group was daubed with H_2O_2-5% every morning. The mice in the control group were treated with 30% ethanol. Minoxidil was the positive control drug. Each treatment group was administered 1.0 mL of drug per day for 14 days.[22]

Evaluating hair growth

The differences in the amount of newborn hair in the mice were determined as follows: the hair in the longer area was extracted with tweezers, the longest three were selected for length measurement, and the newborn hair with an area of 1 cm^2 × 2 cm^2 was cut along the hair root with scissors. The average gross weight of the mice in each group was calculated using an analytical balance, and the length and weight of the hair in each group were compared. The hair growth was evaluated according to Table 1.

Table 1: Hair growth evaluation[23]

Conditions of hair growth	Point
Depilation area showing no hair growth and redness	0
Depilation area exhibiting grey color	1
Depilation area exhibiting black color	2
Depilation area exhibiting black color and a small amount of hair growth	3
The density and length of new hair are approximately half that of non-depilated area	4
The density and length of new hair are consistent with surrounding hair	5

Histology and immunohistochemistry

For further histological and immunohistochemical analysis, skin tissues were harvested and formalin-fixed. The paraffin-embedded skin tissues were sectioned (5 μm thickness) and stained with hematoxylin and eosin (H&E). Skin tissue sections were blocked and incubated with primary antibodies at a 1:200 dilution at 4°C overnight after deparaffinization. After washing with phosphate-buffered saline (PBS) three times, the sections were incubated with diluted horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L) (1:500, GB23303, Servicebio Technology Co. Ltd., Wuhan, China) for 1 hour at 25°C. The primary antibodies were β-catenin (1:1000, GB11015), vascular endothelial growth factor (VEGF) (1:100, GB13034) (Servicebio Technology Co. Ltd., Wuhan, China), and tyrosinase (1:200, AF5491) (Affinity Biosciences, Cincinnati, OH, USA). The sections were stained with 3,3'-diaminobenzidine (DAB) reagent for 10 min. The sections were photographed with 40 × and 100 × magnification on a fluorescence microscope (Olympus IX71, Olympus, Japan).[24] The integral optical density values of the sections were calculated using image analysis software (ImageJ), which indirectly represented the expression levels of inflammatory cytokines. Each experiment was conducted in triplicate.

Immunofluorescence assays

The sliced sections were washed three times with PBS (pH 7.4) for 5 min. Bovine serum albumin was used to block the tissue for 1 hour at 25°C. After shaking off the blocking solution, the first antibody was added to sections placed in a wet box for 4 hours and incubated overnight. The tissues were then incubated with the secondary antibody for 1 hour at 25°C. To stain the nuclei, 4,6-diamidino-2-phenylindole was incubated with sections at 25°C for 10 min. After quenching the spontaneous fluorescence of the tissue, the film was sealed and photographed using a fluorescence microscope (NIKON ECLIPSE C1).[25]

Determination of melanocytes by Masson-Fontana melanin staining

The concentrated ammonia water was added dropwise in 10 mL melanin dye solution until precipitation occurred and then continued adding ammonia water by shaking the solution, until the precipitate dissolved again and the solution became clear. A few drops of melanin dye were added to make the solution slightly turbid. After that, 20 mL of distilled water was added to prepare the melanin working solution. The paraffin sections were dewaxed using water, immersed in the melanin working solution, covered and kept at 4°C for 18 hours, and washed with distilled water three times. After staining again with van Gieson's stain, a transparent film was obtained. Finally, the images were collected and analyzed under a microscope (NIKON ECLIPSE C1).

Western blotting

Total protein from mice skin tissues was extracted with RIPA lysis buffer containing 1.0 wt% phenylmethanesulfonyl fluoride and 1.0 wt% protease inhibitor cocktail. Protein concentrations were determined using a BCA Protein Assay Kit. Proteins from each group were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After blocking for 1 hour using 5% non-fat dried milk, the membrane was incubated with a specific primary antibody at 4°C for 24 hours, followed by incubation with HRP-conjugated goat anti-rabbit IgG (H + L) (1:3000, GB23303) (Servicebio Technology Co. Ltd., Wuhan, China) for 1 hour at 25°C.[26] The primary antibodies were β-catenin (1:800, GB11015) and β-actin (1:1000, GB12001) (Servicebio Technology Co. Ltd., Wuhan, China). Specific protein bands were visualized using an enhanced chemiluminescence advanced western blotting detection kit. The densitometric measurement of band intensity was performed using Quantity One Software (Bio-Rad Laboratories, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from skin tissues and quantified spectrophotometrically. RT-PCR was performed using a cDNA reverse transcription kit and a LightCycler RT-PCR System (Roche Applied Science, Indianapolis, IN, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assayed in parallel as an internal control. PCR amplification was performed according to the following conditions: 95°C for 10 min for the first cycle, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C. mRNA expression levels were measured by cycle threshold (CT) values, normalized to that of GAPDH (ΔCT = CT (GAPDH) − CT (target), and then expressed as 2ΔCT.[27] The resulting fold change in qRT-PCR was calculated from the differences in CTs using the 2ΔCT method. The primer sequences for β-catenin, VEGF, and tyrosinase are shown in Table 2.

Statistical analysis

Data were expressed as the mean ± standard deviation. The difference was analyzed using SPSS (version 18.0 software, Duxbury Press), and the *P* value < 0.05 was considered statistically significant. The experiment has been approved by the ethics committee with an approval date of 2020.12.03.

RESULTS

Effects of GA on the development of mouse hair follicles

The experimental administration lasted for 20 days. From Table 3, the model group had light hair. Hair growth of mice in the Minoxidil, GA-10%, and GA-20% groups was nearly significant. The score from high to low is in the order of GA-1% group > Control group > H_2O_2-5% model group.

From Table 4, the hair density from large to small was in the order of GA-20% group > GA-10% group > minoxidil group > GA-1% group > control group > H_2O_2 5% model group. This indicated that the GA-10% and GA-20% groups were more suitable for hair growth.

Histological observations for the number of hair follicles

The number of hair follicles in the control group was slightly higher than that in the H_2O_2-5% model group, but the number of hair follicles in the GA-10% and GA-20% groups was significantly higher, indicating

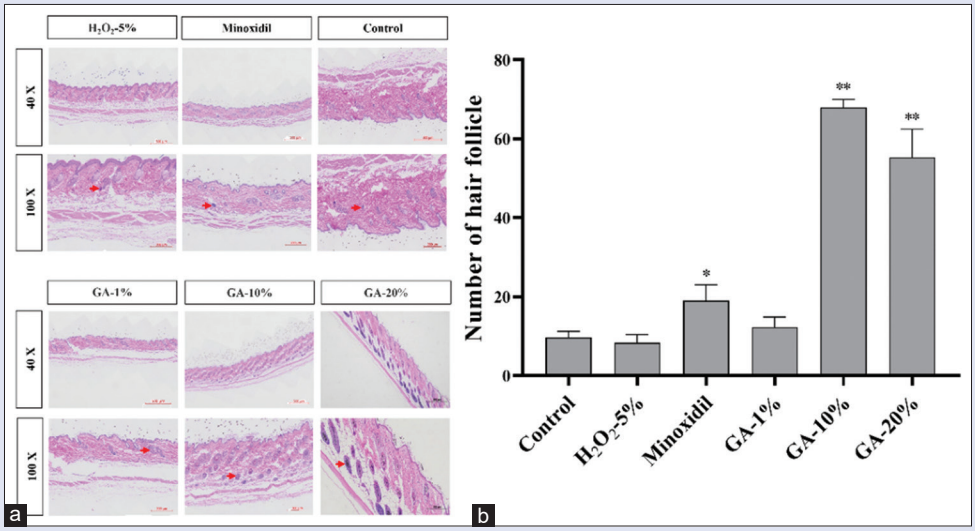

Figure 1: (a) Hair follicles images from hematoxylin and eosin staining. (b) The number of hair follicles was analyzed by ANOVA and Duncan's multiple range test that showed significant difference with the model (*P* < 0.01). Each value represents an average of 10 mice ± SD.

Table 2: Primer sequences for β-catenin, VEGF, and tyrosinase

Gene	Sequence of primer (5′→3′)	
	Forward primer	Reverse primer
β-catenin	GGACCCCAAGCCTTAGTAAACA	TTATATCATCGGAACCCAGAAGC-
VEGF	CACTGGACCCTGGCTTTACTG′	CTCAATCGGACGGCAGTAGC-
Tyrosinase	ACACACTGGAAGTATTTTTGAACA-	TAGGTGCATTGGCTTCTGGG
GAPDH	CCTCGTCCCGTAGACAAAATG- TG	TGAGGTCAATGAAGGGGTCGT′

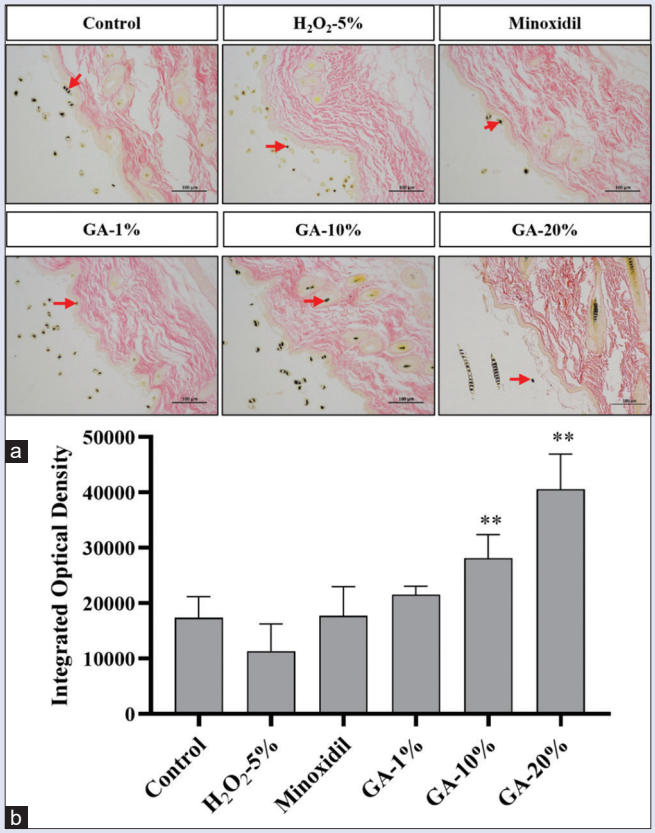

Figure 2: (a) Images of Masson Fontana melanin staining section. (b) Optical densities of the sections stained with Masson Fontana melanin. Each value represents the average of 10 mice ± SD. One-way variance test ($P < 0.01$) was used for significance analysis

a greater difference [Figure 1]. This shows that GA can promote the growth of hair follicle cells and fulfill black hair.

Histological observations for melanin content

Compared to the control group, the melanin content in the skin of the H_2O_2-5% model group was slightly reduced [Figure 2]. Compared to H_2O_2-5% group, the skin melanin contents in GA-1%, GA-10%, GA-20%, and minoxidil groups were higher. There was a significant difference in melanin content between the GA-10%, GA-20%, and H_2O_2-5% groups. It can conclude that GA is an active component in Liquorice that affects melanin production. The melanin content in mice can be increased by exposure to an appropriate concentration of the drug.

Table 3: Evaluation of hair growth

Groups	1d	5d	20d
H_2O_2-5% model group	0.50	0.50	2.50
Control group	0.50	0.74	3.88
Minoxidil group	0.50	0.82	4.35
GA-1% group	0.50	0.83	4.42
GA-10% group	0.50	0.94	4.65
GA-20% group	0.50	1.02	4.78

Table 4: Hair length and weight of mice in each group

Groups	Length of hair (mm)	Weight of hair (g)	Density of hair (g/mm)
H_2O_2-5% model group	2.75	6.40	2.52
Control group	5.87	18.11	2.65
Minoxidil group	6.12	19.21	2.78
GA-1% group	6.42	20.12	2.72
GA-10% group	6.53	20.42	2.87
GA-20% group	6.78	20.68	2.98

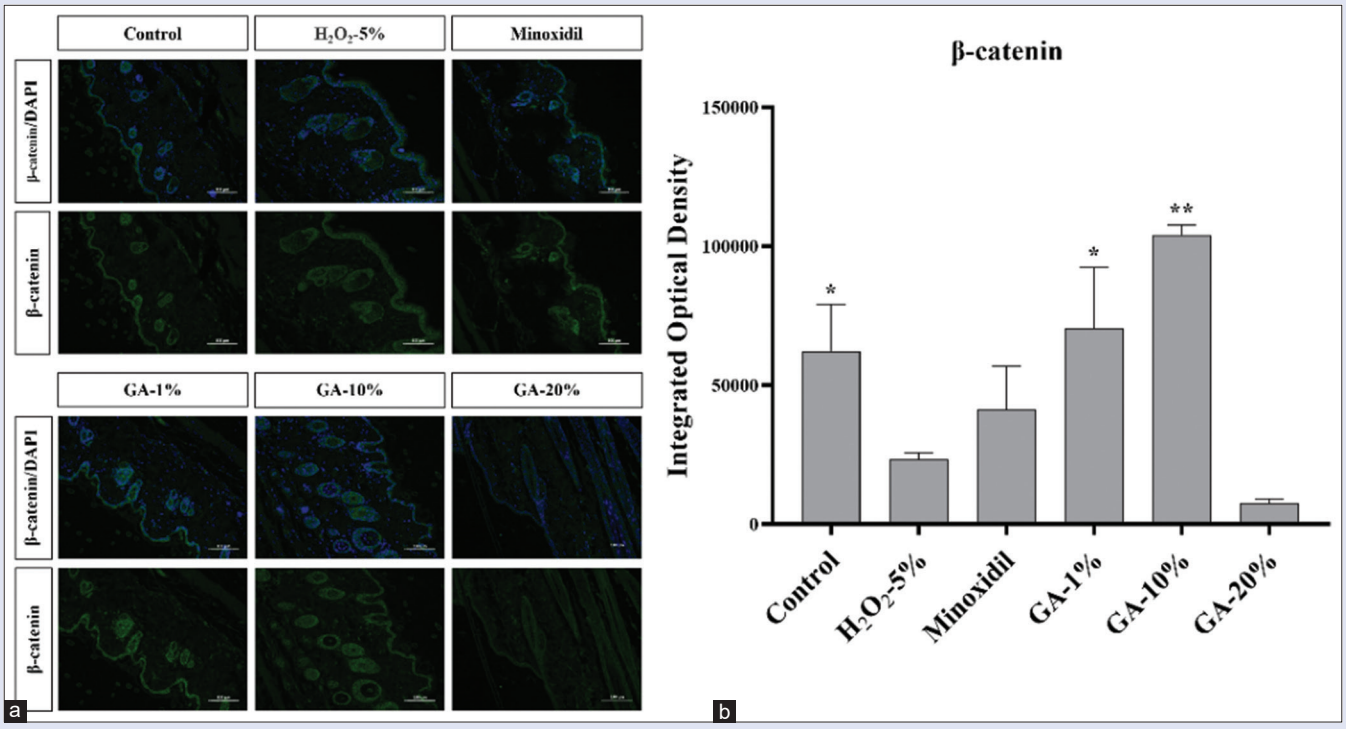

Figure 3: (a) Immunofluorescence images of paraffin sections detected by inverted fluorescence microscope. (b) Optical density of β-catenin determined from the immunofluorescence images. Each value represents the average of 10 mice ± SD. One-way variance test ($P < 0.01$) was used for significance analysis

Histological immunofluorescence

Compared to H_2O_2-5% group [Figure 3], the GA-10% group had better skin quality because the level of β-catenin was significantly higher in the GA-10% group ($P < 0.01$). The expression level of β-catenin in GA-1% and minoxidil groups was significantly higher than H_2O_2-5% group.

Immunohistochemistry

Compared to H_2O_2-5% group [Figure 4], the expression level of tyrosinase in the skin of the minoxidil, GA-10%, and GA-20% groups was significantly higher. Minoxidil, GA-10% and GA-20% groups had better skin quality because the level of β-catenin was significantly higher ($P < 0.01$). Compared to H_2O_2-5% model group, the expression level of β-catenin in GA-1% group was significantly higher than that in the H_2O_2-5% groups ($P < 0.05$). Compared to that in the control group, the expression level of VEGF in the H_2O_2-5% group was significantly lower ($P < 0.05$). Compared to H_2O_2-5% model group, the expression levels of VEGF in the skin of the GA-20% and minoxidil groups were significantly higher ($P < 0.05$). Therefore, GA may promote hair blackening by increasing the secretion of VEGF.

Western blotting analysis

It can be seen from Western Blotting bands in Figure 5, compared to H_2O_2-5% group, the ratio of the grey value of the target band to the grey value of the internal reference band of the expression of β-catenin was increased by varying degrees. The expression of β-catenin in minoxidil group was also significantly higher than H_2O_2-5% group.

Quantitative real-time RT-PCR

Compared to H_2O_2-5% group, the control group, GA-1% group, GA-10% group, and GA-20% group had the same skin β-catenin level [Figure 6]. The amplification multiples of catenin were significantly increased. In addition, the GA-1% and GA-20% groups showed no significant differences. The amplification multiple of catenin was lower than that in GA-10% group. Compared to the blank group, tyrosinase in H_2O_2-5% group decreased significantly. The skin tyrosinase amplification multiple of H_2O_2-5% model group was the lowest among six groups, and that of GA-10% group was the highest. Compared to H_2O_2-5% group, the skin tyrosinase amplification multiple in the control groups, GA-1%, GA-10%, and GA-20%, was significantly higher. Compared to H_2O_2-5%, the amplification multiple of VEGF in the skin of GA-20%, blank and control groups was significantly increased. Compared to H_2O_2-5%

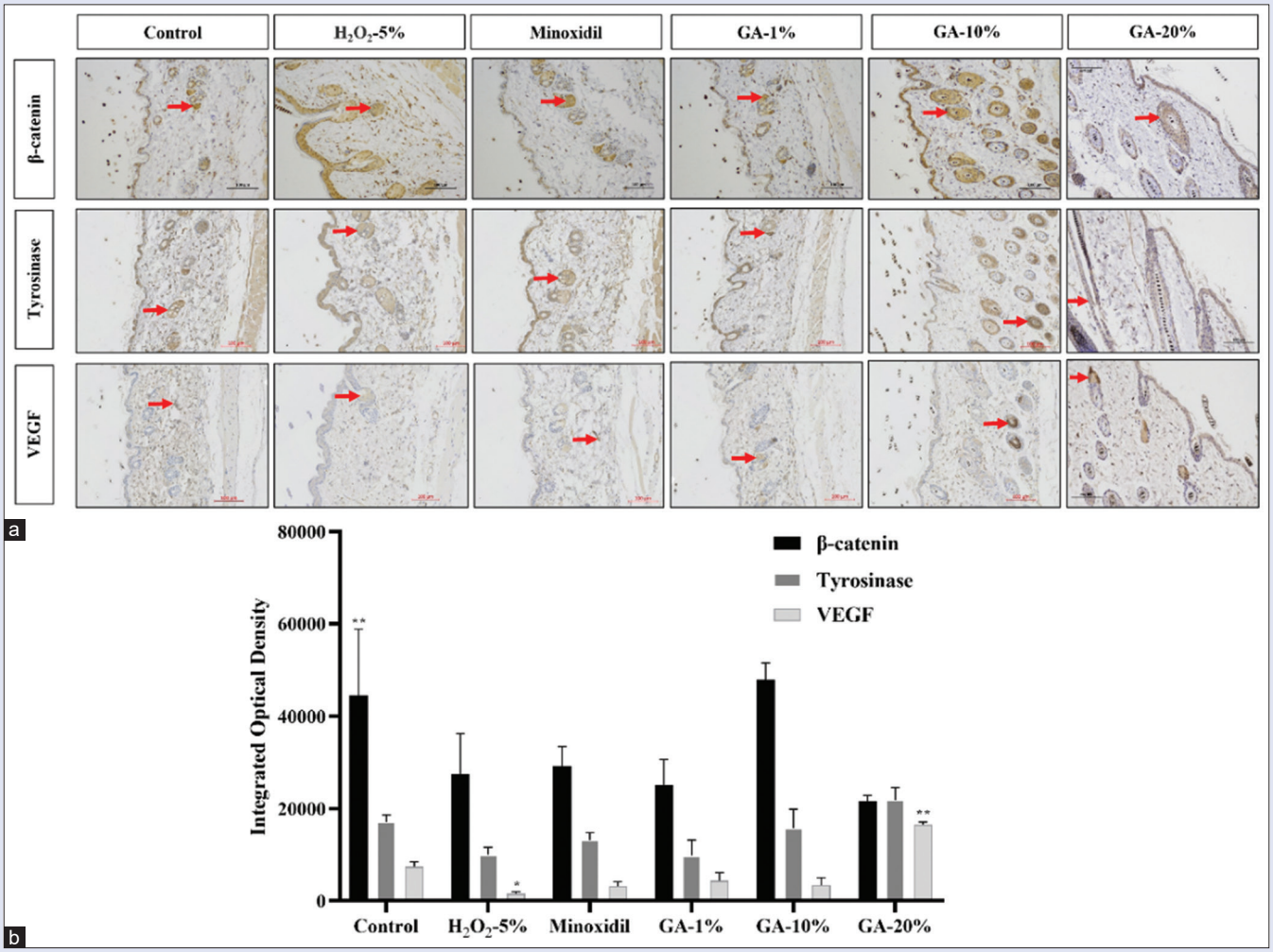

Figure 4: (a) Immunohistochemical staining on β-catenin, tyrosinase, and VEGF in the skin tissues of mice. (b) Optical densities of β-catenin, tyrosinase, and VEGF determined from the immunohistochemical staining. Each value represents the average of 10 mice ± SD. One-way variance test ($P < 0.01$) was used for significance analysis

model group, the amplification multiple of VEGF in the control groups, GA-1% and GA-10%, was lower.

DISCUSSION

Liquorice has anti-inflammatory and antiviral properties, detoxifies the liver, and enhances immune function.[28] GA possesses glucocorticoid-like pharmacological effects. Therefore, it has no serious adverse effects. The compound is widely used in the clinical treatment of various acute and chronic illnesses, such as hepatitis, bronchitis, and AIDS. GA also has anticancer, interferon inducing, and cellular immune modulator functions.[29] GA also has lipid-lowering and anti-atherosclerosis effects.[30] This undoubtedly proves its important pharmacological value.

The primary objective of this study is to determine the effect of GA on hair growth and the importance of hair color regulation. GA reduces the formation of VEGF by inhibiting the mitogen-activated protein kinase (MAPK) signaling pathway.[31] GA reduces collagen deposition and the hypnotic content of liver tissue and suppresses hematopoietic stem cell activation.[32] Therefore, the expression level of β-catenin is also reduced.[33] Furthermore, studies have shown that high concentrations of GA can inhibit the expression of complexaminase, and hence inhibit the melanin expression.[28] The present results confirm that GA is the main active ingredient in liquorice and may be related to melanin production.

In conclusion, this study investigated the biochemical activity and pathological mechanisms of liquorice and the role of GA in macro-regulating hair regeneration. We found that three different concentrations of GA resulted in the growth of black hair in C57BL/2 mice. Based on the animal fur model experiment using hydrogen peroxide, skin tissue showed a significant increase in the number of hair follicles upon treatment with GA-10% and GA-20%, and the expression levels of β-catenin, VEGF, and tyrosinase in the skin varied relative to the hydrogen peroxide model group. Three concentrations of GA increased the expression level of β-catenin in the mouse skin. Compared to the model group, GA increased the expression level of VEGF in mice. In concordance with our predictions, GA upregulated tyrosinase expression during the skin thickening stage. GA could increase melanocyte cells in mouse skin tissue, increase mouse hair growth, promote skin thickening, and enhance the expression of VEGF, tyrosinase, and β-catenin.

Further studies on the mechanism of GA dyeing are required to confirm the application of GA as a hair-blackening agent. Our study has revealed the relationship between the activated signaling pathway and premature greying when using GA. Other traditional Chinese medicines to replenish Qi and promote blood circulation to influence blackening of hair should also be considered.[34] Originally, the traditional Chinese medicine liquorice was used to tonify the spleen, and Qi had been scientifically proven at the tissue and gene levels.[35] For example, after traditional Chinese medicine treatment, hair growth factor expression can be activated, or the related gene expression can be inhibited.[36] In the next step, different Qi Traditional Chinese Medicine (TCMs) and their molecular and macroscopic effects on hair growth will be studied. More functional cosmetics derived from Chinese herbal medicine will also be developed to meet crying demand.

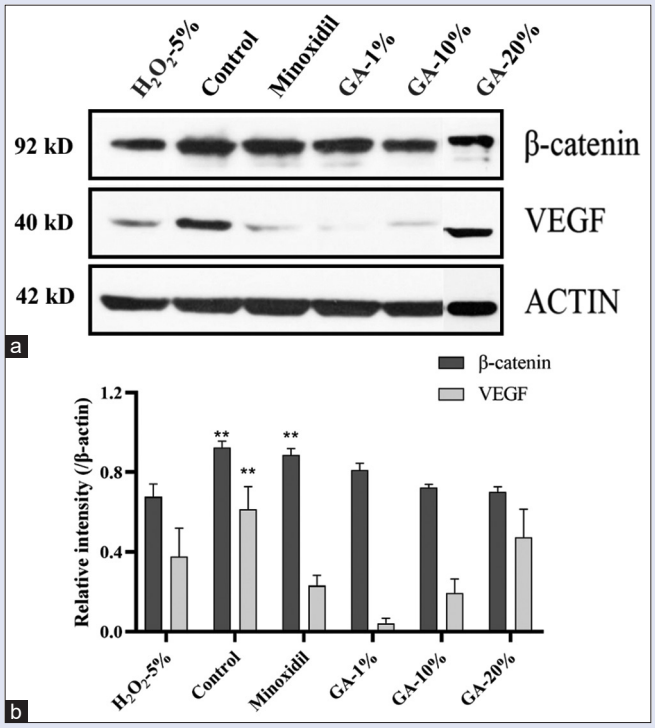

Figure 5: (a) Western blot analysis, actin as internal control adding the same amount of protein (50 μg). (b) Expression levels of β-catenin determined by Western blot analysis. Each value represents an average of 10 mice ± SD. One-way variance test ($P < 0.01$) was used for significance analysis

CONCLUSION

This study investigated the biochemical activity and underlying mechanisms of GA (found in liquorice) on the macro-regulation of hair regeneration. It was found that three different concentrations of GA had a hair blackening effect in C57BL/2 mice, and the skin tissue showed a significant increase in the number of hair follicles after being treated with 10 wt% and 20 wt% of GA.

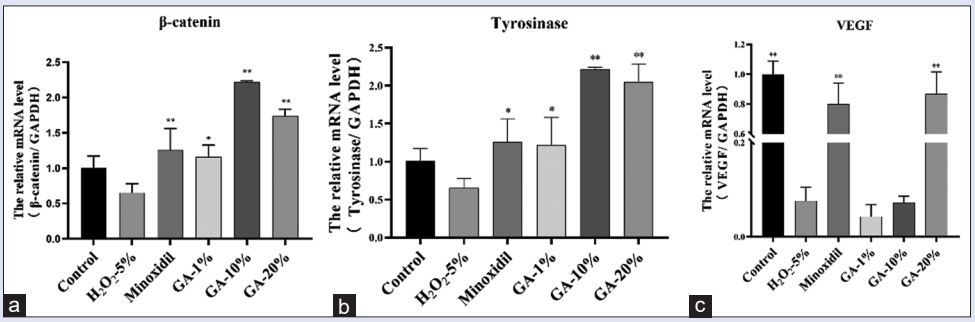

Figure 6: The expression of (a) β-catenin, (b) tyrosinase, and (c) VEGF in skin tissues of mice for each group determined by fluorescence quantitative polymerase chain reaction. Each value represents the average of 10 mice ± SD. One-way variance test ($P < 0.01$) was used for significance analysis

Authors contribution

Bing Zhang and Wenyun Zhang led the overall plan; Jianfang Luo and Jian He led the design experiments; Baoshan Rong and Xiaomin Zheng conducted the total RNA extraction from the skin tissue and the spectrophotometric quantification; Siyang Zhu, Xian Xu, and Yong Ai led the animal experiments and biological activity tests; Lanyue Zhang and Tinggang He conducted the data analysis.

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Conflicts of interest

There are no conflicts of interest.

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