Effects of *Tricholoma matsutake* Extracts on Promoting Proliferation of HaCaT Cells and Accelerating Mice Wound Healing

Wenyu Zhu,[1,2] Yujuan Chen,[1,2*], Kaige Qu,[2] Chunyan Lai,[2] Zhengcheng Lu,[3] Fan Yang,[2] Tuoyu Ju,[2] Zuobin Wang,[2,3*]

[1]School of Life Sciences, Changchun University of Science and Technology, Changchun 130022, China; [2]International Research Centre for Nano Handling and Manufacturing of China, Changchun University of Science and Technology, Changchun 130022, China; [3]JR3CN & IRAC, University of Bedfordshire, Luton LU1 3JU, UK.

*Address all correspondence to: Yujuan Chen, [1]School of Life Sciences, Changchun University of Science and Technology, Changchun 130022, China, [2]International Research Centre for Nano Handling and Manufacturing of China, Changchun University of Science and Technology, Changchun 130022, China, chenyujuan@cust.edu.cn; Zuobin Wang, [2]International Research Centre for Nano Handling and Manufacturing of China, Changchun University of Science and Technology, Changchun 130022, China, [3]JR3CN & IRAC, University of Bedfordshire, Luton LU1 3JU, UK, wangz@cust.edu.cn.

SHORT TITLE: Effects of *T. matsutake* Extracts on Promoting Proliferation of Cells *in-vivo* and i*n-vitro*

ABSTRACT:

Introduction: The edibility and medicinal purpose of *Tricholoma matsutake* are popular in Asian countries. *Tricholoma matsutake* is a precious natural medicinal fungus, and it is widely used in food and biological products. The aim of this study is to explore the mechanism of *Tricholoma matsutake* on promoting proliferation of HaCaT cells and accelerating mice wound healing.

Methods: The MTT assay was used to test the effects of three different *T. matsutake* extracts (0, 62.5, 125, 250, 500 and 1000 μg/mL) on HaCaT cells viability. The HaCaT cells were treated with three different *T. matsutake* extracts (100, 500 μg/mL) and the morphological and biophysical properties were detected by AFM with JPKSPM Data Processing. Western blot were detected Notch signaling pathways of HaCaT cells that were treated with 50%T for 24 h (100, 500 and 1000 μg/mL). The mice wounds were treated with 50%T for 15 days. The effects of wound healing were observed on mice back skin at different time. The quality of wound healing was estimated by histological staining (H&E and Masson's). All data were counted by GraphPad Prism 5 software.

Results: With the concentration increase of *Tricholoma matsutake*, it remarkably promoted the HaCaT cell proliferation. The Young's modulus of HaCaT cells showed the biggest increase from 1.73±0.13 kPa (0 μg/mL) to 4.57±0.16 kPa (500 μg/mL) in the 50%T group. The Notch1/Jagged1 pathways were upregulated with an increase of the concentration (0, 100, 500 and 1000 μg/mL). Moreover, comparing the negative and positive control groups, *Tricholoma matsutake* promoted the wound healing of mice by epidermis regeneration, subepidermal tissue formation and collagen deposition.

Conclusion: The results proved that *Tricholoma matsutake* not only promotes the proliferation of HaCaT cells, but also promotes the wound healing of mice.

KEY WORDS: *Tricholoma matsutake*, HaCaT cells, Young's modulus, Notch1/Jagged1 pathways, wound healing.

ABBREVIATIONS: 50%T, 50% ethanol extract of *T. matsutake*; **95%T**, 95% ethanol extract of *T. matsutake*; **WT**, sterile water extract of *T. matsutake*

I. INTRODUCTION

T. matsutake is a mycorrhizal fungus that grows on the roots of pine and oak.[1,2] It is a rare and precious natural medicinal fungus in the world, and it is one of the second-class endangered species in China.[3] Researches have shown that *T. matsutake* is rich in protein, amino acids, essential trace elements,[4] unsaturated fatty acids, nucleic acid derivatives, peptides and other rare elements.[5] According to reports, *T. matsutake* has many effects, such as anti-cancer,[6] anti-aging, immunity enhancement, treatment of diabetes and cardiovascular diseases, promoting gastrointestinal peristalsis, liver protection, anti-radiation, anti-mutation and anti-bacteria[7] effects.

As the largest organ of the human body,[8] skin is not only the first natural barrier to protect the body from external aggression, but also has other biological functions such as immune surveillance and self-healing.[9] Nonetheless, under the action of some external factors, the skin loses its first natural barrier, which is likely to cause complications such as bacterial infection, subcutaneous blood vessel damage, tissue necrosis and skin ulceration.[10] The repair of skin injury is an extremely complicated course,[11] including the hemostasis of wounds, the occurrence of inflammatory reactions, migration and proliferation of cells, regeneration and remodeling of blood vessels and tissues.[12] At present, the prevention and treatment methods of wound healing mainly focus on external wound dressings[13] and antibiotic ointment.[14] However, clinical practice has proved that these methods have many disadvantages, such as delayed wound healing, toxic side effects and high price.[15] The object of this study is to provide an effective and safe natural product which can promote wound healing.

At the current stage, the research on *T. matsutake* is mainly focused on its chemical composition analysis, cultivation technology and development of functional products.[16] This work is to study the effects of cell proliferation and skin wound healing with *T. matsutake* extract and its mechanism *in vitro* and *in vivo*.

II. MATERIAL AND METHODS

A. Preparation of *T. matsutake* extract

T. matsutake was collected from Yanji, Jilin province, China. Three different extractants were used to extract *T. matsutake*, and they were 50% ethanol (v/v), 95% ethanol (v/v) and sterile water. 150 g *T. matsutake* was extracted and lyophilized by using Soxhlet extractor, rotary evaporators and freeze dryer. They were named 50%T (50% ethanol extract of *T. matsutake*), 95%T (95% ethanol extract of *T. matsutake*) and WT (sterile water extract of *T. matsutake*).

B. Cell culture and cell viability assay

Human immortalized keratinocytes (HaCaT) were cultured in a 5% CO_2 incubator (Sanyo, Japan) at 37°C with the Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented using 10% fetal bovine serum (FBS, ABW, Uruguay) and 1% penicillin-streptomycin solution (antibiotics, Solarbio, China). HaCaT cells were seeded into the 96-well plate with the density of 4×10^3 per well and cultured for 12h. The final concentrations of 50%T, 95%T and WT added into the well were 0, 62.5, 125, 250, 500 and 1000 μg/mL. Then the cells were cultured for 48h. Furthermore, each well will be added 20 μL of Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma-Aldrich, USA). After incubated for 4h, the supernatant was

removed and 150 μL DMSO was added to each well. The OD value was detected using a microplate reader (BioTek, USA) at the wavelength of 490 nm.

C. Atomic force microscopy (AFM)

The HaCaT cells were plated on the coverslips with 2 mL DMEM medium for 12h, and treated with the different concentrations (100, 500 μg/mL) of 50%T, 95%T and WT for 24h. Then the HaCaT cells were detected by atomic force microscope (JPK, Germany), and the MLCT probe (spring constant: 0.07 N/m) was used in a quantitative imaging (QI) mode. The mechanical properties of cell height, adhesion force and Young's modulus were analyzed.

D. Western Blot

The HaCaT cells were treated with the different concentrations of 50%T (0, 100, 500, 1000 μg/mL) for 24h. In order to extract the cells total protein, the cells were forzen and thawed repeatedly for three times in sterile water. Then the proteins were collected by the centrifuge for 10min at 10000 rpm. The protein samples were loaded onto an 8% SDS-PAGE and electro transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% BSA for 2h and incubated overnight with the following goat anti-rabbit antibodies: anti-Beta Tubulin antibody (1:1000, Sangon, China), anti-Notch1 antibody (1:1000, Solarbio, China); goat anti-mouse antibody: anti-Jagged1 antibody (1:1000, Proteintech, China). Then the film was incubated with peroxidase-conjugated secondary anti-body IgG (1:5000, Solarbio, Proteintech, China). At last, the protein bands were visualized with ECL (Millipore, USA) and imaged by multifunctional imager (Analytik Jena, Germany).

E. Treatment of wound healing

Eighteen healthy male Kunming mice were bought from Yisi experimental animal technology company (China), and each mouse weight was 30-35 g. The animal experiments were authorized by Changchun University of Science and Technology ethical committee. All animal experiments conform to 'EU Directive 2010/63/EU for animal experiments' rules. The registration number of permission to perform animal experiment was No.2019081405 and the date of permission was 14th August, 2019. All mice were anesthetized with 10% chloral hydrate (3 mL/kg). Then their back hairs were removed by depilatory cream and sterilized with 75% ethanol. Two full-thickness cutaneous wounds with the diameter of 15 mm were made by a pair of surgical scissors on the back of each mice. The injured mice were randomly divided into 3 groups with 6 mice in each group. Then the three groups were treated with 100 μL stroke-physiological saline solution (negative control, NC), 100 μg Erythromycin Ointment (positive control, PC) and 100 μL 50%T, respectively, for 15 days and photographed on the 0th, 5th, 10th and 15th days to obtain the wound healing area. The treatments were repeated once every day. Then the wound healing rates were calculated according to the wound healing area. Wound healing rate = (original wound area—final wound area) / original wound area×100%.

F. Histological staining

After the experiment, the mice were euthanized. The skin samples were separated and immersed in 10% formaldehyde solution, treated with paraffin and sliced into 5 μm by freezing microtome (Leica, Germany). Next, the skin samples were stained

with hematoxylin and eosin (H&E, Solarbio, China) and Masson's trichrome staining (Solarbio, China), respectively.

G. Statistic analyzes

The experimental data were expressed as mean ± standard error and statistical data (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$) were considered statistically significant. The data were counted by GraphPad Prism 5 software.

III. RESULTS

A. Cell viability

The MTT assay was used to test the effects of three different *T. matsutake* extracts on HaCaT cells viability. The cells were exposed in different concentrations (0, 62.5, 125, 250, 500 and 1000 μg/mL) of three extracts (50%T, 95%T and WT) for 24 h. As shown in **Figure 1**, the proliferation rate of HaCaT cells are increased with the increase of concentrations. The effect of 50%T on the HaCaT cell proliferation was significantly better than the other two extracts.

B. Cell morphological and biophysical properties

As shown in **Figure 2(a)**, the HaCaT cell AFM images were obtained with the designed treatments of 50%T, 95%WT and WT for 24 h. As shown in **Figure 2(b)**, compared with the control group, the average height of the three experimental groups were slightly increased and showed a dose-dependent effect. However, the average adhesion force showed the decreased tread in both three groups with the

increasing concentrations of the extracts. The influence on the cell height and adhesion force by the three extracts were little different.

Interestingly, the Young's modulus of HaCaT cells changed dramatically compared with the control group of 1.73±0.13 kPa and there were obvious different under the treatments of the three extracts. Especially, at the concentration of 500μg/mL, the cell Young's modulus of the HaCaT cells was increased to 4.57±0.16 kPa for the 50%T group, 4.04±0.28 kPa for the 95%T group, and 3.52±0.33 kPa for the WT group. The results proved that the cell Young's modulus had a significant increase after treated with 50%T, 95%T and WT.

In conclusion, all the three *T. matsutake* extracts (50%T, 95%T and WT) could influence the mechanical properties of HaCaT cells. In addition, the 50%T had a greater effect on the Young's modulus than 95%T and WT.

C. Analyses of protein expression

It has been diffusely confirmed that the reason of the scar formation was short of epidermal stem cells, as well as the Notch1/Jagged1 pathway can promote epidermal stem cells proliferation. As shown in **Figure 3(a)**, the protein expression levels of Notch1 and Jagged1 were significantly increased as the concentration of the 50%T increased. And relative protein expression of notch1 and Jagged1 were showed in **Figure 3(b)**.

D. Analyses of mice wound healing

Figure 4(a) shows a representative wound. **Figure 4(b)** shows the pictures of wounds on days 0, 5, 10 and 15. There were different levels of scabs in all three groups on day 5. It can be seen that the wounds are treated with PC and 50%T

groups show the accelerated closure compared with the NC on days 10 and 15. Notably, the 50%T group have better therapeutic effect than the PC, and there are less scabs. As shown in **Figure 4(c)**, the wound healing rates of 50%T group are better than the NC and PC groups at all treatment times (days 5, 10 and 15) by quantitative analyses. The results show that 50%T can obviously accelerate the regeneration of wounds, promote the wound healing and reduce the scabs formation.

E. Analyses of histological staining

As shown in **Figure 5(a)**, in the PC and 50%T groups, the wound areas have begun to close and the epidermal regeneration can be observed on day 5. Nevertheless, the epidermal regeneration is incomplete and the hair follicles are few in the NC group. On day 15, the wounds have not entirely healed in the NC group, however, totally regeneration of the skin structure can be observed in the PC and 50%T groups. Most of all, the amount of granulation tissue is increasing, the hair follicles have reborn and are well-integrated with the surrounding area in the 50%T group. These findings prove that 50%T can accelerate the regeneration of epidermal cells, promote the tissue of granulation and increase the tightness of tissue arrangement during the wound healing.

As shown in **Figure 5(b)**, on day 5, the wounds treated with the 50%T group show much better collagen deposition and appendage density than the NC and PC groups. On day 15, it can be clearly seen that the collagen fibers are the highest in the 50%T group. However, less formation and arrangement of collagen are viewed in the NC and PC groups. Therefore, 50%T can effectively accelerate the skin wound collagen deposition and also facilitate wound healing.

IV. DISCUSSION

T. matsutake is one of the most precious mushrooms because of its taste and nutritive value. With the development of traditional Chinese medicine, more attention has been paid to *T. matsutake* pharmacological action.[17] *T. matsutake* is a kind of nutrition food, and it has no toxic side effects.

This study shows that *T. matsutake* can promote the cell proliferation, and the 50%T have the best effect than the others. It was reported that *T. matsutake* had a relationship with the cell proliferation, differentiation and apoptosis. Hiroki[18] found that methyl cinnamate in *T. matsutake* significantly suppressed the melanogenesis of murine B16-F10 melanoma cells without affecting the cell growth. Hou[19] researched that *T. matsutake* polysaccharide exhibits unique anti-tumor and immunoregulatory properties. *T. matsutake* is rich in protein, amino acids and other rare elements. It could be caused by 50% ethanol can extract more active ingredients from *T. matsutake*. The nutrients may act on a certain target of the cells, thereby promoting the cell proliferation and differentiation.

Biophysics is an interdisciplinary subject combining physics and biology, and is one of the important branches and fields of life science and physics. Physical property of biological cells is one of the acknowledged frontier research topics in biomedical physics.[20] The cell proliferation and differentiation may be related to their physical properties.[21] Kobiela[22] found that AFM analysis showed an increase of the cell stiffness for the cells treated with Acetyl Tetrapeptide-2 (P1) in concentration. In the aspect of cell morphological and biophysical properties, the HaCaT cells were treated with 50%T, 95%T and WT, and the cell height had a slight increase and adhesion force had a slight decrease with the increase of the

concentration. The Young's modulus represented the physical quantity of the material's elasticity, reflecting its resistance to stretching or compression deformation. The HaCaT cells were treated with *T. matsutake*, and the Young's modulus gradually grew larger showing that the cells had less prone to deformation. Thus, *T. matsutake* not only promoted cell proliferation, but also had a good protective effect on cells.

The Notch signaling pathway plays a regulating role in the cell proliferation, differentiation and apoptosis.[23-25] Increased expression of Notch1 and Jagged1 proteins caused cell proliferation. *T. matsutake* can activate the Notch1/Jagged1 pathway in HaCaT cells. This may be caused by the certain chemical constituents of *T. matsutake* which activates some factors, regulates the Notch1/Jagged1 expression, and accordingly up-regulate the Notch1/Jagged1 pathway. Meanwhile, with the up-regulation of Notch1/Jagged1 pathway, it may have a relationship with the cellular physical properties, such as the increasing of the cell height and Young's modulus.

When the skin is injured, it needs to be repaired by itself and medicine.[26] The result of the skin repair is affected by many factors.[27,28] According to the literature, the Notch1/Jagged1 pathway was involved in the mouse wound healing.[29] The mice wounds were treated with the NC, PC and 50%T, and the histological analyses of healing wounds in mice. It was found the healing effect of 50%T group was better than the NC and PC groups. In summary, it could be the extracts of *T. matsutake* that accelerated the regeneration of wounds, promoted wound healing and reduced the scabs formation by increasing the epidermis regeneration, sub-epidermal tissue formation and collagen deposition. In addition, whether *T.*

matsutake activates other healing-related signaling pathways and further mechanisms are still worthy of further study.

As a traditional Chinese medicine, *T. matsutake* comes from natural world, which has a stable effect, less toxic and side effect. It has a lot of advantages in the treatment of some diseases than medicine used in the clinic. This kind of natural products has good application potential on the treatment of skin wounds, such as applied to accelerate repair of the wound of surgical patient.

V. CONCLUSIONS

In this work, we used biological and physical methods to study *T. matsutake* promote the cell proliferation *in vitro* and *in vivo*. In *in vitro* experiment, the HaCaT cells viability and Young's modulus were increased after treated with *T. matsutake*. And *T. matsutake* can activating the Notch1/Jagged1 pathway. In *in vivo* experiment, *T. matsutake* can promote wound healing in mice, and the healing effect of *T. matsutake* was better than control group. In short, this work founds that *T. matsutake* can promote the HaCaT cells proliferation by activating the Notch1/Jagged1 pathway and increasing the cells Young's modulus. And also accelerate the mice wound healing by increasing skin regeneration, subcutaneous tissue collagen formation. As a traditional Chinese medicine, *T. matsutake* has a stable effect, less toxic and side effect. This work proved that *T. matsutake* can be used as a new type of medicine for wound healing in the future.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1. The cell viabilities of HaCaT cells exposed to the different concentrations of 50%T, 95%T and WT. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

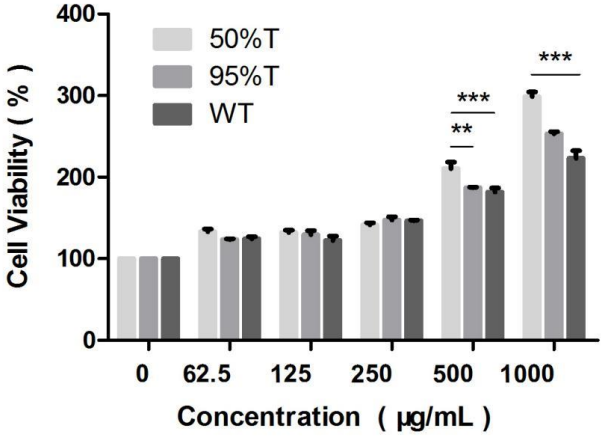
Figure 2. (a): AFM height (A_1-G_1) and adhesion force (A_2-G_2) images of the HaCaT cells incubated with *T. matsutake* extracts at 0 (A_1 and A_2), 50%T at 100 (B_1 and B_2), 50%T at 500 (C_1 and C_2), 95%T at 100 (D_1 and D_2), 95%T at 500 (E_1 and E_2), WT at 100 (F_1 and F_2) and WT at 500 μg/mL (G_1 and G_2). Statistical analyses of height (b), adhesion force (c) and Young's modulus (d) of the HaCaT cells incubated with *T. matsutake* extracts (0 μg/mL), 50%T (100, 500 μg/mL), 95%T (100, 500 μg/mL) and WT (100, 500 μg/mL). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. (a): Western bolt analysis of Notch1, Jagged1 and β-Tubulin. Control: the HaCaT cells without treatment; 1: the HaCaT cells were treated with 100 μg/mL of 50%T; 2: the HaCaT cells were treated with 500 μg/mL of 50%T ; 3: the HaCaT cells were treated with 1000 μg/mL of 50%T. (b): Relative protein expression of notch1 and Jagged1. Significant difference of Notch1 and Jagged1 of groups 1, 2 and 3 were compared to the control groups.

Figure 4. (a): Establishment of wound model in mice; (b): pictures of wound closure after three treatment groups on days 0, 5, 10 and 15, NC: negative control, PC: positive control; (c): wound healing rates quantified and statistically analyzed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 5. (a): Histological analyses of healing wounds in mice. Sections of wound skin at two points in time following three groups were stained with H&E; (b): Histochemical staining of collagens deposition. Sections of wound skin at two points in time following three groups were stained with Masson's trichrome.

FIG 1.


Cell Viability (%)
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200
100
0
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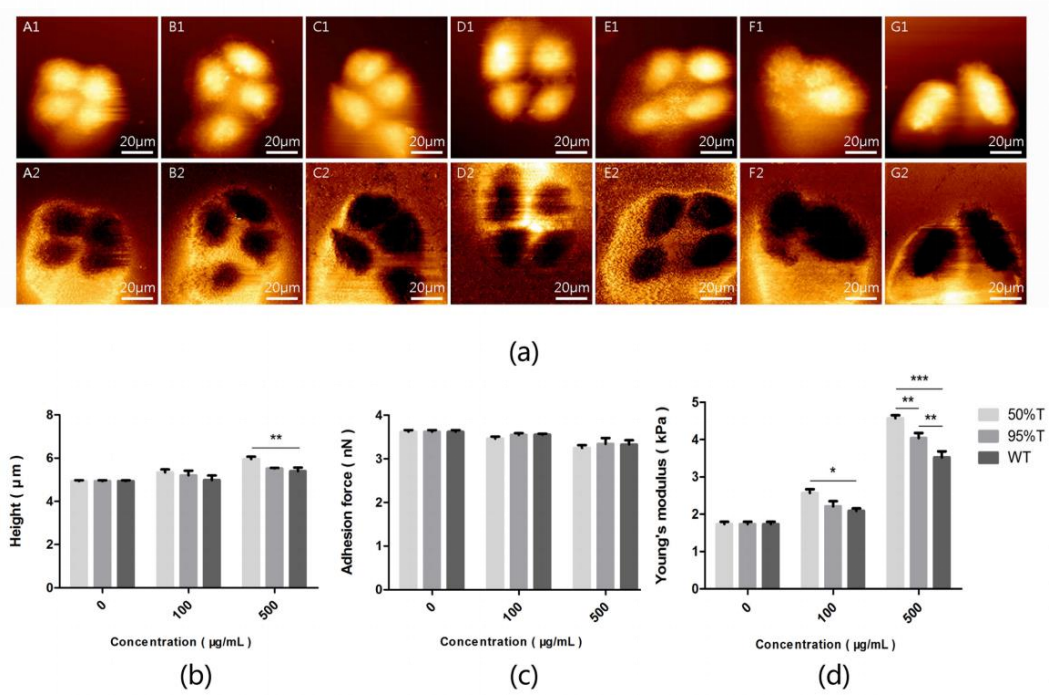
62.5
125
250
500
1000
Concentration (μg/mL)

FIG 2.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Height (μm)
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Adhesion force (nN)
Concentration (μg/mL)
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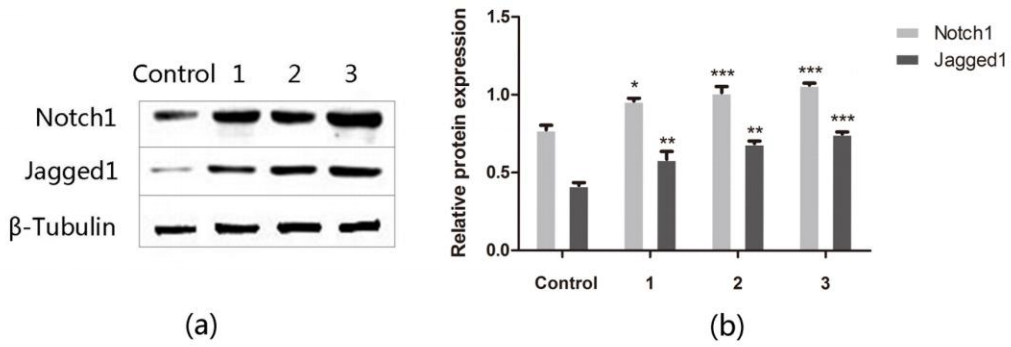
Young's modulus (kPa)
Concentration (μg/mL)
(d)
50%T
95%T
WT

FIG 3.


Control 1 2 3
Notch1
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(a)
Relative protein expression
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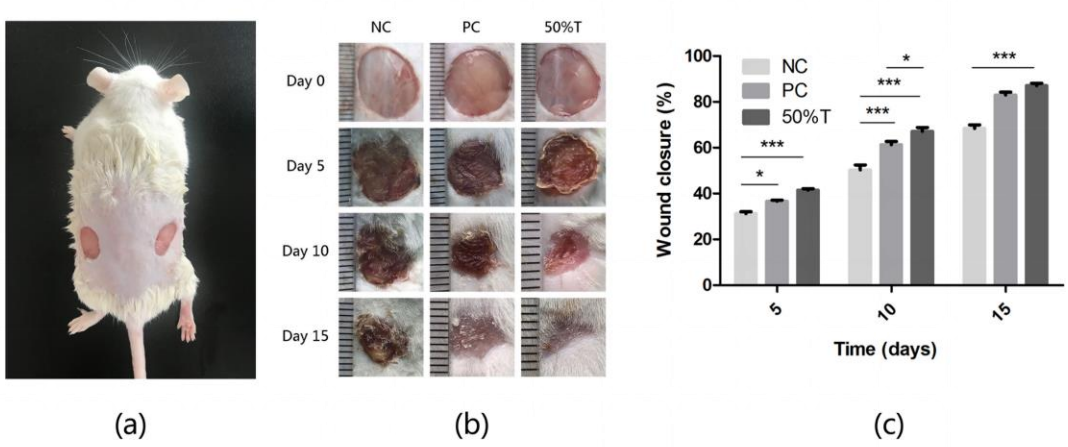
FIG 4.


NC
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50%T
Day 0
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(a)
(b)
Wound closure (%)
100
80
60
40
20
0
NC
PC
50%T
Time (days)
5
10
15
(c)

FIG 5.