# STUDY ON THE EFFECT OF COMPOUND "ZICAO" TEMPERATURE-SENSITIVE GEL ON SKIN WOUND HEALING OF NEW ZEALAND RABBITS

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***ABSTRACT***

*In this study, a temperature-sensitive gel made by our lab was used to evaluate the effect of compound comrador on skin wound healing in New Zealand rabbits. First of all, New Zealand rabbit skin excision injury model was established and four will be randomly divided into treatment group (normal saline negative control group, yunnan baiyao group, the blank group, the compound radix arnebiae seu lithospermi matrix group win min gel), to deal with New Zealand rabbit was detected after 14 d of wound healing rate and hydroxyproline content, HE dyeing observation was carried out on the wound tissue fibroblasts epithelization degree,Masson staining was performed to observe the fibers and inflammatory factors in the wound tissue. The results showed that after 14 days of treatment, the wound healing rate of New Zealand rabbits in compound comrador temperature-sensitive gel group was significantly higher than that in other treatment groups, and the wound healing rate was 92.4%. In addition, the histological analysis of wound tissue of New Zealand rabbits treated with compound comradina thermosensitive gel also showed that the degree of epithelialization was increased and the proliferation of fibroblasts was enhanced. Through the evaluation of its mechanism, the compound thermal-sensitive gel could also increase the content of hydroxyproline. The total hydroxyproline content of wound tissue increased to 3.51μg·mg-1 after the treatment of compound thermal-sensitive gel, which further confirmed that it could protect cells from oxidative damage, thus accelerating wound healing and reducing wound healing time. In conclusion, the topical application of compound thermosensitive gel made by ourselves in this study can promote wound healing in New Zealand rabbits, and provide a new and better choice for wound healing.*

***Keywords:*** *Compound "Zicao" thermosensitive gel, pharmacodynamics, wound healing.*

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## Introduction

Skin tissue is the largest organ in the human body and has many important functions, including protective barrier against etiology and mechanical stress, temperature regulation, touch, pressure, vibration and pain sensation, etc.[1]. However, skin tissue defects or hard-to-heal wounds (mainly chronic wounds) caused by trauma, burns, surgery or complications of diabetes are still a major medical problem.Although modern medicine has made great progress in wound repair, scar removal and muscle generation, wound infection, inflammation and scarring are still the most difficult and key problems in the process of wound repair. These problems bring many inconveniences to patients, such as decreased quality of life, physical limitations, a large number of hospitalizations and the need to change dressings frequently.

It not only brings long-term healing process and cruel psychological harm to patients, but also brings high monetary cost to society[2-3]. It has been noted that more than 20 million people worldwide suffer from this disease and the global wound care market is expected to cost up to $22 billion by 2024[4]. After the skin is damaged, its structure and function must be restored as soon as possible to maintain the stability of the internal environment. So the wound healing process begins almost immediately after skin injury to avoid the risk of bacterial infection.

This process requires many cytokines, inflammatory cytokines, growth factors, and extracellular matrix regulation, and is usually divided into several stages: The stages of coagulation, inflammation, proliferation, and remodeling are usually independent of strict and well-defined time periods and may overlap, while some large, chronic, and infectious skin wounds make it difficult for self-repair mechanisms to effectively rebuild skin wounds[5]. Therefore, it is of great significance in the field of medical application to develop an efficient, stable and durable wound healing drug that can not only protect the wound surface but also promote skin repair.

However, currently commonly used drugs for skin injury mainly include antibiotics, sterols, and fibrocell growth-promoting drugs, etc., which are easy to irritate the skin and cause allergies and other adverse reactions, and large or long-term use of antibiotics will produce certain toxic and side effects on the body, and most of these drugs are expensive[6]. At the same time, traditional Chinese medicine is mostly natural ingredients, which has the advantages of less adverse reactions, less scar formation, convenient materials and low production cost.Therefore, there is an urgent need to develop natural drug resources in clinical practice[7].

Arnebiaeuchroma (Royle) Johnst from Xinjiang is a plant of the genus Arnebiaeuchroma in the family of Arnebiaeuchroma, which was first described in Shennong's Herbal Studies. It is sweet, salt and cold in taste. It has the functions of clearing away heat and cooling blood, promoting blood circulation and detoxification, penetrating rash and eliminating spots, etc. It is often used for poisonous blood heat, purple black rash, impenetrable measles, sore selection, eczema, and scald of fire and water[8]. At the same time, there are many folk remedies to treat wounds, such as Angelica sinensis, Phellodendri phellodendri, Angelica dahuricae, and borneol, which have the functions of nourishing qi and blood, nourishing saprophytic muscle, anti-bacterial dryness, reducing swelling and relieving pain, and promoting wound healing. By referring to relevant materials and consulting Associate Professor He Changliang of Sichuan Agricultural University, we prepared a compound Chinese herbal medicine of sinensis composed of sinensis, angelica sinensis, phellocypress, angelica dahurica, borneol, etc., in order to find a more economic, convenient and effective wound treatment drug[9]. Meanwhile, in recent years, some studies have shown that thermosensitive gel materials can load proteins and peptide drugs, and can prolong the stability of the loaded biologic drugs. Poloxam, as an ABA type block amphiphilic copolymer composed of polyoxyethylene and polyoxypropylene, is a highly studied polymer material for the construction of temperature-sensitive gels, with good safety, stability and biocompatibility[10]. Therefore, in the previous study, our laboratory successfully prepared a temperature-sensitive gel containing poloxam, a temperature-regulating agent, and glycerol, a moisturizing agent, and loaded it with compound shiveria herb solution.

In this study, we, on the basis of previous research in a laboratory, to develop good compound radix arnebiae seu lithospermi win min outside gel is used to construct the model of the New Zealand rabbit skin excision injury, respectively to investigate treatment after 14 d of New Zealand rabbit wound healing rate, hydroxyproline content, tissue repair, etc., to observe the compound radix arnebiae seu lithospermi win min gel in New Zealand rabbit skin wound healing, The aim is to provide the possibility for the development of topical preparations for the treatment of skin wounds in the future.

## Materials and methods

### Materials

#### *Experimental animals*

Clean New Zealand rabbits, aged 3 months, with body weight of 2.0 ~ 2.5kg, were reared in cages with both male and female. Provided by Chengdu Dasuo Experimental Animal Co., Ltd.

The room temperature was 22°C±1°C, the relative humidity was 50%~60%, the indoor light was kept for 12h, and the circulation was kept out of light for 12h.

With rabbit special feed feed, free to eat and drink, feed and drink clean. The rabbits were fed adaptively for one week before the experiment, and their health status was observed. New Zealand rabbits with good health and nutrition status were selected for the wound repair experiment of compound comradina thermosensitive gel.

***Establishment of a full-thickness skin defect model in New Zealand rabbits***

New Zealand rabbits were fasted for 12h before surgery, and water was forbidden for 4 h. After weighing, 3% pentobarbital sodium solution was injected into the ear vein at 30 mg/kg. After the limbs of the New Zealand rabbits were stabilized, they were fixed on the operating table in the prone position, and their back was depilated with a female electric hair shaver (supplemented with 10% sodium sulphide if necessary). The depilation area was about 15 cm×10 cm. After hair removal, the back of the New Zealand rabbit was disinfected. After the completion of disinfection, the back spine of the New Zealand rabbit was taken as the midline. Two circular wounds with a diameter of 2.0 cm were planned on the two sides of the back spine of each New Zealand rabbit from front to back, a total of four wounds were planned (Figure 1). The skin was then removed to form four round full-thickness skin wounds as deep as the musculofascia.

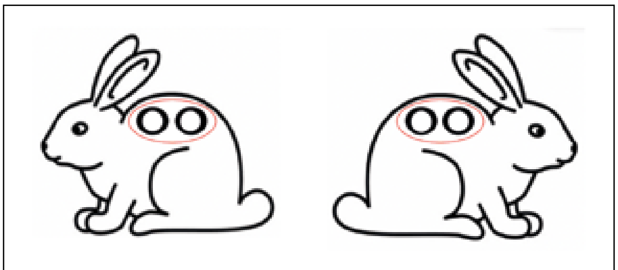

**Figure 1:** New Zealand rabbit back innovation model map.

***Experimental grouping and administration***

Each New Zealand rabbit used self-control method. As shown in Figure 2, the four wound surfaces were corresponding to (1) normal saline negative control group; (2) Yunnan Baiyao positive control group; (3) Gel matrix negative control group; (4) compound "Zicao" thermosensitive Gel group (the content of compound "Zicao" thermosensitive Gel was 8%).

From the first day after modeling, normal saline negative control group was given any treatment with saline Yunnan Baiyao positive control group was treated with Yunnan Baiyao; Gel matrix negative control group was treated with blank gel matrix; compound "Zicao" thermosensitive Gel group was treated with compound "Zicao" thermosensitive Gel. External application was given twice a day (once in the morning and once in the afternoon), 200μL each time, for continuous application for 14 d, and sample was taken 6 h after the last administration.

**Figure 2:** New Zealand rabbit back actual creation map.

***Observation of macroscopic recovery of wound surface***

After modeling, a digital camera was used to take local photos of the wounds of each New Zealand rabbit every day to observe and record the wound conditions, focusing on individual mental state, changes in the wound surface, healing conditions, such as redness and swelling, bleeding, infection, exudation and scab. At the same time, each New Zealand rabbit was weighed daily and the data of weight change was recorded.

***Calculation of wound healing rate***

Building after 24 h (i.e., postoperative day 1), the trauma was covered with cellophane, the trauma edge was marked with a pen, and the cellophane was cut along the edge. then weighed on an analytical balance with a division of 0.0001 g, and converted into an area that was the original, and the area of the unhealed trauma at a certain time point was calculated by the same method on days 2, 4, 6, 8, 10, 12, and 14. The healing rate (M) = (original wound area - unhealed wound area) / original wound area × 100%.

***HE dyed***

The standard hematoxylin-eosin protocol was performed on the sections to study the wound healing process. The specific operations were as follows: tissue block, after fixation, conventional paraffin embedding, sectioning; paraffin sections were dewaxed and hydrated; hematoxylin staining solution was stained for 3-10 min (specific time was adjusted according to the staining results and experimental requirements), rinsed with tap water for 5-10 s; differentiation The staining was carried out in the following way: 1-5 s of differentiation solution, 20-30 s of tap water rinsing, washing off the differentiation solution; 30 s-2 min of eosin staining (the specific time was adjusted according to the staining results and experimental requirements). time adjusted according to staining results and experimental requirements), tap water rinse 1-5 s; dehydrating, transparenting, sealing.

The film was sealed with neutral gum and observed under the microscope.

### *Masson tricolor dyeing*

Masson trichrome staining was used to measure the deposition of collagen fibers at the wound site. The specific operations were as follows: routine dewaxing of sections to water; The prepared Weigert iron hematoxylin staining solution was used for 5 min to 10min. Acidic ethanol differentiation solution differentiated for 5-15 s, washed with water; Masson blue solution returned to blue for 3-5 min, then washed with water; Wash with distilled water for 1 min; Li Chun hong Acid Fuchsin Staining Solution dye for 5-10 min; In the above operation process, according to the ratio of distilled water: weak acid solution = 2:1, prepare weak acid working solution, wash with weak acid working solution for 1min; Wash with phosphomolybdic acid solution for 1-2 min; Wash with the prepared weak acid working solution for 1 min; Directly into aniline blue staining solution for 1-2 min; Wash with the prepared weak acid working solution for 1 min; 95% ethanol rapid dehydration; Dehydrate with anhydrous ethanol for 3 times, 5-10 s each; Xylene transparent 3 times, 1-2 min each; Neutral gum seal.

### *Hydroxyproline (HYP) content calculation*

The hydroxyproline content was estimated by Hydroxyproline Assay Kit (Nanjing Jiancheng Institute of Biological Engineering) according to the instructions for use. Precisely weigh 30~50 mg of skin tissue into a test tube, add 1 mL of hydrolysis solution, mix well, and hydrolyze for 20 min at 95°C or in a boiling water bath (10 min of hydrolysis is to mix once to make the hydrolysis more adequate). Adjust pH value to 6.0~6.8, then add double-distilled water to 10 mL, mix well; take 3~4 mL of diluted hydrolysis solution and add appropriate amount of activated carbon (about 20~30 mg, subject to clarification and colorless after centrifugation of supernatant), mix well, centrifuge at 3500 r/min for 10 min, carefully take 1 mL of supernatant for testing. 0.5ml of reagent 1, reagent 2 and reagent 3 were added to the supernatant respectively. The mixture was mixed in water bath at 60 °C for 15 min, and centrifuged at 3500 r/min for 10 min. The supernatant was taken at 550 nm wavelength and 1 cm optical diameter. The double steam water was set to zero. The absorbance value of each tube was finally determined to calculate the content of hydroxyproline in the tissue.

Hydroxyproline content (μg/mL wet weight) = (measured OD value - blank OD value)/(standard OD value - blank OD value)* standard concentration (5μg/mL)* total volume of hydrolysate (mL)/wet weight of tissue (mg).

### *Statistical and analysis*

One-way analysis of variance (ANOVA) was used to statistically evaluate the results, with a $P<0.05$ indicating a significant difference, All data are expressed as mean ± SD and all data were expressed at least 3 independent evaluations. Excel 2016 was used for data collation, SPSS 23.0. were used for statistical analysis.

## Results and discussion

### *Observation results of macroscopic recovery of wound surface*

The wound area of New Zealand rabbits after treatment with different drugs is shown in Figure 3, which shows representative photographs of the wounds of New Zealand rabbits in different administration groups on days 0, 3, 6, 9, 12, and 14.

As can be seen in Figure 3, in all four treatment groups, plasma exudation was observed from the time of successful modeling until day 2, surface crusting began to form from day 4, and by day 9, crusting became more pronounced, with debridement beginning on day 12 in all treatment groups (e.g., in the group treated with compound "Zicao" thermosensitive Gel on day 12). As the contraction of the wound area occurred in a centripetal fashion, the initially almost circular wound now decreased in size and producing irregular edges with no defined geometry. However, the reduction in area and the contraction of the wound in a gradual and similar manner was observed between the wounds treated with Comfrey Warming Gel and the control, suggesting that there was no impairment of the healing process.

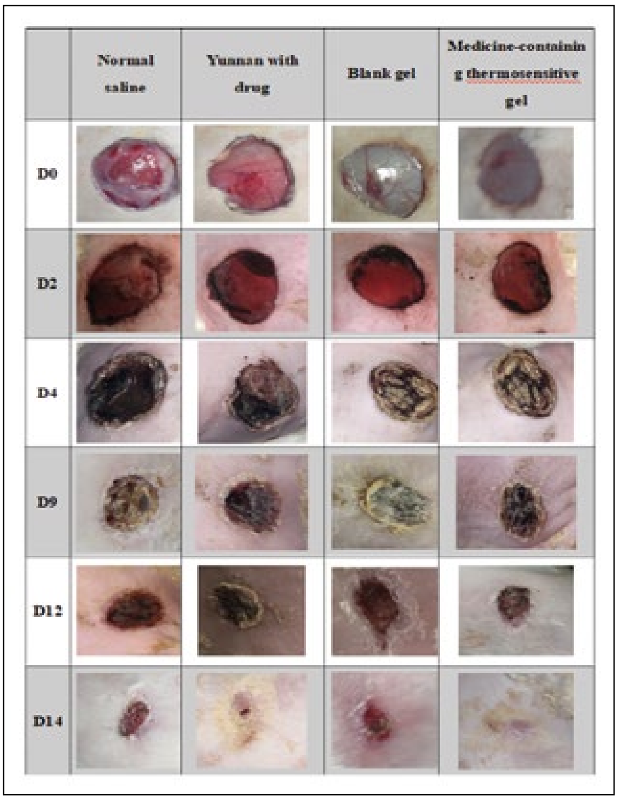


**Figure 3:** Representative images of different treated wounds at days 0 to 14.

### *Skin wound healing rate results*

Wound healing in New Zealand rabbits is shown in Figure 4, which shows the wound healing rates of New Zealand rabbits in different drug administration groups on days 2, 4, 6, 8, 10, 12, and 14 based on the change in wound area before and after drug administration. The wound healing rates of the saline and blank gel treatment groups were comparable, and were smaller than those of the Yunnan Baiyao treatment group and the compound "Zicao" thermosensitive gel treatment group.

The wound healing rate of Yunnan Baiyao treatment group was significantly higher than that of the compound "Zicao" thermosensitive gel treatment group; from day 6, the wound healing rate of Yunnan Baiyao treatment group was comparable to that of the compound "Zicao" thermosensitive gel treatment group; from day 8, the wound healing rate of the compound "Zicao" warm-sensitive gel treatment group was higher than that of the Yunnan Baiyao group, which showed that the compound "Zicao" thermosensitive gel treatment group and Yunnan Baiyao were similar in efficacy and could promote wound healing. The changes of wound area and wound healing rate of New Zealand rabbits were combined to confirm that compound "Zicao" thermosensitive gel promoted wound healing.

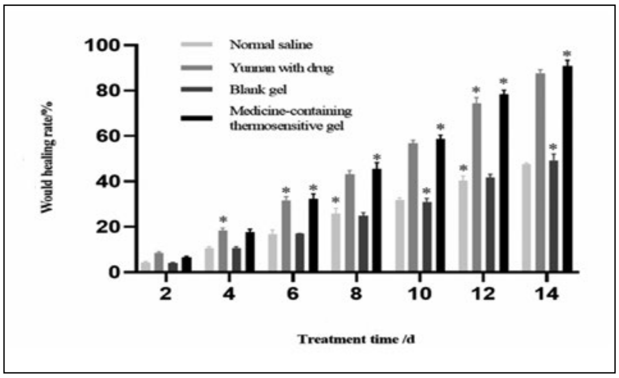


**Figure 4:** Wound healing rate of different treatments from day 0 to day 14.

### *Difference analysis of hydroxyproline in wound healing process*

In order to further verify the effect of compound "Zicao" thermosensitive gel in the wound healing process, the content of hydroxyproline, a precursor ofcollagen, was measured in the wound tissue. According to the data in Figure 5, The hydroxyproline content in the compound "Zicao" warming gel-treated group and Yunnan Baiyao group was significantly higher than that in the saline negative control group and the blank gel matrix-treated group, and there were significant differences. The results showed that the compound "Zicao" thermosensitive gel could promote collagen synthesis in wound healing process, which was similar to Yunnan Baiyao.

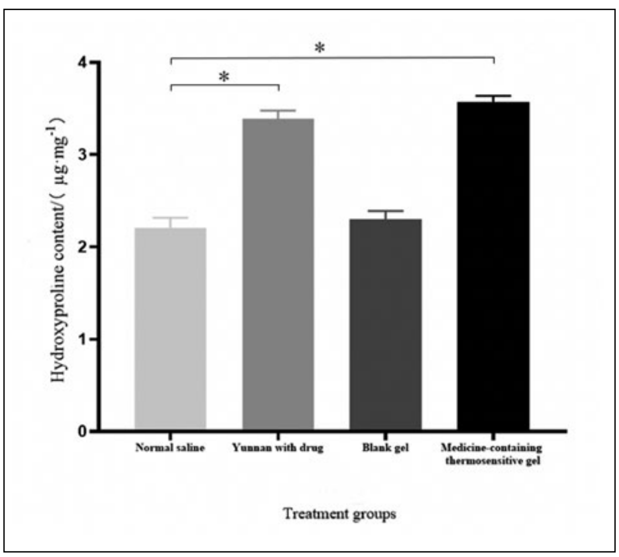


**Figure 5:** Hydroxyproline content in different treatment groups on Day 14.

### *HE staining results*

As shown in Figure 6, the HE staining results showed that on day 14, the physiological saline negative control group and the blank gel matrix group still had inflammatory reactions, and the newly formed fibroblasts (blue arrows) were less than those in the compound "Zicao" thermosensitive gel group. In addition to other factors, the characteristics of wound healing is hemostatic, epithelial and remodeling of the extracellular matrix, again epithelial change is epithelial newer process after injury, involving the epithelial cell proliferation and migration to the center of the wound[11-12]. therefore in the histologic analysis, again epithelial change on the surface of the wound is based on the length and thickness of the assessment, the results show that the treated water gel after 14 days, The wound surface of each group was flattened and covered by new epidermal tissue, and the dermis of the repaired surface was replaced by fibrous tissue. Meanwhile, the fibers of Yunnan Baiyao group and Compound "Zicao" thermosensitive gel group were more tightly arranged and curved like elastic fibers, which indicated that the effect of Compound "Zicao" thermosensitive gel on wound healing was not significantly different from that of Yunnan Baiyao group and was better than that of normal saline negative control group and blank gel matrix group. In addition to skin regeneration, compound "Zicao" thermosensitive gel was observed hair follicles (black arrow), blood vessels (red arrows), such as skin appendages, and at the same time, compared with yunnan baiyao group with compound "Zicao" thermosensitive gel processing wound appears more again epithelial change, shows that compound "Zicao" thermosensitive gel significantly promote the wound healing of skin wound repair and regeneration effect.

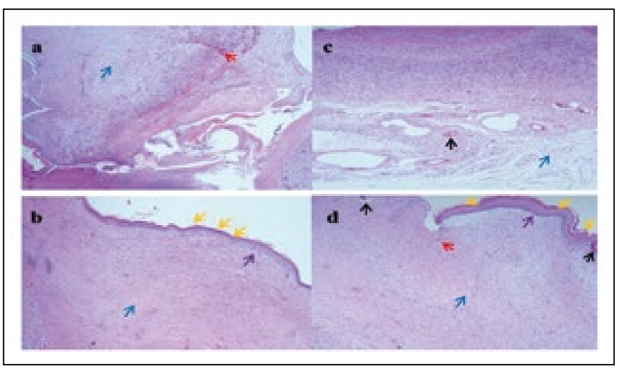


**Figure 6:** Day 14 Microphoto of HE staining results from different treatment groups (newborn epithelium: yellow arrow; inflammatory cells: purple arrow; blood vessels: red arrow; hair follicles: black arrow; fibroblast: blue arrow).
*a: Normal saline. b: Yunnan with drug. c: Blank gel. d: Medicine-containing thermosensitive gel.*

### *Masson tricolor staining results*

Cell proliferation is a key phenomenon in the process of epithelial re-formation during wound healing[13].

After injury, keratinocytes at the wound edges rapidly multiply to form a dense epithelium, and subsequently these keratinocytes migrate to the wound bed to restore the barrier function of the epidermis. The remodeling of skin composition during the final phase of wound repair is therefore a key indicator of wound healing, which can be visualized by the deposition of collagen at the wound site.

During the healing process, collagen deposition intensifies and most cells disappear, and this observation may be attributed to the apoptosis of fibroblasts and endothelial cells and the formation of scar tissue. Collagen synthesis began several hours after the injury, but did not become significant until about a week after the injury.

To assess the level of collagen deposition, Masson trichrome staining was also performed to demonstrate wound healing.

Three color as shown in figure 7, Masson staining results showed that compared with other treatment group, a large amount of collagen dyed blue precipitate appeared in compound "Zicao" thermosensitive gel cut part of the treatment group, other groups of collagen after different treatment is also present different degree of sedimentation, but collagen deposition levels are compound "Zicao" thermosensitive gel processing group is low.

The good collagen deposition indicates the great potential of the compound thermosensitive gel in wound healing[14]. In the future, this hydrogel may provide a new opportunity for more other drugs to be studied in the field of biomedical science.

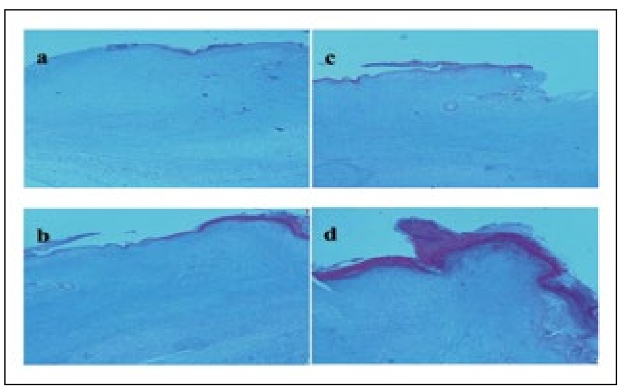


**Figure 7:** Micrographs of Masson staining results in different treatment groups on day 14.
*a: Normal saline. b: Yunnan with drug. c: Blank gel. d: Medicine-containing thermosensitive gel.*

## Conclusion

In this study, the pharmacodynamics test showed that: For 14 consecutive days, the New Zealand rabbits with full-thickness skin defects were treated with compound "Zicao" thermo-sensitive gel prepared by this research institute. by testing the New Zealand rabbit wound healing rate, content of hydroxyproline, HE dyeing observation was carried out on the wound tissue fibroblasts epithelization, Masson staining was carried out on the wound tissue fiber in the watch and inflammatory factors.

The results showed that after 14 days of treatment, the wound healing rate of New Zealand rabbits in compound "Zicao" temperature-sensitive gel group was significantly higher than that in other treatment groups, and the wound healing rate was 92.4%.In addition, the histological analysis of wound tissue of New Zealand rabbits treated with compound "Zicao" thermosensitive gel also showed that the degree of epithelialization was increased and the proliferation of fibroblasts was enhanced. Through the evaluation of its mechanism, it was found that the compound "Zicao" thermal-sensitive gel could also increase the content of hydroxyproline.

After the compound "Zicao" thermal-sensitive gel treatment, the content of total hydroxyproline in the wound tissue increased to 3.51μg·mg-1, which could protect cells from oxidative damage, thus accelerating wound healing and reducing wound healing time.

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