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Comparative Study of Wound Healing After Treatment With Crude Date Extract and Silver Sulphadiazine

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Abstract

Objective: Various naturally occurring substances have been used in wound treatment throughout history. The most common topical agent used in the treatment of wounds includes silver sulphadiazine. Crude date extract (CDE) in comparison with silver sulphadiazine was tested to evaluate their effects on wound healing and their antimicrobial activities against the growth of *Staphylococcus aureus*.

Material and Methods: The effect of different concentrations of crude khudari date extract on the growth of *S. aureus* was studied. Active compound was partially purified using thin layer chromatography technique with different solvents. Evaluation of wound healing activity with CDE was done by creating full-thickness incision wounds in rabbit's skin and using in comparison silver sulphadiazine.

Results: Crude date extract 5% (w/v) and silver sulphadiazine 20% (w/v) inhibited the growth of *S. aureus* by 67% and 87%, respectively. However, 10% and 20% (w/v) date extract showed 100% inhibition. Date extract was fractionated using different solvents with different polarities; all fractions were tested for their antimicrobial activities. Ethyl acetate fraction was found to have inhibitory activity against the growth of *S. aureus*. Preparative TLC was done for ethyl acetate fraction which was further identified to be terpenoidal compound. The histological changes in healing wounds have also been investigated by light microscopy after treatment with 20% and 40% crude date extract, silver sulphadiazine and amoxicillin.
Conclusions: The results of this study showed that 20% CDE treatment has promoted the process of wound healing and stimulates fibroblast, collagen and epithelialization significantly. This effect was comparable with the effect of silver sulphadiazine treatment.

Key words: Date extract, silver sulphadiazine, *Staphylococcus aureus*, wounds.

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Introduction

Proper healing of wounds is essential for restoration of disturbed anatomical continuity and disturbed functional status of the skin. It is a product of the integrated response of several types to injury [1]. Wounds are classified according to various criteria: etiology, duration, morphological characteristics, and the degree of contamination. The management of wounds depends on the stage of the wound healing, which is a complex process involving interaction among a variety of

different cell types. The normal wound repair process consist of three phases: inflammation, proliferation and remodeling, that occurs in a predictable series of cellular and biochemical events [2].

Wound with sufficiently hypoxic and reduced environment are susceptible to colonization by a wide variety of endogenous anaerobic bacteria. However, pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and Beta hemolytic streptococci are the primary causes of delayed healing and infection in both acute and chronic wounds [3-7].

Silver has been used for centuries to prevent and treat a variety of diseases, most notably infection. Both bacterial and fungal infections of wound have promoted the development of variety of different topical agents that may be applied to the wound to reduce the chances of wound infection. The most common topical agents used in the treatment of wounds include silver sulphadiazine (SSD) and silver nitrate [8-9]. The efficacy of (SSD), a topical antimicrobial agent, was confirmed in the prevention and treatment of burns or other surgical wounds. Furthermore, SSD used in clearing staphylococcal carriage [10-11]. Various naturally occurring substances have been used in wound treatment throughout history. Among these substances, sugar (sucrose), honey (main constituents are glucose, fructose and maltose) and molasses (main constituents are sucrose and glucose) are the most common [12-13]. Different plant extracts (*Datura alba, Ixora coccinea , Michauxia spp, Pyrostegia venosta*) were reported recently for wound healings [14-17].

Dates are regarded as high-energy food due to its high sugar content (75%); all sugars in dates consist of a mixture of sucrose, glucose and fructose. Dates contain also protein (2.5%) and lipids (2.5%) on a dry weight basis. They also contain vitamins and significant amount of certain minerals such as iron, calcium and potassium [18]. There are a number of substances in minute quantities in date flesh such as polyphenol and organic acid [19]. The role of phenolic compounds as antimicrobial agents is reported by Prescott et al. [20].

Date extracts were studied for their effects on the growth of *Bacillus subtilis*, *S. aureus*, *Salmonella typhi* and *P. aeruginosa* [21]. Also, the effects of date extracts on *Candida albicans* were investigated [22]. The present study aimed to investigate the effect of crude and partially purified date extract on the growth of *S. aureus*, and to evaluate the wound healing activity of crude khudari date extract in comparison with silver sulphadiazine in rabbit's skin.

Materials and methods

Organism and growth conditions

Staphylococcus aureus (ATCC 29213) was used as control in this study. Nutrient broth and nutrient agar (Oxoid, England) were used to grow and maintain this culture. Incubation of cultures was performed at 37°C for 24h.

Preparation of crude date extract (CDE)

The local date marketing, khudari date *Phoenix dactylifera* was used in its ripe stage. Extracts of dates were prepared by suspending 400 g date in 1000 ml distilled water for 24h, and then homogenized in warring blender at maximum speed. The homogenized extract was filtered by double layer cheesecloth then kept at 4°C, to be used as a stock date extract solution 40%.

Nutrient agar media containing different concentration of CDE, 5%, 10% and 20%were prepared from the stock 40% date extract solution, as final concentration in addition to 20% silver sulphadiazine(Sigma). Media sterilization was done at 121°C for 10 min.

The inoculum of (1.5×10^8) CFU/ml is diluted by serial dilution to obtain 250-350 CFU/ml. 1 ml of bacterial inoculum was added on nutrient agar plates containing different concentration of date extract or silver sulphadiazine. This inoculum was spread according to spread- plate technique (23), also the inoculum was added to a Petri dish containing only nutrient agar, as a negative control. All plates were incubated at 37°C for 24-48h. The number of colonies in each plate was counted.

Fractionation of CDE

The date was fractioned using different extraction solvents with different polarities [23-24]. Five hundred grams of date was suspended in one-liter of petroleum ether for 24-48h with continuous shaking, since this non-polar solvent dissolves fixed oil, fatty acids, fats and other hydrocarbons. Petroleum ether extract was collected after filtration with filter paper. The residual fraction was collected and extracted again with one-liter semi-polar solvent chloroform for 24h with continuous shaking; in order to dissolve lipids and oils [23].

The residue was further collected and extracted again with ethyl acetate (one liter) for 24h with continuous shaking so

that volatile oil, alkaloids, phenols and terpene esters are dissolved [23]. Ethyl acetate extract was collected, and the residue was extracted again with water for 24h with continuous shaking to retain sugars, proteins and ionic solutes. The three organic extracts evaporated by rota vapor under vacuum at 45°C. About 2 ml of each extract were collected.

Identification and isolation of partially purified compound from ethyl acetate extract

Ethyl acetate extract was applied on TLC (Thin layer chromatography) plate, then developed using the following development solvents: (hexane: chloroform: methanol) in a ratio of (2.5: 5.5: 2) and 3-4 drops of acetic acid. Plate was sprayed after that with a spraying agent which consists of 3 g vanillin dissolved in 100 ml of 95% ethanol in addition to 1 ml of 5% sulfuric acid. After spraying, the plate was placed in the oven at 110°C for 4 min., different visualized spots were marked. The fractions were separated on preparative thin layer chromatography, and then each fraction was tested for its antimicrobial activities.

Antimicrobial activity

Agar well diffusion method was used to measure the antimicrobial activity of different fractions (petroleum ether extract, chloroform extract, ethyl acetate extract and the partially purified compound) according to Shanmuga Priya et al. [14] and Roy et al. [17].

Histological study-Animals used

Female New Zealand rabbits 3-4 months of age, weighing 2.5-3.5 kg were used in wound healing model experiments. They were bred at the animal house unit, faculty of medicine, Jordan University of Science and Technology. Food and water were supplied to the animals during the whole period of the experiments.

Healthy rabbits (New Zealand) were first anaesthetized with light ether. Hair was removed by shaving the back of the rabbits and then each rabbit was placed in a separate cage. Immediately before making the wounds, the skin of the animals was washed with soap and cleaned with 70% ethanol. One type of wound, namely surgical incision wound was created of full-thickness type, extending down to the subcutaneous tissue, in the shaved areas of skin.

Incision wounds and induced skin infection with S. aureus

Two incision wounds (2 cm linear for each) were made on the shaved area on the back of each animal by using scalpel blade, one incision wound as a test and the other wound as a control (untreated). Immediately after incision, the area of each wound was infected with 200 µl (10^5 CFU) *S. aureus* using sterile cotton swab. The experimental animals were divided into two groups, each group with two rabbits. Group 1 wound treated topically with 20% CDE and group 2 wound treated topically with silver sulphadiazine 20% [10, 23].

Histological examination

Animals were anaesthetized and sacrified after 6 and 12 days of treatment and a specimen sample of skin tissue from each group of rabbits was taken out from the healed wounds skin for histological analysis. Skin specimens from wounds of all animals were fixed in 10% neutral – buffered formalin, pH 7.2 for at least 48 h, and then washed with 0.1 M phosphate buffer, pH 7.2 three times, dehydrated with graded ethanol series (30, 50, 70, 80, 95, and 100%) then clearing by xylene. The tissues were then infiltrated and later embedded in paraffin wax. 5 µm sections were prepared by standard techniques using a rotary microtome. The thin sections of skin tissue were stained with Mayer's haematoxylin and eosin (16).

Results

Effect of different concentration of CDE on S. aureus in comparison with silver sulphadiazine

The antimicrobial activity of different concentrations of CDE and silver sulphadiazine 20% (w/v) against *S. aureus* are presented in **Table 1**. Date extract (5%) inhibited the growth of *S. aureus* by 67%, whereas 10% and 20% CDE showed 100% inhibition. Silver sulphadiazine inhibited the growth of *S. aureus* by 78% (**Fig. 1**).

Table 1. Effect of different concentrations of CDE, 20% silver sulphadiazine (S) on the growth of S. aureus.

Antimicrobial agents	CFU/ plate*	% Inhibition
Control	309	0%
5% CDE	109	67%
10% CDE	0	100%
20% CDE	0	100%
20% S	72	78%

* Readings represent triplicate trials.

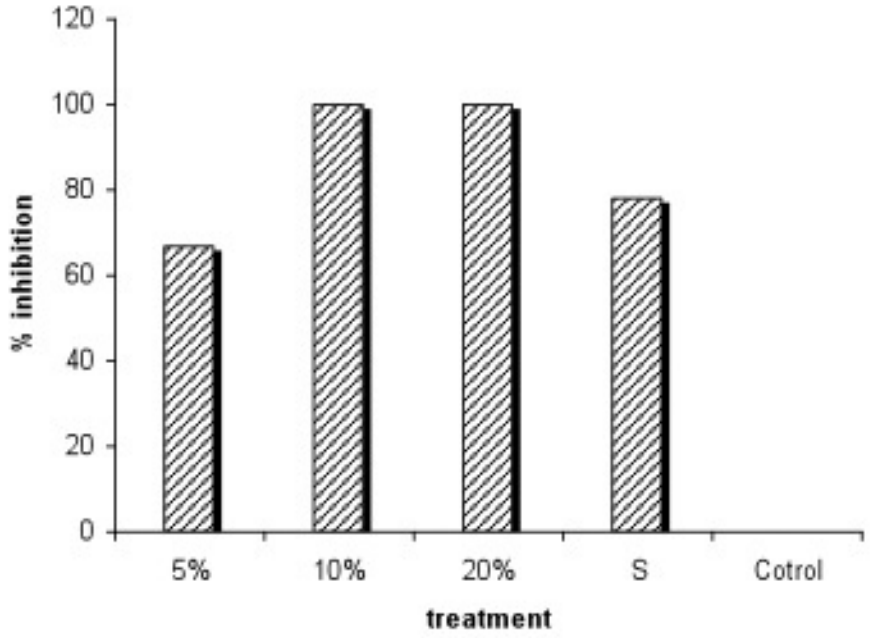

Fig. 1. Effect of different concentrations of CDE on the growth of S.aureus.

Fractionation of CDE and their antimicrobial activities against S. aureus

Crude date extract was fractionated using different solvents with different polarities namely; petroleum ether, chloroform and ethyl acetate. Four fractions were recovered and tested for their inhibitory effects on *S. aureus* growth.

Using agar well diffusion method, ethyl acetate fraction has shown inhibitory activity The diameter of the inhibition zone of ethyl acetate fraction was 14 mm, while other fractions were found to be inactive (no zone of inhibition). Ethyl acetate was tested as a negative control and showed no antimicrobial activity against *S. aureus*.

The isolated active fractions using ethyl acetate were further applied on a preparative TLC. Eleven bands were observed under the UV light. These bands were scrapped and dissolved in ethyl acetate. The antimicrobial activities of the different isolated bands were tested against *S. aureus* using agar well plate methods. Fractions 8, 9, 10, and 11 showed inhibitory activity with 3, 5 , 9 and 3 mm respectively. Other fractions were found to be negative.

Fractions 8 to 11 were further applied to TLC plate (**Fig. 2**) and then were suspected to be terpenoidal compounds, since they reacted with vanillin-sulfuric acid reagent [24].

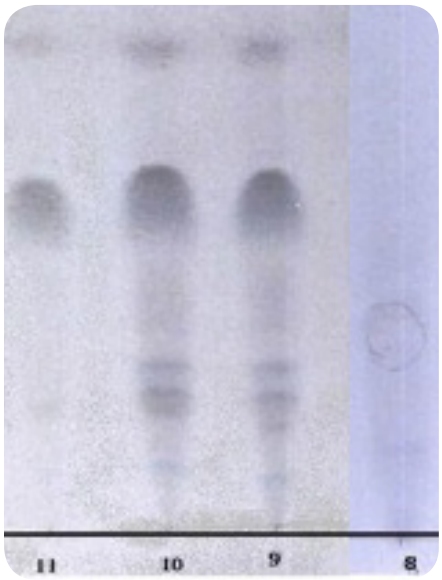

Fig. 2. The TLC plate for the four isolated active ethyl acetate fractions (8, 9, 10 and 11) after spraying with vanillin-sulfuric acid.

Histological study-Day 6

There was quite little variation in the control group and the healing process of the wound, this variation ranges from the irregular epithelium development with certain thickness of the keratin in the surface to highly congested blood vessels in between collagen bundles (**Fig. 3a**). In 20% of CDE group, there was active proliferation of blood vesssels at the area of

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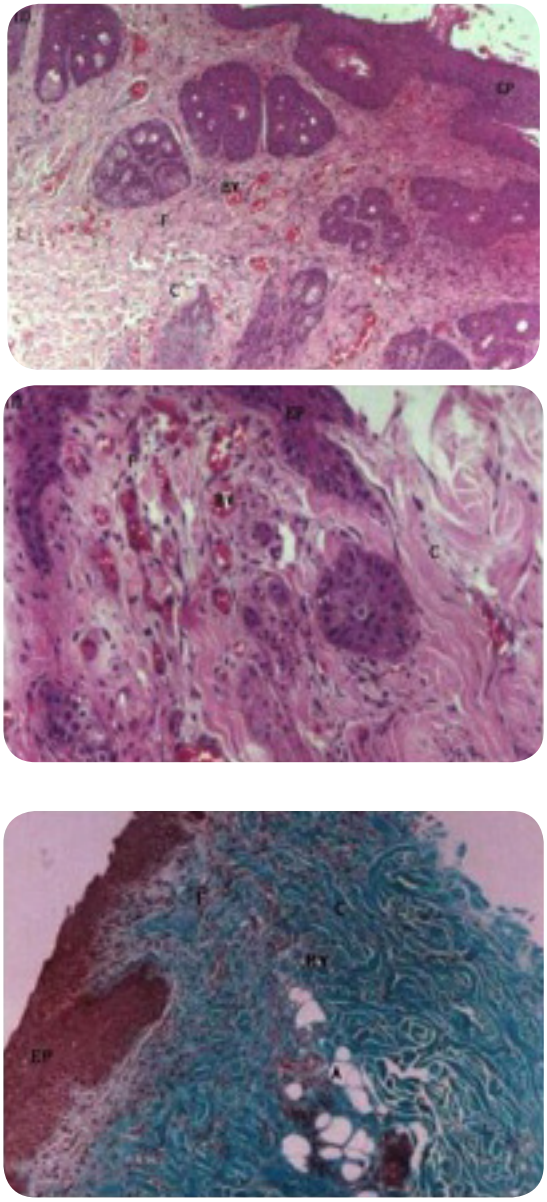

Fig. 3. Photomicrograph of 6-days healing wound, **A**, control, stained with H & E (200X); **B**, 20% CDE treatment, stained with H & E (200X); **C**, 20% silver sulphadiazine treatment, stained with Masson's trichome (80X). F (fibroblast), HF (hair follicle), EP (epithelium), BV (blood vessel), C (collagen) and A (adipocytes).

both wound edges. Colagen bundles were well organized. Bridging of the wound lips was evident with epithelium development; however, blood vessels were still large in number and contain red blood cells (**Fig. 3b**). On the other hand, extention into the wound site was shown in SSD treated group, in addition to some irregularities of the newly formed surface epithelium. Hypercellularity and well organized collagen bundles with few adipocytes are predominant features of the wound site (**Fig. 3c**).

Day 12

Histologically, in the control group there was complete and well developed epithelium in the surface with hypercellularity and increased collagen bundles. Also, there were complete absence of hair follicles and glands in the wound region (**Fig. 4a**). In 20% of CDE group, there were clear epithelialization and less collagen bundles when compared to other regions. Congested blood vessels, fibroblasts and collagen bundles were the predominant feature of this stage.

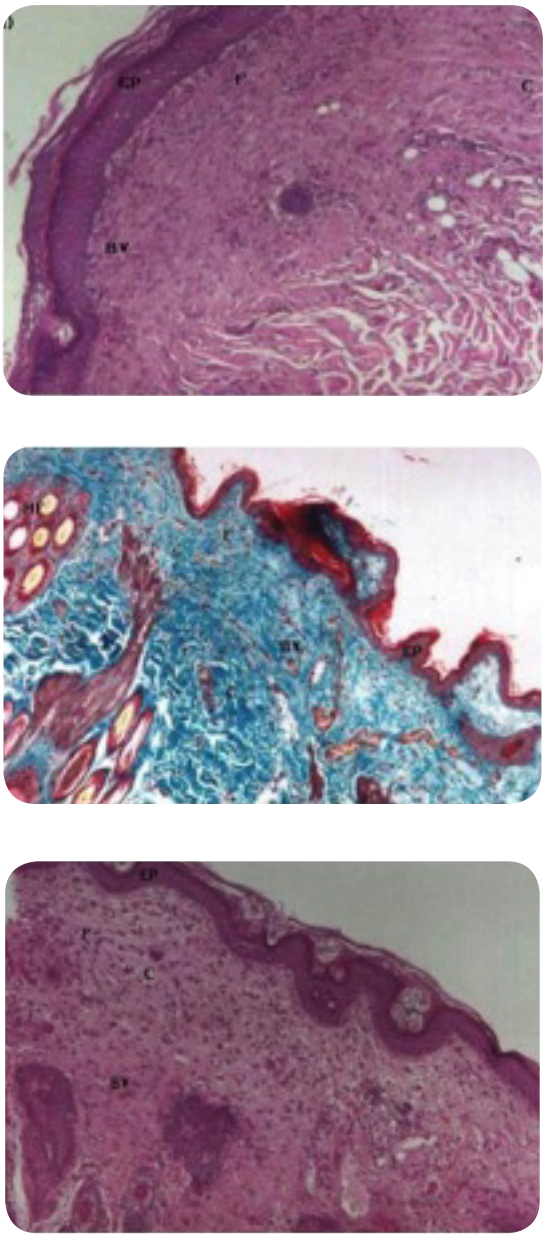

Fig. 4. Photomicrograph of 12-days healing wound. **A**, control, stained with H &v E (200X); **B**, 20% CDE treatment, stained with Masson's trichome (80X); **C**, 20% silver sulphadiazine treatment, stained with H & E (200X). F (fibroblast), HF (hair follicle), EP (epithelium), BV (blood vessel), C (collagen) and A (adipocytes).

Few adipocytes were observed in one section. In other sections, complete healing of the wound with well organized epithelium was detected with few congested blood vessels (**Fig. 4b**).

Well organized and developed epithelium on the surface of the wound. Keratinization of the surface epithelium and the formation of layers of keratin in a whirle shape are the features of the silver sulphadiazine treatment of the wound. The dermis is filled with congested blood vessels (**Fig. 4c**).

Discussion

Date is considered as a staple food in many desert areas, since they contain high sugar content and significant amounts of certain minerals such as potassium and iron, but they are poor sources of vitamins [18].

Date extract was found by Sallal and Ashkenani [21] to inhibit the growth of certain Gram negative and positive bacteria, and also to inhibit spore germination of *Bacillus subtilis*. Growth of *Candida albicans* and germ tube formation was also inhibited by date extract which was compared with certain known antimicrobial agents such as amphotericin B and nystatin [22].

Due to the effect of CDE on *S. aureus*, this study was performed to concentrate on the possible histological effect of CDE in wound healing.

Silver sulphadiazine used in this study for comparative purpose with CDE was found to inhibit the growth of *S. aureus* (**Fig. 1**). Similar to other heavy metals, silver is toxic to microbs by poisining respiratory enzymes and components of the microbial electron transport system as well as impairing some DNA function [9]. This is in accordance with the results reported from previous studies which suggested the use of silver sulphadiazine in treating staphylococcal carriage as an alternative to mupirocin treatment [10-11]. In addition, silver sulphadiazine is widely used for the prophylaxis and treatment of wound infection [11].

Aqueous and dichloromethane extract of *Bolax gummifera* were used for the treatment of wound infected by *P. aeruginosa* and *S. aureus*. The aqueous extract showed 82% inhibition against *S. aureus* but no activity against *P. aeruginosa* [25]. Chemical investigation of genus Buddleja showed that the presence of saponins, flavonoids and other phenolic compounds could contribute to wound healing because of their detergent ability to remove grease, dirt and bacteria from tissue and to act as antimicrobial agent [26-27]. In this study four fractions were recovered from date extract with ethyl acetate and these fractions were tested for their effects against the growth of *S. aureus*. The results showed a good inhibitory activity for the ethyl acetate fraction and no effect for the other tested fractions.

Terschuk et al. [28] reported that ethyl acetate fraction from leaves of *Tagets minuta* had antimicrobial effects. Moreover, the results of this study were similar to some plant extracts that contained diterpens and showed antimicrobial, anti-inflammatory and wound healing effects [29].

Further purification of ethyl acetate fraction resulted in fractions 8, 9, 10 and 11 showed a good inhibitory activity on the growth of *S. aureus*, while other fractions were found inactive. These fractions are possibly terpenes ester since they reacted with vanillin-sulfuric acid [24], and not with ferric chloride and ammonium chloride. Therefore, these fractions are almost neither phenolic nor flavonoidal compounds. Suresh et al. [30] reported the presence of terpens in *Sontalina chamaecyparissus* oil which has antimicrobial activity.

Silver sulphadiazine has been used in clinical settings for more than a century. Typically, the only side effect to its use is a transient discoloration of the treated area as a result of interaction of silver with proteins and chlorides present in the tissue [9]. Silver sulphadiazine is absorbed into the skin, where it forms a reservoir of silver ions, which are then released into the tissue [31].

Well-organized, developed epithelium and formation of keratin layers on the surface of wounds are the features of the silver sulphadiazine treatment in this study (**Fig. 2**). However, silver sulphadiazine enhanced re-epithelialization by silver ions in the presence of minimal infection as it exhibit anti-infammatory effects mediated by matrix metalloproteinases (MMPs) (collagenase inhibition) [31]. Agren [32] has demonstrated that MMPs play an integral role in the degradation of extarcellular proteins in wound sites, allowing re-epithelialization to progress optimally. Additionally, accumulation of silver in rat wound was associated with increased zinc, presumably reflecting higher levels of zinc metalloenzymes, which was associated with increased epidermal cell proliferation and improved re-epithelialization [31]. Also, accelerated wound healing by silver ions may be due to excitation of growth factors, cytokines and chemotactic pathways [33]. These reported effects of silver sulphadiazine are comparable with the results obtained in **Fig 3a** and **b**.

This study showed that topical application of CDE at the concentration of 20% significantly enhanced wound healing by active proliferation of blood vessels and well organized collagen bundles in day 6. After 12 days of treatment with 20% CDE, congested blood vessels, fibroblasts and collagen bundles were the predominant features of these stages, in addition to clear epithelialization. Other studies indicated that the common factor in wound contraction is the activity of fibroblasts that are found in the granulation tissue of healing wound [34]. In conclusion, there is a significant correlation between results of 20% CDE and silver sulphadiazine. Both enhanced healing of dermal wounds in rabbits.

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