Investigation of the effects of *Achillea millefolium* extract in diabetic rats with second-degree Burns

Tulay Diken Allahverdi[1], Ertugrul Allahverdi[2], Pınar Aksu Kilicle[3] and Hasan Ozen[4]
[1]Kafkas University Department of General Surgery, KARS, Turkey
[2]Kafkas University Department of Orthopedics and Traumatology, KARS, Turkey
[3]Kafkas University Department of Biology, KARS, Turkey
[4]Kafkas University Department of Patology, KARS, Turkey

Abstract: Our aim was to investigate the effects of *Achillea millefolium* on wound healing in second-degree burns formed in diabetic rats. This study was conducted at the Kafkas University Experimental Research Center. 20 Sprague Dawley rats were divided into 2 groups. Group I (n=10 rats) was the Control group and contained the diabetic rats with burn injury. Group II (n=10 rats) was the group where burn injury was created and *Achillea millefolium* was administered to diabetic rats. The backs of the rats were shaved so as to include 30% of the body area. A 10% lanolin extract was administered for 14 days after the burn injury was created. Tissue was obtained from the burn area of the rats sacrificed. No significant difference was found in Group II in terms of the severity of the dermatitis and inflammatory cell reactions when compared to Group I. No significant difference was observed between FGFR1immunoreactivity in the epidermis and dermis in Group I. While FGFR1immunoreactivity in 3 rats in Group II was similar to Group I, strong immunore activity that was more prominent in the epidermis was found in 7 rats in Group II. We believe that *Achillea millefolium* contributes to wound healing in burn injury due to its antioxidant and anti-inflammatory properties.

Keywords: *Achillea millefolium*, burn, diabetes, antioxidant.

INTRODUCTION

Burns constitute a public health problem that causes long-term disability, morbidity and mortality worldwide and especially in undeveloped countries. They are complex problems leading to high health care costs, long-term disability, extremity losses, multiple operative procedures, long-term hospitalization, infections or even deaths (Nasiri *et al*., 2017). Approximately 2.5 million people have been reported to suffer from burns each year in a study conducted in the USA. Approximately 100,000 patients were reported to be hospitalized and treated each year in the same study. More than 10.000 of these patients die due to complications of the burn (Zhang *et al*., 2017). Hot water is the most common cause. The necrotic tissues in burn injuries have a negative effect on healing by allowing the growth of microorganisms in the wound, creating an infection source and even leading to sepsis (Durmuş *et al*.,2009). Since hemorrhage will decrease in the affected regions in diabetic cases with burn injury, a complex condition in which the normal course of tissue repair is impaired develops (Fantinati *et al*., 2016). *Achillea millefolium* is a member of the Asteraceae family and is also called Yarrow. It has been used as a folk remedy in skin inflammations, spasmodic gastrointestinal disorders, and hepatobiliary symptoms (Potrich *et al*., 2010). *Achillea millefolium* is known to contain several components including essential oils and flavonoids and phenolic compounds such as phenolcarboxylic acids. The various preparations of *Achillea millefolium* have anti-inflammatory, antibacterial, antioxidant, liver protectant and antitumoral effects (Potrich *et al*., 2010). A gastroprotective effect of the drug with its antioxidant properties and useful effects in wound healing with the antioxidant and anti-inflammatory features have been shown in previous studies (Potrich *et al*., 2010).There are no studies on the effect of *Achillea millefolium* on burn injury in diabetic rats in the literature. Our aim in this study was to investigate the effects of *Achillea millefolium* on wound healing in second-degree burns created in diabetic rats.

MATERIALS AND METHODS

Diabetes creation: We took 21mg/1 ml of the prepared sodium citrate buffer and dissolved streptozocin (STZ) inside it. Since STZ solution could deteriorate under light and could be affected by heat, the tube where it was prepared was wrapped in aluminum foil and kept in an ice-filled beaker during the injection, and the solution was administered freshly to the animals. A 1ml insulin injector was used to inject 50 mg per kg streptozocin according to the weight of the rat intraperitoneally to 30 rats to create diabetes. Fasting blood glucose levels were determined, by using a glucometer, from the blood samples taken from the tail vein of the rats kept fasting for 8 hours before starting the study. Later, blood glucose levels were measured again after 8 hours of fasting on the 3rd day of STZ administration and the rats with glucose levels over 250 mg/dl were included in the study.

**Corresponding author:* e-mail: drtulaydiken@hotmail.com

Extraction Procedure: Achillea millefolium, a perennial herbaceous plant from the daisy family, was harvested from the Kars-Kağızman district in May-June. Its identification was performedat the Kafkas University Faculty of Science and Literature, Department of Biology's Botanic Department. The flower parts of the harvested plant were dried by intermittent spreading in a dark room free of sunshine and light, and with dry airflow. Once the desired flower parts became dry, they were passed through a grinder. The extraction procedure was performed with the help of a Soxhlet device. About 50 grams of dried flower parts, weighed with a sensitivity of 0.1 mg, was placed in a cartridge washed with the extraction solvent. The cartridge was placed in 500 ml of Soxhlet extractor and 650 ml of solvent was placed into the boiling flask. The solvent was extracted (40-45 times) until it became clear.Ethanol was used as the extraction solvent. After the extracts obtained were filtered off the blue band filter paper and the particles removed, the solvent was evaporated at 65-75°C with a rotary evaporator in flasks with constant weight, providing the extract. The obtained extract was weighed and a 10% cream was prepared with lanolin. Chemical composition of the essential oil from *Achillea millefolium* consists of eucalyptol (24.6%), camphor (16.7%), α-terpineol (10.2%), β-pinene (4.2%), and borneol (4.0%).

Burn formation

This study was conducted at the Kafkas University Experimental Research Center (June 2017). 20 male Sprague Dawley rats weighing 180-350 gr were used in the study. Group I (n=10 rats) was the Control group and contained the diabetic rats with burn injury. Group II (n=10 rats) was the group where burn injury was created and *Achillea millefolium* was administered to diabetic rats.The subjects were brought to the research center from the production center one week before the study started and prepared for the experiment by being kept in an environment with a fixed temperature (22ºC) with a 12-hour day and 12-hour night environment and given a standard rat diet. The rats to be used in the experiment were fasted and allowed to drink only water for 12 hours beforehand. After the experimental study protocol received ethical and scientific approval from Kafkas University Medical Faculty's Experimental Research Committee (ethics committee no. KAÜHAYEK / 2017-048), it was conducted in accordance with the rules of the "National Institutes of Health, Guide for the care and use of laboratory animals". The rats used in the experiment were obtained from the Experimental Animal Production Center of Atatürk University Research Center. Rats in all groups received anesthesia with ketamine hydrochloride and xylazine hydrochloride given intraperitoneally following a 12-hour fasting period. The back of the rats were then shaved so as to contain 30% of the body area. Our burn model was a modification of the method previously used by Sawada *et al*. (Sawada, 1997). A Vileda (© Procter & Gamble) sponge cut to a size of 25x10x5 mm was kept in boiling water (100ºC) and applied to a predefined and prepared area on the back for 35 seconds with its own weight. A preliminary study was performed with 5 rats to find the optimum duration. The burn depth was examined histopathologically after burning the rats for 15, 20, 25, 30, and 35 seconds and we found that 35 seconds provided the desired burn depth. Development of a second-degree burn (deep partial thickness burns) was confirmed histopathologically in all burned rats. We then applied 10% lanolin extract to cover the wound for 14 days. Tissues obtained from the burn area after the rats were sacrificed via hypovolemia were sent to the Pathology department for histopathologic investigation on the post-operative 14th day.

Histopathologic investigation

The tissue samples were fixed in 10% formaldehyde buffer and paraffin blocks were prepared. The tissue samples cut from the paraffin blocks were routinely processed, stained with hematoxylin and eosin (HE), and examined under the microscope.

Immunohistochemical evaluation

The avidin-biotin peroxidase method was used to determine Fibroblast Growth Factor Receptor (FGFR1) activity in the tissues. Skin sections cut 4-5 µ thick from the paraffin blocks were treated with 0.3% H_2O_2 for 15 minutes to block endogenous peroxidase activity following deparaffinization and rehydration. We then added 0.1% Trypsin for 15 minutes at 37°C and washed the tissues with phosphate buffer solution (PBS) to expose the antigenic receptors. Non-immune goat serum was applied to the tissues for 30 minutes to block non-specific antibody binding. Subsequently, the tissues were incubated at room temperature for 1 hour with 1:200 diluted rabbit anti-FGFR1 antibody (Abcam Catalog No.: ab10646). Primary antibody binding in the tissues was revealed with diaminobenzidine/H_2O_2 after the application of biotinylated secondary antibody followed by horseradish peroxidase. After routine procedures, the hematoxylin-stained tissues were covered with a coverslip and evaluated in terms of their staining properties under the microscope.

RESULTS

Histopathological investigations revealed moderate dermatitis, accompanied by hemorrhage of varying degrees in certain cases, characterized by lymphocyte, monocyte and neutrophil infiltration in the dermis that was more intensive right under the epidermis in Group I. Irregularly structured connective tissue bundles were also noted in the dermis. The epidermis generally showed an effort to regenerate locally and with varying epidermal thickness and the corneum layer had started to develop in regions close to these areas (fig. 1a). The presence of a

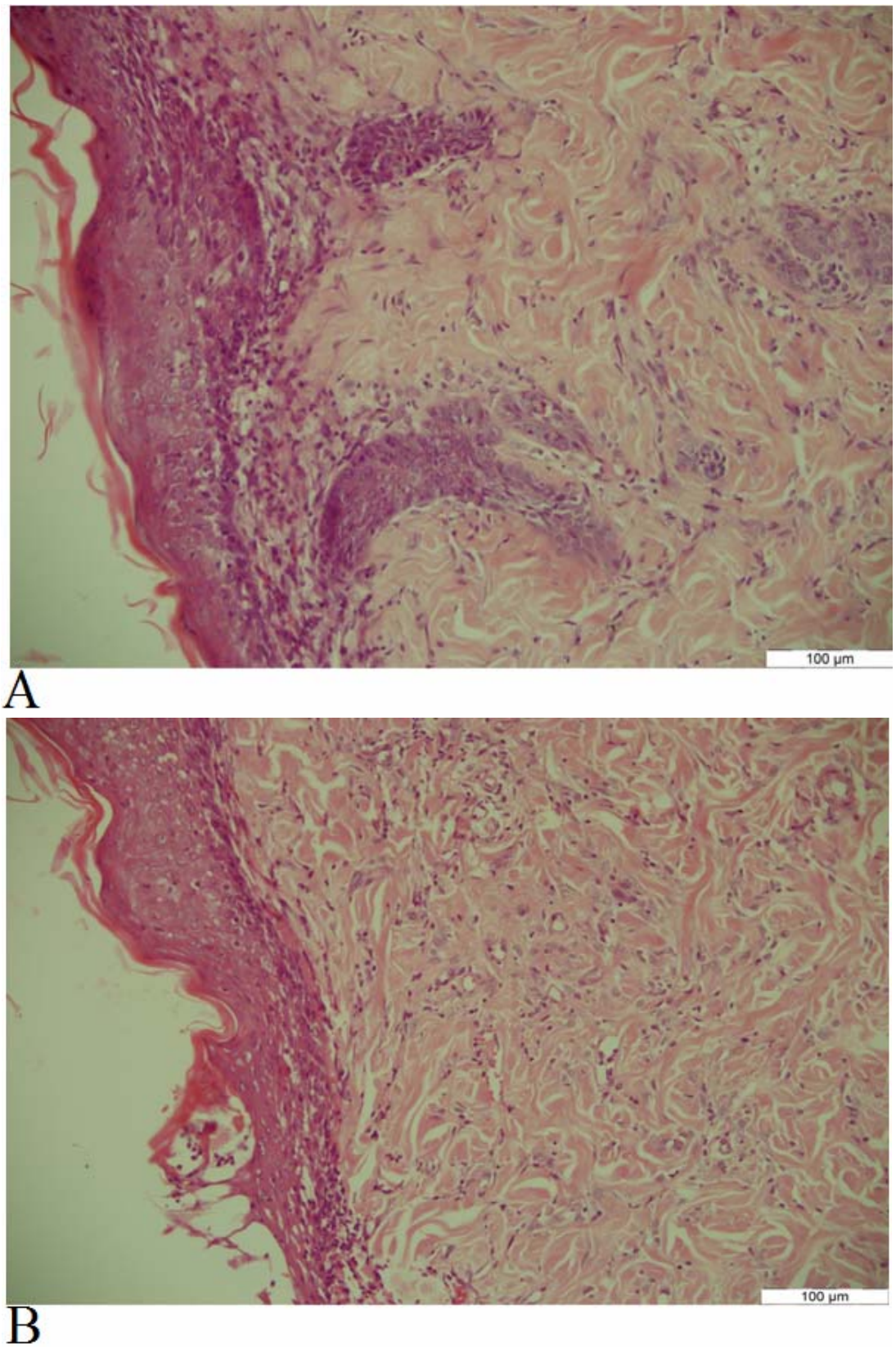

Fig. 1: Mild dermatitis characterized by infiltration with low numbers of lymphocytes, monocytes and neutrophils and irregularly structured connective tissue bundles at the dermis close to the epidermis and regeneration at the epidermis. a) Control group, b) Extract group (HE).Relatively less inflammation is observed in the extract group (HE).

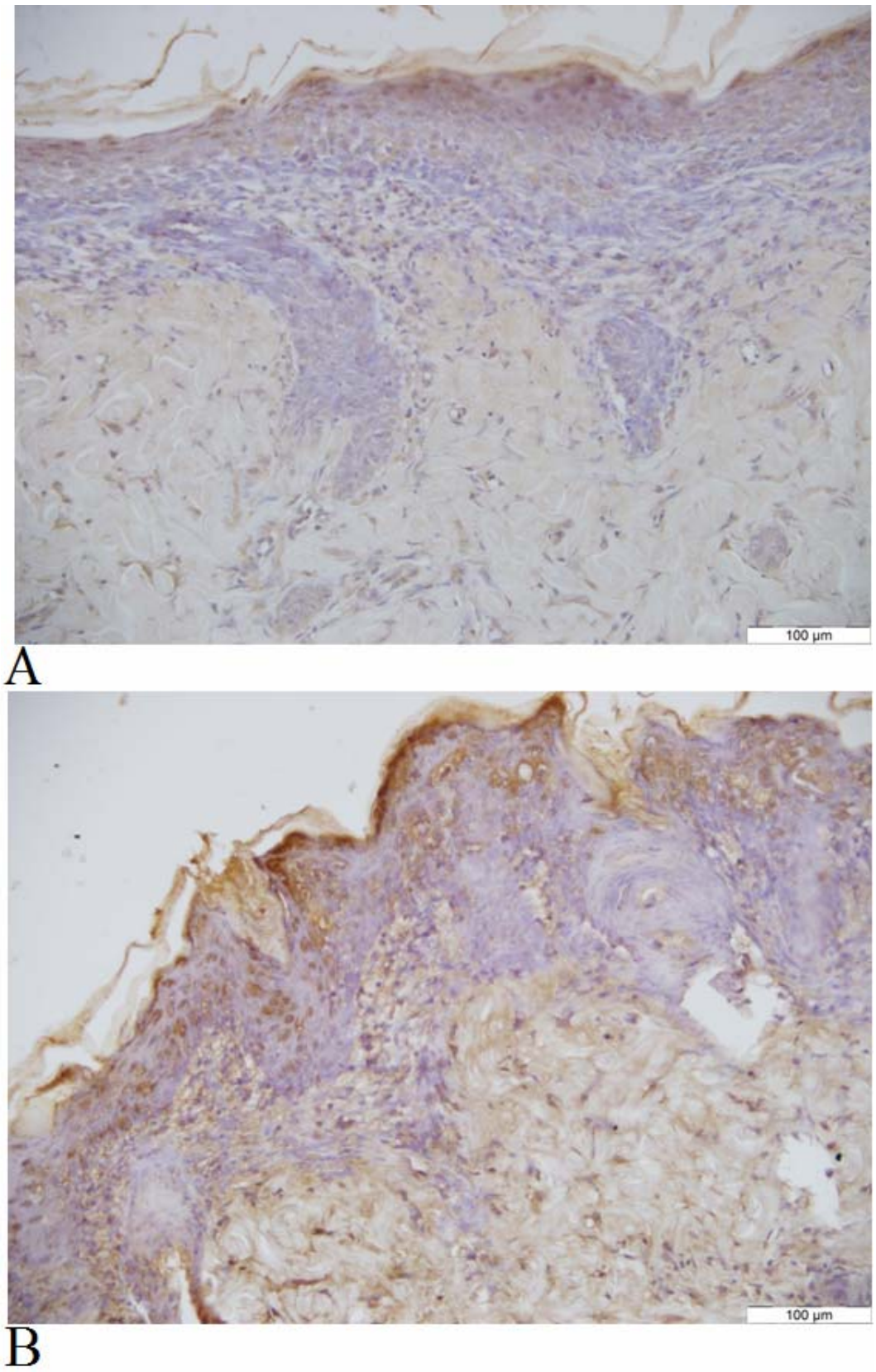

Fig. 2: FGFR1 immune staining in dermis fibroblasts and more prominent staining in the epidermis and the surface epithelium. a) Control group, b) Extract group.While FGFR1immunoreactivity was observed in both the epidermis and the dermis in both groups, the immunoreactivity was more prominent in some rats in the extract group.

crust structure involving necrotic tissue masses was noticed in 6 rats.No significant difference was found in Group II in terms of dermatitis severity and inflammatory cell reactions when compared to Group I. However, the lymphocyte, monocyte and neutrophil infiltration was observed to be relatively less prominent and epidermal regeneration to be more successful in 7 rats in this group (fig. 1b).

It was found that immune staining was more prominent in the fibroblasts of the hair follicles and the fat glands in the dermis and mostly the epithelium that was superficial or close to the surface in the epidermis when FGFR1-stained tissue sections were immunohistochemically evaluated. Dermal immunohistochemical staining was much weaker in areas where epidermal healing was relatively better (fig. 2a). No significant difference was observed in terms of FGFR1immunoreactivity in the epidermis and dermis in Group I (fig. 1 a, b). FGFR1 immunore activity in 3 rats in Group II (fig. 2b) was similar to Group I rats but strong immunoreactivity that was more prominent in the epidermis was found in 7 rats in Group II.

DISCUSSION

Burns lead to major mortality and morbidity worldwide. The main aims of burn treatment are to prevent wound infections and to ensure effective wound healing (Cavalcanti *et al*., 2006). We evaluated the effectiveness of *Achillea millefolium* extract on treating second-degree burn injury created in diabetic rats in this study. The burn process is characterized by an increase in the basal metabolic rate and basal body heat, hyperdynamic circulation, lipolysis, melting of body mass, and a delay in wound healing. As a result, cellular functions deteriorate and the risk of infection increases. After the burn, immunosuppression develops with the suppression of humoral as well as cellular defense mechanisms and there is an increased incidence of infections (Tokyay *et al*., 2005). Diabetes also causes high rates of mortality and morbidity due to its complications. Oxidative stress plays a major role in the pathogenesis of the disorder. The main mechanisms contributing to increased oxidative stress in diabetes are hyperglycemia, non-enzymatic glycolysis of proteins, oxidative DNA damage, oxidative glycolysis, sorbitol pathway activity, metabolic stress due to changes in the energy metabolism, changes in inflammatory mediator levels, inadequate antioxidant defense system, and increased oxidative stress (Baykal, 2000, Gumieniczek, 2003, Halifeoğlu *et al*., 2005). Diabetes causes delays in wound healing due to these mechanisms.

Achillea millefolium has been reported to be a folk remedy used for the treatment of various disorders including gastrointestinal disorders, pain and inflammation in Brazil in the studies conducted by Cavalcanti *et al*. [(Cavalcanti *et al*., 2006) and Baggio *et al* (Baggio *et al*., 2002) and has also been shown to have gastroprotective properties. *Achillea millefolium* has been demonstrated to have a liver protective effect by decreasing the damage caused by carbon tetrachloride and acetaminophenin the liver in a study conducted by Gagdoli *et al* (Gagdoli, 1995). Hemmati *et al*. (Hemmati *et al*., 2002) reported that *Achillea millefolium* helped wound healing by stimulating myofibroblast contraction and that this effect could be more marked during the proliferation and remodeling phase. It was also reported to prevent wound infections in the same study (Hemmati *et al*., 2002). We found moderate inflammatory cell infiltration of the dermis in Group 1 and mild infiltration in Group II. Necrotic areas were present in Group I but not in Group II. The lymphocyte, monocyte and neutrophil infiltration was less prominent, epidermal regeneration was better and dermatitis was milder in Group II than Group I. No significant difference was observed in terms of FGFR1immunoreactivity in the epidermis and dermis in Group I. While FGFR1immunoreactivity in 3 rats in Group II was similar to Group I rats, strong immunoreactivity that was more prominent in the epidermis was found in 7 rats in Group II. *Achillea millefolium* was shown to have positive effects on wound healing in burn injury with these findings in our study.

CONCLUSION

The positive effects of *Achillea millefolium* on burn injury can be attributed to its antioxidant, antibacterial and anti-inflammatory properties. In addition, *Achillea millefolium* had positive effects on wound healing, although wound healing was delayed in the diabetic rats.

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