CREATING A DIABETIC MODEL WITH SKIN WOUNDS IN RATS FOR APPLICATION IN WOUND TREATMENT

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Chronic limb ulcer is one of many dangerous complications of diabetes mellitus. Creating a skin lesion model in rats with diabetes and hyperlipidemia opens the door for testing new treatments in clinical practice. Wistar male rats were induced to have diabetes and dyslipidemia using a high-fat diet for six weeks and low-doses Streptozocin (STZ) injections (35 mg/kg). After that, skin wounds were created by cutting the total skin layer; observation of healing process and histopathological assessment were conducted. The results showed that rats fed with a high-fat diet combined with low-dose STZ injection induced hyperlipidemia and hyperglycemia that remained above 11 mmol/L during the follow-up period. The diabetic and hyperlipidemic rats had longer wound healing time than the normal rats. Thus, this study successfully created an excisional skin wound model on Wistar rats with diabetes and hyperlipidemia which could serve in future testing phases of new wound healing treatments.

Keywords: Wound healing, skin wound, diabetes, hyperlipidemia rat model.

I. INTRODUCTION

According to statistics from the International Diabetes Federation Diabetes Atlas (IDF), in 2021, there were 537 million people with diabetes worldwide. This number is projected to increase rapidly and reach 783 million cases by 2045.[1] Diabetes causes metabolic disorders and dangerous complications, including chronic limb ulcers. Studies have shown that up to 6.3% of diabetic patients have chronic skin ulcer complications.[2] Chronic limb ulcers and slow-wound healing problems affect the health and mobility of patients that cause an economic burden to society. In the United States, the annual cost of treating chronic extremity ulcers in diabetic patients amounts to between $9 and $13 billion; however, the time- and cost-effectiveness expectations are still not achieved.[3] Creating skin lesions models in diabetic animals will enable deeper understanding of the pathogenesis and better testing of potential treatments in the future.

Many studies worldwide have built different models of skin lesions in animals, especially in rats.[4,5] However, in Vietnam, established skin-wound models in diabetic animals are still few and have many limitations. The existing models cannot simulate the pathogenesis of chronic limb ulcers and wound-healing disorders in diabetic patients. Thus, we conducted this study to develop a procedure for creating skin

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wounds and monitoring wound healing in *Wistar* rats with diabetes and hyperlipidemia.

II. SUBJECTS AND METHODS

1. Experimental animals

Adult *Wistar* rats of male gender and weighed 180 - 220 grams were used for the study. Rats were kept at a temperature of 26 ± 2°C, with a 12-hour light-dark cycle, and given a standard diet for 4 days to acclimatize to the conditions in the animal room before the experiment. This study was conducted at University of Medicine and Pharmacy, Vietnam National University.

2. Machines and chemicals

- Machines: Blood glucose monitoring system On Call EZII (ACON Biotech, USA); Animal blood counter Vet Exigo (Boule Medical AB, Sweden); Chemistry analyzer Erba and Test strips: blood triglyceride, LDL-C, HDL-C, cholesterol (Transasia, India); Centrifuge EBA 20 (Hettich, Germany); microscope IX73 (Olympus, Japan); HM 325 Rotary Microtome (Thermo Scientific, USA); HistoStar operator (Thermo Scientific, USA).

- Chemicals: Streptozotocin 1g (Sigma-Aldrich, Singapore), Buffer solution Citrate pH 4.5; Chloral hydrate 500g (BDH Chemical, UK); Formaldehyde solution 500mL (Xilong Scientific, China); HE staining chemicals (Labcoms, China).

3. Research Methods

The study consists of two phases:

Phase 1: Building the diabetic and dyslipidemic rat model

The diabetic rat model used in this study was based on Zhang (2008).[6] Adult healthy *Wistar* rats were randomly divided into two groups:

- Group 1 (normal group, n = 6 animals): Raised on a standard diet (15% lipid, 58% carbohydrates, 27% protein and minerals).

- Group 2 (diabetic/hyperlipidemic group, n = 6 animals): Raised on a high-fat diet (40% lipid, 48% carbohydrates, 12% protein and minerals).

Rats were housed on the described diet for six weeks. After six weeks, rats were taken blood samples to examine serum levels of triglycerides, HDL-C, LDL-C, and cholesterol, and test the fasting glucose concentration with a Blood glucose monitoring system. Then, rats in Group 2 were injected peritoneally with Streptozocin (STZ) at two doses of 35mg/kg daily dissolved in citrate buffer pH 4.5, while rats in the control group were injected with citrate buffer pH 4.5. After three days of injecting STZ, rats were considered to have induced diabetes if their fasting blood glucose was ≥ 11.1 mmol/l (200 mg/dl).

Phase 2: Building the cutting skin wound model in rats with diabetes and dyslipidemia

The cutting skin wound procedure was conducted following Galiano et al.[5] After removing hair from the back of rats with a shaver and hair removal cream, rats were anesthetized with chloral hydrate at a dose of 250 mg/kg. Then we used surgical scissors and forceps to cut two wounds of 2 cm in diameter at the skinned site and fixed the wound with a silicone ring and surgical dressing.

On the 3rd, 5th, 7th, and 13th day after wounding, the wounds were scored using the scale presented in Table 1, and pictures of the wound were taken. The wound area was calculated using ImageJ version 1.8.0 software. The following formula was used to calculate the wound closure rate: Wound closure rate (%) = (original wound area − present wound area)/ original wound area × 100%.

On the 7th and 14th days, skin wound tissue, liver, and pancreas samples of one random rat in each group were harvested for histological examination. The obtained tissue

samples were fixed into a 10% formaldehyde solution. Satisfactory samples were sliced, stained by the improved HE and Masson methods, and observed by software system on an IX73 microscope with 4X, 10X, 20X and 40X magnifications. Other rats would be monitored until the wound completely healed.

Table 1. Scoring criteria for skin wounds in rats

Characteristics of wound	Detail	Score
Congestion	None	0
	Have congestion	1
Swelling	None	0
	Have swelling	1
	Have blistering	2
Secretions	None	0
	Colorless discharge	1
	Yellow or pus discharge	2
	Bleeding discharge	3
Scab formation	Completely	0
	A part	1
	None	2
Epithelization	Completely	0
	A part	1
	None	2
Wound close	Completely	0
	A part	1
	None	2

4. Statistical analysis

Data were recorded using Microsoft Excel 2016 software. Then data were cleaned and analyzed using IBM SPSS Statistics 22 software. Quantitative results are expressed as mean ± standard deviation (X ± SD). Comparison of the mean values between the two groups were done using Student's t test. The difference was considered statistically significant if the p-value is less than 0.05.

III. RESULTS

1. Building the diabetic and hyperlipidemic model in *Wistar* rats

The effectiveness of a lipid disorder model with a high-fat diet was evaluated by the body weight change and blood lipid indexes. The results are shown in Tables 2 and 3:

Table 2. Change in weight of rats during the experiment

	Weight (gram) (X ± SD)	
	Before the experiment	**After six weeks**
Group 1: Normal group (n = 6)	225.00 ± 39.87	240.00 ± 57.50
Group 2: Diabetic and hyperlipidemic group (n = 6)	239.17 ± 32.31	253.33 ± 30.11
p (t-test)	0.51	0.52

Before the experiment, the mean weights of rats in the two groups were similar ($p > 0.05$). After six weeks, the weight of rats in both the normal and the disease group increased. However, there was no difference in the weight of rats fed with a high-fat diet compared to normal rats ($p > 0.05$).

Table 3. Concentrations of serum lipid indexes after feeding with a high-fat diet for six weeks

	Lipid concentration (X ± SD)			
	Cholesterol (mmol/L)	**Triglycerids (mmol/L)**	**HDL-C (mmol/L)**	**LDL-C (mmol/L)**
Group 1: Normal group (n=6)	1.97 ± 0.22	1.02 ± 0.36	0.90 ± 0.16	0.60 ± 0.23
Group 2: Diabetic and hyperlipidemic group (n=6)	2.56 ± 0.38	2.05 ± 1.03	0.75 ± 0.23	0.88 ± 0.59
p (t-test)	0.008	0.045	0.220	0.310

After six weeks of inducing obesity, the concentrations of serum total cholesterol and triglycerides in rats fed with a high-fat diet were higher than that in the rats fed with a standard diet, especially total cholesterol concentration ($p = 0.008$). The HDL-C and LDL-C levels differed between the two groups, but this difference was not statistically significant ($p > 0.05$). The increase in cholesterol and triglyceride levels in Group 2 showed that six weeks of high-fat diet led to hyperlipidemia in rats.

To induce diabetes, the rats in Group 2 were administered two low-doses STZ by peritoneally injections (35 mg/kg). The results of changes in blood glucose concentration after STZ injection are shown in Table 4.

Table 4. Changes in blood glucose concentration before and after STZ injection

	Blood glucose level (mmol/L) (X ± SD)		
	Before injecting STZ (t_0)	**3rd day after injecting STZ**	**14th day after injecting STZ**
Group 1: Normal group (n=6)	4.08 ± 0.98	4.60 ± 1.03	4.77 ± 1.01

	Blood glucose level (mmol/L) (X ± SD)		
	Before injecting STZ (t_0)	3rd day after injecting STZ	14th day after injecting STZ
Group 2: Diabetic and hyperlipidemic group (n=6)	4.99 ± 0.47	19.65 ± 4.72	14.62 ± 4.62
p (t-test)	0.069	0.0001	0.0001

After six weeks of feeding with a high-fat diet but before injecting STZ, the average fasting blood glucose concentration of rats in the disease group were higher than that of the normal rats, but the difference was not statistically significant ($p > 0.05$). After injecting STZ, the diabetic/hyperlipidemic group had significantly higher average blood glucose concentration than the normal group ($p = 0.0001$). On the 14th day after injecting the last dose of STZ, the glucose levels of the diabetic/hyperlipidemic rats remained higher than that of the normal rats.

To further support the evidence of diabetes after high-fat diet feeding and low-doses STZ injection, the histopathological changes in the liver and pancreatic tissues of rats were evaluated. The histopathological results showed that there was fatty degeneration of liver cells in rats fed with the high-fat diet (Figure 1). We also assessed the effect of STZ on pancreatic tissue imaging in the two study groups. The data indicated STZ caused partial destruction of pancreatic islet cells, causing hyperglycemia expression (Figure 2). The microscopic images showed that the size of endocrine pancreatic islets decreased in the rats injected with STZ. Thus, STZ partly destroyed the pancreatic islet cells which led to hyperglycemia (Figure 2).

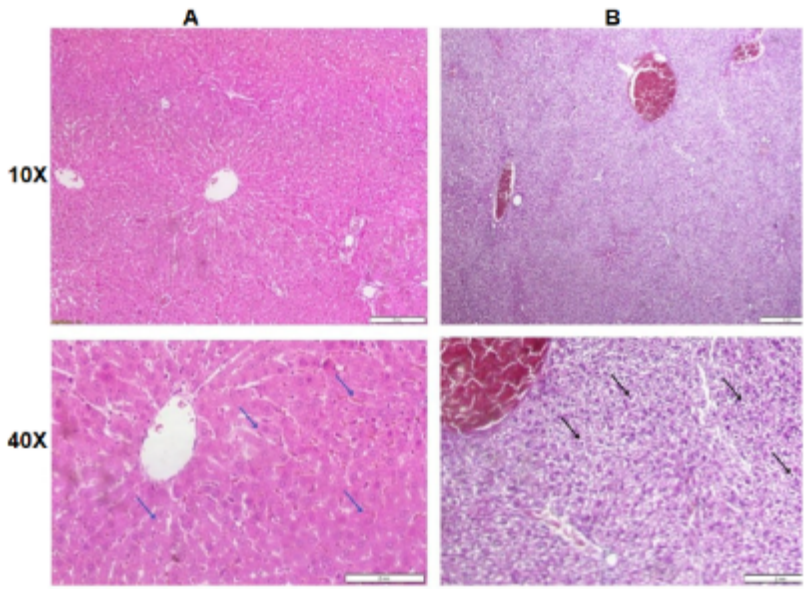

Figure 1. Image of liver tissue of rats at 10X and 40X magnification

(A: Normal group, B: Diabetic/ hyperlipidemic group, ↘ normal liver cells, ↘ fatty degeneration liver cells)

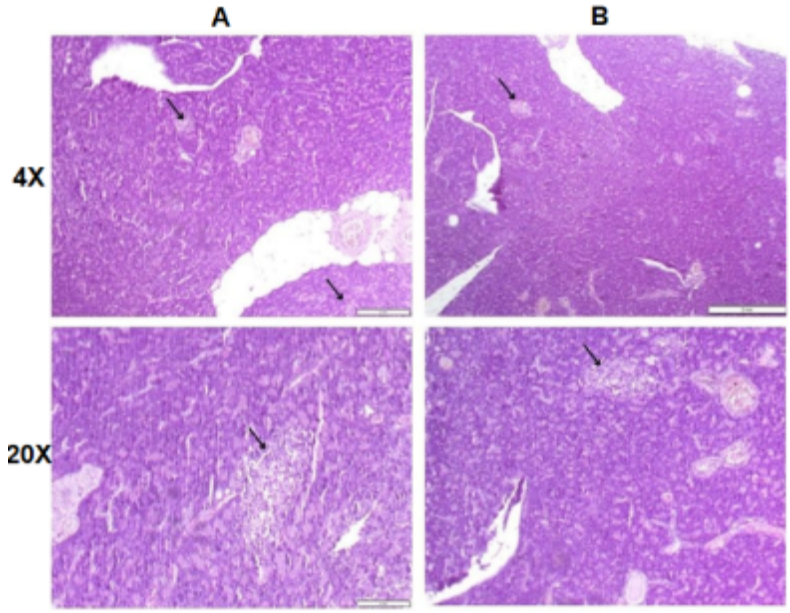

Figure 2. Image of pancreatic tissue of rats at 4X and 20X magnification

(A: Normal group, B: Diabetic/ hyperlipidemic group, pancreatic islets)

3.2. Creating skin excision in the diabetic and hyperlipidemic rat model

After successfully inducing the diabetic/ hyperlipidemic rats, rats in both groups were given skin wounds for 2-centimeters in diameter with two wounds on both sides of the back in each rat. To compare the healing wound process between the diabetic/hyperlipidemic group and the normal group, we scored the wound and calculated the wound area and healing time. The results are shown in Tables 5 and 6 and Figures 3, 4, and 5.

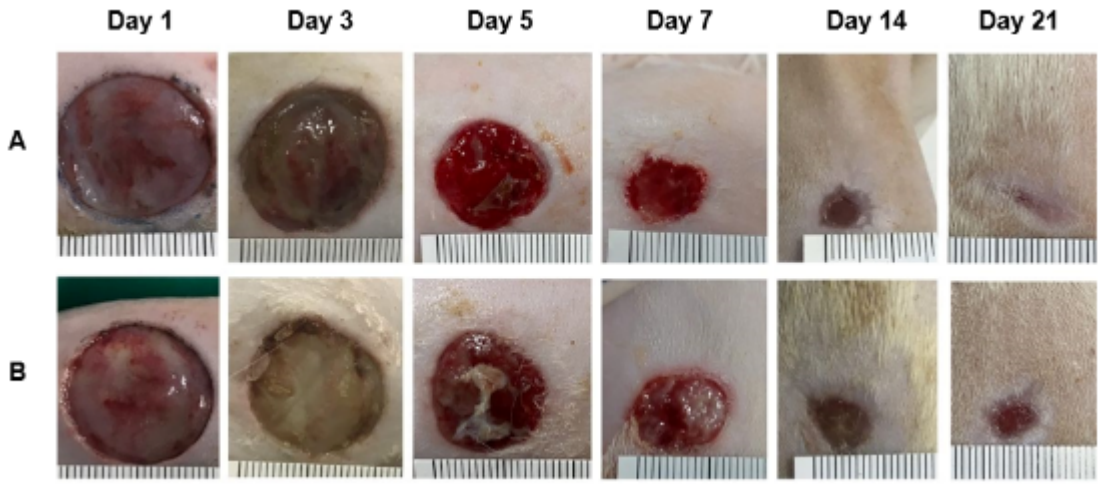

Figure 3. Image of wound healing progress on the skin of rats

(A: Normal group, B: Diabetic/ hyperlipidemic group)

Table 5. Change of injury score during skin wound monitoring

	The total score of wounds (X ± SD)			
	3rd day after wounding	**5th day after wounding**	**7th day after wounding**	**13th day after wounding**
Group 1: Normal group (n=6)	9.13 ± 0.83	4.13 ± 1.13	2.25 ± 0.46	1.50 ± 0.55
Group 2: Diabetic and hyperlipidemic group (n=6)	9.63 ± 0.52	5.50 ± 0.76	3.00 ± 0.76	2.17 ± 0.41
p (t-test)	0.170	0.012	0.031	0.038

On the 3rd day after dermal excision, the lesion scores between the two groups of rats were similar ($p > 0.05$). On the 5th, 7th, and 13th day after wounding, the total scores of the two groups decreased gradually, and on average, the diabetic/hyperlipidemia rat group had a significantly higher score than the normal group ($p < 0.05$). This showed that the macroscopic characteristics of the skin wounds in the diabetic/hyperlipidemiac group were worse than that of the normal group.

Table 6. Change of wound area during the experiment

	Wound area (cm^2) (X ± SD)			
	3rd day after wounding	**5th day after wounding**	**7th day after wounding**	**13th day after wounding**
Group 1: Normal group (n=6)	2.41 ± 0.50	1.64 ± 0.30	0.80 ± 0.12	0.14 ± 0.17
Group 2: Diabetic and hyperlipidemic group (n=6)	3.26 ± 1.15	2.69 ± 0.88	1.60 ± 0.88	0.61 ± 0.47
p (t-test)	0.075	**0.007**	**0.023**	**0.046**

On average, the wound areas in the diabetic/hyperlipidemic group on the 5th, 7th, and 13th days after wounding were larger than that in the normal group. The difference in wound area between the two groups was statistically significant ($p < 0.05$). Also, diabetic/hyperlipidemic rats had slower wound closer rate than normal rats (Figure 3 and 4). On the 13th day, the wound closure rate of the normal group was up to 95.57%, meanwhile, this rate in the diabetic/hyperlipidemic group was only 83.6%. The difference in the wound closure rate between the two groups was statistically significant ($p < 0.05$).

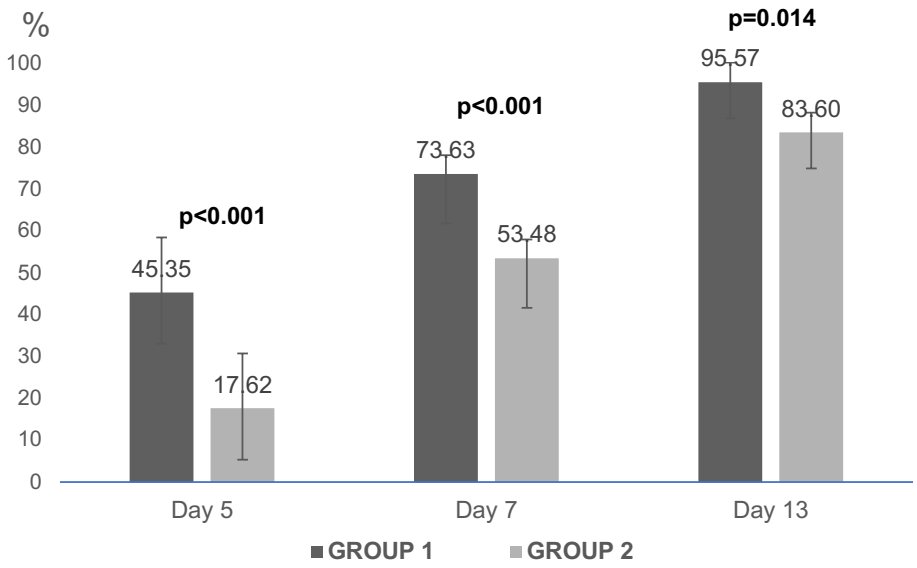

Figure 4. The wound closure rate in the study

(Group 1: Normal group, Group 2: Diabetic/ hyperlipidemic group)

The normal group had significantly faster healing time (15 days on average) than the diabetic/ hyperlipidemic group (20 days on average) ($p < 0.05$) (Figure 5).

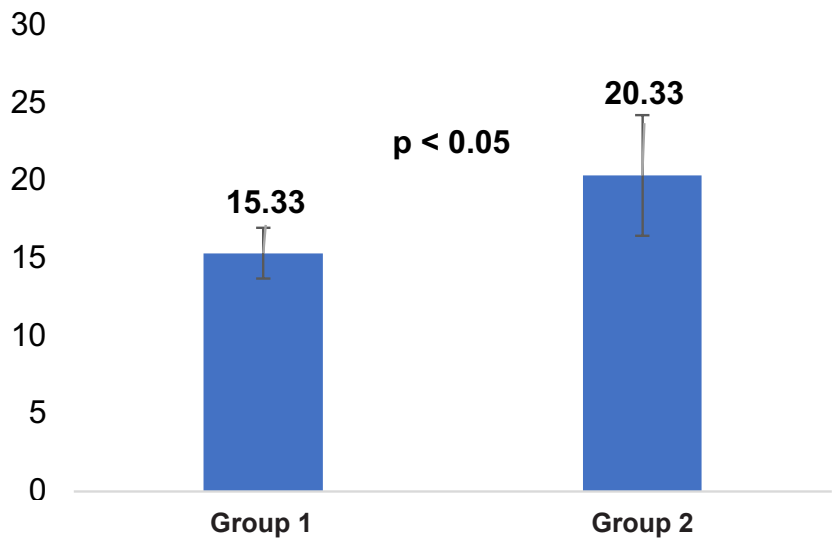

Figure 5. The complete wound healing time of the experimental groups

(Group 1: Normal group, Group 2: Diabetic/ hyperlipidemic group)

To evaluate the microscopic granulation and healing status of skin tissue, samples were collected randomly at the wound at 7 and 14 days after wounding for histology examination (Figure 6).

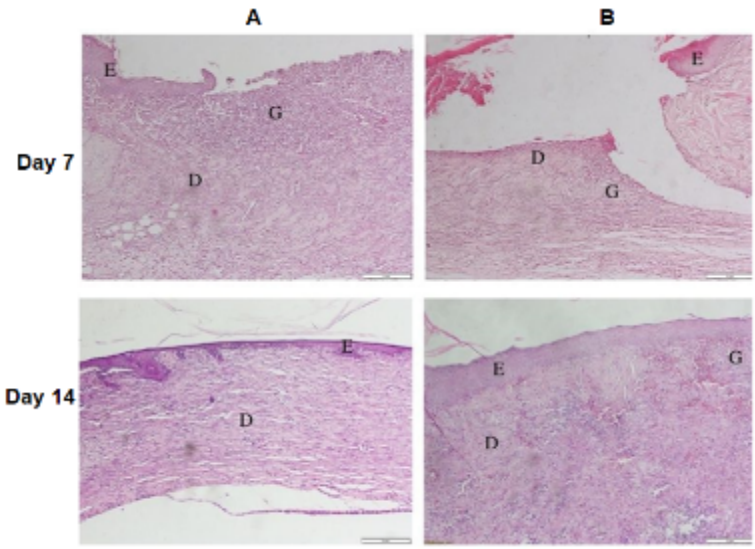

Figure 6. Wound tissue of normal group (A) and diabetic/ hyperlipidemic group (B) at 10X magnification.

(E: epidermis, D: dermis, G: Granulation tissue)

On the 7th, the skin wound tissues of the normal rat composed more granulation tissue and blood vessels than the diabetic/ hyperlipidemic rat. On the 14th, the skin of diabetic/hyperlipidemic rats still had granulation tissue process, while the skin tissue of normal rats already developed the epidermis and dermis layers.

IV. DISCUSSION

Studies worldwide have reported many methods for inducing diabetes in animals. In this study, we selected to induce diabetes/ hyperlipidemia in *Wistar* rats by combining a high-fat diet and repeated low doses of STZ 35mg/kg based on the results of Srinivasan, Magalhaes, and Ming Zhang.[6-8] A high-fat diet will induce obesity and lead to peripheral insulin resistance.[6,8] At the same time, using low-doses STZ injection in rats with insulin resistance will cause partial destruction of pancreatic beta cells, thereby reducing insulin secretion.[9] This is the optimal method, helping inducing a pathological condition in animals similar to the pathogenesis of type 2 diabetes in humans. The average weight gained after six weeks of high-fat diet was not significantly different between the high-fat diet group and the standard diet group. However, the cholesterol and triglyceride levels of rats fed a fatty diet were significantly higher than those of rats fed a standard diet. The histopathological image of the livers also showed that the livers of rats in the fat-raising group had fatty degeneration. This result is similar to other studies by Zhang, Srinivasan, Diego, Nilsson, and Reed.[6-8,10,11]

Many studies have used STZ to induce diabetes in animal models with different doses.[6-10] In this study, we injected STZ at repeated low doses of 35mg/kg to avoid excessive destruction of pancreatic beta cells, causing sudden hypoglycemia and death in rats. The results showed that blood glucose levels increased three days after last dose STZ injection and remained elevated 14 days after the last dose of STZ. This indicates that

repeated low-dose STZ caused diabetes in rats, similar to other studies in the world.[6,8,9]

Observing the histopathological specimen of the model group, it can be seen that the pancreatic islets of the two groups can still easily identify the endocrine islets but observed in the normal group, the islet area was larger than the group causing diabetic and hyperlipidemic model. A possible explanation is that low-doses STZ injections cause only partial destruction of pancreatic islets indicated by a decrease in the area of endocrine islets, but do not cause atrophy or fibrosis of the endocrine pancreas or complete destruction of the islets. This result does not affect the experimental results because the fasting blood glucose index in the model rat group remained >11 mmol/L throughout the study period. Therefore, the models used in this study resulted in the development of diabetes and lipid disorders, similar to the pathogenesis in patients with type 2 diabetes presenting with hyperglycemia, but with unclear pancreatic islets damage and often on the background of hyperlipidemia or obesity.

To induce a skin wound in rats that is similar to chronic limb ulceration in diabetic patients, we fixed the wound with a silicone ring around the wound mouth immediately after excising the skin of the rats. Using silicone rings can help prevent the rapid wound formation in rats caused by *Panniculus Carnosus* muscle contraction instead of granulation tissue formation so that the healing process is similar to human wounds.[5]

To compare the difference in the healing process between the diabetic/hyperlipidemic group and the normal group, we evaluated the wound area, healing time, wound recovery rate, and wound scores over time. The 3rd day after creating the skin wound was selected as a reference point for comparison during the study because, during the first two days, the wound was fixed with a silicone ring to eliminate *Panniculus Carnosus* muscle contraction. Starting from the 3rd day, the average wound area in diabetic rats were larger than that in normal rats, suggesting the wound healing rate was slower than that of the healthy rat.

Our results showed that the healing process occurred faster in the normal group than in the diabetic/hyperlipidemic group. This is consistent with the normal healing process.[12] On the 14th day after wounding in the control group, there was no longer granulosa tissue, and there was observable regenerated three layers of the skin tissue's epidermis, dermis, and hypodermis. In contrast, the diabetic/hyperlipidemic group was still in the healing process, evidently by the presence of a lot of granulosa tissue and proliferating epidermis. It shows that the wound is still in the healing process, the skin tissue structure has not been completely regenerated, and the wound in the diabetes/hyperlipidemia group healed slower than in the normal group. This difference may be due to hyperglycemia increasing oxidative stress, leading to tissue hypoxia, prolonged inflammation, and delayed wound healing.[13] This is the main mechanism causing chronic limb ulceration and wound healing disorders in diabetic patients.

V. CONCLUSION

In this study, the model of skin-cutting wounds has been successfully induced in *Wistar* rats with diabetes and hyperlipidemia. The macroscopic and microscopic characteristics of the skin wound indicated that the diabetic and hyperlipidemic rats had a slower wound healing time than the normal rats. The results from this study can be used to guide future testing of new wound healing treatments.

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