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Original Article

Effects of liraglutide on the survival of random skin flaps in rats: an experimental study

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Abstract: In this work, we aimed to explore the effects and associated mechanisms of liraglutide on the survival of random skin flaps on the backs of rats. A total of 60 SD rats were randomly divided into experimental (liraglutide group, n=30) and control groups (saline group, n=30), which were further classified into two subgroups with 15 SD rats each, 3-days postoperation and 7-days postoperation groups. Random skin flaps with a pedicle on the side of the flap were generated on the backs of SD rats. Rats in the experimental and saline groups were subcutaneously injected with the same amount of liraglutide solution or normal saline. Flap survival rate was measured 7 days after the operation. Flap tissues were obtained and visualized by hematoxylin-eosin (HE) staining and immunohistochemical staining of CD31 and GLP-1R. Moreover, the levels of SOD, MDA, VEGF, TNF-α, and IL-6 in flap tissues were tested. The survival rate of flaps in the liraglutide group was significantly higher than that in the saline group. The liraglutide group had less inflammation and more obvious microvascular hyperplasia than the saline group. GLP-1 receptors were significantly expressed in both groups. Microvessel density (MVD), VEGF and SOD activities in the liraglutide group were higher than those in the saline group. In contrast, lower levels of TNF-α, IL-6, and MDA were observed in the liraglutide group than in the saline group. Liraglutide can promote the survival of random flaps.

Keywords: Random skin flap, survival rate, liraglutide, oxidative stress, inflammatory response

Introduction

A random skin flap is commonly used to repair tissue defects. However, it is limited by the length to width ratio [1]. For instance, once a definite ratio is exceeded, necrosis develops on the distal part of the flap, mainly as a result of microcirculation disorder and ischemia-reperfusion injury [2]. How to effectively improve the survival rate of the flap is a research hotspot in clinical practice. Glucagon-like peptide-1 (GLP-1) receptor agonists belong to a class of hormones called incretin and are secreted by intestinal cells after food stimulation. They can stimulate insulin secretion by exerting a hypoglycemic effect upon binding to the glucagon-like-1 receptor (GLP-1R) and thus have been widely used to treat diabetes in recent years [3]. GLP-1 receptor agonists include exenatide, liraglutide, and benalutide. Recent studies have revealed that GLP-1 receptor agonists can both exert a hypoglycemic effect and effectively reduce ischemia-reperfusion injury of the brain, heart, kidney, and other organs via mechanisms that include the inhibition of inflammation and apoptosis and the reduction of oxidative stress [4-6]. However, the effect of GLP-1 receptor agonists on flap survival remains unclear. Therefore, upon assessing the biological characteristics of the GLP-1 receptor agonist, we investigated its potential role in improving the survival rate of the flap. Here, we established a random skin flap model on the back of rats to evaluate the effects of liraglutide on flap survival and explore the related mechanisms.

Materials and methods

Constructing the animal model

Here, 60 male SD rats (weighting 250 ± 30 g) were randomly divided into the experimental group (liraglutide group, n=30) and the control group (saline group, n=30). Further, each group was classified into two subgroups, either the 3 or 7 days post-operation group, each contained 15 rats. After intraperitoneal injection of anesthesia (2% pentobarbital sodium, 0.3 ml/100

g), a 9 cm × 2.5 cm random skin flap with a pedicle on the side of the flap was generated on the back of the SD rats, using the McFarlane method. We sterilized the operation area, cut the skin to the deep fascia layer according to the design line, separated and lifted the flap, and cut off blood vessels. After completion of hemostasis, the flap was sutured in situ with a 3-0 silk thread. Povidone was used to disinfect areas adjacent to the incision after the operation, and chlortetracycline ointment was applied to prevent infection. The liraglutide group was injected subcutaneously with liraglutide solution (0.2 mg/kg/d, Novo Nordisk, Denmark) 1 hour before the operation and 6 consecutive days postoperation, whereas the control group was subcutaneously injected with the same amount of normal saline with the same timelines. The two groups of rats were injected at the same time every day, and every SD rat was reared in a single cage. All operations were performed by the same investigator. This study was approved by the Animal Protection Ethics Committee of the Second Affiliated Hospital of Soochow University.

Flap general observation

The vital features of the flaps in the rats, including the color, texture, edema, exudation, ulceration, and survival, were observed each day after the operation. To judge flap necrosis, the flap turned black, the texture was stiff, the tissue elasticity was poor, and no bleeding occurred when the tissue was cut. Two investigators observed the above characteristics.

Flap survival rate

On experiment day 7 after the operation, 15 rats from each 7-day subgroup of both the experimental and control groups were anesthetized using the same method as previously described. Images of the back flap were captured using a digital camera, and the survival rate of the flap was calculated via Image Pro-Plus 6.0 software using the formula: Flap survival rate = flap survival area/total flap area × 100% [7].

Histological observation

On day 3 after the operation, 15 rats from each 3-day subgroup of both the experimental and control groups were anesthetized using the same method as previously described. Then, a full-thickness flap tissue was taken 4-5 cm from the tail of the flap. The sampling position was the same for all rats, and the size was approximately 1.0 cm × 1.0 cm. The rats were sacrificed after the operation. On the 7th day post-operation, we obtained 15 rats from each 7-day subgroup of both the experimental and control groups and used the same method to sample the specimens after measuring the survival rate of the flap. The specimens were divided into two portions. One portion was placed in 10% formaldehyde for 24 hours, dehydrated, embedded, and sliced at a thickness of 3 μm. Routine pathological and immunohistochemical examinations were performed. The other part was stored in the refrigerator at -80°C for subsequent analyses.

Microvessel density (MVD)

CD31 immunohistochemical staining was performed using the SP method. Following Weidner's method, the slices were placed under a low-power microscope to observe the highest blood vessel density area. Then, 5 fields were selected under a microscope with 200 × magnification to count the microvessels. The average value was taken as the number of microvessels per unit area (MVD) [8].

GLP-1 receptor (GLP-1R) immunohistochemical staining

With paraffin sections that were taken on the third and seventh days after the operation, GLP-1R immunohistochemical staining was performed via the SP method. Then, 5 fields of view were selected under a microscope (400 ×), and the average value was considered as the number of GLP-1 receptors per unit area [9].

SOD and MDA tests

We obtained the specimens stored at -80°C, prepared the tissue homogenate of flaps, and extracted the supernatant, then followed the operating instructions of the kit (Nanjing Jiancheng Bioengineering Institute, China). We used the hydroxylamine method to measure the activity of SOD, while the TBA method was used to measure the MDA content.

VEGF, TNF-α, and IL-6 test

We obtained the specimens and stored them at -80°C; then, we prepared flap tissue homoge-

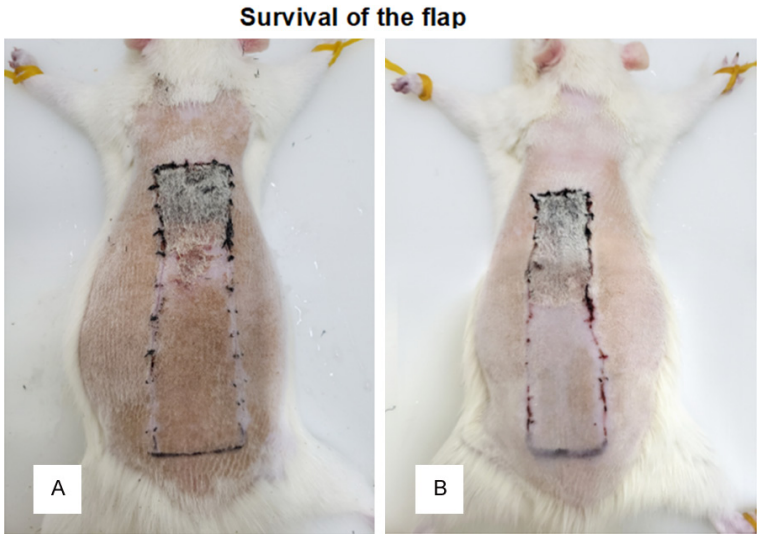

Figure 1. Survival of random skin flaps on the back of the two groups of rats at 7 days post-operation. A. Liraglutide group. B. Saline group.

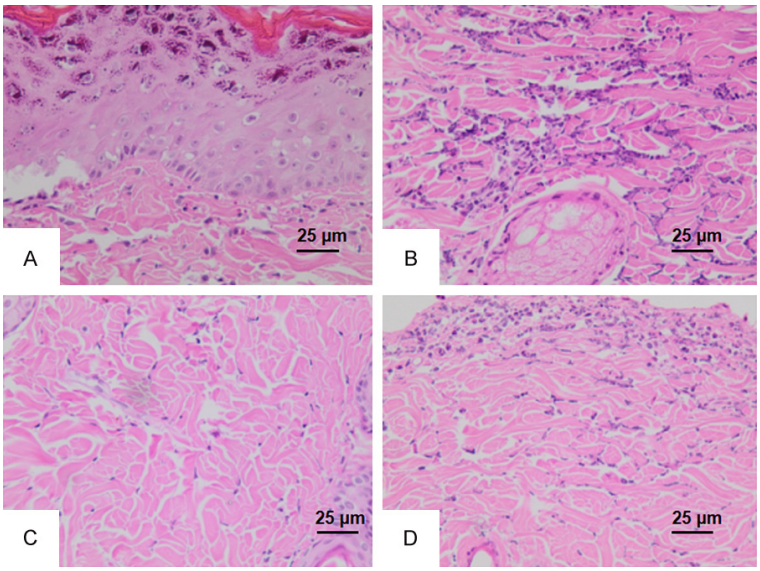

Figure 2. Histological morphology of random skin flaps on the back of rats at 3 and 7 days post-operation. A. Liraglutide group 3 days post-operation. B. Saline group 3 days post-operation. C. Liraglutide group 7 days post-operation. D. Saline group 7 days post-operation. (Magnification: 400 ×, scale bar =25 μm).

nates and extracted the supernatant. An ELISA double antibody sandwich method was used to evaluate the expression of VEGF, IL-6, and TNF-α, following the operating instructions outlined in the kit (Abcam Company, United Kingdom).

Data analysis

SPSS 20.0 statistical software was used to analyze all statistical data, which were expressed as the mean ± standard deviation. Further, independent sample t-test was used for a comparison between two groups. $P<0.05$ was considered statistically significant.

Results

Survival of flaps

All SD rats survived until the end of the experiment. No obvious abnormalities in diet, spirit, or defecation were observed. We did not observe any obvious infection or exudation. On the 7th day after the operation, necrosis of the flaps tended to stabilize; a black scab that was hard to peel off formed, and no bleeding occurred when cutting the tissue. Compared with the saline group, the necrotic area in the liraglutide group was smaller (**Figure 1A** and **1B**). Notably, the survival rate of flaps of rats in the liraglutide group was significantly higher than that in the saline group (69.73% ± 7.51% vs. 53.27% ± 6.06%, $P<0.01$).

Pathological observation of flaps

On post-operative day 3, tissue edema was mild, a small number of inflammatory cells infiltrated, the layer hierarchy was clear, and dilated microvessels were visible in the dermis and subcutaneous layers in the liraglutide group. Whereas in the saline group, tissue edema was more obvious, the structure was disordered, a large number of inflammatory cells were diffusely infiltrated, and fewer dilated microvessels were visible in the dermis and subcutaneous layers (**Figure 2A** and **2B**). On the 7th day after the operation, in the liraglutide group, we observed mild edema of the tissue, a small amount of inflammatory cell infiltration, and dilated microvessels in the dermis and subcutaneous layer. Whereas in the saline group, tissue edema was milder than it

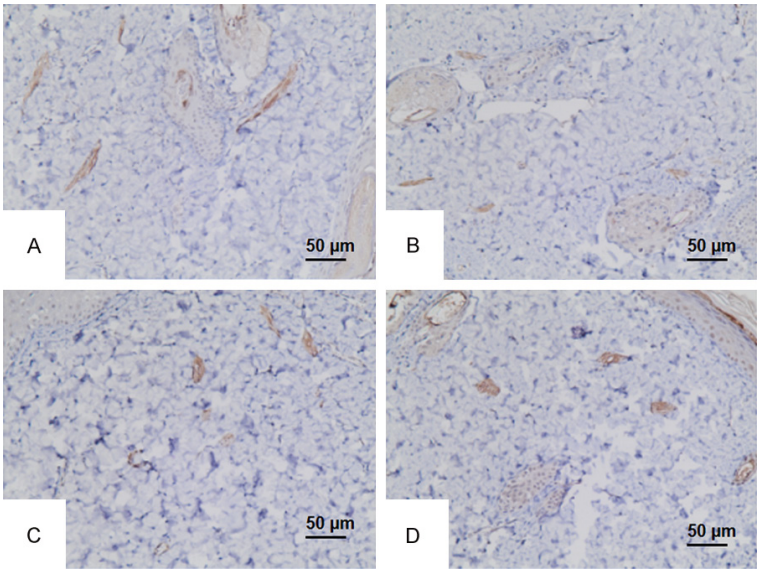

Figure 3. Immunohistochemical expression of CD31 on the back of rats at 3 and 7 days post-operation. A. Liraglutide group 3 days post-operation. B. Saline group 3 days post-operation. C. Liraglutide group 7 days post-operation. D. Saline group 7 days post-operation. (Magnification: 200 ×, scale bar =50 μm).

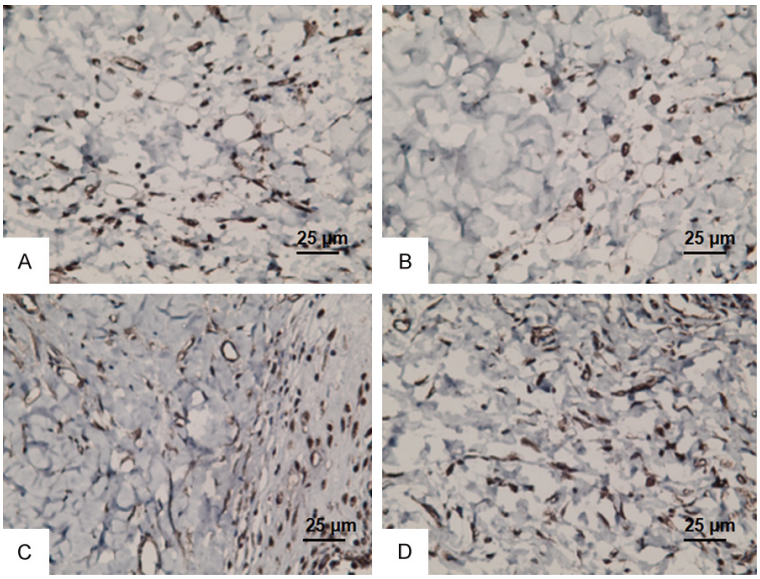

Figure 4. Immunohistochemical expression of GLP-1R in random skin flaps on the back of rats at 3 and 7 days post-operation. The brown-yellow marked parts are GLP-1R positive cells. A. Liraglutide group 3 days post-operation. B. Saline group 3 days post-operation. C. Liraglutide group operation 7 days post-operation. D. Saline group 7 days post-operation. (Magnification: 400 ×, scale bar =25 μm).

was before treatment, inflammatory cell infiltration was obvious, and less dilated microvessels were visible in the dermis and subcutaneous layer (**Figure 2C** and **2D**).

Microvessel density (MVD)

Tissue slices observed under a microscope at 200 × magnification at 3 days post-operation revealed that the MVD of rats in the liraglutide group was significantly higher than that in the saline group (10.38 ± 1.74 vs. 6.73 ± 1.39, P<0.01) (**Figure 3A** and **3B**). At 7 days after the operation, the MVD of rats in the liraglutide group was significantly higher than that in the saline group (10.80 ± 2.17 vs. 7.04 ± 1.54, P< 0.01) (**Figure 3C** and **3D**).

Expression of GLP-1R (GLP-1 receptor)

GLP-1R expression was revealed through immunohistochemical staining of the liraglutide group and saline group on the 3rd and 7th days after the operation. The brown-yellow marked parts depict GLP-1R-positive cells. Using a microscope at 400 × magnification, it was determined that the expression of GLP-1R in the liraglutide group was not significantly different from that in the normal saline group 3 days after the operation (40.47 ± 8.78 vs. 37.27 ± 5.80, P>0.05) (**Figure 4A** and **4B**). In addition, the expression of GLP-1R in the liraglutide group was not significantly different from that in the saline group at 7 days after the operation (44.93 ± 7.04 vs. 40.73 ± 6.30, P>0.05) (**Figure 4C** and **4D**).

SOD and MDA test results

At days 3 and 7 postoperation, the SOD activity of the flap in the liraglutide group was higher than that in the saline group. In contrast, the MDA in the liraglutide group was lower than that in the saline group (**Table 1**).

Table 1. Comparison of SOD activity (U/mgprot) and MDA content (nmol/mgprot) of two groups (n=15, $\bar{x}$ ± sd)

Group	SOD (3 d)	SOD (7 d)	MDA (3 d)	MDA (7 d)
Experimental group	61.07 ± 11.02	49.47 ± 9.05	8.35 ± 1.76	7.22 ± 1.55
Control group	40.27 ± 8.78	34.83 ± 6.81	11.47 ± 2.00	10.17 ± 1.78
P (α=0.05)	<0.01	<0.01	<0.01	<0.01

Table 2. Comparison of the levels of TNF-α and IL-6 between two groups of flaps (n=15, $\bar{x}$ ± sd, pg/ml)

Group	TNF-α (3 d)	TNF-α (7 d)	IL-6 (3 d)	IL-6 (7 d)
Experimental group	33.27 ± 6.43	27.93 ± 6.09	21.47 ± 4.27	17.53 ± 4.69
Control group	51.27 ± 11.29	44.73 ± 13.65	31.07 ± 8.80	26.33 ± 6.96
P (α=0.05)	<0.01	<0.01	<0.05	<0.01

Expression of VEGF, TNF-α, and IL-6

ELISA results demonstrated a higher expression of VEGF in the liraglutide group than in the saline group 3 days after the operation (418.40 ± 53.15 pg/ml vs. 251.27 ± 33.93 pg/ml, P<0.01). In addition, the expression of VEGF in the liraglutide group was higher than that in the saline group at 7 days after the operation (350.87 ± 47.57 pg/ml vs. 219.53 ± 37.55 pg/ml, P<0.01). At 3 and 7 days post-operation, the content of TNF-α and IL-6 in the liraglutide group was lower than that in the saline group (**Table 2**).

Discussion

In this work, we revealed that necrosis occurred at the distal end of the flap 7 days after operation in both experimental and control groups. Furthermore, the necrotic area of the flap in the liraglutide group was significantly smaller than that in the saline group. It is therefore evident that liraglutide potentially promotes the survival of random skin flaps.

Liraglutide, a long-acting GLP-1 receptor agonist, exerts a hypoglycemic effect by activating the GLP-1 receptor (GLP-1R) [10]. GLP-1R is known to be found in islet cells, as well as the gastrointestinal tract, lung, brain, kidney, hypothalamus, cardiovascular system, liver, adipocytes, and skeletal muscle, among others [11]. No reports exist on GLP-1R expression in skin flap tissue. In this experiment, we confirmed that GLP-1R is also found in skin flap tissue, an indication that liraglutide, when integrated with GLP-1R, promotes flap survival. Notably, GLP-1 receptor agonists stimulate insulin secretion via a glucose concentration-dependent mechanism [12, 13], where GLP-1 exerts a hypoglycemic effect only at high blood glucose levels. In contrast, its hypoglycemic effect is suppressed when the blood glucose level normalizes; thus, hypoglycemia rarely occurs. In our experiment, no rats experienced serious complications, such as apparent hypoglycemia or death, due to the use of liraglutide.

In addition, VEGF can specifically act on vascular endothelial cells and promote their proliferation and differentiation, thus accelerating the formation of new blood vessels [14]. Additionally, it aids in reconstructing the blood flow at the base of the flap and the edge of the incision, thereby improving the microcirculation of flaps to promote their survival [15]. We reported a highly significant expression of VEGF in the liraglutide group compared to the saline group, suggesting that liraglutide might promote angiogenesis and improve microcirculation by elevating VEGF expression. Moreover, pathological sections showed that the liraglutide group had obvious vascular hyperplasia post-operation, whereas immunohistoche-mistry revealed higher MVD in the liraglutide group than in the saline group.

TNF-α and IL-6 are key inflammatory cytokines and play an important role in flap injury [16]. For instance, TNF-α can alter the permeability of capillaries, enhance the chemotaxis of inflammatory cells, and initiate the inflammatory response [17]. On the other hand, IL-6 is secreted

by macrophages after injury, and as the main inflammatory mediator, it participates in the body's inflammatory response, thereby causing secondary tissue damage [18]. The expression levels of TNF-α and IL-6 are negatively correlated with the degree of tissue inflammatory injury. In this study, we found lower levels of TNF-α and IL-6 in the liraglutide group, and pathological sections showed a less inflammatory response. This is an implication that liraglutide might reduce the inflammatory damage of flap tissue by lowering the levels of TNF-α and IL-6.

Additionally, oxidative stress is a principal mechanism in flap ischemia-reperfusion injury [19, 20]. SOD, one of the most important antioxidant enzymes in the body, can effectively eliminate oxygen free radicals and reduce tissue damage as a result of oxidative stress [21]. Additionally, MDA, a product of lipid peroxide metabolism, can reflect the degree of tissue lipid peroxidation and is positively correlated with oxygen free radical damage [22]. Based on our findings, the SOD activity in the liraglutide group increased, while the MDA content decreased. This signified that liraglutide may reduce oxidative stress damage to the flap by scavenging oxygen free radicals.

In conclusion, our results affirm that liraglutide, a GLP-1 receptor agonist, promotes the survival of random skin flaps. Mechanistically, it enhances angiogenesis, minimizes oxygen free radical-induced damage, and inhibits the inflammatory response. However, additional in-depth studies are needed to uncover other mechanisms of action of liraglutide in the survival of random skin flaps.

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Disclosure of conflict of interest

None.

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