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Hypoglycemic effect of insulin-loaded hydrogel-nanogel composite on streptozotocin-induced diabetic rats

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At present, how to increase insulin rapidly, availably and stably is still a conundrum in the treatment of diabetes mellitus. *In vitro* studies have shown that insulin can be released from hydrogel-nanogel composite according to the changes of glucose level. This study aimed to observe the glucose-lowering effects and evaluate the safety of the insulin-loaded hydrogel-nanogel composite in diabetic rats. We found that significant glycemic regulation could be observed up to 30 hours after subcutaneous injection, and the fasting blood glucose was reduced effectively. The result of an oral glucose tolerance test showed that the level of insulin expressed a stable increase from 0.5 hours to 3.5 hours, which led to a reduction of glucose with steady steps. Also, compared with Ins group, the Gel+Ins group showed slighter skin and pancreas damage, while the oxidative stress and inflammation response were similar to the normal control group. In conclusion, these results demonstrated that the glucose-lowering action of the insulin-loaded hydrogel-nanogel composite was superior to that of the regular insulin, and might thus become an insulin carrier in the future.

1. Introduction

Absolute or relative deficiency of insulin secretion is the main pathogenesis of diabetes mellitus, so exogenous insulin supplementation is universally used in the treatment of diabetes (Svensson et al. 2020). At present, the dosage forms of insulin are mainly divided into fast-acting insulin, short-acting insulin, intermediate-acting insulin, long-acting insulin and ultra-long-acting insulin according to the duration of action, among which fast-acting insulin is rapidly absorbed and shows a short peak time. However, it needs multiple daily subcutaneous injections. In addition, it is difficult to satisfy the necessity for accurate postprandial glycemic control and may also lead to the occurrence of hypoglycemia (Heise et al. 2017; Schmeisl and Kretzschmar 2016; Zijlstra et al. 2018; Benkhadra et al. 2017). Furthermore, multiple self-administration and injections are accompanied by effects like subcutaneous nodules, drug malabsorption, infections and omission of prescribed doses (Espona-Noguera et al. 2019). To avoid the above-mentioned shortcomings in insulin application, changing the delivery mode of insulin has attracted great interest and has widely been investigated (Veiseh et al. 2015). Besides other approaches, research is focused on self-regulating drug delivery based on glucose-responsive insulin delivery systems (Omami et al. 2017; Volpatti 2020; Li et al. 2019; Wang et al. 2019; Agazzi et al. 2020).

Among various glucose-responsive materials, phenylboronic acid (PBA) and its derivatives can form reversible covalent PBA-diol complexes in which cis-diol units (Matsumoto et al. 2012; Chou et al. 2015) respond to the changes in glucose concentration. So far, PBA-based materials have been broadly considered and utilized in development of the glucose-responsive system for insulin delivery (Kim et al. 2012; Liu et al. 2010), such as nanoparticles (Tang 2019), microgels (Kikuchi et al. 1996), and bulk hydrogels (Zhao et al. 2015). In previous studies, researchers prepared a glucose-responsive PBA-containing polymer hydrogel (Yang et al. 2014), and studied its glucose-responsive insulin release behavior (Yesilyurt et al. 2016). However, bulk hydrogels are usually compromised by weak glucose responsiveness leading to the poor ability of controlling the release of insulin. Recently, in order to achieve notable glucose-responsiveness, various PBA-modified nanoparticles have been studied (Li et al. 2013; Yang et al. 2015; Yang et al. 2015; Ma et al. 2012; Tyrrell et al. 2010). Shi et al. (Ma and Shi 2014; Li et al. 2013; Ma et al. 2015) obtained a series of glucose-responsive polymeric micelles by self-assembled PBA-containing block copolymers and glycopolymers, these micelles displayed good glucose-triggered insulin release behavior with a glucose concentration of 2 g/L at physiological pH 7.4, but unsuitable short-term release time limited the effectiveness of nanoparticle insulin delivery system *in vivo* (Merino et al. 2015).

To obtain better glucose responsiveness and achieve long-term controlled insulin release, a hydrogel-nanogel composite was prepared on the basis of a reversible PBA/galactosyl complex by uniformly dispersing insulin-loaded glucose-responsive nanogels in a glucose-responsive hydrogel matrix. The hydrogel-nanogel composite loaded with insulin can overcome the disadvantages of single application of hydrogel or nanogels in insulin delivery system: (1) The insulin-loaded hydrogel-nanogel composite can rapidly release insulin according to the concentration of glucose, displaying an enhanced glucose-responsiveness; (2) The three-dimensional network structure of the hydrogel can provide a porous and stable scaffold for nanogels and prolong the release of insulin *in vivo*. Nevertheless, the above studies are all done *in vitro*, and the situation *in vivo* is currently unclear. So we utilized fast-acting insulin loaded hydrogel-nanogel composite and carried out a cascade of experiments *in vivo* to evaluate its effect on regulating blood glucose and duration of its action. Simultaneously, this study also assessed the effects of the material's function on the liver, kidneys and pancreas, as well as the degree of oxidative stress and inflammatory response in rats.

2. Investigations and results

2.1. Formation of insulin-loaded hydrogel-nanogel composite and self-regulated release of insulin

A hydrogel-nanogel composite based on PBA was prepared by implanting an insulin-loaded nanogel into the hydrogel matrix,

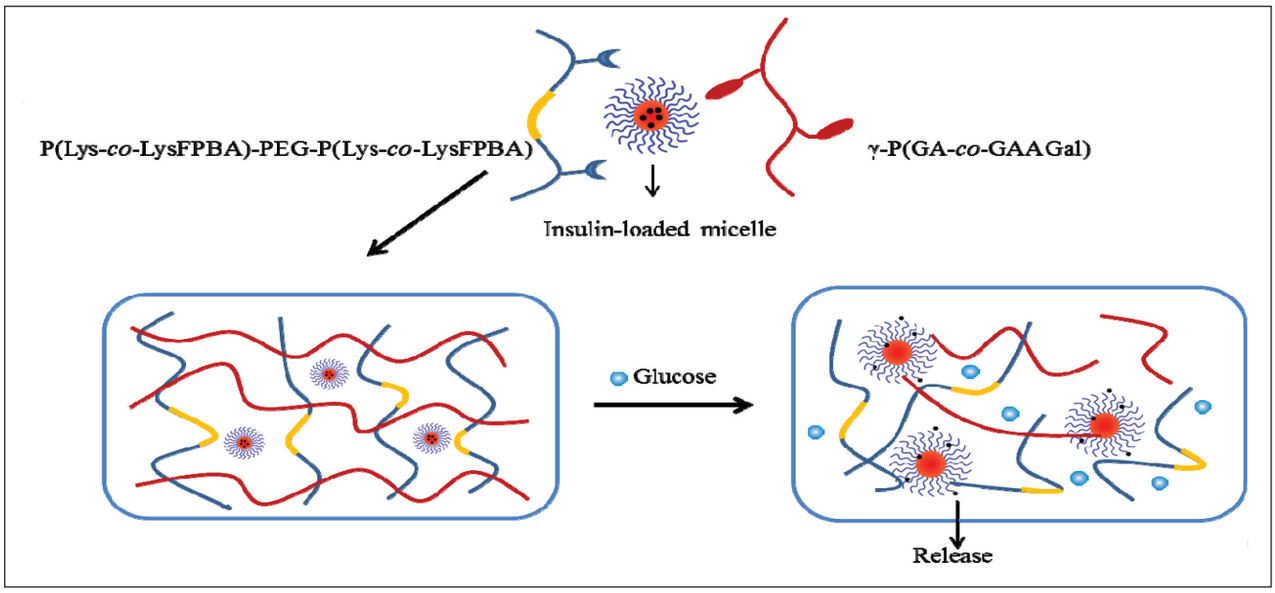

Fig. 1: Schematic formation of hydrogel-nanogel composite loaded with insulin and self-regulated release of insulin.

and a controlled glucose-responsive insulin release system was obtained. The glucose-responsive nanogels acted as reservoirs of insulin, and were incorporated into glucose-responsive hydrogel to prolong the release of insulin *in vivo* (Fig. 1).

2.2. The hydrodynamic diameter distributions f(D_h) of blank nanogels and insulin-loaded nanogels

The hydrodynamic diameter distributions *f*(D_h) of blank nanogels and insulin-loaded nanogels measured by DLS are shown in Fig. 2. The average diameters of blank nanogel and insulin-loaded nanogel were 188 nm and 200 nm, respectively.

2.3. Comparison among different studied groups regarding the changes in blood glucose in rats

To better compare the onset time and the duration of the hypoglycemic effect of the insulin-loaded hydrogel-nanogel composite and the other two groups, the blood glucose level of each group was measured at the same time and marked as 0 h glucose. And then the rats in each group were subjected to pharmacological intervention. As shown in Fig. 3, the change in blood glucose levels within 30 h in the DC group was relatively stable, and the blood glucose value measured each time was around the 0 h glucose, indicating no significant difference (P>0.05). However, the blood glucose of the Ins group showed a different trend that the glucose level dropped 73.6% within the first 3 h compared with the initial concentration and ascended to nearly the initial content at 8 h. After that, the curve was relatively smooth, the change in blood glucose level from 8 h to 27 h was 84.7-96.0% of 0 h glucose. Meanwhile, compared with the DC group, the blood glucose levels of the Ins group were significantly reduced from 1 h to 5 h (P <0.05). The glucose in the Ins and Gel+Ins group dropped to the lowest level significantly at 3 h (P<0.05), but unlike Ins group ,Gel+Ins group had a 73.7% reduction in blood glucose levels within 3 h. Moreover, its blood glucose fluctuated from 49.2 to 55.6% of the initial blood glucose value up to 27 h and returned to the initial point at 30 h. We also observed significantly lower blood glucose levels in the Gel+Ins group than in the Ins group from 4 h to 28 h, except at 12 o'clock (P<0.05).

2.4. The change curve of fasting blood glucose in each group within 14 days

In order to investigate the glucose-lowering effect of insulin-loaded hydrogel-nanogel composite on fasting blood glucose, the following experiments were conducted. After the rats were fasted for 10 h, the fasting blood glucose level of each group was measured each day for 14 d. The diversification of fasting blood glucose in each group is presented in Fig. 4. Firstly, fasting blood glucose in the DC group was maintained at a relatively stable high level (474.1-544.0 mg/dL) throughout the experiment, and there was no significant change in the blood glucose value compared with that at 0 h. And second, the slight reduction of fasting blood glucose in the Ins group exhibited no detectable difference compared with that in the DC group. To our surprise, fasting blood glucose in the Gel+Ins group dropped sharply to 65.3% of the initial value on the first day, and the concentration range was maintained between 25.1 and 48.9% during the following 13d. Certainly, it is not difficult to see a significant decrease compared to the initial level ($p < 0.05$).

2.5. Changes in blood glucose and serum insulin levels after OGTT test in different studied groups of rats

To obtain the change of blood glucose and secretion of serum insulin with subcutaneous injection of insulin-loaded hydrogel-nanogel composite in an oral glucose tolerance test, after a fasting period of 10 h, the rats were provided with drugs and the blood glucose level was measured (0 h blood glucose). After half an hour, the rats went through a period similar to that after a diet by pouring high-concentration glucose into the stomach (2 g/kg). An oral glucose tolerance test was carried out and then the content of blood glucose (Fig. 5 a) and serum insulin (Fig. 5 b) in each group were determined per 0.5 h until 3.5 h. As shown in Fig. 5 a, blood glucose in the NC group preserved a stable level (P>0.05), the curve of glucose reached the peak value (163.2 mg/dL) at 2 h and then declined to the level of 0 h at 3.5 h in the NC group, while the insulin of NC group rose to the maximum at 1.5 h. On the other hand, from the red graph line, we can see that the blood glucose decreased at 1 h, and then remarkably elevated to the climax (527.0 mg/dL) at 1.5 h, subsequently declined to the initial level at 2.5 h, owning to the low-lying curve of insulin till 3.5 h in DC group. Blood glucose was reduced 63.3% at 1.5 h compared with that at 0 h. And then it almost rose to the value of blood glucose close to 0 h at 3.5 h. Meanwhile, a quick rise of insulin level was observed because of the injection of insulin solution, the highest vale at 0.5 h it climbed to the highest value, and then the serum insulin descended to the level of 0 h in the Ins group. Moreover, blood glucose in the Gel+Ins group kept a downtrend noticeably. More precisely, the level of blood glucose showed a slow and steady decrease of 26.8% within 3.5 h compared with its initial content

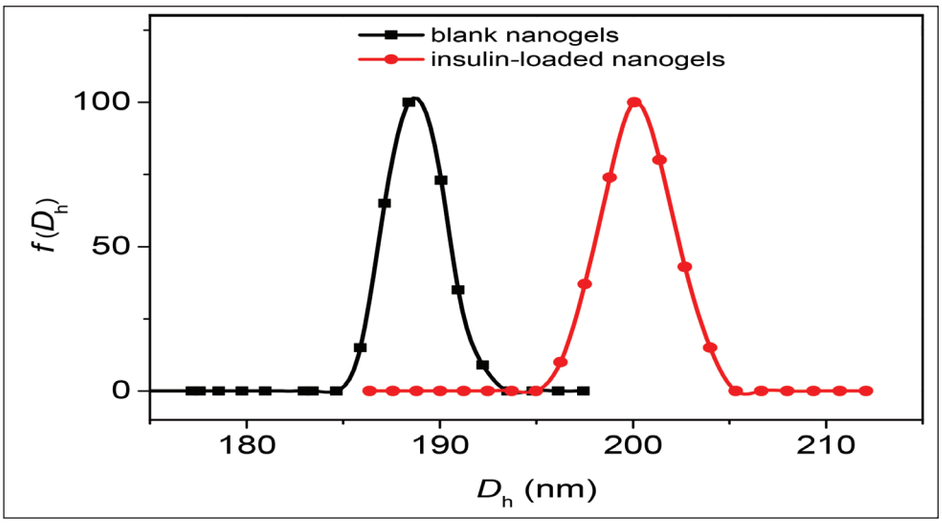

Fig. 2: The hydrodynamic diameter distributions f (D_h) of blank nanogels and insulin-loaded nanogels.

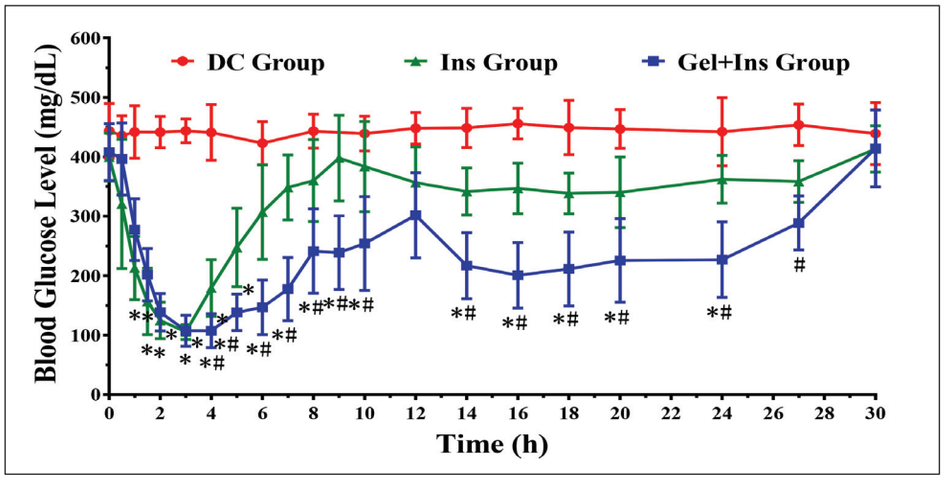

Fig. 3: The changes of blood glucose in each group of diabetic rats (vs the blood glucose at 0h (*P<0.05); vs Ins group (#P<0.05).

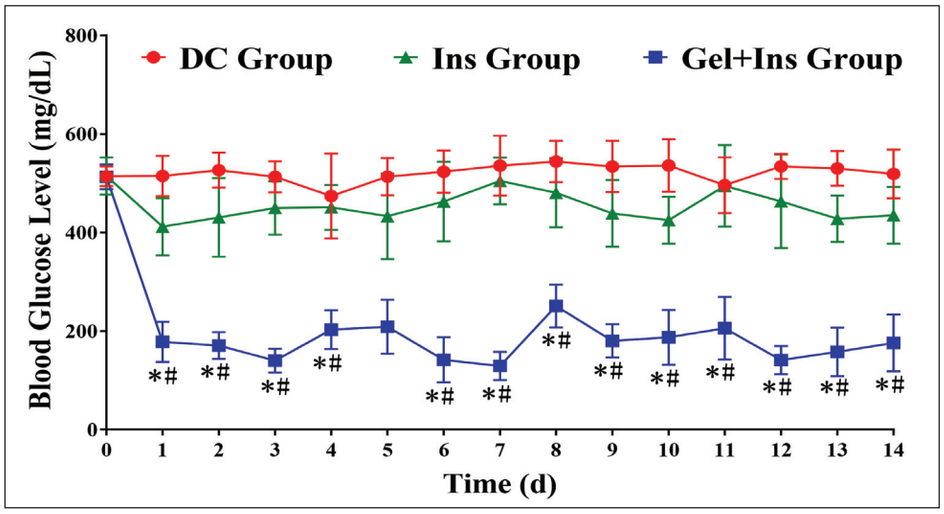

Fig. 4: The graph of fasting blood glucose changes in each group of rats in 14 days. vs the blood glucose at 0d, *P<0.05; vs Ins group, #P<0.05. P>0.05 indicates that there is no significant difference in fasting blood glucose between the groups.

value in the Gel+Ins group (P<0.05). Simultaneously, the content of serum insulin revealed a stable lift from 0.5 to 3.5 h and maintained the content of 150.2-194.4 μg/mL in the Gel+Ins group.

2.6. Evaluation in the changes of liver and renal function of rats in each group

To investigate the hepatic and nephritic security of insulin-loaded hydrogel-nanogel composite, we collected the venous blood and measured the concentration of ALT, AST, CREA and UREA in each group after the injection of the insulin loaded hydrogel-nanogel composite for 14 days. As shown in the Table, the AST concentration in the Gel+Ins group was significantly lower than that in the DC and Ins group, but failed to decline to the level of the NC group (P<0.05). Moreover, the level of ALT in the Gel+Ins group descended in contrast to that in the DC group (P<0.05). While there was no significant difference in CREA among the groups (P>0.05). Besides, the concentration of ALT, CREA and UREA in

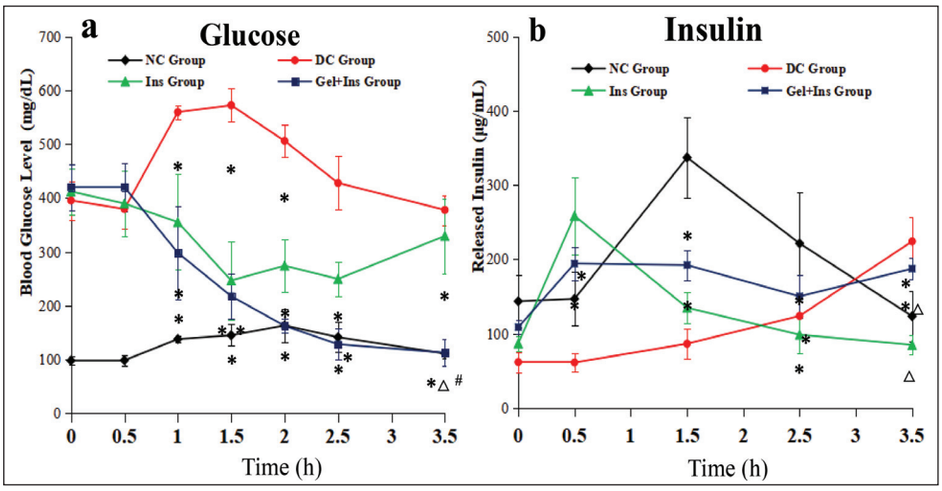

Fig. 5: vs the blood glucose at 0h, *P<0.05; vs DC group , &P<0.05; vs Ins group, #P<0.05. Figure a: A line chart of blood glucose level changes after OGTT experiment in each group of rats. Figure b: The broken line graph of plasma insulin change in different studied groups of diabetic rats in OGTT.

Gel+Ins group had no noticeable difference with those in the NC group respectively.

Table: Changes of liver and renal function of rats in each group [±s]

	ALT (U/L)	AST (U/L)	CREA (umol/L)	UREA (umol/L)
NC Group	38.40±8.73	78.60±14.36	71.00±6.04	7.36±1.63
DC Group	154.5±62.09 &	320.17±21.67 &	78.20±7.36	11.15±2.25 &
Ins Group	86.89±18.60 &#	223.11±34.51 &#	75.80±5.17	9.90±2.72
Gel+Ins Group	66.67±12.94 #	141.67±31.25 &#Δ	70.83±6.59	9.40±1.05
F	13.15	77.36	1.72	3.20
P	<0.01	<0.01	>0.05	<0.05

vs NC group, &P<0.05; vs DC group, ΔP<0.05; vs Ins group, #P<0.05.

2.7. *Oxidative stress response of rats in each group*

To assess the oxidative stress of insulin-loaded hydrogel-nanogels composite, we measured the concentrations of serum SOD and MDA in each group, both of which are the biological markers of oxidative stress. From Fig. 6, although the vitality of blood MDA in DC, Ins and Gel+Ins groups gradually reduced, it was significantly higher than that in NC group (P<0.05). On the contrary, the content of SOD in blood samples from the rats of NC group was obviously higher than that in samples from the other three groups (P<0.05). the activity of serum SOD in the DC group was distinctly lower than Ins and Gel+Ins groups, and compared with the Ins group, the content of SOD in rats from the Gel+Ins groups was significantly increased (P<0.05).

2.8. *Pathological morphology alterations of skin and pancreas tissue of rats in each group*

To observe the inflammatory reaction on the injection site and the impact on the pancreas after subcutaneous injection of the insulin-loaded hydrogel-nanogel composite, we stained the tissue of skin and pancreas. Microscopic inspection of the skin revealed that the inflammatory cells in the Gel+Ins group did not grow obviously after subcutaneous injection of insulin-loaded hydrogel-nanogel composite for 14 days. There was no noticeable difference between the Ins and Gel+Ins group, and the inflammatory cells were evidently lessened compared with DC group (Fig. 7a). As shown in Fig, 7b, a microscopic inspection of the pancreas exhibited that the volume and quantity of pancreatic islets in Ins group declined remarkably compared with that in the Gel+Ins group. The cell density of the island increased, the arrangement of cells was uniform and regular, and the morphology and structure of islets tended to be normal in the Gel+Ins group. Compared with other diabetic rat groups, islet damage was the mildest in the Gel+Ins group (Fig. 7b).

2.9. *The inflammatory reaction of rats in each group*

To research into the inflammatory reaction after subcutaneous injection of insulin-loaded hydrogel-nanogel composite, we stained the tissue of pancreas and observed the inflammatory reaction after 14 days on the basis of the experimental procedure.

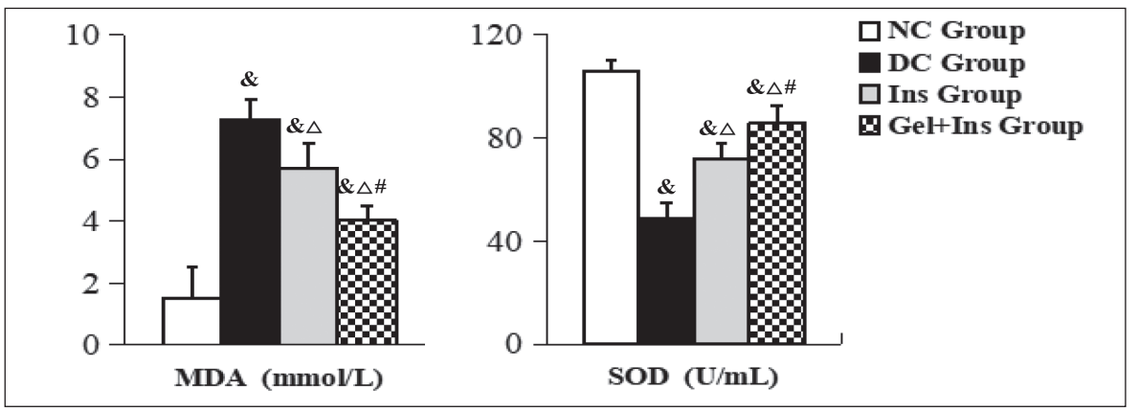

Fig. 6: Effects of the insulin-loaded hydrogel-nanogels composite on oxidative stress. Data represents the mean±standard deviation of SOD and MAD contents in each group of rats for 14 days. vs NC group, &P<0.05; vs DC group, ΔP<0.05; vs Ins group, #P<0.05.

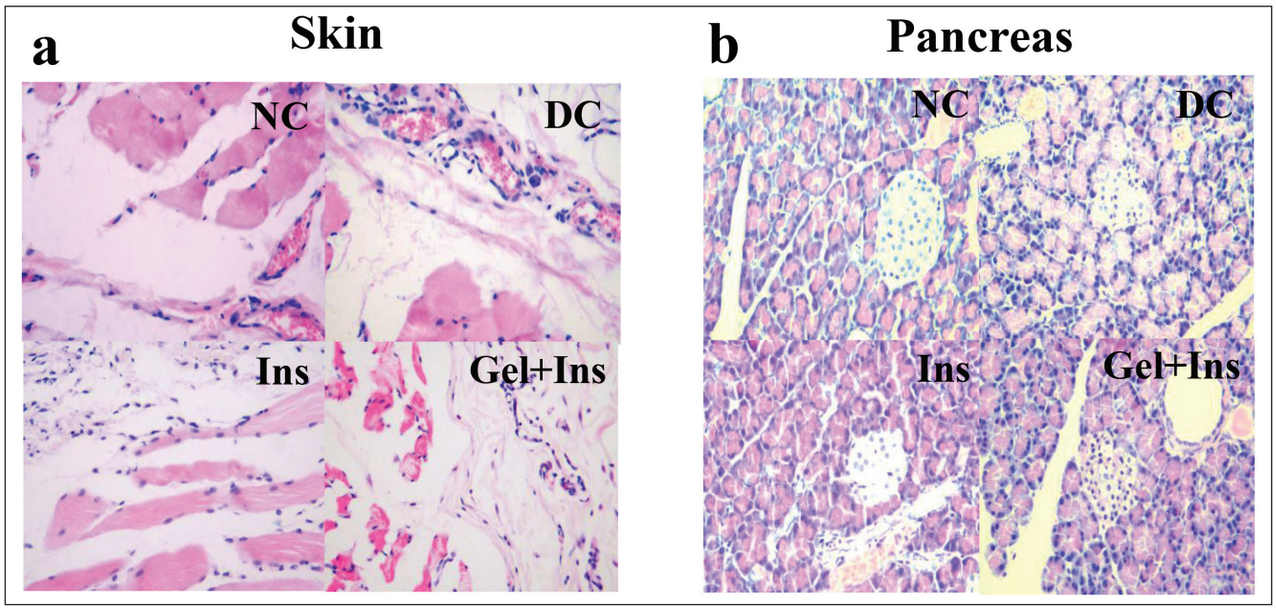

Fig. 7: The pathological and morphological changes of the skin(a) and pancreas(b) of rats in each group

The immunohistochemically-stained positive cells were brown granule cells. Compared with the NC group, pancreas in the DC group dramatically decreased. However, there existed amelioration to some extent in the Ins and Gel+Ins groups. The pancreas expression in Gel+Ins group exceeded that in the Ins group, which verged on the NC group, indicating that the improvement of Gel+Ins group was superior to that of Ins group. Furthermore, the levels of IL-1β and TNF-α (Fig. 8c) significantly decreased in the Gel+Ins group compared with the DC group and Ins group, but exceeded those in NC group.

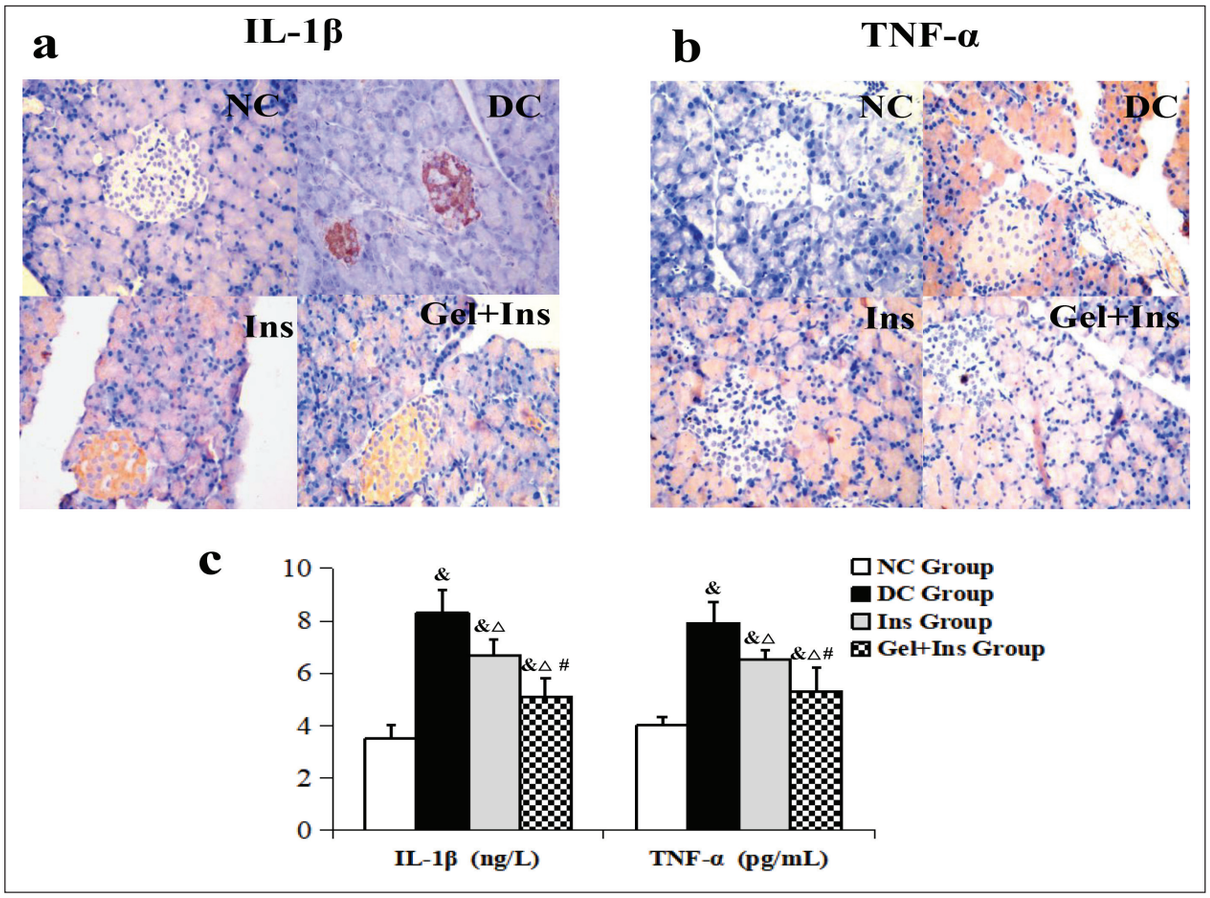

Fig. 8: The inflammatory reaction in each group of diabetic rats. vs NC group, $^{\&}P<0.05$; vs DC group, $^{\triangle}P<0.05$); vs Ins group, $^{\#}P<0.05$. (a) IL-1β;(b) TNF-α;(c) The histogram of the content of IL-1β and TNF-α in each group of rats.

3. Discussion

The purpose of our study was to discuss the effectiveness and safety of insulin-loaded hydrogel-nanogel composite, and to confirm whether the hydrogel-nanogel composite could be a potential carrier of insulin in the clinical treatment of diabetes. The major new findings were as follows: (1) The hydrogel-nanogel composite exhibited glucose-responsive release of insulin, and provided significant glucose-lowering effects for up to 30 h in rats; (2) hydrogel-nanogel composite loaded with insulin presented the effect of steadily reducing fasting and postprandial blood glucose without the occurrence of hypoglycemia; (3) Subcutaneous injection insulin-loaded hydrogel-nanogel composite was an excellent treatment with no effect on liver and kidney function and no damage to the skin and pancreas for diabetic rats; (4) Another feature of the insulin-loaded hydrogel-nanogel composite was ameliorating the oxidative stress and inflammatory reaction.

Nowadays, diabetes has become one of the leading causes of death. In order to prevent the occurrence of fatal acute and chronic diabetes complications, patients need lifelong treatment to preserve glucose concentration at normal levels. Although the daily hypodermic injection of insulin is the most effective way to treat diabetes mellitus, it also brings a lot of inconvenience to diabetics. Therefore, we hope to find an ideal diabetes treatment method that can spontaneously release insulin depending on the changes of blood glucose levels. Currently, new carriers of insulin are under investigation, and a growing number of researchers have explored various forms of carrier material to load insulin. Zhang et al. (2015) administered an oral administration of self-assembled insulin nanoparticles to rats, showing a depression of 48.70% compared with the initial level at 3 h but saw a return after 12 h in blood glucose, failing to be available to diabetic with a disease of digestive tract and fasting. Additionally, Gu et al. (2013) found that insulin delivered by injectable microgels could reduce blood glucose in mice and maintained a normoglycemic state, but unfortunately only for 12 hours. When Chen et al. (2017) injected insulin-loaded poly-γ-glutamic acid subcutaneously into experimental rats, the results exhibited a 46% drop in blood sugar compared with the initial level and the maximum duration of serum insulin at 1 h. Although the experimental material of the above-mentioned load insulin had boosted certain achievements in the hypoglycemic or extended time, there is a certain gap for achieving the glucose-responsive release of insulin and prolonging the hypoglycemic time. Fortunately, Hao et al. (2018) found that hydrogel-nanogel composite had attracted growing scientific attention as exceedingly desirable carriers for glucose-responsive insulin delivery. However, no *in vivo* study of hydrogel-nanogel composite loaded with insulin has been elaborated.

For our study, we subcutaneously injected insulin-loaded hydrogel-nanogel complexes into rats. The curve of blood glucose showed a deep drop and fluctuated at a lower level within 30 h than that in the regular insulin group, in which a glucose lowering effect of only 9 hours was preserved. The results indicated that insulin-loaded hydrogel-nanogel composite had stable and lasting hypoglycemic effects on rats. Furthermore, we observed that a strong glucose-lowering action on fasting blood glucose and the concentration of fasting blood glucose kept in the range of 140-250mg/dL for 14 days without hypoglycemia in the insulin-loaded hydrogel-nanogel composite group. Not only that, but our research demonstrated that the concentration of insulin released by hydrogel-nanogel composite contributed to a steady declining of blood glucose within 3.5 h without hypoglycemia through the imitation of oral glucose tolerance test. The above results further indicated that the insulin-loaded hydrogel-nanogel composite exhibited the desired glucose lowering action, with the fasting glucose and postprandial blood sugar being remarkably declined within a prolonged time. Therefore, we hypothesized that the insulin-loaded hydrogel-nanogel composite may be able to self-regulated insulin release and improve the sensitivity to glucose for response to the fluctuation of blood glucose level, thereby steadily and long-lasting lowering blood glucose in rats.

The hydrogel-nanogel composite revealed great biocompatibility through *in vitro* experiments. Our findings support that insulin loaded hydrogel-nanogels composite did not cause significant impairment of liver and kidney function, and that it is less damaging to the pancreas than the other groups depending on microscopic inspection. Besides, the results of our microscopic examination of the skin exhibited that the inflammatory response caused by the injection site of the insulin-loaded hydrogel-nanogel complex did not differ significantly from that of the conventional insulin injection group. Accordingly, we hypothesized that the hydrogel-nanogel complex did not cause more damage to the skin at the injection site, and that the insulin-loaded hydrogel-nanogel composite may reduce the damage of high sugar to the liver and kidney by maintaining blood glucose smoothly for a long time continuously. On the other hand, the extra insulin effectively lessens the burden of the pancreas and at the same time decreases the toxicity of high glucose to the pancreas. Therefore, lowering glucose by hydrogel-nanogel composite loaded with insulin may be a safe and powerful treatment strategy for diabetic patients.

A number of studies have demonstrated that oxidative stress and lipid peroxidation are critical factors involved in the progression of diabetes mellitus, and oxidative stress will increase in hyperglycemia. The proportions of oxygen free radicals are elevated, and the antioxidant enzymes are continuously consumed by peroxidation, eventually leading to tissue injury (Yaribeygi et al. 2020; Yang et al. 2020). Moreover, SOD and MDA, the antioxidant enzymes, are momentous markers of oxidative stress, which can directly reflect the content of oxygen free radicals and the degree of oxidative damage. Both SOD and MDA are currently recognized as sensitive indicators of oxidative stress (Ming et al. 2020). Our research found that the concentration of SOD in insulin-loaded hydrogel-nanogel composite group was significantly elevated compared with that in insulin group but failed to ascend to the level of the normal control group. However, the results of MDA were opposite to SOD. It turned out that the hydrogel-nanogel composite abated the level of oxidative stress but failed to reach normal level of oxidative stress. In addition, the chronic inflammatory response is the main mechanism of insulin resistance and diabetic complications. Inflammation activates the non-specific immune system. Macrophages, adipose cells and endothelial cells generate a large number of cytokines, mainly include IL-1β, TNF-α, etc. And IL-1β and TNF-α are vital factors leading to the apoptosis of β cells and insulin resistance in diabetic patients, which can reflect the inflammation reaction of the body (Li et al. 2021; Barakat et al. 2017; Wu and Ballantyne 2020; Bifulco et al. 2017). In our study, the concentrations of IL-1β and TNF-α in the insulin-loaded hydrogel-nanogel composite group declined compared with the diabetes control group and regular insulin injection group, and rose noticeably in contrast to that in normal control group. Thus, according to the results above, the insulin-loaded hydrogel-nanogel composite demonstrated an outstanding feature of ameliorating the inflammatory response and decreasing the level of oxidative stress. Based on these, hydrogel-nanogel composite loaded with insulin may be an excellent candidate for the treatment of diabetes.

Several limitations in this study are worth noting. Firstly, because of high costs and strict storage conditions, the experiment only lasted 14 days. It would be even more interesting if we extended the study to observe the effects of hydrogel-nanogel composite on other organs in rats. Secondly, based on the ability to prolong the glucose-lowering effect of regular insulin from the novel carrier, the effect on glucose of hydrogel-nanogel composite loaded with other dosage insulin still needs more researches. And third is that, only 40 type 1 diabetic rats were observed in the experiment. Further research should focus on more rats and those of type 2 diabetes mellitus to explore whether the hydrogel-nanogel composite could be widely applied in clinical practice.

To summarize, the current study manifests that diabetic rats treated with insulin-loaded hydrogel-nanogel composite showed long-term lower blood glucose, better liver and kidney function, less skin and pancreas damage, improved inflammatory response, and increased capacity for oxidative stress. This suggests that the

hydrogel-nanogel composite can convey and discharge insulin in a self-regulated way in response to the change of blood glucose level. As a consequence, the subcutaneous injection of insulin-loaded hydrogel-nanogel composite would be a powerful and potential strategy for building self-regulated insulin delivery systems to treat diabetes, and the carrier ought to also be a highly promising candidate for insulin delivery.

4. Experimental

4.1. Synthesis of hydrogel-nanogel composite forming polymers

Poly(ethylene glycol)-*b*-poly(aspartic acid-*co*-aspartamido phenylboronic acid) (PEG-*b*-P(Asp-*co*-AspAPBA)) and poly(aspartic acid-*co*-aspartic galactose) (P(Asp-*co*-AspGal)) were synthesized according to the literature procedures (Tyrrell et al. 2010). γ-P(GA-*co*-GAAGal) was synthesized by partially conjugating D-amino galactose to the carboxy groups of γ-PGA in the presence of EDC/NHS. Poly (lysine-*co*-lysine-4-carboxyphenylboronic acid)-*b*-poly(ethylene glycol)-*b*-poly (lysine-*co*-lysine-4-carboxyphenylboronic acid)(P(Lys-*co*-LysFPBA)-*b*-PEG-*b*-P (Lys-*co*-LysFPBA)) was composited by partial modification of PLys-*b*-PEG-*b*-PLys with FPBA.

4.2. Preparation of insulin-loaded glucose sensitive hydrogel-nanogel composite

The formation of insulin loaded hydrogel-nanogel composite was simply carried out by mixing PEG-b-P(Asp-co-AspAPBA), P(Asp-co-AspGal) and insulin in aqueous solution, before that the first two block copolymers had been dissolved into pH 10 NaOH solutions and PBS7.4 respectively. After dialyzing against PBS7.4 for 2 days, the insulin loaded hydrogel-nanogel composite solution was lyophilized for the following experiments. The loaded content of nanogels was 8.6±0.4wt%, and encapsulation efficiency was 13.0±0.2 wt%. Next, insulin-loaded hydrogel-nanogel composite was prepared by mixing P(Lys-co-LysFPBA)-b-PEG-b-P(Lys-co-LysFPBA) PBS solution (200 mg/mL) and γ-P(GA-co-GAAGal) PBS solution (200 mg/mL) which both were dissolved lyophilized powder of insulin-loaded hydrogel-nanogel composite .

4.3. Characterization of nanogels and hydrogel-nanogel composite

The nanogels were characterized by dynamic and static laser scattering (DLS and SLS) experiments, which were performed on a laser light scattering spectrometer (BI-200SM) equipped with a digital correlator (BI-9000AT) at 636 nm under room temperature. All samples were first prepared by filtering solutions through a 0.45 μm Millipore filter into a clean scintillation vial. Subsequently, Dynamic rheological analysis of hydrogel and hydrogel-nanogels composite (25mM) was accessed on an AR2000ex rheometer (TA Instrument, America). Parallel plate geometry with a diameter of 40 mm was used in the process, and the temperature was set at 37 °C. The gel sample was allowed to well-formed on the plate for 10 min. Frequency sweeps were performed at 1% strain, then stress sweeps were performed at 10rad s^{-1} by ramping the stress from 0.5 Pa to 1000 Pa.

4.4. In vitro glucose-responsive insulin release from hydrogel-nanogel composite

The *in vitro* release test of FITC-insulin was assessed by dialysis. Firstly, FITC-insulin was loaded into the hydrogel-nanogel composite. And then the insulin-loaded composite was transferred into a dialysis bag (MWCO = 10kDa) and immersed in 15 mL of PBS solution with varying glucose concentrations of 1, 2, and 5 g/L at 37 °C. The release medium (1 mL) outside the dialysis bag was taken out for analysis at a given definite intervals and replaced with fresh buffer. The released FITC-insulin was measured by fluorescence spectrometry at 519 nm upon excitation at 494 nm (Fig. 9).

4.5. Experimental animal model

Forty male (280-320 g) Sprague-Dawley rats (Beijing Vital River Laboratory Animal Technology Co., Ltd; ID: No.11400700218632) were used for these experiments. All experimental procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The project design and the protocol were reviewed and approved by the Institutional Animal Care and Use Committee at Hebei University. The animals were housed under standard laboratory conditions with 12 hours of light and darkness each day and the room temperature (18-20°C) and humidity were controlled. The experimental rats were randomly divided into four groups: normal control group (NC, n=10), diabetes control group (DC, n=10), insulin subcutaneous injection group (Ins, n=10) and insulin loaded hydrogel-nanogel composite subcutaneous injection group (Gel+Ins, n=10). All rats were provided with a regular diet and were allowed to eat and drink freely. One week later, the rats were fasted for 12 h, and after that DC, Ins, and Gel+Ins groups received intraperitoneal infusions containing 1% streptozotocin (STZ: 65mg/kg; pH = 4.0), At the same time, the NC group received equal doses of saline. After three days, their baseline blood samples were obtained via tail vein and blood glucose was measured with a glucometer (MEDISAFE blood glucose instrument). Rats with random blood glucose concentrations over 300 mg/dL, were considered as diabetic, meaning that the diabetes model was successfully created. During the whole process, two rats died (1 in the DC group and 1 in the Ins group) and one rat was eliminated due to failure of molding (1 in DC group). Subsequently, the collected blood samples were immediately centrifuged at 4000 rpm for 7 min. The plasma was drawn off and stored at -80 °C for further analysis.

4.6. Plasma samples measurements

Venous blood was collected to measure the concentration of blood glucose (MEDISAFE blood glucose instrument). After 14 days of the subcutaneous injection of fast-acting insulin loaded nanogels, venous blood (1 mL) was collected for measurements of circulating insulin levels by radioimmunoassay method (Roche, Cobasc 311, Mannheim, Germany). The liver function indexes such as alanine amino transferase (ALT) and aspartate amino transferase (AST) and renal function indexes (creatinine CREA, urea nitrogen UREA) were determined using a DPP full automatic biochemical analyzer (Meso Scale Diagnostics Kit, #K152BZC-3, Meso Scale Diagnostics, MD, USA). Superoxide dismutase (SOD) and malondialdehyde (MDA) content was determined by a reflectance spectrophotometer (XRite Gretag Macbeth, Berlin, Germany). During the high glucose gavage experiment, the serum insulin (μU/mL) levels were detected using the upper layer of serum samples (0.5 ml) from the tail veins of the rats by electrochemical luminescence (Roche electroluminescence instrument E601).

4.7. Histological and immunohistochemistry

At the end of the experimental procedure, all rats were euthanized with sodium pentobarbital (65 mg/kg, i.v., Euthanyl) after 10 h of fasting. Afterwards, the terminal blood samples were taken from the tail vein of rats, and the plasma was collected and stored as described above. Meanwhile, pancreatic and skin tissues were immediately excised and stored for immunohistochemical analysis. First of all, tissues from rats were settled by immersion in 10% formalin buffer and inserted in paraffin for light microscopy studies. Then, all paraffin-embedded tissue sections (5 μm thickness) were stained with Hematoxylin-Eosin (HE), and changes in tissue and cell structure were observed and recorded. Thirdly, these paraffin-embedded sections were immunostained using the streptavidin-peroxidase (SP) method. Briefly, after antigen retrieval, tissue sections were treated with interleukin-1β (IL-1β) (Santa Cruz, USA; pancreas 1:150) and tumor necrosis factor-α (TNF-α) (Santa Cruz, USA; pancreas 1:150) as the primary specific antibodies. In additional sections, the primary antibody was omitted and non-immunized goat serum was used for negative controls. After extensive rinsing, the biotinylated secondary antibody and SP complexes (Bio-high Technology, Hebei, China) were applied. Five randomly selected sites from each section were examined by optical fields with a microscope (400 x). Quantitation of immunoreactivity: yellow-brown granules in the cytoplasm-identified positive

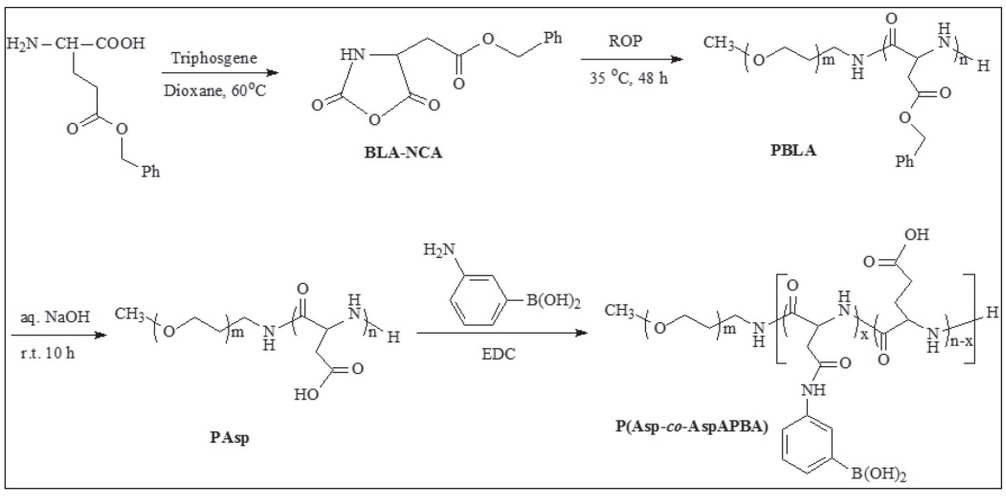

Fig. 9: The construction of FITC-insulin was loaded into hydrogel-nanogel composite.

cells. The immunohistochemical score (IHS) was defined as the function of the percentage of positive cells and the staining intensity of positive cells as follows: A, the percentage of positive cells (no positive cells=0, positive cells accounted for 1-10%=1, 11-50%=2, 51-80%=3, 81-100%=4); B, staining intensity of positive cells (negative=0, weak positive=1, moderately positive=2, strong positive=3), A multiplied by B is the IHS score (Soslow et al. 2008).

4.8. Experimental protocols

In the course of the experiment, all the experimental animals were provided with food and water at the same time. Then only the food was removed in all groups. Then only the food was removed in all groups. Ins group received subcutaneous fast-acting insulin injection once every day (fast-acting insulin used by Biosynthesis of human insulin, Novolin R, Novo Nordisk, 400 IU/10 mL/teams, 25 μL/IU, 2 IU/kg per time injection, once a day); Gel+Ins group were given hydrogel-nanogel composite loaded with fast-acting insulin with an equal dose of insulin at a stable time (2 IU/kg per time injection, once a day); NC and DC group were injected with the same amount of saline. On this basis, we carried out the experimental protocols below: (1) The effective time and the duration of hypoglycemic effect of hydrogel-nanogel composite loaded with insulin were observed; (2) The diversification of fasting blood glucose was monitored; (3)The variety content of blood glucose and plasma insulin in diabetic rats after pouring high-concentration glucose into stomach imitating diet were qualified; (4) Effects of subcutaneous injection of insulin loaded hydrogel-nanogel composite on hepatic and renal, oxidative stress and inflammatory responses in rats were evaluated,

4.9. Statistical analysis

Statistical analyses were carried out with the SPSS version 20.0. All data were displayed as median and range, and comparisons were performed using nonparametric tests. Gene expression levels were presented as mean±standard deviation (SD), the one-way analysis of variance (ANOVA) was performed when the parameters displayed homogeneity of variance. differences between groups were determined using the Newman-Keuls post hoc test. A value of $P<0.05$ was considered statistically significant.

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