Regulatory Effect of Liraglutide on Endoplasmic Reticulum Stress in Non-alcoholic Fatty Pancreas Disease

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Research article

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Abstract

Background The pathophysiology and treatment of non-alcoholic fatty pancreas disease (NAFPD) have not been well established. Thus, the aim is to prove hypotheses that endoplasmic reticulum (ER)stress may take part in the pathogenesis of NAFPD.

Methods The mice were randomly grouped into normal-group, model-group and experimental-group. The normal-group was fed with regular-diet, while model-group and experimental-group were fed with high-fat-diet for 16 weeks. After the NAFPD model was established successfully, the experimental-group began to give liraglutide 0.6 mg·kg -1 ·d -1 for 4 weeks. The normal-group and model-group were received saline only, total pancreatectomy was performed and pancreas samples were divided for western-blot and immunohistochemical, to detect the expression of GRP78、PERK、eLF2α、ATF4、CASPASE12 and CHOP.

Results Western-blot and immunohistochemistry both revealed that the expression of above proteins of model-group were significantly increased compared with normal-group and were also obviously increased compared with experimental-group. (all $P \leq 0.05$). The weight of body and the pancreas of the model-group was markedly higher than that of normal-group, and was also dramatically higher than that of experimental-group (all $P \leq 0.01$).

Conclusion Those results show ER stress may be one of the pathogenesis of NAFPD and liraglutide have the effect on regulating ER stress in NAFPD. Those findings have great significance in figuring out the pathophysiology of NAFPD.

Background

The high calorie intake in today's society could cause ectopic fat infiltration. The excessive fat could be accumulated in different visceral organs, for instance, liver and pancreas,[1] leading to non-alcoholic fatty liver disease (NAFLD) and/or non-alcoholic fatty pancreas disease (NAFPD). NAFPD is range from the ectopic adipose infiltration in pancreatic cells to pancreatic steatosis and inflammation, eventually resulting in fibrosis, that is akin to process of NAFLD.[2] Similar in pathophysiology to how NAFLD causes liver cancer, NAFPD may lead to the pancreatic carcinoma, and may also be associated withβ-cell dysfunction, insulin resistance, type 2 diabetes(T2DM),NAFLD, severity of acute pancreatitis, and pancreaticoduodenal leakage. What's more, NAFPD has instructive significance in the treatment and prognosis of the above diseases. It may also be one of the early intervention indications of metabolic syndrome and T2DM. However, on the contrary to NAFLD，the pathophysiology and treatment of NAFPD have not been well established yet. It is urgent to find a treatment for NAFPD.

Glucagon like peptide-1 (GLP-1), a gastroenteric hormone excreted by the intestinal cell ,[3] stimulates the secretion of glucose-dependent insulin to regulate blood glucose, [3, 4] playing a role in losing weight and improving the NAFLD as well.[5]Liraglutide is an agonist of the GLP-1 receptor and, thereby, occupying a certain position in the treatment of NAFLD.

Innumerable pathological conditions,including excessive accumulation of unfolded proteins in endoplasmic reticulum (ER) and saturated fatty acids[6]in cells could initiate the non-homeostasis of ER and the ER stress. [7]The cellular reaction to ER stress is to activate an signaling cascade known as the unfolded protein response (UPR) pathway[8]which enables the cell to reduce unfolded protein load in ER and restores homeostasis. [9] However the long-term ER stress or acute ER stress could cause chronic activation of UPR or full mobilization of UPR which induces an apoptotic procedure. [10] Cao J et al reported that the suppression of UPR could significantly inhibit apoptosis of hepatocytes in NAFLD. [6]Surveys such as that conducted by Na Ao et al have shown that Liraglutide could decrease the expression of GRP78 PERK CHOP and Caspase12 in mice of NAFLD and improve liver histology. [11] Sandberg MB et al founded that the of ER stress may take a part in the pathogenesis of NAFPD. [7] Given that the liver and pancreas have similar embryological origins [2]and ectopic fat deposition, [1]it is also plausible, as recommended for NAFLD, that GLP-1 could improve NAFPD by inhibiting UPR. Thus, the aim of our research is to prove hypotheses that ER stress may take part in the pathogenesis of NAFPD and the inhibition of GLP-1 on ER stress may improve NAFPD.

Methods

1.Materials

1.1Animals: SPF C57BL/6J male mice of five-week-old, weighing 15±1g, were used in this study, purchased from Shangjie Runyi Experimental Animal Service Department, Minhou County, Fujian Province. The protocol of animal experiments was approved by the animal management committee of the Second Affiliated Hospital of Fujian Medical University and performed strictly according to the guideline on animal experimentation.

1.2 Drugs and Reagents: Liraglutide, specification: 18mg/3ml, batch number: GP51912, Novo Nordisk A/S. Regular-diet and high-fat-diet (60% of calories from fat) were purchased from Minhou County Fujian Province.

2. Experimental methods

2.1 Animal modeling and grouping

Five-week-old SPF male C57BL/6 mice were grouped into normal-group, model-group and experimental-group. After 16-week feeding high-fat-diet and confirming the successful establishment of NAFPD model in the model-group (n = 10) and experimental-group (n = 10) , while the normal-group was fed with regular-diet. Then the experimental-group began to give liraglutide 0.6 $mg \cdot kg^{-1} \cdot d^{-1}$ for 4 weeks. The normal-group and model-group were received saline only.

2.2 Euthanasia and Experimental specimens:

After the intraperitoneal injection and weigh daily in the 4-week period and an overnight fast with water allowed ad libitum, the mice were sedated with an isoflurane-soaked gauze placed in a 2000 cm^3 glass jar. They were then anesthetized with an intraperitoneal injection of xylazine (15 mg/kg) and ketamine (50mg/kg) and were sacrificed by cervical dislocation. After that ,the total pancreatectomy was executed and pancreatic tissues were weighed and then split up for western-blot and immunohistochemistry.

2.3 Western-blot was carried out to discover the expression of GRP78、PERK、eLF2α、ATF4、CASPASE12 and CHOP in pancreatic tissue.

The tissue was cut into fine fragments, and the PMSF was added in the proportion of 100-250 μl lysate per 20mg tissue, and the homogenizer homogenized until completely cracked. After pyrolysis, the samples were centrifuged at 4 ℃ for 12000 rpm for 5 min, and the protein was quantified with BCA working solution. According to the quantitative results of protein, the required protein was added with appropriate amount of sample buffer, and then centrifuged to take the sample after boiling water bath for 5 min. The prepared PAGE gel was put into the electrophoresis cell, added with proper amount of electrophoresis buffer, and then sealed with 5% skimmed milk powder. According to the instructions: rabbit Anti-GRP78 BiP antibody(ab21685) 1: 2000, rabbit Anti-PERK antibody (ab192591) 1: 2000, rabbit Anti-eLF2α antibody (ab28726) 1: 2000, The rabbit Anti-ATF4 antibody(ab31390) 1: 2000, mouse Anti-DDIT3 antibody(ab11419) 1: 2000, Caspase 12 (GTX74429) 1: 2000, GAPDH 1: 5000 diluted antibody, antibody diluted to the required concentration in the blocking solution, incubated at room temperature for the night. The membrane incubated with first antibody was washed with TBST for 3 times, 5 min. each time. Then, according to the dosage, the second antibody labeled with HRP was diluted by 1 ≤ 1000 and incubated with the membrane at 37 ℃ for 1 h. Wash with TBST 3 times, 5 min. each time The ECL luminous solution was prepared. According to the dosage, the ECL luminous liquid A and B were mixed and added to the front dark chamber of the film to avoid light for 5 min. Pour out the color solution, carefully absorb the color solution with the paper, and cover it with a flat layer of transparent paper. The results of protein imprinting were standardized by internal reference GAPGH after gray scanning, and the values were analyzed by SPSS 24.0 software.

2.4 Immunohistochemistry (IHC) was carried out to discover the expression of GRP78、PERK、eLF2α、ATF4、CASPASE12 and CHOP.

The pancreatic tissue of 0.2cm-0.3cm was fixed with 10% neutral formalin for 48 h and then waxed at about 58 ℃. After wax soaking, it was sliced and mounted with silicified glass, then roasted at 60 ℃ for 1 h, immediately rinsed with xylene 10 min with ethanol of different concentrations from high to low (100%-30%), rinsed with tap water for 5 min, at a time, and fresh 3% H_2O_2 was equipped with distilled water. After sealing at room temperature for 10 min, rinse with distilled water for 3 minutes each time. The slices were immersed in 0.01m hydrochloric acid buffer, microwave was adjusted to 98 ℃-100 ℃ to boil, cooled for 5 min, and repeated twice, the slices were naturally cooled to room temperature, and washed with PBS for 3 times for 5 min each time. The excess liquid was removed by sealing solution at room temperature for 20 min, and incubated at 4 ℃ with first antibody (GRP78 1:200, PERK 1: 200, eIF2α

1: 200, ATF4 1: 200, CHOP 1: 200, CASPASE12 1: 200) over night, then washed 3 times with PBS for 3 min each time . After dripping the second antibody，setting 30min at 37 ℃. PBS was washed 3 times, 3min each time. DAB was added to the slice for 5-10 min the staining degree was observed and mastered under microscope. After rinsing with tap water, it was re-dyed with hematoxylin for 2 min, then flushed with tap water again, then sealed with glue and examined by microscope. Using Image-Pro Plus to collect Image Optical density.

3 Data analysis

Results are expressed as means ± SEM. Statistical analysis was done using SPSS version 24.0. The intergroup difference was compared with the independent sample t test and a *P* value≤ 0.05 was deemed as statistically significant. Date was analyzed by GraphPad & Prism version 7.00 software program.

Results

1. Impact of liraglutide on the ER stress in pancreas of mice with NAFPD

Western-blot analysis displayed that the expression of GRP78、PERK、eLF2α、ATF4、CASPASE12 and CHOP in pancreatic tissue of the normal group, compared with the model group, was significantly decreased(all $P \leq 0.05$) (Fig.1 and Table 1).At the same time, immunohistochemical analysis also showed that the expression of those proteins in the normal group was significantly decreased than that in the model group (all $P \leq 0.05$) (Fig.2 and Table 2).Those results suggest that ER stress does take part in the pathogenesis of NAFPD, specially the PERK-eIF2α-ATF4 pathway、CHOP pathway and Caspase12 pathway .

Using western-blot and immunohistochemistry, we found that Liraglutide have the effect on inhibiting ER stress in NAFPD，especially the PERK-eIF2α-ATF4 pathway、CHOP pathway and Caspase12 pathway. After the intervention, the expression of above proteins in the model group was founded to be markedly increased than those in the experimental group by western blot and immunohistochemistry (all $P \leq 0.05$) (as shown in Fig.1、Fig.2、Table 1 and Table 2).

2. Impact of liraglutide on the weight of pancreas of mice with NAFPD

To prove the effect of Liraglutide on losing weight of the pancreas and the body of mice with NAFPD, the weight of the pancreas and the body of mice on the last day were weighed. The weight of body of the normal group、the model group and the experimental group was (29.10 ±2.37), (36.10 ±2.73), (28.50 ±1.58) g, respectively. Compared with the normal group, the weight of the model group was notably higher($P \leq 0.01$). At the same time, the weight of the experimental group was notably decrease than that of the model group ($P \leq 0.01$) (as shown in Fig. 3). The weight of pancreas of the normal group、the model group and the experimental group was (0.11 ±0.01), (0.14 ±0.01), (0.12 ±0.01) g, respectively. Compared with the normal group, the weight of pancreas of the model group was dramatically higher($P \leq 0.01$). Meanwhile, the weight of pancreas of the experimental group was notably decrease than that of the

model group ($P \leq 0.01$) (as shown in Fig. 4). Those results showed that liraglutide could loss the weight of the pancreas and the body of mice with NAFPD.

Discussion

1.ER stress may be part of the cause of NAFPD

UPR,a self-protective response, mainly includes three branches: PERK-eIF2α-ATF4 pathway, IRE1αpathway and ATF6 pathway. [12] Upon the non-stressed circumstance，the these transducers of signaling pathways of UPR are combine with the BiP/GRP78,which suppress these signaling pathways. [12] When non-homeostasis of the ER take place, BiP/GRP78 will be separated from these transducers, leading to the activation of signaling pathways. However，the long-term ER stress or acute ER stress could cause chronic activation of UPR or full mobilization of UPR，which upregulates the expression of CHOP (also known as DDIT3) pathway, Caspase12 pathway and IRE1α-ASK1-JNK pathway and induces an apoptotic procedure. [10]Caspase12, a member of the Caspase family, is the only one in the family could be motivated by URP[13] and subsequently activates the rest of the Caspase family (such as Caspase9, Caspase3, etc.) to promote apoptosis. [13]Elida Lai et al reported that，together with others, [6, 14] the PERK-eIF2α-ATF4 pathway takes a significant position about mediating fat-induced ER stress and pancreatic cell apoptosis. [15] Elizabeth Karaskov et al also showed that the palmitate could increase the expression of the CHOP transcription factor. [14]

Here we report that the expression of GRP78, PERK,eIF2α,ATF4 Caspase-12 and CHOP in pancreatic tissue of the model group was higher than that of the normal group, which indicates that ER stress may be part of the cause of NAFPD,especially the PERK-eIF2α-ATF4，CHOP and Caspase12 pathway. Those findings have great significance in figuring out the pathophysiology of NAFPD, and have great value in the treatment plan for NAFPD.

2. Liraglutide have the effect on regulating the signal pathway of ER stress in pancreatic cell of NAFPD mice

Here we found that the expression of PERK-eIF2α-ATF4-related factors in the experimental group was decreased ,compared with the model group,illustrates that liraglutide have the effect on inhibiting ER stress in NAFPD，especially the PERK-eIF2α-ATF4 pathway，CHOP pathway and Caspase12 pathway. However, the mechanistic relevance of the finding is also unclear, since these could be protective, deleterious or just a bystander effect.

Although, Na Ao et al showed that liraglutide could decrease the expression of GRP78，PERK，CHOP and Caspase12 in mice of NAFLD and improve liver histology. [11] Meanwhile, many studies have confirmed that the effect of drugs on inhibiting the expression of ER stress pathway protein could improve the liver histology of NAFLD. [16–18] The PERK-eIF2α-ATF4 signaling pathway takes a significant position in mediating fat-induced ER stress and pancreatic cell apoptosis. [6, 14, 15]Given that the liver and pancreas have similar embryological origins[2]and ectopic fat deposition[1] it is also plausible, as recommended

for NAFLD, that GLP-1 could improve NAFPD by inhibiting UPR, however,the straight evidence of improvement of NAFPD was not displayed in present study. Hence, further studies are needed.

3. Liraglutide has effect on weight loss of the pancreas and the body of NAFPD mice

Obesity is considered to be associated with NAFPD, recent study suggests that obesity is an independent risk factor. [2] Animal experiments have shown that the accumulation of pancreatic interlobular or pancreatic intralobular fat, accompanied by inflammation and fibrosis, can be found in mice of long-term high-fat chow, leading to the impairment of islets and pancreatic acinar and the destruction of islet cell structure. [19] The pancreatic fat capacity and the amount of intaking fatty acid were markedly decreased in 7 of 10 cases of weight-loss surgery, that were all diagnosed with NAFPD, [20] which suggests that weight loss treatment may be used to treat NAFPD, such as liraglutide. [5] Some studies have shown that liraglutide can reduce the weight of mice fed on high-fat diet, inhibit ER stress, and improve steatosis. [13]Our study reveals that liraglutide not only could reduce the weight but also decrease the weight pancreas in NAFPD mice, and suggest that, together with the above research, the weight loss effect of liraglutide may helpful to improve the fat infiltration of NAFPD.

One of the shortcomings of this study is the absence of the straight evidence of improvement of NAFPD.

Conclusion

In conclusion, the ER stress may be part of the cause of NAFPD,especially the PERK-eIF2α-ATF4CHOP and Caspase12 pathway. and Liraglutide does repress the ER stress and could lighten the pancreas of NAFPD mice which may improve NAFPD. Hence, further studies are needed.

Abbreviations

ATF4:Activating Transcription Factor 4;Caspase12:Cysteme Aspartate Specific Protease 12;CHOP:C/EBP-homologousprotein; ER: Endoplasmic ReticulumEIF2α:Eukaryotic translation Initiation Factor 2α;GLP-1:Glucagon Like Peptide-1;GRP78: Glucose-regulatedprotein78;IHC: Immunohistochemistry; NAFLD non-alcoholic fatty liver disease ; NAFPD non-alcoholic fatty pancreas disease; PERK:R-like endoplasmic reticulum kinase;T2DM:type 2 diabetes; UPR:unfolded protein response

Declarations

Ethics Approval and Consent to Participate: All procedures had in keeping with prescriptive principles for animal experiment and experimental protocols were carried out in accordance with the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH, 80-23) and approved by the local Committee with Laboratory Animal Certificate Number: SYXK(MIN)2016- 0001.

Consent for publication: Not applicable

Availability of data and materials: The datasets supporting the conclusions of this article can be found online at http://dx.doi.org/10.17632/hm32r98hmt.2

Competing interests: "The authors declare that they have no competing interests.

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Authors' contributions: Y.C. and T.F. analyzed the data, designed the study and drafted the manuscript. J.C. W.H.S.H. and Z.C. interpreted the results and reviewed the manuscript. All authors critically revised the manuscript and approved the final version.

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Tables

Table 1 the expression of proteins of three groups after the intervene (Western-blot)			
Proteins	Normal group	Model group	Experimental group
GRP78	$0.47\pm0.35^{*}$	$1.89\pm1.44^{\#}$	0.60±0.35
PERK	$0.14\pm0.42^{**}$	$0.61\pm0.26^{\#\#\#}$	0.21±0.13
eIF2α	$0.20\pm0.12^{*}$	$1.62\pm1.41^{\#}$	0.40±0.19
ATF4	$0.39\pm0.23^{*}$	$1.82\pm1.42^{\#}$	0.52±0.19
Caspase12	$0.99\pm0.53^{**}$	$2.42\pm1.11^{\#\#\#}$	0.96±0.46
CHOP	$0.68\pm0.46^{*}$	$1.91\pm1.49^{\#}$	0.40±0.24

Comparison of the expression of GRP78、p-PERK、eLF2α、ATF4、CASPASE12 and CHOP of three groups after treatment with liraglutide in experimental group for 4 weeks(`x±s) .Compared with model group, $^{*}P \leq 0.05$、$^{**}P \leq 0.01$、Compared with Experimental group、$^{\#}P \leq 0.05$、$^{\#\#\#}P \leq 0.001$. GRP78: Glucose-regulatedprotein78;PERK:R-like endoplasmic reticulum kinase;EIF2α:eukaryotic translation initiation factor 2α; ATF4:activating transcription factor4;Caspase12:cysteme aspartate specific protease 12;CHOP:C/EBP-homologousprotein;

Table 2 the expression of proteins of three groups after the intervene (IHC)

Proteins	Normal group	Model group	Experimental group
GRP78	$2167.85\pm1211.00^{***}$	$6821.27\pm1389.20^{\#\#\#}$	1995.54±579.60
PERK	$1073.45\pm264.99^{***}$	$4131.04\pm1840.32^{\#\#\#}$	1351.66±443.85
eIF2α	$1310.78\pm411.63^{***}$	$5360.33\pm1302.93^{\#\#\#}$	2117.07±502.56
ATF4	$2722.43\pm684.32^{*}$	$5033.27\pm2927.69^{\#\#}$	1047.44±407.41
Caspase12	$2934.61\pm965.82^{***}$	$6393.62\pm1183.75^{\#\#\#}$	2041.62±621.56
CHOP	$2183.27\pm523.76^{***}$	$6214.80\pm1654.93^{\#\#\#}$	1528.48±431.76

Comparison of the expression of GRP78、p-PERK、eLF2α、ATF4、CASPASE12 and CHOP of three groups after treatment with liraglutide in experimental group for 4 weeks(`x±s) .Compared with model group, $^{*}P \leq 0.05$、$^{***}P \leq 0.001$、Compared with Experimental group、$^{\#\#}P \leq 0.01$、$^{\#\#\#}P \leq 0.001$. GRP78:Glucose-regulatedprotein78;PERK:R-like endoplasmic reticulum kinase;EIF2α:eukaryotic translation initiation

factor 2α;ATF4:activating transcription factor4;Caspase12:cysteme aspartate specific protease 12;CHOP:C/EBP-homologousprotein

Figures

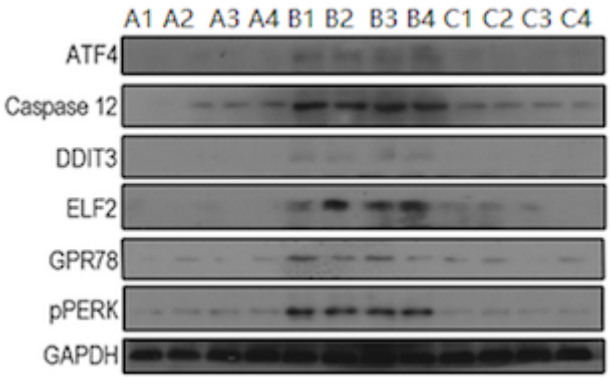

Figure 1

Expression of GRP78、p-PERK、eLF2α、ATF4、CASPASE12 and CHOP in three group:(A1-A4)the Normal-group,(B1-B4) the Model-group and(C1-C4)the Experimental-group. Using GAPDH as standard.

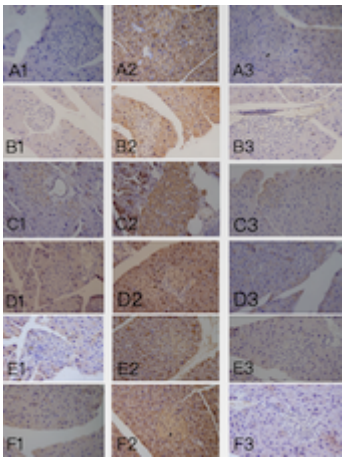

Figure 2

Effect of Liraglutide on ER stress assessed by immunohistochemistry: (A)GRP78, (B) PERK, (C) eIF2α, (D) ATF4,(E) Caspase12, (F) CHOP;(1) the normal-group, (2) the model-group and (3) the experimental-group.

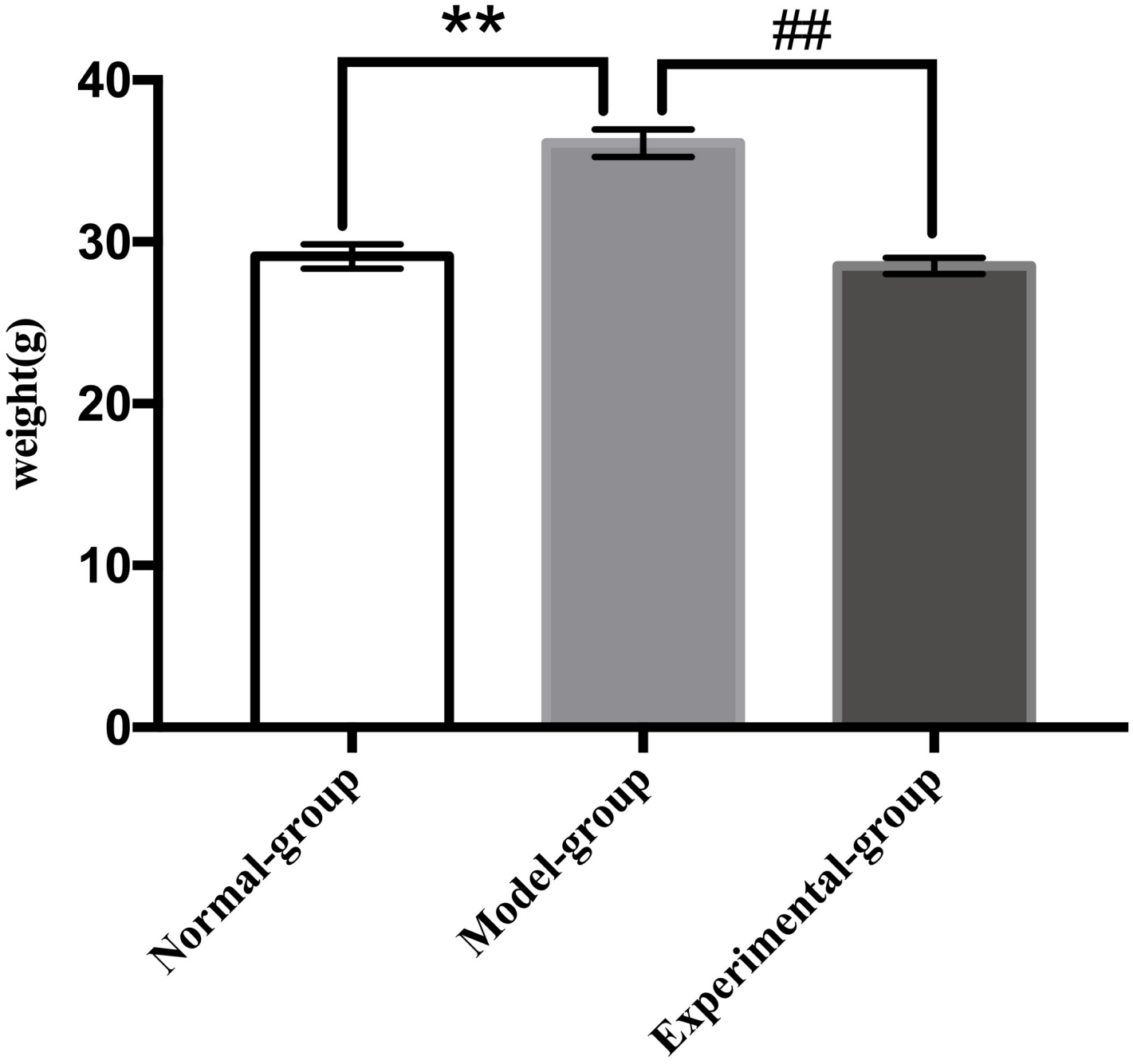

Figure 3

Values are means ± SEM ，n= 10 mice per group. ** P≤0.01 vs. Normal-group / Model-group; ## P ≤0.01 vs. Experimental-group / Model-group.

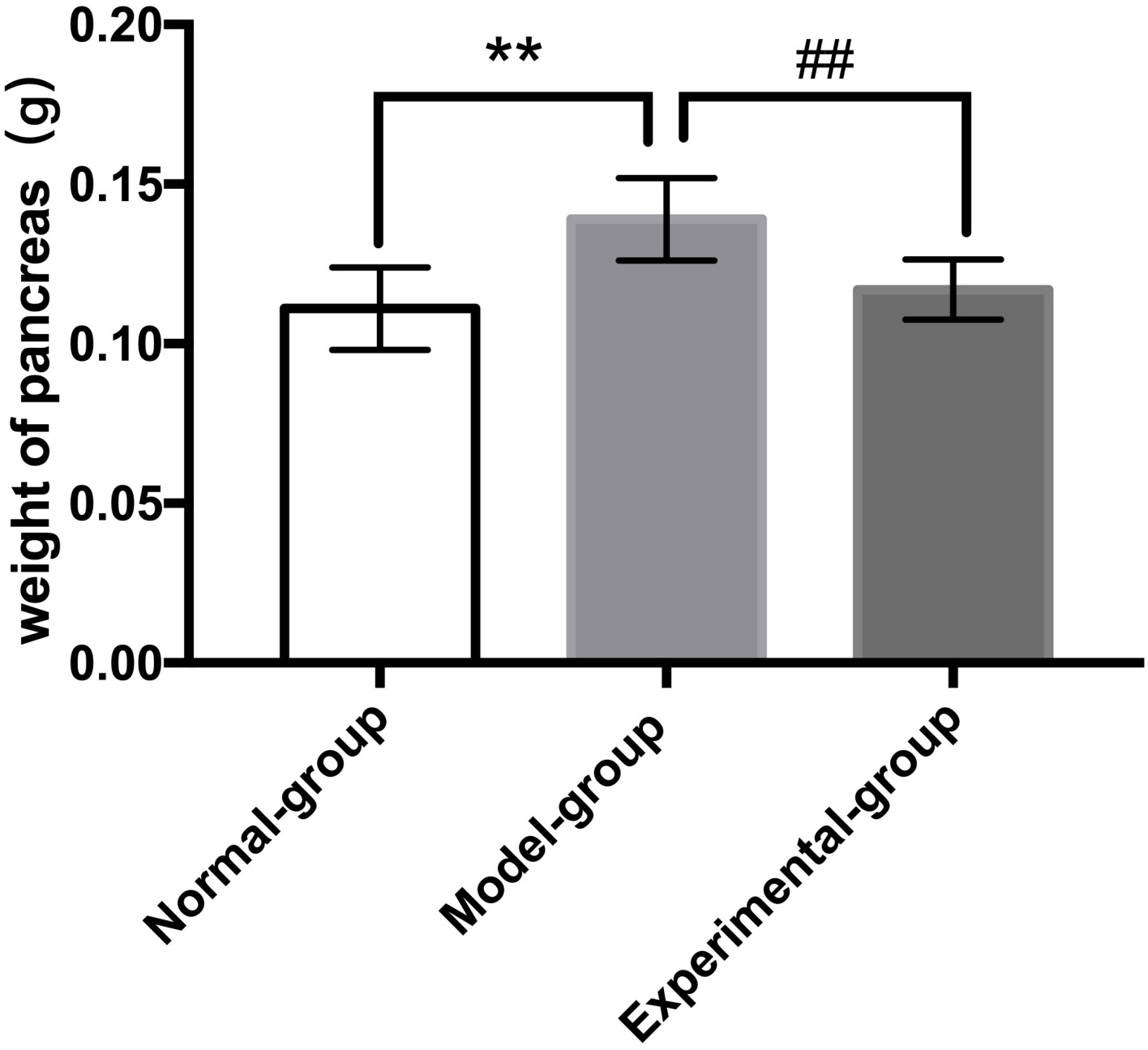

Figure 4

the weight of pancreas in three group** P≤0.01 vs. Normal-group / Model-group; ## P ≤0.01 vs. Experimental-group / Model-group.

Supplementary Files

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