Different Effect of Handle Region Peptide on β-Cell Function in Different Sexes of Rats Neonatally Treated with Sodium L-Glutamate

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BCE 1 Yi-xi Wu*
BCE 1,2 Ru-qiong Sun*
AFG 1 Guo-shu Yin
B 1 Dong-chuan Xu
B 1 Ping Wang
BC 1 Kun Lin
BD 1 Chu-jia Lin
AFG 1 Shao-da Lin*

1 Department of Endocrinology and Metabolism, The First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong, China
2 Department of Endocrinology and Metabolism, Tongxiang First People Hospital, Tongxiang, Zhejiang, China

* These authors contributed equally to this study

Corresponding Authors: Guo-shu Yin, e-mail: yinqinbiao@sina.com and Shao-da Lin, e-mail: linshaoda@126.com
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Background: The (pro)renin receptor ((P)RR) was reported to be expressed in various tissues including the pancreas, and handle region peptide (HRP) is believed to block the function of (P)RR. This study aimed to investigate the effect of HRP on the glucose tolerance status and β-cell function of female rats, neonatally treated with sodium L-glutamate (MSG) and to compare with the previously reported HRP effect on male rats.

Material/Methods: Female MSG rats aged 8 weeks were divided into MSG control group and HRP treated group and the normal SD rats served as control. The MSG rats were treated with HRP by osmotic minipumps with dose of 1 mg/kg per day for total 28 days. Glucose tolerance status was evaluated at the end of the study. Islets α-cell and β-cell were marked with insulin antibody and glucagon antibody respectively. The proliferation of islet cells and expression of subunit of NADPH oxidase P[22phox] were marked by PCNA and P[22phox] antibody. Picrosirius red staining was performed for evaluating fibrosis of islets.

Results: HRP improved the glucose status tolerance with decreasing α-cell mass, islets PCNA-positive cells, expression of P[22phox] and picrosirius red stained areas, and increasing β-cell mass in female MSG rats. The indexes with obviously interacted effect of sexes and HRP for the MSG rats were the AUC of blood glucose concentration ($p<0.01$), α-cell mass ($p<0.05$), proliferation of islet cells ($p<0.01$) and area of picrosirius red staining ($p<0.01$).

Conclusions: HRP improved the glucose tolerance status in the females although it was previously reported to worsen the glucose tolerance in male MSG rats. Different levels of sex hormones may partly account for the disparate effects observed for HRP in different sexes.

MeSH Keywords: Insulin Resistance • Islets of Langerhans • Renin-Angiotensin System

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Background

The (pro)renin receptor ((P)RR) cloned in 2002 was reported to be expressed in various tissues including the pancreas [1]. Its ligand, prorenin, is known to be activated without catalytic conversion into mature renin when combining with the (P)RR to alter the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) [1–4]. Although the renin inhibitor aliskiren theoretically blocks such prorenin-dependent Ang II generation, an alternative way to suppress the Ang source is by infusion of the (P) RR blocker called handle region peptide (HRP) [5,6].

The rats neonatally treated with sodium L-glutamate (MSG) is a model associated with insulin resistance that may occur without the presence of type 2 diabetes, depending on the age at which the animals are studied. The administration of MSG to newborn rats results in distinctive lesions in hypothalamic arcuate nucleus (ARC) neurons. The neuronal loss impairs insulin and leptin signaling and impacts the energy balance [7–11]. We previously reported that functions and structures of islets in MSG rats are abnormal, and that losartan improves the islet function [12].

Recent clinical data also showed that prevalence of type 2 diabetes is lower in premenopausal women, especially diabetic syndromes with insulin deficiency, suggesting that the female hormone 17β-estradiol protects pancreatic β-cell function and may constitute an important regulator of glucose metabolism and homeostasis [13–16]. We previously reported that HRP improved insulin sensitivity but not β-cell function and lead to hyperglycemia in male rats neonatally treated with MSG [12]. This study aimed to report the effect and mechanisms of HRP on the glucose tolerance status in female rats neonatally treated with MSG and to compare these effects to previously published data obtained for HRP in male MSG rats.

Material and Methods

Animals

All animal protocols were approved by the Ethics Committee of Shantou University Medical College. Timed pregnant SD rats were obtained from laboratory animal center of Shantou University Medical College. Neonatal female rats were injected subcutaneously with either 4 mg/g of MSG (Sigma Aldrich, Mo, USA) or NaCl(1.87% solutions) as control (Con group) at age of 2, 4, 6, 8, and 10 days. The experiments about the male rats were conducted at the same time and the results were published before [12]. All of the animals were weaned at age of 3 weeks. The control group of rats was given normal diet, whereas all of the MSG rats were given high-energy diet (445.5 Kcal/100g, Slaccas, Shanghai, China). At age of 8 weeks, the

MSG rats were divided into MSG control group (MSG group, n=5) and HRP treated group (MSG-HRP group, n=5) and the normal SD rats served as control (Con group, n=5). Then (day 0 and day 15) osmotic minipumps (2ML4 ALZET, CA, USA) were implanted subcutaneously under isoflurane anesthesia to infuse vehicle (saline, for MSG group), or HRP (NH2-RILLKKMPSV-COOH, Chinapeptides, Shanghai, China, 1 mg/kg per day for total 28 days, for the MSG-HRP group). At the age of 12 weeks, after OGTT and ITT assays, rats were sacrificed using pentobarbital sodium at the doze of 50 mg/kg body weight. The pancreas was rapidly dissected and opened longitudinally, then embedded in paraffin.

Oral glucose tolerance test and insulin tolerance test

Oral glucose tolerance test (OGTT) was performed after 16-h overnight fasting. Glucose (2 g/kg) was administered orally, and small blood samples (about 100μl) were collected from the tail vein at 0, 30, 60 and 120 min for immediate insulin (ELISA, Cusabio, Wuhan, China) and glucose (with a glucometer, Johnson & Johnson, New Brunswick, USA) measurements. For the insulin tolerance test (ITT), rats were given an intraperitoneal injection of 0.5 U/kg human insulin (Novo Nordisk, Tianjin, China) after 4 h of fasting, and glucose was measured with a glucometer before(0 min) and 15 min after insulin injection. The decreased rate of blood glucose was calculated according to the formula: (glucose (0 min)-glucose (15min))/glucose (0 min).

Measurement of physiological parameters

At 12 weeks of age, body weights and lengths of the animals were measured, and the Lee's index was calculated according to the formula:

$$\text{Lee's index} = \sqrt[3]{Weight(g)} \times 1000 \big/ Length(cm) \ .$$

Picrosirius red staining

Picrosirius red staining was used for evaluating the fibrosis status of pancreatic islets. Four-micron paraffin sections were prepared from 4% paraformaldehyde-fixed, paraffin-embedded rat pancreas. Sections were stained with 0.1% sirius red (Sigma Aldrich, MO, USA) in saturated picric acid (picrosirius red) for 1 h and mounted. The ratio of stained area to that of the whole islet was calculated using the computer-imaging software IPP6.0. Three islets were randomly selective for analysis for each animal (n=5 for each group).

Immunohistochemistry measurement for β-cell mass, α-cell mass, cell proliferation and NADPH oxidase subunit

The expressions of the β-cell marker insulin and α-cell marker glucagon were examined by immunohistochemistry using

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Table 1. Physiological parameters of the animals.

Group	n	Weight (g)	Length (cm)	Lee's index
Con	5	223.21±10.42	21.52±0.55	282.17±3.98
MSG	5	233.12±19.44	18.84±0.82*	326.40±18.69*
MSG-HRP	5	236.01±36.33	19.54±0.85*	316.55±14.41*

* Compared with Con group, P<0.05. Lee's index= $\sqrt[3]{Weight(g)\times 1000}/Length(cm)$.

insulin (sc-9168, 1:1,000; Santa Cruz, Texas, USA) and gluca-gon (sc-13091, 1:100; Santa Cruz, Texas, USA) antibodies, re-spectively. Slides were incubated with primary antibodies for 1 h at room temperature. After washing, secondary antibodies (1:500, biotin-conjugated goat anti-rabbit IgG; Boster, Wuhan, China) were applied for 30 min at room temperature. The ar-eas of islets staining positively (insulin and glucagon respec-tively) and the whole islet were assessed by IPP6.0. And then β-cell mass and α-cell mass were respectively calculated (β-cell mass or α-cell mass=area of insulin or glucagon staining pos-itively/area of the whole islets). Three islets were randomly selective for analysis for each animal (n=5 for each group).

Proliferation of islet cells was assessed by immunohistochem-ical staining for proliferating cell nuclear antigen (PCNA) us-ing PCNA antibodies (sc-7907, 1:100; Santa Cruz, Texas, USA). Slides were incubated with primary antibodies for 1h at room temperature. After washing, secondary antibodies (1:500, bi-otin-conjugated goat anti-rabbit IgG; Boster, Wuhan, China) were applied for 30 min at room temperature. The number of PCNA-positive cells per islet section was counted. Three is-lets were randomly selective for analysis for each animal (n=5 for each group).

To evaluate the expression of NADPH oxidase P[22phox] subunit in islet sections, staining was carried out with P[22phox] antibodies (sc-20781, 1:100; Santa Cruz, Texas, USA). Slides were incubat-ed with the primary antibodies for 1 h at room temperature. After washing, secondary antibodies (1:500, biotin-conjugat-ed goat anti-rabbit IgG; Boster, Wuhan, China) were applied for 30 min at room temperature. The average gray-scale in-tensities of cells staining positively were measured by IPP 6.0. Three islets were randomly selective for analysis for each an-imal (n=5 for each group).

Statistical analysis

Data are shown as mean ±SD. One-way ANOVA and LSD-test were performed to assess differences between groups of the female rats. Two-way ANOVA analysis was performed to make sense of the interacted effect of sexes and HRP for the MSG group (male and female) and the MSG-HRP group

(male and female). A value of P<0.05 was considered sta-tistically significant. All statistical analyses were conducted with SPSS 13.0.

Results

Living status of rats in different groups

The basal characteristics of each group are shown in Table 1. Body weights showed no statistical difference between the Con group and MSG groups. Compared with the Con group, MSG rats had reduced body length. Therefore, the Lee's index, re-flecting the extent of obesity, was increased in MSG rats com-pared with the Con group. No statistical significant difference was observed in body weight, length and Lee's index between the MSG and MSG-HRP groups.

### HRP increases insulin sensitivity and ameliorates the glucose tolerance status

The response of blood glucose to OGTT at week 12 of the ex-perimental period is shown in Figure 1A, 1B. The AUC of blood glucose concentration was higher in the MSG group compared with the Con group, although the difference was not statisti-cally significant. The MSG-HRP group displayed significantly lower AUC of blood glucose concentration than the MSG group (P<0.05), indicating that HRP improved the glucose tolerance status in female MSG rats.

The response of serum insulin to OGTT is shown in Figure 1C, 1D. The serum insulin concentrations in the MSG and MSG-HRP groups at fasting, 30 min and 60 min after oral glucose and the AUCs of serum insulin concentrations were lower than the values obtained for the Con groups. AUC of serum insulin concentration in the MSG-HRP group was higher than in the MSG group, although without statistical significance.

Insulin sensitivity was evaluated according to ITT and the re-sults were shown in Figure 2. The decreased rate of blood glu-cose was smaller in the MSG group compared with the Con group at 15 min after insulin injection (P<0.05) and treatment
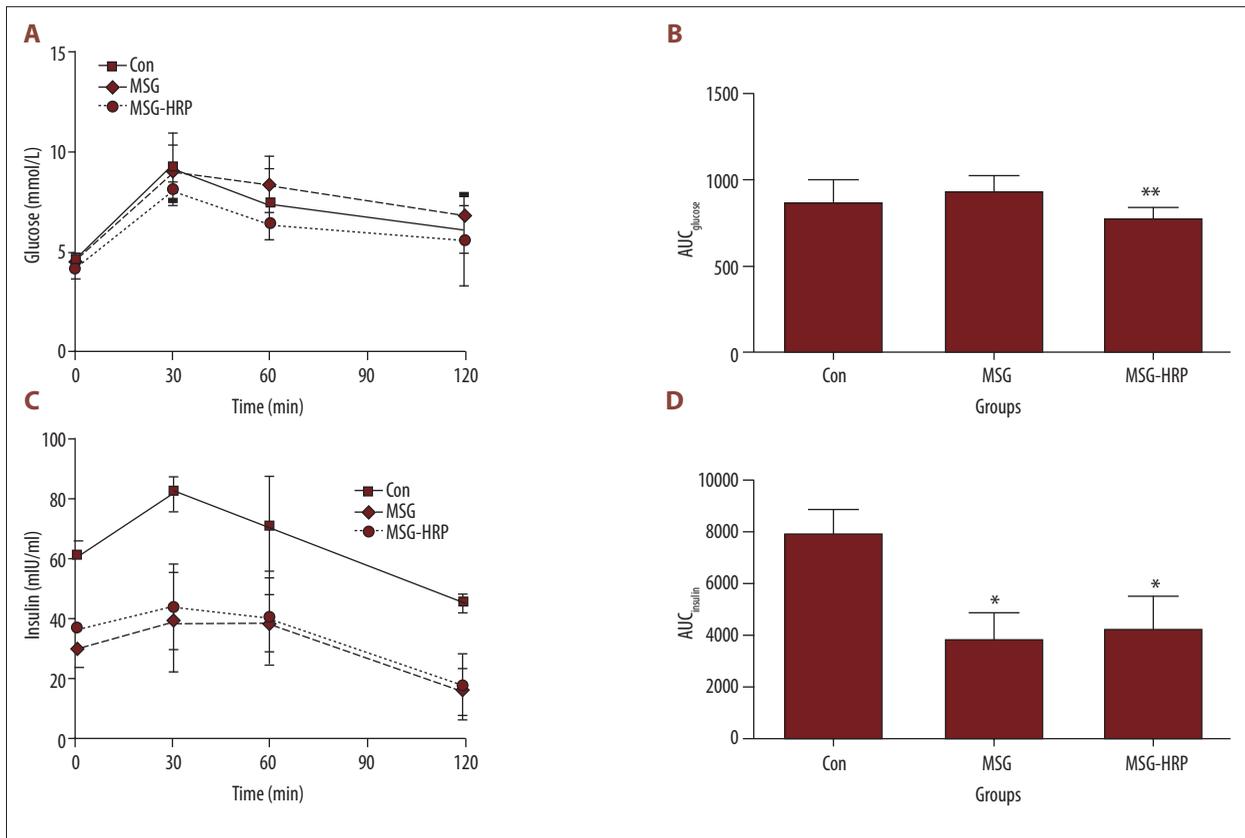
Figure 1. Samples for blood glucose concentration (**A**) and serum insulin concentration (**C**) before and 30 min, 60 min, and 120 min after glucose load were obtained and AUCs were calculated (**B and D**, respectively). * Compared with the Con group, P<0.01; ** Compared with the MSG group, P<0.05.
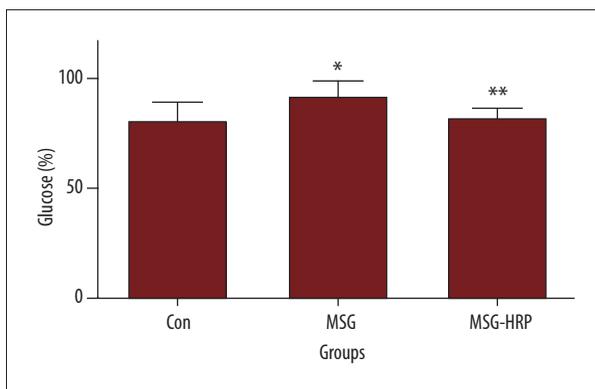
Figure 2. Insulin tolerance test data. The decreased rate of blood glucose was less pronounced in the MSG group compared with the Con group at 15 min after insulin injection. Treatment with HRP resulted in a more pronounced decrease in blood glucose rate in comparison with MSG rats. * Compared with the Con group, P<0.05; ** Compared with the MSG group, P<0.01.

with HRP had higher decreased rate of blood glucose when compared with the MSG group (P<0.01).

HRP increases β-cell mass and decreases α-cell mass

Islets β-cell and α-cell were labeled by insulin and glucagon antibodies, respectively (Figures 3, 4). To quantify the changes in β-cell and α-cell masses, the ratios of respective stained area of insulin and glucagon to whole islet area were calculated, and the results are shown in Figure 3D and Figure 4D. MSG rats treated with HRP increased β-cell mass compared with the MSG group (P<0.05). The α-cell mass in the MSG-HRP group was decreased in comparison with the MSG group (P<0.05).

HRP decreases proliferation of islet cells

Cells staining positively for the PCNA marker in pancreatic islets are shown in Figure 5. The number of PCNA-positive cells per islet was determined. Most PCNA-positive staining cells distributed in the periphery of the islets and were accordance with the location of α cell. The number of PCNA-positive cells was higher in the MSG group compared with the Con group (88.40±7.09 vs. 6.60±1.95, P<0.01). Treatment with HRP significantly decreased the number of PCNA-positive cells (53.20±7.92), compared with the MSG group (P<0.01).
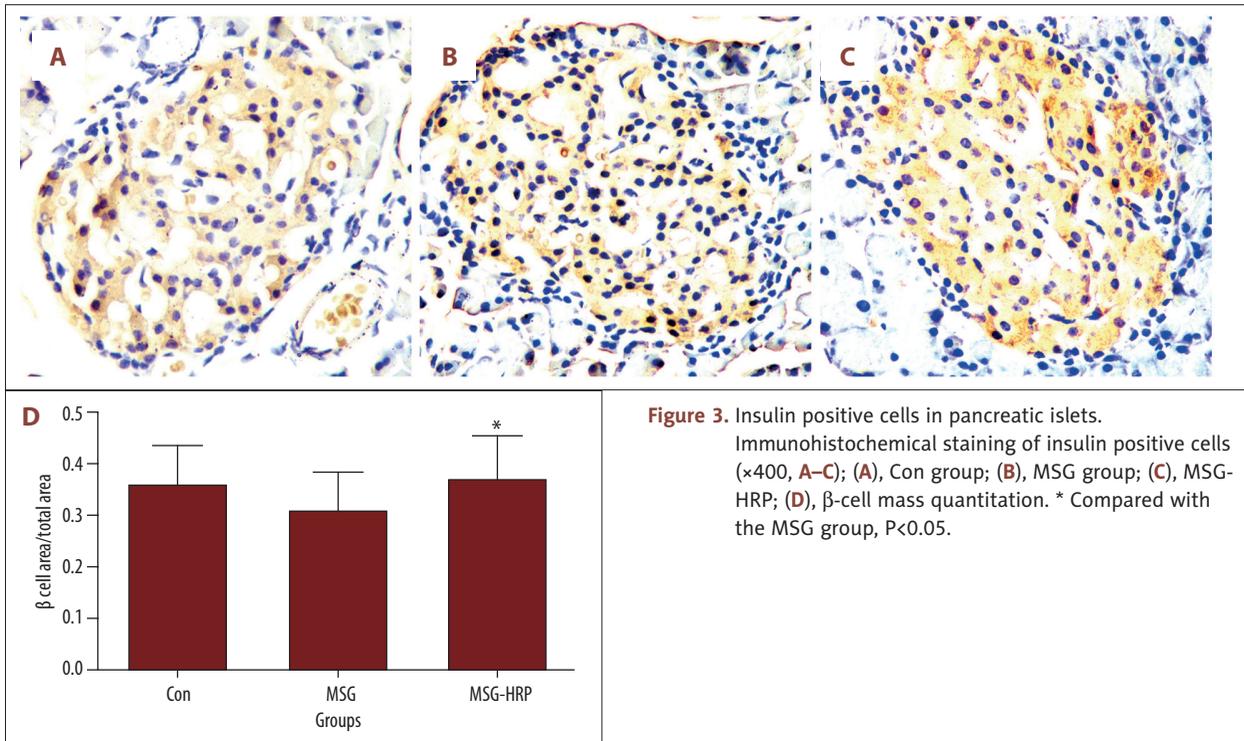
Figure 3. Insulin positive cells in pancreatic islets. Immunohistochemical staining of insulin positive cells (×400, **A–C**); (**A**), Con group; (**B**), MSG group; (**C**), MSG-HRP; (**D**), β-cell mass quantitation. * Compared with the MSG group, P<0.05.
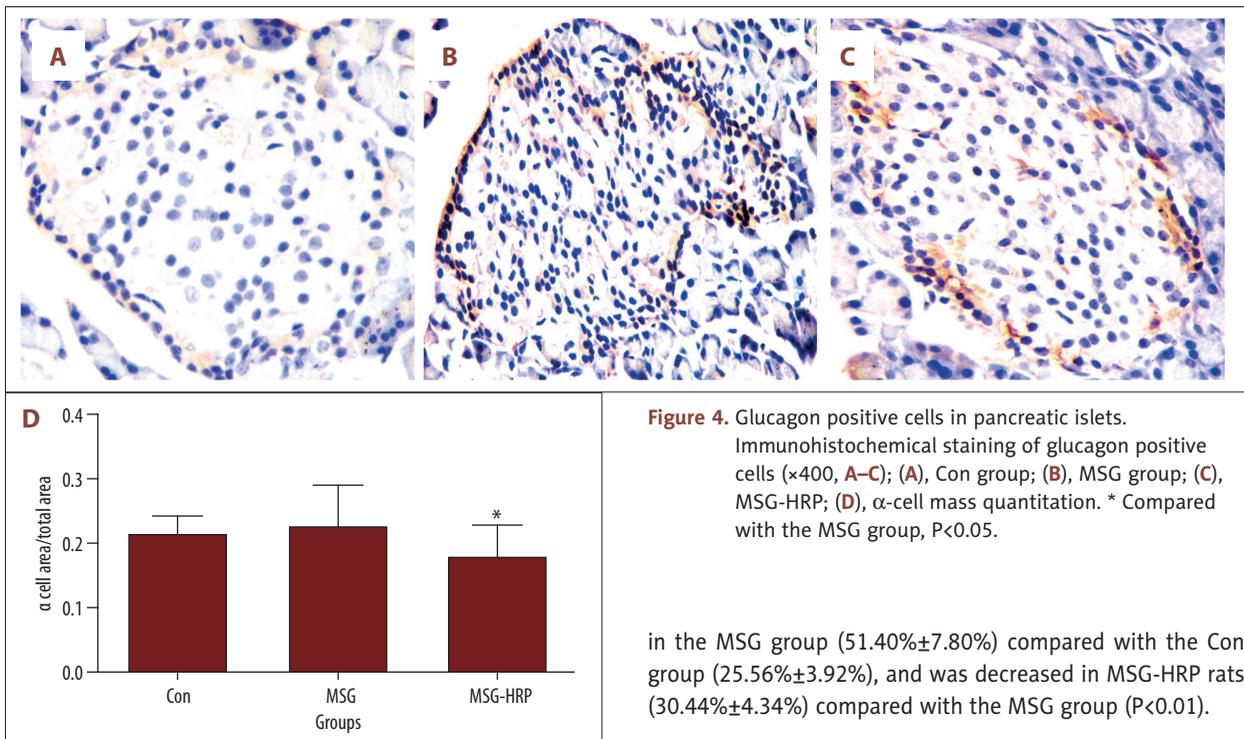
Figure 4. Glucagon positive cells in pancreatic islets. Immunohistochemical staining of glucagon positive cells (×400, **A–C**); (**A**), Con group; (**B**), MSG group; (**C**), MSG-HRP; (**D**), α-cell mass quantitation. * Compared with the MSG group, P<0.05.

in the MSG group (51.40%±7.80%) compared with the Con group (25.56%±3.92%), and was decreased in MSG-HRP rats (30.44%±4.34%) compared with the MSG group (P<0.01).

HRP decreases pancreas oxidative stress

Immunostaining of P^{22phox} in the pancreatic islets is shown in Figure 7, with the average gray-scale intensities in Figure 7D. The immunostaining of P^{22phox} was increased in the MSG group compared with control animals (p<0.05). Treatment with HRP

### HRP ameliorates fibrosis of the pancreatic islets

Fibrosis of the pancreatic islets was evaluated by the picrosirius red staining (Figure 6) and the ratio of stained area to whole islet area was determined. The ratio was higher
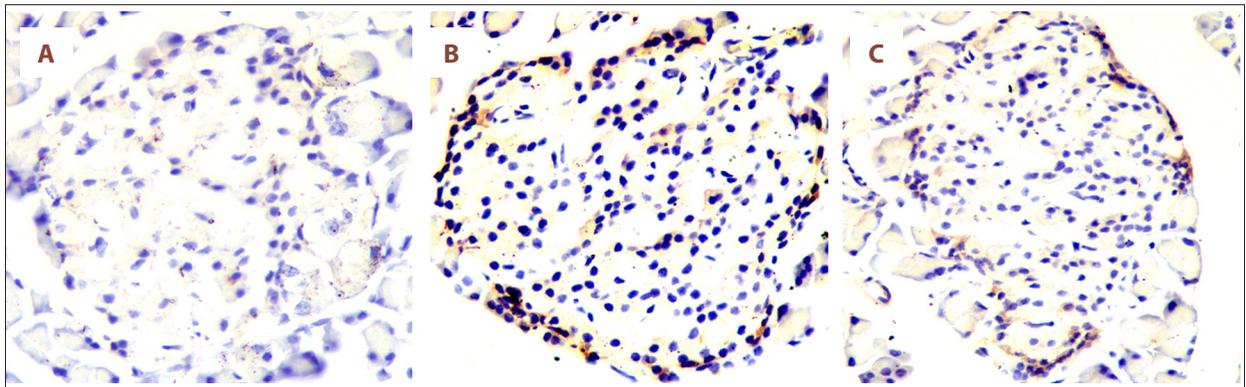
Figure 5. Cells staining positively for the PCNA marker in pancreatic islets (×400). Most PCNA-positive staining distributed in the periphery of the islets in MSG rats. HRP significantly decreased the number of PCNA-positive cells. (**A**); Con group; (**B**), MSG group; (**C**), MSG-HRP group.
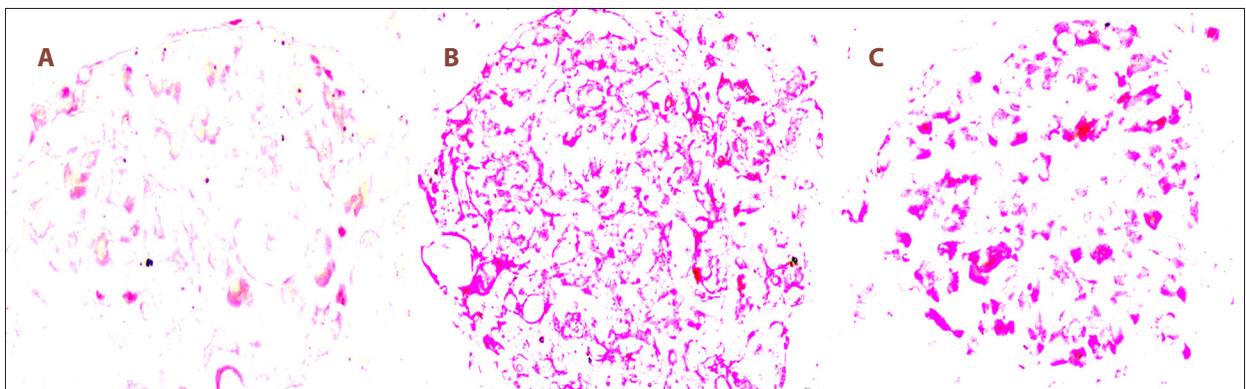
Figure 6. Picrosirius red staining of the pancreatic islets (×400). The staining area was significantly increased in MSG rats compared with Con rats. MSG rats that received HRP treatment showed significantly decreased staining area. (**A**), Con group; (**B**),: MSG group; (**C**), MSG-HRP group.

decreased the average gray-scale intensities of P^{22phox} immunostaining (P <0.01).

**Interacted effect of sexes and HRP for the MSG rats**

Two-way ANOVA analysis was performed to make sense of the interacted effect of sexes and HRP for the MSG rats (the results of the male rats have been published before [12]). The indexes with obviously interacted effect of sexes and HRP for the MSG rats were the AUC of blood glucose concentration (P<0.01), α-cell mass (P<0.05), proliferation of islet cells (P<0.01) and area of picrosirius red staining (P<0.01).

Discussion

Several key renin-angiotensin system (RAS) components were detected in the pancreas tissue [1,2,17,18] and the local RAS are responsive to various physiological and pathophysiological stimuli such as hyperglycemia, leading in turn to aggravation of islet function [19–24]. Recent clinical studies have

shown that treatment with angiotensin type 1 (AT1) receptor blockers or angiotensin-converting enzyme (ACE) inhibitors protects against the development of insulin resistance in hypertensive patients and new onset of type 2 diabetes in "at-risk" patients. Animal studies have indicated that RAS inhibitors improved islet function in various animal models of type 2 diabetes [19,21,22,25]. To our surprise, the effect of HRP on the glucose tolerance status was different from female and male MSG rats. We reported previously that HRP increased the AUC of blood glucose for OGTT with decreasing levels of serum insulin concentration and lead to hyperglycemia in male MSG rats [12]. However, HRP improved the glucose tolerance status in female MSG rats in this study.

Whether HRP ameliorates the architecture of the islet in different sexes of MSG rats may directly account for the distinct effects observed for HRP on glucose metabolism. First, suitable ratio of α-mass and β-mass is an important factor for maintaining islet function. Previously studies showed that RAS inhibitors tend to increase the β-mass [24,25]. Our results indicated that HRP increased β-cell mass and decreased α-cell mass,
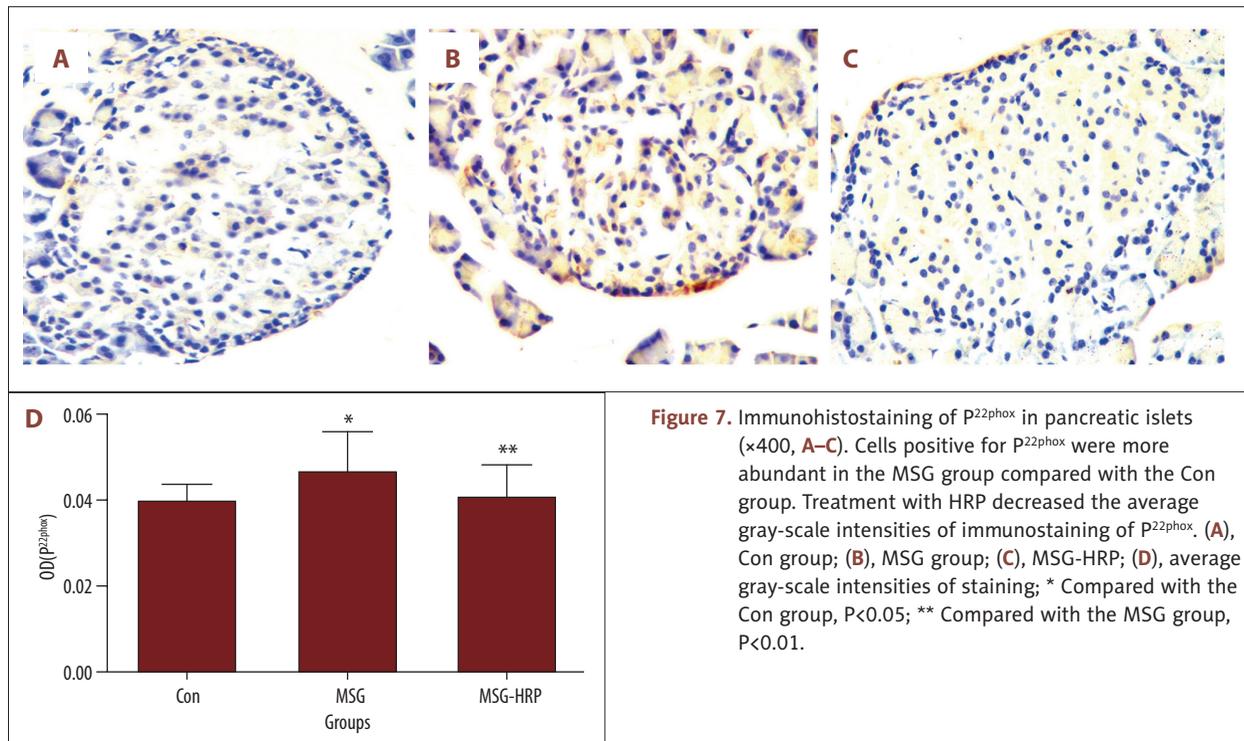
Figure 7. Immunohistostaining of P^{22phox} in pancreatic islets (×400, **A–C**). Cells positive for P^{22phox} were more abundant in the MSG group compared with the Con group. Treatment with HRP decreased the average gray-scale intensities of immunostaining of P^{22phox}. (**A**), Con group; (**B**), MSG group; (**C**), MSG-HRP; (**D**), average gray-scale intensities of staining; * Compared with the Con group, $P<0.05$; ** Compared with the MSG group, $P<0.01$.

in accordance with data obtained for insulin release test in female MSG rats. The regulation of islet cell apoptosis and proliferation is important in maintaining normal ratio of β-cell mass and α-cell mass. HRP decreased the PCNA-positive cells in the periphery of the islets accordance with the location of α cell in female rats, although the results were not accord with male MSG rat data [12]. Second, fibrosis is another factor that can change the specialized architecture of the pancreatic islet, and islet fibrosis was suppressed by RAS inhibitors in animal models of type 2 diabetes [19,21]. Ichihara [26,27] and co-workers showed that HRP decreases the expression of collagen I and III in the heart, and collagen IV in the kidney, in spontaneously hypertensive rats. HRP was shown to ameliorate fibrosis status of islets in female MSG rats but not in the male MSG rats.

Moreover, over activation of oxidative stress in islets was observed in animal models of type 2 diabetes and RAS blockade decreased the activity of NAD(P)H oxidase and markers of oxidative stress [21,24]. As shown above, treatment with HRP decreased expression of the P^{22phox} protein in pancreatic tissue both in male and female MSG rats. These findings support the notion that RAS inhibitors ameliorate including HRP improve islet function by decreasing the activity of oxidative stress.

Therefore, HRP in female and male rats showed some similar effects on islets in MSG animals, including increased β-cell mass and decreased activity of oxidative stress. However, HRP decreased α-mass and amount of proliferation of islet cells, and improved the status of islets fibrosis in female but not in male MSG rats.

The exact mechanisms underlying the sexes specific effects of HRP are unclear. Estrogen is considered an important regulator of glucose metabolism. The estrogen receptor (ER)-α, ER-β, and the G-protein coupled ER are present in β-cells where they enhance islet survival and improve islet lipid homeostasis, insulin biosynthesis and against oxidative stress injury [14,28–30]. It has been shown that ER-β selective agonists enhance glucose-stimulated insulin secretion both in mouse and human islets [31]. Loss of estrogen sulfotransferase, the enzyme responsible for the sulfonation and inactivation of estrogens in female mice improved metabolic functions in various mouse models of type 2 diabetes [32]. Overall, these findings provide a cue that estrogen may promote the protecting effect of HRP on glucose metabolism in female MSG rats and (or) interfere with the signal transduction of (P)RR. However, the exact mechanisms are unclear and need further studies.

Conclusions

HRP ameliorated β-cell function of the female but not in the male MSG rats although insulin sensitivity was ameliorated both in the male and female rats. HRP decreased the α-cell mass and fibrosis status in the islets of the female MSG rat but not in the male rats. Different levels of sex hormones may partly account for the special effect of HRP on different genders.

Conflict of interest

The authors declare that they have no conflicts of interest.

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