

## Original Article

# Butyrate alleviates metabolic impairments and protects pancreatic $\beta$ cell function in pregnant mice with obesity

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**Abstract:** The relative or absolute deficiency of pancreatic  $\beta$ -cell mass function underlies the pathogenesis of diabetes. It is necessary to alleviate the metabolic stress and reduce the demand for insulin to decrease the effects of mutations affecting  $\beta$ -cell expansion. Butyrate is a natural nutrient existed in food and can also be produced physiologically through the intestinal fermentation of fiber. Pregnancy and obesity model would be helpful for understanding how  $\beta$ -cell adapt to insulin resistance and how butyrate alleviate the metabolic impairment and protect pancreatic  $\beta$  cell function in pregnant mice with obesity. C57BL/6J female mice were divided into three groups and fed with high fat food (HF group, 40% energy from fat), high fat with sodium butyrate food (HSF group, 95% HF with 5% butyrate), or control food (CF group, 14% energy from fat), respectively. The feeding would last for 14 weeks before mating and throughout the gestation period. A subset of dams were sacrificed at gestational day (GD) 14.5 to evaluate the changes of metabolism and  $\beta$ -cell function, mass, proliferation and apoptosis, inflammatory reaction of islet from different diet. Pancreases were double immuno-labeled to assess the islet morphology, insulin expression, expression of proliferation gene PCNA and anti-apoptosis gene bcl-2. Moreover, we detected the expression of NF- $\kappa$ B, phosphorylated NF- $\kappa$ B (pNF- $\kappa$ B) to evaluate the islet inflammatory response with immunohistochemistry. Mice fed with HSF showed obviously changes including the decreased values of weight gain, glucose, insulin, triglyceride and total cholesterol level of blood compared with high fat diet group, and the reduced circulating maternal pro-inflammation factors at GD14.5. Mice fed with HF displayed  $\beta$ -cell hyperplasia with a greater  $\beta$ -cell size and  $\beta$ -cell area in pancreas. Furthermore, the higher ratio of apoptosis and inflammatory response were found in HF group compared with HSF and CF group, while the proliferation rates of  $\beta$ -cell increased in HF group, but not in HSF or CF. Butyrate shows an obvious function of anti-obesity, and can alleviate the metabolic stress, maintain the  $\beta$ -cell function and protect them from inflammatory response in pregnant obese mouse without obvious fetus toxicity.

**Keywords:** Sodium butyrate,  $\beta$ -cell, pancreatic islet, diabetes, GDM

## Introduction

Pancreatic  $\beta$ -cell are important for glucose sensing, insulin production and secretion, and diabetes is manifested as hyperglycemia due to a relative or absolute deficiency of the production of insulin by the  $\beta$ -cell [1, 2]. Many physiological and pathophysiological researches showed there were increasing systemic insulin demand in pregnancy and obesity to compensate for the relative insulin deficiency,  $\beta$ -cell dynamically expand their mass [3], at the same time, inflammatory response of  $\beta$ -cell is also an important factor to the compensation and fate

[4], but the most robust physiological stimuli of  $\beta$ -cell expansion is pregnancy [4, 5]. Because of these, pregnancy with obesity would serve as a good model for understanding how  $\beta$ -cell adapts to insulin resistance.

One way to effectively reduce metabolic load or increase the ability of  $\beta$ -cell to successfully adapt to metabolic load is a common theme for preventing and treating diabetes. As a dietary component (i.e. butter and milk product), butyrate is more interesting to us, sodium butyrate (CH<sub>3</sub>CH<sub>2</sub>CH<sub>2</sub>COONa) is a fatty acid derivative found in foods, and can also be produced physi-

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ologically in large amounts (from 40 to 100 mmol) from fermentation of dietary fiber in the large intestine, it can be used as substrates of energy production for intestinal epithelial cells [5, 6]. Butyrate also is a histone deacetylase inhibitor (HDACi), which regulates the gene transcription through modification of histone protein acetylation and is potential therapeutics for metabolic syndrome [6-12]. Most recent studies suggested that butyrate might mediate the effects of diet and gut microbiota on host appetite [7], adiposity [13], and immunity [14, 15], but there is no vivo research to investigate the influence of butyrate on pancreatic islet function. The precise mechanism of  $\beta$ -cell mass expansion including proliferation and apoptosis, and the islet inflammatory response in overload station is still unclear.

In our research, we divided mice into three groups fed with high fat food (HF group, 40% energy from fat), high fat food with sodium butyrate (HSF group, 95% HF with 5% sodium butyrate), or normal food (control group, 14% energy from fat), respectively, during the pregnancy to explore what would change in each groups, and to explore the effects of sodium butyrate on metabolism and  $\beta$ -cell biological behaviors such as proliferation and apoptosis, and inflammatory response compared among 3 groups.

### Materials and methods

#### *Animals and pregnant models with obesity*

Female C57BL/6J (4 weeks old) mice were purchased from the Shanghai Slac Laboratory Animal Co., Ltd. All mice were housed in the animal facility with a 12-h light/dark cycle, 40%-60% humidity and constant temperature (22-24°C), with free access to water and diet. After 1 week quarantine, these mice were randomly divided into three groups fed with three different diets. HF group (n = 20) were fed with research diets [16, 17] (40% fat, 20% protein, 40% carbohydrate,) HSF group (n = 20) were fed with the above high-fat diet and sodium butyrate (303410; Sigma) at 5% wt/wt [10-12]; Control group (n = 20) (CF, 14% fat, 26% protein, 60% carbohydrate) [18] were fed with normal food. The above foods were all purchased from Shanghai Slac Laboratory Animal Co., Ltd. Sodium butyrate was blended into the HSF diet before formed, then sodium butyrate containing diet was pelleted and stored in a -20°C

freezer until usage. For the supplemented diet, mice could receive sodium butyrate at 5 g/kg/day at the normal daily rate of calorie intake [10]. After 14 weeks dietary intervention, breeding was conducted overnight in a 1:2 ratio; mating was confirmed by presence of a vaginal mucous plug the following morning, which represented gestation day GD 0.5 until noon after successful mating. Once the females were pregnant, the males were removed.

All procedures were performed in accordance with national institutes of health guidelines for the care and use of animals and were approved by the institutional animal care and use committee at animal research center of Shanghai 6th People's Hospital.

#### *Measurements of body weight, blood glucose and insulin levels*

Body weight was measured once a week and blood glucose was determined by glucometer (ACCU-CHEK Performa) at indicated time points from cutting tails after overnight fasted 12 h every 2 weeks. Blood samples for insulin were collected by retroorbital sinus puncture without anesthetized after overnight fasted 12 h after 14 weeks dietary intervention. Following immediate centrifugation at 4000 rpm at 4°C, plasma was separated and stored at -70°C until insulin analysis.

#### *Intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT)*

After 14 weeks dietary intervention and before the mating, conventional glucose and insulin tolerance tests were performed on six mice of each group fasted for 12 h and 4 h respectively. Blood sample collected from the tail was detected by the glucometer at baseline (as 0 min), 30, 60, 120 and 180 min after glucose (2 g/kg) loading in intraperitoneal injection [19]; other six mice of each group were fasted for 4 h, then glucose level was measured as the method in IPGTT at the 30, 60, 120 and 180 min after human regular insulin (Novolin R, Novo-Nordisk) intraperitoneal injection at 0.5 units/kg [20].

#### *Collection of blood and tissue samples*

Dams were sacrificed at GD14.5 since the peak of  $\beta$ -cell proliferation during the pregnancy

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occurred on GD14.5 in mice [5]. Six mice of each group gave birth to explore whether the dose of sodium butyrate had toxicity to the infants. After fasting 4 hours, maternal blood was collected by cardiac puncture, allowed to clot, and spun at 4000 rpm to collect the serum. Maternal serum was analyzed for triglyceride and total cholesterol by triglyceride and total cholesterol test kits (Beijing BHKT Clinical Reagent Co. Ltd) according to the manufacturer's instructions. Insulin levels were analyzed by mouse Insulin Batch Kit (F1093, Shanghai Westang Bio-Tech Inc. Ltd), and the levels of TNF- $\alpha$  (12-2720-096, Dakewe Biotech Co., Ltd.), and IL-1 $\beta$  (12-2012-096, Dakewe Biotech Co., Ltd.) was detected by commercially available ELISA kits followed manufacturer's, respectively.

Entire pancreas were removed and weighed quickly, then one part of pancreas was fixed in 4% paraformaldehyde for 24 h, and laid flat for paraffin section, the other part of tissues snapped frozen in Optical Coherence Tomography (OCT) blocks, and stored in 70°C for frozen section.

### *Immunofluorescence staining*

Frozen section of 5  $\mu$ m were mounted on plus microscope slides, then air dry the sections for 30 minutes at room temperature, fixed them with the cold acetone for 15 min, washed with PBS 5 min for 3 times, then sections were blocked in 10% normal donkey serum, and 0.01% Triton X-100 diluted in 0.1 M PBS, pH7.4 for 30 min. Insulin expression as a marker of the  $\beta$ -cell was examined by immunohistochemistry, so incubation Insulin antibody (goat, diluted 1:20; Santa Cruz Biotechnology, SC-7839) with the other primary antibody respectively, PCNA antibody (rabbit, diluted 1:10, SC-7907, Santa Cruz Biotechnology), Bcl-2 antibody (rabbit, 10  $\mu$ g/ml, AF819, R & D Systems), and then performed at 4°C overnight in a wet chamber, added secondary antibody: anti-GQAT IgG Conjugated with FITC (DONKEY, 605-702-002, Rockland) and anti-rabbit IgG conjugated Texas Red (Donkey, 1:100, 611-7902, Rockland) at room temperature for 60min in a wet chamber protected from light, then incubated with DAPI for 1 min, washed with PBS thoroughly. Imaging was performed using microscopes of Olympus BX51.

### *Morphometric analysis*

In brief, three different parts of the pancreatic specimens from each mouse ( $n = 6$  for each group) were analyzed. To determine the insulin expression in situ, we randomly selected five islets in each section. Images were analyzed with Image-Pro Plus software by determining the average pixel value of staining per islet; background staining was subtracted by determining the average pixel value of staining per islet in a published procedure [21, 22]. For each group, about 100 islets from non-serial frozen sections (take one from every 6 serial sections) of pancreatic tissues from 4 animals was analyzed. When the  $\beta$ -cell size was measured,  $\beta$ -cell number per islet was also quantified by counting the number of cell nuclei in the insulin immune-reactive area. The  $\beta$ -cell size was calculated by dividing  $\beta$ -cell area per islet with  $\beta$ -cell number per islet. The ratio of the  $\beta$ -cell area/pancreatic area was digitally quantified as the area of insulin positive area per section as previously described [23]. The ratio of  $\beta$ -cell to pancreatic areas in each section was then multiplied by pancreatic weight to obtain absolute  $\beta$ -cell mass.

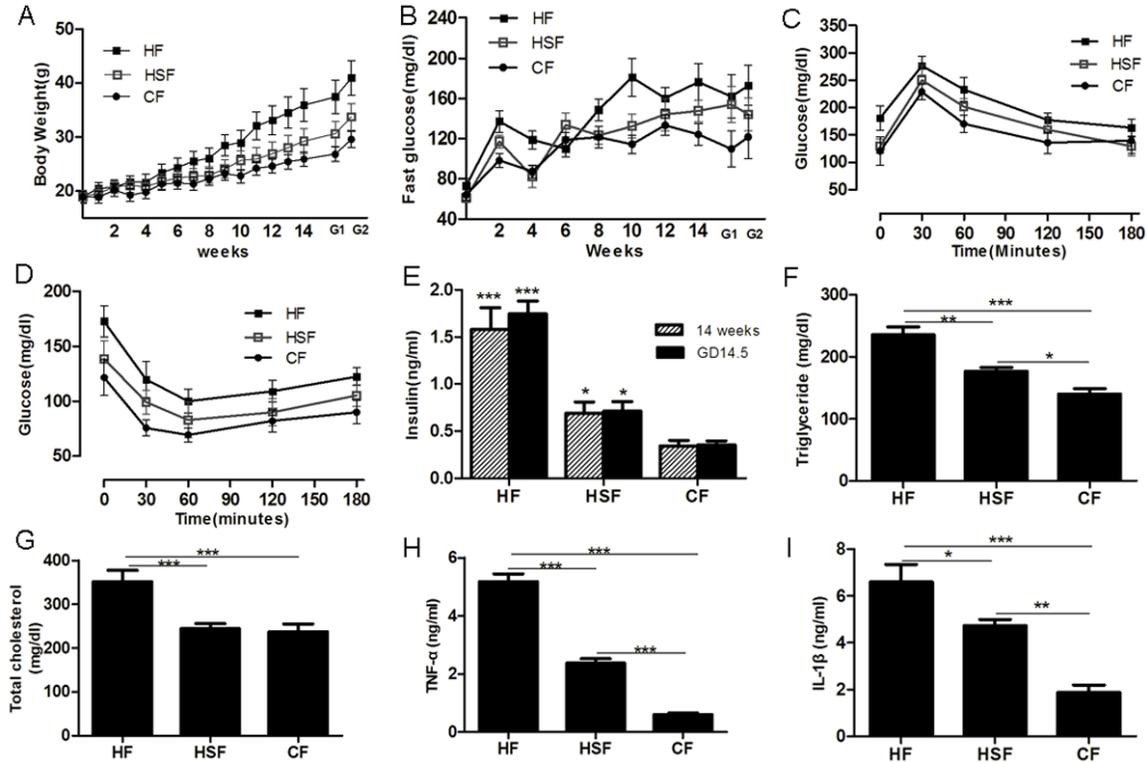
### *Proliferation and apoptosis of $\beta$ -cell*

In double-immunolabeling studies, islet cell replication and anti-apoptosis rates were calculated by detecting proliferating cell nuclear antigen (PCNA) and anti-apoptosis gene bcl-2, respectively [23, 24], and choose 30 islets randomly per mouse pancreas ( $n = 4$ /group) for analysis. Results were showed as the mean number of cells staining positively for PCNA or Bcl-2 in  $\beta$ -cells of islet.

### *Inflammatory response of islet*

The pancreatic sections were subsequently dehydrated in graded concentrations of ethanol, cleared in xylene, and subsequently embedded in paraffin wax at 55°C. The tissues were sectioned at 5  $\mu$ m thickness, mounted on plus microscope slides (158105W, CITOGLAS Company) and dried at 42°C overnight and stored at 4°C until processing. Every 6th section (an average of 6-7 sections per pancreas) was used. The paraffin-embedded sections were deparaffinized/rehydrated in xylene followed by ethanol and PBS serial rehydration. The incubation respectively with the

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**Figure 1.** Metabolic changes in different groups. Body weight (A) was weighed once a week; fast glucose (B) was measured once every two weeks, and G1, G2 in (A and B) means gestation at 1st week and 2nd weeks; IPGTT (C) and IPITT (D) were detected before mating, at the 14th week before mating and at GD 14.5, fast insulin was all measured (E), triglyceride (F) and total cholesterol (G) were detected at GD14.5. Here HF: high fat group, mice were fed with the high fat diet (40% fat, 20% protein, 40% carbohydrate, Research Diets); HSF: (5% sodium butyrate, 38% fat, 19% protein, 38% carbohydrate); CF: Control food group, mice were fed with control diet (14% fat, 26% protein, 60% carbohydrate, Research Diets). Error bars depicted the standard error of the mean. \* $P < 0.05$ , \*\* $P < 0.01$  or \*\*\* $P < 0.001$  compared to control group.

NF- $\kappa$ B antibody (diluted 1:100 SC-372 Santa Cruz Biotechnology), pNF- $\kappa$ B antibody (diluted 1:50, SC-372 Santa Cruz Biotechnology) was performed at 4°C overnight in a wet chamber and immunohistochemistry was carried using labeled streptavidin/peroxidase method and microwave antigen retrieval technique. The reagents (PV9000 and DAB) were obtained from ZSGB-BIO and staining was performed as protocol. All sections were analyzed for staining using light microscopy and digital analysis as detailed above. The assessment was performed by determining the number of positive cells per islet section by Image-Pro Plus software [23, 24]. Results were showed as the mean number of cells staining positively for pNF- $\kappa$ B or NF- $\kappa$ B of islet.

### Statistical analysis

Data were expressed as the mean  $\pm$  standard error of the mean (SEM). The data obtained in

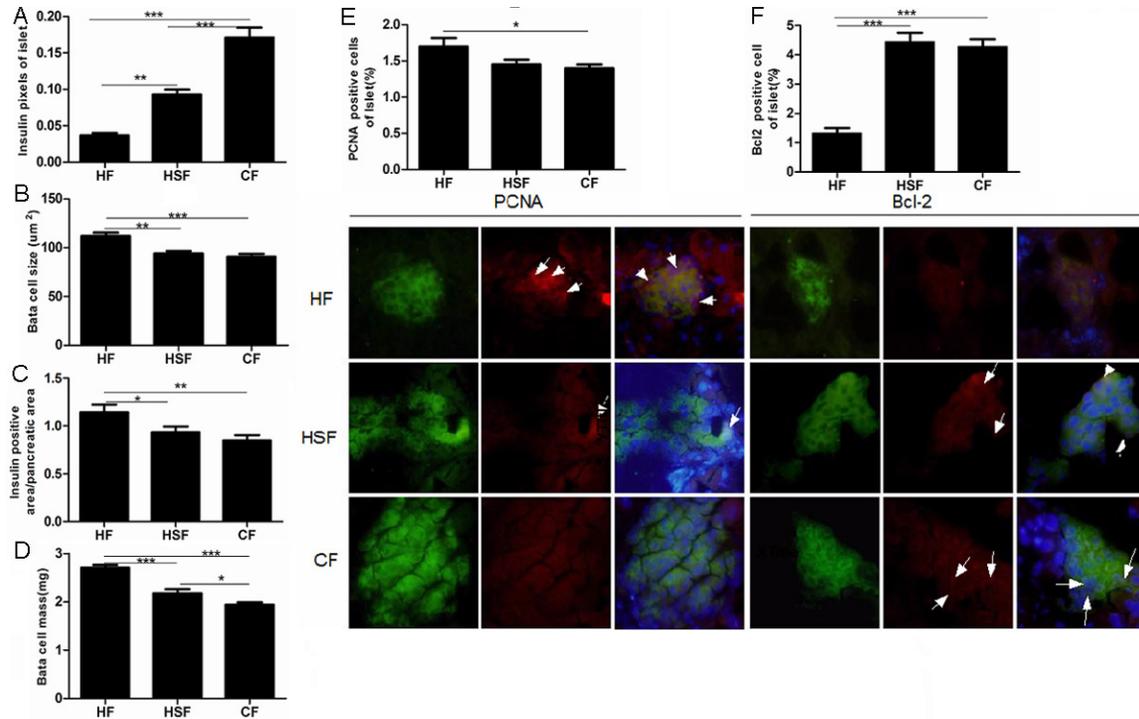
the present study were analyzed using an ANOVA.  $P < 0.05$  was considered to be statistically significant. Data were analyzed using the statistical package SPSS for Windows version 17.0.

### Results

#### *Sodium butyrate alleviated metabolic impairments of high fat diet*

The body weight of HF group ( $20.74 \pm 1.15$  g) became significant higher than the control group ( $19.20 \pm 1.12$  g) from the second week of feeding (**Figure 1A**) ( $P < 0.05$ ), and the body weight of HSF group ( $20.97 \pm 1.16$  g) was significant lower than that of HF group ( $21.67 \pm 1.14$  g) since the third week. There was no significant difference between the body weight of HSF group ( $24.09 \pm 1.68$  g) and the control group ( $23.11 \pm 1.22$  g) until the 9th week of diet intervention (**Figure 1A**). Before pregnancy,

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**Figure 2.** Morphometric analysis of  $\beta$ -cell in groups. A showed results of the average pixel value of staining per islet in three groups; B was average size of  $\beta$  cells in each group; C: The ratio of the  $\beta$ -cell area/pancreatic area was digitally quantified as the area of insulin positive area per section; D showed  $\beta$ -mass by pancreatic weight multiplied by the results of C, respectively; E and F showed PCNA and anti-apoptosis gene Bcl-2 levels of  $\beta$ -cell in each group. Representative images of F and G were showed at the bottom of Figure 2E and 2F, respectively. Original magnification:  $\times 400$ . Arrows in pictures was pointed to positive nucleus of  $\beta$  cells. Error bars showed the standard error of the mean. \* $P < 0.05$ , \*\* $P < 0.01$  or \*\*\* $P < 0.001$  compared to the CF control.

the body weight of HF group increased 19 g, the HSF group gained 10.3 g and the control group increased 7.4 g during 14 weeks (**Figure 1A**). At GD14.5, the maternal body weight of HF group increased 5 g, which was significant higher than the HSF group (4.5 g,  $P < 0.05$ ) and the control group (3.6 g,  $P < 0.001$ ) (**Figure 1A**). These results showed that the butyrate supplementation in the diet could prevent the significant weight gain caused by the high fat diet.

The fasting glucose of HF-fed dams was significantly increased after 10 weeks until GD14.5 (**Figure 1B**) ( $P < 0.001$ , compared to CF), fasting glucose levels in HSF group were elevated compared to CF group after diet intervention, while the fasting glucose levels of HSF-dams were still lower compared to HF and the fast glucose of CF group did not increase during this period (**Figure 1B**).

After 14 weeks dietary intervention and before the mating, IPGTT tests performed and demonstrated that the glucose clearance was significantly retarded in HF group (**Figure 1C**), and the

HF group exhibited much worse glucose clearance response to insulin stimulus at all time-points (30, 60, 120, and 180 min) (**Figure 1C** and **1D**) compared to HSF and CF. HSF had improved insulin resistance somewhat (**Figure 1C** and **1D**), but was still impaired worse than CF.

The fasting insulin test showed there was significant insulin resistance with insulin of ( $1.580 \pm 0.23$ ) ng/ml at 14 weeks ( $P < 0.001$  compared CF and HSF) (**Figure 1E**) in HF group, and in HSF group was ( $0.690 \pm 0.120$ ) ng/ml ( $P < 0.05$  compared CF), and Control group ( $0.343 \pm 0.06$  ng/ml). At GD14.5, the difference still existed. The levels of fasting insulin in 3 groups all showed a little increase compared with the levels of pre-pregnancy, which may be due to insulin resistance caused by pregnancy-associated hormone, but the statistical difference between groups were not changed (**Figure 1E**).

The triglyceride level of HSF group ( $176.6 \pm 13.92$ ) mg/dl was significantly lower than the level of HF group ( $235.21 \pm 29.08$ ,  $P < 0.001$ )

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although it was still higher than control group ( $140.45 \pm 18.02$ ,  $P < 0.05$ ). The total cholesterol level of HSF group ( $244.42 \pm 25.17$ ) mg/dl was significantly lower than the level of HF group ( $351.08 \pm 59.07$ ,  $P < 0.001$ ), and a little higher than control group ( $236.8 \pm 41.8$ ,  $P > 0.05$ ). Hence, our results showed sodium butyrate can clear away blood fat as stated in other studies [10, 11],

In our study, HF group showed a similar result with other studies that obesity increased inflammatory cytokines in blood [25], and sodium butyrate not only decreased the metabolic load in HSF group, but also anti-inflammatory cytokines in peripheral blood. In HF group, the levels of IL-1 $\beta$  ( $6.58 \pm 1.70$ ) and TNF- $\alpha$  ( $5.18 \pm 0.58$ ) significantly increased than the values of IL-1 $\beta$  ( $1.872 \pm 0.71$ ,  $P < 0.001$ ) and TNF- $\alpha$  ( $0.60 \pm 0.14$ ,  $P < 0.001$ ) in control group. However, the IL-1 $\beta$  ( $4.73 \pm 0.59$ ,  $P < 0.001$ ) and TNF- $\alpha$  ( $2.36 \pm 0.34$ ,  $P < 0.001$ ) level in HSF group were significant decreased compared with HF group although these still were significant higher than those in control group (IL-1 $\beta$   $P < 0.01$ ; TNF- $\alpha$   $P < 0.001$  respectively).

### *Sodium butyrate protected islet function*

Morphometric comparison of the pancreases in 3 groups was shown in **Figure 2**. The expression of insulin was measured by calculating the pixel value of staining in islet. Insulin staining was strong and intense in islets of CF group with average pixel value of insulin as ( $0.17 \pm 0.03$ ). HF diet markedly decreased the insulin content with a decreased pixel value of insulin staining of ( $0.037 \pm 0.007$ ). In the HSF group, the average value ( $0.09 \pm 0.01$ ) was 3 times of the HF group ( $P < 0.001$ ) (**Figure 2A**). The increased blood glucose level in HF group may be a result of poorly stored insulin in  $\beta$ -cell.

Increased size of  $\beta$ -cell mass was probably due to cell hypertrophy or hyperplasia [26]. We measured the area of single  $\beta$ -cell and the results showed the average area of each cell was increased in HF mice ( $112.1 \pm 7.18$   $\mu\text{m}^2$ ), compared to HSF group ( $94.5 \pm 4.21$   $\mu\text{m}^2$ ,  $P < 0.01$ ) and control group ( $90.6 \pm 6.73$   $\mu\text{m}^2$ ,  $P < 0.001$ ) as showed in **Figure 2B**. The size of  $\beta$ -cell in HSF group was a little increased compared to CF, but there was no statistically difference between two groups ( $P > 0.05$ ).

We also measured the ratio of the  $\beta$ -cell area/pancreatic area and  $\beta$ -cell area/exocrine area,

which was ( $1.142 \pm 0.18$ )% in HF mice, ( $0.93 \pm 0.15$ )% in HSF, and ( $0.85 \pm 0.13$ )% in CF (**Figure 2C**), respectively. Then, the ratio of  $\beta$ -cells was multiplied by pancreatic weight to obtain absolute  $\beta$ -cell mass. After multiplying by pancreatic weight, the  $\beta$ -cell mass was ( $2.67 \pm 0.17$ ) mg in HF group, ( $2.18 \pm 0.19$ ) mg in HSF and ( $1.94 \pm 0.11$ ) mg in CF (**Figure 2D**), respectively. The  $\beta$ -cell mass of HF mice was significantly different from HSF and CF ( $P < 0.001$ ), and there only small difference between HSF and CF ( $P < 0.05$ ).

Moreover, significant increase in the mass of  $\beta$ -cell for HF group was observed on GD14.5, which was associated with a reduced insulin expression in  $\beta$ -cell. On the other hand, when the  $\beta$ -cell mass coupled with their body weight, HF was very limited in the result of  $\beta$ -cell mass/mouse body weight. Compared to HF mice, the improved glucose level of HSF group was caused by increased  $\beta$ -cell insulin express, but not by enhancing hyperplasia or hyperplasia.

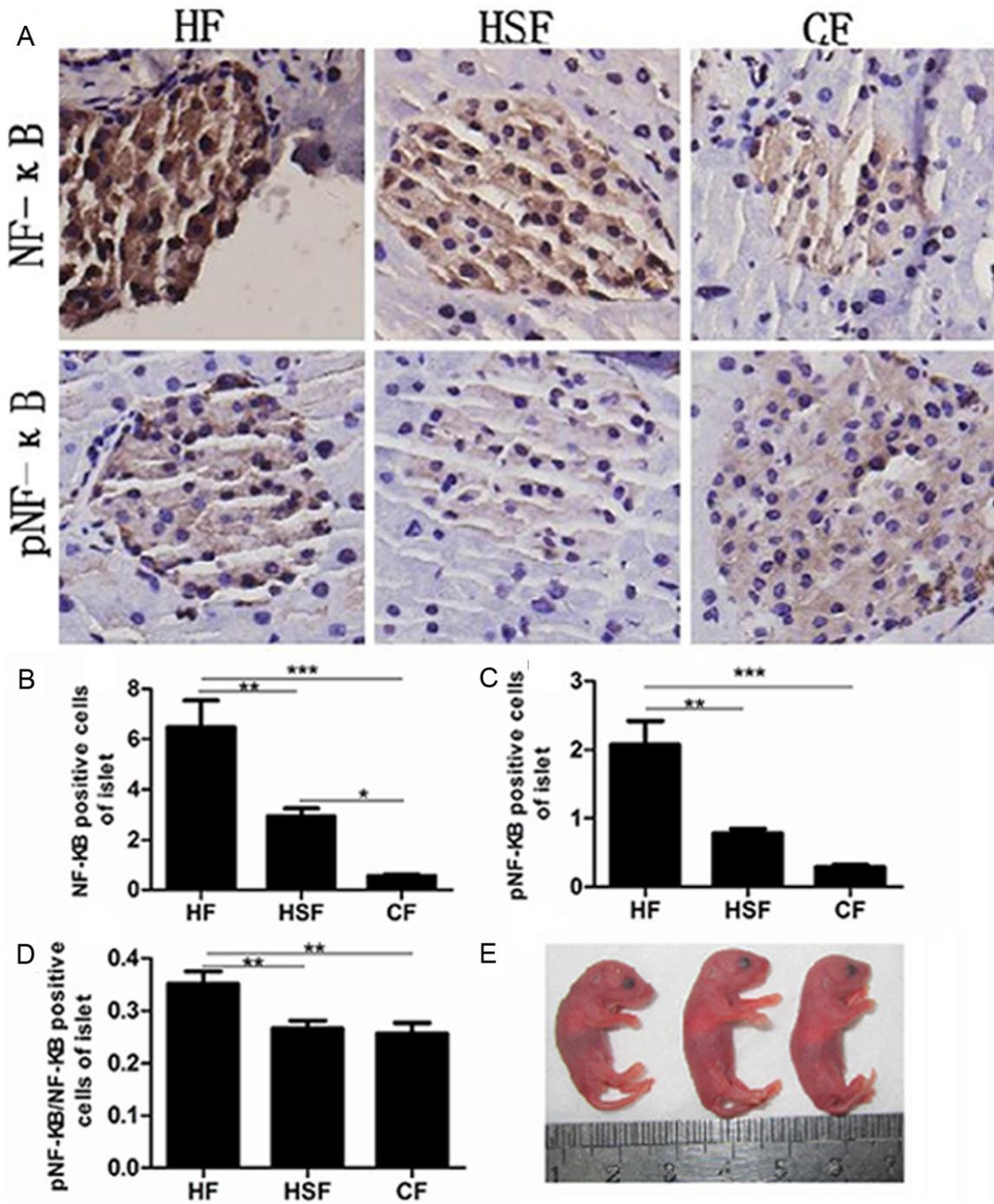
### *Butyrate stimulates proliferation and inhibits apoptosis of $\beta$ -cell*

Apoptosis probably is the main form of  $\beta$ -cell death in the disease [27, 28]. During time of prolonged metabolic demand for insulin, the endocrine pancreas can respond by increasing  $\beta$ -cell mass, both by increasing cell size and by changing the balance between cell proliferation and apoptosis. PCNA and Bcl-2 were respectively used as the markers of proliferation and anti-apoptosis functions of  $\beta$ -cell within islet boundaries.

The percentage of PCNA positive  $\beta$ -cells was very low (below 2%) in CF and HSF groups, with no difference between them. However, the percentage of PCNA positive  $\beta$ -cells was higher in HF ( $P < 0.05$ ) compared with other two groups (**Figure 2E**). On the other hand, the number of Bcl-2 positive  $\beta$ -cells was obviously lower in the HF group ( $1.26 \pm 0.49$ ) at GD14.5 compared to HSF ( $4.32 \pm 0.81$ ,  $P < 0.001$ ) and CF ( $4.46 \pm 0.49$ ,  $P < 0.001$ ) (**Figure 2F**).

### *Sodium butyrate reduces inflammatory response of islet cells*

The microenvironment in islet is very important, where the elevated cytokines can impair  $\beta$ -cell function and impact its survival. Subclinical inflammation is a recently discovered phenomenon in type 2 diabetes [4, 29], and in type 1



**Figure 3.** Inflammatory response in islets of three groups. A-D: The expression of NF- $\kappa$ B and phosphorylated NF- $\kappa$ B (pNF- $\kappa$ B) in islets from groups were analyzed by immunofluorescence staining. Original magnification:  $\times 400$ ; and the mean number of cells staining positively was showed in A and B; the results of positive numbers of NF- $\kappa$ B/pNF- $\kappa$ B were analyzed in C; E showed neonate Of HSF was normal without any visible deformity to the naked eye. Error bars depicted the standard error of the mean. \* $P < 0.05$  or \*\*\* $P < 0.001$  compared to the control group.

diabetes, invading pro-inflammatory cytokines from immune cells are integrant [30]. The activation of NF- $\kappa$ B is an important and classical marker of inflammatory response [31].

The number of NF- $\kappa$ B positive cells in islet of HF group ( $6.48 \pm 2.39\%$ ) was significantly higher than those of HSF group ( $2.92 \pm 0.73\%$ ) and control group ( $0.56 \pm 0.15\%$ ),  $p < 0.001$

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(**Figure 3A**), and the inflammatory response of islet in HSF group was greater than that in CF ( $P < 0.05$ ), as measured by pNF- $\kappa$ B positive cells in islet. The inflammatory response of HF group ( $2.07 \pm 0.78$ ) was increased compared to HSF ( $0.78 \pm 0.15$ ,  $P < 0.005$ ) and CF ( $0.286 \pm 0.08\%$ ,  $P < 0.001$ ) (**Figure 3B**). There was no statistically significant difference between control group and HSF group (**Figure 3B**). HF had a strong inflammatory response and dieting with sodium butyrate could suppress this response and the intensity of each cell nuclei also demonstrated these results. The ratio of pNF- $\kappa$ B to NF- $\kappa$ B positive cells in HF group ( $0.35 \pm 0.052$ ) was higher than those values in HSF ( $0.27 \pm 0.03$ ,  $P < 0.001$ ) and control group ( $0.2666 \pm 0.04$ ,  $P < 0.001$ ) (**Figure 3C**).

*No obviously toxic effect on mothers and infants*

The infants born from HSF group were not different compared with those of control group on the pregnant duration and neonates' weight. Moreover, there was no visible deformity found (**Figure 3D**).

### Discussion

We did not find obvious toxicity of butyrate supplementation at 5% wt/wt in high fat to the fetus and mothers in mice, besides that important and necessary precondition. An important observation was that butyrate could prevent development of dietary obesity by improving glucose and lipids metabolism, as well as it could reduce circulatory inflammatory factors such as IL-1 $\beta$  and TNF- $\alpha$  level from high fat influence. Another important finding was butyrate had an important role of maintaining the  $\beta$ -cell function and preventing  $\beta$ -cell from inflammation attack. So in our study, butyrate and its derivatives might have the possibility in the prevention and treatment of metabolic syndrome in humans even in pregnant women.

Our study showed butyrate could prevent obesity from high fat diet. One mechanism was related to promotion of energy expenditure and induction of mitochondrial function in muscle and liver. The stimulation of peroxisome proliferator-activated receptor (PPAR) co-activator (PGC-1 $\alpha$ ) activity has been suggested as the molecular mechanism of butyrate, and activation of AMPK and inhibition of histone deacetylases

may contribute to the PGC-1 $\alpha$  regulation [10]. In liver, Li et al had suggested that FGF21 was induced by butyrate and involved in the stimulation of fatty acid oxidation, and butyrate enhances FGF21 transcription through inhibition of HDAC3 [11]. Another mechanism of butyrate about anti-obesity may due to regulating leptin secretion from the adipose tissue [12]. On the other hand, recent evidence shows that the global effect of butyrate is to downregulate the expression of nine key genes involved in intestinal cholesterol biosynthesis [32].

The increase in energy metabolism suggests that butyrate may protect mice from weight gain and insulin resistance induced by high-fat diet. Systemic insulin sensitivity was analyzed by fasting glucose, fasting insulin, and IPGTT and IPTT. The fasting glucose was increased significantly on high-fat diet. But in HSF group, the increase was not obviously before and during the pregnancy period. IPGTT and IPTT tests of HSF group exhibited much better response to insulin and glucose at all-time points. Besides the molecular mechanism of insulin sensitization [10, 33], we also found that butyrate played an important role in the remodeling the process by increasing insulin expression and storage in  $\beta$ -cells.

In our study,  $\beta$ -cell mass increased in HF group, but the ratio of  $\beta$ -cells mass/body weight in HF group was still lower than HSF group. And many studies showed butyrate could increase  $\beta$ -cell mass by stimulation of  $\beta$ -cell neogenesis, growth and proliferation, and by inducing the GLP-1 in enteroendocrine cells of the proximal small intestine, GLP-1 also affects  $\beta$ -cell mass by limiting apoptosis, a recent study using freshly isolated human islets showed there was reduction in the number of apoptotic  $\beta$ -cells during in vitro treatment with GLP-1 compared with the control [34, 35]. The above-mentioned results showed  $\beta$ -cell in HSF group might have a better compensation ability on neogenesis, growth and proliferation when the metabolic environment needed.

It has been suggested mechanisms of extra-nutrient and cytokine-induced  $\beta$ -cell death in the progression of type 2 and type 1 diabetes pathology [36-38]. Subclinical inflammation is a recently discovered phenomenon in type 2 diabetes. In the insulinitis lesion of type 1 diabetes, invading immune cells produce cytokines,

such as IL-1 $\beta$ , TNF- $\alpha$ , these cytokines induce  $\beta$ -cell apoptosis via the activation of gene networks under the control of the transcription factors NF- $\kappa$ B. NF- $\kappa$ B activation leads to production of nitric oxide (NO) and chemokines and depletion of endoplasmic reticulum (ER) calcium. The execution of  $\beta$ -cell death occurs through activation of mitogen-activated protein kinases, via triggering of ER stress and by the release of mitochondrial death signals. Thus, cytokines and extra-nutrients trigger  $\beta$ -cell death by fundamentally different mechanisms, namely an NF- $\kappa$ B dependent mechanism that culminates in caspase-3 activation for cytokines [36]. Our study also demonstrated that the NF- $\kappa$ B signaling was downregulated by butyrate in mouse model. Furthermore, several studies in extro with colonocyte, adenocarcinoma cells and murine macrophages had shown that butyrate downregulated NF- $\kappa$ B signaling induced by cytokines or LPS.

However, it is unlikely that glucotoxicity acts alone, the negative contribution of saturated fatty acids [38], circulating and locally produced cytokines will further burn out the  $\beta$ -cell in HF group. These factors will induce apoptosis and/or necrosis, which the presence of pro-inflammatory cytokines may activate specific immunological phenomena and ultimately result in autoimmunity Type 1 and Type 2 diabetes characterized by progressive  $\beta$ -cell failure. At the same time, beneficial effects of butyrate including decreasing deleterious effects of hyperglycemia, blood fat and inflammatory cytokines, are also known to improve  $\beta$ -cell functions.

Many physiological and pathophysiological researches stated that several factors (such as pregnancy, obesity, aging, genetic insulin resistance, and acute illness) increase systemic demand to insulin. To compensate for the relative deficiency of insulin, pancreatic  $\beta$ -cell dynamically expand their mass, one of the most robust physiological stimuli of  $\beta$ -cell expansion is pregnancy, and studies in rats demonstrated that  $\beta$ -cell proliferation dramatically increased during the pregnancy, with a peak occurring about two thirds of the way through gestation [3]. However, until now, there is no research paying an attention on the islet changes in gestation after butyrate supplementation, so we combined obesity and gestation at the GD14.5 to explore the status of  $\beta$ -cell and the changes

of its' function. Meanwhile, it is also a strict method to test the safety of butyrate in mouse.

Since the prevalence of kinds of diabetes and the burgeoning global obesity epidemic, drugs which can be used to achieve the goals of anti-diabetes and anti-obesity are urgently needed; On the other hand, sodium butyrate might be developed urgently in the treatment since it can protect  $\beta$ -cell from potential harm and death caused by obesity and diabetes. Butyrate presents itself as a new therapeutic target, which is a natural nutrient without the known side effects and obvious toxicity and the data from literature and clinical experience of several research groups showed a wide spectrum of possibilities for potential therapeutic use of butyrate by oral administration without having serious adverse events [6-8, 39].

But treatment of tumor cells with sodium butyrate where accumulates acetylated histones in the nucleus, has shown to induce transcriptional activation of critical genes needed for tumor growth arrest, differentiation, or apoptosis, depending on each cellular system [40], and while therapies through improving insulin sensitivity have shown promising effects, but how to promote proliferation, reduce apoptosis and inflammatory reaction by molecular mechanism on functional  $\beta$  cells still needs to be further investigated.

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### Disclosure of conflict of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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## Butyrate protects pancreatic $\beta$ cell function in pregnant mice with obesity

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