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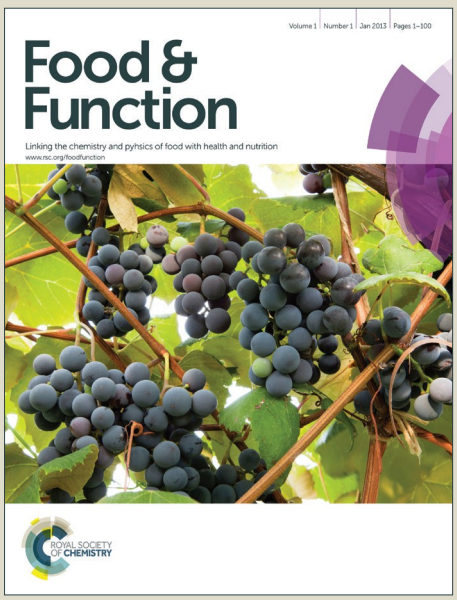

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ARTICLE

Improvement of glycemic control in streptozotocin-induced diabetic rats by Atlantic salmon skin gelatin hydrolysate as the dipeptidyl-peptidase IV inhibitor

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In our previous study, Atlantic salmon skin gelatin hydrolysed with flavourzyme possessed 42.5% dipeptidyl-peptidase (DPP)-IV inhibitory activity at the concentration of 5 mg/mL. The oral administration of hydrolysate (FSGH) at a single dose of 300 mg/d in streptozotocin (STZ)-induced diabetic rats for 5 weeks was evaluated for its antidiabetic effect. During the 5-week experiment, body weight was increased, food and water intake were reduced by FSGH in diabetic rats. The daily administration of FSGH for 5 weeks was effective to lower the blood glucose levels of diabetic rats during oral glucose tolerance test (OGTT). After 5-week treatment, plasma DPP-IV activity was inhibited; plasma activity glucagon-like peptide-1 (GLP-1), insulin and insulin-to-glucagon ratio were increased by FSGH in diabetic rats. The results indicate that FSGH has the function of inhibiting GLP-1 degradation by DPP-IV, resulting in the enhancement of insulin secretion and improvement of glycemic control in STZ-induced diabetic rats

Introduction

It is widely accepted that the dipeptidyl-peptidase (DPP)-IV inhibitors can be used as a novel treatment for type 2 diabetes [1, 2]. DPP-IV inhibitors can protect incretin, especially glucagon-like peptide-1 (GLP-1) which may decrease blood glucose and improve impaired glucose tolerance, against the degradation by DPP-IV [3,4]. There have been several DPP-IV inhibitors, e.g. sitagliptin, vildagliptin, saxagliptin and alogliptin, as the antidiabetic agents approved by United States and Europe governments. These DPP-IV inhibitors were examined their antidiabetic effects *in vivo* by animal and clinical experiments [5-8]. Although the risks of infection and hypoglycemia for long-term gliptin treatment are low, further researches are needed to clarify the possible link on other side effects, e.g. asthenia, cardiac and vascular events [9].

A DPP-IV inhibitor, 1-[2-[(5-cyanopyridin-2-yl) amino] ehtylamino] acetyl-2-cyano-(S)-pyrrolidine monohydrochloride salt (NVP-DPP728) was used to examine its effect on the improvement of glycemic control in obese Zucker rats [10]. The complete inhibition of plasma DPP-IV activity by orally administered NVP-DPP728 (10 μmole/kg) at -30 min of the experiment resulted in 20 and 30% reduction of basal plasma glucose and insulin concentrations, respectively, in obese rats. The same study also reported that during an oral glucose tolerance test, treatment of NVP-DPP728 completely inhibited plasma DPP-IV activity, highly preserved the endogenously secreted GLP-1, augmented the insulin response and all but restored the glucose excursion to normal in glucose intolerant, obese Zucker rats. Another study examined the effects of saxagliptin and sitagliptin on glycemic control in streptozotocin (STZ)-induced type 2 diabetic mice for 45 days [8]. The results showed that the both DPP-IV inhibitors attenuated the increase in water intake, and slightly but significantly improved glycemic control in STZ-treated mice. In addition, improved β-cell mass and morphology were also observed. There were still some animal and clinical studies that evaluated the antidiabetic efficacy of DPP-IV inhibitors, e.g. sitagliptin, vildagliptin and ASP8497 [5,6,11]. These studies have demonstrated that DPP-IV inhibitors could improve the glycemic control mainly attributed to the plasma DPP-IV inhibition, insulin secretion, active GLP-1 level elevation, and glucagon suppression.

However, the antidiabetic effects of DPP-IV inhibitory peptides were evaluated with limited animal and clinical studies, and these studies were almost investigated with the acute effects. Oral administration of trypsin-treated β-lactoglobulin (single dose of 300 mg/kg) or sitagliptin phosphate hydrate (3 mg/kg) 30 min prior to an oral glucose tolerance test (OGTT) significantly lowered blood glucose levels in C57BL/6 mice [12]. A peptide, LPQNIPPL, isolated from gouda-type cheese showed the *in vitro* IC_{50} value of 46 μM against DPP-IV and was used to examine its efficacy *in vivo* in rats [13]. The LPQNIPPL-administered (single dose of 300 mg/kg) group was observed to have significantly lower plasma glucose level but not to show differences in plasma insulin concentration as compared to control group during an OGTT. A previous study reported that administration of a zein hydrolysate (ZeinH; single dose of 500 mg), which induced GLP-1 secretion in mice, resulted in a 2.4-fold increase in plasma insulin concentration and a 23% decrease in plasma glucose level relative to the control (meat hydrolysate) during the ip glucose tolerance test (IPGTT) in rats [14]. In addition, the

administration of ZeinH increased active GLP-1 level and decreased plasma DPP-IV activity by 20-26% from basal levels, and therefore, that would lead to the enhancement of insulin secretion and the prevention of hyperglycemia in rats.

Our previous study has shown the Atlantic salmon skin gelatin hydrolysate possessed great *in vitro* DPP-IV inhibitory activity [15], and two peptides within the hydrolysate were identified as Gly-Pro-Ala-Glu (372.4 Da) and Gly-Pro-Gly-Ala (300.4 Da) with their IC_{50} values against DPP-IV of 49.6 and 41.9 μM. However, the *in vivo* antidiabetic effect of the hydrolysate has not been evaluated. Moreover, the DPP-IV inhibitory peptides were rarely studied by chronic *in vivo* animal studies. The aim of this study was to examine the antidiabetic effects of DPP-IV inhibitory peptides from Atlantic salmon skin gelatin on STZ-induced diabetic rats by a chronic study. This expected to give insight into the possible utilization of Atlantic salmon skin as a potential source in the treatment of type 2 diabetes.

Results and discussion

Body weight, food, water intake and nonfasting blood glucose

At the beginning of the experiment (week 0), body weight of the rats in normal group was higher than that in both DM groups (Fig. 1A). During the 5-week test period, weight gain was observed in all animals, and that of the rats in DM group was fewer than those in normal and DM+FSGH groups. It has been reported that animals receiving STZ subsequently showed an initial weight reduction, followed by slower weight gain compared with STZ- and non-STZ-treated animals [8].

Food intake, as expected, was enhanced in DM group rats, but decreased in DM+FSGH group rats to the levels similar to normal group (Fig. 1B). During the 5-week period, food intake of the rats in DM group increased rapidly since the third week and reached approximately 40% elevation at the end of the test. In addition, there were no significant differences (P>0.05) in food intake between the rats in normal and DM+FSGH group, while food intake increased less than 20% during the test period. Water intake significantly increased in DM group rats compared with DM+FSGH group rats in week 2 to 5, while during the test period, water intake showed insignificant differences (P>0.05) between normal and DM+FSGH group rats (Fig. 1C).

During the 5-week chronic daily dosing study, FSGH significantly decreased nonfasting blood glucose levels from 243 to 178 mg/dL in DM rats (Fig. 1D); while the nonfasting blood glucose levels in DM group rats gradually increased to 300 mg/dL. A previous study showed that ASP8497, a DPP-IV inhibitor, significantly decreased nonfasting blood glucose and HbA_{1c} of STZ-induced mildly diabetic mice after the 4-week chronic experiment [11]. The result indicated that FSGH has the hypoglycemic effect on DM rats.

Oral glucose tolerance test

At the end of the experiment, OGTT blood glucose responses of the rats in all groups were shown in Fig. 2A, and the area under curve (AUC) was shown in Fig. 2B. The blood glucose levels during OGTT test of DM rats were significantly higher than the rats in both normal and DM+FSGH groups (P<0.05). FSGH was potent to lower the blood glucose levels of diabetic rats to less than 200 mg/dL during OGTT. As the results of the plasma glucose AUC, FSGH significantly improved the blood glucose levels of diabetic rats after 5-week administration (P<0.05); meanwhile, the glucose AUC of the rats in normal and DM+FSGH groups was not significantly different (P>0.05). The result was consistent with the previous study, which reported that the cheese peptide LPQNIPPL showed hypoglycemic effect on the female SD rat model [13]. A previous study has reported that the oral and single administration of zein hydrolysates significantly lowered glucose levels of diabetic rats to around 50% of those of diabetic control rats [14]. Therefore, the administration of FSGH for 5 weeks is effective for glycemic control of diabetic rats.

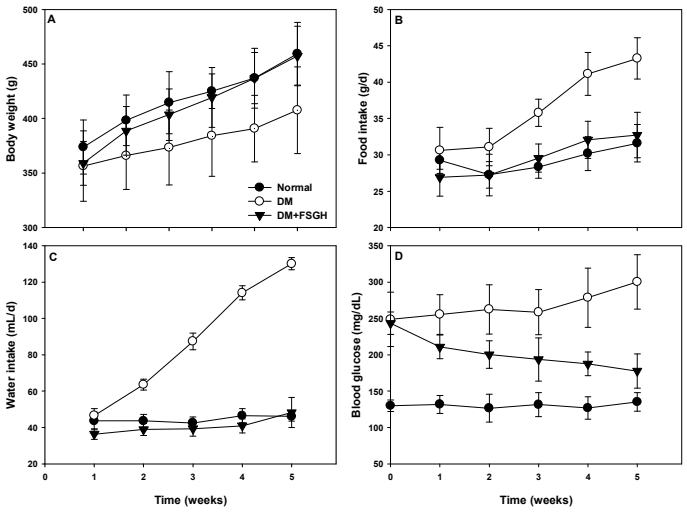

Fig. 1 Weekly average (A) body weight, (B) food intake, (C) water intake, and (D) nonfasting blood glucose of diabetic rats treated with FSGH during 5-week experiment. (n = 12/group)

Plasma DPP-IV activity

The effect of administration of FSGH after 5 weeks on the plasma DPP-IV activity in diabetic rats was shown in Fig. 3. The plasma DPP-IV activity of diabetic control rats was 115.5% and significantly higher than those of the rats in normal and DM+FSGH groups (P<0.05). The DPP-IV activity of diabetic rats administrated with FSGH was only 82.6%, which showed lower levels than normal rats (P<0.05). The result showed that FSGH could reduce the plasma DPP-IV activity in order to improve the glycemic control in diabetic rats. A previous study has indicated that a DPP-IV inhibitor, NVP-DPP728, orally administrated 10 min before the oral glucose challenge significantly and potently inhibited plasma DPP-IV activity throughout the OGTT in obese *fa/fa* rats [10]. For the long term (up to 5 weeks) of DPP-IV inhibitor, sitagliptin, administration in STZ-induced diabetic mouse, the plasma DPP-IV activity was greatly inhibited by approximately 50%, which started from the next day of administration of sitagliptin and lasted until 5 weeks [6].

Plasma total and active GLP-1 levels

After 5-week experiment, the plasma total GLP-1 level of normal rats was 21.34 pM, which was significantly higher than those (16.22 and 17.24 pM) of the rats in DM and DM+FSGH groups (P<0.05) (Fig. 4A). Meanwhile, the total GLP-1 levels

of both DM and DM+FSGH group rats were insignificantly different (P>0.05). The plasma active GLP-1 level of DM+FSGH group rats was significantly higher than those of the diabetic control rats and normal rats (P<0.05) (Fig. 4B). Moreover, the GLP-1 level of the diabetic control rats was similar to that of normal rats without significant differences (P>0.05). Some studies indicated that the daily administration of DPP-IV inhibitors (sitagliptin, ASP8497 and vildagliptin) for up to 1 month resulted in significant increases in plasma levels of active GLP-1 in STZ-induced diabetic mice and resulted in the improvement of glycemic control [6,11]. Previous studies have shown that a zein hydrolysate can increase GLP-1 secretion in both GLUTag cells and normal SD rats models [14,16]. However, in the present study, FSGH can increase active GLP-1 levels but not induce GLP-1 secretion in diabetic rats. In another study, the DPP-IV inhibitor valine pyrrolidide had no significant effect on GLP-1 secretion of the STZ-induced diabetic minipigs, while that resulted in an increase in circulating levels of active GLP-1 [17]. In the similar diabetic animal models induced by STZ presented in this and previous studies, DPP-IV inhibitors showed the protective effects on intact, active GLP-1 from the degradation by DPP-IV [6,11,17], but their effects on the increase of GLP-1 secretion were not observed.

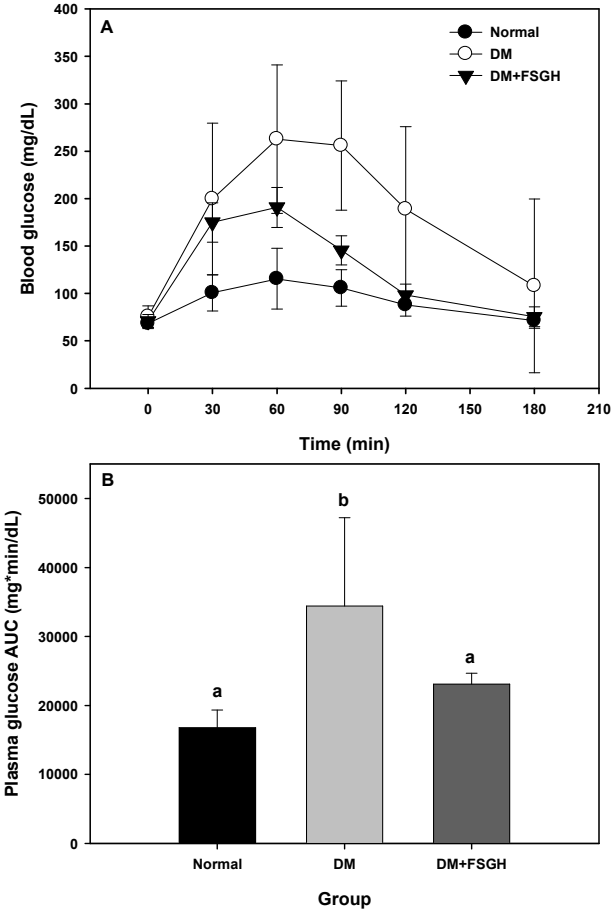

Fig. 2 (A) The blood glucose levels and (B) $AUC_{0\text{-}180}$ values during oral glucose tolerance test of diabetic rats treated with FSGH after 5-week experiment. Bars with different letters are significantly different at P<0.05. (n = 12/group).

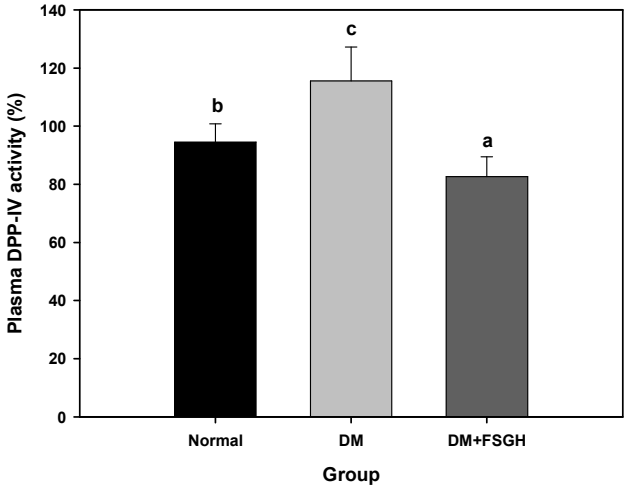

Fig. 3 Effect of daily administration of FSGH on plasma DPP-IV activity of STZ-induced diabetic rats. Bars with different letters are significantly different at P<0.05. (n = 12/group)

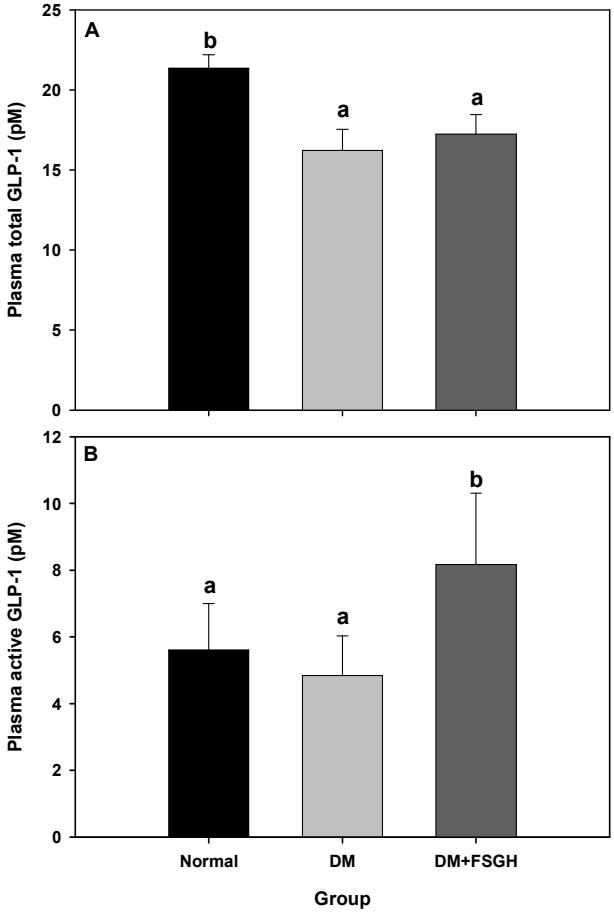

Fig. 4 Effect of daily administration of FSGH on plasma (A) total and (B) active GLP-1 levels of STZ-induced diabetic rats. Bars with different letters are significantly different at P<0.05. (n = 12/group)

Plasma insulin and glucagon levels

The effect of the daily administration of FSGH for 5 weeks on the plasma insulin level in diabetic rats was shown in Fig. 5. The insulin level of normal rats was about 2.25 μg/L and higher than those of diabetic rats in the other two groups (P<0.05). The diabetic rats administrated with FSGH showed their plasma insulin level over 1.50 μg/L and significantly higher (P<0.05) than the diabetic control rats (Fig. 5). The results showed that the long-term administration of DPP-IV inhibitory peptides, FSGH, was potent to improve the insulin secretion in diabetic rats. Streptozotocin may induce the loss of pancreatic β-cells and their functional defects and therefore result in the reduction of postprandial insulin secretion and lead to hyperglycemia [11]. The daily administration of DPP-IV inhibitors, ASP8497 and vildagliptin (3 mg/kg) in STZ-NA-induced diabetic mice for 4 weeks, the glucose-dependent insulin secretion and accompanying blood glucose-lowering effects were identified to lead to economization of insulin and increases in pancreatic insulin content [11]. Additionally, it was shown that long-term (4 to 7-week) treatment of STZ-induced diabetic rats with DPP-IV inhibitors sitagliptin and isoleucine thiazolodide resulted in reduced blood glucose levels and increases in pancreatic insulin content and the number of small islets [6, 18].

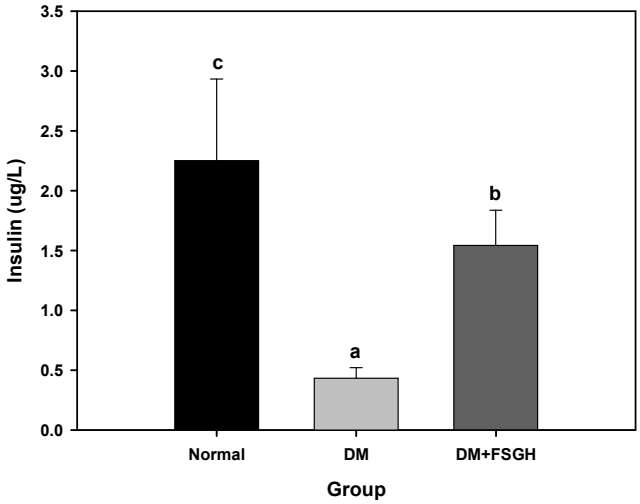

Fig. 5 Effect of daily administration of FSGH on plasma insulin levels of STZ-induced diabetic rats. Bars with different letters are significantly different at P<0.05. (n = 12/group)

The diabetic control rats showed the lowest plasma insulin-to-glucagon ratio in the present study; meanwhile, the normal control rats had the highest value (P<0.05) (Fig. 6). The result showed that the daily administration of FSGH may improve the control of blood glucose levels of STZ-induced diabetic rats. It has been reported that the diabetic patients had relatively high glucagon level that resulted in glycogenlysis, gluconeogenesis and then lead to hyperglycemia [19]. The DPP-IV inhibitor vildagliptin at 100 mg once or twice daily for 4 weeks reduced glucagon response to a mixed meal, and this reduction correlated with the improvement in glucose tolerance [7]; moreover, a study also showed that the reduction in glucagon levels by vildagliptin corresponded to inhibition of hepatic glucose production [5]. The administration of a single dose (4 g/kg) of sitagliptin in STZ-induced mice for 4 weeks retained the relatively high plasma insulin-to-glucagon ratio as compared to the diabetic control rats, reflecting the control of blood glucose levels [6].

The hyperglycemic animal model used in this study is the STZ-NA-induced adult diabetic SD rats. As NA is an antioxidant which exerts protective effect on the cytotoxic action of STZ by scavenging free radicals and causes only minor damage to pancreatic β-cell mass producing hyperglycemia. The chemically induced diabetic animal model shows moderate and stable non-fasting hyperglycemia with partial β-cells necrosis and insulin deficiency. Therefore, this model is found to be an advantageous tool for investigation of insulinotropic agents in the treatment of hyperglycemia [21]. However, this animal model shows some shortcomings. First, the animal model has hyperglycemia which develops primarily by direct cytotoxic action on the β-cells and insulin deficiency rather than consequence of insulin resistance. Second, this model is disputed to be type 1 or 2 diabetes [21,22]. In this study, therefore, we have difficult to determine the effects of the DPP-IV inhibitor on the insulin secretion resulted from the β-cell function when compared with the normal and diabetic groups.

From the above results, the diabetic rats showed slight reduction of GLP-1 secretion (Fig. 4A), greater DPP-IV activity (Fig. 3) to inactivate GLP-1, but the similar active GLP-1 levels (Fig. 4B) as compared to normal rats. Therefore, we demonstrate that the hyperglycemia of the diabetic rats to be contributed by the severe insulin deficiency (Fig. 5 and Fig. 6). The oral administration of FSGH, a DPP-IV inhibitor determined in the previous study [15], significantly decreased the DPP-IV activity and increased the circulating levels of active GLP-1 of the diabetic rats, although the GLP-1 secretion was not enhanced. The high level of active GLP-1 potently stimulated insulin secretion and then resulted in the improvement of glycemic control. Our previous study has shown that the non-diabetic rats administered by the porcine skin gelatin hydrolysate as a DPP-IV inhibitor did not show any unexpected change, especially hypoglycemia, during the 42-day experiment [23].

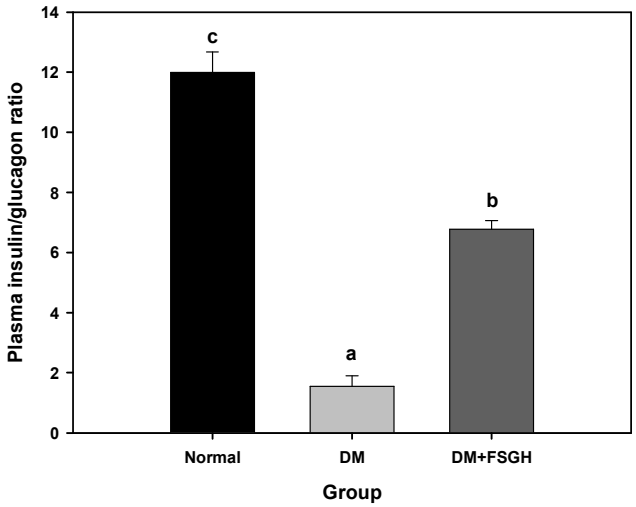

Fig. 6 Effect of daily administration of FSGH on plasma inslulin-to-glucagon ratio of STZ-induced diabetic rats. Bars with different letters are significantly different at P<0.05. (n = 12/group)

Experimental

Materials and reagents

Atlantic salmon (*Salmo salar*) fish skins, the processing byproducts recovered from fresh skin-off fillets, were supplied by Albion Fisheries Ltd. (Vancouver, BC, Canada). The fish skins were transferred on ice to our laboratory, vacuum packed and stored at -25℃ until use. The preparation of the fish skin gelatin hydrolysates was reported in our previous study [15]. Briefly, the extracted gelatin from Atlantic salmon skin was hydrolysed with flavourzyme with the enzyme/substrate ratio of 6% for 4 h, and the hydrolysates were lyophilised and stored at -20℃ until use. Streptozotocin (STZ) and nicotinamide (NA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals and reagents used were analytical grade and commercially available.

Animals

Male Sprague-Dawley rats (LASCO, Taipei, Taiwan), aged 8 weeks and weighing between 230-250 g were used. Diabetes was induced in overnight (>10 h) fasted rats by a single intraperitoneal injection of a citrate buffer (0.01 M, pH 4.5) solution of STZ at a dosage of 65 mg/kg body weight (BW), 15 min after the i.p. administration of 180 mg/kg BW of NA in normal saline. One week after the injection of STZ, animals were considered to be diabetic if they had plasma glucose levels over 200 mg/dL [20] during the oral glucose tolerance test (OGTT). All rats care and procedures were approved by the Institutional Animal Care and Use Committee of China Medical University.

Experimental group and treatment

Animals were divided into 3 groups of 12 rats each, and the rats in experimental groups were administrated samples by oral gavage. The experimental period was 5 weeks. Normal group: normal control rats administered drinking water daily; DM group: diabetic rats administered drinking water daily; DM+FSGH group: diabetic rats administered fish skin gelatin hydrolysates (FSGH; 300 mg/day).

Plasma glucose and oral glucose tolerance test

Nonfasting blood glucose levels were measured in tail blood using a blood glucose meter (TD-4207, Taidoc, New Taipei, Taiwan). The OGTT test was performed in overnight fasted rats from all groups, and the plasma glucose levels were determined at 0, 30, 60, 90, 120 and 180 min after glucose challenge (2 g/kg).

Biochemical determinations

On the morning after final administration, the animals were sacrificed by over dose of CO_2. Blood samples under nonfasting conditions were collected in chilled blood vases containing ethylenediaminetetraacetic acid (EDTA). Samples were centrifuged (3,000*g*, 15 min) and stored at -80℃. Plasma DPP-IV activity was measured using a DPPIV/CD26 assay kit (Enzo Inc., Farmingdale, NY, USA). Plasma total and active GLP-1 concentrations were measured using a glucagon like peptide-1 (total) RIA kit and a glucagon like peptide-1 (active) ELISA kit (Millipore Corp., Billerica, MA, USA), respectively. Plasma insulin concentration was measured using a Mercodia rat insulin kit (Mercodia Inc., Uppsala, Sweden). Plasma glucagon concentration was measured using a glucagon EIA kit (Yanaihara Inc., Shizuoka, Japan).

Statistical analysis

Each data point represents the mean of three samples was subjected to analysis of variance (ANOVA) followed by Duncan's test, and the significance level of $P<0.05$ was employed.

Conclusions

FSGH from Atlantic salmon skin gelatin had a superior antidiabetic effect in STZ-induced diabetic rats, including the improvement of glucose tolerance, inhibition of plasma DPP-IV activity, elevation of active GLP-1 levels, resulting in the enhancement of insulin secretion, the reduction of glucagon levels and finally improvement of glycemic control. This study indicates the Atlantic salmon skin gelatin has the potential to be a functional food for antihyperglycemia.

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Notes and references

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