Original paper

Fucoidan from Cucumaria frondosa Inhibits Pancreatic Islets Apoptosis Through Mitochondrial Signaling Pathway in Insulin Resistant Mice

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Received October 5, 2015; Accepted March 4, 2016

Fucoidan from sea cucumber is reported to exhibit anti-hyperglycaemic effect, but its influence on pancreatic islets is lacking. The effects of fucoidan from Cucumaria frondosa (Cf-FUC) on inhibiting pancreatic islets apoptosis in high-fat high-sucrose diet (HFSD)-induced insulin resistant mice were investigated in this study. The results showed that Cf-FUC significantly prevented HFSD-injured pancreatic islets, decreased blood glucose, insulin and TNF-α levels, and increased adiponectin level. Cf-FUC significantly reduced Bid, Bax, cytochrome c, caspase 9, and caspase 3 mRNA expression levels, and increased Bcl-2 and Bcl-xL mRNA expression levels. Cf-FUC also caused significant down-regulation of t-Bid, Bax, cytochrome c in cytoplasm, caspase 9, and cleaved-caspase 3 proteins, and up-regulation of Bcl-2 and Bcl-xL proteins. Furthermore, Cf-FUC enhanced the effects of RSG. This study demonstrates that Cf-FUC inhibits pancreatic islets apoptosis via inhibiting mitochondrial pathway, which may provide a dietary intervention hyperglycaemia-induced pancreatic islets apoptosis.

Keywords: Cucumaria frondosa, fucoidan, pancreatic islets, apoptosis, mitochondrial pathway, cytochrome c.

Introduction

Diabetes mellitus type 2 is becoming increasingly common in many countries (Shaw, 2009). It is characterized by hyperglycemia in the context of relative deficiency of insulin and insulin resistance (Alberti and Zimmet, 1998; Muoio and Newgard, 2008). The inability of pancreatic β-cell to provide the body with an insufficient insulin supply to compensate for insulin resistance is an early defect in the natural history of type 2 diabetes (Moon et al., 2013). The reduction of β-cell in the pancreas mass has been reported to be a pathological hallmark in the development of type 2 diabetes (Jurgens et al., 2011; Lee et al., 2011). The β-cell mass is reduced to a variable extent in patients with type 2 diabetes (Maedler, 2008). It is also reported that increased β-cell apoptosis can lead to the continuous loss of β-cells (Meier and Bonadonna, 2013). Few of the pharmacological therapies for type 2 diabetes are reported to prevent the progressive decline in β-cell function or inhibition of its apoptosis (Hou et al., 2013). Therefore, therapeutic agents that can halt or prevent pancreatic β-cell apoptosis failure

Abbreviations: ANOVA, one-way analysis of variance; Cf-FUC, Fucoidan from Cucumaria frondosa; ECL, super-enhanced chemiluminescence; HE, hematoxylin and eosin; HFSD, high-fat high-sucrose diet; HOMA-IR, homeostasis model assessment of insulin resistance index; LFSD, low-fat low-sucrose diet; M-MLV, moloney murine leukemia virus; PVDF, polyvinylidene fluoride; QUICKI, quantitative insulin sensitivity check index; RSG, rosiglitazone; RT-PCR, reverse transcriptase-polymerase chain reaction; SD, standard deviation.

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will likely have a major impact on disease progression.

Inflammation plays a critical role in the development of insulin resistance and type 2 diabetes (Kang et al., 2010; Lee, 2013). It has been reported that inflammatory mediators, including tumour necrosis factor-α (TNF-α), are involved in the pathogenesis of β-cell dysfunction of diabetes (Bouzakri et al., 2011; Cai et al., 2011; Marselli et al., 2013). Recently, a specific role has been proposed for TNF-α in impairing insulin secretion and inducing β-cell apoptosis in pancreatic cell lines (Zhang et al., 2011). Adiponectin is an insulin-sensitizing hormone which is negatively associated with insulin resistance (Blesso et al., 2013). Adiponectin plays a crucial role in down-regulating of hyperglycaemia and suppressing pancreatic islet apoptosis (Chai et al., 2011). A recent study indicates that adiponectin prevents pancreatic islet ischemia-reperfusion injury via inhibiting TNF-α production (Eizirik et al., 2009). Therefore, TNF-α and adiponectin are regarded as important factors in terms of β-cell apoptosis.

Sea cucumber is a popular marine food in China, and contains many kinds of bioactive compounds, such as sulphated polysaccharides, saponins, and lipids (Hu et al., 2013; Wu et al., 2013; Zhao et al., 2012; Zhong et al., 2007). Sulphated polysaccharides from sea cucumbers (namely fucoidan and fucosylated chondroitin sulphate) have attracted considerable attention in recent years. Current researches on bioactivities of fucoidan are mainly focused on anti-coagulation and anti-thrombosis (Chen et al., 2012), promotion of neural stem/progenitor cell proliferation (Zhang et al., 2010), alleviation of ethanol-induced gastric damage (Wang et al., 2012), inhibition of adipocyte differentiation (Kim et al., 2009) and osteoclastogenesis (Kariya et al., 2004). Our previous studies have shown that fucoidan could reduce blood glucose level and improves insulin resistance (Hu et al., 2014). However, its effect on pancreatic islets has not been understood. In the present study, we aimed at evaluating the effects of fucoidan isolated form Cucumaria frondosa (C.-FUC) on inhibition of pancreatic islets apoptosis in insulin resistant mice and finding the mechanisms involved in the apoptosis-related genes expression in islets.

Materials and Methods

Materials

Dried Cucumaria frondosa was purchased at a seafood market in Qingdao, China. It was identified by Professor Yulin Liao of the Chinese Academy of Sciences Institute of Oceanography (Qingdao, China). Insulin and TNF-α ELISA assays kits, TRizol reagent were Invitrogen products (Carlsbad, CA, USA). RNase free water, dNTPs, moloney murine leukemia virus reverse transcriptase (tumour), random primer, and PageRuler prestained protein ladder were from TaKaRa Bio Inc (Otsu, Shiga, Japan). Rabbit anti-rat 1-Bid, Bax, cytochrome c, Bel-2, Bel-xL, caspase 3, cleaved-caspase 3, caspase 9, β-actin polyclonal antibodies, and goat-anti rabbit antibody IgG-HRP were Cell Signaling products (Beverly, MA, USA). Glucose test kit was from Biosino Bio-Tec (Beijing, China). Western blot IP lysis buffer, BCA protein concentration kit, and super-enhanced chemiluminescence (ECL) detection kit were Applyugen Technologies Inc products (Beijing, China). The primers of Bid, Bax, Bak, Bel-2, Bel-xL, cytochrome c, caspase 9, caspase 3, and β-actin were synthesized by ShanGon Ltd. Co. (Shanghai, China).

C-FUC preparation C-FUC was prepared from the body wall of wild C. frondosa as previously reported (Yu et al., 2013). The obtained C-FUC was composed of fucose, galactosamine, galactose and glucosamine with a ratio of 1:0.1:0.3:0.17. Its sulphat content was 29.31%. The protein content in C-FUC fraction was 7.84%.

Animals and experimental design Four weeks old male C57BL/6J mice (18 – 20 g) were provided by Vital River Laboratory Animal Center (Beijing, China; Licensed ID: SCXK2009-0007). They were housed in a 12-h light/12-h dark cycle condition at 23 ± 1°C. The use of animals in this study was approved by the Ethical Committee of Experimental Animal Care at Ocean University of China. The insulin resistant model mice were established by fed a high-fat high-sucrose diet (HFSD, Research Diets, New Brunswick, NJ, USA; #D12331), which consisted 20% protein, 25% fat and 20% carbohydrates as previously described (Hu et al., 2013). The mice were randomized into 6 groups of 10 animals each: control, HFSD-fed (HFSD), 1 mg/kg/d RSG-treated (RSG), 80 mg/kg/d C-FUC-treated (C-FUC), 20 mg/kg/d C-FUC plus 1 mg RSG/kg/d RSG-treated (20 C-FUC + RSG), and 80 mg/kg/d C-FUC plus 1 mg RSG/kg/d RSG-treated (80 C-FUC + RSG) groups. Mice in the control group were fed with a low-fat low-sucrose diet (LFSD, #D12328) and the others were fed with HFSD. After 19 weeks of treatment, the 8-h fasting animals were sacrificed. The blood was collected to test fasting blood glucose and adiponik levels. The tail of pancreas was cut off carefully to observe the histological structure of islets of langerhans and to detect apoptosis-related genes expression.

Blood glucose and insulin levels assay Fasting blood glucose level was determined with a commercial kit (Biosino, Beijing, China). Serum insulin level was assessed with insulin ELISA assay kit (Invitrogen, Carlsbad, CA, USA). Insulin resistance was assessed by homeostasis model assessment of quantitative insulin sensitivity check index (QUICKI) and insulin resistance index (HOMA-IR) as follows (Bhuvanaswari and Anuradha, 2012):

\[ \text{QUICKI} = 1/\left(\text{lg(fasting blood glucose level)} + \text{lg(serum insulin level)}\right) \]

\[ \text{HOMA-IR} = \text{fasting blood glucose level} \times \text{serum insulin level}/22.5 \]

Serum adipokine determination Serum adipokine levels, including adiponectin, resistin, leptin, and TNF-α, were determined by ELISA assay kits (Invitrogen, Carlsbad, CA, USA) according to the instructions.

Microscopic structures of islet of Langerhans Pancreas tails were fixed in 10% formalin, paraffin embedded, sectioned, stained
with hematoxylin and eosin (HE). Microscopic structure of islet of langerhans was photographed using an optical microscope (BH-2, Olympus, Japan).

**RT-PCR analysis** The expression levels of apoptosis-related genes, namely Bid, Bax, Bcl-2, Bcl-xL, cytochrome c, caspase 9, and caspase 3 were examined by reverse transcriptase-polymerase chain reaction (RT-PCR). RNA extract and amplification were performed according the methods described in the literatures with modification (Chang et al., 2012; Sikdar S, 2013; Srzentić et al., 2015). Briefly, the total RNA from gastrocnemius or adipose tissue was extracted using TRIzol reagent, and 1 μg RNA was reversely transcribed to cDNA with M-MLV. PCR was carried out in 30 μL system containing 0.5 μg cDNA on a MJ Research thermocycler (TC-96/G/H(b)A; Hangzhou, China). The thermocycling was performed by denaturing at 94°C for 45 s, annealing at the temperature desirable to each primer pair for 45 s and extending at 72°C for 45 s for cycling number set for each primer pair followed by an extra extending at 72°C for 10 min. PCR products were separated in 1% agarose gel buffered with Tris-acetate-EDTA and visualized with ethidium bromide staining. The housekeeping gene β-actin was used as a control. The parameters for RT-PCR are listed in Supplementary Table 1. Bands were quantitated using the Image J program (Version 1.41o; NIH, Bethesda, MA, USA). The mRNA relative expression levels were expressed as the ratio of signal intensity for the target genes to that of β-actin.

**Isolation of cytosolic fraction of pancreas** Cytosolic fraction of pancreas, which was used to measure cytochrome c release, was prepared following the reported method with modification (Karatug and Bolkent, 2013). Briefly, the pancreas tissues were disrupted by Western and IP lysis buffer including 50 mM Tris (pH 7.4), 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na3VO4, and 0.5 μg/mL leupeptin in a glass homogenizer. The homogenate was centrifuged twice at 14,000×g for 30 min at 4°C. Then the supernatant was centrifuged at 100,000×g for 30 min at 4°C again. The yield cytosol supernatant was used for assaying cytochrome c protein expression by western blot (WB).

**WB analysis** To dissolve cellular protein, 0.1 g pancreas tails were lysed in Western and IP lysis buffer. The proteins were resolved by 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes. Members blotted with 5% skimmed milk. Protein was incubated with t-Bid, Bax, cytochrome c, Bcl-2, Bcl-xL, caspase 3, cleaved-caspase 3, caspase 9, or β-actin primary antibodies and HRP-IGG secondary antibodies, and then was detected by ECL (Applygen, Beijing, China). Normalization of protein expression was assayed using β-actin as control.

**Statistical analysis** Data were expressed as mean ± standard deviation (SD). Statistically comparisons were carried out by one-way analysis of variance (ANOVA) followed by Tukey’s test using SPSS version 17.0 software. P<0.05 indicated statistically significant.

**Results**

*Cf-FUC reduced blood glucose level and improved insulin resistance* Table 1 showed the blood glucose and serum insulin levels of the C57BL/6j mice. HFSD significantly increased fasting blood glucose, serum insulin, HOMA-IR, serum resistin and reduced QUICKI when compared to the control group (P < 0.01). *Cf-FUC* treatment decreased HFSD induced elevation in fasting blood glucose, serum insulin, HOMA-IR and serum resistin by 17.5%, 5.2%, 30.8% and 23.0%, and increased QUICKI by 4.5%, respectively. High dose *Cf-FUC* supplement enhanced the role of

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer</th>
<th>Tm (℃)</th>
<th>No. of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bid</td>
<td>F 5'-CCAAATAGCGATGAGAT-3' R 3'-CACCTGGAAATAGGGAGA-5'</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>Bax</td>
<td>F 5'-CTTCTTCAGATGGTGGAG-3' R 3'-CTTTTCTTCAGGGTCCA-5'</td>
<td>59</td>
<td>32</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>F 5'-GTGCTCGTGTCGTCGGTT-3' R 3'-TCAAGGTGCGGTGGCAA-5'</td>
<td>55</td>
<td>34</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>F 5'-TGGGAGACGGTGATGAGA-3' R 3'-CCACGGAATCAGAAAGG-5'</td>
<td>59</td>
<td>32</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>F 5'-ACCCCTAAAGCACTATT-3' R 3'-TCCCTCTGAAACCACTTA-5'</td>
<td>58</td>
<td>32</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>F 5'-CTAATATCTTGGCGAATAGG-3' R 3'-TGGGGACCAGGTCATT-5'</td>
<td>52</td>
<td>34</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>F 5'-TGACTGGAAACCGAAACT-3' R 3'-CTGGATGAAACGAGACC-5'</td>
<td>52</td>
<td>36</td>
</tr>
<tr>
<td>β-actin</td>
<td>F 5’-GTGGCGCTTAACCCCTTCGTTGGTCCA-5’</td>
<td>60</td>
<td>27</td>
</tr>
</tbody>
</table>
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RSG in inhibiting the changes of fasting blood glucose, serum insulin, HOMA-IR, serum resistin and QUICKI caused by HFSD. These results suggest that Cf-FUC can significantly decrease blood glucose level and improve insulin resistance.

Cf-FUC decreased TNF-α level and increased adiponectin level. The inflammatory mediator, TNF-α, is involved in apoptosis of pancreatic acinar cells (Liu et al., 2012). Adiponectin is reported as an adipocytokine that can reduce TNF-α production and inhibit pancreatic islets apoptosis (Du et al., 2013). Table 1 showed that Cf-FUC significantly suppressed both HFSD-induced TNF-α increase and adiponectin reduction. High-dose Cf-FUC further decreased serum TNF-α level and increased adiponectin level when combined with RSG. These results suggest that Cf-FUC can significantly reduce the inflammatory factor in insulin resistant mice.

Cf-FUC repaired islet of Langerhans. Fig.1 showed that various pathologic manifestations such as cell necrosis, atrophy of islet of langerhans, substantial inflammatory infiltration, and nuclear chromatin uneven distribution were observed in islet of langerhans of HFSD mice. These histological changes were alleviated in Cf-FUC-treated mice. When the mice were treated by Cf-FUC + RSG combination, the pathological alleviation was better than that in RSG group. High dose Cf-FUC combined with RSG further normalized the histology, which was approximated to that in the control group. These results suggest that Cf-FUC can repair HFSD-injured pancreatic islets.

Cf-FUC decreased caspase 3 and caspase 9 expression. To clarify whether Cf-FUC repaired HFSD-injured pancreatic islets through inhibiting islets cells apoptosis, caspase 3 and caspase 9 genes expression levels were studied. Caspases play essential roles in apoptosis. Caspase 3 is regarded as an apoptosis marker, and caspase 9 is an initiator of caspase 3 activity (Hou et al., 2013; Saleh et al., 2014). As shown in Fig. 2, Cf-FUC significantly reduced the mRNA expression level of caspase 9 in HFSD-fed mice.

### Table 1. Effects of Cf-FUC on the fasting blood glucose, insulin parameters and adipokines in HFSD-induced insulin resistant mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>HFSD</th>
<th>RSG</th>
<th>Cf-FUC</th>
<th>20Cf-FUC+RSG</th>
<th>80Cf-FUC+RSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>9.27±1.40</td>
<td>13.12±0.82**</td>
<td>10.92±0.69*</td>
<td>10.82±1.41*</td>
<td>10.11±0.92*</td>
<td>9.72±1.24**, ★</td>
</tr>
<tr>
<td>Serum insulin (mU/L)</td>
<td>10.82±0.32</td>
<td>14.68±0.60**</td>
<td>13.36±0.14*</td>
<td>13.92±0.03*</td>
<td>13.01±0.19*</td>
<td>11.94±0.60**, ★★</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>4.53±0.31</td>
<td>8.22±0.95**</td>
<td>5.79±0.62**</td>
<td>5.69±0.57**</td>
<td>5.42±0.70**</td>
<td>4.56±0.48**, ★★</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.50±0.01</td>
<td>0.44±0.01**</td>
<td>0.47±0.00*</td>
<td>0.46±0.00*</td>
<td>0.47±0.00*</td>
<td>0.49±0.01**, ★</td>
</tr>
<tr>
<td>Serum resistin (ng/mL)</td>
<td>5.57±0.10</td>
<td>11.60±0.60**</td>
<td>8.43±0.18**</td>
<td>8.93±0.47**</td>
<td>9.40±0.28*</td>
<td>6.82±0.35**, ★★</td>
</tr>
<tr>
<td>Serum leptin (ng/mL)</td>
<td>0.14±0.01</td>
<td>0.32±0.03**</td>
<td>0.22±0.04*</td>
<td>0.19±0.00**</td>
<td>0.19±0.02**</td>
<td>0.14±0.01**, ★</td>
</tr>
<tr>
<td>Serum TNF-α (pg/mL)</td>
<td>57.0±1.4</td>
<td>100.8±6.5***</td>
<td>77.4±3.1**</td>
<td>80.3±4.5*</td>
<td>75.9±1.8**</td>
<td>66.6±1.4**, ★</td>
</tr>
<tr>
<td>Serum adiponectin (ng/mL)</td>
<td>205±10</td>
<td>110±8**</td>
<td>152±7**</td>
<td>137±4*</td>
<td>167±5**</td>
<td>186±8**, ★★</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD (n=10/group). Multiple comparisons were done using one way ANOVA analysis followed by Tukey’s test.

\( ^{/*} P < 0.01 \) versus the control group.

\( ^{*} P < 0.05, ^{**} P < 0.01 \) versus the HFSD mice.

\( ^{*} P < 0.05, ^{**} P < 0.01 \) versus the RSG-treated mice.

Fig. 1. Effect of Cf-FUC on microstructure of islets of langerhans in insulin resistant mice. (× 400; arrow points to islets of langerhans, n=4/group)
mice pancreatic islets \((P < 0.05)\), but caspase 3 mRNA expression unchanged. Although the protein expressions of caspase 9 and cleaved-caspase 3 were decreased in \textit{Cf}-FUC group, there was no statistically significant. RSG-treated group showed significant decrease in the mRNA expression levels of caspase 9 and caspase 3, and in the protein expression levels of caspase 9 and cleaved caspase 3. These suggest that the activity of RSG was better than \textit{Cf}-FUC. It is dramatical that the mRNA expression levels of caspase 9 and caspase 3 in \(80\textit{Cf}-\text{FUC+RSG}\) group were lower than those in RSG group \((P < 0.05)\). Moreover, the same changes were shown in the protein expression levels \((P < 0.01)\). These results suggest that \textit{Cf}-FUC can synergistically enhance the effect of RSG on inhibiting pancreatic islets apoptosis.

**Cf-FUC inhibited intrinsic mitochondrial pathway** As \textit{Cf}-FUC significantly suppressed apoptosis in pancreatic islets, we examined its effects on mitochondrial pathway which was the pivotal signaling of cell apoptosis (Xiang et al., 2015). Cytochrome \(c\) release is recognized as a key point in this mitochondrial pathway (Zhu et al., 2015). As shown in Fig. 3, HFSD-induced cytochrome \(c\) mRNA expression elevation in the pancreatic islets was reduced by \textit{Cf}-FUC to 74.8\% \((P < 0.05)\). Further, \textit{Cf}-FUC significantly decreased cytochrome \(c\) protein level in cytoplasm by 19.6\% \((P < 0.05)\), which indicated that \textit{Cf}-FUC inhibited mitochondrial cytochrome \(c\) release to the cytoplasm. These beneficial effects were further improved when high dose \textit{Cf}-FUC combined with RSG \((P < 0.05, P < 0.01)\). Bax plays an essential role in the activation of the execution phase of apoptosis by binding to and antagonizing the Bcl-2 protein (Feng et al., 2015; Mignard et al., 2014). Bid, a BH3 only protein, is cleaved to t-bid by activated caspase 8 and subsequently induces Bax assemblage from cytoplasm to mitochondria (Hutcheson, & Perlman, 2008). As shown in Fig. 4, \textit{Cf}-FUC significantly lowered both Bax and Bid mRNA expression levels in the pancreatic islets of insulin resistant mice \((P < 0.05)\). WB results further indicated that Bax and t-Bid protein expression levels were significantly reduced by 26.6 and 34.9\% when treated with \textit{Cf}-FUC \((P < 0.05, P < 0.01)\),

![Fig. 2. Effects of \textit{Cf}-FUC on the mRNA and protein expression of caspase 9 and caspase 3 in pancreatic islets of insulin resistant mice. The same photos of \(\beta\)-actin in mRNA and protein expression were used. A: caspase 9 mRNA expression, B: caspase 9 protein expression, C: caspase 3 mRNA expression, D: cleaved-caspase 3 protein expression. Data are expressed as mean ± S.D. \((n=6/group)\). Multiple comparisons were done using one way ANOVA analysis followed by Tukey’s test. ★\(P < 0.05\) versus control; ★★\(P < 0.05\), ★★★\(P < 0.01\) versus HFSD; *\(P < 0.05\), ★★\(P < 0.01\) versus RSG group.](image-url)
The results of this study demonstrated that Cf-FUC could improve hyperglycemia and increase insulin sensitivity in HFSD-induced insulin resistant mice. Our data further suggested that Cf-FUC could suppress pancreatic islets apoptosis by reducing TNF-α level and increasing adiponectin level. Here, we reported for the first time that the inhibition of apoptosis in islets of langerhans by Cf-FUC depended on the down regulation of mitochondrial pathway.

It has been reported that inflammatory cytokines can induce the death of pancreatic β-cell (Govindaraj and Sorimuthu Pillai, 2015; Tian et al., 2014). The present study indicated that serum level of the pro-inflammatory cytokines, TNF-α, was significantly higher in the HFSD group than that in the control group. Cf-FUC supplement could significantly decrease serum TNF-α level of the HFSD-treated mice. Adiponectin is an adipose tissue-derived mediator with anti-inflammatory and insulin sensitizing properties (Ziemke and Mantzoros, 2010). Previous studies have revealed that adiponectin can suppress glucotoxicity-induced cell apoptosis (Lindmark et al., 2006; Lin et al., 2009). Du et al. (2013) and Thole et al. (2012) reported that TNF-α exacerbated impaired insulin action and destruction of pancreatic β-cell. In this study, histological changes showed that Cf-FUC repaired the injury of the islet of langerhans, indicating that Cf-FUC could protect pancreatic cells from death by reducing TNF-α level in insulin resistant mice.

Cieślak et al. (2015) reported that biological activities of TNF-α were exerted through mitochondrial dysfunction and activation of various downstream signaling effectors. These effectors led to the activation of specific signaling pathways of apoptosis, such as the release of mitochondrial cytochrome c into the cytoplasm (Yang et al., 2014). In this study, HFSD induced a significant increase of cytochrome c mRNA expression in the pancreatic islets, but Cf-FUC could alleviate this induction. Moreover, Cf-FUC also significantly decreased the release of cytochrome c to cytoplasm. Once entering the cytoplasm, cytochrome c could directly activate caspases, which cleaved a series of cellular proteins and thus caused apoptotic changes. Some studies demonstrated that the reduction of cytochrome c release could cause the inactivation of apoptosis related effectors, such as caspase 3 and caspase 9 (Bishayee et al., 2013; Jiang et al., 2013). Our research results were consistent with the previous findings, which showed that caspase 3 and caspase 9 mRNA expression levels were down-regulated, and caspase 3 protein and cleaved-caspase 9 protein expression levels were also reduced in HFSD-treated mice by Cf-FUC. Therefore, it could be speculated that Cf-FUC supplement inhibited cytochrome c release, and subsequently suppressed the activation of caspase 3 and caspase 9, which finally blocked the mitochondrial apoptotic pathway.

Cytochrome c release and mitochondria disruption are controlled by the Bcl-2 family proteins (Vander Heiden and Thompson, 1999). Bcl-2 and Bcl-xL inhibit apoptosis by cleaning up reactive oxygen species, preventing the release of Ca^{2+} from the endoplasmic reticulum and preventing the mitochondrial transitional pore opening by opposing the effect of Bax thereby.

**Discussion**

The effects of Cf-FUC in the pancreatic islets were assessed using Western Blot analysis and semiquantitative RT-PCR, respectively. Both Bcl-2 and Bcl-xL can reduce caspase activity and inhibit cell apoptotic processes (Shi et al., 2015). As shown in Fig. 5, mRNA expression levels of Bcl-2 and Bcl-xL in the HFSD-fed animals, respectively, were statistically significant in Bcl-xL mRNA expression. WB assays further showed that Cf-FUC significantly increased these two protein expression levels by 1.60-fold and 1.74-fold in HFSD-fed mice compared to that of HFSD-fed mice (P < 0.05, P < 0.01). Moreover, the mRNA expression of Bcl-xL and the protein expression of Bcl-2 and Bcl-xL were all significant increased in 80 Cf-FUC+RSG group compared with RSG alone group (P < 0.05, P < 0.01). These results suggest that Cf-FUC can suppress pancreatic islets apoptosis via blocking intrinsic mitochondrial pathway, though its effects were lower than RSG. Cf-FUC can also enhance the effects of RSG when they were combined.

**Fig. 3.** Effects of Cf-FUC on mRNA and protein expression of cytochrome c in pancreatic islets of insulin resistant mice. Cytochrome c protein was detected using the cytosol fraction of pancreatic islets. The same photos of β-actin in mRNA and protein expression were used. A: cytochrome c mRNA expression, B: cytochrome c protein expression. Data are expressed as mean ± S.D. (n=6/group). Multiple comparisons were done using one way ANOVA analysis followed by Tukey’s test. *P < 0.05, **P < 0.01 versus control; ***P < 0.05, **P < 0.01 versus HFSD; *P < 0.05 versus RSG group.
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blocking cytochrome c release and inhibiting caspase activities (Bouzakri et al., 2011; Donovan and Cotter, 2004; Lamothe and Aggarwal, 2002; Sha et al., 2008). In our present study, Bcl-2 and Bcl-xL were significantly decreased by HFSD treatment, but these decreases could be inhibited by Cf-FUC. The results indicated that Bcl-2 family proteins might play a critical role in regulating mitochondrial pathway of cytochrome c release, and Cf-FUC was able to protect pancreatic islets from apoptosis via modulating of Bcl-2 and Bcl-xL expression levels. The key pro-apoptotic protein, Bax, which is a main inhibitor of Bcl-2 and Bcl-xL, is one member of the apoptosis-regulated gene Bcl-2 family (Antonsson et al., 1997). Upon activation, Bax can induce cytochrome c release to cytoplasm by multiple mechanisms (Eskes et al., 1998; Gómez-Crisóstomo et al., 2013). In this study, Cf-FUC significantly inhibited both mRNA and protein expression levels of Bax in HFSD-treated mice. Caspase 8 mediates the intrinsic pathway through cleavage of the pro-apoptotic Bid protein, a BH3-only protein, to a truncated Bid (t-Bid) via translocation from the cytoplasm to the mitochondria, triggering mitochondrial dysfunction (Hutcheson and Perlman, 2009; Park et al., 2013). This always leads to the activation of Bax and opening of mitochondrial membrane (Shamas-Din et al., 2013; Strasser, 2005). It was shown in our research that Cf-FUC significantly decreased Bid mRNA and t-Bid protein expression levels. These results demonstrated that Cf-FUC suppressed pancreatic islets apoptosis via inactivation of Bid protein and consequent inactivation of the intrinsic mitochondrial pathway. The reduction of Bax, activation of Bcl-2 and Bcl-xL by Cf-FUC further inhibited cytochrome c release to the cytoplasm and subsequently inactivated the apoptotic effectors caspase 9 and caspase 3.

FUC from some marine plants was also proved to exhibit the activities of improving insulin resistance and providing pancreatic protection. Jiang et al. (2015) reported that FUC from the sea weed *Fucus vesiculosus* could stimulate insulin secretion and provide...
pancreatic protection via the cyclic adenosine monophosphate (cAMP) pathway in RIN-5F rat insulin-secreting cells and GK rats. Hernández-Corona et al. (2014) reported that FUC from marine plants could promote insulin secretion and improve insulin resistance in overweight or obese adults. Carvalho et al. (2014) proved that FUC reduced the severity of acute pancreatitis in mice by decreasing neutrophil infiltration and systemic inflammation. Our results showed that Cf-FUC also increased serum insulin level, reduced inflammatory cytokines levels, and protected pancreatic islets apoptosis. It was implied to be concerned with the activities of inhibition of α-amylase and α-glucosidase by FUC derived from brown marine algae (Kim, Rioux and Turgeon, 2014). They also reported that the activities of FUC were different from the distinct species.

In the present study, the dosage of Cf-FUC was also examined. The protection effects of structure and insulin secretion of islets in (80 Cf-FUC + RSG)-treated group were significantly improved when compared with the RSG-treated group. When compared with the RSG-treated group, the mitochondrial pathway was significantly inactivated in (80 Cf-FUC + RSG)-treated group but not in (20 Cf-FUC + RSG)-treated group. These results indicate that the dosage of Cf-FUC was crucial for its effect on protecting islets of langedrhans from apoptosis, and it was effective when the dosage of Cf-FUC was up to 80 mg/kg/d. On the other hand, these study were not investigate the effects of a dose dependency of RSG and fucoidan combination on insulin resistance and pancreas apoptosis, and the future study will be focused on it.

**Conclusion**

In the present study, the results demonstrated that Cf-FUC could inhibit HFSD-induced pancreatic islets apoptosis. Activation of Bcl-2 and Bcl-xL, which led to the inactivation of intrinsic mitochondrial pathway, was the underlying mechanism for the protection effects of Cf-FUC. Thus, supplementation with Cf-FUC gives a dietary intervention for pancreatic islets apoptosis development, providing an alternative precaution for insulin

**Fig. 5.** Effects of Cf-FUC on mRNA and protein expression of Bcl-2 and Bcl-xL in pancreatic islets of insulin resistant mice. The same photos of β-actin in mRNA and protein expression were used. A: Bcl-2 mRNA expression, B: Bcl-2 protein expression, C: Bcl-xL mRNA expression, D: Bcl-xL protein expression. Data are expressed as mean ± S.D. (n=6/group). Multiple comparisons were done using one way ANOVA analysis followed by Tukey's test. *P < 0.01 versus control; *P < 0.05, **P < 0.01 versus HFSD; *P < 0.05, **P < 0.01 versus RSG group.
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Acknowledgements  This study was supported by National Natural Science Foundation of China (31071525) and Research Start-up Funding of Zhejiang Ocean University (Q1442 and Q1443). We also thank Yulin Liao (the Chinese Academy of Sciences Institute of Oceanography, Qingdao, China) for his species identification of the sea cucumber Cusumaria frondosa.

References


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