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Upregulation of Wnt Signal Pathway by Myricetin Attenuates β-Cell Dysfunction Induced by Cytokines*

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Abstract We examined the effect of myricetin on cell dysfunction in cytokine-induced pancreatic β cells and assessed whether Wnt signal pathway was the target of myricetin. RIN-m5f β cells were exposed to a combination of tumor necrosis factor-α, interleukin-1β, and interferon-γ, with or without myricetin pretreatment for 48 h. The cell viability, basal and glucose-stimulated insulin secretion and Wnt-signaling proteins were evaluated with methyl thiazolyl tetrazolium assay, radio immunoassay and Western blotting, respectively. The 48 h multiple-cytokine treatment decreased cell viability and glucose-stimulated insulin secretion, while increasing basal insulin secretion. Western blot analysis showed that Wnt-signaling proteins were decreased in cytokine-treated RIN-m5f cells. However, myricetin pretreatment protected against cytokine-induced cell death. In addition, myricetin (20 μmol/L) obviously decreased basal insulin secretion and increased glucose-stimulated insulin secretion in cytokine-treated RIN-m5f cells. Western blot analysis showed that Wnt-signaling proteins were increased after myricetin pretreatment. Therefore, myricetin might attenuate cell dysfunction in cytokine-induced RIN-m5F cells *via* the Wnt signal pathway, and the Wnt signal pathway might be used as a new target for protecting pancreatic β cells against cytokine-induced cell dysfunction and death.

Key words myricetin, cytokine, Wnt signal pathway, pancreatic β cells
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Diabetes is characterized by dysfunction of pancreatic β cells, specifically insufficient insulin production resulting from the absolute or relative inadequate functional β cell mass[1]. Lines of evidence had shown that the toxic effect of inflammatory cytokines had a key role in progression of pancreatic β-cell dysfunction[2–3]. In our previous study, we also found that exposure to the cytokine mixture of tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β) and interferon γ (IFN-γ) decreased cell viability and disturbed insulin secretion in RIN-m5f β cells [4]. Nevertheless, the mechanisms underlying cytokine-induced pancreatic β-cell dysfunction remain unresolved.

The Wnt signal pathway is one of the central pathways that take part in embryogenesis and tumorigenesis. Much attention has been recently drawn toward the role of this pathway in diabetes pathogenesis. Some *in vitro* and animal studies indicated that Wnt-signaling molecules were expressed in murine and human β cells; Wnt signal pathway was also identified as a regulator of β-cell proliferation and insulin secretion[5–6]. Furthermore, studies in humans found that polymorphisms within the gene encoding transcription factor 7 like 2 (TCF7L2, formerly known as TCF-4), the Wnt pathway effector, had been strongly linked to type 2 diabetes [7]. Thus, Wnt signaling may be necessary and sufficient for pancreatic β-cell function. Although recent studies showed an association between cytokines and Wnt signaling[8–10], whether and how Wnt signal pathway

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alters the function of pancreatic β cells during cytokines' treatment still deserve further investigation.

Myricetin is a naturally occurring flavonoid with hydroxyl substitutions at the 3, 5, 7, 3′, 4′ and 5′ positions (Figure 1). It is commonly ingested through our diet in fruits, vegetables, tea, berries and red wine [11]. Recently, there has been an increasing attention to its anti-inflammatory activity. *In vitro* studies demonstrated that myricetin inhibited the expression of cytokines (*e.g.*, IL-1β and IL-6) [12–13]. Furthermore, myricetin also inhibited downstream events of cytokines. For example, treatment of MG-63 cells with myricetin not only inhibited anti-Fas IgM-induced apoptosis, but also blocked the synergetic effect of anti-Fas IgM with TNF-α or IL-1β on cell death [14]. On the other hand, various studies had illustrated that myricetin was a potent anti-diabetic agent [15–18]. However, these studies mainly focused on myricetin as a promising therapeutic agent for the treatment of insulin resistance; little information is available describing its effect on pancreatic β-cell dysfunction.

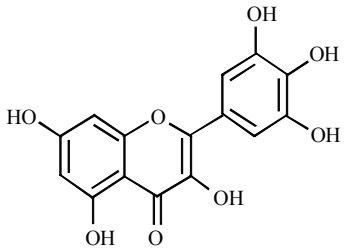

Fig. 1 Chemical structure of myricetin

In the present study, we investigated whether Wnt signal pathway participated in cytokine-induced pancreatic β-cell dysfunction. Meanwhile, given the recent findings that some flavonoids affected Wnt signal pathway[19–20], myricetin was examined to clarify whether it could protect RIN-m5f β cells against cytokine-induced cell dysfunction *via* promoting Wnt signaling. To test cell viability, methyl thiazolyl tetrazolium (MTT) assay was performed. To determine cell function, both basal and glucose-stimulated insulin secretion were assessed. To learn the relevant mechanisms, the expressions of phosphorylated glycogen synthase kinase 3β (p-GSK 3β-Ser9) and β-catenin protein in both cytoplasm and nucleus, as well as the TCF-4 level were determined.

1 Materials and methods

1.1 Reagents

Myricetin (purity ⩾ 95.0%) and MTT were obtained from Sigma-Aldrich (USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and trypsin were from Gibco (USA). TNF-α, IL-1β and IFN-γ were purchased from Peprotech (USA). The antibodies to p-GSK 3β (Ser9) and β-catenin were from Millipore (USA). TCF-4, GAPDH and IgG HRP-linked secondary antibodies were from Santa Cruz (USA). Nuclear and cytoplasmic extraction reagents, BCA protein assay kit, phenylmethyl sulfonylfluoride (PMSF), leupeptin, aprotinin and pepstatin were from Pierce (USA). Super ECL plus detection reagent was purchased from Applygen Technologies Inc. (China).

1.2 Cell culture

The insulin-secreting β cell line RIN-m5f was kindly presented by Dr. ZHOU Chun-Yan (Department of Biochemistry and Molecular Biology, School of Basic Medical Science, Peking University). The cells were maintained in high glucose-DMEM containing 10% FBS at 37℃ in humidified 5% CO_2 and 95% O_2. When grown upon reaching 70% confluence, cells were pretreated with different concentrations of myricetin for 3 h, and then, the combination of cytokines(10 μg/L TNF-α, 5 μg/L IL-1β and 1000 U/ml IFN-γ; referred to as TII) were added. All of the determinations were performed 48 h later.

1.3 Cell viability assay

After designed treatment, cell viability was measured in 96-well plates with MTT. Briefly, 100 μl of MTT solution (1 g/L) was added to each well and the plates were incubated at 37℃ for 4 h. The MTT solution was removed and the colored formazan crystal was dissolved in dimethyl sulfoxide (DMSO). The absorbance at 595 nm was measured by a microplate reader using DMSO as the blank.

1.4 Basal and glucose-stimulated insulin secretion assay

After washed with phosphate buffered solution (PBS), cells were incubated in phenolsulfonphthalein-free DMEM containing 2.8 mmol/L glucose for 1 h at 37℃ and the culture medium was collected. The same cells were then washed with PBS and incubated in phenolsulfonphthalein-free DMEM supplemented with 16.7 mmol/L glucose for 1 h at 37℃, the culture

medium was also collected. Insulin secretion was quantified by radio immunoassay and normalized by the cellular protein content. Results were expressed as μU of insulin per mg of protein.

1.5 Western blot

For whole-cell extracts, cell lysates were prepared in lysis buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1 mmol/L EGTA, 0.1% Triton X-100, 2.5 mmol/L $Na_4P_2O_7$, 1 mmol/L Na_3VO_4, 11 mmol/L β-mercaptoethanol, 250 mmol/L PMSF, 1 mg/L aprotinin, 1 mg/L leupeptin and 1 mg/L pepstatin) for 0.5 h on ice. The lysates were then centrifuged at 12 000 *g* for 5 min at 4℃ , and the supernatants were collected. For differential nuclear and cytoplasmic extraction, a protocol following the instructions of nuclear and cytoplasmic extraction reagents was used. The protein content was determined with a BCA protein assay kit. An aliquot of 40 μg of protein from each sample was then boiled with 5 × sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Following electrophoresis, the proteins were electrotransferred onto 0.45 μm pore size polyvinylidene fluoride membranes (Millipore). The membranes were blocked for 1 h with TBST (50 mmol/L Tris-HCl pH 7.4, 0.15 mol/L NaCl, 0.1% Tween-20) containing 5% non-fat dried milk at ambient temperature. Immunoblots were analyzed using appropriate primary antibodies and secondary antibodies. Protein expression was visualized autoradiographically by using super ECL plus detection reagent.

1.6 Statistical analysis

The data are represented as $\bar{x} \pm SE$. Comparisons between multiple groups were performed by one-way analysis of variance (ANOVA) followed by the LSD test. $P < 0.05$ was considered to be statistically significant.

2 Results

2.1 Myricetin enhanced cell viability in TII-treated RIN-m5f β cells

The cell viability was expressed as the absorbance at 595 nm. No significant difference in cell viability was seen following treatment with different concentrations of myricetin (5, 10 and 20 μmol/L, respectively) for 48 h (Figure 2a). However, cell viability was decreased in the presence of TII alone for 48 h (the absorbance was 0.2222 ± 0.0095), which was significantly lower than that in untreated control cells (the absorbance was 0.5002 ± 0.0172). When cells were pretreated with different concentrations of myricetin for 3 h, and followed by 48 h incubation with TII, the cell viability was increased compared with TII-treated cells (Figure 2b). The absorbance in 5, 10 and 20 μmol/L myricetin treated cells were (0.2612±0.0254), (0.3198±0.0240) and (0.3900±0.0166) respectively. Although 5 μmol/L myricetin had no notable effect on cell viability, 10 μmol/L and 20 μmol/L myricetin significantly enhanced cell viability. These results depicted that myricetin could enhance the cell viability in RIN-m5f β cells induced by TII in a concentration-dependent manner.

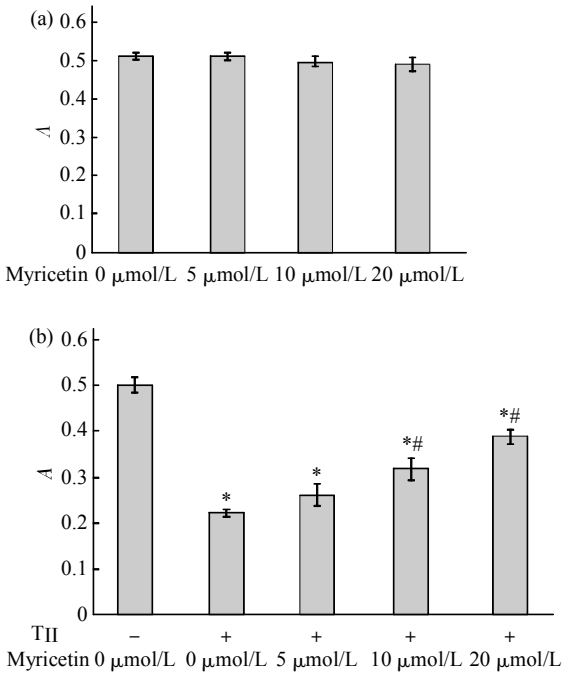

Fig. 2 Effects of myricetin on cell viability in TII-treated RIN-m5f β cells

Data are expressed as the $A_{595} \pm SE$ from six experiments. (a) The cell viability in untreated control cells and myricetin-treated control cells. (b) RIN-m5f β cells were pretreated with myricetin for 3 h and further treated with TII for 48 h. * Designated statistically significant difference from untreated control group, $P<0.05$. #Designated statistically significant difference from TII-alone group, $P<0.05$.

2.2 Myricetin reversed cellular function in TII-treated RIN-m5f β cells

As shown in Figure 3, basal and glucose-stimulated insulin secretion in untreated control cells and 20 μmol/L myricetin-treated control cells did not differ significantly. However, insulin secretion was disturbed by TII treatment. Compared with untreated

control cells, basal insulin secretion in TII-treated cells was significantly increased [(68.9235 ±2.4862) μU/ (mg •h) *vs*. (28.1126 ±1.5460) μU/ (mg •h)], while glucose-stimulated insulin secretion was obviously decreased [(24.7947 ±1.4772) μU/ (mg •h) *vs*. (62.6875 ±2.1548) μU/(mg•h)]. When 20 μmol/L myricetin was pretreated prior to TII, the cellular function was reversed. Compared with TII-treated cells, basal insulin secretion in myricetin-treated cells was significantly decreased [(39.2540 ± 1.8696) μU/ (mg •h)], while glucose-stimulated insulin secretion was obviously increased [(37.4516 ± 2.1717) μU/(mg•h)].

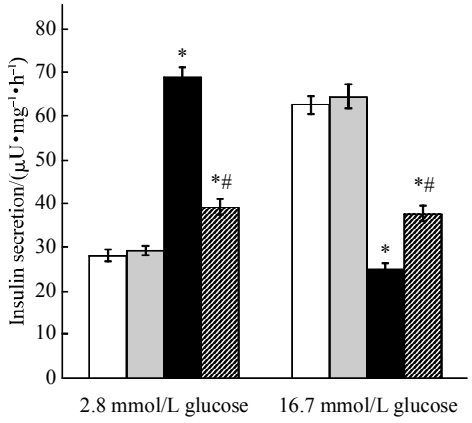

Fig. 3　Basal and glucose-stimulated insulin secretion in RIN-m5f β cells

RIN-m5f β cells were pretreated with or without 20 μmol/L myricetin for 3 h and further added TII for 48 h. The insulin level was quantified by radio immunoassay. Results were represented as μU of insulin per mg of protein of six separate experiments. * Designated statistically significant difference from untreated control group, $P < 0.05$. # Designated statistically significant difference from TII-alone group, $P < 0.05$. □: Control; ▨: Myricetin; ■: TII; ▨ TII+ myricetin.

2.3　Myricetin upregulated the Wnt signal pathway in TII-treated RIN-m5f β cells

Finally, the possible mechanisms were investigated by Western blot analysis. As shown in Figure 4, in contrast to untreated control cells, the levels of β-catenin protein in both cytoplasm and nucleus were decreased after TII treatment. However, the pretreatment with myricetin increased the phosphorylation level of GSK 3β-Ser9 and the expressions of cytoplasmic and nuclear β-catenin under both normal and TII-treated conditions. Additionally, the expression of TCF-4 was also studied (Figure 5). Contrary to untreated control cells, TII treatment significantly decreased the level of TCF-4. Interestingly, 20 μmol/L myricetin slightly upregulated the level of TCF-4 in TII-treated cells; while no obvious change was seen in myricetin-treated control cells. Collectively, these studies indicated that myricetin might upregulate the Wnt signal pathway under both normal and TII-treated conditions.

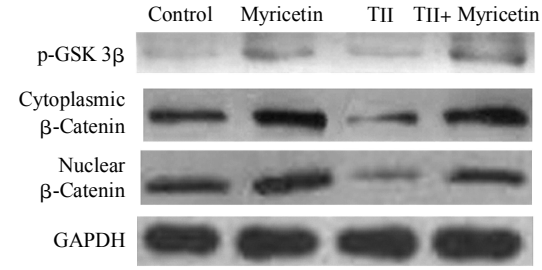

Fig. 4　Effects of myricetin on p-GSK 3β (Ser9) and β-catenin expression in TII-treated RIN-m5f β cells

An aliquot of protein from each sample was subject to Western blot analysis using p-GSK 3β (Ser9) and β-catenin antibodies. GAPDH protein levels were used as a control. The result is one representative example of the three independent experiments.

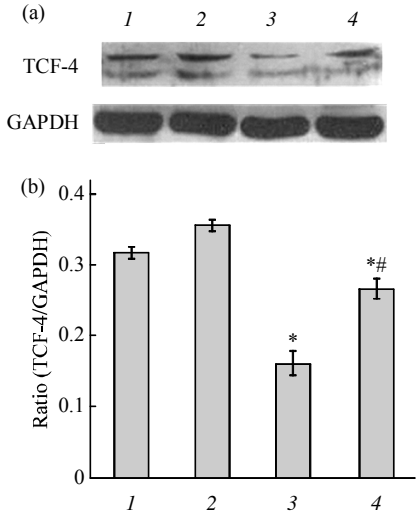

Fig. 5　Effects of myricetin on TCF-4 expression in TII-treated RIN-m5f β cells

An aliquot of protein from each sample was subjected to Western blot analysis using TCF-4 antibody. GAPDH protein levels were used as a control. A ratio between specific protein and GAPDH was calculated as ($\bar{x} \pm SE$) of three experiments. *Designated statistically significant difference from untreated control group, $P<0.05$. #Designated statistically significant difference from TII-alone group, $P < 0.05$. *1*: Control; *2*: Myricetin; *3*: TII; *4*: TII+Myricetin

3 Discussion

The main cytokines of β-cell damage secreted by CD^{4+} T-cells and macrophages are TNF-α, IL-1β and IFN-γ [3, 21]. In our previous study, exposure to combination of cytokines (TNF-α 10 μg/L + IL-1β 5 μg/L+ IFN-γ 1000 U/mL) for 24 h severely impaired RIN-m5f β cells [4]. In this study, we observed that a 48 h exposure of the same dosages of cytokines decreased cell viability and disturbed insulin secretion. These findings demonstrated that it was a suitable *in vitro* model for the study of pancreatic β-cell damage. Remarkably, we found that glucose-stimulated insulin secretion was obviously reduced, whereas basic insulin secretion was markedly upregulated in TII-treated cells. This was in line with recent results from human and mouse islets. The treatment of cytokine mixture (IL-1β 2 μg/L+ IFN-γ 1000 U/mL) for 96 h increased basic insulin secretion and decreased glucose- stimulated insulin secretion [22]. We hypothesize that increased basic insulin secretion is associated with the stimulation of cytokines and may be a stress response, possibly as an indicator of the further β-cell damage and even loss. However, studies are still needed to address the mechanisms of action in this regard.

As mentioned above, the canonical Wnt signal pathway influences pancreatic β-cell functions including insulin secretion, survival and proliferation. The main effector of this signal pathway is the bipartite transcription factor β-catenin/TCF. In a resting cell, cytoplasmic β-catenin is phosphorylated and degraded through the actions of a protein complex including Axin, GSK 3β, and other proteins, among which GSK 3β is an important negative modulator. When activated, Wnt signal pathway is transmitted by an association between Wnt receptors and Dishevelled, which triggers the disruption of the protein complex, thus resulting in the stabilization of β-catenin. β-Catenin then enters the nucleus and interacts with TCF transcription factors to control the activation of β-Catenin/TCF downstream target genes [23]. A previous study showed that the *in vivo* disruption of the Wnt/β-Catenin pathway inhibited β-cell regeneration in diabetic rats [24]. In addition, an *in vitro* study reported that the cytokine mixture with IL-1β (2 μg/L) plus IFN-γ (1000 U/ml) decreased TCF-4 expression in human and mouse islets [22]. In keeping with these reports, we found that cytokines in conjunction decreased the stabilization and nuclear localization of β-catenin and inhibited TCF-4 expression. The results suggest that cytokines might induce RIN-m5f β-cell dysfunction partly through the inhibition of Wnt/β-catenin/TCF-4 signal pathway.

The important finding of the present study was that myricetin could activate Wnt signal pathway in TII-treated RIN-m5f β cells. Indeed, the data concerning the regulation of Wnt signal pathway by phytochemicals were full of inconsistencies. In the study with 3T3-L1 preadipocytes, *Lindera obtusiloba* extract effectively reduced the phosphorylation of β-Catenin, thus stabilizing β-catenin and blocking adipogenesis [25]. Another *in vitro* study observed that naringenin (a citrus flavonoid) enhanced melanogenesis through increasing the intracellular accumulation of β-catenin [19]. However, in the study of cancer, some phytochemicals were used as inhibitors of Wnt/β-catenin signaling. Pterostilbene was found to reduce the protein level of β-catenin and inhibit its nuclear localization in HT-29 colon cancer cells [26]. In addition, quercetin, a similar plant flavonol, was reported as an excellent inhibitor of Wnt signaling in SW480 colon cancer cells, which was due to the decreased nuclear β-catenin and TCF-4 proteins [20]. These contradictory conclusions might be partly explained by the differences in cell types and phytochemical structures. In the present study, myricetin (20 μmol/L) enhanced the stabilization and nuclear localization of β-catenin and increased TCF-4 expression in TII-treated RIN-m5f β cells, which might be one of the mechanisms of its cytoprotective action. Interestingly, compared to control group, treatment of myricetin alone increased the stabilization of β-catenin, but no obvious changes were seen in the cell activity and insulin secretion. It was likely that Wnt signal pathway controlled the pancreatic β-cell function to achieve the most desirable physiological response.

The inhibition of GSK 3β-mediated β-catenin degradation is known to be the key event in Wnt/β-catenin signal pathway. A previous *in vivo* study showed that activation of the Wnt pathway through the inhibition of GSK 3β had a significant stimulatory effect on β-cell regeneration [24]. In the present study, the phosphorylation level of GSK 3β-Ser9 in response to myricetin pretreatment was upregulated in TII-treated cells, which corresponded to the increase in β-catenin levels. We suppose that the

phosphorylation of GSK 3β by myricetin inactivates the activity of GSK 3β, resulting in the intracellular accumulation and nuclear localization of β-catenin. GSK 3β is also a component of the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt) pathway. Some previous studies indicated that these two pathways crosstalked at the GSK 3β level [27]. For instance, the study from Huang *et al*. revealed that naringenin treatment upregulated the phosphorylation of GSK 3β protein in melanoma cells and the naringenin-induced increase in phospho-Akt was prior to the elevation in the level of β-catenin[19]. However, a recent study showed that insulin decreased the activity of GSK 3β by increasing the phosphorylation level of GSK 3β-Ser9, but did not affect the expression of β-catenin [28]. This result indicated that GSK-3β could only be phosphorylated by PI3K when it was not bound to Axin, whereas the protein complex-bound GSK 3β was dedicated to Wnt signaling. Although myricetin was observed to increase the phosphorylation levels of Akt-Ser473 and GSK-3b-Ser9 in C2C12 myotubes in another study from this laboratory [18], it was unclear here whether the same subcellular pool of GSK 3β was targeted by both Wnt and PI3K after myricetin treatment. Additional studies will be needed to address this issue.

Taken together, our data suggested that pancreatic β-cell dysfunction induced by TNF-α, IL-1β and IFN-γ was partially attributed to the inhibition of Wnt signal pathway, especially the decrease in stabilization of β-catenin and TCF-4 expression, whereas myricetin overcame the cytokine-induced β-cell dysfunction by up-regulating the phosphorylation level of GSK 3β-Ser9 and TCF-4 expression, which promoted the signal transduction of Wnt signal pathway. Therefore, the Wnt signal pathway might be used as a new target for protecting pancreatic β cells against cytokine-induced cell dysfunction. In addition, agents such as myricetin could potentially be useful for early intervention in β-cell damage disease like diabetes.

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杨梅黄酮通过 Wnt 通路改善细胞因子诱导的胰岛 β 细胞功能障碍的实验研究 *

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摘要 本文探讨杨梅黄酮对细胞因子诱导的胰岛 β 细胞功能障碍的影响及 Wnt 通路在其中的作用．MTT 法检测细胞的存活率；放射免疫法检测 RIN-m5f β 细胞在基础状态和高糖状态下胰岛素的分泌水平；Western blot 法检测 Wnt 通路相关蛋白的表达情况．肿瘤坏死因子 α、白介素 1β 和 γ 干扰素联合作用细胞 48 h 后，与空白对照组相比，细胞的存活率显著下降；基础状态下胰岛素的分泌量增加至空白对照组的 2.45 倍，而高糖刺激状态下胰岛素的分泌量减少至空白对照组的 39.6%．Western blot 结果显示，细胞因子处理后使 Wnt 通路相关蛋白的表达量下降．而杨梅黄酮作用后，与细胞因子组相比，可使细胞存活率上升．另外，20 μmol/L 杨梅黄酮可逆转细胞因子对胰岛素分泌的影响，使基础状态下胰岛素的分泌减少，高糖刺激状态下胰岛素分泌量显著增加．Western blot 结果显示，杨梅黄酮提高了 Wnt 通路相关蛋白的表达水平．上述结果表明，细胞因子联合作用 48 h 能诱导 RIN-m5f 细胞功能障碍，杨梅黄酮干预后能减少这种细胞损伤，保护机制可能与其激活 Wnt 通路有关．

关键词 杨梅黄酮，细胞因子， Wnt 通路，胰岛 β 细胞

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