

The effects of Sea buckthorn seed protein on glucose metabolism in streptozotocin-induced diabetic ICR mice

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Abstract: In this paper, we investigated the effects of protein from sea buckthorn seed on the expression of genes involved in liver glucose metabolism and on the activation of the AMPK/SIRT1 pathway in streptozotocin (STZ)-induced diabetic ICR mice. The investigated factors included oral glucose tolerance test, insulin resistance, insulin sensitivity index, AMPK and SIRT1 activity and the expression of liver glucose metabolism genes. Seabuckthorn seed protein (SSP) improved the oral glucose tolerance and insulin sensitivity, reduced insulin resistance, suppressed expression of liver glucose metabolism genes and upregulated activation of the AMPK/SIRT1 pathway. Therefore, the results demonstrate that SSP can improve insulin resistance, suppress expression of these genes and upregulate activation on the AMPK /SIRT1 pathway in STZ-induced diabetic ICR mice.

Keywords: Seabuckthorn seed protein, glycometabolism, AMPK/SIRT1, ICR mice.

INTRODUCTION

Sea buckthorn (*Hippophae rhamnoides* L.) is an edible berry rich in nutrients and phytochemicals, including water, lipids and vitamins, and flavonoids including procyanidin. It is utilized around the world for its medicinal and nutritional values (King 2008). It has been found to promote healthy blood circulation and respiratory system. In addition to its pleasant flavor, the intake of sea buckthorn adds additional benefits to remain the function of spleen, stomach and intestinal tract. Moreover, it was found to improve muscle movement. Last but not least, lines of evidence from previous research have also shown the therapeutic values of sea buckthorn intake. These include its anti-inflammatory and antioxidant effects. It greatly modulates our immunity and may be a great food source for patients with tumor, atherosclerosis and hyperlipidemia (Pichiah *et al.*, 2012). The plant has become increasingly accepted as an important potential source of bioactive constituents for nutraceuticals, functional foods and personal care products in recent years.

Diabetes is a metabolic disorder characterized by chronic hyperglycemia and caused by a variety of pathogenic factors (Zheng and Xu, 2012). It has been estimated that in 2008, there are 150 million people worldwide suffering from diabetes, and the number is expected to reach 300 million by 2025 (Tan *et al.*, 2008). Insulin resistance is a major metabolic abnormality of type 2 diabetes, which leads to the insulin regulation of metabolic signals blocked, including glycogen synthesis and related gene expression (Moller 2001; DeFronzo and Ferrannini, 1991).

Currently, AMP-activated protein kinase (AMPK) is one

of many mechanisms for pharmacological treatment of insulin resistance (Ye *et al.*, 2005). AMPK is an important protein kinase receptor that plays an important role in regulating cell and whole body energy balance (Leiter and Reifsnyder, 2004). AMPK has been implicated in insulin signaling based on findings demonstrating that it participates in the inhibition of glycogen synthesis and the regulation of glycolysis in response to insulin resistance, insulin sensitivity or glucose metabolism (Wang and Li, 2007; Halse *et al.*, 2003).

SIRT1 is a NAD⁺-dependent histone deacetylase, which can induce gluconeogenesis genes and hepatic glucose output (Cohen *et al.*, 2004; Rodgers *et al.*, 2005). Studies have shown that SIRT1 expression exist in peripheral white blood cells, and its expression may indirectly reflect the function of islet beta cells and the relationship with T2DM (Song *et al.*, 2011). In the last ten years, numerous studies have shown that SIRT1 exerts a protective effect against the progression of diabetic nephropathy by promoting reconstruction of energy homeostasis, modulating cell redox state, preventing cell apoptosis, inhibiting inflammation and ameliorating renal fibrosis (Hou *et al.*, 2014). SIRT1 overexpression relates to anti-lipogenic effects, including down regulation transcription of lipogenic genes and stimulation of AMPK phosphorylation, which may successively and independently result in lower lipogenic gene expression and enzymatic activities (Song *et al.*, 2011; Hou *et al.*, 2014; Ahn *et al.*, 2008; Feige *et al.*, 2008).

Sugar metabolism disorder is the main pathological factor of diabetes mellitus. The enzymes that are involved in the glucose metabolism play important roles in metabolism disorder. These enzymes include G-6-P, CPT1- α , GP and GSK-3. Amongst all four, GSK-3 enables to regulate the roles of the rest three enzymes in a series of sugar

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metabolism (Mues *et al.*, 2009; Kumar *et al.*, 2010; Macaulay and Woodgett, 2008). Previous studies have reported that the occurrence of IR is related to post insulin receptor signaling transduction disorder (Grzelkowska-Kowalczyk *et al.*, 2013; Maiese *et al.*, 2012).

We analyzed the critical signaling molecules of receptors in liver tissue including G-6-P, CPT1- α , GP and GSK-3 to explore the mechanism of type 2 diabetes. We have previously reported that sea buckthorn seed protein (SSP) has obvious hypoglycemic and anti-inflammatory effects.

Compared to diabetic control mice, diabetic mice treated with SSP had the lower BW, FBG levels, SIN and lipid content (Yuan *et al.*, 2016). In this paper we have taken effective methods to study the effects of SSP on the suppression of genes involved in liver glucose metabolism and on the upregulation of the AMPK/SIRT1 pathway in streptozotocin (STZ)-induced diabetic ICR mice.

MATERIALS AND METHODS

Materials

Seabuckthorn seeds, SPR (33.9%) and SPO (80%) were purchased from Qinghai Kang Pu Biotechnology Co., Ltd. Streptozotocin (STZ) was from Sigma Chemical Co. (Nanjing, China). The glimepiride and buffer solution were purchased from the Pharmaceutical Group Co., Ltd. (Anhui, China). Other reagents and chemicals were analytical grade.

Preparation of SSP

The methods on preparation and purification of SSP were performed with minor modification on the basis of the previous methods (Yuan *et al.*, 2016). Seabuckthorn seeds were crushed to remove oil, flavonoids and procyanidin. To remove oil, the seeds were mashed and passed through an 80 mesh screen, defatted with petroleum ether and dried in vacuum drying box. Flavonoids were detached through incubating seeds with 50% alcohol (1:40 w/v) for 2h at 60°C (Cao, 2004). Then the flavonoid content was determined to be 0.21%. Lastly, by alkaline extraction and acid precipitation, seabuckthorn seed protein (SSP) was prepared. Alkaline extraction was performed by incubating samples in water in a 1:14 ratio at 60°C and pH 11 for 1h, in succession, supernatant was obtained by centrifugation and separation. Acid precipitation was performed at pH 5, then by centrifugal separation, precipitate was achieved. The precipitate was washed several times with deionized water at pH 7, and finally dried by vacuum freeze.

Animals and experimental protocol

One hundred fifty male (23 $\pm$ 2g) ICR mice (SPF mouse) were acquired from the animal experiment center of Anhui Medical University. The animals were raised under

specified conditions (25°C, relative humidity 60 $\pm$ 10% and 12h light/dark cycle with free eating and drinking). Mice need to be acclimatized to diet for seven days under laboratory conditions. The experiments were performed in accordance with the animal experiment Committee of China and conform to national guidelines on the care and use of laboratory animals.

Experimental design

After a one-week adaptation and three-week high-fat feeding period, ICR mice were fasted for 12h and administered STZ (100mg/kg) soluble in cold sodium citrate buffer solution (pH 4.2-4.5) through intraperitoneal injection. Seven days after injecting STZ, hyperglycemia was measured by determining the glucose content of tail vein blood using a Roche Glucometer (Roche Diagnostics GmbH, Mannheim, Germany). In this experiment, the mice were divided into twelve groups on the basis of the fasting blood glucose and body weight (NC (normal control), DC (diabetic model control), PC (positive control), SPH (seabuckthorn seed protein high-dose group), SPM (seabuckthorn seed protein middle-dose group), SPL (seabuckthorn seed protein low-dose group), SPRH (seabuckthorn procyanidin high-dose group), SPRM (seabuckthorn procyanidin middle-dose group), SPRL (seabuckthorn procyanidin low-dose group), SPOH (seabuckthorn polysaccharide high-dose group), SPOM (seabuckthorn polysaccharide middle-dose group) and SPOL (seabuckthorn polysaccharide low-dose group)) in similar initial blood glucose and body weight (n=12 per group).

The mice in the NC and DC groups were supplied 4g/kg distilled water by gavage. The mice from PC group were given 4g/kg Glimepiride. The mice in the low, middle and high dose groups were administered once daily 50, 100, and 200mg/kg SPO, SPR, and SSP aqueous solutions, respectively, for four consecutive weeks. Ensuring that there is enough water in bottle every day, and the mice were weighed once per week. Blood glucose was also measured once a week. After the experiment, the mice were fasted overnight and killed by cervical dislocation. Blood was collected through the orbital venous plexus and rapidly centrifuged to obtain serum. Indicators were measured according to the manufacturer's instructions using a fully automated analyzer. The serum was stored at -20°C for later determination of AMPK, SIRT1 and serum insulin (SI) by double antibody sandwich step ELISA (enzyme linked immunosorbent assay) in strict accordance with the operating method in the ELISA Kit. The livers, spleens and kidneys were fleetly removed, weighed then immediately stored at -80°C.

Oral glucose tolerance test (OGTT)

After 4-week treatment with SSP, SPR and SPO, the mice were arranged to fast for 12h, then gave them oral glucose (2.0g/kg body weight). Blood glucose was determined at

0, 30, 60, 90, and 120 min respectively after oral glucose by determining tail vein glucose content with a Roche Glucometer (Roche Diagnostics GmbH, Mannheim, Germany).

Serum insulin resistance index

Frozen serum samples were thawed at 4°C and serum insulin was measured by using double antibody sandwich ELISA according to the manufacturer Insulin sensitivity index (ISI) was calculated according to the following formula:

$$ISI = \frac{1}{FBG \times FINS}$$

Insulin resistance index (IRI) was calculated based on the computational formula:

$$IRI = \frac{FBG \times FINS}{22.5}$$

Determination of AMPK and SIRT1 activity

Frozen liver samples (0.3g) were homogenized and incubated in 1.5ml of frozen homogenization buffer while centrifuging at 3,000rpm in an ice bath, and then centrifuged at 12,000xg for 5 min at 4°C. The supernatant was collected and used for detection of AMPK and SIRT1. AMPK and SIRT1 activity were determined using AMPK and SIRT1 double antibody sandwich ELISA kits in accordance with its instructions.

Semi-quantitative reverse-transcription (RT)-PCR

Total RNA was extracted from liver tissue using Trizol Reagent (“Invitrogen”, United States) and using a Nano Drop ND-1000 spectrophotometer to quantify RNA concentration. DNase-treated total RNA was converted into cDNA using Transcript First-Strand cDNA Synthesis SuperMix according to the manufacturer’s instructions. RT-PCR for each gene was performed using the following protocol: 95°C for 5 min, 35 cycles of 95°C for 30s, 58°C for 30s and 72°C for 1min, 72°C for 10 min. Primer sequences were designed with primer prime 5 Software. Primer sequences of G-6-P, GP, GSK-3 and CPT1-a gene are shown in table 1.

Table 1: Primer design

Primers		Primer sequences	Length
G-6-P	forward	AGTCGTTCCCATTCGCTT	280
	reverse	GGGTTTCAGAGGCTTTAGTTGTT	
GP	forward	GGGTTTCAGAGGCTTTAGTTGTT	430
	reverse	GTTGGTGTAGGCGAAGGTC	
GSK-3	forward	TACTACCGTGCTCCAGAACTCAT	366
	reverse	ATCGAAGAAGCTGTGGCA	
CPT1-a	forward	GTGTCCAAGTATCTGGCAGTC	318
	reverse	TCAGGGTATTTCTCAAAGTCAA	
β-actin	forward	CACGATGGAGGGGCCGACTCATC	250
	reverse	TAAAGACCTCTATGCCAACACAGT	

STATISTICAL ANALYSIS

Origin 6.1 was used to the present data analysis. All experimental data were statistically evaluated using the Student’s t-test and expressed as the means ± standard deviation. A value of p<0.05 was considered significant.

RESULTS

Total extraction content of seabuckthorn seed protein

The preparation of SSP as described above, after eliminating oil, flavonoids and proanthocyanidins, flavonoid and procyanidin content were determined to be 0.21%, 2%, respectively. Through the Kjeldahl method, the protein content was 70.1%.

Effect of SSP on the oral glucose tolerance test

After 4-week treatment of SSP, SPR and SPO, glucose tolerance was carried out for groups by OGTT method. fig. 1 shows the changes of blood glucose during OGTT (2 g glucose/kg body weight). The DC group showed an obviously stronger hyperglycemic response to oral glucose administration compared to NC mice. The blood glucose level of NC, PC and SSP-, SPR-, SPO-treated groups were significantly reduced (p < 0.05) than the MC group at each time point.

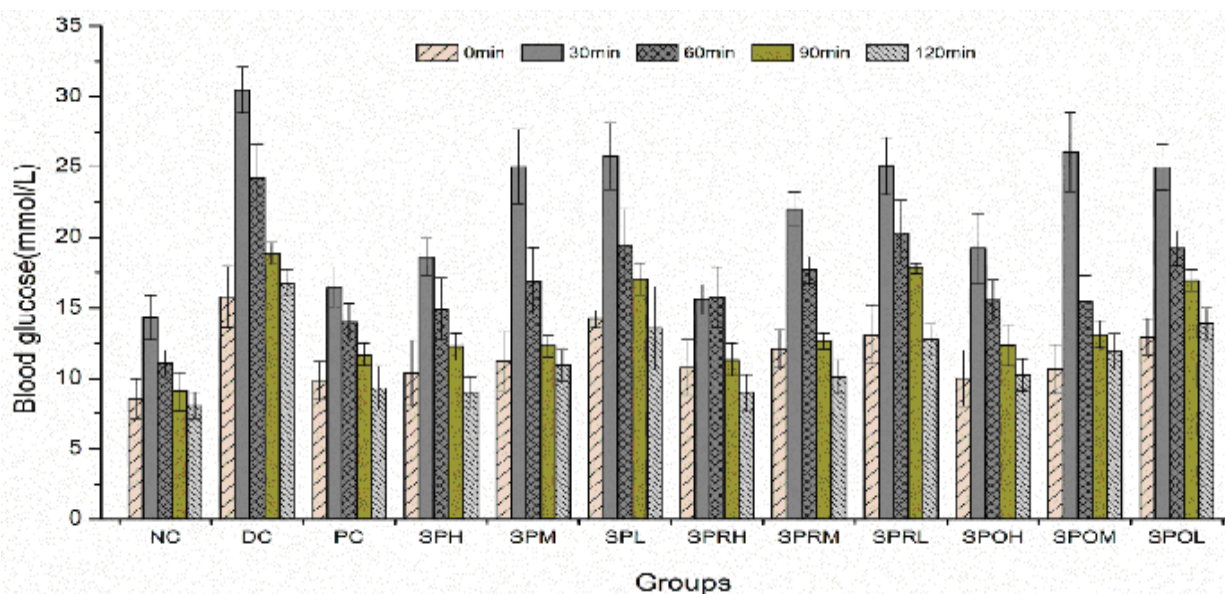
Effect of SSP on serum insulin

The serum insulin, insulin sensitivity index and insulin resistance index of each group is shown in table 2. The results show that the serum insulin level and IRI of the NC group were the lowest among all of the groups, whereas the ISI of this group was the highest. In contrast, the serum insulin level and insulin resistance index of the DC group were higher than in other groups, whereas ISI of this group was the lowest. Serum insulin levels and the insulin resistance index of the PC group were apparently higher than NC mice (P<0.01) and significantly lower than those of the DC group (P<0.01), which is demonstrative of type 2 diabetes in DC mice caused by insulin resistance.

Table 2: Levels of insulin resistance indexes (FINS, ISI and IRI) of the mice

Groups	FINS(mIU·L ⁻¹)	ISI	IRI
NC	16.20±0.33 ^b	-4.86 ±0.07 ^b	5.76±0.44 ^b
DC	34.72±0.73 ^a	-6.80±0.07 ^a	40.13±2.83 ^a
PC	21.75±0.21 ^{a,b}	-5.35±0.06 ^{a,b}	9.41±0.58 ^{a,b}
SPH	21.75±0.21 ^{a,b}	-6.07±0.04 ^{a,b}	19.21±0.86 ^{a,b}
SPM	24.56±0.01 ^{a,b}	-6.21±0.09 ^{a,b}	22.12±1.95 ^{a,b}
SPL	25.81±0.22 ^{a,b}	-6.42±0.06 ^{a,b}	27.43±1.62 ^{a,b}
SPRH	22.60±0.12 ^{a,b}	-6.32±0.04 ^{a,b}	24.61±0.91 ^{a,b}
SPRM	24.94±0.46 ^{a,b}	-6.42±0.06 ^{a,b}	27.35±1.57 ^{a,b}
SPRL	28.43±0.49 ^{a,b}	-6.61±0.06 ^{a,B}	32.92±1.89 ^{a,B}
SPOH	23.42±0.29 ^{a,b}	-6.24±0.06 ^{a,b}	22.86±1.29 ^{a,b}
SPOM	25.75±0.14 ^{a,b}	-6.35±0.09 ^{a,b}	25.41±2.22 ^{a,b}
SPOL	26.91±0.86 ^{a,b}	-6.35±0.06 ^{a,b}	25.51±1.50 ^{a,b}

Each value in the table represents the mean values ± standard deviation (n=12); a indicates (P<0.01) compared to the Normal control; A indicates (P<0.05) compared to the Normal control; b indicates (P<0.01) compared to the Model control; B indicates (P<0.05) compared to the Model control.


Fig. 1: Effect of SSP on OGTT in ICR mice (Data are expressed as means ± SD (n = 12)).

After 4 weeks of Glimepiride drug treatment, symptoms of insulin resistance in the PC group were improved. The serum insulin level and insulin resistance index of SSP, SPR and SPO treatment groups were significantly lower than those of the DC group (P<0.05), but significantly higher than in the NC group (P<0.05). The insulin sensitivity index of SSP, SPR and SPO treatment groups were apparently higher than the DC group, but obviously lower than the NC group.

Effect of SSP on AMPK and SIRT1 activity

The effects of SSP, SPR and SPO on AMPK and SIRT1 levels in liver tissue of diabetic mice are shown in Fig. 2. Compared with other mice, AMPK and SIRT1 activity were apparently higher in the DC group (p<0.01). AMPK

activity was also significantly higher in the SPOH group than in NC mice (p<0.01), while PC, SPRH and SPOM groups had no significant difference in AMPK activity compared with mice in the NC group (p>0.05). SPH, SPM, SPL and SPOL had significantly lower AMPK activity than NC mice (p<0.01). SIRT1 activity was not significantly different in PC, SPRH and SPOH groups compared with mice in the NC group (p>0.05), whereas SPH, SPM, SPL, SPRL, SPRM, SPOM and SPOL were significantly lower (p<0.05).

Effect of SSP on specific expression of G-6-P, GP, GSK-3 and CPT1-α in type 2 diabetic mice

RT-PCR results from liver tissue, shown in fig. 3, reveal that mice in the NC group had the lowest expression

levels of each of these genes, while the DC group had the highest expression levels. G-6-P, CPT1-a, GP and GSK-3 gene expression levels were reduced in SSP-, SPR- and SPO-treated mice. Specifically, gene expression levels in SPH, SPM, SPOM SPOH, SPRM and SPRH groups were significantly lower than in the DC group, and these results showed a dose-dependent response.

DISCUSSION

Fig. 1 stated clearly that SSP showed a marked enhancement in overall glucose response. table 2 indicates that the insulin resistance symptoms improved, but that the effects were not mild. Therefore, SSP, SPR and SPO can improve the insulin resistance symptoms of diabetic mice.

Previous studies on the AMPK/SIRT1/NF- κ B pathway have revealed that in the process of inflammatory response, SIRT1 activation is a result of enhanced NAD⁺ synthetase by AMPK. Subsequently, activated SIRT1 leads to acetylation of lysine residue 310 in NF- κ B RelA/p65 subunit (Cui *et al.*, 2006; Guo *et al.*, 2012). Previous studies have shown that early stage of insulin resistance in T2DM was associated with the occurrence of beta cell damage. The expression of cellular level of AMPK and SIRT1 were also found to be decreased. This reduction is deleterious owing to normal expression of the two are essential in maintaining steady sugar level in metabolic regulation (Chen *et al.*, 2010; Rodgers *et al.*, 2005).

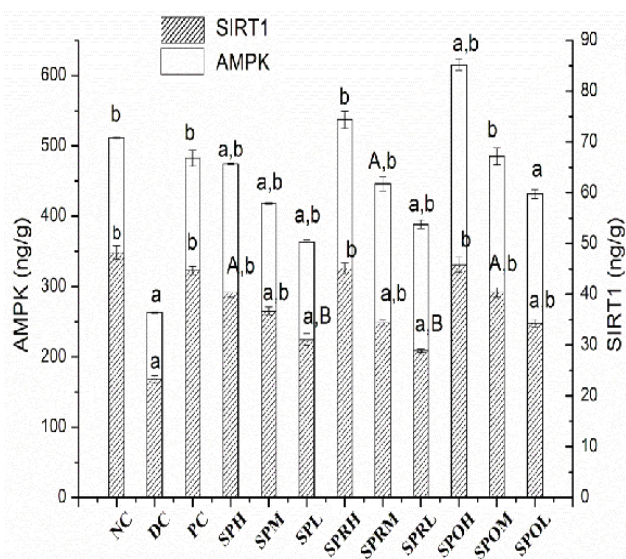


Fig. 2: Effect of SSP on AMPK and Sirt1 activity (n=12)

Each value in the table represents the mean values $\pm$ standard deviation; a indicates ($P < 0.01$) compared to the Normal control; A indicates ($P < 0.05$) compared to the Normal control; b indicates ($P < 0.01$) compared to the Model control; B indicates ($P < 0.05$) compared to the Model control.

Fig. 2 shows that SPO significantly affected the activity of liver AMPK and SIRT1 in diabetic mice. Mice that received SPO appeared to be hyperactive in their living space. The AMPK/SIRT1 pathway may be mediated through upregulating the activation of AMPK by SSP, SPR and SPO, which in turn could promote NAD⁺ synthetase activity and result in the activation of SIRT1. The results of the ELISA study are also consistent with previous animal studies (Yuan *et al.*, 2016; Chen *et al.*, 2010; Rodgers *et al.*, 2005). Therefore, treatment with SSP, SPR and SPO can induce activation of energy-sensing molecules AMPK and SIRT1, leading to the upregulation of the AMPK /SIRT1 pathway.

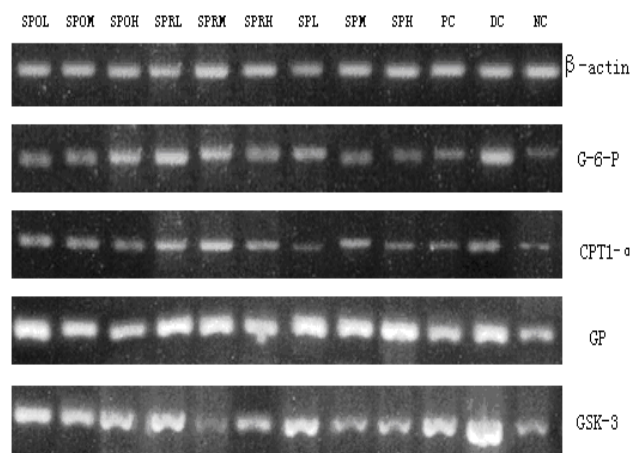


Fig. 3: Effect of SSP, SPR and SPO on gene expression of G-6-P, GP, GSK-3 and CPT1-a in type 2 diabetic mice, means normal control (NC), diabetic control (DC), the treated groups are shown.

Glucose-6-phosphatase (G-6-P) is an important enzyme in the metabolism of sugar and directly determines the steady state of the body's glucose levels. G-6-P is the final and rate-limiting step of gluconeogenesis and glycogen decomposition. Previous studies have shown that increased level of G-6-P gene expression is closely associated with the incidence of diabetes mellitus (Zhao 2006). Glycogen phosphorylase (GP) is a key enzyme affecting the rate of glycogen degradation in the liver, and inhibiting its activity can significantly reduce fasting plasma glucose concentrations in patients with type 2 diabetes (Chen and Yao, 2009). The main role of glycogen synthase kinase (GSK-3) is in regulating glucose transport and glycogen synthesis. Studies have shown that GSK-3 plays a large role in the pathogenesis of diabetes and insulin resistance (Henriksen 2010; Long and Zhou, 2012). Carnitine palmitoyl transferase1-a (CPT1-a) is a rate-limiting enzyme that is responsible for assisting long chain fatty acids to pass into the inner membrane of mitochondria. Decreased CPT1-a activity could result to the reduction of long chain fatty acids within mitochondria, which in turn negatively influences the energy transferring.

To decrease glucose production has become a new approach in treating type 2 diabetes. Since glucose-6-phosphatase catalyses the final step required for output of glucose from the liver, so inhibition of G-6-P would attenuate hepatic glucose production both gluconeogenesis and glycogen catabolic pathways. Inhibition of GSK-3 would stimulate the activation of glycogen synthetase (Moller 2001). GSK-3 can regulate the gene expression of rate-limiting enzyme G-6-P in gluconeogenesis pathway and G-6-P is the central enzyme in maintaining steady glucose level in our body, which in turn directly influences the activity of endogenous glucose (Lochhead *et al.*, 2001). GP is a key enzyme in the degradation of glycogen, and its activity directly affects glucose output. Inhibiting GP can reduce the degradation of glycogen and reduce hepatic production of glucose, which can in turn lower blood glucose. The experimental results show that the SSP, SPR and SPO can adjust the G-6-P, CPT1- α , GP and GSK-3 gene expression and indirectly regulate glycolipid metabolism, with previous findings. This may be related to the molecular mechanism of SSP, SPR and SPO, which improve the symptoms of diabetes in mice.

CONCLUSION

The present study plunks for the traditional utilizing of sea buckthorn (*Hippophae rhamnoides* L.) for treating diabetes. SSP had beneficial health effects in STZ-induced diabetic ICR mice. Significant changes in diabetic ICR mice were found, including improving oral glucose tolerance, lower insulin resistance, suppressing expression of genes involved in liver glucose metabolism, upregulating the AMPK/SIRT1 pathway. In summary, SSP diets can improve the symptoms of diabetes in ICR mice. In our future work, we will study the mechanisms of SSP effects on insulin resistance and the mechanism that toxins influence glucose metabolism in mice.

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