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Glucose-lowering activity of dark tea protein extract by modulating spleen–brain axis of diabetic mice

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Abstract

The present study aims to explore the glucose-lowering effects of the previously characterised dark tea (*Camellia sinensis* L.) protein extract (DTPE) from Heimaojian on the spleen–brain axis of diabetic mice. DTPE was orally administrated (50–100 mg/kg) to alloxan-induced mice for 21 d; a biochemical assay and transcriptome profiling (RNA sequencing (RNA-Seq)) were performed. The results showed that DTPE can improve glucose tolerance. Compared with the model group, at day 21, the fasting blood glucose values were significantly ($P < 0{\cdot}05$) decreased by 44·9 % (13·8 *v.* 7·6 mmol/l) and 51·4 % (13·8 *v.* 6·7 mmol/l) for high dose of DTPE (100 mg/kg) and drug metformin (125 mg/kg) groups, respectively. Subsequently, transcriptome profiling (RNA-Seq) was performed on the spleen and brain of diabetic mice. Totally, fifty-two spleen-derived and forty-seven brain-derived differentially expressed genes related to the synthesis, transport and metabolism of glucose were identified. The regulatory network analysis indicated that DTPE may exert glucose-lowering effects through a thirty-seven-gene sub-network related to metabolism, Parkinson's disease, oxidative phosphorylation and immunity. In summary, for the first time, the present data revealed that dark tea-derived DTPE could exert a potential anti-hyperglycaemic effect by modulating the spleen–brain axis.

Key words: **Dark tea: Protein extracts: Glucose-lowering: RNA sequencing: Spleen–brain axis**

Type 2 diabetes is a chronic endocrine disease characterised by high blood sugar (hyperglycaemia), insulin resistance and relative lack of insulin. Due to the existence of severe side effects for available anti-diabetic drugs, natural products have been considered as effective strategies and alternative medicine for diabetes management[1]. It is documented that tea and tea extracts have protective effects against diabetes by regulating various signalling pathways, including immunity enhancement and alleviating diabetes-induced damages of neural cells. Roghani & Baluchnejadmojarad[2] showed that lower glucose intolerance and higher insulin sensitivity were observed in pre-diabetic rats drinking white tea for 2 months. Islam[3] reported that white tea extract can exhibit anti-diabetic activity by reducing insulin resistance, followed by hyperlipidaemia and oxidative stress. Welch *et al.*[4] investigated the glucose-lowering activities of the bioactive polyphenols in kinkeliba tea (*Combretum micranthum*) by decreasing the expression of phosphoenolpyruvate carboxykinase mRNA and increasing glucose tolerance.

It is noted that the studies on anti-diabetic ingredients in tea and their extracts were mainly involved in polyphenols[5] or polysaccharides[6]; the glucose-lowering effect of tea-derived protein extract was rarely studied. In the previous study, the hypoglycaemic activity of the protein extract from dark tea (fermented tea) Heimaojian (HMJ) was investigated *in vitro*[7]. The results showed that the protein extract had potent enzyme inhibition activities, with IC_{50} (mg/ml) values of 0·0942 (SD 0·0023) and 0·1794 (SD 0·0204) for α-glucosidase and dipeptidyl peptidase, respectively. Moreover, the active peptides were identified from dark tea protein extract (DTPE), for example, TAELLPR, CGKKFVR, AVPANLVDLN VPALLK, VVDLVFFAAAK, MSLYPR and QGQELLPSDFK[8]. However, the *in vivo* activity and the underlying mechanism were not studied. The aim of this study was to investigate the glucose-lowering effects of HMJ protein extract on the spleen–brain axis in alloxan-induced diabetic mice, and the acting mechanism was explored by RNA sequencing (RNA-Seq)-based transcriptomics.

Materials and methods

Materials

Alloxan was purchased from Sigma Chemical Co. Metformin was from Yuanye Biotechnology Co. Ltd. The DTPE from HMJ was prepared in our laboratory[7]. Briefly, the milled (20 mesh)

Abbreviations: DTPE, dark tea protein extract; HMJ, Heimaojian; qPCR, quantitative PCR; RNA-Seq, RNA sequencing.

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HMJ leaves were extracted in ultrapure water bath for 30 min at 95°C, filtrated and concentrated to obtain the extracts, and the solution was subjected to dialysis (500 Da). Then, 5 % (w/v) activated carbon was added at 45°C for 30 min. After filtration, saturated ammonia sulphate solution was added to precipitate the protein at 4°C for 12 h, centrifuged at 6000 ***g*** for 20 min at 4°C and dialysed at room temperature for 24 h to eliminate micro-molecules.

Characterisation of dark tea protein extract

Amino acid composition of DTPE was assayed by an automatic amino acid analyzer (L-8900, Hitach Co. Ltd). Molecular weight distribution of DTPE was measured by gel permeation chromatography (Water Breeze, Waters). The main peptides of DTPE were characterised previously[8].

Animal experiment

Sixty SPF C57BL/6 male mice (8 weeks old, weighing about 22·4 (SD 3) g) were purchased from Jinan Pengyue experimental animal breeding Co. Ltd. and inspected by the Institute of medical experimental animals, Chinese Academy of Medical Sciences, license no. SCXK 2014 0007; feeding environment: temperature: 25°C, humidity: 60 %, light conditions: 12 h light–12 h dark alternately. During the experiment, the mice drank and ate freely and kept the living environment clean; the bedding was dry and sanitary, ventilated regularly and changed every day. After 1 week of adaptive feeding, mice were randomly divided into six groups. In order to examine the effects of DTPE on normal mice, the control (normal) group (0·9 % saline) and DTPE (100 mg/kg per d) + control group are set; other four groups include: model group (diabetic mice group, 0·9 % saline), low-dose DTPE (50 mg/kg per d) + model group, high-dose DTPE (100 mg/kg per d) + model group and positive drug (metformin, 125 mg/kg per d) + model group. Each group has ten mice, every five mice in a cage, with normal drinking and eating. The mice in the last four groups were unable to drink water for 6 h after fasting. After intravenous injection of alloxan (200 mg/kg per d, solved in citric acid–sodium citrate buffer (pH 4·5)) prepared with normal saline, the mice resumed eating and drinking water. After fasting for 6 h on the third day after injection, blood was collected from the tail tip and blood glucose was measured with a blood glucose meter. The diabetic model was successfully established with the blood glucose >11·1 mmol/l.

Subsequently, continuous administration by gavage was performed for 21 d (once per d in the afternoon, 0·2 ml of the gavage volume): 0·9 % saline for control (normal) group, HMJ protein extract (100 mg/kg per d) for DTPE + control group, 0·9 % saline for model group, HMJ protein extract (50 mg/kg per d) for low-dose DTPE + model group, HMJ protein extract (100 mg/kg per d) for high-dose DTPE + model group and metformin (125 mg/kg per d) for positive drug (metformin) + model group. During the experiment, the mice were fed with normal drinking water and their body weight and fasting blood glucose were recorded on the 5th, 7th and 14th day of the administration (fasting for 6 h before measurement). After 3 weeks of continuous administration, the mice were fasted for 12 h, weighed and killed. Five mice in each group of the model group and high-dose administration group were selected, and their brains and spleen were quickly removed. The extracted tissues were washed in normal saline, placed in a centrifuge tube, temporarily stored in dry ice and then transferred to a refrigerator at –80°C for further use.

Determination of glucose tolerance

The mice were fasted for 12 h before administration on the 21st day and then given 2·5 g/kg glucose by gavage after 3 h of administration. The blood glucose was detected by blood glucose detector at 0, 0·5, 1·0 and 2·0 h after administration.

RNA sequencing and bioinformatics analysis

Three biological replicates were used for all RNA-Seq experiments from each treatment. Total RNA were extracted using TRIzol® reagent (Invitrogen Life Technologies). RNA quality was assessed via agarose gel electrophoresis and NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). RNA integrity number was measured using the Agilent 2100 TapeStation system (Agilent Technologies). Libraries for RNA-Seq were prepared with a TruSeq® RNA LT/HT Sample Prep Kit according to the instructions of the manufacturer (Illumina). RNA-Seq was performed on an Illumina HiSeq 2500 with 2 × 100-bp paired-end reads. Quality trimming removed low-quality and ambiguous nucleotides of sequence ends and adapter contamination. The gene expression abundance was normalised by FPKM (fragments per kilobase of exon per million fragments mapped), and differentially expressed genes were analysed by Cufflinks software[9], with the threshold $\log_2$(fold change) >1 and q-value <0·05 as the criteria of significant difference.

Gene ontology and gene enrichment analysis were conducted by a PLAZA web tool (http://bioinformatics.psb.ugent.be/plaza/). The pathways that showed the most differentially expressed genes were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/). The interaction networks of differentially expressed genes were obtained using the STRING version 10 database (http://string-db.org/).

Real-time quantitative PCR verification

To verify the transcriptomic findings, seven genes related to glucose regulation and metabolism were selected for quantitative PCR (qPCR) analysis. The primers for mRNA were designed and synthesised by Sangon Biotech (China). *Rpl15*: Rpl15-F, GTAAACGCCCAGTTCCTAA, Rpl15-R, AATCTTCA CCAA CCCAGTAG; *Srebf2*: Srebf2-F, TCGACGAGATGCTACAGTTTG, Srebf2-R, TGGGACCTGGCTGA-ATGA; *Gdpd2*: Gdpd2-F, GGCAGGAGTGGCATAGTTT, Gdpd2-R, AGCAGC-GGCATCA GGTAG; *Pgam2*: Pgam2-F, CAACTACTACACCTCCATC-AGC, Pgam2-R, TCATTCCAGAAGGGCAAA; *Igfbp4*: Igfbp4-F, GCC TCACAGAG-CCGTACCCA, Igfbp4-R, CCCTGTCTTCCGAT CCACA; *Snca*: Snca-F, TTGTCA-AGAAGGACCAGAT, Snca-R, GCATTTCATAAGCCTCACT; *Hs3st4*: Hs3st4-F, AGGCTATCCG AGTCCACCC, Hs3st4-R, ATCTGCCCATCCAATGTCTT; *β*-actin: *β*-actin-F, AGCCATGTACGTAGCCATCC, *β*-actin-R, CTCTCAGC TGTGGTG-GTGAA. The cDNA synthesis was performed by the HiScript® Q RT SuperMix for qPCR (+gDNA wiper) according to

products manuals (Takara Biotechnology). Then, qPCR measurement was conducted on a CFX Touch™ Real-Time PCR Detection System (Bio-Rad) with ChamQ SYBR qPCR Master Mix (Takara Biotechnology Co. Ltd). The β-actin gene was used as a reference, and the fold change was quantified by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

SPSS 19.0 software was used for one-way ANOVA, and Duncan's model was used to analyse the significant differences between different samples. The results are expressed as means and standard deviations, and $P < 0{\cdot}05$ was considered to be significant.

Results

Amino acid composition and molecular weight distribution

The DTPE from HMJ was prepared in our laboratory[7]. The amino acid composition indicated that the top two high contents of amino acids were glutamic acid and aspartic acid, and basically no methionine. The molecular weight distribution showed that small molecular compounds were above 90 % (Table 1).

Table 1. Amino acid composition of dark tea protein extract

Amino acid composition					
Asp	Aspartic acid	11·7446	Ile	Isoleucine	3·0230
Thr	Threonine	4·6763	Leu	Leucine	5·4887
Ser	Serine	5·4502	Tyr	Tyrosine	2·1593
Glu	Glutamic acid	29·0842	Phe	Phenylalanine	3·2963
Gly	Glycine	8·2830	Lys	Lysine	5·1428
Ala	Alanine	5·7764	His	Histidine	1·3880
Cys	Cysteine	0·4453	Arg	Arginine	5·8297
Val	Valine	4·3732	Pro	Proline	3·8519
Met	Methionine	–			
Molecular weight distribution					
>10 kDa (%)			3–10 kDa (%)		<3 kDa (%)
8·26			53·46		38·28

Effect on body weight

The effects of DTPE on the weights of alloxan-induced hyperglycaemic mice are shown in Table 2. The growth rate of body weight in the model group was obviously slower than that in the normal control group, which was one of the typical symptoms of type 2 diabetes. The body weight growth rate of the control group fed with DTPE was slower than that of the normal control group, and the body weight growth rates of the high-dose group and the low-dose group were slower than that of the model group, which indicated that DTPE could inhibit the weight growth, although no obvious dose dependence was observed.

Effect on fasting blood glucose

The fasting blood glucose assay (Table 2) showed that after 21 d of experiment, the fasting blood glucose values in the high-dose DTPE + model group and the low-dose DTPE + model group were significantly different from that of the model group ($P < 0{\cdot}05$), and there was also significant difference between the model group and the control group ($P < 0{\cdot}05$). Moreover, there was no significant difference in the fasting blood glucose values between the DTPE + control group and the control group, indicating that proper administration of DTPE had no significant effect on the fasting blood glucose of normal mice. On the other hand, the decrease of fasting blood glucose in the low-dose DTPE + model group was smaller than that in the high-dose DTPE + model group, which reflected a certain dose relationship between blood glucose level and DTPE concentration. In particular, compared with the initial value, the fasting blood glucose values at day 21 were decreased by 67 % (23·3 *v.* 7·7 mmol/l), 73·3 % (28·5 *v.* 7·6 mmol/l) and 75·3 % (27·1 *v.* 6·7 mmol/l) for low-dose DTPE, high-dose DTPE and drug metformin groups, respectively. Compared with model group, at day 21, the fasting blood glucose values were decreased by 44·9 % (13·8 *v.* 7·6 mmol/l) and 51·4 % (13·8 *v.* 6·7 mmol/l) for high-dose DTPE and drug metformin groups, respectively. Notably, the used doses for the high-dose DTPE and drug metformin groups were 100 mg/kg and 125 mg/kg, respectively; this hints that the smaller dose of DTPE achieved similar glucose-lowering results to the drug metformin.

Changes in glucose tolerance

The changes of glucose tolerance in hyperglycaemic mice are shown in Table 2. At the beginning (0 h), except for the model group, there was no significant difference in blood glucose values among the groups. In the period of time (0·5–2 h), the DTPE groups exhibited an obvious trend to improve glucose tolerance and the blood glucose in the high-dose DTPE group was even better than that in the drug metformin group. Within 0–2 h, the blood glucose in the high- and low-dose DTPE groups was significantly lower than that in the model group, suggesting that the intake of DTPE was really helpful for the regulation of glucose tolerance in hyperglycaemic mice. Similarly, in the duration of 0–2 h, the blood glucose values in the normal control group were lower than those in the DTPE + normal control group; specially, at the time point of 1 h, the difference was significant (11·1 *v.* 8·5 mmol/l), hinting that the intake of DTPE in normal mice was also good for controlling the rise of blood glucose after eating.

RNA-sequencing analysis

In order to explore the glucose-lowering mechanism of DTPE in diabetic mice, RNA-Seq was performed in spleen and brain tissues of mice. Totally, 51 912 genes were detected in spleen or brain tissues, 175 or 514 of which had significant difference in expression for spleen or brain tissues, respectively. In particular, fifty-two spleen-derived or forty-seven brain-derived genes related to the synthesis, secretion, transport, metabolism and regeneration of sugars were screened out from all differentially expressed genes (Tables 3 and 4). For spleen tissue, twenty-eight genes were up-regulated, such as *Rnasek* (30·28-fold), *Anp32b* (32·67-fold), *mt-Cytb* (34·68-fold), *Rps13-ps1* (39·03-fold) and *Col19a1* (44·74-fold); and twenty-four genes were down-

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Table 2. Changes of body weight, fasting blood glucose and glucose tolerance in mice (Mean values and standard deviations; percentages)

		Body weight								
		0 d		7 d		14 d		21 d		
Group	Gavage dose	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Growth percentage
Normal	Saline (0·9 %)	21·11	0·33	23·96	0·62	25·67	0·33	28·25*	0·41	33·82
Model	Saline (0·9 %)	20·63	1·62	23·14	1·54	25·36	0·66	27·37	1·16	22·93
DTPE + normal	DTPE (100 mg/kg)	21·50*†	2·54	23·03	0·47	25·88	0·24	27·25*	1·54	26·74
High-dose DTPE + model	DTPE (100 mg/kg)	21·96*†	0·25	22·93	1·22	24·33*†	0·62	26·70*†	0·55	21·58
Low-dose DTPE + model	DTPE (50 mg/kg)	21·27	0·66	23·29	0·54	22·45*†	0·41	25·84*†	0·33	21·48
Drug+model	Metformin (125 mg/kg)	20·54	0·29	22·17	0·09	23·87*†	1·09	26·23*†	0·68	27·70

		Fasting blood glucose									
		Pre-model		1 d		7 d		14 d		21 d	
Group	Gavage dose	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Normal	Saline (0·9 %)	8·3	1·3	10·2*	3·2	7·5*	1·5	9·2*	1·8	8·5*	0·5
Model	Saline (0·9 %)	7·4	1·5	24·5†	3·7	17·7†	2·4	16·4†	0·9	13·8†	2·8
DTPE + normal	DTPE (100 mg/kg)	8·1	3·1	9·5*	2·1	8·8*	2·1	9·6*	1·4	8·8*	0·6
High-dose DTPE + model	DTPE (100 mg/kg)	9·4	1·2	28·5†	2·3	16·6†	2·5	9·1*	2·3	7·6*	2·0
Low-dose DTPE + model	DTPE (50 mg/kg)	7·9	2·5	23·3†	2·6	16·5†	1·1	12·0*	2·1	7·7*	0·6
Drug+model	Metformin (125 mg/kg)	8·6	2·4	27·1†	3·3	10·9*	1·6	9·5*	0·5	6·7*	1·1

		Glucose tolerance							
		0 h		0·5 h		1 h		2 h	
Group	Gavage dose	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Normal	Saline (0·9 %)	6·2*	2·2	10·4*	1·8	11·1*	2·4	8·6*	2·0
Model	Saline (0·9 %)	15·7†	1·2	26·5†	2·3	31·4†	2·5	21·9†	2·3
DTPE + normal	DTPE (100 mg/kg)	5·4*	1·8	9·5*	1·8	8·5*†	0·4	7·8*	2·1
High-dose DTPE + model	DTPE (100 mg/kg)	7·4*	0·7	11·8*	1·2	8·4*†	1·2	8·2*	0·8
Low-dose DTPE + model	DTPE (50 mg/kg)	7·8*	2·3	15·9†	1·2	10·4*	0·2	9·0*	1·6
Drug+model	Metformin (125 mg/kg)	6·4*	1·5	11·3*	1·2	11·0*	2·3	9·4*	1·3

DTPE, dark tea protein extract.
* Significant ($P < 0·05$) compared with the model group.
† Significant ($P < 0·05$) compared with the normal group.

regulated, for instance, *Try5* (−63·69-fold), *Try4* (−42·92-fold), *Clps* (−13·64-fold) and *Gp2* (−11·93-fold). For brain tissues, fifteen genes were up-regulated and thirty-two genes were down-regulated, for example, *Ly6g6d* (4·06-fold), *Lingo4* (4·33-fold), *Crybg3* (5·17-fold); *Cpne7* (−8·13-fold), *Pcsk9* (−6·02-fold) and *Lcn2* (−5·87-fold). Thus, DTPE caused more remarkable changes in spleen than in brain, although more differential genes were observed in brain than in spleen.

Gene ontology enrichment and Kyoto Encyclopedia of Genes and Genomes pathway analyses

Gene ontology enrichment analysis of differential genes in spleen presented that the most significantly enriched functions or processes ($P < 0·001$) were cell surface receptor signalling pathway, regulation of multi-cellular organismal development and regulation of developmental process for spleen. Kyoto Encyclopedia of Genes and Genomes pathway analysis indicated that the differential genes were mainly involved in human disease pathway, biological system pathway, metabolic pathway and environmental information processing pathway. In the biological system, genes with significant differences were closely related to immune system, digestive system and endocrine system (Fig. 1(a)). Similarly, for brain, many functions or processes were significantly enriched ($P < 0·001$), primarily related to signalling (single organism signalling, anterograde trans-synaptic signalling, synaptic signalling, cell–cell signalling) and channel activity (ion channel activity, cation channel activity, substrate-specific channel activity). Kyoto Encyclopedia of Genes and Genomes pathway analysis showed similar results to spleen, and in the biological system, the differential genes were closely related to the immune system, digestive system and circulatory system (Fig. 1(b)).

Interacting network analysis

Network analysis demonstrated that the differential genes in spleen were primarily mapped into two sub-networks (Fig. 1(c)): eight-gene sub-network (*Mt-Co3*, *mt-Cytb*, *mt-Nd1*, *mt-Nd2*, *mt-Nd3*, *mt-Nd4*, *mt-Nd5*, *mt-Nd6*) related to Parkinson's disease, thermogenesis, oxidative phosphorylation and metabolic pathways, and sixteen-gene sub-network (*Gp2*, *Clps*, *Try5*, *Try4*, *Retn*, *Mmp8*, *Adamts5*, *Fabp4*, *Acsl1*, *Tnf*, *Ccl22*, *Ltb*, *Bcl3*, *Dydc2*, *Cd79a*, *Vpreb3*) related to metabolism and degradation of the extracellular matrix. The differential genes in brain were primarily mapped into two sub-networks (Fig. 1(d)): four-gene sub-network (*H2-DMb2*, *H2-DMa*, *H2-Ab1*, *H2DMb1*) related to type 1 diabetes mellitus and intestinal immune network for IgA production, and seven-gene sub-network (*Pcsk6*, *Lpl*, *Ldlr*, *Pcsk9*, *Igfbp4*, *Igf1*, *Igfbpl1*) related to

Table 3. Differential genes in spleen

Gene names	C_P	T_P	T_P/C_P	Log2FC (T_P/C_P)	*P*	Significant	Regulation
Try5	8·27	0·13	0·0157	−5·9913	0·0003	Yes	Down
Try4	13·19	0·31	0·0233	−5·4265	0·0000	Yes	Down
Clps	21·01	1·54	0·0733	−3·7701	0·0006	Yes	Down
Gp2	2·19	0·18	0·0838	−3·5765	0·0000	Yes	Down
Adamts5	2·44	0·56	0·2278	−2·1339	0·0000	Yes	Down
Stard13	1·67	0·47	0·2840	−1·8162	0·0001	Yes	Down
Sema3d	2·21	0·83	0·3750	−1·4150	0·0001	Yes	Down
Mmp8	0·77	0·32	0·4131	−1·2755	0·0004	Yes	Down
Crispld1	0·80	0·35	0·4352	−1·2003	0·0001	Yes	Down
Dydc2	0·55	0·24	0·4390	−1·1877	0·0007	Yes	Down
Lrrn1	1·03	0·46	0·4434	−1·1733	0·0001	Yes	Down
Sorbs2	6·33	3·64	0·5748	−0·7988	0·0005	Yes	Down
Flt1	3·08	2·04	0·6613	−0·5967	0·0004	Yes	Down
Acacb	1·47	1·22	0·8299	−0·2689	0·0003	Yes	Down
Sdpr	2·82	2·57	0·9091	−0·1375	0·0001	Yes	Down
Mrc1	5·76	5·66	0·9826	−0·0253	0·0000	Yes	Down
Gsta3	1·43	1·63	1·1422	0·1918	0·0001	Yes	Up
Gdpd2	9·06	10·88	1·2005	0·2637	0·0000	Yes	Up
Adamts1	0·73	0·89	1·2247	0·2924	0·0003	Yes	Up
Errfi1	5·00	7·22	1·4447	0·5307	0·0000	Yes	Up
Lrp1	14·05	20·87	1·4852	0·5706	0·0008	Yes	Up
Bmp2	0·62	0·95	1·5351	0·6183	0·0007	Yes	Up
Gcnt2	6·93	10·77	1·5541	0·6361	0·0000	Yes	Up
Col4a2	3·13	4·90	1·5665	0·6476	0·0000	Yes	Up
Rpl15	8·18	16·64	2·0330	1·0236	0·0002	Yes	Up
Srebf2	3·40	37·14	10·9119	3·4478	0·0003	Yes	Up
Ffar1	0·58	6·51	11·2241	3·4885	0·0000	Yes	Up
Lat2	4·28	54·93	12·8432	3·6829	0·0005	Yes	Up
Ptp4a3	5·09	66·56	13·0773	3·7090	0·0003	Yes	Up
Thy1	8·54	115·40	13·5133	3·7563	0·0008	Yes	Up
mt-Nd5	77·68	1258·10	16·1952	4·0175	0·0006	Yes	Up
Fscn1	1·92	31·42	16·3910	4·0348	0·0003	Yes	Up
Bcl3	1·89	31·19	16·5333	4·0473	0·0005	Yes	Up
Exosc6	0·50	8·84	17·6734	4·1435	0·0002	Yes	Up
Fam189b	1·54	30·04	19·5505	4·2891	0·0005	Yes	Up
mt-Nd4	110·89	2249·90	20·2889	4·3426	0·0003	Yes	Up
Mbd3	2·61	53·55	20·5445	4·3607	0·0005	Yes	Up
mt-Nd1	202·60	4197·58	20·7185	4·3729	0·0002	Yes	Up
Il4i1	2·82	60·22	21·3796	4·4182	0·0003	Yes	Up
Egr3	0·68	15·93	23·4313	4·5504	0·0000	Yes	Up
Ltb	15·02	352·05	23·4387	4·5508	0·0002	Yes	Up
Ccl22	0·25	5·99	23·6478	4·5636	0·0000	Yes	Up
Lsm2	1·42	34·54	24·2652	4·6008	0·0008	Yes	Up
Fbl	4·18	101·80	24·3549	4·6061	0·0005	Yes	Up
Gm28661	532·55	13 214·98	24·8144	4·6331	0·0004	Yes	Up
Cd79a	22·71	613·83	27·0328	4·7566	0·0004	Yes	Up
Vpreb3	2·56	73·35	28·6906	4·8425	0·0000	Yes	Up
Rnasek	3·11	94·27	30·2808	4·9203	0·0008	Yes	Up
Anp32b	4·53	147·90	32·6721	5·0300	0·0001	Yes	Up
mt-Cytb	152·66	5294·19	34·6804	5·1160	0·0001	Yes	Up
Rps13-ps1	19·18	748·66	39·0268	5·2864	0·0001	Yes	Up
Col19a1	37·04	1657·27	44·7426	5·4836	0·0000	Yes	Up

regulation of insulin-like growth factor transport and uptake, and plasma lipoprotein assembly, remodelling and clearance. Interestingly, all the differential genes in spleen and brain were primarily mapped into a big thirty-seven-gene sub-network (Fig. 1(e)) related to metabolism, Parkinson's disease, oxidative phosphorylation and thermogenesis.

Verification of selected genes by quantitative PCR

Finally, among the genes with significant difference in expression, the genes with high expression and high correlation with glucose metabolism and fat metabolism were selected and verified by qPCR. Seven target genes were screened out, including three genes from spleen: *Rpl15*, *Srebf2*, *Gdpd2*; four genes from brain: *Pgam2*, *Igfbp4*, *Snca*, *Hs3st4*; and *β*-actin was the reference gene. Fig. 2 indicates that among the seven target genes, four genes had the same variation trend: *Rpl15*, *Gdpd2*, *Igfbp4*, *Snca*.

Discussion

Plants are considered as one of the best sources of diabetic therapy. An increasing number of studies have demonstrated that plants and their extracts are hypoglycaemic agents. In our

Table 4. Differential genes in brain

Gene names	C_N	T_N	T_N/C_N	Log2FC(T_N/C_N)	*P*	Significant	Regulation
Cpne7	41·1033	5·055	0·1230	−3·0235	0·0034	Yes	Down
Pcsk9	1·1733	0·195	0·1662	−2·5890	0·0027	Yes	Down
Lcn2	8·4867	1·445	0·1703	−2·5541	0·0000	Yes	Down
Pgam2	9·33	2·015	0·2160	−2·2111	0·0000	Yes	Down
Alox12b	3·23	0·745	0·2307	−2·1162	0·0005	Yes	Down
Msantd1	3·85	0·965	0·2506	−1·9963	0·0024	Yes	Down
Igfbpl1	0·9533	0·32	0·3357	−1·5749	0·0004	Yes	Down
Ugt1a6a	2·1533	0·725	0·3367	−1·5705	0·0002	Yes	Down
Igfbp4	39·5767	14·125	0·3569	−1·4864	0·0000	Yes	Down
Hs3st4	21·5767	7·78	0·3606	−1·4716	0·0000	Yes	Down
Lpl	5·1567	1·915	0·3714	−1·4291	0·0003	Yes	Down
Snca	262·7466	98·355	0·3743	−1·4176	0·0000	Yes	Down
Lrg1	6·5533	2·54	0·3876	−1·3674	0·0025	Yes	Down
Lrrc6	3·82	1·515	0·3966	−1·3343	0·0005	Yes	Down
Ccdc87	1·4367	0·58	0·4037	−1·3086	0·0011	Yes	Down
H2-DMa	9·38	4·015	0·4280	−1·2242	0·0003	Yes	Down
Stpg1	2·23	1·025	0·4596	−1·1214	0·0037	Yes	Down
Col25a1	7·46	3·445	0·4618	−1·1147	0·0000	Yes	Down
Plppr1	3·71	1·745	0·4704	−1·0882	0·0002	Yes	Down
Plppr4	37·4233	17·9	0·4783	−1·0640	0·0000	Yes	Down
Pld3	315·2267	151·335	0·4801	−1·0586	0·0000	Yes	Down
Susd1	4·49	2·165	0·4822	−1·0523	0·0000	Yes	Down
Khdrbs3	88·5933	42·73	0·4823	−1·0519	0·0000	Yes	Down
Mir22hg	16·87	8·27	0·4902	−1·0285	0·0000	Yes	Down
Ociad2	44·65	21·995	0·4926	−1·0215	0·0000	Yes	Down
Gcnt2	7·0233	3·49	0·4969	−1·0089	0·0000	Yes	Down
Bend5	9·4767	4·765	0·5028	−0·9919	0·0000	Yes	Down
Adgrb2	112·6433	57·615	0·5115	−0·9672	0·0000	Yes	Down
Dclk3	10·2067	5·38	0·5271	−0·9238	0·0000	Yes	Down
Sh3rf1	8·25	4·37	0·5297	−0·9168	0·0000	Yes	Down
Vstm2b	30·03	16·11	0·5365	−0·8984	0·0000	Yes	Down
Pcsk6	13·7867	32·92	2·3878	1·2557	0·0000	Yes	Up
Trim67	3·13	8·035	2·5671	1·3601	0·0000	Yes	Up
Vwa7	1·69	4·545	2·6893	1·4273	0·0000	Yes	Up
Lrrc2	0·9433	2·545	2·6980	1·4319	0·0001	Yes	Up
Svep1	1·1467	3·165	2·7601	1·4647	0·0001	Yes	Up
Gprc5c	7·8167	22·905	2·9303	1·5510	0·0000	Yes	Up
Ptar1	1·29	3·925	3·0426	1·6053	0·0017	Yes	Up
Skor1	1·7633	5·39	3·0568	1·6120	0·0018	Yes	Up
Gpr63	6·0933	19·25	3·1592	1·6596	0·0000	Yes	Up
Slc5a1	0·96	3·16	3·2917	1·7188	0·0001	Yes	Up
Slc5a1	0·96	3·16	3·2917	1·7188	0·0001	Yes	Up
Ccdc63	1·46	5·47	3·7466	1·9056	0·0003	Yes	Up
Ly6g6d	1·76	7·14	4·0568	2·0203	0·0039	Yes	Up
Lingo4	0·8467	3·67	4·3345	2·1159	0·0000	Yes	Up
Crybg3	0·1567	0·81	5·1691	2·3699	0·0036	Yes	Up

study, the DTPE from HMJ reduced by 44·9 % of blood glucose at 100 mg/kg, which was close to the drug metformin that reduced by 51·4 % at 125 mg/kg, after 21 d of administration to diabetic mice. Previous studies indicated that *Annona muricata* aqueous extract (100 mg/kg) could significantly reduce by 72·6 % of blood glucose level on day 21[10]. The aqueous extract of *Fagonia cretica* caused a 45 % decrease in the plasma glucose level at the end of the experimental period (21 d) with the dose of 500 mg/kg[11]. *Sarcostemma brevistigma* extract (250 mg/kg) was found to reduce by 17·9 % blood glucose at day 21[12]. Green tea consumption (2 g/kg) reduced 14·3 % of blood glucose in 21 d of diabetic rats[13]. After being intragastrically administered with 100 mg/kg of Fuzhuan Brick-Tea extract for 4 weeks, 33 % of the serum glucose levels of diabetic mice were reduced[14]. This means that 5-fold lower dose of DTPE achieved similar result to *F. cretica* extract; 2-fold lower dose of DTPE achieved 2-fold higher glucose-lowering effect than *S. brevistigma* extract; while the same dose of DTPE generated smaller effect than *A. muricata* extract, but compared with their initial values, DTPE possessed similar result to *A. muricata* extract, which reduced by 73·3 % and 73·8 % of blood glucose at day 21 with the dose of 100 mg/kg, respectively. Compared with tea or tea extract, DTPE is similar to Fuzhuan Brick-Tea extract, but much more potent than green tea.

Recently, the role of the gut–brain axis in the management of many diseases has been the focus of much research activity. However, although a few studies reported the effect of plant extracts on microbiota (not gut–brain axis) in the diabetic animal model[15], the anti-diabetic effects of plant extracts based on the gut–brain axis were rarely explored. Duca *et al.*[16] indicated that the widely used drug metformin was shown to suppress glucose production through the gut–brain axis. Xu *et al.*[17] demonstrated that silibinin, the pre-dominant component of silymarin (approximately 80 %) extracted from the seeds of milk thistle (*Silybum*

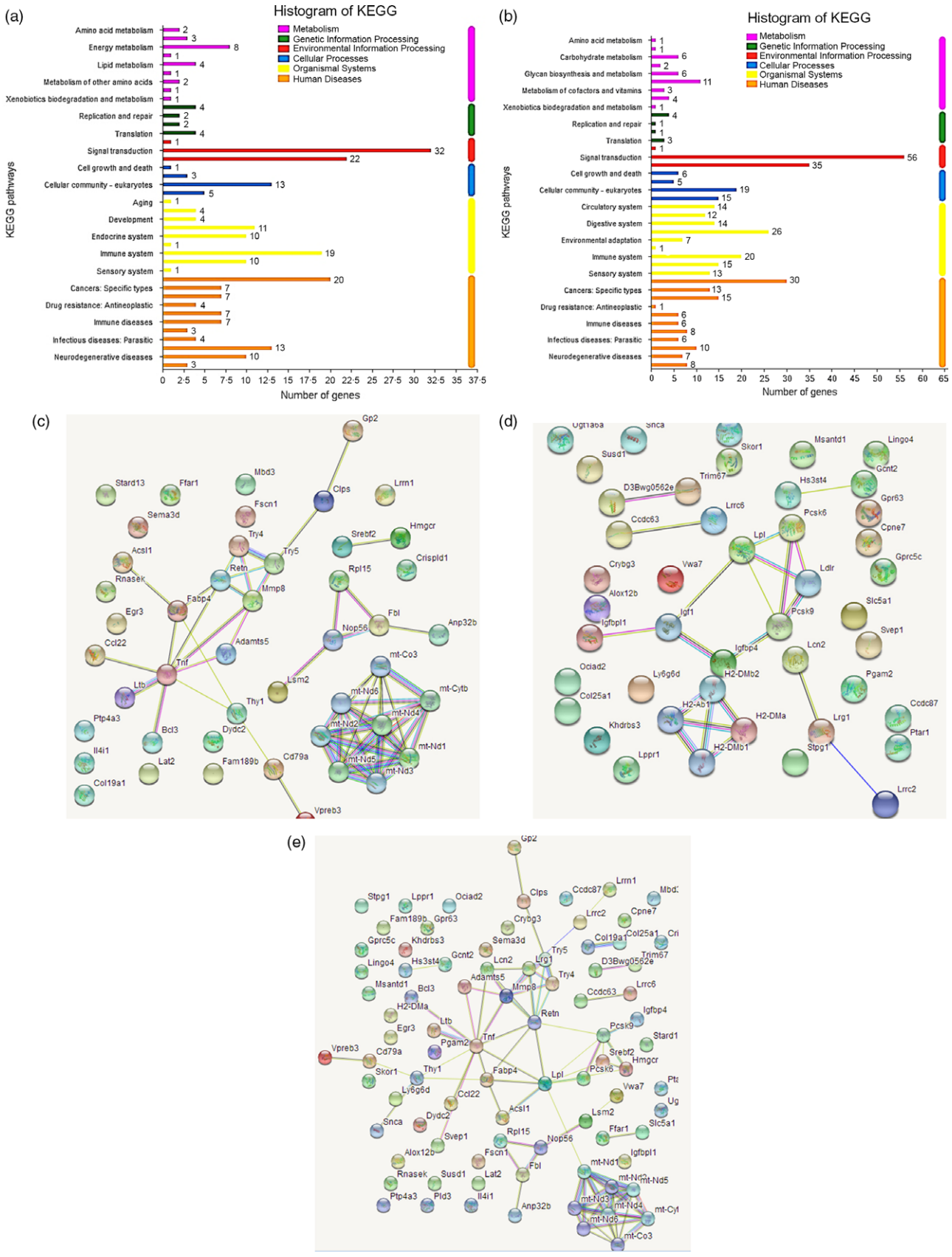

Fig. 1. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway diagrams of differentially expressed genes in spleen (a) and brain (b). Interacting networks of differentially expressed genes in spleen (c), brain (d), both spleen and brain (e).

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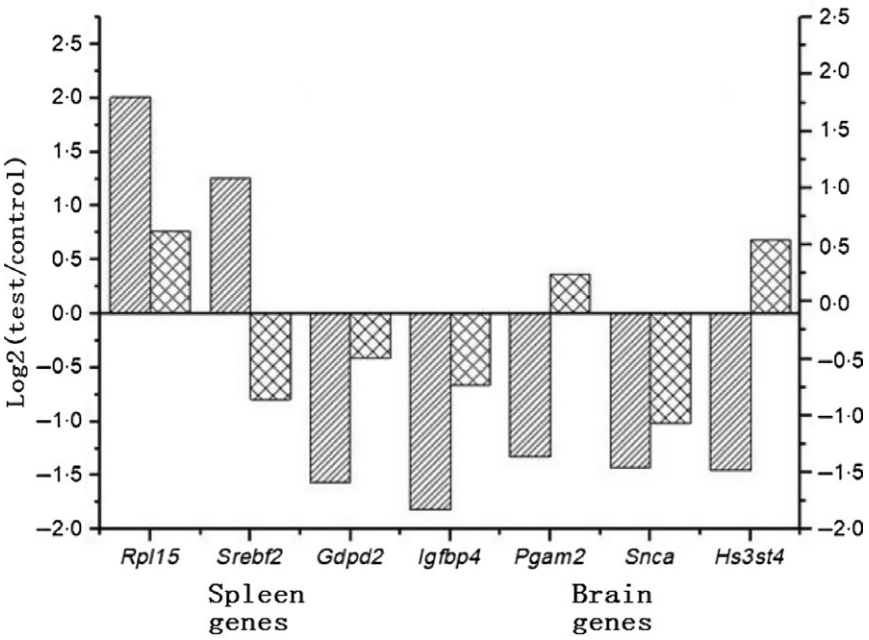

Fig. 2. Comparison between RNA sequencing (RNA-Seq, ▨) and quantitative PCR (▩) for the expressions of seven genes.

marianum), decreased hepatic glucose production through the activation of gut–brain–liver axis in diabetic rats.

A main novelty of this study was that the preliminary acting mechanism of DTPE was explored from the unique perspective of spleen–brain axis, for the first time, using transcriptomic technology (RNA-Seq). Consequently, fifty-two spleen-derived and forty-seven brain-derived differentially expressed genes related to the synthesis, transport and metabolism of glucose were identified. In particular, some markedly changed genes deserve attention. For example, in spleen tissue, the 34·68-fold up-regulated mt-Cytb (cytochrome b) is used for ATP synthesis as a part of the mitochondrial respiratory chain, which was observed to be markedly reduced in the mice of diabetic cardiomyopathy compared with controls[18]; the 32·67-fold up-regulated Anp32b (acidic leucine-rich nuclear phosphoprotein 32 family member B) works as a cell cycle progression factor as well as a cell survival factor, which appears to play a role in regulating adequate adaptive immune responses[19]; the 23·6-fold up-regulated Ccl22 (C-C motif chemokine 22) plays an important role in the collaboration of dendritic cells and B-lymphocytes with T-cells in immune responses, which protects mice from diabetes[20]; in brain tissue, the 5·87-fold down-regulated Lcn2 (neutrophil gelatinase-associated lipocalin-2) is an acute-phase protein known to promote neuroinflammation via the recruitment and activation of immune cells, which was found significantly higher in patients with type 2 diabetes[21] and plays the critical role in the pathogenesis of diabetic encephalopathy due to the fact that induction of diabetes increased the expression of both Lcn2 mRNA and protein in the hippocampus[22]. These results suggest that DTPE exerted anti-diabetic effect partly by modulating energy-related or immune-related genes on the spleen–brain axis, except for diabetes-related genes.

Further gene-interacting network analysis displayed that the obviously distinct networks were observed in spleen and brain tissues; this also reflects the complexity in the pathogenesis of diabetes. Indeed, many pathways were involved: enzyme activity, insulin resistance, hyperglycaemia, oxidative stress, endothelial dysfunction, cytokine expression, immunity and damages of neural cells[5]. Notably, taking all the differential genes in spleen and brain into consideration, they were mapped into a unified network. This suggests that the genes in spleen and brain acted as a whole to synergistically modulate blood glucose in mice in response to DTPE intervention, although these genes were separately located in different tissues. However, the specific communication mechanism between spleen and brain tissues deserves further study.

In summary, the present data confirmed that the protein extract from dark tea HMJ possessed significant glucose-lowering activity in type 2 diabetic mice. However, these results were based on animal model only; the detailed human study is needed. Subsequently, RNA-Seq analysis revealed that the preliminary acting mechanism was associated with differentially gene regulation on the spleen–brain axis. Further investigations are necessary to isolate and purify individual bioactive compounds so as to test their anti-diabetic effect individually and to discover the detailed communication mechanism between spleen and brain.

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The amount of dark tea protein extract used in animal studies could reasonably be expected to be achieved in the human population.

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