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EXPERIMENTAL STUDY

Gene expression profiling of rat livers with *Yin*-deficiency-heat syndrome

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Abstract

OBJECTIVE: To explore the nature of "*Yin* internal heat caused by *Yin*-deficiency," in terms of the theory of Traditional Chinese Medicine, by studying energy metabolism in rats with *Yin*-deficiency-heat syndrome and analyzing the gene expression profile of their livers.

METHODS: A *Yin*-deficiency-heat syndrome model was induced in rats using three Chinese medicinal herbs. Glycogen and triglycerides in blood plasma, and the enzyme activity of ATP in livers were measured colorimetrically. Triiodothyronine (T_3), thyroxine (T_4), and thyroid stimulating hormone levels in blood plasma were also measured with enzyme linked immunosorbent assay. The gene expression profile of livers was detected with gene chip analysis. Differentially expressed genes were screened out and classified according to Gene Ontology. The accuracy of results were examined with reverse transcription-polymerase chain reaction.

RESULTS: Compared with the control group, body weight ($P<0.05$) and hepatic glycogen ($P<0.05$) were significantly lower in the *Yin*-deficiency-heat syndrome group. Moreover, toe temperature ($P<0.01$) and triglyceride ($P<0.05$), Na^+-K^+-ATPase ($P<0.01$), Mg^{2+}-ATPase ($P<0.01$), T_3 ($P<0.05$), and T_4 ($P<0.01$) levels were significantly higher. There were 99 differentially expressed genes in livers from the *Yin*-deficiency-heat syndrome group. Genes were mainly related to sterol synthesis (Pc=0.0392), defense response (Pc=0.0448), and sterol metabolism (Pc=0.0533).

CONCLUSION: Abnormal expression genes in rats with *Yin*-deficiency-heat syndrome prompted the synthesis and metabolism of cholesterol, increased energy consumption, and reduced defense response. This gene expression might be the molecular mechanism underlying "internal heat caused by *Yin*-deficiency" in the rats with *Yin*-deficiency-heat syndrome.

Key Words: *Yin* deficiency; Deficiency heat; Oligonucleotide array sequence analysis

INTRODUCTION

Heat and deficiency syndromes are common in Traditional Chinese Medicine (TCM) practice. *Yin*-deficiency-heat syndrome belongs both to heat and deficiency syndromes. In TCM theory, "physical life is the balance of *Yin* and *Yang*."[1] If *Yin* is deficient and cannot counterbalance *Yang*, it will lead to an excess of *Yang*, and vice versa. Therefore, the excessive consumption of

Yin will lead to the pathological condition "*Yin* deficiency and *Yang* hyperactivity" or "internal heat due to *Yin* deficiency."

Research on *Yin*-deficiency-heat syndrome reported that energy metabolism increased in rats with *Yin*-deficiency-heat syndrome. The rats' production capacity and energy dissipation increased as well.[2] Another study also reveled that cellular immunity decreased in patients with *Yin*-deficiency-heat syndrome.[3] It was also reported that an increase in Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β), and Interleukin-6 (IL-6) levels was associated with vexing heat in the chest, palms, and soles of patients with *Yin*-deficiency heat syndrome.[4] The nature of *Yin*-deficiency-heat syndrome is related to the disturbance of the cytokine network induced by the increase of Interleukin-1(IL-1) and Tumor Necrosis Factor (TNF).[5] In this study, a systematic pharmacological experiment was applied and gene chip technology was used to explore the nature of internal heat induced by *Yin* deficiency.

METHODS

Experimental animals

Twenty SPF Wistar rats, half male and half female, weighing (200±20) g, were purchased from the experimental animal center of Shandong Lukang Pharmaceutical Co. Ltd. (Jining, China). The animal certification number was SCXK (Lu)20080002.

Herbs and reagents

Fuzi (*Radix Aconiti Lateralis Preparata*) was harvested in Jangyou, Sichuan province; Rougui (*Cortex Cinnamomi Cassiae*) in Dayao, Guangxi province; and Ganjiang (*Rhizoma Zingiberis*) in Qianwei, Sichuan province. Plants were identified by an expert of Chinese materia medica. ATPase assay (Lot No. 20090821) and glycogen kits (Lot No. 20100610) were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Triglyceride test was obtained from North of Clinical Reagent Co, Ltd. Hong Thai (Beijing, China). One Touch Ultra blood glucose test strips (Lot No. 2897340) were obtained from Johnson and Johnson Co, Ltd. (New Brunswick, NJ, USA). Rat triiodothyronine (T_3), thyroxine (T_4), and thyroid stimulating hormone (TSH) enzyme linked immunosorbent assay (ELISA) kits were purchased from Alpha Diagnostic Intl., Inc. (San Antonio, Tx, USA). Rat Ref-12 v1 Expression BeadChip (Bar Code 4562019003) was provided by Illumina Co, Ltd. (Santiago, CA, USA).

Procedure and data collection

Animals were randomly divided into a control group (n=10) and a model group (n=10). The decoction was prepared with Fuzi (*Radix Aconiti Lateralis Preparata*), Rougui (*Cortex Cinnamomi Cassiae*), and Ganjiang (*Rhizoma Zingiberis*) in the ratio 1∶1∶1. The decoction was intragastrically administered to the treatment group at 32 $g/kg^{-1} \cdot day^{-1}$ for 14 days. The rectal temperature and toe temperature were measured on the day before the experiment, and the 7th and 14th days after the model establishment.

After the treatment course was finished, the animals fasted for 8 h. Then, blood samples were taken from the abdominal aorta. Hepatic glycogen and plasma triglycerides were examined with a chemical colorimetric method. The levels of Triiodothyronine (T_3), thyroxine (T_4), and thyroid stimulating hormone (TSH) in blood plasma were measured with enzyme-linked immuno sorbent assay (ELISA). The activities of Na^+-K^+-, Mg^{2+}-, Ca^{2+}-, and Ca^{2+}-Mg^{2+}-ATPases were examined colorimetrically.

After sacrifice, the rats' livers were harvested at 4℃ and frozen in liquid nitrogen. The whole genome expression of livers was examined with a gene chip. The total RNA in hepatic tissue was extracted with unizol. The amount and quality of RNA was verified with a GeneQuant Pro and 1% agarose gel electrophoresis. RNA amplification was carried out according to the Illumina® TotalPrep RNA amplification kit.

(a) The first strand complementary Deoxyribonucleic acid (cDNA) was synthesized with the T7 Oligo (dT) by reverse transcription; (b) The second strand cDNA was synthesized by converting the single-stranded cDNA into a double-stranded DNA (dsDNA) template for transcription; (c) cDNA was purified by removing RNA, primers, enzymes, and salts; (d) complementary Ribonucleic Acid (cRNA)was transcribed and synthesized *in vitro*; (e) cRNA was purified and ready for use with Illumina's array kit by removing unincorporated Nucleoside triphosphates (NTPs), salts, and enzymes. The probes were labeled with Cy3-labeled 2'-Deoxyuridine 5'- Triphosphate (Cy3-dUTP). After hybridizing, washing, and staining, the chip was scanned by an Illumina 500 system Beadstation scanner and the acquired image was analyzed using Illumina BeadStudio software (Illumina,Inc., San Diego, CA, USA).

RT1-Ba, Ephx2, and Fos were used as target genes and Actb was used as a housekeeping gene, which was randomly selected for Real-Time Quantitative polymerase chain reaction (RTQ-PCR) to verify the gene chip. RTQ-PCR was performed using an ABI 7900 real-time PCR system. The primer 5.0 software (Premier Biosoft International, CA, USA) was used to design the primer sequences. The primer sequences were synthesized by Shanghai Biostar Genechip, Inc., China. The primer sequences of RT1-Ba were: forward primer, 5'-GGCACCATCTTCATCATTCAA-3'; reverse primer, 5'-AGAGCCACGCACCTTCTTT-3'. The primer sequences of Ephx2 were: forward primer, 5'-CAAGTGGCTGAAGACTGAAATCC -3'; reverse primer, 5'-AGAACGATGCTGCTGGCTGT-3'. The primer sequences of Fos were: forward primer, 5'-GAGCCGGTCAAGAACATTAGC-3'; reverse primer, 5'-AAGGAACCAGACAGGTCCACAT-3'. The relative quantitative $2^{-\Delta\Delta Ct}$ method was used to analyze the results. The experiment was repeated 3 times.

Data analysis

Data were analyzed with SPSS 13.0 (SPSS Inc. Chicago, IL, USA) and one-way ANOVA. P <0.05 were considered significant. Cubic Spline was used for the normalization of data. DiffScore >+ 20 (up-regulation) or DiffScore <– 20 (down-regulation) were the selection criteria for differentially expressed genes according to their functions. The differentially expressed genes were analyzed by Gene Ontology (GO) with Agilent Genespring GX 11 software (Agilent Technologies Co. Ltd., Parrot, CA, USA). P values <0.1 were considered significant in detecting gene function.

RESULTS

Comparison of body weight, rectal temperature, and toe temperature

Before experiment there were no significant differences between the two groups in body weight, rectal temperature, or toe temperature. There was a significant reduction in body weight of the model group on the 7th day after treatment (P=0.00) and the 14th day after model establishment (P=0.03). A significantly lower toe temperature was observed in the model group on the 7th day (P=0.00) and 14th day (P=0.005) compared with the control group. There was also a significant decrease in toe temperature on the 14th day (P=0.007) compared with the day before the experiment in the model group. The difference between the two groups in rectal temperature was insignificant (Figure 1-3).

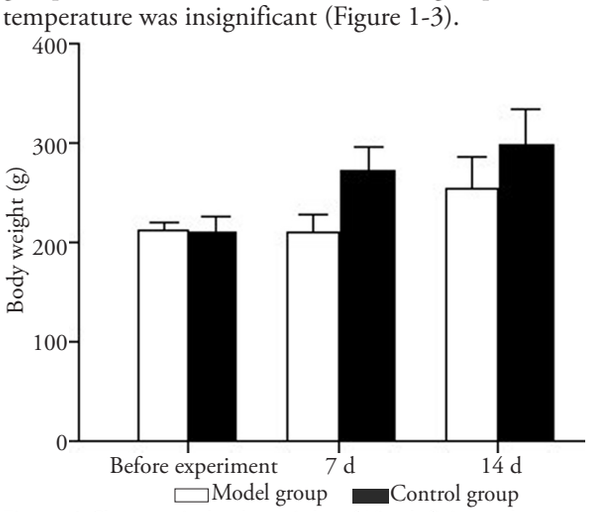

Figure 1 Changes in body weight of *Yin*-deficiency-heat syndrome model group

[a]P<0.01, compared with the control group; [b]P<0.05, compared with the control group.

Comparison of blood sugar, hepatic glycogen and triglyceride in blood plasma

There was significantly lower hepatic glycogen (P= 0.033), significantly higher plasma triglycerides (P= 0.048), and no significant difference in blood glucose between the model and control groups, respectively (Table 1).

Comparison of the activities of Na^+-K^+-, Mg^{2+}-, Ca^{2+}-, and Ca^{2+}-Mg^{2+}-ATPases

As shown in Figure 4, there was a significantly higher

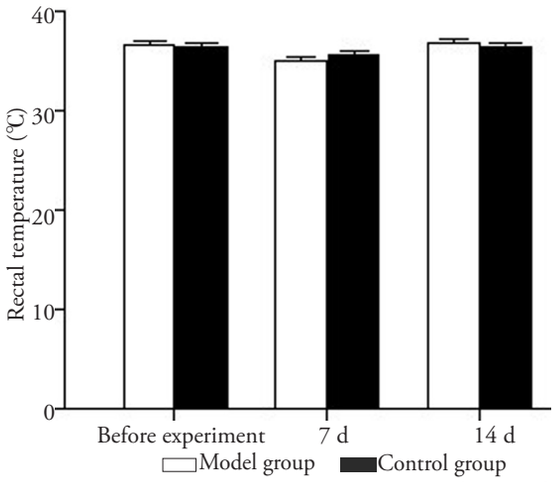

Figure 2 Changes in rectal temperature of *Yin*-deficiency-heat syndrome model group

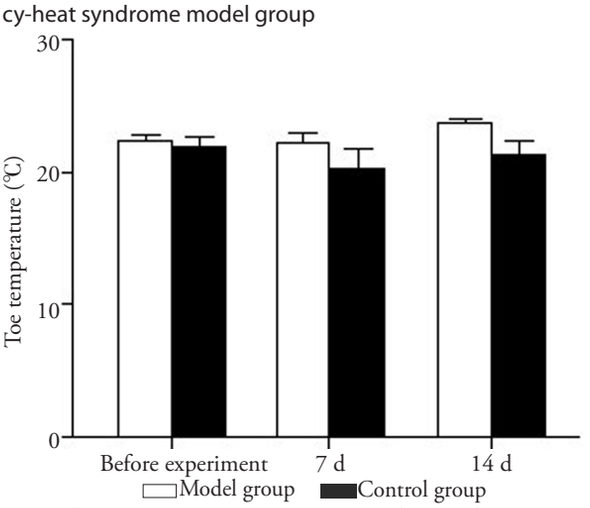

Figure 3 Changes in toe temperature of *Yin*-deficiency-heat syndrome model group

[a]P<0.01, compared with the control group.

Na^+-K^+-ATPase (P=0.007) and Mg^{2+}-ATPase (P=0.006) activity in the model group compared with the control group. There were no differences in Ca^{2+}-ATPase or Ca^{2+}-Mg^{2+}-ATPase activities between the two groups.

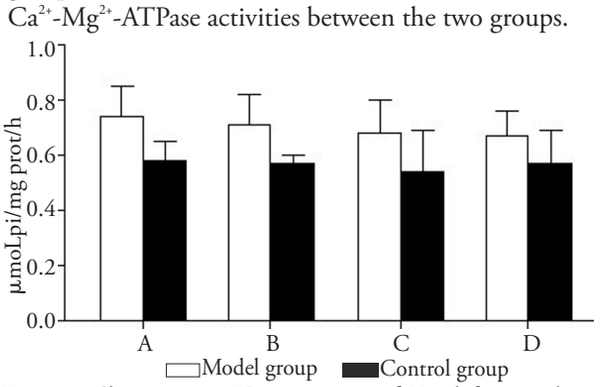

Figure 4 Changes in ATPase activity of *Yin*-deficiency-heat syndrome model group

A: Na^+-K^+-ATPase; B: Mg^{2+}-ATPase; C: Ca^{2+}-ATPase; D: Ca^{2+}-Mg^{2+}-ATPase. [a]P<0.01, compared with the control group.

Comparison of the content of T_3, T_4, and TSH in blood

There was significantly higher level of T_3 (P=0.028) and T_4 (P=0.00) in the model group compared with the control group (Figures 5). The content of TSH in blood of the model group is 0.87 ng/mL, in blood of the control group is 1.36 ng/mL. No significant differ-

ence was found in TSH levels between the two groups.

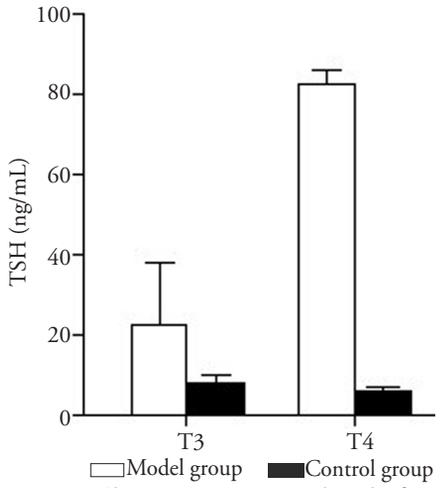

Figure 5 Changes in T3, T4 in bood of *Yin*-deficiency-heat syndrome model group

TSH: thyroid stimulating hormone. T_3: Triiodothyronine, T_4: thyroxine. [a]*P*<0.01, compared with the control group; [b]*P*< 0.05, compared with the control group.

Comparison of the gene expression profile of liver

The total RNA ratio was OD_{260}/OD_{280} >1.8 in each group, and the total RNA had no degradation, as found with 1% agarose gels electrophoresis. Chip scanning figure of the model group and the control group shown in Figure 7. Compared with the control group, there were 99 strips of differentially expressed genes in the model group. Within the 99 genes, there were 60 up-regulated genes and 39 down-regulated genes. Using Agilent Genespring GX 11 analysis software (Agilent Technologies, Santa Clara, CA, USA), the function of each differentially expressed gene was analyzed. Gene Ontology classification was used to analyze gene function. 55 genes were annotated. The software found that three biological processes were the significant gene functions. These processes included sterol biosynthetic process (*P*=0.0392), defense response (*P*=0.0448), and sterol metabolic process (*P*=0.0533). Differentially expressed genes associated with the above-mentioned functions are in Table 2.

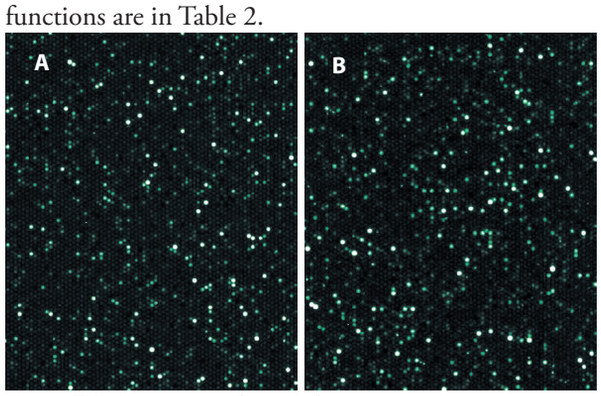

Figure 7 Chip scanning figure of the two groups

A: model group; B: control group.

Results of RT-PCR

The $2^{-\Delta\Delta Ct}$ values of RT1-Ba, Ephx2, and Fos were 0.398 997, 4.904 289, and 0.397 581, respectively. The values, being consistent with the study of gene chip, indicated the reliability of the results.

DISCUSSION

Since the 1980s, Liang *et al* [6] have studied cold and heat syndromes in Beijing Medical University. They noticed that patients with heat syndrome can transform into deficiency-induced cold syndrome if medication with cold-properties are overdosed. Moreover, patients with deficiency-induced cold syndrome could develop into *Yin*-deficiency-heat syndrome if medication with warm-properties are overdosed. Moreover, medication with cold-properties might induce *Yin*-deficiency-heat syndrome or cold syndrome. Therefore, Liang *et al* [7,8] began to produce animal models with cold or heat syndromes by giving mice large doses of medication with cold-properties or heat-properties. According to TCM theory, medication with cold-properties can weaken *Yang*, and *Yang*-deficiency can lead to cold syndrome. Medication with warm-properties is likely to damage *Yin*, which may generate "endogenous pathogenic fire". Chen *et al* [9,10] thought that Liang's animal models could be generally accepted. However, the models could not reach the standard of deficiency-induced cold syndrome if the medication dose was not high enough or treatment course was not long enough. In this experiment, we tried to improve the model by increasing the doses of medication to much larger than Liang's. A few deaths occurred after the rats took the decoction for 2 weeks and mortality increased as time elapsed. This suggested that the proper time for creating a model was 14 days.

In this study, we found that there was a significantly lower body weight and glycogen level, and significantly higher toe temperature, triglyceride level, Na^+-K^+- and Mg^{2+}-ATPase activity, and T_3 and T_4 levels in the model group compared with the control group. This showed that energy metabolism in rats with *Yin*-deficiency-heat syndrome was enhanced, reflecting the state of heat syndrome.

Through analysis of the gene expression profile of the livers, 99 strips of differentially expressed genes between the model and control groups were mainly related to the function of sterol synthesis and metabolism, and the defense response.

Cholesterol synthesis and metabolism in Yin-deficiency-heat syndrome rats were up-regulated

Scd1 coding Stearoyl-Coenzyme A desaturase 1 (SCD1) is a rate-limiting enzyme converting saturated fatty acids (SFA) to monounsaturated fatty acids (MUFA). SCD1 plays an important role in the regulation of fatty acid metabolism and energy balance.[11] Abcg5 coding ATP-binding cassette, sub-family G, member 5 (ABCG5) involves reverse transportation of cholesterol and plays an important role in the decrease of cholesterol absorption in intestine, the increase of steroid excretion in liver, the control of steroid level in plasma and the maintenance of steroid balance in body.[12] Acat2 coding acyl coenzyme A: cholesterol acetyltransferase (ACAT2) is mainly in the liver cells and the epithelial

Table 1 Changes in energy metabolism of *Yin*-deficiency-heat syndrome model group

Group	Blood sugar (mg/dL)	Plasma triglyceride (mg/g)	Glycogen (mg/g)
Model group	70.9±5.6	26.4±5.5[a]	4.0±1.2[a]
Control group	74.0±6.1	21.1±4.2	5.3±0.9

Note: [a]*P*<0.05, compared with the control group.

Table 2 Expression profile of genes in the model group

GenBank accession	Symbol	Definition	DiffScore[a]
Genes about sterol biosynthetic and metabolic			
NM_001013098	Dhrs7	Dehydrogenase/reductase member 7	59.221 53
NM_022936	Ephx2	Epoxide hydrolase 2, cytoplasmic	53.771 42
NM_139192	Scd1	Stearoyl-Coenzyme A desaturase 1	46.195 18
NM_053754	Abcg5	ATP-binding cassette, sub-family G , member 5	39.978 34
NM_001006995	Acat2	Acetyl-Coenzyme A acetyltransferase 2	31.828 24
NM_080886	Sc4mol	Sterol-C4-methyl oxidase-like	25.870 08
NM_017136	Sqle	Squalene epoxidase	20.964 66
NM_001013071	Tm7sf2	Transmembrane 7 superfamily member 2	20.677 04
NM_017268	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	20.113 82
Genes about defense response			
NM_001025004	Vsig4	V-set and immunoglobulin domain containing 4	103.7418
NM_058208	Socs2	Suppressor of cytokine signaling 2	48.831 77
NM_001009488	Ly49s6	Ly49 stimulatory receptor 6	-20.262 38
NM_139089	Cxcl10	Chemokine ligand 10	-21.118 95
NM_021835	Jun	Jun oncogene	-21.2906
NM_017019	Il1a	Interleukin 1 alpha	-21.820 93
NM_053299	Ubd	Ubiquitin D	-22.649 04
NM_001033691	Irf7	Interferon regulatory factor 7	-25.081 14
NM_053960	Ccr5	Chemokine receptor 5	-26.135 97
NM_145672	Cxcl9	Chemokine ligand 9	
NM_001008847	RT1-Da	RT1 class II, locus Da	-28.059 72
NM_020096	Ifit1	Interferon-induced protein with tetratricopeptide repeats 1	-28.756 39
NM_198741	Hla-dma	Major histocompatibility complex, class II, DM alpha	-32.924 63
NM_013069	Cd74	CD74[b] antigen	-36.130 64
NM_022197	Fos	FBJ murine osteosarcoma viral oncogene homolog	-38.560 15
NM_001008884	RT1-Db1	RT1 class II, locus Db1	-45.638 87
NM_022266	Ctgf	Connective tissue growth factor	-46.481 01
NM_031512	Il1b	Interleukin 1 beta	-54.139 23
NM_001008831	RT1-Ba	RT1 class II, locus Ba	-67.188 53

Notes: [a]compared with the control group; [b]Cluster of Differentiation 74.

cells of intestinal villi. It can catalyze long chain fatty acids and cholesterol into cholesterol esters. It also plays an important role in the absorption, transport and storage of cholesterol.[13] Sqle coding squalene epoxidase can catalyze squalene into squalene epoxide, which can turn into lanosterol and create cholesterol. It is a rate-limiting enzyme in cholesterol biosynthesis. Sc4mol coding sterol-C4-methyl oxidase-like protein also takes part in the metabolism of fatty acid and biosynthesis of cholesterol. Ephx2 coding soluble epoxide hydrolase (sEH) mainly takes part in the metabolism of endogenous fatty acid epoxides. The up-regulation of Ephx2 could promote the metabolism of endogenous fatty acid epoxides.[14]

Changes in gene expression related to defense response in rats with Yin-deficiency-heat syndrome

Vsig4 coding V-set and Ig domain-containing 4 (VSIG4) is the costimulator of the B7 clan, which plays an important role in the activation and survival of T cells. It is the only costimulator which shows specific expression on the macrophage surface;[15,16] Ccr5 coding CC-chemokine receptor 5 (CCR5) is the specific receptor of chemokine ligand 3 (CCL3) and chemokine ligand 5 (CCL5). CCR5 is mainly expressed in mononuclear cells, T cells, and other white blood cells. It is thought to be the surface marker of the activation of Thl cells.[17] Cxcl10 codes CXC-Chemokine Ligand 10 (CXCL10). After CXCL10 is combined with CXC-chemokine receptor 3 (CXCR3), the CXCR3 positive cells can activate and migrate to inflammatory lesions. CXCL9 codes CXC-Chemokine Ligand 9 (CXCL9). CXCL9 has a chemotactic effect on activating T lymphocytes and monocytes. CXCL9 plays an important role in a variety of autoimmune diseases, inflammatory diseases, and cancer;[18] Il1α codes interleukin 1α and Il1β codes interleukin 1β. IL-1 is a key cytokine in the initiation of the inflammatory response. IL-1 affects a variety of immune cells and plays an important role in the elimination of foreign microorganisms, inhibition of tumor cell growth, and maintenance of internal environment in balance.[19] Irf7 codes interferon regulatory factor 7 (IRF7). IRF is a multifunctional transcription factor which can regulate the gene expression of interferon. IRF7 is the main regulatory factor which can induce the expression of interferon.[20]

Overall, energy metabolism in rats with *Yin*-deficiency-heat syndrome was enhanced. This reflected, in terms of TCM theory, the pathogenesis of hyperactivity of *Yang* and endogenous heat. The molecular mechanism might be that cholesterol synthesis and metabolism genes were up-regulated. Meanwhile, the genes down-regulating the defense response may be related to the "depletion of vital essence resulting in deficiency syndrome" and "internal heat induced by *Yin*-deficiency." Therefore, *Yin*-deficiency-heat syndrome can be explained as abnormal gene expression related to sterol synthesis, sterol metabolism and defense response.

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