SIMULATED WEIGHTLESSNESS LED TO THE TRANSFORMATION OF GLYCOLIPID METABOLISM IN THE LIVERS OF MICE

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ANTIGRAVIDADE SIMULADA LEVOU À TRANSFORMAÇÃO DO METABOLISMO DE GLICOLIPÍDIOS NO FÍGADO DE CAMUNDONGOS

LA ANTIGRAVEDAD SIMULADA PROVOCÓ LA TRANSFORMACIÓN DEL METABOLISMO DE LOS GLICOLÍPIDOS EN EL HÍGADO DE RATONES

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ABSTRACT

Objectives: The effects of weightlessness on the liver were studied using a tail suspension (TS) male mouse model. Methods: The effects of 0-, 2- and 4-week TS (CON, TS2 and TS4 groups) on glycogen and lipid content, as well as on the molecular processes of the synthesis and degradation pathways, were examined. Results: (1) The number of glycogenosomes under ultrastructure and the glycogen content were considerably larger in the TS4 group than in the other two groups. (2) In the TS4 group, glycogen synthase activity remained constant while glycogen phosphorylase activity dropped, indicating that glycogen breakdown was reduced. (3) The livers of the TS2 group had the highest lipid and triglyceride content, indicating lipid buildup in the liver at this time. (4) In the TS2 group, the activities of the fatty acid synthesis-related factors acetyl-CoA carboxylase and fatty acid synthase increased, while hepatic lipase decreased, indicating that lipid synthesis increased, while decomposition decreased. (5) In the TS2 group, the protein expression of glucose transporters 1 and 2 increased. Conclusions: From TS2 weeks to TS4 weeks, the main energy consumption mode in the livers of mice transitioned from glucose metabolism to lipid metabolism as glucose use decreased. ***Level of evidence II; Comparative prospective study.***

Keywords: Microgravity; Hindlimb suspension; Glycogen; Lipids.

RESUMO

Objetivos: Os efeitos da antigravidade no fígado foram estudados usando um modelo de camundongo macho com a suspensão pela cauda (TS, tail suspension). Métodos: Foram examinados os efeitos da TS em 0, 2 e 4 semanas (grupos CON, TS2 e TS4) sobre o conteúdo de glicogênio e lipídios, bem como nos processos moleculares das vias de síntese e degradação. Resultados: (1) O número de glicogenossomos ultraestruturais e o teor de glicogênio foram expressivamente maiores no grupo TS4 do que nos outros dois grupos. (2) No grupo TS4, a atividade de glicogênio sintase permaneceu constante, enquanto a atividade de glicogênio fosforilase caiu, indicando que a degradação do glicogênio foi reduzida. (3) Os fígados do grupo TS2 tiveram o maior teor lipídico e de triglicérides, indicando acúmulo de lipídios no fígado no momento. (4) No grupo TS2, a atividade dos fatores relacionados com a síntese de ácidos graxos acetil-CoA carboxilase e ácido graxo sintase aumentaram, enquanto a lipase hepática diminuiu, indicando que a síntese de lipídios aumentou, enquanto a decomposição diminuiu. (5) No grupo TS2, a expressão proteica dos transportadores de glicose 1 e 2 aumentou. Conclusões: De TS2 semanas para TS4 semanas, o principal modo de consumo de energia no fígado de camundongos passou do metabolismo da glicose para o metabolismo lipídico, à medida que o uso de glicose diminuiu. ***Nível de evidência II, Estudo retrospectivo comparativo.***

Descritores: *Microgravidade; Elevação dos membros posteriores; Glicogênio; Lipídios.*

RESUMEN

Objetivos: Se estudiaron los efectos de la antigravedad en el hígado utilizando un modelo de ratón macho en prueba de suspensión de la cola (TS, tail suspension). Métodos: Se examinaron los efectos de la TS a las 0, 2 y 4 semanas (grupos CON, TS2 y TS4) sobre el contenido de glucógeno y lípidos, así como sobre los procesos moleculares de las vías de síntesis y degradación. Resultados: (1) El número de glucogenosomas ultraestructurales y el contenido de glucógeno fueron expresivamente más altos en el grupo TS4 que en los otros dos grupos. (2) En el grupo TS4, la actividad de la glucógeno sintasa se mantuvo constante, mientras que la actividad de la glucógeno fosforilasa disminuyó, lo que indica que la degradación del glucógeno se redujo. (3) Los hígados del grupo TS2 presentaron el mayor contenido de lípidos y triglicéridos, lo que indica la acumulación de lípidos en el hígado en ese momento. (4) En el grupo TS2, la actividad de los factores relacionados con la síntesis de ácidos grasos acetil-CoA carboxilasa y ácido graso sintasa aumentó, mientras que la lipasa hepática disminuyó, indicando que la síntesis de lípidos aumentó mientras que la

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descomposición disminuyó. (5) En el grupo TS2, la expresión proteica de los transportadores de glucosa 1 y 2 aumentó. Conclusiones: Desde la semana TS2 hasta la semana TS4, el principal modo de consumo de energía en el hígado de los ratones pasó del metabolismo de la glucosa al metabolismo de los lípidos a medida que disminuía el uso de la glucosa. ***Nivel de Evidencia II, Estudio retrospectivo comparativo.***

Descriptores: *Microgravedad; Suspensión trasera; Glucógeno; Lípidos.*

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INTRODUCTION

One of the fundamental challenges of space biology and medicine is to simulate some of the physiological consequences of weightlessness that occur during space voyages on Earth.[1] The activity of 30 enzymes involved in glycolipid metabolism in the rat liver was altered by the Cosmos 936 biosatellite,[2] and abnormalities of glycolipid metabolism can result in a variety of illnesses.[3] Tail suspension (TS) is a common animal model for simulating the consequences of weightlessness, and it has been found to cause liver damage, including extensive granular degeneration, chronic inflammation, and portal fibrosis, after 10 days and 4 weeks of TS.[4] The quantity of total lipids in rat liver increased significantly after 10 days of TS,[5] while the expression of genes involved in triglyceride metabolism increased significantly after 21 days of TS.[6] It is still unclear whether varied lengths of simulated weightlessness influence liver glycolipid metabolism, and the mechanism behind this influence has yet to be discovered.

Glycogen synthase (GS), a crucial enzyme in glycogen formation, polymerizes UDP-glucose to produce glycogen granules, and its active state is phosphorylated GS (P-GS).[7,8] The rate-limiting enzyme glycogen phosphorylase (GYPL) breaks down glycogen granules to glucose.[9] The direct method of glycogen production necessitates one or more glucose transporters transporting glucose into cells (GLUTs).[10] Studies have shown that the level of glycogen phosphorylase activity in the liver of rats after space flight decreases, but it is unclear how the level of glycogen synthesis changes.[11] Thus, studies on the above factors could help reveal the mechanism related to changes in liver glycogen content under simulated weightlessness.

Acetyl-CoA carboxylase (ACC) is an essential rate-limiting enzyme in fatty acid metabolismo.[12] Fatty acid synthase (FASN) is a vital enzyme that catalyzes the de novo synthesis of long-chain fatty acids.[13] Hepatic lipase (HL) is an enzyme that is made primarily by hepatocytes and hydrolyzes phospholipids and triglycerides of plasma lipoproteins.[14] It is necessary to study lipid-related factors under microgravity.

Based on the above, we hypothesized that different periods of TS lead to the transformation of glucose and lipid metabolism in mouse livers. We also hypothesized that these effects are related to their synthesis and decomposition signal pathways. To test these hypotheses, we first observed the ultrastructure and counted the number of glycogenosomes in the liver of mice after different periods of TS (control, 2 weeks, and 4 weeks). Oil red O staining was used to quantify lipid content. We also determined the protein expression levels of glycogen and fatty acid synthesis and degradation-related signals. We further explored the molecular mechanism related to the effects of simulated weightlessness on the changes in glycolipid metabolism in the liver.

MATERIALS AND METHODS

Animals and groups

SPF Kunming (KM) male mice with 5 weeks old were purchased from Pengyue Experimental Animal Breeding Co., Ltd. (Jinan, China). They were housed with two animals per cage (28 × 18 × 12 cm) at an ambient temperature of 22 ± 2 ℃, relative humidity of 55% ± 5%, and light regime of 12:12 h light/dark (light on from 06:00 to 18:00). Standard mouse chow (Pengyue Experimental Animal Breeding Co., Ltd., China) and water were provided *ad libitum,* and wood shavings were used as bedding.

After two weeks of adaptation with free movement in the laboratory, mice were numbered, weighed, and randomly divided into three groups (n = 16). The three groups included: Control group (CON): Mice moved freely without TS; 2-week TS group (TS2): The mice were suspended by their tail at an angle of 30° (between body and horizontal plane) for 2 weeks (from 9 weeks to 11 weeks old);[15] 4-week TS group (TS4): The mice were TS for 4 weeks (from 7 weeks to 11 weeks old). The three groups were maintained under the same light, temperature, and humidity conditions and were all 11 weeks old at the end of the treatment.

Sample preparation

All animals were sacrificed by CO_2 asphyxiation at 08:00 on the last morning of the day. After rapid removal of the liver, the livers of 8 mice in each group were frozen with liquid nitrogen and stored at −80 °C for enzyme activity and western blot analysis. The livers of the other 8 mice were used for transmission electron microscopy and frozen section experiments. All procedures were carried out in accordance with the approved guidelines.

Transmission electron microscopy (TEM)

The liver was examined via TEM (Hitachi, HT7800, Japan), as described previously.[16] Images were processed with NIH Image-Pro Plus 6.0 and then analysed using the measurement tools provided by this software. Glycogenosome densities were determined within a defined region (1 μm^2 area) at a minimum of three locations within an image taken at 10 000× magnification.

Glycogen and triglyceride content detection

The amount of glycogen in the liver from the three groups was determined with a Glycogen Assay Kit (YX-W-B603, Shanghai Hengyuan Biological Technology, Ltd., China) by the anthrone method.[17] The amount of triglycerides from the three groups was determined with a Triglyceride Assay Kit (YX-W-B408, Shanghai Hengyuan Biological Technology) by spectrophotometry. Glycogen and triglyceride levels were normalized by cell protein concentration measured using the BCA assay.[18]

Oil red O (ORO) staining

ORO staining was used to detect the fat content in the liver.[19] In short, frozen tissue was cut into 10-μm thick sections and dried at room temperature. The slides were colored by soaking in the dye solution of ORO dissolved in 60% isopropanol. The slides were sealed with glycerol gelatin and examined under a microscope. Quantification analysis of the blots was performed using NIH ImageJ software (Image-Pro Plus 6.0).

GS, GYPY, ACC, FASN, HL activity

Samples stored at −80 °C were used to detect the activity of related factors. The GS active was determined with a GS Assay Kit (BC3335, Solarbio, Beijing, China) by the rate of decrease in NADPH. The GYPL activity was determined with a GYPL Assay Kit (BC3345, Solarbio) by the increase rate of NADPH. The ACC activity was determined with an ACC

Assay Kit (BC0410, Solarbio) by the increase rate of inorganic phosphorus. The FASN activity was determined with a Fatty Acid Synthetase Activity Assay Kit (BC0550, Solarbio) by the rate of decrease in light absorption at 340 nm. The HL activity was determined with an HL Activity Assay Kit (BC2385, Solarbio) by the decrease rate of α-naphthol at 595 nm.

Western blot

Total protein was extracted from the tissues, and electrophoresis, film transfer, incubation and exposure methods were the same as before[17]and the total protein content was used as the reference.[20]. Quantification analysis of the blots was performed using NIH ImageJ software (Image-Pro Plus 6.0).

Statistical analyses

SPSS version 22.0 was used for all statistical analyses. The data are presented as the mean ± SD. The data are presented as means ± SD. One-way analysis of variance (ANOVA) was used to determine overall differences, and Fisher's least significant difference (LSD) *post hoc* test was used to determine group differences. ANOVA-Dunnett's T2 method was used when no homogeneity was detected. The results were significant at $P < 0.05$.

RESULTS

Liver organ coefficient of mice

No significant differences in body weight (BW) were observed among the three groups before the experiment. After 4 weeks of different treatments, the BW, body length (BL), and carcass weight (CW) of mice were higher in the CON group than in the two TS groups ($P < 0.05$). However, the liver mass (LM) to BW ratio (LM/BW) and LM to CW ratio (LM/CW) were highest in the TS2 group ($P < 0.05$) (Tabela 1).

Values are means ± SD. $n = 16$. CON, control group; TS2, 2-week tail suspension group; TS4, 4-week tail suspension group. Different letters (such as a and b) indicate significant differences among TS-treated groups ($P < 0.05$).

Ultrastructural changes in liver

Glycogenosomes were distributed in clusters, and the number of glycogenosomes in each cluster was different, ranging from 3 to 30. Glycogenosome clusters and mitochondria were randomly distributed in the cytoplasm (Fig. 1A and B). In the TS4 group, the number of glycogenosomes was greater than that in the CON and TS2 groups ($P < 0.05$) (Figure 1C).

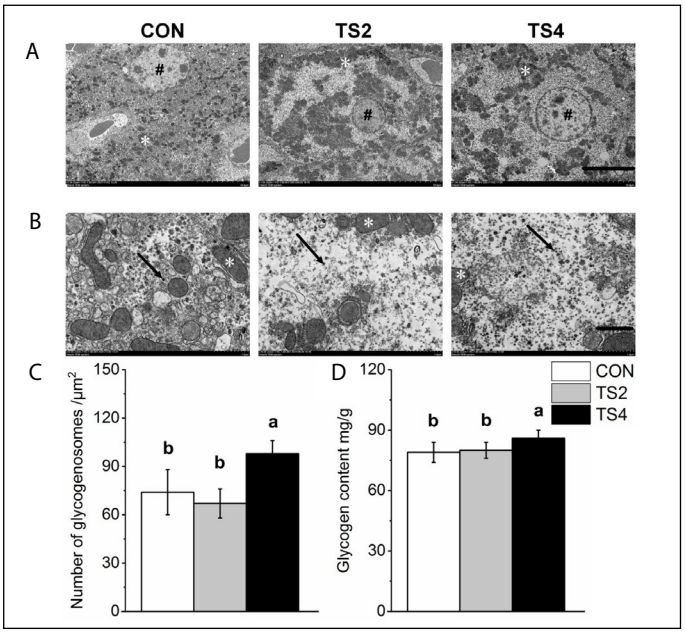

Figure 1. Ultrastructure of glycogenosomes and glycogen content in the livers of mice.

(A) Ultrastructure of liver cells. Scale bar = 10 μm. (B) Ultrastructure of glycogenosome. Scale bar = 1 μm. # shows the nucleus, and * shows the mitochondria with clear cristae and intact membranes. In the CON and TS4 groups, the cluster distribution of glycogenosomes (arrow) was more obvious, while in the TS2 group, glycogenosomes appeared to be less abundant per cluster. (C) Bar graph of number of glycogenosomes. Six figures were analysed in each sample; four samples were analysed in each group. (D) Glycogen content in liver by anthrone method. Values are means ± SD. n=8. CON, control group; TS2,2-week tail suspension group; TS4,4-week tail suspension group. Different letters (such as a and b) indicate significant differences among TS-treated groups ($P < 0.05$).

Table 1. Effects of TS on body weight (BW), body length (BL), carcass weight (CW), liver weight (LW), LW to BW ratio (LW/BW) and LW to CW ratio (LW/CW) in mice.

	CON	TS2	TS4
BW before treatment (g)	31.22 ± 1.61	31.02 ± 1.59	31.32 ± 1.63
BW after treatment (g)	45.67 ± 3.89[a]	41.65 ± 2.87[b]	40.45 ± 2.39[b]
BL after treatment (g)	11.21 ± 0.49[a]	10.38 ± 0.49[b]	10.50 ± 0.71[b]
CW after treatment (g)	31.12 ± 2.89[a]	27.34 ± 2.20[b]	27.42 ± 2.74[b]
LW (g)	2.51 ± 0.31[a]	2.49 ± 0.79[a]	2.22 ± 0.33[b]
LW/BW (mg/g)	55.27 ± 5.31[b]	59.39 ± 5.26[a]	54.97 ± 4.53[b]
LW/CW (mg/g)	81.63 ± 10.40[b]	90.76 ± 21.85[a]	81.47 ± 10.22[b]

Glycogen quantification

Glycogen quantification showed similar trends to the number of glycogenosomes, with significant accumulation in the TS4 group ($P < 0.05$) (Figure 1D).

Lipid content in liver

Both the lipid distribution stained with oil red O and the triglyceride content in the liver showed that the TS2 group was the highest ($P < 0.05$) (Figure 2).

(A) Lipid distribution in liver by Oil red O staining. Scale bar = 50 μm. (B) Lipid and nuclei distribution in liver by Oil red O and hematoxylin staining. Red represents lipids and blue represents nuclei. Scale bar = 50 μm. (C) Lipid content statistics of oil red O staining. (D) Triglyceride content in the liver by spectrophotometry. Values are means ± SD. n =8. CON, control group; TS2,2-week tail suspension group; TS4,4-week tail suspension group. Different letters (such as a and b) indicate significant differences among TS-treated groups ($P < 0.05$).

Changes in glycogen metabolism related factors

The results showed that GS activity in the CON group was significantly higher than that in the TS2 group ($P < 0.05$), but the TS4 group was not significantly different from the CON and TS2 groups. Furthermore, GYPL activity showed a trend of CON > TS2 >TS4 ($P < 0.05$) (Figure 3).

Values are means ± SD. $n = 8$. CON, control group; TS2, 2-week tail suspension group; TS4, 4-week tail suspension group. Different letters (such as a and b) indicate significant differences among TS-treated groups ($P < 0.05$).

Changes in glucose transporter related factors

Among the three groups, GLUT1 and GLUT2 protein expression was highest in the TS2 group ($P < 0.05$) (Figure 4).

Values are means ± SD. $n = 8$. CON, control group; TS2, 2-week tail suspension group; TS4, 4-week tail suspension group. Different letters (such as a and b) indicate significant differences among TS-treated groups ($P < 0.05$).

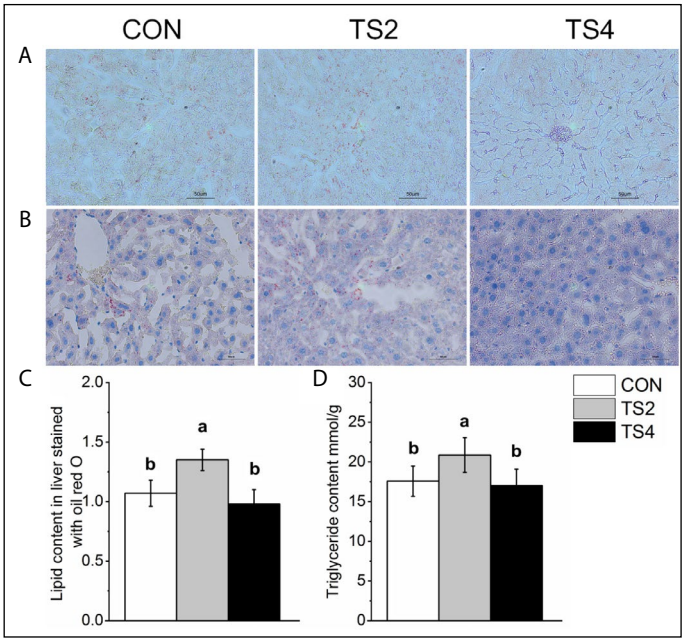

Figure 2. Lipid distribution and triglyceride content in the livers of mice.

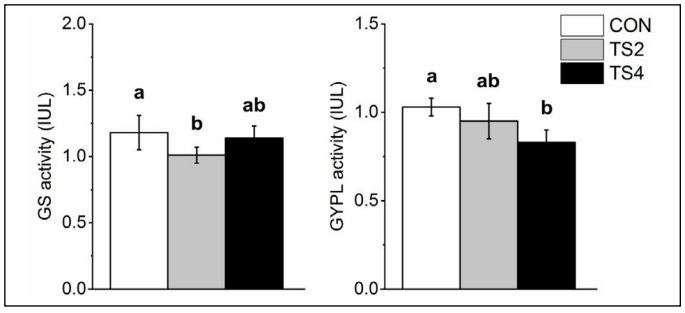

Figure 3. GS and GYPL activity in liver of mice.

Changes in lipid metabolism related factors

ACC and FASN, two key factors of fatty acid synthesis, showed that the enzyme activity levels of the TS2 group were the highest ($P < 0.05$) among the three groups. The trend of HL enzyme activity level was opposite to that of ACC and FASN, which was the lowest in the TS2 group ($P < 0.05$) (Figure 5).

(A) Representative immunoblots of GLUT1 and GLUT2. (B) Representative polyacrylamide gel of total protein. (C) Relative protein expression of GLUT1. (D) Relative protein expression of GLUT2. Values are means ± SD. $n = 8$. CON, control group; TS2, 2-week tail suspension group; TS4, 4-week tail suspension group. Different letters (such as a and b) indicate significant differences among TS-treated groups ($P < 0.05$).

DISCUSSION

We found that the LW/BW and LW/CW of the TS2 group increased significantly, which may mean that TS caused liver hypertrophy in mice. This is similar to a previous study, which showed that two months of tail suspension resulted in a significant increase in the liver to body weight ratio of rats,[4] which may be mainly because the growth rate of animal weight is lower than that of liver mass. The LW/BW and LW/CW of the TS4 group returned to the level of the CON group. Studies have shown that TS can lead to structural remodeling of rat skeletal muscle,[6] thus speculating that the recovery of the liver mass ratio may be caused by simulated weightlessness.

An important finding in this study is that the level of glycogen in the liver remained unchanged in the TS2 group and increased significantly in the TS4 group. The stability of glucose metabolism in liver is the basis

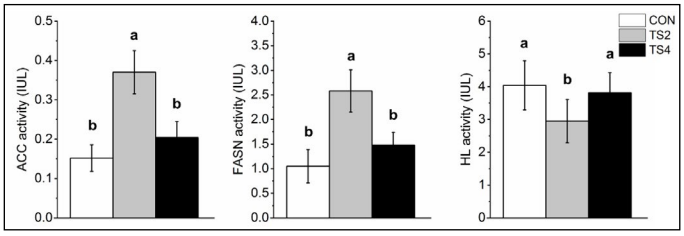

Figure 4. ACC, FASN and HL activity in liver of mice.

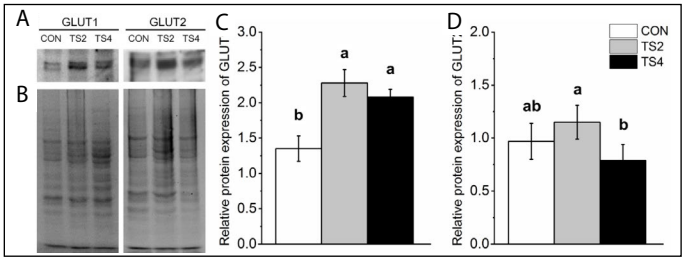

Figure 5. GLUT1 and GLUT2 protein expression in the livers of mice.

of maintaining normal physiological function.[11,21] The ultrastructure showed that the number of glycogenosomes in the liver of mice in the TS4 group was significantly higher than that in the other two groups, and the aggregation degree of glycogen particles seemed to be higher, which was consistent with the glycogen content, indicating that long-term simulated weightlessness did lead to glycogen accumulation in the liver of mice. The aggregation of glycogen particles in the livers of mice in the TS2 group seemed to be weakened, which may suggest that the level of glucose metabolism in the TS2 group was higher than that in the TS4 group.

GS is the limiting enzyme of glycogen synthesis, and GYPL is the key factor of glycogen decomposition.[8,22] The detection of their enzyme activity levels found that the GS activity level decreased and GYPL remained stable in the TS2 group, suggesting that the glycogen synthesis level decreased and the glycogen decomposition level basically remained stable in the livers of mice suspended for two weeks, which may be the reason for the slight decrease in the number of glycogen particles in the ultrastructure. It should be noted that the activity level of GS in the liver of the TS4 group remained unchanged, while GYPL decreased, suggesting the maintenance of glycogen synthesis levels and the reduction of glycogen decomposition levels in the liver of mice around tail suspensions. Therefore, from 2 weeks to 4 weeks, we found that the decrease in glycogen decomposition levels may mean a decrease in glucose metabolism levels. This was verified by the study of GLUT. The protein expression results showed that GLUT1 and GLUT2 in the TS4 group were significantly lower than those in the TS2 group, suggesting that the transport capacity of glucose decreased from 2 weeks to 4 weeks, which may mean a decrease in glucose metabolism levels.

Another important finding was the accumulation of lipid content in the liver in the TS2 group and the recovery of the TS4 group. The liver is the most important organ for the synthesis of lipids (mainly triglycerides), but it is not a storage organ.[23] The increase in lipid content in the liver in the TS2 group may mean that the body's utilization of fat is reduced. At this time, liver hypertrophy may also be caused by lipid accumulation. Fatty acid synthesis is the basis of lipid accumulation. In this study, the activities of ACC and FASN, two key factors related to fatty acid synthesis, were significantly increased in the TS2 group, indicating an increase in fatty acid synthesis levels. The decrease in HL activity indicates a decrease in fatty acid decomposition levels, which may be the main reason for lipid accumulation in the liver. In addition, combined with the change trend of glycogen content, we found that

lipid accumulation and glycogen were stable in the TS2 group, while lipid recovery and glycogen accumulation were stable in the TS4 group. This may mean that with the extension of tail suspension time, the main mode of energy material metabolism in the liver was transformed from glucose metabolism to lipid metabolism.

Ethics Statement

All procedures followed the Laboratory Animal Guidelines for the Ethical Review of Animal Welfare (GB/T 35892-2018) and were approved by the Biomedical Ethics Committee of Qufu Normal University (Permit Number: dwsc 2021050). The authors declare that the study was carried out in compliance with the ARRIVE guidelines.

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