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Study on the Effects of Semen Astragali Complanati *GAD67*, *GABA-T* and *SSADH* Gene Expression in the *Hippocampus* of Exercised Rats

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ABSTRACT

Purpose: The aim of the present study was to investigate the impact of semen astragali complanati (SAC) on GAD_{67}, GABA-T and SSADH gene expression in the hippocampus of exercised rats. Methods: All rats were randomly divided into four average groups: a control group, animals receiving no SAC and no training (NSNT), animals receiving moderate training and no SAC (MTNS), animals receiving intensive training and no SAC (ITNS) and animals receiving intensive training and SAC (ITS). The Trizol homogenate of hippocampus was prepared after 7 weeks training for rats. The products of RT-PCR were separated by 1% agarose gel electrophoresis and quantitatively analysised by UVI gel analysis system. The data of each group was processed and tested by S-N-K of one way ANOVA. Results: The GAD_{67} significantly enhanced in the ITNS group than in the other three groups ($p<0.01$, $p<0.01$, $p<0.01$), and significantly enhanced in the MTNS and ITS group than in NSNT group ($p<0.01$, $p<0.01$). The GABA-T significantly decreased in the ITNS group than in the other three groups ($p<0.01$, $p<0.01$, $p<0.01$). The SSADH significantly decreased in the ITNS group than in the other three groups ($p<0.01$, $p<0.01$, $p<0.01$), and significantly decreased in the ITS group than in MTNS group ($p<0.01$). Conclusions: (1) Intensive exercise significantly up-regulated the expression of GAD_{67} while down-regulated the expression of GABA-T and SSADH in the hippocampus of exercised rats, which promoted the central inhibition and correlated with exercise intensity. Therefore, this study supports the theory of "protective inhibition" in exercise induced fatigue. (2) SAC can significantly down-regulate GAD_{67} expression while up-regulate the expression of GABA-T and SSADH and then reduce Glu converting into GABA, improve the whole excitability of rat hippocampus and delay the central inhibition and central fatigue induced by high intensity exercise. (3) The regulating effects of SAC on GAD_{67}, GABA-T and SSADH in the hippocampus of exercised rats may be related to its effect of tonifying the liver and kidney.

Keywords: Semen astragali complanati; *Hippocampus*; GAD; *GABA-T*; *SSADH*; Gene expression

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INTRODUCTION

World vegetation forms various landscapes (forests, savannah, bushes, steppes etc.) that are rich in spontaneous plants species. For centuries, our ancestors used to relieve their pains and treat themselves with plants. Their medicinal experiences were kept and transmitted from a generation to another [1]. Nowadays, aromatic and medicinal plants remain the basis of alternative medicinal cares despite progression observed in modern health systems [2]. In developing countries, they constitute the main

source of medication when modern medicine is not found [3,4]. Thus, WHO (World Health Organization) showed its importance and recommended its integration into official health systems especially in developing countries [5,6].

Among 500 000 vegetal species recorded on earth, 80 000 are described as medicinal ones [5]. Morocco, as a phytogenetic tank formed with about 4500 vascular species and sub-species, is among mediterranean countries where inhabitants developed a real know- how in the field of medicinal plants [7]. However, bibliographic studies on this rich Moroccan flora showed that regional medicinal plants are scattered. Moreover, this know-how is now kept only by few people [8]. In fact, transformation of this traditional knowledge into a scientific one is an important mean of revalorization and conservation that can lead to a rational use [9]. Therefore, this research, as others in floristic and ecological fields, has the same objectives as programs of international organisations such as World Union for Nature which aim is to promote sustainable uses of natural resources in North Africa and biodiversity conservation with involvement of local communities [10].
Thus, this study performed in *Masmouda* is a contribution to conservation of this traditional knowledge. It focuses on therapeutic uses of three medicinal and aromatic plants: *Satureja nepeta*=Menth, *Myrtus communis*=Rayhan and *Pistacia lentiscus*=Drou [11].

EXPERIMENTAL METHODS

Experimental Design and Subjects

Forty-eight SPF grade (Specific pathogen Free) Sprague-Dawley (SD) healthy male rats of 2-month-old were used weight 210-230 g and were provided care as directed by the Experimental Animal Center of the Medical School, Xi'an Jiaotong University (Animal certificate No: Shannxi Medical Animal No: 08-005). The temperature varied from 20℃ to 25℃, the relative humidity was 40%-60%, the cages were illuminated by natural light, the ambient noise was no higher than 50 dB and all rats had free access to water and basic rodent chow. Shaanxi Hengxintang Pharmaceutical Co., Ltd (Xi'an, China) provided SAC preparation. (60% SAC extractum: sucrose: dextrin=1:3:3, pharmaceutic adjuvant only plays the role of filling, bonding and forming)

The SAC dosage of animals was determined by the reference [5] and the human dose converted by the unit of the body weight. Based on this, the SAC dosage of each rat in ITS group was determined to 1.8 g/kg body weight, once a day for 7 weeks. Each dosage of SAC was dissolved in 2 mL normal saline and then fed to animals at a fixed time of between 9:00-9:30 a.m. during the training weeks. All the rest of the animals (NSNT, MTNS, and ITNS group) were fed with the same dosage of normal saline as placebo.

This study was performed according to the international, national, and institutional rules considering animal experiments, clinical studies and biodiversity rights, and had been approved by Xijing Hospital Ethic Committee in Fourth Military Medical University.

Exercise Protocol

Forty-eight rats were randomly divided into four average groups: a control group, animals receiving no SAC and no training (NSNT), animals receiving moderate training and no SAC (MTNS), animals receiving intensive training and no SAC (ITNS) and animals receiving intensive training and SAC (ITS). After 5 weeks adaptive training, the MTNS, ITNS and ITS group would individually receive a moderate training, an intensive training and an intensive training with SAC, totally for 2 weeks.

A slightly changed Benford motion model was applied. After 2 days adapting to environment, the animals began to be trained according to Table 1 at the fixed time 18:00-18:30, once a day. The treadmill was horizontal, and from the first week of training, the treadmill speed increased weekly to 15 m/min→22 m/min→27 m/min→31 m/min→35 m/min. Each training lasted for 20 minutes. Adaptive training lasted for 5 weeks and then the rats would be trained as the following plan:

In the sixth and seventh weeks, a moderate training applied to MTNS group, at a speed of 35 m/min for 20 min, once a day and an intensive training applied to both ITNS and ITS group, at a speed of 35 m/min for

30 min, once a day. Meanwhile, ITS group was given SAC at a dosage of 1.8 g/kg body weight. The model establishment would be totally lasting for 7 weeks.

Table 1: Training scheme for rats

Weeks	Speed (m/min)	Treadmill slope (°)	Training time (min/d)
1	15	0	20
2	22	0	20
3	27	0	20
4	31	0	20
5	35	0	20
6	35	0	20（30）
7	35	0	20（30）

Main Apparatus and Reagent

A Hettich MIKRO 22R refrigerated centrifuge, DK-98-1A water bath, BP-310S electronic balance, a DSPT-202 treadmill, a LDZX-30KBS autoclave, a BCD-272 refrigerator, Micropipet, Hitachi U-3900 UV spectrophotometer, SW-CJ-2FD superclean bench, Stratgene Mx3000P PCR instrument, DYCP-31E electrophoresis apparatus and Cambridge UVItec Gel analysis system.

Dimethyl sulfoxide; DEPC (diethyl pyrocarbonate) water; Isopropanol; Chloroform; Agarose; RNasin; AMV(*avian myeloblastosis* virus), Reverse Transcriptase; TRIZol RNA kit; RT-PCR kit; PCR Master Mix kit; Primer synthesis.

Dissection and Testing

At the end of the seventh week, after the rats completed the entire training program, all the animals were ether anesthetized and killed by dislocating cervical vertebra. The whole *hippocampus* was isolated from the brain and washed with 4°C normal saline.

TESTING PROCEDURE

The gene expression of *GAD67*, *GABA-T* and *SSADH* in *hippocampus* of rats was measured by semi quantitative RT-PCR.

Total RNA Extraction

Appropriate fresh *hippocampus* tissues was weighed and ground in liquid nitrogen. Then the Trizol homogenate (1 mL Trizol/100 mg tissue) was prepared for RNA extraction.

1. The homogenate was mixed with Trizol (1 mL Trizol/100 mg tissue) and shaken for 5-10 min at room temperature.
2. 1/5 volume chloroform was added into the Trizol homogenate and violently shaken for 15 s, and then let it stand for 5 min.
3. 4℃, the homogenate was centrifuged at 12000 g for 15 min.
4. The supernatant was transferred into another Eppendorf tube (only a small amount of precipitation was retained) and mixed with 0.8 mL isopropanol, then let it stand for 10 min.
5. 4℃, the homogenate was centrifuged at 12000 g for 15 min once again.
6. The precipitation was washed with 1 mL 75% ethanol.
7. 4℃, the homogenate was centrifuged at 10000 g for 10 min and then the RNA was separated for air drying for 10 min.
8. The RNA was dissolved in 50 μL DEPC water and mixed with 20 μL RNasin, preserved in -20℃.

We applied formaldehyde denaturing gel electrophoresis to identify RNA was whether degrading or contaminated by DNA, and applied UV spectrometry to identify RNA purity and perform a quantitative analysis.

1 μL total RNA, 4 μL sample buffer and 1 μL EB (ethidium bromide) were mixed to denature at 65℃ for 10 min, and then performing a 1% agarose (containing 18% formaldehyde) gel electrophoresis at 45℃ for 4 hours to observe the 28 S, 18 S, and 5 S bands are whether clear, degrading or existing DNA contamination. 1 μL RNA sample and 99 μL DEPC water were mixed and measured by UV spectrometry at 260 and 280 nm. The value of A260/A280 in pure RNA should range from 1.8 to 2.0. This method also can be used to quantify RNA.1 A260 unit amounts to 40 μg/mL RNA.

RT-PCR

The cDNA was synthesized according to the kit instructions.

(1) 10 μL RNA, 1 μL random primer and 1 μL H_2O were mixed in the ice box reacting at 70℃ for 5 min.
(2) 4 μL 5×buffer, 1 μL RNasin, 2 μL dNTP (10 mM) were mixed in the ice box reacting at 25℃ for 5 min
(3) 1 μL AMV reverse transcriptase was added to react at 25℃ for 10 min and then to react at 42℃ for 60 min.
(4)The reacting terminated at 70℃ for 10 min and the product cDNA can be used for PCR amplification.

PCR amplification was performed according to the kit instructions. 10 μL cDNA templet, 1 μL upstream and 1 μL downstream 50 pM primers and 12.5 μL 2× MasterMix reaction system were mixed, adding DEPC H_2O to 25 μL, and covered with 50 μL liquid paraffin. The mixture was pre-denatured at 95℃ for 5 min→denatured at 94℃ for 60 s→denatured at 55℃-60℃ for 60 s→denatured at 72℃ for 60 s. After 28 cycles, the PCR products extended at 72℃ for 300 s.

The cDNA sequence of *GAD_{67}*, *GABA-T*, *SSADH* and *β-actin* were obtained from Genbank. The primer of the *GAD_{67}*, *GABA-T*, *SSADH* and the reference gene *β-actin* were designed to be shown in Table 2.

Table 2: Primer design

Gene	Primer sequence	Length（bp）	
GAD_{67}	forward 5′(1345) caa act cag cgg cat aga aag	3′	313
	reverse 5′(1658) tgg cat aga ggt att cag cc	3′	
GABA-T	forward 5′(1067) gca aga aga tga tga ctg g	3′	310
	reverse 5′(1376) ttc ctg gca att agg atg ag	3′	
SSADH	forward 5′(1042) atg tga atg acg cag ttg cca	3′	309
	reverse 5′(1351) aga tca gtc ctt cgt tca cg	3′	
β-actin	forward 5′cca agg cca acc gcg aga aga gga c	3′	591
	reverse 5′agg gta cat ggt ggt gcc gcc aga c	3′	

Electrophoresis Quantitative Analysis

The product of RT-PCR was observed under the 256 nm UV light after performing a 1% agarose gel electrophoresis. We applied UVI Gel analysis system scanning the electrophoresis band of the PCR products, and relatively quantified the mRNA of these three genes through the OD value of *GAD_{67}*/*β-actin*, *GABA-T*/*β-actin*, and *SSADH*/*β-actin* (Table 3 and Figures 1-3).

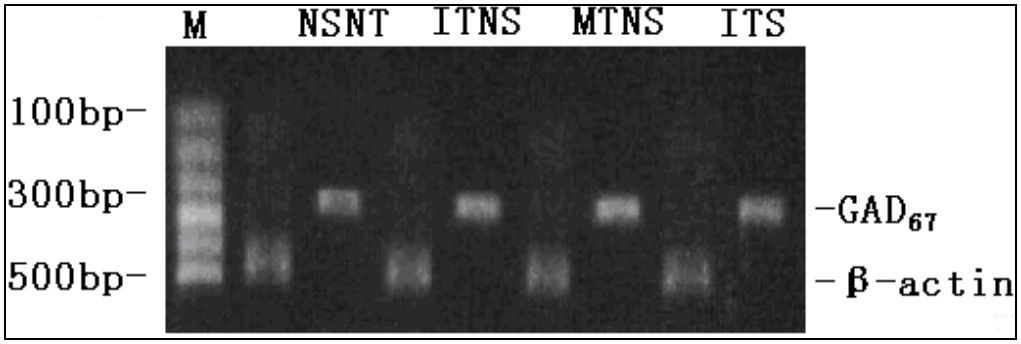

Figure 1: The electrophorogram of *GAD$_{67}$* mRNA in rat *hippocampus* of each group

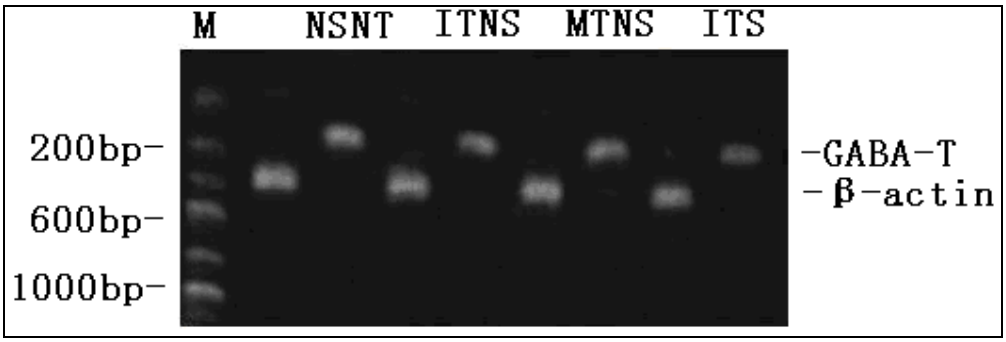

Figure 2: The electrophorogram of *GABA-T* mRNA in rat *hippocampus* of each group

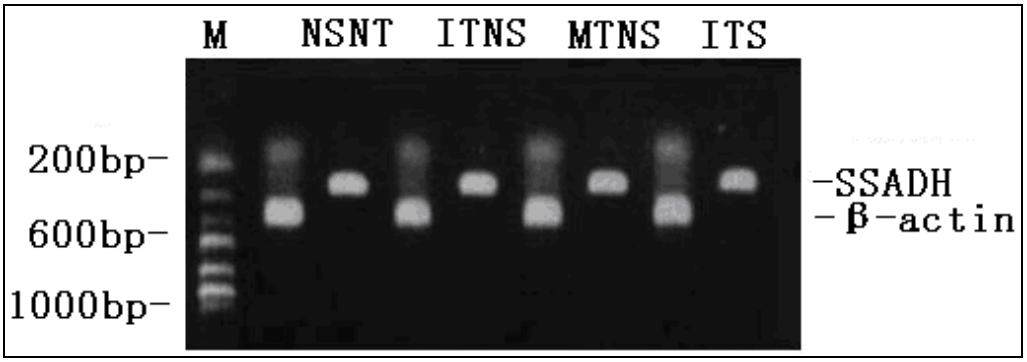

Figure 3: The electrophorogram of *SSADH* mRNA in rat *hippocampus* of each group

Table 3: The change of gene expression in rat *hippocampus* of each group (mean ± SD)

Gene/Groups	NSNT group♠	MTNS group♣	ITNS group♥	ITS group♦
GAD$_{67}$ mRNA	1.14±0.02♥	1.25±0.03♥♠	1.39±0.07	1.28±0.02♥♠
GABA-T mRNA	0.98±0.04♥	0.97±0.02♥	0.94±0.01	0.97±0.01♥
SSADH mRNA	0.97±0.01♥	0.98±0.02♥	0.93±0.02	0.96±0.01♣♥

Note: by S-N-K test of a one way ANOVA, ♥$p<0.01$, compared with the ITNS group; ♠$p<0.01$, compared with the NSNT group; ♣$p<0.01$, compared with the MTNS group

As the results shown in Table 3, the *GAD$_{67}$* significantly enhanced in the ITNS group than in the other three groups ($p<0.01$, $p<0.01$, $p<0.01$. *Cohen's d*=0.92/0.79/0.73), and significantly enhanced in the MTNS and ITS group than in NSNT group ($p<0.01$, $p<0.01$. *Cohen's d*=0.91/0.96); The *GABA-T* significantly

decreased in the ITNS group than in the other three groups ($p<0.01$, $p<0.01$, $p<0.01$. *Cohen's d*=0.57/0.69/0.83); The *SSADH* significantly decreased in the ITNS group than in the other three groups ($p<0.01$, $p<0.01$, $p<0.01$. *Cohen's d*=0.78/0.78/0.69), and significantly decreased in the ITS group than in MTNS group ($p<0.01$. *Cohen's d*=0.53). According to the Cohen's standards, the *t* test has a small effect size, medium effect size and large effect size when Cohen's *d* value=0.2, 0.5, and >0.8.

DISCUSSION

Many studies had shown that amino acid neurotransmitter (AAN) involved in changing in *hippocampus* excitability induced by exercise fatigue. Glu is the strongest excitatory AAN while GABA is the strongest inhibitory AAN in the central nervous system (CNS). The Glu/GABA ratio reflects the state and level of excitability in brain. A high intensity exercise can decrease Glu/GABA ratio in rat *hippocampus*, which enhanced the postsynaptic inhibition and decreased neuron excitatory, and then developed exercise-induced fatigue [6]. The metabolism between Glu and GABA was shown as Figure 4. GABA would accumulate in the brain if GAD activity increased while *GABA-T* activity decreased (Figure 4).

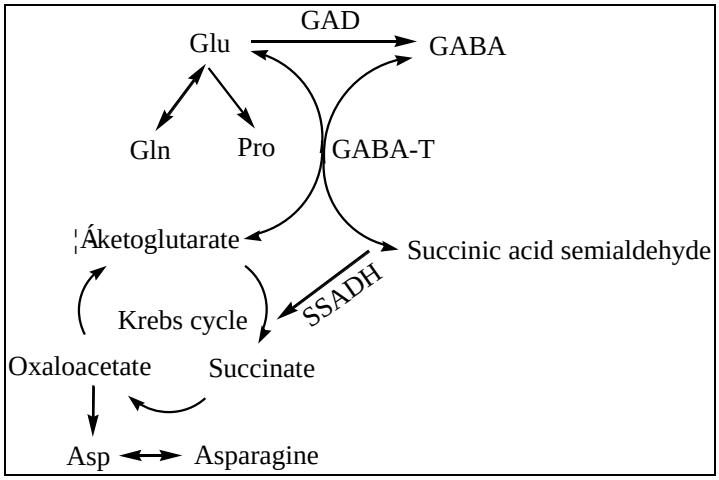

Figure 4: The metabolism of Glu and GABA in the brain

Abbreviations:
Glu=glutamate; Gln=glutamine; Pro=praline; Asp=aspartic acid;
GAD=glutamate decarboxylase; GABA=γ-aminobutyric acid;
GABA-T=GABA transaminase; *SSADH*=succinic semialdehyde dehydrogenase

As the part of CNS structure and the memory loop dominated the formation of motor skills, the excitability level in *hippocampus* can directly affect the completion of sports skills. The decrease of Glu/GABA ratio was a key factor of exercise-induced central fatigue [7,8]. Based on the analysis of the experimental data. We found that SAC has a positive effect on delaying the central fatigue induced by exercise. GAD has GAD_{65} and GAD_{67} two subtypes. The GAD_{65} mainly exists in nerve ending in the form of apoenzyme, which usually shows less active. While the GAD_{67} mainly exists in the neuronal cell body in the form of combination with cofactors, which usually shows more active. So choosing GAD_{67} as observation index would be more sensitive [9]. As shown in Table 3, GAD_{67} expression in rat *hippocampus* enhanced along with the increase of exercise intensity, while the expression of GAD_{67} in ITS group significantly decreased than in ITNS group ($p<0.01$), which indicated that SAC shows an effect of inhibiting the up-regulation of GAD_{67} in rat *hippocampus* under the strenuous exercise.

As the data shown in Table 3, *GABA-T* appeared a down-regulative trend along with the increases of exercise intensity, the expression of *GABA-T* in ITNS group significantly decreased than in the other three groups ($p<0.01$, $p<0.01$, $p<0.01$). After SAC intervention, the *GABA-T* expression in ITS group significantly increase than in ITNS group ($p<0.01$), which indicated that SAC has an effect of up-

regulating *GABA-T* expression, increasing the GABA oxidation and reducing its accumulation in rat *hippocampus*. So SAC shows an effect of anti central fatigue induced by exercise. The similar results were also presented in *SSADH* expression. Intensive training made *SSADH* expression down-regulated, which was positively correlated with exercise intensity. As shown in Table 3, *SSADH* expression in ITS group significantly enhanced than in MTNS group ($p < 0.01$), which indicated that SAC could significantly inhibit the down-regulation of *SSADH* induced by exercise. Because GAD_{67}, *GABA-T* and *SSADH* are the key enzyme in Glu and GABA metabolism in the brain, however, the Glu and GABA are usually difficult to pass through the blood-brain barrier and so their metabolism mainly occurred within the brain. Therefore, these experimental results suggest the following three points: (1) Intensive exercise could up-regulate GAD_{67} expression while down-regulate *GABA-T* and *SSADH* expression in rat *hippocampus*, and there was a significant correlation with the exercise intensity. The total effect on intensive exercise was that Glu was oxidized to GABA in a large amount, while the oxidation pathway of GABA was blocked, which resulted in the accumulation of GABA in *hippocampus*. As a result of a decrease of Glu/GABA ratio, the inhibitory process was enhanced while excitability decreased in *hippocampus*. Because the *hippocampus* is closely related to the formation and automation of motor skills, this change promotes the occurrence of exercise-induced central fatigue. (2) Through the analysis of the experimental data, SAC could down-regulate GAD_{67} while up-regulate *GABA-T* and *SSADH* expressions in the *hippocampus* of exercised rats, and effectively prevent Glu/GABA ratio from declining, so SAC could maintain a higher excitability in the rat *hippocampus* and therefore show an effect of anti central fatigue induced by exercise. (3) According to the theory of "protective inhibition" in sports fatigue, along with the exercise going on, excitatory Glu converted into inhibitory GABA which enhanced central inhibition and eventually developed sport fatigue. The sports fatigue prevents the body from an excessive losing of vital essence, which resulted from a negative feedback regulation of the body and could be seen as a self-protection in the movement [10].

Our previous study also found that exhausted exercise could lead serum aspartate transaminase (AST) and blood usea nitrogen (BUN) significantly increase and seriously damage functions of the liver and kidney in rats [11-14]. The effects of SAC on regulation to GAD_{67}, *GABA-T* and *SSADH* in rat *hippocampus* may be associated with its function of tonifying liver and kidney. Traditional Chinese medicine thinks that SAC is sweet flavor and warm in nature and has the functions of tonifying liver and kidney, controlling nocturnal emission, arresting polyuria and improving eyesight. Traditional Chinese medicine also considers that kidney has the function of being in charge of bone, producing marrow and storing vital essence, so it is the congenital foundation and the place where *archaeus* exist, and the resource of "side force" in the body. Liver has the function of storing blood and controlling dispersion of qi, liver dysfunction would decrease physical function and exercise ability and further develop sports fatigue [15]. The functions of SAC in tonifying liver and kidney and controlling nocturnal emission have been confirmed by modern medicine. A study by Huang CG et al. found that SAC can significantly increase the sperm number and sperm motility, reduce sperm deformity rate and improve the seminal vesicle index in rats after SAC intervention in spermatogenic dysfunction model induced by gossypol acetate and endoxan [16]. Another study by Sun LB et al. confirmed SAC shows a good protective and anti lipid peroxidation effect on rat liver injury induced by carbon tetrachloride [17]. A survey by Wang WX also shows that SAC can significantly improve the symptoms of depressed mice, while depression is losely related to the imbalance of neurotransmitter metabolism in the brain [18].

Glu is the highest up to 68%, in the 14 kinds of amino acid consisting in SAC [19]. We speculated that the distinct Glu content in SAC may be somewhat related to its anti central fatigue effect. Although, Glu in the peripheral blood usually cannot pass through the blood-brain barrier, however, during the exercise especially intensive exercise, cerebral ischemia and hypoxia induced by the redistribution of blood flow would lead to a decrease for pH and an increase for NO and intracellular Ca^{2+}, the latter regulate the proteins conformation of blood-brain barrier such as protein ZO^{-1}, ZO^{-2} and occludin-5 through the protein kinase, protein phosphatase and Rho kinase. As a result, the zipper structure was damaged and the permeability of blood-brain barrier increased [20]. Based on these changes, there may be a small quantity of Glu pass through the blood-brain barrier and affect the excitability of the brain. Meanwhile, cerebral ischemia and hypoxia during exercise also decreased aerobic metabolism of the brain, and further decreased oxidation of succinic acid semialdehyde, eventually lead to GABA accumulation (Figure 4).

CONCLUSION

(1) Intensive exercise significantly up-regulated the expression of GAD_{67} while down-regulated the expression of *GABA-T* and *SSADH* in the *hippocampus* of exercised rats, which promoted the central inhibition and correlated with exercise intensity. Therefore, this study supports the theory of "protective inhibition" in exercise induced fatigue. (2) SAC can significantly down-regulate GAD_{67} expression while up-regulate the expression of *GABA-T* and *SSADH* and then reduce Glu converting into GABA, improve the whole excitability of rat *hippocampus* and delay the central inhibition and central fatigue induced by intensive exercise. (3) The regulating effects of SAC on GAD_{67}, *GABA-T* and *SSADH* in the *hippocampus* of exercised rats may be related to its effect of tonifying the liver and kidney.

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