*Pakistan J. Zool., vol. 46(1), pp. 223-230, 2014.*

# Therapeutic Effect of Huangjingzanyu Optimized Formula on Sperm Quality and Activities of Succinic Dehydrogenase and Lactate Dehydrogenase-$C_4$ in Rat Asthenospermia Model

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**Abstract.-** This study was aimed at investigating the effects of Huangjingzanyu optimized formula on the energy metabolism of sperm and on the activities of mitochondrial marker enzymes. Polyglycoside of Tripterygium wilfordii (GTW) was used to establish rat asthenospermia models. We investigated the mechanism of the action of the Huangjingzanyu optimized formula on mitochondrial energy metabolism by measuring sperm adenosine triphosphate (ATP) content and changes in succinic dehydrogenase (SDH) and lactate dehydrogenase-$C_4$ (LDH-$C_4$) activities, Compared to the model group, the sperm ATP content was significantly increased in the high- and moderate dose Huangjingzanyu optimized formula. The SDH and LDH-$C_4$ activities were also significantly increased after treatment with the high- and moderate dose. No significant difference was noted between the optimized formula small-dose group and the model group. The Huangjingzanyu optimized formula can increase sperm ATP content by improving the activities of SDH and LDH-$C_4$ and other mitochondrial marker enzymes for energy metabolism, thereby improving sperm motility.

**Key words:** Asthenospermia, Huangjingzanyu optimized formula, ATP, SDH, LDH-$C_4$

## INTRODUCTION

Asthenospermia is a type of semen abnormality which accounts for approximately 46% of infertility (Guo and Chang, 2003). Although there are several drug available in the market, but none of these has been approved by FDA with curative effect (Crimmel *et al.*, 2001). Traditional Chinese medicine has specific therapeutic effects on male infertility and has been widely applied in clinical practice with convincing therapeutic efficacy (Zhang, 2012).

The Huangjingzanyu capsule is the first Chinese medicine (National New Drug Certificate: 220010103) used to treat male infertility. The original Huangjingzanyu formula is composed of 20 herbs, whereas Professor Qi Wang developed the Huangjingzanyu optimized formula (Wu, 2012; Chen *et al.*, 2009) using five herb extracts: Rhizoma polygonati, tuber fleeceflower root, wolfberry, red sage root, and dandelion. These compounds have been incorporated to preserve the health benefits of the original herb formula.

Previous studies have demonstrated that Huangjingzanyu capsules can improve sperm motility (Liu *et al.*, 2006). In addition, the ATP content of sperm was significantly increased after treatment with high and moderate doses of the capsules. Further studies studies are therefore, required to investigate the physiological mechanism involved in the improved motility.

Succinic dehydrogenase (SDH) and lactate dehydrogenase-$C_4$ (LDH-$C_4$) are closely related to sperm production, metabolism, and energy acquisition (Li and Li, 2011; Guo *et al.*, 1998; Duan *et al.*, 2003; Cheema *et al.*, 2013). SDH activity represents the physiological conditions of substrate oxidation and energy metabolism, along with mitochondrial function (Ji *et al.*, 2009). LDH-$C_4$ can bind to the shuttle system and can couple with electron transport in mitochondria, thereby generating ATP by oxidative phosphorylation (Xin and Lan, 2008). LDH-$C_4$ has a relatively high affinity for lactate, and energy can be produced

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0030-9923/2014/0001-0223 $ 8.00/0

through oxidative dehydrogenation of lactate. Therefore, SDH and LDH-$C_4$ are the key enzymes for sperm energy metabolism.

Hence, sperm ATP content is directly related to motility, whereas SDH and LDH-$C_4$ serve as marker enzymes for production of ATP in mitochondria. In order to investigate the mechanism of sperm mitochondrial energy metabolism, we have measured in this study the sperm ATP content and changes in SDH and LDH-$C_4$ activities before and after treatment with the drug.

## MATERIALS AND METHODS

*Tested drugs and chemicals*

Huangjingzanyu capsules of Beijing Chinese and Western Integrative Andrology Pharmaceutical Beijing, China, batch number 031105 and polyglycoside of *Tripterygium wilfordii* (GTW) of Zhejiang Apeloa Jiayuan Pharmaceutical Co. Ltd Dongyang, Zhejiang, China, batch number: 0309010 were used.

Three concentrations *viz.*, 0.5, 1 and 2 g/mL of the Huangjingzanyu optimized formula were provided by Beijing China-Japan Friendship Hospital. These drug formulations were suspended in 0.5% carboxymethyl cellulose (CMC) before clinical use.

Besides that Medium 199 (M199) containing Earle's balanced salt solution and L-glutamine produced by Invitrogen, California, USA batch number: 11298800; and the chemicals for preparation of SDH incubation medium (0.1 M sodium succinate, 0.1 M PBS pH 7.4, nitro blue tetrazolium, dimethyl sulfoxide) (Ji *et al.*, 2009) were purchased from Beijing Chemical Reagent Co. Beijing, China.

*Animals*

Male adult Sprague-Dawley, rats (grade II, weighing between 230-235 g) were supplied by Beijing Vital River, A Charles River Company) Certificate Number: SCXK-(Beijing) 2002-0003). The animals were housed separately and provided with necessary water and food *ad libitum*. The animal housing temperature was maintained at 24±1$^o$C, with a natural light/dark cycle.

*Modeling methods*

GTW tablets were crushed suspended in water and given to the rats by gavage feeding at 20 mg/kg once a day continuously for 30 days (Fang *et al*., 2000).

*Animal grouping and drug administration*

Seventy rats were adaptively fed for 1 week and then were randomly divided into 7 groups, each with 10 rats, the control group, the GTW model group, the negative control group, the Huangjingzanyu capsule group, the Huangjingzanyu optimized formula (= optimized formula) high-dose group, the moderate-dose group, and the low-dose group.

GTW at 2 mg/kg.d was administered to GTW model group by gavage feeding for 30 days. The animals were then sacrificed to measure each parameter. The control group was given 2 mL 0.5% CMC once a day for 60 days.

After the animal models were established the negative control group was administered 0.5% CMC at 2 mL/d by gavage feeding for 30 days.

Huangjingzanyu capsules were administered to Huangjingzanyu group at 465.mg/kg.d by gavage feeding for 30 days and different doses of the optimized formula were administered by gavage feeding at 12000 mg/kg.d (high dose), 6000 mg/kg.d (moderate dose) and 3000 mg/kg.d, small-dose groups for 30 days.

The animals in each group were weighed once a week for drug dose adjustment.

*Sperm quality analysis method*

*Sperm collection and processing*

Following Yu *et al*. (2000) with minor adjustments, we used a diffusion method to collect sperm from the tail of the epididymis. After drug administration, the animals were sacrificed. The left tail of the epididymis was obtained and placed in 3 mL of saline at 37$^o$C (preheated). Then, the specimen was cut into small pieces and incubated for 1 min. Next, a 50 µL sperm suspension was added into l mL of M199 medium and placed in a water bath at 37$^o$C for 5 min. Finally, a 13 µL suspension was added to a pre-warmed hemocytometer for sperm quality analysis.

*Sperm quality analysis*

The pre-treated sperm suspension to a counting board of WL-9000 sperm quality analysis system (Computer aided sperm analysis; Beijing WeiliNew Century Science & Tech. Div. Co, Beijing, China) at a test temperature of 37°C, Five fields of view were selected, and the analysis was completed within 2 minutes. The main test parameters included sperm density (× 106/mL), sperm motility (%), sperm viability (%), straight-linear velocity (VSL, μm/s), average path velocity (VAP, μm/s), and curvilinear velocity (VCL, μm/s).

*ATP content assay*

ATP CLS type II bioluminescent kit produced by Roche, Basel, Switzerland was used for ATP content assay (Xue *et al*., 2003). The specimens in each group were first defrosted. Then, a 0.l mL sperm suspension was added to pre-boiled 0.9 mL Tris-EDTA (pH 7.8), placed in 100°C boiling water for 2 min, and centrifuged for 1 min at 10,000 rpm/min. Next, 100 μL of luciferase was added to 100 μL of supernatant and was then placed on ice for further testing. The standard curve of ATP was first generated using standard ATP samples. Then, each specimen was sequentially tested with a bioluminescence detection system (Turner Biosystem, USA), and ATP content was measured based on the ATP standard curves.

*SDH activity assay*

The methods for sperm suspension processing and the biochemical reactions were based on Ruiz-Pesini *et al*. (1998) with a few adjustments for improvement. The specimens in each group were washed with a rinse solution (0.1 M PBS +0.2% BSA) three times and then centrifuged for 8 min at 1500 r/min. After the supernatant was removed, the incubation solution and the sperm suspension was mixed at a ratio of 2:1 and incubated in a water bath at 37°C for 2 h. A 10 μL sample was obtained to make a smear. After air-drying, neutral balsam was used for slide mounting. Quantitative image analysis was then conducted on the smears, using a CMIAS multifunctional true color pathological image analysis system developed by the Air Force General Hospital and the Beijing University of Aeronautics, Beijing, China. Each smear was analyzed at 200 × magnification over 5 randomly selected fields of view. More than 100 sperm cells were tested for integrated optical density (IOD), mean optical density (MOD), and positive area ($\mu m^2$). Because IOD integrates positive area and MOD, IOD was mainly used to represent SDH activity.

*LDH-$C_4$ assay*

LDH-$C_4$ activity was determined in strict accordance with the kit instructions. LDH-C4 assay kit produced by Shenzhen Huakang Biomedical Engineering Co., Ltd., Shenzhen, China was used in this study. After the specimens in each group were defrosted, substrates A and B (750 μL each) were added into the same reaction tube and pre-warmed at 37°C for 5 minutes. Then, a 500-μL aliquot was added to 10 μL of defrosted sperm suspension. After rapid mixing, the samples were tested using a semi-automatic biochemical analyzer (Photometer 5010) (Boehringer Mannheim, Germany).

*Statistical methods*

All data are expressed as means ± standard deviations. The between-group comparison was conducted using a univariate analysis of variance (ANOVA) with SPSS11.0 software, with $P < 0.05$ considered as statistically significant. The correlation between two variables was analyzed using a linear correlation analysis.

## RESULTS

*Modeling results*

Compared with the control group, sperm density, motility, and viability were significantly decreased in the GTW model group. The results are shown in Table I.

**Table I.- Comparison of sperm density, motility, and viability between two groups ( ± s).**

| Groups | N | Sperm concentration ($10^6$/ml) | Sperm motility (%) | Sperm live-rate (%) |
|---|---|---|---|---|
| Normal | 10 | 11.41±7.34 | 12.66±6.15 | 34.85±10.61 |
| Model | 10 | 2.93±0.97* | 4,42±4.07* | 21.92±8.53* |

**Note: Compared with the control group * P<0.05**

*Sperm velocity in the GTW model group*

Compared with the control group, VSL, VAP, and VCL were significantly decreased in the GTW model group (P<0.05). The results are shown in Table II.

**Table II.- Comparison of sperm velocity between two groups (X± s).**

| Groups | n | VSL(μm/s) | VAP (μm/s) | VCL (μm/s) |
|---|---|---|---|---|
| Normal | 10 | 15.19±2.55 | 23.05±2.69 | 52.20±5.56 |
| Model | 10 | 8.75±2.6315* | 15.56±3.63** | 38.80±18.22* |

**Note: Compared with the control group * P<0.05, ** P<0.01 VSL, straight-line velocity; VAP, Average-path velocity; VCL, Curvilinear velocity.**

*Sperm quality*

The effects of the Huangjingzanyu optimized formula on sperm density, motility, and viability. Compared with the GTW model group, sperm density, motility, and viability were significantly increased in groups treated with high and moderate doses of the optimized formula (P<0.05). The therapeutic efficacies of those groups were similar to the Huangjingzanyu capsule group (P>0.05). The sperm density, motility, and viability were increased in the low-dose group. However, the difference was not statistically significant (P>0.05). These results are shown in Table III and Figure 1.

**Table III.- Comparison of sperm density, motility, and viability among groups (X± s).**

| Groups | n | Sperm concentration (106/ml) | Sperm motility (%) | Sperm live-rate (%) |
|---|---|---|---|---|
| Normal | 10 | 11.41±7.34# | 12.66±6.15# | 34.85±10.61# |
| Negative | 10 | 3.81±3.21* | 7.73±5 .56 | 24.96±7.67 |
| Model | 10 | 2.93±0.97* | 4.42±4.07* | 21.92±8.53* |
| HJZY | 10 | 8.87±3 .11# | 12.82±5.95# | 38.23±23.69# |
| Optimized formula high-dose | 10 | 7.28±3.09# | 10.14±5.33# | 34.49±15.34# |

Note: Compared with the control group * P<0.05, **P<0.01; Compared with the GTW model group #P<0.05, ##P<0.05.

*Sperm velocity*

Compared with the GTW model group, VSL, VAP, and VCL were significantly increased in the group treated with a moderate dose of the optimized formula and the group treated with the Huangjingzanyu capsule (P<0.05). VCL was significantly increased in the group treated with the high dose of the optimized formula. VSL and VAP were significantly increased in the group treated with a low dose of the optimized formula. The results are shown in Table IV.

*Sperm ATP content*

Compared with the control group, the sperm ATP content was significantly decreased in the GTW model group; the difference was statistically significant (P<0.05). Compared with the GTW model group, the sperm ATP content was significantly increased in the groups treated with the Huangjingzanyu capsule and the high dose of the optimized formula (P<0.05). No significant difference was noted in the group treated with the low dose of the optimized formula (P>0.05). The results are shown in Table V.

**Table IV.- Comparison of sperm velocity among groups (X±s).**

| Groups | n | VSL(μm/s) | VAP(μm/s) | VCL(μm/s) |
|---|---|---|---|---|
| Normal | 10 | 15.19±2.55## | 23.05±2.69## | 52.20±5.56# |
| Negative | 10 | 9.82±3.67** | 18.15±4.72 | 41.98±8.68 |
| Model | 10 | 8.75±2.63* | 15.56±3.64** | 38.80±18.22* |
| HJZY | 10 | 13.54±6.99# | 18.30±6 .56# | 51.59±11.25# |
| optimized formula high-dose | 10 | 11.92±5.35 | 16. 49±7.75 | 50.76±17.43 |
| optimized formula moderate-dose | 9 | 14.16±3.51# | 22.30±4.17## | 50.67±12.55# |
| optimized formula low -dose | 9 | 13.22±4. 86# | 20.66±4.60# | 48.63±10. 68 |

Note: Compared with the control group * P<0.05, **P<0.01; Compared with the GTW model group #P<0.05, ##P<0.05.

**Table V.- Comparison of sperm ATP content among groups (X ± s).**

| Groups | n | ATP content (pmol/$10^6$) sperm |
|---|---|---|
| Normal | 7 | 5.35±1.61# |
| Negative | 8 | 3.62±2.85 |
| Model | 10 | 1.59±0.48* |
| HJZY | 9 | 5.96±4.75# |
| Optimized formula high-dose | 9 | 5.59±3.45# |
| Optimized formula moderate-dose | 9 | 6.O1±4.38## |
| Optimized formula low -dose | 7 | 4.74±4.32 |

Note: Compared with the control group * P<0.05; Compared with the GTW model group #P<0.05, ##P<0.01.

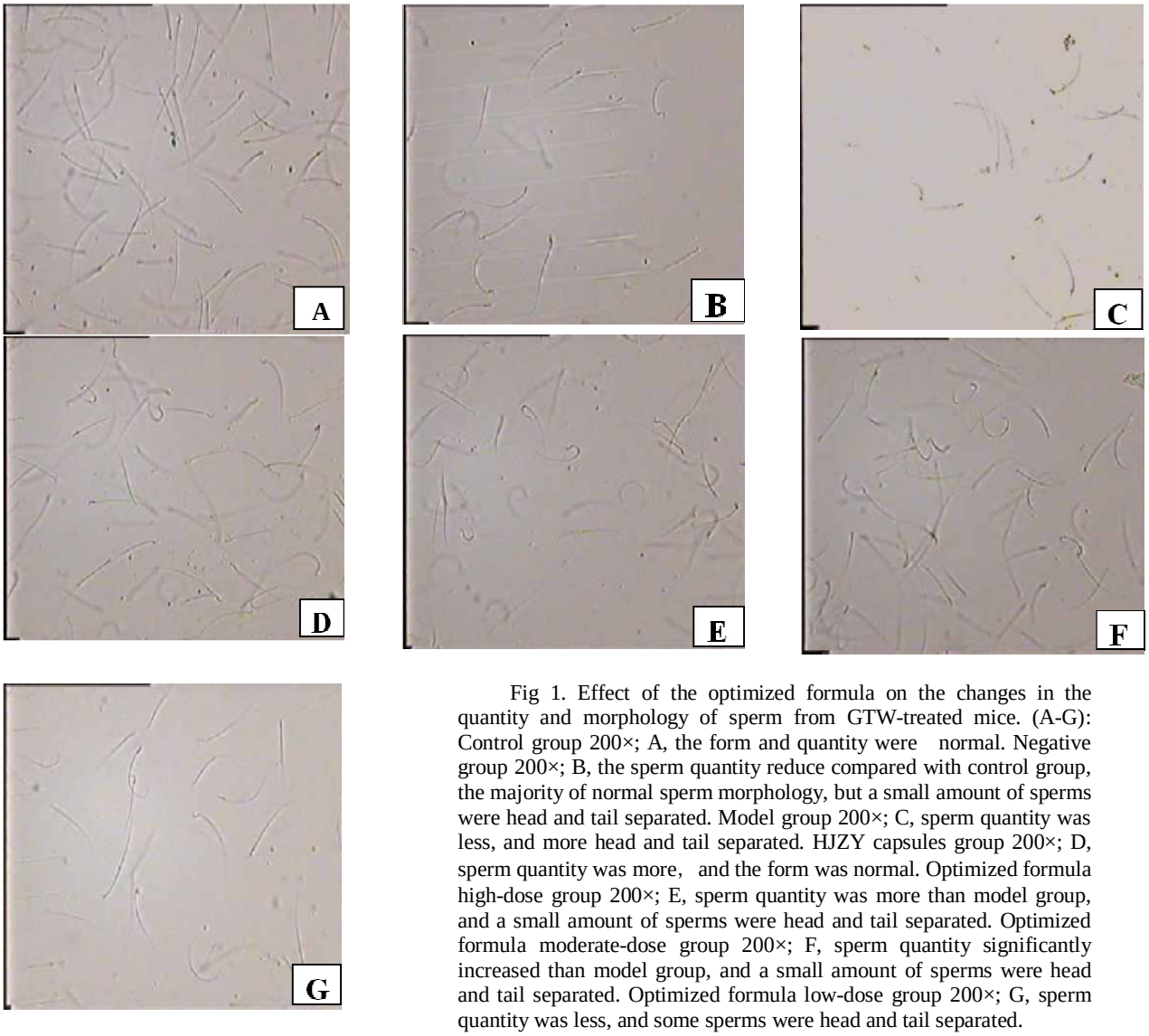


Fig 1. Effect of the optimized formula on the changes in the quantity and morphology of sperm from GTW-treated mice. (A-G): Control group 200×; A, the form and quantity were normal. Negative group 200×; B, the sperm quantity reduce compared with control group, the majority of normal sperm morphology, but a small amount of sperms were head and tail separated. Model group 200×; C, sperm quantity was less, and more head and tail separated. HJZY capsules group 200×; D, sperm quantity was more, and the form was normal. Optimized formula high-dose group 200×; E, sperm quantity was more than model group, and a small amount of sperms were head and tail separated. Optimized formula moderate-dose group 200×; F, sperm quantity significantly increased than model group, and a small amount of sperms were head and tail separated. Optimized formula low-dose group 200×; G, sperm quantity was less, and some sperms were head and tail separated.

*Sperm SDH activity*

Compared with the control group, the SDH activity was significantly decreased in the GTW model group; the difference was statistically significant ($P<0.05$). Compared with the GTW model group, the SDH activity was significantly increased in the groups treated with high and moderate doses of the optimized formula ($P<0.05$). No significant difference was noted in the group treated with the low dose of the optimized formula ($P>0.05$). The results are shown in Table VI.

*Sperm LDH-C4 activity*

Compared with the control group, although the sperm LDH-C4 activity was decreased in the GTW group, the difference was not statistically significant ($P>0.05$). Compared with the GTW model group, the sperm LDH-C4 activity was significantly increased in the groups treated with high and moderate doses of the optimized formula ($P<0.05$). No significant difference was noted in the group treated with the low dose of the optimized

formula (P>0.05). The results are shown in Table VII.

**Table V.- Comparison of sperm ATP content among groups (X± s).**

| Groups | n | ATP content ($pmol/10^6$) sperm |
|---|---|---|
| Normal | 7 | 5.35±1.61# |
| Negative | 8 | 3.62±2.85 |
| Model | 10 | 1.59±0.48* |
| HJZY | 9 | 5.96±4.75# |
| Optimized formula high-dose | 9 | 5.59±3.45# |
| Optimized formula moderate-dose | 9 | 6.O1±4.38## |
| Optimized formula low -dose | 7 | 4.74±4.32 |

Note: Compared with the control group * P<0.05; Compared with the GTW model group #P<0.05, ##P<0.01.

**Table VI.- Comparison of sperm SDH activity among groups (X± s).**

| Groups | n | IOD |
|---|---|---|
| Normal | 6 | 92.30±17.74# |
| Negative | 6 | 50.44±22.26 |
| Model | 6 | 24.99±10.28* |
| HJZY | 6 | 97.60±28.76# |
| Optimized formula high-dose | 6 | 92.48±37.93# |
| Optimized formula moderate-dose | 6 | 118.20±74.60## |
| Optimized formula low -dose | 6 | 61.61±21.04 |

Note: Compared with the control group * P<0.05; Compared with the GTW group #P<0.05, ##P<0.01.

**Table VII.- Comparison of sperm LDH-$C_4$ activity among groups (X ± s)**.

| Groups | n | LDH-C4 ($mU/10^6$ sperm) |
|---|---|---|
| Normal | 8 | 24.52±10.44 |
| Negative | 7 | 32.04±26.96 |
| Model | 7 | 20.64±11.81 |
| HJZY | 7 | 59.64±52.24# |
| Optimized formula high-dose | 7 | 61.75±20.63# |
| Optimized formula moderate-dose | 6 | 81.82±55.41# |
| Optimized formula low -dose | 6 | 41.58±34.10 |

**Note: Compared with the GTW model group #P<0.05.**

*Correlation analysis between ATP and sperm SDH and LDH-C4 activities*

In each group, sperm ATP content was positively correlated with sperm viability and SDH and LDH-C4 activities, respectively: sperm viability: r=0.805, P<0.05; SDH activity: r=0.908, P<0.01; LDH-C4 activity: r=0.866, P<0.05.

## DISCUSSION

Based on the original Huangjingzanyu capsules, the Huangjingzanyu optimized formula is composed of five herbs: Rhizoma polygonati, tuber fleeceflower root, wolfberry, red sage root, and dandelion. A low dose of Rhizoma polygonati decoction can increase serum LDH activity in mice (Li and Zhao, 2010). Tuber fleeceflower root can improve the survival rate and motility of sperm after thawing from frozen samples (Shi and Xu, 2010). Lycium barbarum polysaccharide is the major functional component in wolfberry and has a protective effect on the rat reproductive system (Huang *et al*., 2003a,b; Şereflişan *et al*., 2013).

Red sage root liquid extract can effectively increase sperm motility and can improve sperm quality when sperm are processed in vitro (Zhao *et al*., 1998; Lu *et al*., 2002). In addition, the total salvianolic acid from red sage root extract can increase cellular ATP contents (Gong *et al*., 2013). Dandelion can facilitate antigen engulfment among leukocytes and reticuloendothelial cells, thereby inhibiting immune responses. In addition, dandelion has a relatively strong anti-inflammatory effect in the reproductive tract. With the interactions of different herbal components, the Huangjingzanyu optimized formula promotes the complementary effects of clearing and strengthening, which have the overall effects of “strengthening the kidneys and tonifying essence, activating blood circulation and removing blood stasis, eliminating wet and clearing dampness”, thus promoting sperm production and fertilization.

Our early study showed HJZY optimized formula could restore the continuity of plasma structures of sperm heads, cervical parts and tails, render sperm heads to restore its well-stacked and homogeneous shape and correct the twisting deformity of sperm tails, thus to improve the sperm quality and enhance sperm motility capacity (Chen *et al*., 2008).

Sperm primarily use ATP produced from aerobic oxidation in mitochondria to maintain their motility. ATP content directly reflects mitochondrial functional status (Shi *et al*., 2008). SDH is mainly

present in the mitochondria of germ cells in the testicles and is the key enzyme for aerobic respiration in testicular energy metabolism (Burgos et al., 1995). Reduced SDH enzymatic activity can affect sperm energy metabolism, leading to a significant reduction in sperm motility (Xu *et al*., 2011). SDH can catalyze the transfer of succinate to fumarate, followed by ATP production after dehydrogenation. Therefore, SDH activity is associated with ATP production. LDH-$C_4$ is closely related to sperm production, metabolism, and energy acquisition. LDH-$C_4$ can bind to the shuttle system and couple with electron transport in mitochondria, thereby aiding in the generation of ATP by oxidative phosphorylation. Therefore, mitochondrial ATP content is related to SDH and LDH-$C_4$ activity levels. GTW can inhibit sperm maturation. The extent of sperm mitochondrial damage is positively correlated with drug administration duration and dosage. Short-term drug administration can reduce LDH and SDH activity levels. In this study, sperm motility and viability were significantly reduced in the GTW model group compared with the control group. ATP content was also decreased in the GTW model group. ATP content was significantly increased in the group treated with high and moderate doses of Huangjingzanyu optimized formula, suggesting that an increase in ATP content might be one reason for the improved sperm motility. And correlation analysis further demonstrated that ATP content was significantly correlated with SDH and LDH-$C_4$ activity levels in each group, suggesting that the Huangjingzanyu optimized formula can increase ATP production by increasing the activity levels of mitochondrial marker enzymes related to energy metabolism, such as SDH and LDH-$C_4$, thereby improving sperm motility.

The Huangjingzanyu optimized formula can increase ATP production in sperm flagella by increasing the activity levels of mitochondrial SDH and LDH-$C_4$ in sperm, thereby playing a role in improving sperm motility.

## ACKNOWLEDGEMENTS

The work is supported by the National Basic Research Program of China (973 program)under grant no.2011CB505403, and National Science Foundation of China under no.81273767.

*Conflict of interest declaration.*

There is no conflict of interest or otherwise.

*Policy of Ethics Committee*

This research has been accepted by Ethics Committee of Beijing University of Chiese Medicine.

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*(Received 4 October 2013, revised 20 November 2013)*