Effects of tortoise-shell glue on rat oligoasthenospermia model

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To the Editor: Infertility is a public health concern in modern society. Oligoasthenospermia (OA) is one of the leading causes of male infertility that impacts about 5% of reproductive-aged couples worldwide. The etiology and pathogenesis of OA remain poorly understood. The characteristics of OA include a reduction in the total number (<33 × 10^6 per ejaculate or <12 × 10^9/mL) or concentration of spermatozoa, and low or nil sperm motility (<32%).

Traditional Chinese medicine (TCM) has a long history of treating male infertility by using drugs derived from natural sources, such as herbal or animal tissue-derived materials. Tortoise-shell glue (TSG), a classic TCM, is prominently used as a drug or supplement on multiple health conditions to replenish “Qi”, which is considered to be the energy source of the human body in the TCM theory. A mitochondrion, a cell’s “powerhouse,” is essential for cellular signaling and plays a role similar to that of “Qi” in TCM. The mid piece of sperm is packed with mitochondria; therefore, mitochondrial dysfunction may play a vital causative role in inadequate sperm mobility and male infertility. Mitochondria not only provide the spermatozoa the necessary energy for successful fertilization but are also closely associated with the viability of the spermatozoa via regulating the permeability by the mitochondrial permeability transition pore (mPTP), a transmembrane protein located in the inner membrane of mitochondria. Normally, it remains closed, but the opening of mPTP allows large molecules to pass through the mitochondrial membrane. Malfunction of mPTP leads to cell apoptosis, and in the case of OA, it results in a reduced sperm number and motility. In the current study, we aimed to evaluate the therapeutic effects of TSG on spermatogenesis and reproductive hormones, and also investigate the potential protective effects of TSG on mitochondrial functions.

Twenty-eight male Sprague Dawley (SD) rats (weight: 200 ± 20 g; age: 10 weeks) were purchased from Hunan Silaik Jingda Laboratory Animal Co., Ltd (Changsha, China) and housed one per cage under a 12-h light/dark cycle with 55 ± 5% humidity at 22 ± 1°C. The rats were divided randomly into seven groups, namely normal control, OA model (Model), OA + low dosage TSG (Low-TSG), OA + medium dosage TSG (Medium-TSG), OA + high dosage TSG (High-TSG), Levocarnitine, and TSG + Levocarnitine, with four rats in each group. All the animal study protocols were approved by the Animal Welfare Committee of the Hunan University of Chinese Medicine, Changsha, China (No. 2019-0019). The rats in the normal control group were given saline intragastrically for 50 days. The OA model was established according to the protocol adapted from previous publications showing L-thyroxine-induced OA in rats. To elucidate, the rats were given 330 μg·kg^{-1}·day^{-1} thyroid hormone: L-thyroxine (BioVision Inc., Milpitas, CA, USA) by intragastric administration for 20 days. From day 21, saline solution was given to the OA model group. The low, medium, and high TSG groups daily received 1.0 g/kg, 1.5 g/kg, and 2.0 g/kg TSG (Hunan Dongjian Pharmaceutical Co. Ltd., Changsha, China), respectively. The positive control Levocarnitine group received 330 μg/kg Levocarnitine (BioVision Inc.) per day, and the TSG + Levocarnitine group received 330 μg/kg Levocarnitine + 2 g/kg TSG per day. After 30 days of treatment, blood was extracted for a hormonal test, and the left testis from each rat was harvested for mitochondrial assessments. A part of each right testis was fixed in a 10% formalin solution to carry out histological analysis, and the remaining part was used for sperm counts.

Semen parameters, including sperm motility (%) and sperm concentration (×10^9/mL), were characterized to determine the impact of TSG on OA rats [Figure 1A–B]. L-thyroxine treatment-induced characteristic OA symptoms, including significantly decreased sperm curvilinear velocity (VCL), motility, and concentration as compared to the normal control (P < 0.05). Medium-and high-TSG treatments restored the sperm VCL, motility, and concentration of OA rats as compared to the model group (P < 0.05) and showed similar therapeutic outcomes with the levocarnitine positive control. The TSG + Levocarnitine-tine group exceeded the levocarnitine positive control in promoting VCL and sperm concentration. Testosterone is an important male

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reproductive hormone. A significant reduction in testosterone was noticed in the OA group as compared to the normal controls. TSG treatments restored sperm production gradually in a dose-dependent manner. The medium- and high-TSG groups showed a significant increase in testosterone as compared to the OA group ($P < 0.05$). OA rats that

Figure 1: TSG enhances sperm activities and testosterone level and protects testis and mitochondrial functions in normal rats, OA Model rats, low-, medium-, high-TSG treated rats, Levocarnitine treated rats and TSG + Levocarnitine treated rats. (A) Sperm VCL ($\mu$m/s), motility, and concentration ($10^6$/mL), (B) Hematoxylin and eosin staining of testis tissues, original magnification $\times 400$. (C) Flow cytometry analysis of mPTP functions and (D) Mitochondrial respiratory complex activities. $^\ast P < 0.05$ vs. normal; $^\dagger P < 0.05$ vs. model, $^\ddagger P < 0.05$ vs. levocarnitine. OA: Oligoasthenospermia; mPTP: Mitochondrial permeability transition pore; TSG: Tortoise-shell glue; VCL: Sperm curvilinear velocity.
received high-TSG and Levo-carnitine treatments showed similar levels of testosterone production. The combination treatment of TSG + Levocarnitine achieved the highest level of testosterone production among all OA rats [Figure 1C]. Histological analysis of testis tissue showed that in the normal group, the rat testis revealed a regular arrangement and tightly intact epithelial cells in the seminiferous tubules, as well as a regular density of spermatogenic cells. Testis tissue in the OA model demonstrated remarkable damages, including several shrunken and disrupted tubules and epithelial cell organization, loss of spermatogenic cells, and degeneration of interstitial cells. TSG treatment gradually restored the structural damage of the testis with the increase of dosage. Among all experimental groups, TSG + Levo-carnitine treatment showed the most significant protective effects on the structural integrity of OA testis tissue [Figure 1D].

Thereafter, mitochondrial functions, including mPTP function and mitochondrial respiratory complex activities [Figure 1E], were evaluated. All six parameters were impacted similarly, as the OA group demonstrated a drastic reduction in mitochondrial functions. TSG treatments partially restored the levels of all six parameters with the increase of dosages, and the combination of TSG + Levocarnitine demonstrated the best recovery of the mitochondrial functions. Numerous underlying conditions may cause OA in humans. For example, patients with hyperthyroidism consistently show decreased spermatozoa motility.\(^5\) We established the rat OA model by a prolonged administration of levothyroxine, a thyroid hormone. The rats that received levothyroxine demonstrated structural damage in their testes, and a decrease in sperm density, motility, and testosterone production, which are the main characteristics of OA. Levocarnitine is a well-characterized drug that has been proven to increase sperm quality in OA patients. Therefore, we used levocarnitine treatment as the positive control. The TSG treatments restored the testosterone level, sperm density, and motility in a dose-dependent manner and protected the structural damage in the testis. The TSG-high group even exceeded the positive control group in showing an improvement in semen qualities, thereby indicating the beneficial impacts of TSG had on OA.

Mitochondria play a key role in male fertility because they not only provide energy to the sperm but also play vital roles in sperm motility, hyperactivation, DNA integrity and acrosome reaction, etc. Nowicka-Bauer et al.\(^6\) reported that sperm mitochondrial dysfunction was commonly observed in patients with normozoospermia and asthenozoospermia, and reactive oxygen species (ROS) could be the possible reasons. Some underlying conditions or environmental influences may induce oxidative stress that generates high levels of ROS; high levels of thyroid hormones could be one of the causes of OA.\(^5\) In this study, we observed a significant decrease in mPTP function and mitochondrial respiratory complex activities in Levothyroxine-induced OA rats, indicating severe mitochondrial damage in this model. The Western blotting results showed suppressed expressions of key proteins, including voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT) [Supplementary Digital Content, Figure 1, http://links.lww.com/CM9/A688]. The TSG treatment partially recovered the mPTP level and mitochondrial respiratory complex activities in a dose-dependent manner, suggesting the protective effect of TSG against mitochondrial damage. By combining levocarnitine with TSG, the most significant decrease in testis tissue damage and increase in mitochondrial functions were observed. This indicated that TSG could be used as a supplement to assist regular clinical treatments to achieve better outcomes.

Overall, a high dosage of TSG treatment demonstrated promising therapeutic effects on the spermatogenesis process in OA rats by remarkably increasing sperm density, motility, and testosterone production. The enhanced spermatogenesis by TSG could be attributed to its protective effect against mitochondria damages. Such protective effect was significantly enhanced when both TSG and Levocarnitine were used together. Our results highlighted the critical role of TSG as a drug or a supplement that assists traditional treatments for OA. The limitations of this study include a relatively small animal sample size and the single pathogenesis of the OA model we used. To better illustrate the effectiveness of TSG, OA models other than hyperthyroidism-induced with a larger animal sample size will be used in future studies.

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**Conflicts of interest**

None.

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