

## Original Research Article

# Astragalus polysaccharide relieves reproductive toxicity in phenobarbital-treated epileptic rats

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### Abstract

**Purpose:** To investigate the underlying mechanisms by which *Astragalus polysaccharide* (APS) relieves the reproductive toxicity induced by phenobarbital (PB) treatment in epileptic rats.

**Methods:** Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay kits were used to quantify cell apoptosis in an epileptic rat model. The weight of sex organs and levels of three reproductive hormones, viz, follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone, were measured in order to evaluate the effect of APS administration on reproductive ability. Concentration, motility, morphology as well as fertilization rate of sperms were analyzed as well.

**Results:** Increase in sex organ weight and decrease in apoptosis were both associated with oral APS treatment. In APS-treated group, FSH, LH, and testosterone levels were raised while concentration, motility and normal morphology of sperm also increased. This was consistent with the observed increase in fertilization rate. In addition, hematoxylin and eosin (HE) staining of the testis was performed in the epileptic rat model showed that the size of cell lumen increased in APS-treated group. All APS-associated phenotypes occurred in a concentration-dependent manner.

**Conclusion:** These data suggest that APS lowers reproductive toxicity in PB-treated epileptic rats by regulating the reproductive hormones, FSH, LH and testosterone, and also by altering the concentration, motility, and morphology of sperm. Thus, APS has a potential treatment for minimizing the side effects of antiepileptic drugs.

**Keywords:** *Astragalus polysaccharide*, Reproductive toxicity, Phenobarbital, Epileptic rats

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## INTRODUCTION

Epilepsy is a common neurological disorder characterized by recurring seizures. About two-thirds of seizures are treated with antiepileptic drugs (AEDs), such as the well-known

phenobarbital (PB), which is commonly used in young children and pregnant women [1,2]. Despite the wide usage of AEDs, they impair fertility, bone health and sexuality in human and cause severe side effects including birth defects, cognitive impairment, and reproductive toxicity by

mechanisms which are not well-understood [3,5]. Previous studies suggested that PB treatment should be reserved for patients with resistant disabling myoclonus and seizures [4].

Astragalus polysaccharide (APS), a component found in traditional Chinese herbal medicines, is used to treat a wide variety of body disorders and diseases [6]. In recent years, natural products isolated from such herbal medications have found their clinical applications in treating cancers and inflammatory disease therapy has been actively investigated [6]. It has also been reported that APS is able to inhibit muscle cell atrophy by activating the ubiquitin proteasome pathway (UPP) associating murine chronic kidney diseases [7].

The aim of this research is to investigate the impact of APS on side effects of PB for treating epilepsy as well as to explore the mechanisms of APS for relieving reproductive toxicity in PB-treated epileptic rats.

## EXPERIMENTAL

### Epilepsy rat model

The study was approved by the Animal Ethics Committee of the affiliated Hospital of Hunan University of Traditional Chinese Medicine (approval ref no. 2015-0003). Experiments on rats were in full compliance with the Principles of Laboratory Animal Care [8]. Lithium chloride (127 mg/kg) was injected into the enterocoelia of healthy adult Wistar rats.

Atropine or butylamine scopolamine (1 mg/kg) was injected to the enterocoelia after 24 h. Pilocarpine (1 %, 30 mg/kg) was then injected after 30 min. The epileptic state was sustained for 1 h, after which phenobarbital (30 mg/kg) was injected to stop the epileptic seizure. Rats that survived the process were used as long-term epilepsy rat models.

### Drug treatment

Astragalus polysaccharide (APS) was purchased from Shi feng Biological Co., Shanghai, China. Drugs were administered orally at different concentrations: APS1 [low dose (200 mg/kg/d APS)], APS2 [medium dose (400 mg/kg/d APS)], and APS3 [high dose (800 mg/kg/d APS)]; phosphate-buffered saline (PBS) was used as blank control group, APS and PB were injected concurrently at each dose and continuously for 60 days, as previous studies showed that injection of phenobarbital (PB, 30 mg/kg) over a

course of 60 days could induce reproductive toxicity [9].

### Hematoxylin and eosin (HE) staining

#### *Hydrating testis and epididymis sections*

Slides were dried for 1-2 h at 65°C and placed in a rack. Racks were dipped into four consecutive jars containing xylene, in order to remove paraffin. Racks were then submerged in ethanol for 5 min to remove xylene and then rinsed with the tap water for 5 min to eliminate ethanol.

#### *Staining nucleus with hematoxylin*

Slides with rat tissues were left in hematoxylin for 10 min, and then rinsed with double-distilled water (ddH<sub>2</sub>O). The rinsed slides were immersed in HCl (0.1%), ddH<sub>2</sub>O, NH<sub>4</sub>OH (0.1%) and again ddH<sub>2</sub>O successively.

#### *Staining cytoplasm with dehydrate and eosin*

The slides were left in eosin for 3 min, then immersed in ethanol (100 %), acetone and xylene, successively. Mountant (3 drops) was added onto each slide and a cover slip was applied.

### TUNEL assay to detect apoptosis

To detect cell apoptosis, the paraffin sections of the testis and epididymis and the apoptosis detection kit (Takara Corporation) were used.

### Enzyme-linked immunosorbent assay (ELISA)

The levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were measured by ELISA kit (Thermo Fisher scientific)

### Evaluation of sperm motility and sperm concentration

Epididymis and testes were isolated from rats and suspended in PBS. On removal of vessels and adipose were the tissues added into Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 medium (1 mL). Sperms were allowed to "swim-up" for 1 min. Semen (5 µL) was then used to calculate the sperm concentration. Motility of sperms was determined under microscope [10,11].

### Morphological examination of sperm

To observe sperm morphology, semen (5 µL) was stained with Spermblye according to an established method [12].

## Evaluation of fertilization rate

Two female and one male rat were caged together. Upon formation of a vaginal plug in a female, the caged females were placed with two new females. Each mating experiment lasted 2 weeks, after which the number of vaginal plugs formed and the number of offspring were calculated for each male rat to determine fertilization rate.

## Statistical analyses

Analyses were performed using SPSS v.13.0 (SPSS Inc., Chicago, IL). Data were reported as mean  $\pm$  SEM. Differences were considered statistically significant when  $p < 0.05$ . Differences between groups were evaluated using the Student's t-test and one-way analysis of variance (ANOVA).

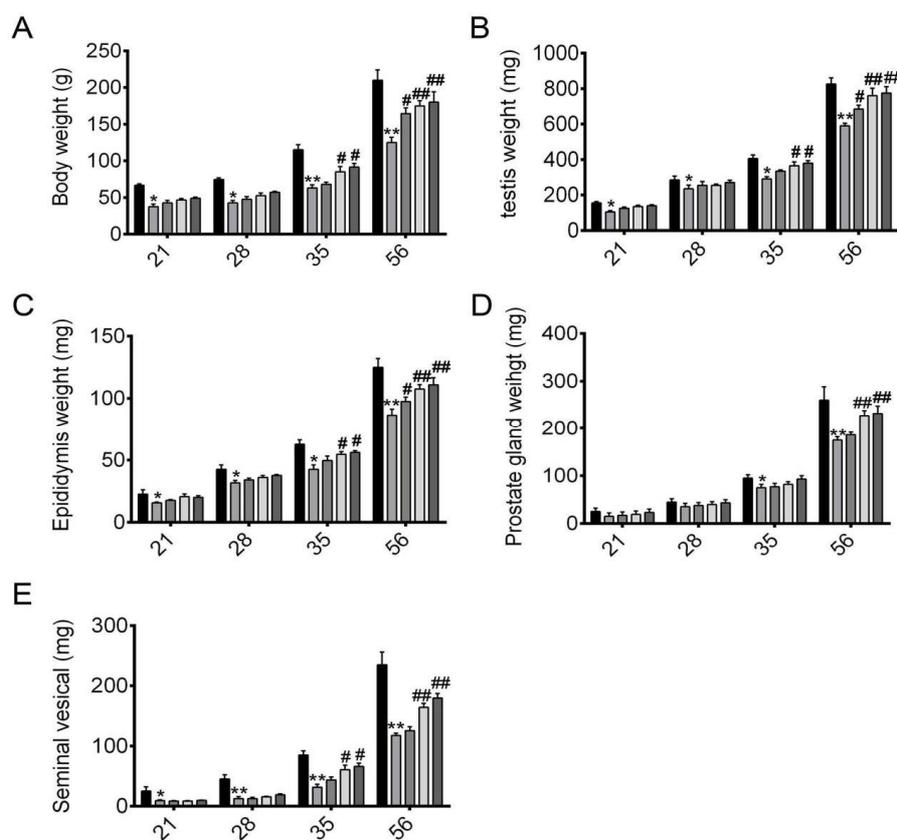
## RESULTS

### APS treatment improved body and sex organ mass in PB-treated rats

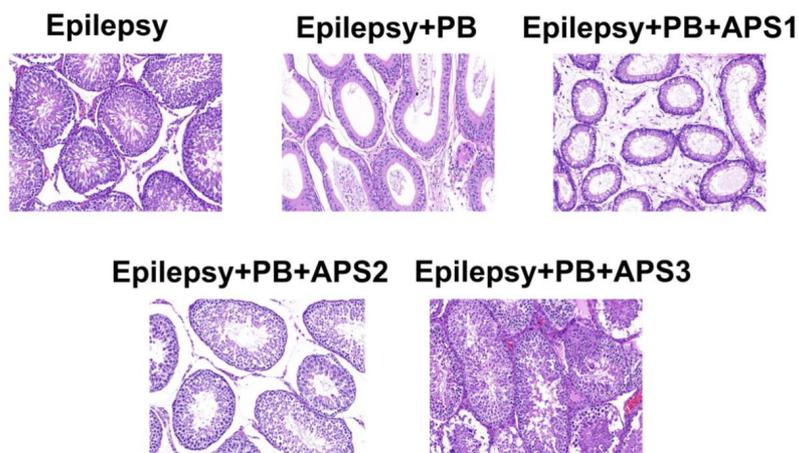
Reproductive ability was evaluated by measuring the mass of total rat body, testis, epididymis, prostate gland, and seminal vesicle. As shown in Figure 1, on the days 35 and 56, body mass and sex organ mass of the APS + PB-treated group increased significantly when compared with the PB-treated group ( $p < 0.05$ ), however the difference was not significant with relative to the control group. In APS3 group (800 mg/kg/d APS), mass of rats raised more than that in the APS1 group (200 mg/kg/d APS). The results indicated that the APS raised both body mass and sex organ mass in PB-treated rats.

### APS treatment increased the lumen size of testis cells and reduced the number of apoptotic cells in the testis of PB-treated rats

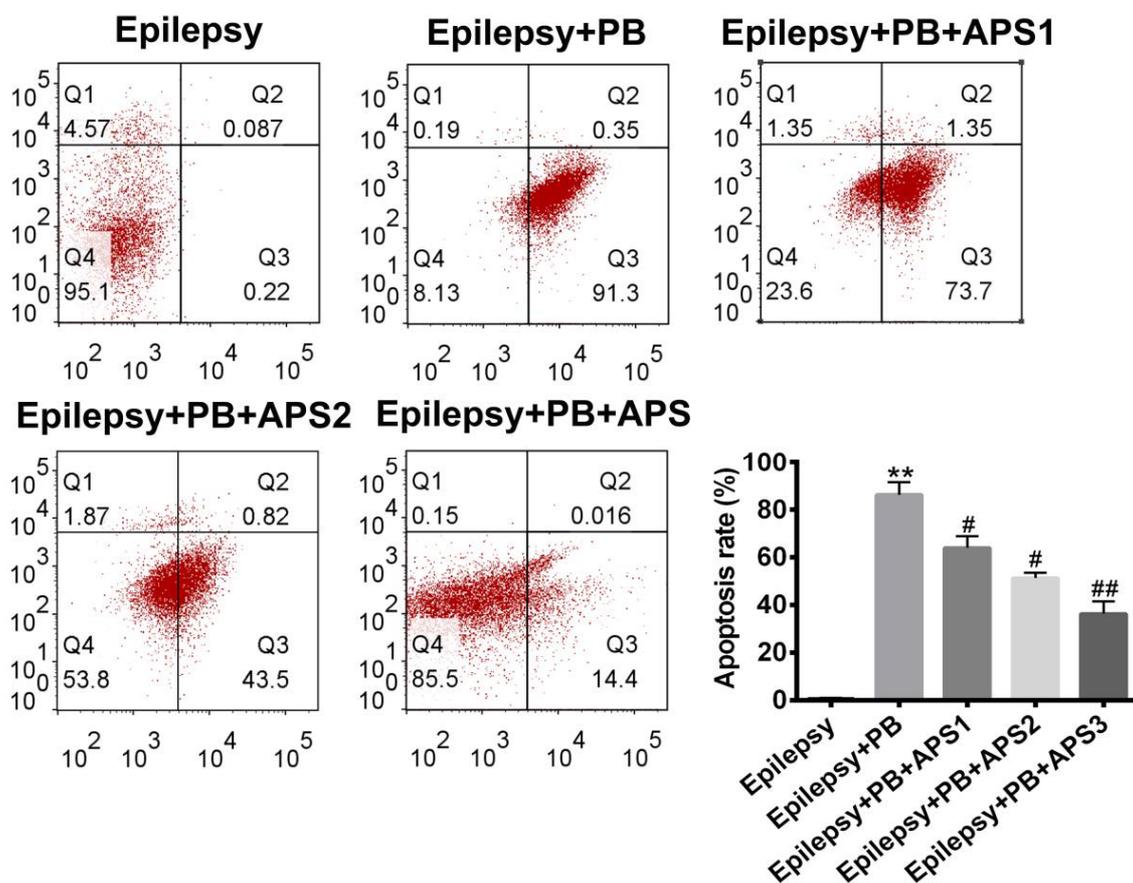
HE staining of testis and TUNEL flow cytometry were performed in the epileptic rats. In the APS-treated groups, the size of cell lumen increased, and the frequency of cell apoptosis was significantly lower than their counterparts in the PB-treated group ( $p < 0.05$ ) (Figure 2 and Figure 3). The alleviating effect of APS on the phenotypes occurred in a concentration-dependent manner (Figure 2 and Figure 3).



**Figure 1:** Mass of total body and sex organs of rats. (A) Total body; (B) testes, (C) epididymis, (D) prostate gland, and (E) seminal vesicle. Results are reported as mean  $\pm$  SEM,  $n = 5$ . ■: Epilepsy; ▒: Epilepsy+PB; ▓: Epilepsy+PB+APS1; ▒: Epilepsy+PB+APS2; ▓: Epilepsy+PB+APS3. \*  $p < 0.01$ , #  $p < 0.05$ , vs. control group; ##  $p < 0.01$ , #  $p < 0.05$ , vs. PB-treated group



**Figure 2:** The HE staining of testis and epididymis. Differences in morphology of cells in different treatment groups were visualized by HE staining (40 x)

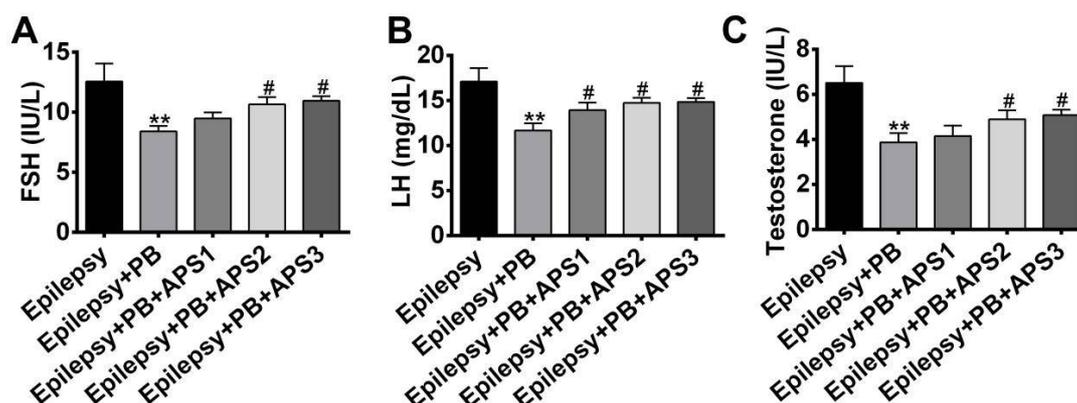


**Figure 3:** Measurement of apoptosis by TUNEL flow cytometry analysis across treatment groups. Results are reported as mean  $\pm$  SEM, n = 5.  $p < 0.01$ ,  $p < 0.05$ , vs. control group;  $###p < 0.01$ ,  $#p < 0.05$  vs. PB-treated group

**Effect of APS treatment on the levels of the reproductive hormones FSH, LH, and testosterone, in epileptic rats**

Levels of the reproductive hormones, FSH, LH, and testosterone, were measured to evaluate the effect of APS administration on reproductive ability [13]. The levels of all three hormones of APS-treated group were significantly higher than

those of the PB-treated group ( $p < 0.05$ ), but lower than those of the control group (Figure 4). Moreover, rats of APS3 (800 mg/kg/d) group expressed higher levels of FSH, LH, and testosterone than rats of APS1 (200 mg/kg/d) group (Figure 4). The results indicated that the APS treatment increased levels of FSH, LH, and testosterone in PB-treated rats in a concentration-dependent manner.



**Figure 4:** Levels of reproductive hormones FSH, LH, and testosterone in different APS treatment groups: (A) FSH levels, (B) LH levels, and (C) testosterone levels. Results are reported as mean  $\pm$  SEM,  $n = 5$ . \*\* $p < 0.01$ ,  $p < 0.05$ , vs. control group; ## $p < 0.01$ , # $p < 0.05$  vs. PB-treated group

**Table 1:** Sperm concentration and motility in epileptic rats of different treatment groups

Group	Sperm concentration ( $10^6$ / ml)	Sperm motility (%)
Epilepsy	0.99 $\pm$ 1.55	90.15 $\pm$ 8.88
Epilepsy+PB	0.54 $\pm$ 0.64*	65.13 $\pm$ 3.89*
Epilepsy+PB+APS1	0.89 $\pm$ 1.22#	70.33 $\pm$ 9.98#
Epilepsy+PB+APS2	1.36 $\pm$ 0.54#	78.88 $\pm$ 4.23#
Epilepsy+PB+APS3	1.56 $\pm$ 0.37#	80.55 $\pm$ 3.71#

Results are reported as mean  $\pm$  SEM,  $n = 5$ ; \* $p < 0.01$ ,  $p < 0.05$  vs. control group; ## $p < 0.01$ , # $p < 0.05$  vs. PB-treated group

#### Effects of APS treatment on sperm concentration, motility and morphology and on fertilization rate in epileptic rats

For male animals, sperm concentration is a reflection of fecundity capacity, and sperm motility is an indication of effective fertilization potential [14]. Sperm concentration and motility obtained during this study are summarized in Table 1. In the APS-treated group, the concentration and motility of sperms were significantly higher ( $p < 0.05$ ) than those in the PB-treated group (Table 1). Furthermore, the

frequency of abnormal sperm morphology decreased in APS-treated groups (Table 2). Fertilization rates and offspring numbers increased in the APS-treated group (Table 3). Additionally, all alleviation of PB-associated phenotypes by APS treatment occurred in a concentration-dependent manner. These data suggested that APS could relieve reproductive toxicity due to PB treatment in epileptic rats both by raising the levels of FSH, LH, and testosterone and by increasing concentration, motility, and morphology of sperms.

**Table 3:** Reproductive capacity of epileptic rats following treatment

Group	Fertilization rate (%)	Number of offspring
Epilepsy	100	200
Epilepsy+PB	50	90
Epilepsy+PB+APS1	58	120
Epilepsy+PB+APS2	65	140
Epilepsy+PB+APS3	68	155

Results are reported as mean value,  $n = 5$

## DISCUSSION

Epilepsy has major effects on many important aspects of human life [5]. Patients with seizures

**Table 2:** Sperm morphology in epileptic rats of different treatment groups

Group	Abnormal sperm (%)	Head abnormality (%)	Tail abnormality (%)	Multiple abnormalities (%)
Epilepsy	28.85 $\pm$ 3.96	10.22 $\pm$ 8.75	11.3 $\pm$ 73.74	4.79 $\pm$ 3.58
Epilepsy+PB	59.32 $\pm$ 9.89	28.84 $\pm$ 3.77	40.55 $\pm$ 2.09	9.90 $\pm$ 1.33
Epilepsy+PB+APS1	51.90 $\pm$ 3.87	22.73 $\pm$ 5.69	35.75 $\pm$ 2.04	7.69 $\pm$ 2.24
Epilepsy+PB+APS2	46.09 $\pm$ 8.85	17.17 $\pm$ 3.33	30.24 $\pm$ 3.34	6.34 $\pm$ 1.11
Epilepsy+PB+APS3	45.55 $\pm$ 3.83	16.98 $\pm$ 5.78	28.77 $\pm$ 8.75	6.11 $\pm$ 1.09

Results are reported as mean  $\pm$  SEM,  $n = 5$

often suffer from underlying brain disease and co-morbidity with depression or other mental disorders.

In both animals and humans, epileptic discharges directly alter hormone levels. Previous studies suggested that approximately two-thirds of seizures could be treated with AEDs [1,2]. However, AEDs have undesired hormonal side effects that can influence such as bone health, fertility, and sexual function. It was found that fertility rates were lower in patients with epilepsy than in general population [15]. Different AEDs may affect hormonal systems differently and cause various types of hormonal imbalances [16-18]. Previous research indicated that PB was effective for epilepsy treatment, however undesired side effects were as well reported [19]. To reconcile the use of PB as therapy for epilepsy with the side effects produced by this drug, we used APS, a component found in traditional Chinese herbal medicines to treat a wide variety of body disorders and diseases.

APS was found to be able to raise mass of total body and sex organs for PB-treated epileptic rats. It could also increase the size of cell lumen of testis and reduce the frequency of cell apoptosis in PB-treated rats. Three reproductive hormones, FSH, LH, and testosterone, were used to evaluate reproductive ability in rats [13]. In male animals, sperm concentration was used to measure the reproductive capacity and sperm motility was to measure effective fertilization potential [14]. Significant differences were observed between APS-treated and control groups regarding the levels of FSH, LH, and testosterone, as well as the concentration, motility, and morphology of sperms.

The results proved that APS treatment relieves reproductive toxicity caused by PB treatment in epileptic rats by regulating levels of FSH, LH, and testosterone and by influencing concentration, motility, and morphology of sperms. Alleviating effects varied with concentrations of APS dose, indicating that APS treatment alleviated reproductive toxicity in a concentration-dependent manner. The research indicated a potential treatment for subduing side effects of AEDs.

Future investigations regarding transferring the scientific research to a feasible clinical therapy will be carried out. Reducing AED side effects will lay a foundation for the treatment of epileptic patients and more effective therapy for these patients will be explored in the future.

## CONCLUSION

The above results concluded that APS treatment can relieve reproductive toxicity induced by PB treatment in epileptic rats by regulating the reproductive hormones (FSH, LH, and testosterone). APS treatment can also influence concentration, motility, and morphology of sperms. The research suggested a potential treatment for subduing side effects of AEDs.

## DECLARATIONS

### *Conflict of Interest*

The authors declare that there is no conflict of interest associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jun Lu designed all the experiments and revised the paper. Hou-pan Song, Qin-hui Tuo and Qi-chang Zeng performed the experiments, Qin Wang and Ya-hui Huang wrote the paper.

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