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Amygdala Kindling Alters Estrus Cycle and Ovarian Morphology in the Rat

Juan Pan[1,5], Lingwu Zhang[1], Feng Wang[2,3], Dan Liu[1], P. Andy Li[4], Tao Sun[2,3]

[1]Department of Gynecology, General Hospital of Ningxia Medical University, Yinchuan, China, 75004

[2]Ningxia Key laboratory of Cerebrocranial Disease, Incubation Base of National Key Laboratory, Ningxia Medical University, Yinchuan, China, 75004

[3]Department of Neurosurgery, General Hospital of Ningxia Medical University, Yinchuan, China, 75004

[4]Department of Pharmaceutical Sciences, Biomanufacturing Research Institute Biotechnology Enterprise (BRITE), North Carolina Central University, Durham, NC 27707, USA

[5]Department of Gynecology, Changqing Oil Field Worker's Hospital, Xi'an, China 710201

*Juan Pan and Linwu Zhang contributed equally

Abstract: The objective of this study is to explore the effects of amygdala kindling on estrus cycle and ovarian morphology. Thirty-five female rats at the age of 8 weeks were randomly designated to electrode kindled, sham-kindled, and normal controls. Kindled rats were implanted with kindling electrodes in the left basolateral amygdala and kindled by brief suprathreshold stimulations with a bipolar electrode. Estrous cycles were daily monitored through vaginal smears. Electrographic and behavioral seizures were recorded and ovarian morphology was evaluated by light and electron microscopies. Our results showed that the kindled rats lost their ovarian periodicity displayed significant ovarian enlargement. H&E staining revealed increased number of growing follicles and total follicles, as well as polycysts in the ovaries of the kindled animals compared to sham and control animals. Ultrastructural study detected numerous apoptotic granulosa cells in growing follicles and thecal cell hyperplasia with secretary granules in the thecal cells in the kindled rats. The results suggest that amygdala kindling is a risk factor for the development of polycystic ovary syndrome.

Keywords: amygdala kindling; endocrinology; epileptogensis; estrus cycle; ovarian morphology; polycystic ovary syndrome; reproductive system

Introduction

Polycystic ovary syndrome (PCOS) is one of the common reproductive endocrine disorders and a main cause of anovulation in women of childbearing age. The pathological features of PCOS include impaired follicle maturation, presence of polycystic ovaries (PCOs), a 2~3-fold increase of pre-antral follicles, and lack of dominant follicles. Clinically, PCOS is characterized by anovulatory menstruation including oligomenorrhea and amenorrhea. The etiology of PCOS is still unclear. In clinic, anovulatory infertility in 50%~70% of patients are cause by PCOS.

PCOS is mainly characterized by oligo-ovulation or anovulation. Laboratory and clinical examinations have shown that PCOS patients have hyperandrogenism and polycystic-like ovaries. The etiology of PCOS is still poorly understood. It may be associated with genetic factors, hypothalamic dysregulation, or ovarian, glucose, and environmental factors. Some researchers have even proposed that

P. Andy Li, Tao Sun (Correspondence)
pli@nccu.edu, suntao@nxmu.edu.cn
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PCOS is a variant of neuropathy or epilepsy [1]. In the present study the rats in the kindling group not only had a significant increase in body weight, but also exhibited marked changes in the estrus cycle and ovaries.

Previous studies have observed that epileptic women, especially those with limbic or temporal lobe epilepsy are more susceptible to menstrual disorders, infertility, PCOS, sexual dysfunction, and other gynecologic diseases than healthy women [2-4]. As such, the incidence of PCOS is 10%~20% in epileptic and 5%~10% in healthy women [5,6].

It is not fully understood why epileptic female has increased chance for PCOS. It has been postulated that either epilepsy itself or long-term administration of anti-epileptic drugs, especially sodium valproate (VPA), cause PCOS [3,4,6,7,8-13]. In the present study, a left amygdala kindle model was established in rats to study the effect of amygdala kindling (epileptogenesis) on ovulation and ovarian morphology.

Materials and Methods

Animals

Eight-week-old female SD rats (n=35), weighing 180~220 g, were purchased from the Experimental Animal Center of Ningxia Medical College and housed individually with free access to food and water. Animal room temperature is controlled between 21~25^{o}C, a humidity of 50%~70%, and a light cycle of 12 h. The estrus cycle of the animals was determined by morning vaginal smears. Rats with 3 normal estrus cycles were included in the present study. Animals were then divided into 3 groups: kindled group, animals with a bipolar electrode implantation and electric stimulation (n=15); sham-kindled group, animals with electrode implantation without electric stimulation (n=10); and control group, normal non-operated animals (n=10)

Establishment of rat kindle model

Electrode implantation: Rats were anesthetized intraperitoneally with sodium pentobarbital. According to the description in the Paxinos and Watson atlas, a bipolar electrode was stereotacsically implanted into the left basolateral nucleus of the amygdala using the coordination below: 2.8 mm posterior to the bregma, 4.9 mm lateral to the midline, and 8.6 mm below the dura mater. Animals were housed individually after the electrode implantation.

Electric stimulation: One week after the electrode implantation, electric stimulation was performed and the after discharge threshold (ADT) changes were detected using the AM-2100 single-channel stimulator. The initial stimulus intensity was 60 μA, with an increment of 20 μA and the intervals between two stimulations were ⩾ 60 s. The brain electrical activity was recorded by using an Alpha-Lab 4-channel Signal Acquisition and Processing System until the spikes lasted ⩾ 3 s, which was defined as an ADT. Thereafter, stimulation was carried out twice daily at an interval of 4 h. The conditions for stimulations were as follows: rectangular square wave, pulse width of 1 ms; 60 Hz, and duration of 1 s [14]. At the same time, the seizure strength was recorded and the changes in the brain electrical activity were observed. The maximal stimulus intensity was ⩽ 400 μA. Figure 1 shows the the EEG characteristic pattern of normal baseline, afterdischarge and seizure activity in freely moving rats.

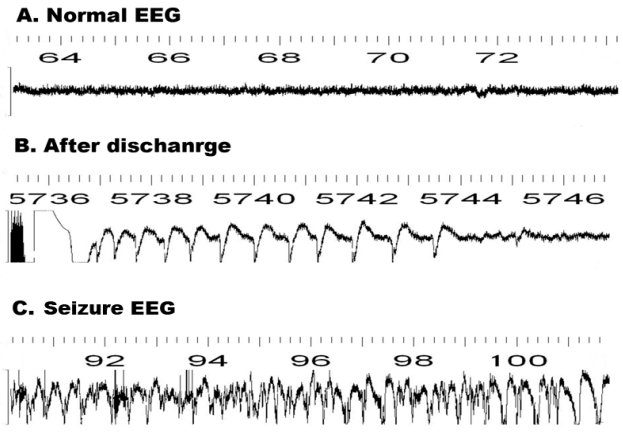

Fig.1. Original EEGs recorded from the left amygdale. **A**, a normal baseline EEG; **B**, evoked afterdischarge; and **C**, electrical seizure activity.

Determination of kindling model: At 10:00 am and 14:00 pm, stimulation was carried out at the ADT and the behaviors of the animals were observed and recorded. Seizure strength was determined according to the Racine staging system [14], Three consecutive seizures of stage IV-V were used to confirm the successful establishment of the kindling model. For kindling rats, electric stimulation was still performed daily at the ADT at 10:00 am with a 400 μA for 20 consecutive days.

Determination of the estrus cycle

The normal estrus cycle in the rat ranges from 4-6 days, which can be determined by cytologic examination of vaginal smears. In the present study, the estrus cycle was checked in the 8-week-old rats daily at 8:30~9:30 am. The estrus cycle was staged based on vaginal cell morphology according to the Freeman's criteria [13].

Sample collection

Twenty days after the establishment of kindling model, rats in diestrus stages were intraperitoneally injected with pentobarbital sodium for subsequent tissue sampling. Control and sham-kindled rats with matched estrus cycle were sacrificed as well. The rats were then perfused with 2% paraformaldehyde and the brains were harvested.

Both ovaries were collected from each rat. The right ovary was used for Haematoxylin and Eosin (H&E) staining. Consecutive ovary section was obtained at the level that has the largest cross-sectional area along the long axis and used for morphological analysis. A fraction of ovaries in the left ovary were randomly selected for ultrathin sectioning, followed by observation of growing follicles under an electron microscope (Hitachi 7650B)

Light microscopy

Images were randomly captured in three microscopic fields at magnification of 200X with an Olympus-DP71 system and analyzed by Image-pro Plus 6.0. The sections with the largest cross-sectional area were used for image analysis. The pre-antral follicles, antral follicles, atretic follicles, and total growing follicles were counted. The section area, the thickness of the granulosa and membranous layers of the growing follicles were measured. The grading of follicles was performed according to the criteria developed by Nestorovic [15] and Borman [16].

Electron microscopic analysis of follicles

Ovarian tissues were fix in 4% glutaraldehyde and embedded Durcopan ACM. Ultrathin sections (70-nm) were cut using a diamond knife and stained with uranyl acetate and lead citrate. Sections were observed under a Hitachi transmission electron microscope.

Statistical analysis

Statistical analysis was performed with SPSS (version 11.5). Parametric data were expressed as the means ± SD and analyzed using one-way analysis of variance (ANOVA) followed by Scheffe's post test. For non-parametric data, Kruskal-Wallis followed by Mann-Whitney's test was used for analysis. A $P<0.05$ was considered statistically significant.

Results

Evaluation of the animal model

A total of 15 rats were performed in the kindled group. Among them, 1 rat died of anesthesia and 3 rats were excluded due to absence of epileptic EEG pattern. Of the remaining 11 rats, 5 developed grade IV seizures after for 7~10 days of kindling, and the remaining 6 rats had grade V seizures. All animals in the sham-kindled and control groups were survived. Figure 1 demonstrates the EEG pattern recorded from a freely moving control rat, a rat with after discharge and a freely moving rat with epileptic discharge**.**

At the end of the study, all animals were perfusion-fixed and brain sections were stained with Nissl's staining to confirm the correct implantation of the electrode in the kindled and sham-kindled groups

(Fig. 2).

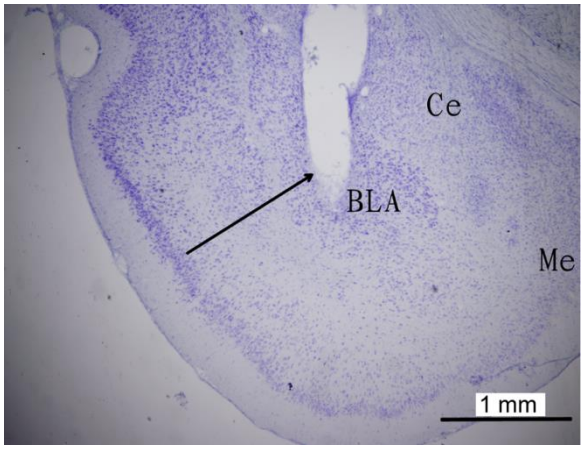

Fig. 2. Nissl's staining showing the position of electrode tip planted in rat brain. Arrow indicates the bipolar electrode tip positioned in anterior part of basolateral amygdaliod nucleus. BLA, basolateral amygloid nucleus; Ce, central amygdaloid nucleus; Me, medial amygdaloid nucleus. medial amygdaloid nucleus. Magnification, 40X. Bar = 1 mm

Changes in the estrus cycle

Alteration of estrus cycle was observed in all kindled rats who developed grade IV~V seizures two weeks after being kindled. The alterations were characterized by prolongation of the epithelial phase to equal or more than 4 days, and shortening of the leukocyte phase to equal or less than 1 day. In two rats, the keratinized epithelial phase lasted 8 days. Following a transient leukocyte phase, the long-lasting epithelial phase occurred on the next day. At the end of the study, the estrus cycle remained abnormal in all rats in the kindled group. The longest epithelial phase was approximately 15 days, followed by the leukocyte phase.

Changes in daily body weight and ovarian weight

All rats were weighted daily and their body weights were recorded. As described in Table 1, kindled rats had an average of daily body weight gain of 4.5 g, while those in sham-kindled and control groups had a weight gain of 1.42 g and 1.72 g ($p<0.05$). The ovarian weights were measured at the end of the experiment in all three groups. There was a significant increase in the kindled group compared with other two groups ($p<0.05$).

Table 1. Average daily weight gain and unilateral ovarian weight in three female experimental groups. Data are presented as means±s.d. *$p<0.05$ *vs.* control and sham kindled rats.

	Weight gain, g	**Ovary weight, g**
Kindled	4.50±2.37*	45.35±11.27*
Sham kindled	1.42±0.69	33.50±5.29
Control	1.72±1.12	33.50±10.90

Ovarian morphology

Gross morphology: In the control and sham-kindled animals, the ovaries were in approximately same size and had a fresh color. Comparing with sham and control groups, the ovaries were enlarged and numerous light pink colored cystic follicles were observed on the surfaces of the ovaries in the kindled animals. In general, the color of the ovaries of kindled rats was lighter compared to the other two groups, but the ovaries were rich in blood supply between the cystic follicles. In two kindled rats, the ovaries displayed light purple color.

Microscopic morphology: Follicles at different phases were observed in all three groups. In the kindled group, numerous pre-antral and antral follicles were noted and the majority of which became atretic. The oocytes in the central follicles had karyopyknosis and cytoplasmic heterogeneity, or became collapsed. The arrangement of granulosa cells was uneven in thickness and some granulosa cells were detached. The closer the follicular cysts were to the granulosa cells, the more disordered the arrangement of granulosa cells. The disordered arrangement of granulosa cells was also present on the surface of the cumulus oophorus that were close

to the follicular cysts. The large cystic follicles were largely deformed or became collapsed and irregular. The cystic wall was continuous and intact; however, signs of ovulation were absent. The thickness of the theca of the follicle in the kindled group increased and became more translucent than the sham and control groups. (Fig. 3)

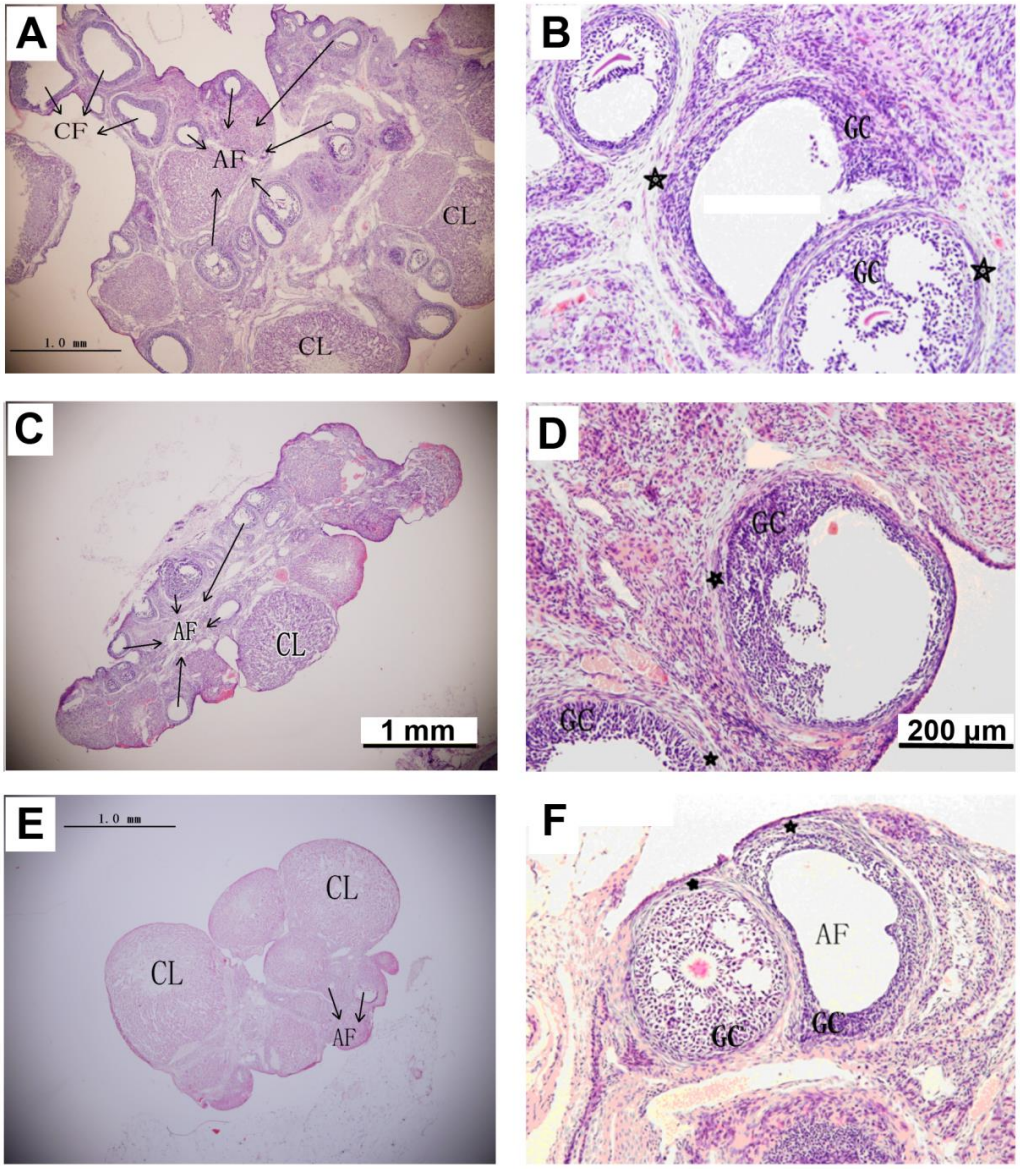

Fig. 3. Representative H&E-stained sections showing ovarian morphology in kindled (A&B), sham kindled (C&D) and control animals (E&F). A, an ovarian section from a kindle rat. Numerous pre-antral and antral follicles were noted. B, follicles from a kindled animal. Uneven thickness of granular cells and detached granular cells were observed. The oocytes in the central follicles had karyopyknosis and cytoplasm heterogeneity, or collapsed. **C**, an ovarian section from a shame-kindled rat, **D**, growing follicles of a sham-kindled rat. **E**, an ovary section of a control rat. F, growing follicles from a control rat. AF, developing antral follicles; CF, cystolic follicle; CL, corpus luteum; GC, granular cell; star, thecal cell. Bar= 1 mm in A,C,E and 200 μm in B,D,F.

Morphomatrics of the ovaries: As shown in Table 2, in the kindled group, the mean area of cross-section in kindled animals was significantly larger than that in the sham-kindled and control groups ($P<0.05$).

Similarly, the thecal cell layer of the follicle was also thicker in the kindled rats than that in the other two groups the control and sham groups (P<0.01). However, the granulosa cell layer in the kindled group was only slightly thinner than that in the other two groups and such difference did not reach statistical significance (P>0.05). Moreover, no marked differences were found between the sham-kindled and control groups with respect to the aforementioned parameters (P>0.05).

Table 2. Comparison of ovarian morphology measure in three groups. Data are presented as means±s.d. **p*<0.05 and ***p*<0.01 *vs.* control and sham kindled animals.

	Kindled	Sham kindled	Control
Section area，mm^2	8.92±3.12*	5.58±1.33	4.98±0.92
Granulosa cell-layer, μm	40.19±13.40	48.95±9.90	51.91±14.07
Thecal cells layer, μm	49.42±10.34**	31.87±8.10	32.08±10.47

Follicle counting

As shown in Table 3, the number of pre-antral follicles was markedly increased in the kindled group, as compared to the sham-kindled and control groups (P<0.05). In addition, the number of atretic follicles, the total number of growing follicles, and the ratio of atretic follicles to growing follicles in the rats of the kindled group were also markedly higher than those in the other two groups (P<0.01); however, the numbers of antral follicles and corpus luteum were not significantly different between the kindled group and the rest groups.

Table 3. Comparison of ovarian morphology count in three group Data are presented as means±s.d. **p*<0.05 and *p*<0.01 *vs.* control and sham kindled rats.

	Kindled	Sham-kindled	Control
Preantral follicles	36.18±20.74*	12.10±3.87	16.70±4.37
Antral follicle	6.27±1.90	4.40±2.68	3.90±3.18
Atretic follicle	9.45±7.17**	1.40±1.51	1.30±1.25
Total number of follicles	43.55±19.96**	16.80±4.44	20.80±5.80
Number of corpus luteum	3.45±1.58	4.10±2.28	4.10±1.79
Atretic follicle / total follicle	0.26±0.21**	0.09±0.09	0.07±0.08

Ultra-microstructural changes of the growing follicles

Transmission electron microscopy revealed tightly arranged granulosa cells and relative thin thecal foliculli interna in normal control and sham-kindled rats (Fig 4A). Cells in the thecal folliculi interna had a few lipid droplets, smooth endoplasmic reticulum and mitochondria. However, in the kindled animals, the granulosa cells were loosely misarranged, the thickness of the thecal foliculli interna increased (Fig 4B) and the thecal cells were rich in lipid drops (Fig 4C), smooth endoplasmic reticulum and mitochondria (Fig 5B). In addition to the above alterations, the external and mesenchymal cells of the thecal folliculi were abundant in collagen fibers (data not shown). Furthermore, apoptotic granulosa cells, characterized by karyopyknosis and karyorrhexis, increased in the kindled animals (Fig 5A). There were no difference among the three groups in number and morphology of the rough endoplasmic reticulum, ribosome, Golgi body, and mitochondrion

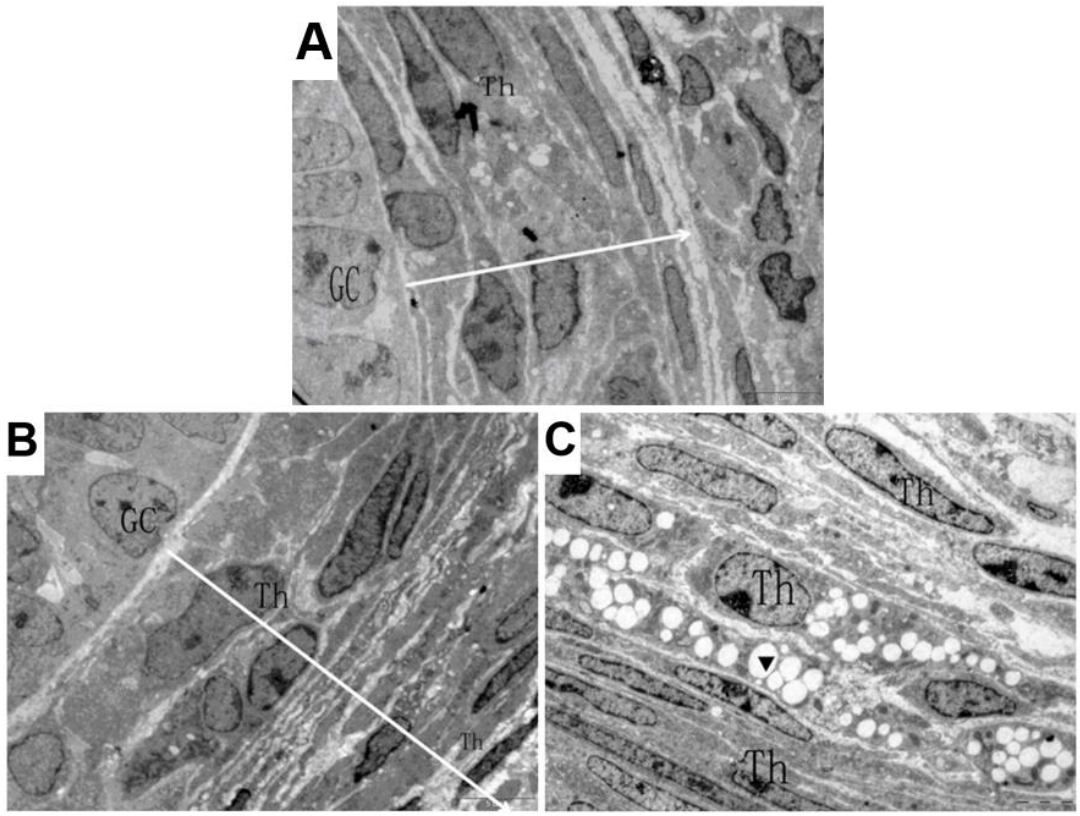

Fig. 4. Ultrastructures of growing follicles in control and kindled rats. **A**, Antral follicle from a control rat. Granular cells (GC) were closely arranged and the theca was relatively thin (length of white arrow). **B**, Antral follicle from a kindled rat. Uneven arrangement of granular cells and increased thecal cell layer (length of white arrow). C, Thecal cells (TH) filled with lipid droplets (filled triangle) in the kindeled rat. Length of the white arrow indicate the thickness of the thecal cell layer. Magnification, 700X.

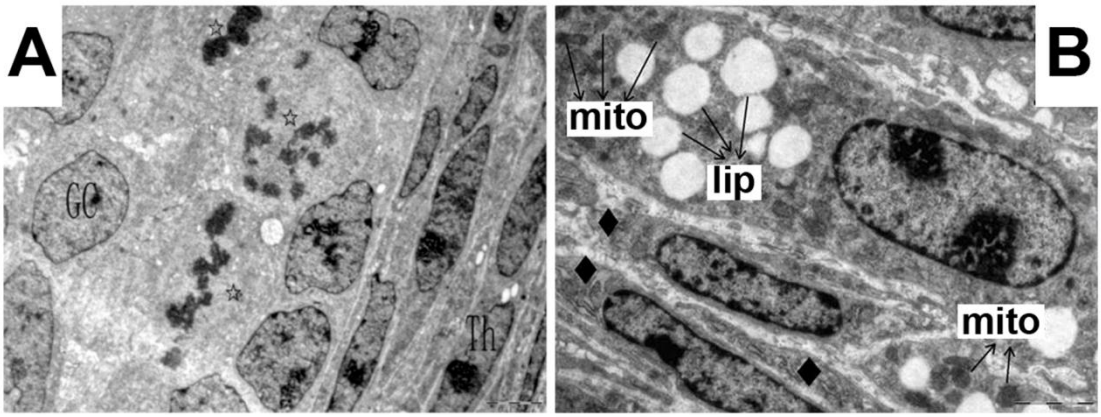

Fig. 5. Ultrastructural changes of an antral follicle from a kindled rat. **A**, Granular cells are loosely arranged and several apoptotic granular cells (starts) were observed. **B**, Thecal cells of an antral follicle with rich lipid droplets, smooth endoplasmic reticulum and mitochondria. GC, granular cell; Th, Thecal cell; mito, mitohcondria; lip, lipid droplet; open star, apoptotic granular cells; filled diamond, smooth endoplasmic reticulum. Magnifications, 700X for **A** and 2000X for **B**

Discussion

Amygdala kindling is a model of early epilepyogesesis that leads to repeated spontaneous seizures, the latter is the main clinical feature of epilepsy. The influence of epilepsy on hypothalamus has been previously explored. It has been reported that hypothalamus loses its ability of regulating hormone secretion, resulting in dysfunction of the hypothalamic-pituitary- ovarian axis [4,7,8,17]. Previous studies have shown that epilepsy may induce PCOS [1,2]. However, it is not known whether amygdala kindling causes disturbance to estrus cycle and ovarian morphological changes. Our present study demonstrated that amygdala kindling not only disturbed the estrus cycle but also resulted in ovarian enlargement and polycyst formation, granulosa cell apoptosis in growing follicles, and proliferation of follicular thecal cells.

Disordered Estrus Cycle

The estrus cycle reflects the function of gonadal glands in female mammals [1]. In the rat, each cycle lasts about 4-5 days. Estrus cycle includes 4 phases. It starts with proestrus phase, which lasts approximately 17~21 h in the rat. In this phase, estrogen level starts to increase and follicles are starting to grow. It is followed by estrus phase that lasts 9~15 h, characterized by a peak level of estrogen. The estrus phase corresponds to the pre-ovulatory and ovulatory phases. In the pre-ovulatory and ovulatory phases, the shedding cells are mainly the epithelial cells and thus this is also known as the epithelial phase. In the post-estrus phase, the estrogen level starts to decrease and this phase lasts approximately 10~14 h, at which time the corpus luteum forms and progesterone is secreted. In the diestrus phase, estrogen reaches a minimal level and this phase usually lasts 60~70 h. The post-estrus and diestrus phases are characterized by an increase in the number of leukocytes, and thus are also known as the leukocyte phase.

In the present study, altered estrus cycle was observed in rats who developed grade IV or V seizures, and in rats after being kindled for 2 weeks The disordered estrus cycles were characterized by the prolongation of the leukocyte phase, lasting 3~15 days. Our findings are slightly at variance from previous reports [18,19]. Edwards and colleagues reported two types of estrus cycle disorders in epileptic rats, a shortened estrus cycle (the epithelial and leukocyte phases were < 2 days), or an arrested estrus cycle in the epithelial phase, also known as persistent vaginal cornification (PVC) [18]. In contrast, Kathryn and co-workers did not observe PVC in the vaginal smears in rats after being kindled for 20 days; however, these rats displayed a prolongation of the epithelial phase as compared to the leukocyte phase or the increased ratio of the epithelial phase to the leukocyte phase [19]. In addition, these researchers proposed that the degree of disordered estrus cycle was associated with the severity of epilepsy and the duration of kindling.

Previous studies have reported that a sudden discharge during epilepsy and the discharges between two epileptic attacks can affect the pulsatile secretion of gonadotropin releasing hormone (GnRH) through excitatory neurotransmitters, which results in dysfunction of the reproductive endocrine axis [15,20,21]. Epilepsy can increase the pulse frequency of GnRH release and subsequently the pulsatile release of luteinizing hormone (LH), without affecting the release of follicular stimulating hormone (FSH). The maturation of oocytes requires the presence of a LH peak. In addition, maintenance of the blood LH and FSH peak for a suitable length of time is necessary critical for ovulation. The abnormal release of LH affects the formation of the LH peak, which then impairs follicular maturation and ovulation.

Ovarian morphological alterations

In the present study the weight and volume of the ovaries in the kindling group were significantly increased when compared to the other two groups. Follicles in different phases were found. However, the pre-antral and atretic follicles in the kindling

group were dramatically increased, with polycyst formation in the ovaries, which were similar to the pathologic features of PCOS and the main cause of ovarian enlargement observed in the kindled rats. In human, 23% of healthy women at child bearing age has PCOS [22]. However, the incidence increased to 26~41% in women with epilepsy [23]. It is speculated that the high rate of PCOS in epileptic women may be caused by increased pulse frequency of GnRH and the increase in LH/FSH [24].

In the present study, the number of antral follicles in the kindling group was not increased comparing to other two groups. This may reflect the follicles becoming atretic before developed into antral follicles or the follicles arrested in the antral phase. In the present study, we observed increases in number of apoptotic oocytes and in granulosa cells, which may be responsible for atresia of growing follicles.

Follicular theca is the main site for synthesis of androgens in the ovary. Our results showed that the thickness of the theca increased in the kindled rats. Electron microscopic study revealed that cells in the theca of follicles were actively proliferating and densely distributed. The layers of the theca increased and thecal cells were rich in the smooth endoplasmic reticulum and tubular mitochondria, suggesting that the synthesis of steroid hormones may be active in the ovaries of epileptic rats. It is possible that amygdala kindling stimulates hypothalamus-pituitary-ovary axis, which subsequently promotes the development of multiple follicles in the primitive follicular phase. As a result, number of growing follicles was increased in the ovaries of the kindled rats, which may attribute to the active synthesis of androgens. Overproduction of androgens in the sinus follicular phase and subsequent phases may suppress the development of follicles, resulting in follicular atresia [25,26].

Conclusion

Our results showed that left amygdala kindling led to a disordered estrus cycle, abnormal ovulation cycle, and polycystic changes in the ovaries as in PCOS. In addition, number of thecal cells in the follicles significantly increased, suggesting enhanced proliferation in the kindled rats. The kindled rats developed similar clinical and pathological features of PCOS. Our results suggest that amygdala kindling is a risk factor for PCOS. Further study to investigate the changes of hormones in amygdala kindling model and epileptic model is warranted.

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Authors Contributions

The work presented here was carried out in collaboration among all authors. Conceived and designed the experiments: LZ, FW, PAL, TS. Performed the experiments: JP, DL, FW. Wrote the manuscript: JP, FW, PAL

Competing Interests

The authors have declared that no competing interested exists

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