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Subtotal hysterectomy causes fewer long-term detrimental effects on ovary tissues than total hysterectomy

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Summary. Background. Hysterectomy is the basic surgical procedure of gynecological surgery. Traditionally, it is divided into total hysterectomy (TH) and subtotal hysterectomy (STH) according to the scope of surgery. The ovary is a dynamic organ appended with the uterus, and the uterus provides vascular supply to the developing ovary. However, the long-term impacts of TH and STH on ovary tissues need to be evaluated.

Method. In this study, rabbit models of different ranges of hysterectomy were successfully created. The estrous cycle of animals was determined by vaginal exfoliated cell smear 4 months after the operation. The apoptosis rate of ovarian cells in each group was determined by flow cytometry, and the morphology of ovarian tissue and granulosa cells in the control group, triangular hysterectomy group and total hysterectomy group were observed under microscope and electron microscope, respectively.

Results. After total hysterectomy, the apoptotic events in ovarian tissues were significantly increased when compared to the sham and triangle hysterectomy group. Increased apoptosis was accompanied with the morphological changes and disrupted organelle structures in ovarian granulosa cells. The follicles in the ovarian tissue were dysfunctional and immature, with more atretic follicles being observed. In contrast, ovary tissues in triangular hysterectomy groups showed no obvious defects on the morphology of ovarian tissue and granulosa cells.

Conclusions. Our data suggest that subtotal hysterectomy may serve as an alternative to total hysterectomy, with fewer long-term detrimental effects on ovary tissues.

Key words: Total hysterectomy, Subtotal hysterectomy, Triangle hysterectomy, Uterus, Ovary tissues, Granulosa cells

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Introduction

As a common gynecological surgery, hysterectomy can be divided into total hysterectomy (TH) and subtotal hysterectomy (STH) according to the scope of operation (Andersen et al., 2015). Common causes of hysterectomy include uterine organic disease, uterine functional disease, uterine prolapse, pelvic floor injury, obstetric emergency bleeding and cervical cancer (Ramdhan et al., 2017). Total hysterectomy, also known as transabdominal extrafascial total hysterectomy (TAETH) has been reported to cause adverse effects, especially the loss of residual ovarian function (Moorman et al., 2011; Singha et al., 2016) and the occurrence of residual ovary syndrome (ROS) after total hysterectomy (Pastore et al., 2007).

The uterus has been recognized as a major nurturing organ for fertilized eggs. However, it can also secrete certain active substances to regulate ovary tissues (Nair et al., 2017; Kelleher et al., 2019). The crosstalk between uterus endometrium and ovary is crucial for a balanced development and maturation of follicles (Park et al., 2020; Gibson et al., 2020). In addition, the ovary is an organ with dual blood supply from the ovarian artery and the ovarian branch of the uterine artery. An early study reported that after total hysterectomy, the blood supply to the ovary can immediately decrease by 52-89% (Janson and Jansson, 1977). Petri et al. (2005) also reported that the ovarian blood flow in patients with total hysterectomy showed a significant decrease of ovarian blood supply during reproductive period. Therefore, a functional uterus seems to support the optimal status of ovary tissues.

During the development of ovary follicles, granulosa cells play a vital role in deciding the fate of follicles and serving molecules and hormones essential for follicular growth (Matsuda et al., 2012; Asaduzzaman et al., 2020). The proliferation and differentiation of granulosa cells are the basic conditions for the continuous development of follicles to maturity, and their apoptosis is an important and direct inducing factor leading to follicular atresia (Zhang et al., 2019; Hoque et al., 2021).

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However, the effects of hysterectomy on ovarian granulosa cells are unclear.

In this study, we aim to establish a subtotal hysterectomy, and evaluate the effects of subtotal and total hysterectomy on ovary tissues respectively. We applied "triangular hysterectomy" as subtotal surgery to preserve the integrity of the vascular network between the uterus, fallopian tube and ovary, as well as the integrity of the main sacral ligament of the uterus. 4 months after operation, the apoptosis rate of ovarian cells in each group was determined by flow cytometry, and the morphology of ovarian tissue and granulosa cells in the control group, triangular hysterectomy group and total hysterectomy group were observed under microscope and electron microscope, respectively. Our data suggest that subtotal hysterectomy may serve as an alternative to total hysterectomy, with fewer long-term detrimental effects on ovary tissues.

Materials and methods

Animals

A total number of 36 adult female New Zealand rabbits (7-8 months old, weighing 4-5kg, unmated) were provided by the medical experimental animal center of Bethune International Peace Hospital, and raised in the medical experimental animal center of Bethune International Peace Hospital at room temperature of 20-28°C. The rabbits were allowed free access to food and drinking water.

Ovary operation procedures

All the experimental procedures were approved by the experimental animal use and care committees of Hebei Provincial People's Hospital. 36 adult female New Zealand rabbits were randomly divided into three groups (n=12 in each group): total hysterectomy group (group A), triangle hysterectomy group (group B) and control group with sham procedure (Group C).

Preparation: All experimental rabbits were fasted for 24 hours and deprived of drinking water for 6 hours before operation. All surgical instruments were disinfected and the depilation of skin in the operation area was performed.

Anesthesia: after weighing, 2% thiopental sodium solution was injected into the ear vein at the rate of 15 mg/kg. If the animal was restless during the operation, 0.5% lidocaine solution was used for local infiltration anesthesia.

Operation procedures: The skin of the operation area was disinfected with iodine and alcohol, and a longitudinal incision with a length of about 8 cm was planned to be made in the middle of the lower abdominal white line. The incision was anesthetized by local infiltration with lidocaine solution. The abdomen layer was cut to fully expose the abdominal cavity, and the uterus and ovary. The uterus of the rabbit is a hard tube, consisting of a neck, a body and two long horns, with the uterine arteries distributed on both sides of the uterus. The ovaries are attached to the transverse abdominal fascia behind the kidney on the inner side of the last rib through the ovarian suspensory ligament. The ovarian veins and arteries are distributed in the mesentery. After probing the uterus and bilateral ovaries, the bilateral uterine horns were pulled and the uterine body was exposed, and the broad ligament of the uterus was flattened for operation (Fig. 1A,B).

Group A (total hysterectomy): the uterine was disconnected from ovarian ligaments. From the uterine horn to the cervix, holes were drilled in the avascular area of the broad ligament. The broad ligament between the uterine horn and the uterine body on both sides were ligated step by step, together with the uterine arteries and veins. Then, the uterine body was separated from the broad ligament of the uterus. The uterine body was pulled to expose the cervix, double clamps were fixed at the junction of cervix and vagina, and the entire uterus was surgically removed (Fig. 1C).

Group B (triangle hysterectomy): The myometrium and endometrium were clamped and fixed successively from the uterine horn to the uterine body, without disconnecting the ovarian and uterine ligaments. The operation did not damage the broad ligament, and the uterine artery, vein, uterine seromyometrium and cervix were preserved. Part of the uterine body was surgically removed (Fig. 1D).

Group C (sham group): the uterus and ovaries were exposed for half an hour without any operational procedures

After the operation, the abdomen was closed layer by layer after checking that there was no active bleeding. The rabbits after operation were closely monitored, and administrated with penicillin 75 units per day by intramuscular injection for three days to prevent infection. During the postoperative feeding period, all the experimental rabbits were in good condition, with no infection or death observed.

Estrous cycle observation

All experimental rabbits were fed for four months after operation before histological examination. In order to confirm the estrous cycle, the exfoliated cells were taken from the rabbit vagina at 4:00 p.m. every day. A cotton swab was inderted into the rabbit vagina after the experimental rabbit was supine and fixed, with some rotation to grip some tissues. The material collected on the swab was evenly applied on the slide, and fixed with 95% ethanol solution. The exfoliated cells were stained with papanicolaou (PAP) dye and observed under the light microscope. The procedures were performed for two estrous cycles continuously. After the validation of each experimental rabbit's estrous cycle, the histological analysis was performed in the early estrous period.

Ovary tissue collection

Anesthesia, fixation, disinfection and laparotomy

were performed as described above. After opening the abdominal cavity, the ovarian tissues were separated from the surrounding tissue. After the left ovary was isolated, the tissues near the ovarian suspensory ligament were cut immediately and cut into 1×1×4 mm^3 tissue blocks, and placed in 4% glutaraldehyde solution for fixation and to be analyzed by electron microscope. The remaining left ovarian tissue was fixed with 70% ethanol solution for flow cytometry analysis. The right ovary was fixed with formaldehyde solution and to be analyzed by HE staining.

Electron microscope

The pre-fixed tissues in each group were cut into 1mm3 size blocks and washed three times with phosphate buffered saline (PBS). Six blocks in each group were randomly selected for slide preparation. The tissues were then fixed with 1% osmic acid for 2h at 4°C. After washing, the tissues were dehydrated and infiltrated with the mixture of acetone and resin (3:1) for 15 minutes, and then infiltrated with pure resin for 30 minutes. Then, the samples were embedded in epoxy resin 812 and 815 embedding solution, followed by incubation in ovens at 37°C, 45°C, and 60°C for 24 hours respectively. The embedded block was cut into the semi-thin section with a thickness of 2~3 μm and stained with toluidine blue to localize the granulosa cells of growing follicles. After that, the part containing growing follicles were cut into unltra-thin sections with a thickness of 50 nm using Leica microtome. Double electro-staining was performed with uranyl acetate for 15min and lead citrate for 30 min. Hitachi H-7500 transmission electron microscope was used to observe the ultrastructure in the ovary sections.

Hematoxylin and eosin (H&E) staining

The right ovarian tissue was fixed in 4% paraformaldehyde. After 24 hours, the fixed tissue block was dehydrated with regular gradient alcohol, soaked in xylene and embedded in paraffin. Serial paraffin sections were made with Leica rm2125 microtome with a thickness of 6 μm. A total of 6 tissue samples were randomly selected from each group. The prepared sections were deparaffinized and dehydrated and then rinsed with distilled water. Samples were stained with Hematoxylin solution for 1 minute and rinsed with tap

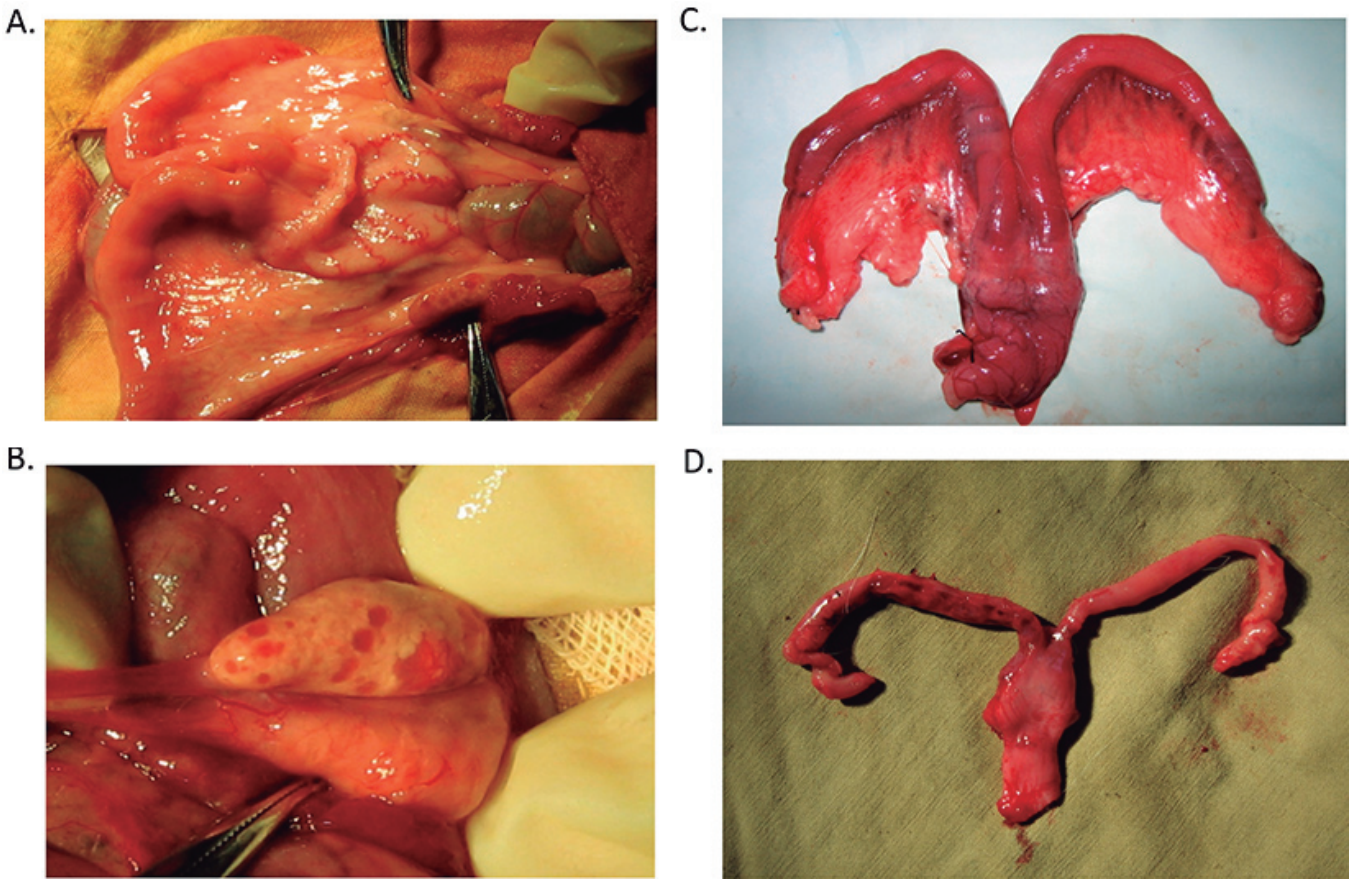

Fig. 1. The establishment of rabbit model of hysterectomy. **A.** Representative image of rabbits' uterus and ovaries. **B.** The exposed ovaries. **C.** The uterine specimen of total hysterectomy. **D.** The uterine specimen of triangle hysterectomy.

water. The sections were further processed by 1% hydrochloric acid for 1 minute, and then stained with Eosin solution for 2 minutes. The sections were dehydrated by conventional alcohol gradient and sealed with neutral gum, and observed under a light microscope.

Flow cytometry analysis

The specimens were fixed with 70% ethanol solution, and then cut into small pieces using ophthalmic scissors, and gently forced with ophthalmic forceps through a 40 μm cell strainer placed on a 50 ml Falcon tube. The forced tissues were washed with normal saline, and the cell suspensions collected were centrifuged at 1000 rpm for 4 minutes, and the supernatant were discarded. The cell pellet was resuspended in PBS and counted using a heamocytometer. 1 million cells were stained with 50 μg/ml propidium iodide (PI) solution and then analyzed on the Epics-x1 flow cytometer (Beckman Coulter, CA, USA) and the DNA content analysis was performed with musicycle AV analysis software.

Statistical analysis

The results of measurement data are expressed in (mean ± standard deviation), and all data are subject to the analysis using SPSS13 Version 0 statistical software. Unpaired Students' t-test or one-way ANOVA was used for statistical analysis. $P<0.05$ was considered to be statistically significant.

Results

The apoptosis rate detection in ovarian tissues

As determined by flow cytometry analysis, the apoptosis rate of ovarian cells in the total hysterectomy group (22.48±5.13%) was significantly higher than that in the sham group (6.91±3.48%), and the difference was statistically significant (Fig. 2A,B, $P<0.001$). The apoptosis rate of ovarian cells in the total hysterectomy

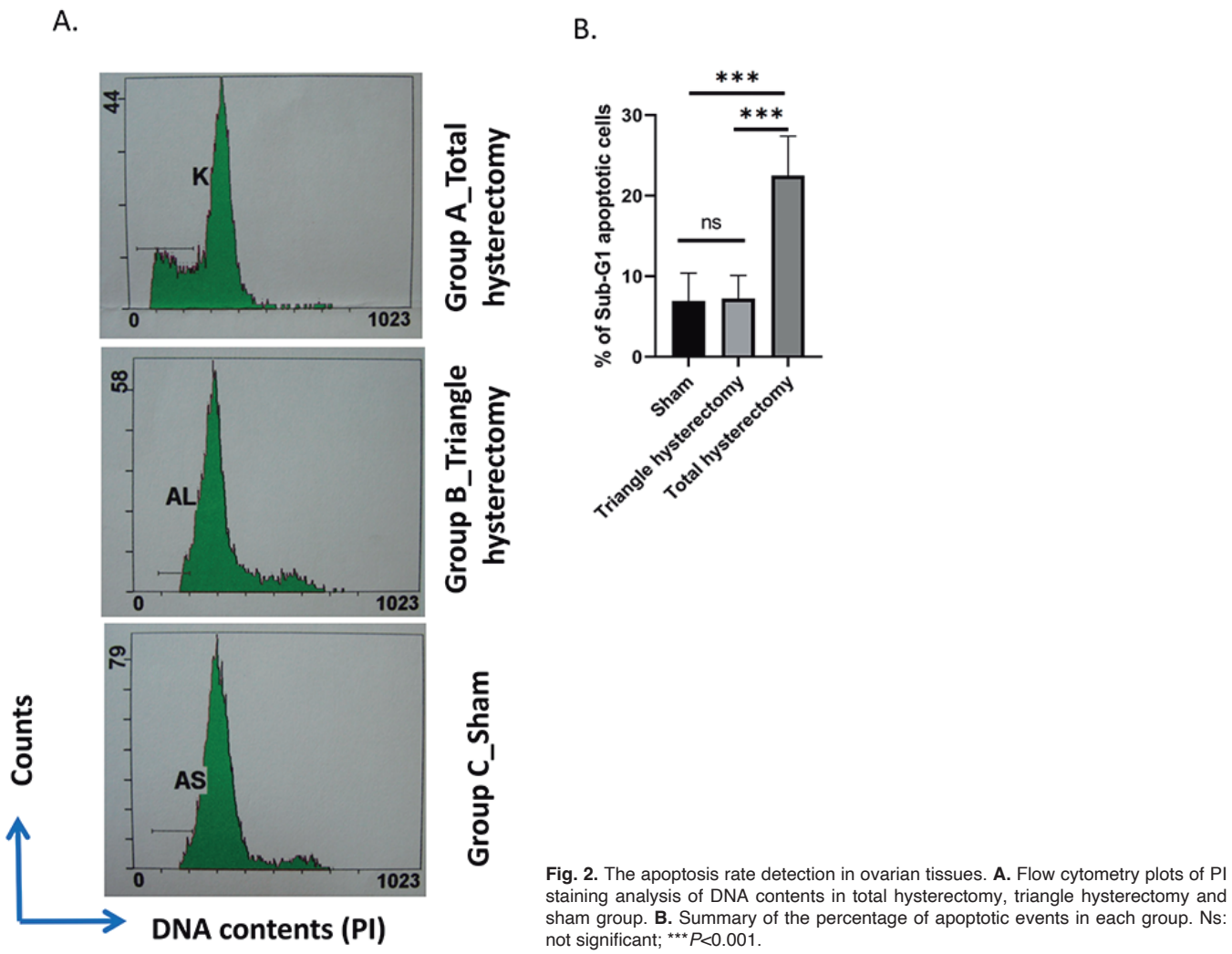

Fig. 2. The apoptosis rate detection in ovarian tissues. **A.** Flow cytometry plots of PI staining analysis of DNA contents in total hysterectomy, triangle hysterectomy and sham group. **B.** Summary of the percentage of apoptotic events in each group. Ns: not significant; ***$P<0.001$.

group was also significantly higher than that of the triangular hysterectomy group (7.20±3.18%), and the difference was statistically significant (Fig. 2A,B, $P<0.001$). There was no significant difference between the triangular hysterectomy group and the sham control group ($P>0.05$).

Ultrastructure of granulosa cells under electron microscope

In sham group (Group C), granulosa cells were irregularly oval-shaped and arranged adjacent to each other in the periphery of the oocyte, with tight junctions connecting the cells (Fig. 3A1). There were sinus-like spaces between cells and there were some collagen fiber filaments in the space (Fig. 3A2). In the cytoplasm, there were abundant organelles including rough endoplasmic reticulum and smooth endoplasmic reticulum, tubular mitochondria with clear outlines of the outer membrane and inner cristae, and abundant lipid droplets (Fig. 3A2,A3).

In Group B (triangle hysterectomy), the arrangement and shape of granulosa cells were normal and intercellular junctions were observed (Fig. 3B1), with abundant cytoplasmic organelles. The structure of each organelle wwas well-preserved, with abundant lipid droplets in the cytoplasm (Fig. 3B2,B3). Basically, the morphologies of the subcellular structures were similar to those in sham group C.

In the total hysterectomy group, the distance between the granulosa cells was larger, with fewer cell junctions observed (Fig. 3C1). The number of the smooth endoplasmic reticulum was significantly reduced, with fewer mitochondria and disrupted cristae

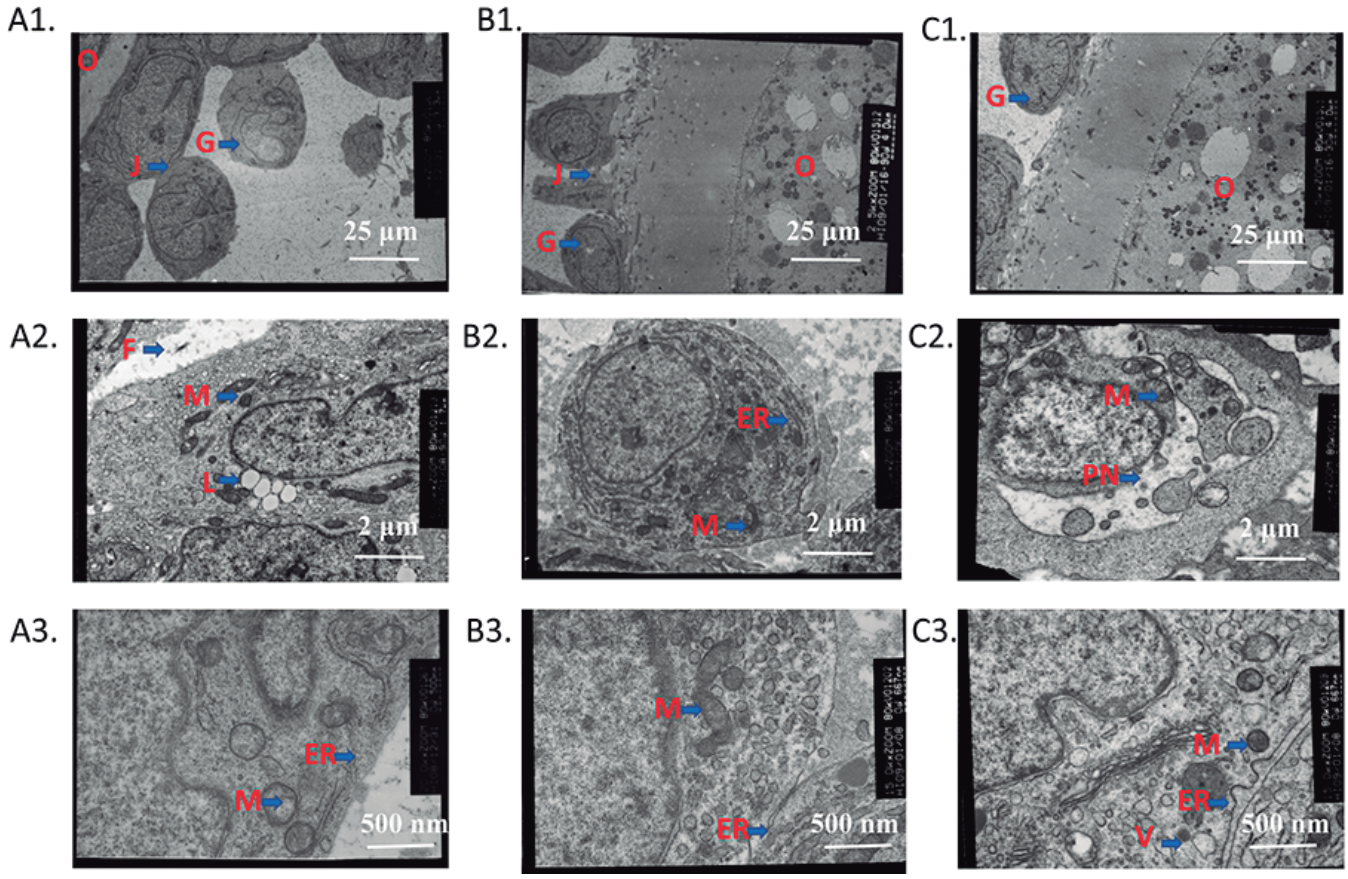

Fig. 3. Ultrastructure of granulosa cells under electron microscope. **A1-A3.** The ultrastructures of granular cells inthe ovary tissues of sham group (Group C). Oval-shaped granulosa cells are arranged adjacent to each other in the periphery of the oocyte, with tight junctions connecting the cells. There are sinus-like spaces between cells and there are some collagen fiber filaments in the space. Abundant organelles are observed in the cytoplasm including rough endoplasmic reticulum and smooth endoplasmic reticulum, tubular mitochondria with clear outlines of the outer membrane and inner cristae, and abundant lipid droplets. **B1-B3.** The ultrastructures of granular cells in the ovary tissues Group B (triangle hysterectomy). The arrangement and shape of granulosa cells are normal and intercellular junctions were observed, with abundant cytoplasmic organelles. The structure of each organelle is well-preserved, with abundant lipid droplets in the cytoplasm. **C1-C3.** The ultrastructures of granular cells in the ovary tissues of group A (total hysterectomy). There is larger distance between the granulosa cells, and the organelles in the cytoplasm are reduced. Some mitochondria show the fusion of mitochondrial cristae and membranes. Local cytoplasmic edema and the expansion of perinuclear space are also observed. The endoplasmic reticulum shows dilated morphology and numerous vacuoles can be seen in the cytoplasm. Abbreviations: G, granulosa cell; J, cell junction; F, extracellular fiber; M, mitochondrion; ER, endoplasmic reticulum; L, lipid droplet; O, oocyte; PN, perinuclear space; V, vacuole.

(Fig. 3C2,C3). Some mitochondria showed swollen morphology, with the fusion of mitochondrial cristae and membranes (Fig. 3C3). Local cytoplasmic edema, defect of nuclear membrane and expansion of perinuclear space were also observed (Fig. 3C2). The endoplasmic reticulum showed dilated morphology and numerous vacuoles could be seen in the cytoplasm (Fig. 3C3).

Ovarian tissue and follicle structure by H&E staining

H&E staining revealed that in the ovarian tissues of sham group (Group C), there were a large number of

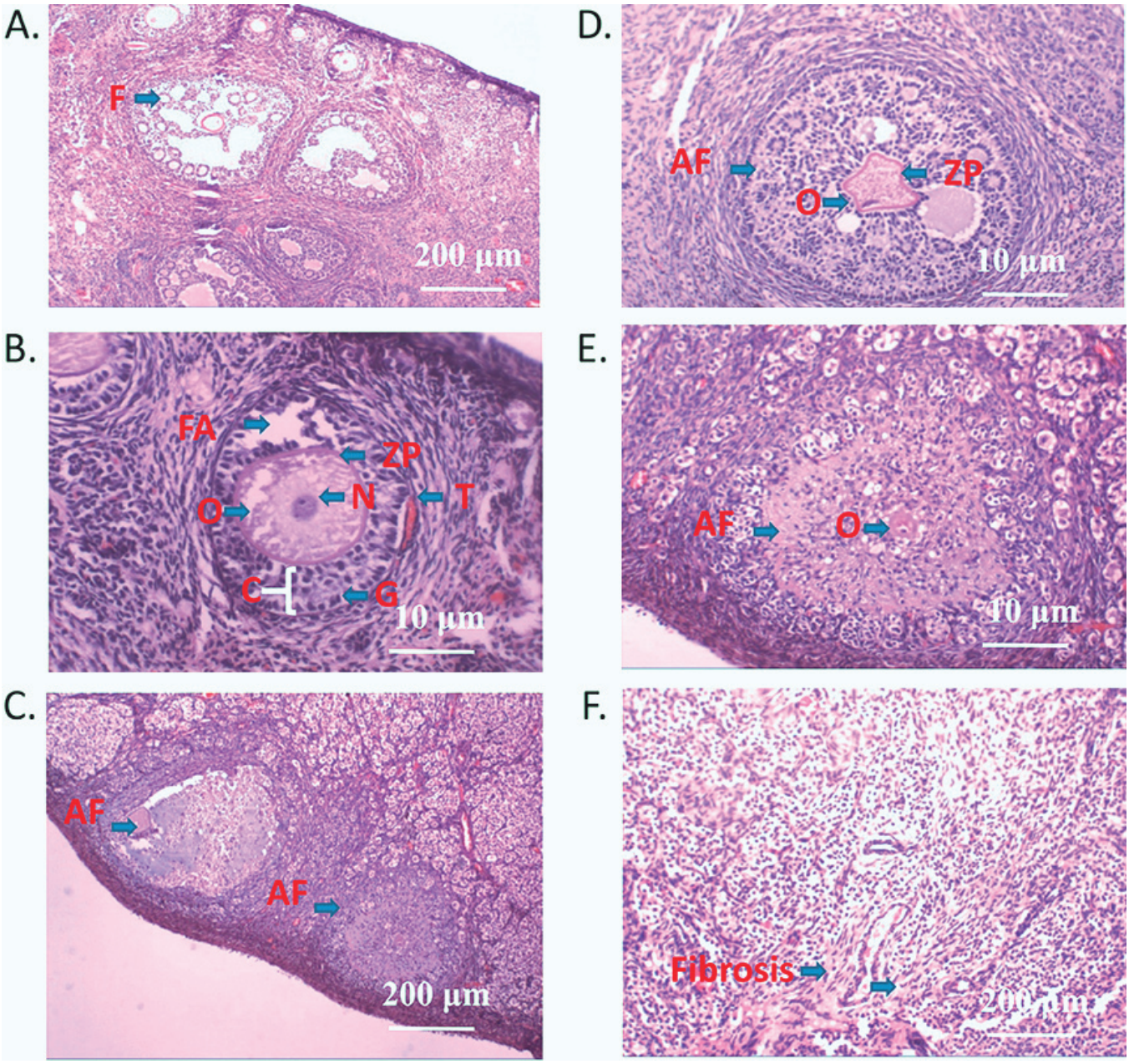

Fig. 4. Ovarian tissue and follicle structure by H&E staining. **A, B.** Ovarian tissue and follicle structure by H&E staining in group C (sham group). In The ovary tissue of the sham group, abundant developing and mature follicles can be observed, with large oocyte and well-defined nucleus (Fig. 4B). The follicles are well-structured with clear follicular antrum, follicular fluid and cumulus. The granulosa cells and theca cells are well-organized around the oocyte and the zona pellucid is well-formed. **C-F.** Ovarian tissue and follicle structure by H&E staining in group A (total hysterectomy). In the total hysterectomy group, there is a reduced number of follicles in the ovarian cortex and most are atretic follicles with irregular shape. The oocytes show pyknosis and the collapse of the zona pellucid, with atrophied granulosa cells and theca cells sparsely arranged in the follicle. There is also interstitial fibrosis. Abbreviations: AF, atretic follicle; C, cumulus; F, follicle; FA, follicular antrum; G, granulosa cell; N, nucleus; O, oocyte; T, theca cell; ZP, zona pellucida.

primordial follicles in the superficial part of ovarian cortex. Abundant developing and mature follicles could be observed (Fig. 4A). Most of the growing follicles had large oocyte and fine-grained nuclear chromatin (Fig. 4B). The follicles were regular in shape, with clear follicular antrum, follicular fluid and cumulus. The granulosa cells and theca cells were well-organized and the zona pellucid was well-formed (Fig. 4B). In the ovarian tissues of Group B (triangular hysterectomy group), the follicle structures were basically the same as that of group C.

However, in Group A (total hysterectomy group), there were reduced number of follicles in the ovarian cortex, including the developing follicles at all levels, especially mature follicles. However, there were more atretic follicles (Fig. 4C). Most of the follicles were irregular in shape, with nuclear disruption and the collapse of the zona pellucid (Fig. 4D). Atrophied granulosa cells and theca cells were sparsely distributed in the follicle without proper organization (Fig. 4E). There were clear interstitial fibrotic changes in ovarian tissue (Fig. 4F).

Discussion

Hysterectomy is the basic operation of gynecological surgery and a widely used operation in gynecology. Traditionally, hysterectomy is divided into total hysterectomy (TH) and subtotal hysterectomy (STH) according to the scope of operation. Total hysterectomy, also known as transabdominal extrafascial total hysterectomy (TAETH), has been a commonly used surgical procedure for many years. However, the adverse effects of this procedure have gradually emerged, especially the loss of residual ovarian function and the occurrence of residual ovary syndrome (ROS) after total hysterectomy (Ramdhan et al., 2017). A large number of studies have found that total hysterectomy in pre-menopausal women can gradually cause ovarian function loss, regardless of whether the ovary is removed or not. After total hysterectomy, follicle stimulating hormone (FSH) and luteotropic hormone (LH) are significantly higher than those of women of the same age who did not undergo total hysterectomy, while estradio (E2) level is reduced (Biro and Eneroth, 1990; Wilson et al., 2019; Krzymowski and Stefanczyk-Krzymowska, 2002; Buckingham et al., 2019). Predominant follicles in patients after total hysterectomy are significantly reduced compared with women of the same age who did not undergo total hysterectomy (Petri et al., 2005). The incidence of perimenopausal symptoms and osteoporosis are significantly higher in patients with hysterectomy than in patients without hysterectomy (Kritz-Silverstein et al., 2000; Rieger and Dietl, 2004).

Subtotal hysterectomy strategies have been formulated, and triangular hysterectomy is one of the representative procedures (Asnafi et al., 2010; Persson et al., 2010). Although previous reports showed that subtotal abdominal hysterectomy results in more rapid recovery and fewer short-term complications (Persson et al., 2010), there is little understanding about how the ovary tissues are affected by total or subtotal hysterectomy. The triangular hysterectomy has the following foreseen advantages: 1. the integrity of the vascular network between the uterus, fallopian tube and ovary is preserved, thus maintaining the original blood supply of the ovary; 2. the integrity of the main sacral ligament of the uterus is preserved. After hysterectomy, the cervix and the seromuscular layers of the uterus on both sides are sutured to make it a "small uterus".

The ultrastructure of follicles is one of the important means to identify the developmental status of follicles, assess their vitality and health status. The follicle consists of a core egg cell surrounded by granulosa cells and theca cells (Baerwald et al., 2012). The granulosa cells of the mature follicle are the largest cell group that constitutes the follicle, play an important role in the oocyte, and are the main source of estrogen and progesterone (Hardy et al., 2018). In this study, ovarian granulosa cells were used as target cells for electron microscopy. The normal ovarian granulosa cells contain a large nucleus with secretory granules in the cytoplasm. Under high magnification, the organelles in the cytoplasm are well-defined. However, in the ovary tissues of total hysterectomy group, granulosa cells showed the following changes: the number of various organelles in the cytoplasm was reduced, the mitochondrial cristae were plate-like and blurred, the endoplasmic reticulum was slightly expanded, and the cytoplasm was slightly enlarged. Multiple vacuoles and local cytoplasmic edema were observed. The nuclear membrane was partially damaged, the perinuclear space was slightly expanded, and local granular cell membrane defects were also seen. In contrast, the ultrastructures of granulosa cells in triangle hysterectomy group showed similar morphology as the normal granulosa cells. These data suggest that total hysterectomy causes morphological changes and severe organelle damage in ovarian granulosa cells. We speculate that the damaged vascular system connecting to ovary tissues and the disrupted hormone levels may both contribute to the defects in granulosa cells.

Ovarian granulosa cells are most closely related to the development and maturation of follicles in the ovarian cycle. The proliferation and differentiation of granulosa cells are the basic conditions for the continuous development of follicles to maturity, and their apoptosis is an important and direct inducing factor leading to follicular atresia (Palma et al., 2012; Zhou et al., 2019). Although apoptosis within the granulosa cells is an integral part of normal development (Irving-Rodgers et al., 2003) granulosa cells undergoing apoptosis are still functioning and can reorganize their cytoplasmic contents for progesterone production (Motta, 1969). Functioning apoptotic granulosa cells were reported to reorganize the cell cytoplasm, create blebs of non-cytoplasmic organelles including

mitochondria, Golgi apparatus and endoplasmic reticulum, and form large fluid filled vacuoles containing steroids, lipids, and proteins (Guraya, 1971; Nottola et al., 2006). Interestingly, we also observed that the granulosa cells in the ovary tissues with total hysterectomy showed disorganized organelles as well as large vacuoles and local cytoplasmic edema. It remains to be determined whether those cells are functional granulosa cells which are able to secrete steroid hormones.

During the periodic follicular development, the atretic follicle at each stage is closely related to the apoptosis of ovarian granulosa cells (Almeida et al., 2018). Excessive apoptosis can lead to degeneration, atrophy and dysfunction of tissue cells (Matsuda et al., 2012; An et al., 2021). Previous studies showed that the DNA of granulosa cells in atretic follicles was fragmented, but in developing follicles the DNA of granulosa cells showed no obvious fragmentation, indicating that most of the granulosa cells in atretic follicles are undergoing apoptosis (Jin et al., 2011; Bhardwaj and Saraf., 2020). In our study, we found that total hysterectomy induced massive apoptosis in ovary tissues, evidenced by the significant increase of sub-G1 cell population in flow cytometry analysis. On the contrary, in the group with subtotal triangle hysterectomy or sham procedures, there is minimal level of apoptosis observed. Consistently, in the ovary of total hysterectomy, primordial follicles can be seen in the ovarian cortex under light microscope, while the mature follicles are very rare, which suggests a defects in follicle maturation. Therefore, apoptosis may be one of the molecular mechanisms that causes the defects in granulosa cells and the failure of maturation of ovary follicles.

We speculate that at least two major factors could contribute to the defects in granulosa cells and ovary follicle development after total hysterectomy. First, blood supply to the ovaries is impaired since the vascular system surrounding the ovary is disrupted after total hysterectomy. The ovary is an organ with dual blood supply from the ovarian artery and the ovarian branch of the uterine artery (Brown and Russell, 2014). The blood supply to the ovary from the ovarian branch of the uterine artery accounts for more than 50% of the total blood supply to the ovary (Mori et al., 2012; Lee et al., 2012). Second, the uterus also serves as an organ for producing certain hormones or active substances to regulate ovary (Kelleher, et al., 2019). However, the above defects need to be further validated in animal models or human clinical samples.

Conclusions

This study successfully established animal models with different ranges of hysterectomy, and examined the apoptosis rate and the histological structures of granulosa cells and ovary follicles. We found that after total hysterectomy, ovarian cell apoptotic events were increased, which was accompanied with the morphological changes and disrupted organelle structures in ovarian granulosa cells. The follicles in the ovarian tissue were dysfunctional and immature, with more atretic follicles. In contrast, ovary tissues in triangular hysterectomy groups showed no obvious defects on the morphology of ovarian tissue and granulosa cells. These observations suggest that subtotal hysterectomy may cause fewer long-term detrimental effects on ovary tissues.

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