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Original Article

GnRH agonist enhances autophagy in a mouse model of adenomyosis

Song Guo[1*], Xiaowei Lu[1*], Ruihuan Gu[2], Di Zhang[3], Yijuan Sun[2], Yun Feng[1]

[1]Reproductive Medicine Center, Department of Obstetrics and Gynecology, Ruijin Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China; [2]Shanghai Ji Ai Genetics & In vitro fertilization Institute, Obstetrics and Gynecology Hospital, Fudan University, Shanghai, China; [3]Department of Gynecology and Obstetrics, Jinan Military General Hospital, Jinan, China. [*]Equal contributors.

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Abstract: Gonadotropin-releasing hormone (GnRH) agonists are effective treatments for adenomyosis. Previous studies have suggested that autophagy in the eutopic endometrium is impaired in patients with adenomyosis. However, whether GnRH agonists act on autophagic mechanisms to exert their therapeutic effect remains unclear. Here we investigated the effect of a GnRH agonist on autophagy in a mouse model of adenomyosis. Mice in the model group showed significantly fewer autophagosomes and less Beclin 1, LC-3 expression in the eutopic endometrium than control mice. However, in model mice that received a GnRH agonist, significantly more autophagosomes and Beclin 1, LC-3 protein expression were observed than in model mice that received vehicle. These results indicate that adenomyosis causes inhibition of autophagy, but that this is reversed by GnRH agonists. The study contributes to an understanding of the roles of autophagy in the treatment of adenomyosis using GnRH agonists, and suggests that targeting aberrant autophagy might be a novel strategy for the treatment of adenomyosis.

Keywords: Adenomyosis, GnRH agonist, autophagy, autophagosome, Beclin 1, LC-3

Introduction

Adenomyosis is a common benign gynecologic disease characterized by the abnormal growth of the endometrium into the myometrium, and myometrial hypertrophy/hyperplasia [1, 2]. Adenomyosis plays an important role in female infertility [3-5], and treatment remains a challenge in reproductive medicine. Present treatment options include medical and surgical approaches [6-8]. Gonadotropin-releasing hormone (GnRH) agonists are frequently used in assisted reproduction as a less invasive approach for adenomyosis than surgery. In addition, Niu et al. found that treatment with GnRH agonists before frozen embryo transfer can improve clinical pregnancy, implantation, and ongoing pregnancy rates [9]. The mechanism of action of GnRH agonists may involve pituitary down regulation, which decreases the size of adenomyotic lesions [9], or antiproliferative and apoptotic effects [10].

Autophagy is an intracellular system in which superfluous cytoplasmic macromolecules are sequestered into double-membrane structures called autophagosomes, which undergo bulk degradation of cytoplasmic components [11, 12]. Recent studies suggested that autophagy is a key mechanism in various pathophysiological processes, including cancer [13] and neurodegeneration [14, 15]. The level of autophagy during the secretory phase of the menstrual cycle is lower in endometriotic tissue than in normal endometrium [11]. Ren et al. found that expression of Beclin 1, an autophagy-related gene, was lower in the eutopic endometrium of women with adenomyosis than in control subjects [16]. However, whether GnRH agonists exert their therapeutic effect in adenomyosis by regulating autophagy remains unclear.

The implantation window is the critical period during which embryo implantation is possible. We therefore chose this period for the present study. In patients undergoing frozen embryo transfer, those with adenomyosis are ready for embryo transfer after GnRH agonist treatment, making it difficult to obtain endometrium sam-

ples from patients during the implantation window. We therefore chose to use a mouse model of adenomyosis to investigate the effect of GnRH agonists on autophagy.

Materials and methods

Animals and treatments

We established models of adenomyosis by administering tamoxifen to neonatal mice [17-20]. Six pregnant ICR strain mice were purchased from Shanghai Laboratory Animal Corporation (Shanghai, P. R. China), and each was housed in a single cage during the perinatal period. Female offspring were selected for use in this study. Twelve of the neonatal mice received tamoxifen (1 mg/kg orally; Shanghai Fudan Forward Science & Technology Co., Ltd, China) suspended in a mixture of peanut oil/lecithin/condensed milk (2:0.2:3, by volume) once daily on postnatal days 1-4. The remaining six mice received vehicle on the same schedule. All mice were housed in an animal care facility under controlled conditions (20°C, 12:12 h light-dark cycle, lights on at 6:00 am), with free access to chow and fresh water. As the mice were neonates, they were housed with their dams (mothers) until post natal day 28 and then moved to their own cages. Four mice were housed together.

Seventy-five days after the final tamoxifen administration [17, 21], the 12 mice exposed to tamoxifen were randomly divided into two groups: six mice received a single intraperitoneal depot injection of the GnRH agonist triptorelin (8 mg) [22], and the remaining six mice received an equivalent volume of vehicle. After 4 weeks, each mouse was allowed to mate with a male from 7:00 p.m. to 7:00 a.m. The following morning, females were checked for the presence of vaginal plug (day 1 of pregnancy). Mice were euthanized by intraperitoneal injection of pentobarbital sodium (200 mg/kg; Kyoritsu Seiyaku Corp., Tokyo, Japan) between 7:00 p.m. and 8:00 p.m. on day 4 of pregnancy. The uterus was removed, weighed, and divided into three parts. One part was fixed in 4% formalin and embedded in a paraffin block for sectioning and hematoxylin-eosin (H&E) staining. Another part was fixed in 2.5% glutaraldehyde solution for electron microscopy. The endometrium of the remaining part was scraped off using a curved needle, then snap-frozen in liquid nitrogen and stored at -80°C for western-blot analysis.

The protocol followed the guidelines of the National Research Council's Guide for the Care and Use of Laboratory Animals and was approved by the institutional experimental animals review board of Ruijin Hospital affiliated to Shanghai Jiaotong University School of Medicine, China.

Histopathology

From each tissue block fixed in 4% formalin and embedded in paraffin, serial sections (4 um thick) were obtained and subjected to H&E staining to confirm pathologic diagnosis of adenomyosis and observe the morphological differences among the three groups. Three randomly selected sections were chosen for H&E staining for histopathology. If the endometrium had been displaced into the myometrium, the adenomyosis model was deemed successful. Only tissue from endometrial meeting this criterion was used for electron microscopy and western-blot analysis.

Transmission electron microscopy

The samples fixed in phosphate-buffered glutaraldehyde (2.5%) were post-fixed in osmium tetroxide (1%) and dehydrated through graded alcohols. The tissue was embedded in an epoxy resin and examined using a transmission electron microscope (CM-120, Philips). Five to eight fields from each of five to eight uterine samples were chosen for analysis.

Western blotting

The frozen endometrial tissue was incubated on ice for 15 minutes in RIPA lysis buffer (Beyotime, China) containing a protease inhibitor cocktail (Merck, Germany). After centrifugation at 12,000 g for 5 minutes at 4°C, the supernatant was collected. Protein concentrations were determined using a CA protein assay (KeyGEN, China) according to the manufacturer's instructions. Equal amounts of total protein (30 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Millipore, Billerica, MA). The membrane was blocked in 5% nonfat milk in Tris-buffered saline containing Tween 20 on a shaker for 2 h, then incubated with 1:1,000 dilution of Beclin 1 (sc-10086, Santa Cruz Biotechnology, Inc., Santa

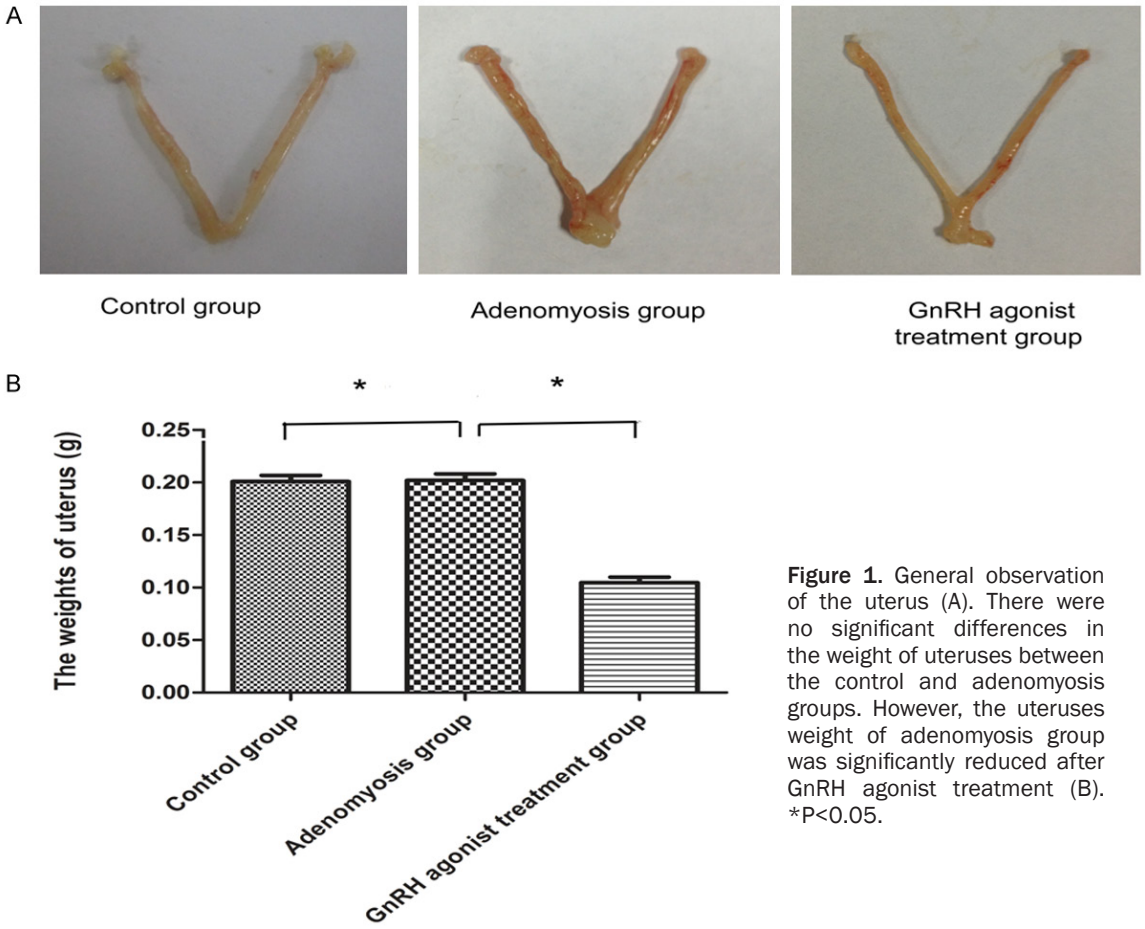

Figure 1. General observation of the uterus (A). There were no significant differences in the weight of uteruses between the control and adenomyosis groups. However, the uteruses weight of adenomyosis group was significantly reduced after GnRH agonist treatment (B). *P<0.05.

Cruz, CA, USA), 1:1,000 dilution of LC3 (ab48394, Abcam Inc., Cambridge MA, USA) and 1:400 dilution of β-actin primary antibody (sc-47778, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C overnight. After three washes with phosphate-buffered saline containing Tween 20 (PBST), the membrane was incubated with the appropriate conjugated secondary antibody at dilution of 1:2000 conjugated to peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h at room temperature and washed three times with PBST. Protein bands were revealed using the ECL kit (Thermo Fisher Scientific, MA, USA). Band intensity was quantified using Quantity One software (Bio-Rad, Hercules, California).

Statistical analysis

Data were analyzed by one-way ANOVA using SPSS17.0 for Windows (SPSS, Chicago, IL). Results are expressed as the mean ± standard deviation (SD). Statistical significance was defined as $P<0.05$.

Results

Uterus weight

No significant difference in uterus weight was observed between the control and adenomyosis groups ($P>0.05$). However, uteri in the group that received the GnRH agonist weighed significantly less than control uteri ($P<0.05$) (**Figure 1**).

Histopathology

At 90 days, all 12 mice treated with tamoxifen during the neonatal period showed adenomyosis. This was characterized by infiltration of the endometrial glands and stroma into the myometrium, and associated hypertrophy and hyperplasia of the adjacent myometrium (**Figure 2**). No control animals showed this pathology.

Tissue ultrastructure

Transmission electron microscopy revealed noticeable differences between the groups in

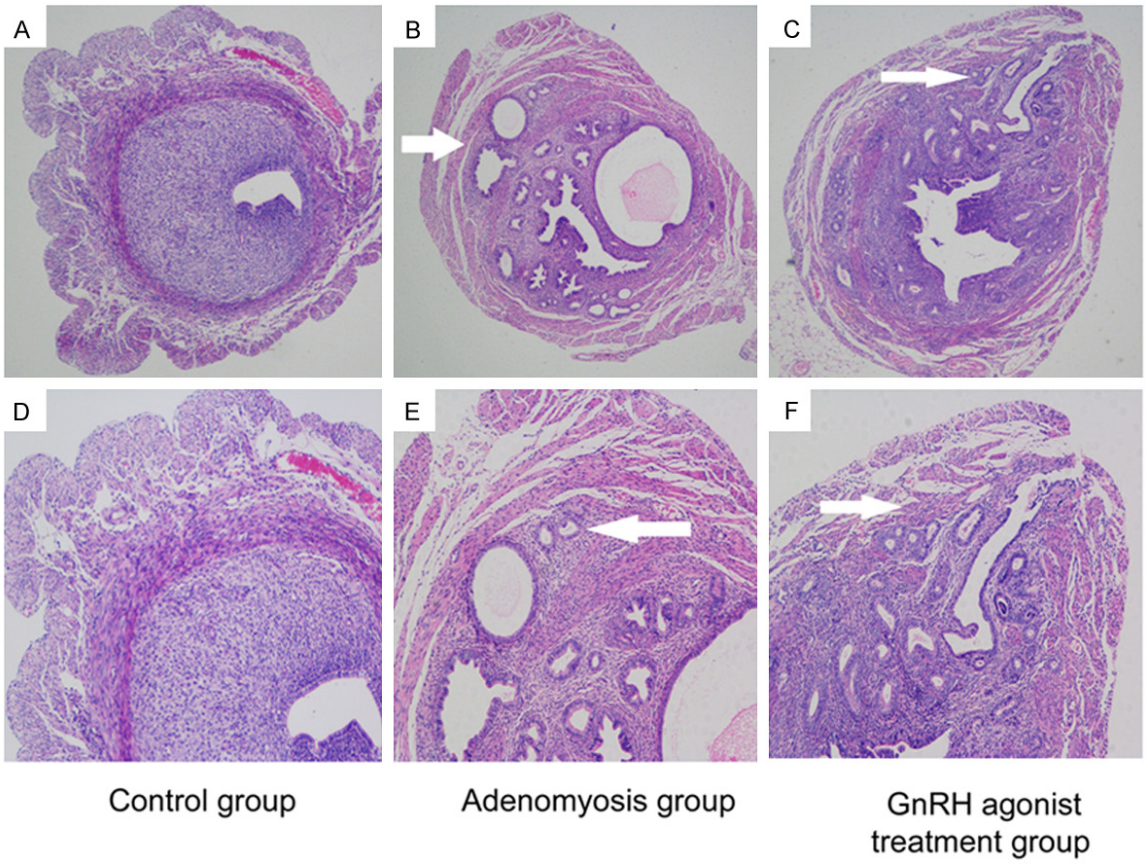

Figure 2. The pathological examinations of the uterus. All mice treated with tamoxifen in the neonatal period showed adenomyosis and none of the untreated group. Adenomyosis group was characterized by the endometrial glands and stroma infiltrated into the myometrium associated with hypertrophy and hyperplasia of adjacent myometrium (arrow). However, the control group showed a well-demarcated core of endometrium surrounded by regular, concentric layers of smooth muscle. (A-C: ×40; D-F: ×100).

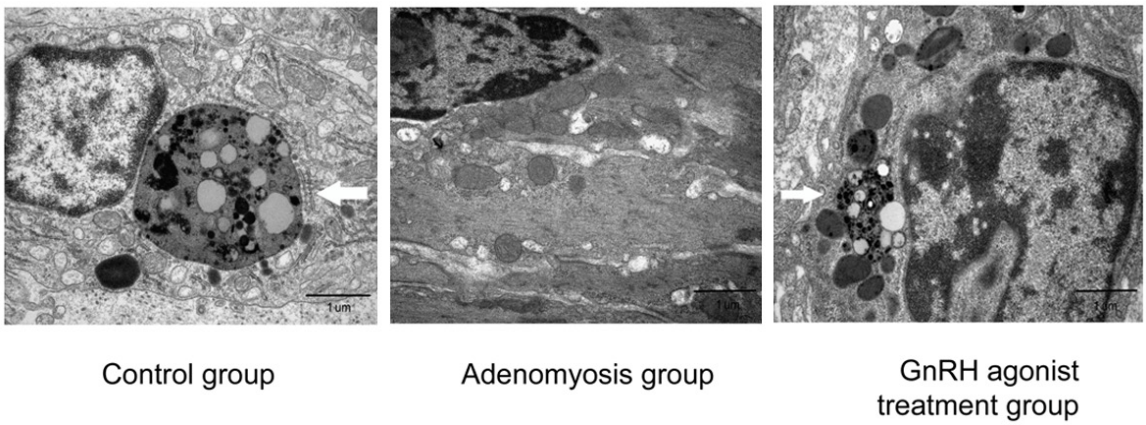

Figure 3. Transmission electron microscope images of endometrial cells among the three groups. Large amounts of autophagosomes were seen in the control group. Additionally, there was no autophagosomes found in the adenomyosis group. However, we found the number of autophagosomes was significantly increased in GnRH agonist treatment group. Arrows indicate representative autophagosomes. Scale bars: 1 um.

the morphology of the autophagic structures (**Figure 3**). The level of autophagy in eutopic endometrial stromal cells of the adenomyosis group was lower than in the control group.

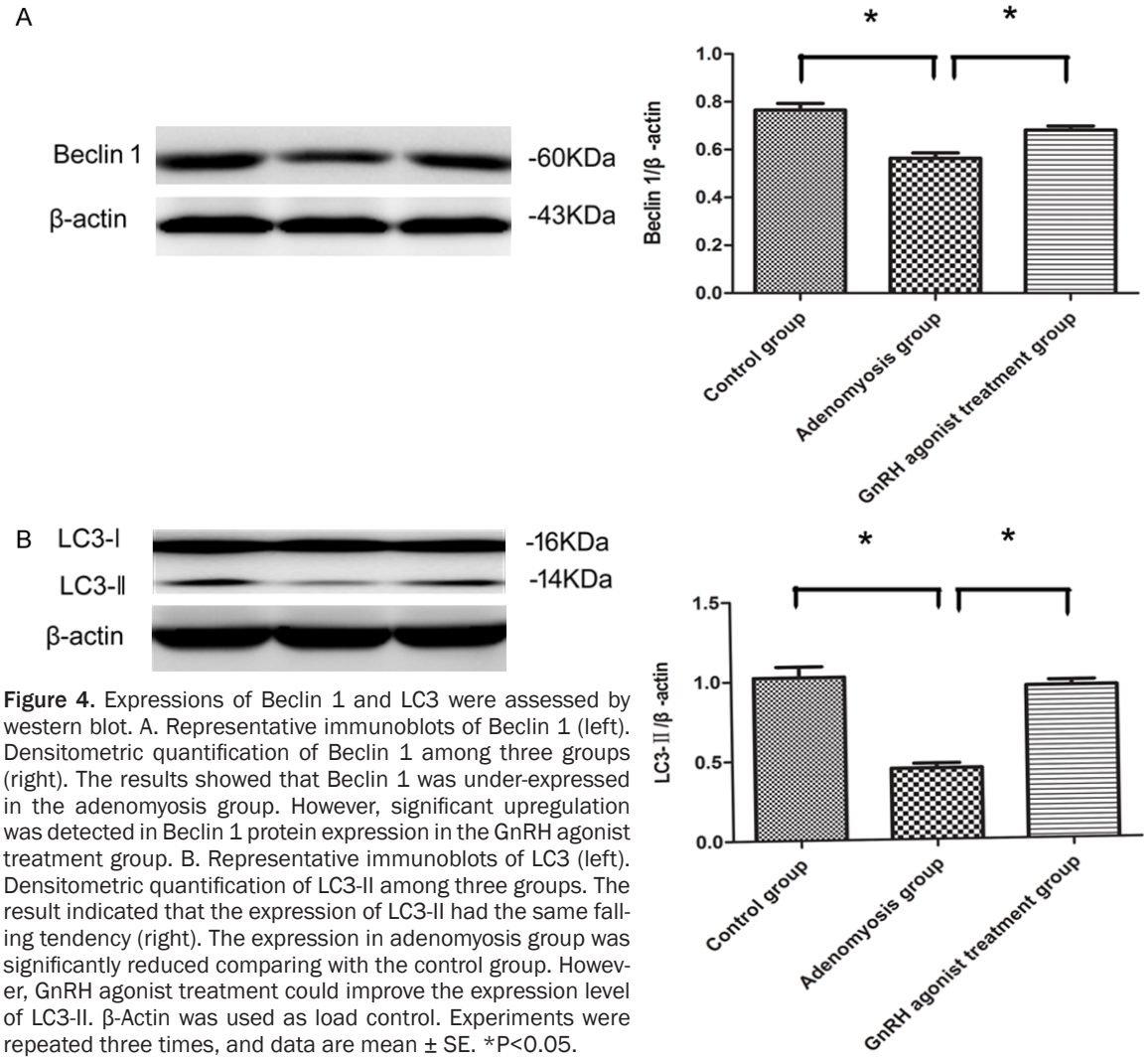

Figure 4. Expressions of Beclin 1 and LC3 were assessed by western blot. A. Representative immunoblots of Beclin 1 (left). Densitometric quantification of Beclin 1 among three groups (right). The results showed that Beclin 1 was under-expressed in the adenomyosis group. However, significant upregulation was detected in Beclin 1 protein expression in the GnRH agonist treatment group. B. Representative immunoblots of LC3 (left). Densitometric quantification of LC3-II among three groups. The result indicated that the expression of LC3-II had the same falling tendency (right). The expression in adenomyosis group was significantly reduced comparing with the control group. However, GnRH agonist treatment could improve the expression level of LC3-II. β-Actin was used as load control. Experiments were repeated three times, and data are mean ± SE. *P<0.05.

However, the level of autophagy was markedly greater in the group that received the GnRH agonist than in the adenomyosis group. The GnRH agonist group showed typical ultrastructural changes associated with autophagy, such as the presence of multiple autophagosomes within cells.

Beclin 1 and LC-3 protein expression in endometrial tissue

Through specific study of autophagy related protein during the implantation window (Day 4), we found that the level of Beclin 1 expression in the adenomyosis group was significantly lower compared with the control group (P<0.05). However, a significant increase of Beclin 1 protein expression was observed after GnRH agonist treatment (P<0.05) (**Figure 4A**). The expression of LC-3 showed the same change, the change difference was significant (P<0.05) (**Figure 4B**).

Discussion

In the present study, we used a mouse model to investigate the relationship between autophagy and GnRH agonist therapy in adenomyosis. Although transmission electron microscopy is an accepted method of identifying autophagy, it is difficult to distinguish autophagic vacuoles from other cellular vacuoles on the basis of morphology alone [23]. Therefore, we used transmission electron microscopy and quantification of autophagy-related proteins to elucidate the relationship between autophagy and the GnRH agonist. To our knowledge, the present study is the first to investigate the role of

autophagy in the treatment of adenomyosis using a GnRH agonist. We found that levels of autophagy were significantly elevated after administration of the GnRH agonist, demonstrated by a higher number of autophagosomes and greater expression of autophagy-related protein.

Autophagy, known as type II programmed cell death, was first discovered more than half a century ago using transmission electron microscopy [24]. Cellular components are sequestered in an autophagosome, which transports damaged organelles and abnormal proteins to lysosomes for degradation [25, 26]. Autophagy has gained considerable research attention for its role in certain diseases, especially cancer, neurodegeneration and endometriosis [27-32]. Many studies on endometriosis have demonstrated that there is less autophagy in endometriotic tissues than in normal endometrium [11, 31, 33]. Consistent with those findings in endometriosis, we also detected autophagy in eutopic endometrial stromal cells during the implantation window. Transmission electron microscopy showed that the adenomyosis group did not show characteristic signs of autophagy. In comparison, the number of autophagic vacuoles and expression of autophagy-related proteins were markedly greater after administration of the GnRH agonist.

Beclin 1, an important autophagy-related protein, participates in the regulation of autophagy and has an important role in differentiation and anti-apoptosis, as well as cancer progression [16]. Ren et al. [34] showed that Beclin 1 expression is decreased in the eutopic endometrium of women with adenomyosis. This decreased expression of Beclin 1 may facilitate the invasion of the endometrial stroma and glands into the myometrium and affect the development of adenomyosis. GnRH agonists might exert their therapeutic action by enhancing the expression of Beclin 1 to control the invasion of the endometrial stroma and glands into the myometrium. The protein expression of LC3 was also measured by Western blot analysis. To the best of our knowledge, this study was the first to explore the association between GnRH agonist and LC3 in adenomyosis treatment. LC3 is another key autophagy-related marker protein. Under autophagy activation, LC3-I is converted to LC3-II [35]. So the presence of LC3 in autophagosomes and the conversion of LC3 to the lower migrating form LC3-II are widely used as a marker of autophagy activation [36, 37]. We found that the level of LC3-II expression in the adenomyosis group was significantly lower compared with the control group. However, a significant increase of LC3-II expression was observed after GnRH agonist treatment. These results further confirmed that GnRH agonist might induce the autophagic cell death in adenomyosis.

Choi et al. found that the steroidal progestin dienogest suppresses Akt and ERK1/2 activity, thereby inhibiting mTOR and inducing autophagy in endometriotic cells [31]. This suggests that dienogest mediates induction of autophagy in endometriotic cells via a proapoptotic mechanism. GnRH agonists might play the same role in the treatment of endometriotic cells by inhibiting mTOR and inducing autophagy, because both agents have anti-estrogen effects. Further research is needed to better understand the relationship between GnRH agonists, mTOR and autophagy.

Together, our data showed that there was a significant increase of autophagy in the adenomyosis group by after administration of a GnRH agonist. GnRH agonists might act by promoting the degradation of harmful cytosol and organelles, thus promoting cell survival by regulating autophagic activity [25].

The main limitation of the present study was that our results were not confirmed in human samples. Results in mice cannot entirely reflect the natural course of the human disease, so it is necessary to validate the results in patients with adenomyosis. Second, because autophagy is a dynamic process and difficult to measure accurately, we did not investigate the changes in autophagy across different stages of the menstrual cycle. Further research is needed to characterize the dynamic changes in autophagy after GnRH agonist administration.

In conclusion, the present study suggests that adenomyosis lead to autophagy inhibition, and this is maybe reversed by GnRH agonists. This sheds light on the role of autophagy in the therapeutic action of GnRH agonist. In addition, our results suggest that aberrant autophagy might be a novel target for the treatment of adenomyosis.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yijuan Sun, Shanghai Ji Ai Genetics & In vitro fertilization Institute, Obstetrics and Gynecology Hospital, Fudan University, Shanghai 200011, China. Tel: +86-21-6345-5468; Fax: 021-33180478; E-mail: yijuansss@163.com; Dr. Yun Feng, Reproductive Medicine Center, Department of Obstetrics and Gynecology, Ruijin Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200025, China. Tel: +86-21-6437-4262; Fax: 021-64374262; E-mail: artruijin1@sina.com

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