Adenomyosis in mice resulting from mechanically or thermally induced endometrial–myometrial interface disruption and its possible prevention

**BIOGRAPHY**
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**KEY MESSAGE**
A mouse model of adenomyosis induced by endometrial–myometrial interface disruption was established, providing evidence for iatrogenically induced adenomyosis in humans. Perioperative administration of an NK1R antagonist or beta-blocker reduced the risk of mice developing adenomyosis. These findings open up new avenues for research into the prevention of adenomyosis.

**ABSTRACT**

Research question: Do uterine procedures potentially disrupting the endometrial–myometrial interface (EMI) induce adenomyosis?

Design: Six prospective, randomized controlled experiments were conducted involving a total of 106 female BALB/c and 12 female C57BL/6 mice. The incidence of adenomyosis was evaluated in these two strains of mouse after mechanically induced EMI disruption (EMID), or thermally induced EMID using electrocoagulation of different intensities. Finally, the incidence was evaluated in mice that had received perioperative administration of aprepitant (an NK1R inhibitor), propranolol (a beta-blocker) or vehicle. Body weight, hot plate latency and grade of myometrial infiltration were evaluated. Histology, immunohistochemistry and histochemistry analyses were also performed.

Results: Mechanical injury to the EMI caused EMID. Adenomyosis developed in the majority of mice in the EMID groups 3 months after mechanically induced EMID but did not develop in the control group (83.3% in C57BL/6 mice, P = 0.015; 100% in BALB/c mice, P = 0.0002). With thermally induced EMID, adenomyosis was found in 30% of the EMID mice 10 weeks later, but the incidence increased to 66.7% if the extent of EMID damage was increased. In mice with perioperative administration of aprepitant or propranolol, the incidence of adenomyosis was reduced from 100% to 58.3% (both P = 0.00034).

Conclusions: This study provides the first piece of experimental evidence that EMID resulting from iatrogenic uterine procedures can substantially increase the risk of developing adenomyosis, with the risk in proportion to the severity of disruption. More intriguingly, perioperative administration of an NK1R antagonist or beta-blocker significantly reduced the risk of developing adenomyosis.

**KEYWORDS**
Adenomyosis
Endometrial–myometrial interface
Epithelial–mesenchymal transition
Fibrosis
Hyperalgesia
Mouse
INTRODUCTION

Adenomyosis is an uterine disease defined as the presence of endometrial glands and stroma infiltrated deep and haphazardly into the myometrium (Bird et al., 1972). Although adenomyosis has traditionally been viewed as a disease affecting mostly multiparous women (Thompson and Davion, 1986), it has now, owing to improved imaging diagnostics, become increasingly diagnosed in younger women of reproductive age (Chapron et al., 2020; Nofalín et al., 2012; Van den Bosch and Van Schoubroeck, 2018). Despite its prevalence, it is often regarded as an enigmatic disease with poorly understood pathogenesis and pathophysiology (Bergeron et al., 2006; Garcia-Solares et al., 2018; Vannuccini et al., 2017; Vannuccini et al., 2019). Presenting with a soft and diffusely enlarged uterus, adenomyosis causes pelvic pain, abnormal uterine bleeding and subfertility (Farquhar and Brosens, 2006; Gords et al., 2018; Harada et al., 2016; Vercellini et al., 2014). Although approximately 35% of cases of adenomyosis are asymptomatic (Benson and Sneeden, 1958), dysmenorrhea is the most prevalent symptom besides abnormal uterine bleeding (Li et al., 2014).

Currently, there are essentially two major prevailing yet competing theories on the pathogenesis of adenomyosis: metaplasia and invagination (Garcia-Solares et al., 2018; Vannuccini et al., 2019). The metaplasia theory posits that adenomyotic lesions may originate from metaplasia of displaced embryonic Mullerian remnants (Ferenczy, 1998). The invagination theory was based largely on the tissue injury and repair theory proposed by Leyendecker and colleagues (Leyendecker and Wildt, 2011; Leyendecker et al., 2009; Leyendecker et al., 2015). The tissue injury and repair theory also attempts to account for the pathogenesis of endometriosis (Leyendecker and Wildt, 2011; Leyendecker et al., 2009; Leyendecker et al., 2015). Despite their popularity no experimental evidence has, however, been published in support of either theory.

Compelling epidemiological data indicate that iatrogenic uterine procedures, such as induced abortion and dilatation and curettage (D&C), are a risk factor for developing adenomyosis (Curtis et al., 2002; Levger et al., 2000; Panganamamula et al., 2004; Parazzini et al., 1997; Parazzini et al., 2009; Taran et al., 2010). However, to the best of the current authors' knowledge, there has been no experimental evidence to demonstrate that iatrogenic disruption of the endometrial–myometrial interface (EMI) can directly cause adenomyosis, although such a possibility has been mentioned (Leyendecker and Wildt, 2011; Leyendecker et al., 2009; Leyendecker et al., 2015).

Based on the recent discovery that activated platelets induce hypoxia and increase oestrogen biosynthesis in endometriotic stromal cells (Qi et al., 2019; Qi et al., 2020), and identification of the role of hypoxia in dramatically changing cellular phenotypes in endometrial cells (Hsiao et al., 2015), this group has recently proposed a new hypothesis on the pathogenesis of adenomyosis (Guo, 2020). The new hypothesis, termed EMI disruption (EMID), revamps the tissue injury and repair theory and postulates that EMI, induced by iatrogenic uterine procedures, can cause adenomyosis later in life. The current study was undertaken to test this hypothesis in mice.

EMID can be elicited mechanically or thermally (as in electrocoagulation). Hence, both modes of EMI need to be evaluated. However, both modes cause tissue injury, which induces substance P secretion and activates the hypothalamic–pituitary–adrenal axis; this results in an increased release of catecholamines such as adrenaline/noradrenaline, which in turn may suppress cell-mediated immunity (Goldfarb et al., 2011). The current authors have shown that perioperative administration of a beta-blocker and a nuclear factor kappa B (NF-κB) inhibitor, androgapholid, can substantially reduce the progression of endometriosis in mice (Long et al., 2016), as well as the recurrence of endometriosis due to incomplete excision of endometriosis in mice (Long et al., 2019). In light of these findings, this study also evaluated the possibility of reducing the risk of developing adenomyosis through perioperative administration of a beta-blocker or an inhibitor of neurokinin 1 receptor (NK1R), also known as tachykinin receptor 1 (TACR1), the main receptor for substance P.

MATERIALS AND METHODS

Animals and chemicals

All procedures were performed at the in-house animal facility in accordance with the guidelines of the National Research Council’s Guide for the Care and Use of Laboratory Animals (Council, 2011) and approved by the Institutional Experimental Animals Review Board of Shanghai Obstetrics and Gynecology Hospital, Fudan University on 30 January 2019. A total of 106 female BALB/c mice and 12 C57BL/6 female mice, all 6–8 weeks old and about 18–20 g in body weight, were purchased from Shanghai LingChang Laboratory Animal Center (Shanghai, China) and used for this study.

Aprepitant capsules were purchased from Merck Sharp & Dohme (Hoddesdon, UK) and propranolol hydrochloride tablets from Jiangsu Yabang Aipusen Pharmaceutical (Yancheng, China). Both aprepitant and propranolol hydrochloride tablets were dissolved in 0.9% saline for oral administration.

Vaginal cytology

A cotton-tipped swab was wetted with saline and inserted into the vagina of the mouse. The swab was gently rolled against the vaginal wall and the attached cells were transferred to a glass slide. The slides were air-dried and stained with haematoxylin–eosin (H&E) for 1 min. The slides were then rinsed with water and a coverslip was laid on each slide. The slides were imaged under bright-field illumination. The oestrus cycle was determined based on the proportion of leukocytes, cornified epithelial cells and nucleated epithelial cells, as previously reported (Byers et al., 2012).

The procedure of mechanically induced EMID

After 2 weeks of acclimatization, body weight was recorded for all the mice and a baseline hot plate test was administered before the adenomyosis-induction procedure (see below). To mimic iatrogenic endometrial injury such as endometrial D&C in humans, microcatheters (STD125-26S, Asahi Intecc, Tambol Bangkadi, Thailand) were used for uterine artery embolization as a tool to induce EMID in one of the two murine uterine horns, chosen at random, through the vaginal canal; the contralateral intact uterine horn was used as the control for each procedure.
However, inserting the microcatheter blindly into the uterus had two problems. First, there was no way to control the location or depth of the injury the catheter could elicit. Second, the procedure could cause unintended uterine rupture in some cases. To circumvent these problems, the mouse’s abdomen was opened up to monitor the procedure. Specifically, 2% chloral hydrate (w/v) was used for animal anaesthesia and then a 2 cm incision was made in the abdominal midline to expose the uterine horns so that the tip of the guidewire could be visualized inside the uterine horn. The tip of the guidewire, 0.53 mm in diameter, was used to elicit EMID by carefully thrusting the tip back and forth a few times against the EMI, making sure that the EMI was damaged. The exact set-up is shown in Supplementary Figures S1 and S2. For each uterine horn, five EMID spots or injuries were made to mimic iatrogenic procedures and to increase the chance of inducing adenomyotic lesions.

In experiment 1, uterine histology in 4 BALB/c mice was evaluated using the uninjured contralateral uterine horns as controls. Twenty-four hours after the EMID procedure, mice were sacrificed by cervical dislocation, the paired horns were collected and the extent of injury was evaluated by H&E staining.

Experiment 2 used 16 BALB/c mice that were randomly divided into two groups: the EMID group (n = 11) and the SHAM group (n = 5). A smaller sample size was intentionally chosen for the SHAM group as mice in that group would not be expected to develop adenomyosis. The mice from the SHAM group received an incision identical to that of the EMID group but without the vaginal insertion of the microcatheter. For all mice, body weight was recorded and the hot plate test was administered every 4 weeks after the procedure and before sacrifice. Three months after induction, all mice were sacrificed by cervical dislocation and their uterine horns were harvested and processed for H&E, immunohistochemistry (IHC) and Masson staining.

To see whether there was any strain difference, experiment 3 was an identical experiment on C57BL/6 mice. Twelve C57BL/6 mice were randomly divided into two equally sized groups, the EMID and the SHAM groups. All methods were the same as for the BALB/c mice.

Thermally induced EMID by electrocoagulation in BALB/c mice

Electrocoagulation procedures are used in many uterine procedures, such as endometrial polypectomy, submucosal myomectomy and endometrial resection. To see whether EMID induced by such procedures could also increase the risk of adenomyosis, experiment 4 involved induction of EMID using electrocoagulation. Twenty BALB/c mice were randomly divided into two equally sized groups, the SHAM and the electrocoagulation (E-EMID) groups. The EMID procedure was performed using a modified electrosurgical scalpel (CV-2000; Conway, Beijing, China).

A conductive enamelled wire with a diameter of 0.5 mm was connected to the unipolar knife with an insulating clip (see Supplementary Figure S3 for the detailed set-up). Chloral hydrate 2% (w/v) was used for animal anaesthesia, and the mouse was then fixed on an electrode plate in a supine position. Next a 2 cm incision was made in the abdominal midline to expose the uterine horns. The enamelled wire was inserted into a microcatheter, which was inserted into one of the horns at random. After the enamelled wire had been run through the sheath of the microcatheter to reach the uterine horn area, the machine was turned on. After trial and error, the electrosurgical power was set at 17 W with a duration of 2 s, and coagulation was performed at five different locations within the same uterine horn, similar to the mechanical EMID experiment. After the procedure, the surface of the uterine horn at the electrocoagulated locations changed colour and seemed paler than the surrounding uterine tissue.

Experiment 5 was conducted to determine whether (i) more extensive electrocoagulation-induced EMI injury/trauma, in terms of more thermal energy and more injuring sites, and (ii) the phase of the oestrous cycle would influence the risk of developing adenomyosis after electrocoagulation injury (E-EMID); the experiment involved E-EMID on mice in the oestrus and dioestrus phases of the cycle. The oestrous cycle was determined using vaginal cytology.

Thirty additional mice were divided into four groups: SHAM mice at oestrus (n = 6), SHAM mice at dioestrus (n = 6), E-EMID mice at oestrus (E-EMID/E; n = 10) and E-EMID mice at dioestrus (E-EMID/D; n = 8). Mice from the SHAM group received the same abdominal opening procedure but without E-EMID. In an attempt to increase the adenomyosis induction rate, the electrosurgical power was increased from 17 W to 20 W, with the same duration of 2 s, and the number of coagulated spots was increased from five to 20 different locations in a single uterine horn. Body weight was measured and the hot plate test was administered every 2 weeks until sacrifice. Ten weeks after the induction procedure, all the mice were sacrificed by cervical dislocation and all uterine horn tissues were harvested and processed for H&E, IHC and Masson staining.

Perioperative administration of NK1R inhibitor and beta-blocker in BALB/c mice

To see whether perioperative intervention could reduce the risk of adenomyosis, a total of 36 female BALB/c mice were divided randomly into three groups of equal sizes for experiment 6. Mechanically induced EMID was performed on one uterine horn of each mouse. Mice from different groups were orally dosed with different drugs 1 h before and 4 h after the induction procedure. Mice in the APT group received aprepitant treatment (25 mg/kg body weight), the PROP group received propranolol hydrochloride treatment (10 mg/kg body weight) and the UNT group received no treatment except normal saline of the same volume. The hot plate test was performed on all mice before and every 4 weeks after the surgery until they were sacrificed. Eight weeks after the EMID procedure, all mice were sacrificed by cervical dislocation and all the uterine horn tissues were harvested and processed for H&E, IHC and Masson staining.

Histochemistry and IHC analyses

All specimens were fixed in 4% paraformaldehyde (w/v), and the horn tissues were then embedded vertically in paraffin wax. The specimens were sliced every 2 mm and cross-sections of 5 μm were fixed onto adhesive slides. The first slides made in this way were used for H&E staining to evaluate whether adenomyosis had been produced, based on the positive identification of endometrial glands and stroma infiltrated into the myometrium. The depth of infiltration of ectopic endometrium into the myometrium was evaluated according
positive and negative controls are shown in Supplementary Figure S4. The representative IHC results for E-cadherin was used instead of primary antibodies. (JieHao Biotechnology). For positive staining, and mouse liver tissue for α-smooth muscle actin (α-SMA) staining, as previously reported (Shen et al., 2016). Briefly, infiltration of ectopic endometrium tissues into the myometrium was classified into three grades – I, II and III – depending on the depth of infiltration, involving the superficial, mid- or beyond mid-myometrium, respectively. For analytic purposes, grade 0 was allocated when no ectopic endometrium was observed in the myometrium, i.e. no adenomyosis was present.

Subsequent slides were deparaffinized in xylene, rehydrated in alcohol of serial concentrations and used for IHC analysis for E-cadherin (rabbit monoclonal antibody, 1:100; Cell Signaling Technology, Boston, MA, USA), alpha-smooth muscle actin (α-SMA) (rabbit polyclonal antibody, 1:100; Abcam, Cambridge, UK) and vimentin (rabbit monoclonal antibody, 1:100; Abcam, Cambridge, UK). The slides were heated at 98°C in citric acid buffer (pH 6.0) for antigen retrieval. The sections were incubated overnight at 4°C and incubated with the horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (JieHao Biotechnology, Shanghai, China) for 30 min at room temperature the following day. Positive staining was visualized using 3,3′-diaminobenzidine (JieHao Biotechnology) and counterstained with haematoxylin (JieHao Biotechnology). For positive controls, mouse kidney tissues were used for vimentin staining. Human breast cancer tissues were used for E-cadherin staining, and mouse liver tissue for α-SMA staining, as previously reported (Shen et al., 2016). For negative controls, rat serum (1:1000; Boster, Wuhan, China) was used instead of primary antibodies. The representative IHC results for positive and negative controls are shown in Supplementary Figure S4.

Five randomly selected images of each sample at 400 × magnification were taken and the mean density of staining intensity was acquired using Image Pro-Plus 6.0 (version 6.0.0.206; Media Cybernetics, Bethesda, MD, USA).

The extent of lesional fibrosis was evaluated by Masson trichrome staining, and the hot plate test was performed to assess the extent of adenomyosis-associated hyperalgesia. The procedures for Masson trichrome staining and hot plate test are described in the Supplementary Methods.

Results

Mechanical injury to the EMI causes EMID

Five randomly selected images of each sample at 400 × magnification were taken and the mean density of staining intensity was acquired using Image Pro-Plus 6.0 (version 6.0.0.206; Media Cybernetics, Bethesda, MD, USA).

To see whether the injury procedure induced the desired EMID, the procedure was performed on one of the two murine uterine horns in four mice, using the contralateral horn as a control. Twenty-four hours after the procedure, the uterine horn was seen to be oedematous on the injured side compared with contralateral one (FIGURE 1A). The extent of injury was also evaluated by H&E staining, showing that, in contrast to the EMI that was disrupted in the injured uterine horn in all the injured mice (FIGURE 1B, C), the endometrial glands and stroma were surrounded by well-defined layers of smooth muscle cells and the EMI was intact and continuous in the contralateral uterine horn (FIGURE 1D). Thus, the EMID procedure that was employed was shown to have caused the EMID as intended.

EMID induces adenomyosis in BALB/c mice

Next the hypothesis that EMID can induce adenomyosis in mice was tested. The EMID procedure was performed as described above in 11 female BALB/c mice. As a control, the same laparotomy was performed without EMID in five mice in the SHAM group. No mouse died during the entire experimental period. Twelve weeks after the EMID procedure, adenomyosis had successfully developed in all mice in the EMID group (100%) but none in the SHAM group, as shown by the presence of endometrial glands and stroma that had infiltrated into the myometrium (P = 0.0002; FIGURE 2A, TABLE 1).

No adenomyotic lesion was found in the uterine horn contralateral to the injured one in EMID mice. As shown in FIGURE 2A, the endometrial tissues invaded the myometrium and reached the uterine serosa in the uterine horns that underwent the EMID procedure, in contrast to the normal endometrium from SHAM mice (FIGURE 2A). In SHAM mice, the EMI was intact and clearly visible, and no adenomyotic lesion was found. In EMID mice, similar findings were observed in the uterine horn contralateral to the injured one (FIGURE 2A, green arrow). However, in EMID-induced adenomyosis, infiltration of endometrial tissue within the myometrium was found (FIGURE 2A). The ectopic endometrial glands and stroma invaded the myometrium and even reached the uterine serosa in the uterine horns of EMID mice (FIGURE 2A, black arrow). These results indicate that EMID is sufficient to induce adenomyosis in mice.
FIGURE 2 Endometrial–myometrial interface disruption (EMID) induces adenomyosis in BALB/c mice. (A) Representative images of uterine tissues from SHAM and EMID groups. Adenomyosis was successfully induced in the injured uterine horn in mice receiving EMID but not in mice from the SHAM group or in the uterine horn contralateral to the injured one in EMID mice. The green arrows point to the endometrial–myometrial interface, while the black arrows point to adenomyotic lesions. \( n = 5 \) mice in the SHAM group; \( n = 11 \) mice in EMID the group. (B) Average body weight in the two groups. (C) Boxplot of the grade of myometrial infiltration in uterine tissues from SHAM mice, and in the uterine horn contralateral to the injured horn and the injured uterine horn from EMID mice. The statistical significance of the difference between the SHAM and contralateral EMID groups could not be calculated as values were all zero in both groups (therefore N/A, not available). (D) Average hot plate latency, tested at the indicated times, between the two groups. The \( P \)-values shown are the result of a multiple regression analysis incorporating time (months since induction) and EMID as the two co-variables. Representative immunostaining results for E-cadherin (E) and vimentin (F) in normal endometrium (SHAM), eutopic endometrium from the uterine horn contralateral to the injured one and ectopic endometrium from the injured ipsilateral uterine horn, along with boxplots summarizing the staining data (right-hand panel). (G) Representative images of Masson trichrome staining in normal, eutopic and ectopic endometrium, along with a boxplot summarizing the staining data (right-hand panel). The nuclei are stained black, smooth muscle cells red and collagen fibres blue. (A) Magnification = 100 ×, scale bar = 200 µm. (E–G) Magnification = 400 ×, scale bar = 50 µm.
As expected, E-cadherin staining was observed mostly in the cell membranes of glandular epithelium, while vimentin staining was observed primarily in the cytoplasm of stromal cells in normal endometrium but also in the epithelial cells in adenomyotic lesions (figure 2b, i). Decreased E-cadherin staining was observed in the glandular epithelium in adenomyotic lesions, compared with normal endometrium in SHAM mice, but the difference did not reach statistical significance (P = 0.18) due, possibly, to the lack of statistical power. However, the lesional E-cadherin staining levels were significantly lower than those in the contralateral uterine horns (P = 0.038, figure 2e). Lesional vimentin staining levels were significantly higher than those of either control endometrium or endometrium from the contralateral uterine horn (P = 0.018 and P = 0.012, respectively; figure 2f). Suggestive of epithelial to mesenchymal transition (EMT). In addition, a dramatic increase in fibroblastic content was found in ectopic endometrium in EMID mice, but not in control endometrium or endometrium from the contralateral horns (P = 0.0032 and P = 0.001, respectively; figure 2g). Compared with control endometrium, the staining levels of E-cadherin and vimentin and the extent of tissue fibrosis in the endometrium of the contralateral uterine horns from EMID mice did not differ (all P-values ≥0.58, figure 2e-g).

These results have provided, to the best of the authors’ knowledge, the first piece of experimental evidence that EMID – similar to iatrogenic uterine procedures in humans – induces adenomyosis in mice. In addition, the resultant adenomyotic lesions show cellular changes consistent with EMT and increased fibrosis, as previously demonstrated in mice and humans (Liu et al., 2016; Shen et al., 2016).

**EMID also induces adenomyosis in C57BL/6 mice**

To determine whether EMID-induced adenomyosis is strain dependent, exactly the same EMID procedure was performed on C57BL/6 mice. Adenomyosis was successfully induced in 5 of 6 (83.3%) mice in the EMID group but none (0.0%) in the SHAM group, and in none of the contralateral uterine horns (P = 0.015, figure 3a, table 1).

No difference in body weight was found between the two groups before induction and 1, 2 and 3 months after induction (figure 2a). The grade of myometrial infiltration of the endometrial tissues in SHAM mice and in the contralateral uterine horns in EMID mice was 0, but the average grade of myometrial infiltration in the injured uterine horns was 2.1 (±0.7, median 2, range 1–3), significantly higher than those in the contralateral horns (P = 0.0014 and P = 0.0032, respectively; figure 2c).

Multiple linear regression analysis indicated that, although hot plate latency in all mice appeared to progressively decrease (P = 1.2 × 10^{-4}) over the course of the experiment, the decrease became significantly more pronounced in EMID mice (P = 2.1 × 10^{-4}, R^2 = 0.35, figure 2d). Indeed, 2 and 3 months after induction, hot plate latency in the EMID mice was significantly shortened compared with that before the induction (P = 0.0098, and P = 0.00098, respectively, figure 2d). By the end of the experiment, EMID mice had significantly shorter latencies than those of SHAM mice (P = 0.003, figure 2d).

IHC analysis of E-cadherin and vimentin and quantitative assessment of the extent of tissue fibrosis by Masson trichrome staining were performed (figure 2e-g). As expected, E-cadherin staining was

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mouse strain</th>
<th>Induction method</th>
<th>Number and sizes of groups</th>
<th>Induction duration (weeks)</th>
<th>Outcome (incidence of adenomyosis) n (%)</th>
<th>Remarks</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>BALB/c</td>
<td>Mechanical EMID</td>
<td>SHAM: 5 EMID: 11</td>
<td>12</td>
<td>SHAM: 0 (0%) EMID: 11 (100%) Highly significant</td>
<td>Grade of infiltration ↑ Hyperalgesia ↑ Lesions show signs of EMT and fibrogenesis</td>
</tr>
<tr>
<td>3</td>
<td>C57BL/6</td>
<td>Mechanical EMID</td>
<td>SHAM: 6 EMID: 6</td>
<td>12</td>
<td>SHAM: 0 (0%) EMID: 5 (83.3%) Statistically significant</td>
<td>Lesions show signs of EMT, FMT and fibrogenesis</td>
</tr>
<tr>
<td>4</td>
<td>BALB/c</td>
<td>Thermal EMID (electrocoagulation)</td>
<td>SHAM: 10 E-EMID: 10</td>
<td>10</td>
<td>SHAM: 0 (0%) E-EMID: 3 (30%) Statistically not significant</td>
<td>Grade of infiltration ↑ Hyperalgesia ↑ Lesions show signs of EMT and fibrogenesis</td>
</tr>
<tr>
<td>5</td>
<td>BALB/c</td>
<td>Thermal EMID (electrocoagulation)</td>
<td>SHAM: 12 E-EMID/E: 10 E-EMID/D: 8</td>
<td>10</td>
<td>SHAM: 0 (0%) E-EMID/E: 7 (70%) E-EMID/D: 5 (62.5%) Highly significant increase compared with SHAM</td>
<td>Grade of infiltration ↑ Hyperalgesia ↑ Increased incidence as injury becomes more extensive and severe Lesions show signs of EMT and fibrogenesis</td>
</tr>
<tr>
<td>6</td>
<td>BALB/c</td>
<td>Mechanical EMID</td>
<td>UNT: 12 APT: 12 PROP: 12</td>
<td>8</td>
<td>UNT: 12 (100%) APT: 7 (58.3%) PROP: 7 (56.3%) Highly significant reduction compared with UNT</td>
<td>Grade of infiltration ↓ Hyperalgesia attenuated Lesions in treated mice show signs of attenuated EMT and fibrogenesis</td>
</tr>
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</table>

EMID, endometrial-myometrial interface disruption; E-EMID, electrocoagulation-induced EMID; E-EMID/E, E-EMID/D, E-EMID/Eeestrus, E-EMID/D, E-EMID/eestrus, APT, aperpitant, PROP, propranolol; UNT, untreated, EMT, epithelial to mesenchymal transition; FMT, fibroblast to myofibroblast transdifferentiation.
FIGURE 3 Endometrial–myometrial interface disruption (EMID) induces adenomyosis in C57BL/6 mice. (A) Representative images of uterine tissues from the SHAM and EMID groups. Adenomyosis was successfully induced in the injured uterine horns of mice receiving EMID but not in mice from the SHAM group, or in the uterine horn contralateral to the injured one in EMID mice. The green arrows point to the EMI, while the black arrows point to adenomyotic lesions. n = 6 mice in the SHAM group; n = 6 mice in the EMID group. (B) Average body weight in the two groups. (C) Boxplot of the grade of myometrial infiltration in uterine tissues from the SHAM and EMID mice. (D) Average hot plate latency, tested at the indicated times, in the two groups. The P-values shown are the result of a multiple regression analysis incorporating time (months since induction) and EMID as the two co-variables. Representative immunostaining results for E-cadherin (E) and alpha (Greek letter) smooth muscle actin (α-SMA) (F) in normal (SHAM) and ectopic endometrium (EMID), along with boxplots summarizing the staining data (right-hand panel). (G) Representative images of Masson trichrome staining in normal and ectopic endometrium, along with a boxplot summarizing the staining data (right-hand panel). The nuclei are stained black, smooth muscle cells red and collagen fibres blue. (A) Magnification = 100 ×, scale bar = 200 µm. (E-G) Magnification = 400 ×, scale bar = 50 µm.
linear regression analysis indicated that, although the hot plate latency appeared to be progressively shortened (P = 9.2 × 10⁻⁵) in all mice over the course of the experiment, the shortening became much more pronounced in the EMID mice (P = 4.4 × 10⁻⁴; R² = 0.55; FIGURE 3D). Hence, by the end of the experiment, hot plate latency in EMID mice was significantly lower than in SHAM mice (P = 0.0087; FIGURE 3D).

Next, IHC analysis of E-cadherin and α-SMA in normal and adenomyotic endometrium, as well as in endometrium from the contralateral uterine horns of EMID mice, was performed. As there was no difference in histology and IHC staining between normal endometrium and endometrium from the contralateral uterine horns of EMID mice (data not shown), only the results for normal endometrium are presented here. Significantly reduced E-cadherin staining but increased α-SMA staining in the glandular epithelial component was observed in ectopic endometrium from EMID mice compared with normal endometrium (P = 0.017 and P = 0.030; FIGURES 3E, F), suggestive of both EMT and fibroblast-to-myofibroblast transdifferentiation. Consistent with this, the extent of lesional fibrosis was significantly higher than that of tissue fibrosis in endometrium from SHAM mice (P = 0.0043; FIGURE 3G).

It should be noted that one mouse from the EMID group showed no adenomyotic lesions. Even though an adenomyotic lesion was not identified by H&E staining, there was still a possibility that this might be a false-negative result simply because any lesion present had not been found. Alternatively, it is possible that the EMID procedure performed did not cause enough injury to later induce adenomyosis.

EMID thermally induced by electrocoagulation also induces adenomyosis

Experiment 4 was conducted to test the hypothesis that E-EMID could cause adenomyosis in BALB/c mice. E-EMID was performed using open abdominal visualization in 10 female BALB/c mice. For controls, only an identical laparotomy was performed, without electrocoagulation injury. Ten weeks after the E-EMID procedure, adenomyosis had been successfully induced in three mice in the E-EMID group (30%) but none in the SHAM group (FIGURE 4A, TABLE 1). No adenomyotic lesion was found in the uterine horn contralateral to the injured one in the EMID mice. Similar to mice with EMID-induced adenomyosis (FIGURES 2 and 3), the endometrial tissues invaded the myometrium and sometimes reached the uterine serosa in uterine horns that had received the E-EMID procedure (FIGURE 4A). In contrast, no adenomyotic lesion was found in the uterine horn contralateral to the injured one in the E-EMID mice (FIGURE 4A).

No difference in body weight was found between the two groups before induction and 2, 4, 6, 8 and 10 weeks after induction (FIGURE 4B). The difference in incidence of adenomyosis between the SHAM and E-EMID groups was not statistically significant (P = 0.21), and the difference in grade of myometrial infiltration also failed to reach statistical significance (P = 0.078; FIGURE 4C); the lack of statistical significance is almost certainly due to a lack of statistical power. This is because the SHAM mice would not be expected to develop adenomyosis, at least not within 10 weeks after a laparotomy. The contralateral uterine horn in the E-EMID mice would not develop adenomyosis either. Hence, had 16 mice been allocated to the SHAM group, the result of observing three mice with adenomyosis among 10 E-EMID mice would have become statistically significant (P = 0.046), as would the difference in myometrial infiltration grade (P = 0.026).

Multiple linear regression analysis indicated that, while overall the hot plate latency in all mice appeared to be progressively shortened (P = 2.8 × 10⁻⁵) during the course of the experiment, the shortening became significantly more pronounced in the E-EMID mice (P = 2.8 × 10⁻⁵; R² = 0.24; FIGURE 4D). By the end of the experiment, hot plate latency in the E-EMID mice was significantly lower than that in the SHAM mice (P = 0.029, FIGURE 4D). In fact, despite the finding that only three mice in the E-EMID group developed histologically detectable adenomyosis, the group as a whole had significantly shorter latency than the SHAM mice even after the three mice with adenomyosis had been removed from the analysis (P = 9.5 × 10⁻⁵; R² = 0.22). This implies that even though those E-EMID mice were not found to have adenomyosis, they had seemingly experienced certain pathological changes, most likely in their uterus, that led to central sensitization manifested as reduced hot plate latency.

Consistently and similar to mechanical injury-induced EMID, we found that E-cadherin staining levels were significantly decreased (P = 0.007; FIGURE 4E) but vimentin staining levels were significantly elevated (P = 0.019 after removing an apparent outlier in the SHAM group; FIGURE 4F) in the epithelial component in three mice found to have adenomyosis, again suggestive of EMT. In addition, the adenomyotic lesions exhibited significantly elevated fibrotic content compared with normal endometrium (P = 0.007; FIGURE 4G). Thus, E-EMID also increases the risk of developing adenomyosis in mice.

To rule out the possibility that E-EMID had led to adenomyosis in only a small proportion of mice because there had not been enough disruption to the EMI, the electrical power was increased in experiment 5 from 17 W to 20 W, and, in addition, the number of trauma spots was increased from five to 20. Moreover, the oestrous cycle was staged and the E-EMID mice subgrouped into oestrous and dioestrous groups.

Ten weeks after the induction, adenomyosis had been successfully induced in seven mice in the E-EMID/oestrous group (70.0%) and five in the E-EMID/dioestrous group (62.5%), but none (0%) in the SHAM group, yielding a total incidence of 66.7% in the two E-EMID groups (P = 0.0009 and P = 0.0027, respectively; FIGURE 5A; TABLE 1). No difference in body weight was found among the three groups before induction and 2, 4, 6, 8 and 10 weeks after induction (FIGURE 5B). The grade of myometrial infiltration in SHAM mice and the contralateral uterine horns in the E-EMID mice was 0 (no adenomyosis), but the average grade of myometrial infiltration in the injured uterine horns was 1.5 (± 1.3, median 1.5, range 0–3) in the oestrous mice and 1.0 (± 1.1, median 1, range 0–3) in the dioestrous mice, significantly higher than in the SHAM mice (P = 0.0009 and P = 0.0027, respectively; FIGURE 5C). The myometrial infiltration grade in EMID mice in the oestrous group was not significantly
FIGURE 4  Electrocoagulation-induced endometrial–myometrial interface disruption (E-EMID) increases the risk of developing adenomyosis in BALB/c mice. (A) Representative images of uterine tissues from the SHAM and E-EMID groups. Adenomyosis was successfully induced in the injured uterine horn in three mice receiving E-EMID but not in mice from the SHAM group, or in the uterine horn contralateral to the injured one in the E-EMID mice. The green arrows point to the EMI, while the black arrows point to adenomyotic lesions. \( n = 10 \) mice in the SHAM group; \( n = 10 \) mice in the E-EMID group. (B) Average body weight in the two different groups. (C) Boxplot of the grade of myometrial infiltration in uterine tissues from SHAM mice and in the injured uterine horns from three E-EMID mice with adenomyosis. (D) Average hot plate latency, tested at the indicated times, in the SHAM and E-EMID groups. The \( P \)-values shown are the result of a multiple regression analysis incorporating time (weeks since induction) and E-EMID as the two co-variables. Representative immunostaining results for E-cadherin (E) and vimentin (F) in normal endometrium from SHAM mice and ectopic endometrium from E-EMID mice with adenomyosis, along with boxplots summarizing the staining data (right-hand panel). An apparent outlier in the SHAM group was not included in the vimentin data. (G) Representative images of Masson trichrome staining in normal endometrium from SHAM mice and ectopic endometrium from E-EMID mice with adenomyosis, along with a boxplot summarizing the staining data (right-hand panel). (A) Magnification = 100 ×, scale bar = 200 µm. (E–G) Magnification = 400 ×, scale bar = 50 µm.
FIGURE 5 Electrocoagulation-induced endometrial–myometrial interface disruption (E-EMID) increases the risk of developing adenomyosis in BALB/c mice in the oestrus and dioestrous phases of the cycle. (A) Representative images of uterine tissues from SHAM mice, from the uterine horn contralateral to the injured one in mice receiving E-EMID and from mice receiving E-EMID in the oestrus (E-EMID/E) and dioestrous (E-EMID/D) phases of the cycle. Adenomyosis was successfully induced in injured uterine horn from some mice receiving E-EMID but not in mice from the SHAM group, or in the uterine horn contralateral to the injured one in the E-EMID mice. The green arrows point to the EMI, while the black arrows point to adenomyotic lesions. \( n = 6 \) mice in the SHAM oestrus group; \( n = 6 \) mice in the SHAM dioestrous group; \( n = 10 \) mice in EMID/E group; \( n = 8 \) mice in the EMID/D group. (B) Average body weight in three different groups. (C) Boxplot of the grade of myometrial infiltration in the uterine tissues of SHAM mice and in the injured uterine horns of E-EMID mice, measured in the oestrus (E-EMID/E) and dioestrous (E-EMID/D) phases of the cycle in mice with adenomyosis. (D) Average hot plate latency, tested at the indicated times, in the three groups. The \( P \)-values shown are the result of a multiple linear regression incorporating time (weeks since induction), E-EMID/E and E-EMID/D as the three co-variables. Representative immunostaining results for E-cadherin (E) and vimentin (F) in normal endometrium from SHAM mice and ectopic endometrium from E-EMID/E and E-EMID/D mice with adenomyosis, along with boxplots summarizing the staining data (right-hand panel). (G) Representative images of Masson trichrome staining in normal endometrium from SHAM mice and ectopic endometrium from E-EMID/E and E-EMID/D mice with adenomyosis, along with a boxplot summarizing the staining data (right-hand panel). (A) Magnification = 100 ×, scale bar = 200 µm. (E–G) Magnification = 400 ×, scale bar = 50 µm.
different from that of the dioestrus group (P = 0.43; FIGURE 5C).

Multiple linear regression analysis indicated that although the hot plate latency in all mice appeared to be progressively shortened overall (P < 2.2 × 10⁻¹⁶) during the course of the experiment, the shortening became significantly more pronounced in the E-EMID mice (the estimated regression slope parameter beta for E-EMID/E and E-EMID/D being −2.18, P = 1.5 × 10⁻⁶, and −1.42, P = 0.0027, respectively; R² = 0.40; FIGURE 5D). Hence, by the end of the experiment, hot plate latency in the E-EMID mice (oestrous and dioestrus groups combined) was significantly lower than that in the SHAM mice (P = 0.044, FIGURE 5D).

Within the epithelial component of the adenomyotic lesions, E-cadherin staining was found to be significantly reduced in both the oestrous and dioestrus groups (P = 0.005 and P = 0.027, respectively; FIGURE 5E) while vimentin staining was significantly elevated (P = 0.0005 and P = 0.006, respectively; FIGURE 5E). No significant difference was found between control endometrium and eutopic endometrium in the contralateral uterine horns of E-EMID mice (both P > 0.55). In addition, the adenomyotic lesions exhibited significantly elevated fibrotic content compared with normal endometrium (P = 0.0002 and P = 0.002, respectively; FIGURE 5E). No significant difference was found between the oestrus and dioestrus groups in E-EMID mice (all P > 0.44).

**Perioperative administration of NKIR inhibitor or beta-blocker reduces the risk of EMID-induced adenomyosis**

Whether elicited by mechanical means (using the tip of a microcatheter) or heat (as in electrocoagulation), EMID, as a form of invasive procedure, causes tissue injury: It was thus wondered whether perioperative administration of NKIR inhibitor or beta-blocker could reduce the risk of EMID-induced adenomyosis.

Compared with the UNT mice, which all developed adenomyosis (100%), seven mice each (58.3%) from the APT and PROP groups developed adenomyosis, significantly fewer than in the UNT group (both P = 0.00034; FIGURE 6A, TABLE 1). The average grade of myometrial infiltration in APT mice was 1.2 (±0.9, median 1, range 0–3), significantly lower than that in the UNT group (2.3±0.9, median 3, range 1–3; P = 0.0087; FIGURE 6C). The average grade of myometrial infiltration in the PROP group was 1.8 (±1.3, median 2, range 1–3), not statistically significant from that of the UNT mice (P = 0.33; FIGURE 6C). The grade in the contralateral uterine horns in all mice was 0 (i.e. no adenomyosis was seen).

Although there was no difference in body weight before and 8 weeks after the induction procedure (P = 0.13, and P = 0.24, respectively; FIGURE 6B), there was a significant difference 4 weeks after induction (P = 0.029; FIGURE 6B). Multiple linear regression analysis indicated that all mice had gained weight over the course of the experimental period (P < 2.2 × 10⁻¹⁶), and that mice in the APT group, in particular, had a higher body weight than the other two groups of mice (P = 0.00023, R² = 0.79; FIGURE 6B).

Multiple linear regression analysis consistently indicated that, while the hot plate latency in all mice appeared to be progressively shortened (P < 2.2 × 10⁻¹⁶) over the course of the experiment, mice in the APT group, but not the PROP group, had a significantly attenuated shortening of latency compared with the UNT mice (P = 0.009, R² = 0.64; FIGURE 6D). Hence, by the end of the experiment, hot plate latency in the APT mice was significantly lower than that in the UNT mice (P = 0.012; FIGURE 6D). There was no difference in latency between the PROP and UNT groups at the end of the experiment (P = 0.29; FIGURE 6D).

IHC staining of E-cadherin and Masson staining of adenomyotic lesions indicated that mice in the APT group had significantly greater E-cadherin staining (P = 0.033) in the glandular epithelial component but reduced fibrotic content (P = 0.039) in lesions compared with the UNT group (FIGURE 6E, F). In contrast, there was no significant difference in E-cadherin staining and fibrotic content between the PROP and UNT groups (P = 0.29 and P = 0.76, respectively; FIGURE 6E, F). Thus, perioperative administration of aprepitant seems to be more effective than propranolol in reducing the risk of developing EMID-induced adenomyosis in mice and in alleviating adenomyosis-associated hyperalgesia.

Hot plate latency was negatively correlated with grade of myometrial infiltration (Spearman’s r = −0.50, P = 0.0018; FIGURE 6G), which, in turn, was positively correlated with the extent of lesional fibrosis (Spearman’s r = 0.70, P = 2.3 × 10⁻⁶; FIGURE 6H). Consistent with the notion that EMT is involved in lesional fibrogenesis, E-cadherin staining was negatively correlated with the extent of lesional fibrosis (r = −0.54, P = 0.0007; FIGURE 6I).

**DISCUSSION**

This study has presented a mouse model of adenomyosis induced by EMID. Unlike the previous rodent models of adenomyosis (discussed below), this model agrees with, and in fact provides the first piece of experimental evidence to support, numerous epidemiological reports linking uterine procedures and increased risk of adenomyosis (Curtis et al., 2002; Levgur et al., 2000; Panganamulula et al., 2004; Porazini et al., 1997). Remarkably, EMID, induced either mechanically or thermally, can result in adenomyosis in mice, and the risk of developing adenomyosis appears to depend on the severity of the EMID. The EMID-induced adenomyotic lesions here apparently underwent EMT; fibroblast to myofibroblast transdifferentiation, fibrogenesis and presumably smooth muscle metaplasia, as previously reported (Liu et al., 2016; Shen et al., 2016). More intriguingly, perioperative administration of an NKIR antagonist or, to a much less extent, a beta-blocker can significantly reduce the risk of developing adenomyosis. This study thus provides a mouse model of adenomyosis that can be used for research purposes, sheds new light onto the pathogenesis of adenomyosis and explains why a prior history of uterine procedures is a risk factor for adenomyosis. More importantly, it suggests a tantalizing yet intriguing possibility of intervention to reduce the risk of developing adenomyosis when performing uterine procedures. Given today’s ubiquity of iatrogenic uterine procedures, such a prevention measure, if proven to be efficacious and safe, has important clinical implications.

Several epidemiological studies have reported the proportion of patients who underwent iatrogenic uterine procedures among those with adenomyosis (TABLE 2). These numbers are seemingly lower than the nearly 100% adenomyosis shown here in mice with EMID or even the 70%
FIGURE 6. Perioperative administration of aprepitant and propranolol reduces the risk of endometrial–myometrial interface disruption (EMID)-induced adenomyosis. (A) Representative images of uterine tissues from the uterine horn subjected to EMID in mice administered perioperative saline (no treatment, or UNT group), aprepitant (APT) or propranolol (PROP) (n = 12 mice per group). The images in the APT and PROP groups are from mice without adenomyosis. (A) Magnification = 100 ×, scale bar = 200 µm. The green arrows point to the EMI, while the black arrows point to adenomyotic lesions. (B) Average body weight in the three groups. (C) Boxplot of the grade of myometrial infiltration in uterine tissues from mice in the UNT, APT and PROP groups. (D) Average hot plate latency, tested at the indicated times, in the three groups. The P-values shown are the result of a multiple linear regression, incorporating time (months since induction), APT and PROP as the three co-variables. Representative immunostaining results for E-cadherin staining (E) and Masson staining (F) in ectopic endometrium from the three groups of mice, along with boxplots summarizing the staining data (right-hand panel). (E, F) Magnification = 400 ×, scale bar = 50 µm. (G) Hot plate latency correlates negatively with the grade of myometrial infiltration (Spearman’s r = –0.50, P = 0.002). (H) Hot plate latency correlates positively with the extent of lesional fibrosis (Spearman’s r = 0.70, P = 2.3 × 10⁻⁶). (I) E-cadherin staining correlates negatively with the extent of lesional fibrosis (r = –0.54, P = 0.0007). The solid line represents the regression line.
in those with E-EMID. Why is there such a discrepancy? Does this gap mean that this mouse model is invalid?

First, the difference is not as large as it is perceived to be. In fact, two studies actually reported the proportion to be around 60% (TABLE 3; Levugur et al., 2000; Toron et al., 2010), which is not substantially different from the 70% as seen in experiment 5. Second, there are vast differences in genetic background, development and physiology between humans and mice, and these differences could also account for the difference in the incidence of adenomyosis caused by EMID between the species. Third, unlike humans, the mice used in this study were genetically, demographically and environmentally homogeneous, with an identical reproductive history. In addition, the EMID procedure that were employed was uniform across all the mice, ensuring that all mice received the same extent of EMID. These homogeneities would ensure more uniformity in outcome. Fourth, the total amount of tissue trauma created is likely to be greater in the current model than in iatrogenic procedures in humans, as at least five spots of EMID were made following each laparotomy. Thus, the procedure itself likely constitutes a greater stress on mice than on humans. Fifth, the current study is prospective, compared with the retrospective nature of human studies, and there was no loss to follow-up and no recall or other biases that can be difficult to avoid in human studies. Finally, all human studies have been based on a patient population that had undergone hysterectomy (TABLE 2). In most cases, these studies reported that patients who underwent hysterectomy specifically for adenomyosis were more likely to have more severe disease (Parazzini et al., 1997; Parazzini et al., 2009), and may well have had a different pathogenesis from that seen with iatrogenic EMID (see FIGURE 7 and the discussion below). All these considerations in fact justify the use of the current mouse model of adenomyosis, which, aside from the difference that mice do not menstruate, recapitulates two key features of iatrogenically induced adenomyosis in humans: that EMID can be caused by uterine procedures, and that adenomyosis ensues some time later.

The increased incidence of adenomyosis induced by E-EMID when both the electric power and the number of injury sites were increased strongly indicates that the risk of developing iatrogenically induced adenomyosis may depend on the extent and severity of EMID. This may explain why not all women who undergo iatrogenic uterine procedures develop adenomyosis. Indeed, if EMID is extensive and severe enough, there should be an increased risk of developing adenomyosis. This, if further confirmed independently, has apparent clinical implications, in that EMID elicited by iatrogenic uterine procedures should be performed with extreme care to minimize its extent and severity in order to reduce the risk of adenomyosis.

Although the current study has not provided any mechanistic explanation for why mechanically induced EMID appears to confer higher risk of adenomyosis than thermally induced EMID, one possible explanation is that mechanical EMID results in more bleeding than E-EMID, and thus entails more platelet aggregation, which in turn results in a greater release of numerous bioactive molecules by the activated platelets. Among these, the most abundant may be transforming growth factor-β1 (TGF-β1), which has been shown to induce EMT in endometrial epithelial cells (Zhang et al., 2016). In addition, tissue injury also results in the release of substance P (Onuoha and Alpor, 1999; Onuoha et al., 2001), which may also induce EMT in endometrial epithelial cells (Yan et al., 2019). As a result of this EMT, these epithelial cells, previously immobile within the endometrium, become mobile and invasive, and can move across the disrupted EMI, infiltrate the myometrium and establish the initial adenomyotic foci.

In contrast, thermally induced tissue injury elicits little haemorrhage, leading to little or no platelet aggregation and thus much less TGF-β1 release. However, substance P is still released (Yan et al., unpublished data), possibly leading to EMT as well. This might be the reason why the perioperative use of NK1R inhibitor was found here to reduce the risk of adenomyosis, probably more effectively than beta-blocker use. As TGF-β1 may be more potent than substance P in inducing EMT, and as mechanical damage induces the release of both TGF-β1 and substance P as opposed to just substance P in thermal damage, mechanical damage may lead to more extensive invasion of endometrial epithelial cells into the myometrium than thermal damage, and consequently may confer a higher risk of developing adenomyosis.

### TABLE 2 OCCURRENCE OF ADENOMYOSIS IN PATIENTS WITH A HISTORY OF VARIOUS IATROGENIC UTERINE PROCEDURES, AS REPORTED BY EPIDEMIOLOGICAL STUDIES OF PATIENTS WHO HAD UNDERGONE HYSTERECTOMY

<table>
<thead>
<tr>
<th>Study</th>
<th>Adenomyosis in patients who had undergone hysterectomy % (n/N)</th>
<th>Age (years)*</th>
<th>Risk factor</th>
<th>Patients with a history of the named procedure in patients with adenomyosis % (n/N)</th>
<th>Odds ratio (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parazzini et al. (1997)</td>
<td>21.2 (150/707)</td>
<td>Median: 50 (19–84)</td>
<td>D&amp;C</td>
<td>14.0 (21/150)</td>
<td>2.1 (1.1–3.8)</td>
</tr>
<tr>
<td>Levugur et al. (2000)</td>
<td>32.4 (36/111)</td>
<td>46.5 ± 1.5</td>
<td>Pregnancy termination</td>
<td>58.8 (10/17)</td>
<td>4.35 (119–5.99)</td>
</tr>
<tr>
<td>Curtis et al. (2002)</td>
<td>199 (368/1850)</td>
<td>Not reported</td>
<td>Induced abortion</td>
<td>171 (63/368)</td>
<td>1.5 (1.1–2.1)</td>
</tr>
<tr>
<td>Panganamamula et al. (2004)</td>
<td>47.2 (412/873)</td>
<td>47.1 ± 10.7</td>
<td>All uterine procedures</td>
<td>48.8 (201/412)</td>
<td>1.37 (1.05–1.79)</td>
</tr>
<tr>
<td>Parazzini et al. (2009)</td>
<td>28.2 (231/820)</td>
<td>51.2 ± 8.5 (at hysterectomy)</td>
<td>Induced abortion</td>
<td>21.6 (50/231)</td>
<td>1.9 (1.2–2.8)</td>
</tr>
<tr>
<td>Taran et al. (2010)</td>
<td>33.3 (76/228)</td>
<td>41.0 ± 6.4</td>
<td>All uterine procedures</td>
<td>60.5 (46/76)</td>
<td>1.8 (1.03–3.10)</td>
</tr>
</tbody>
</table>

* Mean ± SD, unless otherwise specified.

**Note:** The table lists the odds ratios and confidence intervals for the risk of adenomyosis among patients who had undergone hysterectomy, compared to those who had not, for various iatrogenic uterine procedures. The data are extracted from epidemiological studies conducted on human patients who had undergone hysterectomy. The procedures include induced abortion, all uterine procedures, and others. The studies report varying odds ratios and confidence intervals, reflecting the risk of adenomyosis associated with each procedure.
Although this study was able to show that more extensive E-EMID results in a higher risk of adenomyosis, it did not provide similar data for mechanical EMID. However, it is conceivable this may also be the case as more disruptions and more injury would yield increased platelet aggregation and thus increased release of both TGF-β1 and substance P, which, in turn, induce more EMT and prompt more invasion of endometrial epithelial cells into the myometrium, resulting in a higher risk of developing adenomyosis. Future studies are warranted to confirm this.

Of course, the current authors are under no illusion that EMID-induced adenomyosis can account for the pathogenesis of all causes of adenomyosis. Indeed, there must be more than one cause of adenomyosis as many women with adenomyosis do not have such a history. Nonetheless, given that so far there has been no animal model of adenomyosis that is supported by epidemiological data, this mouse model should be useful in future research on adenomyosis. The mechanistic insight that could be gained from this model should hopefully help in cracking the enigma of pathogenesis from other causes.

The relevance of this mouse model to human adenomyosis may be best illustrated by contrast to several other rodent models of adenomyosis. Although prenatal exposure to low-dose diethylstilbestrol can induce adenomyosis (Huseby and Thurlow, 1982), there has so far been no epidemiological data in support of such a link in humans. Fluoxetine administration also has been
reported to induce adenomyosis in rodents (Ficicioglu et al., 1995; Sengupta et al., 2013), but again no such link has been documented in humans. The same is true for the mouse model of adenomyosis induced by intruterine pituitary grafts (Huseby et al., 1985; Mori et al., 1981). Of course, women with adenomyosis often have elevated prolactin concentrations (Taran et al., 2010), and prolactin is shown to be a mitogen for smooth muscle cells in vitro (Bazik et al., 2010; Nowak et al., 1993; Nowak et al., 1999). However, whether an increased prolactin concentration is a cause or a consequence remains to be elucidated.

The mouse model of adenomyosis induced by neonatal feeding of tamoxifen is an interesting model (Parrott et al., 2001) as postmenopausal women with breast cancer who received tamoxifen treatment are reported to have an increased risk of adenomyosis (Cohen et al., 1997). However, there is a vast difference between neonatal and postmenopausal exposure to tamoxifen, since the neonatal period is highly sensitive to endocrine-disrupting chemicals (Street et al., 2018). In addition, adenomyosis usually occurs before menopause, and the majority of postmenopausal women do not take tamoxifen. This animal model also is strain dependent (Mehosseb et al., 2010). More remarkably, neonatal administration of the same dose of tamoxifen to the identical strain of mice via the subcutaneous rather than the oral route produced uterine carcinomas rather than adenomyosis (Newbold et al., 1997). Such a variation in outcome depending on the different in strain as well as the administration route limits the validity or applicability of the model. Indeed, nearly two decades after the first report of the mouse model, it is only known that the ‘disruption of the mesenchymal layers surrounding the endometrium in the neonatal period can give rise to disordered development of uterine stroma, smooth muscle, blood vessels, and possibly its innervation’ (Parrott et al., 2001), but how this relates to the human condition remains unclear.

Although the current mouse model of adenomyosis is simple and economical to establish, it is by no means perfect. In fact, future improvements can be made. For example, laparotomy is currently employed to monitor the EMID procedure, and perhaps ultrasound-guided EMID could be instituted. Of course, this would necessitate an ultrasonography instrument for small animals, which could be expensive.

That perioperative use of NK1R inhibitor reduces the risk of adenomyosis is entirely biologically plausible. Substance P can act as an immune modulator and injury messenger in various peripheral tissues (Hong et al., 2009). It has a broad range of effects mediated through binding to NK1R, including increased production of inflammatory cytokines (Ho et al., 1996) and chemotaxis of immune cells (Carolan and Casale, 1993; Frode-Soleh et al., 1999). Systemic substance P concentrations are elevated when there is tissue injury (Onouha and Alpar, 1999; Onouha and Alpar, 2001), also seen in this mouse model (Yan et al., unpublished data). NK1R antagonism decreases oxidative stress in the peritoneum (Reed et al., 2007), and reduces postoperative adhesion formation (Reed et al., 2004) without interfering with wound healing (Prushk et al., 2007). In future studies, the optimal dosage as well as the treatment duration should be further investigated in order to achieve maximal prevention effect.

The beta-blocker did not work as effectively as the NK1R inhibitor in reducing the grade of myometrial infiltration, even though it seemed to reduce the incidence of adenomyosis. Although both NK1R and ADRB2 (adrenoceptor beta 2) have been reported to be involved in EMT (Nilsson et al., 2020; Yan et al., 2019), it is likely that substance P may be more potent than adrenaline/noradrenaline in inducing EMT. Alternatively, the beta-blocker might need to be used in conjunction with a COX-2 (cyclooxygenase 2) inhibitor (Long et al., 2019) to achieve a better effect. Future research is needed to illuminate this issue.

The seemingly higher success rate in inducing adenomyosis by mechanical, as opposed to thermal, EMID seems to suggest that the two modes of EMID may entail differential risks of developing adenomyosis. This may be consistent with the report that abortion induced by sharp curettage seems to be accompanied by an increased risk of adenomyosis when compared with the situation without induced abortion (Curtis et al., 2002). If this proves to be true, it obviously has clinical implications. Thus, future research is warranted to clarify this issue.

Although this study has shown that EMID can increase the risk of development of adenomyosis in mice, providing experimental support for the link between iatrogenic procedures and adenomyosis, it is unclear whether EMID can also induce endometriosis. Regardless of this, the reduction in risk of developing adenomyosis following perioperative use of a beta-blocker or NK1R inhibitor echoes the finding of a substantial reduction in recurrence of endometriosis in mice with incomplete excision of lesions after perioperative use of a beta-blocker and/or NF-κB inhibitor (Long et al., 2019). In fact, there are preliminary data to show that the perioperative use of an NK1R inhibitor can also achieve a similar reduction in recurrence of endometriosis to that of a beta-blocker plus androgapholide (Chen et al., unpublished data), suggesting that injury-induced release of substance P may also participate in promoting the growth of residual lesions.

The current study has several strengths. Aside from the experimental demonstration that EMID can induce adenomyosis, the hypothesis was testing using two mouse strains and two different modes of EMID, which adds to the validity of the conclusion. In addition, the demonstration that the perioperative administration of an NK1R inhibitor can reduce the risk of developing EMID-induced adenomyosis may open up new lines research in the future.

However, there are also several limitations. First, the study did not provide any mechanistic explanation for why EMID can induce adenomyosis or why perioperative administration of an NK1R inhibitor can reduce the risk of developing EMID-induced adenomyosis. This needs to await further investigations. Second, serum prolactin concentrations were not measured, which could be useful to relate to the mouse model of adenomyosis caused by intruterine pituitary grafts. Again, future studies are warranted to see whether our mouse model of adenomyosis also displays hyperprolactinaemia. Third, it was not specifically investigated whether graded mechanically induced EMID would yield a differential risk of adenomyosis, even though this seems to be entirely
biologically plausible. Future experiments may be needed to validate this.

Finally, the sample sizes in some experiments may be too small to permit more conclusive findings. For example, the oestrus mice in the E-EMID group appeared to be more susceptible to EMID than the dioestrus mice, but the difference did not reach statistical significance (Figure 5C). It is well known that female rodents in the oestrus phase have higher circulating oestrogen but lower progesterone concentrations, which is the opposite of rodents in the dioestrus phase of the cycle (Wood et al., 2007). This is reminiscent of the report that the recurrence risk of endometriosis was two times higher among women who underwent surgery for endometriosis during the luteal phase compared with the follicular phase (Schweppe and Ring, 2002). It is possible that EMID elicited during the oestrus phase in mice coincides with low concentrations of circulating progesterone but higher concentrations of oestrogen, generating a microenvironment that is more conducive to the invasion of endometrial cells into the myometrium. Indeed, it is well documented that oestrogens are mitogens and are the fuel for the proliferation of endometrial cells. In contrast, progesterone is known to suppress endometrial proliferation and inflammation (Li et al., 2016). Hence, depending on the type of prevailing hormone – oestrogen or progesterone – the trauma induced may be boosted if EMID occurs in the oestrus phase, or attenuated if it occurs in the dioestrus phase. Future studies are needed to clarify this issue.

In summary, this study presents a mouse model of adenomyosis induced by EMID, elicited by either mechanical or thermal means. The model is easy and economical to implement, and provides experimental evidence that iatrogenic uterine procedures can increase the risk of adenomyosis. Hopefully, the insights gained from this model would help us to understand the pathogenesis of adenomyosis due to other causes. The demonstration that perioperative administration of an NKIR antagonist or, to a much less extent, a beta-blocker can significantly reduce the risk of developing adenomyosis raises the prospect of perioperative intervention in uterine procedures to reduce the risk of adenomyosis.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2020.07.023.

NOTE ADDED IN PROOF

The authors thank Dr. Dan Martin for stimulating discussions subsequent to acceptance of this paper. Regarding why mechanically induced EMID appears to confer higher risk of adenomyosis than thermally induced EMID, Dr. Martin called my attention to Dr. Kurt Semm’s work in the 1980s. Dr. Semm observed that mechanical techniques resulted in a greater net residual trauma than coagulation because coagulation killed the cells within tissues involved. Mechanical techniques produce ischemia and release local substances that create adhesions. Since dead tissue does not react, the thermally induced EMID would be less likely to cause invasion of endometrial cells into the myometrium, thus conferring lower risk of developing adenomyosis as compared with mechanically induced EMID.

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