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ORIGINAL ARTICLE

Efficient Production of Murine Uterine Damage Model

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Abstract

BACKGROUND: Thin or damaged endometrium causes uterine factor-derived infertility resulting in a failure of embryonic implantation. Regeneration of endometrium is a major issue in gynecology and reproductive medicine. Various types of cells and scaffolds were studied to establish an effective therapeutic strategy. For this type of investigations, production of optimal animal models is indispensable. In this study, we tried to establish various murine uterine damage models and compared their features.

METHODS: Three to ten-week-old C57BL/6 female mice were anesthetized using isoflurane. Chemical and mechanical methods using ethanol (EtOH) at 70 or 100% and copper scraper were compared to determine the most efficient condition. Damage of uterine tissue was induced either by vaginal or dorsal surgical approach. After 7–10 days, gross and microscopic morphology, safety and efficiency were compared among the groups.

RESULTS: Both chemical and mechanical methods resulted in thinner endometrium and reduced number of glands. Gross morphology assessment revealed that the damaged regions of uteri showed various shapes including shrinkage or cystic dilatation of uterine horns. The duration of anesthesia significantly affected recovery after procedure. Uterine damage was most effectively induced by dorsal approach using 100% EtOH treatment compared to mechanical methods.

CONCLUSION: Taken together, murine uterine damage models were most successfully established by chemical treatment. This production protocols could be applied further to larger animals such as non-human primate.

Keywords Uterine damage · Endometrium thickness · Murine model · Embryonic implantation

1 Introduction

Infertility or subfertility is a very important issue in many countries because of its increasing prevalence. In spite of a recent progress in the assisted reproductive techniques such as *in vitro* fertilization, the therapeutic efficiency stays at a plateau without further improvement of pregnancy rates. Among many causes, e.g. anovulation, oligospermia, uterine defects, etc., uterine factor infertility is more retractable.

Fibrosis or adhesion due to damage of endometrium is a leading cause of infertility [1]. In addition, aging of uterine tissue, physiological or chemical factors such as repeated abortion, endocrine disruptor are other causes for the

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failure of embryonic implantation, which ultimately leads to uterine factor-derived infertility [2–4].

Endometrium is a layer that regenerates cyclically, and its thickness is critical for achievement of successful implantation [5–7]. However, the prevalence of damaged thin endometrium, caused by such as Asherman's syndrome, is reported as 13% of infertile women [8]. Currently, uterine factor-derived infertility caused by thinner endometrium than a threshold thickness has very limited treatment options with very low efficiency [5, 8, 9]. Development of more efficient therapeutic strategies is critical. In many studies, the use of stem cells [10] and biological scaffolds has been evaluated in many different uterine damage models [11–13].

However, trials of various potential therapies were not only successful but also difficult to compare due to the lack of standardized model production. Therefore, generation of optimal animal models with uterine damage is a prerequisite for the development of efficient therapeutic strategy. To date, for the establishment of endometrial damage models, various chemical or mechanical methods have been used, but the results were inconsistent and not effective [14, 15]. In some studies, ethanol (EtOH) was even used for treating ovarian tumors [16, 17]. Despite past numerous reports, there has not been a report of proposed efficient protocols in order to induce endometrial damage efficiently and optimally.

In this study, we tried to establish an efficient production protocol for the establishment of murine uterine damage models. The efficacy and safety of various different methods was evaluated and compared, and the most effective concentration of EtOH was searched.

2 Materials and methods

2.1 Animals and anesthesia

Six to ten-week-old C57BL/6 female mice were maintained in cages in a temperature- and light-controlled room (24 ± 2 °C, 40–60% humidity, and 12 h light/-dark cycle). They were allowed to have an access to a commercial pelleted diet and the weight was 200–250 g.

Animals were anesthetized using 5% isoflurane, and maintained at 1% during treatment. After surgery, animals were recovered in warm blanket, returned to cages and remained under intensive veterinary supervision to monitor good health and body condition including attitude, appetite, or behavior. At the end of the experiments, they were sacrificed and their uterine horns were isolated for further analyses (Fig. 1).

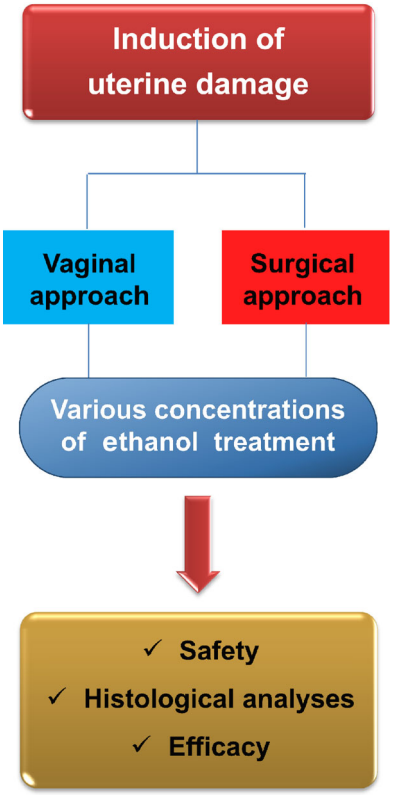

Fig. 1 Schematic presentation of establishing murine uterine damage models

2.2 Chemically methods for uterine damage

2.2.1 Vaginal approach

Anesthetized mice were positioned as supine and the opening of vagina was pre-screened under inverted microscope. A small pipette tip was inserted into vagina and a catheter (22G, BD Biosciences, San Jose, CA, USA) was inserted into either left or right uterine horn (Fig. 2). Fifty microliter of EtOH (Sigma-Aldrich, St. Louis, MO, USA) was injected through the inserted catheter and remained for several minutes. Then, injected EtOH was retrieved and endometrial cavity was washed using Hank's balanced salt solution (HBSS, Invitrogen, Calsbad, CA, USA). The surface of vagina was then treated with 10% povidone-iodine solution (Sigma-Aldrich, St. Louis, MI, USA) using sterilized cotton swab.

2.2.2 Dorsal surgical approach

Back of anesthetized mice was shaved, and skin was sterilized using 70% EtOH solution. The back of renal portion was incised, the fat pad near ovary was drawn, and the uterus horn was distinguished and exposed (Fig. 3). The

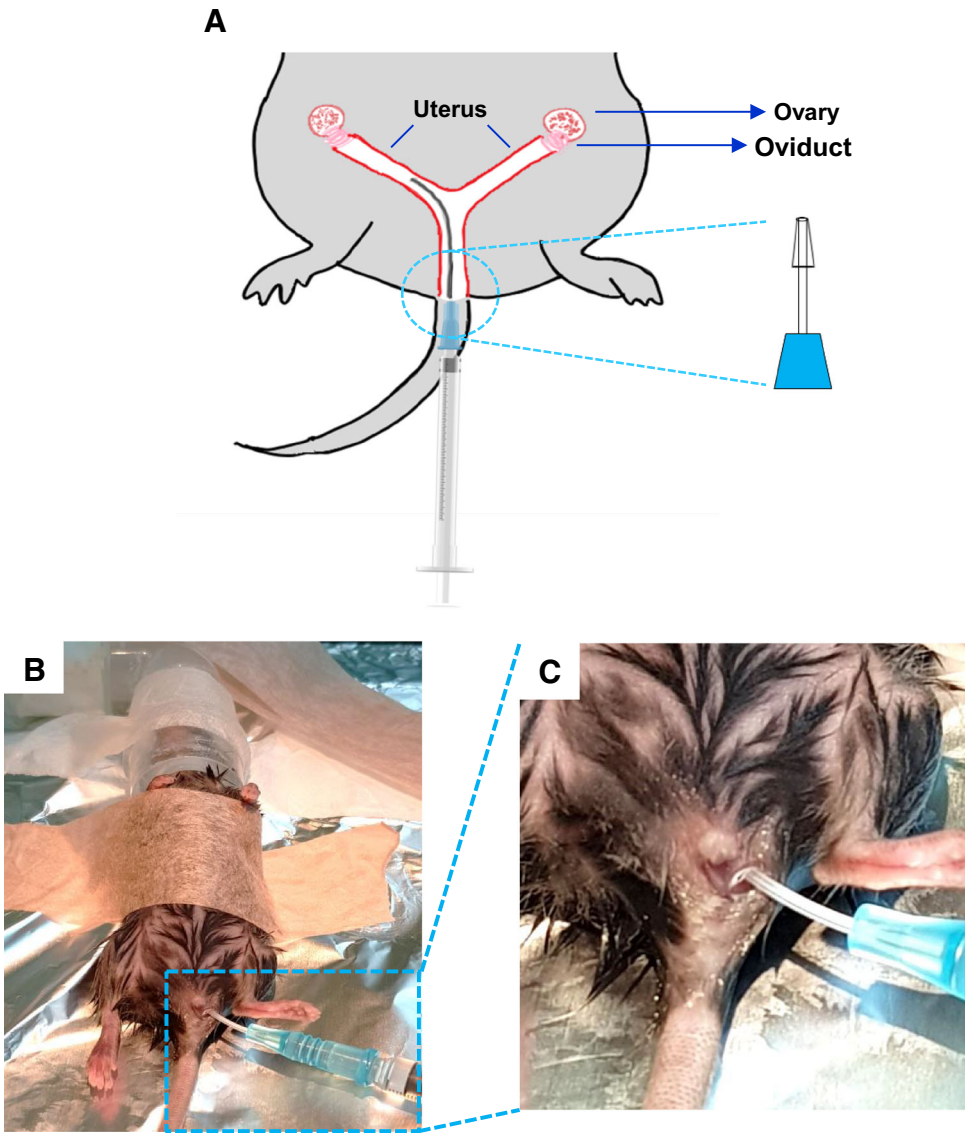

Fig. 2 Procedures of vaginal approach. **A** Vaginal approach: illustration and structural view of guide tip and catheter, **B** insertion of 22G catheter, with guide tip, injecting ethanol through cervix, **C** enlarged image of inserted catheter

exposed uterus horn was clipped and a 30G gauge needle with 1 ml syringe (BD Precision Glide™ needle, BD Biosciences, San Jose, CA, USA) was inserted into the uterus far from ovary. Fifty microliter EtOH was injected and remained several minutes. Then, the injected EtOH was retrieved, and the cavity was washed using HBSS (Invitrogen). The opened skin and muscle layer was sutured using absorbable suture (Vicryl 5.0, Ethicon, Somerville, NJ, USA), and the skin was treated with 10% povidone-iodine solution using sterilized cotton swab.

2.2.3 Mechanical method

Specially designed copper scraper with a diameter 1.8 mm was used for inducing mechanical damage. The approach was vaginal. Liner copper scraper was pre-sterilized and it was inserted with guide tip (Fig. 2A). The scraper was inserted into the uterine cavity to touch the end of cavity wall, and then scraped throughout the inner surface of cavity at least 10 times.

2.3 Morphological and histological analyses

At postoperative 7–10 days, uteri were isolated from sacrificed animals, washed with HBSS (Invitrogen, Calsbad, CA, USA), and fixed using 10% formalin (Sigma-Aldrich, St. Louis, MI, USA) for 12 h. Samples were washed with PBS, and dehydrated in serial EtOH solutions. Then, tissue was embedded with paraffin and sectioned transversely with a 5-μm thickness. The slides were stained with hematoxylin and eosin (H&E), and the thickness of endometrial layer was mainly analyzed at each section. For this measurement, the images were taken using inverted microscope (Nikon, Tokyo, Japan). Diameters of

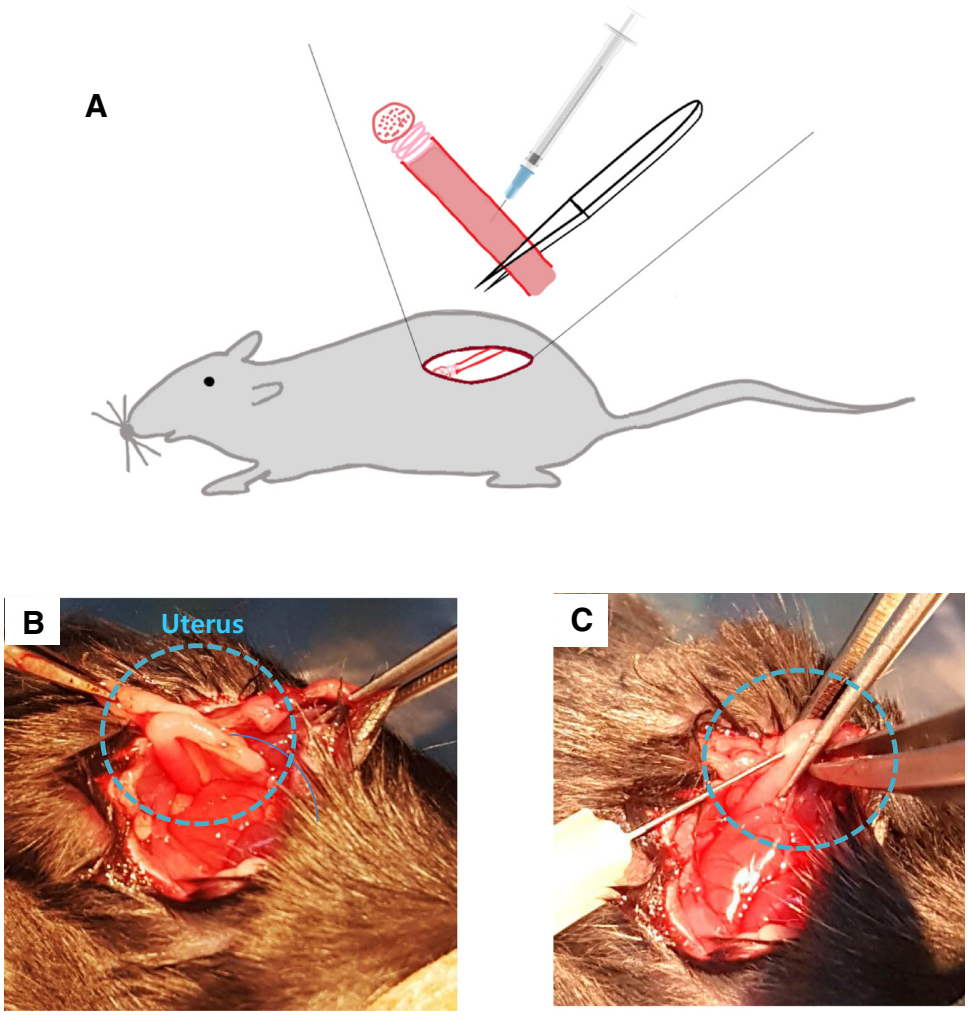

Fig. 3 Procedures of dorsal surgical approach. **A** dorsal surgical approach: illustration and structural view of injection guided by clamping forceps, **B** insertion of 30G syringe containing ethanol into uterine horns exposed, **C** enlarged image of damage induction site

endometrial cavity were evaluated and analyzed using *i*-solution program.

3 Results

3.1 Optimal age of animals for uterine damage induction

Vaginal or dorsal surgical approach was used for chemical or mechanical methods for inducing uterine endometrial damage. Various factors affected the efficiency of damage induction, and they are indicated in Table 1. The age of used animal was ranged from 3 to 10 weeks. The length of uterine horns and diameter was associated with the success of damage induction. The mice younger than 4 weeks had a relatively small diameter compared to those of above 4 weeks. Horns with small diameter did not show sufficiently induced damages, due to their weak tensile strength and tear during procedure. Those above 9 weeks had larger and well-developed uteri, however, they were surrounded by fat pad and were tough to grab during procedure. Therefore, mice between 5 and 8 weeks were used for the comparative analysis.

3.2 Optimal duration and concentration of anesthesia

Duration of anesthesia was significantly affected by age of mice. When duration was longer than 30 min, some animals under 4 weeks, either with vaginal or dorsal surgical approach, were shocked. As for the concentration, when anesthesia was maintained as 3% during procedures, some animals under 4 weeks were shocked, while those above 5 weeks showed normal post-anesthetic recovery. Maintenance of anesthesia with lower percentage was associated with faster recovery, but lower than 1% was not effective due to unintended awakening. Therefore, anesthesia was effectively maintained at 1–1.5% according to the weight of used animals.

Table 1 Comparison of prognostic factors for inducing murine uterine damage model

Anesthesia concentration		Anesthesia duration		EtOH concentration		Age	
5% → 3.0%	+	30–45 min	+	100%	+++	8–10 wks	++
5% → 1.5%	++	15–30 min	++	70%	+++	5–8 wks	+++
5% → 1.0%	+++	0–15 min	+++	50%	++	3–4 wks	+

Factors considered for induction of uterine damage model are listed and their efficiency and safety are indicated. (+: low, ++: intermediate, +++: high)

EtOH ethanol, *min* minutes, *wks* weeks

3.3 Vaginal versus dorsal approach

For vaginal approach, the lower part of 200-μl sterilized pipette tip (guide tip) was cut into 8 mm in diameter (Fig. 2A). The piece was softly inserted into female vagina using micro forceps until the cervix was clearly visualized. And the 22G catheter, connected with 1 ml syringe, containing various concentration of EtOH was inserted through the guide tip and further inserted to either left or right horn of uterus (Fig. 2B). Approximately 2 cm length of catheter was inserted and 50 μl EtOH was injected and remained for 7–8 min (Fig. 2C). Then, injected EtOH was collected and washed with HBSS.

For dorsal surgical approach, anesthetized mice were positioned as prone and shaved. The shaved skin was sterilized, and skin at position 1.2–1.5 cm from midline was opened using micro forceps and scissors (Fig. 3A). The uterus horn was exposed and stretched (Fig. 3B). The exposed horn was clipped. A syringe containing EtOH with 30G needle injected into the middle part of exposed horn and 50 μl of EtOH was injected (Fig. 3C).

Mechanically induced damage showed a moderate level as compared to chemical methods. However, the power of scraping and the diameter of uterine horn were varied. Thus, the degree of damage was relatively inconsistent compared to chemical methods.

3.4 Effects on endometrium thickness

Thickness of damaged endometrium was analyzed on histological observation. Treatment of EtOH significantly reduced the thickness of endometrium resulting in the loss of outer epithelial layers. Several concentrations, 50, 70 and 100%, were tested and the optimal concentration was 70% (Table 1). All concentrations affected the cell distribution of outer layer (Fig. 4A, B). Among the concentrations, 70% EtOH treatment using dorsal surgical approach was the most effective, showing even and wider range of thinner endometrium, as compared to 50 and 100% EtOH (Fig. 4B).

Gross uterine morphology revealed various shapes after EtOH treatment. Upper part of untreated horn was edematous (Fig. 5A, B), the length of uterine horn was not similar between left and right horns (Fig. 5C, D) and even swelled, expanded morphology from cervix to horns (Fig. 5E, F).

4 Discussion

An increase of uterine factor-derived infertility leads to the necessity of developing tailored therapeutic strategies. Most of previous studies evaluated the efficiency of cell sources such as bone marrow-derived, embryonic and induced pluripotent stem cells, uterine stromal stem cells or in combination with variety of biological scaffolds [10–13, 18], which could be used as undifferentiated or differentiated cells [19]. However, majority focused only on the therapeutic effects of cells, scaffolds, and conditions [20, 21]. This non-standardized generation of damage models prevents from the objective and quantitative analysis between attempted treatments. A consistent and efficient production of uterine damage models is a very important pre-requisite to developing novel therapeutics (Table 2).

In this study, we compared the chemical and mechanical methods used for establishing uterine damage with various factors and conditions, may affect the efficiency of damage induction (Table 1). We used mechanical methods using copper scraper, which is widely used because of its toxic effects on endometrium in larger animals such as rats [22] and rabbits [23]. This copper scrapper-induced damage showed moderate degree of damage compared to chemical methods. Its application to a small uterine horn frequently led to the tear of horn or uncontrollable bleeding. This method was more optimal for those with relatively larger uteri.

We compared the safety and efficiency between vaginal and dorsal surgical approach. The vaginal approach has advantages of shorter anesthesia duration and no additional requirements for recovery without the risk of operation-caused adhesion. However, we could not observe the exact insertion of catheter, thus in some cases, the catheter was inserted into abdomen cavity by perforating the cervix, which resulted in sudden deaths in some cases. Dorsal surgical approach had a privilege of selection of damage

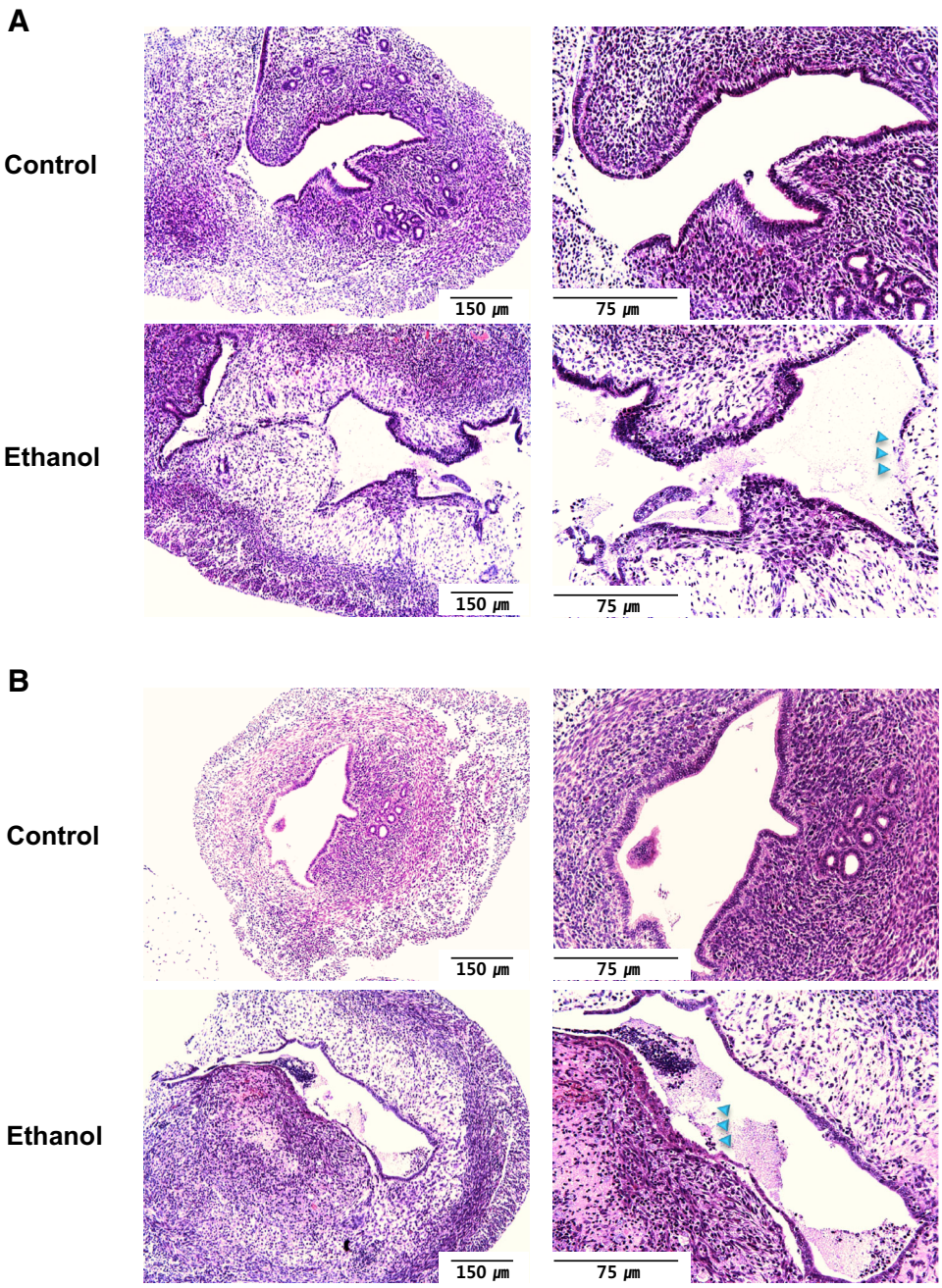

Fig. 4 Histological analyses of damaged uterine tissue. **A** Stained images of damage-induced murine endometrium via vaginal approach demonstrated disrupted and thinner endometrium. Upper panel: control, lower panel: ethanol. Magnification; ×100 and ×200. **B** Stained images of damage-induced murine endometrium via dorsal surgical approach also demonstrated disrupted and thinner endometrium. The disrupted part was wider and even in surgically induced damage models. Upper panel: control, lower panel: ethanol. Magnification; ×100 and ×200. *Blue triangles* indicate damaged, thinned endometrium

induction site. Clamping prevented the unintended leakage of injected EtOH. Small incisions and earlier awakening of anesthesia frequently prevented from successful damage induction when using dorsal surgical approach. If dorsal approach chosen, further application of cell therapy needs re-open of operation site, therefore, minimal operation with fast recovery is important.

A shorter duration of anesthesia demonstrated a higher efficacy regardless of approach in this study. We only used isoflurane as anesthetic agent, however, the safety and efficacy could be affected by the sort of anesthetic agents. The agents are known to affect vascular permeability for uterus [24] and correlation of anesthetic agent with reproductive function has been reported [25, 26].

As for the concentrations of EtOH, we used are 50, 70 and 100%. Previous studies used various types of reagents for denaturation of endometrial outer layer, basal lamina and inner layer. When injected, its denaturation and dehydration could be observed during procedure and flow-through to opening of horn if not optimally clumped. Use of EtOH was simple and efficient for denaturation of thinned endometrial layers (Fig. 4). The most effective concentration was 100% in our study, which is similar to a

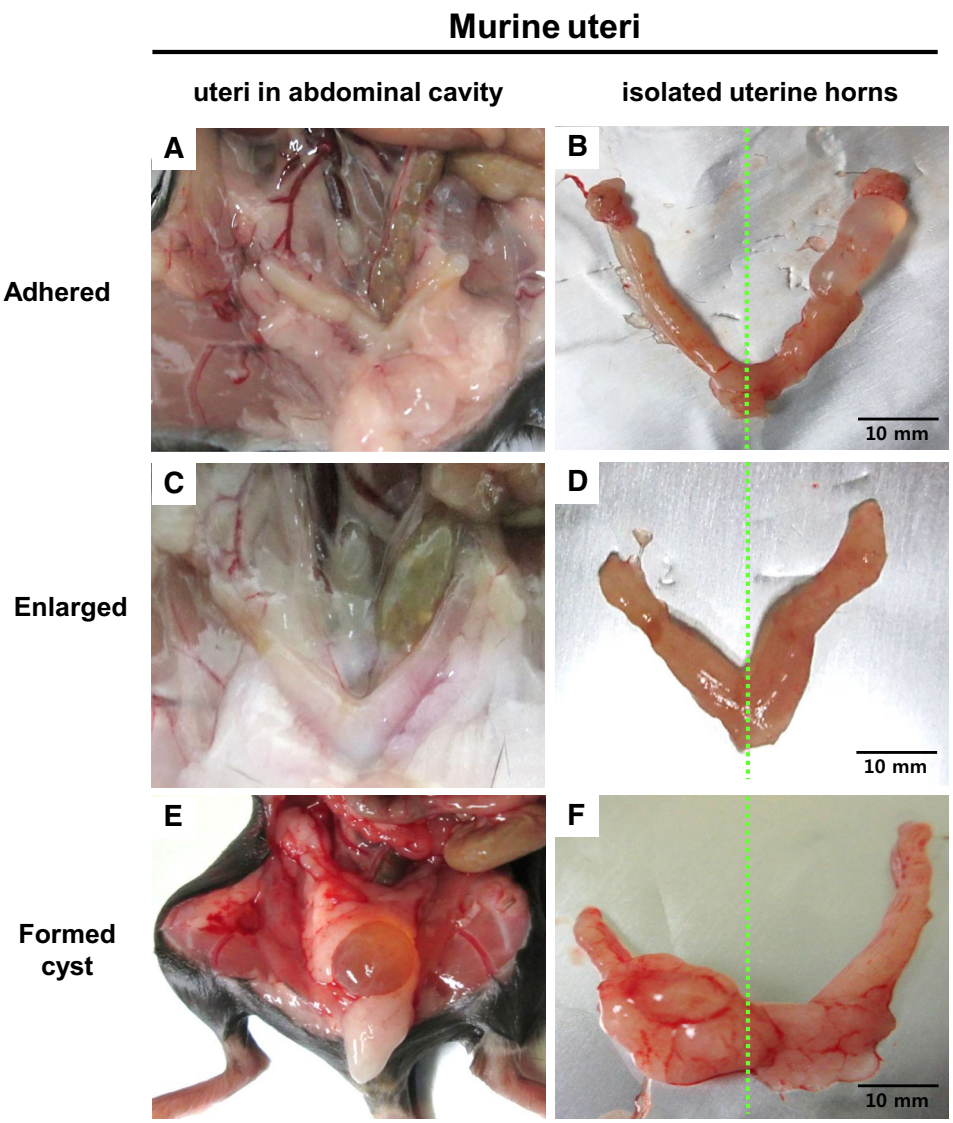

Fig. 5 Gross morphological observation of normal and damaged uteri. Various types of anomalies were observed after induction either by vaginal or dorsal surgical approach. **A** Adhered horn, **B** isolated uteri with adhered horn and partially fluid-filled region, **C** enlarged horns, **D** isolated uteri with enlarged horns, which demonstrated atypical larger diameters, **E** formation of cyst, **F** isolated uteri with cyst, abnormal morphology and adhesion

previous report that elucidated that 95% of EtOH was effective method in a rat model [27].

Complete loss of endometrium may be deemed successful from a certain experimental perspective, however, it should also be considered that endometrium be regenerated when cells are injected. Therefore, controllability regarding the degree of induced damage is important from the perspective of optimal damage induction. In this context, the chemical induction using EtOH can an efficient method for establishing rodent models.

This study has some limitations. Firstly, individual mouse may have different in terms of genetic and epigenetic characteristics. Application of our data should be careful when other strain than C57BL/6 is used. Secondly, animals with larger horns generally have larger diameters of cavity and thicker endometrial layers, therefore, the evaluation and interpretation should also be careful in larger animals. To date, many studies used murine models [28]. Since rodents have different reproductive physiology to humans, it is necessary to expand to the animal models with similar reproductive physiology such as non-human primate [29, 30]. Furthermore, uterine function is closely related to ovarian cellular physiology and hormonal regulation [31–37], therefore, follow-up of maintaining reproductive function after approach or post-hormone treatment should be considered.

In conclusion, we evaluated various methods of uterine endometrial damage induction, and found that the age or size of animals, route of treatments, use of chemical or mechanical inducers are important factors that contribute to the efficiency and safety. The optimal concentrations of EtOH should be tested individually according to the age and size of animals. Further investigations are necessary in larger animal models such as NHP, considering the differences in the anatomy and physiology of reproductive organs.

Table 2 Previous studies on uterine damage with various cell types and scaffolds

Age	Weight	Strain	n	Anesthesia	OP region	Damage	Time	Sacrifice	References
	200–250 g	Sprague–Dawley Rat	66	Ketamine and diazepam (50 mg/kg, i.p., respectively)	Abdominal midline incision	Collagen scaffolds		4 and 12 weeks	[38]
	250–300 g	Sprague–Dawley Rat	92		Low abdominal midline incision	Partial full thickness uterine excision and Collagen scaffolds			[39]
	250–300 g	Sprague–Dawley Rat	81	Ketamine and diazepam (50 mg/kg, i.p.)	Abdominal midline incision	Collagen scaffolds		30–90 days	[11]
	250–300 g	Sprague–Dawley Rat	72	Ketamine and diazepam (50 mg/kg, i.p.)	Abdominal wall incision	Partial of rat uterine horn was excised and left for scar formation		30 days	[4]
10–12 W		Fischer Rat	24 uterine horns	3% inhaled isoflurane	Abdominal midline incision	Uterine horn excision		28 or 90 days	[40]
	25–30 g	Kunming mouse	80		Abdomen	Scratched with a blunt syringe on the right uterine horn or both		Day 8 of pregnancy	[41]
9 W		MRL/Mpj (+/+), C57Bl/6	37	Ketamine (60–80 mg/kg), Xylazine (10–15 mg/kg) i.p.	Abdomen	Uterine incision		3, 5, 15 and 60 days	[42]
	200–230 g	Wistar albino rat	32	Ketamin and xylazine (35 mg/kg, i.p.)	Abdominal wall incision	Full thickness defect by incising a segment		15 or 30 days	[43]
D9–D11 (D1 = the day vaginal plug was found)		C57BL/6		Isoflurane (5% for induction, 1.5% for maintenance) in oxygen		Intrauterine LPS injection or intravaginal LPS administration			[44]
	180–230 g	Wistar albino rat		Ketamin hydrochloride (75 mg/kg), Xylazine hydrochloride (10 mg/kg)	Abdominal midline incision	Monopolar electrocautery	3–5 secs	14 days	[45]
8 W		Kunming mouse	10	Inhaled anesthetics	Vertical incision in the abdominal wall and the uterus	Intrauterine adhesions using mechanical injury		After 2 estrous cycles	[46]
6–7 W		ICR mouse		8% Chloral hydrate (0.1 ml/10 g), i.p.	Vertical incision in the abdominal wall and the uterus	Electrocoagulation	3 secs		[47]

Table 2 continued

Age	Weight	Strain	n	Anesthesia	OP region	Damage	Time	Sacrifice	References
		ICR mouse				Curettage and coagulation		1 week	[48]
		SD Rat	26			95% alcohol injection	5 mins	After 2 estrous cycles	[49]
		C57Bl/6			Mid-abdominal incision	Lower uterine horn and uterine artery using atraumatic vascular clips	30 mins	8 months	[50]
6–10 W		BDF1, TNF-R p55-deficient mouse, C57BL/6	87	Sodium pentobarbital (40 mg/kg)	Mid-abdominal incision	Clamping the uterine horn and uterine artery	5–30 mins		[51]
8–10 W		BALB/c	48			i.p. injection		48 h	[52]
8 W		C57Bl/6 J	40	Isoflurane		Intrauterine injection		After 3 estrous cycles	[53]

i.p. intraperitoneal injection, *mins* minutes, *secs* seconds, *W* weeks

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval All of the animal studies were performed after receiving an approval of the Institutional Animal Care and Use Committee of the Biomedical Research Institute at the Seoul National University Hospital (SNUH-IACUC No. 15-0032).

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