Human Reproduction, Vol.32, No.10 pp. 2026–2035, 2017
Advanced Access publication on August 31, 2017 doi:10.1093/humrep/dex250

human reproduction

ORIGINAL ARTICLE ***Gynaecology***

Allowable warm ischemic time and morphological and biochemical changes in uterine ischemia/reperfusion injury in cynomolgus macaque: a basic study for uterus transplantation

Iori Kisu[1,*], Kiyoko Umeneo[1], Masataka Adachi[1], Katsura Emoto[2], Yuya Nogami[1], Kouji Banno[1], Iori Itagaki[3,4], Ikuo Kawamoto[3], Takahiro Nakagawa[3], Hayato Narita[3], Atsushi Yoshida[3], Hideaki Tsuchiya[3], Kazumasa Ogasawara[3,5], and Daisuke Aoki[1]

[1]Department of Obstetrics and Gynecology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan [2]Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan [3]Research Center for Animal Life Science, Shiga University of Medical Science, Setatsukinowa, Otsu, Shiga 520-2192, Japan [4]The Corporation for Production and Research of Laboratory Primates, Sakura, Tsukuba, Ibaraki 305-0003, Japan [5]Division of Pathology and Disease Regulation, Department of Pathology, Shiga University of Medical Science, Setatsukinowa, Otsu, Shiga 520-2192, Japan

*Correspondence address. Tel: +81-3-3353-1211; Fax: +81-3-3353-0249; E-mail: iori71march@hotmail.co.jp/iori71march@a7.keio.jp

Submitted on May 4, 2017; resubmitted on July 1, 2017; accepted on July 11, 2017

STUDY QUESTION: How long is the allowable warm ischemic time of the uterus and what morphological and biochemical changes are caused by uterine ischemia/reperfusion injury in cynomolgus macaques?

SUMMARY ANSWER: Warm ischemia in the uterus of cynomolgus macaques is tolerated for up to 4 h and reperfusion after uterine ischemia caused no further morphological and biochemical changes.

WHAT IS KNOWN ALREADY: Uterus transplantation is a potential option for women with uterine factor infertility. The allowable warm ischemic time and ischemia/reperfusion injury of the uterus in humans and non-human primates is unknown.

STUDY DESIGN, SIZE, DURATION: This experimental study included 18 female cynomolgus macaques with periodic menstruation.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Animals were divided into six groups of three monkeys each: a control group and groups with uterine ischemia for 0.5, 1, 2, 4 and 8 h. Biopsies of uterine tissues were performed before blood flow blockage, after each blockage time, and after reperfusion for 3 h. Blood sampling was performed after each blockage time, and after reperfusion for 5, 15 and 30 min for measurement of biochemical data. Resumption of menstruation was monitored after the surgical procedure. Morphological, physiological and biochemical changes after ischemia and reperfusion were evaluated.

MAIN RESULTS AND THE ROLE OF CHANCE: Mild muscle degeneration and zonal degeneration were observed in all animals subjected to warm ischemia for 4 or 8 h, but there were no marked differences in the appearance of specimens immediately after ischemia and after reperfusion for 3 h in animals subjected to 4 or 8 h of warm ischemia. There were no significant changes in any biochemical parameters at any time point in each group. Periodical menstruation resumed in all animals with warm ischemia up to 4 h, but did not recover in animals with warm ischemia for 8 h with atrophic uteri.

LIMITATIONS, REASON FOR CAUTION: Warm ischemia in actual transplantation was not exactly mimicked in this study because uteri were not perfused, cooled, transplanted or reanastomosed with vessels. Results in non-human primates cannot always be extrapolated to humans.

WIDER IMPLICATIONS OF THE FINDINGS: The findings suggest that the tolerable warm ischemia time in the uterus is expected to be longer than that in other vital organs.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number 26713050. None of the authors has a conflict of interest to declare.

Key words: uterus transplantation / cynomolgus macaque / ischemia and reperfusion injury / warm ischemia / uterine factor infertility

Introduction

Uterus transplantation (UTx) is a potential option for women with uterine factor infertility to have a child. Brännström *et al.* described the first human delivery after UTx with a living donor in 2014 (Brännström *et al.*, 2015) and has now reported six births of healthy babies (Brännström, 2017). This major achievement has stimulated interest in UTx in several countries, but only a few cases have been reported worldwide. Many scientific issues have still to be resolved before UTx has adequate safety and efficacy, and more basic data have to be accumulated for establishment of UTx.

UTx with the objective of delivering a child is not life-supporting organ transplantation and is viewed as a transient transplantation, since the grafted uterus is removed after delivery to avoid rejection and risk due to use of immunosuppressants. Therefore, priority for organ procurement from deceased donors is given to life-supporting organs such as the heart, liver and kidney, with the uterus being a lower priority (Gauthier *et al.*, 2014). Procedures for removal and transport of life-supporting organs are specified based on the allowable ischemic time by organ, however this time is unknown for the uterus. The total ischemic time for an organ is divided into warm and cold ischemic times, with the warm ischemic time being further defined as the first (donor ischemic time: from clamping the vessels to starting to cool the organ) and second (recipient ischemic time: from taking the organ from the back table to starting reperfusion after vascular anastomosis) warm ischemic times (Halazun *et al.*, 2007). Under cold ischemia, tissue damage can be suppressed for a certain time because of decreased tissue metabolism and oxygen demand. However, a prolonged warm ischemic time increases tissue damage and affects subsequent graft survival (Abt *et al.*, 2004; Merion *et al.*, 2006). In cases of living or brain dead donors, the time for vascular anastomosis in the second warm ischemic time is the main contributor to the warm ischemic time and should be minimized as much as possible.

There are few studies of the warm ischemic time of the uterus in animal models. Díaz-García *et al.* indicated that long warm ischemic periods above 5 h have detrimental effects on survival of the uterus after transplantation in a rat model (Díaz-García *et al.*, 2013). In sheep models described by Wranning *et al.*, the warm ischemic time was about 3 h in a sheep that achieved pregnancy after auto-UTx, which indicates that the allowable warm ischemia time of the uterus in sheep is at least 3 h (Wranning *et al.*, 2010; Brännström M, personal communication). In our studies in cynomolgus monkeys, which are similar to humans anatomically and physiologically, we have achieved natural pregnancy and delivery after auto-UTx (Mihara *et al.*, 2012). In this procedure, the warm ischemic time was 3 h 44 min. In UTx in humans performed by Brännström *et al.*, the warm ischemic time in the uterus was 1 h 13 min in the first delivery case after transplantation (Brännström *et al.*, 2015) and an average of 1 h 23 min in seven subsequent cases (excluding two cases with removed uteri) (Brännström *et al.*, 2014). In five human cases performed by a team in Texas, the warm ischemic times in three cases with failed grafts were 60 min, 68 min and 74 min, and those in two cases with regular menstruation after surgery were 40 min and 67 min (Testa *et al.*, 2017).

In our previous evaluation of histopathological changes in warm ischemia of the uterus in cynomolgus monkey, there were no particular histopathological findings in light and electron microscopy after ischemia for up to 4 h; however, dilated nuclear pores and rough endoplasmic reticulum swelling were found in uterine biopsy specimens, together with mitochondrial swelling and cristae loss after warm ischemia for 8 h (Adachi *et al.*, 2016). Periodical menstruation eventually restarted in all animals with warm ischemia up to 4 h, but not in the animal with warm ischemia for 8 h. This result indicates that the uterus of the cynomolgus monkey tolerates warm ischemia for up to 4 h. However, the study was limited to evaluation of each ischemia time in a single animal. Thus, the results are preliminary and further data are required in a large-scale study.

Ischemia-reperfusion injury (IRI) has a major impact on transplantation success, since it increases the rates of acute and chronic rejection (Howard, 1990; Schwarz, 2005). IRI is a multifactorial process including the cytokine/adhesion molecular cascade, generation of reactive oxygen species (ROS), mitochondrial defects, and inflammatory processes involving leukocytes. Acid-base imbalance and electrolyte disturbance, including pH, HCO_3^-, lactate, base excess (BE), potassium and calcium also play roles in IRI (Kosieradzki and Rowiński, 2008; Kim *et al.*, 2013; Li *et al.*, 2015; Fukazawa *et al.*, 2016). Therefore, IRI prevention is critical in organ transplantation, but the details of IRI in the uterus in humans and non-human primates is unknown. Therefore, the aim of this study was to examine the allowable warm ischemic time of the uterus in cynomolgus monkeys and to evaluate morphological, physiological and biochemical changes after ischemia and reperfusion.

Materials and Methods

Animals

Eighteen female cynomolgus monkeys with periodic menstruation aged 5–9 years with body weights of 3.26 ± 0.39 kg were used in the study. The study was performed in accordance with the Guide for the Care and Use

of Laboratory Animals of the National Research Council. The experimental protocols were approved by the Animal Care and Use Committee of our Research Center (2015-1-6).

Anesthesia and operative management

After sedation with i.m. ketamine and xylazine, a tracheal tube was inserted and anesthesia with ventilation was maintained by isoflurane inhalation (0.5–1.5%; Abbott Japan, Tokyo, Japan). All animals received 25 mg/kg of cefazolin every 3 h as antibiotic prophylaxis from initiation of the operation. To compensate for fluid loss during surgery, each animal received continuous intravenous infusion (10–20 mL/kg/h) of Ringer's solution. Antibiotics and buprenorphine hydrochloride were administered during and after surgery to prevent infection and treat pain. An anticoagulant (heparin, 300–500 IU) was administered intravenously 5 min before vascular clamping. Normothermia was maintained throughout the operation using heating lights and blankets.

Surgical procedures

The animals were operated on as previously described (Adachi *et al.*, 2016). Round ligaments were cut and the broad ligaments of the uterus and retroperitoneum were exposed. After confirming that the ureter crossed the uterine artery and vein on the dorsal side, the uterine artery and vein were dissected from the ureter. The vesicouterine ligament and paracolpium were cut and tied while preserving the uterine artery/vein, and then the urinary bladder and rectum were exfoliated from the uterus. With the uterine artery/vein and ovarian artery/vein connected to the uterus, the vaginal canal was cut. The uterus was kept connected with ovarian and uterine vessels alone (Fig. 1A). After each tissue and blood sampling (see below), the disconnected vaginal canal was anastomosed and suture of the retroperitoneum and repair of the round ligament were performed before closing the abdominal incision.

Uterine tissue sampling

The 18 female cynomolgus monkeys were divided into six groups of three monkeys each: a control group and groups with uterine ischemia for 0.5, 1, 2, 4 and 8 h. As described previously (Adachi *et al.*, 2016), vessels were kept open in the control group after vaginal resection and blood flow from the bilateral uterine and ovarian arteries and veins was blocked using two surgical clips for cerebral aneurysm in each vessel (Sugita Clips, Mizuho Co., Japan) in the other five groups for 0.5, 1, 2, 4 and 8 h, respectively (Fig. 1B). Ovarian arteries and veins were clamped at the tubal origin and ovarian ligaments to preserve ovarian function. After the designated blockage time, blood flow was unblocked and the uterus was reperfused. Smooth muscle tissues of all myometrial layers in the corpus uteri of approximately 10 mm^2 were resected with a surgical knife in biopsies performed before blood flow blockage, after each blockage time, and after reperfusion for 3 h. In control animals, biopsies were performed after resection of the vaginal canal and 3 h later. Uterine tissue samples obtained from each animal were stored in 20% formalin for subsequent histopathological and immunohistochemical evaluations (Table I).

Blood sampling

Blood sampling was performed during surgery for arterial blood gas analysis and measurements of lactic acid, anion gap (AG), base excess (BE) and potassium. A unilateral femoral artery and vein were surgically exposed after skin incision of the groin region. Blood sampling from the femoral artery was performed after each blockage time, and after reperfusion for 5, 15 and 30 min for arterial blood gas analysis and biochemical measurements (Table I). Changes in ventilation and administration of drugs were not performed from clamping of vessels until reperfusion for 30 min, during which time the animals were under general anesthesia with stable O_2 saturation, heart rate, blood pressure and temperature.

Indocyanine green (ICG) fluorescence imaging

After all vessels were clamped, indocyanine green (ICG) (Diagnogreen 0.5%; Daiichi Pharmaceutical, Tokyo, Japan) was intravenously administered to confirm the absence of blood flow in the uterus grossly (Fig. 2A). After the designated blockage time and unblocking of blood flow, ICG fluorescence imaging was used to observe reperfusion of the uterus in real time (Fig. 2B).

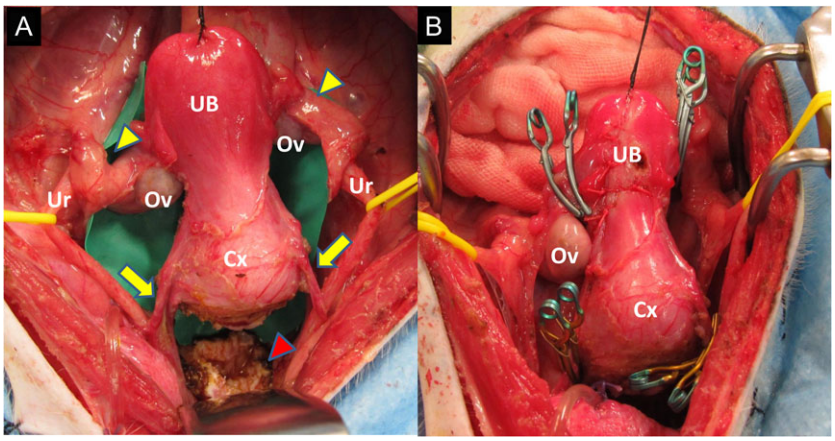

Figure 1 The uterus before and during ischemia. (**A**) Before ischemia. The vaginal canal (red triangle) was cut and the uterus was connected to the pelvis with the bilateral ovarian arteries/veins (yellow triangle) and uterine arteries/veins (yellow arrow) alone. (**B**) During ischemia. Blood flow from the bilateral uterine and ovarian arteries and veins was blocked using two surgical clips. UB, uterine body; Cx, cervix; Ov, ovary; Ur, ureter.

Table I Timing of evaluations performed in the study.

Sample/Method	Evaluation	Pre ischemia (control)	Blockage time (0.5, 1, 2, 4, 8 h)	After reperfusion 5 min	15 min	30 min	3 h
Uterine tissue/histopathological	Hematoxylin-eosin staining	✓	✓				✓
	Caspase staining	✓	✓				✓
Blood/hematological	Biochemical test		✓	✓	✓	✓	
	Arterial blood gas		✓	✓	✓	✓	

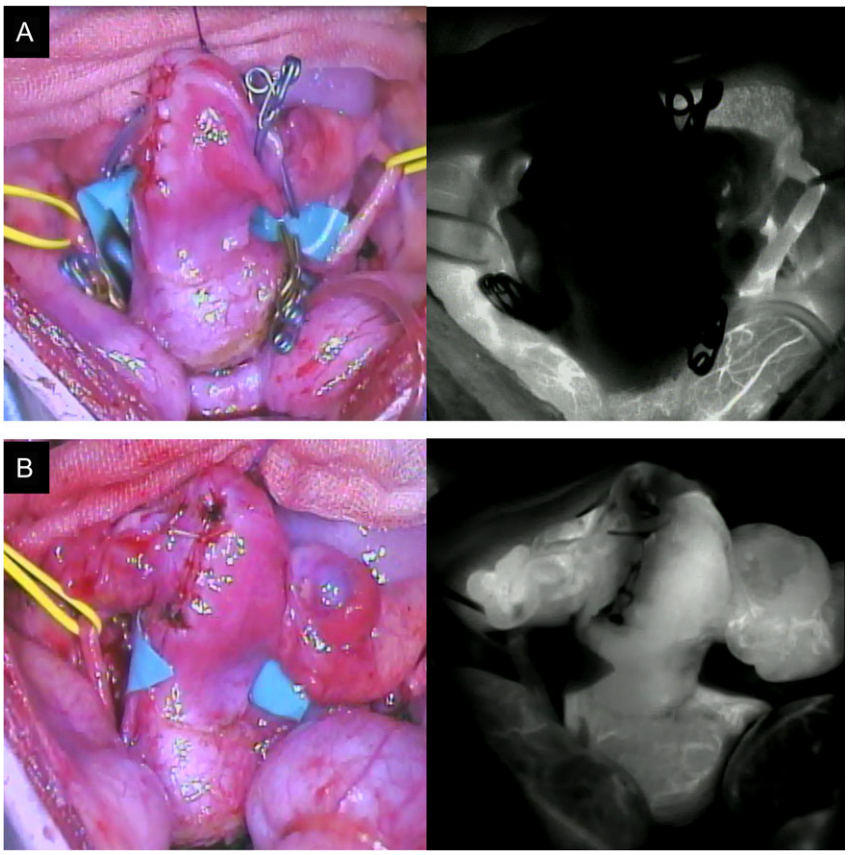

Figure 2 ICG fluorescence imaging of the uterus during ischemia and after reperfusion. (**A**) Enhancement of the uterus was absent during ischemia. (**B**) Recovery of blood flow to the uterus was observed after reperfusion.

Histopathological and immunohistochemical evaluation

Formalin-fixed, paraffin-embedded tissue was sliced at 3 μm and stained with hematoxylin-eosin (HE) for observation by light microscopy. Immunohistochemical staining of the tissue sections was performed using the immunoperoxidase method. Briefly, each section was deparaffinized, rehydrated, and incubated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Antigen retrieval was performed by autoclaving the sections in 10 mM citrate buffer (pH 6.0) for 5 min at 120°C. All sections were incubated for 10 min with normal horse serum to eliminate nonspecific staining, followed by incubation with a primary rabbit monoclonal antibody (diluted at 1:800; Cell Signaling Technology, Beverly, MA, USA) to detect cleaved caspase 3 (CC3). The antibody was applied overnight to the sections at 4°C, followed by incubation with a secondary antibody (ImmPRESS Reagent Kit; Vector Laboratories, Burlingame, CA) for 60 min. Slides were then incubated in diaminobenzidine (DAB)/Tris solution (3 DAB/Tris tablets diluted in 150 mL distilled water; Muto Pure Chemicals, Tokyo, Japan) supplemented with 15 μL of 30% H_2O_2. Finally, the slides were counterstained with hematoxylin. CC3 staining was evaluated in the groups with uterine ischemia for 4 and 8 h, based on our previous finding that the uterus of the cynomolgus monkey can tolerate warm ischemia for up to 4 h.

Biochemical evaluation and blood gas analysis

Biochemical evaluation and arterial blood gas analysis of all blood samples were performed using i-STAT® I Analyzer (Abbott Japan Co., Ltd., Tokyo, Japan) during the operation.

Postoperative observation

After the surgical procedure, the general condition of animals (appetite, bowel movement, vomiting, urination) and the laparotomy wound site were evaluated daily. Resumption of menstruation was also monitored. Uterine size, endometrial thickness and uterine arterial blood flow were evaluated by transabdominal ultrasonography one and two months after the procedure. Animals without menstruation for a long period after surgery or with an obviously atrophic uterus on transabdominal ultrasonography and some animals to be used for other studies underwent a second-look surgery and were then euthanized.

Statistical analysis

Comparisons of biochemical and blood gas data were performed by Kruskal-Wallis test using SPSS ver. 23, with $P < 0.05$ considered significant.

Results

Histopathological findings

There were no marked histopathological findings in HE staining in light microscopy in uterine biopsy specimens of all animals with warm ischemia up to 2 h and after reperfusion for 3 h after each warm ischemic time. In all animals subjected to warm ischemia for 4 h, dilation between smooth muscle bundles and mild muscle degeneration were observed, with weak positive staining for CC3 in narrow areas away from vessels, both after warm ischemia for 4 h and reperfusion for 3 h (Fig. 3). In all animals subjected to warm ischemia for 8 h, zonal and atrophic degeneration of smooth muscles was observed, with positive staining for CC3 after warm ischemia for 8 h and reperfusion for 3 h (Fig. 3). There were no marked differences in the appearance of degenerated smooth muscle of specimens immediately after ischemia compared to that after reperfusion for 3 h in animals subjected to 4 and 8 h of warm ischemia.

Biochemical evaluation and blood gas analysis

Lactic acid, AG, BE and potassium levels from the designated blockage time until after reperfusion for 5, 15 and 30 min in animals with uterine

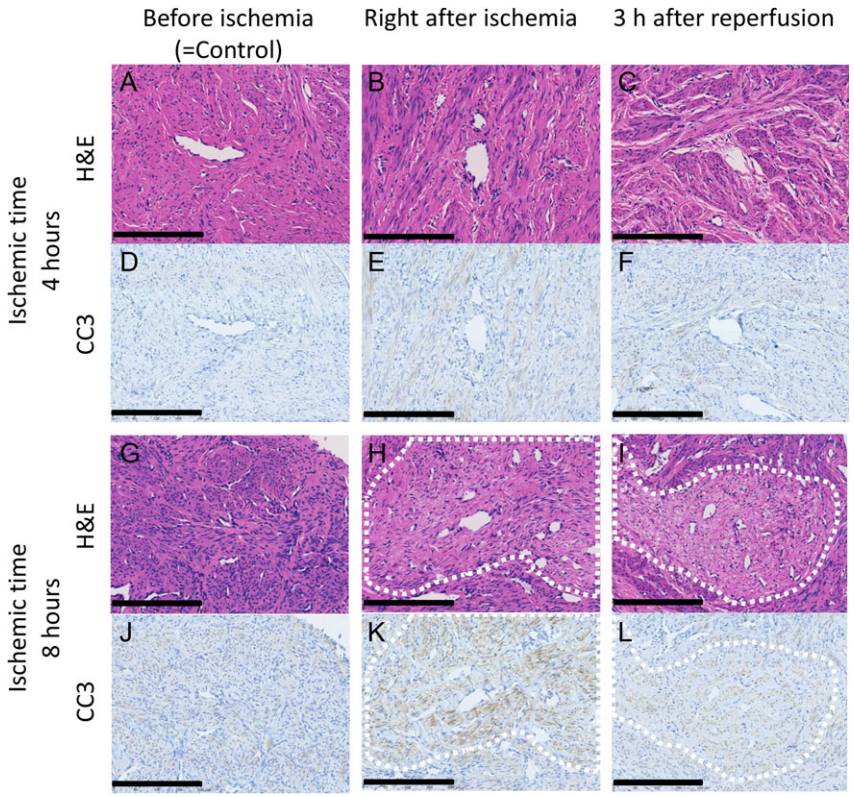

Figure 3 Myometrial biopsy analyses of ischemic effects with hematoxylin-eosin (HE) staining and immunohistochemistry (IHC) for cleaved caspase 3 (CC3) (**A**, **D**) Control specimens with no significant HE or CC3 staining. (**B**, **E**) After 4 h ischemia, HE staining showed dilation between smooth muscle bundles and mild degeneration, and there was weak CC3 staining in narrow areas away from vessels. (**C**, **F**) After 4 h ischemia and 3 h reperfusion, staining was similar to that after 4 h ischemia alone. (**G**, **J**) Control specimens showed no significant HE and CC3 staining. (**H**, **K**) After 8 h ischemia, HE staining showed zonal and atrophic degeneration of smooth muscles, in addition to the findings after 4 h ischemia, and there was positive staining for CC3 (white dots). (**I**, **L**) After 8 h ischemia and 3 h reperfusion, the findings were similar to those after 8 h ischemia alone. (A–L) Bar = 200 μm. (A–C, G–I) HE stain. (D–F, J–L) IHC for CC3.

ischemia for 0.5, 1, 2, 4 and 8 h are shown in Fig. 4. There were no significant changes in any of these biochemical parameters at any time point in each group.

Overall outcome and cyclicity

The general condition of all animals was good for a long period after the surgical procedure. In all animals with warm ischemia up to 4 h, periodical menstruation resumed within three months after surgery. Regular menstruation did not recover in the three animals with warm ischemia for 8 h. Amenorrhea continued in the two of these animals, but irregular menstruation with 2- to 3-month cycles resumed 13 months after surgery in the other animal (Fig. 5). In all animals with warm ischemia up to 4 h, there were normal findings in uteri on transabdominal ultrasonography. In animals with warm ischemia for 8 h, bilateral uterine arterial blood flow was detected by transabdominal ultrasonography at one month after surgery, but atrophic uteri and an unclear endometrium were present. Uterine blood flow was not detected in these animals at two months after surgery. The uterus in the animal with warm ischemia for 8 h in which irregular menstruation resumed remained atrophied on ultrasonography at two years after surgery, but a slight endometrium was detected (Fig. 6).

Macroscopic and histopathological findings in second-look surgery

One animal with warm ischemia for 4 h and two animals with warm ischemia for 8 h in which amenorrhea continued after surgery underwent second-look surgeries. In laparotomy, a uterus with a normal size and mild adhesion to the bladder was found in the animal with 4 h warm ischemia. Atrophic uteri with extensive adhesions to surrounding tissues, especially the bladder and rectum, were found in the two animals with 8 h warm ischemia (Fig. 7). In histopathological findings for uteri removed at autopsy in these animals, no specific findings were observed in the uterine cervix and corpus in the animal with warm ischemia for 4 h, but the uterine lumen of the cervix and corpus was stenosed and focally occluded in the uterine corpus in the two animals with warm ischemia for 8 h. Pseudostratified squamous epithelium in the cervix was thinner and the occlusive area in the corpus showed severe fibrosis and hemosiderin deposition

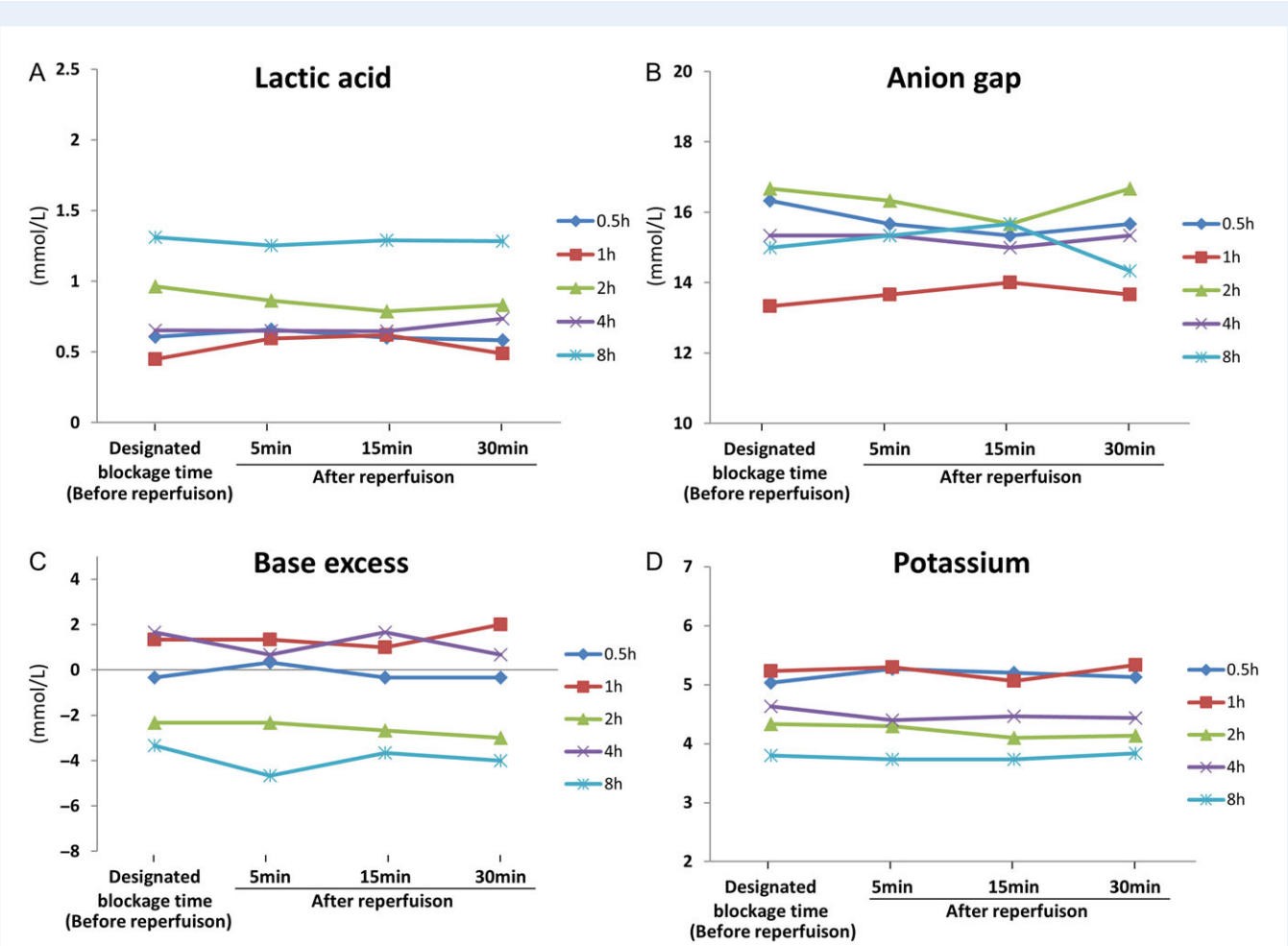

Figure 4 Levels of lactic acid (**A**), anion gap (**B**), base excess (**C**) and potassium (**D**) in groups with uterine ischemia for 0.5, 1, 2, 4 and 8 h from before reperfusion until after reperfusion for 30 min.

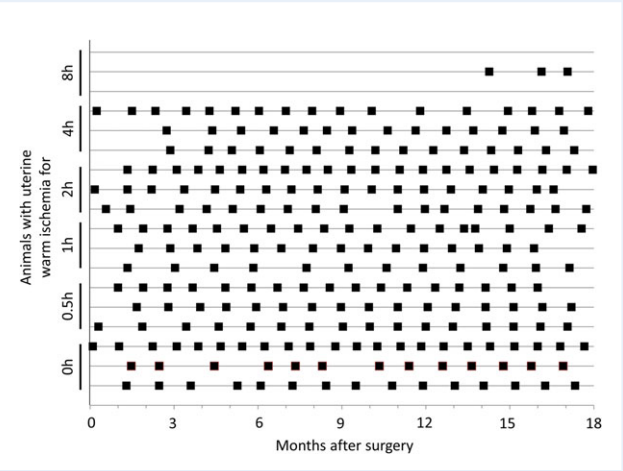

Figure 5 Menstrual bleeding (black boxes) after surgery in all animals with uterine warm ischemia for each indicated time.

without endometrium in the two animals in which menstruation did not recover (Fig. 8).

Discussion

In this study, the effects of warm ischemia time and reperfusion in the uterus on morphological and biochemical parameters were examined in cynomolgus monkeys. To our knowledge, this is the first major report in primates to examine the allowable warm ischemic time of the uterus and to evaluate morphological, physiological and biochemical changes after ischemia and reperfusion.

Warm ischemia causes more serious organ damage than cold ischemia, and the warm ischemic time should be reduced as much as possible in transplantation. The warm ischemia time is prolonged by anastomosis of vessels and the time waiting for a decision on organ donation without organ cooling after cardiac arrest in a non-heart-beating donor. In UTx, two arteries and veins each are anastomosed (Brännström *et al.*, 2014) and this is likely to take longer than anastomosis in kidney and liver transplantation. The optimal warm ischemia times are estimated to be

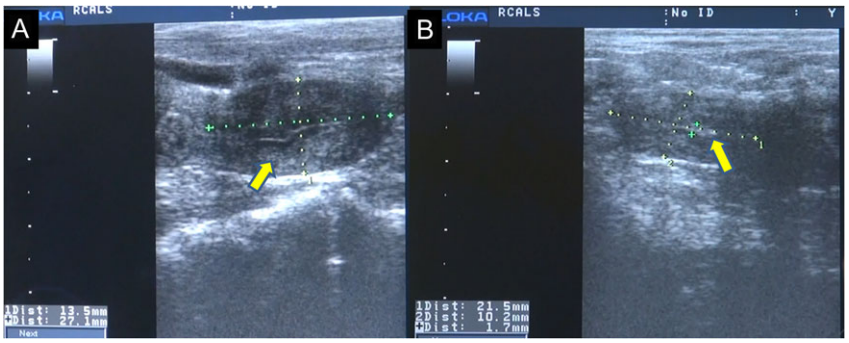

Figure 6 Transabdominal ultrasonography of the long axis of the uterine body. (**A**) Findings in an animal with warm ischemia for 2 h at two months after the procedure. A normal size of the uterine body (27.1 × 13.5 mm: long axis × anteroposterior diameter) and endometrial thickness (yellow arrow) were found, with sufficient uterine blood flow. (**B**) Findings in an animal with warm ischemia for 8 h, in which menstruation resumed irregularly, at 2 years after the procedure. The uterine body (21.5 × 10.2 mm) was atrophic and a thin endometrium (yellow arrow) was detected.

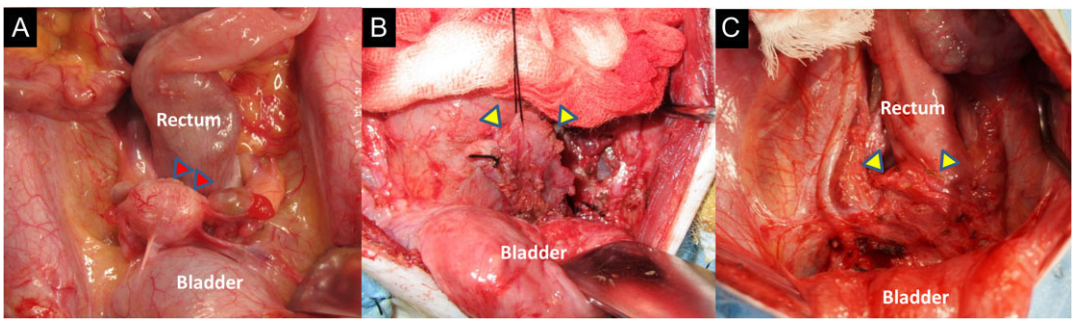

Figure 7 Macroscopic findings in second-look surgery in animals with warm ischemia for 4 h (**A**) and 8 h (**B**, **C**). (A) Normal size uterus (red triangle) with mild adhesion to the bladder. (B, C) An atrophic uterus (yellow triangle) appeared after exfoliation of extensive adhesions to surrounding tissues.

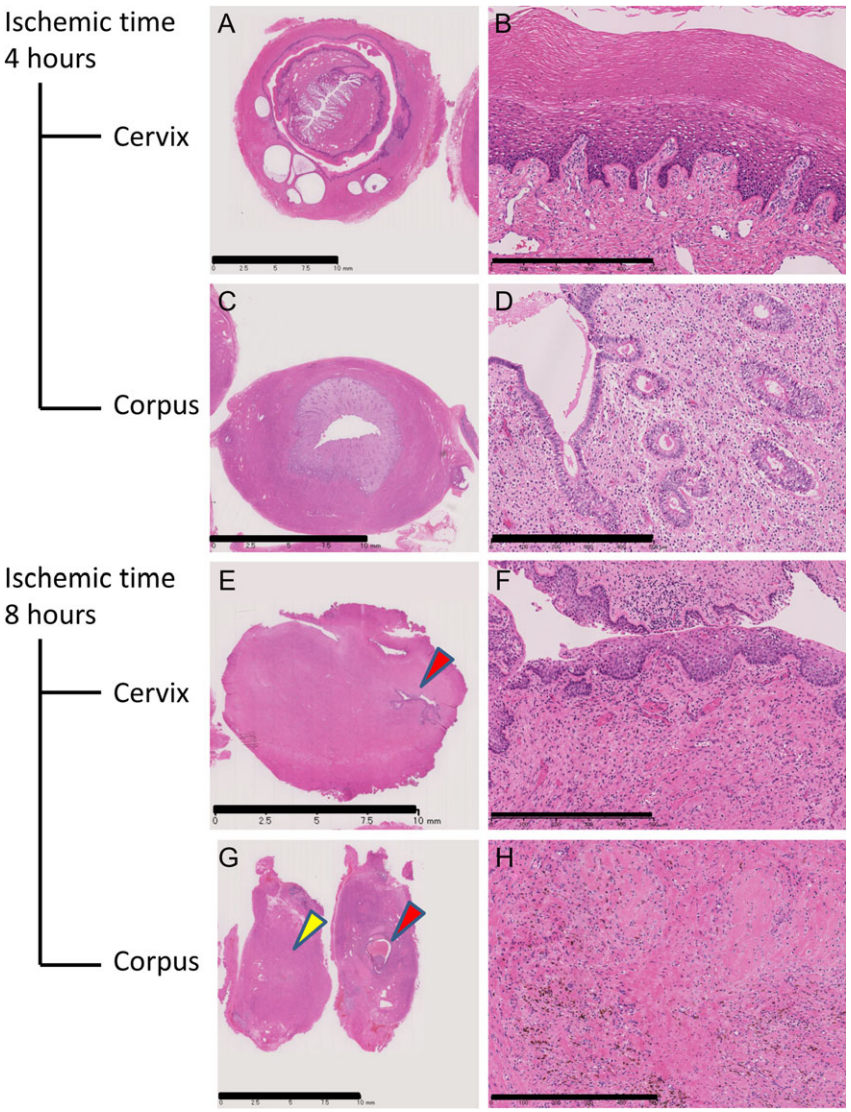

Figure 8 Histopathological findings in HE staining of the uterus in autopsy in animals with warm ischemia for 4 h and 8 h. (**A**–**D**) An animal with warm ischemia for 4 h. There were no specific findings in the uterine cervix (A, **B**) and corpus (**C**, D). (**E**–**H**) An animal with warm ischemia for 8 h and amenorrhea. The uterine lumen was stenosed (red arrowhead) and focally occluded (yellow arrowhead), and the pseudostratified squamous epithelium (**F**) was thin. The occlusive area (H) showed no endometrium, but had severe fibrosis and hemosiderin deposition. (A, C, E, **G**) Bar = 10 mm. Axial section of the uterus. (B, D, F, H) Bar = 500 μm.

less than 15 min for the heart (Fatullayev *et al.*, 2015), 30 min for the kidney (Mir *et al.*, 2016), 30 min for the liver (Nemes *et al.*, 2016) and 60 min for the lung (Van Raemdonck *et al.*, 1998; Baste *et al.*, 2015), whereas the cold ischemia times are less than 6–8 h for the heart (Scheule *et al.*, 2002; Hicks *et al.*, 2006), 8 h for the lung (Hicks *et al.*, 2006; Botha, 2009), 10 h for the liver (Jiménez-Romero *et al.*, 2014; Nemes *et al.*, 2016) and 18 h for the kidney (Barba *et al.*, 2011).

The tolerable warm ischemia time in the uterus is approximately 5 h in rats (Díaz-García *et al.*, 2013) and is expected to be longer than that in other organs (Díaz-García *et al.*, 2013). Brännström *et al.* reported warm ischemia times in humans of about 1 h 30 min (Brännström *et al.*, 2014), but the tolerable warm ischemia time is still unclear. Therefore, we examined warm ischemia times in primates with similar anatomical and physiological features to humans, although results in primates cannot always be extrapolated to humans. Our earlier preliminary study indicated that menstruation restarted in monkeys with a warm ischemia time of less than 4 h, while uterine atrophy was found in those with a warm ischemia time of 8 h, suggesting that the tolerable warm ischemia time was less than 4 h (Adachi *et al.*, 2016). The reproducibility of these results was verified in more monkeys in the current study. No abnormality in the uterus was detected and menstruation restarted in all monkeys with warm ischemia times of less than 4 h, whereas ultrasound results showed uterine atrophy and periodic menstruation did not restart in monkeys after 8 h of warm ischemia.

Histopathological observation of ischemia-induced histological damage in uteri was conducted using biopsy specimens in the preliminary study, but did not identify differences in degeneration between biopsy specimens with different ischemia times. In this study, we focused on regional degeneration based on caspase and HE staining. Slight degeneration was found in regions away from vessels in monkeys with 4 h warm ischemia, and zonal degeneration in the myometrium was seen in monkeys with 8 h warm ischemia. Monkeys with atrophic uteri causing amenorrhea had no unusual general conditions or uterine infection. These results suggest that an atrophic uterus does not induce serious conditions, even if uterus function is lost, similarly to our findings for uteri after rejection in uterine allotransplantation in monkeys (Kisu *et al.*, 2016). Vital organs result in life-threatening conditions if their function is lost, but the uterus is not critical for survival.

In IRI, Na^+-K^+ ATPase activity in the cell membrane is decreased by ischemia, subsequent Na+ retention in the cells causes edema, and ROS generated by hypoxanthine accumulation due to ATP hydrolysis may cause cell dysfunction that is exacerbated by reperfusion (i.e. reperfusion injury) (Laskowski *et al.*, 2000; Kosieradzki and Rowiński, 2008; Chen and Date, 2015). Immunological mechanisms are also involved in IRI (Ioannou *et al.*, 2011). mitochondrial dysfunction-based respiratory metabolic disorder and inflammation caused by free radicals and cytokine production are additionally implicated in IRI, further indicateing the complexity of IRI pathogenesis. To evaluate IRI in uteri of cynomolgus monkeys, histological changes were compared after 4 or 8 h of warm ischemia and after an additional 3 h reperfusion in monkeys, but no differences were found. However, in our preliminary study in cynomolgus monkey, cytological changes in electron microscopy were found after reperfusion in uterine smooth muscle biopsy tissue specimens from animals with warm ischemia for 4 and 8 h, compared with specimens collected before reperfusion (Adachi *et al.*, 2016).

There were also no biochemical changes associated with organ-induced acid-base imbalance or electrolyte disturbance until 30 min after reperfusion. In other organs, metabolic acidosis is caused by reperfusion and adjustment is sometimes required during surgery. The uterus differs from other organs in that it is not involved in metabolism, has a small capacity and is resistant to ischemia, and thus may be unaffected by acid-base imbalance and electrolyte disturbance.

This study has several limitations. First, warm ischemia in actual transplantation is not exactly mimicked by the conditions in the study because the uteri were not perfused, cooled, transplanted and reanastomosed with vessels. Therefore, the warm ischemic time in organ transplantation differs from that defined in this study, although the tolerability of the uterus to warm ischemia was examined. Second, the endometrium of the uterus was not evaluated although Wranning *et al.* showed that the endometrium is more sensitive to cellular damage than the myometrium in cold ischemia-induced changes in human uterine tissue (Wranning *et al.*, 2005). We did not examine the endometrium because we were unable to sample sufficient endometrial tissues for analysis from the very small uterus of cynomolgus monkey and because the endometrium has multiple phases in cyclicity that may lead to complications in evaluation. Third, ATP activity, caspase 3, MDA and GSH were also assayed in uterine biopsy specimens in this study, but the biopsy samples were small and measurement errors were large, which did not allow accurate data to be obtained. Finally, results in non-human primates cannot always be extrapolated to humans for reasons such as the difference in size of the uterus.

In conclusion, warm ischemia in the uterus of cynomolgus monkeys was tolerated for up to 4 h, but not for 8 h; however reperfusion after 8 h of uterine ischemia histopathologically caused no further morphological changes. There were no significant changes in the levels of lactic acid, AG, BE or potassium in ischemia or reperfusion.

Acknowledgements

The authors thank the helpful staff at Research Center for Animal Life Science, Shiga University of Medical Science.

Authors' roles

I.K. was involved in the design, execution and analysis of the study and drafting of the manuscript. K.U., M.A. and Y.N. were involved in the execution of the study. K.E. contributed to the intellectual input in pathology. I.I, I.K., T.N., H.N., A.Y. and H.T. were involved in experimental support in animals. K.B., K.O. and D.A. provided intellectual input and supervision in the overall study design.

Funding

This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number 26713050.

Conflict of interest

None declared.

References

Abt PL, Desai NM, Crawford MD, Forman LM, Markmann JW, Olthoff KM, Markmann JF. Survival following liver transplantation from non-heart-beating donors. *Ann Surg* 2004;**239**:87–92.

Adachi M, Kisu I, Nagai T, Emoto K, Banno K, Umene K, Nogami Y, Tsuchiya H, Itagaki I, Kawamoto I *et al*. Evaluation of allowable time and histopathological changes in warm ischemia of the uterus in cynomolgus monkey as a model for uterus transplantation. *Acta Obstet Gynecol Scand* 2016;**95**:991–998.

Barba J, Zudaire JJ, Robles JE, Tienza A, Rosell D, Berián JM, Pascual I. Existe un intervalo de tiempo de isquemia fría seguro para el injerto renal? [Is there a safe cold ischemia time interval for the renal graft?] (in Spanish. *Actas Urol Esp* 2011;**35**:475–480.

Baste JM, Gay A, Smail H, Noël R, Bubenheim M, Begueret H, Morin JP, Litzler PY. Organotypic lung culture: a new model for studying ischemia and ex vivo perfusion in lung transplantation. *Exp Lung Res* 2015;**41**:564–575.

Botha P. Extended donor criteria in lung transplantation. *Curr Opin Organ Transplant* 2009;**14**:206–210.

Brännström M, Johannesson L, Dahm-Kähler P, Enskog A, Mölne J, Kvarnström N, Diaz-Garcia C, Hanafy A, Lundmark C, Marcickiewicz J *et al*. First clinical uterus transplantation trial: a six-month report. *Fertil Steril* 2014;**101**:1228–1236.

Brännström M, Johannesson L, Bokström H, Kvarnström N, Mölne J, Dahm-Kähler P, Enskog A, Milenkovic M, Ekberg J, Diaz-Garcia C *et al*. Livebirth after uterus transplantation. *Lancet* 2015;**385**:607–616.

Brännström M. Uterus transplantation and beyond. *J Mater Sci Mater Med* 2017;**28**:70.

Chen F, Date H. Update on ischemia-reperfusion injury in lung transplantation. *Curr Opin Organ Transplant* 2015;**20**:515–520.

Díaz-García C, Akhi SN, Martínez-Varea A, Brännström M. The effect of warm ischemia at uterus transplantation in a rat model. *Acta Obstet Gynecol Scand* 2013;**92**:152–159.

Fatullayev J, Samak M, Sabashnikov A, Weymann A, Mohite PN, García-Sáez D, Patil NP, Dohmen PM, Popov AF, Simon AR *et al*. Non-heart-beating donor heart transplantation: breaking the taboo. *Med Sci Monit Basic Res* 2015;**21**:153–156.

Fukazawa K, Vitin AA, Pretto EA Jr. Serum acidosis prior to reperfusion facilitates hemodynamic recovery following liver transplantation. *J Anesth* 2016;**30**:80–88.

Gauthier T, Piver P, Pichon N, Bibes R, Guillaudeau A, Piccardo A, Pesteil F, Tricard J, Gardet E, Laskar M *et al*. Uterus retrieval process from brain dead donors. *Fertil Steril* 2014;**102**:476–482.

Halazun KJ, Al-Mukhtar A, Aldouri A, Willis S, Ahmad N. Warm ischemia in transplantation: search for a consensus definition. *Transplant Proc* 2007;**39**:1329–1331.

Hicks M, Hing A, Gao L, Ryan J, Macdonald PS. Organ preservation. *Methods Mol Biol* 2006;**333**:331–374.

Howard TK. The influence of preservation injury on rejection in the hepatic transplant recipient. *Transplantation* 1990;**49**:103–107.

Ioannou A, Dalle Lucca J, Tsokos GC. Immunopathogenesis of ischemia/reperfusion-associated tissue damage. *Clin Immunol* 2011;**141**:3–14.

Jiménez-Romero C, Caso Maestro O, Cambra Molero F, Justo Alonso I, Alegre Torrado C, Manrique Municio A, Calvo Pulido J, Loinaz Segurola C, Moreno González E. Using old liver grafts for liver transplantation: where are the limits? *World J Gastroenterol* 2014;**20**:10691–10702.

Kim SY, Huh KH, Lee JR, Kim SH, Jeong SH, Choi YS. Comparison of the effects of normal saline versus Plasmalyte on acid-base balance during living donor kidney transplantation using the Stewart and base excess methods. *Transplant Proc* 2013;**45**:2191–2196.

Kisu I, Kato Y, Yamada Y, Matsubara K, Obara H, Emoto K, Adachi M, Umene K, Nogami Y, Banno K *et al*. Clinical features indicating irreversible rejection after uterus allotransplantation in cynomolgus monkey. *Transplantation* 2016;**100**:S457–S458.

Kosieradzki M, Rowiński W. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. *Transplant Proc* 2008;**40**:3279–3288.

Laskowski I, Pratschke J, Wilhelm MJ, Gasser M, Tilney NL. Molecular and cellular events associated with ischemia/reperfusion injury. *Ann Transplant* 2000;**5**:29–35.

Li J, Li RJ, Lv GY, Liu HQ. The mechanisms and strategies to protect from hepatic ischemia-reperfusion injury. *Eur Rev Med Pharmacol Sci* 2015;**19**:2036–2047.

Merion RM, Pelletier SJ, Goodrich N, Englesbe MJ, Delmonico FL. Donation after cardiac death as a strategy to increase deceased donor liver availability. *Ann Surg* 2006;**244**:555–562.

Mihara M, Kisu I, Hara H, Iida T, Araki J, Shim T, Narushima M, Yamamoto T, Moriguchi H, Kato Y *et al*. Uterine autotransplantation in cynomolgus macaques: the first case of pregnancy and delivery. *Hum Reprod* 2012;**27**:2332–2340.

Mir MC, Pavan N, Parekh DJ. Current paradigm for ischemia in kidney surgery. *J Urol* 2016;**195**:1655–1663.

Nemes B, Gámán G, Polak WG, Gelley F, Hara T, Ono S, Baimakhanov Z, Piros L, Eguchi S. Extended criteria donors in liver transplantation Part I: reviewing the impact of determining factors. *Expert Rev Gastroenterol Hepatol* 2016;**10**:827–839.

Scheule AM, Zimmerman GJ, Johnston JK, Razzouk AJ, Gundry SR, Bailey LL. Duration of graft cold ischemia does not affect outcomes in pediatric heart transplant recipients. *Circulation* 2002;**106**:I163–I167.

Schwarz A. Risk factors for chronic allograft nephropathy after renal transplantation: a protocol biopsy study. *Kidney Int* 2005;**67**:341–348.

Testa G, Koon EC, Johannesson L, McKenna GJ, Anthony T, Klintmalm GB, Gunby RT, Warren AM, Putman JM, dePrisco G *et al*. Living donor uterus transplantation: a single center's observations and lessons learned from early setbacks to technical success. *Am J Transplant* 2017. doi:10.1111/ajt.14326. [Epub ahead of print].

Van Raemdonck DE, Jannis NC, De Leyn PR, Flameng WJ, Lerut TE. Warm ischemic tolerance in collapsed pulmonary grafts is limited to 1 hour. *Ann Surg* 1998;**228**:788–796.

Wranning CA, Mölne J, El-Akouri RR, Kurlberg G, Brännström M. Short-term ischaemic storage of human uterine myometrium—basic studies towards uterine transplantation. *Hum Reprod* 2005;**20**:2736–2744.

Wranning CA, Marcickiewicz J, Enskog A, Dahm-Kähler P, Hanafy A, Brännström M. Fertility after autologous ovine uterine-tubal-ovarian transplantation by vascular anastomosis to the external iliac vessels. *Hum Reprod* 2010;**25**:1973–1979.