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Mycophenolate mofetil attenuates uterine ischaemia/reperfusion injury in a rat model

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KEY MESSAGE

Mycophenolate mofetil is a widely used immunosuppressive for organ transplants. Our study additionally revealed its anti-inflammatory, anti-apoptotic and anti-oxidative actions contributing to reduced tissue damage in a uterine ischaemia/reperfusion injury rat model. Its inclusion in uterus transplant induction regimens may help improve clinical outcomes.

A B S T R A C T

This study evaluated the effect of mycophenolate mofetil (MMF) on uterine tissue preservation following ischaemia/reperfusion (I/R) injury. Uterine I/R injury was induced in rats by clamping the lower abdominal aorta and ovarian arteries for 30 min. Group I/R + V (n = 7) received vehicle alone while Group I/R + M (n = 7) received 20 mg/kg/day MMF. Control groups underwent sham surgery and received vehicle (Group C) or 20 mg/kg/day MMF (Group M) (n = 7 for both). Four hours after detorsion, uterine tissue 8-hydroxy-2'-deoxyguanosine (8-OHdG), glutathione, malondialdehyde (MDA), myeloperoxidase (MPO), superoxide dismutase (SOD) and serum ischaemia modified albumin (IMA) concentrations were measured. Histopathological analyses were performed. The I/R + M group showed significant reduction in serum IMA and uterine tissue 8-OHdG, MDA and MPO and significant increase in SOD concentrations compared with the I/R + V group, indicating a protective effect against I/R oxidative damage (P = 0.009, P = 0.006, P = 0.002, P = 0.003 and P = 0.009, respectively). Histopathological evaluation revealed MMF treatment resulted in significantly less tissue and cellular damage and apoptosis compared with the I/R + V group. These results indicate MMF is effective in attenuating uterine tissue damage and preventing apoptosis following uterine I/R injury, probably via anti-inflammatory and anti-oxidative action.

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Introduction

Uterine transplantation is rapidly gaining interest as a new potential alternative to gestational surrogacy for treatment of uncorrectable uterine factor infertility such as congenital absence of the uterus (Mayer–Rokitansky–Küster–Hauser syndrome), previous hysterectomy or severe intrauterine adhesions. This may be especially appealing in many countries where surrogacy is not an option for religious, ethical, social or legal reasons. The first human uterus transplant was performed in Saudi Arabia in 2000 (Fageeh et al., 2002). This attempt failed and the organ had to be removed after 3 months due to prolapse and necrosis. The second attempt at uterine transplantation was performed in Turkey in 2011 from a cadaver donor. The recipient, who had complete Müllerian agenesis, successfully acquired normal menstrual cycles following the transplantation but no live birth was achieved (Ozkan et al., 2013). Recently, a Swedish group has described the world's first live birth after living-donor uterus transplantation (Brannstrom et al., 2015). While this important proof-of-concept study paves the way for uterus transplantation to treat patients with uterine factor infertility around the world, development of effective strategies to minimize allograft dysfunction and rejection would be paramount in optimizing success of this new technology.

Ischaemia/reperfusion (I/R) injury is associated with early allograft dysfunction in organ-transplanted patients. In an experimental rat uterine transplant model, an extended period of ischaemia had detrimental effects on survival of the transplanted uterus (Diaz-Garcia et al., 2013). Previous investigations have shown that I/R injury triggers an inflammatory response with overproduction of reactive oxygen species (ROS) (Liu et al., 2009). These ROS are thought to be central in mediating transplant tissue damage by lipid peroxidation, oxidation of proteins to inactive states and DNA strand breaks, resulting in cell necrosis and apoptosis (Neri et al., 2015). Using immunomodulatory strategies to minimize oxidative stress may lead to attenuation of uterine I/R injury resulting in better clinical outcomes.

Mycophenolate mofetil (MMF) is a powerful immunosuppressive agent that is currently used in organ transplantations such as kidney, liver and heart (Kogiso et al., 2015; Soderlund and Radegran, 2015; Tanriover et al., 2015). MMF is metabolized *in vivo* to mycophenolic acid (MPA). MPA depletes guanosine triphosphate pools in monocytes and lymphocytes and inhibits the de-novo biosynthesis of purins, thereby exerting anti-proliferative effects on these cells (Liu et al., 2009; Ventura et al., 2002). In addition, MPA inhibits the production of cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM). Previous studies have shown that blockade of leukocytes and cell surface adhesion molecules is protective against I/R injury (Haller et al., 1996; Kelly et al., 1994, 1996) and that MMF treatment suppresses the attachment of lymphocytes to ICAM-1, VCAM, E-selectin and P-selectin (Blaheta et al., 1999; Laurent et al., 1996).

As transplanted uteri are subject to I/R injury, the potential protective effects of MMF on uterine I/R injury are of considerable clinical importance. However, to the best of our knowledge there is no study evaluating the impact of MMF on uterine I/R injury. Therefore, the present study aimed to investigate the effects of MMF pretreatment on uterine I/R injury induced by occlusion of distal abdominal aorta and ovarian arteries. Because MMF is known to have antioxidant properties in addition to its immunosuppressive effects in the setting of kidney and liver transplantation (Liu et al., 2009; Ventura et al., 2002), this study also examined its anti-oxidative effects in the uterine I/R model.

Materials and methods

A total of 28 adult female (12-week-old) Wistar albino rats weighing 230–250 g was obtained from Acibadem University Experimental Animal Laboratory. The animals were housed under controlled environmental conditions on a 12-h light/dark cycle with room temperature set at 21 ± 1°C and fed with rat chow *ad libitum*. All experimental procedures and protocols were approved by the Local Animal Ethics Committee of Acibadem University on 10 November 2014 (approval number: 2014/21) and were performed according to the National Health and Medical Research Council guidelines for the care of experimental animals.

Experimental animal model

The rats were randomly assigned into four groups (n = 7/group). Group I (M) underwent a sham surgery and received pretreatment with 20 mg/kg/day MMF (Roche Pharmaceuticals, Basingstoke, UK) once daily dissolved in the drug vehicle (0.5% sodium carboxymethylcellulose); Group 2 (C) underwent a sham surgery and received vehicle alone; Group 3 (IR + V) underwent the uterine I/R procedure and received vehicle alone; Group 4 (IR + M) underwent uterine I/R procedure and received pretreatment with 20 mg/kg/day MMF. All treatments were administered by gavage starting 5 days before surgery and continued until uteri were removed. Doses and administration period of MMF were chosen based on the study by Liu et al. (2009).

All surgical procedures were performed under sterile conditions and general anaesthesia using 60 mg/kg ketamine hydrochloride (Ketasol, Richter Pharma, Austria) and 10 mg/kg xylazine (Rompun, Bayer Healthcare, Germany) intramuscularly. After anaesthesia induction, the surgical field was shaved and disinfected with povidone-iodine solution. A 3-cm midline lower abdominal incision was made and uterine horns, ovaries and distal abdominal aorta were identified. In the sham groups (M, C), distal abdominal aorta and ovarian arteries were dissected but not occluded. In I/R groups (I/R + V, I/R + M), ischaemia was induced by clamping the distal abdominal aorta and ovarian arteries bilaterally with a 20–25 g-pressure microvascular bulldog clamp. The duration of the ischaemia was determined on the basis of a pilot experiment, in which 30 min of ischaemia caused pronounced uterine injury without severe uterine necrosis or death of the animals. After 30 min of uterine ischaemia, the clamps were removed and reperfusion was allowed for 4 h. Following the removal of all clamps, the abdominal incision was closed with a 4–0 silk suture during the reperfusion period. After a 4-h reperfusion period the animals were killed and both uterine horns were harvested. In each animal, one of the uterine horns was collected and stored at −80°C for biochemical analysis. The other horn was transferred into a 10% neutral-buffered formalin solution (10% formaldehyde, 4 g of NaH_2PO_4, 6 g of Na_2HPO_4 in solution of per litre) for histological examination.

Biochemical analyses

Uterine tissue 8-hydroxy-2'-deoxyguanosine (8-OHdG), glutathione (GSH), malondialdehyde (MDA), myeloperoxidase (MPO), superoxide

dismutase (SOD) and serum ischaemia modified albumin (IMA) concentrations were measured.

Tissue 8-OHdG measurement

Tissue samples were collected and genomic DNA was immediately extracted using a commercial PureLink® Genomic DNA Extraction Kit according to the manufacturer's protocol (Invitrogen, USA). DNA concentrations were then measured with nanodrop according to the manufacturer's instructions (Biotek Epoch, USA) and the samples stored at –80°C for determination of 8-OHdG. Measurement of tissue 8-OHdG concentrations was performed via competitive ELISA using a DNA Damage ELISA kit (Cell Biolabs, USA) and detected by absorbance at 450 nm.

Serum IMA measurement

Blood samples were drawn from the jugular vein following the 4-h reperfusion period and allowed to clot for 1 h at room temperature. They were then centrifuged at 2500*g* for 10 min at 4°C. Following centrifugation, sera were separated and stored at –80°C for future analysis of IMA concentrations. Serum IMA concentrations were detected with colorimetric assay using an albumin-cobalt binding method. Serum samples (40 µl) were incubated with 0.1% cobalt(II) chloride (10 µl) for 10 min. After incubation, cobalt(II) binds to amino-acid residues of the N-terminus of albumins. Then 1.5 mg/ml dithiothreitol (10 µl) was added and incubated for 2 min to form a coloured complex with cobalt(II). The reaction was stopped with 0.9% NaCl solution (200 µl) and colour formation was assessed by absorbance at 470 nm using an ELISA reader.

Tissue GSH and MDA measurement

Frozen samples of uterine tissue were homogenized with steel beads in 150 mmol/l KCl buffer and centrifuged at 5000*g* for 10 min at 4°C. The MDA concentrations, reflecting products of lipid peroxidation, were measured by monitoring thiobarbituric acid reactive substance (TBARS) formation as described previously (Buege and Aust, 1978) and results were reported as nmol MDA/mg protein. GSH concentrations were analysed using the modification of the Ellman method (Beutler, 1975). Protein samples were precipitated with metaphosphoric acid and centrifuged at 2000*g* for 10 min. The supernatant was incubated with phosphate-buffered dithiobisnitrobenzoate for 10 min at room temperature. After the incubation absorbance was measured at 412 nm and results were expressed as µmol GSH/mg protein.

Tissue MPO measurement

The MPO activity was measured in uterine tissues using the method described by Hillegass et al. (1990). Uterine samples were homogenized with 50 mmol/l potassium buffer including 0.5% (w/v) hexadecyltrimethylammonium bromide using steel beads. After the homogenization samples were centrifuged at 6000*g* for 10 min at 4°C. The supernatant was collected and sample was incubated for 3 min at 37°C with phosphate buffer containing o-dianisidine and hydrogen peroxide. Enzyme activity was subsequently measured at 460 nm and the results were expressed as U/mg protein.

Tissue SOD measurement

SOD activity was determined using the Mylroie method as previously described (Mylroie et al., 1986). Tissue samples were mixed with potassium phosphate buffer (pH 7.5) containing 0.39 mmol/l riboflavin and 6 mmol/l o-dianisidine-HCl. The mixture was incubated for 8 min under 20 W fluorescent light at 37°C. Following incubation, absorbance was measured at 460 nm and results were expressed as U/mg protein.

Histological analysis

Uterine tissue samples were fixed in 10% formaldehyde solution. After washing with tap water, tissues were dehydrated with ascending ethanol series and cleared with toluene. Following overnight incubation at 60°C, tissues were embedded in paraffin (Leica TP1020). Paraffin tissue sections (5 µm thick) were cut using a rotary microtome (Leica RM2125RT) and mounted on poly-l-lysine slides. The sections were dried overnight at 37°C for light microscopic processes.

All sections were evaluated by a histologist in a blinded manner at ×200 magnification using an Olympus DP72 camera system integrated into an Olympus BX51 light microscope (Olympus, Tokyo, Japan). A semi-quantitative evaluation of the number of infiltrated neutrophils, vasocongestion in endometrial stroma and morphology of glandular cells (disruption of glandular epithelium) were done according to the criteria modified from Davies et al. (2000): 0, none; 1, mild; 2, moderate; 3, severe. Length of epithelial cells was quantified using Image J software (NIH, USA).

Apoptotic cell death was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) technique using the ApopTag® Peroxidase in-situ Apoptosis Detection Kit (EMD Millipore, Darmstadt, Germany). The TUNEL method was applied according to the manufacturer's manual. The slides were counterstained with Mayer haematoxylin, dehydrated in ethanol and cover-slipped with Entellan (Merck, Darmstadt, Germany). Semi-quantitative scoring of TUNEL-positive cells was performed as follows: 0: positive in <5% of the cells, 1: positive in 5–25% of the cells, 2: positive in 26–50% of the cells, 3: positive in >50% of the cells/area.

Electron microscopy

Uterine tissue samples were fixed by immersion in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) (0.1 mol/l, pH 7.2) for 4 h followed by 1% osmium tetroxide in PBS (0.1 M, pH 7.2) for 1 h. Fixed specimens were dehydrated in ascending ethanol series, embedded in Epon 812 (Fluka, Sigma-Aldrich Chemica, Steinheim, Switzerland) and polymerized at 60°C. Semi-thin sections (1 µm) were cut using a diamond knife (Diatome, USA) on a Leica Ultracut R ultramicrotome (Wien, Austria) and stained with toluidine blue. Stained sections were photographed by Olympus BX51 light microscope (Tokyo, Japan). Ultrathin sections (80 – 100 nm) were cut and collected on 200-mesh copper grids (EMS, USA). Grids containing uterine tissues were contrasted with uranyl acetate and lead citrate (Leica EMAC20) and observed under a Jeol 1200 EXII transmission electron microscope. Electron microscopy sections were photographed using the SIS-Morada Soft Imaging System (Olympus, USA).

Statistical analysis

Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) version 17 program (SPSS Inc., Chicago, IL, USA). Distributions of the variables were investigated using the Shapiro–Wilk test. Data with a normal distribution were analysed by one-way ANOVA test while non-normally distributed data were

evaluated by a Kruskal–Wallis test. When overall significance was observed in the ANOVA test, pairwise post-hoc tests were performed using Tukey's test. The Mann–Whitney *U*-test was performed to test the significance of pairwise differences after the Kruskal–Wallis test. $P < 0.05$ was considered statistically significant.

Results

There were no statistically significant differences in serum IMA concentrations, uterine tissue MPO and SOD enzymatic activities or uterine tissue 8-OHdG, GSH and MDA oxidative stress marker levels between MMF and vehicle-treated rats in the sham-operated groups. Uterine I/R injury led to increase in tissue 8-OHdG, MDA, MPO and serum IMA concentrations 4 h after reperfusion (**Figure 1** and **Table 1**). These elevations in 8-OHdG, MDA, MPO and IMA concentrations were significantly attenuated by pretreatment with MMF (I/R + V group versus I/R + M group, $P = 0.006$, $P < 0.001$, $P = 0.003$ and $P = 0.009$, respectively). Moreover, concentrations of the anti-oxidative markers GSH and SOD were decreased following reperfusion in the I/R + V group (**Figure 1** and **Table 1**). The decline in SOD was significantly attenuated by pretreatment with MMF ($P = 0.04$) while no significant difference was noted in GSH concentrations between the I/R + V and I/R + M groups.

Histological examination of uterine tissue of sham groups showed regular endometrial architecture with normal appearing glands and epithelial cell line (**Figure 2a** and **Figure 3a, b, d, e**). In contrast, the uterus of the I/R + V group exhibited large infiltration of neutrophils, vasocongestion and oedema of endometrial stroma with disruption of glandular cells (**Table 1**, **Figure 2b** and **Figure 3g, h**). In the mycophenolate-treated group, less endometrial stroma neutrophil infiltration, oedema and vasocongestion were noted, along with regeneration of endometrial stroma and preservation of glandular cells (**Table 1**, **Figure 2c** and **Figure 3j, k**).

Electron microscopic evaluation of uterine tissue glandular structures demonstrated regular alignment and normal morphology in sham groups (**Figure 3c and f**). In contrast, endometrial glands of the I/R + V group showed degenerative changes, including prominent vacuolization in the cytoplasm of glandular epithelial cells and basal deterioration (**Table 1**, **Figure 3i**). In the mycophenolate-treated group, the glandular epithelial cells displayed cytoplasmic regeneration and less vacuolization compared with the I/R + V group (**Table 1**, **Figure 3l**).

Tissue evaluation of apoptotic cell death revealed that TUNEL-positive cell number was highest in the I/R + V group (**Figure 3g**, **Figure 4** and **Table 1**). The TUNEL-positive cell number was significantly decreased in the MMF-treated group when compared with the I/R + V group ($P = 0.002$) (**Figure 3j**). In sham groups, there were very few TUNEL-positive cells (0 to <5%) (**Figure 3a, d**).

Discussion

The present study investigated whether MMF could mitigate the negative impact of warm ischaemic injury on the uterus using a rat model of uterine I/R. Results from this study demonstrate that pretreatment with MMF confers protection against uterine I/R, as shown by histological findings, significant amelioration of apoptosis, and biochemical markers.

Table 1 – The levels of serum ischaemia modified albumin, uterine tissue 8-hydroxydeoxyguanosine, glutathione, malondialdehyde, myeloperoxidase, superoxide dismutase and histological scores of the experimental groups.

	Sham + V	*Sham + M*	*I/R + V*	*IR + M*	*P-value*
IMA (U/mL)	0.23 ± 0.05 (0.18–0.28)	0.22 ± 0.04 (0.18–0.26)	0.56 ± 0.08 (0.48–0.64)[b,e]	0.41 ± 0.07 (0.34–0.48)[b,e,h]	<0.001
8-OHdG (ng/µg DNA)	1.06 ± 0.12 (0.94–1.17)	1.02 ± 0.19 (0.84–1.20)	1.82 ± 0.40 (1.44–2.20)[b,e]	1.22 ± 0.12 (1.10–1.34)[a,d,h]	0.001
GSH (µmol/mg protein)	2.31 ± 0.35 (1.98–2.63)	2.27 ± 0.21 (2.07–2.48)	1.74 ± 0.23 (1.52–1.96)[b,e]	1.88 ± 0.20 (1.69–2.07)[a,d]	0.001
MDA (mmol/mg protein)	10.53 ± 1.50 (9.14–11.92)	10.67 ± 1.14 (9.61–11.73)	15.96 ± 1.21 (14.84–17.08)[c,f]	12.13 ± 1.14 (11.07–13.19)[j]	<0.001
MPO (U/mg protein)	1.60 ± 0.43 (1.20–2.00)	1.66 ± 0.38 (1.30–2.01)	6.27 ± 2.26 (4.17–8.36)[b,e]	3.05 ± 0.70 (2.39–3.70)[b,e,h]	<0.001
SOD (U/mg protein)	4.95 ± 1.39 (3.66–6.24)	4.30 ± 1.24 (3.15–5.45)	1.98 ± 0.45 (1.56–2.40)[c,e]	3.76 ± 1.30 (2.55–4.97)[g]	0.001
Histological store					
PNL infiltration	0.15 ± 0.19 (–0.01–0.33)	0.28 ± 0.29 (0.01–0.55)	2.75 ± 0.20 (2.56–2.94)[b,e]	1.47 ± 0.32 (1.17–1.77)[b,e,h]	<0.001
Vasocongestion	0.14 ± 0.18 (–0.02–0.31)	0.27 ± 0.29 (0.0–0.54)	2.81 ± 0.19 (2.63–2.99)[b,e]	2.12 ± 0.36 (1.79–2.46)[b,e,h]	<0.001
Disruption	0.05 ± 0.07 (–0.01–0.13)	0.15 ± 0.16 (14.57–15.73)	2.87 ± 0.17 (2.71–3.02)[b,e]	2.02 ± 0.32 (1.73–2.32)[b,e,h]	<0.001
Epithelium length (µm)	14.98 ± 0.73 (14.30–15.66)	15.15 ± 0.62 (14.57–15.73)	40.64 ± 9.70 (31.66–40.62)[b,e]	15.60 ± 1.27 (14.42–16.77)[h]	0.001
TUNEL scoring	0.86 ± 0.37 (0.51–1.21)	0.71 ± 0.48 (0.26–1.17)	2.86 ± 0.37 (2.51–3.21)[b,e]	1.71 ± 0.48 (1.26–2.17)[b,e,h]	<0.001

Data are shown as mean ± SD (95% CI). *P*-values in the column for comparison across all groups.

V, vehicle; M, mycophenolate mofetil; I/R, ischaemia/reperfusion; IMA, ischaemia modified albumin; 8-OHdG, 8-hydroxydeoxyguanosine; GSH, glutathione; MDA, malondialdehyde; MPO, myeloperoxidase; SOD, superoxide dismutase; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labelling.

[a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ versus sham + V (control) group; [d]$P < 0.05$, [e]$P < 0.01$, [f]$P < 0.001$ versus sham + M group; [g]$P < 0.05$, [h]$P < 0.01$, [j]$P < 0.001$ versus I/R + V group.

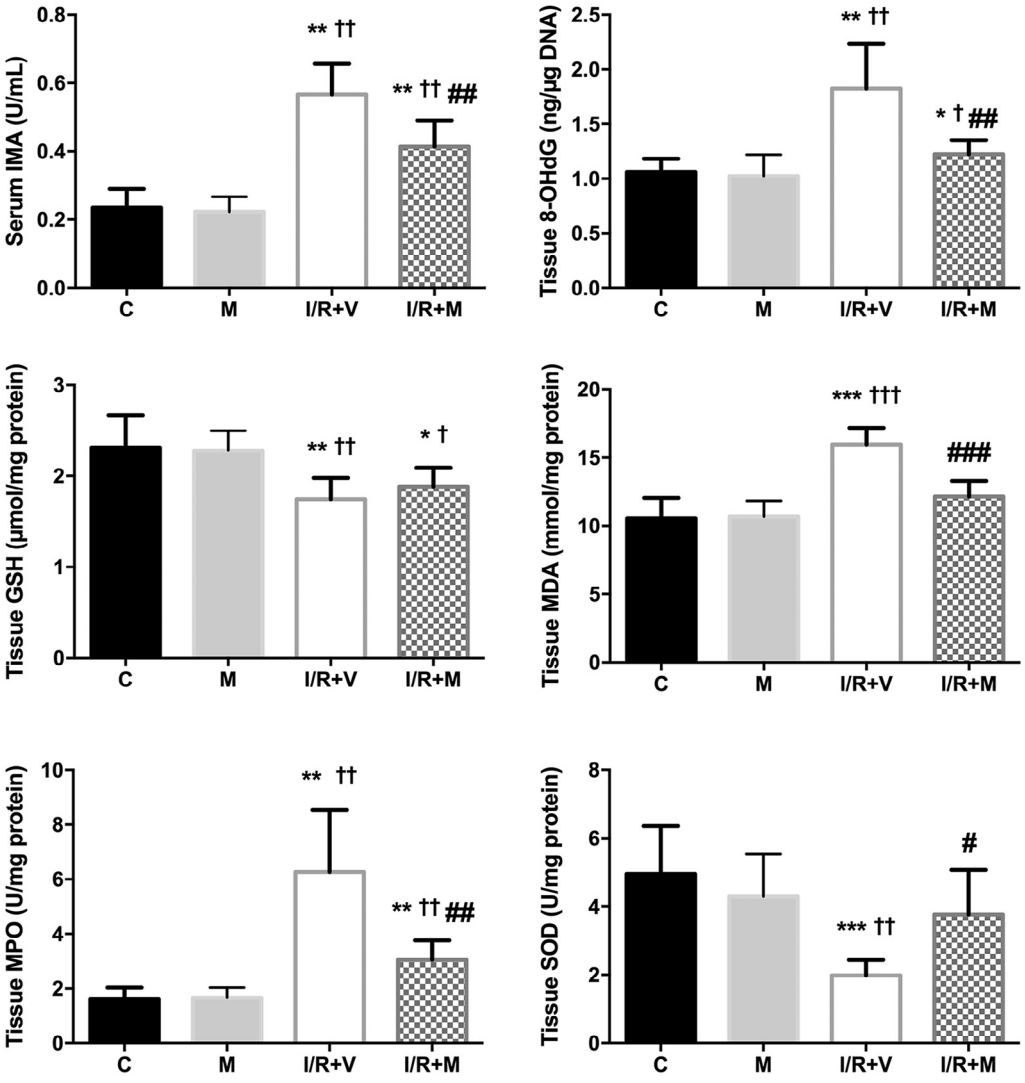

Figure 1 – The concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG), ischaemia modified albumin (IMA), glutathione (GSH), malondialdehyde (MDA), myeloperoxidase (MPO) and superoxide dismutase (SOD), in the sham + vehicle (C), sham + MMF (M), ischaemia/reperfusion + vehicle (I/R + V) and I/R + mycophenolate mofetil (I/R + M) treated groups. Values are expressed as mean ± SD (*n* = 7 rats in each group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus C group; †*P* < 0.05, ††*P* < 0.01, †††*P* < 0.001 versus M group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 versus IR + V group.

I/R injury is associated with increased risk of early allograft dysfunction and failure in solid organ-transplanted patients (Shoskes and Halloran, 1996). In addition, it is also a risk factor for decreased long-term survival of the allograft (Totsuka et al., 2004). Depending on the warm ischaemia period (time between clamping of the vessels until commencing of the cold perfusion and time from end of the cold perfusion until anastomosis is completed), the damage may change from temporary dysfunction of the transplanted organ to graft rejection (Diaz-Garcia et al., 2013; Saikumar et al., 1998). In a rat uterine transplantation model, Diaz-Garcia et al. (2013) showed that prolonged warm ischaemia (>4 h) is associated with reduced survival of the transplanted uterus. The detrimental effects of I/R injury are probably mediated by several mechanisms, including the overproduction of ROS, influx of neutrophils into the transplanted tissue, endothelial dysfunction, microcirculatory disturbances, apoptosis and necrosis (Eickelberg et al., 2002; Kupiec-Weglinski and Busuttil, 2005; Liu et al., 2009). Stimulation of inflammatory cells and endothelium by ROS and pro-inflammatory mediators is a key step in the cascade of I/R injury (Menger and Vollmar, 2000). Following activation, leukocytes adhere to the endothelium and migrate into the allograft (Liu et al., 2009). It is known that blockade of these leukocytes and adhesion molecules can protect the tissue from I/R injury (El-Badry et al., 2007; Kelly et al., 1994). Several studies found that immunosuppressive drugs, which are used to prevent rejection in transplant recipients, also prevent I/R injury in the transplanted organs (Crenesse et al., 2003; Frink et al., 2007; Matsuda et al., 1998).

MMF is an immunosuppressive agent that leads to anti-inflammatory effects via its anti-proliferative action on monocytes and lymphocytes as well as inhibition of the production of cell adhesion molecules. A study of a hepatic I/R model revealed that MMF pretreatment led to decreased numbers of rolling and adherent leukocytes as well as VCAM-1 expression concomitant with reduction in microcirculatory damage and liver cell apoptosis (Liu et al., 2009). Similarly, Ventura et al. (2002) has reported that pretreatment with MMF

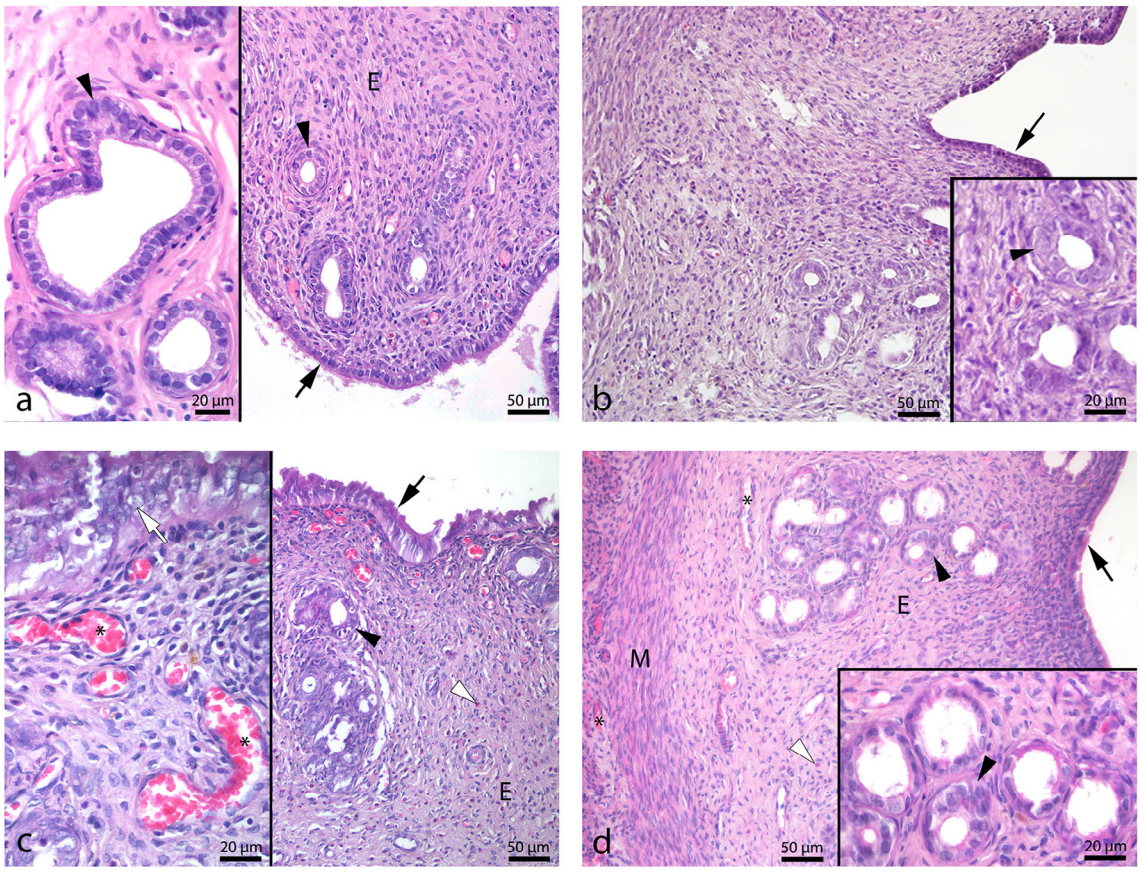

Figure 2 – Histological micrographs of haematoxylin-eosin stained uteri following ischaemia/reperfusion (I/R) injury. (a) Sham + vehicle group. Epithelial cell layer with regular morphology (arrow); normally organized uterine glands (arrowhead); endometrial stroma without cellular infiltrates (E). (b) Sham + MMF group. Epithelial cell layer with regular morphology (arrow); normally organized uterine glands (arrowhead). (c) I/R + vehicle (V) group. Surface epithelium with raised height (black arrow) and increased number of cell layers (white arrow); polymorphonuclear cell infiltration (white arrowhead) into oedematous endometrial stroma (E); congested blood vessels (asterisks); uterine glands showing epithelial cells with cytoplasm loss (black arrowhead). (d) I/R + mycophenolate mofetil (M) group demonstrating abnormally organized surface epithelium (arrow) but less irregularity of uterine glands (black arrowhead), blood vessels with milder vasocongestion (asterisks) and less polymorphonuclear cell infiltration (white arrow) into endometrial stroma (E) compared with I/R + V group; myometrium with regular morphology (M).

resulted in functional protection against renal I/R injury by reducing inflammation. In addition, Farivar et al. (2005) reported that MMF was protective in the setting of lung I/R injury, reducing lung vascular permeability and alveolar leukocyte counts following I/R. This study extends these findings to the uterus, demonstrating that MMF pretreatment results in reduced neutrophil infiltration, stromal oedema and vasocongestion and is protective against degenerative endometrial changes and apoptosis following I/R injury. In addition, previous reports (Henry et al., 2006; Husain and Singh, 2002; Wu et al., 2003) suggest that MMF may have antibacterial and antiviral effects against certain pathogens such as hepatitis C virus, hepatitis B virus and *Pneumocystis jirovecii*. Therefore, antimicrobial activity of MMF, beside its immunosuppressive abilities, may improve the outcomes of patients in the post-transplant period.

Production of ROS following I/R is thought to be central in mediating transplant tissue damage by lipid peroxidation, oxidation of proteins to inactive states and DNA strand breaks, resulting in cell necrosis and apoptosis (Neri et al., 2015). In this study, several biochemical markers were used to evaluate the degree of oxidative damage following uterine I/R injury. MDA concentration, an indicator of ROS-induced lipid peroxidation, and MPO concentration, a marker of neutrophil infiltration, were decreased in the I/R + MMF group compared with the I/R + V group. Similarly, IMA concentrations, which are directly associated with oxygen species-free radicals that are formed during reperfusion injury, were lower in the I/R + MMF compared with the I/R + V group. In addition, the concentrations of 8-OHdG, a well-established biomarker of oxidative deformation in DNA, was significantly lower in the I/R + MMF than the I/R + V group. Moreover, while I/R injury was associated with decreased concentrations of the antioxidants SOD and GSH in the vehicle-treated group, MMF pretreatment resulted in significant increase in SOD concentrations compared with the I/R + V group. These data indicate that MMF pretreatment ameliorated oxidative damage following uterine I/R injury. Consistent with this study's findings, Chauhan et al. (2012) demonstrated that the neuroprotective effects of MMF in the rat experimental ischaemic stroke model were associated with reduction in oxidative damage indices. In addition, Liu et al. (2009) showed that MMF pretreatment led to suppression of ROS production in the rat liver I/R

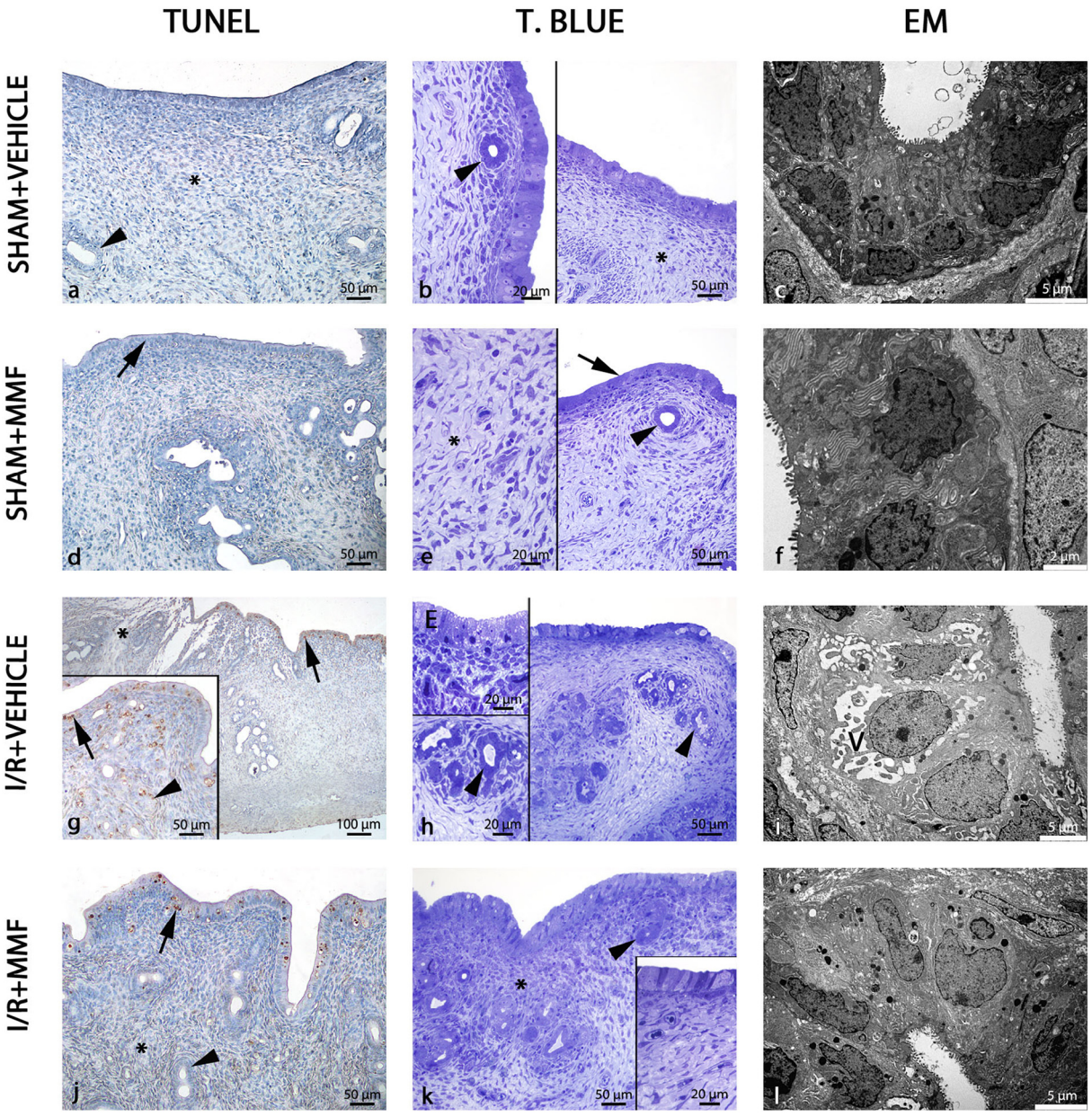

Figure 3 – Micrographs of TUNEL-stained paraffin sections (a, d, g, j) and toluidine blue-stained semi-thin Epon sections (b, e, h, k) at light microscopic level, and contrasted thin sections at electron microscopic level (c, f, i, l) of rat uterine tissue. Control sham groups (a–f); regular contour of endometrial epithelium (arrows), glands (arrowheads) and stroma (asterisks). ischaemia/reperfusion + vehicle (I/R + V) group (g–i) demonstrating numerous TUNEL-positive cells in epithelium (g, arrows) and glands (g, arrowhead) along with glandular degeneration (h, arrowheads). Vacuolization in cytoplasm of endometrial epithelial cells (h, E) and glandular cells was also noted (i, V). I/R + mycophenolate mofetil (M) group (j–l) showing less TUNEL-positive staining in endometrial epithelium (j, arrow) and glands (j, arrowhead), and regular organization of uterine stroma (j and k, asterisks) and glands (k, arrowhead) without cytoplasmic vacuolization (l).

model. Moreover, Treska et al. (2006) reported in a swine renal I/R injury model that MMF pretreatment led to decreased immediate post-transplant ROS and reduction in interstitial inflammation.

Previously, Sahin et al. (2014) evaluated the protective effects of tacrolimus in a rat model of uterine I/R injury and showed that pre-ischaemia administration of tacrolimus decreased uterine MDA concentrations and improved uterine GSH concentrations and catalase activity compared with the I/R only group. In their study, tacrolimus pretreatment led to decreased local inflammatory tissue response and improved uterine histology following I/R injury. Because the combination of tacrolimus and MMF as a pretreatment to prevent kidney I/R injury has been suggested to lead to better protection than either agent alone (Treska et al., 2004), it would be interesting to compare the protective effects of tacrolimus with MMF, alone or in combination, on uterine I/R. In addition, further studies are needed to investigate the molecular mechanisms underlying the protective effects of these agents on the uterus following I/R.

Transplanted organs of different types may vary when it comes to immune response and organ-specific immunosuppression protocols exist. With respect to uterus transplantation and immunosuppression,

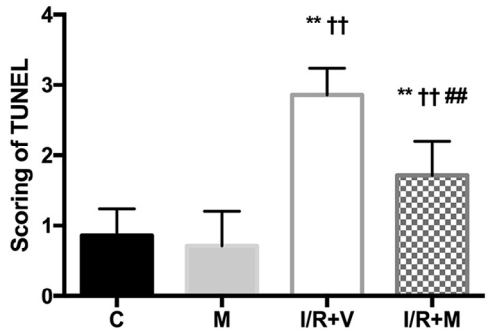

Figure 4 – TUNEL scoring in the sham + vehicle (C), sham + mycophenolate mofetil (M), ischaemia/reperfusion + vehicle (I/R + V) and ischaemia-reperfusion + mycophenolate mofetil (I/R + M) groups. **$P < 0.01$ versus C group; ††$P < 0.01$ versus M group; ##$P < 0.01$ versus IR + V group.

there are several animal studies, also involving non-human primates and a few human cases (Brannstrom et al., 2015; Kisu et al., 2014; Wei et al., 2013). These studies have provided information about allogenic uterine transplantation techniques and immunosuppression protocols. Tacrolimus as monotherapy in allogeneic uterine transplant in rats was shown to be sufficient to avoid graft rejection during pregnancy (Diaz-Garcia et al., 2010). In large animals, allogeneic uterine transplant in sheep was performed with cyclosporine for immunosuppression, and pregnancy and live birth were reported (Ramirez et al., 2011). Others used an induction protocol consisting of anti-thymocyte globulin, prednisolone, tacrolimus and MMF, which is similar to the protocols used to prevent acute rejection after hepatic, renal and cardiac transplantation in humans (Wei et al., 2013). In their study, they successfully performed allogenic uterine transplantation with this treatment protocol in sheep. In a study of uterine transplant in a swine model, tacrolimus was used followed by cyclosporine plus methylprednisolone, reporting long-term survival and graft function in 50% of the animals (Avison et al., 2009). In another study of uterine transplantation in monkeys, it was suggested that the lack of administration of MMF may have caused failure to overcome acute rejection (Kisu et al., 2014). With regard to human cases, 11 human uterus transplantation attempts have been reported thus far (Brannstrom et al., 2015; Fageeh et al., 2002; Ozkan et al., 2013). In the first reported case, immunosuppression was maintained by oral cyclosporine, azathioprine and prednisolone, but the transplant underwent progressive necrosis (Fageeh et al., 2002). In the second case (Ozkan et al., 2013), as well as in the clinical trial of nine women which recently reported the first live birth after transplantation (Brannstrom et al., 2015), induction immunosuppression including anti-thymocyte globulin and methylprednisolone, and maintenance immunosuppression including tacrolimus and MMF, were used.

It is well known that protection of harvested organs against ischaemia until transplantation is crucial. Cold preservation solutions lessen ischaemic damage in the transplanted uterine tissue (Wranning et al., 2006, 2008). However, this study did not include a comparison of MMF with temperature reduction, which is also known to prolong survival of tissues subjected to ischaemia. Future studies should evaluate whether temperature reduction, alone or in combination with agents such as MMF, may provide added protection to the uterus in the setting of I/R injury.

In conclusion, this study suggests that MMF may have protective anti-inflammatory and anti-oxidative effects on the uterus in the setting of I/R injury. Further studies are warranted to assess whether its inclusion in uterus transplant induction regimens may help improve clinical outcomes of this procedure in the future.

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