Author's response to reviews

Title: Mycophenolate Mofetil and FK506 Have Different Effects on Kidney Allograft Fibrosis in Rats that Underwent Chronic Allograft Nephropathy

Authors:

Lei Luo (luolei7710@yahoo.com.cn)
Lin Zhao Sun (szl7710@hotmail.com)
Dong Wei Wu (weidw@163.com)
Heng Guang Luo (luoguangheng1975@hotmail.com)

Version: 3 Date: 7 June 2012

Author's response to reviews: see over
June 7 2012

Dear Dr Hayley,

We wish to resubmit our manuscript entitled “Mycophenolate Mofetil and FK506 Have Different Effects on Kidney Allograft Fibrosis in Rats That Underwent Chronic Allograft Nephropathy” for consideration for publication in BMC Nephrology.

We thank you for giving us the opportunity to address the reviewers’ comments. Our responses are outlined below. We have reformatted the manuscript and it is now compatible with the requirements of the BMC Nephrology. We have enlisted the help of professional copy editors to ensure that the language requirements have been met.

Finally, we declare that this paper reflects original unpublished work, which has not been submitted to any other journal. None of the authors have any competing financial interests to declare.

We hope that the manuscript now meets the high standards required by BMC Nephrology. We look forward to hearing from you at your earliest convenience.

Yours sincerely,

Professor Guangheng Luo
Department of Urology Surgery
Guizhou Province People’s Hospital, Guiyang 550002, PR China
E-mail: luoguangheng1975@hotmail.com
Responses to reviewer’s comments

REVIEWER #1

1. Provide a better summary paragraph on the natural history of CAN in the Introduction

We have provided a summary paragraph about CAN in the introduction as follows below:

“Despite improvements in immunosuppressive protocols, long-term survival of allografts remains a major impediment [1]. Research has demonstrated that more than 50% of all renal transplants have graft failure, ultimately due to end-stage renal failure [2]. This process of renal failure was formerly known as chronic allograft nephropathy (CAN), which was originally coined as a histological grading of the extent of interstitial fibrosis (IF) and tubular atrophy (TA) present in biopsies in 1991. Moreover, other histological characteristics can also be involved in CAN, including transplant vasculopathy and glomerulopathy [3,4]. A significant amount of CAN histological damage can appear from as early as a median of 3 months post-transplant, and the condition is progressive [5]. The incremental IF/TA from immunological and non-immunological causes eventually leads to chronic graft dysfunction [4].”

Although the concept of CAN has disappeared in the recent Banff criteria, our study was based on a previous clinical observation of inhibition of CAN by MMF treatment, and therefore, we used the original concept and criteria for CAN.

References


2. In the methods you need to describe the model not just refer to the publication

We have added a description of the model to the Methods section. In brief, the donor was anesthetized by intraperitoneal injection of urethane (1.0 mL/100 g body weight). The donor left kidneys were isolated and preserved in 0°C to 4°C heparin sodium chloride solution for 1 h to reinforce the cold ischemic injury before transplantation. After left native nephrectomy was performed in male Lewis recipient males, the donor renal artery were anastomosed to the aorta of the recipient using interrupted 9-0 nylon sutures. The donor vein was attached to the inferior vena of the recipient using the cuff technique. Right native nephrectomy was performed on the 10th postoperative day.

3. In methods please state clearly the criteria upon which you decided whether the allograft surgery was valid

We have added the criteria that we used to decide whether the allograft surgery was valid in the Methods as follows below:

“1. When vascular anastomosis is completed, the transplant kidneys immediately become red after circulation returns, with a certain degree of flexibility and hardness. There is no anastomotic bleeding, the renal artery beats well, and there is no renal vein distortion and no congestion. There is visible ureteral peristalsis and urine outflow in the ureteral orifice after 2-3 minutes.”
2. The rats survive for more than 3 days after transplantation.”

4. For E-cadherin and Trichrome results you report results as (p<0.05) How you quantified these end-points is missing from the methods and results

We have added the methods and results of immunohistochemistry quantification as follows below.

1. Methods

The images were captured by a Nikon spot cool CCD. The staining results were analyzed using the image-Pro Plus multimedia color pathological image analysis system.

2. Results

Table 1. IOD values of α-SMA in each group at 4, 8 and 12 w post-transplantation ($\times 10^4$)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>FK506</th>
<th>MMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 w</td>
<td>4.6±0.3</td>
<td>4.8±0.3</td>
<td>4.3±0.4</td>
</tr>
<tr>
<td>8 w</td>
<td>9.1±0.9</td>
<td>8.5±0.7</td>
<td>6.1±0.4*</td>
</tr>
<tr>
<td>12 w</td>
<td>19.6±2.4</td>
<td>20.9±1.8</td>
<td>14.2±1.1*</td>
</tr>
</tbody>
</table>

Table 2. IOD values of E-cadherin in each group at 4, 8 and 12 w post-transplantation ($\times 10^4$)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>FK506</th>
<th>MMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 w</td>
<td>18.1±2.2</td>
<td>17.3±2.1</td>
<td>21.6±1.9</td>
</tr>
<tr>
<td>8 w</td>
<td>7.6±0.6</td>
<td>7.0±0.9</td>
<td>13.5±1.2*</td>
</tr>
<tr>
<td>12 w</td>
<td>4.2±0.9</td>
<td>4.9±0.6</td>
<td>9.1±1.1*</td>
</tr>
</tbody>
</table>

Table 3. IOD values of collagen deposition in each group at 4, 8 and 12 w post-transplantation ($\times 10^4$)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>FK506</th>
<th>MMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 w</td>
<td>6.7±0.5</td>
<td>7.1±0.3</td>
<td>5.2±0.4*</td>
</tr>
<tr>
<td>8 w</td>
<td>12.1±2.1</td>
<td>12.5±2.6</td>
<td>8.2±0.9*</td>
</tr>
<tr>
<td>12 w</td>
<td>25.6±2.9</td>
<td>24.2±3.1</td>
<td>11.2±1.1*</td>
</tr>
</tbody>
</table>
5. E-cadherin in the rat marks out the distal tubule. Therefore all images for E-Cadherin should be taken from the cortex and should include a glomerulus in the field to authenticate this and should also clearly show that the staining is occurring in cells with the typical bulging nuclear morphology of the distal tubule.

We have re-taken the images for E-cadherin in the cortex and have included a glomerulus in the field, which showed that staining occurred in cells, with typical bulging nuclear morphology of the distal tubule.

6. In the discussion

-Provide more of a comment on how the MMF group retains such a comparatively favourable functional picture at 12 weks despite massive histopathological damage

In our study, the histopathological damage (Banff score) was evaluated by glomerular lesions, intimal proliferation lesions, tubular atrophy, interstitial fibrosis and interstitial infiltration of plasma cells.

Renal function was determined by serum creatinine (Scr) levels. Scr changes are mainly determined by glomerular filtration capacity (glomerular filtration rate). The creatinine concentration increased when filtration capacity decreased. Scr was an accurate indicator of renal parenchymal damage, but not a sensitive one. This is because serum creatinine levels significantly increased only when the glomerular filtration rate was reduced to 1/3 of normal levels. In our study, there was massive histopathological damage (Banff score) in the MMF group, but HE and Masson’s trichrome staining results showed that parts of the glomeruli remained functional, and this might be the reason why Scr was maintained at a relatively low level.

-Do not refer to EMT as if it were a 100% proven phenomenon. This is a theory for which the evidence base is increasingly being critiqued. There is no need to base your results around MMF inhibiting EMT.

Our study aimed to determine whether MMF could ameliorate the process of kidney fibrosis in an experimental CAN rat model. EMT might be an important mechanism
in the tubular fibrosis process. However, as commented by the reviewer, since EMT was not a 100% proven phenomenon, we have revised related content in the manuscript.

-You need to be very careful about how you link cause and effect. For example you say on page 8 "MMF effectively prevented fibrosis in kidney allografts by reducing the expression of CTGF..." and on page 9 you say "FK506 dramatically upregulated the expression of fibrosis related genes...."

We appreciate the reviewer’s suggestion. We have revised the sentences as follows: "MMF could effectively prevent fibrosis in kidney allografts, possibly by reducing the expression of CTGF, α-SMA and collagen...." and "FK506 significantly up-regulated the expression of α-SMA and collagen, and down-regulated the expression of E-cadherin...."

-You have not proven this direct cause-effect relationship in either. In the former there is is an association. In the latter in fact FK506 and Vehicle are not significantly different in CTGF mRNA for example. The model therefore is the primary driver of upregulation of gene expression.

In the current study, the transplantation recipients were divided into the vehicle group, the FK506 group and the MMF group.

1) In the (vehicle) placebo group, the recipient rats were no longer treated with immunosuppressive agents after 10 days of CsA treatment. This may have caused cellular and humoral mediated graft immune rejection, leading to an increase in CTGF expression and chronic graft damage, which resulted in CAN development in recipient rats.

2) In the FK506 group, FK506 prevented immune rejection by blocking the post-receptor signal transduction of interleukin-2 (IL-2) by interacting with a family of intracellular binding proteins termed immunophilins FKBP5 [1]. However, the use of FK506 can cause severe renal toxicity, which can enhance renal vascular resistance, reduce renal blood flow and glomerular filtration rate, and increase serum creatinine.
levels. In addition, FK506 inhibits cellular degranulation and apoptosis, blocks the activation of nitric oxide synthetase, and potentiates the cellular effects of steroids. In the current study, these processes could also have induced the up-regulation of CTGF expression, which resulted in CAN development in recipient rats.


On page 9 you state the following "Both alph-SMA and E-cadherin are fundamental extracellular matrix proteins" This is fundamentally factually inaccurate. Revise all such statements

We have revised all such statements as suggested. E-cadherin and α-SMA are extracellular matrix proteins that are correlated with the EMT process.

Reviewer #2

1. Abbreviations (CTGF, α-SMA, HE, PAS) should be defined

The abbreviations (CTGF, α-SMA, HE, PAS) have been defined in the abstract as follows below:

CTGF: Connective tissue growth factor; α-SMA: alpha smooth muscle actin; HE: hematoxylin-eosin; PAS: Periodic acid-Schiff

2. Mechanism of action of MMF and FK506 must be clearly defined.

Mechanism of action of MMF and FK506 have been clearly defined in the
Mycophenolate mofetil (MMF) is the first pharmaceutical prodrug of mycophenolic acid (MPA) and is used in the prevention of acute rejection. MPA inhibits the activity of inosine 5'-monophosphate dehydrogenase (IMPDH, EC1.1.1.205), the rate-limiting enzyme in the de novo pathway of guanosine nucleotide synthesis. The pharmacological inhibition of IMPDH by MPA is considered to cause the depletion of guanosine nucleotides, leading to the suppression of cell proliferation in activated lymphocytes and a decrease in the expression of adhesion molecules.

Similar to cyclosporine, tacrolimus (FK506) is a CNI and acts primarily by interfering with T-cell activation. Tacrolimus binds to a corresponding protein, FK506-binding protein 12 (FKBP12). These tacrolimus-immunophilin complexes inhibit the activity of calcineurin, a calcium-dependent phosphatase. Inhibition of calcineurin impedes calcium-dependent transduction and inactivates transcription factors, particularly NFAT. NFAT is believed to initiate gene transcription for the formation of lymphokines, such as interleukin-2, interferon-c, and others. The clinical result of this NFAT inhibition is immunosuppression.

In addition, tacrolimus inhibits cell degranulation and apoptosis, blocks the activation of nitric oxide synthetase, and potentiates the cellular effects of steroids.

References


3. Material and Methods: The study design is reasonable; however there might be a MMF+FK506 group in order to clarify the combined effect. (It’s recommended to write it in limitation of this study.)

We thank the reviewer for the recommendation. We have mentioned that the lack of a MMF+FK506 group is a limitation of this study in the Discussion.

4. In page 13 the sentences starting “Chronic histological changes were graded………” needs some detailed information about histological changes.

In our study, the definition of chronic histological changes was quantitatively and qualitatively evaluated by glomerular lesions, intimal proliferation lesions, tubular atrophy, interstitial fibrosis and interstitial infiltration of plasma cells. The criteria score was as follows: 0, no lesions; 1, mild lesions; 2, moderate lesions; and 3, severe lesions.

5. The spelling and grammar throughout the entire manuscript needs some attention.

We have enlisted the help of professional copy editors to ensure that the language requirements have been met.

REVIEWER #3

1. Please write out in full mycophenolate mofetil in the title.

We have written out in full “mycophenolate mofetil” in the title. The title is: “Mycophenolate Mofetil and FK506 Have Different Effects on Kidney Allograft Fibrosis in Rats That Underwent Chronic Allograft Nephropathy”.

2. Similarly, please clarify abbreviations in the abstract, e.g. CTGF, #-SMA, PAS, HE
The abbreviations (CTGF, α-SMA, HE, PAS) have been defined in the abstract as follows below:

**CTGF**: Connective tissue growth factor; **α-SMA**: alpha smooth muscle actin; **HE**: hematoxylin-eosin; **PAS**: Periodic acid-Schiff

3. In the introduction, the authors state: “In our previous study, we observed that FK506 up-regulated the expression of TGF-#1 and Smad2 in grafts, and down-regulated the expression of Smad7, while MMF had opposite effects.”. Please add a reference.

We have added a reference to the Introduction:


4. According to the BMC Nephrology website a Background section should be followed by a Methods section. The numbers of the sections appear to be incorrect as section 5. Materials and Methods starts with subsection 2.1 Animals.

We have corrected the numbers of the sections.

5. Each treatment group consisted of 9 rats. The rats were sacrificed to harvest the renal allografts at 4, 8 and 12 weeks post-transplantation. Does that mean each time point consisted of only 3 rats? Please clarify.

Yes, this means each time point consisted of only 3 rats. We have clarified this in the Methods.

6. Discussion, page 9, ........CAN and EMT, however, with no statistical significant difference.

We have corrected the sentence to “..........CAN and EMT; however, with no significant difference.”