Rapamycin attenuates the severity of established nephritis in lupus-prone NZB/W F₁ mice

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Abstract

Background. Rapamycin is a potent immunosuppressive drug with proven efficacy in rejection prophylaxis in solid organ transplantation. By virtue of its immunosuppressive properties, rapamycin might also be useful in the treatment of autoimmune diseases. The aim of this study was to determine the effect of rapamycin on the severity of established nephritis in lupus-prone New Zealand Black/White F₁ (NZB/W F₁) mice.

Methods. Six-month-old female NZB/W F₁ mice with active nephritis (albuminuria > 100 mg/dL) were treated with rapamycin (3 mg/kg body weight) or saline once daily by oral gavage for 4 months. The effect of rapamycin on the severity of nephritis was evaluated by clinical manifestations, biochemical parameters, renal histology, immunohistochemistry and semi-quantitative gene expression studies.

Results. Treatment with rapamycin significantly decreased albuminuria, improved survival, diminished splenomegaly, preserved renal function and reduced serum anti-dsDNA antibody levels. Kidney sections from saline-treated mice revealed marked mesangial proliferation, tubular dilation with intra-tubular protein cast deposition and leukocytic infiltration of the interstitium. The rapamycin-treated mice, in contrast, had relatively mild histological changes in their kidneys. Rapamycin treatment also significantly reduced the amount of immune complex deposition in the glomeruli, suppressed the interstitial infiltration by T-cells, B-cells and macrophages as well as down-regulated the intra-renal expression of RANTES.

Conclusions. We conclude that rapamycin is effective in attenuating the severity of established nephritis in NZB/W F₁ mice. The beneficial effects of rapamycin are mediated, at least in part, through inhibition of lymphoproliferation, reduced RANTES expression and decreased inflammatory cell infiltration in the kidneys. Rapamycin could be of therapeutic value in the treatment of human lupus nephritis.

Keywords: autoimmunity; lupus nephritis; NZB/W F₁ mice; rapamycin

Introduction

Systemic lupus erythematosus (SLE) is a relatively common systemic autoimmune disease which predominantly affects young female individuals [1,2]. Renal involvement or lupus nephritis is one of the most important complications of SLE and might lead to the development of acute or chronic renal failure [3]. Patients with lupus nephritis are usually being treated with immunosuppressive drugs such as corticosteroids, cyclophosphamide, azathioprine and more recently mycophenolate mofetil [4,5]. A certain proportion of patients with severe lupus nephritis, however, might fail to respond to the immunosuppressive therapies currently available. Moreover, some patients might not be able to tolerate the immunosuppressive drugs because of their side effects. It is therefore still necessary to develop newer agents to supplement our armamentarium for the treatment of lupus nephritis.

Rapamycin is a macrolide antibiotic with potent immunosuppressive properties [6]. It acts by inhibiting mammalian target of rapamycin (mTOR), a key protein kinase necessary for cell cycle progression, thereby suppressing the proliferation and clonal expansion of interleukin-2-stimulated T lymphocytes [7]. At present, the main clinical application of rapamycin is for the prevention of acute rejection in solid organ transplant recipients [8].

The New Zealand Black/White F₁ (NZB/W F₁) mouse is a commonly used experimental model of SLE. This strain of mouse spontaneously develops an autoimmune disease at the age of 5–6 months, which is characterized by splenomegaly, lymphadenopathy, autoantibody production and immune-complex-mediated glomerulonephritis [9,10].
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Death as a result of renal failure usually occurs by the age of 10–12 months. By virtue of its immunosuppressive properties, rapamycin might be useful in the treatment of autoimmune diseases. Rapamycin has been shown to be effective in preventing the development of nephritis in the lupus-prone MRL/lpr mice [11] and the NZB/W F1 mice [12]. However, the therapeutic effect of rapamycin on established nephritis in these murine lupus models has not been fully studied. The aim of this study was to determine the effect of rapamycin treatment on the severity of established glomerulonephritis in the NZB/W F1 mice.

Materials and methods

Mice

Female NZB/W F1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained at the Laboratory Animal Unit of the University of Hong Kong under standard conditions with a 12-h light and dark cycle and were allowed access to water and chow ad libitum. All animal protocols were reviewed and approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong.

Drug

Oral liquid formulation of rapamycin (1 mg/mL) was supplied by Wyeth Hong Kong Ltd (Hong Kong SAR, People’s Republic of China).

Experimental design

Six-month-old female NZB/W F1 mice (N = 38) were divided into two groups. One group was treated with rapamycin (3 mg/kg body weight) once daily by oral gavage (N = 17). The other group received equal volume of normal saline on the same schedule and served as the treatment control (N = 21). Both groups of mice had evidence of active nephritis (urine albumin excretion >100 mg/dL) at the commencement of drug treatment. The mice were treated for 4 months (i.e. till 10 month of age). Urinary albumin excretion was determined at baseline and then monthly thereafter. At the time of killing, blood was collected for the measurement of serum urea, creatinine and anti-dsDNA antibody levels. The kidneys were harvested for histological, immunohistochemical and semi-quantitative gene expression studies. The spleen was dissected out for the measurement of spleen weight and for the determination of total splenocyte count.

Albuminuria

Spot urine samples were collected from each mouse by urinary bladder massage at baseline and then once every month after the initiation of drug treatment. Urinary albumin levels were measured by a commercially available ELISA kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer’s instruction.

Renal function and anti-dsDNA antibody measurements

Blood was collected from the mice by cardiac puncture under anaesthesia at the time of killing. Serum urea and creatinine levels were measured by the modified Jaffe’s method using a Beckman CX-5 analyser (Beckman Instruments Inc., Fullerton, CA, USA). Serum levels of anti-dsDNA (IgG) antibody were determined by a commercially available ELISA kit (Alpha Diagnostic International, San Antonio, USA) with reference to the manufacturer’s instruction.

Total splenocyte count

Single cell suspension from spleen was prepared by mashing the whole spleen and straining the resulting suspension through a nylon wool sieve. The cells were then centrifuged at 400 g for 5 min. The pelleted cells were resuspended in a Lysis Buffer to lyse the red blood cells. After washing with PBS, the total number of splenocytes per spleen was counted using a haemocytometer.

Renal histology and immunofluorescence studies

At the time of killing, kidney specimens obtained were fixed in 10% formaldehyde and embedded in paraffin. Four-micrometre sections were prepared and then stained with haematoxylin and eosin. The kidney sections were coded and examined by two independent observers (SLL and KWC) who were blinded to the treatment groups. The histological changes for mesangial proliferation, tubular dilation, protein cast deposition inside the tubules and inflammatory cell infiltration into the interstitium were assessed in a semi-quantitative manner by a scoring system of 0–3+, where 0 = no change; 1+ = mild; 2+ = moderate and 3+ = severe. At least 50 glomeruli were examined for each sample.

For immunofluorescence studies, kidney specimens were snap-frozen in 22-oxycalcitriol (OCT) compound. Five-micrometre cryostat sections of the kidneys were prepared and then stained with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG (Sigma Chemical Co., St Louis, MO, USA) or FITC-conjugated goat anti-mouse C3 antibodies (Sigma). The amount of immune complex deposition in the glomeruli was scored semi-quantitatively by an investigator (SLL) who was blinded to the treatment groups, according to the intensity of the fluorescence on a scale of 0–3+ where 0 = absent, 1+ = mild, 2+ = moderate and 3+ = strong staining. At least 20 glomeruli were examined per microscopic slide.

Immunohistochemical staining

The expression of regulated upon activation, normal T-cell expressed and secreted (RANTES) within the kidney was detected by immunohistochemical staining. Formalin-fixed kidney tissue sections were deparaffinized and then incubated with 0.3% hydrogen peroxide in 100% methanol for 10 min to block the endogenous peroxidase. The sections were incubated with biotin-labelled rat anti-mouse RANTES antibodies (Santa Cruz Biotechnology, Santa
A commercially available kit for total cellular RNA isolation, ToTALLY RNA (Ambion Inc., TX, USA), was used to extract total RNA from snap frozen kidney samples, according to the manufacturer’s instructions. The quality of RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

The equal amounts of total RNA (1 µg) from test and control samples were reversed transcribed into cDNA using Superscript RNase H reverse transcriptase and random hexamers as previously described (Yung et al, FASEB J, published online 18 May 2001 as doi: 10.1096/fj.00–0794fje). PCR amplification was performed in a total volume of 20 µl containing 1 µl RT product and 19 µl master mix comprising 12.8 µl H2O, 0.5 µl 5′ primer (20 µM), 0.5 µl 3′ primer (20 µM), 1.6 µl dNTPs (2.5 mM), 2 µl 10 × PCR buffer, 1.4 µl MgCl2 (2 mM) and 0.2 µl Ampli-Taq Polymerase. The PCR amplification consisted of 2 min of denaturation at 94°C followed by 39 cycles consisting of 94°C for 30 s, 58°C for 60 s, 72°C for 90s and a final extension at 72°C for 5 min. Ten microlitre of each PCR primer mix was then electrophoresed on a flat-bed agarose gel (2%) and stained with ethidium bromide (0.5 µg/ml). The density of the bands was evaluated by a ChemiGenius Gel Documentation System using ChemiGenius analysis software (Syngene, Gene Company, Hong Kong). The results were expressed as the ratio of RANTES to the house-keeping gene β-actin. The primer sequences used are shown in Table 1.

Table 1. Sequences of the PCR primers and size of the amplicons

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Bp</th>
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<tbody>
<tr>
<td>RANTES</td>
<td>Forward 5′-CTGCTGCTTTGCTACCTCT-3′</td>
<td>286</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-GCCCATTTTCCCCAGGAC-3′</td>
<td>364</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward 5′-GGACTCTCTATGGGGTGACGAGG-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-GGGAGAGCATACAATCTCOTGAT-3′</td>
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The effect of rapamycin treatment on the severity of albuminuria is shown in Figure 4. At the commencement of drug treatment and when the mice were around 6 months of age, both the saline and the rapamycin-treated mice had already developed significant albuminuria (urine albumin...
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The amount of albuminuria increased progressively with time in the saline-treated mice. In contrast, the degree of albuminuria in the rapamycin-treated mice remained relatively stable. From the second month after the commencement of drug treatment onwards, the amount of albuminuria was significantly lower in the rapamycin-treated mice than in the saline-treated mice.

Renal function

The NZB/W F₁ mice typically develop progressive deterioration in renal function after the onset of nephritis. At 10 months of age, the serum creatinine and urea levels of the rapamycin-treated mice were significantly lower than those of the saline-treated mice (17.28 ± 3.90 µmol/L versus 30.00 ± 8.10 µmol/L for serum creatinine, \( P = 0.020 \); and 6.61 ± 1.38 mmol/L versus 12.44 ± 5.36 mmol/L for serum urea, \( P = 0.021 \)).

Serum anti-dsDNA levels

Saline-treated mice had very high levels of anti-dsDNA antibodies in the serum. The antibody levels were markedly suppressed in mice treated with rapamycin (620 ± 915 µg/mL versus 71 ± 132 µg/mL, \( P = 0.021 \)).

Renal histology and immune deposition

Kidney sections from the saline-treated mice showed marked mesangial cell proliferation, tubular dilation, tubular protein cast deposition and leukocytic infiltration of the interstitial areas. These histological changes were markedly reduced in the rapamycin-treated mice (Figure 5 and Table 2).

Indirect immunofluorescence studies revealed intense staining for both IgG and C3 in the glomeruli of the saline-treated mice while only weak glomerular staining for IgG and C3 was observed in the rapamycin-treated mice (Figure 6 and Table 3).

Infiltration of inflammatory cells into the kidneys

The number and composition of mononuclear cell infiltration in the kidney sections of saline- or rapamycin-treated mice were determined by immunofluorescence using monoclonal antibodies against B lymphocytes, CD4⁺ T lymphocytes, CD8⁺ T lymphocytes and macrophages. There were numerous B lymphocytes, CD4⁺ T lymphocytes, CD8⁺ T lymphocytes and macrophages infiltrating in the periglomerular, perivascular and interstitial areas of the saline-treated mice. The number of infiltrating cells...
Table 2. Effect of rapamycin treatment on the histological manifestations of glomerulonephritis in NZB/W F1 mice.

<table>
<thead>
<tr>
<th></th>
<th>Saline-treated</th>
<th>Rapamycin-treated</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesangial cell proliferation</td>
<td>2.18 ± 1.13</td>
<td>1.00 ± 0.61</td>
<td>0.003</td>
</tr>
<tr>
<td>Tubular dilation</td>
<td>0.88 ± 1.11</td>
<td>0.18 ± 0.39</td>
<td>0.035</td>
</tr>
<tr>
<td>Protein cast deposition in the tubules</td>
<td>0.82 ± 1.19</td>
<td>0.06 ± 0.24</td>
<td>0.018</td>
</tr>
<tr>
<td>Interstitial lymphocytic infiltration</td>
<td>0.65 ± 0.86</td>
<td>0.06 ± 0.24</td>
<td>0.013</td>
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</table>

*The histological changes for mesangial proliferation, tubular dilation, protein cast deposition and lymphocytic infiltration were scored semi-quantitatively on a 0 to 3+ scale. The scoring was performed by two independent observers, using coded slides. The X values for the various histological changes ranged from 0.68 to 0.79, indicating a very good correlation between the two observers. Six-month-old female NZB/W F1 mice (n = 38) were treated for 4 months with saline or rapamycin (3 mg/kg/day) by oral gavage. Values are means ± SD.

Table 3. Effect of rapamycin treatment on glomerular immune deposition in NZB/W F1 mice with established nephritis.

<table>
<thead>
<tr>
<th></th>
<th>Saline-treated</th>
<th>Rapamycin-treated</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG deposition score</td>
<td>2.60 ± 0.55</td>
<td>0.60 ± 0.55</td>
<td>0.003</td>
</tr>
<tr>
<td>C3 deposition score</td>
<td>2.40 ± 0.55</td>
<td>0.40 ± 0.55</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*The intensity of IgG and C3 deposition in the glomeruli were scored semi-quantitatively on a 0 to 3+ scale where 0 = absent and 3+ = strong. 6-month-old female NZB/W F1 mice (n = 38) were treated for 4 months with saline or rapamycin (3 mg/kg/d) by oral gavage. Values are means ± SD.

was significantly reduced in the rapamycin-treated mice (Table 4).

**RANTES mRNA and protein expression in the kidney**

A number of recent studies have demonstrated that RANTES plays an important role in the pathogenesis of SLE [13,14]. To investigate whether rapamycin could modulate the intra-renal expression of RANTES in lupus nephritis, we examined the effect of rapamycin on RANTES protein and gene expression by immunohistochemical staining and RT-PCR, respectively. Increased immunostaining for RANTES, particularly in the glomeruli, was observed in the saline-treated mice. Moreover, the protein cast within the dilated renal tubules also showed positive immunostaining for RANTES. In contrast, the immunostaining for RANTES in the kidney sections of the rapamycin-treated mice was markedly diminished (Figure 7). Rapamycin treatment also significantly reduced

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Fig. 5. Histopathological changes in the kidneys of NZB/W F1 mice treated with either rapamycin or saline. (A) Low power view of the kidney section of a rapamycin-treated mouse showing relatively normal renal histology. (B) Low power view of the kidney section of a saline-treated mouse showing grossly abnormal renal architecture. (C, E and G) High power view showing normal looking glomerulus, interstitium and renal tubules respectively in a rapamycin-treated mouse. (D, F and H) High power view showing marked mesangial proliferation, interstitial inflammatory cell infiltration (arrow) and tubular dilation with intra-tubular protein cast deposition (*) respectively in a saline-treated mouse. (magnification: A and B ×100; C to H ×400, haematoxylin and eosin stain.)
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**Table 4.** Effect of rapamycin treatment on inflammatory cell infiltration in the kidney of NZB/W F1 mice with established nephritis

<table>
<thead>
<tr>
<th></th>
<th>Saline-treated</th>
<th>Rapamycin-treated</th>
<th>( P ) value</th>
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<tbody>
<tr>
<td>CD4(^+) T-cells</td>
<td>25.0 ± 5.70</td>
<td>6.00 ± 2.92</td>
<td>0.003</td>
</tr>
<tr>
<td>CD8(^+) T-cells</td>
<td>41.20 ± 6.41</td>
<td>9.40 ± 3.36</td>
<td>0.001</td>
</tr>
<tr>
<td>B-cells</td>
<td>24.8 ± 4.15</td>
<td>8.40 ± 1.67</td>
<td>0.001</td>
</tr>
<tr>
<td>Macrophage</td>
<td>20.8 ± 8.17</td>
<td>5.20 ± 0.84</td>
<td>0.016</td>
</tr>
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</table>

The number of CD4\(^+\) T-cells, CD8\(^+\) T-cells, B-cells and macrophages were counted in 20 randomly selected, non-overlapping high power fields in the cortical interstitial area. The cell counts are expressed as cell number/high power field (magnification \( \times 400 \)). Six mice were included in each group. Values are means ± SD.

the intra-renal expression of RANTES mRNA as compared to saline treatment (Figure 8).

**Discussion**

The results of our study indicate that treating 6-month-old female NZB/W F1 mice with rapamycin for 4 months, reduced albuminuria, improved survival, diminished splenomegaly, preserved renal function, lowered anti-dsDNA antibody levels, attenuated the histological severity of nephritis, decreased immune complex deposition in the glomeruli and inflammatory cell infiltration into the interstitium as well as down-regulated the intra-renal expression of RANTES. Our data thus show that rapamycin can attenuate the severity of established nephritis in the NZB/W F1 mice.

Two previous studies utilizing the MRL/lpr mice and the NZB/W F1 mice respectively have shown that rapamycin, when started before the onset of active nephritis, is effective in preventing the subsequent development of lupus nephritis [11,12]. While the preventive approach adopted by these two studies is instrumental in proving the therapeutic potential of rapamycin, it might be more relevant clinically to evaluate the therapeutic effects of rapamycin in lupus mice with established nephritis. The findings of our study, in which rapamycin treatment was initiated in NZB/W F1 mice with clinical evidence of active nephritis, bore closer resemblance to the actual clinical situation in human and confirmed the therapeutic value of rapamycin in the treatment of lupus nephritis. Our findings are in line with another recently published study in which it was shown that rapamycin treatment at 1 mg/kg/day in 5-month-old NZB/W F1 mice for 4 months reduced proteinuria and ameliorated the histological lesions of lupus nephritis [15].

Uncontrolled lymphoproliferation is a characteristic pathological feature of the NZB/W F1 mice, and is responsible for the development of massive splenomegaly, lymphadenopathy, excessive production of auto-antibodies and immune-mediated tissue injury [9,10]. Our observations that the degree of splenomegaly and the total splenocyte count were markedly reduced in the rapamycin-treated
mice suggest that the beneficial effects of rapamycin on lupus nephritis in the NZB/W F1 mice are probably mediated through suppression of lymphoproliferation. This postulation is consistent with the known mechanism of the action of rapamycin, which is the suppression of the proliferation of interleukin-2-stimulated T lymphocytes via inhibition of mTOR [7]. The markedly reduced serum anti-dsDNA antibody levels and intra-renal immune complex deposition in the rapamycin-treated mice are probably a consequence of the suppressed lymphoproliferation.

The beneficial effects of rapamycin in lupus nephritis might also be mediated through modulation of the intra-renal expression of RANTES. Chemokines and chemokine receptors play an important role in the pathogenesis of many forms of kidney diseases [16,17]. Antagonists of chemokines and chemokine receptors have been shown to be of therapeutic potential in experimental models of lupus nephritis [18]. RANTES is a pro-inflammatory CC-chemokine that mediates the recruitment of leukocytes to sites of inflammation and has been implicated in the pathogenesis of lupus nephritis. Up-regulation of RANTES has been demonstrated prior to the development of renal injury in MRL/lpr mice [13,19]. MRL/lpr mice that were deficient in RANTES have marked reduction in lymphadenopathy [14]. In addition, increased serum [20] and urinary [21] levels of RANTES have been shown to correlate with disease activity in lupus patients. Given the important role played by RANTES in the pathogenesis of lupus nephritis, our finding that rapamycin significantly reduced the intra-renal expression of RANTES has important clinical implications and suggests that the inhibitory effect of rapamycin on RANTES expression might represent another mechanism by which rapamycin attenuates lupus nephritis. The substantial reduction in the number of inflammatory cells infiltrating into the kidneys could be attributed to the suppression of RANTES expression by rapamycin.

The results of our study, as well as those of others utilizing animal models of membranous nephropathy [22], mesangioproliferative glomerulonephritis [23] and reduced renal mass [24], have indicated that rapamycin is beneficial in proteinuric nephritis. The safety of using rapamycin in clinical practice to treat patients with proteinuric nephropathy, however, is more controversial. There are recent reports in renal transplant recipients suggesting that withdrawal of calcineurin inhibitor and conversion to rapamycin led to the development of proteinuria [25,26]. Nevertheless, it should be noted that all the patients in these reports have pre-existing chronic allograft dysfunction. It has been argued that the onset of proteinuria in these patients could be related to the glomerular haemodynamic changes following calcineurin inhibitor withdrawal rather than the introduction of rapamycin [27]. It is noteworthy that rapamycin-associated proteinuria has not been reported in renal transplant patients who were given de novo rapamycin without

Fig. 6. Indirect immunofluorescence staining for immune complex deposition in the glomeruli of NZB/W F1 mice after 4 months of treatment with either saline or rapamycin. (A) IgG deposition in a rapamycin-treated mouse; (B) IgG deposition in a saline-treated mouse; (C) C3 deposition in a rapamycin-treated mouse; (D) C3 deposition in a saline-treated mouse. (magnification ×400)
calcineurin inhibitor [28] or following early elimination of calcineurin inhibitor [29]. Clinical studies evaluating the efficacy of rapamycin in the treatment of focal segmental glomerulosclerosis (FSGS) have also generated conflicting results. In one study in which 21 patients with steroid resistant idiopathic FSGS were treated with rapamycin, ~60% of the subjects were able to achieve complete or partial remission [30]. In another study, in contrast, it was found that in patients with idiopathic FSGC who had prolonged disease duration and prior cyclosporine exposure, rapamycin treatment was associated with deterioration in renal function and worsening of proteinuria [31]. It is apparent that our current understanding on the clinical significance and causative mechanism of rapamycin-associated proteinuria in patients with renal transplant or chronic nephritis is far from complete. The effect of rapamycin on proteinuria is probably influenced by multiple factors such as the presence of pre-existing renal damage, timing of administration, dosage of rapamycin used and prior exposure to calcineurin inhibitor. Additional basic and clinical studies are warranted to clarify the issue of rapamycin-associated proteinuria.
In conclusion, the results from our studies show that rapamycin is effective in attenuating the severity of established nephritis in the NZB/W F1 mouse. The beneficial effects of rapamycin are mediated, in part, through suppression of lymphoproliferation, inhibition of intra-renal expression of RANTES and reduction of inflammatory cell infiltration. The potential role of rapamycin in the treatment of human lupus nephritis warrants further investigation.

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Conflicts of interest statement. The donors had no role in the study design, execution, data analysis or interpretation or writing of the report. We declare that the results presented in this paper have not been published previously in whole or part, except in abstract form.

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