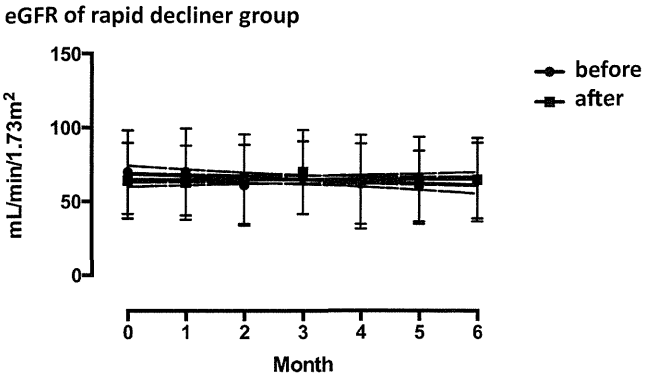


Figure 6. **Comparison of the slopes of the regression lines of the eGFR values in the rapid decliner group before and after treatment.**
Before: Y=-1.296*X+68.79, After: Y=0.08786*X+64.71, p=0.1105

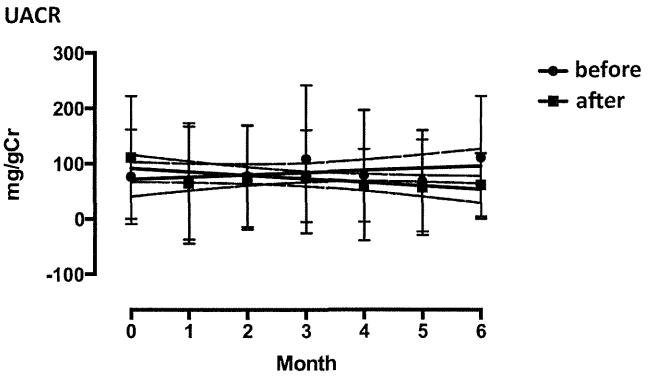


Figure 8. **Comparison of the slopes of the regression lines of the UACR values before and after treatment.**
Before: Y=3.992*X+71.93, After: Y=-6.335*X+91.68, p=0.037

values improved following treatment with alogliptin in four patients and were exacerbated following treatment in four patients. Among the subjects who were not early decliners, the ratio of patients exhibiting improvement to those demonstrating exacerbation was 10:18. Fisher's exact test showed a p value of 0.6832. No significant differences were observed in the rate of decrease in the eGFR between the early decliners and the other subjects (p=0.8267).

A Pearson product-moment correlation analysis performed to examine the correlations between the rate of change in eGFR and each parameter revealed a significant correlation with systolic blood pressure (Y=-0.3683*X+40.71, r= -0.3761, p=0.0238), indicating a higher rate of decrease in eGFR in the subjects with higher systolic blood pressure at the start of treatment with alogliptin. No significant correlations were observed with the other parameters (Fig. 7).

No associations were observed between the presence or absence of the administration of ARBs and the presence or absence of an improvement in the eGFR according to Fisher's exact test (p=1.0000).

The mean UACR decreased to 61.6±57.1 mg/gCr six months after the start of treatment, although the change was

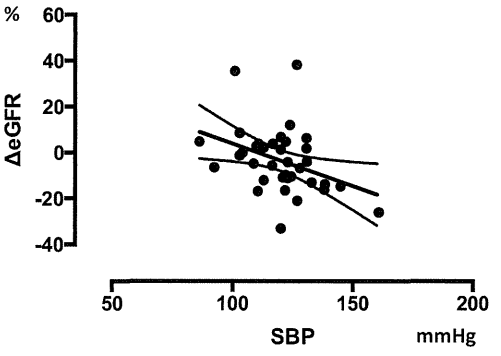


Figure 7. **Correlation between the rate of change in eGFR and the baseline systolic blood pressure.**
Y=-0.3683*X+40.71, r=-0.3761, p=0.0238
ΔeGFR: rate of change in eGFR (%), SBP: systolic blood pressure

not significant (p=0.9869) (Table 1). However, the slope of the regression line (3.992 before treatment with alogliptin) became negative at -6.335 after the start of treatment, showing a tendency to decrease. The difference in the slopes of the regression lines before treatment and after the start of treatment was significant (p=0.037) (Fig. 8).

Adverse events

A slight increase in the transaminase level was recorded in a single patient after the start of treatment with alogliptin; however, the event was not considered to be clinically significant, and the alogliptin treatment was continued.

Discussion

In the present study, we examined the effects of alogliptin in maintaining the renal function in type 2 diabetes patients with accompanying CKD.

Almost no changes in the HbA1c levels were observed following treatment with alogliptin. Possible reasons for the absence of significant changes include: 1) the Hb1Ac levels were controlled to a certain extent by existing therapeutic agents in many subjects; and 2) some subjects experienced extreme exacerbation after the start of the treatment with alogliptin. However, the 1,5-AG values showed a tendency toward improvement. Although data cannot be presented because the blood glucose profiles were not examined in detail, the changes in the 1,5-AG values suggest the possibility that alogliptin improved postprandial hyperglycemia. It is not uncommon that alpha-glucosidase inhibitors (α-GIs) specialized in treating postprandial hyperglycemia improved the 1,5-AG values to approximately 20 μg/mL without changing the HbA1c levels to be within the normal range (28). Based on the results of the present study, we speculate that DPP-4 inhibitors also improve blood glucose variability as drugs suppressing postprandial hyperglycemia. It is particularly noteworthy that a similar effect was observed in the subjects with CKD. Nevertheless, no definitive conclusions can be

drawn because patients with various types of nephropathy were enrolled in this study, which restricts the evaluation of the 1,5-AG values.

When we compared the clinical background characteristics between the improved group and the exacerbation group (defined based on HbA1c), a difference was noted in the TC levels. Therefore, the possibility that patients with higher TC levels are resistant to DPP-4 inhibitors cannot be excluded. There were no differences in the changes in eGFR or UACR between the improved group and the exacerbation group. This result does not support the hypothesis that the renal protective effects of alogliptin observed in this study depended on the blood glucose levels.

The eGFR was found to be a significant parameter correlated with the HbA1c level after the start of treatment with alogliptin. This result suggests that patients with a lower renal function have lower HbA1c levels, a finding that is commonly accepted in the daily treatment of diabetic patients based on the drug metabolism in the kidneys.

Regarding the eGFR, the slope of the regression line was approximately 0 throughout the entire study period, indicating that the eGFR values were stable throughout the 13 months. As many subjects were eventually treated at a dose of 25 mg, it can be speculated that alogliptin is not likely to adversely affect the renal function. In addition, similar results were observed in the subjects with stage 3 or worse CKD in this study, which strongly supports the safety of alogliptin. The disposition of alogliptin has been examined for different levels of the renal function, and the relationship between the level of the renal function and the dose of alogliptin has been clarified. The absence of accumulation has also been shown in simulation experiments, although these studies were based on single-dose administration (15, 29). Moreover, in addition to its hypoglycemic effects, the multifaceted extrapancreatic actions, including the improvement of lipid profiles or hypotensive actions mediated by the inhibition of reabsorption of sodium in the kidneys, of alogliptin have been reported, although in a small number of patients (24, 30, 31). While it is unknown if these actions result in renal protection, no significant changes were observed in the lipid profiles or blood pressure, and neither blood pressure nor the lipid levels were correlated with the rate of change in eGFR in this study.

The natural history of diabetic nephropathy is generally long. However, it is widely known that many patients clearly exhibit more rapid progression of the disease (1, 32). Although the rapidly progressive type has not been clearly defined and its causes are unknown, patients whose GFR decreases by 3-5 mL/min/1.73 mm^2 or more per year are generally considered to have the condition. In our study, we defined subjects whose GFR decreased by 5 mL/min/1.73 mm^2 or more per year as belonging to the rapid decliner group for the analysis. The results showed that the GFR, which continued to decrease before treatment with alogliptin, stopped declining after the start of treatment and showed a tendency, although slight, to increase during the six months of treatment. Comparing the clinical background factors of the subjects in the rapid decliner group with those of the remaining patients, only diastolic blood pressure was found to show a significant difference. Diastolic blood pressure was significantly lower in the rapid decliner group; however, this cannot be considered a sufficient factor to explain the improvement in the speed of decrease in eGFR (33). Moreover, the presence or absence of treatment with ARBs was not related to changes in eGFR. Based on these results, it is possible that alogliptin itself contributed to the improvement of eGFR in the rapid decliner group.

In addition, the analysis of early decliners suggested that the effects of alogliptin are not influenced by the baseline eGFR or the presence or absence of proteinuria.

The correlation between the rate of decrease in eGFR and the baseline systolic blood pressure indicates that patients with higher blood pressure experience more severe exacerbation of the renal function, as supported by various studies. Our results thus confirmed the importance of lowering blood pressure for maintaining the renal function. In many of the 36 subjects examined in this study, the blood pressure was controlled to less than 125 mmHg, according to the Japanese guidelines for CKD. However, we speculate that blood pressure was poorly controlled in some subjects and that the renal function of these subjects tended to deteriorate.

The UACR values continued to increase before treatment with alogliptin, then started to decline after the start of treatment. Considering the renal protective effects of alogliptin, this result provides significant confirmation of the maintenance of the eGFR values in all subjects. Although other DPP-4 inhibitors have been reported to decrease the UACR, to the best of our knowledge, this is the first report of a reduction in UACR due to alogliptin treatment (19, 34, 35).

Many factors of the mechanisms underlying the renal protective effects of DPP-4 inhibitors (including alogliptin) remain unknown. Possible mechanisms include the resolution of glucose toxicity accompanied by an improvement in blood glucose variability (28). Improving the blood glucose level is well known to have a secondary effect of inhibiting the progression of complications (4, 6). Although there are no clear data concerning blood pressure, improvements in the balance of body fluid volume due to the promotion of sodium excretion by alogliptin may also contribute to renal protection (17, 30, 36). According to *in vitro* data, the glucagon-like peptide-1 (GLP-1) receptor is expressed in the kidneys; therefore, the kidney is the organ with the highest DPP-4 activity (37). For these reasons, the kidneys are likely to be a major target of the extrapancreatic effects of incretins. There are reports suggesting that, when activated by DPP-4 inhibitors, the level of GLP-1 is increased locally in the kidneys, producing anti-inflammatory effects and promoting antioxidative actions (16-21, 23, 38-41).

There are only a small number of reports on the direct renal protective effects of DPP-4 inhibitors, including alogliptin, in patients with diabetic nephropathy. Extensive research on these effects should be conducted, including ani-

mal and clinical studies.

In this study, we administered alogliptin in 36 patients with type 2 diabetes accompanying CKD who were receiving treatment with existing diabetes drugs and diet therapy. Neither the blood glucose levels nor renal function were exacerbated before or after the start of treatment. The results of this study suggest that alogliptin can be administered safely in CKD patients. Furthermore, we cannot deny the possibility that alogliptin may delay the progression of diabetic nephropathy, independent of the levels of blood glucose, blood pressure and lipids. However, although we expected alogliptin to exhibit renal protective effects, we were unable to detect statistically significant differences. One reasons for this finding is that there are few registered cases. Additional reports on the renal protective effects of alogliptin are needed.

The authors state that they have no Conflict of Interest (COI).

References

1. Phillips A, Janssen U, Floege J. Progression of diabetic nephropathy. Insights from cell culture studies and animal models. Kidney Blood Press Res **22**: 81-97, 1999.
2. Rossing P, de Zeeuw D. Need for better diabetes treatment for improved renal outcome. Kidney Int **79** (Suppl): S28-S32, 2011.
3. Ismail-Beigi F, Craven T, Banerji MA, et al. Effect of intensive treatment of hyperglycaemia on microvascular outcomes in type 2 diabetes: an analysis of the ACCORD randomised trial. Lancet **376**: 419-430, 2010.
4. Turner RC, Holman RR, Cull CA, et al. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet **352**: 837-853, 1998.
5. Ninomiya T, Perkovic V, de Galan BE, et al. Albuminuria and kidney function independently predict cardiovascular and renal outcomes in diabetes. J Am Soc Nephrol **20**: 1813-1821, 2009.
6. Group AC, Patel A, Macmahon S, et al. Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. N Engl J Med **358**: 2560-2572, 2008.
7. Neumiller JJ, Setter SM. Pharmacologic management of the older patient with type 2 diabetes mellitus. Am J Geriatr Pharmacother **7**: 324-342, 2009.
8. Moen MF, Zhan M, Hsu VD, et al. Frequency of hypoglycemia and its significance in chronic kidney disease. Clin J Am Soc Nephrol **4**: 1121-1127, 2009.
9. Bodmer M, Meier C, Krähenbühl S, Jick SS, Meier CR. Metformin, sulfonylureas, or other antidiabetes drugs and the risk of lactic acidosis or hypoglycemia: a nested case-control analysis. Diabetes Care **31**: 2086-2091, 2008.
10. Munshi MN, Segal AR, Suhl E, et al. Frequent hypoglycemia among elderly patients with poor glycemic control. Arch Intern Med **171**: 362-364, 2011.
11. Slinin Y, Ishani A, Rector T, et al. Management of hyperglycemia, dyslipidemia, and albuminuria in patients with diabetes and CKD: a systematic review for a KDOQI clinical practice guideline. Am J Kidney Dis **60**: 747-769, 2012.
12. Zanchi A, Lehmann R, Philippe J. Antidiabetic drugs and kidney disease: recommendations of the Swiss Society for Endocrinology and Diabetology. Swiss Med Wkly **142**: w13629, 2012.
13. Nisal K, Kela R, Khunti K, Davies MJ. Comparison of efficacy between incretin-based therapies for type 2 diabetes mellitus. BMC Med **10**: 152, 2012.
14. Baetta R, Corsini A. Pharmacology of dipeptidyl peptidase-4 inhibitors: similarities and differences. Drugs **71**: 1441-1467, 2011.
15. Scheen AJ. Pharmacokinetics of dipeptidylpeptidase-4 inhibitors. Diabetes Obes Metab **12**: 648-658, 2010.
16. Panchapakesan U, Mather A, Pollock C. Role of GLP-1 and DPP-4 in diabetic nephropathy and cardiovascular disease. Clin Sci (Lond) **124**: 17-26, 2013.
17. Herzlinger S, Horton ES. Extraglycemic effects of glp-1-based therapeutics: addressing metabolic and cardiovascular risks associated with type 2 diabetes. Diabetes Res Clin Pract **100**: 1-10, 2013.
18. Liu Q, Adams L, Broyde A, Fernandez R, Baron AD, Parkes DG. The exenatide analogue AC3174 attenuates hypertension, insulin resistance, and renal dysfunction in Dahl salt-sensitive rats. Cardiovasc Diabetol **9**: 32, 2010.
19. Hattori S. Sitagliptin reduces albuminuria in patients with type 2 diabetes. Endocr J **58**: 69-73, 2011.
20. Yamagishi S, Matsui T. Pleiotropic effects of glucagon-like peptide-1 (GLP-1)-based therapies on vascular complications in diabetes. Curr Pharm Des **17**: 4379-4385, 2011.
21. Kodera R, Shikata K, Kataoka HU, et al. Glucagon-like peptide-1 receptor agonist ameliorates renal injury through its anti-inflammatory action without lowering blood glucose level in a rat model of type 1 diabetes. Diabetologia **54**: 965-978, 2011.
22. Zhang H, Zhang X, Hu C, Lu W. Exenatide reduces urinary transforming growth factor-β1 and type IV collagen excretion in patients with type 2 diabetes and microalbuminuria. Kidney Blood press Rese **35**: 483-488, 2012.
23. Mima A, Hiraoka-Yamomoto J, Li Q, et al. Protective effects of GLP-1 on glomerular endothelium and its inhibition by PKCβ activation in diabetes. Diabetes **61**: 2967-2979, 2012.
24. Hocher B, Reichetzeder C, Alter ML. Renal and cardiac effects of DPP4 inhibitors: from preclinical development to clinical research. Kidney Blood Press Res **36**: 65-84, 2012.
25. National Kidney Foundation. KDOQI Clinical Practice Guideline for Diabetes and CKD: 2012 Update. Am J Kidney Dis **60**: 850-886, 2012.
26. Seino Y, Nanjo K, Tajima N, et al; The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes mellitus. Report of the Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus. J Diabetes Invest **1**: 212-228, 2010.
27. Matsuo S, Imai E, Horio M, et al. Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis **53**: 982-992, 2009.
28. Del Prato S. In search of normoglycaemia in diabetes: controlling postprandial glucose. Int J Obe Relat Metab Disord **26** (Suppl 3): S9-S17, 2002.
29. Lee B, Shi L, Kassel DB, Asakawa T, Takeuchi K, Christopher RJ. Pharmacokinetic, pharmacodynamic, and efficacy profiles of alogliptin, a novel inhibitor of dipeptidyl peptidase-4, in rats, dogs, and monkeys. Eur J Pharmacol **589**: 306-314, 2008.
30. Chaykovska L, von Websky K, Rahnenfuhrer J, et al. Effects of DPP-4 inhibitors on the heart in a rat model of uremic cardiomyopathy. PLoS One **6**: e27861, 2011.
31. Fukuda-Tsuru S, Anabuki J, Abe Y, Yoshida K, Ishii S. A novel, potent, and long-lasting dipeptidyl peptidase-4 inhibitor, teneligliptin, improves postprandial hyperglycemia and dyslipidemia after single and repeated administrations. Eur J Pharmacol **696**: 194-202, 2012.
32. Kelly KJ, Dominguez JH. Rapid progression of diabetic nephropathy is linked to inflammation and episodes of acute renal failure. Am J Nephrol **32**: 469-475, 2010.
33. Hoefield RA, Kalra PA, Lane B, O'Donoghue DJ, Foley RN, Middleton RJ. Associations of baseline characteristics with evolution of eGFR in a referred chronic kidney disease cohort. QJM

106: 915-924, 2013.

34. Liu WJ, Xie SH, Liu YN, et al. Dipeptidyl peptidase IV inhibitor attenuates kidney injury in streptozotocin-induced diabetic rats. J Pharmacol Exp Ther **340**: 248-255, 2012.

35. Alter ML, Ott IM, von Websky K, et al. DPP-4 inhibition on top of angiotensin receptor blockade offers a new therapeutic approach for diabetic nephropathy. Kidney Blood Press Res **36**: 119-130, 2012.

36. Ogawa S, Ishiki M, Nako K, et al. Sitagliptin, a dipeptidyl peptidase-4 inhibitor, decreases systolic blood pressure in Japanese hypertensive patients with type 2 diabetes. Tohoku J Exp Med **223**: 133-135, 2011.

37. Mentlein R. Dipeptidyl-peptidase IV (CD26): role in the inactivation of regulatory peptides. Regul Pept **85**: 9-24, 1999.

38. Hirata K, Kume S, Araki S-i, et al. Exendin-4 has an anti-hypertensive effect in salt-sensitive mice model. Biochem Biophys Res Commun **380**: 44-49, 2009.

39. Mosenzon O, Raz I, Scirica BM, et al. Baseline characteristics of the patient population in the Saxagliptin Assessment of Vascular Outcomes Recorded in patients with diabetes mellitus (SAVOR)-TIMI 53 trial. Diabetes Metab Res Rev **29**: 417-426, 2013.

40. Ishibashi Y, Nishino Y, Matsui T, Takeuchi M, Yamagishi S. Glucagon-like peptide-1 suppresses advanced glycation end product-induced monocyte chemoattractant protein-1 expression in mesangial cells by reducing advanced glycation end product receptor level. Metabolism **60**: 1271-1277, 2011.

41. Hendarto H, Inoguchi T, Maeda Y, et al. GLP-1 analog liraglutide protects against oxidative stress and albuminuria in streptozotocin-induced diabetic rats via protein kinase A-mediated inhibition of renal NAD(P)H oxidases. Metabolism **61**: 1422-1434, 2012.

Therapeutic Apheresis and Dialysis

Therapeutic Apheresis and Dialysis 2014; 18(2):181–184
doi: 10.1111/1744-9987.12099

Clinical Benefit of the Change of Dialysate Calcium Concentration From 3.0 to 2.75 mEq/L

Yukinao Sakai,[1] Tomoyuki Otsuka,[1] Dai Ohno,[1] Tsuneo Murasawa,[1] Saori Sakai,[2] and Shuichi Tsuruoka[3]

[1]Division of Nephrology, Department of Internal Medicine, Nippon Medical School Musashikosugi Hospital, [2]Department of Internal Medicine, Zenjinkai Maruko-Clinic, Kawasaki and [3]Division of Nephrology, Department of Internal Medicine, Graduate School of Medicine, Nippon Medical School, Tokyo, Japan

Abstract: Because active vitamin D preparations and calcimimetics have been widely used to treat secondary hyperparathyroidism, maintenance of acceptable serum calcium and phosphate levels is important. A 2.75 mEq/L dialysate calcium product, which may bring the calcium balance closer to 0, has recently been launched, and we had an opportunity to examine its possible benefits. We performed a 6-month retrospective review after switching from 3.0 mEq/L to 2.75 mEq/L calcium dialysate in 85 outpatients undergoing chronic hemodialysis. We evaluated blood biochemical parameters, including predialysis and postdialysis serum calcium and phosphate levels, predialysis intact parathyroid hormone (iPTH) levels; dialysis dose (Kt/V); and doses of concomitant active vitamin D preparations, calcimimetics, phosphate binder, and erythropoiesis-stimulating agents. Postdialysis calcium levels were significantly lower and predialysis corrected calcium levels significantly decreased. The change in calcium levels before and after dialysis was smaller after switching of the dialysate than before. iPTH levels significantly increased 1 month after switching of the dialysate. No remarkable changes were observed in phosphate levels or Kt/V. The dose of alfacalcidol, one of the concomitant drugs, somewhat increased, and no remarkable changes in dosage were observed for other concomitant drugs. These results were favorable in terms of calcium balance. However, there may be limitations in interpreting the results, but the resultant calcium levels suggest that switching to 2.75 mEq/L calcium dialysate may improve the control of calcium levels. In addition, it is hoped that the treatment choice of secondary hyperparathyroidism is extended. **Key Words:** Active vitamin D, Calcium concentration, Dialysate, Intact parathyroid hormone, Secondary hyperparathyroidism.

Because active vitamin D preparations and calcimimetics have been widely used to treat secondary hyperparathyroidism, the importance of maintaining acceptable serum phosphate and calcium levels has been widely recognized (1). In 1993, 2.5 mEq/L calcium dialysate was made available. However, poorly corrected acidosis and unacceptably lowered serum calcium level, conditions that were attributable to bicarbonate acid and/or chloride concentrations in the dialysate, were later reported. In 2006, "Guidelines for the management of secondary hyperparathyroidism in chronic dialysis patients" was published by the Japanese Society for Dialysis Therapy (2). The guidelines recommended that calcium concentration in bicarbonate dialysate should be determined, taking calcium load and its possible physiologic effect into consideration. The guideline issued by Kidney Disease: Improving Global Outcomes (KDIGO) (3) suggested that dialysate calcium concentration should be between 2.5 and 3.0 mEq/L, depending on individual cases. There are two concentrations of calcium contained in dialysate products commercially available in Japan: 2.5 and 3.0 mEq/L. The 2.5 mEq/L calcium dialysate products tend to remove calcium, whereas the 3.0 mEq/L calcium dialysate products often cause it to accumulate in the body (4,5). The 3.0 mEq/L calcium dialysate products are routinely used in our hospital. Recently, a 2.75 mEq/L calcium dialysate

Received February 2013; revised June 2013.
Address correspondence and reprint requests to Dr Yukinao Sakai, Chief Assistant Professor, Division of Nephrology, Department of Internal Medicine, Nippon Medical School Musashikosugi Hospital, 1-396 Kosugi-cho, Nakahara-ku, Kawasaki 211-8533, Japan. Email: y-sakai@nms.ac.jp

product (Kindaly 4, Fuso Pharmaceutical Industries, Tokyo, Japan), which may bring the calcium balance closer to 0, has been launched, and we had an opportunity to examine its possible benefit.

PATIENTS AND METHODS

Patients

Our study included 85 patients (65 men and 20 women) who received chronic HD on an outpatient basis. They had a mean age of 66.7 years (SD of 11.4 years) and a mean dialysis period of 82.7 months (SD of 77.5 months). Their underlying diseases were diabetes ($N = 40$), cystic kidney ($N = 2$), chronic glomerulonephritis ($N = 20$), nephrosclerosis ($N = 11$), and others ($N = 12$).

Methods

The patients were examined for 6 months after switching from 3.0 mEq/L to 2.75 mEq/L calcium dialysate. We evaluated blood biochemical parameters, including predialysis and postdialysis serum calcium and phosphate levels, predialysis intact parathyroid hormone (iPTH) levels; dialysis dose (Kt/V); and doses of concomitant active vitamin D preparations, calcimimetics, phosphate binder, and erythropoiesis-stimulating agents. Because the study was conducted in our existing clinical setting, no therapeutic requirements were imposed in relation to their specific treatments except for switching of dialysate. The variations in the laboratory parameter values over time were analyzed using a linear mixed model. The Dunnett multiple comparison test was performed. The parameter values for which no normal distribution could be assumed were transformed into log values for analysis. The doses of concomitant drugs that changed over time were analyzed by performing paired Wilcoxon tests. Included data was obtained over 6 months before switching of the dialysate. The two-sided significance level was set at 5%. The test results are shown together with P-values. Predialysis calcium levels, when the albumin level was less than 4 g/dL, were corrected using Payne's equation (corrected Ca [mg/dL] = Ca [mg/dL] + {4 –albumin [g/dL]}).

RESULTS

Postdialysis calcium levels were significantly lower than values before the switch, 1 month or later after switching of the dialysate. Predialysis corrected calcium levels were significantly lower than values before the switch, 2, 5, and 6 months after switching of the dialysate, and an overall decreasing tendency was observed. In addition, the change in calcium levels before and after dialysis was apparently smaller after switching of the dialysate (Fig. 1). In addition, calcium balance calculated as the difference between the predialysis value and postdialysis value (mean [SD]), decreased significantly from 0.74 (0.56) mg/dL to 0.35 (0.62) mg/dL 1 month after the change, and then remained at the same level for up to 6 months (Fig. 2).

Intact parathyroid hormone levels increased significantly more than values before the switch, 1 month after switching of the dialysate, and were subsequently maintained until the end of the observation period. The iPTH values were plotted over time in a box plot because of their distribution shape. They were tested using their log-transformed values (Fig. 3).

No remarkable changes were observed in phosphate levels or Kt/V before and after switching of the dialysate.

The dose of alfacalcidol, one of the concomitant drugs, somewhat increased more than values before the switch, after switching of the dialysate, and its dose significantly increased at 5 months after switching of the dialysate. When the recipients were

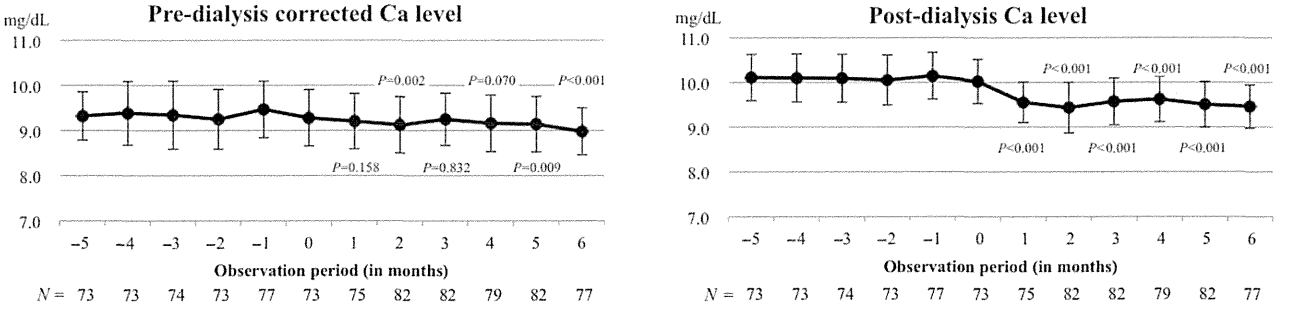


FIG. 1. Changes of predialysis and postdialysis calcium levels. P-values: analysis was performed using linear mixed models. Dunnett multiple comparison was performed on the set for the observation period. Previous value: mean value over 6 months before switching of the dialysate.

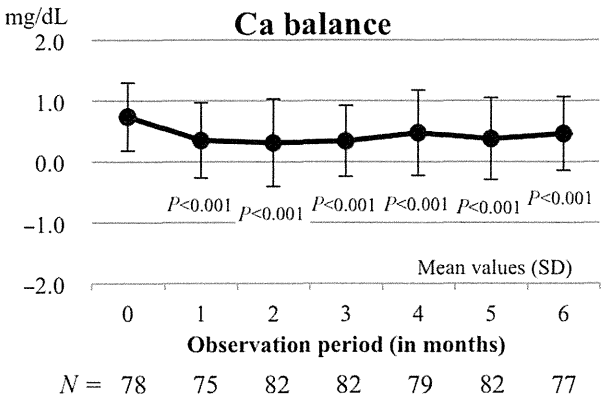


FIG. 2. Change of calcium balance. Calcium balance: difference between postdialysis calcium and predialysis corrected calcium. *P*-values: analysis was performed using linear mixed models. Dunnett multiple comparison was performed on the "0" set for the observation period. 0: Mean value over 6 months before switching of the dialysate.

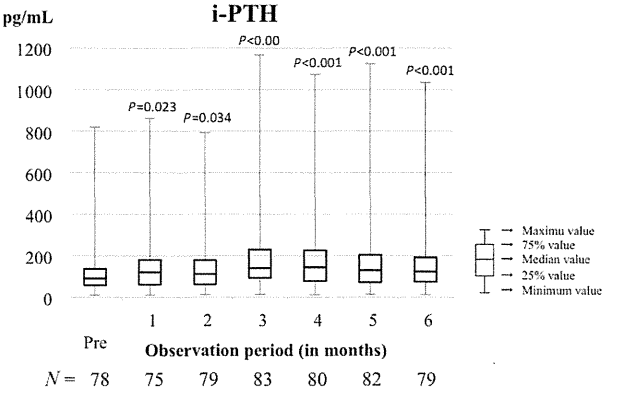


FIG. 3. Changes of intact parathyroid hormone (iPTH) levels. *P*-values: Log-transformed values were analyzed using linear mixed models. Dunnett multiple comparison was performed against the "0" set for the observation period. 0: Median value over 6 months before switching of the dialysate.

classified into "decrease," "no change," and "increase" groups in terms of alfacalcidol doses compared, no consistent tendencies were found for those groups. The number of recipients classified into the "increase" group gradually increased until 6 months after switching of the dialysate. However, the size of the "decrease" group greatly varied over the observation period (Fig. 4). No remarkable changes were observed in the doses of other concomitant drugs.

DISCUSSION

In dialysis recipients, to address the control of serum calcium levels whose importance has been highlighted in the Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD) guidelines (6), we examined the use of 2.75 mEq/L calcium dialysate, which is reported to bring the calcium balance near 0, and reported our findings.

Both postdialysis and predialysis calcium levels significantly decreased after switching of the dialysate. The change in calcium levels before and after dialysis was smaller after switching of the dialysate than before. These results were favorable in terms of calcium balance (7,8). A crossover study of 3.0 and 2.75 mEq/L calcium dialysates (9) showed that both postdialysis and predialysis calcium levels remained decreased, and the change in calcium levels before and after dialysis was smaller when 2.75 mEq/L calcium dialysate was administered than when 3.0 mEq/L calcium dialysate was administered. This is consistent with our findings.

Matsuura et al. (10) observed that switching from 3.0 mEq/L to 2.75 mEq/L calcium dialysate did not

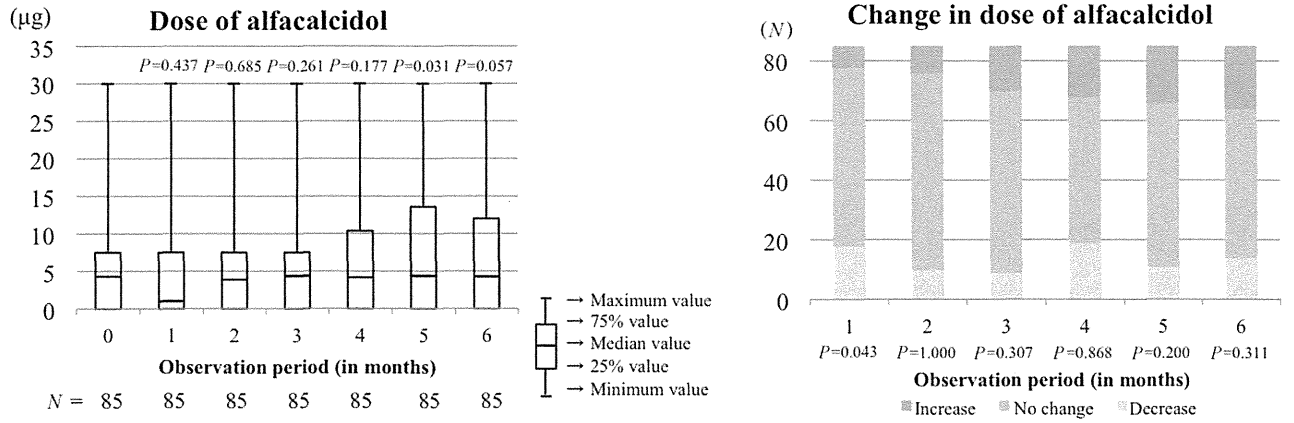


FIG. 4. Changes of alfacalcidol doses. Alfacalcidol dose: the total dose of 1 month, and set to 0 μg for the month of no treatment. *P*-values: paired Wilcoxon test was performed for "0". 0: Median dose value over 6 months before switching of the dialysate, left panel. *P*-values: sign test. Change in doses: change from the median dose value over 6 months before switching of the dialysate, right panel.

affect iPTH levels. However, this finding represents only the patients who experienced no change to their treatment for CKD-MBD. They noted a possibility that iPTH levels would increase in the patients for whom doses of active vitamin D preparations, calcium-containing phosphate binder, or cinacalcet hydrochloride had to be changed. Our study showed that 77 of 85 patients experienced changes in the doses of concomitant drugs for the treatment of CKD-MBD. An increase in iPTH levels was probably associated with these dose changes.

No consistent tendency in dose changes was observed for any of the concomitant drugs, including alfacalcidol, the most popular. To control serum phosphate, calcium, and iPTH levels in dialysis recipients, these individuals are usually treated with a combination consisting of appropriate doses of phosphate binder, active vitamin D drugs, or cinacalcet hydrochloride (6). This would explain why no consistent tendency was detected in the dose changes of these concomitant drugs among our dialysis recipients after their dialysate was changed using the central dialysis fluid delivery system.

CONCLUSION

Our study may contain many biases because it was conducted as a part of routine dialysis practices, where no specific limitations were imposed on dialysis procedures or concomitant drugs. Therefore, there may be limitations in interpreting the results, but the resultant calcium levels, at least, suggest that switching to 2.75 mEq/L calcium dialysate may improve the control of calcium levels. Although Shigematsu et al. (9) and Matsuura et al. (10) covered 6 and 12 weeks of treatment duration, respectively, our study covered 6 months, which allowed us to examine the benefits of the dialysate over a longer period of time. It is important to continue to follow the data on a long-term basis.

CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

REFERENCES

1. Hirakata H, Nitta K, Inaba M et al. Japanese Society for Dialysis Therapy guidelines for management of cardiovascular diseases in patients on chronic hemodialysis. *Ther Apher Dial* 2012;16:387–435.
2. Guideline Working Group, Japanese Society for Dialysis Therapy. Clinical practice guideline for the management of secondary hyperparathyroidism in chronic dialysis patients. *Ther Apher Dial* 2008;12:514–25.
3. KDIGO Guideline. KDIGO clinical practice guideline for the diagnosis, evaluation, prevention, and treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int Suppl* 2009;76:S1–130.
4. Basile C. Calcium mass balances in bicarbonate hemodialysis. *Int J Nephrol* 2011;2011:540592.
5. Karohl C, de Paiva Paschoal J, de Castro MC et al. Effects of bone remodelling on calcium mass transfer during haemodialysis. *Nephrol Dial Transplant* 2010;25:1244–51.
6. CKD-MBD Guideline Working Group, Japanese Society for Dialysis Therapy. Clinical practice guideline for CKD-MBD. *J Jpn Soc Dial Ther* 2012;45:301–56.
7. Yokoyama K, Kagami S, Ohkido I et al. The negative Ca(2+) balance is involved in the stimulation of PTH secretion. *Nephron* 2002;92:86–90.
8. Basile C, Libutti P, Di Turo AL et al. Effect of dialysate calcium concentrations on parathyroid hormone and calcium balance during a single dialysis session using bicarbonate hemodialysis: a crossover clinical trial. *Am J Kidney Dis* 2012;59:92–101.
9. Shigematsu T, Kanayama Y, Ozawa H, Oguchi K, Wakasa M, Hashiguchi T. Clinical examination of 2.75 mEq/L calcium dialysate, K-FT1—Multi-center Phase III Comparison Clinical Study. *Shinyaku to Rinsho* 2012;61:368–87.
10. Matsuura A, Inaguma D, Itawaki D et al. The change of the dialysaste calcium concentration from 3.0 to 2.75 mEq/L did not affect the sarum PTH level in hemodialysis patients. *J Jpn Soc Dial Ther* 2012;45:873–80.

RENAL FAILURE

http://informahealthcare.com/rnf
ISSN: 0886-022X (print), 1525-6049 (electronic)

Ren Fail, Early Online: 1–7
 DOI: 10.3109/0886022X.2013.844622

informa
healthcare

CLINICAL STUDY

Febuxostat for treating allopurinol-resistant hyperuricemia in patients with chronic kidney disease

Yukinao Sakai[1], Tomoyuki Otsuka[1], Dai Ohno[1], Tsuneo Murasawa[1], Naoki Sato[2], and Shuichi Tsuruoka[3]

[1]Division of Nephrology, Department of Internal Medicine, Nippon Medical School Musashikosugi Hospital, Kawasaki, Japan, [2]Division of Cardiology, Department of Internal Medicine, Nippon Medical School Musashikosugi Hospital, Kawasaki, Japan, and [3]Division of Nephrology, Department of Internal Medicine, Graduate School of Medicine, Nippon Medical School, Tokyo, Japan

Abstract

Background: Availability of the novel xanthine oxidase inhibitor febuxostat, which has multiple excretion pathways, enables investigation of the significance of serum uric acid control on renal function in patients with chronic kidney disease (CKD). *Methods*: This was an exploratory, retrospective, observational study conducted at a single Japanese center. Serum uric acid concentrations and serum creatinine levels in the 6 months before and after the start of febuxostat treatment were collected for CKD patients switched from allopurinol after failing to achieve serum uric acid concentrations ≤6.0 mg/dL. *Results*: Evaluable data were available for 60 patients, 67% of whom had advanced CKD (eGFR <30 mL/min/1.73 m^2). Mean dose of febuxostat was 15.9 (±8) mg/day. Mean serum uric acid concentration decreased from 8.4 (±1.4) mg/dL at baseline to 6.2 (±1.2) mg/dL at 6 months; 47.5% of patients achieved a level ≤6.0 mg/dL. The change from baseline in eGFR was positive at all time points during febuxostat treatment and the increase of 2.3 (±5.6) mL/min/1.73 m^2 at 6 months was significant ($p = 0.0027$). Whereas the eGFR slope was negative during allopurinol treatment, it became positive after the switch to febuxostat. The change in eGFR slope before and after febuxostat treatment was significant for all patients ($p < 0.01$), for male patients ($p < 0.05$), and for patients with a baseline eGFR of <15 mL/min/1.73 m^2 ($p < 0.05$). *Conclusions*: In patients with CKD, febuxostat reduces serum uric acid concentrations effectively and may suppress the progressive decline in renal function.

Keywords

Allopurinol, chronic kidney disease, febuxostat, hyperuricemia

History

Received 25 July 2013
Revised 26 August 2013
Accepted 30 August 2013
Published online 23 October 2013

Introduction

An estimated 13.3 million Japanese people – or 1 in 8 adults – have chronic kidney disease (CKD).[1] Treatment of CKD is aimed at slowing the progression to end-stage kidney disease (ESKD) and the onset of cardiovascular disease (CVD). To this end, multimodal treatment ranging from lifestyle and dietary guidance to blood pressure, glucose, and lipid control through to correction of renal anemia and abnormalities in bone and mineral metabolism is essential to prevent or delay a series of pathologies.[1] Recently, serum uric acid has become a target of control as per the 2012 revision of the Japanese Society of Nephrology CKD treatment guideline.[2]

Hyperuricemia is defined as a serum uric acid concentration greater than 7.0 mg/dL.[3] As the dissolution limit of urate in body fluids is around 6.4 mg/dL, persistent hyperuricemia causes formation and deposition of urate crystals in body tissues. The typical resultant disease is gouty arthritis; however, urolithiasis and gouty nephropathy are also known to occur. In gouty nephropathy, crystals deposit in the renal medulla, inducing chronic interstitial nephritis. Moreover, it has recently been considered that oxidative stress due to hyperuricemia induces arteriosclerosis in association with hypertension and glucose/lipid metabolism disorder, resulting in renal hypertension and reduced renal function as pathologies unrelated to urate deposition. Nearly a decade ago already, Iseki and colleagues reported an association between an increased risk of ESKD and serum uric acid concentrations of ≥7 mg/dL in males and ≥6 mg/dL in females.[4]

Few studies to date have investigated the significance of treating hyperuricemia on renal function, likely due to the well-known difficulties of using conventional urate-lowering drugs in patients with reduced renal function. Allopurinol suppresses uric acid production by inhibiting xanthine oxidase, but it has a purine structure and is known to affect other nucleic acid metabolic enzymes. Metabolism of allopurinol by xanthine oxidase produces the active metabolite, oxypurinol, which is excreted almost entirely by the kidneys. Oxypurinol plasma concentrations are easily elevated to the toxic range in patients with impaired renal function; this often precludes administering allopurinol at a

Address correspondence to Yukinao Sakai, Division of Nephrology, Department of Internal Medicine, Nippon Medical School Musashikosugi Hospital, 1-396 Kosugi-cho, Nakahara-ku, Kawasaki, 211-8533, Japan. Tel: +81 44 733 5181; Fax: +81 44 711 8333; E-mail: y-sakai@nms.ac.jp

dose necessary to adequately reduce serum uric acid concentrations. In addition, uricosuric agents such as benzbromarone are not recommended for patients with reduced renal function due to an attenuated effect.

Febuxostat is a novel xanthine oxidase inhibitor introduced in Japan in May 2011. It does not contain purine in its chemical structure and is a highly selective, potent, and continuous inhibitor of xanthine oxidase via a new mechanism of inhibition.[5] Multiple excretion pathways permit its use even in patients with reduced renal function. Febuxostat has shown good efficacy with regard to patients achieving a serum uric acid target level of ≤6.0 mg/dL.[6,7] In this regard a number of well-controlled clinical trials such as APEX,[8] FACT,[9] and EXCEL[10] have shown that febuxostat (80–240 mg/day) was more effective than allopurinol (100–300 mg/day) in lowering and maintaining serum urate levels below 6 mg/dL in patients with hyperuricemia and gout. Moreover, its availability enables investigation of the potential impact of active intervention for hyperuricemia on renal function.

The purpose of this exploratory study was to evaluate the urate-lowering activity of febuxostat and its effect on renal function in patients with CKD.

Materials and methods

This was a retrospective observational exploratory study performed in patients who had attended the Nippon Medical School Musashikosugi Hospital between July 2011 and November 2012. The study population involved patients with CKD (estimated glomerular filtration rate [eGFR] <60 mL/min/1.73 m^2) who had failed treatment with allopurinol (serum uric acid concentrations remained ≥6.0 mg/dL) and who had been treated with febuxostat for 6 months or longer. The trial was performed in accordance with Japanese Good Clinical Practice and Good Postmarketing Surveillance Practice guidelines, and in line with the ethical principles set out in the Declaration of Helsinki.

Data regarding serum uric acid concentrations and serum creatinine levels in the 6-month periods before and after the start of febuxostat treatment were extracted from patients' electronic medical records. The usual laboratory methods employed by the hospital were used throughout the study. Estimated GFR was calculated using a formula based on serum creatinine developed by the Japanese Society of Nephrology for the Japanese population [eGFR (mL/min/1.73 m^2) = $194 \times Cr^{-1.094} \times Age^{-0.287}$ (×0.739 if women).[1] Based on monthly eGFR values a regression line was calculated for each subject to determine the rate of change in eGFR per 6-month period, and this was defined as the slope of the linear relationship between renal function and time for each individual.[11]

Statistical analysis

Changes in serum uric acid concentrations and eGFR in the 6-month periods before and after the switch to febuxostat were the primary assessment parameters. Each measurement was presented as a mean ± standard deviation and a paired *t*-test was used to determine statistical significance. The relationship between change in serum uric acid concentrations and change in eGFR at 6 months after the switch to febuxostat was investigated using Pearson's correlation coefficient and Spearman's rank correlation coefficient. Estimated GFR slopes in the 6 months before and after the switch to febuxostat were compared and the paired *t*-test was used to determine statistical significance. For all statistical analyses $p<0.05$ was considered statistically significant. Analysis software was SAS version 9 in a Windows 7 operating system (SAS® Software, Cary, NC).

Results

Patient characteristics

Sixty patients were able to be followed for 6 months after switching from allopurinol to febuxostat. Baseline characteristics are presented in Table 1. Most patients were male (73%) and the mean age of the cohort was 63.1 (±16.6) years. Mean serum uric acid concentration before switching to febuxostat was 8.4 (±1.4) mg/dL (range: 6.2–13.1 mg/dL). Two-thirds of patients ($n=40$) had advanced CKD (eGFR <30 mL/min/1.73 m^2). Primary conditions underlying renal disease were hypertension ($n=15$), chronic glomerulonephritis ($n=21$), and diabetic nephropathy ($n=10$), while other less common causes were responsible for the remaining 14 cases (Table 1). Four patients had a history of gouty kidney disease of mean duration 6.5 (±2.1) years. In terms of concomitant medications, 45 patients were being treated with an angiotensin converting enzyme (ACE) antagonist or angiotensin II receptor blocker (ARB), none were prescribed a non-steroidal anti-inflammatory drug (NSAID) on a regular basis, and alcohol was used only occasionally (no heavy drinkers were included in this cohort).

Febuxostat was administered at a daily dose of 10 mg in 33 patients, 20 mg in 21 patients, 30 mg in 3 patients, and 40 mg in 3 patients. The mean dose in all patients was 15.9 (±8) mg/day. The mean allopurinol dose prior to the switch was 71.3 (±29.5) mg/day.

Serum uric acid concentrations

Changes in serum uric acid concentrations in the 6-month periods before and after the switch from allopurinol to febuxostat are shown in Figure 1. The mean serum uric acid concentration was significantly ($p<0.001$) reduced from baseline at 1 month after the switch to febuxostat and remained significantly lower throughout treatment. At 6 months, the mean serum uric acid concentration was 6.2 (±1.2) mg/dL. Nearly half the population (47.5%) achieved a serum uric acid concentration ≤6.0 mg/dL. Reduction in the mean serum uric acid concentration was satisfactory irrespective of gender (Figure 2a) or baseline renal function (Figure 2b).

eGFR

After switching to febuxostat, there was a positive change from baseline in the mean eGFR at all time points (Table 2). The increase of 2.3 (±5.6) mL/min/1.73 m^2 at 6 months was significant ($p=0.0027$) versus baseline. No clear correlation between the change in serum uric acid concentrations and change in eGFR after 6 months of febuxostat treatment was found overall or in most patient

Table 1. Baseline characteristics of the study population.

Baseline characteristics	All patients $n=60$	Male patients $n=44$ (73%)	Female patients $n=16$ (27%)
Age (years)			
Mean (±SD)	63.1 ± 16.6	62.8 ± 16.4	63.9 ± 17.7
Range	29–98	30–89	29–98
Serum uric acid concentration (mg/dL)			
Mean (±SD)	8.4 ± 1.4	8.5 ± 1.4	8.2 ± 1.6
Range	6.2–13.1	6.3–13.1	6.2–12.1
<7 mg/dL (*n*, %)	6 (10.0)	4 (9.1)	2 (12.5)
7–8 mg/dL (*n*, %)	16 (26.7)	10 (22.7)	6 (37.5)
8–9 mg/dL (*n*, %)	25 (41.7)	20 (45.5)	5 (31.3)
≥9 mg/dL (*n*, %)	13 (21.7)	10 (22.7)	3 (18.8)
eGFR (ml/min/1.73 m^2)			
Mean (± SD)	27.2 ± 19.2	29.8 ± 20.7	20.2 ± 12.0
Range	3.1–89.8	4.0–89.8	3.1–43.7
<15 mL/min/1.73 m^2 (*n*, %)	18 (30.0)	12 (27.3)	6 (37.5)
15–29 mL/min/1.73 m^2 (*n*, %)	22 (36.7)	15 (34.1)	7 (43.8)
30–44 mL/min/1.73 m^2 (*n*, %)	11 (18.3)	8 (18.2)	3 (18.8)
45–59 mL/min/1.73 m^2 (*n*, %)	4 (6.7)	4 (9.1)	0
≥60 mL/min/1.73 m^2 (*n*, %)	5 (8.3)	5 (11.4)	0
Underlying morbidity			
Hypertension	15 (25.0)	11 (25.0)	4 (25.0)
Chronic glomerulonephritis (*n*, %)	21 (35.0)	16 (36.4)	5 (31.3)
IgA nephropathy (*n*, %)	12 (20.0)	8 (18.2)	4 (25.0)
Membranous nephropathy (*n*, %)	7 (11.7)	6 (13.6)	1 (6.3)
Focal segmental glomerulosclerosis (*n*, %)	1 (1.7)	1 (2.3)	0
Biopsy not performed (*n*, %)	1 (1.7)	1 (2.3)	0
Diabetic nephropathy (*n*, %)	10 (16.7)	6 (13.6)	4 (25.0)
Gouty nephropathy (*n*, %)	4 (6.7)	4 (9.1)	0
Polycystic kidney (*n*, %)	4 (6.7)	3 (6.8)	1 (6.3)
Interstitial nephritis (*n*, %)	2 (3.3)	1 (2.3)	1 (6.3)
Rheumatoid arthritis (*n*, %)	2 (3.3)	1 (2.3)	1 (6.3)
MRSA nephropathy (*n*, %)	1 (1.7)	1 (2.3)	0
Renal cell carcinoma (*n*, %)	1 (1.7)	1 (2.3)	0

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; SD, standard deviation.

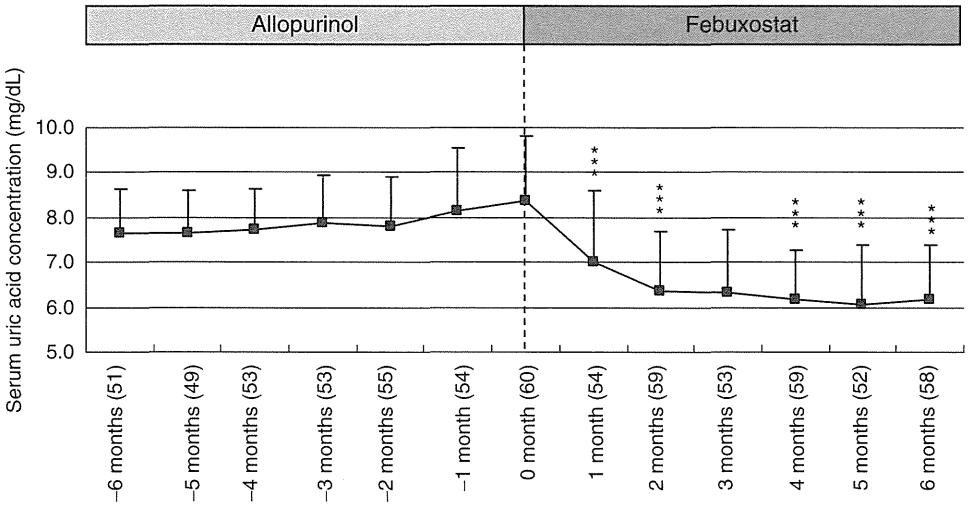


Figure 1. Change in serum uric acid concentration (mean ± SD) before and after the start of febuxostat treatment (number in parentheses indicates number of patients). ***$p<0.001$ versus Month 0 (paired *t*-test).

subgroups stratified by gender or baseline eGFR (Table 3). A significant inverse correlation ($r=-0.70$; $p=0.0013$) was detected only in patients whose baseline eGFR was <15 mL/min/1.73 m^2 (Table 3). Subsequent examination of eGFR before and after the switch to febuxostat in this particular patient group showed that, in contrast to the steady decline in renal function during allopurinol treatment, there was a tendency towards improvement in eGFR during febuxostat treatment (Figure 3).

Accordingly, eGFR slopes in the 6-month periods before and after the switch to febuxostat were evaluated to determine the effect of active reduction of serum uric acid concentrations on renal function. Whereas the eGFR slope was negative during allopurinol treatment, it became positive after the switch to febuxostat (Figure 4) and was positive irrespective of gender (Figure 5a) or baseline renal function (Figure 5b). The change in eGFR slope before and after febuxostat treatment was significant for all patients ($p<0.01$), for male

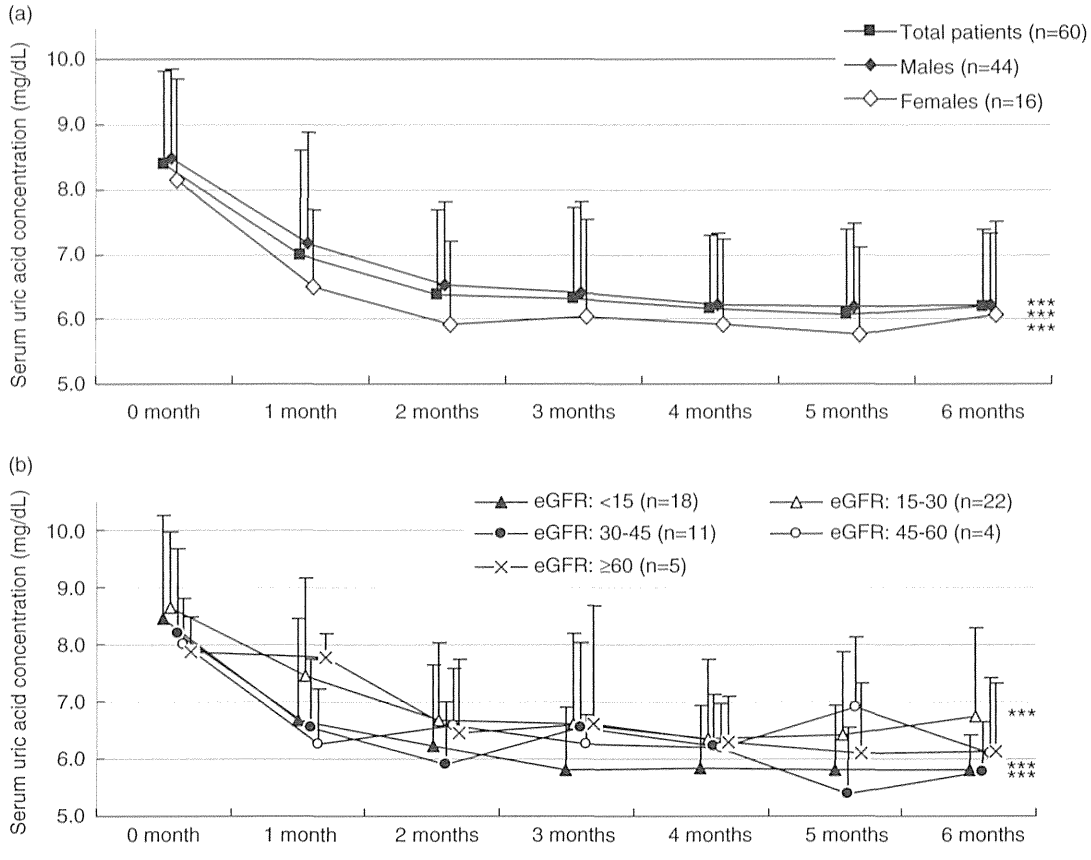


Figure 2. Change in serum uric acid concentrations (mean ± SD) during febuxostat treatment stratified by (a) gender and (b) baseline eGFR (mL/min/1.73 m^2). ***$p < 0.001$ versus Month 0 (paired t-test).

Table 2. Change from baseline in eGFR (mL/min/1.73 m^2) after the start of febuxostat treatment.

	Total			Male			Female		
	n	Mean ± SD	p Value*	n	Mean ± SD	p Value*	n	Mean ± SD	p Value*
Baseline (Month 0)	60	27.2 ± 19.2	–	44	29.8 ± 20.7	–	16	20.2 ± 12.0	–
Month 1	54	1.2 ± 4.1	0.0416	40	0.9 ± 4.1	0.1672	14	1.8 ± 4.0	0.1076
Month 2	59	1.8 ± 6.0	0.0252	44	1.9 ± 6.7	0.0650	15	1.5 ± 3.6	0.1294
Month 3	53	0.7 ± 4.3	0.2308	38	0.6 ± 4.9	0.4638	15	1.1 ± 2.4	0.1082
Month 4	59	0.6 ± 5.7	0.4354	44	0.6 ± 6.2	0.5420	15	0.6 ± 3.7	0.5525
Month 5	52	0.8 ± 4.4	0.2008	38	0.7 ± 4.7	0.3588	14	1.0 ± 3.7	0.3165
Month 6	58	2.3 ± 5.6	0.0027	42	2.5 ± 5.8	0.0074	16	1.7 ± 5.1	0.1998

Symbols: *versus Month 0 (paired t-test).

Table 3. Correlation between the change in serum uric acid concentrations and change in eGFR at 6 months after the start of febuxostat treatment.

Patient group	Pearson correlation coefficient (r)	p Value
Total ($n = 58$)	−0.10	0.4747
Male (42)	−0.02	0.8860
Female (16)	−0.29	0.2696
eGFR at Month 0: <15 mL/min/1.73 m^2 (18)	−0.70	0.0013
eGFR at Month 0: 15–30 mL/min/1.73 m^2 (21)	0.21	0.3694
eGFR at Month 0: 30–45 mL/min/1.73 m^2 (11)	−0.52	0.1022
eGFR at Month 0: 45–60 mL/min/1.73 m^2 (3)	−0.97	0.1685
eGFR at Month 0: ≥60 mL/min/1.73 m^2 (5)	0.71	0.1835

patients ($p < 0.05$), and for patients with a baseline eGFR <15 mL/min/1.73 m^2 ($p < 0.05$).

Adverse events

A slight increase in transaminase levels was recorded in a single patient after switching to febuxostat, but the event was not considered clinically significant and febuxostat treatment was continued as before.

Discussion

Hyperuricemia is frequently associated with hypertension, diabetes, dyslipidemia, and other metabolic disorders.[1] As uric acid is cleared through the kidneys, a decline in renal function can lead to hyperuricemia especially in elderly persons long affected by these lifestyle diseases. In turn, it is

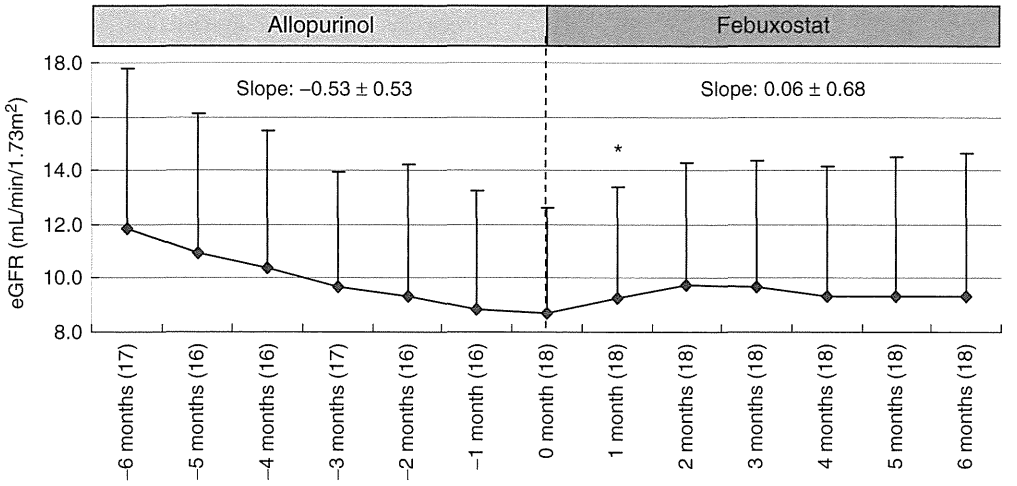
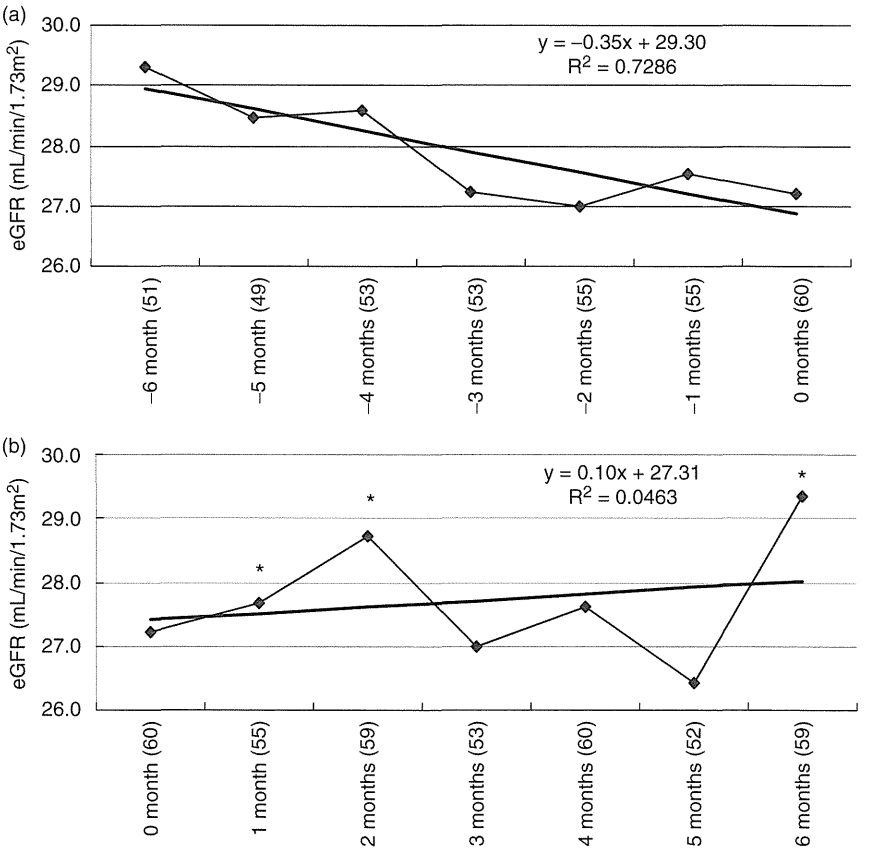


Figure 3. Change in eGFR (mean ± SD) before and after the start of febuxostat treatment in patients with a baseline eGFR <15 mL/min/1.73 m^2 (number in parentheses indicates number of patients). *$p < 0.05$ versus Month 0 (paired t-test).

Figure 4. Change in eGFR slope (mean ± SD) before (a) and after (b) the start of febuxostat treatment (number in parentheses indicates number of patients). *$p < 0.05$ versus Month 0 (paired t-test).



becoming increasingly accepted that hyperuricemia contributes to the development and progression of renal pathology by both direct and indirect mechanisms. In the current study, retrospective analyses were performed to investigate the effects of switching from the conventional urate-lowering agent, allopurinol, to the novel urate-lowering agent, febuxostat, on uric acid control and renal function in patients with CKD.

Febuxostat produced significant reductions in mean serum uric acid concentrations from Month 1 onwards. Nearly half the patient sample (47.5%) reached the target serum uric acid concentration of ≤6.0 mg/dL with a 10–20 mg dose; even greater efficacy could be expected with higher doses. Importantly, the slow but steady decline in eGFR observed with allopurinol in the 6 months prior to the switch was halted by febuxostat and there was a tendency towards improvement. Although data were not presented due to small sample sizes, no major differences were observed in the efficacy of febuxostat according to presence or type of underlying disease (e.g., hypertension, glomerulonephritis, etc). Taken together, these findings suggest that febuxostat may be useful in the maintenance of renal function in patients with CKD.

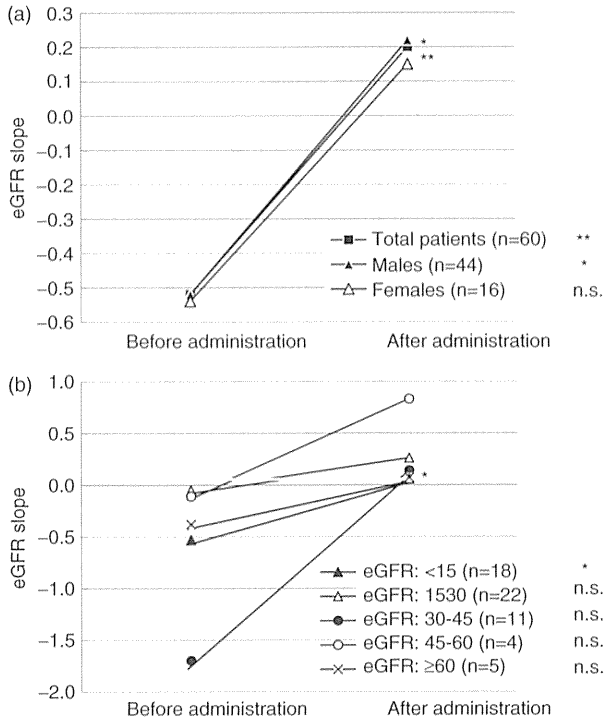


Figure 5. Change in eGFR slope (mean ± SD) before and after febuxostat treatment stratified by (a) gender and (b) baseline eGFR (mL/min/1.73 m^2). *$p<0.05$ and **$p<0.01$ versus before treatment (paired t-test).

A study from the US which examined the long-term (5-year) effects of febuxostat on eGFR found an inverse correlation between the quantitative reduction from baseline in serum uric acid concentrations and maintenance or improvement of eGFR.[12] In the current study, this relationship was detected only in the subgroup with a baseline eGFR <15 mL/min/1.73 m^2. Studies over a longer term and with larger sample sizes should help confirm the inverse association between the magnitude of reduction in serum urate levels and improvement in renal function.

Uric acid is known to be a potent antioxidant. However, studies *in vivo* and *in vitro* have suggested that uric acid may induce oxidative stress and inflammation in vascular endothelial cells leading to reduced production of nitric oxide[13] or that it may induce renal arteriopathy or tubulointerstitial fibrosis through activation of the renin–angiotensin system.[14] Both sets of results suggest that elevation of serum uric acid concentrations can lead to renal impairment, and support the establishment of uric acid as a therapeutic target to prevent the onset and progression of CKD.[2] Indeed, support for uric acid as a target of therapy in CKD derives from several sources. In an investigation of 167 patients who underwent renal biopsy, higher serum uric acid concentrations were associated with a greater degree of hyaline degeneration and arterial wall thickening in the renal artery.[15] Elsewhere, high-dose allopurinol was shown to exhibit hypotensive activity in hyperuricemic adolescents with newly-diagnosed hypertension.[16] Hiramitsu and colleagues reported blood pressure improvement in patients with hyperuricemia treated for 12–16 weeks with febuxostat under general practice conditions.[17] Reducing serum uric acid concentrations may improve vascular endothelial dysfunction, leading to functional and structural improvement of blood vessels. Although the effect of febuxostat on blood pressure was not analyzed in the current study, this same mechanism may underlie the improvement in eGFR observed with febuxostat.

Guidelines for treatment of hyperuricemia/gout recommend a target serum uric acid concentration of ≤6.0 mg/dL,[3] or even <5.0 mg/dL,[18] to achieve durable control of the signs and symptoms of gout. These recommendations stem from the need to maintain serum uric acid concentrations below the dissolution limit of extracellular fluid. In the current study, it was considered that removing urate deposits in the kidney was one of the factors underlying the tendency towards improvement in eGFR seen with febuxostat.

At our center we have experienced other cases in which urate crystals identified on kidney sonogram following insufficient reduction of serum uric acid concentrations with allopurinol disappeared when levels were maintained below 6.0 mg/dL by treatment with febuxostat. In the study of Goicoechea and colleagues, 113 CKD patients with eGFR <60 mL/min/1.73 m^2 were randomly assigned to receive allopurinol 100 mg/day or continue with usual therapy. After a mean follow-up of 24 months, serum uric acid concentrations were reduced and the decline in eGFR was suppressed in the group receiving allopurinol compared to the control group. Serum uric acid concentrations were reduced from 7.8 to 6.0 mg/dL in the allopurinol group but remained unchanged (7.3 to 7.5 mg/dL) in the control group.[19] These findings highlight the benefits that can be achieved by reducing and maintaining serum uric acid concentrations at around 6.0 mg/dL in patients with impaired renal function.

It has been demonstrated clinically that febuxostat inhibits xanthine oxidase more strongly than allopurinol and has a potent uric acid-reducing effect. In addition, its inhibitory effect on xanthine oxidase-dependent reactive oxygen species formation was shown to be 1000-fold greater than that of allopurinol.[20] Recently, Isaka and co-workers reported that febuxostat showed inhibitory activity on oxidative stress markers including nitrotyrosine and thiobarbituric acid reactive substances (TBARS), producing an anti-inflammatory and renal protective effect in animal models with renal ischemia-reperfusion injury and ureteral ligation.[21,22] Thus, the renal protective effect of febuxostat may not only be due to its potent uric acid-reducing effect, but also to inhibition of oxidative stress through potent inhibition of xanthine oxidase in pathological conditions related to reduced renal function.

As this was an exploratory, retrospective study it has a number of limitations. In particular, the number of patients with data available for analysis was small for some subgroup analyses. As such, the findings should be interpreted with caution. The observation period of 6 months was relatively short and precludes extrapolating the results over the longer term. Nevertheless, it was highly encouraging to demonstrate the uric acid-lowering efficacy of febuxostat and the trend towards slowing the progression of renal dysfunction in patients with CKD. Prospective, long-term, large-scale, randomized comparative studies are required to further elucidate the significance of actively treating hyperuricemia in patients with CKD.

Acknowledgments

Assistance with English language was kindly provided by Content Ed Net.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Japanese Society of Nephrology. Evidence-based practice guideline for the treatment of CKD. *Clin Exp Nephrol.* 2009;13(6):537–566.
2. Japanese Society of Nephrology. *CKD treatment guidance 2012* [Article in Japanese]. Tokyo: Tokyo Igakusha; 2012.
3. Yamanaka H; Japanese Society of Gout and Nucleic Acid Metabolism. Japanese guideline for the management of hyperuricemia and gout: second edition. *Nucleosides Nucleotides Nucleic Acids.* 2011;30(12):1018–1029.
4. Iseki K, Ikemiya Y, Inoue T, Iseki C, Kinjo K, Takishita S. Significance of hyperuricemia as a risk factor for developing ESRD in a screened cohort. *Am J Kidney Dis.* 2004;44(4):642–650.
5. Okamoto K. A new-generation uric acid production inhibitor febuxostat – comparison of the XO inhibition mechanism with allopurinol [Article in Japanese]. *Yakuri to Chiryo.* 2010; 38(11):955–962.
6. Kamatani N, Fujimori S, Hada T, et al. An allopurinol-controlled, randomized, double-dummy, double-blind, parallel between-group, comparative study of febuxostat (TMX-67), a non-purine selective inhibitor of xanthine oxidase, in patients with hyperuricemia including those with gout in Japan: phase 3 clinical study. *J Clin Rheumatol.* 2011;17(4 Suppl 2):S13–S18.
7. Becker MA, Schumacher Jr HR, Espinoza LR, et al. The urate-lowering efficacy and safety of febuxostat in the treatment of the hyperuricemia of gout: the CONFIRMS trial. *Arthritis Res Ther.* 2010;12(2):R63. doi: 10.1186/ar2978.
8. Schumacher Jr HR, Becker MA, Wortmann RL, et al. Effects of febuxostat versus allopurinol and placebo in reducing serum urate in subjects with hyperuricemia and gout: a 28-week, Phase III, randomized, double-blind, parallel-group trial. *Arthritis Rheum.* 2008;59(11):1540–1548.
9. Becker MA, Schumacher Jr HR, Wortmann RL, et al. Febuxostat compared with allopurinol in patients with hyperuricemia and gout. *New Engl J Med.* 2005;353:2450–2461.
10. Becker MA, Schumacher HR, MacDonald PA, et al. Clinical efficacy and safety of successful long term urate lowering with febuxostat or allopurinol in subjects with gout. *J Rheum.* 2009; 36(6):1273–1282.
11. Hanratty R, Chonchol M, Dickinson LM, et al. Incident chronic kidney disease and the rate of kidney function decline in individuals with hypertension. *Nephrol Dial Transplant.* 2010; 25(3):801–807.
12. Whelton A, Macdonald PA, Zhao L, Hunt B, Gunawardhana L. Renal function in gout: long-term treatment effects of febuxostat. *J Clin Rheumatol.* 2011;17(1):7–13.
13. Jalal DI, Chonchol M, Chen W, Targher G. Uric acid as a target of therapy in CKD. *Am J Kidney Dis.* 2013;61(1):134–146.
14. Yu MA, Sánchez-Lozada LG, Johnson RJ, Kang DH. Oxidative stress with an activation of the renin–angiotensin system in human vascular endothelial cells as a novel mechanism of uric acid-induced endothelial dysfunction. *J Hypertens.* 2010; 28(6):1234–1242.
15. Kohagura K, Kochi M, Miyagi T, et al. An association between uric acid levels and renal arteriolopathy in chronic kidney disease: a biopsy-based study. *Hypertens Res.* 2013;36(1):43–49.
16. Feig DI, Soletsky B, Johnson RJ. Effect of allopurinol on blood pressure of adolescents with newly diagnosed essential hypertension: a randomized trial. *JAMA.* 2008;300(8):924–932.
17. Hiramitsu S, Ishiguro Y, Matsuyama H, et al. Investigation of the utility of febuxostat (Feburic® tablet) in patients with hyperuricemia – multi-center collaborative study by general practitioners [Article in Japanese]. *Blood Pressure.* 2012;19: 395–402.
18. Khanna D, Fitzgerald JD, Khanna PP, et al. 2012 American College of Rheumatology guidelines for management of gout. Part 1: systematic nonpharmacologic and pharmacologic therapeutic approaches to hyperuricemia. *Arthritis Care Res (Hoboken).* 2012;64(10):1431–1446.
19. Goicoechea M, de Vinuesa SG, Verdalles U, et al. Effect of allopurinol in chronic kidney disease progression and cardiovascular risk. *Clin J Am Soc Nephrol.* 2010;5(8):1388–1393.
20. Malik UZ, Hundley NJ, Romero G, et al. Febuxostat inhibition of endothelial-bound XO: implications for targeting vascular ROS production. *Free Radic Biol Med.* 2011;51(1):179–184.
21. Omori H, Kawada N, Inoue K, et al. Use of xanthine oxidase inhibitor febuxostat inhibits renal interstitial inflammation and fibrosis in unilateral ureteral obstructive nephropathy. *Clin Exp Nephrol.* 2012;16(4):549–556.
22. Tsuda H, Kawada N, Kaimori JY, et al. Febuxostat suppressed renal ischemia–reperfusion injury via reduced oxidative stress. *Biochem Biophys Res Commun.* 2012;427(2):266–272.

PLOS | ONE

RESEARCH ARTICLE

Acute Graft-Versus-Host Disease of the Kidney in Allogeneic Rat Bone Marrow Transplantation

Seiichiro Higo[1,2], Akira Shimizu[2]*, Yukinari Masuda[2], Shinya Nagasaka[2], Yusuke Kajimoto[2], Go Kanzaki[2], Megumi Fukui[1], Kiyotaka Nagahama[2], Akiko Mii[1], Tomohiro Kaneko[1], Shuichi Tsuruoka[1]

1. Department of Nephrology, Nippon Medical School, Tokyo, Japan, **2.** Department of Analytic Human Pathology, Nippon Medical School, Tokyo, Japan

*ashimizu@nms.ac.jp

Abstract

Allogeneic hematopoietic cell or bone marrow transplantation (BMT) causes graft-versus-host-disease (GVHD). However, the involvement of the kidney in acute GVHD is not well-understood. Acute GVHD was induced in Lewis rats ($RT1^l$) by transplantation of Dark Agouti (DA) rat ($RT1^a$) bone marrow cells (6.0×10^7 cells) without immunosuppression after lethal irradiation (10 Gy). We examined the impact of acute GVHD on the kidney in allogeneic BMT rats and compared them with those in Lewis-to-Lewis syngeneic BMT control and non-BMT control rats. In syngeneic BMT and non-BMT control rats, acute GVHD did not develop by day 28. In allogeneic BMT rats, severe acute GVHD developed at 21–28 days after BMT in the skin, intestine, and liver with decreased body weight (>20%), skin rush, diarrhea, and liver dysfunction. In the kidney, infiltration of donor-type leukocytes was by day 28. Mild inflammation characterized by infiltration of CD3+ T-cells, including CD8+ T-cells and CD4+ T-cells, and CD68+ macrophages to the interstitium around the small arteries was noted. During moderate to severe inflammation, these infiltrating cells expanded into the peritubular interstitium with peritubular capillaritis, tubulitis, acute glomerulitis, and endarteritis. Renal dysfunction also developed, and the serum blood urea nitrogen (33.9 ± 4.7 mg/dL) and urinary N-acetyl-β-D-glucosaminidase (NAG: 31.5 ± 15.5 U/L) levels increased. No immunoglobulin and complement deposition was detected in the kidney. In conclusion, the kidney was a primary target organ of acute GVHD after BMT. Acute GVHD of the kidney was characterized by increased levels of urinary NAG and cell-mediated injury to the renal microvasculature and renal tubules.

CrossMark
click for updates

OPEN ACCESS

Citation: Higo S, Shimizu A, Masuda Y, Nagasaka S, Kajimoto Y, et al. (2014) Acute Graft-Versus-Host Disease of the Kidney in Allogeneic Rat Bone Marrow Transplantation. PLoS ONE 9(12): e115399. doi:10.1371/journal.pone.0115399

Editor: Valquiria Bueno, UNIFESP Federal University of São Paulo, Brazil

Received: July 5, 2014

Accepted: November 21, 2014

Published: December 26, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

Funding: The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Allogeneic hematopoietic cell transplantation (HCT) is a clinical treatment for a variety of conditions, including hematologic disorders, metabolic storage diseases, immune deficiencies, and is used as a rescue technique after cancer treatment [1, 2]. Despite improved outcomes following HCT, renal impairments remain a common complication. Acute kidney injury has been reported to manifest in approximately 70% of HCT recipients [3]. Acute kidney injury itself is an important risk factor for the development of chronic kidney disease, and is associated with increased short- and long-term mortality following HCT [4]. Therefore, strategies to preserve renal function in patients receiving HCT should be implemented, given the potential for positive patient outcomes. Often, the accurate etiology of post-transplant renal dysfunction cannot be diagnosed, as renal biopsy is rarely performed in the peri-transplantation period.

In patients with HCT, multiple factors have been linked to the development of renal impairments, including preexisting renal injury, direct effects of conditioning chemotherapy and irradiation, complications of the infused cryopreserved cells, tumor lysis syndrome, calcineurin inhibitor used for graft-versus-host disease (GVHD) prophylaxis, and infection and its treatment [3, 5–7]. Despite the multiple etiologies of post-transplant renal dysfunction, GVHD has rarely been linked to the kidney, and most physicians believe that the kidney is not a target of acute GVHD. However, several recent studies have demonstrated chronic GVHD of the kidney that resulted in nephrotic syndrome [8, 9]. In addition, some studies suggest that acute GVHD may also develop in the kidney after HCT [10, 11].

In the present study, to clarify whether acute GVHD develops in the kidney, we used the major histocompatibility complex–disparate rat allogeneic bone marrow transplantation (BMT) model. We used the already established rat GVHD model, which involves transplantation of bone marrow cells (BMCs) from DA rats ($RT1^a$, $RT1A^a$) into lethally irradiated Lewis rat ($RT1^l$, $RT1A^l$) recipients without immunosuppression [12]. Although, this rat BMT model is different from clinical HCT in human, this model is considered to be useful to evaluate the acute GVHD on the kidney, because severe and acute GVHD develops within 21 days after BMT in this model.

Materials and Methods

Animals

The animal experiments described in this study were approved by the Animal Experiments Ethical Review Committee of Nippon Medical School (protocol no. 26–122). We used inbred male DA and Lewis rats (Charles River Japan, Kanagawa, Japan) that weighed 190–220 g and 220–270 g, respectively. All animals received humane care in compliance with the Guideline by the Committee of Nippon Medical School.

Bone Marrow Transplantation

BMC suspensions were harvested from DA and Lewis rats by flushing the marrow from the femurs and tibias with cold RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 2.5% fetal bovine serum and 25 mM HEPES. Recipient Lewis rats were irradiated with a dose of 10 Gy (MBR 1505R2; type PI-CR-1505R2, type MI-RC-3E, Hitachi Medical Corporation, Japan) prior to BMT. After 2–3 h, 6.0×10^7 BMCs from the DA or Lewis rats were then injected into Lewis rat recipients via the tail vein.

In this model, acute GVHD developed by day 21 to day 28 in allogeneic BMT rats. The growth of transplanted BMCs, body weight, degree of acute GVHD, liver and renal functions, pathology, and cytokines milieu were evaluated by day 28 in allogeneic BMT rats (n=5 at each time point), Lewis-to-Lewis syngeneic BMT control rats (n=5 at each time point), and non-BMT control rats (n=3 at each time point).

Reconstruction of Transplanted BMCs

To examine the reconstruction of transplanted BMCs, blood samples were collected on days 4, 7, 14, 21, and 28 after BMT from the tail vein, to measure the number of white blood cells (Full Automatic Blood Cell Counter, model: PCE-210N, ERMA. INC), and flow cytometry was conducted to assess the expression of $RT1A^a$ (donor-type cells), CD6+ T-cells, CD8+ T-cells, CD4+ T-cells, and CD68+ macrophages. Peripheral blood mononuclear cells were treated with anti-mouse CD16/32 Ab (clone 2.4G2) to block the Fc-receptors followed by direct or indirect staining of fluorochrome-conjugated antibodies (Table 1). Dead cells were identified and excluded using propidium iodide. Cell suspensions were analyzed on a FACSCanto II flow cytometer (BD Biosciences).

Systemic Analysis of GVHD

The degree of systemic GVHD was assessed using a standard scoring system that incorporated five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity [13]. Each parameter was evaluated and graded from 0 to 2. A clinical index was subsequently generated by the sum of the five criteria scores (maximum index =10). The skin, liver, intestine, and kidney from allogeneic BMT rats were examined pathologically at day 28 after BMT. As controls, the skin, liver, intestine, and kidney from non-BMT control Lewis rats and from Lewis-to-Lewis syngeneic BMT control rats were prepared at day 28 after BMT. Blood samples were collected on day 28 from allogeneic and syngeneic BMT rats and non-BMT control rats to examine liver function (total bilirubin [T-Bil], aspartate aminotransferase [AST], and alanine aminotransferase [ALT]), as well as renal function (serum creatinine [Cr], blood urea nitrogen [BUN]), using an autoanalyzer (SRL, Tokyo, Japan). Urine was also collected on day 28, to examine proteinuria and urinary N-acetyl-β-D-glucosaminidase (NAG) levels (SRL, Tokyo, Japan).

Table 1. The kinds of reagents.

*	Reagent	Target	Clone	Conjugate	Company
Flow, IF	Anti-rat RT1A^{a,b} Ab	MHC class I	C3	FITC	BioLegend, San Diego, CA
IF	Anti-rat CD3 Ab	T cell	1F4	FITC	BioLegend, San Diego, CA
IF	Anti-rat CD4 Ab	CD4+ T cell	10B5	Purified	Abcam, Tokyo, Japan
Flow	Anti-rat CD4 Ab	CD4+ T cell	W3/25	APC-Cy7	BioLegend, San Diego, CA
Flow	Anti-rat CD6 Ab	T cell	OX-52	FITC	BD Pharmingen, San Diego, CA
Flow, IF	Anti-rat CD8α Ab	CD8+ T cell	G28	PE	BioLegend, San Diego, CA
IF	Anti-rat CD45 Ab	White blood cell	OX-1	PE-Cy7	BioLegend, San Diego, CA
IHC	Anti-human CD3 Ab	T cell	Rabbit, Polyclonal	Purified	DAKO, Glostrup, Denmark
IHC	Anti-rat CD8α Ab	CD8+ T cell	OX-8	Purified	BioLegend, San Diego, CA
IHC	Anti-rat CD68 Ab	Macrophage	ED1	Purified	BMA BIOMEDICALS, Augst, Switzerland
Flow	Anti-rat Granulocyte Marker Ab	Monocyte, Macrophage	HIS48	Biotin	eBioscience, San Diego, CA
Flow	Streptavidin			PE-Cy7	BioLegend, San Diego, CA
Flow, IF	Anti-mouse CD16/32 Ab	Fcγ receptor III/II	2.4G2	Culture sup	ATCC**, Manassas, VA
IF	Anti-mouse IgG1 Ab	IgG1	Goat, Polyclonal	TRITC***	SouthernBiotech, Birmingham, AL
IF	Anti-rat IgM Ab	IgM	Goat, Polyclonal	FITC	MP Biomedicals, Tokyo, Japan
IF	Anti-rat IgG Ab	IgG	Rabbit, Polyclonal	FITC	MBL, Nagoya, Japan
IF	Anti-rat C3 Ab	C3	Goat, Polyclonal	FITC	MP Biomedicals, Tokyo, Japan
IF	Anti-rat RT1B Ab	MHC class II	OX-6	PE	BioLegend, San Diego, CA

*Assessment: Flow: Flow cytometory; IHC: Immunohistochemistry; IF: Immunofluorescence.
**American Type Culture Collection.
***Tetramethylrhodamine isothiocyanate.

doi:10.1371/journal.pone.0115399.t001

Pathology and Immunohistochemistry

To study the histological features of GVHD, the skin, liver, intestine, and kidney tissues were fixed in 20% buffered formalin and embedded in paraffin for light microscopic examination. Tissues were stained with hematoxylin and eosin (H&E) and periodic and acid-Schiff (PAS) staining for histopathological examination, and naphthol AS-D chloroacetate esterase staining to detect neutrophils. The primary antibodies used for immunohistochemistry are indicated in Table 1. To detect infiltrating CD3+ T-cells, CD8+ T-cells and CD68+ macrophages, 20% -buffered, formalin-fixed, paraffin-embedded tissue sections were stained by the standard avidin-biotin-peroxidase complex technique. Before incubation with the primary antibody, for ED1+ and CD3+ detection, tissue sections were incubated with 0.1% pepsin for 45 min. For CD8+ detection, the sections were treated with 1 mM EDTA (pH 8.0) in hot water at 90℃ for 3 h.

In each kidney sample, the number of infiltrating CD3+ T-cells, CD8+ T-cells, and CD68+ macrophages was examined in 40 randomly selected interstitial fields at ×200 magnification, by an investigator blinded to the clinical or histological findings.

Immunofluorescence study was also performed using frozen tissues. The deposition of IgM, IgG, and C3 was evaluated using the indirect method. To

detect the donor type of leukocytes in the kidney, double stain with fluorescein isothiocyanate (FITC)-conjugated anti-rat $RT1A^{a,b}$ antibody (donor type of $RT1A^{a}$) and phycoerythrin (PE)-conjugated anti-rat CD45 antibody (leukocyte common antigen) was performed. To detect CD8+ T-cells or CD4+ T-cells, double stain with FITC-conjugated anti-rat CD3 antibody and PE-conjugated anti-rat CD8α antibody or anti-rat CD4 antibody was performed. To evaluate the expression of MHC class II in renal tubules, immunostaining with PE-conjugated anti-rat RT1B antibody was performed. In each kidney sample, more than 100 cross-sections of renal tubules were graded semiquantitatively based on the specimens stained for rat RT1B, using the following grading system: absence of MHC class II staining; 0, mild increase of MHC class II staining; 1, moderate increase of MHC class II staining; 2, marked increase of MHC class II staining; 3.

Real-time Reverse Transcription-Polymerase Chain Reaction for Cytokines

Real-time reverse transcription-polymerase chain reaction (Real-time RT-PCR) was performed as described previously [14], to examine the mRNA expression levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, and IL-17 in the kidney. Total renal RNA was extracted using the ISOGEN (Nippon Gene, Japan) according to the manufacturer's protocol. The purified total RNA was 1.9–2.2 of A260/A280. cDNA libraries were created with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol from 2 μg of total RNA. The gene expression levels were analyzed by quantitative RT-PCR using the THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) according to the manual supplied by the manufacturer (ABI PRISM 7900 HT, Applied Biosystems). The normalized value for mRNA expression in each sample was calculated as the relative quantity of relevant primers divided by the relative quantity of the housekeeping gene, β-actin. The RT-PCR primer sequences used in this study are listed in Table 2. Quantification was performed using the SDS 2.3 software program (Applied Biosystems).

Statistical Analysis

The results were expressed as mean ± standard deviation. Differences were evaluated using either the Student *t*-test using an analytical software program (Excel, Microsoft, Redmond, WA) or ANOVA determined by analysis of variance which performed Fisher's one. Differences were considered significant at the 95% confidence intervals ($P<0.05$).