Active vitamin D and its analogue, 22-oxacalcitriol, ameliorate puromycin aminonucleoside-induced nephrosis in rats

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Abstract

Background. Recent studies have demonstrated that podocyte injury, which results in proteinuria, leads to tubulointerstitial fibrosis. Although some studies have revealed that vitamin D administration protects renal structure and function in mesangial cell proliferative and/or excessive matrix productive models, the effects of vitamin D on podocyte injury have remained uncertain.

Methods. In this study, we examined whether administration of active vitamin D (calcitriol) or its analogue, 22-oxacalcitriol (maxacalcitol), is preventative in podocyte injury using the puromycin aminonucleoside nephrosis model with neither mesangial proliferation nor matrix accumulation.

Results. Before the onset of proteinuria, renal 1α-hydroxylase and 24-hydroxylase were markedly down-regulated and up-regulated, respectively, leading to impaired vitamin D activation. Thereafter, serum 25-hydroxyvitamin D decreased along with the increased excretion of vitamin D-binding protein in urine. After confirming that podocytes express vitamin D receptor and all retinoid X receptors (RXRs) except RXR-α, we found that daily administration of calcitriol or its analogue 22-oxacalcitriol ameliorated the nephrotic state by protecting podocytes, as shown by the reduced staining of desmin (podocyte injury marker) and the upregulation of nephrin and podocin. These data suggest that the impairment of the vitamin D system plays a role in increasing proteinuria in podocyte injury.

Conclusions. We demonstrated the breakdown of the vitamin D activation system in podocyte injury, and established a preventative role for vitamin D in podocyte injury.

Keywords: calcitriol; maxacalcitol; nephrin; podocyte; proteinuria

Introduction

Podocyte injury leads to glomerulosclerosis, tubular damage and interstitial fibrosis through perturbation of the slit-diaphragm and resultant proteinuria in many human and experimental kidney diseases [1–4]. Gene-targeted ablation in mice has revealed that mutations in podocyte-associated molecules, including nephrin, podocin, CD2-associated protein and α-actinin-4, result in proteinuria and glomerulosclerosis [5–9]. Thereafter, these podocyte injuries cause tubulointerstitial fibrosis, which is the common pathway leading to end-stage renal disease. Kriz et al. suggested that podocyte loss and the resultant adhesion between the bare glomerular basement membrane and Bowman’s capsule induce renal tubular damage [1–3,10]. As we have reported previously, proteinuria itself also damages proximal tubular epithelial cells [11,12]. These findings indicate that injury to podocytes is a key initial step in the pathogenic sequences of chronic kidney disease (CKD). Therefore, it is important to develop a novel strategy targeting podocytes in order to protect kidney function.

One of the candidates to protect podocytes from injury is vitamin D. When diabetes is introduced by streptozotocin, VDR-null mice develop significantly more severe albuminuria in comparison with wild-type diabetic mice [13]. In addition, transgenic rats constitutively expressing vitamin D 24-hydroxylase [24(OH)ase] also excrete significantly higher amounts of urinary protein than wild-type rats [14,15]. These data suggest that the abnormalities in vitamin D metabolism result in kidney dysfunction. In patients with CKD, deficiency of the most biologically active vitamin D molecule, 1 alpha,25-dihydroxyvitamin D3, [1α,25(OH)2D3], occurs early along with the progression of CKD, as the final hydroxylation step from its substrate 25-hydroxyvitamin D [25(OH)D] is mediated by 1α-hydroxylase [1α(OH)ase] in proximal tubular epithelial cells [16–18]. To date, the complications of 1α,25(OH)2D3 deficiency in CKD patients have been investigated in relation to the development of secondary hyperparathyroidism [19–21]. Although several experimental studies have demonstrated the renoprotective effects of vitamin D in mesangial cell proliferative and/or excessive matrix productive models, the relationship between 1α,25(OH)2D3 deficiency and podocyte injury remains uncertain [22–27].
Here, we examined the podocyte-protective effects of active vitamin D or its analogue, 22-oxacalcitriol, in puromycin aminonucleoside (PAN) nephrosis rats. We found that the vitamin D activation system is severely impaired in this model and investigated the underlying mechanisms for the deficiency of active vitamin D. Moreover, we demonstrated that treatment with vitamin D has preventative effects on podocyte injury in the PAN nephrosis model.

**Subjects and methods**

**Animals**

Six-week-old male Sprague-Dawley rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and were maintained at the animal facility of Osaka University Graduate School of Medicine. The rats were handled in a humane manner, and all procedures were approved by the Animal Committee of Osaka University Graduate School of Medicine. The rats were provided with MF diet (Oriental Yeast, Tokyo, Japan) ad libitum. PAN nephrosis was induced with a single intravenous injection of PAN (Sigma, St Louis, MO, USA) dissolved in saline at a dose of 10 mg/100 g body weight. Subsequently, 1α,25(OH)2D3 or 22-oxacalcitriol dissolved in PBS containing 0.01% Tween 20 and 0.2% ethanol was administrated intraperitoneally at 0.08 μg/kg/day for 2.0 μg/kg/day for 22-oxacalcitriol groups, or at 0.016 μg/kg/day or 0.4 μg/kg/day for 1α,25(OH)2D3 groups, once daily from the day of PAN injection (Day 0) until Day 6 (see Figure 4A). We purchased 1α,25(OH)2D3 (Rocaltrol® injection) from Kirin Pharma Co., Ltd (Tokyo, Japan). Vitamin D analogue 22-oxacalcitriol was kindly provided from Chugai Pharmaceutical Co., Ltd (Tokyo, Japan). At Day 7, rats were anesthetized by intraperitoneal administration of pentobarbital and processed as previously described [28,29].

**RNA extraction and quantitative reverse transcription-PCR**

RNA was extracted from each tissue using TRIZOL reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). To quantify the expression of mRNA, the real-time SYBR-Green assay was performed as previously described [29]. Primer sets were as follows: CYP2R1 forward, 5′-attatggacacctgctgt-3′, CYP2R1 reverse, 5′-atgacaagaaggagccact-3′; CYP27B1 forward, 5′-atcgccttctcagctgggca-3′, CYP27B1 reverse, 5′-cccttgcaagacggctac-3′; CYP24 forward, 5′-agccgctccgcaggt-3′, CYP24 reverse, 5′-gcttcaatttcacgctttg-3′; DBP forward, 5′-ggtcagcagcagcagctggt-3′, DBP reverse, 5′-cagagcagcagcagcagcag-3′, GAPDH forward, 5′-tgacacctcacttgctga-3′, GAPDH reverse, 5′-gccagcaggcagctttg-3′.

**SDS-PAGE and immunoblot analysis**

In order to evaluate serum and urinary protein, serum and urine samples were subjected to SDS-PAGE analysis using the 10–20% Super-Sep polyacrylamide gel (Wako, Osaka, Japan) and were visualized with Coomassie Brilliant Blue. For immunoblot analysis of DBP, the serum samples were diluted (1:200) in Laemmli buffer containing 50 mM DTT and 2% β-mercaptoethanol. An aliquot of diluted serum sample was applied to the diluted (1:200) in Laemmli buffer containing the same reducing agents as mentioned above. The electrophoresed samples were electroblotted onto Hybond P (GE Healthcare, Buckinghamshire, UK). Membranes were incubated in a 5% skim milk/20 mM Tris-HCl-buffered saline (pH 7.6)/0.1% Tween 20, followed by incubation in an anti-DBP antibody at 1:500 (Abcam, Cambridge Science Park, UK) or anti-nephrin antibody at 1:200 (Santa Cruz Biotechnology, CA, USA). To visualize the signals in serum and urine samples, TrueBlot™ (eBioscience, San Diego, CA, USA) was used at 1:5000 in combination with a SuperSignal West Pico Chemiluminescent Substrate (Pierce, MA, USA).

**Immunohistochemical analysis**

Antibodies against specific molecules were obtained as follows: VDR (Chemicon, Billerica, MA, USA), RXRa, RXRβ, RXRγ and WT-1 (Santa Cruz Biotechnology), podocin (IBL, Gunma, Japan) and desmin (Dako, Glostrup, Denmark). The dilution rates of the primary antibody were 1:100 for VDR, podocin and desmin and 1:50 for RXRa, RXRβ and WT-1. An anti-nephrin antibody for immunohistochemical analysis was kindly provided by Dr Hiroshi Kawachi, Niigata University, Niigata, Japan, and was used at 1:1000. The sections were processed as previously described [28,29]. All images were obtained using a Bio-Rad radiance 2100 (Bio-Rad, Hercules, CA, USA) confocal laser microscope.

**Statistical analysis**

Statistical analysis for multiple comparisons was performed with Tukey–Kramer’s test as shown in Figures 1B, C and 2A, B. As shown in Figure 4B and C, Dunnett’s test was performed using the corresponding values of Group 2 as references. The non-paired t-test was used for the comparison of two groups. The significance was defined as P < 0.05. All error bars in graphs represent standard deviation (SD).
Results

Vitamin D deficiency in PAN nephrosis

In order to assess the protective effects of vitamin D on podocytes, we employed PAN nephrosis model rats, in which proteinuria occurs on Day 4 and reaches a maximum level on Day 7 (Figure 1A). We initially examined serum levels of 1α,25(OH)₂D₃ and 25(OH)D. Serum 25(OH)D levels remained stable for 3 days after disease induction, but decreased significantly on Day 7, concomitantly with increased proteinuria (Figure 1B). In contrast, serum 1α,25(OH)₂D₃ levels were markedly reduced on Day 3 before the onset of proteinuria and were further reduced on Day 7 (Figure 1C).

Amelioration of nephrotic state by vitamin D

Given that the reduction of serum 1α,25(OH)₂D₃ preceded the onset of proteinuria, we speculated that vitamin D supplementation may play a favourable role in PAN nephrosis rats. To test this hypothesis, we administered 1α,25(OH)₂D₃ or its analogue 22-oxacalcitriol (maxacalcitol) to PAN nephrosis rats. We show our experimental protocol in Figure 4A. Saline or PAN was intravenously injected on Day 0 (Figure 4A, white arrow). Vehicle, 1α,25(OH)₂D₃ or 22-oxacalcitriol was intraperitoneally administrated once a day from Day 0 to Day 6 (Figure 4A, black arrows). On Day 7, all animals were sacrificed (Figure 4A, bold grey arrow). Within the experimental period, normal control rats did not excrete overt urinary protein (Figure 4B, Group 1). As compared with PAN nephrosis rats treated with vehicle (Figure 4B, Group 2), treatment with high doses of 22-oxacalcitriol (2.0 µg/kg/day; Figure 4B, Group 4) or high doses of 1α,25(OH)₂D₃ (0.4 µg/kg/day; Figure 4B, Group 6) significantly suppressed proteinuria. However, low doses of these compounds did not show any significant differences (Figure 4B, Groups 3 and 5).

Together with the reduction of proteinuria, vitamin D therapy with high-dose 22-oxacalcitriol (Figure 4C, Group 4) or high-dose 1α,25(OH)₂D₃ (Figure 4C, Group 6) improved serum total protein, albumin and total cholesterol levels (Figure 4C a-c), as compared with vehicle-treated PAN nephrosis rats (Figure 4C,

Underlying mechanism for vitamin D deficiency

As we observed decreased serum 25(OH)D on PAN nephrosis Day 7, we examined the metabolism of vitamin D in the liver. Real-time PCR analysis demonstrated that the induction of PAN nephrosis did not affect the mRNA expression of CYP2R1 (25-hydroxylase) (Figure 2A), indicating that the 25-hydroxylation step in the liver is not involved in the decrease in serum 25(OH)D. The vitamin D-binding protein (DBP) expression in the liver was also sustained (Figure 2B). However, along with serum protein (Figure 2C), DBP was lost in urine (Figure 2D).

In contrast to vitamin D metabolism in the liver, real-time PCR analysis revealed that renal CYP27B1 (1α-hydroxylase) and renal CYP24 (24-hydroxylase) decreased and increased, respectively, on Day 3 (Figure 2E and F).

Vitamin D receptor (VDR) and retinoid X receptors (RXRs) in podocytes

Most of the biological roles of 1α,25(OH)₂D₃ require its receptor, VDR. Like other steroid receptors after ligand binding, VDR heterodimerizes with RXRs and regulates target gene expression. To verify whether podocytes express VDR and RXRs, we performed immunohistochemical analysis using normal rat kidney tissue (Figure 3A, C, E and G) and PAN nephrosis Day 7 kidney tissue (Figure 3B, D, F and H). WT-1-positive podocytes were confirmed to express VDR in nuclei (Figure 3A and B, merged yellow). In glomeruli, mesangial cells also expressed VDR (Figure 3A and B, glomerular green nucleus). Podocytes expressed all RXRs, except RXR-α (Figure 3C–H).

Fig. 2. Underlying mechanism in vitamin D deficiency. Real-time PCR demonstrated that CYP2R1 (25-hydroxylase) (A) and vitamin D-binding protein (DBP) (B) expressions in the liver were not affected in PAN nephrosis (N = 4 in each group). (C) SDS-PAGE analysis, after staining with Coomassie Brilliant Blue, showed the serum and urinary protein in PAN nephrosis rats. (D) Immunoblot analysis suggested that serum DBP was lost in urine on Day 7. Real-time PCR showed that CYP27B1 (1α-hydroxylase) (E) and CYP24 (24-hydroxylase) (F) down-regulated and up-regulated, respectively, in kidney of PAN nephrosis on Day 3 (N = 4 in each group: *P < 0.05; **P < 0.01).
Vitamin D protects podocytes

Fig. 3. Vitamin D receptor (VDR) and retinoid X receptors (RXRs) in podocytes. Immunohistochemistry confirmed the expressions of VDR and RXRs in normal rat kidney (A, C, E and G) and PAN nephrosis kidney on Day 7 (B, D, F and H). All sections were double stained with WT-1 (red) to identify podocyte nuclei. Fluorescein signals indicate (A and B) VDR, (C and D) RXR-α, (E and F) RXR-β and (G and H) RXR-γ (original magnification, ×600).

Group 2). We also examined the effects of vitamin D on calcium and inorganic phosphate (IP) homeostasis. The induction of PAN nephrosis decreased serum ionized calcium (Figure 4C (d), Group 2). Treatment with high-dose 22-oxacalcitriol (Figure 4C (d), Group 4) recovered serum ionized calcium to comparable levels as in normal control rats (Figure 4C (d), Group 1). However, treatment with high-dose 1α,25(OH)2D3 (Figure 4C (d), Group 6) significantly increased serum ionized calcium levels, which remained higher than those in normal control rats. While administration of 22-oxacalcitriol did not affect IP levels (Figure 4C (e), Groups 3 and 4), both low and high doses of 1α,25(OH)2D3 therapy increased serum IP (Figure 4C (e), Groups 5 and 6).

**Effects of vitamin D on podocyte injury markers**

Because PAN nephrosis did not show any mesangial cell proliferation or matrix expansion, we hypothesized that administration of vitamin D protected podocytes from PAN nephrosis without involving mesangial cells. Because we previously reported that nephrin is transcriptionally regulated by all-trans retinoic acid (ATRA) and that nephrin expression is inversely correlated with the amount of proteinuria [28,29], we investigated the effects of vitamin D on nephrin expression. As we previously reported, the induction of PAN nephrosis diminished glomerular nephrin expression levels, but high-dose 22-oxacalcitriol or 1α,25(OH)2D3 restored glomerular nephrin expression (Figure 5A and B). To verify the podocyte-protective effects of 22-oxacalcitriol or 1α,25(OH)2D3, we carried out immunostaining for podocin (Figure 6A) and desmin (Figure 6B). Like the expression pattern of nephrin (Figure 5A), the diminished expression levels of podocin by PAN nephrosis induction were partly restored by high-dose 22-oxacalcitriol or 1α,25(OH)2D3 (Figure 6A). The podocyte injury marker desmin exhibited a reciprocal staining pattern (Figure 6B). All of these data suggest the podocyte-protective effects of vitamin D.

**Discussion**

In this study, we examined whether supplementation with 1α,25(OH)2D3 or 22-oxacalcitriol has preventative effects on podocyte injury in a rat PAN nephrosis model. Based on our results, both 1α,25(OH)2D3 and 22-oxacalcitriol appear to have protective functions against podocyte injury. Several key findings support this conclusion: (1) serum 1α,25(OH)2D3 reduction preceded the appearance of overt proteinuria, in accordance with the down-regulation of renal 1α(OH)ase; (2) thereafter, serum 25(OH)D levels decreased significantly on Day 7, concomitant with the increased excretion of DBP into urine; (3) podocytes express VDR and RXRs; (4) active vitamin D or 22-oxacalcitriol therapy ameliorated the nephrotic state in PAN-injected rats; (5) induction of PAN nephrosis reduced glomerular nephrin expression, while administration of 1α,25(OH)2D3 or 22-oxacalcitriol recovered its expression and (6) the slit-diaphragm-associated molecule podocin and podocyte injury marker desmin were restored and diminished, respectively, by vitamin D administration. These observations suggest that vitamin D supplementation has podocyte-protective effects.

We demonstrated that serum 25(OH)D levels decreased concomitantly with the increased proteinuria, while serum 1α,25(OH)2D3 levels were markedly reduced on Day 3 before the onset of proteinuria. Although several types of cell, including podocytes, express 1α(OH)ase, the main contributors to circulating levels of 1α,25(OH)2D3 are renal proximal tubular epithelial cells [22,30]. Locally produced 1α,25(OH)2D3, except in proximal tubular cells, primarily serves as an autocrine/paracrine factor [30]. PAN nephrosis is a well-established podocyte injury animal model with disturbed slit-diaphragm functions, but renal tubular damage apparently preceded podocyte dysfunction, as
Fig. 4. Amelioration of nephrotic state by active vitamin D or its analogue. (A) Experimental protocol to assess the effects of exogenous 1α,25(OH)₂D₃ and its analogue 22-oxacalcitriol is shown. Saline (Group 1) or puromycin aminonucleoside (PAN; Groups 2–6) was injected intravenously on Day 0. Rats received vehicle (Groups 1 and 2), 22-oxacalcitriol (0.08 µg/kg/day in Group 3 and 2.0 µg/kg/day in Group 4) or 1α,25(OH)₂D₃ (0.016 µg/kg/day in Group 5 and 0.4 µg/kg/day in Group 6) intraperitoneally once a day for 7 days (Days 0–6). In all subsequent experiments, statistical significance was defined using the corresponding values of Group 2 as references. (B) Urinary protein creatinine ratio significantly decreased in Group 4 on Days 6 and 7 and in Group 6 on Days 4, 5, 6 and 7 in comparison with the corresponding values of Group 2. (C) High-dose 22-oxacalcitriol (Group 4) or high-dose 1α,25(OH)₂D₃ (Group 6) improved (a) serum total protein, (b) albumin and (c) total cholesterol levels. High-dose 1α,25(OH)₂D₃ (Group 6) induced hypercalcaemia and hyperphosphataemia. All values are expressed as means ± SD (N = 9 in each group: *P < 0.05; **P < 0.01; ***P < 0.001, versus Group 2).
Vitamin D protects podocytes

Fig. 5. Effects of active vitamin D and its analogue on nephrin expression. (A) Immunohistochemistry demonstrated that the linear expression pattern of nephrin in normal glomeruli (a) disappeared upon PAN nephrosis induction (b). Low-dose 22-oxacalcitriol (0.08 µg/kg/day) (e) or low-dose 1α,25(OH)2D3 (0.016 µg/kg/day) (f) was insufficient to up-regulate nephrin. However, high-dose 22-oxacalcitriol (2.0 µg/kg/day) (d) and high-dose 1α,25(OH)2D3 (0.4 µg/kg/day) (f) partially restored nephrin expression. (B) Immunoblot analysis of isolated glomeruli confirmed the recovery of nephrin expression by high-dose 22-oxacalcitriol or high-dose 1α,25(OH)2D3. The grouping is the same as in Figure 4. For loading control, the same sample was stained with Coomassie Brilliant Blue, as commonly used controls, such as β-actin and α-tubulin, are up-regulated in the glomeruli in PAN nephrosis (data not shown).

Serum 1α,25(OH)2D3 decreased on Day 3 before the onset of proteinuria (Figure 1C). Indeed, urinary N-acetyl-β-D-glucosaminidase (NAG) has been reported to increase significantly in the first 2 days following intravenous PAN injection [31].

In CKD patients, 1α,25(OH)2D3 deficiency occurs early along with disease progression due to insufficient 1α(OH)ase in proximal tubular epithelial cells [16–18]. Although it remains uncertain why the induction of PAN nephrosis reduces 1α(OH)ase in proximal tubular epithelial cells, oxidative stress may cause this process, as reactive oxygen metabolites are important mediators in PAN nephrosis and also affect 1α(OH)ase activity [32–34].

DBP is a high-affinity carrier protein of vitamin D metabolites, which preferably binds 25(OH)D and 24,25(OH)2D [35]. Under normal conditions, DBP can partly pass through the glomerular barrier, and filtrated DBP binds endocytosis receptor megalin expressed at the apical membrane of proximal tubular epithelial cells, releasing 25(OH)D for metabolism by 1α-hydroxylase or 24-hydroxylase [30]. Due to the uptake at the proximal tubule under normal conditions, DBP is undetectable in urine. However, overt proteinuria leads to excretion of 25(OH)D-bound DBP in urine, leading to decreased serum 25(OH)D. Substrate deficiency and disordered vitamin D activation in tubules support the rationale for supplementation with vitamin D.

Immunohistochemical analysis demonstrated that podocytes express VDR and RXRs, except for RXR-α, thus suggesting that vitamin D may have a renoprotective function in podocytes, as well as in mesangial cells. Several reports have shown the therapeutic value of vitamin D, focusing on mesangial cells. Panichi et al. showed that 1α,25(OH)2D3 decreases glomerular hypercellularity and urinary proteinuria/creatinine ratio in an anti-Thy-1 model [23]. Hirata et al. reported that 22-oxacalcitriol favourably affects serum urea nitrogen, creatinine and urinary albumin/creatinine ratio with decreased mesangial cell
proliferation and mesangial matrix expansion in subtotally nephrectomized rats [25]. Although PAN can induce mesangial cell proliferation when hemi-nephrectomized rats are treated with repeated PAN administration, our experimental PAN nephrosis model, which undergoes a single intravenous PAN injection without nephrectomy, did not show any mesangial cell proliferation [28,29,36–38]. Therefore, we confirmed the podocyte-protective effects of vitamin D in the model without involvement of mesangial cells or matrix.

Nephrin, an important constituent of the slit-diaphragm, is known to be a key molecule in maintaining barrier function [5,39–41]. Disrupted nephrin expression induces proteinuria, and the recovery of nephrin expression accompanies reductions in proteinuria. In the PAN nephrosis model, treatment with 1α,25(OH)2D3 or 22-oxacalcitriol recovered nephrin expression, which explains the reduction of proteinuria. Kitamura et al. demonstrated that active vitamin D directly binds VDR in podocytes and increases the transcription of nephrin mRNA in cultured murine podocytes [42,43]. Furthermore, additional treatment with 1α,25(OH)2D3 and ATRA synergistically up-regulate nephrin gene expression [42,43].

The observation that podocytes express VDR and RXRs confirms that 1α,25(OH)2D3 can directly protect podocyte function by increasing nephrin expression. However, it is possible that indirect action of vitamin D may participate in nephrin induction in our experiments, as vitamin D affects the renin-angiotensin system (RAS) [44,45]. Renin is strongly induced in VDR-null mouse kidney, and 1α,25(OH)2D3 down-regulates renin gene transcription [44–47]. Recently, Zhang et al. demonstrated that combination therapy with an AT1 receptor blocker and a vitamin D analogue markedly ameliorate renal injury in 24–25-dihydroxyvitamin D3 levels and bone mass in 24-hydroxylase transgenic rats. FASEB J 2003; 17: 737–739

In conclusion, the application of 1α,25(OH)2D3 or 22-oxacalcitriol to kidney injury with proteinuria has a preventative effect with regard to podocyte injury. In other words, vitamin D therapy can prevent injured kidneys from falling into a vicious cycle driven by the impairment of the vitamin D system in proteinuria.

Acknowledgements. We would like to thank Naoko Horimoto for her technical assistance. This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (no. 20790593).

Conflict of interest statement. Enyu Imai is a member of Steering Committee of Chronic Kidney Disease Japan Cohort study sponsored by Kyowa Hakko-Kirin Co. He also received lecture fee from Chugai Pharmaceutical Co. and Kyowa Hakko-Kirin Co.

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Received for publication: 13.11.08; Accepted in revised form: 25.2.09