Paper

Effects of ACE Inhibition and Angiotensin II Receptor Blockade on Glomerular Basement Membrane Protein Excretion and Charge Selectivity in Type 2 Diabetic Patients

Oguzhan Deyneli,† Dilek Yavuz,† Ayliz Velioglu,# Hasan Cacına,# Nihal Aksoy,# Goncagül Haklar,# Yavuz Taga,# Sema Akalın†

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†Section of Endocrinology and Metabolism Department of Internal Medicine, Marmara University, Istanbul, Turkey

#Department of Biochemistry, Marmara University, Istanbul, Turkey

Correspondence to:
Dr Oguzhan Deyneli
Tophanelioglu Cad,
Askorukent Sitesi
C Blok,
Daire 4, Uskudar/
Istanbul
Turkey 34662
Tel: +90 542 416 6375
Fax: +90 216 428 0013
E-mail: odeyneli@marmara.edu.tr

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Abstract

Angiotensin-converting enzyme (ACE) inhibitors may reduce urinary albumin excretion (UAE) by decreasing glomerular pressure and increasing glomerular charge selectivity through preservation of glycosaminoglycans. The effect of Angiotensin II antagonism on glomerular charge selectivity remains to be determined. The aim of this study was to compare the effects of an AT_1 blocker losartan and an ACE inhibitor (ACE-I) enalapril on UAE, extracellular matrix proteins, glycosaminoglycan excretion (U_{GAG}) and red blood cell anionic charge (RBCCh) which are the indirect markers of glomerular basement membrane anionic content in hypertensive Type 2 diabetic patients. Twenty-four patients were randomised into two groups and received either enalapril (5–20 mg/d) or losartan (50–100 mg/d). All parameters were measured at baseline and after six months of treatment. At the end of six months, systolic and diastolic blood pressures (BP), UAE rates, U_{GAG} excretion and RBCCh were significantly and equally reduced in both treatment groups compared with baseline. RBCCh was negatively correlated with UAE ($r=-0.57$, $p<0.0001$) and U_{GAG} excretion ($r=-0.57$, $p<0.0001$); UAE was correlated with U_{GAG} excretion ($r=0.58$, $p<0.0001$). In conclusion, enalapril and losartan treatment were equally effective in reducing BP, UAE as well as U_{GAG} excretion and preserving RBCCh in hypertensive Type 2 diabetic patients. ACE inhibition and AT_1-receptor blockade may have favourable effects on preserving glomerular anionic content in hypertensive diabetic patients.

Introduction

Changes in extracellular matrix components of glomerular basement membrane (GBM) are thought to be involved in the pathogenesis of diabetic nephropathy.[1]

Loss of anionic charged proteoglycans may be responsible for the increased excretion of negatively charged albumin.[2] Heparan sulfate proteoglycan is a strong inhibitor of mesangial growth and reduced content of heparan sulfate in GBM was found to be associated with increased mesengial expansion in experimental models and also in diabetic patients.[3,4]

The major extracellular matrix protein of the basement membrane, collagen IV, was shown to be increased in the glomerular mesangium and GBM of diabetic patients.[1,5] Studies which show increased urinary excretion of collagen IV ($U_{Collagen\ IV}$) in proteinuric diabetic patients suggest that urinary measurement of these proteins could reflect matrix changes and GBM damage in patients with diabetes.[6-8]

Angiotensin-converting enzyme (ACE) inhibitors are known to have antiproteinuric effects on the diabetic kidney.[9] Recent studies indicate that the renoprotective effects of ACE inhibitors (ACE-Is) in diabetes could be at least partly independent of their blood pressure (BP)-lowering effects.[10,11] ACE-Is were shown to preserve anionic content of GBM in experimental diabetic models and limited clinical studies.[12,13]

Other renin-angiotensin system (RAS) inhibitors, Angiotensin II (Ang II) subtype 1 receptor blockers (ARBs) have antihypertensive and antialbuminuric effects comparable to ACE-Is.[9,14,15] Although the major mechanism of antialbuminuric action of ARBs was believed to be due to their glomerular arteriolar pressure lowering effects, recent data indicate that renal protective effects of ARBs may also be independent of the reduction in BP.[16] Their effects on glomerular anionic content are currently unknown.

The aim of this study was to evaluate the effects of ACE-Is and ARBs on glomerular charge selectivity, urinary excretion of extracellular matrix proteins such as collagen IV and fibronectin and urinary N-acetylglucoseaminidase (U_{NAG}) as a tubular marker in hypertensive Type 2 diabetic patients.

Materials and Methods

Patient Selection and Study Design

All male and female patients attending Marmara University Hospital Endocrine and Internal Medicine outpatient clinics with Type 2 diabetes mellitus diagnosed after the age of 30, with mild-to-moderate essential hypertension (according to JNC VI), and microalbuminuria, were assessed for eligibility for the study. Exclusion criteria were secondary hypertension, history of malignant hypertension, myocardial infarction (MI), cerebrovascular disease, heart failure, treatment with anti-aggregants, steroids or other drugs that might effect BP, serum creatinine > 200 µmol/L, urinary tract infection and other systemic disorders. All of the participants underwent initial evaluation by medical history, physical examination, haematological and biochemical tests including measurement of blood glucose, serum electrolytes (sodium and potassium), urea, creatinine, lipids, thyroid function tests and urine analysis. Diabetes was diagnosed according to the American Diabetes Association criteria. Renal ultrasound and Doppler examination was normal in all hypertensive patients. The study was approved by the Marmara University Medical Faculty Ethics Committee and all participants gave written informed consent.

All patients were hypertensive for at least six months according to hospital records and none of them were on antihypertensive treatment. There were 28 eligible Type 2 diabetic hypertensive microalbuminuric patients during the randomisation phase of the study. All patients, except two who refused to attend regular visits, were enrolled.

This study was designed as an open-label, parallel-prospective, randomised study, beginning with a six-week titration phase (aiming at a BP target of less than 130/80 mmHg), and a 24-week maintenance phase comparing the effects of enalapril and losartan. Data obtained from the control group were only used for cross-sectional comparisons with baseline measurements of hypertensive diabetic groups to clarify the situation about the altered milieu in untreated hypertensive diabetic patients. In addition to home blood glucose monitoring, serum glucose levels and office BPs were measured at monthly intervals and HbA_{1C} levels every three months. No significant difference in these parameters was observed during the study period. The drug dosages ranged between 5–20 mg/day (Renitec, Merck Sharp Dohme, Turkey) for Enalapril group and 50–100 mg/day (Cozaar, Merck Sharp Dohme, Turkey) for Losartan group. Lifestyle, diet, oral hypoglycaemic or insulin therapy remained unchanged throughout the study. BP was measured at each visit between 8 and 10 a.m. and 24 hours after medication ingestion, with a standard sphygmomanometer and an appropriately sized cuff. Korotkoff phases I and V were used to determine the systolic and diastolic values, respectively. Three recordings were done in the sitting position after 10 minutes of rest. Patients were randomised into two treatment groups using Arcus QuickStat software. Sequentially numbered opaque, sealed envelopes were prepared according to the list generated by the program and used for the randomisation: 13 patients were given enalapril (Enalapril Group) and 13 were given losartan (Losartan Group). Also 12 age and body mass index (BMI) matched healthy volunteers recruited among hospital staff were included as control group. All control subjects had normal physical findings, a normal electrocardiogram (ECG) and were all free from a family history of hypertension, premature cardiovascular death and diabetes mellitus. Demographic characteristics of the groups are shown in Table 1. Diabetes was well controlled in all patients, with HbA_{1C} levels below 7.0% for at least three months before the study. Two patients were on insulin therapy and all others were on oral antidiabetic drugs (OAD).

Compliance and tolerability were assessed by monitoring spontaneous reports of adverse experiences and pill counts at each visit. Twenty-four-hour urine and fasting venous blood samples were collected at the beginning of the study before titration phase and at the end of the study after 24 weeks of the maintenance phase on antihypertensive therapy, and the following parameters were evaluated.

Red Blood Cell Anionic Charge (RBCCh) Measurement

RBCCh was evaluated with a cationic dye (Alcian blue) according to the method of Levin *et al.*[17] with minor modifications as follows: from citrated venous blood samples, platelets and leukocytes were removed by the method of Beutler *et al.*[18] RBCs were washed three times in saline, and subsequently a fraction of these cells was

Table 1
Demographic characteristics of study groups.

	Enalapril Group n=12	Losartan Group n=12	Controls n=12
Sex (M/F)	2/10	4/8	3/9
Age (years)	52.8±5.5	51.9±6.5	51.3±5.3
BMI (kg/m²)	28.6±8.8	29.3±5.3	29.0±4
Diabetes duration (years)	4.7±3.2	4.6±3.7	-
Diabetic medication (OAD/insulin)	11/1	11/1	-
HbA_{1C} (%)	6.5±0.5	6.4±0.6	-

OAD = oral antidiabetic drugs

resuspended in the same solution containing Alcian blue at a final concentration of 250 mg/L. After 30 minutes incubation at 37°C, the RBC suspension was centrifuged, and the Alcian blue concentration was measured in the supernatant with a Shimatzu UV 2100 spectrophotometer (Shimatzu, Japan) at a wavelength of 650 nm. Each determination represented the mean of two assays. The quantity of Alcian blue bound to RBCs was expressed as nanograms of Alcian blue per 10^6 RBCs. In our experimental conditions the intra-assay and inter-assay coefficients of variation were 5.8 and 7.6%, respectively.

Urinary Glycosaminoglycan Level Determination

Urinary glycosaminoglycan (U_{GAG}) excretion was determined in 24-hour urine samples spectrophotometrically at 520 nm by the addition of dimethyl-methyleneblue (Aldrich Chem Co., USA) and standard bovine renal heparan sulfate (Sigma, USA).[19] In our experimental conditions the intra-assay and inter-assay coefficients of variation were 2.4 and 1.5%, respectively.

Urinary Albumin Excretion

Urinary albumin excretion was measured with a nephelometric method (BN Prospec, Dade Behring Marburg GmbH). The intra-assay and inter-assay coefficients of variation were 4.3 and 4.4% for a mean value of 45 g/L albumin concentration.

Urinary and Serum IgG Levels

These parameters were also measured nephelometrically by BN Prospec (Dade Behring Marburg GmbH). The intra-assay and inter-assay coefficients of variation were 2.1 and 2.7% for IgG measurements. IgG clearance and IgG/albumin ratio were calculated.

Urinary N-Acetyl-ß-D–Glucosaminidase (NAG) Levels

UNAG levels were measured by a colorimetric assay with a kit by Roche Diagnostics GmbH (Mannheim, Germany), the intra and inter-assay coefficients of variation were 4.8% and 5%, respectively.

Urinary Fibronectin

Urinary fibronectin was measured by using ELISA technique. (Takara Shuzo Co. Ltd, Japan). The intra and inter-assay coefficients of variation were 4.7%, and 6%, respectively.

Urinary Collagen IV

Urinary collagen IV was determined by using ELISA Technique (Bio 83).

Statistical Analysis

The analysis of the data was performed with PC compatible Instat-II software. Paired *t*-test and ANOVA were used where appropriate. Correlation analysis was performed with Pearson test. The differences were considered significant at $p<0.05$. The results were given as mean±SD.

Results

There was no significant difference in baseline gender distributions, age, weight, body mass index in control and enalapril or losartan treated diabetic groups. The duration of diabetes and HbA_{1C} levels were similar in both diabetic groups (see Table 1). One patient from the enalapril group had to stop drug treatment because of side effects (cough, dizziness) and one from the losartan group was non-compliant. There were neither deaths nor any cardiovascular events during the course of the study. Twelve patients in each group completed the protocol and were

Table 2
Clinical parameters of all study groups.

	Enalapril Group		Losartan Group		Controls
	Before Treatment	After Treatment	Before Treatment	After Treatment	
SBP (mmHg)	144.1±18.8 δ	125.0±15.6 *	142.5±18.6 δ	122.5±18.3 §	120.4±10.3
DBP (mmHg)	89.5±4.5 ¥	76.2±7.1 †	90.0±6.7 ¥	75.4±4.5 †	74.5±6.8
Creatinine clearance (ml/min)	102.6±22	114.5±30	115.9±23	111.6±28	112.5±28
UAE (mg/day)	83.5±51 δ	17.5±7.4 ψ	80.1±52 δ	19.3±8.4 ψ	8.3±4.1
U_{GAG} (mg/day)	50.2±19 ¥	25.1±17 ψ	58.2±22 ¥	28.5±18 ψ	9.4±5.6
U_{GAG}/cr	0.46±0.1	0.25±0.2 †	0.5±0.2	0.25±0.25 £	0.23±0.1
U_{NAG} (ng/L)	10.4±7 δ	4.9±3.3 ψ	7.7±5.7 ϕ	3.3±2.3 §	2.6±1.8
$U_{Fibronectin}$ (ng/ml)	116.6±75	72.2±40.1ψ	117.6±70.9	87.7±49.3	80.0±32.2
$U_{Collagen\ IV}$ (ng/ml)	3.78±1.5 ϕ	2.4±1.0 ‡	3.5±1.8	1.91±1.1 ψ	2.1±1.0
IgG $_{Clearence}$ (μg/min)	3.81±1.0	1.2±0.5 §	3.7±2.9	0.6±0.3 †	-
U IgG/UAE	0.451±0.19	0.301±0.09 §	0.475±0.23	0.259±0.14‡	-

* p<0.0005 vs. before treatment; y p<0.005 vs. before treatment; d p<0.01 vs. Controls; ¥ p<0.001 vs. Controls;
§ p<0.05 vs. before treatment; † p<0.001 vs. before treatment; f p<0.05 vs. Controls; ‡ p<0.01 vs. before treatment;
SBP: Systolic blood pressure; DBP: Diastolic blood pressure; UAE: Urinary albumin excretion;
U_{GAG}: Urinary glycosaminoglycan excretion; U_{NAG}: Urinary N-Acetyl-ß-D–glucosaminidase

evaluated for statistical analysis. After six months of active treatment, both losartan and enalapril significantly decreased systolic and diastolic BP without a clear-cut difference between the two treatment groups (Table 2). The percentage of subjects who achieved the goal diastolic BP < 80 mmHg at the end of the study was 83.3% in the losartan group and 91.6% in the enalapril group. The doses of enalapril and losartan classified as maximum and minimum were similar in both groups. There was no statistically significant difference between groups in UAE at baseline. The mean decrease in UAE was significant by week 24 in both treatment groups. Albuminuria decreased from 83.5±51 mg/d at baseline to 17.5±7.4 mg/d ($p<0.005$) in the enalapril group, and from 80.1±52 mg/d to 19.3±8.4 mg/d for losartan group ($p<0.005$). Basal albuminuria measurements were significantly lower in the control group (8.3±4.1 mg/d) than in hypertensive patients ($p<0.01$). RBC anionic charge was significantly increased from 166.7±72.4 ng alcian Blue/10^6 RBC to 433.2±52.6 ng alcian blue/10^6 RBC ($p<0.0001$) in enalapril treated patients and from 163.5±74.3 to 368.4±54 ng alcian blue/10^6 RBC for losartan treated patients ($p<0.0005$). Basal measurements of hypertensive groups were significantly lower than controls ($p<0.01$) (Figure 1).

Baseline U_{GAG} and $U_{Collagen\ IV}$ excretions were similar in both diabetic groups but higher than healthy controls ($p<0.05$). At the end of the six-month treatment period U_{GAG} and $U_{Collagen\ IV}$ excretions were significantly decreased compared to baseline levels in both treatment groups ($p<0.005$). A similar trend was observed for $U_{GAG/Cr}$ ratio and $U_{Fibronectin}$ excretion (see Table 2). Prior to treatment U_{NAG} levels were significantly higher in diabetic patients than controls ($p<0.05$). U_{NAG} excretion decreased from 10.4±7 ng/ml at baseline to 4.9±3.3 ng/ml with enalapril treatment ($p<0.005$) and from 7.7±5.7 ng/ml to 3.3±2.3 ng/ml with losartan treatment ($p<0.05$). IgG clearance was significantly decreased at the end of the six-month period compared to baseline levels in both enalapril- ($p<0.05$) and losartan- ($p<0.001$) treated groups. IgG clearance was not calculated in the control group because seven healthy subjects had undetectable urinary IgG excretion and U_{GAG} excretion. There was no significant difference in any of the followed parameters at the end of the 6 months treatment period between enalapril- and losartan-treated patients (see Table 2). UAE was correlated with U_{GAG} ($r=0.58$, $p<0.0001$) and U_{NAG} excretion ($r=0.56$, $p<0.0001$), and inversely correlated with RBC anionic charge ($r=-0.57$, $p<0.0001$). Urinary collagen IV and fibronectin levels had a weak correlation with microalbuminuria ($r=0.31$, $p<0.01$ and $r=0.28$, $p<0.05$ respectively). Diastolic BP measurements were correlated with UAE ($r=0.52$, $p<0.0001$) in all study groups. RBC anionic charge showed a negative correlation with U_{GAG} ($r=-0.57$, $p<0.0001$). A positive correlation was observed between U_{GAG} and U_{NAG} levels ($r=0.45$, $p<0.0005$) in all study groups. IgG clearances were correlated with UAE ($r=0.58$, $p<0.0001$), DBP ($r=0.50$, $p<0.0001$), U_{NAG} levels ($r=0.52$, $p<0.0001$), U_{GAG} levels ($r=0.32$, $p<0.01$), urinary collagen IV ($r=0.34$, $p<0.01$) and negatively correlated with RBC anionic charge ($r=-0.65$, $p<0.0001$) in all study groups. Stepwise linear regression analysis of all univariate significant variables including variables that failed to reach significance (age, BMI, enalapril or losartan dosage) with UAE rate as the dependent variable, showed systolic and diastolic BP values, fibronectin, collagen IV, urinary GAG and RBC anionic charge to be independent risk factors for UAE rate (Coefficient of determination of the model was $r^2=0.507$; $p<0.0005$).

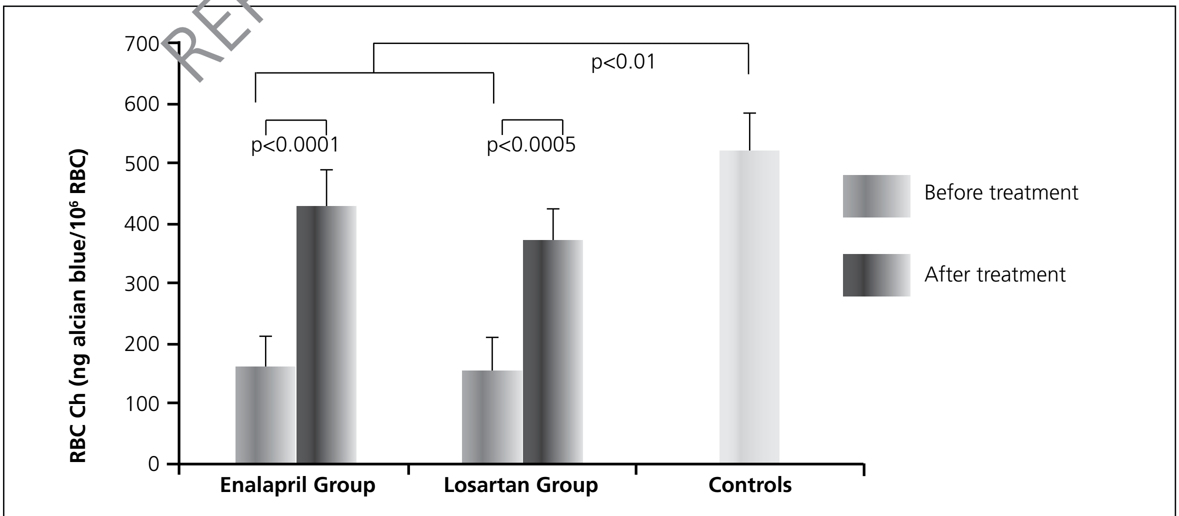

Figure 1
Red blood cell anionic charge measurements before and after treatment in all study groups. Compared with controls pre-treatment values were significantly lower in hypertensive Type 2 diabetic patients. After treatment, there was a significant increase in both enalapril ($p<0.0001$) and losartan ($p<0.0005$) groups.

Discussion

In this study we observed that treatment with losartan and enalapril for six months reduced UAE significantly and similarly in hypertensive Type 2 diabetic patients. This result is in accordance with previous studies that indicate that ACE-Is and ARBs have similar effects in reducing albuminuria in diabetic patients.[1,9]

As a measure of charge selectivity we evaluated IgG clearance and IgG/albumin ratio. IgG clearance was higher and IgG/albumin ratio was lower in diabetic patients compared with healthy controls. IgG clearance decreased and IgG/albumin ratio increased after treatment with both losartan and enalapril.

In a clinical setting, IgG clearance is one of the proven methods in evaluating GBM charge selectivity.[20] In a recent study, Narita *et al*. demonstrated an increase in small-sized plasma protein (IgG, IgG4) excretion in normoalbuminuric Type 2 diabetic patients suggesting the role of impaired glomerular pore size selectivity and increased intra-glomerular pressure in the development of diabetic kidney disease.[21] Animal and human studies indicate the favourable effects of ACE-Is on glomerular charge selectivity,[14,22] but the data about the effects of ARBs on charge selectivity is somewhat unclear. Andersen *et al*. concluded that AT_1-receptor blockage repaired the size selective barrier in Type 1 diabetic patients with early diabetic nephropathy.[23] In a Type 1 diabetes animal model, we also found beneficial effects of ACE-Is and neutral effect of ARB on glomerular basement membrane anionic charges while both agents had similar antiproteinuric effects.[24] Hansen *et al*.[13] have suggested that ACE-Is have favourable effects on glomerular charge selectivity in Type 1 diabetic patients. They found a negative correlation between charge selectivity and urinary albumin excretion. Although captopril treated Type 1 diabetic patients had a higher selectivity index compared with controls, the difference was not statistically significant in their cross-sectional study.

Results of indirect measurements of GBM anionic charge such as RBCCh are in concordance with the results of other studies which show that RBC membrane negative charges are diminished and negatively correlated with the urinary excretion of albumin in patients with diabetes.[25-32] In our study, RBC anionic charges were significantly increased compared with basal levels in both groups. Negative correlation between RBCCh and UAE is in accordance with the findings of Gambaro *et al*. who showed abnormal erythrocyte charge in diabetes mellitus was associated with microalbuminuria.[29]

RBC membrane anionic charges have been assayed in this study by the binding of the cationic dye Alcian blue which is considered to be a reliable estimation of negative charges on RBC surface. Decrease of RBC membrane negative charge in patients with diabetes evidenced by the Alcian blue binding test agrees well with the loss of the surface of electrical charge and reflects reduced GBM anionic content.[17,29-31]

Glycosaminoglycans play an important role in GBM permeability. Increased loss of GAG from basement membrane has been postulated to reduce glomerular charge selectivity which contributes to urinary loss of albumin.[12,33,34] We found increased U_{GAG} excretion in diabetic patients. Although some studies have shown conflicting results, increased U_{GAG} excretion has been similarly documented in diabetic patients by McAuliffe *et al*. and De Muro *et al*.[35,36]

Taken together, these results support the Steno hypothesis which argues that loss of GBM anionic content leads to albuminuria in diabetes.[1] Loss of glycosaminoglycans could be the cause of diminished charge selectivity in diabetic patients. Beneficial effects of ACE-Is and ARBs on charge selectivity could be partly related to decreased loss of GAGs from GBM. Other components of extracellular matrix proteins such as collagen IV and fibronectin are known to be affected by the hyperglycaemic milieu. Increased matrix accumulation in extracellular space and GBM of kidney were reported in diabetes.[1,5] Increased collagen IV excretion accompanies the overproduction of this extracellular matrix protein in the course of diabetic nephropathy.[7,8] In this study untreated diabetic patients have higher urinary collagen IV excretion compared with healthy controls and both ACE-Is and ARB treatment decreased urinary collagen IV excretion to a level similar to healthy controls. Baseline urinary fibronectin levels of diabetic patients were found to be increased compared to healthy controls. Although enalapril-treated diabetics had lower levels compared with baseline, the reduction in the losartan-treated group did not reach statistical significance. Reduced collagen IV excretion might indicate inhibition of collagen IV overproduction by ACE-Is or AT_1-receptor blockers in Type 2 diabetic patients. Although enalapril and losartan have comparable effects in this regard, the effects of enalapril on urinary fibronectin excretion are more pronounced.

In conclusion, ACE-Is and ARBs have comparable effects in preserving GBM anionic content and reducing excretion of collagen IV in Type 2 diabetic patients. Tissue protective effects of RAS inhibition may partly be related to their inhibitory effects on extracellular matrix overproduction and the preservation of anionic charged glycosaminoglycan content of GBM in addition to effective BP control.

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