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Influence of *L*-arginine on glucose mediated collagen cross link precursors in patients with diabetes mellitus

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Summary. Long term complications of diabetes mellitus are largely due to chemical, structural and mechanical changes of connective tissue proteins involving glucose mediated collagen cross links (GMCC). To date there are only experimental therapeutic approaches for preventing long term complications of diabetes mellitus using the toxic substance aminoguanidine. *L*-arginine, a non-toxic substance, has been shown to reduce GMCC in animal models of diabetes mellitus. We have now performed a blind placebo controlled study with crossing over of two treatment periods of three months each in 29 patients with diabetes mellitus in order to examine the effect of treatment with *L*-arginine (2 × 1g daily) on glucose mediated collagen cross links (GMCC). GMCC was evaluated by determining glycosyl lysine (hexosyl lysine) levels in skin punch biopsies. Patients treated by *L*-arginine showed significantly lower GMCC precursors of skin collagen compared with the placebo treated group (difference of hexosyl lysine as counts/mL/ug hydroxyproline between the first and second skin biopsy 0.11 ± 4.44 vs. $4.03 \pm 5.27, t = 2.17, p < 0.05$). The only side effects of *L*-arginine were gastric pain occurring only in patients who did not follow the instructions to take *L*-arginine at meals. We conclude that *L*-arginine could be useful for treating long term complications of diabetes mellitus.

Keywords: Collagen cross links – Glucose mediated cross links – Diabetes mellitus – *L*-arginine – Long term complications – Hexosyl lysines – Glycosylated lysines

Introduction

It is widely accepted that long term complications of diabetes mellitus are at least in part due to nonenzymatic glycosylation of connective tissue proteins. Glucose induced posttranslational modification of collagens, the predominant proteins of connective tissue, has been described by several research groups [1, 2]. Nonenzymatic glycosylation of collagens and their biological consequences are well documented and consists of altered mechanoelastic properties of interstitial and basement membrane collagens, increased aging of collagens in diabetic patients and the relation between glucose induced modification of basement membrane and vascular complications in diabetes mellitus [3–8]. The underlying mechanism for vascular and interstitial collagen accumulation in diabetes mellitus may be explained by nonenzymatic glycosylation of epsilon amino groups of (mainly) lysines in collagen molecules ultimately leading to abnormal glucose mediated cross links [9, 10]. The abnormally cross linked collagen accumulates as it is less soluble and less susceptible to collagenolytic activity [11, 12]. Moreover, modified collagens contribute to impairment of other biological functions and thus to long term complications of diabetes mellitus.

Brownlee and coworkers reported on the pharmacological inhibition of glucose mediated cross links by aminoguanidine, a hydrazine compound [13]. However, aminoguanidine turned out to be toxic. Searching for a less toxic substance, we found that *L*-arginine decreased glucose mediated cross links and reduced basement membrane collagen accumulation in several animal models of diabetes mellitus [14, 15]. Based on these observations and on the fact that *L*-arginine is a nontoxic compound which has been used for many years in paediatric medicine [16], we decided to investigate the influence of this amino acid on glucose mediated cross linking in patients with diabetes mellitus by studying its effect on the crosslink precursor, hexosyl lysine.

Patients and methods

Study design

We performed a blind placebo controlled study with crossing over of two treatment periods of three months. Patients were randomly assigned to treatment groups A or B.

At enrollment, a first blood sample was taken from both groups for evaluation of glycemic control (t1). After a baseline period without treatment of one month, a second blood sample was taken and a skin biopsy was performed (t2). Thereafter the patients of group A were treated with *L*-arginine and the patients of group B with placebo for a period of three months. Then, after drawing a third blood sample (t3), the treatment was crossed over so that group B received *L*-arginine and group A the placebo for a period of three months. Thereafter, the fourth blood sample (t4) was taken and a second skin biopsy was performed. A third skin biopsy after the first three months' treatment period was not performed due to ethical reasons.

The study protocol was evaluated and approved by the Ethical Committee of the University of Vienna and written informed consent was obtained from all patients.

Treatment

Treatment consisted of two daily dosages of 1 g *L*-arginine free base (sugar, salt and starch free, Solgar Inc., Lynbrook, N.Y., USA, FDA approved) in capsules of 500 mg each. Patients were asked to take the capsules at meals (breakfast and dinner) or thereafter with plenty of fluid. Intake of *L*-arginine was controlled by interviewing the patients at regular intervals. Moreover, we assumed that only patients who had taken the drug regularly would have given their consent to the second skin punch biopsy.

Side effects

Side effects were defined as the development of any symptoms, both expected and unexpected. The patients were asked to keep a questionnaire in order to register side effects of the medication throughout the study period.

Patients

40 patients with diabetes mellitus (age 56–78 years) were recruited from the outpatients clinic of the 1st Department of Medicine, University of Vienna. Criteria for inclusion to the study were the diagnosis of diabetes mellitus, the willingness to participate and to accept to take the medication and to take part in the follow-up investigations. Exclusion criteria were other connective tissue disorders, glomerulonephritis and a medication known to influence collagen metabolism such as *d*-penicillamine or cortisone.

At begin of the study there were no significant differences between the two groups concerning sex, age, weight, type and duration of diabetes, associated conditions and their treatment.

Concomitant diseases

Concomitant diseases observed were hypertension ($n = 5$), cirrhosis of the liver ($n = 2$), steatosis of the liver ($n = 1$), asymptomatic cholelithiasis ($n = 1$), osteoporosis ($n = 1$), glaucoma ($n = 1$), cardiac arrhythmias ($n = 1$), stenocardic symptoms ($n = 1$), and bronchial asthma ($n = 1$).

At the begin of the study and at each subsequent follow-up visit, the following data were recorded: weight, medication, diet, concomitant diseases; routine laboratory parameters, blood pressure.

Skin punch biopsies

Skin punch biopsies were performed on the upper arm under local anaesthesia (Xylocain®) using a 6 mm disposable biopsy punch.

Preparation of skin biopsy specimen

Biopsies were finely chopped, blot dry, weighed, washed in PBS (0,15 M NaCl, 0,1 M phosphate buffer, pH 7,4) on Spiramix for 16 hrs. The supernatant was removed, blot dry, weighed and added to 2 ml of PBS. Reduction with borohydride: Aliquots of labelled NaBT4 (125 mCi/mmol) were added to give a collagen/borohydride ratio of 30 : 1 according to the wet weight of the sample. The reduction was allowed to proceed at room temperature for 1 hr, stopped by the addition of acetic acid to a pH of 4.0. The supernatant was removed and the pellet was washed twice with distilled water, freeze dried (15 hrs) and the dry weight was determined. Hydrolysis of samples: Reduced and freeze dried samples were hydrolyzed

in 6 N HCl for 72 hrs. 72 hrs. hydrolysis was employed to ensure maximum and reproducible conversion to hexosyl lysine anhydrides [1]. HCl was removed by freeze drying and twice redissolving in distilled water. The dried hydrolyzates were made up to 1 ml with distilled water, 10 ul were used in the hydroxyproline assay in order to evaluate the collagen content, and 940 ul remained for the determination of hexosyl lysines.

Isolation of reduced cross link components

The reduced hydrolyzates were separated and identified using a single column ion exchange system. The hydrolyzed samples were mixed with 1 ml of an amino acid solution containing 2.5 mg of phenylalanine, tyrosine, lysine, hydroxylysine and leucine/ml 0.01 N HCl before they were applied onto the column (1.2 × 60 cm) filled with Duolite C 225 cation exchange resin, maintained at 60°C by a water jacket. Before packing the column the resin is washed sequentially with hot 0.4 M NaOH, H2O, 1 N HCl, H2O and then converted to the pyridinium form by stirring in 2 M pyridine and finally equilibrated in 0.1 M pyridine-formic acid buffer pH 2.9. The column was eluted with a gradient formed by running 1 M pyridine formate buffer, pH 5.0 into a mixing vessel containing 350 ml of the starting buffer. The flow rate was 60 ml/hr and fractions were automatically collected every 5 min. At the end of each run the column was regenerated by elution with 2 M pyridine, followed by starting buffer.

Fractions were analyzed by counting 2 ml of each fraction in 8 ml scintillation liquid (Scintran, Cocktail T, BDH Chemicals, UK) on an LKB 1215 Rackbeta II liquid scintillation counter. Semiquantitative estimation of reducible collagen cross link content of skin samples was obtained by summation of radioactive counts. Counts of hexosyl lysine and hexosyl lysine anhydride were summed and related to the hydroxyproline content.

Ninhydrin positive peaks corresponding to amino acids were located: aliquots of fractions were spotted onto Whatman 1 paper and peaks were located by spraying with ninhydrin (0.25% w/v in acetone). Glycosylated lysine (hexosyl lysine) levels were expressed as counts/ml/ug hydroxyproline.

Assessment of glycemic control

Blood glucose was determined by glucoseoxydase method. HbAIc was determined after a chromatographic standard method. Fructosamine was assessed by a commercially available photometric kit (Roche), total glycosylated hemoglobin by a commercially available affinity chromatographical assay (Isolab).

Statistical analysis

Data were analyzed by Statistical Analysis Systems (SAS Institute, Cary NC [17]). Differences between groups were analyzed by unpaired *t*-test if appropriate (Shapiro-Wilk statistic), or by Wilcoxon test. Differences within groups were analyzed by paired *t*-test if appropriate (Shapiro-Wilk statistic), or by Friedmans two-way analysis for block design.

Results

From the initially enrolled 40 patients 29 (14 of group A and 15 of group B) terminated the study. Seven patients did not reappear at t2 and one patient did not reappear at t4. Another patient stopped the treatment after infection of the biopsy wound. Two patients decided at t3 that they already had to take so many pills that they would not like to take the arginine capsules in addition.

Four patients did not follow the entire study protocol but were not excluded from the study as the treatment was interrupted only for a short period: patient 16 cancelled the medication for a period of 10 days because of gastric pain. After being advised to take the capsules with fluid, the pain disappeared and he continued the treatment. Patient 14 did not take the medication for 5 days due to gastric pain from taking the drug in the fasting state. Patient 26 did not take the medication for 16 days due to gastric pain after intake without fluid. Patient 31 took only half the dose during the last month of the treatment period.

Hexosyl lysine determinations in skin biopsy material

Mean glycosylated lysine (hexosyl lysine) levels in skin punch biopsy material determined before the first (t2) and after the second treatment period (t4) are given in Table 1 and Fig. 1. After three months of *L*-argine treatment there was a significant reduction of the hexosyl lysine levels in group B whereas there was no difference compared to the baseline values after three months of placebo treatment (group A).

Table 1. Hexosyl lysine determinations in skin biopsies obtained before (t2) and after (t4) the treatment periods

	Mean hexosyl lysine levels (mean SD) (counts/mL/ug hydroxyproline)		
Group	t2	t4	difference t4–t2
A	4.31 ± 3.60	4.21 ± 3.41	−0.11 ± 4.44
B	7.30 ± 6.00	3.27 ± 2.45	−4.03 ± 5.27*

* $t = 2.17$, $p < 0.05$

Side effects

Six patients who did not follow the instructions to take the capsules with fluid and preferably at or after meals complained of moderate [4] or severe gastric pain [2] lasting for hours. This gastric pain was observed only in patients taking *L*-arginine but not during placebo treatment.

Glycemic control

Data on glycemic control are given in Table 2. There were no significant differences throughout the study period in respect to blood glucose, fructosamine, total glycosylated hemoglobin and HbAIc levels.

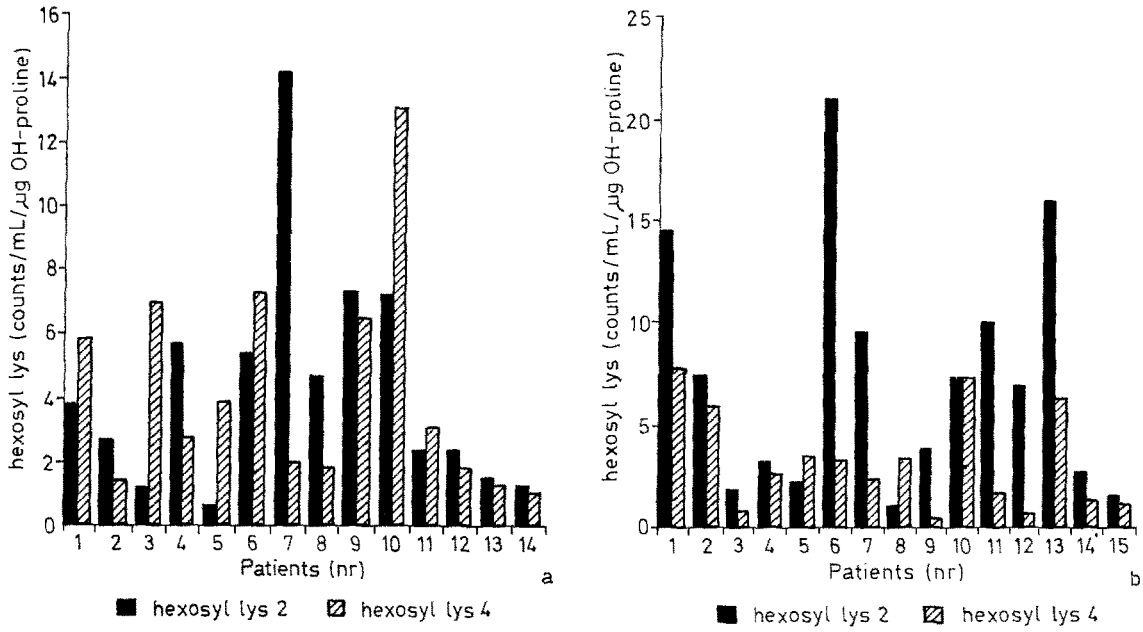

Fig. 1. a Hexosyl lysine levels in skin biopsy material obtained from patients of group A before the first and after the second treatment period. **b** Hexosyl lysine levels in skin biopsy material obtained from patients of group B before the first and after the second treatment period

Table 2. Data on glycemic control

Parameter	Group	t1	t2	t3	t4
Serum glucose (mg/dL)	A	168 ± 51	227 ± 100	207 ± 61	200 ± 89
	B	213 ± 84	193 ± 54	184 ± 97	183 ± 62
HbAIc (%)	A	7.5 ± 1.6	7.0 ± 1.5	7.4 ± 1.5	7.8 ± 1.4
	B	8.0 ± 2.2	8.1 ± 2.2	8.2 ± 1.7	7.8 ± 2.2
Fructosamine (umol/L)	A	366 ± 83	354 ± 55	329 ± 70	316 ± 45
	B	353 ± 96	364 ± 97	321 ± 89	291 ± 75
Total glycosylated hemoglobin (%)	A	10.0 ± 2.2	10.0 ± 2.5	9.7 ± 2.1	9.8 ± 2.3
	B	10.9 ± 3.5	10.5 ± 3.6	10.6 ± 3.3	9.2 ± 3.1

Mean values ± SD

Discussion

A significant reduction of hexosyl lysines reflecting potential glucose mediated cross linking of collagens by *L*-arginine but not by placebo could be demonstrated. It was assumed that in group A the possible lowering of the hexosyl lysine during the first three months had recovered during the second three

months when placebo was used whereas the lowering of hexosyl lysine in group B was due to the effect of *L*-arginine during the last three months.

The positive effect on collagen cross links incriminated as the common pathogenetic factor in (vascular) connective tissue alterations i.e. long term complications, along with the absence of toxicity and poor adverse effects of gastric pain makes *L*-arginine a potentially valuable drug for prevention and treatment of connective tissue complications in diabetes.

The major problem for studying effects on collagens is the availability of tissue: taking serial biopsies from sites as e.g. kidneys is invasive and raises ethical problems. We solved this problem by performing skin punch biopsies. The use of this tissue is appropriate as the nonenzymatic glycosylation is an ubiquitous process in the organism and Monnier and coworkers clearly showed that the degree of collagen accumulation in patients' dermal collagen correlated with the severity of diabetic retinopathy [7].

The reduction in hexosyl lysines on the collagen did not parallel the absence of changes in the level of glycosylated hemoglobin and hemoglobin AIc. The arginine is therefore unlikely to be inhibiting the formation of hexosyl lysines, but may act on the slowly formed Amadori reaction products, which has time to accumulate on a protein of long biological half life. It has been proposed that amino-guanidine inhibits the formation of glucose mediated collagen cross links by reacting with the carbonyle of the Amadori product [11]. The close structural relationship of amino-guanidine and arginine, and the similar effect of both in animal experiments [14] points to an analogous mechanism. Whether this arginine—Amadori complex is cleaved during acid hydrolysis or stabilised during borohydride reduction but not identified on the chromatogram is not clear. However, the end result of the reaction of arginine with the Amadori product is that further reaction to the functionally damaging cross links is inhibited.

Additional effects of *L*-arginine could be responsible for the reduction of glucose mediated cross links: *L*-arginine could have released insulin and improved glycemic control. However, an insulin releasing effect of *L*-arginine in our patients is highly improbable as no influence on glycosylated proteins was observed. Activation of macrophages by *L*-arginine [18] increasing collagen degradation would not have influenced our results because we related hexosyl lysine to the collagen content (expressed as hydroxyproline) of the skin biopsy material. Although these two mechanisms could not be evaluated in our study, it is obvious that both effects could be beneficial to the diabetic patient.

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