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PAMAM G4 dendrimers lower high glucose but do not improve reduced survival in diabetic rats

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ABSTRACT

For nearly a decade poly(amidoamine) (PAMAM) dendrimers G4 were claimed unnegligible cytotoxic agents. Here we monitored whether *in vivo* cytotoxic effect of PAMAM G4 (0.5 μmol kg^{-1} day^{-1}) may be compromised by its ameliorating effect on severe hyperglycaemia in chronic streptozotocin-diabetic Wistar rats. PAMAM G4 significantly reduced the 60-day overall survival in long-term experimental diabetes: treated animals were 6.7 times more likely to die than control animals ($p < 0.025$). PAMAM G4 significantly reduced numerous biochemical parameters in blood, including glucose, glycated haemoglobin or protein oxidation, cholesterol and triglycerides, but apparently unchanged plasma insulin peptide C. Terminal blood glucose in PAMAM-treated animals was significantly higher in survivors, pointing to the possible preventive role of glycation in reducing of PAMAM G4 cytotoxicity.

Our results provide the first *in vivo* evidence that PAMAM G4 is able to lower plasma glucose and suppress long-term markers of diabetic hyperglycaemia. Nevertheless, this beneficial influence cannot override PAMAM G4 cytotoxic effects in the increased mortality of streptozotocin-diabetic rats.

1. Introduction

Poly(amidoamine) (PAMAM) dendrimers (Starburst™ dendrimers) are a new type of promising synthetic polymers characterized by a dendric branched spherical shape and high density surface charge (Malik et al., 2000; Esfand and Tomalia, 2001; Gupta et al., 2006a,b). These dendritic macromolecules with a large number of surface terminal groups, which doubles with each generation, and interior cavities offer a better opportunity for delivery by becoming charged and acting as static covalent micelles (Bielinska et al., 1996; Malik et al., 2000; Esfand and Tomalia, 2001; Gupta et al., 2006b). The defined structure of these molecules and their large number of surface amino groups has led to the interest in dendrimers as substrates for the attachment of antibodies, contrast agents and radionucleotides for applications in a number of different areas of biology and medicine (Roberts et al., 1990; Wiener et al., 1994; Singh et al., 1994; Esfand and Tomalia, 2001; Braun et al., 2005; Tomalia et al., 2007). Due to their surface covered with primary amino groups, dendrimers are able to interact on an electrostatic charge basis with biologically relevant polyanionic macromolecules, such as proteins or nucleic acids, the feature that has been progressively developed and exhaustively studied (Frechet, 1994; Braun et al., 2005; Svenson and Tomalia, 2005). On the other side, due to the presence of numerous free primary amino groups on their surface, poly(amidoamine) dendrimers are strongly nucleophilic molecules, and hence, they appear potential top candidates in the prevention against the modification of macromolecules against carbonylation, acetylation, acylation or glycation. As such they may represent an important group of pharmaceuticals in targeting the excessive post-synthetic modifications of proteins under various clinical conditions. Such a tentative role of PAMAMs, as potential targets for non-enzymatically driven modifications of biomolecules, has never been so greatly appreciated hitherto. The ability of these dendritic polymers to bind large numbers of ligands to their endgroups might lead to novel applications as sequestering agents for metabolic "waste products" under states of imbalance between antioxidant defense and free radical production, like in diabetes, uremia, cancer or aging.

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In vitro and *in vivo* studies in experimental animals using dendrimer conjugates with various biologically active agents have documented these conjugates to be non-toxic and able to target specific cells (Frechet, 1994; Svenson and Tomalia, 2005; Tomalia et al., 2007). On the other hand, numerous reports have shown that amino-terminated PAMAM dendrimers exhibit a considerable cytotoxic activity (Jevprasesphant et al., 2003; Fischer et al., 2003), contrary to anionic compounds (Malik et al., 2000; El Sayed et al., 2002). As far as PAMAM dendrimers are considered for the *in vivo* applications, any studies aimed at establishing various pharmacological and pharmacokinetic parameters, such as biocompatibility and/or any effects on a drug efficacy or an overall survival, seem essential.

In this study we have investigated the ability of PAMAM G4 dendrimers to function as an effective "scavenger" of excessive glucose and targeted modulation of impaired carbohydrate metabolism in animals. To show the scavenging role of poly(amidoamine) dendrimers we have chosen the model of experimental pathology, in which hyperglycaemia and extensive modification of free amino groups by glucose is substantial – streptozotocin diabetes. While these dendrimers are known to be cytotoxic, we aimed at evaluating whether and to which extent such cytotoxic affects might be compromised by the putative beneficial influence of PAMAM G4 against deteriorative implications of hyperglycaemia, oxidative stress and carbonyl stress occurring in chronic untreated experimental diabetes.

The outcomes of this study give us an insight about the Janus face of PAMAM G4 dendrimers *in vivo*. On one hand it supports the previous reports on *in vitro* PAMAM G4 cytotoxicity, on the other it reveals new aspects of their *in vivo* biological activity towards ameliorating impaired carbohydrate metabolism in experimental diabetes.

2. Materials and methods

2.1. Reagents and chemicals

PAMAM G4 dendrimers (methanol solution) as well as all other chemicals were purchased from Sigma–Aldrich (Germany), unless otherwise stated. Potassium chloride, potassium phosphate monobasic and organic solvents for HPLC analysis were from Merck (Merck Sharp & Dohme IDEA, Inc., Bratislava, Slovakia). Trichloroacetic acid (TCA) and lithium hydroxide (LiOH) were from Merck (Darmstadt, Germany). HPLC-grade acetonitrile was from J.T. Baker (Deventer, The Netherlands). Solvents for HPLC were from Merck (Whitehouse Station, NJ). High-sensitivity C-reactive protein (CRP) EIA kit was from Randox Laboratories Ltd. (Crumlin, Co. Antrim, United Kingdom). Linco's Rat C-Peptide Radioimmunoasay Kit was from DRG International Inc. (NJ, USA). Kits for routine biochemical determinations were from Roche Diagnostics (Switzerland) and for glycated haemoglobin (HbA_{1c}) from Drew Scientific Ltd. (Barrow-in-Furness, Cumbria, United Kingdom). Accucheck Active glucose strips were purchased from Roche Diagnostics Polska Ltd. (Warsaw, Poland).

2.2. Animals and study design

In total 80 adult male Wistar rats, weighing between 250 and 300 g, were used in the experiment. Rats were bred in groups of five in accordance with the guidelines of the International Guiding Principles for Biomedical Research Involving Animals of the Council for International Organizations of Medical Sciences (CIOMS 1983), which concurred the principles of respect for life. A standard laboratory chow and tap water were provided *ad libitum*. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1985), as well as with the guidelines formulated by the European Community for the Use of Experimental Animals (L358-86/609/EEC) and the Guiding Principles in the Use of Animals in Toxicology (1989). All experiments were carried out under approval of an appropriate institutional local ethics committee.

According to the estimation of a minimum sample size, we randomly selected 52 rats for the induction of experimental diabetes. Diabetes was induced by an intraperitoneal injection of streptozotocin (STZ) in a dose of 60 mg kg^{-1} body weight. Briefly, STZ was dissolved in 0.1 mol l^{-1} citrate buffer, pH 4.5. Diagnosis of diabetes was made on the basis of the blood glucose concentration (measured in the morning hours, 08:00–09:00 AM). The animals with blood glucose concentrations higher than 16.7 mmol l^{-1}, were considered diabetic and included to the study. Each STZ-injected rat showing hyperglycaemia lower than 16.7 mmol l^{-1} at 72 h after injection was excluded from the study. The experiment on PAMAM G4 effect started after 7 days upon induction of laboratory confirmed diabetes.

According to the estimates based on a pilot study, the follow-up time after recruitment was 60 days and the accrual time during which we recruited animals to experiment was 5 days. Animals with a confirmed diabetes were randomly allocated to one of two groups either receiving or not receiving PAMAM G4 at a dose of 0.5 μmol kg^{-1}. The dose was optimized based on our observations originating from a pilot study showing a marked effect on cardiomyocyte mitochondrial function and lack of hemolytic effects. PAMAM G4 stock dissolved in methanol (10% w/w solution) was diluted in physiological saline 15-fold and given by peritoneal injection daily until the experiment was terminated. Administration of a vehicle (pure methanol 15-fold diluted in physiological saline) served as a control. At the termination of the experiment the survivors in both groups were sacrificed and their blood and organs collected for further biochemical analyses.

Twenty-five non-diabetic rats of the same strain were included in this study to assess the baseline values of the monitored parameters in the group of healthy animals, as the reference control group for diabetic rats.

In-life non-fasting blood glucose (measured always at 09:00–10:00 AM) and body weight were measured once in control non-diabetic animals and monitored weekly in all STZ-diabetic animals under study. Blood was obtained from tail vessels by needle prick and tested using glucose strips or, when exceeding 33.3 mmol l^{-1}, with biochemical analyser. The last determination of blood glucose and body weight were recorded at time preceding the critical event (within a week) and referred respectively to as terminal glucose or terminal body weight.

2.3. Blood collection and biochemical measurements

Animals were anaesthetized with i.m. injection of ketamine•HCl (100 mg kg^{-1} b.w.) and xylazine (10 mg kg^{-1} b.w.). Blood was collected from abdominal aorta of anaesthetized rats (Dobaczewski et al., 2006) and immediately subjected to the separation of blood cells from plasma. All collected plasma samples were frozen at −20 °C, then deep frozen within 6 h, and analysed within 6 months upon sampling.

Routine biochemical determinations were performed with the Hitachi 911 Analyser (Roche Hitachi, Walpole, MA, USA) and plasma C peptide concentration was determined with Rat C Peptide Radioimmunoassay Kit (sensitivity 25 pmol ml^{-1}). Measurements of glycated haemoglobin (HbA_{1c}) in blood samples were made with the DS5 instrument Drew Scientific Ltd. (Barrow-in-Furness,

Cumbria, United Kingdom). To verify the possible interference of PAMAM G4 with determinations of biochemical parameters, the selected samples of whole blood or plasma were *in vitro* supplemented with 0.5 or 5 μmol l^{-1} dendrimer prior to analysis.

2.4. Determination of AGEs and AOPP

Determination of advanced glycation end-products (AGEs) in blood plasma (fluorescent products including pentosidine and carboxymethyllysine) was based on spectrofluorometric detection according to Henle et al. (1999) and Munch et al. (1997) in a modification by Kalousova et al. (2002). Briefly, plasma was diluted 50-fold in a saline (pH 7.4) and fluorescence excited at 350 nm was recorded at 440 nm (PerkinElmer LS50-B, Waltham, MA, USA). Fluorescence intensity was expressed in arbitrary units (a.u.) per g of total plasma protein.

Plasma advanced oxidation protein products (AOPP) were determined spectrophotometrically (340 nm) by microplate assay according to Witko-Sarsat et al. (1998), with chloramine T and acetic acid. AOPP concentration was expressed in chloramine units (μmol l^{-1}).

2.5. Red blood cell hemolysis

The extent of the *in vivo* blood hemolysis in animals administered with PAMAM G4 was monitored in separated plasma as the amount of released hemoglobin at 408 nm (Soret band) and 540 nm (Drabkin method). The *in vitro* hemolysis was monitored likewise, following the incubation of rat whole blood samples (37 °C, in the dark, 4–24 h) with PAMAM G4 at final concentrations of 0.5, 1, 5 or 10 μmol l^{-1}.

2.6. Analyses of dendrimers administered to rats or incubated with glucose

Glycated PAMAM G4 dendrimers were produced in the course of the *in vitro* incubation of 120 μmol l^{-1} PAMAM G4 with 27.8 mmol l^{-1} glucose (37 °C, in dark, 72 h). PAMAM G4 incubated in glucose-free medium served as the control non-glycated dendrimers. Comparative analysis of glycated and non-glycated PAMAM was performed using a combination of high-performance liquid chromatography (HPLC), simple gradient gel electrophoresis (PAGE) and matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) techniques. The reversed phase (RP) HPLC system consisted of a quaternary pump, autosampler, thermostated column compartment, vacuum degasser, and diode-array detector (Hewlett-Packard 1100 Series system, Waldbronn, Germany). For instrument control, data acquisition and data analysis an Hewlett-Packard ChemStation for LC 3D system including single instrument Hewlett-Packard ChemStation software and Vectra color computer was used. UV spectra were recorded on a Hewlett-Packard HP 8453 diode array UV–vis spectrophotometer. The pH of the buffers used was adjusted by potentiometric titration. Water for HPLC analysis was purified using a Millipore Milli-QRG (Vien, Austria) system. The 40 μl aliquot of the final analytical solution was injected onto a 150 mm × 4.6 mm, 5 μm ZORBAX SB C18 column. The mobile phase (flow rate, 1.2 ml/min; temperature, 25 °C) consisted of 0.05 M trichloroacetic acid buffer (solution A), adjusted to pH 2.2 with lithium hydroxide solution of the same concentration, and acetonitrile (solution B). The elution profile was as follows: 0–5 min, 7% B; 5–8 min, 7–35% B; 8–12 min, 35–7% B. Identification of peaks was based on comparison of retention times and diode-array spectra, taken at a real time of analysis, with the corresponding set of data obtained by analyzing authentic compounds. The analytical wavelength was 235 nm. MALDI-TOF mass spectra were acquired using Voyager-Elite spectrometer (PerSeptive Biosystems Inc., Framingham, MA, USA) with the ionizing by pulse laser radiation at 337 nm. The sample was mixed with a matrix solution (α-cyano-4-hydroxycuinnamonic-(*trans*) acid) and further crystallized. PAGE was carried out with the gradient 4–20% gels in non-reducing conditions with Tris–glycine native buffer, pH 8.3 (Bio-Rad Laboratories, CA, USA). Gels were stained with Coomassie Blue R-350 solutions (Shi et al., 2005). The free PAMAM G4 amino groups were evaluated with ninhydrin method (MacFadyen, 1944). HPLC was further used for the detection of dendrimers in rat plasma.

2.7. Statistical analysis

All measurements of biochemical analysis and mitochondrial function were performed in duplicates or triplicates to reason on repeatability. For all quantitative parameters mean with a standard deviation ($\bar{x} \pm$ S.D.) or median (*Me*) and interquartile range (IQR: from lower (25%) quartile (LQ) to upper (75%) quartile (UQ)) were evaluated. Some data with right-skewed distributions were log-transformed prior to analysis. We used standard one-way ANOVA or Student test for the comparison of data showing no departures from normality (according to Shapiro–Wilk's test), and the non-parametric Kruskal–Wallis test or Mann–Whitney *U* test for the remaining variables. Associations between variables were evaluated with the use of non-parametric Spearman rank correlation (R_S) or multiple regression method. Qualitative data were assessed with the exact Fisher's test (Zar, 1999; Armitage et al., 2002).

Time-to-event analysis of data was performed with the use of survival analysis. The event of interest was animal death (overall survival), whereas the explanatory data were the factors believed to be associated with the event or to promote or decelerate its occurrence. The starting point was the date of the first PAMAM G4 injection and in uncensored (complete data) the ending point was animal death. Censored (incomplete) data included rats that remained alive at the termination of this study, or died of causes unrelated to the effect of diabetes and/or PAMAM G4 treatment. The life table method was used to estimate the survival rate and for animals included in the study the requirements of analysis have been met. For the purpose of the time-to-event analysis all animals were randomly allocated to one of two groups according to PAMAM G4 treatment. Log-rank test was used to compare two survival curves. For assessing the associations of between explanatory variables and survival rate we employed Cox proportional hazards regression analysis with the assumption that a ratio risk greater than 1 (lower than 1) denotes an increased (decreased) risk for those with a given characteristic.

Statistical calculations and graphical analysis was made with the use of *Statistica for Windows* (StatSoft Inc., Tulsa, OK, USA) and *Stats Direct* (StatsDirect Ltd., Cheshire, UK).

3. Results

3.1. Metabolic control and other biochemical parameters

All but one STZ-injected rats were characterized by blood glucose exceeding 16.7 mmol l^{-1} at 7 days after STZ injection, so 51 STZ-diabetic animals were included in the further study. Before starting the administration of either PAMAM G4 or a vehicle the rats were randomly allocated to both experimental cohorts, and the final group sizes in the experiment were respectively 25 control rats receiving a vehicle and 26 animals receiving PAMAM G4.

Selected biochemical parameters monitored in blood from healthy non-diabetic rats, as well as diabetic animals receiving either vehicle or PAMAM G4 that survived the experiment

Table 1
Biochemical parameters in blood plasma withdrawn from healthy non-diabetic rats and STZ-diabetic rats treated with either PAMAM G4 or vehicle

Parameter	Healthy (non-diabetic) non-treated ($n = 25$)	Diabetic given vehicle ($n = 20$)	Diabetic given PAMAM G4 ($n = 8$)	Significance ($p<$)
Glucose (mmol l^{-1})	9.4 (9.0; 11.1)	35.1 (33.0; 40.7)	22.2 (19.0; 25.8)	0.0001^{np} 0.0001^{np}
HbA_{1c} (%)	4.5 ± 0.3	7.2 ± 1.5	4.7 ± 0.9	0.0001 0.0001
AGEs (a.u. g^{-1} protein)	1.24 ± 0.24	2.95 ± 0.83	1.81 ± 0.29	0.0001 0.04
AOPP (μmol l^{-1})	162 (134; 193)	233 (199; 302)	145 (50; 191)	0.0003 0.0001
Total protein (g l^{-1})	58.3 (54.6; 65.2)	63.1 (58.3; 64.4)	60.3 (59.5; 61.8)	n.s. ^{np} n.s. ^{np}
Albumin (g l^{-1})	26.2 ± 2.6	23.9 ± 6.8	19.0 ± 5.6	n.s. n.s.
Alkaline phosphatase (μkat l^{-1})	2.5 (1.9; 2.9)	5.2 (3.6; 7.7)	5.0 (3.3; 6.5)	0.0001^{np} n.s. ^{np}
Ala aminotransferase (μkat l^{-1})	1.0 (0.9; 1.1)	7.0 (3.9; 13.9)	1.1 (1.0; 1.4)	0.0001 0.0001
Asp aminotransferase (μkat l^{-1})	1.2 (1.1; 1.4)	12.7 (6.0; 26.7)	1.6 (1.6; 1.9)	0.0001^{np} 0.0001^{np}
Creatinine (μmol l^{-1})	61.4 ± 4.5	37.4 ± 6.1	33.0 ± 3.1	0.0001 n.s.
Uric acid (μmol*l^{-1})	42 (36; 49)	52 (36; 89)	94 (41; 172)	n.s. ^{np} 0.002^{np}
Urea (mmol l^{-1})	7.7 ± 1.8	17.7 ± 6.3	11.6 ± 2.6	0.0001 0.005
Total cholesterol (mmol l^{-1})	1.14 ± 0.40	1.48 ± 0.42	1.08 ± 0.15	0.02 0.05
Triglycerides (mmol l^{-1})	0.64 (0.39; 0.84)	2.20 (1.28; 3.21)	0.90 (0.77; 0.96)	0.0001^{np} 0.01^{np}
C-reactive protein (μg l^{-1})	75 (0; 440)	55 (0; 215)	15 (0; 120)	n.s. ^{np} n.s. ^{np}

All parameters in diabetic rats measured in plasma derived of blood withdrawn from the animals that survived the observation. Mean ± 1 S.D. are given for normally distributed variables, and median (lower quartile; upper quartile) for the remaining parameters; n in parentheses. Significance of differences estimated with one-way ANOVA and the post-hoc multiple comparison Tukey's test or non-parametric Kruskal–Wallis test with the all pairwise comparison Connover–Inman test (^{np}); significance values refer to the comparison between healthy non-treated animals *vs.* STZ-diabetic rats treated with a vehicle (upper) and between diabetic rats treated with a vehicle *vs.* diabetic treated with PAMAM (lower); n.s., considered non-significant if $p \geq 0.05$.

are summarized in Table 1. The majority of parameters (including glycated haemoglobin, AGEs, AOPP, aminotransferases, with the ratios of their activities unchanged, lipids and urea) were greatly elevated in diabetic rats compared to control animals, while the administration of PAMAM G4 normalized these values to normal or near-normal physiological levels. Plasma glucose was vastly increased in diabetic animals receiving a vehicle, whereas PAMAM G4 moderately reduced its level, though it still remained over twofold higher compared to healthy control. The hallmarks of the non-enzymatic protein modification and glycaemic control became completely, like glycated haemoglobin (HbA_{1c}) and advanced oxidation protein products (AOPP), or almost completely normalized, like the advanced glycation end-products (AGEs). Both plasma albumin and uric acid were not different between healthy and diabetic animals, however, the treatment with the dendrimer lowered the first and raised the second. A few other parameters, although altered in diabetic state, did not change significantly upon PAMAM G4 administration (creatinine, alkaline phosphatase). Serum C-reactive protein was decreased over threefold in animals treated with dendrimers, these changes however, remained beyond significance (Table 1). The administration of dendrimer did not influence plasma concentrations of insulin peptide C (1.60 [1.10–1.93] μmol l^{-1} in PAMAM-treated *vs.* 1.73 [0.98–1.97] μmol l^{-1} in non-treated animals, *NS*; reference range in control non-diabetic rats, $n = 69$, $\bar{x} \pm 95\%$CI: 4.80 [4.25–5.30] μmol l^{-1}). None of the analytical methods used for monitoring of biochemical parameters was affected by the *in vitro* sample supplementation with dendrimer at either 0.5 or 5 μmol l^{-1}. Based on the amount of released hemoglobin both groups of animals did not differ in the extent of *in vivo* hemolysis. Also, no significant hemolysis was recorded following the *in vitro* blood incubation with dendrimer up to the concentration of 10 μmol l^{-1}.

The in-life non-fasting blood glucose monitored within a week preceding animal death or at the termination of the experiment, was significantly lower in animals administered with PAMAM G4 (14.4 [8.3; 17.2] mmol l^{-1} *vs.* 19.6 [15.1; 22.0] mmol l^{-1} in controls, $p < 0.0001$), while there were no significant differences in terminal body weights between the groups (200 [200; 213] g *vs.* 220 [180; 230] g in controls, n.s.). Interestingly, in the group of diabetic rats treated with PAMAM G4 the in-life terminal blood glucose, but not terminal body weights, were significantly higher in survivors compared to animals that did not survive the observation period (21.2 [18.9; 22.3] mmol l^{-1} *vs.* 12.5.0 [12.0; 13.1] mmol l^{-1} in non-survivors, $p < 0.0005$). No similar differences were found between survivors and non-survivors with respect to either terminal glucose or terminal body weight in diabetic animals not receiving PAMAM G4.

We revealed positive correlation between survival and terminal glucose ($R_S = 0.588$, $p < 0.0001$), but not for pre-terminal measurements (2 or 3 weeks prior to the terminal determination), when all animals from both groups were included in the analysis. The association remained significant also for the group of PAMAM-treated diabetic animals ($R_S = 0.357$, $p < 0.04$), but not for the excluded group of rats receiving a vehicle ($R_S = 0.208$). There was a significant collinearity of nearly all biochemical parameters, which were different between the groups. However, upon standardizing for other variables in the model, the only significant partial correlation was found between glycated haemoglobin and plasma triglycerides ($r_p = 0.478$, $p < 0.04$). Glycated haemoglobin was the variable that mostly contributed to significant discrimination between the groups of rats treated with either PAMAM G4 or a vehicle (par-

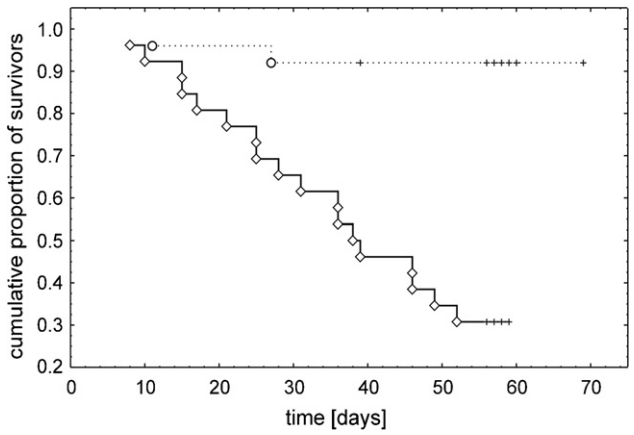

Fig. 1. The Kaplan–Meier curves of survival in groups of Wistar rats given PAMAM G4 or vehicle. The step function of the estimated cumulative proportions of survivors are given for STZ-diabetic rats treated with PAMAM G4 (solid line) or given a vehicle (dashed line). Complete observations are marked with circles or diamonds, respectively for control or PAMAM G4-treated animals; censorships are marked by '+'. Significance of differences between survival curves, given by Wilcoxon–Gehan test, was $p = 0.00005$.

tial Wilks' lambda = 0.623, $p < 0.002$, tolerance = 0.969 according to forward stepwise discriminant analysis).

3.2. PAMAM G4 administration as the prognostic factor for increased mortality

As shown in Fig. 1 the Kaplan–Meier estimates of the interim 30-day survival rates were 61.5% (95%CI: 42.8–80.2%) for the PAMAM-treated animals ($n = 26$) and 92.0% (95%CI: 81.4–102.6%) for the control group ($n = 25$). For twice as long period of observation, the estimates were respectively 30.8% (95%CI: 13.0–48.5%) and 90.2% (95%CI: 79.1–100.2%). Both the generalized Wilcoxon (Gehan–Breslow) test ($p = 0.00005$) and log-rank test ($p = 0.00002$) confirmed a statistically significant difference between the survival rates over time between rats receiving PAMAM G4 and a vehicle (OR = 12.6; 95%CI: 8.2–19.4; $p < 0.001$). These estimates of cumulative survival in both groups of animals clearly show that PAMAM drastically reduced both early survival and late survival. Mean survival time was 38.8 days (95%CI: 31.9–45.7 days) for the PAMAM-administered group and 65 days (95%CI: 57.4–72.6 days) for the reference group. It means that the estimated 50% of animals given PAMAM will die within 39 days after the starting point; the other 50% will either not die or will die later than 39 days after the observation started. PAMAM G4 administration was thus more risky to experience death by Wistar rats with chronic STZ-diabetes. Further investigation with Cox proportional hazards regression analysis, which controlled for the effects of the terminal non-fasting blood glucose and body weight, indicated that animals administered with PAMAM were over 6 times more likely to die than those, which received a vehicle (the hazard or risk ratio = 6.7; 95%CI: 3.3–13.6; $p < 0.025$) (Fig. 1).

3.3. Effects of PAMAM G4 on the fluctuations of in-life blood glucose and body weight

As evidenced by the Cox proportional hazard regression analysis, there was a reciprocal dependence between the terminal blood glucose and rat survival (0.87; 95%CI: 0.86–0.89; $p < 0.0001$ for the unitary increment in glucose, concentration, i.e. 1 mmol l^{-1}), which means that each increment in blood glucose concentration by 1 mmol l^{-1} was accompanied by the reduction in rat survival by approximately 15%. The effect of blood glycaemia was not sustained when controlling for PAMAM administration, which points to collinearity of these two effects. Paradoxically, in the group of animals given PAMAM there was a discriminating effect of blood glucose on survival depending on the monitoring time preceding the animal death. The survival rate was significantly lower in the animals with lower blood glucose when monitored 3 weeks prior to critical event (or the termination of the experiment) (Fig. 2a), the effect was less distinct after 1 additional week (Fig. 2b), and completely vanished for terminal measurements (Fig. 2c). It means that at 3 wk prior to critical event the rats with blood glucose below the median value of the group, i.e. 16.3 mmol l^{-1}, were over 2.3 times more likely to die than those with higher blood glycaemia (OR = 2.33, 95%CI: 1.22–4.44, $p = 0.044$). Respectively, at 2 weeks prior to critical event the hazard ratio decreased a little (OR = 2.03, 95%CI: 1.00–4.13, $p = 0.073$), and the "lethal" effect disappeared when glucose was monitored terminally. We revealed no significant effect of terminal body weight on the incidence of fatal events,

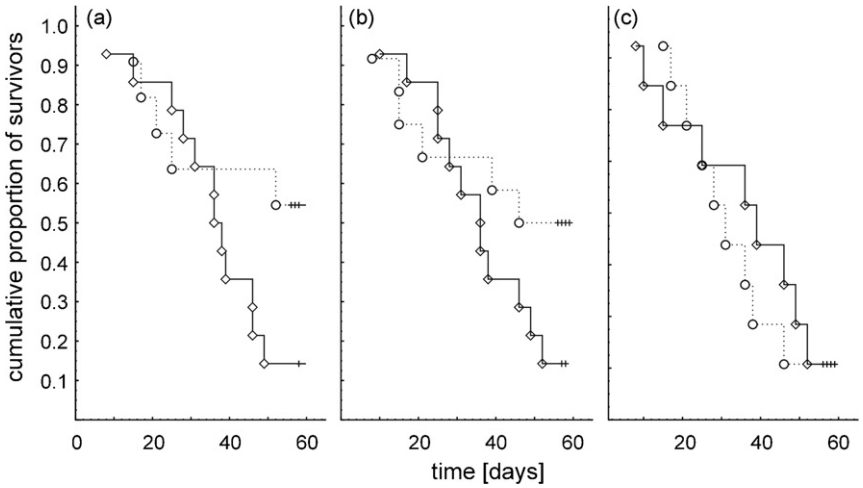

Fig. 2. The Kaplan–Meier curves of survival in PAMAM G4-treated streptozotocin-diabetic Wistar rats in groups divided based on blood glucose concentrations. The step function of the estimated cumulative proportions of survivors for STZ-diabetic rats treated with PAMAM G4 with either high (above median, dashed line) or low (below or equal to median, solid line) blood glucose concentrations monitored (a) 3 weeks ($Me = 16.3$ mmol l^{-1}), (b) 2 weeks ($Me = 15.1$ mmol l^{-1}) or (c) within 1 week prior to critical event ($Me = 14.4$ mmol l^{-1}). Complete observations are marked with circles or diamonds, respectively for animals showing respectively lower or higher glucose; censorships are marked by '+'. Significances of differences between survival curves, given by log-rank test, were (a) $p = 0.044$, (b) $p = 0.073$ and (c) $p = 0.406$.

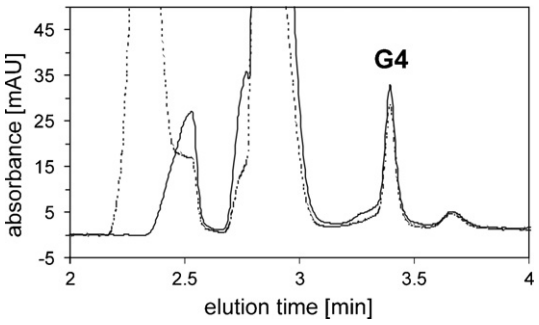

Fig. 3. Chromatograms of amine-terminated non-modified (solid line) and glycated (dashed line) generation 4 dendrimers (PAMAM G4). PAMAM dendrimers were injected separately. The mobile phase flow rate was 1.2 ml min^{-1} (25 °C), and consisted of 0.05 mol l^{-1} TCA (solution A, adjusted to pH 2.2 with LiOH) and acetonitrile. The elution profile was as follows: 0–5 min, 7% B; 5–8 min, 7–35% B; 8–12 min, 35–7% B. The detection wavelength was 235 nm.

when monitored either in both groups of diabetic rats or exclusively in the PAMAM-treated animals.

3.4. PAMAM G4 non-enzymatic modifications and its detection in STZ-diabetic rats

Using the ninhydrin method we were able to detect 12.6% (±0.8%, S.E.M.) of all surface primary amino groups in the commercially available PAMAM G4 preparation used in this study. The prolonged *in vitro* incubation of PAMAM G4 with high concentrations of glucose reduced the number of free ninhydrin-reactive surface amino groups by 89.8 ± 3.2% ($n = 5$). MALDI-TOF showed a considerable heterogeneity of the used PAMAM G4 preparation (12–15 kD, not shown). Neither PAGE nor MALDI-TOF allowed for reliable discrimination between non-modified and glycated PAMAM G4, pointing that there is no detectable size difference between two forms of PAMAM G4 (not shown). Fig. 3 shows the chromatograms of non-modified and the *in vitro* glycated generation 4 dendrimers (PAMAM G4). Under the same gradient conditions, the chromatograms show no marked differences except for the dead volume peaks. The results clearly demonstrate that there is no detectable difference in retention between non-modified and glycated PAMAM G4. Elution profiles of selected samples taken each few days did not show any appreciable change, which confirm the stability of the forms of PAMAM G4 dendrimers.

Due to very low PAMAM G4 concentrations (beyond detection limits of the method) in plasma samples, we were not able to either estimate the real PAMAM concentrations in the circulating blood withdrawn from the monitored animals, or discriminate between various dendrimer forms.

4. Discussion

We demonstrate here for the first time – to the best of our knowledge – the novel activity of full generation of PAMAM dendrimers *in vivo* on the overall survival of laboratory animals with experimental diabetes. One of novelties of our present report is pointing to the possible dual role of polycationic PAMAM dendrimers in the pathological states with massive deterioration of biomacromolecules, like chronic poorly controlled diabetes. PAMAM G4 appears to mimic the action of hypoglycaemic agents in reducing plasma hyperglycaemia and long-term markers of poor metabolic control in diabetes: protein glycation and glycoxidation, as well as other markers of oxidative and carbonyl stress. Although the precise mechanism(s) underlying the PAMAM G4 *in vivo* activity as the agent alleviating carbohydrate metabolism impairments and reducing the impact of long-term diabetic hyperglycaemia in STZ-diabetic rats remain unknown, some plausible targets have been tested. We have shown in this study that PAMAM G4 suppresses plasma hyperglycaemia in diabetic rats, and thus may contribute to reduced glycoxidation and post-synthetic non-enzymatic modification of biomacromolecules in long-term streptozotocin-diabetic rats. Despite glucose, also plasma cholesterol and triglycerides, elevated in diabetic animals, became reduced upon PAMAM G4 administration. Most likely, these beneficial and alleviating effects of dendrimer on diabetes-associated metabolic impairments was not due to its cytoprotective effects on pancreas, since we did not reveal elevated generation of residual insulin in PAMAM-treated STZ-diabetic rats.

Despite these apparently mitigating effects on diabetes-associated complications, the overall impact of the dendrimer on animal mortality was negative, pointing that such a beneficial influence cannot compromise PAMAM's G4 cytotoxicity and its contribution to lowered overall animal survival.

The limitation of this study was that our statistical discrimination with respect to metabolic control variables between PAMAM G4-treated rats and the animals receiving a vehicle is based on one-point measurements performed in survivors of this observational study. It was so because our main objective was to record the overall effect of PAMAM G4 on survival of animals with experimental diabetes. As far as we employed such a design of our study we were not able to collect biological material in an amount sufficient to perform the in-life measurements of a majority of biochemical parameters. Thus we are not able to directly link the possible fluctuations in the recorded metabolic parameters to the declining survival of animals under study. Using such an approach we deliberately chose merely single parameters, which we were able to catch with no interference to the prospective observation of animals.

This paper is the first one concerning the effects of the administration of poly(amidoamine) dendrimers (not conjugated with any drug) in animal models of experimental diabetes. In line with our present findings on the *in vivo* cytotoxicity are earlier studies showing detrimental effects of PAMAM G4, uptaken probably in liver, spleen and kidneys (Wang et al., 2003). To date, the toxicity of dendrimers has been primarily studied *in vitro*, however, a few *in vivo* studies have also been published. Full-generation dendrimers with numerous end amino groups have been repeatedly demonstrated as highly cytotoxic via mechanism(s) depending on cell membrane disintegrating properties (Malik et al., 2000; Gupta et al., 2006a,b). Such a lytic toxicity of all polycationic full generation dendrimers has been claimed a major limitation for their use as drug delivery systems. In our experiment however, we did not record the increased *in vivo* lysis of red blood cells in diabetic rats administered with PAMAM G4 (ca. 1.67 mg kg^{-1}) compared to diabetic animals treated with a vehicle, which remains convergent with the earlier study by Boas and Heegaard revealing the lack of lytic toxicity of PAMAM dendrimers up to the fifth generation, when given at doses of up to 10 mg kg^{-1} to mice (Boas and Heegaard, 2004). Furthermore, in our study we have not noted any considerable hemoglobin release in response to the *in vitro* incubation of rat blood with PAMAM G4 up to the concentration of 10 μmol l^{-1}.

Several *in vivo* and *in vitro* studies published hitherto have provided rather conflicting results, showing either none or significant toxicity (Veronese et al., 1990; Roberts et al., 1996; Malik et al., 2000; Bhadra et al., 2003), and thus gave the rise to the speculations that the differences in outcomes between the *in vitro* and *in vivo* studies might be attributable to the ultimate toxicological profile of any dendrimer given to laboratory animals (Roberts et al., 1996). Regardless of these differences, however, cationic dendrimers are generally claimed to act as unnegligible cytotoxic agents (Malik et al., 2000), and the presence of free amino groups on the sur-

face of plain dendrimers, which may become occupied by the drug molecules in case of dendritic drug complex, are responsible for the toxicity (Veronese et al., 1990; Bhadra et al., 2003). Polycationic dendrimers have been reported to exert immunogenicity and myotoxicity in rats with the severity closely related to the overall molecule positive change (Roberts et al., 1996; Brazeau et al., 1998). The compounding harmful effects of PAMAM dendrimers with their relatively high bioavailability, due to unrestrained solubility, as well as physical and chemical interaction in the immediate surrounding environment (Malik et al., 2000; Gupta et al., 2006b, 2007), could have furnished to provide increased potency of PAMAM G4 dendrimers as the agents reducing overall survival in experimental animal models.

The idea of undertaking this project was to monitor the impact of PAMAM dendrimers as scavengers in animal model of pharmacologically untreated experimental diabetes, where the cumulative diabetes-related and chronic hyperglycaemia-mediated complications are strong life-threatening risk factors, contributing themselves to increased mortality of diabetic animals (Thorpe and Baynes, 1996; Vlassara, 1997; Baynes and Thorpe, 2000; Vlassara and Palace, 2003; Watala et al., 2006; Temel and Akyuz, 2007). In our approach we deliberately used higher dose of streptozotocin (60 mg kg^{-1} compared to 45 mg kg^{-1} used in some earlier studies (Watala et al., 2006; Ulicna et al., 2006)) to induce a severe experimental diabetes. The severity of disease in our experiment was reflected by enormous alterations in the monitored biochemical parameters in diabetic animals, including the typical markers of glycaemic control, like glycaemia, HbA_{1c}, AGEs, AOPP, as well as other parameters showing impaired liver function (aminotransferases, urea) (Loeb, 1997; Nichols, 2003). Interestingly, the present study reveals that increased non-enzymatic modifications of proteins in diabetic rats (glycated haemoglobin, AGEs, advanced oxidation protein products) became vastly reduced in the animals treated with PAMAM G4. Although the reduction in plasma glucose in PAMAM-treated animals may appear moderate, the markers of glycaemic control became almost completely normalized, thus pointing that the glucose lowering by the dendrimer may be merely one of the contributors to reduced protein glycation and oxidation. Encouraging may be the suggestion that PAMAM G4, rich in free primary amino groups, may act as competing targets for non-enzymatically attached adducts and shield the natural protein amino groups against the excessive modification(s) in poor-controlled diabetes. In present study we provide two lines of evidence to support the above reasoning. First, we showed that PAMAM G4 dendrimers incubated *in vitro* with high glucose may undergo non-enzymatic glycosylation at surface primary amino groups, although we were unable to detect such modified dendrimers in the circulating blood of treated animals. Second, we showed that considerable reductions in protein glycation/glycoxidation (HbA_{1c}, AGEs) and protein oxidation (AOPP) associated with PAMAM G4 treatment was much more profound than the dendrimer-mediated reductions in plasma glucose concentrations. Interestingly, due to high contents of negatively charged glycosaminoglycans in extracellular matrix, polycationic dendrimers could accumulate up to high concentrations in vessel wall, where their impact on hampering non-enzymatic modifications of biomolecules might be even underestimated (Sakharov et al., 2003).

One way to reduce the cytotoxicity of cationic dendrimers may reside in partial surface derivatization with chemically inert functionalities, such as PEG or fatty acids (Yoo and Juliano, 2000). Interestingly, also the complexes of PAMAM dendrimers with drugs covalently linked to surface amino groups showed the reduced overall positive charge (Kobayashi et al., 2001) and much lesser toxicity at equivalent concentrations of dendrimer (3.5–7 times) than the plain dendrimers (Bhadra et al., 2005). Herein, we hypothesize that the alternate, pathophysiological but natural modification might be non-enzymatic attachment to dendrimeric free surface amino groups the by-products generated under some states of exaggerated glycoxidation, carbonylation or oxidative stress. Such a non-enzymatic covalent modification may have two possible implications for dendrimer pharmacokinetics. First, the clearance of thus modified full generation cationic PAMAMs would be retarded, retaining their longer bioavailability and making them more effective drug carriers and hence more suitable for targeted delivery (Bhadra et al., 2005). Second, PAMAMs with naturally modified amino groups by glucose, carbonyls, etc., would be expected to appear the less cytotoxic, the more advanced non-enzymatic modifications of their surface amino groups have occurred. While the first implication has been positively verified by making glycodendrimeric carriers for primaquine targeting to liver (Bhadra et al., 2005), the second has been revealed in our study. Paradoxically, higher blood glucose was apparently protective against polycationic PAMAM-mediated cytotoxicity death under conditions of a long-term chronic diabetes. In the present study we showed that dendrimers may undergo excessive glycation following their *in vitro* incubation with high concentrations of glucose. Due to some technical limitations of HPLC assay in a preparative separation of various forms of PAMAM dendrimers (Islam et al., 2005), their considerable size heterogeneity and also because the real concentrations of PAMAM G4 in a circulating blood of animals were extremely low and remained beyond a detection limit, we were not able to determine the contents of glycated dendrimer derivatives in plasma obtained from diabetic rats (Muller et al., 2007).

In summary, by limiting the progression of diabetic sequelae poly(amidoamine) dendrimers could potentially contribute to lesser severity of diabetes and suppressed mortality of diabetic animals. One should consider however, the peculiar condition of a compromise in the appropriate number of surface amino groups: low enough to reduce polycationic cytotoxicity and high enough to shield against non-enzymatic modification of biomacromolecules. Whether such a compromise could be achieved with lower generation of PAMAM dendrimers, remains to be established.

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