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Ambiguous effect of dendrimer PAMAM G3 on rat heart respiration in a model of an experimental diabetes – Objective causes of laboratory misfortune or unpredictable G3 activity?

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ABSTRACT

Poly(amido)amine (PAMAM) dendrimer G3 was investigated for its ability to support the proper functioning of rat heart mitochondria exposed to hyperglycemia, in both the *in vitro* and *in vivo* experiments. The main aims of this study were to check whether PAMAM G3 dendrimer improves the efficiency of the impaired respiration of rat heart mitochondria.

This study showed that mitochondria isolated from animals studied in different seasons respond to G3 (100 μM) exposure to a different extent. Probably, seasonal variations had the impact on rat metabolism and consequently on the received data. The used biological samples formed a heterogenous group and therefore the obtained results were not pooled together but treated separately. Nevertheless, the *in vitro* part of this study revealed that PAMAM G3 could be successfully used in the protection of heart mitochondria against MG-induced impaired respiratory activity. Despite these promising data, the protective effect of G3 was not confirmed in the *in vivo* experiment. This study revealed that dendrimer G3 (20 mg/kg bw) is toxic and very high mortality among the animals administered with G3 did not allow to perform a reliable data analysis.

1. Introduction

World Health Organization (WHO) reports that at present more than 220 million people have diabetes. It is estimated that this number will increase to 366 million in 2030. This disease is associated with a predisposition to the occurrence of chronic complications, such as retinopathy, cataracts, neuropathy, nephropathy and cardiovascular diseases, which are the leading cause of death in patients with diabetes (Rolo and Palmeira, 2006). Emerging evidence suggests that diabetic cardiomyopathy is linked to alterations in myocardial fuel and energy metabolism. Different experimental reports indicate that the mitochondria play a key role in the pathogenesis of insulin resistance and diabetes (Oliveira et al., 2003). The reduced efficiency of mitochondrial respiration has been demonstrated in diabetes, particularly in tissues highly dependent on aerobic metabolism, such as the heart or brain cells. It has been suggested that this decrease is the result of disturbances in the mitochondrial functioning, reductions in the electron transport chain activity and thus, a lower production of ATP. These changes have been attributed to defects in several active sites of the mitochondrial electron transport chain, including all mitochondrial complexes (I, II, III, IV) and the ATP synthase. Therefore, in our study, we have just focused on the respiratory capacity of cardiac mitochondria. Cardiomyocytes are the cells that are greatly influenced by insulin and are characterized by very high demand for energy. Diabetic heart is characterized not only by the reduced ATP formation, but also by the decreased oxidative capacity and the increased production of reactive oxygen species (ROS) (Shen, 2010).

Chronic hyperglycemia is widely recognized as a factor linking diabetes with its complications. Toxic effects of glucose on cells and tissues are underlied by the action of several mechanisms, including increased production of advanced glycation end products (AGEs) and oxidative stress. These processes are related to each other at several molecular levels and biochemical pathways (Solnica, 2006). It is suggested that chronic hyperglycemia can affect the functioning of mitochondria by modification of mitochondrial proteins in the process of glycation (Kang et al., 1996). The main sites of the attack of excessive glucose concern the complexes I, III and IV (Bessman and Mohan, 1997; Craven et al., 2001). The

Abbreviations: AGEs, advanced glycation end products; DM, diabetes mellitus; ETS, electron transfer system; MG, methylglyoxal; OXPHOS, oxidative phosphorylation; PAMAM G3, generation 3 of poly(amido)amine dendrimer; RCR, respiratory control ratio; ROS, reactive oxygen species; STZ, streptozotocin.

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results of these interactions are the respiratory chain damage, ROS overproduction and macromolecules modification (Wallace, 1999). Consequently, in damaged mitochondria the ATP production is significantly limited. Furthermore, mitochondria do not use glucose and lipids in the proper way and it contributes to their accumulation out side the mitochondria. Then, mitochondria start to work in a vicious circle of glycation processes, oxidative stress and the subsequent mitochondrial damage, which often leads to numerous tissue and cell disturbances (Waldbaum and Patel, 2009).

The drug/diet supplement administration in order to prevent protein glycation is an extremely important achievement in the curing of diabetes, its late complications and many other diseases associated with the formation and accumulation of AGEs in the organism. It is highly probable that this potential could be found in the full generations of poly(amido)amine PAMAM dendrimers, containing on their surface free primary amino groups. It was experimentally revealed (in *in vitro* and *in vivo* research) that dendrimers' amine groups have the potency to bind the excess of free glucose and effectively diminish the diabetic complications caused by hyperglycemia (Labieniec and Watala, 2010; Labieniec et al., 2010). Our *in vivo* study has shown that dendrimer PAMAM G4 administered to animals for 60 days at a dose of 0.5 μmol/kg/day successfully reduces the level of hyperglycemia and, decreases the effect of glycooxidation and detrimental biomolecules' modifications in rats with experimentally streptozotocin (STZ)-induced diabetes (Labieniec et al., 2010).

In this paper, we tested the hypothesis that PAMAM dendrimer G3 has a potency to reduce the detrimental effect of experimental diabetes on rat heart mitochondrial respiratory capacity – both under *in vitro* and under *in vivo* conditions. In order to verify this assumption, the following specific main objectives were realized: the estimation whether PAMAM dendrimers of the generation G3 are able to restore the bioenergetics of rat heart mitochondria subjected to detrimental changes caused by: (a) methylglyoxal (MG), the strong reactive dicarbonyl compound participating in the formation of AGEs in the model of *in vitro* formation of diabetic metabolic impairments, and (b) experimentally induced STZ-diabetes – the *in vivo* model of experimental type 1 diabetes. The secondary aim was to investigate whether there is a positive correlation between the outcomes of *in vitro* and *in vivo* studies and whether the *in vitro* experimental design could be helpful in the designing of the *in vivo* study with respect to experimental conditions (concentrations, duration, *etc.*). The assessment of the impact of seasonal variations on mitochondrial respiratory response has been also observed and analyzed as an important confounder of the studied mitochondrial characteristics.

2. Materials and methods

2.1. Chemicals

PAMAM G3 dendrimers 20 wt.% solution in methanol (ethylenediamine core; PAMAM G3, $[NH_2(CH_2)_2NH_2]$:(G=3);dendri PAMAM$(NH_2)_{32}$; molecular weight of this dendrimer and the number of terminal NH_2 groups are 6909 and 32, respectively), succinate, rotenone, oligomycin, FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), antimycin A, dispase II (D 4693), Bicinchoninic Acid Kit and streptozotocin were purchased from Sigma–Aldrich. All other reagents and solvents used in this study were of the highest analytical reagent grade.

2.2. Biological material

Mitochondria were isolated from hearts of Wistar rats (males, 180–300 g). Before the experiment, the animals were housed under standard environmental conditions for 2 weeks (25 °C, with a light/dark cycle of 12 h/12 h). The experiments were conducted 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), 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).

2.3. Isolation of rat heart mitochondria

Mitochondria were isolated according to the procedure described by Ferko et al., with slight modifications (Ferko et al., 2008). Briefly, hearts removed from the animals, were immediately cooled down in ice-cold isolation solution (IS, containing 250 mM sucrose, 0.5 mM EDTA, 10 mM Tris and 1 g/l bovine serum albumin, pH 7.4). The heart tissue was cut into small pieces with scissors and stirred on ice for 20 min in 10 ml of IS containing the addition of dispase II (1.5 mg/ml). Then pieces of heart were gently homogenized using a teflon/glass homogenizer and centrifuged for 10 min at 800 × *g*. The pellet was discarded and the supernatant was centrifuged again for 10 min at 4800 × *g*. Then, the resulting supernatant was removed together with the top layer of the pellet, which contained damaged mitochondria. The pellet was resuspended in the same volume of IS and again centrifuged (4800 × *g*, 10 min). All centrifugations were conducted at 4 °C. Finally, the pellet containing rat mitochondria was gently resuspended in the ice-cold IS and stored on ice prior to the experiments. Mitochondrial protein concentration in each preparation was determined using the Bicinchoninic Acid Kit.

2.4. Mitochondrial respiratory measurements

Oxygen consumption in rat heart mitochondria (~24 mg of protein/ml) was monitored *in vitro* using a polarographic technique with a Clark electrode (Oxygraph-2k, OROBOROS, Innsbruck, Austria). Measurements were performed at 37 °C in respiration medium (MIR05) containing 110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 1 g/l BSA essentially fatty acid-free, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, pH 7.1 and supplemented with catalase 280 IU/ml (MIR06) (Gnaiger, 2009). Mitochondria were added to 2 ml air-saturated respiration medium (MIR06) containing the substrate for complex II – succinate (10 mM) and the inhibitor for complex I – rotenone (0.5 μM). At this stage mitochondria respire slowly – State 2. In order to assess stimulated oxygen consumption, ADP (0.25 mM) was added. In the presence of ADP there is an acceleration of the electron flow through the respiratory chain and the rapid consumption of oxygen – State 3 (or State P). State 4 was achieved by adding oligomycin (2 μg/ml), which blocks the flow of protons by the active ATP synthase. Then ADP phosphorylation is blocked and oxygen consumption decreases. Titration with FCCP (1.5–2 μM) causes the maximum flow of electrons through respiratory chain and maximal oxygen consumption without the oxidative phosphorylation – State E (ETS capacity). This state informs about the respiratory chain condition. Finally, antimycin A (2.5 μM) was used in order to completely inhibit the complex III – State ROX. This state is used to evaluate (a) the oxygen consumption due to the oxidative side reactions and (b) the oxygen consumption remaining after the application of ETS inhibitors. The value of ROX was measured separately for every mitochondrial preparation and then was subtracted from the values of other designated states. Respiration values were calculated as the decrease in the oxygen concentration in time measured in a closed chamber, and expressed per milligram of protein. Finally, the following mitochondrial parameters were calculated:

- RCR (respiratory control ratio) – measured as the ratio of State 3 to State 4. It is the index of the mitochondrial capacity oxygen flux in the presence of ADP;
- P/E (Phosphorylation System Control Ratio) – measured as the ratio of State 3 (State P) to ETS capacity (state E). It is the index of the limitation of OXPHOS capacity by the phosphorylation system;
- L/E ratio (Leak Control Ratio) – measured as the ratio of 'leak' respiration (State 2) to ETS capacity (State E). It is the index of mitochondrial uncoupling or mitochondrial dyscoupling;
- ADP/O (nmol ADP/nAtm O) – the amount of ATP production per 1 atom of oxygen. This state provides an information about the correlation between the mitochondrial respiratory condition and oxidative phosphorylation (Gnaiger, 2009; www.oroboros.at).

2.5. Experimental design of the in vitro study

The experimental scheme was divided into several steps. The main aim of the first step was the selection of the methylglyoxal concentration, which should limit the mitochondrial respiratory capacity to a significant extent in comparison to control mitochondria (mitochondria untreated with MG). The next step concerned the choice of PAMAM G3 concentration which did not affect the mitochondrial functioning and might be safely used in other steps of experiment. In order to achieve this purpose, dendrimer's concentrations were tested in the range of 25–500 μM. Next, the mitochondria isolated from the single rat heart were divided into 6 groups: (1) mitochondria untreated with any of the substances (control); (2) mitochondria incubated with methanol (vehicle for dendrimer); (3) mitochondria incubated with MG (at the selected concentration); (4) mitochondria incubated with dendrimer (at the selected concentration); and mitochondria incubated with MG and PAMAM G3 in two variants differing in the order of substances addition (5) first MG later G3 and (6) first G3, then MG. We used 25 animals for this experiment.

2.6. Experimental design of the in vivo study

Seventy male rats of Wistar strain were used in this experiment. The experimental animals were randomly divided into four groups: (1) healthy rats (non-diabetic) – not supplemented with PAMAM G3 (15 rats); (2) healthy animals that were given PAMAM G3 in a dose of 20 mg/kg bw/day for 60 days (15 rats); (3) untreated diabetic rats (20 rats); (4) diabetic rats treated with PAMAM G3 at a dose of 20 mg/kg bw/day for 60 days (20 rats). In order to induce diabetes in rats, the animals were intraperitoneally injected with the streptozotocin (STZ dissolved in 0.1 mol/l citrate buffer, pH 4.5) at a dose of 70 mg/kg of body weight. The development of diabetes was diagnosed based on the non-fasting blood glucose concentration. Animals with streptozotocin-induced diabetes lasting for 7 days and blood glucose higher than 16.7 mmol/l were considered diabetic and included in the study. Intraperitoneal administration of PAMAM G3 dendrimer [20% (w/w) solution in methanol] diluted in physiological saline was started after 7 days upon the induction of laboratory-confirmed diabetes. Control groups of animals (animals with or without diabetes not treated with PAMAM G3) received a vehicle (pure methanol diluted in physiological saline). After 60 days of administration of dendrimer or methanol, the survived individuals were sacrificed and their hearts were collected for analyses.

2.7. Statistical analysis

All measurements of mitochondrial function were performed in duplicates. Due to occasional data asymmetry in some variables and groups all data were expressed as median and interquartile range (lower–upper quartile, 25–75%). Data normality was checked using the Shapiro–Wilk's test and variance homogeneity was verified with Levene's test. Then, data with evidenced normality were analyzed with parametric tests and these with non-proved normality were analyzed with non-parametric tests. For comparing the groups with heterogeneous variances, the non-parametric Kruskal–Wallis test and non-parametric Conover–Inman test for multiple comparisons were used. The statistical significance between homogenous groups was estimated using one-way ANOVA and *post hoc* Tukey test. For both the *in vitro* and *in vivo* experiments, the number of sample size was estimated for type I and II statistical errors of 0.05 and 0.8, respectively. Furthermore, the power of used tests was also checked for each analysis. The power test below 80% was considered as unbelievable outcome and the constructive conclusions were not formulated. All statistical calculations were made with the use of STATISTICA.PL v.9 (StatSoft) and StatsDirect (StatsDirect Limited).

3. Results

3.1. Changes in mitochondrial parameters measured in different seasons – in vitro evaluation

As shown in Table 1, seasonality affected only one mitochondrial parameter -P/E- in all tested samples except the samples incubated with PAMAM G3. Parameter P/E was significantly increased ($p < 0.005$) in heart mitochondria isolated from rats studied in spring (in control mitochondria, as well as in other samples). It could mean that rat mitochondria isolated from the spring livestock are characterized by the higher level of uncoupling, nevertheless the Leak Control Ratio (L/E) remains at the same level regardless of season. Moreover, the increase in P/E had not impact on such important parameters as RCR (assessment of mitochondrial oxygen flux) or ADP production. In mitochondria from spring rats treated with PAMAM G3, parameter P/E was aggravated in comparison to the samples derived from autumn/winter rats. At the same time RCR and ADP/O increased, indicating improved mitochondrial functionality in rats derived in spring. Interestingly, the increase in P/E parameter had not affected negatively the level of mitochondrial oxygen flux and ATP production. It is highly probably that PAMAM G3 improved the functionality of mitochondria. The obtained results also indicate that seasonal variations can have a considerable impact on mitochondrial response to PAMAM G3 exposure.

3.2. Heart rat mitochondrial respiration upon G3 exposure – the assessment of G3 as a protector against MG-impaired mitochondrial damage in the in vitro study

To determine the effect of PAMAM G3 on rat heart mitochondria impaired by MG (500 μM), the most important parameters, such as RCR, P/E, L/E and ADP/O, were assessed in a standardized experimental regime. Respiratory control ratio (RCR) measured upon the addition of ADP, was decreased for MG ($p = 0.0036$), tested for both combinations, MG + G3 ($p = 0.00012$) and G3 + MG ($p = 0.026$), compared to control. No changes in RCR were observed for G3 (100 μM) and pure methanol, $p = 0.17$ and $p = 0.6$, respectively. These data suggest that methylglyoxal alone, as well as in combination with G3, have significantly reduced the oxygen flux by mitochondria. On the other hand, it has been evidenced that the sequence of the treatment of mitochondria with the compound matters. In the combination with using G3 before MG, the reduction in RCR was significantly lower in comparison to MG + G3 ($p = 0.026$). These promising data indicate that G3 could be effectively used in the decreasing of the detrimental effect(s) of MG on RCR, although

Table 1
The *in vitro* impact of tested compounds on rat heart mitochondrial parameters monitored in different seasons – *in vitro* evaluation.

Sample	Season	Parameters				Statistical significance
		RCR	P/E	L/E	ADP/O	
Mitochondria (control)	Autumn/winter	3.60 (3.13; 4.00)	1.22 (1.13; 1.31)	0.34 (0.3; 0.36)	1.29 (1.06; 1.43)	$p = 0.00029$
	Spring	4.03 (3.47; 4.42)	1.39 (1.31; 1.51)*	0.36 (0.3; 0.4)	1.15 (1.04; 1.74)	
Mitochondria + methanol	Autumn/winter	3.76 (3.43; 3.87)	1.28 (1.12; 1.37)	0.36 (0.30; 0.37)	1.20 (1.03; 1.37)	ns
	Spring	3.53 (3.14; 4.14)	1.29 (1.26; 1.34)	0.36 (0.32; 0.41)	1.23 (1.13; 1.39)	
Mitochondria + methylglyoxal (500 μM)	Autumn/winter	2.90 (2.21; 3.35)	1.37 (1.24; 1.60)	0.49 (0.35; 0.68)	1.42 (1.28; 2.00)	*$p = 0.044$
	Spring	3.26 (2.81; 3.81)	1.46 (1.40; 1.79)*	0.49 (0.39; 0.67)	1.29 (1.24; 1.99)	
Mitochondria + PAMAM G3 (100 μM)	Autumn/winter	2.79 (2.55; 3.67)	1.48 (1.11; 1.53)	0.52 (0.35; 0.62)	1.18 (1.10; 1.32)	*$p = 0.032$
	Spring	3.72 (3.09; 3.93)*	1.70 (1.56; 1.94)**	0.53 (0.39; 0.60)	1.51 (1.26; 1.66)*	**$p = 0.0036$

Data were expressed as median and lower–upper quartile range, $n = 11–23$. Statistical significance is given for samples derived from autumn/winter *vs.* spring. For experimental and statistical details see 'Section 2'.

the improvement of RCR for the combination of G3 + MG has not reached the RCR level characteristic for control (Fig. 1).

As shown in Fig. 2, the effects of the most tested compounds (MG, G3 and both the combinations of MG and G3) on P/E is adverse. The changes in P/E were not observed only in the case of methanol ($p = 0.069$). The increase in P/E under conditions when the state ETS is lowered, evidenced for G3 ($p = 0.0013$) and MG ($p = 0.021$), suggests that both of them contribute to the reduction in OXPHOS capacity. It means that the capability of mitochondrial chain transport system decreased for electrons transport (data not shown). The sequence of reagents adding had not affected P/E. The statistical significance between MG + G3 and G3 + MG was not recorded ($p = 0.83$). The level of P/E changes upon exposure to MG or G3 separately added to mitochondria was not significantly different ($p = 0.19$). Nevertheless, as shown in Fig. 3, although L/E (the index of mitochondrial uncoupling) undesirably increased for MG ($p < 0.0001$), G3 ($p < 0.0001$), as well as for their combinations: MG + G3 ($p < 0.0001$) and G3 + MG ($p = 0.0001$), it has been demonstrated that the sequence of adding reagents (G3 and MG) to mitochondria may have its scientific importance. Mitochondrial uncoupling significantly decreased for the combination of G3 + MG compared to MG + G3 ($p = 0.0067$). The uncoupling properties of MG and G3 were found at the same level ($p = 0.75$). The effect of pure methanol on mitochondrial coupling was not observed.

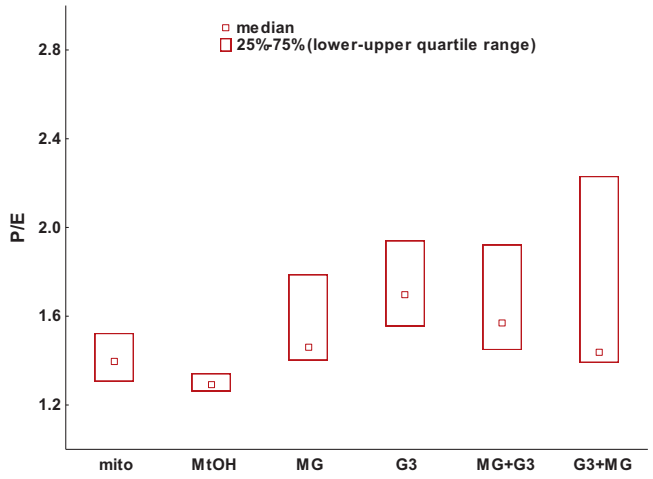

Fig. 2. P/E (Phosphorylation System Control Ratio) of heart mitochondria isolated from Wistar rats after *in vitro* exposure to tested compounds: mito – control (mitochondria alone), MetOH – methanol, MG – methylglyoxal (500 μM), G3 – PAMAM dendrimer G3 (100 μM). Data presented as median and upper–lower quartile range, $n = 9–21$. Significance of differences estimated by means of Kruskal–Wallis and median tests and *post hoc* all-pairwise comparisons Conover–Inman test: MG + G3 *vs.* G3 + MG, $p = 0.83$, ns – no significant; mito *vs.* MG, $p < 0.05$; mito *vs.* G3, $p < 0.005$; mito *vs.* MG + G3, $p < 0.05$; mito *vs.* G3 + MG, $p < 0.05$.
For experimental and statistical details see 'Section 2'.

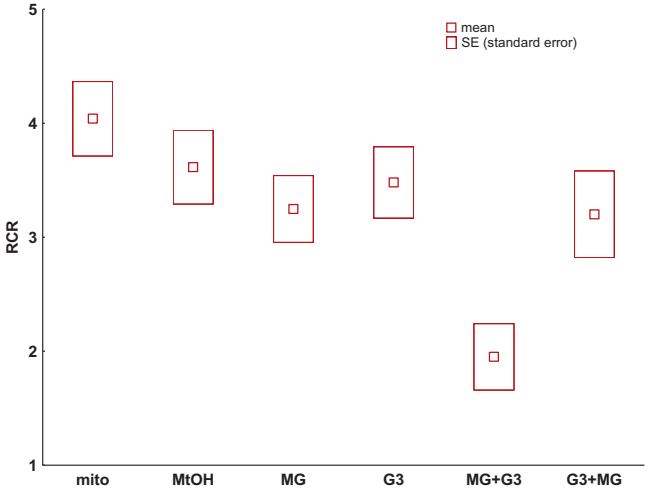

Fig. 1. RCR (respiratory control ratio) of heart mitochondria isolated from Wistar rats after *in vitro* exposure to tested compounds: mito – control (mitochondria alone), MetOH – methanol, MG – methylglyoxal (500 μM), G3 – PAMAM dendrimer G3 (100 μM). Data presented as mean and standard error (SE), $n = 9–21$. Significance of differences estimated by one-way ANOVA and *post hoc* Tukey test for unequal group sizes: MG + G3 *vs.* G3 + MG, $p < 0.001$.
For experimental and statistical details see 'Section 2'.

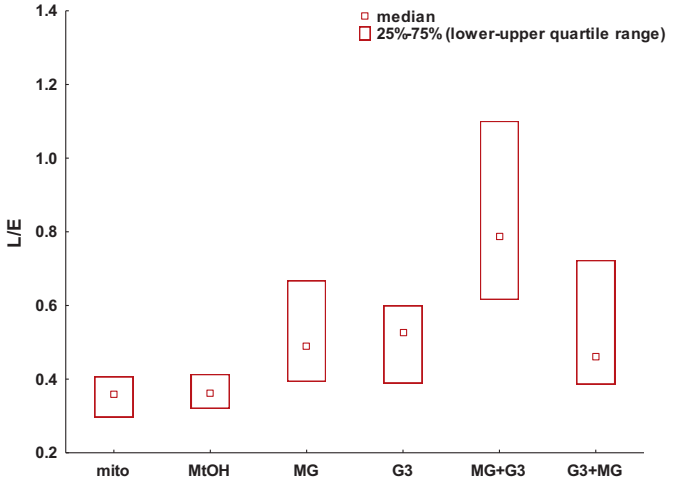

Fig. 3. L/E (Leak Control Ratio) of heart mitochondria isolated from Wistar rats after *in vitro* exposure to tested compounds: mito – control (mitochondria alone), MetOH – methanol, MG – methylglyoxal (500 μM), G3 – PAMAM dendrimer G3 (100 μM). Data presented as median and upper–lower quartile range, $n = 9–21$. Significance of differences estimated by means of Kruskal–Wallis test and *post hoc* all-pairwise comparisons Conover–Inman test: MG + G3 *vs.* G3 + MG, $p = 0.01$; mito *vs.* MG, $p < 0.0001$; mito *vs.* G3, $p < 0.0001$; mito *vs.* MG + G3, $p < 0.0001$; mito *vs.* G3 + MG, $p = 0.0001$.
For experimental and statistical details see 'Section 2'.

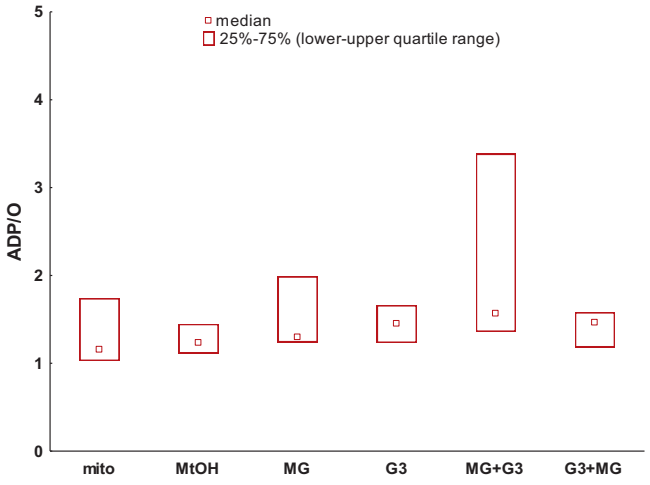

Fig. 4. ADP/O of rat heart mitochondria isolated from Wistar rats after *in vitro* exposure to tested compounds: mito – mitochondria (control), MetOH – methanol, MG – methylglyoxal (500 μM), G3 – PAMAM dendrimer G3 (100 μM). Data presented as median and upper–lower quartile range, $n = 9–21$. Significance of differences estimated by Mann–Whitney test with Bonferroni's correction.
For experimental and statistical details see 'Section 2'.

Interestingly, the effect of all tested compounds on ATP production (ADP/O) by mitochondria was not revealed (Fig. 4). These results clearly indicate that neither MG nor G3 affect mitochondrial respiratory at the level of energy formation.

3.3. Respiratory capacity of rat heart mitochondria after supplementation with G3 – in vivo evaluation

Respiratory activity of mitochondria isolated from rats supplemented with PAMAM G3 at a dose of 20 mg/kg bw/day (for 60 days) was measured in animals that survived the experiment. Surprisingly, as shown in Table 2, dendrimer G3 appeared very toxic for the treated animals. In a group of healthy animals given G3 dendrimer only 2 animals survived the experiment (of 15 animals that started the experiment). The similar situation was observed for diabetic animals treated with G3: in this group only 5 of 20 animals survived the two-month administration of PAMAM G3. Thus, taking into account the high reduction in the animal survival supplemented with dendrimer, the number of rats' heart taken to the respirometric measurements was not sufficient to receive reliable outcomes and derive any reasonable conclusions. As it has been shown in Table 2, the power of statistical analysis was under the value of 45%. The small sample size and the low power of statistical testing made the collected data unreliable.

4. Discussion

The current study was undertaken to examine the impairments in the mitochondrial cardiac bioenergetics investigated in the *in vitro* and the *in vivo* model of experimental diabetes and to consider the opportunity of the use of PAMAM dendrimer G3 in their prevention. One reason for the poor prognosis in patients with both diabetes and heart disease is the enhanced myocardial dysfunction leading to accelerated heart failure (diabetic cardiomyopathy). Thus, patients with diabetes are unusually prone to congestive heart failure (Grundy et al., 1999). Due to the importance the heart mitochondria have in the context of the bioenergetics of the myocardium, it appears logical to explore mitochondrial dysfunction as an important link between hyperglycemia and heart alterations observed in the course of diabetes. Based on what has been described so far,

Table 2
Heart mitochondrial respiratory parameters in rats administered with PAMAM G3 for 60 days.

Mitochondria from:	*n* (number of animals)	Parameters				Statistical significance	Power of test
		RCR	P/E	L/E	ADP/O		
Healthy rats + methanol	15	3.03 (2.46; 3.76)	1.03 (0.88; 1.15)	0.32 (0.29; 0.39)	0.85 (0.68; 1.1)	ns	<45%
Healthy rats + PAMAM G3	2	3.50 (3.48; 3.59)	1.26 (1.13; 1.31)	0.35 (0.35; 0.35)	0.74 (0.69; 0.82)	ns	<7%
Diabetic rats + methanol	15	3.01 (2.68; 3.57)	1.10 (0.88; 1.26)	0.35 (0.33; 0.39)	0.9 (0.71; 1.07)	ns	<35%
Diabetic rats + PAMAM G3	5	2.89 (2.33; 3.40)	1.24 (0.91; 1.30)	0.39 (0.38; 0.40)*	1.17 (0.73; 1.54)	* $p = 0.0025$	<10%

Data were expressed as median and lower–upper quartile range, $n = 2–15$ animals. PAMAM G3 administered at the dose of 20 mg/kg/day. Significance estimated for the multiple comparisons among the groups. The highest revealed statistical power values among all comparisons performed for all tested four parameters are given in the last column. * $p < 0.0025$ for mitochondria isolated from 'healthy rats + methanol' *vs.* 'diabetic rats + PAMAM G3'; ns = non-statistical significance. For more experimental and statistical details see 'Section 2'.

there dominates the feeling that it is still a lot to be learnt about interactions of hyperglycemia with heart mitochondria. Studies on isolated heart mitochondria, especially diabetic heart mitochondria, are not abundant. It may be clearly evidenced that liver mitochondria are more often studied than heart mitochondria. Therefore, there is a recurrent feeling that a clear link between hyperglycemia and cardiac mitochondrial changes is still missing.

In this paper we tried to find the relevance between conclusions drawn on the basis of the *in vitro* studies and designing the conditions for the *in vivo* research. We believed that based on model biological studies (including isolated mitochondria) we would be able to learn about the impact of PAMAM G3 on heart mitochondria and extrapolate this knowledge to the preclinical practice (experiments including animals). Of course, we were aware that the leap from the isolated organelles to the intact body may be considerably large, therefore, our intention was to find out whether *in vitro* evaluations could be relevant to further *in vivo* treatments. The positive answer to this posed uncertain issue could simply orientate our future *in vitro* studies more to elucidate the activity of investigated agents, f.i. dendrimers, at the level of the details of molecular mechanisms. It seems clear that studying of the response and function of isolated mitochondria subjected to certain treatments mimicking the *in vivo* experiments may be of some use to predict the behavior of the diabetic heart and may provide a useful model to study the effects of protective compounds, like antioxidants and others, either by adding them *in vitro* to mitochondria isolated from hyperglycemic animals or by direct treating of hyperglycemic animals.

Unfortunately, in the case of our observations (based on the *in vitro* and the *in vivo* part of the study) it is impossible to confirm the assumptions posed at the beginning of this work. We did not evidenced a positive correlation between the results obtained in the simple *in vitro* experiments and those received in the *in vivo* intervention part of our study. Thus, at this stage it is impossible to conclude on the possibility of "transfering" the data from *in vitro* experiments onto the planning of *in vivo* experiments. The other revealed aspect concerns the differentiated impact of the seasonality on the obtained results. The data obtained in autumn/winter differ considerably from those collected during spring. As shown in Table 1 mitochondrial parameters, such as P/E, RCR and ADP/O (the last two were changed only for the sample of 'mitochondria + PAMAM G3 (100 μM)'), were significantly increased for animals derived in spring. These data have proven that in spring mitochondria are in better condition compared to the ones tested in autumn/winter period. The discrepancy between these seasonal observations may be the first and probably the most important factor limiting the using of such data for planning of *in vivo* experiments. On the other side, the observations of our study do not differ from the literature data published during last few years (Carras et al., 2010; Martin et al., 2008; Mujkosova et al., 2008; Bronson, 2009; Prendergast et al., 2007; Murphy and Ebling, 2011). Some papers have demonstrated the impact of seasonal differences on measured parameters in various tissues and cells of tested organisms. Carras et al. (2010) showed that seasons play an important role in the coordinating the breeding activity of numerous animal species and it may have a negative effect on animal metabolism. Martin et al. (2008) confirmed the hypothesis that seasonal changes have an impact on immune defence in small animals and birds. The authors showed that environmental conditions are in temporal flux over much of the planet. Summer and spring represent conditions, in which individuals of most species can thrive. Winter with its accompanying low temperatures and reduced food availability, however, make breeding and sometimes even survival extremely difficult. Their alternative hypothesis proposes that the costs of immune activity underlie seasonal fluctuations. That is, immune activity is traded off during specific phases of animals' lives, such as breeding, owing to the incompatible costs of simultaneous demanding physiological activities. Both hypotheses are supported by studies performed on multiple taxa and together they explain a significant proportion of the immunological variation seen in animals across the year. However, both hypotheses require refinement. These data, although very interesting, should not be applied unreservedly to the *in vivo* studies, in which we often make a considerable attempt to maintain the same experimental conditions for the housed animals throughout the year. Despite that there are occasional scientific reports showing the effect of seasonality also on animals housed for experimental purposes. Mujkosova et al. (2008) reported that there are numerous data about seasonal variations in function, structure and enzyme activity in rat hearts derived from laboratory breeding. The changes were also revealed in ADP sensitivity and oxidative capacity of heart mitochondria. On the other hand, as Bronson (2009) suggests, the small rodents all over the world may adapt rather easily to seasonal changes, but the longer lived mammals, whose reproduction is regulated by photoperiod, may not do so well. Nevertheless, Prendergast et al. (2007) investigating the laboratory Wistar rats' behavior, showed that the adaptive significance of seasonal changes in immunity – principally the enhancement of peripheral leukocyte numbers and the suppression of sickness responses under winter conditions – may lie in a redistribution of energy towards physiological systems responsible for host defense at a time of year when reproduction is contraindicated. Thus, it is possible that seasonal variations may influence animal metabolism and may become reflected in obtained results. The study of Murphy and Ebling (2011) revealed that most animals, including laboratory animals, display profound seasonal cycles of body weight, reflecting changes in both food intake and energy expenditure. Seasonal cycles in energy metabolism, food intake, body weight, and reproductive ability can be driven in this species simply by changing the light-dark cycle on which the animals are maintained in the laboratory. Long days of over 12.5 h of light promote food intake and development of gonads, whereas short days promote weight loss and regression of reproductive organs. Exposure to short (winter) photoperiods induces a state, in which animals become hypophagic and catabolize abdominal fat reserves such that they may lose up to 40% of total body weight. Therefore, based on literature and our own observations, we suggest that the results obtained from rats treated in various seasons may differ between themselves at the significant statistical level. If this discrepancy is so clear, the aspect of the usefulness of laboratory tests in the creation of reality should be re-considered in the future.

Another interesting aspect of this study concerns the impact of PAMAM G3 dendrimer on mitochondria in health and pathologic conditions (experimental diabetes). To the best of our present knowledge, for the first time in this paper, the effect of G3 dendrimers on mitochondrial bioenergetics has been presented. PAMAM dendrimers are emerging as promising topical antimicrobial agents and as targeted nanoscale drug delivery vehicles. Hence, they have a vital role to play in the developing field of medical nanotechnology. Therefore, it is very important to check their effect on mitochondria as important energy centers of every single cell. So far only few papers appeared describing interactions between dendrimers and mitochondria (Labieniec et al., 2010; Labieniec and Gabryelak, 2008; Labieniec and Watala, 2009; Lee et al., 2009; Thomas et al., 2009; Mukherjee et al., 2010). All of them indicate that PAMAM dendrimers, regardless of the used type of dendrimer (cationic or anionic) or dendrimer generation, significantly affect rat liver (Labieniec et al., 2010; Labieniec and Gabryelak, 2008; Labieniec and Watala, 2009) and as well as rat heart mitochondria (Labieniec et al., 2010), and modulate their functionality. Moreover, recent scientific reports derived from three independent laboratories proved the role of PAMAMs in apoptosis activation

via mitochondria (Lee et al., 2009; Thomas et al., 2009; Mukherjee et al., 2010). Lee et al. (2009), using human lung cells (WI-26 VA4), revealed that dendrimers G4 co-localized with mitochondria and caused the release of cytochrome c. Dendrimers also disrupted the mitochondrial potential and led to activation of caspases 3 and 9. Mitochondria-dependent apoptosis was also shown by Thomas et al. (2009), who using KB cells, observed that PAMAM dendrimers were taken up into the lysosomal compartment, and that they increased the lysosomal pH and cytotoxicity as a function of the number of surface amino groups on the dendrimer. The positively charged, amine-terminated PAMAM dendrimer induced cellular apoptosis, as demonstrated by mitochondrial membrane potential changes and caspase activity measurements. These results suggest that PAMAM dendrimers are endocytosed into the KB cells through a lysosomal pathway, leading to lysosomal alkalinization and induction of mitochondria-mediated apoptosis. The similar results were obtained by Mukherjee et al. (2010), who-using two mammalian cells (HaCat and SW480) and three generations of PAMAM dendrimers (G4, G5 and G6), proved their localization near mitochondria and negative interactions inside the cells. First of all, increased level of oxidative stress and apoptosis, as well as DNA damage, were observed in tested cells upon exposure to dendrimers. In our opinion, these data are not promising and in the future may contribute to qualify dendrimers as compounds, which should not be used in pre- or clinical tests just because of their cytotoxicity or apoptotic activity. On the other hand, we should be very cautious in expressing such an opinion before we do not know the whole truth about mechanisms of action of these compounds. The *in vitro* results demonstrated in this paper (Figs. 1 and 3) showed that PAMAM G3 can effectively protect heart mitochondria against negative activity of methylglyoxal applied as a glycoxidation factor. In this experiment dendrimer G3 was used at the relatively high concentration of 100 μM (a very safe concentration chosen based on the results obtained during autumn/winter tests, data not shown). Using mitochondria from spring livestock it has been demonstrated that G3 at 100 μM can modulate some selected mitochondrial parameters (RCR, P/E and ADP/O). The increase in P/E indicates that mitochondrial OXPHOS capacity could be significantly limited by G3. Theoretically, such an activity should be detrimental for mitochondrial function, but the other parameters, such as f.i. RCR or ADP/O, have been improved in comparison to the samples collected in autumn/winter period. It suggests that this quite a high concentration of G3 can be still regarded as the safe concentration for mitochondria. Interestingly, more important for the final outcomes of this study was the protocol of using tested compounds rather than their concentration(s) or seasonal effect. The dendrimer PAMAM G3 appeared effective in the limiting of destructive impact of MG only for the combination of G3 (first) + MG (second). The administration of these compounds in the reverse order (MG (first) + G3 (second) did not prove the assumption of our hypothesis that G3 can act as the protector against glycooxidation process occurring in the *in vitro* sample (Figs. 1 and 3). This phenomenon is not easy to explain for us because the mitochondrial samples were not pre-incubated with G3 and/or MG before the final bioenergetics measuring. We may only suggest that G3 added as the first agent acts fast enough to compete with the potentially "interesting" targets for MG reactivity in the mitochondrial respiratory chain. In our previous works (Labieniec and Watala, 2010; Labieniec et al., 2008) we reported that dendrimers PAMAM G2 and G4 may be considered effective and safe chemical competitors for the non-enzymatic modifications of biomacromolecules (they prevented modifications of proteins in the course of glycooxidation) and such protection was demonstrated in both the *in vitro* tests (Labieniec and Watala, 2010) and the *in vivo* study (Labieniec et al., 2008). In the present study we do not present detailed molecular mechanisms underlying the action of G3 on mitochondrial respiration. However, based on the outcomes of the *in vitro* experiments we reason that this dendrimer could be potentially considered as a successful cardioprotective agent against glycation and glycoxidation at the cellular level. We suggest that the reactivity of G3 towards mitochondria is significantly reduced compared to MG, therefore, adding G3 as the first agent makes the amino groups of G3 and not those of mitochondria proteins the potential and preferential target of MG attack.

The STZ-treated animal model is by far the most common to study cardiac mitochondrial alterations induced by hyperglycemia. It was shown that cardiac muscle cells from STZ-treated rats presented swollen mitochondria, clearing of mitochondrial matrix and incorporation of lysosomal membranes into mitochondrial matrix. It became apparent that cardiac mitochondria appearance and function would be changed after STZ-treatment (Oliveira, 2005). Therefore, for our *in vivo* study we have chosen the STZ-model of diabetes and we have decided to check the impact of dendrimer G3 on heart mitochondria activity. Dendrimer was given everyday at a dose of 20 mg/kg/day for 60 days. Unfortunately, most of animals treated by dendrimer (but not by its vehicle – methanol) died before the termination of the experiment. It seems that the used dose of dendrimer (20 mg/kg bw) was apparently too toxic for animals, despite of the fact that-according to the published literature, the selected dose did not seem very high. Over the past three decades, dendrimers such as polyamidoamine (PAMAM, *i.e.* Starburst™) have been used widely as vehicles for drug or gene delivery. However, the *in vivo* toxicity of dendrimers has not been systematically investigated (Labieniec and Watala, 2009). Cationic dendrimers of generation 3, generation 5, and generation 7 were given i.p. to mice at the doses ranging from 0.026 to 45 mg/kg, either as a single dose or once a week for 10 weeks. The *in vivo* toxicity was evaluated by behavioral abnormalities 2 h post injection and by body weight changes for up to 6 months. No significant behavioral abnormality or weight loss was observed through the study. However, one out of five mice died at about 24 h after dendrimer injection in the G7 group with the highest dose of 45 mg/kg. These studies suggest that low generation cationic dendrimers (G < 7), even administered at high doses, do not cause adverse effects (Aillona et al., 2009). Nephrotoxicity and hepatotoxicity have been reported consistent with biodistribution studies that revealed high kidney and liver accumulation for G3 PAMAM dendrimers (i.p.), G3 and G4 PAMAM dendrimers (i.v. or i.p.) (Labieniec and Watala, 2009), biotinylated-PAMAM dendrimers (G0–4) (i.v.), and PEG-polyester dendritic hybrids (i.v.) (Roberts et al., 1996; Malik et al., 2000). Detailed studies of Roberts et al. (1996) demonstrated the differences between distribution of three PAMAMs' generations (G3, G5 and G7) in animal organism. The authors revealed that liver activity tended to clear after G5 and G7 administration, but G3 activity continued to increase slightly with time in this organ. Kidney localization was fairly comparable for the three generations, with G3 showing, in general, higher accumulation in that organ. Likewise, spleen activity over time for the three generations was virtually indistinguishable. In contrast, G5 showed very high, but usually not significantly different, localization in the pancreas. G3 and G7 accumulation in this organ tended to be below 10% ID/g with the exception of the 2-h time point after G7 administration (21% ID/g). These data may suggest that dendrimer PAMAM G3 can localize longer than other dendrimers in some important organs of the organism, and therefore, prolonged period of administration of this compound may cause toxic effects in the treated animals or even may contribute to their deaths. In our *in vivo* experiment only single organisms survived – it was too few animals for reliable analysis of obtained data, therefore, we are not able to draw the constructive conclusions concerning the activity of G3 in our *in vivo* model.

5. Conclusions

In summary, our present data revealed the ambiguous effect of PAMAM dendrimer G3 on rat heart mitochondria activity. On one hand, we could observed its effectiveness in the limiting of detrimental impact of methylglyoxal on mitochondrial bioenergetics (*in vitro* evaluation). On the other, we revealed surprisingly the increased mortality among animals treated with G3 (20 mg/kg, 60 days). In our opinion these data suggest that PAMAM dendrimers (particularly, the third generation) demand further investigations. Because of the growing interest in using dendrimers in biological applications, the behavior of these polymers in biological systems is of critical importance. Therefore, the scientists certainly cannot limit their experimental challenges concerning dendrimers only to the *in vitro* studies, but there is a necessitating need to plan their toxicity measurements using also animal models. Otherwise, there is a high probability that results derived merely from the *in vitro* experiments will never have their relevance to pre- and clinical studies. In this work we have also proved the influence of seasonality when carrying of our experiments and acquiring data throughout the year. As it emerges from our experiments, in order to obtain reliable conclusions, the aspect of seasonal changes in laboratory animals has also to be taken into consideration at the experimental designing of the whole study.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

Aillona, K.L., Xiea, Y., El-Gendya, N., Berklanda, C.J., Forresta, M.L., 2009. Effects of nanomaterial physicochemical properties on *in vivo toxicit*. Adv. Drug Deliv. Rev. 61, 457–466.

Bessman, S., Mohan, C., 1997. Insulin as a probe of mitochondrial metabolism in situ. Mol. Cell. Biochem. 174, 91–96.

Bronson, F.H., 2009. Climate change and seasonal reproduction in mammals. Philos. Trans. R. Soc. Lond. B Biol. Sci. 364, 3331–3340.

Carras, M.L., Brenowitz, E., Rubel, E.W., 2010. Peripheral auditory processing changes seasonally in Gambel's white-crowned sparrow. J. Comp. Physiol. Neuroethol. Sens. Neural Behav. Physiol. 196, 581–599.

Craven, P., Melhem, M., Phillis, S., DeRubertis, F., 2001. Over expression of Cu2+/Zn2+ superoxide dismutase protects against early diabetic glomerular injury in transgenic mice. Diabetes 50, 2114–2125.

Ferko, M., Habodaszova, D., Waczulikova, I., Mujkosova, J., Kucharska, J., Sikurova, L., Ziegelhoffer, B., Styk, J., Ziegelhoffer, A., 2008. Endogenous protective mechanisms in remodeling of rat heart mitochondrial membranes in the acute phase of streptozotocin-induced diabetes. Physiol. Res. 57, 67–73.

Gnaiger, E., 2009. Capacity of oxidative phosphorylation in human skeletal muscle: new perspectives of mitochondrial physiology. Int. J. Biochem. Cell. Biol. 41, 1837–1845.

Grundy, S.M., Benjamin, I.J., Burke, G.L., Chait, A., Eckel, R.H., Howard, B.V., Mitch, W., Smith Jr., S.C., Sowers, J.R., 1999. Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association. Circulation 100, 1134–1146.

Kang, Y., Edwards, L., Thornalley, P., 1996. Effect of methylglyoxal on human leukemia 60 cell growth: modification of DNA, growth arrest and induction of apoptosis. Leuk. Res. 20, 397–405.

Labieniec, M., Gabryelak, T., 2008. Preliminary biological evaluation of poli(amidoamine) (PAMAM) dendrimer G3.5 on selected parameters of rat liver mitochondria. Mitochondrion 8, 305–312.

Labieniec, M., Ulicna, O., Vancova, O., Glowacki, R., Sebekova, K., Bald, E., Gabryelak, T., Watala, C., 2008. PAMAM G4 dendrimers lower high glucose but do not improve reduced survival in diabetic rats. Int. J. Pharm. 364, 142–149.

Labieniec, M., Watala, C., 2009. PAMAM dendrimers – diverse biomedical applications. Facts and unresolved questions. Cent. Eur. J. Biol. 4, 34–451.

Labieniec, M., Watala, C., 2010. Use of poly(amido)amine dendrimers in prevention of early non-enzymatic modifications of biomacromolecules. Biochemie 92, 1296–1305.

Labieniec, M., Ulicna, O., Vancova, O., Kucharska, J., Gabryelak, T., Watała, C., 2010. Effect of poly(amido)amine (PAMAM) G4 dendrimer on heart and liver mitochondria in an animal model of diabetes. Cell Biol. Int. 34, 89–97.

Lee, J.H., Cha, K.E., Kim, M.S., Hong, H.W., Chung, D.J., Ryu, G., Myung, H., 2009. Nanosized polyamidoamine (PAMAM) dendrimer-induced apoptosis mediated by mitochondrial dysfunction. Toxicol. Lett. 190, 202–207.

Malik, N., Wiwattanapatapee, R., Klopsch, R., Lorenz, K., Frey, H., Weenerc, J.W., Meijerc, E.W., Paulusd, W., Duncan, R., 2000. Dendrimers: relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of ^{125}I-labelled polyamidoamine dendrimers in vivo. J. Control. Release 65, 133–148.

Martin, L.B., Weil, Z.M., Nelson, R.J., 2008. Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. Philos. Trans. R. Soc. Lond. B Biol. Sci. 363, 321–339.

Mujkosova, J., Ferko, M., Humenik, P., Waczulikova, I., Ziegelhoffer, A., 2008. Seasonal variations in properties of healthy and diabetic rat heart mitochondria: Mg^{2+}-ATPase activity, content of conjugated dienes and membrane fluidity. Physiol. Res. 57, S75–S82.

Mukherjee, S.P., Lyng, F.M., Garcia, A., Davoren, M., Byrne, H.J., 2010. Mechanistic studies of in vitro cytotoxicity of poly(amidoamine) dendrimers in mammalian cells. Toxicol. Appl. Pharmacol. 248, 259–268.

Murphy, M., Ebling, F.J.P., 2011. The role of hypothalamic tri-iodothyronine availability in seasonal regulation of energy balance and body weight. J. Thyroid Res. 2011, 1–7.

Oliveira, P.J., Rolo, A.P., Seica, R., Santos, M.S., Palmeira, C.M., Moreno, A.J., 2003. Reduction in cardiac mitochondrial calcium loading capacity is observable during alpha-naphthylisothiocyanate-induced acute cholestasis: a clue for hepatic-derived cardiomyopathies? Biochim. Biophys. Acta 1637, 39–45.

Oliveira, P.J., 2005. Cardiac mitochondrial alterations observed in hyperglycaemic rats – what can we learn from cell biology? Curr. Diabetes Rev. 1, 11–21.

Prendergast, B.J., Kampf-Lassin, A., Yess, J.R., Galang, J., McMaster, N., Kay, L.M., 2007. Winter day lengths enhance T lymphocyte phenotypes, inhibit cytokine responses, and attenuate behavioral symptoms of infection in laboratory rats. Brain Behav. Immun. 21, 1096–1108.

Roberts, J.C., Bhalgat, M.K., Zera, R.T., 1996. Preliminary biological evaluation of polyamidoamine (PAMAM) Starburst™ dendrimers. J. Biomed. Mater. Res. 30, 53–65.

Rolo, A.P., Palmeira, C.M., 2006. Diabetes and mitochondrial function: role of hyperglycaemia and oxidative stress. Toxicol. Appl. Pharm. 212, 167–178.

Shen, G.X., 2010. Oxidative stress and diabetic cardiovascular disorders: roles of mitochondria and NADPH oxidase. Can. J. Physiol. Pharmacol. 88, 241–248.

Solnica, B., 2006. Molecular mechanisms of the damaging effect of glucose on tissues. Diabetologia Doswiadczalna i Kliniczna [Experimental and Clinical Diabetology] 5, 1–8.

Thomas, T.P., Majoros, I., Kotlyar, A., Mullen, D., Holl, M.M., Baker Jr., J.R., 2009. Cationic poly(amidoamine) dendrimer induces lysosomal apoptotic pathway at therapeutically relevant concentrations. Biomacromolecules 14, 3207–3214.

Waldbaum, S., Patel, M., 2009. Mitochondria, oxidative stress, and temporal lobe epilepsy. Epilepsy Res. 88, 23–45.

Wallace, D., 1999. Mitochondrial diseases in man and mouse. Science 283, 1482–1488.