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Seasonal Variations in Properties of Healthy and Diabetic Rat Heart Mitochondria: Mg^{2+}-ATPase Activity, Content of Conjugated Dienes and Membrane Fluidity

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Summary

Our previous preliminary results pointed to possible seasonal variations in Mg^{2+}-ATPase activity of rat heart mitochondria (MIT). It is not too surprising since seasonal differences were already reported in myocardial function, metabolism and ultrastructure of the intact as well as hemodynamically overloaded rabbit hearts and also in other tissues. The present study is aimed to elucidate whether seasonal differences observed in rat heart MIT Mg^{2+}-ATPase activity will be accompanied with changes in membrane fluidity and in the content of conjugated dienes (CD) in the lipid bilayers of MIT membranes as well as whether the above seasonal differences will also be present in the diabetic heart. Our results revealed that values of Mg^{2+}-ATPase activity in the winter/spring-period (W/S-P) exceeded significantly ($p<0.05$-0.001) those in the summer/autumn-period (S/A-P). Similar trend was also observed in hearts of animals with acute (8 days) streptozotocin diabetes. With the exception of values of CD in the S/A-P, all values of Mg^{2+}-ATPase activities, membrane fluidity and CD concentrations in diabetic hearts exceeded those observed in the healthy hearts. Our results indicate that seasonal differences may play a decisive role in the evaluation of properties and function of rat heart MIT.

Key words

Heart mitochondria • Seasonal variations • Mitochondrial Mg^{2+}-ATPase • Conjugated dienes • Membrane fluidity • Diabetic heart

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Introduction

It is well documented, that hearts of healthy rats, mice and rabbits exhibit considerable seasonal variability in activities of diverse enzymes of aerobic heat production (Wickler 1981), antioxidant enzyme activities and lipid peroxidation (Belló-Klein *et al.* 2000) as well as in certain indicators of cardiac contractile function (Frolov *et al.* 1991) and rhythmicity (Bačová *et al.* 2007, Švorc *et al.* 2007). In addition, in a recent study (Mujkošová *et al.* 2006) we also revealed that heart mitochondria (MIT) of healthy rats exhibit ~30 % higher Mg^{2+}-ATPase activity ($p<0.05$) in the winter/spring period (W/S-P, from November to April) in comparison to that measured in the summer/autumn period (S/A-P, from May to October). In respect to the latter it was interesting to find out whether seasonal differences will be also present in heart MIT Mg^{2+}-ATPase activity of diseased animals. Studies in rats with streptozotocin diabetes indicated that some among the disease-triggered functional and structural changes in myocardial membrane systems, particularly in the sarcolemma and MIT, may not be considered unconditionally as deterioratory. They proved to be associated with induction of endogenous protective mechanisms leading to desirable compensatory changes or even adaptation to the disease (Tribulová *et al.* 1996, Ziegelhöffer *et al.* 1996, 1997, 1999, 2002, 2006, Ravingerová *et al.* 2000, 2001). This also concerns a part of changes observed in diabetic heart MIT particularly the increase in activity of the MIT Mg^{2+}-ATPase, elevation in

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fluidity of the MIT membrane (Ferko *et al.* 2006a,b), and facilitated transmembrane delivery of ATP from the MIT to the cytoplasma (Ziegelhöffer-Mihalovičová *et al.* 1997, Ziegelhöffer *et al.* 2002, Ziegelhöffer 2005). Present study is devoted to elucidation whether seasonal differences observed in MIT Mg^{2+}-ATPase activity will be also accompanied by changes in some chemical and physical properties, such as in the content of conjugated dienes (CD) and fluidity of the lipid bilayer of MIT membrane. A further goal is to find out whether the seasonal changes in properties of the MIT detected in healthy heart will be also present in the diabetic heart and whether they these differences will be consonant with the changes caused by the disease itself.

Material and Methods

The study was conducted in accordance with 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 rules issued by the State Veterinary and Alimentary Administration of the Slovak Republic, based on § 37 (6), piece of legislation No 488/2002 of the Slovak Parliament.

Adult ($230^{\pm}20$ g body weight) male Wistar rats were kept under standard conditions (12 L/12 D regimen, 21-23 °C, three animals per cage). Both the healthy control and the diabetic animals had free access to water and standard laboratory diet.

All reagents and chemicals applied in the study were purchased from Sigma-Aldrich USA, SERVA or Merck (Germany) and had analytical grade quality.

Induction and control of acute diabetes

Diabetes was induced by a single dose of streptozotocin (STZ; 65 mg/kg b.wt., applied i.p., in 0.1 mol/l of citrate buffer, pH=4.5). Development of the disease was monitored daily by estimation of glucosuria using Gluko Phan ® stripes. Metabolic status of animals was investigated at the beginning and at the end of experiment by estimation of glucose (Bio-La-Test) and glycohemoglobin (Burrin *et al.* 1980) in the blood, as well as cholesterol (Watson 1960), triacylglycerols (Fossati and Prencipe 1982) and insulin (commercial RIA kit) in serum. Experiment was terminated at the day 8 after STZ administration.

Isolation of heart mitochondria

Rats were anesthetized by thiopental (60 mg/kg, i.p.) with heparin (500 IU, i.p.). After cervical dislocation the hearts were quickly removed, washed free of blood in ice-cold saline and weight. Subsequently they were transferred to small volume of ice-cold isolation solution (IS) containing in mmol/l: 180 KCl, 4 EDTA and 1 % of bovine serum albumin, pH=7.4 and minced by scissors. Thereafter, the minced tissue was transferred to a teflon/glass homogenizer together with 20 ml of IS containing in addition protease (Sigma P 6141, 2.5 mg/g wet weight) and homogenized gently for 2-3 min. Homogenate was than spunned down at 1000 x g for 10 min. Protease containing supernatant together with a part of MIT being in direct contact with the protease discarded. Pellet was re-suspended in the same volume of IS without protease, again homogenized and spunned down as previously. This supernatant containing now predominantly MIT which were not in direct contact with protease was centrifuged at 5000 x g for 15 min. Finally the pellet containing MIT was again re-suspended in IS which, however, contained no albumin and spunned down at 5000 x g for 15 min. All isolation was performed at 4 °C.

Mg^{2+}- dependent and 2, 4 -dinitrophenol stimulated MIT ATPase

Activity of the Mg^{2+}-dependent ATPase (also termed as oligomycin-sensitive ATPase) of isolated MIT was estimated in 1 ml of incubation medium containing (in mmol/l): 250 imidazol buffer, pH=7.4; 40 $MgCl_2$; 40 ATP-Tris; 50-70 µg of MIT protein (~1 µg/µl). However, membranes of intact MIT are impermeable to Mg^{2+}. Hence, the enzyme activity obtained by direct measurement is only referred to the part of MIT with leaky membranes. To learn the total MIT Mg^{2+}-ATPase activity requires also the rest of MIT membranes in leaky state. This may be achieved by preincubation of the MIT with some uncoupler, in our case 0.1 mmol/l 2,4-dinitro-phenol (DNP). After 10 min preincubation with DNP at 37ºC, the reaction was started with addition of ATP, it was kept running for 20 min and terminated by 1 ml ice-cold 12 % trichloroacetic acid. ATPase activity was measured by estimating the amount of orthophosphate (P_i) liberated by ATP splitting and it was expressed in mmol P_i *per* g of MIT protein *per* h (Máleková *et al.* 2007).

Purity of the MIT preparation

Presence of membranes of the sarcolemma (SL) and sarcoplasmic reticulum (SR) in MIT preparation was

tested by estimation of their marker ATPases: the Na^+, K^+-ATPase for SL and the Mg^{2+}, Ca^{2+}-ATPase for SR in the absence and presence of their specific inhibitors, ouabain and thapsigargin, respectively. The activities of these enzymes were also determined by measuring of P_i liberated by ATP splitting. For further details concerning the estimation of single ATPases, such as the composition of incubation media, concentration of cationic ligands and the inhibitors applied etc., see the original procedures described by Ferko *et al.* (2006) and Máleková *et al.* (2007).

Membrane fluidity

Fluidity of lipid layer of the MIT membrane was assessed by measuring steady-state fluorescence anisotropy of the lipophilic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH, Aldrich) or its cationic derivative (1-[4(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH, Aldrich). The probe incorporates spontaneously into the inside of MIT membrane, depending on fluidity of its lipid layer and that movement is associated with a decrease in the fluorescence signal. Membrane fluidity is than expressed as the reciprocal value of fluorescence anisotropy. Isolated MIT were re-suspended at a final protein concentration of 0.5 mg/ml in an isotonic buffer (containing in mmol/l: NaCl 180, EDTA 4, adjusted to pH=7.4) and labeled with a 0.25 µmol/l solution of DPH or 0.1 µmol/l TMA-DPH dissolved in a mixture of acetone and water in a ratio of 1:250. Samples were incubated at 22±1 ºC for 20 min (10 min with TMA-DPH) to allow complete incorporation of the probes into the membranes. Steady-state fluorescence anisotropies (r) were measured at 22±1 ºC with a Perkin-Elmer LS45 luminescence spectrometer. Fluorescence excitation was set at 360 or 340 nm (10 nm slit width) and emission was detected at 425 or 450 nm. The degree of fluorescence anisotropy and the time course of DPH incorporation, followed by consecutive measurements were estimated as described previously by Waczulíková *et al.* (2007).

Conjugated dienes

Content of CD in membrane lipids was assessed by the original method of Kogure *et al.* (1982) adapted to estimation of CD in membranes of heart MIT. Adaptation concerned the adjustment of optimal conditions for extraction of membrane lipids. Briefly, for extraction of membrane lipids, the fresh isolated heart MIT were re-suspended to concentration ~1µg/µl in a solution containing in mmol/l 180 KCl and 4 Na_2EDTA, adjusted to pH=7.4 by Tris-HCl.

500 µl of the latter membrane suspension was extracted by a mixture of chloroform and methanol in a ratio of 500:1000 µl under vortexing for 30 s. The mixture was than enriched by further 500 µl of chloroform and subsequently again vortexed for 30 s. Extraction was terminated by addition of 500 µl of 15 mmol/l Na_2EDTA containing 4 % NaCl and spunned down for 10 min at 1900xg. Than 600 µl of the lipids containing lower layer of chloroform/methanol was transferred to separate test tube with inert atmosphere (nitrogen) and carefully evaporated at laboratory temperature by means of a continuous stream of nitrogen. The lipids were than dissolved in 3 ml of cyclohexane, vortexed for 30 s and used directly for spectrophoto-metric determination of the CD content (against cyclohexane, λ = 233 nm, ε =29000 $l.mol^{-1}.cm^{-1}$) maintaining all time the inert atmosphere.

Estimation of inorganic phosphate (Pi) and protein

Concentration P_i originating from ATP splitting was determined by the method Taussky and Shorr (1953). Protein concentration was estimated according to Lowry *et al.* (1953) using bovine serum albumin as a standard.

Statistical evaluation

The data were expressed as means ± S.E.M. Statistical significances were ascertained by using the Student´s two-tailed test for unpaired observations with Bonferroni´s correction or by multiple comparisons ANOVA. Seasonal differences between enzyme activities were analyzed by means of Tukey-Kramer and Kruskal-Wallis tests. Comparison of differences in membrane fluidity was performed by means of the Mann-Whitney U test. $P<0.05$ value was considered significant.

Results

Metabolic status of the animals

Diabetes in rats was manifested by significant 367 % increase ($p<0.001$) in blood levels of glucose amounting to 19.98±0.13 mmol/l vs. 5.45±0.07 mmol/l in non-diabetic animals. In comparison with parallel running healthy control rats the diabetic animals further exhibited a 392 % increase in serum levels of triacylglycerols (from 1.17±0.19 to 4.59±0.24 mmol/l) and 178 % elevation of cholesterol (from 2.33±0.11 to 4.14±0.33 mmol/l) (all $p<0.001$). The content of glycohemoglobin (expressed in % of the total Hb content in blood of the non-diabetic animals) showed a significant ($p<0.05$) rise amounting to 173 % (from 4.30±0.05 to 7.43±0.52 %). In addition, diabetic animals were also characterized by decreased

Table 1. Relationship between MIT Mg^{2+}-ATPase activities, content of CD and fluidity of MIT membranes in W/S-P and S/A-P.

	Control W/S-P	Diabetes W/S-P	Control S/A-P	Diabetes S/A-P
Mg^{2+}-ATPase activity	**65.79***	**71.43*****	**51.53**	**53.86***
µmol.Pi/g prot./hod.	2.34	5.54	2.31	3.86
	n=8	n=8	n=8	n=8
Conjugated dienes	**47.33**	**51.93**	**115.22***	**97.05**
nmol/g prot.	50.69	89.63	136.78	140.31
	40.18	40.54	73.41	70.18
	n=4	n=4	n=7	n=5
Membrane fluidity	**3.03**	**3.15$^{o\square}$**	**2.94**	**3.03**
1/anisotropy	3.04	3.25	3.02	3.05
	2.93	3.13	2.91	2.97
	n=7	n=7	n=10	n=9

CD – conjugated dienes, CD and membrane fluidity are given in medians plus upper and lower quartile, ATPase activities are means ± S.E.M; n=number of experiments. $^{*}p<0.05$, $^{***}p<0.001$, ^{o} $p<0.008$ for diabetic group vs. control group in the W/S-P, $^{\square}p<0.01$ for diabetic group in the W/S-P vs. S/A-P.

insulin level amounting to 0.5 vs. 1.0 ng/ml of blood serum in non-diabetic animals.

Purity and Mg^{2+}-ATPase activities of the mitochondrial preparation

Purity of our MIT preparation proved to be satisfactory since judging by the activity of marker ATPases the contamination with membranes of the SL and SR represented only 0.94 % and 1.89 %, respectively.

MIT Mg^{2+}-ATPase activities (Table 1) were evaluated in several ways: i) for seasonal differences within the group of control hearts and the diabetic hearts each separately; ii) for differences in healthy control hearts vs. diabetic hearts in the W/S-P and S/A-P each separately. Results indicate that MIT Mg^{2+}-ATPase activities registered during the W/S-P exceed those in the S/A-P in both the control group of healthy and also in diabetic animals. In diabetic group of animals this difference represented an enhancement in Mg^{2+}-ATPase activity by 32.62 % ($p<0.001$) and in the group of healthy control animals by 28 % ($p<0.05$) against the values in S/A-P (Fig. 1).

Another important observation was that the impulse inducing the increase in MIT Mg^{2+}-ATPase activities in the W/S-P amplified also the effect induced by diabetes. This is manifested by the finding that the difference in enzyme activity between the healthy and diabetic hearts that amounts in the S/A-P 4.52 % ($p<0.05$) increases in the W/S-P to 8.57 % ($p<0.001$), always in favor of the diabetic heart MIT ATPase (Fig. 2).

Variations in conjugated dienes

Content of CD in lipids of cardiac mitochondrial membranes exhibited high inter-individual variations in both groups, the healthy control as well as in diabetic animals. For this reason, it was necessary to evaluate the seasonal and diabetes-induced variations in CD contents by comparison of their medians. Nevertheless, most differences including the increase in CD content that could be anticipated in the diabetic group have not reached the level of significance. Oppositely, in S/A-P the CD levels in MIT from diabetic hearts were significantly ($p<0.05$) below those in the healthy hearts. Analysis of data collected along the whole year further revealed that the values of CD in the S/A-P exceed considerably those in the W/S-P (Table 1), but this relationship also remained out of statistical significance.

Variations in membrane fluidity

In the W/S-P group of diabetic hearts membrane fluidity exceeded significantly ($p<0.01$) that in the S/A-P group. The latter finding seems to be specific for the diabetic heart MIT since in healthy control hearts these organelles failed to exhibit any significant seasonal differences in membrane fluidity.

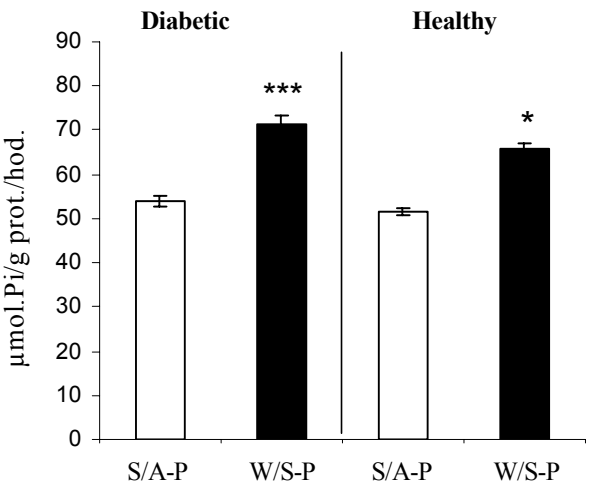

Fig. 1. Seasonal differences of MIT Mg^{2+}-ATPase activities in the W/S and S/A periods: healthy vs. diabetic hearts. W/S-P – winter-spring period, S/A-P – summer-autumn period; ATPase activities are means ± S.E.M; n=8, $^{*}p<0.05$, $^{***}p<0.001$, both for healthy vs. diabetic group.

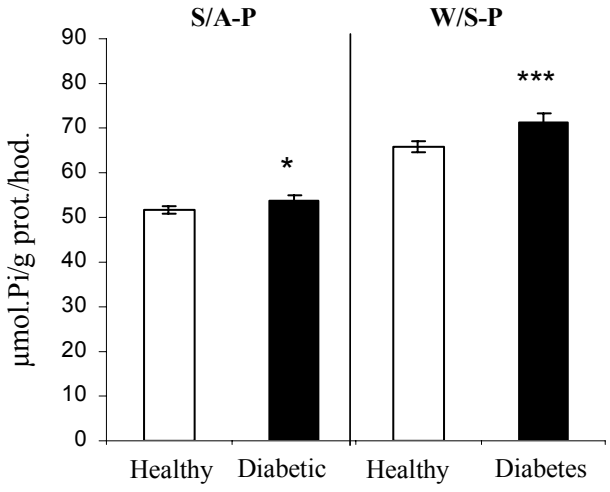

Fig. 2. Seasonal differences of MIT Mg^{2+}-ATPase activities in the groups of healthy and diabetic hearts: W/S-P vs. S/A-P. W/S-P – winter-spring period, S/A-P – summer-autumn period; ATPase activities are means ± S.E.M; n=8, $^{*}p<0.05$, $^{***}p<0.001$, both for the S/A-P vs. W/S-P.

Discussion

In the literature there are numerous reports about seasonal variations in function and structure and enzyme activities in the heart (Belló-Klein *et al.* 2000, Frolov 1984, Frolov *et al.* 1991, Wickler 1981). However, seasonal variability was not restricted to variables in the heart only. Guderley and St. Pierre (1999) confirmed the presence of seasonal differences in ADP sensitivity, and oxidative capacity of MIT from red myotonal muscle of rainbow trout. Marti *et al.* (2007) discovered seasonal changes in the activity of the antioxidant enzyme systems comprising superoxide dismutase, glutathione reductase, glutathione peroxidase and catalase in seminal plasma of the ram. Seasonal differences in activities of catabolic enzymes in the flight muscles of in birds were observed by Lundgren and Kiessling (1985). This study revealed high oxidative capacity and low glycolytic and anaerobic capacity during the migration season against low oxidative and high glycolytic and anaerobic capacity in the breeding season. Further studies indicated that seasonal variations could be also present in pathological conditions. Resistance to acute hypoxia was evaluated by the life span of rats exposed to high altitude hypoxia in different seasons of the year (Khachaturyan and Panchenko 2002). They found that the differences in life span between the low and highly resistant rats were most pronounced in the winter season.

In spite of relatively numerous, convincing observations concerning season-bound variations in diverse metabolic parameters or processes, in different animals, these still neither allow universal nor definitive conclusions about the internal causes of seasonal differences. The reasons for that may be lying, in part, in diversity and specific properties of investigated species (rats, fish, migrating birds, etc.) given by conditions and mode of their life, in part by non-adequate maintenance of exact conditions of their housing during the period of investigation. Even in laboratory rats, the usual declaratory statement that they were kept at constant temperature and in 12 h light and 12 h dark (LD 12/12) regimen is often more expressing a believe of the investigator than a condition documented by exact monitoring that exclude any contamination of the environment by changes in external temperature and/or in the photo-period. Although in present experiments the LD 12/12 and temperature of 21-23 ^{o}C were kept constant, a comparison of our results with those of Belló-Klein *et al.* (2000), Frolov (1984), Frolov *et al.* (1991) or Wickler (1981) requires caution.

MIT Mg^{2+}-ATPase is actually the ATP synthase estimated in reversed reaction i.e., when the enzyme is splitting ATP in presence of Mg^{2+}. However, the membrane of intact mitochondria is impermeable for Mg^{2+}. Therefore, Mg^{2+} may cause ATP splitting only in that part of the investigated population of MIT which has leaky membranes. For the latter reason, in order to learn the total MIT Mg^{2+}-ATPase activity in the preparation, all MIT were made permeable for Mg^{2+} by applying the uncoupler 2,4-DNP. In the literature is MIT Mg^{2+}-ATPase often termed by various synonyms. When estimated by ATP splitting in presence of 2,4-DNP, the

enzyme is often referred to as the Mg^{2+}-dependent and DNP-stimulated MIT ATPase (Cerreijó-Santaló 1967).

MIT Mg^{2+}-ATPase is localized in the inner MIT membrane and is transpassing its lipid bilayer. This makes the enzyme sensitive to fluidity of membrane lipids. However, the latter property of MIT membrane may depend not only on composition of its fatty acids and their oxidation status, but also on conformation state of the whole membrane that may be modulated by protein-protein crosslinks and mainly by membrane contact sites (energy transport pores) formation (Ziegelhöffer-Mihalovičová *et al.* 1997, Ziegelhöffer 2005). For the latter reasons in parallel to Mg^{2+}-ATPase activity we also investigated the fluidity and the content of CD in the MIT membranes.

Results in Table 1 revealed that an increase in MIT membrane fluidity is usually associated with a considerable elevation of the MIT Mg^{2+}-ATPase activity. This mutual relationship is particularly significant when comparing the diabetic group against the control group ($p<0.008$) in W/S-P and the diabetic group in S/A-P against the same group in W/S-P ($p<0.01$). This points to the probability that, at least in the diabetic heart**,** the following sequence of regulations may operate: i) the demonstrated elevation in activity of the MIT Mg^{2+}-ATPase is induced by increased fluidity of the MIT membrane; ii) the elevation in membrane fluidity is a consequence of structural changes in MIT membrane (the parallel bilayer structure is turning to pillow like one) caused by enhanced contact sites formation (Ziegelhöffer 2005); iii) the impulse for increased creation of contact sites is provided by significantly elevated Ca-transients that are characteristic for the diabetic myocardium (Ziegelhöffer-Mihalovičová *et al.* 1997).

The latter regulatory sequence is also consistent with vulnerable energy equilibrium in the diabetic heart (Ferko *et al.* 2006a,b). Diabetic hearts experience pseudo-hypoxia with the energy production slowed down. However, the latter is in part mitigated: i) upstream, by activation of endogenous protective processes which, involve increase in MIT Mg^{2+}-ATPase activity and ii) downstream, by facilitated transfer of ATP from the MIT to the cytosol *via* the energy transfer pores (contact sites) in the MIT membrane (Ziegelhöffer-Mihalovičová *et al.* 1997). This adaptation remodeling of diabetic heart MIT was found to be also associated with decrease in transmembrane potential of the MIT and the latter is in reciprocal relationship to the membrane fluidity (Waczulíková et. al. 2007). Although the described regulatory mechanisms are probably not the only ones that may be acting, they enable to explain why the Mg^{2+}-ATPase activity in diabetic heart MIT in each case exceeds that in the healthy heart. Nevertheless, the explanation offered for regulations triggered by diabetes seems not to work for the seasonal changes. In case of the latter it may be at present only speculated that the differences in MIT Mg^{2+}-ATPase activity might be associated with circannual differences in metabolism that may persist in spite of food, temperature and light regimen kept constant during housing of the animals.

Changes in content of CD exhibited high variability in each experimental situation. Hence, at the given number of experiments, the majority of changes remained not significant. In contrast to expectations based on increased peroxidation processes in heart tissue observed in the spring season (Belló-Klein *et al.* 2000) and also confirmed in part by ourselves, the season-bound changes in content of CD in cardiac MIT seem to exert only minor influence on MIT membrane fluidity. This indicates that peroxidation processes running in myocardial tissue are not distributed evenly. In consequence of that, findings obtained in homogenates of myocardial tissue (Belló-Klein *et al.* 2000) can't be representative for each compartment of the cardiomyocytes (Ziegelhöffer 2007) and may differ considerably from the findings in subcellular organelles such as the MIT.

It may be summarized that heart MIT Mg^{2+}-ATPase activity and membrane fluidity exhibit significant seasonal differences that are more expressed in the W/S-P. This makes the results obtained in W/S-P non-additive with those in the S/A-P. Similarly to healthy heart MIT seasonal differences MIT Mg^{2+}-ATPase activity and membrane fluidity are also present and even amplified in the diabetic heart.

Conflict of Interest

There is no conflict of interest.

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