Virchows Arch [Pathol Anat] (1982) 397:83–91

Virchows Archiv A

Lack of Cardiotoxic Effect of Isoproterenol in Streptozotocin Diabetic Rats

A Morphometric Study of Isoproterenol Induced Fibrosis

Ole Gøtzsche

University Institute of Pathology and Second University Clinic of Internal Medicine, Kommunehospitalet, DK-8000 Aarhus C, Denmark

Summary. The well known cardiotoxic effect of isoproterenol (ISO) was investigated in normal and streptozotocin diabetic rats. Seven days after the subcutaneous injection of ISO (15 mg/kg) the hearts were perfusion fixed and 12 sections from each heart were stained (Masson's trichrome). ISO induced myocardial fibrosis was quantified at the light microscopic level according to established morphometric principles. Pulse rate and ST elevation were recorded by EEC (3 standard leads) before and after the ISO injection. Non-diabetic control animals showed marked fibrosis after ISO, but surprisingly the diabetic animals showed no fibrosis after ISO treatment. These findings were in accordance with an ISO induced ST elevation seen only among control animals although both groups showed the same degree of tachycardia. Insulin treatment prevented the protection against ISO and when streptozotocin was injected 24 h after the ISO a normal quantitative and qualitative appearance of the scar tissue was seen. It thus seems that streptozotocin diabetic rats are protected against the toxic effect of ISO, leaving the haemodynamic response unaffected. Which factor in the diabetic metabolism is reponsible for the present phenomenon is not known, but a defect in the signal transmission from the β-receptor to the adenylcyclase is suggested as a possible explanation.

Key words: Isoproterenol – Myocardial fibrosis – Diabetes

Diabetes is a well known risk factor in the development of myocardial infarction. This has usually been explained by the increased coronary atherosclerosis seen among diabetics. However, during recent years the existence of a specific diabetic heart disease has been suggested. This diabetic cardiopathy, combined with changes due to atherosclerosis has been suggested as an explanation to the increased incidence of cardiac complications in

Offprint requests to: O. Gøtzsche at the above address

0340-1227/82/0397/0083/$01.80

diabetes (Ledet et al. 1979). Epidemiological studies have shown that immediate and long term survival after myocardial infarction is significantly decreased among diabetics and that the incidence of myocardial infarction in a diabetic population is as high in females as in males, even at the lower age levels (Partamian and Bradley 1965; Kannel 1978).

Over the years diabetics develop a characteristic histological picture in the myocardium consisting of an increased amount of interstitial collagen, increased perivascular fibrosis and the existence of small dispersed scars, suggesting earlier microinfarctions (Regan et al. 1975; Ledet 1976). Apart from these small scars the same histological picture has been described after 9 months of streptozotocin diabetes in the rat (Baandrup et al. 1981).

In order to investigate why diabetics are more prone to acute complications like shock and congestive heart failure after myocardial infarction (Partamian and Bradley 1965) we studied the effect of the diabetic state on the myocardial healing process. Myocardial necrosis was induced by isoproterenol (ISO) in high doses. Subcutaneous injection of this strong β-adrenergic stimulator leads to extensive myocardial degeneration especially localized to the endocardium (Rona et al. 1959). Within the next few days the necrosis is eventually replaced by connective tissue. This healing process shows the same characteristics as that following myocardial infarction in humans (Mallory et al. 1939), although it develops much more quickly in the rat.

Somewhat to our surprise ISO in the doses given (15 mg/kg body weight), produced no signs of toxic effect among the streptozotocin diabetic rats 7 days after the injection. The control animals showed marked fibrosis in the myocardium. In order to elucidate this finding we investigated the effect of insulin treatment on this phenomenon and the effect of diabetes on the myocardial healing process, once the necrosis has been established. The present paper reports the quantitative histological measurements of the fibrosis following the isoproterenol induced necrosis in these groups.

Methods

Male Wistar rats weighing 300–400 g were included in the study when they were 3 months old (range $\pm$ 7 days). Diabetes was induced by an intraperitoneal injection of streptozotocin (70 mg/kg) (Upjohn Inc.). Blood glucose was measured twice (glucose oxidase method) in each animal during the experimental period and only animals with a non-fasting blood glucose of 300–450 mg/100 ml were included in the study. None of these animals had ketonuria (Ketostix). At termination 1 ml of blood was obtained from the inferior vena cava, centrifuged and the serum frozen for subsequent chemical measurements of cholesterol (Enzymatic colorimetric method, Boehringer Mannheim, FRG) and triglyceride (Kinetic UV-method, Boehringer Mannheim, FRG). A long acting heat-treated non-commercial ultralente insulin (NOVO) was used for the insulin treatment. An individual dialy dose was given subcutaneously according to the blood glucose level (Dextrostix and Ames Reflectance Meter). All blood glucose estimations and insulin injections were performed between 11 a.m. and 2 p.m.

ISO-treated rats were given 15 mg/kg isoproterenol sulphate (Boehringer, Mannheim, FRG) on two succeeding days. Before each injection the animals were anaesthetized by nembutal (30 mg/kg). Control rats were also anaesthetized and injected with 1 ml of isotonic NaCl instead of ISO.

Pulse rate and ST elevation were estimated by an electrocardiographic recording (Cardioline Electrocardiograph) of the 3 standard leads before and every minute during the first

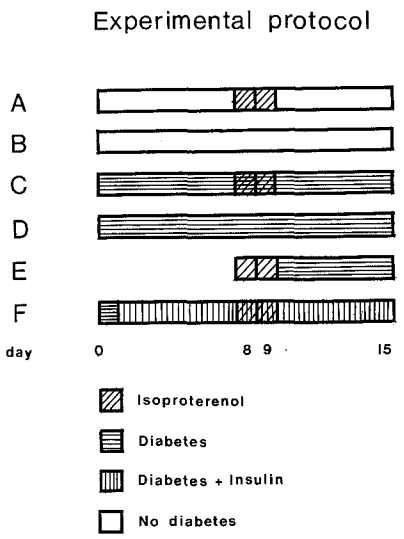

Fig. 1. Schematic outline of the experimental procedures in the 6 groups of Wistar rats (see text for details)

Table 1. Metabolic data and serum values in the 6 groups A–E. The results are means ± SEM. *n* indicates number of animals in each group

	n	Body weight g	Heart weight mg	Heart weight / Body weight $\times 10^{-3}$	Blood glucose mg/dl	Serum triglyceride mg/dl	Serum cholesterol mg/dl
A	7	352± 9	1,037±32	2.95±0.07	112±11	113±11	46±4
B	8	324±18	936±40	2.90±0.13	104±14	108±18	63±2
C	8	270±11	861±27	3.20±0.06	387±15	145±33	63±5
D	6	273± 9	850±29	3.12±0.05	373±10	201±41	70±7
E	7	287± 8	1,025±49	3.57±0.16	365±17	277±55	50±5
F	8	340± 8	1,080±47	3.17±0.07	see Fig. 5	96±26	37±2

15 min after an ISO injection in a group of control ($n=6$) and diabetic animals ($n=7$). ST elevation was said to be present when it exceeded 1 mV in any 1 of the 3 standard leads at any recorded point of time. No histological investigation was performed in these animals.

Experimental Protocol

As shown in Fig. 1 the animals were divided into 6 groups A) control animals (ISO at day 8 and 9, $n=7$), B) control animals (NaCl at day 8 and 9, $n=8$), C) diabetic animals (ISO at day 8 and 9, $n=8$), D) Diabetic animals (NaCl at day 8 and 9, $n=6$), E) control animals (ISO at day 8 and 9 followed by streptozotocin at day 10, $n=7$), F) diabetic animals (ISO at day 8 and 9, receiving insulin treatment from day 2 to day 15, $n=8$).

In order to investigate the effect of strain and diabetes duration on the ISO induced fibrosis 13 animals (Male Lewis rats, initial age 3 months (range ± 7 days), initial weight 250–300 g) of which 7 had had streptozotocin induced diabetes for 6 months (blood glucose 300–500 mg/100 ml measured 3 times during the 6 months in each animal) were divided into 4 groups corresponding to group A–D as described above. These groups were designated I–IV.

Measurement Technique

At the end of the experiment the animals were anaesthetized with nembutal and the hearts removed and weighed. Within 30 s of removal the hearts were fixed by retrograde perfusion through aorta at a constant pressure of 80 mm Hg for 2 min. The perfusion medium consisted of a formaldehyde buffer (pH 7.2) in which the hearts were stored at 4° C until sectioning. Then the atria were removed and 3 blocks (each 3 mm) cut from the apex with a set of fixed razor blades. After mounting the blocks in paraffin, 4 sections (5 μ thick) from each block (at a distance of 200 μ) were cut. In this way 12 sections from the apex towards the atrioventricular plate were available. Masson's trichrome stain was used giving the connective tissue a clear blue colour – in sharp contrast to the red myocardium. ISO induced fibrosis was defined as confluent loose connective tissue replacing normal myocardium and consisting of fibers fibroblasts and dilated capillaries. Interstitial and perivascular connective tissue was not included in the measurements. Tangentially cut lamina adventitia from the intramyocardial arteries could not be discriminated from the ISO induced fibrosis in some instances. However, this relatively small error was taken to be equal in all the groups.

The quantitative measurements were performed by point counting. The same person (O.G.) did all the measurements, unaware of the identity of the sections. Approximately 250 fields of vision, selected at random by a computerized, graded device connected to the switchboard, were investigated per animal. The image was projected onto a vertical screen (final magnification 320×) on which a 48-point grid was mounted. Approximately 10% of the total cross sectional area was examined in this way. The volume fraction of ISO induced fibrosis was calculated for all 12 sections and expressed as a percentage of the myocardium in each animal. This stereological technique showed a coefficient of variation of 19% ($n=6$).

Wilcoxon non-parametric test was used to evaluate differences in ISO induced fibrosis and Student's t-test for other differences with a $2p=0.05$ level of significance.

Results

Figure 2 shows the microscopic appearance of the ISO induced fibrosis. The results from the quantitation of this fibrosis is shown in Figs. 3 and 4. Note that the results are plotted on a semilogarithmic scale. It is seen that group C does not differ from either of the NaCl treated groups, B and D. The difference between groups A and C is significant ($p < 0.01$) as well as the differences between A–B ($p < 0.01$) and A–D ($p < 0.01$). Group C animals and animals that did not receive any ISO (B and D) showed no sign of myocardial necrosis (Haemotoxylin-Eosin and PAS-stain) and the small amounts of fibrosis in these animals represented lamina adventitia as mentioned earlier. In group E streptozotocin was injected the day after the second ISO injection. Within 24 h after the streptozotocin injection the blood glucose was above 300 mg/100 ml in all the animals. This group therefore illustrates the influence of the diabetic state on the myocardial healing process. It is seen that these animals showed the same amount of ISO induced fibrosis as in the non-diabetics injected with ISO.

In order to investigate the effect of insulin-treatment on the ISO induced fibrosis in diabetic animals, another group (F) was treated with insulin starting the day after the injection of streptozotocin. This treatment normalized blood glucose within 2 days (Fig. 5) and the animals did not loose weight. As seen in Fig. 3 they now showed the same amount of ISO induced fibrosis as in group A and E. The possibility that streptozotocin in itself is responsible for the protection against the cardiotoxic effect of ISO seems

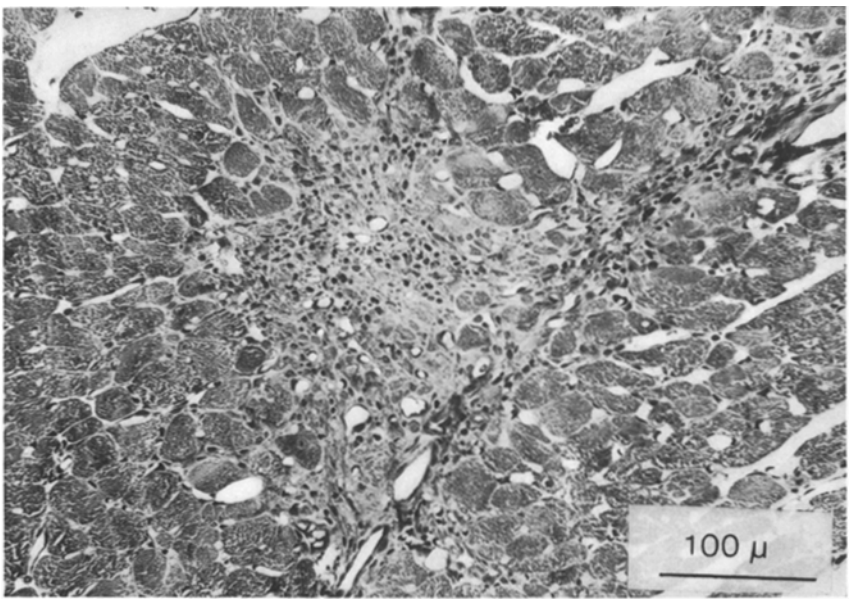

Fig. 2. Light microscopy of ISO induced fibrosis 7 days after the second injection of ISO (Masson's trichrome stain)

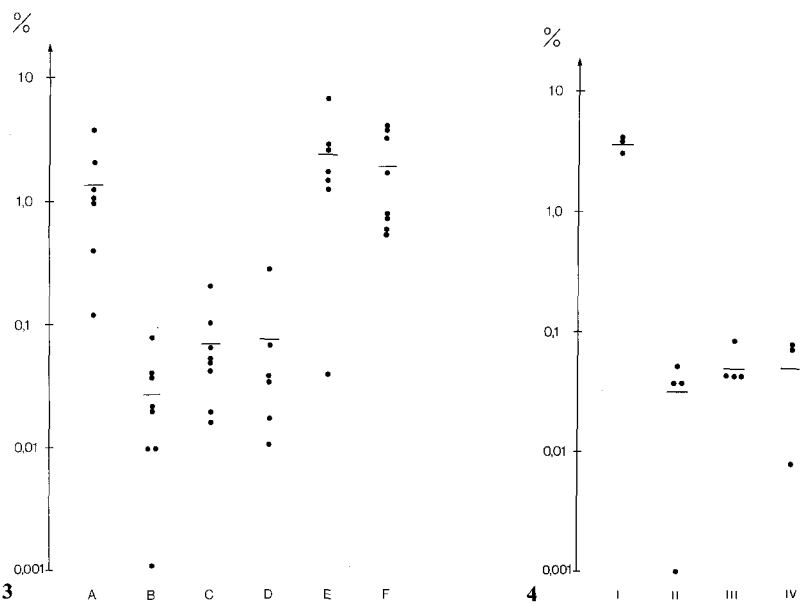

Fig. 3. Per cent ISO induced fibrosis in the 6 groups of Wistar rats. Note the semilogarithmic scale. Bars represent means

Fig. 4. Per cent ISO induced fibrosis in the 4 groups of Lewis rats

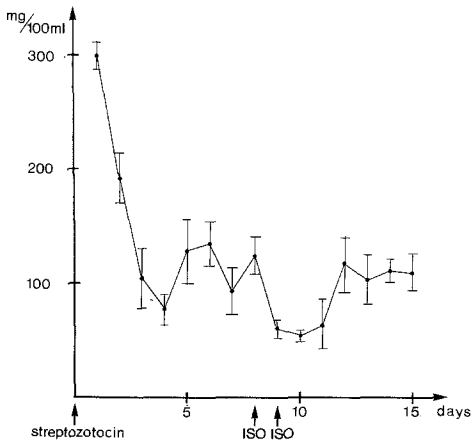

Fig. 5. Blood glucose in the insulin treated animals (group E). Mean ± SEM is indicated for the daily estimations from day 2 to 15 after the injection of streptozotocin

Table 2. Increase in pulse rate (beats/min) in normal and diabetic animals (mean ± SEM). Values are estimated from electrocardiographic recording and represents maximal increase during the first 15 min after isoproterenol

	ΔP	ΔP%	
Normal ($n=6$)	105 ± 19	28.4 ± 6.6	N.S.
Diabetic ($n=7$)	105 ± 16	32.1 ± 4.2	

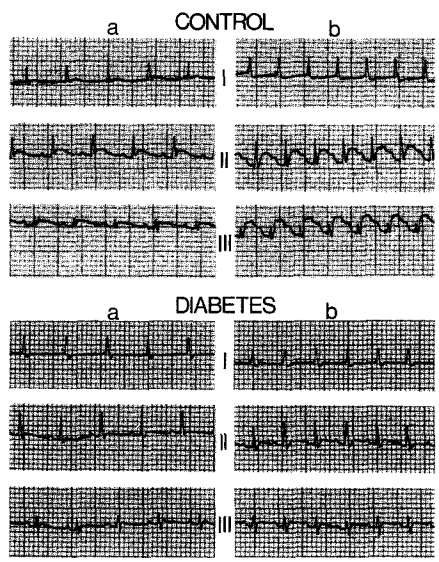

Fig. 6a, b. Electrocardiographic recording of the three standard leads before (**a**) and 5 min after (**b**) the injection of isoproterenol in a control and a diabetic animal. ST elevation (> 1 mV) is seen in the control (paper rate 50 mm/s)

thus unlikely. There was no correlation between mean blood glucose level and amount of ISO induced fibrosis within this group.

The Lewis rats (group I–IV) in which group III–IV had had streptozotocin induced diabetes for 6 months, showed the same distribution of ISO induced fibrosis among the groups as seen in the Wistar rats with diabetes

of recent onset (Fig. 4). The blood glucose level in these animals did not differ from that in the corresponding groups of Wistar rats.

Cholesterol and triglyceride was measured in serum from non-fasting animals at the time of sacrifice (Table 1). Se-triglyceride was increased significantly in diabetes when group C, D and E was compared to the controls (group A and B, (175 ± 89 vs. 110 ± 41 mg/dl), ($p > 0.01$). Se-cholesterol showed no difference.

Electrocardiographic recording after the ISO injections in controls ($n = 6$) and diabetics ($n = 7$) showed the same degree of tachycardia within minutes after the injection (Table 2). An elevation of ST segments exceeding 1 mV in either of the three recorded leads was seen in all the control animals after ISO, but in none of the diabetic animals (Fig. 6).

Discussion

The present investigation shows a remarkable dissociation between the cardiotoxic effect of ISO and its chronotropic effect in normal and streptozotocin diabetic rats. This finding may illustrate the mechanism behind the toxic effect of ISO, but may also demonstrate functional alterations in the myocardial cell in experimental diabetes.

A number of factors seem to account for the cardiotoxic effect of ISO. Haemodynamically, ISO in high doses induces a fall in blood pressure due to peripheral vasodilatation. At the same time it has a strong positive inotrope and chronotrope effect on the heart. In this way a disproportion between oxygen delivery and demand arises, which has been considered to be the cause of the necrosis (Rona et al. 1963). However, when using in vitro perfusion of the rat heart (Dhalla et al. 1978) as well as cultured human heart cells (Hofmann et al. 1977), β-receptor stimulants have been shown to have marked direct effects on the myocardial cell. This action seems to be mediated via the β-receptor – adenylcyclase – cyclic AMP system (Murad et al. 1962). As high doses of ISO induce an influx of calcium leading to toxic concentrations of free calcium (Ca^{2+}) in the cytosol, this has been hold to be responsible for the necrosis (Fleckenstein 1971; Bloom and Davies 1972). This influx appears within minutes after the injection of ISO, at which time the first ultrastructural changes can be detected (Csapo et al. 1972). Pretreatment with propranolol normalized the myocardial calcium content 1 h after the injection of ISO. This pretreatment was, however, not able to prevent some myocardial necrosis (Lehr et al. 1966; Bloom and Davies 1972). Calcium channel blockers such as verapamil have also been reported to protect the myocardium against ISO (Fleckenstein 1971).

A number of factors have been shown to modify the development of ISO induced myocardial necrosis. The sensitivity seems to differ among different strains of animals and to increase with increasing age and weight of the animals (Rona et al. 1959). Moreover a number of pharmacological and dietary interventions can modify the extent of necrosis (Rona 1963). The most remarkable findings in these studies were an increased amount of ISO induced myocardial necrosis when animals were kept on a low potas-

sium, high sodium diet or when given a pretreatment with minerallocorticoid.

In the present study the complete lack of fibrosis after giving ISO to the diabetic animals, contrasts with the normal increase in pulse rate seen within minutes after the injection. Thus, it seems to be the direct toxic effect of ISO from which the diabetic animals are protected, leaving the haemodynamic action unaffected. This is also supported by the fact that no ST-elevation was seen in the diabetic animals, compared with the marked changes seen in the normals. The ionic changes responsible for this electrocardiographic change, did not seem to appear in the diabetic rats. The increase in pulse rate after ISO is in itself a poor variable to evaluate the haemodynamic response. However, the result is in agreement with a recent study using in vitro heart perfusion, showing the same left ventricular pressure development after ISO in diabetic as in control animals (Ingebretsen et al. 1981). Despite this it was also shown that the increase in myocardial c-AMP and protein kinase activity after ISO was clearly diminished in diabetes, a finding that might be of relevance for the lack of ISO toxicity in diabetic rats. There thus seems to be a defect in signal transmission from the β-receptor to adenylcyclase, somehow leaving the haemodynamic response unaffected. ISO increases the uptake of Ca^{2+} via c-AMP (Schneider and Sperelakis 1975) and excessive Ca^{2+} uptake leading to toxic cell death (Katz and Reuter 1979) is held to be responsible for the lesion induced by ISO. It might therefore be of interest to investigate the transport of Ca^{2+} across the sarcolemma in response to ISO in diabetes. Preliminary results from heart perfusion experiments in streptozotocin diabetes rats show a marked decrease in the ISO induced $^{45}Ca^{2+}$ uptake compared with control animals (Gøtzsche 1981). This finding is compatible with the present report and focuses on an abnormality in the diabetic heart.

Of the many alterations in diabetic whole body metabolism, it is difficult to indicate those elements in the environment of the heart responsible for the phenomenon described. Insulin treatment, with all its consequences, could normalize the reaction towards ISO. In the present experimental design it was not possible to detect a negative correlation between blood glucose and the extent of ISO induced fibrosis. However, such a negative correlation would be expected to exist in a group of animals with slightly elevated blood glucose levels. The all or none character of our findings is also against the elevated serum triglyceride per se as a responsible factor because of the overlap seen among controls and diabetic animals with regard to this variable. As none of the animals showed ketonuria, it is hard to believe that ketone bodies in the diabetic animals could desensitize the myocardium to the toxic effect of ISO.

In diabetic patients an increased incidence of myocardial infarction and other cardiovascular complications has been demonstrated. Experimental studies have shown that diabetic rats have a decreased resistence to severe ischaemia (Feurray et al. 1979) or increased work loads (Miller 1979) resulting in rapid ventricular failure. As catecholamines have been suggested to play a role in the development of myocardial infarction (Waldenström et al. 1978) the present report is paradoxical. The finding of resistance to the

cardiotoxic effect of ISO in the streptozotocin diabetic rat might, however, go hand in hand with other functional alterations in the response to catecholamines. Such alterations could be of relevance for the acute cardiovascular complications in diabetes mellitus. The answer to this must await further investigations.

References

Baandrup U, Ledet T, Rasch R (1981) Experimental diabetic cardiopathy preventable by insulin treatment. Lab Invest 45:169–173

Bloom S, Davies DL (1972) Calcium as mediator of isoproterenol-induced myocardial necrosis. Am J Pathol 69:459–470

Csapo Z, Dusec J, Rona G (1972) Early alterations of the cardiac muscle cells in isoproterenol induced necrosis. Arch Pathol 93:356–365

Dhalla NS, Yates JC, Lee SL, Singh A (1978) Functional and subcellular changes in the isolated rat heart perfused with oxidized isoproterenol. J Mol Cell Cardiol 10:31–41

Fleckenstein A (1971) Specific inhibitors and promotors of calcium action in the excitation-contraction coupling of heart muscle and their role in the prevention of production of myocardial lesions. In: Harris P, Poie LH (eds) Calcium and the heart. Academic Press, London p 135

Feuvray D, Idell-Wenger JA, Neely JR (1979) Effect of ischemia on rat myocardial function and metabolism in diabetes. Circ Res 44:322–329

Gøtzsche O (1981). Abnormal myocardial calcium uptake in response to isoproterenol in experimental diabetes. Diabetologia 21:276–277

Hofmann W, Schleich A, Schroeter D, Weidinger H, Wiest W (1977) Der Einfluß von β-Sympathomimetica und sog. Ca^{++}-antagonistischer Hemmstoffe auf den menschlichen Herzmuskel in vitro. Virchows Arch [Pathol Anat] 373:85–95

Ingebretsen WR, Peralta C, Monsher M, Wagner LK, Ingebretsen CG (1981) Diabetes alters the myocardial cAMP-protein kinase cascade system. Am J Physiol 240:H375–H382

Ledet T, Neubauer B, Christensen NJ, Lundbæk K (1979) Diabetic cardiopathy. Diabetologia 16:207–209

Lehr D, Krukovski M, Colon R (1966) Correlation of myocardial and renal necrosis with tissue electrolyte changes. JAMA 197 no. 2:145–152

Kannel WB (1978) Role of diabetes in cardiac disease. Conclusions from populations studies. In: Zoneraich S, Thomas CD, (eds) Diabetes and the heart. Publich, USA p 97

Katz AM, Reuter H (1979) Cellular calcium and cardiac cell death Am J Cardiol 44:188–190

Mallory GK, White PD, Salcedo-Salgar J (1939) The speed of healing of myocardial infarction. Am Heart J 18:647–672

Murad F, Chi YM, Rall TW, Sutherland EW (1962) Adenyl cyclase. III. The effect of catecholamines and choline esters on the formations of adenosine 3′, 5′-phosphate by preparations from cardiac muscle and liver. J Biol Chem 237:1233–1238

Partamian JO, Bradley RF (1965) Acute myocardial infarction in 258 cases of diabetes. New Eng J Med 273:455–461

Regan TJ, Ahmed SS, Levinson GE, Oldewurtel HA, Ahmad MR, Haider B, Lyons MM (1975) Cardiomyopathy and regional scar in diabetes mellitus. Trans Assoc Am Physicians 85:217–223

Rona G, Chappel CI, Balatz T, Gaundry R (1959) The effect of breed, age and sex on myocardial necrosis produced by isoproterenol in the rat. J Gerontol 14:169–173

Rona G, Chappel CI, Kahn DS (1963) The significance of factors modifying the development of isoproterenol-induced myocardial necrosis. Am Heart J 66:389–395

Schneider JA, Sperelakis N (1975) Slow Ca^{++} and Na^{+} responses induced by isoproterenol and methylxanthines in isolated perfused Guinea-pig hearts exposed to elevated K^{+}. J Mol Cell Cardiol 7:249–273

Waldenström AP, Hjalmarson AC, Thornell L (1978) A possible role of noradrenalin in the development of myocardial infarction Am Heart J 95 (1):43–51

Accepted April 20, 1982