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EXPERIMENTAL STUDY

Effect of levosimendan on erythrocyte deformability during myocardial ischaemia-reperfusion injury

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Abstract: Diabetes mellitus (DM) is a chronic metabolic disorder accompanied by an increase in oxidative stress. Ischaemia–reperfusion (IR) injury is a cascade of events initiated by tissue ischaemia. The cellular damage produced by reperfusion leads to an active inflammatory response. Erythrocyte deformability and plasma viscosity are of crucial importance for the perfusion of tissues and organs. The aim of this study was to evaluate the effect of levosimendan on erythrocyte deformability during IR myocardial injury in diabetic rats.
Methods: Twenty-four Wistar albino rats were included in the study after streptozocin (55 mg/kg) treatment for 4 weeks to observe the existence of diabetes. The animals were randomly assigned to one of four experimental groups. In Group C and DC (sham-control group), the coronary artery was not occluded or reperfused in the control rats. Myocardial IR was induced by ligation of the left anterior descending coronary artery for 30 min, followed by 2 h of reperfusion in the diabetes-IR (DIR) and diabetes-IR-levosimendan (DIRL) group. Deformability measurements were performed in erythrocyte suspensions containing Htc 5 % in a phosphate-buffered saline (PBS) buffer.
Results: The deformability index was significantly increased in the diabetic rats. It was similar in Group DC and DIRL It was significantly increased in the DIR group compared to Group C, DIRL and DC. The relative resistance was increased in the IR models.
Conclusion: Erythrocyte deformability was decreased in rats with diabetes and IR injury. This injury might lead to further problems in microcirculation. Levosimendan may be useful in enhancing the adverse effects of this type of injury *(Fig. 2, Ref. 41)*. Text in PDF *www.elis.sk*.
Key words: erythrocyte deformability, myocardial ischaemia reperfusion, experimental diabetes, levosimendan, rat.

Diabetes mellitus (DM) is a complex, chronic disease and an increasingly significant health problem as the incidence increases worldwide. By 2030, the World Health Organization predicts that 366 million people worldwide will have diabetes. DM is a risk factor for coronary heart disease, which is two to four times higher in the diabetes patients. The risk of stroke or peripheral vascular disease also increases considerably in DM patients (1).

A 50 % increase in early mortality following coronary artery bypass grafting has been described in diabetic patients (2). One study demonstrated that the rate of unrecognised myocardial infarction was 39 % in diabetic patients and 22 % in nondiabetic patients (3). Cardiac surgery with cardiopulmonary bypass inevitably causes a systemic inflammatory response and ischaemia–reperfusion (IR) injury, which affects multiple organs (4, 5).

Many tissues and cells can be damaged by free radicals, with red blood cells (RBCs) being one of the most susceptible. During IR, the increased oxidative stress can cause augmented RBC membrane lipid peroxidation, with consequent alteration of cellular deformability. RBC deformability and aggregation have a very important effect on the microcirculation. In the capillaries, where the RBCs must deform to enter and transit vessels smaller than the resting cell diameter, erythrocyte deformability is a crucial factor affecting the flow of blood (6–8).

DM induces several changes in the erythrocyte membranes and in the cytoplasm, leading to alterations in the deformability. A decreasing trend of deformability in diabetes patients has been reported (9, 10). Many studies have shown that DM is associated with increased whole blood viscosity and decreased erythrocyte deformability. Some have suggested that these abnormalities in blood rheology may play a causative role in the pathogenesis of diabetic vascular complications (11, 12).

Levosimendan is a relatively new inotropic and vasodilator agent used in the management of acute and chronic heart failure (13). Its positive inotropic effect is mediated by calcium sensitization of contractile proteins, and its vasodilatory and anti-ischemic effects are mediated by the opening of adenosine triphosphate-sensitive potassium channels in vascular smooth muscle cells (13–16). The protective effects of levosimendan are not limited to cardiac tissue. Some studies have suggested that levosimendan also attenuates IR injury in the spinal cord, lung and renal tissue (17–19).

The effects of levosimendan on myocardial injury induced by left anterior descending (LAD) IR have not yet been investigated.

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The primary aim of this study was to investigate deformability changes and the role of levosimendan in preventing these changes in the erythrocytes of diabetic rats in an experimental model of myocardial IR injury.

Materials and methods

Animals and experimental protocol

This study was conducted in the GUDAM Laboratory of Gazi University with the consent of the Experimental Animals Ethics Committee of Gazi University. All the procedures were performed according to the accepted standards of the Guide for the Care and Use of Laboratory Animals.

In the study, 24 male Wistar albino rats weighing between 225 and 275 g, raised under the same environmental conditions, were used. For at least one week prior to surgery, the animals were housed in standard cages in a pathogen-free environment, with free access to food (until 2 h before the anaesthetic procedure) and water and with a 12 h light/dark cycle. The animals were randomly separated into four groups, each containing six rats.

Diabetes was induced by a single IP injection of streptozotocin (Sigma Chemical, St. Louis, MO, USA) at a dose of 55 $mg.kg^{-1}$ body weight. The blood glucose levels were measured 72 h after this injection. The rats were classified as diabetic if their fasting blood glucose (FBG) levels exceeded 250 $mg.dl^{-1}$, and only animals with FBG levels > 250 $mg.dl^{-1}$ were included in the diabetic groups (diabetes only, diabetes plus IR and diabetes plus levosimendan-IR). The rats were kept alive for four weeks after streptozotocin injection to allow the development of chronic diabetes before they were exposed to IR (20).

The rats were anesthetized with an IP injection of 100 $mg.kg^{-1}$ of ketamine. The trachea was cannulated for artificial respiration. The chest was shaved, and each animal was fixed in a supine position on the operating table. The chest was opened by a left thoracotomy, followed by sectioning the fourth and fifth ribs about 2 mm to the left of the sternum. Positive-pressure artificial respiration was started immediately with room air, using a volume of 1.5 ml/100 g body weight at a rate of 60 strokes/min. Sodium heparin (500 IU/kg) was administered through the peripheral vein in the tail.

After the pericardium had been incised, the heart was exteriorized with gentle pressure on the right side of the rib cage. An 8/0 silk suture attached to a 10 mm micropoint reverse-cutting needle was quickly placed under the left main coronary artery. The heart was then carefully replaced in the chest, and the animal was allowed to recover for 20 min.

Anaesthesia was maintained by repetitive injections of 20 $mg.kg^{-1}$ ketamine if a positive reaction to surgical stress or intermittent tail pinch could be observed.

There were four experimental groups: Group C (control; $n = 6$), Group DC (diabetes control; $n = 6$), Group DIR (diabetes-IR; $n = 6$) and Group DIRL (diabetes-IR-levosimendan; $n = 6$). The DIRL group underwent left thoracotomy and received IP levosimendan (Simdax 2.5 µg/ml, Abbott®, Orion Pharma, Espoo, Finland) 12 $\mu g.kg^{-1}$ diluted in 10 ml of 0.5% dextrose administered intraperitoneally 30 min before ligating the LAD (21). A small plastic snare was threaded through the ligature and placed in contact with the heart. The artery was then occluded by applying tension to the ligature (30 min), and reperfusion was achieved by releasing the tension (120 min) (22). However, after the above procedure, the coronary artery was not occluded or reperfused in the control and diabetic control rats.

Intracardiac blood samples were obtained from all the rats. Heparinized total blood samples were used to prepare erythrocyte packs. Deformability measurements were performed using erythrocyte suspensions with 5% haematocrit in a phosphate-buffered saline (PBS) buffer.

Deformability measurements

Blood samples were carefully taken, and the measurement process was as fast as possible to avoid haemolysis of the erythrocytes. The collected blood was centrifuged at 1000 rpm for 10 min. Serum was removed, in addition to the buffy coat on the erythrocytes. An isotonic PBS buffer was added to the collapsing erythrocytes, and this was centrifuged at 1000 rpm for 10 min. The liquid on the upper surface was removed. Finally, pure red cell packs were obtained from the washing process, which was repeated three times. The erythrocyte packs were mixed with the PBS buffer to generate a suspension with a value of 5 % Htc. These erythrocyte suspensions were used for the measurement of deformability. The collection and the deformability measurements of the erythrocytes were performed at 22 °C.

A constant-current filtrometer system was used in the measurement of the erythrocyte deformability. Samples to be measured were prepared with 10 ml of erythrocyte suspension and PBS buffer. The flow rate was held constant at 1.5 ml/min with an infusion pump. A 28 mm nucleoporin polycarbonate filter with a 5 µm pore diameter was preferred. Pressure changes while the erythrocytes passed through the filter were detected by a pressure transducer, and the data were transferred to the computer with the help of an MP30 data equation system (Biopac Systems Inc., Commat, USA). The calculations were performed with related computer programs by measuring the pressure changes at various times. Pressure calibration of the system was performed before each sample measurement. The buffer (P_T) and the erythrocytes (P_E) were passed through the filtration system, and the changes in pressure were measured. The relative refractory period value (Rrel) was calculated by relating the pressure value of the erythrocyte suspension to the pressure value of the buffer. An increasing Rrel in the deformability index was interpreted as adversely affecting the deformability of the erythrocytes (23, 24).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) 12.0 program was used for the statistical analysis. Variations in blood glucose levels, erythrocyte deformability and rat weights between the study groups were assessed using the Kruskal-Wallis test. The Bonferroni-adjusted Mann-Whitney *U* test was used if the results of the Kruskal-Wallis test were significant to determine which groups differed from the others. The results were expressed as mean ± standard deviation (mean ± SD). Statistical significance was set at a p value of < 0.05 for all the analyses and $p < 0.033$ (0.1/3) for the Bonferroni-adjusted Mann-Whitney *U* test.

Results

Blood glucose measurements were 88.8±9.7, 325.8±47.5, 338.5±56.7 and 333.7±58.5 mg/dL for Group C, DC, DIR and DIRL, respectively. Serum glucose was significantly lower in Group C when compared to Groups DC, DIR and DIRL ($p < 0.0001$) (Fig. 1).

The deformability index was significantly increased in the diabetic rats ($p < 0.0001$). However, it was similar in Group DC and DIRL ($p = 0.976$). It was significantly increased in Group DIR when compared to Groups C, DC and DIRL ($p < 0.0001$, $p = 0.001$, $p = 0.007$, respectively) (Fig. 2). The relative resistance was increased in the IR models.

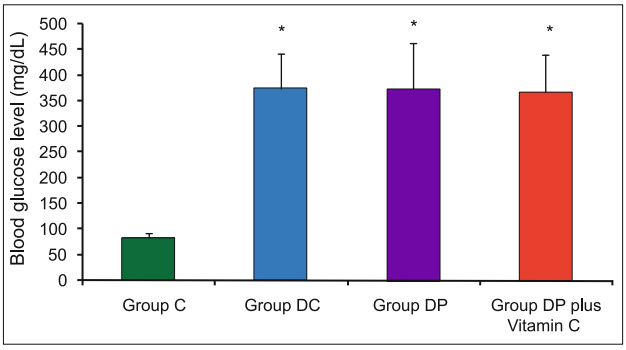

Fig. 1. Blood glucose levels

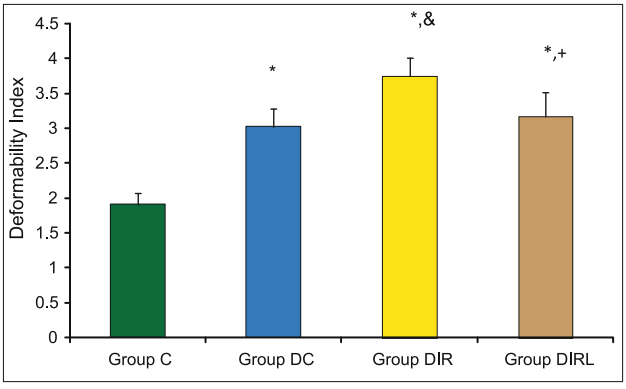

Fig. 2. Erythrocyte deformability values of the groups. Each bar represents the mean ± sd. * p<0.05 compared to Group C; p< 0.05 compared to Group DC; p< 0.05 compared to Group DIR

Discussion

DM makes an individual prone to various complications such as macro- and microvascular disease, hypertension, neuropathy, cardiomyopathy and premature aging, indicating that these complications develop through a similar pathway to that of diabetes (25). It is known that the intracellular calcium concentration is increased in most tissues in subjects with diabetes and that altered intracellular calcium metabolism seems to result from a common, underlying abnormality linking metabolic, cardiovascular, ocular and neural manifestations in the diabetic disease process (26).

The effects of levosimendan on haemodynamics that include increased contractility, improved ejection fraction, increased cardiac output, reduced cardiac filling pressures and reduced systemic, pulmonary and coronary vascular resistance are based on its positive inotropic, lusitropic and vasodilatatory properties (14, 27, 28). Levosimendan is a calcium sensitizer that increases the sensitivity of the myocardium to calcium, thereby increasing myocardial contractility without a rise in intracellular calcium (29). In addition, levosimendan causes adenosine triphosphate-sensitive potassium channels to open in vascular smooth muscle, resulting in smooth muscle relaxation (29, 30). This contributes to vasodilatation and probably anti-ischemic effects (28, 29).

Haemorheological parameters such as haematocrit, plasma proteins, erythrocyte aggregation and erythrocyte deformability are often disturbed in DM (31). For migration of oxygen and vital molecules to the final organ capillaries and clearance of metabolic wastes, erythrocytes must be able to extend and curve and have the capability to move in these areas. This capacity, called 'deformability', is important in the microcirculation. Altered erythrocyte deformability not only changes the oxygen delivery capacity of the erythrocytes it also affects the survival of the circulating erythrocytes (32–34).

It has been suggested that the impaired perfusion at the tissue level observed as a complication of DM is primarily due to reduced erythrocyte deformability (35, 36). Moreover, the impairment of erythrocyte deformability has been attributed to specific changes in the membrane structure. The oxidative stress due to high glucose concentrations causes damage to the erythrocyte membrane proteins, even with a relatively short exposure time (37).

Barnes et al (38) showed that erythrocyte deformability was lower in 14 diabetes patients with extensive micro-angiopathy than in controls or in 22 diabetes patients with slight or no complications. They suggested that hyperviscosity and reduced erythrocyte deformability may be important and potentially treatable factors in the aetiology or progression of microcirculatory disease in diabetes. Similar to these previous studies, we also found that erythrocyte deformability was decreased in rats with induced diabetes.

In this study, for the first time to our knowledge, we have reported that IR of the diabetic rat myocardium results in significant negative changes that can be observed in erythrocyte deformability and that levosimendan, administered at the beginning of myocardial ischaemia, can provide varying degrees of protection against negative effects of variations in erythrocyte deformability.

The administration of levosimendan is also associated with peripheral vasodilation, anti-ischaemic cardioprotection (39), neuroprotection (40) and anti-inflammatory and anti-apoptotic effects (41).

In conclusion, the results of this study clearly demonstrated that erythrocyte deformability is significantly altered in experimental myocardial IR injury in the diabetic rat. This might lead to further problems in microcirculation. Thus, measurement of erythrocyte deformability might have an important impact on the follow-up for IR injury. Additionally, levosimendan administered before the induction of ischaemia had protective effects on these alterations in myocardial IR injury. Other aspects of these findings, including clinical significance and practical applications, merit further experimental and clinical investigation.

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