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# Reduced negative surface charge on arterial endothelium of diabetic rats

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**Summary.** Cationized ferritin binding was used to measure negative surface charge on endothelium of large arteries in streptozotocin-induced diabetic rats and normal control rats. The negative charge was significantly lower in the diabetic animals ($p < 0.01$). This change, possibly related to glycosylation, may lead to altered vascular permeability and may be of importance in the vascular pathology of diabetes.

**Key words:** Cationized ferritin, negative charge, endothelium, diabetic rats.

The endothelial cell surface bears a negative surface charge at physiological pH due to heparin-like sulfated mucopolysaccharides as well as neuraminidase sensitive (sialic acids) and resistant anionic groups [1-3]. It has been suggested that this negative charge exerts an electrostatic repulsion which contributes to the non-reactivity of normal endothelium with circulating blood constituent blood cells and platelets [4]. Changes in endothelial cell surface charge may be important in disease processes such as atherosclerosis and thrombosis, which are especially prevalent in diabetes [5].

It is known that in diabetic mice the concentration of heparan sulfate proteoglycan in the basement membrane is 20% of that in normal mice [6]. Glycosoaminoglycans of the renal glomerular basement membrane contain anionic sites that regulate the charge-selective nature of the glomerular filtration barrier [7, 8]. These glycosaminoglycans are reduced in glomeruli of diabetic rats which may account for the defective function in their glomerular filtration barrier [9]. The erythrocyte surface concentration of sialic residues and, therefore, their surface negative charge is also significantly lower in diabetic patients than in healthy subjects [10, 11].

There seem to be no reports on the endothelial surface negative charge of large vessels in diabetes, the usual site of atheroma; therefore, we performed the study presented here.

## Materials and methods

Twenty randomly bred male sabra rats from the Hebrew University breeding colony, weighing 175-225 g were housed in groups of two at 24° C and 12-h light dark cycles. All the rats were fed with Amrod # 935 (Ambar Food Mills, Israel). This diet contained a minimum of 17.3% protein 4% fat, 47.9% carbohydrate and 8.3% fibre. All the rats had free access to water and food.

Ten animals were rendered diabetic by injection of 50 mg/kg of streptozotocin (Sigma Chemical Co., St. Louis, Mo, USA), buffered in sodium citrate, pH 4.0, into the tail vein. Blood glucose levels were measured 48 h after the injection using the glucose oxidase method [12]. All animals with blood glucose levels under 14 mmol/1 ($n=3$) were excluded from the experiment. The control animals received sodium citrate buffer only.

Body weight, blood glucose, and glycosylated haemoglobin ($HbA_{1c}$) of the rats in both groups were measured after 5, 60 and 120 days. Plasma glucose was measured by glucose oxidase/peroxidase as described [12]. Glycosylated haemoglobin ($HbA_{1c}$) was determined by the thiobarbituric acid colorimetric assay [13] (Normal range 5.5-8%).

Negatively charged surface sites were visualized by electron microscopy using positively charged cationized ferritin [3] which, when applied following aldehyde fixation, electrostatically "stains" surface anionic groups in their native distribution [14].

One-hundred twenty days after the induction of diabetes 5 diabetic rats and 5 control healthy rats were anaesthetised with ether. They were then perfused through the left ventricle with a mixture of equal parts of phosphate buffered saline (PBS) and Karnovsky fixative containing 1% formaldehyde and 3% glutaraldehyde in 0.1 N cocodylate buffer pH 7.4. The perfusion was continued for 20 min under pressure of 110 mmHg. Pieces of thoracic aorta, coronary and renal arteries were dissected and immersed for another hour in 2.5% glutaraldehyde in cocodylate buffer (pH 7.4). The samples were washed 3 times, in PBS and incubated in a solution of

cationized ferritin 0.5 mg/ml (Bio-Yeda, Rehovot, Israel) diluted with PBS. The solution was constantly agitated for 30 min. The specimens were then washed 3 times, 10 min each in PBS and postfixed for 1 h at 4°C in 1% osmium tetroxide in 0.1 mol/l cocodylate buffer. After dehydration in graded alcohols the material was embedded in Araldite. Three blocks from each specimen were sectioned at a thickness of 50 nm. These were examined unstained with a Philips 300 electronmicroscope. Four random areas containing the ferritin labelled endothelial cells were photographed in each block at a magnification of × 12,000. The negatives were enlarged in a microfilm projector to × 360,000. A transparent grid with 500 squares (measuring 1 cm each) was superimposed on the screen and the number of ferritin particles in 16-20 squares was counted on each picture. In this way the mean ± SD per square was derived from about 200 squares in each case. Knowing the exact enlargement the number of bound particles per $\mu m^2$ was calculated.

### *Statistical analysis*

The results for each vessel from the rats in each group were averaged (± SD) and compared for significance of the differences with the Student's t-test.

## Results

Animals administrated streptozotocin lost up to 10% of their initial body weight during the five day period following the injection. Thereafter, the diabetic rats gained weight at a slower rate than the control rats.

The mean weight, blood glucose levels, and $HbA_{1c}$ during 120 days of follow up in the 5 diabetic and 5 control rats used in the experiment are listed in Table 1. It can be seen that during this period the streptozotocin-injected rats had severe uncontrolled diabetes.

Transmission electron microscopy of sections stained with uranyl and lead did not reveal any morphological changes in the diabetic arteries in the time periods studied.

In the unstained sections the cationized ferritin particles adhering to the luminal surface of the endothelial cells were well visible and abundant in slightly tangential sections (Figs. 1a and b). The difference in particle density was not visibly remarkable but proved to be significant in all the arteries checked ($p < 0.01$) on morphometric analysis (Table 2).

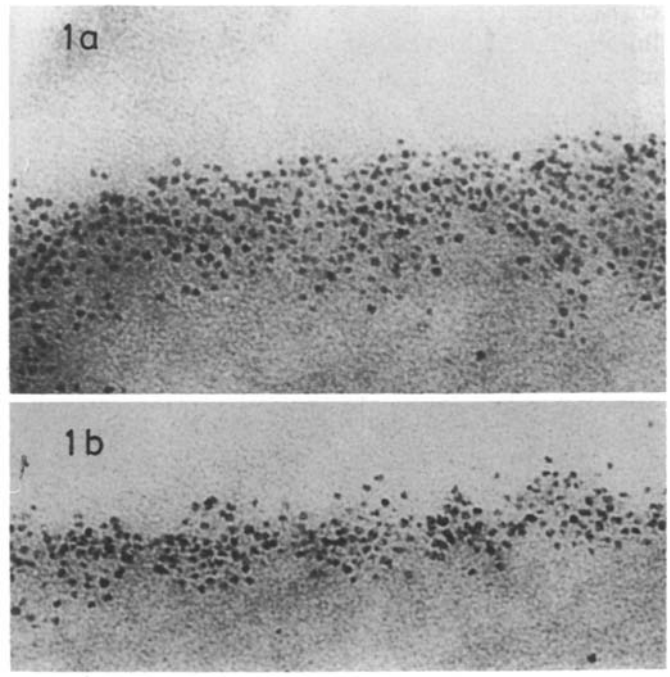


**Fig. 1a and b.** Micrograph of unstained, slightly tangential sections of rat aortas (control, 1a; diabetic, 1b) pretreated (after fixation) with 0.5 mg/ml of cationized ferritin (× 230,000). The number of particles is not obviously different but after counting the diabetic rats proved to have a significantly lower particle density

## Discussion

It is known that the endothelial cell surface bears a negative charge at physiologic pH [3]. This negative surface charge might contribute by electrostatic repulsion to the non-reactivity of the normal endothelial lining with circulating blood cells and platelets [4]. It was found that the uptake of low density lipoprotein (LDL) and fibrinogen from circulating blood was in-

**Table 1.** Weight and glucose homeostasis in diabetic and age matched control rats

| | | Day after streptozotocin injection | | |
|---|---|---|---|---|
| | | 5 | 60 | 120 |
| Weight (g) | Diabetic rats | 204 ± 4.5 | 233 ± 7.9[a] | 282 ± 8.1[a] |
| | Control rats | 210 ± 5.1 | 289 ± 6.6 | 418 ± 12.2 |
| Mean blood glucose (mmol/l) | Diabetic rats | 19.5 ± 1.4 | 22.3 ± 1.0[a] | 21.9 ± 0.9[a] |
| | Control rats | 4.8 ± 0.2 | 5.0 ± 0.2 | 5.5 ± 0.3 |
| $HbA_{1c}$ (%) | Diabetic rats | 4.8 ± 0.3 | | 12.2 ± 0.4[a] |
| | Control rats | 4.7 ± 0.4 | | 4.9 ± 0.4 |

[a] The differences between diabetic and control rats are significant, at $p < 0.001$)

**Table 2.** Number of cationized ferritin particles per $\mu m^2$ in various

| Arteries | Diabetic rats (5) | Control rats (5) |
|---|---|---|
| Aorta | 7690 ± 1310 (5060 – 9300) | 11300 ± 1120 (10000 – 14300) |
| Coronary artery | 8500 ± 1400 (5710 – 9900) | 11720 ± 1600 (9980 – 1480) |
| Renal artery | 9020 ± 2100 (6500 – 11400) | 13510 ± 1520 11900 – 15100) |

Results are mean ± SD from vessels 120 days after streptozotocin or saline injection (the ranges are in brackets). The differences between diabetic and control rats are significant at $p < 0.01$).

creased in arteries which in spite of an intact endothelium had diminished sialic acid moieties on their luminal surfaces [15, 16].

The diabetic state has been shown to cause a decrease in negatively charged molecules such as heparan sulfate and sialic-acid moieties on some cell surfaces. Heparan sulfate is found to be diminished in the glomerular basement membrane [9] and sialyl residues are significantly reduced on the erythrocyte surface of diabetic patients [10, 11]. In diabetic rats and rabbits [17–20] abnormalities in glucosaminoglycan concentration of aorta were also found. However, their distribution in the arterial system of diabetic dogs was uneven and surprisingly increased in some arteries [18].

In the present study we could demonstrate a decreased negative charge in large arteries such as aorta, renal and coronary arteries of diabetic rats.

Diabetes is known to be associated with enhancement and acceleration of atherosclerosis [21]. Since atherosclerosis is associated with the accumulation of LDL-cholesterol and fibrinogen in arterial walls [22], an increased uptake of these substances might occur with reduced negative charge of the endothelium. Therefore, it is likely that the decreased negative charge of the endothelium plays a role in the pathogenesis of diabetic atherosclerosis.

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