

# Correction of Erythrocyte Deformability Defect in ALX-induced Diabetic Rabbits After Treatment With Aminoguanidine

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**To test whether treatment with aminoguanidine, a drug known to prevent cross-linking between glycated proteins, is effective in improving reduced erythrocyte deformability in diabetes, we studied a group ( $n = 6$ ) of ALX-induced long-term ( $12.7 \pm 2.2$  mo of hyperglycemia) diabetic New Zealand white rabbits before and after 20 wk of treatment with aminoguanidine ( $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ). The key findings were as follows: 1) at 12 wk of treatment with aminoguanidine, mean erythrocyte deformability normalized and remained within the normal reference range throughout the period of aminoguanidine administration; 2) 10 wk after discontinuing aminoguanidine in a subset of diabetic rabbits, mean erythrocyte deformability deteriorated by  $\sim 50\%$ ; 3) blood glucose and total GHb did not vary significantly during treatment with aminoguanidine nor after its discontinuation. *Diabetes* 42:590–93, 1993**

**R**BC-df, defined as the passive change in the shape of erythrocytes in response to shear forces, is crucial for normal erythrocyte flow and function in microcirculation (1–6). In diabetes mellitus, marked impairment of RBC-df has been reported in patients with severe micro- and macrovascular disease (7–9). However, Williamson et al. (10) and Rand et al. (11) have reported the absence of changes in RBC-df in diabetes. Nevertheless, previous work by Baba et al. (12) and others (13,14) suggests that diabe-

tes-induced impairment of RBC-df may be the consequence of the formation of AGEs in erythrocytes.

Aminoguanidine is a nucleophilic hydrazine known to block AGE formation by reacting with early glycation products (15,16). Reports of the use of aminoguanidine in animal models of diabetes mellitus for prevention or reversal of diabetic complications have been limited (15–17). The purpose of this study is to assess the effects of aminoguanidine on RBC-df in the ALX-induced diabetic rabbit observed over the long term.

## RESEARCH DESIGN AND METHODS

This investigation was performed on male NZW rabbits that were individually caged and permitted food and water ad libitum. Diabetes was induced in the rabbits by an intravenous injection of ALX (60–120 mg/kg) as described previously (19). After inducement of hyperglycemia, rabbits were left without any further treatment and were permitted continued access to food and water. All animals selected for this study had sustained hyperglycemia ( $\geq 11.1$  mM) for a period of  $\geq 11$  mo ( $12.7 \pm 2.2$  mo). Subsequently, they were given daily doses of aminoguanidine (100 mg/kg) by oral gavage for a period of 20 wk. During treatment with aminoguanidine (20 wk) all diabetic rabbits remained stable. After completion of the course of treatment with aminoguanidine, 3 diabetic rabbits died from infection 2–3 wk after the last dose of aminoguanidine. Observation of the 3 surviving ALX-induced diabetic rabbits continued for an additional 10 wk after aminoguanidine had been withdrawn.

Blood was carefully drawn from a marginal ear vein to avoid stasis at biweekly intervals to measure blood glucose concentration; GHb and RBC-df were analyzed monthly. Blood glucose was measured by glucose oxidase technique on an auto-analyzer (Beckman, Rochester, NY), and GHb was quantitated by affinity chromatography (Isolab, Akron, OH) as described by Abraham et al. (20).

MCV was determined by dividing the hematocrit (mea-

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ALX, alloxan; RBC-df, erythrocyte deformability; AGE, advanced glycation end product; NZW, New Zealand White; MCV, mean corpuscular volume; PBS, phosphate-buffered saline.

sured by centrifugation for 10 min at 12,500 *g*) by the erythrocyte count as determined by Technicon H-1 counter (Technicon, Terrytown, NY). We observed no significant difference in MCV between normal and diabetic rabbits ( $63.3 \pm 5.2$  vs.  $64.4 \pm 4.8$  fL, respectively,  $P = 0.68$ ). Furthermore, no significant change was observed in MCV during treatment with aminoguanidine ( $P = 0.60$ ). This study was conducted in accordance with guidelines set by our laboratory animal research council.

#### Erythrocyte deformability.

**Sample preparation.** After high-speed centrifugation, plasma, buffy coat, and the uppermost layer of erythrocytes were removed. The remaining packed erythrocytes were washed three times with isotonic PBS (NaCl 8.3 g, KCl 0.2 g,  $\text{Na}_2\text{HPO}_4 \cdot 0.7\text{H}_2\text{O}$  0.94 g,  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  0.2 g, and albumin 5 g made up to 1 L (pH = 7.4). Washed erythrocytes, aspirated from the middle of the packed erythrocyte column, were resuspended in isotonic PBS to a final concentration of 4%.

The RBC-df (filterability) was determined by measuring their ability to pass through a 3-micron pore filter (Nuclepore, Pleasanton, CA) under a constant negative pressure of  $-20$  cm  $\text{H}_2\text{O}$ . In each filtration experiment, the time required for 5 ml of the 4% red cell suspension to pass through the filter was determined. A blank value was obtained by recording the time required for 5 ml of plain buffered isotonic saline to pass through the filter. A qualitative measurement of RBC-df is expressed as a deformability (filtration) index, defined as follows: the time required for 5 ml of buffer to filter, divided by the time required for an equal volume of red cell suspension to filter. By this method, the deformability index relates directly to RBC-df (filterability). The deformability index is reported as the average of three repeated tests. A fresh filter (taken from the same batch) was used for each measurement. All measurements were performed at  $37^\circ\text{C}$  and within 1 h of venesection. The filtered erythrocyte suspension was examined by light microscopy to detect evidence of hemolysis or crenation of red cells, neither of which was noted. This method was validated by comparison to prefiltration of whole blood with cotton wool removed from a leukocyte filter (Imugard 500, Terumo, Tokyo, Japan) (21). Deformability experiments were performed in accordance with guidelines set by the International Committee for Standardization in Haematology, Expert Panel on Blood Rheology (22).

Reference means for RBC-df, GHb, and blood glucose concentration were obtained from a group of normal NZW rabbits ( $n = 30$ ), matched for age, sex, and weight.

**Statistical analysis.** Statistical analysis was performed with paired and unpaired, two-tailed Student's *t* test. Statistical significance is indicated by  $P < 0.05$ . Data are expressed as means  $\pm$  SD.

## RESULTS

Before treatment with aminoguanidine, ALX-induced diabetic rabbits had a highly significant elevation in mean blood glucose concentration and GHb compared with normal rabbits ( $16.2 \pm 4.3$  mM,  $P < 0.001$  and  $5.91 \pm 1.31\%$ ,  $P < 0.001$ , respectively). This level of hy-

TABLE 1

Comparison of blood glucose and GHb in normal and ALX-induced diabetic rabbits, before and after treatment with aminoguanidine

	<i>n</i>	Blood glucose (mM)	GHb (%)
Normal rabbits	30	$6.4 \pm 0.8$	$3.15 \pm 0.39$
Diabetic rabbits	6		
Pretreatment		$16.2 \pm 4.3^{\dagger}$	$5.91 \pm 1.31^{\dagger}$
Treatment after 20 wk		$17.2 \pm 5.1^*$	$6.55 \pm 0.84^*$

Data are means  $\pm$  SD.

\* $P < 0.001$  compared with normal rabbits.

$\dagger$ No significant difference in mean blood glucose and GHb before and during aminoguanidine administration.

perglycemia and excessive GHb did not change significantly ( $P > 0.09$  and  $P > 0.07$ , respectively) throughout the period of aminoguanidine administration (Table 1).

**Treatment with aminoguanidine.** Although blood glucose and GHb were stable in ALX-induced diabetic rabbits throughout 20 wk of aminoguanidine treatment, after initiation of aminoguanidine treatment progressive and significant improvement was noted in RBC-df (i.e.,  $P < 0.01$  at 4 wk,  $P < 0.05$  at 8 wk, and  $P < 0.01$  at 12–20 wk (Fig. 1A). By 12 wk of aminoguanidine treatment, mean RBC-df in ALX-induced diabetic rabbits improved to a level equivalent to that measured in normal rabbits ( $0.25 \pm 0.1$  vs.  $0.26 \pm 0.04$ ,  $P = 0.53$ ). Normalization of mean RBC-df in treated ALX-induced diabetic rabbits was sustained throughout the period in which aminoguanidine was administered (Fig. 1A).

**Discontinuation of aminoguanidine.** After 20 wk of treatment, aminoguanidine was withdrawn. Three rabbits

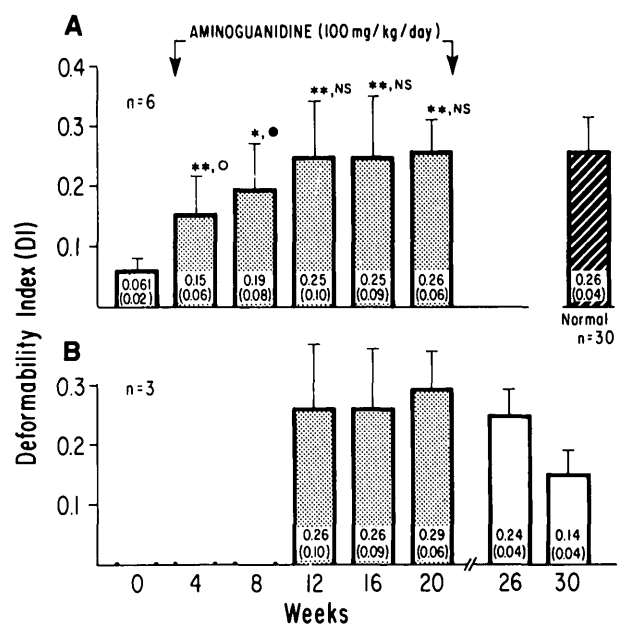


FIG. 1. **A:** Improvement in RBC deformability in ALX-induced diabetic rabbits after treatment with aminoguanidine. \* $P < 0.05$ ; \*\* $P < 0.01$ , compared with pretreatment value.  $\circ P < 0.0001$ ,  $\bullet P = 0.02$ , NS, compared with mean normal deformability index. **B:** Deterioration in RBC deformability of  $\sim 50\%$  10 wk after discontinuing aminoguanidine.

developed infections and therefore were killed. Observations of the 3 surviving healthy rabbits were continued for 10 wk. A marked reduction in mean RBC-df (~50%) was noted by 10 wk after aminoguanidine discontinuation (Fig. 1B), while the changes in mean blood glucose and GHb were negligible (4.0 and 0.1%, respectively) (data not shown).

## DISCUSSION

No consensus has been reached as to the mechanisms underlying reduced RBC-df observed in diabetes. Previously suggested mechanisms include hypoinsulinemia (23), increased internal viscosity (24), increased erythrocyte sorbitol concentration (25), and increased erythrocyte membrane rigidity cause by glycation of erythrocyte membranes (26). Still other investigators have failed to detect any alteration in RBC-df in diabetic patients (27,28).

In this study, orally administered aminoguanidine, a drug previously shown to prevent AGE formation in the glomerular basement membrane (16) and arterial wall connective tissues (15) in diabetic rats, was found to correct impaired RBC-df in ALX-induced diabetic rabbits. Ten weeks after discontinuation of aminoguanidine, a return of significant RBC-df impairment was noted.

The process of nonenzymatic glycation of proteins begins within hours of protein exposure to glucose with formation of a Schiff base. Afterwards, the Schiff base undergoes chemical rearrangement to form a more stable Amadori product. To this point, the process of nonenzymatic glycation is reversible, and the quantity of Schiff base and Amadori products formed are directly related to the time averaged ambient glucose concentration (29).

However, in proteins that have relatively long turnover times, such as albumin, collagen (29), lens crystallins (30), and HbA and erythrocyte membrane proteins (13), nonenzymatic glycation continues with Amadori products undergoing a series of rearrangements to form AGE and glucose-derived protein cross-links.

Formation of AGEs are nonreversible. Once formed they remain throughout the life span of the protein involved (29). Because protein turnover in HbA and the erythrocyte membrane is negligible (13), once AGE formation is initiated, we hypothesize that a steady accumulation occurs throughout the life span of the erythrocyte in the ALX-induced diabetic rabbit. In agreement with this thesis are studies by Schleicher et al. (13) and others (31) noting that poorly controlled diabetic patients, compared with normal volunteers, have a two-fold increase in erythrocyte membrane and HbA glucose specifically bound in ketoamine linkage to lysine residues.

In vitro studies conducted by Lewis and Harding (18) and Edelstein and Brownlee (32) support the premise that aminoguanidine blocks AGE formation by binding covalently to the glucose-derived intermediary Amadori product.

If the presumed accumulated AGE in the erythrocytes of ALX-induced diabetic rabbits is irreversible, then

treatment with the AGE inhibitor, aminoguanidine, should have no effect on relatively rigid circulating mature erythrocytes. Nevertheless, despite persistence of hyperglycemia and excessive GHb, progressive improvement in RBC-df was observed over a period of 12 wk, followed by complete correction of the RBC-df defect during aminoguanidine treatment (Fig. 1A). These data suggest that aminoguanidine may protect against formation of AGEs and stable cross-linkages between glycated erythrocyte proteins in young erythrocytes whereas mature, AGE-replete, and less deformable cells are removed from the circulation by the reticulum endothelial system.

After discontinuation of aminoguanidine, no significant increase was seen in RBC rigidity for several weeks. However, 10 wk after discontinuation of aminoguanidine, severe reduction in RBC-df was again manifested.

On average, 50% of the rabbit erythrocyte population is replaced at 10 wk by new cells; the proportion of surviving cells declines rapidly thereafter (33,34). Based on a previous study (29), we hypothesize that although ambient hyperglycemia persists, deterioration in RBC-df is dependent on the variables of the rate of AGE formation (relative to the ambient glucose concentration) and erythrocyte survival in rabbits (18,32–34).

It is conceivable that aminoguanidine might have a direct hemorheological effect on diabetic erythrocytes by influencing internal viscosity (3), corpuscular volume (35), the erythrocyte membrane cholesterol/phospholipid molar ratio (1,14), or membrane fatty acid composition (36). The action of aminoguanidine, however, has not been associated with any of these mechanisms. Other reported mechanisms of action by which aminoguanidine might affect RBC-df are by effecting membrane lipid peroxidation (37) as suggested by Picard et al. (38) or by inhibition of the inducible isoform of nitric oxide synthase (39). Recent studies by Kumari et al. (40) and Geisen (41) suggest that aminoguanidine inhibits aldose reductase activity and thereby limits erythrocyte sorbitol accumulation. However, previous work by Maeda et al. (9) suggests that sorbitol erythrocyte concentration does not correlate with RBC-df in the ALX-induced rat model of diabetes. They concluded that diabetes-induced RBC-df impairment was caused by increased stiffness of the erythrocyte membrane.

Although this study involved a small sample size, the results, nonetheless, suggest the possibility of total reversal of a diabetes-associated complication by treatment with the AGE inhibitor, aminoguanidine, in an intact animal model of diabetes mellitus.

Studies aimed at expanding these observations and investigating the efficacy of aminoguanidine in the treatment and prevention of other diabetic complications are in progress.

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