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Morroniside protects cultured human umbilical vein endothelial cells from damage by high ambient glucose¹

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KEY WORDS morroniside; umbilical veins; endothelial cells; diabetic angiopathies

ABSTRACT

AIM: To determine whether morroniside, a compound in *Cornus officinalis* Sieb *et* Zucc can prevent cultured human umbilical vein endothelial cells (HUVEC) from damage by high ambient glucose. **METHODS:** HUVEC was incubated in glucose, 5 or 30 mmol/L, either alone or in the presence of morroniside (final concentration 100, 10, and 1 μ mol/L, respectively) for 48 h. The proliferation of HUVEC was quantified by MTT method; its cycle was analyzed by flow cytometry; morphological change was observed with fluorescence microscopy. **RESULTS:** Survival of HUVEC cultured in high ambient glucose was significantly decreased when compared to that in normal concentration of glucose ($P < 0.01$). High ambient glucose also lowered the rate of cells entering into S-phase, along with severe morphological damage. With the intervention of morroniside (final concentration 100 and 10 μ mol/L), the cell survival was significantly recovered ($P < 0.01$, $P < 0.05$, respectively), accompanied with increased S-phase rate and less extent of morphological damage. **CONCLUSION:** Morroniside protected HUVEC against high ambient glucose induced injury, which suggested that morroniside could exert a beneficial effect on preventing diabetic angiopathies.

INTRODUCTION

Vascular endothelial cells and vascular muscle cells are the two major components of the vessel wall. Because endothelial cells are within the intimal layer of the vessel wall, abnormal levels of glucose in blood may trigger initial damages and induce functional and structural alterations, and therefore may represent a prominent factor among the metabolic abnormalities potentially responsible for such diabetic complications. High

ambient glucose concentrations has been reported to be toxic *in vitro* for endothelial cells, as represented by retarded cell proliferation^[1], disturbed cell cycle^[2], increased DNA damage^[3], and slightly accelerated cell death^[2]. Such pathological changes may play important role in causing the microvascular lesions that are both common and prominent in diabetes. Thus prevention of hyperglycemia-triggered endothelial cell lesions may have important implications for pharmacological attempts at preventing diabetes-associated microvascular complications.

The Maillard or browning reaction between reducing sugars and proteins is proposed to be involved in the pathophysiological procedure of aging and diabetic complications^[4]. Our early experiments showed *Cornus officinalis* Sieb *et* Zucc could inhibit non-enzymatic

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glycation of protein *in vitro*^[5], and morroniside (Fig 1), one compound isolated from it, has the similar effect. It suggests that morroniside may be an active component of *Cornus* in improving the chronic complications of diabetes. So in this study we further investigated the effects of morroniside on the change of morphology, cell cycle, and proliferation of endothelial cell cultured in high glucose medium.

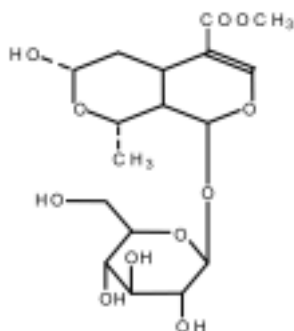


Fig 1. Chemical structure of morroniside.

MATERIALS AND METHODS

Materials Medium RPMI-1640 and fetal calf serum (FCS) were purchased from GIBCO (USA); Typsin, Me₂SO from MERCK (USA); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from FLUKA (Switzerland); Flow cytometer was from FACSCALIBUR BECTON DICKINSON.

Cell culture Human umbilical vein endothelial cells (HUVEC) were isolated by 0.25 % trypsin treatment of umbilical vein. Acquired cells (1×10^8 /L) were cultured in RPMI-1640 supplemented with 20 % FCS, penicillin (100 kU/L), streptomycin sulfate (100 kU/L), and this solution served as basal culture medium, in 24-96 well plastic plates as previously described. When grown to about 80 % confluence, the monolayer cells were washed with RPMI-1640 and then the basal culture medium supplemented with glucose 5 or 30 mmol/L was fed, either alone or in the presence of morroniside or aminoguanidine for 48 h.

Cell survival determination Proliferation of the HUVEC cultured in basal culture medium supplemented with glucose of different concentration, and with or without the interference of different drugs was determined by MTT method. Cells after 80 % confluence reached with a further 48-h culture in 96 well plates were washed again, then RPMI-1640 180 μ L and MTT (5 g/L) 20 μ L were added. After incubating for 4 h at 37 °C, the supernatant was carefully aspirated out, and

then 200 μ L Me₂SO was added to dissolve formazan. Then the 96 well microplate was transferred to the microplate reader for measuring the absorption at 490 nm. A value measured was positively related to the cell survival.

Morphological study Cultured cells of all groups were stained by mixed dye (euchrysin:EB=1:1) for studying the morphological change of stained cells under fluorescence microscope.

Cell cycle analysis For observing the influence of high concentration of glucose on cell cycle and the interventional effect of drugs, the cycle of cultured HUVEC was analyzed with the flow cytometer. Seeded cells cultured for 48 h in two different concentration of glucose (5 mmol/L, 30 mmol/L, respectively) and glucose 30 mmol/L with morroniside or aminoguanidine (final concentration 0.1 mmol/L) were trypsinized from culture plates. Cells were then counted and diluted to about 1×10^8 /L, fixed in 70 % ethanol, stained with propidium iodide (10 mg/L, 30 min at 4 °C) and processed in a flow cytometer, followed by estimating the distribution of cell cycle.

RESULTS

Cell viability and proliferation Cells viability and proliferation assessed by MTT method showed that high concentration of glucose (30 mmol/L) could retard proliferation and decrease cell viability as compared to cells grown in lower glucose medium (5 mmol/L) ($P < 0.01$). However, cells grown in high glucose with morroniside or aminoguanidine (final concentration 100, 10, and 1 μ mol/L, respectively) showed a significant improvement ($P < 0.05$, $P < 0.01$, respectively), but no significant differences were found within different concentration of the drugs. Cells cultured in glucose 5 mmol/L with morroniside did not show any change of the proliferation, which could support that morroniside had no toxic effect on HUVEC cultured *in vitro* (Tab 1).

Morphological analysis By staining with mixed dye, morphological change was observed and photographed under fluorescence microscopy. Cells cultured in normal concentration of glucose assumed cobblestone shape which was typical for the endothelial cells cultured *in vitro*. In contrast, it turned to be asteroid under high ambient glucose, along with decreased cell counting, pyknosis nuclei, and unclear membrane boundary. However cells pretreated with morroniside or aminoguanidine presented lighter lesions than those in glucose 30 mmol/L alone (Fig 2).

Tab 1. The effect of morroniside on HUVEC proliferation in 5, 30 mmol/L glucose medium. $n=3$. Mean \pm SD. ^c $P<0.01$ vs glucose 5 mmol/L. ^e $P<0.05$, ^f $P<0.01$ vs glucose 30 mmol/L.

Additions	Concentration/ $\mu\text{mol}\cdot\text{L}^{-1}$	$A_{490\text{ nm}}$
Glucose 5 mmol/L	-	0.216 \pm 0.019
Glucose 5 mmol/L+morrniside	100	0.215 \pm 0.013
	10	0.208 \pm 0.016
Glucose 30 mmol/L	-	0.149 \pm 0.003 ^c
Glucose 30 mmol/L+morrniside	100	0.187 \pm 0.008 ^f
	10	0.173 \pm 0.010 ^e
	1	0.158 \pm 0.012
Glucose 30 mmol/L+aminoguanidine	100	0.183 \pm 0.010 ^f
	10	0.178 \pm 0.011 ^e
	1	0.154 \pm 0.006

Cell cycle Cell cycle analyses with flow cytometer further proved that cell proliferation rate decreased in the culture medium supplemented with glucose 30 mmol/L, but it could be partially prevented or reversed by morroniside or aminoguanidine. We could simply

come to such a conclusion from the distribution ratio of S-phase of each group (Fig 3).

DISCUSSION

Endothelium dysfunction was proved to mediate the initiation and development of many kinds of diabetic angiopathies. Contraction and relaxation, the basic function of endothelium, was found in disorder partially caused by diminished NO release^[6]. Since endothelial cells were the intimal layer of the vessel wall, it was proposed to be the prominent target of high ambient glucose and other toxic factors under diabetes state. In the light of previous research achievements, we made an insight into finding the effective compound preventing endothelial cells from damage by high ambient glucose.

In an experimental diabetes model induced by streptozotocin, we previously found that the main components of *Cornus*, iridoid total glycoside, could apparently ameliorate endothelium injury. Since morroniside was the most abundant compound in iridoid total glycoside, we proposed that it should be beneficial in protecting endothelial cells from damage induced by high

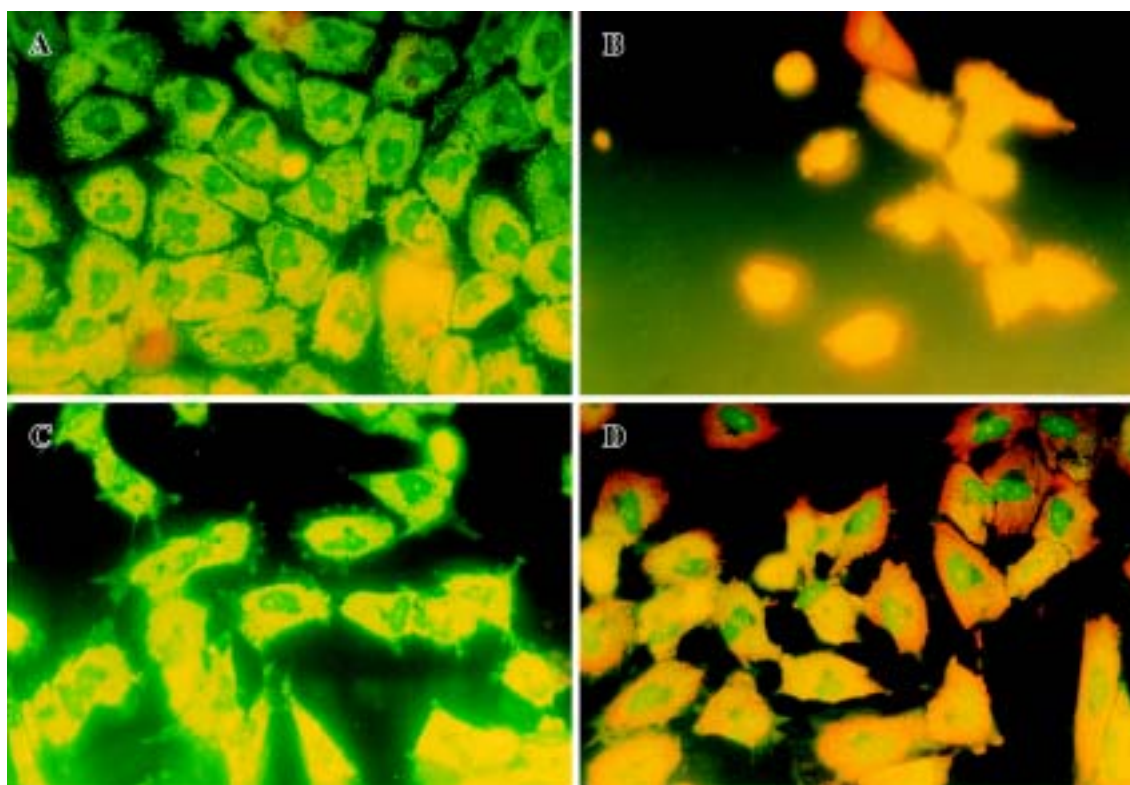


Fig 2. High glucose induced lesion in HUVEC (fluorescence staining, $\times 400$). A: glucose 5 mmol/L; B: glucose 30 mmol/L; C: glucose 30 mmol/L with morroniside; D: glucose 30 mmol/L with aminoguanidine.

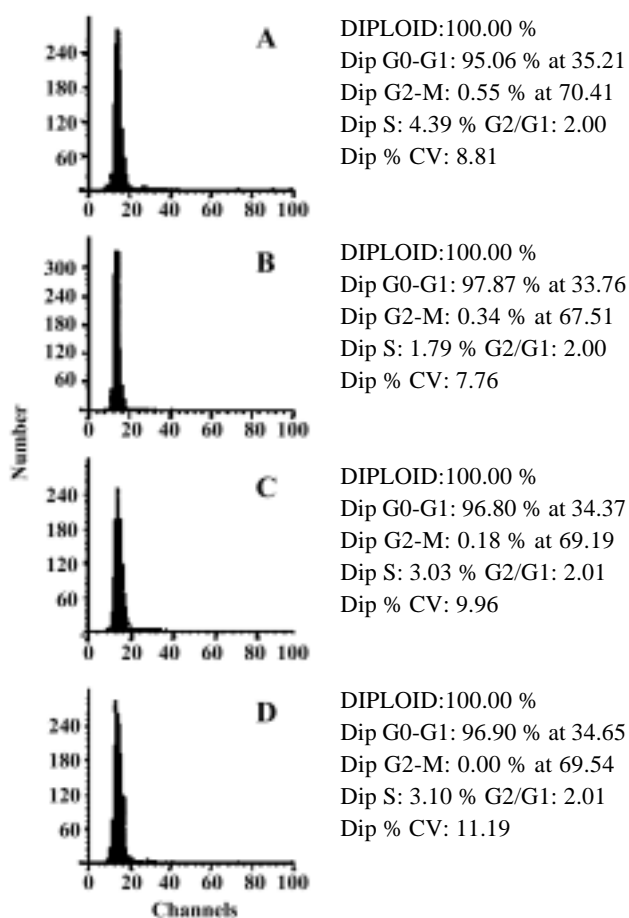


Fig 3. Cell cycle phase distributions of HUVEC. **A:** glucose 5 mmol/L; **B:** glucose 30 mmol/L; **C:** glucose 30 mmol/L with morroniside; **D:** glucose 30 mmol/L with aminoguanidine.

ambient glucose.

High ambient glucose caused HUVEC death, which was proved by the MTT detecting result and morphological evidence. The cell proliferation judged from S-phase distribution was also inhibited by high ambient glucose. Both aminoguanidine and morroniside ameliorated HUVEC damage by the morphological evidence and cell proliferation analysis. Hyperglycemia may exert toxic effect on endothelium through direct path such as oxidative stress or indirect path by the formation of advanced glycation products (AGE). Aminoguanidine was reported to be a specific inhibitor of AGE formation, but its role of inhibiting the formation of AGE could not

explain the present result because of the short incubating time. Since oxidative stress was involved in high ambient glucose toxicity and aminoguanidine was previously reported to be effective in inhibiting oxidative stress induced by high glucose^[7,8], it was believed that aminoguanidine may act on protecting HUVEC from damaging partially through preventing oxidative stress in the present study. In previous study, we found that iridoid total glycoside could increase the superoxide dismutase activity in the experimental diabetes model, thus morroniside protecting HUVEC from damage may also act through preventing oxidative stress induced by high ambient glucose. Although its detailed mechanisms remain unknown, it is evident that morroniside may be beneficial for the therapy of diabetic angiopathies.

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