

# Electron microscopic analysis of glucose-induced endothelial damage in primary culture: Possible mechanism and prevention

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**Summary.** We previously reported that high glucose treated cultured endothelial cells (ECs) showed intercellular gaps by transmission electron microscopy (TEM). These gaps were abrogated with insulin and/or heparin treatment. Our aims were to assess the severity of injury in ECs treated with high glucose for variable duration, and to further study the protective effects of insulin and/or heparin. Cells were also treated with L-buthionine sulfoximine (BSO), a glutathione inhibitor, to help understand the mechanism of high glucose injury. Primary porcine ECs were treated with high glucose (30 mM) for 2, 6 or 10 days; and glucose plus insulin (1 U/ml), glucose plus heparin (5 µg/ml), glucose plus insulin plus heparin for 6 days. ECs were treated with BSO (0.001-0.05 mM) for 2 days. Pellets from trypsinized cells were processed for TEM. High glucose treatment revealed apoptosis or necrosis showing variable cell size, abnormal nuclei, condensation of nuclear chromatin, few mitochondria, cell membrane disruption and needle-shaped structures. Changes increased with duration of exposure. In high glucose plus heparin or insulin treated cultures at least one-half of the cells appeared normal. Most ECs were intact when treated with high glucose plus insulin plus heparin. BSO treatment showed dose-dependent changes with low doses showing apoptosis whereas higher doses revealed necrosis similar to high glucose treatment for 6 or 10 days. High glucose-induced EC injury increased with duration of exposure. These data demonstrate that high glucose injury resembles that of BSO treatment, suggesting that glutathione depletion may be involved in EC injury. Insulin and/or heparin protect against high glucose-induced injury.

**Key words:** Endothelial cells, High glucose, Electron microscopy, Heparin, Insulin

## Introduction

Hyperglycemia is the central initiating factor for diabetic microvascular disease (Ruderman et al., 1992). Levels of glycosylated hemoglobin are directly correlated with incidence of cardiovascular disease even in individuals not yet diagnosed with diabetes (Khaw et al., 2004). Diabetes impairs endothelial function through several proposed mechanisms including hyperglycemia, hyperinsulinemia and oxidative stress (Williams et al., 1996; Hurst and Lee, 2003). Our cell culture studies have demonstrated that treatment of endothelial cells (ECs) with a high concentration of D-glucose (30 mM or 540 mg/dl) causes severe morphological damage to ECs (Mandal et al., 2000).

Apoptosis and necrosis describe two related forms of cell injury. Morphologically, apoptosis is characterized by a decrease in cell size, condensation of nuclear chromatin, and break-up of the plasma membrane with appearance of apoptotic bodies ("budding appearance"), which contain fragments of condensed chromatin and organelles such as mitochondria. Necrosis gives rise to cell swelling, fragmentation of nuclei, break-up of the plasma membrane, and ultimately cell rupture. Cytosolic contents leak from the necrotic cell causing injury and inflammation in the surrounding tissue (Lieberthal and Levine, 1996a). A wide variety of cytotoxic stimuli including ischemia, oxidants, ethanol, which cause cell necrosis, are also capable of producing apoptosis when given at a lower dose (Gottlieb et al., 1994; Thompson, 1995; Lieberthal et al., 1996b). The cytotoxic stimulus used in this study is a high concentration of D-glucose. Some investigators have reported that excess glucose is toxic and is the cause of many of the complications of diabetes (Bouldin et al., 2002). Hyperglycemia also

triggers apoptosis in cultured ECs (Baumgartner-Parzer et al., 1995).

Previous studies demonstrated that treatment of cultured ECs with insulin, heparin and more so by a combination of insulin and heparin prevent the formation of intercellular gaps (Mandal et al., 2000) and the decrease in number of viable cells per culture (Han et al., 2005) when cells were treated with high glucose. Heparin has also been shown to reduce free radical-induced injury in cultured endothelium (Hiebert and Liu, 1990). However, there is much that is not known about EC injury in the presence of high glucose and the possible protective effects of heparin and insulin.

This study was designed to accomplish the following three objectives: 1) To examine the severity of EC injury in relation to duration of exposure to a high concentration of D-glucose in medium; 2) To further examine if addition of insulin, heparin, or a combination of both is effective in preventing glucose-induced EC injury; 3) To elucidate the mechanism of high glucose-induced EC injury. To that effect, ECs were treated with buthionine sulfoximine (BSO), a potent inhibitor of cellular glutathione (GSH).

## **Materials and methods**

### *Culture of endothelial cells*

Porcine aortic ECs were isolated and cultured according to previously described methods (Gotlieb and Spector, 1981). Aorta segments were trimmed and the lumen rinsed in calcium and magnesium-free Dulbecco's phosphate-buffered saline (CMF-DPBS) before being filled with collagenase solution (Type IV, Sigma, St. Louis, Missouri, USA; 1 mg/ml in CMF-DPBS). The collagenase solution was removed after 5 minutes of incubation at room temperature. The aorta was then filled with M199 (GIBCO BRL, Grand Island, New York, USA), supplemented with 5% fetal bovine serum (FBS, GIBCO), 10 U/ml penicillin (Sigma, St. Louis, Missouri, USA), and 10 µg/ml streptomycin (Sigma, St. Louis, Missouri, USA). The media in the vessel was used to gently rinse the luminal surface and was then transferred to 60 mm culture dishes. Cells were incubated at 37°C with 5% CO<sub>2</sub>/95% air in a humidified environment. Endothelial cells were identified by the presence of von Willebrand's factor in initial cultures and their morphology, cobblestone-like flattened cells growing in a monolayer. Before the first passage, any smooth muscle cells or fibroblasts were visually identified using an inverted microscope and were destroyed by aspirating them from the bottom of the dish. Cells were subcultured up to passage 5. Cells were grown in 35 mm dishes for experimental purposes.

### *Addition of test agents*

Glucose (D-glucose, BDH, Inc., Toronto, Canada) was first prepared as a stock solution of 3 M in

phosphate-buffered saline (PBS). Then, 10 µl of the stock solution was added directly to 990 µl of culture medium in the dish to give a final concentration of 30 mM or 540 mg/dl. An aliquot (10 µl) of insulin (Humulin N, 100 U/ml) was added directly to 990 µl of culture medium for a final concentration of 1 µg/ml. Bovine lung heparin (Lot No. 722EH, 151 USP U/mg Upjohn Pharmaceuticals, Kalamazoo, Michigan, USA) was prepared in PBS, 1 mg/ml. Then 5 µl of the stock solution was added to culture medium to give a final concentration of 5 µg/ml.

To determine if high glucose causes cell injury, glucose (30 mM) was added every second day in fresh medium for 2, 6 or 10 days. Glucose addition to cultures every second day was based on our previous studies demonstrating that glucose remains in medium at the same concentration for at least 48 hours (unpublished observations).

To determine if heparin and/or insulin modify EC injury due to high glucose, heparin (5 µg/ml), and/or insulin (1 µg/ml) were added to cultures along with glucose every second day in fresh medium up to 6 days. Cells were harvested 24 hours after the last addition of test agents for transmission electron microscopy (TEM).

BSO was prepared in CMF DPBS and added to medium at concentrations ranging from 0.001 to 0.05 mM. Cells were harvested 48 hours later.

### *Cell harvest*

Cultures were washed twice with CMF DPBS, followed by addition of 300 µl of 0.025 % trypsin for 2.5 min when cells were rounded and detached from the dish. Medium (M 199, 500 µl without serum) was added and the cell suspension was transferred to centrifuge tubes and centrifuged at 3,000 rpm for 5 min. The supernatant was discarded, medium was added to the cell pellet and centrifuged again. The supernatant was removed and 3% glutaraldehyde in 0.1 M cacodylate buffer was added to the pellet and refrigerated at 4°C for later processing.

### *Transmission electron microscopy study of cultured endothelial cells*

Cell pellets stored in glutaraldehyde in 0.1 M cacodylate buffer were centrifuged at 3000 rpm and the supernatant removed. The cell pellet was mixed with 1% agarose (stirring with a stick) and then spun down again. The samples were removed from the tubes and excess agar cut away. The agar pieces containing pelleted material were placed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer and stored at 4°C overnight. The samples were rinsed three times in 0.1 M sodium cacodylate buffer over a 24-h period and post-fixed in 1% osmium tetroxide in 1.25% sodium bicarbonate buffer (pH 7.4) for 1 hour at room temperature. Samples were then dehydrated in a graded ethanol series and en block stained with uranyl acetate. The samples were

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infiltrated with epon/araldite, blocked, and polymerized at 55°C (blocks). From the blocks, ultra-thin sections were cut at 70 nm thickness using a Leica Ultracut UCT ultramicrotome and placed on 200 mesh bare cooper grids. Sections were stained with 0.5% aqueous lead citrate followed by 4% aqueous uranyl acetate. Sections were examined in a LEO EM910 TEM operated at 80 kV. Images were made at various magnifications, using a Gatan 792 BioScan 1024x1024 digital camera.

Attention was focused on the following specific features of EC changes in the TEM study. For each treatment group, ten or more cells were observed in each field at low magnification. Specific changes observed were cell size, presence or absence of nucleus, size and shape of the nucleus, condensation of nuclear chromatin, presence or absence of nucleoli, preponderance and appearance of mitochondria, appearance of endoplasmic reticulum, preponderance and size of vacuolation, appearance of abnormal intrinsic structures, integrity of cellular membrane such as break with extrusion of intrinsic contents and budding appearance. Abnormal intrinsic needle shaped structures were also identified.

### Measurement of cell size

Area of ECs were measured utilizing NIH-image software (Image-J). Measurement was done only in those cells in which a clear nuclear profile and a continuous cell boundary were identified. The images were spatially calibrated utilizing the internal micron marker recorded on each image. Profiles of cells were outlined by hand using the mouse cursor and results are presented as mean  $\pm$  standard error in square micrometers.

## Results

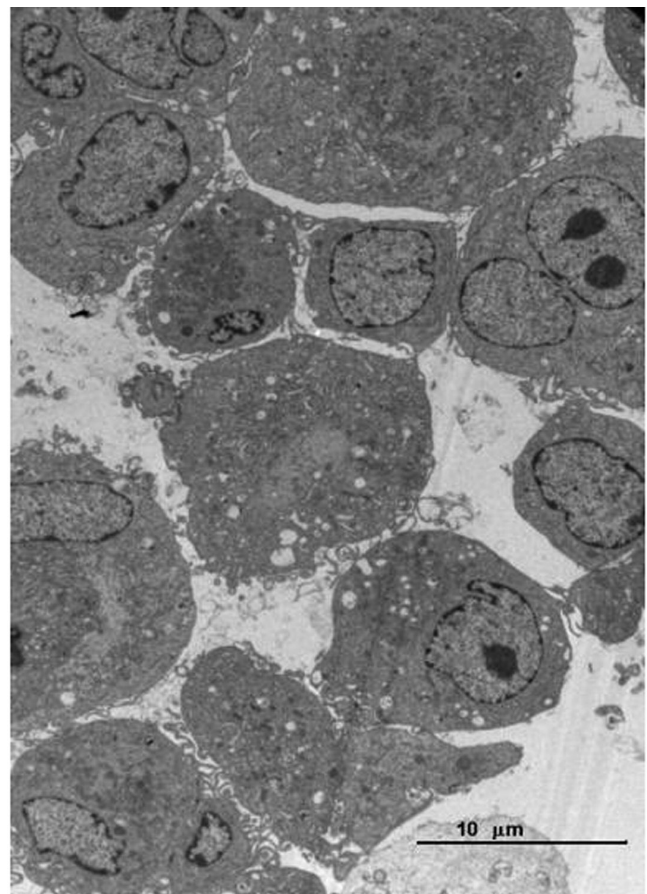
### Control cultures

To establish the appearance of control ECs, porcine aortic ECs in control medium for 2, 6, or 10 days were observed by TEM. ECs in control medium revealed normal appearing nuclei, the presence of mitochondria and endoplasmic reticulum, and an intact plasma membrane (Fig. 1).

### Effect of high glucose treatment for 2, 6 or 10 days

To determine the effects that duration of high glucose treatment had on ECs, cultures were treated with 30 mM glucose for 2, 6, or 10 days. After two days of high glucose treatment, ECs showed a conspicuous feathery appearance of the plasma membrane, absence of nuclei in 50 percent of the cells, with condensation of nuclear chromatin in the other 50 percent of cells, and scarcity of mitochondria in a vast majority of the cells (Fig. 2). These findings are consistent with apoptosis. After 6 days of high glucose treatment (30 mM), ECs were different in size and shape when compared to

controls (Fig. 3). In fact, cell area in glucose treated cultures ( $89.1 \pm 3.0 \mu\text{m}^2$ ) was significantly less than that in control endothelial cells ( $102.7 \pm 2.9 \mu\text{m}^2$ ) when 137 and 134 cells were measured in glucose and control groups respectively (probability  $< 0.0005$ , one-tailed t-test). Two third to three-fourths of the total number of the cells had inconspicuous nuclei while one-quarter of the cells lacked nuclei; some cells were fused intracellularly. Some cells showed prominent intracellular needle-shaped structures, while other cells showed dissolution of the cell membrane and extrusion of intracellular structures with a budding appearance. A few cells appeared necrotic and were beyond identification (Fig. 3). After 10 days of high glucose treatment (30 mM), ECs showed a pronounced appearance of intracellular needle shaped structures (Figs. 4 and 5), dissolution of cytosolic contents, loss of nuclear chromatin and nuclei and fragmentation of the cell membrane (Fig. 4). These changes are consistent with necrosis.



**Fig. 1.** Control cultured porcine aortic endothelial cells. A total of 13 cells are seen in the field. Almost all cells show high contrast suggesting they are healthy cells. Most cells contain one or more nuclei with normal nuclear chromatin, and mitochondria. The cell membrane is intact. A few vacuoles are present in some cells.



### Effects of insulin and heparin on cultures treated with high glucose for 6 days

To determine if insulin and heparin protected high glucose treated ECs, cells were treated with insulin and/or heparin as well as high glucose for 6 days. This treatment duration was selected since considerable injury was present following 6 days of treatment with high glucose alone. After 6 days of high glucose and insulin treatment, almost one-half of the ECs appeared as control ECs (Fig. 6) with less apparent damage than with high glucose treatment alone (Fig. 3). Ten to twenty percent of ECs showed a budding appearance.

EC changes after 6 days of high glucose and heparin treatment are similar to those after 6 days of high glucose and insulin treatment. However, unlike insulin treatment, some ECs showed condensation of nuclear chromatin and increased vacuolation (Fig. 7).

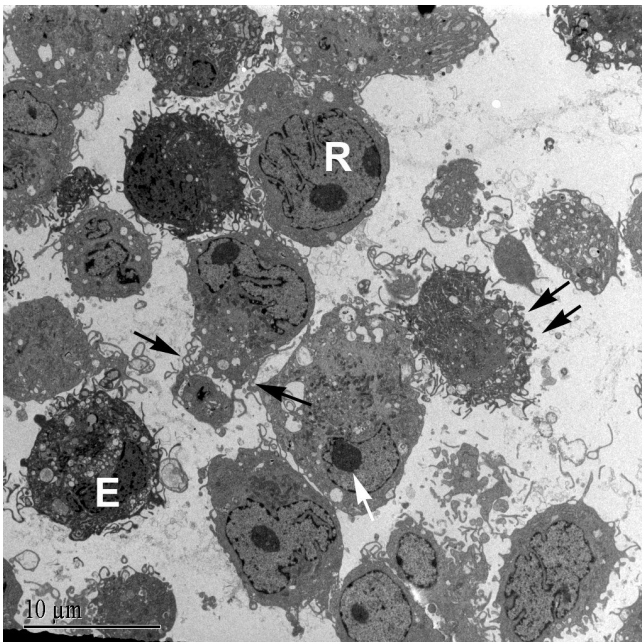
A vast majority of ECs (90-100 percent) after 6 days of treatment with high glucose plus insulin and heparin appeared normal (Figs. 8,9), showing intact cell membrane, intact nuclei, plentiful normal appearing mitochondria, minimal vacuolation and occasional

needle-shaped structures. There is little evidence of apoptosis and no evidence of necrosis.

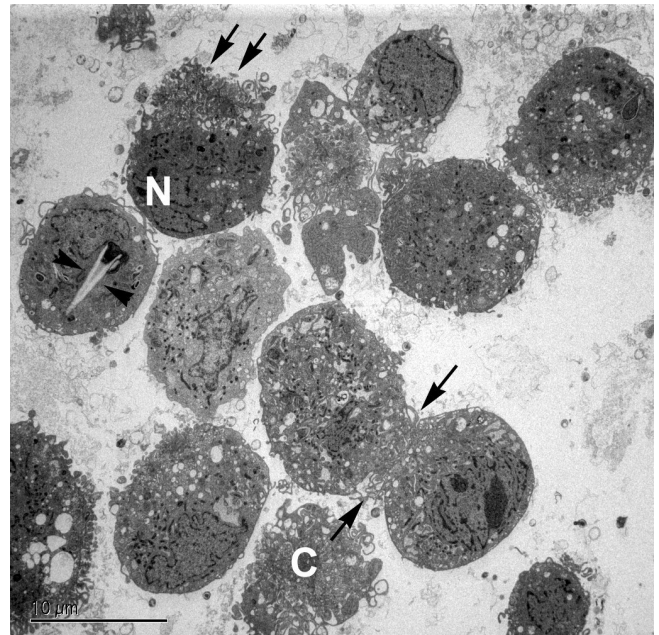
### Buthionine sulfoximine (BSO)-treated endothelial cells

To determine if BSO treated cells showed similar damage to high glucose treatment, BSO was added to EC cultures in three different doses. ECs treated with a low dose of BSO (0.005 mM) showed predominantly a budding appearance. Fifty percent of the cells showed absence of nuclei, condensation of nuclear chromatin and scarcity or conglomeration of mitochondria (Fig. 10). These changes are consistent with apoptosis.

A vast majority of ECs treated with high dose BSO (0.01 mM) showed pronounced condensation of nuclear chromatin, absence of nuclei and mitochondria and dissolution of plasma membrane with extrusion of cytosolic contents in the form of a budding appearance (Fig. 11). These changes are consistent with necrosis. ECs treated with a still higher dose of BSO (0.05 mM) showed complete dissolution of the cellular constituents and are difficult to interpret.

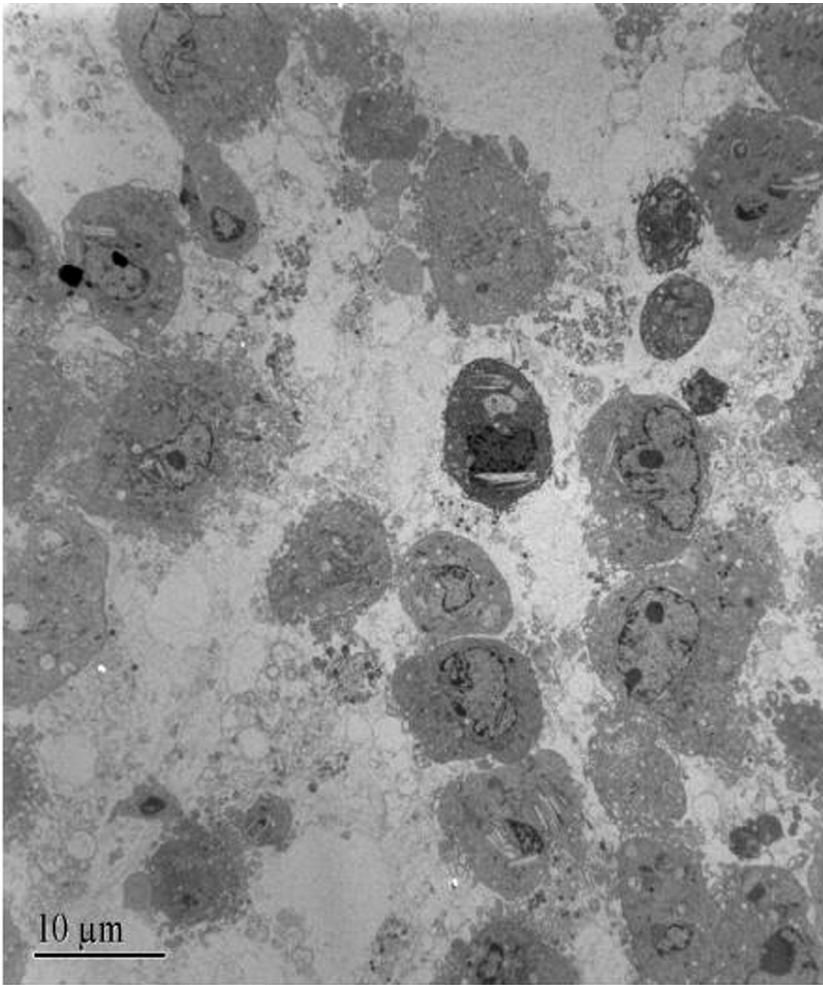


**Fig. 2.** Cultured endothelial cells (ECs) treated with a high concentration of glucose (30 mM) for 2 days show disruption of intercellular connections. The twenty-one ECs shown are variable in size and one-half of the total number of ECs lack nuclei. Where nuclei are present they are variable in size, ragged (R) or eccentric (E) in appearance (arrow). Condensation of nuclear chromatin is evident. A quarter of the ECs show a feathery appearance of the plasma membrane (double arrows). Occasional ECs show disruption of the plasma membrane with extrusion of intracellular contents (between arrows). Most of the cells have no mitochondria, endoplasmic reticulum or centrioles.

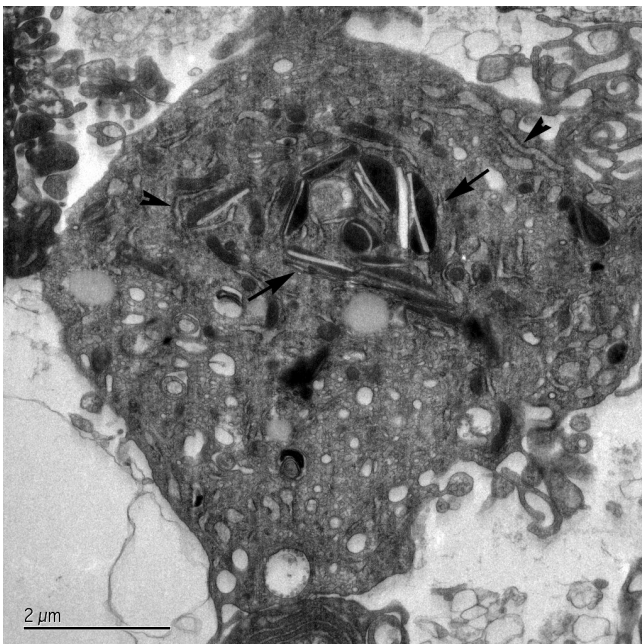


**Fig. 3.** Endothelial cells (ECs) after 6 days of treatment with a high concentration of glucose (30 mM). Thirteen cells are shown. These cells show inconspicuous nuclei (N) in three-quarters of the total cells and absence of nuclei in one-quarter of the ECs. Most of the ECs have few or no mitochondria, endoplasmic reticulum or centrioles and increased numbers of vacuoles. About one-half of the ECs show disruption of the plasma membrane resulting in fusion (between arrows) and a budding appearance (double arrows). Twenty percent of the ECs are severely damaged with a total loss of cellular constituents (C). A conspicuous feature is a needle shaped structures in one of the cells (between arrowheads).

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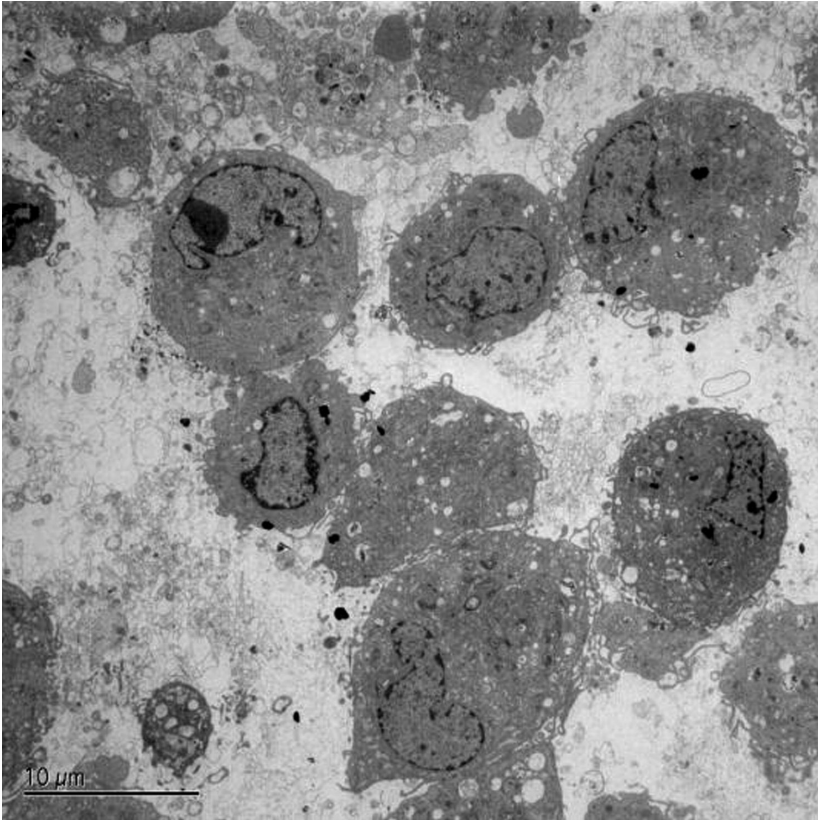
**Fig. 4.** Endothelial cells (ECs) after 10 days of treatment with a high concentration of glucose (30 mM). At least twenty-three cells or cell fragments are shown. One-half of the cells lack a nucleus. Six cells show the presence of needle shaped structures within the cell. Many of the cells are severely damaged with a breakdown of the plasma membrane and loss of cellular contents.



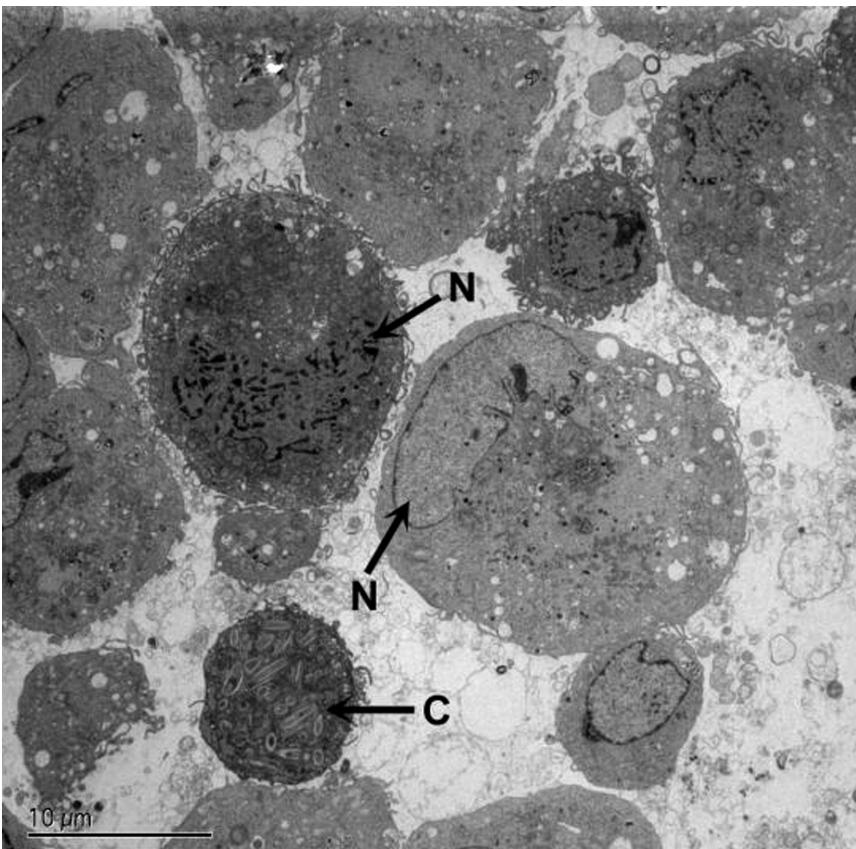
**Fig. 5.** An endothelial cell after 10 days of treatment with a high concentration of glucose (30 mM). The cell shows many needle shaped structures (arrows), many vacuoles, a few dilated endoplasmic reticulum (at arrowheads) and a few mitochondria.



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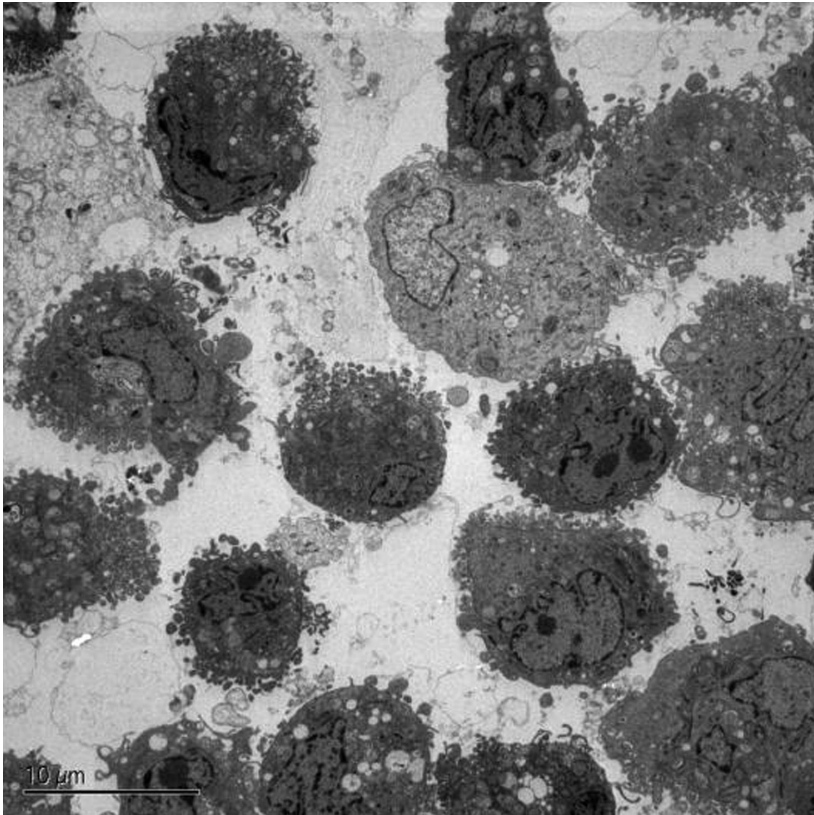


**Fig. 6.** Endothelial cells after 6 days of treatment with a high concentration of glucose (30 mM) and insulin (1 U/ml). Seven whole cells and three partial cells are seen in the field. One out of seven whole cells lack nuclei. Other cells contain one or more nuclei with normal nuclear chromatin. Most cells contain abundant mitochondria. A few vacuoles are seen.

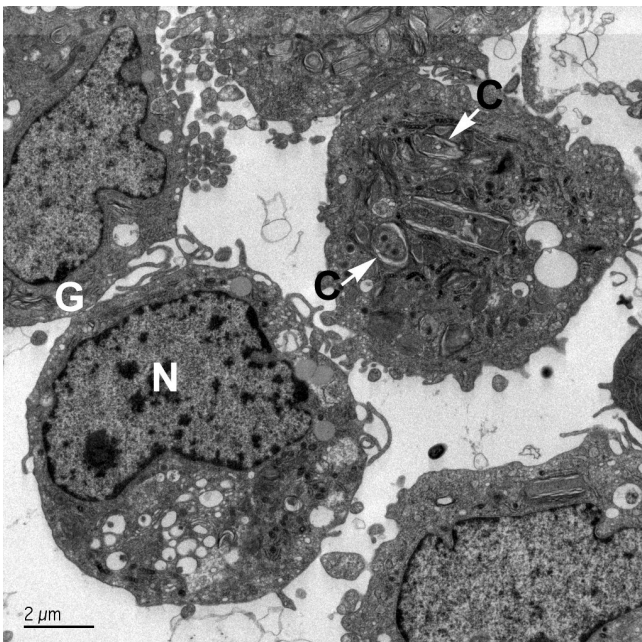


**Fig. 7.** Endothelial cells (ECs) after 6 days of treatment with high-glucose (30 mM) and heparin (5 μg/ml). Ten cells are seen in the field. Slight variability in cell size is seen. The cells are highly contrasting suggesting they are healthy. Nuclei (N) is slightly ragged and pushed to the periphery in two cells, however the cell membrane is intact. One cell contains crystalline structures (C).

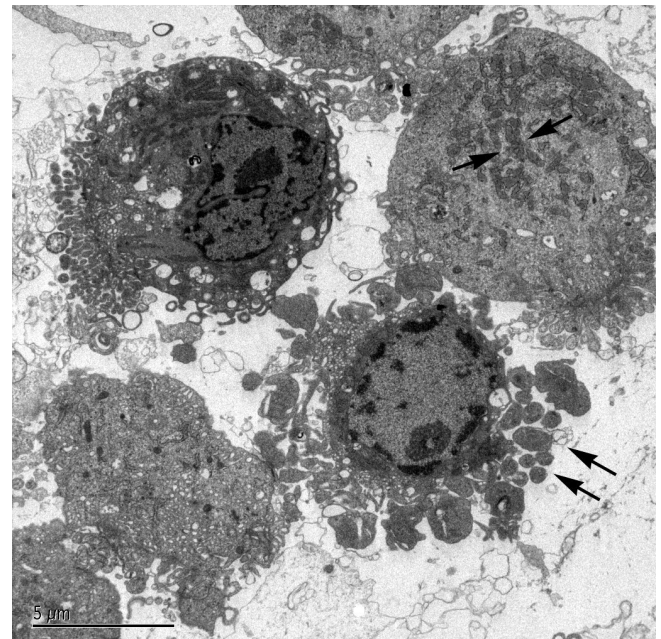
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**Fig. 8.** Endothelial cells (ECs) after 6 days of treatment with high-glucose (30 mM) plus insulin (1 U/ml) and heparin (5 µg/ml). Fifteen endothelial cells are seen, thirteen with visible nuclei. The majority of the cells are intact.

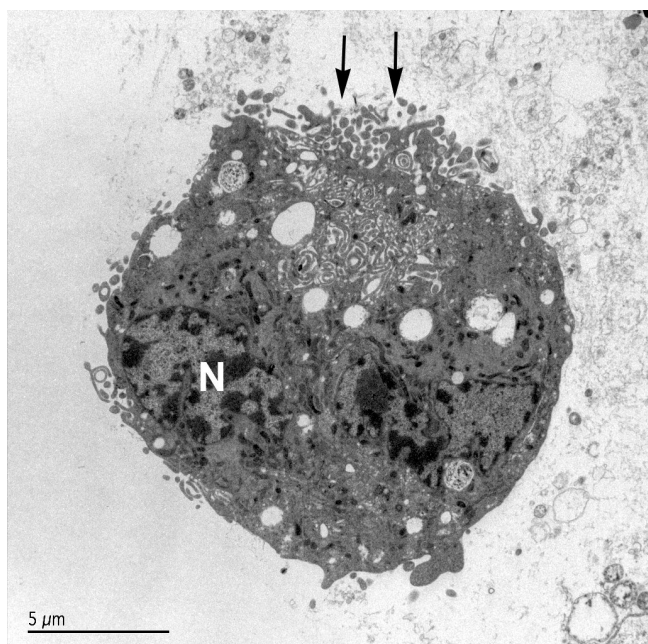


**Fig. 9.** Four endothelial cells (ECs) after 6 days of treatment with high-glucose (30 mM) plus insulin (1 U/ml) and heparin (5 µg/ml). Three ECs are near normal characterized by normal nuclei (N), conspicuous Golgi complex (G) and centriole (C, arrow). One EC shows no nuclei.



**Fig. 10.** Four endothelial cells (ECs) after 2 days of treatment with a low dose of buthionine sulfoximine (0.005 mM). A conspicuous budding appearance (arrows) is shown in two of the four ECs, however nuclei are intact. Two ECs have no nuclei. In one EC, mitochondria show conglomeration (between arrows). Another EC, shows few cell constituents.





**Fig. 11.** An endothelial cell after 2 days of treatment with a high dose of buthionine sulfoximine (0.01 mM). Severe changes are shown characterized by fragmentation of nuclei (N), condensation of nuclear chromatin, and disruption of the plasma membrane with extrusion of cytosolic contents (arrows). Few mitochondria, no endoplasmic reticulum or centriole are seen. Intracellular needle shaped structures are not found.

#### *Intracellular needle-shaped structures*

Intracellular needle-shaped structures were found in a small number of high glucose alone treated ECs for 2 days but in a large number of high glucose alone-treated ECs for 6 days and in a more pronounced fashion after 10 days (Figs. 3,4). These structures were not found in control ECs and found rarely in ECs treated with high glucose and insulin, high glucose and heparin, alone or in combination. These structures were not found in BSO-treated ECs.

#### **Discussion**

This study shows that cultured ECs treated with a high concentration (30 mM) of glucose causes variable injury to the ECs. After 2 days of treatment with glucose alone, EC changes are mainly consistent with apoptosis (Fig. 2), whereas glucose treatment for 6 or 10 days, EC changes are mainly consistent with necrosis (Figs. 3, 4). We have observed that longer exposure to high glucose causes more severe cell injury than shorter exposure to high glucose. Heparin, insulin or a combination of insulin and heparin prevent glucose-induced EC injury when ECs are treated for 6 days. However, salutary effect is most pronounced with insulin and heparin in combination (Fig. 7) confirming our previous study (Mandal et al., 2000).

Our study in cultured ECs examined cell size, presence or absence of nucleus, as well as other parameters indicating cell injury such as the appearance of mitochondria, endoplasmic reticulum, degree of vacuolation, integrity of the cell membrane and abnormal intrinsic structures. Although we acknowledge that cell size and appearance of the nucleus is dependant on the orientation of the cell during sectioning, cells showing no nucleus and diminished size were consistently seen in high glucose alone treated cultures. We have thus recorded these observations here since they may reflect true changes associated with high glucose treated cells. Intracellular needle-shaped structures, observed in 6 and 10 day high-glucose treated cultures, are considered important and likely play a role in glucose-induced EC injury.

A high glucose concentration represents a toxic stimulus to ECs. Although the dose of glucose is the same in all experiments, duration of exposure can be equated to the dose of toxic stimulation. Thus, exposure of ECs to a high-glucose concentration for a shorter duration (2 days) can be considered as a low-dose toxic stimulation, which gives rise to apoptosis (Fig. 2); whereas exposure of ECs to the same concentration of glucose for a longer duration (6 or 10 days) can be considered as a high-dose toxic stimulation, which leads to necrosis (Figs. 3, 4).

The exact mechanism of glucose-induced EC injury has not been elucidated. Our current observations suggest that generation of reactive oxygen species is a plausible mechanism of glucose-induced EC injury. GSH is the most abundant antioxidant in cells and plays a major role in the defense against oxidative stress-induced EC injury (Hall, 1999; Lu, 1999). Previous studies reveal that removal of GSH from different types of cells results in a time-dependent production of apoptosis as well as necrosis (Li et al., 1999). In our study, we have shown that BSO, an inhibitor of GSH, causes apoptosis at a lower dose but necrosis at a higher dose and severe necrosis at a still higher dose. Since EC injury patterns after 2 and 6 or 10 days of glucose treatment resemble those of low dose and high dose of BSO treatments respectively; suppression of GSH by a high concentration of glucose resulting in generation of reactive oxygen species can be considered tantalizing evidence of glucose-induced EC injury.

A previous study showed that exposure of human renal tubule cells to 30 mM glucose for 48 hours resulted in a significant increase in apoptosis compared with 5 mM glucose (Verzola et al., 2002). Antioxidants, such as taurine, glutathione, or N-acetylcysteine attenuated the high glucose-induced apoptosis, suggesting that reactive oxygen species are generated by high glucose (Verzola et al., 2002) supporting the hypothesis suggested above. Therefore, mitigation of glucose-induced EC injury by treatment with insulin, heparin or a combination of insulin and heparin, shown in our present and past studies, could be due to enhancement of antioxidant mechanisms or protection of glutathione levels and



consequently suppression of reactive oxygen species. Indeed our previous study showed that heparin and related polyanions protect ECs from free radical injury (Hiebert and Liu, 1990).

Although the nature of intracellular needle-shaped structures has not been identified, their presence, only in glucose-treated cells, suggests that they are likely to be glucose. Addition of insulin or heparin to high glucose-treated cultured ECs does not bring glucose levels back to normal (unpublished observations) thus it is unlikely that prevention of EC injury by insulin, with or without heparin, is simply due to reduction of the glucose levels in the medium. However, rare needle-shaped structures in insulin or insulin and heparin treated groups, suggest that insulin with or without heparin somehow abrogates the formation of needle-shaped structures inside the ECs and, in doing so, prevents EC injury.

It is understood that our work is limited to cell culture studies and that high glucose levels are likely cyclical. Nevertheless, the findings of these *in vitro* studies are germane to the understanding of the mechanism and prevention of diabetic microvascular complications. EC injury increases with duration of high glucose-treatment, suggesting that diabetic subjects with longer duration of hyperglycemia are more likely to develop severe vascular lesions including glomerular capillary lesions than those with shorter duration of hyperglycemia. Additionally, this work suggests that insulin treatment is most likely to prevent endothelial injury.

Further, EC death and loss of functional endothelium are associated with a reduced thromboresistance and reduced vasodilation of the vessel wall and may be an important risk factor to enhance the likelihood of vascular complications in diabetes (Risso et al., 2001). In our study, when a combination of insulin and heparin was added to glucose in culture medium, fewer than 10 percent of ECs were found to be abnormal compared to nearly 50 percent of ECs showing some abnormalities when either treatment was added alone. Given that scenario, it is prudent to state that heparin is additive to insulin in the prevention of EC injury. However, the mechanism of the additive effect of heparin with insulin is unclear. This mechanism may be related to heparin's ability to protect cultured ECs from free radical injury as previously reported (Hiebert and Liu, 1990). Heparin also upregulates nitric oxide and inhibits endothelin production in cultured cells and thus is protective of endothelium (Yokokawa et al., 1994). Heparin has also been reported to be protective of the renal glomerulus in *in vivo* studies (Mandal et al., 1978).

Finally, our current and previous cell culture studies provide compelling evidence of the detrimental effect of high glucose on ECs, and a near complete protection by a combination of insulin and heparin. The exact mechanism of protection by insulin or heparin of ECs, is not fully elucidated. Nevertheless, the results of our *in vitro* studies provide a novel incentive for further investigation to determine the exact mechanism and

prevention of diabetic vascular lesions by a combination of insulin and heparin.

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