*Original Article*

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# Effect of Glyoxal on Cytotoxicity in Human Umbilical Vein Endothelial Cell Culture

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**Abstract**

*Objectives:* Glyoxal is used as a biocide and disinfecting agent in pharmacy and dye production is released to the environment, air and water with emissions. Furthermore, glyoxal is produced endogenously in non–enzyme-mediated pathways in intracellular metabolism which can be detected frequently in fermented food and beverages. For this purpose, in this study we investigated the effect of glyoxal on cell viability and proliferation in human umbilical vein endothelial cell (HUVEC) line *in vitro*.

*Method:* In our study, cell culture was performed using HUVEC. Cell proliferation and viability were evaluated by spectrophotometry with tetrazolium salt (MTT) by applying different doses of glyoxal to HUVECs. Data were analyzed by SPSS 21.0 program.

*Results:* Glyoxal in doses of 320, 16, 0.8 $\mu$M significantly decreased cell proliferation when compared with control group ($p < 0.05$). The doses of $4 \times 10^{-2}$, $2 \times 10^{-3}$, $1 \times 10^{-4}$, $5 \times 10^{-5}$, $2 \times 10^{-5}$, $1 \times 10^{-6}$, $6 \times 10^{-7}$, $3 \times 10^{-7}$ $\mu$M, were found to increase cell proliferation significantly ($p < 0.05$).

*Conclusion:* According to our study, when compared to human plasma glyoxal level (0.1 – 1 $\mu$M), doses of glyoxal over 1 $\mu$M showed cytotoxic effect on cells, whereas doses below 1 $\mu$M increased the proliferation of endothelial cells. It is accepted as an important intermediate in the formation of advanced glycation end products (AGEs) by binding to the amino groups, nucleotides and lipids of the glyoxal proteins entering the cell. AGEs modification may activate cell proliferation pathways at low doses by altering protein function and influencing intracellular signaling pathways, while at high doses it may affect repair mechanisms and apoptotic processes, leading to cell damage.

**Keywords:** Cytotoxicity; Endothelium; Glyoxal; Proliferation.

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## Introduction

Diabetes mellitus (DM) develops as a result of absolute deficiency of insulin, impaired synthesis and secretion, and/or insufficient effect at the receptor and postreceptor level and characterized by impaired carbohydrate, lipid and protein metabolism and chronic hyperglycemia as such a metabolic disease.[1] Fasting and postprandial hyperglycemia are responsible for acute, early and late complications of diabetes that affect all body organs and systems. Increased incidance in both

type diabetes (Type 1 and Type 2) has a crucial role in morbidity and mortality all over the world. Hyperglycemia is associated with microvascular diseases such as nephropathy, retinopathy, neuropathy as well as macrovascular disease like coronary heart disease, stroke and peripheral artery disease.[2] Chronic hyperglycemia plays a key role in the onset of vascular complications in diabetes.[3] The mechanism underlying the vascular pathology caused by high glucose levels in diabetes is explained by five different pathways, including the polyol pathway leading to sorbitol and fructose accumulation; the pathway for advanced glycation end products (AGEs) formation; protein kinase C (PKC); the hexosamine pathway; and RAS activation but which way is dominant is still controversial.[4-6] In circulation, hypergycemia during diabetes leads to an increased concentration of α-oxoaldehydes.[7] Alpha-oxoaldehydes are highly reactive carbonyles and cause structural and functional failures by binding proteins, nucleic acids and other proteins that leads to several complications such as vascular diorders, atherosclerosis, hypertension and Alzheimer's disease.[8] In literature, complications associated with diabetes attributed to glycation of glucose with proteins. It was shown that α-oxoaldehydes like glyoxal, methylglyoxal and 3-deoxyglucosone are made up of proteins during glucose-mediated glycation.[9]

AGEs are the molecules those formed by non-enzymatic glycation of proteins, lipids or nucleic acids.[10,11] This process is ascribed to Louis Camille Maillard (1878–1936) and so called as "Maillard reaction". Non-enzymatically formed sugars by covalent binding of proteins to aldehyde and ketone groups, reduced to free amino acids leads to labile Schiff bases.[12] After that, more stable ketoamins called "Amadori products" are formed by irreversible reactions (e.g. HbA1c).[13] Amadori products are transformed into highly stable AGE compounds by further structural changes through oxidation, dehydration and degradation.[14] The reaction leads to AGE formation may take weeks or years and can effect long-lived substrates especially like collogen.[15] Increased substrates, high temperature and increaqsed oxidative stress can reduce AGE formation to several hours.[16] Hormones, enzymes, amino acids or lipids are affected short-lived products.[17–21] Although AGEs are known to be by-products of hyperglycemia, foods cooked at high temperatures[22] and cigarette smoke[23] are important exogeneous source of AGEs. Especially, dietary AGEs are important *in vivo* source for circulation.[24–26] AGEs in circulation is thought to play key role in pathogenesis of micro- and macrovascular diseases arised in hyperglycemia.[27–29] Acculumation of AGEs are proportional to age under physiological conditions[30] and this accumulation can be increased by DM, renal failure, cardiovascular disease, Alzheimer's disease, romatoid arthritis; etc.[25,32–34] Inflammation and oxidative stress,[35] increased glycation of LDL and HDL,[36] activation of proinflammatory iNOS[37] and decreaed NO[38] can be the part of underlying mechanism in detrimental effects of AGEs on the vascular system. Another mechanism involves increased monocyte and macrophage migration as well as increased production of cytokines, such as IGF-1 or PDGF, which modify the proliferation of vascular smooth muscle cells.[39,40] Effects caused by AGEs can be classified by impact areas or receptor dependencies. In vasculature, AGEs cause damage in vascular cells by[41] changing intracellular proteins which regulates gene transcription.[18] AGE precursors leave cells by diffusion and change extracellular matrix proteins firstly, then, by changing signaling between matrix and cells leads to cellular disfunction.[42] AGEs and their precursors can change circulatory proteins leading to functional change. AGEs-modified proteins in circulation binds to AGE receptors and activate them thus changes the production of inflammatory cytokines and growth factors that promote cell and tissue damage.[24,39] In fact, activation of certain receptors for AGEs (e.g. RAGE) promote inflammatory response, mainly by nuclear factor kB (NFkB), apoptosis, prothrombotic activity, expression of adhesion molecules, and activation of oxidative stress[43-45] in addition, AGE-RAGE interaction can activate iNOS. iNOS is mainly found in inflammatory cells, regulated by inflammatory cytokines, and can be produced by toxic concentrations of NO when stimulated by NFkB-induced oxidative stress.[46,47] Subsequent reaction with the oxygen radical generates an excessively reactive metabolite called peroxynitrite. Peroxynitrite interacts with protein and DNA to cause nitrative stress, DNA damage, further NFkB, caspase-3 activation, and vascular cell apoptosis.[48] Animal models and *in vitro* studies support that AGEs affect different cells in atherosclerosis such as endothelial cells,[39,49,50] platelets[51] monocytes/macrophages[52] or vascular smooth muscle cells[53] and thus are associated with vascular disease. Interaction of AGEs with endothelial cells expressing RAGE decreases endothelial barrier function with increased permeability and subendothelial lipid entry. AGE-RAGE interaction triggers the expression of adhesion molecules such as VCAM-1[54] and also promotes the formation of Foam cells by promoting

transendothelial migration of monocytes.[54–56] Xu et al. showed that during incubation of human umbilical vein endothelial cells (HUVEC) with AGE-modified albumin without high glucose medium, NOS endothelial isoform was supressed by concentration and time dependent.[57] Naser et al. showed that 5 min incubation with AGE in bovine aortic endothelial cells causes depletion of intracellular $Ca^{2+}$ stores.[58] When angiogenesis is impaired in the peripheral vascular system, it contributes to delayed wound healing, exacerbation of ischemia in the peripheral extremities, and exacerbates cardiac morbidity with reduced collateral vascular development.[59] The mechanism lying under impared angiogenesis induced by AGEs is explained by Liu et al.[60] Researchers have shown that using *in vitro* endothelial cells and mouse aortas, methylglioxaline (highly reactive AGE precursor) decreases endothelial angiogenesis through RAGE-mediated, peroxynitrite-dependent, and autophagy-induced vascular endothelial growth factor receptor 2 (VEGFR2) degredation. VEGFR2 is the main receptor that causes vasodilatation, endothelial cell migration and proliferation in vascular endothelial growth factor signaling.[60,61] Extracellular AGEs are responsible for impaired cell proliferation and adhesion and inhibition of growth by cross-linking with extracellular proteins.[62] The major extracellular proteins targeted for glycation are long-lasting matrix proteins such as collagen Types I, III and IV, elastin, cartilage proteoglycan aggregate, and short-lived plasma proteins such as ApoB, LDL, albumin, immunoglobulins. Glycation of intracellular and extracellular proteins changes protein function and disrupts cellular metabolism. RAGE is an AGE receptor that accumulates in diabetes, the aging process and neurodegenerative processes in Alzheimer's. RAGE is abundant during embryonic development and is thought to be involved in cell migration.[63] Binding of RAGE with different ligands results in activation of the transcription factor NFkB.[64] Binding proteins for RAGE or AGE moieties have been shown in monocyte/macrophages, endothelial cells, pericides, T-lymphocytes, mesengial cells and Type I pneumocytes and osteoblast-like cells.[65–68]

## Materials and Methods

We used Glyoxal 40% solution (Cat No: 820610-1L, Merck) in our study. Glyoxal is used as a biocide and disinfecting agent in pharmacy and paint production, is released to the environment, air and water with emissions. Furthermore, glyoxal is produced endogenously in non–enzyme-mediated pathways in intracellular metabolism which can be detected frequently in fermented food and beverages. In human blood plasma the concentration of glyoxal is 0.1–1 $\mu$mol/L, of course it has higher levels in patients with diabetes or renal failure. Glyoxal, is considered to be an important intermediate in the formation of advanced glycation end products (AGEs) by attacking amino groups of proteins, nucleotides, and lipids. Skin irritation, allergic skin reaction and serious eye irritation are the effects of glyoxal; and causing genetic defects also. In animal models, the acute toxicity of glyoxal is low to moderate, depending on the actual concentration of glyoxal in the tested product. In rats, for 40% glyoxal, the LC50 for a single 4-h inhalation of aerosol is 2440 mg/m$^3$, the oral $LD_{50}$ value ranges from 3000 to 9000 mg/kg body weight (with higher sensitivity in females), and dermal $LD_{50}$ values are >2000 mg/kg body weight. Exposure with inhalation causes local irritations of the eyes and respiratory organs. After oral exposure to glyoxal, macroscopic observations include irritations of the gastrointestinal tract and congestion in the gastrointestinal tract, lung, kidney, and adrenal glands are seen.[70]

We used HUVECs in our study. When the cells were examined with inverted microscope, it was seen that the cells spread and multiplied by holding on the bottom of the culture chamber. The cultured cell medium was changed every 72 hours. The culture of confluent exhibited a typical endothelial cell characteristic of cobblestone morphology with large and dark nuclei inside the cell (Fig. 1). Appearance of HUVECs in light microscopy (X400). Cell proliferation and viability were evaluated by MTT method by culturing from HUVEC cell line. The MTT method is a frequently used cell proliferation test based on the measurement of metabolic activity used to assess cell proliferation, viability and cytotoxicity. With this method, the proportion of living cells in the cell population can be determined quantitatively. When the dehydrogenase enzymes in the mitochondria of intact cells reduce the MTT stain and break down the tetrazolium ring a color change occurs. This change can be evaluated by this method. This reaction depends on the activity of succinate dehydrogenase, a fragile mitochondrial enzyme. As a result of the degradation of tetrazolium ring in living vascular smooth muscle cells, the pale yellow MTT dye becomes dark blue-purple formazone product.[71–73] The method is mainly based on the principle of colorimetric measurement of the absorbance value of the color change resulting from the conversion

of proliferating cells to violet formazone using yellow water soluble tetrazolium with increased dehydrogenase activity. Since proliferating cells are more metabolically active than non-proliferating cells, not only cell viability and cytotoxicity but also cell activation and proliferation are evaluated with this method. The tetrazolium salt is converted to a water-insoluble formazone ring in the presence of a living cell and formazone crystals dissolve and turn purple by the addition of detergent-active solutions such as DMSO. In the proliferation study, HUVECs were seeded in a 96-well culture dish with $5 \times 10^3$ – $10^4$ cells per well. After cells were incubated for 24 hours in an incubator containing 5% $CO_2$ – 95% air at 37°C, glyoxal at doses of 320, 16, 0.8, $4 \times 10^{-2}$, $2 \times 10^{-3}$, $1 \times 10^{-4}$, $5 \times 10^{-5}$, $2 \times 10^{-5}$, $1 \times 10^{-6}$, $6 \times 10^{-7}$, $3 \times 10^{-7}$ $\mu$M were added to each well and incubated again for 24 hours. At the end of the incubation, the solution on the cells was removed. Cells were incubated for 4 hours by adding 100 $\mu$L of MTT (5 mg/mL MTT) solution to each well. At the end of this period, chemicals were removed from the wells by pipette and 100 $\mu$L of DMSO was added. The cells were incubated for 20–30 minutes to dissolve the formazone crystals. It was observed that the pale yellow MTT dye formed as a result of the degradation of the tetrazolium ring turned into a dark blue-violet formazone product. DMSO was used to dissolve MTT and reduction products. After incubation of ninety-six-well cell culture dish with DMSO for 20–30 minutes, color change was evaluated as absorbance at 570 – 630 nm wavelength with spectrophotometer or plate reader. The mean absorbance of each group was calculated by subtracting the absorbance value of each well at 570 nm wavelength from the absorbance value at 630 nm (A570–A630 nm).

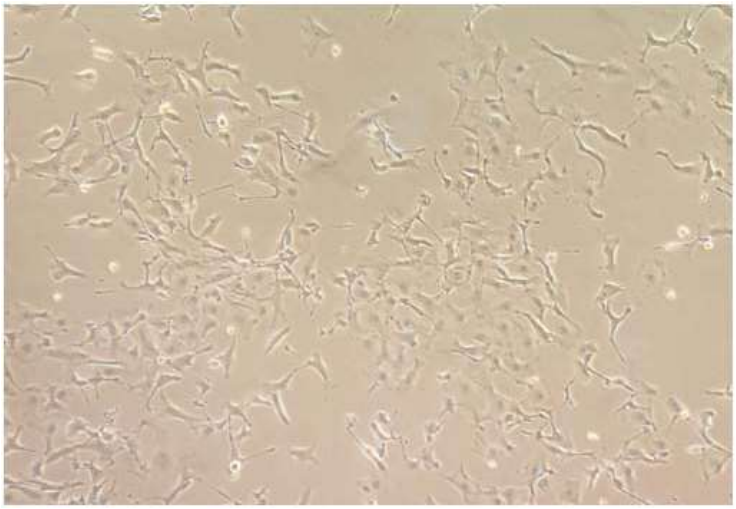

**Fig. 1.** Appearance of HUVECs in invert microscopy (X400).

### *Statistical analysis of data*

SPSS 21.0 package program was used for statistical analysis of the data. Data were expressed as mean ± standard error. In the statistical analysis, after examining whether the variances were homogeneously distributed or not, the parametric test ANOVA was used. In case of difference, post hoc Dunnett test was performed to show which groups were significant among the groups. The statistical significance level was taken as 0.05 in all tests.

## Results

The HUVECs in the cell culture dishes were spread on the surface of 75 $cm^2$ flask and the proliferation and cell morphology were evaluated with an inverted microscope (Fig. 1). Appearance of HUVECs in light microscopy (X400).

Glyoxal at doses of 320, 16, 0.8 $\mu$M significantly decreased cell proliferation compared to control group ($p = 0.000$, $p = 0.000$, $p = 0.000$, repectively). Doses of $4 \times 10^{-2}$, $2 \times 10^{-3}$, $1 \times 10^{-4}$, $5 \times 10^{-5}$, $2 \times 10^{-5}$, $1 \times 10^{-6}$, $6 \times 10^{-7}$, $3 \times 10^{-7}$ $\mu$M, significantly increased cell proliferation ($p = 0.001$, $p = 0.001$, $p = 0.000$, $p = 0.003$, $p = 0.002$, $p = 0.000$, $p = 0.000$, $p = 0.000$, respectively), (Fig. 2), Glyoxal dose proliferation study in HUVEC culture. Data are expressed as mean ± SD, * $p < 0.05$ compared to control. Oneway ANOVA Post hoc Dunnett test ($n = 8$–16).

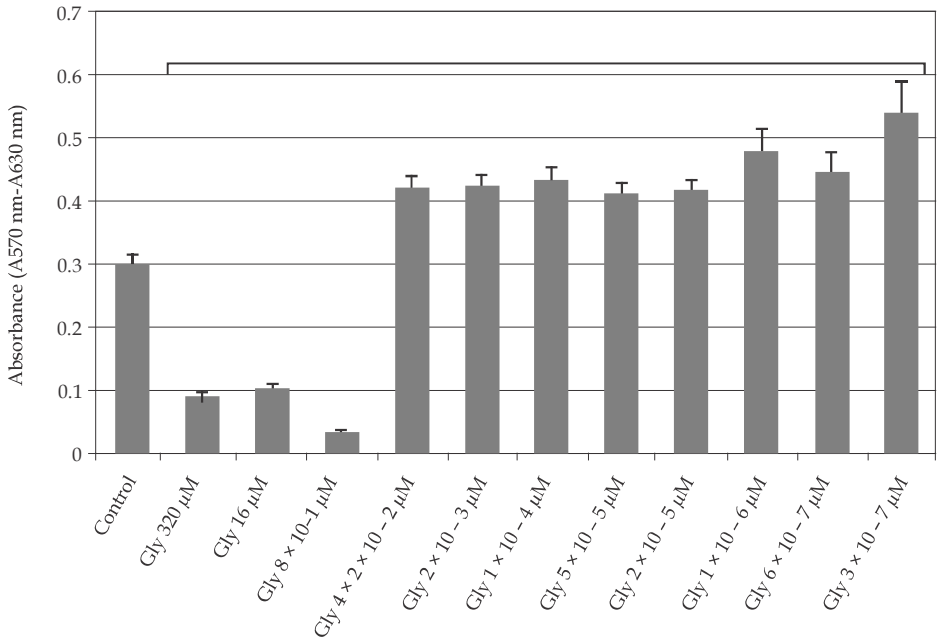


**Fig. 2:** Glyoxal dose proliferation study in HUVEC culture. Data are expressed as mean ± SD
* $p < 0.05$ compared to control. Oneway ANOVA Post hoc Dunnett test ($n$ = 8–16).

## Discussion

Our findings showed that, when compared with human plasma glyoxal level (0.1–1 $\mu$M), doses of glyoxal above 0.8 $\mu$M had cytotoxic effect on cells, but doses below 0.8 $\mu$M increased the proliferation of endothelial cells. Glyoxal, causes cytotoxicity, changes in cell morphology, mitochondrial dysfunction, cell skeleton rearrangements, barrier dysfunction, inhibition of DNA synthesis and cell replication and inhibition of vascular endothelial cells. It is accepted as an important intermediate in the formation of advanced glycation end products (AGEs) by binding to the amino groups, nucleotides and lipids of the glyoxal proteins entering the cell.[74] During diabetes, AGEs are paired with a number of complications such as the pathogenesis seen in cardiovascular diseases like vascular damage including macrovascular and microvascular complications. Cross-linking with AGE has been reported to cause vascular stiffening and endothelial dysfunction.[74] Glyoxal reacts with arginine to form imidazolium and with lysine to form N-carboxymethyl lysine (CML).[75] 40–50% of AGEs are estimated to form Schiff's base originating from glyoxal.[76] Glyoxal adducts with nucleic acids (DNA and RNA) causing mutations. It has been reported that α-oxoaldehydes-mediated glycation plays a role in diabetic vascular damage.[75,77] It is known that vasculopathy during diabetes is the cause of various cardiovascular diseases.[78] Generally, it is attributed to diabetic vascular damage, glycative, glyoxidative, carbonyl and oxidative stress due to increased glucose levels.[79,80] Methylglyoxal exposure revealed diabetes-like microvascular changes and damage in rats.[81] Increased serum levels of AGE in patients with Type 2 diabetes are associated with endothelial dysfunction.[82] High levels of intracellular AGE formation in endothelial cell culture has shown to be induced by heypergylcemia due to ROS.[83] AGEs show their effect on vascular ECs by interacting with RAGE.[84] AGEs also mediate oxidative stress (lipid peroxidation) by altering gene expression and vascular abnormalities by interacting with RAGE in vascular ECs.[85,86] Interacting with RAGE, AGE causes increased EC permeability and vascular hyperpermeability *in vitro*.[55] In the mean while AGE-modified human serum albumin (HSA) has been shown to induce hyperpermeability and actin cytoskeletal reorganization in ECs.[87,90] Both glucose-derived oxoaldehydes and AGE-modified proteins cause EC cytoskeletal rearrangement and hyperpermeability. Albumin-derived AGEs have been shown to disrupt vascular EC junctions associated with increased EC permeability, such as cadherins and catenins.[89] Sliman et al. showed that in BPAECs, glyoxal induces reorganization of tight junction protein ZO-1 and suggested that glucose-derived α-oxoaldehyde is effective in the alteration of EC tight juntions that would cause EC barrier dysfunction and hyperpermeability.[90] Healthy ECs are very important for angiogenesis. The results of

Sliman's study show that glyoxal inhibits *in vitro* angiogenesis (tube formation) in BPAECs culture. As a result glyoxal causes cytotoxicity, changes in cell morphology, mitochondrial dysfunction, inhibition of cell replication and cytoskeletal changes. Glycated basic fibroblast growth factor (FGF-2), similar to AGEs in hyperglycemia, is shown to activate signal transduction pathways[91] also activate specific signaling pathways including oxoaldehydes in vascular ECs including PTyKs, p38 MAPK, extracellular signal-regulated kinase and JNK. shown.[92] Glycation and oxidative stress mediated lipid peroxidation in hyperglycemic conditions contributes to the formation of reactive carbonyl species and induction of carbonyl stress.[93] Glyoxal cytotoxicity in hepatocytes is associated with GSH depletion, oxidative stress and mitochondrial damage.[75] In another study by Shangari et al. 10 mM glyoxal concentration causes oxidative stress.[93] In Sliman's study it was accepted that oxidative stress mediated glyoxal cytotoxicity in BPAECs.[89] Kasper M et al. reported that up to 0.4 mM, glyoxal causes apoptosis in human embryonic lung epithelial cells.[94] In immortalized E1A-NR3 retinal cells, up to 0.8 mM glyoxal causes cytotoxicity, alterations in cell morphology, mitocondrial and DNA damage.[95] Cervantes-Lauren et al.[96] and Sliman's studies in BPAEC model, high concentrations of glyoxal (1–10 mM) could have been drastically lowered due to the formation of Schiff's base adducts, AGEs, and also enzymatic and non-enzymatic degradation at the target sites.

Glyoxalase has shown to metabolically detoxify glyoxal by converting glyoxal to glycolic acid. However, high concentrations of glyoxal have been shown to inhibit glyoxal-metabolizing enzymes. High concentrations of glyoxal were also detected in diabetic conditions (~27.2 $\mu$g/ml). Sliman et al.[89] showed for the first time glyoxal related changes such as cytotoxicity, cytoskeletal changes and barrier dysfunction in vascular ECs. Glyoxal may activate cell proliferation pathways at low doses by altering protein function in endothelial cells and influencing intracellular signaling pathways, while at high doses it may affect repair mechanisms and apoptotic processes, leading to cell damage. These effects are due to the fact that glyoxal activates different receptors or due to disturbance on certain receptors. AGEs are known to play a major role in vascular cell injury, and microvascular and macrovascular complications associated with hyperglycemia increase the cost of treating diabetes. In addition, it causes additional organ damage and extends the spread of the disease in the body, causing damage to many organs and systems. Therefore, it seems to be necessary to plan and produce treatment regimens that inhibit AGE formation and receptor binding.

When it is considered that glyoxaline is also an AGE variant, it seems necessary to clarify its effects at cellular level and the pathological mechanisms it elicits. It is also important to clarify in which signaling pathways glyoxal plays a more active role on receptors. Each new mechanism to be discovered is important in finding solutions for the treatment of diabetes and developing new treatment strategies. For this purpose, additional studies are needed to place the results of our study on a more meaningful basis.

***Conflict of Interest:*** No

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