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Original Article

Evaluation of the Effects of End-Group Aminated Dextran and 1,6-Hexamethylenediamine Dextran on HEK-293 and Vero Cell Lines

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Abstract

Background: Dextran is a polysaccharide that can be chemically modified to produce derivatives with novel properties and applications. End-group aminated dextran can be synthesized by reductive amination using an aminating reagent. This study aimed to prepare an end-group aminated dextran using 1,6-hexamethylenediamine (HMDA) and evaluate its potential cytotoxicity compared to HMDA alone in vitro.

Methods: Dextran was aminated with HMDA using sodium cyanoborohydride. The product was purified by dialysis and lyophilization. Cytotoxicity was assessed in HEK-293 and Vero cell lines and treated with varying concentrations of the aminated dextran or HMDA for 48 hours. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Lactate dehydrogenase (LDH) activity, total antioxidant capacity (TAC), lipid peroxidation (LPO), total thiol groups (TTG), catalase (CAT), and superoxide dismutase (SOD) activity underwent evaluation.

Results: HMDA markedly reduced cell viability in both cell lines in a dose-dependent manner (half-maximal inhibitory concentration = 501.20 ppm and 372.00 ppm for HEK-293 and Vero cells, respectively), while the aminated dextran showed no significant cytotoxicity up to 15 000 ppm. Biochemical assays revealed that HMDA significantly decreased TAC, TTG, CAT, and SOD but increased LPO and LDH release compared to the control. The aminated dextran did not significantly alter these biochemical markers.

Conclusion: Overall, HMDA induced oxidative stress and cytotoxicity in vitro, while the end-group amination of dextran with HMDA blocked its reactive amine groups and prevented the toxic effects. The synthesized aminated dextran derivative exhibited no cytotoxicity and may have potential biomedical applications. Further studies are warranted to fully characterize its safety profile.

Keywords: 1,6-Hexamethylenediamine, Dextran, Modified dextran, Polysaccharides, HEK-293, Vero

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Introduction

Among natural products, polysaccharides as polymeric structures can be considered one of the most abundant and practical materials that have wide applications in different fields, such as the food industry, pharmaceuticals, cosmetics, and health technologies (1).

There are various types of polysaccharides based on the monomer type, number of monomers, bond arrangement, and polysaccharide source. Glucose is one of the well-known building monomers, and plants, fungi, and microorganisms, such as bacteria, can be considered the most significant sources of polysaccharides (2-4). One of the well-known polysaccharides biosynthesized by special species of lactic acid bacteria is dextran, which is formed from D-glucopyranosyl units in a branched fashion (5). Glucose molecules in polysaccharide chains are bound to each other by α1,6 glycosidic bonds and α1,2, α1,3, and α1,4 in branches (6).

Due to its high solubility in water, high biocompatibility, lack of immunogenicity, notable tunability, high relative accessibility, and diversity in molecular weight, dextran is a suitable substance for use in the preparation of food products, pharmaceutical formulations, and biomedical sciences. To improve physicochemical properties (e.g., viscosity, solubility in different solvents, molecular weight and electrical charge, and pharmacological effects) and expand its range of applications, many derivatives have been synthesized by introducing various functional groups on dextran (7,8).

Amination is one of the methods of modifying dextran, which has been the focus of many studies. It can be performed using various reagents and can produce a diverse range of dextran derivatives. Aminated dextran can be used in various fields, such as plasmid delivery as a non-viral vector, RNA delivery (e.g., small interfering RNA), tissue engineering, antimicrobial agents, and drug delivery (9-12).

Due to raising attention to the therapeutic applications of enzymes (increasing the efficiency of targeted delivery of enzymes, improving the pharmacokinetic properties of enzymes, such as increasing their half-life in serum, elevating cellular uptake, and increasing their structural stability against different stresses), designing and synthesizing aminated dextran are among the most important aims (13-15).

The reductive amination method is one of the most useful amination strategies that can effectively aminate dextran or its oxidized derivative, dextran aldehyde. (6,16) With this method, a Schiff-base structure can be synthesized by reacting the primary amine with the aldehyde groups in dextran, which is then converted to the secondary amine group by a reducing agent, such as sodium cyanoborohydride. The 1,6-hexamethylene diamine (HMDA) is one of the commonly used compounds that can be considered for the reductive amination of dextran (13). In addition, HMDA has been utilized for the amination of bovine serum albumin and the preparation of a cationic derivative from it (17,18).

Despite the mentioned applications of HMDA, some studies have demonstrated that this substance can cause some disturbances in cellular functions, such as reducing the ability of cells to proliferate or reducing the survival rate of treated cells (19,20).

The aim of this study is to evaluate the effects of end-group aminated dextran synthesized with HMDA and dextran-HMDA copolymer on human embryonic kidney (HEK 293) and African Green monkey kidney cell lines. They can be used in the fields of drug delivery or gene delivery if they do not have devastating effects.

Materials and Methods

End-Group Aminated Dextran Synthesis

End-group aminated dextran was synthesized using the method reported by Rees with minor modifications (21). Briefly, 2 g of dextran was dissolved in 10 mL of deionized water under constant stirring. In addition, 1 g of HMDA and 400 mg of sodium cyanoborohydride were then added to the reaction vessel. Sodium cyanoborohydride acts to reduce Schiff base/imine intermediates to secondary amine linkages, allowing stable conjugation of HMDA to the dextran backbone. The reaction proceeded under gentle stirring at room temperature for 24 hours. The end-group aminated dextran product was purified by dialysis against distilled water utilizing dialysis tubing with a 3500 Da molecular weight cutoff, thus ensuring the removal of unreacted reagents and byproducts from the final polymer. The purified product solution was subsequently lyophilized for 24 hours to obtain the solid end-group aminated dextran (22). All reagents were of analytical grade and obtained from commercial suppliers. This provides an efficient and reproducible approach to synthesizing amine-functionalized dextran polymers for biomaterials and biomedical applications.

The structure of the synthesized end-group aminated dextran was characterized by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. The end-group aminated dextran sample was dissolved in dimethyl sulfoxide prior to ^{1}H NMR analysis.

Cell Culture

The human embryonic kidney (HEK-293) and African green monkey kidney (Vero) cell lines utilized in this study were obtained from the Pasteur Institute of Iran. The cells were kept in 25 cm^2 cell culture flasks containing Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. The cells were maintained at 37 $^{\circ}$C under a humidified atmosphere of 5% CO_2 and 95% air and then routinely subcultured upon reaching approximately 80% confluence, as estimated by inverted microscopy. For subculturing, cell monolayers were dissociated via trypsinization and reseeded into new flasks at lower densities. Both the HEK-293 and Vero cell lines were continuously assessed for morphology, growth kinetics, and viability to ensure consistency across experiments (23).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

The cytotoxicity induced by various test compounds was assessed in HEK 293 and Vero cell lines using the MTT reduction assay. This colorimetric assay measures the activity of mitochondrial reductase enzymes as a proxy for cell viability. Viable, metabolically active cells reduce the yellow tetrazolium MTT to dark purple formazan crystals via nicotinamide adenine dinucleotide-dependent cellular oxidoreductase enzymes. Briefly, 5000 cells per well were seeded in 96-well microplates for each cell line. After allowing 24 hours for adherence and stabilization of logarithmic growth, the cells were incubated with the serial dilutions of the following experimental compounds in quadruplicate wells:

HMDA (39.06 ppm, 78.12 ppm, 156.25 ppm, 312.5 ppm, 625 ppm, 1250 ppm, 2500 ppm, 5000 ppm) and end-group aminated dextran (312.5 ppm, 625 ppm, 1250 ppm, 2500 ppm, 5000 ppm, 7500 ppm, 10 000, and 15 000 ppm)

Following 48 hours of treatment across the concentration range, 20 µL of the MTT reagent (5 mg/mL in phosphate-buffered saline) was added to each well and incubated for 3 hours at 37 °C. Resultant purple formazan precipitates were solubilized by aspirating media and adding 100 µL of dimethyl sulfoxide per well. The absorbance was quantified at 570 nm using a microplate spectrophotometer (24).

Biochemical Assays

To perform a biochemical assay, the first HEK-293 with a density of 800 000 cells/well was seeded and incubated for 24 hours at 37 °C and 5% CO_2. Then, the cells were treated with HMDA with a concentration equal to half-maximal inhibitory *concentration* and end-group aminated dextran with the maximum dose treated in the MTT cell viability assay. After 48 hours, the culture supernatant was collected for lactate dehydrogenase (LDH) measurement. Further, the cells in the wells were also lysed using lysing buffer, and the cell lysate was used to measure other markers. (24).

Lactate Dehydrogenase Activity Measurement

LDH enzyme activity was determined as an index of cell membrane integrity using a colorimetric method. LDH is a stable intracellular enzyme that is present in extracellular fluids only when severe damage to the cell membrane has occurred. This study quantified LDH activity in cell lysates using a commercially available colorimetric assay kit (Pars Azmoun Company) and spectrophotometric detection. The colorimetric assay utilizes the reduction of NAD + to nicotinamide adenine dinucleotide by LDH, coupled with the reduction of a colorless probe to a colored product that absorbs at 340 nm (24).

Lipid Peroxidation Measurement

LPO levels were quantified by measuring malondialdehyde (MDA), a byproduct, and indicator of LPO, using a colorimetric assay based on the reaction between MDA and thiobarbituric acid to form a chromophore. The absorbance of the resultant chromophore was measured at 532 nm utilizing a spectrophotometric microplate reader (enzyme-linked immunosorbent assay reader). This colorimetric assay provides a quantitative measurement of LPO (25).

Total Antioxidant Capacity

The total antioxidant capacity (TAC) of the cell lysate was determined through a ferric reducing antioxidant power colorimetric assay. This assay works by measuring the ability of antioxidant compounds within a sample to reduce ferric (Fe^{3+}) iron to ferrous (Fe^{2+}) iron. Specifically, the Fe^{3+}-TPTZ complex (2,4,6-tripyridyl-s-triazine) is reduced to the Fe^{2+}-TPTZ form under acidic conditions, resulting in an intense, blue-colored product with absorption maxima at 593 nm. The absorbance of this product is directly proportional to the total reducing/antioxidant power within the sample. By generating a standard curve using known concentrations of Fe^{2+}, the reducing capacity of the cell lysate can be quantified and compared between experimental conditions (25).

Total Thiol Group

Total thiol content was quantified using a colorimetric assay based on the organosulfur reagent 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). This water-soluble compound contains a symmetric disulfide bond that undergoes reductive cleavage in the presence of free thiols, releasing 2-nitro-5-thiobenzoate (TNB^-). TNB^- is a brightly colored anion ($\lambda max = 412$ nm) providing the basis for spectrophotometric thiol detection. Briefly, DTNB was added to freshly prepared cell lysates. Reaction with free protein thiols generated TNB^-, measurable by absorbance at 412 nm. A standard curve using cysteine allowed the quantitative determination of unknown thiol content (24).

Catalase Activity

The catalytic activity of CAT was assayed by measuring the decrease in absorbance at a wavelength of 240 nm. The assay mixture contained potassium dihydrogen phosphate/potassium hydrogen phosphate buffer (pH: 7.5, 50 mM) and hydrogen peroxide as the substrate. The CAT activity unit was calculated from the rate of hydrogen peroxide decomposition, expressed as the µmole of hydrogen peroxide consumed per minute per mL of the enzyme (U/mL) (24,26).

Superoxide Dismutase Activity

Superoxide radicals are generated through the enzymatic reaction between xanthine and xanthine oxidase. The amount of superoxide produced was quantified by measuring the reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrasodium chloride (INT) to a colored formazan dye. INT reacts with superoxide radicals to form the formazan product. y spectrophotometrically computing the absorbance at 550 nm, the degree of inhibition of this reaction was used to assess SOD activity. A standard calibration curve was constructed by plotting the percentage inhibition versus SOD concentration (U/mL) to determine total SOD activity (24,26).

Statistical Analysis

All the obtained data were analyzed using GraphPad Prism software, version 9.0. The results are presented as means ± standard deviations (SD). A P value < 0.05 was considered statistically significant (25).

Results

Effect on Cell Viability

Based on the results (Figures 1–4), HMDA significantly reduced the viability of HEK-293 and Vero cells within

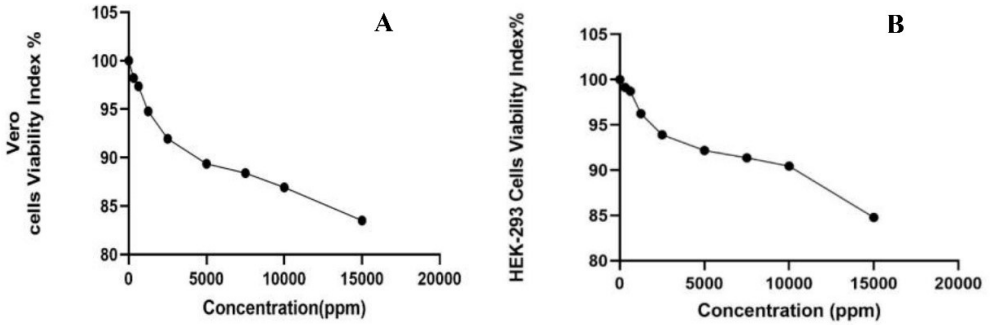

Figure 1. Viability of Vero (A) and HEK-293 (B) Cells at Various Concentrations of End-Group Aminated Dextran

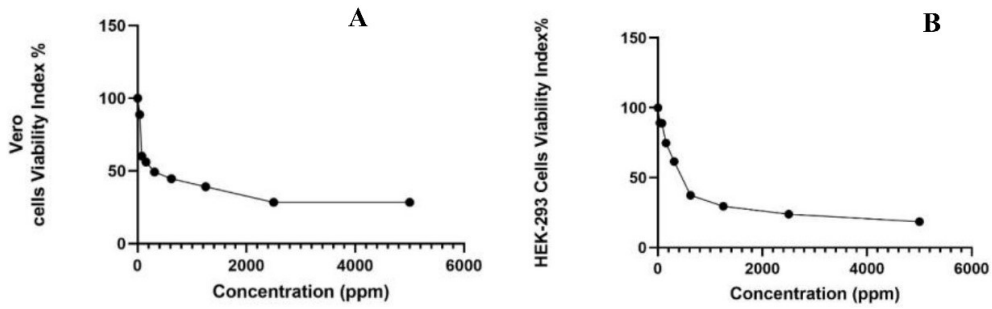

Figure 2. Viability of Vero (A) and HEK-293 (B) Cells at Various Concentrations of HMDA. *Note*. HMDA: 1,6-Hexamethylenediamine

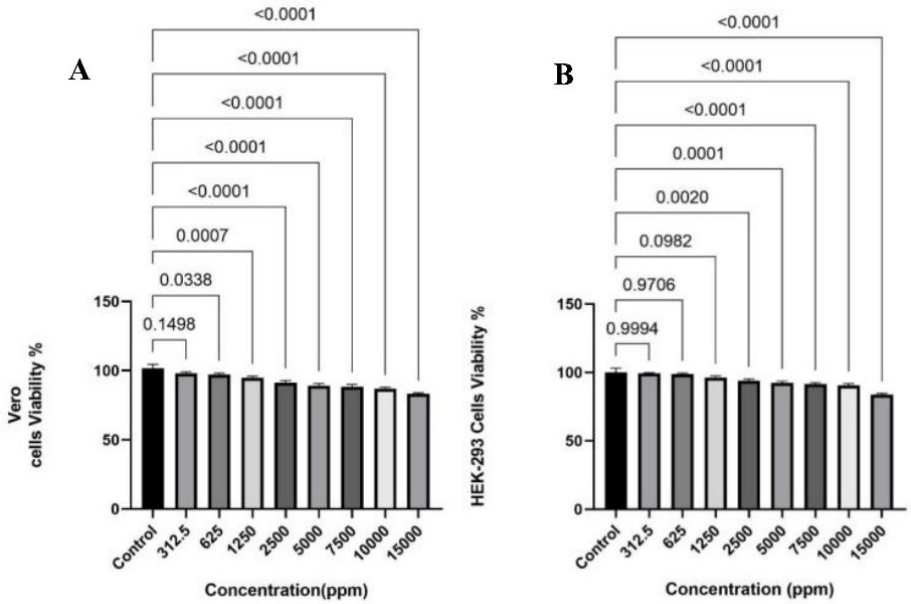

Figure 3. Effect of Different Concentrations of End-Group Aminated Dextran on the Viability of Vero (A) and HEK-293 (B) Cells. *Note*. ANOVA: Analysis of variance. $P<0.05$ is considered statistically significant. Groups were compared with each other using ANOVA and Tukey's test

48 hours. Meanwhile, end-group aminated dextran did not cause a remarkable decrease in the viability of the mentioned cells under similar conditions (Figures 1–4).

Effect of 1,6-Hexamethylenediamine and End-Group Aminated Dextran on Lactate Dehydrogenase Activity

After 48 hours of treatment, HMDA significantly increased LDH activity compared to both the untreated control group and the end-group aminated dextran-treated group ($P<0.0001$). In contrast, end-group aminated dextran did not significantly alter LDH activity compared to the control group ($P=0.7736$). In summary, while HMDA exposure led to marked elevations in LDH activity, possibly indicating cytotoxicity, end-group aminated dextran did not significantly affect enzyme activity levels. Further studies are required to fully elucidate the mechanisms underlying the differential cytotoxic effects of these compounds (Figure 5).

Impact of 1,6-Hexamethylenediamine and End-Group Aminated Dextran on Total Antioxidant Capacity

Following 48 hours of treatment, HMDA could

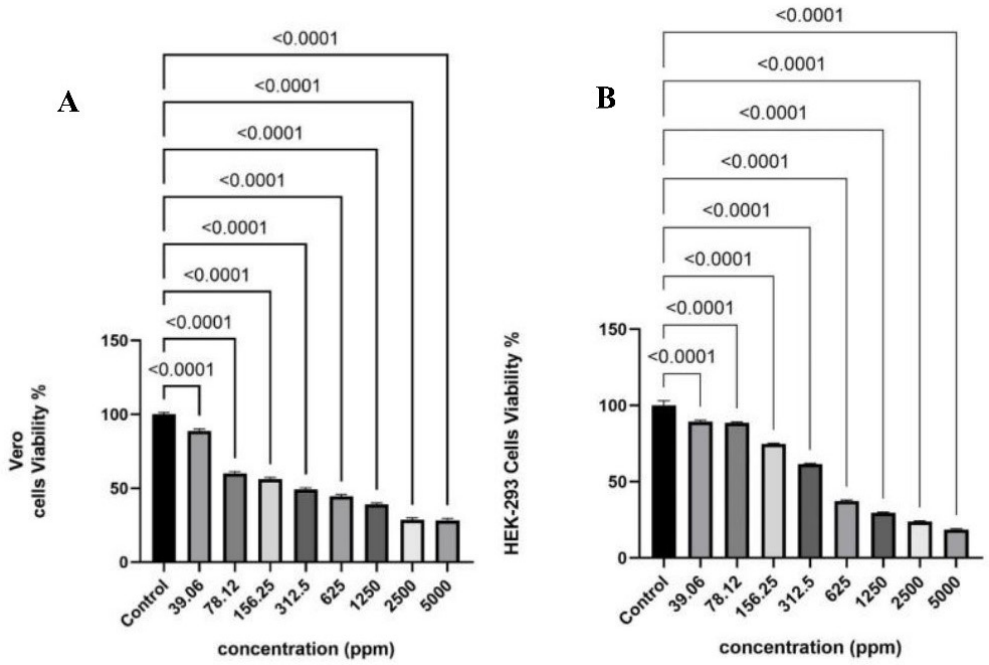

Figure 4. Effect of Different Concentrations of HMDA on the Viability of Vero (A) and HEK-293 (B) Cells. *Note*. HMDA: 1,6-Hexamethylenediamine; ANOVA: Analysis of variance. $P<0.05$ is considered statistically significant. Groups were compared with each other using ANOVA and Tukey's test

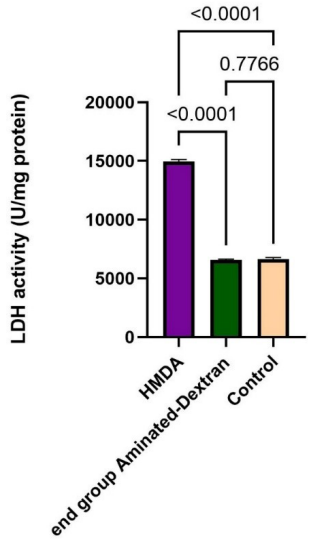

Figure 5. Effect of HMDA and End-Group Aminated Dextran on LDH Activity. *Note*. HMDA: 1,6-Hexamethylenediamine; LDH: Lactate dehydrogenase; SE: Standard error of the mean; ANOVA: Analysis of variance. The data are displayed as mean ± SE. $P<0.05$ is considered statistically significant. Groups were compared with each other using ANOVA and Tukey's test

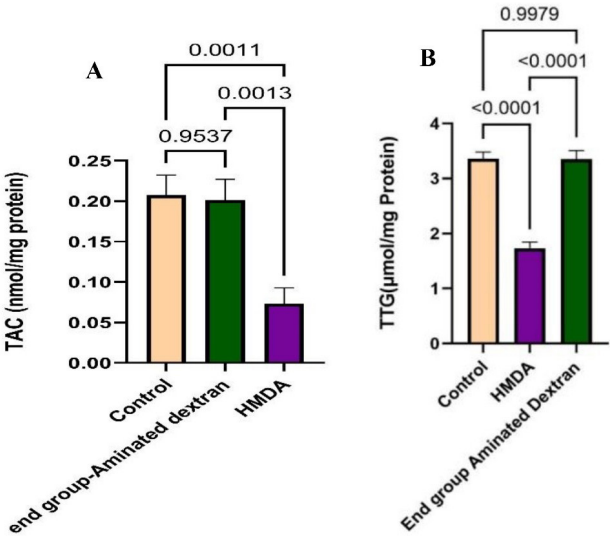

Figure 6. Effect of HMDA and End-Group Aminated Dextran on TAC (A) and TTG (B). *Note*. HMDA: 1,6-Hexamethylenediamine; TAC: Total antioxidant capacity TTG: Total thiol group; SE: Standard error of the mean; ANOVA: Analysis of variance. The data are provided as mean ± SE. $P<0.05$ is considered statistically significant. Groups were compared with each other using ANOVA and Tukey's test

significantly decrease TAC compared to the untreated control group ($P=0.0011$). Conversely, end-group aminated dextran did not cause a significant change in TAC compared to controls ($P=0.9537$, Figure 6).

Effect of 1,6-Hexamethylenediamine and End-Group Aminated Dextran on the Total Thiol Groups

Based on the results, HMDA treatment significantly decreased TTG compared to the untreated control group ($P<0.0001$). Contrarily, end-group aminated dextran caused no statistically significant changes in total thiol levels in comparison to controls ($P=0.9979$, Figure 6).

Impact of 1,6-Hexamethylenediamine and End-Group Aminated Dextran on Lipid Peroxidation

The findings revealed that HMDA treatment remarkably increased MDA production when compared to the untreated control group ($P=0.0002$), indicating elevated LPO. However, end-group aminated dextran led to no statistically significant changes in MDA levels in comparison to controls ($P=0.7796$, Figure 7).

Effect of 1,6-Hexamethylenediamine and End-Group Aminated Dextran on CAT and Superoxide Dismutase Activities

After 48 hours of treatment, HMDA could significantly decrease the activities of both SOD and CAT compared to the untreated control group ($P=0.0002$). In contrast, end-group aminated dextran exposure did not lead to any significant changes in SOD ($P=0.9759$) and CAT ($P=0.8905$) activities (Figure 8).

Discussion

Hexamethylenediamine, which is an aliphatic diamine molecule, can be employed as an amination reagent to modify dextran and increase its application range. Perez et al reported that the use of end-group aminated dextran and its conjugation with the antioxidant enzyme SOD could improve the physicochemical properties and subsequent pharmacokinetic properties (e.g., clearance) to a great extent while reducing paw inflammation in rats (13). So far, few studies have investigated the effect of HMDA on humans and its mechanism of action. Some studies have confirmed the cytotoxic effects of this molecule. For instance, Luebke et al concluded that HMDA can decrease proliferation in T and B lymphocytes isolated from the spleen in a dose-dependent and time-dependent manner in an in vitro study (19). In another study, it has been shown that the treatment of HeLa cells with low concentrations of HMDA decreased viability. (20) In agreement with previous studies, another study demonstrated that this aliphatic amine caused a significant decrease in cell viability in HEK and Vero lines. Changes in evaluated biochemical markers, such as LDH activity, in the collected cell culture medium were indicative of damage to the cell membrane (24,26) and death of cells

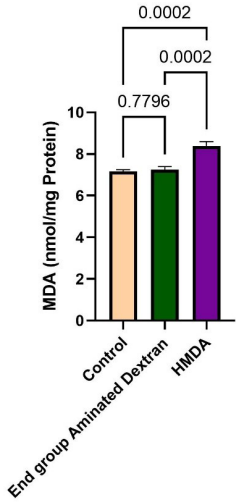

Figure 7. Effect of HMDA and End-Group Aminated Dextran on Lipid Peroxidation. *Note*. HMDA: 1,6-Hexamethylenediamine; SE: Standard error of the mean; ANOVA: Analysis of variance. The data are displayed as mean ± SE. $P<0.05$ is considered statistically significant. Groups were compared with each other using ANOVA and Tukey's test

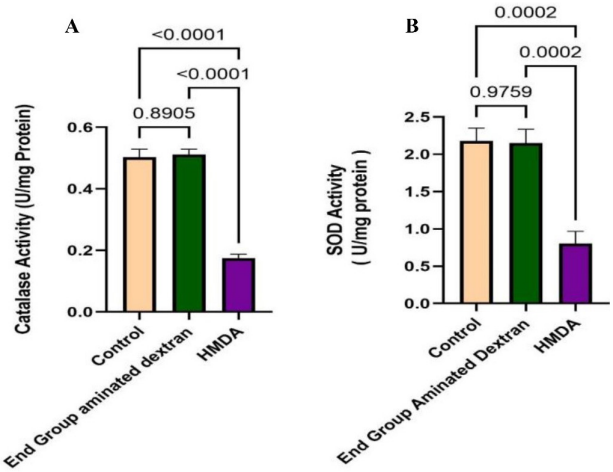

Figure 8. Effect of HMDA and End-Group Aminated Dextran on CAT (A) and SOD (B) Activities. *Note*. HMDA: 1,6-Hexamethylenediamine; CAT: Catalase; SOD: Superoxide dismutase; SE: Standard error of the mean; ANOVA: Analysis of variance. The data are represented as mean ± SE. $P<0.05$ is considered statistically significant. Groups were compared with each other using ANOVA and Tukey's test

Table 1. Different Concentrations of HMDA and Corresponding Viability

	1,6-Hexamethylenediamine							
Concentration (ppm)	39.06	78.12	156.25	312.5	625	1250	2500	5000
Viability % (HEK293)	89.232	88.935	74.776	61.511	37.477	29.506	23.973	18.560
SD	1.516%	1.003%	1.732%	1.425%	1.517%	1.394%	1.286%	1.551%
Viability % (Vero)	88.761	60.232	56.125	49.281	44.640	39.206	28.473	28.412
SD	0.912%	1.087%	0.856%	0.930%	1.100%	0.896%	1.058%	0.973%

Note. HMDA: 1,6-Hexamethylenediamine; SD: Standard deviation.

Table 2. Different Concentrations of End-Group Aminated Dextran and Corresponding Viability

	End-Group Aminated Dextran							
Concentration (ppm)	312.5	625	1250	2500	5000	7500	10000	15000
Viability % (HEK293)	99.126	98.725	96.245	93.915	92.185	91.369	90.456	84.785
SD	0.831%	1.462%	1.639%	1.715%	1.736%	1.512%	1.896%	1.414%
Viability % (Vero)	98.211	97.363	94.791	91.954	89.375	88.418	86.927	83.521
SD	1.059%	1.012%	0.901%	1.125%	1.096%	1.112%	0.991%	0.967%

Note. SD: Standard deviation.

treated with HMDA, which is in concurrence with the results obtained in the MTT assay. Cellular redox status markers, such as TAC, in cells treated with HMDA were significantly lower compared to the untreated control group and cells treated with end-group aminated dextran, indicating a decrease in the level of non-enzymatic antioxidant species, such as molecules possessing thiol groups, including glutathione (27,28).

In the case of oxidative stress, thiol-containing compounds such as glutathione and metallothionein (29,30) are susceptible to being oxidized by oxidants and form disulfide bonds. The results of our study revealed that treatment with HMDA markedly decreased the level of the total thiol group compared to the control group. Meanwhile, no remarkable changes were observed in treatment with end-group aminated dextran in comparison to the control group. Other studied redox indicators, such as the activity of two antioxidant enzymes, CAT and SOD, which play an essential role in neutralizing hydrogen peroxide and superoxide radical anion radicals, respectively (31,32), also showed that the intracellular redox balance was disturbed in the treatment with HMDA and caused oxidative stress. Based on the results, the activity of this enzyme was significantly lower in the treatment with HMDA compared to the control group and the end-group aminated dextran group. If hydrogen peroxide and other oxidizing species are not degraded, the fatty acids that make up the lipids in the cell, especially unsaturated fatty acids, undergo LPO, and small molecules (e.g., MDA) are produced (27,32), which is in line with the results of this study regarding other investigated markers. The level of MDA was statistically higher in treatment with HMDA than in the control group and the end-group aminated dextran group.

Conclusion

This study sheds light on the impact of end-group aminated dextran and HMDA on HEK-293 and Vero cell lines as an in vitro model. Our findings demonstrated that HMDA caused a substantial decrease in the viability of both cells by inducing oxidative damage, while end-group aminated dextran did not alter the cell viability rate and cellular redox balance.

Competing Interests

The authors declared that they have no conflict of interests.

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