Time- and Dose-Dependent Effect of Dexamethasone on Endothelial Cell Apoptosis

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Abstract

Objectives: Endothelial cell (EC) apoptosis plays a critical role in the physiological and pathological vascular regression, remodeling, and angiogenesis. There are several therapeutic agents such as glucocorticoids (GCs), which could influence EC apoptosis, causing coagulation events. Due to the paradoxical effects of GCs on cellular apoptosis, the aim of the current study was to investigate the dose and time in which GCs could initiate and terminate in vitro cellular apoptosis.

Materials and Methods: Dexamethasone (DEXA) was serially diluted 10-folds for 8 serial concentrations (from 1 mM to 0.1 nM) added to cultured human umbilical vein endothelial cells (HUVECs). The cytotoxic effects of DEXA on HUVEC were tested with a rapid colorimetric test using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay. Apoptotic assays based on quantitative polymerase chain reaction was performed for \( Bax \) and \( Bcl-2 \) genes and terminal deoxynucleotidyl transferase dUTP nick end labeling assay.

Results: DEXA at the concentration of 1 µM showed significant cytotoxic effects, more intense anti-apoptotic effects in lower concentrations (1 nM to 100 nM), and anti-apoptotic effects with less intensity in higher concentrations (10 µM to 1 mM). Six hours of treatment by 1 µM of DEXA was estimated as the initial time of DEXA that could remarkably induce HUVECs apoptosis. The maximum significant increase of apoptosis was detected 24 hours after treatment with DEXA.

Conclusions: Our findings suggested that GCs can influence cellular apoptosis in a dose- and time-dependent manner.

Keywords: Glucocorticoids, Steroids, Endothelial cell, Apoptosis, Vascular

Introduction

The integration of vascular endothelium is one of the factors that affects homeostasis and the normal function of body organs. The pathogenesis of various vascular diseases indicates the damage and dysfunction of the vascular endothelium. The results of in vitro studies show that many factors cause endothelial cell (EC) apoptosis. Vascular damage causes a vascular leak, inflammation, and coagulation. Meanwhile, apoptosis EC plays an important role in physiological regression and vascular pathological, vascular regeneration, and angiogenesis. Apoptotic cells detach before engulfment since blood pressure affects ECs. The loss of small amounts of ECs by this process could cause vascular leakage and exposure to the thrombogenic sub-endothelial matrix. Apoptotic ECs are both the precursors of platelets and leukocytes and are procoagulants, causing blood clotting, autoimmunity, or bone/vascular disease (1-3).

Glucocorticoids (GCs) are one of the factors affecting the apoptosis of ECs and are widely used in rheumatoid arthritis, asthma, systemic lupus erythematosus, and cancer, along with transplant patients. Although these drugs have many side effects (4,5), especially in long-term use, the therapeutic benefits outweigh the side effects. Furthermore, the results of distinct studies have shown the existence of a link between GCs and venous thrombosis (6-10). GCs indirectly cause thrombolytic blockage or direct damage to ECs, eventually leading to blood clotting. The use of GCs in the early stages of rheumatoid arthritis treatment causes thromboembolic complications (5,11). In addition to the thromboembolic effect of GCs, studies reported that GCs can increase apoptotic and proliferative processes in ECs depending on the application time and dose (12). Some roles of GCs are known to be mediated by a cytoplasmic receptor, called a glucocorticoid receptor (GR). GCs attach to GRs, causing major changes in the structure. Consequently, the complex is transferred to the nucleus, where it becomes paler. This action causes the nuclear displacement or modulation of transcription factors such as nuclear factor κB, which, in turn, modifies the central mediators of innate immunity and compatibility (13). One of the most well-known pathways of apoptosis is the Fas pathway, which acts through the immune response. The FADD protein (Fas-associated protein with death domain) is absorbed by binding the Fas ligand (FasL) to its receptor (FasR) and receptor oligomerization. This action interacts with caspase-8 and causes caspase cascade and apoptosis (14). There is a relatively rapidly
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Glucocorticoids can influence cellular apoptosis in a dose- and time-dependent manner. Glucocorticoid-induced cellular apoptosis can lead to a better understanding of the molecular pathogenesis of vascular and bone diseases.

induced induction for reducing the planned cell count in dexamethasone (DEXA)-treated T lymphocytes that occurs within a few hours after the treatment (15).

Based on all evidence, it might be concluded that GCs can induce EC apoptosis, which can lead to coagulation events. The apoptotic and proliferative effects of GCs are notably time- and dose-dependent, responding differently in various types of cells (16,17).

Although GC induces apoptosis in T lymphocytes, DEXA as a GC can proliferate and protect cells against cell death under certain conditions. This effect is observed in a variety of cells including corneal epithelial cells, thymocytes and mammary gland epithelial cells, liver cells, endothelium, and keratocytes. This contradictory biphasic effect can be regulated through different pathways and in different cell types or doses in response to the GR gene. EC apoptotic death appears to act as a mechanism for capillary destruction in GC-mediated hypertension (18).

Therefore, GC-induced hypertension in ECs disrupts the blood flow in the arteries and disrupts the healing process (19). On the other hand, an increase in blood pressure in the arteries can lead to an increase in thrombin, which converts fibrinogen to fibrin and eventually fibrin clots (20).

Due to the paradoxical effects of GCs on cellular apoptosis, the aim of this study was to investigate the dose and time in which GCs could initiate and terminate in vitro cellular apoptosis. This study could help better understand the molecular pathogenesis of vascular diseases such as the GC-induced avascular necrosis of the femoral head (ANFH) in which EC apoptosis occurs by steroid administration (7, 21-23).

**Materials and Methods**

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were obtained from the National Cell Bank of Iran-Pasteur Institute (Catalog No. C554). The cells were cultured with 10% fetal bovine serum (FBS) containing 100 IU/mL penicillin and 100 μg/mL streptomycin and then incubated at 37°C and 5% CO₂. The cultured HUVECs were analyzed using phase-contrast microscopy.

DEXA pure powder, as a synthetic analog of GCs, was generously gifted by Iran Hormone Company. It was dissolved in liquid, sterile-filtered dimethyl-sulfoxide (DMSO) and then added to the culture medium. DEXA was prepared in a stock solution of 10 mM and serially diluted from higher to lower concentrations (from 1 mM to 0.1 nM). To study the cytotoxic effect of DEXA from the DMSO effect, similar experiments were performed by DMSO alone.

**MTT Assay**

The rapid colorimetric method and MTT were performed to compare the effects of the untreated control cells of DEXA and/or DMSO cytotoxicity in vitro on HUVEC. The MTT method is highly dependent on the ability of mitochondrial dehydrogenase enzymes for converting 3,4,5-dimethiazole-2,5-diphenyltetrazolium bromide to purple formazan precipitate. Finally, the A570 supernatant was measured by spectrophotometry in the enzyme-linked immunosorbent assay (ELISA) reader (24).

This method can be based on the metabolic reduction and the metabolic reduction of soluble MTT by the activity of live tumor cell mitochondrial enzyme to an insoluble dye formulation product that is measured after dissolution in DMSO. For studying apoptosis in HUVECs, the cells were seeded at a density of 5000 cells/well in 96-well plates in triplicate for 24 hours. The cells were then allowed to attach overnight. On the second day, 100 μL of DMEM containing various concentrations of DEXA (i.e., 10-3, 10-4, 10-5, 10-6, 10-7, 10-8, 10-9, and 10-10 M) or DMSO alone were added into each well for 24 hours. Following the incubation time, cell viability was assessed using the MTT (Sigma, St. Louis, USA) colorimetric method based on the manufacturer’s protocol. In fact, the cells were incubated with 0.5 mg/mL MTT for 4 hours at 37°C. By adding 100 μL of DMSO and stirring gently for 30 minutes, the formalin crystals resulting from the MTT reduction were dissolved under mitochondrial dehydrogenase. Eventually, the ELISA reader and the spectrophotometric method were used to measure the A570 of the supernatant (25).

**Apoptosis Assays**

**Bax and Bcl2 Gene Expression**

This work was designed to shed light on the initiation time of cellular apoptosis, which is induced by 1 μM of DEXA. The Bcl-2 and Bax expression ratio was assessed by the real-time polymerase chain reaction (RT-PCR), which was previously explained (26). Then, a 1 μM concentration of DEXA was selected based on the previous phase of our study.

**RNA Isolation**

HUVECs were seeded in a 6-well plate (15 × 10⁴ cells/well) in 3 mL of DMEM-F12 supplemented with 2% FBS for 24 hours. On the second day, 3 mL of DMEM-F12 containing 1 μM concentration of DEXA was replaced with the medium in each well, then incubated for several time points (i.e., 1.5, 3, 6, 12, 24, 36, and 48 hours). After the incubation time, HUVECs were harvested for total RNA extraction utilizing the AccuZol (Bioneer Inc, Daejeon, Korea), followed by DNase I treatment (Thermo Scientific Inc, Vilnius, Lithuania) as suggested...
by the manufacturer. RNA concentrations were measured by spectrophotometry and integrity was obtained before cDNA synthesis by analysis on 1.5 agarose gels at 18S and 20S scales.

**SYBR-Green RT-PCR**

Total RNA (0.5-5 µg) was used for quantitative reverse transcription polymerase chain reaction (RT-qPCR) with reverse transcriptase (Thermo Scientific Inc, Vilnius, Lithuania) using the oligo-dT primer under thermal conditions as the cycling of 42°C (60 minutes) and 65°C (10 minutes). The protocol of RT-qPCR included 25 µL of AccuPower® 2X GreenStar™ qPCR Master Mix, forward and reverse primers (in a final concentration of 0.8 pmol), and 50 ng of cDNA in a final reaction volume of 50 µL, as well as the initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C (30 seconds), 60°C annealing temperature (30 seconds), and 72°C (45 seconds). The primers for the analysis of Bcl-2, Bax, and GAPDH gene expressions were previously designed (27,28).

GAPDH was used as a reference gene to normalize the expression of Bcl-2 and Bax mRNAs. The initial sequences and annealing temperatures are shown in Table 1. To determine the relative quantity of gene expression, the comparative threshold cycle (ΔCt) method was used and normalized against GAPDH, which was measured by the same method. All PCRs were performed in three versions. Control reactions were regulated by the absence of reverse transcriptase to assess the level of genomic DNA contamination. This method was in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines.

**Measurement of the Termination of Apoptosis by Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay**

To estimate the termination time of apoptosis, in this study, the terminal deoxynucleotidyl transferase dUTP labeling technique was used to label immunoperoxidase DNA fragments (29).

DNA fragmentation occurred in the final stage of apoptosis using TUNEL assay (Roche Applied Science, Penzberg, Germany). HUVECs were seeded on a poly-L-lysine coated glass slide at a density of 11×10³ cells/slide, and then the treated area was covered with a glass cover, and finally, the edges were sealed with clear nail polish. The slides were viewed under a digital microscope (Olympus BX51, Japan).

**Statistical Analysis**

Excel (Version 2013, Chicago, IL) and SPSS (Version 18.0, Chicago, IL) software were used for all statistical analyses. The mean value and standard deviation (SD) were calculated using descriptive statistics. The comparison of means was performed by post hoc Tukey test. The statistical value of P<0.05 was considered to be significantly different.

TUNEL assay results processed using SPOT software, version 1.1.02. In addition, 40 random fields were chosen for all samples, and the number of viable and apoptotic cells per field was counted using rectangular grids, placed randomly on the investigated areas. Finally, the mean of viable and apoptotic positive cell number per unit area (NA) was calculated in different regions using the following formula (30):

\[
NA = \frac{\sum Q}{a \sum P}
\]

In this formula, “ΣQ”, “a/Σ”, and “ΣP” indicate the sum of counted particles appeared in sections, the area associated with each frame, and the sum of frame associate points

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**Table 1. List of the Applied Primers for Real-time PCR Assay**

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Sequence (5’ to 3’)</th>
<th>Product Length</th>
<th>Annealing Temperature</th>
</tr>
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</table>
| **GAPDH**          | F: 5’- CAGCTGCTCATGGGTGTAAC-3’  
                    | R: 5’- CAGCTGCTCATGGGTGTAAC-3’  | 178 bp            | 60°C                   |
| **Bax**            | F: 5’- GGTTCATGACCAAGCAGAC-3’  
                    | R: 5’- GGTTCATGACCAAGCAGAC-3’  | 148 bp            | 60°C                   |
| **Bcl2**           | F: 5’- GCCAGGAGAAATCAAACAGAGG-3’  
                    | R: 5’- GCCAGGAGAAATCAAACAGAGG-3’  | 64 bp             | 60°C                   |

*Note: PCR: Polymerase chain reaction; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.*
hitting the reference, respectively. The statistical analysis was performed using SPSS 11.5 software for windows. The values were compared by an independent t test. The P value of less than 0.05 was considered statistically significant.

Results
Dose-Dependent Effect of DEXA on Cellular Apoptosis
To investigate the influence of different doses of DEXA on cellular apoptosis or proliferation, HUVECs were cultured in DMEM-F12 containing a serial dilution of DEXA (10 mM to 1 nM of DEXA) for 24 hours. The cytotoxicity effect of DEXA on HUVECs was measured by the MTT assay and then compared with untreated controls (31). MTT assay revealed paradoxical results on the dose-dependent effect of DEXA on HUVEC proliferation (Figure 1). As shown in Figure 1A, the viability of the HUVECs without adding DEXA was assumed 100% as the control group. The viability percentage in the serial concentrations of DEXA (i.e., 10^-3, 10^-4, 10^-5, 10^-6, 10^-7, 10^-8, 10^-9, and 10^-10 M) was compared with the control group. DEXA at the concentration of 1 µM showed significant cytotoxic effects while more intense anti-apoptotic effects at lower concentrations (1 nM to 100 nM) and anti-apoptotic effects with less intensity at higher concentrations (10 µM to 1 mM) were observed compared to the control group (P < 0.05 in comparison with the untreated control group).

The statistical significance was evaluated using the two-way analysis of variance (ANOVA) test. Based on ANOVA results, the most significant differences were observed at concentrations of 1 µM and 100 µM of DEXA with more intensity in 1 µM compared with the control group. Based on these data, 1 µM of DEXA was selected and used in the next steps of this study.

Time-Dependent Effect of DEXA on Cellular Proliferation
Quantitation of Apoptosis Initiation Time
Bcl-2 inhibits apoptosis and Bax induces cell death and their expression level assessment allows the detection of changes in the early phases of apoptosis (32-34). Figure 2A illustrates the differential gene expression level of Bax at different time points (i.e., 1.5, 3, 6, 12, 24, 36, and 48 hours) of incubation with 1 µM of DEXA. The specification of each pair of primers was confirmed by melting curve analysis using the RT-qPCR. As displayed in Figure 2A, the maximum significant overexpression for the Bax gene (as a pro-apoptotic gene) and the minimum significant down-regulation for the Bcl-2 gene (as an anti-apoptotic gene, Figure 2B) were observed after 6 hours of treatment by 1 µM of DEXA compared with the control group (P<0.05).

Considering that Bax and Bcl-2 play opposite roles in the mitochondria-dependent apoptotic pathway, two-way ANOVA was used to elucidate if there were significant differences between the treatment groups and the control group and to assess the interaction effect between these two genes. Based on the results in Figure 2, there were significant differences in the Bax expression level in all time points except for 1.5 hours following the treatment by DEXA. The significant Bcl-2 expression level was observed only 6 hours after the treatment by DEXA.

In conclusion, 6-hour treatment by 1 µM of DEXA was estimated as the initial time of DEXA which could remarkably induce HUVEC apoptosis.

Quantitation of Apoptosis Termination Time
For more investigations, a TUNEL assay was applied to detect DNA fragmentations by labeling the terminal end of nucleic acids to estimate the late stage of apoptosis (35). Morphological differences between apoptotic and normal cells are depicted in Figure 3.
The Data showed that the -7
Illustrated Morphological Differences and Characterization
A
DNA are stained dark brown.

The apoptotic nuclei and fragmented
cytoplasm of apoptotic cells are shrank. The apoptotic nuclei and fragmented

Dexamethasone; HUVECs: Human umbilical vein endothelial cells; The
Treatment With 1 µM of DEXA (48). Additionally, the maximum effect of
DEXA on the apoptosis of the human mammary carcinoma cell
line (MCF-7). Likewise, Buxant et al determined the anti-
proliferative effect of 10 nM and 100 nM DEXA
on the apoptosis of HUVEC Preincubated for Different Time Points in the Absence (control) or
Presence of DEXA (1 µM). Note: DEXA: Dexamethasone; HUVECs: Human
umbilical vein endothelial cells; qPCR: Quantitative polymerase chain
reaction; Bar chart A: Data showed that the Bax gene had the maximum
expression level 6 hours after treatment with DEXA; Bar chart B: The
minimum expression level of the Bcl2 gene was observed 6 hours after DEXA
treatment. Based on these data, the initial time of cellular apoptosis 6 hours
after treatment was postulated by DEXA (1 µM). Asterisks indicate significant
P values.

Figure 2. Real-time q-PCR Assays of (A) Bax and (B) Bcl-2 mRNA Expression
in HUVEC Preincubated for Different Time Points in the Absence (control) or
Presence of DEXA (1 µM). Note: DEXA: Dexamethasone; HUVECs: Human
umbilical vein endothelial cells; qPCR: Quantitative polymerase chain
reaction; Bar chart A: Data showed that the Bax gene had the maximum
expression level 6 hours after treatment with DEXA; Bar chart B: The
minimum expression level of the Bcl2 gene was observed 6 hours after DEXA
treatment. Based on these data, the initial time of cellular apoptosis 6 hours
after treatment was postulated by DEXA (1 µM). Asterisks indicate significant
P values.

the TUNEL assay revealed that 1 µM of DEXA 12 and 24
hours after the treatment induced a significant increase
in the apoptotic rate. The maximum significant increase
of apoptosis was detected 24 hours after the treatment
with DEXA. However, no significant changes were found
between 36 hours in comparison with 24 hours after the

treatment. Moreover, there was no significant increase
in HUVEC apoptosis 3 and 6 hours after the treatment
(Figures 4 and 5).

Figure 3. Illustrated Morphological Differences and Characterization
Between Normal HUVECs (A) and Apoptotic HUVECs 24 Hours After
Treatment With 1 µM of DEXA (B) Under Magnification = x100. Note: DEXA:
Dexamethasone; HUVECs: Human umbilical vein endothelial cells; The
cytoplasm of apoptotic cells are shrank. The apoptotic nuclei and fragmented
DNA are stained dark brown.

Discussion
It was demonstrated that DEXA, as a potent GC, could
induce HUVEC apoptosis in a dose- and time-dependent
manner. The results of the present study represented the
dose-dependent effect of DEXA on HUVEC proliferation
with a significant cytotoxicity effect at 1 µM, a high
intense anti-apoptotic effect at low concentrations (1
nM to 100 nM), and a low intense anti-apoptotic effect
at higher concentrations (10 µM to 1 mM). The apparent
paradoxical effect of DEXA was previously observed in
corneal epithelial cells (36) and retinal pigment epithelial
cells (37). The anti-apoptotic effect of DEXA may be
through the inhibition of the mitochondrial apoptotic
pathway (38). There was a threshold dose for the anti-
apoptotic effect of DEXA so that with a higher dose of
DEXA, a significant level of cytotoxicity occurred in our
in vivo studies. The suppressive effect of 10 nM DEXA on
bovine glomerular EC apoptosis was previously reported
by (39). In another study, Messmer et al (40) found the
significat protective effect of 10 nM and 100 nM DEXA
on the apoptosis of the human mammary carcinoma cell
line (MCF-7). Likewise, Buxant et al determined the anti-
proliferative effect of 10⁻⁸ to 10⁻⁴ M of DEXA (41).

Another notable finding of our study is that the
apoptosis of HUVECs was initiated 6 hours after the

treatment by 1 µM of DEXA (estimated with Bax and
Bcl-2 expression levels). There is more evidence showing
the apoptotic than the anti-apoptotic effect of DEXA
and it is more accepted that the prominent role of DEXA
is its apoptotic effect (42-45). Prominent among the
apoptosis regulatory proteins, is the Bcl-2 super-family of
polypeptides comprising of both anti-apoptotic (i.e.,
Bcl-2, Bcl-xl, Bag1, Mcl1, and A1) and pro-apoptotic (i.e., Bax,
Bad, Bak, Bid, and Bcl-xs) members (46). Bcl-2 and Bax
are two key regulator genes in the mitochondrial apoptotic
pathway, as the first evidence of cellular apoptosis (47).
It was also demonstrated that the apoptosis induced by
DEXA was terminated 24 hours after incubation with
1 µM of DEXA. Along with our TUNEL assay results,
Machuca et al suggested that the maximum cytotoxicity
changes occur between 24 and 48 hours after incubation
with DEXA (48). Additionally, the maximum effect of
DEXA on cellular apoptosis was estimated 24 hours after
incubation (37). Thus, DEXA could trigger pro-apoptotic
signals by pre- and/or post-cytochrome c release from
mitochondria 6 hours after its treatment (49).

The direct and indirect effects of GC on cells, especially
epithelium and bone cells have been also studied in bone
and vascular diseases in ex vivo and in vivo models (47-
49). It was concluded that GC use, especially at high doses,
is complicated by its adverse outcomes such as thrombotic
events or the acceleration of inflammatory response in
conditions such as myeloma and osteonecrosis (5). In
several diseases, GC mechanisms mostly result in positive
feedback loops to potentiate the disease process (7).
In bone-vascular diseases such as osteonecrosis, high-
Figure 4. Representative Images of HUVEC Apoptosis in Different Time Points (3, 6, 12, 24, and 36 hours) Compared to the DEXA Free Medium as a Control Group (Magnification = ×400). Note: DEXA: Dexamethasone; HUVECs: Human umbilical vein endothelial cells; The apoptotic index significantly increased over time.

Figure 5. One Micromolar DEXA Increased Cellular Apoptosis Over Time. Note. DEXA: Dexamethasone; There was no significant increase in apoptosis 3 hours after treatment with DEXA compared to the control group. A significant increase in the apoptotic index was observed 12 and 24 hours after the treatment. The maximum apoptotic index was revealed 24 hours after the treatment. No significant difference was observed between 24- and 36-hour time points. *P value >0.05, Not significant.

dose GC could decrease tissue plasminogen activator activity (t-PA) while increasing plasma plasminogen activator inhibitor-1 (PAI-1) antigen levels resulting in a hypercoagulable state (47). Despite the strong association of GC with vascular diseases, the underlying mechanisms of GC remain unclear in many cases, and more in vivo and in vitro studies are needed to elucidate the role of GC, especially in cell apoptosis. Given that HUVEC represents a macrovascular system, comprehensive studies on microvascular systems are recommended as well. This is an interventional study to mimic the pathological events of GC-induced endothelial cell apoptosis. In addition, gene expression and other in vitro studies in this pathological condition can be assessed to understand the molecular pathogenesis of bone and vascular diseases such as ANFH.

Conclusions
Overall, our findings suggested that GCs can affect cellular apoptosis in a dose- and time-dependent manner. Accordingly, identifying the cellular expression profile during different stages of GC-induced cellular apoptosis can lead to a better understanding of the molecular pathogenesis of vascular and bone diseases.

Authors’ Contribution
Conception and design: MAK; Experiments performance: FP; Manuscript writing: FP and MAK; Final approval of manuscript: FP and MAK.

Conflict of Interests
The authors declare that they have no financial interest or conflict of interests regarding the study.

Ethical Issues
The entire procedure was approved by the Ethics Committee of Mashhad University of Medical Sciences (reference No. 910571) and carried out after obtaining the written informed consent of the donors.

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