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中文摘要

角膜內皮細胞是影響角膜含水量的最重要組織。它可以經由細胞間的緊密相連，和 Na-K-ATPase 作用而調節角膜含水量。然角膜內皮細胞雖有重要生理作用，相關研究卻相當缺乏。在角膜受到傷害，如發炎，手術傷害，角膜移植瓣排斥時，類固醇 (Dexamethasone) 是廣泛利用的藥劑。若能了解類固醇在角膜內皮細胞上扮演的生理機轉，不但能擴展知識，甚至可引導至治療層面之上。近年來許多研究指出類固醇在細胞的生理作用上扮演重要的腳色，例如它會影響傷口癒合，細胞分裂，細胞移行，細胞凋零，Na-K-ATPase 活性和細胞骨骼的維持等等。因此，我們利用初步培養的牛角膜內皮細胞做為細胞模式，並加入不同濃度的類固醇 (Dexamethasone)。研究結果顯示，我們利用細胞免疫染色法，RT-PCR 和西方墨點法先證實了牛角膜內皮細胞確實表現類固醇接受器。接著我們利用 MTS 和 flow cytometry 方式偵測類固醇是否影響牛角膜內皮細胞的細胞分裂及細胞凋零，結果顯示在加入高濃度的類固醇確實會降低細胞分裂的能力而增進細胞凋零。我們並利用非同位素的方法來測定類固醇是否影響牛類角膜內皮細胞的 Na-K-ATPase 酵素活性，結果顯示在加入某種濃度的類固醇會降低牛類角膜內皮細胞的 Na-K-ATPase 酵素活性。

關鍵字: 角膜內皮細胞，細胞凋零，類固醇，細胞分裂，Na-K-ATPase 酵素活性

ABSTRACT

Purpose: To assess the in-vitro effects of dexamethasone (DEX) on the proliferation, apoptosis, and Na⁺-K⁺-ATPase activity of bovine corneal endothelial cells.

Methods: Bovine corneal endothelial cells were cultured with DEX ranging from 10⁻¹⁰ to 10⁻³ M. The effect of DEX on the proliferation was analyzed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay. Apoptosis and necrosis were detected by staining with fluorescein-conjugated annexin V and propidium iodide, followed by flow cytometry. The effect of DEX on Na⁺-K⁺-ATPase activity was evaluated using non-isotopic methods.

Results: DEX did not affect cellular proliferation or induce apoptosis/necrosis from 10⁻¹⁰ to 10⁻⁵ M. At 10⁻⁴ and 10⁻³ M, DEX significantly decreased proliferation and increased apoptosis and/or necrosis. DEX significantly increased the Na⁺-K⁺-ATPase activity from 10⁻⁸ to 10⁻⁶ M, with the maximal effect at 10⁻⁶ M ($p < 0.01$); this effect was inhibited by RU38486, an antiglucocorticoid molecule.

Conclusions: Bovine corneal endothelial cells express glucocorticoid receptor (GR) mRNA and protein. DEX decreases cell proliferation and induces cellular apoptosis and/or necrosis at high concentrations. DEX also increases the Na⁺-K⁺-ATPase activity at certain concentrations.

KEYWORDS Corneal endothelial cells; apoptosis; dexamethasone; proliferation; Na⁺-K⁺-ATPase

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INTRODUCTION

Dexamethasone (DEX), a glucocorticoid clinically used as an anti-inflammatory agent and immunosuppressant, exerts dramatic effects on various tissues. The action of DEX is believed to be mediated by glucocorticoid receptors (GR).¹⁻³ DEX inhibits inflammation through multiple mechanisms.⁴⁻⁶ Interestingly, the GR response gene may positively or negatively regulate the actions according to different types of cells and different dosages of DEX treatment.^{7,8} Although not clearly defined, the dual effects of glucocorticoids may be the result of cross-talk between nuclear comodulators, or of interactions of transcription factors.^{9,10} Different types of ocular tissues are known to express GR and are responsive to glucocorticoid treatment.^{7,8,11-13} Although DEX effectively reduces ocular surface inflammation, delays excess corneal wound healing, and reduces pain, prolonged use of DEX induces numerous ocular side effects such as glaucoma and cataract.¹⁴⁻¹⁶ Numerous studies have demonstrated the changes of corneal stromal and epithelial wound healing after DEX use, implying that steroid eyedrops impaired wound healing rather than having no effect.^{17,18} Under particular conditions, DEX also affected proliferation and induced apoptosis and/or necrosis in corneal epithelial cells and keratocytes.^{7,8} Corneal endothelium controls the hydration status of the cornea. The cell density, pumping activity including Na⁺-K⁺-ATPase, and the cell-cell barrier are the major factors maintaining the functional homeostasis of the cornea.¹⁹⁻²¹ Unlike corneal epithelium or keratocytes, human corneal endothelial cells have limited proliferative capacity in vivo.^{21,22} The aging process, trauma, corneal graft rejection, stress caused by certain systemic diseases, endothelial dystrophies, and improper medications may all cause corneal endothelial cell loss and permanent damage.²³⁻²⁵ To avoid this, appropriate medications that diminish damage to corneal endothelial cells and promote corneal endothelial cellular function are extremely important. Clinically, DEX is frequently used for corneal endothelial diseases.²⁶⁻²⁸ Little is known, however, about the direct effects of DEX on these cells. In the present study, we used cultured bovine corneal endothelial cells as the experimental model. Whether or not GR mRNA and its corresponding protein are expressed in bovine corneal endothelial cells and the in-vitro effects of DEX on these endothelial cells, such as proliferation, apoptosis, necrosis, and Na⁺-K⁺-ATPase activity, were assessed.

MATERIALS AND METHODS

Cell Culture of Bovine Corneal Endothelial Cells

Adult bovine corneal buttons were obtained within 3 h after enucleation. Corneal endothelial cells with Descemet's membrane were dissected under the microscope and separated from the stroma after treatment with trypsin-EDTA (Invitrogen-Gibco, Paisley, UK). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (Gibco BRL/Life Technologies, Rockville, MD, USA).

Second passage cells were used in all the experiments. For the proliferation and apoptosis parts of the experiment, we added 2×10^{-5} cells/well in 24-well tissue culture plates (Costar) and waited 1 d before DEX was added. For the Na⁺-K⁺-ATPase activity part of the study, we waited until the cells were confluent before DEX was added in order to mimic in-vivo conditions.

Drug Preparation

DEX, purchased from Sigma-Aldrich (Saint Quentin Fallavier, France), was diluted in 0.1% absolute ethanol before being added to the cultured medium. On the second day, tissue culture medium was replaced by 1 ml medium containing various concentrations of DEX (10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , and 10^{-10} M). The culture media were changed daily for 6d. Mifepristone (RU38486; Roussel Uclaf, Romainville, France), an antiglucocorticoid molecule, was dissolved in absolute ethanol. RU38486 (10^{-5} M) was added daily to the culture medium, alone or in combination with 10^{-6} M DEX, for six days. All experiments were repeated six times.

Glucocorticoid Receptor Analysis

GRs were studied using reverse transcriptase-polymerase chain reaction (RT-PCR). cDNA was prepared from 1 μ g total RNA by RT in a 20 μ l volume, using reagents from a commercially available kit (Promega, Pittsburgh, PA, USA). Primers specific for bovine GR were designed using MacVector 5.0 software (Oxford Molecular Group, Oxford, UK). The primer pairs used were: GR upstream sequence, 5_-GTTTCTGCGTCTTCACCCTCA-3_; downstream sequence, 5_-GTCTCTCCCATATACAGTCCC-3_. PCR was performed in a reaction mixture containing 1 μ g cDNA and 2.5 μ M each of the upstream and downstream primers, plus reagents from a commercially available kit (Gibco). PCR was performed in a thermal cycler. The PCR products were electrophoresed in 2% agarose gels containing ethidium bromide and photographed under UV illumination (665 film; Polaroid, Cambridge, MA, USA). Additionally, the PCR product was sequenced and checked for percent homology with the sequence from 1 to 158 bp of bovine GR, specified as BTU37383 in the Genbank.

Immunocytochemical Localization of Glucocorticoid Receptors

Bovine corneal endothelial cells cultured with 10^{-6} M DEX for 6 d were fixed, permeabilized, blocked, and incubated with primary antibody at room temperature for 2 h. The primary antibody, a rabbit polyclonal antibody (No. PAI-511, Dianova-Immunotech, Hamberg, Germany), was confirmed to have positive immunocytochemical staining for GR in bovine cells.²⁹ The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 37°C. The cells were then washed and mounted in medium containing propidium iodide (PI; Vector Laboratories, Burlingame, CA, USA). Immunocytochemical study was also conducted on transverse cryosections of bovine cornea. The staining patterns were observed by conventional fluorescence microscopy using an Eclipse E800 Nikon

microscope with a VFM Epi-fluorescence attachment (Nikon, Melville, NY, USA) equipped with a Spot digital camera and Spot version 1.1 CE software (Diagnostic Instruments, Sterling Heights, MI, USA).

SDS-PAGE and Western Blotting of Glucocorticoid Receptors

Cultured cells were trypsinized, suspended, and homogenized. Equal amounts of protein were electrophoresed and transferred to a polyvinylidene difluoride membrane (Millipore Corp, Bedford, MA, USA). After blocking, the membranes were incubated with 1:750 polyclonal rabbit anti-human GR antibody (Rabbit, No. PAI-511, Dianova-Immunotech, Hamburg, Germany). The membranes were then washed and incubated with 1:6000 horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology). The signal was detected using enhanced chemoluminescence according to the manufacturer's instructions (Pierce, Rockford, IL, USA) and exposed to autoradiographic film.

MTS Assay

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) and electron coupling reagent were purchased from Promega and Sigma, respectively. After 2, 4, and 6 d of culture, 100 μ l culture medium and 20 μ l reagents were incubated at 37°C for 1 h. In addition, corneal endothelial proliferation was analyzed by staining the nucleus with amidoblack (Sigma) and counted using a hemocytometer and a cell counter (Coulter, Hialeah, FL, USA). To clarify whether the change of living cellular amount was caused by cytotoxicity or change of proliferation, immunocytochemical staining with Ki67 was performed to evaluate the proliferative status.

Apoptosis Assay

The cells, cultured in different concentrations of DEX for 6 d, were washed with cold PBS, and 1×10^6 cells were incubated in 195 μ l annexin-binding buffer containing 5 μ l annexin V and 5 μ l 20 μ g/ml PI for 15 min at room temperature. After incubation, 400 μ l annexin-binding buffer was added. The cells were then analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Rungis, France). At least 20,000 cells were analyzed for each experiment. AV-/PI- cells were considered viable, AV+/PI- cells were considered early apoptotic cells, and AV+/PI+ cells were considered necrotic cells. Bovine corneal endothelial cells cultured in DMEM medium with 0.1% absolute ethanol were used as the control group. All experiments were repeated six times.

Na⁺-K⁺-ATPase Activity

Cells were harvested 2, 4, and 6 d after DEX exposure for the determination of Na⁺-K⁺-ATPase activity. Aliquots of the suspension of bovine endothelium cell homogenates were used. Na⁺-K⁺-ATPase activity was quantified using the method of Hwang and colleagues with minor modifications.^{30–32} In brief, the sample was prepared in reaction buffer. After incubation, ice-cold trichloroacetic acid was added to stop the reaction. The vials were centrifuged, and the supernatant and the phosphate

standard were added with 2.5% acid molybdate solution, 10% SDS, and 0.025% 1-amino-2-naphthol-4-sulformic acid. After incubation, the inorganic phosphate production was measured at 700 nm according to the method described by Peterson.³³ The enzyme activity of Na⁺-K⁺-ATPase was defined as the difference between the inorganic phosphate liberated in the presence and absence of 0.5 mM ouabain in the reaction mixture, and calculated by comparison with the concentration of inorganic phosphate standard. In this study, the percentage of activity compared to the control group was used for analysis.

Statistical Analysis

Experimental data were analyzed using a one-factor analysis of variance (ANOVA), Dunnett's multiple comparison test, and Student's two-tailed *t*-test. The results are expressed as the mean \pm standard error of the mean (SEM). The probability of error $p < 0.05$ was considered to be statistically significant.

RESULTS

Detection of Glucocorticoid Receptor mRNA in Cultured Bovine Corneal Endothelial Cells

RT-PCR demonstrated the existence of GR mRNA in cultured bovine corneal endothelial cells. A unique PCR product of 158 nucleotides was detected with ethidium bromide after gel electrophoresis (Fig. 1). The size of the band was consistent with the expected fragment size, determined from bovine GR cDNA. Additionally, the PCR product was sequenced and exhibited 97% homology with bovine GR BTU37383 in the Genbank.

Detection of Glucocorticoid Receptor Protein in Cultured Bovine Corneal Endothelial Cells

Specific nuclear staining of GRs was observed in cultured cells (Fig. 2 A, B) and corneal endothelial cells on tissue sections (Fig. 2 C, D). The negative control showed no staining (data not shown). The Western blot results also demonstrated the existence of GR protein in the freshly obtained bovine corneal endothelial cells and cultured bovine corneal endothelial cells (Fig. 2E–G).

Effect of DEX on Cellular Proliferation Evaluated by MTS Assay

After six days of culture, DEX did not change the amount of living cells at concentrations ranging from 10^{-10} to 10^{-5} M. In contrast, DEX at 10^{-4} and 10^{-3} M significantly decreased the amount of living cells ($p < 0.01$; Fig. 3). There were no significant effects of DEX on the amount of living cells at Day 2 and Day 4 (data not shown). All of the results of the MTS assays were confirmed by flow cytometry and hemocytometer counting assays (data not shown).

Apoptosis Assay

After six days of culture, the percentage of viable cells, apoptotic cells, and necrotic cells did not change at DEX concentrations ranging from 10^{-5} M to 10^{-10} M. However, the percent viable cells decreased when 10^{-4} and 10^{-3} M DEX was added to the

cultured medium. Corneal endothelial apoptosis and necrosis were significantly enhanced when 10^{-4} and 10^{-3} M DEX was added ($p < 0.01$; Fig. 4). All of the results of the apoptosis/necrosis assay were compatible with the live/dead cell assay (data not shown). There were no significant effects of DEX on the percentage of viable cells, apoptotic cells, and necrotic cells on Day 2 and Day 4 (data not shown).

Effect of DEX and RU38486 on Na⁺-K⁺-ATPase Activity

After six days of culture, we observed a dose dependent effect of DEX on Na⁺-K⁺-ATPase activity of bovine corneal endothelial cells at DEX concentrations of 10^{-8} M and 10^{-6} M. However, at higher (10^{-4} M) or lower (10^{-10} M) concentrations of DEX, there was no significant difference compared to the control group (Fig. 5A). There were no significant effects of DEX on Na⁺-K⁺-ATPase activity on Day 2 and Day 4 (data not shown). The addition of 10^{-5} M RU38486 alone to the culture medium had no significant effect on the Na⁺-K⁺-ATPase activity compared to the effect of the ethanol control treatment. Nonetheless, it significantly decreased the promoting effect of Na⁺-K⁺-ATPase activity at 10^{-6} M DEX (Fig. 5B).

DISCUSSION

Corneal endothelial cells participate in maintaining corneal transparency by barrier and 'pump' functions.²¹ Since human corneal endothelial cells have limited proliferative capacity throughout the human lifespan and may lose the ability to maintain corneal transparency once cellular loss reaches a critical level, any insult that potentially causes corneal endothelial cell damage should be recognized and avoided.^{21,22} DEX is widely used for the treatment of ocular inflammation. The originality of the present work is the possible effect of low-dose DEX on the various important cellular functions of cultured bovine corneal endothelial cells. We first used immunocytochemistry, RT-PCR, and Western blot analysis to show that GR mRNA and protein are expressed in cultured bovine corneal endothelial cells. We also used RU38486 (mifepristone), an 11-substituted 19-norsteroid steroid antagonist with antiprogestone and antigluocorticoid effects,³⁴ to inhibit the function of DEX. RU38486 inhibited the 10^{-6} M DEX-induced increase in Na⁺-K⁺-ATPase activity. This is pharmacologic evidence of the existence of functional GRs in corneal endothelial cells. Cenedella and Fleschner³⁵ reported cholesterol biosynthesis by the rabbit cornea and showed that possible endogenous metabolites synthesized from cholesterol can be found in the cornea. Since GRs were identified in all three layers of corneal cells, it is reasonable to suggest that endogenous steroids may have autocrine and/or paracrine roles in the cornea and that exogenous steroids may have direct functions on these cells.

It has been shown that AP1 components (c-Fos and c-Jun) are expressed in corneal endothelial cells.^{36,37} These proto-oncogenes may play an important role in modulating cell functions. DEX and other steroids could interact through the AP1

proto-oncogenes. GR and AP1 interactions have been described in the glucocorticoid response elements (GREs), where these two transcriptional factors are adjacent.³⁸ We observed non-responsiveness to DEX for endothelial cell proliferation at DEX concentrations from 10^{-10} to 10^{-5} M, and significant inhibition of cell proliferation at higher concentrations (10^{-4} M and 10^{-3} M, $p < 0.01$). A previous study using immunostaining with Ki 67 demonstrated that confluent cultured rat corneal endothelial cells cease to proliferate.³⁹ We also found no proliferation in all experimental groups in this study on Days 2, 4, and 6 (data not shown). The change in the MTS assay result is thus supposed to be caused by cytotoxicity instead of altered proliferation.

The similar response curve for the hemocytometer cellcounts and the MTS assay results provides evidence that they measured similar effects. DEX induced an apparent paradoxical biphasic effect (i.e., proliferation after exposure to low-dose DEX and inhibition of growth after exposure to high-dose DEX) on human corneal epithelial cells, rabbit conjunctival cells, dermal fibroblasts, retinal pigment epithelial cells, and human corneal keratocytes.^{7,8,40,41} It is quite interesting to find that cultured bovine corneal endothelial cells seem to be more refractive to survival/proliferation under treatment of DEX. In vivo, corneal endothelial cells are unique compared to other corneal cells for having a limited proliferative capacity due to various intrinsic and extrinsic factors.²¹ Although an in-vitro system differs from an in-vivo one, it is not surprising that DEX does not increase cellular proliferation of cultured corneal endothelial cells. However, when the concentration of DEX reached 10^{-4} to 10^{-3} M, cell numbers significantly decreased. Since the percentage of apoptotic and necrotic cells also increased at this concentration, we believe that the decreased cell number was mainly due to the significant increase in cellular death.

Apoptosis in the corneal endothelium has been suggested to play a major role in cell loss of various conditions.^{42,43} Glucocorticoid-induced cell death or apoptosis has been described in various cell systems.⁴⁴⁻⁴⁶ However, glucocorticoid was shown to protect other cell types, such as epithelial cells of the mammary gland, hepatocytes, and thymocytes.^{47,48} GR, as a signaling protein, can display protoapoptotic or anti-apoptotic activity depending on cell context. Cross talks between ubiquitous signaling proteins can produce context-specific effects on cell survival.⁴⁹⁻⁵¹ We found no significant change in the apoptosis or necrosis rate of corneal endothelial cells with DEX concentrations from 10^{-10} to 10^{-5} M (10^{-7} to 10^{-8} M, intracameral concentrations after topical usage).^{52,53} In contrast, the same concentrations of DEX did induce apoptosis or necrosis of human keratocytes and corneal epithelial cells.^{7,8} The refractiveness of DEX to induce apoptosis on corneal endothelial cells can be roughly explained by two mechanisms: refractiveness to induce the 'death gene' by activated GR or refractiveness to repress the survival function.⁸ At higher concentrations of DEX (10^{-4} to 10^{-3} M, close to eyedrop concentrations), however, toxicity which caused cellular apoptosis and necrosis was found. This finding differs from those of some other studies. Reinhard and Sundmacher⁵⁴ showed that

intracameral steroid injection seemed to be a safe and helpful therapeutic measure in the treatment of moderate and severe endothelial immune reactions without complications to the human corneal endothelial cells. Wang et al.⁵⁵ used an in-vitro MTT viability assay and demonstrated relatively low complication rates for steroid therapy of porcine corneal endothelial cells compared to some antibiotics. Although their study results are different from ours, Solomon et al.⁵⁶ demonstrated adverse effects of corticosteroid on the morphology of cat corneal endothelial cells after trauma. In spite of the need for in-vivo studies to prove our DEX effects, our results point out the potential side effects of DEX on corneal endothelial cells and the necessity for using DEX with caution.

As for the effect of DEX on Na⁺-K⁺-ATPase activity, we found a small but statistically significant increase in enzyme activity at concentrations of 10⁻⁶ and 10⁻⁸ M (*p* < 0.01). Previously, very few reports demonstrated the possible pharmacologic increase in Na⁺-K⁺-ATPase activity in rabbit corneal endothelial cells,⁵⁷ and our study is the first to demonstrate that DEX increased Na⁺-K⁺-ATPase activity in these cells. There are several studies showing the effects of DEX to regulate Na⁺-K⁺-ATPase gene expression through multiple complex mechanisms, including transcription, posttranscriptional, translational, and enzyme activity in a variety of tissues.⁵⁸⁻⁶² In some cells, one or more of these processes may coexist in the regulatory process. Since we did not examine the sodium pump protein levels or mRNA levels, the mechanisms for DEX to increase enzyme activity of corneal endothelial cells await to be defined. The glucocorticoid-induced increase of Na⁺-K⁺-ATPase in this study, however, suggests a potential contribution of the effectiveness of DEX in treating corneal endothelial dysfunction. Although DEX seemed to increase Na⁺-K⁺-ATPase activity in this study, which may play a beneficial role in the enhancement of the recovery of corneal endothelial pumping dysfunction, DEX can also induce unwanted corneal endothelial cellular necrosis and apoptosis at higher concentrations. Long-term and high-dose DEX administration induces glaucoma, cataract, and other ocular complications.^{17,22-27} Therefore, shortening the treatment course and adjusting the dosage of DEX are important. In addition, the in-vivo effect of DEX on Na⁺-K⁺-ATPase activity still needs to be confirmed.

In conclusion, our experimental results indicate the expression of GRs in cultured bovine corneal endothelial cells. Such receptors may influence bovine corneal endothelial proliferation, apoptosis/necrosis, and Na⁺-K⁺-ATPase activity in a dose-dependent way. Although the in-vitro effects do not necessarily correlate with those found in vivo, human corneal endothelial cells may differ from bovine cells, and young and non-confluent cells may not be similar to old and confluent cells, our results at least suggest that DEX may influence the physiological function of young bovine corneal endothelial cell in vitro instead of having no effect. The in-vivo function of DEX on corneal endothelial cells warrants further study.

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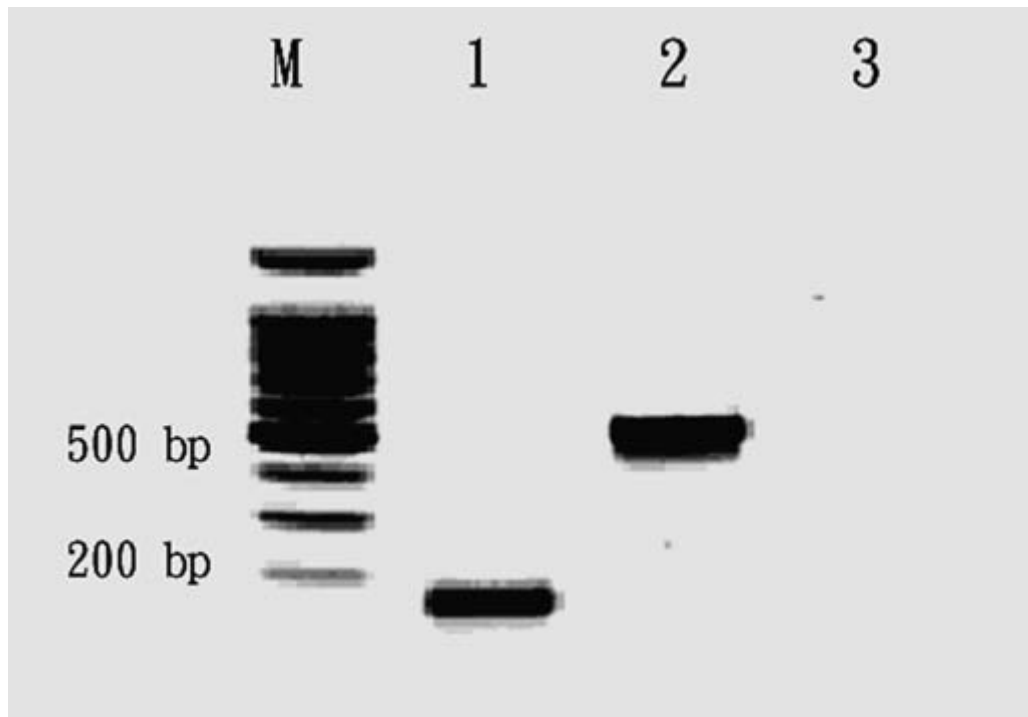


FIGURE 1 Electrophoretic profile of the bovine glucocorticoid receptor product obtained from reverse transcription-polymerase chain reaction (RT-PCR). One microgram of total RNA extracted from cultured corneal endothelial cells was reverse-transcribed using oligo(dN) and oligo(dT) primers. The obtained cDNA was electrophoresed, and the bands were visualized by ethidium bromide staining. Lane 1: cultured bovine corneal endothelial cells showing a length of 158 bp. Lane 2: GAPDH (BG223947. 1M0017C04 Bovine, 2001 in Genbank) as positive control. GAPDH is the product of a bovine skeletal muscle structural gene with 516 bp. Lane 3: PCR negative control. Lane M: 100 bp DNA ladder.

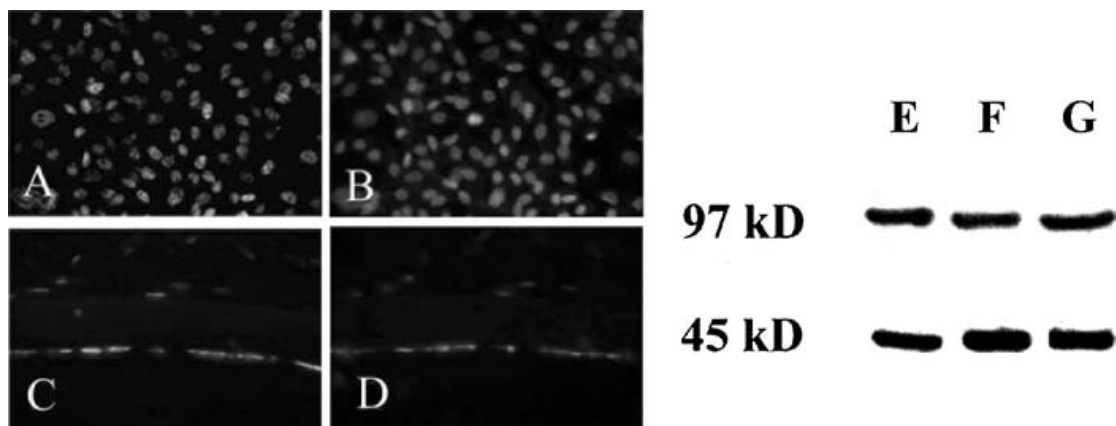


FIGURE 2 Immunocytochemical staining and Western blot analysis demonstrated the expression of GR protein in cultured bovine corneal endothelial cells and fresh obtained corneal endothelial cells. (A,B) Immunocytochemical staining of cultured corneal endothelial cells with antiglucocorticoid receptor antibody. (C,D) Immunocytochemical staining of corneal sections with antiglucocorticoid receptor antibody. Expression of GR was found in the nucleus in both cultured cells and tissue sections. Green: nuclear staining of GR. Red: stained for propidium iodide, which is a nuclear marker. Inverted fluorescent microscopy. Original magnification: $\times 400$. (E–G) Western blot analysis confirmed the existence of GR in cultured bovine corneal in (E) medium with DEX, (F) medium without DEX, and (G) freshly obtained corneal endothelial cells. The band at 97 kD confirmed the expression of GR, while the band at 47 kD was β -actin (internal control).

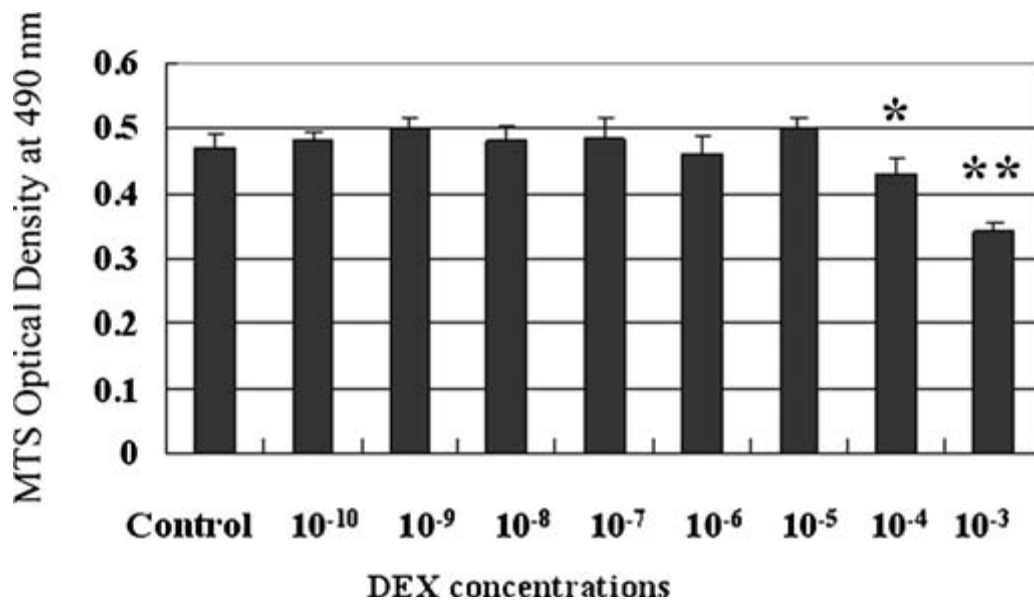


FIGURE 3 Corneal endothelial survival studied by MTS assay. Bovine cornea1 endothelial cells were cultured with various concentrations of DEX (10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, and 10⁻¹⁰ M) diluted in 0.1% absolute ethanol for 6 d. Results of MTS assay are expressed in optical densities, measured spectrophotometrically at 490 nm. Bars represent mean ± SEM (n = 6 in each group). The control consisted of corneal endothelial cells cultured in 0.1% absolute ethanol without DEX. DEX did not affect the number of living cells at DEX concentrations from 10⁻¹⁰ to 10⁻⁵ M. However, 10⁻⁴ and 10⁻³ M DEX had an inhibitory effect on cell proliferation. *p < 0.01, **p < 0.001 by Dunnett's test.

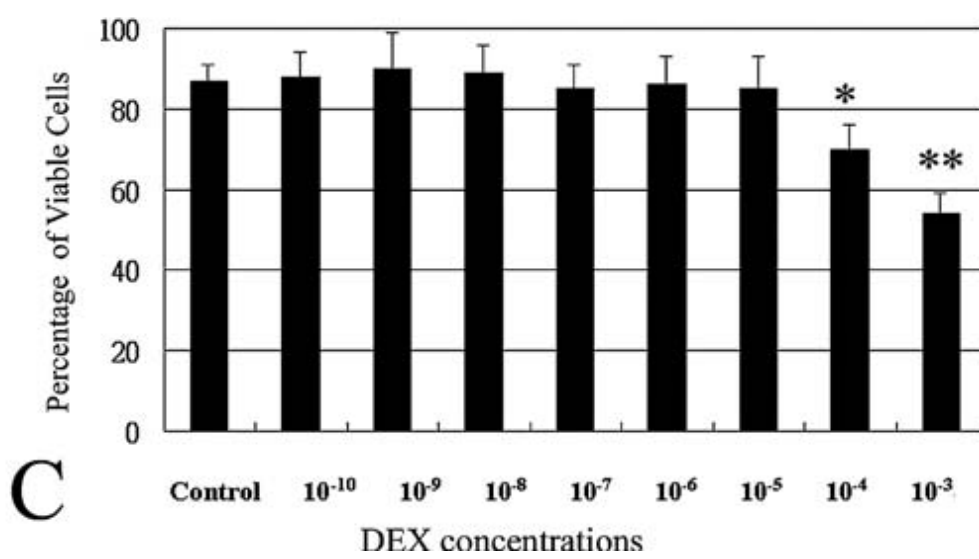
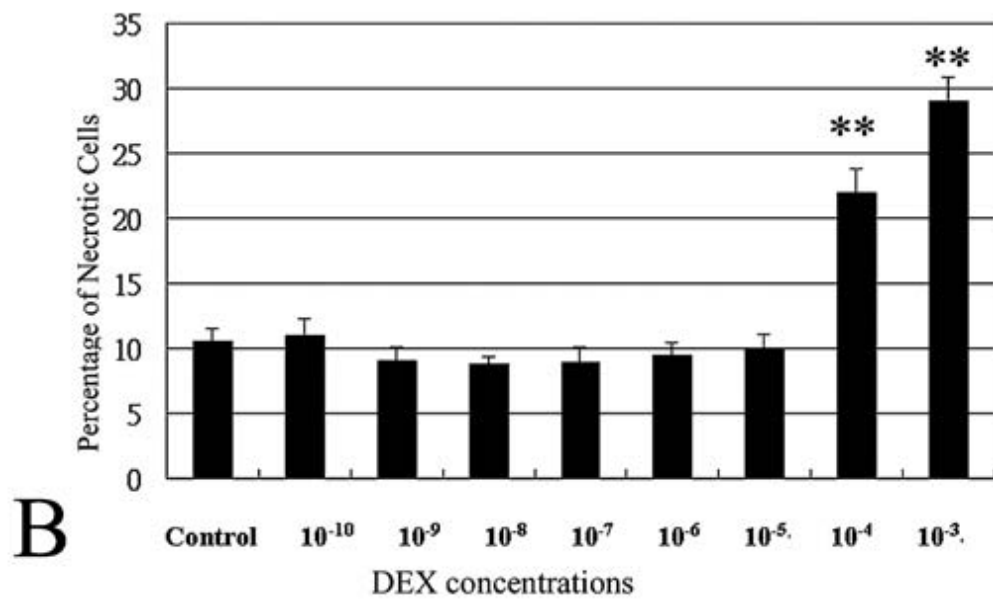
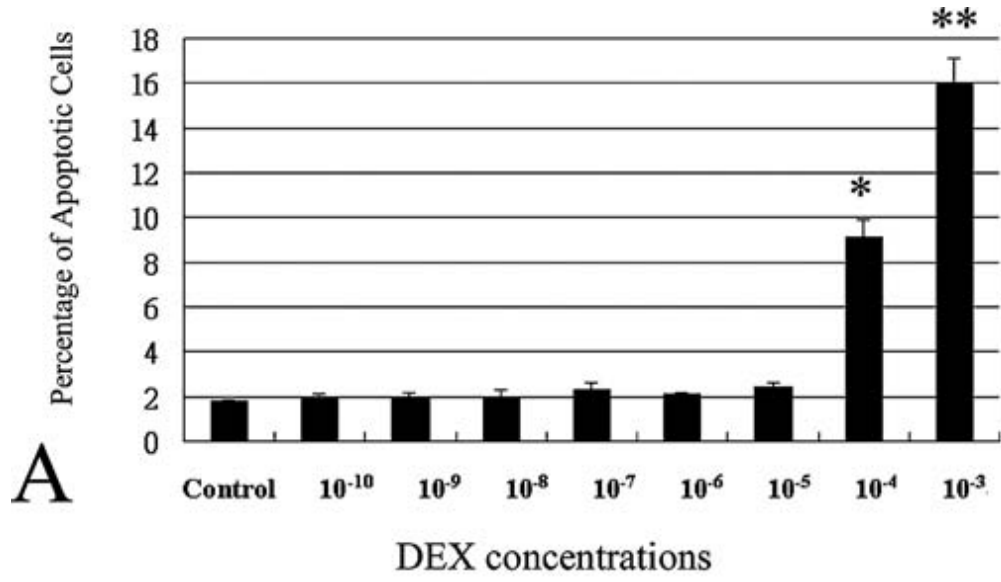


FIGURE 4 Apoptosis, necrosis, and viability assay. Results are expressed for (A) apoptotic, (B) necrotic, and (C) vital cells as the percentage of cells (mean \pm SEM). Apoptosis and necrosis were significantly enhanced by adding 10^{-4} and 10^{-3} M DEX to the culture medium. Viable cells were significantly decreased by adding 10^{-4} and 10^{-3} M DEX to the culture medium. Blot shows mean \pm SEM (n = 6). *p < 0.01, **p < 0.001 by Dunnett's test.

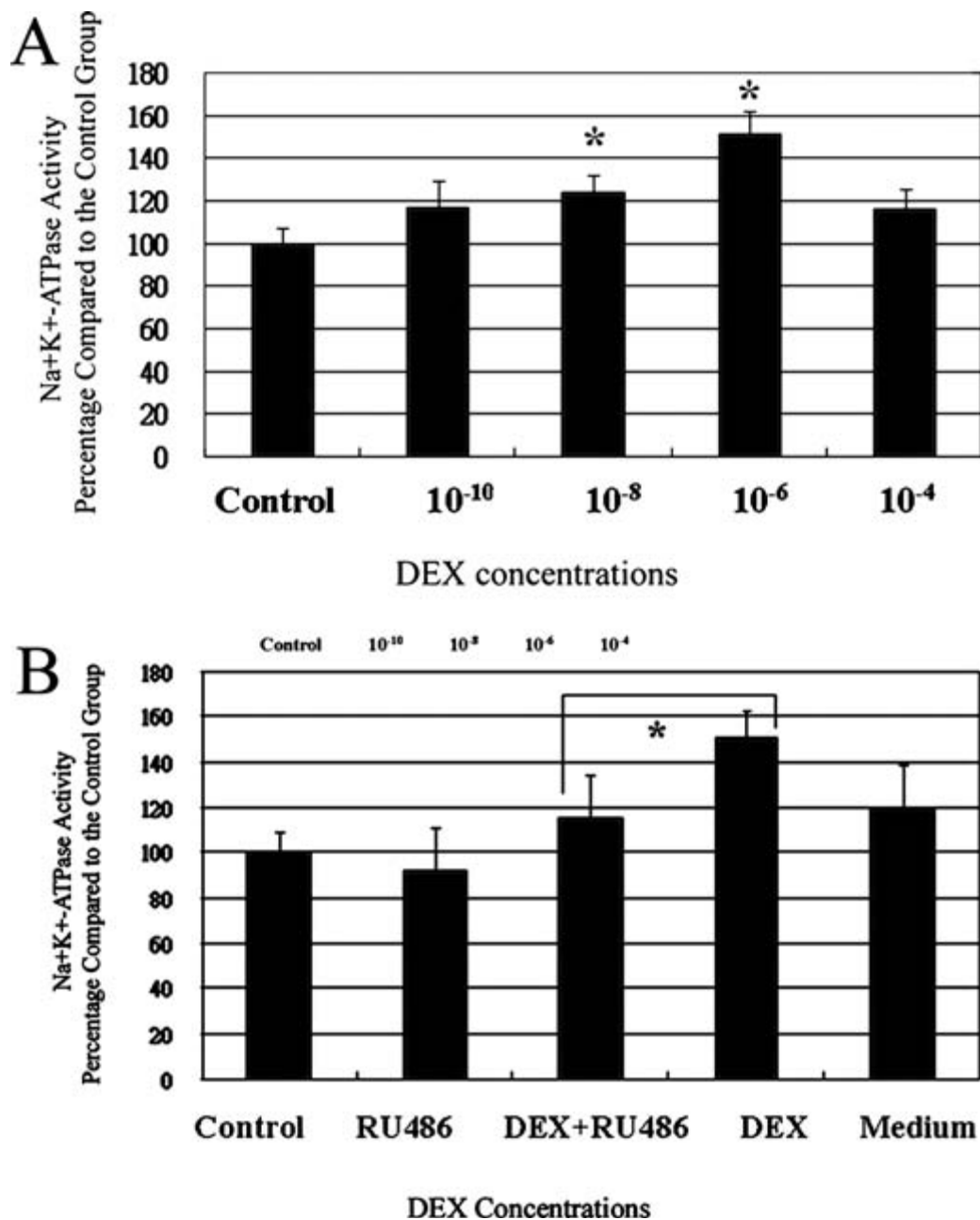


FIGURE 5 (A) Na⁺-K⁺-ATPase activity assay. Na⁺-K⁺-ATPase activity was significantly enhanced by adding 10⁻⁸ M and 10⁻⁶ M DEX to the culture medium. Blot shows mean ± SEM (n = 6). *p < 0.01, by Student's t -test. (B) RU38486 antagonist effect. RU38486 (mifepristone), an 11-substituted 19-norsteroid steroid antagonist, which has antiprogestosterone effects (at low concentrations) and antiglucocorticoid effects (at high concentrations). Bovine corneal endothelial cells were cultured for 6 d. Five groups were compared: control (0.1% final absolute ethanol in medium), RU486 (10⁻⁵ M RU38486 diluted in 0.1% final absolute ethanol), DEX and RU486 (10⁻⁶ M DEX + 10⁻⁵ M RU38486 diluted in 0.1% final absolute ethanol), DEX (10⁻⁶ M DEX diluted in 0.1% final absolute ethanol), and Med (medium without adding ethanol, DEX, or RU38486). The addition of 10⁻⁵ M RU38486 inhibited increased Na⁺-K⁺-ATPase enzyme activity with 10⁻⁶ M DEX treatment. However, the addition of 10⁻⁵ M RU38486 did not significantly inhibit the Na⁺-K⁺-ATPase enzyme activity compared to the effect of ethanol control treatment. Blot shows mean ± SEM (n = 6). (A) *p < 0.01 by Dunnett's test. (B) *p < 0.01 by Student's t -test.

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