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# Diverse effects of synthetic glucocorticoid species on cell viability and stress response of neuroblastoma cells

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ABSTRACT

Glucocorticoids (GCs) are widely used as powerful anti-inflammatory and immunosuppressive therapeutics in multiple pathological conditions. However, compelling evidence indicates that they might promote neurodegeneration by altering mitochondrial homeostatic processes. Although the effect of dexamethasone on cell survival and homeostasis has been widely investigated, the effect of other glucocorticoids needs to be explored in more detail. In this report, we have compared the neurotoxicity induced by dexamethasone, prednisolone, betamethasone, and hydrocortisone in cultured neuroblastoma cells, through the analysis of several parameters such as cell viability, ER stress, oxidative stress, and mitochondrial fusion and fission markers. Interestingly, we have found that synthetic glucocorticoids may impact neuronal viability by affecting different cellular responses, suggesting that their therapeutic use should be consciously decided after careful consideration of benefits and detrimental effects.

## Introduction

Synthetic glucocorticoids (GCs) are largely used as powerful anti-inflammatory and immunosuppressive therapeutics in multiple pathological conditions.

GCs are effective in treating various inflammatory diseases of the central nervous system (CNS), peripheral nerves, neuromuscular junction, and muscles. Additionally, they provide therapeutic benefits in certain neurological conditions via non-inflammatory mechanisms. For example, in CNS malignancies, glucocorticoids alleviate vasogenic edema, while in Duchenne muscular dystrophy (DMD), they are pivotal for maintaining ambulation, predominantly via non-inflammatory pathways (Moxley et al., 2006; Galati et al., 2021). Despite dosing variations based on disease and indication, clinicians must grasp the immunosuppressive consequences, toxicity, and adverse effects of glucocorticoids across neurological disorders. Recent studies indicate that prolonged exposure to GCs might promote the development of both metabolic and neurodegenerative disorders (de Guia, 2020; De Nicola et al., 2020). In particular, dexamethasone has been reported to induce cell death in striatal cells (Haynes et al., 2004), cerebellar neurons (Jacobs et al., 2006), SH-SY5Y cells (Tazik et al., 2009), and in the granule cell layer of the hippocampus (Almeida et al., 2000), and causes steroid-induced glaucoma by promoting the death of retinal ganglion cells (Armaly, 1963; Biedner et al., 1980; Caplan et al., 2017; Roberti et al., 2020). Moreover, scientific evidence indicates that stress-level administration of GCs may exacerbate the advancement of neurodegenerative disorders such as Alzheimer's disease (AD) (Green et al., 2006; Fang et al., 2023).

Neuronal survival requires healthy mitochondria, as deregulation of mitochondrial homeostatic processes may lead to neuronal cell death (Reeve et al. 2018; Intihar et al. 2019). Indeed, the impairment of mitochondrial dynamics is associated with a variety of neurological diseases such as Alzheimer's (Moreira et al. 2010; Moreira et al. 2006), Parkinson's, Huntington's diseases, and also in depressive disorders. Mitochondria dysfunction is mainly due to the increase in reactive oxygen species (ROS) production following exposure to GCs, with subsequent reduction of cellular energy yield and rise of cytosolic $Ca^{2+}$ concentration (Choi and Han, 2021). Such events alter the balance

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between fission and fusion mechanisms (Chen and Chan, 2009) and modify mitochondrial permeability, leading to the apoptotic death of neuronal cells (Cheung et al. 2007). Mitochondrial fission events are required in both dividing cells and during differentiation, as well as in response to new energy demands and toxin exposures (Westermann, 2010; Yang et al., 2020). On the other hand, mitochondrial fusion is crucial during development and has a protective role against apoptosis (Gottlieb, 2006).

Lastly, GCs themselves (Green et al., 2006) or through mitochondria dysfunction, could lead to phosphorylation and aggregation of Tau proteins, whose role in neurodegeneration are well known (Chiasseu et al., 2016). In a recent paper (Choi et al., 2021), it has been shown that stress-induced GCs, such as corticosterone, disrupt mitochondrial function and dynamics in hippocampal neurons, SH-SY5Y cells, and ICR mice, leading to mitochondrial accumulation instead of clearance via NIX-dependent mitophagy. Although the impact of dexamethasone on neuronal survival and mitochondrial dynamics has been investigated (Tazik et al. 2009; Müller et al. 2014; Laane et al. 2007; Suwanjang et al. 2019; Suwanjang et al. 2013), the effect of other glucocorticoids on similar experimental models remains elusive. As damaged mitochondria may promote neurodegeneration, we evaluate and compare the cell viability and organelle stress response to four GCs, dexamethasone, betamethasone, prednisolone, and hydrocortisone in SH-SY5Y cells, an undifferentiated neuroblastoma cell line which has been previously used as a model to investigate mitochondrial dynamics in response to GC-treatment (Suwanjang et al., 2013a).

## Experimental procedures

### *Cell line*

SH-SY5Y neuroblastoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (Biowest, Stockumer Kirchstraße, Düsseldorf, Germany) supplemented with heat-inactivated Fetal Bovine Serum at 10 % (Biowest; Stockumer Kirchstraße), penicillin and streptomycin. Cells were propagated at 37 °C in an atmosphere of 5 % $CO_2$.

### *Cell growth and viability*

Cell Growth was assessed by counting cells exposed to 500 nM of dexamethasone, betamethasone, prednisolone, or hydrocortisone. Firstly, about $10^5$ SH-SY5Y cells were seeded in each well of a 24-well plate and, after attachment, were treated or not in triplicate with different GCs. Every 24 h after the treatment, for a total of 72 h, the cells were washed, trypsinized, and counted on a Neubauer chamber grid. Average cell number was used to generate a growth curve. The viability assay was performed using the Cell Proliferation Kit II – XTT (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, $1.5x10^4$ cells were seeded in each well of a 96-well plate and, the day after, were treated or not with prednisolone, betamethasone, dexamethasone (all purchased from SIGMA-ALDRICH, Saint-Louis, MO, USA) and hydrocortisone (Abcr GmbH, Karlsruhe, Germany) at the indicated concentrations for 24 h and 48 h. After the treatment period, 50 µl of an XTT labeling mixture, formed by XTT labeling reagent and electron-coupling reagent, were added to the medium in each well (final XTT concentration 0.3 mg/ml) and incubated for 4 h. The assay is based on the cleavage of the tetrazolium salt XTT in viable cells, producing a soluble formazan salt. The formazan dye deposition was then quantified by reading absorbance at 450 nm with the microplate reader Seac-Sirio-S and normalized with respect to that in control wells. Two independent experiments were performed in triplicate and significance was assessed by ANOVA.

### *Annexin V/7AAD staining for apoptosis*

Apoptosis was assessed by using the eBioscience Annexin V Apoptosis Detection Kit eFluor 450 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and analyzed following the manufacturer's instructions. Briefly, SH-SY5Y cells were treated with 500 nM of GCs for 24 h. Later, $2*10^5$ cells were harvested and resuspended in a binding buffer. Then Annexin V-eFluor 450 and 7-AAD were added to the samples. Apoptosis of the samples was detected with the BD FACSCelesta™ Cell Analyzer (BD Bioscience, Franklin Lakes, NJ, USA). Annexin V-eFluor 450 only positive cells undergo early apoptosis, whereas 7-AAD-positive only cells are considered necrotic. Both Annexin V-eFluor450 and 7-ADD-positive cells undergo late apoptosis.

### *Western blot*

For protein extraction, cells were washed twice with ice-cold PBS, scraped, and centrifuged at 6000 rpm for 5 min at 4 °C. The pellet was then resuspended in ice-cold NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 % NP40, and 2 mM EDTA) supplemented with protease inhibitors (Roche, Basel, Switzerland). Protein concentration in cleared lysates was assessed by a Bradford Assay, using Quick Start Bradford Dye reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and an equal amount of proteins were used for western blot analysis. Separation by SDS-PAGE, blotting, and incubation with primary and secondary antibodies was performed as described elsewhere (Mazzone et al., 2020). The chemiluminescence reaction was carried out with Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the signal acquisition was performed through the ChemiDoc Xrs + by the Image Lab software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Antisera and monoclonal antibodies used in the present work are the following: anti-cyclinD1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti beta-actin (SIGMA-ALDRICH, Saint-Louis, MO, USA), anti-MFN2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-OPA 1 (ABclonal, Düsseldorf, Germany), anti-DRP1 (ABclonal, Düsseldorf, Germany)anti-P-JNK (Cell signaling, MA, USA), anti-caspase 8 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CHOP (PROTEINTECH), anti-GRP78 (Abcam), anti-PDRP1 (S616) (Cell signaling, MA, USA), anti-PDRP1 (S637) (Cell signaling, MA, USA), anti-ATF4 (GeneTex, Dongmei Rd., Taiwan), anti-ATF6 (Abcam, Cambridge, UK), HRP-conjugated anti-mouse and anti-rabbit (SIGMA-ALDRICH, Saint-Louis, MO, USA).

### *Immunofluorescence*

SH-SY5Y cells were cultured in a 24-well plate on sterile glass coverslips at 37 °C and, the day after, exposed or not to 500 nm of glucocorticoids (GCs) for 24 h. Then, the cells were fixed with methanol (MetOH) for 5 min at −20 °C and permeabilized with 0.2 % Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature, followed by rinsing with PBS three times. Non-specific antibody binding sites on the cells were blocked by incubating with 0.5 % bovine serum albumin (BSA) in PBS for 30 min at room temperature. Subsequently, the cells were incubated with a primary antibodies against OPA1 or DRP1 (1:250 in PBS) at 4 °C overnight, followed by incubation with Alexa 546 anti-rabbit IgG (1:250 in PBS) for 1 h at room temperature. After three washing steps with PBS, the cells were incubated with DAPI (0.1 mg/ml in PBS) for 20 min. Stained slides were mounted using PBS/glycerol (1:1) and observed under a fluorescence microscope at 20x magnification.

### *Gene expression analysis*

Gene expression analysis was performed as previously described (Zerillo et al., 2022). Briefly, total RNA was isolated from SH-SY5Y cells using MicroElute® Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA),

quantified with Thermo Scientific NanoDrop One Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1 μg was reverse transcripted with iScript Advanced cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). cDNA was used in Real-time PCR reactions with the following primers:

CyclinD1 fwd 5′-TCCTCTCCAAAATGCCAGAG-3′;
CyclinD1 rev 5′- TGAGGCGGTAGTAGGACAG-3′.
NRF2 fwd 5′-AGA TTC ACA GGC CTTTCTCG.
NRF2 rev 5′-CAG CTC TCC CTA CCG TTG AG.
HO-1 fwd 5′-CCT GGT GCA AGA TAC TGC CC.
HO-1 rev 5′-GAA GCT GAG AGT GAG GAC CCA.
SOD2 fwd 5′-GTT GGC CAA GGG AGA TGT TA.
SOD2 rev 5′-CTG ATT TGG ACA AGC AGC AA.
xCT fwd 5′-CCT CTA TTC GGA CCC ATT TAG T.
xCT rev 5′-CTG GGT TTC TTG TCC CAT ATA A.
β2 microglobulin fwd 5′-CCA CTG AAA AAG ATG AGT ATG CCT-3′.
β2 microglobulin rev 5′-CCA ATC CAA ATG CGG CAT CTT CA-3′.
β – actin fwd 5′- TCA CCC ACA CTG TGC CCA TCT ACG A.
β – actin rev 5′- CAG CGG AAC CGC TCA TTG CCA ATG G.

Gene expression analysis was carried out with Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Inc.) using β2-microglobulin and β–actin as reference genes for normalization.

### *Total antioxidant capacity (TAC)*

$2x10^4$ cells were seeded in each well of a 96-well plate and, the day after were treated or not with prednisolone, betamethasone, dexamethasone, and hydrocortisone for 24 h or 96 h at indicated concentrations. After the incubation period, cells were washed in PBS and resuspended in water. Antioxidant capacity in SH-SY5Y cells following exposure to GCs was performed with the Total Antioxidant Capacity Kit (SIGMA-ALDRICH, Saint-Louis, MO, USA) according to the manufacturer's instructions. The assay evaluates the capacity of small antioxidant molecules to convert $Cu^{2+}$ ions chelating a colorimetric probe to its reduced form, giving a broad absorbance peak at ~570 nm. The measured absorbance is proportional to the total antioxidant capacity, which is expressed as equivalents of Trolox, used as reference. Experiments were performed in triplicate.

### *Statistical analysis*

All statistics were performed on GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA). The statistical tests employed for each experiment are detailed in the figure legends. A *p-value* $< 0.05$ was considered significant.

## Results

We examined the effect of different GCs on cell proliferation and survival of cultured SH-SY5Y neuroblastoma cells. Cultured cells were treated with 500 nM of betamethasone, dexamethasone, prednisolone, and hydrocortisone, and cell number and viability were determined at different time points. The 500 nM concentration has been chosen based on previous data by Suwanjang et al., 2013 reporting that at 500 nM dexamethasone had negligible effect on cell viability (Suwanjang et al., 2013a).

After 48 h, exposure to betamethasone and hydrocortisone induced a slight, but statistically significant, reduction in cell number compared to control cells (Fig. 1A). Subsequently at 72 h, cells in betamethasone still show slower growth, whereas hydrocortisone-treated cells are comparable to controls. As expected, the exposure to dexamethasone is associated with the highest reduction in cell number after both 48 h and 72 h (Fig. 1A). When performing XTT, we found that cells treated with dexamethasone exhibited a significant reduction of cell viability at both time points (−7,5% after 48 h and –22,7% after 72 h), whilst the exposure to other GCs showed a milder effect only after 72 h, reducing viability by about 10 % Fig. 1B). To investigate whether a reduction in cell viability is due either to a proliferation inhibition or to an increase in stress-induced cell death, we analyzed the expression of Cyclin D1, the activation of the Stress-Activated Protein Kinase (SAPK) c-Jun N-terminal Kinase (JNK) and the expression of Caspase-8. Effectively, dexamethasone-treated SH-SY5Y cells showed a reduction in both *Cyclin D1* mRNA and protein levels (Fig. 1C-E). In addition, the exposure to betamethasone resulted in the down-regulation of *Cyclin D1* expression with respect to control cells, whereas prednisolone and hydrocortisone up-regulated *Cyclin D1* mRNA levels (Fig. 1E). Also, in dexamethasone-treated cells, we observed higher levels of phosphorylated JNK and an increase in the cleaved form of caspase-8 (Fig. 1F).

Cells were treated with 500 nM of dexamethasone, betamethasone, prednisolone, or hydrocortisone. Untreated cells were used as control. (A) Growth curves were obtained by cell counting at 24 h, 48 h, and 72 h after GCs addition. Statistical significance was determined by two-way ANOVA followed by Dunnett's multiple comparison test. At 48 h Dexamethasone vs ctrl $p < 0.0001$; Betamethasone and Hydrocortisone vs ctrl $p = 0.0006$. At 72 h Dexamethasone and Betamethasone vs ctrl $p < 0.0001$. (B) Cell viability was assessed by XTT assay performed at 48 h and 72 h after GCs addition. Statistical significance was determined by two-way ANOVA followed by Dunnett's multiple comparison test. At 48 h Dexamethasone vs ctrl $*p = 0.0122$; at 72 h Dexamethasone vs ctrl $****p < 0.0001$; Betamethasone vs ctrl $**p = 0.0036$; Prednisolone vs ctrl $***p = 0.0003$; Hydrocortisone vs ctrl $**p = 0.0011$. (C) Immunoblot analysis of Cyclin D1 levels in lysates from SHSY-5Y treated with two applications of 500 nM of indicated GCs for 72 h, and (D) densitometric analysis of three independent blots. Statistical significance was determined by one-way ANOVA followed by Dunnett's post-hoc test. Dexamethasone vs ctrl ** $p = 0.0072$. (E) *Cyclin D1* gene expression analysis by RT-qPCR performed on RNA extracted from SHSY-5Y treated as in (C). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison test. Dexamethasone, Betamethasone, Prednisolone and Hydrocortisone vs ctrl **** $p < 0.0001$. (F) Western Blot analysis of P-JNK and Caspase8 expression in lysates obtained from SHSY-5Y treated with indicated GCs. All data are means ± SD (A, B, D) or ± SEM (E) from three independent experiments.

To monitor early apoptosis events induced by GCs, which are not evidenced by the trypan blue technique and XTT, we performed flow cytometer analysis on SH-SY5Y cells labeled with AnnexinV and the viability dye 7-AAD (Fig. 2A). After exposure to GCs for 24 h, we observed a significant induction of early apoptosis (AnnexinV-positive cells) in dexamethasone- and prednisolone-treated cells compared to controls (Fig. 2B). Interestingly, although a percentage of necrotic cells is detectable in all experimental groups, due to repeated washing steps when preparing cells for flow cytometer analysis, necrotic cells (AnnexinV/-negative and 7-AAD-positive cells) are significantly lower upon prednisolone treatment. Finally, only dexamethasone treatment was associated with a statistically significant increase of all dying cells, namely with both necrotic and apoptotic features, respect to controls. In contrast, the exposure to hydrocortisone is associated with a reduction of total dead cells (Fig. 2B).

Cells were treated or not with 500 nM of dexamethasone, betamethasone, prednisolone, or hydrocortisone for 24 h. (A) Cells were stained with AnnexinV/7AAD and analyzed by flow cytometer. (B) Bar diagrammatic representation showing the percentage distribution of early apoptotic, late apoptotic, and necrotic cells in response to different GCs. All data are means ± SD from three independent experiments. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test. *P values* indicate significant differences as compared with the control: * $p \leq 0.05$; *** $p \leq 0.001$; ****$p \leq 0.0001$.

Since Suwanjang et al., 2019 reported an alteration of mitochondrial dynamics in neuronal cells treated with dexamethasone, we compared fusion and fission protein expression in SH-S5Y cells treated for 24 h with GCs. In particular, we observed the accumulation of the fusion factor Optic Atrophy Type 1 (OPA1) in dexamethasone- and

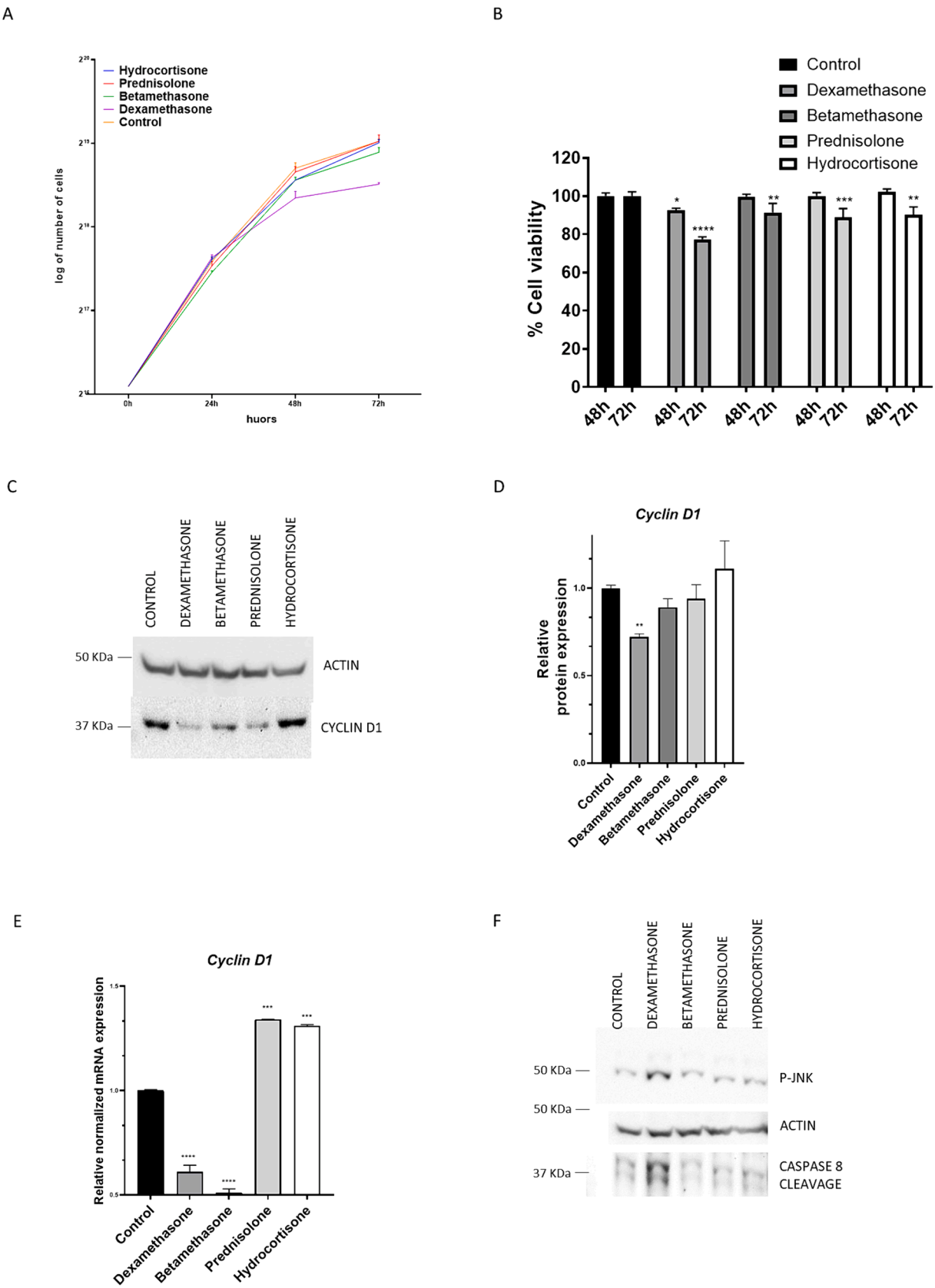


**Fig. 1.** Effect of GCs treatments on SH-SY5Y cells proliferation and viability.

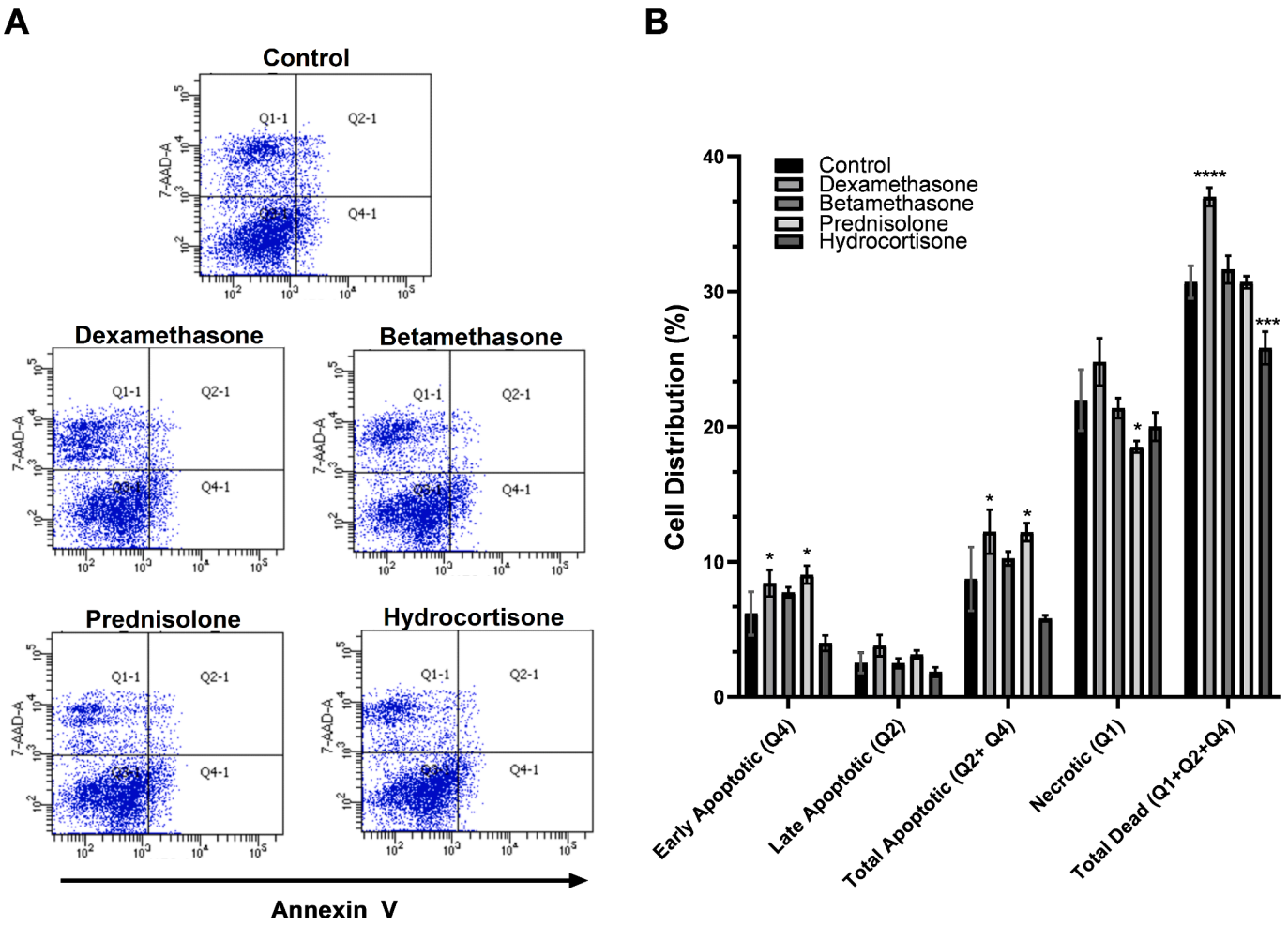


**Fig. 2.** Effect of GCs treatments on SH-SY5Y viability.

betamethasone-treated cells, as confirmed by both immunofluorescence staining (Fig. 3A) and western blot followed by densitometry analysis (Fig. 3B-C). In the same conditions, we also detected higher levels of the fusion factor Mitofusin 2 (MFN2) in cells treated with dexamethasone and betamethasone, compared to those treated with other GCs (Fig. 3B-D), although densitometry analysis revealed that such increase was not statistically significant.

Next, we analyzed the fission factor Dynamin-Related Protein 1 (DRP1) and its phosphorylation status. DRP1 undergoes regulation through multiple phosphorylation sites (Knott et al., 2008). Phosphorylation of DRP1 at Ser637 by PKA inhibits its GTPase activity and mitochondrial fission (Chang and Blackstone, 2007; Cribbs and Strack, 2007). Conversely, phosphorylation of DRP1 at Ser616 by MAPK or during mitosis by CDKs promotes mitochondrial fission (Taguchi et al., 2007). Therefore, to assess DRP1 activity, we examined phosphorylation levels at both Ser637 and Ser616. Interestingly, dexamethasone and betamethasone induced a statistically significant increase in p-DRP1 at Ser637, whereas hydrocortisone did not (Fig. 3E-F). Prednisolone is also associated with higher levels of p-DRP1 at Ser637, although they are not statistically significant (Fig. 3E-F). In contrast, we observed no remarkable variations in the levels of p-DRP1 at Ser616 (Fig. 3E-G).

Moreover, dexamethasone and betamethasone induced a significant reduction in total DRP1 protein levels (Fig. 3E-H), although this result was not as evident in immunofluorescence assays (Supplementary Fig. 1).

Recent findings suggest that excessive mitochondrial fusion results in oxidative stress and the imbalance between ROS production and their effective removal by both non-enzymatic and enzymatic antioxidants could induce cell death. Therefore, we evaluated the capability of dexamethasone, prednisolone, hydrocortisone, and betamethasone in inducing the up-regulation of detoxifying genes in SH-SY5Y cells.

In particular, we analyzed the expression levels of Nuclear Factor-Erythroid 2-related Factor 2 (*Nrf2*), Heme Oxygenase-1a (*HO-1a*), Superoxide Dismutase 2 (*SOD2*), and Cystine/Glutamate Transporter (*xCT*) after exposure to different GCs for 24 h following a single application, and 96 h following two applications.

Interestingly, we found that the duration of the treatment affects the expression pattern of detoxifying genes, indicating that different GCs activate specific transcriptional programs. In particular, SH-SY5Y cells treated with dexamethasone for 24 h do not show any induction of investigated genes (Fig. 4A), which are strongly up-regulated only after 96 h (Fig. 4B). Instead, short exposure to betamethasone is sufficient to induce an antioxidant response through the significant up-regulation of *Nrf2* and *HO-1a* (Fig. 4A), and such a response appears more robust after longer treatments (Fig. 4B). In contrast, prednisolone induces a slight increase in *HO-1a* and *xCT* after 24 h and in *Nrf2* and *SOD2* after 96 h, whereas hydrocortisone does not affect the expression of analyzed genes in our experimental conditions, except for *SOD2,* which is down-regulated after 24 h.

In parallel, we also evaluated the total non-enzymatic antioxidant capacity (TAC) correlated with the exposure of neuroblastoma cells to GCs and we observed that dexamethasone is associated with a significant reduction of the antioxidant effect after short treatment (Fig. 4C). Conversely, longer exposure to both dexamethasone and betamethasone results in a significant increase in non-enzymatic antioxidant capacity (Fig. 4D). Interestingly, prednisolone and hydrocortisone are not

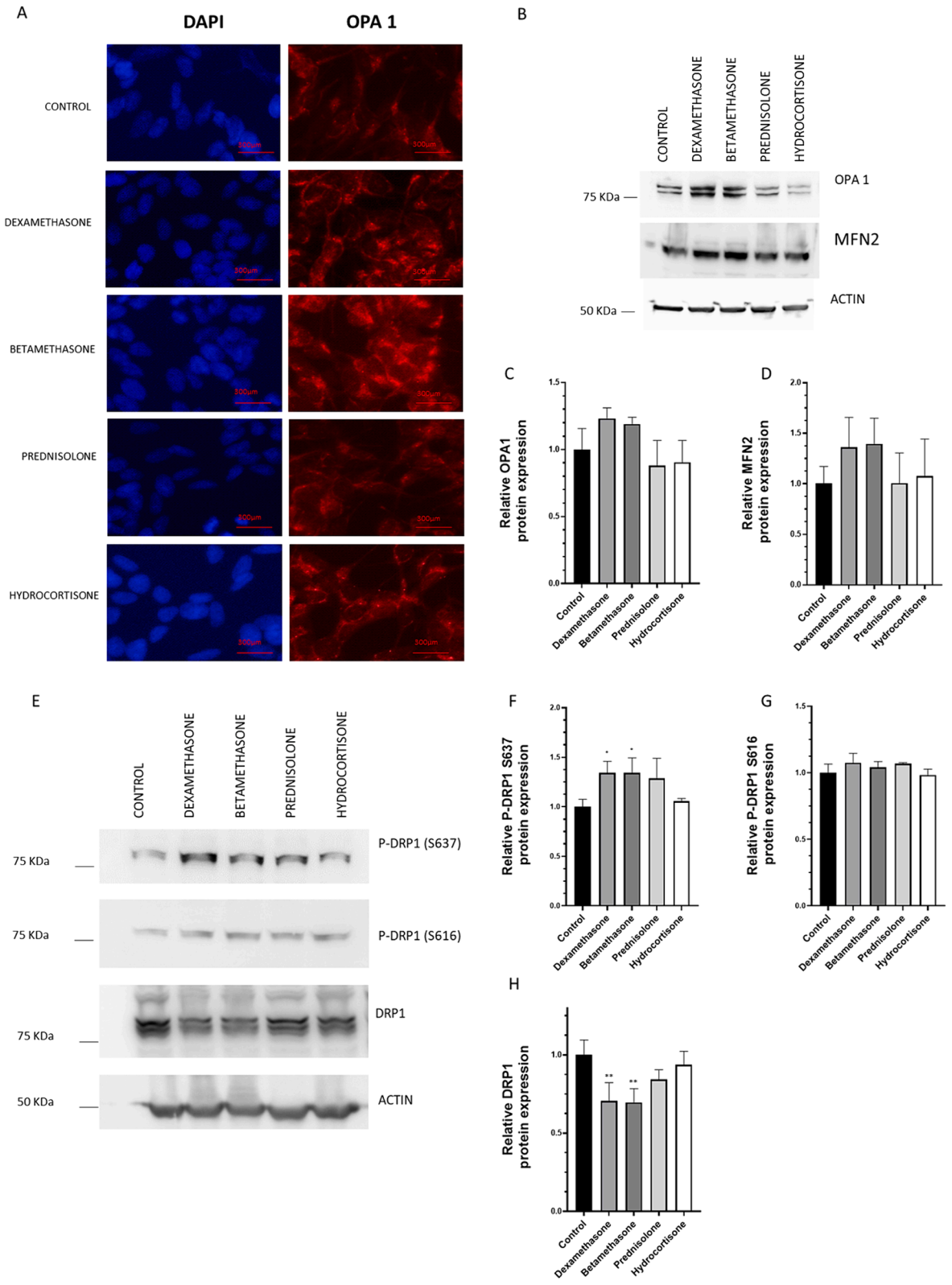


**Fig. 3.** Analysis of mitochondrial fusion and fission factors in SH-SY5Y cells treated with different GCs for 24 h. (A) Detection of OPA1 through immunofluorescence. (B) Protein levels of the fusion factors OPA1 and MFN2 and (C-D) densitometry analysis of three independent blots. Statistical significance was determined by one-way ANOVA. (E) Protein levels of the fission factor DRP1 and its phosphorylated forms at Ser637 and Ser616 with respective densitometry analyses of three independent blots (F-G-H). Statistical significance was determined by one-way ANOVA followed by Dunnett's comparison test. In (F), Dexamethasone and Betamethasone vs ctrl * $p = 0.0295$. In (H), Dexamethasone vs ctrl ** $p = 0.0087$; Betamethasone vs ctrl ** $p = 0.007$. All data are means ± SD from three individual experiments.

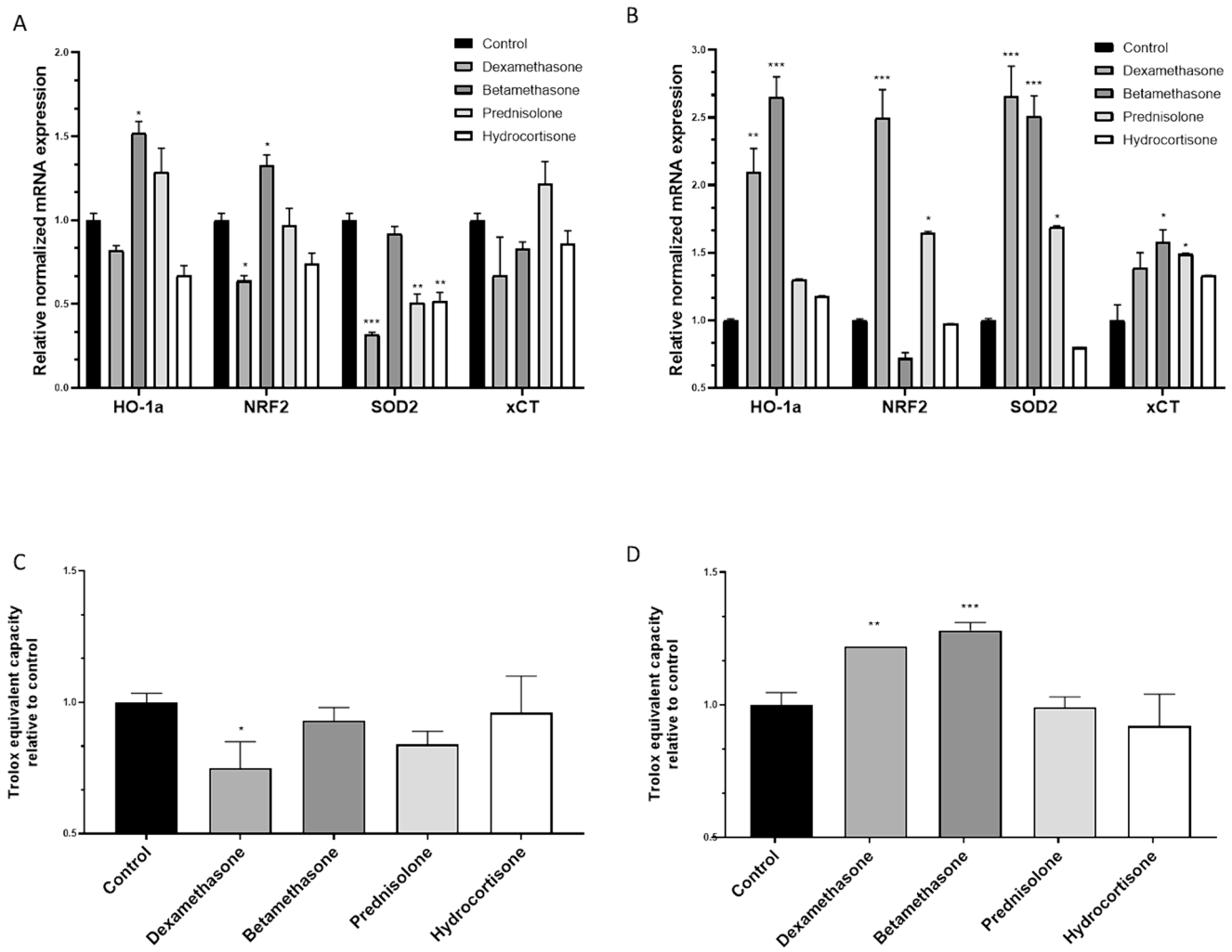


**Fig. 4.** Effect of GCs on redox signaling in SH-SY5Y cells following 24 h (1 application) and 96 h treatment (2 applications). Redox signaling gene expression in SH-SY5Y cells following (A) 24 h (1 application) and (B) 96 h treatment (2 applications). For each gene, statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test. *P values* indicate significant differences as compared with the control: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. All data are means ± SEM from three individual experiments. Total antioxidant capacity measured using TAC Assay kit in SH-SY5Y cells following (C) 24 h (1 application) and (D) 96 h treatment (2 applications). Statistical significance was determined by one-way ANOVA followed by Dunnett's *post-hoc* test. In (C), Dexamethasone vs ctrl * $p = 0.0152$. In (D) Dexamethasone vs ctrl ** $p = 0.0047$; Betamethasone vs ctrl *** $p = 0.0008$. All data are means ± SD.

associated with significant changes in the non-enzymatic antioxidant response of SH-SY5Y cells (Fig. 4C-D).

Next, we asked whether GCs might induce ER stress, which is also affected by cellular redox state. To this aim, we evaluated the protein levels of the ER stress markers Glucose-Regulated Protein 78 (GRP78), Activating transcription factor 4 (ATF4), Activating transcription factor 6 (ATF6), and C/EBP homologous protein (CHOP) in SH-SY5Y treated with 500 nM of different GCs for 24 h. Interestingly, we found that dexamethasone provokes a significant increase in GRP78 protein levels with respect to those in control cells, whereas betamethasone and prednisolone do not (Fig. 5A). In contrast, hydrocortisone-treated cells show very low levels of GRP78 (Fig. 5A).

Dexamethasone, betamethasone, and prednisolone are also associated with a slight increase in ATF6 forms and with higher levels of CHOP, whereas hydrocortisone does not affect such ER stress markers (Fig. 5B). In the same experimental conditions, ATF4 protein levels are not considerably influenced (Fig. 5B).

## Discussion

In the present work, we compare the effect of four different GCs in inducing organelles-associated stress, which in turn might affect the survival of SH-SY5Y cells, which has already been used as a model for the study of glucocorticoid-induced neurodegeneration (Suwanjang et al., 2013a) and (Choi et al., 2021).

Firstly, our findings indicate that glucocorticoids (GCs) differentially influence cell growth and viability. Among them, dexamethasone exhibited the most pronounced effect, as shown by XTT and flow cytometer analyses, confirming previously published data. (Tazik et al., 2009). Consistently, dexamethasone-treated SH-SY5Y cells show inhibition of CyclinD1 expression and activation of the SAPK JNK, whereas SH-SY5Y cells exposed to betamethasone, hydrocortisone, and prednisolone do not, indicating that dexamethasone specifically induces cell stress. Next, we investigated the potential mechanism underlying the toxicity of dexamethasone, by carrying out FACS analysis at an earlier time point (at 24 h), in order to discriminate between apoptotic and necrotic events, which are not evidenced by the trypan blue technique. Consistently, we observed that the percentage of apoptotic cells is significantly higher after exposure to dexamethasone and prednisolone. Additionally, there is a statistically significant increase in the total number of dying cells treated with dexamethasone. This evidence aligns with trypan blue and XTT data and suggests that dexamethasone toxicity is elicited through both apoptotic and necrotic mechanisms. Conversely, the lack of a significant effect of prednisolone on the total number of

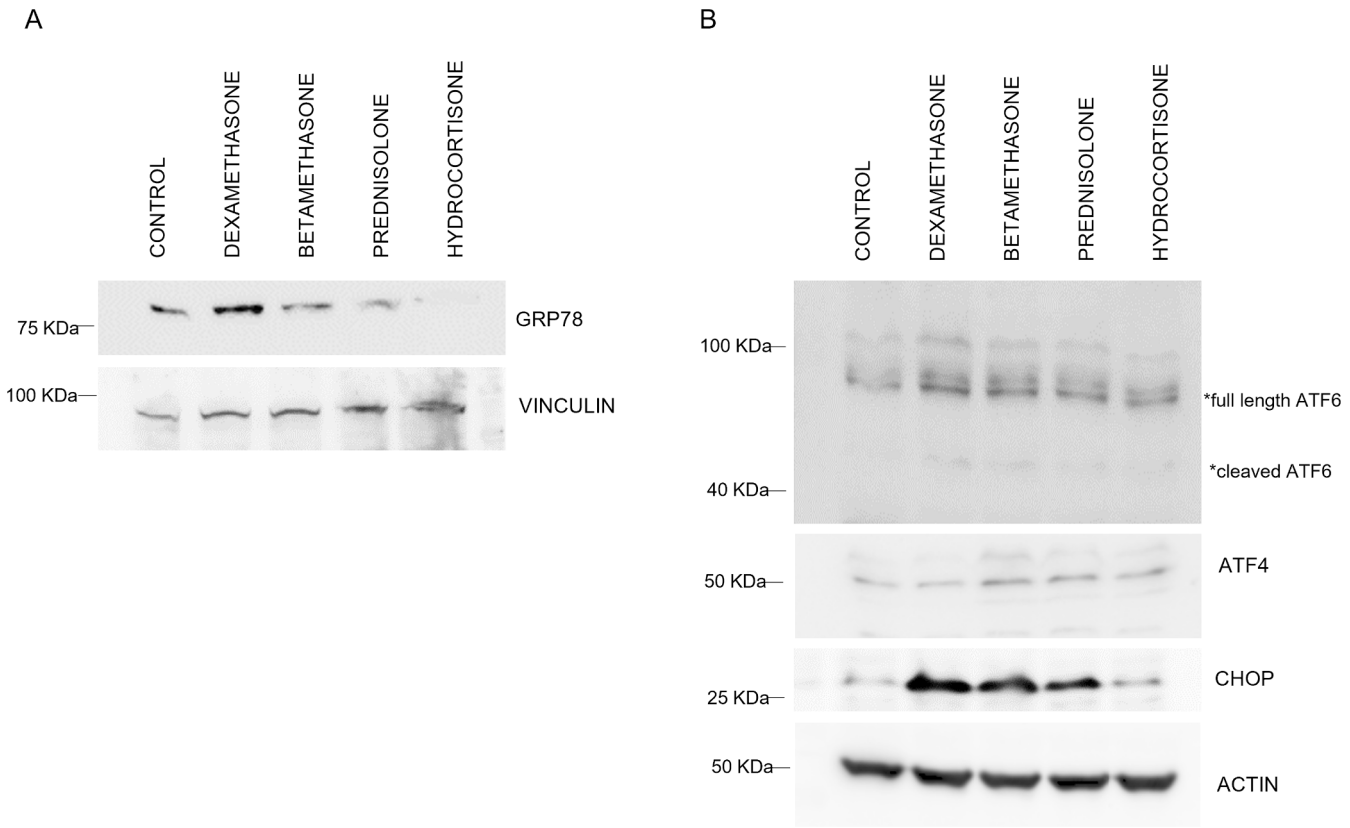


**Fig. 5.** Effect of GCs treatments on SH-SY5Y ER stress. (A) Western blot analysis of GRP78 and (B) of ATF4, ATF6, and CHOP in SH-SY5Y cells treated with different GCs for 24 h.

dying cells may be attributed to its potential to reduce the number of necrotic cells, as measured by FACS. Finally, hydrocortisone treatment is associated with a reduction of total dead cells.

As mitochondrial fission and fusion play critical roles when cells experience metabolic or environmental stress, we have compared the effect of synthetic GCs on mitochondria-associated proteins involved in fusion and fission. Interestingly, we found that exposure to dexamethasone and betamethasone but not to other GCs, induces an increase in factors regulating mitochondrial fusion. Moreover, when evaluating the activity of the fission factor DRP1, only hydrocortisone failed in inducing its phosphorylation at Ser637, which is known to inhibit mitochondrial fission (Chang and Blackstone, 2007; Cribbs and Strack, 2007). On the other hand, the fission-promoting phosphorylation of DRP1 at Ser616 was not affected in all investigated experimental conditions. Altogether, these findings suggest that dexamethasone and betamethasone might promote mitochondrial fusion by both inducing an up-regulation of fusion factors OPA1 and MFN2 and by inhibiting the activity of the fission factor DRP1, reinforcing previously reported data on dexamethasone-induced alteration of mitochondrial dynamics (Suwanjang et al., 2019).

Neuronal cells are particularly susceptible to mitochondrial impairment, as it can promote oxidative stress and ultimately cell death (Knott et al., 2008). Indeed, fusion can be activated as a compensation mechanism that helps to counteract stress by merging the contents of damaged mitochondria, whereas fission generates new mitochondria and drives the clearance of compromised organelles (Westermann, 2010). The imbalance of mitochondrial fission and fusion processes leads to different human disorders through the regulation of the ATP levels, production of ROS, expression of pro- and anti-apoptotic factors, and by modifying mitochondrial membrane potential (Bertholet et al. 2016; Bose and Beal 2016; Knott et al. 2008; Cho et al. 2010; Satapati et al. 2015).

It has been hypothesized that exposure to dexamethasone can cause DNA damage through oxidative stress and can induce apoptosis through the up-regulation of pro-death proteins and/or down-regulation of pro-survival proteins (Abrams et al., 2004; Laane et al., 2007; Suwanjang et al., 2013b, 2013a; Ortega-Martínez, 2015). In addition, some GCs have been shown to promote neuronal death by altering the mitochondrial dynamics (Müller et al. 2014; Haynes et al. 2004; Suwanjang et al. 2019; Suwanjang, Abramov, et al. 2013; Jacobs et al. 2006; Choi et al. 2021).

As the cellular response to ROS production is the activation of both non-enzymatic and enzymatic antioxidant pathways, we evaluated the capability of SH-SY5Y cells to cope adequately with the stress induced by dexamethasone, prednisolone, hydrocortisone, and betamethasone.

In our experimental conditions, we found that hydrocortisone does not affect the expression of the analyzed genes in SH-SY5Y cells, except for *SOD2*, whereas short exposure to betamethasone and prednisolone is sufficient to induce an antioxidant response through the up-regulation of *HO-1a*, *Nrf2,* and *xCt*, although with different timing. In contrast, dexamethasone strongly activates antioxidant genes only following longer treatments. On this basis, we can hypothesize that neuroblastoma cells are not able to timely and appropriately respond to dexamethasone-induced oxidative stress, with consequent detrimental outcomes. While this paper was in preparation, Du et al., 2023 confirmed our findings, showing that intraperitoneal administration of dexamethasone in mice induces an alteration of mitochondrial permeability, a significant increase of ROS, and a decrease of mitochondrial membrane potential through the up-regulation of Cyclophilin D in the hippocampal neurons. More interestingly, they find that GC-induced mitochondrial dysfunction is strictly related to neuronal damage as well as to the accumulation and phosphorylation of Tau, arguing that GC treatment could precipitate Tau pathogenesis and other neurodegenerative disorders such as Alzheimer's disease and glaucoma (Fang et al.,

2023).

Our data show that also the non-enzymatic antioxidant activity is increased upon exposure to betamethasone and dexamethasone, indicating that neuroblastoma cells treated with these GCs suffer an imbalance of the redox state (Baiċc et al. 2007; Costantini et al. 2011; Pereira et al. 1999; Feng and Tang 2014).

We also investigated the involvement of ER stress in SH-SY5Y response to GCs, by evaluating protein levels of ER-stress-associated markers, namely GRP78, ATF6, ATF4, and CHOP. In dexamethasone-, betamethasone-, and prednisolone-treated cells, both ATF6 and CHOP increased, whether only dexamethasone is associated with higher levels of GRP78, indicating that GCs-induced stress also affects ER.

Our data strengthen and extend pre-existing evidence demonstrating that dexamethasone induces ER stress, thereby leading to extensive tissue damage in both *in vitro* and in vivo models of different pathological conditions induced by GCs, such as glaucoma (Zode et al., 2014; Kasetti et al., 2020) and osteoporosis (Liu et al., 2018; Xiao et al., 2022). Further evidence demonstrates that either chemical or genetic inhibition of ER stress reduces dexamethasone-inducing toxicity, ameliorating GCs-caused tissue damage(Kasetti et al., 2020; Xiao et al., 2022). In addition, our data suggest that the ER stress provoked by specific GCs, such as dexamethasone and, to a lesser extent, betamethasone and prednisolone, might be further detrimental due to an inadequate temporal antioxidant response in neuroblastoma cells. Indeed, Xiao et al. observed that the escalation of ER stress triggered by dexamethasone in rat osteoblasts is counteracted by the ER-stress inhibitor Geniposide, which in turn up-regulates *NRF2* gene expression and stimulates an antioxidant response. Similarly, in SH-SY5Y cells treated for 24 h with dexamethasone and prednisolone, we did not detect a prompt up-regulation of *NRF2*, consistently with published data.

A main limitation of the present study is that the involvement of organelle-associated stress in neurodegeneration induced by synthetic GCs has not been investigated in differentiated SH-SY5Y or in other neuronal cells, to verify whether the cytotoxic effect is cell type-dependent. As stated before, undifferentiated neuroblastoma cells are widely used as a model of neurodegeneration for many studies (Xicoy et al., 2017; Alrashidi et al., 2021), especially in studies on mitochondrial dysfunction in Parkinson Disease (Watabe and Nakaki, 2008). Such acceptance depends on the fact that SH-SY5Y cells exhibit a phenotype with dopaminergic features. Although some authors report that, compared to differentiated cells, undifferentiated SH-SY5Y are more appropriate for investigating neurotoxicity and/or neuroprotection to drugs in neurodegenerative diseases (Cheung et al., 2009; Lopes et al., 2010), our findings require further validation on mature neuronal cells.

In addition, it must be considered that the activity of synthetic GCs in vivo is strictly dependent on their heterogeneous bioavailability (Paragliola et al., 2017), and on the variable affinity concerning their cognate receptors GRs and MRs, which are diversely distributed in the brain (Paragliola et al., 2017; Meijer et al., 2023; Yang et al., 2023). Last but not least, synthetic GCs are known to elicit different effects on the regulation of the hypothalamic-pituitary axis (HPA) and, as a consequence, of cortisol levels, influencing the systemic response to prolonged GC therapies (Paragliola et al., 2017).

However, such considerations along with data provided in the present study highlight that the multiple cellular effects of different GCs can no longer be overlooked and that, as therapeutics, one molecule can not be easily substituted by another. On the other hand, further studies are needed to explore in detail additional molecular, cellular, and tissue-specific mechanisms affected by GCs, with particular attention to the experimental system in which such investigation is performed.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethics: All Authors have read and have abided by the statement of ethical standards for manuscripts submitted to Neuroscience

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2024.07.010.

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