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Research Paper

Dexamethasone acts as a radiosensitizer in three astrocytoma cell lines via oxidative stress

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ABSTRACT

Glucocorticoids (GCs), which act on stress pathways, are well-established in the co-treatment of different kinds of tumors; however, the underlying mechanisms by which GCs act are not yet well elucidated. As such, this work investigates the role of glucocorticoids, specifically dexamethasone (DEXA), in the processes referred to as DNA damage and DNA damage response (DDR), establishing a new approach in three astrocytomas cell lines (CT2A, APP.PS1 L.1 and APP.PS1 L.3). The results show that DEXA administration increased the basal levels of gamma-H2AX foci, keeping them higher 4 h after irradiation (IR) of the cells, compared to untreated cells. This means that DEXA might cause increased radiosensitivity in these cell lines. On the other hand, DEXA did not have an apparent effect on the formation and disappearance of the 53BP1 foci. Furthermore, it was found that DEXA administered 2 h before IR led to a radical change in DNA repair kinetics, even DEXA does not affect cell cycle. It is important to highlight that DEXA produced cell death in these cell lines compared to untreated cells. Finally and most important, the high levels of gamma-H2AX could be reversed by administration of ascorbic acid, a potent blocker of reactive oxygen species, suggesting that DEXA acts by causing DNA damage via oxidative stress. These exiting findings suggest that DEXA might promote radiosensitivity in brain tumors, specifically in astrocytoma-like tumors.

Background

Glucocorticoids (GCs) such as dexamethasone (DEXA) are widely known for their anti-inflammatory properties, and are used, as such, in the treatment of inflammatory disorders such asthma [1], rheumatoid arthritis [2] and autoimmune diseases [3]. Moreover, GCs are commonly used as co-medications in cancer therapy [4] due to their effectiveness in treating the secondary effects of the cancer treatments, including inflammation, pain, edema, anorexia, and nauseas [4,5].

These GCs are not only given during chemotherapy treatment but also before and after, depending on the procedure and dose, which may vary for different kinds of tumors. Regardless of the procedure used, the ultimate goal of GC treatment is to reduce acute toxicity in cancer patients, thus offering protection against the long-term effects of genotoxic drugs [5].

Despite the extended use of the GCs, its pro- and anti-apoptotic effects, which depend on the cell type, have only been partially described in recent years. It is known that GCs induce apoptosis mainly in cells of the hematological lineage, as well as in some non-hematologic cells such osteoblasts. GCs promote survival in several non-hematologic tissues, such as gliomas, mammary glands, ovaries, livers, and fibroblasts [6]. In addition, it is known that GCs may have anti- or pro-apoptotic effects within an identical cell type, depending on different external circumstances [7,8].

The most common glucocorticoid prescribed for brain tumors is DEXA [9,10], a synthetic steroidal glucocorticoid. The reason for widespread use of DEXA is its long biological half-life and its low mineralocorticoid activity (sodium retaining) [2]. This GC acts by decreasing the permeability of the blood–brain barrier and lowering regional cerebral blood volume, leading to subsequent improvement in the symptoms of chemotherapy patients [6]. In addition, DEXA may counteract the actions of vascular endothelial growth factor (VEGF) by decreasing edema in the brain tumor [11]. However, not all data obtained from the use of DEXA in brain tumors patients have been positive. In fact, doctors must now weigh the beneficial effects of this treatment in patients with brain tumors against the possibility that it may reduce the efficacy of chemotherapy drugs that act by inducing apoptosis. In this regard, it has been reported that DEXA pre-treatment may interfere with apoptotic death in brain tumor cells via the transcriptional activation of a Bcl-xL gene [6]. Indeed, patients treated with the combination of 1,3-Bis (2-chloroethyl)-1-nitrosourea (BCNU) and a

Abbreviations: DEXA, dexamethasone; GCs, glucocorticoids; IR, Irradiation; DDR, DNA Damage response; NHEJ, non-homologous end-joining pathway; DSBs, double strand breaks; GR, glucocorticoid receptor; MR, mineralocorticoid receptor.

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high-dose of methylprednisolone show less of the apoptotic effect than those treated with BCNU alone [6]. In addition, it has been reported that DEXA induces apoptosis resistance in most solid malignant tumors during co-treatment with chemotherapy agents such as camptothecin (CAM) [6]. The beneficial effects related to the use of DEXA in patients with intracranial tumors have been described extensively in the literature [2,12,13].

The DEXA effects have also been studied in other kinds of tumors, mainly multiple myeloma (MM). Through this research, an antimyeloma effect of DEXA has been partly elucidated as acting via the induction of oxidative stress when DEXA is combined with radiation [14]. This introduces a new paradigm for DEXA usage in MM that can potentially be incorporated into the design of more effective combined modality therapies [14]. Despite this, other studies have shown that DEXA may confer radioresistance in different types of carcinoma [15,16].

In the specific case of brain tumors, different practical guides exist regarding the use of DEXA [1]. In all cases, DEXA is used to minimize neurological symptoms and treatment-related side-effects [17]. Although the best dosages have been established [1,18] in the extended use of steroids in the treatment of brain tumors [19,20], the molecular mechanisms that underlie their beneficial effects are not yet well established.

Interesting, a previous work in adult neurogenesis has elucidated a new role of DEXA causing a decrease in the adult neural stem cell (aNSC) pool, proliferation-dependent, within dentate gyrus (DG) when DEXA was administered postnatally. Indeed, DEXA negatively affected neuronal proliferation [30].

Taking all the data together, it is possible to highlight that despite the clinical relevance of GCs in the co-treatment of cancer, their role in combination with chemotherapy or radiation agents have still not been well explored. In addition, there are multiple questions remaining, as GCs do not act in the same way in all types of cells or with all types of primary treatments (different chemotherapeutic or radiation agents). For this reason, the current work has been focused on clarifying the role of DEXA in the specific case of astrocytoma cell lines (CT2A, APP.PS1 L.1 and APP.PS1 L.3), looking particularly at DNA damage and repair as key mechanisms in the evolution of the cancer process. It was considered essential to uncover whether DEXA itself may have a protective effect against cancer in these cell types and whether combination with irradiation (IR) could help solve the high proliferation present by definition in the oncogenic process. To clarify the role of DEXA in these processes, different mechanisms involved in DNA damage and repair were studied after DEXA was administered in combination with IR or without it. In addition, this work provides a new model for the mechanism by which DEXA may contribute to DNA damage and subsequently cause cell death in three different kinds of astrocytoma cell lines.

Methods

Cell lines

Three astrocytoma cell lines were used. CT2A is a commercial astrocytoma cell line. APP-PS1 L.1 and APP. PS1 L.3 are two different astrocytoma cell lines obtained from a mice APP.PS1 which previously were injected with 20-methylcholantrene (a carcinogen) and which developed different kinds of astrocytoma tumors. These three cell lines were a gift from Dr. Ricardo Martínez-Murillo laboratory (Cajal Institute, Madrid, Spain). These cells were maintained in RPMI medium (12633-012, Gibco) supplemented with 10% fetal bovine serum (10,106-151, Gibco) and 5 mg/mL penicillin/streptomycin (15070-063, Gibco).Cells were cultured in a humidified incubator at 37 °C under 5% CO_2 atmosphere.

Table 1
Description and details of the 26 antibodies used for cell lines characterization.

Antibody	Dilution	Time (h)	Company
NG2	1:500	24	Millipore
A2B5	1:500	24	Millipore
β-III tubulin	1:1000	24	Abcam
MCM2	1:500	24	Abcam
8OHdG	1:250	24	Santa Cruz
NeuroD1	1:100	24	Abcam
Nestin	1:500	24	Millipore
Sox-2	1:100	24	R&D Systems
Sox-9	1:100	24	Abcam
GFAP	1:1000	24	Sigma Aldrich
DCX	1:1000	24	Santa Cruz
IGFR1	1:500	24	Cell Signaling
Prominin-1	1:100	24	Abcam
Calretinin	1:3000	24	Swant
Calbindin	1:500	24	Millipore
MCR	1:500	24	Santa Cruz
Arc	1:500	24	Santa Cruz
p35	1:500	24	Cell Signaling
LEF1	1:500	24	Abcam
GR	1:500	24	Abcam
mAB367	1:500	24	Millipore
AchE	1:500	24	Santa Cruz
AchRα7	1:500	24	Santa Cruz
AchRβ2	1:500	24	Santa Cruz
Glutamate R1 AMPA	1:500	24	Sigma Aldrich
PROX-1	1:500	24	Abcam

DEXA treatment

All treatments with DEXA were performed by incubating cells with DEXA at 50 μM (D4902, Sigma-Aldrich, St. Louis, MO, USA). Aliquots of 1 μg DEXA were resuspended in 1 mL of ethanol and diluted in 49 mL culture medium [RPMI media (1640, Gibco)+10% Fetal Bovine Serum (10,106-151, Gibco)] to a final concentration of 50 μM. 3 mL of this medium was used in DEXA conditions, and 3 mL of culture medium as the vehicle in control conditions. Treatment was carried out at different times indicated in each experiment.

Ionizing radiation

Cells were irradiated with 5 Gy (100 kV, 250 mA, 1 min) for all experiments. The model of the irradiator used was the ´Smart 200 E" 85,034′, supplied by Cajal Institute.Experiments at Oxford University were performed using their own irradiator (5 Gy was the dose used).

Cell characterization

To characterize the cells, the 26 antibodies described in Table 1 were used.

Immunofluorescence

The cells were plated on coverslips, irradiated with 5 Gy if indicated, and collected at varying time intervals. At collection, the cells were fixed with 4% paraformaldehyde and 0.2% Triton X-100 in PBS for 20 min at RT, followed by 3 washes in PBS. After blocking in 1% BSA and 0.1% Triton X-100 in PBS for 1 h at 37 °C, the cells were incubated with 53BP1 (NB100-304, Novus Biologicals), or Anti-phospho-Histone H2AX (Ser139) clone JBW301(05-636, Millipore) antibodies (1:3000 dilution), overnight at 37 °C. Three washes in PBS were carried out followed by incubation for 1 h at 37 °C with the appropriate secondary antibodies. The final

washes in PBS were carried out, the cells were counterstained with DAPI and coverslips were mounted on slides using gervatol mounting medium. Fluorescent micrographs were taken using a Nikon 90i upright microscope. These experiments were performed in triplicate.

Kinetic experiment

The cells were plated overnight. They were irradiated with 5 Gy, and fixed for different periods of time (30, 60, 210, 360 min). The control conditions were not irradiated, only fixed at the beginning of the experiment. 53BP1 Foci were evaluated as indicators of the non-homologous end-joining (NHEJ) pathway. 53BP1 Blocks resection of DNA ends resulting from damage in G1, but its action is antagonized by BRCA1 in the S phase. γH2AX was an indicator of double strand breaks (DSBs) and DNA damage. These experiments were performed in triplicate.

Irradiation+DEXA experiments

The cells were plated in p60 overnight and then irradiated with 5 Gy. DEXA medium (50 μM) was added for the DEXA conditions, while the medium was changed for vehicle conditions. The cells were fixed either 30 or 240 min after irradiation. In control conditions, culture medium or DEXA medium (50 μM) was added without irradiation. The control cells were fixed after 240 min. 53BP1 foci and γH2AX foci were evaluated. These experiments were performed in triplicate.

Irradiation+DEXA+ascorbic acid experiments

The cells were plated in p60 overnight and then irradiated with 5 Gy. DEXA medium (50 μM) was added for the DEXA conditions, along with the combination of DEXA (50 μM) and ascorbic acid (A4403, Sigma-Aldrich, St. Louis, MO, USA) in 0.05 mM concentration. In vehicle conditions, only the medium was changed. The cells were fixed either 30 or 240 min after IR. In control conditions, culture medium, DEXA medium (50 μM), or a combination of DEXA (50 μM) and ascorbic acid (0.05 mM) were added, without IR. The control cells were fixed after 240 min. The γH2AX foci were evaluated. These experiments were performed in triplicate at Oxford University (Dr. Eric O'Neill's lab).

Neutral comet assay

Neutral comet assays were performed using CometSlide assay kits (Trevigen). Cells were treated with either vehicle or DEXA for 2 h. The cells were then irradiated with 5 Gy and incubated at 37 °C for different periods of time (0, 30, 60, 90 and 120 min) to allow DNA damage repair. The cells were embedded in agarose, lysed, and subjected to neutral single cell gel electrophoresis. The agarose was dehydrated and the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) for visualization under a fluorescence microscope. The olive comet moment was determined using the software CometScore Version 1.5 (TriTek). The olive moment was calculated by multiplying the percentage of DNA in the tail and the difference between the means of the head and tail distributions, as described [21]. A total of 50 comets (and two replications) were analyzed per sample for each experiment. These experiments were performed in triplicate.

Cell cycle experiments

1×10^6 cells were plated in 1 cm^2 plate with RPM1+10% FBS. The following day DEXA was added for 6 h. After, cells were washed with PBS and centrifuged, and the pellet was fixed in 70% ethanol in PBS and conserved one day at −20 °C. The next day pellets cells were resuspended in 0.5 mL PBS containing DAPI (1 mg/mL). The cell cycle analysis was performed using a FACStar Plus (Becton, Dickinson). 30,000 Cells per condition (CT2A control, CT2A DEXA, APP-PS1 control, APP-PS1 DEXA, APP-PS1 L.3 control and APP-PS1 DEXA) were evaluated in total.These experiments were performed at Leibniz Institute for Age Research (Dr.Zhao-Qi Wang's lab), and repeated in triplicate at Oxford University (Dr. Eric O'Neill's lab).

Time-lapse experiments

300,000 Cells per condition (CT2A control, CT2A DEXA, APP-PS1 control, APP-PS1 DEXA, APP-PS1 L.3 control and APP-PS1 DEXA) were plated in 6-well plates and cultured with RPMI+10% FBS. 3 Hours after addition of DEXA for all DEXA conditions, beginning to register the behavior of cells using time lapse microscopy over 24 hours in a humidified incubator at 37 °C under a 5% CO_2 atmosphere.

Survival experiments

350,000 Cells per condition (CT2A control, CT2A DEXA, APP-PS1 control, APP-PS1 DEXA, APP-PS1 L.3 control and APP-PS1 DEXA) were plated in 6-well plates and cultured in an IncuCyte microscope (in a humidified incubator at 37 °C under 5% CO_2 atmosphere), with or without DEXA treatment for 3 days. Subsequently, the final confluence in these wells was evaluated using the IncuCyte software. The experiment was evaluated in triplicate.

Statistical analysis

For all the experiments, a "two-tailed" student's *t*-test or ANOVA was used to calculate statistical significance of the observed differences, using SPSS software or GraphPad Prism software. Furthermore, for the neutral comet assay, a repeated measures ANOVA was performed to evaluate the dexamethasone effect as a whole. Microsoft Excel v.2010 was used for the calculations. In all cases, differences were considered statistically significant when $p < 0.05$.

Results

Characterizing astrocytoma cell lines using a battery of twenty-six different markers.

CT2A, is used in literature as a cell line of commercial astrocytoma. APP. PS1 L.1 and APP.PS1 L.3, are two different cell lines, type astrocytoma obtained through APP-PS1 mice, and generated in the laboratory of Dr. Ricardo Martinez-Murillo (Department of Neuroanatomy and Cell Biology, Instituto Cajal, CSIC, Avenida del Doctor Arce 327, 28,002 Madrid, Spain). An analysis was performed to characterize these cell lines by employing 26 different markers (Table 1). The results obtained are described in Fig. 1. Most of the markers analyzed were present in all cell lines. It is important to highlight the presence of the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) in the three cell lines, suggesting that they are susceptible to glucocorticoid and mineralocorticoid action.

By contrast, the main differences found are shown correspond to Acetylcholine Receptor Alpha 7 (AchRα7), Glial Fibrillary Acidic Protein (GFAP) and 8-hydroxydeoxyguanosine (8-oHdG[1]). In the case of AchRα7, the differences found are that this receptor was present in CT2A and APP.PS1 L.1 but it was not present in APP.PS1 L.3. This receptor has been implicated in a lot of process as an

[1] 8-OHdG antibody used was sc-66036. This antibody is recommended for detection of 8-OHdG (8-hydroxy-2′-deoxyguanosine, 8-hydroxyguanine and 8-hydroxyguanosine).

A

ANTIBODY	DESCRIPTION	CT2A	APP.PSI L.1	APP.PSI L.3
NG2	NG2 Chondroitin Sulfate Proteoglycan	+	+	+
A2B5	Anti-Neuron Cell Surface Antigen	+	+	+
β-III TUBULIN	Anti-Neuron specific beta III Tubulin	+	+	+
CLR	Calretinin	+	+	+
CALBINDIN	Calbindin	+	+	+
MCR	Mineralocorticoid Receptor	+	+	+
ARC	Activity-regulated cytoskeleton-associated protein	+	+	+
p35	Neuronal-specific activator of cyclin-dependent kinase 5 (Cdk5)	+	+	+
AchE	Acetylcholinesterase	+	+	+
AchRα7	Acetylcholine receptor alpha 7 subunit	+	+	¿?
LEF-1	Lymphoid enhancer-binding factor 1.	+	+	+
GR	Glucocorticoid Receptor	+	+	+
mAB367	Muscarinic Acetylcholine Receptor m2, clone M2-2-B3	+	+	+
AchRβ2	G protein-linked acetylcholine receptor	+	+	+
Glut.R1 AMPA	This Anti-Glutamate receptor 1, GluR1 subunit	+	+	+
PROX1	Prospero homeobox 1	+	+	+
SOX2	(Sex determining region Y)-box 2, also known as SOX2	¿?	¿?	+
GFAP	Glial fibrillary acidic protein (GFAP)	+	+	-
DCX	Neuronal migration protein doublecortin	¿?	-	-
MCM2	DNA replication licensing factor MCM2	+	+	+
8OHdG	8-hydroxydeoxyguanosine	-	+	+
NEUROD1	Neurogenic differentiation 1 (NeuroD1)	+	+	¿?
NESTIN	Nestin	+	+	+
IGFR1	The Insulin-like Growth Factor 1 (IGF-1) Receptor	+		+
DREBRIN	A neuron-specific, actin binding protein	N.F	N.F	N.F
SOX9	Transcription factor SOX-9	+	+	+
PROMININ-1	Prominin-1/CD133 is a plasma membrane marker	+	+	+

B

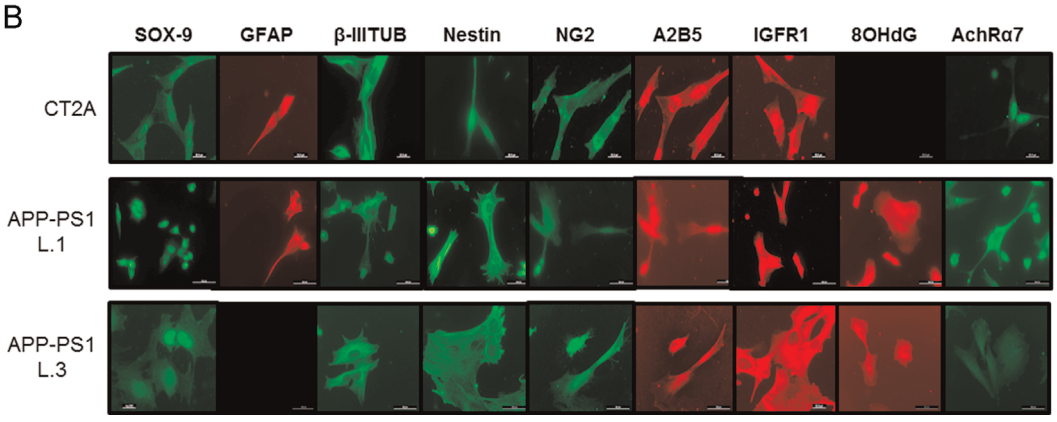

Fig. 1. Summary of results for the characterization of these cell lines. 1A. Table indicating results obtained which each antibody. ¿?Indicates that immunostaining did not reveal a sufficiently good labeling to make a reliable conclusion. N.F. indicates that the antibody did not work. 1B. Example of the immunofluorescence obtained in some relevant antibodies.

essential regulator of inflammation [22], and currently has been described its role in neuroprotection [23]. Besides CT2A and APP.PS1 L.1 also shared the expression of GFAP, which is not found in the APP.PS1 L.3 cell line. This marker is present in neural stem cells (NSCs) and glial cells, but not in neurons. Finally, there was no presence of the marker 8-hydroxy-2′-deoxyguanosine (8-OHdG) in CT2A while this marker was present in astrocytomas cell lines which were obtained from mice APP.PS1 (L.1 and L.3). 8-OHdG is a biomarker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics [24]. The cytoplasmic fine granular

8-OHdG expression was considered to reflect 8-OHdG-positive mitochondrial DNA affecting oxidation stress [25]. The lack of presence of this market is relevant because it suggests that the CT2A cell line does not exhibit oxidative stress as a phenotypically intrinsic cell characteristic (see Fig. 1B).

Evaluating the kinetics of the formation and disappearance of γ-H2AX and 53BP1 foci in astrocytoma cell lines following irradiation (IR).

It is known that following irradiation, cells initiate processes to repair the damage, and at that time it is possible to observe the

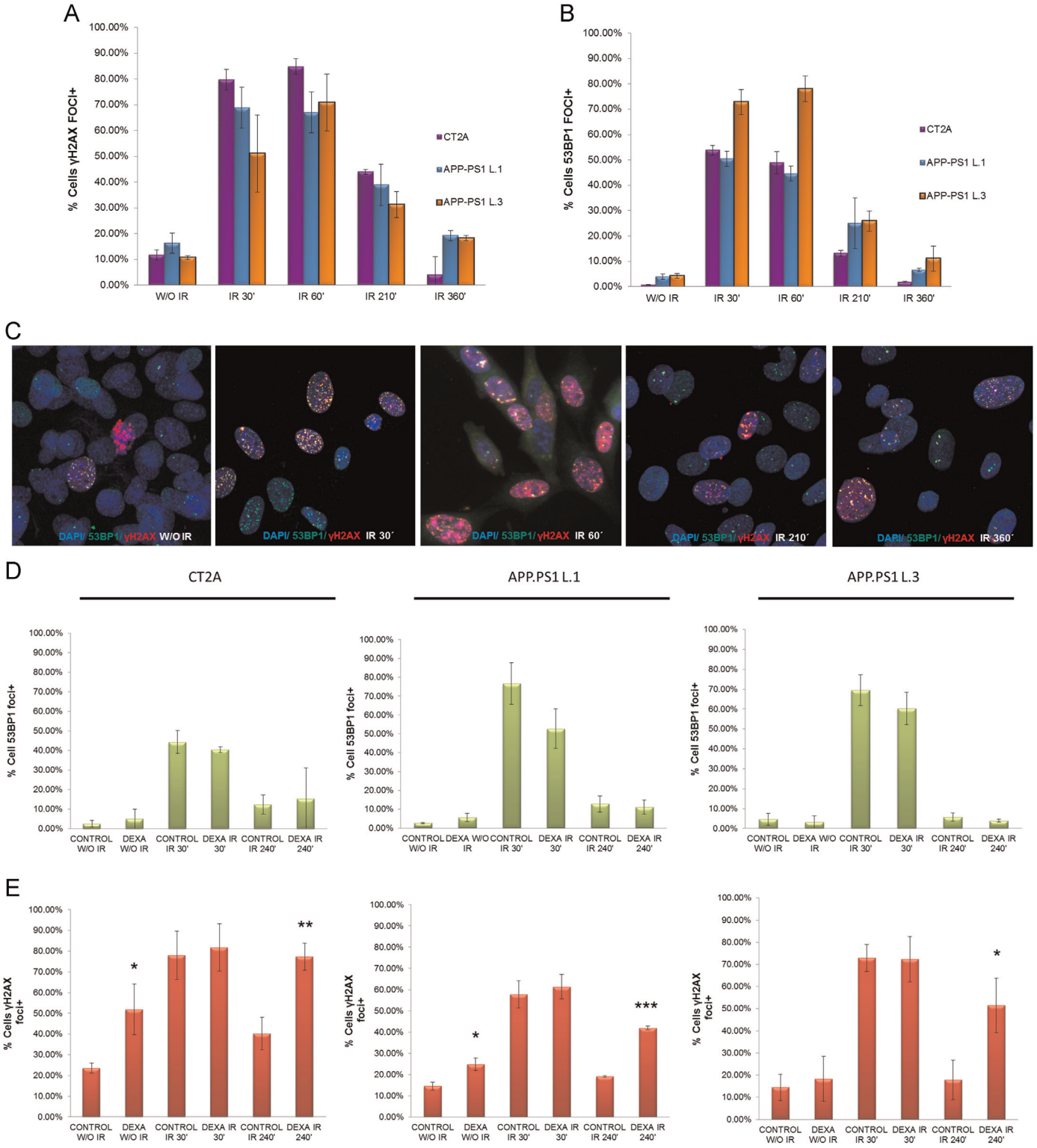

Fig. 2. Foci formation and disappearance. 2A. Percentage of γH2AX-positive cell foci over time. 2B. Percentage of 53BP1-positive cell foci over time. 2C. Example of the immunofluorescence of kinetic experiment, analyzing the formation and disappearance of γ-H2AX and 53BP1 foci. Objective 40x. 2D. Evaluation of 53BP1 foci by cell lines in the Irradiation + dexamethasone condition. 2E. Evaluation of γH2AX foci in the Irradiation + dexamethasone condition. CT2A: DEXA w/o IR ($p = 0.049$), DEXA IR 240′ ($p = 0.011$); APP.PS1 L.1 w/o IR ($p = 0.040$), DEXA IR 240′ (p = 0,000); APP.PS1 L.3 DEXA IR 240′ (p = 0,044).

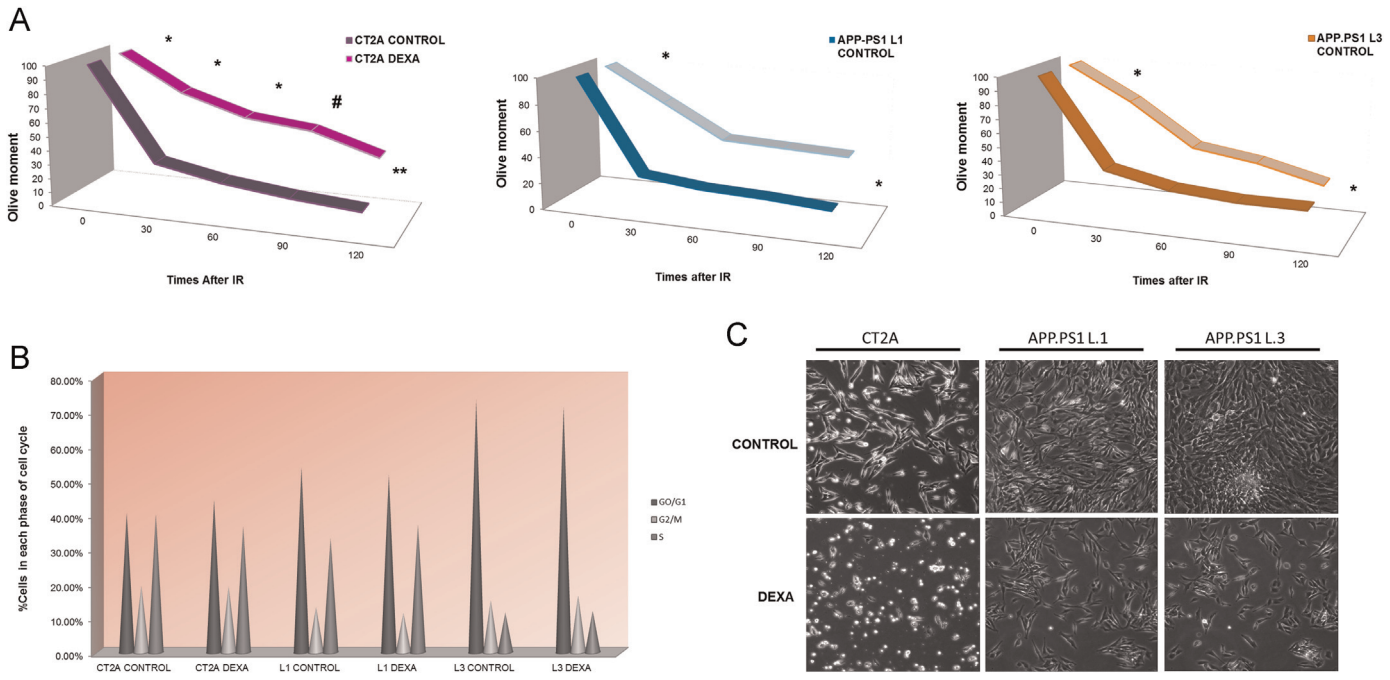

Fig. 3. (A) Neutral comet assay graph obtained for the three cell line with and without IR. CT2A DEXA 30′ ($p=0.031$), 60′ ($p=0.033$), 90′ ($p=0.012$), 120′ ($p=0.052$). Repeated measures test comparing Control to DEXA in CT2A ($p=0.001$). App. PS1 L.1 DEXA 30′ ($p=0.036$). Repeated measures test comparing Control to DEXA in APP.PS1 L.1 ($p=0.022$). App. PS1 L.3 DEXA 30′ ($p=0.023$). Repeated measures test comparing Control to DEXA in APP.PS1 L.3 ($p=0.028$). (B) Cell cycle analysis in three astrocytoma cell lines. No differences in cell phases were observed between Control and DEXA conditions in any cell line 3C. Proliferation and cell death experiment. Evolution of each cell line under Control and DEXA conditions after 24 h. Objective 10 ×.

presence of different types of foci, as is analyzed here. After a length of time, these cells recover their normal state and the foci are no longer present. This time varies depending on the cell line. For that reason, this study aimed to evaluate the baseline process of γ-H2AX and 53BP1 foci formation and disappearance, for later comparison with the different treatment conditions. In all cases, the percentage of γ-H2AX was used as a measure of DNA damage, and the 53BP1 foci was used as a measure of DNA repair by the NHEJ pathway. To determine whether a cell was positive for the presence of foci, it was determined that cells must present at least five foci. When the cells showed a smaller number of foci, they were considered negative.

In this experiment, statistical analysis comparing the different cell lines have not been performed since the main goal of the experiment was to evaluate how each cell line responds to the IR, and how the kinetics of the formation and disappearance of the foci is. The results showed, in general, that without IR all cell lines had a background of foci+cells (53BP1 as well as γ-H2AX) in the ratio 10–15%. 30–60 Min after IR all cell lines increased its percentage of foci+cells, which decrease when it was evaluated 210 min after IR. Then, after 6 h of IR all cell lines presented the initial background of foci+cells (Fig. 2A and B). An example of the immunofluorescence of CT2A cell line is represented in Fig. 2C.

Evaluating the kinetics of the formation and disappearance of γ-H2AX and 53BP1 foci, in astrocytoma cell lines after IR, with or without subsequent addition of glucocorticoid (DEXA, 50 μM).

This experiment aimed to evaluate the effect of GCs, specifically DEXA 50 μM, administered immediately after the IR. For this purpose, the effect in Control conditions without irradiation was first evaluated. In all cases, the percentage of γ-H2AX was used as a measure of DNA damage, and 53BP1 foci as a measure of DNA repair by the NHEJ pathway.

DEXA did not produce any consequence in 53BP1 foci, and there were no significant differences between Control and DEXA conditions when these foci were evaluated in any cell line (Fig. 2D). On the other hand, when γ-H2AX foci were evaluated (Fig. 2E), there were significant differences between Control and DEXA in conditions without irradiation in CT2A ($p=0.049$) and APP.PS1 L.1 ($p=0.040$), and in all cell lines when the cells were irradiated and γ-H2AX foci were evaluated 4 h after the IR (CT2A $p=0.011$; APP.PS1 L.1 $p=0.000$; APP.PS1 L.3 $p=0.054$). There were no significant differences when the cells were irradiated and γ-H2AX foci were evaluated 30 min following IR (CT2A $p=0.079$; APP.PS1 L.1 $p=0.702$; APP.PS1 L.3 $=0.955$).

Analyzing the DNA damage repair kinetics in Control conditions and with addition of DEXA, in astrocytoma cell lines using the neutral comet assay technique.

The purpose of this experiment was to evaluate the kinetics of DNA repair using the neutral comet assay, a technique that analyzes unrepaired DNA over time as a measure of the olive moment, calculated using CometScore software as explained above. In order of evaluate the possible significance of any differences, the statistical software SPSS was used, and student's *t*-tests were performed for Control and DEXA conditions at each time evaluated. In addition, a repeated measures ANOVA was used to evaluate the DEXA effect as a whole.

The results obtained from the kinetic curves show that there were significant differences in the three cell lines, between Control and DEXA treatment, by repeated measures ANOVA (CT2A $p=0.001$; APP.PS1 L.1 $p=0.022$; APP.PS1 L.3 $p=0.028$). Evaluating the differences between treatments at each time point, in all cell lines there were significant differences between Control vs DEXA treatment at 30 min (CT2A $p=0.031$; APP.PS1 L.1 $p=0.036$; APP.PS1 L.3 $p=0.023$), corresponding to the phase in which the DNA is repaired faster and more effectively via the NHEJ pathway. In the other times (60, 90 and 120 min) were not found any significant differences between treatments (Control vs DEXA) in APP.PS1 L.1 and APP.PS1 L.3 cell lines, although DEXA condition presents

always higher levels of DNA unrepaired compared to Control condition. By contrast CT2A cell line presented significant differences in all times analyzed: 60 min ($p=0.033$); 90 min ($p=0.012$), and a trend toward significance (represented by # in Fig. 3A) at 120 min ($p=0.052$). In all cases, the DEXA condition presented higher levels of unrepaired DNA compared to Control conditions (Fig. 3A).

Analyzing DEXA effects in cell cycle profile in astrocytoma cell lines.

This experiment was used to evaluate if DEXA causes or not a problem in the progression of the cells through cell cycle. In all cases, DEXA did not produce any change in the cell cycle profile compared to Control condition suggesting DEXA does not produce an arrest any cell cycle phases (Fig. 3B).

Analyzing cell survival in Control and DEXA conditions using an Incucyte microscope.

The Incucyte microscope was used to evaluate the confluence of cells over time, along with the DEXA effect in astrocytoma cultures. In all cases, DEXA was added at the beginning of the experiment. The results obtained were similar in the three cell lines used. Thus in the Control condition, cells grew over time until reaching confluence. In the DEXA condition, the Incucyte microscope was able to detect cell death over time.

Determining the existence of cell death or cell proliferation with and without the addition of DEXA in the astrocytoma cell lines by time-lapse microscopy.

The goal of this experiment was to determine whether cell

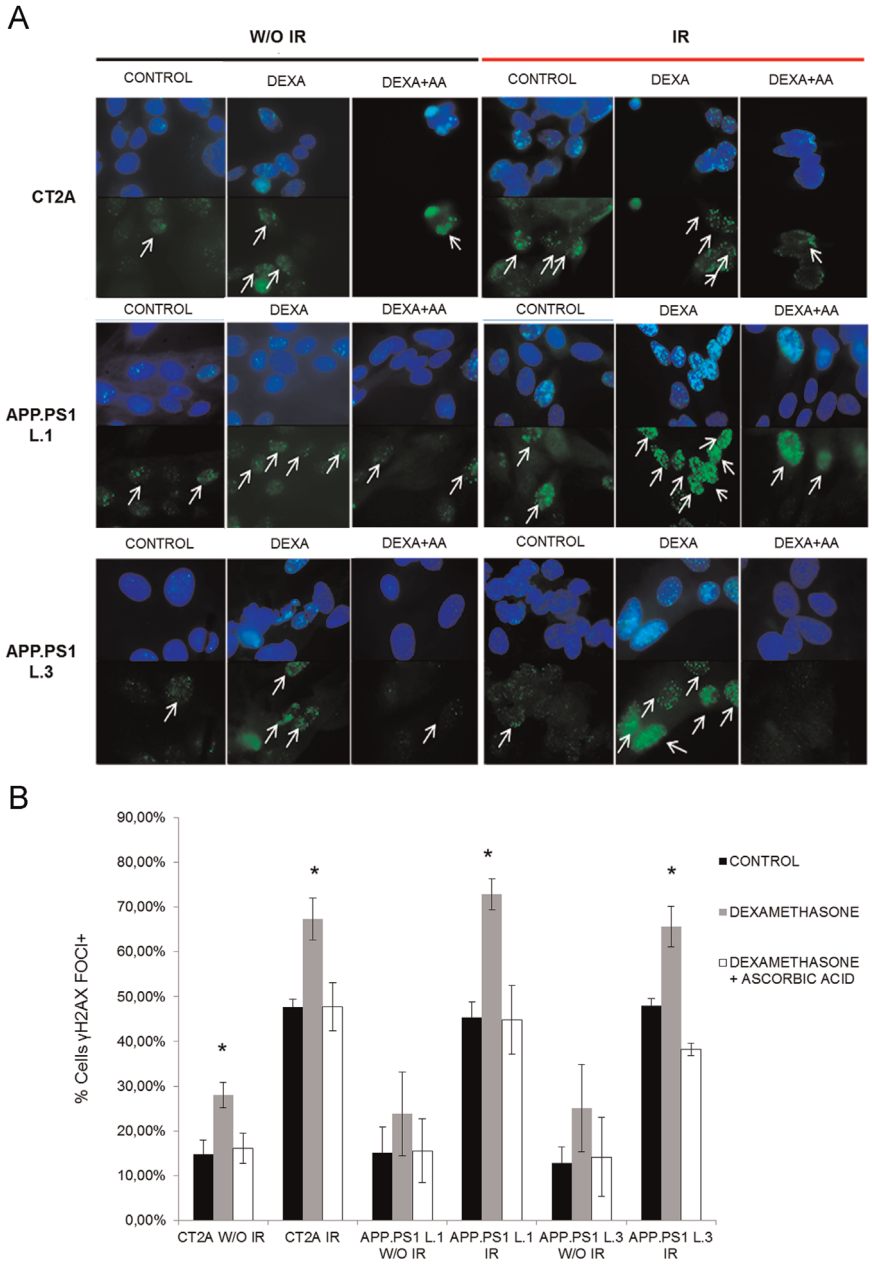

Fig. 4. Ascorbic acid experiment. (A) Immunofluorescence of ascorbic acid experiment, analyzing the formation and disappearance of γ-H2AX foci after addition of DEXA alone and DEXA+ascorbic acid (AA). Arrows indicate γH2AX-positive cell foci. Objective 40×. (B) Percentage of γH2AX-positive cell foci in the different conditions (control, DEXA and DEXA + ascorbic acid). CT2A DEXA w/o IR ($p=0.046$), IR+240 ($p=0.025$). App. PS1 L.1 DEXA IR + 240 ($p=$). App. PS1 L.3 DEXA IR + 240 ($p=0.014$).

death or cell proliferation occurred over time via time-lapse microscopy. Control and DEXA-treated cells were monitored every 5 min for 24 hours. The results were similar in three cell lines and show that the DEXA conditions mainly produced cell death, while the Control conditions showed cell proliferation (Fig. 3C). An example of the CT2A cell line time lapse are shown in video 1 (Control condition) and 2 (DEXA condition).

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2015.06.006.

Potential of ascorbic acid to reverse the DEXA/IR-induced γ-H2AX foci.

To analyze whether the increase in the γ-H2AX foci with DEXA treatment could be reversed, ascorbic acid, an agent that recruits free radicals, was used in a concentration of 0.05 mM. The formation of γH2AX foci without IR and 4 h after the IR were determined in both treatment conditions. Results obtained from each cell line are explained (Fig. 4):

- CT2A: DEXA alone produced an increase in the number of γH2AX foci+cells without IR ($p=0.046$) and 4 h following IR ($p=0.025$). However, when ascorbic acid was added to the DEXA condition, the levels of foci+cells returned to baseline (Fig. 6A and B). This result suggests that DEXA may increase γH2AX foci levels via oxidative stress induced augmentation of free radicals.
- APP. PS1 L.1: DEXA produced an increase in the number of γH2AX foci+cells in the condition 4 h following IR ($p=0.014$). The increase levels of γH2AX foci that occur in the DEXA condition was reversed by adding ascorbic acid, suggesting again that DEXA could exert their action through the increase of free radical.
- APP. PS1 L.3: DEXA only produced an increase in γH2AX foci levels in the 4 h after IR condition ($p=0.014$). Similar to the results obtained in other cell lines, said increase was reversed by adding ascorbic acid, suggesting that DEXA exerts its action by increasing levels of free radicals.

Discussion

The main goal of the present work was to elucidate the effects of DEXA (glucocorticoid) in the specific case of three different astrocytoma cell lines in the context of DNA damage and repair. This premise is essential because the use of GCs is widespread as co-treatment with chemotherapy and radiotherapy for many tumors, including those of the central nervous system [9,10], but how these GCs act in processes such as DNA damage and repair – essential steps in the oncogenic process – remain largely unknown.

A multitude of DNA damage and repair pathways could be crucial as diverse as the progression of cells that have the ability to cause cancer. It is logical to consider that in the oncogenic processes, the mechanisms underlying DNA damage and repair may be altered, conferring upon these cells the difference between the normal and pathological state. There have been many investigations aimed at studying the role of the DDR, or different molecules involved in it, in astrocytoma or glioblastoma cell lines. These studies have been designed, for example, to evaluate the effect of IR on those cell lines [26,27], having as an ultimate goal the elucidations of the mechanisms underlying this type of tumor therapy [28]. The present work has focused on gaining a better basic understanding of the key processes governing the DDR in three astrocytomas cell lines because one of the main problems in the treatment of brain tumors lies in their extreme diversification. For that reason, their characterization is an important key for developing further treatment. Likewise, the study wanted to examine the effects of GCs, specifically DEXA, as such molecules could play a key role in the DNA damage and repair. DEXA is used in the co-treatment of brain tumors, mainly for its anti-inflammatory effects [2,9,17,19]; however, it may have another relevance that has yet to be described.

To achieve these goals, the first step was to characterize these cell lines using twenty-six different markers. While most of the markers analyzed were present in the three cell line, one important result was the absence of the marker 8-hydroxy-2-deoxyguanosine (8-OHdG) just in the case of CT2A cell line while it was present in cell obtained from APP.PS1 mice. This marker is crucial [24] because it indicates that there is no oxidative stress mediated DNA background damage in the CT2A cell line. Other important results, such as the presence of both kinds of steroid receptors (glucocorticoid receptor (GR) and mineralocorticoid receptor (MR)) show that these cell lines are susceptible to the actions of both steroids.

These results are relevant, because they reveal a genetic background that might be involved in how these cells behave with IR, drugs or the DNA-repair pathways. The biological significance of these data are the importance of characterizing tumors. These phenotypic characteristics for specific tumors could be crucial in establishing better therapies. This means that different kinds of tumors may not show the same therapeutic response to treatment.

Another goal was to highlight how a glucocorticoid, specifically DEXA, could influence DNA damage and repair mechanisms. One of the key experiments consisted subjecting astrocytoma cells to a pulse of IR, and then evaluating the evolution of two types of foci (gamma-H2AX and 53BP1) in the absence or presence of DEXA (see Fig. 2D and E). On one hand, DEXA did not seem to influence the formation and persistence of 53BP1 foci in any of the cell lines studied (see Fig. 1D), meaning that, in principle, it does not influence the NHEJ repair pathway at the level of that specific protein. On the other hand, an increase in the basal levels of the gamma-H2AX foci was observed without IR in CT2A and APP.PS1 L.1 (see Fig. 2E). This is extremely significant because it means that DEXA can, by itself, cause DNA damage in some astrocytoma cells. This result could impact the therapeutic implications of DEXA in the treatment of brain tumors, emphasizing the importance of further studies of this effect. Moreover, the DEXA produced higher levels of gamma-H2AX foci for a longer time, 4 hours after IR, while in Control conditions, the gamma-H2AX foci had already returned to nearly basal levels. This result, obtained in all cell lines analyzed, is of great relevance, since it shows that DEXA can increase DNA damage in the astrocytoma cells for a longer period of time when combined with IR.. These data have future biological implications, because GCs may not only act as anti-inflammatory molecules in the treatment of tumors, but they might also influence the mechanisms underlying DNA damage and repair, as key mechanisms in the evolution of oncogenic processes.. This finding is really exciting, as it suggests that DEXA could promote radiosensitivity in brain tumors.

The neutral comet assay was used to analyze whether DEXA indeed changes the kinetics of DNA repair. The results obtained show that DEXA administered 2 hours before IR led to a radical change in the kinetics of the DNA repair curve in all astrocytoma cell lines (see Fig. 3A). The first phase of the comet assay corresponding to the fast track of DNA repair by NHEJ was much less pronounced, and the slow HR repair phase was also modified. The overall result was that DEXA indeed exerted an effect upon both DNA repair pathways. The ability of DEXA to impact DNA repair kinetics following IR suggests that this drug could potentially be used as a radiosensitizer, although future experiments are needed

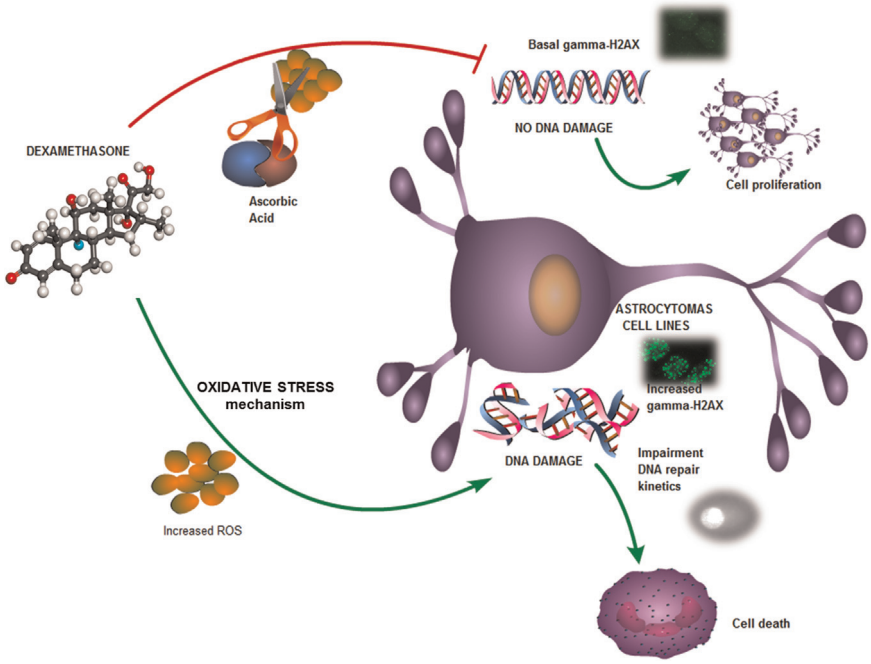

Fig. 5. Model proposal for one of the DEXA mechanism of action, causing DNA damage, via oxidative stress.

to test this hypothesis.

In addition, it was important to assess whether or not DEXA produces impairments in cell proliferation or cell death in astrocytoma cell cultures of these three cell lines. The findings shown in Fig. 3C demonstrate that DEXA caused a decrease in normal cell proliferation, compared with Control conditions. At the same time, DEXA caused an increase in cell death, leading to very low confluence in the cell cultures. DEXA also acts by promoting apoptosis, as has been previously described in different kinds of cell cultures [1,17,18]. Consistent with the fact that DEXA produced cell death are cell cycle results, concluding that DEXA did not produce an arrest in any phase of the cell cycle (see Fig. 3B).

After the evaluation of the DEXA effects in terms of increased DNA damage (seen by the increase in γ-H2AX foci levels), impaired DNA repair kinetics, and cell death in the three cell lines cultures, it was important to analyze the possible mechanisms of action by which DEXA could be exerting its effects. The hypothesis was that the drug might be increasing free radicals via oxidative stress, as has been demonstrated in previous studies [2,19]. This in turn could cause an increase in DNA damage. In order to test this hypothesis, ascorbic acid was used, as it has been shown in the literature to reduce the production of reactive oxygen species (ROS) [29]. The results show that, in fact, when cells were irradiated, addition of DEXA and ascorbic acid led to the return of gamma-H2AX foci to basal levels, suggesting that at least one of the mechanisms of DEXA in the DNA damage pathway is increasing ROS, which would in turn lead to an increase in the levels of gamma-H2AX foci (Fig. 4). This result is relevant because it give us a better means to investigate how glucocorticoids act in the specific case of astrocytoma cell lines. In this regard, this work has proposed a new model for the mechanism of action by which DEXA causes DNA damage, with higher levels of gamma-H2AX foci observed (see Fig. 5).

Conclusions

Taken together, the data presented here establish the importance of looking at the role of GCs as an adjuvant in the treatment of brain tumors, in particular astrocytomas, since this study has demonstrated that they play a key role in increasing DNA damage (seen by higher persistence of gamma-H2AX foci levels) produced by cancer therapies such as IR. At the same time, it is established that DEXA itself cause cell death in astrocytoma cell cultures, as well as producing impairments in the DNA repair pathways, even does not cause arrest in any of the cell cycle phases. Finally and most importantly, this work has elucidated at least one of the mechanisms of action by which DEXA acts: the increase of free radicals in astrocytoma cell cultures via oxidative stress. In that sense, this work marks an important starting point in research regarding a new model of GCs action in the treatment of brain tumor therapies, with all of the future therapeutic implications that includes.

Authors' contributions

SOM has made the conception and experimental design, acquisition of data, and the analysis and interpretation of data. SOM has been involved in writing the manuscript and has given the final approval of the version to be published.

Competing interests

The author has no conflicts of interest to declare in relation to this manuscript.

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