Received: 19 July 2024 | Revised: 31 August 2024 | Accepted: 11 September 2024

DOI: 10.1111/jns.12659

WILEY

RESEARCH REPORT

Oxygen matters: Unraveling the role of oxygen in the neuronal response to cisplatin

Jose Crugeiras[1,2] | Aina Calls[1,2] | Estefanía Contreras[1,2] | Montse Alemany[3] | Xavier Navarro[1,2] | Victor J. Yuste[4] | Oriol Casanovas[5] | Esther Udina[1,2] | Jordi Bruna[2,3]

[1]Department of Cell Biology, Physiology, and Immunology, Institute of Neuroscience, Autonomous University of Barcelona, Bellaterra, Spain

[2]Biomedical Research Center Network on Neurodegenerative Diseases (CIBERNED), Bellaterra, Spain

[3]Unit of Neuro-Oncology, Hospital Universitari de Bellvitge, Bellvitge Institute for Biomedical Research (IDIBELL), Barcelona, Spain

[4]Department of Biochemistry, Institute of Neuroscience, Autonomous University of Barcelona, Bellaterra, Spain

[5]Tumor Angiogenesis Group, ProCURE Program, Catalan Institute of Oncology, OncoBell Program, IDIBELL, Barcelona, Spain

Correspondence
Jordi Bruna, Unit of Neuro-Oncology, Hospital Universitari de Bellvitge-ICO L'Hospitalet, Feixa Llarga s/n 08907, L' Hospitalet, Barcelona, Spain.
Email: 35078jbe@comb.cat

Funding information
Instituto de Salud Carlos III

Abstract

Background and Aims: Cell culture is a fundamental experimental tool for understanding cell physiology. However, translating these findings to in vivo settings has proven challenging. Replicating donor tissue conditions, including oxygen levels, is crucial for achieving meaningful results. Nevertheless, oxygen culture conditions are often overlooked, particularly in the context of chemotherapy-induced neurotoxicity.

Methods: In this study, we investigated the role of oxygen levels in primary neuronal cultures by comparing neuronal performance under cisplatin exposure (1 μg/mL) in supraphysiological normoxia (representing atmospheric conditions in a standard incubator; 18.5% O_2) and physioxia (representing physiologic oxygen conditions in nervous tissue; 5% O_2). Experiments were also conducted to assess survival, neurite development, senescence marker expression, and proinflammatory cytokine secretion.

Results: Under control conditions, both oxygen concentration conditions exhibited similar behaviors. However, after cisplatin administration, sensory neurons cultured under supraphysiological normoxic conditions show higher mortality, exhibit an evolutionarily proinflammatory cytokine profile over time, and activate apoptotic-regulated neuron death markers. In contrast, under physiological conditions, neurons treated with cisplatin exhibited senescence marker expression and an attenuated inflammatory secretome.

Interpretation: These results underscore the critical role of oxygen in neuronal culture, particularly in studying compounds where neuronal damage is mechanistically

Esther Udina and Jordi Bruna are cosenior authors.

linked to oxidative stress. Even at identical doses of evaluated neurotoxic drugs, distinct cellular phenotypic fates can emerge, impacting translatability to the in vivo setting.

KEYWORDS
cell culture, chemotherapy-induced neuropathy, cisplatin, neurotoxicity, oxygen

1 | INTRODUCTION

Cell culture is a widely employed basic tool for comprehending cell physiology and investigating the cellular effects of conditional alterations in the immediate cellular environment. Although this method has proven highly valuable in deconstructing and comprehending cellular transduction pathways, translating the impacts of cell exposure to different agents to animal models or, more significantly, to patients has led to considerable challenges and frequently resulted in unmet expectations.[1–3] The chemotherapy-induced peripheral neuropathy (CIPN) research field serves as a paradigmatic example. Despite more than 40 years of extensive investigation and identification of multiple potential pathogenic mechanisms in preclinical studies, even for the same drug,[4–6] no specific treatments have been able to prevent CIPN in patients thus far.[7]

Classical studies had pointed that cisplatin toxicity is mediated by activation of the apoptotic pathway triggered by inefficient DNA repair mechanisms and the oxidative stress.[8,9] However, these experiments have mainly been conducted in vitro with limited support from in vivo models.[10,11] In contrast, by using an in vivo experimental model, we demonstrated that damaged neurons exposed to cisplatin activate p21 and survive in a senescence-like phenotype.[12]

Both in vitro and in vivo models have strengths and weaknesses, as nicely reviewed by Lehmann.[13] However, the use of living organisms is more expensive, time-consuming, and poses ethical dilemmas compared to in vitro models. In fact, despite their limitations[11] in vitro neuronal cultures also offer several advantages in studying disease conditions and exploring potential treatment effects. Therefore, it is important to optimize in vitro models to enhance the reproducibility and the chances of finding relevant translatable results to clinics. Cell cultures must mimic, as closely as possible, the conditions of the donor tissue.

Extensive research has already been conducted to study the impact of several intrinsic (related to the tissue source) and extrinsic (related to the environmental conditions) culture variables that can influence the molecular phenotype profile, adaptive behavior, and viability of cultured neurons exposed to neurotoxic cytostatic agents.[14–20] However, the role of oxygen, a potentially relevant extrinsic physical variable, in culture has been largely neglected in the chemotherapy-induced neurotoxicity literature.

Thus, it should be desirable to maintain cultures within physiological normoxic limits. Nevertheless, there are inconsistencies among researchers regarding the term 'normoxia' in cell culture experiments.[21,22] Typically, normoxia or supraphysiological normoxia refers to the atmospheric conditions of a standard incubator rather than the true microenvironment to which cells are exposed. For instance, room air at sea level contains 160 mmHg O_2, whereas the air in a humidified CO_2 (5%) cell culture incubator contains 140 mmHg O_2 (20.9% and 18.5% O_2, respectively). Notably, under standard tissue culture conditions (140 mm Hg), cells are exposed to a partial pressure of oxygen even higher than that experienced by lung alveoli (~110 mmHg), which encounter the highest partial pressure of oxygen in the mammalian body. However, the physiologic ("physioxia") O_2 levels in neural tissue range from 21 to 47 mmHg (approximately 5%).[23]

Several biochemical reactions use oxygen as a substrate, and in some instances, oxygen tension can significantly influence chemical reaction flux.[24] Oxygen can also react with metals and cofactors,[25] catalyzing the formation of reactive oxygen species (ROS) and amplifying oxidative damage through ROS. Additionally, developmental and differentiation programs are sensitive to oxygen.[23,26–28] Thus, it is reasonable to hypothesize that oxygen levels in experimental in vitro paradigms can either mask or unmask cellular adaptive phenotypes.

Therefore, the objective of this study was to compare neuronal performance under cisplatin exposure in primary dorsal root ganglia (DRG) cultures under supraphysiological normoxic (standard incubator room) and physioxic conditions.

2 | MATERIALS AND METHODS

2.1 | Study design

To obtain comprehensive insights into the influence of oxygen concentration on dissociated primary sensory neuron cultures, we either treated these cells with cisplatin or vehicle and cultured under supraphysiological normoxic and physioxic conditions, and assessed the following different relevant cellular aspects: (1) neuronal survival, (2) neurite growth, (3) senescence markers, and (4) secretion of proinflammatory cytokines.

All the procedures involving mice were approved by the Ethics Committee of the Universitat Autònoma de Barcelona (procedure: 5382-CEEA-UAB; CEEAH4058R), and all methods were performed in accordance with ARRIVE guidelines and were carried out in strict agreement with EU Directive 2010/63/EU.

2.2 | Dorsal root ganglia cultures

Ten-week-old BALB/c mice (male:female, 1:1) were sacrificed (administering an intraperitoneal overdose of sodium pentobarbital at a

dosage of 200 mg/kg), and all their DRGs were dissected and placed in cold Gey's salt solution (Sigma, Cat #G9779) supplemented with 0.033 M glucose (Sigma, Cat #G7021-1KG). DRGs were enzymatically dissociated in Hank's Balanced Salt Solution (Sigma, Cat #14170-088) supplemented with 10% trypsin (2.5 mg/mL; Sigma, Cat #T-4674), 10% collagenase A (12.5 collagen digestion units/ml; Sigma, Cat #C2674) and 10% DNAse (1 mg/mL; Roche, Cat #11284932001) for 45 minutes at 37°C, followed by mechanical dissociation. After the enzymes were blocked with 10% hiFBS in DMEM (Sigma, Cat #41966052) and centrifuged at 900 rpm for 7 min, the cells were centrifuged again in 15% BSA (Sigma, Cat #A6003) in Neurobasal A (Gibco, Cat #10088022). The supernatant was withdrawn, and 1 mL of culture medium was added. The medium consisted of Neurobasal A supplemented with 2% B27 (Gibco, Cat #17504044), 0.033 M glucose, 1 mM glutamine (Gibco, Cat #35050-038), 1× penicillin/streptomycin (Sigma, Cat #P0781), and 20 mM HEPES (Sigma, Cat #H0887). To culture the cells, 24-well plates were used. The plates were previously coated with 100 µg/mL poly-D-lysine (PDL; Sigma, Cat #P6407) for 2 h at 37°C, washed, dried and further coated with 1 µL/mL laminin for a minimum of 2 hours (Sigma, Cat #L-2020). Then, neurons were plated at a density of 30×10^3 neurons/mL and incubated under two different conditions: 37°C, 5% CO_2, and atmospheric oxygen (supraphysiological normoxia) or, alternatively, 5% O_2 (physioxia).

These conditions were established by employing an nBIONIX Hypoxic Cell Culture Kit (Bulldog Bio, Cat #103881-422). Plates were taken out of the hypoxic chamber each time a manipulation was performed, and placed again inside afterward, to maintain the physioxic condition along with the follow-up.

2.3 | Culture treatments

Twenty-four hours after the neurons were seeded, cisplatin or cis-diaminodicloroplatin (CDDP) (Selleckchem, Cat #S1166) dissolved in a sterile saline solution at a concentration of 0.5 mg/mL and stored at −80°C until its use, was added at a concentration of 3.3 M (1 µg/mL) and incubated for 24 h, after which the medium was changed to the fresh culture medium.

This dose of CDDP was chosen after exploring a range of doses from 0.8 to 13 M (0.25–4 µg/mL) in a pilot study (data not shown, published as thesis: https://www.tdx.cat/handle/10803/673816#page=1). The selected dose was the highest at which neuronal death was not induced compared to control cultures, but it was sufficient to induce DNA damage, which was analyzed 3 days after drug withdrawal (following 5 days in culture) in $n = 3$ independent experiments per condition. The observed fold-change increase in p-H2AX protein levels at 3.3 M of CDDP compared to untreated cultures was 17-fold and 6-fold at 1 and 3 days, respectively, after drug withdrawal (see the previous free access online reference). We also qualitatively checked that this dose was able to increase p-H2AX protein in sensory neurons under both oxygen concentration conditions, serving as an indicator of induced DNA damage (Figure S1).

2.4 | Quantification of neuronal survival

Cell viability was determined using a method similar to the one described by Ta et al.[29] Briefly, the number of viable neurons was quantified in the same field (previously labeled) at days 1, 2, and 4 after CDDP treatment under a light field (Olympus CKS41 inverted microscope). At least four independent experiments were performed under the two conditions of supraphysiological normoxia and physioxia.

2.5 | Immunofluorescence

A total of 3000 cells were plated and cultured for 5 days. Thereafter, the cultures were fixed with 4% paraformaldehyde for 20 min, and the neurons were immunostained with primary antibodies against ß-III-tubulin (1:500; Biolegend, Cat #MMS435P), p-H2AX (1:200; Cell Signaling, Cat #9718) and DAPI (1:1000; Sigma, Cat #D9564-10MG). After washing with PBST, the cells were incubated with anti-mouse 488 (1:200; Invitrogen, Cat #21202). The p-H2AX antibody was amplified via the Tyramide signal amplification (TSA) method with a TSA Biotin System (Perkin Elmer, Cat #NEL700A001KT) following the manufacturer's protocol. The length of the longest neurite from ßIII-tubulin-positive neurons was measured at 100× magnification under an epifluorescence microscope (Nikon ECLIPSE Ni) attached to a digital camera (DS-Ri2). Three different cultures from at least three different wells were analyzed under both conditions. All the editing and analysis of the images were performed using ImageJ software.

2.6 | Real-time quantitative polymerase chain reaction

Total RNA from the cultures was isolated using an RNeasy Micro Kit (Qiagen, Cat #74004) and quantified using a NanoDrop spectrophotometer. A total of 30–100 ng of RNA was reverse transcribed into cDNA using a kit (Applied Biosystems, Cat# 4368813). Polymerase chain reactions (PCRs) were conducted in 10 µL containing 1 µL of cDNA template, 0.5 µL of each primer at 10 µM, and 5 µL of 2× Applied Biosystems Power SYBR Green PCR Mastermix (Thermo Fisher Scientific) in nuclease-free water. PCR was performed on a CFX384 Touch Real-Time PCR Detection System. The geometric mean of the expression levels of GAPDH was used to normalize the expression of the Cp values. The primer sequences are listed in Table 1.

2.7 | Multiplex analysis of cytokine profiles in culture media

At 1 and 4 days after CDDP treatment, culture supernatants were collected, and the proteins were concentrated to a final volume of 50 µL with centrifugal filter units (Merck, Cat #MRCPRT010). The protein

levels of IL-6, CCL11 (Eotaxin), CXCL1 (GROα), CXCL10 (IP-10), CCL3 (MIP-1α) and CCL5 (RANTES) were determined and analyzed using a custom-designed Milliplex Cytokine/Chemokine Magnetic Bead Panel on a MAGPIX system (EMD Millipore, Cat #EPXR260-26088-901) following the manufacturer's instructions. Standard curves were generated using the specific standards supplied by the manufacturer. The data were normalized to the mean of the control samples for each specific time point. Four independent cultures were analyzed per treatment condition and time point of study.

TABLE 1 List of primers.

Gene	Direction	Sequence
GAPDH	Forward	TGGCCTTCCGTGTTCCTAC
	Reverse	GAGTTGCTGTTGAAGTCG
Cdkn1a	Forward	AACATCTCAGGGCCGAAA
	Reverse	TGCGCTTGGAGTGATAGAAA
Cdkn2a	Forward	AATCCTCCGCGAGGAAAGC
	Reverse	GTCTGCAGCGGACTCCAT
Lamin B1	Forward	GGGAAGTTTATTCGCTTAAGA
	Reverse	ATCTCCCAGCCTCCCATT
Caspase 3	Forward	GCTGAGCAGTGAAGCCTATT
	Reverse	GCATGTGCCAGACAGTACAC
IL-6	Forward	CTAAGGCCAACCGTGAAAAG
	Reverse	ACCAGAGGCATACAGGGACA
Glut1A	Forward	CATCCTTATTGCCCAGGTGTTT
	Reverse	GAAGACGACACTGAGCAGCAGA
Carbonic Anhydrase VII	Forward	CAATGACAGTGATGACAGAA
	Reverse	TCCAGTGAACCAGATGTAG

2.8 | Statistical analysis

Survival analysis of sensory neurons was performed by repeated-measures analysis of variance (ANOVA). To compare one variable of independent experiments between multiple groups, two-way ANOVA was used. The Bonferroni post hoc correction was applied for multiple comparisons when needed. For more details on the specific statistical test used for each comparison and the exact value of *n*, please refer to the corresponding figure legend. GraphPad Prism v8.4.0 software was used for statistical inference analysis, and the data are graphically presented as the group mean ± SD. Differences among groups or time points were considered significant at $p < .05$.

3 | RESULTS

3.1 | Survival susceptibility

The following four experimental groups were assessed: a control group and a CDDP group cultured under supraphysiological normoxic conditions (18.5% O_2), and two other respective groups, one control and one CDDP, cultured under physioxic conditions (5% O_2). As shown in Figure 1, no differences in survival were observed according to the experimental oxygen concentration culture conditions between the control groups. However, there was a significant reduction in the survival percentage in both supraphysiological normoxic and physioxic treated groups compared to the control group. This decrease was evident the day after the addition of CDDP to the medium but reached significance only under supraphysiological normoxic conditions. This reduction in survival intensifies over time, becoming significant in both experimental groups compared to their respective controls.

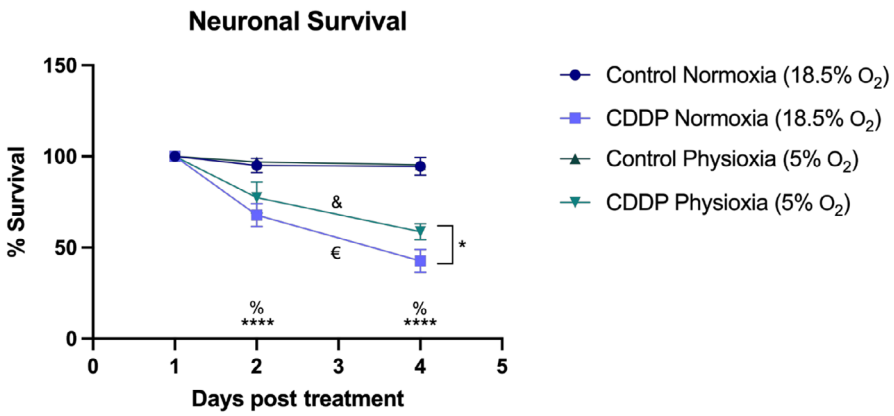

FIGURE 1 Neuronal survival after CDDP administration in DRG cells cultured under different atmospheric conditions (supraphysiological normoxia (18.5% O_2) and physioxia (5% O_2)). The percentage of surviving cells after CDDP treatment was calculated with respect to the basal concentration for each condition. A significant loss of neurons over time was observed in CDDP-treated cultures, and this loss was greater under normoxic (18.5% O_2) conditions than under physioxic (5% O_2) conditions. $n = 4$ cultures. Statistical analyses were performed using repeated measures two-way ANOVA followed by the post hoc Bonferroni correction. ****$p < .001$ CDDP Normoxia (18.5% O_2) versus Control Normoxia (18.5% O_2). % $p < .001$ CDDP Physioxia (5% O_2) versus Control Physioxia (5% O_2). &$p < .001$ CDDP Physioxia (5% O_2) 4th day versus CDDP Physioxia (5% O_2) 1st day. €$p < .001$ CDDP Normoxia (18.5% O_2) 4th day versus CDDP Normoxia (18.5% O_2) 1st day. *$p < .05$ CDDP Physioxia (5% O_2) versus CDDP Normoxia (18.5% O_2). The data are presented as the means ± SDs.

Notably, there were significant differences between the two experimental groups that received CDDP, with the physioxic condition exhibiting a greater percentage of neuronal survival.

3.2 | Neurite growth

Neurons cultured under control conditions showed normal arborization at both oxygen concentrations, although the neurites in the physioxia group had a greater length compared to their respective controls (553.68 ± 69.44 vs. 411.15 ± 57.97; $p = .08$) (Figure 2). However, after CDDP administration, the percentage of decrease was similar between the two oxygen concentration conditions (32.9% and 37.4% for supraphysiological normoxia and physioxia, respectively; $p = .48$). This reduction in length was statistically significant compared to that of the respective controls (Figure 2). In neuron development or maturation programs, CDDP, a cytotoxic drug that does not have a direct cytoskeletal interference mechanism, has the same toxic effect under supraphysiological normoxic and physioxic conditions. However, oxygen concentrations seem to impact the efficiency of these programs under control conditions.

3.3 | Molecular markers of the senescence phenotype

According to previous findings,[12] the administration of CDDP to mice (total cumulative dose of 42 mg/kg for 10 weeks) induces the development of peripheral neuropathy, wherein DRG neurons exhibit a senescent-like phenotype. Here, we assessed the expression of several classic senescence markers in relation to our two oxygen concentration culture conditions. To ensure that physiological conditions did not induce hypoxia in the culture, we examined the expression of two genes upregulated by the hypoxia-inducible factor Glut1a and carbonic anhydrase VII,[30,31] and no changes were observed between the groups (Figure S2). As depicted in Figure 3, no differences in the senescence-related molecular markers analyzed were noted between control supraphysiological normoxic and physioxic conditions. Similarly, both supraphysiological normoxic and physioxic conditions significantly increased Cdkn1a expression after CDDP exposure compared to that in the respective controls, with no changes in IL-6. However, Cdkn2a was significantly upregulated and Lamin B1 was significantly underexpressed under physioxic conditions compared to supraphysiological normoxic conditions. Importantly, caspase-3, a surrogate marker of neuronal apoptosis, was differentially activated under supraphysiological normoxic conditions compared to both the control and physioxic conditions, where no evidence of apoptotic execution was identified.

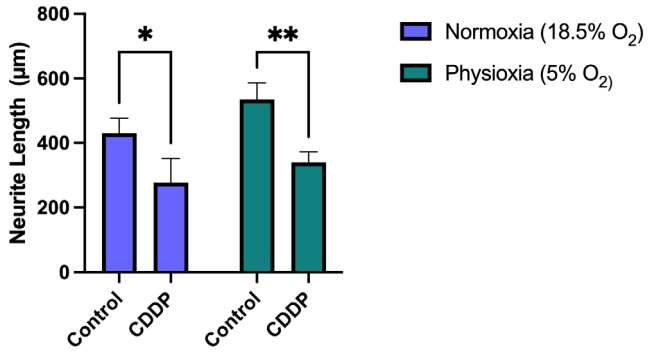

FIGURE 2 Neurite extension after CDDP administration in DRG cell cultures under different atmospheric conditions (supraphysiological normoxia (18.5% O_2) and physioxia (5% O_2)). Quantification of the longest neurites in each condition ($n = 3$–4 experiments per condition). Two-way ANOVA followed by post hoc Bonferroni correction was used for multiple comparisons. *$p < .05$ CDDP Normoxia (18.5% O_2) versus Control Normoxia (18.5% O_2). **$p < .005$ CDDP Physioxia (5% O_2) versus Control Physioxia (5% O_2). The data are expressed as the mean ± SD.

3.4 | Secretome cytokine composition on the basis of oxygen levels

When analyzing the levels of several proinflammatory cytokines (CXCL10, IL-6, CXCL1, CCL5, CCL3, and CCL11) in the culture media (Figure 4), we observed a general tendency for the levels of all cytokines to increase at 24 h after CDDP exposure under supraphysiological normoxic conditions compared to those in the corresponding controls, except for CCL11 (Eotaxin). However, 72 h after exposure, the levels of most cytokines decreased to values similar to those of the control group. However, at this time point, CCL11 expression was significantly greater in CDDP-treated cultures than in control cultures.

Under physioxic conditions, compared with those in the controls, a trend toward an increase in proinflammatory cytokines at 24 or 72 h after CDDP exposure was not observed. When the two experimental oxygen concentration conditions were compared, no changes in cytokine levels were observed in both control groups. However, a significant increase in IL-6, CCL5, and CCL3 was observed 24 h after CDDP exposure under supraphysiological normoxic conditions compared to physioxic conditions. A significant difference in CCL11 expression was also observed between the groups at 72 h.

Sensory neurons exhibit similar cytokine profiles, regardless of the experimental oxygen concentration culture conditions, when not exposed to any stressful stimuli. However, when cultured under supraphysiological normoxic conditions, the cells exhibited a time-dependent increase in inflammation after CDDP exposure, which was not observed under physioxic conditions.

4 | DISCUSSION

Adult mice mixed primary sensory neurons from dissociated DRGs are considered a good in vitro model to test CDDP neurotoxicity, since CIPN primarily affects adult patients, whose sensory neurons are fully mature and in relationship with other supportive DRG cells. However,

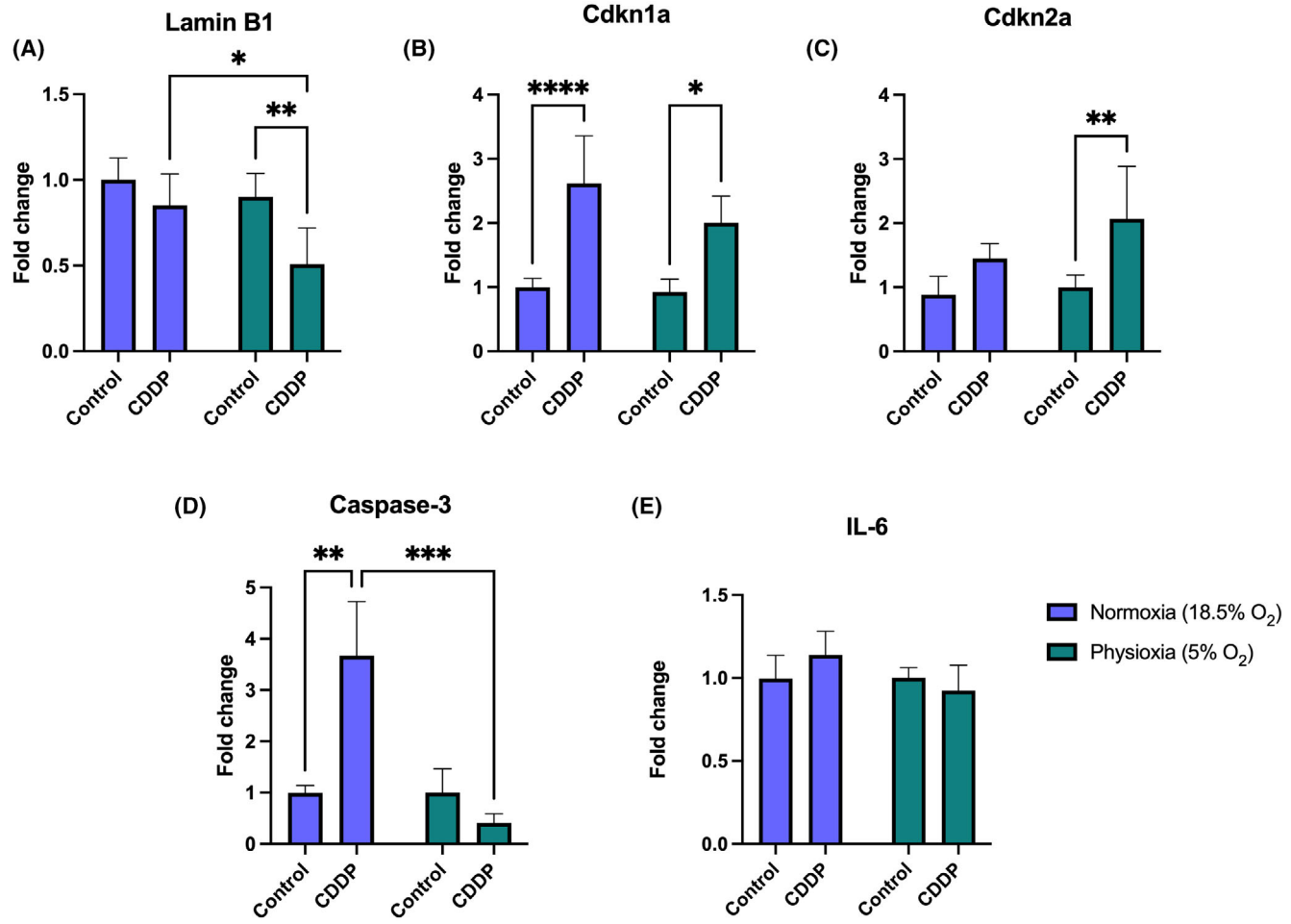

FIGURE 3 Senescence hallmarks after CDDP administration in DRG cells cultured under different atmospheric conditions. The expression of different markers was analyzed via RT-qPCR. (A) Expression level of Lamin B1. ($n = 5–6$ experiments per condition. $^{*}p < .01$ CDDP Normoxia (18.5% O_2) versus CDDP Physioxia (5% O_2). $^{**}p < .05$ CDDP Physioxia (5% O_2) versus Control Physioxia (5% O_2)). (B) Expression levels of Cdkn1a, a senescence inducer ($n = 5–7$ experiments per condition). $^{*}p < .05$ CDDP Physioxia (5% O_2) versus Control Physioxia (5% O_2). $^{****}p < .0001$ CDDP Normoxia (18.5% O_2) versus Control Normoxia (18.5% O_2). (C) Expression levels of Cdkn2a, a senescence inducer ($n = 5–7$ experiments per condition). $^{**}p < .05$ CDDP Physioxia (5% O_2) versus Control Physioxia (5% O_2). (D) Expression level of caspase-3 ($n = 3$ experiments per condition). $^{**}p < .05$ CDDP Normoxia (18.5% O_2) versus Control Normoxia (18.5% O_2). $^{***}p < .0001$ CDDP Physioxia (5% O_2) versus CDDP Normoxia (18.5% O_2)). (E) Expression levels of IL-6 ($n = 3$ experiments per condition). (A–E) Two-way ANOVA followed by post hoc Bonferroni correction for multiple comparisons. The data are expressed as the fold change versus the control condition and are presented as the mean ± SD. RT-qPCR, real-time quantitative polymerase chain reaction.

we underscored the importance of considering oxygen levels in these setting, as they can significantly influence survival, neurite growth, secretion of proinflammatory cytokines, and changes in molecular phenotype, especially when neurotoxic drugs, such as CDDP, are used to induce stress.

Under basal conditions, supraphysiological oxygen tension does not seem to impact the survival of sensory neuron cultures. Cellular and medium antioxidant buffers and the differentiated postmitotic nature of the cell cycle in adult neurons can compensate the excess of oxygen. Conversely, even in nontoxic conditioning media, replicative cells are susceptible to oxygen tension, as evidenced by the 'Hayflick limit' (the maximum number of divisions a normal somatic cell population can undergo before cell division ceases).[32] In fact, cells passaging many more times when cultured under low oxygen tensions than when cultured under higher oxygen tensions.[33]

On the other hand, under stressful or toxic media conditions, compensatory systems for scavenging ROS and cellular repair mechanisms to counteract ROS damage may prove insufficient[34] impacting cell survival and promoting differential adaptive neuron responses, as shown in our results.

The importance of conducting experiments under physiological oxygen concentrations is well recognized in senescence research, where most preclinical in vitro studies are typically carried out to more accurately mimic in vivo tissue oxygen conditions.[35] Therefore, oxygen levels must be considered when studying compounds that damage neurons, especially through mechanisms linked to oxidative stress, such as platinum and paclitaxel drugs.[36–38] However, the majority of preclinical in vitro CIPN research has been conducted under supraphysiologic oxygen concentrations. In these studies, evidence pointed that CDDP neurotoxicity was mediated by the

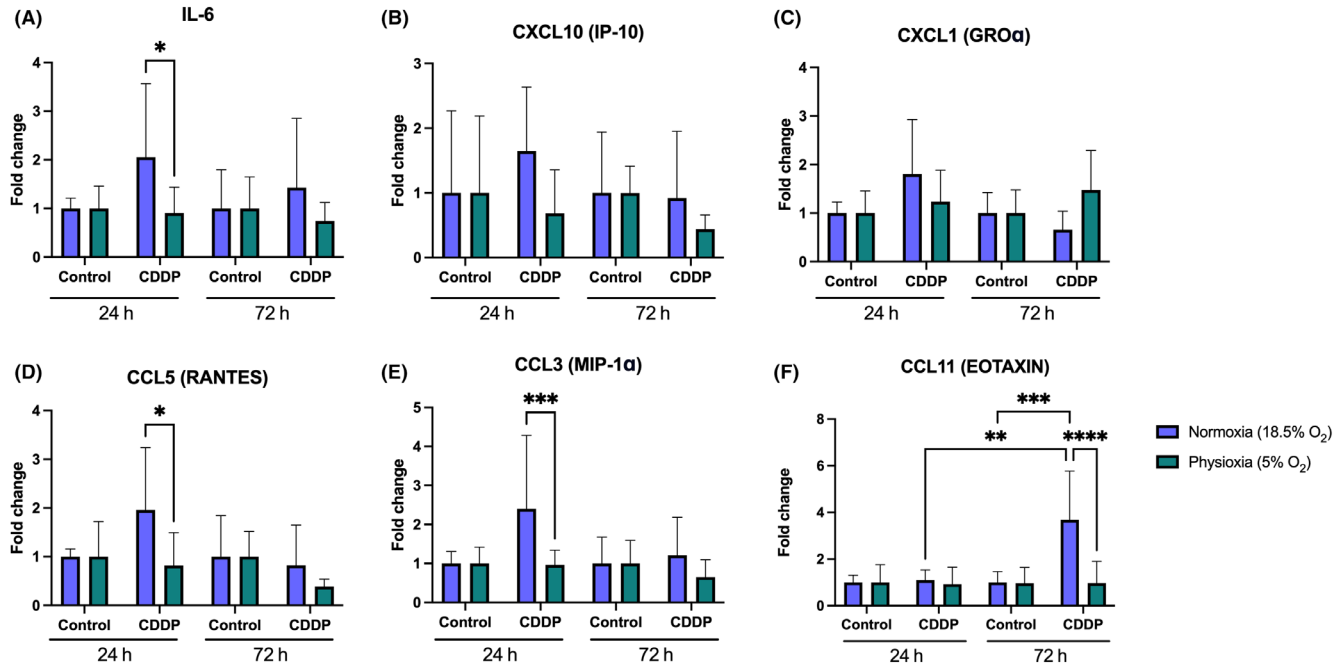

FIGURE 4 Secretome cytokine composition in the supernatant of DRG cultures under different atmospheric conditions after CDDP administration. Measurement of the fold change in the expression of each cytokine in physioxia and normoxia CDDP cultures at two time points. $n = 3–8$ independent experiments for each condition and time point. (A) IL-6. *$p < .05$ CDDP Physioxia (5% O_2) 24 h versus CDDP Normoxia (18.5% O_2) 24 h). (B) CXCL10. (C) CXCL1 (GROα). (D) CCL5 (RANTES) *$p < .05$ CDDP Physioxia (5% O_2) 24 h versus CDDP Normoxia (18.5% O_2) 24 h). (E) CCL3 (MIP-1a) ***$p < .005$ CDDP Physioxia (5% O_2) 24 h versus CDDP Normoxia (18.5% O_2) 24 h). (F) CCL11 (EOTAXIN) **$p < .001$ CDDP Normoxia (18.5% O_2) 72 h versus CDDP Normoxia (18.5% O_2) 24 h. *** < 0.005 CDDP Normoxia (18.5% O_2) 72 h versus Control Normoxia (18.5% O_2) 72 h. ****$p < .0001$ CDDP Physioxia (5% O_2) 72 h versus CDDP Normoxia (18.5% O_2) 72 h). (A–F) Two-way ANOVA followed by post hoc Bonferroni correction for multiple comparisons. The data are expressed as the fold change versus the control condition and are presented as the mean ± SD.

activation of pro-apoptotic mechanisms. In contrast, a senescence-like response rather than apoptosis or oxidative stress has already been described when using a well-characterized mouse model of cisplatin-induced peripheral neuropathy,[12] that was corroborated by another research group.[39]

In fact, we demonstrate in this study that, under normoxic conditions, neuron death induced by CDDP seems triggered by apoptosis, whereas when using physioxic conditions, senescence markers were observed. These include the upregulation of Cdkn1a and Cdkn2a mRNA levels. Both genes encode proteins that, in the absence of apoptosis, are considered markers of early and fully established differentiation senescence programs, respectively.[40] Additionally, a classic senescence molecular marker, Lamin B1, was downregulated.[41,42] In contrast, under supraphysiological normoxic conditions, only Cdkn1a was upregulated. This upregulation, together with the activation of caspase 3, suggests that neural fate is directed toward an apoptotic pathway. All these findings support the notion that oxygen levels play a crucial role in influencing the severity of cellular damage and conditioning the cellular response to noxious stimuli.

Oxygen levels also determine distinct cytokine profiles secreted in the media of our mixed cultures (containing both neurons and glial cells), differences that can impact the interactions between neurons and surrounding cells. In agreement with our findings, a previous study by Demaria's group also revealed that senescent cells express lower levels of detrimental proinflammatory factors in physiologically hypoxic environments.[35] Hence, the intensity of DNA damage, the robustness of DNA damage response pathway activation, and protein stress can differ based on the oxygen concentration. Even at identical doses of neurotoxic agents and DNA damage, distinct cellular outcomes and phenotypic fates can arise.

Obviously, more in-depth and extensive studies can be conducted to comprehend the pathways involved in this differential response to varying atmospheric oxygen concentrations. This may involve measuring ROS levels in cells or identifying the factors that drive the rewiring of transcriptional programs, such as the p53 transcriptional targets that can implement a cellular response (repair, senescence, or apoptosis) depending on the type and intensity of the stress as well as the cellular context, in a way not yet fully understood.[43] However, our primary objective was to emphasize the significance of oxygen conditions, particularly in the context of CIPN research.

5 | CONCLUSION

Undoubtedly, groundbreaking discoveries have been made in neuron cell cultures, even when exposed to supraphysiological oxygen

tensions. However, researchers must remain mindful of how elevated oxygen levels can obscure or reveal certain aspects of biology. Our findings suggest that the choice of oxygen concentration conditions during neuronal culture may impact the translatability of experimental results to in vivo models and clinical scenarios.

AUTHOR CONTRIBUTIONS

Formal analysis, *investigation*, *writing original draft*, *writing-review and editing*, *and visualization*: Jose M. Crugeiras. *Formal analysis*, *investigation*, *and writing-review and editing*: Aina Calls, Estefanía Contreras, and Montse Alemany. *Investigation*, *writing-review and editing*, *and funding acquisition*: Xavier Navarro. *Methodology*, *investigation*, *and writing-review and editing*: Victor J. Yuste and Oriol Casanovas. *Conceptualization*, *validation*, *supervision*, *and writing-review and editing*: Esther Udina. *Conceptualization*, *formal analysis*, *writing original draft*, *writing-review and editing*, *and funding acquisition*: Jordi Bruna.

ACKNOWLEDGMENTS

The authors' research was supported by funds from the CIBERNED and TERCEL networks to XN and by a PI21/00181 grant to JB from the Instituto de Salud Carlos III of Spain, cofunded by the European Union (ERDF/ESF, "Investing in your future"). We also thank the CERCA Programme/Generalitat de Catalunya for institutional support.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets generated during the current study are available from the corresponding author on reasonable request, and the raw data are deposited in https://data.mendeley.com/preview/f89s25xt9c?a=82146a5c-e748-489b-b6ea-cbbe4eae3c9e.

ORCID

Jose Crugeiras https://orcid.org/0009-0008-1564-4462
Jordi Bruna https://orcid.org/0000-0001-6895-5047

REFERENCES

1. Smith PA. Neuropathic pain; what we know and what we should do about it. *Front Pain Res (Lausanne)*. 2023;4. doi:10.3389/FPAIN.2023.1220034
2. Khodosevich K, Dragicevic K, Howes O. Drug targeting in psychiatric disorders—how to overcome the loss in translation? *Nat Rev Drug Discov*. 2024;23(3):218-231. doi:10.1038/S41573-023-00847-7
3. Seyhan AA. Lost in translation: the valley of death across preclinical and clinical divide—identification of problems and overcoming obstacles. *Transl Med Commun*. 2019;4(1):1-19. doi:10.1186/S41231-019-0050-7
4. Calls A, Carozzi V, Navarro X, Monza L, Bruna J. Pathogenesis of platinum-induced peripheral neurotoxicity: insights from preclinical studies. *Exp Neurol*. 2020;325. doi:10.1016/J.EXPNEUROL.2019.113141
5. Staff NP, Fehrenbacher JC, Caillaud M, Damaj MI, Segal RA, Rieger S. Pathogenesis of paclitaxel-induced peripheral neuropathy: a current review of in vitro and in vivo findings using rodent and human model systems. *Exp Neurol*. 2020;324:113121. doi:10.1016/J.EXPNEUROL.2019.113121
6. Geisler S. Vincristine- and bortezomib-induced neuropathies—from bedside to bench and back. *Exp Neurol*. 2021;336. doi:10.1016/J.EXPNEUROL.2020.113519
7. Loprinzi CL, Lacchetti C, Bleeker J, et al. Prevention and management of chemotherapy-induced peripheral neuropathy in survivors of adult cancers: ASCO guideline update. *J Clin Oncol*. 2020;38(28):3325-3348. doi:10.1200/JCO.20.01399
8. Gill JS, Windebank AJ. Cisplatin-induced apoptosis in rat dorsal root ganglion neurons is associated with attempted entry into the cell cycle. *J Clin Invest*. 1998;101(12):2842-2850. doi:10.1172/JCI1130
9. McDonald ES, Randon KR, Knight A, Windebank AJ. Cisplatin preferentially binds to DNA in dorsal root ganglion neurons in vitro and in vivo: a potential mechanism for neurotoxicity. *Neurobiol Dis*. 2005;18(2):305-313. doi:10.1016/J.NBD.2004.09.013
10. Fischer SJ, McDonald ES, Gross L, Windebank AJ. Alterations in cell cycle regulation underlie cisplatin induced apoptosis of dorsal root ganglion neurons in vivo. *Neurobiol Dis*. 2001;8(6):1027-1035. doi:10.1006/nbdi.2001.0426
11. Alaedini A, Xiang Z, Kim H, Sung YJ, Latov N. Up-regulation of apoptosis and regeneration genes in the dorsal root ganglia during cisplatin treatment. *Exp Neurol*. 2008;210(2):368-374. doi:10.1016/j.expneurol.2007.11.018
12. Calls A, Torres-Espin A, Navarro X, Yuste VJ, Udina E, Bruna J. Cisplatin-induced peripheral neuropathy is associated with neuronal senescence-like response. *Neuro Oncol*. 2021;23(1):88-99. doi:10.1093/NEUONC/NOAA151
13. Lehmann HC, Staff NP, Hoke A. Modeling chemotherapy induced peripheral neuropathy (CIPN) in vitro: prospects and limitations. *Exp Neurol*. 2020;326. doi:10.1016/J.EXPNEUROL.2019.113140
14. I G, RD K, SB M, J C. Growth responses of different subpopulations of adult sensory neurons to neurotrophic factors in vitro. *Eur J Neurosci*. 1999;11(10):3405-3414. doi:10.1046/J.1460-9568.1999.00756.X
15. Ng WP, Lozano AM. Neuronal age influences the response to neurite outgrowth inhibitory activity in the central and peripheral nervous systems. *Brain Res*. 1999;836(1–2):49-61. doi:10.1016/S0006-8993(99)01588-7
16. Genç B, Ulupinar E, Erzurumlu RS. Differential Trk expression in explant and dissociated trigeminal ganglion cell cultures. *J Neurobiol*. 2005;64(2):145-156. doi:10.1002/NEU.20134
17. Zheng JH, Walters ET, Song XJ. Dissociation of dorsal root ganglion neurons induces hyperexcitability that is maintained by increased responsiveness to cAMP and cGMP. *J Neurophysiol*. 2007;97(1):15-25. doi:10.1152/JN.00559.2006
18. Ponce A, Castillo A, Hinojosa L, Martinez-Rendon J, Cereijido M. The expression of endogenous voltage-gated potassium channels in HEK293 cells is affected by culture conditions. *Physiol Rep*. 2018;6(8):e13663. doi:10.14814/PHY2.13663
19. Schwaid AG, Krasowka-Zoladek A, Chi A, Cornella-Taracido I. Comparison of the rat and human dorsal root ganglion proteome. *Sci Rep*. 2018;8(1). doi:10.1038/S41598-018-31189-9
20. Zhuo M, Gorgun MF, Englander EW. Neurotoxicity of cytarabine (Ara-C) in dorsal root ganglion neurons originates from impediment of mtDNA synthesis and compromise of mitochondrial function. *Free Radic Biol Med*. 2018;121:9-19. doi:10.1016/J.FREERADBIOMED.2018.04.570
21. Place TL, Domann FE, Case AJ. Limitations of oxygen delivery to cells in culture: an underappreciated problem in basic and translational research. *Free Radic Biol Med*. 2017;113:311-322. doi:10.1016/J.FREERADBIOMED.2017.10.003
22. Keeley TP, Mann GE. Defining physiological normoxia for improved translation of cell physiology to animal models and humans. *Physiol Rev*. 2019;99(1):161-234. doi:10.1152/PHYSREV.00041.2017

23. Ast T, Mootha VK. Oxygen and mammalian cell culture: are we repeating the experiment of Dr. Ox? *Nat Metab*. 2019;1(9):858-860. doi:10.1038/S42255-019-0105-0
24. Alva R, Gardner GL, Liang P, Stuart JA. Supraphysiological oxygen levels in mammalian cell culture: current state and future perspectives. *Cells*. 2022;11(19). doi:10.3390/CELLS11193123
25. Imlay JA. Iron-sulphur clusters and the problem with oxygen. *Mol Microbiol*. 2006;59(4):1073-1082. doi:10.1111/J.1365-2958.2006.05028.X
26. Bell EL, Klimova TA, Eisenbart J, Schumacker PT, Chandel NS. Mitochondrial reactive oxygen species trigger hypoxia-inducible factor-dependent extension of the replicative life span during hypoxia. *Mol Cell Biol*. 2007;27(16):5737-5745. doi:10.1128/MCB.02265-06
27. Kamphorst JJ, Cross JR, Fan J, et al. Hypoxic and Ras-transformed cells support growth by scavenging unsaturated fatty acids from lysophospholipids. *Proc Natl Acad Sci U S A*. 2013;110(22):8882-8887. doi:10.1073/PNAS.1307237110
28. Nisimoto Y, Diebold BA, Constentino-Gomes D, Lambeth JD. Nox4: a hydrogen peroxide-generating oxygen sensor. *Biochemistry*. 2014;53(31):5111-5120. doi:10.1021/BI500331Y
29. Ta LE, Espeset L, Podratz J, Windebank AJ. Neurotoxicity of oxaliplatin and cisplatin for dorsal root ganglion neurons correlates with platinum-DNA binding. *Neurotoxicology*. 2006;27(6):992-1002. doi:10.1016/J.NEURO.2006.04.010
30. Potter C, Harris AL. Cell cycle hypoxia inducible carbonic anhydrase IX, marker of tumour: hypoxia, survival pathway and therapy target Christian Potter. *Cell Cycle*. 2004;3(2):164-167. doi:10.4161/cc.3.2.618
31. Kierans SJ, Taylor CT. Regulation of glycolysis by the hypoxia-inducible factor (HIF): implications for cellular physiology. *J Physiol*. 2021;599(1):23-37. doi:10.1113/JP280572
32. Hayflick L. The limited in vitro lifetime of human diploid cell strains. *Exp Cell Res*. 1965;37(3):614-636. doi:10.1016/0014-4827(65)90211-9
33. Packer L, Fuehr K. Low oxygen concentration extends the lifespan of cultured human diploid cells. *Nature*. 1977;267(5610):423-425. doi:10.1038/267423A0
34. Levings DC, Pathak SS, Yang YM, Slattery M. Limited expression of Nrf2 in neurons across the central nervous system. *Redox Biol*. 2023;65. doi:10.1016/J.REDOX.2023.102830
35. van Vliet T, Varela-Eirin M, Wang B, et al. Physiological hypoxia restrains the senescence-associated secretory phenotype via AMPK-mediated mTOR suppression. *Mol Cell*. 2021;81(9):2041-2052.e6. doi:10.1016/J.MOLCEL.2021.03.018
36. Melli G, Taiana M, Camozzi F, et al. Alpha-lipoic acid prevents mitochondrial damage and neurotoxicity in experimental chemotherapy neuropathy. *Exp Neurol*. 2008;214(2):276-284. doi:10.1016/J.EXPNEUROL.2008.08.013
37. Janes K, Doyle T, Bryant L, et al. Bioenergetic deficits in peripheral nerve sensory axons during chemotherapy-induced neuropathic pain resulting from peroxynitrite-mediated post-translational nitration of mitochondrial superoxide dismutase. *Pain*. 2013;154(11):2432-2440. doi:10.1016/J.PAIN.2013.07.032
38. Duggett NA, Griffiths LA, McKenna OE, et al. Oxidative stress in the development, maintenance and resolution of paclitaxel-induced painful neuropathy. *Neuroscience*. 2016;333:13-26. doi:10.1016/J.NEUROSCIENCE.2016.06.050
39. Acklin S, Zhang M, Du W, et al. Depletion of senescent-like neuronal cells alleviates cisplatin-induced peripheral neuropathy in mice. *Sci Rep*. 2020;10(1):14170. doi:10.1038/S41598-020-71042-6
40. Stein GH, Drullinger LF, Soulard A, Dulić V. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol Cell Biol*. 1999;19(3):2109-2117. doi:10.1128/MCB.19.3.2109
41. Adams PD, Ivanov A, Pawlikowski J, et al. Lysosome-mediated processing of chromatin in senescence. *J Cell Biol*. 2013;202(1):129-143. doi:10.1083/JCB.201212110
42. Salama R, Sadaie M, Hoare M, Narita M. Cellular senescence and its effector programs. *Genes Dev*. 2014;28(2):99-114. doi:10.1101/GAD.235184.113
43. Vousden KH, Lane DP. p53 in health and disease. *Nat Rev Mol Cell Biol*. 2007;8(4):275-283. doi:10.1038/nrm2147

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Crugeiras J, Calls A, Contreras E, et al. Oxygen matters: Unraveling the role of oxygen in the neuronal response to cisplatin. *J Peripher Nerv Syst*. 2024;29(4):528-536. doi:10.1111/jns.12659