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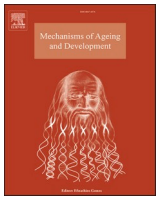

Doxorubicin-induced senescence in normal fibroblasts promotes *in vitro* tumour cell growth and invasiveness: The role of Quercetin in modulating these processes

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ABSTRACT

Ageing is a complex biological phenomenon representing the major risk factor for developing age-related diseases, such as cardiovascular pathologies, neurodegenerative diseases, and cancer. Geroscience, the new vision of gerontology, identifies cellular senescence as an interconnected biological process that characterises ageing and age-related diseases. Therefore, many strategies have been employed in the last years to reduce the harmful effects of senescence, and among these, the most intriguing ones use nutraceutical compounds. Here we show that a pre-treatment with Quercetin, a bioactive flavonoid present in many fruits and vegetables, increasing cellular antioxidant defence, can alleviate Doxorubicin (Doxo)-induced cellular senescence in human normal WI-38 fibroblasts.

Furthermore, our work demonstrates that Quercetin pre-treatment, reducing the number of senescent cells and the production of the senescence-associated secretory phenotype (SASP) factors, can decrease the pro-tumour effects of conditioned medium from Doxo-induced senescent fibroblasts on osteosarcoma cells. Overall, our findings are consistent with the hypothesis that targeting senescent cells can be an emerging strategy for cancer treatment, especially in elderly patients, in which senescent cells are already abundant in several tissues and organs.

1. Introduction

The increase in human life expectancy and the consequent ageing of the population will lead to an increase in age-related diseases (ARDs), like cardiovascular disease, cancer, neurodegenerative diseases, diabetes, arthritis, osteoporosis, sarcopenia, and several other chronic conditions, for which ageing represents the major risk factor. Thus, Geroscience, the new vision of gerontology, aims to understand the relationship between ageing and ARDs and improve the mechanisms that can prevent, delay, and/or counteract them. Therefore, it is likely that the future trend of medicine will try to decrease the prevalence of diseases by slowing the ageing rate, thus allowing people to stay healthy and active as long as possible (Franceschi et al., 2018; Sierra and Kohanski, 2015). However, because ARDs are so different, it is likely that one or a few mechanisms involved in ageing drive or favour the development and progression of these pathologies. One of the most intriguing candidates for this role is cellular senescence, a process described first by Hayflick (Hayflick and Moorhead, 1961; Hayflick, 1965) that showed that normal human fibroblasts in culture underwent irreversible proliferative arrest after a finite number of divisions due to the progressive shortening of telomeres. Subsequent studies have shown that in addition to "replicative-induced senescence", other types of stimuli, such as oxidative stress, exposition to ionising radiations, activated oncogene and chemotherapy, can trigger a "premature senescence" (Campisi, 2001; Campisi and D'Adda Di Fagagna, 2007). In addition to the proliferation arrest, other typical features characterise senescent cells, *i.e.* enlarged cellular morphology, expression of senescence-associated β-galactosidase activity (SA-β-Gal), formation of senescence-associated heterochromatin foci (SAHF), apoptosis resistance, as well as the secretion of a vast spectrum of cytokines, chemokines, proteases, and growth factors, referred to as "senescence-associated secretory phenotype" (SASP) (Campisi and D'Adda Di Fagagna, 2007; Coppé et al., 2008; Hernandez-Segura et al., 2018; Muñoz-Espín and Serrano, 2014). However, none of these features

Abbreviations: ARDs, Age-related diseases; CM, conditioned media; Doxo, Doxorubicin; OS, Osteosarcoma; SASP, Senescence-associated secretory phenotype.

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unequivocally identify senescent cells independently, but only a combination of these markers can define senescence. Moreover, not all senescent cells and not all types of senescence-induced stimuli lead to the expression of the same hallmarks (Rodier and Campisi, 2011).

In the last years, several studies have shown an accumulation of senescent cells in tissues and organs of aged people and mainly in ARDs, such as cardiovascular diseases (Erusalimsky and Kurz, 2005), osteoarthritis (Price et al., 2002), cancer (Ana Krtolica et al., 2001), and neurodegenerative pathologies (Bhat et al., 2012; Chinta et al., 2018; Salminen et al., 2011). Senescent cells drive ageing and ARDs, and it is crucial to understand how they accumulate with age and how it is possible to prevent their deleterious effects. First, the damaging effects of senescent cells can be due to their altered gene expression profile and morphology, compromising tissue function and limiting the regenerative potential of tissues with high cellular turnover (Rodier and Campisi, 2011). Second, the effects of SASP factors, which include several inflammatory cytokines and chemokines (Freund et al., 2010; Hernandez-Segura et al., 2017), may contribute to establishing a low-level chronic inflammation, called *inflammaging*, that drives the development of the major ARDs (Cevenini et al., 2013; Franceschi et al., 2007).

Concerning cancer, SASP factors can play a role in carcinogenesis and promote cancer cell proliferation, invasion, and metastasis (Coppé et al., 2010; Yang et al., 2021). Furthermore, since different chemotherapy drugs can induce cell senescence in both cancer and normal cells, these therapy-induced senescent cells can drive and increase the development of the malignant phenotype in tumour residual living cells (Mongiardi et al., 2021; Saleh et al., 2020). Consequently, it can be essential to identify substances capable of reducing therapy-induced senescence that can be used as adjuvants in association with the anti-cancer drugs to prevent or decrease cancer recurrence and/or progression.

Osteosarcoma (OS) is a high-grade primary bone tumour with a high mortality rate and a bimodal incidence, with a first peak occurring at the age of puberty, and a second peak in the elderly over 75 years (Whelan and Davis, 2018). Currently, OS treatment involves a combination of surgery and chemotherapy, carried out with three common cytotoxic agents, Cisplatin, Doxorubicin, and Methotrexate (Casali et al., 2018). Nowadays, Doxorubicin (Doxo) is counted among those chemotherapeutic drugs that can induce the so-called therapy-induced senescence (TIS) in both cancer and normal cells (Bielak-Zmijewska et al., 2014; Demirci et al., 2021; Hou et al., 2019; Maejima et al., 2008), as fibroblasts that may constitute cancer stroma. This off-target effect could be a determinant factor in cancer progression and metastatic spread (Demirci et al., 2021; Lecot et al., 2016), that together with therapy toxicity, are the principal causes of OS treatments failure. In this scenario, an important role is played by tumour stroma, constituted by different types of cells, including fibroblasts, that contribute to OS progression through fundamental features for neovascularization, homing, microvesicles and soluble factors secretion, paracrine feeding, and immune modulation (Cortini et al., 2017). Therefore, it is crucial to analyse the mechanisms and actors involved in metastatic progression and investigate new therapeutic approaches that can be used as adjuvants to traditional therapies to improve efficiency and reduce toxicity.

We hypothesised that the treatment with Doxo may induce in fibroblasts the senescent phenotype, which promotes OS cells growth and invasiveness through the production and release of SASP factors. This effect could be more evident in old patients, who already present an age-related accumulation of senescent cells and a concomitant systemic inflammaging. Moreover, we proposed the use of Quercetin (3,3', 4'5,7-pentahydroxyflavone), a natural bioactive flavonoid found in a wide variety of fruits and vegetables (Andres et al., 2018; Boots et al., 2008; D'Andrea, 2015), known for its antioxidant, anti-inflammatory (Boots et al., 2008; Santos and Mira, 2004), and anti-cancer properties (Salehi et al., 2020), to alleviate Doxo-induced senescence and minimise the tumour-promoting effects of SASP factors on cancer cells.

Our results show that the pre-treatment with Quercetin can reduce Doxo-induced senescence in human fibroblasts *in vitro,* enhancing cellular antioxidant defences. Moreover, we demonstrated that conditioned medium from Doxo-induced senescent fibroblasts increases OS cells growth, migration and invasiveness and that the pre-treatment with Quercetin, reducing Doxo-induced senescence, decreased the protumorigenic effects of the conditioned medium. These data indicate that Quercetin pre-treatment can be considered a good adjuvant to Doxo treatment to contrast the adverse effects of therapy-induced senescence in the OS microenvironment.

2. Materials and method

2.1. Reagents

Doxorubicin (#5927S Cell Signalling Technology) was dissolved in Dimethyl Sulfoxide (DMSO, D8418, Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 10 mM and stocked at − 20 °C until use. Quercetin (purity ≥ 95 %, Q4951) powder was purchased from Sigma-Aldrich and was resuspended in DMSO to a concentration of 30 mg/mL (99 mM). 5-Bromo-4-Chloro-3-Indolyl B-D-*Galactop B4252 was purchased from Sigma-Aldrich.

2.2. Cell lines and culture conditions

Human foetal lung fibroblasts WI-38 (AG06814-N, Coriell Institute for Medical Research, Camden, New Jersey), and human OS cell line U2OS (pRB +/+, p53+/+) (HTB-93, ATCC, Manassas, VA, USA) were grown in complete medium (DMEM High supplemented with 10 % of heat-inactivated FBS, 100 U/mL Penicillin, 100 μg/mL Streptomycin, 0.25 μg/mL Amphotericin B, and 2 mM L-Glutamine) at 37 °C in 5 % CO_2 humidified incubator. WI-38 cells with Population Doubling Level (PDL) < 30 were used in this study to avoid replicative senescence.

2.3. Induction of senescence and Quercetin pre-treatment

2.3.1. Induction of senescence

Before starting experiments, an adequate number of WI-38 cells (3×10^4 for well) were plated for 24 h in complete medium at 37 °C in a 5 % CO_2 humidified incubator. Subsequently, WI-38 cells were treated with Doxo 50 nM for 48 h to induce senescence, and then cultured in complete medium for 3 days. The control was represented by cells incubated for 48 h in complete medium with 0.0005 % of DMSO (used as diluent for Doxo) and then maintained in fresh complete medium for the following 3 days. At this time, senescence markers were analysed. The dose for Doxorubicin treatment was chosen after many experiments with different doses and times of treatment to find the correct one that mainly induced senescence and not cell death (data not shown).

2.3.2. Quercetin pre-treatment

Based on the range of doses of Quercetin used for *in vitro* studies in literature, from 10 to 40 μM, we have optimised our protocol with several experiments (data not shown) to prevent Doxo-induced senescence. WI-38 cells were pre-treated with Quercetin 40 μM for 24 h in complete medium, washed and exposed to Doxorubicin for 48 h and then cultured in fresh complete medium for 3 days. Control samples were represented by WI-38 cells treated with DMSO (C) or Quercetin (Q) for 24 h and then cultured in fresh complete medium.

2.4. Conditioned medium preparation and treatment of U2OS and WI-38 cells

WI-38 cells after the pre-treatment with Quercetin (24 h) and the treatment with Doxo (48 h) were cultured for 3 days with fresh complete medium. The conditioned medium (CM) was collected, centrifuged, and used to treat U2OS and WI-38 cells.

2.5. Cell proliferation

Trypan Blue exclusion assay was used to determine cell growth and vitality. Briefly, WI-38 or U2OS cells were trypsinized and stained with Trypan Blue. Then, live cells and dead cells were counted using a Bürker chamber under a light microscope.

2.6. Senescence-associated β-galactosidase staining

SA-β-Gal staining was performed as described previously (Dimri et al., 1995). WI-38 were plated 8×10^3 cells/cm^2 and assessed for SA-β-Gal activity at various times after the different treatments. A minimum of 300 cells for each group was counted. Positive (blue) cells were expressed as a percentage of the total cell number.

2.7. Flow-cytometric analysis of cell cycle

Cell cycle analysis was performed at different times with Propidium Iodide staining. Briefly, WI-38 cells were trypsinized, counted, and aliquoted in FACS tubes. The cells were washed in PBS 1X and then incubated in a hypotonic solution of Propidium Iodide (0.1 % w/v Sodium citrate tribasic, 0.1 % Triton X-100, and 50 μg/mL Propidium Iodide) at 4 °C for 30 min. The cell cycle was analysed using FACS CANTO II and BD FACSDIVA Software (Becton, Dickinson and Company). The data were analysed using FlowJo Software.

2.8. Western blotting

The whole-cell lysate was obtained using Laemmli Buffer, while nuclear-cytoplasmic fractions were obtained using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific), following manufacturer's instruction. Protein concentration was determined using BCA Protein assay kit (ThermoFisher Scientific). 50 μg of protein per sample were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to nitrocellulose membrane (Amersham Protran, GE Healthcare Life Science) by electroblotting. After being blocked with Bovine Serum Albumin (BSA) 5 %, the membrane was incubated with primary antibody against phospho(Ser807/811)-RB (#8516, Cell Signalling Technology), Lamin B1 (ab16048, Abcam), p21 (sc-817, Santa Cruz Biotechnology), p16 (sc-56330, Santa Cruz Biotechnology), Fibrillarin (sc-166001, Santa Cruz Biotechnology), NF-κB p65 (#4764, Cell Signalling Technology), SOD-1 (GTX100659, Genetex), SOD-2 (ADI-SOD-110, Enzo Life Sciences) and α-tubulin (sc-32293, Santa Cruz Biotechnology), respectively, at 4 °C overnight. Washed membranes were incubated for 1 h at room temperature with IRDye800CW–conjugated or IRDye680–conjugated secondary antibody (LI-COR Biosciences), and antibody-coated bands were visualised by Odyssey Infra-red Imageing System Densitometry (LI-COR Biosciences).

2.9. Immunostaining

WI-38 cells were plated on sterile coverslips placed insight 12-well plates, and the treatments were performed as described above. At the end of experiments, cells were fixed in PFA 4 % for 10 min or in iced Methanol 100 % at − 20 °C for 15 min, permeabilised with 0.2 % Triton X-100 for 5 min, quenched with Sodium Borohydride, washed, and blocked for 1 h with 1 % BSA 10 % horse serum at room temperature. Slides were incubated overnight at 4 °C with primary antibodies in BSA 1 % (p21: sc-817, Santa Cruz Biotechnology; Lamin B1: #68591, Cell Signalling Technology; Histone Macro H2A.1: ABE215, Merck Millipore; γH2AX: #9718, Cell Signalling Technology). Slides were washed three times and incubated at room temperature for 40 min with secondary antibodies conjugated with Alexa Fluor 488 or Cy3 (Goat anti-Rabbit and Goat anti-Mouse, respectively, IgG (H+L) Cross-Adsorbed Secondary Antibody, Thermofisher Scientific). Nuclear DNA was stained with ProLong™ Gold Antifade Mountant with DAPI (Thermofisher Scientific). Slides were visualised using Leica SP8 Confocal Microscope.

2.10. ROS assessment by flow cytometry analysis

ROS production was assessed in WI-38 fibroblasts after 24 and 48 h of Doxo-treatment. Briefly, cells were trypsinised, washed with PBS1X, and incubated with H2DCF-DA 2.5 μM (Invitrogen, Carlsbad, CA, USA) in DMEM High glucose without serum and phenol red for 30 min at 37 °C 5 % CO_2. After labelling, the samples were examined immediately using FACS CANTO II flow cytometer, and the data were analysed using FACSDiva software (Becton-Dickinson, San Jose, CA, USA).

2.11. Total antioxidant capacity (TAC) assay

The oxygen radical absorbance capacity (ORAC) method was performed as previously described (Emmi et al., 2019; Sofi et al., 2018) on WI-38 fibroblasts protein lysates extracted 24 h after Quercetin treatment. Briefly, fluorescein solution (6 nM) was prepared in 75 mM sodium phosphate buffer (pH 7.4), and Trolox (250 μM) was used as standard. 100 μL of fluorescein was pre-incubated with 70 μL of each sample for 30 min at 37 °C, in each well, before rapidly adding AAPH solution (19 mM final concentration). Fluorescence was then measured using Synergy H1 microplate reader (BioTek, Winooski, VT), and the results were expressed as Trolox Equivalents (μM) and then normalised for protein concentration.

2.12. Wound healing assay

The wound-healing assay was performed to evaluate U2OS and WI-38 cell migration under the effect of CM from Quercetin pre-treated and Doxo-treated fibroblasts. U2OS and WI-38 cells were plated on 24 well plates 72 h before the treatment. A vertical line was scratched on adherent cell monolayer in the centre with a sterile 200 μL micropipette tip. Then, cells were washed with PBS 1X and incubated with CM. Wound closure was monitored at 6, 24, 48 h. The scratch closure area was measured using ImageJ software.

2.13. Invasion assay

U2OS cells invasiveness was evaluated using Boyden chamber assay. The method is based on the passage of the cells across 8 μm pore size polyvinyl pyrrolidone (PVP)-free polycarbonate filters (Neuro Probe, Inc., USA) precoated with Matrigel (50 μg/filter), placed between the two wells of the Boyden chamber. CM from Doxo-treated fibroblasts (SM), Quercetin pre-treated Doxo-treated fibroblasts (QSM), Quercetin pre-treated fibroblasts (QM), and control cells (CM) were placed in the bottom wells of the chamber. U2OS cells resuspended in complete DMEM High supplemented with 2 % FBS were seeded in the upper chamber (2×10^4 cells/well) and incubated overnight. The filters were removed and fixed in Methanol for 1 h. Non-migrated cells attached to the upper surface of the filter were scraped, while migrated cells, adherent on the lower filter surface, were stained with Crystal violet 0.01 % and counted using a light microscope (40 × magnification). Each point was performed in triplicate, and mean values of migrated cells for each point were calculated and expressed in relation to control.

2.14. Colony formation assay

U2OS colony formation under treatment with CM was evaluated as follows: 2×10^3 cells were seeded into a 60 mm cell culture dish in complete medium, and after 72 h, the medium was removed, and the cells were incubated with SM, QSM, CM, or QM for 7 days. Then, cells were washed with PBS 1X and stained with 0.1 % Crystal Violet for 10 min. After washing twice with water, the dishes were left to dry overnight. The next day, a picture of the whole plates was taken, and the

number and area of colonies were evaluated by ImageJ software.

2.15. RNA extraction and quantitative real-time PCR

Total RNA extraction was performed 3 days after Doxo treatment with a Total RNA Purification kit (Norgen Biotek Corp., Thorold, ON, Canada) following the manufacturer's instruction. cDNA synthesis was carried out using ImProm-IITM Reverse Transcription System (Promega Corporation, Madison, WI, USA), and quantitative real-time PCR (qPCR) was performed using GoTaq® qPCR Master Mix (Promega). For primer sequences, see Table 1. The qPCR analysis was carried out in triplicate using CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). mRNA was quantified with the ΔΔCt method, and mRNA levels were normalised to GAPDH as endogenous control.

2.16. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as mean ± SD calculated on at least three independent experiments. P values were calculated using the Student's t-test or one-way analysis of variance (ANOVA). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Doxorubicin-induced senescence in WI-38 fibroblasts

We used Doxorubicin (50 nM) for 48 h to induce senescence in WI-38 fibroblasts, and we analysed senescence markers at 72 h after the treatment with Doxo (Fig. 1**A**). Our results showed that WI-38 cells after 48 h of Doxo treatment significantly decreased their growth compared to control (C), and the proliferation arrest was maintained even for the following 72 h (Fig. 1**B**). Furthermore, cytofluorimetric analysis of cell cycle showed that Doxo-treated cells exhibited a significantly predominant block in G2/M phase at both times 64.4 % and 71.5 %, respectively) compared to the control (21.7 % and 16.6 %, respectively) (Fig. 1**C**). Concurrently, 72 h after Doxo-treatment, we observed that cells were enlarged in size, and at least 60 % were positive for SA-β-Gal activity compared to control cells (Fig. 1**D**). We have also analysed in Western Blot the expression of the two crucial pathways closely related to cellular senescence, p21–p53, p16-pRB and the expression of Lamin B1, a structural component of the nucleus reduced in senescent cells. Fig. 1**E** shows that treated cells significantly reduced phospho-Rb and Lamin B1 and increased p21 and p16 expression compared to control cells. Concomitantly, we have analysed by confocal microscopy the presence in Doxo-treated cells of γH2AX nuclear foci, a marker of DNA double-strand breaks (DSB) frequently present in senescent cells, and macroH2A1, a histone variant component of SAHF. Notably, most of the treated cells (71.3 %) were positive for both p21 and γH2AX foci, and 75 % of cells were simultaneously negative for Lamin B1 and positive for macroH2A1 (Fig. 1**F**). The control showed a low presence of cells positives for p21 and γH2AX foci (4.4 %) and cells simultaneously negative for Lamin B1 and positive for macroH2A1 (13.2 %). These results indicated that after Doxo-treatment, most WI-38 cells were senescent.

3.2. Quercetin pre-treatment reduces Doxo-induced senescence in WI-38 fibroblasts

To verify the ability of Quercetin to reduce Doxo-induced senescence, WI-38 fibroblasts were pre-treated with the flavonoid for 24 h before the induction of senescence with Doxo (Fig. 2**A**). As described above, Doxo-treated cells underwent a proliferative arrest, and, on the contrary, Quercetin pre-treated cells partially recovered their proliferative capacity from day 3, and their growth continued for the following 10 days after Doxo treatment (Fig. 2**B**). Indeed, at day 3, Doxo-treated cells presented an accumulation in G2/M phase (61.5 %) along with a minimal S phase fraction (8.5 %), and reduced G0/G1 phase (30 %), while Quercetin pre-treated cells showed similar values for G0/G1 phase (30.4 %), but a reduced G2/M phase (50.4 %) and a significantly increased S fraction (19.2 %) compared to Doxo-treated cells ($p < 0.05$). On day 10, Doxo-induced senescent fibroblasts did not resume cell cycle and presented similar percentages to day 3 values (35.9 % G0/G1, 5.6 % S, 58.5 % G2/M). Conversely, as shown in Fig. 2**C**, the cell cycle of Quercetin pre-treated fibroblasts varied with respect to day 3, and at day 10 the cells showed an increased G0/G1 and S phase (45 % and 13 %, respectively) and a significant reduction in G2/M fraction (41.7 %) compared to Doxo-induced senescent cells ($p < 0.05$).

Notably, the analysis of Sa-β-Gal activity showed that at day 3, cells pre-treated with Quercetin presented a significantly lower number of positive cells (16.8 %) than Doxo-treated cells (60.8 %). On day 10, although the percentage of positive cells increased in the pre-treated sample (36.8 %), this was still significantly lower than Doxo-treated cells (91.5 %) (Fig. 3).

Next, to verify the effect of the Quercetin on senescent phenotype, we evaluated the changes in the expression of senescent molecular markers. Phospho-RB and Lamin B1 expression was significantly up-regulated, while p21 and p16 significantly decreased in Quercetin pre-treated cells compared to Doxo-treated cells (Fig. 4).

Moreover, by confocal microscopy, we have analysed the presence of γH2AX and macroH2A1 foci combined with p21 and Lamin B1 expression, respectively. As shown in Fig. 5, p21-γH2AX positive cells and Lamin B1 negative-macroH2A1 foci positive cells were significantly reduced in Quercetin-pretreated cells (30.2 % and 16 %, respectively) compared to Doxo-treated cells (79 % and 73 %, respectively).

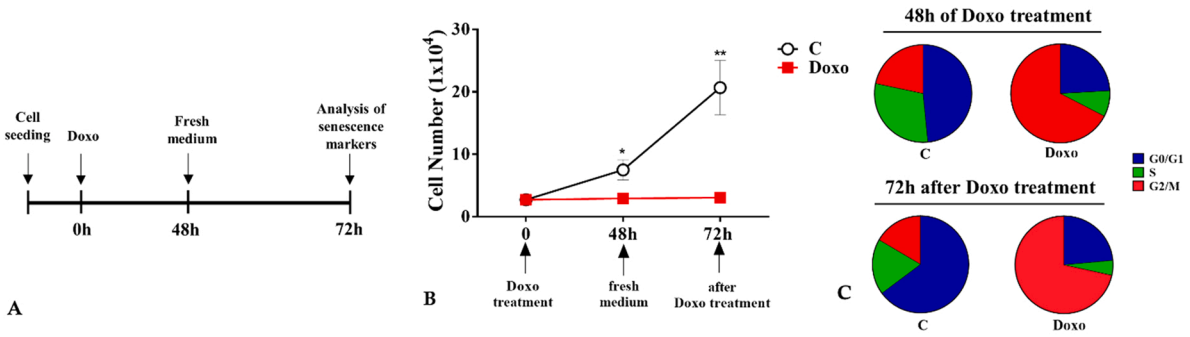
In conclusion, our results showed that Quercetin pre-treated cells partially recovered their proliferative capacity, reduced G2/M arrest, decreased Sa-β-Gal activity, and reduced formation of γH2AX and macroH2A1 foci, suggesting that Quercetin pre-treatment could protect the cells from Doxo-induced senescence.

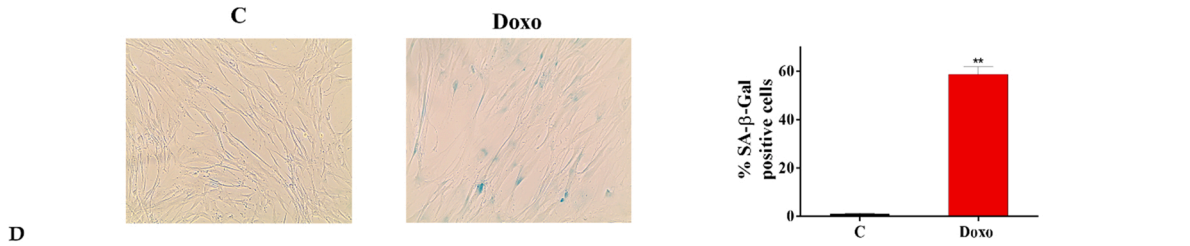
3.3. Quercetin pre-treatment increases cellular antioxidant defences

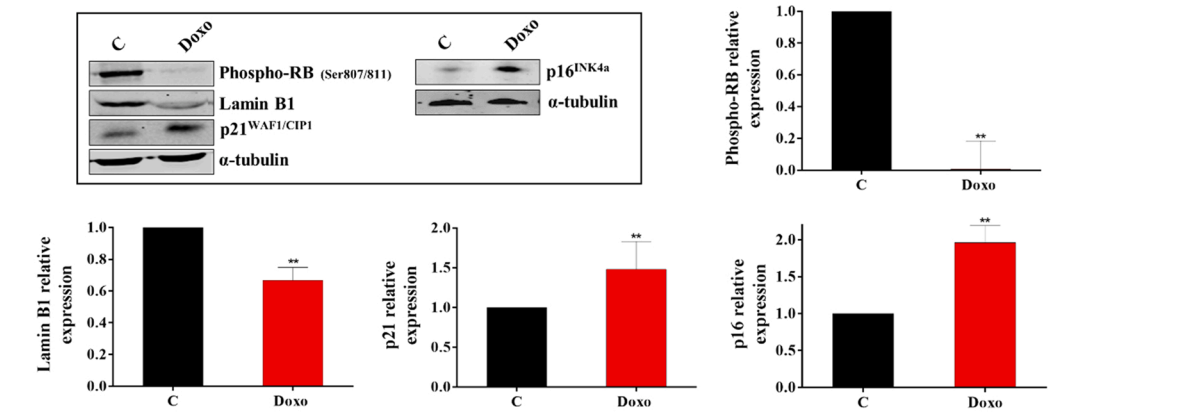
To investigate the mechanisms underlying the protective effect of Quercetin pre-treatment, we evaluated its effect on cellular antioxidant systems. As shown in Fig. 6**A,** the Total Antioxidant Capacity of WI-38 cells exposed for 24 h to Quercetin increased significantly. Moreover, we demonstrated that Quercetin pre-treatment significantly up-regulated SOD-1 and SOD-2 enzyme expression (Fig. 6**B**). We next assessed ROS production after 24 and 48 h of Doxo-treatment, and

Table 1
Primer sequences used for qPCR evaluation of the indicated gene expression.

Gene	Forward primer	Reverse primer
IL-6	5'-AGACAGCCACTCACCTCTTCAG-3'	5'-TTCTGCCAGTGCCTCTTTGCTG-3'
IL-8	5'-GAGAGTGATTGAGAGTGGACCAC-3'	5'-CACAACCCTCTGCACCCAGTTT-3'
GROα	5'-AGCTTGCCTCAATCCTGCATCC-3'	5'-TCCTTCAGGAACAGCCACCAGT-3'
IL-1α	5'-TGTATGTGACTGCCCAAGATGAAG-3'	5'-AGAGGAGGTTGGTCTCACTACC-3'
MMP2	5'-AGCGAGTGGATGCCGCCTTTAA-3'	5'-CATTCCAGGCATCTGCGATGAG-3'
CXCL12	5'-CTCAACACTCCAAACTGTGCCC-3'	5'-CTCCAGGTACTCCTGAATCCAC-3'
TNFα	5'-CTCTTCTGCCTGCTGCACTTTG-5'	5'-ATGGGCTACAGGCTTGTCACTC-3'
GAPDH	5'-GTCTCCTCTGACTTCAACAGCG-3'	5'-ACCACCCTGTTGCTGTAGCCAA-3'







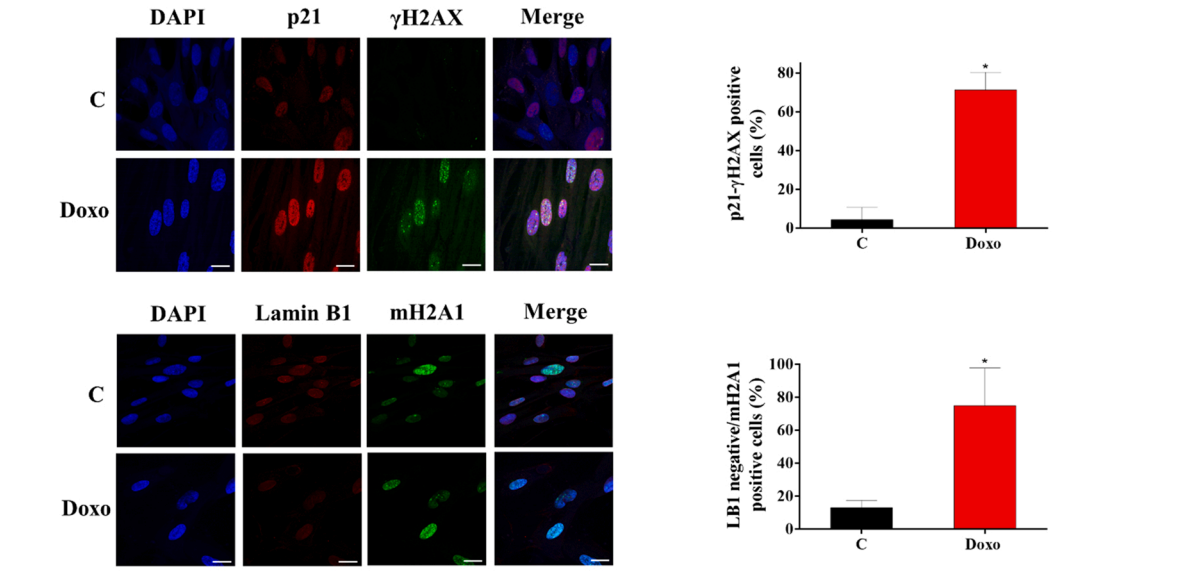


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Fig. 1.. Doxorubicin treatment induces senescence in WI-38 fibroblasts. **(A)** Experimental scheme. Cells were exposed to Doxorubicin 50 nM for 48 h and then cultured in fresh complete medium for the following 72 h. **(B)** Trypan Blue exclusion assay was performed at 48 h of Doxo treatment, and the following 72 h, in Doxo-treated cells (Doxo) and control cells (C). Data are expressed as the mean of at least four independent experiments ± standard deviation (SD). P-value refers to differences with respect to control C (*$p < 0.05$; **$p < 0.001$). **(C)** Doxo treatment induces a cell cycle arrest in G2/M phase in most treated cells. Each pie chart section represents the percentage of cells in a specific cell cycle phase. Data are expressed as the mean of at least four independent experiments. The differences among G0/G1, S, and G2/M phases of Doxo sample and control resulted significant ($p < 0.001$ for all three phases) at 48 h of Doxo treatment and 72 h after Doxo treatment ($p < 0.001$, $p < 0.05$, $p < 0.001$, respectively). **(D)** Representative images of SA-β-Gal activity (left). The percentage of SA-β-Gal positive cells (blue ones) compared to the total number of cells/well was evaluated in three independent experiments, and the results are represented as the mean ± SD (right). P-value refers to difference compared to control C (**$p < 0.001$). **(E)** Immunoblot representative image (left) and quantification of the expression of phosho-RB, Lamin B1, p21 and p16 in Doxo-treated (Doxo) and control (C) WI-38 fibroblasts. Lysates were collected 72 h after Doxo treatment. Protein band intensity was normalised to α-tubulin and expressed in relation to control. Data are expressed as the mean ± SD of triplicate experiments. P-value refers to differences compared to control (**$p < 0.001$). **(F)** Confocal microscopy images of indirect immunofluorescence of γH2AX foci (green) and p21 (red), and macroH2A1 foci (green) and Lamin B1 (red), in control cells (C) and Doxo-induced senescent fibroblasts (Doxo). DNA was counterstained with DAPI (blue). Scale bars: 20 μm. Confocal images from three independent experiments were analysed to quantify p21-γH2AX positive cells and Lamin B1 negative-macroH2A1 positive cells with respect to the total number of cells. P-value refers to differences regarding control (*$p < 0.05$). Abbreviations: *C*: control; *Doxo*: Doxo-induced senescent fibroblasts.

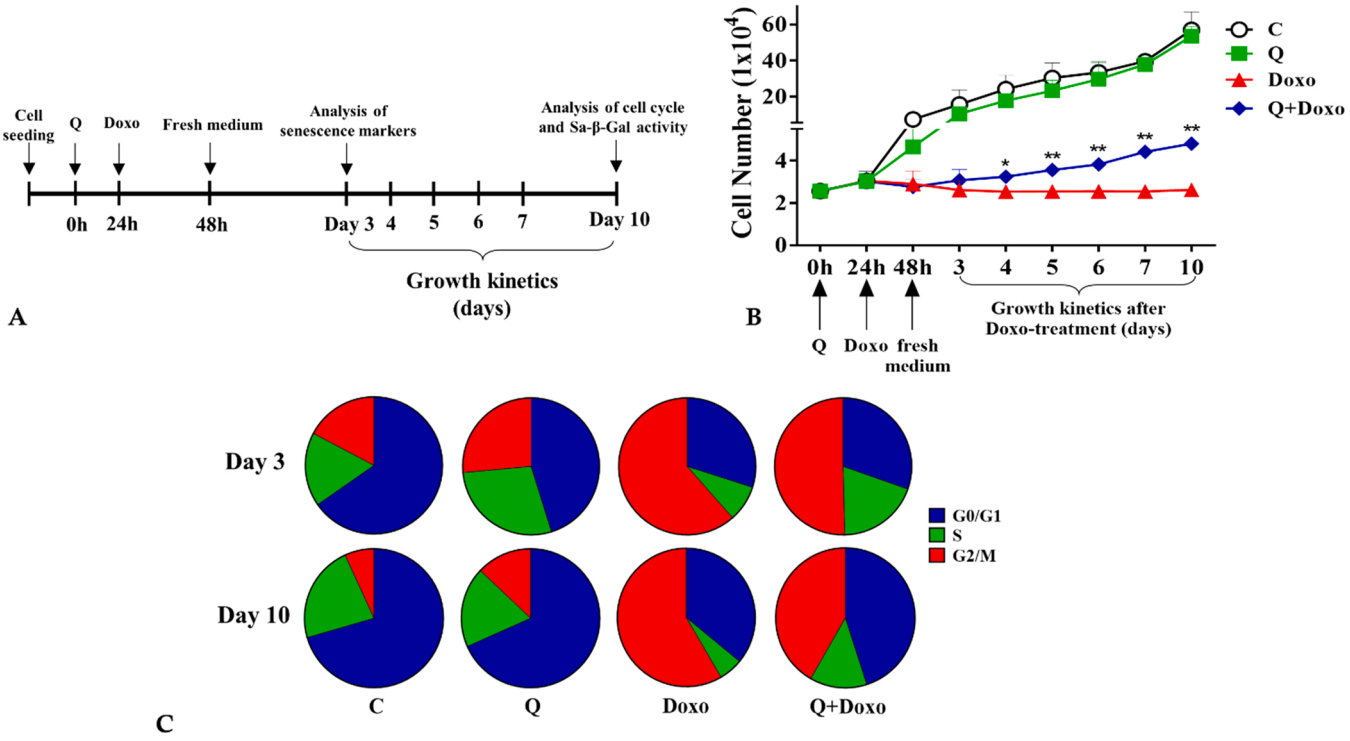

Fig. 2. Quercetin pre-treatment reduces Doxorubicin-induced senescence in WI-38 fibroblasts. (A) Experimental scheme. Cells were pre-treated with Quercetin 40 μM for 24 h and then exposed to Doxo-induced senescence. **(B)** Trypan Blue exclusion assay was performed at different times during the treatments, and a growth curve was done from day 3 onwards. Data are expressed as the mean ± SD of at least three independent experiments. The differences in cell proliferation between Doxo-treated cells (Doxo) and control (C) or Quercetin treated fibroblasts (Q) resulted significant at all times ($p < 0.001$). P-value, shown in the figure, refers to differences between Quercetin pre-treated Doxo-treated fibroblasts (Q + Doxo) and Doxo-treated cells (Doxo) (*$p < 0.05$; **$p < 0.001$). **(C)** Cell cycle analysis was performed on day 3 and 10 from the end of Doxo treatment. Each pie chart section represents the percentage of cells in a specific cell cycle phase. Data are expressed as the mean of at least three independent experiments. The differences between G0/G1, S, and G2/M phase of Doxo sample and control resulted significant at both times ($p < 0.001$, $p < 0.05$, and $p < 0.001$, respectively). Quercetin pre-treated Doxo-treated fibroblasts (Q+Doxo) presented a significant increased S phase ($p < 0.05$) at day 3, and a significant decreased G2/M phase ($p < 0.05$) at day 10 compared to Doxo-treated cells (Doxo). Abbreviations: C: control; Q: Quercetin treated fibroblast; Doxo: Doxo-induced senescent fibroblasts; Q + Doxo: Quercetin pre-treated Doxo-induced senescent fibroblasts.

cellular ROS levels were significantly increased by 1.3 and 1.7 times, respectively, compared to control (Fig. 6**C**). Conversely, Quercetin pre-treatment significantly reduced ROS production compared to Doxo-treated cells (0.7 at 24 h and 0.8 at 48 h). These results suggested that Quercetin pre-treatment could potentiate cellular antioxidant defences, which in turn countered Doxo-induced oxidative stress and protected the cells from damage and senescence.

3.4. Quercetin pre-treatment reduces NF-κB nuclear translocation and gene expression of some SASP factors

To verify the activity of NF-κB, one of the major transcription factors that control the production of SASP molecules, we have analysed the expression of p65 in the nuclear fraction of the cells. As shown in Fig. 7**A,** Doxo-treated cells presented a significant increase of NF-κB nuclear translocation compared to control and Quercetin pre-treated cells.

Moreover, gene expression levels of some SASP factors, named IL-1α, IL-6, IL-8, GROα, MMP2, TNFα, and CXCL12, were evaluated 3 days after Doxo treatment. Real-Time PCR experiments indicated that IL-6 and IL-8, two of the most prominent SASP products, IL-1α, MMP2, TNFα, and CXCL12, were significantly up-regulated in Doxo-induced senescent fibroblasts (Fig. 7**B**). Although the pre-treatment with Quercetin reduced the expression of all 6 genes, only for IL-6, IL-8, CXCL12,

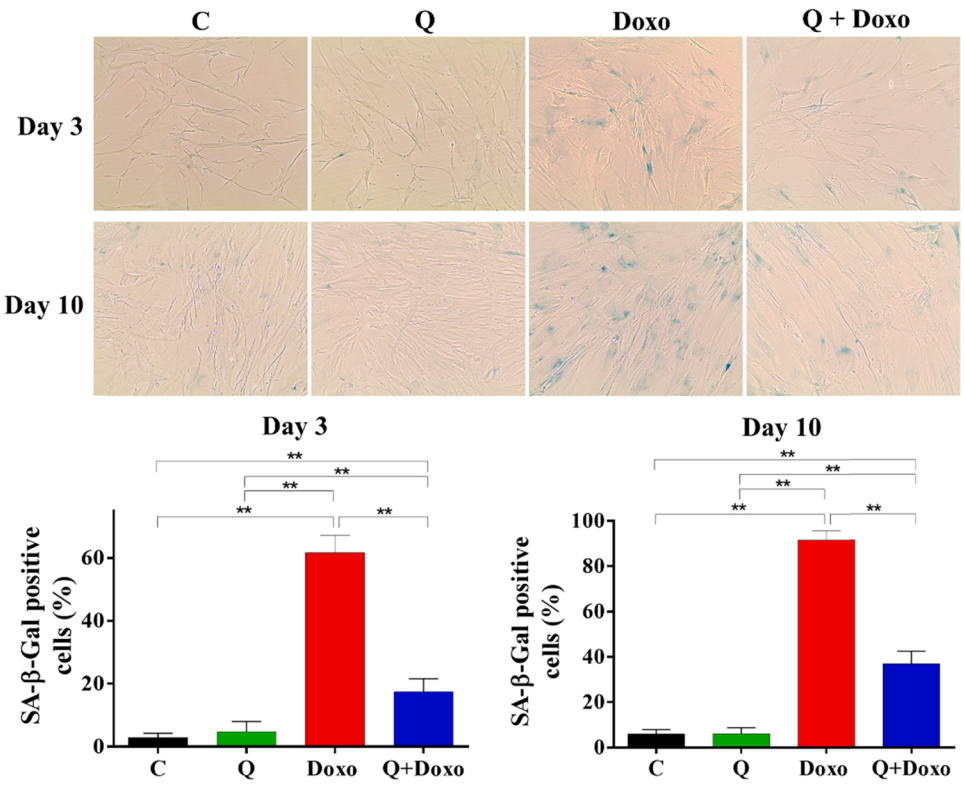

Fig. 3. Quercetin pre-treatment reduces the number of SA-β-Gal positive cells. Representative images of SA-β-Gal activity (upper) performed 3 and 10 days after Doxo treatment. The percentage of SA-β-Gal positive cells (blue ones) with respect to the total number of cells/well (down) was evaluated in at least three independent experiments, and the results are represented as the mean ± SD (**$p < 0.001$). Abbreviations: C: control; Q: Quercetin treated fibroblast; Doxo: Doxo-induced senescent fibroblasts; Q + Doxo: Quercetin pre-treated Doxo-induced senescent fibroblasts.

and IL-1α, the differences were significant ($p < 0.05$). Moreover, the expression of GROα was not modified in Doxo-induced senescent fibroblasts compared to control but was significantly reduced in both samples treated with Quercetin, with respect to control and to Doxo-induced senescent fibroblasts. These results suggest that Quercetin pre-treatment could decrease SASP production, maybe through the reduction of NF-κB activation.

3.5. Effects of Conditioned Medium from Doxo-induced senescent fibroblasts and Quercetin pre-treated Doxo-induced senescent fibroblasts on osteosarcoma cells (U2OS)

After verifying the increase in gene expression of some SASP factors by Doxo-induced senescent fibroblasts and the preventing action of Quercetin, we verified whether the conditioned medium from Doxo-induced senescent fibroblasts (SM) and Quercetin-pretreated Doxo-treated fibroblasts (QSM) could have effects on U2OS proliferation and invasion capacity. SM significantly enhanced U2OS proliferation over time (Fig. 8**A**) and colonies number and area (Fig. 8**B** and **C**) compared to QSM.

Moreover, the wound healing assay showed that SM induced a significant increase in U2OS cell migration ($p < 0.05$), while as expected, QSM reduced this effect (Fig. 9**A**). Similar results were obtained by evaluating the invasion capacity of U2OS cells (Fig. 9**B**). SM induced a 1.6-fold increase of invasive index, while U2OS treated with QSM showed an invasive grade like those of controls ($p < 0.05$). Overall, these results demonstrate that SM, but not QSM, could increase the proliferation, migration, and invasiveness of U2OS cells. On the other hand, Quercetin pre-treatment, reducing the senescent cells and modulating the expression and secretion of SASP factors, diminishes SM cancer-promoting effects.

3.6. Effects of Conditioned Medium from Doxo-induced senescent fibroblasts and Quercetin pre-treated Doxo-induced senescent fibroblasts on WI-38 fibroblasts

To verify whether SM increased aggressiveness was specific to cancer cells or a general effect, we analysed its effect on proliferation and migration of non-tumoral cells as WI-38 fibroblasts. Interestingly, SM significantly reduced WI-38 cell proliferation, especially at 72 h ($p < 0.05$) and 96 h ($p < 0.001$), compared to CM and QSM (Fig. 10**A**). This reduction of the proliferation induced by SM could be due to the induction of paracrine senescence, even if our preliminary findings did not demonstrate it (data not shown). Furthermore, the migration analysis did not show significant differences between samples (Fig. 10**B**). These results underline that the promoting effects of CM from Doxo-induced senescent fibroblasts are specific to OS cells and did not affect normal fibroblasts.

4. Discussion

Ageing is a complex biological phenomenon characterised by the progressive accumulation of unrepaired or damaging modifications occurring in molecules, cells, tissues, and organs, which the body tries to counteract by implementing a series of adaptive mechanisms that become an essential part of the ageing process (Ostan et al., 2008). Moreover, it represents the major risk factor for several pathologies that occur more frequently in people getting older, named age-related diseases (ARDs). Geroscience identified cellular senescence as one of the nine selected and interconnected biological processes representing the critical pillars of ageing and ARDs (López-Otín et al., 2013). Senescent cells accumulate in different aged tissues over time and contribute to the development and progression of several ARDs, such as cardiovascular diseases, neurodegenerative diseases, and cancer (Collado et al., 2007; He and Sharpless, 2017). Different studies showed that senescent cell clearance *in vivo* extends lifespan and healthspan and attenuates age-related pathologies (Baker et al., 2016, 2011; Childs et al., 2016;

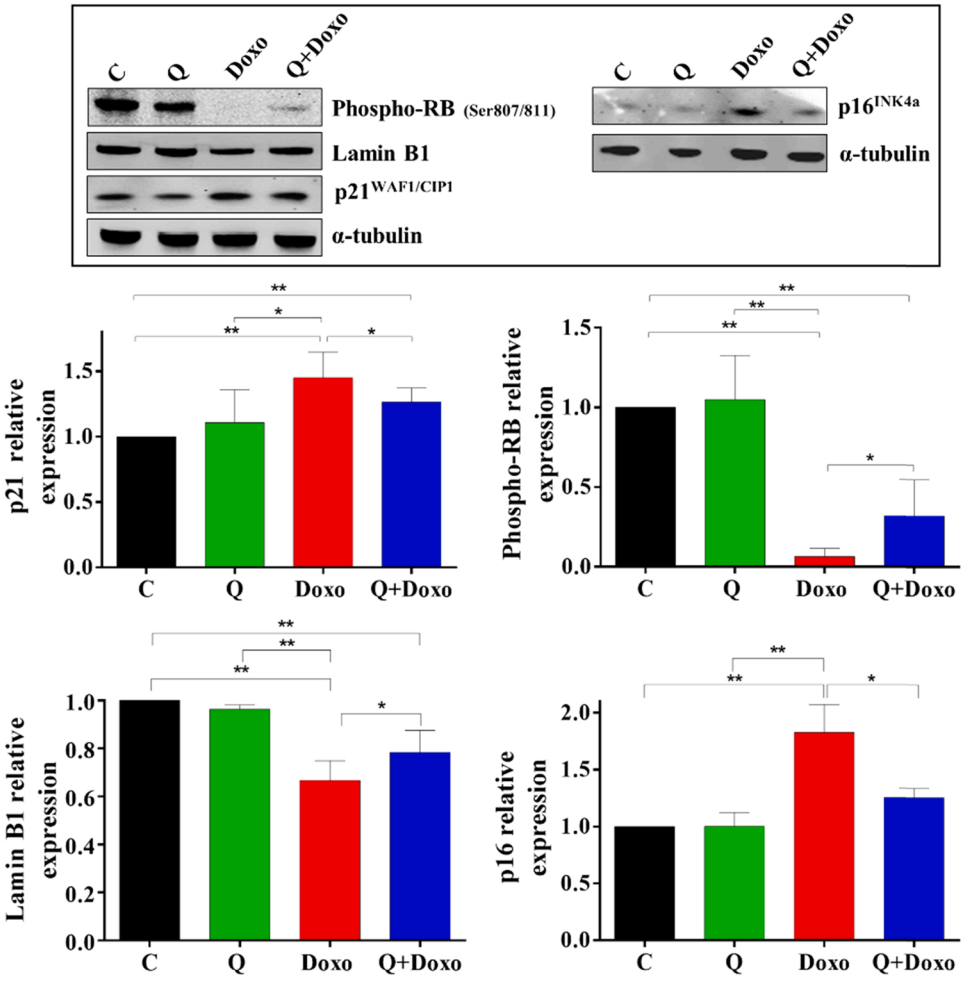

Fig. 4. Quercetin pre-treatment changes the expression of senescence markers. Immunoblot representative image (upper) and quantification of the expression of phosphor-RB, Lamin B1, p21 and p16 in control (C), Quercetin treated (Q), Doxo-treated (Doxo) and Quercetin pre-treated Doxo-treated (Q+Doxo) WI-38 fibroblasts. Lysates were collected 3 days after Doxo treatment. Protein band intensity was normalised to α-tubulin and expressed in comparison to control. Data are represented as the mean ± SD of at least three independent experiments (*$p < 0.05$; **$p < 0.001$). Abbreviations: C: control; Q: Quercetin treated fibroblast; Doxo: Doxo-induced senescent fibroblasts; Q + Doxo: Quercetin pre-treated Doxo-induced senescent fibroblasts.

Chinta et al., 2018). The deleterious biological effects of senescence are driven mainly by the SASP (Coppé et al., 2010), which contributes to inflammaging, creating a microenvironment favourable for developing different ARDs, including cancer (Collado et al., 2007; He and Sharpless, 2017; López-Otín et al., 2013). Different stimuli can induce cellular senescence and among these are also several chemotherapy drugs (Demaria et al., 2017; Ewald et al., 2010; Saleh et al., 2020). The induction of the so-called therapy-induced senescence (TIS) in cancer cells may be considered a positive outcome for the chemotherapy because it arrests tumour growth, resulting in inhibitory or growth-neutral tumour effects (Ewald et al., 2008; Xue et al., 2007). However, recent studies have demonstrated that senescence can be reversible for a subpopulation of tumour cells and allow cancer resurgence and progression (Bojko et al., 2019; Gerashchenko et al., 2016; Mosieniak et al., 2015; Saleh et al., 2018). Furthermore, different studies have suggested that one of the central mechanisms leading to chemotoxicity is the premature and excessive induction of senescence in non-malignant cells. Findings demonstrated that genetic or pharmacological senescent cells removal could alleviate therapy-induced toxicities, such as fatigue, myelosuppression, cardiomyopathy, bone loss and frailty, and cancer progression and relapse (Baar et al., 2017; Demaria et al., 2017; Murali et al., 2018; Sun et al., 2012; Yao et al., 2020). In this study, we showed that the treatment with Doxorubicin successfully induced cellular senescence in WI-38 normal lung fibroblasts, as evidenced by the acquisition of large and flattened morphology, the arrest of the cell cycle, the down-regulation of phospho-RB and Lamin B1, and the increase of p21 and p16 expression, SA-β-Gal activity, and SAHF formation. Our results were consistent with several studies that reported the ability of Doxo to induce senescence in different types of cells (Bielak-Zmijewska et al., 2014; Hou et al., 2019; Kozhukharova et al., 2018; Maejima et al., 2008; Piegari et al., 2013), including WI-38 fibroblasts (Liu et al., 2019). Doxo, belonging to the anthracycline family, acts by interacting with DNA by intercalation, causing the inhibition of topoisomerase II and generating free radical species that induce DNA double-strand breaks and cellular damages (Rivankar, 2014). Conversely to what happens in replicative senescence, in which cells preferentially accumulate in the G1 phase of cell cycle, our data, according to other studies (Bielak-Zmijewska et al., 2014; Chang et al., 2002; Roger et al., 2021; Yang et al., 2012), showed that Doxo-induced senescent fibroblasts preferentially accumulate in G2/M phase. This phenomenon could be explained by considering that cells in the S phase, the most targeted by the drug, are damaged and blocked in the subsequent phase, the G2/M. Interestingly, the arrest of the cell cycle in this phase was likely reinforced by the observed high level of p21, which seems to inhibit the mitotic cyclin B1-CDK1 complex, and lead to the reduced phosphorylation of pRB protein with consequent repression of E2F-dependent G2/M regulators (Cherrier-Savournin et al., 2004; Krenning et al., 2014).

Due to the proven important role of cellular senescence in ageing and ARDs, recent advances encouraged a search for strategies to reduce its damaging impact on tissues and organs. This work demonstrated that a pre-treatment with Quercetin could partially protect the cells from Doxo-induced senescence and consequently reduce the SASP. Quercetin is a bioflavonoid, widely distributed in fruits and vegetables, known for its antioxidant, anti-inflammatory, and anti-cancer activity (Andres et al., 2018; D'Andrea, 2015; Salehi et al., 2020). Our findings demonstrated that Quercetin pre-treatment reduced the number of SA-β-Gal

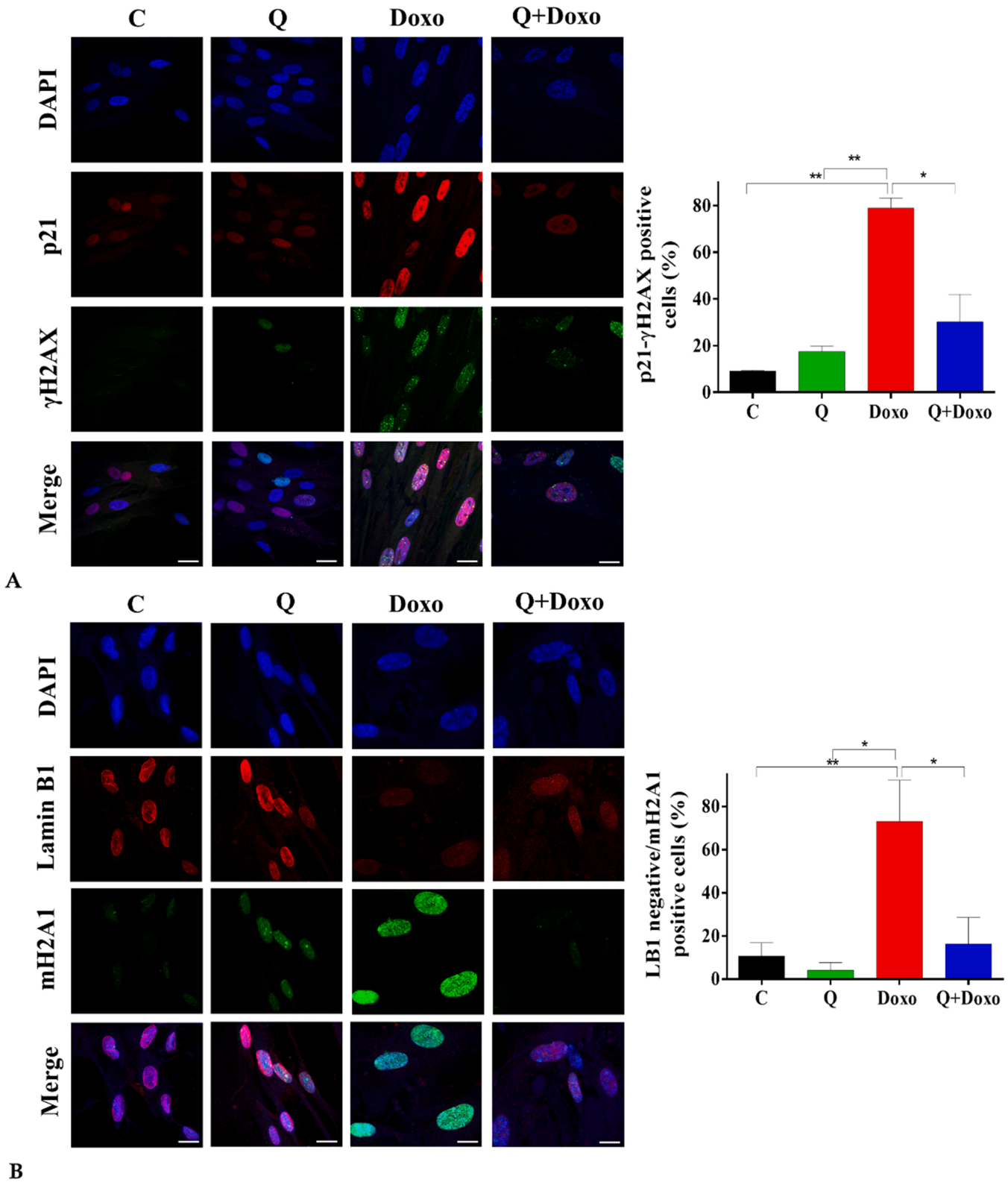

Fig. 5. Quercetin pre-treatment reduces SAHF. (A) Confocal microscopy images (left) of indirect immunofluorescence of γH2AX foci (green) and p21 (red) and the percentage of p21/γH2AX positive cells (right), in control (C), Quercetin treated cells (Q), Doxo-induced senescent fibroblasts (Doxo), and Quercetin pre-treated Doxo-treated fibroblasts (Q+Doxo). DNA was counterstained with DAPI (blue). Scale bars: 20 μm. Confocal images from three independent experiments were analysed to quantify p21-γH2AX positive cells with respect to the total number of cells (*$p < 0.05$; **$p < 0.001$). **(B)** Confocal microscopy images (left) of indirect immunofluorescence of macroH2A1 foci (green) and Lamin B1 (red), and the percentage of Lamin B1 negative/macroH2A1 positive cells (right). DNA was counterstained with DAPI (blue). Scale bars: 20 μm. Confocal images from three independent experiments were analysed to quantify Lamin B1 negative-macroH2A1 positive cells with respect to the total number of cells (*$p < 0.05$; **$p < 0.001$). Abbreviations: C: control; Q: Quercetin treated fibroblast; Doxo: Doxo-induced senescent fibroblasts; Q + Doxo: Quercetin pre-treated Doxo-induced senescent fibroblasts.

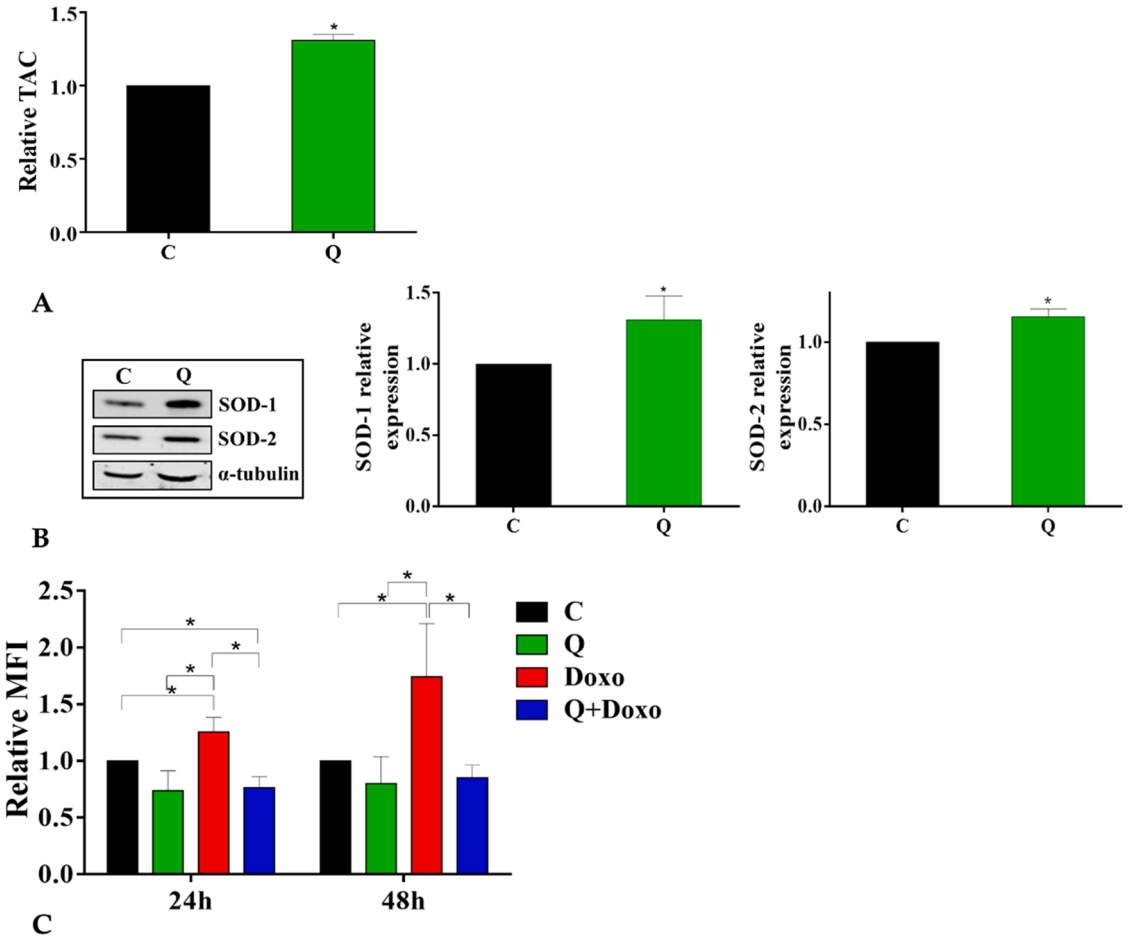

Fig. 6. Quercetin pre-treatment increases cellular antioxidant defences. (A) Total antioxidant capacity (TAC) of protein lysates collected after 24 h of Quercetin treatment. P-value refers to differences with respect to control C (*p < 0.05). **(B)** Immunoblot representative image (left) and quantification of the expression of SOD-1 and SOD-2 enzymes in control (C) and Quercetin treated cells (Q). Data are represented as the mean of three independent experiments ± SD. P-value refers to differences with respect to control C (*p < 0.05). **(C)** Analysis of ROS production by H2DCF-DA assay, after 24 and 48 h of Doxo-treatment. Data are represented as the mean of Median Fluorescent Intensity (MFI) relative to control of three independent experiments ± SD (*p < 0.05). Abbreviations: C: control; Q: proliferating fibroblasts treated with Quercetin; Doxo: Doxo-induced senescent fibroblasts; Q + Doxo: Quercetin pre-treated Doxo-induced senescent fibroblasts.

positive cells, decreasing cell cycle arrests and proliferation block, increasing the expression of phospho-RB, and reducing p21 and p16 levels in Doxo-treated fibroblasts. Moreover, WI-38 pre-treated fibroblasts showed an increased Lamin B1 expression and a reduction of γH2AX and macroH2A1 foci. Since the formation of SAHF is associated with the stable repression of genes involved in cell cycle progression, and it seems to be strictly related to the hypo phosphorylation of RB (Narita et al., 2003), the increased expression of phospho-RB in Quercetin pre-treated cells is likely related to the decreased presence of SAHF and consequently to the increased proliferation. Some other works have investigated the role of Quercetin as a geroprotective agent that can delay or reduce the onset of replicative senescence in human cells (Chondrogianni et al., 2010; Geng et al., 2019). Chondrogianni et al. have shown that Quercetin treatment on human embryonic fibroblasts for several days delayed the appearance of replicative senescence, and restored in part their proliferation, maybe due to its antioxidant and proteasome activating properties (Chondrogianni et al., 2010).

Moreover, in an *in vitro* model of accelerated ageing, Quercetin reduces replicative senescence onset, restoring heterochromatin architecture and decreasing ROS levels (Geng et al., 2019). For the first time, our study demonstrates that a pre-treatment with Quercetin could reduce Doxo-induced senescence and DNA damage by exerting its protective effects to enhance the total cellular antioxidant activity, particularly SOD-1 and SOD-2 expression. Moreover, we showed that Quercetin pre-treatment decreased Doxo-induced ROS production. Consistent with our data, such evidence from human and animal studies demonstrated that the antioxidant activity of Quercetin is mainly exerted through its effects on antioxidant enzymes, including the upregulation of SOD activity (Dong et al., 2014), signal transduction pathways, and ROS (Xu et al., 2019).

Furthermore, we evaluated whether Quercetin pre-treatment could also inhibit the SASP, which is the principal responsible for the damaging activities of senescent cells (Coppé et al., 2006, 2008; Dilley et al., 2003; Gonzalez-Meljem et al., 2018; Krtolica et al., 2001; Liu and Hornsby, 2007). Accordingly, in Doxo-induced senescent and Quercetin pre-treated fibroblasts, we have investigated the expression of NF-κB, one of the most important transcription factors driving the SASP program (Salminen et al., 2012). Our data showed that NF-κB nuclear translocation was significantly enhanced in Doxo-treated fibroblasts and that Quercetin pre-treatment prevented this increase. These findings are in accord with other studies showing that Quercetin exerts an anti-inflammatory action by suppressing NF-κB expression and its nuclear translocation in different cells (Chen et al., 2020; Cheng et al., 2019; Lee et al., 2018). We have also investigated the expression of several SASP-related molecules, including IL-6, IL-8, IL-1α, TNF-α, MMP2, and CXCL12, which significantly increased in Doxo-induced senescent cells while are reduced in Quercetin pre-treated fibroblasts. Interestingly, the production of IL-1α seems to happen in the early stage of senescence, when it plays a fundamental role in the development and maintenance of the SASP (Acosta et al., 2008; Roger et al., 2021). Thus,

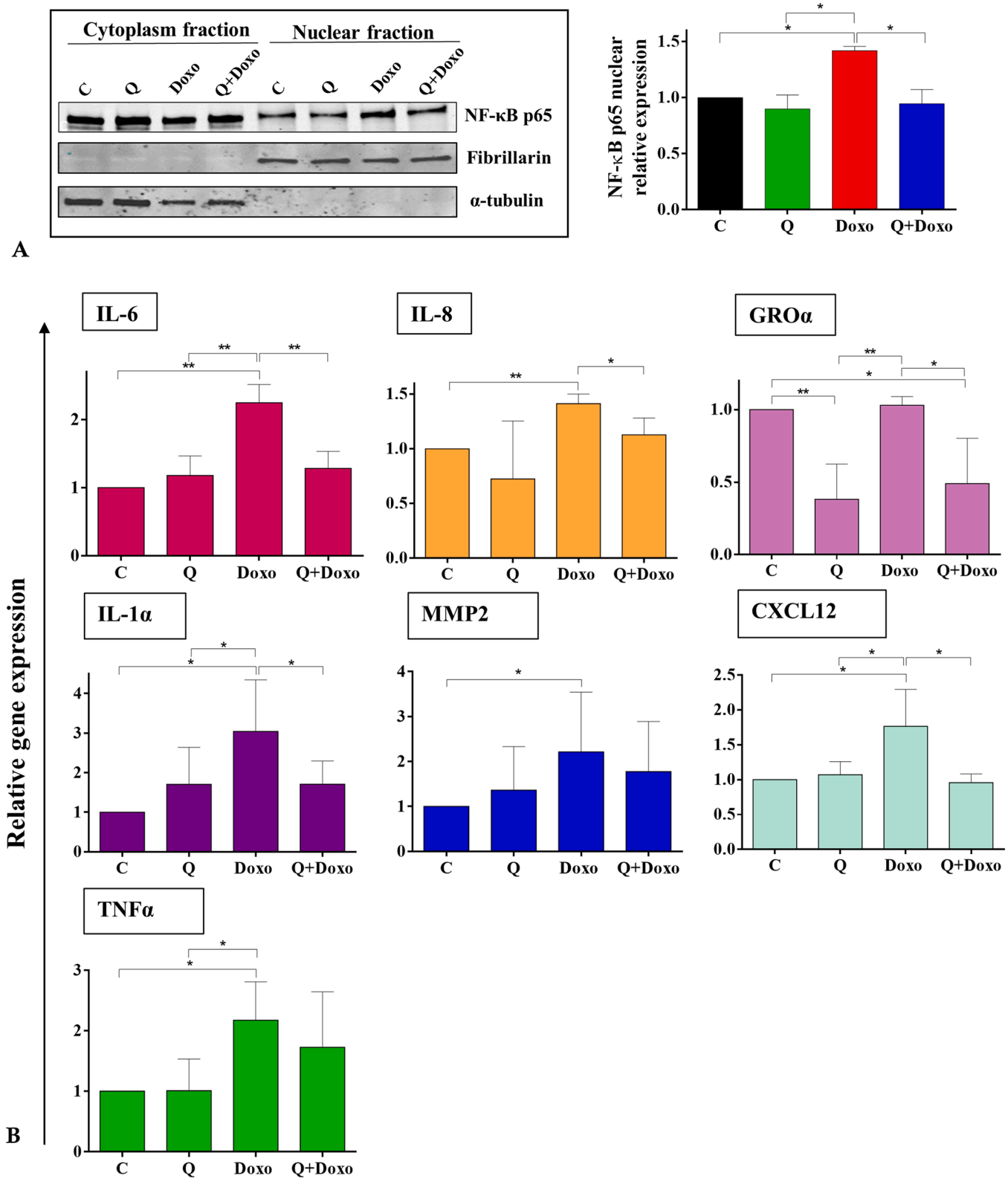

Fig. 7. NFkB p65 relative nuclear expression. (A) Immunoblot representative image (left) and quantification of the expression of NF-κB p65 in the nuclear fraction of in control (C), Quercetin-treated (Q), Doxo-treated (Doxo) and Quercetin pre-treated Doxo-treated (Q+Doxo) WI-38 fibroblasts. Protein band intensity was normalised to Fibrillarin and expressed in relation to control. Data are represented as the mean ± SD of three independent experiments (*$p < 0.05$). **(B)** Gene expression analysis was performed on control (C), Quercetin-treated cells (Q), Doxo-induced senescent fibroblasts (Doxo), and Quercetin pre-treated Doxo-treated fibroblasts (Q+Doxo) 3 days after Doxo treatment. GAPDH was used as the housekeeping gene, and data were normalised to control C (assumed as value 1). Data are expressed as the mean ± SD of triplicate experiments (*$p < 0.05$; **$p < 0.001$). Abbreviations: C: control; Q: proliferating fibroblasts treated with Quercetin; Doxo: Doxo-induced senescent fibroblasts; Q + Doxo: Quercetin pre-treated Doxo-induced senescent fibroblasts.

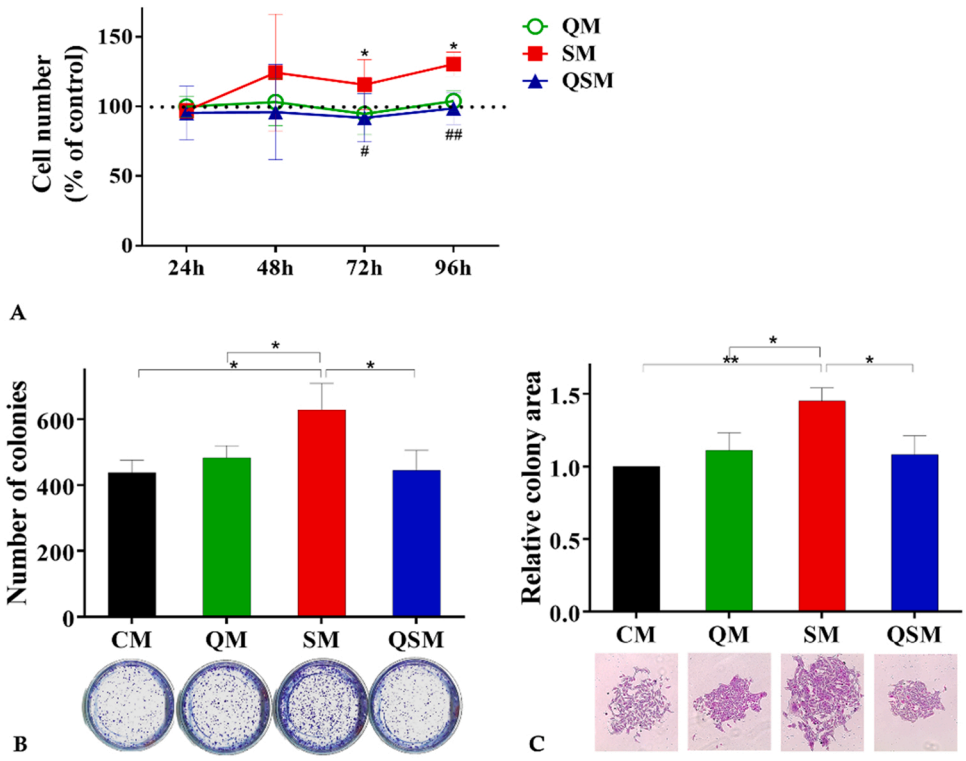

Fig. 8. Effect of Conditioned Medium from Doxo-induced senescent fibroblasts and Quercetin pre-treated Doxo-induced senescent fibroblasts on U2OS growth. (A) Trypan Blue exclusion assay on U2OS cells treated with conditioned medium from Quercetin treated cells (QM), Doxo-induced senescent fibroblasts (SM), and Quercetin pre-treated Doxo-treated fibroblasts (QSM). Data are shown as the mean percentage of three independent experiments ± SD relative to U2OS treated with control conditioned medium (CM). P-value refers to differences respect to control (*$p < 0.05$) and to SM ($^{\#}p < 0.05$; $^{\#\#}p < 0.001$). (B) Representative images (down) and calculated number of U2OS colonies (upper) formed after 7 days of treatment with conditioned media. Data represented the mean of three independent experiments ± SD (*$p < 0.05$). (C) Colonies area (representative images below) was calculated with Image J Software, and data are expressed relative to control (CM) and expressed as the mean of three independent experiments ± SD (*$p < 0.05$; **$p < 0.001$). Abbreviations: CM: conditioned medium from control proliferating fibroblasts; QM: conditioned medium from Quercetin treated fibroblasts; SM: conditioned medium from Doxo-induced senescent fibroblasts; QSM: conditioned medium from Quercetin pre-treated Doxorubicin-treated fibroblasts.

the reduced expression of this interleukin in Quercetin pre-treated fibroblasts can reinforce SASP suppression by the flavonoid.

Subsequently, we measured the ability of conditioned medium of Doxo-induced senescent fibroblasts (SM) to promote human osteosarcoma (OS) cell growth and invasiveness. OS is a primary malignant bone tumour with a high mortality rate and a bimodal incidence observed worldwide, with the first peak in puberty, and the second peak in old age, mainly in the seventh and eighth decades of life (Mirabello et al., 2009a). The appearance of OS in the elderly requires aggressive treatment with both neoadjuvant and adjuvant chemotherapy, and it is frequently associated with a greater incidence of metastasis and worse prognosis (Ek et al., 2006; Mirabello et al., 2009b; Savage and Mirabello, 2011). Doxorubicin is one of the mainstay drugs in the treatment of OS, and our data confirmed its known role as a senescence inducer genotoxic agent in cells that belong to tumour stroma, such as fibroblasts or mesenchymal cells as well as OS cells (Czarnecka et al., 2020). Consistent with this, our results showed that SM increased proliferation and colony formation of U2OS cells significantly and that QSM did not modify OS cells growth compared to the control. In accord with our data, different studies have shown that co-culture of senescent fibroblasts, induced by various stimuli, can promote the growth of pre-neoplastic and malignant cells from several types of tumours (Bhatia et al., 2008; Gonzalez-Meljem et al., 2018; Krtolica et al., 2001; Liu and Hornsby, 2007; Luo et al., 2011; Pazolli et al., 2009). Overall, the pro-tumorigenic action of SM that we have seen on U2OS cells could probably be due to the SASP, particularly to those factors we have investigated. Indeed, both IL-6 and IL-8 were shown to be essential factors in the malignant transformation of OS cells due to their ability to increase proliferation, colony formation, migration and invasiveness of U2OS and MG-63 cells (Cortini et al., 2016; Gross et al., 2018; Lin et al., 2013; Zhang et al., 2019).

Furthermore, some studies showed that IL-6, IL-8, and TNFα serum levels were higher in OS patients than in controls (Kushlinskii et al., 2014; Xiao et al., 2014; Zhang et al., 2019) and that IL-6 increased level was associated with worse TNM stages, and presence of metastasis, while TNFα and IL-8 levels correlated with bigger tumour size and maybe the progression of the disease (Xiao et al., 2014).

The increased mortality and worse prognosis observed in older people affected by cancer could be fuelled by a chronic, low-level inflammatory state typical in the elderly, named “inflammaging”, and could represent one of the links between ageing and cancer. Considering these data, the further increase of pro-inflammatory cytokines due to Doxo-induced senescent fibroblasts could increase the inflammaging of elderly patients, potentially favouring the creation of a pro-tumour microenvironment that could contribute to the worse prognosis observed in older. In this perspective, Quercetin, reducing IL-6, IL-8 and TNF-α expression and consequently the pro-tumorigenic activity of Doxo-induced senescent fibroblasts, could potentially be used to counteract the detrimental effect of therapy-induced senescence in cancer treatment.

Furthermore, we have also demonstrated that SM stimulated U2OS migration and invasiveness and that QSM, did not induce the same aggressive behaviour. Accordingly, different studies showed that senescent cells had been found to contribute also to the acquisition of invasive and metastatic properties of several types of cancer cells (Alspach et al., 2013; Coppé et al., 2008; Farsam et al., 2016; Hassona et al., 2014; Kim et al., 2017).

Our results showed that Doxo-induced senescent fibroblasts overexpressed CXCL12, a chemokine that functions as a chemoattractant, confirming what was seen in other senescent cell types (Choi et al., 2021; Jiang et al., 2019; Kim et al., 2017). Several studies have shown the importance of CXCL12/CXCR4 (C-X-C motif chemokine receptor 4) pathway in OS progression and prognosis (Laverdiere et al., 2005; Lu et al., 2015; Neklyudova et al., 2016; Oda et al., 2006; Perissinotto et al., 2005; Pollino et al., 2019), and the overexpression of CXCR4 was detected in OS patients, with high-grade disease, and also in two different lines of OS cells, 143-B and U2OS (Pollino et al., 2019). Thus, the proven upregulation of CXCR4 in U2OS cells (Pollino et al., 2019) and the overexpression of its ligand CXCL12 by Doxo-induced senescent fibroblasts identifies this axis as one of the best candidates for driving SM effects on U2OS cells migration and invasiveness. Conversely, the reduction by Quercetin pre-treatment of CXCL12 expression maybe

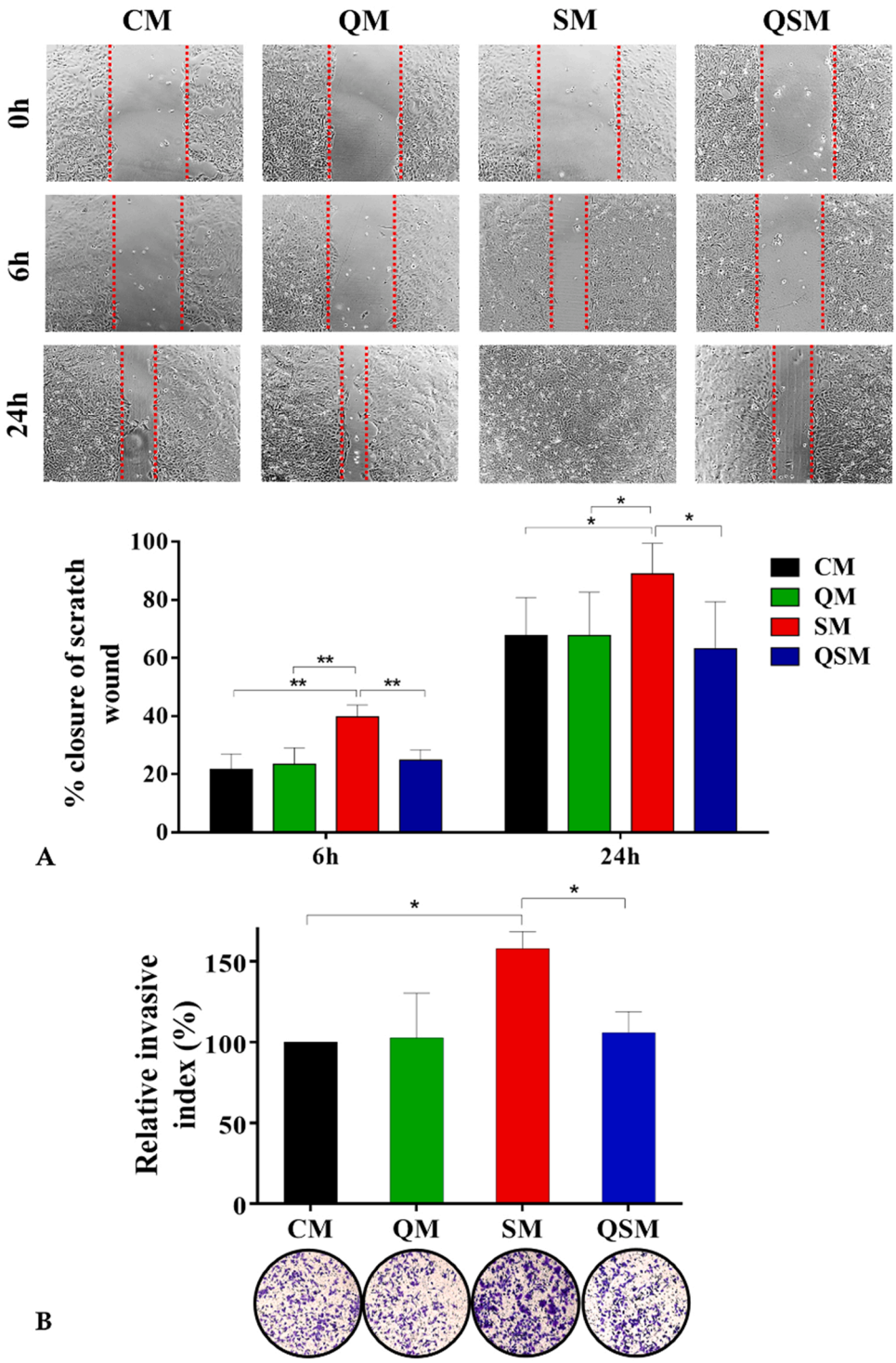

Fig. 9.. Effect of Conditioned Medium from Doxo-induced senescent fibroblasts and Quercetin pre-treated Doxo-induced senescent fibroblasts on U2OS cell migration and invasiveness. (A) Representative images (upper) and the percentage of scratch wound area (down) of Wound Healing Assay of U2OS cells treated with conditioned media. Data are shown as the mean percentage of three independent experiments ± SD of wound closure area with respect to initial scratch (*$p < 0.05$; **$p < 0.001$). (B) Boyden chamber invasion test on U2OS cells. Conditioned media from control fibroblasts (CM), Quercetin-treated fibroblasts (QM), Doxo-induced senescent fibroblasts (SM), and Quercetin pre-treated Doxo-treated fibroblasts (QSM) were placed in the bottom well of the chamber. Data are represented as a relative invasive index with respect to control, and the mean of three independent experiments ± SD (*$p < 0.05$). Representative images of invasive cells are shown below the graph. Abbreviations: *CM*: conditioned medium from control proliferating fibroblasts; *QM*: conditioned medium from Quercetin treated fibroblasts; *SM*: conditioned medium from Doxo-induced senescent fibroblasts; *QSM*: conditioned medium from Quercetin pre-treated Doxo-treated fibroblasts.

explains the reduced aggressiveness of U2OS cells incubated with QSM. Otherwise, another important SASP factor that can promote the invasive activity of several cancer cells, including U2OS (Hassona et al., 2014; Liu et al., 2016; Malaquin et al., 2013), is MMP2. MMP2 belongs to the family of metalloprotease, and it can be synthesised by different cell types, including tumour stromal cells (Egeblad and Werb, 2002) and several senescent cells (Hassona et al., 2014; Malaquin et al., 2013). Accordingly, Doxo-induced senescent fibroblasts up-regulated MMP2 expression and could contribute to the increased invasiveness of U2OS, although Quercetin pre-treatment did not significantly reduce its expression.

Furthermore, in accord with other studies (Hou et al., 2019; Krtolica et al., 2001), the promoting effects of senescent fibroblasts did not affect normal human cells, in which SM did not alter the migration while significantly reduced the proliferation. This reduction in proliferation could be due to the induction of senescence *via* "bystander effect", as shown in other works (Acosta et al., 2013; da Silva et al., 2019; Nelson et al., 2018, 2012).

These data suggest that the senescent microenvironment can synergise only with neoplastic cells favouring their malignant features. Therefore, Quercetin pre-treatment, acting as a preventive agent, can alleviate the effect of Doxo-induced senescent fibroblasts both on OS

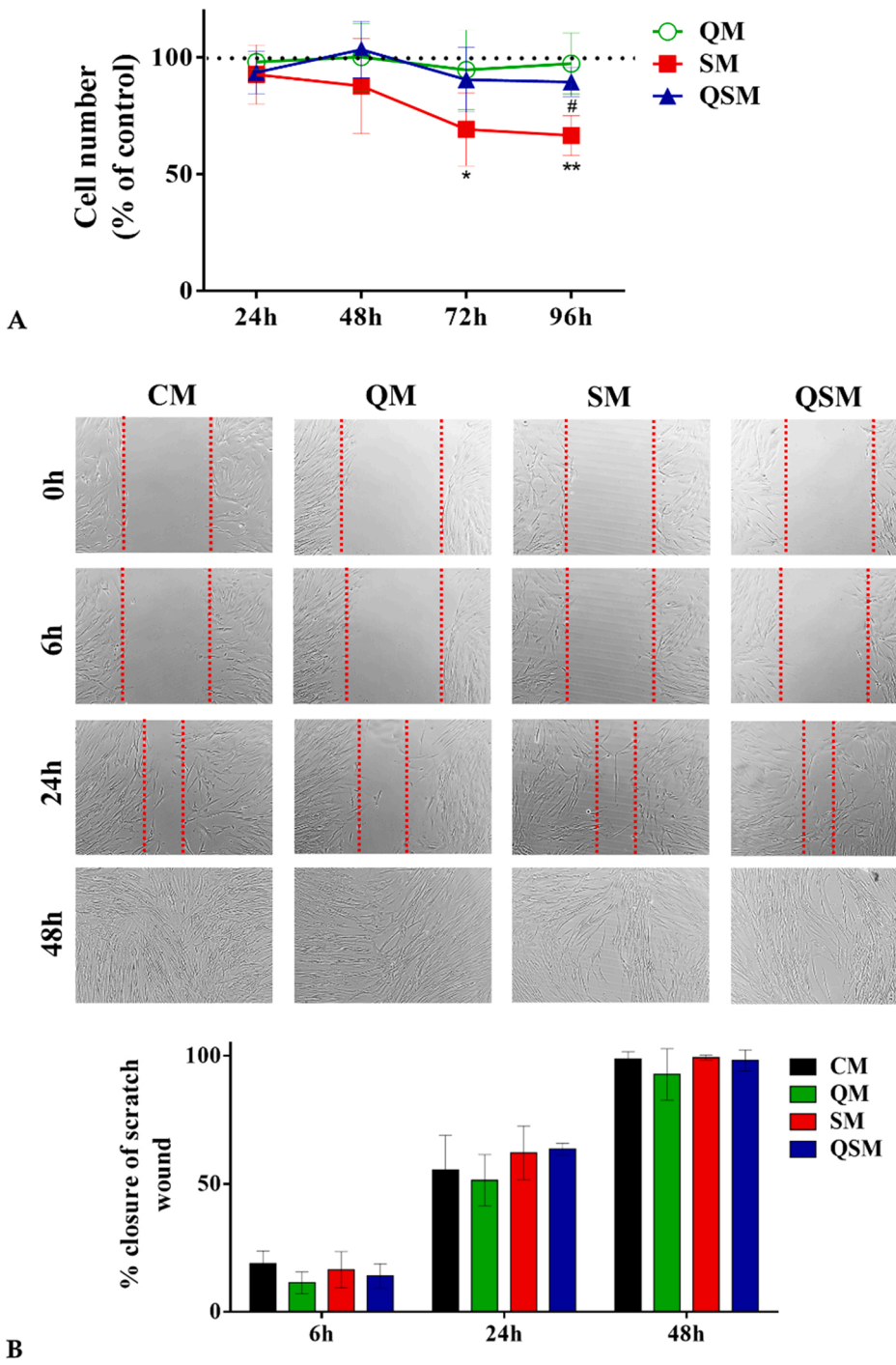

Fig. 10. Effect of Conditioned Medium from Doxo-induced senescent fibroblasts and Quercetin pre-treated Doxo-treated fibroblasts on WI-38 fibroblasts. (A) Trypan Blue exclusion assay on WI-38 cells treated with conditioned medium from Quercetin treated cells (QM), Doxo-induced senescent fibroblasts (SM), and Quercetin pre-treated Doxo-treated fibroblasts (QSM). Data are shown as the mean percentage of three independent experiments ± SD relative to WI-38 treated with control conditioned medium (CM). P-value refers to differences with respect to control (*$p < 0.05$; **$p < 0.001$) and to SM ($^{\#}p < 0.05$). (B) Representative images (upper) and the percentage of scratch wound area (down) of Wound Healing Assay of WI-38 cells treated with conditioned media. Abbreviations: CM: conditioned medium from control proliferating fibroblasts; QM: conditioned medium from Quercetin treated fibroblasts; SM: conditioned medium from Doxo-induced senescent fibroblasts; QSM: conditioned medium from Quercetin pre-treated Doxo-treated fibroblasts.

cells, reducing their aggressiveness and maybe blocking the spread of senescence on normal fibroblasts.

5. Conclusion

For the first time, this study shows that Quercetin, a natural bioactive flavonoid, can prevent Doxo-induced senescence, alleviating the effects of the SASP on U2OS and normal cells. Furthermore, by increasing cellular antioxidant defence, Quercetin pre-treatment can protect normal fibroblasts from the damaging effect of ROS produced by Doxo treatment, decreasing SAHF formation, Lamin B1 loss, NF-κB nuclear translocation, and consequently the levels of some SASP molecules. Although other studies are necessary to deepen the role of Quercetin as a geroprotective agent, this work may pave the way to investigate the use of Quercetin as an adjuvant in the treatment of OS, trying to reduce the damaging effects of therapy-induced senescence, especially in elderly patients, in which senescent cells are already abundant in several tissues and organs.

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Declarations of interest

None.

Data availability

The data that has been used is confidential.

References

Acosta, J.C., Banito, A., Wuestefeld, T., Georgilis, A., Janich, P., Morton, J.P., Athineos, D., Kang, T.W., Lasitschka, F., Andrulis, M., Pascual, G., Morris, K.J., Khan, S., Jin, H., Dharmalingam, G., Snijders, A.P., Carroll, T., Capper, D., Pritchard, C., Inman, G.J., Longerich, T., Sansom, O.J., Benitah, S.A., Zender, L., Gil, J., 2013. A complex secretory program orchestrated by the inflammasome controls paracrine senescence. Nat. Cell Biol. 158 (15), 978–990. https://doi.org/10.1038/ncb2784.

Acosta, J.C., O'Loghlen, A., Banito, A., Guijarro, M.V., Augert, A., Raguz, S., Fumagalli, M., Da Costa, M., Brown, C., Popov, N., Takatsu, Y., Melamed, J., d'Adda di Fagagna, F., Bernard, D., Hernando, E., Gil, J., 2008. Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell 133, 1006–1018. https://doi.org/10.1016/J.CELL.2008.03.038/ATTACHMENT/EFFC016E-F0D0-4775-B9AE-9F1E19F7DF02/MMC1.PDF.

Alspach, E., Fu, Y., Stewart, S.A., 2013. Senescence and the pro-tumorigenic stroma. Crit. Rev. Oncog. 18, 549–558. https://doi.org/10.1615/CritRevOncog.2014010630.

Andres, S., Pevny, S., Ziegenhagen, R., Bakhiya, N., Schäfer, B., Hirsch-Ernst, K.I., Lampen, A., 2018. Safety aspects of the use of quercetin as a dietary supplement. Mol. Nutr. Food Res. 62, 1700447 https://doi.org/10.1002/mnfr.201700447.

Baar, M.P., Brandt, R.M.C., Putavet, D.A., Klein, J.D.D., Derks, K.W.J., Bourgeois, B.R.M., Stryeck, S., Rijksen, Y., van Willigenburg, H., Feijtel, D.A., van der Pluijm, I., Essers, J., van Cappellen, W.A., van IJcken, W.F., Houtsmuller, A.B., Pothof, J., de Bruin, R.W.F., Madl, T., Hoeijmakers, J.H.J., Campisi, J., de Keizer, P.L.J., 2017. Targeted apoptosis of senescent cells restores tissue homeostasis in response to chemotoxicity and aging. Cell 169, 132–147. https://doi.org/10.1016/J.CELL.2017.02.031/ATTACHMENT/C16B304A-4968-479C-9E6E-663AB2AADE43/MMC5.MP4.

Baker, D.J., Childs, B.G., Durik, M., Wijers, M.E., Sieben, C.J., Zhong, J., Saltness, A., Jeganathan, R., Verzosa, K.B., Pezeshki, G.C., Khazaie, A., Miller, K., Van Deursen,, J.M., 2016. Naturally occurring p16 Ink4a-positive cells shorten healthy lifespan. Nature 530, 184–189. https://doi.org/10.1038/nature16932.

Baker, D.J., Wijshake, T., Tchkonia, T., LeBrasseur, N.K., Childs, B.G., van de Sluis, B., Kirkland, J.L., van Deursen, J.M., 2011. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature 479, 232–236. https://doi.org/10.1038/nature10600.

Bhat, R., Crowe, E.P., Bitto, A., Moh, M., Katsetos, C.D., Garcia, F.U., Johnson, F.B., Trojanowski, J.Q., Sell, C., Torres, C., 2012. Astrocyte senescence as a component of Alzheimer's disease. PLoS One 7, e45069. https://doi.org/10.1371/journal.pone.0045069.

Bhatia, B., Multani, A.S., Patrawala, L., Chen, X., Calhoun-Davis, T., Zhou, J., Schroeder, L., Schneider-Broussard, R., Shen, J., Pathak, S., Chang, S., Tang, D.G., 2008. Evidence that senescent human prostate epithelial cells enhance tumorigenicity: cell fusion as a potential mechanism and inhibition by p16INK4a and hTERT. Int. J. Cancer 122, 1483–1495. https://doi.org/10.1002/IJC.23222.

Bielak-Zmijewska, A., Wnuk, M., Przybylska, D., Grabowska, W., Lewinska, A., Alster, O., Korwek, Z., Cmoch, A., Myszka, A., Pikula, S., Mosieniak, G., Sikora, E., 2014. A comparison of replicative senescence and doxorubicin-induced premature senescence of vascular smooth muscle cells isolated from human aorta. Biogerontology 15, 47–64. https://doi.org/10.1007/s10522-013-9477-9.

Bojko, A., Czarnecka-Herok, J., Charzynska, A., Dabrowski, M., Sikora, E., 2019. Diversity of the senescence phenotype of cancer cells treated with chemotherapeutic agents. Cells 8, 1501. https://doi.org/10.3390/CELLS8121501.

Boots, A.W., Haenen, G.R.M.M., Bast, A., 2008. Health effects of quercetin: from antioxidant to nutraceutical. Eur. J. Pharm. 585, 325–337. https://doi.org/10.1016/j.ejphar.2008.03.008.

Campisi, J., 2001. Cellular senescence as a tumor-suppressor mechanism. Trends Cell Biol. 11, S27–S31. https://doi.org/10.1007/BF02711481.

Campisi, J., D'Adda Di Fagagna, F., 2007. Cellular senescence: when bad things happen to good cells. Nat. Rev. Mol. Cell Biol. 8, 729–740. https://doi.org/10.1038/nrm2233.

Casali, P.G., Bielack, S., Abecassis, N., Aro, H.T., Bauer, S., Biagini, R., Bonvalot, S., Boukovinas, I., Bovee, J.V.M.G., Brennan, B., Brodowicz, T., Broto, J.M., Brugières, L., Buonadonna, A., De Álava, E., Dei Tos, A.P., Del Muro, X.G., Dileo, P., Dhooge, C., Eriksson, M., Fagioli, F., Fedenko, A., Ferraresi, V., Ferrari, A., Ferrari, S., Frezza, A.M., Gaspar, N., Gasperoni, S., Gelderblom, H., Gil, T., Grignani, G., Gronchi, A., Haas, R.L., Hassan, B., Hecker-Nolting, S., Hohenberger, P., Issels, R., Joensuu, H., Jones, R.L., Judson, I., Jutte, P., Kaal, S., Kager, L., Kasper, B., Kopeckova, K., Krákorová, D.A., Ladenstein, R., Le Cesne, A., Lugowska, I., Merimsky, O., Montemurro, M., Morland, B., Pantaleo, M.A., Piana, R., Picci, P., Piperno-Neumann, S., Pousa, A.L., Reichardt, P., Robinson, M.H., Rutkowski, P., Safwat, A.A., Schöffski, P., Sleijfer, S., Stacchiotti, S., Strauss, S.J., Sundby Hall, K., Unk, M., Van Coevorden, F., Van Der Graaf, W.T.A., Whelan, J., Wardelmann, E., Zaikova, O., Blay, J.Y., 2018. Bone sarcomas: ESMO–PaedCan–EURACAN CLinical Practice Guidelines for Diagnosis, Treatment and Follow-up†. Ann. Oncol. 29, iv79–iv95. https://doi.org/10.1093/ANNONC/MDY310.

Cevenini, E., Monti, D., Franceschi, C., 2013. Inflamm-ageing. Curr. Opin. Clin. Nutr. Metab. Care 16, 14–20. https://doi.org/10.1097/MCO.0b013e32835ada13.

Chang, B.D., Swift, M.E., Shen, M., Fang, J., Broude, E.V., Roninson, I.B., 2002. Molecular determinants of terminal growth arrest induced in tumor cells by a chemotherapeutic agent. Proc. Natl. Acad. Sci. USA 99, 389. https://doi.org/10.1073/PNAS.012602599.

Chen, T., Zhang, X., Zhu, G., Liu, H., Chen, J., Wang, Y., He, X., 2020. Quercetin inhibits TNF-α induced HUVECs apoptosis and inflammation via downregulating NF-kB and AP-1 signaling pathway in vitro. Medicine 99, e22241. https://doi.org/10.1097/MD.0000000000022241.

Cheng, S.C., Huang, W.C., Pang, J.H.S., Wu, Y.H., Cheng, C.Y., 2019. Quercetin inhibits the production of IL-1β-induced inflammatory cytokines and chemokines in ARPE-19 cells via the MAPK and NF-κB signaling pathways. Int. J. Mol. Sci. 20, 2957. https://doi.org/10.3390/IJMS20122957.

Cherrier-Savournin, F.B., Chateau, M.-T., Gire, V., Sedivy, J., Piette, J., Dulic, V., 2004. p21-mediated nuclear retention of Cyclin B1-Cdk1 in response to genotoxic stress. Mol. Biol. Cell 15, 3965–3976. https://doi.org/10.1091/mbc.E03.

Childs, B.G., Baker, D.J., Wijshake, T., Conover, C.A., Campisi, J., Van Deursen, J.M., 2016. Senescent intimal foam cells are deleterious at all stages of atherosclerosis. Science 354, 472–477. https://doi.org/10.1126/science.aaf6659.

Chinta, S.J., Woods, G., Demaria, M., Rane, A., Zou, Y., McQuade, A., Rajagopalan, S., Limbad, C., Madden, D.T., Campisi, J., Andersen, J.K., 2018. Cellular senescence is induced by the environmental neurotoxin paraquat and contributes to neuropathology linked to Parkinson's disease. Cell Rep. 22, 930–940. https://doi.org/10.1016/j.celrep.2017.12.092.

Choi, Y.W., Kim, Y.H., Oh, S.Y., Suh, K.W., Kim, Y.S., Lee, G.Y., Yoon, J.E., Park, S.S., Lee, Y.K., Park, Y.J., Kim, H.S., Park, S.H., Kim, J.H., Park, T.J., 2021. Senescent tumor cells build a cytokine shield in colorectal cancer. Adv. Sci. 8, 1–20. https://doi.org/10.1002/advs.202002497.

Chondrogianni, N., Kapeta, S., Chinou, I., Vassilatou, K., Papassideri, I., Gonos, E.S., 2010. Anti-ageing and rejuvenating effects of quercetin. Exp. Gerontol. 45, 763–771. https://doi.org/10.1016/j.exger.2010.07.001.

Collado, M., Blasco, M.A., Serrano, M., 2007. Cellular senescence in cancer and aging. Cell. https://doi.org/10.1016/j.cell.2007.07.003.

Coppé, J.-P., Kauser, K., Campisi, J., Beauséjour, C.M., 2006. Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. J. Biol. Chem. 281, 29568–29574. https://doi.org/10.1074/jbc.M603307200.

Coppé, J.P., Desprez, P.-Y., Krtolica, A., Campisi, J., 2010. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu. Rev. Pathol. 5, 99–118. https://doi.org/10.1146/annurev-pathol-121808-102144.

Coppé, J.P., Patil, C.K., Rodier, F., Sun, Y., Muñoz, D.P., Goldstein, J., Nelson, P.S., Desprez, P.-Y., Campisi, J., 2008. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol. 6, 2853–2868. https://doi.org/10.1371/journal.pbio.0060301.

Cortini, M., Avnet, S., Baldini, N., 2017. Mesenchymal stroma: role in osteosarcoma progression. Cancer Lett. 405, 90–99. https://doi.org/10.1016/j.canlet.2017.07.024.

Cortini, M., Massa, A., Avnet, S., Bonuccelli, G., Baldini, N., 2016. Tumor-activated mesenchymal stromal cells promote osteosarcoma stemness and migratory potential via IL-6 secretion. PLoS One 11, e0166500. https://doi.org/10.1371/JOURNAL.PONE.0166500.

Czarnecka, A.M., Synoradzki, K., Firlej, W., Bartnik, E., Sobczuk, P., Fiedorowicz, M., Grieb, P., Rutkowski, P., 2020. Molecular biology of osteosarcoma. Cancers 12, 2130. https://doi.org/10.3390/CANCERS12082130.

D'Andrea, G., 2015. Quercetin: a flavonol with multifaceted therapeutic applications? Fitoterapia 106, 256–271. https://doi.org/10.1016/j.fitote.2015.09.018.

da Silva, P.F.L., Ogrodnik, M., Kucheryavenko, O., Glibert, J., Miwa, S., Cameron, K., Ishaq, A., Saretzki, G., Nagaraja-Grellscheid, S., Nelson, G., von Zglinicki, T., 2019. The bystander effect contributes to the accumulation of senescent cells in vivo. Aging Cell 18, e12848. https://doi.org/10.1111/acel.12848.

Demaria, M., O'Leary, M.N., Chang, J., Shao, L., Liu, S., Alimirah, F., Koenig, K., Le, C., Mitin, N., Deal, A.M., Alston, S., Academia, E.C., Kilmarx, S., Valdovinos, A., Wang, B., de Bruin, A., Kennedy, B.K., Melov, S., Zhou, D., Sharpless, N.E., Muss, H., Campisi, J., 2017. Cellular senescence promotes adverse effects of chemotherapy and cancer relapse. Cancer Discov. 7, 165–176. https://doi.org/10.1158/2159-8290.CD-16-0241.

Demirci, D., Dayanc, B., Mazi, F.A., Senturk, S., 2021. The jekyll and hyde of cellular senescence in cancer. Cells 10, 1–24. https://doi.org/10.3390/CELLS10020208.

Dilley, T.K., Bowden, G.T., Chen, Q.M., 2003. Novel mechanisms of sublethal oxidant toxicity: induction of premature senescence in human fibroblasts confers tumor promoter activity. Exp. Cell Res. 290, 38–48. https://doi.org/10.1016/S0014-4827(03)00308-2.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Al, E., 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. USA 92, 9363.

Dong, Q., Chen, L., Lu, Q., Sharma, S., Li, L., Morimoto, S., Wang, G., 2014. Quercetin attenuates doxorubicin cardiotoxicity by modulating Bmi-1 expression. Br. J. Pharm. 171, 4440–4454. https://doi.org/10.1111/BPH.12795.

Egeblad, M., Werb, Z., 2002. New functions for the matrix metalloproteinases in cancer progression. Nat. Rev. Cancer 23 (2), 161–174. https://doi.org/10.1038/nrc745.

Ek, E.T.H., Ojaimi, J., Kitagawa, Y., Choong, P.F.M., 2006. Outcome of patients with osteosarcoma over 40 years of age: is angiogenesis a marker of survival? Int. Semin. Surg. Oncol. 3, 7. https://doi.org/10.1186/1477-7800-3-7.

Emmi, G., Mannucci, A., Argento, F.R., Silvestri, E., Vaglio, A., Bettiol, A., Fanelli, A., Stefani, L., Taddei, N., Prisco, D., Fiorillo, C., Becatti, M., 2019. Stem-cell-derived circulating progenitors dysfunction in Behçet's syndrome patients correlates with oxidative stress. Front. Immunol. 10, 2877. https://doi.org/10.3389/FIMMU.2019.02877.

Erusalimsky, J.D., Kurz, D.J., 2005. Cellular senescence in vivo: its relevance in ageing and cardiovascular disease. Exp. Gerontol. 40, 634–642. https://doi.org/10.1016/j.exger.2005.04.010.

Ewald, J.A., Desotelle, J.A., Almassi, N., Jarrard, D.F., 2008. Drug-induced senescence bystander proliferation in prostate cancer cells in vitro and in vivo. Br. J. Cancer 98, 1244–1249. https://doi.org/10.1038/sj.bjc.6604288.

Ewald, J.A., Desotelle, J.A., Wilding, G., Jarrard, D.F., 2010. Therapy-induced senescence in cancer. J. Natl. Cancer Inst. 102, 1536–1546. https://doi.org/10.1093/jnci/djq364.

Farsam, V., Basu, A., Gatzka, M., Treiber, N., Schneider, L.A., Mulaw, M.A., Lucas, T., Kochanek, S., Dummer, R., Levesque, M.P., Wlaschek, M., Scharffetter-Kochanek, K., 2016. Senescent fibroblast-derived Chemerin promotes squamous cell carcinoma migration. Oncotarget 7, 83554. https://doi.org/10.18632/ONCOTARGET.13446.

Franceschi, C., Capri, M., Monti, D., Giunta, S., Olivieri, F., Sevini, F., Panourgia, M.P., Invidia, L., Celani, L., Scurti, M., Cevenini, E., Castellani, G.C., Salvioli, S., 2007. Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. Mech. Ageing Dev. 128, 92–105. https://doi.org/10.1016/J.MAD.2006.11.016.

Franceschi, C., Garagnani, P., Morsiani, C., Conte, M., Santoro, A., Grignolio, A., Monti, D., Capri, M., Salvioli, S., 2018. The continuum of aging and age-related diseases: common mechanisms but different rates. Front. Med. 5, 61. https://doi.org/10.3389/FMED.2018.00061.

Freund, A., Orjalo, A.V., Desprez, P.-Y., Campisi, J., 2010. Inflammatory networks during cellular senescence: causes and consequences. Trends Mol. Med. 16, 238–246. https://doi.org/10.1016/j.molmed.2010.03.003.

Geng, L., Liu, Z., Zhang, W., Li, W., Wu, Z., Wang, W., Ren, R., Su, Y., Wang, P., Sun, L., Ju, Z., Chan, P., Song, M., Qu, J., Liu, G.H., 2019. Chemical screen identifies a geroprotective role of quercetin in premature aging. Protein Cell 10, 417–435. https://doi.org/10.1007/s13238-018-0567-y.

Gerashchenko, B.I., Salmina, K., Eglitis, J., Huna, A., Grjunberga, V., Erenpreisa, J., 2016. Disentangling the aneuploidy and senescence paradoxes: a study of triploid breast cancers non-responsive to neoadjuvant therapy. Histochem. Cell Biol. 145, 497–508. https://doi.org/10.1007/S00418-016-1415-X/FIGURES/5.

Gonzalez-Meljem, J.M., Apps, J.R., Fraser, H.C., Martinez-Barbera, J.P., 2018. Paracrine roles of cellular senescence in promoting tumourigenesis. Br. J. Cancer 118, 1283–1288. https://doi.org/10.1038/s41416-018-0066-1.

Gross, A.C., Cam, H., Phelps, D.A., Saraf, A.J., Bid, H.K., Cam, M., London, C.A., Winget, S.A., Arnold, M.A., Brandolini, L., Mo, X., Hinckley, J.M., Houghton, P.J., Roberts, R.D., 2018. IL-6 and CXCL8 mediate osteosarcoma-lung interactions critical to metastasis. JCI Insight 3, e99791. https://doi.org/10.1172/JCI.INSIGHT.99791.

Hassona, Y., Cirillo, N., Heesom, K., Parkinson, E.K., Prime, S.S., 2014. Senescent cancer-associated fibroblasts secrete active MMP-2 that promotes keratinocyte dis-cohesion and invasion. Br. J. Cancer 111, 1230. https://doi.org/10.1038/BJC.2014.438.

Hayflick, L., Moorhead, P.S., 1961. The serial cultivation of human disploid cell strains. Exp. Cell Res. 25, 585–621.

Hayflick, L., 1965. The limited in vitro lifetime of human diploid cell strains. Exp. Cell Res. 37, 614–636. https://doi.org/10.1016/0014-4827(65)90211-9.

He, S., Sharpless, N.E., 2017. Senescence in health and disease. Cell 169, 1000–1011. https://doi.org/10.1016/J.CELL.2017.05.015.

Hernandez-Segura, A., De Jong, T.V., Melov, S., Guryev, V., Campisi, J., Demaria, M., 2017. Unmasking transcriptional heterogeneity in senescent cells. Curr. Biol. 27, 2652–2660. https://doi.org/10.1016/j.cub.2017.07.033.

Hernandez-Segura, A., Nehme, J., Demaria, M., 2018. Hallmarks of cellular senescence. Trends Cell Biol. 28, 436–453. https://doi.org/10.1016/j.tcb.2018.02.001.

Hou, J.-G., Jeon, B.-M., Yun, Y.-J., Cui, C.-H., Kim, S.-C., 2019. Ginsenoside Rh2 ameliorates doxorubicin-induced senescence bystander effect in breast carcinoma cell MDA-MB-231 and normal epithelial cell MCF-10A. Int. J. Mol. Sci. 20, 1244. https://doi.org/10.3390/ijms20051244.

Jiang, S., Song, C.S., Chatterjee, B., 2019. Stimulation of prostate cells by the senescence phenotype of epithelial and stromal cells: implication for benign prostate hyperplasia. FASEB BioAdvances 1, 353–363. https://doi.org/10.1096/fba.2018-00084.

Kim, Y.H., Choi, Y.W., Lee, J., Soh, E.Y., Kim, J.H., Park, T.J., 2017. Senescent tumor cells lead the collective invasion in thyroid cancer. Nat. Commun. 8, 15208. https://doi.org/10.1038/NCOMMS15208.

Kozhukharova, I., Zemelko, V., Kovaleva, Z., Alekseenko, L., Lyublinskaya, O., Nikolsky, N., 2018. Therapeutic doses of Doxorubicin induce premature senescence of human mesenchymal stem cells derived from menstrual blood, bone marrow and adipose tissue. Int. J. Hematol. 107, 286–296. https://doi.org/10.1007/S12185-017-2346-6/FIGURES/6.

Krenning, L., Feringa, F.M., Shaltiel, I.A., vandenBerg, J., Medema, R.H., 2014. Transient activation of p53 in G2 phase is sufficient to induce senescence. Mol. Cell 55, 59–72. https://doi.org/10.1016/j.molcel.2014.05.007.

Krtolica, Ana, Parrinello, S., Lockett, S., Desprez, P.Y., Campisi, J., 2001. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc. Natl. Acad. Sci. USA 98, 12072–12077. https://doi.org/10.1073/PNAS.211053698.

Kushlinskii, N.E., Timofeev, Y.S., Solov'ev, Y.N., Gerstein, E.S., Lyubimova, N.V., Bulycheva, I.V., 2014. Components of the RANK/RANKL/OPG System, IL-6, IL-8, IL-16, MMP-2, and calcitonin in the sera of patients with bone tumors. Bull. Exp. Biol. Med. 1574 (157), 520–523. https://doi.org/10.1007/S10517-014-2605-Y.

Laverdiere, C., Hoang, B.H., Yang, R., Sowers, R., Qin, J., Meyers, P.A., Huvos, A.G., Healey, J.H., Gorlick, R., 2005. Messenger RNA expression levels of CXCR4 correlate with metastatic behavior and outcome in patients with osteosarcoma. Clin. Cancer Res. 11, 2561–2567. https://doi.org/10.1158/1078-0432.CCR-04-1089.

Lecot, P., Alimirah, F., Desprez, P.Y., Campisi, J., Wiley, C., 2016. Context-dependent effects of cellular senescence in cancer development. Br. J. Cancer 11411 (114), 1180–1184. https://doi.org/10.1038/bjc.2016.115.

Lee, H.N., Shin, S.A., Choo, G.S., Kim, H.J., Park, Y.S., Kim, B.S., Kim, S.K., Cho, S.D., Nam, J.S., Choi, C.S., Che, J.H., Park, B.K., Jung, J.Y., 2018. Anti-inflammatory effect of quercetin and galangin in LPS-stimulated RAW264.7 macrophages and DNCB-induced atopic dermatitis animal models. Int. J. Mol. Med. 41, 888. https://doi.org/10.3892/IJMM.2017.3296.

Lin, Y.M., Chang, Z.L., Liao, Y.Y., Chou, M.C., Tang, C.H., 2013. IL-6 promotes ICAM-1 expression and cell motility in human osteosarcoma. Cancer Lett. 328, 135–143. https://doi.org/10.1016/J.CANLET.2012.08.029.

Liu, C., Zhao, P., Yang, Y., Xu, X., Wang, L., Li, B., 2016. Ampelopsin suppresses TNF-α-induced migration and invasion of U2OS osteosarcoma cells. Mol. Med. Rep. 13, 4729–4736. https://doi.org/10.3892/MMR.2016.5124.

Liu, D., Hornsby, P.J., 2007. Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. Cancer Res. 67, 3117–3126. https://doi.org/10.1158/0008-5472.CAN-06-3452.

Liu, Y., Pan, J., Pan, X., Wu, L., Bian, J., Lin, Z., Xue, M., Su, T., Lai, S., Chen, F., Ge, Q., Chen, L., Ye, S., Zhu, Y., Chen, S., Wang, L., 2019. Klotho-mediated targeting of CCL2 suppresses the induction of colorectal cancer progression by stromal cell senescent microenvironments. Mol. Oncol. 13, 2460–2475. https://doi.org/10.1002/1878-0261.12577.

López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., Kroemer, G., 2013. The hallmarks of aging. Cell 153, 1194. https://doi.org/10.1016/J.CELL.2013.05.039.

Lu, Y., Hu, B., Guan, G.F., Chen, J., Wang, C., qiu, Ma, Q., Wen, Y.H., Qiu, X.C., Zhang, X., ping, Zhou, Y., 2015. SDF-1/CXCR4 promotes F5M2 osteosarcoma cell migration by activating the Wnt/β-catenin signaling pathway. Med. Oncol. 32, 194. https://doi.org/10.1007/S12032-015-0576-0.

Luo, X., Ruhland, M.K., Pazolli, E., Lind, A.C., Stewart, S.A., 2011. Osteopontin stimulates preneoplastic cellular proliferation through activation of the MAPK pathway. Mol. Cancer Res. 9, 1018. https://doi.org/10.1158/1541-7786.MCR-10-0472.

Maejima, Y., Adachi, S., Ito, H., Hirao, K., Isobe, M., 2008. Induction of premature senescence in cardiomyocytes by Doxorubicin as a novel mechanism of myocardial damage. Aging Cell 7, 125–136. https://doi.org/10.1111/J.1474-9726.2007.00358.X.

Malaquin, N., Vercamer, C., Bouali, F., Martien, S., Deruy, E., Wernert, N., Chwastyniak, M., Pinet, F., Abbadie, C., Pourtier, A., 2013. Senescent fibroblasts enhance early skin carcinogenic events via a paracrine MMP-PAR-1 axis. PLoS One 8, 63607. https://doi.org/10.1371/JOURNAL.PONE.0063607.

Mirabello, L., Troisi, R.J., Savage, S.A., 2009a. International osteosarcoma incidence patterns in children and adolescents, middle ages, and elderly persons. Int. J. Cancer 125, 229. https://doi.org/10.1002/IJC.24320.

Mirabello, L., Troisi, R.J., Savage, S.A., 2009b. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the surveillance, epidemiology, and end results program. Cancer 115, 1531. https://doi.org/10.1002/CNCR.24121.

Mongiardi, M.P., Pellegrini, M., Pallini, R., Levi, A., Falchetti, M.L., 2021. Cancer response to therapy-induced senescence: a matter of dose and timing. Cancers 13, 1–16. https://doi.org/10.3390/cancers13030484.

Mosieniak, G., Sliwinska, M.A., Alster, O., Strzeszewska, A., Sunderland, P., Piechota, M., Was, H., Sikora, E., 2015. Polyploidy formation in doxorubicin-treated cancer cells can favor escape from senescence. Neoplasia 17, 882. https://doi.org/10.1016/J.NEO.2015.11.008.

Muñoz-Espín, D., Serrano, M., 2014. Cellular senescence: from physiology to pathology. Nat. Rev. Mol. Cell Biol. 15, 482–496. https://doi.org/10.1038/nrm3823.

Murali, B., Ren, Q., Luo, X., Faget, D.V., Wang, C., Marie, R., Gruosso, T., Flanagan, K.C., Fu, Y., Leahy, K., Alspach, E., Su, X., Ross, M.H., Burnette, B., Weilbaecher, K.N., Park, M., Mbalaviele, G., Monahan, J.B., Stewart, S.A., 2018. Inhibition of the stromal p38MAPK/MK2 pathway limits breast cancer metastases and chemotherapy-induced bone loss. Cancer Res. 78, 5618–5630. https://doi.org/10.1158/0008-5472.CAN-18-0234.Inhibition.

Narita, Masashi, Nũnez, S., Heard, E., Narita, Masako, Lin, A.W., Hearn, S.A., Spector, D. L., Hannon, G.J., Lowe, S.W., 2003. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell 113, 703–716. https://doi.org/10.1016/S0092-8674(03)00401-X.

Neklyudova, O., Arlt, M.J.E., Brennecke, P., Thelen, M., Gvozdenovic, A., Kuzmanov, A., Robl, B., Botter, S.M., Born, W., Fuchs, B., 2016. Altered CXCL12 expression reveals a dual role of CXCR4 in osteosarcoma primary tumor growth and metastasis. J. Cancer Res. Clin. Oncol. 142, 1739–1750. https://doi.org/10.1007/S00432-016-2185-5.

Nelson, G., Kucheryavenko, O., Wordsworth, J., von Zglinicki, T., 2018. The senescent bystander effect is caused by ROS-activated NF-κB signalling. Mech. Ageing Dev. 170, 30–36. https://doi.org/10.1016/J.MAD.2017.08.005.

Nelson, G., Wordsworth, J., Wang, C., Jurk, D., Lawless, C., Martin-Ruiz, C., von Zglinicki, T., 2012. A senescent cell bystander effect: senescence-induced senescence. Aging Cell 11, 345–349. https://doi.org/10.1111/j.1474-9726.2012.00795.x.

Oda, Y., Yamamoto, H., Tamiya, S., Matsuda, S., Tanaka, K., Yokoyama, R., Iwamoto, Y., Tsuneyoshi, M., 2006. CXCR4 and VEGF expression in the primary site and the metastatic site of human osteosarcoma: analysis within a group of patients, all of whom developed lung metastasis. Mod. Pathol. 195 (19), 738–745. https://doi.org/10.1038/modpathol.3800587.

Ostan, R., Bucci, L., Capri, M., Salvioli, S., Scurti, M., Pini, E., Monti, D., Franceschi, C., 2008. Immunosenescence and immunogenetics of human longevity. Neuroimmunomodulation 15, 224–240. https://doi.org/10.1159/000156466.

Pazolli, E., Luo, X., Brehm, S., Carbery, K., Chung, J.J., Prior, J.L., Doherty, J., Demehri, S., Salavaggione, L., Piwnica-Worms, D., Stewart, S.A., 2009. Senescent stromal-derived osteopontin promotes pre-neoplastic cell growth. Cancer Res. 69, 1230–1239. https://doi.org/10.1158/0008-5472.CAN-08-2970.

Perissinotto, E., Cavalloni, G., Leone, F., Fonsato, V., Mitola, S., Grignani, G., Surrenti, N., Sangiolo, D., Bussolino, F., Piacibello, W., Aglietta, M., 2005. Involvement of chemokine receptor 4/stromal cell-derived factor 1 system during osteosarcoma tumor progression. Clin. Cancer Res. 11, 490–497.

Piegari, E., De Angelis, A., Cappetta, D., Russo, R., Esposito, G., Costantino, S., Graiani, G., Frati, C., Prezioso, L., Berrino, L., Urbanek, K., Quaini, F., Rossi, F., 2013. Doxorubicin induces senescence and impairs function of human cardiac progenitor cells. Basic Res. Cardiol. 108, 1–18. https://doi.org/10.1007/s00395-013-0334-4.

Pollino, S., Palmerini, E., Dozza, B., Bientinesi, E., Piccinni-Leopardi, M., Lucarelli, E., Righi, A., Benassi, M.S., Pazzaglia, L., 2019. CXCR4 in human osteosarcoma malignant progression. The response of osteosarcoma cell lines to the fully human CXCR4 antibody MDX1338. J. Bone Oncol. 17, 100239 https://doi.org/10.1016/j.jbo.2019.100239.

Price, J.S., Waters, J.G., Darrah, C., Pennington, C., Edwards, D.R., Donell, S.T., Clark, I. M., 2002. The role of chondrocyte senescence in osteoarthritis. Aging Cell 1, 57–65. https://doi.org/10.1046/j.1474-9728.2002.00008.x.

Rivankar, S., 2014. An overview of doxorubicin formulations in cancer therapy. J. Cancer Res. Ther. 10, 853–858. https://doi.org/10.4103/0973-1482.139267.

Rodier, F., Campisi, J., 2011. Four faces of cellular senescence. J. Cell Biol. https://doi.org/10.1083/jcb.201009094.

Roger, L., Tomas, F., Gire, V., 2021. Mechanisms and regulation of cellular senescence. Int. J. Mol. Sci. 22, 13173.

Saleh, T., Bloukh, S., Carpenter, V.J., Alwohoush, E., Bakeer, J., Darwish, S., Azab, B., Gewirtz, D.A., 2020. Therapy-induced senescence: an "old" friend becomes the enemy. Cancers 12, 822. https://doi.org/10.3390/CANCERS12040822.

Saleh, T., Tyutynuk-Massey, L., Cudjoe, E.K., Idowu, M.O., Landry, J.W., Gewirtz, D.A., 2018. Non-cell autonomous effects of the senescence-associated secretory phenotype in cancer therapy. Front. Oncol. 8, 164. https://doi.org/10.3389/FONC.2018.00164.

Salehi, B., Machin, L., Monzote, L., Sharifi-Rad, J., Ezzat, S.M., Salem, M.A., Merghany, R.M., El Mahdy, N.M., Kılıç, C.S., Sytar, O., Sharifi-Rad, M., Sharopov, F., Martins, N., Martorell, M., Cho, W.C., 2020. Therapeutic potential of quercetin: new insights and perspectives for human health. ACS Omega 5, 11849–11872. https://doi.org/10.1021/acsomega.0c01818.

Salminen, A., Kauppinen, A., Kaarniranta, K., 2012. Emerging role of NF-κB signaling in the induction of senescence-associated secretory phenotype (SASP). Cell. Signal. 24, 835–845. https://doi.org/10.1016/j.cellsig.2011.12.006.

Salminen, A., Ojala, J., Kaarniranta, K., Haapasalo, A., Hiltunen, M., Soininen, H., 2011. Astrocytes in the aging brain express characteristics of senescence-associated secretory phenotype. Eur. J. Neurosci. 34, 3–11. https://doi.org/10.1111/j.1460-9568.2011.07738.x.

Santos, M.R., Mira, L., 2004. Protection by flavonoids against the peroxynitrite-mediated oxidation of dihydrorhodamine. Free Radic. Res. 38, 1011–1018. https://doi.org/10.1080/10715760400003384.

Savage, S.A., Mirabello, L., 2011. Using epidemiology and genomics to understand osteosarcoma etiology. Sarcoma 2011, 548151. https://doi.org/10.1155/2011/548151.

Sierra, F., Kohanski, R., 2015. Advances in geroscience. Adv. Gerosci. https://doi.org/10.1007/978-3-319-23246-1.

Sofi, F., Dinu, M., Pagliai, G., Cesari, F., Gori, A.M., Sereni, A., Becatti, M., Fiorillo, C., Marcucci, R., Casini, A., 2018. Low-calorie vegetarian versus mediterranean diets for reducing body weight and improving cardiovascular risk profile: CARDIVEG study (cardiovascular prevention with vegetarian diet). Circulation 137, 1103–1113. https://doi.org/10.1161/CIRCULATIONAHA.117.030088.

Sun, Y., Campisi, J., Higano, C., Beer, T.M., Porter, P., Coleman, I., True, L., Nelson, P., 2012. Treatment-induced damage to the tumor microenvironment promotes prostate cancer therapy resistance through WNT16B Yu. Nat. Med. 18, 1359–1368. https://doi.org/10.1038/nm.2890.Treatment-induced.

Whelan, J.S., Davis, L.E., 2018. Osteosarcoma, chondrosarcoma, and chordoma. J. Clin. Oncol. 36, 188–193. https://doi.org/10.1200/JCO.2017.75.1743.

Xiao, H., Chen, L., Luo, G., Son, H., Prectoni, J.H., Zheng, W., 2014. Effect of the cytokine levels in serum on osteosarcoma. Tumor Biol. 35, 1023–1028. https://doi.org/10.1007/s13277-013-1136-x.

Xu, D., Hu, M.J., Wang, Y.Q., Cui, Y.L., 2019. Antioxidant activities of quercetin and its complexes for medicinal application. Molecules 24, 1123. https://doi.org/10.3390/MOLECULES24061123.

Xue, W., Zender, L., Miething, C., Dickins, R.A., Hernando, E., Cordon-cardo, C., Lowe, S. W., Harbor, C.S., Harbor, C.S., 2007. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature 445, 656–660. https://doi.org/10.1038/nature05529.Senescence.

Yang, J., Liu, M., Hong, D., Zeng, M., Zhang, X., 2021. The paradoxical role of cellular senescence in cancer. Front. Cell Dev. Biol. 9, 722205 https://doi.org/10.3389/FCELL.2021.722205.

Yang, M.-Y., Lin, P.-M., Liu, Y.-C., Hsiao, H.-H., Yang, W.-C., Hsu, J.-F., Hsu, C.-M., Lin, S.-F., 2012. Induction of cellular senescence by doxorubicin is associated with upregulated miR-375 and induction of autophagy in K562 cells. PLoS One 7, e37205. https://doi.org/10.1371/journal.pone.0037205.

Yao, Z., Murali, B., Ren, Q., Luo, X., Faget, D.V., Cole, T., Ricci, B., Thotala, D., Monahan, J., Van Deursen, J.M., Baker, D., Faccio, R., Schwarz, J.K., Stewart, S.A., 2020. Therapy-induced senescence drives bone loss. Cancer Res. 80, 1171. https://doi.org/10.1158/0008-5472.CAN-19-2348.

Zhang, C., Ma, K., Li, W.Y., 2019. IL-6 promotes cancer stemness and oncogenicity in U2OS and MG-63 osteosarcoma cells by upregulating the OPN-STAT3 pathway. J. Cancer 10, 6511–6525. https://doi.org/10.7150/jca.29931.