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## Resveratrol prevents the expression of glaucoma markers induced by chronic oxidative stress in trabecular meshwork cells

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### Abstract

Elevated intraocular pressure (IOP) constitutes the best characterized risk for primary open-angle glaucoma (POAG). Elevated IOP is believed to result from an increase in aqueous humor outflow resistance at the level of the trabecular meshwork (TM)/ Schlemm's canal (SC). Malfunction of the TM in POAG is associated with the expression of markers for inflammation, cellular senescence, oxidative damage, and decreased cellularity. Current POAG treatments rely on lowering IOP, but there is no therapeutic approach available to delay the loss of function of the TM in POAG patients. We evaluated the effects of chronic administration of the dietary supplement resveratrol on the expression of markers for inflammation, oxidative damage, and cellular senescence in primary TM cells subjected to chronic oxidative stress (40% O<sub>2</sub>). Resveratrol treatment effectively prevented increased production of intracellular reactive oxygen species (iROS) and inflammatory markers (IL1 $\alpha$ , IL6, IL8, and ELAM-1), and reduced expression of the senescence markers sa- $\beta$ -gal, lipofuscin, and accumulation of carbonylated proteins. Furthermore, resveratrol exerted antiapoptotic effects that were not associated with a decrease in cell proliferation. These results suggest that resveratrol could potentially have a role in preventing the TM tissue abnormalities observed in POAG.

### Keywords

Resveratrol; oxidative stress; glaucoma; trabecular meshwork

### 1. Introduction

Primary Open-Angle Glaucoma (POAG) is an age-related-disease affecting millions of people worldwide. This optic neuropathy is characterized by loss of retinal ganglion cell axons and remodeling of the optic nerve head that is accompanied by progressive visual field loss that can result in irreversible blindness. The main risk factor for POAG is elevated intraocular pressure (IOP) and lowering IOP is currently the only therapeutic approach available to delay the progression of the disease (Armaly, et al., 1980; Leske, et al., 2003). The trabecular meshwork (TM) is the tissue responsible for draining most of the aqueous humor from the anterior chamber of the eye, and the increase in IOP observed in POAG is believed to result from an increase in resistance to aqueous humor outflow at the level of the TM/ Schlemm's canal (SC) (Maepea and Bill, 1992; Moses, 1977).

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The specific mechanisms leading to the failure of the TM to maintain normal levels of outflow resistance in POAG are not completely understood. However, it is known that the TM from glaucoma donors is characterized by the sustained activation of a stress response that results in expression of pro-inflammatory markers such as ELAM-1 (Wang, et al., 2001). In a comparative analysis between control and POAG tissues, several genes associated with inflammation and an acute-phase response, including ELAM-1, were up-regulated (Liton, et al., 2006). Glaucomatous TM cells produce constitutively IL1 $\alpha$  that has been shown to lead to the up-regulation of ELAM-1 and other inflammatory mediators (Wang, et al., 2001; Zhang, et al., 2006). In addition, a polymorphism in IL1 $\alpha$  that leads to increased IL1 expression has been reported to be a risk factor for POAG (Wang, et al., 2006). Together with the sustained activation of a pro-inflammatory response, the TM from glaucoma donors is also known to show some decrease in cellularity and an increase in the expression of the cellular senescence marker sa-beta-galactosidase (sa- $\beta$ -gal) (Alvarado, et al., 1984; Liton, et al., 2005), which suggests that apoptosis and cellular senescence may contribute to the loss of function of the TM.

Oxidative stress may also be a contributing factor in the observed alterations of the TM in glaucoma. Acute treatment of TM cells with H<sub>2</sub>O<sub>2</sub> has been shown to induce the expression of the glaucoma marker ELAM-1 (Zhou, et al., 2007) and chronic H<sub>2</sub>O<sub>2</sub> treatment results in a sustained activation of a stress response similar to that observed in TM cells from glaucoma donors (Li, 2007). Furthermore, oxidative stress has been suggested to contribute to the loss in cellularity in the TM by inducing apoptosis (Alvarado, et al., 1981; Alvarado, et al., 1984) and is also known to contribute to an increased expression of sa- $\beta$ -gal (Caballero, et al., 2003). The TM is subjected to relatively high oxygen concentration from the aqueous humor, between 5% and 6% (Helbig, et al., 1993), higher than in most tissues (around 3%). The pathogenic role of reactive oxygen species (ROS) in glaucoma is supported by additional experimental findings including the induction of TM degeneration and increase in resistance to aqueous humor outflow by hydrogen peroxide (Kahn, et al., 1983; Nguyen, et al., 1988); reduction in the antioxidant capacities of the TM with age (De La Paz and Epstein, 1996) and positive correlation between oxidative DNA damage in the TM with visual-field loss and increased IOP (Sacca, et al., 2005). Recently, the increased expression of the oxidative stress marker sPLA<sub>2</sub>-IIA has also been reported in association with glaucoma (Zhou, et al., 2007).

Although there is great deal of research aimed at preventing the loss of ganglion cells using neuroprotective agents (Cheung, et al., 2008; Weber, et al., 2008), current treatments for POAG rely on lowering the IOP and, at the present, there is no therapy that can help to prevent or delay the loss of function of the TM in POAG patients. Resveratrol, a naturally occurring polyphenol found in berries, nuts, and red wine, can enhance stress resistance and extend the life span of various organisms from yeast to vertebrates (Bauer, et al., 2004; Baur, et al., 2006; Howitz, et al., 2003; Valenzano, et al., 2006). It has been reported to exert anti-inflammatory, anti-oxidant, and anti-apoptotic effects, and there is experimental evidence supporting the beneficial effects of resveratrol in preventing or slowing down a wide variety of age related diseases (Baur and Sinclair, 2006; Cucciolla, et al., 2007; Holme and Pervaiz, 2007; Kahn, et al., 1983). Therefore, we hypothesized that resveratrol may help to prevent the alterations induced by chronic oxidative stress in TM cells. To evaluate the potential of resveratrol to protect TM function we analyzed the effects of chronic resveratrol treatment on the expression of markers for inflammation, oxidative damage, and cellular senescence in primary TM cells subjected to chronic oxidative stress, as well as on the levels of apoptosis induced by acute oxidative injury.

## 2. Material and Methods

### 2.1. Cell culture

Primary porcine TM cells were obtained from pig eyes, and cultured as previously described (Stamer, et al., 1995). Cell cultures were maintained at 37°C in 5% CO<sub>2</sub> in media (low glucose Dulbecco's Modified Eagle Medium with L-glutamine, 110mg/mL sodium pyruvate, 10% fetal bovine serum, 100µM non-essential aminoacids, 100 units/mL penicillin, 100µg/mL streptomycin sulfate and 0.25µg/mL amphotericin B). All the reagents were obtained from Invitrogen Corporation (Carlsbad, CA).

### 2.2. Chronic Oxidative stress and resveratrol treatment

For most of the experiments, confluent primary porcine TM cells were submitted to chronic treatment with resveratrol (25µM in DMSO, from Sigma, Saint Louis, MO) or vehicle (1µl DMSO/mL cell culture media) every three days for 15 days. Cells under resveratrol or vehicle treatment were incubated under oxidative stress conditions (40% oxygen). Additional control cultures were treated with vehicle and incubated at physiological oxygen concentration (5%). After 15 days cells were subjected to different assays.

### 2.3 Cytotoxicity Assay

Cytotoxicity was measured using the Cyto Tox 96® Non-Radioactive Cytotoxicity assay (Promega, Madison, WI) following the manufacturer's instructions. Briefly the release of lactate dehydrogenase (LDH) is converted into a red formazan product proportional to the number of lysed cells.

### 2.4. Senescence associated beta-galactosidase (sa-β-gal) activity

Activity of sa-β-gal was measured by flow cytometry using the fluorogenic substrate C<sub>12</sub>FDG (Molecular Probes, Eugene, OR) as previously described (Fiering, et al., 1991). Briefly, cells were incubated with 300µM of chloroquine for 30 minutes to modulate the intracellular pH. Cell cultures were then washed with phosphate buffered saline (PBS), trypsinized, resuspended in PBS, and incubated for 5 minutes at 37°C in a water bath, mixed gently with 2mM of FDG, and incubated for 1 minute. The cell suspension was placed on ice, diluted 10 times with cold PBS, and incubated on ice until flow cytometry analysis (FACScaliber, Becton Dickinson, CA, USA). The average number of cells analyzed for each experiment was 10,000.

### 2.5. Cell proliferation

Cell proliferation was assayed using 10µM BrdU (5-bromo-2'-deoxyuridine, from Sigma, Saint Louis, MO). Briefly, after two weeks of treatment, cells in 6 well plates were trypsinized and seeded in 10cm plates, incubated 48 hours and labeled with BrDu for 5 hours. After labeling, cells were trypsinized and fixed in cold methanol overnight. Cells were washed in PBS/BSA, denaturated with 2M HCl, neutralized with 0.1M sodium borate, and hybridized with anti BrdU-FITC antibody (Santa Cruz Biothecnology, Santa Cruz, CA), and resuspended in propidium iodine (10µg/mL) containing 300µg/mL RNase (Sigma, MO, USA). BrdU incorporation was calculated from 10,000 cells by flow cytometry.

### 2.6. Analysis of autofluorescence

Cell autofluorescence (lipofuscin-like or ceroid-like material) was determined by flow cytometry at 563-607nm. The average number of cells analyzed for each experiment was 10,000.

## 2.7. Intracellular Reactive Oxygen Species (iROS) measurement

Cells were loaded with 10 $\mu$ M of 2',7'-dichlorofluorescein diacetate (DCFDA) (Calbiochem, La Jolla, CA) incubated for 30 minutes in PBS and washed with PBS. This was followed by incubation for 20 minutes in media and trypsinization. Finally, the cells were collected in PBS and kept on ice until analyzed by flow cytometry. The average number of cells analyzed for each experiment was 10,000.

## 2.8. RNA extraction

For RNA extraction, porcine primary TM cells were washed with PBS and immediately submerged in RNA-later (Ambion Inc, Austin, TX). Total RNA was then isolated using RNeasy kit (Qiagen Inc. Valencia, CA) according to the manufacturer's instructions and treated with DNase. RNA yields were determined using RiboGreen fluorescent dye (Molecular Probes Inc. Eugene, OR).

## 2.9. Quantitative real-time PCR (RT-PCR)

First strand cDNA was synthesized from total RNA (1 $\mu$ g) by reverse transcription using oligo-dT and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. RT-PCR reactions were performed in 20 $\mu$ L mixture containing 1 $\mu$ L of the cDNA preparation, 1X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), using the following PCR parameters: 95 $^{\circ}$ C for 5 minutes followed by 50 cycles of 95 $^{\circ}$ C for 15 seconds, 65 $^{\circ}$ C for 15 seconds, and 72 $^{\circ}$ C for 15 seconds. The fluorescence threshold value (Ct) was calculated using the iCycle system software. The absence of nonspecific products was confirmed by both analysis of the melting curves and electrophoresis in 3% Super agarose gels.  $\beta$ -actin was used as an internal standard of mRNA expression. Primers for RT-PCR were designed using Primer3 (v. 0.4.0) software (Rozen, 2000) and are showed in Table 1.

## 2.10. Protein extraction and protein carbonylation assay

For protein extraction cells were washed in PBS and lysated in 1X RIPA buffer, and protein concentration was determined using Micro BCA Protein Assay Kit (Pierce, Rockford, IL). The OxyBlot<sup>TM</sup> Protein Oxidation Detection Kit (Chemicon, Temecula, CA) was employed for the comparative analysis of total protein carbonylation according to the manufacturer's instructions. Briefly, 2,4-dinitrophenylhydrazine (DNPH) derivatization was carried out followed by SDS-PAGE under reducing conditions and blots were incubated with anti-DNP antibody. Blots were developed using a chemiluminescence detection system (ECL-Plus from Amersham, Buckinghamshire, UK). The comparison between the samples was calculated using the ratio between densitometric values of the total oxyblot bands and the total proteins stained with Commassie Blue on the same blot.

## 2.11. DNA damage assay

DNA damage quantification kit (Dojindo, Gaithersburg, MD) detects DNA modifications that result in the formation of aldehyde groups, which are the open ring form of the abasic sites (AP sites). Briefly, an excess of Aldehyde Reactive Probe (ARP) conjugated to avidin was incubated with DNA samples. After labeling, the AP sites were quantified by a colorimetric reaction using anti avidin antibody conjugated to peroxidase, following the manufacturer's instructions.

## 2.12. Proteasome activity assay

Chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (PGPH) activities of the proteasome were assayed using the fluorogenic peptides (from Sigma, Saint Louis, MO) N-Succinyl-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC at 70 $\mu$ M), Z-Leu-Leu-Glu-AMC

(ZLLG-AMC at 70 $\mu$ M) and Boc-Leu-Arg-Arg-AMC (BOLAA at 200 $\mu$ M). Briefly, cells were lysated (20mM Tris-HCl, pH 7.5; 10% glycerol; 5mM ATP; 0.2% Np40) and 20 $\mu$ g of protein extract diluted in assay buffer (50mM Hepes/ KOH pH 7.5). Assays were carried out at 37°C for 30 minutes. The fluorescence of the samples was evaluated using a BIO-TEK Synergy HT spectrofluorimeter at excitation/emission wavelengths of 360/460. Activity was determined as the difference between total activity of crude extracts and the remaining activity with 20 $\mu$ M MG132 (Sigma, St Louis, MO), a proteasome inhibitor.

### 2.13. Apoptotic analysis

Apoptosis was measured using the Vybrant Apoptosis Assay kit (Invitrogen, Eugene, OR). Briefly, apoptosis was induced using several concentrations of H<sub>2</sub>O<sub>2</sub> (0, 200, 400 and 800 $\mu$ M). Cells were treated with 25 $\mu$ M of resveratrol or vehicle 24 hours before H<sub>2</sub>O<sub>2</sub> treatment and during the treatment. Cells were trypsinized four hours after H<sub>2</sub>O<sub>2</sub> treatment, washed with cold PBS, resuspended in PBS with YO-PRO and propidium iodine dyes, incubated on ice for 30 minutes, and analyzed by flow cytometry. The average number of cells analyzed for each experiment was 10,000.

### 2.14. Statistical analysis

Results are expressed as mean value  $\pm$  SD in at least three independent experiments. The SAS Software package (SAS/STAT, version 8, Cary, NC: SAS Institute Inc., 1999) was used for the statistical analysis. P-values were calculated by a non paired two sided t-test and by the non parametric test Kruskal-Wallis (that is the same as the Wilcoxon test when there are only two groups). In the figures “\*” denoted p-values based on t-test and “#” p-values based on the Kruskal-Wallis test.

## 3. Results

### 3.1. Cytotoxicity of chronic treatment with resveratrol in TM cells

In order to evaluate potential cytotoxic effects of resveratrol, TM cell cultures incubated at physiologic oxygen concentration (5%) were subjected to chronic treatment with different concentrations of resveratrol or vehicle for 15 days. A single treatment with high concentrations of resveratrol (200 and 400 $\mu$ M) resulted in extensive cell death in less than 48 hours. However, chronic treatment with resveratrol at concentrations of 100 $\mu$ M or lower did not show any increase in cytotoxicity compared to the vehicle treated controls. Treatment with resveratrol at concentrations of 50 and 100 $\mu$ M resulted in a small but significant decrease in cytotoxicity compared to control cultures treated with vehicle (Figure 1).

### 3.2. Resveratrol inhibits intracellular ROS production

To evaluate the effects of resveratrol on the induction of intracellular ROS production mediated by chronic oxidative stress, confluent primary cultures of porcine TM cells were subjected to chronic oxidative stress (15 days at 40% oxygen) and chronic treatment with resveratrol or vehicle (every three days during 15 days). Non-stressed control cultures were incubated in parallel at 5% oxygen concentration. The induction of endogenous ROS production in this model was significantly decreased by resveratrol treatment (4 fold) compared to cells treated with vehicle. The amount of intracellular ROS in resveratrol-treated cells under oxidative stress was similar to that of non-stressed control cells incubated at physiological oxygen concentration (5%) (Figure 2).

### 3.3. Resveratrol reduces the activation of inflammatory markers

The induction of mRNA expression of the inflammatory markers IL1 $\alpha$ , IL6, IL8 and ELAM-1 after chronic oxidative stress was significantly inhibited by chronic treatment with resveratrol.

As shown in figure 3, resveratrol inhibited the induction of these inflammatory markers in samples incubated at 40% oxygen.

### 3.4. Resveratrol reduces the accumulation of senescence markers

Cells treated with vehicle and incubated under oxidative stress showed a significant increase in autofluorescence (2.4 fold) and sa- $\beta$ -gal (1.8 fold) when compared to cells in the same condition treated with resveratrol. Cells under oxidative stress, treated with vehicle or resveratrol, showed higher levels of autofluorescence (3.1 and 1.2 fold respectively) and sa- $\beta$ -gal (3.5 and 1.9 fold respectively) when compared to cells incubated at more physiological oxygen concentration (Figure 4A and 4B).

### 3.5. Decreased accumulation of carbonylated proteins with no change in proteasomal activity after resveratrol treatment

The accumulation of carbonylated proteins induced by oxidative stress was significantly lower in resveratrol-treated samples compared to samples treated with vehicle under oxidative stress, as well as to that in cells incubated at physiological oxygen concentrations (Figures 5A, B and C). However, this decrease in oxidized proteins was not associated with an increase in proteasomal activity. The three proteolytic activities of the proteasome (chymotrypsin-like, trypsin-like and caspase-like activity) showed decreased activity in both resveratrol treated and non-treated cells incubated at 40% oxygen compared to the non-stressed controls incubated at physiologic oxygen concentrations (Figure 5D).

### 3.6. Resveratrol protects against apoptosis in an acute model of oxidative stress

Cells treated with resveratrol showed protection against apoptosis after acute oxidative stress (200, 400, and 800 $\mu$ M of H<sub>2</sub>O<sub>2</sub>), when compared to cells treated with vehicle. Cells treated with vehicle showed a linear correlation between H<sub>2</sub>O<sub>2</sub> concentration and apoptosis; cells treated with resveratrol exhibited protection against apoptosis in all H<sub>2</sub>O<sub>2</sub> concentrations (Figure 6).

### 3.7. Proliferation and DNA damage are not significantly affected by resveratrol treatment in a chronic model of oxidative stress

Resveratrol treatment did not result in significant changes on proliferation measured by BrDU incorporation when compared to cells treated with vehicle (Figure 7A). Similarly, no significant changes were observed in the amount of DNA damage under oxidative stress. Cells treated with vehicle or resveratrol under oxidative stress showed similar levels of DNA damage that were higher than those in cells incubated at physiological oxygen concentration (Figure 7B).

## 4. Discussion

Our results demonstrated that chronic treatment of cultured TM cells with resveratrol effectively prevented the upregulation of markers induced by oxidative stress.

Resveratrol has been shown to reduce inflammation *in vivo* via inhibition of cyclooxygenase-2 and NF- $\kappa$ B activities (Holmes-McNary and Baldwin, 2000; Martin, et al., 2006; Tsai, et al., 1999). However, we have recently reported that the induction of the inflammatory markers IL1 $\alpha$ , IL-6, IL-8, and ELAM-1 in TM cells subjected to chronic oxidative stress is dependent on the activation of iROS generated by the mitochondria. This increase in iROS is not dependent on cyclooxygenase and is responsible for the chronic activation of NF- $\kappa$ B. One of the more clear effects of chronic treatment with resveratrol in TM cells was the inhibition of iROS production that results from oxidative stress. Therefore the inhibitory effect of resveratrol



on the production of iROS may be responsible for the concomitant inhibition of the sustained stress response. Although resveratrol has been shown to induce the production of iROS in some cancer cell lines (Heiss, et al., 2007; Shankar, et al., 2007), our results are consistent with those reported in other primary cells including HUVEC cells (Chow, et al., 2007; Ungvari, et al., 2007), primary cortical astrocytes (de Almeida, et al., 2007; de Almeida, et al., 2007), RAW264.7 macrophages (Ciz, et al., 2007; Jang, et al., 1999), RPE cells (King, et al., 2005), and dopaminergic neurons (Okawara, et al., 2007).

Chronic treatment of TM cells with resveratrol also resulted in a significant decrease in the accumulation of fluorescent pigments, carbonylated proteins, and the expression of the cellular senescence marker sa- $\beta$ -gal, that are induced by oxidative stress. A decrease in lipofuscin and sa- $\beta$ -gal upon resveratrol treatment has also been observed in the short-lived fish *N. furzeri*. In this small vertebrate resveratrol treatment not only reduces the expression of senescence markers but increases life span and decreases aggregated protein in elderly fish brains (Valenzano and Cellerino, 2006; Valenzano, et al., 2006). In our model, treatment with resveratrol did not prevent the loss of proteasome activity induced by oxidative stress. Therefore, the observed decrease in accumulation of oxidized proteins and fluorescent pigments is not likely to result from increased proteasomal degradation of damaged proteins, but rather from other factors such as resveratrol ROS scavenging properties (Ciz, et al., 2007; Lorenz, et al., 2003); an increase in antioxidant enzymes (Baur and Sinclair, 2006; Karaoglan, et al., 2008; Robb, et al., 2007; Ungvari, et al., 2007); activation of autophagy pathways (Opipari, et al., 2004; Trinchieri, et al., 2007); and/or the observed inhibition of iROS production (Chow, et al., 2007; de Almeida, et al., 2007; Jha, et al., 2008; King, et al., 2005).

In addition to the protective effects in our chronic oxidative stress model, resveratrol also demonstrated a significant anti-apoptotic effect after acute oxidative injury. Resveratrol has received a great deal of attention because of its pro-apoptotic effects in cancer cells (Kaindl, et al., 2007; Kalra, et al., 2007; Park, et al., 2007; van Ginkel, et al., 2007). However, its anti-apoptotic effects in several primary cell lines have been well documented (Csaki, et al., 2008; Jha, et al., 2008; Kumar, et al., 2007). Interestingly, our results showed no negative effects of chronic resveratrol treatment on the proliferation of TM cells. This is somewhat surprising, since resveratrol has been reported to exert antiproliferative effects, mainly in cancer cells, but also in other non-cancerous cell types (Lee and Moon, 2005; Poussier, et al., 2005). RPE cells treated with resveratrol at pre-confluence levels (80%) showed a significant decrease in proliferation that was not observed when they were confluent (King, et al., 2005). In our experimental model, cells were treated at confluence levels and this difference might explain our observed effect of resveratrol on proliferation. Since the decrease in cellularity of the TM associated with aging and POAG has been hypothesized to result, at least in part, from oxidative damage, our results suggest that chronic treatment with resveratrol could be effective in delaying the loss of cells in the TM by acting to prevent apoptosis without inhibiting cell proliferation. One important point that deserves future investigation is whether resveratrol can reverse, at least in part, some of the effects of chronic oxidative stress. Preliminary data suggest that short-term treatment with resveratrol may not be helpful to reverse the damage that has already been produced (data not showed). However, a definitive answer to this question will require longer term experiments aimed at evaluating the effects of resveratrol treatment on the progression of oxidative damage for an extended period of time after chronic oxidative injury.

In conclusion, the inhibition of iROS production by resveratrol may help to prevent the induction of inflammatory and senescence markers in TM cells after chronic oxidative stress, as well as possibly prevent the decrease in cellularity observed in the TM of POAG patients. These results suggest that resveratrol could potentially have a novel role in preventing or delaying some of the abnormalities of the TM observed in POAG.

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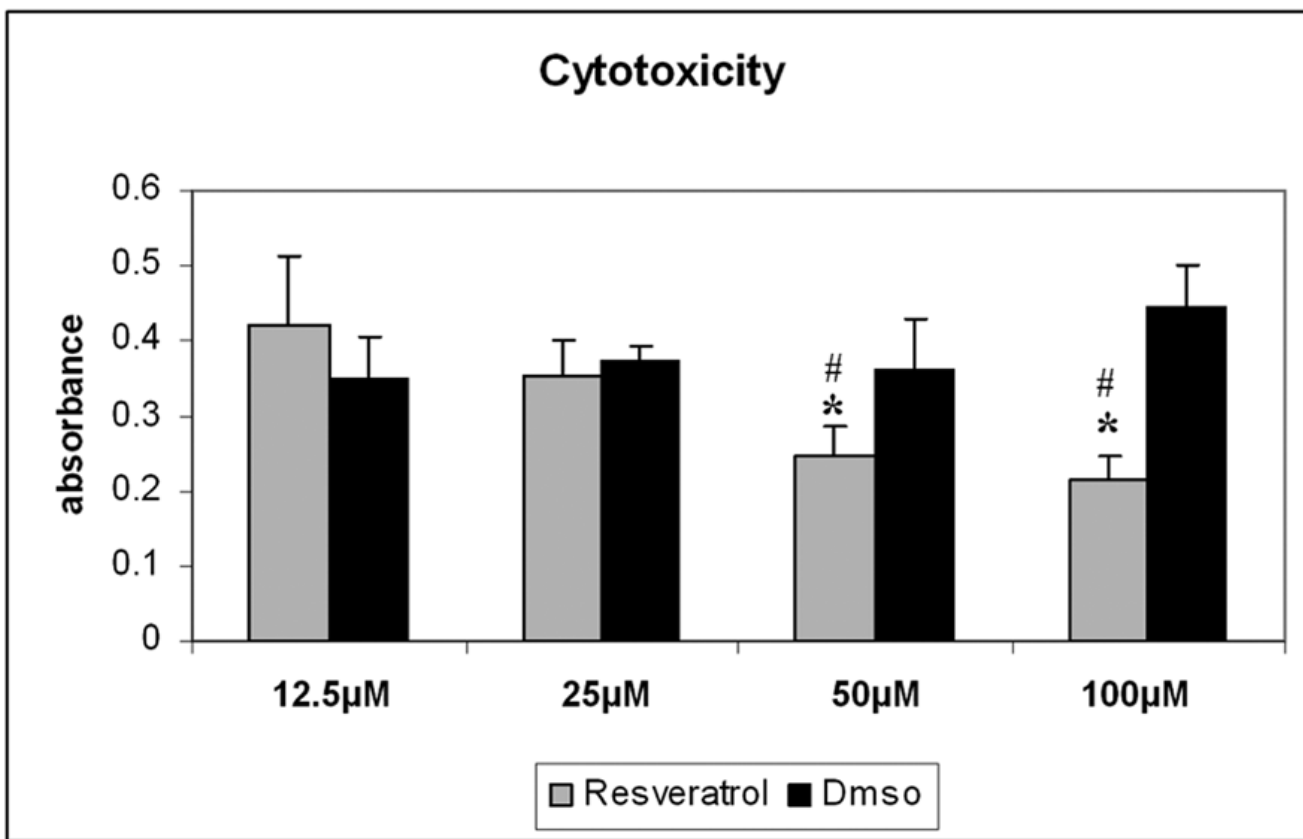
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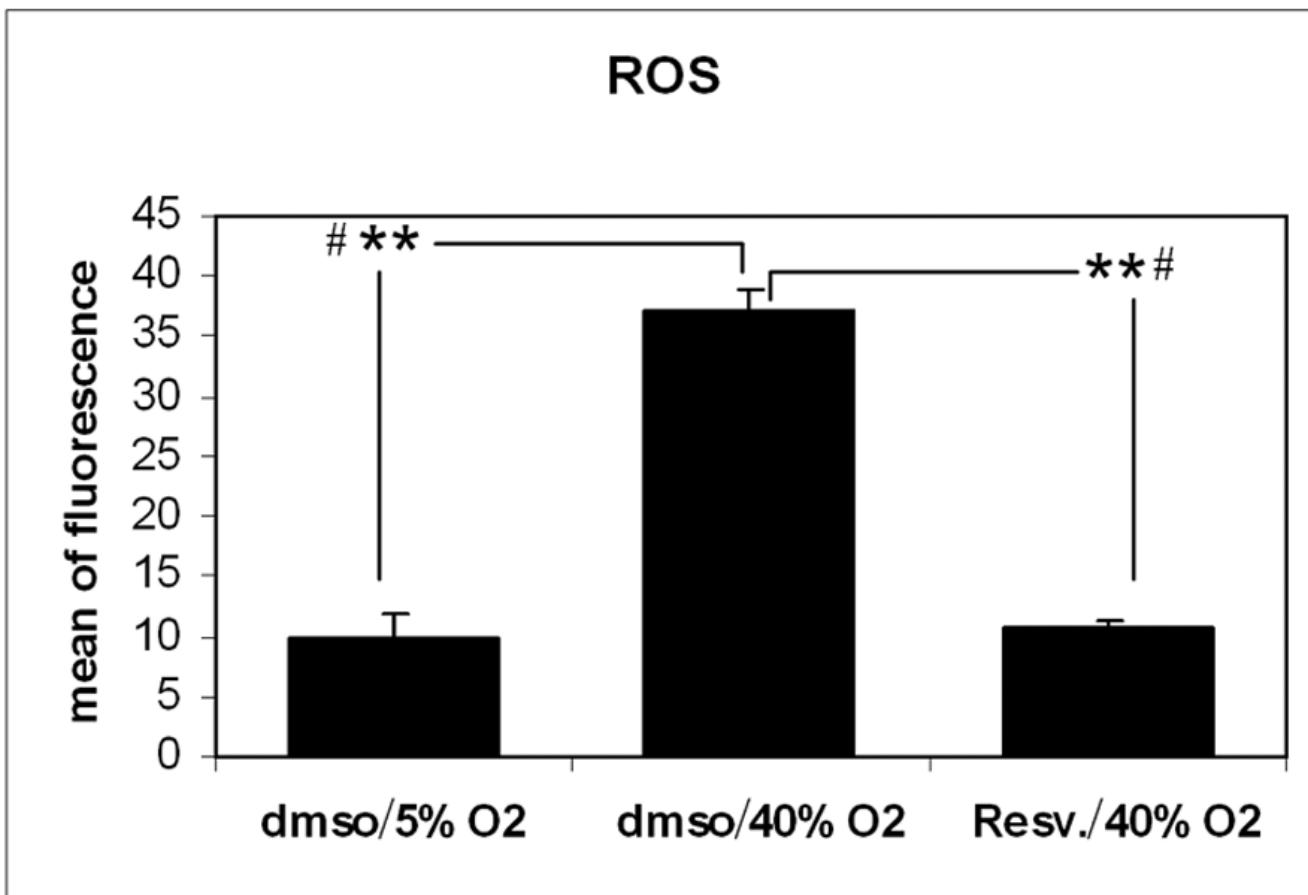
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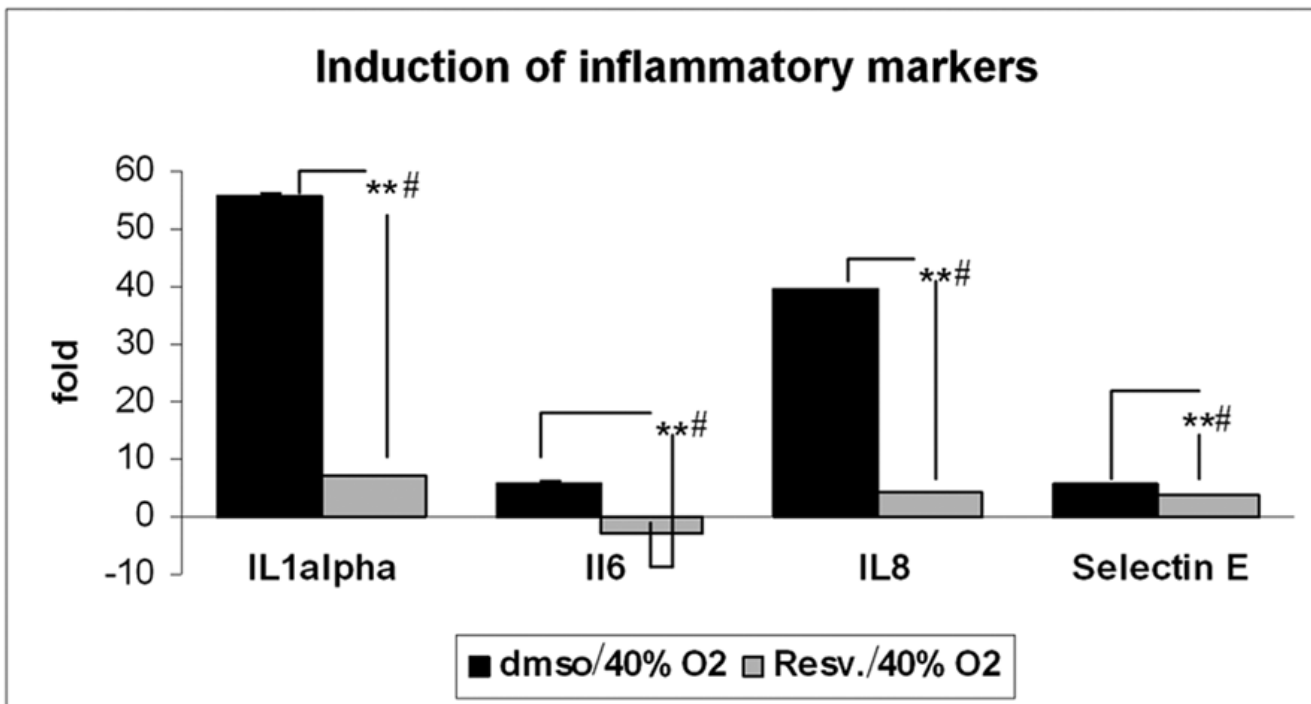
**Figure 1.**

Cytotoxic effects of chronic treatment with resveratrol in TM cells incubated at physiological oxygen concentration (5%). Cytotoxicity was evaluated using Cyto Tox 96 assay after 15 days of chronic treatment with different concentrations of resveratrol (12.5 to 400µM) or vehicle. A single treatment with high concentrations of resveratrol (200 and 400µM) resulted in extensive cell death in less than 48 hours. Since these cultures did not survive a chronic treatment for 15 days, cytotoxicity data for these concentrations is not included in the figure (N=3, t-test \*P ≤ 0.05, # Kruskal-Wallis test P ≤ 0.049).



**Figure 2.**

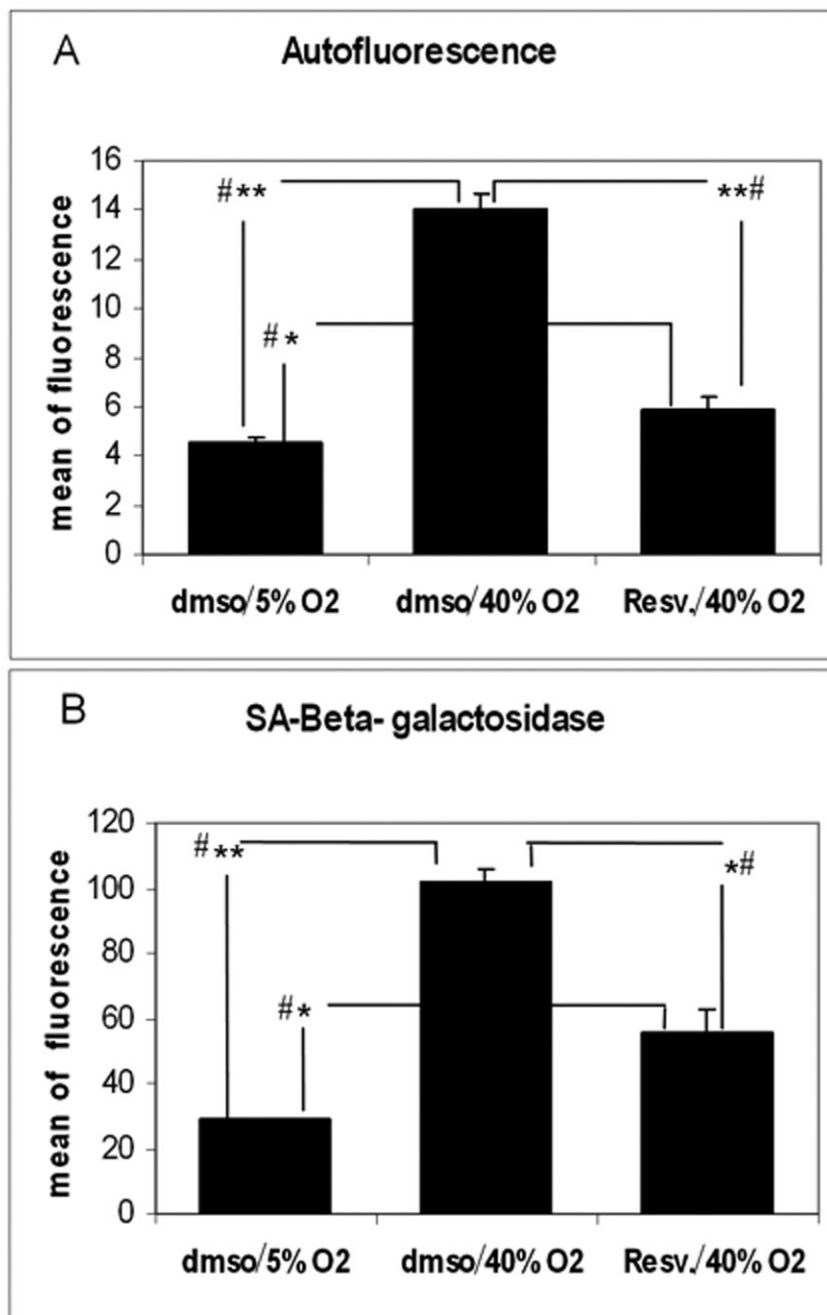
Effect of resveratrol treatment on the induction of iROS mediated by chronic oxidative stress in pTM cells. Levels of iROS were measured by H<sub>2</sub>DCFDA from cells incubated at 40% oxygen and treated with resveratrol or vehicle and compared to cells incubated at physiological oxygen conditions (5%) (N=3, t-test \*\*P ≤ 0.001, # Kruskal-Wallis test P ≤ 0.049).



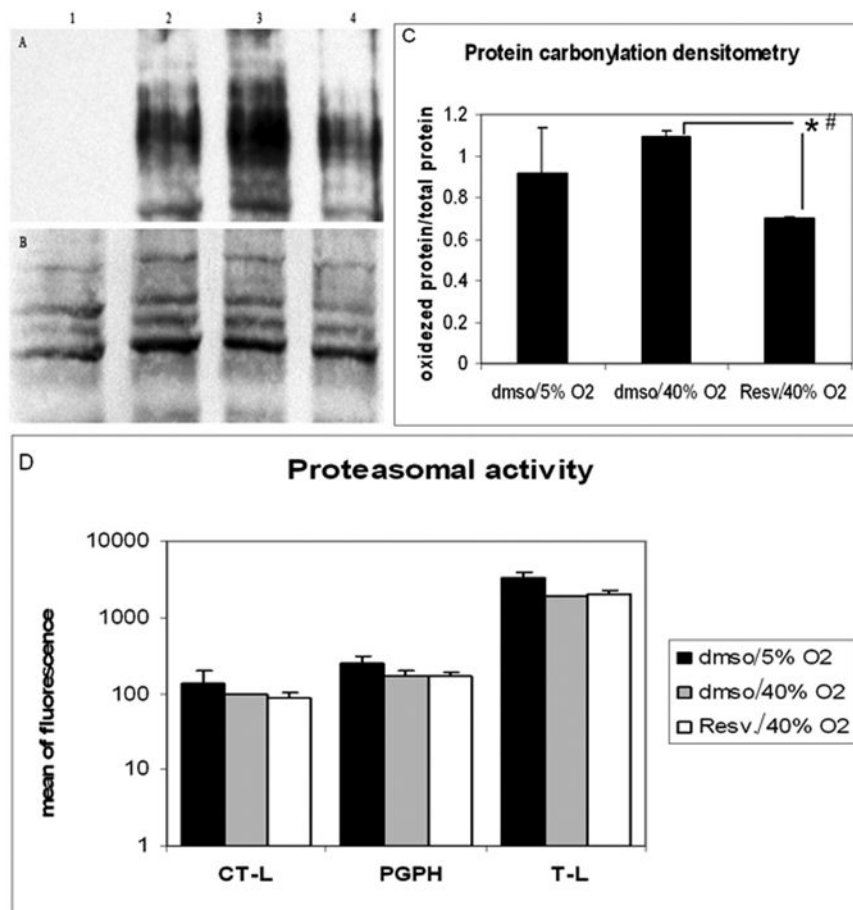
**Figure 3.**

Effect of resveratrol treatment on the expression of inflammatory markers induced by chronic oxidative stress. The changes in mRNA expression of IL1 $\alpha$ , IL6, IL8 and ELAM-1 induced by incubation at 40% oxygen compared to control cultures incubated at 5% oxygen were measured by Real Time-PCR in cells treated with either resveratrol or dms0.  $\beta$ -actin was used as an internal control (N=3, t-test \*\*  $P \leq 0.001$ ; # Kruskal-Wallis test  $P \leq 0.049$ ).

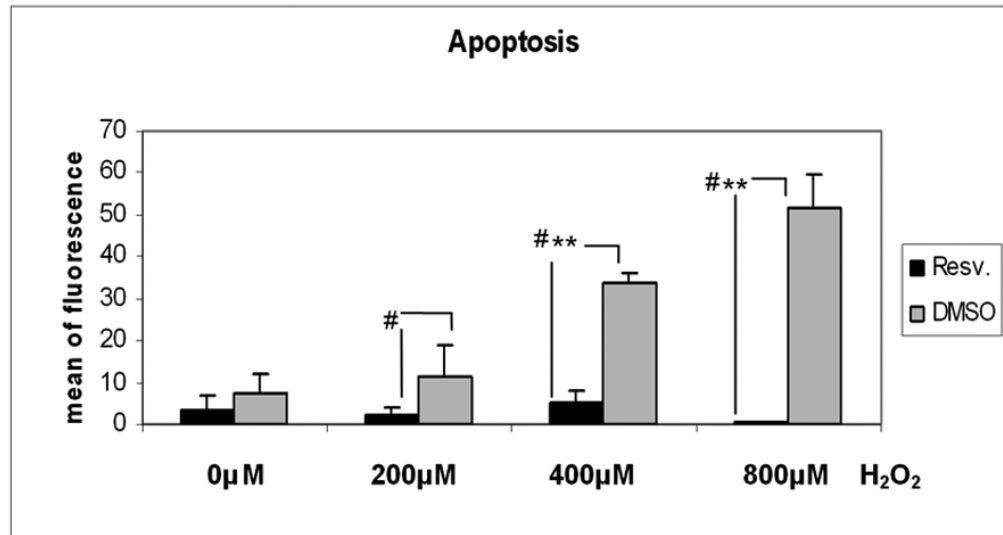




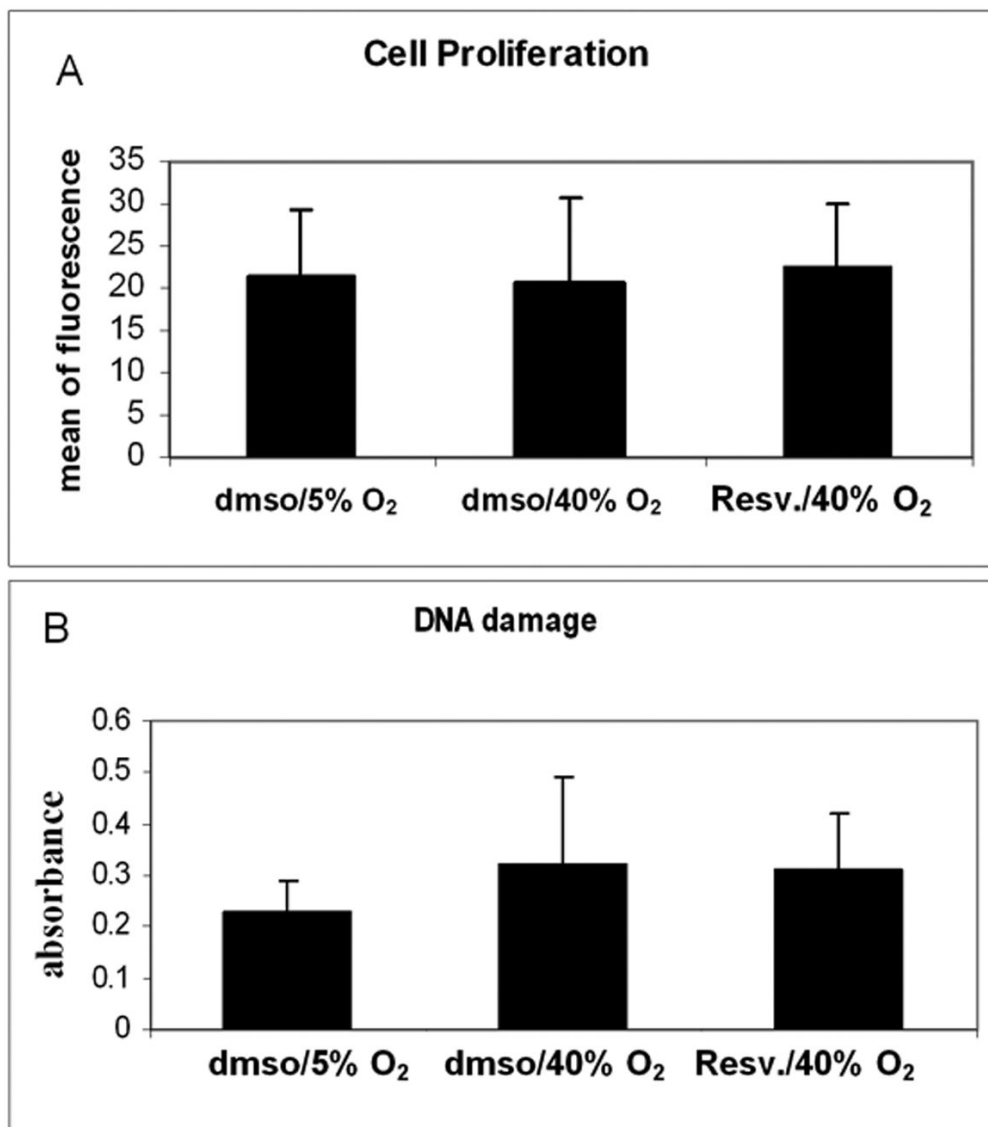
**Figure 4.** Effect of resveratrol treatment on the expression of the senescence markers autofluorescence (A) and sa- $\beta$ -gal (B). Autofluorescence and sa- $\beta$ -gal activity were determined by flow cytometry in cells subjected to chronic oxidative stress (40% oxygen) treated with either resveratrol or vehicle and compared to cells incubated at physiological oxygen concentration (N=3, t-test, \*  $P \leq 0.05$ , \*\*  $P \leq 0.001$ ; # Kruskal-Wallis test  $P \leq 0.049$ ).



**Figure 5.** Effects of resveratrol treatment on the accumulation of carbonylated proteins and proteasome activity in pTM cells after chronic oxidative stress. Total protein carbonylation and proteolytic activity were measured in cells incubated at 40% oxygen treated with either resveratrol or vehicle and compared to cells incubated at 5% oxygen. Representative Western Blot from oxidized proteins is shown in panel A (1= negative control for carbonylation; 2= DMSO/ 5% oxygen; 3= DMSO/ 40% oxygen; 4= resveratrol/ 40% oxygen) and total proteins stained with Commassie Blue in panel B. Densitometry summarizing 3 different experiments is showed in panel C. Panel D shows the proteolytic activities of the proteasome subunits: CT-L (chymotrypsin like), T-L (trypsin like), and PGHP (caspase like) (N=3, t-test, \*  $P \leq 0.05$ ; # Kruskal-Wallis test  $P \leq 0.049$ ).



**Figure 6.** Protective effect of resveratrol against apoptosis induced by acute oxidative challenge. Cells treated with resveratrol or vehicle were subjected to different concentrations of H<sub>2</sub>O<sub>2</sub> (200, 400, and 800 μM) for 4 hours and assayed for apoptosis (N=3, t-test, \*\* P ≤ 0.001; # Kruskal-Wallis test P ≤ 0.049).



**Figure 7.** Effects of resveratrol treatment on cell proliferation and DNA damage. Cells incubated at 40% oxygen and treated with resveratrol did not show any significant difference in proliferation quantified by BrdU incorporation (A) or DNA damage analyzed by measuring the formation of aldehyde groups (B) (Panel A, N=5; panel B, N=3).

**Table 1**

Name	Genebank Acc #	Start position	Forward 5'-3'	Reverse 5'-3'
IL-1 $\alpha$	NM_14029	842	AAGTGTGACAGGCCGTATG	TACCAGACTTCGCTCCCTCT
ELAM-1	NM_214268	697	CCCATGGAACACAACCTGTGCATT	AGCTTTACACGTTGGCTTCTTGCC
IL-8	NM_213867	92	AAACTGGCTGTTGCCTTCTT	ATTATGCACTGGCATCGAA
IL-6	NM_214399	365	GCTTCCAATCTGGGTTCAAT	CTAATCTGCACAGCCTCGAC
$\beta$ -actin	AY550069	1063	AAGATCAAGATCATCGCGCTCCA	TGGAATGCAACTAACAGTCCGCCT