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Fátima Leal Seabra

Effect of resveratrol in stress-induced premature senescent human fibroblasts

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Effect of resveratrol in stress-induced premature senescent human fibroblasts

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Summary

Fibroblasts in culture have been used as models for the study of aging. Under standard culture conditions or after the action of known stressors as oxidants, cells exhibit several features of which cell cycle arrest is the loss-of-function hallmark. Resveratrol, shown to prevent age-related effects in different organisms, is currently under active study and is employed in cell ageing models. Early passage WI-38 fibroblasts grown in medium with or without resveratrol were submitted to subcytotoxic levels of H_2O_2. Cell proliferation, measured by MTT assays for day 2 upon to day 5, exhibited a dose dependent reduction, which was intensified by H_2O_2. Similarly, senescence associated - β - galactosidase (SA-β-gal) positively stained cells increased. Resveratrol attenuated H_2O_2 cytotoxic effect but enhanced sirtuin 1 expression, manganese superoxide dismutase, p21 and Cyclin D1. Exposure to H_2O_2 resulted in SIRT1 expression decrease but the effect was reverted in cells pre-treated with resveratrol. In H_2O_2-induced SIPS, resveratrol blunted MnSOD, p21 and Cyclin D expression. The results show that resveratrol results in the expression of features resembling replicative senesncence, which are enhanced by the exposition to H_2O_2.

Introduction

Human aging results in progressive functional disability and susceptibility to a wide spectrum of pathological entities such as heart disease, diabetes, osteoarthritis, dementia or cancers. It is influenced by environmental or genetic factors that may be unrelated to each other. Different patterns of functional disability are observed in different organs and cells. In any case, the result is similar, i.e., a progressive accumulation of damage or a reduced clearing process.

At the cellular level, the most common model of aging was described by Hayflick and Moorehead in 1961, when they observed that human diploid fibroblasts (HDFs) underwent a finite number of cell divisions in culture, before entering in a cell cycle arrest state termed replicative senescence (Hayflick & Moorhead 1961). In this condition, fibroblasts appear large, flat and stelate. They also present molecular and biochemical features such as shortened telomeres, appearance of senescence-associated-β-galactosidase (SA-β-gal) activity and overexpression of p16, p21 (Adams 2009), Fibronectin, Apolipoprotein J, Transforming Growth Factor beta-1 and Insulin Growth Factor Binding Protein-3 (Dierick *et al.* 2002). Despite these changes and their unresponsiveness to growth factors, senescent fibroblasts are not functionally inactive for they continue secreting molecules such as collagen and metalloproteinase.

There is considerable evidence indicating that telomere attrition is the cause for replicative senescence but other conditions may lead to the appearance of the senescent phenotype referred as Stress-Induced Premature Senescence (SIPS) (Frippiat *et al.* 2001; Adams 2009). They include oncogene activation (Campisi 2001), UV radiation, and oxidative stress challenges, hyperoxia or *tert*-butylhydroperoxide (Toussaint *et al.* 2000). Similarly, when fibroblasts are exposed to H_2O_2, they exhibit a senescent

phenotype too and overexpress collagenase, matrix-metalloproteinase-1, IFN-γ and MnSOD (Toussaint *et al.* 2000). As in replicative senescence, H_2O_2-treated HDFs are blocked mainly in the G1 phase of the cell cycle, likely by the Cyclin-Dependent Kinase Inhibitors (CDKIs) p21 and p16 which are overexpressed (Toussaint *et al.* 2000). Replicative senescence is considered an advantage because it ensures that cells with potentially oncogenic damage are incapable of cell division and hence neoplastic growth (Campisi 2001), in contrast, for example, with other tumor suppressor mechanisms as apoptosis, that eliminate permanently cells that acquired extensive damage.

The contribution of cell senescence to organism aging is still uncertain but its verification would consolidate the point strongly. Some researchers state that cell senescence appears to parallel organismal aging (Sasaki *et al.* 2006), and some others emphasized that, although some variation from organ to organ may exist, the number of cells that express one or more senescence markers appears to increase from young to aged organism (Campisi & di Fagagna 2007). The study of primates appears to be one such example (Jeyapalan *et al.* 2007) but there is not consensus (Macieira-Coelho 2010). Their likely contribution to aging and their cancer preventive properties make of human senescent cells an important model to study various biological processes.

Sirtuins are NAD-dependent deacetylases and are thus important regulators of gene expression at the transcriptional level. In mammalian cells, they regulate a wide variety of processes from apoptosis to cell survival (Michan & Sinclair 2007) and it was recently reported that sirtuin 1 (SIRT1), antagonizes senescent features of HDFs (Huang *et al.* 2008). In mammals, SIRT1 is a member of a family of seven different sirtuins (1 to 7) but it has orthologs in other species which include worms (Viswanathan *et al.* 2005), flies (Wood *et al.* 2004) and yeasts (Howitz *et al.* 2003) where its overexpression

results in lifespan extension. As they are important modulators of aging and longevity (Huang *et al.* 2008), sirtuin regulators notably resveratrol have attracted considerable attention.

Resveratrol and calorie restriction activate similar SIRT1 – mediated pathways whose actions are thought to result in prevention of common age-related diseases and life extension (Markus & Morris 2008). Resveratrol is a polyphenol found in skin of red wine grapes and other dark red fruits, seeds and nuts. Some studies reveal that resveratrol increases lifespan in lower organisms and prevents age-related diseases and lifespan shortening in obese mice (Marques *et al.* 2010). As there is evidence that SIRT1 activity is decreased in SIPS induced by H_2O_2 (Alcain & Villalba 2009), it was hypothesized that resveratrol would attenuate the appearance of senescence biomarkers in H_2O_2-induced SIPS eventually by the enhancement of SIRT1 expression. The aim of this study was to evaluate the appearance of senescence biomarkers and SIRT1 expression in H_2O_2-SIPS HDFs pre-treated with resveratrol.

Results

Cell Proliferation

To assess the effect of resveratrol on cell proliferation, cells were cultured in complete medium supplemented with either 0,2μM or 2μM resveratrol. Control cells were treated in the same conditions without resveratrol and quantification was made using MTT assay at different time-points as shown in Figure 1. In 5 days, cell number presented a 3,5-fold increase in control cells, while cells submitted to 0,2μM or 2μM resveratrol showed less than 3,0-fold increase in cell number. This data indicated that resveratrol reduced cell proliferation slightly at 0,2μM and more intensively at 2μM.

The treatment of control cells with 75μM or 100μM H_2O_2 blunted cell proliferation along the time-points of study (Figure 2A). At day 5, after H_2O_2 stress, cells presented only 2-fold or 1,6-fold increase in number for 75μM and 100μM H_2O_2, respectively. Similar results were found for cells which were pre-treated either with 0,2μM (Figure 2B) or 2μM resveratrol (Figure 2C). As expected, H_2O_2 was able to provoke cell cycle arrest. Therefore, regarding cell proliferation, resveratrol seems to be unable to attenuate H_2O_2 effect.

H_2O_2 cytotoxicity

To address H_2O_2 cytotoxicity, cell survival was measured by MTT assay immediately after stress. The higher concentration of H_2O_2 used (100μM) elicited an unexpected strong decrement in cell survival, down to 20% compared to controls (Figure 3), suggesting that subcytotoxic levels had been exceeded; 75μM H_2O_2 caused a substantial reduction too, down to approximately 40% compared to control. Maintaining

these culture conditions may require a review of H_2O_2 dose in future experiments. However, when resveratrol was present the decrement was considerably reduced when cells were treated either with 75μM or 100μM H_2O_2. For the above reasons, in the following studies, we decided to emphasize on 75μM when using H_2O_2.

Senescence associated β - galactosidase (SA-β-gal) activity

As expected for the cells cultured without resveratrol, there was an increase in the number of cells positive for SA-β-gal after treatment with 75μM or 100μM H_2O_2 (Figure 4). The addition of 2μM resveratrol to the medium also led to a significant increase in SA-β-gal when compared with control (p<0,05), but this effect was further enhanced when 2μM resveratrol treated cells were exposed to H_2O_2. The lower dose of resveratrol (0,2μM) led to an increase, although not significant, of cells positive for SA-β-gal; when exposed to H_2O_2 the number did not change. This indicates that only at lower concentrations, resveratrol might be able to attenuate the appearance of the senescence biomarker SA-β-gal induced by H_2O_2.

SIRT1 expression

Sirtuin 1 protein expression was semi-quantified by Western blotting in two independent experiments (Figure 5). Human fibroblasts grown in 0,2μM resveratrol presented no changes in SIRT1 expression when compared to control cells (Figure 6A). However, the addition of 2μM resveratrol resulted in ~ 1,5-fold increase in SIRT1 protein levels compared to control. When cells were submitted to 75μM H_2O_2, there was a slight 0,7-fold decrease of SIRT1 expression in control cells (Figure 6B). When

cells were grown with any dose of resveratrol, SIRT1 decrement induced by H_2O_2 was attenuated to values similar to BME treated cells.

Expression of MnSOD antioxidant enzyme and p21 and Cyclin D1 cell cycle regulators

Protein expression levels of MnSOD, p21 and cyclin D1 were semi-quantified by Western blotting (Figure 7).

MnSOD is a mitochondrial antioxidant enzyme involved in detoxification of reactive oxygen species. Cells pre-treated with both concentrations of resveratrol presented 4,6 or 4,7-fold increase in MnSOD protein expression, respectively, when compared to control cells, although this variation was not statistically significant (Figure 8A). It is known that MnSOD is overexpressed in the H_2O_2-induced SIPS model (Toussaint *et al.* 2000). As expected, cells exposed to 75μM H_2O_2 presented a 1,6-fold increase in MnSOD protein levels when compared to cells treated with BME (Figure 8B). The addition of resveratrol weakened the effect of H_2O_2 in increasing MnSOD. Moreover, cells grown in 2μM resveratrol showed a slight decrease in MnSOD expression after exposure to 75μM H_2O_2 compared to BME treated cells.

The cyclin-dependent kinase inhibitor p21 binds to and inhibits the activity of Cyclin Dependent Kinase (CDK) 2/4 complexes and thus functions as a negative regulator of cell cycle progression at G1. It is considered a senescence biomarker and is increased in H_2O_2–SIPS. As we show in Figure 9A, p21 expression seems to increase with the presence of resveratrol in a dose-dependent manner. Cells pre-treated with 0,2μM resveratrol revealed a 1,8-fold increase while 2μM resveratrol cells had a 2,4-

fold increase in p21 levels when compared to control cells. As one should expect, H_2O_2 stressed cells, in the absence of resveratrol, appears to increase p21 expression (Figure 9B), as already described for H_2O_2-SIPS model. The addition of 0,2µM resveratrol seems to be able to blunt p21 increment induced by H_2O_2. However, 2µM resveratrol was not so effective as the lower dose in preventing such increase.

Cyclin D1 is a member of cyclin family of cell cycle regulators and is required in combination with CDK4/6 for cell cycle G1/S transition. Cells treated with 2µM resveratrol appear to have a 1,6-fold increase in the expression of cyclin D1, when compared with control cells (Figure 10A). Treatment of control cells with 75µM H_2O_2 resulted in a slight increase in cyclin D1 expression (Figure 10B). The lower dose of resveratrol was not able to reduce such effect; however 2µM resveratrol attenuated cyclin D1 increased expression induced by H_2O_2.

Discussion

Resveratrol is a stilbene member of a large family of compounds referred as polyphenols. It became an important molecule due to its property as activator of sirtuins, an evolutionarily conserved family of deacetylases with important effects on gene activation and expression. Yet, other mechanisms are likely to be involved in resveratrol action (Baur 2010), including its antioxidant properties.

A remarkable effect of sirtuins is the extension of lifespan in a number of species. In fact, overexpression of sirtuin results in life extension of yeasts (Kaeberlein *et al.* 1999), flies (Wood *et al.* 2004) and worms (Viswanathan *et al.* 2005). Possibly through the activation of SIRT1, resveratrol also provides health benefits in mammals, to include cancer prevention (Jang *et al.* 1997) and it also restores normal lifespan of mice fed a high calorie diet (Baur *et al.* 2006). Therefore, it is not surprising that this compound attracted much attention in recent times, especially because most of the aging research is not aimed to extend lifespan of organisms, but to improve their health. Moreover, the most important experimental, not genetically manipulated, model of lifespan extension and health quality improvement is calorie restriction. It appears that sirtuins are important mediators of the effects of calorie restriction in yeast (Lin *et al.* 2000), worms (Wang & Tissenbaum 2006) and flies (Rogina & Helfand 2004).

The effect was also approached at the cellular level employing various cell lines and verifying phenotypic and genotypic variation related to aging.

In SIRT1 transfected 2BS fetal lung fibroblasts (Huang *et al.* 2008), sirtuin overexpression leads to increased cell lifespan and reduced SA-β-gal staining, i.e, features characterizing antagonism of senescence. In addition, when challenged by resveratrol 0,2 and 2μM, the same cells evidenced reduced expression of the senescence

biomarker p16. However, employing established human cell lines and different resveratrol doses resulted in different outcomes, including no change or substantial cell proliferation inhibition (Lu *et al.* 2001; Sgambato *et al.* 2001; Joe *et al.* 2002). Therefore, the effect of resveratrol on human cells is not entirely predictable, for they may evidence some discrepancies. Most current data point to a reduced rate when compared with controls, a finding that was observed in the present study with WI-38 fibroblasts.

The use of H_2O_2 in the setting of aging cell studies is intended to challenge cells with an oxidative stress; it results in cell structural and molecular features similar to replicative senescence and therefore, H_2O_2 is probably the most often used inducer of stress-induced premature senescence (Toussaint *et al.* 2000). In the present report H_2O_2 exerted a profound effect on fibroblast survival which led us to focus on 75µM concentration. However in any dose of H_2O_2 (75µM or 100µM) a adding resveratrol to the medium resulted in partial preventive effect on survival, similarly to previous observations (Robb *et al.* 2008), possibly due to its antioxidant properties (De Salvia *et al.* 2002).

Hydrogen peroxide effect on proliferation was also marked. In any experimental condition the increase in number did not exceed 1,7-fold, compared to 3,5-fold in controls and 3,0 or 2,3 -fold observed when the medium contained resveratrol. This inhibiting effect of H_2O_2 was similarly observed in human dermal fibroblasts (Gonzalez-Espinosa *et al.* 2007), human lens epithelial B3 cells (Seomun *et al.* 2005) or mouse embryo fibroblasts (Chua *et al.* 2005). Therefore, H_2O_2 not only inhibits cell proliferation, but it also blunts the small proliferative effect of resveratrol. Data from this study and from others show that the effect goes beyond the cell cycle arrest, to include features as the increasing number of SA-β-gal positive cells and p21 enhanced

expression, two important markers of replicative senescence or SIPS (Frippiat *et al.* 2001; Seomun *et al.* 2005).

Cyclin D1 is a cyclin D member whose major part is to bind CDK and promote G1/S cell cycle transition. In our cells, cyclin D1 level of expression increased slightly with resveratrol in a dose dependent-fashion, an effect which further enhanced by H_2O_2. This marginal increase of cyclin D1 after resveratrol contrasts with previous publications where no change (Della Ragione *et al.* 1998) or even a decrement (Joe *et al.* 2002) were observed. However, it should be mentioned that in any of those studies, neoplastic cell lines were used and the dose of resveratrol employed reached 100-fold our own. It is thus difficult to compare the results. More consistent is the increase in cyclin D1 expression when cells are challenged with H_2O_2. In this case the likely reason is the reduced degradation due to inhibition of proteosome, a mechanism that was strongly evidenced in replicative senescence (Chondrogianni *et al.* 2003). A similar conclusion was taken from experiments with mouse fibroblasts (Munoz *et al.* 2001).

The current results indicate that resveratrol or H_2O_2 enhance MnSOD expression and, when combined the effect appears to maintain only at a small resveratrol concentration. Such outcome was previously noted on the human cell line MRC-5 (Robb *et al.* 2008) but not in WI 38 cells. MnSOD expression in this context, likely results from an indirect effect of resveratrol. In fact, resveratrol has been shown to enhance the sirtuin-catalyzed deacetylation of FOXO3a, which stimulates the migration of FOXO to the nucleus (Robb *et al.* 2008) and enhances its transcriptional activity. MnSOD is known to be a target of FOXO3a and indeed MnSOD expression is strongly induced in cells overexpressing FOXO3a (Kops *et al.* 2002; Robb *et al.* 2008). The mechanism for the effect of H_2O_2 on MnSOD expression is uncertain but, most likely it does not involve SIRT1 activation, because actually we noticed its inhibition, which

was partially removed only when the medium contained resveratrol. Therefore, other pathways, eventually sensitive to high levels of resveratrol, may be sensing the strong effect of H_2O_2 and act upon to enhance MnSOD transcription. This effect was recognized in SIPS (Toussaint *et al.* 2000).

Resveratrol leads to a reduction of WI38 proliferation, which is intensified by H_2O_2. In one case and the other, p21 expression is enhanced, as might be expected when cell cycle is slowed. Interestingly, the presence of cells exhibiting SA-β-gal increases, as well as MnSOD, conditions which resemble replicative senescence but it is speculative to associate them, unless other studies of gene expression are made.

Experimental procedures

Reagents and antibodies

WI-38 fetal lung human diploid fibroblasts were purchased from European Collection Cell Cultures –ECACC/Sigma-Aldrich, Porton Down, United Kingdom. Cell culture supplies (Basal-Medium-Eagle, BME and fetal bovine serum, FBS) were from Biochrom AG, Berlin, Germany. Resveratrol and SA-β-gal were obtained from Calbiochem, Darmstadt, Germany. Hydrogen Peroxide was from Merck, Darmstadt, Germany. MTT and BSA were from Sigma-Aldrich, St Louis, USA. Bradford reagent and nitrocellulose membrane were from Bio-Rad Laboratories, Munich, Germany and Enhanced chemiluminescence reagents used for the immune-Western Blot was from Thermo Scientific, Rockford, USA. Non-fat dry milk was from Molico Nestlé, Vevey, Switzerland. Primary antibodies used in present study were purchased from Cell Signaling Technology, Danvers, USA (p21 and Cyclin D1) and Santa Cruz Biotechonology, Santa Cruz, USA (SIRT1 and secondary antibodies). The MnSOD was obtained from Stressgen, Michigan, USA. Other chemicals were from Merck, Darmstadt, Germany.

Cell culture and H_2O_2 cytotoxicity assay

WI-38 fetal lung human diploid fibroblasts were grown in complete medium composed by BME supplemented with 10% FBS at 37°C and 5% CO_2. Cells at early passages (below 30 passages) were used in cell experiments to avoid complications of replicative senescence. For the induction of premature senescence, subconfluent WI-38 cells were exposed to 75µM or 100µM H_2O_2 for 2 hours. The cells were washed twice

with prewarmed (37ºC), phosphate buffer solution to remove H_2O_2 and recultured in fresh complete medium. H_2O_2 cytotoxicity was measured immediately after stress using MTT assay. Briefly, cells were incubated with 1mg MTT/mL media for 3 hours at 37ºC and 5% CO_2. MTT solution was removed, and cells were lysed using 1M HCl in 2-propanol, and respective absorbance at 570nm was measured. To assess the effect of resveratrol in H_2O_2 treated cells media were supplemented with 0,2µM or 2µM resveratrol two passages before stress.

Cell proliferation assay

Cell proliferation was assessed by MTT assay as already described above. WI-38 cells (2×10^4cells/well) were seeded into wells of 24-well plates and cultured in complete medium 24h after stress. The MTT assay was preformed daily till 5 days after stress in order to evaluate cell growth.

Senescence-Associated beta-galactosidase (SA-β-gal) assay

The senescent status of cells was verified by *in situ* staining for SA-β-gal activity, performed 72h after stress as already described (Dimri *et al.* 1995). In brief, cells were fixed and incubated in SA-β-gal staining solution at 37ºC for 16h. Senescent cells were identified as blue stained cells by standard light microcopy, and a total of 400 cells were counted in random fields to determine the percentage of SA-β-gal positive cells.

Western blot analysis

WI-38 cells submitted to the different treatments were lysed on ice in a lysis buffer (50mM Tris, pH 7,4, 10mM NaCl, 5mM EDTA, 0,25% Triton X-100). The protein concentration of the cell extracts was quantified using Bradford assay (Bradford 1976). An equal amount of protein (20µg/lane) from each cell extract was loaded into 10%, 14% and 16% polyacrylamide gels, and was then submitted to SDS-PAGE. After electrophoresis, proteins were blotted onto a nitrocellulose membrane. The membranes were blocked either with 5% nonfat dry milk or 5% BSA in Tris buffered saline containing 0,1% Tween-20 (TBS-T). They were subsequently probed with specific primary antibodies at a predetermined optimal concentration, overnight at 4ºC. Following extensive washing with TBS-T, the immunoblots were then incubated with an appropriated peroxidase-conjugated secondary antibody for 1h at room temperature. After rinsing, peroxidase complex was detected using chemiluminescent substrate. Simultaneous detection of tubulin was used as the loading control. The semi-quantification of immunoreactive bands was performed using "Scion Image" software (Scion Corporation ®).

Statistical analysis

All analyses were performed using Microsoft Excel. Quantitative data were expressed as the mean value ± SEM, when at least three independent experiments were analyzed. The non-parametric Mann-Whitney U test was used for the comparative analysis between two groups. A p value <0,05 was considered statistically significant.

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Author contributions

Conceived and designed the experiments: LM and HA. Performed the experiments: FL-S and LM. Analyzed the data: FL-S, LM, HA. Contributed reagents/ materials/analysis tools: DN. Wrote the paper: FL-S, LM and HA.

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Figure legends

Figure 1 – Resveratrol addition to the media results in a small reduction of cell proliferation. Cell proliferation index was determined after cell viability quantification with MTT assay during 4 consecutive days, assuming that day 2=1. CTL condition corresponds to cells cultured in the absence of resveratrol. Each data point represents mean values ± SEM from three independent experiments. The proliferative index obtained for cells treated with 2µM resveratrol was significantly decreased comparing to CTL (Mann Whithey U test, $^{*}p<0,05$).

Figure 2– H_2O_2 exerts a considerable inhibitory effect on cell proliferation even in the presence of resveratrol. Cells grown without resveratrol **(A),** with 0,2µM **(B)** or 2µM resveratrol **(C)** were submitted to 0µM, 75µM or 100µM H_2O_2. BME condition represents cells treated with 0µM H_2O_2. Cell proliferation was assessed using MTT assay daily till 5 days after stress, assuming day 2=1. Each data point represents mean values ± SEM from three independent experiments. Both doses of H_2O_2 were able to cause a significant inhibition in cell proliferation comparing to BME, for all resveratrol concentrations tested (Mann Whithey U test, $^{*}p<0,05$).

Figure 3 – Resveratrol is able to attenuate H_2O_2 cytotoxic effect. Cytotoxicity was measured using MTT assay immediately after 2h of H_2O_2 challenge, assuming that BME (0µM H_2O_2)=100%. Each bar represents mean values ± SEM from three independent experiments. Both concentration of H_2O_2 greatly affects cell viability in the absence of resveratrol. When resveratrol is present such effect is attenuated. *p<0,05 comparing to the respective BME.

Figure 4 – 0,2µM resveratrol was able to thwart SA-beta-galactosidase (SA-β-gal) expression induced by H_2O_2. SA-β-gal activity was quantified 72h after H_2O_2 stress. Each bar represents mean values ± SEM from three independent experiments. H_2O_2 is able to increase SA-β-gal in the absence of resveratrol. The lower dose (0,2µM) of resveratrol attenuates such effect while 2µM is unable to do so. *$p<0,05$ comparing to BME as indicated in the plot. n.s. – not significantly different.

Figure 5 – SIRT1 descreases after exposure to H_2O_2 and is enhanced with resveratrol. Western blot of extracts from cells submitted to different conditions. Bands shown here are representative of two independent experiments.

Figure 6 – Resveratrol seems to increase SIRT1 expression and to attenuate H_2O_2 effect. Resveratrol influence on SIRT1 protein levels (A) presented as relative densitometry units, assuming that CTL (0µM resveratrol)=1. The effect of H_2O_2 in SIRT1 expression either in the presence or absence of resveratrol (B), assuming that BME (0µM H_2O_2)=1. Tubulin was used as loading control. Each bar represents mean values from two independent experiments.

Figure 7 – MnSOD, p21 and Cyclin D1 protein expression varies according to the different conditions. Extracts from cells submitted to different treatments were analysed by Western blotting. Bands shown here are representative of two or three independent experiments.

Figure 8 – Resveratrol enhances MnSOD expression and appears to blunt H_2O_2 effect. Resveratrol influence on MnSOD protein levels (A) presented as relative densitometry units, assuming that CTL (0µM resveratrol)=1. The effect of H_2O_2 in MnSOD expression either in the presence or absence of resveratrol (B), assuming that BME (0µM H_2O_2)=1. Tubulin was used as loading control. When three independent experiments were analysed, data are expressed as mean values ± SEM.

Figure 9 – Resveratrol augments p21 expression and seems to attenuate H_2O_2 effect. Resveratrol influence on p21 protein levels (A) presented as relative densitometry units, assuming that CTL (0µM resveratrol)=1. The effect of H_2O_2 in p21 expression either in the presence or absence of resveratrol (B), assuming that BME (0µM H_2O_2)=1. Tubulin was used as loading control. When three independent experiments were analysed, data are expressed as mean values ± SEM.

Figure 10 – Resveratrol seems to improve Cyclin D1 expression and to minimize H_2O_2 effect. Resveratrol influence on Cyclin D1 protein levels (A) presented as relative densitometry units, assuming that CTL (0µM resveratrol)=1. The effect of H_2O_2 in Cyclin D1 expression either in the presence or absence of resveratrol (B), assuming that BME (0µM H_2O_2)=1. Tubulin was used as loading control. Each bars represents mean values from to independent experiments.

Figures

Figure 1

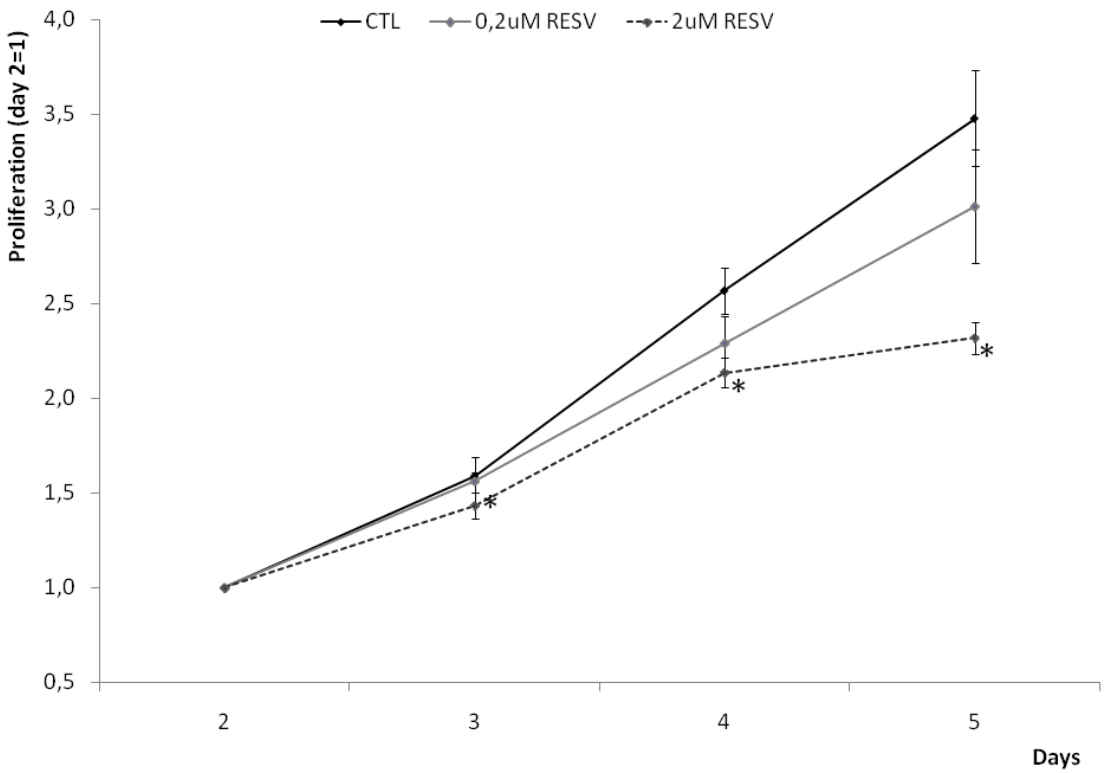
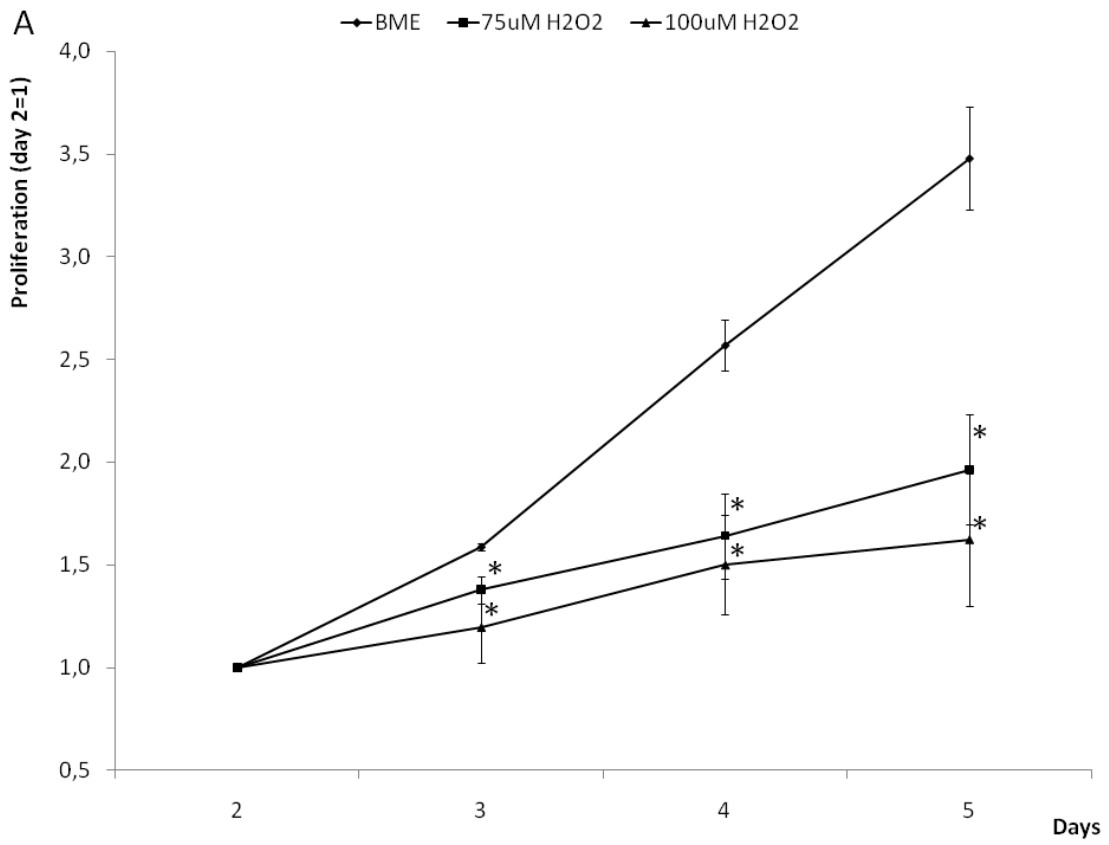
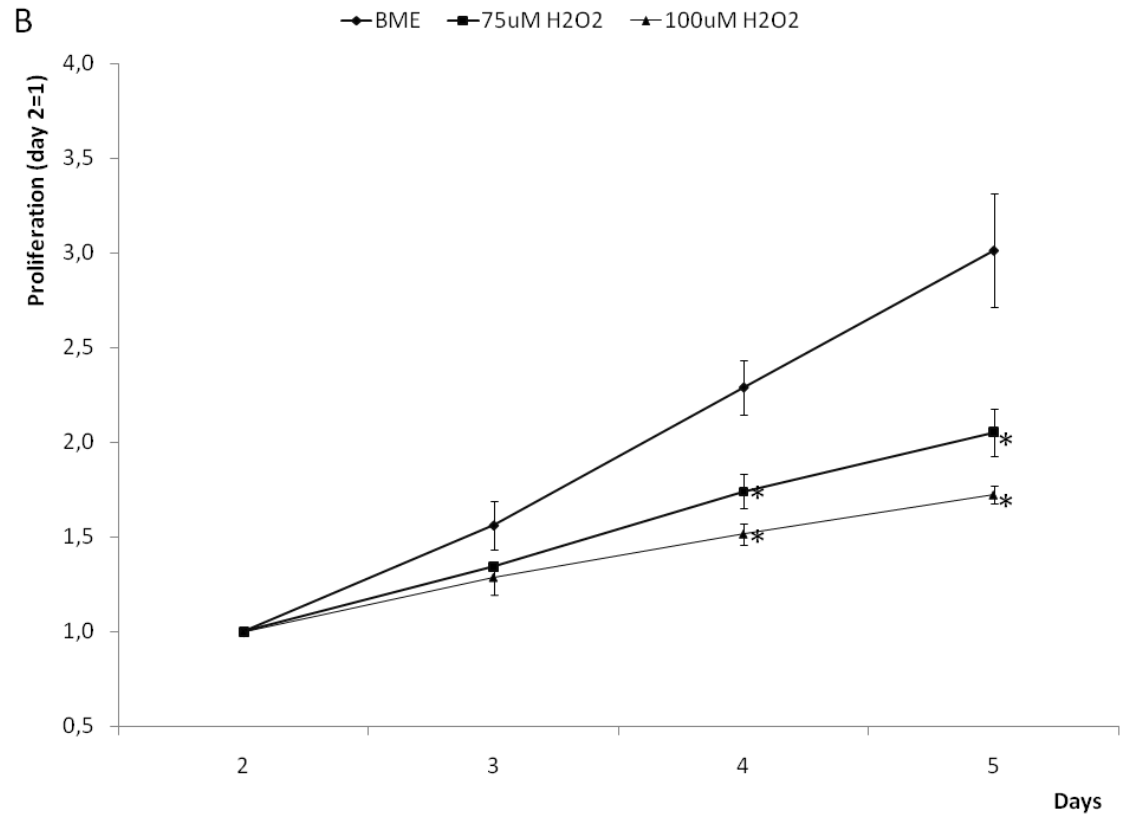


Figure 2


A
BME
75uM H2O2
100uM H2O2
Proliferation (day 2=1)
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
2
3
4
5
Days
*


B
BME
75uM H2O2
100uM H2O2
Proliferation (day 2=1)
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
2
3
4
5
Days
*

C

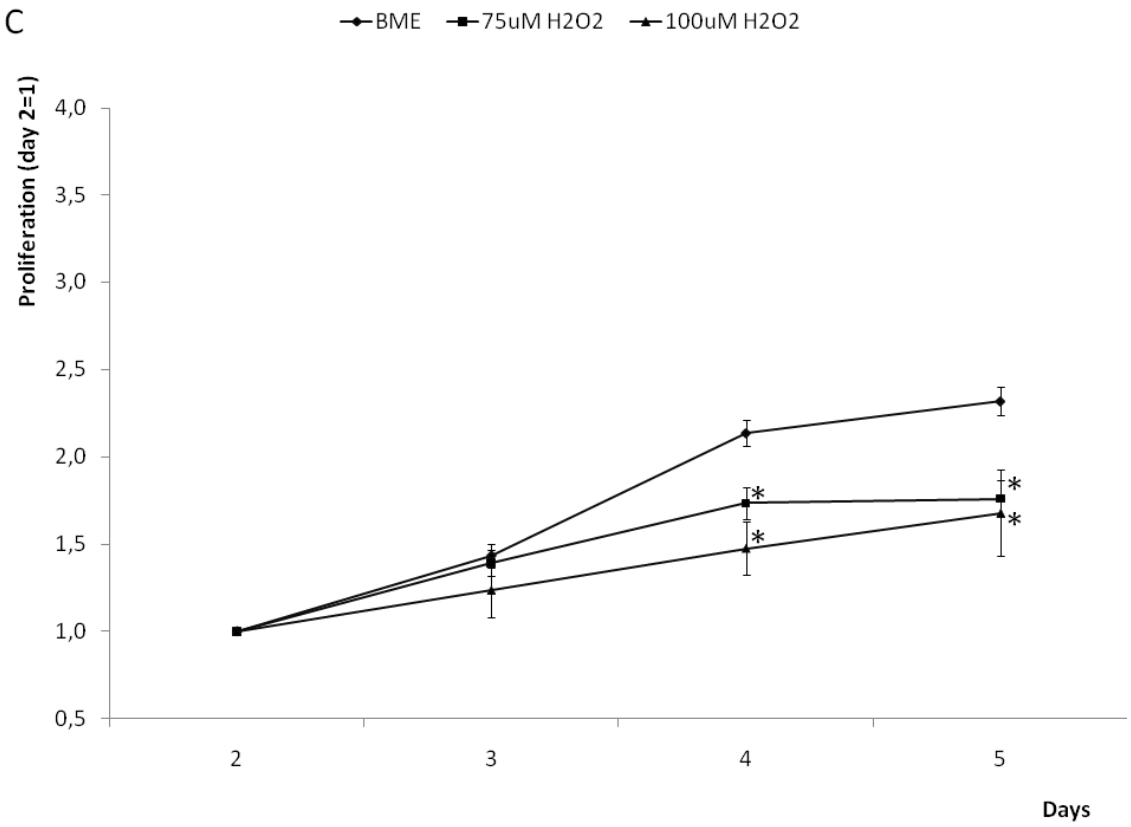

BME
75uM H2O2
100uM H2O2
Proliferation (day 2=1)
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
2
3
4
5
Days
*
*
*

Figure 3

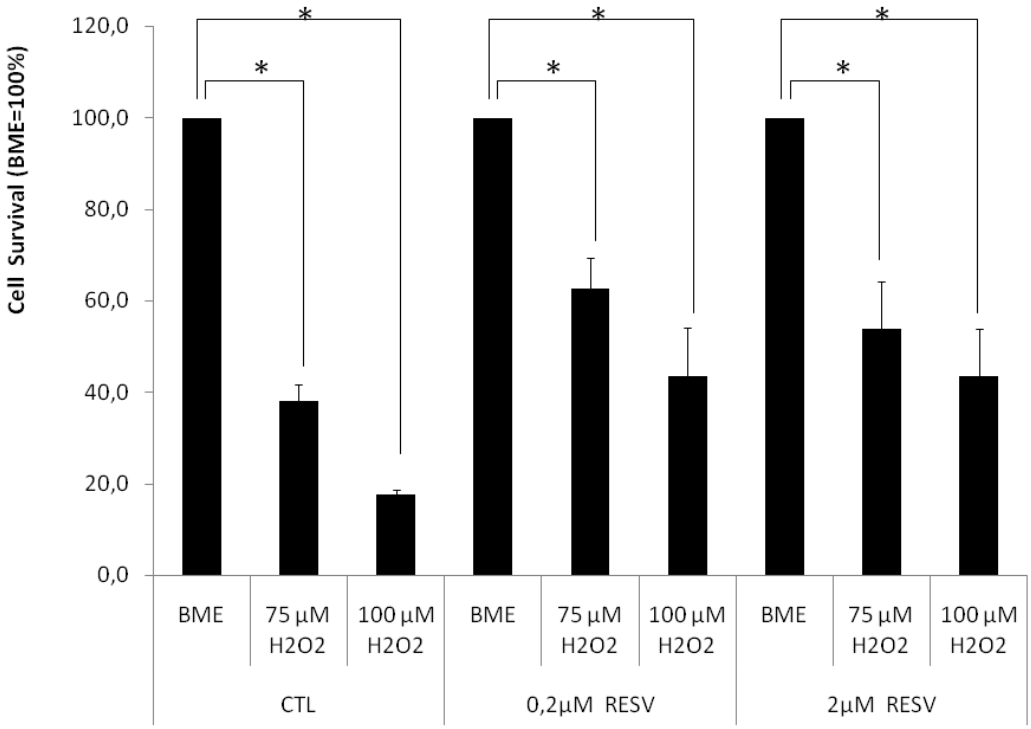

Cell Survival (BME=100%)
120,0
100,0
80,0
60,0
40,0
20,0
0,0
*
*
*
*
*
*
BME
75 µM H2O2
100 µM H2O2
BME
75 µM H2O2
100 µM H2O2
BME
75 µM H2O2
100 µM H2O2
CTL
0,2µM RESV
2µM RESV

Figure 4

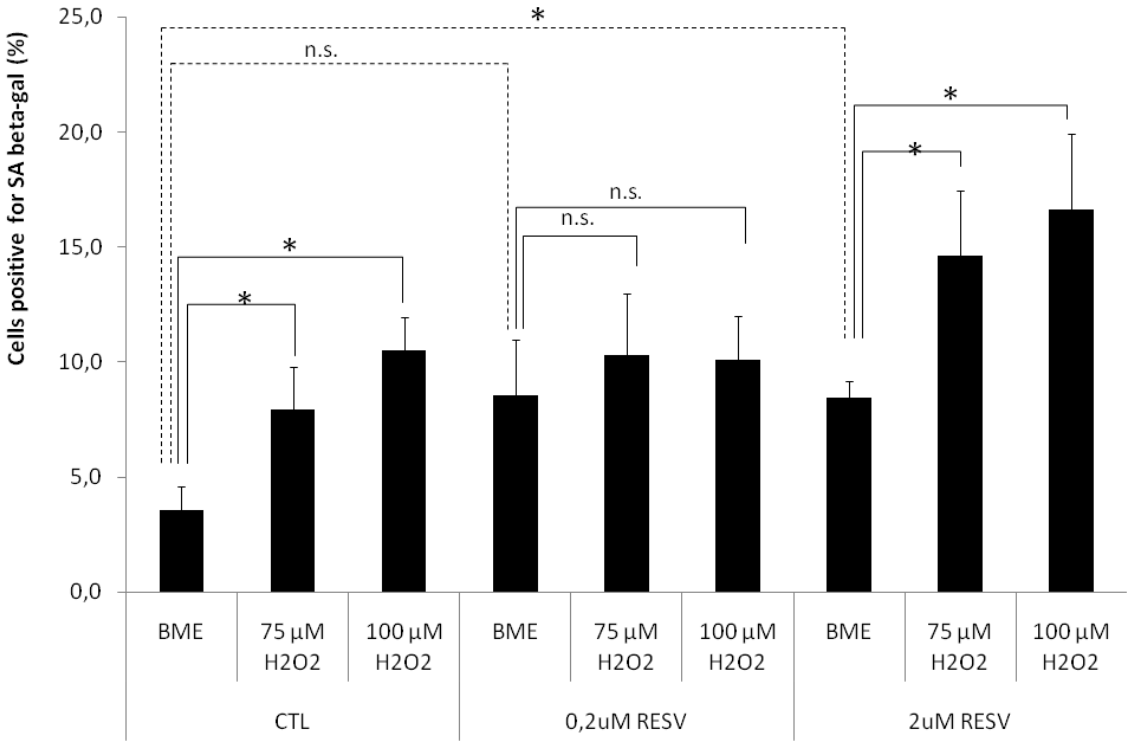

Cells positive for SA beta-gal (%)
25,0
20,0
15,0
10,0
5,0
0,0
*
n.s.
*
*
n.s.
n.s.
*
*
BME
75 µM H2O2
100 µM H2O2
BME
75 µM H2O2
100 µM H2O2
BME
75 µM H2O2
100 µM H2O2
CTL
0,2uM RESV
2uM RESV

Figure 5

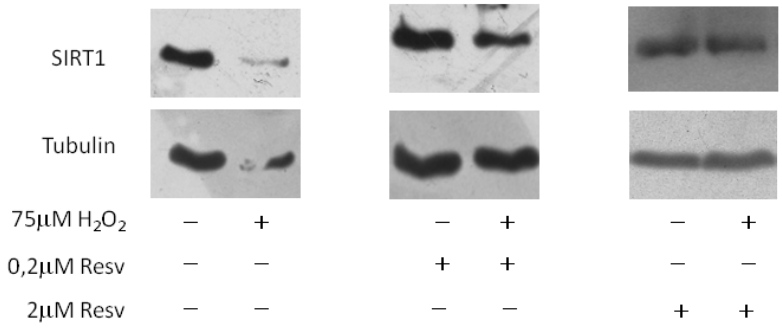


Figure 6

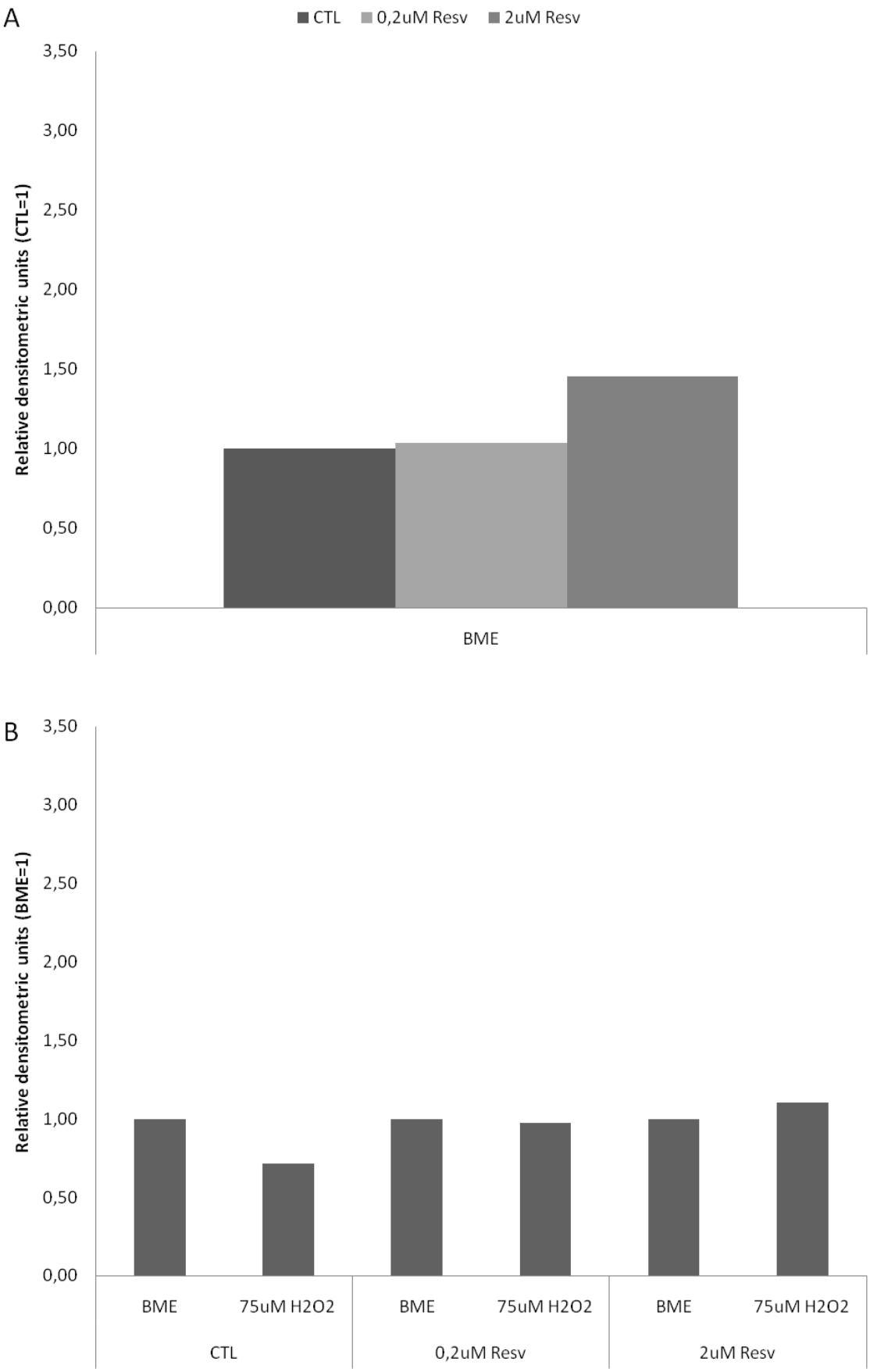

A
CTL
0,2uM Resv
2uM Resv
Relative densitometric units (CTL=1)
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
BME
B
Relative densitometric units (BME=1)
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
BME
75uM H2O2
BME
75uM H2O2
BME
75uM H2O2
CTL
0,2uM Resv
2uM Resv

Figure 7

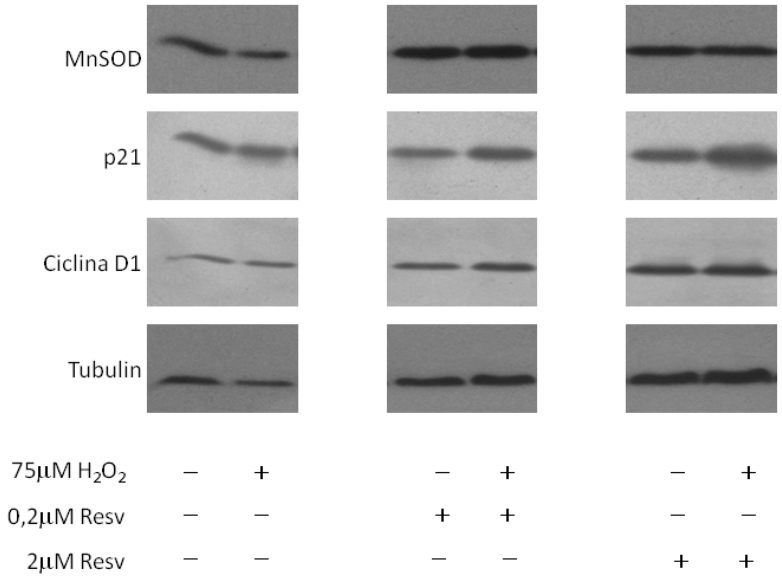


Figure 8

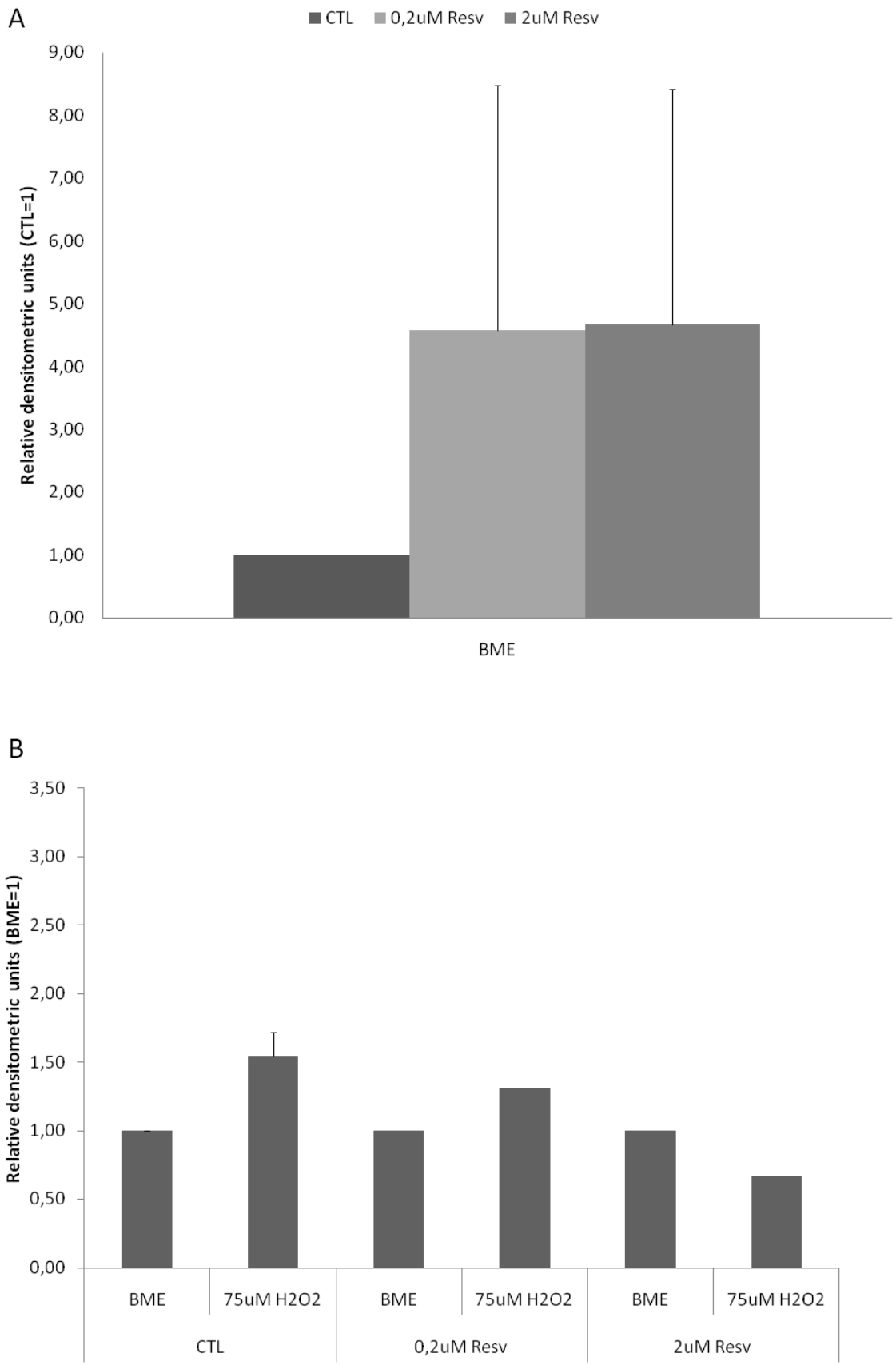
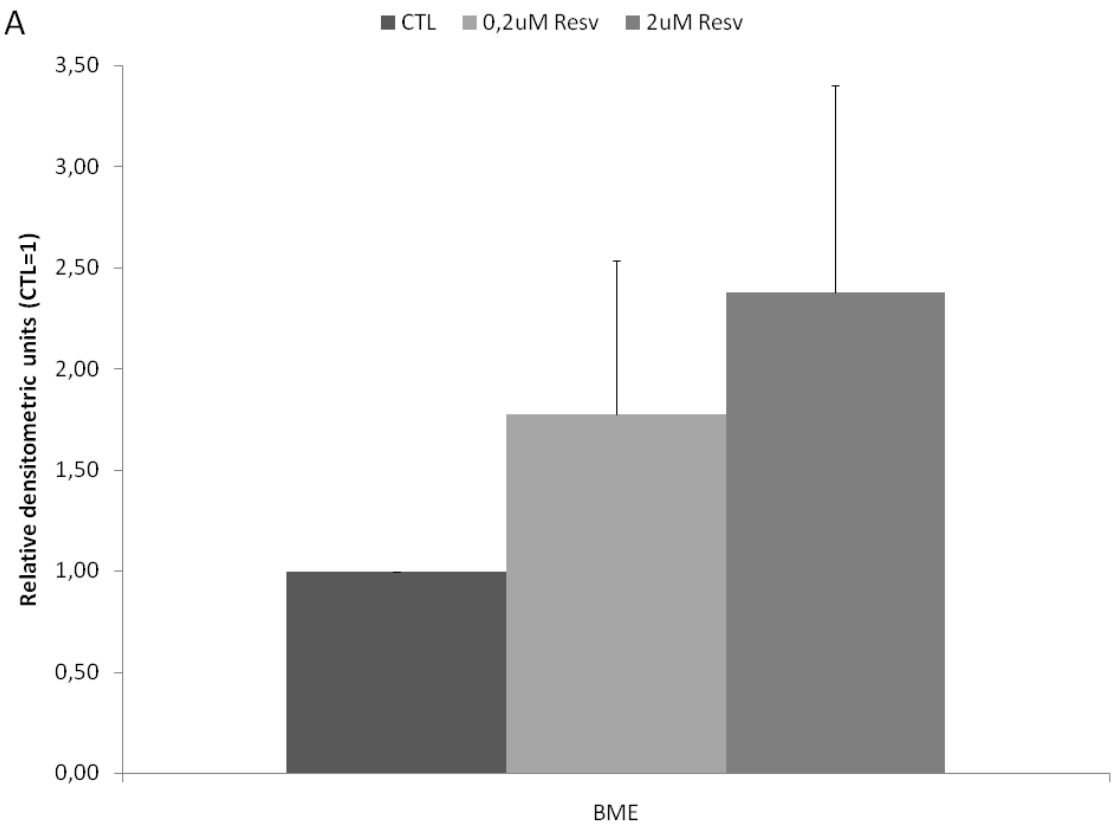

A
CTL
0,2uM Resv
2uM Resv
9,00
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
Relative densitometric units (CTL=1)
BME
B
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
Relative densitometric units (BME=1)
BME
75uM H2O2
BME
75uM H2O2
BME
75uM H2O2
CTL
0,2uM Resv
2uM Resv

Figure 9


A
CTL
0,2uM Resv
2uM Resv
Relative densitometric units (CTL=1)
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
BME

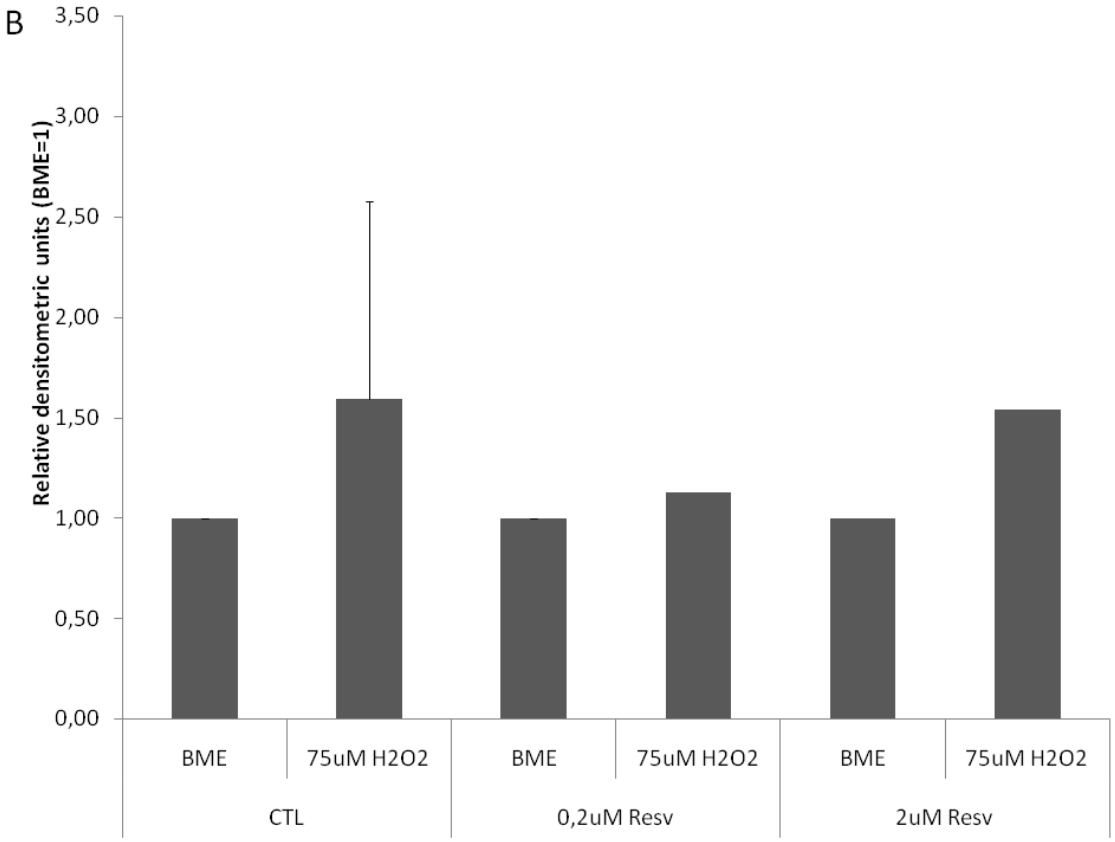
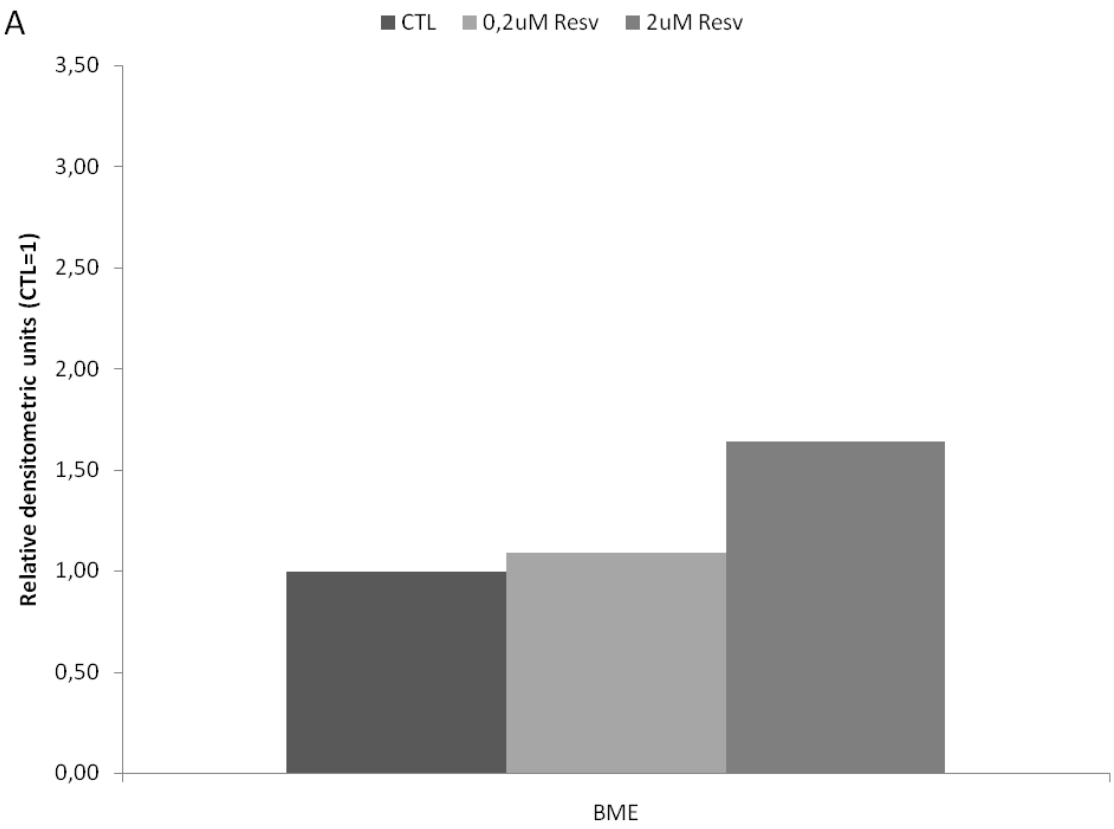
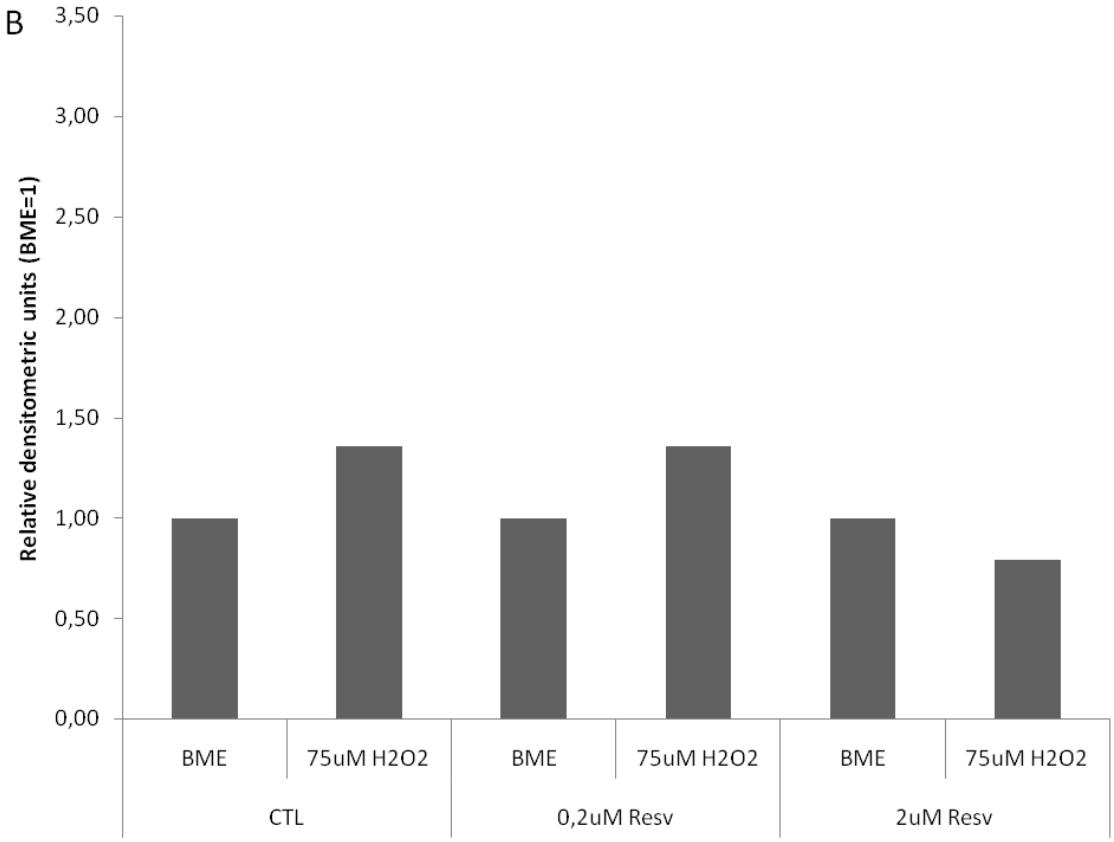

B
Relative densitometric units (BME=1)
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
BME
75uM H2O2
BME
75uM H2O2
BME
75uM H2O2
CTL
0,2uM Resv
2uM Resv

Figure 10


A
CTL
0,2uM Resv
2uM Resv
Relative densitometric units (CTL=1)
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
BME


B
Relative densitometric units (BME=1)
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
BME
75uM H2O2
BME
75uM H2O2
BME
75uM H2O2
CTL
0,2uM Resv
2uM Resv