UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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DISCIPLINA DE TRABALHO DE CONCLUSÃO DE CURSO DE FARMÁCIA

NFκB NA MODULAÇÃO REDOX DO CRESCIMENTO CELULAR EM CÂNCER DE PULMÃO DE NÃO-PEQUENAS CÉLULAS

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PORTO ALEGRE, JUNHO DE 2011.

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TRABALHO DE CONCLUSÃO DE CURSO - FARMÁCIA
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TITLE PAGE

Title: NFκB in the redox-modulation of cell growth in nonsmall cell lung cancer

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Running title: NFκB in redox-modulation of lung cancer growth

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Abreviations:

H_2O_2 – hydrogen peroxide;

CAT- catalase;

NSCLC- non-small cell lung cancer;

NFκB- nuclear factor kappa B;

AdC – adenocarcinoma;

LCC - large cell carcinoma;

RS - reactive species;

IL-1- interleukin-1;

TNF - tumour-necrosis factor;

SRB - sulforhodamine B;

TRAP - radical-trapping antioxidant potential;

SH- sulfhydryl groups;

GST - glutathione S-transferase

TBARS - tiobarbituric acid reactive species

Keywords: A549, catalase, NSCLC, tumor aggressiveness

ABSTRACT

Lung cancer is the most lethal malignant disease worldwide with limited efficacy of current therapeutics and dismal prognostic. Approximately 80% of the cases are non-small cell lung cancer (NSCLC). NFκB is a major transcription factor associated with tumor progression that responds to stressful stimuli, such as oxidative stress, influencing cell survival and chemoresistance. Since NSCLC aggressiveness is associated with higher intracellular oxidative stress, this study evaluated the involvement of NFκB in the redox modulation of tumor aggressiveness in the human NSCLC cell line A549. Treatment with the antioxidant enzyme catalase (CAT) (1000 U/mL) for 96h inhibited cell proliferation and when the enzyme was withdraw of the enzyme restored cell proliferation rates. In addition, catalase treatment decreased intracellular thiol levels (SH) and non-enzymatic antioxidant potential (TRAP). This redox modulation could be explained by the antioxidant contribution provided by high dose of exogenous CAT and was also reverted when the enzyme was withdraw. In agreement with the decreases in antioxidant defenses, the activation of the redox-sensitive transcription factor NFκB was decreased in catalase-treated cells as assessed by Western blotting for the nuclear content of the NFκB member p65. Taken together, data presented here suggest that decreases in the pro-oxidant status of lung cancer cells with catalase treatment can inhibit cell proliferation and activation of tumor-associated signaling pathways, providing a new therapeutic strategy for NSCLC therapy.

INTRODUCTION

Lung cancer remains the most common and lethal cancer-related pathology [1], with approximately 1.1 million deaths annually worldwide [2]. There are two main types of lung cancer with epithelial origin: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [2]. The latter comprises more than 80% of all cases of lung cancer [3] and is divided in three major histological subtypes: adenocarcinoma (AdC), large cell carcinoma (LCC) and squamous cell carcinoma [4]. Despite progress in molecular research, the therapeutic armamentarium remains poor [5] and unable to improve NSCLC patients outcome, which have a 5 year survival of only 15% [1]. All steps involved in malignant transformation are influenced by the redox metabolism [6,7] and previous studies demonstrated that NSCLC aggressiveness is associated with elevated intracellular oxidative stress. On the other hand, it was already demonstrated that exogenous treatment with the antioxidant enzyme catalase (CAT) (Motta et. al, unpublished data) and CAT overexpression attenuated tumor aggressiveness [8].

Free radicals and other reactive species (RS) are generated physiologically in every cells and its homeostasis is maintained by antioxidant systems [9]. Despite their harmful effects, RS can also enhance cellular proliferation, migration, differentiation, and survival [8,10]. Recently RS have been described as mediators of oncogenic phenotype activating transcription factors such as NFκB and AP-1 [6].

NFκB is a major transcription factor that modulates gene expression in response to growth factors, proinflammatory agents such as interleukin-1 (IL-1), tumor-necrosis factor (TNF), oxidants and cytotoxic drugs. [11-14]. The NFκB family contains five members, which form homo and heterodimers and exist in the cytoplasm in an inactive state bound to inhibitory protein IκB. The most common form of NFκB is the p65/p50 heterodimer that, when activated, translocates to the nucleus, where if activates transcription of target genes [15]. Substantial studies indicate that NFκB regulates oncogenesis and tumor progression since NFκB can induce cell proliferation, cell-cycle progression, angiogenesis, metastasis by enhancing the transcription of anti-apoptotic and

antioxidant genes including Bcl-xL c-FLIP, SOD2, glutathione S-transferase, and ferritin heavy chain [12,14,16-18].

This way, the present study aimed to investigate if NFκB could be involved in the cytostatic effect of CAT treatment in NSCLC. For this purpose, the human NSCLC cell line A549 was treated with exogenous CAT, and the proliferative behavior, intracellular redox status and p65 nuclear and cytosolic immunocontent were evaluated. It was found that CAT inhibits cell proliferation, reduces intracellular antioxidant capacity and decreases p65 nuclear immunocontent. Thus, redox modulation of NSCLC aggressiveness might rely on NFκB activation, and NFκB activation/inhibition could provide a future target to adjuvant therapeutics interventions.

MATHERIAL AND METHODS

Cell Lines and Cell Culture

Exponential growing human non-small cell lung cancer cell line A549, obtained from NCI-Frederick Cancer DCTD cell line repository, was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mg/L of amphotericine B and 50 mg/Lof garamicin, in a humidified atmosphere of 5% of CO_2 in air at 37°C.

CAT Treatment and Growth Inhibition Assays

The intracellular levels of H_2O_2 can be reduced with exogenous addition of CAT (E.C. 1.11.1.6), once H_2O_2 diffuses through membranes [19].Cells ($2x10^4$) were seeded in a 12 well plate and, after adherence, their redox state was modulated by addition of purified active or heat-inactivated CAT (1,000 U/mL). The cell number was evaluated each 24h for 120h of treatment using Neubauer chamber cell counting and Sulforhodamine B (SRB) assay [20]. In SRB assay, cells were fixed with cold 10% TCA for 1h at 4°C, washed with

distilled water and dried at room temperature. Then, cells were stained with 0.2% SRB (w/v) in 1% acetic acid (v/v) at room temperature for 20 min followed by five times washing with 1% acetic acid, before air-drying. Bound dye was solubilized with 10 mM Tris-base solution (pH 10.5) and plates were left on a plate shaker for 10 min. Absorbance was measured at 490 nm. Data is expressed as "cell number" with a standard curve (cell number X SRB_{abs}). To revert CAT effects in A549 cells, the enzyme was washed out after 48h of treatment.

Samples Preparation for Redox Profile Analysis

Cells (2×10^4) were cultivated in 12 well plates, washed with PBS and frozen-and-thawed in 10 mM PBS three times prior to harvesting and then centrifuged (400*g* /6 min). The supernatant protein concentration was determined by Lowry's [21] for data normalization.

Redox Parameters

The total antioxidant capacity of cells was evaluated by total radical-trapping antioxidant potential (TRAP) assay. This assay is based on luminol-chemoluminescence measurement induced by ABAP (2,2'-Azo-bis(2-amidinopropane) in glycine buffer (pH 8.6) [22]. After system stabilization, sample was added (20 µg of protein) and the chemoluminescence decreases proportionally to the amount of non-enzymatic antioxidants monitored (Wallace 1450 MicroBetaTriLux Liquid Scintillation Counter & Luminometer, Perkin Elmer). A time per chemoluminescence curve was obtained and the relative "area under the curve" (AUC) in the recovery phase used to analysis [22]. Sulfhydryl groups (-SH) level was measured as an indicative of the intracellular redox state, as previously described [23]. Samples (35 µg) were diluted in PBS 10 mM and buffer (10 mM boric acid, 0.2 mM EDTA pH 8.5). DTNB 10 mM (5,

5'-dithionitrobis 2-nitrobenzoic acid) was added and, after 60 min, it was read at 412 nm. Results are expressed as nmol SH/mg protein.

Cytoplasmic and Nuclear fractions

For extraction of cytoplasmic and nuclear fractions, cells were scraped, harvested and collected by centrifugation (4,000 rpm/ 4 min). The cells were then lysed by resuspension in hypotonic buffer (10mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, proteases inhibitor cocktail (Roche®), 1 mM sodium orthovanadate, 5 mM sodium fluoride) and incubated on ice for 15 min. After, 10% IGEPAL® was added and cells were disrupted by vortexing (every 15 s for 5 min). The resulting suspension was centrifuged at (14,000 rpm/30 seg), and the supernatant (cytoplasmic extract) was separated and stored at -80°C. The nuclear pellet was washed with cytoplasmic extraction buffer and then resuspended in high salt buffer consisting 20 mM HEPES (pH 7.9), 400 mM NaCl, 1.5 mM $MgCl_2$, 0.25 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM sodium orthovanadate, proteases inhibitor cocktail (Roche®), and 25% glycerol and incubated for 40 min in ice and vortexed for 15 s every 5 min to releasing soluble proteins from the nuclei. After, the nuclear fraction was centrifuged (12,000*g*/ 10 min) and supernatant containing soluble nuclear proteins was stored at -80°C until experiments. The protein concentration was determined by Bradford assay [24].

Western Blot

Cytoplasmic and nuclear proteins extracts were separated by sodium dodecyl sulfate-polyacrylamide gel eletrophoresis (SDS-PAGE) and then transferred onto nitrocelulose membrane. Thereafter, nonspecific binding was blocked with 5% of BSA in TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20 - pH 7.6) for 1h at room temperature. Membranes were than incubated overnight at 4°C with primary antibodies anti-p65 (1:1000) from Santa Cruz® or

anti-β-actin (1:1000) from Cell Signaling®. After washing in TBS-T, blots were incubated with horseradish peroxidase-linked anti-immunoglobulin G (IgG) secondary antibodies for 2h at room temperature. Bands were visualized with Super Signal West Pico Chemislumiscense Substrate from PIERCE® (Rockford, II,USA) and quantified using Image-J® software.

Statistical analysis

Data are expressed as means ± S.E.M. (standard error of mean) of at least 3 independent experiments in triplicate with significance level considered $p < 0.05$. Statistical analysis and graphs were obtained with GraphPad Software Inc., San Diego, CA, USA version 5.0.

RESULTS

NSCLC Growth Inhibition by CAT

Exogenous addition of catalase caused inhibition of cellular proliferation in human NSCLC A549 cells (Fig. 1). This effect was observed in 24h of treatment and maintained over 120h of treatment. This growth inhibition was related to a cytostatic, not cytotoxic, effect of intracellular H_2O_2 consumption, since CAT washout readily restored cellular proliferative rate similar to control A549 cells (Fig.1). Based on the abovementioned data, we used 48 and 96h of treatment for the following experiments.

CAT influence in NSCLC intracellular redox state

CAT-treated cells presented lower antioxidant potential and thiol levels when compared to untreated cells (Fig. 2). Notably, the decrease in antioxidant

potential was time dependent for both groups, while thiol levels were not. CAT washout was able to restore both thiol and antioxidant potential levels.

NFκB Activation

Time course experiments showed that CAT treatment for 48h caused a decrease in tumor cell proliferation, decreased intracellular antioxidant capacity and thiol levels. When we evaluated NFκB activation status (p65 subcellular compartmentalization/distribution), we found decreases in the nuclear immunocontent of the NFκB subunit p65, suggesting a decreased in NFκB activation in CAT-treated A549 cells (Fig. 3).

DISCUSSION

Among malignancies, lung cancer ranks as one of the most common and lethal. Although chemotherapy presents efficacy for some patients with lung cancer, effective therapeutic options for advanced stages of the disease remain limited and cure rates are low [25]. Extensive evidence has shown that cancer cells have an imbalance in redox parameters compared to normal cells, which are related to carcinogenesis and tumor progression [26].

CAT is one of the most effective antioxidant enzymes and is very efficiently to promote the conversion of hydrogen peroxide to water and molecular oxygen [6]. Controversial results have been reported regarding the involvement of H_2O_2 in cell proliferation [8]. Thus, we treated A549 cells with CAT and, once the H_2O_2-scavenger caused inhibition of cellular proliferation, H_2O_2 was confirmed as a necessary mediator of A549 proliferation. CAT washout readily restored cellular proliferative rate compared to control cells. Therefore, this was related to a cytostatic, not cytotoxic effect of intracellular H_2O_2 consumption. The difference in cellular proliferation was statistically significant at 48h and 96h of treatment. Thus, we analyzed the intracellular thiol levels and antioxidant potential in these incubation times to address potential relationship between cellular proliferative rates with intracellular redox

state. Once CAT treatment was effective in inhibit cellular proliferation, we aimed to determine if this cytostatic effect was related to changes in intracellular antioxidant defenses. Our results demonstrated that CAT treatment significantly decreased endogenous antioxidant potential.

Since H_2O_2 diffuses through membranes [19], the addition of CAT may be generating a gradient of H_2O_2 out of the cell. Our data suggest that in response to a reduction in intracellular H_2O_2 concentration, cells decreased their endogenous production of antioxidant defense. Here, catalase washout also restores the redox state. This could explain the cytostatic effect of CAT, once cellular proliferation can be influenced by alterations in intracellular redox environment through modulation of cell survival signals mediated by redox-sensitive transcription factors [6].

Reactive species are predominantly known for causing cell damage. However cumulative information has related increases in basal levels of RS in cancer cells with tumor progression [8], since RS play a major physiological role in the control of redox-sensitive signaling pathways as NFκB. However, the mechanism for activation of NFκB by reactive species is not yet clear [6]. We observed a decrease in nuclear immunocontent of the NFκB subunit p65 in CAT-treated cells. This reduction in NFκB activation status in CAT-treated cells could be related to the global decrease in antioxidant capacity found in this study, since the expression of several antioxidant genes are regulated by this transcription factor. In this way, the evaluation of Bcl-xL, SOD2 and GST immunocontent, and specifically the levels of some oxidative stress markers (*e.g.*: TBARS, carbonyl groups), in CAT-treated cells could confirm this hypothesis. Therefore, as important as the demonstration that CAT treatment was effective in inhibit cellular proliferation and CAT washout restored the proliferation rates, this protocol can be used as a model to assess the role of RS in chemotherapy efficacy, analyzing whether pre-treatment or co-treatment of CAT with well known cytotoxic drugs (*e.g.*:cisplatin, carboplatin, VP-16, vimblastine, taxol,…) is able to increase the effectiveness of chemotherapy.

Also, this redox modulation of NSCLC aggressiveness might rely on NFκB activation. Thus, understanding the relationship between the activity of NFκB and the intracellular redox environment can provide us potential targets

for future research and further development of new therapies based on redox modulation of cell signaling pathways. Ongoing studies in our laboratory are evaluating these hypotheses.

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FIGURES LEGEND

Figure 1: CAT treatment causes reversible growth inhibition in human NSCLC cell line A549. (A) Exogenous CAT addition (1000 U/mL) to A549 cell culture inhibited cellular proliferation when compared to control. Growth inhibition is reverted by catalase washout after 48h. Cell number was quantified by SRB assay as described in *Material and Methods* section. (B) Representative phase contrast images of growth inhibition assay (10X magnification). Data are presented as mean ± S.E.M. **P* < 0.05 compared with control using t-test.

Figure 2: CAT treatment decreased intracellular antioxidant capacity in human NSCLC cell line A549. (A) Thiol levels and (B) total antioxidant potential were significantly lower in CAT-treated cells. Both parameters were reverted by CAT washout after 48h. Two-way ANOVA showed that antioxidant potential was affected by time of incubation. (C) Representative TRAP assay demonstrating reduction of luminol oxidation by peroxyl radicals (chemoluminescence emission) after sample addition. The area under the curve (AUC) in the recovery phase (demarked by vertical dotted lines) is used in statistical analysis. Data are presented as mean ± S.E.M. **P* < 0.05 using two-way ANOVA and tukey post-test among groups.

Figure 3: CAT treatment reduces p65 nuclear immunocontent in human NSCLC cell line A549. Densitometric analysis of p65 subunit immunocontent and representative bands of (A) cytosolic and (B) nuclear fraction. Data are presented as mean ± S.E.M.. **P* < 0.05 compared with control using t-test.

Figure 1

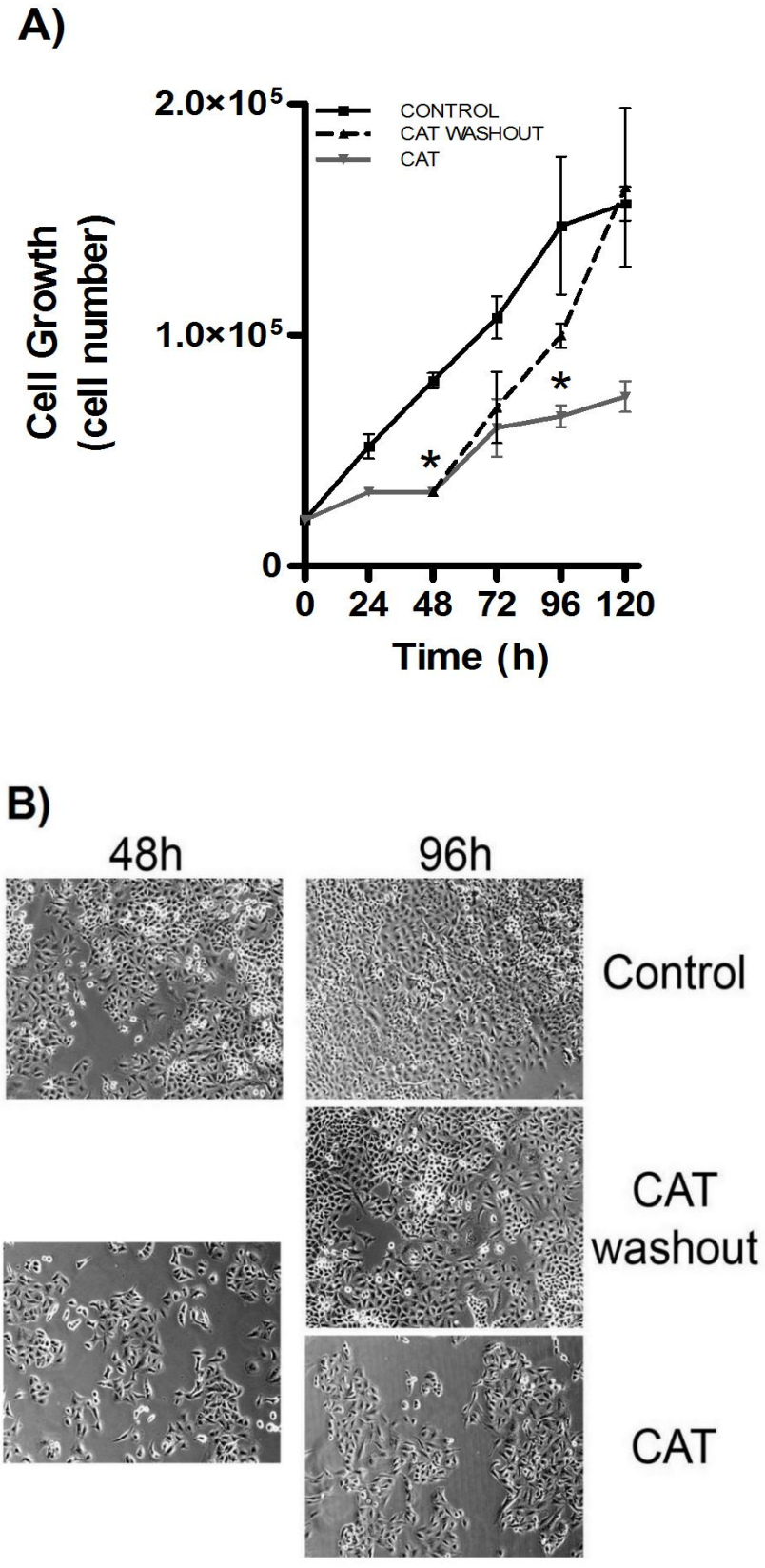
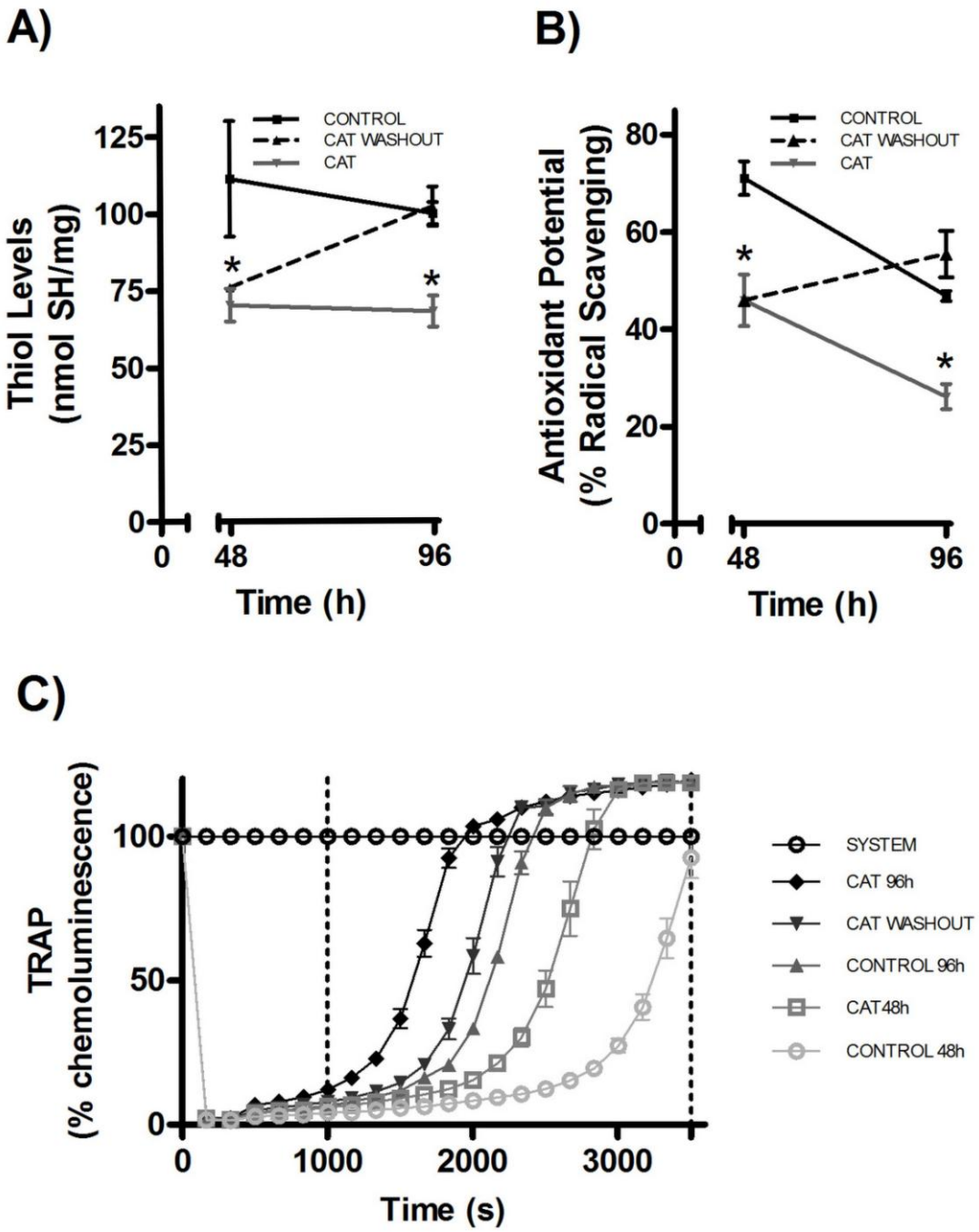

A)
2.0×10⁵
1.0×10⁵
0
Cell Growth
(cell number)
CONTROL
CAT WASHOUT
CAT
*
*
0 24 48 72 96 120
Time (h)
B)
48h
96h
Control
CAT
washout
CAT

Figure 2


A)
Thiol Levels (nmol SH/mg)
125
100
75
50
25
0
0
48
96
Time (h)
CONTROL
CAT WASHOUT
CAT
*
*
B)
Antioxidant Potential (% Radical Scavenging)
80
60
40
20
0
0
48
96
Time (h)
CONTROL
CAT WASHOUT
CAT
*
*
C)
TRAP (% chemoluminescence)
100
50
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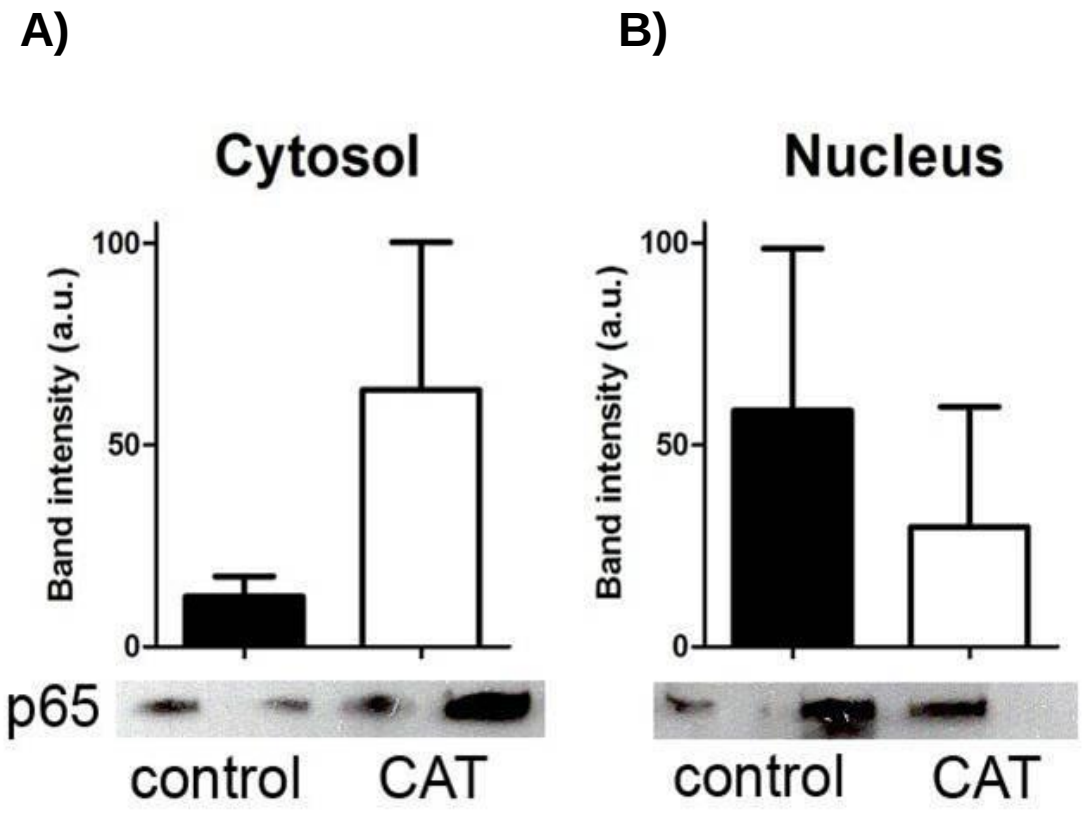
1000
2000
3000
Time (s)
SYSTEM
CAT 96h
CAT WASHOUT
CONTROL 96h
CAT48h
CONTROL 48h

Figure 3


A)
B)
Cytosol
Nucleus
Band intensity (a.u.)
100
50
0
p65
control
CAT
control
CAT

ANEXO 1: NORMAS DO PERIÓDICO MOLECULAR CARCINOGESIS

GUIDELINESOF JOURNAL MOLECULAR CARCINOGESIS

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1. Sherman ML, Shafman TD, Kufe DW. Modulation of cyclic AMP levels and differentiation by adenosine analogs in mouse erythroleukemia cells. J Cell Physiol 1988;134:429–436.

Book chapter

2. Gilmore ML, Rouse ST, Heilman CJ, Nash, NR, Levey AI. Receptor fusion proteins and analysis. In: Ariano MA, editor. Receptor localization. New York: Wiley-Liss; 1998. p 75–90.

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3. Voet D, Voet JG. Biochemistry: text book for students. New York: John Wiley & Sons, Inc.; 1990. 1223 p.

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