UNIVERSIDADE ESTADUAL DE CAMPINAS

Claudia Lumy Yano

ESTUDO DOS EFEITOS CITOTÓXICOS E DO ESTRESSE OXIDATIVO INDUZIDO PELO CLORETO DE CÁDMIO ASSOCIADO OU NÃO AO SULFATO DE ZINCO EM CÉLULAS MUSCULARES ESQUELÉTICAS E NEOPLÁSICAS.

Tese apresentada ao Instituto de Biologia para a obtenção de Título de Doutor em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientadora: Profa. Dra. Maria Cristina Cintra Gomes Marcondes

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BANCA EXAMINADORA

Profa. Dra. Maria Cristina Cintra Gomes Marcondes (Orientadora) _____
Assinatura

Prof. Dr. Antonio Carlos Boschero _____
Assinatura

Prof. Dr. Hernandes Faustino de Carvalho _____
Assinatura

Prof. Dr. Miguel Arcanjo Areas _____
Assinatura

Prof. Dr. Stephen Hyslop _____
Assinatura

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Assinatura

Prof. Dr. Paulo Pinto Joazeiro _____
Assinatura

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"Um trabalho de pesquisa não se faz só. Apesar de muitas vezes se encontrar... é preciso uma boa retaguarda e perseverança".

Índice

I- RESUMO

Metais pesados como o cádmio são considerados agentes tóxicos devido sua extensiva utilização nas indústrias e agropecuária e, como conseqüência, são amplamente dispersados no meio ambiente. No entanto, o cádmio tem sido foco, também, de inúmeras pesquisas relacionadas a exposição humana e suas conseqüências patológicas como o câncer. Estudos, claramente, caracterizam as relações de tumor de pulmão com a inalação do cádmio e mostram a possível participação deste metal tanto na iniciação quanto na progressão tumoral. Por outro lado, são raros os relatos da literatura envolvendo o mecanismo de ação do cádmio em tecido muscular, uma vez que já foi observado acúmulo desse metal em musculatura esquelética de animais. A administração do cloreto de cádmio, metal pesado designado como carcinogênico, em linhagem de células musculares esqueléticas C_2C_{12} promoveu lesões consistentes com estresse oxidativo, observado pela diminuição da viabilidade celular, aumento da peroxidação de lipídios (conteúdo de malondialdeído) e conseqüente diminuição da enzima antioxidante glutationa transferase (GST). O estresse oxidativo, possivelmente, alterou a adesão celular e, conseqüentemente, houve retração dos miotúbulos, observada através de microscopia de luz e microscopia eletrônica de varredura (Capítulo I- Trabalho publicado no periódico *Free Radical Biology & Medicine, 2005*). A atenuação das lesões promovidas pelo cloreto de cádmio em linhagem de células C_2C_{12} foi verificada com o pré-tratamento com o sulfato de zinco antecedendo o tratamento com cloreto de cádmio. Os efeitos protetores foram observados através da preservação da viabilidade celular, da GST, e diminuição do conteúdo de malondialdeído. A ação protetora foi verificada, também, na maior preservação da adesão celular, principalmente, contra as maiores concentrações de cádmio (Capítulo II- Trabalho a ser submetido ao periódico *Free Radical Biology & Medicine*). Por outro lado, a exposição crônica de células tumorais, linhagem de adenocarcinoma de cólon MAC13, ao cloreto de cádmio promoveu alterações morfológicas associadas ao aumento da atividade mitocondrial, interferência quanto à atividade lisossomal e diminuição da viabilidade celular, principalmente, na maior concentração de cádmio, após 24hs de exposição (Capítulo III- Trabalho a ser submetido ao periódico *International Journal of Cancer).*

ABSTRACT

The heavy metals as cadmium are a toxic agent since it is extensively utilized in industry and can be amply distributed in environment. The cadmium is research focused as its pathological consequences in human exposure as it has been classified as carcinogenic agent. This fact is evident since the cadmium inhalation can be related to lung tumour and many studies show the possible participation of the cadmium on tumoral cells initiation and progression. However, few studies observed that cadmium can be accumulated in animal skeletal muscle cells and its action mechanisms are not completed known. The cadmium chloride exposure promoted oxidative stress and morphologic changes in C_2C_{12} myotubes cell, *in vitro*, associated to decrease on cellular viability, high lipid peroxidation (increase on malondialdehyde content, MDA) and decrease on glutathione-S-transferase (GST) activity. The cadmium chloride produced chances on the cellular adhesion, integrity and retraction in C_2C_{12} myotubes cells. These effects could be attenuated by zinc sulphate pre-treatment, which maintained the cellular viability, GST activity, reducing the MDA content. The zinc sulphate pre-treatment preserved the cellular adhesion, especially in high cadmium chloride concentration. Additionally, the tumoral cells (colon adenocarcinoma MAC 13) chronically exposed to cadmium chloride showed increase on the mitochondrial activity, and reduction on lysosomal and cellular viability, especially at high cadmium chloride concentration after 24h of treatment, probably indicating the tumoral cell changes.

Introdução Geral

Nas últimas décadas, estudos na área molecular sobre o câncer foram intensos e realizados para a maior compreensão sobre alvos que identificam tanto eventos precoces, como tardios, do processo carcinogênico, permitindo, deste modo, o desenvolvimento de novas e efetivas terapias (Bertram, 2001).

O processo pelo qual uma célula normal começa a se transformar em malígna é bem conhecido. Requer aquisições seqüenciais de mutações, que chegam como conseqüência de danos ao genoma. Estes danos podem ser o resultado de processos endógenos tais como erro na replicação do DNA, da instabilidade química intrínseca de certas bases de DNA, ou a partir do ataque de radicais livres gerados durante o metabolismo. Danos ao DNA podem, também, resultar da interação com agentes exógenos tais como radiação ionizante, radiação UV e carcinógenos químicos (Bertram, 2001). Paralelamente, o estresse oxidativo tem sido relacionado à patogênese de várias doenças degenerativas, incluindo o próprio câncer (Jones, 1985; Kappus, 1985; Sies, 1985). Sabe-se que baixos níveis de oxidantes podem modificar proteínas de sinalização celular, ocasionando alterações funcionais. Estas proteínas são, também, alvos importantes de antioxidantes quimiopreventivos, que bloqueiam a sinalização induzida por oxidantes e, dentre as funções desses agentes antioxidantes, inibem as respostas celulares dependentes de proteína kinase C (PKC). A proteína kinase C pode ser ativada por estresse oxidativo que, por sua vez, regula vários processos celulares incluindo mitose, adesão celular, apoptose, angiogênese, invasão e metástase (Gopalakrishma & Jaken, 2000).

O câncer é foco de inúmeras pesquisas relacionadas às exposições a agentes tóxicos e metais pesados, como o cádmio; este, por sua vez, possui ampla distribuição e extensiva utilização nas indústrias e agropecuaria. Estudos mostram a possível participação do cádmio tanto na iniciação como na progressão de tumor (Pearson & Prozialek, 2000; Waalkes et al., 1992). Embora o mecanismo carcinogênico do cádmio não esteja bem definido, evidências *in vitro* mostram o potencial do cádmio quanto à progressão de células tumorais (Waalkes et al., 2000; Olabarrieta et al., 2001) e evidências experimentais sugerem que a tolerância à toxicidade ao cádmio está relacionada à participação da metalotionina (MT) que promove o seqüestro dos íons cádmio, quando em baixas concentrações (Klaassen, et al., 1999). Outras hipóteses sobre o efeito carcinogênico do cádmio incluem a ação direta com a cromatina,

promovendo quebras, *crosslinks*, e alterações estruturais do DNA, ou forma indireta através do desequilíbrio do sistema antioxidante e conseqüente aumento do peróxido de hidrogênio (H_2O_2). O aumento de H_2O_2 resultaria na catálise de reação de oxi-redução dos íons ferro/cobre, aumentando os níveis de radicais livres interferindo na sinalização de moléculas, indução da expressão gênica e apoptose (Hatcher et al. 1995; Hassoun & Stohs, 1996; Hussain et al., 1987; Manca et al., 1991).

No organismo, metais como o zinco, cobre, cálcio, crômio e ferro são considerados metais essenciais relacionados às várias funções moleculares. O zinco (Zn) exibe propriedades anti-apoptóticas, através da atuação com enzimas do metabolismo do DNA e fatores de transcrição potencialmente ativados na apoptose (Valee & Auld, 1990; Hainaut & Milner, 1993; Wellinghausen et al., 1997). A possível propriedade antioxidante do zinco, também, estaria envolvida na interferência da via apoptótica, atuando sobre as espécies reativas de oxigênio (EROs) que são mediadores apoptóticos (Bray & Better, 1990; Szuster-Ciesielska et al.,1999). Estudos relatam que a suplementação com zinco, tanto *in vivo* como *in vitro*, previne a apoptose induzida pelo cádmio (Chai et al., 1999).

Assim a avaliação dos mecanismos envolvidos em processos carcinogênicos são de extrema importância para o melhor conhecimento dos prognósticos de patologias como o câncer e, também, dos tratamentos a serem utilizados.

Objetivos

O presente trabalho de pesquisa teve como objetivo avaliar o possível mecanismo de toxicidade e o efeito carcinogênico do cádmio em linhagem de célula muscular esquelética (C_2C_{12}), a participação desse metal pesado sobre possíveis alterações do estresse oxidativo, avaliando-se as alterações citotóxicas e morfológicas *in vitro* (Capitulo I), e os efeitos da utilização do sulfato de zinco em relação aos efeitos antioxidantes e possível atenuação dos efeitos oxidativos, produzidos pelo cloreto de cádmio em linhagem de células muscular esquelética (C_2C_{12}) (Capitulo II).

Foi, também, avaliado o efeito do cádmio em células tumorais MAC 13, considerando o tumor pré-estabelecido (MAC13) e possíveis alterações morfológicas nessas células (Capitulo III).

Capítulo I

"Estresse oxidativo induzido por cloreto de cádmio em células musculares *in vitro***"**

Trabalho publicado no periódico *Free Radical Biology & Medicine* (**FRBM 39: 1378-1384, 2005.)**

Os efeitos do cloreto de cádmio ($CdCl_2$) sobre o estresse oxidativo em linhagem de células do músculo esquelético C_2C_{12} foram analisados. Mioblastos foram diferenciados em miotúbulos e tratados com $CdCl_2$ (1, 3, 5, 7.5, 10, e 12.5 μM) pelos períodos de 24, 48, e 72 h. Células homogenizadas foram utilizadas para os ensaios de MTT (3-(4,5-*dimethylthiozol*-2-yl)-2,5-*diphenyltetrazolium bromide*) vermelho neutro e conteúdo de ácido nucléico. Citotoxicidade do Cd em células C_2C_{12} ocorreu de maneira concentração dependente. A atividade da GST (nmol μg de proteína^{-1} min^{-1}) foi aumentada em 1 e 3 μM $CdCl_2$ (36,9 ± 5,6 e 32,1 ± 6,0, respectivamente) comparado a células controle (21,8 ± 1,5), mas diminuída nas maiores concentração (7,5 μM = 15,9 ± 3,3, 10 μM = 15,9 ± 4,6, e 12,5 μM = 10,5 ± 2,8). Aumento do conteúdo de malondialdeído (nmol μg de proteína^{-1} min^{-1}, observado principalmente em alta concentração $CdCl_2$ (controle = 7,3 ± 0,5; $CdCl_2$: 7,5 ± μM =11,2 ± 3,1; 10 μM = 14,6 ± 3,8 e 12,5 μM = 20,5 ± 6,5) mostra aumento da peroxidação de lipídios. Análises morfológicas de microscopia de luz e microscopia eletrônica de varredura mostraram perda concentração dependente da adesão celular e formação de vesículas indicativas de morte celular. Os resultados indicam que $CdCl_2$ promoveu o aumento do estresse oxidativo em células C_2C_{12} comprometendo provavelmente a adesão celular e o mecanismo de defesa antioxidante.

"Cadmium chloride-induced oxidative stress in skeletal muscle cells *in vitro*."

Abstract

The effects of cadmium chloride ($CdCl_2$) on oxidative stress in the skeletal muscle cell line C_2C_{12} were investigated. Myoblast cells that differentiated into myotubes were treated with $CdCl_2$ (1, 3, 5, 7.5, 10, and 12.5 μM) for 24, 48, and 72 h. Subsequent assay of cell homogenates for MTT (3-(4,5-*dimethylthiozol*-2-yl)-2,5-*diphenyltetrazolium bromide*) reduction, neutral red uptake and nucleic acid content showed that cadmium was toxic to C_2C_{12} cells in a concentration-dependent manner. Glutathione-S-transferase activity (nmol μg of protein^{-1} min^{-1}) was increased with 1 and 3 μM $CdCl_2$ (36.9 ± 5.6 and 32.1 ± 6.0, respectively) compared to control cells (21.8 ± 1.5), but decreased at higher concentrations (7.5 μM = 15.9 ± 3.3, 10 μM = 15.9 ± 4.6, and 12.5 μM = 10.5 ± 2.8). An increase in malondialdehyde content (nmol Ag of protein^{-1}), especially at high $CdCl_2$ concentrations (control = 7.3 ± 0.5; $CdCl_2$: 7.5 μM =11.2 ± 3.1, 10 μM = 14.6 ± 3.8, and 12.5 μM = 20.5 ± 6.5) indicated that there was enhanced lipid peroxidation. Light and scanning electron microscopy showed that there was a concentration-dependent loss of adherent cells and the formation of vesicles indicative of cell death. These results indicated that $CdCl_2$ increased oxidative stress in C_2C_{12} cells, and this stress probably compromised cell adhesion and the cellular antioxidant defense mechanisms.

Keywords: Cadmium chloride; Myotubes; Oxidative stress; Skeletal muscle cells

Introduction

Cadmium (Cd) is an environmental and industrial pollutant with a wide variety of toxic manifestations, including lung fibrosis, kidney tubular dysfunction, hypertension, osteoporosis, and cancer [1–4]. Studies in animals have shown that exposure to Cd can lead to the formation of a variety of malignancies, including sarcomas [5], leukemia [6], and lung and prostate cancers [7]. Other studies have suggested a correlation between exposure to Cd and some types of human cancers [8], indicating that Cd can also promote carcinogenesis [9]. The promoter activity of Cd may involve oxidative stress, disruption of intercellular gap junction communication (IGJC) and alteration of the cytoskeleton [10–12]. Since Cd is generally a poor mutagen [13], the carcinogenic potential of this metal is unknown, but could contribute to nongenotoxic or indirectly genotoxic events that may enhance cell proliferation, depress apoptosis, and/or alter DNA repair [14]. Such injuries caused by Cd or other noxious agents probably lead to cell death [15]. Alternatively, Cd may act indirectly by attenuating cellular antioxidant defenses, thereby increasing the intracellular levels of hydrogen peroxide. The latter can in turn produce free radicals capable of breaking or crosslinking DNA or triggering lipid peroxidation. This indirect action of Cd may trigger a process associated with the formation of mutagenic adducts in DNA. Finally, Cd may interact with the metal- binding sites of proteins involved in DNA transcription, DNA replication, and DNA repair [15–17].

Although there have been marked advances in our understanding of how organic toxic agents can affect living organisms, the mechanisms by which toxic metals such as Cd produce their biochemical effects are still largely unknown [18,19]. The role of oxidative damage in the cytotoxicity, genotoxicity, and carcinogenicity of Cd has not been fully elucidated. The specific antioxidative response of tissues appears to be dependent not only on the nature of the reactive oxygen species (ROS), but also on the specific tissue and oxidative agent involved [17]. In muscle, for example, variations in the activities of antioxidant enzymes have been reported under different pathological conditions associated with free radical injury [20]. Differences in the mechanisms regulating antioxidant defenses

in muscle may explain the phenotypic variability among muscle disorders in which ROS play a pathogenic role [21]. In this context, cellular metabolism, biosynthetic pathways, and cell adhesion molecules may be targets for metal toxicity [22–24]. Although the liver and kidney are specific target organs for the bioaccumulation of metals, Seidki et al. [25] have also reported high levels of Cd in skeletal muscle. Several investigations have examined the effects of Cd on skeletal and smooth muscle function [26–29]. Since Cd can induce lipid peroxidation, one of the main signs of oxidative damage and [24], and since oxidative stress is one of the main processes in a wide variety of muscle diseases and pathologies [20,30], as well as in protein wasting in skeletal muscle [31], in this work we examined the ability of $CdCl_2$ to alter the levels of oxidative stress in myotubes of cultured C_2C_{12} skeletal muscle cells.

Materials and methods

Cell culture

Myoblast C_2C_{12} cells were generously provided by Dr. Michael J. Tisdale (Laboratory of Cancer Research, Aston University, Birmingham, England). The cultures were grown in tissue culture flasks (Corning, NY) in DMEM medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Sigma), 1% penicillin, and 1% streptomycin (Sigma) at 37°C in a humidified atmosphere of 5% CO_2. All of the experiments were initiated using cells grown to 90– 100% confluence. To induce differentiation, the growth medium was replaced by medium supplemented with 2% horse serum. $CdCl_2$ (Sigma), prepared freshly for each experiment, was used at final concentrations of 1, 3, 5, 7.5, 10, and 12.5 μM and left in contact with the cells for 24 h.

Cytotoxicity assays

The viability of control and $CdCl_2$-treated C_2C_{12} myotubes was assessed based on MTT reduction, neutral red uptake (NRU) and nucleic acid content (NAC). The MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a sensitive, quantitative colorimetric assay that measures cell viability based on the ability of mitochondrial succinyl dehydrogenase in living cells to convert the yellow substrate MTT into a dark blue formazan product. For the assay, the medium containing $CdCl_2$ was

removed and a solution containing 0.01% MTT was added to each well. After incubation for 10 min at 37°C, the medium was removed and the formazan solubilized in ethanol. The plate was shaken for 30 min and the absorbance was measured at 570 nm [32]. The NRU assay is a cell viability test based on the incorporation of dye into the lysosomes of viable cells following incubation with the test agents. After removal of the medium from the plates, a solution of 0.05% neutral red was added to each well followed by incubation for 3 h at 37°C. The cells were then washed with phosphate-buffered saline containing calcium (PBS-Ca^{2+}), followed by the addition of 1% glacial acetic acid and 50% ethanol to each well to fix the cells and extract the neutral red incorporated into the lysosomes. The plates were shaken for 20 min and the absorbance was measured at 540 nm [33]. For the NAC assay, monolayers of cells were solubilized with 0.5 N NaOH at 37°C for 1 h and the absorbance was measured at 260 nm; the results were expressed as a percentage of the control [34].

Analytical methods

After 24 h of treatment with $CdCl_2$, the cells were washed with cold PBS and collected in homogenization buffer (HB) (20 mM Tris, 1 mM DTT, 2 mM ATP and 5 mM $MgCl_2$, pH 7.2), and centrifuged at 10,000 rpm for 15 min at 4°C. Aliquots of homogenate supernatants were analyzed for glutathione-S-transferase (GST) activity based on the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione and the activity was expressed in nanomoles per microgram of protein per minute, using an extinction coefficient of 9.6, as described by Habig et al. [35]. The lipid peroxidation product malondialdehyde (MDA) was determined using MPO (N-methyl-2-phenylindole) as the substrate. The resulting absorbance was measured at 590 nm and the results were expressed in nanomoles per milligram protein [31]. The protein content was measured by the method of Lowry et al. [36].

Light (LM) and scanning electron (SEM) microscopy

Myotubes were cultured on coverslips and treated with various concentrations of $CdCl_2$ for 24 h prior to analysis by LM (Leica DMLM, Wetzlar, Germany). For SEM, other cells were fixed in 2.5% paraformaldehyde/glutaraldehyde (Sigma) in 0.1 M PBS, pH 7.4,

and then washed in PBS followed by postfixation with 1% osmium tetroxide (Sigma) and dehydration in a graded ethanol series. The cells were then critical-point-dried (CPDO030–Balzers, BAL-TEC AG, Wiesbaden, Germany) and gold-sputtered (SCD050–Balzers) before being analyzed in a scanning electron microscope (JSM-5800LV, JEOL, Peabody, MA) operated at 1 kV.

Statistical analysis

The results were expressed as the mean ± SE. One-way ANOVA followed by Bonferroni's test for multiple comparisons [37] was used to compare the $CdCl_2$ treatments with the controls. A value of $P < 0.05$ indicated statistical significance.

Results

In this study, C_2C_{12} myotubes were treated with various concentrations of cadmium chloride ($CdCl_2$) (1–12.5 μM) for 24, 48, and 72 h to assess the toxicity of this metal to these cells. After a 24-h exposure, $CdCl_2$ decreased the cell viability only at the highest concentration (12.5 μM), whereas after 48 and 72 h, a reduction in cell viability was seen at all $CdCl_2$ concentrations, especially at ≥7.5 μM after 72 h (Figure 1A). The NRU assay also showed a significant decrease in the viability of C_2C_{12} myotubes after a 24-h exposure at all concentrations of $CdCl_2$ (Figure 1B). In agreement with these results for MTT and NRU, the nucleic acid content (NAC) of C_2C_{12} myotube cells started to decrease after a 24-h exposure to the highest concentrations (10 and 12.5 μM) of $CdCl_2$; a similar response was also seen after 48 and 72 h (Figure 1C). The effects of $CdCl_2$ on C_2C_{12} myotubes were also assessed by measuring the glutathione-S-transferase activity (GST), lipid peroxidation (MDA formation), and protein content. The results again clearly indicated that $CdCl_2$ caused oxidative cellular damage to C_2C_{12} cells. Figure 2A shows that the GSTactivity of myotubes increased at low concentrations (1 and 3 μM) of $CdCl_2$ (around 1.7- and 1.4-fold higher, respectively) and decreased at 5, 7.5, 10, and 12.5 μM compared to control cells. In contrast, there was a significant increase in MDA levels at 10 and 12.5 μM $CdCl_2$ (around 2- and 2.8-fold higher, respectively, compared to control cells) (Figure 2B). The protein content was not significantly altered at any of the $CdCl_2$ concentrations (Figure 2C).

Light microscopy showed that there were morphological changes in C_2C_{12} myotubes treated with 3, 5, 7.5, 10, and 12.5 μM of $CdCl_2$ for 24 h (Figure 3). Cells grown in complete medium in the absence of $CdCl_2$ had a normal, elongated shape (Figure 3A). However, after a 24-h incubation with $CdCl_2$, morphological changes that included a loss of cell to- cell contact with subsequent cell detachment, retraction, and a change in shape were seen. This loss of contact with neighboring cells was particularly evident at $CdCl_2$ concentrations ≥7.5 μM (Figs. 3D–F). Morphological changes were also seen in SEM. Numerous membranous vesicles (Figure 4B), as well as cell detachment and changes in shape as a consequence of cell retraction, were seen after incubation at all concentrations of $CdCl_2$ (Figs. 4B–F) when compared with untreated cells (Figure 4A). This morphological damage induced by $CdCl_2$ probably resulted in irreversible cell injury.

Discussion

Studies in several systems have shown that Cd can affect various metabolic processes, especially energy metabolism, membrane transport, and protein synthesis, and may act on DNA directly or indirectly by interfering with genetic control and repair mechanisms [38]. Cadmium induces the formation of ROS and causes damage consistent with oxidative stress [28,39,40]. The production of ROS may also be associated with Cd toxicity [40], and may induce oxidative stress by depleting intracellular antioxidants such as glutathione, or by inhibiting the activity of superoxide dismutase [40]. Cadmium may adversely affect enzyme activities [40,41], enhance lipid peroxidation [24], alter mitochondrial functions [41,43], and break DNA [44,45]. The exhaustion of GSH stores during acute intoxication by Cd may result in an increase in oxidative stress to produce superoxide anions and nitric oxide [46]. Gaubin et al. [47] showed that exposure to a low concentration (1–10 μM) of Cd resulted in increased glutathione levels. Mehlen et al. [48] suggested that there was a correlation between the increase in the expression of heat shock proteins (HSP) and the increase in the cellular content of glutathione such that small HSP may modulate intracellular glutathione levels. In agreement with these authors, we observed an increase in GST activity after exposure to low $CdCl_2$ concentrations, but this was probably insufficient to overcome the oxidative stress generated by the metal. In contrast, high $CdCl_2$ concentrations generated ROS, in addition to causing oxidative stress,

and consequently reduced the glutathione level. Since oxidative stress in skeletal muscle cells, even under physiological conditions, has been implicated in a wide variety of muscle diseases and pathological conditions [49], the reduced viability of C_2C_{12} myotubes following exposure to $CdCl_2$ indicated that Cd adversely affected cellular metabolism and, consequently, muscle tissue function. The decrease in GST activity seen here with increasing $CdCl_2$ concentrations could be explained by an ROS production that exceeded the catalytic capacity to reduce glutathione. The increase in GST activity seen with 1 and 3 μM $CdCl_2$ was probably related to the production of GSH, which acts as a scavenger and/or a cofactor in the metabolic detoxification of ROS during defense against oxidative damage and free radical generation [48]. Mehlen et al. [48] also observed that high Cd concentrations (10– 100 μM) significantly reduced the glutathione levels. In addition, Yang et al. [50] showed that the treatment of CHO cells with cadmium acetate (4 μM for 4 h) decreased glutathione peroxidase (47%), glutathione reductase (40%), and catalase (22%) activities. This inhibition of protective enzymes and the disappearance of glutathione trapped by Cd suggested that there was little or no inactivation of H_2O_2 and lipid hydroperoxide products by glutathione peroxidase and/or catalase. Elevated cellular peroxidation depends on the intracellular content of free radical oxygen. This increased level of ROS may result from the overproduction of these species or a reduced ability to destroy them [50]. Xenobioticgenerated ROS initiate peroxidation by interacting with unsaturated fatty acids [18], and an increased level of lipid peroxidation stimulates mitochondrial activity, which is an important source of ROS [41]. Several reports that have investigated the effect of Cd on tissue glutathione (GSH) levels have shown a strong correlation between the endogenous GSH pool and protection against xenobiotics [42]. The effect of the coadministration of antioxidants on the toxicity of Cd has also been studied. As shown here, exposure to $CdCl_2$ resulted in increased lipid peroxidation (detected as the product MDA) in myotubes (Figure 2), in agreement with other studies. Hussain et al. [51] reported that Cd increased lipid peroxidation by a direct effect or by decreasing the glutathione content. Furthermore, decomposition of the products of lipid hydroperoxides, such as malondialdehyde and 4-hydroxynonenal [52], may contribute to cell damage by forming Schiff bases with cell membrane proteins [51], thereby destabilizing the membrane structure. The overproduction of reactive species after exposure to $CdCl_2$ may also be

associated with a reduced ability of GST to catalyze the formation of conjugates with glutathione. Cadmium chloride caused a loss of cell-to-cell contact and cell retraction, and also increased the number of membranous vesicles in C_2C_{12} myotubes. After exposure to 3–12.5 μM $CdCl_2$ for 24 h, the cells separated from each other and detached from their substrate. In the presence of 3μM $CdCl_2$, the cells began to separate from each other (Figure 3B) and assumed a round shape with 5μM $CdCl_2$ (Figure 3C). The greatest changes in the monolayers were seen with 7.5–12.5μM $CdCl_2$, with marked cell detachment from the substrate surface and the formation of clusters of cells. The latter event appeared to be associated with cell death, as also observed by Prozialeck and coworkers [18,19,53]. Cadmium produces a variety of cytotoxic effects in epithelial cells and can damage epithelial cell-to-cell junctions in some tissues and cultured cells, probably by disrupting E-cadherin-dependent cell-cell junctions. In skeletal muscle, Cd may interfere with the normal function of the extracellular matrix (ECM) through cell adhesion molecules by disrupting the cell-surface proteins that act as structural mechanical components that maintain cell-to-cell and cell-to-substrate attachment [53]. The results described here show that Cd adversely affected skeletal muscle cells, possibly by increasing the levels of ROS and causing disarrangement of the extracellular matrix. However, this damage may also occur independently of any cytotoxic effects or may be part of an integrated cascade of events leading to severe cell injury and death [27–29]. In conclusion, our results indicate that $CdCl_2$ induced oxidative damage in C_2C_{12} cells that compromised cell adhesion and resulted in cellular lesions and morphological changes similar to those reported by Aoki and Hoffer [54] and Hew et al. [55] for endothelial cells and Sertoli cells, respectively. Additional investigations are needed to understand the sequence of cellular events that lead to this damage in muscle cells after exposure to $CdCl_2$, and to assess the general impact of heavy metals on cell adhesion and molecules associated with signaling pathways.

Acknowledgments

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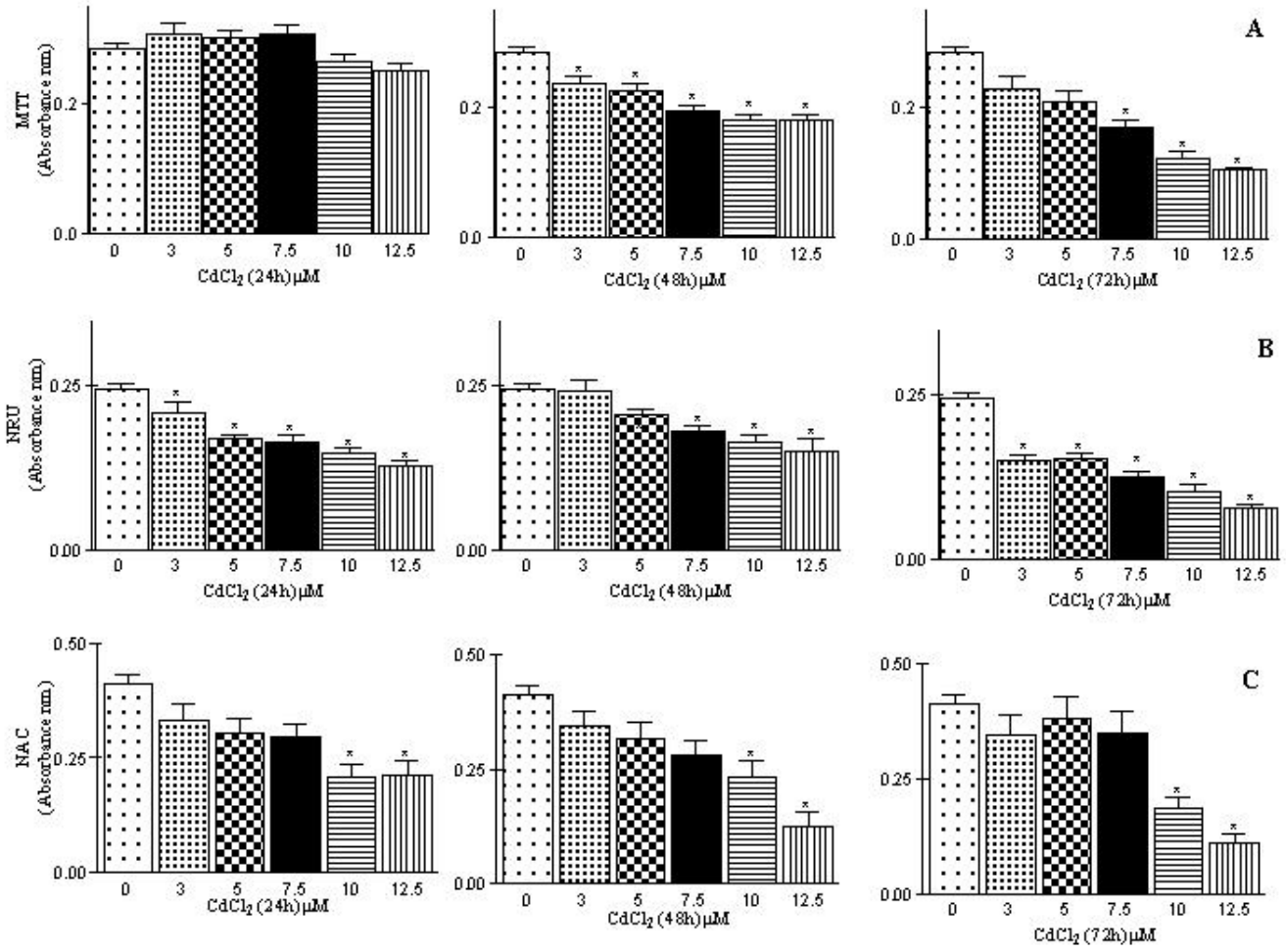
Figures Legend

Figure 1. Effect of $CdCl_2$ on C_2C_{12} cell viability assessed by the MTT reduction (A), NRU (B), and NAC (C) assays. C_2C_{12} cells differentiated into myotubes were incubated with 1, 3, 5, 7.5, 10, and 12.5 μM of $CdCl_2$ for 24, 48, and 72 h. The columns are the mean ± SE of triplicate experiments. *$P < 0.05$ compared to untreated cells (ANOVA).

Figure 2. Effects of $CdCl_2$ on glutathione-S-transferase activity (A), lipid peroxidation (B), malondialdehyde (MDA) levels, and protein content (C). The columns are the mean ± SE of triplicate experiments. Different letters indicate significant ($P < 0.05$) differences.

Figure 3. Light microscopy of C_2C_{12} skeletal muscle cell myotubes after a 24-h incubation with $CdCl_2$ (3, 5, 7.5, 10, and 12.5 μM). (A) Control (untreated) cells showing confluent cell growth. (B) C_2C_{12} myotubes treated with 3 AM $CdCl_2$. Note the detached cells and nonconfluent cell layer (arrows). (C) Morphological changes in C_2C_{12} myotubes treated with 5 μM $CdCl_2$. (D) C_2C_{12} myotubes treated with 7.5 μM $CdCl_2$. Note the loss of cell-to-cell contact and cell retraction. (E) Morphological changes in C_2C_{12} myotubes treated with 10 μM $CdCl_2$. Note the round shape and detachment from the surface. (F) C_2C_{12} myotubes treated with 12.5 μM $CdCl_2$. Note the extensive loss of cell contact and the increased number of round cells.

Figure 4. Scanning electron micrographs of C_2C_{12} myotubes treated with 3, 5, 7.5, 10, and 12.5 μM $CdCl_2$. See Material and methods for details. (A) Untreated (control) C_2C_{12} myotubes. (B) C_2C_{12} myotubes treated with 3 μM $CdCl_2$, showing spaces between cells (arrows) and irregular cell membrane. (C) C_2C_{12} myotubes treated with 5 μM $CdCl_2$, showing several vesicles (arrows). (D) C_2C_{12} myotubes treated with 7.5 μM $CdCl_2$. (E) C_2C_{12} myotubes treated with 10 μM $CdCl_2$. (F) C_2C_{12} myotubes treated with 12.5 μM $CdCl_2$.


A
B
C
MTT (Absorbance nm)
NRU (Absorbance nm)
NAC (Absorbance nm)
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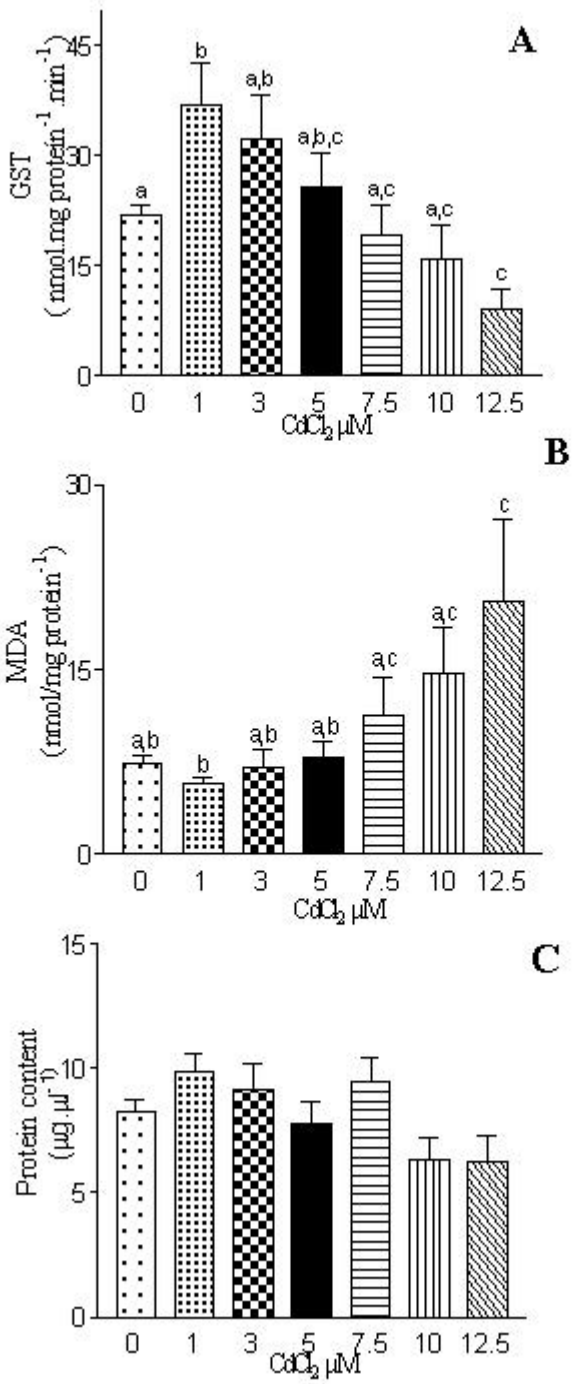
0.25
0.50
0 3 5 7.5 10 12.5
CdCl2 (24h)μM
CdCl2 (48h)μM
CdCl2 (72h)μM

Figure 1


A
GST
(nmol.mg protein-1.min-1)
45
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15
0
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0 1 3 5 7.5 10 12.5
CdCl2 µM
B
MDA
(nmol/mg protein-1)
30
15
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0 1 3 5 7.5 10 12.5
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Protein content
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Figure 2

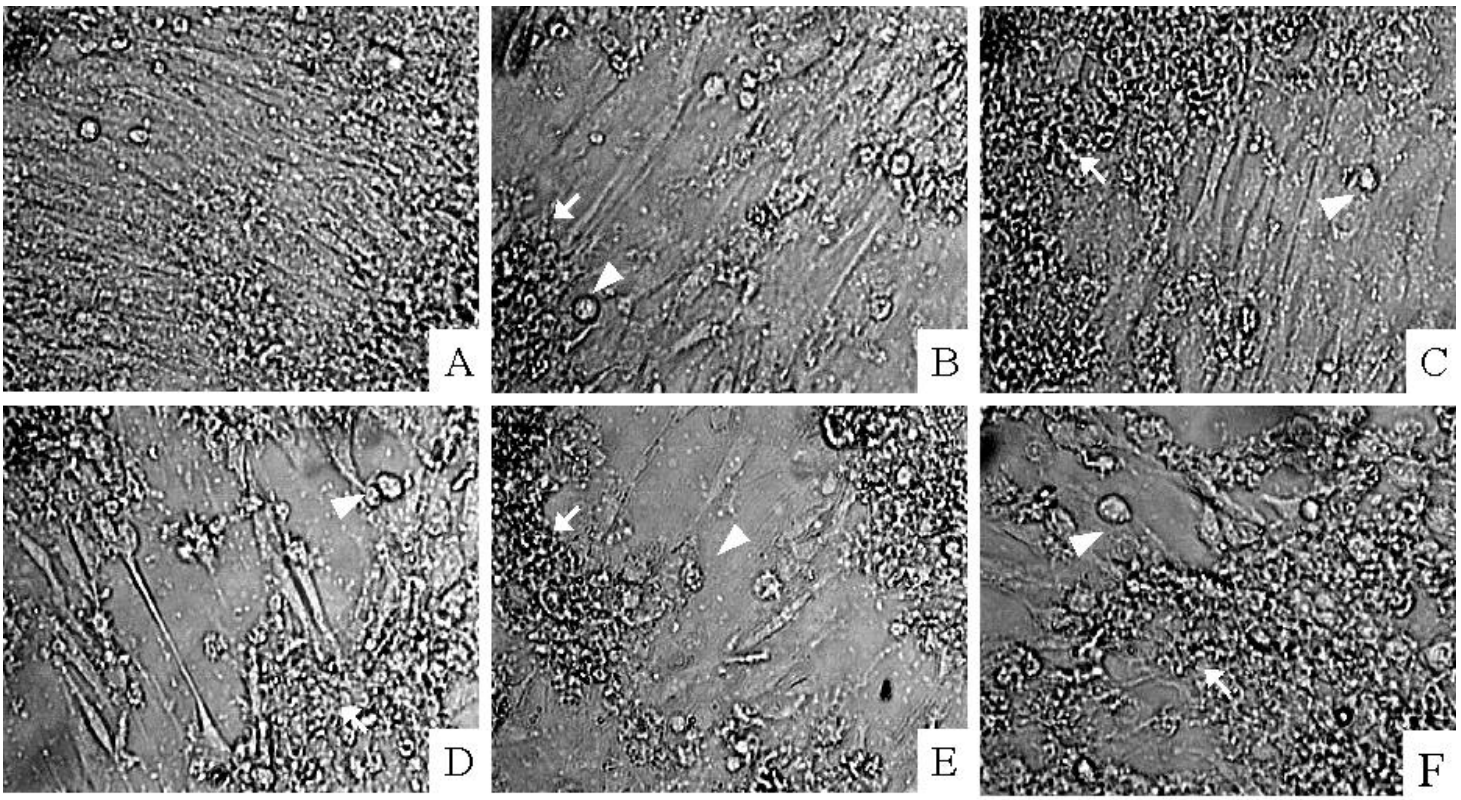

Figure 3

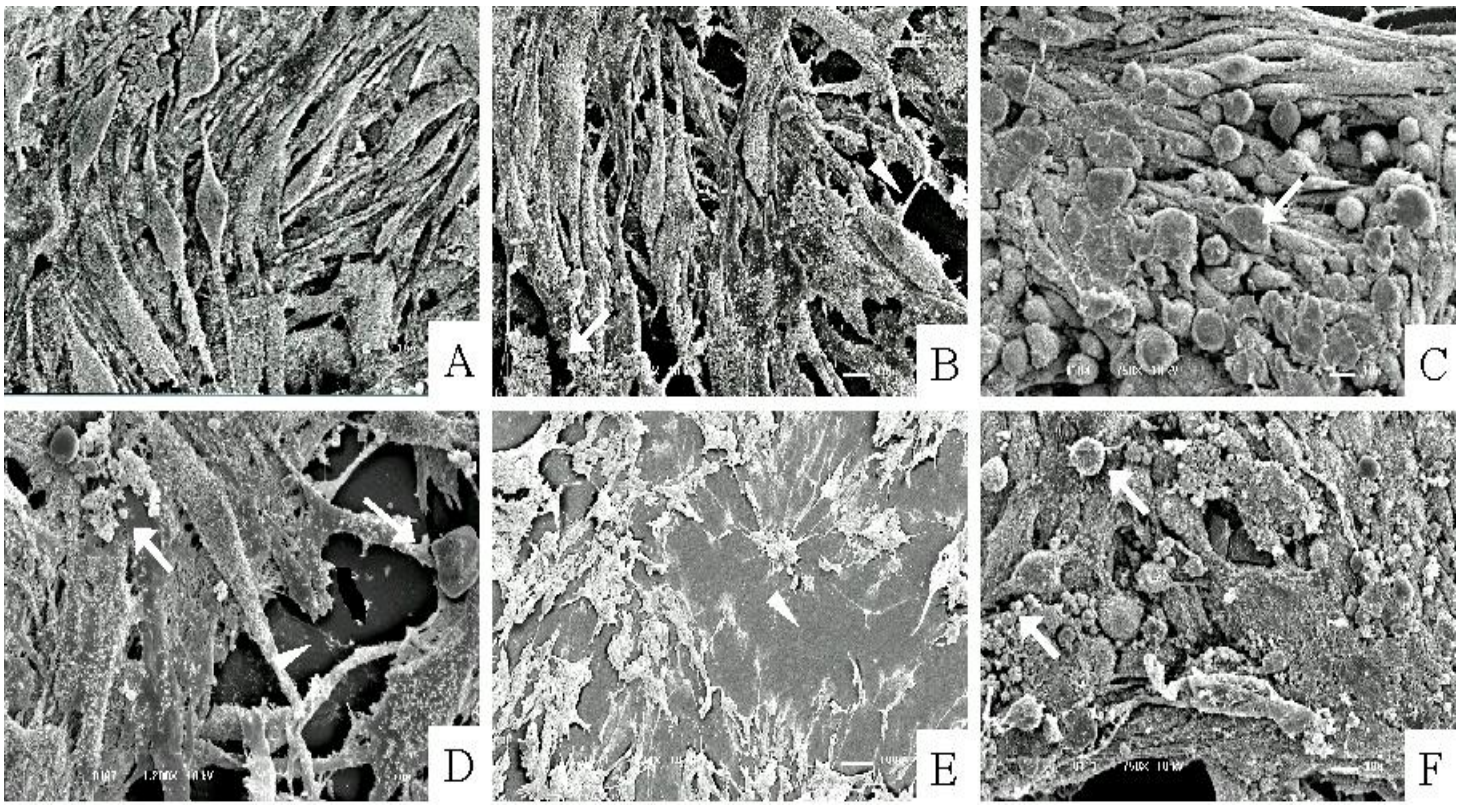

Figure 4

Capítulo II

"Efeito protetor do zinco contra citotoxicidade produzida pelo cádmio em células musculares- Linhagem C_2C_{12}- *in vitro*."

Trabalho a ser submetido ao periódico *Free Radical Biology & Medicine.*

No presente trabalho foram avaliados os efeitos protetores do sulfato de zinco ($ZnSO_4$) sobre o estresse oxidativo promovido pelo cloreto de cádmio ($CdCl_2$) em linhagem de células de músculo esquelético C_2C_{12}. Mioblastos foram diferenciados em miotúbulos e pré-tratados com diferentes concentrações de sulfato de zinco (10, 20 e 40μM) pelo período de 24h, e tratadas com diferentes concentrações de cádmio (1, 3, 5, 7,5; 10 e 12,5μM) pelo período de 24, 48 e 72h. Células homogenizadas foram utilizadas para os ensaios de MTT (3-(4,5-*dimethylthiozol*-2-yl)-2,5-*diphenyltetrazolium bromide*) vermelho neutro e conteúdo de ácido nucléico nas células musculares, encontrando-se efeitos benéficos nas concentrações de 20 e 40μM de sulfato de zinco logo após 24h de tratamento com cloreto de cádmio. A atividade enzimática da GST (nmol μg de proteína^{-1} min^{-1}) foi preservada nos pré-tratamentos com sulfato de zinco e tratamentos com cloreto de cádmio. Aumento do conteúdo de malondialdeído (nmol μg de proteína^{-1} min^{-1}) foi observado com o pré-tratamento $ZnSO_4$ 40μM após 24h e tratamento com cloreto de cádmio. Após 48h, houve o aumento das lesões oxidativas em células C_2C_{12} tratadas com cloreto de cádmio 7,5μM (Cd= 66,4 ± 10,9) comparado com as células tratadas apenas com cádmio. Análises morfológicas de microscopia de luz e microscopia eletrônica de varredura mostraram significativa preservação da adesão celular com o pré-tratamento com sulfato de zinco 20μM e 40μM principalmente em alta concentração de cloreto de cádmio (10μM e

12,5μM). Os resultados indicam que o sulfato de zinco diminui o estresse oxidativo em células C_2C_{12} preservando a adesão celular e o mecanismo de defesa antioxidante por período mais longo de exposição ao cloreto de cádmio.

"PROTECTIVE EFFECT OF ZINC AGAINST CADMIUM CITOTOXICITY IN SKELETAL MUSCLE CELLS *IN VITRO*"

Claudia Lumy Yano; Emilianne Miguel Salomão; Maria Cristina Cintra Gomes-Marcondes.

Departamento de Fisiologia e Biofísica e Departamento de Biologia Celular, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, 13083-970, Campinas, São Paulo, SP, Brazil

Abstract

The protective effects of zinc sulfate ($ZnSO_4$) against cadmium chloride ($CdCl_2$) on oxidative stress in the skeletal muscle cell line C_2C_{12} were investigated. Myoblasts cells differentiated into myotubes were pretreated with different zinc sulfate concentrations (10, 20 and 40μM) for 24h, and further treated with different cadmium concentrations (1, 3, 5, 7.5, 10 and 12.5μM) for 24, 48 and 72h. Subsequent assay of cell homogenates for MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide) reduction, neutral red uptake and nucleic acid content showed that zinc sulfate pretreatment protected myotubes against cadmium chloride's toxicity. Glutathione-S-transferase activity (nmol μg of protein^{-1} min^{-1}) showed no differences in all pretreatment with zinc sulfate and treatment with cadmium chloride. Increase in malondialdehyde content (nmol μg of protein^{-1}) was observed with pretreatment zinc sulfate 40μM after 24h in cadmium treatment 10μM (Cd= 20.6 ± 0.9) and treatrement with cadmium chloride alone (Cd= 18.7± 1.4) compared to control cells (control= 14.6 ± 1.1). After 48h increase the oxidative damage in C_2C_{12} treatment with cadmium chloride at 7.5μM (Cd= 66.4 ± 10.9) was compared with cadmium chloride alone (Cd= 22.6 ± 4.7). Light and scanning electron microscopy showed significant preservation of

the cellular adhesion in pretreatment with zinc sulfate 20μM and 40μM mainly at high cadmium chloride concentration 10μM and 12.5μM. These results indicated that zinc sulfate decrease oxidative stress in C_2C_{12} cells, preserved cell adhesion and maintained the cellular antioxidant defense mechanisms by the longest period against cadmium chloride $CdCl_2$.

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Keywords: zinc; cadmium; myotubes; oxidative stress; skeletal muscle cells
*Correspondence: MCC Gomes-Marcondes: cintgoma@unicamp.br

Introduction

Cadmium is a heavy metal, which is widely used in industry and listed by the US Environmental Protection Agency as the one of 126 priority pollutants. In most studies, the cadmium's half-life is estimated to be 15 to 20 years in humans [1]. Environmental and occupational exposure to cadmium is implicated in a number of clinical complications, primarily renal dysfunction, bone disease, and also some cancers [2]. First observation of human cadmium contamination was reported in Japan, and been responsible for severe disease (Itai–Itai disease) characterized by severe pain, bone fractures, proteinuria and severe osteomalacia, which appeared mainly among women [3]. However excessive Cd^{2+} exposure causes renal, skeletal, vascular and respiratory disorders and furthermore International Agency for Research on Cancer (IARC) has classified Cd^{2+} as a group 1 carcinogen in humans [4]. Although the carcinogenic mechanism of cadmium (Cd) is not well defined, recent *in vitro* and *in vivo* evidence indicated that this metal may also enhance progression of tumor cells and enhanced invasiveness and metastasis potential of the ensuing tumors may have important implications in chronic exposures to Cd, or in cases of co-exposure of Cd with organic carcinogens, as in tobacco smoking [5-8]. In the last two decades there has been an explosive interest in the role of oxygen-free radicals, especially in carcinogenesis experimental and clinical medicine [9]. Oxidative damage accumulates during the life cycle, and radical-related to DNA, proteins and lipids damage has been proposed to play a key role in the development of age-dependent diseases such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders and other conditions [9]. Studies have demonstrated that cadmium induced reactive oxygen species (ROS)

production, and caused consistent oxidative stress damage [10-12]. This may induce oxidative stress, by depleting intracellular antioxidants, such as glutathione, or inhibiting the active of superoxide dismutase [13, 14]. Cadmium also increases the levels of lipid peroxidation in myotubes cells [15], and liver mitochondria of exposed rats [16], and in cultured rat hepatocytes [10, 17]. On the other hand, zinc (Zn) treatment induces tolerance to the toxicity of cadmium [18], but the protective mechanisms of Zn ions on cadmium toxicity is still unknown [19]. Cadmium and zinc are both effective inducers of metallothioneins (MT) synthesis, a metal-binding protein, with recognized function of detoxification of heavy metals such as cadmium and mercury [20, 21]. Zinc plays an important protect role on cellular components from oxidation and damage of DNA [22], receiving increase attention how it can benefit and increase the anti-oxidative protection in cancer patients [23]. Zinc deficiency results in great sensitivity to oxidative stress [24] and may, in part, account for the mechanism by which zinc deficiency increases the risk for cancer development. Thereby, zinc supplementation strategies have also been shown to be beneficial against oxidant damage and the progression of ROS-induced diseases [25]. Knowing this facts, the aim of the present study is to evaluate the possible therapeutic effect the zinc sulfate against cytotoxicity of cadmium on myotubes C_2C_{12} , since the skeletal muscle was the main target in a wide variety of muscle diseases and pathologies [26,27], as well in protein wasting disease such as cancer and aging [28] and recently verified in oxidative damage [15].

Materials and Methods

Cell culture

Myoblast C_2C_{12} cells, generously provided by Dr.Michael J. Tisdale (Laboratory of Cancer Research, Aston University, Birmingham, England), were grown in tissue culture flasks (Corning, NY) in DMEM medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Sigma), 1% penicillin, and 1% streptomycin (Sigma) at 37°C in a humidified atmosphere of 5% CO_2. All of the experiments were initiated using cells grown to 90–100% confluence. Myotubes differentiation was induced replacing the initial medium by supplemented medium with 2% horse serum. Myotubes C_2C_{12} were pretreated

with zinc sulfate at 10, 20 and 40μM/well for 24 h. After 24h, the medium was replaced and the myotubes were now exposed to $CdCl_2$ (Sigma), prepared freshly for each experiment, at final concentrations of 1, 3, 5, 7.5,10, and 12.5 μM for 24h, 48h and 72h.

Cytotoxicity assays

The viability of control and $CdCl_2$-treated C_2C_{12} myotubes was assessed based on MTT reduction, neutral red uptake (NRU) and nucleic acid content (NAC). The MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a sensitive, quantitative colorimetric assay that measures cell viability based on the ability of mitochondrial succinyl dehydrogenase in living cells to convert the yellow substrate MTT into a dark blue formazan product. For the assay, the medium containing $CdCl_2$ was removed and a solution containing 0.01% MTT was added to each well. After incubation for 10 min at 37ºC, the medium was removed and the formazan solubilized in ethanol. The plate was shaken for 30 min and the absorbance was measured at 570 nm [15]. The NRU assay is a cell viability test based on the incorporation of dye into the lysosomes of viable cells following incubation with the test agents. After removal of the medium from the plates, a solution of 0.05% neutral red was added to each well followed by incubation for 3 h at 37°C. The cells were then washed with phosphate-buffered saline containing calcium (PBS-Ca^{2+}), followed by the addition of 1% glacial acetic acid and 50% ethanol to each well to fix the cells and extract the neutral red incorporated into the lysosomes. The plates were shaken for 20 min and the absorbance was measured at 540 nm [15]. For the NAC assay, monolayer of cells were solubilized with 0.5 N NaOH at 37ºC for 1 h and the absorbance was measured at 260 nm; the results were expressed as a percentage of the control [15].

Analytical methods

After 24h of pretreatment with zinc sulfate and treatment with $CdCl_2$ for further 24, 48 and 72h, the cells were washed with cold PBS and collected in homogenization buffer (HB) (20 mM Tris, 1 mM DTT, 2 mM ATP and 5 mM $MgCl_2$, pH 7.2), and centrifuged at 10,000 rpm for 15 min at 4°C. Aliquots of homogenate supernatants were analyzed for

glutathione-S-transferase (GST) activity based on the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione and the activity was expressed in nmoles per microgram of protein per minute, using an extinction coefficient of 9.6, as described by Habig et al. [29]. The lipid peroxidation product malondialdehyde (MDA) was determined using MPO (N-methyl-2-phenylindole) as the substrate. The resulting absorbance was measured at 590 nm and the results were expressed in nmoles per milligram protein [28]. The protein content was measured by the method of Lowry et al. [30].

Light (LM) and scanning electron (SEM) microscopy

Myotubes were cultured on cover slips and pretreated with zinc sulfate for 24h and further treated with different concentrations of $CdCl_2$ for 24 h to access the light microscopy analysis (LM) (Leica DMLM, Wetzlar, Germany). New myotubes, treated as described above, were fixed in 2.5% paraformaldehyde/glutaraldehyde (Sigma) in 0.1 M PBS, pH 7.4, and then washed in PBS followed by post fixation with 1% osmium tetroxide (Sigma,) and dehydration in a graded ethanol series. The cells were then critical-point-dried (CPDO030–Balzers, BAL-TEC AG, Wiesbaden, Germany) and gold-sputtered (SCD050–Balzers) before being analyzed in a scanning electron microscope (JSM-5800LV, JEOL, Peabody, MA) operated at 1 kV. For SEM.

Statistical analysis

The results were expressed as the mean ± SE. One-way ANOVA followed by Bonferroni's test for multiple comparisons [31] was used to compare the $CdCl_2$ treatments with the controls. Statistical significance was considered as a P value below 5%.

Results

In the present study, the protective effect of zinc sulfate against the toxicity effects of cadmium chloride in myotubes C_2C_{12} cells line were evaluated by MTT , NRU and nucleic acid content, showed in Figures 1, 2 and 3. The results showed that mitochondrial activity, demonstrated by MTT assay, was maintained in all cadmium chloride concentrations in myotubes C_2C_{12} pretreated with zinc sulfate (10μM) compared with cadmium chloride alone (Cd) after 24, 48 and 72h (Figure1A). The pretreatment with

20μM zinc sulfate alone increased the mitochondrial activity, however after 48 and 72h the MTT values maintained similar to control. The treatment with different cadmium chloride exposure pretreated with 20μM zinc sulfate maintained the mitochondrial activity in all times(Figure 1B). The similar data was verified in the pretreatment with 40μM zinc sulfate and exposure with different cadmium chloride concentrations, except after 72h at high cadmium chloride concentration (12.5μM) when compared to control (Figure1C). The results show that zinc sulfate maintained the mitochondrial activity in the myotubes exposed to cadmium chloride. The lysosomal activity, verified by neural red uptake, also was maintained in cells pretreated with 10μM zinc sulfate followed exposure to different cadmium chloride doses after 24, 48 and 72h (Figure 2A). The NRU assay showed the myotubes viability were preserved in all cadmium concentrations after 24h, 48, and 72h, following the increase the viability cellular in cadmium chloride 7.5μM after 48h, decreased the viability cellular in zinc sulfate control were decreased after 24h and maintain the viability cellular when compared with control untreated cell after 48h and 72h (Figure2A). After 24h, the pretreatment of 20μM zinc sulfate showed preservation of the cellular viability in all cadmium chloride concentrations, however, after 72h there was a decrease on the cellular viability especially at 10 and 12.5μM cadmium concentrations (Figure 2B). The C_2C_{12} cells pretreated with 40μM zinc sulfate showed that the lysosomal activity could be preserved even in all cadmium concentrations only after 24h; there was a deep decrease on NRU value in zinc pretreated myotubes in all cadmium doses after 72h (Figure 2C). The nucleic acid content (NAC) was preserved in myotubes after pretreatment 10μM zinc sulfate followed cadmium exposure and this parameter decreased after 48 and 72h, however, statistically significant at high cadmium concentration (7.5 to 12.5μM $CdCl_2$) after 72h exposure (Figure 3A). The pretreatment with 20μM and 40μM zinc sulfate were efficient on preservation of the cellular viability in all cadmium concentrations (Figs. 3B, 3C). The effects of pretreatment of zinc sulfate against toxic effect cadmium chloride on C_2C_{12} myotubes were also assessed by measuring the glutathione-S-transferase activity (GST) and lipid peroxidation (MDA formation). The results indicated none significant difference in the GST activity of myotubes pretreated with zinc sulfate at 10μM and 20μM treated with different cadmium chloride

concentrations after 24, 48h (Figure 4 A and B). However, after 72h pretreatment of 40μM zinc sulfate there was deep decrease on GST activity in all cadmium concentrations (Figure 4C). The data showed enhanced myotubes' lipid peroxidation on pretreatment of 40μM zinc sulfate after 24h cadmium chloride exposure only at 10μM (Figure 5C). The pretreatment with 40μM zinc sulfate after 48h showed the increase the oxidative damage in C_2C_{12} myotubes treated with cadmium chloride at 7.5μM when compared to cadmium chloride alone. After 72h, there was expressive increase on MDA content in all cadmium doses (Figure 5C).

Light microscope (Figure 6) shows typical morphology of skeletal muscle cells in non Cd treatment (Figure A). The C_2C_{12} cells differentiated in myotubes pretreated with zinc sulfate at 10μM, 20μM and 40μM after 24h (Figure 6B, C, D) showed similar morphology to $CdCl_2$ untreated cells. The myotubes treated with cadmium chloride at 10μM and 12.5μM after 24h (Figure 6E, F), showing the severe injury to the cellular adhesion with loss of cell-to-cell contact and detachment and alteration of shape cell. The C_2C_{12} cells pretreated with 10μM zinc sulfate followed to cadmium chloride treatment at 10μM and 12.5μM after 24h (Figure 6G,H), showed preservation of cellular adhesion against $CdCl_2$ effect at 10μM when compared the cells pretreated with zinc sulfate 10μM and $CdCl_2$ 12.5μM, or the treatment with $CdCl_2$ 10μM alone. Myotubes pretreated with 20μM zinc sulfate followed the cadmium chloride treatment at 10μM and 12.5μM after 24h (Figure 6I,J), showed protection against to $CdCl_2$ toxic effect at high concentrations (10μM and 12,5μM, respectively) when compared to $CdCl_2$ alone. The pretreatment with 40μM zinc sulfate followed the cadmium chloride treatment at 10μM and 12.5μM after 24h (Figure 6K, L, respectively), showed that myotubes could preserve the cellular adhesion when compared with the pretreatment zinc sulfate at 10μM, 20μM or $CdCl_2$ alone. Morphological changes were also seen in SEM. After 24h the zinc sulfate pretreatment (20μM) followed cadmium chloride treatment (10 and 12.5 μM) for 24h, there were many cellular vesicles and characteristic of cellular death process (Figure 7E-F, respectively). The most preservation of cellular adhesion can be observed in pretreatment with 40μM zinc sulfate and high cadmium chloride concentrations (10 and 12.5μM) after 24h when compared with cadmium chloride treatment 10 μM alone (Figure 7G,H,

respectively). The myotubes treated only with zinc sulfate (20 and 40μM, Figure 7C, D, respectively) showed the typical feature of skeletal muscle with elongated shape (Figure 7 A).

Discussion

Cadmium is a heavy metal, which is widely used in industry, affecting human health through occupational and environmental exposure [32]. Acute toxicity induced by $CdCl_2$ may be due to the exhaustion of GSH stores and the increase on oxidative stress [33]. Protection against these acute $CdCl_2$ effects can be achieved through the antioxidant systems [33]. In the present study, the protective effects of zinc sulfate ($ZnSO_4$) against oxidative stress induced by cadmium chloride ($CdCl_2$) on in the skeletal muscle cell line C_2C_{12} were investigated by MTT, NRU, and NAC viability assay, GST activity, lipid peroxidation verifying the malondialdehyde content (MDA) and morphological analysis. Pretreatment in all zinc sulfate concentrations showed an effective maintenance of the mitochondrial and lysosomal activity in the myotubes C_2C_{12} exposed to cadmium chloride. This confirmation is observed through NAC assay that show greater preservation of the DNA integrity mainly in zinc sulfate pretreatment at 20μM and 40μM and high cadmium chloride concentrations (10μM and 12.5μM) for long time (72h). Studies verified that cadmium stimulated the mitochondrial ROS production in liver, brain, and heart [34]. The increase in cellular activity was postulated by Probs et al (1977) who observed greater activity in cells pretreated with 20μM and 40μM Zn and with low cadmium concentration 3μM suggesting that the protective effect was due to induction of metallothionein synthesis by zinc (Probs et al., 1977) [35]. However, an alternative mechanism inducing cells' tolerance to cadmium may be related to non-metallothionein systems such as a reduction of cadmium uptake [36] and other cadmium-binding proteins [37]. Additionally, the way which zinc could induce tolerance to cadmium cytotoxicity via non-metallothionein mechanisms was not clear [38]. Mishima et. al. (1997) demonstrated that in vascular endothelial cells zinc was not an effective inducer of metallothionein but protects against cadmium cytotoxicity mainly via a decrease in the intracellular accumulation of cadmium

[38]. Those studies suggested that intracellular zinc mimicked the cadmium and contributed to the balance between intracellular and extracellular cadmium concentration. The present study, none significant difference was showed on the GST activity of myotubes pretreated with zinc sulfate at 10μM and 20μM, except at 40μM associated to high cadmium concentrations, after 72h, indicating that pretreatment with zinc sulfate could preserve the GST activity in myotubes exposed to cadmium. The increase on the oxidative damage (high MDA content, Figure 5C) in C_2C_{12} myotubes pretreated with zinc sulfate was higher only at 7.5μM and 10μM cadmium concentration after 48 and 72h when compared to cadmium chloride alone, suggesting a correlation with the low GST activity in this situation (Figure 4C and.5C). The mechanisms of zinc sulfate protective effect can be due the action on mitochondrial integrity, antioxidant function and metallothionein induction, metal-binding proteins, with recognized function of detoxification of heavy metals such as cadmium and mercury [20,21]. Metallothionein induction appears to be the most effective mechanism, since pretreatment with high levels of zinc induced a stronger tolerance to cadmium [39]. The morphological analysis of the myotubes C_2C_{12} cells pretreated with zinc sulfate at 10μM, 20μM and 40μM showed effective preservation of cellular adhesion compared to cadmium treatment alone. This was also observed in SEM after 24h the cadmium chloride treatment. The hypothesis how the zinc protective effect could preserve the membrane integrity remains to be elucidated, probably by stabilizing the membrane structure [40], that the intracellular zinc served as an antioxidant [22] or increase the intracellular glutathione content and then protected against lipid peroxidation which could be induced by cadmium [41]. Alternatively, the intracellular zinc may have competed with cadmium directly at the sites where cadmium exhibits its toxicity within the cells or the zinc somehow augmented the physiological functions of the cells such as proliferation [42]. Possibly zinc may block the intracellular cadmium uptake via voltage-sensitive calcium channels [43]. Studies have demonstrated that zinc possesses antioxidant proprieties [44], protecting against the hepatotoxicity [10] and the nephrotoxicity [45]. Therefore, further studies are necessary and underway in our laboratory to elucidate the real benefit cadmium-zinc interaction and how this interaction could preserve the oxidative damage in skeletal muscle cells, as verified previously Yano & Gomes-Marcondes (2005),

that has been implicated in a wide variety of muscle diseases and pathological conditions [46], moreover, have also reported Cd accumulation in animals skeletal muscle [47].

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Figures Legend

Figure 1: C_2C_{12} cells viability assessed by MTT assay. C_2C_{12} cells differentiated into myotubes were pretreated with zinc sulfate at 10µM (A), 20µM (B) and 40µM/well concentration (C) for 24h and following treatment with cadmium chloride at 1, 3, 5, 7.5, 10 and 12.5µM/well concentration for 24h, 48h and 72h. Each experiment was made in triplicate. *Significantly different from untreated cells by ANOVA, $p<0.05$.

Figure 2: C_2C_{12} cells viability assessed by NRU assay. C_2C_{12} cells differentiated into myotubes were pretreated with zinc sulfate at 10µM (A), 20µM (B) and 40µM/well concentration (C) for 24h and following treatment with cadmium chloride at 1, 3, 5, 7.5, 10 and 12.5µM/well concentration for 24h, 48h and 72h. Each experiment was made in triplicate. *Significantly different from untreated cells by ANOVA, $p<0.05$.

Figure 3: C_2C_{12} cells viability assessed by NAC assay. C_2C_{12} cells differentiated into myotubes were pretreated with zinc sulfate at 10µM (A), 20µM (B) and 40µM/well concentration (C) for 24h and following treatment with cadmium chloride at 1, 3, 5, 7.5, 10 and 12.5µM/well concentration for 24h, 48h and 72h. Each experiment was made in triplicate. *Significantly different from untreated cells by ANOVA, $p<0.05$.

Figure 4: C_2C_{12} cells viability assessed by GST assay. C_2C_{12} cells differentiated into myotubes were pretreated with zinc sulfate at 10µM (A), 20µM (B) and 40µM/well concentration (C) for 24h and following treatment with cadmium chloride at 1, 3, 5, 7.5, 10 and 12.5µM/well concentration for 24h, 48h and 72h. Each experiment was made in triplicate. *Significantly different from untreated cells by ANOVA, $p<0.05$.

Figure 5: C_2C_{12} cells viability assessed by MDA assay. C_2C_{12} cells differentiated into myotubes were pretreated with zinc sulfate at 10µM (A), 20µM (B) and 40µM/well concentration (C) for 24h and following treatment with cadmium chloride at 1, 3, 5, 7.5, 10

and 12.5μM/well concentration for 24h, 48h and 72h. Each experiment was made in triplicate. *Significantly different from untreated cells by ANOVA, $p<0.05$.

Figure 6: Light microscopy analysis of myotubes C_2C_{12} cells, after 24 hours of pretreatment with zinc sulfate at 10μM, 20μM and 40μM followed treatment with cadmium chloride $CdCl_2$ at (0, 10 and 12.5 μM/mL) concentrations for 24 hours. A, Cd untreated cells. B, C_2C_{12} cells treated with 10μM Zn alone, as control zinc. C, treatment with 20μM Zn alone, as control zinc. D, and treatment with 40μM Zn alone, as control zinc. Cd untreated cells as well as control zinc showed cells grown as monolayer with long fuse shape, characteristic of skeletal muscle cell. E, C_2C_{12} cells treated with 10μM $CdCl_2$ showing cell-to-cell contact lost and cells retractions. F, C_2C_{12} cells treated with 12.5μM $CdCl_2$ showing significant injure the entire monolayer. G, C_2C_{12} cells after pretreatment with zinc at 10μM followed treatment with cadmium at 10μM and H, 12.5μM showing protection of zinc at 10μM concentration in preservation the monolayer against effects of cadmium at 10μM and 12.5μM concentrations. I, C_2C_{12} cells after pretreatment with zinc at 20μM followed treatment with cadmium at 10μM and J, 12.5μM showing also the preservation of morphologic cell. K, C_2C_{12} cells after pretreatment with zinc at 20μM followed treatment with cadmium at 10μM and L, 12.5μM showing better preservation of adhesion cellular. (x400)

Figure 7: Scanning electron micrographs of C_2C_{12} myotubes after 24 hours of pretreatment with zinc sulfate at 20μM and 40μM followed treatment with cadmium chloride $CdCl_2$ at (0, 10 and 12.5 μM/mL) concentrations for 48 hours. A, Cd untreated cells. B, C_2C_{12} cells treated with 10μM $CdCl_2$ showing cellular retraction (arrow). C, 20μM Zn alone, as control zinc. D, treatment with 40μM Zn alone, as control zinc. None apparent morphological difference with untreated cells was observed. E, C_2C_{12} cells after pretreatment with zinc at 20μM followed treatment with cadmium at 10μM and F, 12.5μM showing the cellular death (arrow) and many cellular vesicles (arrow) G, C_2C_{12} cells after pretreatment with zinc at 40μM followed treatment with cadmium at 10μM and H, 12.5μM showing also protection of zinc at 40μM concentration in preservation the monolayer against effects of cadmium at 10μM and 12.5μM concentrations.

Abbreviations: Cd, cadmium; $CdCl_2$, cadmium chloride; Zn, zinc; $ZnSO_4$, zinc sulfate; CDNB, 1-chloro-2,4- dinitrobenzene; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GST, glutathione-S-transferase; LPO, lipid peroxidation; MDA, malondialdehyde; MPO, N-methyl-2-phenylindole; MTT, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, nucleic acid content; NRU, neutral red uptake; PBS, phosphate-buffered saline; PBS-Ca^{2+}, phosphate buffered saline calcium; ROS, reactive oxygen species. HB, homogenization buffer.

Figure 1

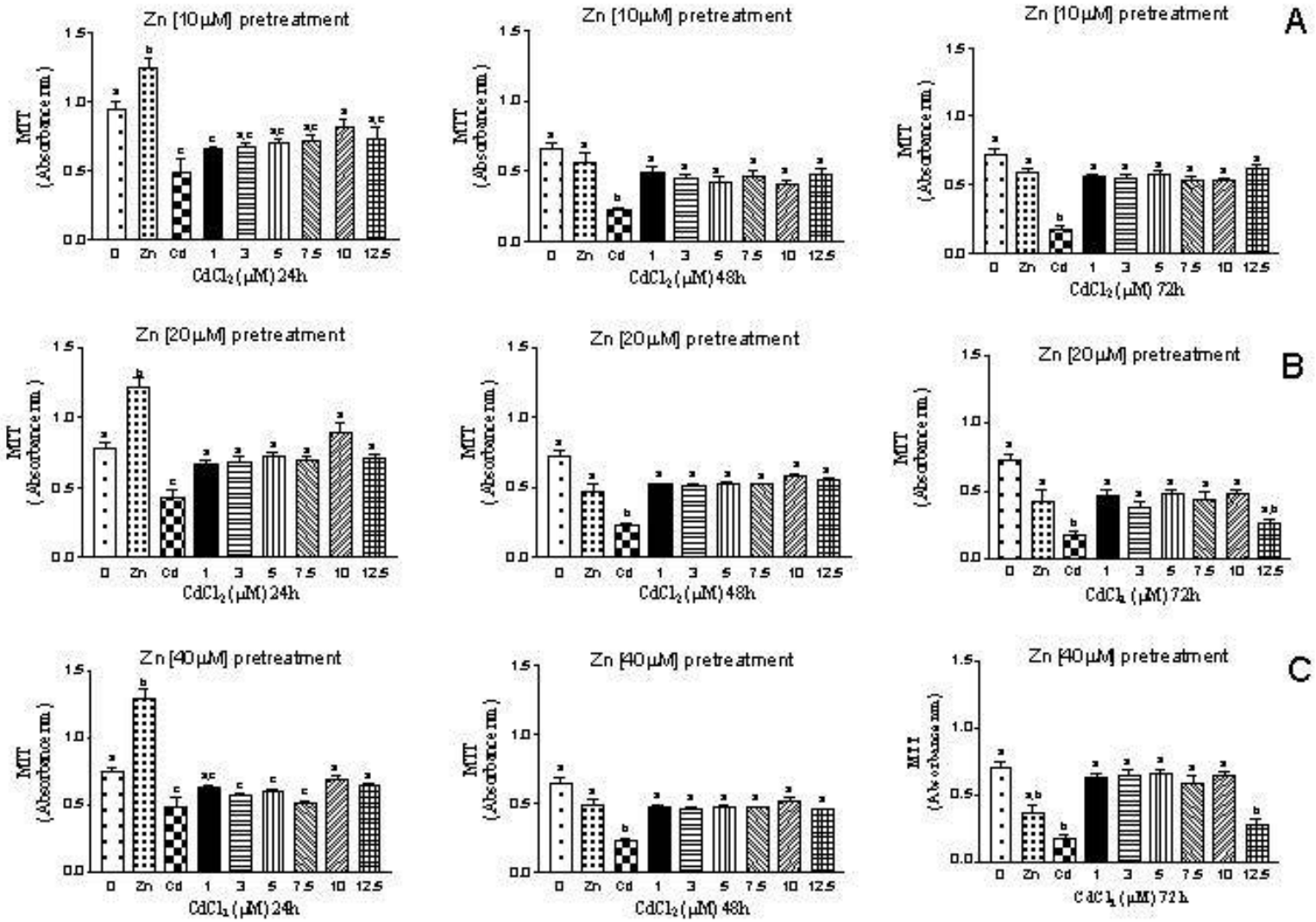

Zn [10μM] pretreatment
Zn [10μM] pretreatment
Zn [10μM] pretreatment
A
Zn [20μM] pretreatment
Zn [20μM] pretreatment
Zn [20μM] pretreatment
B
Zn [40μM] pretreatment
Zn [40μM] pretreatment
Zn [40μM] pretreatment
C
MTT (Absorbance nm)
0 Zn Cd 1 3 5 7.5 10 12.5
CdCl2 (μM) 24h
CdCl2 (μM) 48h
CdCl2 (μM) 72h

Figure 2

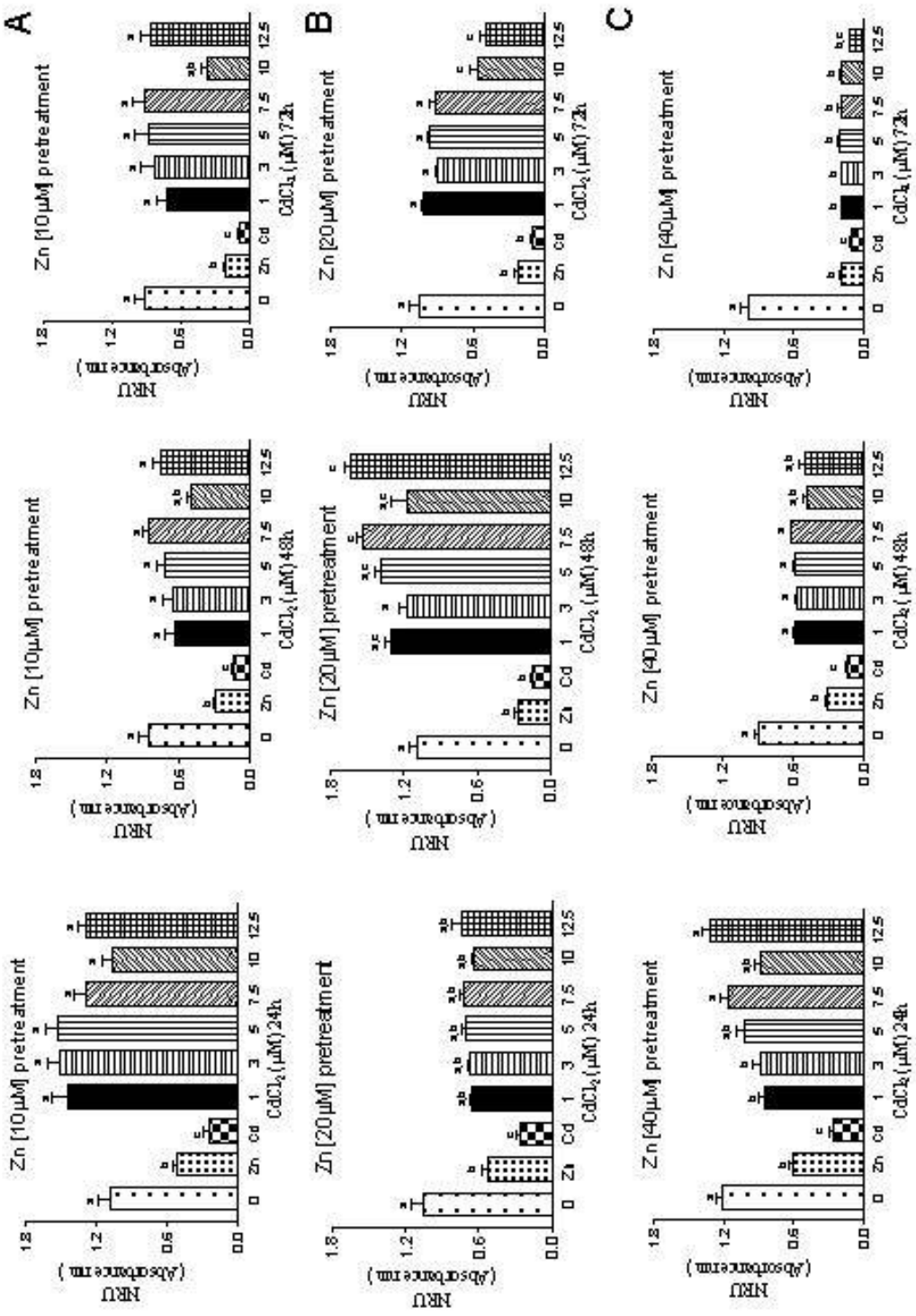
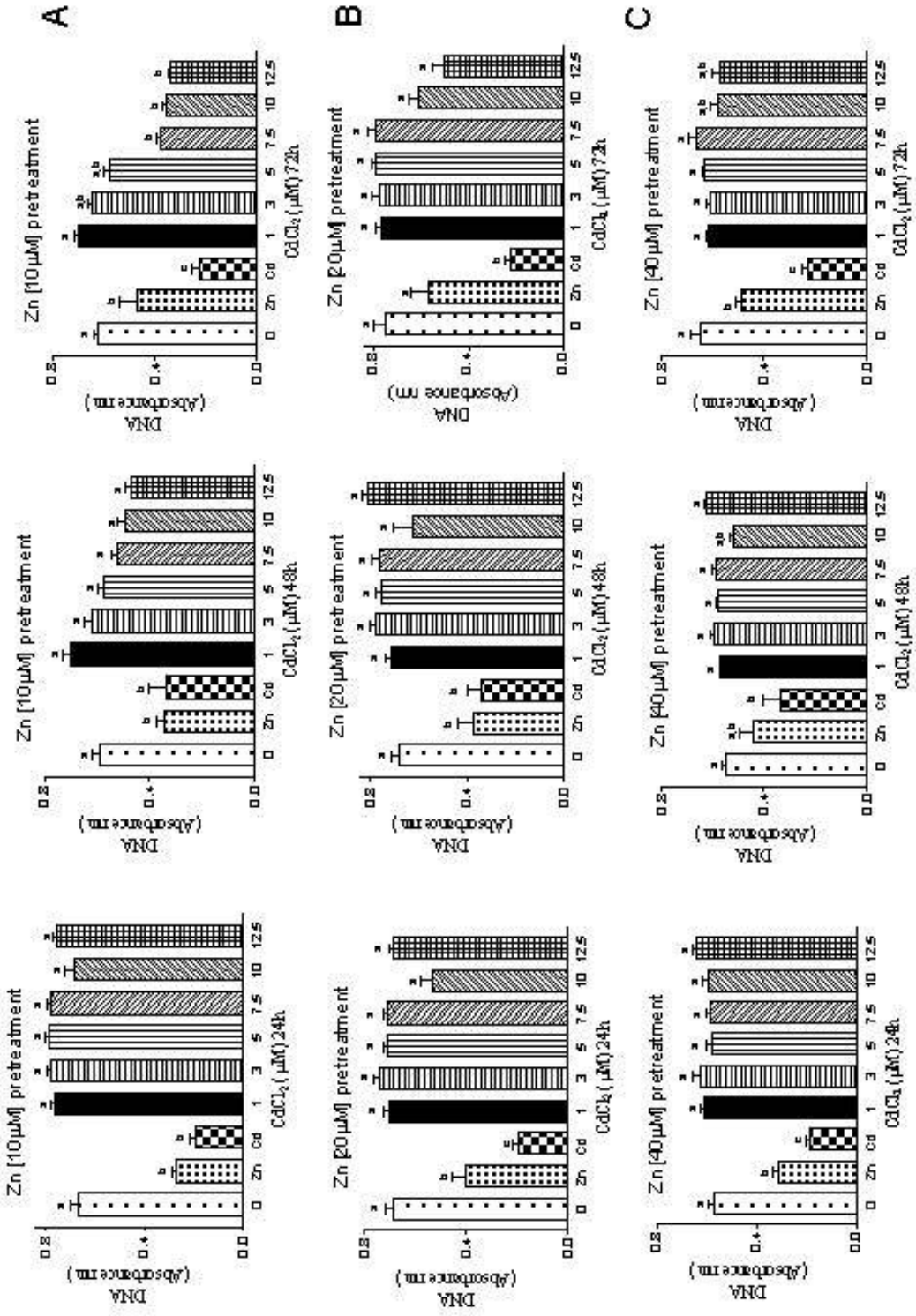

A
B
C
Zn [10μM] pretreatment
Zn [20μM] pretreatment
Zn [40μM] pretreatment
NRU (Absorbance nm)
CdCl2 (μM) 24h
CdCl2 (μM) 48h
CdCl2 (μM) 72h
0 Zn Cd 1 3 5 7.5 10 12.5

Figure 3


A
B
C
Zn [10μM] pretreatment
Zn [20μM] pretreatment
Zn [40μM] pretreatment
DNA (Absorbance nm)
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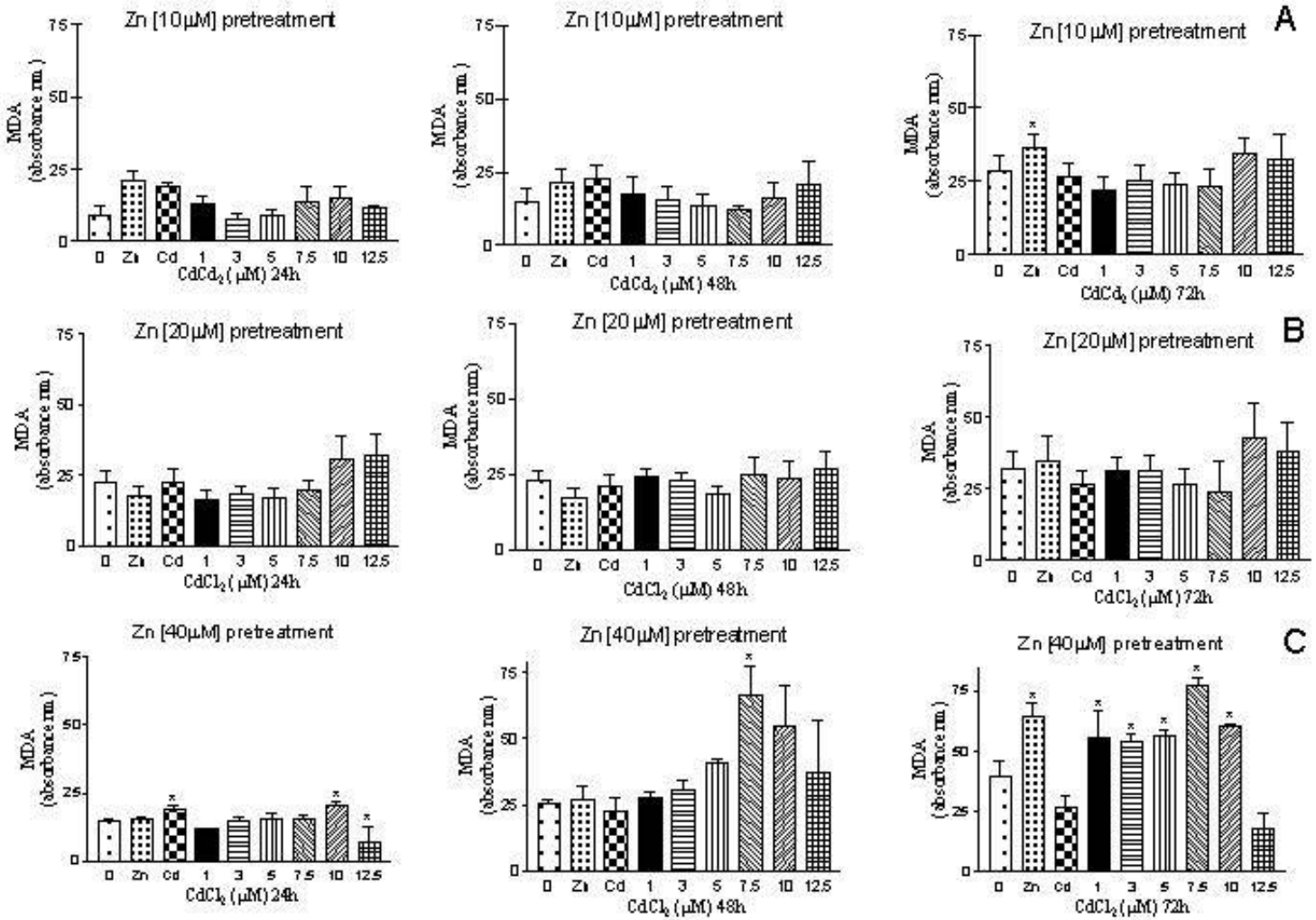
CdCl2 (μM) 24h
CdCl2 (μM) 48h
CdCl2 (μM) 72h

Figure 4


Zn [10μM] pretreatment
MDA (absorbance nm)
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CdCd2 (μM) 24h
Zn [10μM] pretreatment
CdCd2 (μM) 48h
Zn [10 μM] pretreatment
A
CdCd2 (μM) 72h
Zn [20μM] pretreatment
CdCl2 (μM) 24h
Zn [20 μM] pretreatment
CdCl2 (μM) 48h
Zn [20μM] pretreatment
B
CdCl2 (μM) 72h
Zn [40μM] pretreatment
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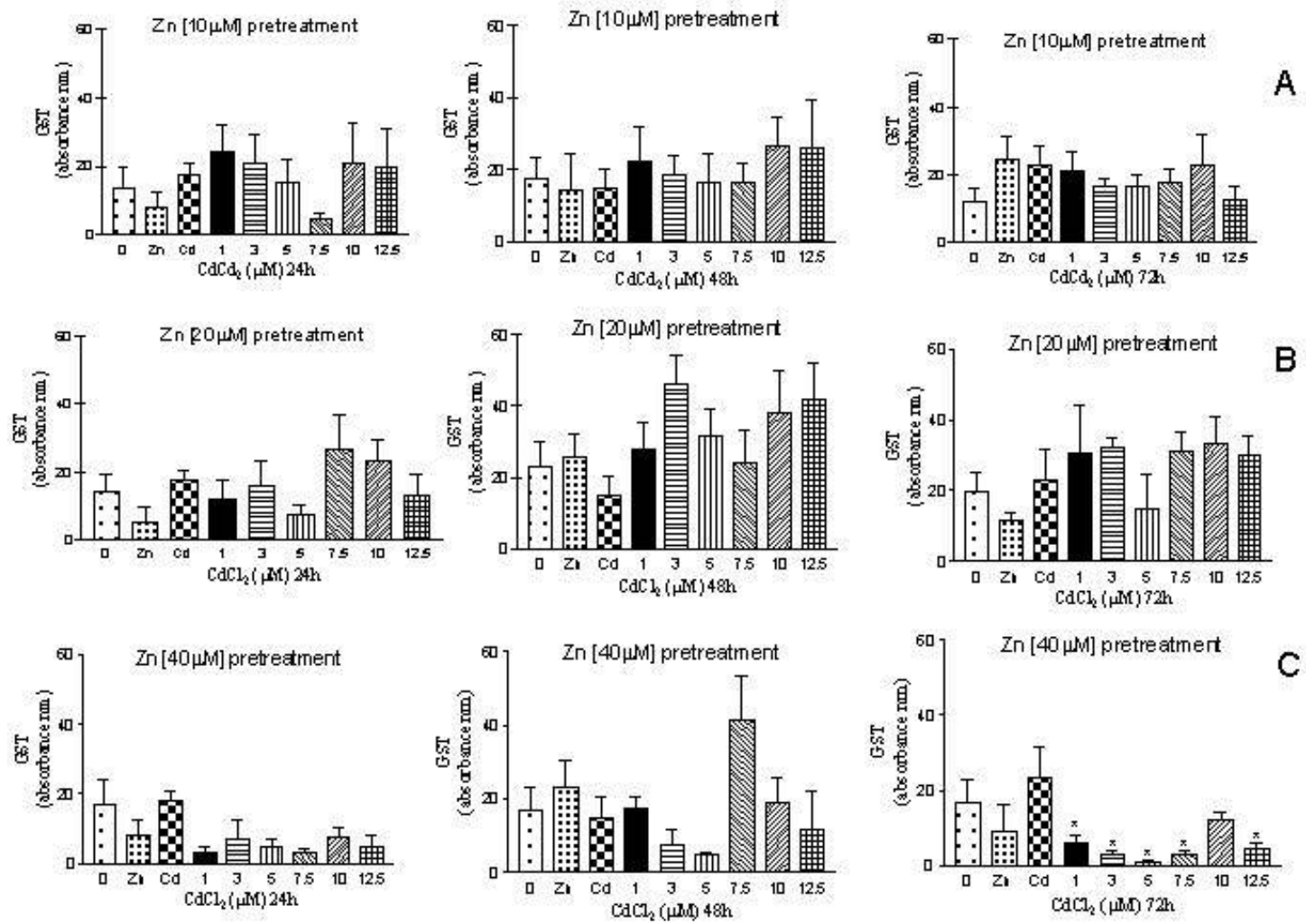
Zn [40μM] pretreatment
CdCl2 (μM) 48h
Zn [40μM] pretreatment
C
CdCl2 (μM) 72h

Figure 5


Zn [10μM] pretreatment
GST (absorbance nm)
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CdCl2 (μM) 24h
Zn [10μM] pretreatment
CdCl2 (μM) 48h
Zn [10μM] pretreatment
CdCl2 (μM) 72h
A
Zn [20μM] pretreatment
CdCl2 (μM) 24h
Zn [20μM] pretreatment
CdCl2 (μM) 48h
Zn [20 μM] pretreatment
CdCl2 (μM) 72h
B
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Zn [40μM] pretreatment
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Zn [40 μM] pretreatment
CdCl2 (μM) 72h
C

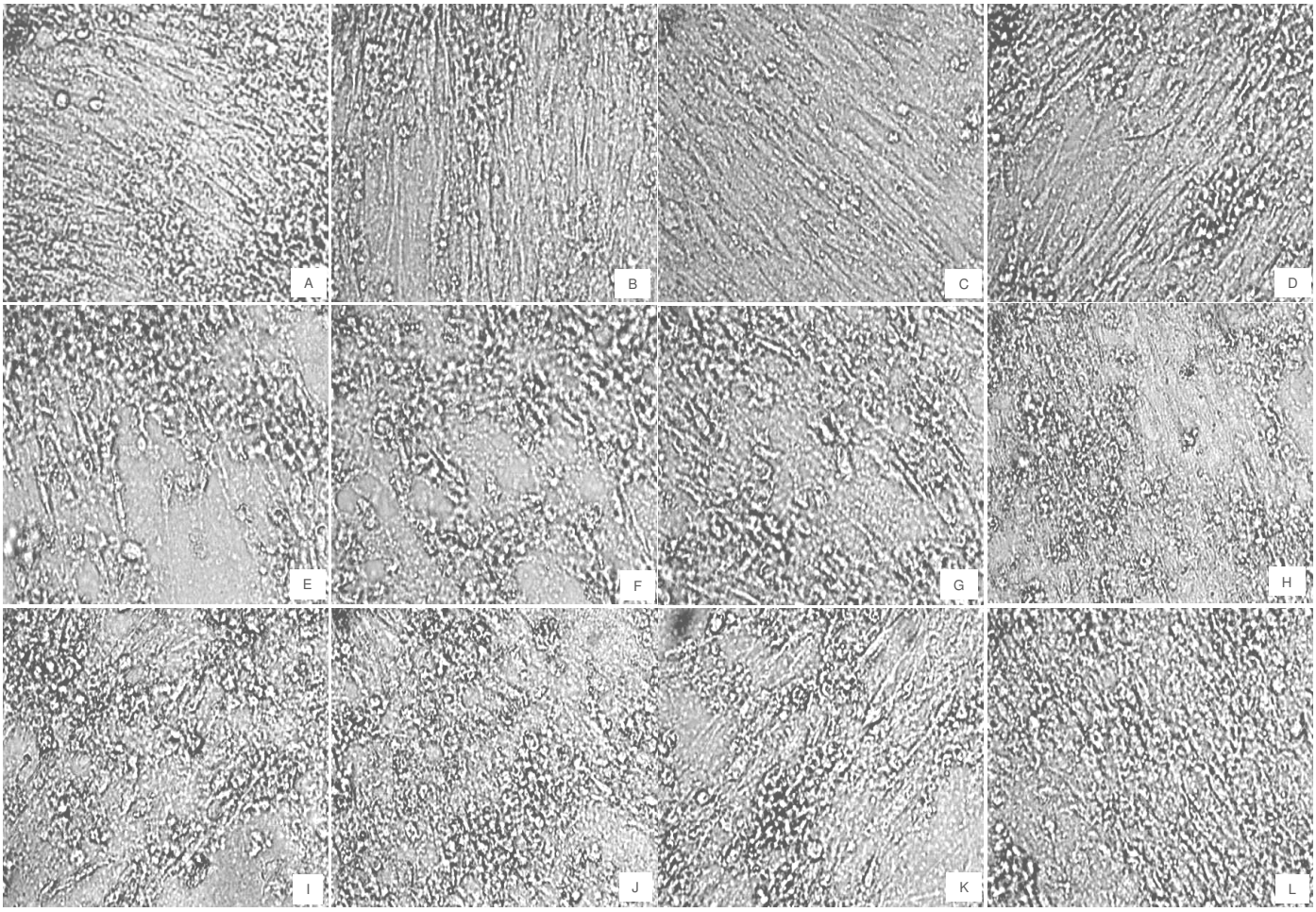

Figure 6

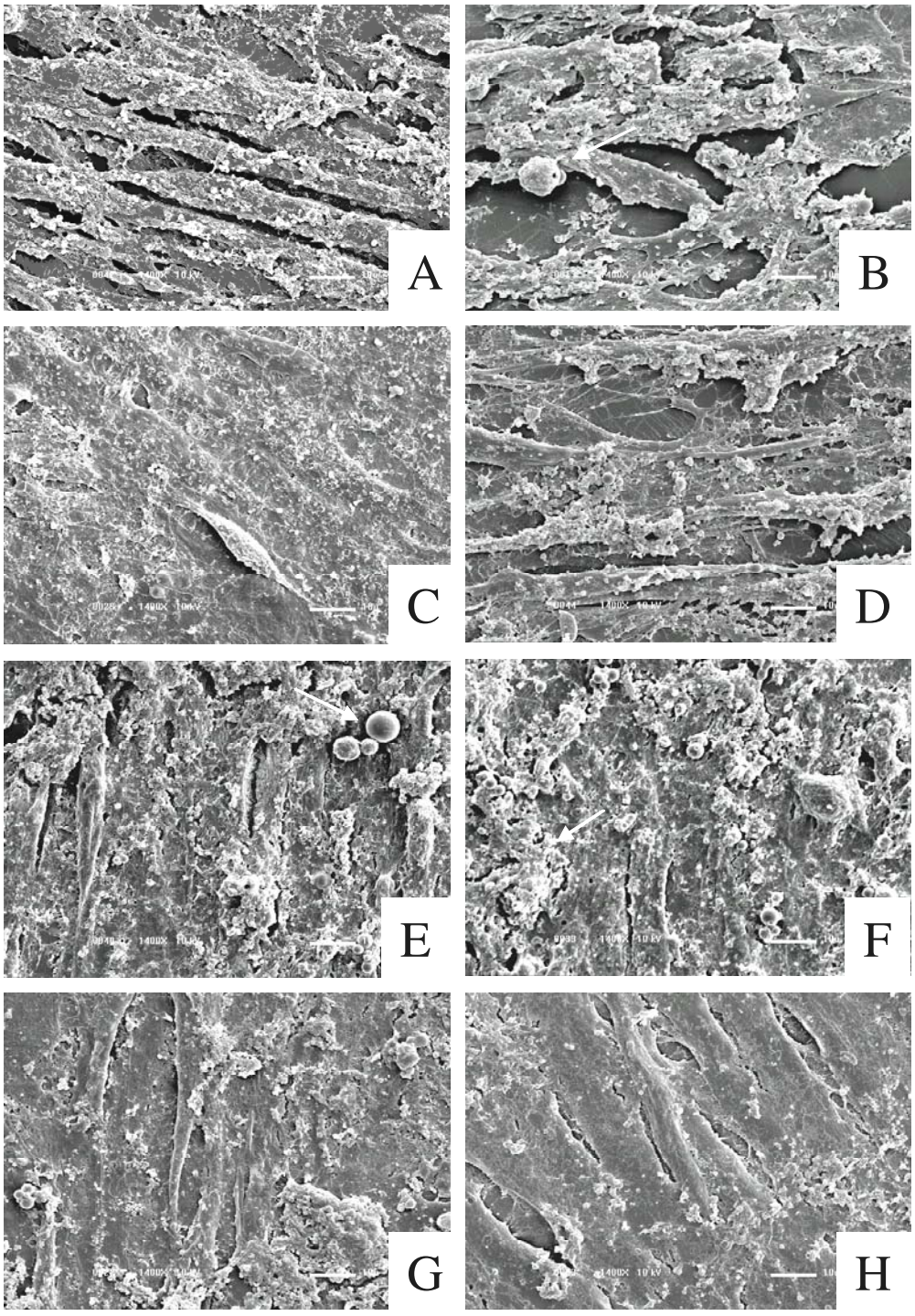
A
B
C
D
E
F
G
H

Figure 7

Capítulo III

"Cloreto de cádmio altera a morfologia de células tumorais de adenocarcinoma de cólon - linhagem MAC 13."

Trabalho a ser submetido ao periódico *International Journal of Cancer* .

No presente trabalho foram analisados os efeitos em células tumorais *in vitro* à exposição crônica ao cloreto de cádmio. Células do adenocarcinoma de cólon MAC13 foram tratadas com $CdCl_2$ nas concentrações de 1; 3; 5; 7,5; 10; e 12,5 μM, pelo período de 24, 48, e 72 h, e análises quanto aos ensaios de MTT (3-(4,5-*dimethylthiozol*-2-yl)-2,5-*diphenyltetrazolium bromide*), captação do vermelho neutro e conteúdo de ácido nucléico. Além disso, avaliação da atividade enzimática da glutationa-S-transferase (GST) foi diminuída nas concentrações de $CdCl_2$ 3, 5 e 10μM após 24hs e 5 a 12,5 μM, após 48 horas, comparado às células controle. Contudo, após 72h ocorreu diminuição do conteúdo de malondialdeído nas concentrações de 5 a 12,5μM de cloreto de cádmio. Análises da morfologia dessas células, observadas através de microscopia de luz e microscopia eletrônica de varredura, mostraram características típicas de possíveis alterações da atividade celular tumoral.

"CADMIUM CHLORIDE ALTERS THE PHENOTYPE OF MAC13 TUMOR CELLS."

Claudia Lumy Yano, Maria Cristina Cintra Gomes Marcondes.
Departamento de Fisiologia e Biofísica e Departamento de Biologia Celular, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, 13083-970, Campinas, São Paulo, SP, Brazil.

Abstract

This study was designed to determine the effects of chronic cadmium chloride $CdCl_2$ exposures on tumor cell and evaluate the possible changes in cellular activity *in vitro*. Colon adenocarcinoma MAC13 cells were treated with $CdCl_2$ (1, 3, 5, 7.5, 10, and 12.5 μM) for 24, 48, and 72 h. After this the cell homogenates were subsequently assessed for MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide) reduction, neutral red uptake and nucleic acid content showing decrease on lysosomal activity (at 5 and 12.5μM cadmium chloride) and nucleic acid content (at 3 to 12.5 μM $CdCl_2$) after 72h. Glutathione-S-transferase activity (nmol μg of protein^{-1} min^{-1}) was decreased at 5 to 12.5μM when compared to control cells, after 72h. An increase in malondialdehyde content (nmol μg of protein^{-1}) was observed at 3, 5 and 10μM after 24h and 5 to 12.5 μM $CdCl_2$ concentrations after 48h compared to the control cells. However, after 72 h of treatment, a decreased on malondialdehyde content was verified at 5 to 12.5μM cadmium chloride concentrations. Light and scanning electron microscopy showed morphological alteration, especially after 72h, with feature typical of cellular progression and aggressive behavior.

Keywords: Cadmium chloride; Mac13 tumor cells, oxidative stress, progression

* Corresponding author.

E-mail address: cintgoma@unicamp.br (M.C.C.G. Marcondes).

Introduction

Cancer remains the curse of modern society and one of the most challenger research fields. It is initially a localized disease that can be often treated well at a very early stage, however the vast majority of cancer deaths result from a pernicious progression of the disease, the development of distant metastases. Thus, the short-term goal the pharmacological prevention is thus elongate the survival time of the cancer patients with a maximum of life quality, for this goal the understanding of the growth and metastasis development are the critical parameters [1]. Cadmium (Cd) is classified as a known human carcinogen [2] and highly toxic agent [3,4], and its half-life in humans is estimated to be between 15 and 20 years [5], which would be clear drawbacks to any pharmaceutical application. However, cadmium can be effective as an anti-tumor agent in mice even when given well after tumors were formed through what appears to be a tumor-specific effect [6]. Cadmium-induced tumor suppression could be accomplished with doses that were not overtly toxic [6], which would be a positive attribute for any cancer chemotherapeutic. Otherwise, has been suggested that cadmium, under certain circumstances, may act as a 'tumor progressor'[7], considerable evidence indicates that cadmium may be involved in the initiation and/ or progression of some types of cancer, but the specific mechanisms still not understood [8]. The association between multiple Cd exposures and enhanced metastatic potential of the ensuing tumors may have important implications in industrials workers exposed to Cd, or in cases of co-exposure of Cd with organic carcinogens, as in tobacco smoking [6]. Cancer chemotherapy has gradually improved with the development of novel anti-tumor drugs and with positive results when applied to many hematologic malignancies, some solid tumours and childhood malignancies [9]. Effective cancer chemotherapy may be impaired severely by the presence of drug-resistant cells within a tumour population. Some malignant tumours are intrinsically resistant to standard anti-neoplastic agents, whereas others respond initially to chemotherapy and then relapse [10]. Medicinal application of metals was stimulated by the discovery of cisplatin that dominated the treatment of various cancers by chemical agents [11]. Despite the success of cisplatin, however, it lacks selectivity for tumor tissue, which leads to severe side effects, which are only partially reversible when the treatment is

stopped. The pharmaceutical use of metal complexes therefore has excellent potential to clinical therapeutic the some tumors [12]. Cadmium, as a class of anti-neoplastic drugs generally has a very narrow therapeutic index with a greater potential for harmful side-effects than most other categories of pharmaceuticals [13]. In fact, many cancer chemotherapeutics are also potential human carcinogens, including cisplatin [14]. Cadmium is a toxic heavy metal with pro-apoptotic potential in various cells *in vivo* and *in vitro* [15,16]. However, several studies have demonstrated that cadmium can also be anti-apoptotic in some circumstances [17,18]. Apoptosis is a cellular process by which damaged cells actively facilitate their own demise without damaging their neighbors, thus selectively removing themselves from the cellular population [19]. This selective nature is the preferred mode of action of cancer chemotherapeutics [20]. Another important factor in apoptosis is the excessive generation of reactive oxygen species (ROS) [21]. Studies have demonstrated cadmium induced reactive oxygen species production, and caused damage consistent with oxidative stress [22,23]. Thus, this study was designed to determine the effects of cronic Cd exposures on MAC 13 colon tumor cell and possible inhibitory potential and/or tumoral invasive *in vitro*.

Materials and Methods

Cell culture

MAC 13 colon adenocarcinoma cells were generously provided by Dr. Michael J. Tisdale (Laboratory of Cancer Research, Aston University, Birmingham, UK). The cultures were grown in tissue culture flasks (Corning, NY) in RPMI medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Sigma), 1% penicillin, and 1% streptomycin (Sigma) at 37°C in a humidified atmosphere of 5% CO_2. All of the experiments were initiated using cells grown to 90–100% confluence. MAC 13 cells were treated with chronic cadmium chloride $CdCl_2$ (Sigma), 1, 3, 5, 7.5,10, and 12.5 μM for 24h, 48h and 72h.

Cytotoxicity assays

The viability of control and MAC 13 cell was assessed based on MTT reduction, neutral red uptake (NRU) and nucleic acid content (NAC). The MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a sensitive, quantitative colorimetric assay that measures cell viability based on the ability of mitochondrial succinyl dehydrogenase in living cells to convert the yellow substrate MTT into a dark blue formazan product. For the assay, the medium containing MAC 13 was removed and a solution containing 0.01% MTT was added to each well. After incubation for 10 min at 37ºC degrees, the medium was removed and the formazan solubilized in ethanol. The plate was shaken for 30 min and the absorbance was measured at 570 nm [24]. The NRU assay is a cell viability test based on the incorporation of dye into the lysosomes of viable cells following incubation with the test agents. After removal of the medium from the plates, a solution of 0.05% neutral red was added to each well followed by incubation for 3 h at 37°C degrees. The cells were then washed with phosphate-buffered saline containing calcium (PBS-Ca^{2+}), followed by the addition of 1% glacial acetic acid and 50% ethanol to each well to fix the cells and extract the neutral red incorporated into the lysosomes. The plates were shaken for 20 min and the absorbance was measured at 540 nm [25]. For the NAC assay, monolayers of cells were solubilized with 0.5 N NaOH at 37°C degrees for 1 h and the absorbance was measured at 260 nm; the results were expressed as a percentage of the control [26].

Analytical methods

After 24, 48 and 72h of treatment with $CdCl_2$ for 24, 48 and 72h, the cells were washed with cold PBS and collected in homogenization buffer (HB) (20 mM Tris, 1 mM DTT, 2 mM ATP and 5 mM $MgCl_2$, pH 7.2), and centrifuged at 10,000 rpm for 15 min at 4ºC. Aliquots of homogenate supernatants were analyzed for glutathione-S-transferase (GST) activity based on the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione and the activity was expressed in nmoles per microgram of protein per minute, using an extinction coefficient of 9.6, as described by Habig et al. [27]. The malondialdehyde (MDA) content, a lipid peroxidation product, was determined

using MPO (N-methyl-2-phenylindole) as the substrate, the absorbance was measured at 590 nm and the results were expressed in nmoles per milligram protein [28]. The protein content was measured by the method of Lowry et al. [29].

Light (LM) and scanning electron (SEM) microscopy

MAC 13 were cultured on coverslips and treated with various concentrations of $CdCl_2$ for 24, 48 and 72h prior to analysis by LM (Leica DMLM, Wetzlar, Germany). For SEM, other cells were fixed in 2.5% paraformaldehyde/glutaraldehyde (Sigma) in 0.1 M PBS, pH 7.4, and then washed in PBS followed by postfixation with 1% osmium tetroxide (Sigma) and dehydration in a graded ethanol series. The cells were then critical-point-dried (CPDO030–Balzers, BAL-TEC AG, Wiesbaden, Germany) and gold-sputtered (SCD050–Balzers) before being analyzed in a scanning electron microscope (JSM-5800LV, JEOL, Peabody, MA) operated at 1 kV.

Statistical analysis

The results were expressed as the mean ± SE. One-way ANOVA followed by Bonferroni's test for multiple comparisons [30] was used to compare the MAC 13 cells treatments with the control. A value of $P < 0.05$ indicated statistical significance.

Results

In this study, MAC 13 colon adenocarcinoma cells were treated with various concentrations of cadmium chloride ($CdCl_2$) (3–12.5 μM) for 24, 48, and 72 h to assess the effect of this metal. Increased mitochondrial activity after treatment cadmium chloride was observed after 24h at 3, 5, 7.5,10 and 12.5μM, however after 48h was observed the decrease in higher cadmium chloride concentration 12.5μM and none significative difference after 72h through MTT assay (Figure 1A).Otherwise, NRU assay showed the decrease on lysosomal activity in treatment cadmium chloride at 5 to 10μM after 48 and 72 h, respectively (Figure 1B). The inhibition of growth cell measured by NAC was verified in 12.5μM cadmium chloride treatment after 24h (Figure 1C). These results reflected a

greater toxicity towards lysossomes. The effects of $CdCl_2$ in MAC 13 cells were also assessed by measuring the glutathione-S-transferase activity (GST) and lipid peroxidation (MDA formation).The results clearly indicated that chronic $CdCl_2$ exposure induced changes on cellular activity of MAC 13 tumor. The GST activity in Mac 13 tumor cells was statistically decreased after cadmium chloride exposure for 72hs (Figure 2A). The increase the MDA was significative after 48h (Figure 2B) and protein content was decrease at 7.5 and 12.5µM of $CdCl_2$ after 48h (Figure2C). The light microscopy images showed that there were no morphological changes in MAC 13 treated with 3, 5, 7.5, 10, and 12.5 µM of $CdCl_2$ for 24h (Figure 3) and apparently decreased the viability cellular after 48h at 5, 7.5, 10 and 12.5 µM $CdCl_2$ concentrations (Figure 4). The marked morphologic alterations could be observed after 72h at all chronic cadmium chloride exposure. MAC 13 cells showed presence of cell vacuoles and many cellular fragments, typical feature the apoptosis process (Figure 5B, C). Morphological changes were also seen in SEM images showing that Mac 13 cells had rounded shape and colony cellular growth (Figure 6A). The treatment with chronic cadmium chloride at 10 µM (Figure 6B,C) and 12.5 µM (Figure 6D-F) for 72h showed cells with morphological feature equivalent to control cells, however the apparent decrease on cell proliferation, and marked difference was observed through the extracellular matrix (ECM) alteration (Figure 6 B-F). This alteration of ECM is probable indicative of the invasiveness and aggressiveness of tumor cells.

Discussion

Resistance to chemotherapy is the major concern in treatment of the most solid tumours [31, 32]. The drug resistance can also be associated with decreased cell proliferation, cell-cell contact and adhesion of cancer cells to extracellular matrix. In addition, the microenvironmental stress conditions may select tumor cells that have decreased apoptotic potencial through genetic alterations, thereby leading to the resistance to apoptosis induction by antitumor drugs [9]. Additionally, stress conditions also induce drug resistance without genetic alterations in tumor cells [9]. In fact, many tumors are intrinsically resistant to many of the more potent cytotoxic agents used in cancer therapy. Other tumors, initially sensitive, became recurrent and are resistant not only to the initial therapeutics agents, but also to other drugs [9]. The chronic cadmium chloride exposure to

Mac 13 tumor cells induced reactive oxygen species production and a later decrease. This is supported by decreased intracellular concentrations of ROS scavengers, such as glutathione (GSH). Cd^{+2} ions mobilize GSH, compromising the cellular defense mechanism against oxidative stress, many times associated with mutagenesis and carcinogenesis [33]. However, either continuous exposure or exposure to toxic doses of cadmium may overwhelm the cellular supply of GSH and the related defense system so as the result to toxicity, including carcinogenesis [3]. Mac 13 tumor cells shows high mitochondrial function, increase RSO and glutathione (GST) depletion, these results indicate escape to cell death process and possible adaptive tolerance or increased resistance to cadmium chloride. This resistance can contribute to a more aggressive behavior. Cells develop tolerance to cell death, generally due to perturbation of the JNK signaling pathway and the nonresponsiveness of JNK phosphorylation reverting cadmium-sensitive phenotype in adapted cells [34]. Jin et al. (2003) [35] observed that chronic exposure of environmentally relevant concentrations of cadmium can result in extreme hypermutability. Most information regarding mechanisms of resistance derives from *in vitro* models of cells selected by exposure to extremely high levels of drugs that are not of clinical relevance. These studies have shown many mechanisms of resistance and that resistance is often multifactorial. Selection of cells in vitro for resistance to a variety of anticancer drugs may result in the development of cross-resistance to other, structurally unrelated drugs [10]. Resistance is often multifactorial, and a tumour does not consist of completely sensitive or completely resistant cells but a continuous spectrum of cells with different levels of sensitivity [36]. Several reports have suggested an important role of glutathione in human multidrug resistance-associated protein MRP1-mediated drug efflux [38]. It is possible that glutathione as well as anticancer drugs interact directly with MRP1 and that this interaction is necessary for transport [39]. However, the mechanism by which glutathione facilitates transport has not yet been fully elucidated [38]. The induction of apoptosis by cadmium is not necessarily protective against malignant transformation [15]. Achanzar et al. (2000) [15] treated normal human prostate cells with cadmium and observed the induction of the proto-oncogenes c-jun and c-myc, and the tumor suppressor gene p53. Only a fraction of the cells underwent apoptosis, whereas 35% of the cells exhibited increased metallothionein and stayed viable, suggesting a selection of apoptosis-

resistant cells. Further evidence for an acquired apoptotic resistance to cadmium-adapted fraction of prostate cells is indicated by the down-regulation of apoptotic caspases and the increased expression of the antiapoptotic protein Bcl-2 [40]. Cadmium-adaptation may also inhibit apoptosis allowing the accumulation of critical mutations and favoring the genes expression and stress response genes of pre-neoplastic cells towards tumor development [41]. An inverse relationship has been noticed between the metallothioneins content and sensitivity of cultured cells and tissues of animals to cadmium exposure [42] and susceptibility to apoptosis [43]. Intracellular localization of metallothionein has been reported to be an important determinant of resistance to oxidative stress [44]. Koropatnick & Pearson (1993) [45] showed that perturbation of both drug resistance and cellular homeostasis occurs in cells with genetically altered metallothionein biosynthesis. With relation to the aggressive behavior of Mac13 tumor cells, recently the theory to explain cadmium carcinogenesis has been the correlation between the loss of E-cadherin expression or function and tumor cell metastasis and invasion [46]. (Beavon, 2000) since cadmium could bind to a polypeptide which corresponds to one of the extracellular Ca^{+2}-binding regions of mouse E-cadherin, changing its conformation [47], damaging the E-cadherin-dependent junctions between cells [48]. In contrast, calcium may act as an anticarcinogen [49], activating the E-cadherin and suppressing the β-catenin [50], the displacement of calcium from E-cadherin by cadmium possibly contributes to abnormal differentiation and malignant progression. The disruption of cell–cell adhesion caused by cadmium binding to this protein could represent a crucial step in both the initiation of cancer and in tumor promotion [51]. The present results are in agreement with Waalkes et al. (2000) [7], who indicate that repeated exposures to the carcinogenic, inorganic Cd can result in the more rapid onset of more highly aggressive tumors and more experiments are underway to clear the pathway which cadmium could act as potent carcinogen. The mechanism of this effect is yet undefined, but this could have an important impact on hazards posed by multiple Cd exposures alone or in combination with exposure to other carcinogens.

Acknowledgments

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Figures Legend

Figure 1. Effect of $CdCl_2$ on MAC 13 cell viability assessed by the MTT reduction (A), NRU (B), and NAC (C) assays. MAC 13were incubated with 1, 3, 5, 7.5, 10, and 12.5 µM of $CdCl_2$ for 24, 48, and 72 h. The columns are the mean ± SE of triplicate experiments. *P < 0.05 compared to untreated cells (ANOVA).

Figure 2. Effects of MAC 13 on glutathione-S-transferase activity (A), lipid peroxidation (B), malondialdehyde (MDA) levels, and protein content (C). The columns are the mean ± SE of triplicate experiments. Different letters indicate significant (P < 0.05) differences.

Figure 3. Morphological observation of confluent cultures of MAC13 tumor cells after treatment with different cadmium chloride concentration for 24h. (A) Untreated confluent cultures of MAC13 tumor cells. (B) MAC13 tumor cells were treated with cadmium chloride at 3µM/mL (C) MAC13 tumor cells were treated with cadmium chloride at 5µM/mL. (D) MAC13 tumor cells were treated with cadmium chloride at 7.5µM/mL. (E) MAC13 tumor cells were treated with cadmium chloride at 10µM/mL. (F) MAC13 tumor cells were treated with cadmium chloride at 12.5µM/mL. None morphological alteration is observed in all cadmium chloride treatments. Note the confluent adhesion cellular (x 400).

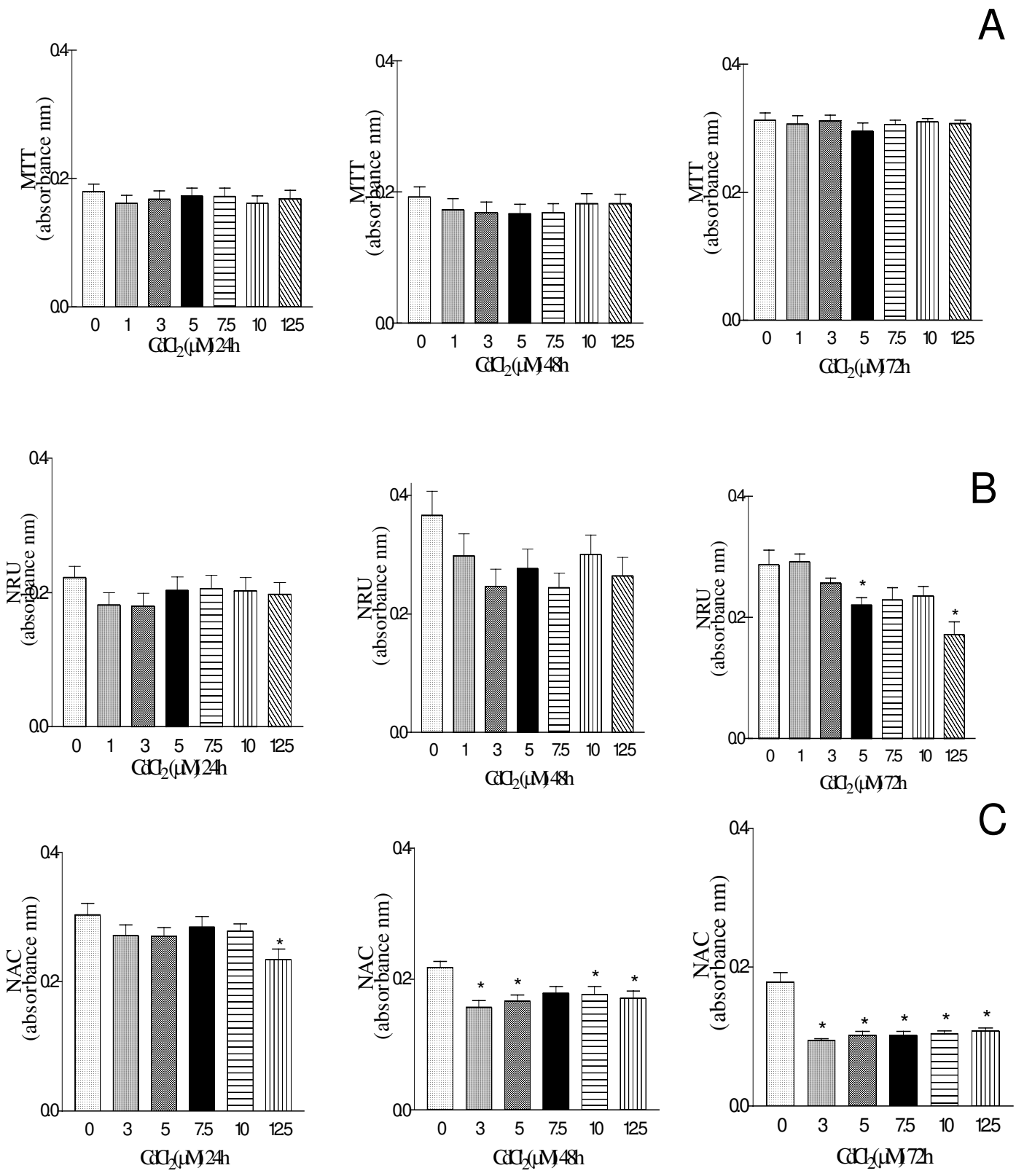
Figure 4. Morphological observation of confluent cultures of MAC13 tumor cells after treatment with different cadmium chloride concentration for 48h. (A) Untreated confluent cultures of MAC13 tumor cells. (B) MAC13 tumor cells were treated with cadmium chloride at 3µM/mL (C) MAC13 tumor cells were treated with cadmium chloride at 5µM/mL. (D) MAC13 tumor cells were treated with cadmium chloride at 7.5µM/mL. Note the cellular division (arrow). (E) MAC13 tumor cells were treated with cadmium chloride

at 10μM/mL. (F) MAC13 tumor cells were treated with cadmium chloride at 12.5μM/mL (x 400).

Figure 5. Morphological observation of confluent cultures of MAC13 tumor cells after treatment with different cadmium chloride concentration for 72h. (A) Untreated confluent cultures of MAC13 tumor cells. (B) MAC13 tumor cells were treated with cadmium chloride at 3μM/mL. Presence of vacuole cellular; typical feature the apoptotic process. (arrow) (C) MAC13 tumor cells were treated with cadmium chloride at 5μM/mL. Note the presence the fragmented cells (D) MAC13 tumor cells were treated with cadmium chloride at 7.5μM/mL. Cellular division presence, that can be indicative the possible cadmium chloride exposure resistance. (arrow).(E) MAC13 tumor cells were treated with cadmium chloride at 10μM/mL. Still having the presence the many fragments cellular. (arrow). (F) MAC13 tumor cells were treated with cadmium chloride at 12.5μM/mL. Note the morphological alteration with feature the possible invasion tumoral (x 400).

Figure 6: Scanning electron micrographs of Mac 13 colon adenocarcinoma cells after 72 hours of chronic cadmium chloride exposure at (0, 10 and 12.5 μM/mL). (A), Cd untreated cells shows the Mac 13 cells rounded shape and growth cellular in colony. (B, C), The treatment with chronic cadmium chloride at 10 μM showed the cells with feature morphological equivalent to control cells (arrow) and the significative extracellular matrix (ECM) alteration. (D-F), Mac 13 cells treated with 12.5μM $CdCl_2$ showing also ECM alteration (arrow) and presence the cells with morphological shape normal (arrow). This alteration of ECM is probable indicative the invasiveness/ aggressive tumor cells.

Abbreviations: Cd, cadmium; $CdCl_2$, cadmium chloride; CDNB, 1-chloro-2,4-dinitrobenzene; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GST, glutathione Stransferase; LPO, lipid peroxidation; MDA, malondialdehyde; MPO, N-methyl-2-phenylindole; MTT, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, nucleic acid content; NRU, neutral red uptake; PBS, phosphate-buffered saline; PBS-Ca^{2+}, phosphatebuffered saline calcium; ROS, reactive oxygen species. HB, homogenization buffer; MRP1, multidrug resistance-associated protein ; HSPs, heat-shock proteins; SOD,superoxide dismutase; MT, Metallothioneins ; ECM, extracellular matrix.


A
MTT (absorbance nm)
0.4
0.2
0.0
0 1 3 5 7.5 10 125
CdCl2 (μM) 24h
CdCl2 (μM) 48h
CdCl2 (μM) 72h
B
NRU (absorbance nm)
*
C
NAC (absorbance nm)
*

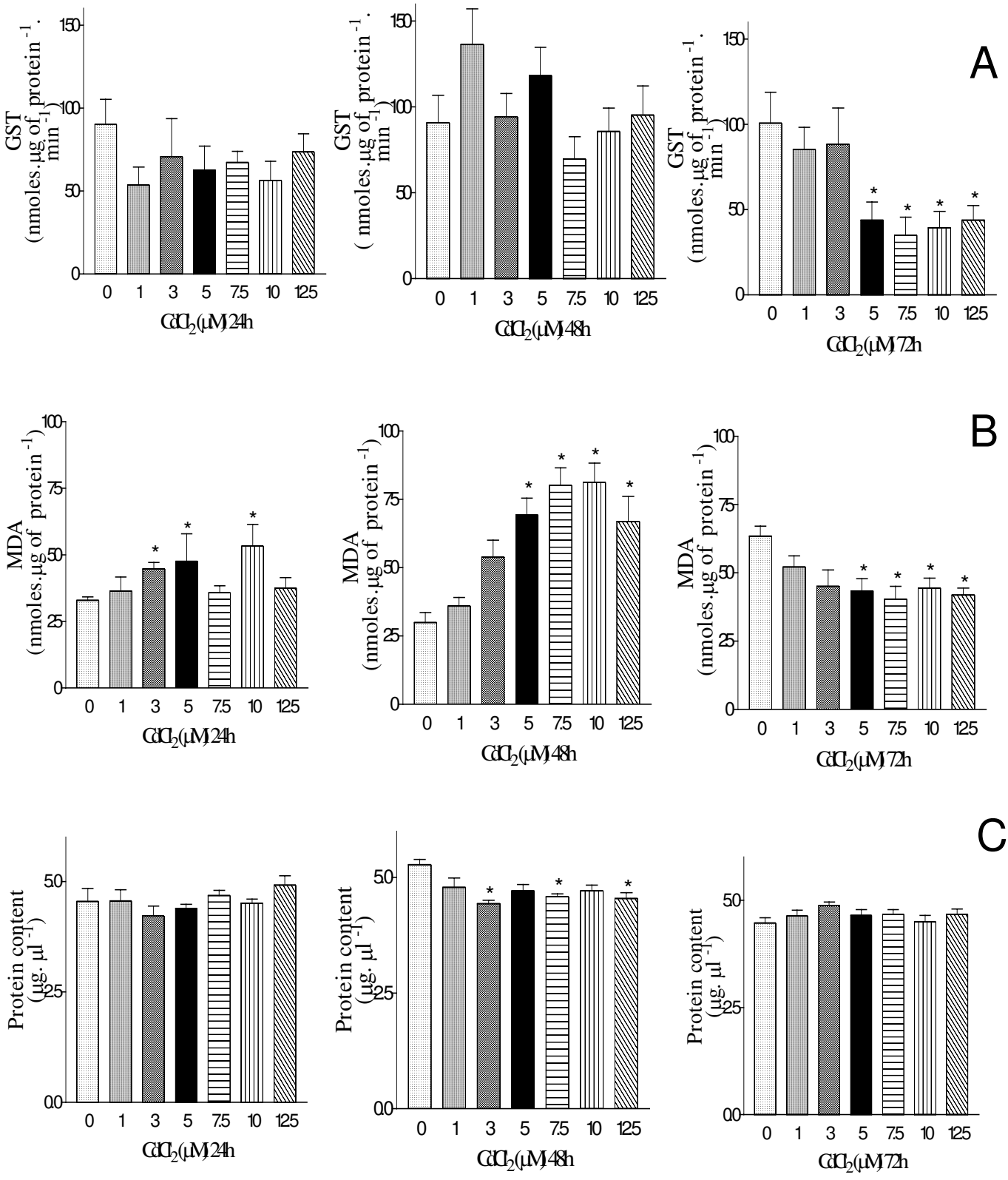

A
GST (nmoles.µg of protein-1. min-1)
0 1 3 5 7.5 10 125
CdCl2(µM),24h
CdCl2(µM),48h
CdCl2(µM),72h
B
MDA (nmoles.µg of protein-1)
C
Protein content (µg. µl-1)

Figure 3

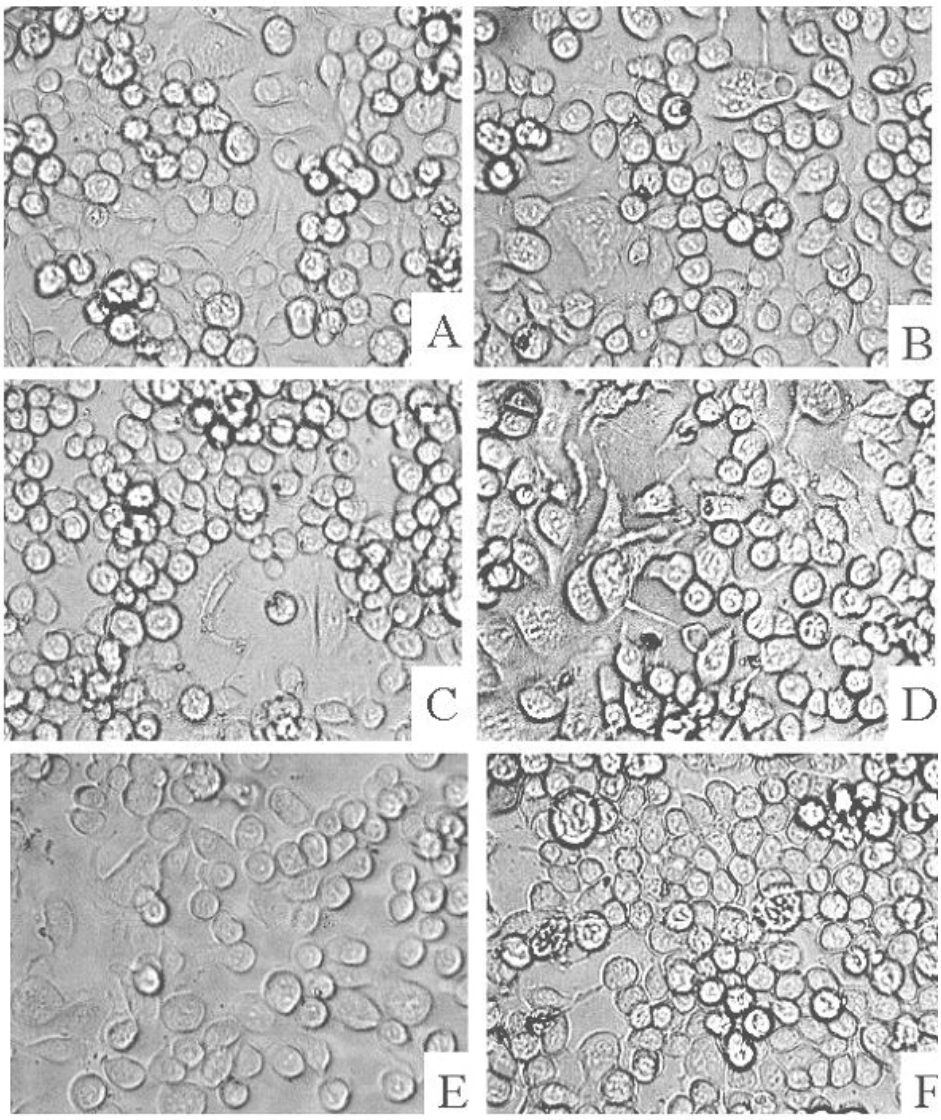


Figure 4

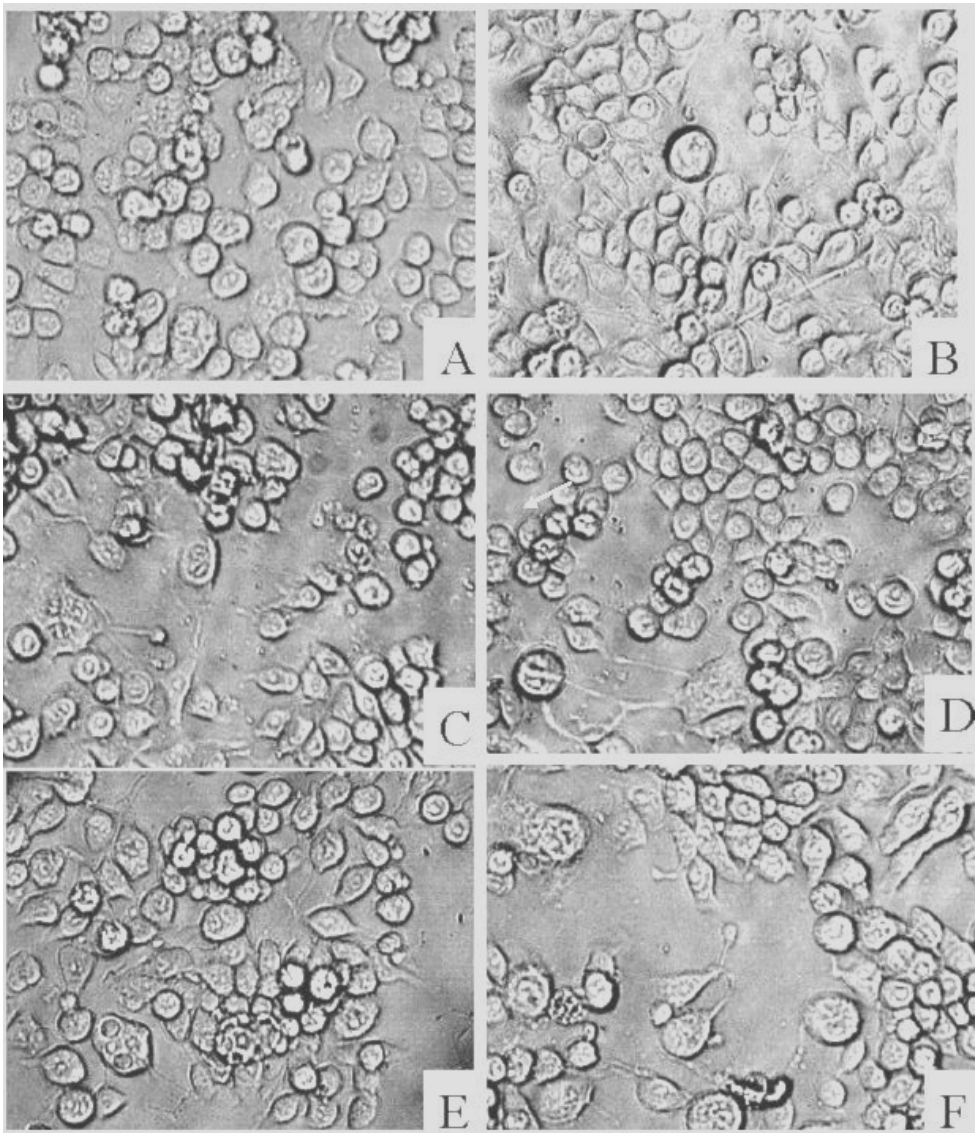


Figure 5

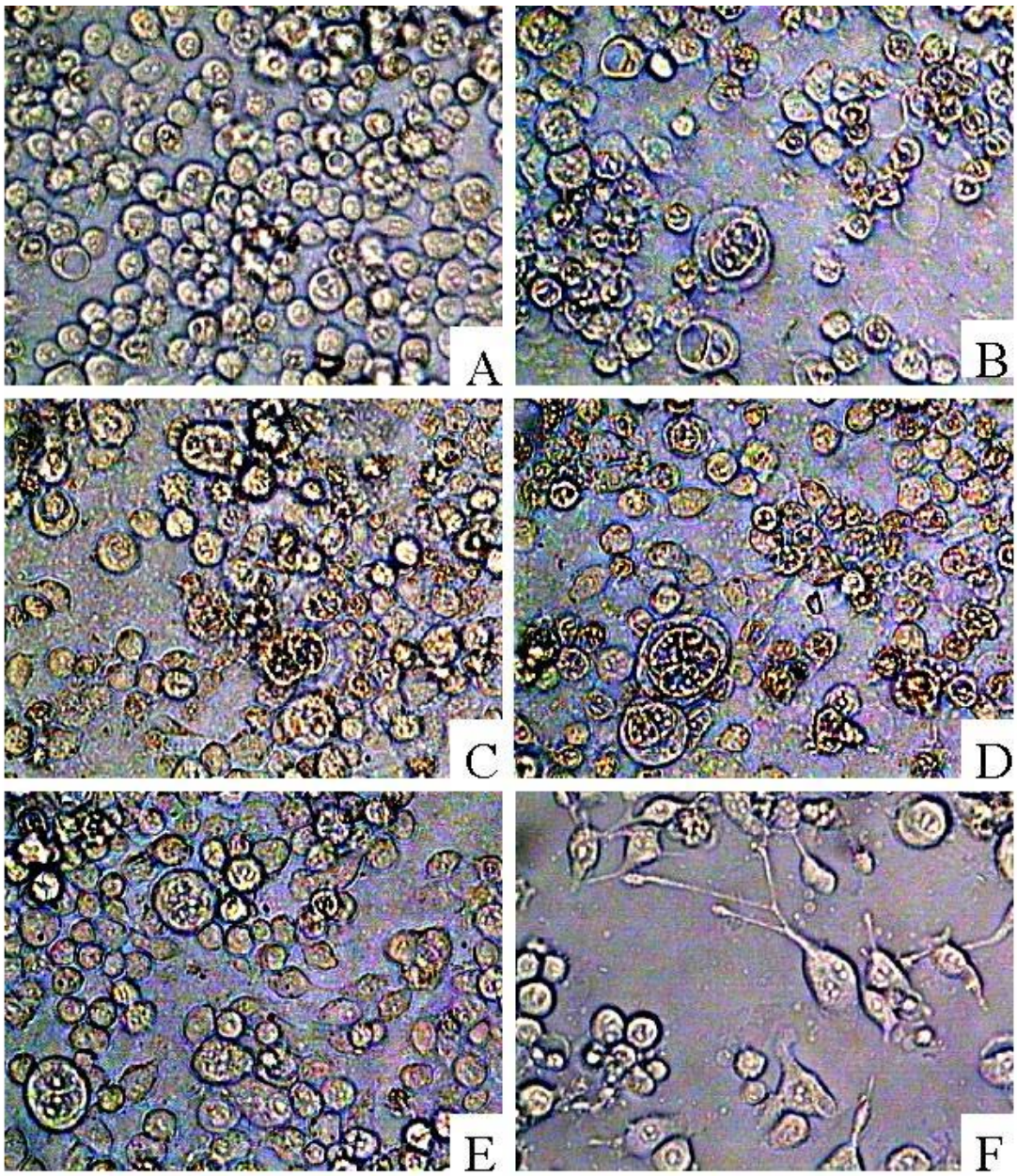
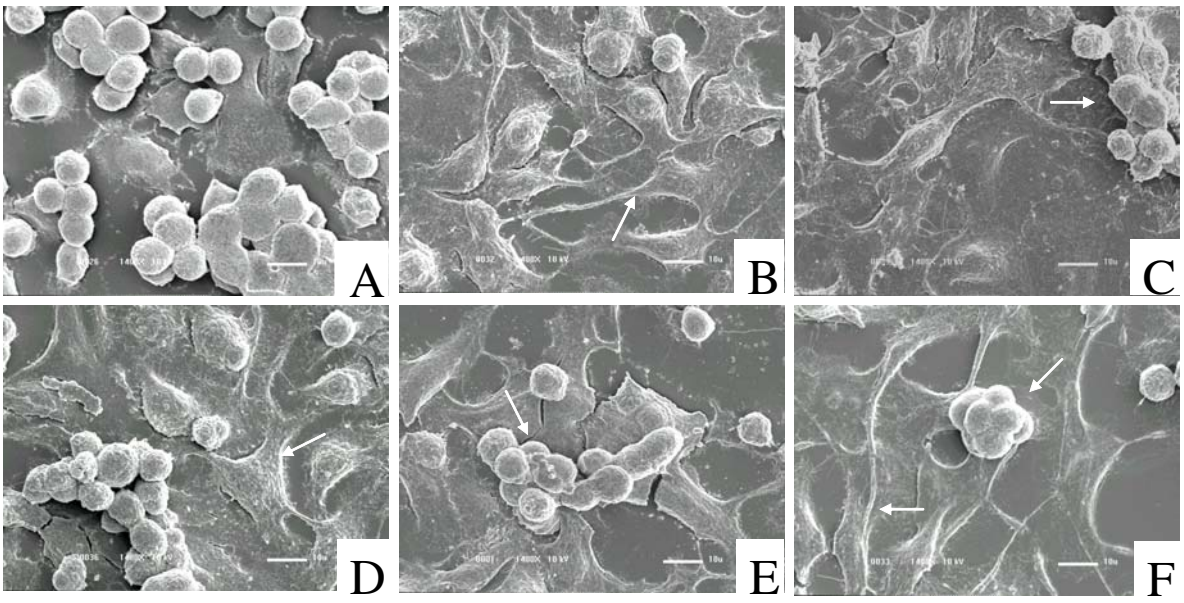


Figure 6



Conclusões Gerais

Os dados da literatura conduzem-nos à importância de pesquisas sobre os poluentes ambientais como o cádmio. Por outro lado, cabe-nos, também, enfocar a necessidade de estudos relacionados aos efeitos protetores de certas substancias (como o sulfato de zinco) para atenuar ou, ate mesmo, impedir as ações citotóxicas, além de carcinogênicas, já verificadas pela atuação do cádmio. Desse modo, neste trabalho de tese concluímos que:

- O cloreto de cádmio induz lesões em células muscular esquelética (C_2C_{12}), consistentes com o estresse oxidativo. Esses resultados complementam os dados da literatura, que abordam principalmente os efeitos citotóxicos do cádmio em outros tecidos, como gonadal, hepático, pulmonar e renal.

- A literatura pertinente mostra o alto índice de exposição da população a fatores citotóxicos, como o cádmio, associado à deficiência em zinco, principalmente nos paises em desenvolvimento. Assim, no presente trabalho conclui-se que o sulfato de zinco possui ação protetora em tecidos de extrema importância, como a musculatura esquelética, células C_2C_{12}, contra os efeitos citotóxicos do cloreto de cádmio, possivelmente, devido a sua ação antioxidante, nas concentrações de 20μM e 40 μM, mas principalmente na de 20μM.

- O tratamento crônico com cloreto de cádmio altera a atividade e morfologia de células do adenocarcinoma de cólon Mac 13, levando-nos a enfocar a importância de estudos com metais pesados, em células já inicializadas ou tumorais, e a prevenção desses efeitos com substâncias antioxidantes.

Assim, este trabalho abre novas perspectivas para estudos futuros relacionados aos tratamentos alternativos e preventivos à citotoxicidade e carcinogênese

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Apêndice

Apresentação de Trabalhos em Congresso

"EFEITO CITOTÓXICO DO CÁDMIO EM CÉLULAS MUSCULARES ESQUELÉTICAS" – Trabalho apresentado na XVII Reunião Anual da Federação de Sociedades de Biologia Experimental - Curitiba – PR - Agosto/2003.

Abstract

O objetivo do trabalho foi verificar a toxicidade do cádmio (Cd) em células muscular esquelética (C_2C_{12}). Estudos mostram que este metal atua na indução de morte celular pelo processo de apoptose em células germinativas e hepatomas. Mioblastos (C_2C_{12}) (1,5 x 105 células) foram cultivados em meio DMEM contendo antibióticos (100 U penicilina G/ml;100 µg estreptomicina/ml) e suplementado com 10% soro fetal eqüino em 5%CO_2 a 37 ºC (Renzi et al., 1993). Após 90-100% de confluência, mioblastos foram diferenciados em miotúbulos (4-5 dias, DMEM com 5% soro eqüino). Miotúbulos foram tratados com cloreto de cádmio nas concentrações (1, 3, 5, 7,5, 10 e 12 µM) para análise de viabilidade celular através do ensaio de MTT e alterações morfológicas através de microscopia eletrônica de varredura (MEV). As análises dos efeitos do cloreto de cádmio foram observadas após 24 e 48hs após o tratamento. Após 48hs houve significativa diferença quanto à viabilidade celular em todos os tratamentos exceto para 1µM (0,5446 ± 0,018), quando comparado ao grupo controle (C 0,6819 ± 0,015, Cd 3µ M 0,5139 ± 0,19, Cd 5µM 0,5039 ± 0, 22, Cd 7,5µM 0,4241 ± 0,017 e Cd 10 µM 0,4784 ± 0,017). Análises morfológicas (MEV) mostram a perda da adesão celular e a presença de vesículas indicativas de possível morte celular. Os resultados mostram o aumento da toxicidade do cádmio quanto à proliferação dos miotúbulos, o que comprometeria mecanismos de controle e adesão celular e provavelmente à viabilidade de forma irreversível.

Pesquisa com suporte: Capes, Fapesp, CNPq, FAEP-UNICAP

"EFEITOS DO 'FATOR WALKER' (FW) SOBRE A ATIVIDADE DE CÉLULAS C_2C_{12} DIFERENCIADAS EM MIOTÚBULOS" -Trabalho apresentado na XIX Reunião Anual da Federação de Sociedades de Biologia Experimental – Águas de Lindóia – SP - Agosto/2004.

Em pacientes com câncer há intensa mobilização de substratos dos tecidos da carcaça do hospedeiro. Essa mobilização decorre, preferencialmente, da depleção de proteína muscular em função do aumento da degradação e/ou diminuição da síntese protéica no músculo.

Objetivo: Elucidar o efeito do FW em células de músculo esquelético (C_2C_{12}).

Métodos e Resultados: Cultura de C_2C_{12} foram diferenciadas em miotúbulos e tratadas com diferentes concentrações de FW, 3.0μg, 5.0μg, 10.0μg, 15.0μg, 20.0μg e 25.0μg /mL, durante 24, 48 e 72 horas. Analisou-se MTT, vermelho neutro (VN), conteúdo de DNA, MDA (malondialdeído) e atividade da glutationa-S-transferase, fosfatase e chymotrypsina-like, bem como análise morfológica em microscopia de luz (ML). Os resultados mostraram redução da resposta celular para atividade mitocondrial (MTT), lisossomal (VN) e da viabilidade celular (DNA) nas concentrações de 20μg e 25μg/mL. Houve aumento, de produtos da peroxidação de lipídeos, MDA, nas concentrações de 5 e 10μg/mL, após 24 horas de exposição do FW; menor atividade da GST, indicando redução do mecanismo de proteção celular, em 48 e 72horas; houve aumento da atividade da chymotrypsina-like nas concentrações de 15 e 20μg/mL. Morfologicamente (ML), verificou-se que o tratamento com o FW promoveu retração dos tapetes celulares e ocorrência de diversas células em suspensão, nas altas concentrações (15 - 25μg/mL).

Conclusão: Com base nos resultados obtidos sugerimos que os efeitos deletérios do FW, sejam, possivelmente semelhante à atuação do fator de indução de proteólise (PIF), já descrito na literatura como principal responsável pelo desenvolvimento da caquexia no câncer.

Pesquisa com suporte: Capes, Fapesp, CNPq, FAEP-UNICAP

Lista de Abreviaturas

Cd, cádmio;

$CdCl_2$, cloreto de cádmio;

CDNB, 1-cloro-2,4- dinitrobenzeno;

DMEM, *Dulbecco's modified Eagle's medium*;

FCS, soro fetal bovino;

GST, glutationa - S - transferase;

LPO, perixidação de lipídeos;

MDA, malondialdeído;

MPO, N-metil-2-fenilindol;

MTT, 3-(4,5-*dimethylthiozol*-2-yl)-2,5-*diphenyltetrazolium bromide*;

NAC, conteúdo de acido nucléico;

NRU, captação de vermelho neutro;

PBS, tampão salina-fosfato;

PBS-Ca^{2+}, tampão salina-fosfato cálcio;

EROs ou ROS, espécies reativas de oxigênio.

HB, tampão de homogeneização;

MRP1, proteína associada a resistência à multidrogas;

HSPs, proteínas *heat-shock*;

SOD, superóxido dismutase;

MT, metalotionina;

ECM, matriz extracelular.