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Modified citrus pectin confers a preventative effect on cancer-related pathways in $CdCl_2$-treated *C. elegans*

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ABSTRACT

Cadmium chloride ($CdCl_2$) is a toxic compound found as a pollutant in the environment due to agricultural and industrial sources. Exposure to Cd^{2+} is known to promote malignant tumors such as lung cancer and leukemia. While the current medications for cadmium toxicity focus on treatments to promote the excretion from the body, treatments to improve health after cadmium exposure are less well studied. Modified citrus pectin (MCP) is a polysaccharide derived from citrus peels that has been shown to induce natural killer cell activity in myeloid leukemia cells and also act as a natural chelation agent to help excrete toxic metals from healthy human subjects. We hypothesized that MCP might have a counteracting effect against $CdCl_2$ toxicity through cancer-related pathways. This study investigates the effects of MCP on $CdCl_2$ toxicity in *C. elegans*, which shares a number of cancer-related pathways with mammals. The results indicated that MCP was able to significantly counter the toxic effects of $CdCl_2$ on *C. elegans* lifespan and development. Our studies suggest that the beneficial effects of MCP may result from its ability to mitigate the effects of $CdCl_2$ on gene expression, particularly in conserved pathways associated with apoptosis, tumor induction and suppression and inflammation-related pathways.

1. Introduction

Cancer, a condition where abnormal cells divide uncontrollably and spread throughout parts of the body, is the second leading cause of death in the United States as stated by the Centers of Disease Control and Prevention (CDC) (CDC, 2021). The National Cancer Institute (NCI) has also estimated that by 2040, there will be a rise of 29.5 million new cancer cases per year (NCI, 2015). Compounds that cause or promote cancer are defined as carcinogenic. A genotoxic carcinogen is defined as a chemical that causes cancer by altering cells' genetic material directly (Hayashi, 1992). Cadmium chloride ($CdCl_2$) is a genotoxic carcinogen that is found as a toxic pollutant in the environment due to agricultural and industrial sources (Bertin & Averbeck, 2006; Mei et al., 2017). Current treatment for cadmium toxicity involves the use of chemical chelation agents, such as penicillamine, dimercaprol and dithiocarbamates (Rahimzadeh et al., 2017). The increased presence in the environment and tumor promoting effects of $CdCl_2$ (Du et al., 2022) prompted us to investigate its carcinogenic effects in the model system *C. elegans*, in order to better understand its toxicity and the mechanisms via which it adversely impacts conserved regulatory pathways related to tumor formation and growth.

Because the incidence of some cancers is correlated with diet, there has been a considerable effort to identify and characterize natural products that might help lower the incidence of cancer. In traditional Chinese medicine (TCM), Chenpi (CP), aged citrus peel, is used to treat indigestion, diarrhea and nausea (Yu et al., 2018). CP has a high concentration of flavonoids as well as insoluble fibers, which contribute to its potential anti-cancer effects (Olano-Martin et al., 2003; Qian et al., 2021; Yu et al., 2022). Furthermore, citrus peel pulp contains citrus pectic oligosaccharides (POS) which are non-digestible oligosaccharides that have been shown to possess prebiotic potential (Manderson et al., 2005) and anti-Shiga toxin-producing *Escherichia coli* (STEC) properties (Di et al., 2017). One specific type of POS is modified citrus pectin (MCP), which has been shown to inhibit the growth of breast cancer cells, attenuate carbohydrate-mediated angiogenesis in mice, induce natural killer cell activity in myeloid leukemia cells and slow the progression of prostate cancer in humans (Guess et al., 2003; Nangia-Makker et al., 2002; Ramachandran et al., 2017; Wang et al., 2022).

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Significantly, cadmium urinary excretion has been shown to increase following oral administration of MCP in a small clinical trial of healthy humans (Eliaz et al., 2006). The present study assesses the ability of MCP to ameliorate or counter the toxic effects of $CdCl_2$ on conserved cancer-related pathways in *C. elegans*.

In this study, we utilized *C. elegans* as an animal model system. Their simplicity and similarity to higher organisms make these nematode worms an excellent *in vivo* model to test the effects of treatments and interventions on lifespan, cellular signalling pathways and gene expression. While *C. elegans* do not develop cancer or tumors within their short lifespan, we can nonetheless use this system to gain an understanding of how carcinogens such as $CdCl_2$ impact these conserved pathways and lead to their dysfunction. While *C. elegans* somatic adult cells do not undergo further proliferation when they reach adult stage, cancer studies in *C. elegans* can take advantage of their invariant cell linage during development to study the proliferation, death, and differentiation of cells and to examine the mechanisms leading to tumor progression (Cerón, 2023). One example of a mechanism that is highly conserved in *C. elegans* and mammals that is dysregulated in cancer is the apoptosis pathway (Cerón, 2023; Conradt & Horvitz, 1998; Kyriakakis et al., 2015). Another advantage of studying cancer related conserved pathways in *C. elegans* is that these pathways are usually easier to analyse due to the presence of typically fewer members within the gene families, compared to mammals, and thus reducing the complexities associated with genetic redundancy (Kirienko et al., 2010). In this study, we have exploited these features to follow the expression of $CdCl_2$-regulated *C. elegans* genes whose dysfunctional regulation in mammalian systems is associated with cancer.

Apoptosis is a form of ordered programmed cell death pathway that occurs naturally and continually in healthy cells (Elmore, 2007). Studies have shown that $CdCl_2$ exposure causes DNA damage, oxidative stress, inflammation and programmed cell death (Skipper et al., 2016; Zhao et al., 2006), all of which are associated with cancer (Reuter et al., 2010). This study aims to investigate whether MCP might exert a protective effect against $CdCl_2$ toxicity via prevention or alleviation of damages resulting from exposure to Cd^{2+} by analysing how MCP modulates the expression of genes related to apoptosis, tumor induction and suppression, inflammation and oxidation in *C. elegans*.

2. Material and methods

2.1. MCP and C. elegans acquisition and compound preparation

MCP powder was obtained from EcoNugenics (Santa Rosa, CA USA). Wild type N2 *C. elegans* were obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota. MCP at 2 mg/ml was utilized for all treatments. $CdCl_2$ at 100 μM was utilized for the lifespan analysis and 200 μM $CdCl_2$ for all other assays to observe the acute effect of $CdCl_2$ exposure in *C. elegans*.

2.2. Lifespan assay

C. elegans were maintained on nematode growth media (NGM) in 60 mm petri dishes. The food source for *C. elegans* was *E. coli strain* OP50. Worms were synchronized by harvesting the eggs from the adult worms using the bleach method (Nigon & Félix, 2018). The eggs were washed twice with M9 buffer and then once with S buffer. The isolated eggs were plated on NGM without MCP or $CdCl_2$ until the worms reached the L4 stage. At the L4 stage, worms were then transferred to the 96-well filter plate containing liquid medium with the specific treatments. For each treatment, a total of 30 worms were used. S-complete medium was prepared as shown in the WormBook (Nigon & Félix, 2018). 5'-Fluoro-2-deoxyuridine (FUDR), a compound used to inhibit egg production, was included in the growth medium (Sutphin & Kaeberlein, 2009).

The treatments (control, 100 μM $CdCl_2$, 2 mg/ml MCP and 100 μM $CdCl_2$ + 2 mg/ml MCP) were carried out in liquid S-complete medium containing OP50 + FUDR and 96-well receiver plates fitted with a MilliporeSigma™ MultiScreen™-Mesh Filter (250 μl/well). For lifespan analysis, four-to-five synchronized worms at the L4 stage were pipetted into single wells of 96-well filter plates containing the various treatments listed above. About 30 worms were observed over 18 days to record their viability. The liquid medium and treatments were refreshed every day for 18 days. We conducted three biological replicates of this experiment, and the results were presented in a line graph.

2.3. Length and area assay

C. elegans were synchronized and treated in S-complete medium and exposed to specific treatments (above) from the egg stage up until the L4 stage (approximately 2 days). At the L4 stage, 30 worms exposed to each treatment were transferred to individual NGM plates. Pictures of each worm were taken under the microscope next to a transparent ruler using a DinoEye Digital Eye Piece Camera. Image J software was used to measure both the length (mm) and area (mm^2) of the worms.

2.4. RNA isolation and RT-qPcCR

For the RT-qPCR analysis, *C. elegans* were synchronized and treated in S-complete medium and exposed to specific treatments from the egg stage to the L4 stage (approximately 2 days). Synchronized eggs mixture in S-buffer (100 μl) were pipetted into each treatment and grown to the L4 stage. At the L4 stage, the worms were transferred into 1.5 ml Eppendorf tubes and washed with water. Trizol was then added to the washed worms to isolate RNA by the freeze-cracking method (Nigon & Félix, 2018). The RNA concentration was determined at OD_{260} using a Nanodrop spectrophotometer (Thermo Fisher).

The reverse transcription reaction was performed as specified in the Applied Biosystems High-Capacity cDNA Reverse Transcriptase Kit. The primers used for amplifying actin, inflammation and oxidation cDNA are identical to those described in a previous publication (Cai Shi et al., 2023). Primers for genes involved in apoptosis, tumor suppression and tumor induction were designed using PrimeExpress (Applied Biosystems). These primer sequences are listed in Table 1. The RT-qPCR analysis was performed using the 2 × SYBR Green master mix (Applied Biosystems) with the respective primers to analyze the gene expression levels. The relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Actin was commonly used as the single endogenous control gene in *C. elegans* (Cai Shi et al., 2023; Zhang et al., 2012). Four biological replicates were conducted, and two technical replicates were used during each RT-qPCR analysis.

2.5. H_2O_2 assay for C. elegans

Synchronized L4 -stage worms were treated with 200 μM $CdCl_2$, 2 mg/ml MCP or 200 μM $CdCl_2$ + 2 mg/ml MCP in a 24-well plate containing S-complete with FUDR and placed on a shaker for 3 days. Approximately 50 worms from each treatment were then collected into 100 μL 1 × PBS and 1 % Tween-20 in Eppendorf tubes. The worms were sonicated and prepared for H_2O_2 assay, as described previously (Cai Shi et al., 2023). Three biological replicates were conducted, and three technical replicates were used during each assay.

2.6. Histological assay for apoptosis

C. elegans were synchronized and treated in S-complete medium and exposed to specific treatments starting from the egg stage up until the L4 stage before being transferred to solid medium containing 35 mm NGM + OP50 plate, together with the reagents required for apoptosis staining. The apoptosis staining was performed as described by Lant and Derry (2013). Acridine orange (AO) at 10 mg/ml was used to stain the worms. AO stock solution (7.5 μl) was added to 1 ml of M9 buffer and 200 μl of this diluted AO solution was pipetted onto the surface of the bacterial

Table 1
Oligonucleotide primers for apoptosis, tumor suppression and tumor induction genes used in the RT-qPCR assay. (list all primers).

Genes	Forward primer	Reverse primer	Accession number
act-1	CTCCACGCGCCGTGTT	CATACCGACCATGACTCCTTGA	T04C12.6.1
egl-1	CAGCAGCATCGGCTACGA	TCGAAGTCATCGCACATTGC	F23B12.9.1
ced-9	GCTCGCAGTGCCCAGAA	TTCCAACCGTCCGAACCA	T07C4.8.1
ced-4	ACACGGCCGAGCTGGAT	TTGGTCAGATTTCGAAAGAGCTT	C35D10.9b.1
ced-3	TGGAACGGTTCGCGAGAA	CCCGTCGTTGCACTGCTT	C48D1.2a.1
let-60	ATGCCTCCTCGACATATTGGA	CACGCATCGCCGAATATTC	ZK792.6.1
let-23	AGAATTTGCCTCATTCCATCATC	CTCCGGCTCCCAGCTTTT	ZK1067.1d.1
age-1	TCACACGAATCAGCACTCTTGTC	CACGCAGTTTCATCGTAGCAA	B0334.8a.1
daf-16	CCGCCGGATGGAAGAACT	CGCATGAAACGAGAATGAAGAG	R13H8.1h.1
daf-18	CCCGCAATCATGTGCTTCT	CACGACGCTCGATTTGCA	T07A9.6.1
sod-1	GCCGGAGCCCATGGAT	CGGCCTTACAGTACTTGGTGATG	C15F1.7a.1
gpx-1	GCGAGGGAGTCGGAGACAA	GAGCTCCGGCGTTTCCA	F26E4.12.1
ctl-1	GACCGAATTTGAACGCGTATC	TCGCGTTGATCCAGACTTTGT	Y54G11A.6.1
gst-10	ACAAAAAGGATGGTCTCGAAGTTC	TGGTTCCTGACCGGCAAA	Y45G12C.2a.1
trf-1	TGTCAACATGATCGGGCAAA	TCAAAAGTGCAAACGACTGGAA	F45G2.6.1
F22E5.6	TCCCCATACGAAACAACACA	CTCCTCCCAGCTTTTCCACAA	F22E5.6.1
ZC239.12	CCAGAAGAATCCCCATACGA	TCCTCCTCCAACTTTTCCAAA	ZC239.12.1
hsp-16.2	GGTGCAGTTGCTTCGAATCTT	TCTTCCTTGAACCGCTTCTTTC	Y46H3A.3a.1
hsp-16.41	AAACAAAATCGGAACATGGATACTT	TGGAGCCTCAATTTGGAGTTTTC	Y46H3A.2.1

lawn containing the treated worms. The AO-containing plates were stored in the dark for 1 h at room temperature (22 ℃) Afterwards, the worms were transferred to a fresh plate containing only OP50 and incubated in the dark for another 3 h to destain excessive AO from the worms' intestines.

Coverslips were prepared with 4 % (w/v) agar pads to prevent the coverslips from crushing the worms, while the worms were destaining. After a 3 h incubation, five treated worms were carefully picked from their plates into a 2.5 μl drop of 20 mM tetramisole to anesthetize the worms on the surface of the thin agar pad. The slides were viewed at 10 × magnitude under the GFP filter of an EVOS M5000 fluorescence microscope.

2.7. Caspase-3 activity assay

Synchronized L4 *C. elegans* grown from the egg-stage in S-complete medium in the presence of specific treatments were collected and washed with M9 buffer. The synchronized eggs in 100 μl S-buffer were pipetted into each treatment and grown to the L4 stage. The worms were centrifugated for 2 min at 300 g and most of the M9 buffer was removed. The caspase-3 activity colorimetric assay kit was purchased from Elabscience® (cat. #E-CK-A383). The worm lysate was prepared as described by the colorimetric assay kit protocol with modifications as previously published (Bahaji et al., 2012). The worm pellet was resuspended in cold lysis buffer and then homogenized in lysis buffer using an ultrasonic bath homogenizer. The samples underwent three rounds of sonication for 15 s each, followed by a 5 min incubation in an ice bath. After the third sonication, the homogenized samples were incubated in an ice bath for an additional 30 min. Samples were then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was transferred to a new tube and put on ice. Protein determinations were made spectrophotometrically at OD_{280} using a Nano-Spectrophotometer.

The samples were prepared according to the caspase-3 activity assay kit manufacturer's protocol. Prepared samples were added to a 96-well plate and incubated at 37 °C overnight. The OD value of the 96-well plate was read using the Agilent BioTek Synergy H1 Hybrid Multi-Mode Reader at 37 °C with an absorbance set at 405 nm. The caspase 3 activity was calculated as follows $(OD_{Sample} - OD_{Blank})/(OD_{Negative} - OD_{Blank})$ and plotted the relative activity of the treatments compared to the control on a graph. M9 buffer was used as the negative control while the lysis buffer was used as the blank. Approximately 100 μg of protein was used for each sample. Samples were stored at –80 ℃. Three biological replicates were conducted for this experiment.

2.8. Statistical analysis

The logrank test was performed to assess the statistical significance between the treatments in the lifespan analysis. The student *t*-test was used to assess the statistical significance between the control and the compound treated worms in RT-qPCR analysis, length and area analysis, H_2O_2 assay and caspase-3 activity assay. All data were presented as the mean ± SD. It was considered statistically significant when the differences were $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

3. Results and discussion

3.1. MCP effects on the lifespan of $CdCl_2$-treated C. elegans

Lifespan analysis was carried out over a period of 18 days on L4 stage N2 worms grown from egg to L4 stage in solid medium without treatment and then transferred to liquid S-complete medium containing MCP and $CdCl_2$. Lifespans for different worm populations were determined by calculating the day at which 50 % viability was reached using a polynomial regression line for each of the curves. The results (Fig. 1) show that the lifespan of $CdCl_2$-treated *C. elegans* was reduced to 10.36 days, compared to worms fed with only OP50 whose lifespan was 14.77 days ($p = 3 \times 10^4$). MCP extended the lifespan of $CdCl_2$-fed N2 worms from 10.36 days to 12.29 days at 50 % viability ($p = 0.02$). MCP alone, in the absence of $CdCl_2$ did not greatly affect the lifespan of worms, as the 50 % viability of N2 worms fed without any compounds was 14.77 days, while

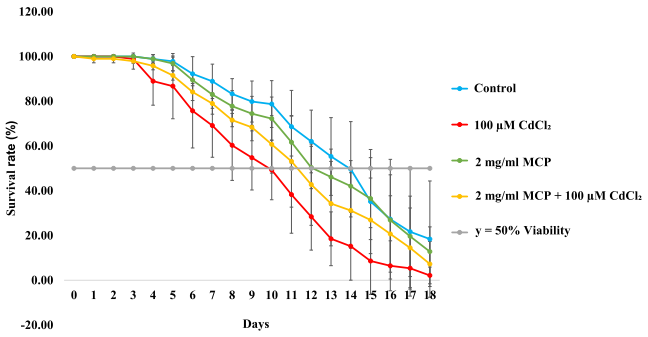

Fig. 1. Lifespan of MCP and $CdCl_2$-treated N2 worms. Synchronized N2 worms at the L4 stage were fed with either $CdCl_2$ at 100 μM, 2 mg/ml MCP or both. The percentage viability of the worms was plotted from day 0 to day 18. Three biological replicates were performed with standard deviations shown for each time point. Statistical significance was compared to the worms fed with only OP50 (control) and described by the logrank test.

the 50 % viability of N2 worms fed with MCP was 13.65 days ($p = 0.8$). These results are consistent with previous research that demonstrated that $CdCl_2$ toxicity decreases the lifespan of *C. elegans* (Turner, 2021). It was concluded that $CdCl_2$ significantly decreased the lifespan of the N2 worms and that MCP treatments could ameliorate this deleterious effect, extending the lifespan of $CdCl_2$-treated worms by about 2 days.

3.2. MCP effect on the length and body area of $CdCl_2$-treated *C. elegans*

Our preliminary experiments showed that *C. elegans* treated with 200 μM $CdCl_2$ from the egg stage were significantly smaller than those in the control group or those exposed to MCP treatment alone. We examined the length and body area of L4 worms treated from the egg stage on, and then exposed in either $CdCl_2$, MCP or both treatments. Our results revealed that the length of $CdCl_2$-treated worms (Fig. 2A) was 46.9 % smaller (0.341 mm) than the control worms (0.643 mm) ($p = 7.59 \times 10^{-21}$) and their body area (Fig. 2B) was 71.2 % less (0.008 mm^2) than the control worms (0.029 mm^2) ($p = 9.99 \times 10^{-18}$). These data support those from a previous report (Cui et al., 2007) which showed that $CdCl_2$ exposure affected nematode growth. The results also showed that the length of MCP-treated worms (Fig. 2A) was 9.6 % smaller (0.581 mm) than the control worms (0.643 mm) ($p = 5.35 \times 10^{-3}$) and their body area (Fig. 2B) was 19.3 % less (0.023 mm^2) than the control worms (0.029 mm^2) ($p = 2.84 \times 10^{-3}$). The slightly shorter length and reduced body area of the MCP-treated worms compared to the control were possibly due to MCP's dietary fiber nature. Dietary fibers are known to stimulate weight loss (Lattimer & Haub, 2010). We also observed that the length of MCP + $CdCl_2$-treated worms (Fig. 2A) was 20.8 % shorter (0.509 mm) than the control worms (0.643 mm) ($p = 6.57 \times 10^{-6}$) and their body area (Fig. 2B) was reduced by 43 % (0.016 mm^2), compared to control worms (0.029 mm^2) ($p = 1.63 \times 10^{-8}$). These data demonstrate that MCP treatment was capable of remediating the detrimental effect of $CdCl_2$ on N2 worms to certain levels.

3.3. Effects of MCP on the expression of genes involved in apoptosis, tumor induction, tumor suppression, inflammation and oxidation

We decided to study the effects of MCP and $CdCl_2$ on the expression of *C. elegans* genes involved in apoptosis, tumor induction, tumor suppression, inflammation and oxidation to see how they might contribute to the effects on lifespan. The regulation of apoptosis in *C. elegans* is less complex than it is in humans. However, the function of the four key apoptotic genes (*egl-1, ced-9, ced-4* and *ced-3*) remains the same for both organisms (Kokel & Xue, 2006). Additionally, it has been shown that homologs of the *C. elegans* genes *let-23, let-60* and *age-1* are involved in tumor induction in humans (Bae et al., 2012; Mihaylova et al., 1999; Zhi et al., 2014), while the genes *daf-16* and *daf-18* are related to two human tumor-suppressor genes (Liu & Chin-Sang, 2015; Yanase et al., 2013). We also included in this study several oxidation- and inflammation-related genes whose expression has been associated with the beneficial effects of mogrosides in vitamin B12-deficient *C. elegans* (Cai Shi et al., 2023). Table S1 summarizes the functions of the human genes and their *C. elegans* orthologs studied in this research.

3.3.1. Effects of MCP on the expression of genes involved in apoptosis

RT-qPCR was used to investigate the effects of MCP on the expression of *egl-1, ced-9, ced-4* and *ced-3* in $CdCl_2$-treated *C. elegans*. *Ce egl-1* is known to initiate the cascade of the apoptosis pathway in *C. elegans* (Nehme & Conradt, 2008). Its human ortholog is *BCL-2* homology region 3 and its function is conserved in *Ce egl-1* (Conradt & Horvitz, 1998). Fig. 3A shows the expression of *egl-1* on worms treated with either $CdCl_2$, MCP or both. Worms treated with $CdCl_2$ increased the expression of *egl-1* gene by 3.9-fold (p=0.0381) compared to untreated control worms. The expression of *egl-1* in worms fed with only MCP was unchanged, compared to the control worms. However, when $CdCl_2$-treated worms were fed with MCP, the expression of *egl-1* went up by only 1.8-fold (p=`0.0237), indicating that MCP significantly attenuated the induction of *egl-1* by $CdCl_2$. It has been shown previously that the loss of function of *egl-1, ced-4* and *ced-3* and the gain of function of *ced-9* prevents all somatic programmed cell death (Galvin et al., 2011). Our results, displayed in Fig. 3B, showed that *ced-9* gene expression was significantly reduced by 43 % (p=0.0082) in $CdCl_2$-treated worms, compared to the control worms. *Ce ced-9* is known to encode apoptotic regulators that are members of the Bcl-2 family, which protect the cells from programmed cell death (Galvin et al., 2011). The down regulation of *ced-9* gene expression suggests that $CdCl_2$ toxicity in worms may be manifested, in part, through the modulation of this apoptosis-related gene. However, MCP did not seem to have any effect on *ced-9* expression as MCP alone did not affect its steady-state level with MCP + $CdCl_2$ treatment reducing the expression of *ced-9* by 40 % ($p = 0.00005$) compared to the control worms, which was similar to the 43 % reduction caused by $CdCl_2$ treatment (Fig. 3B).

Additionally, our results (Fig. 3C) indicate that $CdCl_2$, MCP and MCP + $CdCl_2$ treatments did not change the expression of *ced-4*, compared to control worms. Ce *ced-4* gene facilitates the activation of the protein CED-3 (Galvin et al., 2011). *Ce ced-3* gene is known to encode for a caspase that is essential for apoptosis (Chen et al., 2016). The results in Fig. 3D show that $CdCl_2$ significantly decreased *ced-3* expression by 48 % ($p = 0.0084$) compared to the control worms. The addition of MCP did not counteract the downregulation of *ced-3* gene expression caused by

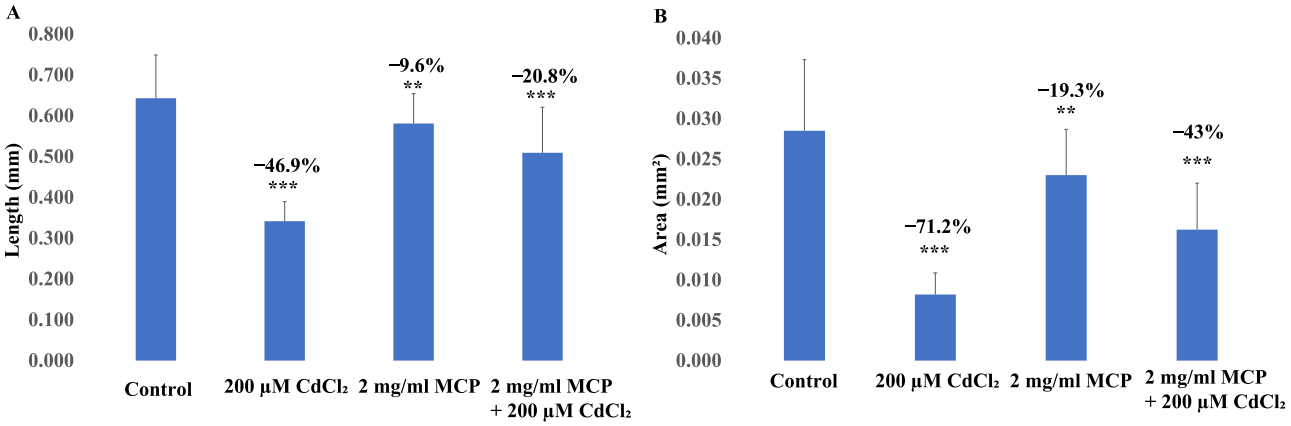

Fig. 2. Length (A) and body area (B) of MCP and $CdCl_2$-treated N2 worms. Synchronized eggs from N2 worms were treated with either $CdCl_2$ at 200 μM, 2 mg/ml MCP or both. The worms were grown for approximately 2 days until L4 stage. Standard deviations were obtained from the measurements of 30 individual worms per treatment. All treatments were compared to the worms fed with only OP50 (control). The student-*t* test was performed to assess statistical significance ($p < 0.01$** and $p < 0.001$***).

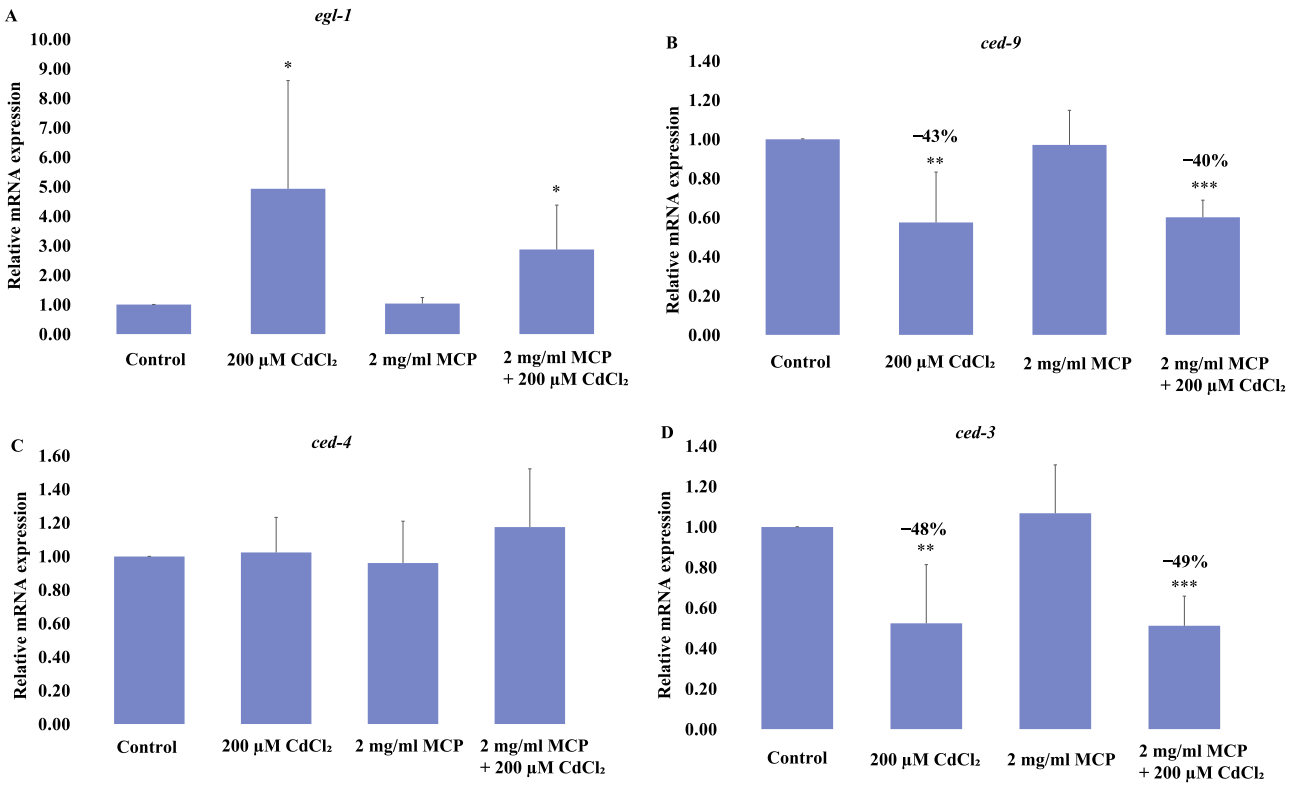

Fig. 3. Expression of *egl-1* (A), *ced-9* (B), *ced-4* (C) and *ced-3* (D) genes on MCP and $CdCl_2$-treated N2 worms. The fold-change ($2^{-\Delta\Delta Ct}$) and standard deviations of the gene expression were calculated from four biological replicates. All treatments were compared to the worms fed with only OP50 (control). The student-*t* test was performed to assess statistical significance ($p < 0.05$*, $p < 0.01$** and $p < 0.001$***).

$CdCl_2$. MCP treatment alone did not have any effect on *ced-3* expression.

3.3.1.1. Apoptosis staining of C. elegans treated with MCP and $CdCl_2$. To further investigate the effects of $CdCl_2$ and MCP on the apoptosis pathway, AO staining was used to visualize apoptosis in worms. In the control worms, we observed that the AO stain was distributed evenly on the surface of the worm and within the intestinal tract (Fig. 4A). The bright fluorescence at the end tip of the worms was due to germ cell corpses associated with spermatocytes located closer to the uterus area that take up AO dye (Lant & Derry, 2013). In contrast, the $CdCl_2$-treated AO-stained worms displayed a granular distribution of fluorescence along the body of the worm (Fig. 4B), indicating the presence of cells undergoing apoptosis throughout the worm's tissues. Additionally, it was noted that the $CdCl_2$-treated worms were significantly smaller than the control, MCP- and MCP + $CdCl_2$-treated worms. This result is consistent with the previous research on the effect of $CdCl_2$ toxicity in *C. elegans* (Cui et al., 2007; Skipper et al., 2016).

Similar to the control, MCP-treated AO-stained worms showed the same uniform distribution of the AO dye within the intestinal tract and the surface of the worms (Fig. 4C). In MCP + $CdCl_2$-treated worms (Fig. 4D), the AO stain distribution was similar to those observed in the control and in MCP-treated worms. Additionally, measurement of the length and body area of worms revealed that the size of the MCP + $CdCl_2$-treated worms was greater than were $CdCl_2$-treated worms. These results suggest that MCP could counteract the many of the adverse effects of $CdCl_2$ on growth, development and cell death of *C. elegans*.

3.3.1.2. Caspase-3 assay of C. elegans treated with MCP and $CdCl_2$. The caspase-3 activity of *C. elegans* was analyzed to see if it correlated with results from histological staining for apoptosis. The results showed that worms treated with $CdCl_2$ increased their caspase-3 activity by about 66 %, relative to the control worms ($p = 0.00013$) (Fig. 5). Worms treated with only MCP show a very slight relative increase of 3 % in their caspase-3 activity, compared to the control worms ($p = 0.321$). The addition of MCP to the $CdCl_2$-treated worms reduced the caspase-3 activity significantly, with a 21 % increase, compared to the control ($p = 0.439$), suggesting that MCP has a counteracting effect against the effects of $CdCl_2$ on caspase-3. These results are consistent with the previous AO staining assay results, which confirmed that $CdCl_2$ causes apoptosis in *C. elegans* and that MCP could counteract the toxic effect of $CdCl_2$.

3.3.2. Effects of MCP on the expression of genes involved in tumor induction

The effects of MCP on the expression of tumor-inducing genes, *let-60, let-23* and *age-1* were also analyzed on $CdCl_2$-treated *C.* elegans. The role of *let-60* in *C. elegans* is to control the initiation of vulva formation (Beitel et al., 1990). Its human ortholog, the *RAS* gene, has a key role of converting normal cells to malignant cells (Taira et al., 2016). The results show that $CdCl_2$ treatment increased the expression of *let-60* gene by 0.8-fold ($p = 0.00182$). Interestingly, while MCP alone did not increase *let-60* expression, it also did not reduce the up-regulation of *let-60* caused by $CdCl_2$ (Fig. 6A).

The second tumor induction gene studied was *let-23. Ce let-23* is the human ortholog of *EGFR* and plays a significant role in the signalling pathway of the worms' vulval precursor cells (Hajnal et al., 1997). In this study, it was observed that the *C. elegans* worms treated with $CdCl_2$ had a reduced length and body area; however, we did not observe any phenotypic effect on vulval morphology. The results show that, similar to the *let-60* results (Fig. 6A), $CdCl_2$ increased the expression of *let-23* by 0.9-fold ($p = 0.028$) and MCP alone did not have much an effect on either control or on $CdCl_2$-treated worms (Fig. 6B).

The third tumor induction gene studied was *age-1*, which is known to participate in the suppression of dauer larva formation. Inactivation of *age-1* causes worms to go into dauer larva formation, extending the lifespan of the worms (Mihaylova et al., 1999). $CdCl_2$ treatment

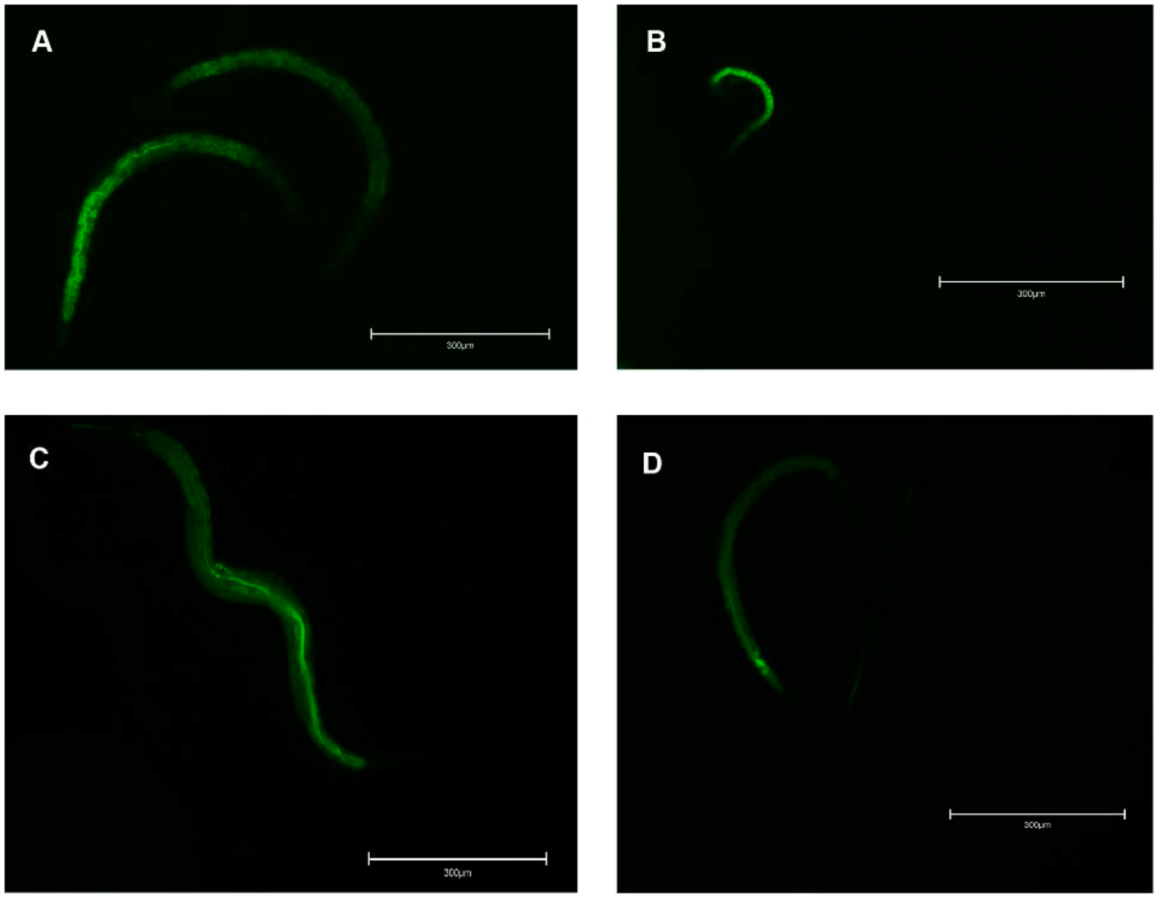

Fig. 4. Apoptosis staining of *C. elegans* treated with no compound (A), $CdCl_2$ (B), MCP (C) and MCP + $CdCl_2$ (D). Apoptosis was detected by acridine orange (AO) staining (Lant and Derry, 2013). All pictures were taken at 10× magnitude under the GFP filter of an EVOS M5000 fluorescence microscope.

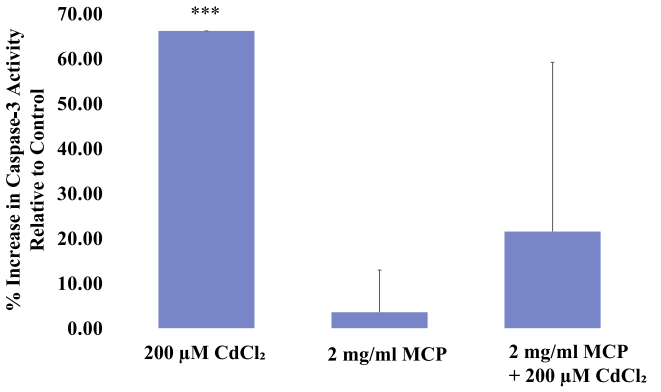

Fig. 5. Relative percentage of increase in caspase-3 activity on worms treated with $CdCl_2$, MCP and MCP + $CdCL_2$ compared to the control worms fed with only OP50. The standard deviations of the relative % increase difference were calculated from three biological replicates. The student-*t* test was performed to assess statistical significance ($p < 0.001$***).

decreased the expression of *age-1* by 50 % ($p = 0.000412$) compared to the control worms (Fig. 6C). MCP alone did not have any effect on *age-1* expression, yet it reduced the downregulation of this gene caused by $CdCl_2$ to 30 % ($p = 0.000006$), relative to the control.

3.3.3. Effects of MCP on the expression of genes involved in tumor suppression

We also investigated the effect of MCP on the expression of the *C. elegans* orthologs of two mammalian tumor suppression genes. *Ce daf-16* is involved in the regulation of metabolic processes and defense responses against bacterial pathogens (Miyata et al., 2008; Zečić & Braeckman, 2020). Fig. 7A shows that $CdCl_2$ and MCP + $CdCl_2$ treatments increased the expression of *daf-16* gene by 2.1-fold ($p = 0.0214$) and 1.8-fold ($p = 0.0002$), compared to the control worms, respectively, while worms fed with only MCP slightly decreased the expression of *daf-16* by 18 % ($p = 0.0061$). $CdCl_2$ treatment alone increased the expression of this gene, possibly due to the stress responses that are activated as a response to the toxicity of this compound and its downstream effects on metabolism and cell death. The presence of MCP alone did not trigger worms' defense responses, as indicated by the slight decrease in *daf-16* expression.

The second tumor suppression gene studied was *daf-18*, an ortholog of human *PTEN*, which is highly conserved with *C. elegans* and which has essential functions in DNA repair and in the development, suppression, and metabolism of tumors (Liu & Chin-Sang, 2015). Our results show that $CdCl_2$ treatment decreased the expression of *daf-18* by 39 % ($p = 0.0345$) (Fig. 7B). While MCP alone did not affect *daf-18* expression, it was able to reduce it by 27 % ($p = 0.0041$) in $CdCl_2$-treated worms.

3.3.4. Effects of MCP on the expression of genes involved in inflammation

Since inflammation is activated by the immune system, we evaluated the effect of MCP on *C. elegans* genes involved in inflammation and the immune responses. *Ce trf-1* is involved in the regulation of worms' innate immune response (Tenor & Aballay, 2008). Its human ortholog is *TRAF4*, which encodes a tumor necrosis factor involved in the innate immune response (Miller, 2002). In this study, worms fed with MCP alone did not show any change in the expression of *trf-1*, compared to the control worms (Fig. 8A). However, in the $CdCl_2$-treated and MCP + $CdCl_2$-treated worms, the *trf-1* expression was significantly increased by

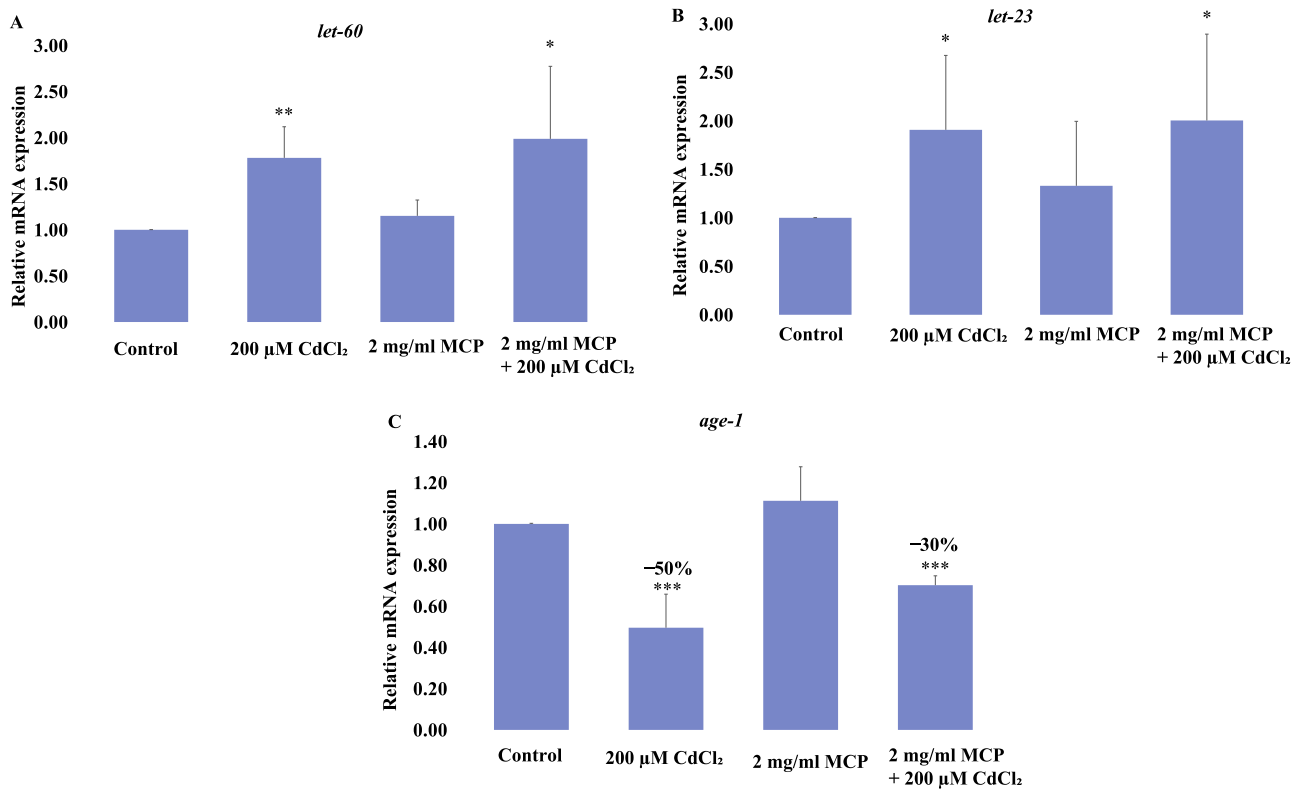

Fig. 6. Expression of *let-60* (A), *let-23* (B) and *age-1* (C) genes in MCP and $CdCl_2$-treated N2 worms. The fold-change ($2^{-\Delta\Delta Ct}$) and standard deviations of the gene expression were calculated from four biological replicates. All treatments were compared to the worms fed with only OP50 (control). The student-*t* test was performed to assess statistical significance ($p < 0.05$*, $p < 0.01$** and $p < 0.001$***).

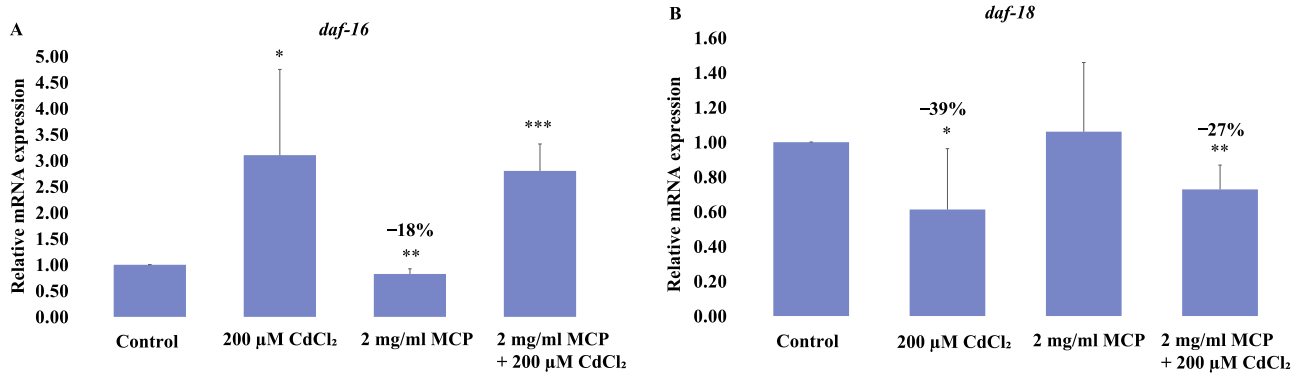

Fig. 7. Expression of *daf-16* (A) and *daf-18* (B) genes on MCP and $CdCl_2$-treated N2 worms. The fold-change ($2^{-\Delta\Delta Ct}$) and standard deviations of the gene expression were calculated from four biological replicates. All treatments were compared to the worms fed with only OP50 (control). The student-*t* test was performed to assess statistical significance ($p < 0.05$*, $p < 0.01$** and $p < 0.001$***).

7.8-fold ($p = 0.0212$) and 4.6-fold ($p = 0.0214$), respectively. The observed upregulation of *trf-1* expression in response to $CdCl_2$ exposure suggests an adaptive response to this compound's toxicity in *C. elegans*, and that treatment with MCP can ameliorate some of the deleterious effects induced by $CdCl_2$.

F22E5.6 and *ZC239.12* are homologs of the human *TNFA1P1* genes involved in stress response and inflammation (Xin et al., 2013). Fig. 8B and C show that $CdCl_2$ significantly increased the expression of *F22E5.6* and *ZC239.12* by 7.5-fold ($p = 0.00205$) and 3.4-fold ($p = 0.00248$), respectively. The expressions of *F22E5.6* and *ZC239.12* were not changed by MCP alone. Rather, they increased by14.5-fold ($p = 0.000055$) and 17.1-fold ($p = 0.0183$) in worms treated with $CdCl_2$ or with MCP + $CdCl_2$, respectively, indicating that MCP could not attenuate the stress response and inflammation caused by $CdCl_2$ in *C. elegans* and may even enhance some aspects of this response.

This study also investigated the effect of MCP and $CdCl_2$ on *hsp-16.2* and *hsp-16.41*, which are two small heat shock proteins that are induced in *C. elegans* due to stress response (Hong et al., 2004). The worms fed with only MCP did not change the expression of *hsp-16.2* (Fig. 8D) nor *hsp-16.41* (Fig. 8E), compared to the control worms. Fig. 8D shows that $CdCl_2$ and MCP + $CdCl_2$ significantly increased the expression of *hsp-16.2* gene by 83.4-fold ($p = 0.000050$) and 109.2-fold (Fig. 8D) ($p = 0.002529$), respectively. Similarly, $CdCl_2$ and MCP + $CdCl_2$ significantly increased the expression of *hsp-16.41* gene by 140.5-fold ($p = 0.000001$) and 130.2-fold ($p = 0.000787$), respectively (Fig. 8E). The upregulation of *hsp-16.2* and *hsp-16.41* expression by $CdCl_2$ indicates that the worms mount a stress response to counteract the toxic effects of this compound. The slight decrease of *hsp-16.41* in MCP + $CdCl_2$ worms suggests a

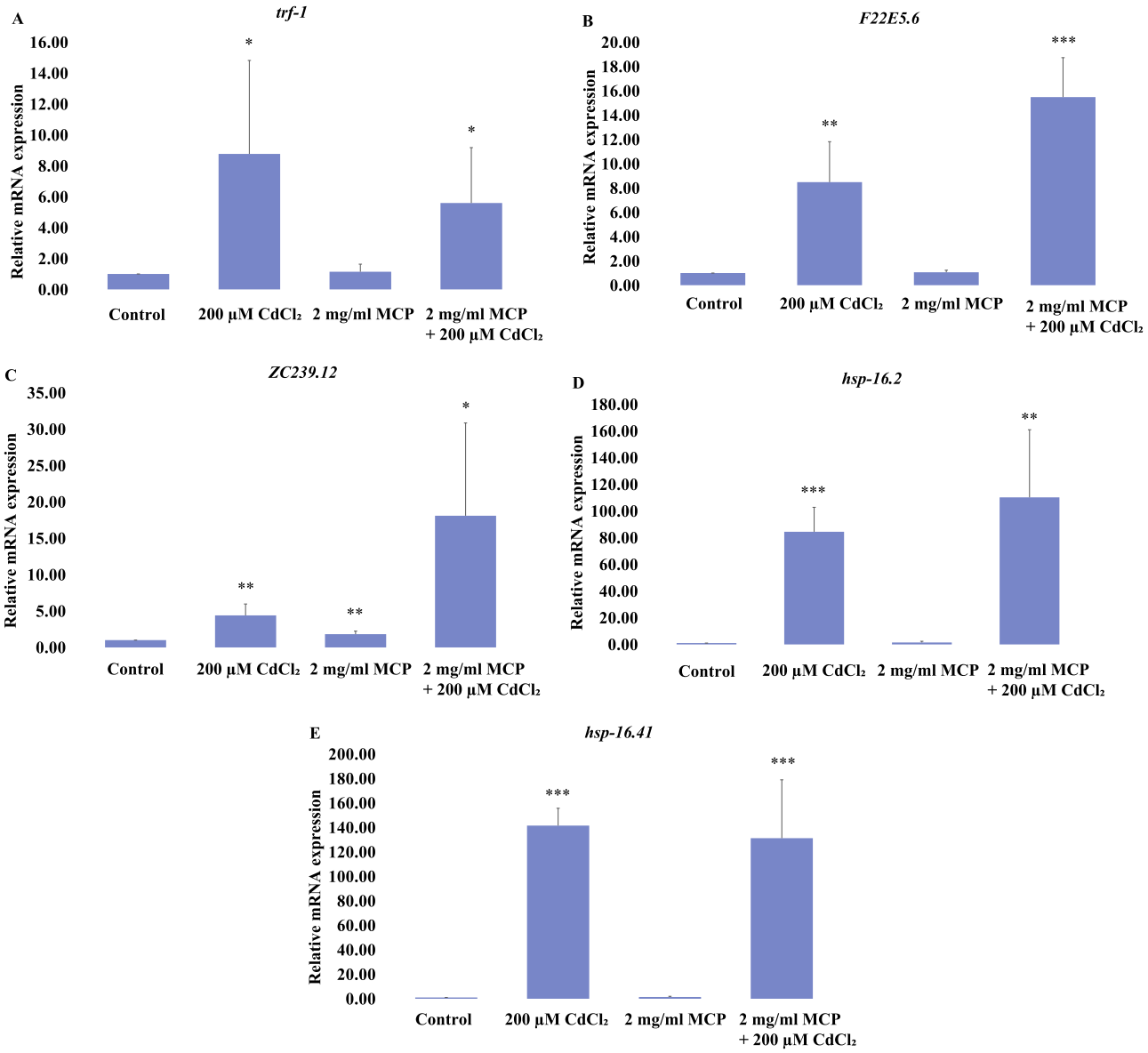

Fig. 8. Expression of *trf-1* (A), *F22E5.6* (B), *ZC239.12* (C), *hsp-16.2* (D) and *hsp-16.41* (E) genes on MCP and $CdCl_2$-treated N2 worms. The fold-change ($2^{-\Delta\Delta Ct}$) and standard deviations of the gene expression were calculated from four biological replicates. All treatments were compared to the worms fed with only OP50 (control). The student-*t* test was performed to assess statistical significance ($p < 0.05$*, $p < 0.01$** and $p < 0.001$***).

possible effect of MCP in countering the toxic effect of $CdCl_2$ and restore protein homeostasis.

3.3.5. *Effects of MCP on the expression of genes involved in the response to oxidative stress*

Oxidative stress in humans can lead to inflammatory stress, which plays a major role in diseases such as diabetes, neurodegenerative, cancer and cardiovascular disease (Reuter et al., 2010). This study assessed the effects of MCP and $CdCl_2$ on the expression of four oxidative stress-related genes in *C. elegans*. *Ce sod-1* encodes a superoxide dismutase in *C. elegans*, which converts superoxide into the less toxic compounds hydrogen peroxide and oxygen (Horspool & Chang, 2017). Fig. 9A shows that $CdCl_2$ significantly decreased the expression of the *sod-1* gene by 28 % ($p = 0.00931$), compared to the control worms. MCP alone and the addition of MCP to $CdCl_2$-treated worms did not significantly change the expression of *sod-1*.

Ce gpx-1 is one of the genes that comprise the glutathione peroxidase (GPx) family, which utilizes reduced glutathione to reduce a variety of organic hydroperoxides to the corresponding alcohol or H_2O (Ferguson & Bridge, 2019). $CdCl_2$ and MCP + $CdCl_2$ treatments increased the expression of *gpx-1* gene by 3.3-fold ($p = 0.0026$) and 4.1-fold ($p = 0.0241$), respectively, compared to the control worms (Fig. 9B). However, MCP alone did not change the expression of *gpx-1*.

Ce ctl-1 encodes one of the catalase genes in *C. elegans* (Schiffer et al., 2020). Fig. 9C shows that $CdCl_2$ and MCP + $CdCl_2$ treatments increased the expression of the *ctl-1* gene by 1.2-fold ($p = 0.0147$) and 1.3-fold ($p = 0.000249$), respectively, while MCP alone did not have much effect on *ctl-1* expression. The fourth gene involved in oxidative response was *gst-10*. Our results showed that MCP and $CdCl_2$ did not have any effect on this particular gene.

3.3.5.1. Antioxidant activity of MCP on $CdCl_2$-treated C. elegans. To further investigate the antioxidant activity of MCP, the H_2O_2 levels of worms treated with MCP, $CdCl_2$ and MCP + $CdCl_2$ were analyzed. Fig. 10 shows that in $CdCl_2$-treated worms, there was a slight reduction (9 %, $p = 0.0196$) in H_2O_2 levels, compared to the control worms. MCP alone significantly reduced H_2O_2 levels by 40 % ($p = 0.0236$) compared to the control worms, confirming MCP's antioxidative activity as

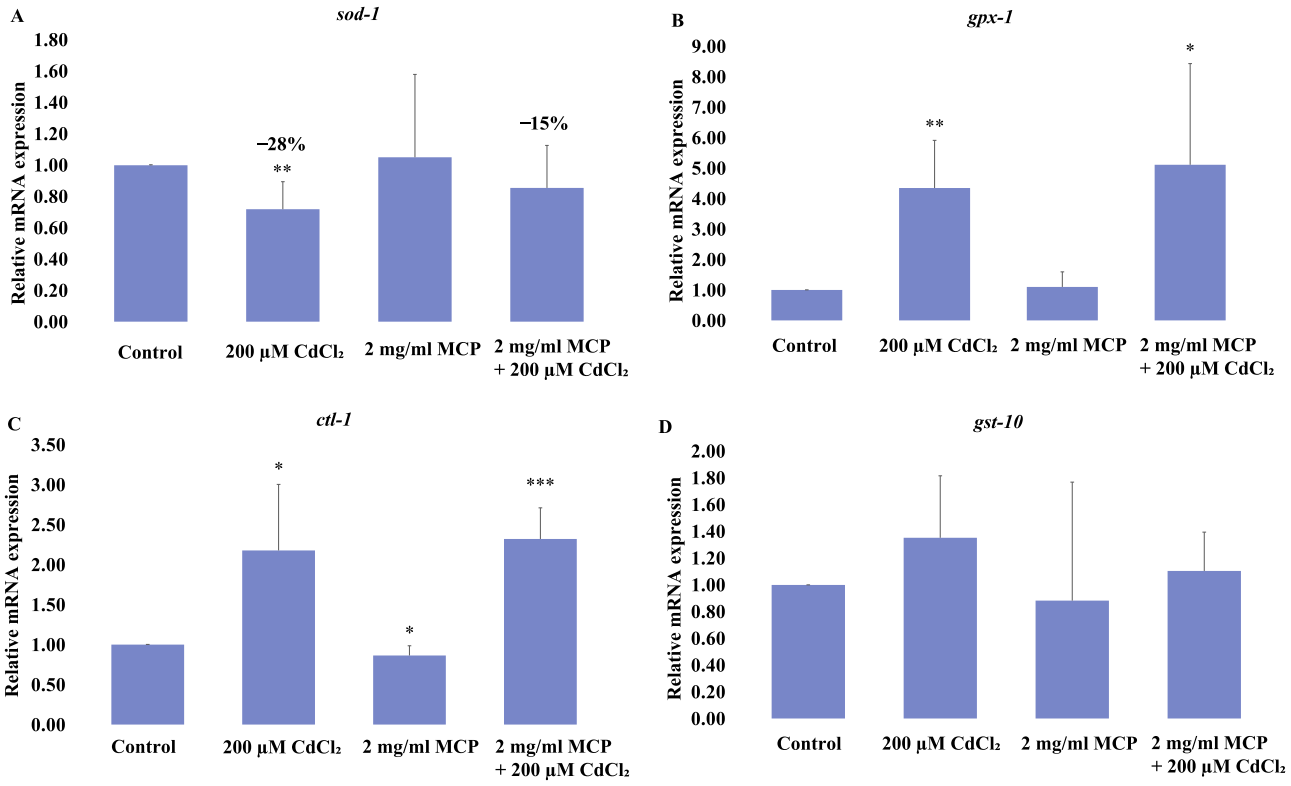

Fig. 9. Expression of *sod-1* (A), *gpx-1* (B), *ctl-1* (C) and *gst-10* (D) genes on MCP and $CdCl_2$-treated N2 worms. The fold-change ($2^{-\Delta\Delta Ct}$) and standard deviations of the gene expression were calculated from four biological replicates. All treatments were compared to the worms fed with only OP50 (control). The student-*t* test was performed to assess statistical significance ($p < 0.05$*, $p < 0.01$** and $p < 0.001$***).

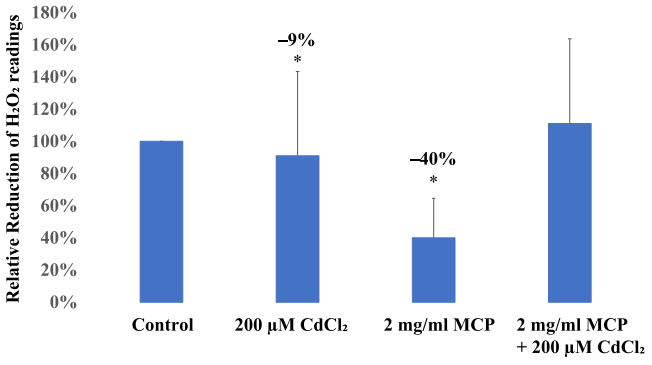

Fig. 10. Antioxidant activity of MCP and $CdCl_2$ in *C. elegans*. Synchronized worms at L4 stage were treated with 200 µM $CdCl_2$, 2 mg/ml MCP or 200 µM $CdCl_2$ + 2 mg/ml MCP in a 24-well plate containing S-complete with FUDR and placed on a belly shaker for 3 days. Worms were collected after 3 days of being treated and assayed for ROS. Standard deviations were calculated from three biological replicates. All treatments were compared to the worms fed with only OP50 (control). The student-*t* test was performed to assess statistical significance ($p < 0.05$*).

previously reported (Ramachandran et al., 2017). However, the H_2O_2 level was not reduced by MCP in the presence of $CdCl_2$.

3.3.6. Discussion summary

We hypothesized that MCP may exert anti-cancer proprieties in humans and animals by modulating gene expression and pathways associated with apoptosis, tumor induction, tumor suppression, oxidative stress responses and inflammation. Using the *C. elegans* model system to study these highly conserved cellular pathways, our results show that exposure of worms to $CdCl_2$ caused the upregulation of genes related to inflammation, stress responses and oxidative stress. Additionally, we observed that $CdCl_2$ increased the expression of *egl-1*, activating the apoptosis pathway, while MCP alone did not influence this pathway. $CdCl_2$ is known to induce apoptosis *in vitro* and *in vivo* in many cell and tissue types (Skipper et al., 2016). Worms treated with both MCP and $CdCl_2$ had a slight increase in the expression of *egl-1* but levels were not as elevated as they were in the worms treated only $CdCl_2$ alone. Based on the upregulation of *egl-1*, we expected that *ced-3* would be also up-regulated. *Ce ced-3* is the ortholog of the human *CASPASE*-3 and its function is highly conserved in *C. elegans* (Pinan-Lucarre et al., 2012). While *ced-3* was not upregulated in response to $CdCl_2$ in *C. elegans*, we did observe down-regulation of *ced-9* and *ced-3*. It is possible that MCP and $CdCl_2$ may have post-transcriptional and post-translational effects on compounds involved in apoptosis that would not be revealed in the present study.

Researches using modified sugar beet pectin, citrus, apple pectin and POS have shown that these preparations can induce apoptosis in colon cancer cells and prostate cancer cells (Olano-Matin et al., 2003; Jackson et al., 2007; Maxwell et al., 2016). MCP has many reported effects, some of which may reflect different methods of production (pH, heat and enzyme), Differences in MCP structure could be responsible for the different apoptosis results that we observed in *C. elegans* in response to $CdCl_2$, relative to previous reports, since the commercial MCP that we used and the MCP used by Olano-Martin et al. (2003), Jackson et al. (2007) and Maxwell et al. (2016) were produced by four different methods. Nonetheless, preparations of MCP characterized by a low molecular mass, a low degree of esterification and the presence of abundant arabinogalacto-oligosaccharide side-chains have been shown to be consistently associated with anti-cancer activities (including apoptosis) in mammalian cells (Eliaz & Raz, 2019).

Previous research has shown that $CdCl_2$ induces apoptosis through the generation of reactive oxygen species (ROS), which causes oxidative damage to cellular components, leading to the activation of various

signalling pathways that ultimately trigger apoptosis (Cui et al., 2007). We observed that MCP treatment alone did not significantly increase of caspase-3 activity, compared to controls, which is consistent with the known antioxidant properties of MCP (Ramachandran et al., 2017). Based on the results of oxidation-related gene expression, we found that $CdCl_2$ treatment decreased the expression of *sod-1*, which encodes a superoxide dismutase in *C. elegans* (Horspool & Chang, 2017). The addition of MCP mitigated the negative effects of $CdCl_2$ by bringing the expression of *sod-1* back down to the same level as controls. However, MCP did not mitigate the effect of $CdCl_2$ on the other oxidative stress-related genes studied here. Additionally, our H_2O_2 assay demonstrated that while MCP had a demonstrable and potent antioxidant activity, it was not able to attenuate the effects of $CdCl_2$ by modulating H_2O_2 levels. This initially suggests that the antioxidant properties of MCP may not play a major role in the attenuation of $CdCl_2$ toxicity observed in the apoptosis, tumor induction, tumor reduction and inflammation gene expression results. However, it is also possible that the ability of MCP to attenuate $CdCl_2$ was overwhelmed, and that we might yet observe some beneficial effects of MCP at lower concentrations of $CdCl_2$.

The MCP chelating effects on $CdCl_2$ may have also played a role on the attenuation effects observed in this study (Eliaz et al., 2006; Z. Y. Zhao et al., 2008). MCP is known to increase the excretion of cadmium in adult humans by 150 % after 6 days of oral administration and the secretion of other toxic metals (Eliaz et al., 2006). The effects shown in our study might be due to the MCP + $CdCl_2$-treated worms being exposed to less Cd^{2+} from the chelation by MCP. However, MCP's effects on *C. elegans* gene expression should not be excluded since both the chelation effect and gene expression effects could play a synergistic role in protecting worms against $CdCl_2$ toxicity. Further research would help tease apart the relative contributions of these effects and their relationship in protecting against the toxic effects of exposure to $CdCl_2$.

4. Conclusion

Modified citrus pectin (MCP), well known for its broad health-promoting properties, can inhibit the growth of breast cancer cells and reduce the extent of carbohydrate-mediated angiogenesis in mice (Nangia-Makker et al., 2002; Wang et al., 2022). MCP is also known to have anti-inflammatory and antioxidant properties *in vitro*, can induce natural killer cell activity in myeloid leukemia cells and slow the progression of prostate cancer in humans (Guess et al., 2003; Ramachandran et al., 2011, 2017). In order to better understand how MCP might affect cancer development in mammalian systems, we turned to the model organism *C. elegans*. While nematode worms do not develop tumors, *per se*, they do contain a substantial number of key regulators and effectors that have been implicated in the onset and progression of tumors in mammals, and in particular, in humans. This approach provides an opportunity, to tease out the specific effects of MCP on cytotoxic stress, such as those induced by $CdCl_2$, a widely distributed heavy metal pollutant, exposure to which is associated with an increased incidence of malignant tumors, such as lung cancer and leukemia.

This study confirms that treatment of worms with 2 mg/ml MCP can mitigate the toxic effects associated with exposure to $CdCl_2$ including its adverse effects on apoptosis, tumor inducing and tumor suppressing gene expression, inflammation-related gene expression, lifespan, growth, development and maturation. Additionally, our results demonstrated that MCP alone did not have harmful effects on *C. elegans*, as measured by lifespan, length, body area and gene expression, compared to the control worms. Together, these findings suggest that MCP can counter many of the toxic effects induced by $CdCl_2$ exposure, specifically those related to apoptosis, tumorigenesis and inflammation genes in *C. elegans*. These studies suggest that it would be worthwhile examining these responses and gene expression levels in a mammalian system, so that beneficial effects of MCP can be related to the incidence and severity of tumorigenesis and to survival outcomes. Despite the limitations of the *C. elegans* system as a model for cancer, it provides a useful system in which the individual pathways invoked by tumor-inducing agents can be delineated and in which the mechanism of action of potential anti-cancer compounds and extracts can be associated with specific cellular pathways. These studies provide insight into the potential mode-of-action of MCP on $CdCl_2$ in nematodes, and potentially provide a path for developing anti-cancer interventions in more complex organisms.

Ethical statement

This is to declare that this study does not contain any research conducted on human or animal subjects.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fhfh.2023.100161.

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