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ASCORBIC ACID SUPPLEMENTATION SUPPRESSES CADMIUM-DERIVED ALTERATIONS IN THE FISSION YEAST *SCHIZOSACCHAROMYCES POMBE*

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ABSTRACT
Cadmium (Cd) a highly toxic environmental pollutant, that does not have any physiological function in the organism, represents a great concern for human health as it can be easily transported from its environmental sources to the food chain. Food, water, and air are the major sources of Cd exposure to the population. Cd-mediated impairments of the basic cellular properties largely depend on its ability to enhance the formation of reactive oxygen species (ROS) and thus triggers oxidative stress to the cell. With the use of fission yeast *Schizosaccharomyces pombe* (*S. pombe*) as the model organism, we have analyzed the impact of Cd on the cell growth intensity, as it represents the fundamental feature of all living organisms. Cells were incubated with different Cd concentrations for 3, 6, and 9 hours to investigate the effect of Cd on cell growth in a time and dose-dependent manner. Further possible Cd-derived alterations, as the peroxidation of membrane lipids or the functional impairment of the enzymatic antioxidant protection mechanisms, were investigated by determination of the MDA content and via catalase (CAT) activity detection. Moreover, ascorbic acid (AsA) pre-treatment was subjected to investigate the assumed positive effect of AsA against Cd toxicity. We show here on one hand that cells suffer under the influence of Cd, but on the other hand, they substantially profit from AsA supplementation. Because *S. pombe* is known to shares many molecular, and biochemical similarities with higher organisms, the effect of AsA in cadmium toxicity elimination might be expected to a similar extent also in other cell types.

Keywords: cell; cadmium; ascorbic acid; oxidative stress; contamination

INTRODUCTION
As the problem of environmental contamination affects the entire ecosystem, it represents a global concern. Heavy metal contamination in soils and water may lead to severe human health alterations due to the metals' toxicity.

Although toxic ions naturally occur in the earth´s crust, anthropogenic activities including mining, industrialization, agricultural activities, waste disposal, etc. enhance their presence in the environment **(Okereafor et al., 2020)**. To the most threatening elements belongs cadmium (Cd) a non-essential toxic metal that is ranked at 7th position among the 20 highest toxins. Its natural occurrence in the earth's crust represents approximately 0.1 mg.kg^{-1}, but in sediments and marine phosphates its concentration may increase to 15 mg.kg^{-1} **(Abedi and Mojiri, 2020; Hamid et al., 2019)**. Environmental contamination by Cd derives particularly from the use of phosphate fertilizers, recycling of electronic waste, smelting, and refining of metals, and combustion of fossil fuel **(Genchi et al., 2020)**. In such environments elevated Cd amounts deteriorate agricultural soils leading to Cd accumulation in crops ultimately reaching the human body. Scientists all around the globe evaluate food quality in terms of heavy metals contamination, including Cd. Rice is considered as the main Cd source worldwide, followed by seafood, vegetables, seaweed, grains, potatoes, and other types of food such as mushrooms, or raw cow milk **(Boudebbouz et al., 2021; Chiocchetti et al., 2020; Ćwieląg-Drabek et al., 2020; Huang et al., 2017; Jarosz-Krzemińska, Mikołajczyk and Adamiec, 2020; Kosečková et al., 2020; Liu et al., 2020; Satapathy, Panda and Jena, 2019; Suwatvitayakorn et al., 2019)**. Additionally, as Cd is naturally highly accumulated by the tobacco plant (*Nicotiana tabacum*), smokers incorporate a large amount of Cd from cigarettes **(Ganguly et al., 2018)**.

The adverse effects of Cd on human health are related to its long degradation period, unsuitability for decomposition, and its ability to accumulate in the kidneys, liver, and gut **(Fatima et al., 2019; Tinkov et al., 2018)**. Moreover, Cd is a proven (group I) human carcinogen according to International Agency for Research on Cancer classification. Cadmium exposure of either occupational or environmental origin can lead to apoptotic or necrotic events mediated through the cadmium-induced enhancement of reactive oxygen species (ROS) formation, excessive accumulation of Ca^{2+} ions, and/or irregular expression of caspase 3, bcl2, and p53. Additionally,

cadmium was shown to interfere with the activity of antioxidant enzymes, such as catalase or superoxide dismutase **(Genchi et al., 2020)**. Cadmium-mediated cell damage due to oxidative stress induction suggests that antioxidants may be able to reduce cadmium toxicity. One of the prominent antioxidant supplements, ascorbic acid (vitamin C) has been shown to prevent oxidative impairments of the cell membrane induced by free radicals **(Erdogan et al., 2005)**. Recently **Darwish et al. (2019)** have shown that ascorbic acid supplementation helped HepG2 cells to recover from oxidative damage caused by food-relevant concentrations of Cd. In the presented study we investigated the effect of ascorbic acid (AsA) pre-treatment on Cd-mediated cellular impairments. A nonpathogenic yeast *Schizosaccharomyces pombe* was used as the model organism for the experimental analyses. *S. pombe* is a widely used model system for eukaryotic biology as it possesses great similarity of genome organization with mammals and shares many features with higher eukaryotes **(Awan, Manfredo and Pleiss, 2013; Fawcett et al., 2014)**. We assume that because of this high level of similarity the obtained results might predict the effect of AsA in the elimination of Cd toxicity in other cell types. Figure 1 represents the positive effect of AsA against Cd-induced toxicity.

Scientific hypothesis

We predict that ascorbic acid due to its high antioxidant property will help cells to suppress the negative impact of Cd.

MATERIAL AND METHODOLOGY

Samples

Biological Material

Yeast strain

For all analyses, the prototroph wild-type strain of the yeast *Schizosaccharomyces pombe* was used (JG 15459).

Cultivation and growth conditions

The overnight culture of the yeast cells was performed according to **Forsburg and Rhind (2006)** in the complete, liquid YES medium which contains 3% glucose, 0.5% yeast extract (YE), and supplements (S) containing 225 $mg.L^{-1}$ of amino acids: adenine, L-histidine, L-leucine, L-lysine, and uracil (AppliChem GmbH, Darmstadt, Germany). Incubation was performed at 30 °C under aerobic conditions ensured by stirring at 150 rpm.

Chemicals

Ascorbic acid (AsA), Sigma-Aldrich, St. Louis, MO.

CdCl2, Sigma-Aldrich, St. Louis, MO.

Bovine serum albumin (BSA), Sigma-Aldrich, St. Louis, MO.

Instruments

GloMax Multi Detection System Promega Corporation, Madison, WI, USA.

Laboratory Methods

Growth rate determination

According to **Forsburg and Rhind (2006)**.

Growth intensity ratio (GIR) assessment

Determination of catalase activity

According to **Aebi (1984)**.

Measurement of MDA content

A slightly modified methodology of **Pekmez et al. (2008)** according to **Garre et al. (2010)** was used.

Protein concentration

According to Bradford assay **(Bradford, 1976)**.

Description of the Experiment

Growth rate determination

For the experimental analyses, yeast cells from the overnight culture were either pre-treated with ASA (Sigma-Aldrich, St. Louis, MO) or left untreated. Cell cultivation was performed under stirring conditions at 150 rpm and 30 °C. The experimental group of yeast cells was pre-treated with 10 mM of AsA for 30 min under aerobic conditions at 30 °C. Cell concentration was determined as optical density measured at 600 nm by the use of the GloMax Multi Detection System (Promega Corporation, Madison, WI, USA). Cells were diluted to an OD_{600} = 0.3 and subsequently transferred to 24-well plates where indicated final concentrations of Cd were added: 0, 1, 5, 10, 20, 40, 60, 100, 200, 300, and 400 μM of $CdCl_2$. Cells were incubated at 30 °C with shaking and after 3 hours of incubation, OD_{600} was measured. The difference between the OD_{600} value in the time point 3 h and the time point 0 h represents the growth intensity (GI) of the yeast cells after 3 hours of incubation. From the obtained data, the half critical concentration of Cd (IC_{50}) causing cell growth retardation to 50% in the presence or absence of AsA pre-treatment, was calculated. Freely available software Quest Graph™ IC50 Calculator was used to calculate the IC_{50} value.

Growth intensity ratio (GIR) was calculated from the growth intensity (GI) values obtained after 3, 6, and 9 hours of incubation in the presence of different Cd concentrations with (+AsA) and without (-AsA) ascorbic acid treatment. Growth intensity of AsA pre-treated cells was expressed as a percent ratio from the AsA untreated cells according to the formula:

$$GIR = 100 \, . \, GI^{+AsA} / GI^{-AsA}$$

The GIR value above 100 represents more intense cell growth of the AsA treated cells compared to AsA untreated cells.

Growth rate determination at time points 3, 6, and 9 h after Cd treatment was selected because the fission yeast cell cycle takes, on average, 3 h. Hence, analyses of two and three live cycles of the yeast served as experimental proof of the long-term Cd impact on the cell growth ability. Moreover, the effect of AsA pre-treatment on Cd-induced impairments on this process was assessed.

Analysis of oxidative stress and antioxidant defense

The antioxidant activity of catalase (CAT), referring to H_2O_2 decomposition, was determined by the use of Agilent Cary 60 UV/VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) as the drop of absorbance at 240 nm for 90 s as described by Aebi **(Aebi, 1984)**. The analysis was initiated by the addition of 50 μL of 30 mM H_2O_2 to the reaction solution containing 100 μL of the sample reaching the final volume of 600 μL. The molar absorption coefficient (36 $mM^{-1}.cm^{-1}$) was used to define the specific catalase activity.

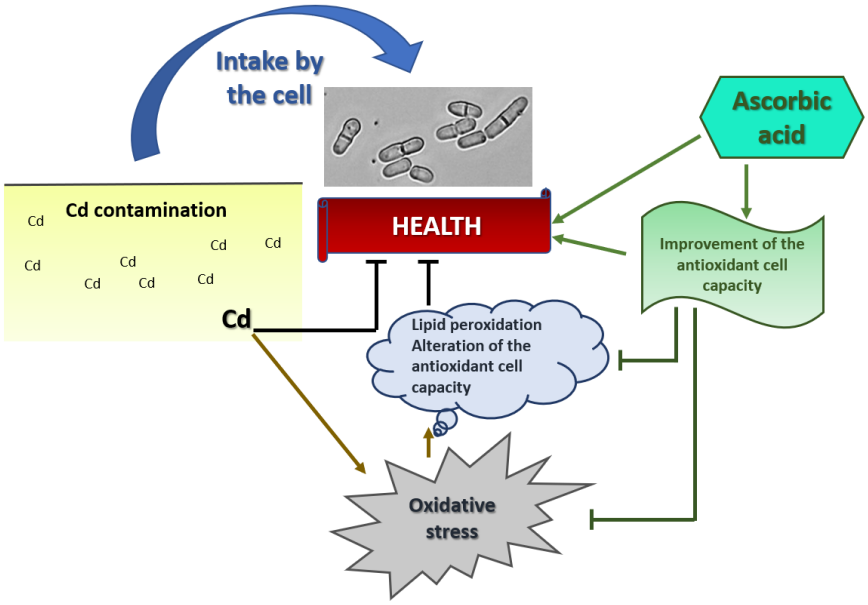

Figure 1 Schematic draw represents the positive effect of AsA against Cd-induced toxicity.

Lipid peroxidation was estimated by measurement of malondialdehyde (MDA) content. Briefly, to 300 µL of supernatant from each sample, 600 µL of thiobarbituric acid (TBA) solution containing 15% trichloroacetate (TCA) supplemented by 0.375% (w/v) TBA, was added and boiled at 95 °C for 30 min. Subsequently, the sample was cooled on ice and centrifuged at 8500 rpm for 60 s. The light absorption of the supernatant at 532 and 600 nm was measured with the use of an Agilent Cary 60 UV/VIS spectrophotometer. For calculation of the MDA content, we used the molar absorption coefficient 153 $mM^{-1}.cm^{-1}$, results were expressed as nmol per µg protein. Determination of protein concentration with the bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as a standard was performed at 600 nm according to Bradford assay **(Bradford, 1976)**.

Sample preparation: Yeast cell material (sample) for each treatment were collected from the yeast culture as described in the cultivation methodology. For biochemical analysis, the samples were stored at -80 °C until analyses.

Number of samples analyzed: Each treatment was analyzed using seven individual samplex (n = 7).

Number of repeated analyses: Spectrophotometrical measurements were performed 3 times for each sub-sample.

Number of experiment replication: The number of repetitions of each experiment to determine one value was triple.

Statistical Analysis

All data from the analyses are presented as the mean ± standard deviation (*SD*). Statistical analyses were performed using the Statistica 10 software (StatSoft Inc., Tulsa, OK, USA). The significance of the obtained differences was analyzed by ANOVA through Duncan and Fisher's LSD post hoc test.

Homogeneity and normality distribution of experimental data were evaluated by Lavene and Cochran's test. The statistical significance was set up to p <0.05 *, 0.01 **, and 0.001 *** for the detected statistically significant differences.

RESULTS AND DISCUSSION

Ascorbic acid mitigates Cd-induced cell growth impairments

As it has been previously reported on various model systems, Cd induces alterations of cellular processes on different levels resulting in cell growth retardation **(Yamada et al., 2009; Sandalio et al., 2001; Xu et al., 2019)**. To evaluate the effect of Cd on the growth ability of *S. pombe*, cells were subjected to various Cd concentrations supplemented in the culture media. Increasing Cd concentration led to sequential abolishment of the cell growth (Figure 2 red symbols). The effect of ascorbic acid on Cd-mediated cellular impairments was investigated based on previous reports showing that ascorbic acid, as a strong antioxidant compound plays a significant role in alleviating various abiotic stresses **(Venkatesh and Park, 2014)**. Additionally, external compounds such as salicylic acid or selenium, through scavenging reactive oxygen species, can mitigate Cd toxicity **(Liu et al., 2016; Lin et al., 2012)**. Indeed, culture pre-treatment with 10 mM AsA largely improved the growth of cells exposed to Cd (Figure 2 green symbols). Figure 2 depicts the growth intensity of cells treated for 3 hours with various Cd concentrations and the effect of AsA supplementation. AsA markedly enhanced cell growth, additionally acknowledged by the IC_{50} value. Cells pre-incubated with AsA display a 2.8 times higher IC_{50} value meaning that in the presence of AsA cells require almost three times the amount of Cd to inhibit cell growth to 50% compared to AsA untreated cells.

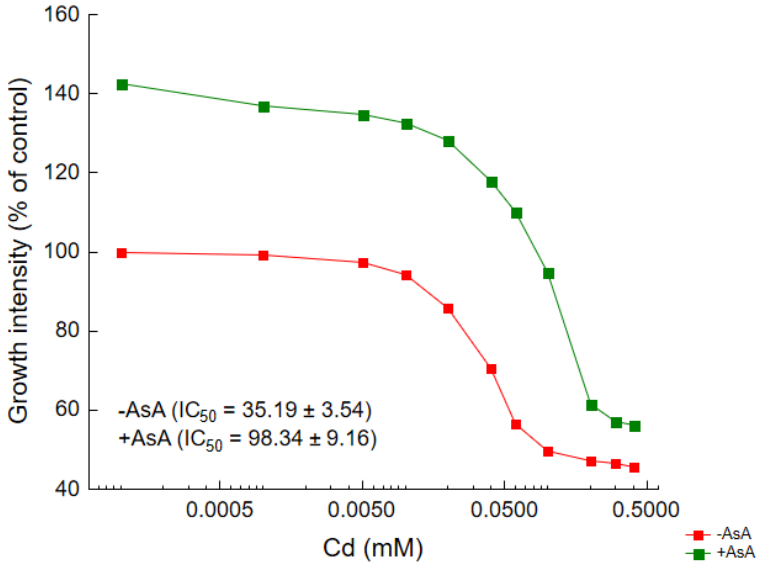

Figure 2 AsA eliminates Cd-derived growth intensity alterations.
Note: Depicted curves represent growth intensity of cells treated (green symbols) and untreated (red symbols) with AsA upon increasing Cd concentrations after 3 h of incubation expressed as % of control. IC_{50} values represent concentrations of Cd necessary for 50% cell growth inhibition without AsA supplementation (-AsA) or with AsA supplementation (+AsA).

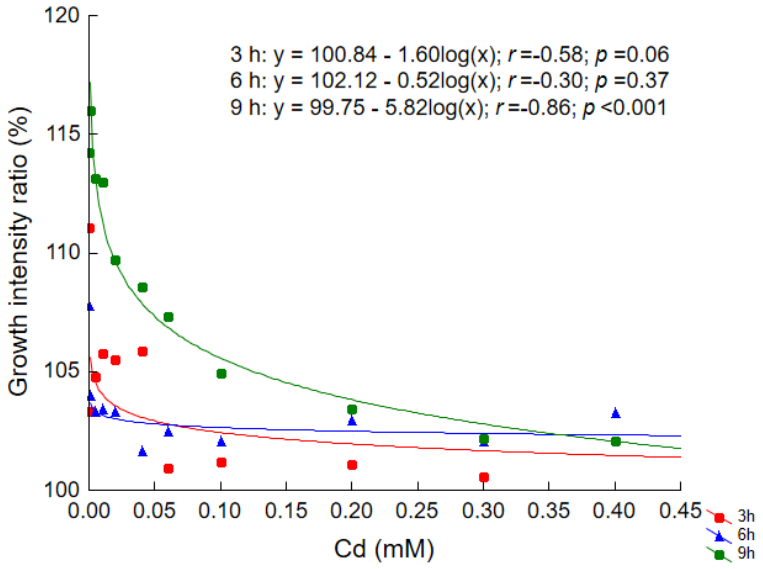

Figure 3 Growth intensity ratio of AsA treated cells.
Note: Time dependent growth intensity ratio represents proportional increase in the cell density of AsA treated cells. Growth intensity ratio (GIR) is expressed in percent of AsA treated cells growth intensity to the growth intensity of AsA untreated cells. If GIR value equals 100 it would represent identical growth intensity for both, AsA treated and untreated cells. GIR value greater than 100 represents higher growth intensity of AsA treated compared to untreated cells. The red line and symbols represent GIR after 3 hours, blue line and symbols represent GIR after 6 hours, green line and symbols represent 9 hours of cells incubation with Cd (0, 0.001, 0.005, 0.01, 0.02, 0.04, 0.06, 0.1, 0.2, 0.3, 0.4 mM).

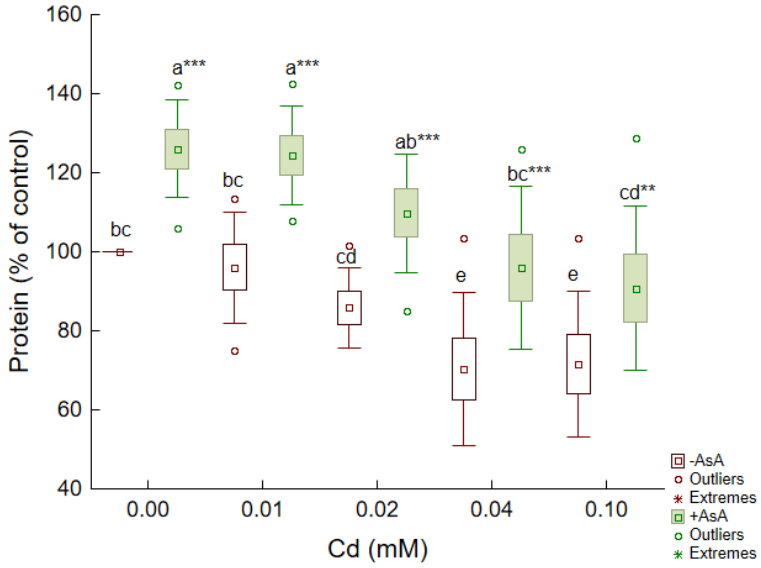

Figure 4 Protein mass gain.
Note: The difference of the protein mass gain represents the difference between the cell density increase of AsA untreated (brown symbols) and AsA treated (10 mM) (green symbols) cells after three hours of incubation with different Cd concentrations (0, 0.01, 0.02, 0.04, 0.1 mM). Individual values are expressed as % of control, n = 7. Number of * indicate statistical significance (p <0.05 *, 0.01 **, 0.001 ***), identical letters above bars denote that these values possess no significant difference.

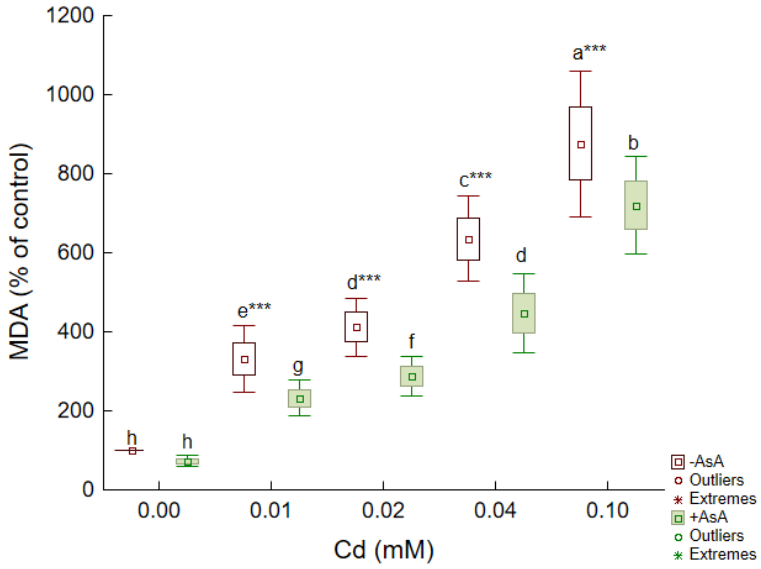

Figure 5 AsA alleviates Cd-mediated lipid peroxidation.
Note: Measurement of the MDA content expressed as % of control represents the difference of the MDA production between AsA untreated (brown symbols) and AsA treated (10 mM) (green symbols) cells after 3 hours of incubation with different Cd concentrations (0, 0.01, 0.02, 0.04, 0.1 mM), n = 7. Number of * indicate statistical significance (p <0.05 *, 0.01 **, 0.001 ***), identical letters above bars denote that these values possess no significant difference.

Moreover, growth curves of Cd-treated cells expressed as growth intensity ratio of the control show dose and time-dependent impact of cadmium and AsA on the cell proliferation (Figure 3).

Growth curves in Figure 3 show dose and time-dependent Cd-derived growth retardations and the significant positive impact of AsA pre-treatment. Hence, the data show that AsA pre-treatment mitigates the negative effect of Cd on cell growth. The statistical significance of the single and the mutual impact of Cd addition and AsA supplementation on the cell growth after 3, 6, and 9 hours of incubation was evaluated by the two-way ANOVA and for more accurate interpretation is depicted in Table 1 (After 3 h, After 6 h, After 9 h).

Another confirmation of the positive effect of AsA against Cd-mediated cell impairment is the protein content measurement that represents cell mass gain. As it is shown in Figure 4, AsA significantly enhanced the amount of protein in the presence of all Cd concentrations but also even in its absence. This implies that AsA widely improves cellular condition resulting in enhanced growth ability represented by elevated mass gain ability. Statistical evaluation by two-way ANOVA of the single and the mutual impact of Cd addition and AsA supplementation on the protein level is shown in Table 1 (Protein). Concomitant with our data a recently published study by **Zhang et al. (2019)** although on the different model system shows that exogenous application of AsA eliminates the negative effect of Cd on the growth of maize. Moreover, studies with guinea pigs also demonstrate that sufficient doses of ascorbic acid help to restore Cd-induced reduction of immune cells or eliminate nephrotoxicity **(Nagyova, Galbavy and Ginter, 1994; Kubová et al., 1993)**.

Ascorbic acid protects cells against Cd-mediated oxidative stress

To further investigate Cd-mediated toxicity to our model system and its possible elimination by ascorbic acid, the Cd-mediated oxidative stress and antioxidant defense mechanisms have been analyzed. Numerous studies on various model organisms confirmed the cadmium-derived enhancement of ROS production. **Wang et al. (2019)**, **Singh et al. (2018)**, **and Li et al. (2019)** detected increased free radical (as H_2O_2, O_2^-) formation in the lettuce and tomato seedlings, respectively upon Cd treatment. Moreover, Cd-induced cytotoxicity by excessive ROS was demonstrated by **Zhuang et al. (2019)** in duck renal tubular epithelial cells, **Modareszadeh et al. (2021)** in *Arabidopsis*, and interestingly by **Khojastehfar et al. (2015)** also in MCF-7 human breast cancer cell line and by **Wang et al. (2015)** in PC-12 cells. Although Cd does not induce elevation of ROS generation directly **(Liu, Qu and Kadiiska, 2009)**, its impact on excessive free radical formation is unquestionable. Hence, we were interested if *S. pombe* cell exposure to Cd will cause oxidative stress to the yeast cells. As the end product of the membrane lipid peroxidation is malondialdehyde (MDA), its increasing content indicates an escalation of oxidative stress **(Requena et al., 1996)**. In our experimental conditions, increasing Cd concentration enhanced MDA production in a dose-dependent manner (Figure 5 brown symbols). AsA pre-treatment significantly reduced MDA content (Figure 5 green symbols) suggesting its protective role against Cd-mediated oxidative stress. Culture treatment with AsA did not significantly affect MDA content in the control cells without Cd supplementation, while the addition of all Cd concentrations led to the dramatic increase of MDA amount. However, cells pre-incubated with AsA were largely protected against Cd toxicity. More detailed statistical analyses by two-way ANOVA of the significance of the single and mutual impact of Cd addition and AsA supplementation on MDA content are shown in Table 1 (MDA). Our observation is supported by other studies, revealing that antioxidant additives such as *Senna alexandrina* extract or salicylic acid or components enhancing the antioxidant capacity of the cell such as selenium or silicon might serve as cells protectors against Cd-mediated oxidative stress **(Wang et al., 2020; Liu et al., 2016; Tang et al., 2015; Lin et al., 2012)**.

To confirm the protective role of AsA through its antioxidant property against Cd toxicity, the activity of catalase (CAT) the antioxidant enzyme responsible for H_2O_2 scavenging, was analyzed. Lower Cd concentration (0.01 mM) led immediately to the enhancement of CAT activity of cells without AsA treatment, while AsA pre-incubation was sufficient to protect cells against Cd-mediated oxidative stress without CAT support. Increasing Cd concentrations in AsA untreated cells at first further enhanced CAT activity, however high Cd concentrations (0.04, and 0.1 mM) consistently interrupted CAT activity, and thus subjected cells to oxidative stress. Strikingly, AsA pre-treatment promoted cellular CAT activity even under exposure to high Cd concentrations leading to extended cell protection (Figure 6). Statistical evaluation by two-way ANOVA of the significance of the single and mutual impact of Cd addition and AsA supplementation on the CAT activity is shown in Table 1 (CAT). Similarly, while CAT activity in Cd-treated roots of *Brassica napus* has dramatically increased, concomitant AsA supplementation brought CAT activity to a normal state hence triggering decreased sensitivity of the plant towards Cd **(Jung et al., 2020)**. Interestingly, similar to our observations, AsA supplementation to Cd-exposed rats largely improved their antioxidant defense status **(Chen et al., 2018)**.

A presented study reveals that the impact of Cd on the exposed organism, through oxidative stress induction, is highly conserved among species and is evident to a similar extent from yeast to higher organisms such as plants, animals, or humans. Contamination of food, water, and air represents the major pathway of human exposure to Cd including Cd exposure to children through breast milk leads to Cd-mediated adverse effects of human health through oxidative stress induction **(Wu et al., 2016; Kippler et al., 2012; Chunhabundit, 2016)**. Thus, we assume that according to our observation, people living in areas exposed to critical amounts of Cd in the environment that are at high risk of oxidative stress should consider supplementation of their nutrition with AsA.

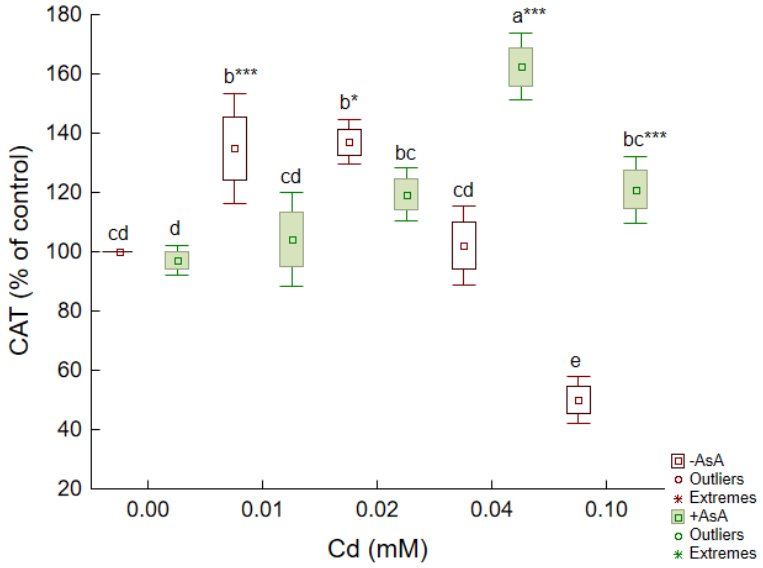

Figure 6 Cadmium mediated impairments of the CAT activity are restored by AsA supplementation.
Note: CAT activity determination expressed as % of control depicts the differences of the CAT activity between AsA untreated (brown symbols) and AsA treated (10mM) (green symbols) cells after three hours of incubation with different Cd concentrations (0, 0.01, 0.02, 0.04, 0.1 mM), n = 7. Number of * indicate statistical significance (p <0.05 *, 0.01 **, 0.001 ***), identical letters above bars denote that these values possess no significant difference.

Table 1 Statistical analyses by two-way ANOVA for changes in the cell growth and biochemical parameters. AsA – AsA treatment, Cd – different Cd concentration, AsA×Cd – interaction of AsA and Cd effects.

Trait	Effect	*SS*	*MS*	*F*	*p*
			Growth intensity		
After 3 h	AsA	0.0745	0.0745	38.0	0.000000
	Cd	3.2725	0.3273	167.0	0.000000
	AsA×Cd	0.0509	0.0051	2.6	0.009611
After 6 h	AsA	0.1269	0.1269	18.95	0.000045
	Cd	27.4592	2.7459	410.12	0.000000
	AsA×Cd	0.0964	0.0096	1.44	0.181151
After 9 h	AsA	0.0675	0.0675	5.95	0.017271
	Cd	62.0007	6.2001	546.18	0.000000
	AsA×Cd	0.0732	0.0013	7.02	0.000000
			Biochemistry		
Protein	AsA	0.15156	0.15156	217.29	0.000000
	Cd	0.61470	0.15367	220.32	0.000000
	AsA×Cd	0.03920	0.00980	14.05	0.000001
MDA	AsA	2.9769	2.9769	204.522	0.000000
	Cd	49.1648	12.2912	844.446	0.000000
	AsA×Cd	0.6322	0.1580	10.858	0.000015
CAT	AsA	0.0116	0.0116	0.0187	0.892672
	Cd	10.8949	2.7237	4.3866	0.010434
	AsA×Cd	11.6358	2.9089	4.6849	0.007852

Table 2 The effect of Cd and AsA supplementation on protein level, MDA content and CAT activity in absolute values.

Cd (mM)	Protein (mg.mL^{-1})		MDA (mg.mL^{-1})		CAT (μM.mg^{-1}.min^{-1})	
	-AsA	+AsA	-AsA	+AsA	-AsA	+AsA
0	0.95 ±0.11	1.18 ±0.08***	0.47 ±0.06	0.34 ±0.02nd	2.98 ±0.85	2.89 ±0.12nd
10	0.90 ±0.07	1.17 ±0.08***	1.51 ±0.11	1.07 ±0.04***	4.31 ±0.67	2.81 ±0.23**
20	0.80 ±0.05	1.02 ±0.10***	1.90 ±0.08	1.33 ±0.09***	6.37 ±0.96	3.35 ±0.22***
40	0.65 ±0.09	0.89 ±0.09***	2.93 ±0.20	2.05 ±0.11***	3.64 ±0.61	5.94 ±0.62***
100	0.66 ±0.07	0.84 ±0.07**	4.32 ±0.15	3.31 ±0.06***	1.79 ±0.61	4.42 ±0.53***

CONCLUSION

A surrounding environment contaminated with increasing Cd concentration causes dose-dependent cell growth intensity alteration. Cell pretreatment with 10 mM AsA considerably improved the growth of cells resulting in a significantly increased gain of protein mass (p <0.001). Cd-mediated oxidative stress led to lipid peroxidation resulting in the consistent increase of the MDA content (p <0.001) upon rising Cd concentration. The strong antioxidant property of AsA caused that its pre-treatment markedly decreased MDA content (p <0.001) suggesting cell membrane lipid preservation. Enzymatic protection against oxidative stress ensured by CAT was significantly altered by Cd (p <0.05), while AsA pre-treatment restored CAT activity (p <0.01) thereby protecting cells against Cd-induced oxidative stress (summarized in Table 2).

Taken together, our results demonstrate the positive effect of AsA against cellular impairments resulting from Cd toxicity upon its contamination. We show here that cells subjected to Cd contamination substantially profit from AsA supplementation. However, further studies are required to understand the exact protective nature of AsA on the overall cellular parameters under conditions of the contaminated environment.

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