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Astaxanthin Provides Cytoprotection in Response to Oxidative Stress in an Autophagy-Dependent Manner

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ABSTRACT

Objective: Astaxanthin (ATX) is a well-known antioxidant and cytoprotective natural xanthophyll carotenoid that exhibits protective effects against a wide range of pathological conditions. However, its underlying mechanisms and the contribution of autophagy remain unclear. This study aimed to investigate the potential role of autophagy in ATX-mediated cytoprotection under oxidative stress conditions through a genetic approach.

Materials and Methods: Wild-type and autophagy-deficient (*atg8Δ*) *Saccharomyces cerevisiae* strains were exposed to 1, 3, or 8 mM H_2O_2 for up to three hours. Cell viability and intracellular reactive oxygen species (ROS) levels were measured using the colony-forming unit assay and H_2DCFDA staining, respectively. To evaluate the contribution of autophagy to ATX-mediated cytoprotection, wild-type and *atg8Δ* cells were pretreated with or without 30 µM ATX for three hours, followed by exposure to 3 mM H_2O_2 for one hour. Viability and ROS levels were compared between wild-type and *atg8Δ* strains.

Results: H_2O_2 reduced cell viability in both strains in a dose- and time-dependent manner, with *atg8Δ* cells showing increased sensitivity. ROS levels also showed a dose-dependent increase in both strains and were higher in *atg8Δ* cells after prolonged H_2O_2 exposure. ATX pretreatment effectively reduced intracellular ROS levels in both strains. While ATX improved viability in wild-type cells, it failed to enhance viability in autophagy-deficient cells.

Conclusion: Autophagy may not be required for the ROS-reducing effects of ATX but may be essential for protection against oxidative stress-induced cell death, highlighting the importance of autophagy in ATX-mediated cytoprotection.

Keywords: Astaxanthin, autophagy, oxidative stress, *Saccharomyces cerevisiae*, *atg8*.

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INTRODUCTION

Oxidative stress, defined as an imbalance between oxidant molecules and antioxidant defense mechanisms in favor of the former, is a major contributor to numerous disorders, including neurological, cardiovascular, and respiratory diseases, diabetes mellitus, and even cancer.[1] Reactive oxygen species (ROS) are the most prevalent and reactive forms among oxidant molecules, and while physiological concentrations of ROS are beneficial for cells by acting as signaling molecules,

elevated levels can alter cell signaling and oxidatively damage biomolecules such as proteins, lipids, and nucleic acids.[2] ROS generation in cells may result from radiation, toxins, inflammation, as well as regular metabolic processes. Consequently, antioxidant molecules play a crucial role in maintaining cellular homeostasis by neutralizing excessive ROS and preventing oxidative damage.

Natural antioxidant compounds have been used in therapies for centuries and have gained scientific attention in recent decades for their ability to protect cells against oxidative stress and related damage, as well as for their pharmaceutical potential. Among them, astaxanthin (ATX) is a natural xanthophyll carotenoid synthesized by various microorganisms and marine organisms, and is known for its strong antioxidant capacity.[3] The antioxidant activity of ATX is reported to be approximately 6,000 times stronger than that of vitamin C, 11 times more potent than β-carotene, and about 50 times more effective than vitamin E.[4] These properties position ATX among the most powerful natural antioxidants identified to date. ATX exerts its antioxidant effects by directly scavenging ROS and by modulating cellular stress-response and redox-sensitive signaling pathways such as Nrf2 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). In addition to its antioxidant effects, ATX exhibits anti-apoptotic, anti-proliferative, and anti-invasive properties in cancer cells, as well as anti-inflammatory effects in several diseases,[4] making it a promising multifunctional agent for the prevention and treatment of chronic diseases.

Autophagy is a fundamental cellular process that degrades damaged or redundant cellular components in response to various stressors, including nutrient deficiency, misfolded proteins, and oxidative/nitrosative stress.[5] Thus, autophagy serves as an important protective mechanism that maintains cellular homeostasis and survival. The role of ATX in the regulation of autophagy is disease- and context-specific, generally promoting cellular health. Several studies have shown that ATX suppresses autophagy in doxorubicin-induced myocardial damage[6] and cerulein-induced acute pancreatitis in mice.[7] In contrast, ATX improves metabolic homeostasis and reduces lipid accumulation in the liver by promoting autophagy in high-fat-diet rats[8] and increases autophagy markers such as mTOR, beclin-1, and LC3B in patients with type 2 diabetes, thereby improving metabolic homeostasis.[9]

The objective of this study was to determine the potential contribution of autophagy to the protective role of ATX against oxidative stress by using a genetic approach in wild-type (WT) and autophagy-related protein 8 (Atg8)-deficient (*atg8Δ*) *Saccharomyces cerevisiae* strains.

KEY MESSAGES

- Autophagy is required for the cytoprotective effect of astaxanthin under oxidative stress.
- Reduction of intracellular ROS levels by astaxanthin may be independent of autophagy.
- Survival of autophagy-deficient cells under oxidative stress is not enhanced by astaxanthin pretreatment.

MATERIALS AND METHODS

Yeast Strains, Culture Conditions, and Experimental Treatments

Wild-type (WT) (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*), and *atg8Δ* (MAT*a*; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; atg8::KANMX4) *Saccharomyces cerevisiae* strains derived from the BY4741 genetic background were used. All strains were cultured on Yeast extract Peptone Dextrose (YPD) plates (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) and incubated at 30°C for two days. Following incubation, saturated yeast cultures were obtained from single colonies of each strain by inoculating them into liquid YPD media (1% yeast extract, 2% peptone, and 2% glucose) and culturing at 30°C with shaking at 175 rpm for two days. A small volume of each saturated culture was then inoculated into fresh YPD medium and incubated overnight at 30°C with shaking at 175 rpm. The cultures were grown to either early (OD_{600}≈0.3) or mid-late logarithmic phase (OD_{600}≈1.0), depending on the experimental design. To induce oxidative stress, hydrogen peroxide (H_2O_2) was applied to cultures grown to the mid-late logarithmic phase. Based on our previous studies,[10–12] 1 mM H_2O_2 for one hour was used to model mild oxidative stress, 3 mM for one hour represented moderate stress, and 8 mM for three hours simulated severe oxidative stress. For ATX pretreatment, WT and *atg8Δ* cells grown to early logarithmic phase (OD_{600}≈0.3) were divided into two groups and incubated with or without 30 µM ATX for three hours at 30°C with shaking. Following pretreatment, cells were harvested, washed, resuspended in fresh YPD medium, and used for downstream oxidative stress experiments.

Experimental Design

To assess the role of autophagy under oxidative stress conditions, WT and *atg8Δ S. cerevisiae* strains (T0) were exposed to H_2O_2 at concentrations of 1 mM for one hour (T1), 3 mM for one hour (T1), and 8 mM for three hours (T3) to evaluate cell viability. Intracellular ROS levels were measured under all three concentrations (1, 3, and 8 mM H_2O_2) at both 1-hour and 3-hour time points (T1 and T3). To assess the cytoprotective effect of ATX and the contribution of autophagy under oxidative stress conditions, ATX-pretreated and untreated WT and *atg8Δ S.*

cerevisiae strains were exposed to 3 mM H_2O_2 for one hour (T1) to induce oxidative stress. Cell viability and intracellular ROS levels were assessed at T1 in the following four experimental groups: WT + ATX, WT – ATX, *atg8Δ* + ATX, and *atg8Δ* – ATX. All experiments were conducted in biological triplicates (n=3). Cell viability and ROS levels were measured using colony-forming unit (CFU) assays and H_2DCFDA-based fluorescence analysis, respectively.

Colony-Forming Unit Assay

The CFU assay was performed as described previously.[13] For experiments assessing the involvement of autophagy in oxidative stress, samples were collected before (T0) and after H_2O_2 induction (T1 or T3). For ATX experiments, samples were taken immediately after the 3-hour ATX incubation (T0) and following 1 and 3 hours of incubation in fresh liquid YPD medium containing 1 µM or 8 µM H_2O_2. Briefly, 10-fold serial dilutions of each sample were prepared in sterile dH_2O. A 100 µL volume of the appropriate dilution of each sample was spread onto a YPD plate and incubated at 30°C for two days. To determine the number of CFU/mL in the sample, colonies on each plate were counted and divided by the plated volume (0.1 mL) and the dilution factor. CFU fold changes were calculated by dividing the CFU/mL value by that of the initial sample (T0). The average CFU fold change values from three experiments were plotted with standard deviation (SD).

Intracellular Reactive Oxygen Species Detection

ROS accumulation was determined using 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA). Briefly, 200 µL of each sample were centrifuged, resuspended in fresh YPD medium, and incubated with 10 µg/mL H_2DCFDA (Molecular Probes) at 30°C for 40 minutes in the dark. After incubation, at least 200 cells from each sample were examined under a fluorescence microscope (Leica DM1000 LED, Leica Microsystems, Germany) and categorized as "DCF-positive" or "DCF-negative." The percentage of fluorescent cells was calculated by normalizing the number of DCF-positive cells to the total number of cells counted in the culture. The average percentage of fluorescent cells from three experiments for each strain was presented with SD.

Statistical Analysis

All experiments were performed in triplicate (n=3), and data are presented as the mean with standard deviation. No formal sample size calculation was performed; the number of replicates was based on standard practices in similar biological studies. The normality of data distribution was assessed using the Shapiro-Wilk test. As the data did not meet the assumption of normality (Shapiro-Wilk test, $p<0.05$), non-parametric statistical methods were applied. Non-parametric comparisons between two independent groups were performed using the Mann-Whitney U test. For non-parametric comparisons involving multiple independent groups, the Kruskal-Wallis test was used. When significant, pairwise comparisons were conducted using Dunn's post hoc test with Bonferroni correction, performed with SPSS version 25.0 (IBM Corp., Armonk, NY, USA). A p-value <0.05 was considered statistically significant. Effect sizes for pairwise comparisons were calculated using Cohen's d. Interpretation of effect sizes was based on the guidelines by Sawilowsky[14] (2009), where d=0.01 is considered very small, 0.20 small, 0.50 medium, 0.80 large, 1.20 very large, and 2.00 huge.

RESULTS

Effects of Autophagy Inhibition on Cell Viability Under H_2O_2-Induced Oxidative Stress

To assess the contribution of autophagy to oxidative stress-induced cell death, CFU fold changes were compared between WT and autophagy-deficient (*atg8Δ*) cells under three different oxidative stress conditions induced by H_2O_2 (Fig. 1a, b). Pairwise comparisons between WT and *atg8Δ* strains under identical oxidative stress conditions, conducted using the Mann-Whitney U test, did not reveal statistically significant differences ($p>0.05$). Mild oxidative stress (1 mM H_2O_2, 1 hour) resulted in a notable decrease in the viability of autophagy-deficient cells, with CFU fold changes of 0.456 and 0.269 for WT and *atg8Δ* cultures, respectively (Fig. 1a). Although no statistical significance was observed, the effect size (d=2.5756) indicated a large biological difference between the groups. The sensitivity of *atg8Δ* cells became more pronounced under moderate oxidative stress conditions (3 mM H_2O_2, 1 hour), with CFU fold changes of 0.138 for WT and 0.007 for *atg8Δ* cells, corresponding to a very large effect size (d=3.5382) (Fig. 1a). Consistently, the average CFU fold change ratio of *atg8Δ* cultures was 0.008, compared to 0.004 for WT cultures, under the most severe oxidative stress conditions (8 mM H_2O_2, 3 hours), while the large effect size indicated an important biological difference (d=1.6330) (Fig. 1b). Together, these findings show that suppression of autophagy leads to reduced cell viability upon H_2O_2 exposure compared to wild-type cells under both oxidative stress conditions examined. This implies that active autophagy may provide protection against oxidative stress-induced cell death.

Intracellular ROS Accumulation in Wild-Type and Autophagy-Deficient Cells Under Oxidative Stress

After 1 and 3 hours of H_2O_2 exposure, both strains showed a gradual increase in ROS levels (Fig. 2). Pairwise comparisons using the Mann-Whitney U test revealed no statistically significant differences between the strains under the same H_2O_2 concentration and exposure duration ($p>0.05$); however, effect size analysis using Cohen's d indicated varying degrees of biological relevance. Intracellular ROS levels were below 1% for

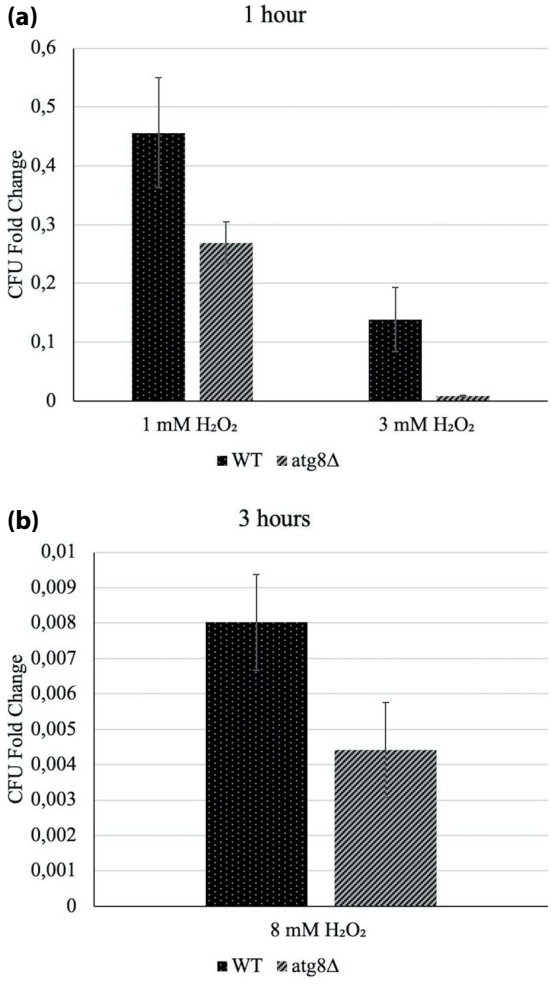

Figure 1. Effects of autophagy deficiency on the viability of Saccharomyces cerevisiae cells in response to oxidative stress. **(a)** Colony-forming unit (CFU) fold change of wild-type (WT) and autophagy-deficient (*atg8Δ*) *S. cerevisiae* cells after 1-hour exposure to 1 mM or 3 mM H_2O_2. **(b)** CFU fold change after 3-hour exposure to 8 mM H_2O_2. Bars represent the mean±standard deviation (SD) of three independent experiments (n=3).

both WT and *atg8Δ* cultures in the absence of H_2O_2, indicating no substantial oxidative stress under normal conditions. Intracellular ROS accumulation in the WT strain in response to 1, 3, and 8 mM H_2O_2 treatment for 1 hour was 6.59%, 28.24%, and 70.2%, respectively. Similarly, the percentages of *atg8Δ* cells treated with 1, 3, and 8 mM H_2O_2 for 1 hour were 13.29%, 28,53%, and 69.58%, respectively. The differences were not significant compared to WT cultures at the same concentrations (p>0.05). A large effect size was only observed in the comparison for the 1 mM H_2O_2 treatment (d=1.6696), indicating biological relevance between WT and *atg8Δ* cells.

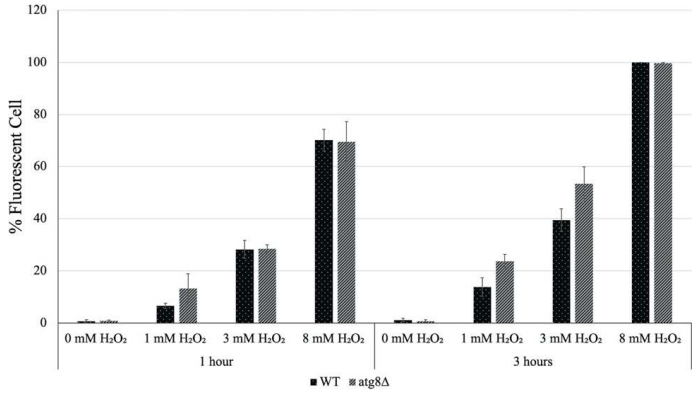

Figure 2. Intracellular reactive oxygen species (ROS) accumulation in wild-type (WT) and autophagy-deficient (*atg8Δ*) cells under oxidative stress. Bars represent the mean±standard deviation (SD) of the percentage of fluorescent cells from three independent experiments (n=3).

However, the biological relevance of the differences between WT and *atg8Δ* was very low in response to 3 and 8 mM H_2O_2 treatments (d=0.1088 and d=0.1008, respectively). After three hours of exposure to 1 and 3 mM H_2O_2, intracellular ROS levels of *atg8Δ* cultures were higher compared to WT. The percentages of ROS accumulation for cells treated with 1, 3, and 8 mM H_2O_2 were 13.84%, 39.5%, and 100% for WT cells, and 23.66%, 53.38%, and 99.66% for *atg8Δ* cells, respectively. While the differences between strains exposed to the same concentrations of H_2O_2 were not statistically significant (p>0.05), the extremely large effect sizes for the 1 and 3 mM H_2O_2 groups reflect a profound biological impact (d=3.1417 and d=2.4755, respectively). The difference in ROS% between WT and *atg8Δ* cultures in response to 8 mM H_2O_2 was minimal, and the effect size (d≈0.00) indicates no biological relevance under this condition. These results suggest that autophagy deficiency leads to increased ROS accumulation under prolonged or mild oxidative stress, whereas severe stress conditions (8 mM H_2O_2) may eliminate this effect, as ROS levels are already extremely high and viability is markedly reduced in both strains.

Effects of Astaxanthin on the Viability of WT and *atg8Δ* Cells in Response to H_2O_2-Induced Oxidative Stress

A significant variation in CFU fold changes across all experimental groups was identified by Kruskal-Wallis analysis (p=0.016) (Fig. 3). ATX pretreatment resulted in a higher CFU fold change in WT cells in response to 3 mM H_2O_2 exposure for one hour, with the ratios being 0.248 for ATX-pretreated and 0.186 for ATX-untreated cells. Although the increase in CFU fold change was not statistically significant (p>0.05), the large effect size (d=2.4842) indicates important biological significance among these groups. The increase in CFU fold

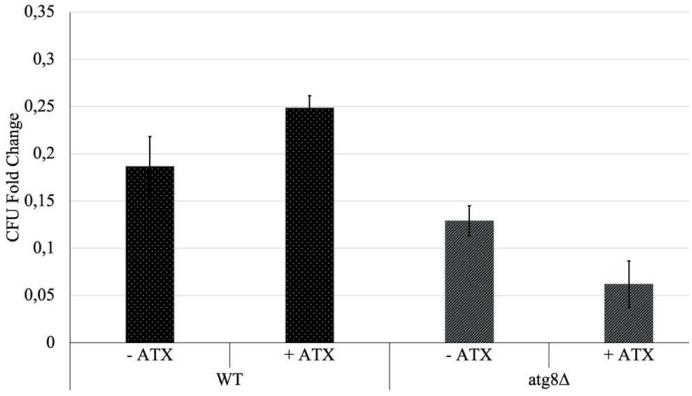

Figure 3. Effects of 3-hour astaxanthin (ATX) pretreatment on the viability of wild-type and autophagy-deficient (*atg8Δ*) cells under 3 mM H_2O_2 exposure for one hour. Bars represent the mean±standard deviation (SD) of the colony-forming unit (CFU) fold change from three independent experiments (n=3). Significant differences were found among the groups, as determined by Kruskal-Wallis analysis (p=0.016).

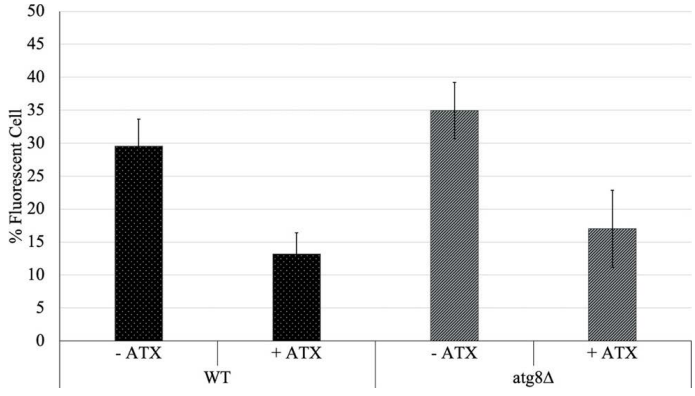

Figure 4. Effects of 3-hour astaxanthin (ATX) pretreatment on intracellular reactive oxygen species (ROS) levels in wild-type and autophagy-deficient (*atg8Δ*) cells under 3 mM H_2O_2 exposure for one hour. Bars represent the mean±standard deviation (SD) of the percentage of fluorescent cells from three independent experiments (n=3). Overall group differences were statistically significant, as determined by Kruskal-Wallis analysis (p=0.021).

change suggests enhanced resistance to oxidative stress-induced cell death. In contrast, resistance to oxidative stress-induced cell death was not observed in autophagy-deficient cells in response to ATX pretreatment. The CFU fold change ratios for ATX-untreated and ATX-pretreated *atg8Δ* cells were 0.128 and 0.062, respectively. Furthermore, the reduction in CFU fold change observed in ATX-pretreated autophagy-deficient cells was biologically important, with a high effect size (d=3.5753), but it was not statistically significant (p<0.05). These data indicate that ATX pretreatment not only fails to improve cell viability in the absence of autophagy but also exacerbates the oxidative stress-induced decrease in CFU fold change. These findings suggest that the protective effect of ATX on cell viability under oxidative stress may depend on the presence of functional autophagy.

Effects of Astaxanthin on Intracellular ROS Levels in WT and *atg8Δ* Cells Under Oxidative Stress

Comparison across all experimental groups using Kruskal-Wallis analysis showed a statistically significant difference in intracellular ROS levels (p=0.021). Consistent with its well-known antioxidant properties, ATX pretreatment decreased intracellular ROS levels in both WT and *atg8Δ* strains in response to 3 mM H_2O_2 exposure. Intracellular ROS levels were reduced from 29.57% to 13.2% in WT cells and from 34.94% to 17.01% in *atg8Δ* cells following ATX pretreatment (Fig. 4). Although these differences were not statistically significant, effect sizes were very large in both strains, indicating a substantial reduction in oxidative stress (p>0.05, d=4.4854 for WT and d=3.5164 for *atg8Δ*). These findings suggest that ATX effectively reduces intracellular ROS levels in both wild-type and autophagy-deficient cells under moderate oxidative stress, supporting its ROS-reducing potential independently of autophagy.

DISCUSSION

In this study, we investigated the potential contribution of autophagy to the cytoprotective effect of ATX against oxidative stress. In the absence of ATX, intracellular ROS levels were elevated in *atg8Δ* cells compared to WT, particularly under mild and prolonged oxidative stress conditions, such as 1 mM H_2O_2 for 1 hour and both 1 and 3 mM H_2O_2 for 3 hours. These findings suggest that autophagy may contribute to ROS clearance under low-to-moderate and prolonged oxidative stress conditions. In contrast, no difference was observed between WT and *atg8Δ* strains under 8 mM H_2O_2, likely due to excessive ROS accumulation that surpasses the cellular detoxification capacity in both strains.

Taken together, the absence of ATG8 consistently resulted in reduced cell viability under all oxidative stress conditions tested, suggesting that autophagy plays a role in protecting against oxidative stress-induced cell death. Similarly, *atg8Δ* cells generally exhibited higher ROS levels, particularly under mild and prolonged oxidative stress, indicating a partial role of autophagy in mitigating ROS accumulation. These observations collectively point to a protective role for autophagy not only in maintaining cell viability but also in limiting intracellular ROS accumulation during oxidative stress. Our findings align with previous studies demonstrating the

regulatory interplay between oxidative stress and autophagy. Filomeni et al.[15] (2015) reported that autophagy plays a critical role in safeguarding cells during oxidative stress by removing oxidatively damaged biomolecules. Xu et al.[16] (2021) showed that iron overload induces both apoptosis and autophagy in osteoblasts, and that inhibition of autophagy exacerbates oxidative stress and cell death. In their study, inhibition of autophagy led to higher ROS accumulation and greater cell death, emphasizing autophagy's role in ROS clearance and cytoprotection. Similarly, in our study, autophagy-deficient (*atg8Δ*) cells exhibited significantly reduced viability and elevated intracellular ROS levels under oxidative stress compared to wild-type controls. These results suggest that functional autophagy not only supports cellular survival but also contributes to redox balance by facilitating the clearance of ROS-damaged components. Collectively, our data and previous studies support the conserved role of autophagy in protecting cells from oxidative damage, particularly under sustained or moderate stress conditions.

Next, we evaluated the cytoprotective properties of ATX and the potential contribution of autophagy to its function. ATX is a powerful antioxidant known to neutralize free radicals and protect various cell types from oxidative damage. Numerous studies have shown that ATX pretreatment improves cell viability under oxidative stress in multiple cell types, including mesenchymal stem cells,[17] hippocampal neurons,[18] retinal ganglion cells,[19] and neuroblastoma cells.[20] In wild-type *S. cerevisiae* cells, we found that ATX pretreatment preserved cell viability under oxidative stress, consistent with its established antioxidant and protective effects. However, in autophagy-deficient (*atg8Δ*) cells, ATX pretreatment did not confer similar protection. These results suggest that the protective effect of ATX against oxidative stress-induced cell death may require functional autophagy. Similarly, in hepatic ischemia-reperfusion injury, ATX pretreatment significantly reduced both apoptotic and autophagy-associated cell death by attenuating ROS accumulation and modulating mitogen-activated protein kinase (MAPK) pathway signaling.[21] ATX has also been shown to enhance cellular homeostasis in neurodegenerative diseases through mechanisms such as lysosomal clearance, restoration of mitochondrial membrane potential, and reduction of toxic protein aggregates, all of which critically depend on autophagy.[22,23] Mechanistically, ATX has been shown to regulate several conserved stress-response pathways that are essential for autophagy. It promotes phosphorylation of AMP-activated protein kinase (AMPK), which suppresses mechanistic target of rapamycin (mTOR) activity and ultimately enhances autophagy.[9,24] ATX also modulates the MAPK cascade, including kinases such as p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK), which are important in both oxidative stress responses and autophagy regulation.[25] Previous studies have reported that ATX reduces apoptosis and induces autophagy by modulating MAPK signaling in mammalian cells.[24] Although our study does not directly examine these signaling pathways, the reduced cell viability observed in *atg8Δ* cells under oxidative stress suggests that the ability of ATX to enhance viability under such conditions may depend on autophagy, possibly through effects on these conserved stress response pathways. In our experimental setting, due to its strong antioxidant capacity, ATX pretreatment significantly reduced intracellular ROS levels in both autophagy-deficient and wild-type cells, indicating that the antioxidant activity of ATX may be independent of the autophagy mechanism. Previous studies have shown that ATX promotes the nuclear translocation of Nrf2, thereby enhancing the transcription of a diverse set of cytoprotective genes involved in redox homeostasis (e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx), heme oxygenase-1 (HO-1)) and detoxification (e.g., NAD(P)H quinone dehydrogenase 1 (NQO1), glutathione S-transferases (GSTs)).[26] Furthermore, ATX not only supports cellular detoxification by activating redox-sensitive signaling pathways involved in antioxidant defense, but also directly exerts ROS-scavenging activity by neutralizing ROS through direct chemical quenching.[27] It has also been reported that ATX localizes to cell membranes and protects them from lipid peroxidation by physically quenching singlet oxygen and other ROS species.[28] Therefore, ATX may retain its ROS-reducing capacity in both strains through direct ROS-scavenging.

Our findings collectively suggest that while the ROS-reducing activity of ATX may be autophagy-independent, its ability to promote cell survival under oxidative stress may require functional autophagy. Consistent with previous studies, our findings in the *S. cerevisiae* model provide clear genetic evidence that autophagy represents an important mechanism in the cytoprotective action of ATX.

CONCLUSION

In conclusion, our results underscore the pivotal role of autophagy in mediating the protective effects of ATX against oxidative stress in *S. cerevisiae* through a genetic approach. Although ATX significantly reduces intracellular ROS levels even in the absence of autophagy, this antioxidant activity alone is not sufficient to maintain cell viability under oxidative stress. The lack of protection from oxidative stress-induced cell death in autophagy-deficient strains highlights the important contribution of autophagy to the cytoprotective role of ATX. Thus, ATX exerts its cytoprotective effects through both its antioxidant properties and the autophagy mechanism. Future studies should investigate how these two major protective mechanisms triggered by ATX coordinate, particularly under prolonged oxidative stress.

Ethics Committee Approval: Ethical approval was not required for this study, as it did not include human subjects, human-derived cells or tissues, animal experiments, or any use of clinical or patient data.

Informed Consent: Since no human subjects or patient-related materials were used in this study, informed consent was not applicable.

Conflict of Interest: The authors have no conflict of interest to declare.

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