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Quercetin Protects Yeast *Saccharomyces cerevisiae pep4* Mutant from Oxidative and Apoptotic Stress and Extends Chronological Lifespan

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Abstract
The yeast *Saccharomyces cerevisiae PEP4* gene encodes vacuolar endopeptidase proteinase A (Pep4p), which is a homolog of the human *CTSD* gene that encodes cathepsin D. Mutation of *CTSD* gene in human resulted in a number of neurodegenerative diseases. In this study, we have shown that yeast *pep4* mutant cells are highly sensitive to oxidative and apoptotic stress induced by hydrogen peroxide and acetic acid, respectively. *pep4*Δ cells also showed accumulation of reactive oxygen species (ROS), apoptotic markers, and reduced chronological lifespan. In contrast, quercetin pretreatment protected the *pep4* mutant from oxidative and apoptotic stress-induced sensitivity by scavenging ROS and reducing apoptotic markers. The percentage viability of quercetin-treated *pep4*Δ cells was more pronounced and increased stress resistance against oxidant, apoptotic, and heat stress during chronological aging. From our experimental results, we concluded that quercetin protects yeast *pep4* mutant cells from oxidative stress and apoptosis, thereby increasing viability during chronological aging.

Introduction

The yeast *Saccharomyces cerevisiae PEP4* gene encodes vacuolar aspartyl protease, proteinase A (Pep4p), which is highly homologous to the mammalian cathepsin D (*CTSD*) gene which codes for lysosomal aspartyl protease, cathepsin D (Cat D) [39]. Mammalian Cat D is very essential for normal development and/or maintenance of neurons in the central nervous system (CNS). It plays a critical role in the degradation of proteins linked to neurodegenerative diseases, such as vasopressin in familial neurohypophyseal diabetes insipidus [6], alpha-synuclein in Parkinson's disease [29], polyQ-huntingtin in Huntington disease [15], lipofuscins in neuronal ceroid lipofuscinosis [32], amyloid protein precursor, tau, and ApoE proteins in Alzheimer's disease [31]. Thus its deficiency results in several catastrophic neurological disorders characterized by extreme neuronal degeneration with severely shortened life span in man, sheep, and mouse [25].

Yeast Pep4p is a major vacuolar endopeptidase that is required for the in vivo processing of a number of vacuolar zymogens as well as for the proteolysis of oxidized proteins [21] and plays an important role in the life cycle of yeast. The *pep4* mutation results in a 90–95% reduction in the activity of vacuolar hydrolases such as proteinases A and B, carboxypeptidase Y, RNase(s), and alkaline phosphatase [12], this leads to the accumulation of ubiquitin–protein conjugates in the vacuole [9]. It has been reported that the yeast Pep4p plays an important role in mitochondrial degradation and acetic-induced apoptosis, suggesting a complex regulation and interplay between mitochondria and the vacuole in yeast programmed cell death [26]. It has been also reported that the deletion of *PEP4* resulted in both apoptotic and necrotic cell death and reduced chronological life span in aging [5]. Several yeast genes have human homologues or at least one conserved domain with human genes [34] and about 27.5% of positionally cloned human disease genes match yeast homologue [2]. The yeast *S. cerevisiae* is the best-understood, readily analyzed simplest eukaryote which facilitates understanding of molecular pathways that underpin several human neurodegenerative disorders and also one of the most widely used model system to study aging [13].

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The *S. cerevisiae* has directly or indirectly contributed to the identification of more mammalian genes that affect aging than any other model organism. Identifying and characterizing the specific disease genes associated with aging is pivotal to elucidate mechanism underlying the pathogenesis and to develop potential new therapeutics.

Recently, phytochemicals have gained a lot of attention for their beneficial effects on human health. Various studies have reported the use of *S. cerevisiae* as a model organism to evaluate the antioxidant and anti-aging potential of various phenolic compounds [23]. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is one of the naturally occurring dietary flavonoids, found in a variety of vegetables and fruits such as onions, apples, berries, potatoes, broccoli, grapes, citrus fruits, and tea. It has been reported to possess potential antioxidant effects as free radical scavengers, hydrogen-donating compounds, singlet oxygen quenchers, and metal ion chelators [30]. It has been demonstrated to possess multiple pharmacological effects and is found to extend the lifespan of various model systems such as human fibroblasts, yeast, and nematode [3, 4, 16]. However, the activity of quercetin in protecting neurodegeneration-associated human homolog yeast *pep4* mutant has not been studied. Therefore in the present study, we used a human neurodegenerative-associated homolog gene deleted yeast *pep4*Δ strain to evaluate the protective effect of quercetin from oxidant and apoptotic cell death. We also examined the protective role of quercetin in extending the short chronological life span associated with yeast *pep4*Δ cells. Our results suggest that quercetin treatment protects yeast *pep4*Δ cells from oxidant and apoptotic stress-induced cell death. We also showed that quercetin prolonged chronological life span of the *pep4* mutant cells. The antioxidant and anti-apoptotic activity of quercetin may be at least partly responsible for protecting yeast *pep4* mutant strain under various stresses.

Materials and Methods

Yeast extract, peptone, dextrose, yeast nitrogen base w/o ammonium sulfate, complete synthetic mixture, dimethyl sulfoxide (DMSO), quercetin, and all other chemicals were purchased from Himedia, Mumbai, India. 2′,7′-dichlorofluorescin diacetate (H_2DCFDA), 4′,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), and 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)) were purchased from Sigma-Aldrich (USA).

Saccharomyces cerevisiae Strains and Growth Conditions

Yeast strains such as wild-type (BY4741) (WT) and *pep4* mutant strain (*pep4*Δ), were purchased from Fischer Scientific, USA. Unless and otherwise stated yeast cells were grown in YPD medium and incubated at 160 rpm, 30 °C. For aging studies yeast cells were inoculated in minimal medium containing yeast nitrogen base medium, 5% ammonium sulfate, and complete synthetic mixture.

Semi-Quantitative Spot Assays

Exponentially growing yeast cells wild type (WT) and *pep4*Δ (OD 0.5–0.6) were harvested and about 10^7/mL were pretreated with quercetin (QT; 200 µM) or equal volume of DMSO (control) for 1 h under shaking at 160 rpm at 30 °C. Following incubation, 20 µL of samples was serially diluted ten times up to 10^{-6} dilution and spotted (5 µL) on to the YPD plates and YPD plates containing different concentrations of hydrogen peroxide (H_2O_2; 2 mM and 3 mM), tertiary butyl hydroperoxide (t-BHP; 2 mM), and acetic acid (60 mM and 80 mM) along with YPD control plate. Plates were incubated for 2–3 days at 30 °C and photos were taken.

Hydrogen Peroxide and Acetic Acid Stress Resistance

Hydrogen peroxide (H_2O_2) stress resistance assay was performed according to the method of Vilaça et al. [38]. Yeast cells were treated with Quercetin or DMSO as described above and subsequently washed with PBS and treated with 1 mM hydrogen peroxide under shaking at 160 rpm for 60 min at 30 °C. The cell suspensions were then serially diluted and plated onto YPD plates. Colonies were counted after 48 h at 30 °C. Cell viability was calculated as the percentage of the colony-forming units (CFU).

Acetic acid stress resistance was performed according to the method of Ludovico et al. [18]. Yeast cells were treated with Quercetin or DMSO as described above. Then cells were washed with PBS and incubated in YPD medium (pH 3.0) containing 60 and 80 mM acetic acid under shaking at 30 °C for 100 min. The cell suspensions were then serially diluted and plated onto YPD plates. Colonies were counted after 48 h at 30 °C. Cell viability was calculated as the percentage of the colony-forming units.

Fluorescence Microscopy

Detection of Intracellular Reactive Oxygen Species (ROS)

Intracellular oxidation level was monitored using 2,7-dichlorofluorescein diacetate (H_2DCFDA). Yeast cultures were pretreated with quercetin or DMSO as described above and subsequently treated with 1 mM H_2O_2 for 1 h at 30 °C. Then cells were washed 3 times with phosphate buffered saline. Samples were incubated with H_2DCFDA

(10 µM) for 30 min. Then cells were washed with PBS and observed under fluorescence microscope.

DAPI and PI Staining

Nuclear staining protocol with DAPI was adapted from Madeo et al. [20]. Propidium iodide (PI) staining was used to monitor cell membrane integrity as described previously [35].

Chronological Life Span Assay (CLS)

CLS assay was carried out in SDC medium (0.18% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, and 0.173% complete amino acid mix) as described by Smith et al. [33] with slight modifications. Briefly, yeast cultures were inoculated into SDC medium at a flask volume/medium volume ratio of 5:1. Quercetin was added at a final concentration of 200 µM and incubated at 30 °C with shaking at 160 rpm. Cultures reached stationary phase by 2nd day and the 3rd day cultures were considered as day 0 for CLS assay. From the starting of 0th day of CLS assay, aliquots were removed at 0, 3, 6, 9, 12, 15, and 20 days, diluted in sterile water, spreaded on to the YPD plates in duplicate, and allowed to grow for 3 days. Percent cell survival was assessed by counting colony-forming units (CFUs/mL). At indicated time points, serially diluted cultures were also spotted on YPD plates and YPD plates containing different stressors H_2O_2 (2 mM), and acetic acid (60 mM), or caffeine (12 mM). A set of YPD plates spotted with cultures were incubated at 37 °C for heat stress.

Visualization of Mitochondrial Morphology Using DiOC6(3) Staining

Aged (day 0 and day 4 old) cells were washed and suspended in 1 mL of 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer containing 0.1 mM $MgCl_2$ and 2% (w/v) glucose, pH 6.0, and DiOC6(3) was added (100 ng/mL) and incubated in dark for 30 min at 30 °C. After the incubation, cells were washed and observed under fluorescence microscope [14].

Statistical Analysis

Statistically significant differences were determined using the one-way analysis of variance test for comparisons between the various treated groups using SPSS software. Values with $P < 0.05$ were considered as statistically significant.

Results

Quercetin Protects Yeast *pep4Δ* Cells from Oxidant-Induced Cell Death

Yeast *PEP4* gene is a homolog of the human *CTSD* gene (cathepsin D). Deletion of *CTSD* gene resulted in a number of neurodegenerative diseases such as neuronal ceroid lipofuscinosis in human [24]. Yeast *PEP4*-deficient cells showed increased sensitivity to hydrogen peroxide [21] and shortened lifespan [8, 17] suggesting the imperative role of Pep4p in mediating a protective response of yeast against oxidative stress. Therefore, we first attempted to protect *pep4*Δ cells under oxidative stress using quercetin. From the Fig. 1a, it is observed that control *pep4*Δ cells showed sensitivity to both H_2O_2 and t-BHP compared to WT control. Pretreatment with quercetin augmented the oxidative stress resistance of *pep4*Δ cells and therefore increased viability compared to the control cells. The sensitivity of both wild-type and *pep4*Δ cells at high concentration of H_2O_2 (3 mM) was also protected by quercetin compared to its control.

Quercetin has been reported to exert an antioxidant effect in WT yeast strain (YPH250) [3]. In this study, we performed the CFU assay to calculate the percentage viability of *pep4*Δ cells in the presence of quercetin pretreatment followed by hydrogen peroxide treatment in liquid YPD medium. Exposure to 1 mM H_2O_2 resulted in a significant decrease in percent viability of yeast *pep4*Δ cells (42.66%) ($P < 0.05$) compared to its control cells. No significant difference in percentage viability among wild-type samples was observed. In contrast, quercetin pretreatment significantly increased the percent viability of *pep4*Δ cells (75.28%) compared to respective H_2O_2-treated cells (Fig. 1b).

To find out the protective mechanism of quercetin from oxidant-induced yeast *pep4*Δ cell death, we investigated the ROS accumulation using H_2DCFDA. The *pep4*Δ cells treated with H_2O_2, resulted in accumulation of high amount of ROS compared to control cells represented by the green fluorescent cells (Fig. 1c). We also observed control *pep4*Δ cells with basal level ROS accumulation. Upon quercetin pretreatment, accumulation of ROS was reduced drastically in control cells also. In addition, number of ROS-positive cells were counted from all the samples. The percentage of ROS-positive cells was significantly high in H_2O_2-treated *pep4*Δ cells (54%) compared to control cells (34%). The increased percentage of ROS-positive cells upon H_2O_2 treatment were scavenged by quercetin pretreatment (30%) (Fig. 1d). Lowering of ROS accumulation in the quercetin-treated *pep4*Δ cells, suggesting that the antioxidant role of quercetin might have protected the oxidative-mediated cell death in *pep4* mutant cells.

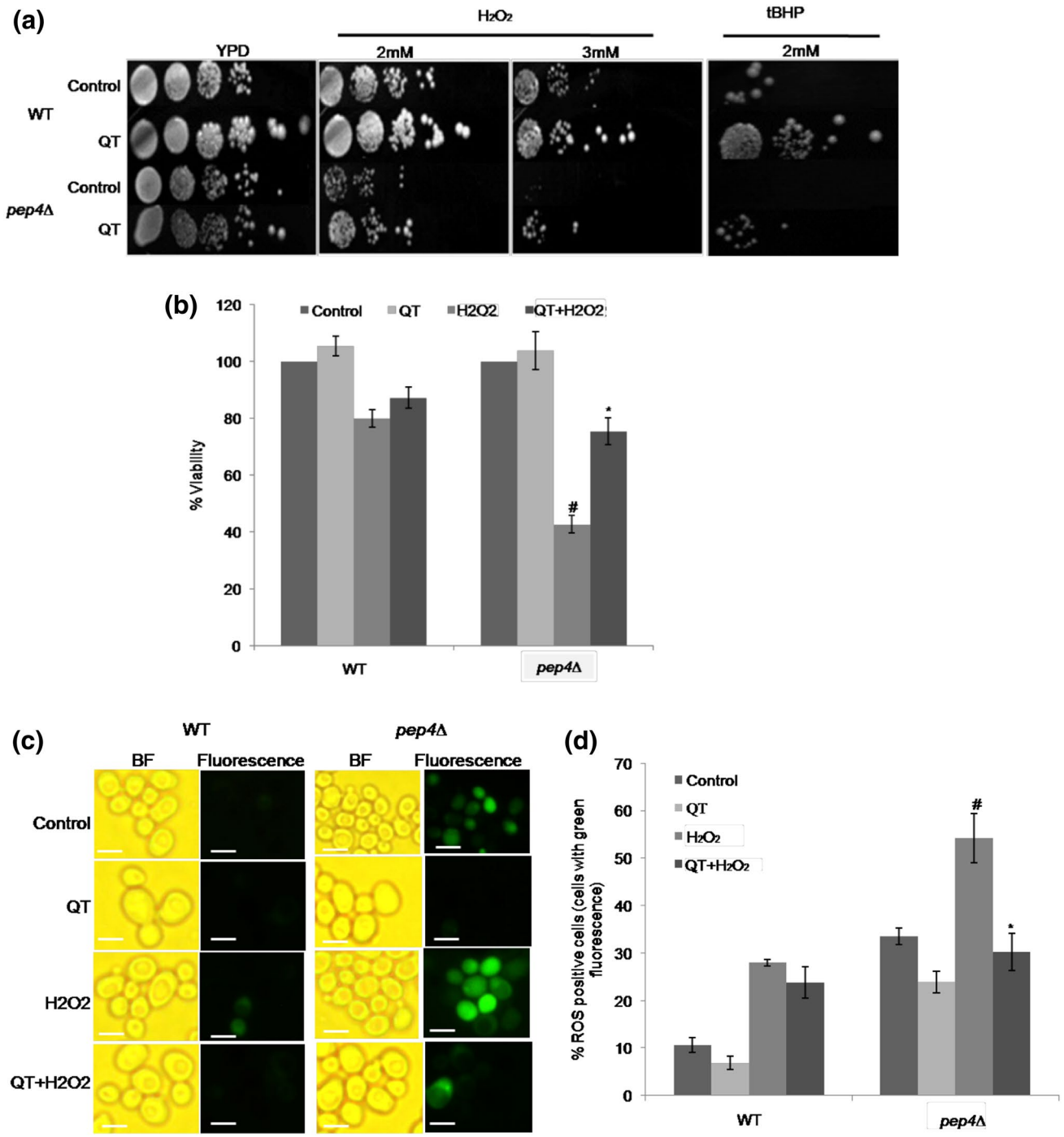


Fig. 1 Quercetin protects yeast *pep4* mutant cells from oxidative stress. **a** Semi-quantitative spot assay. Exponentially growing WT and *pep4*Δ cells were preincubated with QT or DMSO for 1 h. After incubation, yeast cells were serially diluted and spotted onto YPD plates or YPD plates supplemented with H_2O_2 (2 and 3 mM) and t-BHP (2 mM). Spotted plates were incubated at 30 °C for 3 days and photos were taken. Representative images are shown from three independent experiments. **b** Colony-forming unit assay. Exponentially growing yeast cells were treated as described above and subsequently incubated with 1 mM H_2O_2 for another 1 h. Following incubation, yeast cells were spread on to the YPD plates, incubated for 3 days, and percent viability was calculated. Values are means ± SD of three independent experiments. # represents significant decrease in percent viability in H_2O_2-treated *pep4* culture compared to *pep4* control. * Represents significant increase in percent viability in QT + H_2O_2-treated compared to H_2O_2-alone-treated cells ($P < 0.05$). **c** ROS detection by H_2DCFDA staining. Exponentially growing yeast cells were treated as described above and treated with H_2DCFDA. Representative images are shown from at least three independent experiments. Scale bars, 50 µm. **d** Percentage ROS-positive cells detected by H_2DCFDA. At least 100 cells were examined. Data represent an average ± SD of three independent experiments. *BF* Bright field. # represents significant increase in ROS-positive cells in H_2O_2 treatment compared to *pep4* control and * Represents significant decrease in ROS-positive cells for QT + H_2O_2-treated cells compared to H_2O_2 alone ($P < 0.05$)

Quercetin Protects Yeast *pep4Δ* Cells from Acetic Acid-Induced Cell Death

Acetic acid is known to induce apoptosis in yeast cells similar to mammalian cells. It has been reported that yeast (W303 strain background) *pep4*Δ cells undergo acetic acid-induced apoptotic cell death [27]. Therefore, in the present study, we investigated the anti-apoptotic effect of quercetin against the acetic acid-induced cell death in yeast (BY4741 strain background) *pep4*Δ cells. Results showed that *pep4*Δ cells spotted on to the acetic acid-containing plates were highly sensitive in a dose-dependent manner at 60 and 80 mM, respectively, compared to that of wild type. Whereas, quercetin-pretreated *pep4*Δ cells were resistant to acetic acid-induced cell death (Fig. 2a) compared to its control cells. Quercetin pretreatment enhanced the resistance of *pep4*Δ cells against acetic acid stress.

Further, CFU assay was performed to calculate the percentage cell viability of quercetin-pretreated or control cells against acetic acid stress. As shown in Fig. 2b, increase in the percentage viability of wild type upon quercetin pretreatment was found to be 74.29 and 62.56% compared to 66.42 and 43.15% in acetic acid-alone-treated cells at 60 and 80 mM acetic acid concentrations, respectively. Whereas, a significant increase in the percentage viability of *pep4*Δ cells upon quercetin pretreatment was found to be 61.97 and 46.25% compared to 45 and 31.83% in acetic acid-alone-treated cells at 60 and 80 mM acetic acid concentrations, respectively.

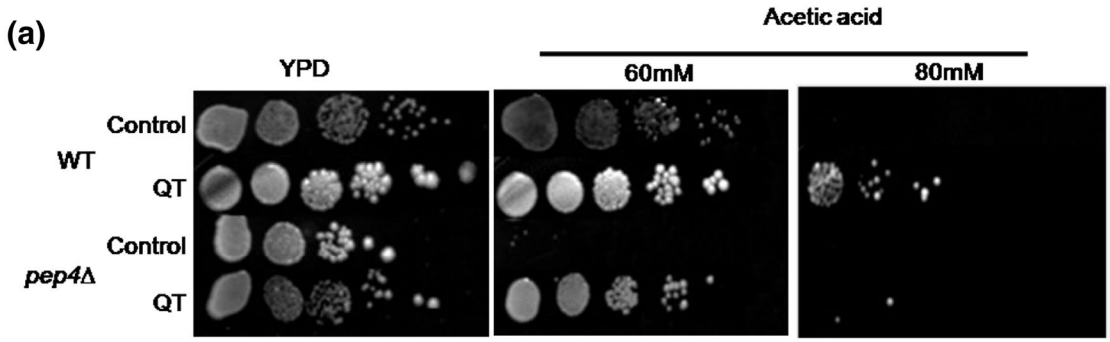
Fluorescence microscopy was performed to confirm the acetic acid-induced apoptosis and its protection by quercetin in yeast *pep4*Δ cells. Apoptotic markers such as chromatin condensation and plasma membrane integrity were investigated using fluorescent dyes such as DAPI and PI, respectively. Acetic acid exposed *pep4*Δ cells displayed, loose nuclei with kidney, or ring-shaped, or condensed chromatin (diffused staining) characteristic of apoptotic cells compared to control cells with well-defined nucleus (Fig. 2c). Acetic acid treatment increased the percentage of DAPI-positive cells in WT (41%) and *pep4*Δ (86%) compared to that of respective control cells (15% for WT and 53% for *pep4*Δ). In contrast, quercetin pretreatment decreased the percentage of DAPI-positive cells in wild-type (31%) and *pep4*Δ (72%) cells compared to acetic acid-treated cells, suggesting the anti-apoptotic role of quercetin against acetic acid-induced cell death in *pep4*Δ cells (data not shown). Quercetin pretreatment resulted in the restoration of acetic acid-induced nuclear alterations where nuclei were homogeneous in shape and density. Similarly, quercetin pretreatment decreased the PI-positive cells (data not shown). It is also observed from the Fig. 2c that quercetin treatment preserved membrane integrity as shown by PI staining, where less PI-positive cells were observed.

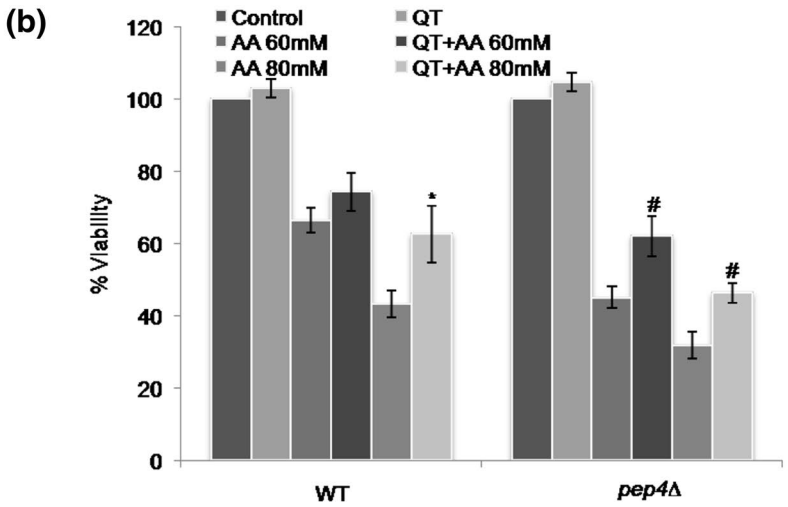
Quercetin Extended the CLS of Yeast *pep4Δ* Cells by Reducing Oxidative and Apoptotic-Mediated Cell Death

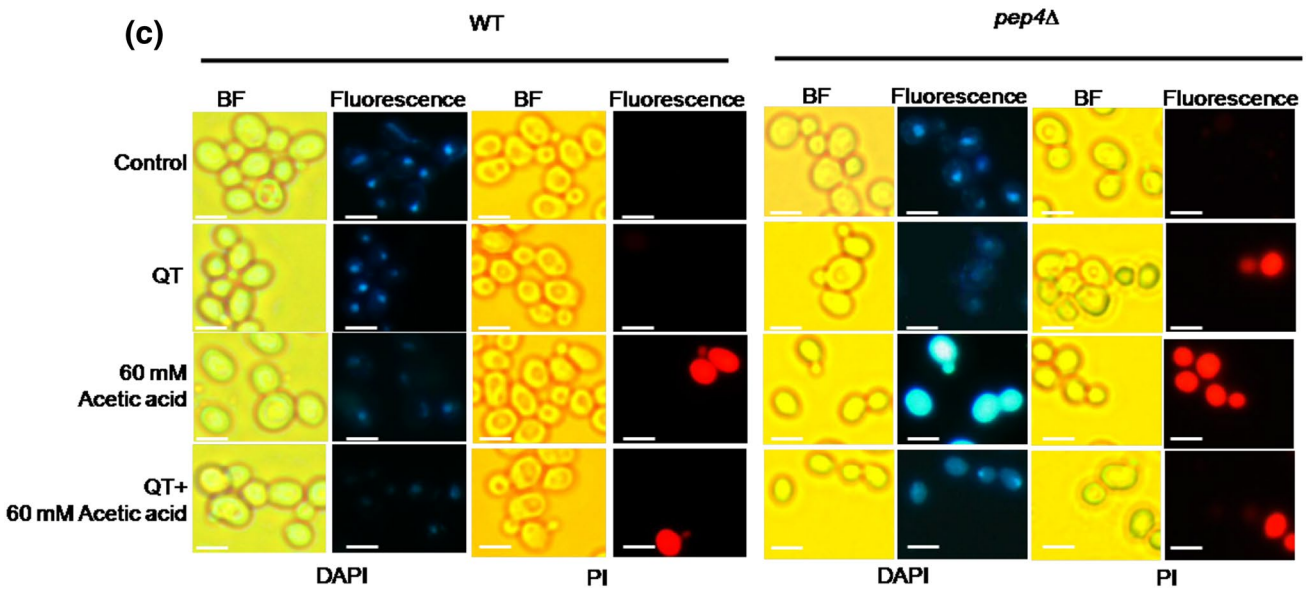
Quercetin has also been shown to increase longevity in wild-type yeast and has anti-neurodegenerative property in human cells [4, 7, 38]. In the present study, we investigated the anti-aging effects of quercetin on the CLS of yeast *pep4* mutant. Yeast deficient in Pep4p has been reported to have a very short lifespan [5]. Therefore we investigated the survival of *pep4*Δ chronologically aged cells in comparison with wild type for 20 days in the presence and absence of quercetin. The survival curves of wild-type and *pep4*Δ cells are shown in Fig. 3a. As expected, it was observed that *pep4*Δ cells died considerably sooner (12 days). After day 3, the survival rate of *pep4*Δ cells started to decrease rapidly (32.1%), and in day 12 we observed that only a small number of *pep4Δ* cells (1.1%) stayed alive. In contrast, the percentage viability of wild-type cells reaches 50% at day 5 and begins to die around day 12. However, quercetin treatment resulted in increased percent viability of *pep4*Δ cells (up to 20 days) compared to its control cells during CLS.

In addition, yeast cells from the CLS experiment (0, 3, 6, 9, 12, 15, and 20 days old) were spotted on YPD plates and YPD plates containing different stressors such as H_2O_2 (oxidative stressor), acetic acid, and caffeine (apoptotic stressors) and heat stress (37 °C) for days 9, 15, and 20 culture. Studies have shown that caffeine induced apoptosis in yeast and other eukaryotic cells [10, 22] and heat stress also induced apoptosis in yeast aging. These experiments were carried out to check the oxidative and apoptotic stress resistance induced by the quercetin during CLS of *pep4Δ*. The spot assay results showed that the chronologically aging yeast *pep4Δ* cells (Fig. 3b, right panel) showed rapid reduction in the ability to grow on H_2O_2-containing YPD from day 12 onwards. Yeast *pep4Δ* cells showed sensitivity to acetic acid and caffeine-containing YPD plates, and heat stress (at 37 °C) as early as day 9 of aging compared to the wild-type cells, where cells survive up to 12–15 days (Fig. 3b, left panel). These results suggest that quercetin has induced resistance to oxidative and apoptotic stresses by scavenging oxidative radicals generated during aging and enhanced the CLS of *pep4Δ* cells.

Chronologically aged yeast cells are characterized by the accumulation of ROS and apoptosis markers leading to increased cell death [11]. Therefore, we investigated the effect of quercetin on the ROS accumulation using H_2DCFDA and apoptosis using DAPI and PI in chronologically aged yeast cells. We observed that basal level of ROS accumulation was more pronounced in control *pep4Δ* cells compared to that of WT cells during aging. These results suggest that ROS-induced oxidative stress in *pep4Δ* cells may be responsible for the short lifespan of *pep4*Δ during


(a)
Acetic acid
YPD
60mM
80mM
Control
WT
QT
Control
pep4Δ
QT


(b)
Control
AA 60mM
AA 80mM
QT
QT+AA 60mM
QT+AA 80mM
% Viability
120
100
80
60
40
20
0
*
#
#
WT
pep4Δ


(c)
WT
pep4Δ
BF
Fluorescence
BF
Fluorescence
BF
Fluorescence
BF
Fluorescence
Control
QT
60 mM
Acetic acid
QT+
60 mM Acetic acid
DAPI
PI
DAPI
PI

◂**Fig. 2** Quercetin protects yeast *pep4* mutant cells from acetic acid-induced apoptosis. **a** Semi-quantitative spot assay. Exponentially growing yeast cells were treated as described above. Following incubation, cells were ten-fold serially diluted and spotted onto YPD-only plates or YPD plates supplemented with different concentrations of acetic acid (60 and 80 mM). Spotted plates were incubated at 30 °C for 3 days and photos were taken. Representative images are shown from at least three independent experiments. **b** Colony-forming unit assay. Exponentially growing yeast cells were incubated with QT or DMSO for 1 h and then incubated with 60 and 80 mM acetic acid (AA) for 100 min. Then the cells were spread on to the YPD plates, incubated for 3 days, and the percent viability was calculated. Values are means ± SD of three independent experiments. * Represents significant increase in WT percent viability of QT + AA 80 mM treatment compared to acetic acid (80 mM) alone treatment and # represents significant increase in *pep4*Δ percent viability of QT + AA 60 mM and QT + AA 80 mM treatment compared to acetic acid (60 and 80 mM) alone treatment, respectively. **c** DAPI and PI staining. Exponentially growing yeast cells were treated with quercetin as described above. Then the yeast cells were incubated with DAPI or PI for 15 min. Then the cells were washed three times with PBS and observed by fluorescence microscope. Representative images are shown from at least three independent experiments. Scale bars, 50 µm

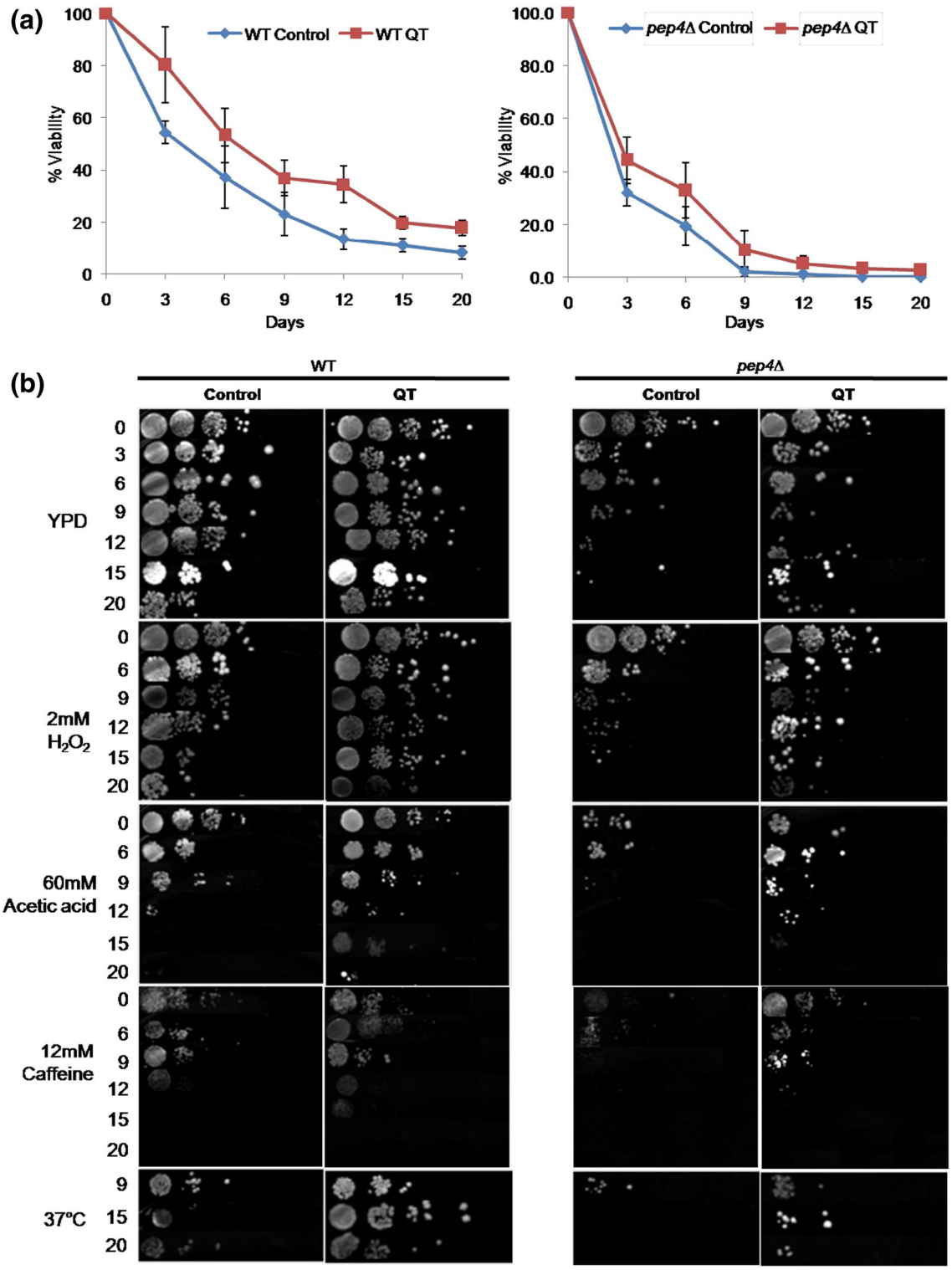
CLS (Fig. 3c upper panel). Chromatin condensation is one of the apoptosis morphology and it was studied in aging cells using DAPI staining. The chromatin condensation in the *pep4Δ* cells was increased at different days (0, 3, and 6 days) of CLS compared to that of wild-type strain. The results showed that 95% of control *pep4Δ* cells showed increased chromatin condensation on day 6 of aging than the wild-type cells (25%) (Fig. 3c, middle panel). In contrast quercetin-treated cells, showed chromatin as a single round spot in the middle of the cell by the end of day 6 (20% in wild-type and 75% in *pep4Δ* cells). Concomitantly yeast cells were also checked for plasma membrane integrity in aged wild-type and *pep4Δ* cells using PI staining (Fig. 3c lower panels). Control *pep4Δ* cells showed more intense red fluorescence than the wild type on day 3 and day 6, suggesting that the loss of membrane integrity in *pep4* mutant was more compared to wild-type cells. However, loss of membrane integrity is restored during aging in quercetin-treated cells represented by reduced red fluorescence *pep4Δ* cells.

It is evident that yeast aging is closely associated with mitochondrial integrity [1]. Therefore we investigated the mitochondrial membrane potential using a specific dye called DiOC6(3). As shown in the Fig. 3d, e, there was a decrease in DiOC6(3) staining and the percentage of stained cells from 64% (day 0) to 50% (day 4) for WT control cells and from 47% (day 0) to 31% (day 4) for *pep4Δ* cells during CLS. In contrast, quercetin treatment resulted in increased DiOC6(3) staining and tubular mitochondrial structure in WT and tubular to punctiform mitochondrial structure in *pep4*Δ cells. Quercetin treatment resulted in increased percentage of DiOC6(3) cells for both WT (day 0, 75%; day 4, 58%) and *pep4Δ* (day 0, 54%; day 4, 49%). Results indicate the maintenance of mitochondrial membrane potential and structure by quercetin increased the viability of *pep4* mutant cells during early days (day 4) of CLS.

Discussion

Increasing evidence suggests oxidative stress as a contributing factor in aging and other age-associated diseases [28]. A significant amount of research has shown that somatic nuclear mutation burden with age resulting in cancer and neurodegeneration [37]. Human cathepsin D gene mutation is one among the somatic mutations known to cause age-associated neurodegeneration in neuronal ceroid lipofuscinosis. Yeast *Pep4* is a homolog to the human cathepsin D, which plays an important role in protein turnover after oxidative damage. Pep4p-deficient cells decreased cell viability due to increased oxidative stress [21, 36]. Yeast *pep4* mutant undergoes both apoptosis and necrosis during chronological aging. In this study, we investigated the role of quercetin in protecting the yeast *pep4Δ* cells from oxidative and apoptotic-induced cell death as well as cell death during aging. We showed that *pep4Δ* cells were found to be highly sensitivity to H_2O_2 toxicity compared to WT. The quercetin pretreatment enhanced the percentage viability of yeast *pep4Δ* cells compared to respective control cells (Fig. 1a, b). It has also been reported that quercetin scavenged the ROS in WT yeast cells and induced oxidative stress resistance [4]. Quercetin is a potent scavenger of ROS and has been shown to counteract oxidative stress-induced cellular damage in various cell types. Our data are consistent with the previous report that quercetin pretreatment reduces the ROS-positive cells leading to increased percent viability of yeast *pep4Δ* cells after H_2O_2 treatment, suggesting the ROS scavenging ability of quercetin protected the oxidative-mediated cell death of *pep4*Δ cells.

Apoptosis in yeast model provides a better understanding of certain aspects of apoptosis in mammals. *S. cerevisiae, CDC48* mutant cells showed key morphological characteristics of mammalian apoptosis such as phosphatidyl serine externalization, chromatin condensation, release of mitochondrial cytochrome c, nuclear fragmentation, and reduction of the mitochondrial membrane potential [19, 40]. These apoptotic markers are increasingly associated with

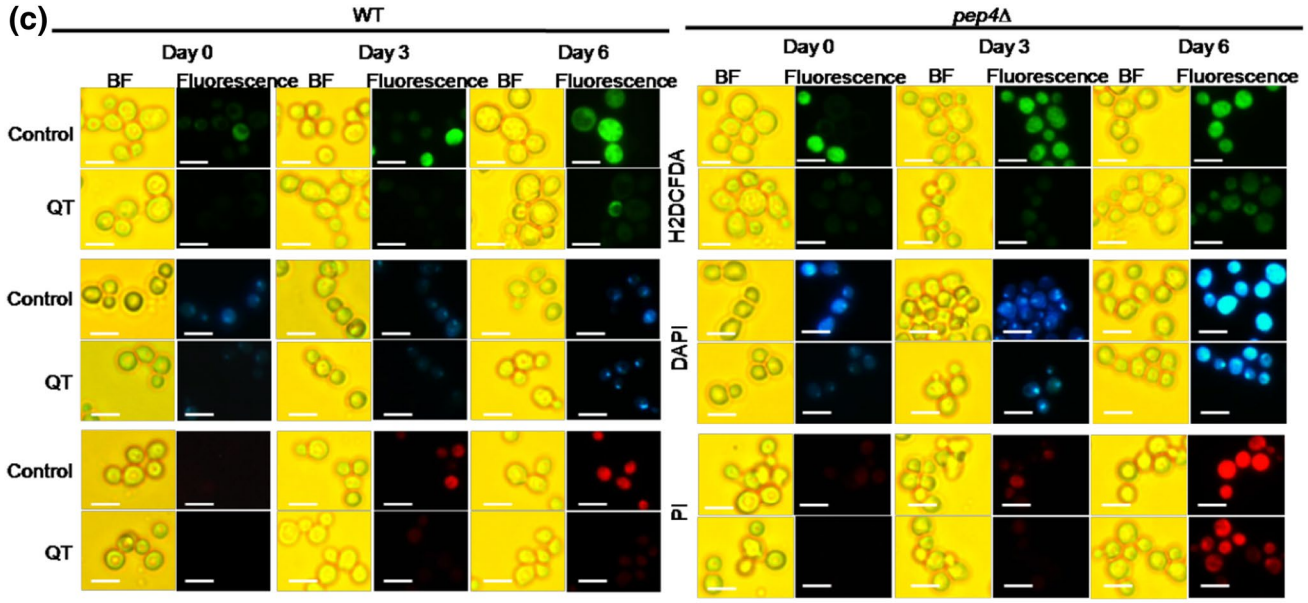

(a)
WT Control
WT QT
% Viability
Days
pep4Δ Control
pep4Δ QT
(b)
WT
pep4Δ
Control
QT
YPD
2mM H2O2
60mM Acetic acid
12mM Caffeine
37°C

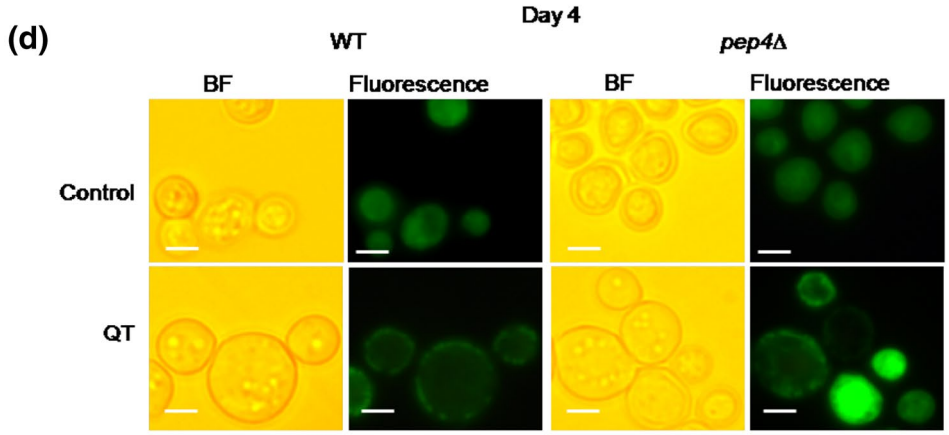
◂**Fig. 3** Quercetin extends the CLS of yeast cells. **a** Percent viability of WT (left panel) and *pep4*Δ (right panel). Yeast cultures were supplemented with quercetin and incubated at 30 °C. Aliquots were plated on to the YPD plates at indicated time points. Percent cell survival was evaluated by calculating colony-forming units per milliliter (CFUs/mL). Values are means ± SD of three independent experiments. **b** Spot assay. WT and *pep4*Δ aging cultures were spotted onto YPD plates containing H_2O_2 (2 mM), acetic acid (60 mM), and caffeine (12 mM). Yeast aging cultures were also subjected to heat stress at 37 °C. Plates were incubated at 30 °C for 3 days and photos were taken. Representative images are shown from at least three independent experiments. **c** Fluorescence microscopy during chronological aging of WT and *pep4*Δ cells. Accumulation of ROS was analyzed using a ROS sensing dye H2DCFDA (Upper panel), and the nuclear condensation and plasma membrane integrity were detected by staining with DAPI (middle panel) and PI (lower panel), respectively. Representative images are shown from at least three independent experiments. Scale bars, 50 µm. **d** Detection of mitochondrial membrane potential. Yeast cells at day 0 and 4 of CLS were incubated with DiOC6(3) and observed under fluorescence microscope (only day 4 cells were shown). **e** Percentage of DiOC6(3)-positive cells. At least 100 cells were examined on day 0 and 4 of the CLS. Data represent an average of three independent experiments with standard deviation. *BF* Bright field. * Represents significant increase in DiOC6(3)-positive cells for QT-treated cells compared to control *pep4*Δ cells on day 4 of CLS ($P < 0.05$). Scale bars, 50 µm

several human diseases. Acetic acid is known to induce apoptosis in yeast as in the case of mammals. In the present study, acetic acid treatment resulted in decrease in *pep4Δ* cell viability due to increase in apoptosis rate which is indicated by increase in the DAPI- and PI-stained cells (Fig. 2). The proteolytic activity of Pep4p is required to mitigate the apoptotic cell death while the non-proteolytic part of this protein is involved in its anti-necrotic function. In addition, both yeast Pep4p as well as mammalian CatD, showed a protective role in acetate-induced apoptosis in yeast and colorectal cancer cells, respectively. In our study, we show that acetic acid-mediated *pep4Δ* cell death was protected by quercetin treatment as shown in the spot and microscopic study (Fig. 2). Pep4 is an anti-apoptotic protein, its function is very much essential during aging to reduce the cell death via apoptosis. Similarly, CatD protein is required to reduce apoptotic cell death in neuron cells and thereby to protect from neurodegenerative diseases. The deficiency of cathepsin D has been recently revealed to provoke a novel type of lysosomal storage disease associated with massive neurodegeneration.

The yeast chronological aging shares similar features to mammalian post-mitotic aging [11]. Increased protein oxidation and aggregation of damaged proteins in *S. cerevisiae* and human CNS is extremely similar [36]. The chronological aging in budding yeast is heavily associated with ROS accumulation and programmed cell death [11]. ROS-mediated cell death has been linked to serious human pathologies as well as in the aging process. In our study, we observed decrease in cell viability with increase in ROS levels and apoptotic cell death during the CLS of *pep4*Δ cells compared to WT cells (Fig. 3). In contrast, quercetin-treated *pep4*Δ cells showed increased longevity (CLS assay), increased resistance to oxidative stress (spot assay), apoptotic stress, and heat stress of CLS, suggesting that Pep4p activity is also required for *S. cerevisiae* to survive. Age-associated ROS accumulation may occur as the result of increased leakage of electrons from the mitochondrial electron transport chain or as the result of decreased antioxidant capacity [1]. In *S. cerevisiae*, respiring mitochondria form an elaborate network of tubular membranes located near the cell periphery. The mitochondrial membrane potential (MMP) was studied using a lipophilic dye, DiOC6 which preferentially stains actively respiring mitochondria in yeast cells [14]. We analyzed the mitochondrial morphology of day 4 aged cells along with day 0 cells and found that mitochondrial fragmentation was increased in day 4 *pep4*Δ cells compared to WT cells (Fig. 3d, e). Whereas, mitochondrial fragmentation is reduced in quercetin-treated *pep4*Δ cells indicating the efficiency of quercetin in protecting the aged cells from age-associated decrease in MMP and mitochondrial fragmentation. These results suggest that anti-aging potential of quercetin is protecting the *pep4* mutant during CLS by scavenging ROS and reducing apoptotic cell death. Studies of post-mitotic aging in *S. cerevisiae* have provided an insight into the genetic regulation of post-mitotic lifespan. Although there are many human homolog genes in yeast that are associated with neurodegenerative disease, the direct relevance of these genes to neuronal aging are unclear at the present time; it is likely that such findings will help in understanding the genetic regulation of post-mitotic neuron aging in the CNS.

In summary, our results confirmed that quercetin successfully protected the *pep4*Δ cells from different stressors such as oxidative and apoptotic stress. In addition, we showed that quercetin enhanced resistance to oxidative, apoptotic, and heat stress in *pep4* mutant cells, resulted in increase in the longevity of CLS. From our yeast experimental results, we conclude that the quercetin can be developed as a potential therapeutic molecule that may help in reducing the age-associated decrement in post-mitotic cell aging of CNS and also in treating the neurodegenerative diseases associated with cathepsin D gene mutation in human.





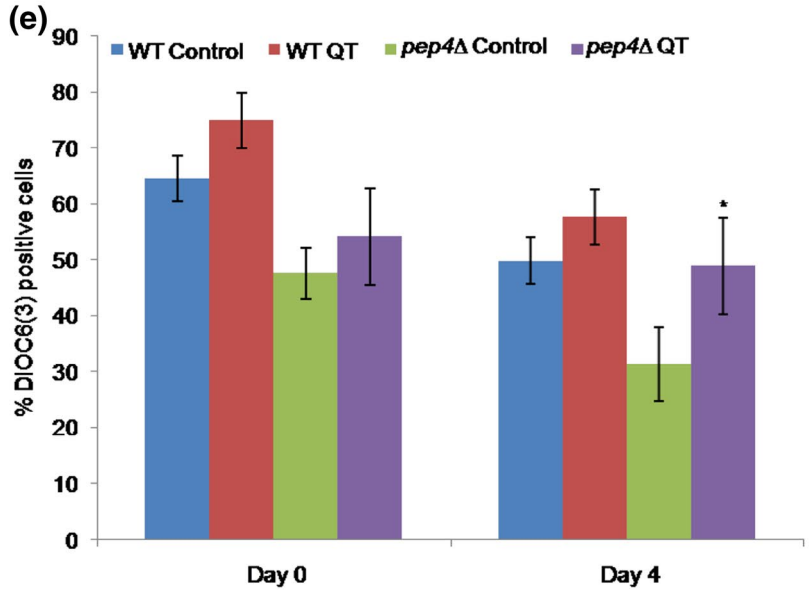


Fig. 3 (continued)

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Compliance with Ethical Standards

Conflict of interest Authors declare that there is no conflict of interest.

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