

High-throughput transcriptome sequencing reveals the protective role of adenosine receptor-related genes in paraquat-exposed *Caenorhabditis elegans*

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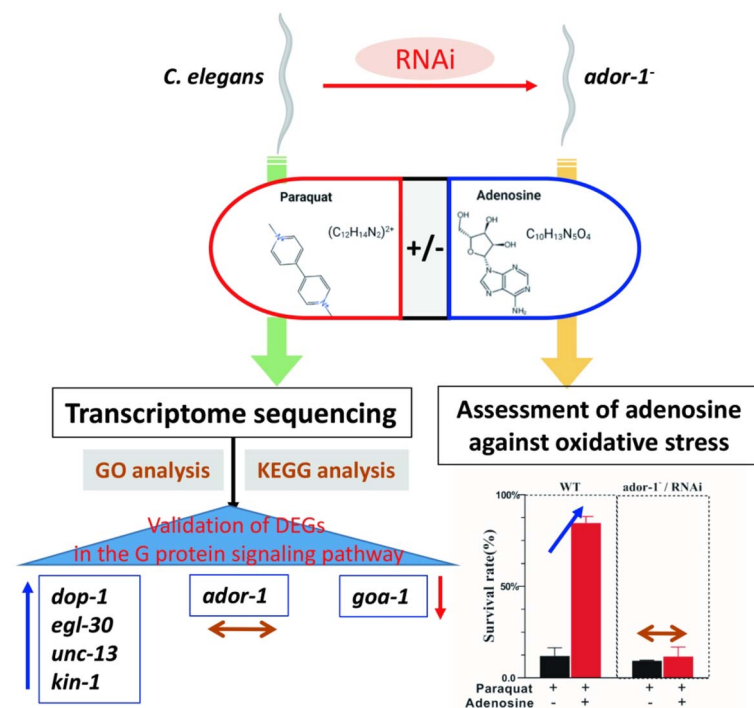
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This study sought to identify the genes associated with adenosine's protective action against paraquat (PQ)-induced oxidative stress via the adenosine receptor (ADOR-1) in *Caenorhabditis elegans* (*C. elegans*). The *C. elegans* was divided into 3 groups—2 groups exposed to PQ, one in presence, and one in absence of adenosine—and a control group that was not treated. Each group's total RNA was extracted and sequenced. When the transcriptomes of these groups were analyzed, several genes were found to be differently expressed. These differentially expressed genes were significantly enriched in adenosine-response biological processes and pathways, including gene ontology terms related to neuropeptide and kyoto encyclopedia of genes and genomes pathways associated to cAMP pathway regulator activity. Quantitative reverse-transcription PCR confirmed that G-protein-coupled receptors signaling pathway involving *dop-1*, *egl-30*, *unc-13*, *kin-1*, and *goa-1* genes may play crucial roles in modulating adenosine's protective action. Interestingly, there are no significant variations in the expression of the *ador-1* gene across the 3 treatments, thereby indicating that adenosine receptor exerts a consistent and stable influence on its related pathways irrespective of the presence or absence of PQ. Furthermore, the wild-type group with *ador-1* gene has higher survival rate than that of the *ador-1*⁻/RNA interference group while treated with PQ in the presence of adenosine. Conclusively, our study uncovered a number of novel PQ-response genes and adenosine receptor-related genes in *C. elegans*, which may function as major regulators of PQ-induced oxidative stress and indicate the possible protective effects of adenosine.

Graphical Abstract



Key words: GO analysis; KEGG; GPCRs; qPCR; RNAi.

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Introduction

Presently, oxidative stress has been linked to the emergence and progression of a variety of diseases, including cancers, Parkinson's disease, atherosclerosis, and Alzheimer's disease.^{1,2} Oxidative stress typically results from the pathological conditions of particular diseases or external causes, such as toxins.³ Paraquat (PQ; also known as 1,1'-dimethyl-4,4'-bipyridinium dichloride) undergoes an in vivo, NADPH-dependent reduction, producing a stable PQ radical that reacts with oxygen to generate a superoxide anion, a reactive oxygen species, allowing it to cause immediate damage via direct contact, ingestion, or inhalation.^{4,5} Therefore, PQ has been as an effective chemical for establishing a model of oxidative stress in the laboratory.

Adenosine (ADO, adenine nucleoside) plays an essential function in the physiological and pathological conditions of the body (such as oxidant injury in neural cells) through combining several types of adenosine receptors (ADORs) that are widely distributed throughout the body's tissues.⁶ ADORs, which belong to the superfamily of G-protein-coupled receptors (GPCRs), have 4 distinct ADOR isoforms, including A1, A2A, A2B, and A3 in mammals.⁷ It is well documented that ADO functions via signaling pathways regulated by the adenosine receptor.⁸

Recently, *Caenorhabditis elegans* has been used as an alternative in the in vivo animal model for toxicological analyses of several chemicals.^{9,10} Recent research from our laboratory indicates that a certain dosage of adenosine has protective effects on *C. elegans* exposed to PQ by reducing its incidence of mortality.¹¹ However, little is known about its protective mechanism against PQ-induced oxidative damage. Notably, ADOR-1, which is encoded by the *ador-1* gene, is the only homolog of the adenosine receptor in *C. elegans* and is known to be an ortholog of human adenosine A2b receptor. Although ADOR-1 has not been characterized in terms of function, adenosine has been identified to have a protective effect against oxidative stress as a neurotransmitter.¹² RNA interference (RNAi) is a valuable technique to determine gene function. In *C. elegans*, RNAi can be achieved by feeding nematodes bacteria carrying a plasmid expressing double-stranded RNA targeting a gene of interest.¹³ Therefore, in the present study, transcriptome and RNAi data from *C. elegans* groups treated with PQ in the presence or absence of adenosine were examined to identify PQ-response genes and ADOR-1-related genes for adenosine's potential protective action.

Materials and methods

Culture and treatment of *C. elegans*

Wild-type (WT) Bristol (N2) *C. elegans* used in the present study was originally obtained from the *Caenorhabditis* Genetic Center (Minneapolis, United States). They were maintained at 20 °C on nematode growth medium (NGM) plates inoculated with *Escherichia coli* (*E. coli*) OP50 according to protocol.¹⁴

As previously described,¹⁵ age-synchronized populations of L4-larval nematodes were obtained at 20 °C for ~48 h. Individually, approximate 1,000 age-synchronized young adults were treated individually with PQ (150 mM) and PQ (150 mM) in the presence of adenosine (3,000 μM), which were abbreviated as PQ group and PQ_AD group. Nematodes treated with sterilized M9 buffer solution (Na₂HPO₄ 6 g, KH₂PO₄ 3 g, NaCl 5 g, MgSO₄•7H₂O .25 g) was used as the control (CK group). PQ and adenosine (provided by Sigma-Aldrich; Merck KGaA) were, respectively, dissolved into 150 mM and 3,000 μM with 10 mL of sterilized M9 buffer according to the previous study.¹¹

Three groups of nematodes were exposed to the respective conditions for 5 min before being washed 3 times with M9 buffer solution to eliminate the bulk of the residual bacteria. Total RNA was extracted immediately using TRIzol (Invitrogen) according to the manufacturer's instructions.

RNA transcriptome sequencing analysis

The RNA transcriptome sequencing analysis was performed by BGI Genomics Corporation (Beijing, China) using HiSeq technology. Reference genome and gene model annotation files were downloaded from WormBase (WS269). To evaluate alterations of molecular signaling pathway after PQ treatment in the presence or absence of adenosine in *C. elegans*, the data were divided into 2 comparisons, containing CK versus PQ (comparison A) and PQ versus PQ_AD (comparison B). An index of the reference genome was created using Hisat2 v2.5, and paired-end clean reads were aligned with the reference genome using Hisat2 v2.5.^{16–18} FeatureCounts v1.5.0-p3 was used to count the read numbers mapped to each gene.¹⁹ The FPKM of each gene was then computed based on the length of the gene and the number of mapped reads. Differential expression analysis of 2 groups was performed via the R package DESeq2 (1.16.1) based on the negative binomial distribution.²⁰

The method of Benjamini and Hochberg was utilized to modulate the obtained *P*-values in order to control the false discovery rate. As a criterion for significantly differential expression, a corrected value of FDR ≤ .001 and fold change ≥ 2 was chosen. The clusterProfiler R package was used to perform the gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) with an adjusted value of *P* < 0.05.²¹ Fisher's exact test was also applied to these DEGs lists to identify gene sets that significantly overlapped with the kyoto encyclopedia of genes and genomes (KEGG) pathway gene sets.

Quantitative reverse-transcription PCR

On the basis of comparison of major differential genes between CK versus PQ and PQ versus PQ_AD in the transcriptome, quantitative reverse-transcription PCR (qRT-PCR) assay was utilized to validate the differential genes among the mRNA expression of G-protein signaling pathway genes. Primers for each genes are listed in Table 1. PrimeScript RT reagent Kit with gDNA Eraser was used to generate cDNA from total RNA (TaKaRa Bio, Japan). Comparative qRT-PCR was performed using Takara TB GREEN Premix Ex Taq II reagent (TaKaRa Bio, Japan) on Bio-Rad CFX 384 to amplify single-stranded cDNA. The PCR cycles were as follows: 95 °C for 60 s; 40 cycles of 95 °C for 5 s; 40 cycles of 60 °C for 30 s. Relative gene expression levels were calculated using the 2^{-ΔΔCt} method.²² The ΔCt value was computed for each sample using *act-1* as an endogenous control gene. The qRT-PCR gene expression was statistically compared using the Student's *t*-test.

The *ador-1* RNAi and assessment of adenosine against oxidative stress

The nematodes grown on *ador-1* RNAi bacteria were prepared exactly as described previously.²³ Briefly, *E. coli* HT115 RNAi clones were grown overnight in LB with 50 μg/mL carbenicillin and 12.5 μg/mL tetracycline hydrochloride, then seeded into the NGM agar plates containing 2 mM isopropylthiogalactoside and 25 μg/mL carbenicillin. Plates were kept for 7 days before feeding and then L4-stage larvae were transferred to the NGM plates seeded with RNAi-induced bacteria. Nematodes which were feed with HT115 *E. coli* bacteria, transformed with the L4440

Table 1. qRT-PCR primers for G-protein signaling pathway-related genes.

Symbol	WormBase ID	KOG (other description) ^a	Primer sequence(5'-3')	Size (bp)
<i>dop-1</i>	WBGene00001052	7 transmembrane receptor, enables dopamine neurotransmitter receptor activity, coupled via Gs	F:CCTTGTTCCTTTGGCCGTGT R:GAACGGCCAGTATCCCAAGA	91
<i>egl-30</i>	WBGene00001196	G-protein subunit Galphaq/Galphay, small G-protein superfamily	F:ACTCGCATCTCGCTGACTAC R:CGCGCAGGTAAAATGAGAGT	140
<i>unc-13</i>	WBGene00006752	Neurotransmitter release regulator, UNC-13	F:TGTGACCACTTTGGAACCCC R:TAGAACTGCATCGGCTTCCG	121
<i>ador-1</i>	WBGene00011878	7 transmembrane receptor	F:GCGCTCACGGATTTTCTAGC R:CACAAAGAGGCACCCGTAGA	99
<i>goa-1</i>	WBGene00001648	G-protein alpha subunit (small G-protein superfamily)	F:TTTTGCGAGCCATGAGCAAC R:TCTGTGTCCTCCATTCTGTC	103
<i>kin-1</i>	WBGene00002189	cAMP-dependent protein kinase types I and II, regulatory subunit	F:AAGCATAAGCAGTCGGGCAA R:GATAGCCTGGAGAATGCGCT	114
<i>act-1</i>	WBGene00000063	Actin	F:CTTCCTCTCCACCTTCCAAC R:GCTGGTGGTGACGATGTTT	144

^aGenes are categorized based on their molecular function (KOG or other description, <http://www.wormbase.org>).

vector (empty vector or carrying specific fragments of cDNA), were divided into WT and *ador-1*⁻¹/RNAi type. For the oxidative stress assay, approximately, 1,080 age-synchronized young adults were transferred to 96-well plates. Three groups of nematodes (CK, PQ, and PQ_AD) were treated with M9 buffer solution, PQ (150 mM) and PQ (150 mM) in the presence of adenosine (3,000 μM), respectively. The survival rate of each cohort was recorded individually at 6-hour intervals. All experiments were repeated for 3 times.

Results

RNA sequencing

RNA-seq data quality assessment results are summarized in [Supplementary Table S1](#). Three groups of samples were processed, and each group's clean reads were >40 M. On average, the number of bases with a sequencing error rate of ≤0.1% (Q30) comprised >85% of the total number of bases. These results indicated that the sequences and their respective alignments were of high quality. Sequencing information was submitted to BioProject database as PRJNA809866, and the website is '<http://www.ncbi.nlm.nih.gov/bioproject/809866>'.

Differential gene expression in RNA-seq data

Using statistical inference based on DESeq, fold changes of *C. elegans* RNA-seq data involved in CK group, PQ group, and PQ_AD group is shown in [Fig. 1](#). In pair-wise comparisons of the 2 groups, a total of 2,269 genes were found to be differentially expressed, including 1,878 upregulated and 391 downregulated genes in CK versus PQ group (comparison A, [Fig. 1A](#)); and 213 upregulated and 975 downregulated genes in PQ versus PQ_AD group (comparison B, [Fig. 1B](#)); 922 genes were discovered to be coregulated between CK versus PQ group and PQ versus PQ_AD group.

GO-based enrichment analysis

The DEGs from the RNA-seq data in the comparison A (CK vs. PQ) and comparison B (PQ vs. PQ_AD) clustered into 3 categories, including biological process, cellular component, and molecular function. There are a total of 1,566 and 830 DEGs in comparison A and comparison B, respectively ([Fig. 2A](#) and [B](#)).

[Figure 3A](#) and [3B](#) display the top 20 GO terms resulting from the GO enrichment of 2 comparisons. Additionally, it was discovered that the GO terms shared by both comparisons comprised five categories, namely signaling (GO:0023052),

extracellular region (GO:0005576), G-protein-coupled receptor signaling pathway (GO:0007186), cell communication (GO:0007154), and cell signal transduction (GO:0007165) ([Table 2](#)).

According to the enrichment results, the gene classes containing >3 genes in the GO terms are considered to have a high frequency. The obtained high-frequency gene families and their functions in the 5 GO classifications are displayed in [Supplementary Table S2](#) (all data are based on Wormbase). Additionally, it was discovered that certain gene families may be associated with the protective effect of adenosine, mainly clustering in neural-related function. These gene families include the *dop* genes family, neuropeptide-related genes family (such as *flp*, *ins*, and *nlp*), peptidase activity regulation-related genes family (such as *sbt*), neuropeptide receptor genes family (such as *npr*), and acetylcholine release-related *unc* genes family. [Figure 4](#) depicts DEGs of the neural-related function in 3 groups.

KEGG pathway-based enrichment analysis

The pathway types and trend of the DEGs were similar between comparison A (CK vs. PQ) and comparison B (PQ vs. PQ_AD), but the number of genes differed ([Fig. 5A](#) and [B](#)). It appears that exposure to PQ altered the pathways of DEGs, which were partially repaired or responded with adding adenosine due to its protective effects.

It demonstrated that amyotrophic lateral sclerosis, amoebiasis, and complement and coagulation cascade pathways have the largest difference in comparison A (CK vs. PQ) ([Fig. 6A](#)). As for PQ treatment in the presence of adenosine in comparison B (PQ vs. PQ_AD), the complement and coagulation cascade pathways and amoebiasis were the most significantly altered ([Fig. 6B](#)).

Two pathways, namely the complement and coagulation cascade pathways and amoebiasis pathway, were identified as the most significant by pathway enrichment of common differential genes ([Fig. 7](#)). Additional investigation revealed that 11 of 12 differential complement and coagulation cascade pathways genes encoded the homologs of human tissue factor pathway inhibitor (TFPI) ([Supplementary Table S3](#)). Moreover, among the 43 differential genes involved in the amoebiasis pathway, 34 genes encode homologs of the mucin family member mucin-2 (MUC2) ([Supplementary Table S3](#)). It was postulated that these 2 components may play a crucial function in the injury and recovery of nematodes.

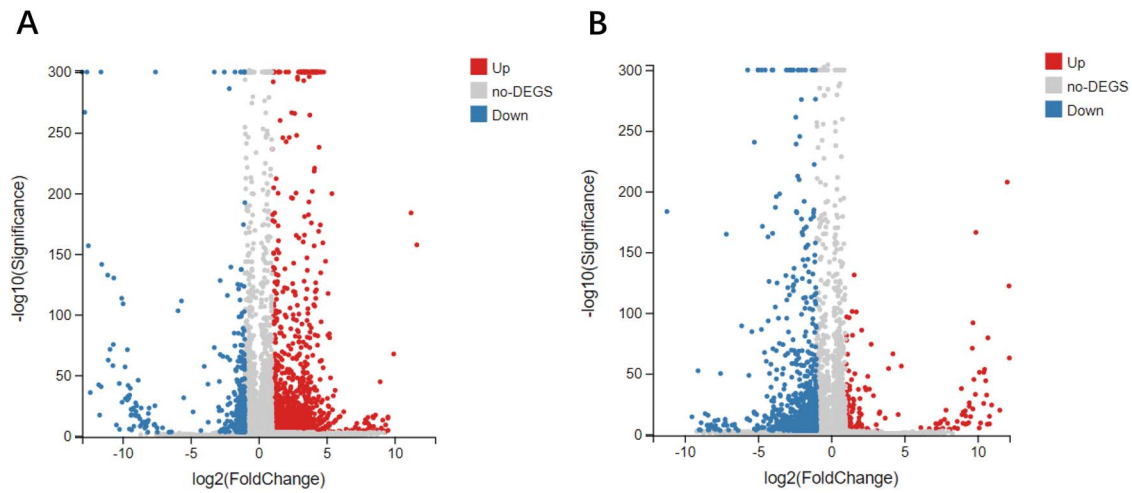


Fig. 1. Volcano plot of fold changes and *P*-values of *C. elegans* RNA-seq data following treatment with sterile M9 buffer solution (CK group), 150 mM PQ group, and 150 mM PQ in present of 3,000 μ M adenosine(PQ_AD group). A) CK versus PQ group; B) PQ versus PQ_AD group. Notes: The X-axis represents the fold of difference value after \log_2 transformation, and the Y-axis represents the significance value after $-\log_{10}$ transformation. Red color represents upregulated DEGs, blue color represents downregulated DEGs, and gray color represents non-DEGs.

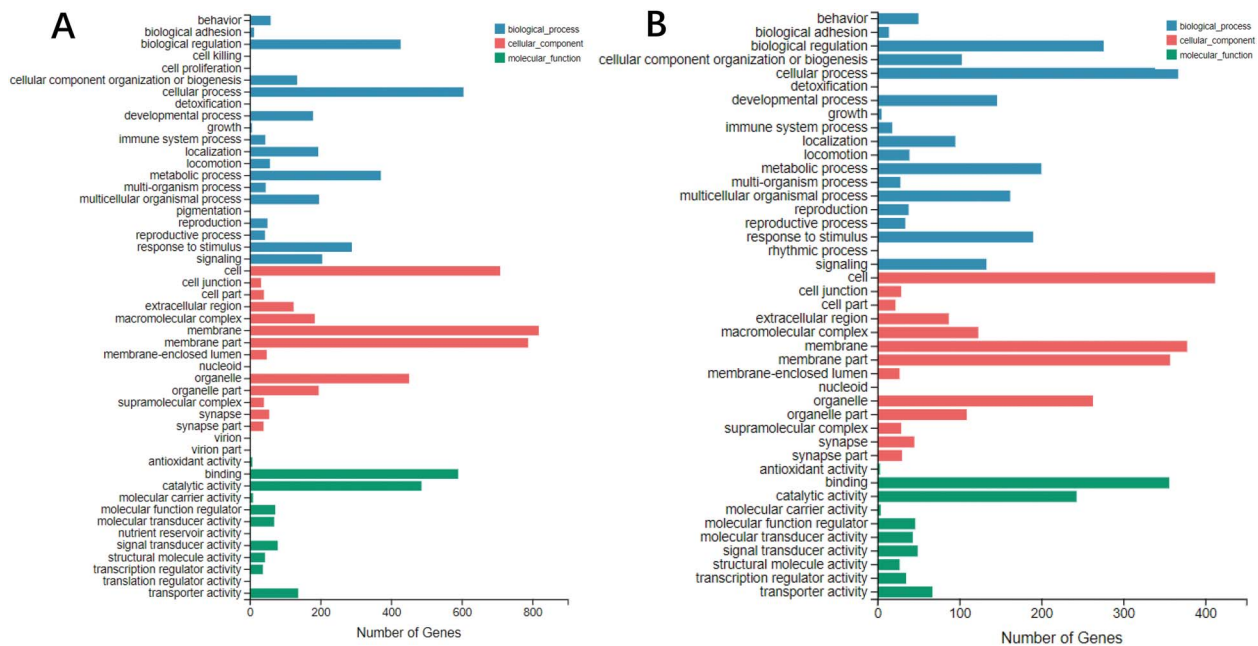


Fig. 2. GO functional annotation classification statistics of DEGs in comparison A (CK vs. PQ) and B (PQ vs. PQ_AD). Note: The X-axis represents the number of genes annotated to GO entries, and the Y-axis represents the GO functional classification.

Table 2. GO terms coexisting in CK versus PQ versus PQ_AD.

GO term ID	GO term	(CK vs. PQ) rich ratio	(CK vs. PQ) Q value	(PQ vs. PQ_AD) rich ratio	(PQ vs. PQ_AD) Q value
GO:0023052	Signaling	0.157	1.16E-12	0.102	1.60E-09
GO:0005576	Extracellular region	0.202	3.34E-11	0.142	1.83E-13
GO:0007186	GPCR signaling pathway	0.340	3.52E-19	0.197	1.80E-09
GO:0007154	Cell communication	0.155	2.82E-12	0.101	1.90E-09
GO:0007165	Signal transduction	0.151	1.79E-09	0.094	4.02E-06

DEGs associated with the adenosine receptor signal transduction pathway

On the basis of pathway analysis of the common DEGs between 2 comparisons, the genes clustering in the signal transduction pathway are most affected by exposure to PQ in the presence or absence of adenosine. The *ador-1* is the homologous gene of the

adenosine receptor in *C. elegans*. It is well-documented that *ador-1* mainly functions via the cAMP signaling pathway. Consequently, clustering of DEGs in the cAMP pathway were calculated based on KEGG pathway analysis (Fig. 8, Supplementary Table S4). PQ groups have higher expression levels of GPCR (*C24A8.6*, *F10D7.1*, *ador-1*, *dop-1*, *gcy-9*, *gcy-11*, *gcy-19*, *gcy-22/daf-37*, *dmsr-8*, *dop-2*,

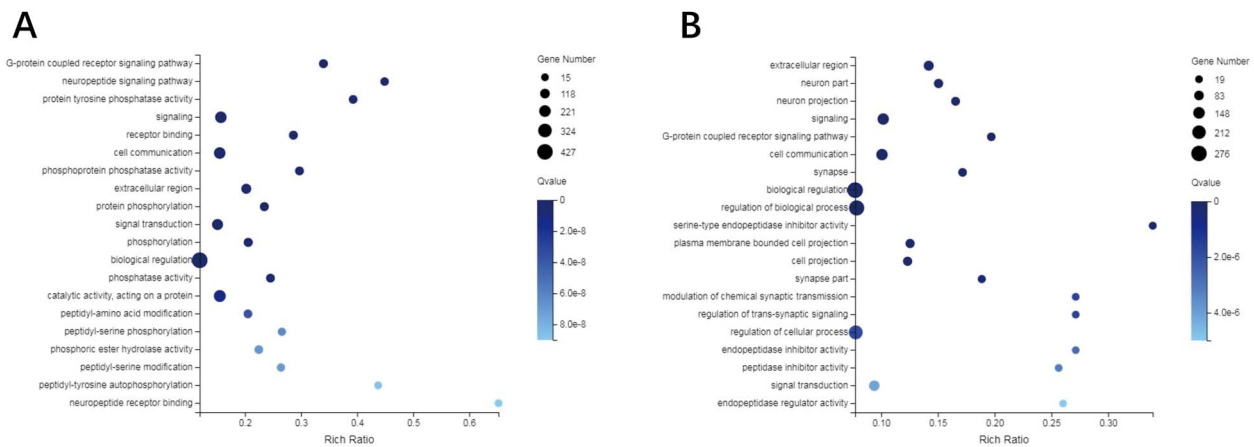


Fig. 3. Top 20 GO terms in GO enrichment of (A) CK versus PQ and (B) PQ versus PQ_AD.

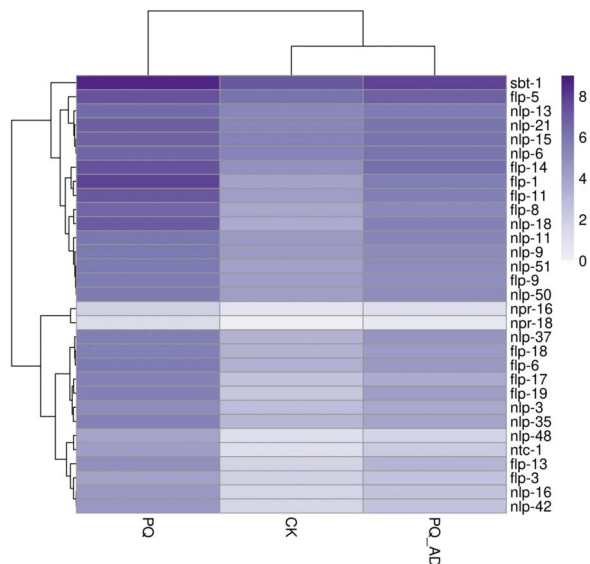


Fig. 4. Heatmap of DEGs of neural-related function for adenosine's protective effects.

dop-3, *dop-5*, *frpr-17*, *npr-16*, *npr-18*, *npr-25*, *npr-29*, and *npr-32*), AC (*acy-2*), cyclic nucleotide gated channel (CNGC) (*K05F1.9*, *T06E4.10*, *T22B3.3*, *ZK354.7*, and *cec-1*), calmodulin (CaM) (*C50C3.5*, *K03A1.4*, *cal-3*, *cal-4*, *cal-8*, and *tnc-2*), PDE (*ZC443.4*), phosphatidylinositol phospholipase C, epsilon (PLC ϵ) (*frm-9*), PP1 (*C06A1.3*, *C24H11.2*, *C27B7.6*, *F20D6.6*, *F40E3.5*, and *F42G8.8*), and MYPT1 (*K02F6.3*) and have lower expression levels of Gi (*gpa-16*), GLI3 (*sup-35*), ACO (*acox-1.4*, *acox-1.5*, and *acox-3*), and ATP (*catp-2*) than the CK group. In addition, PQ_AD group have lower expression levels of GPCR (*C24A8.6*, *F10D7.1*, *dop-6*, *gcy-11*, *gcy-18*, *gcy-19*, *gcy-22*/*C24A8.6*, *daf-37*, *dmsr-7*, *dop-2*, *dop-3*, *dop-5*, *dop-6*, *frpr-5*, *gbb-1*, and *npr-18*), Gs (*gsa-1*), AC (*acy-2*), CNGC (*T06E4.10*), CaM (*C50C3.5*, *cal-3*, *cal-4*, and *tnc-2*), VDCC (*unc-2*), and PDE (*ZC443.4*), JNK (*jnk-1*) but have higher expression levels of Gi (*gpa-16*) than the PQ group.

Validation of DEGs in the G-protein signaling pathway

The implicated genes, which were known to play a role in *C. elegans* motor neuron G-protein signaling pathway, were screened out via transcriptome analysis, and these are demonstrated in Table 1. All the qPCR validation results matched the transcriptome results. In the qPCR validation results, the genes

related to promote acetylcholine release pathway (such as *dop-1*, *egl-30*, and *unc-13*) and the gene related to promote neuropeptide release pathway (such as *kin-1*) exhibited a similar trend in that their expression were upregulation during PQ-treated but downregulation during PQ_AD-treated (treatments with PQ in present of adenosine) (Fig. 9). In addition, the expression of these 4 genes was significantly increased when treated with PQ compared to CK and PQ_AD ($P < 0.01$). Interestingly, although the expression of *ador-1* gene follows the same pattern as that of the *dop-1*, *egl-30*, *unc-13*, and *kin-1* genes, there was no significant change among the 3 treatments CK, PQ, and PQ_AD ($P > 0.05$). It indicated that adenosine receptor has a consistent and steady action on its respective pathways regardless of whether or not PQ is present. Furthermore, qPCR was used to access the dominance of GPCR signaling pathway for adenosine protection effect by validating the gene (*goa-1*) that suppressed the mentioned pathways, including acetylcholine release pathway and neuropeptide release pathway. The expression of the gene *goa-1* followed the exact opposite pattern, decreasing by 65.3% after the PQ-treating and increasing to $(74.5 \pm 8)\%$ following adenosine addition.

Assessment of adenosine against oxidative stress

The knockdown of *ador-1* changed the oxidative-stress resistance of *C. elegans* in the presence of adenosine. The survival rates of WT and *ador-1*-RNAi nematodes treated with CK, PQ, and PQ_AD are counted in Fig. 10. There was no significant difference between WT and *ador-1*-RNAi while treated with M9 buffer solution and PQ. By contrast, RNAi of genes encoding ADOR-1 proteins exhibited significant negative effects on survival under oxidative stress conditions in PQ_AD treatment ($P < 0.01$). Comparing PQ treatment with PQ_AD treatment, there was no significant difference in the survival rate among the RNAi groups, whereas there was a significant increase among the WT groups ($P < 0.01$). Thus, it indicated that the presence of *ador-1* gene played an important role in improving the survival rate when nematodes were treated with adenosine under oxidative stress conditions.

Discussion

In the present study, we performed RNA-seq analysis of *C. elegans* on PQ exposed in the presence/absence of adenosine and performed the GO and KEGG pathway analyses on the DEGs in order to investigate the mechanism of adenosine's protective effects.

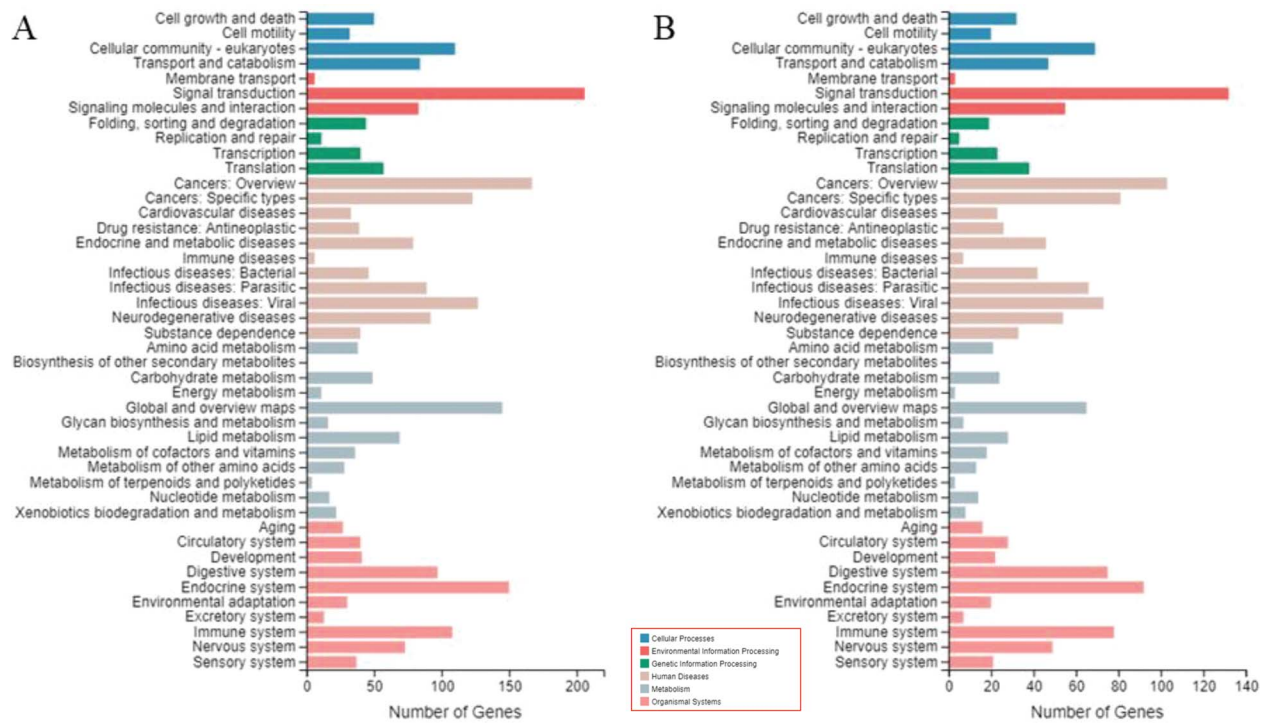


Fig. 5. KEGG pathway analysis of A) CK versus PQ and B) PQ versus PQ_AD.

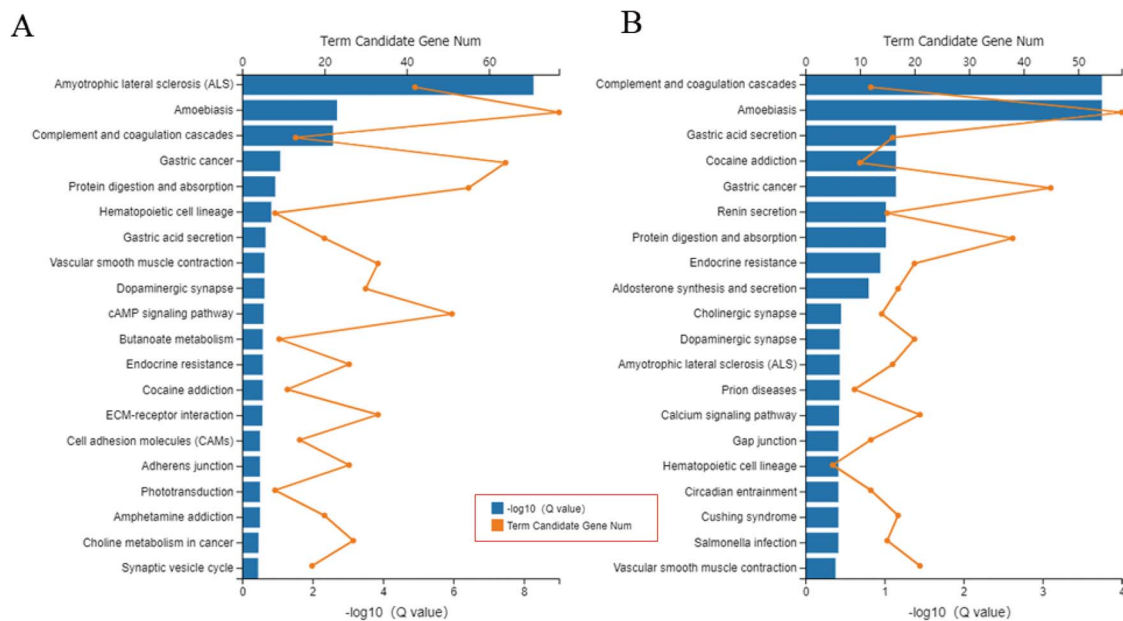


Fig. 6. KEGG pathway enrichments of A) CK versus PQ and B) PQ versus PQ_AD.

In comparison A (CK vs. PQ), 82.8% of the DEGs of PQ group were upregulated relative to the CK group. By contrast, in comparison B (PQ vs. PQ_AD), 82.1% of the DEGs of PQ_AD group were downregulated relative to the PQ group. It appeared that the protective impact of adenosine on nematodes exposed to PQ has an expression mechanism that is inverse to that of PQ exposure.

Intriguingly, in the comparison of CK versus PQ and PQ versus PQ_AD, the DEGs were clustered in 3 identical GO categories: biological process, cellular component, and molecular function (Fig. 2). Considering our prior finding,¹¹ it is possible to predict that the survival rate was improving in *C. elegans* exposed to PQ

in the presence of adenosine due to a significant portion of the gene expression being recovered by the addition of adenosine. Surely, the extent to which genes play a critical role in this process requires more investigation.

GO enrichment analysis identified several gene families associated with the protective effects of adenosine, including *abu*, *dop*, *daf*, *flp*, *nlp*, *npr*, *gcy*, *nhr*, etc. Furthermore, the neuropeptide gene family comprised the majority. Although previous reports on neuropeptides are less about resistance to oxidative stress and are directly affecting the survival of nematodes, most of the neuropeptide genes and its signaling pathway discovered

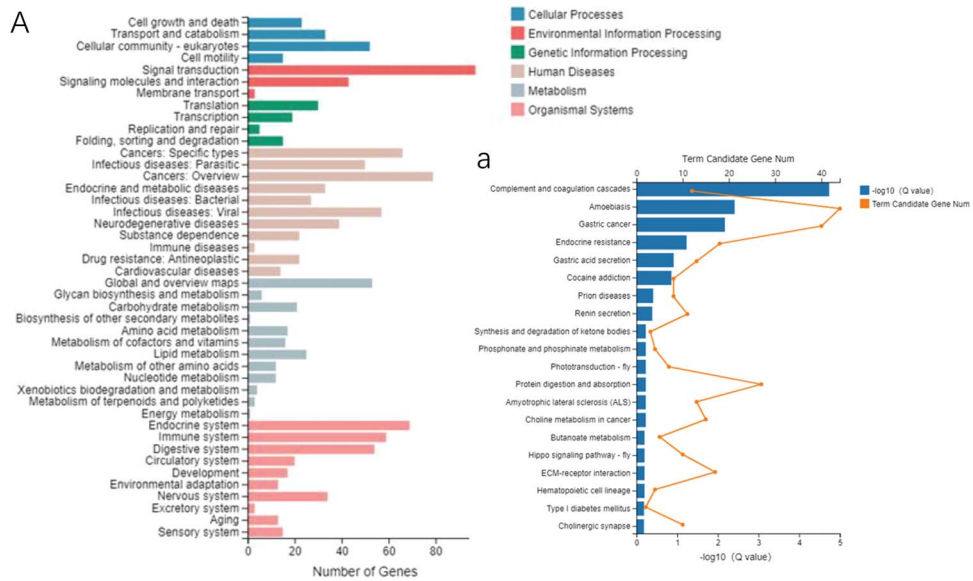


Fig. 7. (A) KEGG pathway analysis and (a) enrichment of common DEGs.

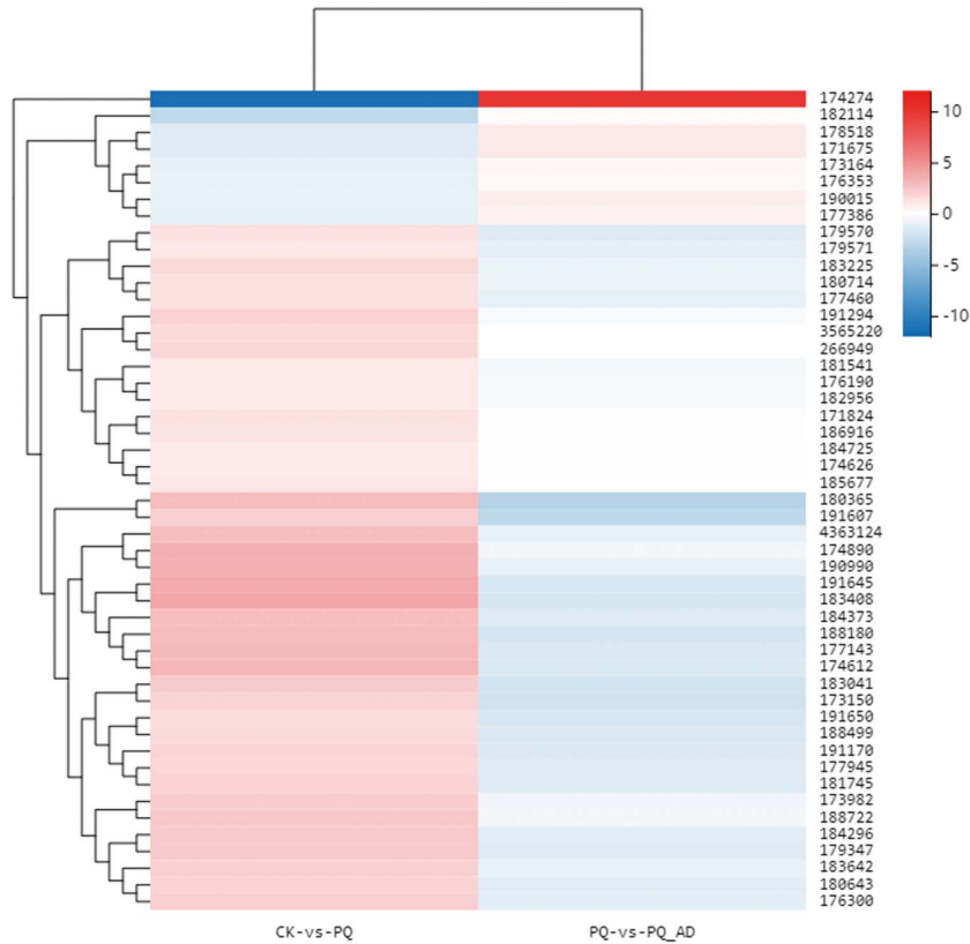


Fig. 8. Differential expression clustering of DEGs in cAMP signal pathway between 2 comparisons (CK vs. PQ and PQ vs. PQ_AD).

this study may play a crucial role in the survival of nematodes. As the largest neuropeptide family in *C. elegans*, the *flp* gene family expressed FMRF-like amide peptides that serve as sensory, motor, and interneurons in >50% of the nervous system.^{24,25} The nematode *flp* gene has neuropeptide receptor binding activity and

functions in the neuropeptide signaling pathway to regulate sleep, movement, mating regulation, action potential regulation, oviposition regulation, etc.²⁶ In the signal transduction system, FLP can function as a neurotransmitter, bind to the transmembrane receptor on the cell membrane, and activate the adjacent G-protein;

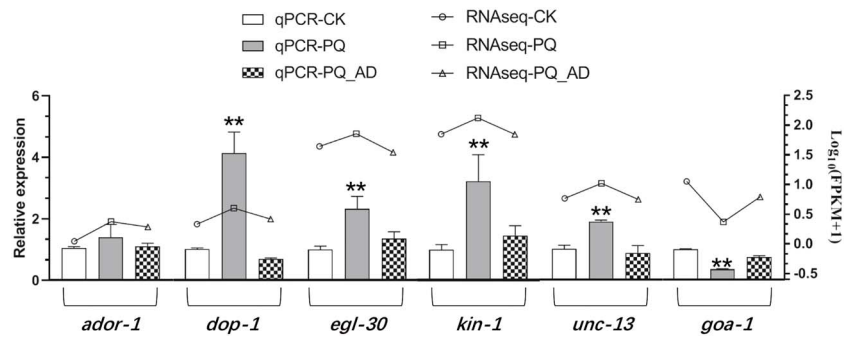


Fig. 9. qPCR validation of G-protein signaling pathway genes in *C. elegans* following treatment with sterile M9 buffer solution (CK group), 150 mM PQ group, and 150 mM PQ in presence of 3,000 μ M adenosine (PQ_AD group).

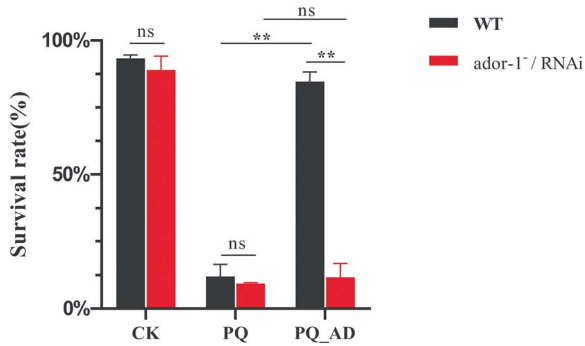


Fig. 10. The survival rate of *C. elegans*, including WT and *ador-1*^{-/-}/RNAi type. CK represents treatment with sterile M9 buffer solution, PQ represents treatment with 150 mM PQ, and PQ_AD represents treatment with 150 mM PQ in the presence of 3,000 μ M adenosine.

and the activated G-protein can activate or inhibit a specific enzyme (such as cAMP, cGMP, etc.), which as a second messenger, activates protein kinase, which in turn activates phosphokinase, converts ATP into ADP, and mediates related reactions; activated G-protein have the ability to activate ion channels, such as Ca²⁺ channels, thereby causing changes in the membrane potential, ultimately leading to the regulation of various physiological activities.

Notably, the *nlp* gene family initially recorded as being resistant to oxidative stress is now predicted to have neuropeptide hormone activity in the Wormbase database. Similar to the *flp* gene family, *nlp* genes are widely expressed in neuronal and nonneuronal tissues.²⁷ The *nlp-7* has been reported to affect lifespan extension through dietary restriction.²⁸ The expression of *nlp-29*, 31 can induce defense responses.^{29,30} The *nlp-24*, 25, 27, 28, 29, 30, 31, and 33 can function as antimicrobial peptides,³¹ and antimicrobial peptides are widely used in innate immunity,²⁹ while *nlp-27*, 29, and 31 may also function as neuromodulators.²⁷ In addition, the expression product of the *npr* gene family is the neuropeptide receptor family, a G-protein-coupled receptor.

KEGG pathway-based enrichment analysis of common differential genes revealed that TFPI in the complement and coagulation cascade pathway and MUC2 in the amebiasis pathway may play a significant role in the process of nematode's injury and recovery. TFPI is an endogenous serine protease inhibitor produced and released by endothelial cells, as is well known. TFPI inhibits a variety of serine proteases, such as trypsin, α -chymotrypsin, plasmin, and cathepsin G, with quite wide inhibitory effects.³² TFPI inhibits the progression of atherosclerosis in humans via its effects on endothelial cell activation, vascular smooth muscle cell proliferation and

migration, inflammatory cell recruitment, and extracellular matrix.³³ TFPI also plays a significant role in the development and treatment of hemophilia.³⁴ In *C. elegans*, the homologous gene of TFPI has not been thoroughly explored, but we hypothesize that its function is strongly related to oxidative stress in *C. elegans* and adenosine's protective impact.

MUC2 is a member of the mucin family, which are high molecular weight glycoproteins produced by numerous epithelial tissues and are the major proteins in the intestinal mucus. MUC2 functions as the intestinal mucus barrier, inhibiting the entry of intestinal pathogens in mice³⁵ and preserving cell viability to reduce oxidative stress in the intestinal epithelial cells.³⁶

cAMP pathway analysis of comparison A (CK vs. PQ) revealed that the expression of GPCRs, including adenosine receptors and dopamine receptors, rose in PQ group, but Gi protein dropped. Together, their expression encouraged the upregulation of cAMP. As the second messenger in the G-protein-coupled signaling pathway, cAMP regulates the expression of CNGC on the cell membrane, increases the influx of Ca²⁺, and passes CaM forms a positive feedback regulation on AC. On the other hand, it transmits the signal to the downstream Epac (RAPGEF3, Rap guanine nucleotide exchange factor) through PLC ϵ -DAG/IP3 pathway further regulates Ca²⁺.

cAMP pathway analysis of comparison B (PQ vs. PQ_AD) revealed that the expression of several of the aforementioned pathways is reversed in PQ_AD group, particularly the expression of GPCR. In the protective impact of adenosine, the expressions of Gs, Gi, AC, CNGC, CaM, and PDE were reversed, indicating that the regulation of cAMP by GPCR, CaM, and PDE represented the restoration of cell activity and the function of mitochondrial cells. The maintenance of intracellular Ca²⁺ homeostasis is important, which is associated with opening of the mitochondrial permeability transition pore, loss of membrane potential, and impairment of energy metabolism and cellular function.^{37,38}

PLC ϵ , MYPT1, ATP, and ADOR-1 were among those whose expressions did not reverse in comparison B, indicating that these genes remained to function or could not be restored from the cell damage to recovery. The regulation of PLC ϵ on Ca²⁺ obviously continues in the protective effect of adenosine, whereas the continuous inhibition of myosin regulatory light chain (MLC) by MYPT1 leads to the tension of nematode endothelial cells and smooth muscle cells; MLC phosphorylation is essential for muscle contraction function, and its decrease in phosphorylation is 1 of the key reasons for the impairment of contraction function.³⁹ This might be due to the tight state of the nematode's body when it is exposed to PQ, and it appears that the protective action of adenosine does not properly ameliorate this condition.

The decrease in Na⁺/K⁺-transporting ATPase subunit expression in PQ-treated cells may be attributable to a malfunction in ion control caused by a decrease in cell activity. In rats with cerebral ischemia, the activity of Na⁺/K⁺-transporting ATPase decreases with the increase of oxidative stress, and the ability of antioxidant protection is destroyed at the same time.⁴⁰ Since powerful antioxidants can protect its activity, we hypothesized that nonantioxidant adenosine cannot directly restore its activity. The elevated expression level of ADOR-1 in the PQ group indicates its antistress action in an environment of oxidative stress. Among all GPCR genes in this pathway, only *ador-1* did not undergo callback in the PQ_AD group ($P = 0.264$). It demonstrated that it continued to contribute to adenosine's protective action on the nematode cells.

Meanwhile, qPCR validation yielded identical results. The *C. elegans* neuron GPCR signaling pathway involving *dop-1*, *egl-30*, *unc-13*, *kin-1*, and *goa-1* genes may play crucial roles in modulating adenosine's protective action (Table 1, Fig. 9). Intriguingly, there are no significant expression differences between the PQ group and PQ_AD group for *ador-1* gene despite a large number of genes in the PQ group being downregulated by the addition of adenosine (Fig. 9). It suggested that the adenosine receptor homolog ADOR-1 may continue to play a consistent role in the protective action of adenosine regardless of whether or not PQ is present. Furthermore, the WT group with *ador-1* gene has a higher survival rate than that of the *ador-1*⁻/RNAi group when they were treated with PQ in the presence of adenosine (Fig. 10). It further demonstrated that *ador-1*-relative genes were of great significance for improving the survival rate of nematodes under oxidative stress. Cytotoxicity induced by PQ has been shown to lead to an increase in mitochondrial membrane permeability, which is manifested as a decrease in mitochondrial membrane potential ($\Delta\Psi$).⁴¹ Furthermore, PQ also has a strong oxidizing ability, which can lead to a decrease in antioxidant enzymes (such as superoxide dismutase, glutathione peroxidase, and total superoxide dismutase). Therefore, the mitochondrial function and antioxidant system should be explored further in *C. elegans*, which were treated with PQ in the presence or absence of adenosine.

Conclusion

Our findings indicated that the adenosine's protective action against PQ-induced oxidative stress in *C. elegans* is mediated via adenosine receptor (ADOR-1)-related genes. Several novel GO and KEGG pathway analyses were carried out to investigate the DEGs in 2 comparisons (CK vs. PQ and PQ vs. PQ_AD). Furthermore, GO enrichment analysis revealed several neuropeptide gene families associated with the protective effect of adenosine, such as *abu*, *dop*, *daf*, *flp*, *nlp*, *npr*, *gcy*, *nhp*, etc. cAMP pathway analysis and qPCR demonstrated that the expression of GPCRs, including adenosine receptors and dopamine receptors, may play a role in the protective effect of adenosine on nematode cells, and this needs further investigation. RNAi of *ador-1* genes indicate that ADOR-1 plays an important role during adenosine against PQ-exposed condition. Overall, our study provides a new insight into the biological basis of the adenosine's protective effect against PQ-induced oxidative stress via RNA-seq and may lead to a greater understanding of the molecular mechanism of the adenosine receptor (ADOR-1).

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Authors' contributions

Lingmei Ma, Chunyan Ling, and Shuning Hu: Methodology, experiment, data analysis, writing; Sudan Ye: conceptualization, review, and editing; Chun Chen: supervision, conceptualization, review, and editing.

Supplementary material

Supplementary material is available at TOXRES Journal online.

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