

2,6-Dichloro-1,4-Benzoquinone Induces Oxidative Stress, Mitophagy-Related Gene Upregulation, and Insulin Signaling Pathway Suppression in T24 Bladder Cancer Cells

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How to cite this paper: Fang, D.K. (2026) 2,6-Dichloro-1,4-Benzoquinone Induces Oxidative Stress, Mitophagy-Related Gene Upregulation, and Insulin Signaling Pathway Suppression in T24 Bladder Cancer Cells. *Occupational Diseases and Environmental Medicine*, 14, 163-176.

<https://doi.org/10.4236/odem.2026.143015>

Received: May 9, 2026

Accepted: July 5, 2026

Published: July 8, 2026

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Abstract

2,6-Dichloro-1,4-benzoquinone (2,6-DCBQ), a disinfection by-product frequently detected in drinking water, exhibits significant toxicity. However, it remains unregulated due to a lack of toxicological data. This study aims to generate toxicological data on 2,6-DCBQ and elucidate its mechanisms of toxicity in T24 bladder cancer cells. T24 cells were exposed to 10 - 250 μ M 2,6-DCBQ. Cell viability was assessed using the CCK-8 assay to calculate the IC_{50} . Cytotoxic effects at concentrations approximating 1/4 IC_{50} , 1/2 IC_{50} and IC_{50} concentrations were evaluated using a lactate dehydrogenase (LDH) cytotoxicity detection kit. Oxidative stress levels were quantified via malondialdehyde (MDA) assay. RNA-seq was employed to analyze changes in gene expression and signaling pathways in T24 cells following 2,6-DCBQ exposure. The results showed that 2,6-DCBQ exposure resulted in a dose-dependent reduction in T24 cell viability and increased mortality. Higher doses induced oxidative damage. Transcriptomic analysis revealed abnormal upregulation of mitophagy-related genes and downregulation of genes associated with the insulin signaling pathway in T24 cells. In conclusion, exposure to 2,6-DCBQ induces oxidative damage leading to cell death in T24 cells, with mitophagy-related pathways and the insulin signaling pathway identified as key contributors. These findings are limited to the bladder cancer cell model and further studies are needed to extrapolate to normal bladder tissue or human health.

Keywords

Disinfection By-Products, Halobenzoquinone, 2,6-DCBQ, RNA-Seq, Mitophagy

1. Introduction

Drinking water disinfectants possess strong oxidizing properties and can chemically react with naturally occurring organic matter (NOM) to produce disinfection by-products (DBPs) [1]-[3]. The human body primarily absorbs DBPs through drinking water and skin contact (e.g., showering and swimming) [4]-[6]. Exposure to DBPs has been linked to numerous adverse human health effects, including developmental defects and the risk of bladder cancer [7]-[10]. However, only a few DBPs, such as trihalomethanes (THMs) and haloacetic acids (HAAs), are regulated [11]. Recent studies have indicated that many unregulated DBPs may be the culprits driving DBP toxicity [12] [13].

As an emerging class of DBPs, halobenzoquinones (HBQs) have garnered more attention due to their higher toxicity compared to THMs and HAAs. Quantitative structure-toxicity relationship (QSTR) analysis predicts that HBQs are potential bladder carcinogens [14] [15]. It is estimated that the lowest observed adverse effect level (LOAEL) for HBQs is 10,000 times lower than that of some regulated DBPs [16]. To date, 12 HBQs have been identified in drinking water, among which 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ) has been proven to be the most toxic and abundant DBP [16] [17]. Multiple studies have found that the cytotoxicity and genotoxicity of 2,6-DCBQ are significantly higher than those of other HBQs [18] [19].

Chen *et al.* evaluated the effects of 2,6-DCBQ exposure on zebrafish and found that it disrupts the homeostasis of the antioxidant system, causing tissue edema and inflammatory cell infiltration in the brain and heart, and altering the VEGF and NOD-like receptor signaling pathways in adult zebrafish [20]. In mammals, exposure to 2,6-DCBQ leads to reduced antioxidant enzyme activity in mice and inhibits the transcription of key genes in the Nrf2-KEAP1 pathway, resulting in increased oxidative stress levels [21]. Thus, exposure to 2,6-DCBQ can cause an imbalance between oxidation and antioxidation in the body, leading to oxidative damage. However, its potential mechanism of action remains unclear.

Transcriptomic sequencing can reveal the effects of toxins on gene expression by studying gene transcription and transcriptional regulation patterns in cells, screening differentially expressed genes and key signaling pathways, and thus exploring the toxicity mechanisms of toxins more deeply. Given the high detection rate and high toxicity of 2,6-DCBQ, elucidating its key mechanism of damage to the body is an important public health task. Therefore, this study evaluated the toxic effects of 2,6-DCBQ on the bladder cancer cell line T24, and conducted transcriptomic sequencing on T24 cells after exposure to 2,6-DCBQ to identify key signaling pathways involved in its toxic effects.

2. Materials and Methods

2.1. Standard Product

The 2,6-DCBQ standard was purchased from Aladdin (China). It was dissolved in

methanol (HPLC grade, Fisher Scientific) to a stock concentration of 100 mM and stored in a sterile brown glass vial at -20°C . The final methanol concentration in all treatment groups (including the control) was adjusted to 0.1% (v/v) by diluting the stock with culture medium. The control group received 0.1% methanol without 2,6-DCBQ to exclude solvent effects.

2.2. Cell Culture

The human bladder epithelial cancer cell line T24 was obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, China) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Procell, Wuhan, China) and 1% penicillin/streptomycin (Invitrogen, USA), and placed in a cell incubator at 37°C with 5% CO_2 .

2.3. Cell Viability

According to the manufacturer's instructions, the cell viability was determined using the CCK8 cell proliferation-toxicity assay kit (DOJINDO, Japan). Specifically, T24 cells were seeded at a density of 7500 cells per well and cultured for 24 hours before cell viability testing. Based on previous research results [22], cells were treated with 10 - 250 μM 2,6-DCBQ for 24 hours, followed by the addition of CCK8 reagent and continued incubation in a 37°C incubator for 1 hour. The absorbance was measured at 450 nm using a microplate reader. Based on the cell viability assay results, a curve depicting the changes in T24 cell viability under different concentrations of 2,6-DCBQ exposure was plotted, and the IC_{50} was calculated. According to the IC_{50} results, the subsequent exposure doses of 2,6-DCBQ were determined, with the high-dose group (H group) at IC_{50} , the intermediate-dose group (M group) at $1/2 \text{ IC}_{50}$, and the low-dose group (L group) at $1/4 \text{ IC}_{50}$.

T24 cells were seeded at a density of 7500 cells per well (100 μL), and after 24 hours of culture, the medium containing three different doses of 2,6-DCBQ was replaced and treated for another 24 hours. According to the manufacturer's instructions, the cytotoxicity of 2,6-DCBQ was measured using a lactate dehydrogenase cytotoxicity assay kit (Beyotime, Shanghai, China).

2.4. Detection of Cellular Oxidative Stress Levels

The levels of malondialdehyde (MDA) adducts in T24 cells from three 2,6-DCBQ-treated groups were determined using a lipid oxidation (MDA) detection kit. T24 cells were seeded at a density of 7500 cells per well (100 μL) and cultured for 24 hours before the medium was replaced with one containing different doses of 2,6-DCBQ. After 24 hours of exposure to 2,6-DCBQ, cell samples were pretreated and MDA adducts were detected according to the instructions provided in the kit.

2.5. Cellular Transcriptomics Sequencing

Transcriptome sequencing and data analysis were completed by Wuhan Kangce Technology Co., Ltd. (Wuhan, China).

2.5.1. RNA Extraction, Library Preparation, and Sequencing

Seed T24 cells in a 10 cm cell culture dish and replace the medium with one containing three different doses of 2,6-DCBQ after 24 hours of culture. After 24 hours of exposure to 2,6-DCBQ, total RNA was extracted from T24 cells using TRIzol Reagent (Invitrogen, USA) according to the method described by Chomczynski *et al.* [22]. After RNA extraction, DNaseI was used for DNA digestion. The integrity of RNA was determined by measuring the A260/A280 ratio using a Nanodrop™ OneC spectrophotometer (Thermo Fisher Scientific Inc, USA) and confirmed by 1.5% agarose gel electrophoresis. Finally, qualified RNA was quantified using the Qubit3.0 and Qubit™ RNA Broad Range Assay kit (Life Technologies, USA).

According to the manufacturer's instructions, the KCTM Stranded mRNA Library Prep Kit for Illumina® (Wuhan Kangce Technology Co., Ltd., China) was used to prepare a stranding RNA sequencing library using 2 µg of total RNA. The PCR products corresponding to 200 - 500 bps were enriched and quantified, and finally sequenced on a PE150 model DNBSEQ-T7 sequencing instrument (MGI Tech, China).

2.5.2. Transcriptome Data Analysis

1) Filtering and annotation of raw sequencing data

The raw sequencing data were first filtered using Trimmomatic (version 0.36) to discard low-quality reads and trim reads contaminated by adapter sequences. Clean reads were then mapped to the reference genome of GRCh 38.87 using STRA software (version 2.5.3a). Reads mapped to the exon regions of each gene were counted using featureCounts (Subread-1.5.1; Bioconductor), and the number of reads per kilobase per million mapped sequences (RPKM) for each gene was calculated.

2) Differentially expressed gene screening

Use the edgeR package (version 3.12.1) in R language to screen genes with differential expression between groups, using raw count data as input. Genes with a false discovery rate (FDR) < 0.05 and $|\log_2 \text{fold change}| > 1$ were considered significantly differentially expressed. The statistical significance of gene expression differences is determined by $P < 0.05$ and $\log_{FC} > 1$ or $\log_{FC} < -1$.

3) Pathway analysis

GO functional enrichment analysis and KEGG pathway analysis were conducted using KOBAS software (version: 2.1.1). GO enrichment annotations and KEGG pathway analysis results were obtained from biological process (BP), molecular function (MF), and cellular component (CC). Statistically significant enrichment was determined with an FDR < 0.05 (replacing the original *P*-value-only threshold).

3. Results

3.1. Effect of 2,6-DCBQ on the Viability of T24 Cells

To test the effect of 2,6-DCBQ on the viability of T24 cells and to explore suitable

exposure concentrations for subsequent experiments, we assessed the changes in T24 cell viability after exposure to 10 - 250 μM 2,6-DCBQ for 24 hours, based on previous research findings. The results are shown in **Figure 1(a)**. Exposure to 2,6-DCBQ significantly reduced the cell viability of T24 cells, and this reduction was dose-dependent. The IC_{50} calculated from the T24 cell viability curve after 24 hours of exposure to 2,6-DCBQ was 114.0 (109.6 - 118.0) μM . To set approximate low, medium, and high exposure doses, we selected 25 μM , 50 μM , and 100 μM , which roughly correspond to $1/4 \text{ IC}_{50}$ (28.5 μM), $1/2 \text{ IC}_{50}$ (57 μM), and IC_{50} (114 μM), respectively, given the convenience of dose preparation and consistency with previous studies. These doses are referred to as low-dose (L, 25 μM), medium-dose (M, 50 μM), and high-dose (H, 100 μM) groups throughout the study, and subsequently measured lactate dehydrogenase (LDH) activity. The results are shown in **Figure 1(b)**. As the concentration of 2,6-DCBQ increased, the activity of LDH gradually increased ($P < 0.01$), indicating that within the exposure range of 0 - 100 μM , 2,6-DCBQ caused T24 cell death in a dose-dependent manner. In summary, 2,6-DCBQ has a significant toxic effect on T24 cells, and this effect is dose-dependent.

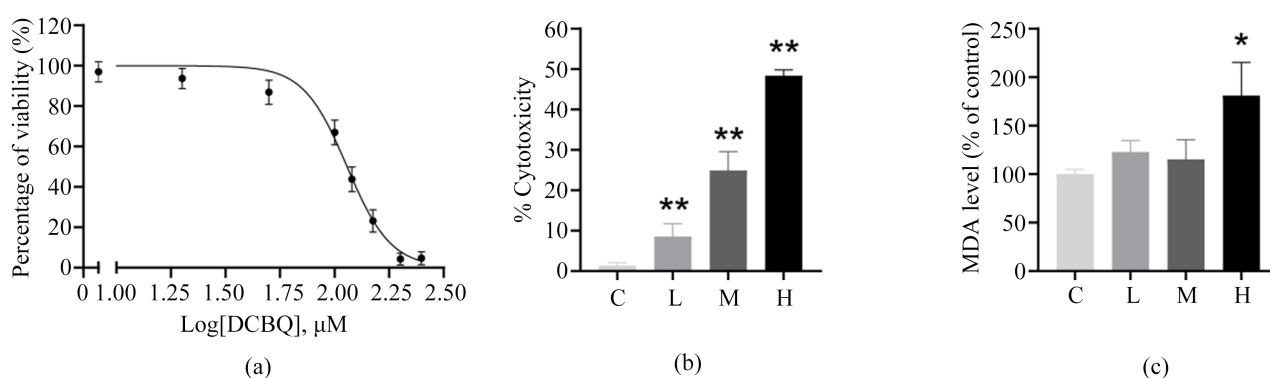


Figure 1. Reduced viability and oxidative damage in T24 cells after 24 hours of exposure to 2,6-DCBQ. Note: (a) represents the viability curve of T24 cells after exposure to 2,6-DCBQ at different concentrations. (b) illustrates the percentage of cytotoxicity calculated based on LDH enzyme activity after exposure to 2,6-DCBQ, where the percentage of cytotoxicity (%) = (absorbance of treated sample – absorbance of sample control well)/(absorbance of maximum enzyme activity of cells – absorbance of sample control well) $\times 100$. (c) shows the changes in MDA adduct levels in each exposure group relative to the control group after exposure to 2,6-DCBQ. All values are expressed as mean $\pm$ standard deviation. Each experiment was set up with six biological replicates, and one-way ANOVA was used for inter-group difference analysis, followed by Dunnett's test for pairwise comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3.2. 2,6-DCBQ Causes Oxidative Damage to T24 Cells

We further examined the content of malondialdehyde (MDA) adducts in T24 cells after exposure to 2,6-DCBQ. The results are shown in **Figure 1(c)**. Compared with the control group (1.00 ± 0.05), the MDA adduct levels in the H group (1.81 ± 0.34) were significantly increased ($P < 0.05$), while there was no statistical difference in MDA adduct levels between the L and M groups. This result suggests that at high doses, 2,6-DCBQ causes lipid peroxidation damage in T24 cells, but such direct oxidative damage was not detected at lower doses under the present assay.

conditions.

3.3. Exposure to 2,6-DCBQ Causes Changes in the Transcriptomic Profile of T24 Cells

Transcriptome sequencing was performed on T24 cells exposed to different doses of 2,6-DCBQ for 24 hours. The quality of the sequencing data is shown in **Table 1**. The clean reads obtained from each sample ranged from 69,082,156 to 199,819,894, and the proportion of bases with quality greater than Q30 in the clean reads was greater than 97.8%.

Table 1. Summary of transcriptome sequencing data.

Sample	Original sequence number (bp)	Original sequence Q20 (%)	Original sequence Q30 (%)	Original sequence GC content (%)	Quality control sequence number (bp)	Original sequence Q20 (%)	Original sequence Q30 (%)	Quality control sequence GC content (%)	efficient (%)
C1	134051482	98.58	95.76	49.72	121,859,674	99.49	97.94	49.51	90.91
C2	156750638	98.60	95.78	49.75	142,669,500	99.47	97.88	49.57	91.02
C3	171627734	98.62	95.89	49.69	156,445,236	99.49	97.95	49.51	91.15
L1	219894194	98.57	95.74	49.69	199,819,894	99.47	97.88	49.54	90.87
L2	156642100	98.62	95.89	49.81	142,982,156	99.49	97.96	49.55	91.28
L3	151420592	98.60	95.84	49.66	137,770,448	99.49	97.96	49.54	90.99
M1	127163540	98.60	95.81	49.64	115,680,686	99.49	97.95	49.41	90.97
M2	169330836	98.60	95.83	49.43	154,103,978	99.49	97.96	49.38	91.01
M3	117864454	98.61	95.85	49.69	107,433,556	99.49	97.94	49.38	91.15
H1	143433254	98.63	95.88	49.61	130,746,082	99.49	97.95	49.49	91.15
H2	76344352	98.52	95.58	49.40	69,082,156	99.46	97.84	49.2	90.49
H3	137823086	98.61	95.84	49.69	125,551,130	99.48	97.94	49.58	91.10

Under the principal component scores of 21.57% and 16.11%, it can be observed that the control group and the three 2,6-DCBQ exposure groups are clearly separated, suggesting that 2,6-DCBQ exposure causes changes in the transcriptome of T24 cells. Cluster analysis was performed on the differentially expressed genes of all samples. Firstly, the samples from each group were well clustered together. Additionally, we found that the direction of differential gene expression changes in Group L and Group M was similar, while it differed from the gene expression pattern in the highest dose Group H. Volcanoes plots were used to show the changes in gene expression under different doses of 2,6-DCBQ exposure. Compared to the control group, 963, 1180, and 979 genes were significantly up-regulated, and 366, 409, and 795 genes were significantly downregulated ($FDR < 0.05$, $|\log_2FC| > 1$) after exposure to low, intermediate, and high doses of 2,6-DCBQ, respectively. In summary, we obtained high-quality gene expression data of T24 cells through transcriptome sequencing, and found that 2,6-DCBQ expo-

sure leads to significant changes in the transcriptome of T24 cells.

3.4. Exposure to 2,6-DCBQ Induces Upregulation of Mitophagy-Related Genes and Downregulation of Insulin Signaling Pathway Genes

To further investigate the alterations in biological functions of T24 cells after exposure to DCBQ, we conducted GO functional classification annotation and KEGG pathway analysis on the differentially expressed genes of T24 cells after exposure to 2,6-DCBQ. In the GO functional classification annotation, the upregulated differentially expressed genes in Group L, M, and H were enriched in 304, 261, and 310 gene-related functions, respectively, while the downregulated differentially expressed genes were enriched in 273, 281, and 339 gene-related functions, respectively. Venn diagrams were used to statistically analyze the common gene-related functions enriched by the upregulated and downregulated differentially expressed genes in the three exposure groups. Among them, there were 42 common gene-related functions enriched by the upregulated differentially expressed genes in the three exposure groups, and 49 common gene-related functions enriched by the downregulated differentially expressed genes. Among the 42 common gene-related functions enriched by the upregulated differentially expressed genes, we identified multiple functions related to mitochondrial autophagy, such as mitochondrial autophagy, positive regulation of autophagy, autophagosome, autophagosome assembly, macroautophagy, and autophagy. Among the 49 common gene-related functions enriched by the downregulated differentially expressed genes, we identified multiple functions related to insulin, such as insulin-like growth factor I binding, insulin-like growth factor II binding, regulation of the insulin-like growth factor receptor signaling pathway, negative regulation of the insulin-like growth factor receptor signaling pathway, positive regulation of the insulin-like growth factor receptor signaling pathway, and proliferation of type B pancreatic cells.

Similarly, in the KEGG pathway analysis, the upregulated differential genes in Group L, Group M, and Group H enriched 24, 25, and 31 gene-related pathways, respectively, while the downregulated differential genes enriched 24, 25, and 21 gene-related pathways, respectively. Venn diagrams were used to statistically analyze the common gene-related pathways enriched by the upregulated and downregulated differential genes in the three exposure groups. Among them, the three exposure groups had 6 common gene-related pathways enriched by the upregulated differential genes and 3 common gene-related pathways enriched by the downregulated differential genes. The common gene-related pathways enriched by the upregulated differential genes included mitochondrial autophagy-animal, ferroptosis, autophagy-animal, ABC transporter, ECM-receptor interaction, complement, and coagulation cascade, while the common gene-related pathways enriched by the downregulated differential genes included mitochondrial autophagy-animal, autophagy-animal, and pathogenic *Escherichia coli* infection. In summary, through GO functional enrichment analysis and KEGG pathway anal-

ysis, we found that after exposure to 2,6-DCBQ, the expression of mitochondrial autophagy-related genes in T24 cells was upregulated, while the expression of insulin-related genes was downregulated (Figure 2).

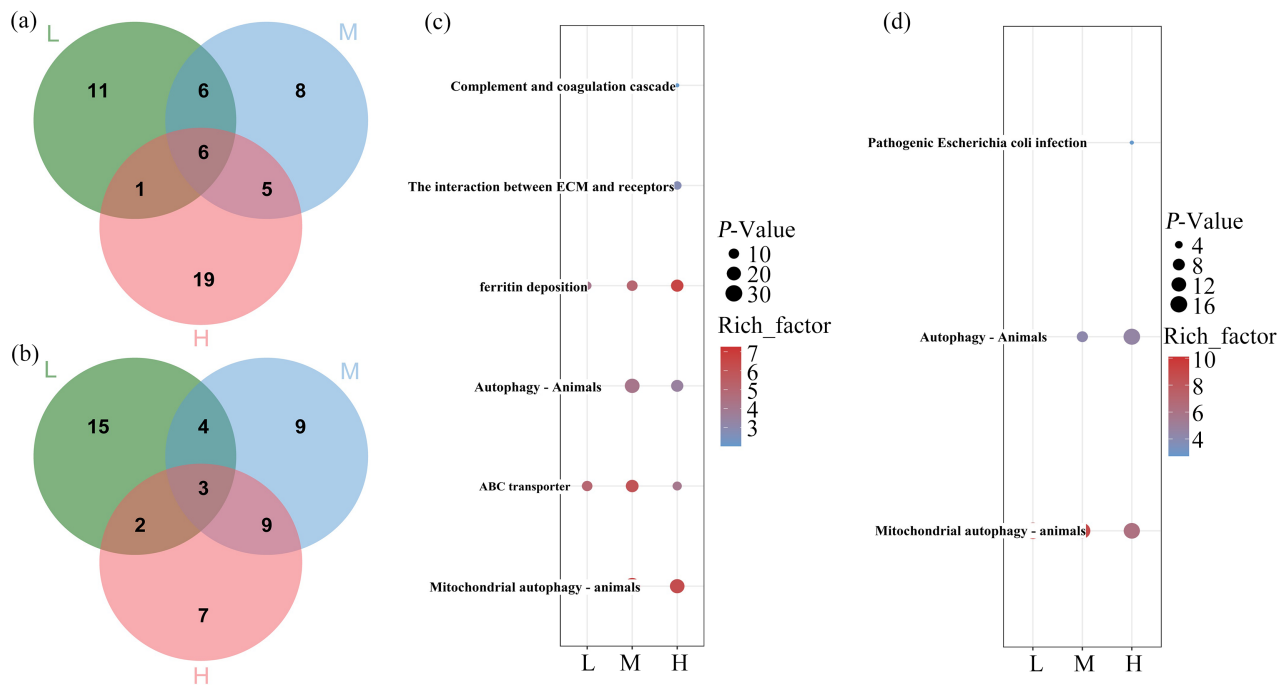


Figure 2. GO functional enrichment analysis of each dose group. Note: (a) represents the Venn diagram for the statistical analysis of the number of KEGG pathways associated with upregulated differential genes across various dose groups. (b) represents the Venn diagram for the statistical analysis of the number of KEGG pathways associated with downregulated differential genes across various dose groups. (c) represents the KEGG pathway analysis diagram for upregulated differential genes across various dose groups. (d) represents the KEGG pathway analysis diagram for downregulated differential genes across various dose groups. L: Low-dose 2,6-DCBQ exposure group; M: Medium-dose 2,6-DCBQ exposure group; H: High-dose 2,6-DCBQ exposure group.

4. Discussion

It is important to note that these findings are derived from a bladder cancer cell line; extrapolation to normal bladder tissue or human health outcomes requires further validation.

Given the high detection rate and high toxicity of 2,6-DCBQ, its potential harm to human health cannot be ignored. In this study, we established a cell exposure model using human bladder cancer T24 cells and systematically evaluated the toxic effects of 2,6-DCBQ. Our results demonstrated that 2,6-DCBQ significantly reduces T24 cell viability in a dose-dependent manner, induces oxidative damage at high doses, and profoundly alters the transcriptomic profile even at lower doses where direct lipid peroxidation was not detected. Through bioinformatic analysis, we identified that mitophagy-related pathways were abnormally upregulated, while the insulin signaling pathway was downregulated, providing new insights into the molecular mechanisms underlying 2,6-DCBQ toxicity.

First, we determined the IC₅₀ of 2,6-DCBQ on T24 cells to be 114.0 (109.6 - 118.0) μ M after 24 h of exposure, which is close to the findings reported by Du *et*

al. [23]. Based on this result, three exposure concentrations (25, 50, and 100 μM) were selected to represent low, medium, and high doses. LDH release assays further confirmed a dose-dependent increase in cytotoxicity. These results indicate that T24 cells are sensitive to 2,6-DCBQ, suggesting that exposure to this disinfection by-product may pose a risk to bladder health. The consistency of our cytotoxicity data with previous studies [23] supports the reliability of our experimental model.

Oxidative stress is considered a major mechanism underlying the toxicity of many environmental pollutants, including 2,6-DCBQ [24]–[26]. To evaluate oxidative damage, we measured the level of malondialdehyde (MDA) adducts, a marker of lipid peroxidation. Interestingly, a significant increase in MDA was observed only in the high-dose group (100 μM), but not in the low- or medium-dose groups. This finding suggests that 2,6-DCBQ may preferentially induce DNA or protein damage rather than lipid peroxidation at lower concentrations. As discussed by previous researchers, MDA is not always the most sensitive biomarker for all types of oxidative stress [27]. Therefore, future studies should consider measuring 8-hydroxy-2'-deoxyguanosine (8-OHdG) for DNA oxidative damage or protein carbonyl content for protein damage to better capture the full spectrum of 2,6-DCBQ-induced oxidative injury.

Transcriptome sequencing is a powerful tool to uncover the molecular events triggered by exogenous chemicals [28]. Our RNA-seq analysis revealed that 2,6-DCBQ exposure led to marked changes in the transcriptomic landscape of T24 cells. Principal component analysis clearly separated control and treated groups, and clustering analysis showed that the low- and medium-dose groups exhibited similar gene expression patterns, while the high-dose group displayed a distinct profile. This divergence may indicate that under higher concentrations, surviving T24 cells activate adaptive or tolerance responses, leading to a different transcriptional program.

GO and KEGG enrichment analyses of differentially expressed genes provided functional insights. Notably, genes involved in mitophagy (mitochondrial autophagy) were consistently upregulated across all exposure groups. Mitophagy is a selective autophagic process that eliminates damaged or dysfunctional mitochondria, thereby maintaining cellular energy homeostasis and redox balance [29]. However, excessive or dysregulated mitophagy can be detrimental. It may reduce mitochondrial quality and quantity, impair energy supply, and trigger the release of mitochondrial DNA and other pro-inflammatory components, leading to cellular dysfunction, inflammatory responses, or even cell death [29]–[32]. Moreover, abnormal mitophagy can influence apoptosis and necrosis pathways, altering the mode of cell death [33]. The upregulation of mitophagy-related genes in our study suggests that excessive mitophagy may contribute to the cytotoxic effects of 2,6-DCBQ in T24 cells. This is consistent with a previous study by Liu *et al.*, who reported that 2,6-DCBQ induced oxidative stress and promoted the mitochondrial apoptosis pathway in human neuroblastoma SH-SY5Y cells [24].

In contrast, genes related to the insulin signaling pathway were significantly downregulated in all treatment groups. The insulin signaling pathway plays a central role in regulating glucose, lipid, and protein metabolism, as well as cell growth and differentiation [34]. Inhibition of this pathway is frequently observed upon exposure to oxidative stress-inducing agents, such as arsenic [35]. Mechanistically, oxidative stress can promote serine/threonine phosphorylation of insulin receptor substrate (IRS) proteins, reducing tyrosine kinase activity of the insulin receptor and impairing downstream signal transduction [36]. It can also activate stress-sensitive kinases (e.g., PKC, JNK, NF- κ B, IKK), which interfere with key mediators like PI3K, Akt, mTOR, and FOXO [37]. Furthermore, oxidative stress reduces the expression and membrane translocation of GLUT4, decreasing insulin-stimulated glucose uptake [38], and increases lipid metabolites such as DAG and ceramide, which further inhibit insulin signaling and promote lipolysis and fatty liver [39]. Thus, the downregulation of insulin-related genes observed in our study is likely a consequence of 2,6-DCBQ-induced oxidative stress, and this may represent a key pathway linking DBP exposure to metabolic dysregulation. Supporting this notion, Yang *et al.* also found that 2,6-DCBQ exposure in zebrafish embryos led to significant downregulation of insulin-related signaling pathways [40].

This study has several limitations. First, all experiments were conducted in the T24 bladder cancer cell line; normal bladder epithelial cells or *in vivo* models are needed to confirm the physiological relevance of our findings. Second, the tested concentrations, while informative for mechanistic studies, are higher than typical environmental levels of 2,6-DCBQ. Third, our conclusions regarding “mitophagy activation” and “insulin signaling suppression” are based on transcriptomic enrichment analyses and lack direct functional validation (e.g., measurement of autophagic flux, mitochondrial membrane potential, or insulin-stimulated glucose uptake). Therefore, our wording has been carefully chosen to reflect upregulation of mitophagy-related genes and downregulation of insulin pathway genes rather than confirmed functional activation or suppression. Future studies employing pharmacological inhibitors, gene knockdown, or rescue experiments are warranted to establish causality.

In summary, this study provides comprehensive toxicological data on 2,6-DCBQ using the human bladder cancer cell line T24. We demonstrate that 2,6-DCBQ reduces cell viability and increases mortality in a dose-dependent manner, with high-dose exposure inducing oxidative damage. Transcriptomic analysis further reveals that 2,6-DCBQ dysregulates gene expression, leading to abnormal upregulation of mitophagy-related genes and suppression of the insulin signaling pathway. These findings advance our understanding of the molecular mechanisms underlying 2,6-DCBQ toxicity in bladder cancer cells and highlight potential biomarkers and therapeutic targets. Given the widespread presence of 2,6-DCBQ in drinking water, our results also provide scientific evidence to support future regulatory risk assessments and policy formulation, while recognizing the need for validation in normal cells and at environmentally relevant concentrations.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

References

- [1] Adusei-Gyamfi, J., Ouddane, B., Rietveld, L., Cornard, J. and Criquet, J. (2019) Natural Organic Matter-Cations Complexation and Its Impact on Water Treatment: A Critical Review. *Water Research*, **160**, 130-147.
<https://doi.org/10.1016/j.watres.2019.05.064>
- [2] Forster, A.L.B., Wiskur, S.L. and Richardson, S.D. (2025) Formation of Eight Classes of DBPs from Chlorine, Chloramine, and Ozone: Mechanisms and Formation Pathways. *Environmental Science & Technology*, **59**, 15594-15611.
<https://doi.org/10.1021/acs.est.5c02208>
- [3] Koley, S., Dash, S., Khwairakpam, M. and Kalamdhad, A.S. (2024) Perspectives and Understanding on the Occurrence, Toxicity and Abatement Technologies of Disinfection By-Products in Drinking Water. *Journal of Environmental Management*, **351**, Article ID: 119770. <https://doi.org/10.1016/j.jenvman.2023.119770>
- [4] Marumure, J., Simbanegavi, T.T., Makuvara, Z., Karidzagundi, R., Alufasi, R., Goredema, M., *et al.* (2024) Emerging Organic Contaminants in Drinking Water Systems: Human Intake, Emerging Health Risks, and Future Research Directions. *Chemosphere*, **356**, Article ID: 141699.
<https://doi.org/10.1016/j.chemosphere.2024.141699>
- [5] Kujlu, R., Mahdavianpour, M. and Ghanbari, F. (2020) Multi-Route Human Health Risk Assessment from Trihalomethanes in Drinking and Non-Drinking Water in Abadan, Iran. *Environmental Science and Pollution Research*, **27**, 42621-42630.
<https://doi.org/10.1007/s11356-020-09990-9>
- [6] Zhang, D., Dong, S., Chen, L., Xiao, R. and Chu, W. (2023) Disinfection By-products in Indoor Swimming Pool Water: Detection and Human Lifetime Health Risk Assessment. *Journal of Environmental Sciences*, **126**, 378-386.
<https://doi.org/10.1016/j.jes.2022.05.003>
- [7] Helte, E., Söderlund, F., Säve-Söderbergh, M., Larsson, S.C. and Åkesson, A. (2025) Exposure to Drinking Water Trihalomethanes and Risk of Cancer: A Systematic Review of the Epidemiologic Evidence and Dose-Response Meta-Analysis. *Environmental Health Perspectives*, **133**, Article ID: 16001.
<https://doi.org/10.1289/ehp14505>
- [8] Helte, E., Säve-Söderbergh, M., Ugge, H., Fall, K., Larsson, S.C. and Åkesson, A. (2022) Chlorination By-Products in Drinking Water and Risk of Bladder Cancer—A Population-Based Cohort Study. *Water Research*, **214**, Article ID: 118202.
<https://doi.org/10.1016/j.watres.2022.118202>
- [9] Deiana, G., Filippini, T., Dettori, M., Vinceti, M. and Azara, A. (2025) Exposure to Disinfection By-Products and Risk of Birth Defects: A Systematic Review and Dose-Response Meta-Analysis. *Science of The Total Environment*, **985**, Article ID: 179693.
<https://doi.org/10.1016/j.scitotenv.2025.179693>
- [10] Kancherla, V., Rhoads, A., Conway, K.M., Suhl, J., Langlois, P.H., Hoyt, A.T., *et al.* (2024) Maternal Periconceptional Exposure to Drinking Water Disinfection By-products and Neural Tube Defects in Offspring. *Birth Defects Research*, **116**, e2370.
<https://doi.org/10.1002/bdr2.2370>
- [11] Fernández-Pascual, E., Droz, B., O'Dwyer, J., O'Driscoll, C., Goslan, E.H., Harrison, S., *et al.* (2023) Fluorescent Dissolved Organic Matter Components as Surrogates for

- Disinfection By-product Formation in Drinking Water: A Critical Review. *ACS ES&T Water*, **3**, 1997-2008. <https://doi.org/10.1021/acsestwater.2c00583>
- [12] Dai, P., Kaplan-Bekaroglu, S.S., Uzun, H. and Karanfil, T. (2026) From the Source to Tap: Exploring the Nationwide Occurrence and Calculated Cytotoxicity of Regulated and Unregulated DBPs in U.S. Water Systems. *Environmental Science & Technology*, **60**, 9481-9495. <https://doi.org/10.1021/acs.est.5c13253>
- [13] Allen, J.M., Plewa, M.J., Wagner, E.D., Wei, X., Bokenkamp, K., Hur, K., *et al.* (2022) Drivers of Disinfection By-product Cytotoxicity in U.S. Drinking Water: Should Other DBPs Be Considered for Regulation? *Environmental Science & Technology*, **56**, 392-402. <https://doi.org/10.1021/acs.est.1c07998>
- [14] Li, X. and Mitch, W.A. (2018) Drinking Water Disinfection Byproducts (dbps) and Human Health Effects: Multidisciplinary Challenges and Opportunities. *Environmental Science & Technology*, **52**, 1681-1689. <https://doi.org/10.1021/acs.est.7b05440>
- [15] Tu, N., Liu, H., Li, W., Yao, S., Liu, J., Guo, Z., *et al.* (2022) Quantitative Structure-Toxicity Relationships of Halobenzoquinone Isomers on DNA Reactivity and Genotoxicity. *Chemosphere*, **309**, Article ID: 136763. <https://doi.org/10.1016/j.chemosphere.2022.136763>
- [16] Li, J., Wang, W., Moe, B., Wang, H. and Li, X. (2015) Chemical and Toxicological Characterization of Halobenzoquinones, an Emerging Class of Disinfection By-products. *Chemical Research in Toxicology*, **28**, 306-318. <https://doi.org/10.1021/tx500494r>
- [17] Zhao, Y., Anichina, J., Lu, X., Bull, R.J., Krasner, S.W., Hrudey, S.E., *et al.* (2012) Occurrence and Formation of Chloro- and Bromo-Benzoquinones during Drinking Water Disinfection. *Water Research*, **46**, 4351-4360. <https://doi.org/10.1016/j.watres.2012.05.032>
- [18] Wu, H., Long, K., Sha, Y., Lu, D., Xia, Y., Mo, Y., *et al.* (2021) Occurrence and Toxicity of Halobenzoquinones as Drinking Water Disinfection By-products. *Science of the Total Environment*, **770**, Article ID: 145277. <https://doi.org/10.1016/j.scitotenv.2021.145277>
- [19] Wang, Y., Wang, F., Li, L., Zhang, L., Song, M. and Jiang, G. (2024) Comprehensive Toxicological Assessment of Halobenzoquinones in Drinking Water at Environmentally Relevant Concentration. *Environmental Science & Technology*, **58**, 9125-9134. <https://doi.org/10.1021/acs.est.4c03308>
- [20] Xiao, C., Wang, C., Zhang, Q., Yang, X., Huang, S., Luo, Y., *et al.* (2021) Transcriptomic Analysis of Adult Zebrafish Heart and Brain in Response to 2, 6-Dichloro-1, 4-Benzoquinone Exposure. *Ecotoxicology and Environmental Safety*, **226**, Article ID: 112835. <https://doi.org/10.1016/j.ecoenv.2021.112835>
- [21] Wu, W., Liu, Y., Li, C., Zhuo, F., Xu, Z., Hong, H., *et al.* (2022) Oxidative Stress Responses and Gene Transcription of Mice under Chronic-Exposure to 2,6-dichlorobenzoquinone. *International Journal of Environmental Research and Public Health*, **19**, Article 13801. <https://doi.org/10.3390/ijerph192113801>
- [22] Chomczynski, P. and Sacchi, N. (1987) Single-step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. *Analytical Biochemistry*, **162**, 156-159. [https://doi.org/10.1016/0003-2697\(87\)90021-2](https://doi.org/10.1016/0003-2697(87)90021-2)
- [23] Du, H., Li, J., Moe, B., McGuigan, C.F., Shen, S. and Li, X. (2013) Cytotoxicity and Oxidative Damage Induced by Halobenzoquinones to T24 Bladder Cancer Cells. *Environmental Science & Technology*, **47**, 2823-2830. <https://doi.org/10.1021/es303762p>

- [24] Liu, T., Chen, X., Li, W., Zhang, X., Wang, G., Wang, J., *et al.* (2023) Oxidative Stress as a Key Event in 2,6-Dichloro-1,4-Benzoquinone-Induced Neurodevelopmental Toxicity. *Ecotoxicology and Environmental Safety*, **263**, Article ID: 115357. <https://doi.org/10.1016/j.ecoenv.2023.115357>
- [25] Wang, C., Yang, X., Zheng, Q., Moe, B. and Li, X. (2018) Halobenzoquinone-induced Developmental Toxicity, Oxidative Stress, and Apoptosis in Zebrafish Embryos. *Environmental Science & Technology*, **52**, 10590-10598. <https://doi.org/10.1021/acs.est.8b02831>
- [26] Liu, T., Wang, J., Dang, X., Wan, S., Luo, X., Tang, W., *et al.* (2023) Investigation of the Nephrotoxicity of 2,6-Dichloro-1,4-Benzoquinone Disinfection By-Product in Mice through a 28-Day Toxicity Test. *Toxicology*, **487**, Article ID: 153459. <https://doi.org/10.1016/j.tox.2023.153459>
- [27] Poljšak, B., Jamnik, P. and Milisav, I. (2025) The Importance of Multifaceted Approach for Accurate and Comprehensive Evaluation of Oxidative Stress Status in Biological Systems. *Antioxidants*, **14**, Article 1083. <https://doi.org/10.3390/antiox14091083>
- [28] Meier, M.J., Harrill, J., Johnson, K., Thomas, R.S., Tong, W., Rager, J.E., *et al.* (2025) Progress in Toxicogenomics to Protect Human Health. *Nature Reviews Genetics*, **26**, 105-122. <https://doi.org/10.1038/s41576-024-00767-1>
- [29] Wang, Q., Sun, Y., Li, T.Y. and Auwerx, J. (2026) Mitophagy in the Pathogenesis and Management of Disease. *Cell Research*, **36**, 11-37. <https://doi.org/10.1038/s41422-025-01203-7>
- [30] Newman, L.E. and Shadel, G.S. (2023) Mitochondrial DNA Release in Innate Immune Signaling. *Annual Review of Biochemistry*, **92**, 299-332. <https://doi.org/10.1146/annurev-biochem-032620-104401>
- [31] Liao, S., Luo, J., Kadier, T., Ding, K., Chen, R. and Meng, Q. (2022) Mitochondrial DNA Release Contributes to Intestinal Ischemia/Reperfusion Injury. *Frontiers in Pharmacology*, **13**, Article 854994. <https://doi.org/10.3389/fphar.2022.854994>
- [32] Li, S., Zhang, J., Liu, C., *et al.* (2021) The Role of Mitophagy in Regulating Cell Death. *Oxidative Medicine and Cellular Longevity*, **2021**, Article ID: 6617256.
- [33] Chu, T., Huang, Z. and Ma, W. (2025) Mitophagy: A Double-Edged Sword in Tumor Cell Death Regulation and Therapeutic Response. *Biochemical and Biophysical Research Communications*, **777**, Article ID: 152254. <https://doi.org/10.1016/j.bbrc.2025.152254>
- [34] Burchfield, J.G., Diaz-Vegas, A. and James, D.E. (2025) The Insulin Signalling Network. *Nature Metabolism*, **7**, 1745-1764. <https://doi.org/10.1038/s42255-025-01349-z>
- [35] Bibha, K., Akhigbe, T.M., Hamed, M.A. and Akhigbe, R.E. (2024) Metabolic Derangement by Arsenic: A Review of the Mechanisms. *Biological Trace Element Research*, **202**, 1972-1982. <https://doi.org/10.1007/s12011-023-03828-4>
- [36] Liu, Y., Wang, W., Liang, B., Zou, Z. and Zhang, A. (2025) NLRP3 Inflammasome Activation and Disruption of IRS-1/PI3K/AKT Signaling: Potential Mechanisms of Arsenic-Induced Pancreatic Beta Cells Dysfunction in Rats. *Ecotoxicology and Environmental Safety*, **289**, Article ID: 117504. <https://doi.org/10.1016/j.ecoenv.2024.117504>
- [37] Newsholme, P., Keane, K.N., Carlessi, R. and Cruzat, V. (2019) Oxidative Stress Pathways in Pancreatic β -Cells and Insulin-Sensitive Cells and Tissues: Importance to Cell Metabolism, Function, and Dysfunction. *American Journal of Physiology-Cell Physiology*, **317**, C420-C433. <https://doi.org/10.1152/ajpcell.00141.2019>

- [38] Singh, A., Kukreti, R., Saso, L. and Kukreti, S. (2022) Mechanistic Insight into Oxidative Stress-Triggered Signaling Pathways and Type 2 Diabetes. *Molecules*, **27**, Article 950. <https://doi.org/10.3390/molecules27030950>
- [39] Jani, S., Da Eira, D., Hadday, I., Bikopoulos, G., Mohasses, A., de Pinho, R.A., *et al.* (2021) Distinct Mechanisms Involving Diacylglycerol, Ceramides, and Inflammation Underlie Insulin Resistance in Oxidative and Glycolytic Muscles from High Fat-Fed Rats. *Scientific Reports*, **11**, Article No. 19160. <https://doi.org/10.1038/s41598-021-98819-7>
- [40] Yang, X., Wang, C., Yang, L., Zheng, Q., Liu, Q., Wawryk, N.J.P., *et al.* (2022) Neurotoxicity and Transcriptome Changes in Embryonic Zebrafish Induced by Halobenzoquinone Exposure. *Journal of Environmental Sciences*, **117**, 129-140. <https://doi.org/10.1016/j.jes.2022.03.042>