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Research Article

Anti-aging effects of deuterium-depleted water on Mn-induced toxicity in the Fibroblast cell model

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ABSTRACT

Objectives: In recent decades, deuterium-depleted water (DDW) has been explored as a supplement for enhancing longevity. This study aimed to investigate the effects of DDW on aging-related factors, including the FOXO gene family and oxidative stress. Additionally, the study examined the impact of DDW on cellular toxicity induced by manganese, a heavy metal.

Methods: The HNNFPi8 cell line was used as an experimental model and incubated in specific DDW media with deuterium concentrations of 30 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm, and 150 ppm, along with 0.01 to 5 mM MnCl2 for up to 72 hours. Cell proliferation and the activities of catalase and superoxide dismutase (SOD) antioxidants were assessed using MTT and colorimetric methods, respectively. RT-PCR was employed to measure FOXO3A gene expression.

Results: The increase in $MnCl_2$ concentration resulted in dose-dependently reduction in the viability of the cells. However, the decrease in the cell viability in the treated groups with DDW was found to be significantly lower in concentrations of 50ppm to 125 ppm DDW. DDW at concentrations of 100 and 125 ppm could significantly upregulate the expression of FOXO3A gene in the cells treated with different concentrations of $MnCl_2$. In addition, DDW at concentrations of 75, 100 and 125 ppm were able to increase the activities of antioxidant enzymes in the cells treated with different concentrations of $MnCl_2$.

Conclusion: The findings suggest that DDW, particularly at concentrations of 100 and 125 ppm, is effective in mitigating the toxic effects of $MnCl_2$.

Keywords: Deuterium depleted water, FOXO genes family, oxidative stress, manganese, aging

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Introduction

The adult human body is composed of approximately 60–70% water, and with aging, this percentage gradually declines. Water plays a crucial role in cellular metabolism and serves as a medium for various enzymatic and chemical reactions (1).

Significant attention has been given to the isotopic composition of water. Hydrogen (H) has three naturally occurring isotopes: ^{1}H, ^{2}H (deuterium), and ^{3}H (tritium) (2). Deuterium is a natural, stable, and non-radioactive isotope of hydrogen, with a concentration of approximately 150 ppm in natural water. Due to the substantial mass difference between hydrogen and deuterium, their chemical and physical properties vary significantly. High concentrations of deuterium in water lead to the formation of heavy water, which has harmful effects on the environment and living organisms (2).

In this regard, Somlyai et al. first reported in 1993 that depleting deuterium in water affects living organisms (3). In vitro studies have shown that deuterium depletion induces apoptosis, exhibits anti-proliferative effects on tumor cells, and influences the functions of tumor suppressor genes and proto-oncogenes. It may also weaken the expression of genes activated by carcinogens. Additionally, a relationship has been identified between aging and deuterium concentration (4, 5). Notably, the effects of lower natural deuterium concentrations on longevity present an intriguing avenue for further investigation (6).

Human longevity is a complex phenotype influenced by multiple genetic and environmental factors. Extensive research has demonstrated that the aging process is largely genetically regulated. Variations in single genes or molecular pathways can significantly affect lifespan. One key example is the insulin/IGF-1 signaling pathway. Mutations that either enhance SIR-2 activity or reduce insulin/IGF-1 signaling have been shown to extend the lifespan of C. elegans by activating the DAF-16/FOXO protein (7).

Sirtuin1 (SIRT1), an NAD-dependent protein deacetylase, plays a crucial role in aging by modulating several downstream transcription factors, including the Forkhead box (FOXO) family. Mammals have four FoxO isoforms: FoxO1, FoxO3, FoxO4, and FoxO6. FoxO1, FoxO3, and FoxO4 are regulated through Akt-dependent phosphorylation at three specific sites in response to growth factor and insulin stimulation (8). FoxO factors have emerged as a critical convergence point for signaling pathways involved in growth stimulation and oxidative stress responses (9).

Manganese (Mn) is a naturally occurring trace element essential for various biochemical reactions in cells (10). As a vital micronutrient, Mn is required for several physiological and homeostatic processes, including reproduction, connective tissue and bone formation, carbohydrate and lipid metabolism, and brain function (11).

Within cells, Mn preferentially accumulates in mitochondria, where it disrupts ATP generation via oxidative phosphorylation and contributes to reactive oxygen species (ROS) production (12). Excessive ROS generation can lead to oxidative damage, including membrane polyunsaturated fatty acid oxidation, resulting in various lipid peroxidation end products.

Material and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA), dimethyl sulfoxide (DMSO), and 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide (MTT) were purchased from Sigma-Aldrich. Deuterium-depleted water (DDW) was obtained from the Atomic Energy Organization of Iran (Tehran, Iran). The HNNFPi8 cell lines were acquired from the Pasteur Institute of Iran.

Cell culture

The HNNFPi8 cell line was cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics. The cells were incubated at 37°C with 5% CO_2, and the medium was changed every 24–48 hours. Once the cells reached 70% confluency, 0.25% trypsin was used to detach them from the flask. After washing with complete media, the cells were transferred to a 6-well plate. Fibroblast-containing wells were treated with complete media, and 24 hours later, each well received specific DDW media with deuterium concentrations of 30 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm, and 150 ppm.

Mn Chloride Treatments

$MnCl_2$ stock solution was freshly prepared at a concentration of 10 mM in serum-free medium and diluted to final concentrations ranging from 0.01 to 5.0 mM. The cells were incubated in the presence or absence of $MnCl_2$ at 37°C with 5% CO_2 in a humidified atmosphere for durations of 24 to 96 hours.

MTT-based cytotoxicity assay

HNNFPi8 cells were seeded in 96-well plates at a density of 5,000 cells per well in 100 µL DMEM medium supplemented with 10% FBS and gentamicin. The following day, the cells were treated for 24, 48, and 72 hours with DDW containing deuterium concentrations of 30 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm, and 150 ppm, either alone or in combination with $MnCl_2$ concentrations

of 0.0 mM, 0.1 mM, 0.5 mM, 1 mM, and 5 mM.

After 24, 48, and 72 hours, 20 µL of MTT (5 mg/mL) was added to each well, and plates were incubated for an additional four hours. The remaining supernatant was removed, and 200 µL of DMSO was added to dissolve the insoluble formazan crystals. Absorbance was immediately recorded at 570 nm. The absolute absorbance values were converted to survival fraction data, representing the percentage of living cells relative to the control.

Antioxidant enzymes assay

Catalase (CAT) and superoxide dismutase (SOD) activities were determined in HNNFPi8 cell hemolysates using colorimetric methods.

Gene Expression assays

Reverse transcription PCR was used to measure FOXO3A gene expression. Each sample was normalized to glyceraldehyde3-phpsphate dehydrogenase (GAPDH) expression level. RNeasy Mini Kit from Qiagen was used to extract RNA from the cells by using the manufacture's protocol. A First Strand cDNA synthesis kit from the Thermo Scientific was used to transcribe the RNA into a cDNA according to the manufacturer's protocol. Primer sequences were: FOXO3A Forward: AGCCAGTCTATGCAAACCCT, FOXO3A Reverse: TTCCCCACGTTCAAACCAAC, GAPDH Forward: CAAGGTCATCCATGACAACTTTG, GAPDH Reverse: GTCCACCACCCTGTTGCTGTAG.

Amplification was performed for 3min at 95 °C and 30 cycles at 95 °C for 30s (denaturation), 61°C for 30s (annealing), and 72 °C for 1min (extension), finally for 5 min at 72 °C. The samples were analyzed on a 2% agarose gel electrophoresis and visualized by UVI-TEC.

Statistical Analysis

Statistical analysis was conducted using SPSS software, employing two-way ANOVA. A p-value of less than 0.05 was considered statistically significant. All data were expressed as mean ± standard deviation (SD).

Results

Evaluation of the cell viability by different concentrations of DDW and $MnCl_2$ in HNNFP-I8 cells

Figures 1–3 illustrate the percentage of cell viability at various DDW concentrations in the presence of different $MnCl_2$ concentrations over 24, 48, and 72 hours.

As shown in Figure 1, increasing $MnCl_2$ concentrations from 0.05 mM to 5 mM resulted in a dose-dependent reduction in cell viability. However, the decrease in cell viability in DDW-treated groups was significantly lower at concentrations ranging from 50 ppm to 125 ppm. Notably, 100 and 125 ppm DDW provided the highest protection against $MnCl_2$ toxicity. As shown in the Figure 1, groups (A-E) at a concentration of 150ppm deuterium plus various concentrations of the manganese chloride show the highest percentage of the cell death compared with the control group which were 14.25%, 24.12%, 26.16%, 20.4% and 25.02%, respectively.

Figure 2 presents cell viability following 48-hour treatment with DDW and various $MnCl_2$ concentrations. The results indicate that the lowest survival rate belongs to the 30ppm groups, while in the cells treated with 50 to 125 ppm of DDW, a steady increase in viability is observed and then, at a concentration of 150 ppm deuterium, we face a sudden reduction in the survival rate.

Figure 3 shows the effects of DDW and $MnCl_2$ treatments at 72 hours. In all concentrations of the $MnCl_2$, the highest percentage of the cell survival was associated with the cell groups treated with deuterium-depleted water at a concentration of 100ppm. Importantly, the strongest protective effect of DDW was observed in cells treated with 100 ppm.

The expression of FOXO3a gene in HNFFP-I8 cells treated with DDW

Figures 4 and 5 demonstrate that increasing $MnCl_2$ concentrations were associated with a significant reduction in FOXO3A gene expression. However, DDW at concentrations of 100 and 125 ppm significantly upregulated FOXO3A gene expression in cells exposed to different $MnCl_2$ concentrations.

The effects of DDW treatment on SOD and catalase activities

As shown in Figure 6, while 50 ppm DDW reduced the activities of SOD and catalase, DDW at concentrations of 75, 100, and 125 ppm enhanced the activity of these two antioxidant enzymes in cells treated with various concentrations of $MnCl_2$. Comparatively, 150 ppm DDW exhibited lower enzymatic activity than DDW at concentrations of 75, 100, and 125 ppm.

Discussion

Aging is a physiological process that leads to the gradual decline in tissue function. Several factors contribute to the aging process, including both genetic and non-genetic influences. Non-genetic factors such as diet, physical activity, healthy habits, and psychological well-being significantly impact lifespan. However, genetic factors play a dominant role in regulating aging (13).

Among genetic contributors, FOXO family transcription factors are directly and indirectly involved in aging. The role of FOXO genes in lifespan regulation has

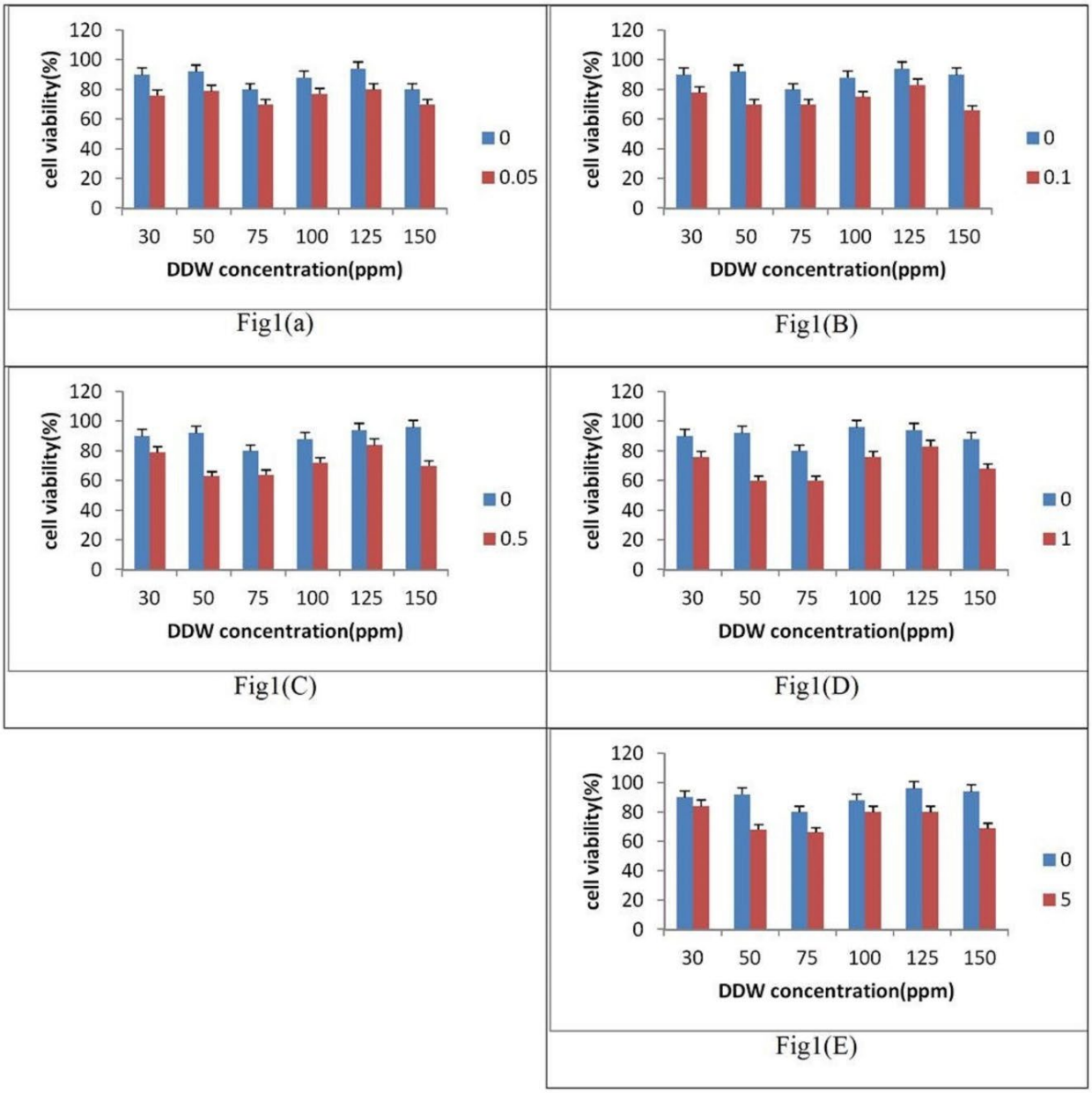


Figure 1: Cell survival analysis 24 hours after treatment of cells with DDW and $MnCl_2$. (A) Mn concentration of 0.05 M. (B) Concentration of 0.1mM $MnCl_2$. (C) Concentration of 0.5mM of $MnCl_2$. (D) Concentration of 1mM of $MnCl_2$. (E) Concentration of 5mM $MnCl_2$. Each column had an average of 3 independent experiments and the difference between groups was calculated using graph pad software, two ways ANOVA.

been demonstrated in multiple studies (14). It has been suggested that common variations in FOXO genes and their associated pathways can influence aging (15, 16). For instance, a study conducted on a group of Japanese men confirmed the association between FOXO3 gene variations and increased lifespan (15). Additionally, FOXO transcription factors regulate genes encoding superoxide dismutase and catalase, thereby reducing intracellular reactive oxygen species (ROS). Increased FOXO expression helps protect cells from oxidative damage induced by ROS (17). Collectively, previous studies suggest that two key pathways involved in aging include the FOXO3A gene and the antioxidant defense system.

Research has indicated that various compounds influence the aging process by modulating FOXO3A gene expression and antioxidant activity. Deuterium-depleted water is one such compound, with its potential effects on living organisms first reported in 1958. One

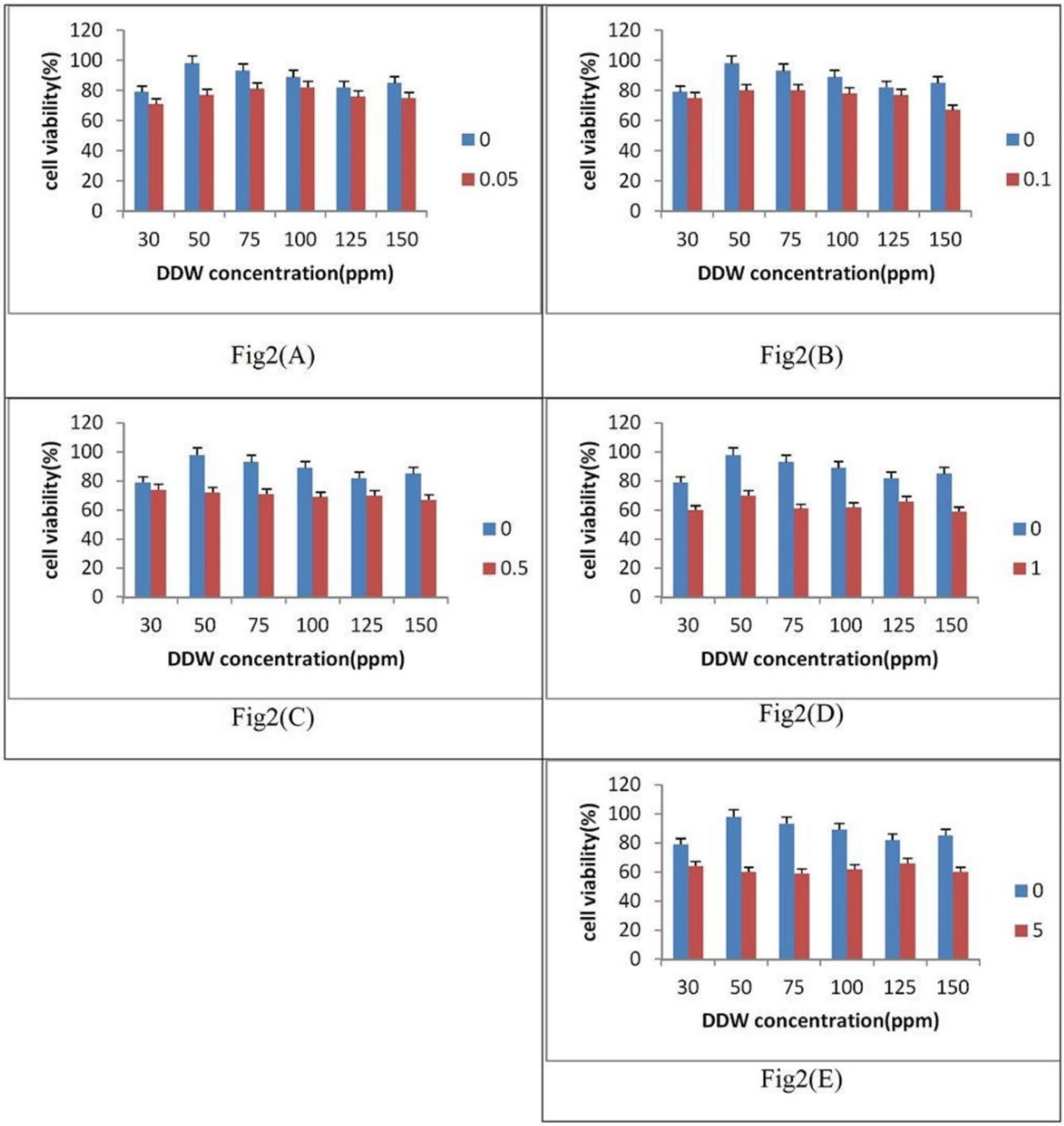


Figure 2: Evaluation of cell survival in 48 hours after treatment with DDW and $MnCl_2$. (A) Concentration of 0.05mM $MnCl_2$. (B) Concentration of 0.1mM $MnCl_2$. (C) Concentration of 0.5mM $MnCl_2$. (D) Concentration of 1mM of $MnCl_2$. (E) Concentration of mM5 $MnCl_2$. Each column had an average of 3 independent experiments and the difference between groups was calculated using graph pad software, two ways ANOVA.

study found that mice exhibited increased lifespan after consuming water with 30% deuterium depletion (18). Additionally, a study by Avila DS et al. demonstrated that lower deuterium levels mitigate the toxic effects of manganese in algae and C. elegans, effectively reversing longevity reduction (7).

In this study, we examined the effects of deuterium-depleted water (DDW) on the aging process and cellular toxicity induced by heavy metals in fibroblast cells. The HNFFP-I8 fibroblast cell line was selected as an appropriate model for aging studies (19), with manganese serving as an external stressor.

The results demonstrated that DDW exhibited no toxic effects on fibroblast cells. Cells were treated with varying concentrations of manganese chloride according to the established protocol. Morphological analysis confirmed

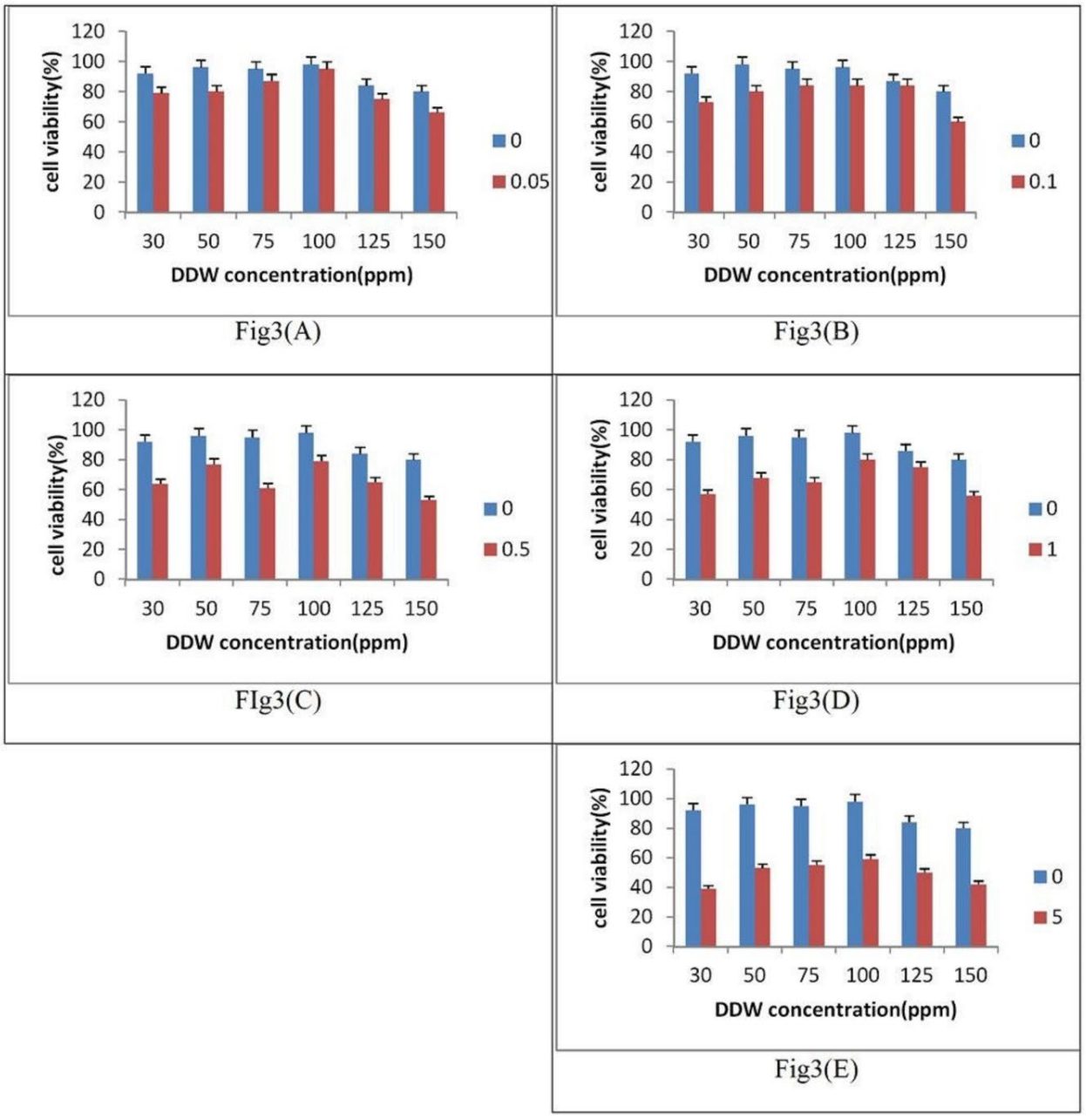


Figure 3: Cell survival evaluation in 72 hours after treatment of cells with DDW and $MnCl_2$. (A) concentration of 0.05mM manganese chloride. (B) Concentration of 0.1mM $MnCl_2$. (C) Concentration of 0.5mM of $MnCl_2$. (D) Concentration of 1mM of $MnCl_2$. (E) Concentration of mM 5 $MnCl_2$. Each column had an average of 3 independent experiments and the difference between groups was calculated using graph pad software, two ways ANOVA.

the toxic effects of manganese, with high concentrations (5 mM $MnCl_2$) leading to complete destruction of large cell colonies.

MTT assay results indicated that DDW at concentrations of 100 and 125 ppm significantly enhanced cell proliferation compared to other groups. The most notable effects of DDW were observed in $MnCl_2$-treated cells, where 100 and 125 ppm DDW markedly improved cell survival rates.

To further investigate the mechanism underlying DDW's effects on fibroblast cells, FOXO3A gene expression was assessed using RT-PCR. The findings revealed that DDW at 100 and 125 ppm significantly upregulated FOXO3A gene expression compared to other treatment groups. Additionally, antioxidant enzyme activity was evaluated by measuring superoxide dismutase (SOD) and catalase levels. The highest rates of cell proliferation were associated with increased antioxidant enzyme activity.

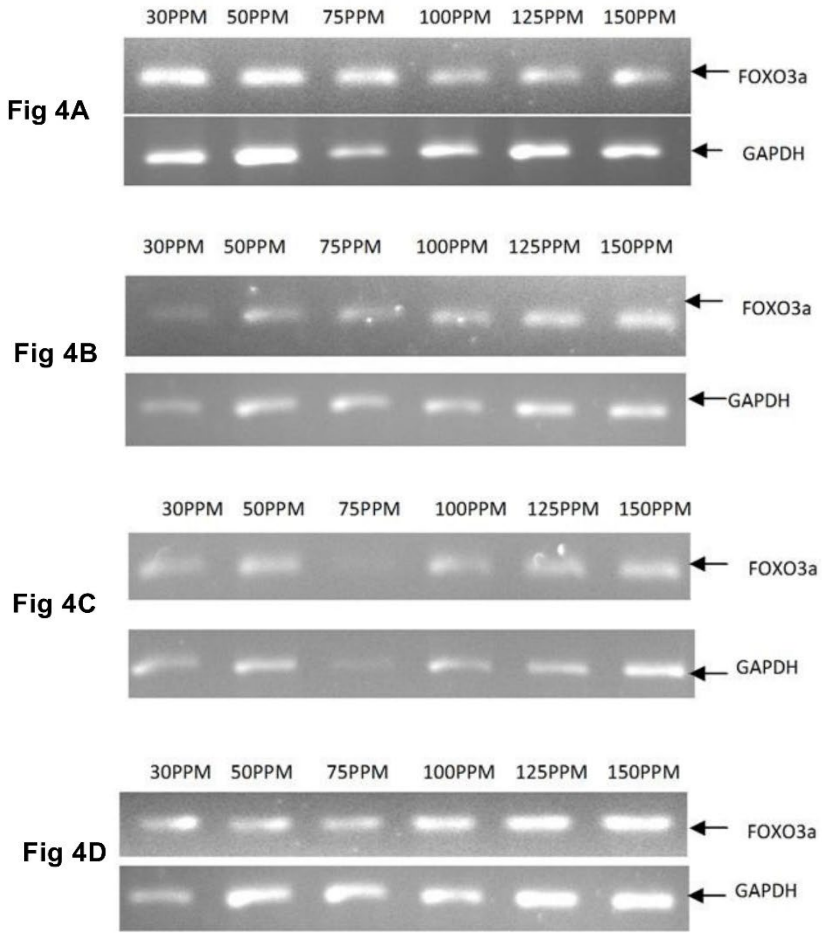


Figure 4: 4A FOXO3a gene expression in treated fibroblast cells with different concentrations of DDW and zero concentrations of $MnCl_2$ (GAPDH as an internal control), **4B:** Expression of FOXO3a gene in fibroblast cells treated with different concentrations of DDW with a concentration of 0.1mM $MnCl_2$. **4C:** Expression of FOXO3a gene in fibroblast cells treated with different concentrations of DDW with a concentration of 0.5mM $MnCl_2$. **4D:** Expression of FOXO3a gene in fibroblast cells treated with different concentrations of DDW with a concentration of 1mM MnCl2.

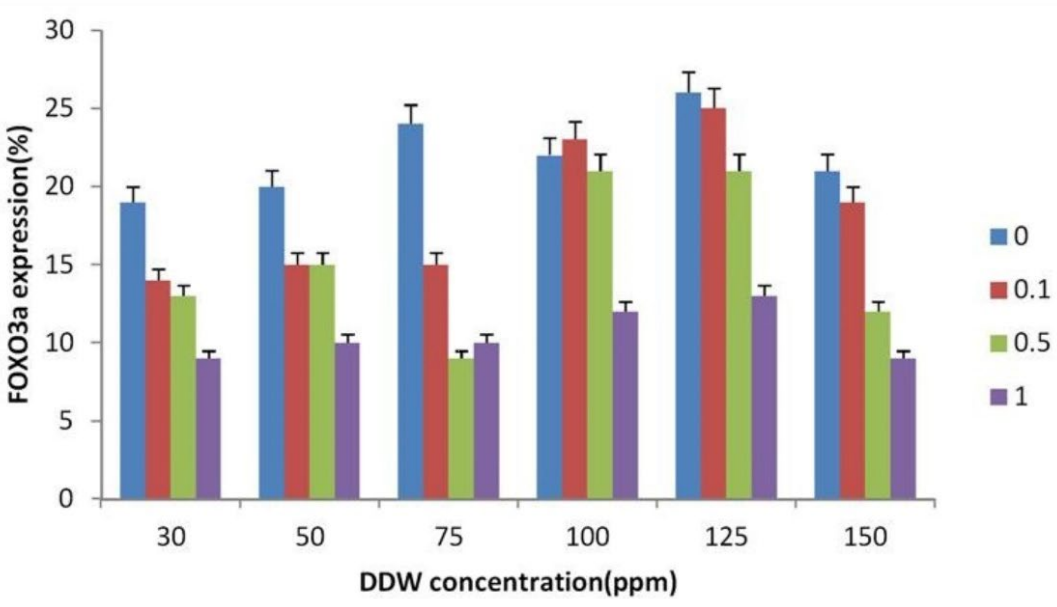


Figure 5: Expression of FOXO3a gene in fibroblast cells treated with different concentrations of DDW with concentrations of 0.1, 0.5 and 1mM of $MnCl_2$.

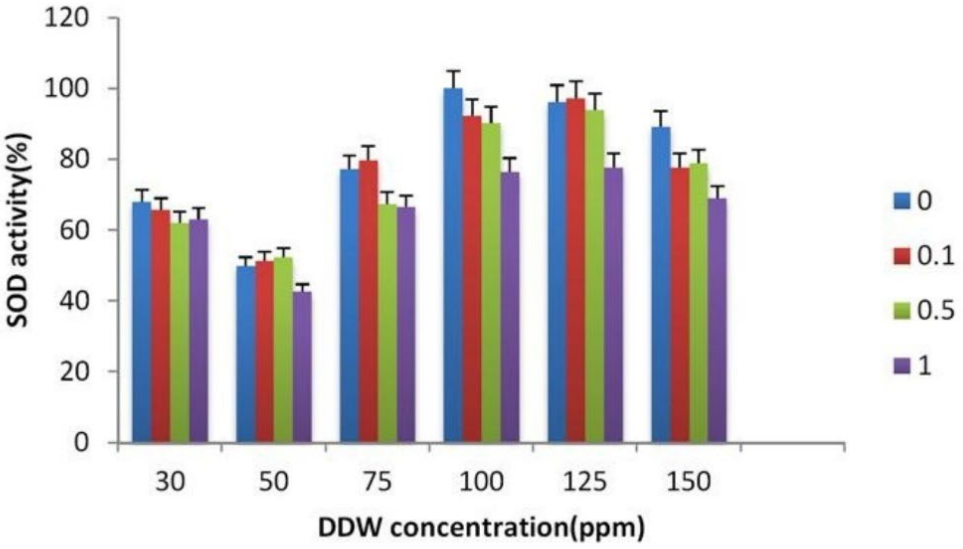


Figure 6: Percentage of SOD activity in fibroblast cells treated with different concentrations of DDW and 0, 0.1, 0.5 and 1mM of $MnCl_2$.

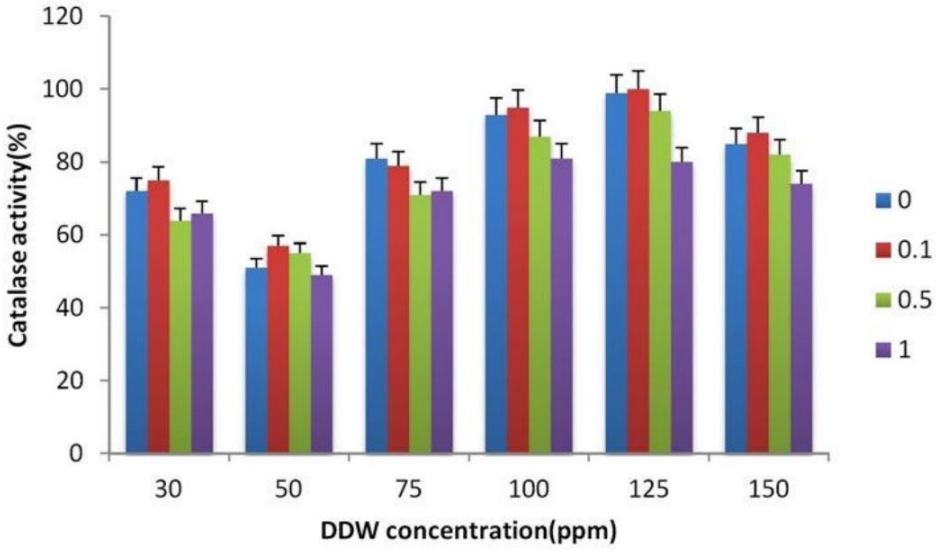


Figure 7: Percentage of Catalase activity in treated fibroblast cells with different concentrations of DDW and concentrations of 0, 0.1, 0.5 and 1mM $MnCl_2$.

Conclusion

In summary, 100 and 125 ppm DDW appear to be the optimal concentrations for promoting cell growth and offering protective effects against aging-related stressors. DDW at these concentrations enhances FOXO3A gene expression, a key regulator of cellular aging. The observed upregulation of FOXO3A is accompanied by increased antioxidant enzyme activity, which helps mitigate oxidative stress within cells.

Conflict of Interest

The authors have nothing to declare.

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