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A Study of Menadione-Induced Cytotoxicity Reduction in Yeast Cells by Selected Enzymes

Linda Yao 26 June 2010 5,503 views 10 Comments

Abstract:

The experiment tested whether Catalase and superoxide dismutase (SOD) would keep yeast cells exposed to menadione (Vitamin K3) alive by reducing the oxidative stress caused by menadione-induced cytotoxicity. Catalase catalyzes the decomposition of hydrogen peroxide into water and oxygen, while SOD converts superoxide anions into hydrogen peroxide and

oxygen. Because SOD produces additional hydrogen peroxide, Catalase was hypothesized to be more effective than SOD in reducing menadione-induced cytotoxicity. Throughout the experiment, method of transcriptional and translational (MTT) assays measured absorbances using a spectrophotometer at 570 nm to test the extent of menadione-induced cytotoxicity in yeast cells, or the cell viability, the determination of living or dead cells. Yeast cells with active mitochondria reduce MTT (3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) effectively into colored formazan crystals and have higher absorbances than yeast cells with mitochondria damaged by menadione. To determine the protective effect of SOD and Catalase, these enzymes were added to yeast suspensions before adding menadione. MTT assays were then conducted after menadione was added. In the experiment, the yeast suspensions exposed to menadione showed reduced cell viability (lower absorbances). When the menadione concentration was 0.025 mM, the addition of both SOD and Catalase decreased menadione-induced cytotoxicity. When the menadione concentrations were 0.1 mM and 0.5 mM, however, only Catalase significantly decreased cytotoxicity. This experiment supports the hypothesis that both SOD and Catalase reduce oxidative stress and consequently menadione-induced cytotoxicity, but Catalase is more effective than SOD is. This demonstrates the possibility of combating menadione-induced cytotoxicity and Vitamin K3 poisoning through the use of Catalase and SOD.

Introduction:

Menadione, known as Vitamin K3, is a synthetic Vitamin K supplement used to control blood clotting and synthesize the liver protein required for blood clotting. Vitamin K is also essential for bone repair, preventing osteoporosis; treating Vitamin K deficiency, preventing hemorrhage disease in newborns; stabilizing anticoagulant therapy like the Warfarin drug; and preventing recurrent hepatocellular carcinoma[1].

People with Vitamin K deficiency often consume Vitamin K supplements. Those who may develop Vitamin K deficiency include people with chronic malnutrition and conditions that limit absorption of dietary vitamins, as well as those taking drugs that reduce Vitamin K levels as a side effect[1].

Menadione, however, if consumed in too large a quantity, induces oxidative stress by releasing reactive oxygen species, including hydrogen peroxide and superoxide anions[2]. Accumulated reactive oxygen species damage mitochondrial DNA and protein, eventually destroying the mitochondria and killing the cell. The oxidative stress later causes complications such as heart disease, neurodegenerative disease, arthritis, and cancer[3].

Because menadione induces cytotoxicity by increasing oxidative stress, the addition of antioxidant enzymes Catalase and SOD to cells exposed to menadione may keep cells alive by reducing oxidative stress. Catalase catalyzes the decomposition of hydrogen peroxide into water and oxygen while superoxide dismutase (SOD) converts superoxide anions into hydrogen peroxide and oxygen[4].

The questions proposed are the following: Can Catalase and superoxide dismutase (SOD) reduce menadione-induced cytotoxicity in mitochondria of yeast cells? Which enzyme is most effective in reducing menadione-induced cytotoxicity? The purpose of this project was not only to determine whether antioxidant enzymes can reduce oxidative stress, but also to determine whether Catalase is the more effective enzyme in accomplishing this purpose.

The hypothesis tested is as follows: If Catalase and SOD are added to the yeast cells exposed to menadione, then Catalase and SOD will both reduce menadione-induced cytotoxicity, but Catalase will be more effective. To test this hypothesis, the extent of menadione-induced cytotoxicity in yeast cells was evaluated by MTT assays with absorbances of yeast suspensions measured at 570 nm by a spectrophotometer. Yeast cells with active mitochondria reduce MTT effectively to colored formazan crystals and thus have higher absorbances than yeast cells with mitochondria damaged by menadione-induced cytotoxicity. To determine the protective effect of SOD and Catalase, the enzymes were added to yeast suspensions before adding menadione, and MTT assays were later performed on these suspensions to determine the effect of the enzymes on the absorbances of the suspensions.

An MTT assay accomplishes this purpose by color change. Originally, MTT

is a pale yellow substrate. When cleaved to an active mitochondria with active mitochondrial reductase enzymes, the substrate changes structure and turns a dark blue color. This color change is quantified by an absorbance value measured by a spectrophotometer. Higher absorbance values show that the color of the substrate has changed, which indicates that the mitochondria is functioning because cell viability has not been reduced. Lower absorbance values, on the other hand, show that the color of the substrate has remained pale yellow or nearly pale yellow, which indicates the mitochondrial enzymes are not active because cell viability has been reduced.

Experimental Design:

Materials:

The experimental unit used was Fleischman's yeast. Catalase and Superoxide Dismutase were the test enzymes used. Chemicals used include menadione (2-Methyl-1, 4-naphthoquinone, Vitamin K3), DMSO (Dimethyl sulfoxide), MTT (3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide), isopropanol (2- Propanol), MES hydrate (2-morpholinoethanesulfonic acid), 1 Normal NaOH, 1 Normal HCl, and 10 Normal Phosphate Buffered Saline. Containers and measuring instruments used include a 1000 ml flask, 150 ml flask, 50 ml conical tubes, 15 ml conical tubes, a 100 ml graduated cylinder, a 100 ml bottle, test tubes and test tube racks, 1.5 ml micro-centrifuge tubes and micro-centrifuge tube rack, and a micropipette. Instruments used include an analytical scale, magnetic stirrer and stir bar, vortex, shaker, laboratory oven, refrigerator, pH electrode, centrifuge, micro-centrifuge, and spectrophotometer.

Solution Preparation:

In a 50 ml conical tube, 1.72 mg/ml 10 mM menadione stock solution was prepared by dissolving 86 mg of menadione in a small amount of DMSO and then adding the DMSO to the 50 ml mark, vortexing the solution to mix. The tube of solution was wrapped with aluminum foil to prevent light exposure and then stored at 4?C.
In a 15 ml conical tube, 4 mg/ml MTT stock solution was prepared by

dissolving 40 mg MTT in 7 ml of
distilled water and then adding water to the 10 ml mark, vortexing to mix. This tube was also wrapped in aluminum foil and stored at 4?C.
In a 100 ml bottle, acid isopropanol was prepared by adding 96 ml of isopropanol to 4 ml of 1 N HCl.
Catalase Solution was created by measuring 30 mg of Catalase into a 1.5 ml micro-centrifuge tube and then adding 1 ml phosphate buffered saline. The solution was agitated with a vortex and filled with PBS to 1.5 ml and vortexed again.

Yeast Preparation:

A 1000 ml flask was filled with 600 ml of distilled water. One gram (dry weight) of yeast was added to the water. A magnetic stir bar was placed in the flask, which was then covered with alumninum foil. The flask was left on a magnetic stirrer in slow, continuous agitation overnight at room temperature. The following day, the yeast suspension was centrifuged at 2,500 rpm for 5 minutes at room temperature. The supernatant was discarded, and the pellet was weighed and re-suspended at a 1g: 1 ml ratio.

Menadione Cytotoxicity Assay:

Two sets of test tubes were prepared as specified in Table 1. Test tubes were agitated with a vortex to mix well and then wrapped in aluminum foil to avoid light exposure. The test tubes were then placed in a test tube rack on the shaker in a 30?C oven with slow to medium speed shaking for one hour. An MTT assay was performed on each test tube when shaking was completed.

Table 1

Menadione Concentration	**Tube 1** **0 mM**	**Tube 2** **0.025 mM**	**Tube 3** **0.1 mM**	**Tube 4** **0.2 mM**	**Tube 5** **0.5 mM**
0.1 M MES buffer	**250 µl**	**250 µl**	**250 µl**	**250 µl**	**250 µl**

10 mM menadione	**0 μl**	**5.625 μl**	**22.5 μl**	**45 μl**	**112.5 μl**
Water	**1.975 ml**	**1.97 ml**	**1.95 ml**	**1.93 ml**	**1.86 ml**
Yeast Suspension	**25 μl**	**25 μl**	**25 μl**	**25 μl**	**25 μl**
Total Volume	**2.25 ml**	**2.25 ml**	**2.25 ml**	**2.25 ml**	**2.25 ml**

MTT Assay for Cytotoxicity:

Six-hundred μl from each test tube were transferred into 1.5 ml micro-centrifuge tubes, and 75 μl of 4 mg/ml MTT solution were added to each tube. The tubes were mixed well with vortex and then placed on a shaker in a 30?C oven for one hour. Six-hundred μl of previously prepared acid isopropanol were then added to each tube to dissolve the insoluble formazan crystals. The tubes were vortexed and placed in the micro- centrifuge and then spun at 11,600 rpm for 2 minutes. The supernatants were transferred into spectrophotometer containers, and the absorbances of the supernatants were measured at 570 nm using the spectrophotometer. The absorbance values were expressed as percentages of the absorbance value for untreated cells (control sample), calculated by dividing the absorbance value by the absorbance value for untreated cells: The percentage of the absorbance = (Absorbance of treated-sample / Absorbance of untreated sample) x 100%.

SOD and Catalase Effect on Menadione-Induced Cytotoxicity:

Test tubes were prepared as specified in Table 2 without adding menadione first. Then the test tubes were agitated with vortex to mix well, and the test tubes were placed on a shaker in a 30?C oven with slow to medium speed shaking for 30 minutes. After 30 minutes, menadione was added to the test tubes according to Table 2 and wrapped in alumninum foil to avoid light exposure. The test tubes were then placed back on the shaker in the 30?C oven for one hour. After shaking, an MTT Assay was performed on each tube.

Table 2

	Tube 1 Control	Tube 2 0.025 mM Menadione	Tube 3 0.1 mM Menadione	Tube 4 0.5 mM Menadione	Tube 5 100 units/ml SOD 0.1 mM Menadione	Tube 6 500 units/ml SOD 0.1 mM Menadione	Tube 7 100 units/ml SOD 0.5 mM Menadione	Tube 500 units/ SOD 0.5 m Mena
0.1 M MES	250 µl	250 µl	250 µl	250 µl	250 µl	250 µl	250 µl	250 µ
SOD	0	0	0	0	23.5 µl	117.4 µl	23.5 µl	117.4
Catalase	0	0	0	0	0	0	0	0
10 mM Menadione	0 mM	5.625 µl	22.5 µl	112.5 µl	22.5 µl	22.5 µl	112.5 µl	112.5
Water	1.975 ml	1.969 ml	1.9525 ml	1.8625 ml	1.929 ml	1.8351 ml	1.839 ml	1.745
Yeast Suspension	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
Total Volume	2.25 ml	2.25 ml	2.25 ml	2.25 ml	2.25 ml	2.25 ml	2.25 ml	2.25

Analysis of Results:

MTT assay for Menadione Cytotoxicity

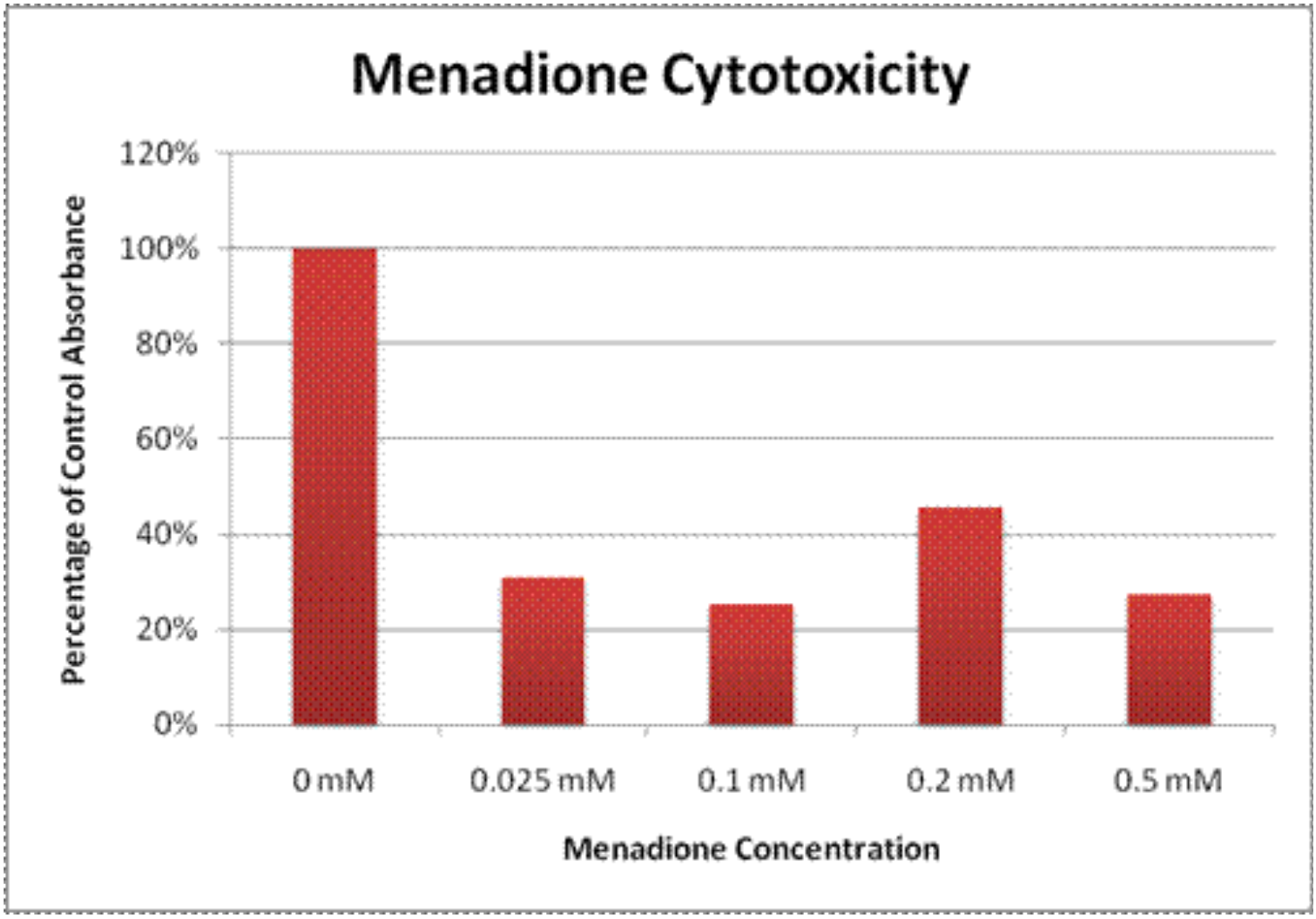
Menadione Cytotoxicity
Percentage of Control Absorbance
120%
100%
80%
60%
40%
20%
0%
0 mM
0.025 mM
0.1 mM
0.2 mM
0.5 mM
Menadione Concentration

Figure 1: Menadione-Induced Cytotoxicity

Table 1: Menadione Effect on Absorbance (570 nm)

Menadione Concentration	Absorbance (570 nm)	Percentage of Control Absorbance
0 mM (Control)	0.099	100%
0.025 mM	0.0305	30.81%
0.1 mM	0.025	25.25%
0.2 mM	0.045	45.45%
0.5 mM	0.027	27.27%

(Figure 1 and Table 1 show the absorbances of yeast suspensions when different concentrations of menadione were placed into the suspensions. The corresponding percentages of control absorbance, correlating to cell viability, are also shown.)

MTT assays were used to measure menadione-induced cytotoxicity to yeast cell. Figure 1 and Table 1 show that menadione decreased the absorbances of the yeast suspensions even with the lowest menadione concentration of 0.025 mM. Increasing menadione concentration, however, does not increase its cytotoxicity to yeast cells. The concentrations selected to be tested in this experiment using the test enzymes were 0.025 mM, 0.1 mM, and 0.5 mM.

SOD and Catalase Effect on Menadione-induced Cytotoxicity:

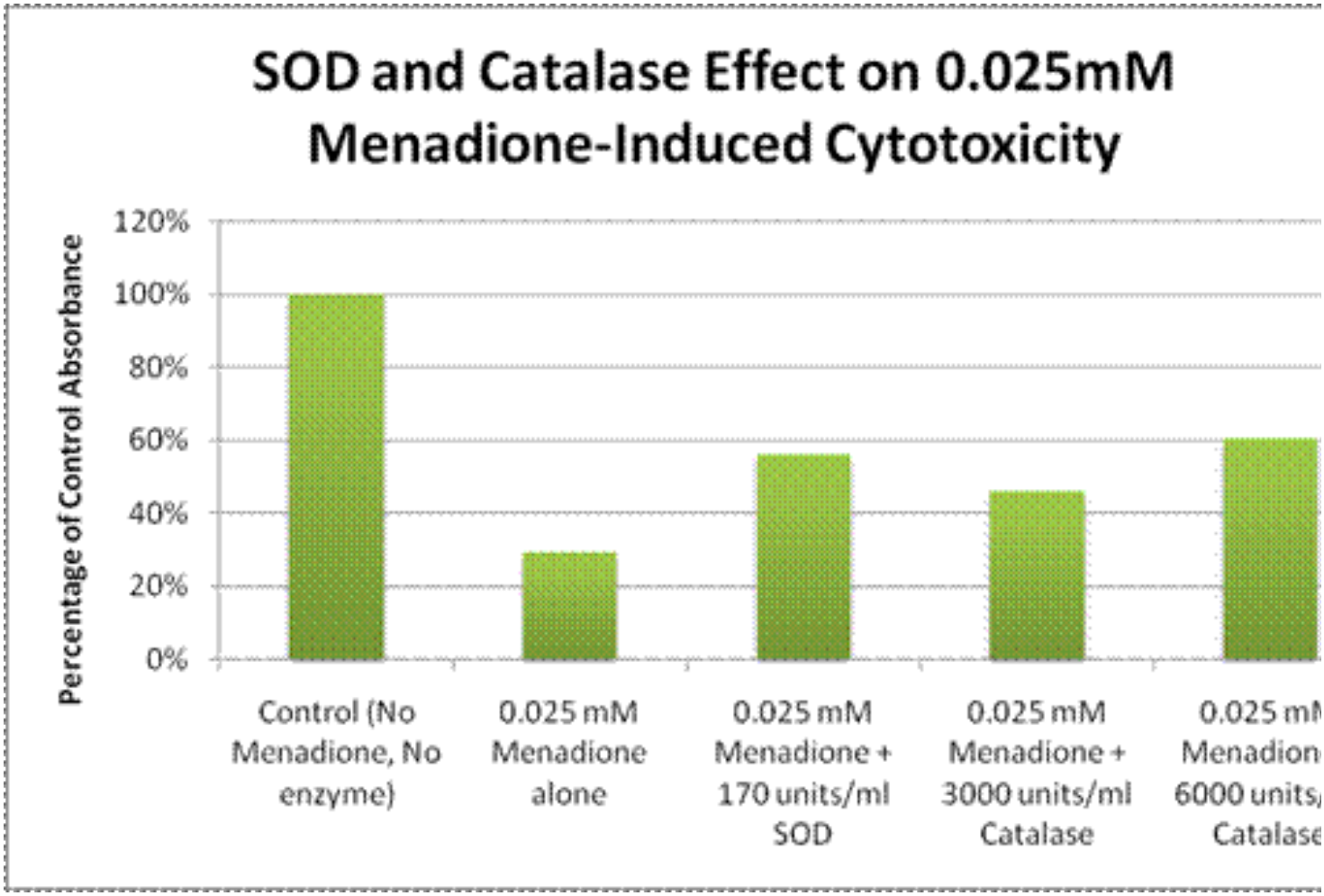
SOD and Catalase Effect on 0.025mM Menadione-Induced Cytotoxicity
Percentage of Control Absorbance
120%
100%
80%
60%
40%
20%
0%
Control (No Menadione, No enzyme)
0.025 mM Menadione alone
0.025 mM Menadione + 170 units/ml SOD
0.025 mM Menadione + 3000 units/ml Catalase
0.025 mM Menadione + 6000 units/ml Catalase

Figure 2: SOD and Catalase Effect on 0.025mM Menadione-Induced Cytotoxicity

Table 2: SOD and Catalase Effect on 0.025 mM Menadione-Induced Cytotoxicity

	Absorbance	Percentage of Control Absorbance
Control (no menadione, no enzyme)	0.048	100%
0.025 mM menadione alone	0.014	29.17%
0.025 mM menadione + 170 units/ml SOD	0.027	56.25%
0.025 mM menadione + 3000 units/ml Catalase	0.022	45.83%
0.025 mM menadione + 6000 units/ml Catalase	0.029	60.42%

(Figure 2 and Table 2 show the absorbances of yeast suspensions when the menadione concentration was 0.025 mM and different concentrations of enzymes were added. The corresponding percentages of control absorbance, correlating to cell viability, are also shown.)

The percentages of the control absorbance were used to determine the effectiveness of SOD or Catalase at certain concentrations in reducing menadione-induced cytotoxicity. Figure 2 and Table 2 show the SOD and Catalase effect on 0.025 mM menadione-induced cytotoxicity. The MTT assay showed that the percentage of control absorbance was significantly increased by both SOD and Catalase at 0.025 mM menadione concentration. The most significant reduction in cytotoxicity was produced by 6000 units/ml Catalase. The yeast suspension to which it was added had the highest percentage of control absorbance (60.42%) when compared to yeast suspensions with 3000 units/ml Catalase and 170 units/ml SOD. Those suspensions had percentages of control absorbance of 45.83% and 56.25%, respectively. The percentage of control absorbance of the yeast suspension without enzymes was 29.17%.

The results indicate that at 0.025 mM menadione concentration, SOD (170

units/ml) and Catalase (3000 units/ml and 6000 units/ml) are both effective in reducing menadione-induced cytotoxicity in the yeast suspensions.

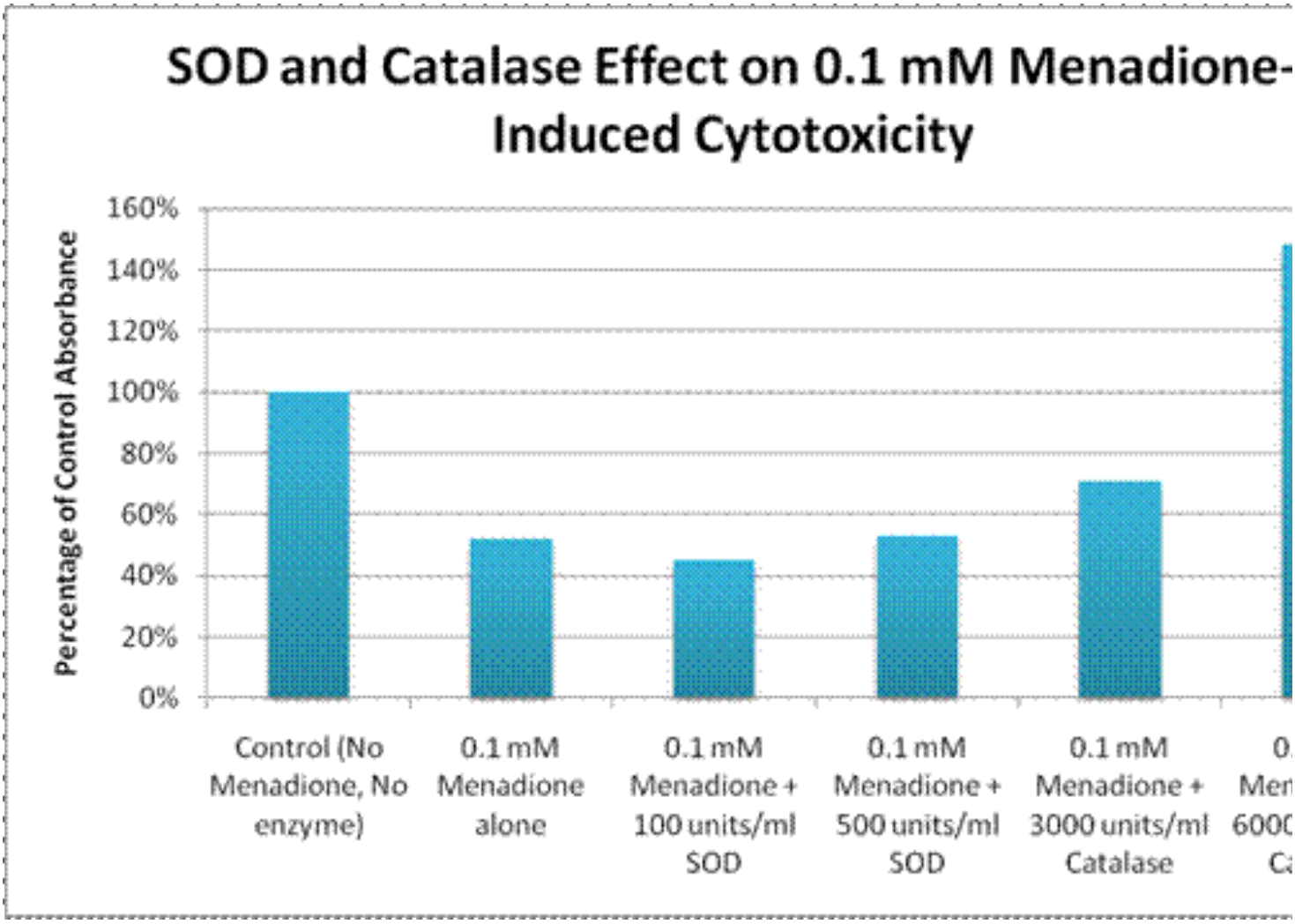

Figure 3: SOD and Catalase Effect on 0.1mM Menadione-Induced Cytotoxicity

Table 3: SOD and Catalase Effect on 0.1 mM Menadione-Induced Cytotoxicity

	Absorbance	Percentage of Control Absorbance
Control (no menadione, no enzyme)	0.048	100%
0.1 mM menadione alone	0.025	52.08%
0.1 mM menadione + 100 units/ml SOD	0.0215	44.79%
0.1 mM menadione + 500 units/ml SOD	0.0255	53.13%
0.1 mM menadione + 3000 units/ml Catalase	0.034	70.83%
0.1 mM menadione + 6000 units/ml Catalase	0.071	147.92%

(Figure 3 and Table 3 show the absorbances of yeast suspensions when the menadione concentration was 0.1 mM and different concentrations of enzymes were added. The corresponding percentages of control absorbance, correlating to cell viability, are also shown.)

The percentages of the control absorbance were used to determine the effectiveness of SOD or Catalase at certain concentrations in reducing menadione-induced cytotoxicity. Figure 3 and Table 3 show the SOD and Catalase effect on 0.1 mM menadione-induced cytotoxicity. The most significant reduction in cytotoxicity was produced by 6000 units/ml Catalase at 0.1 mM menadione. This yeast suspension had an absorbance of 147.92% of the absorbance of the control yeast suspension.

This percentage, however, should not have exceeded 100%, since the control absorbance is the absorbance when no menadione exposure has occurred. Theoretically speaking, that absorbance should be the highest possible absorbance for the yeast suspensions. The fact that this percentage exceeds 100% could be due to a variety of possible measurement errors. The control absorbance reading may have been slightly inaccurate, causing it to be lower than it should be, or the absorbance reading for that yeast suspension with the added Catalase may have been slightly lower in actuality than what the spectrophotometer measured. The menadione- induced cytotoxicity also could have failed to take full effect.

In both test tubes, however, the percentage of the control absorbance exceeds 100%, indicating, that this concentration of Catalase not only counters the damage to the mitochondrial reductase that reduces the MTT formazan but also increases the activity of the reductase to be greater than that without Catalase.

Significant reductions in menadione-induced cytotoxicity were also produced by 3000 units/ml Catalase. This yeast suspension had an absorbance reading that was 70.83% of the control absorbance, showing significant effectiveness of Catalase when the suspension is compared to the suspension with only menadione added, which had an absorbance that was 52.08% of the control absorbance.

There was no significant reduction in menadione-induced cytotoxicity in the yeast suspensions containing SOD, as their percentages of the control absorbance were 44.79% and 53.13%. These results indicate that at 0.1 mM menadione concentration, Catalase is most effective in reducing menadione-induced cytotoxicity, while SOD at concentrations of 100 and 500 units/ml has no effect.

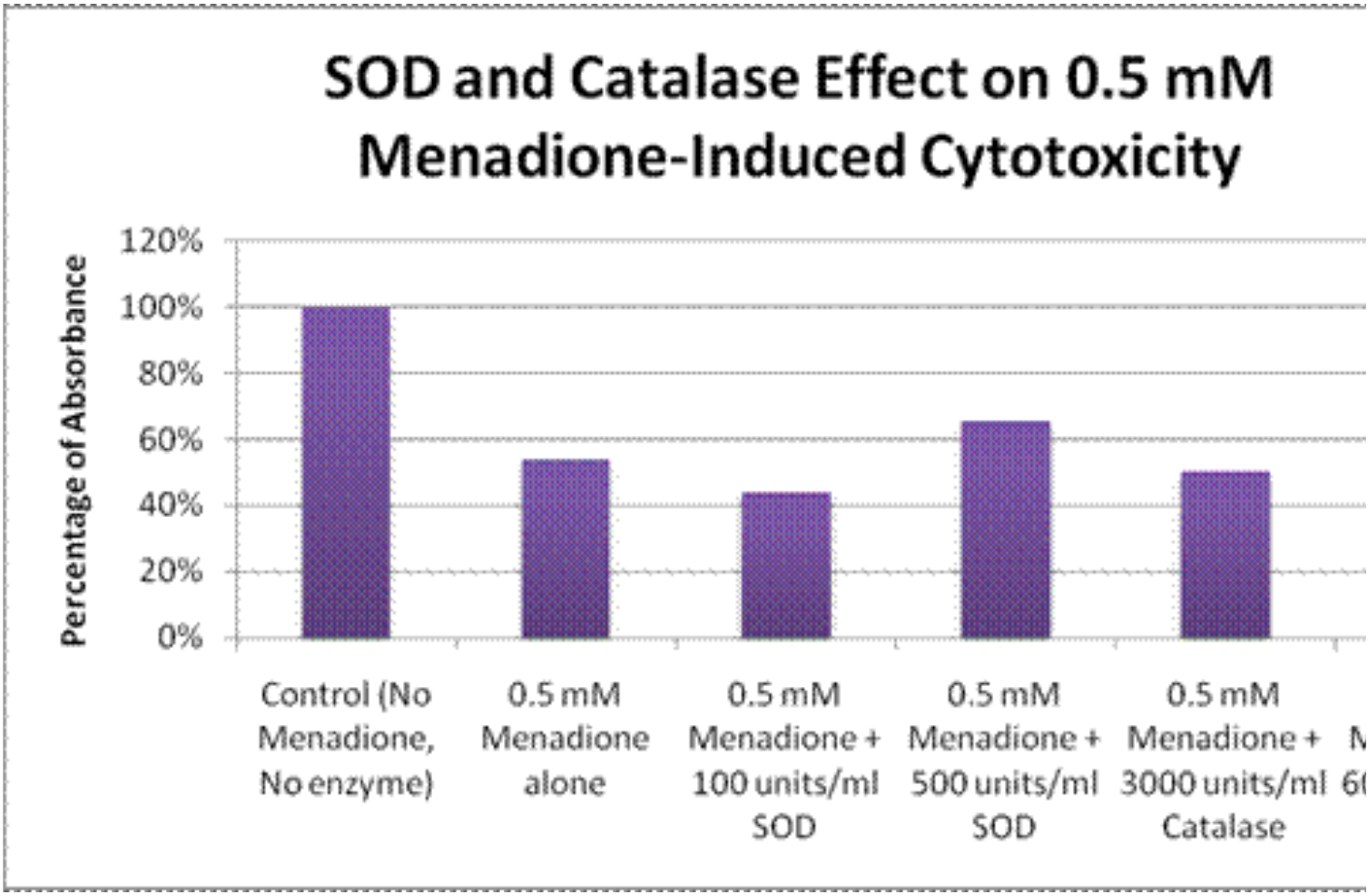

Figure 4: SOD and Catalase Effect on 0.5mM Menadione-Induced Cytotoxicity

Table 4: SOD and Catalase Effect on 0.5 mM Menadione-Induced Cytotoxicity

	Absorbance	Percentage of Control Absorbance
Control (no menadione, no enzyme)	0.048	100%
0.5 mM menadione alone	0.026	54.17%
0.5 mM menadione + 100 units/ml SOD	0.021	43.75%
0.5 mM menadione + 500 units/ml SOD	0.0315	65.63%
0.5 mM menadione + 3000 units/ml Catalase	0.024	50%
0.5 mM menadione + 6000 units/ml Catalase	0.041	85.42%

(Figure 4 and Table 4 show the absorbances of yeast suspensions when the menadione concentration was 0.5 mM and different concentrations of enzymes were added. The corresponding percentages of control absorbance, correlating to cell viability, are also shown.)

The percentages of the control absorbance were used to determine the effectiveness of SOD or Catalase at certain concentrations in reducing menadione-induced cytotoxicity. Figure 4 and Table 4 show the effect of SOD and Catalase on 0.5 mM menadione-induced cytotoxicity. The most significant reduction in cytotoxicity was produced by 6000 units/ml Catalase at 0.5 mM menadione. This yeast suspension had an absorbance of 85.42% of the control absorbance. A significant reduction in menadione-induced cytotoxicity was also produced in the yeast suspension containing 500 units/ml SOD, as it had an absorbance reading of 65.63% of the control absorbance.

There is no reduction in menadione-induced cytotoxicity in the yeast suspensions with 3000 units/ml Catalase or 100 units/ml SOD, which means higher enzyme concentrations are necessary in reducing cytotoxicity. These results indicate that when the menadione concentration is 0.5 mM, 6000 units/ml Catalase is most effective in reducing menadione-induced cytotoxicity, while 500 units/ml SOD can also reduce cytotoxicity but with a

smaller effect.

Conclusion:

The first part of the experiment showed menadione can induce cytotoxicity in yeast cells. MTT assays were used to evaluate the cytotoxicity induced by menadione. Menadione did damage the yeast's mitochondria, affecting their ability to reduce MTT to colored formazan crystals and thus decreasing the absorbance at 570 nm (Figure 1 and Table 1).

When SOD and Catalase were added to the cells at certain concentrations, the oxidative stress induced by menadione was reduced, thus increasing the ability of yeast cells to reduce MTT. Both SOD and Catalase had an effect in reducing 0.025 mM menadione-induced cytotoxicity (Figure 2 and Table 2). However, when the menadione concentration was 0.1 mM, only Catalase was effective in reducing menadione-induced cytotoxicity, while SOD did not show an effect (Figure 3 and Table 3). When the menadione concentration was 0.5 mM, only 6000 units/ml of Catalase was strong enough to reduce menadione-induced cytotoxicity; 500 units/ml of SOD also produced an effect, but the effect was not as strong as the one produced by the Catalase.

These results show that Catalase is more effective in preserving the mitochondria of the cells, as it more consistently reduced menadione-induced cytotoxicity, shown by the higher absorbances of the yeast suspensions. The reason why SOD was less significant may be that SOD converts superoxide anions to hydrogen peroxide, which can inhibit SOD activity.

This experiment supports the hypothesis that if Catalase and SOD are added to yeast cells exposed to menadione, then Catalase and SOD will both reduce menadione-induced cytotoxicity, but Catalase will be most effective.

This project demonstrates the possibility of combating menadione-induced cytotoxicity through use of antioxidant enzymes such as Catalase and SOD. Because Vitamin K is necessary for bone repair and formation, converting glucose to glycogen, and blood clotting, menadione is often used as a Vitamin K supplement. Overdose and subsequent menadione-induced

cytotoxicity is thus a possibility. The menadione-induced oxidative stress can damage DNA and protein of mitochondria, causing the death of cells.

The oxidative stress that menadione induces by releasing reactive oxygen species can be combated by injection or consumption of antioxidants such as Catalase, the preferred enzyme over SOD. The death of cells due to accumulated reactive oxygen species that damage DNA and protein, eventually destroying the mitochondria, can be prevented in some cells by adding Catalase to reduce the menadione-induced cytotoxicity. Such treatment can be used to prevent Vitamin K3 poisoning that leads to heart disease, neurodegenerative disease, arthritis, and cancer, oftentimes the results of free radical damage caused by menadione-induced cytotoxicity.

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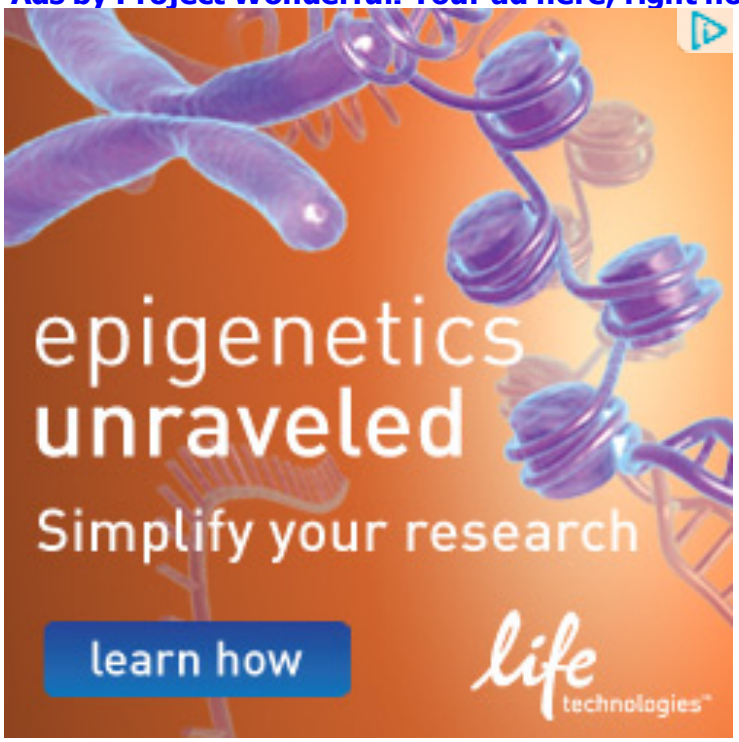

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