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Cytotoxic effects of menadione on normal and cytochrome c-deficient yeast cells cultivated aerobically or anaerobically

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ABSTRACT

Cytotoxic effects of menadione on normal and cytochrome c-deficient yeast cells were examined on the basis of the cell growth rate, NAD(P)H concentration, reactive oxygen production, plasma membrane H^+-ATPase activity, and ethanol production. In aerobically or anaerobically cultured yeast cells, NAD(P)H concentration decreased with increasing concentration of menadione, and the recovery of NAD(P)H concentration was proportional to the cell growth rate. However, there was no relationship among the inhibition of the cell growth and reactive oxygen production, plasma membrane H^+-ATPase activity, and ethanol production. Among them, ethanol production showed resistance to the cytotoxicity of menadione, suggesting the resistance of glycolysis to menadione. The growth inhibitory effect of menadione depended on the rapid decrease and the recovery of NAD(P)H rather than production of reactive oxygen species regardless of aerobic culture or anaerobic culture and presence or absence of mitochondrial function. The recovery of NAD(P)H concentration after the addition of menadione might depend on menadione-resistant glycolytic enzymes.

1. Introduction

When menadione acting as a redox mediator is administered to cells, reactive oxygen species are generated and exhibits cytotoxicity. This phenomenon is common to animal cells, yeast and bacteria. Targets of menadione are DNA [1,2], proteins [3,4] and lipids [5,6], which cause oxidative degradation. Menadione causes a wide range of oxidative stress and induces necrosis or apoptosis [7,8]. Therefore, its use as an anticancer agent is also progressing [9,10].

However, the major cytotoxicity of menadione as an electron transfer mediator is unknown. Although the damage of cell components by reactive oxygen species is well known as mentioned above, the mechanism of cell growth inhibition by reactive oxygen species is also in speculation. In the case of anaerobically cultured yeast cells, the extracellular superoxide dismutase and catalase limitedly blocked the growth-inhibitory effect of menadione [11] which produced reactive oxygen species through NAD(P)H:quinone reductase in cytosol and plasma membrane [12]. These facts suggest that extracellular reactive oxygen species are the cytotoxic factors for anaerobically cultured yeast cells. In addition to reactive oxygen species, NAD(P)H concentration is also considered to control the cell growth in the presence of menadione, because the resistance of yeast cells to the growth inhibition by menadione was observed in the medium to keep NAD(P)H concentration [13].

This study investigated whether the change in NAD(P)H concentration induced by menadione is linked to the proliferation of aerobically or anaerobically cultured yeast cells. Furthermore, normal and cytochrome c-deficient yeast cells were used to investigate the effects of menadiones on the mitochondrial electron transport chain of aerobically cultured yeast cells. This study suggests that the change in NAD(P)H concentration after addition of menadione is an index to predict the cell growth on the basis of the comparison of changes in growth rate, NAD(P)H concentration, production of reactive oxygen species, glucose-induced proton release, and ethanol fermentation.

2. Materials and methods

2.1. Yeast strains and culture conditions

The two strains of *Saccharomyces cerevisiae* used in this study were the cytochrome c-deficient mutant (ATCC18789) and normal strain (ATCC18790).

These strains were grown on at 30 °C overnight. The medium

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composition under aerobic culture conditions was 2% glycerin, 1% malt extract and 0.5% yeast extract. Shaking culture was performed. The composition of the medium under anaerobic culture conditions was 2% glucose, 1% peptone 0.5% yeast extract. The culture was performed in a sealed container.

2.2. Preparation of mitochondria

Yeast mitochondria of aerobically cultured yeast cells were prepared with the improved method described in Ref. [14]. Zymolyase-100T was used for the preparation of spheroplast, and buffer was composed of 0.7 M or 1.2 M sorbitol and 0.1 M EDTA (pH 7.0).

2.3. Difference spectra of mitochondria

Difference spectra due to reduced and oxidized mitochondria was determined as follows. Mitochondria were suspended in the buffer composed of 1.2 M sorbitol and 0.1 M EDTA (pH 7.0), and the final protein concentration of mitochondria was 0.7 mg/ml. This suspension was aerobically incubated in reference cuvette. In sample cuvette crystal dithionite was anaerobically added to the above suspension. After that the difference spectra between oxidized and reduced mitochondria was determined at the range from 500 to 650 nm.

2.4. Protein concentration

The protein concentration of mitochondria was determined by Bradford method using bovine serum albumin as the standard [15].

2.5. Growth rate

The growth rate of yeast cells was determined on the basis of the increase in the absorbance at 600 nm. The initial absorbance of yeast cell suspension was adjusted to 0.1 with the medium used under aerobic or anaerobic conditions, and aerobic and anaerobic culture were performed according to the above conditions.

2.6. Fluorescence intensity due to NAD(P)H concentration in yeast cell suspension

The absorbance of yeast cell suspension was adjusted to 0.4 at 600 nm with 0.1 M phosphate buffer (pH7.0). Fluorescence intensity of yeast suspension was measured with excitation light 365 nm and fluorescence 430 nm.

2.7. Determination of reactive oxygen species

The absorbance of yeast cell suspension was adjusted to 0.5 at 600 nm with 0.1 M Tris/HCl (pH 7.0), and 0.25 ml of the suspension and 0.25 ml of 0.6 mM menadione were mixed and incubated at 30 °C for 10 min. After that, 0.5 ml of luminol solution was injected into the mixture, and luminescence intensity was determined for 5 s. The composition of menadione solution and luminol solution is described in Refs. [16].

2.8. Glucose-induced proton release

Proton release by yeast cells was determined according to methyl red test [17].

The absorbance of yeast suspension was adjusted to 1.0 with pure water after two washing by centrifugation. After 1 ml of yeast scell uspension was mixed with 8 μl of 10 mM methyl red dissolved in dimethyl sulfoxide, 20 μl of 1 M KCl, and 1.2 or 2.4 μl of 0.1 N NaOH, the mixture was incubated for 1 min at 25 °C. The change in the absorbance at 527 nm was recorded after the addition of 20 μl of 1 M glucose, and the proton efflux by yeast cells was calculated on the basis of the relationship between the absorbance and hydrogen ion concentration described in Ref. [17].

2.9. Determination of ethanol production

The absorbance of yeast cell suspension was adjusted to 1.0 at 600 nm with 20 mM phosphate buffer (pH 7.0), and 50 mM glucose and menadione dissolved in dimethyl sulfoxide were added to 5 ml of yeast cell suspension. After the incubation at 30 °C for 1h, the supernatant was obtained by centrifugation of the suspension. The supernatant was diluted 100 fold with pure water, and 0.5 ml of the diluted solution was mixed with 0.5 ml of buffer, 0.1 ml NAD solution (6 mg/ml), 0.1 ml thiazolyl blue tetrazolium bromide (MTT) solution (1 mg/ml), 0.005 ml of 1-methoxy-5-methylphenazinim methylsulfate (1-methoxy PMS) solution (0.024%) and 0.1 ml of alcohol dehydrogenase solution (50U/ml). After the incubation of the mixture at 30 °C for 15 min, the absorbance at 570 nm was determined. The concentration of ethanol in the diluted solution was calculated on the basis of the absorbance of standard ethanol solution. The component of buffer was 10g $Na_2P_2O_2 \cdot 10H_2O$, 2.5g semicarbazide/HCl, 0.5g glycine, and 300 ml pure water. NAD and alcohol dehydrogenase were dissolved in buffer.

2.10. Chemicals

Chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation.

2.11. Statistical analysis

The mean and the standard deviation were calculated by using Office Excel.

3. Results

3.1. Difference spectra of yeast mitochondria

ATCC18789 which is homozygous for the cy1-1 contains exclusively iso-2-cytochrome c at the concentrations of 5–10% of the total cytochrome c found in normal strains, and the basal rate of respiration is supported by the small of this cytochrome [18].

Fig. 1 shows the difference spectra of yeast mitochondria. The obvious reduction of cytochrome c + c1 at 520 nm and 550 nm was observed in mitochondria suspension of ATCC18790 after reduction by

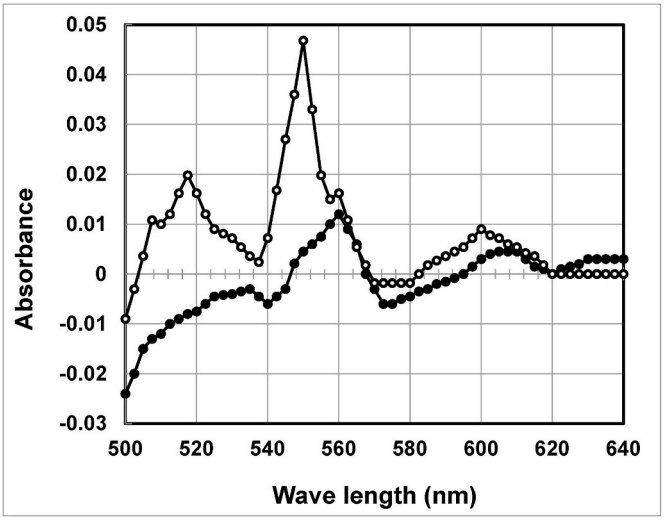

Fig. 1. Difference spectra of mitochondria between reduced and oxidized state. Symbols ○ and ● show the difference spectra of mitochondria in normal and cytochrome c-deficient yeast cells, respectively.

dithionate. On the other hand, ATCC18789 showed little difference spectra due to cytochrome c+ c_1. These facts indicated that ATCC18789 and ATCC18790 showed a clear difference in cytochrome c content under the culture conditions of this study.

3.2. Growth and NAD(P)H concentration of aerobically cultured yeast cells

Since aerobically cultured yeast cells had mitochondrial function, it was expected that menadione affected both cytoplasm and mitochondria. The effect of menadione on mitochondria was also expected to be observed by comparing the effect of menadione on the normal yeast cells and the yeast cells lacking part of mitochondrial function.

The growth rate of cytochrome c-deficient yeast cells was suppressed with increasing concentration of added menadione under aerobically culture condition using glycerin as carbon source (Fig. 2 A). The fluorescence intensity due to NAD(P)H concentration also decreased with increasing concentration of added menadione and was almost constant after the addition of menadione of 100 μM or less (Fig. 2C). On the other hand, the normal yeast cells were more sensitive to the growth inhibitory effect of menadione than the cytochrome c-deficient yeast cells (Fig. 2A and B). After the addition of menadione, the fluorescence intensity due to NAD(P)H concentration continued to decrease with time (Fig. 2 D).

The above facts suggest that menadione-mediated electron transfer from NADH to cytochrome c [19] induces rapid oxidation of NADH in normal yeast to inhibit the cell growth. On the other hand, cytochrome c-deficient yeast cells might show the resistance to the growth inhibitory effect of menadione by suppressing menadione-mediated electron transfer from NADH to cytochrome c.

3.3. Growth and NAD(P)H concentration of anaerobically cultured yeast cells

The growth rate of yeast cells was measured in glucose-based medium.

The growth of cytochrome c-deficient yeast cells was inhibited in the proportion to the concentration of added menadione (Fig. 3 A) and was more inhibited than that of aerobically cultured yeast cells (Figs. 2 A and Fig.3 A). The fluorescence intensity due to NAD(P)H concentration also decreased in the proportion to the concentration of added menadione and remained almost constant after the addition of menadione (Fig. 3C). Menadione-mediated consumption of NAD(P)H in anaerobically cultured yeast cells was greater than that in aerobically cultured yeast cells (Figs. 2C and Fig.3C).

In normal yeast cells, the growth inhibition by menadione was smaller in anaerobically cultured yeast cells than in aerobic cultured yeast cells (Figs. 2 B and Fig.3 B). The fluorescence intensity due to NAD (P)H concentration decreased in the proportion to the concentration of added menadione and increased with time after the addition of menadione (Fig. 3 D), suggesting the recovery of NAD(P)H.

The above facts suggest that the growth of yeast cells depends on the change in NAD(P)H level after addition of menadione regardless of aerobic culture or anaerobic culture and presence or absence of mitochondrial function.

3.4. Generation of reactive oxygen species

It was reported that menadione promoted the production of reactive oxygen species with NAD(P)H: menadione reductase [12], and that the growth of anaerobically cultured yeast cells inhibited by menadione was partially restored by the addition of SOD or catalase [11]. In this study menadione-mediated production of reactive oxygen species was determined with aerobically or anaerobically cultured yeast cells.

Cytochrome c-deficient yeast cells produced more reactive oxygen species than normal yeast cells when cultivated aerobically, but produced less reactive oxygen species than normal yeasts when cultivated anaerobically (Fig. 4). In both aerobic and anaerobic cultures, menadione-mediated production of reactive oxygen species was not proportional to the inhibition of growth rate (Figs. 2–4), suggesting that reactive oxygen species were not major inhibitors of the cell growth. Yeast cells keeping high NAD(P)H concentration might have ability to

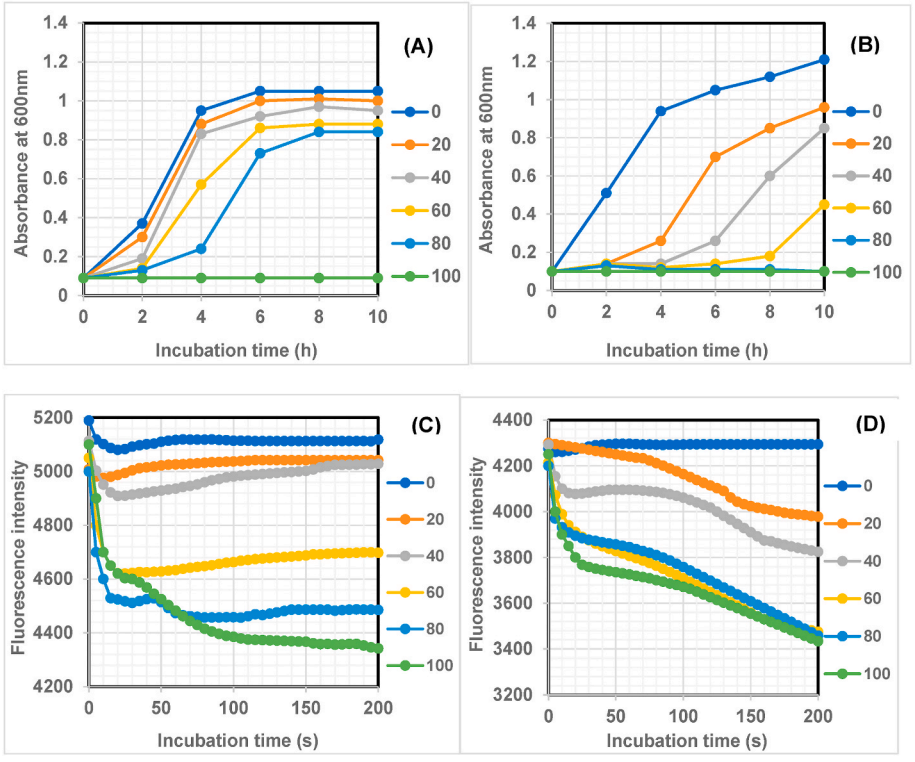

Fig. 2. Cell growth and NAD(P)H concentration of aerobically cultured yeast cells, Fig.2 (A) and (B) show the cell growth of cytochrome c-deficient and normal yeast cells, respectively. Fig.2 (C) and (D) show the fluorescence intensity due. to NAD(P)H concentration of cytochrome c-deficient and normal yeast cells, respectively. The number on the right in each figure is the concentration of menadione (μM). Each value is the mean of three different determinations, and the standard deviation was below 5% of the mean.

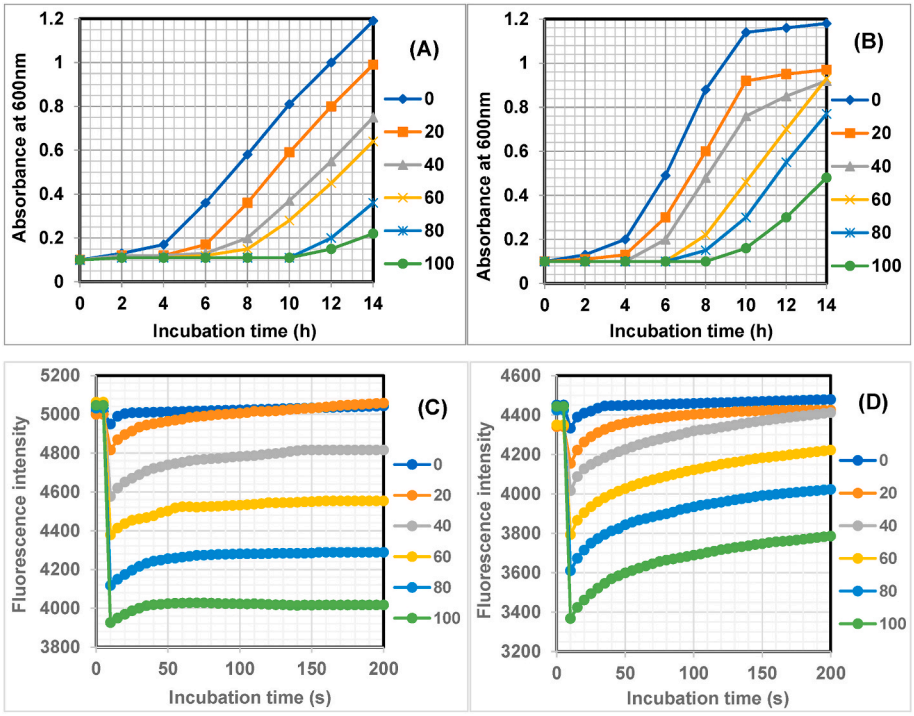

Fig. 3. Cell growth and NAD(P)H concentration of anaerobically cultured yeast cells Fig.3 (A) and (B) show the cell growth of cytochrome c-deficient and normal Yeast cells, respectively. Fig.3 (C) and (D) show the fluorescence intensity due to NAD(P)H concentration of cytochrome c-deficient and normal yeast cells, respectively. The number on the right in each figure is the concentration of menadione (μM). Each value is the mean of three different determinations, and the standard deviation was below 5% of the mean.

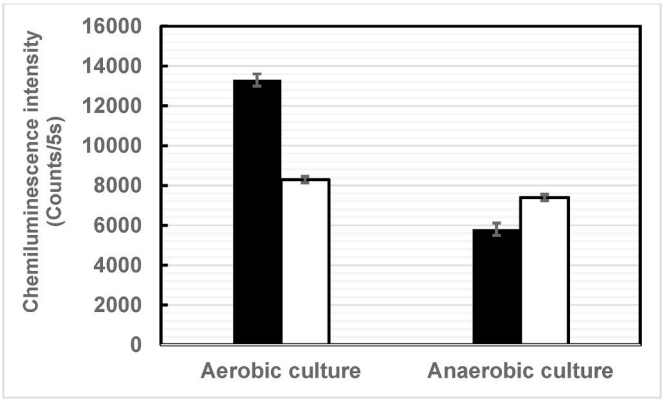

Fig. 4. Production of reactive oxygen species by cytochrome c-deficient and normal yeast cells Black bar and white bar show the chemiluminescence intensity of cytochrome c-deficient and normal yeast cells, respectively. Large bars and small bars represent the mean and the standard deviation, respectively. Each. value is the mean of three different determinations.

keep both the cell growth and the production of reactive oxygen species by supplying NADH to both glycolysis enzymes and NAD(P)H:quinone reductase.

3.5. Glucose-induced acidification

As NAD(P)H: menadione reductase was present in the yeast plasma membrane and produces reactive oxygen species [12], this enzyme was expected to affect various plasma membrane enzymes by producing reactive oxygen species. For example, H^+-ATPase is present in the plasma membrane and is involved in glucose-induced proton release regulating intracellular pH [20]. In this study, the inhibitory effect of menadione on glucose-induced proton release was determined. Even in aerobic and anaerobic cultures, normal yeast cells were more susceptible to inhibitory effect of menadione on glucose-induced proton release than cytochrome c-deficient yeast cells (Fig. 5A and B). Therefore, there was no clear correlation between the inhibition of glucose-induced proton release and the inhibition of cell growth.

3.6. Ethanol fermentation

Menadione and glucose were added to the yeast cell suspension, and the production of ethanol was determined 1h later.

In both aerobic and anaerobic culture, menadione had little inhibitory effect on ethanol production of normal and cytochrome c-deficient yeast cells (Fig. 6), suggesting that glycolytic enzymes were resistant to the cytotoxic effect of menadione.

4. Discussion

Menadione mediated the production of reactive oxygen species by reacting with NAD(P)H:menadione reductase in yeast plasma membrane and cytosol [12]. In yeast cells, NADH was oxidized more than NADPH by addition of menadione [12], suggesting a role of NADH:menadione reductase in the production of reactive oxygen species. The reactive oxygen species produced in this way have been considered to decompose DNA, proteins, and lipids by oxidative or peroxidative reactions, but his reaction between reactive oxygen species and biomolecules is considered to cause secondary cytotoxicity.

In this study the inhibition of yeast cell growth was depending on the concentration of menadione added, and the decrease in NAD(P)H concentration induced by menadione was proportional to the inhibition of yeast cell growth by menadione (Figs. 2 and 3). When yeast cells were aerobically cultured, mitochondrial NADH: coenzyme Q reductase was considered to promote electron transfer from NADH to cytochrome c [19]. This suggestion is supported by the mechanism that menadione transports electrons from Complex III involving an antimycin A-sensitive site to the cytochrome c + c_1 region [19]. On the other hand, cytochrome c-deficient yeast cells might suppress the above electron transport to reduce the consumption of NADH and might be resistant to growth inhibition by menadione.

In anaerobically cultured yeast cells, no persistent NAD(P)H oxidation was observed after the rapid decrease in NAD(P)H concentration by menadione, and the inhibition of yeast cell growth was proportional to

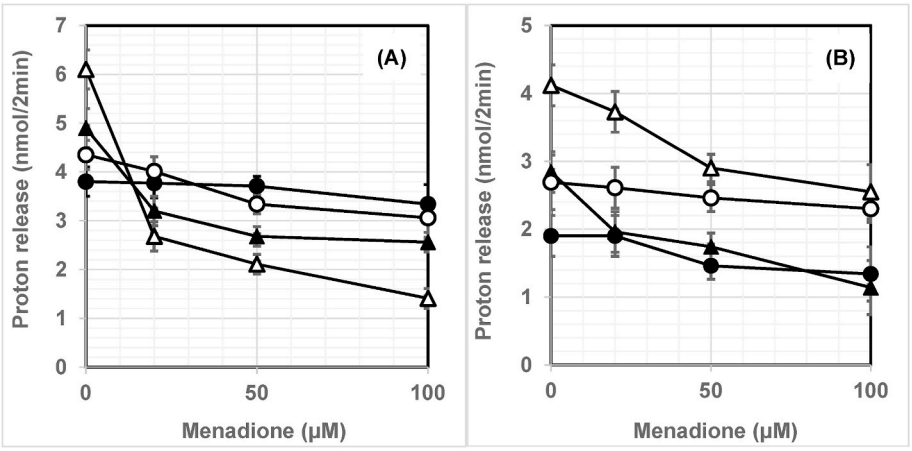

Fig. 5. Glucose-induced proton release Fig.5 (A) and (B) represent glucose-induced proton release by aerobically cultured yeast cells and anaerobically cultured yeast cells, respectively. Symbols ● and ▲ represent glucose-induced proton release by cytochrome c-deficient yeast cells and normal yeast cells, respectively, after the addition of menadione. Symbols ○ and △ represent glucose-induced proton release by cytochrome c-deficient yeast cells and normal yeast cells, respectively, after 1 h-incubation with menadione at 30 °C. Small bars represent the mean and the standard deviation, respectively. Each value is the mean of three different determinations.

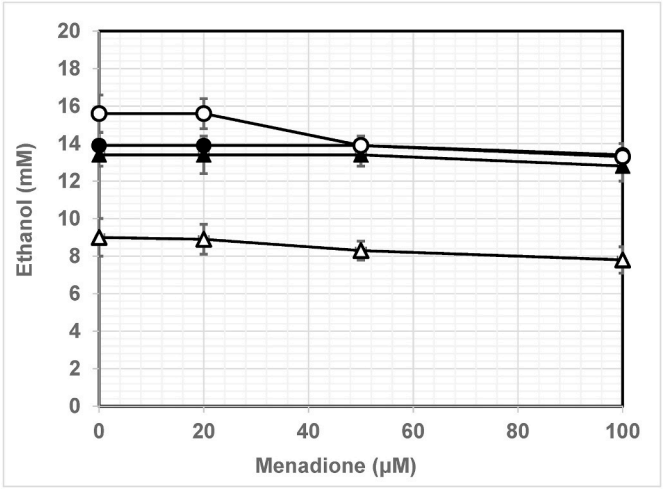

Fig. 6. Production of ethanol. Symbols ○ and △ represent the ethanol production by aerobically cultured cytochrome c-deficient yeast cells and normal yeast cells, respectively. Symbols ● and ▲ represent the ethanol production by anaerobically cultured cytochrome c-deficient yeast cells and normal yeast cells, respectively. Small bars represent the mean and the standard deviation, respectively. Each value is the mean of three different determinations.

the decrease in NAD(P)H concentration. Yeast cells in which NAD(P)H concentration was recovered in the presence of menadione showed resistance to cell growth inhibition by menadione. This suggests that yeast cell growth is controlled in the initial stage of addition of menadione.

Aerobically cultured yeast cells were expected to increase reactive oxygen species in mitochondrial electron transfer system, but production of reactive oxygen species was lower than that of cytochrome c-deficient yeast cells. The opposite phenomenon was observed in anaerobically cultured yeast cells. Although the involvement of reactive oxygen species in cell growth inhibition cannot be ruled out, the production of reactive oxygen species might not be associated with the lethal cytotoxicity.

It is known that NAD(P)H: menadione reductase is present in the plasma membrane and that reactive oxygen species are generated by the addition of menadione to plasma membrane fraction in yeast cells [12]. Therefore, plasma membrane proton pumping ATPase (H^+-ATPase) was expected to be inhibited by menadione. However, whether in aerobic or anaerobic culture, cytochrome c-deficient yeast cells were more resistant to the inhibitory effect of menadione on glucose-induced proton release than normal yeast cells, and the inhibition of glucose-induced proton release was not associated with the inhibition of cell growth.

Since ethanol production was little inhibited by menadione (Fig. 6), glycolysis enzymes might be little affected by menadione-mediated decrease in NAD(P)H concentration and involved in the gradual recovery or the maintenance of NAD(P)H concentration as shown in Figs. 2 and 3.

Immediately after the administration of menadione to the yeast cell suspension, the complex formation with SH compounds were observed [21,22], and the cytotoxic effect of menadione was proposed to depend on the exhaustion of glutathione and cysteine. The formation of S-glutathione conjugate was proposed to be a major pathway of menadione metabolism in yeast cells and that this reaction induced the removal of menadione from yeasts and a decrease of total glutathione concentration [21,22]. Furthermore, reduced glutathione was simultaneously oxidized to GSSG in the presence of menadione. However, it is unclear how the decrease in glutathione contributes to cell growth inhibition.

One interesting study of menadione is that it induces G1 arrest, resulting in resistance to menadione toxicity [23]. However, since it is not clear whether it depends on the concentration of menadione, future studies are awaited.

From the results and discussion, this study proposes that NAD(P)H concentration after the addition of menadione controls the cell growth and that yeast cell proliferation is evaluated on the basis of NAD(P)H concentration. Glycolysis enzymes that are tolerant to the cytotoxicity of menadione might contribute to the recovery of yeast cell proliferation. These suggestions might be applicable to the research of anti-cancer activity of menadione to cancer cells keeping strong glycolysis activity [24–27].

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CRediT authorship contribution statement

Shiro Yamashoji: Methodology, Investigation, Writing - original draft. **Arafat Al Mamun:** Writing - review & editing, Validation. **Latiful Bari:** Writing - review & editing.

Declaration of competing interest

There is no conflict of interest among authors.

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