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Heat-Treated Mineral-Yeast as a Potent Post-irradiation Radioprotector

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Radioprotector/Post-irradiation administration/Mineral-yeast/Mice.

In vivo radioprotection of C3H mice by i.p. administration of Zn-, Mn-, Cu-, or Se-containing heat-treated *Saccharomyces serevisiae* yeast sample was examined. The 30-day survival of the group treated 30 min before 7.5 Gy whole-body X-irradiation with mineral-containing yeast powders suspended in 0.5% methylcellulose was significantly higher than that of control group. When mineral-yeast was administered immediately after irradiation, the survival rate was even higher and Zn- or Cu-yeast showed the highest rate (more than 90%). Although treatment with simple yeast showed a high survival rate (73%), it was significantly lower than that obtained by the Zn-yeast treatment. The effects of Zn-yeast were studied further. When the interval between irradiation and administration was varied, the protective activity of Zn-yeast decreased gradually by increasing the interval but was still significantly high for the administration at 10 h post-irradiation. The dose reduction factor of Zn-yeast (100 mg/kg, i.p. administration immediately after irradiation) was about 1.2. When the suspension of Zn-yeast was fractionated by centrifugation, the insoluble fraction showed a potent effect, while the soluble fraction had only a moderate effect. In conclusion, mineral-yeast, especially Zn-yeast, provides remarkable post-irradiation protection against lethal whole body X-irradiation. The activity is mainly attributable to the insoluble fraction, whereas some soluble components might contribute to the additional protective activity.

INTRODUCTION

Radiotherapy is one of the most effective treatments for cancer and its importance has increased in recent years. For effective treatment, the target tissue should be irradiated with a sufficient dose, which always increases the risk of radiation injury to surrounding normal tissues other than the target cancer cells. Therefore, finding ways to decrease the risk to normal tissues is a fundamental requirement to improve the outcome of radiation therapy. Using radioprotective agents is one way of decreasing damage to normal tissues, and various radioprotective compounds have been reported.[1–3)] We have previously reported on compounds such as edaravone[4)] and PROXYLs[5)] as pre-radiation radioprotectors.

Another group of radioprotector/mitigator compounds are those used for protection against accidental overexposure.[6)] Since individuals are unintentionally exposed in accidents, agents to reduce radiation damage to normal tissues can only be given after receiving radiation exposure. Relatively few agents have been reported so far to be effective when administered after irradiation. Among these agents are OK-432,[7,8)] heat-killed *Lactobacills casei*,[9,10)] and tocopherol-monoglucoside (TMG).[11–14)]

Although normal tissues are critical in both radiation therapy for cancer and accidental overexposure, the approach and compounds to reduce normal tissue damage may differ.[15)] It was shown that treatment with essential metalloelement (Zn, Fe, Mn, and Cu) chelates facilitate tissue repair processes required for recovery from radiation injury, including survival of lethally irradiated mice and rats.[16)] In the present study, we examined a new type of mineral containing compounds, heat-killed mineral-yeasts. These yeast preparations showed remarkable enhancement of survival of mice when administered after X-irradiation, suggesting that they may be useful for accidental overexposure.

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MATERIALS AND METHODS

Chemicals

A variety of mineral-containing heat-killed yeast powders were obtained from Omnica Co. Ltd. (Tokyo, Japan). According to the supplier's information, *Saccharomyces cerevisiae* was cultured in medium containing steam-sterilized syrup, wort, phosphate, ammonium sulfate, potassium chloride, and zinc sulfate (in the case of Zn-yeast). The cultured yeast suspension was purified by repeated centrifugation and resuspension in water to yield pure yeast suspension. The yeast suspension was sterilized at 95°C for 1–3 h. After ultra-sonic drying of the heat-killed yeast suspension, it was pulverized by a spray dryer. Selenium methionate, manganese sulfate, or copper gluconate were added instead of zinc sulfate for Se-yeast, Mn-yeast, and Cu-yeast, respectively. The content of Zn, Mn, Cu, and Se in Zn-, Cu-, Mn-, and Se-yeast was reported by the supplier to be more than 10%, 5%, 5%, and 0.2%, respectively. We examined the content of Zn and Cu in Zn- and Cu-yeast, respectively, by X-ray absorption analysis and confirmed their content. Methylcellulose was obtained from Wako Co. Ltd. (Osaka, Japan). Other reagents were of analytical grade and used without further purification. The yeast powder was pulverized finely with a mortar and pestle, 0.5% methylcellulose solution was added to the powder, and the suspension was mixed well. The turbid suspension was directly administered i.p. to mice.

Fractionation of Zn-yeast Suspension

In one experiment, the Zn-yeast suspension in 0.5% methylcellulose was fractionated into two fractions by centrifugation. The suspension (8.33 mg/ml) was centrifuged at 1,500 × g for 10 min at room temperature. The supernatant was removed and the insoluble fraction was re-suspended in the same volume of 0.5% methylcellulose solution. Both supernatant (soluble fraction) and re-suspended fractions (insoluble fraction) were used for survival experiments.

Animals

The mice used in the present study were treated and handled according to the Recommendations for the Handling of Laboratory Animals for Biomedical Research compiled by the Committee for Safety and Handling Regulations for Laboratory Animal Experiments in our institute. Male C3H mice were obtained from Japan SLC Co. (Hamamatsu, Japan). The mice, received at 8 weeks of age, were housed five per cage and allowed free access to a commercial diet (MB-1, Funabashi Farm Co., Funabashi, Japan) and acidified water during the experimental period. The animal rooms were maintained on a 12 h light-dark cycle, at an air temperature of 23 ± 1°C, and a humidity of 55 ± 5%. The mice were 65–75 days old at the time of irradiation and weighed 24–29 g.

X-irradiation of Mice

Each mouse was weighed and an average weight was calculated for each injection group. Usually, a 0.3 ml volume of yeast suspension was administered i.p. before or after X-irradiation. A group of 10 mice were transferred to a round Lucite container (12 rooms, 23 cm in diameter, 4 cm high). The container was placed on the stand of an irradiator (Pantak HF-320, Shimadzu, Kyoto, Japan), and the mice were irradiated at various whole-body doses with X-rays at 200 kV and 20 mA with a filter (0.5 mm Cu/0.5 mm Al). The radiation dose was determined with a dose meter placed in a compartment of the container. The dose rate used was about 0.55 Gy/min. Throughout most of our experiments, the radiation dose was set at 7.5 Gy. Immediately after irradiation, the mice were separated into groups of 5 and assessed daily for survival for 30 days.

Statistical Analysis

Data were statistically analyzed using the Stat View J4.5 software (Abacus Concepts, Berkeley, CA, USA). For the survival data (Figs. 1, 2 and 3), Kaplan-Meier plots were analyzed with Breslow-Gehan-Wilcoxon's test. For the dose dependency data (Fig. 4), unpaired t-test was used. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Figure 1 shows a Kaplan-Meier plot of survival of mice after 7.5 Gy whole-body X-ray irradiation. The mice were i.p. injected with mineral-yeasts 30 min prior to irradiation. The control group (methylcellulose solution injected group) showed a typical pattern of bone marrow death. The survival at 30 days was about 7%. Intraperitoneal administration of several mineral-yeasts (100 mg/kg body weight (bw)) 30 min prior to irradiation significantly delayed the incidence of death and increased the survival fraction at 30 days. The effect was highest for Zn- and Cu-yeast (88 and 89%, respectively), while the effect was moderate for Mn- and Se-yeast (68 and 63%, respectively).

Figure 2 shows the 30-day survival of mice after 7.5 Gy whole-body X-ray irradiation, where mineral-yeast or simple yeast (without additive-mineral) was i.p. injected immediately after irradiation. Surprisingly, Zn- and Cu-yeast showed very potent effects even by post-irradiation administration and the survival fractions at day 30 were 90% and 91%, respectively. These values were even higher than the values obtained for the mice administered 30 min prior to irradiation as shown in Fig. 1. In addition, the effect of Zn-yeast was significantly higher than that of the simple yeast ($p < 0.05$, Breslow-Gehan-Wilcoxon test), although the simple yeast still showed high activity (30 day survival of 72%).

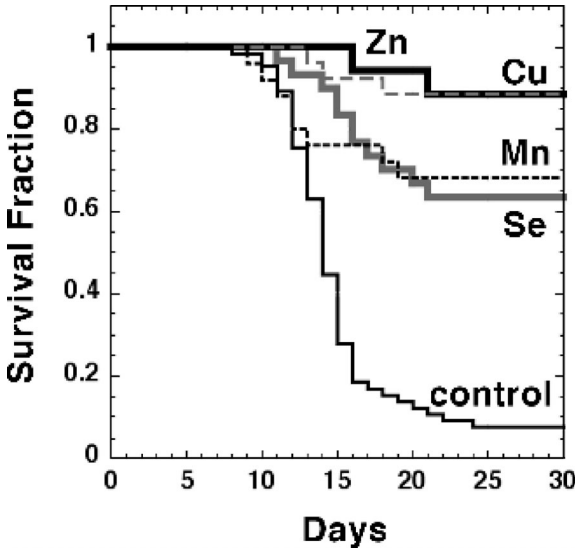

Fig. 1. Survival curve of mice after 7.5 Gy whole-body X-irradiation with intraperitoneal administration of various mineral-yeasts 30 min prior to the irradiation. The 30-day survival of control mice (without injection of mineral-yeast) was about 7%. The survival of mice to which mineral-yeast was administered 30 min before irradiation was 63–89%. The number of mice used was 17, 26, 25, 30, and 65 for Zn-, Cu-, Mn-, Se-yeast, and methylcellulose control, respectively.

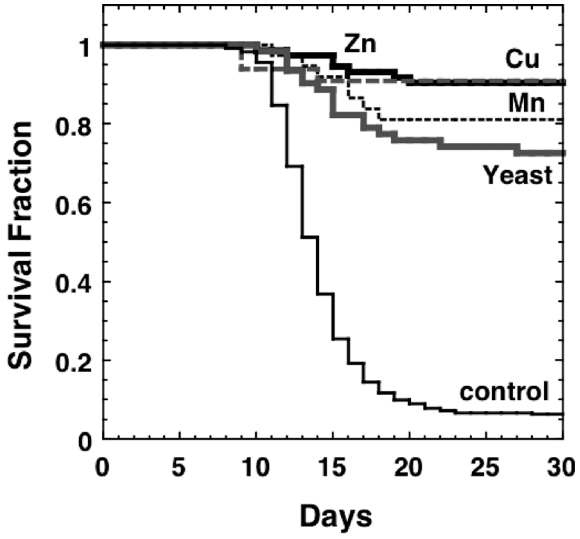

Fig. 2. Survival curve of mice after 7.5 Gy whole-body X-irradiation with intraperitoneal administration of various mineral-yeasts immediately after irradiation. The 30-day survival of control mice was about 6%. The survival of mice to which mineral-yeast was administered immediately after irradiation was 81%, 91%, and 90% for Mn-, Cu-, and Zn-yeast, respectively. Even the mice administered with yeast with no additive-mineral showed a high survival rate (73%). The survival of the Zn-yeast treated group is significantly higher than that of the yeast treated group ($p < 0.05$, Breslow-Gehan-Wilcoxon's test). The number of mice used was 73, 33, 37, 62, and 334 for Zn-, Cu-, Mn-yeast, yeast, and methylcellulose control, respectively.

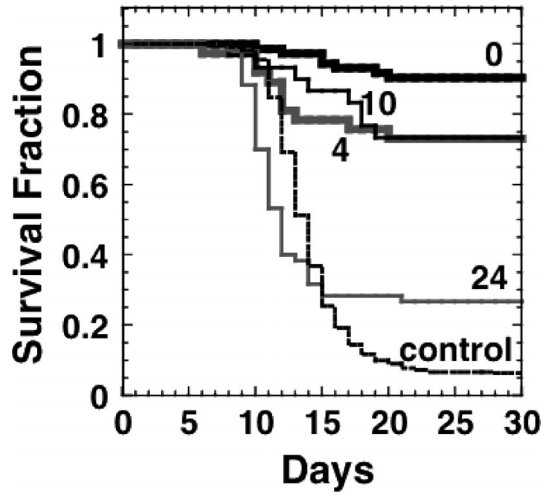

Fig. 3. Survival curve of mice after 7.5 Gy whole-body X-irradiation with intraperitoneal administration of Zn-yeast at various times after irradiation. The 30-day survival of control mice was about 6%. The survival rates of mice to which Zn-yeast was administered immediately (0 h), 4 h, 10 h, and 24 h after irradiation were 90%, 73%, 73%, and 27%, respectively. The survival of the groups treated with Zn-yeast at 0, 4, 10, and 24 h after irradiation was significantly higher than that of the control group ($p < 0.001$, Breslow-Gehan-Wilcoxon's test). The number of mice used was 113, 37, 30, and 60 for the groups of Zn-yeast administration at 0 h, 4 h, 10 h, and 24 h, respectively. The number of mice used for the methylcellulose control was 398.

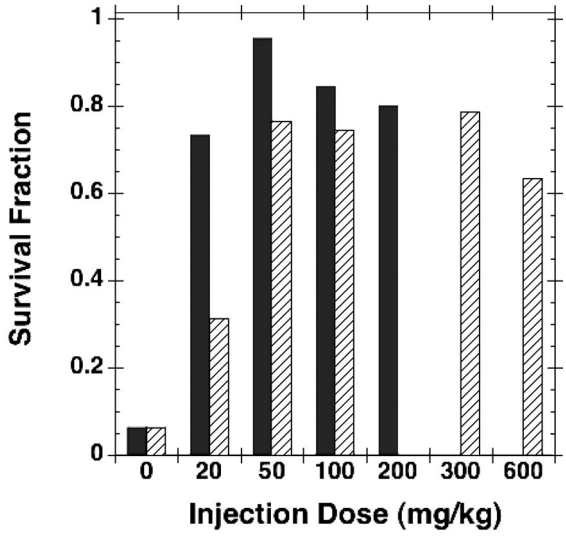

Fig. 4. Effect of dose of Zn- or Se-yeast on 30-day survival of mice. Zn-yeast (closed bar) or Se-yeast (hatched bar) was injected i.p. immediately after whole-body irradiation of 7.5 Gy. The survival of the groups treated with Zn-yeast at 50 and 100 mg/kg was significantly higher ($p < 0.05$) than that of the 20 mg/kg group. The survival of the groups treated with Se-yeast at 50, 100, and 300 mg/kg was significantly higher ($p < 0.05$) than that of the 20 mg/kg group. The number of mice used was 45, 22, 73, and 20 for the groups of Zn-yeast at 20, 50, 100, and 200 mg/kg, respectively, and 16, 17, 37, 47, and 82 for Se-yeast at 20, 50, 100, 300, and 600 mg/kg, respectively.

The effects of Zn-yeast were studied further. We examined the effect of time interval between irradiation and administration of Zn-yeast. As shown in Fig. 3, Zn-yeast was still significantly effective when administered 10 h after irradiation. Even the administration 24 h after irradiation gave higher survival than the control.

Figure 4 shows the effect of injection dose of Zn-yeast and Se-yeast administered immediately after irradiation on survival. The dependency is relatively flat for both Zn-yeast and Se-yeast and the maximum was around 50–100 mg/kg bw.

Mice were irradiated with various doses of X-rays, and Zn-yeast (100 mg/kg bw) was administered i.p. immediately after irradiation. For probit plot, the values of the survival fraction (x%) at various doses were converted to log (x/(100–x)) and plotted as a function of radiation dose (Fig. 5). When x = 50%, the value of log (x/(100–x)) is zero; therefore, the crossing point of the zero line and the linear regression line of the data shows the dose of 50% survival at 30 days ($LD_{50/30}$). The Zn-yeast treated group showed significantly higher $LD_{50/30}$ (8.15 Gy) than the control group (6.69 Gy). The slope of the two groups was similar. Based on these $LD_{50/30}$ values, the dose reduction factor (DRF) of Zn-yeast administered immediately after irradiation at 100 mg/kg is about 1.2.

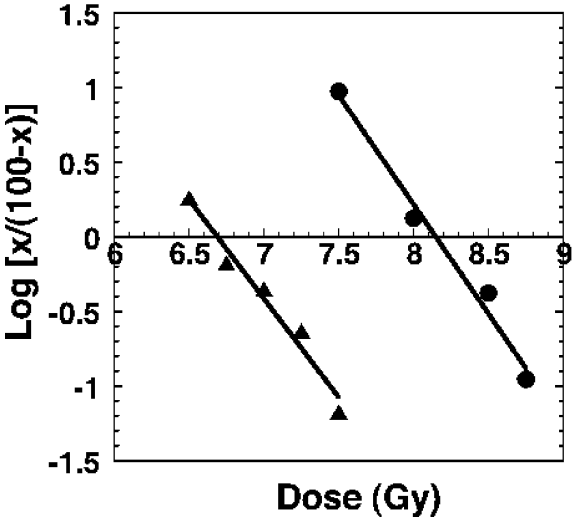

Fig. 5. Dependency of Survival on Radiation Dose. (●) Zn-yeast (100 mg/kg) was administered i.p. immediately after irradiation. (▲) Methylcellulose solution (0.3 ml) was administered i.p. to the control group. For probit plot, the 30-day survival (x%) was converted to log [x/(100–x)] for the vertical axis. In this plot, x = 50% corresponds to 0 on the vertical axis. $LD_{50/30}$ can be estimated at the crossing point of the horizontal axis and the linear regression line. $LD_{50/30}$ of Zn-yeast and control were 8.15 and 6.69 Gy, respectively. The number of mice for each point of Zn-yeast was 73, 28, 27, and 10 for 7.5, 8.0, 8.5, and 8.75 Gy, respectively (r = 0.984). The number of mice for each point of the methylcellulose control was 17, 30, 81, 20, and 334 for 6.5, 6.75, 7.0, 7.25, and 7.5 Gy, respectively (r = 0.983).

To address the mechanism of action of the mineral-yeasts, the Zn-yeast suspension was separated into soluble and insoluble fractions by centrifugation. Figure 6 shows the effect of each fraction in comparison to the control. The insoluble fraction showed significantly higher activity than the soluble fraction. The soluble fraction showed only moderate radioprotection, which was about a half that of the insoluble fraction.

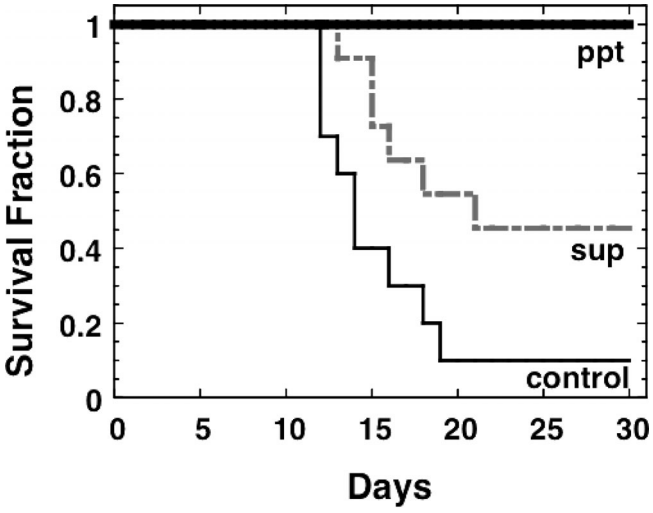

Fig. 6. Survival curve of mice after 7.5 Gy whole-body X-irradiation with intraperitoneal administration of fractionated Zn-yeast. The suspension of Zn-yeast in 0.5% methylcellulose (8.33 mg/ml) was fractionated by centrifugation. The supernatant fraction was removed and the precipitate fraction was re-suspended in the same volume of methylcellulose solution. The supernatant fraction (sup; dotted line) or the re-suspended precipitate fraction (ppt; bold solid line) corresponding to 100 mg/kg of Zn-yeast suspension was administered i.p. to mice immediately after 7.5 Gy whole-body X-irradiation. For reference, unfractionated Zn-yeast or methylcellulose (control) was administered immediately after X-irradiation. The survival curve after administration of unfractionated Zn-yeast suspension (100 mg/kg) overlaps the curve of bold solid line. The curve obtained after administration of methylcellulose solution was shown as narrow solid line. The survival of mice to which the supernatant fraction, precipitated fraction, unfractionated Zn-yeast and methylcellulose solution were administered was 45%, 100%, 100%, and 10%, respectively. The number of mice used was 11, 11, 11, and 10, respectively.

DISCUSSION

In the present study, we showed that mineral yeasts, especially Zn-yeast, are potent post-irradiation protector/mitigators acting to prevent bone marrow death. Only a few compounds have been reported so far to show radioprotection when administered after irradiation, for example glucan,[3] heat-killed *Lactobacillus casei*,[9] and tocopherol-monoglucoside.[14] In addition, the activity of Zn-yeast as a

post-irradiation protector is remarkably high, showing that mineral-yeasts are interesting subjects for further study and for practical use.

Among the mineral-yeasts, Zn- and Cu-yeasts showed the most potent activities. It was reported that pre-treatment with Zn(DL-asparatate)$_2$ (40 mg/kg = 92 μmol/kg) protect mice against the lethal effects of radiation, raising the LD_{50} from 8 Gy to 12.2 Gy.[17] This radiation protective effect was reportedly equivalent to cysteamine and slightly inferior to S,2-aminoethylisothiourea (AET). It was also reported that $ZnCl_2$ (10–20 mg/kg = 73–146 μmol/kg) were effective but about 3–4 fold less than with optimal doses of Zn(DL-aspartate)$_2$. We made a plan to examine radiation protector/mitigator effects of $ZnCl_2$, $ZnSO_4$, and Zn(gluconate)$_2$. Since Zn-yeast contained 10% Zn and 100 mg/kg of Zn-yeast was mainly used, the concentration of 21 mg/kg $ZnCl_2$, 44 mg/kg $ZnSO_4$•$7H_2O$, or 70 mg/kg Zn(gluconate)$_2$ was chosen for the experiments (corresponding to 10 mg Zn/kg = 153 μmol/kg) and these Zn-salts were i.p. administered to mice (5 to each Zn salts) in the preliminary experiment to know acute toxicity. All mice died within 19 days, showing that the Zn salts were toxic. Therefore, we did not perform further experiments to examine radiation protection/mitigation effects of these Zn salts. It was reported that Cu(aspartate)$_2$ was ineffective for radioprotection.[17] In contrast, Cu-yeast in the present study was very effective having similar activity with Zn-yeast. In addition, Zn(aspartate)$_2$ was reportedly ineffective when treated after irradiation,[16] whereas both Zn- and Cu-yeast when treated after irradiation were effective in the present study. All together, it is reasonable to say that the reaction mechanism of Zn-yeast and Zn(aspartate)$_2$ is different.

Simple yeast powder containing no additive minerals also showed significant post-irradiation radioprotection. Furthermore, the precipitated fraction of the suspension of Zn-yeast had very high radioprotective activity. Combining these findings, it is plausible that some insoluble components of the Zn-yeast suspension (possibly cell wall components) are mainly responsible for the radioprotective activity of Zn-yeast. Although the major part of the radioprotective activity of Zn-yeast was derived from the insoluble fraction, the soluble fraction also showed moderate activity (Fig. 6). This is consistent with the finding that Zn-yeast had significantly higher activity than simple yeast. We might be able to postulate a factor, possibly some Zn-related compounds, which produce the additive radioprotective activity in Zn-yeasts.

The DRF of 1.2 obtained for Zn-yeast is impressive considering that it is obtained for administration immediately after irradiation. The DRF value has seldom been reported for post-irradiation radioprotectors.

It was reported that glucan (β-1,3-polyglucose), a component of the cell wall of yeast, has radioprotective activity[3] and it is effective even after irradiation. However, no precise data had been shown for the post-irradiation activity. Since insoluble fraction had very high activity in the present study, glucan is a possible component for the activity. Glucan was reported to act as an immunomodulator, and a macrophage-mediated mechanism contributed to the survival enhancement.[3] It is possible that a main reaction mechanism of mineral-yeasts might also be as an immunomodulator to modulate macrophage-mediated reactions.

Methylcellulose as well as carboxymethylcellulose is often used to stably disperse water-insoluble drugs in water. Methylcellulose used in the present study has a poly-glucose structure (β-1,4) like glucan (β-1,3). However, methylcellulose had no radioprotective effect by itself; the $LD_{50/30}$ value (6.69 Gy, Fig. 5) was almost equal to that of saline (6.6 Gy).[4] The difference in the β-1,3 structure (glucan) and β-1,4 structure (methylcellulose) appears to be critical for the radioprotective activity.

To develop mineral-yeasts, especially Zn-yeast, for practical use, more studies are necessary to reveal the exact reaction mechanism. In addition, several other routes of administration should be examined, and these are now under investigation.

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