REACTIVE OXYGEN SPECIES, NITRIC OXIDE, AND INTRACELLULAR Ca^{2+} PARTICIPATE IN FLUORIDE-INDUCED YEAST CELL DEATH

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ABSTRACT: Numerous studies have indicated that the chronic intake of an excessive dose of fluoride has harmful physiological effects and fluorosis has been confirmed in some models. However, the mechanism underlying fluorosis has not been elucidated. In this study, we investigated the mechanism of fluoride-induced yeast cell death. For this, cultured yeast cells were treated with sodium fluoride (NaF) at concentrations of 0, 15, 30, 60, 90, 120, and 150 mM NaF for up to 24 hr. The results showed that NaF inhibits yeast cell growth, reduces cell viability, inhibits cell division, and increases the levels of reactive oxygen species (ROS), nitric oxide (NO), and intracellular Ca^{2+} in yeast cells. The inhibitory effect of NaF on yeast cells is blocked by antioxidants (ascorbic acid), Ca^{2+} antagonists (EGTA), and NO scavengers (hemoglobin). Taken together, these results indicate that intracellular ROS, NO, and Ca^{2+} participate in NaF-induced yeast cell death. Our findings elucidate the signal transduction pathways mediating the toxicity of fluoride, a common drinking water contaminant, and provides strong evidence that is relevant tor the study of the effects of fluoride on humans and the protection of humans from fluoride damage.

Keywords: Ca^{2+}; Cell death; NaF; NO; ROS; Yeast.

INTRODUCTION

Fluoride is widely distributed in the soil as well as in plants and animal tissues.[1] The main sources of fluorine are (i) drinking water, which, in many countries involves environmentally contaminated ground water, and in other countries, such as the USA and Canada, drinking water to which fluoride has been added for the purpose of reducing dental decay, (ii) air, and (iii) food.[2] An excessive intake of fluoride produces DNA damage and affects a variety of cell functions, including signal transduction and the cell cycle, resulting in the induction of cell death.[3-7] Furthermore, a number of studies have indicated that fluoride induces cell death in human and animal cell cultures.[8,9]

Yeast is a fungus that exists primarily as a single-celled organism.[10] In multicellular organisms, single-cell controlled suicide is of vital importance in growth and homeostasis as a mechanism for the removal of unwanted cells.[11,12] This mechanism may additionally enable the removal of injured cells that may damage biocompatibility.[11,12] *Saccharomyces cerevisiae* (*S. cerevisiae*), a budding yeast, is one of the most extensively researched eukaryotes at the cellular and molecular levels.[13]

S. cerevisiae, which is used to elucidate the molecular mechanisms underlying cell death, is a popular eukaryotic model owing to the numerous similarities between yeast and human cells.[14,15] Several studies have demonstrated that apoptosis in *S.*

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cerevisiae may be activated by various factors such as exposure to hydrogen peroxide (H_2O_2), acetic acid, pheromones, and aging inductors such as DNA copy stress, DNA injury repair defects, aging, and replication.[16-20] Yeasts and other eukaryotes exhibit ready transition from cell multiplication to cell death as a result of the action of several biochemical factors.[21]

Reactive oxygen species (ROS), nitric oxide (NO), and intracellular calcium ions (Ca^{2+}) are important signaling messengers, excessive levels of which lead to cell death.[22,23] In addition, arsenic-induced apoptosis of yeast cells has been shown to be associated with an increase in intracellular ROS and Ca^{2+}.[24] Fluorine and arsenic are considered critical inorganic pollutants of drinking water in China and around the world.[25] Our previous studies have shown that increased ROS levels are associated with fluoride-induced cardiomyocyte death.[26]

However, it remains unclear whether NaF-induced cytotoxicity is related to the accumulation of ROS, NO, and Ca^{2+}. Further, it is not known how ROS, NO, and Ca^{2+}signals participate in fluoride-induced yeast cell death. In this study, we investigate the mechanism by which fluoride induces *S. cerevisiae* death.

MATERIALS AND METHODS

Media and cell growth measurement: In this work, *S. cerevisiae* (CGMCC2.1882) (purchased from the Institute of Microbiology, Chinese Academy of Sciences) was used. This strain was cultured in yeast extract peptone dextrose (YPD) medium, which includes 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. Single colonies stored on the slopes were selected for activation in YPD liquid medium; then, an appropriate amount of the activated yeast solution was shaken in fresh liquid medium at 28ºC, 178 rpm, to log phase. The light absorption value of each concentration medium at OD_{600nm} was measured every 2hr, and a growth curve was obtained. The original OD_{600nm} of each medium was equal to or less than 0.1. The blank culture (0 mM NaF) was used as the control. The OD_{600nm} measurements of each culture were used to generate growth curves.

Cell viability assay: The cell survival rate was determined by methylene blue staining. The yeast suspension and methylene blue dye were mixed (v/v 1:1). Appropriate suspension drops were added to the blood count plates. After staining for approximately 3 min, the numbers of colored cells and total cells were counted using a light microscope, and the cell viability was calculated as follows:

$$\text{Cell viability (\%)} = 1 - \frac{\text{Number of colored cells}}{\text{Total number of cells}} \times 100$$

Measurement of cell resistance to NaF: Appropriate cultures, in the logarithmic phase, were cultured in liquid medium containing different concentrations (0, 30, 90, and 150 mM) of NaF at 28ºC with shaking at 178 rpm. Physiological saline-diluted NaF liquid medium (10-fold gradient dilution of 1, 10, 10^2, and 10^3) was prepared. Five microliters of each sample was spotted onto a solid medium plate (from high to low concentration). After the plate was incubated at 30ºC for 48 hours, colony growth was observed and photographed. The plates were incubated at 30ºC for 48 hr and colony growth was observed and photographed.

ROS measurement: ROS levels were measured using the fluorescent indicator dichlorofluorescein diacetate (DCFH-DA) according to the method of Chen et al.[27] *S. cerevisiae* were incubated with DCFH-DA (final concentration, 5 μM) in the dark at 29ºC for 28 min. Intracellular ROS production was detected by flow cytometry. Following co-treatment of the yeast cells with 90 mM NaF and 0.5 mM ascorbic acid (AsA), the OD_{600nm} of the yeast cells was measured using a spectrophotometer. In addition, the effect of inhibitor and NaF co-treatment on *S. cerevisiae* growth was determined by plate spotting.

NO assay: NO levels in *S. cerevisiae* were measured using a NO fluorescent probe (DAF-FM). Yeast cells were suspended in DAF-FM (final concentration, 5 μM) in the dark at 30ºC for 18 min, and intracellular NO fluorescence intensity was measured using flow cytometry. In addition, *S. cerevisiae* was exposed to 90 mM NaF and 2.0 μM hemoglobin, and the OD_{600nm} of the yeast cells was measured with a spectrophotometer. The plate spot method was also used to determine the effect of the inhibitor on the NaF tolerance of yeast cells.

Detection of intracellular Ca^{2+}: Intracellular Ca^{2+} levels was detected with the fluorescent indicator Fluo-3AM. Yeast cells were incubated with Fluo-3AM (final concentration, 5 μM) in the dark at 29ºC for 28 min. Intracellular Ca^{2+} production was detected by flow cytometry. In addition, *S. cerevisiae* was co-exposed to 90 mM NaF and 0.5 μM ethylenebis tetraacetic acid (EGTA), and the OD_{600nm} of the yeast cells was measured using a spectrophotometer. The effect of inhibitor and NaF co-treatment on *S. cerevisiae* growth was determined by plate spotting.

Statistical analysis: The data, which were expressed as mean ± SD, was the average number of individual studies which were carried out at least in triplicate. The Student's *t*-test was used to determine significant differences between the control and treatment groups, as well as between the remission (NaF + inhibitor) and NaF-alone treatment groups. $p<0.05$ was deemed to represent a significant difference, as denoted by *; $p< 0.01$ was considered to indicate an extremely significant difference, as denoted by **.

RESULTS

Effect of NaF on S. cerevisiae *growth:* The effects of various concentrations of NaF on the growth of *S. cerevisiae* were studied by determining the OD_{600nm} of blast cells. The growth of yeast cells was negatively correlated with the concentration of NaF (Figure 1). After 24 hours of exposure to 120 and 150 mM NaF, the growth of yeast cells was completely inhibited. This result is in contrast to the density of cells treated with low concentrations of NaF. These findings indicate that NaF inhibits *S. cerevisiae* growth in a concentration-dependent manner.

NaF-induced yeast cell death: S. cerevisiae cell death was detected following exposure to NaF (30, 90, and 150 mM) for 6–24 hr. NaF inhibited the viability of *S. cerevisiae* in a time- and concentration-dependent manner (Figure 2).

Yeast cell resistance to NaF: S. cerevisiae cell growth decreased with increasing fluoride concentrations (Figure 3). Exposure to 30, 90, and 150 mM NaF inhibited yeast growth. However, when NaF was diluted 10, 10^2, and 10^3-fold, fewer colonies appeared on the medium. In particular, exposure to 150 mM NaF (diluted 10^2–10^3-fold) completely inhibited yeast growth, and only small colonies grew on the

medium. These results indicate that NaF inhibits cell division and development. With increasing fluoride concentrations, the inhibitory effect was enhanced.

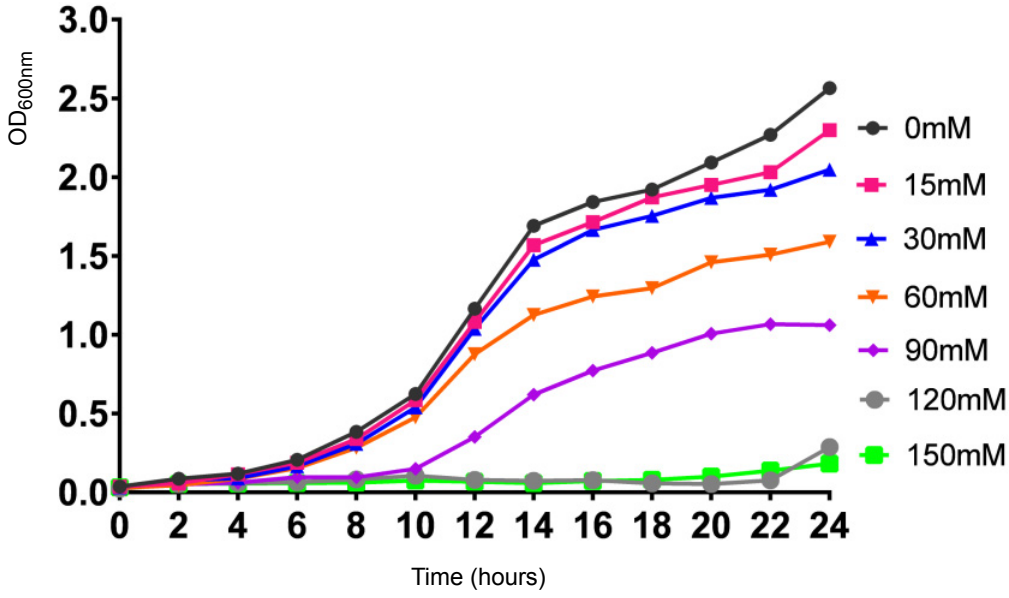


Figure 1. Growth curve of yeast cells exposed to various concentrations of sodium fluoride.

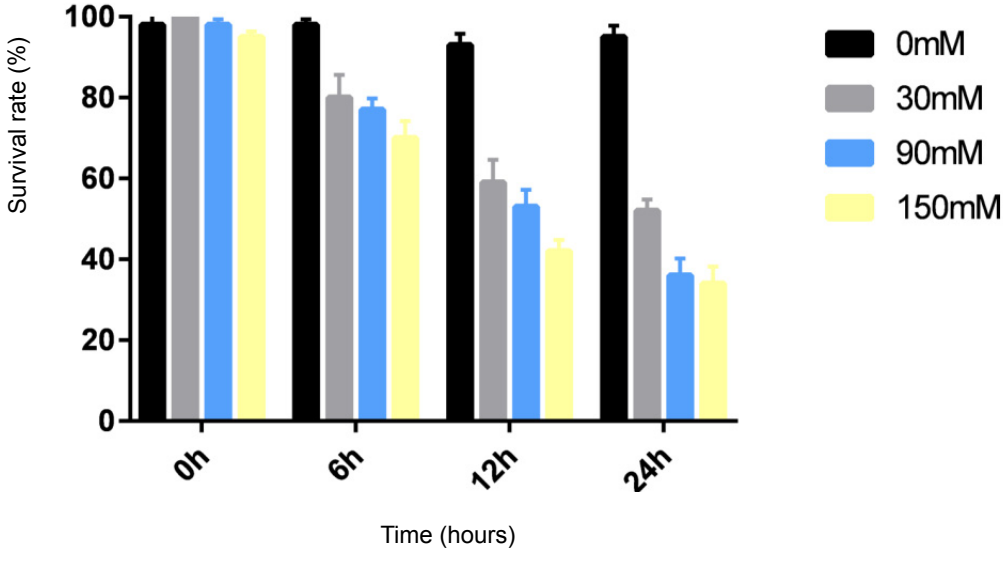


Figure 2. Effect of different concentrations of NaF and varying exposure time on yeast cell survival.

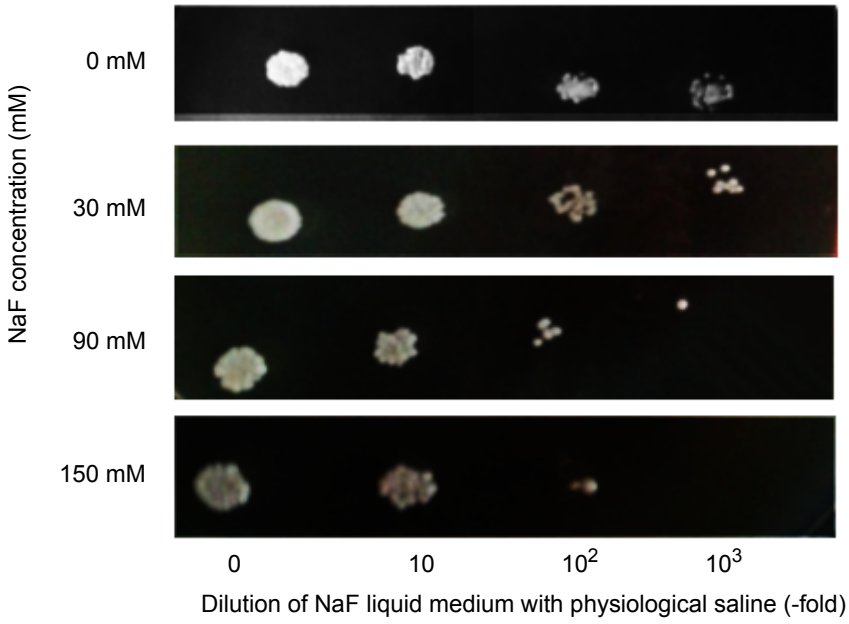


Figure 3. Tolerance of yeast to various concentrations of NaF.

NaF-induced intracellular ROS production in yeast cells: ROS production was elevated as the NaF concentration increased. After 24 hours of exposure to 30, 90, and 150 mM NaF, significant differences were found between the control and treated groups (Figure 4A).

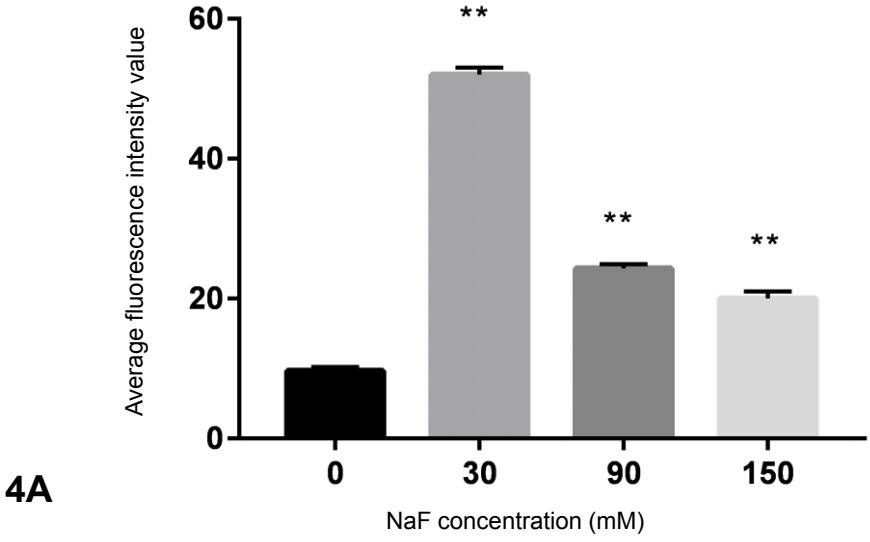
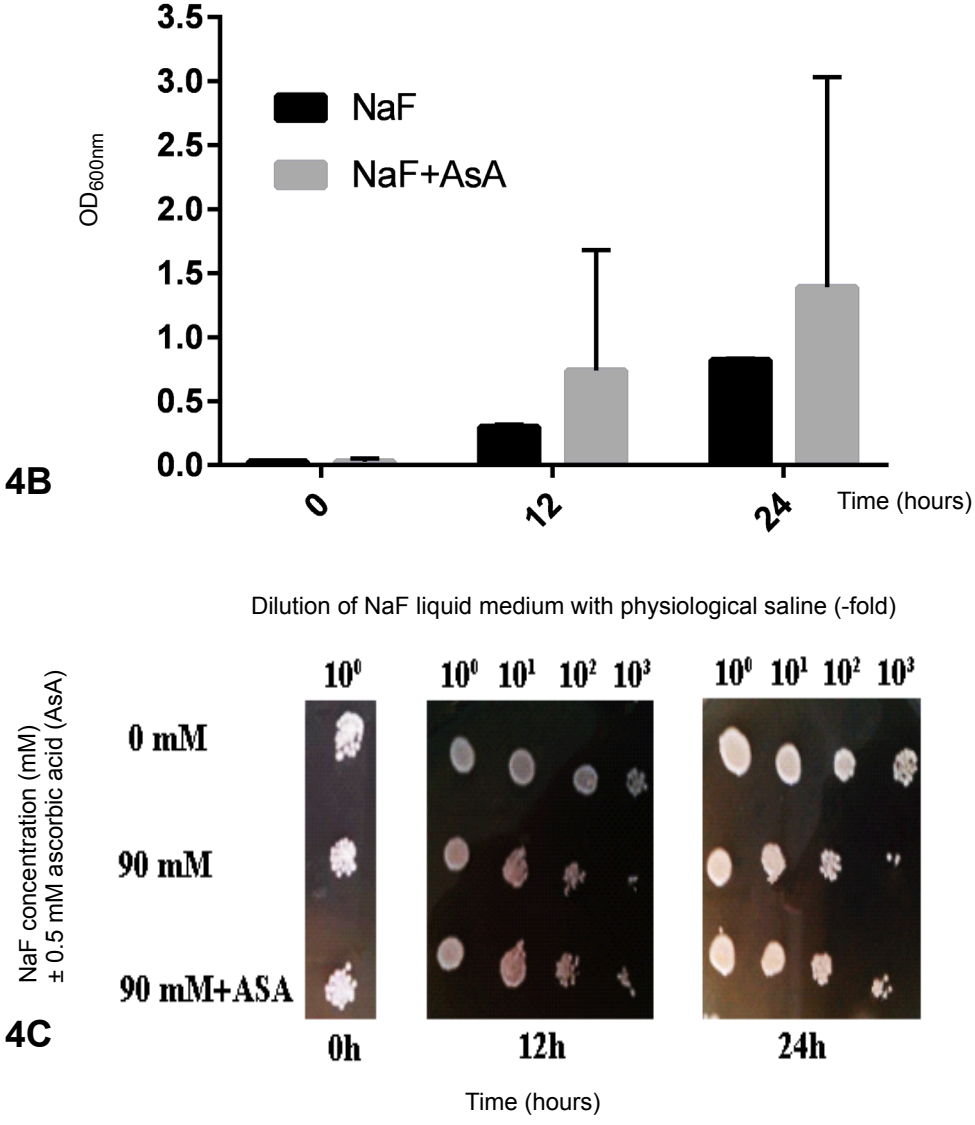


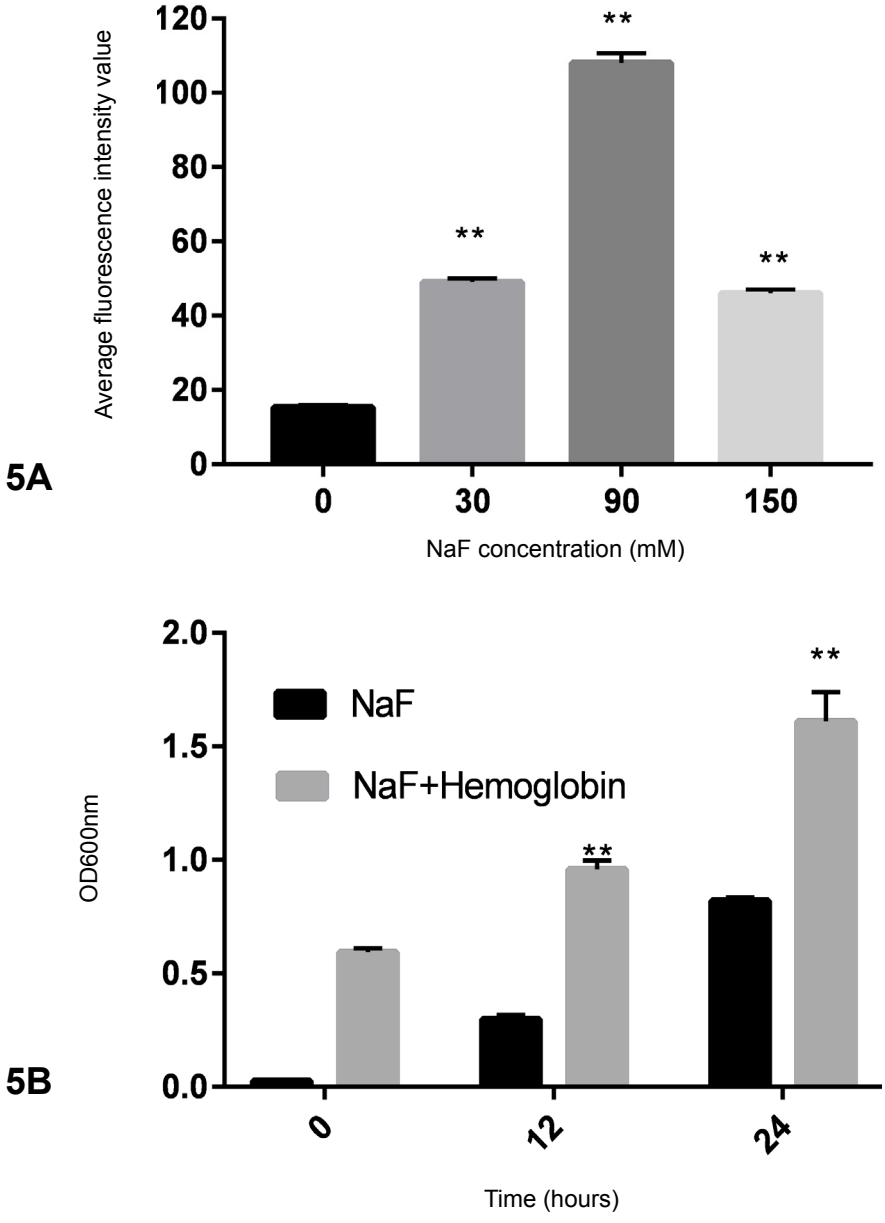
Figure 4A. ROS levels in yeast cells after 24 hours of treatment with NaF are expressed as mean fluorescence intensity. Compared with the control group: **p<0.01.

When 0.5 mM AsA was added together with 90 mM NaF, fluoride-induced cell death (including effects on yeast colony growth) was effectively prevented (Figures 4B and 4C). These data show that the ROS level in *S. cerevisiae* cells is positively correlated with cell death, indicating that elevated ROS levels significantly affect fluoride-induced *S. cerevisiae* death.

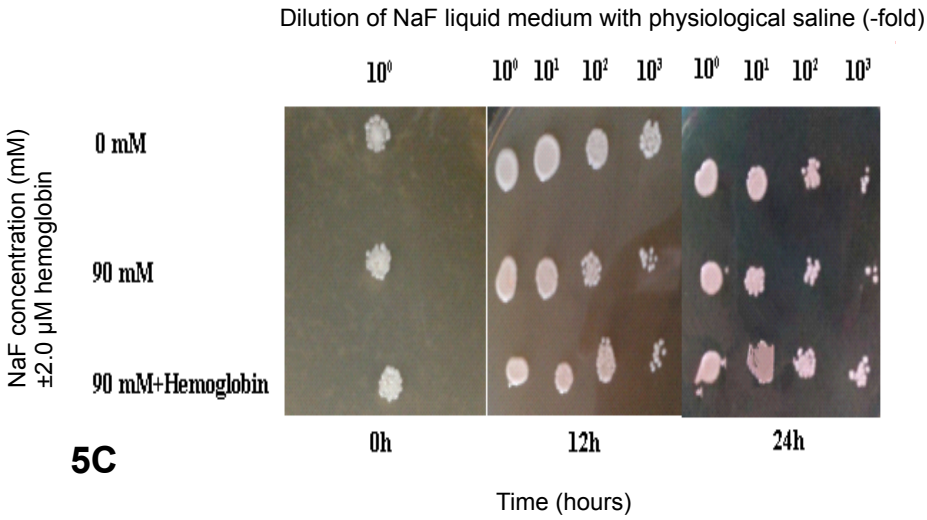


Figures 4B and 4C. Changes in the OD_{600nm} and colony growth of yeast cells exposed to 90 mM NaF with or without 0.5 mM ascorbic acid (AsA).

NaF-induced NO levels in yeast cells: NO production was elevated as the NaF concentration increased. After 24 hours of exposure to 30, 90, and 150 mM NaF, significant differences were observed between the control and treated groups (Figure 5A). When *S. cerevisiae* was treated with 90 mM NaF and 2.0 μM hemoglobin, fluoride-induced cell death (including effects on yeast colony growth) was effectively blocked (Figures 5B and 5C). These findings demonstrate that fluoride-induced *S. cerevisiae* death is associated with elevated NO levels.



Figures 5A and 5B. 5A: Average fluorescence intensity indicating NO levels in yeast cells after 24 hours of treatment with NaF; 5B: Changes in the OD_{600nm} of yeast cells exposed to 90 mM NaF with and without 2.0 μM hemoglobin. Compared with the control group: **p<0.01.



Figures 5C. Changes in the colony growth of yeast cells exposed to 90 mM NaF with and without 2.0 μM hemoglobin.

NaF-induced intracellular Ca^{2+} elevation in yeast cells: After 24 hours of treatment with 90 mM and 150 mM NaF, the average fluorescence intensity of intracellular Ca^{2+} was observably increased in *S. cerevisiae* (Figure 6A).

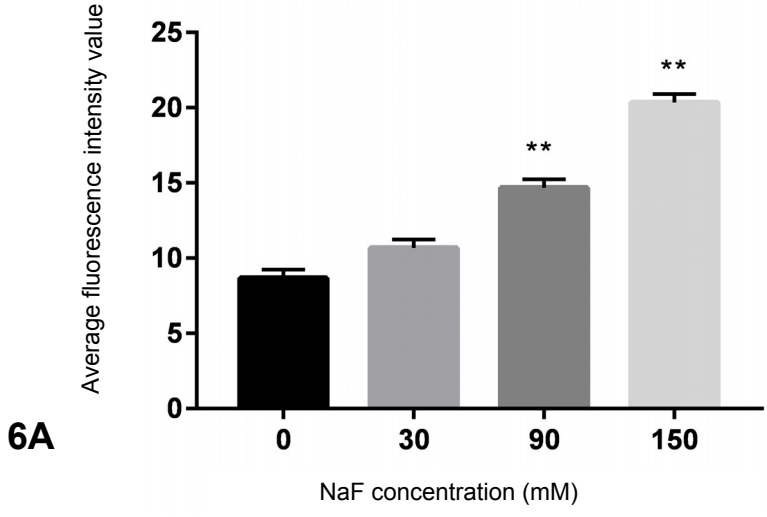
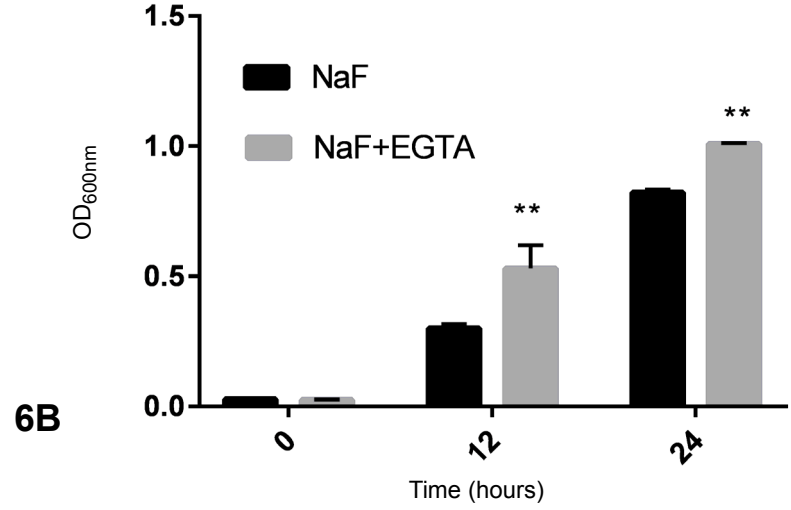
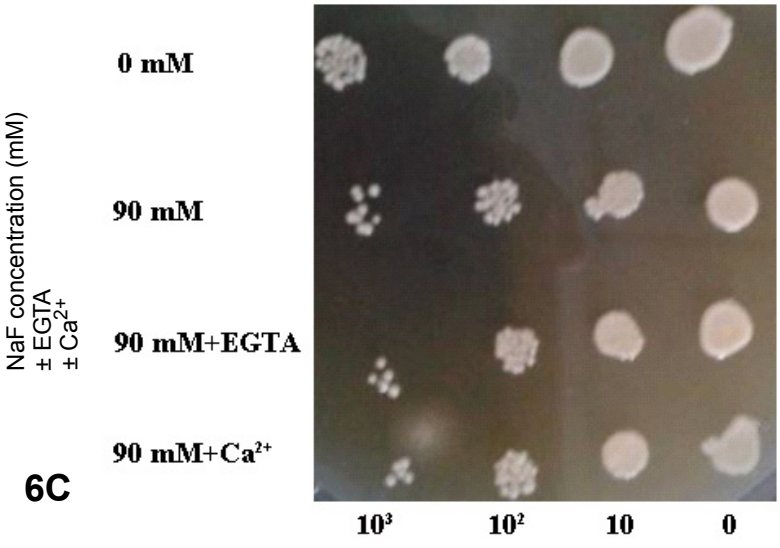


Figure 6A. Average fluorescence intensity indicating Ca^{2+} levels in *S. cerevisiae* after 24 hours of treatment with NaF. Compared with the control group: **p<0.01.

When *S. cerevisiae* was treated with 90 mM NaF and 0.5 μM EGTA, NaF-induced cell death (including effects on yeast colony growth) was blocked (Figures 6B and 6C). These results indicate that elevated calcium levels play a role in NaF-induced cell death in *S. cerevisiae*.





Figures 6B and 6C. Changes in the OD_{600nm} and colony growth of yeast cells exposed to 90 mM NaF with and without 0.5 μM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, egtazic acid] and Ca^{2+}. Compared with the control group: **p<0.01.

DISCUSSION

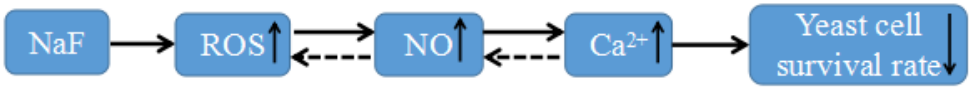
The present work aimed to obtain insights into the mechanism by which fluoride damages yeast cells and causes fluorosis. ROS and NO are cell metabolites whose synthesis is inducible by biological and abiotic stresses.[28,29] ROS, NO, and intracellular messenger Ca^{2+} play an significant role in many cellular functions involved in survival and proliferation.[30] The present work additionally shows that intracellular ROS, NO, and Ca^{2+}play an important role in fluoride-induced death in *S. cerevisiae*.

The results of this study indicate that there is a correlation between ROS, NO, and Ca^{2+} levels and sodium fluoride-induced yeast cell death, with an upstream-downstream relationship involved. As shown here, ROS levels were significantly increased at an NaF concentration of 30 mM, and NO and Ca^{2+} levels were significantly increased at NaF concentrations of 90 and 150 mM. These findings indicate that the possible signal transduction pathways of the three factors involved in yeast cell death are as follows: NaF exposure in yeast cells induces an increase in intracellular ROS levels, further inducing an increase in NO and Ca^{2+} levels.

Previous research has shown that ROS is generated before NO; then, NO induces Ca^{2+} release,[31, 32]which is consistent with the present findings. However, a number of studies have also found that Ca^{2+} is located upstream of ROS, and that there is an interaction between ROS and NO.[33,34] Therefore, it is hypothesized that the upstream signal promotes an increase in the downstream signal level, while the downstream signal exerts feedback regulation on the upstream signal, further stimulating the synthesis of the upstream signal.

CONCLUSIONS

In summary, our results indicate that ROS, NO, and Ca^{2+} are involved in NaF-induced cell death in yeast. A possible molecular mechanism by which NaF induces yeast cell death is as follows: ROS→NO→Ca^{2+}; this in turn activates downstream signal transduction pathways, thus regulating cell death. However, the mechanisms underlying NO and Ca^{2+} feedback regulation of ROS require further verification.



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CONFLICTS OF INTEREST

There are no conflicts of interest.

ETHICAL STATEMENT

In this research, none of the authors performed any studies with human participants or animals.

REFERENCES

1 Jha SK Mishra VK, Sharma DK, Damodaran T. Fluoride in the environment and its metabolism in humans. Reviews of Environmental Contamination & Toxicology 2011;211:121-42.

2 Dec K, Łukomska A, Maciejewska D, Jakubczyk K, Baranowska-Bosiacka I, Chlubek D, Wąsik A, Gutowska I.The influence of fluorine on the disturbances of homeostasis in the central nervous system. Biological Trace Element Research 2017;177:224-34.

3 Barbier O, Arreolamendoza L, Del Razo LM. Molecular mechanisms of fluoride toxicity. Chem Biol Interact 2010;188:319-33.

4 Whitford GM.The physiological and toxicological characteristics of fluoride. Journal of Dental Research 1990;69:539–49.

5 Jeng JH, Hsieh CC, Lan WH, Chang MC, Lin SK, Hahn LJ, Kuo MY. Cytotoxicity of sodium fluoride on human oral mucosal fibroblasts and its mechanisms. Cell Biology & Toxicology 1998;14:383-9.

6 Yang S, Wang Z, Farquharson C, Alkasir R, Zahra M, Ren G, Han B. Sodium fluoride induces apoptosis and alters bcl-2 family protein expression in MC3T3-E1 osteoblastic cells. Biochemical & Biophysical Research Communications 2011;410:910-5.

7 Nguyen Ngoc TD, Son YO, Lim SS, Shi X, Kim JG, Heo JS, Choe Y, Jeon YM, Lee JC. Sodium fluoride induces apoptosis in mouse embryonic stem cells through ROS-dependent and caspase- and JNK-mediated pathways. Toxicology & Applied Pharmacology 2012;259:329-37.

8 He LF, Chen JG. DNA damage, apoptosis and cell cycle changes induced by fluoride in rat oral mucosal cells and hepatocytes. World J Gastroenterol 2006;12:1144-8.

9 Tu W, Zhang Q, Liu Y, Han L, Wang Q, Chen P, Zhang S, Wang A, Zhou X. Fluoride induces apoptosis via inhibiting SIRT1 activity to activate mitochondrial p53 pathway in human neuroblastoma SH-SY5Y cells. Toxicol Appl Pharmacol 2018;3:122-4.

10 Zhao R Y, Zhao R Y. Yeast for virus research. Microbial Cell 2017;4:311-30.

11 Wang Y, Lo WC, Chou CS. A modeling study of budding yeast colony formation and its relationship to budding pattern and aging. Plos Computational Biology 2017;13:e1005843.

12 Azzopardi M, Farrugia G, Balzan R. Cell-cycle involvement in autophagy and apoptosis in yeast. Mechanisms of Ageing & Development 2016;161:211-24.

13 Rego A, Duarte AM, Azevedo F, Sousa MJ, Côrte-Real M, Chaves SR. Cell wall dynamics modulate acetic acid-induced apoptotic cell death of *Saccharomyces cerevisiae*. Microbial Cell 2014;1:303-14.

14 Eid R, Boucher E, Gharib N, Khoury C, Arab NT, Murray A, Young PG, Mandato CA, Greenwood MT. Identification of human ferritin, heavy polypeptide 1 (FTH1) and yeast RGI1 (YER067W) as pro-survival sequences that counteract the effects of Bax and copper in Saccharomyces cerevisiae. Experimental Cell Research 2016; 342:52-61.

15 Carmonagutierrez D, Bauer M A, Zimmermann A, et al. Guidelines and recommendations on yeast cell death nomenclature. Microbial Cell 2018;5:4-31.

16 Fabrizio P, Longo VD. The chronological life span of *Saccharomyces cerevisiae*. Methods in Molecular Biology 2007;371:89-95.

17 Kaeberlein M. Lessons on longevity from budding yeast. Nature 2010;464:513-9.

18 Walter D, Wissing S, Madeo F, Fahrenkrog B. The inhibitor-of-apoptosis protein Bir1p protects against apoptosis in S. cerevisiae and is a substrate for the yeast homologue of Omi/ HtrA2.Journal of Cell Science 2006;119:1843-51.

19 Madeo F, Herker E, Maldener C, Wissing S, Lächelt S, Herlan M, Fehr M, Lauber K, Sigrist SJ, Wesselborg S, Fröhlich KU. A caspase-related protease regulates apoptosis in yeast. Molecular Cell 2002;9:911-7.

20 Belanger KD, Walter D, Henderson TA, Yelton AL, O'Brien TG, Belanger KG, Geier SJ, Fahrenkrog B. Nuclear localisation is crucial for the proapoptotic activity of the HtrA-like serine protease Nma111p. Journal of Cell Science 2009;122:3931-41.

21 Lee M, Spokes KC, WC Aird, MR Abid. Intracellular Ca^{2+} can compensate for the lack of NADPH oxidase-derived ROS in endothelial cells. Febs Letters 2010;584:3131-6.

22 Wu SJ, Wu JY. Extracellular ATP-induced NO production and its dependence on membrane Ca^{2+} flux in *Salvia miltiorrhiza* hairy roots. Journal of Experimental Botany 2008;59:4007-16.

23 Nakamura T, Lipton SA. Preventing Ca^{2+}-mediated nitrosative stress in neurodegenerative diseases: possible pharmacological strategies. Cell Calcium 2010;47:190-7.

24 Wu L, Yi H, Zhang H. Reactive oxygen species and Ca^{2+} are involved in sodium arsenite-induced cell killing in yeast cells. Fems Microbiology Letters 2013;343:57-63.

25 Jiang S, Su J, Yao S, Zhang Y, Cao F, Wang F, Wang H, Li J, Xi S. Fluoride and arsenic exposure impairs learning and memory and decreases mGluR5 expression in the hippocampus and cortex in rats. Plos One 2014;9:e96041.

26 Yan X, Yang X, Hao X, Ren Q, Gao J, Wang Y, Chang N, Qiu Y, Song G. Sodium fluoride induces apoptosis in H9c2 cardiomyocytes by altering mitochondrial membrane potential and intracellular ROS level. Biological Trace Element Research 2015;166:210-5.

27 Chen SR, Dunigan DD, Dickman MB. Bcl-2 family members inhibit oxidative stress-induced programmed cell death in *Saccharomyces cerevisiae*. Free Radical Biology & Medicine 2003;34:1315-25.

28 Carraro M, Bernardi P. Calcium and reactive oxygen species in regulation of the mitochondrial permeability transition and of programmed cell death in yeast. Cell Calcium 2016;60:102-7.

29 Nasuno R, Aitoku M, Manago Y, Nishimura A, Sasano Y, Takagi H. Nitric oxide-mediated antioxidative mechanism in yeast through the activation of the transcription factor Mac1. Plos One 2014;9:e113788.

30 Kania E, Roest G, Vervliet T, Parys JB, Bultynck G. IP3 receptor-mediated calcium signaling and its role in autophagy in cancer. Frontiers in Oncology 2017;7:140-7.

31 Wang G, Coleman CG, Glass MJ, Zhou P, Yu Q, Park L, Anrather J, Pickel VM, Iadecola C. Angiotensin II type 2 receptor-coupled nitric oxide production modulates free radical availability and voltage-gated Ca^{2+} currents in NTS neurons. Am J Physiol Regul Integr Comp Physiol 2012;302:R1076.

32 Gao K, Liu M, Cao J, Yao M, Lu Y, Li J, et al. Protective effects of *Lycium barbarum* polysaccharide on 6-OHDA-induced apoptosis in PC12 cells through the ROS-NO pathway. Molecules 2014; 20:293-308.

33 Chen LD, Liu ZH, Zhang LF, Yao JN, Wang CF. Sanggenon C induces apoptosis of colon cancer cells via inhibition of NO production, iNOS expression and ROS activation of the mitochondrial pathway. Oncology Reports 2017;38:2123-31.

34 Zhao J, Fujita K, Sakai K. Reactive oxygen species, nitric oxide, and their interactions play different roles in *Cupressus lusitanica* cell death and phytoalexin biosynthesis. New Phytol 2007;175:215-29.