*Acta Medica Okayama*

*Volume* 44, *Issue* 6 1990 *Article* 2

DECEMBER 1990

# Effects of antioxidants on V79 Chinese hamster cells treated with ferric nitrilotriacetate.

Yoshiki Takehara* Tamotsu Yoshioka†

Masayoshi Namba‡

*Kurashiki Center for Adult Diseases,

†Kurashiki Center for Adult Diseases,

‡Okayama University,

# Effects of antioxidants on V79 Chinese hamster cells treated with ferric nitrilotriacetate.*

Yoshiki Takehara, Tamotsu Yoshioka, and Masayoshi Namba

## Abstract

The cytotoxic effects of ferric nitrilotriacetate (Fe-NTA) have been considered to be caused by free radicals produced by the drug. The present study was carried out to determine whether or not cytotoxic effects of Fe-NTA on cell growth and lipoperoxide formation of Chinese hamster cells were reduced by antioxidants. Using a spin trapping technique, we found that hydroxyl radical formation in the cells increased in the presence of Fe-NTA. Antioxidants, with the exception of superoxide dismutase, slightly inhibited production of the hydroxyl radical. Mannitol significantly reduced lipoperoxide formation, but other antioxidants did not. However, the growth inhibitory effects of Fe-NTA were not attenuated by these antioxidants. These results indicated that the cytotoxic effects of Fe-NTA may be mostly due to unknown factors other than oxygen free radicals.

**KEYWORDS:** V79 cells, ???NTA, antioxidants, cytotoxicity, free radicals

---

*PMID: 1963729 [PubMed - indexed for MEDLINE]

Acta Med Okayama 44 (6) 287-291 (1990)

# Effects of Antioxidants on V79 Chinese Hamster Cells Treated with Ferric Nitrilotriacetate

**Yoshiki Takehara, Tamotsu Yoshioka and Masayoshi Namba*,[a]**

*Medical Science Laboratory, Kurashiki Center for Adult Diseases, Kurashiki 710, Japan and [a]Department of Pathology, Institute for Cancer Research, Okayama University Medical School, Okayama 700, Japan*

**The cytotoxic effects of ferric nitrilotriacetate (Fe-NTA) have been considered to be caused by free radicals produced by the drug. The present study was carried out to determine whether or not cytotoxic effects of Fe-NTA on cell growth and lipoperoxide formation of Chinese hamster cells were reduced by antioxidants. Using a spin trapping technique, we found that hydroxyl radical formation in the cells increased in the presence of Fe-NTA. Antioxidants, with the exception of superoxide dismutase, slightly inhibited production of the hydroxyl radical. Mannitol significantly reduced lipoperoxide formation, but other antioxidants did not. However, the growth inhibitory effects of Fe-NTA were not attenuated by these antioxidants. These results indicated that the cytotoxic effects of Fe-NTA may be mostly due to unknown factors other than oxygen free radicals.**

*Key words :* **V79 cells, Fe-NTA, antioxidants, cytotoxicity, free radicals**

The ferric nitrilotriacetate (Fe-NTA) complex has been reported to have cytotoxic effects. Repeated administration of Fe-NTA to the rat peritoneum has caused the accumulation of iron in various organs such as the liver, spleen, kidney and pancreas and their dysfunction (1). Fe-NTA has induced tumors in the rat kidney and neoplastic transformation of cultured rat liver cells (2,3). In addition, Fe-NTA caused 6-thioguanine sensitive V79 Chinese hamster cells to mutate into 6 TG-resistant cells (4). Although the precise mechanisms of these biological effects of Fe-NTA are not yet well understood, free radicals produced by Fe-NTA may be involved (5). In fact, Fe-NTA induced lipid peroxidation of cell membranes and cosequently displayed cell toxicity (6), while radical scavengers such as $\alpha$-tocopherol or superoxide dismutase (SOD) suppressed the cytotoxicity (7). We did not, however, detect any protective effects of these scavengers against the cytotoxity. In this communication, we describe the effects of other radical scavengers on hydroxyl radical formation, lipid peroxidation of cell membranes, and the cell growth of V79 cells which were treated with Fe-NTA.

## Materials and Methods

*Materials.* 5,5-Dimethyl-1-pyrroline-N-oxide

* To whom correspondence should be addressed.

(DMPO) was purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan. Superoxide dismutase (SOD) and catalase were obtained from Sigma Chemical Co., Ltd., St. Louis, USA. Butylated hydroxytoluene and mannitol were purchased from Nakarai Tesque Co., Ltd., Tokyo, Japan. $\alpha$-Tocopherol was kindly provided by Eizai Co., Ltd., Tokyo, Japan.

*Preparation of Fe-NTA.* The Fe-NTA solution was prepared just before use. Fe $(NO_3)_3 \cdot 9H_2O$ and NTA were dissolved in distilled water and adjusted to pH 7.4 with 1M $Na_2CO_3$. The final iron concentration was 1 mg/ml and the molar ratio of Fe to NTA was 1 : 3.

*Assay of effects of Fe-NTA and antioxidants on colony formation frequency of V79 cells.* Two hundred cells were seeded into 60-mm dishes in the presence of various concentrations of Fe-NTA and antioxidants, either alone or in combination. The concentrations of each drug are shown in results. The cells were cultured for 7 days in Eagle's minimum essential medium (MEM; Nissui Seiyaku, Tokyo, Japan) supplemented with 10 % heat inactivated fetal calf serum (FCS). Cultures were maintained at 37 °C in a humidified incubator with 5 % $CO_2$. Dishes were fixed with 100 % methanol for 5 min, stained with 1 % Giemsa solution for 30 min, and colonies having more than 30 cells were counted. Triplicate dishes were used at each point in these assays. The cytotoxic effects of each chemical on colony formation of cells were expressed as the percentage of colony number in the untreated control cultures. Based on these data, concentrations of each antioxidant used in Figs 2 to 5 were at the highest showing no cytotoxicity.

*Radical analysis.* When cells in culture flasks (25 $cm^2$) became semiconfluent (*ca.* $3 \times 10^6$ cells), the medium was changed, and Fe-NTA was added to cultures with or without antioxidants. After 48 h, the cells were washed twice with 0.9 % NaCl and suspended in the NaCl solution by pipetting. Then we added 20 $\mu$l of DMPO to 200 $\mu$l of this cell suspension, placed the assay sample in a quartz cell (volume 160 $\mu$l, JEOL Ltd., Tokyo, Japan) and 1 min later determined electron spin resonance (ESR) spectra with a JOEL ESR spectrometer JES-FE1XG. The ESR spectrometer was used under the following conditions: field intensity 3343 + 50 gauss, power 8.0 mW, modulation 100 kHz, modulation width 2 × 1 gauss, response 0.1 sec, sweep time 0.5 min, at room temperature.

*Determination of lipoperoxides.* Lipoperoxides of the culture medium and cells were determined by the fluorometric assay of Yagi (8) using the "Lipoperoxides-test" (Wako Co., Osaka, Japan). We added 4.0 ml of 1/12 N $H_2SO_4$ to the sample (0.5 ml), mixed it well, and added 0.5 ml of 10 % (w/v) phosphotungstic acid to the assay mixture. After standing at room temperature for 5 min, the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was discarded and the precipitate was mixed with 2.0 ml of 1/12 N $H_2SO_4$ and 0.3 ml of 10 % phosphotungstic acid, and centrifuged at 3,000 rpm for 10 min. Then the precipitate was suspended in 4.0 ml of distilled water, and 1.0 ml of thiobarbituric acid reagent was added to it. After the mixture was heated in a boiling water bath for 60 min and cooled, 5.0 ml of n-butanol was added to it, and the mixture was shaken vigorously. After centrifugation at 3,000 rpm for 10 min, the organic layer was collected for fluorometric determination at an excitation wavelength of 515 nm and an emission wave length of 535 nm.

## Results

Fig. 1 shows the effects of Fe-NTA on the colony formation frequency of V79 cells. The colony formation was dose-dependently inhibited by Fe-NTA. Since treatment of cells with 50 $\mu$g/ml of Fe-NTA showed about 50 % reduction of the colony formation, we used this concentration in the following experiments.

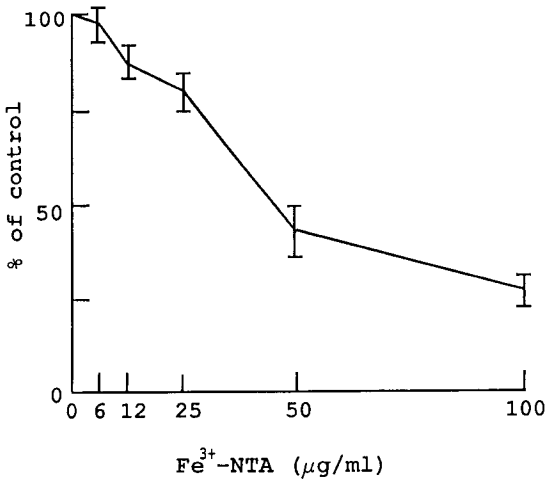


**Fig. 1** Effects of ferric nitrilotriacetate (Fe-NTA) on colony formation of V 79 cells. Ordinate: No. of colonies untreated with Fe-NTA/No. of colonies treated with Fe-NTA × 100 (%).

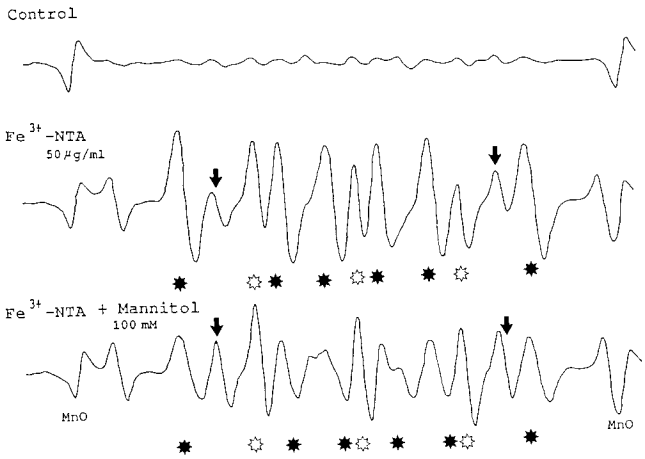


**Fig. 2** Electron spin resonance (ESR) spectra of 5.5-dimethyl-1-pyrroline-N-oxide (DMPO) spin adducts formed in V 79 cells. ↓; hydroxy radical, ✱; 6 line signal, ☼; nitroxide radical. Fe-NTA: See Fig. 1.

Hydroxyl radicals were detected in the cells treated with Fe-NTA by the spin trapping technique employing DMPO. The production of hydroxyl radicals in the cellls treated with Fe-NTA was 11 times greater than that in the control cells (Fig. 2). In the presence of Fe-NTA, catalase, mannitol, $\alpha$-tocopherol, and BHT caused a slight reduction (about 70%) in the production of hydroxyl radicals, but SOD had no effect at all. Three experiments were done and all of them showed the identical results. A representative of them is shown in Fig. 3.

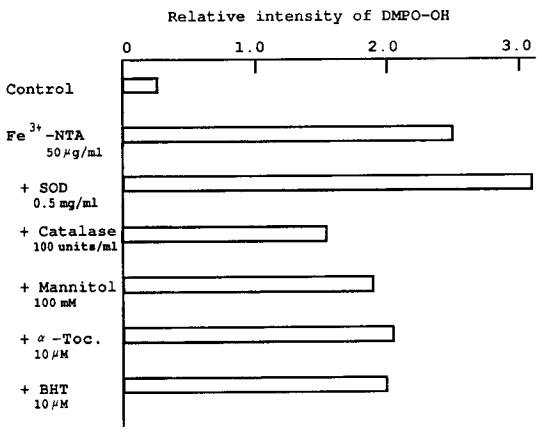


**Fig. 3** Effects of antioxidants on production of hydroxyl radicals in cells treated with Fe-NTA. Concentrations of the antioxidants which were not cytotoxic were determined by examining colony formation of cells. SOD: superoxide dismutase. Other abbreviations: See Figs 1 and 2.

When the cells were cultured with Fe-NTA for 48h, their lipoperoxide concentrations were found to be three times greater than those of the control cultures without Fe-NTA. These findings indicated that the lipid peroxidation in the cells was caused by oxygen radicals produced by Fe-NTA. The lipid peroxidation of Fe-NTA was reduced almost to the control level by mannitol, but was not significantly affected by other antioxidants such as SOD, catalase and BHT. $\alpha$-Tocopherol was relatively effective for prevention of the lipid peroxidation. The lipoperoxide in the medium with Fe-NTA, however, increased only 1.2 times over the control medium. The data of one of two experiments which exhibited similar results are shown in Fig. 4.

To determine whether or not antioxidants can reduce the cytotoxic effects of Fe-NTA, the colony formation of V79 cells was examined in medium containing Fe-NTA in the presence of each antioxidant. The result of three experiments

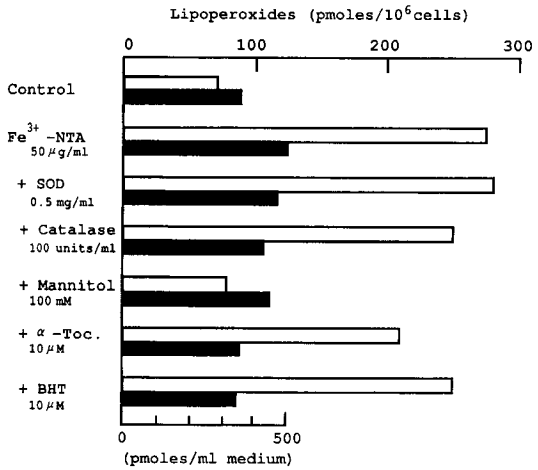


**Fig. 4** Effects of antioxidants on lipoperoxides produced by Fe-NTA. Lipoperoxides were in the cells (■), and in the cultured medium (□). Abbreviations: See Figs 1 and 3.

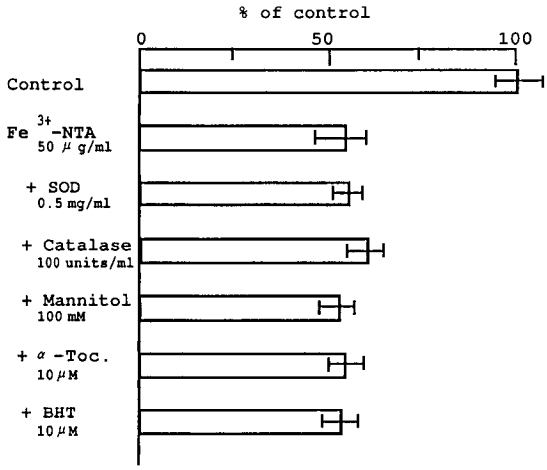


**Fig. 5** Effects of antioxidants on colony formation frequency of V79 cells treated with Fe-NTA. Each bar represents a standard deviation. Abbreviations: See Figs 1 and 3.

showed that no antioxidant attenuated the cytotoxicity of Fe-NTA (Fig. 5). These results indicated that the cytotoxic effects of Fe-NTA on cell growth might be due to mostly unknown factors other than the oxygen radicals produced by Fe-NTA.

## Discussion

The effects of antioxidants on hydroxyl radical formation, lipid peroxidation and the colony formation efficiency of V79 Chinese hamster cells treated with Fe-NTA were investigated. The antioxidants used in the present experiments were classified in terms of action as follows: SOD as scavenger of superoxide radicals, catalase as a scavenger of hydrogen peroxide, mannitol as one of hydroxyl radicals, and $\alpha$-tocopherol and BHT as scavengers of free radicals. Although the cytotoxic effects of Fe-NTA have been considered to be due to oxygen radicals produced by iron in the drug (5), none of the antioxidants used attenuated the cytotoxicity of Fe-NTA in terms of colony formation. However, catalase significantly reduced hydroxyl radical formation and mannitol strongly inhibited the production of lipoperoxides.

The cytotoxicity of Fe-NTA has been reported to be due to the generation of superoxide radicals (7). In addition, Aruoma *et al.* described that Fe-NTA induced the damage of the bases in DNA and this damage was reduced by SOD (9). Our present data showed that SOD did not reduce the DMPO-OH spin adduct but other antioxidants did . Klebanoff *et al.* also reported that the radical formation (DMPO-OH adduct) by $Fe^{2+}$ chelated with deferoxamine was not reduced by SOD (10). These findings suggested that superoxide radicals were not necessary for the formation of DMPO-OH spin adduct. In fact, Kawabata *et al.* described that Fe-NTA produced neither free-state superoxide radicals nor their protonated form, -OOH, while it yielded hydroxyl radicals in the presence of hydrogen peroxide (5).

As mentioned above, if the cytotoxicity of Fe-NTA is due to superoxide radicals, SOD should have attenuated the toxicity, but it did not. Even if SOD cannot enter the cells, it can metabolize superoxide radicals produced outside the cells and prevent the peroxidation of cell membranes. However, the lipoperoxides of the

cells treated with Fe-NTA were not reduced by SOD. The hydroxyl radicals metabolized from superoxide radicals by SOD may have caused the lipid peroxidation. If that is the case, the combined use of SOD and catalase may be effective in reducing the cytotoxicity.

The inhibitory effects of Fe-NTA on the colony formation of V79 cells were not reduced by the antioxidants used in this study, although some of them lowered hydroxyl radicals and lipoperoxides in the cells treated with Fe-NTA. These findings indicate that the cytotoxic effects of the drug may be mostly due to unknown factors other than oxygen free radicals. Since DMPO cannot trap all hydroxyl radicals produced under the present experimental conditions, the remaining radicals may be enough to inhibit the cell growth. It is also likely that the large amount of iron introduced into cells might disturb the balance of the intracellular levels of metal cations. This would modify the organization of chromatin and alter the activity of enzymes involved in DNA replication and repair, leading to genotoxic effects.

Acknowledgment. We thank Dr. Midori Hiramatsu of Okayama University Medical School for her support and detection of DMPO-OH ESR. We also thank Dr. Teruyuki Kawabata of Okayama University Medical School for reading this manuscript and his valuable suggestions.

This work was supported by funds from the Japan Keirin Association.

## References

1. Awai M, Narasaki M, Yamanoi, Y and Seno, S: Introduction of diabetes in animals by parenteral administration of ferric nitrilotriacetate: A model of experimental hemochromatosis. Am J Pathol (1979) **95**, 663–674.
2. Ebina Y, Okada S, Hamazaki S, Ogino F, Li L and Midorikawa O: Neophrotoxicity and renal cell carcinoma after use of iron-and aluminum- nitrilotriacetate complexes in rats. J Natl Cancer Inst (1986) **76**, 107–113.
3. Yamada M, Awai M and Okigaki T: Rapid *in vitro* transformation system for liver epithelial cells by iron chelate, Fe-NTA. Cytotechnology (1990) **3**, 149–156.
4. Nakatsuka S, Tanaka H and Namba M: Nutagenic effects of ferric nitrilotriacetate (Fe-NTA) on V79 Chinese hamster cells and its inhibitory effects on cell-cell communication. Carcinogenesis (1989) **11**, 257–260.
5. Kawabata T, Awai M and Kohno M: Generation of active oxygen species by iron nitrilotriacetate (Fe-NTA). Acta Med Okayama (1986) **40**, 163–173.
6. Yamanoi Y, Matsuura R and Awai M: Mechanism of iron toxicity in the liver and pancreas after a single injection of ferric nitrilotriacetate. Acta Haematol Jpn (1982) **45**, 1229–1235.
7. Yamada M, Okigaki T and Awai M: Role of superoxide radicals in cytotoxic effects of Fe-NTA on cultured normal liver epithelial cells. Cell Struct Funct (1987) **12**, 407–420.
8. Yagi K: A simple fluorometric assay for lipoperoxide in blood plasma. Biochem Med (1976) **15**, 212–216.
9. Aruoma OI, Halliwell B, Gajewski E and Dizdaroglu M: Damage to the bases in DNA induced by hydrogen peroxide and ferric ion chelates. J Biol Chem (1989) **264**, 20509–20512.
10. Klebanoff SJ, Waltersdorph AM, Michel BR and Rosen H: Oxygen-based free radical generation by ferrous ions and deferoxamine. J Biol Chem (1989) **264**, 19765–19771.

Received June 29, 1990; accepted August 1, 1990.