Korea J. of Toxicology
Vol. 4, No. 1, pp. 37-45, 1988

CYTOTOXICITY OF T-2 TOXIN ON PRIMARY CULTURES OF RAT HEPATOCYTES

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ABSTRACT: *Primary cultures of adult rat hepatocytes were used to study in vitro cytotoxic effects of T-2 toxin on liver cells. When T-2 toxin was added to the culture, a significant depression of the hormonal induction of α-aminoisobutyric acid (AIB) uptake and tyrosine aminotransferase (TAT) activity was observed. However, T-2 toxin did not affect the uptake of ouabain into hepatocytes. Protein synthesis was inhibited by T-2 toxin, but RNA synthesis was not severely affected. The inhibitory effects of T-2 toxin on protein synthesis was diminished rapidly with culture time and the hepatocytes culture maintained control level of protein synthesis within 24 hrs.*

Key words: *T-2 toxin, cytotoxicity, primary rat hepatocytes culture.*

INTRODUCTION

T-2 toxin is a trichothecene mycotoxin produced by a number of species of *Fusarium*. It has been suspected to be associated with several outbreaks of mycotoxicosis in both humans and animals and was shown to cause skin lesions, hemorrhagic lesions of internal organs, emesis, vomiting, leukopenia, and radiomimetic cellular injury (Bamburg and Strong, 1971; Joffe, 1971). Tissue distribution experiments in mouse and rat showed that the T-2 toxin administered orally was distributed in many organs shortly after the administration. The highest uptake was observed in the liver followed by kidneys and spleen, and the radioactivity in these tissue has disappeared rapidly without significant accumulation (Masumoto *et al.*, 1978: Nakano *et al.*, 1979). In cellular level, T-2 toxin was reported to inhibit macromolecular synthesis expecially of protein synthesis. The major toxic targets of T-2 toxin are considered to be the actively dividing cells of spleen, bone marrow, thymus, lymph nodes and small intensine (Sato *et al.*, 1975; Ueno *et al.*, 1973).

In vivo and *in vitro* metabolism studies have revealed that T-2 toxin is metabolized

to many deacetylated and hydroxylated derivates via the actions of microsomal carboxyesterase and monooxygenase before excretion (Ohata *et al.*, 1978; Yoshizawa *et al.*, 1980). Since these detoxification pathways are more abundunt in liver than in any other organs, the liver appeared to be less affected by the T-2 toxin even though highest uptake of T-2 toxin occurred in the liver after oral administration. In the present study, we used primary cultures of adult rat hepatocytes to investigate the cytotoxicity of T-2 toxin on liver cells and the time course of its detoxification. Uptake of ouabain, hormonal induction of α-aminoisobutyric acid (AIB) transport and tyrosine aminotransferase (TAT) activity, RNA and protein synthesis were assessed.

MATERIALS AND METHODS

Primary Hepatocyte Cultures and T-2 Toxin Treatment

Male Sprague-Dawley rats (200-250 g) were used. Hepatocytes were isolated by a collagenase perfusion technique and maintained as monolayers on 60 mm collagen-coated plates as reported previously (Yang *et al.*, 1983). Cells were initially cultured in a modified serum-free Waymouth's MB 752/1 medium at 37°C in a humidified incubator gassed with 5% CO_2 and 95% air mixture. After 24 hr, the medium was changed to Swim's S-77 medium containing 4 mM glutamine and gentamycin (50 ug/ml). T-2 toxin was dissolved in dimethylsulfoxide (DMSO) and added directly to the culture at the designated time. The final concentration of DMSO in the culture did not exceed 0.5% (v/v).

AIB and Ouabain Uptake Determination

Uptake of ouabain and hormonal induction of α-aminoisobutylic acid (AIB) transport were measured by the method of Kletzien *et al.*, (1976). AIB transport was preinduced in the cultured hepatocytes by addition of 1 μM dexamethasone 24 hrs after initial plating and 0.2 μM glucagon at 42 hr (Pariza *et al.*, 1976). At 48 hr, the culture medium was aspirated off and the plate was rinsed with warm (37°C) Hank's-Hepes salt solution. The AIB uptake was measured by incubating the cells in 2 ml of Hank's complete-Hepes medium (8 mM glucose added to Hank's-Hepes salt solution) containing 1 mM α-aminoisobutyric acid and α-amino (^{14}C) isobutyric acid (0.2 μCi/ml medium) for 4 minutes at 37°C. Incubation was terminated by aspiration of the medium and rinsing the cells several times with a total of about 20 ml of cold Hank's-Hepes salt solution. Cells were digested in 0.2N NaOH and radioactivity was estimated with a liquid scintillation counter.

Uptake of ouabain was determined similarly as the AIB transport experiment except that no hormonal induction was made. Tritiated ouabain (0.1 μCi/ml medium) was diluted with nonradioactive ouabain octahydrate (1 mM).

TAT Activity Determination

Tyrosine aminotransferase (TAT) was preinduced by an addition of 10 μM dexamethasone 42 hr after the initial plating. At 48 hr, the medium was drawn off and the plate was rinsed once with 3 ml of saline (0.85% NaCl). Then 1 ml of cold homoganiz-

ing buffer (0.2M potassium phosphate, 10 mM α-ketoglutarate, 0.04 mM pyridoxal phosphate, 1 mM EDTA, pH 7.3) was added, and the plates were immediately frozen in deep freezer at $-70°C$. They were subsequently thawed and the cells were scrapped into a test tube and frozen in the freezer and thawed twice. The resulting suspension was centrifuged at 48,200 g for 30 min. and the supernatant fluid served as the source for enzyme assay. The enzyme activity was determined by the method of Diamondstone (1966).

Estimation of Protein and RNA Synthesis

The amount of protein synthesized was determined by radiolabelling (Bonney *et al.*, 1974). At 24 hr, culture medium was aspirated off and fresh medium containing ^{3}H-leucine (5 μCi/plate, 0.38 mM) was added to the culture. After 2 hrs, the medium was removed and cells were scrapped into a test tube and same volume of 20% trichloroacetic acd (TCA) was added. This was centrifuged at 2,000 g for 10 min. and the pellet was washed twice with 10% TCA. The final pellet was digested in 1 ml of 1 N NaOH and radioactivity was determined.

RNA synthesis was determined by measuring the incorporation of ^{3}H-orotic acid (15 μCi/plate, 10 μM). The methods was similar to that of protein synthesis but radioactivity was determined in KOH hydrolyzable fraction.

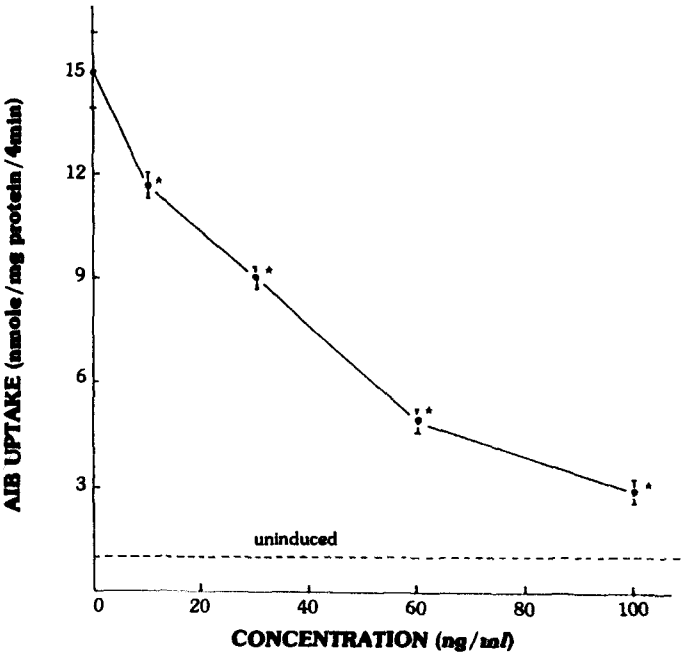

Fig. 1. Effect of T-2 toxin on hormonal induction of α-aminoisobutyric acid (AIB) uptake in primary rat hepatocytes culture. AIB uptake was preinduced by addition of dexamethasone (1 μM) and glucagon (0.2 uM), 24 and 42 hr after initial plating, respectively. T-2 toxin was added at 42 hr and AIB uptake was assayed at 48 hr. All points represents the mean ± SE of triplicate plates. An asterisk indicates values significantly different from induced control ($p < 0.05$).

Statistical Analysis

Results were expressed as the mean ± S.E. and the significance of the difference between mean values was assessed by Student t-test ($p < 0.05$).

RESULTS

Fig. 1 shows the effects of T-2 toxin on hormonal induction of AIB uptake. When T-2 toxin was added to the culture medium simultaneously with glucagon at 43 hr, AIB uptake was markedly depressed at 10 ng/ml concentration. The depression was more significant if the concentration of T-2 toxin was increased up to 100 ng/ml. The inhibition of AIB uptake by T-2 toxin appeared to be not related to the viability of hepatocytes since most of cells excluded tryphane blue at the highest dose of T-2 toxin (100 ng/ml).

Fig. 2 shows the effects of T-2 toxin on the induction of TAT activity by dexamethasone. Similar to AIB uptake, treatment of T-2 toxin caused a significant inhibition of TAT induction in hepatocyte culture. The magnitude of inhibition was related to the dose of T-2 toxin.

The cardiac glycoside, ouabain is known to be transported by an active transport system specific for organic neutral compounds, and is not metabolized by hepatocytes. It has been widely used to test the damage of the plasma membrane associated active

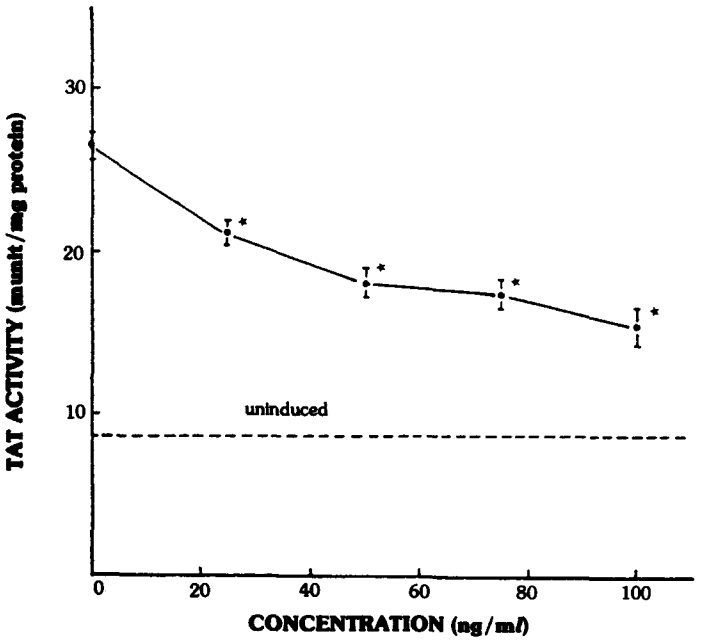

Fig. 2. Effect of T-2 toxin on hormonal induction of tyrosine aminotransferase (TAT) activity in primary rat hepatocytes culture. TAT activity was preinduced by addition of dexamethasone (10μM), 42 hr after initial plating. T-2 toxin was added at 42 hr and TAT activity was assayed at 48 hr. All points represents the mean ± SE of triplicate plates. An asterisk indicates values significantly different from induced control ($p < 0.05$).

Table 1. Effect of T-2 toxin on ouabain uptake in primary rat hepatocyte cultures.[1]

Exposed Time	Ouabain uptake (n moles/mg protein/4 min)
0 hours	1.64 ± 0.08
6 hours	1.67 ± 0.12
24 hours	1.74 ± 0.05

[1]. T-2 toxin (100 ng/ml) was added to culture 24 hr after the initial plating. Values were expressed as the mean ± SE of triplicate plates.

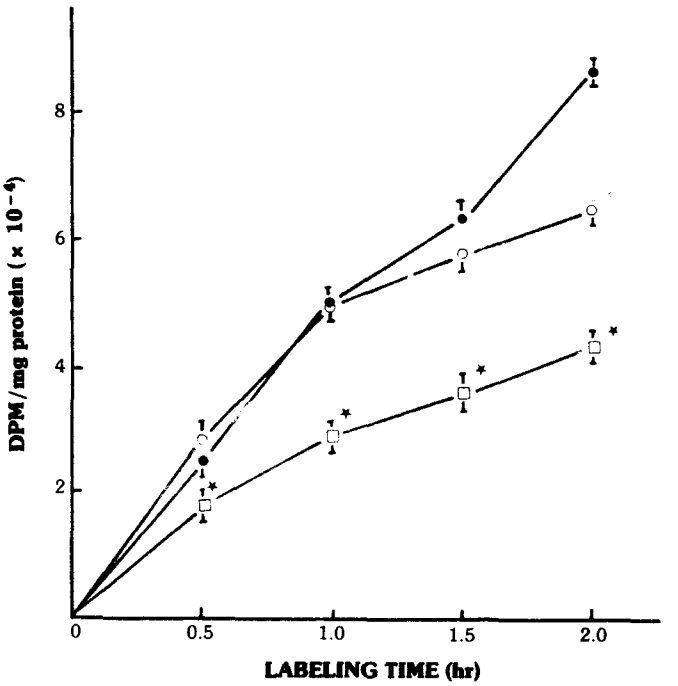

Fig. 4. Effect of T-2 toxin on protein synthesis in primary rat hepatocytes culture. T-2 toxin and ^{3}H-leucine was added to the culture 24 hr after initial plating. All values represents the mean ± SE of triplicate plates. An asterisk indicates values significantly different from the control ($p < 0.05$). Control (--●--), T-2 toxin, 10 n/gm/ (--○--), T-2 toxin, 25 ng/l (--□--).

transport system. As shown in Table 1, ouabain uptake was not significantly affected by T-2 toxin up to 24 hr treatment.

Fig. 3. shows the effects of T-2 toxin on protein synthesis in primary culture of hepatocytes. The synthesis of protein was linear up to 2 hrs in control cultures. At 10 ng/ml of T-2 toxin, protein synthesis was significantly lower than control after 2 hr treatment. At 25 ng/ml of T-2 toxin, however, protein synthesis was significantly inhibited after 30 min treatment.

Fig. 4 shows time course of inhibition of protein synthesis by T-2 toxin. In this experiment, 50 ng/ml of T-2 toxin was added to culture and ^{3}H-leucine pulse labeling was performed every two hour intervals. The maximum inhibition of protein synthesis

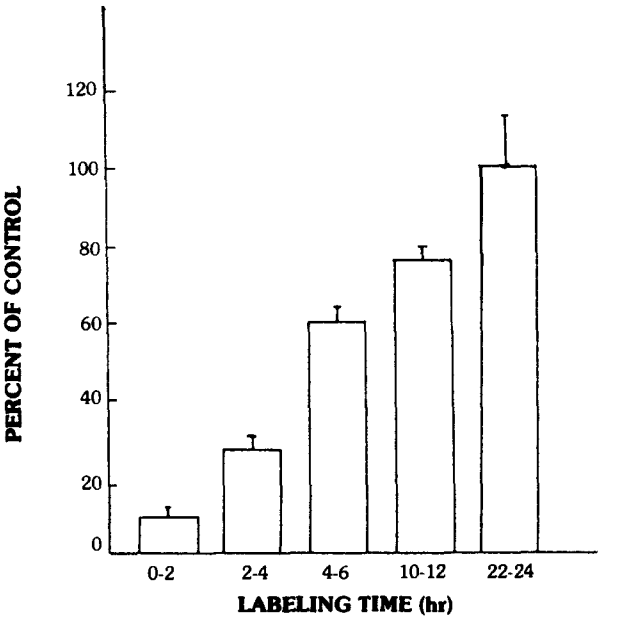

Fig. 4. Time course effects of T-2 toxin and protein synthesis in primary rat hepatocytes culture. T-2 toxin (25 ng/ml) was added to the culture 24 hr after initial plating and ^{3}H-leucine was pulse labeled at 2 hr intervals after addition of T-2 toxin. Each bar represents the mean ± SE of triplicate determinations.

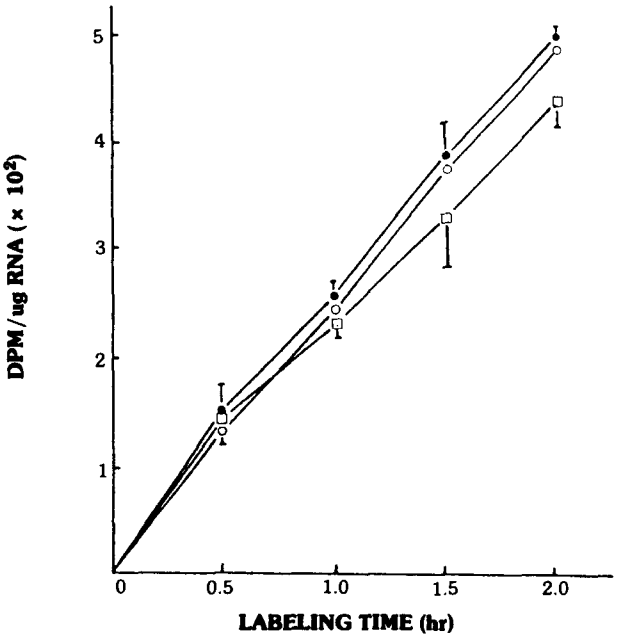

Fig. 5. Effect of T-2 toxin on RNA synthesis in primary rat hepatocytes cultures. T-2 toxin and ^{3}H-orotic acid was added to the culture 24 hr after initial plating. All values represents the mean ± SE of triplicate plates. Control (--●--), T-2 toxin, 10 ng/ml (--○--), T-2 toxin, 25 ng/ml (-- □--).

by T-2 toxin was observed within first two hours and the rate of inhibition was gradually decreased with culture time. After 24 hrs, hepatocytes were fully recovered from the inhibitory effects on protein synthesis by T-2 toxin.

Fig. 5 shows the effect of T-2 toxin on RNA synthesis in hepatocytes culture. Unlike protein synthesis, RNA synthesis was not severely affected by the treatment of T-2 toxin. At 25 ng/ml of T-2 toxin, RNA synthesis was slightly depressed after 1.5 and 2 hr treatment. The effects, however, were not significant statistically.

DISCUSSION

The results of the present study demonstrated that treatment of T-2 toxin to heaptocyte culture caused a dose- and treatment-time related inhibition of the hormonal induction of AIB uptake and TAT activity. These effects of T-2 toxin were not related to the cell death. Meanwhile, uptake of cardiac glycoside, ouabain was not affected by T-2 toxin. The protein synthesis was inhibited by T-2 toxin, but RNA synthesis was not affected severely. Sinece the hormonal induction of AIB transport and TAT activity is known to be inhibited by protein synthesis inhibitors, puromycin and cycloheximide (Pariza *et al.*, 1975; Bonney *et al.*, 1974), the effects of T-2 toxin on AIB uptake and TAT activity appeared to be of similar mechanism. Meanwhile, the uptake of ouabain may not require *de novo* protein synthesis, and so it was not affected by T-2 toxin. The results are in accord with the previous report that the effects of T-2 toxin in the human fibroblasts were largely on DNA and protein synthesis and no apparent effect on existing enzyme protein activity (Oldham *et al.*, 1980).

It has been reported that T-2 toxin is metabolized to more non-toxic, hydrophilic metabolites and excreted in urine and feces. HT-2 toxin, neosolaniol, deacetylneosolaniol, T-2 tetraol were found in excreta, and these were deacetylated metabolites produced by microsomal carboxyesterase (Ohata *et al.*, 1978, Yoshizawa *et al.*, 1980). Recently, new metabolites were isolated and identified as 3′-hydroxy T-2 and HT-2 toxins (Yoshizawa *et al.*, 1982). These metabolites were also formed by liver homogenates of mice and monkey in the presence of NADPH generating system. So, it was suggested that the cytochrome P-450 is catalyzing the hydroxylation at C-3′ position of T-2 and HT-2 toxins (Yoshizawa *et al.*, 1984). The metabolities formed by these pathways had less toxic effect and these two different detoxicating pathways are abundunt in the liver (Ehrlich and Daigle, 1985; Wei and Chu, 1985). In the present study, the hepatocytes culture was recovered from the inhibitory effect of T-2 toxin on protein synthesis rapidly and maintained normal level within 24 hr. Since the primary hepatocyte cultures are known to maintain some drug metabolizing enzyme activities, the recovery of protein synthesis appears to be due to the metabolism of T-2 toxin by the hepatocytes culture. No attemps were made in the present study to identify the metabolites of T-2 toxin in hepatocytes culture.

ACKNOWLEDGEMENTS

This work was supported by the Korea Science and Engineering Foundation Research Grant.

REFERENCES

Bamburg, J.R., Strong, F.M. (1971). 12,13-Epoxytrichothecenes, in "Microbial Toxins", Vol. VII, S. Kadis, A. Ciegler and S.J. Ail, eds., *Academic Press, Inc., New York*, p. 207.

Bonney, R.J., Becker, J.E., Walker, P.R. and Potter, V.R. (1974). Primary monolayer culture of adult rat liver parenchymal cells suitable for study of the regulation of enzyme synthesis, *In Vitro*, 9: 399-413.

Burmeister, H.R. (1971). T-2 toxin production by *Fusarium tricinctum* on solid substrate, *Appl. Microbiol.* 21: 739-742.

Diamondstone, T. (1966). Assay of tyrosine transaminase by conversion of p-hydroxyphenylpyruvate to p-hydroxybenzeldehyde. *Anal. Biochem.* 16: 395-401.

Ehrlich, K.C. and Daigle, K.W. (1985). Protein synthesis by mammalian cells treated with C-3-modified analogs of the 12,13-epoxytrichothecenes T-2 and T-2 tetraol, *Appl. Environ. Microbiol.* 50: 914-918.

Joffe, A.Z. (1971). Alimentary toxic aleukia, in "Microbial Toxins," Vol. VII, S. Kadis, A. Ciegler and S.J. Ail, eds., *Academic Press, Inc., New York*, p. 139.

Kletzien, R.F., Pariza, M.W. Becker, J.E., Potter, V.R. and Butcher, F.R. (1976). Induction of amino acid transport in primary cultures of adult rat parenchymal cells by insulin. *J. Biol. Chem.* 251, 3014-3020.

Matsumoto, H., Ito, T. and Ueno, Y. (1978). Toxicological approaches to metabolites of *Fusaria*. XII. Fate and distribution of T-2 toxin in mice, *Japan J. Exp. Med.* 48: 393-399.

Nakano, N., Nagahara, A., Shimizu, T., Aibara, K., Fujimoto, Y., Morooka, N. and Tatsuno, T. (1979). The tissue distribution and the pattern of excretion of [^{14}C]-13-labeled 12,13-labeled 12,13-epoxytrichothe C-9-ene in mice and rats, Japan *J. Med. Sci. Biol.*, 32: 269-279.

Ohata, M., Matsumoto, H., Ishii, K. and Ueno, Y. (1978). Metabolism of trichothecene mycotoxins. II. Substrate specificity of microsomal deacetylation of trichothecenes, *J. Biochem.* 84: 697-706.

Oldham, J.W., Allred, L.E., Milo, G.E. Kindig, O. and Capen, C.C. (1980). The toxicological evaluation of the mycotoxins T-2 and T-2 tetraol using nomal human fibroblast *in vitro*. *Toxicol. Appl. Pharmacol.* 52: 159-168.

Pariza, M.W., Butcher, F.R. Kleitzien, R.F., Becker, J.E. and Potter, V.R. (1976). Induction and decay of glucagon-induced amino acid transport and primary cultures of adult rat liver cels: Paradoxical effects of cycloheximide and puromycin, *Proc. Natl. Acad. Sci. USA*, 73: 4511-4515.

Sato, N., Ueno, Y. and Enomoto, M. (1975). Toxicological approaches to the toxic metabolites of *Fusaria*. VIII. Acute and subacute toxicities of T-2 toxin in cats, *Japan. J. Pharmacol.*, 25: 263-270.

Ueno, Y., Nakajima, M., Sakai, K.,Ishii, K., Sato, N. and Shimada, N. (1973). Comparative toxicology of trichothecmycotoxins: Inhibition of protein synthesis in animal cells, *J. Biochem.*, 74: 285-296.

Wei, R.D. and Chu, F.S. (1985). Modification of *in vitro* metabolism of T-2 toxin by esterase inhibitors, *Appl. Environ. Microbiol.*, 50: 115-119.

Yang, K.H., Choi, E.J., and Choe, S.Y. (1983). Cytotoxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in primary cultures of adult rat hepatocytes, *Arch. Enviorn. Contam. Toxicol.* 12: 183-188.

Yoshizawa, T., Sakamoto, T., Ayano, Y. and Mirocha, C.J. (1982). 3'-Hydroxy T-2 and 3'-hydroxy HT-2 toxins: New metabolites of T-2 toxin, a trichothecene mycotoxin, in animals, *Agric. Biol. Chem.*, 46: 2613-2615.

Yoshizawa, T., Sakamoto, T. and Okamoto, K. (1984). *In vitro* formation of 3'-hydroxy T-2 and 3'-hydroxy HT-2 toxins from T-2 toxin by liver homogenates from mice and monkeys. *Appl. Environ. Microbiol.* 47: 130-134.

Yoshizawa, T., Swanson, S.P. and Mirocha, C.J. (1980). *In vitro* metabolism of T-2 toxin in tats, *Appl. Environ. Microbiol. 40:* 901-906.