Bull. Environ. Contam. Toxicol. (1993) 51:889–894

Environmental Contamination and Toxicology

Toxicity of Verrucarin A to Gametes and Embryos of the Purple Sea Urchin (*Arbacia punctulata*)

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Mycotoxins represent a group of toxic secondary metabolities of various filamentous fungi. These fungi can invade the food supply during production, processing, transport and storage (CAST 1989). Of this very diverse class of toxins, the aflatoxins and the trichothecene mycotoxins are the most important because of their acute toxicity and, for the aflatoxins in particular, their potency as carcinogens (Ayres et al. 1971; Ohtsubo 1983).

The mode of action of trichothecenes is believed to be that of protein synthesis inhibition. The type and position of the esterifying acids present on the molecule dictate the level of protein synthesis inhibition. This diversity of chemical structures is responsible for the varying biological effects of this toxin (Hiessling 1986).

The study focuses on Verrucarin A (Fig. 1), a macrocyclic trichothecene from the fungus Myrothecium verrucaria. Macrocyclic trichothecenes appear to be most cytotoxic to human epithelial cells and are thus chosen for this study (Robb and Norval 1985). This investigation reports the effects of Verrucarin A on cleavage efficiency and pluteus development in the purple sea urchin, Arbacia punculata.

The sea urchin, A. punculata, has been widely used for studying the different facets of development. The chronology of these events are predictable under controlled conditions and reproduction is rapid and simple thus making the sea urchin an attractive system with which to study the effects of macrocyclic trichothecenes on the fertilization and developmental processes (Costello and Henley 1971; Adams 1983; Adams and Slaughter-Williams 1988).

This study looks at the effects of Verrucarin A on the cleavage efficiency of Arbacia following preinsemination exposure to various concentrations of the toxin for 30 minutes.

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MATERIALS AND METHODS

The toxin was emulsified with reagent grade acetone to a final toxin stock concentration of 500,000 ppb. All treatment mixtures were made via direct dilution. Verrucarin A was introduced as a component of the culture medium. A FSW (filtered seawater) and FSW-acetone control were employed. Cleavage was assessed at 105 minutes post-insemination so as to make identification of cleaving eggs (third cleavage stage) unambiguous. This study was conducted during the summer of 1991 and repeated during the summer of 1992.

Sea urchins were collected weekly by the Marine Resources Department, Marine Biological Laboratory, Woods Hole, Massachusetts. They were maintained in a 1.5 m(l) x 0.9m (w) x0.6 (h) sea table supplied with continuous seawater flow from nearby Buzzards Bay.

Urchins were initially stimulated to shed gametes by passing an alternating current of 10 volts through the animal in order to determine the sex of the organism (Costello and Henley 1971). Two females were then induced to shed their complement of eggs into 600 ml of filtered sea water (FSW).

Just prior to insemination, two drops of undiluted sperm were collected and thereafter diluted in 10 ml of FSW. Six drops of the sperm dilution were added to each treatment mixture (eg: 200 ml eggs/FSW/toxin) with this sperm concentration being sufficient to give 90% or better fertilization efficiency and still avoid polyspermy (Just 1939; Adams and Slaughter-Williams 1988).

Cleavage efficiency was determined by scoring the total number of cells and number of cleaving cells from two random fields of view in six samples. Each sample was counted on a depression slide using a compound microscope at 4x magnification. Point-to-point comparisons of control and experimental data were performed using Duncan's Multiple Range Test with a 95% degree of confidence.

RESULTS AND DISCUSSION

In performing our initial toxicity screening with Verrucarin A, we employed exponential concentrations of the toxin (5, 50, 500, 5000 ppb). The results of that initial screening is seen in Table 1. Cleavage was inhibited by 39% in the 5 ppb concentration of Verrucarin A and by 100% in all other concentrations.

We followed up the initial screening by using 20% dilutions between 10 ppb and 50 ppb. This study was repeated during the summers of 1991 and 1992, with four replicates performed each summer. The data obtained is depicted in Table 2 and Table 3 respectively. Toxin

concentrations of 15 ppb and higher resulted in significant inhibition of cleavage during the summer of 1991, and 10 ppb and higher during the summer of 1992.

Arrested zygotes were in the "streak stage" indicating death at around 35 minutes post-insemination (at 20°C) (Costello and Henley 1971). The LC_{50} is 19.10 ppb for both studies demonstrating the highly reproducible nature of our results.

Table 1. Fertilization efficiency in Arbacia eggs treated 30 minutes prior to insemination and continuously for 1.75 hrs with Verrucarin A (V-A)/ Summer 1991

Treatment	1st observation X% ± S.D	2nd observation X% ± S.D	cumulative X% ± S.D
FSW control	93.65±5.22 N=749	89.20±4.15 N=712	93.44+4.36 N=1461
ACE control	78.7±15.94 N=436	80.35±13.66 N=526	79.53±13.77 N= 962
5ppb V-A	60.3±7.49 N=695	61.55±2.19 N=638	60.92±4.56 N=1333
50ppb V-A	0 N=562	0 N=531	0 N=1093
500ppb V-A	0 N=498	0 N=504	0 N=1002
5000ppb V-A	0 N=507	0 N=494	0 N=1001

Abbreviations: ACE, acetone; FSW, filtered seawater

Table 2. Fertilization efficiency in Arbacia eggs treated 30 minutes prior to insemination and continuously for 1.75 hrs with Verrucarin A (V-A)/ Summer 1991

Treatment	1st observation X% ± S.D	2nd observation X% ± S.D	cumulative X% ± S.D
FSW control	89.75±7.76 N=411	92.00±7.52 N=299	90.87±7.18 N=710
ACE control	94.50±5.56 N=285	95.75±3.50 N=288	92.12±4.35 N= 573
15ppb V-A	61.25±20.56* N=274	65.5±15.02* N=246	63.37±16.82* N=520
25ppb V-A	28.5±26.33* N=326	38.5±32.17* N=246	33.5±27.33* N=572
35ppb V-A	18.25±24.54* N=303	18.5±28.61 N=281	18.37±24.68* N=584
45ppb V-A	4.49±8.34* N=342	3.35±5.56 N=350	4.02±6.62* N=692

*Significantly different from the control, p=5% or less (Duncan's)
Abbreviations: ACE, acetone; FSW, filtered seawater

Table 3. Fertilization efficiency in Arbacia eggs treated 30 minutes prior to insemination and continuously for 1.75 hrs with Verrucarin A (V-A)/ Summer 1992

Treatment	1st observation X% ± S.D	2nd observation X% ± S.D	cumulative X% ± S.D
FSW control	96.25±5.67 N=242	97.00±3.82 N=206	96.62±4.50 N=448
ACE control	96.00±3.36 N=284	95.50±2.38 N=269	95.75±2.71 N= 553
10ppb V-A	83.25±7.36* N=240	79.25±8.65* N=187	81.25±7.74* N=427
20ppb V-A	48.75±10.91* N=228	45.50±18.85* N=272	7.12±14.39* N=500
30ppb V-A	11.75±8.84* N=339	11.25±8.13* N=240	11.50±7.80* N=579
40ppb V-A	1.75±1.70* N=293	3.50±3.00* N=228	2.62±2.44* N=521
50ppb V-A	2.00±2.44* N=269	1.00±1.15* N=202	1.50±1.85* N=471

*Significantly different from control, p=5% or less (Duncan's)
Abbreviations: ACE, acetone; FSW, filtered seawater

The embryos which were observed to cleave at 105 minutes post-insemination were examined after 24 hours of incubation to assess possible sublethal toxic effects. Fifty-five percent (55%) mortality was observed in the 10 ppb concentration of Verrucarin A with the rest of the cells arrested in cleavage. Forty-five percent (45%) mortality was observed in the 20 ppb concentration of the toxin incubated for 24 hours with 56% of the cells arrested in cleavage. The controls showed no mortality with 64% of the control embryos reaching the gastrula stage and 35% reaching the early pluteus stage (Table 4).

Table 4. 24 hr Examination of Verrucarin A (V-A) Treated Embryos/Summer 1992

Treatment	T/D X%+ S.D	Gastrula X%+S.D	Pluteus X%+S.D	Cleavage X%+S.D
FSW control	-	64.5±0.707 N=44	35.0±0.0 N=24	-
ACE control	-	62.5±2.12 N=20	37.5±2.12 N=12	-
10ppb V-A	55.0±20.5 N=33	-	-	33.4±16.13 N=22
20ppb V-A	45.5±15.19 N=23	-	-	54.0±15.04 N=30

Abbreviations: T/D, Transparent/disintegrating; ACE, acetone, FSW, filtered seawater

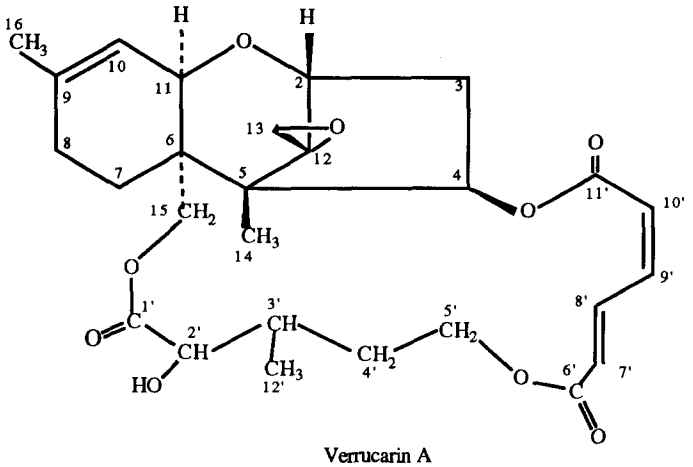

Figure 1. Chemical structure of the toxin.

The results reported in this paper indicate that Verrucarin A is very toxic toward the gametes and the embryos of the sea urchin, A. punculata. Previous studies have set a LD_{50} for Verrucarin A between 0.5 and 1.5 ppm (Busby and Wogan 1978). Our studies show that this toxin is more than two orders of magnitude more effective at poisoning gametes and embryos upon direct exposure. The greater sensitivity of fertilized eggs is completely logical given the mode of action of trichothecenes, eg. inhibition of protein synthesis. Protein synthesis is a major activity of the fertilized egg and is crucial to its function and survival.

Acknowledgments. This work was supported in part by NIH-NIEHS Grant No. 1 R15 E59567, NSF-OCE Grant No. 9016480, and DOC-NOAA Contract No. 0002. The authors thank Jasmine Adams for continued inspiration during the conduct of this study.

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Received November 23, 1992; accepted June 13, 1993.