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*A Research Note*

# An Evaluation of the Genotoxicity of O-Tyrosine, a Proposed Marker for Irradiated Food

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**ABSTRACT**

V79 Chinese hamster cells and Molt-4 human cells have been used to test the cellular and genetic toxicities of o-tyrosine addition at concentrations far in excess of that found after irradiation of chicken to 10 kGy (~1.3 μg/ml). No toxicity, as measured by sister chromatid exchange, micronuclei formation, or reproductive death, was seen in cells exposed to concentrations up to 100 μg/ml o-tyrosine. After 2 d in 200 μg/ml o-tyrosine, Molt-4 cells had a higher percentage of dead cells in the population, but the growth rate and micronuclei formation were not affected.

o-Tyrosine has a number of characteristics that make it attractive as a potential marker for irradiated food: it is present at very low levels in untreated meat, it is relatively easy to quantify, and it is produced in a dose-dependent fashion by irradiation (*1*). The isomers, o-, m-, and p-tyrosine, are formed by the reaction of hydroxyl radicals from the radiolysis of water with the amino acid, phenylalanine. o-Tyrosine has been proposed as the marker because it is easier to quantify than the other isomers (*2*). For these reasons, o-tyrosine has been used as a specific marker for hydroxyl radicals generated in biosystems (*7,8*) and irradiated food (*4,6-8*).

Since o-tyrosine is produced by radiation exposure of protein in meats, it is important to check whether the increased amounts of o-tyrosine represent any health hazard. We have used V79 Chinese hamster cells and Molt-4 human t-lymphocytes to test the cellular and genetic toxicities of o-tyrosine at concentrations in great excess of that expected in food products irradiated to 10 kGy: the maximum dose currently accepted.

## MATERIALS AND METHODS

D,L-o-tyrosine, obtained from Sigma, was dissolved in autoclaved double-distilled water as a stock solution at a concentration of 0.1 mg/ml.

V79 Chinese hamster cells were maintained at 37°C with 2% $CO_2$ in 30 ml of Ham's F12 medium with 10% fetal bovine serum (FBS). Molt-4 human lymphocyte cells were maintained at 37°C with 5% $CO_2$ in 30 ml of RPMI-1640 medium with 10% FBS.

On the day before the sister-chromatid-exchange (SCE) experiment began, 75-$cm^2$ flasks were inoculated with 3 x $10^5$ V79 cells in 30 ml of medium with 10% FBS. On the day of the experiment, the appropriate concentration of the stock o-tyrosine and 1 x $10^{-5}$ mol/L 5-bromodeoxyuridine in double-distilled water was added. After a 20-h incubation, 0.1 μg/ml of colcemid was added and the cells were incubated for an additional 2 h; the flasks were then shaken to dislodge mitotic cells. They were collected and pelleted. The cell pellet was resuspended in 5 ml of 0.56% KCl at 37°C and incubated at 37°C for 15 min. The cells were fixed in methanol (MEOH): acetic acid (3:1). A drop of cell suspension and an additional drop of fixative or MEOH, depending upon the degree of chromosome condensation, were dropped on a washed slide and dried over a flame. The sister chromatid exchanges were differentiated using the method of Goto et al. (*3*).

Molt-4 cells were subcultured at 5 x $10^4$ cell/ml one day before the micronuclei-formation experiment. On the day of the experiment, o-tyrosine and 3.0 μg/ml of cytochalasin B were added and the cells were cultured for 24 h. They were then pelleted, resuspended for 8 min in 0.56% KCl at 37°C, pelleted again, and resuspended in fresh 0.56% KCl. Drops of the cell suspension were allowed to run down the slide and dry. The slides were fixed in 100% MEOH for 3 min and air-dried. The slides were stained with acridine orange by the method of Hayashi et al. (*5*). Binucleated cells were counted for micronuclei.

V79 Chinese hamster cells were plated for toxicity testing at four replicates per dose point with 300 cells per 60-mm culture dish in 2 ml of F12 medium plus FBS and left to attach for 4 h. The o-tyrosine was added in 3 ml of medium for a final volume of 5 ml per dish. After 6 d of incubation, the colonies were stained with 1% methylene blue and counted for relative survival. Molt-4 cells were inoculated at 5 x $10^5$ cells per 25-$cm^2$ flask in medium containing o-tyrosine. After 24 and 72 h, the cell density and percentage of trypan blue excluding cells were measured with a hemocytometer.

The S9 microsomal fraction was purchased from Microbiological Associates (Rockville, MD) and stored in a liquid nitrogen refrigerator. When S9 was used, the cells were allowed to attach for 4 h before o-tyrosine and dilutions of a 10% S9 mix were added to the V79 cells for 2 h. The cells were washed in phosphate-buffered saline before fresh medium was added. The

S9 mix was prepared and used following the procedures of Venitt et al. (9).

## RESULTS

o-Tyrosine was not toxic to V79 Chinese hamster cells at concentrations ranging from 8.9 μg/ml to 106.8 μg/ml, the highest dose tested. There was no change in survival with concentration: the survival at 106.8 μg/ml o-tyrosine relative to the untreated control, for example, was 1.04 ± 0.06 and a linear-regression fit to the data had a slope that was not significantly different from zero. Molt-4 cells were also tested for survival after o-tyrosine exposure. Since they grow in suspension, cellular toxicity was quantified by measuring cell growth and the number of dead cells in the population. The results, averaged from two experiments (Table 1), show that o-tyrosine addition did not affect the survival of Molt-4 cells. The range of values shown for log-cell-number and percent-dead-cells was within the variation for both experiments, except for 6.2% dead cells on day 3 at 200 μg/ml. This value was statistically significant; however, we considered it anomalous since the concentration was so high; there was no dose-dependent increase in killing and the log-cell-number did not show a corresponding decrease.

The average number of micronuclei per 1000 cells in four measurements of Molt-4 cells exposed to 200 μg/ml of o-tyrosine, 0.2 μg/ml mitomycin C, and normal medium was 5.0 ± 0.4, 28.0 ± 1.6, and 5.8 ± 0.8, respectively. The o-tyrosine-treated cells did not have a significantly different number of micronuclei from cells in normal medium; however, the cells treated with mitomycin C, a positive control treatment, had a significantly greater number.

Genetic toxicity was tested by observing sister chromatid exchanges in V79 cells and micronuclei formation in Molt-4 cells. There was no effect of o-tyrosine on SCE production in V79 cells at any concentration (Table 2). Mitomycin C was the positive control. The reproductive index (RI), RI = $(n_1 + 2n_2 + 3n_3)/(n_1 + n_2 + n_3)$ where $n_i$ is the number of cells in the i'th generation, was also measured to ensure that o-tyrosine did not decrease the cellular growth rate. The experiments were designed to give a RI of approximately two, and it did not change significantly with o-tyrosine addition.

Some mutagens require activation before they are effective. The S9 fraction from rat liver was used to determine if o-tyrosine can be activated. The results indicate (Table 2, experiment 3) that S9-mix addition did not activate o-tyrosine into a form that could cause an increase in SCE or the reproductive index.

## DISCUSSION

The amount of o-tyrosine formed by 10-kGy irradiation is small, but it is large enough to be used as a marker for food irradiation in chicken and other meat products (*1*). There is also a small residual amount of o-tyrosine present in unirradiated meat, probably produced by the enzyme tyrosine hydroxylase: the amount of o-tyrosine reported in unirradiated chicken breast meat was 0.12 μg/g (wet weight)

TABLE 1. *The Survival of MOLT-4 cells after exposure to exogenous o-tyrosine.*

| Time (day) | o-Tyrosine concentration (μg/ml) | Log cell No. (%) | Dead cells (%) |
|---|---|---|---|
| 1 | 200.0 | 5.63 | 2.8 |
| | 100.0 | 5.63 | 2.6 |
| | 50.0 | 5.66 | 1.8 |
| | 25.0 | 5.50 | 2.7 |
| | 5.0 | 5.53 | 1.7 |
| | 0.5 | 5.50 | 1.7 |
| | 0.05 | 5.56 | 2.3 |
| | 0.0 | 5.56 | 2.0 |
| 3 | 200.0 | 6.00 | 6.2 |
| | 100.0 | 6.07 | 2.1 |
| | 50.0 | 6.12 | 1.9 |
| | 25.0 | 6.00 | 1.4 |
| | 5.0 | 6.10 | 1.4 |
| | 0.5 | 6.07 | 2.0 |
| | 0.05 | 6.06 | 2.1 |
| | 0.0 | 6.06 | 1.5 |

TABLE 2. *Effect of o-tyrosine addition on sister chromatid exchange and reproductive index (RI) in V79 Chinese hamster cells.*

| Exp. No. | o-Tyrosine concentration (μg/ml) | S9 concentration (μl/ml) | SCE | RI | No. of cells |
|---|---|---|---|---|---|
| 1 | 200.0 | 0.0 | 8.53 ± 0.36 | 1.96 | 50 |
| | 100.0 | 0.0 | 8.28 ± 0.37 | 1.97 | 50 |
| | 10.0 | 0.0 | 7.98 ± 0.36 | 2.03 | 50 |
| | control | 0.0 | 8.00 ± 0.35 | 2.03 | 50 |
| 2 | 200.0 | 0.0 | 9.60 ± 0.41 | 2.00 | 50 |
| | 100.0 | 0.0 | 9.78 ± 0.41 | 2.05 | 50 |
| | control | 0.0 | 8.96 ± 0.44 | 2.26 | 50 |
| | mitomycin C | 0.0 | 23.7 ± 0.79 | 2.05 | 50 |
| 3 | 100.0 | 50.0 | 8.72 ± 0.37 | 2.05 | 50 |
| | 100.0 | 20.0 | 8.80 ± 0.38 | 2.04 | 50 |
| | 100.0 | 10.0 | 9.06 ± 0.37 | 2.06 | 50 |
| | 100.0 | 5.0 | 9.86 ± 0.45 | 2.02 | 50 |
| | 100.0 | 0.5 | 9.02 ± 0.40 | 2.06 | 50 |
| | 100.0 | 0.0 | 9.24 ± 0.45 | 2.09 | 50 |

(*1*). After irradiation to 10 kGy (the maximum dose that can be used for food under current regulations and a dose in excess of that proposed for use with chicken), the amount of o-tyrosine formed was ~0.26 mg per 200-g portion of chicken, which corresponds to a concentration of approximately 1.3 μg/ml (*1*). Concentrations of o-tyrosine of up to 100 μg/ml, approximately 100 times the concentration expected to be produced by industrially proposed doses, did not produce cellular toxicity in V79 Chinese hamster cells or Molt-4 human t-lymphocytes; nor did it increase the number of SCE in V79 cells or micronuclei in Molt-4 cells. The RI in V79 cells and cell growth in Molt-4 cells was also not affected by o-tyrosine addition. At one concentration and time (200 μg/ml and 3 d), the percentage of dead cells in the Molt-4 population was greater than in

controls or other o-tyrosine-treated cells. Since there was no dose-dependent increase in killing, the log-cell-number did not show a corresponding decrease and there was no increase in micronuclei; we believe that this one value at a very high concentration is not biologically significant.

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