# Experimental Design and Predictive Computational Modeling for the Toxicity of Nanomaterials on Human Epidermal Cells

**Natarajan Meghanathan**                    natarajan.meghanathan@jsums.edu
*Assistant Professor, Department of Computer Science*
*Jackson State University*
*Jackson, MS 39217, USA*


**Raphael D. Isokpehi**                    raphael.isokpehi@jsums.edu
*Associate Professor, Department of Biology*
*Jackson State University*
*Jackson, MS 39217, USA*


**Hari Har Parshad Cohly**                    hari.cohly@jsums.edu
*Assistant Professor, Department of Biology*
*Jackson State University*
*Jackson, MS 39217, USA*

## Abstract

Nanomaterials are becoming more commonly used in everyday life, where human beings are becoming exposed to such materials. However, the toxicity of the materials being introduced in our environment is not fully studied. We are currently working on a pilot project to develop computation models that can predict the toxicity of nanomaterials on cell types based on empirical data obtained through monoculture experiments and co-culture experiments. Our hypothesis is that computational approach can be utilized to predict the toxicity and model the intercellular interactions in co-culture studies. The uniqueness of this approach is that we propose to employ computational methods to predict the outcome of co-culture experiments and test the validity of the predictions with cellular biology assays results from co-culture experiments. Human skin cell types such as keratinocytes, melanocytes and dendritic cell lines will be used to mimic the cellular elements of the epidermis. Cytoxicity, genotoxicity and lipid peroxidation assays will be used to measure cytoplasmic, DNA, and lipid membrane damage respectively. The expected results are that the computational approach will use the results from monoculture experiments to generate a preliminary model that will predict the outcome of co-culture experiments. The preliminary model will be further trained using the co-culture experiments conducted to validate the predicted results.

**Keywords:** Computational Modeling, Toxicity, Nanomaterials, Human Epidermal Cells, Co-culture

## 1. INTRODUCTION

During the manufacturing process of nanomaterials, several metals (e.g. Iron) are being used as catalysts. Recent studies (e.g. [1]) have shown that if these metals are not completely purged

from the nanomaterials, exposure of human beings to nanomaterials could be toxic to the human skin. Thus, safety is a major concern in the use of nanomaterials. Our research aims at studying and modeling the toxicity of nanomaterials on the cells constituting the epidermal layer of human skin. This study specifically focuses on the following three cells that are part of the epidermis: (i) Keratinocytes (abbreviated as 'K' cells) – epithelial cells constituting a major portion of the basal layer of the epidermis and act as a barrier between the body and environment; (ii) Melanocytes (abbreviated as 'M' cells) – cells that produce a pigment called melanin which is responsible for the color of human skin; and (iii) Dendritic cells (abbreviated as 'D' cells) – cells that are responsible for the generation of microbial antigens as part of the immune system of the skin. We target to study the following types of toxicity using assays that are available for each of them: (i) Cytotoxicity (abbreviated as 'C' toxic) – affecting the cytoplasm of the cells; (ii) Genotoxicity (abbreviated as 'G' toxic) – affecting the genetic makeup (nucleus) of the cells; (iii) Lipid Peroxidation (abbreviated as 'L' toxic) – affecting the cell membrane, which consists mainly of lipids.

The three primary objectives of our research project are:
1. Conduct experiments on various monoculture cell lines of the epidermal layer of the skin
2. Build a computational model using results from monoculture experiments
3. Validate and train the predictability of computational models using data from co-culture experiments

## 2. LITERATURE REVIEW

Nanomaterials have been earlier used in studies involving keratinocytes [2], melanocytes [3] and dendritic cells [4]. It has been also reported that titanium dioxide ($TiO_2$)-based nanomaterials when exposed directly to in-vitro cell cultures, exert significant and cell-type dependent effects on such cellular functions as viability, proliferation, apoptosis and differentiation [5]. Studies on the effects of two or more nanomaterials on a particular cell type of skin have been documented [6]. However, co-culture studies involving single nanomaterials on more than one cell type of the skin have not been conducted. A three-dimensional, computational fluid dynamics (CFD) model has been used to simulate inhaled airflow and to calculate nasal deposition efficiency on the rat nasal passages [7]. Computational methods have also been used to investigate human H4 neuroglioma cells exposed to CuO nanomaterials [8].

Very few studies combine computational models with the experimental data related to the effects of nanomaterials on the skin. Our approach is novel and unique, because it employs computational models to predict the outcome of a co-culture experiment, and it then tests the validity of the models using experimental data. The model whose predicted values match closely with the experimental data will be further trained with more co-culture experimental data.

## 3. RESEARCH PLAN

Safety issue in nanaomaterials is of primary concern. Skin is the largest organ in the body. Investigation on the toxicity of nanomaterials on the cellular elements of the skin is of great importance. There is a need to develop computational models that would predict the toxicity of nanomaterials on cell culture systems. Individual toxicity of the 3 cell types of the skin including keratinocytes, melanocytes and dendritic cells have been studied earlier [9]. The novelty of our research is that it is translational in nature as it will use the experimental data produced in the laboratory to develop and train computational models that will predict the toxicity of nanomaterials on the epidermal cells of human skin.

Figure 1 illustrates the flow diagram of our research plan. We will use established human cell lines (1 x$10^5$ cells/ml) of keratinocytes (HaCaT), melanocytes (CRL1675), and dendritic cells (THP-1 + A23187). Cells will be grown in Dulbecco's Minimum Essential Medium for HaCaT, RPMI 1640 for THP-1 (dendritic cells), and Vitacell medium for melanocytes (CRL 1675).
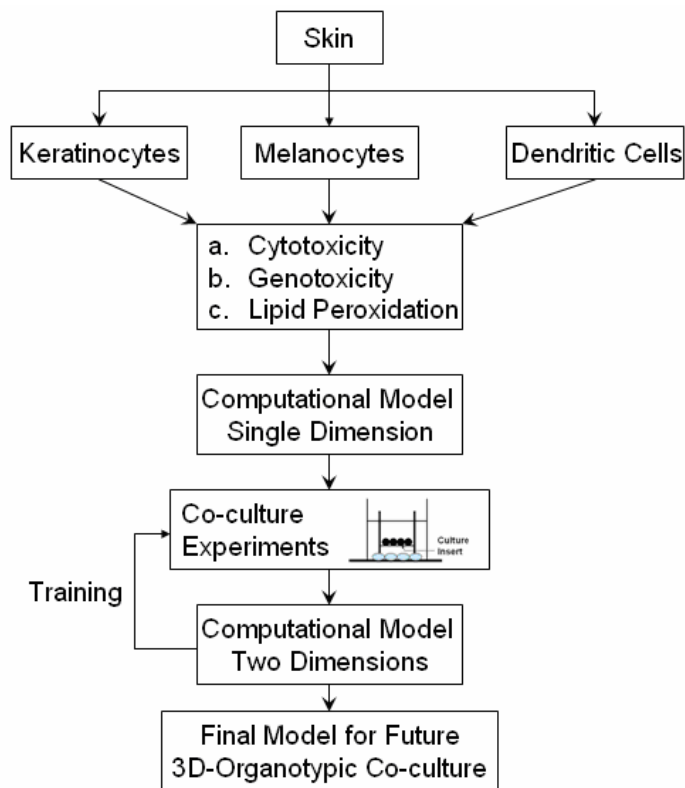
**FIGURE 1:** Flowchart of our Research Plan

The following three assays will be used to study the toxicities of nanomaterials on monocultured epidermal cells:

- Cytotoxicity will be assessed using the Lactate Dehydrogenase assay after 72 hrs of exposure.
- Genotoxicity will be measured through the DNA damage using single cell gel electrophoresis (Comet assay).
- Membrane toxicity will be evaluated using lipid peroxidation.

The results from the toxicity assays for the three different cell types will be used to generate a computational model that will predict the outcome of co-culture experiments. We will additionally use data generated from our labs (Research Center for Minority Institutions, RCMI-funded project by NIH) on the toxicity of arsenic trioxide on skin keratinocytes. Arsenic trioxide can serve as a positive control as it is a known carcinogen and an agent that causes changes in keratinocytes activation and differentiation [10].

## 4. EXPERIMENTAL DESIGN

**Nanomaterial Sonification in Different Media**
Commercially obtained nanomaterials will be suspended in sterile pyrogen-free phosphate buffer and probe-sonicated for 5 seconds and then incubated with the reaction mixture in a water bath in the dark at 37 ℃ for 15 min. Nanomaterial stock solutions will be prepared and supplemented with (1, 5 and 10%) FBS containing 1% penicillin and streptomycin in (i) phosphate buffered saline, (ii) Dulbecco's Minimum Essential Medium (iii) RPMI 1640, (iv) Vitacell medium and briefly probe sonicated (15 seconds) [11][12]. Prior to adding the diluted nanomaterials to the cells, the

medium will be aspirated from each well and the nanomaterials in buffer (250 µL) will be gently layered over the cell monolayer; the cells will be allowed to take up the particles in buffer for the period of 10 min, during which the plates will be periodically agitated. After this initial uptake period, medium with 10% FBS will be added back to each well (total of 1 mL). All incubations will be at 37 ℃, 5% $CO_2$. For the uptake studies, cells will be exposed to the nanomaterials (different shapes and sizes) for 10 min or 24 h. Uptake will be evaluated via EM (Electron Microscopy) and ICP-MS (Inductively Coupled Plasma Mass Spectrometry) analyses.

For EM analyses, the culture supernatant will be removed from each well at the end of the exposure period and glutaraldehyde (2.5%) in Millonig's buffer will be added. Cells will be fixed in this buffer for the period 1 h, after which they will be removed from the culture dishes by scraping and placed in a 15-mL conical tube on its side at 4 ℃ for 3 more days of fixation. Cells will be rinsed and then post-fixed in phosphate-buffered 1% $OsO_4$. After more rinsing, the cells will be trapped in 3% agarose and then cooled so that the material will be cut into 1 mm cubes. These agarose cubes will be stained with 0.25% uranyl acetate in 50% ethanol, dehydrated and infiltrated with araldite resin. The epoxy resin blocks will be sectioned (70 nm) onto 200 mesh grids and the grids will be stained with uranyl acetate and lead citrate.

The grids will be examined for electron-dense particles using a Hitachi 7100 transmission electron microscope; images will be captured digitally. Cell pellets and culture supernatants will be processed for separate analyses of nanomaterial content via ICP-MS analysis. After the 24-h exposure period, cell culture supernatants will be removed and placed in Teflon vials. Each well will be washed 3 times with Hank's balanced salt solution (HBSS), adding each wash to the same vial as the supernatant. The wells will not be acid-washed to remove the nanomaterials that may have adhered to the well material. The cell pellets will be trypsinzed to remove them from the culture wells and placed in another Teflon vial. The wells will then be washed 3 times with HBSS, saving the washes in the same vial as the main pellet. The contents of each vial will be dried down and dissolved in aqua regia with heating and this process repeated until the nanomaterial pellet is no longer visible. The dried samples will be re-suspended in 0.5 N HCl/aqua regia and an aliquot of this solution will be spiked with iridium as an internal standard. Nanomaterial concentrations will be determined in a magnetic sector high resolution Thermo-Finnigan Element 1 ICP-MS (Thermo Fisher Scientific, Waltham, MA). ICP-MS certified standards from Inorganic Ventures (Lakewood, NJ) will be used for the external standardization.

### Electrostatic Potential
We will also determine the zeta potentials (a widely used measure of the electrostatic potential at the nanomaterial surface double layer) as a feature for each nanomaterial. Nanomaterial sizing will be determined by dynamic light scattering and zeta potential measurement will be determined by laser-Doppler electrophoresis.

### Cytotoxicity Assay
Briefly, cells will be counted (20,000cells/well) and re-suspended in complete medium. Aliquots of 100µl of cell suspension will be placed in wells of microtiter plates, and 100µL of different concentrations of nanomaterials (0 to 200µg/mL) will be used to treat the cells. The plates will be incubated for 24, 48, and 72 hours, respectively. In order to assess cytotoxicity, we will measure the release of lactate dehydrogenase (LDH) into the culture supernatant using a kit from Sigma-Aldrich. The use of THP-1 +A23187 to mimic dendritic cells [13] is due to the fact that dendritic cells will be found in the surface epithelium along with keratinocytes and melanocytes.

### Cell Treatment for Genotoxicity
Cells will be counted (10,000 cells/well) and re-suspended in media with 10% FBS. Aliquots of 100µL of the cell suspension will be placed in 96 well plates, treated with arsenic trioxide concentrations at doses of $LD_{10}$ and $LD_{25}$ determined from the cytotoxicity assay data, and incubated in a 5% $CO_2$ at $37^{o}C$ for 72 hrs. After incubation, the cells will be centrifuged, washed with PBS (Phosphate Buffered Saline), and re-suspended in 100 µL PBS. In a 2 mL tube, 20 µL

of the cell suspension and 200 µL of melted agarose will be mixed and 75µL pipetted onto a pre-warmed slide. The slides will be placed in a refrigerator at 4°C for 10-20 min and placed in chilled lysis buffer for 45 min. Slides will be washed twice for 5 min with TBE (Tris-Borate-Edta Buffer) and electrophoresed in a horizontal gel apparatus at 25V for 10 min. Slides will be placed in 70% ethanol for 10 min, removed, tapped to remove excess ethanol, and placed in an alkaline solution containing 99mL $H_2O$, 100µL of 0.1mM $Na_2EDTA$ (Ethylene Diamine Tetraacetic Acid) and 1g NaOH for 45 min. Slides will be air dried for 2.5 hrs, stained and allowed to set 4 hrs. The slides will be viewed with an Olympus fluorescence microscope and analyzed using LAI's Comet Assay Analysis System software (Loates Associates, Inc. Westminster, MD).

### Liquid Peroxidation

Supernatants from the culture plates will be removed and frozen immediately and stored at -80°C until the assay will be started. For assessment of lipid peroxidation products, the supernatant will be mixed with 20% trichloroacetic acid and 0.67% thiobarbituric acid and then heated for 15 min in boiling water. The concentration of thiobarbituric acid-reactive substances (TBARS) extracted with *n*-butanol will be estimated by absorption at 530 nm. TBARS will be expressed as malondialdehyde (MDA) amounts, using freshly produced MDA as standard prepared from 1,1,3,3-tetramethoxypropane with HCl [14][15].

## 5. PREDICTIVE COMPUTATIONAL MODELING

For a given toxicity assay and concentration *C-NMi* of a nanomaterial *NM-i*, let $\%Viability_X^{C-NMi}$ be the *% Viability* brought about by *NM-i* on a cell type *X* mono-culture (where *X* can be a Keratinocyte – K or Melanocyte – M or Dendritic Cell - D). Let $(X/Y)_{co-culture}$ represent a co-culture where *X* is the inactive cell and *Y* is the active cell. The concentration of cell *Y* is kept constant with the exposure of the nanomaterial, while the different values for the concentration of the inactive *X* cell would be (1, 2, 4, 8, 10)*$10^5$ cells/mL. Let *nX* and *nY* be the concentrations of the *X* and *Y* cells respectively in a $(X/Y)_{co-culture}$ and $(ratio)_{X/Y}$ = *nX/nY* represent the ratio of the concentrations of the two cell types *X* and *Y* in the $(X/Y)_{co-culture}$. The *% Viability* of the $(X/Y)_{co-culture}$ for a concentration *C-NMi* of a nanomaterial *NM-i* represented as $\%Viability_{X/Y}^{C-NMi}$ is a function of the four variables: $\%Viability_X^{C-NMi}$, $\%Viability_Y^{C-NMi}$, *C-NMi,* and the $(ratio)_{X/Y}$. Our task is to predict $\%Viability_{X/Y}^{C-NMi}$ using different possible functions of these four variables. Some of the sample functions (F) that are currently being considered for evaluation are as follows:

F1: $$\%Viability_{X/Y}^{C-NMi} = \%Viability_X^{C-NMi} + \left[\%Viability_Y^{C-NMi}\right]^{\left\{\frac{1}{(C-NMi)*(ratio)_{X/Y}}\right\}}$$

F2: $$\%Viability_{X/Y}^{C-NMi} = \%Viability_X^{C-NMi} - \left[\%Viability_Y^{C-NMi}\right]^{\left\{\frac{1}{(C-NMi)*(ratio)_{X/Y}}\right\}}$$

F3: $$\%Viability_{X/Y}^{C-NMi} = \frac{\%Viability_X^{C-NMi}}{\left[\%Viability_Y^{C-NMi}\right]^{\left\{\frac{1}{(C-NMi)*(ratio)_{X/Y}}\right\}}}$$

F4: $$\%Viability_{X/Y}^{C-NMi} = \%Viability_Y^{C-NMi} + \left[\%Viability_X^{C-NMi}\right]^{\left\{\frac{1}{(C-NMi)*(ratio)_{X/Y}}\right\}}$$

F5: $$\%Viability_{X/Y}^{C-NMi} = \%Viability_Y^{C-NMi} - \left[\%Viability_X^{C-NMi}\right]^{\left\{\frac{1}{(C-NMi)*(ratio)_{X/Y}}\right\}}$$

F6:
$$\%Viability_{X/Y}^{C-NMi} = \frac{\%Viability_Y^{C-NMi}}{\left[\%Viability_X^{C-NMi}\right]^{\left\{\frac{1}{(C-NMi)*(ratio)_{X/Y}}\right\}}}$$

In addition to the above functions, we will also formulate and evaluate several different functions that will predict the *% Viability* of a $(X/Y)_{co\text{-}culture}$ for a given concentration of the nanomaterial. The accuracy of the *% Viability* values predicted by each of these functions will be tested by using the co-culture experimental data obtained from the laboratory. The function whose predictive value matches closely with the experimental value will be chosen for further training in order to develop a more accurate and well-trained 2-dimensional prediction model for a $(X/Y)_{co\text{-}culture}$ system. We will then develop a 3-dimensional working model for an organotypic $(X/Y\ /Z)_{co\text{-}culture}$ system involving all the three cell types. The formulation and training approach for the 3-diemsnional working model will be on similar lines as that of the 2-dimensional model.

## 6. EXPECTED RESULTS

A cell is considered toxic if the viability has reached 50%. For a particular type of toxicity, we expect different concentrations of nanomaterials to cause 50% viability. In the co-culture experiments, the varied concentrations of the inactive cells and the released products from the inactive cells will influence the toxicity of the active cell whose concentration is fixed. The computational model developed and trained to predict the *% Viability* of a co-culture system will capture the intercellular interaction and match closely to the experimental data. Our empirical models will be useful to predict the behavior of multi-dimensional co-culture systems. Figures 2 and 3 illustrate samples of %viability results expected from mono-culture studies and from co-culture modeling and experimental studies respectively.
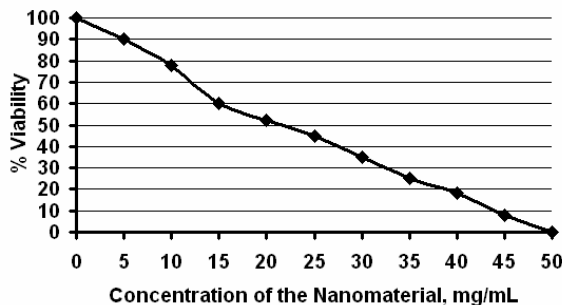


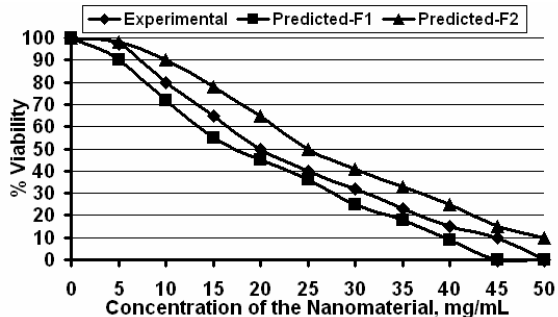**FIGURE 2:** Sample Result from Mono-Culture Studies

**FIGURE 3:** Sample Result from Co-Culture Modeling and Experimental Studies

## 7. CONCLUSIONS AND FUTURE WORK

Our research brings together experimental analysis and computational modeling in the context of the toxicity of nanomaterials on human epidermal cells. The significance of this study is that we develop two-dimensional computational models that can extract information from empirical data obtained through monoculture experiments and co-culture experiments. These computational models can be further trained using more experimental data such that the models will form the basis for future 3-dimensional organotypic culture studies. These co-culture studies can predict outcomes that mimic the in vivo paradigm of human skin, which is the largest organ of the body.

N. Meghanathan, R. D. Isokpehi and H. H. P. Cohly

## 8. ACKNOLWEDGMENTS

## 9. REFERENCES

1. A. R. Murray, E. Kisin, S. S. Leonard, S. H. Young, C. Kommineni, V. E. Kagan, V. Castraova and A. A. Shvedova, "*Oxidative Stress and Inflammatory Response in Dermal Toxicity of Single-walled Carbon Nanotubes*," Toxicology, 257(3): 161-171, 2009.

2. E. Sabbioni, S. Fortaner, S. Bosisio, M. Farina, R. Del Torchio, J. Edel and M. Fischbach, "*Metabolic Fate of Ultratrace Levels of GeCl$_4$ in the Rat and In Vitrio Studies on its Basal Cytotoxicity and Carcniogenic Potential in Balb/3T3 and HaCaT Cell Lines*," Journal of Applied Toxiciology, 30(1): 34 – 41, 2010.

3. L. Xiao, K. Matsubayashi and N. Miwa, "*Inhibitory Effect of the Water-soluble Polymer-wrapped Derivative of Fullerene on UVA-induced Melanogenesis via Downregulation of Tyrosinase Expression in Human Melanocytes and Skin Tissues,*" Archives of Dermatological Research, 299(5-6): 245-257, 2007.

4. V. Manolova, A. Flace, M. Bauer, K. Schwarz, P. Saudan and M. F. Bachmann, "*Nanomaterials Target Distinct Dendritic Cell Populations according to their Size*," European Journal of Immunology, 38(5): 1404-1413, 2008.

5. B. Kiss, T. Biro, G. Czifra, BI Toth, Z. Kertesz, Z. Szikszai, A. Z. Kiss, I. Juhasz, C. C. Zouboulis and J. Hunyadi, "*Investigation of Micronized Titanium Dioxide Penetration in Human Skin Xenografts and its Effect on Cellular Functions of Human Skin-derived Cells*," Experimental Dermatology, 17(8): 659-667, 2008.

6. S. Bastian, W. Busch, D. Kuhnel, A. Springer, T. Meissner, R. Holke, S. Scholz, M. Iwe, W. Pompe, M. Gelinsky, A. Potthoff, V. Richter, C. Ikonomidou and K. Schirmer, "*Toxicity of Tungsten Carbide and Cobalt-doped Tungsten Carbide Nanomaterials in Mammalian Cells In Vitro*," Environmental Health Perspectives, 117(4):530-536, 2009.

7. G. J. Garcia and J. S. Kimbell, "*Deposition of Inhaled Nanomaterials in the Rat Nasal Passages: Dose to the Olfactory Region*," Inhalation Toxicology, 21(14):1165-1175, 2009.

8. F. Li, X. Zhou, J. Zhu, J. Ma, X. Huang and S. T. Wong, "*High Content Image Analysis for Human H4 Neuroglioma Cells Exposed to CuO Nanomaterials*," BMC Biotechnology, 7(66), 2007.

9. B. Graham-Evans, H. H. P. Cohly, H. Yu and P. B. Tchounwou, "*Arsenic-Induced Genotoxic and Cytotoxic Effects in Human Keratinocytes, Melanocytes and Dendritic Cells*," International Journal of Environmental Research and Public Health, 1(2):83 – 89, 2004.

10. C. Huang, Q. Ke, M. Costa and X. Shi, "*Molecular Mechanisms of Arsenic Carcinogenesis*," Molecular and Cellular Biochemistry, 255(1-2):57 – 66, 2004.

11. A. Elder, H. Yang, R. Gwiazda, X. Teng, S. Thurston, H. He and G. Oberdorster, "*Testing Nanomaterials of Unknown Toxicity: An Example Based on Platinum Nanomaterials of Different Shapes*," Wiley Advanced Materials, 19(20):3124 – 3129, 2007.

N. Meghanathan, R. D. Isokpehi and H. H. P. Cohly

12.  S. Y. Shaw, E. C. Westly, M. J. Pittet, A.Subramanian, S. L. Schreiber and R. Weissleder, "*Perturbational Profiling of Nanomaterial Biologic Activity*," In Proceedings of the National Academy of Sciences of the United States of America, 105(210):7387 – 7392, 2008.

13.  F. Laco, M. Kun, H. J. Weber, S. Ramakrishna and C. K. Chan, "*The Dose Effect of Human Bone Marrow-derived Mesenchymal Stem Cells on Epidermal Development in Organotypic Co-culture*," Journal of Dermatological Science, 55(3):150-160, 2009.

14. H. Inano, M. Onoda, N. Inafuku, M. Kubota, Y. Kamada, T. Osawa, H. Kobayashi and K. Wakabayashi, "*Chemoprevention by Curcumin during the Promotion State of Tumorigenesis of Mammary Gland in Rats Irradiated with Gamma-rays*," Carcinogenesis, 20(6): 1011-1018, 1999.

15. J. A. Buege and S. D. Aust, "*Microsomal Lipid Peroxidation*," Methods in Enzymology, 52: 302-310, 1978.