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Reflections in Mutation Research

The comet assay: Reflections on its development, evolution and applications☆

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A R T I C L E I N F O

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The study of DNA damage and its repair is critical to our understanding of human aging and cancer. This review reflects on the development of a simple technique, now known as the comet assay, to study the accumulation of DNA damage and its repair. It describes my journey into aging research and the need for a method that sensitively quantifies DNA damage on a cell-by-cell basis and on a day-by-day basis. My inspirations, obstacles and successes on the path to developing this assay and improving its reliability and sensitivity are discussed. Recent modifications, applications, and the process of standardizing the technique are also described. What was once untried and unknown has become a technique used around the world for understanding and monitoring DNA damage. The comet assay's use has grown exponentially in the new millennium, as emphasis on studying biological phenomena at the single-cell level has increased. I and others have applied the technique across cell types (including germ cells) and species (including bacteria). As it enters new realms and gains clinical relevance, the comet assay may very well illuminate human aging and its prevention.

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1. Introduction

By the time I began research, it was already accepted that to understand the process of aging and its causes, one had to see DNA. The scientists who laid the foundations for our field were scientists who found a way to see: whether Sutton [1] and Boveri who saw that genes had to be located on chromosomes, Franklin and Gosling [2] who saw the structure of DNA, or Tjio and Levan [3] who saw the true number of human chromosomes. The desire to see human aging with as much clarity as I could was always my main mission, and the development of the comet assay was a result of this desire. I always felt that, once seen, the secret of aging and its prevention could be found.

2. Scientific foundation in India

As a child, I thought that I would find the secret to aging and make my parents immortal, but I had no knowledge about research and no intention to pursue it. In July 1967, when I entered King George's Medical College (KGMC) in Lucknow, India, it was with the goal of becoming a family doctor in a village like the one that I had just left or a small town clinic. But KGMC was a unique place. Set on the Gomti River, it is a famously beautiful campus in a city known for its culture and courtliness. At the time, it was the top medical college in India, and its alumni, called Georgians, were top physicians, surgeons and researchers. It was also very well funded. I was exposed to new fields, taught by experts, and I had the opportunity to be in a lab. I stayed there for nearly ten years as a student, then post-graduate and finally as faculty.

During my post-graduate studies in the Department of Anatomy, I had the privilege of establishing a laboratory where I could study chromosomes under the microscope. My childhood desire to find the secret of aging was within my reach! I used to soak red kidney beans in water for 2 to 3 h, then blend and centrifuge them. I would remove the top supernatant layer using an ordinary pipet and syringe. This solution was rich in phytohemagglutinin and was used to stimulate human lymphocytes to divide. After using colchicine to arrest the cell cycle at metaphase, I could see a cell frozen in the midst of division. Finally, I had a chance to look at chromosomes, 46 of them. I ended up writing my thesis on chromosomal aberrations observed after treatments with hormones and antibiotics. During my Master's program, my supervisor, Professor Avinash Chandra Das, Chair of the Department of Anatomy, found funding to create a cytogenetics

☆ This article is part of the Reflections in Mutation Research series. To suggest topics and authors for Reflections, readers should contact the series editor, G.R. Hoffmann (ghoffmann@holycross.edu).

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laboratory and I was only too eager to set it up. This was the beginning of my journey into DNA damage and aging research.

Our conditions were not perfect: the room was a converted processing area for anatomy specimens and body parts. We were missing some key equipment but we found substitutes—I took a pressure cooker from our kitchen at home and this served as the autoclave for our glassware. Without having fully sterile conditions, I used to lose 90% of my cultures to contamination. I had a UV light and a glass chamber that I sterilized using the light. I had a water bath, light microscope and electric centrifuge but no incubator. Electricity outages were common, almost everyday occurrences, and they interrupted many experiments. Still, by aspirating rabbit bone marrow directly, using colchicine to arrest cell division in metaphase, and staining with Wright's or Giemsa stain, we were able to visualize chromosomes. I found effects of antibiotics (tetracycline, chloramphenicol) but not of hormones (testosterone, estrogen and progesterone) on rabbit chromosomes after 7 days of daily injections [4].

Eventually, I wanted to see DNA, not just chromosomes, but this goal exceeded the resources and knowledge at KGMC. In the fall of 1977, I visited the labs of Drs. Geeta Talukedar and Archana Sharma in Calcutta to learn autoradiography and unscheduled DNA synthesis (UDS). The incorporation of radioactive bases into damaged DNA during UDS allowed for the estimation of repair in DNA by visual grain counting. In 1978, I traveled to Bhabha Atomic Research Center in Bombay to learn mutagenesis in bacteria—the Ames test—with Drs. A.S. Aiyar and P.S. Chauhan. This allowed me to quantify the number of mutations induced by environmental chemicals. Still, even at Bhabha, they were not studying DNA damage directly. By the time I left Bombay, I had the notion that I would try to make an assay to directly measure a cell's DNA damage.

Wanting to work with DNA directly, I read any article that I could find on DNA damage, sister-chromatid exchange (SCE), alkaline elution and chromosomal aberrations. The medical library at KGMC had few scientific journals, so I used to read articles in the well-stocked archives of the National Botanic Garden and Central Drug Research Institute, both in Lucknow. On countless occasions, my wife would copy the articles by hand so that I could read and replicate experiments in the lab. After I had left Lucknow and arrived in America, I showed these handwritten copies of articles to their original authors. Ronald Hart was incredulous and amusedly took these papers around the labs at the National Center for Toxicological Research (NCTR). Painstakingly copied in blue ink were the articles of Drs. Nathan Shock, Ed Schneider, George Martin and Dr. Hart himself. I gained a lot of knowledge from this published work, and it inspired me toward new research directions and even life style changes. While I was still in India, Lester Packer's work on vitamin E's effect on WI-38 cells, making them immortal [5], inspired me to buy a bottle of vitamin E oil for daily ingestion.

3. Research training in the United States

Having taken advantage of all the resources available in India for studying DNA damage, I began to look for a post-doctoral fellowship. I wrote letters to every author outside of India whose work I had read and respected. Two positive responses came: one first from Dr. Hart and then one from Dr. Ed Schneider. I accepted Dr. Hart's offer as he was more of a basic researcher. The airplane ticket was equivalent to six months of my salary as a demonstrator in KGMC's anatomy department, where teaching medical students was my main job. I had to borrow money from my father and a fellow "Georgian," the co-author of my first publication, Dr. M.K. Tolani. I had never left India before, but a month after Dr. Hart's letter arrived, I traveled 12,500 miles—exactly half way around the earth.

I arrived at Ohio State University on the 10th of October, 1979, as a post-doctoral fellow. I had less than a hundred dollars in cash, a letter from Dr. Hart, and a suitcase filled with cashew nuts and raisins. As a vegetarian, I had no idea what I would find to eat in the United States. I was fortunate to have the best possible guide into American life; like a kindly grandmother, Mrs. Helen Dixon hosted many foreign postdocs in her large home near campus. She was my good friend and host for my entire time at OSU. My supervisor and the head of our lab, Dr. Hart was tall and vibrant with a booming laugh that conveyed positivity and progress. My main project at OSU was studying the effects of known carcinogens in rat tissue. The animals were sacrificed to estimate DNA damage in various organs. My approach was initially limited to mincing the organs with scalpels in a crisscrossing motion on frosted glass to get single-cell suspensions of the tissues that were then used for a variety of assessments. I spent many contented hours in the lab. I emerged to use OSU's playing fields and swimming pools, trying American-style football, diving or tennis. Many weekends were spent in the immigration offices of Cincinnati, where I struggled to obtain a temporary or permanent status that would allow me to stay in the country.

As I was finishing my postdoctoral fellowship at OSU, I was offered a position in Jefferson, Arkansas, in January of 1981. Dr. Hart had been appointed director of the NCTR, and he asked me to be part of his team. He had ambitious goals. Alongside Drs. Ming Chang and Angelo Turturro, I worked on assessing the effects of asbestos in vivo and in vitro [7]. I also developed a novel technique to infuse BrdU using an intraperitoneal catheter *in utero* in rats [8]. We then found stage-dependent effects of toxic agents on fetal development by studying SCEs in various tissues in embryos at various stages of development [9].

4. Formative ideas for the comet assay

When my appointment as a visiting scientist at NCTR ended, Dr. Steve D'Ambrosio offered me a position as Visiting Assistant Professor back at OSU in 1982. Returning to OSU resulted in my long-lasting research collaboration and friendship with Dr. Ralph Stephens. I learned more about staining DNA working with Dr. Stephens than I had ever known and that was a starting point for developing a new technique. We even published a methods paper showing differences in staining between live and dead cells [10]. By this time, I was familiar with several techniques for assessing DNA damage, including the alkaline sucrose gradient technique, which I had learned from Dr. Hart, and the UDS assay. As a postdoctoral fellow, I also became proficient in the nucleoid sedimentation technique, thanks to the guidance of Drs. Philip Lipetz and Ralph Stephens. In this technique, the nonionic detergent Triton X-100 was added to a high salt (2.5 M) solution for rapid lysis of cells.

Learning these techniques and knowing their drawbacks laid the foundation of ideas for a new technique. While I was still a postdoc, Dr. Douglas Brash, by chance, gave me a book chapter by Rydberg and Johanson [6]. Rydberg and Johanson's technique involved embedding lymphocytes in agarose gel, lysing cells with a solution of detergent (SDS) and EDTA on microscope slides, air drying cells in agarose, treating with an alkaline solution, and then immersing cells and gels in a neutralizing solution before staining with acridine orange. I studied the work overnight, and the next day Dr. Brash told me how to make agarose, mix it with the cells and solidify it on microscope slides. In this technique, the alkaline solution unwinds the DNA, which, after staining, appears as a halo in damaged cells. The intercalation of dye in double-stranded DNA is responsible for the green fluorescence, and the red fluorescence is due to the association of acridine orange along the single stranded DNA. Quantification of the ratio between green and red

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