Global Journal of Biotechnology and Biomaterial Science

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Dates: **Received:** 21 April, 2015; **Accepted:** 23 May, 2015; **Published:** 25 May, 2015

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Keywords: Molecular biomarkers; Genetic damage; Comet assay; Genotoxicity test; Monitoring; Pollution; Cancer

Research Article

Use of Comet Assay in Human Lymphocytes as a Molecular Biomarker for Simultaneous Monitoring of Genetic Damage and Genotoxicity in Residents Who Lived Nearby the Santiago River, Mexico, in 2012

Abstract

Most aquatic and health management authorities need assessing the potential genotoxicity of contaminated effluent to avoid the effects of a lot chemical agents found as environmental contaminants. Previous studies report the presence of some pesticides and heavy metals in waters of Santiago River and suggest a genetic risk for residents, who lived nearby this river, so it is important determine its mutagenic risk. The objective of this study was to use of comet assay in human lymphocytes as a molecular biomarker of genetic damage in residents who lived nearby the Santiago River, México, in 2012 and monitoring simultaneous of genotoxicity of these waters in unexposed persons. The genotoxic activity of water samples taken from the Santiago River was evaluated in human lymphocytes from persons unexposed and the evaluation of genetic damage were studied in people living near the river. The results indicated the existence of mutagens in the water ($p \leq 0.05$), genetic damage statistically significant ($p \leq 0.05$) in residents who lived nearby the Santiago River and carcinogenic risk to human health. The comet assay system in human lymphocytes can be used as a molecular biomarker for simultaneous monitoring of genetic damage and genotoxicity in aquatic environments contaminated with genotoxic, additionally this bioassay is reliable and inexpensive.

Background

Human populations discharge great quantities of residues to the rivers, contaminating them in the process [1-4] in addition, many industries also release genotoxic substances which are incorporated to the organism through direct ingestion, inhalation, or the consumption of plants that have absorbed those substances, making them dangerous for human and other organisms health inducing genetic damage [1,5]. Ohe et al. [6] studied water quality, and demonstrated that some rivers in the world, especially in Europe, Asia and South America, are contaminated with potent direct and indirect frame shift-type and base substitution-type mutagens towards Salmonella strains TA98 and TA100. These studies demonstrated that these environmental mixtures contained many toxicants, which may have carcinogenic potential.

A variety of animals and plants are available for use as environmental and aquatic pollution monitors [7]. However, sometimes it is possible to perform monitoring studies on individuals directly affected by pollution, in this case, the human population. The Santiago River is born east of Chapala lake (20.3032º, -102.7630º latitude, longitude in México) and is located in the west central region of México. It is a portion of the Lerma-Chapala-Santiago basin and has a length of 82 Km and it has 28 Km wide [8]. Its basin covers an area of 191,899 km2 (63.9 %) and crosses Jalisco and Nayarit states and ends in the Pacific Ocean, nearby to San Blas Nayarit, México (21.5397º, -105.2855º). The Santiago River is the major freshwater source for industry and agriculture, as well as the source of the drinking water of millions of residents living along it [9].

The high pollution of the Santiago River is due to industrial wastewater and domestic sewage. In a previous study gas chromatography/mass spectrometry detected pesticides residues and heavy metals [10,11]. The presence of mercury in the hair of the inhabitants that lived on the borders of the lake and its mutagenic potential are reported [12]. Eleven persistent organic pollutants (POPs) were detected in the Santiago River by gas chromatography [13]. There are no epidemiological studies associating Santiago River pollution with increased cancer in the population, but similarly, an epidemiological investigation indicated that the organic contamination in the Songhua River was a risk factor for tumours development among the residents who lived along the Songhua River [14]. The results in this study also showed that there is a relationship between cancer mortalities and organic contamination of the Songhua River.

Because organic pollutant compounds are not known to produce many genotoxic activities such as damaging DNA or chromatids or causing whole cell transformation, the genotoxic effects from environmental contamination are monitored with a large gamut of *in vivo* or *in vitro* system tests [15,16]. The comet assay test has been extensively used as a simple, sensitive, and quick tool to evaluate the DNA damage [17] and it is used in diverse fields of genetic toxicology [15,18-20].

The objective of this study was to use of comet assay in human lymphocytes as a molecular biomarker of genetic damage in residents who lived nearby the Santiago River, México, in 2012 (*in vivo* study) and monitoring of genotoxicity of these waters in unexposed persons (*in vitro* study: lymphocytes exposed directly to contaminated water).

Materials and Methods

Water samples

Five litres of Santiago River water samples was collected directly in May 2012 from Juanacatlán waterfall, Jalisco, México on a well clean glass flask and then it was carefully transported to the laboratory in a time of less than 2 hours. To eliminate the possibility of a bacteriological contamination, the water was filtered with a 0.22 µm membrane [21].

Blood samples for studies of water genotoxicity polluted

13 young (from cities Guadalajara and Zapopan) males (8) and females (5) volunteers with less than 20 years of age who had no history of smoking or drinking and never before exposed to chemical substances, participated as blood donors (information obtained from a previously applied questionnaire in May 2012). 5 drops of blood were collected by puncture of the ring finger and were placed on test tubes with 5 ml of a cold phosphated buffering agent (160 mM NaCl, 8 mM Na2HPO4, 4 mM NaH2PO4, EDTA 50 mM, pH 7). Then, the test tubes were immediately transported to the laboratory.

Blood samples for studies of genetic damage in exposed persons to polluted waters

Blood samples of people living near the river were collected from 14 volunteers through a household visit to the inhabitants of Juanacatlán, Jalisco. The purpose of the study was explained to them and next a blood sample donation was requested. Straight after, 5 blood drops were collected using the same procedure before mentioned.

Obtaining of lymphocytes

The test tubes with the blood samples were centrifuged at 3,000 rpm for 10 min, the supernatant was eliminated and next the pellet was suspended on 500 µl of a phosphate buffer. The cellular suspension was kept at 4 ºC until the moment of use.

The Trypan Blue Test was performed on 20 ml of whole blood suspension to determine the percentage of viable cells for every individual. The mean percentage viability calculated for each group was above 89 % in both tissues.

Comet assay

The obtained lymphocytes were subjected to the alkaline comet assay test as described by Singh et al. (1988). The slides were covered with Normal Melting Point (NMP) agarose at 1 %, it was allowed to solidify and then it was removed to have a completely clean surface. After this, 300 µl of Low Melting Point (LMP) agarose at 0.6 % was placed on the slide. Straight after, 95 µl of LMP agarose at 0.5 % were mixed with 5 µl of the cellular suspension and the mixture was placed over the first layer. Finally, a third layer of 100 µl of LMP agarose at 0.5 % was placed over the second layer.

In vitro study for the evaluation of genotoxic activity from Santiago river water

Lymphocytes of young individuals were placed on agarose gel, as described previously and next were exposed to polluted water. The slides with the lymphocytes of the 13 juvenile and healthy persons not exposed were submerged for 24 hours in 200 ml of the contaminated water at room temperature. When the exposition ended, the slides were washed with distilled water during 5 min. Two slides were used per individual and the experiment was realized as a duplicate. Finally, the alkaline comet assay test was immediately applied.

In vivo study for evaluation of genetic damage in lymphocytes of persons exposed daily to Santiago river water

Lymphocytes of 14 volunteers underwent directly to the comet assay. Lymphocytes from 8 individuals young and healthy not exposed to polluted waters were used as both negative and positive witnesses (cells exposed along 3 hours to N-nitrosodiethylamine (NDEA, CAS No. 55-18-5) 5 mM).

All slides from *in vitro* and *in vivo* studies were submerged in a lysis solution (NaCl 2.5 M, EDTA 100 mM, Tris-base 10 mM, Lauroyl sarcosinate 1%, Triton X-100 1% and DMSO 10%, pH 10) for 3 hours at 4 ºC and then were washed with distilled water. To allow the DNA unfolding, the slides were placed in a horizontal electrophoresis system (Biorad, model A6) immersed in the electrophoresis buffer (NaOH 300 mM, EDTA 1mM, pH 13) for 45 min at 4 ºC. Electrophoresis was carried out for 10 min at 1.0 V/cm with an accompanying amperage of approximately 300 mA (Labconco, model 4333280) and once it finished, the slides were washed with distilled water and were immediately dyed with 0.1 ml of ethidium bromide (20 µl / 1 ml) for 5 min. Soon after, the slides were washed five times with distilled water and a cover slip was placed over the gel for its observation in the fluorescence microscope (Zeizz, model axioskop 40, with excitation filter of 515-560 nm).The evaluation was made based on the measurements of the Tail Length (Comet assay system II). The experiment was repeated twice too.

Statistical analysis

The data obtained was submitted to one-way analysis of variance test (ANOVA) using the Co Stat program [22]. The Dunnett test was used for comparing negative and positive witnesses with data from both human lymphocytes exposed to Santiago River polluted water and inhabitant lymphocytes from Juanacatlán, Jalisco. 100 cells were

used each individual. Results were considered statistically significant at $p \leq 0.05$.

Results

Comet assay *in vivo* study

Data tail length in human lymphocytes from 14 persons who were living nearby of Santiago River are shown in Figure 1. As is observed the mean of the tail length in all lymphocytes was significantly different ($p \leq 0.05$) compared to the negative control: lymphocytes of people who living nearby to Santiago Rivers 36.94 ± 7.6; lymphocytes of young and healthy people expose to polluted water 46.87 ± 8.81; positive control 54.4 ± 6.7 and negative control 16.02 ± 3.3).

Comet assay *in vitro* study

Data of tail lengths in human lymphocytes from 13 young students non-exposed previously to chemical agents are too shown in Figure 1. It is plain that the lymphocytes exposed directly to the polluted water samples showed the highest level of genetic damage compared to the negative control ($p \leq 0.05$).

Both studies: *in vivo* (average tail length 36.54 ± 7.6) and in *in vitro* (average tail length 46.87 ± 8.81), the tail averaged is nearby to the average of the positive control tail (54.4 ± 6.7 µm) and it is very different to negative control (16.02 ± 3.3). In some cases, the genetic damage in the lymphocytes of people living nearby to Santiago River was also high (49.33 ± 6.8 and 49.25 ± 8.0 µm, for example) like an *in vitro* exposition. However is observed genetic damage increased in the *in vitro* study.

Discussion

Heavy pollution of the Santiago River is attributed to industrial

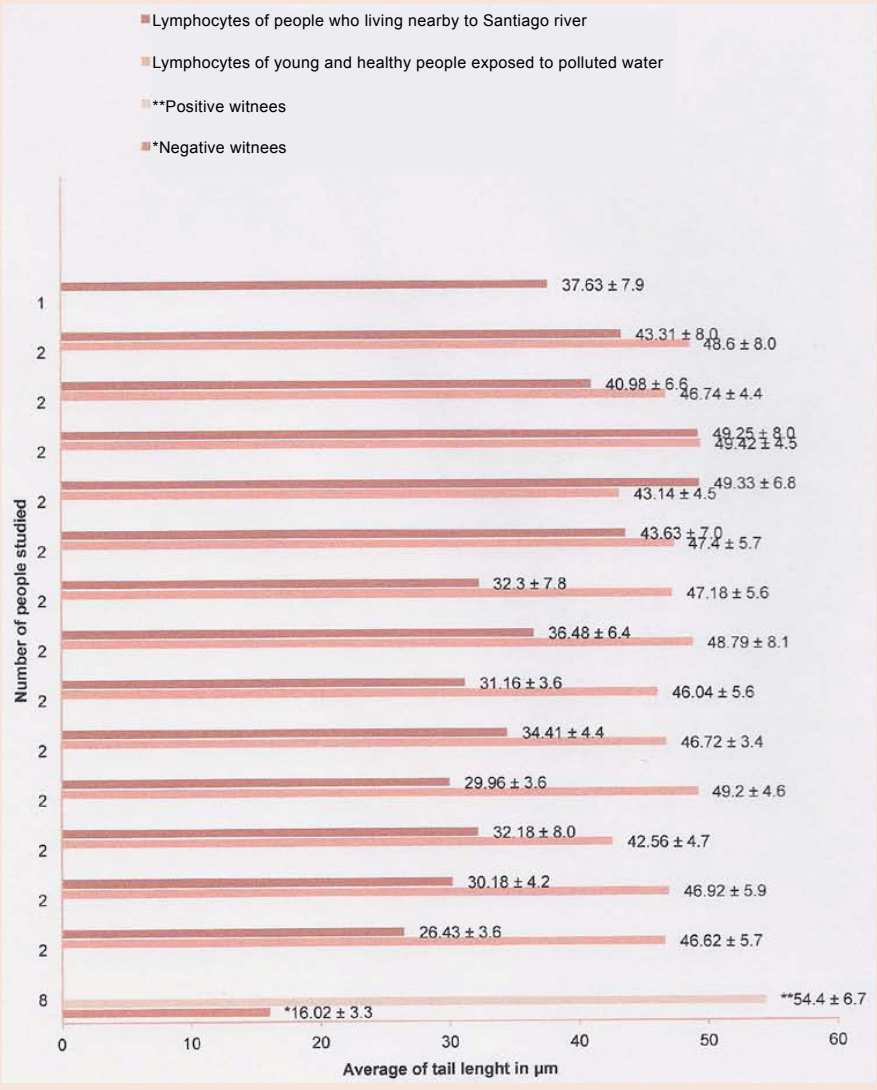

Figure 1: Average of tail length lymphocytes of people living nearby of Santiago River and lymphocytes of healthy young volunteers exposed *in vitro* to polluted waters. $P \leq 0.05$.

Citation: *Alvarez-Moya C, Reynoso-Silva M (2015) Use of Comet Assay in Human Lymphocytes as a Molecular Biomarker for Simultaneous Monitoring of Genetic Damage and Genotoxicity in Residents Who Lived Nearby the Santiago River, Mexico, in 2012. Glob J Biotechnol Biomater Sci 1(1): 004-008.*

waste water and domestic sewage along the Lerma-Chapala-Santiago basin [9,23] but it is particularly higher and evident in Juanacatlán city, Jalisco (20.1133º, -104.2556º) due to the discharge of waste and industrial waters from Guadalajara, the second largest city in México. Previously in Chapala Lake some genotoxic were detected such as organoclorated pesticides [10,11,23,24].

Among various techniques used to estimate the genotoxicity of xenobiotic, the comet assay is becoming an increasingly popular tool for the measurement of DNA damage in individual cells. In recent years, this method has been widely used for studies in genetic toxicology [25-27] because it is a fast, simple and sensitive and it can be applied with just a couple of hundreds of cells, what makes the participation of people much easier because it only requires a couple drops of blood.

As shown in Figure 1, the significant increase in DNA length, using comet assay in human lymphocytes exposed directly to polluted water from Santiago River (*in vitro* study) indicates the presence of chemical agents, which have genotoxic activity and so, exist risk of carcinogenic for human health [1]. In order to obtain reliable results for the *in vitro* study we used only cells of individuals with less exposure to genotoxic agents. Simultaneously, the increased genetic damage (*in vivo* study) observed in lymphocytes from people living nearby to Santiago River water (Figure 1) could be related with a different contact pathway to waste water [12]. Although people living along Santiago River avoid directly ingesting the river water, there are many water wells near the river intended to human use that might be contaminated because of water filtration, additionally many persons have, constantly, dermal contact with foam from a cascade of the river and they indicate an increased in stench, respiratory diseases and incidence of cancer in recent years [28-30]. *In vitro* study did it evident the presence of genotoxic agents in Santiago River. These agents were able to increase the length of the tail in lymphocytes of unexposed people and although it was not possible to identify specifically, each genotoxic agent are highly likely that these same chemicals cause the genetic damage in the inhabitants of El Salto [23,28]. It is clear that the use of comet assay in both directions: *in vivo* and *in vitro* can be used simultaneously as a marker for genetic damage or indicator of the presence of genotoxic and may alert the risk of carcinogenic to human health, particularly people living near water contaminated. It is obvious that detection of genotoxic agents and genetic damage is complementary and can be performed in any other type of animal cell.

Conclusion

The studies *in vivo* and *in vitro* with the comet assay in human lymphocytes were used as a molecular biomarker for simultaneous monitoring of genetic damage and genotoxicity in aquatic environments contaminated with genotoxic. The detection of genotoxic agents and genetic damage is complementary and can be performed in any other type of animal cell. Our data indicates that the Santiago River water in the study area is polluted with substances capable of inducing DNA damage in human cells. Therefore, the direct and indirect exposure to this contaminated water may cause mutagenic/carcinogenic changes in exposed individuals affecting the population of Juanacatlan, Jalisco, Mexico.

Acknowledgments

This work has been partially supported by the University of Guadalajara-COECYTJAL Mexico 2009-401.

References

1. Liu JR, Dong HW, Tang XL, Sun XR, Han XH, et al. (2009) Genotoxicity of water from the Songhua River, China, in 1994-1995 and 2002-2003: Potential risks for human health. Environ pollut 2: 357-364.
2. Zhou JL, Zhang ZL, Banks E, Grover D, Jiang JQ (2009) Pharmaceutical residues in wastewater treatment works effluents and their impact on receiving river water. J Hazard Mat 166: 655–661.
3. Mayo DO, Miranda SG, Rojas SF, Coras MP, Hahn Schlan F et al. (2006) Monitoreo de la calidad del agua en el río Texcoco mediante sensores selectivos de iones. Agrociencia 3: 277-287.
4. Watanabe T, Hirayama T (2001) Genotoxicity of soil. J Health Sci 47: 433-438.
5. Krishnamurthi K, Saravana D, Hengstler JG, Hermes M, Kumar K, et al. (2008) Genotoxicity of sludges, wastewater and effluents from three different industries. Arch Toxicol 12: 965-971.
6. Ohe T, Watanabe T, Wakabayashi K (2004) Mutagens in surface waters: a review. Mutat Res 567: 109-149.
7. Adedeji OB, Okerentugba PO, Okonko IO (2012) Use of molecular, biochemical and cellular biomarkers in monitoring environmental and aquatic pollution. Nat Sci 10: 83.104.
8. Paré L (1989) Los pescadores de Chapala y la defensa del lago. 1ª edition. Edited by UNAM / ITESO / COLJAL, México 144-160.
9. Guzmán AM, Peniche CS, Valdés ZA (2003) La cuenca del río Lerma y el lago de Chapala. In Chapala una crisis programada, 1a edition. Edited by Guzmán AM, Grupo Parlamentario del Congreso de la Union, México 12-58.
10. Trujillo-Cárdenas JL, Nereida P, Saucedo-Torres P, Zárate DV, Nely RD, et al. (2010) Speciation and Sources of Toxic Metals in Sediments of Lake Chapala Mexico. J Mex Chem Soc 54: 79-87.
11. Alvarez MC y, Arévalo HA (2007) COP´S y su Bioacumulación. In Lago de Chapala, Contaminación y Riesgo Genético, 1ª edition. Edited by Alvarez MC, Universidad de Guadalajara, México 59-77.
12. González MI, Cifuentes E, Cortés JE, Ríos C, Rothenberg SJ, et al. (2006) Concentraciones de mercurio total en sangre y cabello por ingesta de pescado en niños, mujeres embarazadas y en edad reproductiva residentes de los municipios aledaños al lago de Chapala, Jalisco-México. Acta Toxicol Argent 14: 22-24.
13. Reynoso SM (2006) Bioacumulación de COP´S y daño genético en el hígado de Goodea atripinnis del Lago de Chápala. MC thesis Universidad de Guadalajara, Biology and Molecular Departament.
14. Zhu ZG, Gan HF, Guo DL, Xie X, Pang YX, et al. (1985) Study on carcinogenic potentiality of organic contamination of the Songhua River. China Environ Sci 5: 7-11.
15. Frenzilli G, Nigro M, Lyons BP (2009) The Comet assay for the evaluation of genotoxic impact in aquatic environments. Mutat Res 681: 80-92.
16. Grant WF, Lee HG, Logan DM, Salamone MF (1992) The use of Tradescantia and Vicia faba bioassays for the in situ detection of mutagens in an aquatic environment. Mutat Res 2: 53-64.
17. Singh N, McCoy M, Tice RR, Schneider EL (1988) Simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175: 184-191.
18. Alvarez-Moya C1, Silva MR, Arámbula AR, Sandoval AI, Vasquez HC, et al. (2011) Evaluation of genetic damage induced by glyphosate isopropylamine salt using Tradescantia bioassays. Genet Mol Biol 34: 127-130.
19. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, et al. (2000) Single

cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35: 206-221.

20. Tice RR (1995) The single cell gel/comet assay: a microgel electrophoretic technique for detection of DNA damage and repair in individual cells. In Environmental Mutagenesis. 1a edition. Edited by Phillips DH and Venitt S, Bios Scientific Publishers, Oxford 315-339.

21. Bekaert C, Rast C, Ferrier V, Bispo A, Jourdain M, et al. (1999) Use of in vitro (Ames and Mutation tests) and in vivo (Amphibian Micronucleus test) assays to assess the genotoxicity of leachates from contaminated soil. Org Geo Chem 30: 953–962.

22. Ma TH, Cabrera GL, Cebulska-Wasilewska A, Chen R, Loarca F, et al. (1994) Tradescantia stamen hair mutation bioassay. Mutat Res 310: 211-220.

23. Arévalo HA, Reynoso SM y Alvarez MC (2011) Compuestos organo-persistentes y daño genético en núcleos hepáticos de Goodea atripinnis del lago de Chapala. Scientia-CUCBA 13: 1-8.

24. Alvarez MC, Arévalo HA, Alvarez BS y Guerrero QL (2007) Contaminación en Chapala. In Lago de Chapala, Contaminación y Riesgo Genético, 1ª edition. Edited by Alvarez MC, Universidad de Guadalajara, México 37-48.

25. Jha AN (2008) Ecotoxicological applications and significance of the comet assay. Mutagenesis: 23: 207-221.

26. Kassie F, Parzefall W, Knasmuller S (2000) Single cell gel electrophoresis assay: a new technique for human biomonitoring studies. Mutat Res 463: 13–31.

27. Mitchelmore CL, Chipman JK (1998) DNA strand breakage in aquatic organisms breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutat Res 399: 135–147.

28. Online Contaminación En El Salto (2012) (OCEES).

29. Online El Salto (2013) México…Cascada, Río Santiago (OESMCRS).

30. (2013) Online Muerte Lenta del Río Santiago por Contaminación (OMLRSC).

Citation: *Alvarez-Moya C, Reynoso-Silva M (2015) Use of Comet Assay in Human Lymphocytes as a Molecular Biomarker for Simultaneous Monitoring of Genetic Damage and Genotoxicity in Residents Who Lived Nearby the Santiago River, Mexico, in 2012. Glob J Biotechnol Biomater Sci 1(1): 004-008.*